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Padma V. Devarajan
Prajakta Dandekar
Anisha A. D'Souza
Editors

Targeted Intracellular Drug Delivery by Receptor Mediated Endocytosis

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Foreword

I am delighted to write this foreword for the book titled *Targeted Intracellular Drug Delivery by Receptor Mediated Endocytosis*, by a team of academicians and industry professionals, from the Institute of Chemical Technology (ICT), Mumbai, a deemed university of national and international repute. The book focuses on a very important thrust area in global healthcare, namely, cancer and infectious diseases. Global statistics reflect staggering increase in patient numbers, evolution of drug resistance, and severe adverse effects, all of which pose serious challenges in therapy. Strategies relying on nanomedicine-enabled targeted delivery which could provide viable solutions are highlighted in this book.

I would like to compliment the multidisciplinary team of experts in nanomedicine and biotechnology who have put together a very exciting and topical theme in an easy-to-comprehend format. The contents of the book reflect a comprehensive coverage of the receptor-mediated endocytic approach for targeted delivery of drugs into afflicted cells to overcome challenges of conventional therapy. Integration of science and technology as a vital aspect of the book ensures the book could cater to wide readership.

My compliments to the team for engaging and training a large number of young scientists during the course of this project.

My best wishes to the editorial team.

Manju Sharma
Former Secretary to the Government of India,
Department of Biotechnology,
Distinguished Women Scientist Chair,
The National Academy of Sciences,
Allahabad, India

Preface

Modernization of society, despite revolutionary developments in medicine, has resulted in manifold increase in ailments as evident from the staggering statistics. This has triggered accelerated developments in drug discovery, with an ever-increasing focus on newer delivery approaches of established drug molecules. This typical approach of "packing old wine in new bottles" has led to path breaking developments of more efficacious and safer drug delivery systems. Despite numerous challenges, targeted drug delivery is making slow but steady progress as an enabler in expanding the horizons of nanomedicine. Major applications of nanomedicine-based targeted delivery are focused on treatment of intracellular afflictions. Two major afflictions in this arena include cancer and intracellular infectious diseases, both of which are rampant and growing at an alarming rate. Among various approaches for intracellular targeting of nanocarriers, this book as reflected by the title is thematic and focuses on one of the major and highly selective approaches of targeting, namely, intracellular delivery by *receptor-mediated endocytosis (RME)*.

We consider this subject as timely and relevant due to the ever-escalating discovery of various receptors and the unfolding of their intricate involvement in the pathology of cancer and infectious diseases. The intent is to juxtapose an understanding of the receptors with rational developments in nanomedicine for targeted delivery with a prime focus on two of the major and challenging therapeutic areas, namely, cancer and infectious diseases, where we believe such developments would have immense practical relevance.

The book commences with an overview on the intracellular endocytic pathways. Citing these natural uptake mechanisms through examples, the chapter highlights the application of these natural biological processes for targeted intracellular drug delivery. The role of phagocytosis, the natural defense mechanism of the body for uptake of pathogens, is elucidated with an emphasis on bypassing the challenges to enable effective drug delivery. The major focus of the chapter however is on the various receptor-mediated endocytic pathways. The differences in the various uptake mechanisms are highlighted along with methods to effectively exploit these pathways through rational nanocarrier design. With this introduction, the subsequent

chapters deal with in-depth discussions on receptors, their biology, the receptor domains for ligand attachment, and intracellular endocytosis. Receptors involved in the pathology of cancers and infectious diseases have been carefully selected. Targeted delivery to the receptors using nanocarriers is highlighted through diverse examples. The clinical trial status presented at the end of each of these chapters signifies their potential for societal relevance. A section on in vitro and in vivo evaluation models and a chapter on cellular assays provide hands-on training to young and nouveau researchers in the field of targeted nanomedicine.

Although the book would be of specific relevance to researchers and scientists working in targeted drug delivery, the book has been carefully planned to cater to the interest of receptor biologists, drug discovery scientists, nanotechnologists, medical doctors, and the pharmaceutical industry. Importantly, as the book is both authored and edited by the editorial team, it has been structured in a manner that permits easy readability and understanding, which could serve as a reference manual and textbook for graduate students and doctoral and postdoctoral fellows.

The editorial team takes great pride in stating that this book has been a stepping stone in enhancing comprehension and writing skills of over 30 young research students and has begun its journey by serving as an educational research tool.

We do hope the book enlightens you on this important aspect of targeted nanomedicine.

Mumbai, India Padma V. Devarajan
Mumbai, India Prajakta Dandekar
Mumbai, India Anisha A. D'Souza

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Prajakta Dandekar received her PhD from the Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai. Her doctoral research involved the development of polymeric nanoparticles for natural therapeutic agents. Later, she joined the Department of Macromolecular Organic Chemistry, University of Saarland, Saarbrucken, as a postdoctoral researcher and was involved in formulating nanoparticles of new hydrophobic starch polymers for encapsulating hydrophobic anticancer activities. She was the first woman scientist from India to be awarded the European Respiratory Society-Marie Curie Joint Postdoctoral Fellowship (long-term) and conducted the associated research involving formulation of nanoparticles for intracellular siRNA delivery at the Department of Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrucken,

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Anisha A. D'Souza is currently working as research formulation scientist at Piramal Enterprises Limited, Mumbai. She received her PhD (Tech.) in Pharmaceutics from the Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai (ICT, formerly known as UDCT). She was the recipient of the most coveted and prestigious "Prof. M. M. Sharma Doctoral Fellowship" at ICT. Subsequently, during her postdoctoral tenure at IIT-Bombay, she has worked on delivery of micronutrient-encapsulated nanoparticles incorporated in different cosmetics for improving mother and child health. Her research interest encompasses colloidal drug delivery systems, namely, polymeric and solid lipid nanoparticles for enhanced bioavailability, controlled delivery, and targeting. She has published research papers and reviews in peer-reviewed journals and has coauthored a number of book chapters.

Part I
Introduction and Overview

Chapter 1
Intracellular Delivery: An Overview

Dhanashree H. Surve, Prajakta Dandekar, Padma V. Devarajan, and Anil B. Jindal

Abstract Targeted intracellular delivery is gaining importance, especially for improved therapy of cancer and intracellular infections. Endocytosis or cellular internalization, a physiological process for intracellular delivery of nutrients or destruction of pathogens, involves two major pathways, namely phagocytosis or cell eating, which enable uptake of solid particles and pinocytosis or cell drinking. Pinocytosis includes fluid-phase endocytosis (macropinocytosis/micropinocytosis) and receptor-mediated endocytosis (RME). While phagocytosis, macropinocytosis, and micropinocytosis are nonselective, RME is a selective process of internalization, which is triggered by association of the receptor with specific ligands. Among endocytic processes, phagocytosis and RME are relied on for nanocarrier-based targeted drug delivery. This chapter describes various pathways with emphasis on phagocytosis and strategies to bypass lysosomal destruction of drugs. A major focus, however, is RME with a detailed discussion on clathrin, caveolin, and clathrin- and caveolin-independent pathways, and also provides a list of receptors based on the internalization pathway. Targeted delivery of drugs to subcellular organelles is also discussed. The discussion on the impact of nanocarrier properties on cellular internalization of nanocarriers throws light on factors to be addressed during nanoparticle design. This chapter thereby enables a comprehensive understanding of intracellular uptake in the context of targeted drug delivery.

Keywords Clathrin · Caveolin · Endosome · Intracellular delivery · Phagocytosis · Pinocytosis · Receptor-mediated endocytosis

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Abbreviations

AP2	Activating protein 2
BSA	Bovine serum albumin
CALM	Clathrin assembly lymphoid myeloid leukemia protein
CAM	Cell adhesion molecule
CavME	Caveolae-mediated endocytosis
Cdc42	Cell division control protein 42 homolog
CDR	Circular dorsal ruffle
CLIC	Clathrin-independent tubulovesicular carriers
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
CTX	Cholera toxin
Dia2	Dynamin 2
DOX	Doxorubicin
DRF	Diaphanous-related formins
EEA1	Early endosome antigen 1
EGF	Endothelial growth factor
EPR	Enhanced permeation and retention
EPS15	Epidermal growth factor receptor pathway substrate 15
ESCRT	Endosomal sorting complex required for transport
FA-M-β-CD	Folate-appended methyl-β cyclodextrin
FCH	Fer/cdc42 interacting protein 4 (CIP4) homology
FcR	Fc receptors
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEEC-GPI	AP-enriched early endosomal compartments
GEF	Guanine nucleotide exchange factor
GRAF1	GTPase regulator associated with focal adhesion kinase 1
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HUVEC	Human umbilical vascular endothelial cells
Ig	Immunoglobulin
IHME	The Institute of Health Metrics and Evaluation
ITAM	Immunoreceptor tyrosine-based activation
LAMP	Lysosomal-associated membrane proteins
LDL	Low-density lipoprotein
MMP	Matrix metalloproteinase
NCF	1-Neutrophil cytosol factor 1/p47-phox
NCF-2	Neutrophil cytosol factor 2/p67-phox
NCF-4	Neutrophil cytosol factor 4/p40-phox
NO^-	Nitric oxide radicals
NOS2	Nitric oxide synthase 2
NRAMP-1	Natural resistance-associated macrophage protein 1

PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PIP2	Phosphatidyinositol-3,4-bisphosphonate
PIP3	Phosphatidylinositol-3,4,5-phosphate
PLGA	Poly(lactic-co-glycolic)acid
Rac1	Ras-related C3 toxin protein
RBC	Red blood cell
RES	Reticuloendothelial system
RILP	Rab interacting lysosomal protein
RME	Receptor-mediated endocytosis
ROS	Reactive oxygen species
RTK	Rho tyrosine kinase
SMTP	Spontaneous membrane translocating peptide
SV40	Simian virus 40
Syk	Spleen-associated tyrosine kinase
TGF	Tumor growth factor
TPP	Triphosphonium ion
V-ATPase	Vacuolar type H^+-ATPase
Vpu	Viral protein U
VSP	Vegetative storage protein
WASP	Wiskott-Aldrich syndrome protein
WHO	World Health Organization

1 Introduction

Intracellular drug delivery construes a vibrant thrust area of research, especially for cancer and intracellular infections, which dictate the need for high intracellular drug concentration for effective therapy. Cancer and infectious diseases are rampant and associated with high morbidity and mortality rates worldwide. The Institute of Health Metrics and Evaluation (IHME) recorded 42 million cases of cancer globally with 8.9 million deaths in 2017. The top echelon of cancer types include breast cancer, colon and rectal cancer, prostate cancer, and lung cancer as per 2016 statistics [1]. Similarly, infectious diseases, and in particular intracellular infections, continue to be a leading cause of deaths across the globe. The beginning of twenty-first century has witnessed the evolution from multiple drug-resistant infections to totally drug-resistant infections signifying immense threat to life. Around 95% of deaths caused due to infectious diseases were from low- and middle-income countries [2].

A number of anticancer and antiinfective agents including small molecule drugs as well as large molecules like DNA, siRNA, miRNA, proteins, and peptides exert their therapeutic effect by acting on targets located intracellularly. For instance, most of the anticancer drugs target the central dogma of molecular biology, which includes DNA to RNA to protein synthesis occurring intracellularly within the

nucleus, mitochondria as target for proapoptotic drugs, and lysosome as target for lysosomal enzymes [3, 4]. Similarly, antiinfective drugs have their targets located intracellularly like the malarial parasite in red blood cells, HIV in CD4 T-cells, and mycobacterium tuberculosis in alveolar macrophages [5].

Intracellular delivery of drugs could have far-reaching effects especially for treatment of cancer and infectious diseases by providing quantum improvement in efficacy with simultaneous diminution in toxicity. This chapter presents an overview of the various pathways of cell internalization and important considerations in effecting intracellular delivery including delivery to subcellular sites. A major focus, however, is on the receptor-mediated endocytic pathways of intracellular delivery.

2 Cellular Internalization Pathways

Cellular internalization is achieved by endocytosis, a process enabling substances to enter cells. The general method adopted by the living system involves surrounding the substance to be internalized by a region of the cell membrane which is then pinched off inside the cell to form a vesicle containing the ingested material. The fate of the vesicle and its contents is decided by the endocytic pathway involved. Two major endocytic pathways are delineated: phagocytosis or cell eating, and pinocytosis or cell drinking, and both could be considered as forms of active transport. In phagocytosis or "cellular eating," the cell plasma membrane surrounds a macromolecule or even an entire cell from the extracellular environment and buds off to form a food vacuole or phagosome inside the cell.

Pinocytosis includes two pathways, namely, fluid-phase uptake and receptor-mediated endocytic uptake. The two major mechanisms of fluid-phase uptake are micropinocytosis and macropinocytosis [6]. Receptor-mediated endocytosis (RME) is a more selective process which is initiated when a cell surface receptor recognizes a particular moiety. Such uptake is responsible for intracellular delivery of a large number of nutrients into cells, classic examples being folic acid and iron mediated through the folate and transferrin receptors, respectively [7]. The various uptake pathways are elucidated in the following text.

2.1 Macropinocytosis and Micropinocytosis

Macropinocytosis is an actin-driven endocytic mechanism wherein extracellular fluid and cell debris are nonspecifically internalized by large heterogenous membrane ruffles. These ruffles collide into the plasma membrane to form vesicles called macropinosomes in the plasma membrane [8–10]. Activation occurs when the cell membrane is subjected to surface ruffling, or structural modifications, wherein the physical stimulation results in vesicle formation and their subsequent internalization. Trafficking of the macropinosome into the cell involves Ras and PIP3 signaling.

Ras activates PIP3 once ligand binds to the plasma membrane, leading to the formation of membrane projection or ruffles, which due to actin polymerization close to form macropinosomes that vary in size between 1 μm and 5 μm. The PIP3 converts to phosphatidylinositol-3,4-bisphosphophonate (PIP2) after closure of the vesicle, which upon vesicle detachment converts to the monophosphate form (PIP) [11]. Macropinocytosis is a receptor-independent process triggered by antigen-presenting dendritic cells and is a major endocytic pathway in epithelial and tumor cells. It is also considered as a major endocytic pathway for gene and drug delivery using cell-penetrating peptides (CPP) which mainly comprise positively charged lysine and arginine-rich molecules. Interaction of positively charged CPP with the negatively charged plasma membrane for long duration culminates in internalization by macropinocytosis [12]. Macropinocytosis utilized by cancer cells for uptake of nutrients by Ras activation pathway has been relied on for delivery of siRNA into cancer cells [13].

Internalization through circular dorsal ruffles (CDR) is also a form of macropinocytosis. CDR are membrane protrusions which appear at the cell periphery or dorsal surface of cell. This pathway is widely used for tyrosine kinase receptor trafficking which is involved in cell growth, mitosis, motility, and invasion [14]. It involves a membrane driven-wave to trigger endocytosis of cargo. The formation of CDR involves activation of RTK by various cargoes like EGF, PDGF, and hepatocyte growth factor (HGF) leading to RTK dimerization and phosphorylation of its cytoplasmic part creating docking site for Src-2 class protein. This triggers a protein cascade involving adaptor and kinase proteins leading to actin polymerization. Actin polymerization leads to membrane curvature by concentric movements. Further, curved protein like TUBA binds to dynamin and activate Bin-Amphiphysin-Rvs domain which senses and induces membrane curvature to form dorsal ruffles. CDR is initiated at the site where spontaneous curvature could be developed involving proteins from concave and convex class. For instance, TUBA is concave curved activator of N-WASP, while BAR protein is convex curved activator protein [15]. Other proteins involved in the cascade include GTPase, Rac Rab5, Ras, non-RTKs, c-Abl and c-Src, serine-threonine kinases and PIP3, MMP, Paxillin, Vincullin of cytoskeleton, kinases and adhesion class. Consequently, the CDR formed have the ability to clear off growth factor-activated membrane receptors within few minutes forming many endosomes containing CDR tubules which encompass more than one cargo [14, 16]. CDR differ from macropinocytosis in engulfment of specific cargo of tyrosine kinase class as opposed to macropinocytosis which generally encompass nonselective large fluid cargo [15]. Characteristic features of CDR include tyrosine kinase-induced membrane tubules activated by various growth factors, large amount of internalization up to 50–60% ligand-receptor complex of tyrosine kinase class, without formation of coated vesicles independent of clathrin, AP2, or caveolin within 15–20 minutes, with the site of ruffle formation similar to dynamin involved in clathrin-mediated endocytosis [14].

Micropinocytosis, on the other hand, enables internalization of small macromolecules (<0.2 μm in diameter) via both clathrin-coated and uncoated vesicles. Macropinocytosis, although less selective, is among the more efficient internalization

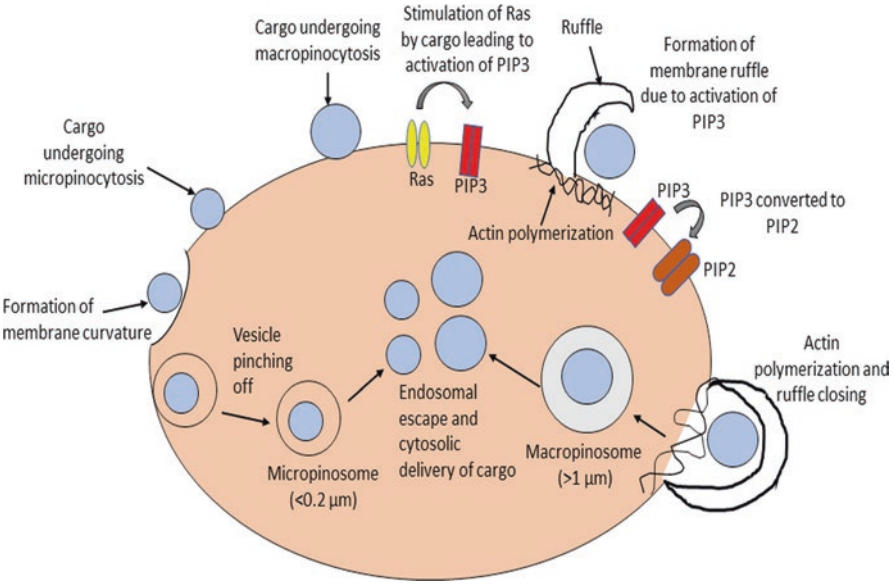

Fig. 1.1 Macropinocytosis and micropinocytosis

processes due to ingestion of larger molecules [17]. Cellular internalization by macropinocytosis and micropinocytosis is depicted in Fig.1.1.

Among the various endocytic pathways, targeted delivery employing nanocarriers, which is the central theme of this book, is achieved by two principle uptake mechanisms: phagocytosis and receptor-mediated endocytosis, which are dealt with in detail in the subsequent part of the chapter.

3 Phagocytosis

Phagocytosis or cell eating is a process exhibited majorly by special cells in the body which belong to the reticuloendothelial system (RES) called phagocytes which serve as the primary defense system of the body. Phagocytes are classified as "professional" and "nonprofessional"; however, only the professional phagocytes exhibit active involvement in phagocytosis. Professional phagocytes include the monocytes, macrophages, neutrophils, tissue dendritic cells, and mast cells. The nonprofessional phagocytes which comprise the epithelial cells, endothelial cells, fibroblasts, and mesenchymal cells participate in a limited manner and are therefore less important in the context of phagocytosis [18].

The process of phagocytosis occurs in distinct stages as described in the following text.

3.1 Opsonization

Adsorption of plasma proteins onto the surface of foreign particles to aid their recognition by opsonic receptors is termed as opsonization and the adsorbed proteins are called opsonins. This is the first step in the recognition of foreign particles by the host. Opsonization is a critical step which facilitates interaction of opsonin-coated foreign particles with receptors on the macrophage. The opsonins that play a role in phagocytosis are discussed in the following text.

3.1.1 Antibodies

Antibodies are generated in response to antigen trigger by B cells. Antibodies identified include immunoglobulin G and M class antibody, antitype II antibody, and heat-labile opsonins like 7S immunoglobulin [19, 20]. While the antibody Fab region exhibits binding to the antigen, the antibody Fc region exhibits binding to an Fc receptor on the phagocyte. Such binding facilitates phagocytosis [21]. Although immunoglobulin M (IgM) does not exhibit binding with Fc receptor on macrophages, it is considered as an opsonin since it activates the complement effectively [20].

3.1.2 Complement Proteins

The role of complement includes initiation of opsonization, inflammation, lysis, and antimicrobial action. Among the various complements, C3b, C4b, and C1q play an important role as opsonins. The complement cascade can be spontaneously activated as part of the alternative complement pathway to convert C3 to C3b, which then plays the role of an opsonin when adsorbed on an antigen [22]. Antibodies can also trigger activation of complement by the classical pathway to enable deposition of C3b and C4b on the surface of the antigen [23]. Once coated on the antigen, both C3b and C4b are recognized by complement receptor 1 which is expressed by all phagocytes on their surface. Unlike C3b and C4b, C1q interacts with a member of the C1 complex which exhibits interaction with the Fc region of antibodies and hence the Fc receptor [22].

3.1.3 Circulating Proteins

The secreted Pattern Recognition Receptors, namely the pentraxins [24], the collectins [25], and the ficolins [25] are also capable of functioning as opsonins. They can coat particles/microbes as opsonins and enhance the reactivity of neutrophils against them.

3.2 Recognition of Particles by Cell Surface Receptors

Recognition of particles is initiated by opsonic receptors which recognize the opsonins or host-derived soluble molecules that adsorb onto the surface of particles and help in their ingestion. In the absence of opsonization, phagocytosis is not triggered. Important opsonic receptors include F_c receptors (FcR) and the complement receptors (CR). F_c receptors bind to the F_c portion of IgG or IgA while complement receptor binds to the iCR3b [21]. F_c receptors follow "tethering and tickling" process, wherein few receptors bind to the ligand and "tether" it to the cell surface and the receptors "tickle" the cell and cause internalization, which ultimately leads to ingestion of particles [21]. For instance, immunoglobulin-G-coated antigen binds to immunoglobulin receptors which activate rearrangement of actin filaments leading to cell membrane distortion to form cup-shaped invagination [9]. Various complement receptors which enable recognition of ligands by phagocytes include CR1, CR4 which bind C3b, iC3b, C1q, and β-integrin [26].

3.3 Particle Internalization

Interaction of foreign particles with phagocytic receptors initiates a signal cascade which triggers actin polymerization and thereby facilitates particle internalization [27]. Following attachment to the phagocytic cell, polymerization of the actin cytoskeleton aids complete coverage of the particle by the plasma membrane to effect phagocytosis [28]. When IgG ligand binds to F_c receptor, Src family kinases are activated which phosphorylate immunoreceptor tyrosine-based activation (ITAM) motifs of F_c receptors. Actin nucleation complex Arp2/3 is regulated by GTPase Rac. Syk recruits enzyme phosphatidylinositol 3-kinase for regulation of lipid phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the phagocytic cup. Rac activation and contractile protein myosin are also regulated by PIP_3.

3.4 Phagosome Formation

Engulfing of foreign invasion/particles necessitates changes in the actin cytoskeleton and plasma membrane to form extensions called pseudopodia. Pseudopodium, also known as pseudopod, represents an arm-like projection of the cell membrane which is generated temporarily to aid coating and ingestion of particles by phagocytes. They are filled with cytoplasm and chiefly comprise actin filaments. They may, in addition, contain microtubules and other intermediate filaments. Complete coverage of the particles is important for phagocytosis. Pseudopodia formation is initiated by disruption of linear actin fibers present in the phagocytic plasma membrane by action of coronins (F-actin debranching proteins) and cofilin and gelsolin

(F-actin-severing proteins). Thereafter, F-actin polymerization takes place by Arp 2/3 protein complex to extend the pseudopodia around the particles. Subsequent depolymerization of actin filaments results in curving of the phagosome base membrane which is controlled by phosphatidylinositol 3-kinase [9, 28].

3.5 Phagolysosome Maturation

Phagosomes once inside the cell are transformed into phagolysosomes by fusion with lysosomes, to destroy foreign particles by the very acidic environment and degradative enzymes present in the lysosomes. The process of formation of phagolysosome is called phagolysosome maturation and occurs in four stages: early, intermediate, late, and ends with the phagolysosome formation which is the final stage.

In the early stage, phagosomes fuse with sorting and recycling endosomes to form early endosomes. The fusion process is controlled by GTPase Rab5 which functions through recruitment of several proteins, namely, early endosome antigen 1 (EEA1) and class III PI-3 K human vacuolar protein-sorting 34 [29]. Early endosomes become slightly acidic (pH 6.1–6.5) due to the gradual accumulation of V-ATPase onto the membrane surface [30]. The intermediate stage involves conversion of Rab5 present on the membrane to Rab7 which mediates fusion of the phagosomes with late endosomes. The membrane of the phagosomes now starts inward growth to form vesicles [31]. In the late stage, Rab7 recruits Rab-interacting lysosomal protein to mediate phagosome fusion with lysosomes [30]. During this stage, the phagosome lumen becomes more acidic (pH 5.5–6.0) due to the accumulation of more number of V-ATPase molecules on the membrane [30]. In addition, several other proteins including Rab-interacting lysosomal protein (RILP) which facilitates contact between phagosomes and lysosomes, lysosomal-associated membrane proteins (LAMPs) responsible for fusion of lysosomes with phagosomes, and luminal proteases are involved in the phagolysosome formation. The final stage relates to microbicidal activity of phagolysosomes which is affected by several mechanisms. Phagolysosomes are highly acidic (pH 4.5) due to the presence of a large number of V-ATPase molecules on the membrane. Low pH inside phagolysosomes due to translocation of protons (H^+) by V-ATPase in the lumen using cytosolic ATP is the most destructive antimicrobial effector. Acidic pH interferes with normal metabolic processes of microorganisms including uptake of essential nutrients and can also activate certain hydrolytic enzymes which enable destruction of microorganisms [32]. Presence of degradative enzymes (lipases, proteases, and lysozymes) and scavenger molecules (lactoferrin and NADPH) also contributes to the microbicidal action of phagolysosomes [33, 34].

Reactive oxygen and nitrogen species also contribute to the antimicrobial effect of phagolysosomes. Reactive oxygen species (ROS) is produced by NADPH oxidase by association of three cytosolic components: NCF-4 (neutrophil cytosol factor 4/p40-phox), NCF-1 (neutrophil cytosol factor 1/p47-phox), and NCF-2

(neutrophil cytosol factor 2/p67-phox). These ROS destroy biological macromolecules including proteins and nucleotides, leading to antimicrobial effect [35]. Furthermore, nitric oxide radicals ($NO^{\cdot}$) are generated by inducible nitric oxide synthase 2 (NOS2) after the conversion of L-arginine and oxygen into L-citrulline and $NONO^{\cdot}$ which react with O_2^- to form peroxynitrite ($ONOO^-$) which is ultimately responsible for destruction of proteins and nucleotides [35].

Elimination of essential nutrients from the phagosome sequestering molecules inside phagosomes or transporters onto the surface of membrane can also induce killing. For instance, depletion of divalent cations Fe^{2+} and Mn^{2+} by sequestering through lactoferrin, a nonheme Fe^{2+}-binding glycoprotein present in neutrophils, or transport of divalent cations (Fe^{2+}, Zn^{2+}, and Mn^{2+}) out of phagolysosomes by the transporter natural resistance-associated macrophage protein 1 (NRAMP-1) can also contribute to microorganism destruction [36]. Antimicrobial peptides, namely defensins and cathelicidins, present inside phagolysosomes also contribute to the antimicrobial effect [37]. Additionally, lysozyme and lysosomal hydrolases disrupt the bacterial cell wall by degrading peptidoglycan which is an essential cell wall component. Further, enzyme Phospholipase A2 can degrade anionic phospholipids such as phosphatidylglycerol, which are important cell membrane components, thereby leading to complete cell wall lyses [32].

3.6 Preventing Phagolysosomal Fusion

Phagocytosis is an important physiological process that can internalize nanoparticles loaded with therapeutic cargoes for macrophage targeting [38]. Nevertheless, the aggressive phagolysosomal environment can prove a serious impediment in ensuring drug stability. Approaches for prevention of phagolysosomal fusion and/or phagolysosomal maturation could be integrated during nanosystem design to maximize therapeutic benefits of macrophage targeting [39].

Shuttle system including proteins such as Wortmannin, Concanamycin-A exhibit the ability to prevent phagosomal maturation by interfering with PI3K, Rab, EEA1, and V-ATPase pathways. Further, they recruit digestive proteases which degrade the lysosome. Concancamycin-A is a V-ATPase inhibitor and hence can prevent acidification of the vesicle and eventually their maturation. Chloroquine, a basic compound, can also prevent phagolysosomal fusion by preventing acidification of the phagosome. Wortmannin, on the other hand, has the ability to interact with the PI3K pathway and inhibit phagolysosomal fusion due to C-20 lysine derivative. Designing nanoparticle with such established shuttle systems could prevent phagolysosomal escape [40]. The steps involved in phagocytosis are depicted in Fig. 1.2.

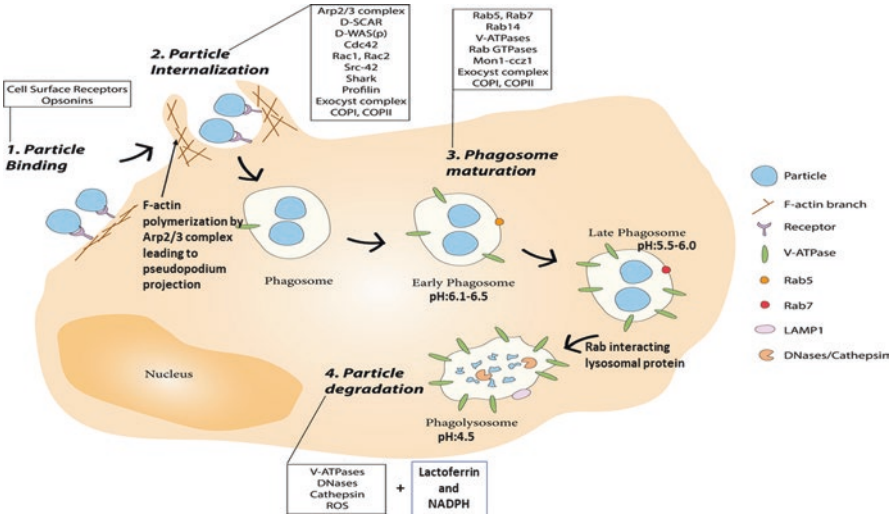

Fig. 1.2 Steps involved in phagocytosis. (Reproduced from ref. no. [41])

4 Receptor-Mediated Endocytosis (RME)

Receptor-mediated endocytosis is a selective process of cell internalization and necessitates recognition of a receptor on the cell membrane prior to internalization. In case of phagocytosis, opsonins serve as ligands recognized by the macrophages irrespective of the nanocarrier or the cargo. Receptors involved in RME express very specific recognition domains and hence exert a high degree of selectivity [42]. However, some receptors like the cytokine receptors may be more promiscuous than others. These receptors are expressed on cells to perform various cellular functions including delivery of nutrients to cells, for instance, folate [43] and transferrin receptors [44] or scavenging endogenous debris, for instance, the asialoglycoprotein receptor [45], or even protecting the body from virulent attacks by microorganisms, for example, mannose receptor, scavenger receptor [46], and toll-like receptors [47].

Unlike phagocytosis which follows a similar endocytic process irrespective of the macrophage involved or the cargo internalized, receptor-mediated endocytosis can follow different pathways. Furthermore, all receptors are not ubiquitous. Although receptors are expressed based on a physiological need, some of these receptors may be overexpressed in pathological conditions. Such overexpression is exploited for targeted delivery of actives into specific cells mediated through these receptors [48]. As a general example, cancer cells overexpress a number of receptors and the same have been widely explored for targeting drugs intracellularly. Activated macrophages also overexpress certain receptors which could also serve as targets especially for targeted therapy of infections.

A large number of receptors have been explored for targeted drug delivery and more receptors are being discovered and recruited for such purposes. Receptors that can contribute significantly for therapeutic advancement are discussed in detail in the subsequent chapters of the book. Each chapter includes an in-depth discussion of the receptor physiology, the domains for ligand attachment, various possible ligands, and targeted drug delivery strategies employed including those that have reached clinical trials.

An important purpose of this chapter is to provide an insight into the various different pathways of receptor-mediated uptake exhibited by cells. The chapter would also throw light on factors that influence such uptake. Targeted drug-delivery strategies employed for efficiently harnessing such uptake for improved therapeutic goals are discussed separately for each receptor in the respective chapter.

4.1 Internalization by RME

RME is adapted by the body for intracellular delivery of various endogenous compounds and nutrients, including cholesterol, iron, vitamin B_{12}, insulin, and epidermal growth factor [7]. Different processes may, however, be followed depending on the receptor under consideration. The general steps, irrespective of the pathway, include binding of proteins to specific cell surface receptor, internalization of the receptor-ligand complex into a vesicle and intracellular trafficking of the receptor-ligand complex to the lysosome or other subcellular organelles [6]. The intake mechanism is determined by the receptor constitution which dictates the pathway based on the various proteins involved in vesicle formation [49]. Nevertheless, intracellular trafficking can be altered through nanocarrier design offering opportunity for tailored intracellular delivery. The various RME uptake pathways are detailed in the following text.

4.1.1 Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis is an important endocytic pathway for cellular internalization in multicellular organisms. It involves internalization of receptors which regulate neurotransmission, signal transduction, cellular homeostasis, growth differentiation, growth control, synaptic transmission, and regulation of plasma membrane activities [50]. Several essential nutrients are also internalized by clathrin-mediated endocytosis. For instance, cholesterol enters into cells as low-density lipoprotein (LDL) via LDL receptor or iron enters into cells via transferrin receptors.

4.1.2 Mechanism of Clathrin-Mediated Endocytosis

The mechanism of clathrin-mediated endocytosis is initiated when binding of a ligand to receptors on the cell membrane triggers clustering of the pioneer proteins (AP2, CALM, FCH01, and EPS15) and adaptor proteins located on the cellular membrane. Thereafter, these interact with a few cellular membrane lipids to form a coat called as "clathrin-coated pits." Clathrin is a three-legged structure known as triskelion which is formed by association of three clathrin heavy chains and three clathrin light chains. The clathrin triskelions associate together in pentagonal and hexagonal fashion with 12 pentagons and 4 hexagons to form an icosahedral cage-like structure [51]. The loading of cargo into the clathrin-coated pits involves a "cargo check point mechanism" which initiates the process of formation of clathrin vesicles only if a specific number of cargoes interact with the cell membrane, else the process is aborted. Membrane bending is essential during endocytosis for the pit to covert to bud following which scission proteins including emphiphysin, endophilin, sorting nexin 9 of BAR domain, and dynamin, with the help of actin polymerization, excise the pits to form clathrin-coated vesicles which are trafficked within the cells [52]. Dissociation of the receptor-ligand complex releases clathrin to form an early endosome, which is mediated by adaptor proteins present in clathrin. These adaptor proteins function in anterograde and retrograde directions between transmembrane Golgi apparatus and endosome [53]. The early endosome comprises microtubules, microfilaments, and other molecular motor proteins and V-ATPase generating protons, thereby exhibiting acidic pH in the range of 5.5–6. The early endosome carrier vesicle detaches from early endosome to form the late endosome with gradual decrease in pH to <5.3 providing favorable environment for acid hydrolase (lysozyme). Fusion of the late endosome with lysosomes budded from trans-Golgi-apparatus results in the formation of the endolysosome which provides a harsh environment of enzymes and low pH [54]. Depending upon the receptor type, the receptor endocytosed in endosome can be recycled back to the plasma membrane for homeostasis or could be directed toward lysosomes. For example, LDL receptors are recycled back to plasma membrane and insulin or epidermal growth factor receptors are subjected to lysosomal degradation [49, 55].

Low pH of late endosome leads to dissociation of ligand from the receptor. Further, it leads to the formation of recycling tubules containing receptors to be recycled either to the plasma membrane or delivered to trans-Golgi network. Collection of recycling tubule forms endosomal recycling compartment. Thereafter, the late endosome fuses with the lysosome wherein the cargo is lysed to obtain various components for cell processing [56]. For example, low-density lipoproteins are broken down to cholesterol, amino acid, and fatty acids which are required for maintaining cell function. Figure 1.3 is a schematic representation of clathrin-mediated endocytosis by LDL receptor.

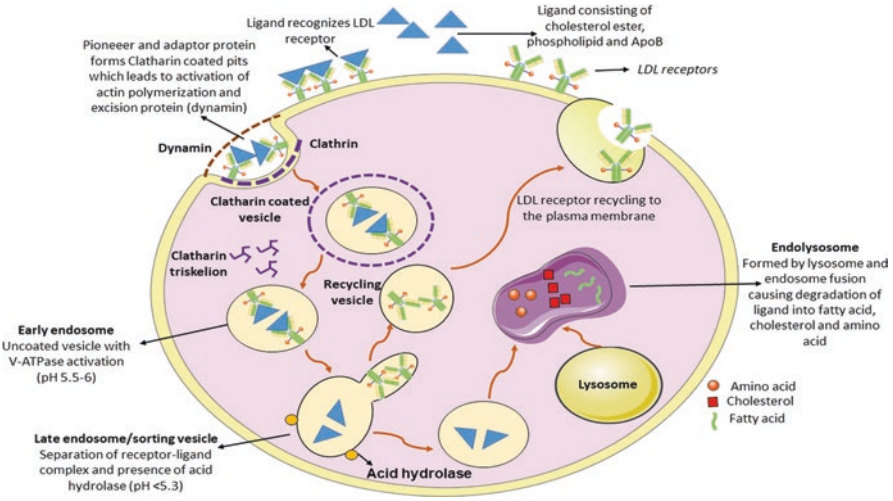

Fig. 1.3 Clathrin-mediated endocytosis by LDL receptors

4.1.3 Caveolae-Mediated Endocytosis

Caveolae-mediated endocytosis is considered the second most important cellular internalization pathway which also plays a key role in regulating cell signaling, lipid regulation, and vesicular transport [51]. Among the receptors that have been evaluated for targeted drug delivery, the folate, endothelial growth factor, and vascular endothelial growth factor receptor are among those that exhibit caveolae-mediated endocytosis. Albumin as well as pathogens like ganglioside-bound cholera toxin, SV40 virus, CTX, polyomavirus, HIV, etc. are internalized via caveolae-mediated endocytosis and transcytosis [54, 57].

Caveolae, the site for caveolae-mediated endocytosis, are flask-shaped membrane invaginations of 60–80 nm and 10–50 nm base and neck diameter, respectively. They are also responsible for vesicle formation and lipid-raft stabilization. Caveolae-mediated endocytosis is initiated by interaction of ligands with receptor domains present abundantly in caveolae. Src-kinase-induced phosphorylation of Caveolin-1, a member of the caveolin protein family, initiates formation of caveolae. The subsequent signaling cascade involves oligomerization of Caveolin-1 with Caveolin-2, aided by actin filaments leading to formation of a caveosome which is mediated by the recruitment of dynamin to the caveolae. Cargoes are then delivered into the cytosol and the actin filaments are recycled back to the plasma membrane. Unlike the clathrin-mediated pathway which goes through stages of the early and late endosome and fusion with the lysosome, delivery of cargo by the caveosomes in the cytosol permits their subcellular accumulation either in endoplasmic reticulum or Golgi bodies. Targeting to the mitochondria or nucleus is also facilitated. Caveloae-mediated uptake through the folate receptor is depicted in Fig. 1.4. The folic acid released from the caveosome is polyglutamated and stored inside the cell.

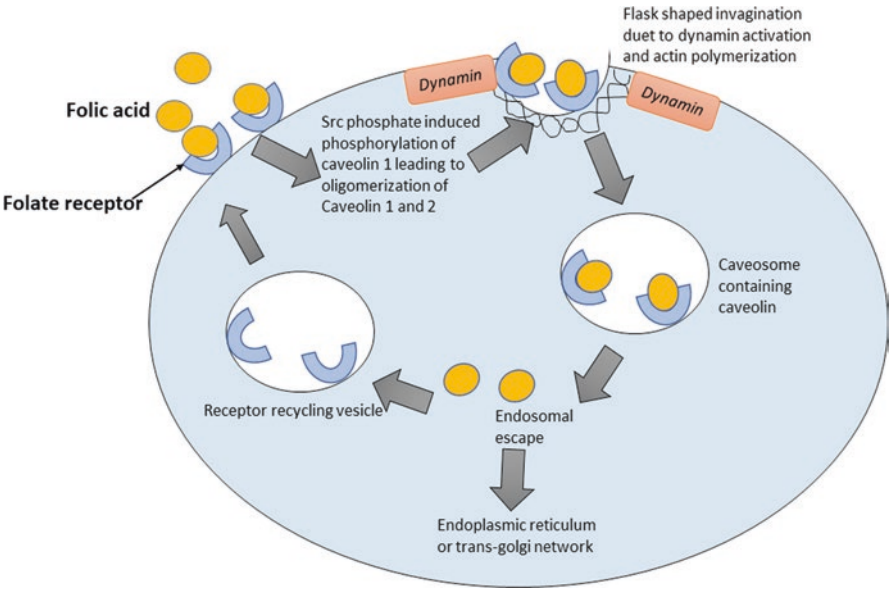

Fig. 1.4 Caveolae-mediated endocytosis of folate receptor

Molecular proteins directing the caveosomes to either endoplasmic reticulum or Golgi bodies are either present during the formation of caveosome or appear after its formation [54, 58]. Caveolae-mediated endocytosis avoids the conventional endosomal and endolysosomal degradation. This pathway is therefore considered most suitable for intracellular delivery of drugs.

4.1.4 Clathrin- and Caveolin-Independent Endocytosis

Clathrin- and caveolin-independent endocytosis may be constitutive or triggered by various signals that do not utilize either clathrin or caveolin for endocytosis or their molecular machinery involved. Both dynamin-dependent and dynamin-independent pathways may be involved. Various clathrin- and caveolin-independent pathways are described briefly in this section.

Flotillin-Mediated Endocytosis

Flotillin-mediated endocytosis is a clathrin-independent cellular internalization pathway which highlights the role of plasma membrane microdomains comprising of flotillin proteins in endocytosis. The mechanism involves formation of caveolae-like microdomains with dominant presence of flotillin-1 and flotillin-2. Flotillin-mediated endocytosis mechanism further involves oligomerization of

membrane-bound proteins flotillin-1 and flotillin-2 which forms mobile microdomains in the plasma membrane and activates immunoreceptors initiating endocytosis which further buds into the cell. These microdomains are sensitive to ligands linked to GPI-anchored proteins. Endocytic trigger by flotillin microdomains is regulated by Fyn-mediated phosphorylation and Src tyrosine kinases [59, 60]. Flotillin aids in regulation of many physiological processes including cell adhesion, endocytosis, signal transduction, regulation of plasma cholesterol, uptake of dopamine and glutamate transporters, and neutrophil migration by interaction with cortical cytoskeleton [61, 62].

Since flotillin-dependent pathway is initiated via lipid-raft, highly hydrophobic and charged moieties are endocytosed via flotillin-mediated endocytosis. Nonviral DNA polyplexes generally undergo flotillin-dependent endocytosis via charged interaction between cationic polyplexes and anionic plasma membrane [63].

RhoGTPase-Dependent Pathway

RhoGTPase belongs to the RasGTPase superfamily which regulates a variety of cellular processes including vesicle formation, apoptosis, viral transport, and cellular transport. Three well-known Rho family members include cdc42, RhoA, and Rac-1 [64]. Regulation of GTPase activity involves various proteins like guanine nucleotide exchange factor (GEF), GTPase activating protein (GAP), and guanine nucleotide dissociation inhibitor (GDI) (Fig. 1.5). Activation of GTPase acts downstream to initiate different cellular protein activation [64]. RhoGTPase triggers actin polymerization by activating the WASP/WAVE (Wiskott-Aldrich syndrome protein) and/or WASP family (Verproline homologous protein), Diaphanous-related formins (DRF), and LIM-K-initiated coffillins. Actin polymerization leads to the formation of membrane vesicles called lamellipodia which are sheet-like protrusions formed by network of actin filaments and filopodia or finger-like protrusions formed by parallel actin filaments. Rho-A has always been associated with lamellipodia and cdc42 with filopodia [65]. This pathway enables delivery of cargo into the cytosol permitting their subcellular accumulation either in endoplasmic reticulum or in Golgi bodies.

Reports have established that the cellular transmission and infection of HIV-1 may occur through Rho-A-dependent pathway which is applicable for natural substances like cholesterol. For instance, virus-infected cells lead to activation of Rho-A which in turn activates Dia2 which binds to microtubules and activates their dynamics leading to transfer of the virus to the nucleus [64]. Rho-A deactivation or protein isoprenylation blockers like statins may therefore delay viral infection and propagation [66]. HIV-1 fusion domain-derived CPP containing large arginine groups like TAT and MPG-α could trigger intracellular delivery of cargoes [67].

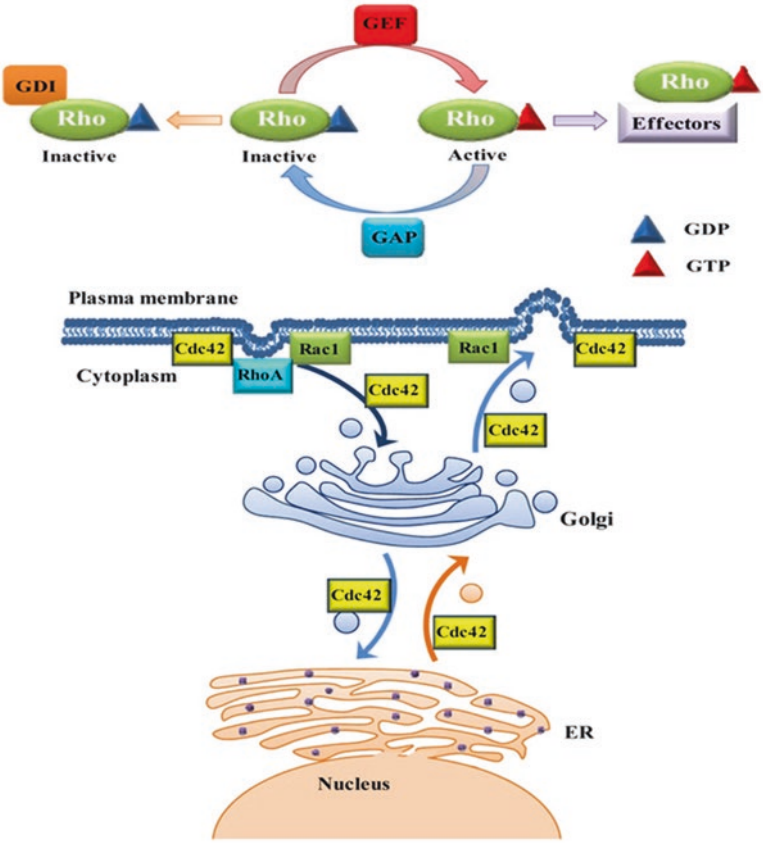

Fig. 1.5 (**a**) Role of GEF, GAP, and GDI in cell signaling. (**b**) Various Rho family members involved in intracellular trafficking to endoplasmic reticulum and Golgi apparatus. (Reproduced from [64])

CLIC/GEEC and Arf6 Endocytic Pathway

CLIC/GEEC pathway involves glycosylphosphatidylinositol anchored protein (GPI-AP) which undergoes fluid-phase endocytosis via dynamin-independent pathway. The GPI-AP fuses with early endosome which are termed as GPI-AP-enriched early endosomal compartments (GEEC) or clathrin-independent tubulovesicular carriers (CLIC) endocytosis pathway. This pathway is insensitive to amilioride and is constitutive unlike macropinocytosis [68]. CLIC involves various molecular machinery including GTPase regulator associated with focal adhesion kinase 1 (GRAF1) and guanine nucleotide exchange factor (GEF) for Arf1, RhoGAP, and RhoGTPase Cdc42. CLIC-mediated endocytosis was reported to be sensitive to concentration of membrane cholesterol. Further, several inherent proteins including CD44, dysferlin, GPI-bound proteins including cell adhesion molecule (CAM),

Table 1.1 Summary of various endocytic pathways

	Characteristics		
Endocytic pathways	Vesicles	Vesicle size	Intracellular fate of cargo
Phagocytosis	Phagosome	0.5–10 μm	Lysosomal degradation
Clathrin-mediated endocytosis	Clathrin-coated vesicle	60–300 nm	Lysosomal degradation
Caveolae-mediated endocytosis	Caveosome	60–80 nm could be enlarged	Lysosomal bypass and delivery to endoplasmic reticulum, golgi apparatus, and nucleus
Clathrin and caveolae independent	Lamellipodia, filopodia, clathrin-independent tubulovesicular carrier	90 nm	Lysosomal bypass and delivery to endoplasmic reticulum, golgi apparatus, and nucleus
Macropinocytosis	Macropinosome	1–5 μm	Lysosomal degradation

CD48, CD14, and folate receptors are endocytosed via this pathway [69]. Thus, ligands specific to GPI-anchored protein and cholesterol-functionalized nanoparticles could be utilized for intracellular delivery via this pathway. For instance, folate-appended methyl-β cyclodextrin (FA-M-β-CD) was developed as a novel anticancer agent for intracellular delivery via CLIC/GEEC pathway in folate-overexpressing cancer cells [70].

While Arf6 is a protein present on cell surface or in some cases on endosomes of clathrin-independent cargoes, many inherent proteins like GP-anchored proteins, nutrient transport protein (GLUT, CD98, LAT1), and proteins responsible for immune function undergo endocytosis via this route. The Arf6 endosome ultimately fuses with Rab-5 positive sorting endosomes which may either be degraded or recycled. Due to its resemblance to CLIC/GEEC pathway, it is considered as a part of this pathway [71].

A brief summary of different endocytic pathways is depicted in Table 1.1.

Table 1.2 depicts the endocytic pathway exhibited by receptors. As covering all receptors is beyond the scope of this book, the list is restricted to the receptors discussed in various chapters of this book.

5 Intracellular Drug Delivery to Subcellular Compartments

Delivery of actives in the cytosol following receptor-mediated endocytic pathways can be challenging, especially if the actives are fragile molecules like siRNA, genes, plasmids, peptides, and proteins. Delivery of molecules that exhibit limited stability in the acidic endosomal and lysosomal pH environment, or are degraded by lysosomal enzymes, is also challenging. For therapeutics that need to localize in specific cellular compartments to elicit activity, specialized approaches may be required. Such approaches are detailed in this section.

Table 1.2 Endocytic pathways and receptors

Endocytic pathways	Receptors
Phagocytosis	Complement receptors
	Dectin-1
	Fc receptors
	IgG receptor FcγRI CD64
	Integrin
	Mannose
	Scavenger receptor, MARCO
	Toll-like receptor
Clathrin-mediated endocytosis	Endothelial growth factor (EGF)
	Estrogen, progesterone, and androgen
	Gonadotropin-releasing hormone
	Insulin-like growth factor
	LDL
	Mannose
	Peroxisome proliferator activated receptors (PPAR)
Caveolae-mediated endocytosis	Adrenergic
	EGFR
	Folate
	Insulin-like growth factor
	Interleukin-2
	Platelet-derived growth factor
	Vascular endothelial growth factor (VEGFR)
Clathrin and caveolae independent	Dopamine
	EGFR
	Integrin
	Thrombin-mediated GPCR
	Transferrin
	Tyrosine kinase

5.1 Cytosolic Delivery

Cytosolic delivery includes delivery of therapeutics to the mild cytoplasmic environment of the cell, particularly for acid- and enzyme-labile drugs. A large number of intracellular infections harbor pathogenic organisms in the cytosol. Effective cytosolic delivery of therapeutic agents in active form is a vital need in such infections. The delivery of cargoes into the cytosol is challenging due to their high molecular weight, hydrophilicity, and endosomal uptake of macromolecules [72]. Methods to achieve cytosolic delivery are elucidated in the following text.

5.1.1 Cell-Penetrating Peptides

Cell-penetrating peptides (CPP) consist of short-chain amino acid sequences which facilitate cellular uptake of various cargoes into the cell due to large abundance of arginine or lysine or other charged or hydrophobic amino acids. Cell-specific peptide-drug conjugates, covalent nanoconjugates, and supercharged peptides for cytosolic delivery have been investigated [72, 73]. CPP can be either conjugated with the drug or over the surface of the nanoparticles encapsulating the drug for delivery into the cytoplasm [74]. pH-sensitive lysine-enriched STP peptide-conjugated doxorubicin (DOX) liposome showed enhanced drug concentration in cytoplasm. Furthermore, STP-conjugated DOX liposome revealed enhanced uptake at pH 5.8 as compared to unmodified DOX-loaded liposome. Uptake of STP-LS-DOX decreased in 293 T cells when the pH changed to 7.4 (Fig. 1.6) [75]. Similarly, Ritonavir-loaded Tat-conjugated lipid nanoparticles also exhibited cytoplasmic

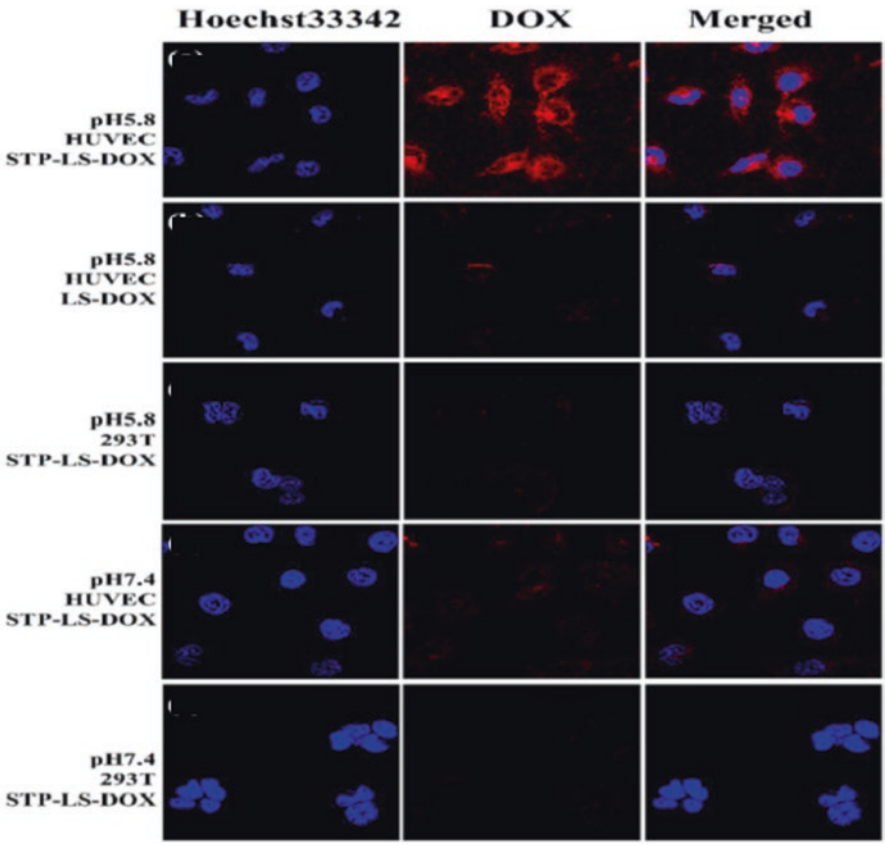

Fig. 1.6 Confocal microscopic image of STP-LS-DOX cell uptake studies in HUVEC and 293 T cells. Blue fluorescence represents Hoechst33342 dye for nucleus of cell while red represents Doxorubicin. (Reproduced from [75])

delivery in brain cells [76]. Cell-penetrating peptides including penetratin, arginyl-glycylaspartic acid (RGD), and octaarginine have also been reported for cytosolic delivery of therapeutic agents [77, 78].

5.1.2 Endolysosomal Escape

Most pathogens harbor safely in the cytosol due to endolysosomal escape or by preventing endolysosome formation. Evasion of endolysosomal fusion by delaying phagosome maturation, phagosome bursting, utilizing back fusion mechanism, and disturbing molecular machineries are some strategies adopted by the pathogens [79–82]. Pathogens including *L. monocytogenes* secrete cholesterol-dependent toxin listeriolysine (O) to form pores in the endosomal membrane. Inhibition of endolysosomal fusion by interfering with or inhibiting protein cascades including ESCRT-I, TGF101, and VPS class required for docking and tethering of endosome to lysosome is also reported [82]. Anthrax and VSV nucleocapsid prevented formation of multivesicular bodies at low pH by fusion of viral envelop with endosomal membrane and delivery of capsid/lethal edema factor respectively, into the cytoplasm to enable endolysosomal escape [79, 80]. Pathogens like *Brucella* and *Leishmania donovani* destroyed the lipid-raft of endosome which consists of various proteins involved in endosomal fusion. Secretion of β-1,2 glycan and GPI-anchored glycolipid lipophosphoglycan from *Brucella* and *L. Donovani*, respectively, led to disorganization of lipid raft and endolysosomal fusion leading to cytosolic delivery [81, 83]. *M. tuberculae* secrete mycobacterial phosphatase. Few pathogens inhibit the lipid-scaffolding protein called phosphoinositide. Secretion of mycobacterial acid phosphatase (SapM) and inositol phosphatase (SopB) by *M. tuberculae* and *S. enterica* respectively, inhibits phosphoinositide-2-phosphate and phosphoinositide-3,5-phosphate respectively, which interferes with endosomal protein sorting process. HIV when in multivesicular body hijacks the endosomal sorting complexes required for transport (ESCRT) protein complex and releases as exosome by fusion of the vesicle with cell membrane instead of meeting with the lysosome [84]. Moreover, pathogens including *M. tuberculosis*, *M. Leprae*, and HIV prevent phagolysosomal fusion by luminal acidification of phagosome and activation of lysosomal hydrolase by preventing the conversion of Rab family protein [85].

Drug-delivery strategies for endolysosomal escape try to mimic one or more of the natural mechanisms exhibited by pathogens by the approaches elucidated in the following text.

- *Fusogenic peptides*

Fusogenic peptides are hydrophobic glycine-rich amino-terminal peptides with either α- or β-conformation with an N-terminal fusogenic moiety and C-terminal helical membrane anchor [86]. Upon contact with the endosomal membrane, they change their conformation from energetically downhill to penetration and kinetically unfavorable fusion with the membrane-phospholipid bilayer, leading to dehy-

dration of lipid headgroup and possible pore formation in the membrane [87]. Fusogenic peptides, due to their membrane penetration coupled with binding ability to DNA, have been widely explored for gene delivery. Various lysine- and arginine-rich peptides with membrane destabilization property can be utilized as fusogenic peptides. Fusogenic hemagglutinin-derived peptides including DiINF 7, INF 6 or KALA, GALA, and JTS1 with amphiphilic and fusogenic property may lead to endolysosomal escape [88–90]. For instance, DiINF 7 which at low pH may induce membrane destabilization by conformational changes and protonation has been utilized for endolysosomal escape [88]. Sabrina Oliveira et al. conjugated fusogenic peptide diINF-7 to siRNA for improving its cellular uptake and cytosolic delivery [91].

- *Cationic ligands*

The membrane of late endosome is enriched with anionic bis(monoacylglycerol) phosphate. Cationic moieties like lipids and cell-penetrating peptides including TAT and penetratin enable membrane fusion due to ionic interaction. This coupled with enhanced permeation, leading to leaky membrane, permits cytosolic delivery [92]. Cationic lipid-based nanoparticles could bind with the anionic membrane of endosome, leading to enhanced cytosolic delivery by membrane destabilization [93]. For instance, cationic lipid (DLinKC2-DMA)-based siRNA nanoparticles led to effective GAPDH silencing in spleen and peritoneal cavity after intravenous administration [94]. Cationic lipoprotein-derived SLN incorporating PEG-c-MET siRNA could efficiently deliver siRNA to glioblastoma due to endosomal escape property of cationic SLN [95].

- *Pore-forming agents*

The formation of pore within the endosomal membrane depends on membrane tension which governs the pore opening and line tension which governs the closing of pore. Pore formation within the endosomal membrane follows the barrel-stave or toroidal models. Cationic amphiphilic peptides leads to increase in membrane tension by orienting in the lipidic membrane and forming stave which on aggregation forms a barrel-like structure perpendicular to the lipidic membrane to form a hole [96]. For instance, spontaneous membrane translocating peptide (SMTP) delivers fluorescent TAMRA dye to the cytosol by pore-forming property of the peptide [97], or frog-derived magainin peptide also possesses the pore-forming ability [98].

- *Proton sponge effect*

It involves inclusion of high buffering capacity substances with pKa lying between physiological and lysosomal acidic pH, wherein protonation leads to high inflow of ions and water resulting in bursting of the endosome causing cytosolic delivery. Tertiary amine-containing polyethylene imine, histidine-containing moieties, and polyamidoimine nanoparticles may be protonated due to amine groups [96]. For instance, PAMAM saporins led to enhanced cytotoxicity of saporins by 14- and 11-fold in Ca9-22 and KJ-1 cells, respectively, compared to free saporins due to proton sponge effect elicited by PAMAM enhancing cytosolic delivery of saporins [99].

5.1.3 Miscellaneous Approaches

Lipid nanocarriers exhibit cytosolic delivery of drugs by fusion of lipids with cell membrane via lipid-raft pathway [100]. For instance, $\alpha_v b_3$ integrin-linked liquid perflurocarbon emulsion showed deposition of nanoparticles on plasma membrane. Further, cell uptake studies in C32 cells under biochemical energy depletion (4 °C, in presence of filipin) revealed that the accumulation of nanoparticles on plasma membrane was maintained but the cytoplasmic delivery of drug was reduced [101]. Fusogenic lipids exhibited cytosolic delivery of drugs by fusion or destabilization of nanoparticles due to phase transition of lipids at acidic pH. Similarly, fusogenic peptides including KALA-, GALA-, and INF-7-conjugated nanoparticles enabled cytosolic delivery of drugs by endosomal destabilization of nanoparticles [74, 102]. Plasmid DNA-encapsulated phosphatidylcholine and phosphatidylethanolamine liposomes with different surface density of R8-arginine were internalized via macropinocytosis and avoided endosomal degradation [77]. Irregular-shaped nanocarriers have also shown enhanced cytosolic delivery of drugs via endosomal rupture. For instance, Zhiqin Chu et al. reported negatively charged prickly nanodiamonds (100 nm) of irregular shape for gene delivery to cytosol due to rapid rupturing of the endosomal membrane [103].

5.2 Mitochondrial Delivery

Mitochondria are also an important site for delivery of therapeutic agents in cancer and infectious diseases. Specialized anatomical mitochondrial features including highly hydrophobic and porous outer membrane, tightly packed hydrophobic internal mitochondrial membrane, and negative charge (−160 to −180 mV) due to the presence of cardiolipin and the electron transport chain provide opportunities for nanocarrier-based targeting to mitochondria. Triphosphonium ion (TPP, 10 μM) has been used as ligand for targeting to mitochondria due to its lipophilic and delocalized cationic charge. Similarly, dequlinium ion has also been reported for mitochondrial targeting. For instance, 4-carboxybutyl triphenylphosphonium bromide-conjugated glycolated micelles showed enhanced drug concentration in mitochondria of cancer cells [104]. Similarly, TPP-modified polyethyleneglycol (PEG) and poly-Ɛ-caprolactone micelles exhibited enhanced mitochondrial delivery of Gambogic acid in A549 cell line [105]. Small mitochondrial-penetrating peptides like gramicidin sequence, Szeto Schiller peptides, P11LRR, and octaarginine (R8) also served as mitochondria-targeting ligands. Mitochondrial-localizing sequence (MLS) peptide-conjugated PEGylated folic acid-decorated nanodiamonds (MLS-FA-PEG-ND) displayed mitochondrial delivery of DOX in cancer cells. These MLS-conjugated nanodiamonds exhibited endosomal escape to release nanodiamonds in the cytoplasm which was further delivered to mitochondria [106].

Several nanocarriers including liposomes [107], polymeric nanoparticles [108], and inorganic nanoparticles [109] have been investigated for mitochondrial targeting. For instance, DOX-loaded MITO-porter liposome containing 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), sphingomyelin (SM), and cholesterol (Chol) in 9:2:1 ratio 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and stearylated octarginine (STR-R8) localized in the mitochondria while free DOX and unconjugated DOX liposomes accumulated in the nucleus [110]. Dual-targeting triphenylphosphine- and folic acid-conjugated mesoporous silica nanoparticles for mitochondrial targeting exhibited selective uptake in the mitochondria of cancer cell lines. Further cell uptake was doubled when nanoparticles were conjugated with folic acid alone and triplicated when both folic acid and TPP were co-conjugated on mesoporous silica nanoparticles [111].

5.3 Nuclear Delivery

DNA and proteins present in the nuclear matrix are targets for many anticancer drugs. For instance, fludarabine phosphate which blocks DNA replication by inhibiting RNA primer and Okazaki fragments, VM-26 or m-AMSA specifically targets nuclear matrix-associated topoisomerase-II [112]. Integrase inhibitors like raltegravir, dolutegravir, and elvitegravir need to be translocated to the nucleus to elicit their anti-HIV effect. Therefore, selective nuclear delivery of these agents to diseased cells is the need of the hour. Several drug-delivery strategies employ virus-derived peptides including Tat and KKKRKV peptide conjugated to nanocarriers, lipoplexes, polyplexes, and pH-sensitive nanoparticles for nuclear targeting of nanocarriers [113]. Hexapeptide (anti-HIV peptide)-bound Tat-conjugated gold nanoparticles located in the cytoplasm and perinuclear region of TZM-b1 cells [114]. The ability of Tat-conjugated nanoparticles for nuclear drug delivery was found to be dependent on particle size, with nanoparticles (25–50 nm) exhibiting enhanced nuclear delivery. Tat peptide conjugated to MSN enhanced cellular uptake in HeLa cell lines compared to free DOX and DOX-MSN with enhanced nuclear delivery while free DOX and DOX-MSN were observed near the periphery [115]. Similarly, dual-targeting strategy was observed for hydrophobic HA2 membrane fusion peptide-grafted hydroxypropyl methacrylate (HPMA) copolymeric micelles of peptide H1-conjugated retinoic acid. Lysosomal destruction by HA2 peptide enabled cytosolic delivery of micelles, which were then directed to the nucleus due to retinoic acid [116]. 2,3-Dimethylmaleic anhydride (DA)-Tat peptide-decorated micelles of polyethylene glycol and poly-Ɛ-caprolactone displayed long circulation as the positive charge of Tat peptide was masked by dimethylmaleic anhydride. In the acidic environment of the cell, the negative charge was again converted to positive charge and micelles elicited nuclear uptake [117].

5.4 Endoplasmic Reticulum and Golgi Apparatus Targeting

The endoplasmic reticulum is a subcellular organelle which functions to maintain protein folding, homeostasis, and calcium balance. The Golgi apparatus is actively involved in protein trafficking of folded proteins [118, 119]. Many proteins of *M. hominis* are located in endoplasmic reticulum which can be targeted to inhibit initiation of cancer [120]. New anticancer drugs like Tunicamycin, Thapsigargin, and Brevefeldin A are known to regulate endoplasmic reticulum homoeostasis [121]. HIV-1 virus causes downregulation of CD4 receptors with the aid of Vpu protein and endoplasmic reticulum-associated degradation machinery [122]. Therefore, delivery of therapeutic agents specifically to endoplasmic reticulum and golgi apparatus along with other subcellular organelles like cytoplasm, nucleus, and mitochondria could be an important strategy in treatment of cancer and infectious diseases.

Various strategies including inorganic nanoparticles [123], surface-modified polymeric nanoparticles [124], and peptide drug conjugates [125] have been investigated for targeted drug delivery to endoplasmic reticulum and golgi apparatus. Meng Ya Chang et al. exploited gold nanoparticles coupled with radiation therapy as a strategy for treatment of cancer wherein inorganic gold nanoparticles were localized in the endoplasmic reticulum and golgi apparatus in B16F10 radiosensitized melanoma cells after 20 h [126]. Using small sequence peptide-conjugated nanocarriers is another important strategy which has been reported for endoplasmic reticulum and golgi apparatus targeting. For instance, peptide (KAAAAK)-decorated PLGA nanoparticles showed enhanced concentration in endoplasmic reticulum compared to unconjugated PLGA nanoparticles in DC2.4 cells [124]. Interleukin-2 (IL-24) conjugated to Tat peptide and endoplasmic reticulum-specific peptide (KDEL (lys-asp-glu-leu)) revealed enhanced cellular uptake as compared to IL-24 and Tat, wherein Tat peptide facilitated cellular entry and KDEL enabled enhanced endoplasmic reticulum concentration of interleukin in T24, MCF-7, H460, and NHLF cells. Furthermore, TAT-IL-24-KDEL was found to be present in endoplasmic reticulum after 12 h in cancer cells [125].

6 Physicochemical Properties of Nanocarriers on Cellular Internalization

Physicochemical properties of nanocarriers exert a huge impact on targeting to desired sites. Their in vivo disposition can be tailored for specific needs dictated by the targeting objective. The physicochemical properties can play an important role at every stage during the receptor-mediated endocytosis process. The various properties and their implications in targeted drug delivery are discussed in the following text.

6.1 Particle Size

Particle size exerts a strong impact on nanoparticle-cell interaction forces involved in the endocytosis. In case of non-specific adhesion of the nanoparticles to the cells, bending energy, adhesion energy, and membrane deformation energy play a significant role. Bending energy is independent of nanoparticle size, while adhesion energy and membrane deformation energy are strongly dependent on particle size and define the lower limit of size which is required for endocytosis. Nanoparticles below the radius of 5 nm cannot be internalized by endocytosis. However, such small particles could be endocytosed after agglomeration in clusters due to effective increase in size. On the other hand, upper size limit depends upon wrapping energy during the nanoparticle-cell interaction and could be of the same length scale of the cell, whereas, in case of specific nanoparticles-cell interaction, chemical energy released from ligand-receptor pair and receptor density bound to the nanoparticle surface is affected by the size. Some reports suggest that particle size of around 30–50 nm would be optimal for receptor-mediated endocytosis. Lower particle size nanoparticles (<30 nm) would lead to inefficient interaction with cell membrane receptors, while higher particle size (70–240 nm) would lead to inefficient membrane wrapping causing inefficient endocytosis [127].

Phagocytosis is enhanced upon increase in particle size of nanocarrier. Generally, particle size >500 nm is highly prone to phagocytosis [128]. Therefore, for targeting to tumor and other infectious sites, particle size of nanocarrier is carefully designed to avoid the reticuloendothelial system (RES) [129]. Although higher sized nanoparticles (>500 nm) are phagocytosed, the effectiveness decreases proportionally with increase in particle size. This could be correlated with the higher binding energy involved in nanoparticle and receptor binding as well its release [127].

Clathrin-mediated endocytosis is more prominent for nanoparticles <200 nm, while nanoparticles between 200 and 500 nm predominantly internalize via caveolae-mediated endocytosis. Therefore, 30–500 nm particle size is favorable for drug delivery to subcellular organelles except lysosome [130]. If the flexibility with respect to size of clathrin and caveolae is compared, caveolae can internalize nanoparticles with size greater than their own diameter but the uptake efficiency decreases with increase in particle size. For instance, it was observed that a number of BSA-coated nanoparticles per caveosome decreased in ascending order of particle size, namely 20 nm > 40 nm > 100 nm in bovine lung microvascular endothelial cells [57]. However, lower particle size aided in better penetration to subcellular organelles, including endoplasmic reticulum, golgi apparatus, and nucleus, while bigger size particles localized in the cytoplasm [131].

Further, although nanoparticles remain stable in buffered saline, they tend to aggregate in blood serum [132]. The interaction of aggregated nanoparticle with the cellular membrane depends on the cell type. For instance, transferrin-coated gold nanoparticles (26, 49 and 98 nm) when aggregated in presence of blood serum depicted 25% lower uptake compared to their non-aggregated counterparts in HeLa and A549 cells. However, in MDA-MB-435 cells, the uptake of 98 nm aggregated

nanoparticles was enhanced compared to non-aggregated. This is attributed to receptor-mediated uptake in HeLa and A549 cells and entry through other pathways in MDA-MB-435 cell lines [133].

In general, nanocarriers <100 nm are considered optimal for delivery in cancer and nanocarriers >200 nm for delivery to the RES.

6.2 Stealth

The innate defense system of the body is the RES or the mononuclear phagocytic system (MPS). Nanoparticles which enter into the systemic circulation are opsonized and recognized by the opsonic receptors present on the phagocytes leading to their phagocytosis [134, 135]. In order to escape RES, the nanoparticles may be surface-modified to increase their hydrophilicity and impart neutral charge. Such surface modification which enables nanocarriers to resemble water bodies and thereby escape RES detection is called stealth [134].

Polyethylene glycol (PEG) is among the first stealth agents successfully employed in the design of stealth liposomes that are currently marketed; for example, Doxil-PEGylated doxorubicin liposomes. The stealth property of coated nanoparticle depends on conformation, thickness, flexibility, and amphiphilic nature of PEG. PEG with conformation in between mushroom and brush-like appearance and coating thickness of 10% could impart stealth properties to nanoparticles [136]. However, PEGylation can lead to steric repulsion of opsonins to enhance the circulation time of nanoparticles in the blood. Limitations include highly polydisperse nature of PEG, anti-PEG immunological response [137], and inability to escape endolysosomal fusion [138]. The detection of the PPES syndrome [139], a severe toxicity, in patients raised numerous questions on the safety of PEG. Stealth agents as alternatives to PEG are listed in Table 1.3.

Table 1.3 Stealth agents for nanodrug delivery

	Agent	Reference
Hydrophilic polymers	Poloxamine polyoxazolines	[140]
	Poly(aminoacids)	[141]
	N-(2-hydroxypropylmethacrylamide)	[142]
	Polybetaines	[143]
	Poly (glycerols)	[144]
Polysaccharides	Dextran	[145]
	Heparin	[146]
	Chitosan	[147]
	Hyaluronic acid	[108]
	Pullulan	[148–150]
	Arabinogalactan	[151]
	Polysaccharide combinations	[152, 153]

In addition to imparting the stealth properties, heparin can inhibit angiogenesis, tumor growth, and metastasis, while cationic nature of chitosan could facilitate receptor-mediated endocytosis [154]. RBC-mimicking vesicles and exosomes have been investigated as next-generation stealth vehicles [155]. In a combination of chitosan and erythrocyte vesicles, the erythrocyte coat enabled longer circulation half-life and polymeric nanoparticles contributed to high drug loading [156]. Erythrocyte- coated polymeric nanoparticles as delivery vehicle to increase the residence time of the nanoparticles in the blood are called as CD47 self-marker camouflaging system.

Stealth property of nanocarriers coupled with small particle size could result in longer circulation of nanoparticles due to phagocytic escape, enabling access to tumor vasculature. Nanocarriers due to their small size can gain access to tumors through the leaky vasculature and be retained by the tumor due to "enhanced permeation and retention (EPR)" effect. Furthermore, stealth nanoparticles attached with ligands undergo receptor-mediated endocytosis due to the ligand. Ligands for specific receptors attached to PEG chain support an energetically favorable conformation during membrane bending, receptor binding, and internalization [157]. More importantly, imparting nanocarriers with stealth is a vital requirement to ensure their availability at the receptor site, especially for non-RES targeting using the receptor-mediated targeting approach.

6.3 Shape

Recently, particle shape, in addition to particle size and surface morphology, has been an avenue of interest for desired intracellular delivery [158] The particle-cell adhesion and strain energy for surface wrapping which determine cellular internalization are widely dependent on particle shape. Elongated nanoparticle can bind to the cell membrane in rocket position or submarine position, both of which require reorientation of the particle for complete wrapping and endocytosis. This requires more energy which is unfavorable. Hence, elongated nanoparticles are endocytosed to lesser extent compared to spherical nanoparticles [159]. Due to lesser strain energy required for internalization of nanodisc compared to nanorod, polyethylene glycol diacrylate nanodiscs were internalized in human umbilical vascular endothelial cells (HUVEC) up to 40–60% compared to nanorods [160]. Further, spherical gold nanoparticles of 74 and 14 nm were internalized more via receptor-mediated endocytosis compared to rod-shaped nanoparticles [161]. Cell uptake studies in MDM cell lines of NanoART, that is, crystalline indinavir, atazanavir, efavirenz, and ritonavir, showed enhanced uptake of polygonal spheroid as well as rod-shaped particles up to 8 h, which were retained in MDM cell lines until 15 days. Spherical-shaped indinavir nanosuspension and ritonavir thick rod were endocytosed within 4 hours while thin rods and ellipsoids of atazanavir and efavirenz took longer time up to 8 hours [162]. The differential uptake was correlated with the energetically

favorable membrane wrapping and endocytosis depending upon the shape and surface area.

6.4 Hydrophobicity

Hydrophobic nanoparticles can interact with and rearrange the cellular membrane lipids, thereby leading to their rapid cellular internalization. Hydrophilic nanoparticles, on the other hand, exhibit RES bypass due to incomplete wrapping of the cellular membrane over the nanoparticles due to the higher energy barrier compared to hydrophobic nanoparticles [163]. Furthermore, hydrophobic nanoparticles predominantly internalize via phagocytosis due to adsorption of opsonins which exhibit high affinity for phagocytic receptors [164]. Nevertheless, small hydrophobic nanocarriers could be leveraged to enable their penetration within the cell nucleus, as demonstrated with pH-sensitive siRNA diblock polymeric nanoparticles consisting of dimethyl aminoethyl methacrylate with hydrophobic core and self-assembling property [165]. Further, caveolae-mediated endocytosis is also enhanced with increase in hydrophobicity of nanoparticles. Polyelectrolyte-modified nanoparticles with poly(styrene sulfonate) (log P − 1.211) exhibited enhanced endocytosis compared to poly(acrylic acid) (Log P − 2.227) and poly(vinyl sulfonic acid) (Log P − 3.283) [166].

6.5 Surface Charge

Charge on nanoparticles can enhance their interaction with cell membranes and increase their cellular uptake [167], depending on the cell type [168, 169]. In a study, positively charged nanoparticles exhibited higher macrophage uptake as compared to neutral or negatively charged nanoparticles after opsonization with mouse serum [175]. Further, positively charged particles can demonstrate both phagocytic and nonphagocytic uptake into cell lines [170–173]. Further, protein adsorption studies of cerium oxide nanoparticles as a function of their zeta potential revealed lower protein adsorption on anionic nanoparticle compared to cationic, and hence enabling RES bypass and enhanced cellular uptake in lung cancer cells [174].

Negatively charged nanoparticles can also increase interaction with phagocyte cell membrane leading to increased phagocytosis [175]. Negatively charged nanoparticles internalize via scarce cationic sites on plasma membrane by nonspecific binding and clustering of nanoparticles by endocytosis [167]. In vivo biodistribution studies in mice bearing SKOV-7 human ovarian cancer xenograft revealed that negatively charged nanoparticles (+/− 0 to 17) were highly concentrated in cancer cells but highly negative or positive charged nanoparticles (> +/−17) were concentrated in the liver due to reticuloendothelial cell uptake [176].

6.6 *Elasticity*

Particle elasticity as a property for cellular internalization revealed that stiffer particles are easily phagocytosed compared to flexible nanoparticles. Therefore, flexible nanoparticle can exhibit long circulation enabling target-specific drug delivery [177]. Flexible nanoparticles would escape phagocytes and therefore provide great opportunity for targeted delivery in cancer therapy [178].

The summary of impact of physicochemical properties of nanocarriers on cellular internalization is presented in Table 1.4.

7 Conclusion

Targeted drug delivery is a promising field particularly in expanding the horizons of nanomedicine. Harnessing nanocarriers for specific targeted therapies can be achieved using the receptor-mediated endocytosis approach. A clear understanding of different approaches to deliver nanocarriers intracellularly, in particular the receptor-mediated approaches, were the major objective of this chapter. The subsequent chapters discuss various receptors associated with major cancers and infectious diseases and comprehensively address the receptor along with aspects important to relate the receptor to targeted delivery of drugs and drug-loaded nanocarriers. Through these chapters, the gamut of possibilities of targeted delivery via receptor-mediated endocytosis are elucidated.

Table 1.4 Impact on physicochemical properties of nanocarriers on cellular internalization

Property	Drug	Agent/nanosystem	Study outcome	Reference
Stealth	Polycationic DNA	PEGylated liposome	The liposomes escaped the phagocytes and accumulated in tumor vasculature with lower liver uptake	[179]
	Adriamycin	Biotinylated pullulan acetate self-assembled nanoparticles	The nanoparticles accumulated at the tumor site by EPR owing to small size (80 ± 46–123 ± 83 nm)	[180]
Shape	–	Rod-shaped gold nanoparticles	10- to 40-fold higher uptake in A549 and HeLa cancer cell lines	[161]
	–	TAT-peptide-functionalized gold nanostars	Enhanced anticancer effect in BT549 breast cancer cells following internalization via lipid-raft macropinocytosis pathway	[181]

(continued)

Table 1.4 (continued)

Property	Drug	Agent/nanosystem	Study outcome	Reference
Hydrophobicity	Zidovudine	Hydrophobic PLA nanoparticles	Efficient phagocytosis via polymorphonuclear leucocytes	[175]
	Pentenyl isoniazid and rifampicin	Hydrophobic PLGA nanoparticles	Sixfold increased therapeutic efficacy compared to free drugs	[182]
Surface charge	–	Anionic citrate surface-modified gold nanoparticles	Enhanced phagocytosis as compared to unmodified nanoparticles	[167]
	Rifampicin	PEI-coated cationic mesoporous silica nanoparticles	Enhanced efficacy against *Mycobacterium tuberculosis* in THP-1 cell lines attributed to the cationic nature of PEI which aided higher macrophage uptake	[170]
	–	Positively charged cholanic acid-modified glycol chitosan nanoparticles	Enhanced uptake in HeLa cells via both clathrin- and caveolae-mediated pathway	[171]
	–	Positively charged mesoporous silica nanoparticles surface-modified with N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride	Clathrin- and actin-dependent cellular uptake of nanoparticles in 3 T3-L1 and human mesenchymal cells	[172]
	–	Cerium oxide nanoparticles	Lower protein adsorption on anionic nanoparticle compared to cationic, enabling RES bypass and enhanced cellular uptake in lung cancer cells	[174]
Elasticity	–	Nanolipogel	Significantly enhanced tumor targeting in human breast cancer cells MDA-MB-231 and MCF-7 compared to the nonelastic counterpart	[178]

References

1. R MRHR. Cancer. 2018. [cited 2018 May 27]. Available from: https://ourworldindata.org/cancer.
2. Fact sheets: Infectious diseases. [cited 2018 May 27]. Available from: http://www.who.int/topics/infectious_diseases/factsheets/en/.
3. Karnofsky DA. Mechanism of action of anticancer drugs at a cellular level. CA Cancer J Clin. 2009;18(4):232–4.
4. Torchilin VP. Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. Annu Rev Biomed Eng. 2006;8:343–75.
5. Zazo H, Colino CI, Lanao JM. Current applications of nanoparticles in infectious diseases. J Control Release. 2016;224:86–102.
6. Iversen T, Skotland T, Sandvig K. Endocytosis and intracellular transport of nanoparticles : Present knowledge and need for future studies. Nano Today. 2011;6(2):176–85.
7. Goldstein J, Anderson R, Brown M. Receptor-mediated endocytosis and the cellular uptake of low density lipoprotein. In: Membrane recycling. Ciba: Pitman Books Ltd; 1982. p. 77–95.
8. Salatin S, Yari Khosroushahi A. Overviews on the cellular uptake mechanism of polysaccharide colloidal nanoparticles. J Cell Mol Med. 2017;21(9):1668–86.
9. Yameen B, Il CW, Vilos C, Swami A, Shi J, Farokhzad OC. Insight into nanoparticle cellular uptake and intracellular targeting. J Control Release. 2014;190:485–99.
10. Maruyama K. Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. Adv Drug Deliv Rev. 2011;63(3):161–9.
11. Bloomfield G, Kay RR. Uses and abuses of macropinocytosis. J Cell Sci. 2016;129:2697–705.
12. Jones AT. Macropinocytosis: Searching for an endocytic identity and role in the uptake of cell penetrating peptides. J Cell Mol Med. 2007;11(4):670–84.
13. Huang JL, Jiang G, Song QX, Gu X, Hu M, Wang XL, et al. Lipoprotein-biomimetic nanostructure enables efficient targeting delivery of siRNA to Ras-activated glioblastoma cells via macropinocytosis. Nat Commun. 2017;8(May):1–18.
14. Orth JD, Mcniven MA, Orth JD, Mcniven MA. Get off my back ! Rapid receptor internalization through circular dorsal ruffles. Cancer Res. 2006;66:11094–6.
15. Hoon J, Wong W, Koh C. Functions and regulation of circular dorsal ruffles. Mol Cell Biol. 2012;32(21):4246–57.
16. Peleg B, Disanza A, Scita G, Gov N. Propagating cell-membrane waves driven by curved activators of actin propagating cell-membrane waves driven by curved activators of actin polymerization. PLoS One. 2011;6(4):1–11.
17. Ekholm R, Ericson LE. The structural basis of thyroid function. In: Thyroid diseases: basic science, pathology, clinical and laboratory diagnoses. Boston: Butterworths; 1994. p. 17–51.
18. Rabinovitch M. Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 1995;5:85–7.
19. Kaminskas LM, Boyd BJ. Nanosized drug delivery vectors and the reticuloendothelial system. In: Intracellular delivery: fundamentals and applications, fundamental biomedical technologies. Dordrechts: Springer;2011.
20. Winkelstein JA. Opsonins: their function, identity, and clinical significance. J Pediatr. 1973;82(5):747–53.
21. David U, Goodridge H. Information processing during phagocytosis. Nat Rev Immunol. 2017;12(7):492–502.
22. Charles A Janeway, Jr, Paul Travers, Mark Walport and MJ Schlomchik. The complement system and innate immunity. In: Immunobiology: the immune system in health and disease 5th ed. New York: Garland Science; 2001.
23. Celik I, Stover C, Botto M, Thiel S, Tzima S, Walport M, et al. Role of the classical pathway of complement activation in experimentally induced polymicrobial peritonitis. Infect Immun. 2001;69(12):7304–9.
24. Moalli F, Jaillon S, Inforzato A, Sironi M, Bottazzi B, Mantovani A, et al. Pathogen recognition by the long pentraxin PTX3. J Biomed Biotechnol. 2011;2011:1–15.

25. Lu J, Teh C, Kishore U, Reid KBM. Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochim Biophys Acta Gen Subj. 2002;1572:387–400.
26. Frank MM, Fries LF. Complement interactions and functions. Immunol Today. 1991;12(9):322–6.
27. Park H, Chan MM, Iritani BM. Hem-1: putting the '" WAVE "' into actin polymerization during an immune response. FEBS Lett. 2010;584:4923–32.
28. Gordon S. Phagocytosis: an immunobiological process. Immun Rev. 2016;44(3):463–75.
29. Jovic M, Sharma M, Rahajeng J, Caplan S. The early endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol. 2010;25(1):99–112.
30. Guerra F, Bucci C. Multiple Roles of the Small GTPase Rab7. Cells. 2016;5(34):1–28.
31. Vieira OV, Bucci C, Harrison RE, Trimble WS, Lanzetti L, Gruenberg J, et al. Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. Mol Cell Biol. 2003;23(7):2501–14.
32. Rosales C, Querol EU. Control of phagocytosis by microbial pathogens. Front Immunol. 2017;8:1–23.
33. Flannagan RS, Cosío G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol. 2009;7(May):355–66.
34. Rosales C, Uribe-querol E. Phagocytosis: a fundamental process in immunity.BioMed Research International. 2017;2017:1–18.
35. Slauch JM. How does the oxidative burst of macrophages kill bacteria? Still an open question. Mol Microbiol. 2012;80(3):580–3.
36. Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015;264:182–203.
37. Lehrer RI, Bevins CL. Defensins and other proteins. In: Mucosale barrier: development and physiology of mucosal defense; 2003. p. 95–110.
38. Ponzoni M, Pastorino F. Targeting macrophages as a potential therapeutic intervention: impact on inflammatory diseases and cancer. Int J Mol Sci. 2018;19:1–17.
39. Visser JG, Du A, Van Staden P, Smith C. Harnessing macrophages for controlled-release drug delivery: lessons from microbes. Front Pharmacol. 2019;10:1–18.
40. Visser JG, Smith C. Development of transendothelial shuttle by macrophage modification. J Tissue Eng Regen Med. 2018;12(4):e1889–98.
41. Melcarne C, Lemaitre B, Kurant E. Phagocytosis in drosophila: from molecules and cellular machinery to physiology. Insect Biochem Mol Biol. 2019;109:1–12.
42. Mayorga LS, Piit A, Stahl PD. Receptor-mediated endocytosis and phagocytosis share common determinants for intracellular trafficking. In: Mononuclear phagocytes. Switzerland: Springer nature; 1992. p. 214–23.
43. Antony AC. Folate receptors. Annu Rev Nutr. 1996;16:501–21.
44. Abbaspour N, Hurrell R, Kelishadi R. Review of iron and its importance on human health. J Res Med Sci. 2014;19:164–74.
45. Nigel E. Kupffer cells and their function. Liver. 1987;7(4):63–75.
46. Gordon SB, Read RC. Macrophage defences against respiratory tract infections. Br Med Bull. 2002;61:45–61.
47. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1:135–46.
48. Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci. 2009;66:2873–96.
49. Brown V, Greene M. Molecular and cellular mechanisms of receptor-mediated endocytosis. DNA Cell Biol. 1991;10(6):399–409.
50. Canton I, Battaglia G. Molecular mechanism and physiological functions of clathrin – mediated endocytosis. Nat Publ Gr. 2011;12(8):517–33.
51. Battaglia ICG. Endocytosis at the nanoscale. Chem Soc Rev. 2012;41:2718–39.
52. Kaksonen M, Roux A. Mechanisms of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol. 2018:1–14.

53. Hinners I, Tooze SA. Changing directions: clathrin-mediated transport between the Golgi and endosomes. J Cell Sci. 2003;4:763–71.
54. Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev. 2007;59(8):748–58.
55. Davidson APJM. Nanovehicular intracellular delivery systems. J Pharm Sci. 2008;97(9):3518–90.
56. Klumperman J, Raposo G. The complex ultrastructure of the endolysosomal system. Cold Spring Harb Perspect Biol. 2014;6:4–20.
57. Wang Z, Tiruppathi C, Minshall RD, Malik AB. Size and dynamics of caveolae studied using nanoparticles in living endothelial cells. ACS Nano. 2009;3(12):4110–6.
58. Nabi IR, Le PU. Caveolae/raft-dependent endocytosis. J Cell Biol. 2003;161(4):673–7.
59. Kumari S, Mg S, Mayor S. Endocytosis unplugged: multiple ways to enter the cell. Cell Res. 2010;(3):256–75.
60. Otto GP, Nichols BJ. The roles of flotillin microdomains – endocytosis and beyond. J Cell Sci. 2011;124:3833–940.
61. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B Biointerfaces. 2010;75(1):1–18.
62. Meister M, Tikkanen R. Endocytic trafficking of membrane-bound cargo: a flotillin point of view. Membranes (Basel). 2014;4(3):356–71.
63. Vercauteren D, Piest M, Van Der Aa LJ, Al Soraj M, Jones AT, Engbersen JFJ, et al. Flotillin-dependent endocytosis and a phagocytosis-like mechanism for cellular internalization of disul fi de-based poly (amido amine)/DNA polyplexes. Biomaterials. 2011;32(11):3072–84.
64. Chi X, Wang S, Huang Y, Stamnes M, Chen J. Roles of Rho GTPases in intracellular transport and cellular transformation. Int J Mol Sci. 2013;14(4):7089–108.
65. Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. 2006;16(10):522–9.
66. Mellado M, Jose MR-F, Martinez-Alonzo C, Manes S, del Real G. Prevention of HIV-1 infection by inhibition of Rho-mediated reorganization and/or content alteration of cell membrane raft. 2007;1:1–9.
67. Gerbal-chaloin S, Gondeau C, Aldrian-herrada G. First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling. Biol Cell. 2007;99:223–38.
68. Howes MT, Kirkham M, Riches J, Cortese K, Walser PJ, Simpson F, et al. Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells. J Biol Cell. 2010;190(4):675–91.
69. Waneck L, Brown D. Glycosyl-phosphatidylinositol-anchored membrane proteins. J Am Soc Nephrol. 1992;3:895–906.
70. Onodera R, Motoyama K, Okamatsu A, Higashi T, Arima H. Potential use of folate-appended methyl- b -Cyclodextrin as an anticancer agent. Scientific Reports. 2013; 3:1–9.
71. Mayor S, Parton RG, Donaldson JG. Clathrin-independent pathways of endocytosis. Cold Spring Harb Perspect Biol. 2014; 6(6):1–20.
72. Fu A, Tang R, Hardie J, Farkas ME, Rotello VM. Promises and pitfalls of intracellular delivery of proteins. Bioconjug Chem. 2014;25(9):1602–8.
73. Chou LYT, Ming K, Chan WCW. Strategies for the intracellular delivery of nanoparticles. Chem Soc Rev. 2011;40(1):233–45.
74. Biswas S, Torchilin VP. Nanopreparations for organelle-specific delivery in cancer. Adv Drug Deliv Rev. 2014;66:26–41.
75. Han Q, Wang W, Jia X, Qian Y, Li Q, Wang Z, et al. Switchable liposomes: targeting-peptide-functionalized and pH-triggered cytoplasmic delivery. ACS Appl Mater Interfaces. 2016;8(29):18658–63.
76. Rao SK, Reddy MK, Horning JL, Labhasetwar V. TAT-conjugated nanoparticles for the CNS delivery of anti-HIV drugs. Biomterials. 2008;29(33):4429–38.
77. Khalil IA, Kogure K, Futaki S, Harashima H. High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. J Biol Chem. 2006;281(6):3544–51.

78. Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, et al. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res. 2004;32(19):1–10.
79. Abrami L, Lindsay M, Parton RG, Leppla SH, Van Der Goot FG. Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. J Cell Biol. 2004;166(5):645–51.
80. Le Blanc I, Luyet P, Pons V, Ferguson C, Emans N, Petiot A, et al. Endosome-to-cytosol transport of viral nucleocapsids. Cell Biol. 2005;7(7):653–64.
81. Dermine J, Goyette G, Houde M, Turco SJ, Desjardins M. Leishmania donovani lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. Cell Microbiol. 2005;7(9):1263–70.
82. Schnupf P, Zhou J, Varshavsky A, Portnoy DA. Listeriolysin O secreted by Listeria monocytogenes into the host cell cytosol is degraded by the N-end rule pathway. Infect Immun. 2007;75(11):5135–47.
83. Ciocchini E, Arellano-reynoso B, Lapaque N, Salcedo S, Briones G, Gorvel J, et al. Cyclic b -1, 2-glucan is a brucella virulence factor required for intracellular survival. Nat Immunol. 2005;6(6):618–25.
84. Gruenberg J. Mechanisms of pathogen entry through the endosomal compartments. Nat Rev Mol Cell Biol. 2006;7:495–504.
85. Deretic V, Singh S, Master S, Harris J, Roberts E, Kyei G, et al. Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cell Microbiol. 2006;8:719–27.
86. Epand RM. Fusion peptides and the mechanism of viral fusion. Biochimica. 2003;1614:116–21.
87. Donald JE, Zhang Y, Fiorin G, Carnevale V, Slochower DR, Gai F. Transmembrane orientation and possible role of the fusogenic peptide from parainfluenza virus 5 (PIV5) in promoting fusion. PNAS. 2011;108(10):3958–63.
88. Munyendo WLL, Lv H, Benza-ingoula H, Baraza LD, Zhou J. Cell penetrating peptides in the delivery of biopharmaceuticals. Biomolecules. 2012;2:187–202.
89. Nakase I, Kogure K, Harashima H. Application of a fusiogenic peptide GALA for intracellular delivery. Methods Mol Biol. 2011;683:525–33.
90. Wang T, Yang S, Petrenko VA, Torchilin VP. Cytoplasmic delivery of liposomes into MCF-7 breast cancer cells mediated by cell-specific Phage fusion coat protein. Mol Pharm. 2010;7(4):1149–58.
91. Oliveira S, van Rooy I, Kranenburg O, Storm G, Schiffelers RM. Fusogenic peptides enhance endosomal escape improving siRNA-induced silencing of oncogenes. Int J Pharm. 2007;331(2):211–4.
92. Ju E, Park K, Su K, Kim J, Yang J, Kong J, et al. Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. J Control Release. 2010;141(1):2–12.
93. Hafez I, Maurer N, Cullis P. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther. 2001;8:1188–96.
94. Basha G, Novobrantseva TI, Rosin N, Tam YYC, Hafez IM, Wong MK, et al. Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells. Mol Ther. 2011;19(12):2186–200.
95. Jin J, Bae KH, Yang H, Lee SJ, Kim H, Kim Y, et al. In vivo specific delivery of c-Met siRNA to glioblastoma using cationic solid lipid nanoparticles. Bioconjug Chem. 2011;22:2568–72.
96. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. J Control Release. 2011;151(3):220–8.
97. He J, Kauffman WB, Fuselier T, Naveen SK, Voss TG, Hristova K, et al. Direct cytosolic delivery of polar cargo to cells by spontaneous membrane-translocating peptides. J Biol Chem. 2013;288(41):29974–86.
98. Villegas E. Pore-forming peptides from spiders. Toxin Rev. 2005;24:347–59.
99. Lai P, Pai C, Peng C, Shieh M, Berg K, Lou P. Enhanced cytotoxicity of saporin by polyamidoamine dendrimer conjugation and photochemical internalization. J Biomed Mater Res. 2007; Part A: 87(1)147–55.

100. Alonso MA, Millán J. The role of lipid rafts in signalling and membrane trafficking in T lymphocytes. J Cell Sci. 2001;114(Pt 22):3957–65.
101. Partlow KC, Lanza GM, Wickline SA. Exploiting lipid raft transport with membrane targeted nanoparticles: a strategy for cytosolic drug delivery. Biomaterials. 2008;29(23):3367–75.
102. Jaspreet K, Vasir VL. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2008;59(8):718–28.
103. Chu Z, Miu K, Lung P, Zhang S, Zhao S, Chang HC, et al. Rapid endosomal escape of prickly nanodiamonds: Implications for gene delivery. Sci Rep. 2015;5:1–8.
104. Tan Y, Zhu Y, Zhao Y, Wen L, Meng T, Liu X, et al. Mitochondrial alkaline pH-responsive drug release mediated by Celastrol loaded glycolipid-like micelles for cancer therapy. Biomaterials. 2017; Available from: https://doi.org/10.1016/j.biomaterials.2017.07.036.
105. Xu Y, Wang S, Chan HF, Liu Y, Li H, He C, et al. Triphenylphosphonium-modified Poly (ethylene glycol) -poly (ε -caprolactone) Micelles for Mitochondria- targeted Gambogic Acid Delivery. Int J Pharm. 2017; Available from: https://doi.org/10.1016/j.ijpharm.2017.01.064
106. Chan MS, Liu LS, Leung HM, Lo PK. Cancer-cell-specific mitochondria-targeted drug delivery by dual-ligand-functionalized nanodiamonds circumvent drug resistance. ACS Appl Mater Interfaces. 2017;9(13):11780–9.
107. Zhou J, Zhao W, Ma X, Ju R, Li X, Li N, et al. The anticancer efficacy of paclitaxel liposomes modified with mitochondrial targeting conjugate in resistant lung cancer. Biomaterials. 2013;34:3626–38.
108. Wang T, Hou J, Su C, Zhao L, Shi Y. Hyaluronic acid-coated chitosan nanoparticles induce ROS-mediated tumor cell apoptosis and enhance antitumor efficiency by targeted drug delivery via CD44. J Nanobiotechnol. 2017;15(1):1–12.
109. Qu Q, Ma X, Zhao Y. Targeted delivery of doxorubicin to mitochondria using mesoporous silica nanoparticle nanocarriers. Nanoscale. 2013; 7(40):1–9.
110. Yamada Y, Munechika R, Kawamura E, Sakurai Y, Sato Y, Harashima H. Mitochondrial delivery of doxorubicin using MITO-porter kills drug-resistant renal cancer cells via mitochondrial toxicity. J Pharm Sci. 2017;106(9):2428–37.
111. López V, Villegas MR, Rodríguez V, Villaverde G, Lozano D, Baeza A, et al. Janus mesoporous silica nanoparticles for dual targeting of tumor cells and mitochondria. ACS Appl Mater Interfaces. 2017;9(32):26697–706.
112. Bukrinsky M. A hard way to the nucleus. Mol Med. 2004;10(1):1–5.
113. Rajendran L, Knölker HJ, Simons K. Subcellular targeting strategies for drug design and delivery. Nat Rev Drug Discov. 2010;9(1):29–42.
114. Singh L, Kruger HG, Maguire GEM, Govender T, Parboosing R. Development and evaluation of peptide-functionalized gold nanoparticles for hiv integrase inhibition. Int J Pept Res Ther. 2018;0(0):1–12.
115. Pan L, He Q, Liu J, Chen Y, Zhang L, Shi J. Nuclear-targeted drug delivery of TAT peptide-conjugated monodisperse mesoporous silica nanoparticles. J Am Chem Soc. 2012;134:5722–5.
116. Zhou Z, Liu Y, Wu L, Li L, Huang Y. Enhanced nuclear delivery of anti-cancer drugs using micelles containing releasable membrane fusion peptide and nuclear-targeting retinoic acid. J Mater Chem B. 2017;5(34):7175–85.
117. Jing Y, Xiong X, Ming Y, Zhao J, Guo X, Yang G. A multifunctional micellar nanoplatform with pH-triggered cell penetration and nuclear targeting for effective cancer therapy and inhibition to lung metastasis. Adv Healthc Mater. 2018;1700974:1–13.
118. Cerrato CP, Künnapuu K, Langel Ü. Cell-penetrating peptides with intracellular organelle targeting. Expert Opin Drug Deliv. 2017;14(2):245–55.
119. Ozcan L, Tabas I. Role of endoplasmic reticulum stress in metabolic disease and other disorders. Annu Rev Biochem. 2005;74:317–28.
120. Zakariah M, Khan S, Choudhary AA, Rolfo C, Ben Ismail MM, Alotaibi YA. To decipher the mycoplasma hominis proteins targeting into the endoplasmic reticulum and their next-generation sequencing data. Molecules. 2018; 23(5):1–12.

121. Boelens J, Lust S, Offner F, Bracke ME, Vanhoecke BW. Review. The endoplasmic reticulum: a target for new anticancer drugs. In Vivo. 2007;21(2):215–26.
122. Inoue T, Tsai B. How viruses use the endoplasmic reticulum for entry, replication, and assembly. Cold Spring Harb Perspect Biol. 2013;5(1):1–17.
123. Pan T, Song W, Gao H, Li T, Cao X, Zhong S, et al. miR-29b-loaded gold nanoparticles targeting to the endoplasmic reticulum for synergistic promotion of osteogenic differentiation. Appl Mater Interfaces. 2016;8(30):19217–27.
124. Sneh-Edri H, Likhtenshtein D, Stepensky D. Intracellular targeting of PLGA nanoparticles encapsulating antigenic peptide to the endoplasmic reticulum of dendritic cells and its effect on antigen cross-presentation in vitro. Mol Pharm. 2011;8(4):1266–75.
125. Zhang J, Sun A, Xu R, Tao X, Dong Y, Lv X, et al. Cell-penetrating and endoplasmic reticulum-locating TAT-IL-24-KDEL fusion protein induces tumor apoptosis. J Cell Physiol. 2016;231(1):84–93.
126. Chang MY, Shiau AL, Chen YH, Chang CJ, Chen HHW, Wu CL. Increased apoptotic potential and dose-enhancing effect of gold nanoparticles in combination with single-dose clinical electron beams on tumor-bearing mice. Cancer Sci. 2008;99(7):1479–84.
127. Behzadi S, Serpooshan V, Tao W, Hamaly MA, Mahmoud Y, Dreaden EC, et al. Cellular uptake of nanoparticles: journey inside the cell. Chem Soc. 2018;46(14):4218–44.
128. Waters KM, Masiello LM, Zangar RC, Tarasevich BJ, Karin NJ, Quesenberry RD, et al. Macrophage responses to silica nanoparticles are highly conserved across particle sizes. Toxicol Sci. 2009;107(2):553–69.
129. Cooley M, Sarode A, Hoore M, Fedosov DA, Mitragotri S, Sen Gupta A. Influence of particle size and shape on their margination and wall-adhesion: implications in drug delivery vehicle across nano-to-micro scale. Nanoscale. 2018; 10(32):15350–64.
130. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J. 2004;169:159–69.
131. Huang K, Ma H, Liu J, Huo S, Kumar A, Wei T, et al. Size-dependent localization and penetration of ultrasmall gold nanoparticles in cancer cells, multicellular spheroids, and tumors in vivo. ACS Nano. 2012;6(5):4483–93.
132. Rausch K, Reuter A, Fischer K, Schmidt M. Evaluation of nanoparticle aggregation in human blood serum. Biomacromolecules. 2010;11(11):2836–9.
133. Albanese A, Chan WC. Effect of gold nanoparticle aggregation on cell uptake and toxicity BT. ACS Nano. 2011;5(7):5478–89.
134. Salmaso S, Caliceti P. Stealth properties to improve therapeutic efficacy of drug nanocarriers. J Drug Deliv. 2013;2013:1–18.
135. Storm G, Belliot S, Daemenb T, Lasic DD. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. Adv Drug Deliv Rev. 1995;17(95):31–48.
136. Owens DE, Peppas NA. Opsonization, biodistribution and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006;307:93–102.
137. Mcsweeney MD, Versfeld ZC, Carpenter DM, Lai SK. Physician awareness of immune responses to polyethylene glycol-drug conjugates. Clin Transl Sci. 2018;11:162–5.
138. Amoozgar Z, Yeo Y. Recent advances in stealth coating of nanoparticle drug delivery systems Zohreh. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2013;4(2):219–33.
139. Podlekareva K, Akmal F. Palmar-plantar erythrodysesthesia associated with chemotherapy and its treatment. Case Rep Oncol. 2011;4:229–35.
140. Von Erlach T, Zwicker S, Pidhatika B, Konradi R, Textor M, Hall H, et al. Formation and characterization of DNA-polymer-condensates based on poly (2-methyl-2-oxazoline) grafted poly (L -lysine) for non-viral delivery of therapeutic DNA. Biomaterials. 2011;32(22):5291–303.
141. Metselaar JM, Bruin P, De Boer LWT, De Vringer T, Snel C, Oussoren C, et al. A novel family of L -amino acid-based biodegradable polymer – lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. Bioconjug Chem. 2003;14:1156–64.

142. Lammers T, Subr V, Ulbrich K, Peschke P, Huber PE, Hennink WE, et al. Simultaneous delivery of doxorubicin and gemcitabine to tumors in vivo using prototypic polymeric drug carriers. Biomaterials. 2009;30(20):3466–75.
143. Sun J, Zeng F, Jian H, Wu S. Conjugation with betaine: a facile and effective approach to significant improvement of gene delivery properties of PEI. Biomacromolecules. 2013;14:728–36.
144. Siegers C, Biesalski M, Haag R. Self-assembled monolayers of dendritic polyglycerol derivatives on gold that resist the adsorption of proteins. Chem Eur J. 2004;10:2831–8.
145. Alhareth K, Vauthier C, Bourasset F, Gueutin C, Ponchel G, Moussa F. Conformation of surface-decorating dextran chains affects the pharmacokinetics and biodistribution of doxorubicin-loaded nanoparticles. Eur J Pharm Biopharm. 2012;81(2):453–7.
146. Vonarbourg A, Passirini C, Saulnier P, Simard P, Leroux JC, Benoit JP. Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J Biomed Mater. 2006; 78(3):620–8.
147. Yoo HS, Lee JE, Chung H, Kwon IC, Jeong SY. Self-assembled nanoparticles containing hydrophobically modified glycol chitosan for gene delivery. J Control Release. 2005;103:235–43.
148. Guhagarkar SA, Majee SB, Samad A, Devarajan PV. Evaluation of pullulan-functionalized doxorubicin nanoparticles for asialoglycoprotein receptor-mediated uptake in Hep G2 cell line. Cancer Nanotechnol. 2011;2:49–55.
149. Guhagarkar SA, Gaikwad RV, Samad A, Malshe VC, Devarajan PV. Polyethylene sebacate – doxorubicin nanoparticles for hepatic targeting. Int J Pharm. 2010;401(1–2):113–22.
150. Guhagarkar SA, Shah D, Patel MD, Sathaye SS, Devarajan PV. Polyethylene sebacate-silymarin nanoparticles with enhanced hepatoprotective activity. J Nanosci Nanotechnol. 2015;15:4090–3.
151. Souza AAD, Devarajan PV. Bioenhanced oral curcumin nanoparticles: role of carbohydrates. Carbohydr Polym. 2016;136:1251–8.
152. Pranatharthiharan S, Patel MD, Malshe VC, Padma V, Pranatharthiharan S, Patel MD, et al. Polyethylene sebacate doxorubicin nanoparticles: role of carbohydrate anchoring on in vitro and in vivo anticancer efficacy. Drug Deliv. 2016;7544:1–10.
153. Pranatharthiharan S, Patel MD, Malshe VC, Gorakshakar A, Madkaikar M, Ghosh K, et al. Asialoglycoprotein receptor targeted delivery of doxorubicin nanoparticles for hepatocellular carcinoma Asialoglycoprotein receptor targeted delivery of doxorubicin nanoparticles for hepatocellular carcinoma. Drug Deliv. 2017;7544:20–9.
154. Doh K-O, Yeo Y. Application of polysaccharides for surface modification of nanomedicines. Ther Deliv. 2013;3(12):1447–56.
155. Milosevits G, Krol S. Exosomes: potential model for complement- stealth delivery systems structure of exosomes. Eur J Nanomed. 2015;7(3):207–18.
156. Fan W, Yan W, Xu Z, Ni H. Erythrocytes load of low molecular weight chitosan nanoparticles as a potential vascular drug delivery system. Colloids Surf B Biointerfaces. 2012;95:258–65.
157. Li Y, Kroger M, Liu Kam W. Endocytosis of PEGylated nanoparticles accompanied by structural and free energy changes of the grafted polyethylene glycol. Biomaterials. 2014;35:8467–78.
158. Jindal AB. The effect of particle shape on cellular interaction and drug delivery applications of micro- and nanoparticles. Int J Pharm. 2017;532(1):450–65.
159. Dasgupta S, Auth T, Gompper G. Shape and orientation matter for the cellular uptake of nonspherical particles. Nano Lett. 2014;14:687–93.
160. Agarwal R, Singh V, Jurney P, Shi L, Sreenivasan SV, Roy K. Mammalian cells preferentially internalize hydrogel nanodiscs over nanorods and use shape-specific uptake mechanisms. Proc Nat Acad Sci. 2013;110(43):1–6.
161. Chithrani BD, Ghazani AA, Chan WCW. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett. 2006;6(4):662–8.
162. Nowacek AS, Balkundi S, Mcmillan J, Roy U, Martinez-skinner A, Mosley RL, et al. Analyses of nanoformulated antiretroviral drug charge, size, shape and content for uptake,

drug release and antiviral activities in human monocyte-derived macrophages. J Control Release. 2011;150(2):204–11.
163. Li Y, Chen X, Gu N. Computational investigation of interaction between nanoparticles and membranes: hydrophobic/hydrophilic effect. J Phys Chem B. 2008;112(51):16647–53.
164. Moyano DF, Goldsmith M, Solfiell DJ, Landesman-milo D, Miranda OR, Peer D, et al. Nanoparticle hydrophobicity dictates immune response. J Am Chem Soc. 2012;134(9):3965–7.
165. Malcolm DW, Freeberg MAT, Wang Y, Sims KR Jr, Awad HA, DSW B. Diblock copolymer hydrophobicity facilitates efficient gene silencing and cytocompatible nanoparticle- mediated siRNA delivery to musculoskeletal cell types. Biomacromolecules. 2017;18(11):3753–65.
166. Voigt J, Christensen J, Shastri VP. Differential uptake of nanoparticles by endothelial cells through polyelectrolytes with affinity for caveolae. PNAS. 2014;111(8):2942–7.
167. Verma A, Stellacci F. Effect of surface properties on nanoparticle – cell interactions. Small. 2010;1:12–21.
168. He C, Hu Y, Yin L, Tang C, Yin C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials. 2010;31(13):3657–66.
169. Ricarda M, Holzapfel V, Musyanovych A, Nothelfer K, Walther P, Frank H, et al. Uptake of functionalized, fluorescent-labeled polymeric particles in different cell lines and stem cells. Biomaterials. 2006;27:2820–8.
170. Clemens DL, Lee B, Xue M, Thomas CR, Meng H, Ferris D, et al. Targeted intracellular delivery of antituberculosis drugs to mycobacterium tuberculosis -infected macrophages via functionalized. 2012;2535–45.
171. Nam HY, Kwon SM, Chung H, Lee SY, Kwon SH, Jeon H, et al. Cellular uptake mechanism and intracellular fate of hydrophobically modified glycol chitosan nanoparticles. J Control Release. 2009;135(3):259–67.
172. Chung T, Wu S, Yao M, Lu C, Lin Y, Hung Y, et al. The effect of surface charge on the uptake and biological function of mesoporous silica nanoparticles in 3T3-L1 cells and human mesenchymal stem cells. Biomaterials. 2007;28:2959–66.
173. Deng L, Que F, Wei H, Xu G, Dong X, Zhang H. Solubilization of tea seed oil in a food-grade water-dilutable microemulsion. PLoS One. 2015;10(5):1–12.
174. Patil S, Sandberg A, Heckert E, Self W, Seal S. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. Biomaterials. 2007;28(31):4600–7.
175. Lourenc I, Mainardes RM, Palmira M, Gremia D. Zidovudine-loaded PLA and PLA – PEG blend nanoparticles: influence of polymer type on phagocytic uptake by polymorphonuclear cells. J Pharm Sci. 2009;98(1):257–67.
176. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, et al. The effect of surface charge on in vivo biodistribution of PEG-oligocholic acid based micellar nanoparticles. Biomaterials. 2011;32(13):3435–46.
177. Beningo KA, Wang Y. Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. J Cell Sci. 2002;115:849–56.
178. Guo P, Liu D, Subramanyam K, Wang B, Auguste DT, Moses MA. Nanoparticle elasticity directs tumor uptake. Nat Commun. 2018; 9:1–9.
179. Li S-D, Huang L. Nanoparticles evading the reticuloendothelial system: role of the supported bilayer. Biochim Biophys Acta. 2010;1788(10):2259–66.
180. Na K, Lee TB, Park K, Shin E, Lee Y, Choi H. S elf-assembled nanoparticles of hydrophobically-modified polysaccharide bearing vitamin H as a targeted anti-cancer drug delivery system. Eur J Pharm Sci. 2003;18:165–73.
181. Yuan H, Fales AM, Vo-dinh T. TAT peptide-functionalized gold nanostars: enhanced intracellular delivery and efficient NIR photothermal therapy using ultralow irradiance. J Am Chem Soc. 2012;134:11358–61.
182. Edagwa BJ, Guo D, Puligujja P, Chen H, Mcmillan J, Liu X, et al. Long-acting antituberculous therapeutic nanoparticles target macrophage endosomes. FASEB J. 2014;28:1–12.

Part II
Receptors and Receptor Mediated Endocytosis in Cancer

Chapter 2
Brain Cancer Receptors and Targeting Strategies

Rijo John, Heero Vaswani, Prajakta Dandekar, and Padma V. Devarajan

Abstract Brain cancer is regarded as the most widespread cancer of the central nervous system. The complexity of the glial cell tumor renders the survival and prognosis of glioma difficult, even after conventional chemotherapy, radiotherapy, and surgery treatments. The major concern is the formidable blood–brain barrier (BBB) that guards the entry of all exogenous moieties into the brain. This chapter addresses in brief the various approaches to bypass the BBB. Among different strategies, receptor-oriented drug delivery takes advantage of receptors on the BBB to traverse into the brain and if appropriately designed can enter the cancer cells through receptors overexpressed on their surface. This chapter will also summarize the various receptors, their physiology, ligands for the receptor, and drug-delivery strategies that could improve brain cancer therapy.

Keywords Brain cancer · Receptor-mediated endocytosis · Low-density lipoprotein · Integrin · Interleukin · Lactoferrin

Abbreviations

ADC	Antibody–drug conjugate
ADMIDAS	Adjacent to MIDAS
AP1	CRKRLDRNC peptide
Apo B	Apolipoprotein B

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Apo E	Apolipoprotein E
AQP4	Aquaporin-4
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EGF	Epidermal growth factor
EGFR	Epithelial growth factor receptor
EPR	Enhanced permeability and retention
GBM	Glioblastoma
IC_{50}	Half-maximal inhibitory concentration
IL	Interleukin
IL-13Rα2	Interleukin-13 receptor subunit alpha-2
IL-2Rγc	Interleukin-2 receptor common gamma chain
Kd	Dissociation constant
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
Lf	Lactoferrin
LfR	Lactoferrin receptor
LRP	Low-density lipoprotein receptor-related protein
MIDAS	Metal ion-dependent adhesion Site
mPEG	Methoxy polyethylene glycol
MTT	(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NPs	Nanoparticles
PE	Paired end
PEG	Polyethylene glycol
PI3 kinase	Phosphoinositide-3-kinase
PLA	Poly lactic acid
PTX	Paclitaxel
RAP	Receptor-associated protein
RGD	Arginine–glycine–aspartic acid
RNA	Ribonucleic acid
STAT 6	Signal transducer and activator of transcription 6
Tf	Transferrin
TPGS	D-α-tocopheryl polyethylene glycol succinate
VLDLR	Very low-density lipoprotein receptor

1 Introduction

Brain cancers, the uncontrolled growth of cells in brain, are among the most feared malignancies owing to their fast development and poor diagnosis. These are either primary or secondary metastasized. Primary brain cancers arise from a single brain

cell type and most common are gliomas, meningiomas, medulloblastomas, and primary central nervous system (CNS) lymphomas [1]. Metastatic brain cancers originate from cancerous cells that are transported through the blood stream from distant areas located in other regions of the body. Although efforts in drug discovery have resulted in newer drugs for glioma, crossing the blood–brain barrier (BBB) constitutes the major limiting factor in the effective treatment of brain cancers. This section describes the BBB and discusses briefly the different opportunities of crossing the same. A specific focus is the endocytic pathway through the receptors on the BBB and the various strategies for targeting drugs and drug-loaded nanocarriers into the brain by receptor-mediated endocytosis.

2 Blood–Brain Barrier

The blood–brain barrier is a discriminating barrier endowed with the ability to block about 98% of molecules with low molecular weight and almost 100% of larger molecules [2]. The endothelial cells of brain capillary with the interspersed tight junctions, supported with basal lamina, pericytes, and astrocytes regulate the transport of all molecules across the brain [3]. This ensures adequate delivery of nutrients and other endogenous moieties while restricting delivery of foreign substances. The interface between blood and brain constitutes the brain endothelium that is interspersed with tight junctions formed by intimate connection of the endothelial cells and astrocytes. .These tight junctions serve as gatekeepers for the paracellular pathways through the BBB. The endothelial lining consists of two discrete sides, the abluminal (brain side) and luminal (blood side). Abluminal side is supported by pericytes which are attached at irregular intervals and is surrounded by the basal lamina (30–40 nm) comprising proteoglycans, type IV collagen, fibronectin, laminin, heparin sulfate, and other matrix proteins which are extracellular. The basal lamina is closely linked with the astrocytes end feet. These collectively constitute the protective BBB which prevents the transport of molecules from the luminal portion to the abluminal portion and into the brain [4]. The astrocytes end feet play a crucial role in regulating the BBB permeability. However, there are a number of pathways through the BBB that allow vital compounds to be transported into the brain [5]. These are discussed in brief and schematically depicted in Fig. 2.1.

2.1 Transport Mechanisms across Across the Blood–Brain Barrier

2.1.1 Transport of Water, Ions, and Nutrients

The movement of water in the brain is regulated through Aquaporin-4 (AQP4) membrane channels which help in osmotic regulation. The transport of ions across the BBB is generally through ion channels. Optimal ion concentrations are important to

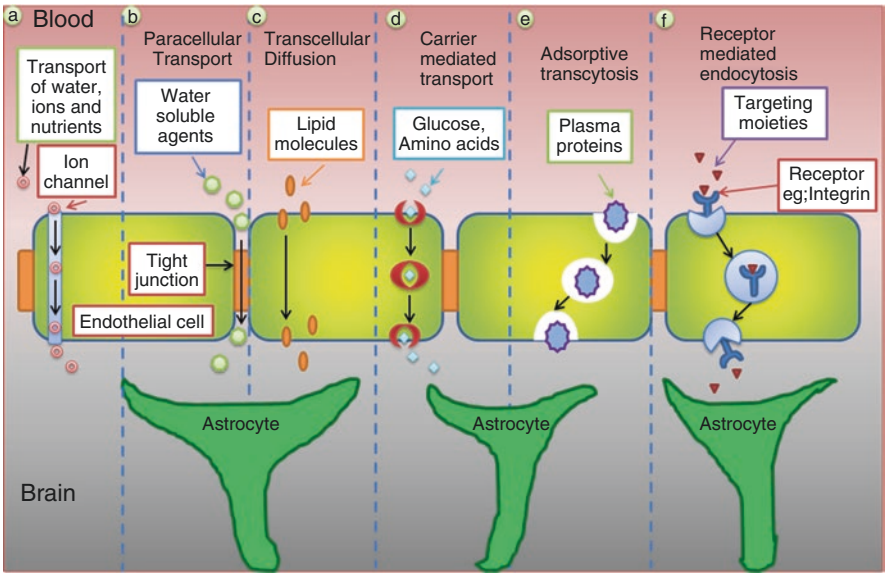

Fig. 2.1 Transport mechanisms across the blood–brain barrier

maintain proper environment for synaptic and neural function. Astrocytes play a significant role in maintaining the blood–brain barrier (BBB) by ensuring ion homeostasis and monitoring metabolism of the amino acid neurotransmitters, as well as assisting neurons with their energy and nutrient requirements [6].

2.1.2 Paracellular Transport

Paracellular transport is a mechanism by which transport of small molecules occurs across the endothelial cell. By this way, it bypasses a group of substrate-specific transport systems that are designed to mediate transport across the BBB [7]. The transport of gaseous molecules like O_2 and CO_2 along with some smaller molecules having sufficient hydrophilicity and lipophilicity such as ethanol and caffeine can occur via paracellular pathway. The entry of larger molecules is limited by this mechanism [8]. Some strategies reported to enhance the paracellular transport of the drug molecule include chemical or physical modification, alteration of physicochemical properties, and the use of compounds modulating tight junctions such as surfactants and chelating agents [9].

2.1.3 Transcellular Diffusion

The transport of drugs or endogenous molecules by transcellular diffusion is a process dependent on concentration gradient, allowing a wide range of low molecular weight lipophilic molecules to cross the BBB based on Lipinski's rule [10]. Two important criteria governing this transport are the lipophilicity (Log *P* between −0.2 and 1.3) and molecular mass (<400 Da) of the compound. Lipophilicity is directly proportional while molecular weight is inversely proportional to the transport of drug across BBB [11]. Other factors that can influence the diffusion of drug across the BBB are the ionization, shape, protein complexation, and hydrogen bonding [12]. Significant limitations of transcellular diffusion have demanded alternative strategies for transport of drugs across the BBB.

2.1.4 Carrier-Mediated Transport

Carrier-mediated efflux is critical for exchange of nutrients, neurotransmitters, and metabolites crossing the BBB. These carriers belong to the Solute Carrier (SLC) superfamily of transporters which are accountable for the transport of specific substrates. Some carriers are very selective in their stereochemical substrate requirements, such as glucose transporter type 1 for glucose, monocarboxylic acid transporter 1 for monocarboxylic acids, and for large neutral and cationic amino acids, the large neutral amino acid transporter I and cationic amino acid transporter I, respectively. The highest carrying capacity is shown by hexose and large neutral amino acid carriers which can serve as effective transporters of cargo to the brain [13].

2.1.5 Adsorptive Endocytosis

Adsorptive endocytosis involves the interaction of the ligand with the cell surface and is activated by electrostatic interaction with micromolecules or positively charged proteins with the negatively charged plasma membrane. Adsorptive endocytosis is nonspecific, occurs via clathrin-mediated mode, and provides the opportunity for movement of molecules across the BBB via endothelial cytoplasm [14]. Cell-penetrating peptides and cationic proteins exhibit adsorptive endocytic uptake. The method adopts the brain endothelial cell technique for binding and absorbing cationic molecules from the blood vessels into the brain via molecular exocytosis [15].

2.1.6 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis (RME) involves binding of transmembrane receptors with ligands. These receptors are expressed on the apical plasma membrane of the endothelial cells. Receptors on the BBB are proteins residing on the membrane of postsynaptic neurons receiving neurochemicals released by brain cells from synapses. Receptors bind with neurotransmitters using a lock and key metaphor, and can be classified based on their location as transmembrane receptors and intracellular receptors [16]. Cells with numerous receptors bind with ligands to activate or inhibit a specific receptor-mediated biochemical pathway. Endocytosis can occur by one or more of the mechanisms detailed in Chap. 1. While clathrin-mediated endocytosis is the major pathway, receptors depending on their constitution may exhibit other pathways of internalization. The brain uptake of nutrients such as leptin [17], iron, and insulin [18] occurs by receptor-mediated transport mechanism. Other receptors present on the BBB are generally exploited for transport across BBB for chemotherapy of brain tumors [19]. Receptor-mediated endocytosis relies on specific and selective affinity of receptors for certain ligands which can be effectively utilized to target therapeutics into the brain through the BBB [20]. Nanocarriers have been successfully delivered using such strategies.

Once delivered inside the brain by one or more of the above strategies, nanocarriers can diffuse out through leaky blood vessels in tumors enabling delivery and concentration buildup through enhanced permeability and retention (EPR) effect [21]. This enables a second stage of RME, wherein nanocarriers are internalized into brain cancer cells through overexpressed receptors. EPR relies on pathophysi-

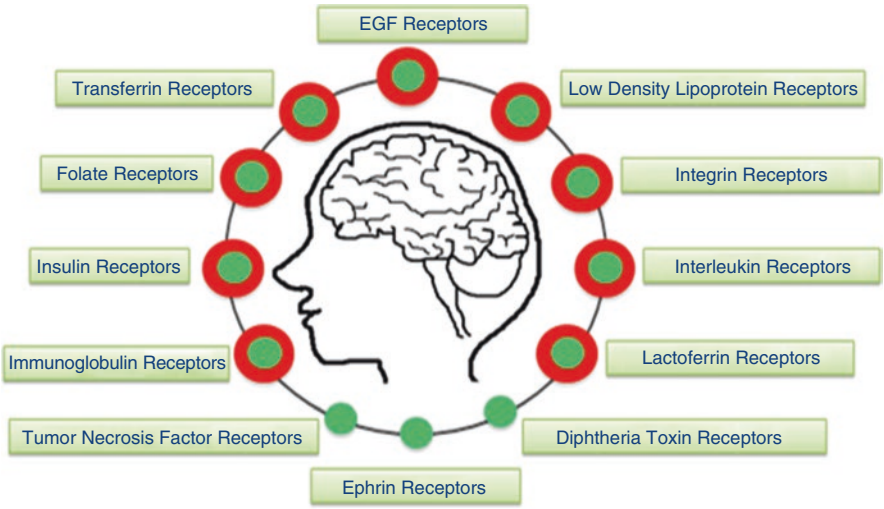

Fig. 2.2 Receptors expressed on the BBB (●) and on the BBB and brain cancer cells (◉)

ological characteristics of tumor tissue, wherein the fenestrated vasculature permits extravasation of materials of nanometer size. This supported by the lack of lymphatic drainage aids in the enhanced and effective accumulation of nanocarriers in tumors [22].

3 Targeting Receptors for Brain Cancer

The BBB has many receptors which can be exploited for drugs to enter the brain. Figure 2.2 depicts the receptors on the BBB and those overexpressed in brain cancers. Interestingly, most of these receptors are overexpressed in brain cancers with the exception of diphtheria toxin receptor and the tumor necrosis factor receptor. The cascade receptor-targeting strategy is therefore appropriate for the therapy of brain cancers, wherein a single ligand can be used for targeting. In the first stage, the ligand would ensure receptor-mediated transport of drug-loaded nanocarriers across the BBB, and in the second stage serve as ligand for receptors, thereby boosting drug concentration to enhance efficacy.

Among the receptors that are majorly studied for targeted drug delivery, this chapter focuses on low-density lipoprotein receptor, integrin receptor, interleukin receptor, and lactoferrin receptor. Other receptors are explicitly discussed in other chapters of book and therefore not dealt with here. The ephrin, immunoglobulin, and insulin receptors are minimally studied and hence not considered under the purview of this chapter.

3.1 Low-Density Lipoprotein Receptors in Brain Cancer

3.1.1 Occurrence of Low-Density Lipoprotein Receptors

Low-density lipoprotein receptors (LDLR) along with their allied proteins 1 (LRP 1) and 2 (LRP 2) mediate the movement of lipoproteins and ligands across the blood–brain barrier by receptor-mediated endocytosis. These receptors are densely localized in specific areas with 80% covering only 2% of the cell surface. LDLs such as cholesterol, tocopherol, and apolipoprotein can bind to the LDLR, located in the caveolae membrane fraction and go across the BBB to reach inside brain tissue through caveolin-mediated endocytosis. Apolipoproteins (Apo B and Apo E) [23] mediate particle interaction with lipoprotein receptors including LRP1, LRP2 and LDLR [24]. The significantly high amount of LDLR on BBB and glioma cells [25] is exploited to carry anticancer drugs through BBB using nanocarriers as vehicles.

3.1.2 Structure and Signal Transduction of LDLR

Low-density lipoprotein receptor consists of spherical particles of 220 Å in diameter having a nonpolar core of approximately 1500 molecules of cholesterol that are esterified by means of fatty acids like oleate and linoleate. LDLR is a transmembrane glycoprotein with single chain [26]. It has five distinct domains, including a ligand-binding domain, EGF precursor-homology domain, O-linked sugar domain, membrane-spanning domain, and cytoplasmic tail [27]. Receptors contain different endocytosis signals that mediate their interactions with the endocytic machinery, and these receptor cytoplasmic tails appear to bind many cytosolic adapters and scaffold proteins that add to signal transduction [28].

3.1.3 Recognition Domain of Low-Density Lipoprotein Receptor

The region of LDLR binding to ligand includes cysteine-rich repeats of 40 amino acids which are seven in number followed by EGF precursor-homology domain consisting of three units of EGF-like repeats of 40 amino acids. The third region, O-linked sugar domain, is enriched with residues of inserin as well as threonine that act as acceptors for O-linked sugars. A hydrophobic 24 amino acids membrane-spanning domain is responsible for the attachment of LDLR to the lipid bilayer. The cytoplasmic tail regulates the intracellular transport and endocytosis across the LDLR. The cysteine units located at amino terminus of receptor mediate the folding of each repetition into a compact structure by forming three disulfide bridges with the bunch of negatively loaded residues with tripeptide surface. The linkers play a significant role by offering the domain with flexibility to accommodate distinct size lipoprotein ligands. LDL particles constitute an outer phospholipid surface layer covered by single protein apoB-100 [29]. The surface exposing the proteins allows recognition of receptor by the amino acid residues and serves as LDLR binding domain. Intercalation of free cholesterol with chains of phospholipid fatty acids imparts some amount of stiffness to the outer monolayer of LDL [30].

3.1.4 Binding of Ligand and Receptor Pathway

The internalization of LDL that binds to LDLR occurs via clathrin-dependent pathway. However, internalization of LDL particles can occur by receptor-mediated and receptor-independent processes. On an average, 30–40% of complete LDL plasma pool is cleaned from body every day, with about two-thirds of the circulating LDL pool removed by receptor-mediated uptake [31]. The receptor-binding sequences present on apoB-100 are highly cationic, while on the cell surface, complementary anionic sequences are present. Electrostatic interactions between them enable binding of LDL particles to LDLR implanted in clathrin-coated pits on the cell membrane [27]. The ligand-binding sites in LDLR are contained in clusters, suggesting a functional duplication within receptor and high-affinity binding of most ligands

needs multiple ligand-binding repeats. Some ligands can sequentially identify distinct combinations of these repeats, while others seem to identify repeats from different clusters [32]. The LDL receptor carries particular macromolecules, primarily cholesterol-rich lipoprotein LDL, through receptor-mediated endocytosis into cells.

3.1.5 Ligands for LDLR

LDL mainly contains cholesterol (approximately 50%) in free and esterified form, 25% proteins, 22% phospholipids, and 5% triacylglycerol [33]. Native LDL exhibits high affinity for LDLR; nevertheless, difficulty in isolation on large scale and high variability in composition and size and difficulty in purification limit its use. Studies on several ligands and their use to transport nanocarriers, antibodies, and drugs through BBB are reported [34]. Aprotinin, angiopep-2, ApoE3 mimetic, and p97 can be used to target LDL receptor family [35]. Targeting must preferably not affect receptor function, as this may result in toxicity or other side effects and ligands should also not be immunogenic [36]. Thus, synthetic LDL analogs prepared as replacements of native LDL could have better applications.

3.2 Integrin Receptors in Brain Cancer

3.2.1 Occurrence of Integrin Receptors

Integrins are powerful heterodimeric transmembrane receptors on the cell surface which play a significant part in cell-to-stroma interaction. They are expressed in significant numbers in tumor vasculature, angiogenic endothelial cells, and tumor cells in gliomas. It is proposed that integrins play a major role in initiation with advancement in cancer [37]. In various cancers, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are reported to be essential for angiogenesis caused by fibroblast growth factor and tumor necrosis factor specifically in malignant gliomas. Integrin $\alpha_v\beta_3$ is expressed in angiogenic endothelial cells [38]. Immunohistochemistry validation by molecular imaging using tracer [^{18}F]Galacto-RGD confirmed that $\alpha_v\beta_3$ integrin expression was primarily restricted to the tumor region and was absent in ordinary tissues [39]. Integrin plays a significant role in encouraging glioma development, invasion, and angiogenesis, and hence can be used as a target for improving cancer therapy.

3.2.2 Structure and Signal Transduction by Integrin Receptor

Integrins are 18α and 8β subunits that are assembled into receptors. Twenty-four integrin receptors are recognized at different locations on nucleated cells with separate features. Each α/β subunit comprises 1000 amino acids with a single membrane-spreading helix and 750 amino acids with a short cytoplasmic tail [40].

Integrin alpha (α) subunit is formed by seven-bladed β-propellers linked to a thigh. The leg structures formed by a calf-1 and a calf-2 domain support the integrin head domain. The end three or four blades of β-propeller contain EF hand domain which connects calcium on the lower side facing away from the ligand-binding surface. Ca^{2+} binding to these sites allosterically affects ligand binding [41].

The integrin beta (β) subunit includes four domains, a β1 domain, a plexin semaphorin integrin domain (PSI), a hybrid domain and a β tail domain with four EGF repeats which are cysteine-rich [42]. The β1 domain also shows the presence of a Mg^{2+}-coordinating MIDAS (Metal-ion-dependent adhesion site) and an adjacent MIDAS site (ADMIDAS) that binds a Ca^{2+} ion inhibitor. Binding of ADMIDAS site to Mg^{2+} ion enables integrins in the active form [41]. Integrin receptors are enzymatically inactive and require linkage with certain components of cell to generate signal transduction. The phenomena known as "outside-in signaling" is associated with integrins, for transducing signals within the cells after binding to extracellular matrix. This signaling is necessary for polymerization of actin cytoskeleton during cell linkage and for controlling cell migration, division, and survival. Phosphorylation of proteins is the first event detected in response to integrins stimulation. Other responses consist of cytosolic kinases induction, phosphoinositides metabolism stimulation, Ras/MAPK activation, and protein kinase C (PKC) pathway as well as regulation of Ras homologous (Rho) GTPases. Integrins have an impact on cell survival as it regulates programmed cell death, which is a tyrosine phosphorylation-dependent response. The signaling pathways of integrins synergize with pathways of other receptors to elevate or decline signals produced by each of the receptors [43]. Thus, a challenging aspect here is the identification of signaling molecules that arbitrate cross talk between the different receptor pathways.

3.2.3 Recognition Domain of Integrin Receptor

The RGD tripeptide in ligands serves as the identification site for different integrins such as α_v integrins, β1 integrins α_5 and α_8, and $\alpha_{IIb}\beta_3$. Some of crystal structures complexed with RGD ligands disclosed the same atomic essentials for interaction [44]. RGD shows binding on the α and β subunit interface domain where basic residues fit the cleft in the b-propeller module subunit, while cation-coordinated acid residue shows binding to b-I domain [45]. RGD-binding integrins are most promising among the receptor family, particularly binding β3 integrins to large amounts of extracellular matrix and soluble vascular ligands. While subsets of integrins share many ligands, the affinity order of ligand differs, probably reflecting accuracy of ligand RGD fit with particular α,β active site pockets.

3.2.4 Binding of Ligand and Integrin Receptor Pathway

Integrins are known to follow multiple internalization pathways including clathrin-mediated endocytosis, caveolae-mediated endocytosis, and sometimes by both clathrin- and caveolae-mediated endocytosis. These pathways occur depending on factors such as the cell environment, cell condition, and cell type [46]. However, adhesion is extremely dynamic, with cells continually sampling their pericellular environment and reacting by quickly altering their position and differentiation status, offering an extremely responsive mechanism for receptor activation. The formation of explained complexes is achieved in two ways, by clustering receptors, which increases molecular interactions resulting in increased binding rate of effector molecules, and by inducing conformational modification in receptors that create or expose binding sites. Conformational regulation is generally the main mode for integrin regulation to affinity [47]. Thus, suitable mechanisms to convey conformational changes over a comparatively large distance between cytoplasmic tails and ligand-binding head domain (20 nm) are important [48].

3.2.5 Ligands for Integrin Receptors

The various ligands with their respective partner integrins include the following: bone sialoprotein that interacts with $\alpha_v\beta_3$ and $\alpha_v\beta_5$; collagens that interact with $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, and $\alpha_{Ib}\beta_3$; fibronectin that interacts with $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha_{IIb}\beta_3$; matrix metalloproteinase-2 that interacts with $\alpha_v\beta_3$; and plasminogen that interacts with $\alpha_{IIb}\beta_3$ [49].

3.3 Interleukin Receptors in Brain Cancer

3.3.1 Occurrence of Interleukin Receptors

Interleukin is a proinflammatory cytokine that promotes malignant actions by activating different cells to upregulate major oncogenic event molecules. Upregulation of cytokines IL-1b, IL-4, IL-6, and IL-8 are majorly observed in patient samples and glioma cell lines. Cell line and xenography studies hypothesize that these inflammatory mediators may be valuable in brain tumor treatment. Interleukin 13 receptor has a key role in immune response regulation coupled with immune microenvironment modulation. IL13Rα2 set a well-defined, fresh glioma therapy. Even before the receptor was characterized, the therapeutic advantage of IL-3-truncated PE fusion chimera protein (IL13PE38QQR) was identified in gliomas [50].

3.3.2 Structure and Signal Transduction by Interleukin Receptor

The receptor consists of 2 distinct chains IL-13Rα1 and IL-13Rα2 which are paired with heterodimer complex IL-4Rα and IL-4Rβ forming the main IL-4 binding protein. This complex acts as a transcription factor activator. Binding triggers cell signals which lead to activation of transcription factors. Cytoplasmic areas of both type II receptors IL-13Rα1 and IL-4Rα chains communicate with different tyrosine kinases, which eventually interact with PI3 kinase and STAT6. Endocytosis is, however, triggered only by additional binding of IL-13 to the IL-13Rα2 chain, found only in type IL-13R. Studies showing high levels of IL-13Rα2 discovered within the intracellular vesicles confirmed that this form IL-13R is in endocytic conduct. In addition, affinity of IL-13 for IL-13Rα2 is higher when compared to IL-13Rα1. IL-13Rα1 activates intracellular signaling in tumor cells, endothelial cells, fibroblasts, and immune cells via Janus-activated kinase/signal transducers and transcription pathway activators. In addition, these ILs are powerful signaling pathway activators that control cell survival and encourage chemotherapy resistance. Therefore, in conjunction with cytotoxic agents, targeting inflammatory cytokines in GBM microenvironment with kinase inhibitors could bring therapeutic benefits to patients with persistent GBM [51].

3.3.3 Recognition Domain of Interleukin Receptor

The extracellular portions of IL-13Rα1 and IL-13Rα2 are composed of three fibronectin type III domains D1, D2, and D3. Each domain includes 100 amino acid residues, developing a β sandwich structure. Cysteine residues in fibronectin domain form two disulfide bonds that play a major role in positioning and binding of ligands to receptor. Domains D2 and D3 comprise the cytokine receptor homology module, a common structure of cytokine receptor family. However, there has been no information about the role of D1 domain in IL-13Rα1 and IL-13Rα2 binding to IL-13 and critical amino acids of cytokine receptor homology modules of IL-13Rα1 and IL13Rα2 [52]. IL13Rα2 gene encodes for a 380-amino-acid protein that consist of a 26-amino-acid signaling sequence and a short 17-amino-acid intracellular domain. This expresses about 30,000 binding sites for IL-13 in brain cancer.

3.3.4 Binding of Ligand with Interleukin Receptor Pathway

The IL-13 ligand binds at diverse cytokine receptor homology sites in IL13Rα1 compared with IL13Rα2; and furthermore, IL13Rα1 binding requires heterodimerization with IL4Rα for a high-affinity bond. A definite sequence between the glutamic acid at position 9 of IL-4 and that at position 13 of IL-13 is believed to be involved in the binding of IL-13 to IL4Rα. IL-13 first gets bound to IL-13Rα1 chain through interactions involving amino acid residues namely, arginine, lysine, and glutamic acid on IL-13 αD helix; this interaction generates an IL-4Rα chain to bind

to IL-13/IL-13Rα1 complex at residues on IL-13 helices completing type II IL-13R formation. Once IL-13 is linked to equal receptor chains, its affinity improves dramatically, suggesting binding. Internalization occurs through a distinctive clathrin-independent endocytosis pathway. IL-13Rα2 is a membrane-bound protein with high affinity to IL-13. Furthermore, it was found that only interactions involving IL-13 and IL-13R could cause internalization owing to existence of the distinctive IL-13Rα2 chain of receptors [53].

3.3.5 Ligand for Interleukin Receptors

IL-13 receptors are recognized as a prospective target for glioblastoma of high grade. IL-13 ligands have four alpha helices of which helix D is mainly accountable for its IL-13Rα2 interaction. Additional mutations of arginine to aspartic acid, serine to aspartic acid, and lysine to arginine at various positions enhance specificity of IL-13 ligand to IL-13RA2.

3.4 Lactoferrin Receptors in Brain Cancer

3.4.1 Occurrence of Lactoferrin Receptors

Lactoferrin (Lf) plays numerous physiological roles especially in host defenses against infections and serious inflammation. This wide range of biological activities is based on Lf's interaction with a large number of cells, which mediates the absorption of Lf into cells. It occurs not only in distinct species on the BBB, but also on glioma cell surface, making it a prospective cascade-targeting ligand for brain cancer. Lf's adsorption is controlled by LDL, which are excessively expressed in glioma cells, allowing Lf to mediate transcytosis of carriers into glioma cells.

3.4.2 Structure of Lactoferrin Receptor

Lactoferrin (Lf) is a transferrin (Tf) family mammalian cationic iron-binding glycoprotein. It shows the presence of two identical lobes that are bound to each other by an extended helical loop that is a highly sensitive site for protease cleavage. Lactoferrin constitutes a 25 amino acid peptide that includes two cysteine residues connected by a disulfide bridge and also contains positively charged hydrophobic residues. Lf exists in iron-free form in biological fluids making them susceptible to proteolysis [54].

3.4.3 Binding of Ligand and Lactoferrin Receptor Pathway

The cerebral lactoferrin receptor has two types of binding sites [55]. The endogenous Lf is lower than the receptor's peak binding constant, and hence cannot bind in sizeable amount to LfR receptor. It is unable therefore to competitively inhibit exogenous lactoferrin. LRP 1 receptor, belonging to the LDL receptor family engaged in internalizing and subsequent degradation of remaining chylomicron particles, mainly internalizes Lf. It is found that Lf is physically associated with cluster of differentiation 14 (CD14), lipopolysaccharide (LPS), and lipopolysaccharide-binding protein (LBP), which signifies interaction of Lf with accessory molecules involved in the Toll-like receptor (TLR4) pathway [56]. LfR located on the plasma membrane is responsible for the uptake of Lf through clathrin-mediated and caveolae-mediated endocytosis [57].

3.4.4 Ligand for Lactoferrin Receptors

The major ligand binding to LfR is lactoferrin. Thus, the different ligands of Lf are categorized based on the different species in which they bind to different tissues. These include human Lf (hLf), bovine Lf (bLf), mouse Lf (mLf), and piglet Lf (pLf) [54].

4 Receptor-Mediated Targeting Strategies

Developments in chemotherapy, radiotherapy, and surgery have resulted in several benefits for brain tumor treatment. While they have demonstrated significant advantages beneficial for primary cancers, brain cancers of metastatic origin demand newer therapeutic options to improve survival rates and quality of life. Novel promising approaches intended for brain cancer therapy should focus on increasing drug accumulation at the tumor site while ensuring minimal toxic effects to other parts of the brain. The overexpression of different receptors as discussed above with continuous changes in tumor microenvironment and vascular characteristics are features that can drive such developments. Among many approaches, receptor-mediated endocytosis which is the focus of this chapter is defined as a mature strategy for targeted brain delivery [58] with high specificity, affinity, and selectivity. Nanotechnology strategies in particular elicit great promise for receptor-mediated delivery [59]. The different targeting approaches are detailed in the following text.

4.1 Prodrugs

Prodrugs are inactive molecules consisting of inert moieties generally lipophilic, such as fatty acids, glycerides, or phospholipids which are covalently bonded to the parent drug moiety. This renders the molecule the desired lipophilicity to cross the BBB. On reaching the brain through blood, the active agent is released by reaction with enzymes present on the surface of blood–brain barrier [60]. The prodrug approach enables delivery of hydrophilic drugs to CNS without modification in their pharmacological activity [61]. A lactoferrin-modified pH-sensitive prodrug comprising hyaluronic acid-doxorubicin exhibited highest cytotoxicity to C6 cells and great promise in C6 glioma-bearing nude mice model.

Different techniques are employed for prodrug targeting such as prodrugs selective for hypoxia, antibody-directed enzyme prodrug therapy (ADEPT), gene-directed enzyme prodrug therapy (GDEPT), and virus-directed enzyme prodrug therapy (VDEPT). All these strategies can be used for prodrug targeting with optimized treatment of brain tumor. For greater details, readers are directed to the following review [62].

4.2 Antibody–Drug Conjugates

Antibody–drug conjugates (ADC) are basically antibodies linked with a cytotoxic drug, which allows immediate delivery of the payload to tumor cells based on the specificity of the targeted antibody. The critical factors in development of antibody–drug conjugate involve the antibody, the cytotoxic drug, and linker that joins the other two moieties to form the antibody–drug conjugate. Antibody attached must be specific for an antigen which is overexpressed selectively on cancer cells in comparison to normal cells [63]. The conjugate must exhibit adequate stability in the blood to enable delivery at the target site [64]. Conjugates can be classified as either cleavable, where the chemical bond between drug and linking site on antibody is cleaved intracellularly [63] or noncleavable where the conjugate itself is released inside cells, and the drug released by proteolytic degradation within the cell lysosome [65]. The selection of optimal concentration of antibody, ideal linker, and optimal ratio of drug linked to the antibody are key factors to maximize efficacy of the conjugates [66]. Immunotoxins have generally been delivered as ADCs. The toxins evaluated include bacterial toxins such as diphtheria toxin, and *Pseudomonas aeruginosa* exotoxin A (PE) which are uptaken by receptor-mediated endocytosis. Inside the lysosomal compartments, these immunotoxins get degraded, causing the toxin payload to be released [67]. Studies have revealed that systemically administered drugs are delivered in higher concentrations by the anti-EGFR ADCs in glioma. Advancement in ADC technology has increased their demand in the treatment of patients with glioblastoma [68]. Nevertheless, because of their elevated specificity, resistance to ADC is likely to occur as a consequence of tumor heterogeneity,

which needs to be addressed by including other strategies simultaneously in glioma treatment. The major benefit with ADC is their ability to cross the BBB which is a key requirement for promising therapeutic outcomes.

4.3 Nanocarriers for Brain Targeting

Nano-drug delivery systems have great potential for targeting drugs to brain by crossing BBB [69]. Various nanosystems explored are liposomes, nanoparticles, nanosuspensions, nanoemulsions and microemulsions, micelles, and others [70]. Such systems when designed to have sizes <100 nm or are surface-modified to escape detection by the reticuloendothelial system (RES) can reach the BBB in adequate concentration for delivery into the brain. The structure and characteristics of nanoparticles can be modified to carry active therapeutic molecules of different physicochemical properties that can control release pattern of drugs in the tumor cell [71].

4.3.1 Liposomes

Liposomes are described as a potential system for better therapeutic impacts in glioma therapy. Their distinctive physicochemical features and high level of safety have resulted in their investigation as carriers for glioma therapy. Daunomycin on conjugation with monoclonal OX26 antibody-loaded PEG sterically stabilized liposome had a prominent effect on the brain targeting [72]. A significant rise in distribution volume at a steady state was associated with high brain tissue accumulation of OX26 immunoliposomes. Liposome with topotecan modified using Tamoxifen and wheat germ agglutinin designed for dual-targeting strategy showed a substantial enhancement in brain tumor-bearing rats' general survival relative to free liposomes [73]. A concise summary of the liposome-based receptor-targeted therapies for brain cancer using other ligands is described in Table 2.1.

4.3.2 Polymeric Nanoparticles

Salient features of nanoparticles include better solubility, nanosize, greater opportunities for surface modification, and multifunctionality which could increase capability of particles to interact with cellular functions in new ways [86]. Nanoparticles for brain delivery have been designed to target various receptors on the BBB. In most cases, the cascade receptor strategy has been relied on. Table 2.2 depicts studies on ligand-mediated targeted delivery.

Table 2.1 Liposomes for targeted delivery in brain cancer

Ligand/nanosystem	Study outcome	Ref.
ApoE peptide/curcumin-loaded sphingomyelin, cholesterol, mal-PEG-PhoEth liposome	Show prominent antiproliferative effect by suppressing C6 glioma cell proliferation	[74]
Apolipoprotein-A1/doxorubicin-loaded DSPE, PEG, DPPC, DPPG liposome	Improved penetration of doxorubicin through BBB cells in U87 cells	[75]
Lactoferrin/doxorubicin-loaded egg phosphotidylcholine, cholesterol procationic liposome	Significant cytotoxic effects on C6 glioma and prolonged survival time compared with other groups	[76]
Lactoferrin/DSPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{3400}-MAL PEGylated liposome	Three times better uptake efficiency in vitro and two-fold higher brain uptake in vivo	[77]
Interleukin-13/doxorubicin-loaded DPPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{2000} maleimide liposome	Significant reduction in the intracranial tumor volume in tumor model	[78]
cRGD/PTX-loaded SPC, cholesterol, DSPE-PEG_{2000}-OMe, DSPE-PEG_{2000}-Mal liposome	Median survival time of tumor-bearing mice was significantly longer	[79]
Transferrin RGD/paclitaxel-loaded SPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{2000}-RGD liposome	Distribution occurs prominently in glioma cells than normal brain cells with higher cytotoxic effects on C6 glioma cells	[80]
Transferrin/cisplatin-loaded DSPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{2000}-COOH liposome	Higher potent cytotoxic action on C6 glioma cell with IC_{50} values four times lower than free drug	[81]
Transferrin/vincristine- and tetrandrine-coloaded EPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{2000}-NHS liposome	Significantly prolonged elimination half-life and highest median survival time in glioma-bearing mice	[82]
Transferrin/daunorubicin-loaded EPC, cholesterol, PEG_{2000}-DSPE, COOH-PEG_{2000}-DSPE, NH_2-PEG_{2000}-DSPE liposome	Strongest inhibitory effect of C6 glioma cells with IC_{50} value 1.39 times lower than free daunorubicin and prolonged survival time in tumor-bearing rats	[83]
Transferrin folate/doxorubicin-loaded DSPC, cholesterol, DSPE-PEG_{2000}, DSPE-PEG_{2000}-COOH, DSPE-PEG2000-F liposome	Longer median survival time as compared to free drug, with less average tumor volume in C6 glioma brain tumors with highest apoptotic activity	[84]
Folate/doxorubicin-loaded DSPC, cholesterol, DSPE-PEG_{2000} liposome	Higher doxorubicin content in the tumor compared to normal brain tissue	[85]

4.3.3 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles comprise solid lipids and are particularly efficient in carrying hydrophobic drugs. When modified with liquid lipids to enhance drug loading and improve stability, they are called as nanostructured lipid carriers. Such lipidic nanocarriers demonstrate a greater capacity to be trapped by the reticuloendothelial system (RES). However, modifying specific primary features including size and surface can impart stealth feature to enable brain targeting and enhanced therapeutic efficacy. Size preferably less than 200 nm, sphericity, and appropriate deformability

Table 2.2 Polymeric nanoparticles for targeted delivery in brain cancer

Ligand/nanosystem	Study outcome	Ref.
Low-density lipoprotein receptor		
Peptide-22/paclitaxel-loaded PEG-PLA NP	Apoptosis of C6 glioma cells and prolonged median survival time of glioma-bearing mice	[87]
Polysorbate-80/gemcitabine poly-butyl-cyanoacrylate NP	Significantly decreased proliferation rate in a dose-dependent manner on C6 glioma cells, with longer survival time in tumor-bearing rats	[88]
Polysorbate-80/bacoside-A-loaded PLGA NP	Tenfold more bacoside-A delivery into the brain when compared to the free drug solution	[89]
ApoE/human serum albumin NP	Involvement of LDLR family members in cellular binding of modified nanoparticles compared to that of unmodified in crossing BBB	[90]
Lactoferrin receptor		
Lactoferrin/doxorubicin-loaded bovine serum albumin mPEG2000 NP	Enhanced cytotoxicity in C6 glioma cell lines with a significant increase in the accumulation of DOX in the brain	[91]
Lactoferrin/curcuminoid polysaccharide (chitosan/hyaluronic acid/PEG_{2000}) NP	Suggested good safety profile of coated formulation in BCECs and C6 cells with significantly lower IC_{50} values	[92]
Integrin and interleukin receptor		
RGD/docetaxel-loaded DSPE-PEG_{2000}–PLGA hybrid NP	Shows strongest inhibitory effect on the proliferation of C6 cells with significantly prolonged survival time in GBM-bearing rats	[93]
I_6P_7 peptide/succinoyl tetraethylenepentamine-histidine DNA NP	Significantly prolonged the survival time of orthotopic U87 glioma-bearing mice	[94]
RGD/Interleukin-13/PEG-PCL NP	3.82-fold uptake than that of plain NPs, with most of particles transported to GBM site	[95]
Transferrin receptor		
Transferrin/methotrexate polysorbate 80-coated PLGA NP	Significant reduction in tumor volume in C6 glioma tumor-bearing rat model which was consistent with higher in vitro cytotoxicity	[96]
Transferrin/temozolomide-loaded PEGylated NP	Higher in vitro cytotoxicity with about 63% growth inhibitions at concentration of 100 μg/ml in brain	[97]
Transferrin/doxorubicin- and paclitaxel-loaded magnetic silica PLGA NP	1.8-fold reduction in IC_{50} value in U-87 cells with high fluorescence signal in vivo at the brain tumor site in U-87 tumor-bearing mice.	[98]
Transferrin/capecitabine-loaded magnetic dextran-spermine NP	Higher cytotoxic effect of the modified formulation on the U87 glioma cell lines	[99]
Transferrin/fluorescein-loaded magnetic NP	Enhanced targeting to brain by crossing BBB and diffuse into brain neurons, and distribute in the cytoplasm, dendrites, axons, and synapses of neurons	[100]
Transferrin/PLGA NP	In vivo targeting of healthy brain was found higher with Tf-NPs	[101]

Table 2.3 SLN/NLC for targeted delivery in brain cancer

Ligand/nanosystem	Study outcome	Ref.
Solid lipid nanoparticles		
Angiopep-2/docetaxel-loaded GMS, SL, stearic acid SLN	Enhanced inhibition and greater induction of apoptosis in U87MG and GL261 cell lines	[104]
ApoE/resveratrol-loaded cetyl palmitate SLN	Higher concentration induces apoptosis and mitochondrial dysfunction, significant decrease in glioma cell viability	[105]
Aprotinin-melanotransferrin/doxorubicin-loaded compritol 888, tripalmitin, stearic acid SLN	Enhanced ability of DOX to infuse the BBB and to inhibit the growth of GBM	[106]
Melanotransferrin/tamoxifen and etoposide-loaded DPPC, cocoa butter, hexadecanoic acid, DSPE-PEG_{2000} SLN	Higher efficiency in permeating the BBB and docking melanotransferrin on GBM.	[107]
Transferrin/temozolomide-loaded HSPC, DSPE, cholesterol, triolein SLN	Enhanced uptake of Tf-SLNs in brain tissue compared with unconjugated SLNs.	[108]
Anti-EGFR/doxorubicin-loaded cocoa butter, stearic acid, HTMAB, DSPE-PEG_{2000} SLN	High specificity in recognizing EGFR on U87MG cells and inducing growth-inhibition effect.	[109]
Nanostructured lipid carriers		
Transferrin/artemisinin-loaded cholesterol, compritol, oleic acid, stearylamine, soy lecithin NLC	Cytotoxicity on U87 cells was intensively greater than free drug at much lower concentrations.	[110]
Transferrin/paclitaxel-loaded cholesterol, triolein, stearylamine, soy lecithin NLC	Increased cellular uptake on the surface of U-87 cells and showed higher cytotoxicity.	[111]
Polysorbate 80/curcumin-loaded phosphatidyl choline, cholesterol oleate, glycerol trioleate NLC	Ex vivo imaging studies confirmed 2.38 times greater accumulation in brain.	[112]
RGD/temozolomide-loaded 888 ATO, Cremophor ELP, SPC NLC	IC_{50} value ten-fold over free drug, with four times higher tumor growth inhibition.	[113]
Lf-RGD/temozolomide and vincristine-coloaded SPC, 888 ATO, Cremophor ELP NLC	Highest tumor growth rate inhibition in U87 and T98G glioma cell lines, inhibited tumor growth in tumor xenograft mouse model	[114]

are essential characteristics to guarantee escape from primary RES organs like the liver and spleen. The surface coating of developed SLN with specific proteins like Apo E, Apo C-II, immunoglobulin, and albumin can prevent opsonization and hence play a critical role in site-specific targeting to brain. Several studies confirmed that SLN can penetrate more efficiently into the cytoplasm of cancer cells with in vitro evaluations on respective U87 human glioblastoma and U373 human astrocytoma cell lines [102]. Angiopep-2, an LRP 1 receptor ligand overexpressed in endothelial cells of both brain and glioma, was grafted to SLN surface for improved delivery of docetaxel. In vivo studies of SLN in glioblastoma-induced C57BL/6 mice model exhibited a considerable targeting effect [103]. A brief summary of different SLN/NLC used for receptor-mediated targeting is provided in Table 2.3.

Table 2.4 Polymeric micelles for targeted delivery in brain cancer

Ligand/nanosystem	Study outcome	Ref.
Candoxin/paclitaxel-loaded modified PEG-PLA micelles	Significantly longer median survival in intracranial U87 glioblastoma-bearing nude mice	[117]
Angiopep-2/PEG-PLA micelles	Highest uptake in brain was obtained at 0.5 h postinjection	[118]
Cyclic RGD/docetaxel-loaded mPEG-PLA micelles	Showed marked antiglioma activity in U87MG malignant glioma tumor	[119]
c(RGDyK)/paclitaxel-loaded-PEG PLA micelle	Increased intracranial tumor accumulation with 2.5 times enhanced cytotoxicity in U87MG human glioblastoma cells	[120]
c(RGDyK)/doxorubicin and Paclitaxel coloaded pluronic micelles	Induced late cell apoptosis with higher in vitro cytotoxicity on the proliferation U87MG cells	[121]
Transferrin c[RGDfK]/paclitaxel-loaded hybrid micelles	Show most potent G2/M arrest in the cell cycle of U-87 MG glioma cells and prolonged survival time of 42 days in glioma-bearing mice	[122]
Transferrin/paclitaxel-loaded polyphosphoester hybrid micelles	1.8 times higher accumulation in brain than unmodified micelles and prolonged the survival time of intracranial U-87 MG glioma-bearing mice	[123]
Transferrin/docetaxel-loaded TPGS-chitosan micelles	IC_{50} values demonstrated 248 folds more effective than docetaxel after 24 h treatment on C6 glioma cells	[124]
Transferrin-biotin/PEG-PLA copolymeric micelles	Accumulation in tumors observed in brain sections from C6 glioma tumor-bearing rat model	[125]
Folic acid/doxorubicin-loaded pluronic P105 micelles	Dual-targeting causes increase in toxicity to the C6 glioma cells with 7.5 times decrease in tumor volume than unmodified micelles	[126]

4.3.4 Polymeric Micelles

Polymer micelles, core–shell nanoparticles formed by the self-assembly of block copolymers, are broadly accepted as promising nanocarriers for targeted brain cancer therapy. Studies have shown that anti-TF antibody fragment-conjugated micelles containing epirubicin and DACHPt have been effectively internalized by TF-overexpressing cancer cells and have shown higher in vitro and in vivo antitumor activity [115]. Antibody and its fragments are beneficial as targetable ligands for active targeting of micelles. Immunomicelles conjugated with antibodies were developed for targeting EGFR receptors [116]. A brief summary of various polymeric micelles used for receptor-based targeted delivery for brain cancer is given in Table 2.4.

4.3.5 Microemulsions and Nanoemulsions

Microemulsions and nanoemulsions are characterized by small globule size (<100 nm). While microemulsions are thermodynamically stable and spontaneously forming, nanoemulsions require energy input and exhibit kinetic stability.

Table 2.5 Microemulsions/nanoemulsions for targeted delivery in brain cancer

Ligand/nanosystem	Study outcome	Ref.
Polysorbate 80/piperine-loaded microemulsion	Enhanced delivery to the brain resulting in better therapeutic outcome compared to the free drug	[129]
Polysorbate 80/ Cefuroxime-loaded nanoemulsion	Improved pharmacokinetic profile in vivo as compared to the free cefuroxime solution and enhanced cefuroxime delivery to the brain	[130]
Polysorbate 80/indinavir-loaded nanoemulsion	Significantly higher brain concentration than produced by administration of a drug solution or control nanoemulsion	[131]
Flax seed oil/saquinavir–loaded nanoemulsion	Oral bioavailability and distribution to the brain were significantly enhanced	[132]

Functionalization of microemulsions and nanoemulsions with ligands for specific receptors can aid in targeted delivery as confirmed by targeting studies. Microemulsions and nanoemulsions coated with apolipoprotein E as ligand on surface would be taken into cells through receptor-mediated endocytosis and release active agent targeting tumor cells. The high concentration of DHA in capillary endothelium of brain indicates that DHA is absorbed from diet by DHA transporters including particular fatty acid-binding lipoprotein transporters. Therefore, DHA introduced the option of overcoming the BBB through receptor-mediated endocytosis (RME). Microemulsions with DHA-rich oil hence could be a constructive vehicle for the solubilization, increased bioavailability, and improved drug delivery to the brain. Curcumin microemulsion displayed enhanced targeting efficiency to brain due to DHA-mediated transport overcoming BBB with prolonged drug retention in the brain [127]. A novel microemulsion formulation was developed using folate ligand for tumor targeted therapy with antibiotic aclacinomycin [128]. The study proved that folate alteration on emulsions is an efficient way to target tumor cells in the brain. A brief summary of microemulsions/nanoemulsions used for receptor-based targeted delivery for brain cancer is given in Table 2.5.

4.4 Dendrimers

Dendrimers are an evolving class of hyper-branched macromolecules that confer distinctive characteristics such as important molecular size control, elevated branching density, nanoscale size, and elevated surface functionality. HAIYPRH is a peptide having high affinity for Tf receptor and has been functionalized with PEGylated PAMAM dendrimers loaded with doxorubicin [133]. Compared to free drug, the complex exhibited elevated internalization indicating targeting of brain tumor cells. A brief summary of various dendrimers used for receptor-based targeted delivery for brain cancer is given in Table 2.6.

Table 2.6 Dendrimers for targeted delivery in brain cancer

Ligand/Nanosystem	Study outcome	Ref.
Transferrin/PEG-PAMAM DNA dendrimer	Median survival time of rats (28.5 days) was longer than that of rats treated with Unmodified (25.5 days) and temozolomide (24.5 days).	[134]
Transferrin/PEGylated poly(amidoamine) dendrimer	Delivered 13.5% of DOX in a period of 2 h, demonstrating an enhanced transport ratio as compared to ratio of free DOX	[135]
Transferrin/PEG-PAMAM-DNA dendrimer	Brain gene expression of the complex was two-fold higher than unmodified complexes.	[136]
Transferrin/doxorubicin, tamoxifen-coloaded PEG-PAMAM dendrimer	In vitro accumulation in the avascular C6 glioma spheroids made the tumor volume effectively reduced	[137]
Transferrin/polypropylenimine DNA complexed dendrimer	Gene expression was three-fold higher in the brain and was at its highest at 24 h following the injection of the treatments.	[138]
OX26/PEG-G4.5-DPDPE dendrimers	Enhanced permeation suggesting CNS therapeutic properties.	[139]
Transferrin/temozolomide-loaded PAMAM-PEG dendrimer	Inhibited tumor growth along with delayed tumor recurrence in orthotopic glioma mice model	[140]
Angiopep/PAMAM-PEG-DNA dendrimer	Higher efficiency in crossing BBB than unmodified NPs confirmed by in vitro BBB model, and accumulated in brain more in vivo	[141]
Lactoferrin/polypropylenimine dendriplex-DNA plasmid	Increased gene expression in the brain, by more than 6.4-fold compared to that of DAB dendriplex	[142]
RVG29/PAMAM-PEG DNA dendrimer	Gene expression was observed in the brain, and significantly higher than unmodified NPs	[143]

4.5 Aptamers

AS1411 aptamer and a phage-displayed TGN peptide, that are particular BBB and cancer cell ligands, were respectively combined with nanoparticles to develop a cascade delivery system (AsTNP) for brain glioma. In vivo imaging showed maximum tumor distribution was achieved by AsTNP [144]. Two ssDNA aptamers, GBM 128 and GBM 131, exhibited selective binding to U118-MG glioma cells, and in segments of human tissues, these aptamers revealed binding only to glioma tissue and not to regular brain tissue, suggesting possible diagnostic applications of these aptamers [145]. Nanoparticles decorated with AS1411 (Ap) exhibited high binding to nucleolin highly expressed in plasma membrane of tumor cells, to facilitate delivery of paclitaxel (PTX). The Ap-PTX-NP displayed longer circulation time and increased accumulation of PTX at tumor site, which eventually resulted in considerably prominent tumor inhibition in mice carrying C6 glioma xenografts and prolonged survival rates in rats carrying intracranial C6 gliomas compared to PTX-NP and Taxol [146].

4.6 Carbon Nanotubes and Carbon Dots

Carbon nanotubes and carbon dots are promising method for selective delivery of therapeutic moiety at the tumor cells. Multiwalled PEGylated carbon nanotubes integrated with angiopep-2 as multitargeting delivery system for therapy of brain glioma proved that DOX accumulation in glioma for DOX-ANG was more than control DOX at 2 h postinjection and maintained up to 24 h postinjection [147]. Multiwalled carbon nanotubes with iron oxide magnetic nanoparticles and loaded with doxorubicin exhibited a significant decrease in IC_{50} compared to that of plain DOX [148]. Studies on carbon dots suggest its ability to cross the BBB when modified with transferrin, showing that the transport occurs through transferrin receptor-mediated transcytosis which was confirmed by the zebrafish model [149]. Further conjugates of carbon dots loaded with doxorubicin were functionalized using transferrin for delivery of doxorubicin to treat pediatric brain tumors. The conjugates show significantly more cytotoxic effect on CHLA-266, SJGBM2, and CHLA-200 brain tumor cell lines as compared to doxorubicin alone [150].

4.7 Diagnostics and Theranostics

4.7.1 Diagnostics

The utilization of macromolecular agents focused on dendrigraft poly-L-lysines (DGLs) with chlorotoxin (CTx) as ligand proved promising results in field of clinical diagnosis of brain tumors. Results revealed that mice treated with CTx-modified contrast had signal enhancement which reached peak level at 5 min for glioma, considerably higher than control [151]. Study on transferrin-conjugated superparamagnetic iron oxide nanoparticles proved considerable contrast enhancement of brain glioma following 48 h postinjection, suggesting Tf-SPIONs as a prospective targeting MR contrast agent for brain glioma [152]. Another work on lactoferrin-conjugated superparamagnetic iron oxide nanoparticles revealed appropriate diagnosis of brain tumors with enhanced signal intensity [153]. Nanoparticles attached with multiple imaging/targeting agents for optical imaging enables higher sensitivity, quicker acquisation time and lesser running cost than MRI. G5 dendrimer labelled with angiopep-2 through a polyethylene glycol linker showed a higher fluorescence intensity and significantly high T/N (tumor/normal tissue) ratio of nanoprobes in brain [154]. RGD peptide-labelled quantum dots administered in glioma bearing mice reported to have contrast enhancement and higher T2 relaxivity signifying integrin targeted optical imaging and cancer detection [155].

4.7.2 Theranostics

Theranostics is a combined form of imaging and therapy where imaging will not only suggest the possibility to noninvasively detect tumor, but also provide quantitative approach to assess the delivery of therapy in tumor cells. A study on PEG-free porphyrin-mimicking lipoproteins demonstrated the possibility for intraoperative fluorescence-guided surgery and tumor-specific PDT [156]. Dual-targeted (EGFpep+Tfpep)-AuNPs-Pc 4 nanoparticles with a photosensitizer phthalocyanine 4 revealed high cellular association and increased cytotoxicity with in vivo studies proving accumulation of nanoparticles in tumor regions [157]. ApoE3-dependent release of porphyrin from lipid nanoparticles in orthotopic U87-GFP tumor-bearing animals revealed reduction in glioblastoma with selective uptake of porphyrin in malignant tissue [158]. Combination treatment of CTX-NP-siMGMT with chlorotoxin as ligand resulted in reduction of tumor growth that was detected by MRI [159]. SLNs conjugated with c(RGDyK) was developed as carriers to enhance the targeted delivery of IR-780 to the tumors. In vitro assays and in vivo PTT treatment defined the eradication of tumor by applying SLN under laser irradiation [160]. Quantum dots (QDs) and apomorphine were incorporated into liposomes to improve brain targeting. Higher fluorescence intensity was observed in mouse brains treated with liposomes compared to that of free QDs with 2.4-fold improved accumulation [161].

5 Receptor-Mediated Delivery in Clinical Trials

The primary therapy for high-grade brain cancer patients is multimodal, including tumor removal followed by radiation and chemotherapy. Research on novel molecularly targeted therapies reflects an improvement in treatment of patients with brain cancer. Targeted chemotherapeutic agent treatment in conjunction with carrier ligands has progressed with some conjugates reaching clinical trials for treating malignant brain tumors. Receptor-mediated delivery of drugs targeting brain cancer in clinical trials is depicted in Table 2.7.

6 Advantages and Limitations

Brain cancer continues to be a deadly disease. Nevertheless, the challenges in the treatment of brain cancer through chemotherapy can find viable solutions through receptor-mediated targeting approaches. The multiple receptors and variety of ligand possibilities coupled with advancements in nanotechnology can harness existing drugs for significantly improved therapy. Nevertheless, directing drugs to the brain in high concentration must be handled with caution. A sensitive organ like the brain may be deleteriously affected if drug concentrations are not titrated to

Table 2.7 Drugs under clinical trials for targeting brain cancer

Conditions	Study title	Clinical trial status	Reference
Brain and central nervous system tumors	Cytotoxic T cells and interleukin-2 in treating adult patients with recurrent brain tumours	Phase I	[162]
Brain metastases Breast cancer	ANG1005 in breast cancer patients with recurrent brain metastases	Phase II	[163]
Gliomas Anaplastic astrocytoma Anaplastic oligodendroglioma	Cellular immunotherapy study for brain cancer	Phase I	[164]
Glioblastoma	IL-4(38–37)-PE38KDEL immunotoxin in treating patients with recurrent malignant astrocytoma	Phase I	[165]
Glioblastoma multiforme	Study of therapy with TransMID™ compared to best standard of care in patients with glioblastoma multiforme	Phase III	[166]
Brain tumor Recurrent brain tumor Refractory	T cells expressing HER2-specific chimeric antigen receptors(CAR) for patients with HER2-positive CNS tumours	Phase I	[167]
Brain and central nervous system tumors	Chemotherapy and vaccine therapy followed by bone marrow or peripheral stem cell transplantation and Interleukin-2 in treating patients with recurrent or refractory brain cancer	Phase II	[168]
Adult brain glioblastoma Adult gliosarcoma Recurrent brain neoplasm	EGFRBi-armed autologous T cells in treating patients with recurrent or refractory glioblastoma	Phase I/ phase II	[169]
Glioblastoma	A study of ABT-414 in subjects with newly diagnosed glioblastoma (GBM) with epidermal growth factor receptor (EGFR) amplification	Phase III	[170]

remain at safe concentration. Such off-site brain toxicity is to be handled with caution.

7 Conclusion and Critical Comments

Receptor-mediated endocytosis is a promising approach for improved therapy of brain cancer, provided toxicity concerns are appropriately addressed.

References

1. Wrensch M, Minn Y, Chew T, Bondy M, Berger MS. Epidemiology of primary brain tumors: Current concepts and review of the literature. Neuro Oncol. 2002;4(4):278–99.
2. Deeken JF, Loscher W. The blood-brain barrier and cancer: transporters, treatment, and Trojan horses. Clin Cancer Res. 2007;13(6):1663–74.
3. Pardridge WM. Drug transport across the blood–brain barrier. J Cereb Blood Flow Metab. 2012;32(11):1959–72.
4. Dong X. Current strategies for brain drug delivery. Theranostics. 2018;8(6):1481–93.
5. Lalatsa A, Butt AM. Physiology of the blood–brain barrier and mechanisms of transport across the BBB. In: Nanotechnology-based targeted drug delivery systems for brain tumors. Academic Press, USA. 2018;49–74.
6. Simard M, Nedergaard M. The neurobiology of glia in the context of water and ion homeostasis. Neuroscience. 2004;129(4):877–96.
7. Wong AD, Ye M, Levy AF, Rothstein JD, Bergles DE, Searson PC. The blood-brain barrier: an engineering perspective. Front Neuroeng. 2013;6:7.
8. Laksitorini M, Prasasty VD, Kiptoo PK, Siahaan TJ. Pathways and progress in improving drug delivery through the intestinal mucosa and blood–brain barriers. Ther Deliv. 2014;5(10):1143–63.
9. Salamat-Miller N, Johnston TP. Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium. Int J Pharm. 2005;294(1–2):201–16.
10. Shinde RL, Jindal AB, Devarajan PV. Microemulsions and nanoemulsions for targeted drug delivery to the brain. Curr Nanosci. 2011;7(1):119–33.
11. Karanth H, Murthy RS. Nanotechnology in brain targeting. Int. J. Pharm. Sci. Nanotechnol. 2008;1:10–24.
12. Bellettato CM, Scarpa M. Possible strategies to cross the blood–brain barrier. Ital J Pediatr. 2018;44(2):131.
13. Liu F, Li X, Zhang L-Y, Song Q-R, Zhang M, Zhao C-X, et al. Stimuli-responsive Nanocarriers for drug delivery to the central nervous system. CNANO. 2015;12(1):4–17.
14. Hervé F, Ghinea N, Scherrmann J-M. CNS delivery via adsorptive transcytosis. AAPS J. 2008;10(3):455–72.
15. Xiao G, Gan L-S. Receptor-mediated endocytosis and brain delivery of therapeutic biologics. Int J Cell Biol. 2013;2013:1–14.
16. Gabathuler R. Approaches to transport therapeutic drugs across the blood–brain barrier to treat brain diseases. Neurobiol Dis. 2010;37(1):48–57.
17. Golden PL, Maccagnan TJ, Pardridge WM. Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. J Clin Investig. 1997;99(1):14–8.
18. Duffy KR, Pardridge WM. Blood-brain barrier transcytosis of insulin in developing rabbits. Brain Res. 1987;420(1):32–8.
19. Akhtar MJ, Ahamed M, Alhadlaq HA, Alrokayan SA, Kumar S. Targeted anticancer therapy: overexpressed receptors and nanotechnology. Clin Chim Acta. 2014;436:78–92.
20. Wei X, Chen X, Ying M, Lu W. Brain tumor-targeted drug delivery strategies. Acta Pharm Sin B. 2014;4(3):193–201.
21. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65(1–2):271–84.
22. Maeda H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. Bioconjug Chem. 2010;21(5):797–802.
23. Wang Y-Y, Lui PC, Li JY. Receptor-mediated therapeutic transport across the blood–brain barrier. Immunotherapy. 2009;1(6):983–93.
24. Lajoie JM, Shusta EV. Targeting receptor-mediated transport for delivery of biologics across the blood-brain barrier. Annu Rev Pharmacol Toxicol. 2015;55(1):613–31.
25. Zhang B, Sun X, Mei H, Wang Y, Liao Z, Chen J, et al. LDLR-mediated peptide-22-conjugated nanoparticles for dual-targeting therapy of brain glioma. Biomaterials. 2013;34(36):9171–82.

26. Xu J, Potenza MN, Calhoun VD. Spatial ICA reveals functional activity hidden from traditional fMRI GLM-based analyses. Front Neurosci. 2013;7:154.
27. Brown M, Goldstein J. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986;232(4746):34–47.
28. Li Y, Cam J, Bu G. Low-density lipoprotein receptor family. Mol Neurobiol. 2001;23:15.
29. Prassl R, Laggner P. Molecular structure of low density lipoprotein: current status and future challenges. Eur Biophys J. 2009;38(2):145–58.
30. Nikanjam M, Blakely EA, Bjornstad KA, Shu X, Budinger TF, Forte TM. Synthetic nano-low density lipoprotein as targeted drug delivery vehicle for glioblastoma multiforme. Int J Pharm. 2007;328(1):86–94.
31. Cassidy SM, Strobel FW, Wasan KM. Plasma lipoprotein distribution of liposomal nystatin is influenced by protein content of high-density lipoproteins. Antimicrob Agents Chemother. 1998;42(8):1878–88.
32. Lee CK, Brown C, Gralla RJ, Hirsh V, Thongprasert S, Tsai C-M, et al. Impact of EGFR inhibitor in non–small cell lung Cancer on progression-free and overall survival: a meta-analysis. JNCI J Nat Cancer Inst. 2013;105(9):595–605.
33. Orlova EV, Sherman MB, Chiu W, Mowri H, Smith LC, Gotto AM. Three-dimensional structure of low density lipoproteins by electron cryomicroscopy. Proc Natl Acad Sci. 1999;96(15):8420–5.
34. Wang Y-Y, Lui PC, Li JY. Receptor-mediated therapeutic transport across the blood–brain barrier. Immunotherapy. 2009;1(6):983–93.
35. Papademetriou IT, Porter T. Promising approaches to circumvent the blood–brain barrier: progress, pitfalls and clinical prospects in brain cancer. Ther Deliv. 2015;6(8):989–1016.
36. Muro S. Challenges in design and characterization of ligand-targeted drug delivery systems. J Control Release. 2012;164(2):125–37.
37. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer. 2010;10(1):9–22.
38. Taga T, Suzuki A, Gonzalez-Gomez I, Gilles FH, Stins M, Shimada H, et al. alpha v-Integrin antagonist EMD 121974 induces apoptosis in brain tumor cells growing on vitronectin and tenascin. Int J Cancer. 2002;98(5):690–7.
39. Beer AJ. Positron emission tomography using [18F]Galacto-RGD identifies the level of integrin v 3 expression in man. Clin Cancer Res. 2006;12(13):3942–9.
40. Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 2010;339(1):269–80.
41. Humphries M. Mapping functional residues onto integrin crystal structures. Curr Opin Struct Biol. 2003;13(2):236–43.
42. Lee J-O, Bankston LA, Arnaout MA, Liddington RC. Two conformations of the integrin A-domain (I-domain): a pathway for activation? Structure. 1995;3(12):1333–40.
43. Clark E, Brugge J. Integrins and signal transduction pathways: the road taken. Science. 1995;268(5208):233–9.
44. Arnaout MA, Goodman SL, Xiong J-P. Coming to grips with integrin binding to ligands. Curr Opin Cell Biol. 2002;14(5):641–52.
45. Xiao X. Modeling gross primary production of temperate deciduous broadleaf forest using satellite images and climate data. Remote Sens Environ. 2004;91(2):256–70.
46. Shin S, Wolgamott L, Yoon S-O. Integrin trafficking and tumor progression. Int J Cell Biol. 2012;2012:1–7.
47. van der Flier A, Sonnenberg A. Structural and functional aspects of filamins. Biochim Biophys Acta Mol Cell Res. 2001;1538(2–3):99–117.
48. Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligand binding to integrins. J Biol Chem. 2000;275(29):21785–8.
49. Humphries JD. Integrin ligands at a glance. J Cell Sci. 2006;119(19):3901–3.
50. Mintz A, Gibo DM, Slagle-Webb B, Christensent ND, Debinski W. IL-13Rα2 is a glioma-restricted receptor for interleukin-13. Neoplasia. 2002;4(5):388–99.

51. Kosmopoulos M, Christofides A, Drekolias D, Zavras PD, Gargalionis AN, Piperi C. Critical role of IL-8 targeting in gliomas. Curr Med Chem. 2018;25(17):1954–67.
52. Arima K, Sato K, Tanaka G, Kanaji S, Terada T, Honjo E, et al. Characterization of the interaction between interleukin-13 and interleukin-13 receptors. J Biol Chem. 2005;280(26):24915–22.
53. Joshi BH, Kawakami K, Leland P, Puri RK. Heterogeneity in interleukin-13 receptor expression and subunit structure in squamous cell carcinoma of head and neck: differential sensitivity to chimeric fusion proteins comprised of interleukin-13 and a mutated form of pseudomonas exotoxin. Clin Cancer Res. 2002;8(6):1948–56.
54. Legrand D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J. Lactoferrin structure and functions. In: Bösze Z, editor. Bioactive components of milk. 2008. p. 163–94.
55. Suzuki YA, Lopez V, Lönnerdal B. Lactoferrin: mammalian lactoferrin receptors: structure and function. Cell Mol Life Sci. 2005;62(22):2560–75.
56. Curran CS, Demick KP, Mansfield JM. Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways. Cell Immunol. 2006;242(1):23–30.
57. Jiang R, Lopez V, Kelleher SL, Lönnerdal B. Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in caco-2 cells. J Cell Physiol. 2011;226(11):3022–31.
58. Sharma P, Debinski W. Receptor-targeted glial brain tumor therapies. Int J Mol Sci. 2018;19(11):3326.
59. Large DE, Soucy JR, Hebert J, Auguste DT. Advances in receptor-mediated, tumor-targeted drug delivery. Adv Ther. 2019;2(1):1800091.
60. Kratz F, Müller IA, Ryppa C, Warnecke A. Prodrug strategies in anticancer chemotherapy. ChemMedChem. 2008;3(1):20–53.
61. Rautio J, Laine K, Gynther M, Savolainen J. Prodrug approaches for CNS delivery. AAPS J. 2008;10(1):92–102.
62. Xu G, McLeod HL. Strategies for enzyme/prodrug Cancer therapy. Clin Cancer Res. 2001;7(11):3314–24.
63. Chari RVJ, Miller ML, Widdison WC. Antibody-drug conjugates: an emerging concept in cancer therapy. Angew Chem Int Ed. 2014;53(15):3796–827.
64. Lambert JM, Morris CQ. Antibody–drug conjugates (ADCs) for personalized treatment of solid tumors: a review. Adv Ther. 2017;34(5):1015–35.
65. Erickson HK, Widdison WC, Mayo MF, Whiteman K, Audette C, Wilhelm SD, et al. Tumor delivery and in vivo processing of disulfide-linked and thioether-linked antibody−maytansinoid conjugates. Bioconjug Chem. 2009;21(1):84–92.
66. Sharkey RM, Goldenberg DM. Use of antibodies and immunoconjugates for the therapy of more accessible cancers. Adv Drug Deliv Rev. 2008;60(12):1407–20.
67. Parakh S, Parslow AC, Gan HK, Scott AM. Antibody-mediated delivery of therapeutics for cancer therapy. Expert Opin Drug Deliv. 2016;13(3):401–19.
68. Razpotnik R, Novak N, Čurin Šerbec V, Rajcevic U. Targeting malignant brain tumors with antibodies. Front Immunol. 2017;8:1181.
69. Muntoni E, Martina K, Marini E, Giorgis M, Lazzarato L, Salaroglio I, et al. Methotrexate-loaded solid lipid nanoparticles: protein functionalization to improve brain biodistribution. Pharmaceutics. 2019;11(2):65.
70. Masood F. Polymeric nanoparticles for targeted drug delivery system for cancer therapy. Mater Sci Eng C. 2016;60:569–78.
71. Chen Y, Liu L. Modern methods for delivery of drugs across the blood–brain barrier. Adv Drug Deliv Rev. 2012;64(7):640–65.
72. Huwyler J, Wu D, Pardridge WM. Brain drug delivery of small molecules using immunoliposomes. Proc Natl Acad Sci. 1996;93(24):14164–9.
73. Du J, Lu W-L, Ying X, Liu Y, Du P, Tian W, et al. Dual-targeting topotecan liposomes modified with tamoxifen and wheat germ agglutinin significantly improve drug transport

across the blood−brain barrier and survival of brain tumor-bearing animals. Mol Pharm. 2009;6(3):905–17.
74. Re F, Cambianica I, Zona C, Sesana S, Gregori M, Rigolio R, et al. Functionalization of liposomes with ApoE-derived peptides at different density affects cellular uptake and drug transport across a blood-brain barrier model. Nanomedicine. 2011;7(5):551–9.
75. Pinzón-Daza M, Garzón R, Couraud P, Romero I, Weksler B, Ghigo D, et al. The association of statins plus LDL receptor-targeted liposome-encapsulated doxorubicin increases *in vitro* drug delivery across blood-brain barrier cells: new strategy for drug delivery into brain tumours. Br J Pharmacol. 2012;167(7):1431–47.
76. Chen H, Qin Y, Zhang Q, Jiang W, Tang L, Liu J, et al. Lactoferrin modified doxorubicin-loaded procationic liposomes for the treatment of gliomas. Eur J Pharm Sci. 2011;44(1–2):164–73.
77. Huang F-Y, Chen W-J, Lee W-Y, Lo S-T, Lee T-W, Lo J-M. In vitro and in vivo evaluation of lactoferrin-conjugated liposomes as a novel carrier to improve the brain delivery. IJMS. 2013;14(2):2862–74.
78. Madhankumar AB, Slagle-Webb B, Wang X, Yang QX, Antonetti DA, Miller PA, et al. Efficacy of interleukin-13 receptor-targeted liposomal doxorubicin in the intracranial brain tumor model. Mol Cancer Ther. 2009;8(3):648–54.
79. Shi K, Long Y, Xu C, Wang Y, Qiu Y, Yu Q, et al. Liposomes combined an integrin $\alpha_v\beta_3$ -specific vector with pH-responsible cell-penetrating property for highly effective Antiglioma therapy through the blood–brain barrier. ACS Appl Mater Interfaces. 2015;7(38):21442–54.
80. Qin L, Wang C-Z, Fan H-J, Zhang C-J, Zhang H-W, Lv M-H, et al. A dual-targeting liposome conjugated with transferrin and arginine-glycine-aspartic acid peptide for glioma-targeting therapy. Oncol Lett. 2014;8(5):2000–6.
81. Lv Q, Li L-M, Han M, Tang X-J, Yao J-N, Ying X-Y, et al. Characteristics of sequential targeting of brain glioma for transferrin-modified cisplatin liposome. Int J Pharm. 2013;444(1–2):1–9.
82. Song X, Liu S, Jiang Y, Gu L, Xiao Y, Wang X, et al. Targeting vincristine plus tetrandrine liposomes modified with DSPE-PEG 2000 -transferrin in treatment of brain glioma. Eur J Pharm Sci. 2017;96:129–40.
83. Ying X, Wen H, Lu W-L, Du J, Guo J, Tian W, et al. Dual-targeting daunorubicin liposomes improve the therapeutic efficacy of brain glioma in animals. J Control Release. 2010;141(2):183–92.
84. Gao J-Q, Lv Q, Li L-M, Tang X-J, Li F-Z, Hu Y-L, et al. Glioma targeting and blood–brain barrier penetration by dual-targeting doxorubincin liposomes. Biomaterials. 2013;34(22):5628–39.
85. McNeeley KM, Annapragada A, Bellamkonda RV. Decreased circulation time offsets increased efficacy of PEGylated nanocarriers targeting folate receptors of glioma. Nanotechnology. 2007;18(38):385101.
86. Alam MI, Beg S, Samad A, Baboota S, Kohli K, Ali J, et al. Strategy for effective brain drug delivery. Eur J Pharm Sci. 2010;40(5):385–403.
87. Zhang B, Sun X, Mei H, Wang Y, Liao Z, Chen J, et al. LDLR-mediated peptide-22-conjugated nanoparticles for dual-targeting therapy of brain glioma. Biomaterials. 2013;34(36):9171–82.
88. Wang C-X, Huang L-S, Hou L-B, Jiang L, Yan Z-T, Wang Y-L, et al. Antitumor effects of polysorbate-80 coated gemcitabine polybutylcyanoacrylate nanoparticles in vitro and its pharmacodynamics in vivo on C6 glioma cells of a brain tumor model. Brain Res. 2009;1261:91–9.
89. Jose S, Sowmya S, Cinu TA, Aleykutty NA, Thomas S, Souto EB. Surface modified PLGA nanoparticles for brain targeting of Bacoside-A. Eur J Pharm Sci. 2014;63:29–35.
90. Wagner S, Zensi A, Wien SL, Tschickardt SE, Maier W, Vogel T, et al. Uptake mechanism of ApoE-modified nanoparticles on brain capillary endothelial cells as a blood-brain barrier model. Deli MA, editor. PLoS One. 2012;7(3):e32568.
91. Su Z, Xing L, Chen Y, Xu Y, Yang F, Zhang C, et al. Lactoferrin-modified poly(ethylene glycol)-grafted BSA nanoparticles as a dual-targeting carrier for treating brain gliomas. Mol Pharm. 2014;11(6):1823–34.

92. Xu Y, Asghar S, Yang L, Li H, Wang Z, Ping Q, et al. Lactoferrin-coated polysaccharide nanoparticles based on chitosan hydrochloride/hyaluronic acid/PEG for treating brain glioma. Carbohydr Polym. 2017;157:419–28.
93. Shi K, Zhou J, Zhang Q, Gao H, Liu Y, Zong T, et al. Arginine-glycine-aspartic acid-modified lipid-polymer hybrid nanoparticles for docetaxel delivery in glioblastoma multiforme. J Biomed Nanotechnol. 2015;11(3):382–91.
94. Wang S. Antitumoral cascade-targeting ligand for IL-6 receptor-mediated gene delivery to glioma. Mol Ther. 2017;25(7):1556–66.
95. Gao H, Xiong Y, Zhang S, Yang Z, Cao S, Jiang X. RGD and interleukin-13 peptide functionalized nanoparticles for enhanced glioblastoma cells and Neovasculature dual targeting delivery and elevated tumor penetration. Mol Pharm. 2014;11(3):1042–52.
96. Jain A, Jain A, Garg NK, Tyagi RK, Singh B, Katare OP, Webster TJ, Soni V. Surface engineered polymeric nanocarriers mediate the delivery of transferrin–methotrexate conjugates for an improved understanding of brain cancer. Acta Biomater. 2015;24:140–51.
97. Jain A, Chasoo G, Singh SK, Saxena AK, Jain SK. Transferrin-appended PEGylated nanoparticles for temozolomide delivery to brain: in vitro characterisation. J Microencapsul. 2011;28(1):21–8.
98. Cui Y, Xu Q, Chow PK-H, Wang D, Wang C-H. Transferrin-conjugated magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel for brain glioma treatment. Biomaterials. 2013;34(33):8511–20.
99. Ghadiri M, Vasheghani-Farahani E, Atyabi F, Kobarfard F, Mohamadyar-Toupkanlou F, Hosseinkhani H. Transferrin-conjugated magnetic dextran-spermine nanoparticles for targeted drug transport across blood-brain barrier: transferrin-conjugated magnetic dextran-spermine nanoparticles. J Biomed Mater Res A. 2017;105(10):2851–64.
100. Yan F, Wang Y, He S, Ku S, Gu W, Ye L. Transferrin-conjugated, fluorescein-loaded magnetic nanoparticles for targeted delivery across the blood–brain barrier. J Mater Sci Mater Med. 2013;24(10):2371–9.
101. Chang J, Paillard A, Passirani C, Morille M, Benoit J-P, Betbeder D, et al. Transferrin adsorption onto PLGA nanoparticles governs their interaction with biological systems from blood circulation to brain cancer cells. Pharm Res. 2012;29(6):1495–505.
102. Brioschi AM, Calderoni S, Zara GP, Priano L, Gasco MR, Mauro A. Solid lipid nanoparticles for brain tumors therapy: state of the art and novel challenges. In: Progress in brain research. 2009;180:193–223.
103. Kadari A, Pooja D, Gora RH, Gudem S, Kolapalli VRM, Kulhari H, et al. Design of multifunctional peptide collaborated and docetaxel loaded lipid nanoparticles for antiglioma therapy. Eur J Pharm Biopharm. 2018;132:168–79.
104. Kadari A, Pooja D, Gora RH, Gudem S, Kolapalli VRM, Kulhari H, et al. Design of multifunctional peptide collaborated and docetaxel loaded lipid nanoparticles for antiglioma therapy. Eur J Pharm Biopharm. 2018;132:168–79.
105. Neves AR, Queiroz JF, Lima SAC, Reis S. Apo E-functionalization of solid lipid nanoparticles enhances brain drug delivery: uptake mechanism and transport pathways. Bioconjug Chem. 2017;28(4):995–1004.
106. Kuo Y-C, Lee I-H. Delivery of doxorubicin to glioblastoma multiforme in vitro using solid lipid nanoparticles with surface aprotinin and melanotransferrin antibody for enhanced chemotherapy. J Taiwan Inst Chem Eng. 2016;61:32–45.
107. Kuo Y-C, Wang I-H. Enhanced delivery of etoposide across the blood–brain barrier to restrain brain tumor growth using melanotransferrin antibody- and tamoxifen-conjugated solid lipid nanoparticles. J Drug Target. 2016;24(7):645–54.
108. Jain A, Singhai P, Gurnany E, Updhayay S, Mody N. Transferrin-tailored solid lipid nanoparticles as vectors for site-specific delivery of temozolomide to brain. J Nanopart Res. 2013;15(3):1518.
109. Kuo Y-C, Liang C-T. Catanionic solid lipid nanoparticles carrying doxorubicin for inhibiting the growth of U87MG cells. Colloids Surf B: Biointerfaces. 2011;85(2):131–7.

110. Emami J, Yousefian H, Sadeghi H. Targeted nanostructured lipid carrier for brain delivery of artemisinin: design, preparation, characterization, optimization and cell toxicity. J Pharm Pharm Sci. 2018;21(1s):225s–41s.
111. Emami J, Rezazadeh M, Sadeghi H, Khadivar K. Development and optimization of transferrin-conjugated nanostructured lipid carriers for brain delivery of paclitaxel using box–Behnken design. Pharm Dev Technol. 2017;22(3):370–82.
112. Meng F, Asghar S, Xu Y, Wang J, Jin X, Wang Z, et al. Design and evaluation of lipoprotein resembling curcumin-encapsulated protein-free nanostructured lipid carrier for brain targeting. Int J Pharm. 2016;506(1–2):46–56.
113. Song S, Mao G, Du J, Zhu X. Novel RGD containing, temozolomide-loading nanostructured lipid carriers for glioblastoma multiforme chemotherapy. Drug Deliv. 2016;23(4):1404–8.
114. Zhang J, Xiao X, Zhu J, Gao Z, Lai X, Zhu X, et al. Lactoferrin- and RGD-comodified, temozolomide and vincristine-coloaded nanostructured lipid carriers for gliomatosis cerebri combination therapy. Int J Nanomedicine. 2018;13:3039.
115. Ahn J, Miura Y, Yamada N, Chida T, Liu X, Kim A, et al. Antibody fragment-conjugated polymeric micelles incorporating platinum drugs for targeted therapy of pancreatic cancer. Biomaterials. 2015;39:23–30.
116. Noh T, Kook YH, Park C, Youn H, Kim H, Oh ET, et al. Block copolymer micelles conjugated with anti-EGFR antibody for targeted delivery of anticancer drug. J Polym Sci A Polym Chem. 2008;46(22):7321–31.
117. Zhan C, Li B, Hu L, Wei X, Feng L, Fu W, et al. Micelle-based brain-targeted drug delivery enabled by a nicotine acetylcholine receptor ligand. Angew Chem Int Ed. 2011;50(24):5482–5.
118. Shen J, Zhan C, Xie C, Meng Q, Gu B, Li C, et al. Poly(ethylene glycol)-block-poly(D, L -lactide acid) micelles anchored with angiopep-2 for brain-targeting delivery. J Drug Target. 2011;19(3):197–203.
119. Li A-J, Zheng Y-H, Liu G-D, Liu W-S, Cao P-C, Bu Z-F. Efficient delivery of docetaxel for the treatment of brain tumors by cyclic RGD-tagged polymeric micelles. Mol Med Rep. 2015;11(4):3078–86.
120. Zhan C, Gu B, Xie C, Li J, Liu Y, Lu W. Cyclic RGD conjugated poly(ethylene glycol)-co-poly(lactic acid) micelle enhances paclitaxel anti-glioblastoma effect. J Control Release. 2010;143(1):136–42.
121. Huang Y, Liu W, Gao F, Fang X, Chen Y. c(RGDyK)-decorated pluronic micelles for enhanced doxorubicin and paclitaxel delivery to brain glioma. Int J Nanomed. 2016;11:1629.
122. Zhang P, Hu L, Yin Q, Feng L, Li Y. Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy. Mol Pharm. 2012;9(6):1590–8.
123. Zhang P, Hu L, Yin Q, Zhang Z, Feng L, Li Y. Transferrin-conjugated polyphosphoester hybrid micelle loading paclitaxel for brain-targeting delivery: synthesis, preparation and in vivo evaluation. J Control Release. 2012;159(3):429–34.
124. Agrawal P, Sonali, Singh RP, Sharma G, Mehata AK, Singh S, et al. Bioadhesive micelles of d -α-tocopherol polyethylene glycol succinate 1000: synergism of chitosan and transferrin in targeted drug delivery. Colloids Surf B Biointerfaces. 2017;152:277–288.
125. Ren W, Chang J, Yan C, Qian X, Long L, He B, et al. Development of transferrin functionalized poly(ethylene glycol)/poly(lactic acid) amphiphilic block copolymeric micelles as a potential delivery system targeting brain glioma. J Mater Sci Mater Med. 2010;21(9):2673–81.
126. Niu J, Wang A, Ke Z, Zheng Z. Glucose transporter and folic acid receptor-mediated Pluronic P105 polymeric micelles loaded with doxorubicin for brain tumor treating. J Drug Target. 2014;22(8):712–23.
127. Shinde RL, Devarajan PV. Docosahexaenoic acid–mediated, targeted and sustained brain delivery of curcumin microemulsion. Drug Deliv. 2017;24(1):152–61.
128. Muzaffar F, Singh UK, Chauhan L. Review on microemulsion as futuristic drug delivery. Int J Pharm Pharm Sci. 2013;5(3):39–53.

129. Etman SM, Elnaggar YSR, Abdelmonsif DA, Abdallah OY. Oral brain-targeted microemulsion for enhanced Piperine delivery in Alzheimer's disease therapy: in vitro appraisal, in vivo activity, and nanotoxicity. AAPS PharmSciTech. 2018;19(8):3698–711.
130. Harun S, Amin Nordin S, Abd Gani SS, Shamsuddin AF, Basri M, Bin BH. Development of nanoemulsion for efficient brain parenteral delivery of cefuroxime: designs, characterizations, and pharmacokinetics. Int J Nanomedicine. 2018;13:2571.
131. Prabhakar K, Afzal SM, Surender G, Kishan V. Tween 80 containing lipid nanoemulsions for delivery of indinavir to brain. Acta Pharm Sin B. 2013;3(5):345–53.
132. Vyas TK, Shahiwala A, Amiji MM. Improved oral bioavailability and brain transport of Saquinavir upon administration in novel nanoemulsion formulations. Int J Pharm. 2008;347(1–2):93–101.
133. Han L, Huang R, Liu S, Huang S, Jiang C. Peptide-conjugated PAMAM for targeted doxorubicin delivery to transferrin receptor overexpressed tumors. Mol Pharm. 2010;7(6):2156–65.
134. Hao B, Gao S, Li J, Jiang C, Hong B. Plasmid pORF-hTRAIL targeting to glioma using transferrin-modified polyamidoamine dendrimer. Drug Des Devel Ther. 2016;10:1.
135. He H, Li Y, Jia X-R, Du J, Ying X, Lu W-L, et al. PEGylated poly(amidoamine) dendrimer-based dual-targeting carrier for treating brain tumors. Biomaterials. 2011;32(2):478–87.
136. Huang R-Q, Qu Y-H, Ke W-L, Zhu J-H, Pei Y-Y, Jiang C. Efficient gene delivery targeted to the brain using a transferrin-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. FASEB J. 2007;21(4):1117–25.
137. Li Y, He H, Jia X, Lu W-L, Lou J, Wei Y. A dual-targeting nanocarrier based on poly(amidoamine) dendrimers conjugated with transferrin and tamoxifen for treating brain gliomas. Biomaterials. 2012;33(15):3899–908.
138. Somani S, Blatchford DR, Millington O, Stevenson ML, Dufès C. Transferrin-bearing polypropylenimine dendrimer for targeted gene delivery to the brain. J Control Release. 2014;188:78–86.
139. Yuan Q, Fu Y, Kao WJ, Janigro D, Yang H. Transbuccal delivery of CNS therapeutic nanoparticles: synthesis, characterization, and in vitro permeation studies. ACS Chem Neurosci. 2011;2(11):676–83.
140. Sun T, Wu H, Li Y, Huang Y, Yao L, Chen X, et al. Targeting transferrin receptor delivery of temozolomide for a potential glioma stem cell-mediated therapy. Oncotarget. 2017;8(43):74451.
141. Ke W, Shao K, Huang R, Han L, Liu Y, Li J, et al. Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. Biomaterials. 2009;30(36):6976–85.
142. Somani S, Robb G, Pickard BS, Dufès C. Enhanced gene expression in the brain following intravenous administration of lactoferrin-bearing polypropylenimine dendriplex. J Control Release. 2015;217:235–42.
143. Liu Y, Huang R, Han L, Ke W, Shao K, Ye L, et al. Brain-targeting gene delivery and cellular internalization mechanisms for modified rabies virus glycoprotein RVG29 nanoparticles. Biomaterials. 2009;30(25):4195–202.
144. Gao H, Qian J, Cao S, Yang Z, Pang Z, Pan S, et al. Precise glioma targeting of and penetration by aptamer and peptide dual-functioned nanoparticles. Biomaterials. 2012;33(20):5115–23.
145. Delač M, Motaln H, Ulrich H, Lah TT. Aptamer for imaging and therapeutic targeting of brain tumor glioblastoma: aptamers in glioblastoma. Cytometry. 2015;87(9):806–16.
146. Guo J, Gao X, Su L, Xia H, Gu G, Pang Z, et al. Aptamer-functionalized PEG–PLGA nanoparticles for enhanced anti-glioma drug delivery. Biomaterials. 2011;32(31):8010–20.
147. Ren J, Shen S, Wang D, Xi Z, Guo L, Pang Z, et al. The targeted delivery of anticancer drugs to brain glioma by PEGylated oxidized multi-walled carbon nanotubes modified with angiopep-2. Biomaterials. 2012;33(11):3324–33.
148. Lu Y-J, Wei K-C, Ma C-CM, Yang S-Y, Chen J-P. Dual targeted delivery of doxorubicin to cancer cells using folate-conjugated magnetic multi-walled carbon nanotubes. Colloids Surf B: Biointerfaces. 2012;89:1–9.

149. Li S, Peng Z, Dallman J, Baker J, Othman AM, Blackwelder PL, et al. Crossing the blood–brain–barrier with transferrin conjugated carbon dots: a zebrafish model study. Colloids Surf B: Biointerfaces. 2016;145:251–6.
150. Li S, Amat D, Peng Z, Vanni S, Raskin S, De Angulo G, et al. Transferrin conjugated non-toxic carbon dots for doxorubicin delivery to target pediatric brain tumor cells. Nanoscale. 2016;8(37):16662–9.
151. Huang R, Han L, Li J, Liu S, Shao K, Kuang Y, et al. Chlorotoxin-modified macromolecular contrast agent for MRI tumor diagnosis. Biomaterials. 2011;32(22):5177–86.
152. Jiang W, Xie H, Ghoorah D, Shang Y, Shi H, Liu F, et al. Conjugation of functionalized SPIONs with transferrin for targeting and imaging brain glial tumors in rat model. Brechbiel MW, editor. PLoS One. 2012;7(5):e37376.
153. Xie H, Zhu Y, Jiang W, Zhou Q, Yang H, Gu N, et al. Lactoferrin-conjugated superparamagnetic iron oxide nanoparticles as a specific MRI contrast agent for detection of brain glioma in vivo. Biomaterials. 2011;32(2):495–502.
154. Yan H, Wang J, Yi P, Lei H, Zhan C, Xie C, et al. Imaging brain tumor by dendrimer-based optical/paramagnetic nanoprobe across the blood-brain barrier. Chem Commun. 2011;47(28):8130–2.
155. Cai W, Shin D-W, Chen K, Gheysens O, Cao Q, Wang SX, et al. Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. Nano Lett. 2006;6(4):669–76.
156. Cui L, Lin Q, Jin CS, Jiang W, Huang H, Ding L, et al. A PEGylation-free biomimetic porphyrin nanoplatform for personalized cancer theranostics. ACS Nano. 2015;9(4):4484–95.
157. Dixit S, Miller K, Zhu Y, McKinnon E, Novak T, Kenney ME, et al. Dual receptor-targeted theranostic nanoparticles for localized delivery and activation of photodynamic therapy drug in glioblastomas. Mol Pharm. 2015;12(9):3250–60.
158. Rajora MA, Ding L, Valic M, Jiang W, Overchuk M, Chen J, et al. Tailored theranostic apolipoprotein E3 porphyrin-lipid nanoparticles target glioblastoma. Chem Sci. 2017;8(8):5371–84.
159. Yoo B, Ifediba MA, Ghosh S, Medarova Z, Moore A. Combination treatment with theranostic nanoparticles for glioblastoma sensitization to TMZ. Mol Imaging Biol. 2014;16(5):680–9.
160. Kuang Y, Zhang K, Cao Y, Chen X, Wang K, Liu M, et al. Hydrophobic IR-780 dye encapsulated in cRGD-conjugated solid lipid nanoparticles for NIR imaging-guided photothermal therapy. ACS Appl Mater Interfaces. 2017;9(14):12217–26.
161. Fang J-Y, Wen C-J, Zhang LW, Al-Suwayeh SA, Yen T-C. Theranostic liposomes loaded with quantum dots and apomorphine for brain targeting and bioimaging. Int J Nanomed. 2012;7:1599.
162. Cytotoxic T cells and interleukin-2 in treating adult patients with recurrent brain tumors – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT00002572.
163. ANG1005 in breast cancer patients with recurrent brain metastases – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT02048059.
164. Cellular immunotherapy study for brain cancer – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT01144247.
165. IL-4(38-37)-PE38KDEL immunotoxin in treating patients with recurrent malignant astrocytoma – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT00003842.
166. Study of therapy with TransMID™ compared to best standard of care in patients with glioblastoma multiforme – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT00083447.
167. T cells expressing HER2-specific chimeric antigen receptors(CAR) for patients with HER2-positive CNS tumors – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT02442297.

168. Chemotherapy and vaccine therapy followed by bone marrow or peripheral stem cell transplantation and interleukin-2 in treating patients with recurrent or refractory brain cancer – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT00014573.
169. EGFRBi-armed autologous T cells in treating patients with recurrent or refractory glioblastoma – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT02521090.
170. A study of ABT-414 in subjects with newly diagnosed glioblastoma (GBM) with epidermal growth factor receptor (EGFR) amplification – full text view – ClinicalTrials.gov [Internet]. [cited 2019 Jun 16]. Available from: https://clinicaltrials.gov/ct2/show/NCT02573324.

Chapter 3
Breast Cancer Receptors and Targeting Strategies

Ashish Pandit, Lalit Khare, Padma V. Devarajan, Ratnesh Jain, and Prajakta Dandekar

Abstract Breast cancer (BC) is the most common cause of death among women worldwide. Characterized by heterogeneous nature, treatment of breast cancer becomes very challenging considering its late detection. Causes for breast cancer and their treatment modalities have been well identified; still management of disease is an uphill task due to complex pathophysiology associated with it. Chemotherapy has remained the mainstay for treatment of BC, although with advent of novel targeted therapies, a paradigm shift is seen in treatment options available for BC. Targeted regimes toward receptors expressed on tumorous surfaces are developed that deploy antibodies and peptides for treating BC. Moreover, with avoidance of side effects of chemotherapy with concomitant annihilation of cancerous cells, it is very imperative to understand the underlying mechanisms of receptors governing the process. Therefore, with an attempt toward comprehensive understanding of the subject, this chapter explores some of the important receptors involved in breast cancer such as estrogen receptor, progesterone receptor, and human epidermal receptor-2. Special emphasis is given for the modulation of their signal transduction mechanism attaining desired goals. Various formulation aspects that are currently undertaken toward BC management are also discussed in brief.

Keywords Breast cancer · Progesterone receptor · Estrogen receptor · Triple-negative breast cancer · Human epidermal receptor

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Abbreviations

ABC	ATP-binding cassettes
ADCC	Antibody-dependent cell-mediated cytotoxicity
BC	Breast cancer
EGFR	Epidermal growth factor receptors
ER	Estrogen receptor modulators
FISH	Fluorescent in situ hybridization assay
HER-2	Human epidermal growth factor receptor-2
IDC	Invasive ductal carcinomas
IHC	Immune Histochemical studies
ILC	Invasive lobular carcinoma
MISS	Membrane-assisted steroid signaling
NDDS	Novel drug delivery systems
P-gp	P-glycoprotein
PR	Progesterone receptor
SERMs	Selective estrogen receptor
TDM-1	Trastuzumab emtansine
TNBC	Triple-negative breast cancer

1 Introduction

Breast cancer (BC) is one of the most commonly occurring cancers in women. Nearly 1.7 million new cases of BC were diagnosed in 2012 alone, and it has been associated with an increasing number of cancer-related deaths among the female population, worldwide [1]. BC is characterized by an uncontrolled growth emanating from the breast tissue, more frequently from the milk ducts and lobules, and if left unrestrained, it can metastasize to the other parts of the human body. The early stages of BC are mostly asymptomatic. A hard painless lump that can be felt by touch is an important symptom of the disease progression. Persistent changes in breast size, distortion of breasts with spontaneous discharge, skin irritation, and retraction of nipples are the other less prevalent symptoms. Although the tumor may be completely treated at this preliminary stage, the aforementioned manifestations are often overlooked by the patients [2]. This results in advancement of the cancer to a secondary or terminal stage, when its treatment is often difficult or impossible. Nowadays, the advent of mammographic screening tests have enabled an early-stage detection of BC, although its appropriate implementation during diagnosis should be sought, in order to decrease the number of women being diagnosed in later stages of the disease [3].

Several risk factors such as mutation of the BRCA1 and BRCA2 genes, and extraneous elements, like use of oral contraceptives, exposure to radiation, etc., are linked with the emergence of BC. Occurrence of BC is particularly high in women

from developed countries, wherein women belonging to the older age group are at a higher risk. Women who bear children at a young age possess a lower risk of having BC. Incidence of BC is also high in females with early menarche and delayed menopause. Risk factors for BC can be further distinguished into two broad categories. The first category includes inherited traits like sex, genetic makeup, age, and familial history of the neoplastic disease, while the second one includes lifestyle-related extrinsic factors like smoking, excessive consumption of alcoholic beverages, and medical interventions such as the hormonal replacement therapy [1]. In comparison to women, the prevalence of BC among men is negligible. Male BC constitutes only 1% of all cases of BC, diagnosed worldwide. Hormonal imbalance, testicular maldescent, congenital inguinal hernia, orchitis from mumps, and testicular injury are some of the predisposing factors that cause BC in men [4, 5].

1.1 Classification and Clinical Subtypes of BC

BC is classified into distinct categories, based on the several, specific distinguishing aspects. The histopathological studies, molecular pathology, genetic analysis, or gene expression profiling are carried out to distinguish between different BC types, as represented in Fig. 3.1. According to the WHO, different histological subtypes of BC have been identified. Invasive ductal carcinomas (IDC) exist in about 50–80% of all the diagnosed cases, whereas the invasive lobular carcinoma (ILC) is the second most common type of BC, affecting about 15–20% of the patients [6]. A low-grade breast tumor is characterized by well-differentiated cancerous cells,

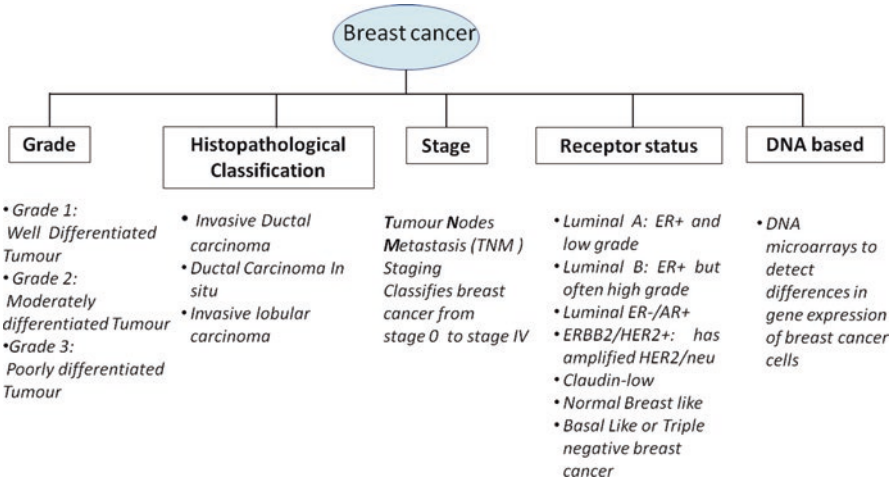

Fig. 3.1 Various classifications of BC

while in case of intermediate grade and high-grade tumors, the cells are moderately and poorly differentiated, respectively. Based on the immunohistochemical analysis of the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor-2 (HER-2), the BC tumors are classified as the luminal (ER+/PR+/HER-2−), HER-2+ (ER−/PR−/HER-2+), or triple-negative (TN; ER−/PR−/HER-2−). Staging of BC according to TNM classification (T: tumor; N: lymph node; M: has tumor metastasized or not) is a common procedure to determine the exact state of the disease progression [7]. BC staging is based on the primary tumor size, the involvement of the sentinel lymph node, and the existence of metastases. The stage advances gradually from 0 to IV, where carcinoma in situ tumors are of stage 0, while a stage IV tumor has already metastasized to a distant location. The stage of the disease is the main driving factor in prognosis of BC [8]. With the advent of new techniques, DNA microarray studies are employed to measure the level of expression of different genes, to differentiate between normal cells and tumorous growths of the breast. More than 90% of tumorous cells exhibit overexpression of genes, such as BRCA1, BRCA2, p53, PTEN, ATM, NBS1, and LKB1 [9].

2 Overview of Role of Receptors in BC

BC cells are distinguished from the normal cells by the prominent overexpression of some of the receptors; for example, mutations in the HER-2/neu gene govern the expression of HER-2 receptor. The upregulated receptor type, confirmed via the immunohistochemical assays, is often correlated with lymph node involvement and tumor grade. These receptors bind to their respective ligands, mostly chemokines secreted by the tumor cells, and certain hormones, to boost the proliferation of cells through their signal transduction mechanisms. Cancer therapy, as a whole, depends on blockage of this receptor–ligand binding or the arrest of their signal transduction pathways. Modulation of receptors pathways represents an attractive therapeutic strategy to treat BC. Furthermore, literature points that the number of cases of BC, with estrogen-positive receptors, is on rise. HER-2 receptor, a subtype of EGFR family, along with the progesterone receptor, is also responsible for the advancement of BCs [9]. Resistance for agents like tamoxifen that target the estrogen receptors in breast cancer is on rise according to recent reports [10]. Hence, there is an immense need to recognize alternate receptors for targeting BC. This chapter will primarily discuss the role of HER-2 receptor, Estrogen receptor (ER), Progesterone receptor (PR), and the regulation of their signal transduction mechanisms, to treat BC as depicted in Fig. 3.2. Also, different treatment modalities to treat triple-negative BC have been discussed. A brief overview has been presented on the results of preclinical studies, ex vivo studies, and mechanisms of HER-2, PR, and ER receptors.

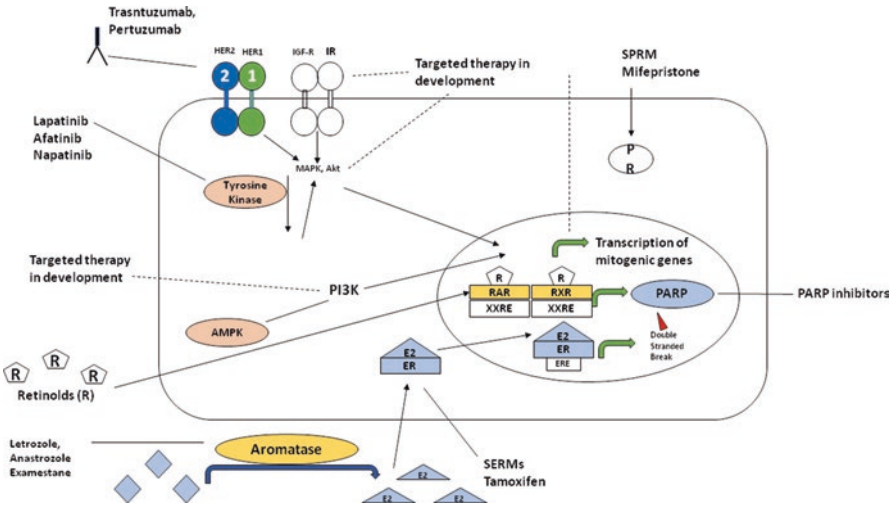

Fig. 3.2 Role of receptors in BC and agents for selective targeting. (Abbreviations: AMPK 5'-AMP-activated protein kinase, PR Progesterone receptor, ER Estrogen receptor, PI3K Phosphoinositide 3-kinases, PARP Poly(ADP-ribose) polymerase, SPRM Selective progesterone receptor modulator, MAPK Mitogen-activated protein kinases (MAPKs), SERM Selective estrogen receptor modulators, RARs Retinoic acid receptors, RXR Retinoid X receptors)

2.1 Involvement of HER-2 Receptor in BC

2.1.1 Occurrence of HER-2 Receptor in BC

The human epidermal growth factor receptor-2 (HER-2) belongs to the superfamily of transmembrane receptors, the epidermal growth factor receptors (EGFR), that possess tyrosine kinase activity. These receptors modulate signal transduction pathways required for normal cell growth and differentiation. HER-2 receptor is encoded by a proto-oncogene HER-2/neu (Erb-B2) present on the long arm of chromosome number 17. Overexpression of HER-2 is a striking feature that distinguishes between the normal and cancerous cells [11]. Ovarian cancers, lung cancer, and cancer of endometrium are other illustrations that are marked with abnormal presence of HER-2. This makes the receptor an important biomarker, as far as prediction and prognosis of cancers is concerned. Nearly 25–30% of BCs cases are characterized by the overexpression of HER-2 receptor. Evaluation of breast tumors for HER-2 expression can facilitate selection of treatment regimens for the patients. Therefore, diagnosis of HER-2 + BCs is being conducted using diverse techniques like immunohistochemical (ICH) assays, fluorescent in situ hybridization (FISH), enzyme-linked immunosorbent assay (ELISA), Southern blotting, etc., as per the recommendation of regulatory agencies like the American Society of Clinical Oncology (ASCO) and the World Health Organization (WHO) [12].

2.1.2 Signal Transduction by HER-2

The structural conformation of HER-2 is similar to that of any other transmembrane receptor. Typically, it contains an extracellular domain that is equipped with an amino terminal and comprises four parts, namely, two ligand-binding domains (LD1 and LD2) and two cysteine-rich domains that aid in ligand binding [13]. Furthermore, the intracellular domain is categorized into two regions, namely, a catalytic tyrosine kinase, having an active phosphorylation site, and a carboxy terminal chain. Upon binding with the specific ligands, these receptors undergo either homodimerization or heterodimerization, leading to trans-phosphorylation of their intracellular domain. Phosphorylation triggers the downstream activities of a variety of secondary messengers, which mediate diverse biological effects. HER-2 is a preferred choice for other subtypes of HER to form heterodimeric complexes, and the complex formed between HER-2 and HER-3 receptors displays the highest efficacy to elicit a pharmacological response, among all the receptors or receptor complexes of the EGFR family. Moreover, HER-2 dimers are less prone to enter the degradation pathways and tend to escape the internalization process as complexes formed with HER-2 are stable and do not dissociate readily [14, 15]. HER-2 complexes can activate the RAS/RAF/MAPK cascade, which regulates the cellular proliferation, and the PI3K/Akt signaling that is responsible for cell survival [16]. Consequently, treatment of patients with HER-targeting agents like lapatinib, trastuzumab, and pertuzumab has considerably improved their pathological complete response rates. Emerging trials are also increasingly pointing toward the efficiency of treatment regimens wherein chemotherapy has been coupled with HER-targeting agents [17].

2.1.3 Strategies to Target HER-2 in BC

Spatial targeting of the HER-2 receptor is gaining momentum owing to its importance in BC. Antibodies like trastuzumab (Herceptin®, Genetech) and pertuzumab (Perjeta® Genetech), antibody drug conjugates like trastuzumab emtansine (TDM-1 Genetech), small molecule tyrosine kinase inhibitors like lapatinib, afatinib, napatinib, etc. have been developed, considering the incongruous behavior of HER receptors in BC. These agents act by diverse mechanisms and operate by disrupting heterodimerization of HER, impeding HER pathways, and are known to exert antibody-dependent cell-mediated cytotoxicity (ADCC) and inhibit cleavage of HER or promote its endocytosis [18]. Heat shock protein (HSP90) inhibitors are found to promote degradation of HER-2 receptor. For example, tanesipimycin, a first-generation HSP 90 inhibitor, has significant activity against HER-2+ BC. Molecular chaperon HSP 90 is required for the stability of various substrate proteins, known as client proteins. Increased client proteins results into blockade of the apoptotic pathway and cause inhibition of tumor cell inactivation. Tanesipimycin interacts with HSP 90 and destabilizes it to induce anticancer

activity. Another two HSP90 inhibitors, namely retaspimycin and AUY922, are currently undergoing clinical investigations, as monotherapy or combination therapy with trastuzumab [19]. Combination therapies of HER-2-targeting agents, along with chemotherapeutic agents, are specifically recommended for metastatic BC. For example, trastuzumab, when combined with lapatinib, increased apoptosis in HER-2 overexpressing SKBR-3 cells. Tyrosine kinase inhibitors, like lapatinib, have been administered along with paclitaxel, anthracycline, and docetaxel. Dramatic improvement in clinical benefit rate was observed upon co-administering trastuzumab and lapatinib [20]. However, in geriatric patients, the combination of the HER-2 + targeting agents and anthracycline-based chemotherapeutic agents was found to be troublesome. Concomitant chemotherapy, along with targeting HER-2+, for prolonged durations was observed to predispose the patients to irreversible heart damage [21].

2.1.4 Resistance in HER-2+ Targeted BC Therapies

Although early and advanced stages of BC were successfully treated by trastuzumab, increasing proportions of patients have been reported to possess intrinsic resistance or have acquired resistance to this biotherapeutic. Tumor cells are known to evade trastuzumab targeting via HER-2 dimerization. This knowledge further led to the development of pertuzumab, a humanized monoclonal antibody that disrupts the formation of HER-2 homodimers. Pertuzumab (Perjeta® Genetech) was approved by the USFDA, in 2013, for use in neoadjuvant setting, as well as in metastatic BCs, along with trastuzumab and taxanes [22]. In some instances, interaction between trastuzumab and HER was blocked. A recent study indicated that epitopes on HER-2 are covered by a transmembrane protein, named MUC4, which caused a significant reduction in the efficacy of trastuzumab [23]. Yet another theory suggested the failure of the immune system in responding to the polymorphism or dysregulation of the Fc receptor. Upregulation of downstream pathways or activation of alternate signaling pathways has been suggested as other contributing factors toward resistance development. Upregulation of signal transduction can occur due to the loss of phosphatase and tensin homolog and presence of P95, a truncated form of HER-2 receptor that lacks the outer cell attachment domain. Also, transactivation of HER-2 signaling can occur through other tyrosine kinases, like IGF-1R and PI3K mutation, thus leading to a resistance against trastuzumab [24]. Further, point mutations in the kinase domain (KD) are responsible for resistance development toward tyrosine kinase inhibitors. T3151 mutations are peculiar characteristic of the KD in the Bcr-Abl fusion gene of the cancerous cells. As a result of this mutation, binding of inhibitor molecules to tyrosine kinase is prohibited. Moreover, it can also result in destabilization of the enzyme inhibitor complex. Additional mechanisms involved with resistance toward TKI inhibitors, like lapatinib, imatinib, etc., include activation of alternate signaling pathways and epigenetic changes in the genome [25].

2.2 Involvement of Estrogen Receptor (ER) in BC

2.2.1 Occurrence of ER in BC

Adjuvant hormonal therapy for BC involves targeting the estrogen receptor signaling pathway. Therefore, determination of the status of the ERs is very crucial for the prognosis of BC. The current techniques that are used to determine the status of ERs include immunohistochemical (IHC) studies that are often coupled with the fluorescent in situ hybridization (FISH) assay, for validation of the results. There are two different types of ER receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β), that are encoded by two different genes, belonging to the steroid hormone super family of nuclear receptors [26, 27]. Under normal conditions, ER β is more profoundly expressed than the ER α. Several studies have pointed toward distinct expression of ER in ductal and lobular BC. In case of high-grade lobular cancer, the ER β expression is lost, while the ER α is expressed in abundance. However, in case of invasive ductal carcinoma, both ER α and ER β are underexpressed. Treatment of BC using tamoxifen is rather doubtful, when both ER α and ER β are co-expressed. Tamoxifen acts as an agonist of ER β at the activator protein sites (AP sites) and therefore would nullify the antitumorigenic response of the tamoxifen–ER α complex. Therefore, tamoxifen is more useful in later stages of lobular BC and cautious use of agents, which act as agonists of ER β, has been recommended by researchers to check the disease progression [28].

2.2.2 Signal Transduction by the ERs in BC

ERs are omnipresent in the cytosol or nucleus and regulate their functions at the genomic level. Moreover, numerous literature reports point toward the presence of membrane-associated ERs. The hormone estrogen selectively binds to the ER and forms a complex. This complex is responsible for the maintenance and regulatory activities like cardiovascular protection, preservation of bone, neuroprotection, and cellular proliferation [29]. The ER complexes, typically, employ two distinct mechanisms for signal transduction. The first is the genomic pathway via the nucleus-initiated steroid signaling (NISS), which involves dimerization of the ER complex and its translocation into the nucleus to elicit a suitable response. The second pathway is the nongenomic pathway or the membrane-assisted steroid signaling (MISS), which exerts its action by rapidly triggering the cytoplasmic signal transduction pathways [29]. Estrogen, as such, can initiate very rapid cellular actions, indicating the presence of a strong MISS. Nongenomic pathways are involved in many other events, including mobilizing secondary messengers and interaction with transmembrane receptors like insulin-like growth factor receptors (IGF-1R) and epidermal growth factor receptor (EGFR) [30]. The presence of a seven-transmembrane G-protein-coupled receptor, called GPR-30, responds to estrogen via rapid cellular signaling. Trans-activation of EGFR signaling pathway, with the involvement of MAP kinase, is mediated by the estrogen-activated GPR-30 pathway. Consequently,

GPR-30 can also activate the adenylyl cyclase pathway. Tamoxifen can activate PI3K via GPR-30, and not ER α [31]. Estrogen and estrogen receptor agonists are known to inhibit the mitochondrial respiratory complexes. Rapid production of intracellular reactive oxygenated species is stimulated by estrogen via the respiratory chains, at the cellular levels. This ROS production is independent of the presence of estrogen receptors, as certain cell lines devoid of ER, like MDA-MB 468, have exhibited near-equal concentrations of ROS. These reactive species can reversibly regulate the cysteine-based phosphatases, including the protein tyrosine phosphatase (PTP) and lipid phosphatase [32]. In addition, ROS also activates MAPK- and PI3K-dependent pathways, thus leading to the activation of AP-1, NF-κB, and NRF1. Therefore, estrogen-backed ROS production is detrimental for genomic integrity of the epithelial cells [33].

2.2.3 Strategies to Target the ERs

Endogenous and exogenous estrogens are known to stimulate the proliferation and growth of BC cells leading to the development of "selective estrogen receptor modulators" (SERMs). Tamoxifen, a well-known SERM, has emerged as a gold standard for clinical treatment of the BCs. The overexpression of ERs is the major indication of the possible response of the BCs toward the endocrine therapy, as approximately 70% of the BCs are estrogen receptor positive and hormone-dependent. Marginal improvement in postsurgical survival rates and reduction in relapses are seen with the introduction of systemic adjuvant therapy for estrogen receptor-positive BC. This adjuvant therapy (AT) that is administered along with chemotherapy or radiotherapy is beneficial to women in early stages of BC. AT primarily comprises i) ovarian suppression, ii) employment of selective estrogen receptor modulators (SERMs), and iii) employment of aromatase inhibitors.

Ovarian Function Suppression (OFS)

Ovarian function suppression (OFS) is either achieved by surgical intervention or through the use of gonadotropin agonists (GNRh). GNRh agonists like goserelin, triptorelin, buserelin, and leuprolide are decapeptides that share structural resemblance with the native GNRh. Continuous administration of GNRh agonists causes significant drop in the circulating levels of follicle-stimulating hormone (FSH) and leutinizing hormone (LH). Studies conducted on ovarian steroidogenesis and GNRh agonists have reported contrasting results. Some researches claim a direct intervention by the agonists in decreasing the production of estrogen and progesterone, while certain reports state that the agonists work by controlling the signaling mechanisms, triggered by the estrogen and progesterone, upon activation of their respective receptors. These agonists, along with the standard adjuvant therapy, are more effective than the adjuvant therapy alone and reduce the recurrence rate and mortality by 25% in BC patients who have not received the standard chemotherapy [34, 35].

Selective Estrogen Receptor Modulators (SERMs)

SERMs can be either ER agonists or antagonists, depending on their interaction with the ER, the target tissues, and the posttranslational effects. These are further categorized into three groups:

(i) Triphenylethylene nonsteroidal derivatives, for example, tamoxifen, toremifene, droloxifene, and idoxifene
(ii) Benzothiophene-based nonsteroidal derivatives, for example, raloxifene, toremifene, and arzoxifene
(iii) Indole, tetrahydronaphthalene, and naphthol SERMs, for example, lasofoxifene and bazedoxifene

Oncostatic mechanism of tamoxifen is believed to be mediated through the reduced production of estrogen. Also, hydroxyl-tamoxifen, an active metabolite of tamoxifen, increases the serum levels of estrogen. Tamoxifen acts on mammary serine protease inhibitor (SERPINB5) to elevate estrogen levels [36]. SERPINB5 gene is downregulated in many of the metastatic and primary BC cells. Toremifene has a β-chlorine attached to its basic structure, which prevents its metabolism by hydroxylation reactions. Thus, preclinical investigations have indicated this molecule as a safer SERM than Tamoxifen [37]. Prolonged treatment of patients with tamoxifen can lead to bone loss or osteoporosis, particularly in premenopausal women. Therefore, Lasofoxifene and bazedoxifene have been designed based on prior pharmacological knowledge of having a favorable impact on lipid and skeletal metabolism, relatively safe on uterine and mammary tissues, and also proven to be neutral toward hot flashes.

Aromatase Inhibitors

Aromatase is an enzyme encoded by the CYP19 gene. It is usually expressed in the granulose of the ovarian cells, in premenopausal women, and in the adipose tissues of the postmenopausal women. The main role of aromatase is in the formation of progesterone and androgens. Typically, two types of aromatase inhibitors exist, based on their mechanism of action. The first category is steroidal aromatase inhibitors like testolactone (first generation), formestane (second generation), and exemestane (third generation), which cause permanent inhibition of aromatase. The second category is the nonsteroidal aromatase inhibitors like aminoglutethimide (first generation); fadrozole (second generation); and anastrozole and letrozole (third generation) that are reversible, competitive inhibitors of the enzyme. As the mechanism of action suggests, the steroidal aromatase inhibitors are not preferred for treatment in premenopausal women. Steroidal inhibitors like examestane bind to the active sites of the aromatase enzyme like a false substrate. This binding is irreversible and thus production of new estrogen requires formation of new aromatase molecules. This leads to a decreased production of estrogen and progesterone creating a temporary hormonal imbalance in patients [38, 39].

2.2.4 Resistance to ER-Targeted Therapies

Considering the complex nature of the ER and its signaling pathways, several mechanisms of resistance have been identified. An epigenetic mutation in gene encoding for ER is one of the major factors governing development of resistance. Modification of the posttranslational events, alteration in the hormone-binding domains, differential recruitment of co-regulators, and influence of the tumor microenvironment are some of the other factors responsible for the resistance to ER-targeting. Two major signaling pathways, namely, the CyclinD1/CDK4/6 pathway and PI3K/AKT/mTOR pathway, have been identified as important for therapeutic arbitrations, considering the resistance developed by the ERs to the endocrine therapy [40]. The PI3K/AKT/mTOR pathway controls the maintenance of cellular functions, like glucose metabolism, protein synthesis, and cellular proliferation. Frequent alterations of growth-signaling PI3K/AKT/mTOR pathway are proposed as a probable mechanism for resistance to endocrine therapy and the estrogen-mediated signaling, along with alteration in the PIK3CA gene. Obliteration of the PI3K pathway can lead to an increased activity of ER, thus aiding in development of resistance. Inhibition of mTOR leads to activation of AKT and the extracellular signal-regulated kinase (ERK). This phenomenon suggests that inhibition of the PI3K/AKT/mTOR pathway may lead to reduction in cellular proliferation [41]. According to some recently reported preclinical studies, a novel molecule AZD5363 was observed to inhibit AKT and resensitize the cells toward tamoxifen, in vitro. This molecule was found to act in synergy with fulvestrant [42].

2.3 Involvement of Progesterone Receptor (PR) in BC

2.3.1 Occurrence of PR in BC

The progesterone receptor (PR), also recognized as NR3C3 (nuclear receptor subfamily 3, group C, member 3), is a protein complex, comprising 933 amino acids that is found inside the human cells [43, 44]. Its activity is triggered by the hormone, progesterone. The PR displays two distinct binding sites, one possessing a significantly higher affinity for its ligands than the other [45]. The human PR has two main isoforms namely, PR-A and PR-B. Translation of PR-A and PR-B starts at two different AUG codons [45]. The PR contains a central DNA-binding domain, with the ligand-binding region being present at the carboxy terminal. Furthermore, it contains multiple activation functions (AFs) and inhibitory elements. One of the AFs, namely, AF-1, is confined to a 91-amino acid region within the NH_2 terminus of PR, while another AF, namely, AF-2, is confined to the C-terminal ligand-binding domain [44]. Both, AF-1 and AF-2 are contained within the PR-A and PR-B. However, it has been shown that sequences within the B-specific region (B164) are required for the maximum activity of AF-1. Recently, a third activation domain (AF-3), which functions only in a restricted cell and promoter context, has

been found within the B164 region of the PR [44–47]. The PR-B contains AF-3, in addition to the AF1 and AF2. A third, lesser-known isoform of PR is the PR-C. The PR-B is the positive regulator of the effects of progesterone, while PR-A and PR-C antagonize the effects of PR-B [48]. Based on immunohistochemical analysis, the PR-A and PR-B are expressed at a ratio of 1:1. The BC tumors contain elevated levels of PR-A, relative to PR-B. Predominant PR-A expression signifies a poor outcome of the hormonal therapies, while predominance of PR-B signifies a poor outcome of the chemotherapy. Thus, deregulation of the PR signaling pathway is the hallmark of BCs [49].

2.3.2 Signal Transduction by the PR

When progesterone is absent, the PR stays within the nucleus of the target cells in a dormant form, in association with a heavy complex comprising heat shock proteins (HSP) like HSP90, HSP72, and HSP59, and other proteins. In cells where both PR-A and PR-B are present, three different dimers (A:A, A:B, and B:B) are formed [50]. The activated receptor dimers strongly interact with specific progesterone response elements (PRE), located within the target genes, and cause modulation in the transcription of these genes. In addition to promoting the formation of receptor dimers, the interaction of the PRs with the hormone facilitates an increase in their overall phosphorylation state [51]. This occurs in two separate steps, with one phosphorylation event occurring upon dislodgment of HSPs, while the second occurring following the association of the receptor with DNA. The specific role of phosphorylation is currently unknown. Also, the mechanism by which the PRs can modulate the transcription of target genes is currently not determined [46, 50].

2.3.3 Strategies to Target the PR

Several antiprogestins have been developed and are being used for the treatment of a variety of endocrine-related disorders. Two of these compounds, namely, RU486 (mifepristone) and ZK98299 (onapristone), are derived from 19-nor-testosterone, which strongly interact with the hormone-binding domain of the PR. Mifepristone is a partial agonist of the receptor, while onapristone is an antagonist and has never been marketed [48]. Telapristone, vilaprisan, and lonaprisan are synthetic, steroidal selective progesterone receptor modulators (SPRM), related to mifepristone. These are being evaluated for the treatment of BC, endometriosis, and uterine fibroids and are in phase II of clinical trials. However, the trails involving vilaprisan were stopped due to safety concerns. Telapristone and lonaprisan are in phase III of clinical trials. In addition to their activity as an SPRM, these drugs also exhibit antiglucocorticoid activity. ORG-31710 and ORG-31806 are the new antiprogestins, which are more potent than mifepristone and onapristone, and are presently in preclinical studies. No new molecules targeting the PR have been reported since 1990 [52].

2.4 Triple-Negative BC (TNBC)

Triple-negative breast cancer is the one that is characterized by the absence of any specific endocrine target receptors, like the ER, PR, and HER-2. This subtype of BC accounts for 15–20% of all the newly diagnosed cases, worldwide, and is of common occurrence among the women of African-American and Hispanic origin [53]. Both TNBC and basal-like BC (BL-BC) exhibit identical features like large tumor size, stromal lymphocytic infiltration, geographic necrosis, and pushing borders of invasion. The TNBC is further grouped into various subtypes, depending on the genetic profiling of the BC patients, which include basal-like (39–54%), claudin low (25–39%), HER-2-enriched/molecular apocrine (7–14%), luminal B (4–7%), luminal A (4–5%), and normal breast-like (1%). When a combination of genome-wide serum miRNA expression and real-time PCR was employed for analyzing the serum samples of TNBC patients, a group of four miRNAs consisting of miR-18b, miR-103, miR-107, and miR-652 was identified as the trait of the disease relapse and overall reduced survival rate. This group of miRNAs is thus considered as the characteristic prognostic feature of TNBC, which can be identified using a minimally invasive technique [54]. Most of the TNBC cases display higher relapse rates, a phenomenon commonly known as the TNBC paradox; therefore, apart from chemotherapy, another promising approach to treat TNBC is cancer immunotherapy [54]. Several studies have marked an increase in the presence of tumor-infiltrating lymphocytes (TIL's) at the site of the tumors. This phenomenon is exaggerated in TNBC tumors, as compared to the HER-2+ cancer. Programed cell death receptors (PD-1) are expressed on the activated T lymphocytes and play a pivotal role in immunological invasion of the tumors. Most of the cancer cells secrete PD-L1, a specific ligand for PD-1 receptors present on the lymphocytes, which bocks their functions. Altogether, it can be concluded that there is a strong rationale to therapeutically target TNBC along the PD-1/PD-L1 axis [55].

2.4.1 Chemotherapy for TNBC

TNBC is the most susceptible to treatment with chemotherapeutic agents. The current strategies employed for alleviating TNBC involve targeting of the DNA repair complexes, using taxanes and platinum agents, and controlling the cellular proliferation with help of anthracycline-based chemotherapeutics. A strong association exists between the BRCA1 mutations and dysfunctional DNA repair and both are regarded as the hallmark of the disease. Therefore, there exists an enormous scope for platinum agents, due to their increased sensitivity in TNBC, as reported through many preclinical and clinical findings. A higher susceptibility has been noted for DNA-strand-disrupting agents like etoposide and bleomycin. p53 mutation is also a very peculiar attribute of TNBC, rendering TNBC susceptive toward anthracycline therapy. The reports are quite controversial as resistance of p53 BCs against anthracycline therapy has also been reported [56].

Neoadjuvant Therapy in TNBC

Neoadjuvant treatment regimen enables estimation of the treatment efficiency, in small patient populations, in a limited time frame. Higher response rates (RR) and complete pathological response (pCR) have been consistently reported in case of the neoadjuvant therapy. In case of neoadjuvant anthracycline- and taxane-based regimens, patients with TNBC demonstrated significantly higher pCR rates, as compared to the patients without TNBC. Also, the patients who achieved pCR had a prolonged disease-free survival (DFS). The neoadjuvant treatment with platinum agents has attracted lot of attention in TNBC patients who are positive for BRCA1. There exists a very strong histological similarity between BRCA1 and TNBC [57, 58]. Preoperative clinical trials conducted with a combination of paclitaxel and cisplatin clearly depicted a relapse rate of only 28%, while a partial relapse rate of 63% was reported in patients who were ER and HER-2 negative [59]. Some newer semi-synthetic analogs of the anticancer agent epothilone B, like ixabepilone, have also been explored in neoadjuvant therapies. Ixabepilone has a tendency to bypass resistance related to drug efflux pumps and β tubulin-associated paclitaxel resistance [60, 61].

Adjuvant Therapy for TNBC

TNBC has a very rapid reoccurrence period of less than three years and can metastasize to the CNS due to the nonvalidated targeted therapies. High-dose regimen of anthracyclines and cyclophosphamide (AC), in tandem, have led to a five-year, event-free survival rates in 71% of patients, as compared to only 26% in TNBC patients treated with only the conventional therapy. On other hand, patients with high expression of HER-2 benefited from the anthracycline-based treatment options; however, their efficacy in TNBC is questioned due to higher relapse rates [62, 63]. Furthermore, a combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) was more effective than the combination of cyclophosphamide, epirubicin, and fluorouracil (CEF), in the adjuvant setting, wherein the CMF arm displayed a distinct improvement [64]. When the combination of AC and paclitaxel was evaluated in patients with node-positive operable BC, it resulted in about 17% reduction in the risk of recurrence and about 18% reduction in risk of death due to the disease. Some other chemotherapeutic agents, like capecitabine, have been underutilized in TNBC. When the standard adjuvant chemotherapy of CMF or AC was compared with capecitabine, in women over 65 years of age, capecitabine was observed to be less efficacious mainly because of the heterogeneous nature of TNBC [17]. However, addition of capecitabine to taxanes and anthracyclines, as an adjuvant, was found to be effective in TNBC patients, as indicated by the subgroup analysis of two large randomized trials. Poly (ADP-ribose) polymerase 1 (PARP1) has been explored as a novel target against TNBC. The PAPR1 gene controls the expression of chromatin-associated enzymes involved in the modification of various nuclear proteins [65, 66]. Inhibition of PAPR1 thus leads to breakage of DNA

strands. Under normal conditions, these are corrected by BRCA1 and BRCA2. Therefore, cells with dysfunctional BRCA1 and BRCA2 are more sensitive toward PAPR1 inhibitors like iniparib [38].

2.4.2 Targeted Therapy in TNBC

Mammalian targets of rapamycin are known to improve outcomes in several types of cancers, including renal cancer. TNBC exhibits a higher prevalence of phosphatase and tensin homolog, PTEN, with consequent mTOR activation. This provides a strong rationale for employing mTOR inhibitors in TNBCs that exhibit a loss of PTEN. According to the recent in vitro studies, addition of everolimus to cisplatin increased the latter's efficiency against TNBC [67]. Antibodies like cetuximab are specific against EGFR and diminish its signaling in several types of cancer. Cetuximab has shown little advantage as a monotherapy in advanced clinical investigations for TNBC. Therefore, the role of cetuximab in TNBC is being evaluated in combination with carboplatin, in the metastatic setting [68, 69]. Genomic profiling studies have confirmed that TNBC is a heterogeneous disease. Based on the RNA and DNA profiling, TNBC has been classified as luminal-AR (LAR), mesenchymal (MES), basal-like immune-suppressed (BLIS), and basal-like immune-activated (BLIA) TNBCs. The targets for these specific disease subtypes include the androgen receptor and the cell surface mucin, MUC1, for LAR, the growth factor receptors (PDGF receptor A; c-Kit) for MES, an immune suppressing molecule (VTCN1) in case of BLIS, and Stat signal transduction molecules and cytokines in case of BLIA [70].

3 Ongoing Clinical Trials for BC

Development of a holistic therapy for BC exploiting underutilized potential of the aforementioned receptors remains an uphill task. However, some novel targets and new permutations of known therapeutic moieties are being investigated in clinical settings to enhance survival rate in BC patients. Some of these recent clinical trials, along with their outcomes, have been provided in Table 3.1.

4 Formulation Approaches for Targeting BC

Majority of the deaths in BC are due to distant metastasis of the tumor, via blood stream or lymphatic system, to other visceral organs like lungs, liver, and lymph nodes. Incomplete eradication of tumorous cells and responsive toward standard line of treatment can cause manipulation of cellular genome, promoting the development of resistance. The chances of disease relapse are higher in such cases,

Table 3.1 Ongoing clinical trials that target different receptors for developing newer therapeutic interventions against BC

Sr.No	Phase/design	Background of trial	Regimen	Accrual goal	Status	Outcome	Ref.
A. HER-2+ BC							
1	Chemo-endocrine therapy vs endocrine therapy alone, in hormone receptor-positive BC *(Phase III trial)*	A randomized trial of the endocrine therapy (ET) versus chemo-endocrine therapy (CET) in women with a mid-range RS	Women with a mid-range RS of 11–25 were randomized to receive endocrine therapy (ET) or chemo-endocrine therapy (CET)	10,253 eligible women were enrolled with Hormone receptor (HR)-positive, HER-2-negative, axillary node (AN)-negative BC	Complete	Women with HR-positive, HER-2-negative, AN-negative BC, and an RS of 11–25, adjuvant endocrine therapy was comparable to chemo-endocrine therapy with respect to recurrence rate	[71]
2	Copanlisib in combination with Trastuzumab *(Phase Ib/II open-label, single-arm adaptive, multicenter trial)*	Copanlisib(a PI3K inhibitor) is known to resensitize trastuzumab-resistant cell line to trastuzumab	Copanlisib IV on days 1, 8 and 15, of a 28 day cycle, with trastuzumab being administered at a fixed dose of 2 mg/kg, weekly	Currently, nine patients are being recruited for the study	Ongoing	The endpoint the of study is to determine the maximum tolerated dose	[72]
3	FEC chemotherapy, trastuzumab, and pertuzumab (*noncomparative, phase II, neoadjuvant, randomized study*)	Immunomodulation in HER-2 (+) BC by interference in different pathways of immune system, administration of trastuzumab via different routes	FEC chemotherapy for 3 cycles, with docetaxel and pertuzumab and IV trastuzumab/SC trastuzumab	Present accrual is for about 60 patients, 34 have been recruited	Recruiting	To evaluate variation of host immune response parameters to trastuzumab, via SC/IV route, in combination with pertuzumab and chemotherapy	[73]

B. Estrogen Receptor							
4.	Ribociclib + Endocrine therapy (ET) *(Phase III)*	Combination of ET with ribociclib is being evaluated for early BC patients	1:1 to oral ribociclib and ET or placebo and ET	4000 women and men with fully resected, intermediate risk, (HR+), HER-2–, EBC	Ongoing	Primary endpoint is evaluation of invasive disease-free survival, while secondary endpoints include recurrence-free survival	[74]
5.	Palbociclib, in combination with ET (*phase II, open-label, multicenter study*)	Efficacy of palbociclib, as monotherapy in BC, was not extensively studied. The only available clinical data was limited to heavily pretreated patients	The monotherapy arm received single-agent oral palbociclib, 125 mg once daily, for 3 weeks, while the combination arm received the same dose with palbociclib	A total of 115 patients were recruited	Complete	Palbociclib was effective as monotherapy in ER+ and HER-2− BC and could reverse the resistance developed against the endocrine therapy	[75]
6.	Pembrolizumab (P) and gemcitabine (G) *(Phase II)* ClinicalTrials.gov Identifier: NCT03025880	Combination of (P) that targets PD-1 and (G), a cytotoxic drug, is being evaluated for the first time in advanced BC	Treatment consists of 21-day cycles, with 200 mg P on day 1, and G on days 1 and 8	Maximum of 65 patients will be recruited	Ongoing	It is hypothesized that these agents may synergize to induce responses, with long-term clinical benefit, in advanced BC cancer	[76]
C. Progesterone receptor							
7.	Darolutamide TRIO030	Darolutamide potently inhibits binding of testosterone to AR. Understanding the biological impact of AR-blockade when darolutamide targets the AR in BC should provide foundational rationale for its development in BC	Darolutamide at a dose of 600 mg b.i.d. (daily dose of 1200 mg), for a minimum duration of 14 days, until the day prior to BC surgery (recommended duration of 21 days, maximum 35 days)	60 women with early-stage invasive BC. BC patients with triple-negative, or ER+/HER-2 negative, or HER-2 positive disease will be enrolled in the study	Ongoing	Identification of molecular changes occurring in BC tissue following short-term exposure to darolutamide, in women with early BC. Secondary objectives include assessing the safety and tolerability of short-term darolutamide in women with early BC	[77]

(continued)

Table 3.1 (continued)

Sr.No	Phase/design	Background of trial	Regimen	Accrual goal	Status	Outcome	Ref.
8.	Mifepristone OT1-04-02	Evaluating the effect of mifepristone treatment for BC patients, wherein expression of progesterone receptor isoform A (PR-A) will be higher than those of isoform B (PR-B)	Patients were treated for 14 days with MFP per os (by mouth) 200 mg	20 patients were enrolled	Complete	Evaluation of Ki-67 staining, pretreatment – and posttreatment and evaluating expression of proliferation/apoptosis/PR signaling markers, in core and surgical biopsy samples	[78]
D. TNBC							
9.	ENMD-2076 (*Single-arm, two-stage, phase II clinical trial*)	ENMD-2076 is orally bio-available inhibitor of aurora and angiogenic kinases, with proapoptotic antiproliferative activity in preclinical models of TNBC	Patients were treated with ENMD-2076 – 250 mg (per os by mouth) PO daily, with continuous dosing in 4-week cycles	41 patients	Complete	ENMD-2076 was found to have durable clinical activity	[79]
10.	MK-3475 *(Single-arm phase II study)*	MK-3475is selective humanized mAb blocking interaction of PD-1 and its ligands PD-L1/PD-L2. It is being investigated against MIBC or non-IBC TNBC	Patients will receive MK-3475 200 mg, IV, every 3 weeks for up to 2 years	Up to 35 patients with HER-2 negative MIBC or metastatic TN-IBC will be evaluated	Ongoing	MK-3475 as single agent should have a disease control rate of about 30%. A disease control rate of 10% or lower will be considered as treatment failure	[80]

11.	Olaparib and durvalumab *(*ClinicalTrials.gov *Identifier: NCT03167619)*	Olaparib, either alone or in combination with the PD-L1 inhibitor, durvalumab, in TNBC subjects who have responded to platinum-based chemotherapy	Arm 1 patients will receive olaparib orally, 300 mg, BID continuously, arm 2 patients will receive olaparib orally, 300 mg BID, in combination with durvalumab, 1500 mg IV, every 4 weeks	Approximately 60 subjects with mTNBC (30 in each arm)	Recruiting	The primary endpoint is progression-free survival; the key secondary endpoint is overall survival	[81]
12.	Gemcitabine (G) and pembrolizumab (P) (*Phase II clinical trial*) ClinicalTrials.gov Identifier: NCT03025880	To assess efficacy of the combination of gemcitabine (G) and pembrolizumab (P) in advanced BC	Treatment will consist of 21-day cycles, with 200 mg P on day 1, and G on days 1 and 8	A maximum of 65 patients will be included	Recruiting	It is hypothesized that the combination of agents will provide a synergistic effect. Primary objective is to find the objective response rate (ORR) and evaluate safety and tolerability	[82]

which usually become disease-resistant and pose a serious threat to the limited treatment modalities available. The major hurdles in BC prognosis and treatment arise due to the presence of certain ATP-binding cassettes (ABC) and higher expression of certain proteins like P-glycoprotein (P-gp/ABCB1), ABCG2, and BCRP, which confer multidrug resistance to the tumorous cells. There are two obvious approaches to enhance the outcomes of drug therapy in case of BC; first, the development of new and better drugs, and second, more effective and safer use of the drugs. The second solution is more promising and practical, due to which the development of novel delivery systems and techniques is at the forefront of research and development. Various drug-delivery systems like nanocapsules, nanospheres, polymeric and inorganic nanoparticles, liposomes, and other micellar formulations are being investigated to tackle BC. A brief overview of such systems is provided in Table 3.2. Apart from antibodies and proteins for selective targeting of BC, several drugs have been approved by FDA, till date. These may be classified into three main categories:

4.1 *Drugs that prevent BC*: Raloxifen hydrochloride (Evista), tamoxifen citrate (Nolvadex, Soltamox)

4.2. *Drugs approved to treat BC*: Fulvestrant (Faslodex), doxorubicin hydrochloride, letrozole (Femara), etc.

4.3 *Drug combinations for BC*: **AC** (doxorubicin hydrochloride (adriamycin) and cyclophosphamide), **AC-T** combination (doxorubicin hydrochloride (adriamycin) and cyclophosphamide with paclitaxel (Taxol), **CAF** (cyclophosphamide, doxorubicin hydrochloride (adriamycin), fluorouracil), **CMF** (cyclophosphamide, methotrexate, and fluorouracil), **FEC** (fluorouracil, epirubicin hydrochloride, cyclophosphamide), and **TAC** (docetaxel (taxotere), doxorubicin hydrochloride (adriamycin), cyclophosphamide).

5 Conclusion

Receptors are employed as a means of selective targeting, in the management of BC. Estrogen receptor, progesterone receptor, and HER-2 receptor are well-known targets involved in endocrine therapy against BC. The complex and heterogeneous nature of the disease makes it imperative to identify and exploit novel targets for treatment of patients. It has been reported by many researchers that combined targeting of two or more receptors at a time, like HER-2 and ER, can arrest cellular growth in BC and can check the cancer progression. Upsurge in the number of drug-resistant cases, as also in certain cases of receptor-targeted therapies, further signifies the need to explore alternate receptors to treat BC. Innovative treatment methods involving exploration of delivery platforms using fabricated protein structures, which can exploit tumor-specific physiology for targeting, have been underutilized. Moreover, a consolidated plan for disease diagnosis and therapy is the need of this hour. Discoveries related to receptor targeting of BC should be

Table 3.2 Different formulation platforms against BC

	Composition	Size	Drug	Target/pathology/purpose	Significance	Ref.
A.	*Micelles and nanoparticulate systems*					
1.	Two block polymers:poly(L-lactide) (PLLA)-b-poly(ethylene glycol) (PEG) –folate	94 nm	Doxorubicin	4 T1 mouse mammary tumor cell line and xenografts of murine carcinoma in murine model	Treatment by DOX-loaded mixed micelles demonstrated no apparent metastasis till 28 days	[83]
3.	Poly(bis(2-hydroxylethyl)-disulfide-diacrylate-b-tetraethylenepentamine)	60–110 nm	Doxorubicin	MCF7 and MCF7/ADR cells, MCF7/ADR xenograft murine model	Polymer lowered the IC50 value of doxorubicin (DOX) in MDR tumor cells, to a comparable level, to that of a drug-sensitive cell line	[84]
4.	Poly(ethylene glycol)-block poly (ε-caprolactone)	19.6 ± 0.4 nm	Paclitaxel	MCF-7 cells, MDA-MB-468 cell xenograft –murine model	Delayed release of drug was achieved, along with significant tumor suppression	[85]
B.	*Dendrimers*					
5.	PAMAM dendrimers with diaminobutane core	31.6 ± 2.1 nm	Docetaxel (DTX)	MDA-MB-453 human BC cells and HER-2-negative MDA-MB-231 human BC cells	Dramatic improvement in pharmacokinetic profile of DTX was observed, upon conjugation with the nanosystem	[86]
6.	Polyester dendrimer composed of glycerol and succinic acid	–	Hydroxyl-camptothecin derivatives	MCF7 cell line was used to study the cytotoxic effect of dendrimers	Increased cellular uptake, with enhanced cellular retention of drugs, was reported	[87]
7.	Polyamidoamine (PAMAM) dendrimer fifth generation (G5)	–	Anti-HER-2 mAb	Monoclonal antibody for targeting HER-2 receptor was coupled to dendrimers	Specific targeting of HER-2 positive cells was achieved	[88]
C.	*Liposomes*					
8.	PEGylated liposomal doxorubicin HCl (CAELYX™) and conventional doxorubicin	–	Doxorubicin	Safety and efficacy of PEGylated liposomes of doxorubicin was compared with native doxorubicin, in women with metastatic BC	Reduction in cardiotoxicity, myleosuppresion was noted for PEGylated liposome	[89]

(continued)

Table 3.2 (continued)

	Composition	Size	Drug	Target/pathology/purpose	Significance	Ref.
9.	TLC D-99 (Myocet – liposome-encapsulated doxorubicin citrate)	–	Doxorubicin	The efficacy and toxicity of liposome-encapsulated doxorubicin (TLC D-99) and conventional doxorubicin were compared	Antitumor activity of TLC D-99 was comparable to doxorubicin, but the formulation exhibited reduced cardiotoxicity	[90]
10.	PEGylated liposomal doxorubicin (PLD)	–	Doxorubicin, Docetaxel	Comparison PEGylated liposomal doxorubicin (PLD) and docetaxel, to significantly prolong time for disease progression, as compared with docetaxel alone	Combination of PLD-docetaxel was more effective than docetaxel alone, in women with metastatic BC	[91]
D.	*Inorganic nanoparticulate delivery*					
11.	Polymer-coated surface-functionalized iron oxide nanoparticles	5–10 nm core	Magnetic core was surface functionalized with urokinase plasminogen activator and doxorubicin	Iron oxide nanoparticles for theranostic applications providing site-specific targeting of urokinase plasminogen activator receptor were prepared	Synthesized drug-loaded nanoparticles maintain contrast effect upon internalization in mouse mammary tumor 4 T1 and human breast cancer MDA-MB-231 cells and can be also employed for imaging	[92]
12.	Magnetic nanocapsules	80–150 nm	Camptothecin, doxorubicin	The nanocapsules were encapsulated in polystyrene nanocapsules to provide a powerful magnetic vector, under moderate gradient magnetic fields	Nanocapsules selectively penetrate deeper tumors and provide drug cargo via remote radiofrequency	[93]
13.	Surface-modified gold nanoparticles	~30 nm	Doxorubicin (DOX)	Gold nanoparticles were surface-modified with PEG, and doxorubicin was attached to it via acid-labile spacer. Toxicity was checked in MCF-7/ADR cancer cells	The nanoparticles were insensitive to P-gp transporter-based efflux and their retention in MDR cells was enhanced	[94]

14.	Gold nanostars (NS)	~80 nm	Nanostars were functionalized with TAT-peptide via PEG linkage	BT549 cells were employed to check intracellular uptake of modified gold nanoparticles	Enhanced intracellular delivery and efficient photothermolysis of TAT-NS were observed	[95]
15.	Hollow manganese oxide nanoparticles	64.9 nm	Herceptin antibody	Cationic polyethylenimine-DOPA-herceptin conjugates were proposed to selectively target cancer cells	Functionalization of nanoparticles enabled the targeted detection of cancer cells in T1-weighted MRI and also enabled efficient intracellular delivery of siRNA	[96]
16.	Discoidal porous silicon particles	500–2600 nm, heights from 200–700 nm	–	Reproducible and scalable fabrication of discoid porous silica particles as biodegradable nanovectors for cancer therapy were aimed to deliver higher payloads of actives to the site of tumor	High accumulation of discoid particulate nanovectors into the orthotopically-grown breast tumors in mice	[97]
17.	Upconverting nanoparticles (UCNPs)	20.1 ± 3.0 nm	O-Nitro benzyl (ONB) derivative of 5-FU (ONB − FU)	UCNPs composed of a core of β-$NaYF_4$: 4.95% Yb, 0.08% Tm, and β-$NaYF_4$ shells were developed as platforms capable for targeted chemotherapy	Upon triggered release, targeted delivery of ONB-FU conjugate (77%) was obtained and the rate of drug release could be tuned with the laser power output	[98]
E.	*Protein-based nanoformulation*					
18.	Lipoprotein-like nanoparticles	66.8 nm	Lapatinib	Nanoparticles were fabricated to enhance the water solubility and elevate the antitumor effect of lapatinib in BT-474 cells and murine xenograft model	Nanoparticles passively diffused into the tumor mass and exhibited enhanced retention, leading to an increased antitumor activity	[99]

(continued)

Table 3.2 (continued)

	Composition	Size	Drug	Target/pathology/purpose	Significance	Ref.
19.	Elastin-based protein polymer nanoparticles	100 nm	Rapamycin	Elastin-like polypeptides of sequence $(Val\text{-}Pro\text{-}Gly\text{-}Xaa\text{-}Gly)_n$ were synthesized to deliver rapamycin, in MDA-MB-468 cells	Increase in release and half-life of rapamycin was observed. Toxicity of rapamycin was reduced in murine model	[100]
20.	Nanoparticle made up of albumin	130 nm	Paclitaxel	Approved by USFDA for the treatment of BC, after failure of taxane-based combination chemotherapy, for metastatic disease	Nab-paclitaxel was more effective in combination with either biological agent and/or other cytotoxic chemotherapeutic agents	[101]

prioritized for clinical evaluation which would bring about a paradigm shift in treatment approaches. On the other hand, understanding the biological heterogeneity of disease will give personalized approach for treatment of BC, realizing the goal of long-term survival and improved quality of life in BC patients.

References

1. Dieterich M, Reimer T, Berling A. Influence of lifestyle factors on breast cancer risk. Breast Care. 2014;9(6):407–14.
2. Care P. Assessment of suspected cancer. InnovAiT. 2008;1:94–107.
3. Carney PA, Miglioretti DL, Yankaskas BC, Kerlikowske K, Rosenberg R, Rutter CM, et al. Individual and combined effects of age, breast density, and hormone replacement therapy use on the accuracy of screening mammography. Ann. Intern. Med. 2015;138:168–175
4. Gui G. Male breast cancer: aetiology in the first of a two-part. Trends in Urology& Men's Health. 2012;(3):29–31.
5. Yalaza M, İnan A, Bozer M. Male breast cancer. J Breast Health 2016;(4):1–8.
6. Reed AEM, Kutasovic JR, Lakhani SR, Simpson PT. Invasive lobular carcinoma of the breast: morphology, biomarkers and 'omics. Breast Cancer Res. 2015;17:1–11.
7. Turashvili G, Brogi E. Tumor heterogeneity in breast cancer. Front Med (Lausanne). 2017;4:227.
8. Übersichtsarbeit RA, Sinn H, Kreipe H. Breast care of breast tumors. 4th ed. Focusing. 2013;149–54.
9. Kumar R, Sharma A, Tiwari RK. Application of microarray in breast cancer: an overview. J Pharm Bioallied Sci. 2012;4(1):21–7.
10. Chang M. Tamoxifen resistance in breast cancer. Biomol Ther (Seoul). 2012;20(3):256–67.
11. Athmanathan NP, Ilous AMIB. HER2 testing in breast cancer: an overview of current techniques and recent developments. Pathology. 2012;44(7):587–95.
12. Furrer D, Sanschagrin F, Jacob S. Advantages and disadvantages of technologies for HER2 testing in breast cancer specimens. Am J Clin Pathol. 2015;144(5):686–703.
13. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007;26(45):6469–87.
14. Graus-porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16(7):1647–55.
15. Tzahar E, Waterman H, Chen X, Levkowitz GIL, Karunagaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol. 1996;16(10):5276–87.
16. Tai W, Mahato R, Cheng K. The role of HER2 in cancer therapy and targeted drug delivery. J Control Release. 2010;146(3):264–75.
17. Wahler J, Suh N. Targeting HER2 positive breast cancer with chemopreventive agents. Curr Pharmacol Rep. 2015;1(5):324–35.
18. Tsang RY, Finn RS. Beyond trastuzumab: novel therapeutic strategies in HER2-positive metastatic breast cancer. Br J Cancer. 2012;106(1):6–13.
19. Zagouri F, Bournakis E, Koutsoukos K, Papadimitriou CA. Heat shock protein 90 (Hsp90) expression and breast cancer. Pharmaceuticals (Basel). 2012;5(9):1008–20.
20. Cameron DA, Stein S. Drug insight: intracellular inhibitors of HER2 — clinical development of lapatinib in breast cancer. Nat Clin Pract Oncol. 2008;5(9):512–20.
21. Chadha J, Stuart S. Targeted agents for HER2-positive breast cancer: optimal use in older patients. Drugs Aging. 2015;32(12):975–82.
22. Tanner M, Kapanen AI, Carraway KL, Nagy P, Friedla E, Isola J, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res. 2005;65(2):473–83.

23. Vu T, Claret FX. Trastuzumab: updated mechanisms of action and resistance in breast cancer. Front Oncol. 2012;2:1–7.
24. Gagliato DDM, Leonardo D, Jardim F, Pereira MS, Hortobagyi GN. Mechanisms of resistance and sensitivity to anti-HER2 therapies in HER2 + breast cancer introduction: pathways to trastuzumab. Oncotarget. 2016;7(39):64431–46.
25. Rosenzweig SA. Therapeutics E. HHS public access. 2018;1–24.
26. Murphy L, Cherlet T, Lewis A, Banu Y, Watson P, Murphy L, et al. New insights into estrogen receptor function in human breast cancer. Ann. Med. 2015;3890:614–631.
27. Fisher ER, Osborne CK, Mcguire WL, Redmond C, Sc D, Knight WA, et al. Correlation of primary breast cancer histopathology and estrogen receptor content. Breast Cancer Res Treat. 1981;1:37–41.
28. Huang B, Omoto Y, Iwase H, Yamashita H, Toyama T, Charles R. Differential expression of estrogen receptor α, β1, and β2 in lobular and ductal breast cancer. Proc Natl Acad Sci. 2013;111:2–7.
29. Zhou Z, Qiao JX, Shetty A, Wu G, Huang Y, Davidson NE, et al. Regulation of estrogen receptor signaling in breast carcinogenesis and breast cancer therapy. Cell Mol Life Sci. 2014;71(8):1549.
30. Voudouri K, Berdiaki A, Tzardi M, Tzanakakis GN, Nikitovic D. Insulin-like growth factor and epidermal growth factor signaling in breast cancer cell growth: focus on endocrine resistant disease. Anal Cell Pathol. 2015;2015:1–10.
31. Wei W, Chen Z, Zhang K, Yang X, Wu Y, Chen X, et al. The activation of G protein-coupled receptor 30 (GPR30) inhibits proliferation of estrogen receptor- negative breast cancer cells in vitro and in vivo. Cell Death Dis. 2014;5(132):e1428–e1428.
32. Manuscript A. NIH Public Access. 2013;318(16):1–14.
33. Okoh V, Deoraj A, Roy D. Biochimica et Biophysica Acta Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. BBA Rev Cancer. 2011;1815(1): 115–33.
34. Puhalla S, Bhattacharya S, Davidson NE. Hormonal therapy in breast cancer: a model disease for the personalization of cancer care. Mol Oncol. 2012;6:222–36.
35. Miller WR. Biological rationale for endocrine therapy in breast cancer. Best Pract Res Clin Endocrinol Metab. 2004;18(1):1–32.
36. Manuscript A. NIH Public Access. 2011;280(40):34210–34217.
37. Ndm ON, Borges S, Desta Z, Li L, Skaar TC, Ward BA, et al. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther. 2006;80(1):61–74.
38. Manuscript A, Inhibitors A, Cancer B. NIH Public Access. 2012;125(410):13–22.
39. Brueggemeier RW, Hackett JC, Diaz-cruz ES. Aromatase inhibitors in the treatment of breast cancer. Endocr Rev. 2005;26(3):331–45.
40. Spring L, Bardia A, Modi S, Hospital G, Kettering S. HHS Public Access. 2017;21(113):65–74.
41. Araki K, Miyoshi Y. Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor – positive , HER2 – negative breast cancer. Breast Cancer. 2017;(0123456789).
42. Marsden R. AKT antagonist AZD5363 influences estrogen receptor function in endocrine resistant breast cancer and synergises with fulvestrant (ICI182780). Mol. Cancer Ther. 2015;44(0):2035–2048.
43. Guiochon-Mantel A, Milgrom E. Role of Progestins and progesterone receptors in breast cancer biology. Endocrinology of breast cancer. 11:245–259.
44. Cui X, Schiff R, Arpino G, Osborne CK, Lee AV. Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy. J Clin Oncol. 2018; 23(30):7721–35.
45. Abderrahman B, Jordan VC. Steroid receptors in breast cancer. 5th ed. The breast. Comprehensive management of benign and malignant diseases. Elsevier Inc. 2017;2:p. 272–81.e2.
46. Kariagina A, Aupperlee MD, Haslam SZ. NIH Public Access 2010;18(1):11–33.

47. Tung L, Melville MY, Hovland AR, Takimoto GS, Sartorius CA, Horwitz KB. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol. 2014;8(10):1347–60.
48. Aupperlee M, Kariagina A, Osuch J, Haslam SZ. Progestins and breast cancer. Breast Dis. 2006;24:37–57.
49. Knutson TP, Lange CA. Tracking progesterone receptor-mediated actions in breast cancer. Pharmacol Ther. 2014;142(1):114–25.
50. Mcdonnell DP. Unraveling the human progesterone receptor signal transduction pathway insights into antiprogestin action. Trends Endocrinol Metab. 1995;6(4):133–8.
51. Obr AE, Edwards DP. Molecular and cellular endocrinology the biology of progesterone receptor in the normal mammary gland and in breast cancer. Mol Cell Endocrinol. 2012;357(1–2):4–17.
52. Kolanska K. Progesterone receptor modulators: current applications and perspectives 2019;2:622–627.
53. Chavez-macgregor M, Brown P. HHS Public Access. 2015;15(13).
54. Manuscript A, Onlinefirst P. Downloaded from clincancerres.aacrjournals.org on January 12, 2015. © 2014 American Association for Cancer Research. 2014.
55. Denkert C, Liedtke C, Tutt A, Von Minckwitz G. Breast cancer 3 molecular alterations in triple-negative breast cancer. Lancet. 2016;6736(16):1–13.
56. Berrada N, Delaloge S, Andre F. Treatment of triple-negative metastatic breast cancer: toward individualized targeted treatments or chemosensitization? Ann Oncol. 2010;21(Suppl 7):30–5.
57. Cancer B. HHS Public Access. 2018;7(1):1–12.
58. Omarini C. Neoadjuvant treatments in triple-negative breast cancer patients: where we are now and where we are going. Cancer Manag Res. 2018;10:91–103.
59. Ezzat AA, Ibrahim EM, Ajarim DS, Rahal MM, Raja MA, Tulbah AM, et al. Phase II study of neoadjuvant paclitaxel and cisplatin for operable and locally advanced breast cancer: analysis of 126 patients. Br J Cancer. 2004;90(5):968–74.
60. Goodin S. Novel cytotoxic agents: epothilones. Am J Health Syst Pharm. 2008;65(10 Suppl 3):10–5.
61. Kubista E, Verrill M, Ciruelos E, Steger GG, Manikhas G, Xu L-A, et al. Phase II genomics study of ixabepilone as neoadjuvant treatment for breast cancer. J Clin Oncol. 2008;27(4):526–34.
62. Harbeck N, Gottschalk N, Nitz U, Liedtke C, Gluz O, Pusztai L. Triple-negative breast cancer – current status and future directions. Ann Oncol. 2009;20(12):1913–27.
63. Sawka CA, Pritchard KI, Narod SA, Lickley LA, Kahn HK, Rawlinson E, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13(15):4429–34.
64. Cheang M, Chia SK, Tu D, Jiang S, Shepherd LE, Pritchard KI, Nielsen TO. Anthracyclines in basal breast cancer: the NCIC-CTG trial MA5 comparing adjuvant CMF to CEF. J Clin Oncol. 2009;27(15S):Abstract 519.
65. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. 2005;434(7035):236–9.
66. Slovin SF, Wang X, Hullings M, Arauz G, Bartido S, Lewis JS, Schöder H, Zanzonico P, Scher HI, Riviere I. Chimeric antigen receptor (CAR+) modified T cells targeting prostrate – specific antigen (PSMA) in patients (pts) with castrate metastatic prostrate cancer (CMPC). J Clin Oncol. 31:917–921.
67. Lane HA, Fumagalli S, Ruetz S, Beuvink I, Zilbermann F, O'Reilly T, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell. 2005;120(6):747–59.
68. Tanei T, Choi DS, Rodriguez AA, Liang DH, Dobrolecki L, Ghosh M, et al. Antitumor activity of Cetuximab in combination with Ixabepilone on triple negative breast cancer stem cells. Breast Cancer Res [Internet]. 2016;18(1):1–9. Available from: https://doi.org/10.1186/s13058-015-0662-4.

69. Cancer B, Service M, Nazionale I. Trople-negative breast cancer: an unmet medical need. Oncologist. 2011;16(suppl 1):1–11.
70. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Fuqua SAW, Savage MI, et al. HHS public access. Clin Cancer Res. 2016;21(7):1688–98.
71. Jotte R. IMpower 131. 2018;(18):10–3.
72. Hernando A, Kelly C, Morris P, Bulger K, Gullo G, Egan K, et al. Abstract OT3-06-05: a phase Ib/II trial of coPANlisib in combination with tratuzumab in pretreated recurrent or metastatic HER2-positive breast cancer "PantHER.". Cancer Res. 2018;78(4 Supplement):OT3-06-05-OT3-06–05.
73. Gianni L, Partesotti G, Generali D, Montemurro F, Musolino A, Bisagni G, et al. Abstract OT1-03-03: phase II, open label, randomized, biomarker study of immune-mediated mechanism of action of neoadjuvant subcutaneous trastuzumab in patients with operable or locally advanced/Inflammatory HER2-positive breast cancer. ImmunHER trial on. Cancer Res. 2018;78(4 Supplement):OT1-03-03-OT1-03–03.
74. Alba E, Montemurro F, Hortobagyi G, O'Shaughnessy J, Im S-A, Dieras V, et al. Abstract OT3-05-06: EarLEE-2: a phase 3 study of ribociclib + endocrine therapy (ET) for adjuvant treatment of patients with hormone receptor-positive (HR+), human epidermal growth factor receptor 2-negative (HER2–), intermediate-risk, early breast cancer. Cancer Res. 2018;78(4 Supplement):OT3-05-06-OT3-05–6.
75. Bernardo A, Malorni L, Criscitiello C, Martignetti A, Boni L, Moretti E, et al. Palbociclib as single agent or in combination with the endocrine therapy received before disease progression for estrogen receptor-positive, HER2-negative metastatic breast cancer: TREnd trial. Ann Oncol. 2018;29(8):1748–54.
76. Benito S, Galluzzi L, Escudero M, Berraondo P, Rojo F, de la Cruz L, et al. Abstract OT1-01-02: a multicenter phase II trial to evaluate the efficacy and safety of pembrolizumab and gemcitabine in patients with HER2-negative advanced breast cancer: GEICAM/2015-04 PANGEA-Breast. Cancer Res. 2018;78(4 Supplement):OT1-01-02-OT1-01–2.
77. Mackey JR, Eiermann W, Fresco R, Fung H, Carrez S, Lopez C, et al. Abstract OT1-05-03: TRIO030: a presurgical tissue-acquisition study to evaluate molecular alterations in human breast cancer tissue following short-term exposure to the androgen receptor antagonist darolutamide. Cancer Res [Internet]. 2018;78(4 Supplement):OT1-05-03 LP-OT1-05–03. Available from: http://cancerres.aacrjournals.org/content/78/4_Supplement/OT1-05-03.abstract.
78. Liguori M, Lanari C, Gass H, Rojas P, Elia A, Martinez Vazquez P, et al. Abstract OT1-04-02: Mifepristone treatment for breast cancer patients expressing levels of progesterone receptor isoform A (PRA) higher than those of isoform B (PRB). Cancer Res [Internet]. 2018;78(4 Supplement):OT1-04-02 LP-OT1-04–02. Available from: http://cancerres.aacrjournals.org/content/78/4_Supplement/OT1-04-02.abstract.
79. Pitts T, Tentler J, Elias A, Miller K, Borges V, Gao D, et al. Abstract PD3-16: clinical safety and efficacy of the aurora and angiogenic kinase inhibitor ENMD-2076 in previously treated, locally advanced or metastatic triple-negative breast cancer. Cancer Res 2018;78(4 Supplement):PD3-16-PD3-16.
80. Reuben J, Liu D, Scoggins M, Willey J, Valero V, Dryden M, et al. Abstract OT1-02-01: a phase II study of anti-PD-1 (MK-3475) therapy in patients with metastatic inflammatory breast cancer (MIBC) or non-IBC triple negative breast cancer (non-IBC TNBC) who have achieved clinical response or stable disease to prior chemot. Cancer Res. 2018;78(4 Supplement):OT1-02-01-OT1-02–01.
81. Blackwell K, Traina T, Kim S-B, Tan T, Im Y-H, Dent R, et al. Abstract OT3-04-02: the DORA trial: a non-comparator randomised phase II multi-center maintenance study of olaparib alone or olaparib in combination with durvalumab in platinum treated advanced triple negative breast cancer (TNBC). Cancer Res. 2018;78(4 Supplement):OT3-04-02-OT3-04–02.
82. Reeder-Hayes KE, Carey LA, Sikov WM. Clinical trials in triple negative breast cancer. Breast Dis. 2010;32(1–2):123–36.

83. Gao ZG, Tian L, Hu J, Park IS, Batist G. Prevention of metastasis in a 4T1 murine breast cancer model by doxorubicin carried by folate conjugated pH sensitive polymeric micelles. J Control Release [Internet]. 2011;152(1):84–9.
84. Yin Q, Shen J, Chen L, Zhang Z, Gu W, Li Y. Overcoming multidrug resistance by co-delivery of Mdr-1 and survivin-targeting RNA with reduction-responsible cationic poly(β-amino esters). Biomaterials [Internet]. 2012;33(27):6495–506.
85. Blanco E, Sangai T, Hsiao A, Ferrati S, Bai L, Liu X, et al. Multistage delivery of chemotherapeutic nanoparticles for breast cancer treatment. Cancer Lett [Internet]. 2013;334(2):245–52.
86. Kulhari H, Pooja D, Shrivastava S, Kuncha M, Naidu VGM, Bansal V, et al. Trastuzumab-grafted PAMAM dendrimers for the selective delivery of anticancer drugs to HER2-positive breast cancer. Sci Rep [Internet]. 2016;6:1–13.
87. Wani MC, Carnahan MA, Oberlies NH, Morgan MT, Grinstaff MW, Kroll DJ, et al. Dendrimer-encapsulated camptothecins: increased solubility, cellular uptake, and cellular retention affords enhanced anticancer activity in vitro. Cancer Res. 2006;66(24):11913–21.
88. Desai AM, Baker JR, Thomas TP, Patri AK, Peters JL, Kukowska-Latallo J, et al. HER2 specific tumor targeting with dendrimer conjugated anti-HER2 mAb. Bioconjug Chem. 2006;17(5):1109–15.
89. O'Brien MER, Wigler N, Inbar M, Rosso R, Grischke E, Santoro A, et al. Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX™/Doxil®) versus conventional doxorubicin for first-line treatment of metastatic breast cancer. Ann Oncol. 2004;15(3):440–9.
90. Belt R, Azarnia N, Winer E, Navari R, Rovira D, Harris L, et al. Liposome-encapsulated doxorubicin compared with conventional doxorubicin in a randomized multicenter trial as first-line therapy of metastatic breast carcinoma. Cancer. 2002;94(1):25–36.
91. Bondarenko IN, Tjulandin SA, Oliynychenko GP, Balashova OI, Yuan Z, Xiu L, et al. Pegylated liposomal doxorubicin plus docetaxel significantly improves time to progression without additive cardiotoxicity compared with docetaxel monotherapy in patients with advanced breast cancer previously treated with neoadjuvant-adjuvant anthracyclin. J Clin Oncol. 2009;27(27):4522–9.
92. Yang L, Cao Z, Sajja HK, Mao H, Wang L, Geng H, et al. Development of receptor targeted magnetic iron oxide nanoparticles for efficient drug delivery and tumor imaging. J Biomed Nanotechnol. 2008;4(4):439–49.
93. Jin S, Lal R, Lee JH, Karin M, Brammer K, Zhang W, et al. Magnetically vectored nanocapsules for tumor penetration and remotely switchable on-demand drug release. Nano Lett. 2010;10(12):5088–92.
94. Wang F, Wang YC, Dou S, Xiong MH, Sun TM, Wang J, et al. Doxorubicin-tethered responsive gold nanoparticles facilitate intracellular drug delivery for overcoming multidrug resistance in cancer cells. ACS Nano. 2011;5(5):3679–92.
95. Yuan H, Fales AM, Vo-Dinh T. TAT peptide-functionalized gold nanostars: enhanced intracellular delivery and efficient NIR photothermal therapy using ultralow irradiance. J Am Chem Soc. 2012;134(28):11358–61.
96. Bae KH, Lee K, Kim C, Park TG. Surface functionalized hollow manganese oxide nanoparticles for cancer targeted siRNA delivery and magnetic resonance imaging. Biomaterials [Internet] 2011;32(1):176–184. Available from: https://doi.org/10.1016/j.biomaterials.2010.09.039.
97. Liu X, Srinivasan S, Alexander JF, Chiappini C, Ferrari M, Godin B, et al. Discoidal porous silicon particles: fabrication and biodistribution in breast cancer bearing mice. Adv Funct Mater. 2012;22(20):4225–35.
98. Fedoryshin LL, et al. Near-infrared triggered anti-cancer drug release from upconverting nanoparticles. ACS Appl Mater Interfaces. 2014;6(16):13600–6.
99. Gao H, Cao S, Chen C, Cao S, Yang Z, Pang Z, et al. Incorporation of lapatinib into lipoprotein-like nanoparticles with enhanced water solubility and anti-tumor effect in breast cancer. Nanomedicine. 2013;8(9):1429–42.

100. Shi P, Aluri S, Lin YA, Shah M, Edman M, Dhandhukia J, et al. Elastin-based protein polymer nanoparticles carrying drug at both corona and core suppress tumor growth in vivo. J Control Release [Internet]. 2013;171(3):330–338. Available from: https://doi.org/10.1016/j.jconrel.2013.05.013.
101. Montero AJ, Adams B, Diaz-Montero CM, Glück S. Nab-paclitaxel in the treatment of metastatic breast cancer: a comprehensive review. Expert Rev Clin Pharmacol. 2011;4(3):329–34.

Chapter 4
Cancer of Reproductive System: Receptors and Targeting Strategies

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Abstract Carcinogenesis in the different organs of the reproductive system, particularly, prostate, ovarian, and cervical tissues, involves aberrant expression of various physiological receptors belonging to different superfamilies. This chapter provides insights into the physiological receptors that are associated with the genesis, progression, metastasis, management, as well as the prognosis of the cancers of the male and female reproductive systems. It also highlights the structural and binding characteristics of the highly predominant receptors, namely, androgen, estrogen, progesterone, and gonadotropin-releasing hormone (GnRH) receptors, which are overexpressed in these cancers and discusses various strategies to target them.

Keywords Reproductive · Cancers · Prostate · Ovarian · Cervical · Receptors · Structure · Binding · Target

Abbreviations

5-A DHT	5A-di-hydro testosterone
AD	Adenovirus
ADC	Antibody–drug conjugates
AIs	Aromatase inhibitors
ARE	Androgen response elements

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BPH	Benign prostate hyperplasia
CDK	Cyclin-dependent kinase
CNS	Central nervous system
CRPC	Castrate-resistant prostate cancer
DBD	DNA-binding domain
DOX	Doxorubicin
E2	Estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EOC	Endometrial ovarian cancer
ER	Estrogen receptor
ERE	Estrogen response elements
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GLU	Glutamate
GNRH	Gonadotropin-releasing hormone
GNRHR	Gonadotropin-releasing hormone receptor
GPCR	G-protein-coupled receptor
GPER	G-protein estrogen receptor
HER-2	Human epidermal growth factor receptor-2
HPG	Hypothalamic–pituitary gonadal (axis)
HPV	Human papilloma virus
HRE	Hormone response elements
HSP	Heat shock proteins
i.m.	Intramuscular
LBD	Ligand-binding domain
LBP	Ligand-binding pocket
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
mAbs	Monoclonal antibodies
MCRPC	Metastatic castrate-resistant prostate cancer
NLS	Nuclear localization signal
NTD	N-terminal transcription regulational domain
P4	Progesterone
PR	Progesterone receptor
PRB	Retinoblastoma protein
PRE	Progesterone response elements
PSA	Prostate-specific antigen
PSGR	Prostate-specific G-protein-coupled receptor
PSMA	Prostate-specific membrane antigen
PSMAA	Prostate-specific membrane aptamers
RES	Reticulo-endothelial system
RP2D	Recommended phase 2 dose

s.c.	Subcutaneous
SAR	Structure–activity relationships
SARM	Selective androgen receptor modulators
SCID	Severe combined immunodeficiency
SERM	Selective estrogen receptor modulators
SMA	Styrene–maleic scid
SMA-RAL	SMA-encapsulated raloxifene
SPRM	Selective progesterone receptor modulators
TMD	Transmembrane domain

1 Introduction: Reproductive System-Related Cancers

Cervical and ovarian cancers are the fourth most and the seventh most common cancers in women, with a global prevalence of approximately 3.7 percent and 1.7 percent, respectively [1–3]. The prostate cancer is considered as the fifth leading cause of cancer-associated mortality in men, with a global prevalence of about 7.9 percent [3, 4]. The risk factors for cancers related to reproductive system include but not limited to endogenous factors such as genetic history, race, aging, hormonal imbalance, and exogenous factors such as inappropriate diet, unhealthy lifestyle, and environmental and occupational factors. Moreover, cervical cancer risk factors are extended to the infection of human papilloma virus (HPV), extended usage of contraceptives, age of menarche and menopause, unsafe sexual activities such as sexual intercourse at an early stage and multiple sexual partners [1, 5–12]. This chapter provides a detailed overview of the structural, pharmaceutical, and clinical aspects of the agents discovered to target the dominant receptors involved in the development, treatment, and prognosis of the reproductive neoplasia.

2 Overview: Receptors Associated with the Cancers of the Reproductive System

Table 4.1 lists the receptors that are ubiquitous in various cancers of the reproductive system, specifically during their prognosis, diagnosis, progression, and therapy.

The subsequent discussion is focused on four principal receptors that display a significant expression pattern during the genesis, diagnosis, treatment, and prognosis of the reproductive neoplasia.

Table 4.1 Receptors associated with the prognosis, diagnosis, progression, and therapy of the cancers of the reproductive system

Type of receptors	Cervical cancer	Ovarian cancer	Prostate cancer
Ion channel receptors (inotropic)	P2 receptors: P2X4, P2X7 [13–15]	P2 receptors: P2X7 [13, 14]	P2 receptors: P2X4, P2X5, P2X7 [13, 14] NMDA (N-methyl-D-aspartate) receptor [16] GABAa receptor [17]
G-protein-coupled receptors (GPCRs) (metabotropic)	Endothelin-1: (ET_AR) [18–20] Protease-activated receptor-2 (PAR-2) [21] Gastrin-releasing peptide receptor (GRPR) [22, 23] G-protein-coupled estrogen receptor-1 (GPER-1) – prognostic maker for early-stage cancer [7] Folate receptor subtype alpha (FRα) [24]	Endothelin-1: (ET_AR) [19, 25, 26] Protease-activated receptor-1 and 2 (PAR 1 and 2) [27, 28] β-Adrenergic receptor [29] Gastrin-releasing peptide receptor (GRPR) [30, 31] G-protein-coupled estrogen receptor-1 (GPER-1) [32, 33] Folate receptor – FRα and Reduced Folate Carrier (RFC) [34] Follicle-stimulating hormone receptor (FSHR) Luteinizing hormone receptor (LHR) Gonadotropin-releasing hormone receptor (GnRHR) Thyroid-stimulating hormone receptor (TSHR) Kisspeptin receptor Angiotensin II type 1 receptor [2] Serotonin (5-HT) receptors – 5-HTR1A, 5-HTR1RB, 5-HTR2B, 5-HTR4 [35]	Endothelin-1: (ET_AR) [19, 36, 37] Protease-activated receptor-1, 2, and 4 (PAR-1, 2, and 4) [38–40] β-Adrenergic receptor [29, 41] Gastrin-releasing peptide receptor (GRPR) [31, 42] G-protein-coupled estrogen receptor-1 (GPER-1) [33] Prostate-specific G-protein-coupled receptor (PSGR)-PSGR2 [43, 44] G-protein-coupled receptor-158 (GPR158) [45] Lysophosphatidic acid (LPA)-1 receptor [46] Gonadotropin-releasing hormone receptor (GnRHR) [47] Serotonin (5-HT) receptors – 5-HTR1A, 5-HTR1RB, 5-HTR2B, 5-HTR4 [48, 49]

(continued)

Table 4.1 (continued)

Type of receptors	Cervical cancer	Ovarian cancer	Prostate cancer
Tyrosine kinase receptors	Human epidermal growth factor receptor (HER-2)/neu [50] Epidermal growth factor receptor (EGFR) [51] Insulin-like growth factor-I receptor (IGF-IR) [52] Vascular endothelial growth factor (VEGF) receptor [53] Prolactin receptor (PRLR) [54] Hepatocyte growth factor/(cMET) [55] Fibroblast growth factor receptor (FGFR) [56]	HER-2/neu receptor [57] Epidermal growth factor receptor (EGFR) [58] Insulin-like growth factor-I receptor (IGF-IR) [59] Vascular endothelial growth factor (VEGF) receptor [53] Hepatocyte growth factor/(cMET) [60] Fibroblast growth factor receptor (FGFR) [61]	HER-2/neu receptor [62] Epidermal growth factor receptor (EGFR) [63] Insulin-like growth factor-I receptor (IGF-IR) [64] Vascular endothelial growth factor (VEGF) receptor [65] Hepatocyte growth factor/(cMET) [60] Fibroblast growth factor receptor (FGFR) [66]
Nuclear receptors	Estrogen receptor (ERα and ERβ) Progesterone receptor (PR): PR-A, PR-B [1] Vitamin-D receptor (VDR) [67] Retinoic acid receptor β [68]	Estrogen receptor (ERα and ERβ) Progesterone receptor (PR): PR-A, PR-B [1, 69] Androgen receptor (AR) Vitamin-D receptor (VDR) [67] Retinoic acid receptor [70]	Androgen receptor (AR) [71] Estrogen receptor (ERβ) [72] Progesterone receptor (PR) [73] Peroxisome proliferator-activated receptor-Gamma (PPARγ) [74]

3 Predominant Receptors in Reproductive System-Related Cancers

3.1 Nuclear Receptors

Nuclear receptors comprise a family of transcription factors that get activated due to the binding of the lipophilic ligands, to carry out reproduction, development, homeostasis, and metabolism. They act by responding to the signals generated by the steroid hormones and regulate the expression of the target genes [75–77].

3.1.1 Steroid Sex Hormone Receptors

The organs of the reproductive system serve as the primary sites of action of sex steroid hormones, such as the estrogen, progesterone, and androgen. These hormones are responsible for mediating the developmental activities and physiological

functions of the male and female reproductive systems. They exert their functions through the action of steroid hormone receptors, namely, the estrogen (ER), progesterone (PR), and androgen (AR) receptors, respectively. Aberrations in their expression and/or in the factors regulating them, termed as coregulators, lead to either activation or suppression of their transcription machinery, eventually impacting their physiological functions. These abnormalities trigger a cascade of pathological changes in vivo, thereby resulting into carcinogenesis [78, 79].

ER-subtype α (ERα) and PR receptors play a pivotal role in the pathophysiology of cervical cancer. The PRs were found to exhibit tumor-suppressive properties in cervical cancer [1, 7]. In the case of ovarian malignancy, ERα expression provided a better prognosis, while the role of ERβ was insignificant. On the other hand, an elevated PR expression was observed to improve the survival rate in patients with endometrial ovarian cancer (EOC) [80]. The activity of AR has been closely linked to the prostatic carcinogenesis. The biochemical pathway of AR, the principal regulator of prostatic cancer, is perturbed during the carcinogenesis. Castration-resistant prostate cancer (CRPC), an advanced stage of the disease which is nonresponsive to hormone deprivation therapy, occurs due to increase in sensitivity of the AR to the agonists, mutations in the receptor, ligand-independent activation of the ARs, etc. [71]

3.2 G-Protein-Coupled Receptors

3.2.1 Gonadotropin-Releasing Hormone Receptor (GnRHR)

GnRH-I, produced in vivo, by the GnRHR (located in the hypothalamus) stimulates the secretion of the gonadotropins, namely the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), which further regulate the in vivo levels of sex hormones [81]. The GnRHR is also expressed in the CRPC [82]. Primary cultures of ovarian carcinomas and biopsy specimens of malignant ovarian tissue have revealed the predominant expression of the GnRHR receptor (>80%), signifying its role in the genesis of malignancy and metastasis. The GnRHR has also been speculated to be associated with the early phases of ovarian carcinogenesis, including cell migration and invasion [83]. It was observed that administration of GnRH-1 agonists and antagonists lead to the downregulation and inactivation of GnRHR, respectively. As a result, the GnRH agonists cause inhibition of cell proliferation, metastasis, and angiogenesis. Moreover, the GnRH antagonists also possess antineoplastic activity [81, 82]. Thus, exploration of the structure and regulation of the GnRHRs in cancers of the reproductive system may enhance their applicability, as a target receptor, for the discovery of new-age anticancer therapeutics.

Understanding the structure and the binding chemistries of these receptors and reviewing the potential of targeting it may pave the way to the discovery of the breakthrough anticancer therapies in the near future.

4 Predominant Receptors in Cancers Related to Reproductive System: Structural Attributes

4.1 Androgen Receptor

Androgen receptor (AR), a 110-kDa protein, is a ligand-activated transcription factor belonging to the family of steroid hormone nuclear receptors [71]. AR, expressed in prostate, is activated by binding of endogenous androgens, such as testosterone and 5α-dihydrotestosterone (5 α-DHT). Functional AR is responsible for in vivo male sexual differentiation and occurrence of pubertal changes [84].

This receptor mediates normal growth and development of the prostate gland and also plays a vital role in the prostatic carcinogenesis and its progression to an androgen-independent disease. Androgen-independent stage of prostate cancer (e.g., CRPC) has been observed due to the activation of the AR receptor by overexpression of gene and cofactors, gene mutations, splice variants, and intracrine synthesis of androgen [85, 86]. In addition, AR is also expressed in the different subtypes of ovarian and cervical cancers [87, 88].

4.1.1 Androgen Receptor: Recognition Domain and Receptor Pathway

The AR modular protein consists of four distinct domains, namely, the ligand-binding domain (LBD), the hinge domain, the DNA-binding domain (DBD), and the N-terminal transcriptional regulation/amino-terminal domain (NTD). NTD is the most variable region, whereas DBD and LBD are highly conserved among different receptors of the steroid hormone nuclear receptor family. The LBD, the key recognition domain of AR, is arranged in three layers and comprises eleven α-helices, particularly, H1–H11, except H2, which results into formation of an antiparallel "α-helical sandwich." The ligand-binding pocket (LBP) is formed by H5, C-terminal of H10 and H11, and N-terminal of H3. The activation function (AF)-1, located at the N-terminal, is not conserved in the sequence and is ligand-independent, whereas AF-2, at the C-terminal, is conserved and ligand-dependent. The nuclear localization signal (NLS) is located between the DBD and the hinge region [71, 89, 90].

The Genomic Pathway of AR

Unbound AR exists in an inactive state in the cytoplasm, in complexation with the heat shock proteins (HSP) such as HSP90. Upon binding to a ligand/agonist, it gets activated and dissociates from the HSP and undergoes dimerization and phosphorylation. Binding of an agonist leads to the formation of the AF-2 region on the surface of LBD. Upon ligand binding, AF-2 interacts with the amino-terminal motifs of the receptor, which leads to an establishment of N/C intradomain crosstalk, thereby leading to receptor stabilization, enhanced DNA-binding affinity, and

reduced ligand dissociation, a phenomenon exclusively observed in the AR, unlike other steroid receptors. AF-2 domain also recruits coregulatory proteins to an activated AR, thereby contributing to its overall function. H12, the core structure of AF-2, acts as a lid to close LBP, upon binding of the agonist. Further, the NLS gets exposed upon the ligand/agonist binding and interacts with importin-α. This leads to translocation of AR from cytoplasm to the nucleus. DBD facilitates interaction of the translocated receptor with the DNA at specific recognition sites. These sites, located in the promoter and enhancer regions of the AR target genes, consist of consensus sequences and are termed as androgen response elements (ARE). Access of AR to the target chromatin requires concerted action of certain transcription factors. AF-1 and AF-5 of NTD mediate the transcriptional activity by recruitment of coactivator complexes and transcription machinery, essential to regulate the expression of the target genes. Selective recognition of specific ARE sequences is regulated by the ligand-binding and/or presence of other transcription factors [84, 89–91]. Figure 4.1a provides a schematic layout of the structure of the steroid receptor (SR) and its pathway of transactivation after binding of the ligand.

4.1.2 Ligands and Their Structure–Activity Relationships (SAR) for Selective Binding to AR

Ligands modulate their action by binding to the LBP of LBD. The AR is capable to accommodate a large variety of ligands by modifying the volume of LBP [71]. Testosterone (Fig. 4.2a) and DHT (Fig. 4.2b) are the physiological ligands of the AR [89]. Carbon atoms of the testosterone skeleton have been numbered in order to provide basis for the SARs with various ligands and their derivatives [71]. Synthetic derivatives of testosterone have been prepared to enhance oral bioavailability. It is essential for the ligand (natural/synthetic) to contain a steroidal skeleton for retaining the androgenic activity. Hydrophobic amino acid residues in the LBP interact with the steroid scaffold. A/B ring junction usually has “trans” stereochemistry. 17β-OH atom is essential for ligand–receptor interaction via formation of a hydrogen bond with the amino acid residues [71, 89].

Fig. 4.1 (**a**) Schematic of the structure and genomic pathway of the Steroid Receptor (SR) activated upon binding of the ligand (e.g., hormone). Binding of the ligand causes activation of the HSP-90-bound SR that undergoes nuclear translocation, dimerization, and then binds to the hormone response elements (HRE) by the action of cofactors/coactivators, thus resulting into transactivation and transcription of the target genes. The enlarged view of the SR reveals different structural domains, namely, N-terminal domain (NTD), DNA-binding domain (DBD), Hinge region (H), and ligand-binding domain (LBD), wherein each domain is capped by N-termini and C-termini amino acid residues. Presence of NLS, AF-1, and AF-2 domains has also been indicated. (**b**) Schematic representation of GnRHR and intracellular pathway activated on binding of GnRH. Binding of the ligand activates the receptor. The latter binds to GTP-linked proteins, or G-proteins. Gαq/11, a subunit of G-proteins, activates phospholipase C, which then hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), further leading to cascade of intracellular events, operating through activation of phospholipase A2, ultimately leading to Gonadotropin biosynthesis and release

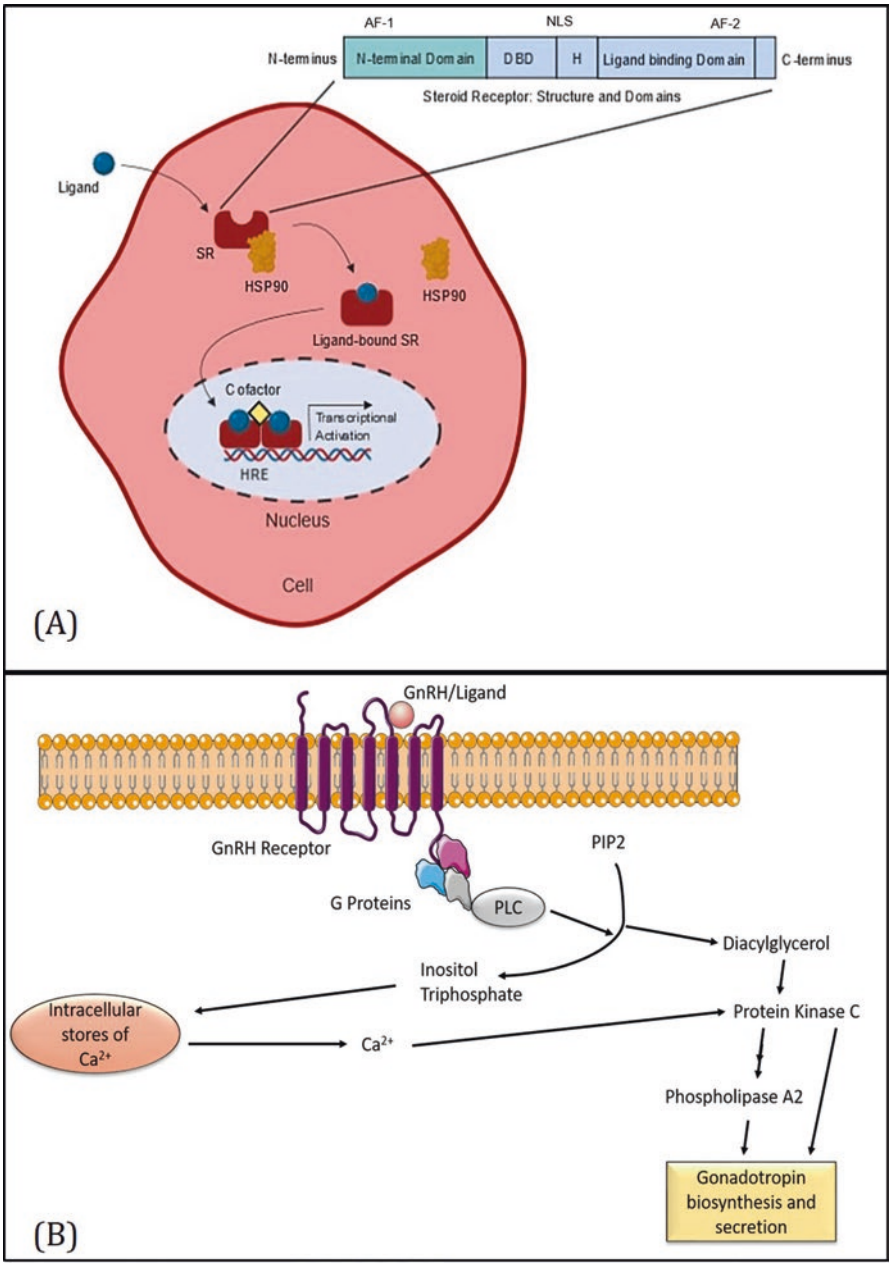

Fig. 4.1 (Caption on p. 116)

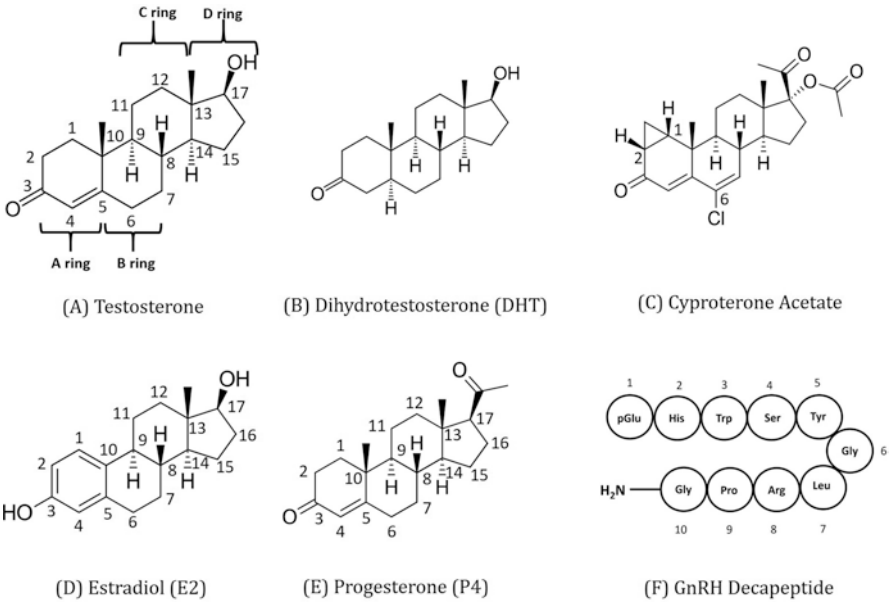

Fig. 4.2 Structure of ligands/chemical agents and action on the predominant receptors of cancers of the reproductive system. Carbon atoms and hydrophobic rings have been numbered wherever necessary. (**a**) Testosterone, (**b**) Dihydrotestosterone, (**c**) Cyproterone acetate, (**d**) Estradiol (E2), (**e**) Progesterone (P4), and (**f**) GnRH decapeptide

4.2 Estrogen Receptor

Estrogens naturally occur in three different forms in females, namely, estradiol (E2) (Fig. 4.2d), estriol, and estrone. It exerts its action through the estrogen receptors (ERs), which belong to the steroid hormone superfamily of nuclear receptors. ERs, occurring in two forms, ER-α and ER-β, act as ligand-activated transcription factors, upon binding to the endogenous ligands [92–94]. Expression of estrogen and its receptors have been very well documented in prostate, ovarian, and cervical cancers. ER-α and ER-β have been reported to play oncogenic and antioncogenic roles in the pathogenesis of prostate cancer. The aberrant expression of the enzymes involved in steroid biosynthesis and metabolism, such as aromatase and 5-α reductase, has also been implicated in prostatic malignancy [72, 95]. This chapter will further provide insights into the structural aspects of the receptor and its SAR with the ligands.

4.2.1 Recognition Domain and Receptor Pathway of ER

ER-α (66-kDa protein) is predominantly expressed in the reproductive tract, whereas ER-β (54-kDa protein) primarily occurs in the vascular endothelial cells, bones, and male prostatic tissues. The ER receptor consists of an NTD, DBD,

hinge region, and LBD. The NTD stimulates transcription from particular estrogen-responsive promoters via AF-1. DBD binds to estrogen response elements (ERE) in the target DNA, while the hinge region contains nuclear NLS. The LBD and AF-2 activate the gene expression in response to ligand binding. The classical genomic pathway results in the formation of estrogen-dependent, nuclear ER homo- or heterodimers, such as ER-α/ER-α, ER-β/ER-β, and ER-α/ER-β, respectively. Further, these dimers subsequently bind to the estrogen response element (ERE) sequences located in the promoter region of estrogen-responsive genes, resulting in the recruitment of coregulatory proteins (coactivators or corepressors) to the promoter. This leads to either an enhancement or reduction in the mRNA levels, further impacting the production of associated proteins and eventually the physiological response [89, 96].

4.2.2 Ligands and Their Structure–Activity Relationships for Selective Binding to ER

A vast array of compounds acting as ligands for the ER-α and ER-β receptors have been classified as endo-estrogens (E2), phytoestrogens (Resveratrol), xenoestrogens (Mestranol), metalloestrogens (copper (Cu^{2+}), etc. [89, 97, 98].

Recognition of the binding of endo-estrogen (E2) to ER is achieved partly by intermolecular hydrogen binding and van der Waals interactions with the receptor [97]. The aromatic A ring, C-3 and 17β hydroxyl groups and the distance between them, and planar hydrophobic structure are essential for estrogenic activity. On the other hand, substitution at C-1, hydroxylation at C-6, 7, and 11, removal of oxygen from C-3 or C-17, and epimerization of 17-β-hydroxyl group of E2 to α-configuration lead to reduction in the activity [89, 99].

4.3 Progesterone Receptor

Progesterone (P4; represented in Fig. 4.2e) and progesterone receptors (PRs) are necessary for the development of hormone-responsive tissues such as breasts and other organs of the reproductive tract. It is responsible for ovulation, embryo implantation, pregnancy, development of the mammary gland, and sexual differentiation and behavior. The hormone inhibits the proliferative action of estrogen in the reproductive tissues, such as endometrium and ovary, thus preventing them from undergoing neoplastic transformation [100, 101]. PRs, existing as two isoforms, namely PR-A ((molecular weight of 94 kDa) and PR-B (molecular weight of 114 kDa), are ligand-activated transcription factors, belonging to the superfamily of steroid hormone nuclear receptors. PR-B is referred to as full length and dominant receptor, while PR-A is the N-terminal truncated version [102, 103]. Both the isoforms suppress proliferation of the prostate stromal and cervical cancer cells [1, 104]. PR overexpression is associated with favorable prognosis in women with ovarian malignancies [105].

4.3.1 Recognition Domain and Receptor Pathway of PR

The PR receptors share common structural elements with other steroid receptors, namely, NTD, DBD, hinge region, and LBD. The NTD is responsible for ligand-independent transcriptional activation and harbors a highly variable AF-1 domain. The DBD binds to the progesterone response elements (PREs) located in the target DNA. The hinge region contains NLS, while the LBD and a highly conserved AF-2 domain are responsible for the ligand-mediated transactivation of the gene expression, via the genomic pathway. The LBD or the primary recognition domain comprises a hydrophobic LBP, to facilitate ligand binding. The genomic pathway operates on binding of P4, which causes conformational change in the PR, thereby transforming it into an active transcriptional factor. As a result, receptor phosphorylation occurs and the PR undergoes dimerization (homo/hetero) and nuclear translocation, to further interact with the PREs. This also leads to the recruitment of coactivators that mediate gene transcription. It has been reported that PR-A and PR-B possess opposite transcriptional activities and the overall response of P4 is dependent on the relative in vivo levels of PR-A and PR-B. 5α-reductase and 20α-hydroxysteroid dehydrogenase are responsible for the metabolic conversion of P4 to a more active or less active form, before interaction with the receptors in the target cells [89, 102, 106].

4.3.2 Ligands and Structure–Activity Relationships for Selective Binding to PR

P4 is an endogenous ligand of the PR receptors. The progestin activity is confined to the molecules having a steroid nucleus. The synthetic progestins have been categorized into two classes, namely, the androgens (19-norandrostane or estrane derivatives), and 17 α-hydroxyprogesterones. In case of the compounds belonging to the androgen category, 17α-substituents like ethyl, methyl, etc., lead to increase in the oral bioavailability (e.g., Ethisterone). Removal of the methyl group at C19 position and chlorination at C21 or methylation at C18 provided norethisterone, whose activity was further enhanced by chlorine substitution at C21 or by the addition of methyl group at C18 (e.g., Norgestrel). Acylation of 17β-hydroxyl group of Norethisterone extended the duration of its action. Synthetic progestins include medroxyprogesterone acetate and norethisterone (first generation), norgestrel and levonorgestrel (second generation), etonogestrel and nosgestimate (third generation), drospirenone and trimegestone (fourth generation), etc. [89].

4.4 Gonadotropin-Releasing Hormone Receptor (GnRHR)

The GnRH (Fig.4.2f) is a decapeptide that plays a pivotal role in regulating the reproductive functions by functioning through the hypothalamic–pituitary gonadal (HPG) axis. The action of GnRH is mediated by the action of GnRHR, which

belongs to the rhodopsin-like GPCR superfamily. GnRHR is expressed in various reproductive cancers, such as the prostate, ovarian, endometrial, and the breast cancer, as well as the nonreproductive cancer types. In these tumors, the GnRH functions as a paracrine–autocrine growth factor and displays a strong anticancer activity. The GnRHR (for 3D structure, refer to Flanagan C.A. et al.) consists of seven transmembrane (TM) domains, as well as an extracellular amino-terminal domain that contains 35 amino acids, along with two putative glycosylation sites. However, it lacks the carboxy-terminal cytoplasmic tail resulting in slow internalization and desensitization of the receptor. The membrane-spanning segments are highly conserved, while the loops and the termini constitute to be the variable regions [47, 82, 107, 108].

4.4.1 Gonadotropin-Releasing Hormone Receptor: Recognition Domain and Receptor–Ligand Interactions

The receptor (R) exists in an equilibrium between an inactive R, which does not activate G proteins and is stabilized by an antagonist, and an active R^* conformation, which activates G proteins and is stabilized by agonists, depending on the presence or absence of the ligand. The ligands of the receptor interact with the variable, extracellular half of the receptor molecule. The membrane-spanning receptor domain transmits the signal generated upon ligand binding to the cytosolic receptor surface, which further interacts with the G protein. The GnRHR must exist in a silent state that does not activate the G protein, in order to transduce the signals mediated by agonists across the cell membrane. Binding of the agonist causes transition from the silent state and leads to the binding and activation of G proteins, situated on the opposite side of the cell membrane. Thus, agonists like GnRH act as allosteric activators of the receptor. The primary features of the inactive form of the receptor include closed G-protein-binding pocket, a hydrogen-bonding network, and a hydrophobic barrier. Binding of the ligand activates the receptor, causing rotation and change in the interfaces of specific TM segments, leading to opening of the hydrophobic barrier, movement of specific amino acid residues toward the interior of the TM bundle, and ultimately opening of the cytoplasmic surface cleft that allows contact and binding of the G-proteins [108]. The structure of the recognition domain or LBD of the receptor, involved in binding, depends on the type of the ligand such as a neurotransmitter, a glycoprotein hormone, and a peptide. In the case of a neurotransmitter, the TM domains (TMDs) themselves form the LBP to facilitate ligand binding. The amino-terminal domain of the receptor, encompassing the high-affinity ligand-binding site, has been reported to be recognition domain for glycoprotein hormones. A high-affinity binding site for the peptide-based ligands include both extracellular and TM residues [109].

4.4.2 Gonadotropin-Releasing Hormone Receptor Pathway

Binding of the hormone causes coupling of GnRHR to $G_{\alpha q/11}$ protein, which stimulates the phospholipase Cβ (PLCβ) activity. This leads to the enhancement of intracellular levels of inositol triphosphate (IP3) and diacylglycerol (DAG), further causing intracellular mobilization of Ca^{2+} ions and activation of the protein kinase C (PKC). These downstream effects lead to the activation of various signaling pathways, which operate through the MAPK reaction cascade. Phospholipase D and phospholipase A2 are also activated in a sequential manner. These biochemical pathways are vital in eliciting the GnRH-mediated downstream effects, such as gonadotropin synthesis and secretion [47]. Figure 4.1b provides a schematic representation of the GnRHR, a GPCR, and the intracellular pathway activated upon binding of GnRH to the receptor.

4.4.3 Structure–Activity Relationships for Selective Ligand Binding to GnRHR

Amino- and carboxy-terminal residues are critical for receptor binding and activation. The presence of achiral glycine (Gly) or D-amino acids is essential at position 6, to facilitate active conformation in the folded state. His^2, Trp^3, as well as $pGlu^1$ of the GnRH decapeptide possess significant roles in receptor activation. Substitution of amino acid residues located outside the amino-terminal domain is speculated to affect the receptor activation, due to the conformational changes in the ligand that may occur upon binding to the receptor [110].

The subsequent section of the chapter highlights the approaches explored for receptor targeting as well as an overview of mechanisms involved therein.

5 Approaches for Receptor Targeting: Relevance to Cancers of Reproductive System

Table 4.2 provides an overview of drug molecules developed to target receptors predominantly expressed in the cancers of the reproductive system. Fig.4.3 provides a schematic layout of the classification of the agents (ligands, agonist, antagonists, modulators, etc.), either endogenously present and/or synthesized for targeting to the aforementioned receptors.

5.1 Mechanisms of Receptor Targeting and Implications in Cancers of Reproductive System

Several mechanisms employed for targeting receptors predominantly expressed in the cancers of reproductive system have been summarized and supported by relevant case studies in this section.

Table 4.2 Overview of drug molecules developed to target receptors predominantly expressed in the reproductive neoplasia

Sr. No.	Class	Description	Examples	Ref
1	Selective androgen receptor modulators (SARM)	The nonsteroidal compounds, referred to as Selective androgen receptor modulators (SARM), demonstrate weak agonistic or antagonistic activity in prostate cancer, while they exhibit agonistic effects in the peripheral tissues. They consist of four principal pharmacophores, namely, N-aryl propionamide, bicyclic hydantoins, quinolinones, tetrahydroquinoline analogs, benzimidazoles, butanamides, etc. These nonsteroidal compounds were found to be superior to their steroidal counterparts, with respect to their receptor selectivity and pharmacokinetic properties.	Ostarine, LGD2226, BMS564929, etc.	[89, 111]
2	Androgen receptor antagonists	AR antagonists, also referred to as antiandrogens, act by competitive inhibition, thus affecting binding of a ligand to the AR. These agents, extensively used for treating prostate cancer, are structurally classified as steroidal and nonsteroidal compounds as well as reversible and irreversible ligands. Reversible ligands undergo interaction with the AR via noncovalent bonds, such as hydrogen, electrostatic, and hydrophobic bonds, whereas irreversible ligands undergo permanent attachment with the receptors via covalent interactions. Different classes of the steroidal antiandrogens include testosterone and DHT derivatives, synthetic progestins, steroidal sulfonyl heterocycles, 4-Azasteroids and Des-A-steroidal antiandrogens. The nonsteroidal antiandrogens categorized as nitrotrifluorotoluenes, trifluoromethylbenzonitriles, quinoline derivatives, cyclocymopol analogs, phthalimide derivatives, etc., have been studied for their AR antagonistic activities. Peptidomimetic pyrimidines (inhibitors of cofactor binding), sintokamide A and oxindole I (inhibitors of AR transactivation), etc., are some of the few recently reported inhibitors.	*Steroidal antiandrogen:* Cyproterone Acetate (Androcur®) (Fig. 4.2c) *Nonsteroidal antiandrogens:* Flutamide (Eulexin®) and Nilatumide (Nilandron®), Bicalutamide (Casodex®)	[112–118]
3	Natural agents targeted to AR	Drugs isolated from natural products have also been found to have a substantial impact on reducing the growth of prostate cancer. Natural drug products are usually preferred as they are regarded as safe and effective in cancer treatments and exhibit lower side effects than the synthetic counterparts.	Selenium, Soy isoflavones, Epi-brassinolide (EBR), Curcumin, etc.	[119]

(continued)

Table 4.2 (continued)

Sr. No.	Class	Description	Examples	Ref
4	Selective estrogen receptor modulators (SERM)	It includes the agents interfering with the activation of ER receptor and the ones limiting the biosynthesis of the estrogens (e.g., aromatase inhibitors). They bring about conformational changes in the LBD that are slightly different from those triggered by the binding of E2 to the receptor. Change in the orientation and binding pattern of the helices and LBP located in the LBD, as well as inhibition of the recruitment of transcription coactivators, leads to the overall antagonistic effect.	Tamoxifen, Raloxifene, Droloxifene	[89]
5	Antiestrogens	They prevent nuclear translocation of the ligand–ER complexes and interfere with the binding of DBD of ER to the ERE elements located on the chromatin.	Enclomiphene, Zuclomiphene, Fulvestrant (ICI) (Faslodex®)	[89]
6	Aromatase inhibitors (AIs)	They are useful either as a mono or as a combination therapy and act by blocking the synthesis of estrogen by inhibiting the aromatase activity.	Letrozole (Femara®), Anastrozole (Arimidex®)	[120, 121]
7	Selective progesterone receptor modulators (SPRMs)	SPRMs act as partial agonists/antagonists and are majorly employed for emergency contraception, termination of pregnancy, dysmenorrhea, premenstrual syndrome, etc. However, due to the associated safety concerns, their usage has been discontinued.	Asoprisnil, Ulipistal acetate (UPA), Vilaprisan	[122]
8	Progesterone receptor antagonist (PRA)	PRAs or antiprogestins compete with P4 for its receptor and eventually prevent the latter from binding to and activating the receptor.	Mifepristone, Onapristone	[89, 123]
9	GnRHR agonists	They act by interfering with the activities of the epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) and inducing cell apoptosis. These agents are 60 times more potent than the native molecule. They are characterized by prolonged half-life and enhanced receptor binding, in comparison to the natural ligand. These agonists have been utilized to treat advanced prostate cancer along with management of ovarian and endometrial cancers.	Goserelin (Zoladex®), Leuprolide (Eligard®), Leuprorelin (Prostap®), triptorelin (Decapeptyl®), and histrelin (Vantas®)	[47, 82, 124]

10	GnRHR antagonists	Discovered to mitigate the flare phenomenon encountered upon administration of the agonists, these agents bind to the receptor in a competitive manner, without activating the intracellular signaling cascade. They act in a time- and dose-dependent manner, to exhibit a cytotoxic effect on the GnRHR-expressing tumor cells, and their cellular action mimics that of the agonists. Administration of these agents in prostate cancer leads to rapid reduction in the levels of LH, testosterone, DHT, and prostate-specific antigen (PSA). Structurally, the GnRH antagonists involve five or more amino acid substitutions, compared to the natural decapeptide. Alterations at positions 1, 6, and 10 affect the receptor binding, while substitutions at positions 2 and 3 affect the gonadotropin release. Administration of GnRHR antagonists was found to be beneficial, as compared to the combination therapy of GnRH agonists and antiandrogens for prostate cancer. The GnRH antagonists offer several benefits over the agonists, which include absence of flare, rapid pituitary downregulation periods, possibility of being administered alone, and availability in depot or subcutaneous formulations. Thus, they are safer and cost-effective, and at the same time can be targeted directly to the primary and secondary tumor cells. This is anticipated to achieve speedy recovery of the pituitary–gonadal function after withdrawal of the treatment.	Cetrorelix, Abarelix, Degarelix, and Ganirelix (Commercially available); Antarelix, Antide, Azaline B, Acyline, and Ozarelix (under clinical investigation)	[47, 82, 125]

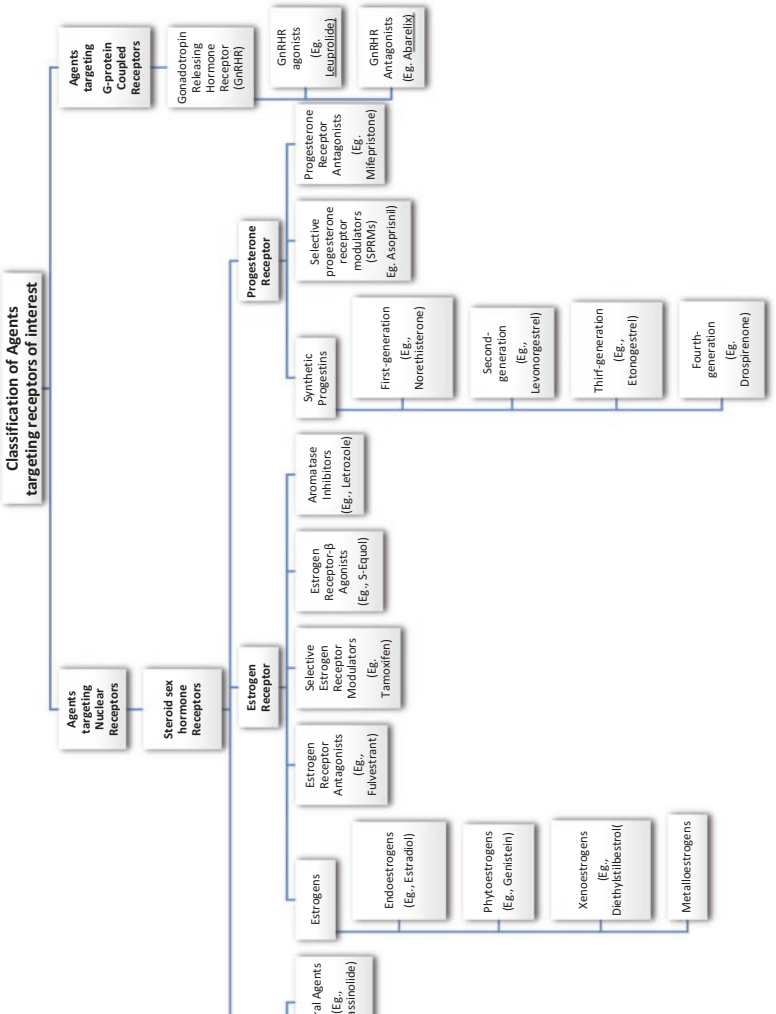

Fig. 4.3 Overview of the classification of several classes of agents, either endogenous or synthesized and used for targeting nuclear receptors and GPCRs, predominantly expressed in the cancers of the reproductive system

5.1.1 Prodrug Approach

The limitations of chemotherapy, such as toxicity and lack of selectivity, can be addressed with different approaches that selectively target the existing drugs to the malignant cells and through the development of nontoxic forms of anticancer agents, which may be specifically activated in the tumor tissues. Selective activation of prodrugs into active anticancer agents, in the vicinity of tumor tissues, can be mediated either by metabolic activity or by spontaneous chemical breakdown. Investigations have been carried out for targeting advanced mCRPC by prodrug approach by means of targeting prostate cancer-specific antigens such as prostate-specific antigens (PSA), prostate-specific membrane antigens (PSMA), CD147, heat shock proteins (HSPs), leutinizing hormone-releasing hormone (LHRH) receptor, epithelial cell adhesion molecules, etc., and prostate-specific enzymes such as cathepsin or matrix metalloproteinase. Numerous PSMA-targeting molecules, which include monoclonal antibodies (mAbs), antibody–drug conjugates (ADCs), antibody fragments (Fabs), peptides, and aptamers, have been developed in the form of prodrugs or nanoparticles. In recent times, a prodrug, namely vinblastine-N-oxide (CPD100), was developed for the treatment of ovarian cancer by Cascade Prodrug, a US-based pharmaceutical company. This compound, formulated as sphingomyelin-cholesterol liposomes, is converted into its parent compound, vinblastine, under hypoxic conditions. This formulation was found to be highly successful in the preliminary studies and if successful in clinical trials, it will be the first-of-its-kind of chemotherapeutic agent to demonstrate anticancer activity due to the hypoxic microenvironment present in the solid tumors [126–128].

5.1.2 Indirect Targeting of the Receptor

Malignancies specific to prostate gland can also be controlled by indirect targeting of AR-targeted drugs such as agonists, antagonists, partial agonists, and antagonists. Indirect targeting approach can also be considered such as targeting small molecules like 17α-hydroxylase involved in the de-novo synthesis of androgens by drugs such as Abiraterone. Abiraterone, in combination with prednisone, was approved in 2012 as the first-line therapy against mCRPCs, before commencing the administration of the conventional chemotherapy. Tamoxifen is the oldest and the most extensively studied SERM. It is a prodrug with low ER affinity, and gets converted into active metabolites such as endoxifen or afimoxifene through metabolism in the liver. These active metabolites demonstrate 30–100 times greater affinity toward the target receptors, as compared to tamoxifen. Substances involved in the synthesis of estrogen, such as aromatase, can also be considered as alternative targets for the treatment of ovarian cancers. The AIs blocks the protein involved in estrogen synthesis, but their usage is limited for the postmenopausal women, as they do not inhibit estrogen synthesis in the ovaries of the premenopausal women. Fulvestrant (Faslodex®), Anastrozole (Arimidex®), Letrozole (Femara®), and Exemestane (Aromasin®) are examples of the AIs, which are useful either as a monotherapy or combinatorial therapy, during the treatment of ovarian cancer [121, 129–131].

5.1.3 Molecular-Based Targeting Approach: Gene Therapy

Gene therapy has been explored as one of the advanced ER-targeting strategies. Recently, an innovative effort provided a strong confirmation that enhanced estrogen signaling is responsible for the growth of the cervical tumor. Enhanced expression of Cyclin D1, ERs, and aromatase is significantly associated with the tumor growth. Hence, blocking the estrogen pathway, particularly to decrease the ER activity, can be a rational approach to activate p53 and retinoblastoma protein (pRb), along with lowering the expression of HPV E6 and E7. This approach has been utilized to block ER-mediated tumor growth in cervical cancer cells, by transfection using adenovirus (AD) as a gene carrier [132].

5.1.4 Novel/Nano Drug Delivery Systems

Different approaches of drug delivery are being employed to target receptors expressed in reproductive neoplasia. Promising results of raloxifene in clinical trials has suggested for an improvement in the efficacy of this drug by exploring novel delivery systems like nanoformulations or through the development of raloxifene analogs. Raloxifene has been effectively encapsulated in nanoparticles, such as styrene–maleic acid (SMA) micelles, which demonstrated superior pharmacokinetic profile than the free drug [95, 133].

As discussed in Table 4.2, Progesterone and GnRH receptors can be directly targeted by means of agonists, antagonists, or partial agonists and antagonists. Extensive research is in progress for finding numerous other potential approaches for targeting these receptor for the treatment of malignancies related to organs of reproductive system.

6 Receptor-Targeted Ligands in Clinical Development: Preclinical and Clinical Studies

6.1 Preclinical Studies

Table 4.3 describes the preclinical studies for drugs targeted to the receptors predominantly expressed in cancers of the reproductive system.

6.2 Clinical Trials

Table 4.4 gives an overview of the clinical trials that have been conducted to target the receptors relevant to the cancers of the reproductive system.

Table 4.3 Preclinical studies conducted to target the drugs to the receptors relevant to the cancers of the reproductive system

Sr. No.	Drug category	Name of the drug	Type of cancer in study	In vitro model	In vivo model	Endpoints/outcomes of study	References
1	Androgen antagonist	ONC1-13B	Prostate cancer	LnCAP cell line	CB17-SCID mice	Reduction in the expression of PSA and Ki67	[134]
2	Progesterone antagonist	Mifepristone in combination with cisplatin	Cervical cancer	CaSki and HeLa cell lines	Human xenografts of cervical tumors	Antiproliferative effect, Synergistic effect with cisplatin	[135]
3	Antiprogestin + Antiestrogen + Cytotoxic agent + Radiation therapy	Mifepristone + ICI 182, 783 + Cisplatin + Radiation therapy	Cervical cancer	HeLa cell line	Cervical xenografts	Cytotoxic effect of combination therapy	[136]
4	Estrogen antagonist	Fulvestrant	High-grade serous ovarian cancer (HGSOC)	HGCOC cells (2D, 3D and forced suspension culture)	Patient-derived xenografts	Blockade of ER-α expression, effect of 3D culture, and ER-α on cellular proliferation	[137]
5	LHRH agonist conjugated with cytotoxic agent	Zoptarelin doxorubicin (AN-152)	Prostate cancer	–	Mice xenografted with androgen-sensitive (LNCaP and MDA-Pca-2b) and androgen-independent (C4-2) cells	Antiproliferative effect	[138]

Table 4.4 Overview of the clinical trials conducted for drugs developed to target receptors relevant to the cancers of the reproductive system

Sr. No.	Drug/Drug combination	Mechanism of action	Phase	Age	Conditions	Dosage	Organization/ sponsor	Trial sites and year	Clinical trial identifier	Ref
Ovarian Cancer										
1	Ribociclib + Letrozole	A) CDK4/6 inhibitor B) Aromatase inhibitor	II	≥18 years	Estrogen receptor positive, postmenopausal and recurrent fallopian tube carcinomas; recurrent ovarian carcinoma;recurrent primary peritoneal carcinoma; recurrent uterine corpus carcinoma	NA	Mayo Clinic and National Cancer Institute (NCI)	United States, (2016–21)	NCT02657928	[139, 140]
2	Onapristone	Antiprogestin	I and II	≥18 years	Progesterone receptor positive tumor	Stage 1 (36 patients): Six dose cohorts, 5 using the ER formulation (10–50 mg BID) and 1 using the IR tablet formulation 100 mg RP2D: 50 mg Stage 2A: 10 patients with RP2D dose Stage 2B: 19 patients, dosing as per response obtained from Stage 2A	Arno Therapeutics	France (2014–16)	NCT02052128	[141]

3	Enzalutamide	Antiandrogen	II	≥18 years	Advanced epithelial ovarian, recurrent epithelial ovarian, fallopian tube, and primary peritoneal carcinoma	NA	Memorial Sloan Kettering Cancer Center and Medivation, Inc.	United States (2013–19)	NCT01974765	[142]
Cervical Cancer										
1	Tamoxifen and Letrozole	Adjuvant therapy of SERM and aromatase inhibitor	II	30–85	Uterine cervical neoplasm, squamous carcinoma of cervix	Phase 2: open-labeled, randomized trial of Tamoxifen (20 mg/day) and Letrozole (2.5 mg) with 44 patients followed 3 monthly for 12 months.	China Medical University Hospital, National Cheng-Kung University Hospital, Taiwan Ministry of Science and Technology, among others	Taiwan (2015–17)	NCT02482740	[143]
2	Metformin hydrochloride and Doxycycline	Blocking enzymes essential for cell growth	II	≥18 years	Breast carcinoma, Endometrial, clear cell adenocarcinoma, endometrial, serous adenocarcinoma, uterine corpus cancer, uterine corpus carcinosarcoma	Metformin hydrochloride, oral, daily, on days 1–3 and BID starting from day 4. Doxycycline oral, every 12 h, starting on day 1. Treatment was repeated for 7 days	Sidney Kimmel Cancer Center at Thomas Jefferson University	United States, Pennsylvania (2016–18)	NCT02874430	[144]

(continued)

Table 4.4 (continued)

Sr. No.	Drug/Drug combination	Mechanism of action	Phase	Age	Conditions	Dosage	Organization/ sponsor	Trial sites and year	Clinical trial identifier	Ref
Prostate Cancer										
1	Enzalu-tamide	Androgen receptor inhibitor	II	≥18 years	Localized prostate cancer	NA	The Netherlands Cancer Institute and Astellas Pharma Inc.	Details not available (2014–17)	NCT03297385	[145]
4	Niraparib + Abiraterone acetate and Prednisone	Niraparib: inhibition of PARP (Poly ADP ribose polymerase) enzyme and Abiraterone: Antiandrogen	I	≥18 years	Prostatic neoplasia, mCRPC	Open trial with dosage as follows: Niraparib – 200 mg/daily Abiraterone acetate – 1000 mg/daily Prednisone – 20 mg/daily	Janssen Research & Development, LLC	United states (2016–18)	NCT02924766	[146]

7 Conclusion and Critical Comments

Carcinogenesis in the different organs of the reproductive system, particularly, prostate, ovarian, and cervical tissues, involves aberrant expression of various physiological receptors belonging to different superfamilies. Structural and pharmacological role of four predominant receptors, namely, AR, ER, PR (sex steroid nuclear receptors), and GnRHR (GPCRs) has been highlighted in this chapter. Moreover, strategies and molecules developed for targeting these receptors, for formulating clinically relevant anticancer therapeutics, have been put forth and supported by the ongoing preclinical and clinical studies. We speculate that a combinatorial therapy comprising receptor-targeted ligands/agents, with clinically acceptable cytotoxic drugs, as well as targeting moieties such as antibodies (antibody–drug conjugates) and use of nano- and novel carriers for drug delivery, will enhance the overall antineoplastic effect. Considerable research has been conducted in deciphering the role of the AR and GnRHR in the cancers of the reproductive system and efforts to target these receptors have been commenced. We anticipate similar investigations to be conducted for ER and PR receptors in the near future.

References

1. Chung S-H. Targeting female hormone receptors as cervical cancer therapy. Trends Endocrinol Metab. 2015;26(8):399–401.
2. Zhang Q, Madden NE, Wong AST, Chow BKC, Lee LTO. The role of endocrine G protein-coupled receptors in ovarian cancer progression. Front Endocrinol. 2017;8:66.
3. Data on specific cancers: World Cancer Research Fund International; [cited 2018 July 20]. Available from: https://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers.
4. Sharifi N, Salmaninejad A, Ferdosi S, Bajestani AN, Khaleghiyan M, Estiar MA, et al. HER2 gene amplification in patients with prostate cancer: evaluating a CISH-based method. Oncol Lett. 2016;12(6):4651–8.
5. Ginsburg O, Bray F, Coleman MP, Vanderpuye V, Eniu A, Kotha SR, et al. The global burden of women's cancers: a grand challenge in global health. Lancet. 2017;389(10071):847–60.
6. Townsley C. Cervical cancer. Elsevier Inc; 2007. Ontario, Canada.
7. Friese K, Kost B, Vattai A, Marmé F, Kuhn C, Mahner S, et al. The G protein-coupled estrogen receptor (GPER/GPR30) may serve as a prognostic marker in early-stage cervical cancer. J Cancer Res Clin Oncol. 2018;144(1):13–9.
8. Gharwan H, Bunch K, Annunziata C. The role of reproductive hormones in epithelial ovarian carcinogenesis. Endocrine Relat Cancer. 2015;22(6):R339–63.
9. Lukanova A, Kaaks R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Prev Biomarkers. 2005;14(1):98–107.
10. Bowtell DD. The genesis and evolution of high-grade serous ovarian cancer. Nat Rev Cancer. 2010;10(11):803.
11. Daniilidis A, Karagiannis V. Epithelial ovarian cancer. Risk factors, screening and the role of prophylactic oophorectomy. Hippokratia. 2007;11(2):63.
12. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, et al. Human prostate cancer risk factors. Cancer. 2004;101(S10):2371–490.

13. White N, Burnstock G. P2 receptors and cancer. Trends Pharmacol Sci. 2006;27(4):211–7.
14. Roger S, Jelassi B, Couillin I, Pelegrin P, Besson P, Jiang L-H. Understanding the roles of the P2X7 receptor in solid tumour progression and therapeutic perspectives. Biochim Biophys Acta. 2015;1848(10):2584–602.
15. Ramírez A, Camacho J. The human papilloma virus–ion channel link in cancer: an alternative opportunity for diagnosis and therapy. Human papillomavirus and related diseases-from bench to bedside-research aspects. InTech; 2012. Belgium.
16. Abdul M, Hoosein N. N-methyl-D-aspartate receptor in human prostate cancer. J Membr Biol. 2005;205(3):125–8.
17. Abdul M, Mccray SD, Hoosein NM. Expression of gamma-aminobutyric acid receptor (subtype A) in prostate cancer. Acta Oncol. 2008;47(8):1546–50.
18. Venuti A, Salani D, Manni V, Poggiali F, Bagnato A. Expression of endothelin 1 and endothelin A receptor in HPV-associated cervical carcinoma: new potential targets for anticancer therapy. FASEB J. 2000;14(14):2277–83.
19. Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G. Endothelin B receptor, a new target in cancer immune therapy. Clin Cancer Res. 2009;15(14):4521–8.
20. Cirilli A, Simeone P, Muller A, Bagnato A, Venuti A. Targeting endothelin receptor type A in human cervical carcinoma cells. J Cardiovasc Pharmacol. 2004;44:S72–5.
21. Sánchez-Hernández PE, Ramirez-Dueñas MG, Albarran-Somoza B, García-Iglesias T, del Toro-Arreola A, Franco-Topete R, et al. Protease-activated receptor-2 (PAR-2) in cervical cancer proliferation. Gynecol Oncol. 2008;108(1):19–26.
22. Cornelio DB, Meurer L, Roesler R, Schwartsmann G. Gastrin-releasing peptide receptor expression in cervical cancer. Oncology. 2007;73(5–6):340–5.
23. Cornelio DB, Meurer L, Schwartsmann G, Roesler R. The gastrin-releasing peptide receptor as a marker of dysplastic alterations in cervical epithelial cells. Oncology. 2012;82(2):90–7.
24. Liu C, Ding L, Bai L, Chen X, Kang H, Hou L, et al. Folate receptor alpha is associated with cervical carcinogenesis and regulates cervical cancer cells growth by activating ERK1/2/c-Fos/c-Jun. Biochem Biophys Res Commun. 2017;491(4):1083–91.
25. Bagnato A, Salani D, Di Castro V, Wu-Wong JR, Tecce R, Nicotra MR, et al. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer Res. 1999;59(3):720–7.
26. Bagnato A, Spinella F, Rosano L. Emerging role of the endothelin axis in ovarian tumor progression. Endocr Relat Cancer. 2005;12(4):761–72.
27. Lin C, Majoor CJ, Roelofs JJ, de Kruif MD, Horlings HM, Borensztajn K, et al. Potential importance of protease activated receptor (PAR)-1 expression in the tumor stroma of non-small-cell lung cancer. BMC Cancer. 2017;17(1):113.
28. Jahan I, Fujimoto J, Alam SM, Sato E, Sakaguchi H, Tamaya T. Role of protease activated receptor-2 in tumor advancement of ovarian cancers. Ann Oncol. 2007;18(9):1506–12.
29. Tang J, Li Z, Lu L, Cho CH, editors. β-Adrenergic system, a backstage manipulator regulating tumour progression and drug target in cancer therapy. Seminars in cancer biology. Elsevier; 2013. Hong Kong, China.
30. Fleischmann A, Waser B, Reubi JC. Overexpression of gastrin-releasing peptide receptors in tumor-associated blood vessels of human ovarian neoplasms. Anal Cell Pathol. 2007;29(5):421–33.
31. Cornelio D, Roesler R, Schwartsmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. Ann Oncol. 2007;18(9):1457–66.
32. Filardo EJ, Thomas P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. Endocrinology. 2012;153(7):2953–62.
33. Qian H, Xuan J, Liu Y, Shi G. Function of G-protein-coupled estrogen receptor-1 in reproductive system tumors. J Immunol Res. 2016;2016:1.
34. Siu MK, Kong DS, Chan HY, Wong ES, Ip PP, Jiang L, et al. Paradoxical impact of two folate receptors, FRα and RFC, in ovarian cancer: effect on cell proliferation, invasion and clinical outcome. PLoS One. 2012;7(11):e47201.

35. Henriksen R, Dizeyi N, Abrahamsson P-A. Expression of serotonin receptors 5-HT1A, 5-HT1B, 5-HT2B and 5-HT4 in ovary and in ovarian tumours. Anticancer Res. 2012;32(4):1361–6.
36. Gohji K, Kitazawa S, Tamada H, Katsuoka Y, Nakajima M. Expression of endothelin receptor a associated with prostate cancer progression. J Urol. 2001;165(3):1033–6.
37. Nelson JB, Udan MS, Guruli G, Pflug BR. Endothelin-1 inhibits apoptosis in prostate cancer. Neoplasia. 2005;7(7):631–7.
38. Black PC, Mize GJ, Karlin P, Greenberg DL, Hawley SJ, True LD, et al. Overexpression of protease-activated receptors-1,-2, and-4 (PAR-1,-2, and-4) in prostate cancer. Prostate. 2007;67(7):743–56.
39. Yu G, Jiang P, Xiang Y, Zhang Y, Zhu Z, Zhang C, et al. Increased expression of protease-activated receptor 4 and trefoil factor 2 in human colorectal cancer. PLoS One. 2015;10(4):e0122678.
40. Yuan T-C, Lin M-F. Protease-activated receptor 1: a role in prostate cancer metastasis. Clin Prostate Cancer. 2004;3(3):189–91.
41. Braadland PR, Ramberg HA, Grytli HH, Taskén KA. β-adrenergic receptor signaling in prostate cancer. Front Oncol. 2015;4:375.
42. Nagasaki S, Nakamura Y, Maekawa T, Akahira J, Miki Y, Suzuki T, et al. Immunohistochemical analysis of gastrin-releasing peptide receptor (GRPR) and possible regulation by estrogen receptor βcx in human prostate carcinoma. Neoplasma. 2012;59(2):224–32.
43. Cao W, Li F, Yao J, Yu J. Prostate specific G protein coupled receptor is associated with prostate cancer prognosis and affects cancer cell proliferation and invasion. BMC Cancer. 2015;15(1):915.
44. Weng J, Wang J, Hu X, Wang F, Ittmann M, Liu M. PSGR2, a novel G-protein coupled receptor, is overexpressed in human prostate cancer. Int J Cancer. 2006;118(6):1471–80.
45. Patel N, Itakura T, Jeong S, Liao C-P, Roy-Burman P, Zandi E, et al. Expression and functional role of orphan receptor GPR158 in prostate cancer growth and progression. PLoS One. 2015;10(2):e0117758.
46. Guo R, Kasbohm EA, Arora P, Sample CJ, Baban B, Sud N, et al. Expression and function of lysophosphatidic acid LPA1 receptor in prostate cancer cells. Endocrinology. 2006;147(10):4883–92.
47. Limonta P, Manea M. Gonadotropin-releasing hormone receptors as molecular therapeutic targets in prostate cancer: current options and emerging strategies. Cancer Treat Rev. 2013;39(6):647–63.
48. Dizeyi N, Bjartell A, Hedlund P, Tasken K, Gadaleanu V, Abrahamsson P-A. Expression of serotonin receptors 2B and 4 in human prostate cancer tissue and effects of their antagonists on prostate cancer cell lines. Eur Urol. 2005;47(6):895–900.
49. Dizeyi N, Bjartell A, Nilsson E, Hansson J, Gadaleanu V, Cross N, et al. Expression of serotonin receptors and role of serotonin in human prostate cancer tissue and cell lines. Prostate. 2004;59(3):328–36.
50. Oh D-Y, Kim S, Choi Y-L, Cho YJ, Oh E, Choi J-J, et al. HER2 as a novel therapeutic target for cervical cancer. Oncotarget. 2015;6(34):36219.
51. Soonthornthum T, Arias-Pulido H, Joste N, Lomo L, Muller C, Rutledge T, et al. Epidermal growth factor receptor as a biomarker for cervical cancer. Ann Oncol. 2011;22(10):2166–78.
52. Kuramoto H, Hongo A, Liu YX, Ojima Y, Nakamura K, Seki N, et al. Immunohistochemical evaluation of insulin-like growth factor I receptor status in cervical cancer specimens. Acta Med Okayama. 2008;62(4):251–9.
53. Frumovitz M, Sood AK. Vascular endothelial growth factor (VEGF) pathway as a therapeutic target in gynecologic malignancies. Gynecol Oncol. 2007;104(3):768–78.
54. Lopez-Pulido EI, Muñoz-Valle JF, Del Toro-Arreola S, Jave-Suárez LF, Bueno-Topete MR, Estrada-Chávez C, et al. High expression of prolactin receptor is associated with cell survival in cervical cancer cells. Cancer Cell Int. 2013;13(1):103.
55. Peng J, Qi S, Wang P, Li W, Liu C, Li F. Diagnosis and prognostic significance of c-Met in cervical cancer: a meta-analysis. Dis Markers. 2016;2016:1.

56. Choi CH, Chung J-Y, Kim J-H, Kim B-G, Hewitt SM. Expression of fibroblast growth factor receptor family members is associated with prognosis in early stage cervical cancer patients. J Transl Med. 2016;14(1):124.
57. Amler LC, Wang Y, Hampton G. HER2 as a therapeutic target in ovarian cancer. Ovarian cancer-clinical and therapeutic perspectives. InTech; 2012. Croatia.
58. Mehner C, Oberg AL, Goergen KM, Kalli KR, Maurer MJ, Nassar A, et al. EGFR as a prognostic biomarker and therapeutic target in ovarian cancer: evaluation of patient cohort and literature review. Genes Cancer. 2017;8(5–6):589.
59. Gotlieb WH, Bruchim I, Gu J, Shi Y, Camirand A, Blouin M-J, et al. Insulin-like growth factor receptor I targeting in epithelial ovarian cancer. Gynecol Oncol. 2006;100(2):389–96.
60. Matsumoto K, Umitsu M, De Silva DM, Roy A, Bottaro DP. Hepatocyte growth factor/MET in cancer progression and biomarker discovery. Cancer Sci. 2017;108(3):296–307.
61. Crickard K, Gross JL, Crickard U, Yoonessi M, Lele S, Herblin WF, et al. Basic fibroblast growth factor and receptor expression in human ovarian cancer. Gynecol Oncol. 1994;55(2):277–84.
62. Omar N, Yan B, Salto-Tellez M. HER2: an emerging biomarker in non-breast and non-gastric cancers. Pathogenesis. 2015;2(3):1–9.
63. Day KC, Hiles GL, Kozminsky M, Dawsey SJ, Paul A, Broses LJ, et al. HER2 and EGFR overexpression support metastatic progression of prostate cancer to bone. Cancer Res. 2017;77(1):74–85.
64. Wu J, Yu E. Insulin-like growth factor receptor-1 (IGF-IR) as a target for prostate cancer therapy. Cancer Metastasis Rev. 2014;33(2–3):607–17.
65. Yang J, Wu HF, Qian LX, Zhang W, Hua LX, Yu ML, et al. Increased expressions of vascular endothelial growth factor (VEGF), VEGF-C and VEGF receptor-3 in prostate cancer tissue are associated with tumor progression. Asian J Androl. 2006;8(2):169–75.
66. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocr Relat Cancer. 2004;11(4):709–24.
67. Deuster E, Jeschke U, Ye Y, Mahner S, Czogalla B. Vitamin D and VDR in gynecological cancers—a systematic review. Int J Mol Sci. 2017;18(11):2328.
68. Ivanova T, Petrenko A, Gritsko T, Vinokourova S, Eshilev E, Kobzeva V, et al. Methylation and silencing of the retinoic acid receptor-β2 gene in cervical cancer. BMC Cancer. 2002;2(1):4.
69. Voutsadakis IA. Hormone receptors in serous ovarian carcinoma: prognosis, pathogenesis, and treatment considerations. Clin Med Insights Oncol. 2016;10:17–25.
70. Kaiser P, Körner M, Kappeler A, Aebi S. Retinoid receptors in ovarian cancer: expression and prognosis. Ann Oncol. 2005;16(9):1477–87.
71. Tan ME, Li J, Xu HE, Melcher K, Yong E-L. Androgen receptor: structure, role in prostate cancer and drug discovery. Acta Pharmacol Sin. 2015;36(1):3.
72. Christoforou P, Christopoulos PF, Koutsilieris M. The role of estrogen receptor β in prostate cancer. Mol Med. 2014;20(1):427.
73. Chen R, Yu Y, Dong X. Progesterone receptor in the prostate: a potential suppressor for benign prostatic hyperplasia and prostate cancer. J Steroid Biochem Mol Biol. 2017;166:91–6.
74. Elix C, Pal SK, Jones JO. The role of peroxisome proliferator-activated receptor gamma in prostate cancer. Asian J Androl. 2018;20(3):238.
75. Hongmao S. A practical guide to rational drug design. Woodhead Publishing; 2015. Maryland, USA
76. Robinson-Rechavi M, Garcia HE, Laudet V. The nuclear receptor superfamily. J Cell Sci. 2003;116(4):585–6.
77. Zuo H, Wan Y. Nuclear receptors in skeletal homeostasis. Curr Top Dev Biol. 2017;125:71–107. Elsevier.
78. Gao X, Loggie BW, Nawaz Z. The roles of sex steroid receptor coregulators in cancer. Mol Cancer. 2002;1(1):7.
79. de Almeida Chuffa LG, Lupi-Júnior LA, Costa AB, de Arruda Amorim JP, Seiva FRF. The role of sex hormones and steroid receptors on female reproductive cancers. Steroids. 2017;118:93–108.

80. Mungenast F, Thalhammer T. Estrogen biosynthesis and action in ovarian cancer. Front Endocrinol. 2014;5:192.
81. Park S, Han JM, Cheon J, Hwang J-I, Seong JY. Apoptotic death of prostate cancer cells by a gonadotropin-releasing hormone-II antagonist. PLoS One. 2014;9(6):e99723.
82. Limonta P, Marelli MM, Mai S, Motta M, Martini L, Moretti RM. GnRH receptors in cancer: from cell biology to novel targeted therapeutic strategies. Endocr Rev. 2012;33(5):784–811.
83. Cheung LW, Yung S, Chan T-M, Leung PC, Wong AS. Targeting gonadotropin-releasing hormone receptor inhibits the early step of ovarian cancer metastasis by modulating tumor-mesothelial adhesion. Mol Ther. 2013;21(1):78–90.
84. Gao W, Bohl CE, Dalton JT. Chemistry and structural biology of androgen receptor. Chem Rev. 2005;105(9):3352–70.
85. Lonergan PE, Tindall DJ. Androgen receptor signaling in prostate cancer development and progression. J Carcinog. 2011;10.
86. Jernberg E, Bergh A, Wikström P. Clinical relevance of androgen receptor alterations in prostate cancer. Endocr Connect. 2017;6(8):R146–R61.
87. Zhu H, Zhu X, Zheng L, Hu X, Sun L, Zhu X. The role of the androgen receptor in ovarian cancer carcinogenesis and its clinical implications. Oncotarget. 2017;8(17):29395.
88. Munoz J, Wheler JJ, Kurzrock R. Androgen receptors beyond prostate cancer: an old marker as a new target. Oncotarget. 2015;6(2):592.
89. Foye WO. Foye's principles of medicinal chemistry. Lippincott Williams & Wilkins; 2008. Philadelphia, USA.
90. Centenera MM, Selth LA, Ebrahimie E, Butler LM, Tilley WD. New opportunities for targeting the androgen receptor in prostate cancer. Cold Spring Harb Perspect Med. 2018:a030478.
91. Davey RA, Grossmann M. Androgen receptor structure, function and biology: from bench to bedside. Clin Biochem Rev. 2016;37(1):3.
92. Deroo BJ, Korach KS. Estrogen receptors and human disease. J Clin Invest. 2006;116(3):561–70.
93. Lee H-R, Kim T-H, Choi K-C. Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. Lab Anim Res. 2012;28(2):71–6.
94. Chen GG, Zeng Q, Tse GM. Estrogen and its receptors in cancer. Med Res Rev. 2008;28(6):954–74.
95. Di Zazzo E, Galasso G, Giovannelli P, Di Donato M, Castoria G. Estrogens and their receptors in prostate cancer: therapeutic implications. Front Oncol. 2018;8:2.
96. Sato T, Miyagawa S, Iguchi T. Estradiol-17β. Handbook of hormones. Elsevier; 2015. p. 520-e94G-4. Japan.
97. Farooq A. Structural and functional diversity of estrogen receptor ligands. Curr Top Med Chem. 2015;15(14):1372–84.
98. Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci. 1997;94(25):14138–43.
99. Jordan VC, Mittal S, Gosden B, Koch R, Lieberman ME. Structure-activity relationships of estrogens. Environ Health Perspect. 1985;61:97.
100. Diep CH, Daniel AR, Mauro LJ, Knutson TP, Lange CA. Progesterone action in breast, uterine, and ovarian cancers. J Mol Endocrinol. 2015;54(2):R31–53.
101. Valadez-Cosmes P, Vázquez-Martínez ER, Cerbon M, Camacho-Arroyo I. Membrane progesterone receptors in reproduction and cancer. Mol Cell Endocrinol. 2016;434:166–75.
102. Sato T, Miyagawa S, Iguchi T. Progesterone. Handbook of hormones. Elsevier; 2015. p. 507-e94A-3. Japan.
103. Daniel AR, Hagan CR, Lange CA. Progesterone receptor action: defining a role in breast cancer. Expert Rev Endocrinol Metab. 2011;6(3):359–69.
104. Yu Y, Liu L, Xie N, Xue H, Fazli L, Buttyan R, et al. Expression and function of the progesterone receptor in human prostate stroma provide novel insights to cell proliferation control. J Clin Endocrinol Metabol. 2013;98(7):2887–96.

105. Lee P, Rosen DG, Zhu C, Silva EG, Liu J. Expression of progesterone receptor is a favorable prognostic marker in ovarian cancer. Gynecol Oncol. 2005;96(3):671–7.
106. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update. 2014;21(2):155–73.
107. Wang L, Chadwick W, Park S-S, Zhou Y, Silver N, Martin B, et al. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol Disord Drug Targets. 2010;9(5):651–60.
108. Flanagan CA, Manilall A. Gonadotropin-releasing hormone (GnRH) receptor structure and GnRH binding. Front Endocrinol. 2017;8:274.
109. Sealfon SC, Millar RP. Functional domains of the gonadotropin-releasing hormone receptor. Cell Mol Neurobiol. 1995;15(1):25–42.
110. Sealfon SC, Weinstein H, Millar RP. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocr Rev. 1997;18(2):180–205.
111. Bhasin S, Jasuja R. Selective androgen receptor modulators (SARMs) as function promoting therapies. Curr Opin Clin Nutr Metab Care. 2009;12(3):232.
112. Reid P, Kantoff P, Oh W. Antiandrogens in prostate cancer. Investig New Drugs. 1999;17(3):271–84.
113. Singh SM, Gauthier S, Labrie F. Androgen receptor antagonists (antiandrogens) structure-activity relationships. Curr Med Chem. 2000;7(2):211–47.
114. Haendler B, Cleve A. Recent developments in antiandrogens and selective androgen receptor modulators. Mol Cell Endocrinol. 2012;352(1–2):79–91.
115. Yang SH, Song C-H, Van HTM, Park E, Khadka DB, Gong E-Y, et al. SAR based design of nicotinamides as a novel class of androgen receptor antagonists for prostate cancer. J Med Chem. 2013;56(8):3414–8.
116. Tesei A, Leonetti C, Di Donato M, Gabucci E, Porru M, Varchi G, et al. Effect of small molecules modulating androgen receptor (SARMs) in human prostate cancer models. PLoS One. 2013;8(5):e62657.
117. Hwang DJ, Yang J, Xu H, Rakov IM, Mohler ML, Dalton JT, et al. Arylisothiocyanato selective androgen receptor modulators (SARMs) for prostate cancer. Bioorg Med Chem. 2006;14(19):6525–38.
118. Banuelos CA, Tavakoli I, Tien AH, Caley DP, Mawji NR, Li Z, et al. Sintokamide A is a novel antagonist of androgen receptor that uniquely binds activation function-1 in its amino-terminal domain. J Biol Chem. 2016;291(42):22231–43.
119. Farooqi AA, Sarkar FH. Overview on the complexity of androgen receptor-targeted therapy for prostate cancer. Cancer Cell Int. 2015;15(1):7.
120. Li Y, Hu W, Fu S, Li J, Liu J, Kavanagh J. Aromatase inhibitors in ovarian cancer: is there a role? Int J Gynecol Cancer. 2008;18(4):600–14.
121. Ho S-M. Estrogen, progesterone and epithelial ovarian cancer. Reprod Biol Endocrinol. 2003;1(1):73.
122. Ali M, Al-Hendy A. Selective progesterone receptor modulators for fertility preservation in women with symptomatic uterine fibroids. Biol Reprod. 2017;97(3):337–52.
123. Chabbert-Buffet N, Meduri G, Bouchard P, Spitz IM. Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update. 2005;11(3):293–307.
124. Hormone therapy for ovarian cancer: American Cancer Society; [cited 2018 October 20]. Available from: https://www.cancer.org/cancer/ovarian-cancer/treating/hormone-therapy.html.
125. Erdkamp F, Boone N, Janknegt R, Zambon V. GnRH agonists and antagonists in prostate cancer. Gene Biosimilars Initiative J. 2014;3:133–42.
126. Ast G. Drug-targeting strategies for prostate cancer. Curr Pharm Des. 2003;9(6):455–66.
127. Shah VM, Nguyen DX, Al Fatease A, Patel P, Cote B, Woo Y, et al. Liposomal formulation of hypoxia activated prodrug for the treatment of ovarian cancer. J Control Release. 2018;291:169–83.

128. Barve A, Jin W, Cheng K. Prostate cancer relevant antigens and enzymes for targeted drug delivery. J Control Release. 2014;187:118–32.
129. Imamura Y, Sadar MD. Androgen receptor targeted therapies in castration-resistant prostate cancer: bench to clinic. Int J Urol. 2016;23(8):654–65.
130. Li Z, Bishop AC, Alyamani M, Garcia JA, Dreicer R, Bunch D, et al. Conversion of abiraterone to D4A drives anti-tumour activity in prostate cancer. Nature. 2015;523(7560):347.
131. Kothari R, Argenta P, Fowler J, Carter J, Shimp W. Antiestrogen therapy in recurrent ovarian cancer resulting in 28 months of stable disease: a case report and review of the literature. Arch Oncol. 2010;18(1–2):32.
132. Au WW, Abdou-Salama S, Al-Hendy A. Inhibition of growth of cervical cancer cells using a dominant negative estrogen receptor gene. Gynecol Oncol. 2007;104(2):276–80.
133. Sundararajan V, Chen S, Rosengren R. Raloxifene: promises and challenges as a drug treatment for castrate resistant prostate cancer. Enliven Toxicol Allied Clin Pharmacol. 2017;4(1):001.
134. Ivachtchenko AV, Mitkin OD, Kudan EV, Rjahovsky AA, Vorobiev AA, Trifelenkov AS, et al. Preclinical development of ONC1-13B, novel antiandrogen for prostate cancer treatment. J Cancer. 2014;5(2):133.
135. Jurado R, Lopez-Flores A, Alvarez A, García-López P. Cisplatin cytotoxicity is increased by mifepristone in cervical carcinoma: an in vitro and in vivo study. Oncol Rep. 2009;22(5):1237–45.
136. Segovia-Mendoza M, Jurado R, Mir R, Medina LA, Prado-Garcia H, Garcia-Lopez P. Antihormonal agents as a strategy to improve the effect of chemo-radiation in cervical cancer: in vitro and in vivo study. BMC Cancer. 2015;15(1):21.
137. Andersen CL, Sikora MJ, Boisen MM, Ma T, Christie A, Tseng G, et al. Active estrogen receptor-alpha signaling in ovarian cancer models and clinical specimens. Clin Cancer Res. 2017;23(14):3802–12.
138. Letsch M, Schally AV, Szepeshazi K, Halmos G, Nagy A. Preclinical evaluation of targeted cytotoxic luteinizing hormone-releasing hormone analogue AN-152 in androgen-sensitive and insensitive prostate cancers. Clin Cancer Res. 2003;9(12):4505–13.
139. Tripathy D, Bardia A, Sellers WR. Ribociclib (LEE011): mechanism of action and clinical impact of this selective cyclin-dependent kinase 4/6 inhibitor in various solid tumors. Clin Cancer Res. 2017;23(13):3251–62.
140. Ribociclib and letrozole in treating patients with relapsed ER positive ovarian, fallopian tube, primary peritoneal, or endometrial cancer [Internet]. Mayo Clinic. 2016 [cited October 25, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02657928?term=Ribociclib+and+Letrozole&cond=ovarian+cancer&rank=2&view=record.
141. Phase 1–2 study of onapristone in patients with progesterone receptor expressing cancers [Internet]. Arno Therapeutics. 2014 [cited October 25, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02052128?term=progesterone+receptor&cond=Ovarian+Cancer&draw=2&rank=2.
142. Enzalutamide in patients with androgen receptor positive (AR+) ovarian, primary peritoneal or fallopian tube cancer and one, two or three prior therapies [Internet]. Memorial Sloan Kettering Cancer Center. 2013 [cited October 26, 2018]. Available from: https://clinicaltrials.gov/ct2/show/study/NCT01974765?term=androgen+receptor&cond=Ovarian+Cancer&rank=2.
143. A trial of tamoxifen and letrozole in recurrent and persistent squamous cell carcinoma of the cervix (TGOG1005) [Internet]. Buddhist Tzu Chi General Hospital. 2015 [cited October 26, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02482740?term=NCT02482740&rank=1.
144. Metformin hydrochloride and doxycycline in treating patients with localized breast or uterine cancer [Internet]. Sidney Kimmel Cancer Center at Thomas Jefferson University. 2016 [cited October 27, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02874430?term=progesterone+receptor&cond=Cervical+Cancer&rank=2.

145. Dynamics of androgen receptor genomics and transcriptomics after neoadjuvant androgen ablation (DARANA) [Internet]. The Netherlands Cancer Institute. 2017 [cited October 27, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT03297385?term=androgen+receptor&cond=Prostate+Cancer&phase=012&draw=2.
146. A safety and pharmacokinetics study of niraparib plus an androgen receptor-targeted therapy in men with metastatic castration-resistant prostate cancer (BEDIVERE) [Internet]. Janssen Research & Development, LLC. 2016 [cited October 28, 2018]. Available from: https://clinicaltrials.gov/ct2/show/record/NCT02924766?term=Erleada+%28apalutamide%29&cond=Prostate+Cancer+Metastatic&rank=6.

Chapter 5
Receptors for Targeting Gastrointestinal Tract Cancer

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Abstract Cancers of the gastrointestinal tract (GIT) are among the most prevalent and fatal cancers. Historically, surgical resection was the only effective treatment of operable GIT tumors. However, more than half of these patients present locally advanced, recurrent, or metastatic disease, necessitating development of alternate strategies for possible therapy. Cellular receptors are instrumental in controlling the basic traits of a cell. Binding of specific ligands to these receptors results in changes in gene expression and increase in cell metabolism, cell growth, or cell death. The therapeutic prospects of ligands for somatostatin receptors (SSTRs), c-Kit, and peroxisome proliferator-activated receptors (PPARs) along with receptor-mediated strategies have been discussed in this chapter. Ligands for these receptors include peptides, small molecules, and oligonucleotides that can be delivered using nanoparticulate delivery systems tailored for specific application. Some important drug candidates undergoing clinical trials have also been mentioned to convey the potential of these receptors as targets for GIT cancer therapy.

Keywords Gastrointestinal tract · Cancer · Receptor · Somatostatin · c-Kit · PPAR

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Abbreviations

CML	Chronic myeloid leukemia
CXC	C-X-C motif chemokine
DBD	DNA-binding domain
DRIP	vitamin D3 receptor-interacting protein
EGFR	Epidermal growth factor receptor
GEP-NET	Gastroenteropancreatic neuroendocrine tumors
GIST	Gastrointestinal stromal tumors
GIT	Gastrointestinal tract
LBD	Ligand-binding domain
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NCoR	Nuclear receptor corepressor
NF-κB	Nuclear factor kappa light chain
NPs	Nanoparticles
PDGFR	Platelet-derived growth factor receptor
PGC-1	PPARγ coactivator-1
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PL	Phospholipase
PPAR	Peroxisome proliferator-activated receptors
PRI	Peptide receptor imaging
PRRT	Peptide receptor radionuclide therapy
PTP	Phosphotyrosine phosphatases
PTX	Pertussis toxin
QDs	Quantum dots
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
SCF	Stem cell factor
SFK	Src family of tyrosine kinases
SH2	Src homology 2
SHP	Small heterodimer partner
SRIF	Somatotropin release-inhibiting factor
SS	Somatostatin
SSTR	Somatostatin receptors
TLR	Toll-like receptors
TMD	Transmembrane domains
TRAP	Thyroid hormone receptor-associated protein
TZD	Thiazolidinedione
USFDA	The food and drug administration
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Cancers of the Gastrointestinal Tract

Cancers of the gastrointestinal tract (GIT) constitute a major class of cancers that account for one of the highest number of disease-related deaths, in both genders, worldwide. According to a recent survey, cancers of the GIT are the most common in males and second most common in females (only next to breast cancer) in the list of the five most common cancers occurring in each gender [1]. The GIT cancers comprise of the malignancies of the alimentary tract, viz., the oral cavity, the esophagus, stomach, small intestine, large intestine (colon), rectum, and the accessory organs such as liver and pancreas. Colorectal cancer is the most prevalent among these, and it is interesting to note that though the small intestine constitutes the major part of the GIT, it presents the least occurrences of tumor malignancies. The associated risk factors, pattern of incidence, prevalence, and prognosis vary among the organ sites. Some of the risk factors can be abated, while in some cases, the cancers can be prevented with proper screening. A large number of the GIT cancers can be cured, when detected and treated at an early stage, thus reducing the associated mortality rates.

2 Overview of Receptors Associated with the GIT Cancers

Several receptors including the growth factor receptors, the nuclear hormone receptors, the toll-like receptors, and the chemokine receptors and their ligands regulate the development of GIT cancers. Receptors provide a very precise means of communication between the cells, triggering a cascade of pathways that affect the cellular responses and interactions, at varied levels. While many of these receptors are discussed in great detail elsewhere in this chapter, a few notable examples are mentioned here.

Vascular endothelial growth factor (VEGF) receptors (VEGFR-1, VEGFR-2, and VEGFR-3) are expressed in varying degrees in colon, pancreatic, and gastric cancers, along with increased levels of VEGF. The VEGFR-1 specifically promotes anchorage-independent growth of cells, as well as antiapoptotic signaling in colorectal cancers [2]. The epidermal growth factor receptor (EGFR), its family members Her-2/ErbB-2, Her-3, Her-4, and their ligands are involved in over 70% of all the cancers and expressed in about 43% of the gastric cancers [3]. The next important class of receptors is that of chemokines. The correlation between the expression of C-X-C motif chemokine 12 (CXCL12) and its receptor (CXCR4), as well as CXCL8-CXCR2, has been established for esophageal cancer, where it is associated with increased lymph node metastasis, invasiveness, and reduced patient survival [4]. Elevated expression of CXCL12-CXCR4 has also been demonstrated in gastric cancers, where it stimulates aggressive invasion and metastasis in cancer cells. Most of the chemokines are upregulated when the normal colonic mucosa becomes cancerous and the cancer becomes more malignant [5]. Toll-like receptors (TLRs)

belonging to the family of pattern recognition receptors also play a noteworthy role in GIT cancers. A few examples of the TLRs overexpressed in GIT tumors include TLR2 (oral, stomach), TLR4, TLR5, and TLR9 (stomach, colorectum). Interestingly enough, TLRs are found to have both tumorigenic and antitumor effects in various instances. The TLR polymorphisms (functional, sequential, and SNP) have been strongly associated with an increased risk of gastric cancer. Additionally, the TLR deficiency in tumor cells is found to result in tumor regression in some isolated studies. The microbial antitumor agent, OK-432 (penicillin-killed and lyophilized low-virulence strain of Streptococcus pyogenes), and a TLR9-targeted oligonucleotide sequence, CpG-ODN, developed for the treatment of colorectal cancer, are some examples of TLR-based immunotherapy in GIT cancers [6].

In addition to these receptors that have been extensively discussed in the other chapters of this book, many alternate receptors have shown potential for being studied as targets for newer anticancer therapeutics. In this chapter, we have discussed three such receptors that are important in the context of various malignancies of the GIT. These are the somatostatin receptors (SSTRs), the "KIT proto-oncogene receptor tyrosine kinase" or c-Kit, and a subfamily of nuclear receptors, the peroxisome proliferator-activated receptors (PPARs).

3 Somatotropin Release-Inhibiting Factor Receptor

The somatotropin release-inhibiting factor (SRIF) or somatostatin (SS) was isolated in 1973, from ovine hypothalamic tissue by Brazeu et al. It was a tetra-decapeptide (SS-14) that inhibited secretion of the growth hormone in rat pituitary cells at a concentration $\geq$1 nM [7]. Later, a form of somatostatin that was extended at the N-terminal and comprised of 28 peptides was isolated from the porcine intestine and was referred as SS-28 [8].

SS mediates its physiological actions by binding to its plasma membrane receptors – the somatostatin receptors (SSTs). Till date, five SST subtypes have been identified (SST1 to SST5) in target tissues such as brain, pituitary gland, pancreas, and gastrointestinal tract. Of these, SST2 exhibits two isoforms, SST2A (long) and SST2B (short), that differ in their cytoplasmic tail and hence further signaling pathways [9]. In the GIT, SSTs are expressed in the gastrointestinal mucosa, peripheral nervous system, and gut-associated lymphoid tissue [10]. GIT is both a major source and the main target organ of SS that contains approximately 65% of the total SS content of the human body [11]. The inhibitory effect of SS depends on the receptor subtype as well as tissue localization. SS modulates various functions of the GIT through its receptors, like gastric emptying, gallbladder contractility, and propulsive activity of the small and large intestines. Several studies have been conducted to elucidate the distribution and role of SSTs in normal and diseased (inflamed/tumor) GIT.

3.1 Recognition Domain and Structure-Activity Relationship of SSTs

The SSTs belong to the superfamily of rhodopsin like G-protein-coupled receptors. All SSTs (SST1-SST5) are characterized by seven transmembrane domains (TMDs) and share a structurally common feature, the conserved sequence (YANSCANPILY) in the transmembrane region 7. They all contain (i) a consensus motif for interaction with the type I PDZ domains at the intracellular C termini and (ii) the DRY motif (aspartic acid-arginine-tyrosine) for coupling with the G proteins in the second intracellular loop. The PDZ domains consist of 80–90 amino acids that facilitate anchoring of the cell surface receptors on the cell membrane, while the DRY motif governs the conformation of the receptor. Alteration in these regions results in a change in the downstream signaling of the SSTs.

Two high-affinity SS agonists, MK678 (hexapeptide) and SMS-201-995 (octapeptide), were studied to understand the ligand-binding domains of the SST2 [12]. It was found that a sequence containing four amino acids (FDFV), at the border of the third extracellular loop (ECL) and the TMD7 is crucial for their binding. Moreover, the interaction of these peptides with SST2 varies in terms of the essential amino acids that are involved. In case of the SST1 mutant $SST1_{S305F}$ (Ser-to-Phe conversion), the binding of hexapeptides was distinguished depending upon the presence of Phe or Tyr at position 2. Thus, a single hydroxyl group hindered the binding of the hexapeptide to $SST1_{S305F}$. Replacement of Asp-124 with Glu rendered the mutated rat $SST3_{D305E}$ incapable of binding to somatostatin 14 with a high affinity [13]. In this case, though the replaced amino acid was negatively charged, the additional methylene group interfered with the binding efficiency of the agonist, suggesting that apart from charge, the size of the binding pocket was also crucial for ligand binding. Greenwood et al. developed mutants for the three ECLs of SST5 and evaluated their binding affinity for ^{125}I-LTT SRIF-28. While binding of ECL1 and ECL3 mutants to the ligand was similar to wild-type SST5, the ECL2 mutant failed to bind to the ligand [14]. Thus, receptor binding was affected by the type of amino acid residue present at a particular position in a peptide. Receptor binding was also a consequence of the number of receptors present on the surface of a cell, at any given time. The status of a receptor, that is, whether it was present in an active or inactive configuration, also played an important role in receptor-ligand binding. An interesting study identified a disulfide-linked cyclic octapeptide antagonist of SS, comprising a DL-cysteine pair at positions 2 and 7. Substitution of D-cysteine at position 2, with an L-cysteine, converted the antagonist into an agonist [15]. This suggested that not only the position but also the chirality of the amino acids altered the interaction of the ligand with the receptor, thus altering the subsequent signaling pathways.

3.2 Binding of Ligand with Receptor and Pathway

All somatostatin receptors, upon stimulation, inhibit hormone secretion, cell proliferation, cell migration, and angiogenesis. These inhibitory actions are mediated by their association with the $G_{i/o}$ proteins, which are the members of the heterotrimeric guanine-nucleotide-binding protein family that is characterized by sensitivity to the pertussis toxin (PTX). Coupling of the ligand with its receptor results in inhibition of adenylate cyclase and, thus, reduction in cAMP accumulation. Ligand binding also reduces Ca^{2+} concentrations, either directly or indirectly through the opening of G-protein gated inwardly rectifying potassium channels. This reduces the intracellular Ca^{2+} concentrations, which results in membrane hyperpolarization and ultimately reduction of Ca^{2+} influx, through voltage-operated calcium channels. The synergistic effects of reduction in both cAMP and Ca^{2+} result in inhibition of hormone release.

The SST receptor subtypes may undergo homo (SST3, SST5) or hetero dimerization with a particular SST subtype (SST1–SST5) or other receptors, after binding with their ligands. Tyrosine kinases and phosphatases are recruited by the activated SSTs to trigger downstream signaling events. For example, SST1 activates phosphotyrosine phosphatases (PTPs) that inhibit cell proliferation by dephosphorylating tyrosine kinase receptors and/or downstream effectors, such as platelet-derived growth factor receptors [16], or by inducing cell cycle arrest via upregulation of p21 (cip1/Waf1) expression [17]. SST2 induces cell cycle arrest and subsequent cell proliferation through upregulation of the cyclin-dependent kinase inhibitor p27/Kip1 [18] and the zinc finger protein (Zac1) [19]. Regulation of both SST1 and SST2 depends on phosphorylation events at their C-terminal tail [20]. SST3-dependent induction of apoptosis involves p53 and Bax.SST4 also plays a role in cell growth arrest, wherein it causes prolonged activation of p38 MAPK (mitogen-activated protein kinase), resulting in stimulation of the cell cycle inhibitor p21 (cip1/Waf1) [21]. SST5 activates tyrosine phosphatases, SHP1, and SHP2, which inhibit the mammalian target of rapamycin (mTOR) pathway, thereby decreasing cell proliferation and growth. Thus, all the subtypes contribute in cell proliferation inhibition. The intracellular trafficking of the receptors varies according to the receptor subtype and they may be degraded in lysosomes or may be recycled back to cell surface [22, 23]. A pictorial representation of the structure of SSTs and their downstream effects is shown in Fig. 5.1.

3.3 Ligands Explored for SSTs

Natural ligands of all the SSTs include somatostatin 14 and 28 and cortistatin 14 and 17. Somatostatin is chemically unstable (circulating half-life <3 min) and is broken down in the body, shortly after its release [24]. A number of synthetic agonists have been developed, namely (i) metabolically stable short chain analogues, with specificity for one subtype, that can be further modified for universal binding

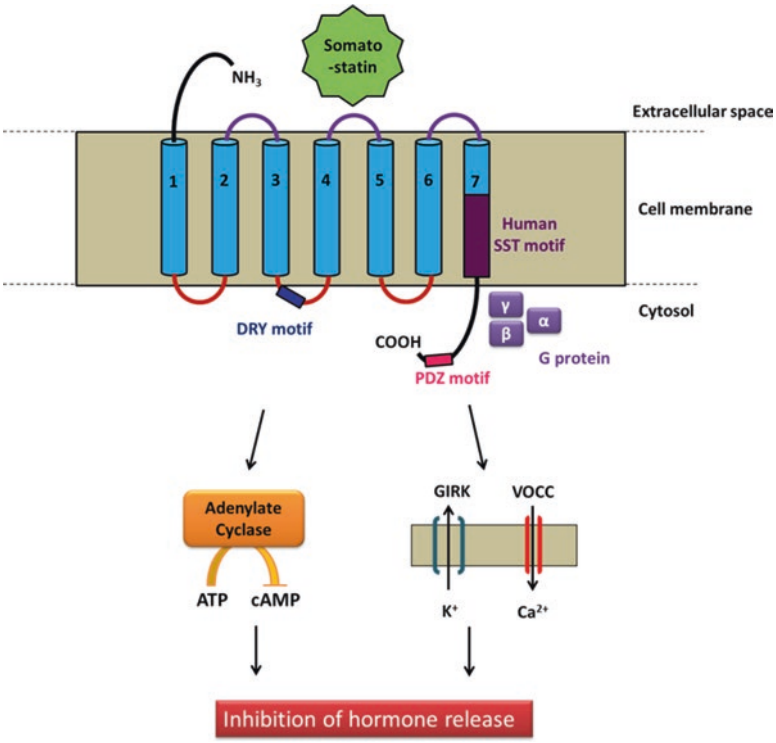

Fig. 5.1 Structure of human SSTs showing common structural features and effects

to all subtypes and (ii) large chain ligands that universally bind to all subtypes and are later chemically modified for metabolic stabilization. They can be grouped into peptide, nonpeptide, and radioligands. Peptides may be cyclic octapeptides (octreotide), cyclohexapeptide-based agonists (MK678), compounds containing aminoheptanoic acid bridging group (L-362), those based on nonpeptide scaffold (KE108), adamantine cyclopeptides (SDZ 222-100), etc. The receptor-ligand binding is influenced by the stability of the β-turn, ring chemistry, and size and position of the bridging units that impart enhanced stability to the resulting complex [25]. Peptide agonists were followed by the advent of nonpeptide ligands, since they provide resistance against proteolysis. Nonpeptide agonists (L-054,522) were synthesized based on a spiroindene-containing lead structure [26]. Some were synthesized by cyclization of the urea backbone of acyclic urea precursors or by using monosaccharides as the scaffolds [27]. Their affinity and selectivity for each receptor subtype could be modulated, thus imparting novel pharmacological, pharmacokinetic, and physicochemical properties to the agonists. Diagnosis and therapy of SST-positive tumors has hugely benefitted from the use of radiolabeled SS agonists. They usually comprise the SS agonist conjugated to a chelating agent, which in turn is tightly bound to the selected radioisotope. Medically significant isotopes of

Iodine (^{125}I), Indium (^{111}In), and Yttrium (^{90}Y) have been used as SS radiolabeled agonists for therapeutic purposes [28]. The evolution of SS agonists from natural cyclic peptides to radiolabeled compounds has paved a way for better diagnosis and possible therapy of SST-related diseases.

Similar to the agonists, antagonists can also be grouped into peptide, nonpeptide, and radiolabeled compounds. The peptide antagonist, CYN-154806, which is a cyclic octapeptide, binds to SST2 with nanomolar affinity and exhibits intermediate affinity for SST5 [15]. Another antagonist, PRL-2970, is a cyclic disulfide octapeptide that is structurally related to CYN-154806 [29]. The octapeptide ODN-8, with an N-methyl-amino-2-napthoyl aminoglycine in its β-turn, selectively binds to SST3 with high affinity [30]. The nonpeptide antagonist SRA-880, based on octahydrobenzo[g]quinoline backbone, displays selectively high affinity for SST1 [31]. BN-81674, a tetrahydro-β-carboline derivative, acts as a competitive antagonist for blocking SS14-mediated inhibition of cAMP accumulation in SST3-expressing CHO-K1 cells [32]. A study conducted by Ginj et al. showed that radiolabeled SS antagonists were preferable over agonists for in vivo targeting of tumors expressing SST [33]. ^{111}In labeled agonists (^{111}In-DOTA-NOC for sst_3 and ^{111}In-DTPA-TATE for sst_2) and antagonists (sst_3-ODN-8 and sst_2-ANT) were injected in mice expressing SST2 and SST3 tumors, wherein it was found that uptake was higher for antagonists in both the cases. The higher affinity of the agonists toward the receptors and their internalization into the target cells makes them an ideal choice for tumor targeting and treatment. Moreover, most of the antagonists do not undergo internalization and hence exhibit limited therapeutic scope. Nevertheless, radiolabeled antagonists may label a higher number of receptor binding sites than agonists and thus improve the sensitivity of diagnostic procedures and the efficacy of receptor-mediated radiotherapy.

Among the several investigational ligands, octreotide, pasireotide, vapreotide, and lanreotide have been approved by the USFDA. A detailed list of agonists and antagonists of somatostatin receptor subtypes can be found on guide to pharmacology [34].

3.4 Receptor-Mediated Targeting Strategies

Overexpression of certain genes during cancer progression offers an opportunity to target these for therapeutic purposes. A comprehensive characterization of the components of SST in gastroenteropancreatic neuroendocrine tumors (GEP- NETs) was performed by Martinez et al. Disease progression and prognosis after 2–10 years was assessed and the expression levels of the components of this SST system were evaluated. SST1, SST2, and SST5 were expressed in both the tumor and the adjacent nontumor tissue; however, their expression was markedly increased in the tumorous tissues. Most of the currently available SS analogues target SST2 since it is highly overexpressed in well-differentiated GEP-NETs. SST2 is also the most frequently expressed SS receptor in the mid-gut and the hind-gut carcinoid tumors. Overexpression of SST3 has been observed in less differentiated and more

aggressive tumors. SST3 agonists were more effective in reducing cell survival in BON-1 cell line, as compared to the treatment with SST2/5-specific agonists. However, a completely opposite effect of SST3 agonists was observed in QGP-1 cell line, suggesting that the response to different agonists was cell-type-dependent, resulting due to the dysregulation in expression, differential activation, or interaction of the SST subtypes [35].

SS analogues like octreotide and lanreotide were reported to improve the symptoms like diarrhea, flushing, bronchial constriction, and elevated levels of urinary-5 hydroxyindoleacetic acid and serotonin, associated with such carcinoid tumors. Insulinomas, gastrinomas, glucagonomas, somatostatinomas, and VIP-omas have been subjected to octreotide treatment, which resulted in varying degrees of success in improving the disease-related symptoms. Octreotide has also been used in combinatorial therapy with interferon [36] and everolimus [37] to target the SSTs and lead to symptomatic improvement as well as direct antitumor effects in GEP-NET patients. SS analogues thus serve as an important palliative tool for the patients diagnosed with metastatic disease, since surgery is rarely curative in such cases.

SST expressing tumors can be visualized by injecting radiolabeled somatostatin analogues, in vivo, followed by imaging using a scintillation counter, a technique known as scintigraphy. SST scintigraphy provides information about the site of tumor, suitability of the SS analogue to be used for treatment and the possibility of using surgery or radioactive therapy for the treatment. Synthesis of SS-based radiopharmaceuticals began with preparation of radioiodinated Tyr^3 analogue of octreotide by Krenning et al. in 1989. This study proved to be a starting point for the development of several other peptide receptor imaging (PRI) analogues [38]. The same research group further developed [^{111}In]pentetreotide, which was the first peptide-based imaging radiopharmaceutical to be approved by the USFDA in 1994. The advancement of PRI to peptide receptor radionuclide therapy (PRRT) was more effective when ^{90}Y-yttrium, a more efficient β-emitter than ^{111}In-indium, was used as the therapeutic radiometal. ^{90}Y was replaced with ^{177}Lu-lutetium for renal protection in patients and has since become the PRRT agent of choice. Several structural modifications of octreotide to increase its affinity toward the SSTs, enhance its tumor uptake, and render it more patient friendly gave rise to [^{177}Lu]DOTATOC. This compound is being evaluated in a multinational phase III trial of the NET therapy, at 41 global sites [39]. Retention of radiopharmaceutical after internalization of receptor is critical for efficient PRI and PRRT. In this context, antagonists were surprisingly found to be more potent, even though their affinity for SSTs was lower than the agonists. This was because the antagonists could label receptors in both active and inactive states, whereas the agonists could only label the receptors in an active conformation. The [^{177}Lu] and [^{68}Ga] labeled antagonists have been found to be more effective than the agonists in terms of image contrast, sensitivity, and diagnostic accuracy [40, 41].

While PRRT is an attractive technique for therapy, switching radiolabeled analogues with fluorescent probes enables ultrasensitive optical detection of the ligands. Organic fluorophores can provide explicit ligand localization; however, they exhibit low chemical stability, toxicity, and photobleaching. The development

of quantum dots (QDs) has greatly refined the field of fluorescence-based imaging. QDs comprise a semiconductor nanocrystal core, which may or may not be capped with an auxillary polymer layer. QD systems offer bright fluorescence, possibility of multiplexed imaging strategies, and low toxicity. QD-based fluorescent somatostatin probe, enabling specific targeting of the SSTs, was developed by Sreenivasan et al. They developed an in situ two-step strategy wherein the SS-biotin complex was administered first, followed by the streptavidin-QDs. This strategy could enable flexible fluorescent tagging of the SRIF for investigating the molecular trafficking inside the cells and targeted delivery in live animals [42]. QD nanocrystals were used as immunocytochemical markers for visualization of somatostatin in somatostatinomas and could be observed by super-resolution light microscopy and immunoelectron microscopy. High quantum efficiency in the emission of photons and increased photostability compared with organic fluorophores such as fluorescein isothiocyanate (FITC) makes QDs a desirable option for immunolabeling [43].

Apart from probes, nanocarrier-based delivery of SS agonists and antagonists has been explored for better and specific penetration of therapeutic and diagnostic substances within the body, with a reduced risk. Gold nanoparticles conjugated to [Tyr^3]Octreotide peptide improved their capacity to be recognized by the protein receptor and enhanced their fluorescence properties [44]. Octreotide-loaded poly(ε-caprolactone)/poly(ethyleneglycol) nanoparticles (NPs) were evaluated for their in vitro and in vivo efficacy toward neuroendocrine pancreatic tumors. The nanoparticulate delivery system allowed targeted delivery into the tumor tissue and reduced the associated side effects. Octreotide NPs were found to be more effective than the native drug and lead to a significant reduction in the tumor volume in tumor-bearing mice [45]. Abdellatif et al. coated octreotide, substituted at N-terminal with 11-mercaptoundecanoic acid onto gold NPs. They observed that the uptake of the NPs was higher than the unmodified ones [46]. SS-14 forms high ordered self-assemblies at the concentration range of 20–60 mM. This tendency of progressive structuring was exploited to form aggregates of SS around gold and silver plasmonic NPs. Adsorption of SS on silver NPs was predicted through an ionic pair interaction, whereas its anchoring on the gold NPs was speculated to be through direct binding, in which the metal atom and the nitrogen of the tryptophan residue were involved. This unique technique thus allowed a control over the coordination and binding sites of the peptides and proteins on plasmonic colloids [47].

The evolution of receptor-mediated targeting of somatostatin using peptide, non-peptide, and radioligands to the recently developed QDs and nanocarriers has facilitated better imaging and therapy of the tumors overexpressing the SSTs. An important part of any therapeutic molecule is its in vivo half-life and its elimination from the body. A majority of analogues are eliminated through biliary or renal clearance, while some are cleared via urine and feces. Whether agonists or antagonists are more suited for a particular treatment, the type of SS analogue to be used and effectiveness in evading cases of relapse are some of the issues that can be addressed by exploiting the current broad knowledge in the field of SS chemistry and biology.

4 c-Kit

In 1986, Besmer et al. discovered a viral oncogene v-kit, which displayed partial homology with a protein kinase oncogene [48]. Its cellular homolog, c-Kit (CD117), is localized on chromosome 4 and expresses a single, 5 kb transcript that encodes a transmembrane glycoprotein. The transduction and truncation of c-Kit is responsible for generating v-kit that lacks extracellular and transmembrane sequences [49]. Several studies were thereafter conducted to elucidate the role of c-Kit in normal and diseased tissues.

c-Kit is a type III receptor tyrosine kinase (RTK), which is found in various cell types like hematopoietic cells, germ cells, mast cells, melanoma cells, and the gastrointestinal tract Cajal cells. About 95% of the gastrointestinal stromal tumors (GIST) express c-Kit, a majority of which exhibit an activating mutation in the c-Kit [50]. This activating mutation in c-Kit was described by Hirota et al. wherein the researchers transiently introduced a mutated c-Kit cDNA into the human embryonic kidney cells. The study revealed that the mutation was present in the region between the transmembrane and the tyrosine kinase domains and enabled c-Kit to dimerize in absence of its ligand, the stem cell factor (SCF). The mutated c-Kit was phosphorylated at the tyrosine residue, in absence of SCF, and thus allowed constitutive activation of the receptor.

SCF, also known as the Kit ligand, activates c-Kit which then undergoes phosphorylation and initiates signal transduction to facilitate cell survival, migration, and proliferation [51]. The SCF comprises of extracellular, transmembrane, and intracellular domains. Alternative splicing generates two variants, a longer soluble and a shorter membrane-bound isoform. N-glycosylated SCF exists in homodimeric conformation and its dimerization plays a regulatory role in dimerization and activation of c-Kit [52].

4.1 Recognition Domain and Structure Activity Relationship of c-Kit

The receptor tyrosine kinases are a family of cell surface receptors that bind to growth factors, cytokines, hormones, etc. and are essential for signal transduction. The type III RTK family includes c-Kit, colony-stimulating factor-1, platelet-derived growth factor α and β receptors (PDGFR), and Fl cytokine receptor. The human c-Kit proto-oncogene is 90 kb long and resides on chromosome 4q11~12. The gene encodes for a 976 amino acid protein [49], which can be divided into three domains, viz., (i) the extracellular domain containing 519 aa and consisting of 5 Ig-like domains (Ig-1 to Ig-5), (ii) a single spanning transmembrane domain consisting of 23 aa, and (iii) intracellular domain comprising 433 aa, starting with a juxtamembrane, followed by a tyrosine kinase domain. The first three Ig-like domains of the extracellular region bind to the SCF, whereas domains 4 and 5

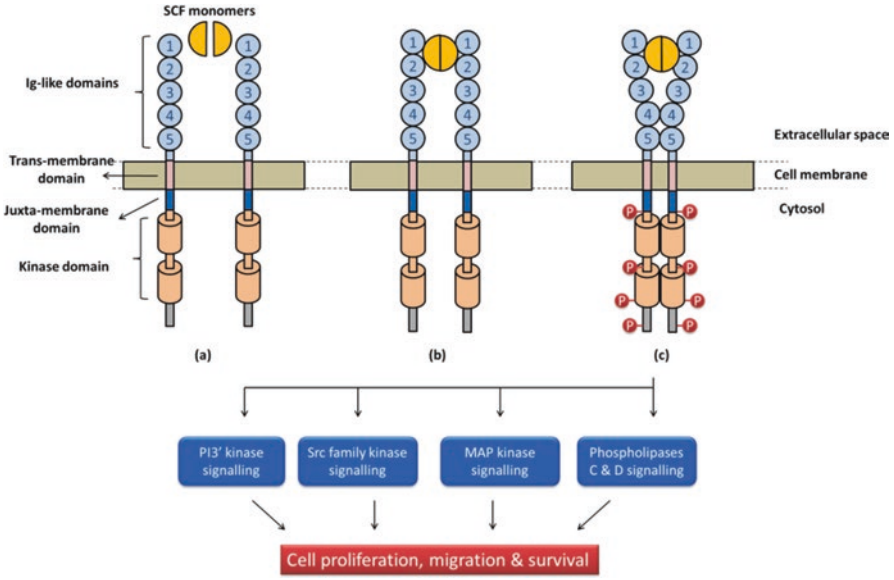

Fig. 5.2 Schematic representation of c-Kit dimerization and activation

participate in the dimerization process [53]. These Ig-like domains may or may not be followed by a Gly-Asn-Asn-Lys (GNNK) tetrapeptide sequence, which differentiates between the two isoforms of c-Kit [54]. Adjacent to GNNK is the transmembrane domain that spans the plasma membrane once and is further connected to the juxtamembrane domain, a stretch of ~30 aa that harbors regulatory function. The kinase domain that follows this 30 aa stretch is interrupted by a kinase insert sequence, thus dividing it into two sub-domains. The last 50 aa form the COOH terminal tail of the receptor.

The crystal structure of the SCF-c-Kit complex shows a 2:2 stoichiometry comprising two receptor-ligand complexes [53]. The binding of SCF and c-Kit is a result of electrostatic interactions between SCF and Ig-1, Ig-2, and Ig-3, followed by dramatic rearrangement of Ig-4 and Ig-5 which aids in receptor dimerization and finally activation (Fig. 5.2).

4.2 Binding of Ligand with Receptor and Pathway

Several events governing cell survival and proliferation occur when SCF activates c-Kit. In the inactive state, the juxtamembrane domain forms a hairpin loop that inserts in the active site, disrupting the regulatory control helix and the phosphate-binding loop, thereby suppressing the kinase activity. It contains two tyrosine residues, Tyr-568 and Tyr-570, which get phosphorylated in response to SCF binding followed by receptor dimerization. This releases the juxtamembrane domain from

its auto-inhibitory configuration and allows a conformation that enables catalytic activity in the kinase domain [55, 56]. Mutations within the juxtamembrane domain and the kinase domain lead to structural changes in the activation loop, resulting in aberrant signaling events.

Autophosphorylation of c-Kit activates class I_A of PI3-K by interacting with the Src homology 2 (SH2) domain of the subunit p85. This interaction results in a conformational change in the enzymatic subunit p110 and its activation [57]. The activated kinase now translocates to the plasma membrane from the cytoplasm and catalyzes the conversion of phosphatidylinositol 4,5 biphosphate to the second messenger, phosphatidylinositol 3,4,5 triphosphate (PIP3). PIP3 recruits proteins with pleckstrin homology domain to the membrane. One such protein, Akt, a serine/threonine kinase, thus docks to the membrane and promotes cell survival by interfering with apoptosis [58]. Akt phosphorylates the proteins Bad and Fox and activates nuclear factor kappa light chain, the enhancer of the activated B cells (NF-κB), resulting in the downstream antiapoptotic events. Activation of the serine/threonine kinase mTOR, the Tec family member Btk, and production of reactive oxygen species have all been speculated to promote cell survival through SCF-mediated activation of c-Kit.

The Src family of tyrosine kinases (SFK), in its inactive conformation, forms a closed structure that does not allow effective catalysis. Phosphorylated c-Kit interacts with the SH2 domain in the SFK, opening the closed structure and hence facilitating its catalytic activity [59]. Lyn is important for phosphorylation and activation of Stat3. The latter is involved in cell cycle regulation, but may negatively regulate the PI3-K/Akt pathway that stimulates cell survival [60]. It also promotes G_1/S phase transition and proliferation of cells as shown in a study involving the megakaryocytic cell line, Mo7e [61]. While the SFK Lyn activates ERK1/2 and JNK MAP kinases, the adaptor protein Lnk negatively regulates both by associating with the Y568 of c-Kit and thereby interfering with SFK binding [62]. Phospholipases (PLs) catalyze the cleavage of phospholipids and convert them into lipid mediators and secondary messengers that play a key role in signal transduction, membrane trafficking, cell proliferation, and apoptosis [63]. The membrane-bound and soluble isoform of SCF induce cell proliferation through PLC-γ and phospholipase D (PLD), respectively [64, 65].

The signal transduction occurring downstream of c-Kit activation is thus complex and encompasses various pathways that are interconnected. This mechanism of action has been pictorially represented in Fig. 5.2.

4.3 Ligands Explored for c-Kit

The fact that tyrosine kinases are often found mutated and constitutively active in various forms of human malignancies has prompted the development of selective inhibitors of these enzymes. A multitude of such inhibitors have been developed and are either in clinical trials or already in the market.

There are two classes of ligands that bind to class III RTKs, viz., (i) the four-helix bundle-type cytokines and (ii) the VEGF-like cysteine-knot type growth factors. The former include the SCF, the macrophage colony-stimulating factor, and the FLT3L. Similarly, the dimerized RTK/ligand complexes can also be divided into two categories, namely, the dimeric ligand-driven and monomeric ligand-driven. The SCF/c-Kit complex belongs to the category of complexes that dimerize due to the dimeric nature of their ligands. Of the other members belonging to this category, the SCF/c-Kit complex displays certain unique characteristics that distinguish it from other receptor/ligand complexes. SCF/c-Kit is the only complex that uses a four-helix bundle ligand; each SCF binds to a separate c-Kit and therefore stands for the most simplified paradigm of receptor dimerization. In addition to this, the c-Kit uses three domains (Ig-1, Ig-2, and Ig-3) to bind to the SCF [66]. SCF is the only natural ligand for c-Kit.

Gleevec (imatinib) was the first tyrosine kinase inhibitor to be developed for clinical use. Imatinib, an antineoplastic agent, is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of tyrosine kinase enzymes. It inhibits c-Kit in GIST patients and Bcr-Abl in chronic myelogenous leukemia. Imatinib shows a mean absolute bioavailability of 98%, with 95% bound to albumin and α-1-acid glycoprotein. Its metabolism primarily occurs in the liver, while its elimination is through feces. Resistance to imatinib could be observed as it only binds to the kinase in its inactive form. In case of GISTs, mutations in the juxtamembrane domain, but not in other parts of the receptor, are sensitive to imatinib treatment. c-Kit mutants greatly differ in their sensitivity to imatinib with substitutions in Asp-816 generating complete resistance to the drug. Another factor for resistance is the mutation in Tyr-670, which forms hydrogen bond with imatinib within the ATP-binding pocket. Consequently, a second-generation inhibitors were developed, viz., sprycel (dasatinib) and tasigna (nilotinib), to overcome the resistance to imatinib, in cases involving alternate mutations in c-Kit signaling. These belong to the class of aromatic anilides that bind to the target receptor in both active and inactive conformation. Their metabolism and elimination from the body is similar to that of imatinib. Apart from c-Kit_{V560D}, c-Kit_{L576P}, and c-Kit_{K642E}, the c-Kit mutants which are sensitive to imatinib, dasatinib, and nilotinib can inhibit c-Kit with mutations at Asp-816 and Asn-822. Dasatinib targets more than 20 tyrosine kinases, whereas imatinib and nilotinib inhibit six enzymes. Secondary mutations occurring in exon 13 and 14 give rise to amino acid substitutions at Val-654 to Ala and Tyr-670 to Ile, respectively. These residues are present at the entrance to the ATP-binding cleft, and substitutions at these positions hinder drug binding, resulting in resistance to imatinib, nilotinib, and even dasatinib. Thus, another drug, sunitinib that does not extend to the catalytic site of c-Kit and can thus inhibit receptors with mutations in this region was developed. However, it binds to c-Kit in its inactive form and thus fails to inhibit Asp-816 mutants. An indolocarbozole compound, Rydapt (midostaurin) is a semi-synthetic derivative of staurosporine, an alkaloid from the bacterium *Streptomyces staurosporeus*. It potently inhibits multiple receptor tyrosine kinases. It is majorly metabolized into CGP62221 and CGP52421 by the hepatic enzymes.

It has been approved by the USFDA for the treatment of acute myeloid leukemia in patients who possess FLT3 mutation.

The ligands approved by the USFDA are apatinib, axitinib, dasatinib, imatinib, midostaurin, pazopanib, sorafanib, and sunitinib. A detailed list of agonists and antagonists of somatostatin receptor subtypes can be found on guide to pharmacology [67].

4.4 Receptor-Mediated Targeting Strategies

In general, receptor-mediated strategies aim either for the selective inhibition or for multitargeted inhibition of kinases. While selective inhibition is suitable for tumors that are specifically dependent on a particular kinase, multitargeted approach might provide an overall activity against several signal transduction pathways that coexist in a tumor. On the other hand, efficacy against multiple kinases may give rise to unwanted side effects. Thus, enhanced efficacy, along with minimum side effects, needs to be considered while designing an effective strategy for targeting tyrosine kinases. A great proportion of the expressed receptors reside inside the cells, which limits their accessibility and renders antibody mediated targeting an unsuitable option.

While all the ligands described in the previous section are specific for c-Kit receptor, ligands directed at specific structures present in the promoter region of c-Kit have also been developed for effective gene regulation. G-quadruplexes are higher order structures formed when guanine-rich regions interact with certain cations. Rankin et al. first described the presence of a putative G-quadruplex structure in the human c-Kit oncogene [68]. Subsequently, several small molecules that bind to and stabilize these structures have been identified, resulting in the downregulation of c-Kit expression. Naphthalene diimide derivatives [69], benzo[a]phenoxazine derivatives [70], 6-substituted indenoisoquinolines [71], and quinazolone derivatives [72], are some of the small molecules that have been shown to downregulate c-Kit gene expression and arrest the growth of GIST cell lines in vitro.

Imatinib has demonstrated significant activity and tolerability in the treatment of malignant unresectable or metastatic GIST, inducing tumor shrinkage of 50% or more or disease stabilization in most patients. Being the first inhibitor to be used in clinical trials, imatinib has been extensively explored and used in free as well as in nanoparticulate form. Many patients develop resistance to the therapeutic ligands due to secondary mutations in c-Kit and PDGF receptors. This necessitates the use of alternative approaches to combat the resistant tumors. Systems incorporating imatinib, such as nanoparticles, microcapsules, microspheres, and liposomes, have been reported to increase the bioavailability and reduce the associated side effects. Imatinib mesylate (STI571) was incorporated in poly(lactic-co-glycolic) acid for suppression of vein graft neointima formation [73] and was also found to be more effective than free imatinib in reducing off-target cardiotoxicity, while at the same time increasing its anticancer efficacy [74] in rats. Imatinib loaded, sterically

stabilized liposomes were found to reduce the interstitial fluid pressure in tumors, which is a major obstacle in chemotherapy. These liposomes were hence used for efficient delivery of doxorubicin to potentiate chemotherapy [75]. A triple targeting system, comprising transferrin (cellular targeting), imatinib, and Bcr-Abl mRNA (molecular targeting), was adopted by Mendonca et al. to silence Bcr-Abl oncogene in chronic myeloid leukemia (CML). Similarly, imatinib loaded, layer-by-layer self-assembled microparticles were found to effectively target CML stem cells. Gold nanoparticles coated with chitosan and RGD tripeptide were used for the encapsulation and delivery of sunitinib into tumor vasculature [76].

miRNAs (microribonucleic acids) are 18–25 nucleotide long, noncoding RNA molecules that play important roles in regulating the gene expression in cancers. miRNAs regulate gene expression and modulate cell functions, such as cell cycle, proliferation, differentiation, stem cell maintenance, metabolism, and apoptosis. Since miRNA expression levels are dysregulated in cancer, efforts are being directed at using these molecules as therapeutic agents. Of the several miRNAs showing an inverse relationship to c-Kit in GISTs, miR-494 was studied by Kim et al. In this study, it was proposed that miR-494 regulated c-Kit expression by showing that (i) miR-494 expression was inversely correlated with c-Kit expression in GIST tissues, (ii) exogenous miR-494 induced downregulation of c-Kit, and (iii) inhibition of endogenous miR-494 increased c-Kit expression [77]. Chemically modified miRNAs, aka miRNA mimics (miR-mimics), are being developed to increase the stability and efficiency of miRNAs. Like miR-494, levels of miRNAs 221 and 222 are also inversely proportional to c-Kit expression in GISTs. The reduced levels of miR-221/222 in GISTs were restored by administering chemically modified miR-mimics for miR-221/222. These were synthesized to possess phosphorothioate (PS) and/or 2′O-methyl RNA (2′-OMe) inside and outside the seed region. These mimics proved to be effective inhibitors of c-Kit gene expression and demonstrated increased stability in rat plasma. Their transfection in GIST48 cells showed significant effects on cellular migration, proliferation, and apoptosis, that is on all processes where c-Kit played a functional role in tumor development [78]. Combining the advantages of miRNAs and nanoparticulate drug delivery systems, Tu et al. developed O-carboxymethylchitosan (OCMC)-tocopherol polymer conjugate-based nanoparticles and loaded them with miR-218 to increase their therapeutic efficacy against GIST. This novel nanocarrier could successfully transfect the miR-218 and enhance its therapeutic efficacy by acting as a tumor suppressor in human GIST cell line (GIST882) [79].

Since it is mainly the activating mutations in c-Kit that result in GIT cancers, receptor-mediated strategies have been developed to downregulate or inhibit c-Kit. However, inhibition of tyrosine kinase can be tricky, considering the number of different kinases that exist in a cell and the interconnected network of pathways triggered by them. From the initial 2-phenylaminopyrimidine derivative to the recent miR-mimics and drug-loaded carriers, the c-Kit inhibitors have come a long way in treating GISTs and related cancers.

5 The Peroxisome Proliferator-Activated Receptors

Nuclear receptors are a family of 48 receptors responsible for the transcriptional regulation of a vast majority of genes, encoding various metabolic enzymes, and thus ultimately regulating the energy homeostasis. They are basically transcription factors and bind to small lipophilic ligands like the hormones, vitamins, and metabolites (prostaglandins, oxidized phospholipids, etc.). The peroxisome proliferator-activated receptors (PPARs) are the members of group C, of the subfamily 1, of the nuclear receptors superfamily and consist of three members, viz., PPARα or NR1C, PPARδ (also known as PPARβ) or NR1C2, and PPARγ or NR1C3. Fatty acid metabolism (FA oxidation and lipogenesis), which is triggered in response to nutritional and physiological stimuli, is partially regulated by the PPARs [80]. Upon activation, the PPARs may interact with transcription factors such as NFκB, STAT-1, and activator protein-1 and lead to transcriptional repression, through a DNA-independent mechanism [81].

The three members of the family are encoded by three different genes. PPARγ is the most extensively studied. It is highly conserved across all the species, with 95% homology in the amino acid sequences found between the human and murine receptors. This subtype further exhibits three isoforms in humans (PPARγ1, PPARγ2, and PPARγ3), which arise due to the presence of different promoters. The PPARγ1 and PPARγ3 proteins are essentially the same, but the PPARγ2 receptor contains additional 28 amino acids at the N-terminus. These three isoforms are differentially distributed throughout the body, with PPARγ1 having the broadest distribution. The PPARγ is critical in regulating adipocyte differentiation and also modulates a number of other genes involved in energy storage and utilization [82]. The human PPARα on the other hand displays 91% similarity in the amino acid sequences, when compared to the mouse receptor. Its mRNA levels are controlled by the endogenous glucocorticoids and it was highly expressed in metabolically active tissues, such as the liver, heart, kidneys, and muscles in rodents as well as humans [82]. The last subtype is PPARδ, which has been known by a variety of names (PPARβ, NUCI, and fatty acid activated receptor (FAAR)), since the time of its discovery. The human and rodent receptors are about 90% homologous and it is expressed in the human tissues that are involved in lipid metabolism, such as the liver, intestine, kidneys, abdominal adipose tissue, and the skeletal muscles [82].

5.1 Recognition Domain of the PPARs

The domain structure of the nuclear receptors is common to most of the members of this family. The PPARs exhibit a modular structure consisting of six functional domains, namely, A/B, C, D, and E/F (Fig. 5.3). The C region consists of about 66 amino acids and is responsible for targeting specific DNA sequences or the response elements. Hence, it is also known as the DNA-binding domain (DBD). This region is stabilized by the presence of zinc atoms, each of which is attached to four

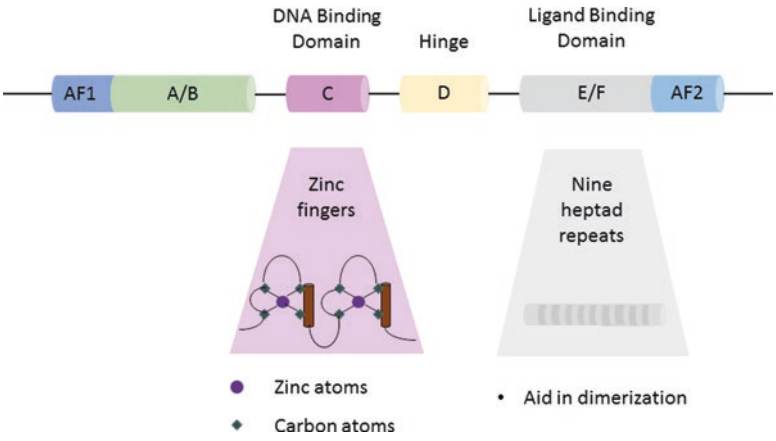

Fig. 5.3 Structure of PPAR showing different domains

cysteine residues. An α-helical structure follows each of these zinc finger complexes. One of these helices is the major recognition sequence that makes base-specific contacts within the major groove of the core site [83, 84]. DBD is reported to house two conserved sets of functionally important amino acid residues, viz., the P-box and the D-box. The P-box is present on the first zinc finger, whereas the D-box is on the second one. The specific interactions between the receptor and DNA are determined by the P-box. The D-box, on the other hand, is linked to protein-protein interactions, like receptor dimerization [85].

The E region, which participates in ligand binding, is a multifunctional domain and is relatively larger than the other domains. Apart from ligand binding, it is involved in dimerization, nuclear localization, ligand-dependent transactivation, intermolecular silencing, intramolecular repression, and association with heat-shock proteins [86]. Dimerization is aided by a region of nine heptad repeats in the E region. The activation domains of the PPARs, which help in ligand binding, are conserved regions across the superfamily of nuclear receptors. Two of them have been identified, with the first one, the constitutive activating function 1 (AF-1), being localized in the A/B domain and a second α-helical ligand-dependent activating domain (AF-2) being localized in the C-terminal region of the E-domain. The two domains act independently or cooperatively, depending on the promoter. The transcriptional activities of the ligand-binding domain (LBD) are thus controlled by ligand binding and dimerization. In fact, ligand binding and dimerization are also mutually dependent, since the ligand-induced conformational change is hypothesized to unmask the major dimerization region in the LBD [87]. In this way, the two phenomena confer transcriptional activities on the heterodimeric receptors that are distinct from those of the component monomers. It is interesting to note that because of this arrangement, a limited number of receptors are able to generate a diverse set of transcriptional responses, to multiple hormonal signals. Even though the overall domain structure is highly conserved in the nuclear receptor family, the LBDs across the different subtypes of PPARs are less conserved and thus bind to similar but not identical ligands [88].

5.2 Binding of Ligand with Receptor and Pathway

The subtypes of PPAR elicit different responses when activated by binding to a ligand. Among these, the activation of PPARα modulates plasma lipids by increasing the levels of high-density lipoprotein cholesterol and apolipoprotein A1, reducing the levels of very low-density lipoprotein triglycerides, and increasing fatty acid oxidation. In case of PPARγ, activation of the receptor improves insulin sensitivity and glucose control and lowers the circulating levels of fatty acids and the proinflammatory markers of the cardiovascular diseases and atherosclerosis.

Activation of the PPARs can occur via direct interaction and stabilization of the components of the transcription machinery. Additionally, sometimes it also recruits various adaptors, coactivators, corepressors, and accessory proteins. These proteins either inhibit or enhance gene transcription, depending upon the nature of the protein and the conformational state of the receptor [88]. The corepressor proteins, like the nuclear receptor corepressor (NCoR), interact with the unliganded receptor and thus downregulate the activation of PPAR, in a normal state. However, they dissociate upon ligand binding, due to the conformational change that occurs in the PPAR structure. Simultaneously, PPAR undergoes heterodimerization with another nuclear receptor, the retinoid X receptor (RXR). This PPAR-RXR heterodimer is instrumental in the recruitment of coactivator proteins like the PPARγ coactivator-1 (PGC-1), p160/SRC-1, and the vitamin D3 receptor-interacting protein (DRIP) or thyroid hormone receptor-associated protein (TRAP)-220 complexes and acts as a scaffold. This scaffold, in turn, recruits histone acetyl transferases and the RNA polymerase complex, which together initiate chromatin relaxation to permit transcription of the target genes [89].

The recruitment of coactivators to the PPARs, based on their ligand binding, has been studied for both PPAR-α and PPAR-γ, which has revealed their contribution to transcription potency and efficacy. The transactivation of the target genes widely differs, depending on the specific pattern of cofactors recruited to the PPAR-RXR complexes, in response to the ligands. The ability of PGC-1 to potently activate the uncoupling protein-1, but not the aP2 gene is an excellent example of target gene selectivity of cofactors. Several cell-based studies have highlighted another interesting feature of the ligand binding in case of PPARs. Some PPAR-γ activators act either as partial agonists or as full agonists, depending on the cell type in question. One possible reason for this behavior is the variations present in the cofactor profile or amount. The action of ligands may vary from being a full agonist in engaging/disengaging of one cofactor (NCoR) to acting as a partial effector on other cofactors (PGC1-α and DRIP/TRAP220), even within the same cell type. This is an important reason why some PPAR agonists display significant side effects while achieving an accurate on-target delivery. This has been studied using the drugs developed for diabetes, another significant condition that relies heavily on the PPAR expression patterns. Each ligand-PPAR receptor complex adopts a different three-dimensional structure that drives a distinct cofactor recruitment pattern and gene signature profile. Studies have also documented differential but overlapping patterns of gene expression in various tissues for different PPAR-γ ligands or dual-PPAR-γ/α agonists, even for compounds that are structurally highly similar [89].

5.3 Ligands Explored for PPARs

Fatty acids are among the most important natural ligands for all the three subtypes of PPARs. Palmitic acid, oleic acid, linoleic acid, and arachidonic acid have been reported to be endogenous activators of the rat PPARα [82]. Apart from these natural ligands, synthetic ligands have also been explored, of which hypolipidemic fibrate drugs are an important class. Wy-14643, clofibrate, fenofibrate, and bezafibrate were developed as hypolipidemic agents, through optimization of their lipid-lowering activity in rodents, before the discovery of the PPARs. Clofibric acid and fenofibric acid, the active metabolites of clofibrate and fenofibrate, are dual activators of PPARα and PPARγ, with a tenfold higher selectivity for PPARα, while bezafibrate activates all three PPAR subtypes at comparable doses [90]. These molecules, when investigated in rodent models of hyperlipidemia, were found to be active at lower doses [91]. Chemical libraries of ureidofibrates have been synthesized using solid-phase synthesis, which has enabled a rapid optimization of their activity upon binding with the human PPAR receptors [92].

A class of antidiabetic agents known as thiazolidinediones (TZDs) or "glitazones" are the first compounds reported as high-affinity PPARγ agonists [93]. Empirical compound screening in rodent models of insulin resistance over a span of almost 15 years had led to the development of TZDs [94]. TZDs, in general, have a higher selectivity for PPARγ than the PPARα and PPARδ subtypes. The TZDs show a range of activity that depends on the cellular model and the response element used in the transactivation assay. This can culminate in either partial or full activation of the PPARs. Some TZDs have also known to mediate their antidiabetic activity through mechanisms that do not involve PPARγ; however, there is still a possibility that the metabolites of these drugs cause the activation of PPARγ. An alternative explanation is that these compounds are able to produce tissue- or promoter-specific modulation of the PPARγ target genes, which may not be detected by the standard PPARγ transactivation assays. Should this latter hypothesis prove valid, it will add a new dimension to the development of PPARγ ligands, similar to the estrogen receptor ligands that are known to be stimulated by selective receptor modulators in case of osteoporosis [82].

Unlike the drugs for the other subtypes (PPARα and PPARγ), there are no known drugs that work through PPARδ. Thus, a part of the challenge in determining the functions of PPARδ has been the identification of potent and selective ligands for use as chemical tools [82].

5.4 Receptor-Mediated Targeting Strategies

Numerous studies report the association of PPARs with the cancers of the GIT although there is still some debate on the intricacies of the molecular mechanisms at play. Further, to complicate the matter, conflicting studies have been reported with regard to the tumor-suppressive and procarcinogenic roles of the PPARs in the

GIT cancers. These contradictions arise due to the experimental differences among the study protocols. However, the antiproliferative and proapoptotic effects shown by these receptors support their role in tumor suppression. The PPAR agonists provoke several physiological modifications influencing lipid metabolism, glucose homeostasis, and inflammation signaling cascade. Imbalances in these processes are highly instrumental in the development of pathological conditions like obesity, metabolic derangement, and chronic inflammatory bowel disease that act as the major risk factors for GIT cancers. The PPARs agonists have been suggested as promising candidates, as well as components of combination treatments, through various in vitro and in vivo models of cancer.

PPARγ, in particular, has been strongly associated with majority of the GIT cancers. An increase in the expression of PPARγ has been reported in the case of a developing esophageal adenocarcinoma with a decreased level of differentiation of the cancerous cells [95]. The same has also been observed for gastric carcinoma and adenocarcinoma. Interestingly, PPARγ agonists exhibit a dose-dependent inhibitory effect on gastric cancer cell lines, which is augmented by the simultaneous addition of 9-cis retinoic acid. Induction of apoptosis along with cell cycle arrest in the G1 phase has been suggested as one of the possible mechanisms of this antiproliferative effect of PPARγ activation [96]. According to a few studies in mice, a normal mucosa of the colon and the rectum exhibit a high expression of PPARγ, whereas deficiency in the intestinal PPARγ is associated with enhanced tumorigenicity. Mutations in the PPARγ gene not only downregulate the expression of the receptor but also impair its function by causing the ligand-binding domain to lose its ability to bind to the ligands and control the gene expression [97]. The antidiabetic glitazones (rosiglitazone, troglitazone, and pioglitazone) have been shown to induce apoptosis in human colon cancer cells. This involves inhibition of glycogen synthase kinase-3β, followed by inhibition of NF-κB, which ultimately results in poor cell survival. Further, glitazone also arrests cells in G_0/G_1 phase, with a parallel decrease in G_0/G_1 phase regulatory proteins like Cdk2, Cdk4, cyclin B1, D1, and E, thus inducing apoptosis. Concomitantly, there is a decrease in the antiapoptotic protein Bcl-2 and an increase in the expression of the proapoptosis-associated proteins, like caspase-3, caspase-9, and Bax [98]. As earlier described for gastric cancer cell lines, the effect of these agonists is augmented by the simultaneous addition of the RXRα ligand and 9-cis retinoic acid. On the other hand, in another study, cyclic phosphatidic acid, a PPARγ antagonist and a structural analogue of lysophosphatidic acid, inhibited PPARγ, consequently preventing the proliferation of human colon cancer HT-29 cells [99]. Synthetic and physiological agonists of PPARγ and PPARδ were also found to induce the expression of VEGF, a crucial growth receptor in cancer development, in the colorectal tumor cell lines SW480 and HT29 [100]. On a slightly different note, PPARδ agonists are known to promote terminal differentiation, making it a promising drug target. However, once again, there are contradictory reports stating pro- or anticarcinogenic effects of PPARδ in different cancer models. Moreover, PPARδ hampers the ability of PPARγ agonists to induce cell death in colorectal cancer cell lines, effectively reducing the potency of PPARγ agonists [101].

The PPAR agonists have also been studied in the rarer types of GIT cancers, like the hepatocellular carcinoma and pancreatic adenocarcinoma. The TZDs have been abundantly reported to arrest cancer growth in both these types of cancers in vitro studies. Accordingly, troglitazone was reported to inhibit the growth and induce apoptosis in HepG2 cells, which are hepatic cancer cells, in a dose-dependent manner. TZD also attenuated the growth of pancreatic cancer cells, in vitro, possibly through G_1 cell cycle arrest, cell differentiation, and increased apoptosis [102]. These molecules have also been suggested to reduce the invasiveness of the tumor cells. An independent study supported the role of PPARγ as an ideal partner with the standard therapy based on gemcitabine, since the anticancer effect of gemcitabine could be enhanced using ligands of PPARγ, such as pioglitazone and rosiglitazone. This study concluded that PPAR ligand-based drugs like pioglitazone, that were originally developed against metabolic diseases like diabetes, have great potential as anticancer agents. However, the adverse effects associated with TZDs, such as weight gain, macular edema, bone loss, and heart failure, in susceptible individuals, should be used as the basis for investigating new analogues of the PPARγ agonists in order to harness their beneficial effects to the fullest.

6 Preclinical and Clinical Studies on GIT Receptors

Among the four receptors described here, somatostatin and c-Kit have been extensively studied with respect to the cancers of the GIT. Therefore, several clinical studies support the efficacy of their ligands as anticancer therapies. In case of PPAR, numerous experimental studies have established the potency of their ligands in the cancers of the lung, breast, and colon. However, clinical trials investigating their efficacy against the GIT cancers are scanty. Nevertheless, the fact that these ligands have been successfully employed in clinical trials of several major types of cancers warrants their use against the cancers of the GIT. The theoretical base for this possibility has already been set by the numerous experimental and preclinical reports describing the efficacy of ligands of somatostatin and c-Kit in the GIT cancers. The following tables (Tables 5.1, 5.2, and 5.3) summarize the ongoing trials for drugs that are being evaluated against the cancers of the GIT.

Table 5.1 Ongoing drug trials for SSTs

Drug	Phase	Duration	Study aim
Lanreotide, Y-90 microspheres	Phase 2	Jul 2017–Mar 2020	Study of lanreotide in patients with metastatic gastrointestinal neuroendocrine tumors who are undergoing liver-directed radio-embolization with Yttrium-90 microspheres
Cetuximab nanoparticles decorated with somatostatin analogue	Phase 1	Feb 2019–Jan 2021	Targeted polymeric nanoparticles loaded with cetuximab and decorated with somatostatin analogue to treat colon cancer

(continued)

Table 5.1 Ongoing drug trials for SSTs

Drug	Phase	Duration	Study aim
Vandetanib	Phase 2	Dec 2013–Aug 2023	Phase II Trial of vandetanib in children and adults with wild-type gastrointestinal stromal tumors
Lutathera		Nov 2018–Dec 2024	Postauthorization long-term safety study of Lutathera
Lanreotide and Metformin	Early Phase 1	Apr 2016–Apr 2022	The Met NET-2 trial
Satoreotide trizoxetan	Phase 2	Sep 2017–Mar 2020	To evaluate the optimal dose of 68Ga-OPS202 as a PET (positron emission tomography) imaging agent in subjects with gastroenteropancreatic neuroendocrine tumor (GEP-NET)
Octreotide acetate, saline solution, paracetamol, sulfasalazine		Jul 2016–Jul 2019	Gastrointestinal nutrient transit and enteroendocrine function after upper gastrointestinal surgery (EndoGut)
Somatuline Depot, Keytruda	Phase 2	Jul 2017–Jun 2020	Study of pembrolizumab with lanreotide depot for gastroenteropancreatic neuroendocrine tumors

Table 5.2 Ongoing drug trials for c-Kit

Drug	Phase	Duration	Study aim
Famitinib	Phase 2	Mar 2012–Jun 2019	A study of famitinib in patients with gastrointestinal stromal tumor
AUY922	Phase 2	Oct 2011–Oct 2019	A study of AUY922 for GIST patients
PLX9486, PLX3397, sunitinib	Phase 2	Mar 2015–Mar 2020	Use of PLX9486 as a single agent and in combination with PLX3397 or PLX9486 with sunitinib in patients with advanced solid tumors
Imatinibmesylate, ipilimumab	Phase 1	Feb 2013–Feb 2020	Ipilimumab and imatinib mesylate in treating participants with metastatic or unresectable solid tumors
Ponatinib 30 MG	Phase 2	Mar 2017–Mar 2020	POETIG Trial – ponatinib after resistance to imatinib in GIST
Apatinibmesylate tablets, Bevacizumab injection	Phase 2	Oct 2017–Dec 2022	Apatinib versus bevacizumab in second-line therapy for colorectal cancer (ABST-C)
Famitinib, placebo	Phase 2	Apr 2012–Oct 2014	A study of famitinib in patients with advanced colorectal cancer
Famitinib, placebo	Phase 3	Jan 2015–Jun 2019	Safety and efficacy study of famitinib in patients with advanced colorectal adenocarcinoma (FACT)
Imatinib	Phase 2	Apr 2016–Mar 2020	Imatinib as preoperative anticolon cancer targeted therapy
Apatinib/capecitabine		May 2018–Oct 2023	Apatinib combined with capecitabine in second-line treatment of advanced gastric cancer: a single-arm exploratory clinical pilot trial

Table 5.3 Ongoing drug trials for PPAR

Drug	Phase	Duration	Study aim
Pioglitazone, nivolumab, treosulfan, clarithromycin	Phase 2	Jan 2016–Jul 2020	A trial with metronomic low-dose treosulfan, pioglitazone, and clarithromycin versus standard treatment in NSCLC
Metformide hydrochloride/ pioglitazone hydrochloride extended release tablet	Phase 2	Aug 2017–Mar 2019	ACTOplus Met XR in treating patients with stage I–IV oral cavity or oropharynx cancer undergoing definitive treatment

7 Conclusion and Perspectives

The factors affecting cancers include the tumor microenvironment, consisting of the extracellular matrix to the interconnected network of multiple pathways that participate in the normal functioning of a cell. An in-depth knowledge of the molecular mechanisms underlying these processes can be used to design newer therapeutic approaches. Unregulated checkpoints in the cell cycle can cause initial loss of control over the cell growth. Also, receptors for hormones, growth factors, and small molecules play a crucial role in the dysregulation of normal cellular processes, like cell survival and proliferation, thus resulting in tumorigenesis. For example, receptor tyrosine kinases, like c-Kit, exhibit constitutive activation due to certain mutations in their exons that lead to uncontrolled growth signaling. Also, the process of endocytosis is deeply associated with cell programming, wherein it participates in cell cycle regulation, mitosis, and apoptosis. It contributes to signal diversification and specificity and also acts as a platform for assembling of the signaling complexes, thus providing a link between endocytosis and signaling. Targeting the proteins involved in this machinery might lead to the identification of newer molecular therapies for the treatment of cancer, since endocytosis dictates the intracellular targeting and recycling of receptors.

Among the three receptors that have been elaborately described in this chapter, the SSTs and c-Kit offer possibilities of many clinically relevant anticancer therapies for the GIT. On the other hand, PPAR is known to have definite role in cancer, but lack data regarding effective ligands. Also, ligands against receptors, like the growth factor receptors, chemokine receptors, and hormone receptors, are clinically effective against the cancers of the GIT. Figure 5.4 gives an overview of the receptor-mediated strategies explored for the treatment of the malignancies affecting the various organs of the GIT.

Peptide and nonpeptide ligands, sometimes labeled radioactively or fluorescently, have been explored both individually and as a part of combinatorial therapy. Monoclonal antibodies, chemotherapeutic agents, and small molecules are all being simultaneously investigated to evaluate their synergistic effects if any. Nevertheless, these molecules exhibit some adverse effects like GI toxicity, skin rashes, diarrhea, nausea, fatigue, and hematological malignancies. These unwanted effects can be mitigated using ligand-based therapies, which may further be combined with nanoparticulate delivery systems, for exploiting the benefits of the latter. Alternatively,

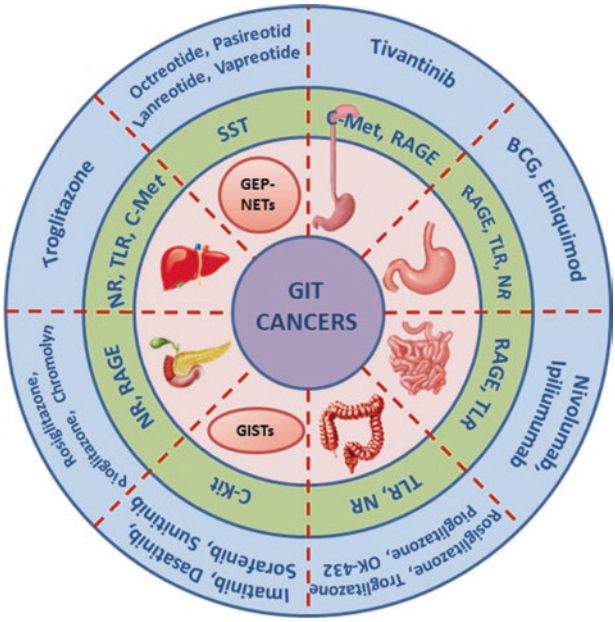

Fig. 5.4 Overview of receptor-mediated strategies for GIT cancers. BCG Bacillus Calmette Guerin, GEP-NETs gastroenteropancreatic neuroendocrine tumours, GISTs gastrointestinal stromal tumours, NR nuclear receptors, RAGE receptor for advanced glycation end-products, SST somatostatin receptors, TLR toll-like receptors

newer approaches like miR-mimics are gaining popularity as means of controlling gene regulation and thus combating cancers. The treatment regime against GIT cancers can be further improved by identifying ancillary targets involved in processes like endocytosis and trafficking, which may be more potent in combating these malignancies and simultaneous development of newer drugs and delivery systems.

References

1. WHO (2014) World cancer report.
2. Dallas NA, Fan F, Gray MJ, Van Buren G, Lim SJ, Xia L, et al. Functional significance of vascular endothelial growth factor receptors on gastrointestinal cancer cells. Cancer Metastasis Rev. 2007;26(3–4):433.
3. Atmaca A, Werner D, Pauligk C, Steinmetz K, Wirtz R, Altmannsberger H-M, et al. The prognostic impact of epidermal growth factor receptor in patients with metastatic gastric cancer. BMC Cancer. 2012;12(1):524.
4. Ogura M, Takeuchi H, Kawakubo H, Nishi T, Fukuda K, Nakamura R, et al. Clinical significance of CXCL-8/CXCR-2 network in esophageal squamous cell carcinoma. Surgery. 2013;154(3):512–20.
5. Itatani Y, Kawada K, Inamoto S, Yamamoto T, Ogawa R, Taketo M, et al. The role of chemokines in promoting colorectal cancer invasion/metastasis. Int J Mol Sci. 2016;17(5):643.

6. Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Arch Pharm Res. 2012;35(8):1297–316.
7. Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, et al. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science. 1973;179(4068):77–9.
8. Pradayrol L, Chayvialle J, Carlquist M, Mutt V. Isolation of a porcine intestinal peptide with C-terminal somatostatin. Biochem Biophys Res Commun. 1978;85(2):701–8.
9. Patel Y, Greenwood M, Kent G, Panetta R, Srikant C. Multiple gene transcripts of the somatostatin receptor SSTR2: tissue-selective distribution and cAMP regulation. Biochem Biophys Res Commun. 1993;192(1):288–94.
10. Reubi J-C. Somatostatin receptors in the gastrointestinal tract in health and disease. Yale J Biol Med. 1992;65(5):493.
11. Shulkes A. 9 Somatostatin: physiology and clinical applications. Baillieres Clin Endocrinol Metab. 1994;8(1):215–36.
12. Liapakis G, Fitzpatrick D, Hoeger C, Rivier J, Vandlen R, Reisine T. Identification of ligand binding determinants in the somatostatin receptor subtypes 1 and 2. J Biol Chem. 1996;271(34):20331–9.
13. NEHRUNG RB, MEYERHOF W, RICHTER D. Aspartic acid residue 124 in the third transmembrane domain of the somatostatin receptor subtype 3 is essential for somatostatin-14 binding. DNA Cell Biol. 1995;14(11):939–44.
14. Greenwood MT, Hukovic N, Kumar U, Panetta R, Hjorth SA, Srikant CB, et al. Ligand binding pocket of the human somatostatin receptor 5: mutational analysis of the extracellular domains. Mol Pharmacol. 1997;52(5):807–14.
15. Bass RT, Buckwalter BL, Patel BP, Pausch MH, Price LA, Strnad J, et al. Identification and characterization of novel somatostatin antagonists. Mol Pharmacol. 1996;50(4):709–15.
16. Duluc C, Moatassim-Billah S, Chalabi-Dchar M, Perraud A, Samain R, Breibach F, et al. Pharmacological targeting of the protein synthesis mTOR/4E-BP1 pathway in cancer-associated fibroblasts abrogates pancreatic tumour chemoresistance. EMBO Mol Med. 2015;7(6):735–53. https://doi.org/10.15252/emmm.201404346.
17. Florio T, Yao H, Carey KD, Dillon TJ, Stork PJ. Somatostatin activation of mitogen-activated protein kinase via somatostatin receptor 1 (SSTR1). Mol Endocrinol. 1999;13(1):24–37.
18. Pagès P, Benali N, Saint-Laurent N, Estève J-P, Schally AV, Tkaczuk J, et al. sst2 somatostatin receptor mediates cell cycle arrest and induction of p27 (Kip1). Evidence for the role of SHP-1. J Biol Chem. 1999;274(21):15186–93.
19. Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, et al. Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. Cancer Res. 2006;66(3):1576–82.
20. Hukovic N, Rocheville M, Kumar U, Sasi R, Khare S, Patel YC. Agonist-dependent up-regulation of human somatostatin receptor type 1 requires molecular signals in the cytoplasmic C-tail. J Biol Chem. 1999;274(35):24550–8.
21. Alderton F, Humphrey PP, Sellers LA. High-intensity p38 kinase activity is critical for p21 cip1 induction and the antiproliferative function of Gi protein-coupled receptors. Mol Pharmacol. 2001;59(5):1119–28.
22. Tulipano G, Stumm R, Pfeiffer M, Kreienkamp H-J, Höllt V, Schulz S. Differential β-arrestin trafficking and endosomal sorting of somatostatin receptor subtypes. J Biol Chem. 2004;279(20):21374–82.
23. Smalley K, Koenig J, Feniuk W, Humphrey P. Ligand internalization and recycling by human recombinant somatostatin type 4 (h sst4) receptors expressed in CHO-K1 cells. Br J Pharmacol. 2001;132(5):1102–10.
24. Bethge N, Diel F, Rösick M, Holz J. Somatostatin half-life: a case report in one healthy volunteer and a three month follow-up. Horm Metab Res. 1981;13(12):709–10.
25. Gazal S, Gelerman G, Ziv O, Karpov O, Litman P, Bracha M, et al. Human somatostatin receptor specificity of backbone-cyclic analogues containing novel sulfur building units. J Med Chem. 2002;45(8):1665–71.

26. Rohrer SP, Schaeffer JM. Identification and characterization of subtype selective somatostatin receptor agonists. J Physiol Paris. 2000;94(3–4):211–5.
27. Hirschmann R, Nicolaou K, Pietranico S, Salvino J, Leahy EM, Sprengeler PA, et al. Nonpeptidal peptidomimetics with beta-D-glucose scaffolding. A partial somatostatin agonist bearing a close structural relationship to a potent, selective substance P antagonist. J Am Chem Soc. 1992;114(23):9217–8.
28. Weckbecker G, Lewis I, Albert R, Schmid HA, Hoyer D, Bruns C. Opportunities in somatostatin research: biological, chemical and therapeutic aspects. Nat Rev Drug Discov. 2003;2(12):999.
29. Hocart SJ, Jain R, Murphy WA, Taylor JE, Coy DH. Highly potent cyclic disulfide antagonists of somatostatin. J Med Chem. 1999;42(11):1863–71.
30. Reubi JC, Schaer J-C, Wenger S, Hoeger C, Erchegyi J, Waser B, et al. SST3-selective potent peptidic somatostatin receptor antagonists. Proc Natl Acad Sci. 2000;97(25):13973–8.
31. Hoyer D, Dixon K, Gentsch C, Vassout A, Enz A, Jaton A, et al. NVP-SRA880, a somatostatin sst1 receptor antagonist promotes social interactions, reduces aggressive behaviour and stimulates learning. Pharmacologist. 2002;44(2 Suppl 1):A254.
32. Poitout L, Roubert P, Contour-Galcéra M-O, Moinet C, Lannoy J, Pommier J, et al. Identification of potent non-peptide somatostatin antagonists with sst3 selectivity. J Med Chem. 2001;44(18):2990–3000.
33. Ginj M, Zhang H, Waser B, Cescato R, Wild D, Wang X, et al. Radiolabeled somatostatin receptor antagonists are preferable to agonists for in vivo peptide receptor targeting of tumors. Proc Natl Acad Sci. 2006;103(44):16436–41.
34. Stefan Schulz CB, Castano Justo, Culler M, Epelbaum J, Hofland L, Hoyer D, Reubi J-C, Schmid H, Schonbrunn A, Feniuk W, Harmar A, Humphrey PPA, Meyerhof W, O'Carroll A-M, Patel YC, Reisine T, Schindler M, Taylor JE, Vezzani A, Hills R (2018) Somatostatin receptors [cited 2018]. Available from: http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=61.
35. Herrera-Martínez AD, Gahete MD, Pedraza-Arevalo S, Sánchez-Sánchez R, Ortega-Salas R, Serrano-Blanch R, et al. Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors. Endocrine. 2018;59(2):426–37.
36. Janson EMT, Ahlström H, Andersson T, Öberg KE. Octreotide and interferon alfa: a new combination for the treatment of malignant carcinoid tumours. Eur J Cancer. 1992; 28(10):1647–50.
37. Pavel ME, Hainsworth JD, Baudin E, Peeters M, Hörsch D, Winkler RE, et al. Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. Lancet. 2011;378(9808):2005–12.
38. Krenning E, Kwekkeboom DJ, Bakker W, Breeman W, Kooij P, Oei H, et al. Somatostatin receptor scintigraphy with [111 In-DTPA-D-Phe 1]-and [123 I-Tyr 3]-octreotide: the Rotterdam experience with more than 1000 patients. Eur J Nucl Med. 1993;20(8):716–31.
39. Strosberg J, El-Haddad G, Wolin E, Hendifar A, Yao J, Chasen B, et al. Phase 3 trial of 177Lu-Dotatate for midgut neuroendocrine tumors. N Engl J Med. 2017;376(2):125–35.
40. Nicolas GP, Mansi R, McDougall L, Kaufmann J, Bouterfa H, Wild D, et al. Biodistribution, pharmacokinetics, and dosimetry of 177Lu-, 90Y-, and 111In-labeled somatostatin receptor antagonist OPS201 in comparison to the agonist 177Lu-DOTATATE: the mass effect. J Nucl Med. 2017;58(9):1435–41.
41. Nicolas GP, Schreiter N, Kaul F, Uiters J, Bouterfa H, Kaufmann J, et al. Comparison of 68Ga-OPS202 (68Ga-NODAGA-JR11) and 68Ga-DOTATOC (68Ga-Edotreotide) PET/CT in patients with gastroenteropancreatic neuroendocrine tumors: evaluation of sensitivity in a prospective phase II imaging study. J Nucl Med. 2017;59(6):915–21.
42. Sreenivasan VK, Kim EJ, Goodchild AK, Connor M, Zvyagin AV. Targeting somatostatin receptors using in situ-bioconjugated fluorescent nanoparticles. Nanomedicine. 2012;7(10):1551–60.

43. Killingsworth MC, Lai K, Wu X, Yong JL, Lee CS. Quantum dot immunocytochemical localization of somatostatin in somatostatinoma by widefield epifluorescence, super-resolution light, and immunoelectron microscopy. J Histochem Cytochem. 2012;60(11):832–43.
44. Surujpaul PP, Gutierrez-Wing C, Ocampo-García B, Ramirez FM, de Murphy CA, Pedraza-Lopez M, et al. Gold nanoparticles conjugated to [Tyr3] octreotide peptide. Biophys Chem. 2008;138(3):83–90.
45. Dubey N, Varshney R, Shukla J, Ganeshpurkar A, Hazari PP, Bandopadhaya GP, et al. Synthesis and evaluation of biodegradable PCL/PEG nanoparticles for neuroendocrine tumor targeted delivery of somatostatin analog. Drug Deliv. 2012;19(3):132–42.
46. Abdellatif AA, El Rasoul SA, Osman S. Gold nanoparticles decorated with octreotide for somatostatin receptors targeting. J Pharm Sci Res. 2015;7(1):14.
47. López-Tobar E, Hernández B, Gómez J, Chenal A, Garcia-Ramos JV, Ghomi M, et al. Anchoring sites of fibrillogenic peptide hormone somatostatin-14 on plasmonic nanoparticles. J Phys Chem C. 2015;119(15):8273–9.
48. Besmer P, Murphy JE, George PC, Qiu F, Bergold PJ, Lederman L, et al. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature. 1986;320(6061):415.
49. Yarden Y, Kuang W-J, Yang-Feng T, Coussens L, Munemitsu S, Dull T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6(11):3341–51.
50. Tabone S, Théou N, Wozniak A, Saffroy R, Deville L, Julié C, et al. KIT overexpression and amplification in gastrointestinal stromal tumors (GISTs). Biochim Biophys Acta Mol Bas Dis. 2005;1741(1–2):165–72.
51. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem cell factor is encoded at the SI locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell. 1990;63(1):213–24.
52. Zhang Z, Zhang R, Joachimiak A, Schlessinger J, Kong X-P. Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. Proc Natl Acad Sci. 2000;97(14):7732–7.
53. Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell. 2007;130(2):323–34.
54. Zhu W-M, Dong W-F, Minden M. Alternate splicing creates two forms of the human kit protein. Leuk Lymphoma. 1994;12(5–6):441–7.
55. Mol CD, Lim KB, Sridhar V, Zou H, Chien EY, Sang B-C, et al. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem. 2003;278(34):31461–4.
56. Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN, et al. Structural basis for the autoinhibition and STI-571 inhibition of c-kit tyrosine kinase. J Biol Chem. 2004;279(30):31655–63.
57. Klippel A, Escobedo JA, Hirano M, Williams LT. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. Mol Cell Biol. 1994;14(4):2675–85.
58. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91(2):231–41.
59. Lennartsson J, Blume-Jensen P, Hermanson M, Pontén E, Carlberg M, RoÈnnstrand L. Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene. 1999;18(40):5546.
60. Shivakrupa R, Linnekin D. Lyn contributes to regulation of multiple Kit-dependent signaling pathways in murine bone marrow mast cells. Cell Signal. 2005;17(1):103–9.
61. Sherry M, LINNEKIN D. Lyn is activated during late G1 of stem-cell-factor-induced cell cycle progression in haemopoietic cells. Biochem J. 1999;342(1):163–70.
62. Simon C, Dondi E, Chaix A, de Sepulveda P, Kubiseski TJ, Varin-Blank N, et al. Lnk adaptor protein down-regulates specific Kit-induced signaling pathways in primary mast cells. Blood. 2008;112(10):4039–47.

63. AOCS (2018) Lipid library [cited 2018]. Available from: http://lipidlibrary.aocs.org/Biochemistry/content.cfm?ItemNumber=39190.
64. Trieselmann N, Soboloff J, Berger S. Mast cells stimulated by membrane-bound, but not soluble, steel factor are dependent on phospholipase C activation. Cell Mol Life Sci. 2003;60(4):759–66.
65. Koike T, Hirai K, Morita Y, Nozawa Y. Stem cell factor-induced signal transduction in rat mast cells. Activation of phospholipase D but not phosphoinositide-specific phospholipase C in c-kit receptor stimulation. J Immunol. 1993;151(1):359–66.
66. Liu H, Chen X, Focia PJ, He X. Structural basis for stem cell factor–KIT signaling and activation of class III receptor tyrosine kinases. EMBO J. 2007;26(3):891–901.
67. PHARMACOLOGY Gt. Type III RTKs: PDGFR, CSFR, Kit, FLT3 receptor family: KIT proto-oncogene receptor tyrosine kinase [cited 2018]. Available from: http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1805.
68. Rankin S, Reszka AP, Huppert J, Zloh M, Parkinson GN, Todd AK, et al. Putative DNA quadruplex formation within the human c-kit oncogene. J Am Chem Soc. 2005;127(30):10584–9.
69. Gunaratnam M, Swank S, Haider SM, Galesa K, Reszka AP, Beltran M, et al. Targeting human gastrointestinal stromal tumor cells with a quadruplex-binding small molecule. J Med Chem. 2009;52(12):3774–83.
70. McLuckie KI, Waller ZA, Sanders DA, Alves D, Rodriguez R, Dash J, et al. G-quadruplex-binding benzo [a] phenoxazines down-regulate c-KIT expression in human gastric carcinoma cells. J Am Chem Soc. 2011;133(8):2658–63.
71. Bejugam M, Gunaratnam M, Müller S, Sanders DA, Sewitz S, Fletcher JA, et al. Targeting the c-Kit promoter G-quadruplexes with 6-substituted indenoisoquinolines. ACS Med Chem Lett. 2010;1(7):306–10.
72. Wang X, Zhou C-X, Yan J-W, Hou J-Q, Chen S-B, Ou T-M, et al. Synthesis and evaluation of quinazolone derivatives as a new class of c-KIT G-quadruplex binding ligands. ACS Med Chem Lett. 2013;4(10):909–14.
73. Kimura S, Egashira K, Nakano K, Iwata E, Miyagawa M, Tsujimoto H, et al. Local delivery of imatinib mesylate (STI571)-incorporated nanoparticle ex vivo suppresses vein graft neointima formation. Circulation. 2008;118(14 suppl 1):S65–70.
74. Marslin G, Revina AM, Khandelwal VKM, Balakumar K, Prakash J, Franklin G, et al. Delivery as nanoparticles reduces imatinib mesylate-induced cardiotoxicity and improves anticancer activity. Int J Nanomedicine. 2015;10:3163.
75. Fan Y, Du W, He B, Fu F, Yuan L, Wu H, et al. The reduction of tumor interstitial fluid pressure by liposomal imatinib and its effect on combination therapy with liposomal doxorubicin. Biomaterials. 2013;34(9):2277–88.
76. Saber MM, Bahrainian S, Dinarvand R, Atyabi F. Targeted drug delivery of Sunitinib Malate to tumor blood vessels by cRGD-chitosan-gold nanoparticles. Int J Pharm. 2017;517(1–2):269–78.
77. Kim WK, Park M, Kim Y-K, You KT, Yang H-K, Lee JM, et al. MicroRNA-494 downregulates KIT and inhibits gastrointestinal stromal tumor cell proliferation. Clin Cancer Res. 2011;17:7584.
78. Durso M, Gaglione M, Piras L, Mercurio ME, Terreri S, Olivieri M, et al. Chemical modifications in the seed region of miRNAs 221/222 increase the silencing performances in gastrointestinal stromal tumor cells. Eur J Med Chem. 2016;111:15–25.
79. Tu L, Wang M, Zhao W-Y, Zhang Z-Z, Tang D-F, Zhang Y-Q, et al. miRNA-218-loaded carboxymethyl chitosan-Tocopherol nanoparticle to suppress the proliferation of gastrointestinal stromal tumor growth. Mater Sci Eng C. 2017;72:177–84.
80. Moreno M, Lombardi A, Silvestri E, Senese R, Cioffi F, Goglia F, et al. PPARs: nuclear receptors controlled by, and controlling, nutrient handling through nuclear and cytosolic signaling. PPAR Res. 2010;2010:1.
81. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: a family of nuclear receptors role in various diseases. J Adv Pharm Technol Res. 2011;2(4):236.

82. Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. J Med Chem. 2000;43(4):527–50.
83. Luisi BF, Xu W, Otwinowski Z, Freedman L, Yamamoto K, Sigler P. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature. 1991;352(6335):497.
84. Schwabe JW, Chapman L, Finch JT, Rhodes D. The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell. 1993;75(3):567–78.
85. Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988;240(4854): 889–95.
86. Tsai M-J, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem. 1994;63(1):451–86.
87. Allan GF, Leng X, Tsai S, Weigel N, Edwards D, Tsai M-J, et al. Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. J Biol Chem. 1992;267(27):19513–20.
88. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res. 1996;37(5):907–25.
89. Wright MB, Bortolini M, Tadayyon M, Bopst M. Minireview: challenges and opportunities in development of PPAR agonists. Mol Endocrinol. 2014;28(11):1756–68.
90. Gaw A, Packard C, Shepherd J. Fibrates. In: Principles and treatment of lipoprotein disorders. Berlin, Heidelberg: Springer; 1994. p. 325–48.
91. Hawke RL, Chapman JM, Winegar DA, Salisbury JA, Welch RM, Brown A, et al. Potent hypocholesterolemic activity of novel ureido phenoxyisobutyrates correlates with their intrinsic fibrate potency and not with their ACAT inhibitory activity. J Lipid Res. 1997;38(6):1189–203.
92. Brown PJ, Hurley KP, Stuart LW, Willson TM. Generation of secondary alkyl amines on solid support by borane reduction: application to the parallel synthesis of PPAR ligands. Synthesis. 1997;1997(07):778–82.
93. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J Biol Chem. 1995;270(22):12953–6.
94. Hulin B, McCarthy PA, Gibbs EM. The glitazone family of antidiabetic agents. Curr Pharm Des. 1996;2(1):85–102.
95. Wang W, Wang R, Zhang Z, Li D, Yu Y. Enhanced PPAR-γ expression may correlate with the development of Barrett's esophagus and esophageal adenocarcinoma. Oncol Res Featur Preclin Clin Cancer Ther. 2011;19(3–4):141–7.
96. Sato H, Ishihara S, Kawashima K, Moriyama N, Suetsugu H, Kazumori H, et al. Expression of peroxisome proliferator-activated receptor (PPAR) γ in gastric cancer and inhibitory effects of PPARγ agonists. Br J Cancer. 2000;83(10):1394.
97. Dai Y, Wang W-H. Peroxisome proliferator-activated receptor γ and colorectal cancer. World J Gastrointest Oncol. 2010;2(3):159.
98. Ban JO, Kwak DH, Oh JH, Park E-J, Cho M-C, Song HS, et al. Suppression of NF-κB and GSK-3β is involved in colon cancer cell growth inhibition by the PPAR agonist troglitazone. Chem Biol Interact. 2010;188(1):75–85.
99. Tsukahara T, Hanazawa S, Kobayashi T, Iwamoto Y, Murakami-Murofushi K. Cyclic phosphatidic acid decreases proliferation and survival of colon cancer cells by inhibiting peroxisome proliferator-activated receptor γ. Prostaglandins Other Lipid Mediat. 2010;93(3–4):126–33.
100. Röhrl C, Kaindl U, Koneczny I, Hudec X, Baron DM, König JS, et al. Peroxisome-proliferator-activated receptors γ and β/δ mediate vascular endothelial growth factor production in colorectal tumor cells. J Cancer Res Clin Oncol. 2011;137(1):29–39.
101. Wang D, Ning W, Xie D, Guo L, DuBois RN. Peroxisome proliferator-activated receptor δ confers resistance to peroxisome proliferator-activated receptor γ-induced apoptosis in colorectal cancer cells. Oncogene. 2012;31(8):1013.
102. Koga H, Selvendiran K, Sivakumar R, Yoshida T, Torimura T, Ueno T, et al. PPARγ potentiates anticancer effects of gemcitabine on human pancreatic cancer cells. Int J Oncol. 2012;40(3):679–85.

Chapter 6
G-Protein Coupled Receptors in Cancer and Targeting Strategies

Aditya Narvekar, Ashu Srivastav, Aparna Tripathi, Padma V. Devarajan, Ratnesh Jain, and Prajakta Dandekar

Abstract G-protein-coupled receptors (GPCRs) play a significant role in a myriad of physiological processes. Therefore, deregulation of GPCR function is implicated in various cancers, making them a suitable target for targeted therapy. Many studies have indicated a key role of GPCRs in cancer initiation, progression, tumorigenesis, and metastases. This manuscript will discuss a few examples of GPCRs involved in cancer with respect to their structure activity, mechanism of binding, ligands explored, and antagonists, along with the targeting strategies. Many clinical trials with different GPCR targeting drugs are ongoing and are expected to contribute to existing anticancer therapeutics. The research in the area of targeting GPCR is anticipated to exploit their potential as pharmacologically important targets.

Keywords G-protein-coupled receptors (GPCRs) · Targeting · Protease-activated receptors · Lysophosphatidic acid receptor · Frizzled receptor

Abbreviations

APC	Activated protein C
APC	Adenomatous polyposis coli
ASA	Acetylsalicylic acid

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AXIN	Axis inhibition protein
CAD	Coronary artery diseases
cAMP	Cyclic adenosine monophosphate
CaR	Calcium sensing
CBP	CREB-binding protein
CK1	Casein kinase 1
CRD	Cysteine-rich domain
DAG	Diacylglycerol
DKK	Dickkopf
DSH	Dishevelled
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly (ethylene glycol)
ECL	Extracellular loops
EDG	Endothelial differentiation gene
EMT	Epithelial-mesenchymal transition
EPCR	Endothelial protein C receptor
FRET	Fluorescence resonance energy transfer
FZD	Frizzled receptor
FZD7-NS	FZD7 antibody-nanoshell conjugate
G2A	G2 accumulation
GDP	Guanosine diphosphate
GPCRs	G-protein-coupled receptors
GSK	Glycogen synthase kinas
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
IC	Inhibitory concentration
ICL	Intracellular loops
IP3	Inositol triphosphate
LEF/TCF	Lymphoid enhancer factor/T-cell factor
LPA	Lysophosphatidic acid
LRP	Lipoprotein receptor-related proteins
LRR	Leucine-rich repeat-containing receptors
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MMP	Matrix metalloproteases
mTORC2	Mammalian target of rapamycin complex 2
NDP gene	Norrie disease protein gene
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NIR	Near-infrared radiation
NT	N-terminal sequence
OMP-18R5	Vantictumab, a monoclonal antibody
OMP-54F28	A novel recombinant fusion protein
PAP	Protease activable prodrugs
PAR	Protease-activated receptor
PC	Planar cell polarity
PEG	Polyethylene glycol

PI3K-AKT	Phosphatidylinositol 3-kinase-protein kinase B
PIP 2	Phosphoinositol
PK	Protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLC-β	Phospholipase C-β
PORC	Porcupine
RAC	Ras-related C3 botulinum toxin substrate
RAF-MEK-MAPK	Rapidly accelerated fibrosarcoma-MAPK ERK kinase-mitogen-activated protein kinase
Rho	RAS homologous protein family
Ryk and Ror2	Tyrosine kinase receptor
SFRP	Secreted frizzled-related protein
sFZD7	Soluble frizzled receptor 7 antagonist decoy receptor
TcdB	*Clostridium difficile* toxin B
TLS	Tethered ligand sequence
TM	Transmembrane
TSH	Thyroid-stimulating hormone
UCNP	Up-conversion nanoparticles
VEGF	Vascular endothelial growth factor;
WNT	Wingless type protein
XWnt	*Xenopus* wingless type protein

1 Introduction

G-protein-coupled receptors (GPCRs) are the most widely targeted receptors in the case of endocrine tumors, with the associated therapeutics being already approved by the USFDA. However, numerous researchers have now established the participation of GPCRs even in other tumor types, due to which they are being looked upon as the "druggable" and novel targets for the treatment of cancers [1]. GPCRs regulate a myriad of physiological functions through the G-proteins, by transduction of a wide and diverse array of external stimuli. They are thus involved in a wide variety of physiological processes like vision, taste, smell, pain, mood regulations, etc. As a consequence, dysregulation of GPCRs is commonly observed in numerous human diseases, rendering them as important targets for numerous therapeutic molecules [2]. Currently, more than 60% of the marketed anticancer drugs are based on GPCRs and almost 34% of all the pharmaceutical prescriptions in the current market target the GPCRs [3].

GPCRs constitute the largest family of cell surface receptors that are involved in the transmission of cellular signals. These include more than 800 proteins, which are encoded by more than 2% of the total human genome [4]. Although historically all the GPCRs were believed to mediate their signaling in the aforementioned way, as depicted in Fig. 6.1, it is now well established that they can also signal via other mechanisms and even function independently of ligands. Recent studies have

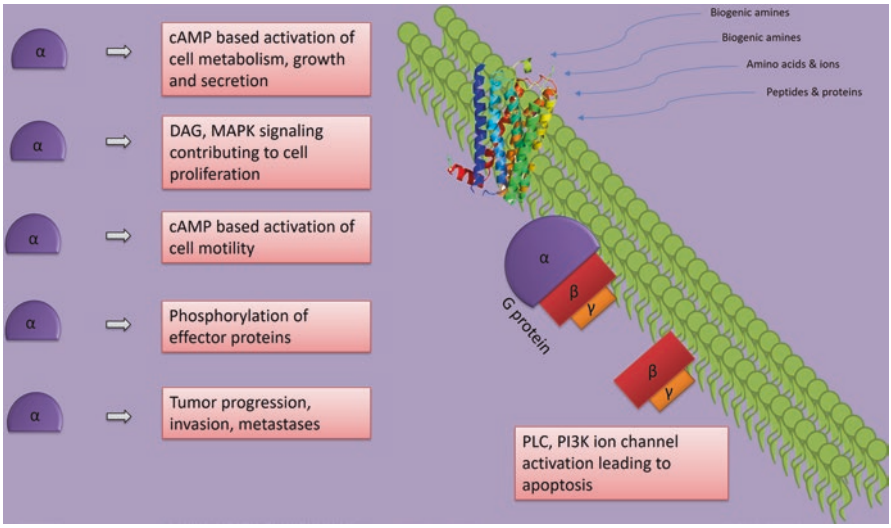


Fig. 6.1 Signaling mediated by GPCR

reported that β-arrestins, known for their function in receptor desensitization, can also serve as independent signaling molecules and as scaffolds for various signaling modules [5].

Along with the regular physiological processes, numerous evidences have suggested the role of GPCRs in cancer progression and development. GPCRs control many features of tumorigenesis, such as invasion, proliferation, and survival at the secondary sites. A few from the 130 orphan GPCRs have been implicated in cancer development and progression. An elevated expression of an orphan GPCR, GPR49, is involved in the formation and proliferation of the basal cell carcinoma, while GPR18 is involved in melanoma metastases. High levels of GPR87 have been reported in lung, cervix, skin, urinary bladder, testis, head, and neck squamous cell carcinomas. On the other hand, GPR56 has been shown to inhibit the progression of prostate cancer and suppress the tumor growth and metastasis in melanomas. It also inhibits vascular endothelial growth factor (VEGF) production in the melanoma cells and thus prevents angiogenesis and growth. Therefore, deciphering the role of GPCRs in the development of cancer may lead to a better understanding of the cancer pathogenesis, as well as provide specific targets for the development of targeted therapeutics. Extensive studies on these receptors are anticipated to provide the basis for designing newer drugs in different types of human cancers. A few of these GPCRs and their involvement in cancer have been summarized in Table 6.1 [6].

In the subsequent sections, we will discuss some of the important GPCRs with respect to their structure, functions, scope of targeting, the advantages and limitations of targeting them, and the preclinical and clinical outcomes of targeting these receptors for alleviating various types of cancers.

Table 6.1 Types of GPCRs and their involvement in cancer [6]

Sr. no.	Type of GPCRs	Activity in cancer
1.	Chemokine receptors	Induce the proliferation of cancer cells and prevent apoptosis
2.	Protease-activated receptors (PARs)	Promote angiogenesis
3.	Lysophospholipid (LPA) receptors	Prevent apoptosis and promote cell proliferation
4.	E-prostanoid (EP) receptors	Tumor promoter
5.	Smoothened (SMO) receptor	Carcinogenesis
6.	Frizzled (FZD) receptors	Wnt signaling
7.	Leucine-rich repeat-containing receptors (LRR)	Gastric cancer
8.	G2 accumulation (G2A) receptor	Oncogenesis
9.	Thyroid-stimulating hormone (TSH) receptor	Growth and survival of pituitary cancer
10.	Calcium-sensing (CaR) receptor	Growth of parathyroid gland cancer

The chapter will include elaborate discussion about protease-activated receptors (PAR), lysophosphatidic acid receptors, and frizzled receptors. PARs are expressed on nearly all cell types in the blood vessel walls, such as endothelial cells, fibroblasts, myocytes and blood cells (platelets, neutrophils, macrophages, and leukemic white cells), except the red blood cells. They are uniquely stimulated by a tethered ligand located within the N-terminus of the receptor itself, rather than by an independent ligand [7]. They play important roles in vascular physiology, neural tube closure, hemostasis, and inflammation. All of these promote cancer invasion and metastasis, at least partially, by facilitating the tumor cell migration, angiogenesis, and interaction with the vascular cells such as platelets, fibroblasts, and the EC lining [8]. On the other hand, activation of the lysophosphatidic acid receptor is mediated through LPA, which results in aberrant expression patterns of various GPCRs. LPA production occurs from lysophosphatidylcholine, in presence of the enzyme autotaxin (ATX). This LPA-ATX-LPAR (Lysophosphatidic acid receptor) signaling axis has an enormous significance in progression of various human cancers. Thus, overexpression of ATX or LPARs has been correlated with malignancies, metastases, and chemo-resistance of tumors, in various cancer types [9]. Finally, the frizzled receptors (FZD1–10) bind to the secreted Wnt proteins, and by activating Wnt signaling, they play a crucial role in the progression of various cancers [10]. It has been observed that blockade of the FZDs by antibodies or by small molecule inhibitors can inactivate the Wnt signaling, thereby hampering the initiation and progression of various human cancers [11]. These GPCRs have thus been highlighted in this chapter. Chemokine receptors, the most significant and the well-documented GPCRs, and which are intricately involved in various cancer types, will be elaborately discussed in Chap. 9.

2 Conditions Characterized by Overexpression of GPCRs

The expression of GPCRs in a variety of cancers has been analyzed using the *in silico* approach. The potential roles of GPCRs, in different stages of cancer progression, have been identified. Here, we have discussed about lung cancer which shows overexpression of GPCRs.

Lung cancer is generally categorized into small-cell lung (SCLC) and nonsmall-cell lung cancer (NSCLC). The most dominant histological subtype of NSCLC is the adenocarcinoma. Microarray studies of adenocarcinomas have exhibited overexpression of GPCRs. Overexpression of a number of GPCRs including several chemokine receptors, neuropeptide receptors, hormone receptors, lysophosphatidic acid receptors etc. is known to occur in lung adenocarcinomas. Many other GPCRs, such as neurotensin receptor, PAR-3, and Edg-2, have been observed in the later stages of cancer development and their levels are upregulated in the stage III of lung cancer [12]. Therefore, the role of various GPCRs in cancers renders them as novel pharmacological targets in the treatment of cancer patients. Among the several GPCRs implicated in various malignancies, a few of whose significance was highlighted in the previous section will be discussed in detail in this chapter.

3 Protease-Activated Receptors

G-protein-coupled protease-activated receptors (PARs), which are uniquely activated by proteolysis, account for approximately 2% of the human genome. These receptors perform diverse biological functions. Four PARs, with distinct N-terminal cleavage sites and tethered ligand pharmacology have been identified till date. These are activated by the action of serine proteases, such as thrombin, which acts on PARs 1, 3, and 4, and trypsin, which acts on PAR 2. These enzymes cleave the N-terminus of the receptors [13]. Thrombin is an established signaling protease that exerts many of its effects through PAR 1 and PAR 3. Trypsin may function as a signaling molecule in the lumen of the small intestine, by cleaving and activating the PAR 2 on enterocytes [14]. The PARs are predominantly expressed in the vascular, immune, and epithelial cells, astrocytes, and neurons. They transmit cellular responses to the coagulant proteases as well as the other proteases expressed in distinct tissues. Further, they play a critical role in homeostasis, thrombosis, embryonic development, wound healing, inflammation, and cancer progression [15]. The PARs represent excellent examples of GPCRs that are regulated by distinct desensitization and endocytic sorting mechanisms. The human PARs, 1–4, include sequences like SFLLRN-NH2, SLIGKV-NH2, TFRGAP-NH2, and GYPGQV-NH2, respectively (represented in Fig. 6.2). Except for PAR3, these peptide sequences are able to act as receptor agonists.

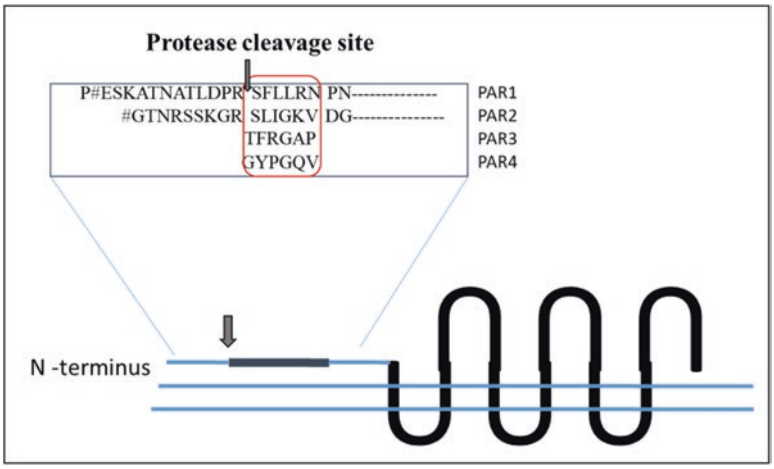


Fig. 6.2 Protease-activated receptor (PAR 1, PAR 2, PAR 3, and PAR 4) amino acid sequence with NH_2-terminus. Arrow indicates the cleavage site, while the box residues show the ligand domains

3.1 Recognition Domain of the PAR

PAR 1 is a GPCR consisting of 415 amino acids and includes five functional domains, namely, the extracellular N-terminal, the extracellular loop, the seven hydrophobic transmembrane domains, the intracellular loop, and the intracellular C-terminal [16]. The extracellular N-terminal contains the cryptic tethered ligand (TL) and an N-terminal sequence (NT) that is essential for PAR1 signaling. The three extracellular loops (ECL1, 2, 3), three intracellular loops (ICL1, 2, 3), as well as a fourth putative loop (ICL4) can be formed transiently, via reversible palmitoylation of a C-terminal cysteine residue and the C-terminal tail of the receptor, which is involved in signal termination/desensitization and trafficking. The TLS is unmasked by the proteolytic activation, which leads to the putative docking of the TL domain near the extracellular loop-2 (ECL2). Residues in the N-terminal extracellular sequence play an important role in the activation of PAR1. The G-protein coupling is mediated via the intracellular loops 2 and 3 (ICL 2 and 3), along with the putative fourth loop formed by a palmitoylated cysteine residue in the C-terminal domain. The C-terminal domain plays an important role in receptor signaling kinetics, desensitization/internalization, and trafficking. The PAR system is energy expensive because the cell is required to synthesize new PAR receptors following each PAR signaling event [17].

3.2 Binding of Substrate/Ligand with PAR Receptor

The PARs are activated through a unique mechanism of enzymatic activation, through the proteolysis of the receptor. This proteolytic cleavage is mediated by a well-characterized family of enzymes that require the serine proteases within the

active site. Receptor activation involves several steps, which are common for most of the receptors. The first step involves interaction of ligands with its receptor. Serine proteases activate PARs by recognition and cleavage of the receptor at the specific enzymatic site within the extracellular N-terminal and finally expose the new N-terminus that acts as a tethered ligand. This further binds to and activates the cleaved receptor molecules. The PARs are considered as specialized peptide receptors because their ligands are physically a part of the cleaved receptor molecules. The second step of GPCR activation is a change in the conformation of the receptor. Finally, receptors in an active conformation interact with the heterotrimeric G-proteins in the plasma membrane, which leads to signal transduction. The PARs couple with several diverse G-proteins. In case of $G_{\alpha q}$, activation of PLC-β (phospholipase C-β) and PIP2 (phosphatidylinositol 4,5-bisphosphate) takes place, which forms IP3 (inositol triphosphate) and DAG (diacylglycerol) [18]. This leads to calcium ion mobilization and activation of protein kinase C (PKC). They also couple with Gi proteins, which inhibit adenylyl cyclase and suppress the formation of cAMP.

3.3 Antagonists of Ligand Binding

The antagonist of PARs is known to induce conformational changes within the transmembrane helices. PARs are activated via peptide-ligand interactions with the residues in the second extracellular loop of the receptor. Vorapaxar (FDA-approved) is a highly specific, irreversible PAR1 antagonist. In a Phase III clinical trial, Vorapaxar was found to be effective against recurrent myocardial infarction [19]. YD-3 ([1-benzyl-3(ethoxycarbonylphenyl)-indazole), a new synthetic indazole derivative, possesses an inhibitory effect on platelet aggregation. PAR1 and PAR4 contribute to human platelet activation, in the presence of high concentrations of thrombin, and inhibition of both these receptors is required to overcome thrombin-induced platelet aggregation. YD-3 was found to competitively inhibit the thrombin-induced platelet aggregation, with little or no inhibitory effect on the actions of other platelet activators [20]. Atopaxar, a PAR 1 antagonist, is an antiplatelet agent that has undergone Phase I and II clinical trials for patients with coronary artery disease. Parmoduin is a small molecule antagonist that targets PAR1. It binds to the intracellular face of PAR1 and interferes with activated GPCR. Pepducin is a unique GPCR antagonist. Its function is to disrupt the interaction between the receptor and heterotrimeric G-protein. It consists of a peptide sequence, corresponding to a region in the intracellular loop or C-terminus of the target GPCR. GB88 (5-isoxazoyl-Cha-Ilespiroindene-1, 4-piperidine) is a potent reversible antagonist that blocked PAR2 activation by endogenous proteases and by the synthetic peptide agonists both in *in vitro* and *in vivo* studies. This compound was found to attenuate the inflammation in rat model of colitis. It blocked only certain PAR2-stimulated signaling pathways. Further, it was found to be selective for Ca^{2+} mobilization, but not for MAP kinase activation [21].

3.4 Targeting Strategies for PAR

The GPCRs represent a superfamily of receptors that have been linked to various disorders of diverse organs. Thrombin was the first protease that cleaved PAR1 and hence was also referred to as the thrombin receptor. While majority of the GPCRs are stimulated by the association of small, soluble peptides with the deep binding pockets on their extracellular face, PAR1 is activated by cleavage at its N-terminus, which exposes a tethered ligand [22]. The mechanism of intramolecular activation leads to conformational changes that transmit signals to the cognate G-proteins and β-arrestin. Matrix metalloproteases (MMPs), such as MMP1, MMP2, MMP9, and MMP13, also cleave PAR 1 at a site other than that cleaved by the serine proteases [23]. Thus, proteolysis of PAR 1 by different proteases and at distinct cleavage sites leads to multiple cellular responses due to a single receptor. In addition to the cleavage at the designated site, different proteases interact with PAR1 through distinct mechanisms. Thrombin binds via exosite I to a hydrophobic hirudin-like sequence DK51YEPF55, on the extracellular face of PAR1. It facilitates the interaction of the cleavage site of PAR1 with the active site of thrombin. After cleavage of PAR1 by thrombin, the receptor serves as a protease responsive guanine nucleotide exchange factor, replacing the GDP with the G_{α} of GTP. In APC-mediated cleavage, the PAR first binds to the endothelial protein C receptor (EPCR), which is compartmentalized in lipid rafts, and leads the protease to the endothelial surface. Binding of APC to EPCR recruits APCR kinase 5, which phosphorylates the cytoplasmic tail of PAR1 and facilitates the recruitment of β-arrestin [24].

3.4.1 PAR Activity in the Reticuloendothelial System

In human endothelial cells, activation of PAR1 by thrombin promotes its coupling with the α subunit of $G_{12/13}$ and $G_{\alpha q}$, and hence activation of the Ras homolog gene family member A (RhoA), as well as other signaling effectors [25]. Activation of PAR1 by coagulant protease thrombin results in RhoA activation, disassembly of the adherens junction, and disruption of the endothelial barrier [24]. In contrast, activation of PAR1 through anticoagulant protease-activated protein C (APC) results in the activation of RecA and enhances the endothelial barrier protection [26]. Cytoprotective signaling by APC-activated PAR1 is mediated through the recruitment of β-arrestin and activation of the dishevelled-2. In human endothelial cells, PAR1 and β-arrestin form a preassembled complex and co-segregate in caveolin-1-enriched fraction. It was found that reduction in the expression of β-arrestin via RNA interference resulted in the loss of APC-induced activation of Rec1, but did not affect thrombin-stimulated Rho signaling. APC also failed to protect against thrombin-induced endothelial barrier permeability in cells deficient in β-arrestin expression [27].

3.4.2 Prodrugs and Drug Complexes Used to Target the PARs

The prodrugs, administered in the inactive form, are bio-transformed to their active form. In most cases, these are simple chemical derivatives which can be converted into the active drug by enzymatic or chemical reactions. Increased expression or altered localization of proteases is typical in many cancers. Among the proteases, cathepsins are known to be upregulated in several tumor tissues. Overexpressed cathepsins are important biomarkers for cancers and can serve as key targets for prodrugs to induce the release of anticancer drugs into the tumor tissue or inside the tumor cells [28]. Cathepsin B is a lysosomal cysteine protease that is overexpressed in various tumor types and is associated with the exterior of cell surfaces. For example, amino acid and dipeptide-daunorubicin conjugates were administered for leukemia. It was found that the conjugates led to superior suppression of tumor growth, as compared to the drug alone. *N*-L-leucyl-doxorubicin (Leu-DOX) was developed as a prodrug of DOX. Leu-DOX prodrugs were also developed to lower the cardiotoxicity and improve the therapeutic index of DOX. The complex exhibited superior antitumor activity as compared to DOX due to its enhanced hydrophobicity and proteolysis in the tumor tissue by the cathepsins. MMP (metalloproteinase)-activated prodrugs have been widely studied as they are found in the extracellular and pericellular areas of the cell. These have been developed by the chemical conjugation of MMP-cleavable peptide substrate (Glu-Pro-Cit-Gly-Hof-Tyr-Leu) to DOX. The peptide substrates are specifically cleaved by MMP-2, 9, and 14. Addition of MMP-2 to the albumin-peptide-DOX conjugate released the tetrapeptide-DOX, which was further converted into DOX. This conjugate showed superior anticancer effect as compared to the native DOX at an equi-toxic dose. Prodrugs have been categorized into cathepsin B-activated prodrugs, antibody-directed enzyme prodrugs, protease cleavable peptide-thapsigargin conjugates, and protease-activated cytotoxic receptor ligands [29]. Overall, the protease-activated prodrugs (PAPs) have shown superior pharmacokinetic properties as compared to the free drugs due to their improved chemical stability, hydrophilicity, and circulation time in the bloodstream. Table 6.2 lists the details of various prodrugs that are currently being investigated for different cancer types.

Table 6.2 Prodrugs in clinical trials

Sr. No.	Name	Protease	Indication	Status
1.	Brentuximab	Cathepsin B	Hodgkin lymphoma	Approved
2.	OPAXIOTM	Cathepsin B	NSCLC	Phase III
3.	PKI	Cathepsin B	Cancer	Phase I/II
4.	PK2	Cathepsin B	Liver cancer	Phase I/II5.
5.	L-377,202	PSA (Prostate specific antigen)	Prostate cancer	Phase II

3.4.3 Nanocarriers for PARs

Protease-activated nanomaterials have been extensively developed and have shown significant targeting ability as improved cancer theranostics. Protease-activated nanoparticles, such as MMP sensing nanoparticles, cathepsin sensing nanoparticles, and other protease-activated nanomaterials, have been developed (listed in Table 6.3). Graphene, dual fret quenching nanoprobes, liposomes, nanoworms, polymeric nanoparticles, up-conversion nanoparticles, etc. are the most common types of nanocarriers that have been developed to target various cancers. Up-conversion nanoparticles (UCNPs) attracted considerable attention due to their tunable fluorescence and near-infrared irradiation. MMP2-sensing UCNP conjugated to gold nanoparticle was fabricated and named as UCNP@p-Au. The gold nanoparticle neutralized the multifluorescence emitted by UCNP upon excitation by NIR irradiation, and this fluorescence-quenched UCNP was evaluated in head and neck cancer cell lines expressing MMP2 proteases. Further, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) DSPE-PEG and cathepsin B-sensitive peptides were used to produce a lipidated cathepsin B inhibitor [30]. Thrombin upregulation promotes cancer metastasis by increasing the malignant cell adhesion. Thrombin-sensitive peptide substrates were conjugated to 40 nm iron oxide nanoworms to detect thrombosis. These nanoworms carried a fluorescent probe attached to a thrombin-sensitive peptide. After thrombosis, fluorescent probes were released and detected in a 96-well plate by ELISA [31]. In case of cathepsin, it has been observed that a lipidated cysteine cathepsin B inhibitor incorporated with a therapeutic drug and a fluorophore to form a liposomal nanocarrier showed efficacy for tumor theranostics in an animal model.Ru@SiO2 nanoparticle monitored cell apoptosis by detecting caspase 3 activity. Table 6.3 states the various protease-activated nanomaterials that are being explored for anticancer therapy.

Table 6.3 Protease-activated nanomaterials for targeted cancer theranostics

Sr. No.	Nanocarriers	Protease	Target
1.	Graphene	MMP14	Mouse squamous cell carcinoma
2.	Dual FRET quenching nanoprobe	MMP2	Mouse squamous cell carcinoma
3.	Liposomal	Cathepsin B	Polyoma middle T oncoprotein mouse
4.	Nanoworm	Thrombin	Mouse lung, urine analysis
5.	Nanomaterials	Caspase 3	Human bone marrow cell line
6.	Polymeric nanoparticle	MMP2	Colorectal cancer
7.	Polymeric nanoparticle	Cathepsin B	Human pancrease/colon/gastric cancer cell line
8.	Up-conversion nanoparticles	MMP2	Head and neck cancer

4 Lysophosphatidic Acid Receptor

Lysophosphatidic acid (LPA-1-acyl-2-lyso-sn-glycero-3-phosphate) is a growth factor like, the simplest glycerophospholipid abundantly present in the biological fluids. It mediates diverse cellular responses, such as stimulation of DNA synthesis, cell proliferation, differentiation, inflammation, angiogenesis, cell survival, and platelet aggregation. It is a bioactive lipid mediator that interacts with G-protein-coupled transmembrane receptors to mediate a wide range of biological actions. At least six receptors, such as LPA1-6, have been identified so far. They belong to the endothelial differentiation gene (EDG) family of receptors. The cell type decides the expression patterns of LPA receptors. While LPA1 is ubiquitously expressed, the expression levels of the other receptors vary with the physiological conditions in the tissues. Every LPA receptor exerts diverse effects in the cancer cells [32].

4.1 Recognition Domain of the LPAR

The carboxyl terminus of the LPAR is important for receptor signaling and recognition. The extreme end of the C-terminus possesses the PDZ domain, which is of canonical type and consists of 362–364 amino acids. PDZ is an acronym derived from the names of the first three proteins in which this domain was observed, namely, the postsynaptic density protein 95 (PSD-95), Drosophila disc large tumor suppressor (Dlg1), and zona occludens 1 (ZO-1). This domain plays a significant role during LPA-induced cell proliferation and activation of the Rho family of GTPases through PDZ-Rho guanine nucleotide exchange factors. The canonical PDZ domains generally consist of six β strands, namely, βA–βF, with a short αA and a long αB helix. It is a highly conserved fold with a varying secondary structure length. The N and the C termini are in close proximity to each other, on the side opposite to the peptide binding site, in a groove between the short and long α helix. Deletion of the PDZ domain prevents signal attenuation, which controls the LPAR activation and cell proliferation [33].

4.2 Cascades of Binding of the Ligands to the LPAR

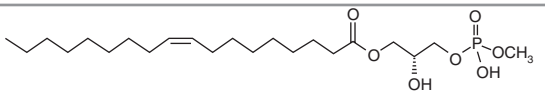
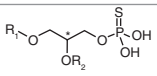
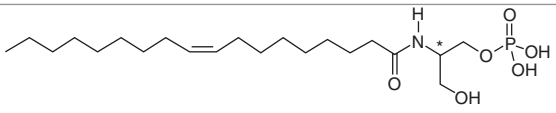
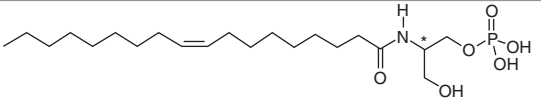
The heterotrimeric components of the LPA receptors, such as the G-proteins, trigger responses that result in diverse outcomes, depending on the context and the cell type [34]. They result in the activation of proteins involved in pathways, such as the RHO, which regulates cell migration, invasion, and cytoskeletal re-adjustments. Similar processes are also regulated by RAC (Ras-related C3 botulinum toxin substrate) activation, which occurs downstream of Gα-i/o-PI3K [35]. However, the most significant function of LPA-induced Gα-i/o is mitogenic signaling, through the

rapidly accelerated fibrosarcoma-MAPK ERK kinase-mitogen-activated protein kinase (RAF-MEK-MAPK) cascade, and survival signaling, through the phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) pathway. Gα-q/11 regulates Ca^{2+} homeostasis through phospholipase C (PLC) and secondary messengers such as diacylglycerol (DAG) and inositol triphosphate (IP3) [36]. Lastly, $G_{\alpha S}$ can increase cAMP concentration, upon LPA-mediated activation of adenylyl cyclase [37].

4.3 Structure-Activity Relationship of the Ligands for Selective Binding to the LPAR

Characterization of the three EDG families of LPARs has led to investigations to understand the structure-activity relationship (SAR) of the LPA agonists [38]. The SAR studies have not been extended to the more recently discovered receptors, which fall out of the EDG family. Table 6.4 enlists analogs that have been extensively studied for their agonistic activity toward both EDG and non-EDG families of receptors. The phosphate monomethyl ester, the closest analog to LPA [39], was tested on all the LPA receptors. It activated LPA1-5 with potencies 5–20 times lower than that of the native LPA. The ethyl and butyl esters, which were characterized for their activity toward LPA1-4, showed a poor potency. The decreased potency of the methyl ester was attributed to the steric effect arising due to the bulky methyl group, which is not present in the LPA, or the electrostatic effects that arise due to the change in the overall charge of the phosphate group. The lysophosphatidic acid moiety is negatively charged by virtue of the phosphate group attached to the acyl chain. The addition of the methyl ester group decreases the overall negative charge of LPA, thus causing electrostatic repulsive interactions with the receptor [40].

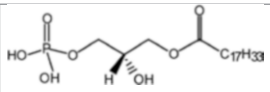
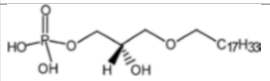
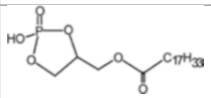
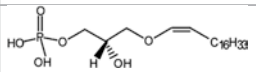
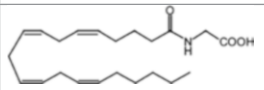
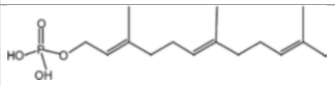
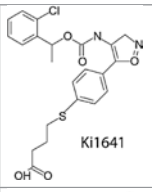
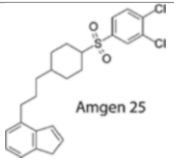
Table 6.4 Compounds studied for their agonistic activity toward the EDG and non-EDG families of LPA receptors

Sr. No.	Compound	Structure
1	LPA-methyl phosphomonoester	
2	Phosphorothioates	
3	VPC31143 ∗R	
4	VPC31144 ∗S	

4.4 Ligands Explored for the LPARs

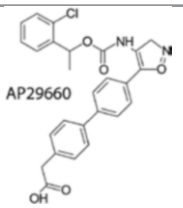
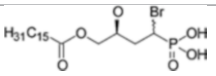
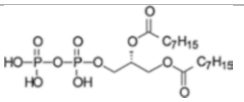
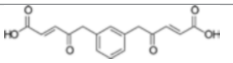
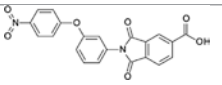
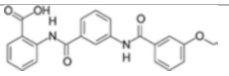
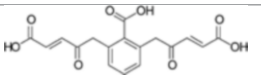
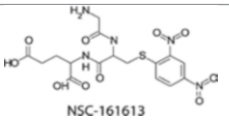
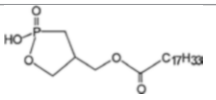
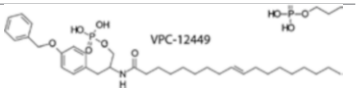
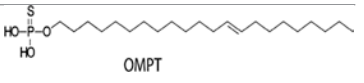
LPA and its naturally occurring analogs interact with the LPARs on the cell surface or the nuclear hormone receptor within the cell. These ligands regulate various physiological processes, such as cell survival, apoptosis, growth, differentiation, transcription, and malignant transformation, through interaction with various LPARs. Several naturally occurring ligands have been listed in Table 6.5 [41] which are analogs of LPA and are known to regulate these physiological processes. Similarly, numerous synthetic ligands have also been explored for regulating different malignancies and have been listed in Table 6.5 [41].

Table 6.5 Ligands of the LPARs

Sr. No.	Chemical name	Structure
Naturally occurring ligands		
1.	LPA 18:1, Oleoyl-lysophosphatidic acid	
2.	AGP 18:1, 1-O-octadecyl glycerophosphate	
3.	CPA 18:1, Oleoyl-cyclic phosphatidic acid	
4.	ALKENYL-GP, Alkenylglycerophosphate	
5.	NAG, N-arachidonoyl glycine	
6.	FMP, Farnesyl monophosphate	
Synthetic ligands		
7.	Ki1641	
8.	Amgen25	

(continued)

Table 6.5 (continued)

Sr. No.	Chemical name	Structure
9.	AP29660	
10.	LPA bromo-phosphonate	
11.	Diacylglycerol pyrophosphate	
12.	H2L515099	
13.	H2L5765834	
14.	H2L5987411	
15.	NSC-47091	
16.	NSC-161613	
17.	Tetradecyl-phosphonate	
18.	2Carba CPA 18:1	
19.	VPC 12449	
20.	D-*sn*-1-O-oleyl-2-O-methyl-glyceryl-3-phosphothionate OMPT	

4.5 Antagonists for the LPARs

An antagonist may be a drug or a ligand that diminishes or blocks the physiological responses by binding to or blocking a receptor, as opposed to an agonist that triggers a response. While elevated levels of LPA cause tumor progression and invasion,

Table 6.6 Antagonists selective to LPAR subtypes

Sr. No.	Receptor type	Antagonist
1	LPA1	BMS-986020, AM-966, ONO- 7300243, AM095
2	LPA2	Dodecyl phosphate, decyldihydrogen phosphate, GRI977143
3	LPA3	Dioctanoylglycerol pyrophosphate
4	LPA5	TCLPA5

they have a protective effect at low doses. The antagonists of LPAR 1 and 3 have therapeutic potential as they inhibit inflammation, neoplasms, and ischemic reperfusion injury [42]. Antagonists like VPC12249, that is specific to a particular subfamily of LPARs (LPAR 1 and 3), have exhibited a protective effect in various pathological conditions. This was discovered by Heasely et al. when a series of 2-substituted N-acyl ethanolamine phosphoric acid derivatives were synthesized and evaluated at the LPARs. VPC12249 possesses a bulky benzyl-4-oxybenzyl substitution, at the 2-position, in the linker region [43]. A series of VPC analogs were prepared and analyzed for their structural components, such as the N-acyl moiety, the outermost benzyl substituent, and their stereochemistry. The SAR indicated that the phospholipid chain length played an important role in the binding of the antagonist to its receptor. It was observed that the compounds active at the LPAR 1 contained long chain unsaturated phospholipids. Palmitoyl derivatives displayed activity at LPAR 3, while linoleoyl amide exhibited activity at the LPAR1. When the benzyl moiety of the receptor was replaced with small alkyl substituents, the inhibitory activity of the antagonists diminished. Among the various stereoisomers evaluated at the receptors, the R stereoisomers were found to be more potent than the S counterparts [42]. Antagonists that are selective to the different LPAR subtypes are enlisted in Table 6.6.

4.6 Targeting Strategies for LPA Receptors

Fells et al. reported that activation of the LPAR 2 protects the intestinal crypt cells from both radiation-induced and chemotherapy-induced apoptosis. Longer survival of irradiated mice was demonstrated in *in vivo* studies, even when the mice were treated with an agonist after irradiation. A nonlipid agonist GRI977143 was recently evaluated through animal studies. It rescued apoptotically attacked cells from high-dose γ-irradiation-induced injury, in *in vitro* and *in vivo* investigations. It exhibited features of a radiomitigator, as it was effective in rescuing mice from deadly levels of radiation. It activated ERK1/2 prosurvival pathway, effectively reduced Bax translocation to the mitochondrion, attenuated the activation of initiator and effector caspases, reduced DNA fragmentation, and inhibited PARP-1 cleavage associated with γ-irradiation-induced apoptosis. LPA agonists were therefore, considered as

promising starting points for development of lead compounds with therapeutic potential. LPAR antagonists have also been investigated as anticancer moieties. Antagonists, which are analogs of LPA and contain phosphate or cyclic thiophosphonate carbon linker regions and fatty acyl chains, have been examined in *in vivo* studies. Other antagonists, which have been tested *in vivo*, share limited resemblance with the LPA, consist of a carboxylate or a carboxylate ester group at one end, and are considered as a phosphate bioisostere. The LPARs are thus broad-spectrum targets for both chemotherapy and combination therapy with the existing anticancer drugs [44, 45].

The α-bromophosphonate analog of LPA displayed the ability to antagonize LPA1-5 and also inhibited the LPA, at a very preliminary stage, during its production by autotaxin. This compound was able to reduce the size of tumor xenografts derived from breast, colon, and nonsmall-cell lung cancer cells, relative to the untreated controls. Vascularization in 3D lung cancer xenograft model was also reduced by this compound [46, 47]. The LPA1 and LPA3 receptors were antagonized by carbocyclic phosphothionate derivative of cyclic phosphatidic acid (ccPA-phosphothionate), which also inhibited autotaxin activity, but had no impact on the LPA2 activity. Lung metastases, developed by injecting B16F10 cells in the tail veins of mice, were reduced after injecting ccPA-phosphothionate, at concentrations ranging from 50 to 100 μM. The role of LPA in the development of bone cancer pain has been probed in animals, by using LPA1 and LPA3 antagonist, VPC32183. After being injected with the cancer cells, the mice exhibited attenuation of mechanical allodynia and thermal hyperalgesia. This study involved targeting of the LPA signaling pathway for the treatment of cancer-related symptoms, as compared to the other studies that targeted the signaling pathway for eliminating the cancer itself [10, 48].

5 Frizzled Receptor

There are ten frizzled receptor (FZD) receptors, FZD1-10, which are encoded by the FZD genes [49]. The common feature of these receptors is that they all carry an N-terminal signal sequence, a highly conserved cysteine-rich domain (CRD) in extracellular region, a seven-pass transmembrane domain, and an intracellular C-terminal domain [11]. Frizzled receptors are linked to the Wnt signaling pathways to transmit signals to cells. Wnt (wingless type) proteins are cysteine-rich, secreted glycoproteins, which contain about 350 amino acid residues and are involved in Wnt signaling pathways. There are 19 different types of Wnt proteins that can bind to the cell surface complex containing the FZD receptors. Activated Wnt pathways activate several downstream transcription factors that are crucial for stem cell regulation, cell polarity, embryonic development, cell proliferation, differentiation, and tumorigenesis [11].

5.1 Recognition Domain of the Frizzled Receptors

All the FZD receptors have an amino terminal cysteine rich domain(CRD) that allows the binding of FZD receptors to Wnt proteins [50]. Signaling is initiated at the cell surface in all.

FZD receptors, wherein the CRD of the FZD receptors interacts with the secreted, lipid-modified Wnt glycoproteins. This association of the Wnt ligands with the FZD receptors takes place via two different interactions; one is a protein-fatty acyl interaction, while the other is a protein-protein interaction. In Wnt-FZD receptor interactions, the cis-unsaturated fatty acyl groups of the Wnt ligand protein is recognized by the CRD of the FZD receptors. X- ray crystallography studies by Nile et al. revealed common mechanism for fatty acyl recognition by multiple FZD receptors and suggested that binding of Wnt to the FZD receptors mediates its dimerization. The fatty acid of Wnt binds with two CRD molecules in the FZD receptors. This forms the lipid-binding groove of the FZD receptor, which adopts flexible U-shaped geometry, and accommodates the fatty acid of Wnt [51].

5.2 Mechanism of Binding of the Ligands to the FZD Receptors and Pathways Related to the FZD Receptors

The FZD receptors participate in three different Wnt signaling pathways: the planar cell polarity (PCP) pathway, the canonical Wnt β-catenin pathway, and the Wnt/calcium pathway. Among these, the PCP pathway deals with the polarity of the cells during cell development and also plays a role in cell metastasis. The canonical Wnt/β-catenin pathway is involved in the stabilization of β-catenin, after binding of the ligand to the receptor, while the noncanonical, Wnt/calcium pathway has the ability to control calcium levels in the intracellular region of the cell [52]. Among all the FZD receptors, FZD7 has been the most extensively studied in cancer research [11]. Breuhahn et al. reported the involvement of FZD7 in the Wnt/β-catenin signaling pathway that is responsible for the progression of human hepatocellular carcinogenesis. When FZD7, along withLRp5/6, were phosphorylated in the dishevelled (DSH; a family of proteins involved in Wnt signaling), it resulted in the inhibition of amino phosphorylation of serine/threonine of the cytoplasmic β-catenin, by casein kinase-1α and glycogen synthase kinase-3b (GSK-3b). Further, axin-1/axin-2 (tumor suppressor proteins) and adenomatous polyposis coli (APC) interacted with these kinases and led to the phosphorylation of β-catenin, recognition of the E3-ubiquitin ligase receptor b-TrCP, and polyubiquitination and subsequent proteasomal degradation of β-catenin. In case of tumor growth and progression, aberrant nuclear accumulation of β-catenin occurs. When β-catenin forms complexes with the LEF/TCF transcription factors and recruits chromatin-remodeling proteins, such as CBP/p300, it leads to the expression of numerous transcriptional targets (e.g., MYC), antiapoptotic proteins (e.g., survivin), invasion proteins (e.g.,

matrix metalloproteinases; MMPs), and angiogenesis factors (e.g., VEGF). Thus, activation of Wnt via the FZD receptor not only regulated tissue development and regeneration but was also observed to influence tumor growth and progression [53].

5.3 Structure-Activity Relationship of the Ligand for Selective Binding to the FZD Receptor

Wnt signaling through FZD receptors requires low-density lipoprotein receptor-related proteins 5 and 6 (Lrp5/6), as co-receptors, and receptors Ryk and Ror2. The CRD at the N-terminal region of the FZD receptor acts as the Wnt-binding domain. According to Janda et al., the crystal structure of xenopus (XWnt) protein, as a complex with FZD8 cysteine-rich domain, is a two-domain Wnt structure, which looks like a structure extended to grasp the FZD8-CRD at two different binding sites. At one binding site, the lipid group of palmitoleic acid of XWnt projects from serine187 into a deep groove in the FZD8-CRD binding domain. At the second binding site, conserved residues present on the tip of Wnt connect with the hydrophobic amino acids of XWnt, forming a depression on the opposite side of the FZD8-CRD domain. The ligand-receptor cross-reactivity of Wnt-FZD is accelerated by the preservation of amino acids at both the interfaces of Wnt. Understanding the interaction of Wnt with the FZD receptor is important for understanding the functional pleiotropy of the former and for the development of anticancer new molecules. The conserved amino acid sequence of Wnt, present at the opposite end of the FZD-CRD binding region, is a site where low-density lipoprotein receptor 5/6 (LRP5/6) binds with Wnt. This interaction enables bridging of Wnt with the FZD to form a ternary complex [54].

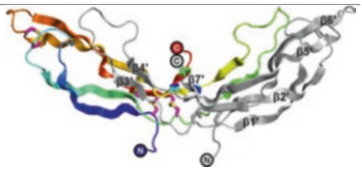
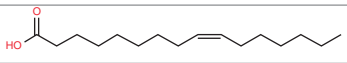
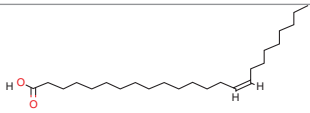
5.4 Antagonists for the FZD Receptors

FZD is an important target for regulating upstream Wnt signaling. Wnt signaling is initiated when the Wnt glycoproteins interact with the CRD domain of the FZD receptor, as well as with the extracellular domains of the LPR5/6, to form Wnt/FZD/LRP complex. Based on the structural information about FZD-Wnt binding, different compounds have been selected as antagonists for their ability to inhibit Wnt signaling. Table 6.7 lists the antagonists which are linked to the FZD receptors [55]. Monoclonal antibody OMP-18R5 was developed by Gurney et al. This antibody could bind to the conserved epitope of the extracellular domains of FZDs 1, 2, 5, 7, and 8 and thereby prevented binding of the Wnt ligands to the FZD receptors. This consequently inactivated the canonical Wnt pathway. It was observed that treatment with OMP-18R5 resulted in reduced growth of human tumor xenograft in mice and in several types of human cancers. Soluble (sFZD7) antagonist was developed by Wei et al. using extracellular peptide of FZD7. sFZD7 acted as a decoy receptor, which was assumed to bind to the Wnt ligands, hindering the binding of Wnt ligands to FZD7 and thereby inhibiting Wnt/β-catenin signaling in hepatocellular carcinoma.

Table 6.7 Antagonists for binding of Wnt to the FZD receptors

Sr. No.	Receptor	Antagonists
1.	FZD1	OMP-18R5
2.	FZD2	OMP-18R5
3.	FZD5	OMP-18R5
4.	FZD7	OMP-18R5
5.	FZD8	OMP-18R5
6.	FZD10	OMP-18R5
7.	FZD7	sFZD7

Table 6.8 Ligands for the frizzled receptors

Sr. No.	Chemical name	Structure
Natural ligands		
1.	Norrin (protein ligand)	
2.	Palmetoleic acid (PAM) CH3(CH2)5CH═CH(CH 2)7COOH	
3.	Nervonic acid C24H46O2	
4.	Toxin B (protein ligand)	
Synthetic ligands		
5.	B12-DKK1	Peptide ligand
6.	FZD-Lrp5/6	Peptide ligand

5.5 *Ligand Explored with the FZD Receptors*

5.5.1 Natural Ligands for the FZD Receptor

Besides Wnts, norrin, a cysteine-knot protein, encoded by the NDP gene, also acts as ligand for the FZD receptors. Mutations in the NDP gene cause Norrie disease, which is characterized by hypovascularization of the retina and a severe loss of visual function. The FZD receptors can also bind to *Clostridium difficile* toxin B (TcdB). This toxin is a critical virulence factor in causing diseases after infection by *Clostridium difficile*. TcdB can bind to the CRD of several FZD family members with varying affinities. The TcdB can compete with Wnt for binding to the FZD receptors and thus block the Wnt signaling [56]. The natural ligands for FZD receptors have been listed in Table 6.8 [57–60].

5.5.2 Synthetic Ligands for the FZD Receptor

Janda et al. developed a surrogate Wnt agonist, which is a water-soluble FZD-Lrp5/6 heterodimerizer. B12-DKK1 is another protein ligands screened by yeast. It binds to CRD of FZD receptors via electrostatic interaction. The synthetic ligands are enlisted in Table 6.8 [58, 61].

5.6 Strategies to Target the FZD Receptors

It was discovered in 1991 that mutations in the tumor suppressor adenomatous polyposis coli (APC) were related to majority of sporadic colorectal cancers, as these mutations caused uncontrolled activation of Wnt signaling. Thus, Wnt signaling was targeted to treat various types of cancers. Loss of function in both the alleles of APC is the main cause of tumorigenesis [62]. Cysteine palmitoylation of Wnt glycoprotein by porcupine (PORC) is required in Wnt ligand secretion and pathway activation in Wnt/β catenin pathway. In the absence of Wnt ligands, β-catenin levels are controlled by the destruction complexes containing APC and axis inhibition protein (XIN). This situation is called "off state," where β-catenin is maintained at low levels in the cytoplasm and in the nucleus. In the "on" state, the Wnt ligand binds to the extracellular CRD of the FZD receptor and the LRP5/6. The destruction complex, along with the activated dishevelled (DSH), is transferred to the plasma membrane. Phosphorylation of LRP5/6 by GSK-3 and CK1 causes binding and degradation of AXIN, at the cytoplasmic tail of LRP5/6. Due to degradation of AXIN, GSK-3 is unable to phosphorylate β-catenin. These events cause accumulation of β-catenin in the nucleus and activation of the downstream targets [63]. Therefore, the method of blocking the upstream Wnt signals, by preventing the binding of ligands to the receptors or targeting the FZDs receptors, is a promising strategy for the treatment of cancers [11]. It was found that Wnt signaling can be inhibited when secreted frizzled-related protein (SFRP) binds to the Wnt ligands, due to their sequence homology to the CRD domain of the FZD receptors [63]. Tumor suppression in melanoma, sarcoma, colorectal cancers, nonsmall-cell lung carcinoma, and mesothelioma was observed, when monoclonal antibodies developed against Wnt-1 and Wnt-2 were employed to inhibit Wnt signaling. OMP-18R5 (Vantictumab), a monoclonal antibody has been reported to target 5 of the 10 FZD receptors. A novel recombinant fusion protein, OMP-54F28, binds to the Wnt ligands and blocks the Wnt signaling through its domain on the extracellular part of the human FZD receptor 8 [64].

5.7 Nanocarrier-Mediated Receptor Targeting

Antibody-based therapies are invaluable tools to study as well as to treat malignancies, but their application is limited by their high production costs and high dosages. These factors render these treatment modalities expensive and risky. Conjugation

can make antibodies multivalent. Many researchers have demonstrated that these multivalent antibodies have increased affinity for their targets, as compared to the native antibodies. Riley et al. have shown that FZD7 antibody-nanoshell conjugates (FZD7-NS) are significantly more effective in inhibiting the Wnt signaling in triple-negative breast cancer cells, as compared to the FZD7 antibodies. Additionally, researchers found that the cells treated with FZD7-NS exhibited decreased viability, as compared to the cells treated with the native antibodies. These results indicated that antibody-functionalized nanoparticles allowed lower doses of antibodies, for research as well as for the treatment of malignancies [65].

6 Clinical Developments in GPCR Targeting

GPCRs are the most intensively studied drug targets, particularly due to their substantial involvement in human pathophysiology. There are currently 475 drugs (~34% of all drugs approved by the FDA) that act on 108 unique GPCR targets. Approximately, 321 agents are currently in clinical trials, of which about 20% target the 66 potentially novel GPCR targets that are not currently approved. The 224 (56%) non-olfactory GPCRs that are yet to be explored in clinical trials have a broad therapeutic potential, specifically in genetic and immune system disorders. Drugs that target the GPCRs account for approximately 27% of the global market share of therapeutic drugs. They constitute the largest human membrane protein family comprising about 800 members. Modulation of GPCRs via allosteric sites, distinct from the binding sites for endogenous ligands, can alter their structure, dynamics, and function of the receptor, to achieve a potential therapeutic advantage, such as increased spatial and temporal selectivity.

The U.S. National Library of Medicine enlists a few clinical studies which involve targeting of GPCRs. The first study involves investigation of the expression of cannabinoid receptors in mucosal biopsies of the colon cancer, by comparing with healthy individuals using polymerase chain reaction, western blot, and flow cytometry. The purpose of this study was to investigate the phospholipid ligands and their receptors involved in colon cancer. Colonic endoscopic biopsies and blood samples were collected from colon cancer patients and healthy individuals. Blood samples were immediately processed for flow cytometric measurement. Mass spectrometry was used to measure the phospholipid content in the serum and colonic mucosa biopsy samples. The lipids and their responsive receptors were used to build a pathway, which was potentially involved in the development of inflammation and cancer. The study type was observational, based on a cohort-based model. The outcome of this study provided information of the cannabinoid receptors and the assessment of malignancy in colon cancer [66].

Another study involved small molecule, ONC201, targeting the GPCR dopamine receptor 2 (DRD2). Integrated stress response was activated by ONC201, downstream of the target engagement in tumor cells, leading to the inactivation of Akt and the extracellular signal regulated kinase (ERK) signaling and induction of the TRAIL pathway. It also activated the DRD2 involved in antitumor responses.

Treatment with ONC201 was well tolerated in Phase I studies involving patients with advanced malignancies, due to its differential antiproliferative and proapoptotic response in tumor cells. Thus, the recommended dose of ONC201 in Phase II trials was set at 625 mg, every 3 weeks. Additional dose escalation study in Phase II is being evaluated for weekly dosage, instead of the 3 weekly regimen in patients with advanced tumors and multiple myeloma. However, the Phase I data suggests a possible clinical benefit in patients with advanced chemorefractory endometrial cancers. The study is now in Phase II trial [66].

7 Conclusion

GPCRs play a very significant role in a myriad of pathophysiological functions, including various cellular and physiological processes and diseased conditions, such as cancer, as discussed in this chapter. Despite the clinical significance, very few anticancer compounds directed toward GPCRs are currently in market. The evidences of aberrant GPCR activity have been demonstrated in various diseased conditions. The ligand-GPCR interaction, which results in the desired effect, is complex and indubitably links ligand molecules to the development and progression of cancer. This evidently makes GPCRs and their downstream effectors as a promising target for the development of innovative strategies to tackle cancer and tumor development. The research in the area of GPCR targeting is gaining impetus, where investigators have deciphered the activity of various ligands and GPCRs in different types of cancers. This chapter focuses on a few representative GPCRs with respect to their structure-activity relationship, ligand interaction and pathway, drug development, delivery strategies, and clinical trials. The current developments and clinical trials show enormous promise and unprecedented opportunities for modulators of GPCRs in cancer therapy. Constant efforts in the area of understanding GPCR driven cancer progression would highlight their potential as oncology drug targets and place them at the forefront of cancer drug development.

References

1. Insel PA, Sriram K, Wiley SZ, French RP, Lowy AM. GPCRs as novel potential therapeutic targets in cancer. FASEB J. 2017;31(1_supplement):671.10.
2. Zalewska M, Siara M, Sajewicz W. G protein-coupled receptors: abnormalities in signal transmission, disease states and pharmacotherapy. Acta Pol Pharm. 2014;71(2):229–43.
3. Hauser AS, Chavali S, Masuho I, Jahn LJ, Martemyanov KA, Gloriam DE, et al. Pharmacogenomics of GPCR drug targets. Cell. 2018;172(1–2):41–54. e19.
4. Pierce KL, Premont RT, Lefkowitz RJ. Signalling: seven-transmembrane receptors. Nat Rev Mol Cell Biol. 2002;3(9):639.
5. DeFea K, Vaughn Z, O'bryan E, Nishijima D, Dery O, Bunnett N. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex. Proc Natl Acad Sci. 2000;97(20):11086–91.

6. Bar-Shavit R, Maoz M, Kancharla A, Nag J, Agranovich D, Grisaru-Granovsky S, et al. G protein-coupled receptors in cancer. Int J Mol Sci. 2016;17(8):1320.
7. Déry O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Phys Cell Phys. 1998;274(6):C1429–C52.
8. Wojtukiewicz MZ, Hempel D, Sierko E, Tucker SC, Honn KV. Protease-activated receptors (PARs)—biology and role in cancer invasion and metastasis. Cancer Metastasis Rev. 2015;34(4):775–96.
9. Lee S-C, Fujiwara Y, Tigyi GJ. Uncovering unique roles of LPA receptors in the tumor microenvironment. Receptors Clin Investig. 2015;2(1).
10. Altman MK, Gopal V, Jia W, Yu S, Hall H, Mills GB, et al. Targeting melanoma growth and viability reveals dualistic functionality of the phosphonothionate analogue of carba cyclic phosphatidic acid. Mol Cancer. 2010;9(1):140.
11. Zeng C-M, Chen Z, Fu L. Frizzled receptors as potential therapeutic targets in human cancers. Int J Mol Sci. 2018;19(5):1543.
12. Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, et al. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci. 2001;98(24):13790–5.
13. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev. 2001;53(2):245–82.
14. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000; 407(6801):258.
15. Gieseler F, Ungefroren H, Settmacher U, Hollenberg MD, Kaufmann R. Proteinase-activated receptors (PARs)–focus on receptor-receptor-interactions and their physiological and pathophysiological impact. Cell Commun Signal. 2013;11(1):86.
16. Lerner DJ, Chen M, Tram T, Coughlin SR. Agonist recognition by proteinase-activated receptor 2 and thrombin receptor Importance of extracellular loop interactions for receptor function. J Biol Chem. 1996;271(24):13943–7.
17. Nguyen C, Coelho A-M, Grady E, Compton SJ, Wallace JL, Hollenberg MD, et al. Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. Can J Physiol Pharmacol. 2003;81(9):920–7.
18. Wong SK-F. G protein selectivity is regulated by multiple intracellular regions of GPCRs. Neurosignals. 2003;12(1):1–12.
19. Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, et al. High-resolution crystal structure of human protease-activated receptor 1. Nature. 2012;492(7429):387.
20. Wu CC, Huang SW, Hwang TL, Kuo SC, Lee FY, Teng CM. YD-3, a novel inhibitor of protease-induced platelet activation. Br J Pharmacol. 2000;130(6):1289–96.
21. Hamilton JR, Trejo J. Challenges and opportunities in protease-activated receptor drug development. Annu Rev Pharmacol Toxicol. 2017;57:349–73.
22. Flaumenhaft R, De Ceunynck K. Targeting PAR1: now what? Trends Pharmacol Sci. 2017;38(8):701–16.
23. Austin KM, Covic L, Kuliopulos A. Matrix metalloproteases and PAR1 activation. Blood. 2013;121(3):431–9.
24. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science. 2002;296(5574):1880–2.
25. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front Endocrinol. 2014;5:67.
26. Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1–phosphate receptor-1 cross activation. Blood. 2005;105(8):3178–84.
27. Soh UJ, Trejo J. Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds. Proc Natl Acad Sci. 2011;108(50):E1372–E80.
28. Weidle UH, Tiefenthaler G, Georges G. Proteases as activators for cytotoxic prodrugs in antitumor therapy. Cancer Genomics Proteomics. 2014;11(2):67–79.

29. Choi KY, Swierczewska M, Lee S, Chen X. Protease-activated drug development. Theranostics. 2012;2(2):156.
30. Chan Y-C, Chen C-W, Chan M-H, Chang Y-C, Chang W-M, Chi L-H, et al. MMP2-sensing up-conversion nanoparticle for fluorescence biosensing in head and neck cancer cells. Biosens Bioelectron. 2016;80:131–9.
31. Chan Y-C, Hsiao M. Protease-activated nanomaterials for targeted cancer theranostics. Nanomedicine. 2017;12(18):2153–9.
32. Yung YC, Stoddard NC, Chun J. LPA receptor signaling: pharmacology, physiology, and pathophysiology. J Lipid Res. 2014;55(7):1192–214.
33. Murph MM, Radhakrishna H. LPAR1 (lysophosphatidic acid receptor 1). Atlas of Genetics and Cytogenetics in Oncology and Haematology. 2010.
34. Fukushima N, Ishii S, Tsujiuchi T, Kagawa N, Katoh K. Comparative analyses of lysophosphatidic acid receptor-mediated signaling. Cell Mol Life Sci. 2015;72(12):2377–94.
35. Xiang SY, Dusaban SS, Brown JH. Lysophospholipid receptor activation of RhoA and lipid signaling pathways. Biochim Biophys Acta (BBA) Mol Cell Biol Lipids. 2013;1831(1):213–22.
36. Hildebrandt J-P. Lysophosphatidic acid induces inositol phosphate and calcium signals in exocrine cells from the avian nasal salt gland. J Membr Biol. 1995;144(1):49–58.
37. Fang X, Yu S, LaPushin R, Lu Y, Furui T, Penn LZ, et al. Lysophosphatidic acid prevents apoptosis in fibroblasts via G (i)-protein-mediated activation of mitogen-activated protein kinase. Biochem J. 2000;352(Pt 1):135.
38. Parrill AL, Tigyi G. Integrating the puzzle pieces: the current atomistic picture of phospholipid–G protein coupled receptor interactions. Biochim Biophys Acta (BBA) Mol Cell Biol Lipids. 2013;1831(1):2–12.
39. Endo T, Kano K, Motoki R, Hama K, Okudaira S, Ishida M, et al. Lysophosphatidylmethanol is a pan lysophosphatidic acid receptor agonist and is produced by autotaxin in blood. J Biochem. 2009;146(2):283–93.
40. Parrill AL. Design of anticancer lysophosphatidic acid agonists and antagonists. Future Med Chem. 2014;6(8):871–83.
41. Tigyi G. Aiming drug discovery at lysophosphatidic acid targets. Br J Pharmacol. 2010;161(2):241–70.
42. Lynch KR, Macdonald TL. Structure–activity relationships of lysophosphatidic acid analogs. Biochim Biophys Acta (BBA) Mol Cell Biol Lipids. 2002;1582(1–3):289–94.
43. Tokumura A, Kume T, Fukuzawa K, Tsukatani H. Cardiovascular effects of lysophosphatidic acid and its structural analogs in rats. J Pharmacol Exp Ther. 1981;219(1):219–24.
44. Kiss GN, Fells JI, Gupte R, Lee S-C, Liu J, Nusser N, et al. Virtual screening for LPA2-specific agonists identifies a nonlipid compound with antiapoptotic actions. Mol Pharmacol. 2012;82(6):1162–73.
45. Kiss GN, Lee S-C, Fells JI, Liu J, Valentine WJ, Fujiwara Y, et al. Mitigation of radiation injury by selective stimulation of the LPA2 receptor. Biochim Biophys Acta (BBA) Mol Cell Biol Lipids. 2013;1831(1):117–25.
46. Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, et al. Dual activity lysophosphatidic acid receptor pan-antagonist/autotaxin inhibitor reduces breast cancer cell migration in vitro and causes tumor regression in vivo. Cancer Res. 2009;69(13):5441–9.
47. Xu X, Prestwich GD. Inhibition of tumor growth and angiogenesis by a lysophosphatidic acid antagonist in an engineered three-dimensional lung cancer xenograft model. Cancer. 2010;116(7):1739–50.
48. Xu Y, Jiang G, Tsukahara R, Fujiwara Y, Tigyi G, Prestwich GD. Phosphonothioate and fluoromethylene phosphonate analogues of cyclic phosphatidic acid: novel antagonists of lysophosphatidic acid receptors. J Med Chem. 2006;49(17):5309–15.
49. Huang H-C, Klein PS. The Frizzled family: receptors for multiple signal transduction pathways. Genome Biol. 2004;5(7):234.
50. http://atlasgeneticsoncology.org/Genes/FZD5ID47614ch2q33.html

51. Nile AH, Mukund S, Stanger K, Wang W, Hannoush RN. Unsaturated fatty acyl recognition by Frizzled receptors mediates dimerization upon Wnt ligand binding. Proc Natl Acad Sci. 2017;114(16):4147–52.
52. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017;36(11):1461.
53. Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene. 2006;25(27):3787.
54. Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC. Structural basis of Wnt recognition by Frizzled. Science. 2012;337(6090):59–64.
55. Lee H-J, Bao J, Miller A, Zhang C, Wu J, Baday YC, et al. Structure-based discovery of novel small molecule Wnt signaling inhibitors by targeting the cysteine-rich domain of frizzled. J Biol Chem. 2015;290(51):30596–606.
56. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell. 2017;169(6):985–99.
57. Zhang X, Dong S, Xu F. Structural and druggability landscape of Frizzled G protein-coupled receptors. Trends Biochem Sci. 2018;43:1033.
58. Chang T-H, Hsieh F-L, Zebisch M, Harlos K, Elegheert J, Jones EY. Structure and functional properties of Norrin mimic Wnt for signalling with Frizzled4, Lrp5/6, and proteoglycan. elife. 2015;4:e06554.
59. ChemSpider.
60. PubChem.
61. Janda CY, Dang LT, You C, Chang J, de Lau W, Zhong ZA, et al. Surrogate Wnt agonists that phenocopy canonical Wnt and β-catenin signalling. Nature. 2017;545(7653):234.
62. Kim Y-M, Kahn M. The role of the Wnt signaling pathway in cancer stem cells: prospects for drug development. Res Rep Biochem. 2014;4:1.
63. Le PN, McDermott JD, Jimeno A. Targeting the Wnt pathway in human cancers: therapeutic targeting with a focus on OMP-54F28. Pharmacol Ther. 2015;146:1–11.
64. Krishnamurthy N, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: update on effectors and inhibitors. Cancer Treat Rev. 2018;62:50–60.
65. Riley RS, Day ES. Frizzled7 antibody-functionalized nanoshells enable multivalent binding for Wnt signaling inhibition in triple negative breast cancer cells. Small. 2017;13(26):1700544.
66. clinicaltrials.gov. U.S. National Library of Medicine. NIH.

Chapter 7
Receptors for Targeting Growth Factors for Treatment of Cancers

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Abstract Growth factor receptors (GFR) are expressed on cell membranes or in the cytoplasm and play a major role in cell growth, survival, angiogenesis, and metastasis. Tumor growth and cell survival are composed of dodging apoptotic signals in cancer cells. The growth of cells is further supported by angiogenesis and metastasis to distant organs. Elevated expression of growth factor receptors contributes to the development of drug resistance. Therefore, therapeutics to target GFRs is a potentially attractive molecular approach to treat cancer more effectively. In this review, we have discussed the contribution of growth factor receptors to cancer development and thereby their subsequent molecular targets for novel drugs developed leading to inhibition of growth factor receptor-mediated pathways.

Keywords Receptor-ligand interaction · Recognition domain · Extracellular domain · Transformation · Drug target

Abbreviations

BMP	Bone morphogenetic protein
CDK	Cell cycle-regulated kinases
CRC	Colorectal cancer
DOX	Doxorubicin

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ECD	Extracellular domain
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
FST	Follistatin
GAB	Grb2-associated binding protein
GAS	Growth arrest specific protein
GBM	Glioblastoma multiforme
GH	Growth hormone
GMP	Gemcitabine monophosphate
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
ILGF	Insulin-like growth factor
IONP	Iron oxide nanoparticles
IPT	Immunoglobulin-like plexin-transcription
IR	Insulin receptor
JMD	Juxtamembrane domains
JNK	Jun N-terminal kinase
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSN	Mesoporous silica nanoparticles
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
NFkβ	Nuclear factor kappa-light-chain-Enhancer of activated β cells
NMOF	Amino-triphenyl dicarboxylate-bridged Zr4+ metal-organic framework nanoparticles
PDGF	Platelet-derived growth factor
PEI	Polyethylenimine
PI3k	Phospho-inositol 3 kinase
PlGF	Placental growth factor
PSI	Plexin-semaphorin-integrin
PTK	Protein tyrosine kinase
RSK2	Ribosomal protein S6 kinase 2
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SEMA	Structural domain of semaphorins
SH2	Src-homology-2 domain
SHC	Src-homology-2 domain
SPARC	Secreted protein acidic and rich in cysteine
SPIO	Superparamagnetic iron oxide
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TMD	Transmembrane domain
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

1 Introduction

The dynamics of cell growth and commitment to a specific lineage is generally governed by the growth factors [1]. As the name suggests, these factors are responsible for determining the fate of the cells, with regard to their division and differentiation. Apart from differentiation, these proteins also have a crucial impact on normal cellular processes, their transformation, regulation, as well as the programmed cell death (apoptosis). Imbalance and overexpression of growth factors can thus modulate a normally dividing cell into an unconditionally dividing cancerous cell, causing further dysfunction in the human body. Such cellular transformations occur due to transcriptional upregulation or via ligand overproduction and signaling through autocrine or paracrine model. These changes impact morphological and mechanical attributes, such as the membrane strength of the cells to form junctions and altered cytoskeletal arrangements, thereby affecting their motility [2]. The transformation of a healthy cell into a highly malignant cell occurs due to the genetic changes caused by the intracellular and extracellular factors, which further leads to invasion and metastasis. The root cause of such transformations can be traced back to the loss of tumor suppressor genes and the gain of oncogenic genes that synthesize oncogenic proteins, in an un-regulated manner [3]. Eradication of cancer cells has been conducted using chemotherapy and radiation therapy, both of which are associated with numerous side effects, especially due to their nonspecificity. The targeted therapy focuses on directing the therapeutics to specific sites or molecules on the cell membrane, thereby inhibiting cellular proliferation. While the traditional therapies generally exert cytotoxic effects, the targeted therapies are predominantly cytostatic (inhibiting the cell proliferation), which provides them with greater specificity, by overcoming resistance toward cytokines and growth factors, and safety [4]. These specific therapies halt cellular proliferation by affecting particular signaling cascades involved in this process and targeting drugs toward receptors to block the downstream signaling pathways and leading to collapse in the growth cone [5].

In this chapter, we have described various cancers that arise due to alteration in normal cell signaling pathways. Further, the downstream signaling molecular targets that are hampered due to this mutational change have also been elaborately discussed. Further, this manuscript also focuses on the predominant targets of specific growth factor receptors that exhibit the potential to form transformed cells that exhibit uncontrolled proliferation, invasion, and metastasis. Finally, the discussion also emphasizes on the structure and mediation of the signaling pathways and their interaction with various natural and synthetic ligand molecules that may present a significant therapeutic role. The structure and downstream signaling pathways of receptors have been depicted in Figs. 7.1 and 7.2. The list of endogenous ligands and malignancies associated with misfunction of these receptors are stated in Table 7.1.

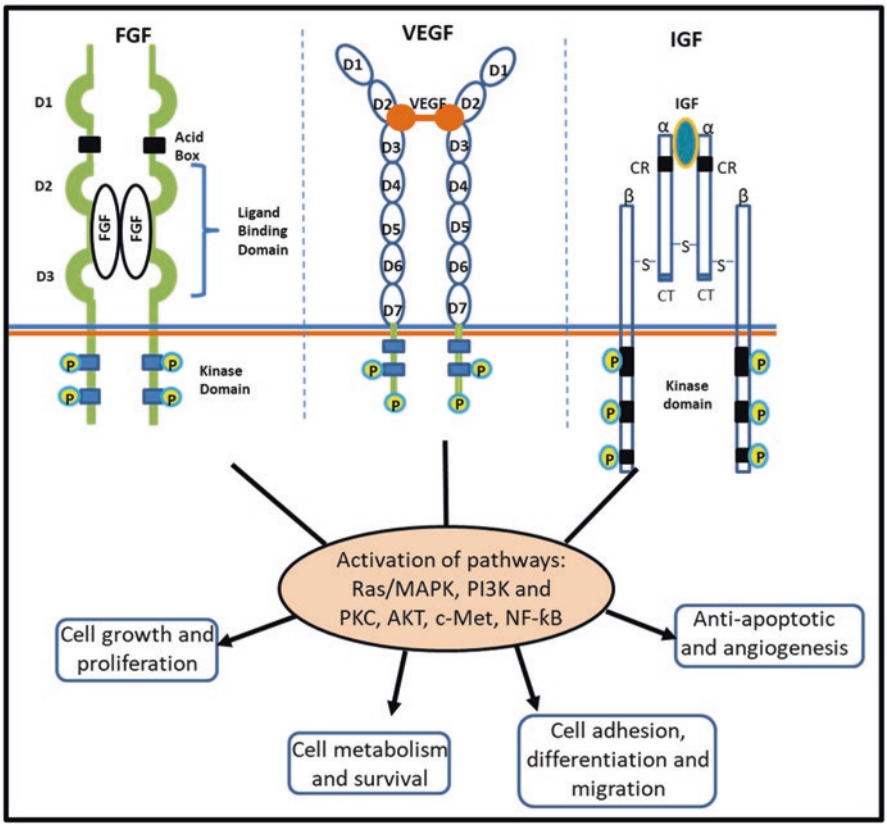

Fig. 7.1 Pictorial depiction of the receptor structure of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF). Ligand-binding domains and phosphorylation sites have been shown encompassing the downstream activation signal for various cellular processes. Constitutively, active pathways can lead to malignancies and various deleterious effects

2 Hepatocyte Growth Factor Receptor (HGF)

Hepatocyte growth factor, also known as scatter factor (SF), by is released the mesenchymal cells, like fibroblasts and some smooth muscle cells. HGF secretion activates Met protein signaling in a paracrine fashion. The receptor responsible for triggering the response mediated by HGF is the c-Met tyrosine kinase receptor, a single-pass heterodimer transmembrane receptor. Overexpression of HGF and its binding to the receptor has been reported to lead to oncogenesis and tumor propagation, thereby causing liver cancer, colorectal cancer, gastric cancers, and various other solid tumor malignancies. The receptor also accounts for cellular processes like mitogenesis, morphogenesis, survival, and motility in various cell types like the endothelial cells, neurons, epithelial cells, hematopoietic cells, and the hepatocytes [16, 17].

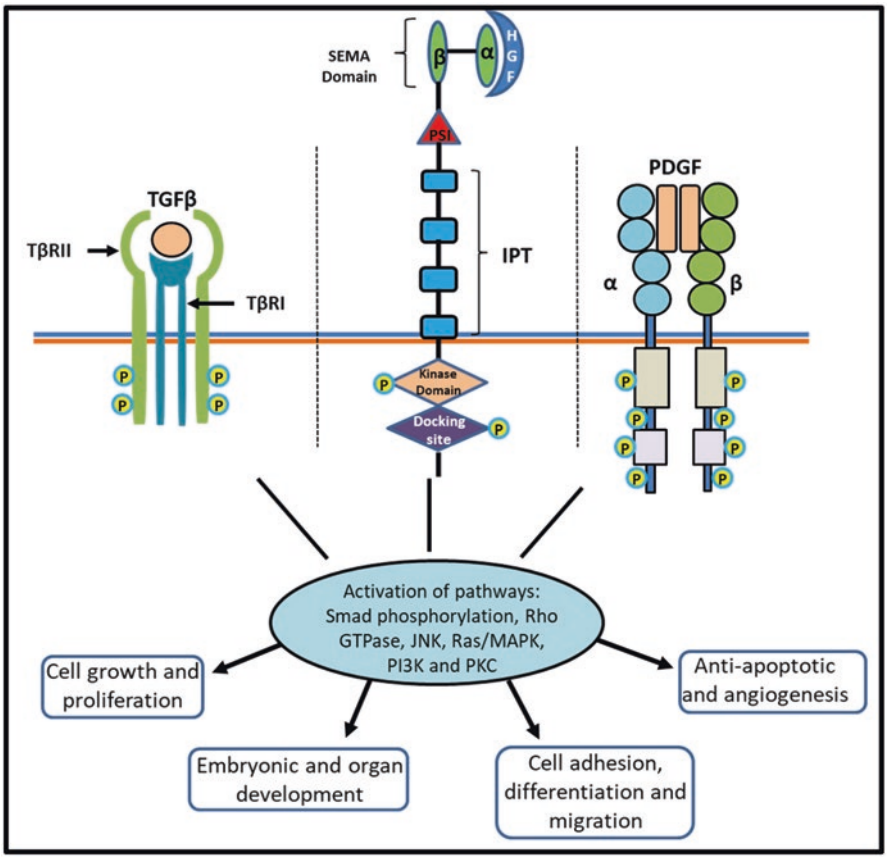

Fig. 7.2 Depiction of the receptor structure of transforming growth factor-B (TGFβ), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDFR). Ligand-binding domains and phosphorylation sites have been shown encompassing the downstream activation signal for various cellular processes. Constitutively, active pathways can lead to malignancies and various deleterious effects

2.1 Recognition Domain of HGFR

Ligand delivery in case of the receptor Met tyrosine kinase (TK) is controlled in a paracrine manner, allowing activation of the binding site and leading to receptor internalization and degradation. Receptor c-Met is transcribed from chromosome 7 into a fully functional 120 kb in length with molecular weight of 190 kDa (50 kDa α chain and a 140 kDa β chain connected together by a disulfide bond). The extracellular region of c-Met contains SEMA domain (structural domain of semaphorins) that directly binds to the ligand, a PSI domain (plexin- semaphorin-integrin) and four immunoglobulin-like plexin-transcription (IPT) domains [18].

Table 7.1 List of growth factors and their respective receptors, physiological functions, along with the endogenous ligands which trigger their intracellular signaling for causing various cancer types

Growth factor	Growth factor receptors	Endogenous ligands	Antagonist		Associated cancer
			Natural	Synthetic	
Hepatocyte growth factor receptor	c-MET receptor	Hepatocyte growth factor (HGF), scatter factor (SF)	NK2, NK3, NK4, SR 144782	MetMab, m224G11, hz224G11, ABT-700, AMG-337 [6]	Neuroblastoma, glioblastomas, osteosarcomas, brain cancer [7] Oesophageal, gastric, colorectal cancer [8], multiple myelomas, and T-cell leukemia [9]
Insulin-like growth factor – 1	IGF2R, IGF2R	Insulin IGF-I (Somatomedin C), IGF-II	GH, Somavert (R), pegvisomant	Linsitinib	Breast, melanoma, squamous cell carcinoma of lung [10]
Platelet-derived GF	PDGFRα, PDGFRβ	PDGF-A, PDGF-B, PDGF-C, PDGF -D	GAS1, SPARC, BM40, osteonectin	Imatinib, sorafenib, dasatinib, sunitinib, neutralizing PDGFR antibodies, GFB-111	Gastrointestinal tumors, chronic eosinophilic leukemia, prostate cancer, nonsmall-cell lung cancer [11]
Transforming growth factor-β	TGF-β1, TGF-β2, TGF-β3	Bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-Mullerian hormone (AMH), activin, nodal, and TGF-β, TGF-β includes - TGF-β1, TGF-β2, TGF-β3	FST, noggin, chordin, BMP-3	Galunisertib	Colon, gastric, neck, and pancreatic cancer [12]
Vascular endothelial growth factor	VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt-4), Neuropilin1, Neuropilin-2 (nonenzymatic Receptors)	VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor, thrombospondins (TSPs)	sFlt-1, AUF1-RGG peptides	Axitinib, cabozantinib, lenvatinib, sorafenib, sunitinib, pazopanib, bevacizumab, nivolumab	Bladder, brain, breast [13], colon, gastric, lung, ovarian, prostate cancer
Fibroblast growth factors	FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, FGFR4	FGF1 (acidic FGF), FGF-2 (basic FGF), FGF-6, FGF-8	FGF23	Dovitinib (TKI258), AZD4547, Ki23057, E7080, brivanib alaninate, nintedanib, ponatinib, MK-2461, and E-3810 [14]	Chronic myeloid leukemia (CML) [15], metastatic prostate cancer, breast cancer, glioblastoma [15]

2.2 Binding of Ligand with HGFR

The receptor binding primarily includes the N-terminal domain of a 69-kDa HGF ligand comprising of α chain. The N-terminal of the ligand contains a hairpin loop, comprising α and β sheets, and has two disulfide linkages that are responsible for proper conformation of the protein. The innermost residues of the ligand, Cys74–Cys84, constitute the disulfide linkage, connecting the C-terminal of the α helix and the N-terminal of the β sheet. On the other hand, the outermost disulfide linkage includes Cys70–Cys96 and joins the middle regions of the α helices and β strands [7]. The binding of the ligand to the receptor is not directly dependent on the N-terminal α sheet (amino acids 25–307) and β sheet (amino acids 308–519). The cysteine-rich domain, comprising amino acids 520–561 along with the C-terminal residues 562–932 (containing four Ig domains), participates in the proper binding of the ligand. The ligand-binding domain of c-Met consists of β-propeller fold. The amino terminal in the juxtamembrane (JM) region encompasses two protein phosphorylation sites, viz., S985 and Y1003. Phosphorylation of S985 negatively regulates the kinase activity [19, 20], while phosphorylation of Y1003 recruits c-Cbl, a ubiquitin protein ligase, which ubiquinates Met, thereby resulting in its internalization and degradation [20, 21].

2.3 Structure-Activity Relationship of Selective Binding of HGF with HGFR

The HGF contains mainly immunoglobulin-like domains. Here, two c-Met heterodimers dimerize, leading to the consecutive autophosphorylation of two tyrosine residues, one within the catalytic loop (Tyr1234–Tyr1235) and in the C-terminal domain (Tyr1349–Tyr1356), thereby providing a docking site for the recruitment of other downstream molecular interactions. A sequence consisting of 13 amino acids, in the binding region, mediates the interaction between Gab1 and c-Met, thus creating a docking platform [22]. This induces morphological changes in the receptor's intracellular protein tyrosine kinase (PTK) domain and thereby activating the receptor. The Grb2-associated binding protein 1 (Gab1) triggers autophosphorylation and provides binding platform for Src-homology-2 domain (SH2)-containing effectors, like the SH2-transforming protein (SHC), the phosphoinositide 3 kinase (PI3K), the SH2-domain-containing protein tyrosine phosphatase (SHP2), the phospholipase Cγ1 (PLCγ1), the signal transducer and activator of transcription 3 (STAT3), and the Ras GTPase p120 [23, 24]. Ras thereby activates Raf, MEK, MAPKs, ERK, JNK (Jun N-terminal kinase), and p38 (HOG). Thereafter, the activated MAPKs enter the nuclei to activate transcription factors Elk1, Etsl, and c-Myc, through further phosphorylation. Abnormal signal transduction, in turn, interferes with the cell cycle and induces cell transformation, consequently promoting carcinogenesis. MAPKs are known to induce degradation of proteins and matrix, thereby promoting cell migration and proliferation of solid tumors.

2.4 Strategies to Target HGFR

The current therapies directed against Met involve the use of kinase inhibitors, ligand- or dimerization-blocking antibodies that inhibit the tumor growth and eliminate the tumor. Thus, in order to improve anti-Met therapy, cross-linked albumin nanoparticles have been linked with anti-HGFR or Met nanobodies (anti-Met-NANAPs). In vitro studies indicated that these lysosome-targeted nanoparticles were designed to bind the cells expressing Met and were further internalized, resulting in lysosomal degradation and hence downregulation of the Met protein [25, 26]. Superparamagnetic iron oxide (SPIO) nanoparticles were combined with polyethylenimine (PEI) to form cationic complexes and bind with c-Met siRNA, *forming* nanoparticles. Galactose (Gal)-modified magnetic nanoparticles were used to target the asialoglycoprotein receptors. SPIOs modified with PEI and Gal were found to protect c-Met siRNA and mediate its cellular uptake and thus can be effectively targeted via Gal-modified PEI-SPIO to inhibit the tumor growth [27].

Human embryonic kidney 293T (HEK293T) cells-derived exosomes were used as delivery vehicles for anti-HGF siRNA. Human umbilical vein endothelial cells (HUVEC), co-cultured with SGC-7901 cells treated with exosomes, loaded with anti-HGF siRNA indicated effectively the delivery of anti-HGF siRNA, suppressing the cellular proliferation and vascular ring formation in HUVEC. The inhibitory effect of siRNA on tumor growth and angiogenesis in gastric cancer resulted in a marked downregulation of HGF expression [28].

3 Insulin-Like Growth Factor Receptor

The insulin-like growth factor (ILGF) receptor is a transmembrane protein tyrosine kinase receptor that transduces the signal through the MAPK and PI3K signaling pathway. The receptor-ligand interactions govern the cell growth and survival. In addition to regulating the normal cellular processes, the binding is also responsible for tumor formation and development and survival of malignant cells [29, 30]. The insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF1R) are members of the RTK family of cell surface receptors [31].

3.1 Recognition Domain of IGFR

ILGF binds to two receptor types, with different affinities. The first type of receptor is the IGF-R1, which plays a crucial role in regulating the growth of normal and malignant cells. The second receptor, namely, the insulin receptor (IR), regulates the cellular differentiation and metabolism [32]. Both the receptors share sequences

and exhibit a high morphological similarity (about 70%). The receptors belong to the glycoprotein category, which comprise 2α- and 2β-subunits. These subunits are evenly spanned into the membrane. The α-subunits are exposed toward the extracellular region to enable binding to the ligand, while the β-subunits are distributed in the transmembrane and intracellular domains. Being protein tyrosine kinase receptors, binding of ligands like IGF-I or IGF-II induces a conformational change in the receptor, due to the autophosphorylation of the three tyrosine residues in the catalytic domain of the C-terminal in the β-subunit, thereby leading to the downstream signaling. The structure of receptor and pathway has been depicted in Fig. 7.2. The insulin receptor is flexible in structure, encoded by various exons, wherein alternative splicing of exon 11 results in two isoforms (A and B) of the insulin receptor that differ in the presence or absence of a 12-residue sequence (717–729). The two isoforms have slightly different affinity for insulin, but the A isoform has significantly higher affinity for IGF-I (40 nM vs. 350 nM) and IGF-II (close to that of insulin). The IGF-I receptor binds to IGF-II with a lower affinity than for IGF-I and to insulin with a 500-fold lower affinity [33].

3.2 Binding of Ligand with IGF Receptor

The C-terminal half of the receptors consists of three fibronectin type III (FnIII) domains, each comprising a seven-stranded β-sandwich structure. The second FnIII domain comprises the C-terminal part of the α-subunit and the N-terminal part of the β-subunit and contains a large insert domain of ~120–130 residues. The structure of this insert domain is largely unknown, but includes a site of cleavage between the α- and β-subunits. The intracellular portion of the β-subunit contains the kinase catalytic domain (980–1255), flanked by two regulatory regions. These comprise a juxtamembrane region involved in docking insulin receptor substrates (IRS), IRS 1–4, and Shc, as well as in receptor internalization. The regulatory regions also contain a C-terminal tail comprising two phosphotyrosine-binding sites. The detailed organization of the modular domains of the insulin receptor has been depicted in Fig. 7.2. The IGF-I receptor has a very similar organization, with the sequence homology varying between 41% and 84%, depending on the domain. Maximum sequence homology has been observed in the kinase domain [33].

3.3 Structure-Activity Relationship of Selective Binding of Ligand with IGFR

The QSAR analysis of the ILGF receptor depicts several carbon atoms being connected to one hydrogen atom and the presence of two aromatic bonds (SaaCHcount) that are detrimental to the receptor activity. The harmful heteroaromatic rings, containing multiple nitrogens, like triazine, were anticipated to result in better

inhibition, as compared to the aromatic rings, like benzene, pyridine, and pyrimidine. Further, the electrotopological state indices of NH_2, connected to one single bond, were conducive for bioactivity, leading to the speculation that primary amines resulted in effective inhibition. This was most likely due to the hydrogen bonding, either by donating or accepting hydrogen atoms, provided that the nitrogen atom was connected to the electropositive groups. The docking poses of the IGF molecule at e MET 1112 at the receptor active site (IGF-1R) exhibited interaction of the fragment R1 with MET 1126 and ARG 1128, and of the fragment R3 with MET 1052 and GLU 1050. The amide group of the fragment R1 donates hydrogen to Met 1126 and Arg 1128. No hydrophobic interactions were observed between R1 and the active site of IGF-1R. Nonhydrophobic substituents, having branching and low NH count, at the fragment R1, were found to be essential for enhanced activity. Scaffolds of the molecules that formed the fragment R2 could be modified by decreasing the number of oxygen atoms and increasing the number of hydrogen bond donors. GQSAR studies suggested that substituents at R3 that contained lower number of aromatic carbons and higher content of NH_2 groups were responsible for majority of the activity [34].

3.4 Strategies to Target the IGFR

Targeted co-delivery of IGF-1R-specific siRNA and docetaxel (DTX) to SKBR3 cells was performed using anti-mucin 1 aptamer (Apt)-conjugated chitosan nanoparticles. Augmentation pathways involved in tumorigenesis and metastasis of breast cancer were studied. The nanoparticles with size of 110–118 nm and zeta potential of 14 mV loaded siRNA and DTX. The Apt-conjugated nanoparticles enhanced the cellular uptake of siRNA into the SKBR3 cells and reduced the genetic expression of IGF-1R, activators of transcription 3 (STAT3), and matrix metalloproteinases [35]. Co-delivery of IGF-1R-specific siRNA and doxorubicin (DOX) using chitosan nanoparticles resulted in a synergistic effect on the DOX-induced cytotoxicity and apoptosis of the tumor cells, when compared with only DOX. This resulted in decreased migration and expression of MMP9, VEGF, and STAT3, in A549 lung cancer cell lines. The loaded chitosan nanoparticles exhibited a size of about 176 nm size, zeta potential of 11 mV, and polydispersity index of 0.3 and possessed the capacity to simultaneously deliver several therapeutic agents. It also favored a controlled release of drugs or siRNA at the acidic pH of the tumor microenvironment [36]. Magnetic resonance imaging (MRI)-guided and IGF-1R-targeted theranostic iron oxide nanoparticles (IONPs) were found to be effective as they overcame the stromal barriers in tumor microenvironment. Pancreatic cancer, featuring enriched tumor stroma intravenously administered IGF1-IONPs resulted in excellent tumor penetration with better inhibition of the growth of pancreatic tumors. The intratumoral nanoparticle delivery was detected by MRI [37]. In female A/J mice, picropodophyllin was administered via nasal inhalation, demonstrating a good bioavailability in lungs and plasma. In human lung cancer cell lines, it inhibited cell

proliferation and phosphorylation of IGF-1R downstream targets, resulting in increased apoptosis and reduced cellular invasion. It is suggested that picropodophyllin can be potential chemopreventive agent [38].

4 Platelet-Derived Growth Factor Receptor

The platelet-derived growth factor (PDGF) isoforms bind to two distinct class III receptor tyrosine kinases, PDGFRα and PDGFRβ. The binding of the ligand leads to autophosphorylation of the receptors on tyrosine residues and this event induces activation of several signaling molecules [39]. Individual PDGF chains have different affinities for the two receptors. PDGFRα has high affinity for PDGF-A, PDGF-B, and PDGF-C, whereas PDGFRβ has high affinity for PDGF-B and PDGF-D. These interactions can be demonstrated in vitro, but it is not known if all are effective in vivo [40]. Ligand binding to receptors induces receptor dimerization, which leads to activation of the intrinsic tyrosine kinase domain and subsequent recruitment of SH-2-domain-containing signaling proteins [41]. Finally, activation of these pathways leads to cellular responses like proliferation and migration. Expression of activated p21Ras in cells influences PDGFRβ signaling at multiple levels. Two separate mechanisms occur for defective PDGFRβ signaling, namely, the transcriptional downregulation of PDGFRβ expression and inhibition of ligand-induced PDGFRβ by a factor of the cell membrane, in p21Ras-expressing fibroblasts [42]. Reversion of the cell phenotype results in the recovery of the PDGFRβ kinase activity. Disruption of the fibroblast cytoskeleton leads to a loss of PDGFRβ function.

4.1 Recognition Domain of the PDGFR

The ligand dimer has a flat shape with β-strands forming a super sheet, leaving the inter-strand loops at the ends of these strands. These loops are not only used for propeptide binding but also for receptor binding. There is a significant steric incompatibility between binding of receptor to PDGFs and the binding of pro-peptides to PDGFs. When the PDGF-A/pro-peptide complex and the PDGF-B/PDGFRβ complex are superimposed, with the backbones of the growth factor domains overlaid, it is apparent that these two binding events are mutually exclusive. The same hydrophobic residues important for pro-peptide association are also used for receptor binding. Consequently, receptor binding can displace the pro-peptide that is bound at the same site [43]. The two arms of the ligand clamp the PDGFR perpendicularly near the receptor's D2–D3 boundary. For PDGFRβ, the D2–D3 linker uses an extended conformation to open a large cleft for contacting PDGF-B. The overall shape of the PDGF-B:PDGFRβ recognition complex resembles other class III RTKs, such as Kit and FMS [44–46]. The positions of the D3 domains are similar in the SCF/Kit complex, the M-CSF/FMS complex, and the PDGF-B/PDGFRβ

complex, despite the positions of the D1 and D2 domains being dramatically different [47]. The PDGF family consists of five members, viz., the disulfide-bonded dimers of homologous A-, B-, C-, and D-polypeptide chains, and the AB heterodimer [48]. The PDGF-α receptor binds to all the PDGF chains, except the D chain, whereas the β receptor binds to PDGF-B and PDGF-D; thus, the different PDGF isoforms can induce αα-, αβ-, or ββ-receptor dimers. The ligand-binding sites are located in Ig-like domains 2 and 3 [43, 49]; however, the ligand-induced receptor dimerization is stabilized by direct receptor-receptor interactions in Ig-like domains 4 and 5 [50]. The latter interactions are important because they orient the receptors so that their activation by autophosphorylation in *trans* is facilitated.

4.2 Binding of the Ligand with PDGFR

PDGF-induced receptor dimerization leads to the autophosphorylation of certain tyrosine residues in the intracellular parts of the receptors. Thus, the α and β receptors have 10 and 11 autophosphorylation sites, respectively [51]. The autophosphorylation serves two important functions, viz., it leads to changes in the conformation of the intracellular parts of the receptors, promoting their activation, and it provides docking sites for the SH2-domain-containing signal transduction molecules. There are at least three mechanisms involved in the activation of the PDGF receptor kinases. Like most tyrosine kinase receptors, the PDGF receptors are autophosphorylated in the activation loop of the kinases (residues Tyr849 and Tyr857 in the α and β receptors, respectively). Phosphorylation of this residue of the β receptor is necessary for the full activation of the receptor kinase [52]. Phosphorylation causes a change in conformation of the activation loop, which opens the active site of the kinase and allows access of ATP and the protein substrate. Moreover, truncation of the carboxy-terminal tail of the β receptor causes receptor activation. The C-terminal is folded over the kinase domain that keeps kinase inactive further leading to autophosphorylation. The juxtamembrane domain of several tyrosine kinase receptors inhibits the kinase domain, causing a change in conformation led by autophosphorylation [53].

4.3 Structure-Activity Relationship of Binding of the Ligand with PDGFR

Autophosphorylation of the PDGF receptors allows binding of the signaling molecules containing the SH2 domains, which recognize phosphorylated tyrosine residues. Different SH2 domains have different preferences regarding the three to six amino acid residues downstream from the phosphorylated tyrosine, and there is a certain specificity in binding. The PDGF receptors are known to bind to about 10 different families of SH2-domain-containing molecules, which initiate the activation

of different signaling pathways. Because the autophosphorylation pattern of the PDGF-α and PDGF-β receptors differs, depending on whether the receptors occur in homo- or heterodimeric complexes, each of the three dimeric PDGF receptor complexes has distinct signaling properties [48]. Much efforts have been dedicated toward elucidating the signaling pathways that mediate the effects of PDGF on cells (i.e., cell proliferation, survival, chemotaxis, and actin reorganization). In general, the PI3 kinase has been found to be important for the antiapoptotic and motility responses of the PDGF, though differences between various cell types have also been reported. Src, via activation of the transcription factor Myc and Ras via activation of the ERK MAP kinase pathway are important for the growth-stimulating effects. However, it should be noted that there is an extensive cross talk between different signaling pathways. Thus, each of the many signaling pathways, induced by the activated receptor, can contribute to most of the cellular effects of the PDGF to different extents and in a cell type-specific manner.

4.4 Strategies to Target PDGFR

The PDGF receptors expressed in cervical cancer cells have been targeted using mesoporous silica nanoparticles (MSN), loaded with the anticancer prodrug cisplatin (cis-DDP) with an affinity probe of poly-acrylic acid (PAA). These PAA-MSNs are specifically taken up by the endothelial cells. The mean particle sizes and zeta potential ranged between 60 and 100 nm and −26.4 to +20.3 mV, respectively [54]. NH3+ groups present on MSNs-cis-DDP complexes interacted with the –OH group of the PAA; thus, the unreacted carboxylic groups had affinity to bind with the receptor [55].

Insufficient therapeutic agents are available to glioblastoma multiforme (GBM) tumor, after crossing the blood-brain barrier. The chemotherapeutic temozolomide is converted to 5-(3-methyltriazen-1-yl) imidazole4-carboxamide [56]. pH-responsive micelles loaded with TMZ and composed of distearoyl phosphoethanolamine-PEG-2000-amine and N-palmitoyl homocysteine and functionalized with PDGF peptide and Dylight 680 fluorophore showed uptake and increased cytotoxicity in glial cells. In vivo studies in orthotopic gliomas implanted in mice demonstrated selective accumulation of PDGF-micelles containing TMZ, with reduced systemic toxicity [57].

Ultrasound-mediated delivery using thermosensitive polymer (TSP)-based liposomes, modified with DNA aptamers, was targeted to PDGFR ligands on cancer cells (APT/TSP liposomes). These liposomes were formulated for breast cancer, using copolymer of N-isopropylmethacrylamide (NIPMAM) and N-isopropylacrylamide (NIPAM) forming TSP liposomes. The APT/TSP liposomes had binding affinity toward the MDA-MB-231 human breast cancer cells due to the presence of PDGFR aptamers. Cancer cell injury assay showed that using DOX-loaded APT/TSP liposomes and ultrasound irradiation, cell viability was 60%, which was lower than that with ultrasound irradiation and DOX-loaded TSP liposomes or with DOX-loaded

APT/TSP liposomes alone [58]. In aptamer-assisted targeting, inhibitory PDGF aptamers and PDGF β-receptors antagonist enhanced antitumor effect of Taxol on subcutaneous KAT-4 tumors in SCID mice and increased the antitumor effects of 5-fluorouracil on subcutaneous PROb tumors in BDIX rats [59].

5 Transforming Growth Factor Receptor

Transforming growth factor-β (TGF-β) regulates various cellular processes like mitotic inhibition or stimulation. The TGFβ pathway, by itself, controls various cellular functions that may lead to differing cellular phenotypes. The receptor model of TGF-β consists of heteromeric complexes of type I and II receptors. Upon ligand binding, the type I receptor is phosphorylated in the GS domain, located upstream in serine/threonine kinase domain and acts as an important regulatory domain for TGF signal transduction. This phosphorylation activity is assisted by the serine/threonine kinase of the type II receptor [60]. Phosphorylation of the GS domain is proposed to activate the type I receptor, resulting in signal propagation to the downstream effector molecules. In addition, specific residues in the nearby regions have also been suggested to have both positive and negative regulatory functions [60–63].

5.1 Recognition Domain of the TGF-β Receptor

The TGF-β family members facilitate signal transduction via binding to the dual specificity kinase receptors, at the surface of the target cells. The receptor family has similar structural characteristics for both serine/threonine and tyrosine kinases. Even though literature refers the receptor family as serine/threonine kinase receptors, they carry dual specificity kinases [64]. There are seven type I human receptors and five type II receptors; individual members of the TGF-β family bind to characteristic combinations of type I and type II receptors. The receptors have a rather small cysteine-rich extracellular domain, a transmembrane domain, a juxtamembrane domain, and a kinase domain; however, except for the BMP type II receptor and in contrast to tyrosine kinase receptors, parts of the carboxy terminal of the kinase domains are very short. Ligand-induced oligomerization of type I and type II receptors promotes phosphorylation of the type II receptors with the help of type I receptors, in a region of the juxtamembrane domain that is rich in glycine and serine residues (GS domain), thus *causing* activation of its kinase [64, 65]. The three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) bind to a single type II receptor (TβRII). Prior to ligand binding, TβRI and TβRII are in form of monomers, homodimers, and heterodimers.

5.2 Binding of the Ligand with TGF-β Receptor

Binding of ligand to receptor forms heterotetrameric complex of two TβRI and two TβRII molecules [63, 66, 67]. Structural studies have reported that the ligand-receptor binding occurs in the ratio of 2:2:2, as a complex, wherein a dimeric TGF-β binds to two TβRI and two TβRII molecules. The TGF-β molecule resembles a hand-like structure, containing a disulfide linkage and the finger-like TβRI and TβRII receptors. The receptor-receptor interaction enhances the stability of the ligand-receptor complex [68]. Binding induces phosphorylation in the GS domain. Phosphorylation of the GS domain, furthermore, enhances interaction with R-Smads, which promotes their phosphorylation [69]. Reports suggest TβRII is tyrosine phosphorylated that leads to the possibility of binding to SH2- or the PTB-domain-containing signaling molecules. The phosphorylation of Tyr284 has been shown to promote binding of the adaptors Shc and Grb2; Grb2 forms a complex with Sos1, a nucleotide exchange factor for Ras, which in turn activates the Erk1/2 MAP kinase pathway.

5.3 Structure-Activity Relationship for Selective Binding of Ligands with TGF-β Receptor

Activation of receptors TβRI and TβRII are regulated via various phosphorylation events. Upon ligand-receptor binding, structural alignment of the complex is induced, wherein TβRII phosphorylates TβRI in the GS domain located in the upstream region of the kinase domain [70]. The phosphorylation occurs on several closely located residues (i.e., Thr186, Ser187, Ser189, and Ser191); wherein no single residue is of crucial importance for activation, but phosphorylation needs to reach to a certain threshold for activation of the TβRI kinase. This phosphorylation leads to a conformational change that causes release of the 12 kDa-immunophilin FK506-binding protein (FKBPI2), which binds to the GS domain and inhibits the TβRI kinase [71–73]. TβRI can be phosphorylated at the Ser165, in the juxtamembrane domain. Interestingly, this phosphorylation modulates TGF-β signaling; growth suppression and matrix production are enhanced after mutation of Ser165, whereas the pro-apoptotic effect is decreased. Similar to TβRII, the kinase domain of TβRI has structural elements similar to both serine/threonine and tyrosine kinases [64]. Like TβRII, TβRI undergoes autophosphorylation on the serine/threonine residues, as well as on the tyrosine residues. The phosphorylated tyrosine residue(s) form docking site(s) for the adaptor molecule Shc via its PTB-domain, followed by its phosphorylation and the recruitment of the Grb2/Sos1 complex, and activation of Ras and the Erk MAP kinase pathways. The phosphorylation of TGF-β receptors is counteracted by several phosphatases. Thus, GADD34, a regulatory subunit of the protein phosphatase 1 (PP1) was found to bind to Smad7, which in turn binds to TβRI; the PP1 catalytic activity is thereby recruited in TβRI and dephosphorylates the receptor [63, 74].

5.4 Strategies to Target the TGF-β Receptor

Administration of TβR-I inhibitor (LY364947) [75] alters the tumor microenvironment along with an enhanced EPR (enhanced permeability and retention) effect. Doxil and a polymeric micelle incorporating ADR have demonstrated the effect of low-doses of the TβR-I inhibitor in xenografts in nude mice, developed using BxPC3 human pancreatic adenocarcinoma cell line [76]. This TβR-I inhibitor has exhibited success in pancreatic adenocarcinoma and gastric cancer, characterized by hypovascularity and thick fibrosis in the tumor microenvironment. Low dose decreased the pericyte coverage of the endothelium and promoted the accumulation of anticancer nanocarriers [77]. Heterogeneous drug distribution-induced regional insufficient chemotherapy accelerates the process of epithelial-mesenchymal transition (EMT) and thus accelerates tumor metastasis. Since TGF-β plays an essential role in EMT to eliminate the insufficient chemotherapy promoted metastasis [12], a combination of DOX and TGF-β receptor inhibitor LY2157299 was investigated in an in vivo study incorporating TGF-β receptor inhibitor along with hydroxyethyl starch-polylactide HES-PLA nanoparticles. The co-delivery of DOX and LY2157299, using HES-PLA nanoparticles, was found to be effective [78]. N-terminal of TTB, TGF-β receptor blocker, was fused with the RGD (arginine-glycine-asparagine) is a peptide of amino acids Arginine-Glycine-Asparagine, to target the tumor. In xenograft models, TTB resulted in distinct neutralization of TGF-β and inhibited cancer cell migration. TTB also attenuated the TGF-β1-induced Smad2 phosphorylation and EMT and suppressed breast cancer metastasis, indicating blocking of the TGF-β-induced pathogenesis [79]. An alternative approach to avert TGF-β signaling was the employment of recombinant Fc-fusion proteins, containing the soluble ectodomain of either TβRII (TβRII-Fc) or the type III receptor, betaglycan [80]. Zebin et al. developed recombinant oncolytic adenoviruses as a potential new class of antitumor agents [81]. These have been hypothesized to kill the tumor cells and simultaneously target the TGF-β pathways, to treat bone metastasis of prostate cancer. Further, Hu et al. also evaluated systemic administration of the TβRII-Fc coupled with an oncolytic adenovirus (Ad.sTβRII-Fc), in a nude mouse model of breast cancer bone metastases. Their study demonstrated that intravenous delivery of Ad.sTβRII-Fc resulted in viral replication and expression of TβRII-Fc in skeletal tumors, as well as a signification reduction of primary tumor growth and osteolytic bone destruction [82].

6 Vascular Endothelial Growth Factor Receptor

The VEGF binds to VEGFR to induce receptor homodimerization or heterodimerization, leading to the activation of tyrosine kinase and autophosphorylation of the tyrosine residues in the intracellular domains of the receptor. The phosphor-tyrosines and the surrounding amino acid residues constitute the binding sites for the adapter molecules, which initiate various intracellular signaling pathways. These pathways mediate immediate responses, such as vascular permeability and long-term responses

that require gene regulation, such as endothelial cell survival, migration, and proliferation. Noncanonical VEGFR signaling is initiated by non-VEGF-dependent activation of VEGFRs [83, 84]. The VEGFR signaling is tightly regulated at numerous different levels, including at the receptor expression level, with respect to the availability and affinities of binding to its different ligands, the presence of VEGF-binding co-receptors, non-VEGF-binding auxiliary proteins and inactivating tyrosine phosphatases, the rate of receptor cellular uptake, the extent of degradation, and the speed of recycling. VEGFR-mediated endocytosis and trafficking regulate the specificity, as well as the duration and amplitude of the signaling output. Once they are in the cytoplasm, the VEGFRs are either shuttled to lysosomes for degradation or recycled back to the membrane, via fast or slow recycling pathways. In case of VEGFR2, activation of ERK1/2 signaling, which is essential for the biology of VEGFR2, is dependent on the speed of the receptor's intracellular trafficking.

6.1 Recognition Domain of the VEGFR

The positively charged domain of VEGF, encompassing the Arg82, Lys84, and His86 residues and located in a hairpin loop, is responsible for the receptor-ligand binding, while the negatively charged residues like Asp63, Glu64, and Glu67 are associated with VEGFR-1. The VEGFR-1 binds to VEGF with 50-fold higher affinity than VEGFR-2 [85], which governs its angiogenic response [86] and is therefore of great therapeutic interest. Only a small number of VEGF residues are important for its binding to the VEGF receptors [87, 88]; thus, several molecules are able to modulate the biological activities of VEGF [89]. Many VEGF mimetic peptides, having antiangiogenic activity, have been described, while only a few of these molecules are known to exhibit a pro-angiogenic activity [90]. Copper stimulates VEGF [91, 92] and is required for the activation of the hypoxia-inducible factor-1, a major transcription factor regulating the expression of VEGF [93]. The activity of copper is VEGF-dependent; the metal ion perturbs the distribution of VEGF receptors, switching the signaling pathways from VEGFR-2 to VEGFR-1, which is associated with the inhibition of the growth of cardiomyocytes and regression of hypertrophy [94].

6.2 Binding of the Ligand with VEGFR

The binding of ligands to the VEGFR is thought to induce receptor dimerization. However, in vitro studies show pre-formed VEGFR2 dimers, with a certain level of kinase activity. The dimer, upon ligand binding, is stabilized by the receptor via binding at specific points. Moreover, binding of the ligands induces a slight conformational change in the transmembrane domains, which is accompanied by rotation of the dimers that is of critical importance for the full activation of the kinase

functions. Different ligands can influence the degree of rotation of the receptor molecules to different extents, and thereby the extent of receptor activation. For example, VEGFβ has been shown to lack the ability to optimally rotate its receptor, VEGFR1, as compared to PlGF. Thus, VEGFβ is a weaker activator of VEGFR1 signaling. In addition to the classical VEGF ligands, the alternatively spliced VEGFβ contains a unique exon 8b that confers it with antiangiogenic effect. However, the VEGFβ variants are also weak VEGFR2 agonists; thus, the mechanism of their antiangiogenic effect is still unclear.

6.3 Structure-Activity Relationship of Binding of the Ligands with VEGFR

The VEGFRs are related to the fibroblast growth factor (FGF) receptors, the colony-stimulating factor 1 (CSF-1) receptors, the stem cell factor (SCF) receptor, c-Kit, and the platelet-derived growth factor (PDGF) receptors. The extracellular domain (ECD) of classes III, IV, and V of the RTKs consists of several Ig-like subdomains and the linkers connecting them. The extracellular Ig-like subdomains have been attributed with three distinct *functions as follows:* (i) they form the ligand-binding domain, (ii) they participate in receptor dimerization, after or concomitant with ligand binding, and (iii) they maintain receptors in the monomeric state in the absence of the ligand. The ligand-binding ability of ECD is documented at the biochemical and the structural level. The VEGFR-1 subdomain 2 is sufficient for binding to the VEGF, while binding to VEGFR-2 involves subdomains 2 and 3 [95–97]. Subsequent to ligand binding, the receptor monomers form dimers that are further stabilized by homotypic interactions of the domains that are in proximity of the plasma membrane Ig-like domains 4 and/or 5, as surveyed for PDGFR-b and Kit [45, 50]. The domains immediately adjacent to the lipophilic membrane, in which the receptors are anchored, the so-called juxtamembrane domains (JMDs), have been shown to regulate the kinase activity in multiple ways [98, 99]. Evident in both extracellular as well as the intracellular JMD [100, 101], which played essential roles in kinase activation, either by properly positioning the kinase monomers relative to each other or by direct interaction with the activation loop. Phosphorylation of the JMDs at specific tyrosine residues disrupts this interaction thereby promoting reorientation of the activation loop and inducing an enzymatically active conformation [101, 102]. The RTKs are activated upon ligand-mediated dimerization or *higher-order* multimerization. Receptor multimerization is not only mediated via ligand binding but also requires additional homotypic interactions in the ECD, the JMD, and the TMD. Dimerization results from interaction between specific epitopes in the N- and the C-lobe of kinase monomers. RTK activation is suggested to rely on mechanisms as deduced for soluble intracellular kinases, for example, cell cycle-regulated kinases (CDKs). Activation of CDKs requires their binding to a regulatory subunit, the cell cycle-regulated cyclins. It remains to be shown whether this model also applies to the other RTK family members [101].

6.4 Strategies to Target VEGFR

Antiangiogenic drugs, in particular those focusing on blocking the VEGF pathway, are a part of the standard therapy for which various drug delivery strategies are applied to target VEGFR. Some of the strategies are discussed in this section.

Liposomal drug delivery systems with the use of highly soluble cisplatin analogue, cis-diammine dinitrato platinum (II), demonstrated a high binding affinity to the glioma cells. Pharmacokinetic study on glioma C6-bearing rats revealed prolonged blood circulation time of the liposomal formulation, due to reticuloendothelial bypass [103]. VEGF-targeted siRNA and gemcitabine monophosphate (GMP) were co-formulated into a single cell-specific, targeted lipid/calcium/phosphate (LCP) nanoparticle. This delivery system enforces eightfold reduction in tumor cell proliferation and a significant decrease of tumor microvessel density (MVD) as compared to therapy with either anti-VEGF siRNA or GMP alone. Further, anisamide (AA) was added to the LCP surface to specifically target the sigma receptors that are overexpressed in many human cancer cells [104]. A novel nanocomposite comprising bevacizumab (Bev) modified SiO_2@LDH nanoparticles (SiO_2@LDH-Bev) loaded with DOX was explored to exhibit an improved cellular uptake and demonstrated targeting of DOX to the brain tumors. This resulted in enhancement of both antineuroblastoma and antiangiogenesis efficiency and also reduced the side effects caused by DOX [105]. Amino-triphenyl dicarboxylate-bridged Zr^{4+} metal-organic framework nanoparticles (NMOFs) were modified with a nucleic acid complementary to the VEGF aptamer. The nucleic acid-functionalized NMOFs were loaded with the anticancer drug, DOX, and were capped by hybridization with the VEGF aptamer that yielded the VEGF-responsive duplex nucleic acid gates. In addition, conjugation to the AS1411 aptamer sequence that binds to nucleolin receptors resulting in the construction of cancer cell-targeted VEGF-responsive DOX-loaded NMOFs. The system demonstrated selective permeation with a twofold enhanced uptake along with the selective apoptosis of the MDA-MB-231 cancer cells, as compared to the normal MCF-10A breast cells [106]. Further, combination of Ang2 inhibitor (recombinant peptide-Fc-fusion protein called peptibody) and VEGF inhibitor (humanized mAb bevacizumab) permitted vascular normalization at significantly reduced doses and avoided excessive vessel regression [107].

7 Fibroblast Growth Factor Receptor

Fibroblast growth factor family comprises 22 identified molecules, of which only 18 function as FGF ligands (exceptions are FGF 11–14). FGF are secreted glycoproteins and they carry strong affinity for the cell surface proteoglycans, which include glycosaminoglycan side chains. Thus, due to their ability to adhere, they are trapped on the surface of the cells which secrete them or the cells in proximity, enhancing their action to mediate short-range signal transduction [108, 109].

The FGF receptor is a transmembrane tyrosine kinase receptor that belongs to the immunoglobulin (Ig) superfamily. Humans consist of four genes encoding for the FGF receptors, a family of receptors responsible for the expression of transmembrane RTKs (FGFR1–4). The FGFR monomers consist of an extracellular domain, including a ligand-binding site, three immunoglobulin loops coded by alternative splicing, an acidic box containing glutamic acid and aspartic acid residues in the IgI–IgII linker region, a transmembrane domain, and a split tyrosine kinase domain constituting the C-terminal cytoplasmic domain. The first Ig-like domain and the acid box forming the N-terminal are reported to play a role in receptor autoinhibition. The second and the third Ig-like domains are known for FGF ligand binding and are responsible for binding to the FGFR subtypes [110–112]. The intracellular portion consists of a juxtamembrane domain, a split tyrosine kinase domain, and a carboxy-terminal tail [113].

7.1 Recognition Domain and Binding of Ligands with the FGFR

The extracellular ligand-binding domain has a hydrophobic signal peptide-containing region and two or three immunoglobulin (Ig)-like domains (D1–D3). The bridge between D1 and D2 comprises 30 serine residues. Signal transduction from extracellular region to the cytoplasmic domain is facilitated by the transmembrane domain. The C-terminal lies in the juxtamembrane region, which emerges from the cytoplasmic membrane and has a split tyrosine kinase domain [114, 115]. This receptor system contains heparan sulfate proteoglycans and the related heparin-like molecules necessary for FGF-FGFR binding and receptor activation. Binding of FGF to the FGFRs induces receptor dimerization, leading to conformational changes within the FGFR structure, thereby leading to trans-phosphorylation of the tyrosine residues in the intracellular part of the receptor, including the kinase domain and the C-terminus [108, 116, 117]. There are seven autophosphorylation sites in FGFR1, Y463 (juxtamembrane), Y583/Y585 (kinase insert), Y653/Y654 (the activation loop), Y730 (kinase domain) and Y766 (C-terminal tail) [118]. Transphosphorylation of the tyrosine residue Y653, in the activation loop, leads to the activation of the kinase by 50–100-fold, thereby autophosphorylating the tyrosine residues in the juxtamembrane (Y463), the split kinase insert (Y583/Y585), and the C-terminal (Y766). These autophosphorylations induce structural changes, thereby presenting the cytoplasmic domain as a docking site for the downstream signaling molecules. Finally, phosphorylation of tyrosine in the activation loop (Y654) leads to further enhancement in the kinase activity by tenfold [119]. The binding of the docking proteins to the FGFRs leads to activation of multiple signal transduction pathways, including the four main downstream pathways, Ras-Raf-MapK, PI3K-Akt, Stats, and PLCγ [108].

7.2 Structure-Activity Relationship for Binding of Ligands with the FGFR

Upon ligand binding, the tyrosine residue 463 in the juxtamembrane is phosphorylated, followed by Crk phosphorylation, to instigate formation of a complex between FGFR and Crk. SOS (activated by Crk) further 42 activates JNK, via Ras [116] and Rac pathway [120]. In addition to Rac, cdc42, a cell cycle regulator has also been reported as an intermediate to the JNK and p38 activation cascades [116]. Direct interaction of DOCK180 with Rac1 occurs, thereby activating JNK in a manner that is dependent on factors like Cdc42Hs and SEK and increasing the amount of GTP-bound Rac1 [121]. Receptor activation also phosphorylates the docking protein, FGFR substrate 2 (FRS2), which further employs Shp2 and enhances association between the growth factor receptor-bound 2 (Grb2) and SOS, thus triggering the induction of Ras/MEK/MAPK signaling pathways [74, 122, 123]. In addition, activation of the PI3-kinase pathway takes place via tyrosine phosphorylation in FRS2α and recruitment of Grb2 and Gab1 [124]. Additionally, interaction of the accessory proteins (SH2 domain-containing adaptor protein B (Shb) and SH2 domain-containing collagen (Shc)) with the FGFRs facilitates the signal transduction [120, 125]. Binding of Shb2 induces tyrosine phosphorylation (Y766), thereby activating the Ras/MEK/MAPK pathway. FGFR binds to the signal transducers and activators of transcription (STAT) and ribosomal protein S6 kinase 2 (RSK2). Further, STAT3 binds to the phosphorylated Tyr^{677} of the FGFR1. In addition, tyrosine activation of STAT3 requires overexpression of FGFR1 or FGFR2 [122]. In cancer cells, when the FGFRs bind to different FGF ligands, the FGFRs can cause abnormal upregulation of the Ras-dependent mitogen-activated protein kinase (MAPK), Ras-independent phosphoinositide3-kinase-protein kinase B/Akt (PI3K-PKB/Akt) pathway, and signal transducer and activator of transcription (STAT)-dependent signaling pathways, which are closely associated with the development of multiple cancers.

7.3 Strategies to Target the FGFR

This section gives an overview of different drug delivery strategies like prodrug complex, nanoparticle delivery to target FGFR for anticancer therapies. Conjugation of truncated human FGF1 (residues 21–154) (FGF1V) with monomethyl auristatin E (MMAE) was developed for potent and specific cytotoxic effect. FGF1V contains three-point mutations (Q40P, S47I, and H93G) and an N-terminal four-amino acid linker (CGGG), which increases its stability. The FGF1V-valine-citrulline-MMAE conjugate showed targeted and efficient release of MMAE at lower concentration than the native MMAE [126]. Further, brivanib alaninate is an L-alanine ester prodrug of brivanib with enhances aqueous solubility of drugs and enables their oral administration. It is a selective dual inhibitor of FGF and VEGF signaling.

Brivanib alaninate has reduced 76% tumor cell proliferation and tumor vascular density in xenograft models [127]. Further, disulfide-stabilized diabody (ds-Diabody) against antibasic fibroblast growth factor (bFGF) was constructed by site-directed mutation and overlap extension PCR (SOE-PCR), at VH44 and VL100, in single-chain variable fragment (scFv) antibody. It inhibited the bFGF-induced activation of the downstream signaling regulators and also decreased the densities of microvessels and lymphatic vessels in the tumor tissue.

8 Clinical Development of Growth Factor Receptor Antagonists

An overview of the clinical development of various growth factor receptor antagonists for cancer treatment has been concisely presented in Table 7.2.

9 Conclusion

Cancer possesses numerous growth alteration mechanisms and compromised cell surface receptors that govern and regulate the cellular functions, enhancing malignant behavior. These cell surface receptor families consist of tyrosine kinases and serine/threonine kinases, which control the cellular expression of the growth factors. There are eight types of growth factors that participate in the controlled development normal cells. In this chapter, seven of the growth factor receptors (HGF, ILGF, PDGF, TGFβ, VEGF and FGF) have been elaborated, while the growth factor receptor activation and signal transduction of the epidermal growth factor receptor has been explained in Chap. 7. This document contains elaborate details about the growth factor receptors, with respect to their structure, binding with the ligands, their binding domains, the signal transduction pathway triggered upon ligand binding, and their downstream signaling mechanisms. Their respective receptors are majorly responsible for the transduction of downstream signaling pathways. These pathways, upon interaction with abnormally expressed ligands, exert continued signaling cascades that transform normal cells to cancerous ones. The factors dictating the transformation of cells into a cancerous can be controlled via regulations in the signaling of growth factor receptors upstream. The conventional drugs used in chemotherapy can be conjugated with various newly designed molecules like small peptides and monoclonal antibodies. Advancements in these molecules are their mode of delivery. Organ specificity of the drugs can be achieved via entrapment in different carrier molecules like liposomes or polysaccharide-based nanoparticles. The drugs and their targeting strategies developed are currently being tested for their stability and preventing the metastasis. Certain genetically engineered variants of ligand have also been studied for the receptor regularization. These ligand variants, drug complexes, and delivery techniques are still in their clinical trial phases.

Table 7.2 Overview of the recently completed clinical trials studies that have been conducted to target the cancer therapy

Drug/drug combination	Phase	Targeted cancer	Study outcome	Clinical trial identifier
HGFR				
Rilotumumab	Phase II	Ovarian epithelial cancer, fallopian tube cancer, primary peritoneal cancer	Well tolerated in the early phase clinical trials and inhibited the HGF-/MET-driven activities in recurrent and resistant cancer cells	NCT01039207 [128]
Crizotinib	Phase II	Solid tumors or anaplastic large cell lymphoma (ALCL) in children	The pharmacokinetics of oral crizotinib in children is similar to that in adults	NCT00939770 [129]
Cabozantinib	Phase III	Hepatocellular carcinoma	Longer overall survival and progression-free survival with cabozantinib than with placebo	NCT01908426 [130]
IGFR				
AMG 479	Phase I	Chemorefractory ewing sarcoma	Absence of severe toxicities and demonstrated promising single-agent activity	NCT00562380 [131]
R1507	Phase II	Chemorefractory ewing sarcoma	Active as monotherapy, but acquired resistance was commonly observed upon continued therapy	NCT00642941 [75]
Ridaforolimus + Dalotuzumab	Phase I	ER+/highly proliferative breast cancers	Inhibited (mTOR), prevented the activation IGFR signaling	NCT01234857 [132]
PDGFR				
Imatinib mesylate	Phase II	Recurrent meningioma	CYP3A4 inducers decreased the plasma concentration of imatinib	NCT00045734 [133]
Olaratumab + doxorubicin	Completed	Soft tissue sarcoma	Improved progression-free survival and overall survival	NCT02326025 [134]
Sunitinib (SU11248)	Phase I/II	Gastrointestinal stromal tumor (GIST)	Overall survival was improved	NCT00457743 [135]
TGF-β receptor				
LY2109761	Preclinical	Colon adenocarcinoma cells	Reduced oncogenic effects of TGF-β on cell migration, invasion, and tumorigenicity in CT26 cells	NA

(continued)

Table 7.2 (continued)

Drug/drug combination	Phase	Targeted cancer	Study outcome	Clinical trial identifier
Galunisertib (LY2157299 monohydrate)	Preclinical	Glioblastoma, pancreatic cancer	Downregulates the phosphorylation of Smad2, abrogating the activation of the canonical pathway	NA [136]
Belagenpumatucel-L	Phase III	Squamous cell carcinoma, large-cell lung cancer	Increased overall survival	NCT00676507
Fresolimumab (GC-1008)	Phase I	Metastatic melanoma, renal cell carcinoma	Safe and well tolerated	NCT00356460 [137]
VEGFR				
Bevacizumab + 5-fluorouracil, oxaliplatin, leucovorin or 5-fluorouracil, irinotecan, leucovorin	Phase III	Metastatic colorectal cancer	Reduced febrile neutropenia incidence	NCT00911170 [138]
Apatinib (YN968D1)	Preclinical	Advanced gastric adenocarcinoma	Improved safety and efficacy	NA [139]
Apatinib (YN968D1)	Phase III	Metastatic gastric carcinoma	Improved the progression-free or overall survival, who failed second-lines of chemotherapy	NCT01512745 [140]
Vatalanib + gemcitabine	Phase I/II	Advanced pancreatic cancer	Combination is well tolerated with antitumor response	NCT00185588 [141]
FGFR				
Dovitinib (TKI258)	Phase III	Metastatic renal cell carcinoma	Lesser activity than sorafenib in patients with progressed on VEGF-targeted therapies/ mTOR inhibitors	NCT01223027 [142]
AZD4547 + fulvestrant	Phase II	Breast cancer	Improved safety in efficacy in combination therapy	NCT01202591 [143]
Lucitanib (E3810)	Phase II	Breast cancer	Daily dosing provide durable clinical responses	NCT02202746 [144]
Lenvatinib	Phase II	Endometrial cancers	Tolerable and clinical benefits in advanced endometrial cancer	NCT01111461 [145]

The combinatorial therapies need a lot of in vitro research base with respect to proving its synergism and effect up on the linked signaling pathways. The pharmacodynamic studies reveal that these drugs carrying specific targets can be useful in designing personalized medicine. These therapies are currently under various phases of clinical trials. Their clinical success will lead to the availability of more specific, effective, and safer therapies for the treatment of a variety of cancers. Multiple clinical trials described in the document have passed phase II depicting the success ratio of the upcoming promising therapy with a drug delivery system. Research attempts directed toward these receptors are anticipated to provide interdisciplinary insights on their implication in the development, progression, and treatment of the cancers caused by overexpression of growth factor signals.

References

1. Cross M, Dexter TM. Growth factors in development, transformation, and tumorigenesis. Cell. 1991;64(2):271–80.
2. Nakanishi T, Markwald R, Baldwin H, Keller B, Srivastava D, Yamagishi H. Extracellular matrix remodeling in vascular development and disease. In: Etiology and morphogenesis of congenital heart disease: from gene function and cellular interaction to morphology; 2016. Springer, Tokyo.
3. Zhang X, Nie D, Chakrabarty S. Growth factors in tumor microenvironment. Front Biosci. 2010;15:151.
4. Tannock IF. Conventional cancer therapy: promise broken or promise delayed? Lancet. 1998;351:SII9–SII16.
5. Xiao Y, Tian Q, He J, Huang M, Yang C, Gong L. MiR-503 inhibits hepatocellular carcinoma cell growth via inhibition of insulin-like growth factor 1 receptor. Onco Targets Ther. 2016;9:3535.
6. Gonzalez A, Broussas M, Beau-Larvor C, Haeuw JF, Boute N, Robert A, et al. A novel antagonist anti-cMet antibody with antitumor activities targeting both ligand-dependent and ligand-independent c-Met receptors. Int J Cancer. 2016;139(8):1851–63.
7. Birchmeier C, Birchmeier W, Gherardi E, Woude GFV. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4(12):915.
8. Matsumoto K, Umitsu M, De Silva DM, Roy A, Bottaro DP. Hepatocyte growth factor/MET in cancer progression and biomarker discovery. Cancer Sci. 2017;108(3):296–307.
9. Rampa C, Tian E, Våtsveen TK, Buene G, Slørdahl TS, Børset M, et al. Identification of the source of elevated hepatocyte growth factor levels in multiple myeloma patients. Biomark Res. 2014;2(1):8.
10. Pavelic J, Krizanac S, Kapitanovic S, Pavelic L, Samarzija M, Pavicic F, et al. The consequences of insulin-like growth factors/receptors dysfunction in lung cancer. Am J Respir Cell Mol Biol. 2005;32(1):65–71.
11. Heldin C-H. Targeting the PDGF signaling pathway in tumor treatment. Cell Commun Signal. 2013;11(1):97.
12. Massagué J. TGFβ in cancer. Cell. 2008;134(2):215–30.
13. Gasparini G. Prognostic value of vascular endothelial growth factor in breast cancer. Oncologist. 2000;5(Suppl 1):37–44.
14. Katoh M, Nakagama H. FGF receptors: cancer biology and therapeutics. Med Res Rev. 2014;34(2):280–300.
15. Savage NM, Johnson RC, Gotlib J, George TI. Myeloid and lymphoid neoplasms with FGFR1 abnormalities: diagnostic and therapeutic challenges. Am J Hematol. 2013;88(5):427–30.

16. Jin M, Kleinberg A, Cooke A, Gokhale PC, Foreman K, Dong H, et al. Potent and selective cyclohexyl-derived imidazopyrazine insulin-like growth factor 1 receptor inhibitors with in vivo efficacy. Bioorg Med Chem Lett. 2011;21(4):1176–80.
17. Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. Clin Cancer Res. 2006;12(12):3657–60.
18. Safaie Qamsari E, Safaei Ghaderi S, Zarei B, Dorostkar R, Bagheri S, Jadidi-Niaragh F, et al. The c-Met receptor: implication for targeted therapies in colorectal cancer. Tumor Biol. 2017;39(5):1010428317699118.
19. Gandino L, Longati P, Medico E, Prat M, Comoglio PM. Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. J Biol Chem. 1994;269(3):1815–20.
20. Cecchi F, Rabe DC, Bottaro DP. The hepatocyte growth factor receptor: structure, function and pharmacological targeting in cancer. Curr Signal Transduction Ther. 2011;6(2):146–51.
21. Adriaenssens E, Vanhecke E, Saule P, Mougel A, Page A, Romon R, et al. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res. 2008;68(2):346–51.
22. Sachs M, Brohmann H, Zechner D, Müller T, Hülsken J, Walther I, et al. Essential role of Gab1 for signaling by the c-Met receptor in vivo. J Cell Biol. 2000;150(6):1375–84.
23. Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 1994;77(2):261–71.
24. Montagner A, Yart A, Dance M, Perret B, Salles J-P, Raynal P. A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation. J Biol Chem. 2005;280(7):5350–60.
25. Heukers R, Altintas I, Raghoenath S, De Zan E, Pepermans R, Roovers RC, et al. Targeting hepatocyte growth factor receptor (Met) positive tumor cells using internalizing nanobody-decorated albumin nanoparticles. Biomaterials. 2014;35(1):601–10.
26. Alibakhshi A, Kahaki FA, Ahangarzadeh S, Yaghoobi H, Yarian F, Arezumand R, et al. Targeted cancer therapy through antibody fragments-decorated nanomedicines. J Control Release. 2017;268:323–34.
27. Yang Z, Duan J, Wang J, Liu Q, Shang R, Yang X, et al. Superparamagnetic iron oxide nanoparticles modified with polyethylenimine and galactose for siRNA targeted delivery in hepatocellular carcinoma therapy. Int J Nanomedicine. 2018;13:1851.
28. Zhang H, Wang Y, Bai M, Wang J, Zhu K, Liu R, et al. Exosomes serve as nanoparticles to suppress tumor growth and angiogenesis in gastric cancer by delivering hepatocyte growth factor si RNA. Cancer Sci. 2018;109(3):629–41.
29. Kalus W, Zweckstetter M, Renner C, Sanchez Y, Georgescu J, Grol M, et al. Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. EMBO J. 1998;17(22):6558–72.
30. Keyhanfar M, Booker GW, Whittaker J, Wallace JC, Forbes BE. Precise mapping of an IGF-I-binding site on the IGF-1R. Biochem J. 2007;401(1):269–77.
31. Cabail MZ, Li S, Lemmon E, Bowen ME, Hubbard SR, Miller WT. The insulin and IGF1 receptor kinase domains are functional dimers in the activated state. Nat Commun. 2015;6:6406.
32. Lee J, Pilch PF. The insulin receptor: structure, function, and signaling. Am J Phys Cell Phys. 1994;266(2):C319–C34.
33. De Meyts P, Sajid W, Palsgaard J, Theede A-M, Gauguin L, Aladdin H, et al. Insulin and IGF-I receptor structure and binding mechanism. In: Mechanisms of insulin action. Springer; 2007. p. 1–32. Landes Bioscience, Austin.
34. Abdullahi AD, Abdualkader AM, Abdulsamat NB, Ingale K. Application of group-based QSAR and molecular docking in the design of insulin-like growth factor antagonists. Trop J Pharm Res. 2015;14(6):941–51.
35. Jafari R, Majidi Zolbanin N, Majidi J, Atyabi F, Yousefi M, Jadidi-Niaragh F, et al. Anti-Mucin1 Aptamer-conjugated Chitosan nanoparticles for targeted co-delivery of Docetaxel and IGF-1R siRNA to SKBR3 metastatic breast cancer cells. Iran Biomed J. 2019;23:21.

36. Shali H, Shabani M, Pourgholi F, Hajivalili M, Aghebati-Maleki L, Jadidi-Niaragh F, et al. Co-delivery of insulin-like growth factor 1 receptor specific siRNA and doxorubicin using chitosan-based nanoparticles enhanced anticancer efficacy in A549 lung cancer cell line. Artif Cells Nanomed Biotechnol. 2018;46(2):293–302.
37. Zhou H, Qian W, Uckun FM, Wang L, Wang YA, Chen H, et al. IGF1 receptor targeted theranostic nanoparticles for targeted and image-guided therapy of pancreatic cancer. ACS Nano. 2015;9(8):7976–91.
38. Zhang Q, Pan J, Lubet RA, Wang Y, You M. Targeting the insulin-like growth factor-1 receptor by picropodophyllin for lung cancer chemoprevention. Mol Carcinog. 2015;54(S1):E129–E37.
39. Magnusson PU, Looman C, Åhgren A, Wu Y, Claesson-Welsh L, Heuchel RL. Platelet-derived growth factor receptor-β constitutive activity promotes angiogenesis in vivo and in vitro. Arterioscler Thromb Vasc Biol. 2007;27(10):2142–9.
40. Batut J, Schmierer B, Cao J, Raftery LA, Hill CS, Howell M. Two highly related regulatory subunits of PP2A exert opposite effects on TGF-β/Activin/Nodal signalling. Development. 2008;135(17):2927–37.
41. Bai A, Meetze K, Vo NY, Kollipara S, Govek E, Winston WM, et al. GP369, an FGFR2-IIIb specific antibody, exhibits potent antitumor activity against human cancers driven by activated FGFR2 signaling. Cancer Res. 2010;70:7630. canres. 1489.2010.
42. Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, et al. Inhibition of pulmonary and skeletal metastasis by a transforming growth factor-β type I receptor kinase inhibitor. Cancer Res. 2006;66(13):6714–21.
43. Shim AH-R, Liu H, Focia PJ, Chen X, Lin PC, He X. Structures of a platelet-derived growth factor/propeptide complex and a platelet-derived growth factor/receptor complex. Proc Natl Acad Sci. 2010;107(25):11307–12.
44. Chen X, Liu H, Focia PJ, Shim AH-R, He X. Structure of macrophage colony stimulating factor bound to FMS: diverse signaling assemblies of class III receptor tyrosine kinases. Proc Natl Acad Sci. 2008;105(47):18267–72.
45. Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell. 2007;130(2):323–34.
46. Liu H, Leo C, Chen X, Wong BR, Williams LT, Lin H, et al. The mechanism of shared but distinct CSF-1R signaling by the non-homologous cytokines IL-34 and CSF-1. Biochim Biophys Acta-Proteins and Proteomics. 2012;1824(7):938–45.
47. Chen P-H, Chen X, He X. Platelet-derived growth factors and their receptors: structural and functional perspectives. Biochim Biophys Acta-Proteins and Proteomics. 2013;1834(10):2176–86.
48. Ekman S, Thuresson ER, Heldin C-H, RoÈnnstrand L. Increased mitogenicity of an αβ heterodimeric PDGF receptor complex correlates with lack of RasGAP binding. Oncogene. 1999;18(15):2481.
49. Heidaran M, Pierce J, Jensen R, Matsui T, Aaronson S. Chimeric alpha-and beta-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the alpha-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem. 1990;265(31):18741–4.
50. Yang Y, Yuzawa S, Schlessinger J. Contacts between membrane proximal regions of the PDGF receptor ectodomain are required for receptor activation but not for receptor dimerization. Proc Natl Acad Sci. 2008;105(22):7681–6.
51. Heldin C-H, Östman A, Rönnstrand L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta-reviews on cancer. 1998;1378(1):F79–F113.
52. Baxter RM, Secrist JP, Vaillancourt RR, Kazlauskas A. Full activation of the platelet-derived growth factor β-receptor kinase involves multiple events. J Biol Chem. 1998;273(27):17050–5.
53. Hubbard SR. Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat Rev Mol Cell Biol. 2004;5(6):464.
54. Deng ZJ, Liang M, Toth I, Monteiro MJ, Minchin RF. Molecular interaction of poly (acrylic acid) gold nanoparticles with human fibrinogen. ACS Nano. 2012;6(10):8962–9.

55. Rejeeth C, Vivek R, NipunBabu V, Sharma A, Ding X, Qian K. Cancer nanomedicine: from PDGF targeted drug delivery. MedChemComm. 2017;8(11):2055–9.
56. Patil R, Portilla-Arias J, Ding H, Inoue S, Konda B, Hu J, et al. Temozolomide delivery to tumor cells by a multifunctional nano vehicle based on poly (β-L-malic acid). Pharm Res. 2010;27(11):2317–29.
57. Miller K, Dixit S, Bredlau A-L, Moore A, McKinnon E, Broome A-M. Delivery of a drug cache to glioma cells overexpressing platelet-derived growth factor receptor using lipid nanocarriers. Nanomedicine. 2016;11(6):581–95.
58. Ninomiya K, Yamashita T, Kawabata S, Shimizu N. Targeted and ultrasound-triggered drug delivery using liposomes co-modified with cancer cell-targeting aptamers and a thermosensitive polymer. Ultrason Sonochem. 2014;21(4):1482–8.
59. Pietras K, Rubin K, Sjöblom T, Buchdunger E, Sjöquist M, Heldin C-H, et al. Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res. 2002;62(19):5476–84.
60. Wieser R, Wrana J, Massague J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J. 1995;14(10):2199–208.
61. Charng M-J, Kinnunen P, Hawker J, Brand T, Schneider MD. FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-β receptors. J Biol Chem. 1996;271(38):22941–4.
62. Souchelnytskyi S, Ten Dijke P, Miyazono K, Heldin C. Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. EMBO J. 1996;15(22):6231–40.
63. Heldin C-H, Moustakas A. Signaling receptors for TGF-β family members. Cold Spring Harb Perspect Biol. 2016;8(8):a022053.
64. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912–34.
65. Saitoh M, Nishitoh H, Amagasa T, Miyazono K, Takagi M, Ichijo H. Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-β. J Biol Chem. 1996;271(5):2769–75.
66. Zhang B, Halder SK, Zhang S, Datta PK. Targeting transforming growth factor-β signaling in liver metastasis of colon cancer. Cancer Lett. 2009;277(1):114–20.
67. Ehrlich M, Gutman O, Knaus P, Henis YI. Oligomeric interactions of TGF-β and BMP receptors. FEBS Lett. 2012;586(14):1885–96.
68. Radaev S, Zou Z, Huang T, Lafer EM, Hinck AP, Sun PD. Ternary complex of TGF-β1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. J Biol Chem. 2010;285:14806. jbc.M109.079921.
69. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-β receptor. Nature. 1994;370(6488):341.
70. Wang T, Li B-Y, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, et al. The immunophilin FKBP12 functions as a common inhibitor of the TGFβ family type I receptors. Cell. 1996;86(3):435–44.
71. Chen YG, Liu F, Massagué J. Mechanism of TGFβ receptor inhibition by FKBP12. EMBO J. 1997;16(13):3866–76.
72. Huse M, Chen Y-G, Massagué J, Kuriyan J. Crystal structure of the cytoplasmic domain of the type I TGF β receptor in complex with FKBP12. Cell. 1999;96(3):425–36.
73. Huse M, Muir TW, Xu L, Chen Y-G, Kuriyan J, Massagué J. The TGFβ receptor activation process: an inhibitor-to substrate-binding switch. Mol Cell. 2001;8(3):671–82.
74. Gotoh N, Laks S, Nakashima M, Lax I, Schlessinger J. FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts. FEBS Lett. 2004;564(1–2):14–8.
75. Sawyer JS, Anderson BD, Beight DW, Campbell RM, Jones ML, Herron DK, et al. Synthesis and activity of new aryl-and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-β type I receptor kinase domain. J Med Chem. 2003;46(19):3953–6.

76. Bae Y, Nishiyama N, Fukushima S, Koyama H, Yasuhiro M, Kataoka K. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. Bioconjug Chem. 2005;16(1):122–30.
77. Kano MR, Bae Y, Iwata C, Morishita Y, Yashiro M, Oka M, et al. Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-β signaling. Proc Natl Acad Sci. 2007;104(9):3460–5.
78. Zhou Q, Li Y, Zhu Y, Yu C, Jia H, Bao B, et al. Co-delivery nanoparticle to overcome metastasis promoted by insufficient chemotherapy. J Control Release. 2018;275:67–77.
79. Zhou C, Li J, Lin L, Shu R, Dong B, Cao D, et al. A targeted transforming growth factor-beta (TGF-β) blocker, TTB, inhibits tumor growth and metastasis. Oncotarget. 2018;9(33):23102.
80. Nacif M, Shaker O. Targeting transforming growth factor-β (TGF-β) in cancer and non-neoplastic diseases. J Cancer Ther. 2014;5(07):735.
81. Toth K, Dhar D, Wold WS. Oncolytic (replication-competent) adenoviruses as anticancer agents. Expert Opin Biol Ther. 2010;10(3):353–68.
82. Hu Z, Zhang Z, Guise T, Seth P. Systemic delivery of an oncolytic adenovirus expressing soluble transforming growth factor-β receptor II–Fc fusion protein can inhibit breast cancer bone metastasis in a mouse model. Hum Gene Ther. 2010;21(11):1623–9.
83. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. J Biochem. 2013;153(1):13–9.
84. Koch S, Tugues S, Li X, Gualandi L, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Biochem J. 2011;437(2):169–83.
85. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science. 1992;255(5047):989–91.
86. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin C-H. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994;269(43):26988–95.
87. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med. 2012;2:a006502.
88. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, De Vos AM. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci. 1997;94(14):7192–7.
89. D'andrea LD, Del Gatto A, Pedone C, Benedetti E. Peptide-based molecules in angiogenesis. Chem Biol Drug Des. 2006;67(2):115–26.
90. Finetti F, Basile A, Capasso D, Di Gaetano S, Di Stasi R, Pascale M, et al. Functional and pharmacological characterization of a VEGF mimetic peptide on reparative angiogenesis. Biochem Pharmacol. 2012;84(3):303–11.
91. Sen CK, Khanna S, Venojarvi M, Trikha P, Ellison EC, Hunt TK, et al. Copper-induced vascular endothelial growth factor expression and wound healing. Am J Phys Heart Circ Phys. 2002;282(5):H1821–H7.
92. Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, et al. Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood. 2005;105(12):4613–9.
93. Feng W, Ye F, Xue W, Zhou Z, Kang YJ. Copper regulation of hypoxia-inducible factor-1 activity. Mol Pharmacol. 2009;75(1):174–82.
94. Zhou Y, Bourcy K, Kang YJ. Copper-induced regression of cardiomyocyte hypertrophy is associated with enhanced vascular endothelial growth factor receptor-1 signalling pathway. Cardiovasc Res. 2009;84(1):54–63.
95. Christinger HW, Fuh G, de Vos AM, Wiesmann C. The crystal structure of placental growth factor in complex with domain 2 of vascular endothelial growth factor receptor-1. J Biol Chem. 2004;279(11):10382–8.
96. Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM. Crystal structure at 1.7 Å resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell. 1997;91(5):695–704.

97. Li B, Fuh G, Meng G, Xin X, Gerritsen ME, Cunningham B, et al. Receptor-selective variants of human vascular endothelial growth factor GENERATION AND CHARACTERIZATION. J Biol Chem. 2000;275(38):29823–8.
98. Davis TL, Walker JR, Loppnau P, Butler-Cole C, Allali-Hassani A, Dhe-Paganon S. Autoregulation by the juxtamembrane region of the human ephrin receptor tyrosine kinase A3 (EphA3). Structure. 2008;16(6):873–84.
99. Zou J, Wang YD, Ma FX, Xiang ML, Shi B, Wei YQ, et al. Detailed conformational dynamics of juxtamembrane region and activation loop in c-Kit kinase activation process. Proteins. 2008;72(1):323–32.
100. Chan PM, Ilangumaran S, La Rose J, Chakrabartty A, Rottapel R. Autoinhibition of the kit receptor tyrosine kinase by the cytosolic juxtamembrane region. Mol Cell Biol. 2003;23(9):3067–78.
101. Stuttfeld E, Ballmer-Hofer K. Structure and function of VEGF receptors. IUBMB Life. 2009;61(9):915–22.
102. Walter M, Lucet IS, Patel O, Broughton SE, Bamert R, Williams NK, et al. The 2.7 Å crystal structure of the autoinhibited human c-Fms kinase domain. J Mol Biol. 2007;367(3):839–47.
103. Shein SA, Kuznetsov II, Abakumova TO, Chelushkin PS, Melnikov PA, Korchagina AA, et al. VEGF-and VEGFR2-targeted liposomes for cisplatin delivery to glioma cells. Mol Pharm. 2016;13(11):3712–23.
104. Zhang Y, Schwerbrock NM, Rogers AB, Kim WY, Huang L. Codelivery of VEGF siRNA and gemcitabine monophosphate in a single nanoparticle formulation for effective treatment of NSCLC. Mol Ther. 2013;21(8):1559–69.
105. Zhu R, Wang Z, Liang P, He X, Zhuang X, Huang R, et al. Efficient VEGF targeting delivery of DOX using Bevacizumab conjugated SiO2@ LDH for anti-neuroblastoma therapy. Acta Biomater. 2017;63:163–80.
106. Chen W-H, Sung SY, Fadeev M, Cecconello A, Nechushtai R, Willner I. Targeted VEGF-triggered release of an anti-cancer drug from aptamer-functionalized metal–organic framework nanoparticles. Nanoscale. 2018;10(10):4650–7.
107. Coutelle O, Schiffmann L, Liwschitz M, Brunold M, Goede V, Hallek M, et al. Dual targeting of Angiopoietin-2 and VEGF potentiates effective vascular normalisation without inducing empty basement membrane sleeves in xenograft tumours. Br J Cancer. 2015;112(3):495.
108. Ahmad I, Iwata T, Leung HY. Mechanisms of FGFR-mediated carcinogenesis. Biochim Biophys Acta-Molecular Cell Research. 2012;1823(4):850–60.
109. Grose R, Dickson C. Fibroblast growth factor signaling in tumorigenesis. Cytokine Growth Factor Rev. 2005;16(2):179–86.
110. Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, et al. Receptor specificity of the fibroblast growth factor family. J Biol Chem. 1996;271(25):15292–7.
111. Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. Receptor specificity of the fibroblast growth factor family, part II. J Biol Chem. 2006;281(23): 15694–15700
112. Eswarakumar V, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16(2):139–49.
113. Ornitz DM, Herr AB, Nilsson M, Westman J, Svahn C-M, Waksman G. FGF binding and FGF receptor activation by synthetic heparan-derived di-and trisaccharides. Science. 1995;268(5209):432–6.
114. Thisse B, Thisse C. Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol. 2005;287(2):390–402.
115. Citores L, Khnykin D, Sorensen V, Weschle J, Klingenberg O, Wiedlocha A, et al. Modulation of intracellular transport of acidic fibroblast growth factor by mutations in the cytoplasmic receptor domain. J Cell Sci. 2001;114(9):1677–89.
116. Knights V, Cook SJ. De-regulated FGF receptors as therapeutic targets in cancer. Pharmacol Ther. 2010;125(1):105–17.
117. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov. 2009;8(3):235.

118. Mohammadi M, Honegger A, Rotin D, Fischer R, Bellot F, Li W, et al. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. Mol Cell Biol. 1991;11(10):5068–78.
119. Powers C, McLeskey S, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer. 2000;7(3):165–97.
120. Mohammadi M, Dikic I, Sorokin A, Burgess W, Jaye M, Schlessinger J. Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol Cell Biol. 1996;16(3):977–89.
121. Furdui CM, Lew ED, Schlessinger J, Anderson KS. Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. Mol Cell. 2006;21(5):711–7.
122. Tiong KH, Mah LY, Leong C-O. Functional roles of fibroblast growth factor receptors (FGFRs) signaling in human cancers. Apoptosis. 2013;18(12):1447–68.
123. Kouhara H, Hadari Y, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I, et al. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell. 1997;89(5):693–702.
124. Ong S, Hadari Y, Gotoh N, Guy G, Schlessinger J, Lax I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc Natl Acad Sci. 2001;98(11):6074–9.
125. Foehr ED, Raffioni S, Fuji R, Bradshaw RA. FGF signal transduction in PC12 cells: comparison of the responses induced by endogenous and chimeric receptors. Immunol Cell Biol. 1998;76(5):406–13.
126. Szlachcic A, Zakrzewska M, Lobocki M, Jakimowicz P, Otlewski J. Design and characteristics of cytotoxic fibroblast growth factor 1 conjugate for fibroblast growth factor receptor-targeted cancer therapy. Drug Des Devel Ther. 2016;10:2547.
127. Bhide RS, Lombardo LJ, Hunt JT, Cai Z-w, Barrish JC, Galbraith S, et al. The antiangiogenic activity in xenograft models of brivanib, a dual inhibitor of vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 kinases. Mol Cancer Ther. 2010;9:369. 1535-7163. MCT-09-0472.
128. Martin LP, Sill M, Shahin MS, Powell M, DiSilvestro P, Landrum LM, et al. A phase II evaluation of AMG 102 (rilotumumab) in the treatment of persistent or recurrent epithelial ovarian, fallopian tube or primary peritoneal carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol. 2014;132(3):526–30.
129. Balis FM, Thompson PA, Mosse YP, Blaney SM, Minard CG, Weigel BJ, et al. First-dose and steady-state pharmacokinetics of orally administered crizotinib in children with solid tumors: a report on ADVL0912 from the Children's Oncology Group Phase 1/Pilot Consortium. Cancer Chemother Pharmacol. 2017;79(1):181–7.
130. Abou-Alfa GK, Meyer T, Cheng A-L, El-Khoueiry AB, Rimassa L, Ryoo B-Y, et al. Cabozantinib in patients with advanced and progressing hepatocellular carcinoma. N Engl J Med. 2018;379(1):54–63.
131. Tolcher AW, Sarantopoulos J, Patnaik A, Papadopoulos K, Lin C-C, Rodon J, et al. Phase I, pharmacokinetic, and pharmacodynamic study of AMG 479, a fully human monoclonal antibody to insulin-like growth factor receptor. Clin Oncol. 2007;25:1390.
132. Di Cosimo S, Sathyanarayanan S, Bendell JC, Cervantes A, Stein MN, Braña I, et al. Combination of the mTOR inhibitor ridaforolimus and the anti-IGF1R monoclonal antibody dalotuzumab: preclinical characterization and phase I clinical trial. Clin Cancer Res. 2015;21(1):49–59.
133. Wen PY, Yung WA, Lamborn KR, Dahia PL, Wang Y, Peng B, et al. Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. Clin Cancer Res. 2006;12(16):4899–907.
134. Shirley M. Olaratumab: first global approval. Drugs. 2017;77(1):107–12.
135. Younus J, Verma S, Franek J, Coakley N. Sunitinib malate for gastrointestinal stromal tumour in imatinib mesylate–resistant patients: recommendations and evidence. Curr Oncol. 2010;17(4):4.

136. Brandes AA, Carpentier AF, Kesari S, Sepulveda-Sanchez JM, Wheeler HR, Chinot O, et al. A phase II randomized study of galunisertib monotherapy or galunisertib plus lomustine compared with lomustine monotherapy in patients with recurrent glioblastoma. Neuro-Oncology. 2016;18(8):1146–56.
137. Morris JC, Tan AR, Olencki TE, Shapiro GI, Dezube BJ, Reiss M, et al. Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. PLoS One. 2014;9(3):e90353.
138. Pinter T, Klippel Z, Cesas A, Croitoru A, Decaestecker J, Gibbs P, et al. A phase III, randomized, double-blind, placebo-controlled trial of pegfilgrastim in patients receiving first-line FOLFOX/bevacizumab or FOLFIRI/bevacizumab for locally advanced or metastatic colorectal cancer: final results of the pegfilgrastim and anti-VEGF evaluation study (PAVES). Clin Colorectal Cancer. 2017;16(2):103–14. e3.
139. Zhang Y, Han C, Li J, Zhang L, Wang L, Ye S, et al. Efficacy and safety for Apatinib treatment in advanced gastric cancer: a real world study. Sci Rep. 2017;7(1):13208.
140. Qin S. Phase III study of apatinib in advanced gastric cancer: a randomized, double-blind, placebo-controlled trial. Am Soc Clin Oncol. 2014;32:15:4003–4003.
141. Kuo T, Cabebe E, Koong A, Norton J, Kunz P, Ford J, et al. An update of a phase I/II study of the VEGF receptor tyrosine kinase inhibitor vatalanib and gemcitabine in patients with advanced pancreatic cancer. J Clin Oncol. 2008;26(15_suppl):15571.
142. Motzer RJ, Porta C, Vogelzang NJ, Sternberg CN, Szczylik C, Zolnierek J, et al. Dovitinib versus sorafenib for third-line targeted treatment of patients with metastatic renal cell carcinoma: an open-label, randomised phase 3 trial. Lancet Oncol. 2014;15(3):286–96.
143. Perez-Garcia J, Muñoz-Couselo E, Soberino J, Racca F, Cortes J. Targeting FGFR pathway in breast cancer. Breast. 2018;37:126–33.
144. Sobhani N, Ianza A, D'Angelo A, Roviello G, Giudici F, Bortul M, et al. Current status of fibroblast growth factor receptor-targeted therapies in breast cancer. Cell. 2018;7(7):76.
145. Vergote I, Teneriello M, Powell MA, Miller DS, Garcia AA, Mikheeva ON, et al. A phase II trial of lenvatinib in patients with advanced or recurrent endometrial cancer: angiopoietin-2 as a predictive marker for clinical outcomes. Am Soc Clin Oncol. 2013;31:15:5520–5520.

Chapter 8
Lung Cancer Receptors and Targeting Strategies

Uday Koli, Anomitra Dey, P. Nagendra, Padma V. Devarajan, Ratnesh Jain, and Prajakta Dandekar

Abstract Lung cancer still remains the leading cause of cancer-related deaths worldwide. Till now, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) have effectively responded to conventional therapy. However, because of cancer nature and subsequent side effects of conventional therapy, inventing novel drug targets for lung cancer therapies has become essential. The disease management recently has seen a paradigm shift with the advent of next-generation sequencing, which has extensively affected the disease prognosis and hence led to newer targeted therapies. Receptors particularly have played an important role as molecular targets and hence presented new opportunities for intracellular targeting of drug delivery systems. Such approach for therapy not only improves the efficacy of the drug but also reduces the overall systemic cytotoxicity. This chapter extensively focuses on such receptors targeted for lung cancer therapy. Further, the role of receptors like epidermal growth factor receptor (EGFR), c-MET, and vascular endothelial growth factor (VEGF) has been discussed with respect to their appropriate ligand(s) binding and developed nanocarrier system for targeting. In addition, this chapter presents the current status of clinical outcomes of conventional drugs in targeting these receptors and thus improving the overall survival rate in patients suffering from this dreaded disease.

Keywords Lung cancer · epidermal growth factor receptor (EGFR) · ligand targeting · c-MET receptor · vascular endothelial growth factor receptor

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Abbreviations

ADC	Adenocarcinomas
ALK	Anaplastic lymphoma kinase
AREG	Amphiregulin
ADCC	Antibody-mediated cellular cytotoxicity
ATP	Adenosine triphosphate
ADAM	A disintegrin and metalloproteinase
BTC	Betacellulin
CRR	Confirmed response rate
EGF	Epidermal growth factor
EPG	Epigen
EPR	Epiregulin
EGFR	Epidermal growth factor receptor
Grb2	Growth factor receptor-bound protein 2
HAP	Hypoxia-activated prodrugs
HIF-1α	Hypoxia inducible factor-1α
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HB-EGF	Heparin-binding EGF
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
NSCLC	Non-small cell lung cancer
NRG	Neuregulins
ORR	Objective response rate
PI3K	Phosphatidylinositol 3′-kinase
PLC	Phospholipase C
PLC-γ	Phospholipase C-γ
PEI	Polyethylenimine
RTKs	Receptor tyrosine kinases
SCC	Squamous cell carcinomas
SCLC	Small cell lung cancer
STATs	Signal transducers and activators of transcription
SAR	Structure activity relationship
ScFv	Single chain variable fragment
TGF-α	Transforming growth factor alpha
TKIs	Tyrosine kinase inhibitors
TGFβ1	Transforming growth factor beta 1
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

1 Introduction

Lung cancer has been one of the major cancer types that are associated with a high-mortality rate, all over the globe. Lung cancer is more commonly observed in male patients than in females, and has a higher prevalence in the geriatric population [1]. Over the past century, there has been tremendous advancement in the pathophysiological understanding about lung cancer. However, the major drawback associated with this disease is its poor prognosis, often leading to an inoperable condition. Nevertheless, considerable progress is being made in the development of newer strategies against lung cancer, especially with regard to the discovery of newer therapeutic targets, development of various therapeutic molecules, either small molecules or macromolecules like the antibody-based options, and also advanced delivery approaches like the targeted delivery systems or combinatorial therapies.

The molecular basis of lung cancer is complex and heterogeneous. Therefore, it is important to understand the molecular alterations at multiple levels, namely, genetic, epigenetic, and protein expression, and their functional significance, which may have the potential to impact the diagnosis, prognosis, and treatment of lung cancer. Lung cancers may develop through multistep processes involving several genetic and epigenetic alterations, particularly activation of growth-promoting pathways and inhibition of tumor suppressor pathways. A greater understanding of these biochemical pathways is thus crucial for the development of treatment strategies that can target the molecular aberrations underlying lung cancer, as well as their downstream pathways.

1.1 Classification of Lung Cancer

Based on their histology, the lung cancers are classified into two main types, which include the non-small cell lung cancer (NSCLC) and the small cell lung cancer (SCLC). Among these, the NSCLC is more predominant and demonstrates an occurrence of almost 85%, as shown in Fig. 8.1 [2]. The NSCLC is further classified into three major types, namely, the *adenocarcinoma*, the squamous cell carcinoma, and the large-cell carcinoma. The origin of the NSCLC is mostly epithelial, whereas that of the SCLC is neuroendocrine [3, 4]. The main characteristics of the different kinds of lung cancers with respect to their origin, occurrence, and their prominent features have been listed in Table 8.1.

2 Brief Overview of the Receptors Associated with Specific Forms of Lung Cancer

Receptor-mediated tumor targeting has received considerable attention in the field of anticancer therapeutics due to their specific action. Targeting the receptors, overexpressed in cancers, has opened new opportunities for intracellular targeting of

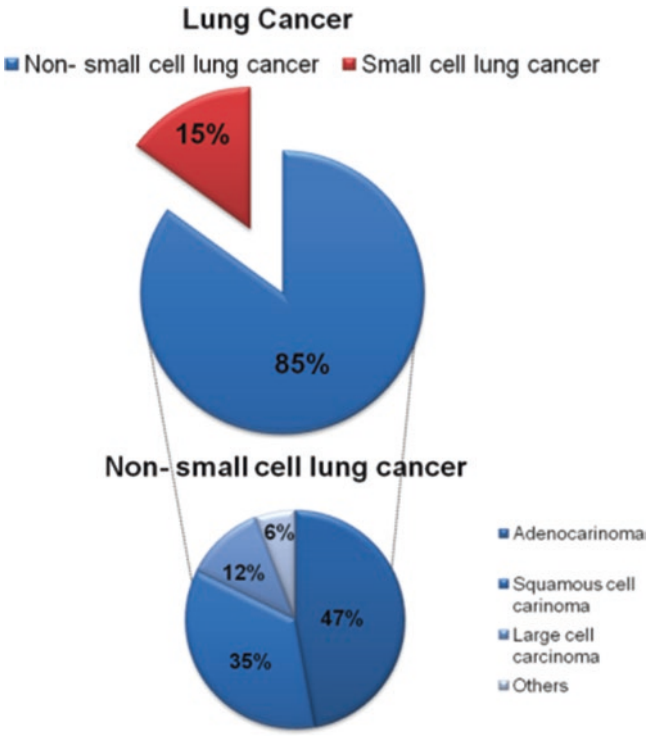


Fig. 8.1 Statistics for the occurrence of different types of lung cancer

drugs and delivery systems that are conjugated with targeting moieties, that is, the ligands. This receptor-mediated targeting of anticancer drugs, especially using nano-sized carrier systems, protects them from the degrading body environment and improves their pharmacokinetic properties by extending their circulation time within the body. Moreover, it also helps to overcome the systemic toxicity and adverse effects that arise due to the nonselective nature of most of the current anticancer therapeutic agents [5].

Recently, a large number of molecular changes, such as mutations and gene amplifications, have been found to be responsible for tumor survival and cancer prognosis [6]. The targeted anticancer therapies also aim to focus on these common cellular modulations that take place at the molecular level. Targeting of these modulations enhances the survival rates in patients, which may not be possible in nonsurgical stages [7]. Personalized therapy can be used to target the cancers, according to the patients' predisposition to them, according to the individuals' genomic profiles, and can hence deliver appropriate drugs, at the correct dose and at the right time [8]. Some of the mediators that may play a predominant role in the treatment of lung cancer include the epidermal growth factor receptor (EGFR), the

Table 8.1 Characteristics of various forms lung cancer

Sr. No	Cancer type	Characteristics
1.	Small cell-lung cancer	Neuroendocrine in origin Highly metastatic and subsequent relapse observed Rarely found in nonsmokers Difficulty in surgical resection
2.	Non-small cell lung cancer	Common form of lung cancer Easily removed by surgical resection, by standard care for localized occurrence
(a) Adenocarcinoma		Major type of NSCLC Caused due to exposure to radiation and carcinogens Originates from peripheral tissue of the lungs, mostly mucus-secreting cells Spreads at a lower rate Bronchioloalveolar carcinoma is adenocarcinoma
(b) Squamous cell carcinoma		Second common type of NSCLC Originates in the airway lining of the lung cells
(c) Large-cell carcinoma		Difficult to treat Originates in the central part of the lungs, may have neuroendocrine origin Quick in growth and spreads rapidly Mostly discovered at later stages

vascular endothelial growth factor (VEGF), the anaplastic lymphoma kinase (ALK), etc. [9]. A comprehensive list of all the prognostic factors or receptors that are significant during the development of lung cancer has been stated in Table 8.2.

Out of these receptors, EGFR, c-MET, and VEGFR have been discussed in details in relevance to lung cancer as they have been extensively studied and exploited for cancer therapy.

3 The EGFR Receptor

The EGFR is highly expressed in almost all types of lung cancers. The receptor has been extensively studied, specifically for targeting the NSCLC, since mutations of EGFR in SCLC patients are rare [36]. This growth factor triggers signaling through the EGFR receptor tyrosine kinase (RTK), which promotes cell growth and eventually leads to the metastasis of lung cancer. The strategies employed for inhibiting EGFR include inactivation of the TK signaling cascade or the use of antibodies to neutralize the EGFR and its associated ligands. There have been several reports about drugs and monoclonal antibodies that have been successfully used against the EGFR. However, the major concern with these therapies is the eventual development of resistance by the receptor, which has necessitated combination therapies, using dual drug systems or drug-antibody systems [10, 37].

Table 8.2 Different receptors features expressed in lung cancer

No.	Receptor name	Family/class	Type of cancer	Role/remarks	Current drugs/target therapy	References
1	Epidermal growth factor receptor (**EGFR**)	ErbB family of receptors	SCLC, NSCLC	Triggers proliferation of malignant lung cells, promotes antiapoptosis, and lung cancer metastasis	Cetuximab, bevacizumab, gifitinib, erlotinib, icotinib, and afatinib	[10]
2	Folate receptor alpha (**FRα**)	Folate receptor (FOLR) family	NSCLC	FRα is an acquired marker for the proliferation of tumor cells, tumor biology, and for patient prognosis	Vintafolide or MK-8109, farletuzumab, FRA-specific CAR-modified T-cells	[11]
3	Human epidermal growth factor receptor 2 (**HER2/neu**)	ErbB family of receptors	NSCLC	Involved in signal transduction pathways, leading to cell growth, and differentiation	Afatinib, rapamycin, trastuzumab, and trastuzumab/paclitaxel combination	[12, 13]
4	TNF-related apoptosis-inducing ligand (TRAIL) death receptors DR4 & DR5	Tumor necrosis factor receptorsuperfamily (TNFRSF)	NSCLC	These are plasma membrane proteins containing the intracellular death domains essential for the transmission of the death signals upon binding of TRAIL	rhTRAIL	[14, 15]
5	Tetraspanins CD151	Transmembrane 4 superfamily (TM4SF) proteins	NSCLC	CD151 has a key role in cell proliferation, migration, colony formation, and signal transduction	CD151-siRNA, anti-CD151 mAb (1A5)	[16–18]
6	Mesenchymal–epithelial transition (MET)	Belongs to tyrosine kinase receptor family	NSCLC and mostly in lung adenocarcinoma	Amplification of MET leads to overexpression of HGFR, which is involved in cancer cell proliferation, migration, invasion, and metastasis. MET amplification commonly causes resistance of EGFR TKI	Therapies for MET are in clinical trial phase. Onartuzumab, a monoclonal antibody against MET has shown good results in phase II clinical trials. Crizotinib is a drug being developed against MET. Also, combination therapies with EGFR TKI, are being studied: erlotinib ± cabozantinib, erlotinib ± tivantinib, gefitinib + capmatinib, etc., are also used	[19, 20]

7	Estrogen receptors (**ERα and ERβ**)	Steriod hormone superfamily of nuclear receptors	NSCLC	ERβ activates PI3K/AKT/Bcl-XL and the RAS/RAF/MEK/ERK signaling pathways to regulate, cell proliferation, invasion, metastasis, mitochondrial biogenesis, and antiapoptosis	Fulvestrant, aromatase inhibitors, letrazole, exemestane, and tamoxifen	[21]
8	G-protein-coupled estrogen receptors	Steroid hormone superfamily of nuclear receptors	NSCLC	Involved in the cancer cell proliferation, migration, and invasion and acts as a modulator of the neoplastic transformation	Antagonists/modulators of the classical estrogen receptors, such as tamoxifen, raloxifen, and fulvestrant, were found to be the GPER agonists	[22]
9	Progesterone receptors (**PRA and PRB**)	Steroid hormone superfamily of nuclear receptors	NSCLC	Promotes tumor angiogenesis	Progesterone	[23]
10	Peroxisome proliferator-activated receptor gamma (**PPARγ**),	Nuclear receptor subfamily	NSCLC	PPARγ activationcan inhibit nuclear factor-κB and COX-2 expression in NSCLC	Rosiglitazoneand troglitazone, thiazolidinedione class (TZDs)	[24]
11	Somatostatin	G-protein-coupled receptor family	SCLC, NSCLC	SSTRs signal through phosphotyrosine phosphatases to induce apoptosis, as well as to decrease cell proliferation	Peptide-receptor radiation therapy (PRRT)	[25, 26]
12	Neuropilins receptor proteins (**NRP**)	Belongs to family semaphorins, which are associated with the secreted, transmembrane and GPI-linked proteins for axonal guidance and development of nervous system	NSCLC	NRPS are associated with increased tumor vascularization and poor diagnosis. These receptors interact with the VEGFs and other growth factors and also c-MET. Targeting NRPs results in both,greater vascular defects and death	Anti-NRP antibodies have been injected in animal models and have resulted in inhibition of tumor angiogenesis. Sulfated polysaccharideslike dextran sulfate and fucoidan have been used to induce internalization of NRP1. This blocks the function of SEMA3A and VEGF165 in the endothelial cells and promotes their internalization into the lysosomes	[3, 27–29]

(continued)

Table 8.2 (continued)

No.	Receptor name	Family/class	Type of cancer	Role/remarks	Current drugs/target therapy	References
13	Echinoderm microtubule-associated protein-like 4 -Anaplastic lymphoma kinase (**EML4-ALK**)	Belongs to tyrosine kinase receptor, a part of the superfamily of insulin	NSCLC	Overexpression in NSCLC due defect in the ALK receptor gene. It inhibits apoptosis, which promotes the proliferation of tumor cells	Crizotinib and ceritinib are FDA-approved drugs for the treatment in patients with ALK-positive lung cancer. Other drugs like brigatinib,alectinib, and lorlatinib are in clinical trial stages. However, limitation with these is that they are susceptible to resistance	[9, 30]
14	Vascular endothelial growth factor receptor (**VEGFR**)	Belongs to tyrosine kinase receptor	NSCLC	Overexpression in tumor promotes angiogenesis along with tumor progression	Monoclonal antibody bevacizumab is used to neutralize VEGFR isoforms. Further, dual therapy is used for inhibition of EGFR and VEGFR, using erlotinib and bevacizumab	[9, 31]|
15	Cluster differentiation 44 **(CD44)**	Transmembrane glycoprotein	NSCLC and squamous metaplasia	Progression of tumor with malignant and metastatic features. It also contributes to drug resistance in NSCLC	Various therapies are under review for CD44. But none of the therapies has made its way to the clinical trials. Interaction of CD44 with hyaluronan is under review along with RNAi technologies	[32, 33]
16	CD24	Cancer stem cell-associated membrane protein, a glycosylphosphatidyl-inositol-anchored molecule	Highly expressed in SCLC, but rarely in NSCLC	Increases cell invasion via biomechanical processes, increases tumor hypoxia by promoting production of H1F1α	Monoclonal antibody SWA11 and doxorubicin conjugated to SWA11	[34]
17	Bombesin receptors (**BBR1, BBR2, and BBR3**)	Group of G-protein-coupled receptors, which bind bombesin	SCLC, NSCLC	Involved in SCLC invasion and metastasis	GRPR antagonist RC-3095 with gemcitabine and temozolomide, cytotoxic agents like 5-FU, irinotecan, silencing of GRPR/GRP by siRNA-delivery	[34, 35]

18	Bradykinin receptors	Group of G-protein-coupled receptors	SCLC	Diverse functions including cell proliferation, leukocyte activation, cell migration, endothelial cell activation, and nociception	Bradykinin antagonist dimerCU201, in combination with doxorubicin, etoposide, cisplatin,vinorelbine, and paclitaxel increases SCLC growthinhibition	[34]
19	Oxytocin and vasopressin receptors	Tissue-specific G protein-coupled receptors	SCLC, NSCLC	ERK 1/2 phosphorylation and thesubsequent p90 ribosomal S6 kinase phosphorylation	Monoclonal antibody MAG-1	[34]
20	Insulin-like growth factor 1 receptor	Tyrosine kinase receptors	SCLC	Associated with apoptosis inhibition and proliferation stimulationthrough downstream signaling pathways including PI3K-Akt andMAPK	OSI-906	[34]
21	Fibroblast growth factor receptors	Members of the fibroblast growth factor family of proteins	SCLC	Promotes cancer progression, neoangiogenesis, and resistance to targeted treatments	Engineered soluble FGF receptor 1 Fc fusion protein, FP-1039 binds tightly to the mitogenic FGFs,inhibits FGF-stimulated cell proliferation, blocks FGF- and VEGF-induced angiogenesis and inhibits tumor growth FGFR pathway inhibition remains an active area of investigation in the SCLC and other tumors, in which this pathway is dysregulated	[34]

The EGFR is a 178 kDa transmembrane protein belonging to the receptor tyrosine kinase (RTK) family of proteins. The family consists of four members, namely, EGFR (Erb1, Her1), Erb2 (neu, Her2), Erb3 (Her3), and Erb4 (Her4). The receptor plays an important role in various cellular functions, including cell proliferation, survival, differentiation, and motility, and is necessary for the normal development of the organism [38].

3.1 Recognition Domain of the EGFR Receptor

All the aforementioned EGFR receptors share a basic structure, as depicted in Fig. 8.2, which consists of an extracellular binding domain that interacts with the ligands, the transmembrane domain traversing the lipid bilayer and the tyrosine kinase domain, on the cytoplasmic side, along with –COOH terminal tail containing several phosphorylation sites [39].

The extracellular region of the Erb family of receptors consists of 621 amino acids and includes two ligand-binding homologous domains (I and III) and two cysteine-rich domains (II and IV).

The transmembrane domain is made up of a single alpha helix containing 23 amino acids. The cytoplasmic domain consists of 542 amino acids that form the juxtamembrane cytoplasmic domain, a tyrosine kinase domain, followed by the carboxyl group terminal tail that encompasses multiple phosphorylation sites.

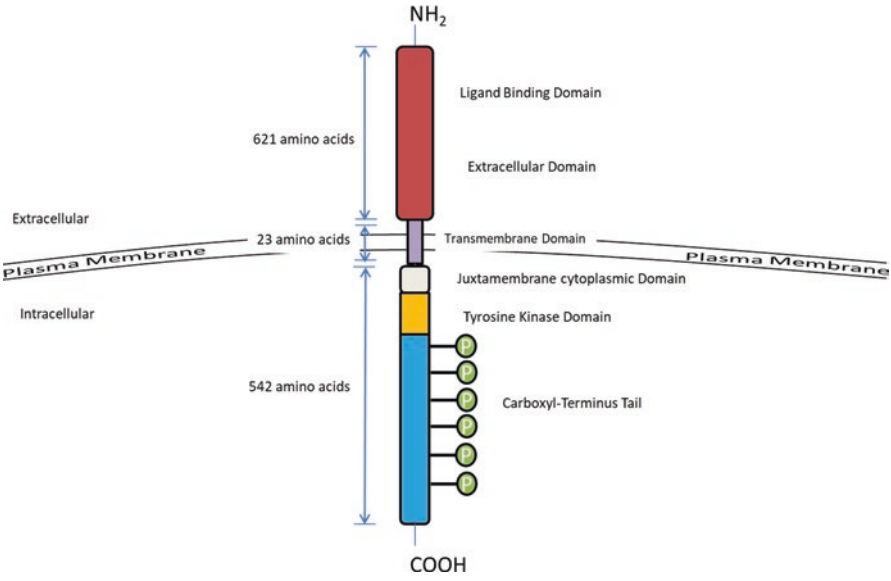


Fig. 8.2 The general structure of EGFR comprising the ligand-binding domain, transmembrane domain, tyrosine kinase domain, and carboxy terminal tail

The intracellular domain of the receptor has 20 tyrosine residues, out of which 12 are known to undergo phosphorylation. These phosphorylation sites serve as binding sites for the membrane-bound or soluble effector molecules, upon activation of the receptor.

Besides the membrane-bound forms, the Erb receptors are also found in soluble forms. The latter do not possess the transmembrane and the cytoplasmic domains and may be generated by proteolytic cleavage of the membrane-bound receptor or by alternative splicing [40].

Activation of the EGFR is controlled by its ligands. Upon binding with its ligand, a single molecule of EGFR dimerizes with another similar EGFR molecule (homodimerization) or with another member of the EGFR family (heterodimerization), preferably Erb2. Upon activation, the cytoplasmic side having the tyrosine kinase domains on both members of the dimer undergoes activation and is autophosphorylated at selective tyrosine residues in the tail region. The autophosphorylation sites serve as docking sites, directly or indirectly, for small signaling molecules such as Grb2, Grb7, Shc, Crk PLC-γ, SRC, PI-3K, and protein phosphatases – SHP1 and SHP2 and E3 ubiquitin ligase Cbl. Other molecules like STAT1, STAT3, STAT5, and PLD participate indirectly by playing a role in signaling. The activation of EGFR further stimulates several other pathways, which have been summarized in Fig. 8.3.

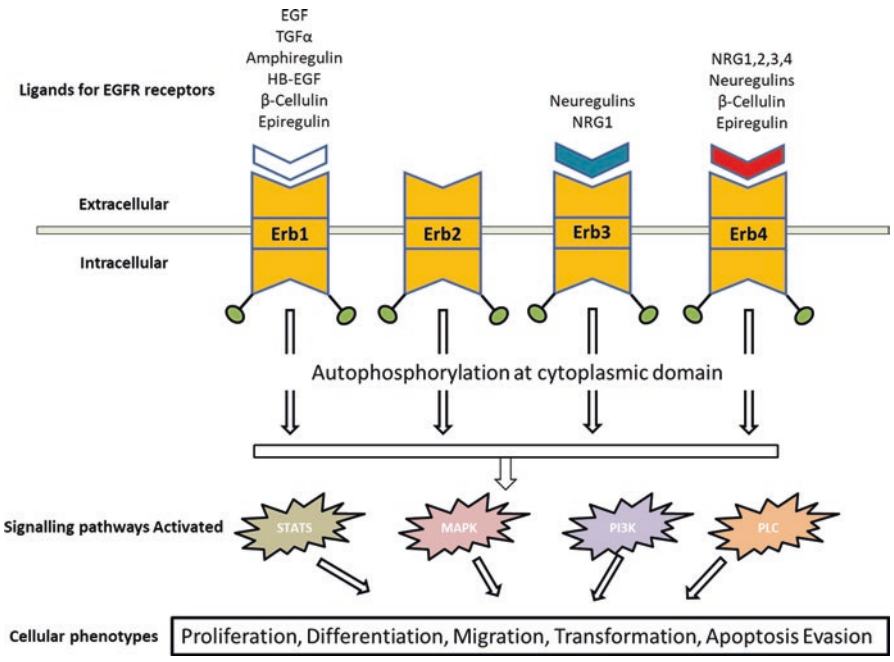


Fig. 8.3 The EGFR signaling network. MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; PLC, phospholipase C; STATs, signal transducers and activators of transcription

It is likely that the Erb2 receptor is necessary for the induction of tumor growth. The heterodimerization of Erb2 receptors with rest of the family members is an important mechanism for the oncogenic transformation of various types of tumors. This was studied using NIH3T3 cell line, which did not express any of the EGFR receptors. The influence of heterodimerization on tumor growth was assessed by transfecting various combinations of EGFR receptors in NIH3T3 cell line [41]. Those cells which expressed homodimers of Erb2, Erb3, and Erb4 did not induce any tumor growth, whereas cells which expressed only Erb1 had moderate tumorigenic characteristics. Interestingly, the Erb2/Erb3 pair was able to induce tumor growth, whereas the Erb1/Erb3 and Erb1/Erb4 pairs did not. On the contrary, the Erb1/Erb2 heterodimer pair was able to produce an aggressive tumorigenic phenotype in the NIH3T3 cells. Coexpression of Erb1 and Erb2 synergistically heightened the cellular response of the EGFRs and increased the overall expression of the proliferative markers [42].

In addition to this ligand-induced dimerization model for EGFR activation, EGFR can be activated by another model of ligand binding, known as the "rotational model." According to this model, the EGFR exists in an inactive, unliganded dimeric form. Once the ligand binds to the extracellular domain, a rotation is induced in its transmembrane domain, in a direction parallel to the plane of the lipid membrane of the inactive, dimeric form. This conformational change rearranges the intracellular kinase domain that leads to the conversion of the inactive symmetric receptor to an active asymmetric form.

3.2 Binding of Ligands with EGFR

As mentioned earlier, the dimerization of EGFR receptors is due to their ligands. There are 11 known ligands associated with the EGFR receptors. These can be divided into three groups, namely, (i) those that activate ErB1, namely, the epidermal growth factor (EGF), the transforming growth factor (TGF)-α, amphiregulin (AREG), and epigen (EPG), (ii) those which are formed by ligands that are bispecific to ErB1 and ErB4, namely, betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), and (iii) those which are formed by neuregulins (NRG), which can bind to both, ErB3 and ErB4 (NRG1 and NRG3) or only ErB4 (NRG3 and NRG4). There are no known ligands that bind to the Erb2 receptor, which forms heterodimers with the other members of the EGFR receptor family, and its overexpression in cells causes ligand-independent cell transformation [43].

Each of these ligands has an EGF-like core domain, consisting of ~60 amino acids, which is responsible for facilitating their biological activity [44]. They are manufactured as type 1 transmembrane precursors that are usually cleaved from the extracellular domain to soluble forms, which then bind to the EGFRs and activate them. This cleavage is promoted by the proteins of a disintegrin and metalloproteinase family (ADAM), which form the soluble peptides, containing at least one EGF-like domain and spatially arranged cysteine residues, and are capable of EGFR

activation. These soluble factors bind to the EGFRs on the cells present at a distance from the release site (endocrine), the neighboring cells (paracrine) or the EGFRs present on the same cells (autocrine). Although the separation of EGFR-specific ligands seems to be an important step for receptor activation, many of the ligands such as HB-EGF, TGF-α, AREG, and BTC, are capable of activating the EGFRs even when they are hitched to the plasma membrane (juxtacrine) [40].

Upon ligand binding, the activated EGFR cluster is internalized via clathrin-coated, receptor-mediated endocytosis, where E3 ubiquitin ligase induces lysosomal degradation. The internal EGFR signaling and trafficking differs according to the various ligands of the receptor. HB-EGF and BTC signal continuous phosphorylation, ubiquitination, and degradation of EGFR. On the other hand, binding of TGF-α leads to temporary phosphorylation, minimum ubiquitination, and complete recycling of the endosomes containing the EGFRs. Inside the early endosomes, the TGF-α dissociates more readily from the receptor, due to the slightly acidic environment, which causes differential trafficking, and recycles the unbound EGFR back to the membrane [40, 45].

The EGFR receptors are internalized not only via clathrin-mediated endocytosis, but also via the caveolae. The mode of endocytosis is determined by the concentration of the ligand present, with a higher concentration inducing continuous phosphorylation and receptor degradation, leading to clathrin-independent endocytosis. On the other hand, lower ligand concentrations lead to clathrin-dependent endocytosis, along with receptor recycling [40].

3.3 Antagonists for Ligand Binding

Over the last decade, research in targeting of lung cancer, especially the NSCLC has been revolving around the use of two major receptor-targeting strategies. First is the use of immune inhibitors, namely, the anti-EGFR antibodies that bind to the extracellular domain and are highly specific for the receptor. The second strategy involves the use of small-molecule inhibitors that compete reversibly with the ATP to bind to the intracellular tyrosine kinase domain of EGFR, thus restricting autophosphorylation and blocking the downstream signaling. The mechanism of action and the biological effect of mAbs and small-molecule tyrosine kinase inhibitors (TKIs) depend on the route of administration, their bio-distribution, induction of EGFR downregulation, and activation of other immune functions. Despite their varied mechanisms of action, EGFR inhibition leads to some common antitumor effects such as inhibition of cancer cell proliferation by arresting the cell cycle in G0/G1 phase, induction of apoptosis, reduced production of the angiogenic growth factors, prohibition of cellular invasion and metastasis, and sensitization of the tumors to cytotoxic drugs and radiotherapy [46].

Among the anti-EGFR mAbs such as cetuximab, specifically bind to the extracellular region of the EGFR in its inactive form and thus obstruct the ligand-binding sites and block the activation of tyrosine kinase [47]. Apart from blocking the

signaling pathways, mAbs also display antitumor action through antibody-mediated cellular cytoxicity (ADCC) and complement-mediated toxicity [48].

Cetuximab is one of the most extensively studied anti-EGFR antibodies for targeting advanced NSCLC. It is a chimeric human murine IgG1 monoclonal antibody, obtained from the myeloma cell line. It consists of murine Fv EGFR-binding region and human IgG1 heavy and light chain Fc regions, collectively having an approximate molecular weight of 152 kDa. It binds to the ligand-binding domain III of the EGFR, with a high affinity (dissociation constant Kd of 1.8 nM, ~10-fold higher than its ligand), and thereby restricts the activation of downstream intracellular signaling, particularly mitogen-activated kinases pathway, by inhibiting the receptor dimerization. It has been observed in certain studies that cetuximab enhances the cellular internalization of the receptor thereby reducing the number of receptors available for ligand binding [49].

Other early competitors of cetuximab included panitumumab and matuzumab. However, they failed in phase II clinical trials as their combination with chemotherapeutic agents did not demonstrate a benefit to patients compared to the chemotherapy alone [50]. Other antibodies targeted toward EGFR include nimotuzumab, pertuzumab, trastuzumab, and necitumumab. Necitumumab has been recently approved by the USFDA for the treatment of squamous cell lung cancer, based on the results of the SQUIRE trial [51]. Its role in targeting of lung cancer, as well as the clinical efficacy in targeting various lung cancer conditions, has been elaborated [52].

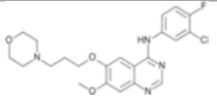
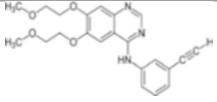
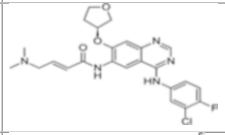
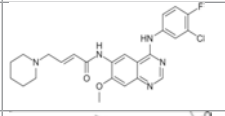
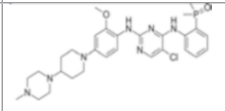
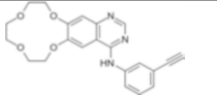
Among the TKIs, gefitinib (ZD1839, Iressa), was the first drug developed to inhibit the EGFR. This molecule competes reversibly with ATP to bind to the intracellular domain of the EGFR and blocks autophosphorylation and downstream signaling. It is an orally administered, low molecular weight, anilinoquinazoline tyrosine kinase inhibitor. A dose of about 250 mg/day is administered for the inhibition of the EGFR and its downstream signaling processes [53]. Gefitinib selectively binds to the EGFR tyrosine kinases and does not inhibit serine threonine kinases [54]. It is capable of arresting the cell cycle in the G1 phase and it reduces the levels of important angiogenesis factors like the VEGF [55, 56].

Other EGFR TKIs include erlotinib, icotinib, afatinib, dacomitinib, osimertinib, rociletinib, brigatinib, olmutinib. Out of these, GILOTRIF (afatinib), IRESSA (gefitinib), TAGRISSO (osimertinib), TARCEVA (erlotinib), VIZIMPRO (dacomitinib) have been approved for the first-line treatment of NSCLC with EGFR mutation.

3.4 Significant Inhibitors of EGFR

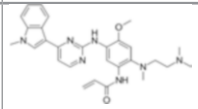
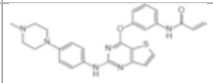
Till date, various drugs/inhibitors have been evaluated for their therapeutic effect in lung cancer. These have been enlisted in Table 8.3, along with their mechanisms of action and structures.

Table 8.3 Inhibitors/drugs for the treatment of lung cancer

Sr.No	Compound	Company	Structure	Mechanism of action	Developmental phase	References
Small molecule: tyrosine kinase inhibitors (TKI)						
1	Gefitinib	AstraZeneca/Teva		First inhibitor of EGFR tyrosine kinase; inhibits by binding to the adenosine triphosphate (ATP)-binding site of the enzyme; this leads to the inhibition of the Ras signal transduction pathway which is "antiapoptotic"; thus, the malignant cells are arrested	Approved	[57]
2	Erlotinib	Genentech/OSI Pharmaceuticals/Roche		Specific inhibitor of (EGFR) tyrosine kinase binds to the ATP-binding site in a reversible manner that inhibits the formation of phosphorylated residues in EGFR and arrests the signaling cascade	Approved	[58, 59]
3	Afatinib	Boehringer Ingelheim GmbH		First-generation TKI, irreversibly inhibits the EGFR by covalently binding to the cysteine 797 of the EGFR It also acts on the T790M mutants of the NSCLC	Approved	[60]
4	Dacomitinib	Pfizer		It is selective to EGFR and binds irreversibly to the receptor	III	[61]
5	Brigatinib	ARIAD Pharmaceuticals		First-generation potent ALK inhibitor of the target protein and of the mutant. Second-generation EGFR inhibitor that acts by deactivating the EGFR or its T790M mutant	III	[62]
6	Icotinib	Beta Pharma		First-generation TKI for the EGFR. Inhibits by competing against the ATP; binds to the ATP-binding site reversible and deactivates the EGFR	Approved	[63]

(continued)

Table 8.3 (continued)

Sr.No	Compound	Company	Structure	Mechanism of action	Developmental phase	References
7	Osimertinib	AstraZeneca		Third-generation EGFR TKI. It binds irreversibly to EGFR proteins expressed by the EGFRs with a T790M mutation; also binds irreversibly to EGFRs with L858R mutation and with an exon 19 deletion	Approved	[64]
8	Olumitinib	Hanmi Pharmaceutical/ BoehringerIngelheim		It is the second-line treatment for NSCLC, having T790M mutation of EGFR It covalently binds to the cysteine residue near the kinase domain of the EGFR	Approved	[65]
Monoclonal Antibodies (MAb)						
1	Cetuximab	Bristol-Myers Squibb/ Merck KGaA	Chimeric (mouse/ human) monoclonal antibody	Inhibits EGFR by interfering with the KRAS signaling cascade	Approved	[66]
2	Zalutumumab	Genmab	Fully human IgG1 monoclonal antibody	It binds to the EGFR Domain III on the cell surface. This locks the receptor in an inactive conformation, competing against the EGF, thus arresting dimerization and promoting apoptosis. It has also has shown ADCC activity	III	[67]
3	Nimotuzumab	Biocon/TheraCIM/ CIMYM Biosciences/ Theraloc/CIMAher	Humanized monoclonal antibody	Its binding affinity is toward the extracellular region of the EGFR, which blocks the ligand-binding region and thus arrests the EGFR activity.	III	[68]
Vaccines						
1	CimaVax-EGF	Centre of Molecular Immunology, Havana	Recombinant human EGF conjugated to a protein carrier	It is an active vaccine that produces antibodies against the EGF	I/II	[69]

3.5 Receptor-Mediated Targeting Strategies

Although various types of lung cancers have been treated using different mAbs and small-molecule TKIs, new strategies for actively targeting the EGFR are being researched by scientists around the globe.

3.6 Prodrugs/Drug Complexes

Prodrugs are medicines or compounds that upon administration are metabolically converted into pharmacologically active drugs. A prodrug can be designed to selectively interact with the cells or processes that are not its direct targets. It may help to improve the specific availability of the drug at the disease site and thus reduce the associated adverse effects.

Prodrugs have been widely used in targeted drug delivery systems to unload the cytotoxic compound into the tumor cells. They offer various strategies for their activation chemistry and can thus act against diverse types of cancers. The current trends in the development of prodrugs for cancer therapy include the use of macromolecules, such as drug-antibody conjugates, polymer-drug conjugates, and other self-assembling macromolecules, such as lipids that form liposomal or micellar nanoparticles. Various chemotherapeutic agents, including paclitaxel, doxorubicin, carboplatin, etc. have been conjugated with polymers, such as PLGA, PEG, etc. to synthesize prodrugs for different types of lung cancer.

The two strategies used for the conversion of prodrugs into active drugs include (i) passive approaches that exploit the basic physicochemical or physiological changes (for e.g., reduced pH, hypoxia, overexpression of the surface receptor) and (ii) active strategies that utilize prodrugs that may be activated by a site-directed enzyme, thus aiding in specialized activation chemistry for the prodrug conversion.

Many enzymes are known to be upregulated in cancer. DT-diaphorase (DTD) is elevated in many cancers including NSCLC [70]. This is a cytosolic enzyme that reduces two electron containing quinone substrates and activates mytomycin C, the DNA cross-linker. DTD can be targeted by alkylating agents, such as RH1 that causes the bioreduction of the attached quinone to selectively activate the aziridine rings in the cancer cells [71]. Also, cytosolic phospholipase A2α, which plays an important role in cell cycle regulation, has been targeted by researchers. Elevated levels of PLA2α increase the production of eicosanoids that results in the promotion of tumor growth and metastatic activity of the tumor. Further, its inhibition is known to suppress the proliferation of tumor cells by inducing apoptosis. Subsequently, a nanodrug delivery system consisting of mesoporous silica nanoparticles containing pyrrolidone-2, and decorated by EGFR receptor-targeted antibody (EGFRAb) was developed. Silica nanoparticles (SN) are nontoxic and pyrrolidone-2, a potent inhibitor of PLA2α, blocks the production of prostaglandins E2 and leukotriene. EGFRAb

was employed to direct the silica nanoparticles specifically to the cancer cells. In vitro studies in H460 lung cancer cells showed the potency of pyrrolidone-2-loaded SN-EGFRAb nanoparticles, by reducing the activity of PLA2α, decreasing the levels of arachidonic acid and limiting the cell proliferation. Furthermore, this nanoparticulate system showed better antitumor activity (38%) with enhanced tumor inhibition rate in a subcutaneous model of NSCLC. Also, the EGFR antibody helped in targeting the nanoparticles specifically to the tumors cells as compared to the native nanoparticles [72].

The second approach involves the passive targeting of prodrugs to the tumor. This approach exploits the physicochemical characteristics of the cancer cells, such as the tumor microenvironment. The solid tumors in general are hypoxic due to deregulated cell growth and poor vascularization. Due to the hypoxic conditions, the cancer cells resist cell death, induce angiogenesis and interfere with energy metabolism of the cells. This enhances the cancer aggressiveness and metastasis. Under such conditions, due to falling oxygen levels, an important transcription factor called hypoxia-inducible factor-1α (HIF-1α) promotes the expression of genes responsible for the suppression of apoptosis, angiogenesis, invasion, and motility [73]. In NSCLC, HIF-1α expression causes resistance to radiotherapy, chemotherapy, and EGFR TKIs [74–77]. Since hypoxia is connected to the resistance in NSCLC therapy, researchers have targeted cancers using hypoxia-activated prodrugs (HAP). One classic example of HAPs is that of tarloxotinib bromide, a bioreductive pan-EGFR inhibitor. Under hypoxic conditions, tarloxotinib undergoes metabolism via one-electron reduction to a fragment and releases a potent EGFR TKI that exerts antiproliferative activity. Tarloxotinib bromide was designed to release an EGFR TKI, erlotinib under hypoxic conditions. Efficient metabolism of tarloxotinib was demonstrated in a range of human NSCLC cell lines and it was shown to be more effective than erlotinib in wild-type and EGFR-mutant NSCLC xenografts [78, 79]. Targeting cancer with tarloxotinib/erlotinib had reached phase II clinical trials in patients with NSCLC; however, poor response rates led to the discontinuation of these trials [80].

Recently, scientists have designed and synthesized an active tumor targeting prodrug, gefitinib (PPG), which is a polyamine analog, for precision therapy in NSCLC. This macromolecule containing an EGFR TKI was not only successful in inhibiting the growth of PPG-sensitive PC9 cells, but was also efficient in killing the PPG-resistant H1650 cells [81].

3.7 Nanocarriers Targeting EGFR Receptor for Lung Cancer Therapy

The nanocarrier approach offers the ability to target the drugs accurately to the tumorous tissue, which may reduce the toxicity of chemotherapeutic agents. Nanoparticles can be targeted via active or passive approaches. Three types of nanoparticles have been explored for the treatment of lung cancer, namely, (1)

natural nanoparticles (2) organic nanoparticles, and (3) inorganic nanoparticles. Of these, many have been used to target the EGFR for specific delivery of various therapeutic compounds and have been listed in Table 8.4.

4 The Receptor: c-MET

c-MET receptor is overexpressed in lung cancer as an outcome of the resistance developed through the EGFR inhibitors, which leads to c-MET amplification. Therefore, c-MET is an important receptor in NSCLC. It is a transmembrane tyrosine kinase receptor (RTK), which is activated by the ligand, the hepatocyte growth factor (HGF). Activation of c-MET RTK drives a plethora of molecular events in the cells, thus rendering it as an ideal target for therapy. Amplification of c-MET in NSCLC leads to proliferation, invasion, metastasis, and angiogenesis of the cancerous cells. As NSCLC has a poor prognosis and is highly malignant due to the overexpression, amplification, and association of c-MET, the receptor can act as a useful target for treating this cancer type. Thus, various therapies and drugs targeting c-MET are currently being tested either alone or in combination with monoclonal antibodies. Various monoclonal antibodies like emibetuzumab, ficlatuzumab, and rilotuzumab along with tyrosine kinase inhibitors, (TKI) such as crizotinib, tepotinib, cabozantinib, and capmatinib, are currently under evaluation. These studies have resulted in an improvement in the overall survival rate of NSCLC patients [80, 89]. Further, investigators have explored c-MET and EGFR for developing combination therapy against NSCLC, as c-MET is known to have considerable cross-talks with the other signaling pathways. Therefore, a comprehensive study of this receptor is anticipated to impart significant knowledge regarding its role in NSCLC [20].

4.1 Recognition Domain of c-MET

c-MET or the hepatocyte growth factor receptor (HGFR) is a protein tyrosine kinase like the EGFR and belongs to the family of oncogenes that regulate important cellular processes, such as differentiation, proliferation, cell cycle, motility, and apoptosis [90].

c-MET is a transmembrane receptor tyrosine kinase (RTK), which is a 150-kDa polypeptide. Upon glycosylation, the receptor is activated and a forms 190 kDa glycoprotein. The receptor comprises a transmembrane β-chain (140 kDa) that is extracellularly attached to the α-chain (50 kDa) via a disulfide linkage. This constitutes the binding site for the ligand at the N-terminal of the c-MET receptor [20, 91, 92]. The receptor is activated by its ligand, namely, the hepatocyte growth factor (HGF), which is a member of the plasminogen-related growth factor family. The precursor of HGF is mainly produced by the cells of mesenchymal origin. There are

Table 8.4 Summary of nanocarriers conjugated with various anti-EGFR molecules for the treatment of lung cancer

Sr. No.	Nanoparticle material	Targeting molecule	Cell line or animal model	Remarks	Ref
1	Gold nanoparticles as contrast agents	Cetuximab (C225) and Llama heavy chain variable region antibody fragments (VHH domains)	4–6-week-old female athymic nude mice were injected with A431 cells to develop tumors having volume of 5 cc	The nanoparticle system allowed effective tumor imaging by computed tomography (CT) with enhanced uptake because of cetuximab	[82]
2	Silica nanoparticles	Anti-EGFR monoclonal antibody	In vitro studies (A549) and BALB/c nude mice induced with A549 tumors	Silica nanoparticles (~100 nm) containing mAb and methylene blue complex were developed as probes for lung cancer detection	[83]
3	Gold nanoparticles	C225	Xenograft models prepared with nude mice injected with A549 and H1299 cells	Gold nanoparticles (~14 nm) efficiently delivered C225 and increased the cytotoxic effect in EGFR-positive NSCLC	[84]
4	Chitosan nanoparticles cross-linked with γ -poly(glutamic acid) loaded with Docetaxel	C225	A549 cells	The drug delivery system showed superior antiproliferative activity over untagged docetaxel chitosan nanoparticles. The cell cycle was arrested in G2/M phase and resulted in the induction of apoptosis	[85]
5	PLGA Nanoparticles loaded with Docetaxel	C225	A549 cells and xenograft mice models bearing A549 tumors	Sustained cytoplasmic delivery of docetaxel was achieved	[86]
6	Liposomes loaded with doxorubicin	GE11 (short peptide specific to EGFR)	A549 cells, male BALB/c nude mice induced with A549 tumors	Liposomes with 10% GE11 had the highest tumor cell killing activity and a 2.6-fold lower IC50 than that of the nontargeted carriers. GE11-modified liposomes showed enhanced accumulation and prolonged retention in tumor tissue	[87]
7	1,2-distearoyl-sn -glycero-3-phosphoethanolamine-N-(amino(polyethylene glycol)-2000 (PEG 2000/ DSPE)	Human epidermal growth factor (EGF)	A549-T24 human lung adenocarcinoma cells	Cellular uptake study revealed that EGF-targeted micelles afforded higher intracellular delivery of paclitaxel as compared to the nontargeted micelles in both resistant and sensitive cell lines	[88]

six domains in the HGF, namely, the N-terminal domain, four kringle domains and the C-terminal domain, which is a catalytic domain that is structurally similar to the serine proteases. The HGF binds to the c-MET in 2:2 ratio, that is, two HGFs bind to the dimerized form of c-MET/HGFR [93], via the semaphorin domain at the N-terminal. The tyrosine kinase domain is located intracellularly in the β chain near the C-terminal end. This end is essential for binding to the substrate and subsequent downstream signaling [92]. The binding of the HGF to c-MET is known to activate several signaling cascades like the growth factor receptor-bound protein 2 (Grb2), mitogen-activated protein (MAP) kinase, phosphoinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ). This receptor–ligand interaction is known to control morphogenesis, motility, mitogenesis, and proliferation in epithelial and endothelial cells [94, 95]. These pathways can promote cell survival and proliferation along with migration, motility, invasion, and angiogenesis, and can bring about transition of epithelial cells to mesenchymal cells [20, 96].

4.2 Interaction of the Receptor with Ligands

The receptor contains an extracellular region, the semaphorin domain that is a cysteine-rich immunoglobulin domain and an intracellular juxtamembrane domain, a tyrosine kinase catalytic domain and a carboxy terminal docking site (Fig. 8.4) [20, 96]. The HGF binds to the c-MET at the N-terminal domain, which is also known as the semaphorin domain or the sema domain. The sema domain is made up of seven β sheets that form a bladed propeller structure having seven arms. Here, the second and the third sheets bind to the active site region of the ß-chain of HGF

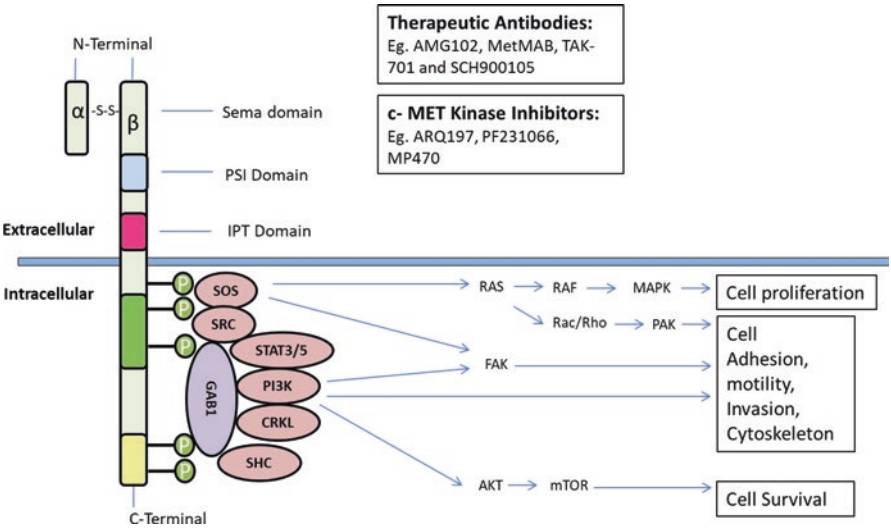


Fig. 8.4 Activation of c-MET and signaling cascade associated with its activation

[97, 98] The tyrosine kinase (TK) region is present at the C-terminal domain, which envelopes multiple sites for the phosphorylation of tyrosine. Upon binding with the c-MET receptor tyrosine kinase, the HGF activates the receptor by dimerization. Homodimer formation further transactivates the TK and the juxtamembrane domains. This cross-transactivation results in autophosphorylation of the three conserved tyrosine residues in the activation loop of the TK domain of the c-MET receptor [99, 100].

Phosphorylation of the c-MET receptor can be mediated either via the HGF or via various RTKs. This further activates various signaling cascades, which bring about changes at the molecular level, through the recruitment of various proteins that play a role in signaling. These signaling cascades govern various biological actions, such as regulation of transcription and gene expression, survival, reduced apoptosis, and regulation of cytoskeletal function along with cell growth and differentiation [90, 101].

However, the main difference in the expression of c-MET in normal and oncogenic cells is that the receptor activation is mediated through the ligands only in case of normal cells, which does not occur in the oncogenic cells [93].

4.3 Antagonists for c-MET

The molecules that mimic the HGF are natural antagonist of c-MET. These include modifications of the HGF that have shown antagonistic activity against the natural HGF. These molecules compete for the binding site at the c-MET without bringing about the required conformational change required for dimerization during the receptor activation. The most common molecule is the pro-HGF, which is also the precursor of HGF and is known to bind to the receptor without bringing about its activation. Further, NK2 and NK4, the HGF α-chain variants, bind to the c-MET without activating the receptor and thus act as antagonists. The NK2 and NK4 consist of a hairpin N-terminal domain and 2–4 kringle domains (2 in case NK2 and 4 in case of NK4) which compete with the HGF. NK2 may act as an antagonist or partial agonist to c-MET, and occurs naturally. On the other hand, NK4 is produced by the proteolytic digestion of HGF and has exhibited a better therapeutic value since its structure is similar to angiostatin that downregulates angiogenesis [89, 102, 103]. Along with these antagonists, there are certain c-MET decoys that have an ability to inhibit the receptor. The above-discussed are naturally available ligands for c-MET.

4.4 Ligands of c-MET

Most of the synthetic ligands of the c-MET receptor target the DFG motif. The DFG motif comprises aspartic acid (D), phenylalanine (F), and glycine (G) residues. It is present at the N-terminal, near the “activation loop” that covers the catalytic site,

the latter being important for the regulation of the receptor. A conformational change in the receptor modulates the kinase activity from DFG-in to DFG-out, that is, active to inactive state [104]. This property of the receptor has been explored for its inhibitory action and has resulted in three main classes of c-MET inhibitors that differ in their structure–activity relationship. The class of small molecules that act on DFG-in state are termed as Class I inhibitors, comprising small molecules like PF-2341066 (Pfizer) and SU11274 (Sugen) [105, 106]. The Class II or AM-like inhibitors bind to the inactive state of DFG-out. These are mainly derived from urea and are either ring-based or non-ring-based structures [107]. They interact with the hydrophobic pocket in the region between the hinge and the C-helix, thus assuming an unphosphorylated conformation of c-mET. These two classes of compounds are competitive ATP inhibitors. Majority of the inhibitors target via competitive inhibition; however, noncompetitive ATP inhibitors have also been explored. ARQ197 (Tivantinib) is a small molecule that inhibits c-MET by interfering with the ATP binding noncompetitively. In vitro, this small molecule binds to dephosphorylated c-MET and is a bisindolylmaleimide. However, its exact mechanism of action still remains unclear, though it has been observed to be safe to the cells [108]. Various ligands have been explored for inhibiting the c-MET receptor and have been listed in Table 8.5. The inhibitors of c-MET mostly compete for ATP-binding sites either in a competitive or noncompetitive manner.

Table 8.5 Ligands explored for inhibition of c-MET

No.	Compound	Developer	Mode of action	Development phase	References
1	ARQ197	ArQule/ Daiichi Sankyo	Noncompetitive; selective, mechanism not clear. Administered with erlotinib for NSCLC	II	[109]
2	PF-2341066	Pfizer	ATP-competitive; c-MET and ALK inhibitor	III	[105]
3	PF-4217903	Pfizer	ATP-competitive; selective	I	[110]
4	JNJ-38877605	Johnson & Johnson	ATP-competitive; selective; for solid tumors	I	[111]
5	XL184/ BMS907351	Exelixis/ BMS	Nonselective inhibitor of tyrosine kinase, effective against c-MET in cases of NSCLC	II	[112]
6	AMG102/ rilotumumab	Amgen	Humanized antihuman HGF IgG2 for SCLC and adenocarcinoma	II	[113]
7	MetMAb	Roche	Humanized antihuman c-MET monovalent antibody, for NSCLC	II	[114]
8	AMG-458	Amgen	ATP-competitive, c-MET, and Ron inhibitor	Preclinical	[115]

4.5 Receptor-Mediated Targeting Strategies

A drug's efficiency is determined by its ability to target the specific site of action. Currently, various nanocarrier-based systems are being explored to enhance the receptor-targeting efficiency. However, only a few systems targeting c-MET have been designed for the therapy of lung cancers as their therapeutic status in progression of lung cancer is still being investigated.

A novel theranostic system was developed by Lu et al. wherein the researchers conjugated quantum dots with human single chain variable fragment (scFv) antibodies. The scFv antibody targeted against c-MET was used to decorate the surface of PEGylated liposomes for delivering doxorubicin in in vitro and in vivo investigations. These liposomes could selectively deliver the drug for treating metastases of lung cancer [116]. Further, another system comprising an adenoviral vector, along with the RGD cell-penetrating peptide, induced with NK4 antagonist of HGF, in mesenchymal stem cells. When this system was tested in a murine model of lung metastasis (C-26), it resulted in an increase in the survival rate of the treated mice. Thus, this drug delivery carrier was able to reduce angiogenesis in tumors and induced apoptosis in the tumorigenic cells, thus prolonging the survival of C-26 mice. The system was thus proposed for the treatment of multiple lung metastatic cancer [117]. Similar therapies have been used for treating solid tumors, glioblastomas, and hepatocytic carcinomas due to the upregulation of c-MET observed in these cancers. As c-MET is a pleiotropic receptor, therefore, inhibitors of c-MET give best results when used in combination with other receptor inhibitor drug. Thus, combination therapy can help to overcome the drug resistance along with arresting of metastasis.

5 VEGF (Vascular Endothelial Growth Factor)

VEGF is a heparin-binding homodimeric glycoprotein, which belongs to the family of growth factors. VEGF exerts its action through the interaction with two highly related tyrosine kinase receptors, VEGFR1 and VEGFR2, which are predominantly expressed in cancer cells. VEGF is the main driver of angiogenesis and is overexpressed NSCLC [31]. A variety of environmental factors (hypoxia), growth factors, and genetic/epigenetic factors (oncogenes/tumor suppressor genes) regulate the expression of VEGF in lung cancer. Along with cytokines and metalloproteinases, the transforming growth factor beta 1 (TGFβ1) and tumor necrosis factor alpha (TNFα) also stimulate the production of VEGF in the lung cancer cells. The NSCLC cells can produce and secrete VEGF, promoting the formation of pleural effusion, angiogenesis, and tumor metastatic progression. Current strategies of inhibiting the VEGF pathway include two main approaches, monoclonal antibodies for targeting the VEGF or VEGFRs and tyrosine kinase inhibitors. Currently, bevacizumab and ramucirumab have been approved for treating the NSCLC patients receiving chemotherapy. On the other hand, the tyrosine kinase inhibitor, nintedanib, in combination

with docetaxel, is the only multikinase antiangiogenic agent that has been approved for treating lung cancer patients with advanced adenocarcinoma, after first-line chemotherapy. Thus, targeting VEGF is foreseen as a promising strategy for the treatment and diagnosis of lung cancer [31, 118].

VEGF plays an important role in tumor development by mediating angiogenesis. It is highly expressed in tumor cells and has implications in both NSCLC and SCLC [119]. The main function of VEGF is to promote tumor growth through neoangiogenesis, lymphangiogenesis, and lymph nodal dissemination. The structure, function, ligand binding, and recognition domain of this receptor have been elaborately discussed separately (Chap. 8). Here, we have discussed about the role of VEGF in lung cancer and how this receptor may be employed as a therapeutic target for treating lung cancer.

5.1 Natural and Synthetic Ligands for VEGFR

Various ligands have been explored for inhibiting the VEGFR. Their mode of action, current developmental phase, and the companies involved in their development have been stated in Table 8.6.

Table 8.6 Various ligands explored for inhibition of VEGF in their developmental phase for lung cancer [120, 121]

No.	Compound	Developer	Mode of action	Developmental phase	References
1	Bevacizumab	Avastin; Genentech	Recombinant humanized IgG1 mAb. It blocks angiogenesis by inhibiting VEGF-A	Approved	[122]
2	Ramucirumab	ImClone Systems Inc.	Fully human IgG1 monoclonal antibody targeting the extracellular domain of VEGFR-2	Approved	[123]
3	Sorafenib	Bayer	Inhibits RTKs including VEGFR	III	[124, 125]
4	PTK787 (Vatalanib)	Novartis	Oral inhibitor of VEGFR-1, -2, and -3 tyrosine kinases	III	[126]
5	Cediranib	AstraZeneca	Inhibits VEGFR-1 and/or VEGFR-2; multikinase inhibitor that has been studied as the first-line therapy for advanced NSCLC	II/III	[127, 128]
6	Nintedanib	Boehringer Ingelheim	Potent TKI having anti-VEGFR-2 activity	III	[129]
7	Neovastat (AE-941)	Æterna	Inhibits VEGF binding and VEGF TK activity	III	[130]

5.2 *Receptor-Mediated Targeting Strategies*

siRNA has proven to be a promising molecule for treating various cancers. However, the molecule requires a robust delivery carrier owing to its extreme fragility. Antiangiogenic therapies were designed by Kim et al., where antiangiogenic siRNA was conjugated with nanoparticles having polyethylenimine (PEI) core and a PEG shell. This was employed for downregulating VEGF expression in animal tumor models. This system was effective in treating lung cancer systemically and locally [131]. Another antiangiogenic system was designed to contain docetaxel and an anti-VEFG intraceptor, and was further decorated with RGD peptide for cell penetration. This combination therapy was tested in H1299 lung cancer cells and in xenografts in athymic nude BALB/c mice. This combination therapy resulted in a higher inhibition of VEGF, promoted apoptosis and arrested angiogenesis [132]. Further, nanocarriers were effectively used for delivering a highly hydrophobic drug, possessing known multitarget antiangiogenic effects. Here, albumin nanoparticles were developed along with polymeric micelles and were administered together. The polymeric micelles resulted in a strong inhibition of angiogenesis, while the albumin nanoparticles demonstrated retardation of tumor growth. Thus, the dual carriers provided a novel combination therapy for tumor regression [133].

6 Drug Resistance in Lung Cancer

Lung cancer is often associated with unprecedented reoccurrence of the disease, probably due to the ineffectiveness of the therapies, most of which are associated with drug resistance. Most of the EGFR-mutant NSCLCs actively respond to the EGFR inhibitors. But, a vast majority of these tumors ultimately become resistant to the drug treatment. About 50% of this resistance is due to the occurrence of a secondary mutation in EGFR (T790M) [134–137]. The T790M mutation mostly occurs due to the first-generation EGFR inhibitors. This mutation is also referred to as the “gatekeeper” mutation [136]. Further, this mutation also triggers MET amplification, which signals through ERBB3 and is characterized by gene amplification of a kinase that is not a direct or downstream target of gefitinib or erlotinib [136]. These findings may have important clinical implications for patients who develop acquired resistance to gefitinib, erlotinib, and afatinib. Hence, combination therapies involving MET kinase inhibitors and irreversible EGFR inhibitors have been recommended for patients whose tumors become resistant to gefitinib or erlotinib [134]. Regales et al. have suggested that dual targeting with cetuximab and a second-generation EGFR TKI can effectively overcome the T790M-mediated drug resistance. Though the combination of afatinib and cetuximab is associated with a response rate of 29% (32% among patients with EGFR T790M and 25% among patients without it), it is associated with side effects such as substantial skin toxicity (20% of grade 3 or higher) and gastrointestinal toxicity (6% of grade 3 or higher)

[135]. AZD9291 is an oral, potent, irreversible EGFR tyrosine kinase inhibitor, developed by AstraZeneca that is selective for the EGFR tyrosine kinase inhibitor–sensitizing mutations and the T790M resistance mutation. The USFDA approval was granted to this drug after it demonstrated efficacy in 411 NSCLC patients with T790M mutations, who exhibited an overall objective response rate (ORR) of 59%. AZD9291 is a monoanilino-pyrimidine compound that is structurally distinct from the other third-generation EGFR TKIs and offers a pharmacologically differentiated profile from the previous generation EGFR TKIs. During the preclinical studies, this drug has been shown to potently inhibit the signaling pathways and cellular growth in both EGFRm+ and EGFRm+/T790M mutant cell lines, in vitro studies. A lower activity against was reported with this molecule in wild-type EGFR cell lines, translating into profound and sustained tumor regression in EGFR mutant tumor xenograft and transgenic models [137].

7 Combination Therapy in Lung Cancer

Lung cancer can be activated through the upregulation of multiple receptors that are responsible for regulating numerous pathways and hence treatments employing monotherapies have been observed as largely ineffective. Thus, combinatorial therapies that simultaneously target different pathways have been foreseen to be promising for treating various forms of this cancer [138]. Combination therapies rely on combining two or more anticancer drugs with the purpose of eliminating the cancer cells. Such an approach is advantageous because the drug combination acts in a synergistic or additive manner on the key target pathways responsible for cancer phenotypes.

The platinum-based chemotherapy is the first-line approach for patients with advanced NSCLC, which results in a median overall survival rate of 8–12 months. Biological molecules, such as bevacizumab and cetuximab, have led to only modest differences in the survival, which has necessitated newer therapeutic paradigms [139]. Paclitaxel/carboplatin has been regarded as a standard drug for combination therapies due to their frequent usage and efficacy in NSCLC patients. Lynch et al. assessed the activity of ipilimumab, which is an anticytotoxic T-cell lymphocyte-4 monoclonal antibody in patients with lung cancer. A randomized phase II study was conducted to compare ipilimumab along with paclitaxel and carboplatin as compared to the drugs combination alone [140]. The study resulted in an improved immune-related progression-free survival rate in patients receiving ipilimumab as compared to those receiving the drug combination without ipilimumab (median 12.9 vs. 9.9 months) [139, 140]. Pirker et al. conducted a phase III study to assess the efficacy and safety of the EGFR-targeted monoclonal antibody, cetuximab in combination with cisplatin/vinorelbine (CV) and compared the effects in NSCLC patients receiving only CV. They found that the combination of cetuximab with CV resulted in superior survival of the patients with advanced EGFR-detectable NSCLC [141].

Another study involved combination therapy with trastuzumab and pertuzumab in Calu-3 and KPL-4 xenograft models. This resulted in tumor regression and a complete inhibition of metastatic tumor spread in animals. Pertuzumab is a HER2 dimerization inhibitor that binds to a different epitope on HER2 than trastuzumab and inhibits the formation of dimers of HER2 with other HER family members, such as HER3 and HER1. The combination of trastuzumab and pertuzumab demonstrated enhanced antitumor effects and promoted tumor regression in xenograft models of HER2-positive breast cancer and NSCLC. Although both these agents could actively induce ADCC, their complementary mechanisms of action resulted in the significantly enhanced antitumor activity [142]. Ramalingam et al. carried out a phase II randomized, double-blinded, and placebo-controlled study to assess the efficacy of vorinostat, in combination with carboplatin and paclitaxel, as a first-line therapy for advanced NSCLC. Vorinostat, a histone deacetylase inhibitor, exerted anticancer effects by both histone and nonhistone-mediated mechanisms. A confirmed response rate (CRR) of 34% was recorded in 94 patients and the overall survival increased from 9.7 months to 13.0 months [143]. A few combination therapies involving mAbs are currently in various phases of clinical trials and have been stated in Table 8.7.

8 Clinical Studies

Over the past decades, lung cancer has been regarded as one of the leading cause of cancer-related mortality in both men and women. Several mutations, like the occurrence of inversions in the short arm of the chromosome that juxtaposes echinoderm microtubule-associated protein-like 4 (EML4) with ALK and produces EML4-ALK–fusion tyrosine kinases, substitution of threonine at 790 to methionine (T790M), escaping the elimination by immune system through programmed death (PD-1) pathway, etc., have been commonly encountered in various phases of clinical studies [155–159].

Crizotinib, a multitargeted TKI was approved by the USFDA in August 2011 for the treatment of advanced NSCLC. The drug exhibited activity against c-MET, ALK, and ROS1 in advanced NSCLC cases that were positive for the ALK rearrangements. About 65–74% of the patients benefitted from this therapy and demonstrated a median progression-free survival rate of 7.7–10.9 months [155, 160]. Other small-molecule TKIs, such as crizotinib, imatinib, erlotinib, and gefitinib, were also approved for the treatment of lung cancer. But, these drugs exhibited low cerebrospinal fluid (CSF)-to-plasma ratios since the central nervous system (CNS) remains one of the dominant sites of progressive tumor burden during chemotherapy with crizotinib and other molecules [155, 160]. The first-generation TKI's, gefitinib, and erlotinib are reversible small-molecule ATP analog, originally designed to inhibit the tyrosine kinase activity of the wild-type EGFR. These were found to be most effective in advanced NSCLC, with a median overall survival period of approximately 19–36 months. But, these first-generation TKIs were associated with side

effects like skin rash and diarrhea due to the inhibition of wild-type EGFR present in the skin and gastrointestinal organs. Furthermore, amplification in HER2 and c-MET, mutation in PIK3CA and BRAF, and loss of NF1, T790M were observed as the most common mechanisms of resistance by the tumor cells in more than 50% of the patients exhibiting disease progression. The T790M mutation is believed to provide resistance against the reversible first-generation TKIs through steric hindrance and increased affinity toward ATP. The second-generation, irreversible EGFR TKIs, such as afatinib and dacomitinib, have proven effective against untreated, EGFR mutant lung cancer. But, as a monotherapy, they have failed to overcome the T790M-mediated resistance in patients, because the concentrations at which these irreversible TKIs overcome the T790M activity in preclinical trials cannot be achieved in humans due to the dose-limiting toxicity related to the nonselective inhibition of the wild-type EGFR. AstraZeneca (Macclesfield, UK) developed an oral, third-generation, irreversible, small-molecule inhibitor (AZD9291) to target the T790M-resistant mutant forms (EGFRm+) with selectivity over the wild-type EGFR. AZD9291 has a chemical structure distinct from the other third-generation TKIs, WZ4002 and CO-1686. This drug acts by binding to the EGFR kinase and targeting the cysteine-797 residue in the ATP-binding site through the formation of an irreversible covalent bond. In the phase I of clinical trials, the drug was found to be 200 times more potent against the T790M mutant than the wild-type EGFR [156].

Several next-generation ALK inhibitors that are more potent than crizotinib, have entered various clinical studies and can overcome the most common mutations conferring resistance to ALK such as Leu1196Met. Among the eight next-generation ALK inhibitors that have entered the clinical trials, three molecules, namely, ceritinib, alectinib, and brigatinib have demonstrated a robust activity in patients with ALK-positive NSCLC. Alectinib has also shown its antitumor activity in patients resistant to crizotinib. 125 subjects were screened during a phase II study in patients with NSCLC, wherein 87 ALK-positive candidates whose disease progressed after crizotinib, were enrolled. The results of this study showed that alectinib was effective in patients suffering from ALK-positive NSCLC and was well tolerated, resulting predominantly in grade 1 or 2 adverse events with improved quality of life. Alectinib also exhibited several potential advantages in terms of both efficacy and tolerability. The median duration of response was prolonged with alectinib (13.5 months) as compared to ceritinib (8.2 months) and brigatinib (9.3 months), respectively. Thus, patients who did not respond to the treatment with crizotinib could be treated with the aforementioned ALK inhibitors, alectinib also resulted in intracranial disease control in 85% and 56% of the patients, at 12 and 24 weeks, respectively [160].

Apart from the mutations occurring in lung cancer, tumors can also escape elimination by the immune system through the activation of inhibitory feedback loops (also known as immunological brakes), which are essential to avoid autoimmune events, and can thus bypass tumor rejection and T-cell activation. The PD-1 and B7.1, also known as CD80 receptors, follow this inhibitory pathway and their activation has been observed in several cancer types including the lung cancer [157–159]. Nivolumab is a fully human, IgG4 immune checkpoint inhibitor antibody, which

Table 8.7 Combination therapy with monoclonal antibodies against EGFR

Sr. no.	mAb	Type/class	EGFR-binding domain	Development phase	In combination with	Therapy	References
1	Cetuximab	Chimeric human murine IgG1	Domain III	III	Only cetuximab	For maintenance after platinum-based chemotherapy during first-line treatment of NSCLC	[144]
					With cisplatin and vinorelbine	First-line treatment of advanced NSCLC	[145]
					With docetaxel and pemetrexed	Treatment of recurrent or progressive NSCLC	[146]
					With carboplatin, docetaxel, and paclitaxel	First-line treatment of patients with advanced/ metastatic NSCLC	[147]
2	Nimotuzumab	Humanized IgG1 obtained from murine IgG2a mAb	Domain III	II	Nimotuzumab plus gefitinib	Randomized Phase II Study of gefitinib and nimotuzumab versus gefitinib in patients with advanced non-small cell lung cancer: dual-agent molecular targeting of EGFR (DATE)	[148]
3	Panitumumab (ABX-EGF)	Recombinant fully human IgG2 mAb	Binds EGFR and inhibits EGF induced RTK phosphorylation	II	In combination with paclitaxel and carboplatin	Treatment of advanced non-small cell lung cancer (NSCLC)	[149]
					Carboplatin, pemetrexed, and panitumumab	Patients with advanced nonsquamous k-ras wild-type NSCLC	[150]
4	Pertuzumab	Recombinant humanized mAb	Domain II of HER2	II	Erlotinib and pertuzumab	Patients with relapsed non-small cell lung cancer	[151]
					Only pertuzumab	Patients with advanced non-small cell lung cancer, which has progressed after prior chemotherapy	[152]
5	Necitumumab	Recombinant human IgG1 monoclonal antibody used as an antineoplastic	Domain III	II	Gemcitabine-cisplatin chemotherapy plus necitumumab	First-line treatment of participants with squamous lung cancer	[153]
6	Trastuzumab Emstatine	–	–	II	Only trastuzumab and emstatine	Participants with human epidermal growth factor receptor (HER)2 immunohistochemistry (IHC)-positive, locally advanced or metastatic non-small cell lung cancer (NSCLC)	[154]

binds to the PD-1 receptors on activated immune cells and thereby inhibits its interaction with PD-L1 and PD-L2 ligands. This event attenuates inhibitory signals and promotes antitumor responses by the host. A phase II clinical study employing 140 patients was conducted, in which, 117 (84%) patients were treated with nivolumab (Bristol-Myers Squibb, Princeton, NJ, USA), as an injectable solution (100 mg; 10 mg/mL) for a period of 100 days. Patients received nivolumab as an intravenous infusion at the concentration 3 mg/kg, every 2 weeks (1 cycle) until the disease progression or unacceptable toxic effects appeared. Nivolumab showed activity in patients with advanced, refractory, and squamous NSCLC and was associated with a manageable safety profile [157].

An early phase clinical trial was initiated with an engineered, humanized IgG1 monoclonal anti-PD-L1 antibody, atezolizumab (MPDL3280A; F Hoffmann-La Roche/Genentech). This antibody acts by blocking PD-L1–PD-1 and PD-L1–B7.1 interactions, which results in the overhauling of T-cell activity and enhancing T-cell priming. POPLAR, a multicenter, randomized, open-label, all comer phase II trial, was carried out at 61 academic medical centers and community oncology practices, across 13 countries in Europe and North America. It was primarily designed to investigate the efficacy and safety of atezolizumab versus docetaxel as the second-line and third-line treatments in NSCLC, and to further assess the predictive value of PD-L1 expression level in tumor cells and tumor-infiltrating immune cells. Accordingly, patients received intravenous atezolizumab (1200 mg fixed dose) or docetaxel (75 mg/m^2) every 3 weeks, on day 1 of each 3-week cycle. Docetaxel was given until disease progression or unacceptable toxicity was observed. No docetaxel-to-atezolizumab crossover was allowed. Results indicated that patients with either squamous or nonsquamous NSCLC showed significant improvement in their overall survival upon treatment with atezolizumab as compared with patients who received docetaxel. Also, atezolizumab was well tolerated and exhibited a safety profile that was consistent with the previous studies [158].

Further, a combination of immunotherapy and chemotherapy was evaluated for its potential to synergistically improve the anticancer activity of the individual drugs. Currently, the standard first-line therapy for patients with advanced nonsquamous NSCLC is platinum-doublet chemotherapy. With the exception of bevacizumab, the addition of a third agent to the platinum-doublet chemotherapy has not improved the progression-free or overall survival rate as compared to the platinum-doublet chemotherapy alone in randomized studies. A study was carried out with pembrolizumab, a humanized, monoclonal antibody against PD-1 that prevents PD-1 from binding to its ligands, PD-L1 and PD-L2. A randomized KEYNOTE-021, phase II study was carried out at 26 academic medical centers in the USA and Taiwan, in patients with chemotherapy-naive, advanced nonsquamous NSCLC. A combination of pembrolizumab and pemetrexed was administered to the patients, wherein the subjects received four cycles of pembrolizumab (200 mg), over 30 min. Further, the chemical drug, pemetrexed was administered at a concentration of 500 mg/m^2 over 10 min, and carboplatin at a dose of 5 mg/mL per min was administered over 15–60 min, intravenously every 3 weeks in the order listed, followed by pembrolizumab for 24 months and optional indefinite pemetrexed maintenance therapy. 123 (56%) patients from the

USA and Taiwan met the eligibility criteria and were randomly distributed for different treatment regimes. 60 patients (49%) were treated with pembrolizumab along with carboplatin and pemetrexed, while 63 patients (51%) were treated with carboplatin and pemetrexed alone. Addition of pembrolizumab to carboplatin and pemetrexed followed by pembrolizumab for 2 years and indefinite pemetrexed maintenance therapy significantly improved the proportion of patients who achieved an objective response as compared to those receiving carboplatin and pemetrexed alone. The median progression-free survival time in the pembrolizumab plus chemotherapy group was 13 months, while the progression-free survival recorded in the chemotherapy group was 8.9 months [159].

9 Conclusion

Lung cancer has been a long-term challenge and still demands newer treatment modalities for its eradication. Availability of safe and effective treatment options has been hampered due to drug resistance and concurrent mutations at various levels. However, research over decades has offered various therapies that have yielded promising results in preclinical and clinical trials. Today, our understanding about cancers has reached greater depths and has enabled the prognosis of various cancer types. A greater understanding of the molecular biomarkers of lung cancer as well as an in-depth understanding of specific receptors overexpressed in this form of cancer will enable the provision of personalized therapies for eradicating this dreadful disease.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7–30. https://doi.org/10.3322/caac.21442.
2. O'Brien TD, Jia P, Aldrich MC, Zhao Z. Lung cancer: one disease or many. Hum Hered. 2018;83(2):65–70.
3. Potiron VA, Roche J, Drabkin HA. Semaphorins and their receptors in lung cancer. Cancer Lett. 2009;273(1):1–14.
4. Vergnenègre A, Chouaïd C. Review of economic analyses of treatment for non-small–cell lung cancer (NSCLC). Expert review of pharmacoeconomics & outcomes research. 2018(just-accepted).
5. Mohanty C, Das M, Kanwar JR, Sahoo SK. Receptor mediated tumor targeting: an emerging approach for cancer therapy. Curr Drug Deliv. 2011;8(1):45–58.
6. Tovar I, Expósito J, Jaén J, Alonso E, Martínez M, Guerrero R, et al. Pattern of use of radiotherapy for lung cancer: a descriptive study. BMC Cancer. 2014;14(1):697.
7. Lamelas IP, Arca JA, Pérez JLF. Directed therapies in lung cancer: new hope? Arch Bronconeumol (English Edition). 2012;48(10):367–71.
8. Słodkowska J, Rojo MG. Digital pathology in personalized cancer therapy. Folia Histochem Cytobiol. 2011;49(4):570–8.

9. Pinho L, Mendes F, Rodrigues M, Estrela J, Teixo R. Molecular targets in lung cancer therapy: a current review. J Integr Oncol. 2015;4(148):2.
10. Liu T-C, Jin X, Wang Y, Wang K. Role of epidermal growth factor receptor in lung cancer and targeted therapies. Am J Cancer Res. 2017;7(2):187.
11. Shi H, Guo J, Li C, Wang Z. A current review of folate receptor alpha as a potential tumor target in non-small-cell lung cancer. Drug Des Devel Ther. 2015;9:4989.
12. Liu S, Li S, Hai J, Wang X, Chen T, Quinn MM, et al. Targeting HER2 aberrations in non–small cell lung cancer with osimertinib. Clin Cancer Res. 2018;24:2594–604.
13. Takenaka M, Hanagiri T, Shinohara S, Kuwata T, Chikaishi Y, Oka S, et al. The prognostic significance of HER2 overexpression in non-small cell lung cancer. Anticancer Res. 2011;31(12):4631–6.
14. Ukrainskaya V, Stepanov A, Glagoleva I, Knorre V, Belogurov A, Gabibov A. Death receptors: new opportunities in cancer therapy. Acta Naturae (англоязычная версия). 2017;9(3):55–63.
15. Spierings DC, de Vries EG, Timens W, Groen HJ, Boezen HM, de Jong S. Expression of TRAIL and TRAIL death receptors in stage III non-small cell lung cancer tumors. Clin Cancer Res. 2003;9(9):3397–405.
16. Vences-Catalán F, Levy S. Immune targeting of tetraspanins involved in cell invasion and metastasis. Front Immunol. 2018;9:1277.
17. Kwon MJ, Seo J, Kim YJ, Kwon MJ, Choi JY, Kim T-E, et al. Prognostic significance of CD151 overexpression in non-small cell lung cancer. Lung Cancer. 2013;81(1):109–16.
18. Li P, Zeng H, Qin J, Zou Y, Peng D, Zuo H, et al. Effects of tetraspanin CD151 inhibition on A549 human lung adenocarcinoma cells. Mol Med Rep. 2015;11(2):1258–65.
19. Chan BA, Hughes BG. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Transl Lung Cancer Res. 2015;4(1):36.
20. Drilon A, Cappuzzo F, Ou S-HI, Camidge DR. Targeting MET in lung cancer: will expectations finally be MET? J Thorac Oncol. 2017;12(1):15–26.
21. Hsu L-H, Chu N-M, Kao S-H. Estrogen, estrogen receptor and lung cancer. Int J Mol Sci. 2017;18(8):1713.
22. Jacenik D, Cygankiewicz AI, Krajewska WM. The G protein-coupled estrogen receptor as a modulator of neoplastic transformation. Mol Cell Endocrinol. 2016;429:10–8.
23. Ishibashi H, Suzuki T, Suzuki S, Niikawa H, Lu L, Miki Y, et al. Progesterone receptor in non–small cell lung cancer—a potent prognostic factor and possible target for endocrine therapy. Cancer Res. 2005;65(14):6450–8.
24. Choudhary R, Li H, Winn RA, Sorenson AL, Weiser-Evans MC, Nemenoff RA. Peroxisome proliferator-activated receptor-γ inhibits transformed growth of non-small cell lung cancer cells through selective suppression of Snail. Neoplasia. 2010;12(3):224–34.
25. Lapa C, Hänscheid H, Wild V, Pelzer T, Schirbel A, Werner RA, et al. Somatostatin receptor expression in small cell lung cancer as a prognostic marker and a target for peptide receptor radionuclide therapy. Oncotarget. 2016;7(15):20033.
26. Callison JC, Walker RC, Massion PP. Somatostatin receptors in lung cancer: from function to molecular imaging and therapeutics. J Lung Cancer. 2011;10(2):69–76.
27. Takashima S, Kitakaze M, Asakura M, Asanuma H, Sanada S, Tashiro F, et al. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci. 2002;99(6):3657–62.
28. Liang W-C, Dennis MS, Stawicki S, Chanthery Y, Pan Q, Chen Y, et al. Function blocking antibodies to neuropilin-1 generated from a designed human synthetic antibody phage library. J Mol Biol. 2007;366(3):815–29.
29. Narazaki M, Segarra M, Tosato G. Sulfated polysaccharides identified as inducers of neuropilin-1 internalization and functional inhibition of VEGF165 and semaphorin3A. Blood. 2008;111(8):4126–36.
30. Hallberg B, Palmer R. The role of the ALK receptor in cancer biology. Ann Oncol. 2016;27(suppl_3):iii4–iii15.
31. Frezzetti D, Gallo M, Maiello MR, D'Alessio A, Esposito C, Chicchinelli N, et al. VEGF as a potential target in lung cancer. Expert Opin Ther Targets. 2017;21(10):959–66.

32. Hu B, Ma Y, Yang Y, Zhang L, Han H, Chen J. CD44 promotes cell proliferation in non-small cell lung cancer. Oncol Lett. 2018;15(4):5627–33.
33. Penno MB, August JT, Baylin SB, Mabry M, Linnoila RI, Lee VS, et al. Expression of CD44 in human lung tumors. Cancer Res. 1994;54(5):1381–7.
34. Teicher BA. Targets in small cell lung cancer. Biochem Pharmacol. 2014;87(2):211–9.
35. Moreno P, Ramos-Álvarez I, Moody TW, Jensen RT. Bombesin related peptides/receptors and their promising therapeutic roles in cancer imaging, targeting and treatment. Expert Opin Ther Targets. 2016;20(9):1055–73.
36. Shiao T-H, Chang Y-L, Yu C-J, Chang Y-C, Hsu Y-C, Chang S-H, et al. Epidermal growth factor receptor mutations in small cell lung cancer: a brief report. J Thorac Oncol. 2011;6(1):195–8.
37. Bethune G, Bethune D, Ridgway N, Xu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. J Thorac Dis. 2010;2(1):48.
38. Bakker J, Spits M, Neefjes J, Berlin I. The EGFR odyssey–from activation to destruction in space and time. J Cell Sci. 2017;130(24):4087–96.
39. Zeng F, Wang Y, Kloepfer LA, Wang S, Harris RC. ErbB4 deletion predisposes to development of metabolic syndrome in mice. Am J Physiol Endocrinol Metab. 2018;315:E583.
40. Chen J, Zeng F, Forrester SJ, Eguchi S, Zhang M-Z, Harris RC. Expression and function of the epidermal growth factor receptor in physiology and disease. Physiol Rev. 2016;96(3):1025–69.
41. Cohen BD, Kiener PA, Green JM, Foy L, Fell HP, Zhang K. The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. J Biol Chem. 1996;271(48):30897–903.
42. Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J. 1996;15(10):2452–67.
43. Dawson JP, Berger MB, Lin C-C, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. Mol Cell Biol. 2005;25(17):7734–42.
44. Mouse H. Epidermal growth factor. 1992.
45. McClintock JL, Ceresa BP. Transforming growth factor-α enhances corneal epithelial cell migration by promoting EGFR recycling. Invest Ophthalmol Vis Sci. 2010;51(7):3455–61.
46. Normanno N, Bianco C, De Luca A, Maiello M, Salomon D. Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer. 2003;10(1):1–21.
47. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med. 2008;358(11):1160–74.
48. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med. 2000;6(4):443.
49. Mazzarella L, Guida A, Curigliano G. Cetuximab for treating non-small cell lung cancer. Expert Opin Biol Ther. 2018;18(4):483–93.
50. Pirker R, Filipits M. Monoclonal antibodies against EGFR in non-small cell lung cancer. Crit Rev Oncol Hematol. 2011;80(1):1–9.
51. Thatcher N, Hirsch FR, Luft AV, Szczesna A, Ciuleanu TE, Dediu M, et al. Necitumumab plus gemcitabine and cisplatin versus gemcitabine and cisplatin alone as first-line therapy in patients with stage IV squamous non-small-cell lung cancer (SQUIRE): an open-label, randomised, controlled phase 3 trial. Lancet Oncol. 2015;16(7):763–74.
52. Reck M, Socinski MA, Luft A, Szczęsna A, Dediu M, Ramlau R, et al. The effect of necitumumab in combination with gemcitabine plus cisplatin on tolerability and on quality of life: results from the phase 3 SQUIRE trial. J Thorac Oncol. 2016;11(6):808–18.
53. Albanell J, Rojo F, Averbuch S, Feyereislova A, Mascaro JM, Herbst R, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. J Clin Oncol. 2002;20(1):110–24.

54. Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res. 2002;62(20):5749–54.
55. Di Gennaro E, Barbarino M, Bruzzese F, De Lorenzo S, Caraglia M, Abbruzzese A, et al. Critical role of both p27KIP1 and p21CIP1/WAF1 in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. J Cell Physiol. 2003;195(1):139–50.
56. Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, et al. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin Cancer Res. 2001;7(5):1459–65.
57. Sebastian M, Schmittel A, Reck M. First-line treatment of EGFR-mutated nonsmall cell lung cancer: critical review on study methodology. Eur Respir Rev. 2014;23(131):92–105.
58. Cappuzzo F, Ciuleanu T, Stelmakh L, Cicenas S, Szczesna A, Juhasz E, et al. SATURN: a double-blind, randomized, phase III study of maintenance erlotinib versus placebo following nonprogression with first-line platinum-based chemotherapy in patients with advanced NSCLC. J Clin Oncol. 2009;27(15_suppl):8001.
59. Raymond E, Faivre S, Armand JP. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. Drugs. 2000;60(1):15–23.
60. Dey N, Leyland-Jones B, De P. HER2 signaling network in advanced breast cancer: opportunities for combination therapies. In: PI3K-mTOR cancer and cancer therapy. Cham: Springer; 2016. p. 231–61.
61. Ramalingam SS, Blackhall F, Krzakowski M, Barrios CH, Park K, Bover I, et al. Randomized phase II study of dacomitinib (PF-00299804), an irreversible pan–human epidermal growth factor receptor inhibitor, versus erlotinib in patients with advanced non–small-cell lung cancer. J Clin Oncol. 2012;30(27):3337.
62. Kim D-W, Tiseo M, Ahn M-J, Reckamp KL, Hansen KH, Kim S-W, et al. Brigatinib in patients with crizotinib-refractory anaplastic lymphoma kinase-positive non-small-cell lung cancer: a randomized, multicenter phase II trial. J Clin Oncol. 2017;35(22):2490–8.
63. Shi Y, Zhang L, Liu X, Zhou C, Zhang S, Wang D, et al. Icotinib versus gefitinib in previously treated advanced non-small-cell lung cancer (ICOGEN): a randomised, double-blind phase 3 non-inferiority trial. Lancet Oncol. 2013;14(10):953–61.
64. Ayeni D, Politi K, Goldberg SB. Emerging agents and new mutations in EGFR-mutant lung cancer. Clin Cancer Res. 2015:clincanres. 1211.2015.
65. Liao B-C, Lin C-C, Lee J-H, Yang JC-H. Update on recent preclinical and clinical studies of T790M mutant-specific irreversible epidermal growth factor receptor tyrosine kinase inhibitors. J Biomed Sci. 2016;23(1):86.
66. Food, Administration D. Class Labeling Changes to anti-EGFR monoclonal antibodies, cetuximab (Erbitux) and panitumumab (Vectibix): KRAS Mutations. Food and Drug Administration2011Class Labeling Changes to Anti-EGFR Monoclonal Antibodies, Cetuximab (Erbitux) and Panitumumab (Vectibix): KRAS Mutations. http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm172905.htm Accessed 2011;7.
67. van Bueren JJL, Bleeker WK, Brännström A, von Euler A, Jansson M, Peipp M, et al. The antibody zalutumumab inhibits epidermal growth factor receptor signaling by limiting intra- and intermolecular flexibility. Proc Natl Acad Sci. 2008;105(16):6109–14.
68. Vacchelli E, Aranda F, Eggermont A, Galon J, Sautes-Fridman C, Zitvogel L, et al. Trial watch: tumor-targeting monoclonal antibodies in cancer therapy. Oncoimmunology. 2014;3(1):e27048.
69. Rodriguez PC, Neninger E, García B, Popa X, Viada C, Luaces P, et al. Safety, immunogenicity and preliminary efficacy of multiple-site vaccination with an Epidermal Growth Factor (EGF) based cancer vaccine in advanced non small cell lung cancer (NSCLC) patients. J Immune Based Ther Vaccines. 2011;9(1):7.
70. Danson S, Ward TH, Butler J, Ranson M. DT-diaphorase: a target for new anticancer drugs. Cancer Treat Rev. 2004;30(5):437–49.

71. Hargreaves R, Hartley JA, Butler J. Mechanisms of action of quinone-containing alkylating agents: DNA alkylation by aziridinylquinones. Front Biosci. 2000;5:E172–E80.
72. Sundarraj S, Thangam R, Sujitha MV, Vimala K, Kannan S. Ligand-conjugated mesoporous silica nanorattles based on enzyme targeted prodrug delivery system for effective lung cancer therapy. Toxicol Appl Pharmacol. 2014;275(3):232–43.
73. Carmeliet P, Dor Y, Herbert J-M, Fukumura D, Brusselmans K, Dewerchin M, et al. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. 1998;394(6692):485.
74. Iida H, Suzuki M, Goitsuka R, Ueno H. Hypoxia induces CD133 expression in human lung cancer cells by up-regulation of OCT3/4 and SOX2. Int J Oncol. 2012;40(1):71–9.
75. Liu Y, Song X, Wang X, Wei L, Liu X, Yuan S, et al. Effect of chronic intermittent hypoxia on biological behavior and hypoxia-associated gene expression in lung cancer cells. J Cell Biochem. 2010;111(3):554–63.
76. Wohlkoenig C, Leithner K, Deutsch A, Hrzenjak A, Olschewski A, Olschewski H. Hypoxia-induced cisplatin resistance is reversible and growth rate independent in lung cancer cells. Cancer Lett. 2011;308(2):134–43.
77. Minakata K, Takahashi F, Nara T, Hashimoto M, Tajima K, Murakami A, et al. Hypoxia induces gefitinib resistance in non-small-cell lung cancer with both mutant and wild-type epidermal growth factor receptors. Cancer Sci. 2012;103(11):1946–54.
78. Patterson AV, Silva S, Guise C, Abbattista M, Bull M, Hsu H-L, et al. The hypoxia-activated EGFR-TKI TH-4000 overcomes erlotinib-resistance in preclinical NSCLC models at plasma levels achieved in a phase 1 clinical trial. AACR. 2015.
79. Patterson AV, Silva S, Guise C, Bull M, Abbattista M, Hsu A, et al. TH-4000, a hypoxia-activated EGFR/Her2 inhibitor to treat EGFR-TKI resistant T790M-negative NSCLC. Am Soc Clin Oncol. 2015;33:e13548.
80. Salgia R. MET in lung cancer: biomarker selection based on scientific rationale. Mol Cancer Ther. 2017;16(4):555–65.
81. Song X, Han X, Yu F, Zhang X, Chen L, Lv C. Polyamine-targeting gefitinib prodrug and its near-infrared fluorescent theranostic derivative for monitoring drug delivery and lung cancer therapy. Theranostics. 2018;8(8):2217.
82. Ashton JR, Gottlin EB, Patz EF Jr, West JL, Badea CT. A comparative analysis of EGFR-targeting antibodies for gold nanoparticle CT imaging of lung cancer. PLoS One. 2018;13(11):e0206950.
83. Wan J, Wu W, Zhang R, Liu S, Huang Y. Anti-EGFR antibody conjugated silica nanoparticles as probes for lung cancer detection. Exp Ther Med. 2017;14(4):3407–12.
84. Qian Y, Qiu M, Wu Q, Tian Y, Zhang Y, Gu N, et al. Enhanced cytotoxic activity of cetuximab in EGFR-positive lung cancer by conjugating with gold nanoparticles. Sci Rep. 2014;4:7490.
85. Maya S, Sarmento B, Lakshmanan V-K, Menon D, Seabra V, Jayakumar R. Chitosan cross-linked docetaxel loaded EGF receptor targeted nanoparticles for lung cancer cells. Int J Biol Macromol. 2014;69:532–41.
86. Patel J, Amrutiya J, Bhatt P, Javia A, Jain M, Misra A. Targeted delivery of monoclonal antibody conjugated docetaxel loaded PLGA nanoparticles into EGFR overexpressed lung tumour cells. J Microencapsul. 2018;35(2):204–17.
87. Cheng L, Huang F-Z, Cheng L-F, Zhu Y-Q, Hu Q, Li L, et al. GE11-modified liposomes for non-small cell lung cancer targeting: preparation, ex vitro and in vivo evaluation. Int J Nanomedicine. 2014;9:921.
88. Gill KK, Kamal MM, Kaddoumi A, Nazzal S. EGFR targeted delivery of paclitaxel and parthenolide co-loaded in PEG-Phospholipid micelles enhance cytotoxicity and cellular uptake in non-small cell lung cancer cells. J Drug Delivery Sci Technol. 2016;36:150–5.
89. Lawrence RE, Salgia R. MET molecular mechanisms and therapies in lung cancer. Cell Adh Migr. 2010;4(1):146–52.
90. Salgia R, editor. Role of c-Met in cancer: emphasis on lung cancer. Seminars in oncology. Elsevier; 2009.

91. Skead G, Govender D. Gene of the month: MET. J Clin Pathol. 2015;68(6):405–9.
92. Porter J. Small molecule c-Met kinase inhibitors: a review of recent patents. Expert Opin Ther Pat. 2010;20(2):159–77.
93. Sattler M, Hasina R, Reddy MM, Gangadhar T, Salgia R. The role of the c-Met pathway in lung cancer and the potential for targeted therapy. Ther Adv Med Oncol. 2011;3(4):171–84.
94. Birchmeier C, Birchmeier W, Gherardi E, Woude GFV. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4(12):915.
95. Zhang YW, Woude GFV. HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem. 2003;88(2):408–17.
96. Organ SL, Tsao M-S. An overview of the c-MET signaling pathway. Ther Adv Med Oncol. 2011;3(1_suppl):S7–S19.
97. Stamos J, Lazarus RA, Yao X, Kirchhofer D, Wiesmann C. Crystal structure of the HGF β-chain in complex with the Sema domain of the Met receptor. EMBO J. 2004;23(12):2325–35.
98. Gherardi E, Sandin S, Petoukhov MV, Finch J, Youles ME, Öfverstedt L-G, et al. Structural basis of hepatocyte growth factor/scatter factor and MET signalling. Proc Natl Acad Sci. 2006;103(11):4046–51.
99. Maulik G, Madhiwala P, Brooks S, Ma P, Kijima T, Tibaldi E, et al. Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer. J Cell Mol Med. 2002;6(4):539–53.
100. Ma PC, Kijima T, Maulik G, Fox EA, Sattler M, Griffin JD, et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res. 2003;63(19):6272–81.
101. Sattler M, Ma PC, Salgia R. Therapeutic targeting of the receptor tyrosine kinase Met. In: Molecular targeting and signal transduction. Boston: Springer; 2004. p. 121–38.
102. Otsuka T, Jakubczak J, Vieira W, Bottaro DP, Breckenridge D, Larochelle WJ, et al. Disassociation of met-mediated biological responses in vivo: the natural hepatocyte growth factor/scatter factor splice variant NK2 antagonizes growth but facilitates metastasis. Mol Cell Biol. 2000;20(6):2055–65.
103. Du W, Hattori Y, Yamada T, Matsumoto K, Nakamura T, Sagawa M, et al. NK4, an antagonist of hepatocyte growth factor (HGF), inhibits growth of multiple myeloma cells: molecular targeting of angiogenic growth factor. Blood. 2007;109(7):3042–9.
104. Meng Y, Lin YL, Roux B. Computational study of the "DFG-flip" conformational transition in c-Abl and c-Src tyrosine kinases. J Phys Chem B. 2015;119(4):1443–56.
105. Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S, et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. Cancer Res. 2007;67(9):4408–17.
106. Wang X, Le P, Liang C, Chan J, Kiewlich D, Miller T, et al. Potent and selective inhibitors of the Met (hepatocyte growth factor/scatter factor (HGF/SF) receptor) tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. Mol Cancer Ther. 2003;2(11):1085–92.
107. Mughal A, Aslam HM, Sheikh A, Khan AMH, Saleem S. c-Met inhibitors. Infect Agents Cancer. 2013;8(1):13.
108. Hiscox S, Jordan NJ, Jiang W, Harper M, McClelland R, Smith C, et al. Chronic exposure to fulvestrant promotes overexpression of the c-Met receptor in breast cancer cells: implications for tumour–stroma interactions. Endocr Relat Cancer. 2006;13(4):1085–99.
109. Mekhail T, Rich T, Rosen L, Chai F, Semic-Suka Z, Savage R, et al. Final results: a dose escalation phase I study of ARQ 197, a selective c-Met inhibitor, in patients with metastatic solid tumors. J Clin Oncol. 2009;27(15S):3548.
110. Timofeevski SL, McTigue MA, Ryan K, Cui J, Zou HY, Zhu JX, et al. Enzymatic characterization of c-Met receptor tyrosine kinase oncogenic mutants and kinetic studies with aminopyridine and triazolopyrazine inhibitors. Biochemistry. 2009;48(23):5339–49.
111. Perera T, Lavrijssen T, Janssens B, Geerts T, King P, Mevellec L et al. JNJ-38877605: a selective Met kinase inhibitor inducing regression of Met-driven tumor models. AACR; 2008.
112. Salgia R, Hong D, Camacho L, Ng C, Janisch L, Ratain M, et al. A phase I dose-escalation study of the safety and pharmacokinetics (PK) of XL184, a VEGFR and MET kinase

inhibitor, administered orally to patients (pts) with advanced malignancies. J Clin Oncol. 2007;25(18_suppl):14031.
113. Giordano S. Rilotumumab, a mAb against human hepatocyte growth factor for the treatment of cancer. Curr Opin Mol Ther. 2009;11(4):448–55.
114. Merchant M, Zheng Z, Romero M, Huang A, Adams C, Moffat B et al. One-armed 5D5 (OA5D5) is a potent humanized HGF-blocking anti-c-Met monovalent antibody that inhibits HGF-dependent activity in vitro and demonstrates anti-tumor efficacy in vivo. AACR; 2007.
115. Underiner TL, Herbertz T, Miknyoczki SJ. Discovery of small molecule c-Met inhibitors: evolution and profiles of clinical candidates. Anticancer Agents Med Chem. 2010;10(1):7–27.
116. Lu R-M, Chang Y-L, Chen M-S, Wu H-C. Single chain anti-c-Met antibody conjugated nanoparticles for in vivo tumor-targeted imaging and drug delivery. Biomaterials. 2011;32(12):3265–74.
117. Kanehira M, Xin H, Hoshino K, Maemondo M, Mizuguchi H, Hayakawa T, et al. Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. Cancer Gene Ther. 2007;14(11):894.
118. Chen Y, Mathy NW, Lu H. The role of VEGF in the diagnosis and treatment of malignant pleural effusion in patients with non-small cell lung cancer. Mol Med Rep. 2018;17(6): 8019–30.
119. Vázquez S, Anido U, Lázaro M, Santomé L, Afonso J, Fernández O, et al. Angiogenesis and lung cancer. In: Oncogenesis, inflammatory and parasitic tropical diseases of the lung. Croatia: InTech; 2013.
120. Herbst RS, Onn A, Sandler A. Angiogenesis and lung cancer: prognostic and therapeutic implications. J Clin Oncol. 2005;23(14):3243–56.
121. Hall RD, Le TM, Haggstrom DE, Gentzler RD. Angiogenesis inhibition as a therapeutic strategy in non-small cell lung cancer (NSCLC). Transl Lung Cancer Res. 2015;4(5):515.
122. Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol. 2004;22(11):2184–91.
123. Doebele RC, Spigel D, Tehfe M, Thomas S, Reck M, Verma S, et al. Phase 2, randomized, open-label study of ramucirumab in combination with first-line pemetrexed and platinum chemotherapy in patients with nonsquamous, advanced/metastatic non–small cell lung cancer. Cancer. 2015;121(6):883–92.
124. Blumenschein GR, Saintigny P, Liu S, Kim ES, Tsao AS, Herbst RS, et al. Comprehensive biomarker analysis and final efficacy results of sorafenib in the BATTLE trial. Clin Cancer Res. 2013;19(24):6967–75.
125. Dingemans A-MC, Mellema WW, Groen HJ, Van Wijk A, Burgers SA, Kunst PW, et al. A phase II study of sorafenib in patients with platinum-pretreated, advanced (stage IIIb or IV) non–small cell lung cancer with a KRAS mutation. Clin Cancer Res. 2013;19(3):743–51.
126. Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res. 2000;60(8):2178–89.
127. Scagliotti G, Vynnychenko I, Park K, Ichinose Y, Kubota K, Blackhall F, et al. International, randomized, placebo-controlled, double-blind phase III study of motesanib plus carboplatin/paclitaxel in patients with advanced nonsquamous non-small-cell lung cancer: MONET1. J Clin Oncol. 2012;2012:2829–36.
128. Kubota K, Ichinose Y, Scagliotti G, Spigel D, Kim J, Shinkai T, et al. Phase III study (MONET1) of motesanib plus carboplatin/paclitaxel in patients with advanced nonsquamous nonsmall-cell lung cancer (NSCLC): Asian subgroup analysis. Ann Oncol. 2014;25(2):529–36.
129. Reck M, Kaiser R, Mellemgaard A, Douillard J-Y, Orlov S, Krzakowski M, et al. Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. Lancet Oncol. 2014;15(2):143–55.

130. Bukowski RM. AE-941, a multifunctional antiangiogenic compound: trials in renal cell carcinoma. Expert Opin Investig Drugs. 2003;12(8):1403–11.
131. Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Control Release. 2008;129(2):107–16.
132. Sundaram S, Trivedi R, Durairaj C, Ramesh R, Ambati BK, Kompella UB. Targeted drug and gene delivery systems for lung cancer therapy. Clin Cancer Res. 2009;15:7299–308. https://doi.org/10.1158/1078-0432.CCR-09-1745.
133. Zhang L, Liu Z, Yang K, Kong C, Liu C, Chen H, et al. Tumor progression of non-small cell lung cancer controlled by albumin and micellar nanoparticles of itraconazole, a multitarget angiogenesis inhibitor. Mol Pharm. 2017;14(12):4705–13.
134. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316(5827):1039–43.
135. Regales L, Gong Y, Shen R, de Stanchina E, Vivanco I, Goel A, et al. Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer. J Clin Invest. 2009;119(10):3000–10.
136. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med. 2011;3(75):75ra26.
137. Jänne PA, Yang JC-H, Kim D-W, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor–resistant non–small-cell lung cancer. N Engl J Med. 2015;372(18):1689–99.
138. Mokhtari RB, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, et al. Combination therapy in combating cancer. Oncotarget. 2017;8(23):38022.
139. Lynch TJ, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non–small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J Clin Oncol. 2012;30(17):2046–54.
140. Reck M, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer: results from a randomized, double-blind, multicenter phase 2 trial. Ann Oncol. 2012;24(1):75–83.
141. Pirker R, Szczesna A, Von Pawel J, Krzakowski M, Ramlau R, Park K, et al. FLEX: a randomized, multicenter, phase III study of cetuximab in combination with cisplatin/vinorelbine (CV) versus CV alone in the first-line treatment of patients with advanced non-small cell lung cancer (NSCLC). J Clin Oncol. 2008;26(15_suppl):3.
142. Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, Hasmann M. Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. Cancer Res. 2009;69:9330–6. https://doi.org/10.1158/0008-5472.CAN-08-4597.
143. Ramalingam SS, Maitland ML, Frankel P, Argiris AE, Koczywas M, Gitlitz B, et al. Carboplatin and paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non–small-cell lung cancer. J Clin Oncol. 2010;28(1):56.
144. Heigener DF, Pereira JR, Felip E, Mazal J, Manzyuk L, Tan EH, et al. Weekly and every 2 weeks cetuximab maintenance therapy after platinum-based chemotherapy plus cetuximab as first-line treatment for non-small cell lung cancer: randomized non-comparative phase IIIb NEXT trial. Target Oncol. 2015;10(2):255–65.
145. Pirker R, Pereira JR, Szczesna A, Von Pawel J, Krzakowski M, Ramlau R, et al. Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial. Lancet. 2009;373(9674):1525–31.
146. Kim ES, Neubauer M, Cohn A, Schwartzberg L, Garbo L, Caton J, et al. Docetaxel or pemetrexed with or without cetuximab in recurrent or progressive non-small-cell lung cancer after platinum-based therapy: a phase 3, open-label, randomised trial. Lancet Oncol. 2013;14(13):1326–36.

147. Al-Farsi A, Ellis PM. Treatment paradigms for patients with metastatic non-small cell lung cancer, squamous lung cancer: first, second, and third-line. Front Oncol. 2014;4:157.
148. Kim HR, Jang JS, Sun J-M, Ahn M-J, Kim D-W, Jung I, et al. A randomized, phase II study of gefitinib alone versus nimotuzumab plus gefitinib after platinum-based chemotherapy in advanced non-small cell lung cancer (KCSG LU12-01). Oncotarget. 2017;8(9):15943.
149. Blumenschein G Jr, Sandler A, O'rourke T, Eschenberg M, Sun Y, Gladish G, et al. Safety and pharmacokinetics (PK) of AMG 706, panitumumab, and carboplatin/paclitaxel (CP) for the treatment of patients (pts) with advanced non-small cell lung cancer (NSCLC). J Clin Oncol. 2006;24(18_suppl):7119.
150. Registry IGCT. A phase II trial of carboplatin, pemetrexed, and panitumumab in patients with advanced non-squamous K-ras wild type non-small-cell lung cancer carboplatin, pemetrexed, and panitumumab in patients with advanced non-squamous K-ras wild type NSCLC. https://ichgcp.net/clinical-trials-registry/NCT01042288. Accessed 26 Jan 2019.
151. Hughes B, Mileshkin L, Townley P, Gitlitz B, Eaton K, Mitchell P, et al. Pertuzumab and erlotinib in patients with relapsed non-small cell lung cancer: a phase II study using 18F-fluorodeoxyglucose positron emission tomography/computed tomography imaging. Oncologist. 2014;19(2):175–6.
152. Medicine USNLo. Safety and effect of pertuzumab in patients with advanced non-small cell lung cancer, which has progressed after prior chemotherapy. https://clinicaltrials.gov/ct2/show/NCT00063154. Accessed 26 Jan 2019.
153. Medicine USNlo. A study of gemcitabine-cisplatin chemotherapy plus necitumumab in the first-line treatment of participants with squamous lung cancer. https://clinicaltrials.gov/ct2/show/NCT01788566. Accessed 26 Jan 2019.
154. Medicine USNLo. A study of trastuzumab emtansine in participants with human epidermal growth factor receptor (HER)2 immunohistochemistry (IHC)-positive, Locally Advanced or Metastatic Non-Small Cell Lung Cancer (NSCLC).
155. Costa DB, Shaw AT, Ou S-HI, Solomon BJ, Riely GJ, Ahn M-J, et al. Clinical experience with crizotinib in patients with advanced ALK-rearranged non–small-cell lung cancer and brain metastases. J Clin Oncol. 2015;33(17):1881.
156. Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. Cancer Discov. 2014;4:1046–61. CD-14-0337.
157. Rizvi NA, Mazières J, Planchard D, Stinchcombe TE, Dy GK, Antonia SJ, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. Lancet Oncol. 2015;16(3):257–65.
158. Fehrenbacher L, Spira A, Ballinger M, Kowanetz M, Vansteenkiste J, Mazieres J, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet. 2016;387(10030):1837–46.
159. Langer CJ, Gadgeel SM, Borghaei H, Papadimitrakopoulou VA, Patnaik A, Powell SF, et al. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. Lancet Oncol. 2016;17(11):1497–508.
160. Shaw AT, Gandhi L, Gadgeel S, Riely GJ, Cetnar J, West H, et al. Alectinib in ALK-positive, crizotinib-resistant, non-small-cell lung cancer: a single-group, multicentre, phase 2 trial. Lancet Oncol. 2016;17(2):234–42.

Part III
Receptors and Receptor Mediated Endocytosis in Infectious Diseases

Chapter 9
Role of Chemokines and Chemokine Receptors in Infectious Diseases and Targeting Strategies

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Abstract Chemokine receptors, a family of G-protein-coupled receptors (GPCRs), bind in a specific manner to chemokines and elicit cellular responses. Their involvement in inflammatory diseases is predominant. Although the main function of chemokine receptors is enrolment of leukocytes at the site of inflammation, they are also widely explored as drug discovery targets. This is due to the fact that blockage of chemokine receptor may provide novel therapeutic interventions. This chapter discusses the various chemokine receptors, involvement of chemokine receptors in the pathogenesis of various diseases, and receptor-mediated strategies to tackle such afflictions.

Keywords Chemokines · G protein-coupled receptor · Receptor-mediated targeting · Signaling · Ligands · Cytokines · Transmembrane

Abbreviations

ACKR	Atypical chemokine receptor
AIDS	Acquired immune deficiency syndrome
AS	Atherosclerosis
BLR1	Burkitt's lymphoma receptor 1
cAMP	Cyclic adenosine monophosphate

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CD4	Cluster of differentiation 4
CHO	Chinese hamster ovary
CM	Cerebral malaria
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
DAG	Diacylglycerol
DARC	Duffy antigen receptor for chemokines
EBV	Epstein–Barr virus
ELC	EBI1 ligand chemokine
GCP	Granulocyte chemotactic Protein
GDP	Guanosine diphosphate
GPCR	G-protein-coupled receptors
GTP	Guanosine triphosphate
GvHD	Graft versus host disease
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HIVE	HIV encephalitis
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IC 50	inhibitory concentration 50
IFN	Interferon
IL-1/IL-8	Interleukin
I-TAC	interferon-inducible T-cell alpha chemoattractant
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic/chemoattractant protein
MIG	monokine induced by gamma interferon
MIP1α/1β	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
MTC	Medullary thyroid carcinomas
NAP	Neutrophil-activating peptide
NK	Natural killer
PIP2	Phosphatidylinositol 4, 5-biphosphate
PLC	Phospholipase C
PLGA	Polylactic-co-glycolic acid
PM	Placental malaria
PTC	Papillary thyroid carcinomas
RA	Rheumatoid arthritis
RANTES	Regulated on activation normal T-cell expressed and secreted
RR-MS	Relapsing–remitting multiple sclerosis
SDF	Stromal-derived Factor
SDP	Spirodiketopiperzine
SLC	Secondary lymphoid tissue chemokine
TECK	Thymus-expressed chemokine
Th2	T lymphocytes
TxP	Threonine x proline
WNV	West Nile virus

1 Introduction

Chemokines are secretory and transmembrane proteins which are structurally related, and whose main functions are to recruit leukocyte populations employing specific receptors into the target. Based on their function chemokines can be classified into two major classes, inflammatory chemokines and immune chemokines. Inflammatory chemokines attract and activate monocytes and neutrophils, thereby playing a major role in inflammatory conditions that are acute [1]. Promiscuity of the receptor and significant redundancy of ligands, which is their major characteristic, facilitates recruitment of inflammatory cells in severe conditions. The immune chemokines play a significantly different role by attracting dendritic and lymphoid cells reflecting their involvement in immune reactions and inflammatory diseases which are chronic. This suggests their great promise and involvement in the therapy of immunological and inflammatory diseases [2]. The receptor has also been studied for therapeutic applications through targeted drug delivery strategies. This chapter discusses chemokine receptors, importance and targeted drug delivery strategies, and their potential clinical applications.

2 The Chemokine Receptors

Chemokines are small signaling peptide molecules with molecular weight 8–10 kDa secreted by the cells of the immune system in the presence of endogenous stimuli like IFN, IL-1, and tumor necrosis factor (TNF) and external stimulus like bacterial lipopolysaccharide (LPS) or viruses [3]. These chemokines cause migration of immune cells and help in recruiting them to the site of action through a variety of mechanisms. The process by which activated immune cells move toward the site of infection is called chemotaxis, that is, the movement of cells in response to chemical stimuli. Chemokines are therefore chemotactic cytokines comprising of >50 small secretory proteins that exhibit their effect on target tissues by interacting with seven heterotrimeric transmembrane (7TM) spanning GPCR family [4]. Chemokine receptors present on the surface of immune cells that interact with chemokine, a type of cytokine, are also known as cytokine receptors [5]. They belong to the large family of GPCR which includes receptors for inflammatory mediators, neurotransmitters, paracrine substances, odorant molecules, calcium ions, proteinases, hormones, and even photons [6].

Immune cells such as leukocytes primarily express chemokine receptors. Chemokines bind to GPCR giving rise to responses such as change in conformation in the receptor, triggering intracellular events that drive cell polarization, migration, and adhesion. This results in the induction of homing and leukocyte trafficking [7]. There are two major classes of chemokine receptors, namely, (CC) containing adjacent cysteines and (CXC) containing cysteines separated by amino acid. The CC receptors bind to CC chemokines and are named CCR1 to 9, and the CXC

Table 9.1 Chemokine receptor classification and various ligands

Class	Subtype	Ligands	Expression on cells
CC	CCR1	MIP-1α, RANTES, MCP-2, MCP-3	Immature DC, mesangial cells
	CCR2A/B	MCP-1, MCP-2, MCP-3, MCP-4, IL-2, IL-10	Immature DC, endothelium, fibroblast
	CCR3	Eotaxin, eotaxin 2, RANTES, MCP-3, MCP-4, MIP-5	T(Th2), eosinophils, basophils, macrophage, dendritic cells
	CCR4	TARC, MDC	T(Th2), basophils, immature dendritic cells
	CCR5	MIP-1α, MIP-1β, RANTES, MCP-2, MIP-1, IL-10	NK cells, thymocyte, dendritic cells
	CCR6	LARC, MIP-3 α, IL-2	T cells, B cells, immature dendritic cells
	CCR7	ELC, SLC	T cells, B cells, dendritic cells
	CCR8	I-309	Neutrophils, thymocyte, T cells, B cells
	CCR9	TECK	T thymocyte, dendritic cells
CXC	CXCR1	IL-8, NAP-2, ENA-78, GCP-2	Neutrophils, T cells, NK cells, mast cells, macrophage, dendritic cells, fibroblasts
	CXCR2	IL-8, GROα, GROβ, GROγ, NAP-2, ENA-78, GCP-2	Monocytes, lymphocytes, neutrophils, T cells, eosinophils, endothelium
	CXCR3	MIG, IP-10, I-TAC, eotaxin, SLC	Neutrophils, Th1, dendritic cells, eosinophils
	CXCR4	SDF-1	Neutrophils, B cells, dendritic cells, macrophage, astrocyte
	CXCR5	BCA-1/BCL-1	T cells, mature B cells
C	XCR1	Lymphotactin	T cells
CX_3C	CX3CR1	Fractalkine	Neutrophils, macrophage, T cells, NK cells, monocyte, neurons, dendritic cells
	D6	MIP-1, RANTES, MCP-1	Alveolar macrophage, innate like B cells
Duffy	DARC (ACKR1)	IL-8, NAP-2, GRO, I-309, RANTES, MCP-1	Erythrocytes, endothelial cells, T lymphocytes

chemokines selectively bind to the CXC receptors termed CXCR1 to CXCR5. The Duffy antigen chemokine receptor (DARC) is known to bind both CXC and CC chemokine receptors as illustrated in Table 9.1 [3, 8–10]. Inflammatory mediators such as basophils, eosinophils, macrophage, and dendritic cells primarily express CC chemokine receptors, neutrophils mainly express CXC chemokine receptors, whereas lymphocytes express both CC and CXC types of cytokines receptors. Another study has shown that chemokine receptors that have been differently expressed cause nonimmunogenic antigens to become immunogenic. This "differently expressed" receptor is in fact the fusion of chemokine with antigen-presenting cells [11]. Chemokine targeting is now seen as a novel target for the treatment of atherosclerosis. This may be possible due to interference with disease progression at a particular stage of disease [12].

2.1 CXCR1 and CXCR2

CXCR1 receptor contains 350 amino acids, N-terminal domain-containing N-linked glycosylation site and all other features similar to that of a 7TM GPCR. IL-8, a CXC chemokine shows high affinity toward CXCR1 and CXCR2 [13]. CXCR1 binds to neutrophil-activating peptide 2 (NAP-2), granulocyte chemotactic protein 2 (GCP-2), and epithelial neutrophil-activating peptide 78 (ENA-78) with low affinity, while binding to IL-8 is highly selective. Growth-related oncogene family are the additional chemokines which bind to CXCR2 [14]. IL-8, a potent chemoattractant, displays its role in acute inflammation by activating CXCR2 and CXCR1 expressed on the surface of neutrophils [15]. Literature reports in vitro production of IL-8 by various cells like keratinocytes, fibroblasts, mast cells, neutrophils, monocytes, endothelial cells, and macrophages [16, 17]. CXCR1 and CXCR2 are expressed in all cell types including CD56+ NK cells, some CD8+ T cells, mast cells, monocytes, and granulocytes. Neutrophils express equal amount of CXCR1 and CXCR2, while expression of CXCR2 is predominant in monocytes and positive lymphocytes compared to CXCR1. TNF and LPS are known to downregulate expression of both CXCR1 and CXCR2 upon activation of tyrosine kinase-dependent signaling pathway. On the contrary, CXCR1 and CXCR2 expression is upregulated by bacterial-derived molecule fmlp and granulocyte colony-stimulating factor [18, 19].

2.2 CXCR3

CXCR3, a member of CXC chemokine receptor family whose primary role is in T-cell trafficking, is expressed majorly on effector T cells. It shares 41% similar amino acids to that of CXCR1 and CXCR2 receptors [20]. CXCR3 shows high expression of Th1-type CD4+ T cells, effector CD8+ T cells. Three interferon-inducible ligands activating CXCR3 are CXCL9 which is also referred as monokine induced by gamma-interferon or MIG, CXCL10 referred as interferon-induced protein of 10 kDa or IP-10 and I-TAC, that is, interferon-inducible T-cell alpha chemoattractant, or CXCL11. It is also worth noting that dendritic cells of plasma and subset of B cells show CXCR3 expression which could play a critical role in favoring their movement into inflamed lymph nodes [21].

2.3 CXCR4

The stromal-derived factor 1 (SDF-1) which is a CXC chemokine, attaches to CXCR4 and activates it leading to stimulation of cellular migration and polymerization of actin in a dose-dependent manner. It has been recently reported to be a vital HIV-1 coreceptor. SDF1α is a highly promising lymphocyte chemical attractant

which also inhibits CD4+ permissive HIV-1 infection depending on CXCR4 expression pattern. A recent study suggests IL-4 causes overexpression of CXCR4 on resting T cells, while T-cell stimulation by CD28, CD3, and CD2 causes its downregulation [22, 23].

2.4 CXCR5

CXCR5 also known as Burkitt's lymphoma receptor 1 (BLR1) is a 7TM domain G protein-coupled receptor which under normal conditions shows predominant expression by follicular helper T cells and mature B cells and controls their migration in the secondary lymphoid organs. CXC chemokine CXCL13 binds to CXCR5 and causes activation of multiple intracellular signaling pathways which regulates cellular functions like cell proliferation, survival, and migration [24].

2.5 XCR1

The receptor for lymphotactin is XCR1. This receptor is expressed strongly in placenta and weakly in spleen and thymus, which correlates with the expression of lymphotactin in these tissues.

2.6 CX3CR1

A CXC chemokine called as fractalkine is known to bind CX3CR1 chemokine receptor. Fractalkine, a glycoprotein attached to membrane with the chemokine, appears perched on an elongated mucin-like strand. CX3CR1 shows high-affinity binding with fractalkine [25]. In the bound form, fractalkine promotes adhesion of monocytes, T lymphocytes, natural killer cells to dendritic, endothelial, and epithelial cells [26].

2.7 CCR1

A cytomegalovirus protein, US28, binds to CCR1 receptor. CCR1 receptor is 33% homologous to the 7-transmembrane protein US28 [27]. Expression of CCR1 receptor causes increase in the F actin content, inhibition of cAMP formation, and basal migration of leukocytes. CCR1 receptor responds to binding of MIP-1a, MCP-2, RANTES (regulated on activation normal T-cell expressed and secreted), along with MCP-3 show specific binding toward HCC1 and CK8b. T-cell

expression of chemokine receptor was induced by IL-2 and IL-15, whereas selective upregulation of CCR1 in human monocytes was facilitated by IL-10 [28].

2.8 CCR2A and CCR2B

Charo et al. produced two clones of CCR2 receptor, that is, CCR2A and CCR2B. Structurally CCR2A and CCR2B contain similar transmembrane and 58 untranslated regions but they differ in their C-terminus. A 36% similarity was observed between carboxy tail of CCR1 and CCR2B, whereas CCR2A bears no such similarity [29]. Lipopolysaccharide and IFN-γ have been reported to decrease expression of CCR2 receptors, whereas IL-2 causes increased expression. Expression of CCR2 in monocytes is enhanced by IL-10 [30].

2.9 CCR3

Eotaxin and eotaxin-2, two CC chemokines, represent the primary ligands for the 7TM GPCR CCR3 and bind them with greatest affinity hence, serve as predominant eosinophil activators which represent a variety of pathological conditions like hypereosinophilic syndrome asthma and urticaria [31, 32]. Thus, CCR3 plays a central role in controlling eosinophil migration. CCR3 has been reported in progressing M tropic HIV-1 infection of permissive cells in conjunction with CD4. CCR3 receptor shows 63% resemblance to CCR1 and 51% to CCR2B and is involved in binding of several CC chemokines. Chemokines which show specific binding toward CCR3 receptor includes MCP-4, RANTES, eotaxin, MCP-3, eotaxin-2, and MIP-5, which altogether are important in eosinophil recruitment and activation [33, 34].

2.10 CCR4

CCL17 or the thymus and activation-regulated chemokine and a macrophage-derived chemokine CCL22, both CC chemokine ligands bind specifically to CCR4. These two chemokine ligands are known to selectively activate CD4+ Th2 T lymphocyte. T-cell receptor and CD28 predominantly enhance expression of CCR4 on Th2 cells [35, 36].

2.11 CCR5

CCR5 shows affinity toward CC chemokines RANTES, MIP-1, MCP-2, MIP-1α, and MIP-1β [37]. CCR5 shows resemblance to CCR2B receptors with 71% identical amino acids. R5 strains of the human immunodeficiency virus (HIV-1 and -2)

enter permissive cells aided by CCR5 as co-receptor in association with CD4. IL-10, an immunosuppressive and anti-inflammatory cytokine, causes upregulation of CCR5 in human monocytes by activation of MAP and STAT kinases [38, 39].

2.12 CCR6

CCR6 receptor shows selective affinity toward LARC (liver and activation-regulated chemokine) and macrophage inflammatory protein (MIP)-3α//Exolus-1/CCL20 [40]. B-lymphocytes, memory T cells, and dendritic cells show CCR6 expression, while peripheral blood leukocytes do not. IL-2 has been reported to cause upregulation of CCR6 mRNA [41]. However, contradictory results have been reported [42]; hence, the expression is not completely elucidated.

2.13 CCR7

CCR7 shows activation upon binding of a CC chemokine ligand CCL21. Dendritic cells and lymphocytes express CCR7 in lymph node, the site for medullary (MTC) and papillary thyroid carcinomas (PTC). T- and B-lymphocytes and dendritic cells show CCR7 expression. CCR7 is highly upregulated in herpesvirus-infected T cells and B cells infected with Epstein–Barr virus [43].

2.14 CCR8

I-309, a CC chemokine, is the only ligand which binds to CCR8. Monocytes and T lymphocytes are mainly activated by I-309 [44]. Type 2 T lymphocyte (Th2)-polarized cells show preferential expression of CCR8 receptor thus postulating that Th2 responses are mainly restricted to CCR8. CCR8 in association with CD4+ acts as a coreceptor for chemokine ligand I-309 of M tropic HIV-1 strains and is reported to be a binding and fusion inhibitor of HIV-1 [45].

2.15 CCR9

CCR9 is the recently identified chemokine receptor specific for the β chemokine thymus-expressed chemokine (TECK)/CCL25 [46]. Activation of dendritic cells and thymocytes by TECK indicates the role played by this CC chemokine in T-cell development [47]. Expression of CCR9 receptor is predominant in thymus, whereas lymph node and spleen show relatively less expression of this receptor [48].

2.16 D6

D6 receptor lacks intracellular signaling on binding of ligand and therefore it appears to be nonfunctional [49]. Monocyte chemoattractant protein (MCP-1 and -2) binds to D6 with high affinity, whereas some researchers reported the receptor to be promiscuous due to its ability to bind several chemokines with similar affinity [50].

2.17 Duffy Antigen Receptor for Chemokines (DARC)

DARC shows affinity toward both CC chemokine and CXC chemokine ligand MCP-1 and IL-8. Other ligands include MCP-3, MCP-4, GRO-α, RANTES, I-309, and eotaxin [51]. DARC is primarily involved in the pathogenesis of malaria. It shows predominant expression on erythrocytes but has also been detected on the capillary endothelium of kidney and spleen. CXCL8-binding proteins have properties similar to human erythrocyte blood group antigen known as duffy that facilitates entry of Plasmodium vivax to the malarial parasite. Duffy antigen displays promiscuity to CC and CXC chemokines. Based on these findings, duffy antigen DARC was renamed as atypical chemokine receptor 1 (ACKR1). Also, binding of chemokine to DARC caused inhibition of Plasmodium vivax infection [52].

3 Chemokine Receptor in Pathogenesis of Diseases

Chemokine receptors participate in the progression of various diseases either by causing overexpression of the receptors or by facilitating access of virus into the target tissues to develop infection. They take part in leucocyte trafficking, recirculation, and recruiting. The chemokine receptor redundancy allows specific receptor to bind several chemokines signaling through 7 TM GPCR. Infectious diseases like malaria and HIV have been shown to use chemokine receptors as entry receptors and coreceptors, respectively. Studies showing the genetics of these receptors and their significance in the pathogenesis of infectious diseases have been possible due to the genetic mutations in these receptor genes that encode the entry receptors for the two pathogens [53].

3.1 Chemokine Receptors as Virus Entry Mediators in HIV Infection

An interesting finding with respect to HIV infection is the fact that leukocyte recruitment and its regulation is central to the process [54].

HIV envelope proteins interact with CD4+ cell surface receptor to ensure efficient binding of virus. The conformational changes in the virus envelope are seen upon interaction with the CCR5 and CXCR4 coreceptors to produce fusion between the viral and cell membrane of the host [55]. Different coreceptors used by different HIV 1 isolates help understand their biological variability. Discovery of coreceptors has caused the change in the nomenclature of the HIV strain [56]. HIV virus strain using CXCR4 as coreceptor are named as X4, strains which involve CCR5 as coreceptor are named as R5, whereas strains which use both the receptors are named as X4R5 virus strains. In progressive stages of AIDS, large percentage of T cells express CXCR4 than CCR5 which allows more cells to be infected and destroyed [57].

Stromal cell-derived factor 1 (SDF-1) exhibits affinity for CXCR4, whereas RANTES, MIP-1 beta, and MIP-1 alpha which belong to β chemokine family are ligands for CCR5, MCP-5 promotes binding of (MCP-1) to CCR2. Eotaxin 1 and 2, MCP-4, and MCP-3 readily bind to CCR3. Thus, R5 strain of virus can be inhibited by ligands which bind CCR5. SDF-1 acts as a specific inhibitor of CXCR4. Hence, chemokine receptor expression in a specific cellular type can be coined as inducible or constitutive [58]. RANTES, MIP-1alpha, and MIP-1beta, the ligands of CCR5, are known to inhibit HIV-1 infection. In a particular study, a chemokine selective for eosinophil was isolated from allergen-challenged guinea pigs and was called "eotaxin." This eotaxin has two crucial roles that it allows for recruitment of eosinophils at the local site and also enhances movement of eosinophils of the bone marrow [59].

Eotaxin's receptor is CCR3. Eotaxin has been known to be a selective ligand for CCR3 but it can also interact with CCR2 and CCR5. Eotaxin is expressed on eosinophils, basophils, and Th2 lymphocytes. It has been understood from a study that eotaxin has antagonistic effect on CCR3. In addition, it has been shown eotaxin is a CCR2 antagonist [30]. A study indicated that eotaxin-3 has modulatory function and not inflammatory as suggested previously [60].

3.2 Malaria

Malaria, a severe parasitic infection of *Plasmodium* species, the most common being *P. falciparum,* is easily transmitted to humans hosts by the female anopheles mosquito. Species of the Plasmodium family that can cause malaria include *P. falciparum, P. malariae, P. ovale*, and *P. vivax*. Progression of the disease is mainly regulated by RANTES expression [61]. A study has shown that RANTES levels were upregulated at the peak of malarial infection in mouse. Hence, it was concluded that RANTES mediates inflammation that is a crucial part of malarial pathogenesis or in other words, the leukocyte recruitment helps in the pathogenesis of the disease. The leukocytes are recruited by chemokines and their receptors [62]. It was also shown that CCR1, CCR3, and CCR5 the receptors for RANTES which are expressed by macrophages, basophils, etc., also have a role in malaria upregulation.

In case of cerebral malaria (CM), the blood–brain barrier is affected causing the release of chemokines and variety of inflammatory cells inside the brain [63]. As mentioned before, CCR1, CCR3, and CCR5 have been implicated in the

progression of malaria. According to a study, CCR2 does not play any role in the development of CM (caused by *P. falciparum*) [64].

Another way to look at the CM pathogenesis is to see the CD8+ T-cell proliferation and activation that causes cerebral damage. These CD8+ T cells are activated via chemokine receptors such as CCR5 and CXCR3. CCR5 that binds CCL3, CCL4, and CCL5 is revealed as a potent chemoattractant for CD8+ T cell. A study has shown that CCR5 deficiency reduces the likeliness of CM occurrence [65]. CXCR3 has also been given attention and in a study, mice lacking CXCR3 were seen less likely to be suffering from CM with fewer T cells implying that lesser chemokine production did not lead to recruitment of T cells. Interestingly, the same study concluded that mice with CM showed increased release of chemokines that bind to CXCR3 [66]. Furthermore, a study illustrates that in the mice with CM, NK cells aid in enhanced recruitment of CXCR3+ T cells [30]. CXCR3 knockouts were protected remarkably from CM, hence confirming the important role of CXCR3 in CM. Continuing on CXCR3, a study suggested that CXCR3 and its ligand CXCL9 recruit T cells [67].

An interesting finding by a study conducted in 1998 was interaction between HIV and malaria [68]. It was later shown that placentas of malaria-infected mothers often contain macrophages in a high concentration [69]. A study concluded that the coinfection state in pregnant Malawi women could be linked to CCR5 expression (in turn, the macrophages recruited by them) which led to higher malarial parasitemias as well as the fact that it was associated with HIV infection [70].

In yet another coinfection study of two *Plasmodium* species, it was found that the coinfection state led to reduced levels of chemokine receptor and its ligands leading to lesser accumulation of CD8+ T cells, and hence prevents CM in such mice. A study with children suffering from acute malaria showed that there is dysregulation of the CC chemokines that is MIP-1alpha and MIP-1beta levels were high, while RANTES level was low at the mRNA and protein level [71].

Looking at another major type of malaria, the placental malaria (PM), a study wherein the investigators tested the chemokine concentration in placental intervillous blood plasma of four different types, that is, {HIV+ PM+/PM−, HIV− PM+ PM−} found that MIP-1beta (belonging to chemokine CC subfamily) was higher in PM+ women than in HIV+ PM- and was not related to their HIV status. The MIP-1alpha levels were invariable though [72]. CCR5 being a coreceptor for macrophage-trophic HIV-1, it can be concluded that CCR5 upregulation can indeed be used in treating coinfection of HIV and PM. A study suggests that CCR3 and CCR5 both help/promote infection of HIV-1 in the central nervous system [73].

3.3 Atherosclerosis

There exists comprehensive literature on the role of chemokines in atherosclerosis (AS) and presently, several in vivo studies have suspected the involvement of several chemokine ligands and receptors in the process of AS [74]. AS is a disease that is known to cause high fatality worldwide. It was known that CCR5 had a role to

play in AS but what effect it had was clearly assumed only after a study that stated that CCR5 expression is usually upregulated during AS plaques [75]. CCR1 and CCR5 have been known to bind to those cell types that are associated with AS. RANTES, CCL3, and CCL4 have been present in monocytes/macrophages/Th1 that have implications in the disease. Met-RANTES is an antagonist for RANTES which is a peptide receptor. This Met-RANTES has been studied in mice and has shown reduction in AS-related lesions. Earlier reports have been tentative, as reports have said that CCR5 deletion led to decline in the formation of atherosclerotic lesions. Another study stated that mice with CCR5 deletion enhanced the plaque quality but had no effect on its size. Thus, a study was performed to give a conclusion to the same and it was found that deficiency in CCR5 protected the cells against the formation of lesions and the accumulation of cells that was associated with the disease. It has been reported that genetic deletion of CX3CR1 and CCR2 decreases AS [76].

3.4 Miscellaneous

The chemokine receptor plays an important role in various other ailments. The same is summarized in Table 9.2 [28, 77–83].

4 Chemokine Receptor Structure

Chemokine receptors, a superfamily of GPCR, are known to bind chemokines in a specific manner and to elucidate a cellular response. They are membrane-bound molecules comprising of parallel strands of 7TM domain that couple with G proteins. Literature reports 18 human chemokine receptors till date. Due to their promiscuous nature, certain CXC chemokines show selective binding toward CXCR1 to CXCR5 receptors. In addition, nine receptors (CCR1 to CCR9) belong to CC chemokine receptor family. CX3CR1 and CXCR1-specific chemokine, Fractalkine has been identified. DARC binds indiscriminately to both CC and CXC chemokine [84]. A schematic representation of a chemokine receptor is illustrated in Fig. 9.1.

4.1 Structural Requirements for Chemokine Receptor Binding

4.1.1 Cysteine

The presence of cysteine residue on the extracellular loop is essential for proper alignment of the receptor on the cell membrane to ensure receptor signaling [85]. Out of the four conserved cysteine residues, one is present on the N-loop and other

Table 9.2 Involvement of chemokine receptors in various pathologies

Sr No.	Disease	Chemokine receptor	Ligand	Pathophysiology	References
1	HCMV virus	US28	CC chemokines, MCP-1, MCP-3, MIP1-a, and MIP1-b, RANTES. Soluble forms of the CX3C chemokine, fractalkine.	US28 overexpression causing smooth muscle cell (SMC) proliferation and migration thus causing disease progression	[28]
2	Herpes simplex virus	CCR1, CCR2, CCR5	CCL3, CCL5, CXCL1, CXCL2, CXCL8, and CXCL9	Chemokine expression due to TNFα, PMNs, macrophage, and NK cells suppresses local HSV-1 infection	[77, 78]
3	CMV encephalitis	CCR1, CCR2, CCR5, CXCR3	Increased RANTES, MIP-1α, Fractalkine, IP-10, and lymphotactin	IP-10 overexpression leads to infiltration of monocytes and lymphocytes which has been linked to CMV infection	[79, 80]
4	HIV encephalitis	CCR5, CCR1, CCR3, CXCR4	MCP-1, MIP-1α, and RANTES	CCR5 appears to be most important for monocytes, macrophages, and microglia causing recruitment of inflammatory infiltrates and formation of microglial nodules leading to disease progression	[81]
5	Multiple sclerosis	CCR1, CCR2, CCR5, CXCR3	CCL3, CXCL12, and CXCL13	CXCL13 has a strong linkage to immunoglobulin levels and also maintains B and T cells in lesions. CXCL12 plays role in leukocyte extravasation	[82]
6	Psoriasis	CCR1-CCR5, CCR6, CXCR3	CCL20	Overexpression on monocytes, macrophages, and T cells leading to chemotaxis of monocytes, macrophage and T cells	[10]
7	Allergy	CCR3, CCR4, CCR8	Thymus- and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC) and I-309, eotaxin, eotaxin-2, MCP-3, MCP-4	CCR3 elevation causes enhanced levels of eotaxin and MCP-4 causing infiltration of eosinophils implicating role of these chemokines in asthmatic lung inflammation	[83]

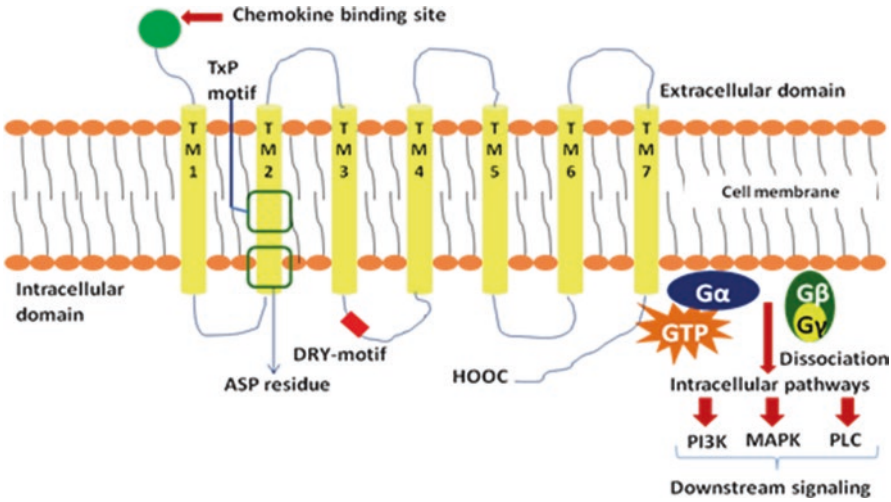

Fig. 9.1 Schematic representation of a chemokine receptor depicting receptor activation and signaling (MAPK Mitogen-activated protein kinases, DRY Asp-Arg-Tyr motif)

three on each of the three extracellular loops. Formation of disulfide bond on loop 1 and 2 is a prerequisite to elicit a cellular response. Formation of disulfide bond between N-loop and extracellular loops of CCR6 receptor is absent. However, in CCR5, all four cysteines are essential for functioning of chemokine receptor [86].

4.1.2 Sulfated Tyrosine

The HIV progression is initiated by the presence of sulfated tyrosine in the N-terminal loop. Posttranslational sulfation of tyrosine in Golgi apparatus affects ligand-binding affinity of chemokine receptors. CCR5 receptor exists in two forms, namely, sulfated and nonsulfated CCR5 based on the presence and absence of sulfated tyrosine. Interaction of N-loop of CCR5 containing sulfated tyrosine with the HIV envelope protein gp120 facilitates the progression of HIV infection, whereas entry of HIV virus is restricted in nonsulfated N-loop due to the absence of interaction with CD4 complexes/gp120 thus causing inhibition of binding and fusion [87].

4.2 Chemokine Receptor Activation and Signaling

Chemokine receptors are stimulated by several ligands, demonstrating that activation is not necessarily due to similar modes of ligand binding but due to similar molecular mechanisms. Though some chemokine receptors show monogamous binding to their ligand, majority of them show promiscuous binding which is however restricted to the same chemokine class [88].

Upon binding of chemokine ligand to the chemokine receptor conformational changes occur in the 7TM domain of the receptor, thereby triggering downstream processes by heterotrimeric (αβγ) G proteins bound to the intracellular loops which in turn leads to activation of intracellular signaling [89, 90]. In an inactive state, G alpha subunit is attached to guanosine diphosphate (GDP) which contains a GTPase domain which promotes hydrolysis and binding of guanosine triphosphate (GTP). Exchange of GDP for GTP takes place which ultimately causes cleavage of the G alpha subunit from the βϒ subunit of the heterotrimer upon activation of receptor by the ligand. The G alpha subunit then interacts with the Gβγ subunit in heterodimer. The dimer can act as an inhibitor for Gα because it facilitates interaction between Gα and GDP. Gα subunit then dissociates from Gα-GTP and Gβγ heterodimer complex of which the latter participates in the signaling cascade [2, 91–93]. G alpha subunits are of four types depending on their sequence and function. Phospholipase C (PLC) is stimulated by Gαq to facilitate intracellular Ca+ mobilization, membrane-associated enzyme phospholipase C2 (PLC2) is activated by the Gβγ heterodimer which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) resulting in the formation of two products intracellularly, namely, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Calcium from intracellular stores is triggered by IP3, while DAG can also activate protein kinase C (PKC) isoforms among other targets [94]. PKC stimulation eventually leads to physiological response. There are extensive reports suggesting downstream signaling of low molecular weight Rho and Ras proteins, tyrosine kinase, phosphatidylinositol 3-kinase, phospholipase A2, and the MAP kinase pathway. The signaling event involves phosphorylation of amino acid residues of threonine and serine in the carboxy loop of the receptor by GPCR kinases sequestration of receptor by internalization [95–97]. The nature of the ligand dictates the conformation changes which are likely to occur on the exterior of the receptor. As the chemokine ligands are large molecules, the shift in 7TM domain which occurs due to binding of ligand is different from β2 adrenergic receptor, another GPCR which binds small agonists or rhodopsin. Small molecules like rhodopsin lack proline residues which constitute the 7TM domain of chemokine receptors. Proline produces kinks and bends in 7TM helix which affects its folding and orientation of intra- and extracellular loop in chemokine receptors. Researchers postulated that CCR2 and CCR5 binding is determined by the presence of the motif threonine-x-proline (TxP) in TM2 [98–100].

Binding of IL-8, the agonist for chemokine receptor CXCR1/CXCR2 to its receptor, results in stimulation of the receptor and causes GDP/GTP exchange which further hydrolyzes the G alpha protein subunit from the heterodimer (GβY). Phospholipase D is activated giving rise to the signaling cascade, namely, MAP kinase pathway and phosphorylation of various amino acid residues like serine/threonine on the carboxy-terminal of the receptor through secretion of various secondary messengers like DAG and IP3 which trigger the intracellular calcium pool. A series of events which follow intracellular calcium mobility include movement of chemotactic cytokines, release of inflammatory granules and free radicals, and finally modification of avidity of cell adhesion molecules like integrin [101, 102].

5 Ligand Binding

Several mutagenesis studies have demonstrated the ligand-binding region of chemokine receptor. The studies revealed that N terminals were important especially for certain receptors, for example, CCR2, CCR3, CCR5, and CXCR1 [84]. Further receptors bind to specific chemokines which could be considered as their ligand partners (CCR-CC, CXCR-CXC). In contrast, DARC exhibits high affinity for inflammatory chemokines of different subfamilies. Chemokine receptors involved in leukocyte migration exhibit high promiscuity. One such receptor is CCR3 which can bind to 10 different chemokines. Furthermore, such binding elicits varied cellular responses which can be very complex [85, 103]. For instance, internalization is triggered when CCL19 binds to CCR7, while the same is not observed when CCL21 binds to CCR7 [13]. In a similar manner, interaction of CCR4 with CCL22 induces internalization, while the same is not observed with CCL17. On the other hand, chemokine receptors which play a role in homeostatic function bind to single chemokines as illustrated by CCR9 which binds only to CCL25, while CXCR5 exhibits binding to CXCL13 [15]. Various ligands for chemokine receptors are depicted in Table 9.1.

Similarly, CXCR1 exhibits specific affinity for CXCL8, CXCR2 is less selective and can bind also to many CXC. More involved studies have demonstrated the role of specific regions of the receptor in binding and have associated the first extracellular loop in ligand binding by CXCR2, and have demonstrated related differences in the affinity of ligands to CXCR2 by eliciting their mechanisms. This has been substantiated by other studies. A multisite binding model has therefore been proposed for CCR1 and CCR3 [22]. Further, in case of CCR1, the role of second extracellular loop in ligand binding has been established using the cross-linkable macrophage inflammatory protein (MIP)-1α. Similarly for CCR2, the relevant site for ligand binding is the amino terminus [104].

Promiscuous chemokine ligand–receptor relationships are common. As a result, defining the chemokine receptor responsible for stimulus–response coupling in primary cells is often not straightforward due to overlapping specificities of receptors for ligands and leukocytes, and a paucity of receptor subtype-selective blocking agents. Although anti-receptor monoclonal antibodies and mice with targeted gene disruptions are now being used to resolve ligand-binding specificities in vivo, problems of interpretation persist due to the inequality of chemokine and chemokine receptor repertoires, tissue distribution, and biological usage among species [8].

6 Antagonists for Ligand Binding

A plethora of diseases have been identified which has postulated the role of chemokine receptors in their progression. Though chemokines have been evolved as regulators of immune response, their improper exploitation has contributed toward many

afflictions. This has led to the discovery of a wide array of antagonists. Specific chemokine receptors like CXCR4 and CCR5 on cell surface of CD4+ T cells bind with HIV-1 envelope proteins, act as entry portals for HIV-1 virus which has raised significant interest by pharmaceutical companies to develop small molecules and antibodies against chemokine receptors [105, 106]. Numerous studies report molecules that potentially inhibit the interaction between gp120 proteins and chemokine receptors, thus preventing internalization. Amino oxypentane (AOP) RANTES an isoform of RANTES which act as a potential inhibitor for the eradication of HIV virus is been identified. In the same context, a series of CCR5 inhibitors have been developed for the treatment of HIV [107–109].

Though a number of biologics and GAG-based therapeutics which possess significant potential in inhibition of chemokine receptor function already exist, research efforts have also aimed toward the discovery of low molecular weight therapeutics with ability to act as chemokine receptor antagonists. CXCR4 and CCR5 have been identified as potential targets due to their roles in HIV entry [110].

A sequence of events follows the entry of human immunodeficiency virus (HIV-1) into the host.

Viral replication cycle starts with the formation of gp120/CD4+ complexes following the interaction between viral envelope protein and host cell membrane. The complex activates the chemokine receptors by undergoing conformational modifications, followed by a series of events which cause fusion between host and viral cell membranes [106]. T-tropic strains of HIV gain entry into the host predominantly through CXCR4, whereas CCR5 is essential for invasion of M-tropic HIV [68, 111, 112]. D6 receptor which lacks intracellular signaling is also reported to play a role [113]. This has led to the development of a number of CXCR4/CCR5 antagonists which have entered clinical trials and are depicted under the Sect. 8 on clinical studies later in this chapter.

7 Receptor-Mediated Targeting Strategies

Nanocarriers have been the major vehicles for receptor-mediated targeting due to the manifold advantages they offer. While they can enable passive targeting which is influenced by their physicochemical properties, for example, size, shape, surface charge, and hydrophobicity, attachment of ligands which can recognize cell membrane components can facilitate active cellular targeting. Using ligands for chemokine receptors can enable such active targeting via receptor-mediated endocytosis. However, despite major studies in the development of new drugs as chemokine agonists and antagonists, exploitation of chemokine receptor-based targeted drug delivery based on nanocarrier strategies is limited.

7.1 Liposomes

Immunoliposomes loaded with siRNA have been successfully employed for systemic targeting of LFA-1 (lymphocyte function-associated antigen-1) integrin present exclusively on leukocytes and other immune cells that act as mediators of HIV-1 infection [114].

An anti-HIV liposomal composition containing cardiolipin as phospholipid exhibited anti-HIV activity by inhibiting the binding and fusion of gp 120 with cell surface receptors CCR5 and CXCR4 of host cells [115]. Non-phospholipidic cationic liposomes containing free fatty acids, their monoesters and cholesterol, namely, Novasomes® 7474 loaded with a combination of 2 RANTES (a CCR5-specific inhibitor) and fusion inhibitor sifuvirtide enabled downregulation of CCR5 [116, 117].

7.2 Nanoparticles

Nanoparticles containing a specific siRNA sequence showed a marked reduction in the expression of CD4+ and CCR5 in explants of HIV-1 negative female, which was evident by decrease in the biomarker CD45. This proposed the ability of the nanoparticles to cause suppression of receptor-specific genes [118]. Intravenous infusion of nanoparticles encapsulated with CCR5-specific siRNA complexed with CD4+ T-cell-specific antibody downregulated expression of CCR5 receptors in preclinical mice model confirming their anti-HIV potential [119].

PLGA nanoparticles incorporating PSC-RANTES (amino terminus-modified synthetic analog of RANTES) showed greater mucosal penetration of the protein and improved activity as HIV-1 entry inhibitors in rhesus macaque model compared to their non-polymeric counterparts [120, 121]. PEG-stabilized gold nanoparticles are reported to be effective HIV-1 fusion inhibitors causing inhibition of binding of HIV envelope protein gp120 and CD4+ T cell. The gold nanoparticles showed efficient inhibition against X4, R5, and X4R5 virus strains of HIV-1 infection [122].

Gold nanoparticles coated with multiple sulfate-modified amphiphilic ligand showed great promise as HIV-1 inhibitor by competitive inhibition of gp 120 glycoproteins on virus and inhibit binding of the virus to dendritic cells and subsequent transfer to T lymphocytes (CCR8), thereby preventing viral replication [123]. Anchoring neutralizing antibodies (NABs) on silver nanoparticles induced the ability to neutralize HIV-1, not observed when NAB alone was used [124].

7.3 Miscellaneous

Atherosclerosis, an inflammatory process is strongly influenced by chemokine/chemokine receptor-like CCR2 which is majorly involved in leukocyte recruitment to the atherosclerotic plaque. Dextran nanoparticles encapsulating siRNA were

employed to silence mRNA, thus downregulating CCR2 expression. Dextran nanoparticles labeled with Zr^{89} also served as a targeted theranostic. When injected in ApoE knockout mice, the nanoparticles showed reduced PET/MRI signal used to spot macrophage in the atherosclerosis-inflamed tissue, suggesting the role of dextran nanoparticles in targeting atherosclerotic plaque [125].

8 Clinical Studies

CXCR4 and CCR5 being the main hallmark for progression of HIV-1 infection have prompted several pharmaceutical players to rapidly develop potent inhibitors against these receptors which could block the selective pathway. Selective inhibition of receptors has given rise to drug-resistant strains of HIV due to the peculiarity of the virus to switch tropism. Hence, current research efforts are aimed to develop antagonists against dual tropic strains of virus. Among the specific inhibitors of HIV-1 infection, CCR5 antagonist has gained much of interest as HIV-1 inhibitor, though it plays a role in the progression of autoimmune diseases like type 1 diabetes and multiple sclerosis. Clinical studies using CCR5/CXCR4 antagonists are depicted in Table 9.3. The role of chemokines in other non-infectious diseases has led their evaluation for other applications. Clinical trials for such applications are recorded in Table 9.4.

9 Conclusion and Future Prospects

Chemokine receptors play a multifaceted role in the progression of infectious diseases like HIV, malaria, atherosclerosis, and multiple sclerosis. A number of antagonists are developed and are under clinical trials. Nevertheless, synergizing such

Table 9.3 Chemokine receptor antagonists under clinical trials for anti-HIV activity

Receptor	Antagonists	Company	Clinical trial Status	References
CCR5	Maraviroc	Pfizer	Available as Selzentry®	[126]
	HGS004	Human genome sciences	Phase I	[127]
	Cenicriviroc/TAK 652	Takeda	Phase IIb	[128]
	PRO140	Progenics pharma	Phase II	[129]
	Aplaviroc/AK602/ GW873140/ONO4128	GSK	Phase II	[130]
	INCB9471	Incyte	Phase II	[131]
	Vicriviroc/SCH D	Schering-Plough	Phase II	[132]
	SCH C	Schering-Plough	Phase II	[133]
CXCR4	AMD11070	ANORMED	Phase I	[134]
	AMD3100	ANORMED	Discontinued	[135]

Table 9.4 Chemokine receptor antagonists under clinical trials for other applications

Receptor	Disease indication	Antagonists	Company	Clinical trial Status	References
CCR1	Rheumatoid arthritis (RA)	CP-481	Pfizer	Phase II	[136]
	RA	MLN3897	Millennium/ Sanofi	Phase II	[136]
	Multiple sclerosis (MS)	BX471	Berlex	Phase II	[136]
	COPD	AZD-4818	Astra-Zeneca	Phase II	[136]
CCR2	RA-MS	MK-0812	Merck	Phase II	[137]
	Diabetes neuropathy	BMS-741672	BMS	Phase II	[138]
	Kidney disorder	CCX140	ChemoCentryx	Phase II	[139]
CCR3	Allergic rhinitis	CAT 213	CAT/Astrazeneca	Phase II	[140]
	Asthma	GSK 766994	GSK	Phase III	[141]
	Asthma	SCH 527123	Schering-Plough	Phase II	[142]
	Pulmonary disease	SB-656933	GSK	Phase I	[143]

developments with targeted drug delivery strategies using nanocarriers could play a major role in harnessing chemokine receptor-based targeting for improved therapeutic outcomes.

References

1. Solari R, Pease JE. Targeting chemokine receptors in disease – a case study of CCR4. Eur J Pharmacol. 2015;763:169–77.
2. Murphy PM. Chemokine receptors: structure, function and role in microbial pathogenesis. Cytokine Growth Factor Rev. 1996;7(1):47–64.
3. Mahalingam S, Karupiah G. Chemokines and chemokine receptors in infectious diseases. Immunol Cell Biol. 1999;77(6):469–75.
4. Gao J-L, Murphy PM. Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. J Biol Chem. 1994;269(46):28539–42.
5. Mukaida N. The roles of cytokine receptors in diseases. Rinsho Byori Jpn J Clin Pathol. 2000;48(5):409–15.
6. Moser B, Willimann K. Chemokines: role in inflammation and immune surveillance. Ann Rheum Dis. 2004;63:ii84–i9.
7. Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012;36(5):705–16.
8. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol. 2007;25:787–820.
9. Le Y, Zhou Y, Iribarren P, Wang J. Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. Cell Mol Immunol. 2004;1(2):95–104.
10. Murdoch C, Finn A. Chemokine receptors and their role in inflammation and infectious diseases. Blood. 2000;95(10):3032–43.

11. Biragyn A, Ruffini PA, Coscia M, Harvey LK, Neelapu SS, Baskar S, et al. Chemokine receptor-mediated delivery directs self-tumor antigen efficiently into the class II processing pathway in vitro and induces protective immunity in vivo. Blood. 2004;104(7):1961–9.
12. Koenen RR, Weber C. Therapeutic targeting of chemokine interactions in atherosclerosis. Nat Rev Drug Discov. 2010;9(2):141–53.
13. Baggiolini M, Loetscher P, Moser B. Interleukin-8 and the chemokine family. Int J Immunopharmacol. 1995;17(2):103–8.
14. Murphy PM, editor. Neutrophil receptors for interleukin-8 and related CXC chemokines. Semin Hematol. 1997;34(4):311–8.
15. Ahuja SK, Murphy PM. The CXC chemokines growth-regulated oncogene (GRO) α, GROβ, GROγ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem. 1996;271(34):20545–50.
16. Kaplanski G, Farnarier C, Kaplanski S, Porat R, Shapiro L, Bongrand P, et al. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. Blood. 1994;84(12):4242–8.
17. Hashimoto S, Yoda M, Yamada M, Yanai N, Kawashima T, Motoyoshi K. Macrophage colony-stimulating factor induces interleukin-8 production in human monocytes. Exp Hematol. 1996;24(2):123–8.
18. Khandaker MH, Mitchell G, Xu L, Andrews JD, Singh R, Leung H, et al. Metalloproteinases are involved in lipopolysaccharide–and tumor necrosis factor–mediated regulation of CXCR1 and CXCR2 chemokine receptor expression. Blood. 1999;93(7):2173–85.
19. Lloyd AR, Biragyn A, Johnston JA, Taub DD, Xu L, Michiel D, et al. Granulocyte-colony stimulating factor and lipopolysaccharide regulate the expression of interleukin 8 receptors on polymorphonuclear leukocytes. J Biol Chem. 1995;270(47):28188–92.
20. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med. 1996;184(3):963–9.
21. Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res. 2011;317(5):620–31.
22. Hesselgesser J, Liang M, Hoxie J, Greenberg M, Brass LF, Orsini MJ, et al. Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. J Immunol. 1998;160(2):877–83.
23. Jourdan P, Abbal C, Nora N, Hori T, Uchiyama T, Vendrell J-P, et al. Cutting edge: IL-4 induces functional cell-surface expression of CXCR4 on human T Cells1. J Immunol. 1998;160(9):4153–7.
24. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12(2):121–7.
25. Jones BA, Beamer M, Ahmed S. Fractalkine/CX3CL1: a potential new target for inflammatory diseases. Mol Interv. 2010;10(5):263–70.
26. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell. 1997;91(4):521–30.
27. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J Exp Med. 1998;188(8):1413–9.
28. Chee MS, Satchwell SC, Preddie E, Weston KM, Barrell BG. Human cytomegalovirus encodes three G protein-coupled receptor homologues. Nature. 1990;344(6268):774–7.
29. Bartoli C, Civatte M, Pellissier JF, Figarella-Branger D. CCR2A and CCR2B, the two isoforms of the monocyte chemoattractant protein-1 receptor are up-regulated and expressed by different cell subsets in idiopathic inflammatory myopathies. Acta Neuropathol. 2001;102(4):385–92.
30. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006;354(6):610–21.

31. Elsner J, Petering H, Kluthe C, Kimmig D, Smolarski R, Ponath P, et al. Eotaxin-2 activates chemotaxis-related events and release of reactive oxygen species via pertussis toxin-sensitive G proteins in human eosinophils. Eur J Immunol. 1998;28(7):2152–8.
32. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, et al. A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell. 1996;85(7):1149–58.
33. Daugherty BL, Siciliano SJ, DeMartino JA, Malkowitz L, Sirotina A, Springer MS. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. J Exp Med. 1996;183(5):2349–54.
34. Dairaghi DJ, Oldham ER, Bacon KB, Schall TJ. Chemokine receptor CCR3 function is highly dependent on local pH and ionic strength. J Biol Chem. 1997;272(45):28206–9.
35. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem. 1997;272(23):15036–42.
36. D'Ambrosio D, Iellem A, Bonecchi R, Mazzeo D, Sozzani S, Mantovani A, et al. Cutting edge: selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. J Immunol. 1998;161(10):5111–5.
37. Ruffing N, Sullivan N, Sharmeen L, Sodroski J, Wu L. CCR5 has an expanded ligand-binding repertoire and is the primary receptor used by MCP-2 on activated T cells. Cell Immunol. 1998;189(2):160–8.
38. Raport CJ, Gosling J, Schweickart VL, Gray PW, Charo IF. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1β, and MIP-1α. J Biol Chem. 1996;271(29):17161–6.
39. Makuta Y, Sonoda Y, Yamamoto D, Funakoshi-Tago M, Aizu-Yokota E, Takebe Y, et al. Interleukin-10-induced CCR5 expression in macrophage like HL-60 cells: involvement of Erk1/2 and STAT-3. Biol Pharm Bull. 2003;26(8):1076–81.
40. Baba M, Imai T, Nishimura M, Kakizaki M, Takagi S, Hieshima K, et al. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. J Biol Chem. 1997;272(23):14893–8.
41. Greaves DR, Wang W, Dairaghi DJ, Dieu MC, Saint-Vis B, Franz-Bacon K, et al. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3alpha and is highly expressed in human dendritic cells. J Exp Med. 1997;186(6):837–44.
42. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. J Immunol. 1999;162(1):186–94.
43. Yoshida R, Imai T, Hieshima K, Kusuda J, Baba M, Kitaura M, et al. Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. J Biol Chem. 1997;272(21):13803–9.
44. Endres MJ, Garlisi CG, Xiao H, Shan L, Hedrick JA. The Kaposi's sarcoma-related herpesvirus (KSHV)-encoded chemokine vMIP-I is a specific agonist for the CC chemokine receptor (CCR)8. J Exp Med. 1999;189(12):1993–8.
45. Horuk R, Hesselgesser J, Zhou Y, Faulds D, Halks-Miller M, Harvey S, et al. The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. J Biol Chem. 1998;273(1):386–91.
46. Carramolino L, Zaballos A, Kremer L, Villares R, Martin P, Ardavin C, et al. Expression of CCR9 beta-chemokine receptor is modulated in thymocyte differentiation and is selectively maintained in CD8(+) T cells from secondary lymphoid organs. Blood. 2001; 97(4):850–7.
47. Vicari AP, Figueroa DJ, Hedrick JA, Foster JS, Singh KP, Menon S, et al. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. Immunity. 1997;7(2):291–301.
48. Zaballos A, Gutierrez J, Varona R, Ardavin C, Marquez G. Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. J Immunol. 1999;162(10):5671–5.

49. Graham GJ. D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. Eur J Immunol. 2009;39(2):342–51.
50. Nibbs RJ, Wylie SM, Yang J, Landau NR, Graham GJ. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. J Biol Chem. 1997;272(51):32078–83.
51. Szabo MC, Soo KS, Zlotnik A, Schall TJ. Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor. J Biol Chem. 1995;270(43):25348–51.
52. Horuk R. The Duffy antigen receptor for chemokines DARC/ACKR1. Front Immunol. 2015;6(279):1–3.
53. Suresh P, Wanchu A. Chemokines and chemokine receptors in HIV infection: role in pathogenesis and therapeutics. J Postgrad Med. 2006;52(3):210–7.
54. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995;270(5243):1811–5.
55. Stone MJ, Hayward JA, Huang C, EHuma Z, Sanchez J. Mechanisms of regulation of the chemokine-receptor network. Int J Mol Sci. 2017;18(2) https://doi.org/10.3390/ijms18020342.
56. Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, et al. A new classification for HIV-1. Nature. 1998;391(6664):240.
57. Simmons G, Wilkinson D, Reeves JD, Dittmar MT, Beddows S, Weber J, et al. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. J Virol. 1996;70(12):8355–60.
58. Wu L, LaRosa G, Kassam N, Gordon CJ, Heath H, Ruffing N, et al. Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J Exp Med. 1997;186(8):1373–81.
59. Bartels J, Maune S, Meyer JE, Kulke R, Schluter C, Rowert J, et al. Increased eotaxin-mRNA expression in non-atopic and atopic nasal polyps: comparison to RANTES and MCP-3 expression. Rhinology. 1997;35(4):171–4.
60. von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, et al. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation. 2001;103(13):1772–7.
61. Greenwood B. Malaria mortality and morbidity in Africa. Bull World Health Organ. 1999;77(8):617–8.
62. Dunst J, Kamena F, Matuschewski K. Cytokines and chemokines in cerebral malaria pathogenesis. Front Cell Infect Microbiol. 2017;7(324):1–16.
63. Belnoue E, Costa FT, Vigario AM, Voza T, Gonnet F, Landau I, et al. Chemokine receptor CCR2 is not essential for the development of experimental cerebral malaria. Infect Immun. 2003;71(6):3648–51.
64. Sarfo BY, Armah HB, Irune I, Adjei AA, Olver CS, Singh S, et al. Plasmodium yoelii 17XL infection up-regulates RANTES, CCR1, CCR3 and CCR5 expression, and induces ultrastructural changes in the cerebellum. Malar J. 2005;4(63):1–13.
65. Chaisavaneeyakorn S, Moore JM, Mirel L, Othoro C, Otieno J, Chaiyaroj SC, et al. Levels of macrophage inflammatory protein 1 alpha (MIP-1 alpha) and MIP-1 beta in intervillous blood plasma samples from women with placental malaria and human immunodeficiency virus infection. Clin Diagn Lab Immunol. 2003;10(4):631–6.
66. Chandramohan D, Greenwood BM. Is there an interaction between human immunodeficiency virus and Plasmodium falciparum? Int J Epidemiol. 1998;27(2):296–301.
67. Clark C, Phillips R. Cerebral malaria protection in mice by species-specific Plasmodium coinfection is associated with reduced CC chemokine levels in the brain. Parasite Immunol. 2011;33(11):637–41.
68. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science. 1996;272(5263):872–7.
69. Hansen DS, Bernard NJ, Nie CQ, Schofield L. NK cells stimulate recruitment of CXCR3+ T cells to the brain during Plasmodium berghei-mediated cerebral malaria. J Immunol. 2007;178(9):5779–88.

70. Hochman S, Kim K. The impact of HIV coinfection on cerebral malaria pathogenesis. J Neuroparasitol. 2012;3:235547.
71. Ochiel DO, Awandare GA, Keller CC, Hittner JB, Kremsner PG, Weinberg JB, et al. Differential regulation of β-chemokines in children with Plasmodium falciparum malaria. Infect Immun. 2005;73(7):4190–7.
72. Pollina E, Chaluluka E, Carr R, Lucas S, Molyneaux M, Rogerson S. Monocytic infiltration of the placenta in malaria and its relation to HIV. Am J Trop Med Hyg. 1999;61:1–11.
73. Sarfo B, Singh S, Lillard J, Quarshie A, Gyasi R, Armah H, et al. The cerebral-malaria-associated expression of RANTES, CCR3 and CCR5 in post-mortem tissue samples. Ann Trop Med Parasitol. 2004;98(3):297–303.
74. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AE, et al. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. Circ Res. 2004;94(2):253–61.
75. Boisvert WA, Curtiss LK, Terkeltaub RA. Interleukin-8 and its receptor CXCR2 in atherosclerosis. Immunol Res. 2000;21(2–3):129–37.
76. Reape TJ, Groot PH. Chemokines and atherosclerosis. Atherosclerosis. 1999;147(2):213–25.
77. Whitley RJ, Roizman B. Herpes simplex virus infections. Lancet. 2001;357(9267):1513–8.
78. Sorensen LN, Paludan SR. Blocking CC chemokine receptor (CCR) 1 and CCR5 during herpes simplex virus type 2 infection in vivo impairs host defence and perturbs the cytokine response. Scand J Immunol. 2004;59(3):321–33.
79. Kopp SJ, Banisadr G, Glajch K, Maurer UE, Grunewald K, Miller RJ, et al. Infection of neurons and encephalitis after intracranial inoculation of herpes simplex virus requires the entry receptor nectin-1. Proc Natl Acad Sci U S A. 2009;106(42):17916–20.
80. Beisser PS, Laurent L, Virelizier J-L, Michelson S. Human Cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. J Virol. 2001;75(13):5949–57.
81. Sanders VJ, Pittman CA, White MG, Wang G, Wiley CA, Achim CL. Chemokines and receptors in HIV encephalitis. AIDS. 1998;12(9):1021–6.
82. Cheng W, Chen G. Chemokines and chemokine receptors in multiple sclerosis. Mediat Inflamm. 2014;2014:1–8.
83. Kaplan AP. Chemokines, chemokine receptors and allergy. Int Arch Allergy Immunol. 2001;124(4):423–31.
84. Kufareva I, Salanga CL, Handel TM. Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. Immunol Cell Biol. 2015;93(4):372–83.
85. Blanpain C, Lee B, Vakili J, Doranz BJ, Govaerts C, Migeotte I, et al. Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity. J Biol Chem. 1999;274(27):18902–8.
86. Lagane B, Ballet S, Planchenault T, Balabanian K, Le Poul E, Blanpain C, et al. Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis. Mol Pharmacol. 2005;67(6):1966–76.
87. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, et al. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. Cell. 1999;96(5):667–76.
88. Jensen PC, Rosenkilde MM. Activation mechanisms of chemokine receptors. Methods Enzymol. 2009;461:171–90.
89. Preininger AM, Hamm HE. G protein signaling: insights from new structures. Sci STKE. 2004;2004(218):re3.
90. Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, et al. Insights into G protein structure, function, and regulation. Endocr Rev. 2003; 24(6):765–81.
91. Bokoch GM. Chemoattractant signaling and leukocyte activation. Blood. 1995;86(5):1649–60.
92. Wu D, LaRosa GJ, Simon MI. G protein-coupled signal transduction pathways for interleukin-8. Science. 1993;261(5117):101–3.
93. Kuang Y, Wu Y, Jiang H, Wu D. Selective G protein coupling by CC chemokine receptors. J Biol Chem. 1996;271(8):3975–8.

94. Lefkowitz RJ. Historical review: a brief history and personal retrospective of seven-transmembrane receptors. Trends Pharmacol Sci. 2004;25(8):413–22.
95. Turner SJ, Domin J, Waterfield MD, Ward SG, Westwick J. The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2α. J Biol Chem. 1998;273(40):25987–95.
96. Huang R, Lian JP, Robinson D, Badwey JA. Neutrophils stimulated with a variety of chemoattractants exhibit rapid activation of p21-activated kinases (Paks): separate signals are required for activation and inactivation of paks. Mol Cell Biol. 1998;18(12):7130–8.
97. Mellado M, Rodriguez-Frade J, Aragay A, Del Real G, Martin A, Vila-Coro A, et al. The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. J Immunol. 1998;161(2):805–13.
98. Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, Kobilka BK. Agonists induce conformational changes in transmembrane domains III and VI of the β2 adrenoceptor. EMBO J. 1997;16(22):6737–47.
99. Arias DA, Navenot J-M, Zhang W-B, Broach J, Peiper SC. Constitutive activation of CCR5 and CCR2 induced by conformational changes in the conserved TXP motif in transmembrane helix 2. J Biol Chem. 2003;278(38):36513–21.
100. Govaerts C, Blanpain C, Deupi X, Ballet S, Ballesteros JA, Wodak SJ, et al. The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation. J Biol Chem. 2001;276(16):13217–25.
101. Kamp M, Liu Y, Kortholt A. Function and regulation of heterotrimeric G proteins during chemotaxis. Int J Mol Sci. 2016;17(1):90.
102. Karnik SS, Gogonea C, Patil S, Saad Y, Takezako T. Activation of G-protein-coupled receptors: a common molecular mechanism. Trends Endocrinol Metab. 2003;14(9):431–7.
103. Baldwin JM. Structure and function of receptors coupled to G proteins. Curr Opin Cell Biol. 1994;6(2):180–90.
104. Pease JE, Wang J, Ponath PD, Murphy PM. The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1α and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. J Biol Chem. 1998;273(32):19972–6.
105. Wells TN, Power CA, Shaw JP, Proudfoot AE. Chemokine blockers–therapeutics in the making? Trends Pharmacol Sci. 2006;27(1):41–7.
106. Olson WC, Rabut GE, Nagashima KA, Tran DN, Anselma DJ, Monard SP, et al. Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. J Virol. 1999;73(5):4145–55.
107. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995;270(5243):1811–5.
108. Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, et al. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science. 1997;276(5310):276–9.
109. Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, et al. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. J Exp Med. 1998;187(8):1215–24.
110. Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao S-H, Goetz MB, Daar ES, et al. A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J Exp Med. 1997;186(8):1395–400.
111. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, et al. CC CKR5: A RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science. 1996;272(5270):1955–8.
112. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, et al. Identification of a major co-receptor for primary isolates of HIV-1. Nature. 1996;381(6584):661.
113. Neil SJ, Aasa-Chapman MM, Clapham PR, Nibbs RJ, McKnight A, Weiss RA. The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. J Virol. 2005;79(15):9618–24.

114. Kim S-S, Peer D, Kumar P, Subramanya S, Wu H, Asthana D, et al. RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. Mol Ther. 2010;18(2):370–6.
115. Malavia NK, Zurakowski D, Schroeder A, Princiotto AM, Laury AR, Barash HE, et al. Liposomes for HIV prophylaxis. Biomaterials. 2011;32(33):8663–8.
116. Kish-Catalone T, Pal R, Parrish J, Rose N, Hocker L, Hudacik L, et al. Evaluation of-2 RANTES vaginal microbicide formulations in a nonhuman primate simian/human immunodeficiency virus (SHIV) challenge model. AIDS Res Hum Retrovir. 2007;23(1):33–42.
117. Franquelim HG, De-Sousa FF, Veiga AS, Santos NC, Castanho MA. Cationic liposomes are possible drug-delivery systems for HIV fusion inhibitor sifuvirtide. Soft Matter. 2011;7(23):11089–92.
118. Asin SN, Eszterhas SK, Rollenhagen C, Heimberg AM, Howell AL. HIV type 1 infection in women: increased transcription of HIV type 1 in ectocervical tissue explants. J Infect Dis. 2009;200(6):965–72.
119. Kumar P, Ban H-S, Kim S-S, Wu H, Pearson T, Greiner DL, et al. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. Cell. 2008;134(4):577–86.
120. Ham AS, Cost MR, Sassi AB, Dezzutti CS, Rohan LC. Targeted delivery of PSC-RANTES for HIV-1 prevention using biodegradable nanoparticles. Pharm Res. 2009;26(3):502–11.
121. Fumakia M, Yang S, Gu J, Ho EA. Protein/peptide-based entry/fusion inhibitors as anti-HIV therapies: challenges and future direction. Rev Med Virol. 2016;26(1):4–20.
122. Vijayakumar S, Ganesan S. Gold nanoparticles as an HIV entry inhibitor. Curr HIV Res. 2012;10(8):643–6.
123. Di Gianvincenzo P, Marradi M, Martínez-Ávila OM, Bedoya LM, Alcamí J, Penadés S. Gold nanoparticles capped with sulfate-ended ligands as anti-HIV agents. Bioorg Med Chem Lett. 2010;20(9):2718–21.
124. Lara HH, Ixtepan-Turrent L, Treviño ENG, Singh DK. Use of silver nanoparticles increased inhibition of cell-associated HIV-1 infection by neutralizing antibodies developed against HIV-1 envelope proteins. J Nanobiotechnol. 2011;9(1):38.
125. Majmudar MD, Keliher EJ, Heidt T, Leuschner F, Truelove J, Sena BF, et al. Monocyte-directed RNAi targeting CCR2 improves infarct healing in atherosclerosis-prone mice. Circulation. 2013;127(20):2038–46.
126. https://clinicaltrials.gov/ct2/show/NCT00098306
127. http://www.natap.org/2006/ICAAC/ICAAC_28.htm
128. https://clinicaltrials.gov/ct2/show/NCT02128828?term=TAK+652+AND+Cenicriviroc
129. https://clinicaltrials.gov/ct2/show/NCT02483078?term=pro+140&rank=1
130. https://clinicaltrials.gov/ct2/show/NCT00102778?term=aplaviroc&rank=1
131. https://clinicaltrials.gov/ct2/show/NCT00393120?term=INCB9471&rank=1
132. https://clinicaltrials.gov/ct2/show/NCT00686829?term=VICRIVIROC&rank=1
133. https://hepcassoc.org/news/article127.html
134. https://clinicaltrials.gov/ct2/show/NCT00361101?term=AMD11070&rank=1
135. https://clinicaltrials.gov/ct2/results?cond=HIV&term=AMD3100&cntry=&state=&city=&dist=&Search=Search
136. Gladue RP, Brown MF, Zwillich SH. CCR1 antagonists: what have we learned from clinical trials. Curr Top Med Chem. 2010;10:1268.
137. https://clinicaltrials.gov/ct2/show/NCT00542022?term=mk0812&rank=2
138. https://clinicaltrials.gov/ct2/show/NCT00699790?term=BMS+741672&rank=1
139. https://clinicaltrials.gov/ct2/show/NCT01440257?term=CCX140&rank=3
140. https://clinicaltrials.gov/ct2/show/NCT00638755?term=CAT+213&rank=4
141. https://clinicaltrials.gov/ct2/show/NCT00990327?term=DPC+168&rank=1
142. https://clinicaltrials.gov/ct2/show/NCT00688467?term=SCH527123&rank=3
143. https://clinicaltrials.gov/ct2/show/NCT00504439?term=Sb656933&rank=1

Chapter 10
Scavenger Receptor and Targeting Strategies

Amit S. Lokhande, Priyanka Jahagirdar, Prajakta Dandekar, and Padma V. Devarajan

Abstract Scavenger receptors constitute a group of receptors on the cell surface that attach to various ligands and remove the targets that are non-self or altered. Signaling, transport, endocytosis, phagocytosis, and adhesion resulting in the removal of harmful and degraded substances are some functions of these receptors. Scavenger receptors bind a large repertoire of ligands indicating their involvement in homeostasis and multiple disease pathologies. In this chapter, we describe the role of scavenger receptor group in the pathogenesis of infections and cancer. In addition, we present a variety of ligands with their scavenger receptor binding strategies through different examples of targeted drug delivery systems.

Keywords Cancer · Infections · Nanosystems · Polyanionic ligand · Scavenger receptor · Targeted drug delivery

Abbreviations

AcLDL	Acylated low-density lipoprotein
Aco-HSA	Polyaconitylated-human serum albumin
AGE	Advanced glycation end products
AgNPs	Silver nanoparticles
BBB	Blood–brain barrier
BSA	Bovine serum albumin

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CFUs	Colony-forming units
CR	Cysteine rich
CXC	Chemokine receptor 16
DCP	Dicetylphosphate
DCs	Dendritic cells
EDCs	Endothelial cells
EGF	Epidermal growth factor
Fe_2O_3	Iron oxide
FEEL	Fasciclin EGF-like, and lamin-type EGF-like domains
GPI	Glycosyl-phosphatidylinositol
HDL	High-density lipoprotein
Hsp	Heat shock proteins
LAMP	Lysosome-associated membrane glycoprotein
LCO	Lithocholic oleate
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LOX-1	Lectin-like oxidized LDL receptor-1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MARCO	Macrophage receptor with collagenous structure
MBSA	Maleylated albumin
MTX	Methotrexate
NMs	Nanomedicines
NK cells	Natural Killer cells
OxLDL	Oxidized low-density lipoprotein
PAS	p-amino salicylic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
POPC	Palmitoyloleoyl-phosphatidylcholine
PS	Phosphatidylserine
RBCs	Red blood cells
ROS	Reactive oxygen species
S_1-CLP	Stabilin-1 interacting chitinase-like protein
SCARA-5	Scavenger receptor class A member 5
SiRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SPARC	Secreted protein acidic and rich in cysteine
SR	Scavenger receptor
SRCL	Scavenger receptors with C-type lectin
SRPSOX	Scavenger receptor that binds phosphatidylserine and oxidized lipids
TAMs	Tumor-associated macrophages
TiO_2	Titanium dioxide
UGPR	Uteroglobin-related protein
VLDL	Very low-density lipoproteins
ZnO	Zinc oxide

1 Introduction

Scavenge means to clear, accordingly the role played by the scavenger receptors is clearing the body of a variety of moieties, for instance, modified low-density lipoprotein (LDL), bacteria or infected RBCs, apoptotic cells, etc. [1]. The receptor was first identified by Brown and Goldstein in macrophages and they observed that while the receptor internalized and degraded modified and oxidized low-density lipoprotein (OxLDL) or acetylated LDL, native LDL was spared by these receptors. Intracellular internalization of modified LDL may be due to foam cell formation [2]. Such foam cells loaded with cholesterol are integral to the atherosclerotic plaques and are also located in the lesions of blood vessel walls [3, 4]. While the scavenger receptors play a crucial physiological role, they can also perform as mediators in various pathologies. This chapter details the receptor with special emphasis on exploiting the endocytic property of this receptor in the targeted therapy of various diseases.

2 Scavenger Receptors

Scavenger receptors encompass a group of membrane proteins along with isoforms and soluble secreted extracellular domain isoforms. Although scavenger receptors are divided into 12 classes A-L (Fig.10.1), a term superfamily is not bestowed, as the receptors reveal no structural homology among the different classes [5]. They are more aptly termed as a supergroup [6]. Though structurally dissimilar, the scavenger receptors show affinity for similar ligands comprising of polyions including

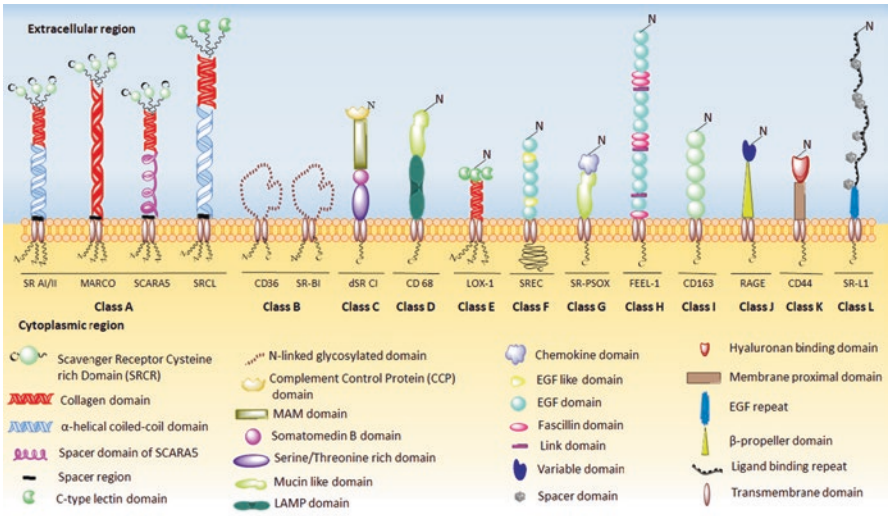

Fig. 10.1 Schematic representation of scavenger receptor classes and their recognition domains

lipoproteins, phospholipids, cholesterol ester, apoptotic cells, carbohydrates, proteoglycans, and ferritin. A structural similarity is evident among different members of a class. Owing to their diverse ligand binding ability, the scavenger receptors represent a significant part of the pattern recognition receptors [7, 8]. A schematic representation of scavenger receptor classes and their recognition domains is depicted in Fig.10.1. A major focus of this chapter is on Class A and B scavenger receptors, the receptors that play a role in infections and cancer.

2.1 Scavenger Receptors' Recognition Domains

2.1.1 Class A

Class A scavenger receptor comprises of type II transmembrane proteins. A cytoplasmic N-terminal domain (40–55 amino acids) is linked to the transmembrane region (26 amino acids). The extracellular domain comprises of three domains, namely, α-helical coiled-coil, C-terminal cysteine-rich (CR), and collagen-like domain and mediates ligand recognition. The unique collagen-like domain has positively charged amino acid residues that bind to polyanions [9–11]. The SR-AI/II, MARCO, SCARA5, and SRCL are most widely studied members of this class (Fig.10.1). SR-AI and AII display identical affinity for collagen-rich region [12]. MARCO exhibits an extended collagen-rich domain and expresses cysteine-rich domain as ligand-binding site [13]. SCARA-5 and MARCO receptors reveal a similar ligand binding. The coiled-coil domain is absent in these two receptors [14]. SRCL comprises of a C-terminal lectin-type domain while it lacks cysteine-rich domain [15].

2.1.2 Class B

The members of this class usually contain type III transmembrane proteins of 450–500 amino acid residues. They mainly express 2 transmembrane regions which contain closely placed short intracellular N- and C-terminals with the central extracellular loop comprising N-linked glycosylated domain of 400–450 amino acid residues, involved in ligand recognition [6]. The CD36 and SR-BI are two major members, which are largely glycosylated and fatty acylated [16, 17].

The structural dissimilarity is evident among different classes of scavenger receptors. Class C is not expressed in humans [18]. Class D scavenger receptors contain lysosome-associated membrane glycoprotein (LAMP) domains and mucin-like domains [19], whereas lectin-like LDLR-1, the only member of class E, shows C-type lectin domain. The C-terminal of this domain is connected by transmembrane domain to the cytoplasmic domain of N-terminal [20]. Class F scavenger receptors revealed growth factor domains, while class G receptors exhibit along with a chemokine domain and a mucin-like glycosylated stem as extracellular domain for ligand binding [21]. Class H scavenger receptors comprise of fascillin,

epidermal growth factor (EGF) like, and lamin-type EGF-like (FEEL) domain [22]. While class I scavenger receptors consist of multiple group B cysteine-rich domains in their extracellular domain [23], class J contains a single transmembrane domain that connects the amino-terminal ligand recognition and binding ectodomain with a short cytoplasmic domain [24]. The class K scavenger receptor consists of hyaluronan binding domain [25, 26] and class L scavenger receptor consists of ligand-binding repeat, EGF repeat, and β propeller domain [27, 28]. A detailed description of these classes can be accessed from the literature [1, 5, 29].

3 Ligand Binding

Although majority of polyanionic ligands bind to scavenger receptors, their specificity depends on scavenger receptor domains. The broad range of specificity of the scavenger receptors prompted scientists to study the active site of these receptors. The positively charged C-terminal of the collagenous domain is essential for binding of ligands. Binding studies suggest that the collagenous domain is responsible for the broad specificity of the receptor [30]. A sticky surface is provided by the collagenous domain that enables selective binding of polyanions with high affinity. The positively charged residues of this domain are important for binding of polyanions. Presence of few negatively charged residues repels polyanions with low affinity and binds only those with high affinity. A direct or indirect effect on ligand binding is shown by other extracellular domains [31].

Although structurally homologous, SR-A1 and MARCO exhibit ligand uptake by discrete mechanisms. Studies suggest that removal of the cysteine-rich domain of MARCO curbs the internalization, whereas an enhanced uptake was seen following CR domain deletion of SR-A1 [32]. A difference in domain charge may have resulted in this consequence. A negatively charged CR domain is predicted by in silico studies. However, some studies report a mixed positive and negative charge for CR domain in MARCO. Such differences in charges could impact the recognition of pathogens and particulate carriers. Ligand receptor binding of MARCO is dependent on metal ions like calcium. Calcium binding and reduced electrostatic potential at the acidic amino acids enable interaction of MARCO with polyanions [33]. Electrostatic potential changes can also alter the stationing of MARCO domains, in turn affecting ligand binding. A high affinity of CD36 of class B to long-chain fatty acids enables fatty acid transport [29, 34].

4 Intracellular Internalization

Scavenger receptors based on their class exhibit different endocytic mechanisms. While SR-A receptors follow clathrin-dependent pathways, LOX-1 proceeds via clathrin-independent pathways. Lipid raft-mediated mechanisms are shown by class

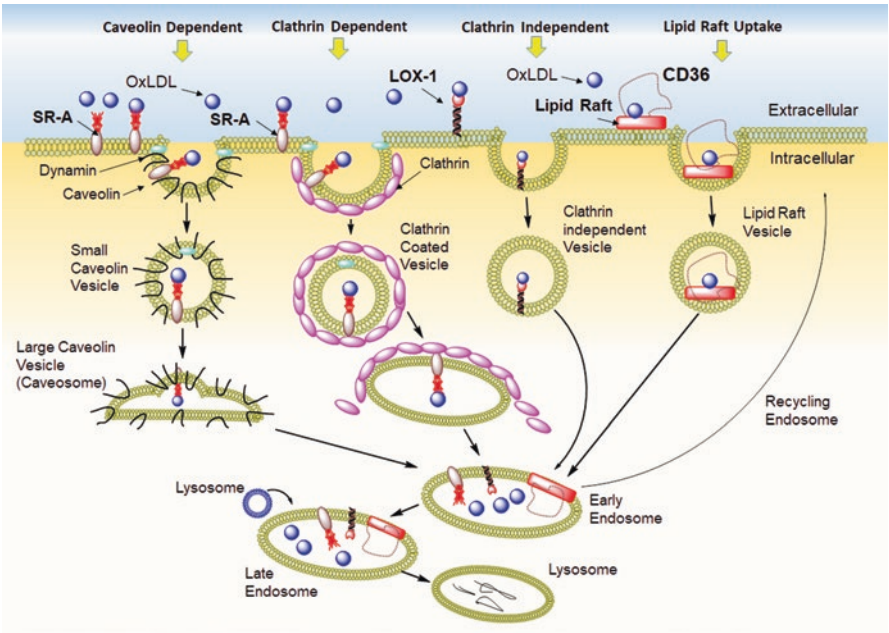

Fig. 10.2 Schematic overview of scavenger receptor-mediated endocytic pathways

B scavenger receptors (Fig.10.2). This endocytosis diversity of scavenger receptors is mainly associated with their sequence diversity and a wide variety of endocytic motifs present in cytoplasmic domains of each scavenger receptor.

The ligand binding to scavenger receptor mediates receptor-mediated endocytosis of this scavenger receptor–ligand complex, followed by intracellular trafficking via endosome lysosome system resulting in the metabolism of ligand. Scavenger receptor-mediated endocytosis of ligands stimulates the cascade of intracellular signaling. This leads to apoptosis, lipid peroxidation, and endothelial cell dysfunction. Monocyte infiltration accompanied by differentiation, which leads to foam cell formation, suggest a role in atherosclerotic plaque formation [6].

4.1 Caveolae/Clathrin-Dependent Pathway

A phagocytic cascade is triggered following internalization of modified LDL by SR-A. In the absence of ligands, unlike LDL receptor, the SR-A does not follow continuous cycling through a metabolic pathway. N-terminal cytoplasmic domain of SR-A contains di-leucine motif at amino acid residues 31 and 32, phosphorylation sites have been involved in ligand internalization and adhesion. This internalization of ligands follows classical coated pit pathway (Fig.10.2) [35].

4.2 Clathrin-Independent Pathway

The class E scavenger receptor LOX-1 binds to OxLDL, apoptotic bodies, and phospholipids and endocytoses via clathrin-independent pathway [36].

4.3 Lipid Raft Uptake

Class B scavenger receptor CD36 follows lipid rafts/caveolae-dependent pathway. Lipid rafts are mainly membrane domains containing lipids such as cholesterol, sphingolipids, glycosyl-phosphatidylinositol (GPI)-anchored proteins, and protein-tyrosine kinases of acylated src family. Caveolae present specialized raft subdomain for uptake mechanisms in some cells [37, 38].

5 Scavenger Receptor Location, Expression, and Function

Scavenger receptors are expressed mainly in endothelial cells (EDCs) and myeloid cells, but others are also expressed in epithelial cells. The SR-AI and AII are mostly expressed on macrophages, EDCs, epithelial cells, astrocytes, dendritic cells (DCs), mast cells, smooth muscle cells and mediates lipid metabolism, clearance of modified host components, pathogens, apoptotic cells, B cell–macrophage interactions, antigen presentation, binding of macrophages to extracellular matrix, and intracellular signaling [39–41]. MARCO is expressed by macrophages, EDCs, DCs, and astrocytes. Infectious stimuli express MARCO in most tissue macrophages. In DCs, antitumor response induces high-level expression of MARCO. MARCO also regulates the clearance of pathogens, necrotic dead cells, unopsonized particles, and enhances B cell–macrophage interaction [42]. SRCLI/II is mostly expressed by EDCs, stromal cells, astrocytes, and microglia, but not by macrophages. SRCL induces adherence of Lewis X-positive cells to vascular endothelium and elicits clearance of desialylated glycoproteins and β-amyloid [15]. Moreover, SCARA-5, a class A receptor is mostly expressed on epithelial cells of testis, airways, thymus, and adrenal glands. SCARA-5 lacks ability to recognize modified LDL and thus not involved in its endocytosis [14].

CD36 is mostly expressed by myeloid cells, platelets, adipocytes, and EDCs. Monocyte differentiation upregulates CD36 level, a mechanism similar to SR-A. The class B receptors induce lipid transfer activity, clearance of apoptotic cells, and *P. falciparum*-infected erythrocytes. SR-BI is found on monocytes, DCs, liver cells, and adrenal glands [43]. Class C is not found in humans and expressed on macrophages of the Drosophila and Mosquitoes [44]. The class D CD68 scavenger receptor shows intracellular expression in macrophages, and surface expression on dendritic cells and osteoclasts [45]. Moreover, scavenger receptor class E (LOX-1)

is mostly found on EDCs, in various diseased conditions is expressed in smooth muscle cells. Furthermore, LOX-1 is involved in induction of apoptosis of EDCs, monocyte adhesion to EDCs, release of proinflammatory cytokines, and increase in ROS production [46]. The class F receptors are expressed over EDCs, macrophages and are involved in clearance of modified host components, antigen clearance and cross-presentation [47]. The class G scavenger receptors are expressed over macrophages, dendritic cells, and also expressed in multiple organs [48]. The class H scavenger receptors are mostly expressed in EDCs of liver, spleen, and lymphatic system, whereas macrophages only express FEEL-1. The class H facilitates lymphocyte adhesion and transmigration, clearance of modified lipoproteins and apoptotic cells, induces angiogenesis, and is involved in intracellular trafficking [49]. However, Class I CD163 receptor is mainly expressed on myeloid cells and mediates clearance of hemoglobin (Hb):haptoglobin (Hp) complexes, and aids erythroblast adhesion to macrophages [6]. Other classes of scavenger receptors such as class J, K, and L are still in research stage, in which class J is mainly expressed on neurons, class K on macrophages, and class L on kidney proximal tubule cells, lung, thyroid, gallbladder, neuroepithelium, epididymis, prostate, ovaries, uterus, and blood–brain barrier. They are mainly involved in the clearance of extracellular matrix ligands [5]. Although different types of scavenger receptors are expressed at the same site, they show diversity in intracellular trafficking and consequently elicit different responses.

6 Pathophysiological Features

SR-AI/II plays a major role in innate immunity against bacterial infections, where they recognize polyanionic cell wall products of bacteria including lipopolysaccharide (LPS) and lipoteichoic acid (LTA). They mediate unopsonized phagocytosis of Gram-positive bacteria. This innate immune response stimulates scavenger receptor and enhances recognition and rapid internalization of pathogenic materials, thereby playing a role in the host defense mechanism [50].

SR-BI is involved in several processes such as apoptosis, binding and internalization of pathogens, and signaling for induction of anti-inflammatory response. Microorganisms supported by anti-inflammatory activity of SR-BI undergo internalization via multimolecular pathways. This was elucidated based on observations in infectious diseases caused by Gram-positive and -negative bacteria, as also infections caused by dengue virus, hepatitis C virus, *Plasmodium* species, and many other infectious agents. SR-BI is also involved in the clearance of microbial end products. Binding of SR-BI to lipopolysaccharide is reported [50].

Involvement of scavenger receptors in the regulation of cancer tumor growth and associated immune reactions is reported. Tumor-associated macrophages (TAMs) show elevated levels of SR-A. An overexpression of SR-BI on cancer cell lines is observed. This results in increased lipid uptake in tumor cells, thus promoting growth [51]. Yet another interesting mechanism by which SR-BI increases tumor

proliferation is the intracellular signaling cascade involving activation of the PI3K/AKT pathway, thereby causing tumor growth [52, 53].

Scavenger receptors are extensively studied in atherosclerosis, where SR-A and CD36 induce modified LDL uptake which is associated with foam cell formation [54]. On the other hand, SR-BI mediates cholesterol transport which is responsible for its anti-atherogenic role [55]. Although cells present in atherosclerotic lesions expressed LOX-1 and CD68, their exact role in response to atherogenesis is yet to be confirmed.

Interestingly, scavenger receptors are reported to play an important role in the pathophysiology of Alzheimer's disease, where they exhibited potential endocytosis of β-amyloid fibrils [56].

7 Ligands

Majority of scavenger receptors are able to bind bacteria, virus, and cell surface components. They showed effective binding with polynucleotides, sulfated polysaccharides, and long-chain fatty acids [40]. Almost all scavenger receptors mediate modified LDL uptake. The members of this supergroup such as SR-A, SR-B, and SR-E show efficient binding to both OxLDL and AcLDL, whereas SR-H only mediate AcLDL uptake. Among all, class B receptors bind to unmodified LDL, HDL, and VLDL. Such differential binding dictates their functional diversity in the clearance of modified LDL. The scavenger receptor expresses positively charged amino acids cluster (arginine or lysine) which thereby facilitates ionic interaction with negatively charged polyanionic ligands, lipoprotein particles, and pathogenic materials such as lipopolysaccharide and lipoteichoic acid. The ligand-binding potential of scavenger receptors increases with enhanced oligomer expression [29, 31, 41]. The numerous ligands for different scavenger receptor classes are summarized in Table 10.1.

Class A recognizes lipidic and apolipoprotein functionalities expressed by modified LDL [57]. This class of scavenger receptors exhibits neuronal cytotoxicity by mediating the uptake of β-amyloid fibrils in microglia and thereby contributes to the pathophysiology of Alzheimer's disease [58]. The affinity of extracellular ligands biglycan and decorin to SR-A induces association of macrophages in the extracellular matrix of smooth muscle cells involved in atherosclerotic plaque formation. Similarly, advanced glycation end products (AGE) efficiently endocytosed by SR-A are released during inflammation. On the other hand, SR-A also mediates uptake of glycated proteins such as glycated collagen IV. Expression of SR-A on adenocarcinoma cells mediates internalization of T-cell tumor antigen and thus plays an important role in cancer pathology. During lung inflammation, MARCO mediates uptake of uteroglobin-related protein-1 (UGRP-1). Besides MARCO another member of class A, SRCL-I recognizes Lewis-X trisaccharides with high affinity and dictates its role in recognizing desialylated glycoproteins. SRCL-I also recognizes β-amyloid peptide in Alzheimer's patients [5].

Table 10.1 Scavenger receptor class and their ligand molecules

Class	Receptor	Endogenous ligands	Pathogenic ligands	Exogenous ligands
A	SR-AI/II	AcLDL, OxLDL, lysophosphatidylcholine, ApoA-I, Apo E, cholesterol, modified collagen type I, III, and IV, biglycan, decorin, AGE-BSA, β-amyloid fibrils, calreticulin, gp96, Hsp70, CpG DNA	*N. meningitidis* surface proteins, Gram-positive and -negative bacteria, C-reactive protein, hepatitis C virus, LPS, LTA	Polyacrylic acid, phosphatidic acid-modified albumin, calciprotein particles, maleylated LDL
A	MARCO	AcLDL, OxLDL	*N. meningitidis* surface proteins, Gram-positive and -negative bacteria, LPS	TiO_2, Fe_2O_3, Latex beads, and $CSiO_2$
A	SCARA5	L-ferritin, haptoglobin, hemoglobin	Gram-positive and -negative bacteria	Fe_2O_3
A	SRCL	OxLDL, β-amyloid, desialylated Lewis X-containing glycoproteins, Lacto-ferrin, matrix metalloproteinases 8, 9	Yeast, Gram-positive and -negative bacteria	Fe_2O_3, modified glycoproteins, modified polysachharides
B	CD36	AcLDL, OxLDL	Gram-negative bacteria, *Cryptococcus neoformans* and *P. falciparum*, LTA	Phosphatidylserine, β-glucan, A diacylated, lipopeptides
B	SR-BI	AcLDL, OxLDL, native LDL, native HDL, VLDL, apoptotic cells	Gram bacteria, *M. fortuitum*, hepatitis C virus, *P. falciparum*, LPS	Sulfated polysaccharides
D	CD68/ Macrosialin	OxLDL	ICAM-L (Leishmania surface protein)	Phosphatidylserine-rich liposomes
E	LOX-1	OxLDL, acLDL, fibronectin, and pancreatic bile salt-dependent lipase Hsp60, Hsp70	Gram-negative and -positive bacteria	Modified LDL, lipoprotein particle, phospholipids, sulfated polysaccharides, poly(I), AGEs
F	SRECI/II	AcLDL, OxLDL, glucose-regulated protein 170, Hsp70, Hsp90, Hsp110	Gram bacteria, hepatitis C virus, fungal pathogens, zymogen granule protein 2	Carbamylated LDL, calreticulin
G	SRPSOX/ CXCL16	OxLDL	Bacteria	Phosphatidylserine

(continued)

Table 10.1 (continued)

Class	Receptor	Endogenous ligands	Pathogenic ligands	Exogenous ligands
H	FEEL-1/ Stabilin1/ CLEVER1	AcLDL, AGE, SPARC, Hsp70, SICLP, Placental lactogen, and GDF-15	Gram-negative and -positive bacteria	Phosphatidylserine, heparin sulfate
H	FEEL-2/ stabilin-2/ HARE	AcLDL, AGE, and GDF-15	Gram-negative and -positive bacteria	Procollagen, hyaluronic acid, phosphatidylserine, heparin
I	CD163	Hb:Hp, TWEAK a TNF superfamily cytokine	Gram-positive and -negative bacteria	Not known
J	RAGE	AGEs, HMGB, S-100 protein	Not known	Modified AGE
K	CD44	Hyaluronan, growth factors, cytokines, and matrix metalloproteinases	Bacteria, proteoglycans	Hyaluronic acid, glycosaminoglycans
L	SR-L1	Cholesterol, Apo-EI	Not known	Not known
L	SR-L2	Leptin, insulin, and amyloid β peptide	Not known	Not known

Class B receptors show diverse ligand specificity where they bind native lipoprotein particles and hypochlorite modified LDL which are found in atherosclerotic lesions. CD36 present in vascular endothelial cells mediates hexarelin uptake and causes vasoconstriction. Furthermore, this CD36 also binds collagen type I, AGE-modified BSA, and β-amyloid fibrils. It also recognizes oxidized phospholipids expressed on apoptotic cells, thereby mediating macrophage clearance. SRBI receptor of class B, binds AcLDL with greater affinity. It also mediates uptake of native lipoprotein particles and recognizes expressed apolipoprotein components. It also binds AGE-BSA and β-amyloid fibers [5, 29, 57, 58].

Class C receptor binds to AcLDL and pathogens, whereas class D (CD68) binds to OxLDL and negatively charged phosphatidylserine-rich liposomes. The Class E (LOX-1) binds to OxLDL, fibronectin, phosphatidylserine, AGE-modified protein and clears apoptotic cells. LOX-1 mediates Hsp70 internalization in dendritic cells, which is not endocytosed by class A & class B receptors. Both Class F receptors, SREC-I and SREC-II, are involved in the uptake of modified LDL, and mediate recognition of other ligands such as calreticulin, molecular chaperones, gp96, and tumor released heat shock proteins (Hsp70), whereas they lack recognition for AGE-modified proteins.

Class G (SRPSOX) receptor-like LOX1 (Class E) binds to OxLDL but not to AcLDL and it also acts as chemokine ligand for CXC chemokine receptor 16, thereby mediating adhesion of DCs to T cells and NK cells. In class H, FEEL-1 receptor binds to extracellular SPARC (secreted protein acidic and rich in cysteine) glycoprotein and SI-CLP (stabilin-1 interacting chitinase-like protein) sorted in macrophages and Hsp70, while they show poor recognition for AGE-BSA. However, FEEL-2 recognizes hyaluronic acid and AGE-BSA with high affinity [5, 6, 29].

Scavenger receptors bind to foreign ligands including bacteria, fungi, virus, and parasites. In case of pathogens, their extracellular expression containing lipopolysaccharides, lipoteichoic acid, C-reactive protein, endotoxins, and numerous other surface proteins are recognized by these receptors [59]. Researchers have studied various other ligands based on functionality for scavenger receptor targeting, which is summarized in Table 10.2.

Table 10.2 List of scavenger receptor ligands based on functionality

Ligand functionality	Ligands	Reference
Polyacids	Polyacrylic acid	[60]
	Polyitaconic acid	[61]
	Poly-D glutamic acid	[62]
Phospholipids	Phosphatidylserine	[63]
	Phosphatidylglycerol	[64]
	Phosphatidylinositol	[37]
	Phosphatidic acid	[37]
	Oxidized phospholipids	[65]
	Cardiolipin	[66]
Polysaccharides	Dextran sulfate	[67, 68]
	Heparin and heparan sulfate	[69, 70]
	Keratan sulfate	[71]
	Dermatan sulfate	[72]
	Chondroitin sulfate	[73]
	Glucoronate oligosaccharide	[74]
	Hyaluronate	[75]
	Carrageenan	[68, 76]
	Carboxymethyl dextran	[77, 78]
	Carboxymethyl cellulose	[79, 80]
	Fucoidan	[81, 82]
	Glycosaminoglycans	[83]
Polynucleotides	Polyinosinic acid poly (I)	[84]
	Poly (G), poly (G:I), polyxanthinylic acid, telomere models $[d(G_4T_4)_5]$	[85]
Fatty acids	Stearic acid	[86]
	Myristic acid, polyunsaturated fatty acids	[87]
Inorganic particles	Fe_2O_3	[33, 88]
	TiO_2, ZnO	[89]
	Silica	[90]
	Asbestos crocidolite	[91, 92]
Modified Proteins	Maleylated BSA	[93, 94]
	Malonaldehyde LDL	[95]
	Calciprotein	[96]
	Procollagen propeptides	[97]
	Heat shock proteins (Hsp)	[98]
	Major vault protein (MVP)	[99]
Others	Bovine sulfatides	[100]

8 Receptor-Mediated Targeting Strategies

Endocytic uptake mechanism of scavenger receptor suggests a simplistic way of receptor-mediated targeted drug delivery. The drug gets released intracellularly after efficient internalization of the ligand–receptor complex. This interaction also induces the cascade of inflammatory responses. Although there exist many different scavenger receptor classes, till date, only A and B scavenger receptors have been studied for nanomedicine-mediated response. These scavenger receptors serve as a unique nanomedicine target also for many theranostic applications. Research directed towards site-specific targeted drug delivery through scavenger receptor using various nanoformulations relies on receptor-specific ligands carrier compositions mainly involving polyanions [101].

8.1 Drug–Ligand Conjugates

The chemical coupling of a suitable drug to a scavenger receptor-specific ligand such as maleylated albumin (MBSA) increases recognition by scavenger receptors for high-affinity binding, thereafter it undergoes internalization and metabolically degraded in lysosomes to release free drug for activity. Almost 100-fold enhanced efficacy was observed with such drug–ligand conjugate when studied in leishmaniasis, tuberculosis, and neoplastic conditions. Coupling of methotrexate (MTX) to MBSA exhibited rapid internalization inside leishmania-infected hamster peritoneal macrophages and demonstrated 100-fold antileishmanial effect compared to free drug. It also eliminated intracellular amastigotes of *L. donovani* and L. mexicana *amazonesis*. However, in case of *M. tuberculosis*-infected macrophages the targeting of anti-tubercular drug p-amino salicylic acid (PAS) and MBSA conjugate resulted in only 50% reduction of colony-forming units (CFUs). However, compared to free drug which exhibited CFU reduction of 0.5%, the enhancement in efficacy was nearly 100-fold. In neoplastic condition, the conjugation of drug Daunomycin with MBSA exhibited 100-fold cytotoxicity over free Daunomycin when tested at low concentration of 0.1 μM. Similar cytotoxic results were found with Doxorubicin-MBSA conjugate when tested on human histiocytic lymphoma cells [102].

8.2 Liposomes

Negatively charged phospholipids such as phosphatidylserine (PS) and phosphatidylglycerol (PG) are efficiently recognized by macrophages [103]. The studies confirmed that negatively charged liposomes are efficiently taken up by macrophage scavenger receptors over neutral or cationic liposomes [104–107]. When macrophage cells which expressed scavenger receptor were treated with negatively charged PS-liposomes and neutral PC-liposomes, the former exhibited 5.3-fold

enhanced macrophage uptake over PC liposomes [108]. Incorporation of dicetylphosphate (DCP) also induced net negative surface charge over liposomes [109]. Polyaconitylated-human serum albumin (Aco-HSA) surface anchored liposomes showed effective anti-HIV 1 activity due to high uptake by scavenger receptors expressed on sinusoidal cells. This conjugation of Aco-HSA to liposomes enhanced liver uptake 17-fold, as compared with control liposomes, and the Aco-HSA liposomes were mostly found in liver EDCs and kupffer cells. Further, in this study, reduced liver uptake (24%) of Aco-HSA was found post-injection of polyinosinic acid, which is a known SR ligand [110].

In case of stealth liposomes, endocytic CD163 scavenger receptor enhanced uptake of monoclonal antibody loaded pegylated liposomes in CD163 transfected cells and macrophages [111]. Palmitoyloleoyl-phosphatidylcholine (POPC)-apoE liposomes functionalized using different apoE proteins (apoE2, apoE3, apoE4, apoE165, apoE202, apoE229, and apoE259) enhanced scavenger receptor B binding affinity and were thought to regulate brain cholesterol metabolism [112]. Liposomes carrying fluorescently labeled cholesterol when tested on HepG2 cells (model system for human hepatocytes) showed 20% binding for class B scavenger receptor and only 10% recognition was confined to low-density lipoprotein receptor (LDLR) which provided additional insights for scavenger receptor-mediated uptake of liposomes [113]. It was reported that, in certain cells, liposome uptake is not inhibited by known scavenger receptor ligands suggesting their uptake was not scavenger receptor-mediated. PS-containing liposomes showed enhanced uptake in an African green monkey kidney cell line (CVI) compared to phosphatidylcholine (PC) liposomes, independent of inhibition by known competitors for scavenger receptor [polyinosinic acid or dextran sulfate]. On the other hand, in case of murine macrophage cell line, PS-liposome uptake was inhibited competitively by polyinosinic acid, but not by polycytidylic acid [114]. The liposomes for targeted scavenger receptor delivery in various diseased conditions are described in Table 10.3.

8.3 Nanoparticles

8.3.1 Lipoprotein Particles

In one study, it was found that OxLDL exhibited stronger CD36 binding and HDL has stronger SR-BI binding ability among all lipoproteins [124]. Synthesized HDL nanoparticles also revealed high affinity for SR-BI-rich cancer cells. Furthermore, HDL nanoparticles mediated delivery of siRNA to the cancer tumors overexpressed with SR-BI. Similarly, these HDL nanoparticles exhibited SR-BI-mediated paclitaxel delivery to prostate cancer cells. Such studies proved the potential of SR-BI-mediated targeting of nanoparticles and their subsequent involvement in many disease states [125]. Administration of acylated LDL particles loaded with muramyl tripeptide mediated antitumor efficacy through the scavenger receptors [126]. In one study, the antinociceptive activity of apo lipoprotein functionalized loperamide-

Table 10.3 Liposomes for scavenger receptor targeting

Active	Ligand	Study outcome	Reference
Tuberculosis			
Rifampicin	Maleylated bovine serum albumin	Higher lung retention in rats compared to free drug	[109]
Rifampicin	Tuftsin	2000-times more effective in lowering lung CFU compared to free drug	[115]
Rifampicin and isoniazid	Dicetylphosphate (DCP)	Decreased bacterial load in lung, liver, and spleen	[116]
Hepatitis			
No drug	L-α-phosphatidylinositol (PI) and L-α-phosphatidylserine (PS)	Improved antiviral efficacy by reducing infected cell cholesterol level	[117]
Leishmaniasis			
Pentavalent antimony	PS	16-fold more efficacy compared to free drug	[118]
Buparvaquone	PS	>90% efficacy in liver and spleen found	[119]
HIV			
No drug	PI and PS	Suppressed mean viral secretion by 22% and infectivity by 55%	[117]
Iminosugar N-butyl-deoxynojirimycin	PI and PS	Decreased viral secretion by 62% and infectivity by 86%	[120]
Cancer			
CPX-351 (cytarabine and daunorubicin 5:1 molar ratio)	PS	SR-BI mediated efficient uptake of CPX-351 in K562 leukemia cells	[121]
Doxorubicin	Polyethylene glycol	CD163-targeted pegylated liposomes showed 50% cell killing over Doxil	[111]
Atherosclerosis			
Fumagillin	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-7-nitro-2-1, 3-benzoxadiazol-4-yl (DPPE-NBD), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl (DPPE-Biotin)	Diminished atherosclerotic lesion	[122]
Dexamethasone	Decadeoxyguanine linked to lithocholic oleate (LCO-dA2dG10)	Enhanced macrophage uptake	[123]

loaded albumin nanoparticles was assessed. Three apo lipoproteins E3, A-I, and B-100 exhibited 95%, 65%, and 50% antinociceptive activity, respectively, whereas plain loperamide solution showed no effect, which showed uptake of such particles through SR-BI receptor expressed at BBB [127].

8.3.2 Inorganic Nanoparticles

Dextran sulfate-mediated macrophage uptake of silver nanoparticles (AgNPs) through scavenger receptor is reported. Scavenger receptor-mediated uptake of AgNPs resulted in their intracellular accumulation and thereby apoptosis [128]. Furthermore, protein functionalization of AgNPs reduced its uptake due to decreased surface charge [129, 130]. Inhalation of ZnO nanoparticles induced enhanced expression of both SR-A and SR-B and thereby influenced atherosclerotic disease progression. However, TiO_2 nanoparticles did not exhibit the same mechanism [89]. The macrophage phagocytic activity was diminished when subjected to superparamagnetic iron oxide nanoparticles exposure. These iron oxide nanoparticles inhibited macrophage activation for M1 to M2 state and enhanced TNF-α production [33, 88].

8.3.3 Miscellaneous

Gadolinium-containing nanomedicines with anti-CD36 antibodies were efficiently taken up by macrophages in vitro compared to nanomedicines without CD36 antibodies [131]. The scavenger receptor A class member MARCO also exhibited interaction with carbon nanotubes [132] and polystyrene nanoparticles [133]. Furthermore, when scavenger receptor A was overexpressed in human embryonic kidney 293 (HEK293) cells, a cell line which is normally devoid of scavenger receptor expression, elicited enhanced uptake of amorphous silica nanoparticles, demonstrating role of scavenger receptor in uptake of nanomedicines [101,134]. The miscellaneous nanoparticles targeting scavenger receptors are given in Table 10.4.

Table 10.4 Other nanosystems for scavenger receptor targeting

Nanomaterials	Study outcome	Reference
Inorganic		
Dextran-coated superparamagnetic iron oxide nanoparticles (SPIO)	Promotes SPIO uptake by embryonic kidney cells (HEK293T) overexpressing SR-AI and MARCO	[33]
Silver nanoparticles	Inhibition of SR-BI caused reduced uptake of AgNPs in endothelial and epithelial cells	[129]
Silver nanoparticles	Decreased uptake in MARCO-deficient alveolar macrophages	[135]
Miscellaneous		
Multiwalled carbon nanotubes	Efficient binding to MARCO in macrophages	[132]
Fluorescent labeled polystyrene particles	Enhanced macrophage association of nanoparticles through MARCO	[133]
Silica nanoparticles	Enhanced nanoparticles uptake by human embryonic kidney 293 (HEK293) cells with overexpression of SR-A	[134]

9 Clinical Trials

Although targeting to scavenger receptor is still in its nascent stage, very few scavenger receptor-mediated delivery systems have reached clinical trials. Herein we discuss such clinical studies with their outcome, demonstrating the role of scavenger receptors and targeting strategies. A study exploring targeting of pegylated interferon α2 plus ribavirin therapy to SR-BI receptor encoded by SCARB1 gene for hepatitis C virus studied the association of single nucleotide polymorphism (SNP) of SCARB1 gene and its response to therapy, where they found SNP may increase antiviral therapy outcome [136, 137]. Interestingly another study was conducted to understand underlying molecular mechanisms causing disruption to HDL regulation through scavenger receptor (SR-BI) in various metabolic diseases including atherosclerosis, where genotype modification affects HDL metabolism and cholesterol homeostasis [138]. One study was conducted to assess the role of scavenger receptor ligands as biomarkers for cardiovascular disease diagnosis, where oxidized phospholipids and apolipoprotein B identification by antibodies can be detected to predict cardiovascular disease state 15 years in advance. The receptor studied here was CD36 [139]. Studies were also conducted for anti-hepatitis C virus efficacy testing of a new molecule ITX 5061 by blocking the virus uptake through scavenger receptor (SR-BI) expressed on hepatocytes to reduce the infection chances in liver transplant patients [140]. No clinical trials are however evident on scavenger receptor-targeted drug delivery.

10 Advantages and Limitations

Targeting scavenger receptors offers great promise for improved therapeutic efficacy. This receptor has recognition specificity for pathogenic materials and plays an important role in various disease conditions. Intracellular delivery of actives can be achieved through scavenger receptor-mediated drug delivery, as the majority of infections are intracellular.

The major limitation of targeting these receptors is their broad ligand binding and recognition including both endogenous and exogenous molecules, which will compete for receptor-mediated endocytosis. Another major challenge is immunogenicity as these receptors are involved in inflammation and expressed on immune cells. Furthermore, they are widely expressed on majority of cell types; hence, specificity is a challenge.

11 Future Perspectives

Recently, newer classes of scavenger receptors were found and many more are still to be discovered, hence targeting to these receptors can provide newer avenues in site-specific drug delivery. Exploitation of scavenger receptor-mediated drug delivery

is an option of the future. SR-BI due to its overexpression is reported as biomarker for human nasopharyngeal carcinoma [141]. The role of scavenger receptor as a biomarker for diagnosing various other disease conditions needs to be explored.

12 Conclusion

Scavenger receptors play a multifaceted and dynamic role in various cell-signaling pathways in the human body and are involved in metabolic regulation of macrophages for improved immune response. Scavenger receptors facilitate uptake of a broad spectrum of ligands including endogenous and foreign molecules. However, this receptor poses a challenge in stealth delivery of nanomedicines due to its inherent ability of scavenging numerous components. Targeted drug delivery using scavenger receptor is still in its nascent stage and can be further exploited for the treatment of infections and cancer.

References

1. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol. 2013;13(9):621–34.
2. Brown MS, Goldstein JL. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. Proc Natl Acad Sci. 1979;76(7):3330–7.
3. Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RG. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J Cell Biol. 1979;82(3):597–613.
4. Su T, Zhao L, Ruan X, Zuo G, Gong J. Synergistic effect of scavenger receptor A and low-density lipoprotein receptor on macrophage foam cell formation under inflammatory stress. Mol Med Rep. 2013;7(1):37–42.
5. PrabhuDas MR, Baldwin CL, Bollyky PL, Bowdish DM, Drickamer K, Febbraio M, Herz J, Kobzik L, Krieger M, Loike J, McVicker B. A consensus definitive classification of scavenger receptors and their roles in health and disease. J Immunol. 2017;198(10):3775–89.
6. Zani I, Stephen S, Mughal N, Russell D, Homer-Vanniasinkam S, Wheatcroft S, Ponnambalam S. Scavenger receptor structure and function in health and disease. Cell. 2015;4(2):178–201.
7. Ashraf MZ, Sahu A. Scavenger receptors: a key player in cardiovascular diseases. Biomol Concepts. 2012;3(4):371–80.
8. de Winther MP, van Dijk KW, Havekes LM, Hofker MH. Macrophage scavenger receptor class A: a multifunctional receptor in atherosclerosis. Arterioscler Thromb Vasc Biol. 2000;20(2):290–7.
9. Wada Y, Doi T, Matsumoto A, Asaoka H, Honda M, Hatano H, Emi M, Naito M, Mori T, Takahashi K, Nakamura H. Structure and function of macrophage scavenger receptors. Ann N Y Acad Sci. 1994;748(1):226–38.
10. Bowdish DM, Gordon S. Conserved domains of the class A scavenger receptors: evolution and function. Immunol Rev. 2009;227(1):19–31.
11. Resnick D, Chatterton JE, Schwartz K, Slayter H, Krieger M. Structures of class A macrophage scavenger receptors electron microscopic study of flexible, multidomain, fibrous proteins and determination of the disulfide bond pattern of the scavenger receptor cysteine-rich domain. J Biol Chem. 1996;271(43):26924–30.

12. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. Nature. 1990;343(6258):531–5.
13. Elomaa O, Sankala M, Pikkarainen T, Bergmann U, Tuuttila A, Raatikainen-Ahokas A, Sariola H, Tryggvason K. Structure of the human macrophage MARCO receptor and characterization of its bacteria-binding region. J Biol Chem. 1998;273(8):4530–8.
14. Jiang Y, Oliver P, Davies KE, Platt N. Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. J Biol Chem. 2006;281(17):11834–45.
15. Nakamura K, Funakoshi H, Tokunaga F, Nakamura T. Molecular cloning of a mouse scavenger receptor with C-type lectin (SRCL), a novel member of the scavenger receptor family. Biochim Biophys Acta. 2001;1522(1):53–8.
16. Thorne RF, Meldrum CJ, Harris SJ, Dorahy DJ, Shafren DR, Berndt MC, Burns GF, Gibson PG. CD36 forms covalently associated dimers and multimers in platelets and transfected COS-7 cells. Biochem Biophys Res Commun. 1997;240(3):812–8.
17. Reaven E, Cortez Y, Leers-Sucheta S, Nomoto A, Azhar S. Dimerization of the scavenger receptor class B type I formation, function, and localization in diverse cells and tissues. J Lipid Res. 2004;45(3):513–28.
18. Pearson A, Lux AL, Krieger M. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. Proc Natl Acad Sci. 1995;92(9):4056–60.
19. Jiang Z, Shih DM, Xia YR, Lusis AJ, de Beer FC, de Villiers WJ, van der Westhuyzen DR, de Beer MC. Structure, organization, and chromosomal mapping of the gene encoding macrosialin, a macrophage-restricted protein. Genomics. 1998;50(2):199–205.
20. Yamanaka S, Zhang XY, Miura K, Kim S, Iwao H. The human gene encoding the lectin-type oxidized LDL receptor (OLR1) is a novel member of the natural killer gene complex with a unique expression profile. Genomics. 1998;54(2):191–9.
21. Ishii J, Adachi H, Aoki J, Koizumi H, Tomita S, Suzuki T, Tsujimoto M, Inoue K, Arai H. SREC-II, a new member of the scavenger receptor type F family, trans-interacts with SREC-I through its extracellular domain. J Biol Chem. 2002;277(42):39696–702.
22. Adachi H, Tsujimoto M. FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. J Biol Chem. 2002;277(37):34264–70.
23. Rodamilans B, Muñoz IG, Bragado-Nilsson E, Sarrias MR, Padilla O, Blanco FJ, Lozano F, Montoya G. Crystal structure of the third extracellular domain of CD5 reveals the fold of a group B scavenger cysteine-rich receptor domain. J Biol Chem. 2007;282(17):12669–77.
24. Ibrahim ZA, Armour CL, Phipps S, Sukkar MB. RAGE and TLRs: relatives, friends or neighbours. Mol Immunol. 2013;56(4):739–44.
25. Naor D, Sionov RV, Ish-Shalom D. CD44: structure, function and association with the malignant process. Adv Cancer Res. 1997;71:241–319. Academic Press.
26. Dzwonek J, Wilczynski GM. CD44: molecular interactions, signaling and functions in the nervous system. Front Cell Neurosci. 2015;9:175.
27. Oh SJ, Kim TH, Lim JM, Jeong JW. Progesterone induces expression of Lrp2 in the murine uterus. Biochem Biophys Res Commun. 2013;441(1):175–9.
28. Bartolome F, Antequera D, Tavares E, Pascual C, Maldonado R, Camins A, Carro E. Obesity and neuroinflammatory phenotype in mice lacking endothelial megalin. J Neuroinflammation. 2017;14(1):26.
29. Plüddemann A, Neyen C, Gordon S. Macrophage scavenger receptors and host-derived ligands. Methods. 2007;43(3):207–17.
30. Ashkenas J, Penman M, Vasile E, Acton S, Freeman M, Krieger M. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. J Lipid Res. 1993;34(6):983–1000.
31. Stephen SL, Freestone K, Dunn S, Twigg MW, Homer-Vanniasinkam S, Walker JH, Wheatcroft SB, Ponnambalam S. Scavenger receptors and their potential as therapeutic targets in the treatment of cardiovascular disease. Int J Hypertens. 2010;2010:646929.

32. Ojala JR, Pikkarainen T, Tuuttila A, Sandalova T, Tryggvason K. Crystal structure of the cysteine-rich domain of scavenger receptor MARCO reveals the presence of a basic and an acidic cluster that both contribute to ligand recognition. J Biol Chem. 2007;282(22):16654–66.
33. Chao Y, Makale M, Karmali PP, Sharikov Y, Tsigelny I, Merkulov S, Kesari S, Wrasidlo W, Ruoslahti E, Simberg D. Recognition of dextran–superparamagnetic iron oxide nanoparticle conjugates (Feridex) via macrophage scavenger receptor charged domains. Bioconjug Chem. 2012;23(5):1003–9.
34. Ibrahimi A, Abumrad NA. Role of CD36 in membrane transport of long-chain fatty acids. Curr Opin Clin Nutr Metab Care. 2002;5(2):139–45.
35. Chen Y, Wang X, Ben J, Yue S, Bai H, Guan X, Bai X, Jiang L, Ji Y, Fan L, Chen Q. The di-leucine motif contributes to class a scavenger receptor-mediated internalization of acetylated lipoproteins. Arterioscler Thromb Vasc Biol. 2006;26(6):1317–22.
36. Murphy JE, Vohra RS, Dunn S, Holloway ZG, Monaco AP, Homer-Vanniasinkam S, Walker JH, Ponnambalam S. Oxidised LDL internalisation by the LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2. J Cell Sci. 2008;121(13):2136–47.
37. Zeng Y, Tao N, Chung KN, Heuser JE, Lublin DM. Endocytosis of oxidized low density lipoprotein through scavenger receptor CD36 utilizes a lipid raft pathway that does not require caveolin-1. J Biol Chem. 2003;278(46):45931–6.
38. Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL. A CD36-dependent signaling cascade is necessary for macrophage foam cell formation. Cell Metab. 2006;4(3):211–21.
39. Hughes DA, Fraser IP, Gordon S. Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. Eur J Immunol. 1995;25(2):466–73.
40. Zingg JM, Ricciarelli R, Azzi A. Scavenger receptors and modified lipoproteins: fatal attractions. IUBMB Life. 2000;49(5):397–403.
41. Platt N, Gordon S. Scavenger receptors: diverse activities and promiscuous binding of polyanionic ligands. Chem Biol. 1998;5(8):R 193–203.
42. Elomaa O, Kangas M, Sahlberg C, Tuukkanen J, Sormunen R, Liakka A, Thesleff I, Kraal G, Tryggvason K. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell. 1995;80(4):603–9.
43. Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, van Berkel TJ. Hepatic and extrahepatic scavenger receptors: function in relation to disease. Arterioscler Thromb Vasc Biol. 2000;20(8):1860–72.
44. Rämet M, Pearson A, Manfruelli P, Li X, Koziel H, Göbel V, Chung E, Krieger M, Ezekowitz RA. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity. 2001;15(6):1027–38.
45. Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. Proc Natl Acad Sci. 1996;93(25):14833–8.
46. Moriwaki H, Kume N, Sawamura T, Aoyama T, Hoshikawa H, Ochi H, Nishi E, Masaki T, Kita T. Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. Arterioscler Thromb Vasc Biol. 1998;18(10):1541–7.
47. Berwin B, Delneste Y, Lovingood RV, Post SR, Pizzo SV. SREC-I, a type F scavenger receptor, is an endocytic receptor for calreticulin. J Biol Chem. 2004;279(49):51250–7.
48. Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T, Yonehara S. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. J Biol Chem. 2000;275(52):40663–6.
49. Prevo R, Banerji S, Ni J, Jackson DG. Rapid plasma membrane-endosomal trafficking of the lymph node sinus and high endothelial venule scavenger receptor/homing receptor stabilin-1 (FEEL-1/CLEVER-1). J Biol Chem. 2004;279(50):52580–92.
50. Vasquez M, Simões I, Consuegra-Fernández M, Aranda F, Lozano F, Berraondo P. Exploiting scavenger receptors in cancer immunotherapy: lessons from CD5 and SR-B1. Eur J Immunol. 2017;47(7):1108–18.

51. Yu X, Guo C, Fisher PB, Subjeck JR, Wang XY. Scavenger receptors: emerging roles in cancer biology and immunology. Adv Cancer Res. 2015;128:309–64.
52. Danilo C, Gutierrez-Pajares JL, Mainieri MA, Mercier I, Lisanti MP, Frank PG. Scavenger receptor class B type I regulates cellular cholesterol metabolism and cell signaling associated with breast cancer development. Breast Cancer Res. 2013;15(5):R87.
53. Twiddy AL, Cox ME, Wasan KM. Knockdown of scavenger receptor class B type I reduces prostate specific antigen secretion and viability of prostate cancer cells. Prostate. 2012;72(9):955–65.
54. Kamada N, Kodama T, Suzuki H. Macrophage scavenger receptor (SR-A I/II) deficiency reduced diet-induced atherosclerosis in C57BL/6J mice. J Atheroscler Thromb. 2001;8(1):1–6.
55. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science. 1996;271(5248):518–20.
56. Husemann J, Loike JD, Anankov R, Febbraio M, Silverstein SC. Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. Glia. 2002;40(2):195–205.
57. Terpstra V, Bird DA, Steinberg D. Evidence that the lipid moiety of oxidized low density lipoprotein plays a role in its interaction with macrophage receptors. Proc Natl Acad Sci. 1998;95(4):1806–11.
58. El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC, Loike JD. Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. Nature. 1996;382(6593):716–9.
59. Dunne DW, Resnick D, Greenberg J, Krieger M, Joiner KA. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc Natl Acad Sci. 1994;91(5):1863–7.
60. Fujiwara M, Baldeschwieler JD, Grubbs RH. Receptor-mediated endocytosis of poly (acrylic acid)-conjugated liposomes by macrophages. Biochim Biophys Acta. 1996;1278(1):59–67.
61. Cordes T, Michelucci A, Hiller K. Itaconic acid: the surprising role of an industrial compound as a mammalian antimicrobial metabolite. Annu Rev Nutr. 2015;35:451–73.
62. Wang G, Simberg D. Role of scavenger receptors in immune recognition and targeting of nanoparticles. Rev Cell Biol Mol Med. 2006;1(3):166–89.
63. Minami M, Kume N, Shimaoka T, Kataoka H, Hayashida K, Yonehara S, Kita T. Expression of scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) in human atheroma. Ann N Y Acad Sci. 2001;947(1):373–6.
64. Gu BJ, Saunders BM, Petrou S, Wiley JS. P2X7 is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. J Immunol. 2011;187(5):2365–75.
65. Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugiu B, Fox PL, Hoff HF. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. J Biol Chem. 2002;277(41):38503–16.
66. Balasubramanian K, Maeda A, Lee JS, Mohammadyani D, Dar HH, Jiang JF, Croix CM, Watkins S, Tyurin VA, Tyurina YY, Klöditz K. Dichotomous roles for externalized cardiolipin in extracellular signaling: promotion of phagocytosis and attenuation of innate immunity. Sci Signal. 2015;8(395):ra95.
67. Tsubamotoa Y, Yamada N, Watanabe Y, Inaba T, Shiomi M, Shimano H, Gotoda T, Harada K, Shimada M, Ohsuga JI, Kawamura M. Dextran sulfate, a competitive inhibitor for scavenger receptor, prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. Atherosclerosis. 1994;106(1):43–50.
68. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem. 1983;52(1):223–61.
69. Urano K, Haba M, Yuasa H, Watanabe J. Internalization of fractionated 3H-heparin by the scavenger-like receptor in rat liver parenchymal cells in primary culture. Drug Deliv. 1997;4(3):181–5.
70. Harris EN. Heparin clearance by liver scavenger receptors. Biochem Anal Biochem. 2012;1:e114.

71. Funderburgh JL, Mitschler RR, Funderburgh ML, Roth MR, Chapes SK, Conrad GW. Macrophage receptors for lumican. A corneal keratan sulfate proteoglycan. Invest Ophthalmol Vis Sci. 1997;38(6):1159–67.
72. Harris EN, Weigel PH. The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E. Glycobiology. 2008;18(8):638–48.
73. Sobal G, Sinzinger H. Binding of [99mTc] chondroitin sulfate to scavenger receptors on human chondrocytes as compared to binding of oxidized [125I] LDL on human macrophages. J Recept Sig Transd. 2002;22(1–4):459–70.
74. Fang W, Bi D, Zheng R, Cai N, Xu H, Zhou R, Lu J, Wan M, Xu X. Identification and activation of TLR4-mediated signaling pathways by alginate-derived guluronate oligosaccharide in RAW264. 7 macrophages. Sci Rep. 2017;7(1):1663.
75. McCourt PA, Smedsrød BH, Melkko J, Johansson S. Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. Hepatology. 1999;30(5):1276–86.
76. Kelley JL, Ozment TR, Li C, Schweitzer JB, Williams DL. Scavenger receptor-A (CD204): a two-edged sword in health and disease. Crit Rev Immunol. 2014;34(3):241–61.
77. Harada M, Imai J, Okuno S, Suzuki T. Macrophage-mediated activation of camptothecin analogue T-2513–carboxymethyl dextran conjugate (T-0128): possible cellular mechanism for antitumor activity. J Control Release. 2000;69(3):389–97.
78. Tokuda H, Masuda S, Takakura Y, Sezaki H, Hashida M. Specific uptake of succinylated proteins via a scavenger receptor-mediated mechanism in cultured brain microvessel endothelial cells. Biochem Biophys Res Commun. 1993;196(1):18–24.
79. Hoang B, Ernsting MJ, Roy A, Murakami M, Undzys E, Li SD. Docetaxel-carboxymethylcellulose nanoparticles target cells via a SPARC and albumin dependent mechanism. Biomaterials. 2015;59:66–76.
80. Martino JV, Van Limbergen J, Cahill LE. The role of carrageenan and carboxymethylcellulose in the development of intestinal inflammation. Front Pediatr. 2017;5:96.
81. Iesaki T, Takeuchi T, Okano M, Hashimoto R, Kakigi R, Ishii Y, Okada T. Fucoidan, a ligand of scavenger receptor class a, causes vascular relaxation through a nitric oxide/cGMP-mediated pathway in rat aorta. Atherosclerosis. 2014;235(2):e36.
82. Thelen T, Hao Y, Medeiros AI, Curtis JL, Serezani CH, Kobzik L, Harris LH, Aronoff DM. The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages. J Immunol. 2010;185(7):4328–35.
83. Rost MS, Sumanas S. Hyaluronic acid receptor stabilin-2 regulates Erk phosphorylation and arterial-venous differentiation in zebrafish. PLoS One. 2014;9(2):e88614.
84. Marshall-Clarke S, Downes JE, Haga IR, Bowie AG, Borrow P, Pennock JL, Grencis RK, Rothwell P. Polyinosinic acid is a ligand for toll-like receptor 3. J Biol Chem. 2007;282(34):24759–66.
85. Pearson AM, Rich A, Krieger M. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. J Biol Chem. 1993;268(5):3546–54.
86. Zeng J, Zhang Y, Hao J, Sun Y, Liu S, Bernlohr DA, Sauter ER, Cleary MP, Suttles J, Li B. Stearic acid induces CD11c expression in proinflammatory macrophages via epidermal fatty acid binding protein. J Immunol. 2018;200(10):3407–19.
87. Loison C, Mendy F, Sérougne C, Lutton C. Dietary myristic acid modifies the HDL-cholesterol concentration and liver scavenger receptor BI expression in the hamster. Br J Nutr. 2002;87(3):199–210.
88. Chao Y, Karmali PP, Mukthavaram R, Kesari S, Kouznetsova VL, Tsigelny IF, Simberg D. Direct recognition of superparamagnetic nanocrystals by macrophage scavenger receptor SR-AI. ACS Nano. 2013;7(5):4289–98.
89. Suzuki Y, Tada-Oikawa S, Ichihara G, Yabata M, Izuoka K, Suzuki M, Sakai K, Ichihara S. Zinc oxide nanoparticles induce migration and adhesion of monocytes to endothelial cells and accelerate foam cell formation. Toxicol Appl Pharmacol. 2014;278(1):16–25.

90. Iyer R, Hamilton RF, Li L, Holian A. Silica-induced apoptosis mediated via scavenger receptor in human alveolar macrophages. Toxicol Appl Pharmacol. 1996;141(1):84–92.
91. Murthy S, Larson-Casey JL, Ryan AJ, He C, Kobzik L, Carter AB. Alternative activation of macrophages and pulmonary fibrosis are modulated by scavenger receptor, macrophage receptor with collagenous structure. FASEB J. 2015;29(8):3527–36.
92. Resnick D, Freedman NJ, Xu SH, Krieger M. Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos. J Biol Chem. 1993;268(5):3538–45.
93. BEPPU M, HORA M, KIKUGAWA K. A simple method for the assessment of macrophage scavenger receptor-ligand interaction: adherence of erythrocytes coated with oxidized low density lipoprotein and modified albumin to macrophages. Biol Pharm Bull. 1994;17(1):39–46.
94. Takata K, Horiuchi S, Morino Y. Scavenger receptor-mediated recognition of maleylated albumin and its relation to subsequent endocytic degradation. Biochim Biophys Acta. 1989;984(3):273–80.
95. Greaves DR, Gordon S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res. 2009;50(Supplement):S282–6.
96. Herrmann M, Schäfer C, Heiss A, Gräber S, Kinkeldey A, Büscher A, Schmitt MM, Bornemann J, Nimmerjahn F, Herrmann M, Helming L. Clearance of fetuin-A–containing calciprotein particles is mediated by scavenger receptor -A. Circ Res. 2012;111(5):575–84.
97. Melkko J, Hellevik T, Risteli L, Risteli J, Smedsrød B. Clearance of NH2-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells. J Exp Med. 1994;179(2):405–12.
98. Facciponte JG, Wang XY, Subjeck JR. Hsp110 and Grp170, members of the Hsp70 superfamily, bind to scavenger receptor-A and scavenger receptor expressed by endothelial cells -I. Eur J Immunol. 2007;37(8):2268–79.
99. Ben J, Zhang Y, Zhou R, Zhang H, Zhu X, Li X, Zhang H, Li N, Zhou X, Bai H, Yang Q. Major vault protein regulates class A scavenger receptor-mediated tumor necrosis factor-α synthesis and apoptosis in macrophages. J Biol Chem. 2013;288(27):20076–84.
100. Takahashi T, Suzuki T. Role of sulfatide in normal and pathological cells and tissues. J Lipid Res. 2012;53(8):1437–50.
101. Shannahan JH, Bai W, Brown JM. Implications of scavenger receptors in the safe development of nanotherapeutics. Recept Clin Invest. 2015;2(3):e811.
102. Basu SK, Majumdar S, Mukhopadhyay B, Mukhopadhyay A. Receptor-mediated drug delivery to macrophages. Proc Indian Natl Sci Acad Part B. 1994;60:345–56.
103. Scherphof GL, Kamps JA. Receptor versus non-receptor mediated clearance of liposomes. Adv Drug Deliv Rev. 1998;32(1–2):81–97.
104. Semple SC, Chonn A, Cullis PR. Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo. Adv Drug Deliv Rev. 1998;32(1–2):3–17.
105. Liu D, Liu F, Song YK. Recognition and clearance of liposomes containing phosphatidylserine are mediated by serum opsonin. Biochim Biophys Acta. 1995;1235(1):140–6.
106. Devine DV, Wong K, Serrano K, Chonn A, Cullis PR. Liposome complement interactions in rat serum: implications for liposome survival studies. Biochim Biophys Acta. 1994;1191(1):43–51.
107. Fidler IJ, Raz A, Fogler WE, Kirsh R, Bugelski P, Poste G. Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. Cancer Res. 1980;40(12):4460–6.
108. Chonn A, Semple SC, Cullis PR. Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. J Biol Chem. 1992;267(26):18759–65.
109. Vyas SP, Kannan ME, Jain S, Mishra V, Singh P. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Int J Pharm. 2004;269(1):37–49.
110. Kamps JA, Morselt HW, Swart PJ, Meijer DK, Scherphof GL. Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells. Proc Natl Acad Sci. 1997;94(21):11681–5.

111. Etzerodt A, Maniecki MB, Graversen JH, Møller HJ, Torchilin VP, Moestrup SK. Efficient intracellular drug-targeting of macrophages using stealth liposomes directed to the hemoglobin scavenger receptor CD163. J Control Release. 2012;160(1):72–80.
112. Li X, Kan HY, Lavrentiadou S, Krieger M, Zannis V. Reconstituted discoidal ApoE-phospholipid particles are ligands for the scavenger receptor BI The amino-terminal 1–165 domain of ApoE suffices for receptor binding. J Biol Chem. 2002;277(24):21149–57.
113. Sakai-Kato K, Sakurai M, Takechi-Haraya Y, Nanjo K, Goda Y. Involvement of scavenger receptor class B type 1 and low-density lipoprotein receptor in the internalization of liposomes into HepG2 cells. Biochim Biophys Acta. 2017;1859(11):2253–8.
114. Lee KD, Pitas RE, Papahadjopoulos D. Evidence that the scavenger receptor is not involved in the uptake of negatively charged liposomes by cells. Biochim Biophys Acta. 1992;1111(1):1–6.
115. Pinheiro M, Lúcio M, Lima JL, Reis S. Liposomes as drug delivery systems for the treatment of TB. Nanomedicine. 2011;6(8):1413–28.
116. Labana S, Pandey R, Sharma S, Khuller GK. Chemotherapeutic activity against murine tuberculosis of once weekly administered drugs (isoniazid and rifampicin) encapsulated in liposomes. Int J Antimicrob Agents. 2002;20(4):301–4.
117. Pollock S, Nichita NB, Böhmer A, Radulescu C, Dwek RA, Zitzmann N. Polyunsaturated liposomes are antiviral against hepatitis B and C viruses and HIV by decreasing cholesterol levels in infected cells. Proc Natl Acad Sci. 2010;107(40):17176–81.
118. Tempone AG, Perez D, Rath S, Vilarinho AL, Mortara RA, de Andrade Jr HF. Targeting Leishmania (L.) chagasi amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine. J Antimicrob Chemother. 2004;54(1):60–8.
119. da Costa-Silva TA, Galisteo AJ, Lindoso JA, Barbosa LR, Tempone AG. Nanoliposomal buparvaquone immunomodulates Leishmania infantum-infected macrophages and is highly effective in a murine model. Antimicrob Agents Chemother. 2017;61(4):e02297–16.
120. Pollock S, Antrobus R, Newton L, Kampa B, Rossa J, Latham S, Nichita NB, Dwek RA, Zitzmann N. Uptake and trafficking of liposomes to the endoplasmic reticulum. FASEB J. 2010;24(6):1866–78.
121. Di Y, Wasan EK, Cawthray J, Wasan KM. Scavenger receptor class BI (SR-BI) mediates uptake of CPX-351 into K562 leukemia cells. Drug Dev Ind Pharm. 2019;45(1):21–6.
122. Pont I, Calatayud-Pascual A, López-Castellano A, Albelda EP, García-España E, Martí-Bonmatí L, Frias JC, Albelda MT. Anti-angiogenic drug loaded liposomes: Nanotherapy for early atherosclerotic lesions in mice. PLoS One. 2018;13(1):e0190540.
123. Rensen PC, Gras JC, Lindfors EK, van Dijk KW, Jukema JW, van Berkel TJ, Biessen EA. Selective targeting of liposomes to macrophages using a ligand with high affinity for the macrophage scavenger receptor class A. Curr Drug Discov Technol. 2006;3(2):135–44.
124. Gan C, Wang K, Tang Q, Chen Y. Comparative investigation on the sizes and scavenger receptor binding of human native and modified lipoprotein particles with atomic force microscopy. J Nanobiotechnol. 2018;16(1):25.
125. Zhang X, Huang G. Synthetic lipoprotein as nano-material vehicle in the targeted drug delivery. Drug Deliv. 2017;24(2):16–21.
126. Shaw JM, Futch WS, Schook LB. Induction of macrophage antitumor activity by acetylated low density lipoprotein containing lipophilic muramyl tripeptide. Proc Natl Acad Sci. 1988;85(16):6112–6.
127. Kreuter J, Hekmatara T, Dreis S, Vogel T, Gelperina S, Langer K. Covalent attachment of apolipoprotein AI and apolipoprotein B-100 to albumin nanoparticles enables drug transport into the brain. J Control Release. 2007;118(1):54–8.
128. Singh RP, Ramarao P. Cellular uptake, intracellular trafficking and cytotoxicity of silver nanoparticles. Toxicol Lett. 2012;213(2):249–59.
129. Shannahan JH, Podila R, Aldossari AA, Emerson H, Powell BA, Ke PC, Rao AM, Brown JM. Formation of a protein corona on silver nanoparticles mediates cellular toxicity via scavenger receptors. Toxicol Sci. 2014;143(1):136–46.

130. Aldossari AA, Shannahan JH, Podila R, Brown JM. Scavenger receptor B1 facilitates macrophage uptake of silver nanoparticles and cellular activation. J Nanopart Res. 2015;17(7):313.
131. Lipinski MJ, Frias JC, Amirbekian V, Briley-Saebo KC, Mani V, Samber D, Abbate A, Aguinaldo JG, Massey D, Fuster V, Vetrovec GW. Macrophage-specific lipid-based nanoparticles improve mri detection and characterization of human atherosclerosis. J Am Coll Cardiol Img. 2009;2(5):637–47.
132. Hirano S, Kanno S, Furuyama A. Multi-walled carbon nanotubes injure the plasma membrane of macrophages. Toxicol Appl Pharmacol. 2008;232(2):244–51.
133. Kanno S, Furuyama A, Hirano S. A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol Sci. 2007;97(2):398–406.
134. Orr GA, Chrisler WB, Cassens KJ, Tan R, Tarasevich BJ, Markillie LM, Zangar RC, Thrall BD. Cellular recognition and trafficking of amorphous silica nanoparticles by macrophage scavenger receptor A. Nanotoxicology. 2011;5(3):296–311.
135. Hamilton R, Buckingham S, Holian A. The effect of size on Ag nanosphere toxicity in macrophage cell models and lung epithelial cell lines is dependent on particle dissolution. Int J Mol Sci. 2014;15(4):6815–30.
136. Hsu CS, Hsu SJ, Liu WL, Chen DS, Kao JH. Association of SCARB1 Gene polymorphisms with virological response in chronic hepatitis C patients receiving pegylated interferon plus ribavirin therapy. Sci Rep. 2016;6:32303.
137. SR-BI and Antiviral Treatment Response in HCV [Internet]. Available from: https://clinicaltrials.gov/show/NCT02714712. Accessed on 14th Jun 2019.
138. Genetic and Metabolism of Post-prandial HDL Particles (HDL-PP) [Internet]. Available from: https://clinicaltrials.gov/show/NCT03109067. Accessed on 14th Jun 2019.
139. Mechanisms of Chronic Kidney Disease (CKD)-Induced Foam Cell Formation [Internet]. Available from: https://clinicaltrials.gov/show/NCT01671605. Accessed on 14th Jun 2019.
140. A Study of Immunological Biomarkers as Predictors of Cardiovascular Events (BIOKID) [Internet]. Available from: https://clinicaltrials.gov/show/NCT02894060. Accessed on 14th Jun 2019.
141. Zheng Y, Liu Y, Jin H, Pan S, Qian Y, Huang C, Zeng Y, Luo Q, Zeng M, Zhang Z. Scavenger receptor B1 is a potential biomarker of human nasopharyngeal carcinoma and its growth is inhibited by HDL-mimetic nanoparticles. Theranostics. 2013;3(7):477–86.

Chapter 11
Toll-Like Receptor-Mediated Endocytosis in Infectious Disease

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Abstract Toll-like receptors (TLR) present a crucial first line of defense to attack by pathogens. In addition, the diversity and widespread localization of TLRs in the human body make them prime candidates for the development of therapeutics. TLRs are known to function by inducing the release of cytokines that result in an inflammation state. However, this release of cytokines needs to be tightly regulated in order to prevent adverse reactions such as sepsis. In this chapter, we describe the role of TLRs in pathogenesis and present various strategies that have been developed to target TLRs, including formulations and administration routes, with the help of various examples.

Keywords Toll-like receptors · Infectious diseases · Innate and adaptive immunity · Pathogenesis · Targeted drug delivery

Abbreviations

AAGPs	Aminoalkylglucosaminide 4-phosphates
AMPs	Antimicrobial peptides
AP-1	Activator protein 1

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APCs	Antigen-presenting cells
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation
CpG	Cytosine phosphate guanine
CQ	Chloroquine
CT	C-terminal
CYLD	Cylindramatosis protein
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
EBV	Ebola virus
ECD	Extracellular domain
ERK	Extracellular signal-regulated kinases
FDA	Food and Drug Administration
GNP	Gold nanoparticle
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HCQ	Hydroxychloroquine
HDL	High-density lipoproteins
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1
HSP	Heat-shock protein
I/R	Ischemia–reperfusion injury
IFN	Interferon
IKK	I Kappa B kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IROs	Immuno-regulatory oligonucleotides
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAL	MyD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MD-2	myeloid differentiation factor-2
MHC	Major histocompatibility complex
MLA	Monophosphoryl lipid
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88
NAHNP	Non-anticoagulant heparin nanoparticle
NF-kB	Nuclear factor kappa-light-chain-enhancer of B cells
NK	Natural killer
NP	Nanoparticle
NSCLC	Non-small-cell lung cancer

NT	N-terminal
ODN	oligodeoxynucleotide
OVA	Fagellin-ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PMN	Polymorphonuclear leukocytes
RA	Rheumatoid arthritis
RIP	Receptor interacting protein-1
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RSV	Rous sarcoma virus
SAR	Structure–activity relationship
siRNA	small interfering RNA
SLE	Systemic lupus erythematosus
SMI	Small molecule inhibitors
SS	Systemic sclerosis
ssRNA	Single-stranded RNA
TACAs	Tumor-associated carbohydrate antigens
TAK-1	Transforming growth factor-β-activated kinase-1
TBK-1	TRAF family member-associated NF-kappa-B activator 1
TGF-β	Transforming growth factor-β
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TMD	Transmembrane domain
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
TNF-α	Tumor necrosis factor-α
TRADD	TNF receptor type 1-associated death domain
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cells
TRIF	Toll-interferon response factor
VIPER	Viral Inhibitory peptide of TLR-4
VSV	Vesicular stomatitis virus

1 Introduction

The innate immunity, earlier considered a rudimentary segment of the immune system, played a major role in initiating immune responses when a group of receptors, known as the Toll-like receptors (TLRs), were discovered. These receptors are present on immune cells, including macrophages, dendritic cells, natural killer cells, and the T and B lymphocytes, and function by detecting specific structural patterns, known as pathogen-associated molecular patterns (PAMPs),

present only on pathogens. TLRs also sense damage-associated molecular patterns (DAMPs), which include host entities such as components of the dead or dying cells [1]. Currently, there are ten TLRs in humans (TLR 1–10) and twelve in mice (TLR 1–9 and TLR 11–13) [2]. Each TLR binds to multiple ligands, and some ligands bind to multiple TLRs, thus increasing their diversity [3]. A comprehensive list of all the TLRs and their corresponding ligands and functions is presented in Table 11.1.

Since their discovery, TLRs have been associated with preventing various diseases, primarily infections. However, it was soon observed that TLRs could behave like double-edged swords, when their involvement in the pathogenesis of Alzheimer's disease was discovered [7, 8].

This observation further propelled the search for therapeutics, including agonists and antagonists, targeting TLRs for the control of myriad diseases ranging from infections to cancer. Since TLRs are at the front line of defense, they serve as prime targets for developing effective therapeutics. Furthermore, since TLRs are present ubiquitously in the human body on immune cells, as compared to specific tissue cells in the case of other receptors, they can be used to target a wider range of diseases. TLRs thus prove to be a prime receptor candidate for drug delivery, as will be discussed in detail with examples through this chapter.

2 Pathophysiological Role of TLRs

As discussed earlier, TLRs are at the front line of the immune response and thus play a crucial role in combating infections. The role of TLRs in some critical examples of infections, such as the lung and skin as well as autoimmune disorders, is explained in the following text.

2.1 Pathogenesis in Bacterial Lung Infections

TLRs play a key role in the pathogenesis of infectious and non-infectious lung diseases. They regulate the immune responses toward *M. tuberculosis*, right from the initial host–pathogen interaction, through disease progression [9]. Activation of TLRs in response to mycobacterial ligands leads to the production of proinflammatory mediators, namely, TNF-α, IL-12, and nitric oxide, which act against mycobacterial defenses [10]. *Legionella pneumophila* that causes the legionnaires disease is recognized by TLR-2, TLR-4, TLR-5, and TLR-9, which leads to increased production of interferon-γ (IFN-γ), TNF-α, IL-12, and nitric oxide [11, 12]. In the case of *Streptococcus pneumoniae*, a leading cause of pneumonia, various TLR-2 and TLR-2/6 ligands have been reported, which result in NF-κB translocation, TNF-α, and macrophages production [13, 14].

Table 11.1 Summary of human TLRs: localization, distribution, and targets

TLR	Cellular localization	Expression on immune cells	Foreign ligand	Endogenous ligand	Source of foreign ligand
TLR-1; TLR-1/TLR-2 (dimer)	Plasma membrane	Most cells including DCs and C cells	Triacylated lipoprotein	Unknown	Bacteria, mycoplasma
TLR-2; TLR-2/TLR-6 (dimer)	Plasma membrane	PMNs, monocytes DCs, T cells, B cells, NK cells	Peptidoglycan, diacyl lipopeptide, lipoteichoic acid, zymosan, porins, lipoarabinomannan	HSP-60, -70, -96; HMGB1, urate, hyaluronan	Bacteria, viruses, parasites
TLR-3	Endolysosome	DCs, NK cells, T cells	dsRNA	mRNA	Viruses
TLR-4	Plasma membrane	Macrophages, DCs, T cells	LPS, mannan, phospholipids, viral envelope proteins (MMTV, RSV)	HSP-22, -60, -70, -96; HMGB1, fibrinogen, hyaluronan fragments, β-defensin, fibronectin, urate, heparan sulfate, surfactant protein A	Bacteria, viruses
TLR-5	Plasma membrane	DCs, monocytes, NK cells, T cells	Flagellin		Bacteria
TLR-6	Plasma membrane	B cells, DCs, monocytes, NK cells	Diacyl lipopeptide, lipoteichoic acid		Bacteria, viruses
TLR-7	Endolysosome	T cells, B cells, DCs, monocytes	ssRNA (viral)	ssRNA/protein complexes	Viruses
TLR-8	Endolysosome	DCs, monocytes, NK cells, T cells	ssRNA (viral)	ssRNA/protein complexes	Viruses
TLR-9	Endolysosome	DCs, B cells, monocytes, macrophages, NK cells, microglial cells	CpG DNA (bacterial/viral)	DNA immune complexes	Bacteria, viruses, protozoa
TLR-10	Plasma membrane	T cells, B cells, DCs, monocytes	Unknown	Unknown	Unknown

References: [4–6]

PMN polymorphonuclear leukocytes, *DC* dendritic cell, *NK* natural killer, *dsRNA* double-stranded RNA, *ssRNA* single-stranded RNA, *MMTV* mouse mammary tumor virus, *RSV* respiratory syncytial virus, *HSP* heat-shock protein, *HMGB1* high mobility group box 1

2.2 Pathogenesis in Viral Infections

Various members of the TLR family are involved in responses to viral infections [15]. The TLR-2 is specifically involved in action against a number of DNA viruses [16]. The envelope proteins of the human cytomegalovirus (HCMV) are recognized by the TLRs-1/2, which leads to the secretion of proinflammatory cytokines [4]. Also, TLRs-2/6 are important for stimulating cytokine production against the replication of the respiratory syncytial virus (RSV) and cause migration of neutrophils to the lung and activation of dendritic cells [17].

Vesicular stomatitis virus (VSV) and Ebola virus (EBV) activate the PI3 pathway via TLR-4, leading to the expression of type I IFN and proinflammatory cytokines, which in turn leads to immunopathogenesis [18]. TLRs-7 and 8 recognize ssRNA of viral genomes. Further, the TLR-9 recognizes hypomethylated CpG motifs present on the microbial DNA. These TLRs are responsible for the production of high levels of type I IFNs, for induction of type I T-helper responses and also for facilitating the B-cell class switching [5].

2.3 Pathophysiology in Skin Infections

TLRs are also expressed on the skin, which provides a major physical and cellular barrier for innate immune responses. TLR-3, in association with TLR-5, produces CCL27, which promotes the recruitment of memory T cells, specifically to the skin, as part of the adaptive immune response [6]. Antimicrobial peptides (AMPs) stimulate cytokine and chemokine responses, through the activation of TLRs. TLRs 2/4 get activated by cathelicidin and produce inflammatory cytokines [19].

2.4 Pathophysiology in Autoimmune Diseases

The role of TLRs has been elucidated in various autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [20]. The PAMPs associated with commensal flora or infectious bacteria like bacterial DNA, the peptidoglycans, and the LPSs act as TLR ligands. SLE affects multiple organs. In this condition, auto-antibodies are produced by the hyper-stimulated B cells, mainly against dsDNA or DNA/RNA bound proteins, which results in the formation of IC (DNA and anti-DNA antibody complex) [21]. This IC interacts with TLR-9, resulting in the activation of dendritic cells and consequently activating B cells, T cells, and other proinflammatory cytokines. Thus, TLR signaling activates cytokines, chemokines, co-stimulatory molecules, and orchestrates between innate and adaptive immune responses, resulting in clearance of majority of the disease-causing agents.

3 The Toll-Like Receptor

TLRs exist in homomeric and multimeric forms, as illustrated in Table 11.1, and have different cellular localizations. While TLRs -1, -2, -4, -5, -6, and -10 are located on the plasma membrane and later migrate to the phagosome, upon activation, TLRs -3, -7, -8, and -9 are only expressed on intracellular components, such as the endosome/lysosome or the endoplasmic reticulum [22, 23]. In this section, we take a deeper look into the structure and function of TLRs.

3.1 Structure of TLRs

The TLR consists of three domains, namely, (i) an extracellular domain (ECD) made up of leucine-rich repeats (LRR) that are the main recognition domains for PAMPs; (ii) a single transmembrane domain (TMD), and (iii) an intracellular Toll-interleukin-1 receptor (TIR) (Fig. 11.1) [24]. The N-terminal ectodomains (ECDs) comprise glycoproteins that consist of 550–800 amino acids. The TLR ECD contains 19–25 LRRs, which form horseshoe structures. The leucine-rich repeats (LRRs) of the TLR ECD are 22–29 residues in length, with the hydrophobic residues placed at intervals.

Overall, the ECDs have a planar structure, which is important in ligand binding and activation. The ligand binding occurs through the lateral surface of the TLR ECD, which is devoid of N-linked glycan and freely interacts with the ligand [25]. The ECDs of the ten human TLRs differ in certain parameters, namely, the number of LRRs and the level of N-linked glycosylation [26, 27]. All the TLR signaling complexes have an "m" shaped TLR dimer, which specifically interacts with the

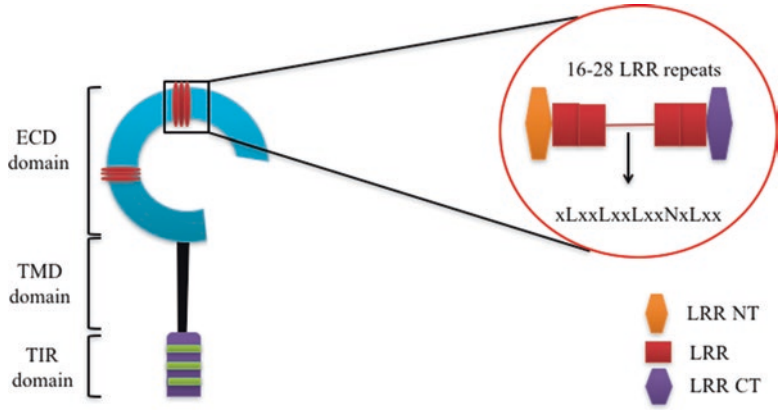

Fig. 11.1 A schematic representation of the TLR structure. The TLRs consist of three domains: (i) an extracellular domain, (ii) a single transmembrane domain, and (iii) an intracellular Toll-interleukin-1 receptor (TIR) domain

N and C termini such that the ECD-N termini diverge to the opposite end, while their interaction with the C-termini is in the middle of TLR and ligand [25].

Structural analysis of the ECDs of TLRs -1, -2, and -6 revealed that they contained three distinct subdomains, the N terminal, the central, and the C-terminal subdomains [28, 29]. There are four different types of protein–ligand interactions for TLR -1, -2, and -6 complexes, namely, the hydrophobic bonding with the TLR-2 pocket and TLR-1 channel, the hydrogen bonding of the peptide head groups, and the hydrophobic interaction with the conserved cysteines from the peptide head groups [25]. The TLR-1 and -2 interact with the triacylated lipopeptide ligand Pam_3CSK_4 which in turn enables dimerization of TIR domains on the inner side of the plasma membrane, facing the cytoplasm. The ECD of TLR-3, which is localized in endosomes and is known to recognize dsRNA, forms dimers, as it binds to the 45 bp segment of the dsRNA and this binding occurs only at pH 6.5 or lower [30].

TLR-4 recognizes the LPS along with its co-receptor MD-2 that is supported by hydrogen bonds on the lateral and concave surfaces of TLR4-ECD that regulates the LPS response and enhances it by binding to TLR-4/MD-2 complex [31, 32]. TLR-5 recognizes the bacterial flagellin. TLR-5-ECD is assumed to contain 20 LRRs and consists of a conserved D1 domain, which is exposed to and interacts with the bacterial flagellin [33]. The TLRs 7, 8, and 9 are located in the endosomes and recognize nucleic acids. The ECDs of the TLRs 7–9 contain 25 LRRs and are heavily glycosylated. The LRRs 2, 5, and 8 loop out from the dimerization surfaces of the ECDs.

The TMD of the TLRs functions to anchor them and also plays a pivotal role in oligomerization. Tight coupling of the ECD and the TIR domains with TMD is an essential requirement for signaling [34]. The dimerization of the TIR domain is a prerequisite for the TLRs to function, which in turn is initiated by TMD. The heterotypic interaction between TLR-2/6 and TLR-10/2 was due to the similarity in sequence among their TMDs, making these interactions similar to homotypic interaction [35, 36]. It was also observed that the TMDs of TLR-2, -7, and -9 contained a Small-xxx-Small motif, where “Small” residues were contemplated to be Gly, Ala, or Ser [53].

3.2 TLR Binding and Signaling

TLRs do not bind to the ligand directly. Binding is facilitated by additional receptors, called co-receptors, and some auxiliary molecules, known as the adaptor proteins. The family of cluster of differentiation (CD) molecules are common co-receptors. The TLRs also work with several adaptor proteins that mediate binding of the ligand to the TLRs and initiate subsequent downstream signaling [1]. These adaptor proteins include MyD88, toll-interferon response factor (TRIF), TIR-domain-containing adaptor protein/MyD88 adaptor-like protein (TIRAP/MAL) and TRIF-related adaptor molecule (TRAM) [37]. MyD88 is utilized by all

TLRs, except TLR-3, while TRIF is utilized only by TLR-3 and TLR-4 [1, 38]. Dimerization of receptors initiates the downstream signaling through the major adaptor proteins, MyD88 and TRIF, as the MyD88-dependent and MyD88-independent (or TRIF-dependent), respectively. Both pathways, however, culminate in the activation of transcription factors, which consequently induce the expression of genes that produce proinflammatory agents, such as type 1 IFN and inflammatory cytokines.

3.2.1 MyD88-Dependent Signaling

In the MyD88-dependent pathway, receptor dimerization triggers the recruitment of TIRAP/MAL, which acts as a sorting adaptor for the subsequent recruitment of MyD88. Most TLR dimers utilize a sorting adaptor, except TLR-5 homodimer, which directly binds MyD88. Subsequently, MyD88 triggers a sequence of downstream events that results in the activation of NF-κB pathway and the mitogen-activated protein kinase (MAPK) pathways. However, the agents involved in these events may differ between the TLRs. An example of TLR-4 signaling via the MyD88-dependent pathway is elaborately discussed in Sect. 3.2.3.

3.2.2 MyD88-Independent (or TRIF-Dependent) Signaling

The TRIF-dependent pathway is exclusively utilized by the TLR-3 and also by the TLR-4, alongside the MyD88-dependent pathway. In this pathway, the bound receptors recruit the sorting adaptor TRAM, analogous to TIRAP in the MyD88-dependent pathway, which facilitates the recruitment of TRIF subsequently resulting in a series of signaling events, which activate the IRF-3 pathway, as well as the NF-κB and the MAPK pathways [1, 39]. The TRIF-dependent signaling of TLR-4 and TLR-3 is explained in Sect. 3.2.3.

3.2.3 Examples of TLR Signaling

TLR-3 and -4 signaling is elaborated here in order to delineate distinctions between pathways. For TLR-4, which functions via both pathways, the co-receptor CD-14 assists by delivering the bacterial LPS ligand to TLR-4, which is already in complex with the accessory molecule, myeloid differentiation factor-2 (MD-2). This complex dimerizes with another TLR-4/MD-2 complex and then recruits TRIF or MyD88 for intracellular signaling [39]. Representative schematic mechanisms of the MyD88-dependent and MyD88-independent pathways are illustrated in Fig. 11.2. In the MyD88-dependent pathway, the TLR-4/MD-2 complex recruits TIRAP and MyD88, as explained in Sect. 3.2.1, leading to the formation of the complex, Myddosome (MyD88, TIRAP, and the interleukin-1 receptor-associated kinase (IRAK) family). IRAK components activate TNF receptor-associated

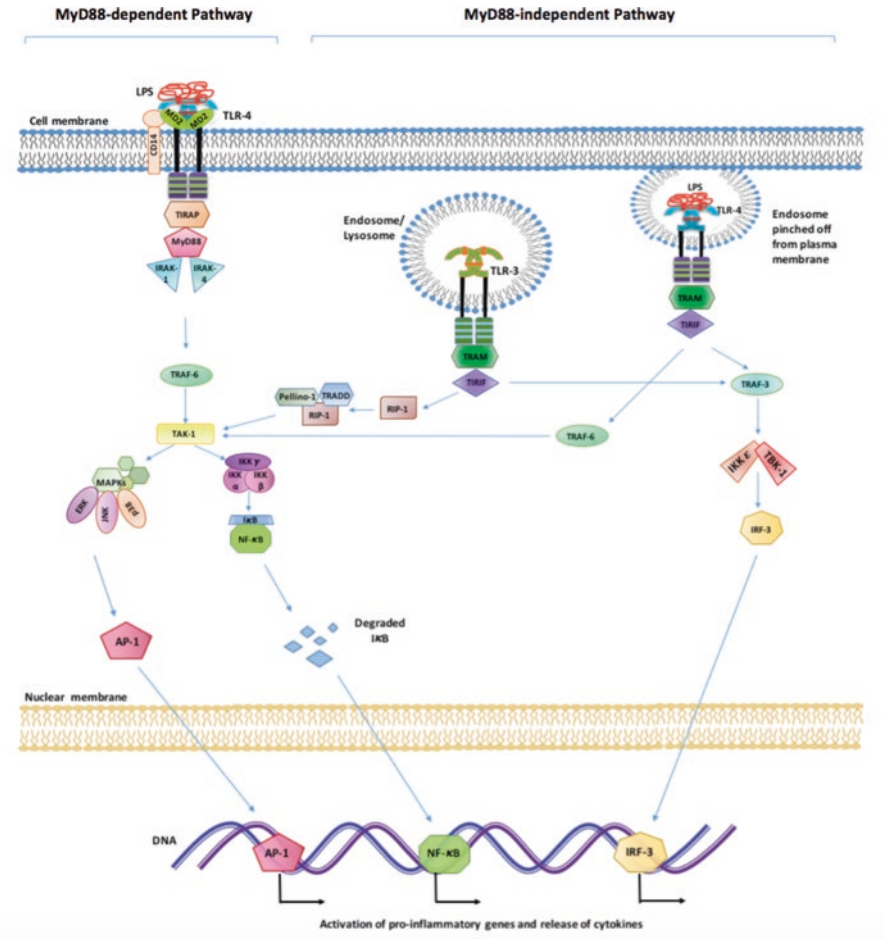

Fig. 11.2 Intracellular signaling mechanisms of TLR-4 (membrane-bound and endosomal) and TLR-3 (endosomal) via the MyD88-dependent and MyD88-independent pathways

factor-6 (TRAF-6) and transforming growth factor-β-activated kinase-1 (TAK-1) protein kinase complex, which further leads to the activation of the IKK-NF-κB pathway and the MAPK pathway. These pathways proceed through a series of downstream events culminating in the translocation of NF-κB and activator protein 1 (AP-1) into the nucleus to induce the expression of inflammatory genes [1, 39].

Alternatively, the MyD88/MD-2 complex bound to LPS is internalized and forms an endosome. The sorting adaptor TRAM is recruited, which in turn recruits TRIF. TRIF recruits either TRAF-6 or TRAF-3, which upon activation stimulates TAK-1 and NF-kB pathways (in a similar manner to that of MyD88) or recruitment and activation of TRAF family member-associated NF-kappa-B activator (TBK-1) and IKKε, respectively. This complex catalyzes the phosphorylation of IRF-3,

which moves into the nucleus and activates genes responsible for the production of interferon-β [39].

In the case of TLR-3, TRIF recruits another adaptor molecule, the receptor-interacting protein-1 (RIP-1). RIP-1 activation is accompanied by the recruitment of TNF receptor type 1-associated death domain (TRADD) and Pellino-1, an E3 ubiquitin ligase. This complex activates TAK-1, leading to the stimulation of MAPK and NF-kB pathways. TRIF also activates IRF3 and production of interferon-β. For this, TRIF recruits TRAF-3, which in turn recruits and activates TBK-1 and IKK-i. This complex activates IRF-3, which translocates to the nucleus, and activates genes responsible for the production of interferon-β [39].

3.3 Structure–Activity Relationship Between TLRs and Its Ligands

In previous sections, we have discussed the structure of TLR, their binding to various ligands and their signaling mechanisms. In the following sections, we will discuss natural and synthetic ligands targeting TLRs, as well as several classes of drugs that have been developed to regulate the activity of TLRs during disease conditions. Also, with the help of modern technologies, it is possible to enhance the effects of these drugs, thereby making better quality therapeutics. The structure–activity relationship (SAR) studies are one such approach that involves studying the effect of structural modifications of a drug on its activity. In the case of TLRs, such structural modifications aid in improving recognition or binding of the drug to TLRs, consequently upregulating or downregulating the activation of genes responsible for the release of cytokines, depending on whether the drug is an agonist or antagonist. In this section, we showcase some attempts made to improve the therapeutic effects of TLR-targeting drugs through SAR studies.

3.3.1 SAR Studies on Lipoproteins and Lipopeptides

Lipoproteins and lipopeptides induce signaling through TLR-2/TLR-1 heterodimers, as stated in Table 11.1. A study by Spohn et al. involved screening of a lipohexapeptide amide collection during combinatorial analysis. Investigations were performed on the structural elements of various lipopeptides to induce the release of interleukin 8 (IL-8), via activation of TLR-2. In the in vitro IL-8 induction assay, 19 proteinogenic amino acids in the peptide moiety of the Pam3Cys-lipopeptides that bind to TLR-2 led to an increase in activity [40]. In another study involving modifications of lipopeptides, acylation was performed at the N termini of the bacterial lipoproteins, using (S)-(2,3-bisacyloxypropyl) cysteinyl residue. These modified lipopeptides activated the heightened innate immune responses through TLR-2, without leading to toxicity in animal investigations [41].

3.3.2 SAR Studies on TLR-4 Antagonists and Agonists

Molecular modeling studies related to the synthesis of TLR-4 antagonists having a glucosamine core, linked to two phosphate esters and two linear carbon chains, indicated that antagonists containing 10, 12, and 14 carbon chains led to a stable MD-2/TLR-4 complex. They were represented as C10, C12, and C14, respectively. On the other hand, the C16 variant, having 16 carbon chains, did not form a stable complex with MD-2/TLR-4. During the MyD88-dependent pathway, the TLR-4 binds to its ligand, LPS, with MD-2 and co-receptors and recruits TIRAP and MyD88, as explained in Sect. 3.2. In this case, due to modifications in the C16 variant, it inhibited the LPS-stimulated TLR-4 signaling [42]. In an alternative study, the length of the acyl chains attached to the aminoalkyl glucosamine phosphate groups, which play a critical role in the agonist activity of TLR-4 receptors, was varied. The aminoalkyl glucosaminide phosphates, which mimicked the lipid A compounds, could be modified by varying the length of the secondary acyl chains, wherein the secondary acyl chains containing less than eight carbons were shown to have drastically reduced activity [43].

3.3.3 SAR Studies on TLR-7 and TLR-7/8 Antagonists and Agonists

TLR-7-agonistic imidazoquinolines were used as vaccine adjuvants. Minor changes in the structure of these compounds led to antagonistic effects, which were useful for managing conditions such as the immune exhaustion observed in patients with human immunodeficiency virus (HIV) infection. Modifications in the secondary amine of the C2 ethylaminomethylene side chain, in one of the compounds, were not tolerated. The retention of 4-amino groups played important roles in maintaining the activity. For example, replacement of the imidazole ring of the scaffold, with triazole or cyclic urea, led to a complete loss of activity [44].

In one study conducted, the importance of imidazo groups in imidazoquinolines was studied for their agonistic activity on TLR-7/8. Here, pyridoxal was used as an aldehyde component, which resulted in the formation of various furo[2,3-c]pyridine-based compounds, instead of imidazo(1,2-a)pyridines. The structure and activity of each compound were then assessed. Results showed that these new compounds containing furo groups were capable of activating TLR-8 via the NF-κB signaling pathway, similar to their imidazo counterparts. Further, within this library of furo[2,3-c] pyridines, a different relation was observed with respect to the activity, when the substituents at the C2 position were modified. For example, in human peripheral blood mononuclear cells (PBMCs), these compounds did not induce the release of proinflammatory cytokines, like most other agonists; however, they upregulated several genes responsible for the release of chemokines [45].

These and many other studies demonstrate that by inducing structural modifications and evaluating their impact on activity, the repertoire of TLR-targeting ligands can be largely expanded. This can aid in the development of more effective drugs and possibly even new compounds for TLR-mediated therapy.

3.4 TLR Antagonists

An uncontrolled release of proinflammatory cytokines, upon TLR activation, can lead to severe inflammatory conditions such as septic shock. Certain autoimmune conditions also involve unregulated release of cytokines, upon TLR stimulation. Thus, modulation of the activity of TLRs is crucial in order to effectively control the pathogen, while simultaneously preventing excessive inflammation. TLR antagonists decrease such inflammatory responses by inhibiting or suppressing the signaling pathways, and can thus be used to control conditions such as autoimmune disorders and inflammation. Several approaches have been explored for blocking the TLR signaling pathway [46]. In this section, we have discussed a few examples of the TLR antagonists and have briefly described their mechanisms of action.

3.4.1 TLR-2 Antagonists

Dysregulated PAMP/DAMP recognition by TLR-2 can result in the release of inflammatory cytokines, which can result in the onset of autoimmune and inflammatory diseases. TLR-2 antagonists are useful in such cases to control inflammation. An example of a TLR-2 antagonist is OPN-305, which is a humanized TLR-2 antagonistic antibody, that prevents ischemia–reperfusion (I/R) injury associated with organ transplantation [46]. In T-cell and/or monocyte-activated pathological conditions, OPN-305 acts by inhibiting the TNF-α secretion from the macrophages, by preventing the activation of TLR-2 and TLR-4 in response to the lipoteichoic acid and LPS [46].

In some cases, computer-aided screening studies have been performed to elucidate the BB loop pocket within the TLR TIR domain, which is critical for mediating certain protein–protein interactions. The computer screenings identified an antagonist, C29, and its derivative ortho-vanillin, both of which blocked the interactions of TLR-2 and MyD88 [47]. Another example of a TLR-2 antagonist is the tumor suppressor protein, cylindramatosis protein (CYLD), which inhibits TRAF-6 and TRAF-7 induction in the TLR-2 signaling pathway. Thus, it reduces TLR response such as the secretion of tumor necrosis factor superfamily member 10 (TNFSF10), which is a type of cytokine produced during inflammation. It also stabilizes IκB-α, which prevents the activation of NF-κB and hence causing subsequent downregulation of the immune responses [48].

3.4.2 TLR-3 Antagonists

Severe pathogenesis and death during viral infections can occur as a result of dysregulated TLR-3-mediated immune response. TLR-3 antagonists reduce activation of NF-kB and subsequently reduce IFN-γ production, thus help in curbing such undesired effects. Certain monoclonal antibodies have been used as TLR-3-specific

antagonists as a result of their ability to reduce NFκB activation, subsequently reducing the production of cytokines such as IL-6 and IFN-γ [125,124]. Another example of a TLR-3 antagonist is an inducible cytoplasmic protein, A20, which inhibits TNF-α-induced NF-κB activity [49] It also plays an important role in LPS-induced signaling and thus downregulates the innate immune responses. Accurate delivery to the endosomal compartment through carriers/conjugates possessing efficacy in safety can be evaluated for TLR-3 antagonists that are currently in clinical trials, such as the A20.

3.4.3 TLR-4 Antagonists

TLR-4 is primarily involved in Gram-negative bacterial infections and modulation of its activity is crucial in order to avoid excessive inflammatory responses. One such example of TLR-4 antagonist is the TLR-4 homolog, the radio-protective protein 105 (RP105), which has conserved extracellular leucine repeat. This repeat lacks a TIR domain and has 6–11 amino acids that are intra-cytoplasmic [50]. RP105 forms a complex with MD-1, which then interacts directly with the TLR-4, inhibiting its binding to the LPS [48]. Another such example is that of VIPER (derived from vaccinia virus protein A46), a novel peptide inhibitor, which inhibits TLR-4 signaling via preventing the TIR–TIR domain interactions [51].

Opioid ligands, such as morphine, serve as crucial ligands for neuroinflammatory responses. Morphine is recognized by TLR-4 and elicits a strong proinflammatory response. Blocking the TLR-4-MD2 complex using antagonists would thus aid in treating the morphine-induced proinflammation. SAR studies were used to design small molecule inhibitors of the TLR-4/MD-2 complex, which could potentiate morphine analgesia and block opiate-induced TLR-4 activation. The dextro-enantiomers of the opioid/TLR-4 antagonists like naltrexone, naloxone, and nalmetrene are thus used for the treatment of neuropathic and nociceptive pain [46].

3.4.4 TLR-5 Antagonists

Flagellin, a protein present on the bacterial flagella, is a natural agonist for TLR-5. It induces proinflammatory cytokines, like NF-kB, which leads to inflammation. Thus, treatment with synthetic antisense oligonucleotides, using complementary base pairing mechanism, was used to mediate downregulation of human TLR-5 gene expression, thereby keeping inflammation in check. Antagonists in the form of immunomodulatory compounds, targeting the TLR-5-flagellin binding interface, is an unexplored area due to the difficulty in disrupting the protein–protein interactions [52]. However, recently, a small-molecule inhibitor, TH1020, was observed to bind to the TLR-5 with high affinity and disrupted its association with the TLR-5 flagellin [53].

3.4.5 TLR -6, -7, -8, and -9 Antagonists

Inflammation in the central nervous system is regulated by the TLRs -6, -7, -8, and -9. The TLRs present on the glial cells act as a crucial target for TLRs modulation, to control the inflammation. One study reported that (+)-Morphinan compounds bind to the TLR-9 blocking activation of glial cells. Another opioid antagonist for TLR-9 receptor, COV08-0064, showed greater bioavailability and specificity for TLR-9, than (+)-Morphinan [54]. Yet another opioid, naltrexone (LDN), which acts by inhibiting the TLR-9 signaling, was explored in lower doses to treat diseases like multiple sclerosis and Crohn's disease [55].

In an alternative study, immunoregulatory oligonucleotides (IROs) were explored as TLR-7 and/or TLR-9 inhibitors. These were designed to contain 2′-O-methyl or methoxyethyl sugar modifications, which have unique inhibitory sequences for TLR-7, -8, and -9, and were developed for suppression of autoimmune diseases, as well as inflammatory diseases [46]. They blocked TLR activation through targeting the different domains of the TLRs and hence indirectly inhibited the activation of several TLRs.

Another synthetic TLR-7 antagonist, E5531, was derived from the structure of *Rhodobacter capsulatus* endotoxin, which is a first-generation lipid A antagonist. The role of this synthetic antagonist has been mentioned in Sect. 3.5.2. The 2′-O-methyl-modified RNA part of E5531 drug reduced the production of IFN-α and IL-6 in TLR-7 agonist-treated murine dendritic cells, human PBMCs and in mice as mentioned in the earlier paragraph. It was thus considered to be a potent TLR-7 antagonist [56].

3.5 Natural and Synthetic Ligands of TLRs

Pathogens and dead or dying cells from within our own body constitute natural ligands. However, ever since TLRs gained reputation in immune responses, several synthetic ligands have been developed, and many more are under development, which have been designed to specifically bind TLRs or TLR signaling molecules, in order to either elicit an inflammatory response or curb it. In the following sections, we discuss examples of natural and synthetic TLR ligands, along with their mechanisms of action.

3.5.1 Natural Ligands for TLRs

The natural ligands of TLRs are the inherently occurring extracellular or intracellular features on foreign or endogenous cells. As outlined in Table 11.1, some TLRs recognize bacteria, protozoa, and fungi through surface-borne patterns, while other TLRs identify viruses through signature sequences in their genetic material.

TLR-2 identifies diacylated and triacylated lipoproteins, present on the cell envelope of bacteria, by the acylated N-terminal cysteine residue. TLR-2 itself cannot discriminate between these two classes of lipoproteins, but is able to do so upon hetero-dimerization with TLR-6 and TLR-1, respectively. The TLR-2/TLR-6 heterodimer also detects fungi via the polysaccharide, zymosan [29]. LPS present on the surface of Gram-negative bacteria is the principal ligand for the TLR-4. Binding and signaling of LPS to TLR-4 are the most extensively studied TLR–ligand combination and have been elaborately explained in Sect. 3.2 of this chapter. In addition to LPS, TLR-4 also binds with bacterial mannans and viral envelope proteins. The TLRs also bind to a range of endogenous molecules originating from apoptotic cells mostly including extracellular matrix components, such as fibrinogen, fibronectin, hyaluronan fragments, etc., primarily by TLRs-2 and -4. Like foreign ligands, the TLRs-3, -7, -8, and -9 detect nucleic acid material from the host cells such as siRNA, mRNA, etc. [57]. Mammalian DNA and RNA form immune complexes and are highly potent self-antigens to the TLRs-7 and -9, resulting in induction of autoimmune diseases such as SLE. The TLRs-7 and -8 bind to their respective ligands, synergistically, that is, a single guanosine or its derivatives binds to one site on the TLR-7, while the ssRNA binds to the second site. Although guanosine binding is sufficient to induce receptor dimerization, the subsequent binding of ssRNA enhances the affinity for guanosine. The TLR-8 functions in a similar way, except that uridine is the preferred ligand in the first binding site. None of the two ligands, alone, can activate the TLRs-7 and -8 [58].

3.5.2 Synthetic Ligands for TLRs

Synthetic ligands that target TLRs are chemically synthesized to structurally mimic a natural ligand that is known to stimulate or block TLR signaling. Therapeutic applications of TLRs, till date, have included such synthetic TLR ligands modified to improve their activity [23]. The development of synthetic ligands stems from the need to develop a strong acquired immunity to various infections and thus these have been mainly developed for use as adjuvants in vaccines (elaborated in Sect. 3.6.1). Most synthetic ligands target the TLRs-2, -4, and -9. The monophosphoryl Lipid A (MPL™) developed by Corixa, USA, is once such adjuvant that is derived from the LPS of *Salmonella minnesota* R595 and stimulates TLR-4 signaling. Since LPS is toxic and can cause a hyper-immune reaction, LPS containing only a single phosphate group, monophosphoryl lipid A (MLA), was isolated and was found to be less toxic and pyrogenic and eventually led to the development of the MPL adjuvant, widely used in vaccines today. Various other synthetic lipid A mimetics have also been developed [59].

The TLR-9 is known to be stimulated by CpG motifs on nucleotides. One study showed that synthetic CpG oligodeoxynucleotide (ODN) agonists increased the production of hybridoma cells from memory B cells by 30–100%, as compared to 1–2% without them. CpG ODN-targeting of the TLR-9 has been utilized in vaccines via mucosal, conjunctival, and transcutaneous routes [60].

3.6 Strategies for Targeting the TLRs

Genetic studies provide convincing evidence that the TLRs make important therapeutic targets as their role in numerous diseases, ranging from infections to cancers, has been sufficiently deciphered [61]. As a result, researchers worldwide have been focusing on developing novel strategies to target the TLRs for treating various diseases. In this section, we discussed a few strategies being developed as TLR-based therapies.

3.6.1 Adjuvants in Vaccines

The TLR ligands, in association with antigens, have been used as adjuvants for the development of novel vaccines. Coupling the TLR ligands with antigens and administering them simultaneously stimulates immune responses by allowing the antigen-presenting cells (APCs) to distinguish between the complexes and the self-apoptotic bodies. This enhances the presentation of antigens by the major histocompatibility complexes (MHCs), increases the uptake of the antigen–ligand complexes by the immune cells and prevents the overuse of adjuvants [62]. Furthermore, TLR agonists are also being conjugated with cationic liposomes to produce enhanced vaccine adjuvants, which will elicit cross-presentation by MHC class I molecules to the CD8-T cells [63]. Also, the TLR ligands activate the CD8 memory cells, which presents the possibility of combining the TLR ligands with agonists, to boost the memory T-cell responses [64]. Some examples of antigen–adjuvant complexes targeting TLRs are described below.

Lipopeptides containing Pam3Cys, Pam2Cys, or lipo-amino acids are the most utilized ligands that serve as adjuvants for TLR-2. Pam3Cys and Pam2Cys are recognized by the TLR-1/2 and TLR-2/6 heterodimer complexes, respectively, and are used to induce dendritic cell maturation, T-helper immune cell responses and cytotoxic T lymphocyte-mediated immune responses [65]. They have also been combined with the tumor-associated carbohydrate antigens (TACAs), which can be employed as antitumor vaccines [66].

The monophosphoryl Lipid A (MPL™), described in Sect. 3.5.2, is the most widely used adjuvant in vaccines targeting TLRs. Further, GM3, a TACA expressed by tumor cells, chemically linked to MPL, has been observed to accelerate the production of IgM and IgG antibodies providing rapid resistance against infections like listeria and influenza [67].

Vaccines containing recombinant flagellin stimulate a strong immune response via TLR-5. For example, the recombinant flagellin–ovalbumin (OVA) fusion protein (STF2.OVA) elicits antigen-specific B- and T-cell responses [68]. This has been used for developing vaccines against the malarial species, *Plasmodium vivax* and *Plasmodium yoelii* and also vaccinia virus [69–71]. VAX102, a recombinant fusion protein that carries influenza antigen M2e and the C terminus of flagellin, was combined to form an influenza A vaccine [72].

Synthetic oligonucleotides (ODNs) having unmethylated CpG motifs (CpG ODN) function via the TLR-9-mediated dendritic cell activation, which induces antigen-specific antibody responses and T-helper cell 1 and 2 immune responses [73]. For example, the vaccine CpG 7909, marketed as Vaximune™, increases immune response by enhancing the T-helper cell-based cytokine response and the CTL responses and increases IFN-γ production [105].

These examples illustrate that TLR-based adjuvants are effective immunotherapeutic agents in vaccine development. Further, targeting TLR signaling is anticipated to boost the vaccine efficacy and will generate avenues for further research.

3.6.2 Prodrugs and Drug Complexes

A prodrug is an inactive form of a drug, which is metabolized by enzymes in the body to an active form. This approach is used to increase the bioavailability of poorly soluble drugs, and is thus most suitable for oral drugs that exhibit limited bioavailability due to first-pass metabolism. TLR-targeting prodrugs can act as agonists or antagonists, for example, isatoribine activates TLR-7 and regulates the expression of proinflammatory genes [48].

Prodrug complexes of oligonucleotide-based antagonists to TLR-7/8 and TLR-9 induce a broad range of cytokines. An example is that of imiquimod, which has the ability to activate TLR-7 and resiquimod that has an ability to activate TLR-7- and TLR-8-mediated signaling. Imiquimod enhances T-cell responses, which are found to be antigen-specific, and thus results in antibody secretion. Prodrugs of imiquimod and resiquimod, namely R-848 (Resiquimod) or R-837 (imiquimod), respectively, thus demonstrate broad-spectrum antiviral activity [74].

Thus, such prodrugs are useful antiinfective, antiviral agents, and antitumor agents, since they have better activity than their original drug counterparts [75].

3.6.3 Nanocarriers and Drug Complexes

Nanodevices such as nanoparticles act as carriers for drugs and interact with cells or tissues, where the drugs need to be transported. Nanocarriers have the advantage of better bio-distribution and sustained circulation, due to their favorable nanometric sizes [76, 77]. Such nanocarriers may carry drugs that modulate TLR signaling and thus prove to be a useful strategy for conditions such as sepsis or inflammation, where nanoparticles loaded with TLR inhibitors can dampen the intensity of TLR signaling in a controlled-release manner. Therefore, nanocarriers are a promising next-generation remedy for improved TLR targeting.

One such approach involved the fusion of a high density-like nanoparticle (HDL-like NP) consisting of a gold NP core and a modified HDL coating [78]. The HDL-like NPs inhibited the TLR-4 signaling that was triggered by various LPS sources and Gram-negative bacteria when the experiments were performed in human cell lines and exhibited higher antiinflammatory activity. In another approach, TNF-α-bound to gold nanoparticles (GNPs) were developed by CytImmune Sciences, Italy.

Such GNP-based formulations acted as potential agents for downregulating the TLR-4 expression and LPS-induced NF-kB activation. A new GNP-glycolipid formulation was developed that comprised a cationic glycolipid coat, which binds to CD14, and the TLR-MD2 pocket inhibited the LPS-induced TLR-4-MD2 activation and was thus shown to be an effective strategy for reducing inflammation [79].

3.6.4 Regulation of TLR-Induced Inflammation

Upon infection by pathogens, the TLRs elicit strong inflammatory responses to eliminate the pathogen. If the infection cannot be controlled by a local response, a systemic response is initiated, which includes the release of IL-1, TNF-α, and other cytokines. Sometimes, a dysregulated inflammatory response occurs, which may give rise to serious conditions such as autoimmunity and septic shock, and can occasionally result in death [80]. A prime example is the induction of septic shock caused by excessive release of IL-1, IL-6, and TNF-α, due to high levels of bacterial LPS in the blood [81]. Septic shock is often fatal even after treatment with antibiotics, while those who survive become immuno-compromised. Thus, although the release of proinflammatory agents is required to prevent a disease state, their prolonged release needs to be restrained in order to prevent adverse effects.

Several negative regulations of the TLR signaling pathways are already in place. For example, in addition to the proinflammatory cytokines, LPS induction also stimulates the release of antiinflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β), and activates the hypothalamus–pituitary axis, leading to the release of corticosteroids [80]. The cumulative effect of these negative regulators leads to a phenomenon known as the "LPS tolerance," where repeated or prolonged exposure to nonlethal doses of LPS results in resistance to the shock induced by a subsequent lethal dose, with reduced inflammatory response by cytokines [82]. Such tolerance is mediated by the cells of the reticuloendothelial system (RES), such as the macrophages. These cells are responsible for the initial pyrogenic response, as well as the following nonpyrogenic response.

Therefore, although TLR induction is a very crucial requirement for eliciting an immune response toward foreign invasion, the intensity of the response needs to be controlled. Though inherent negative control mechanisms exist, immunemodulators such as TLR antagonists may be required to curb the undesirable effects. Such regulation is even more critical in conditions such as autoimmune disorders, as uncontrolled TLR responses can lead to sepsis and even death.

4 Preclinical and Clinical Investigations on TLR-Targeting Compounds

The TLR agonists and antagonists exhibit immense therapeutic potential as a standalone or a combination therapy based on their ligands, as chemotherapeutics, adjuvant small molecules, and antibodies that may be administered via different routes.

Various preclinical and clinical trials are being conducted for establishing the safety and efficacy of the TLR-based therapeutics. Results from these trials have highlighted the release of TLR-activating DAMPs, by the perishing cancer cells, leading to the stimulation of anticancer immune responses. Also, several TLR agonists have been approved as therapeutic interventions by the US Food and Drug Administration (FDA) and other agencies. For example, bacillus Calmette-Guerin (BCG) has been licensed for the treatment of carcinomas of bladder [83], imiquimod has been approved for the topical treatment of actinic keratosis [84], monophosphoryllipid A (MPL) is being used against cervical carcinoma, etc. [85, 86].

The TLR agonists that are administered intravenously and which are currently in clinical trials mainly target the TLRs-7 and -9. Modification of the oligonucleotide backbone of CpG ODNs, by converting the natural phosphodiester bond to a phosphorothioate bond, stabilizes these molecules. These have been shown to prolong the survival of dendritic cells precursors, promote NK cell-mediated lysis, and induce the proliferation of B cells via TLR-9 when administered in mice [87].

A strategy to protect the ssRNA that is recognized by TLR-7 that has stabilized and increased its efficacy is through its complexation with lipoplexes or the polycationic peptide, protamine. Administration of these complexes in mice was observed to stimulate the peripheral and intra-tumoral NK cells and lead to increased IFN-α production [88]. Both of these complexes are in preclinical stages and have been replaced by another successful molecule, namely 852A, which possesses improved stability for intravenous administration [89]. 852A or (N-[4-(4-amino-2-ethyl-1H-imidazo[4,5c]quinolin-1-yl)butyl] methanesulfonamide, 3M-001) is a synthetic TLR-7 agonist that is related to imiquimod and has been administered in patients diagnosed with advanced cancers, that is, in patients with retinal cell carcinoma and melanoma during Phase I and Phase II clinical trials [90].

The most common TLR-3 agonist, the polyribosinic: polyribocytidylic acid consisting of uracil and guanosine residues (poly[I:C_{12}U]) is a synthetic dsRNA trademarked as Ampligen. This product was observed to induce T-helper-1 cells and IL-12 production; during Phase I clinical trials, it was found that the patients exhibited lower frequency of developing category C HIV infection [91].

Subcutaneous injection of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), which is used as a preventive vaccine for tuberculosis and bladder cancer, stimulated the TLR-2. BCG induced TNF-mediated apoptosis that promoted a delayed progression of bladder cancer. A combination therapy, consisting of IFN-α2b and BCG, was used in clinical studies against bladder cancer. It was found that this combination therapy was effective in many patients who possessed a high risk for disease recurrence. It was noted that approximately 55% of the patients could be rendered disease-free by treatment with the combination. Hence, larger Phase II and Phase III trials of this drug combination are currently ongoing [92].

Various small molecule inhibitors (SMI) that inhibit signal transduction of the TLRs are in preclinical and clinical trials. Chloroquine (CQ) and hydroxychloroquine (HCQ) that target the TLRs-7, 8, and 9 and are associated with autoimmune disorders, that is, arthritis and systemic lupus erythematosus (SLE) are being evaluated in animal studies. These SMIs, which are amphipathic in nature, can cross the

cell membranes because of their small size and interfere with the TLRs signaling pathways. They act as weak bases and tend to accumulate in the acidic intracellular compartments like endosomes and lysosomes, thereby altering the pH in these vesicles. This lowering of the pH leads to inhibition of the autoantigen presentation and decrease in cytokine production. In preclinical studies, administration of CQ in ischemic rats demonstrated improved cerebral ischemia symptoms and thus indicated the potential of CQ in patients with cardiovascular diseases. HCQ was also shown to suppress hypertension and aortic endothelial dysfunction in an SLE animal model. These studies suggest that the SMIs are potential drugs for treating a wide range of inflammatory and autoimmune diseases [93, 94].

NI-101 is an anti-TLR-4 antibody that has entered the clinical development phase. This antibody blocks the TLR-4 dimerization and prevents flu-like symptoms against LPS, which is a ligand for the TLR-4. NI-101 is also employed in rheumatoid arthritis patients [95].

Oligonucleotides that act as TLR antagonists are being mainly employed for inflammatory disorders. Some of the oligonucleotides that are in preclinical and clinical phases of development include IRS-954, DV-1179, and IMO-3100. These act against TLR-7/9, and prototypes like IRS-661, which is TLR-7-specific, and IRS-869, which is TLR-9-specific, have been marketed by Dynayax Technologies, California, USA, for SLE. They are found to inhibit the production of IFN-α, by human plasmacytoid predendritic cells, against the DNA or RNA of viruses and immune complexes in SLE patients [21, 96].

A special class of drug Eritoran (E5564), which is a synthetic lipid A analog of *Rhodobacter sphaeroides*, is in the clinical development stage. In Phase I clinical trials, administration of eritoran rapidly decreased IL-6 and TNF-α levels in the blood, while in Phase II clinical trials, it reduced the mortality rate of critically ill septic patients [97].

Although the TLR-based agonists and antagonists that act against specific ligands have shown great promise, they have not yet been established for administration. Combinatorial therapies may have to be developed to decelerate the immune reactions upon their administration at nonspecific sites. Moreover, their toxicity and dosing regimens are still under investigation, thus postponing their application in clinics.

5 Limitations of TLR-Mediated Therapy

There are several limitations of the TLR-based therapeutics that are mainly due to their undesirable immunological effects, their short half-life and their suboptimal delivery at the target sites. As discussed in Sect. 3.6.4., regulation of TLR induction is vital in order to avoid adverse effects, as uncontrolled stimulation can lead to severe inflammation, sepsis, and even death. It is thus necessary for the TLR-based therapeutics to effectively eliminate the pathogens, while at the same time prevent exacerbation. Thus, not all TLR-targeted drugs have been successful in clinical

trials. For example, the use of the CpG ODNs was limited to Phase I trials, as it caused serious adverse events, including loss of various types of blood cells, that is, anemia, thrombocytopenia, and neutropenia [98]. E5564, which is a TLR-4 antagonist candidate, failed in the Phase II clinical trials, as it did not reduce the mortality rates of patients [99].

In another study, an intracranially administered CpG-based agonist of TLR-9 developed for patients with recurrent glioblastoma was withdrawn, as it was observed that patients suffered from severe neurological conditions and fever. Furthermore, another drug candidate, CpG 7909, employed against non-small-cell lung cancer (NSCLC), was also withdrawn, as it was observed that almost all the patients developed severe hematologic toxicity [100].

A potential TLR-7 targeting drug, 852A, thought to be a more potent activator of TLR-7 than imiquimod, was employed for the treatment of advanced cancers like the renal cell carcinoma and melanoma in Phase I and Phase II clinical trials. This candidate, although possessed highly desirable formulation properties and lower metabolism than imiquimod, displayed adverse effects in Phase I clinical trials, such as fever and fatigue.

Another major limitation is the routes of administration of these newer drugs and subsequently their nonspecific targeting. As intravenous administration delivers the drugs throughout the body, they also often affect the healthy tissues. Orally administered TLR agonists have not achieved clinical success due to their low bioavailability and hence, lower therapeutic efficacy. For example, studies conducted with oral imiquimod formulations revealed a plethora of adverse reactions like vomiting, hepatic and renal impairment, fatigue, lymphopenia, and fever. In order to combat these side effects, two derivatives were developed, that is, ANA975 and 852A. However, trials for both ANA975 and 852A were discontinued, as they were unable to induce sufficient cytokine production and consequently displayed inadequate therapeutic indices [100].

Therefore, the future success of the TLR-based therapeutics depends on appropriate optimization of the dosage and dosing regime, SAR studies for the formulations of more effective drug derivatives, and trials with newer formulation strategies (e.g., lipid encapsulation and with homing peptides) and delivery routes.

6 Conclusion

Owing to their importance in disease progression and prevention, targeting the TLRs has become a pioneering therapeutic strategy for a range of diseases, including infections, cancers, and autoimmune disorders. Several agonists and antagonists have been developed to target TLRs, and are being evaluated through various formulations such as vaccines, nanocarriers, and prodrugs. Although only a few candidates have been approved for therapy, many more are still in the preclinical and clinical pipeline. As discussed in the later sections of this chapter, the regulation of TLR-mediated signaling is of great importance. While SAR studies can help in

developing better activators, merely focusing on improving the potency of a TLR inducer is unwise, as higher activities are usually accompanied by the uncontrolled release of proinflammatory cytokines, which can result in adverse reactions. Another area that demands more focus is the search for more appropriate administration routes. Alternative routes to oral and intravenous, such as mucosal delivery, need more attention. Finally, the dosing regimen of such drugs should also be carefully designed in order to avoid toxicity.

In conclusion, although TLRs prove to be highly promising targets for a wide range of diseases, the strategies adopted to target TLRs need significant optimization. Investment of additional efforts to address the abovementioned limitations will help in rendering the TLR-based therapeutics as the mainstay in the treatment of a large range of diseases.

References

1. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461.
2. Gay NJ, Gangloff M. Structure and function of Toll receptors and their ligands. Annu Rev Biochem. 2007;76:141–65.
3. Kitchens RL. Role of CD14 in cellular recognition of bacterial lipopolysaccharides. CD14 in the inflammatory response: Karger Publishers; 2000. p. 61–82. Basel
4. Juckem LK, Boehme KW, Feire AL, Compton T. Differential initiation of innate immune responses induced by human cytomegalovirus entry into fibroblast cells. J Immunol. 2008;180(7):4965–77.
5. Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. Adv Drug Deliv Rev. 2008;60(7):813–23.
6. Krutzik SR, Ochoa MT, Sieling PA, Uematsu S, Ng YW, Legaspi A, et al. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat Med. 2003;9(5):525.
7. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol. 2005;5(6):446.
8. Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. Int Immunopharmacol. 2007;7(10):1271–85.
9. Krutzik SR, Modlin RL, editors. The role of Toll-like receptors in combating mycobacteria. Seminars in immunology. Elsevier; 2004. Amsterdam
10. Underhill DM, Ozinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci. 1999;96(25):14459–63.
11. Akamine M, Higa F, Arakaki N, Kawakami K, Takeda K, Akira S, et al. Differential roles of Toll-like receptors 2 and 4 in in vitro responses of macrophages to Legionella pneumophila. Infect Immun. 2005;73(1):352–61.
12. Kikuchi T, Kobayashi T, Gomi K, Suzuki T, Tokue Y, Watanabe A, et al. Dendritic cells pulsed with live and dead Legionella pneumophila elicit distinct immune responses. J Immunol. 2004;172(3):1727–34.
13. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, et al. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun. 2004;72(2):788–94.
14. Knapp S, Wieland CW, van't Veer C, Takeuchi O, Akira S, Florquin S, et al. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. J Immunol. 2004;172(5):3132–8.

15. Carty M, Bowie AG. Recent insights into the role of Toll-like receptors in viral infection. Clin Exp Immunol. 2010;161(3):397–406.
16. Sørensen LN, Reinert LS, Malmgaard L, Bartholdy C, Thomsen AR, Paludan SR. TLR2 and TLR9 synergistically control herpes simplex virus infection in the brain. J Immunol. 2008;181(12):8604–12.
17. Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, Tripp RA, et al. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J Virol. 2009;83(3):1492–500.
18. Schabbauer G, Luyendyk J, Crozat K, Jiang Z, Mackman N, Bahram S, et al. TLR4/CD14-mediated PI3K activation is an essential component of interferon-dependent VSV resistance in macrophages. Mol Immunol. 2008;45(10):2790–6.
19. Yang D, Chertov O, Oppenheim JJ. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). J Leukoc Biol. 2001;69(5):691–7.
20. Duffy L, O'Reilly SC. Toll-like receptors in the pathogenesis of autoimmune diseases: recent and emerging translational developments. Immunotargets Ther. 2016;5:69.
21. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, et al. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med. 2005;202(8):1131–9.
22. Beutler BA. TLRs and innate immunity. Blood. 2009;113(7):1399–407.
23. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med. 2007;13(5):552.
24. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1(2):135.
25. Botos I, Segal DM, Davies DR. The structural biology of Toll-like receptors. Structure. 2011;19(4):447–59.
26. Matsushima N, Tanaka T, Enkhbayar P, Mikami T, Taga M, Yamada K, et al. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC Genomics. 2007;8(1):124.
27. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, et al. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci. 2005;102(27):9577–82.
28. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik S-G, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007;130(6):1071–82.
29. Kang JY, Nan X, Jin MS, Youn S-J, Ryu YH, Mah S, et al. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity. 2009;31(6):873–84.
30. Leonard JN, Ghirlando R, Askins J, Bell JK, Margulies DH, Davies DR, et al. The TLR3 signaling complex forms by cooperative receptor dimerization. Proc Natl Acad Sci. 2008;105(1):258–63.
31. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol. 2003;3(2):169.
32. Kim HM, Park BS, Kim J-I, Kim SE, Lee J, Oh SC, et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell. 2007;130(5):906–17.
33. Donnelly MA, Steiner TS. Two nonadjacent regions in enteroaggregative Escherichia coli flagellin are required for activation of toll-like receptor 5. J Biol Chem. 2002;277:40456.
34. Treeby M, Vašl J, Ota P, Friedrich J, Jerala R. Different functional role of domain boundaries of Toll-like receptor 4. Biochem Biophys Res Commun. 2009;381(1):65–9.
35. Kruithof EK, Satta N, Liu JW, Dunoyer-Geindre S, Fish RJ. Gene conversion limits divergence of mammalian TLR1 and TLR6. BMC Evol Biol. 2007;7(1):148.
36. Kang JY, Lee J-O. Structural biology of the Toll-like receptor family. Annu Rev Biochem. 2011;80:917–41.
37. Jiménez-Dalmaroni MJ, Gerswhin ME, Adamopoulos IE. The critical role of toll-like receptors—from microbial recognition to autoimmunity: a comprehensive review. Autoimmun Rev. 2016;15(1):1–8.
38. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell. 2006;125(5):943–55.

39. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373.
40. Spohn R, Buwitt-Beckmann U, Brock R, Jung G, Ulmer AJ, Wiesmüller K-H. Synthetic lipopeptide adjuvants and Toll-like receptor 2—structure–activity relationships. Vaccine. 2004;22(19):2494–9.
41. Wu W, Li R, Malladi SS, Warshakoon HJ, Kimbrell MR, Amolins MW, et al. Structure–activity relationships in toll-like receptor-2 agonistic diacylthioglycerol lipopeptides. J Med Chem. 2010;53(8):3198–213.
42. Facchini FA, Zaffaroni L, Minotti A, Rapisarda S, Calabrese V, Forcella M, et al. Structure–activity relationship in monosaccharide-based toll-like receptor 4 (TLR4) antagonists. J Med Chem. 2018;61(7):2895–909.
43. Stöver AG, Correia JDS, Evans JT, Cluff CW, Elliott MW, Jeffery EW, et al. Structure-activity relationship of synthetic toll-like receptor 4 agonists. J Biol Chem. 2004;279(6):4440–9.
44. Shukla NM, Mutz CA, Malladi SS, Warshakoon HJ, Balakrishna R, David SA. Toll-like receptor (TLR)-7 and-8 modulatory activities of dimeric imidazoquinolines. J Med Chem. 2012;55(3):1106–16.
45. Salunke DB, Yoo E, Shukla NM, Balakrishna R, Malladi SS, Serafin KJ, et al. Structure–activity relationships in human Toll-like receptor 8-active 2, 3-diamino-furo [2, 3-c] pyridines. J Med Chem. 2012;55(18):8137–51.
46. Patra MC, Choi S. Recent progress in the development of Toll-like receptor (TLR) antagonists. Expert Opin Ther Pat. 2016;26(6):719–30.
47. Mistry P, Laird MH, Schwarz RS, Greene S, Dyson T, Snyder GA, et al. Inhibition of TLR2 signaling by small molecule inhibitors targeting a pocket within the TLR2 TIR domain. Proc Natl Acad Sci. 2015;112:5455. 201422576.
48. Krishnan J, Lee G, Choi S. Drugs targeting Toll-like receptors. Arch Pharm Res. 2009;32(11):1485.
49. Opipari A, Boguski M, Dixit V. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. J Biol Chem. 1990;265(25):14705–8.
50. Divanovic S, Trompette A, Atabani SF, Madan R, Golenbock DT, Visintin A, et al. Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. Nat Immunol. 2005;6(6):571.
51. Lamphier M, Zheng W, Latz E, Spyvee M, Hansen H, Rose J, et al. Novel small molecule inhibitors of TLR7 and TLR9: mechanism of action and efficacy in vivo. Mol Pharmacol. 2014;85(3):429–40. https://doi.org/10.1124/mol.113.089821.
52. Thompson AD, Dugan A, Gestwicki JE, Mapp AK. Fine-tuning multiprotein complexes using small molecules. ACS Chem Biol. 2012;7(8):1311–20.
53. Yang H, Kozicky L, Saferali A, Fung S-Y, Afacan N, Cai B, et al. Endosomal pH modulation by peptide-gold nanoparticle hybrids enables potent anti-inflammatory activity in phagocytic immune cells. Biomaterials. 2016;111:90–102.
54. Hoque R, Farooq A, Malik A, Trawick BN, Berberich DW, McClurg JP, et al. A novel small-molecule enantiomeric analogue of traditional (−)-morphinans has specific TLR9 antagonist properties and reduces sterile inflammation-induced organ damage. J Immunol. 2013:1202184.
55. Karlsson L, Sun S, Rao NL, Venable J, Thurmond R. TLR7/9 antagonists as therapeutics for immune-mediated inflammatory disorders. Inflamm Allergy Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy). 2007;6(4):223–35.
56. Patel MC, Shirey KA, Pletneva LM, Boukhvalova MS, Garzino-Demo A, Vogel SN, et al. Novel drugs targeting Toll-like receptors for antiviral therapy. Futur Virol. 2014;9(9):811–29.
57. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. J Cell Mol Med. 2010;14(11):2592–603.
58. Majer O, Liu B, Barton GM. Nucleic acid-sensing TLRs: trafficking and regulation. Curr Opin Immunol. 2017;44:26–33.
59. Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR. Enhancement of antigen-specific immunity via the TLR4 ligands MPL™ adjuvant and Ribi. 529. Expert Rev Vaccines. 2003;2(2):219–29.

60. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov. 2006;5(6):471.
61. Hennessy EJ, Parker AE, O'neill LA. Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov. 2010;9(4):293.
62. Lahiri A, Das P, Chakravortty D. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine. 2008;26(52):6777–83.
63. Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol. 2006;176(12):7335–45.
64. Wille-Reece U, Flynn BJ, Loré K, Koup RA, Miles AP, Saul A, et al. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. J Exp Med. 2006;203(5):1249–58.
65. Metzger JW, Beck-Sickinger AG, Loeit M, Eckert M, Bessler WG, Jung G. Synthetic S-(2, 3-dihydroxypropyl)-cysteinyl peptides derived from the N-terminus of the cytochrome subunit of the photoreaction centre of Rhodopseudomonas viridis enhance murine splenocyte proliferation. J Pept Sci. 1995;1(3):184–90.
66. Hewitt MC, Seeberger PH. Solution and solid-support synthesis of a potential leishmaniasis carbohydrate vaccine. J Org Chem. 2001;66(12):4233–43.
67. Cluff CW, Baldridge JR, Stöver AG, Evans JT, Johnson DA, Lacy MJ, et al. Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. Infect Immun. 2005;73(5):3044–52.
68. Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine. 2007;25(4):763–75.
69. Bargieri DY, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espíndola NM, et al. New malaria vaccine candidates based on the Plasmodium vivax Merozoite Surface Protein-1 and the TLR-5 agonist Salmonella Typhimurium FliC flagellin. Vaccine. 2008;26(48):6132–42.
70. Braga CJ, Massis LM, Sbrogio-Almeida ME, Alencar BC, Bargieri DY, Boscardin SB, et al. CD8+ T cell adjuvant effects of Salmonella FliCd flagellin in live vaccine vectors or as purified protein. Vaccine. 2010;28(5):1373–82.
71. Delaney KN, Phipps JP, Johnson JB, Mizel SB. A recombinant flagellin-poxvirus fusion protein vaccine elicits complement-dependent protection against respiratory challenge with vaccinia virus in mice. Viral Immunol. 2010;23(2):201–10.
72. Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008;26(2):201–14.
73. Heit A, Schmitz F, Haas T, Busch DH, Wagner H. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. Eur J Immunol. 2007;37(8):2063–74.
74. Dowling JK, Mansell A. Toll-like receptors: the swiss army knife of immunity and vaccine development. Clin Transl Immunology. 2016;5(5):e85.
75. Averett DR. Administration of TLR7 ligands and prodrugs thereof for treatment of infection by hepatitis C virus. Google Patents; 2009.
76. Li F, Shen A, Amanullah A. Cell culture processes in monoclonal antibody production. Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing. 2010:1–38.
77. Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat Biotechnol. 2015;33(9):941.
78. Foit L, Thaxton CS. Synthetic high-density lipoprotein-like nanoparticles potently inhibit cell signaling and production of inflammatory mediators induced by lipopolysaccharide binding Toll-like receptor 4. Biomaterials. 2016;100:67–75.
79. Rodriguez Lavado J, Sestito SE, Cighetti R, Aguilar Moncayo EM, Oblak A, Lainšček D, et al. Trehalose-and glucose-derived glycoamphiphiles: small-molecule and nanoparticle Toll-like receptor 4 (TLR4) modulators. J Med Chem. 2014;57(21):9105–23.
80. Foster SL, Medzhitov R. Gene-specific control of the TLR-induced inflammatory response. Clin Immunol. 2009;130(1):7–15.

81. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003;348(2):138–50.
82. Beeson PB. Tolerance to bacterial pyrogens: II. Role of the reticulo-endothelial system. J Exp Med. 1947;86(1):39.
83. Hoffman ES, Smith RE, Renaud RC Jr. TLR-targeted therapeutics: Nature Publishing Group; 2005. London
84. Huang S-W, Kao J-K, Wu C-Y, Wang S-T, Lee H-C, Liang S-M, et al. Targeting aerobic glycolysis and HIF-1α expression enhance imiquimod-induced apoptosis in cancer cells. Oncotarget. 2014;5(5):1363.
85. Lehtinen M, Paavonen J. Sound efficacy of prophylactic HPV vaccination: basics and implications. Oncoimmunology. 2012;1(6):995–6.
86. Iribarren K, Bloy N, Buqué A, Cremer I, Eggermont A, Fridman WH, et al. Trial Watch: Immunostimulation with Toll-like receptor agonists in cancer therapy. Oncoimmunology. 2016;5(3):e1088631.
87. Sester DP, Naik S, Beasley SJ, Hume DA, Stacey KJ. Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. J Immunol. 2000;165(8):4165–73.
88. Bourquin C, Schmidt L, Lanz A-L, Storch B, Wurzenberger C, Anz D, et al. Immunostimulatory RNA oligonucleotides induce an effective antitumoral NK cell response through the TLR7. J Immunol. 2009;183(10):6078–86.
89. Harrison LI, Astry C, Kumar S, Yunis C. Pharmacokinetics of 852A, an imidazoquinoline Toll-like receptor 7-specific agonist, following intravenous, subcutaneous, and oral administrations in humans. J Clin Pharmacol. 2007;47(8):962–9.
90. Dudek AZ, Yunis C, Harrison LI, Kumar S, Hawkinson R, Cooley S, et al. First in human phase I trial of 852A, a novel systemic toll-like receptor 7 agonist, to activate innate immune responses in patients with advanced cancer. Clin Cancer Res. 2007;13(23):7119–25.
91. Thompson K, Strayer D, Salvato P, Thompson C, Klimas N, Molavi A, et al. Results of a double-blind placebo-controlled study of the double-stranded RNA drug polyI: polyC12U in the treatment of HIV infection. Eur J Clin Microbiol Infect Dis. 1996;15(7):580–7.
92. O'donnell MA, Krohn J, DeWOLF WC. Salvage intravesical therapy with interferon-α2B plus low dose bacillus Calmette-Guerin is effective in patients with superficial bladder cancer in whom bacillus Calmette-Guerin alone previously failed. J Urol. 2001;166(4):1300–5.
93. Cui G, Ye X, Zuo T, Zhao H, Zhao Q, Chen W, et al. Chloroquine pretreatment inhibits toll-like receptor 3 signaling after stroke. Neurosci Lett. 2013;548:101–4.
94. Gómez-Guzmán M, Jiménez R, Romero M, Sánchez M, Zarzuelo MJ, Gómez-Morales M, et al. Chronic hydroxychloroquine improves endothelial dysfunction and protects kidney in a mouse model of systemic lupus erythematosus. Hypertension. 2014;64(2):330–7.
95. Hatterer E, Shang L, Simonet P, Herren S, Daubeuf B, Teixeira S, et al. A specific anti-citrullinated protein antibody profile identifies a group of rheumatoid arthritis patients with a toll-like receptor 4-mediated disease. Arthritis Res Ther. 2016;18(1):224.
96. Zhu F, Yu D, Kandimalla E, La Monica N, Agrawal S, editors. Treatment with IMO-3100, a novel TLR7 and TLR9 dual antagonist, inhibits disease development in lupus prone NZBW/F1 mice. Keystone Symposia: Dendritic Cells and the Initiation of Adaptive Immunity; 2011.
97. Lynn M, Wong YN, Wheeler JL, Kao RJ, Perdomo CA, Noveck R, et al. Extended in vivo pharmacodynamic activity of E5564 in normal volunteers with experimental endotoxemia. J Pharmacol Exp Ther. 2004;308(1):175–81.
98. Readett DR, Denis LJ, Krieg AM, Benner RJ, Hanson DC. PD3-1-6: PF-3512676 (CPG 7909), a toll-like receptor 9 agonist-status of development for non-small cell lung cancer (NSCLC). J Thorac Oncol. 2007;2(8):S461.
99. Gao W, Xiong Y, Li Q, Yang H. Inhibition of toll-like receptor signaling as a promising therapy for inflammatory diseases: a journey from molecular to nano therapeutics. Front Physiol. 2017;8:508.
100. Engel AL, Holt GE, Lu H. The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system. Expert Rev Clin Pharmacol. 2011;4(2):275–89.

Part IV
Receptor Mediated Endocytosis in Cancer and Infectious Diseases

Chapter 12
Asialoglycoprotein Receptor and Targeting Strategies

Saugandha Das, Pawan Kudale, Prajakta Dandekar, and Padma V. Devarajan

Abstract The asialoglycoprotein receptor is a calcium-dependent, carbohydrate-specific, type C lectin, which is majorly present in the plasma membrane of liver cells (hepatocytes). The high liver specificity, efficient internalization rate, and ready accessibility to the vasculature present great potential of this receptor for use in site-specific targeting of nano-delivery systems for diagnostic and therapeutic applications in liver diseases with negligible nontarget toxicity. This chapter provides a detailed discussion on the receptor expression, structure, binding, ligands, and asialoglycoprotein receptor-mediated hepatocyte-targeted delivery systems. It also discusses the clinical relevance of the receptor in numerous liver afflictions along with its application in liver diagnostics.

Keywords Asialoglycoprotein · ASGP-R · Hepatocyte · Receptor-mediated targeting · Nano-delivery system

Abbreviations

5-FU	5-Fluorouracil
AA	Antibiotin antibody
AF	Asialofetuin
AG	Arabinogalactan
AI-CAH	Autoimmune-type chronic active hepatitis

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AIH	Autoimmune hepatitis
ApoE	Apolipoprotein E
ASGP-R	Asialoglycoprotein receptor
asODN	Antisense oligonucleotides
ASOR	Asialoorosomucoid AuNPs@PDA Gold nanoparticles@ Polydopamine
BDICA	Triiodobenzene compound
BNLCL2	Mouse embryonic liver cells
BSA	Bovine serum albumin
C4-Chol	Cholesten-5-yloxy-N-(4-((1-imino-2-d-thiogalactosylethyl)amino) butyl)formamide
CDE	Cyclodextrin conjugated PAMAM dendrimer
CMC	Carboxymethyl chitosan
CMD	Carboxymethyl-dextran
CPDC	Cationic polycarbonate diblock copolymer
CRD	Carbohydrate recognition domain
CTG	Cholesterylated thiogalactoside
DDMAP	Dimyristoyl diacyltrimethylammonium propane
DOX	Doxorubicin
DPEA	Dioleoylphosphatidyl ethanolamine
DTPA	Diethylenetriamine pentaacetic acid
EGFR-ERK	Epidermal growth factor receptor-extracellular signal regulated kinases
Gal	Galactose
GalNAc	N-acetylgalactosamine
GDPA	Gadolinium diethylenetriamine pentaacetic acid
GO	Graphene oxide
H-bond	Hydrogen bond
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HepG2	Hepatocellular carcinoma cell line
HSA	Human serum albumin
IgA	Immunoglobulins A
IL	Interleukin
KC	Kupffer cell
LA	Lactobionic acid
Lac	Lactosaminated
LDL	Low-density lipoproteins
LSP	Liver-specific protein
MHV	Mouse hepatitis virus
MRI	Magnetic resonance imaging
N-HPMA	N-(2hydroxypropyl) methacrylamide
N-LDLPTA	N-lactosyl-dioleoylphosphatidylethanolamine
NPs	Nanoparticles

p(VLA-co-VNI-co-V_2DTPA)	Poly(vinylbenzyl-O-b-D-galactopyranosyl-D-gluconamide)-co-N-p-vinylbenzyl-6-(2-(4-dimethylamino)benzaldehydehydrazono) nicotinate-co-5,8-bis(carboxymethyl)-3-oxo-11-(2-oxo-2-((4-vinylbenzyl)amino)ethyl)-1-(4-vinylphenzyl)-2,5,8,11-tetraazatridecan-13-oic acid
PAA	Poly(amidoamine)
PAE/PLGA	Poly(β-amino ester)/poly(lactic-co-glycolic acid)
PEC	Poly(ε-caprolactone)
PEG	Polyethylene glycol
PEG-PEC	Poly(ethylene glycol)-poly(ε-caprolactone)
PEI	Polyethylenimine
PGA	Poly-γ-glutamic acid
PGEA	Poly(glycidyl-ethanolamine)
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
PLL	Poly-L-lysine
PNIPAm	Poly (N-isopropylacrylamide)
PTX	Paclitaxel
RBV	Ribavarin
siRNA	Small interfering RNA
SPECT	Single-photon emission computed tomography
TAB	Tetraacetylbromo
TNF	Tumor necrosis factor
TPGS	Tocopherol polyethylene glycol succinate

1 Introduction

Asialoglycoprotein receptor (ASGP-R) is a type C, carbohydrate-binding lectin, which mediates clearance of various defective plasma glycoprotein molecules from the body. These receptors also identify and bind to pathogen membrane glycoproteins to efficiently clear them from circulation by receptor-mediated endocytosis and play a pivotal role in preventing hepatocyte infections, inflammation, and cancer [1]. The exclusive expression and high density of ASGP-R on the hepatocytes (1.35×10^8/g of liver) [2] with negligible extrahepatic presence suggest the receptor as ideal for targeted delivery with minimum concerns of nontarget toxicity. However, the success of designing targeted therapy relies on a detailed understanding of the receptor and its physiology. Thus, this chapter initially provides an understanding of the receptor structure and biology, its function and expression in normal state and diseased state, and receptor–ligand interactions, which can improve liver-targeting strategies. It further discusses the targeted drug delivery and diagnostic approaches utilized in the therapy of infectious diseases and cancer in preclinical and clinical stages.

2 ASGP-R Receptor Physiology

Cell surface carbohydrate lectins (protein that binds oligosaccharide chains) are known to orchestrate a variety of cellular and intercellular functions by specific recognition and binding to various receptors [3]. Of these, the first mammalian cell membrane-bound lectin receptor discovered was ASGP-R or "Ashwell–Morell receptor" which was identified in 1965 by Gilbert Ashwell and Anatol Morell [4]. During one of their studies on metabolism of a serum glycoprotein (ceruloplasmin), they found that modification of N-linked oligosaccharides by deleting the terminal sialic acid residues initiated their rapid clearance from circulation [3]. Today, after years of research, the role of ASGP-R has been extensively evaluated for its implications in various types of liver infections and cancers [3, 5].

2.1 Location

ASGP-R is dominant in the liver with abundant expression on sinusoidal, basolateral membranes of mammalian parenchymal hepatocytes [6] with an approximate density of 100,000–500,000 receptors per hepatocyte [5, 7]. In vitro, various cell lines have been reported to express ASGP-R at varying density per hepatocyte, for example, human hepatoma cell line HepG2 (76,000), HepAD38 (17,000), HuH-7, HuH-6, hepatoma subclones, BNLCL2, and HuH-5-2 (~3000) [8]. Inside the hepatic cell, ASGP-R is generally associated with endocytic organelles and Golgi [9]. Minimal expression of ASGP-R is seen in extrahepatic locations, such as peritoneal macrophages, peripheral blood monocytes, dendritic cells, thyroid, intestinal epithelial cells, testes, primary renal proximal tubular epithelial cells [1], and on some extrahepatic origin tumor cells [10]. However, lower binding affinity to the extrahepatic ASGP-R makes it highly specific for hepatocyte targeting.

2.2 Function

The primary physiological function of ASGP-R is specific binding, endocytosis, and clearance of a wide range of circulating desialylated serum glycoproteins [4] by receptor-mediated endocytosis. Altered or reduced expression of this receptor results in elevated plasma levels of these glycoproteins. ASGP-R also plays an immunomodulatory role and promotes binding, cellular uptake, and clearance of major plasma proteins like transferrin, enzymes like alkaline phosphatase, immunoglobulins (IgA), prothrombotic components, activated lymphocytes, fibronectin and platelets, hepatic low-density lipoproteins, chylomicron remnants, and apoptotic cells [6]. Thus, sialic acid removal facilitates identification and clearance of various kinds of defective molecules from the blood by the liver [11]. The specific

localization and high density of ASGP-R on the liver cells and subsequent recognition and interactions with cellular components of pathogens play a pivotal role in preventing hepatocyte infections, inflammation, and cancer [1]. Thus, adequate function and density of the ASGP-R can protect hepatocytes against various disease-causing pathogens and toxins.

2.3 Pathophysiological Role of ASGP-R in Liver Diseases

Hepatotropic viruses can enter the hepatocytes through indirect virus/receptor interaction with IgA [12] or fibronectin [13], which is trapped by ASGP-R and triggers complement activation and uptake. Some enveloped viruses like hepatitis A [12], B [14, 15], C [16] and Marburg virus [17] have been reported to work as "opportunistic endocytic ligands," which bind to the ASGP-R proteins and undergo endocytic uptake. Inside the endosome, specific viral membrane proteins integrate into the host genome and facilitate replication and infection of the host leading to chronic viremia and liver inflammation [14]. *S. pneumonia* pathogen has been found to induce desialylation and ASGP-R-mediated clearance of platelets and coagulation factors from circulation to establish lethal thrombocytopenia seen in sepsis [18]. ASGP-R has also been studied for its potential involvement in tumor progression where it was shown to promote cancer metastasis by activating the EGFR–ERK pathway [19].

2.4 Immunological Role of ASGP-R in Liver Disease

Inflammation of liver is associated with immune cell activation, cytokine release, and induction of specific immune responses [11]. Cytokines have been reported to modulate expression, synthesis, and function of ASGP-R [6], where inflammatory cytokines (IL-1, IL-6, and TNF) demonstrated augmented synthesis of the receptor [20]. ASGP-R is also recognized to act as a self-antigen in the presence of inflammatory diseases, liver injury, or toxins. Poralla et al. [21] were the first to identify ASGP-R of human hepatocellular surface as a target for humoral and cell-mediated autoimmune reactions in autoimmune-type chronic active hepatitis (AI-CAH). Seminal studies by McFarlane et al. [22] identified ASGP-R as the major component of liver-specific protein (LSP) with antigenic epitopes, which was known to induce autoimmune hepatitis (AIH). Several investigators reported generation of anti-ASGP-R antibodies in patients with chronic liver diseases [11]. Due to disease-specific variability in expression level of this receptor, researchers recommend ASGPR-based targeting strategies for disease management to be clubbed with ASGP-R level diagnosis to improve therapy [23]. Since anti-ASGP-R antibody levels correlate with ASGP-R density in liver disease [24], these antibodies provide clinical prediction of relapse of autoimmune disease [6].

2.5 ASGP-R Expression in Liver Disease

Since ASGP-R is exclusively present in liver, its expression, synthesis, and binding activity are altered by liver inflammatory diseases [25, 26], liver injury, diabetes [27], and toxins [1]. In normal hepatocytes, ASGP-R orients toward the sinusoidal and basolateral membrane. In contrast, during liver inflammation, loss of polarity results in higher expression of ASGP-R in the canalicular membrane compared to sinusoidal and basolateral surfaces [6]. This abnormal distribution of ASGP-R results in impaired binding and clearance and, hence, increased serum levels of asialoglycoproteins, which serve as a marker for liver disease [28, 29]. For example, ASGP-R expression level is around 80% in well-differentiated tumors, while its level in poorly differentiated hepatocellular carcinoma (HCC) is around 20% [30].

3 ASGP-R Receptor

3.1 Structure and Recognition Domain of ASGP-R

ASGP-R is composed of water-soluble glycoprotein made up of carbohydrates and sialic acid [15]. In humans, the major H1 subunit is 46 kD, while the minor H2 subunit is 50 kD with 58% sequence identity [31–33]. Despite the slight interspecies differences in size and number, each subunit presents highly conserved protein sequences indicating similar genetic lineage [33]. Thus, the functional ASGP-R consists of two homologous polypeptide subunits arranged as a hetero-oligomer, encoded by structurally different ASGR1 and ASGR2 genes [34]. Henis et al. demonstrated that immobilization of any one of the subunits resulted in destabilization of the hetero-oligomer [35]. H1 polypeptide when expressed in the absence of H2 matures and forms homo-oligomers normally which are carried to the cell surface [36]. Ishibashi et al. found that mice deficient in H2 (minor) subunit resulted in significantly reduced expression of H1 and almost complete disruption of plasma clearance of asialoorosomucoid [37]. However, H2 rapidly degrades in the absence of H1 subunit [38] and hence mandatory for H2 expression [39]. Thus, a functional ASGP-R requires expression of both subunits in order to achieve ligand binding and internalization at the cell surface. Each of these subunits further comprises four functional domains: a cytoplasmic domain (40 amino acid residues), transmembrane domain (1 amino acid residue) and C terminus (~230 amino acid residues), stalk (~80 amino acid residues), and extracellular carbohydrate recognition domain (CRD) (~150 amino acid residues) [5, 32] (Fig. 12.1). Structurally, the cytoplasmic domain of the H2 subunit includes an additional 18-amino-acid chain when compared to H1 subunit [33].

The CRD of these subunits belong to the calcium-dependent, C-type lectin superfamily and is responsible for specific recognition, interaction, and clearance of glycoproteins. Meier et al. [32] presented the first crystallographic structure of the

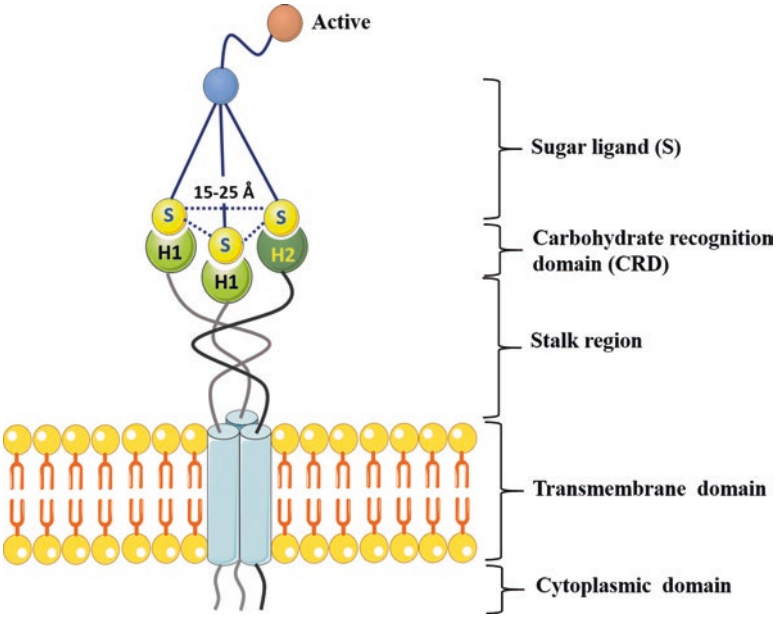

Fig. 12.1 Schematic representation of ASGP-R, illustrating the hetero-oligomer composed of two H1 and one H2 subunit, each of them containing four domains

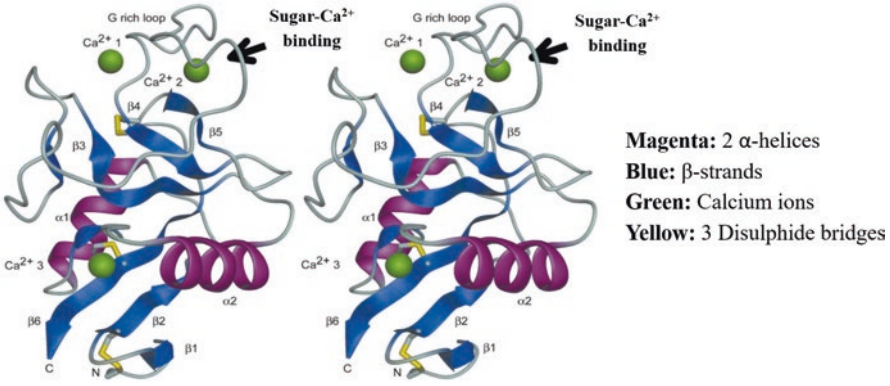

Fig. 12.2 Ribbon diagram of the H1-CRD. (Reproduced from Meier et al. [32], Copyright Elsevier 2000)

CRD of H1 subunit of ASGP-R. It comprises of a globular protein structure containing six long β-strands, two short, conserved β-strands, and two α-helices. As a long-form CRD, it contains three conserved disulfide bridges and a prominent glycine-rich loop, which protrudes from the receptor surface as part of the sugar-binding site (Fig. 12.2) [32]. The cytoplasmic domain and individual tyrosine residues of the receptor are also essential for internalization [40].

3.2 Ligand Receptor Binding and Internalization

ASGP-R has been classically known to recognize desialylated, natural or synthetic Gal-type ligands with high degree of specificity to form receptor–ligand complexes, which are cleared by receptor-mediated endocytosis [41]. Complex formation triggers clathrin-mediated endocytosis of ligand–receptor complex into endosomes with a half-life of approximately 3 min [42]. Ligand release from receptor is more sensitive to Ca^{2+} levels in early endosomes [43]. The turnover time of each ASGP-R unit in hepatoma cell lines has been reported to be 20 min and can be reused up to 200 times, with a half-life of 20 h in the plasma membrane [1]. This rapid recycling of the receptor helps maintain the normal cell surface receptor concentration between 100,000 and 500,000 per cell.

The ASGP-R subunits can exist in various forms and configurations, which contribute to functional differences like substrate specificity or rate of endocytosis [35, 44]. It is, thus, essential to understand the focal points of receptor–ligand binding to understand ASGP-R activity. The trimeric ASGP-R is most abundant and comprises of two H1 and one H2 and exhibits strongest binding affinity [4, 45] to a triantennary oligosaccharide ligand [46]. The carbohydrate-binding domains are positioned on top of the alpha-helical coiled region enabling exposure for ligand binding [32, 47] (Fig. 12.2). An active sugar-binding site on the H1 subunit comprises of the amino acids, aspartic acid 241, aspartic acid 265, asparagine 264, glutamic acid 252, glutamine 239, and tryptophan 243. Of the three Ca^{2+} binding sites on the CRDs, 1 and 2 sites are high-affinity binding sites and mediate cooperative binding with Ca^{2+} present at site 2 which is directly involved in ligand binding [43]. Ligand binding is initiated by coordination of specific amino acids in the receptor with Ca^{2+} to bind with hydroxyl moieties in sugars. Such binding induces structural changes in the CRD and enables ligand–receptor interactions (Fig. 12.3) [48].

ASGP-R ligand binding is influenced by (i) number and position of terminal galactose residues on the ligand; (ii) optimum Ca^{2+} concentration of 0.1–2 M; and (iii) a pH above 6.5 [49]. Massarelli et al. [41] highlighted the structural characteristics for specific binding of ligands to CRDs: (i) proximity of Ca^{2+} to two oxygen atoms (preferably 3-OH and 4-OH) of the sugar to allow coordinate bond formation; (ii) pyranose ring of sugar oriented to allow maximum hydrophobic interaction between Trp 243 amino acid and carbon atoms of the ligands (C3-C6); iii) numerous H bonds mediated stabilization of ligands at binding site. Alterations in amino acid residues of CRD affect sugar specificity [1], while ligand-binding activity is lost following removal of its terminal sialic acid residues [9].

3.3 ASGP-R Ligands

Natural or synthetic glycoproteins or sugars constitute high-affinity ligands of ASGP-R (Fig. 12.4). The ASGP-R binding site is known to be specific for D-galactose (Gal), D-N-acetyl-galactosamine (GalNAc), and other galactosides [9].

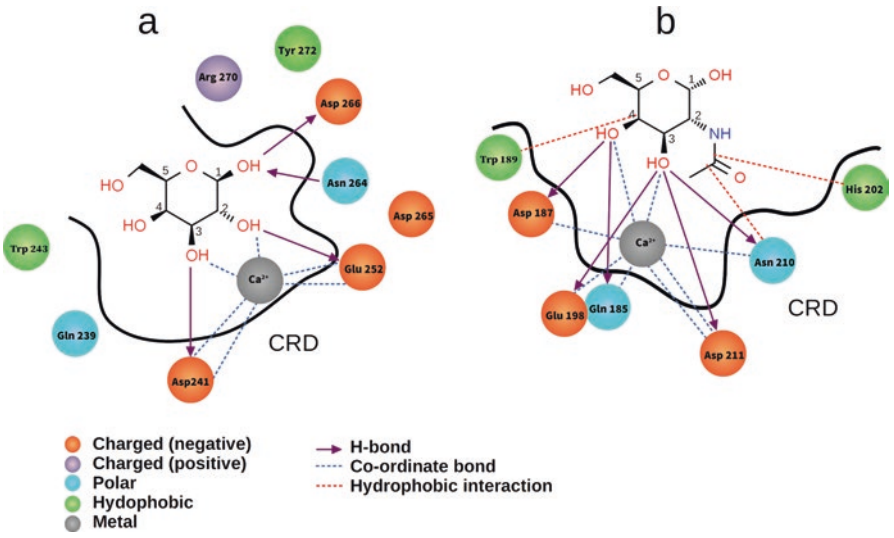

Fig. 12.3 Molecular interaction between (**a**) D-galactose and (**b**) acetyl-galactosamine at the active binding site (CRD) of ASGP-R

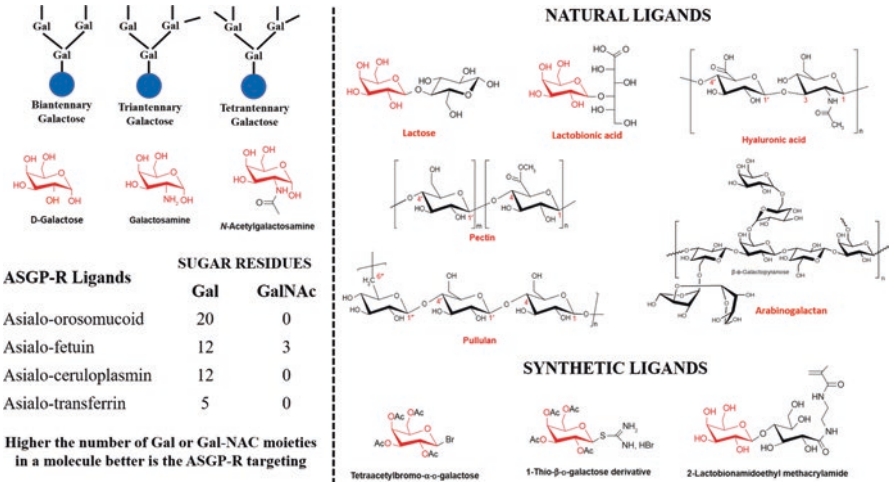

Fig. 12.4 Ligands of ASGP-R

The molecular basis of ASGP-R specificity for galactose versus mannose ligands has been explained [50]. In silico studies have predicted that oligosaccharides that are tri- and tetraantennary exhibit greater binding affinity to ASGP-R than biantennary oligosaccharides. Triantennary GalNAc exhibits highest affinity with ASGP-R when the mutual distance is ~20 Å (Fig. 12.1). Also, small modifications in the geometry of ligands result in significant decrease in binding affinity [46]. Binding

exhibited by ligands is dictated by the physicochemical properties of ligands like isomer form, galactose density and branching, spatial geometry, calcium binding, and hydrophilic–lipophilic balance and has been reviewed in detail [1, 51]. Many of these ligands are also known to trigger pro-inflammatory responses, providing disease protection [52, 53].

3.3.1 Natural ASGP-R Ligands

Glycoproteins and Carbohydrates

A number of naturally occurring soluble and membrane-bound glycoproteins and glycolipids express terminal galactosyl and N-acetylgalactosaminyl residues [15]. Of these, asialoorosomucoid (ASOR possessing 5 branched-chain carbohydrate units) and asialofetuin (AF possessing 3 triantennary carbohydrates with terminal N-acetyllactosamine residues) have been extensively explored for studying ASGP-R-ligand binding and for protein and gene delivery [54]. Lactoferrin is another iron-binding cationic glycoprotein which presents high-affinity ASGP-R binding and internalization by hepatocytes through a galactose-independent mechanism [1].

ASGP-R-specific biocompatible carbohydrate ligands like lactose, galactosamine, dextran, lactobionic acid (LA), and sterylglucoside (β-Sitosterol-β-D-glucoside) can enhance hepatocyte uptake [55], and few have also been used in delivery systems as contrast agent for liver diagnosis [56]. More recently, biocompatible and biodegradable linear carbohydrates like hyaluronic acid, pectin, arabinogalactan (AG), and pullulan have also attracted attention as ASGP-R ligands and are used in various drug delivery systems. AG, a galactose-based polysaccharide with a high Gal density of ~80%mol, has reported rapid, dose-dependent, ASGP-R-mediated hepatocyte internalization, which was 14-fold greater than AF. Though considered safe for oral use, chronic, intravenous administration of AG presents toxicity concerns due to slow dissociation and negligible breakdown within hepatocytes and needs more conclusive studies to validate clinical usage. Pullulan, a glucose-based polysaccharide, also reported dose-dependent, hepatocyte-specific targeting, which was much lower than AG due to its lack of discrimination between Gal and D-glucose. However, both AG and pullulan could be completely inhibited in the presence of AF [1].

3.3.2 Synthetic ASGPR Ligands

Synthetic galactosylations of polymers and lipids minimize Kupffer cell (KC) clearance and have been successfully exploited for designing hepatocyte-targeted drug/gene delivery systems, which have been discussed in detail in receptor-mediated drug delivery section. Various modified mono- or multivalent polymers and lipids like pluronic P123-coupled tetraacetyl bromoα-d-galactose, galactosylated chitosan

conjugated polymers, Gal-poly-glutamic acid, Gal-poly-L-lysine, pullulan derivatives, Gal-DOPE, Gal-cholesterol, etc. have been exploited for ASGPR targeting. Detailed description of ligands used for ASGP-R-mediated liver targeting has been presented in various reviews [1, 51, 57]. The structures of common ASGP-R ligands studied are depicted in Fig. 12.4.

4 ASGP-R-Mediated Targeting Strategies

4.1 Drug–Ligand Conjugate and Complexes

These systems are designed by direct conjugation/complexation of a chemotherapeutic drug to a high-affinity homing ligand which is triggered to release the drug at a desired site, and have been widely used for delivery of anticancer molecules and nucleic acids in cancer therapeutics. Poly(L-lysine) (PLL) is the earliest known polycation to form complexes with DNA but is rapidly cleared from circulation after intravenous administration. Various natural and synthetic galactose ligands have been coupled with these polymers in order to improve biocompatibility and targeting effects of such polyelectrolyte complexes [58, 59]. Lectin-directed enzyme-activated prodrug approach, which releases active form of drug to specific cell type, has shown therapeutic efficacy in a hepatocellular carcinoma (HepG2) disease model [60]. Gal-NAc-siRNA conjugates and doxorubicin-lactosaminated human albumin (L-HSA) conjugates have been successfully studied for liver targeting and are currently in various stages of clinical trials [61–63]. However, despite their usefulness, possibilities of drug inactivation during chemical conjugation [64], in vivo instability [65], nontarget release of the drug [66], and rapid clearance [67] are limitations associated with conjugates. These limitations have been addressed by redox-responsive and pH-sensitive, glycopolymer–drug conjugate NPs, which can target and program drug release in the reductive, acidic tumor microenvironment [68]. Table 12.1 summarizes the more recent drug–ligand conjugates for treatment of various diseases and provides a brief on the outcome.

4.2 Drug Nanoparticles

Colloidal nanocarrier properties like particle size, surface charge, hydrophilicity imparting stealth, and ligand concentration decide uptake, targeting potential, and fate in vivo [1]. Galactosylated NPs were shown to be taken up by clathrin or caveolae-mediated endocytosis. Despite discrepancies, it is generally accepted that nanocarriers with size >70 nm are not recognized or processed by ASGP-R. PEGylated-Gal-NPs for gene delivery of size 50 nm were actively taken up by hepatocytes, while 140-nm particles were taken up by KC [84]. Positively

Table 12.1 Preclinical studies of drug–ligand conjugates for ASGP-R targeting

Ligand/formulation	Study outcome	Reference
Cancer		
AF-DNA oligonucleotide conjugate	Targets primary hepatocytes and human HepG2 cells	[69]
α-L-rhamnopyranoside prodrug of DOX	60-fold increase in hepatocyte selectivity and reduced tumor burden in HCC disease model	[60]
Gal-DOX covalent conjugate	Higher cell affinity to tumor cells than normal liver cell with long tumor retention	[70]
Triantennary GalNAc-based ligand siRNA conjugate	Retained siRNA activity despite greater than 50% reduction in ASGP-R levels	[71]
Chondroitin/DOX-polyelectrolyte complex-loaded gelatin NP	Enhanced cytotoxicity and hepatic targeting in comparison with free DOX	[72]
Pectin-5-FU-loaded pectin NP	Size-induced prolonged circulation as well as ASGP-R targeting ability in HCC cells and rat model	[73]
Multivalent GalNAc siRNA conjugate	Hepatotropic with sustained dose-dependent gene silencing for over 9 months with no adverse effects in rodents	[74]
Gal-CPDC/DNA complexes	Higher gene expression in ASGP-R-positive HepG2 cells, useful as nonviral gene vector for hepatocyte targeting	[75]
Pullulan-PGEA-DNA polycation complex	Higher gene transfection efficiency and cellular uptake rates in HepG2 than in HeLa cell lines	[76]
β-Gal-PEI-DNA conjugate	Higher transfection efficiencies in HepG2 cells with reduced cytotoxicity	[77]
Hepatitis		
Lac-PLL-Ribavirin conjugate	Inhibition of MHV replication at lower drug dose in chronic type C hepatitis	[78]
Gal-jetPEI-siRNA conjugate	Synergistic effects in inducing hepatoma cell apoptosis to inhibit HBV replication in HepG2 cell line	[79]
Malaria		
Triantennary GalNAc primaquine, PGA conjugate	Conjugate taken up over 7 h and binds to rat hepatocytes and completely degraded over 24 h	[80]
Miscellaneous		
Gal-PLGA vitamin K5 conjugate	Prolonged coagulant activity	[81]
Gal-CMD-cytosine β-D-arabinoside conjugate	Specific high affinity to liver parenchymal or nonparenchymal cells without any affinity to other tissues	[82]
Gal-amphiphilic cyclodextrins plasmid DNA complex	Transfection levels increased with increase in linker length	[83]

charged nanocarriers have been reported to readily interact with the negatively charged ASGP-R-binding sites as demonstrated with lactosaminated cationic PLL, which reported 98% hepatocyte uptake after intravenous administration [85]. Stealth coating of nanocarriers has shown improved serum circulation and enhanced

hepatocyte uptake. PEGylated Gal-liposomes and lipoplexes have demonstrated higher hepatocyte uptake compared to nonpegylated carriers, which can be improved by increasing degree of pegylation [86, 87]. ASGP-R-specific ligand coating of nanocarriers could reduce rapid systemic clearance and improve hepatocyte targeting. However, an optimum ligand concentration is essential for desired hepatic uptake since higher concentration of ligands can cause ASGP-R saturation, resulting in increased uptake of nanocarriers by other Gal receptors like KC. GalNAc bearing doxorubicin–HPMA conjugate (PK2) when administered at higher doses caused partial ASGP-R saturation [81, 88]. Few natural carbohydrates like pullulan and AG can function as both a stealth agent and ASGP-R ligand [48]. The recent polymeric nanocarriers, liposomes, and micellar systems designed for liver targeting have been enlisted in Tables 12.2, 12.3, and 12.4, respectively. Exhaustive reviews on ASGP-R targeted delivery systems are also available for reference [1, 54, 57, 62, 89].

Table 12.2 Preclinical studies of drug–NPs for ASGP-R targeting

Polymeric NPs		
Ligand/formulation	**Study outcome**	**Reference**
Cancer		
Lactose-PEG-appended α-CDE gene and siRNA NPs	Hepatocyte-selective gene and siRNA transfer activity in vitro and in vivo	[90]
Lac-DOX mesoporous silica NPs	Effective inhibition of HepG2 and SMMC7721 cells in a time and concentration-dependent manner	[91]
LA-Sorafenib and siVEGF mesoporous silica NPs	Improves the tumor targeting and siVEGF transfection efficiency for HCC therapy in ASGPR-overexpressing Huh7 cells	[92]
Pullulan-PAE/PLGA-paclitaxel, combretastatin A4 NPs	Improved tumor inhibition and angiogenesis pH-responsive, charge reversible NPs	[93]
Gal-carboxymethyl chitosan RASSF1A gene-loaded iron oxide NP	Specific accumulation of NPs in HCC tissue especially with aid of an external magnetic field	[94]
Gal-TPGS-PLA-Docetaxel NPs	Potential application in targeted delivery for liver cancer	[95]
LA-Etoposide TPGS conjugate	Enhanced cytotoxicity and efficient accumulation at tumor site in vivo	[96]
SS-LA-PEC-DOX reduction-sensitive shell-sheddable NPs	Efficiently taken up by ASGPR-overexpressing HepG2 cells with high antitumor activity	[97]
Lac-Norcantharidin-N-trimethyl chitosan NPs	Significantly enhanced drug absorption, penetrates plasma membrane of Caco-2 cells and translocate into cytoplasm and nucleus	[98]
β-galactosidase-polymer/DNA NPs	High gene delivery efficiency into ASGPR expressing hepatocytes	[99]
Galactosamine-PGA and PLA-loaded PTX NPs	Specific interaction with HepG2 cells resulting in arrest in G2/M phase	[100]

(continued)

Table 12.2 (continued)

Gal-carboxymethyl chitosan RASSF1A gene-loaded iron oxide NP	Specific accumulation of NPs in HCC tissue especially with aid of an external magnetic field	
Gal-chitosan oligosaccharide-loaded ATP NPs	Low cytotoxicity and enhanced uptake by HepG2 cells	[101]
Galactopeptide-DOX self-assembled NPs	Higher antitumor activity toward HepG2 cells in vitro and in vivo	[102]
Glycyrrhizin-PTX-O-carboxymethyl chitosan NPs	10-fold higher liver cancer cell internalization and significantly superior targeting to tumor	[103]
Pectin-methotrexate conjugate ionotropic gelation NPs	Enhanced cytotoxicity and improved delivery to cancer cells	[104]
Pullulan-lipoic acid-loaded PTX core cross-linked NPs	Efficient endocytosis and distribution in cytoplasm, long systemic retention time	[105]
Polyethylene sebacate-Gantrez DOX NPs	High hepatocyte: nonparenchymal cell ratio of 85:15, sustained tumor volume reduction in mice, extensive tumor necrosis, reduced collagen content, reduction in HCC biomarker	[106]
Hepatitis		
Lactose-acyclovir-N-succinyl chitosan NP	Excellent liver targeting potential with high drug concentration even after 24 h	[107]
Lactose functionalized amphiphilic random copolymer-Ribavirin conjugate	Hepatocyte targeting, self-assembled NPs	[108]
Malaria		
Pullulan nanocarboplex of primaquine phosphate	Increase in drug t ½ with high hepatocyte/nonparenchymal cell ratio of 75:25	[109]
Miscellaneous		
Kappa carrageenan/AG/pullulan curcumin Gantrez NPs	AG and pullulan revealed maximum hepatic accumulation following IV administration of NPs to rats	[48]
Solid–lipid NPs		
Ligand/formulation	**Experimental outcome**	**Reference**
Cancer		
LA-5-FU nanostructured lipid carriers	Galactosylated NLCs were cytotoxic on HepG2 cells at half dose of 5-FU	[110]
LA-DPEA-loaded DOX SLN	Increased cellular uptake and drug accumulation in hepatoma cells, low systemic toxicity	[111]
GalNAc-PEG siRNA lipid NPs	Effective hepatocyte targeting and gene silencing following SC administration	[112]
GalNAc cluster-siRNA ApoE-loaded lipid NPs	High hepatic targeting in HeLa cell line and mice	[113]

Table 12.3 Preclinical studies of drug–liposomes for ASGP-R targeting

Ligand/formulation	Experimental outcome	Reference
Cancer		
Gal-DOX/vimentin siRNA liposome	Co-delivery of DOX and siRNA, enhanced transfection efficacy, synergistic antitumor therapy	[114]
N-lactosyl-DPEA-DOX encapsulating calcein liposomes	Long blood circulation time, higher uptake significantly stronger tumor inhibitory activity and accumulation of drug within tumor site	[115]
Bifunctional glycolipid–liposome DNA complex	High affinity for ASGP-R and transfection activity	[116]
CTG-lipid-protamine sulfate and plasmid DNA cationic liposomes	Improved levels of gene expression in HepG2 and SMMC-7721, length of spacer between anchor and galactose residues important for ASGP-R recognition	[117]
TAB-Gal-mitoxantrone dual-functional liposome	Increased antitumor activity and selectivity in BALB/c mice bearing orthotopic xenograft HCC tumors	[118]
Hepatitis		
β-Sitosterol-β-D-glucoside modified cationic asODN liposomes	High transfection efficiency by endocytosis and membrane fusion for HBV infection	[119]
β-Sitosterol glucoside modified cationic siRNA DDMAP liposomes	siRNA in liposome protected from lyases, ultimate suppression of HCC	[120]

Table 12.4 Preclinical studies of drug–micelles and other drug delivery systems for ASGP-R targeting

Micelles		
Ligand/formulation	**Experimental outcome**	**Reference**
Cancer		
Gal-Amphiphilic block glycopolymers-DOX-loaded micelles	Enhanced uptake of DOX-loaded Gal-micelles by HepG2 cells, significantly increased cytotoxicity as compared to HEK293	[121]
Gal-PEG-PEC copolymer DOX micelles	Ligand-directed, reduction-sensitive, shell-sheddable targeted cancer chemotherapy	[122]
Lactose-Rhodamine B micelles	Stronger endocytosis by SMMC7221 human liver cancer cells, remarkable liver-targeting effect in mice	[123]
Lactose-asODN micelles	Free asODN released into cell interior with decreased pH in endosomes, better uptake into HuH-7 cells	[124]
Gal-PTX micelles	Enhanced apoptosis and tumor growth inhibition, less damage to liver and kidney	[125]
LA-PTX micelles	High antitumor activity in HepG2 cells, inhibited growth of human hepatoma	[126]
Hepatitis		
Gal-PLA-PAA conjugated RBV tripalmitate-loaded micelles	Specificity toward HepG2 cells	[127]

(continued)

Table 12.4 (continued)

Emulsion		
Ligand/formulation	**Experimental outcome**	**Reference**
Cancer		
Gal-chitosan coating on DOX-PVA microbubbles	Higher HepG2 uptake than normal fibroblasts, retained cytotoxic activity which increased upon ultrasound exposure	[128]
Gal-chitosan loaded 10-hydroxy-camptothecin nanocrystallites	Nanocrystallites stabilized formulation in aqueous medium, enhanced cellular internalization	[129]
Gal-chitosan-graft-PNIPAm nanogels	Higher antitumor activity which increased with increase in number of galactose moieties	[130]
Gal-C4-Chol emulsions	Enhanced uptake and internalization by HepG2 cells but not much by NIH3T3 cells	[131]
Dendrimers		
Cancer		
GalNAc G5 PAMAM dendrimers	Internalization of targeted carriers increased with the increase in G5 concentration and incubation time	[132]
GalNAc-DOX-loaded G5 PAA-PAMAM dendrimers	HCC-bearing NOD SCID gamma mice achieves a 2.5-fold inhibition of tumor growth, apoptosis induction, without DOX cardiotoxicity	[133]
AF-epirubicin loaded chitosan-PLGA NPs	Co-delivery of epirubicin with tocotrienols as antioxidant, significantly reduced angiogenesis, lower cardiotoxicity, and higher apoptosis level	[134]

4.3 Nanoparticle Diagnostics and Theranostics

4.3.1 Diagnostics

Novel galactosylated fluorescent probes like fluorescent silica NPs [135], manganese ferrite NPs for targeted MR imaging [136], pegylated quantum dots [137], and lactose functionalized magneto liposomes [138] have been successfully used for hepatocyte-targeted bio-imaging with enhanced sensitivity [139]. Various studies have used albumin as a substrate for ASGP-R ligands like galactose or lactose for hepatocyte-specific targeting of imaging agents like ^{99m}Tc diethylenetriaminepentaacetic acid galactosyl (DTPA-Gal-HSA) human serum albumin [140], ^{99m}Tc DTPA-Gal-neoglycoalbumin [141], and ^{99m}Tc neolactosylated HSA in clinical tests [142]. Further, Kim et al. used chitosan as a backbone to synthesize ^{99m}Tc hydrazinonicotinamide-galactosylated chitosan (HGC), which can be used for specific and rapid imaging for evaluation of hepatic function [139]. Multimodal imaging techniques merging complementary imaging techniques have been reported to overcome limitations of single imaging techniques. A hepatocyte-targeted, multifunctional nanoplatform consisting of galactosyl conjugated P_{123} (Gal-P_{123})-modified NIRF783-Fe3O4 (GPC@NIRF783-Fe_3O_4) NP was developed for noninvasive,

fluorescence, and MRI-based dual-mode imaging of tumors. The ligand-coated NPs demonstrated 5.4-fold improvement in relative MR signal enhancement compared to uncoated NPs [143]. Kouda et al. investigated ^{99m}Tc-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (^{99m}Tc-DTPA Gal-HSA) as a novel ASGP-R-specific scintigraphic agent to evaluate hepatic function. Their results reported a significant increase (53.2%) in ^{99m}Tc-DTPA Gal-HSA uptake per hepatocyte during recovery stage after a hepatic injury. This augmented uptake might be triggered by hepatotrophic factors as a compensation for the decreased hepatocyte number [144]. A list of diagnostic agents is summarized in Table 12.5.

Table 12.5 Diagnostic agents for ASGP-R targeting

Targeted diagnostic agent	Application	Experimental outcome	Reference
LA conjugated fluorescent silica NPs	Cell labeling and differentiation as novel fluorescent probes	Excellent photostability, biocompatibility, and significant signal amplification	[135]
[^{68}Ga] Gal-DTPA-HSA	Use with PET and planar imaging for functional hepatocellular mass	Higher activity concentration in blood compared to marketed	[145]
^{64}Cu-Lac-HSA	Use for molecular imaging of hepatocytes with PET	Serial PET imaging and autoradiography revealed higher accumulation in HepG2 tumors	[146]
^{125}I-ASOR-BDICA	A bile duct diagnostic small molecule CT contrast agent	Nontoxic, X-ray attenuation properties and high ASGPR affinity	[147]
GDPA-LA-chitosan	Use for molecular imaging of hepatocytes by MRI	High specificity and enhanced images in MRI of normal rat hepatocytes compared to signal strength for rat liver cancer cells	[148]
LA-modified superparamagnetic magnetite NPs	Use as contrast agent for liver diagnosis with MRI	Selective accumulation in rabbit hepatocytes by MRI	[56]
^{99m}Tc-p(VLA-co-VNI-co-V_2DTPA)	Useful for SPECT imaging for evaluating and staging liver fibrosis in vivo	ASGP-R could be a useful marker in stage of liver fibrosis. Liver uptake value decreased with disease progression	[149]
AA-coated with biotinylated AF labelled Magnetic beads	Tool useful in diagnosis and monitoring of HCC	Both positivity rate and number of CTCs were significantly correlated with tumor size, portal vein tumor thrombus, differentiation status, and disease extent	[150, 151]

(continued)

Table 12.5 (continued)

Targeted diagnostic agent	Application	Experimental outcome	Reference
^{111}In-LDTPA Gal-BSA	In vivo probe to understand interaction of radiolabeled glycoprotein with ASGPR transport system	Normal mice showed 70% of radioactivity in hepatocyte, whereas ASGPR-deficient mouse had equal activity in hepatocyte and hepatic endothelial cells	[152]
^{99m}Tc-Fluorocarbon chain LA sugar-DTPA chelate	Hepatocyte targeting, imaging with PET in hepatic fibrosis	Biodistribution by scintigraphy traced a majority of compound in rat liver	[153]
^{99m}Tc-Gal-HSA	Estimation of extent of ASGPR expression for remnant liver function for hepatic surgery	Estimated remnant liver function was significantly correlated with postoperative liver function parameters	[154]
[^{111}In]-labelled Gal-BSA	Effects of number of galactose residues per protein molecule on receptor binding and internalization for ASGP-R targeting systems	Receptor binding and internalization are regulated by number of galactose residues per BSA during hepatic uptake	[155]
^{99m}Tc-labelled Gal-serum albumin	Multicompartmental analysis of hepatic scintigraphy for evaluating function reserve capacity of liver	The multicompartmental model analysis permitted comparable results to different gamma cameras	[156]
^{99m}Tc-Gal-neoglycoalbumin	Diagnostic performance of a receptor-binding radiopharmacokinetic model	Receptor concentration $[R]_0$ and metrics were most accurate index of hepatic function	[157]

4.3.2 Theranostics

Gal-Dox has been used as a theranostic system for targeting ASGP-R on colon cancer cells followed by Dox release and enzyme-stimulated fluorescence activation allowing both drug localization and monitoring [158]. Lactobionic acid-targeted multifunctional theranostic Lipid-AuNPs@PDA nanohybrid NPs have been reported for dual-mode, MRI/CT imaging with photothermal therapy for HCC [159]. Supramolecular glycocomposites constructed by co-assembling a glycoprobe and anticancer drug onto graphene oxide(GO) surface resulted in excellent imaging ability, while the material demonstrated improved toxicity toward ASGP-R overexpressed liver cancer cells [160]. Iodoazomycin arabinofuranoside loaded into galactose-based thermosensitive nanogels could be used for imaging and radiotherapy of solid hypoxic tumors [161]. Disulfide-based fluorescent drug delivery multifunctional conjugates have been reported to undergo thiol triggered disulfide bond cleavage to deliver the drug and the fluorescent probe at targeted sites [162]. A list of theranostic NPs is summarized in Table 12.6.

Table 12.6 Theranostics for ASGP-R targeting

Ligand/formulation	Application	Reference
Cancer		
Iodoazomycin arabinofuranoside Gal-nanogels	Hypoxia-selective multimodal theranostic applications to manage and image hypoxic solid tumors	[161]
LA-indocyanine green in core-shell gold NPs	Multifunctional theranostic agent for targeted MRI/CT dual-mode imaging and photothermal therapy of HCC	[159]
Gal-DOX	Cancer-specific activation with Dox release with a fluorescence turn-on response for colorectal cancer theranostic	[158]
Glycoligand-GO	GO greatly enhances cellular and tissue imaging ability of a small-molecule fluorescence glycoprobe and serves as a clustering platform to reinforce interaction of glycoprobe with selective receptor on cancer cell	[160]
Gal, folate, biotin, RGD peptide sequence-DOX, gemcitabine, camptothecin multifunctional fluorescent drug conjugates	Site-specific cleavage by endogenous thiols serves to release cytotoxic drug and produce an easy-to-monitor change in fluorescence signature of cell	[162]

5 ASGP-R Ligands in Clinical Development

A Phase II clinical trial of galactosamine-modified polymer-linked doxorubicin (PK2) has demonstrated enhanced liver-specific doxorubicin delivery with specific targeting to hepatocytes [163]. Pfizer discovered ASGP-R ligands, PF-06853291 and PF-06868566 (trivalent form of GalNAc), have demonstrated rapid serum clearance followed by hepatic accumulation, suggesting widespread applications in hepatocellular delivery [164, 165]. Huang has presented an exhaustive review of the various preclinical and clinical progress made by GalNAc conjugated nucleic acid therapeutics for targeted gene delivery [61]. Various ASGP-R ligands in different phases of clinical trials have been presented in Table 12.7.

6 Advantages and Limitations of ASGP-R Targeting

Despite the exciting opportunities reported by ASGP-R-targeted drug delivery systems in numerous preclinical studies, these systems have failed in clinical trials. The clinical failure might be due to variability in performance and expression of the receptor among various species and stage of disease. Also, since most preclinical studies are performed in healthy animals, successful targeting might not be an accurate

Table 12.7 ASGP-R ligand–drug conjugates in clinical development

Ligand	Active/formulation	Phase	Application	Reference
Galactosamine	DOX-linked HPMA copolymers bearing galactosamine, PK2	Phase II	Liver-specific targeting in primary HCC tumors, represents the first ligand-targeted system to reach clinics	[163]
Triantennary GalNAc (GN3)	Second-generation gapmer antisense oligonucleotides (ASOs)	Phase I/II	Conjugate is metabolized to liberate parent ASO in hepatocytes but not in plasma	[166]
PF-06853291 and PF-06868566 (trivalent version of natural ASGP-R GalNAc)	Alexa Fluor® 647	–	Increased ASGPR-directed HCC uptake and prolonged retention in vivo compared to trivalent GalNAc	[164]
ALN-HBV (GalNAc)	siRNA	Terminated	Liver-specific targeting for hepatitis B virus infection	[167]

indicator of successful treatment. Off-target toxicities at certain doses have been observed due to ASGP-R expression in both normal hepatocytes and HCC cells. Terada et al. [168] have addressed this issue by designing matrix metalloproteinase-2 cleavable DOPE/PEG liposomes for HCC-specific delivery. In addition, variability in levels of ASGP-R expression in response to time, disease condition, and stage of progression present significant obstacles to effective targeting. Further, surface-modified ASGP-R delivery systems involve complicated preparation methods and can evoke immunogenicity and autoantibody generation, resulting in dire consequences.

7 Conclusion and Future Prospects

To summarize, the exclusive liver expression of ASGP-R presents huge scope in liver-targeted delivery of small molecules, nucleic acids, and diagnostic agents, which has been amply demonstrated in numerous research findings. Novel ASGPR-ligand anchored siRNA delivery systems are an indicator of progress made in the development of ASGP-R-specific targeting. However, restrictions that have previously resulted in failure of these systems in clinical studies need to be validated to mimic specific disease physiology, which include verifying the receptor density and saturation, immune activity and selection of a proper experimental model, and delivery system for each study. Successful targeting to this receptor could have huge implications in the development of novel effective targeted systems for treatment and diagnosis of liver infections and cancer.

References

1. D'Souza AA, Devarajan PV. Asialoglycoprotein receptor mediated hepatocyte targeting - Strategies and applications. J Control Release. 2015;203:126–39.
2. Kedderis GL, Held SD. Prediction of furan pharmacokinetics from hepatocyte studies: comparison of bioactivation and hepatic dosimetry in rats, mice, and humans. Toxicol Appl Pharmacol. 1996;140(1):124–30.
3. Ashwell G, Harford J. Carbohydrate-specific receptors of the liver. Annu Rev Biochem. 1982;51(1):531–54.
4. Grewal PK. The Ashwell–Morell receptor. In: Methods in enzymology, vol. 479: Academic Press; 2010. p. 223–41. United States of America.
5. Spiess M. The asialoglycoprotein receptor: a model for endocytic transport receptors. Biochemistry. 1990;29(43):10009–18.
6. Roggenbuck D, Mytilinaiou MG, Lapin SV, Reinhold D, Conrad K. Asialoglycoprotein receptor (ASGPR): a peculiar target of liver-specific autoimmunity. Auto Immun Highlights. 2012;3(3):119.
7. Weigel PH. Characterization of the asialoglycoprotein receptor on isolated rat hepatocytes. J Biol Chem. 1980;255(13):6111–20.
8. Li Y, Huang G, Diakur J, Wiebe LI. Targeted delivery of macromolecular drugs: asialoglycoprotein receptor (ASGPR) expression by selected hepatoma cell lines used in antiviral drug development. Curr Drug Deliv. 2008;5(4):299–302.
9. Schwartz AL, Ashwell G. The hepatic asialoglycoprotein receptor. Crit Rev Biochem. 1984;16(3):207–33.
10. Park JH, Cho EW, Shin SY, Lee YJ, Kim KL. Detection of the asialoglycoprotein receptor on cell lines of extrahepatic origin. Biochem Biophys Res Commun. 1998;244(1):304–11.
11. Rigopoulou EI, Roggenbuck D, Smyk DS, Liaskos C, Mytilinaiou MG, Feist E, Conrad K, Bogdanos DP. Asialoglycoprotein receptor (ASGPR) as target autoantigen in liver autoimmunity: lost and found. Autoimmun Rev. 2012;12(2):260–9.
12. Dotzauer A, Gebhardt U, Bieback K, Göttke U, Kracke A, Mages J, Lemon SM, Vallbracht A. Hepatitis A virus-specific immunoglobulin A mediates infection of hepatocytes with hepatitis A virus via the asialoglycoprotein receptor. J Virol. 2000;74(23):10950–7.
13. Yang J, Wang F, Tian L, Su J, Zhu X, Lin L, Ding X, Wang X, Wang S. Fibronectin and asialoglyprotein receptor mediate hepatitis B surface antigen binding to the cell surface. Arch Virol. 2010;155(6):881–8.
14. Treichel U, zum Büschenfelde KH, Stockert RJ, Poralla T, Gerken G. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. J Gen Virol. 1994;75(11):3021–9.
15. Stockert RJ. The asialoglycoprotein receptor: relationships between structure, function, and expression. Physiol Rev. 1995;75(3):591–609.
16. Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J Virol. 2003;77(1):546–59.
17. Becker S, Spiess M, Klenk HD. The asialoglycoprotein receptor is a potential liver-specific receptor for Marburg virus. J Gen Virol. 1995;76(2):393–9.
18. Grewal PK, Uchiyama S, Ditto D, Varki N, Le DT, Nizet V, Marth JD. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. Nat Med. 2008;14(6):648.
19. Ueno S, Mojic M, Ohashi Y, Higashi N, Hayakawa Y, Irimura T. Asialoglycoprotein receptor promotes cancer metastasis by activating the EGFR–ERK pathway. Cancer Res. 2011;71(20):6419–27.
20. Nakaya R, Kohgo Y, Mogi Y, Nakajima M, Kato J, Niitsu Y. Regulation of asialoglycoprotein receptor synthesis by inflammation-related cytokines in HepG2 cells. J Gastroenterol. 1994;29(1):24–30.

21. Poralla T, Treichel U, Löhr H, Fleischer B. The asialoglycoprotein receptor as target structure in autoimmune liver diseases. Semin Liver Dis. 1991;11(03):215–22. Thieme Medical Publishers, Inc.
22. McFarlane IG, McFarlane BM, Major GN, Tolley P, Williams R. Identification of the hepatic asialo-glycoprotein receptor (hepatic lectin) as a component of liver specific membrane lipoprotein (LSP). Clin Exp Immunol. 1984;55(2):347.
23. Witzigmann D, Quagliata L, Schenk SH, Quintavalle C, Terracciano LM, Huwyler J. Variable asialoglycoprotein receptor 1 expression in liver disease: implications for therapeutic intervention. Hepatol Res. 2016;46(7):686–96.
24. Villalta D, Mytilinaiou MG, Elsner M, Hentschel C, Cuccato J, Somma V, Schierack P, Roggenbuck D, Bogdanos DP. Autoantibodies to asialoglycoprotein receptor (ASGPR) in patients with autoimmune liver diseases. Clin Chim Acta. 2015;450:1–5.
25. Sawamura T, Nakada H, Hazama H, Shiozaki Y, Sameshima Y, Tashiro Y. Hyperasialoglycoproteinemia in patients with chronic liver diseases and/or liver cell carcinoma: asialoglycoprotein receptor in cirrhosis and liver cell carcinoma. Gastroenterology. 1984;87(6):1217–21.
26. Virgolini I, Müller C, Klepetko W, Angelberger P, Bergmann H, O'Grady J, Sinzinger H. Decreased hepatic function in patients with hepatoma or liver metastasis monitored by a hepatocyte specific galactosylated radioligand. Br J Cancer. 1990;61(6):937.
27. DODEUR M, DURAND D, DUMONT J, DURAND G, FEGER J, AGNERAY J. Effects of streptozotocin-induced diabetes mellitus on the binding and uptake of asialoorosomucoïd by isolated hepatocytes from rats. Eur J Biochem. 1982;123(2):383–7.
28. Burgess JB, Baenziger JU, Brown WR. Abnormal surface distribution of the human asialoglycoprotein receptor in cirrhosis. Hepatology. 1992;15(4):702–6.
29. Marshall JS, Green AM, Pensky J, Williams S, Zinn A, Carlson DM. Measurement of circulating desialylated glycoproteins and correlation with hepatocellular damage. J Clin Invest. 1974;54(3):555–62.
30. Trere D, Fiume L, De Giorgi LB, Di Stefano G, Migaldi M, Derenzini M. The asialoglycoprotein receptor in human hepatocellular carcinomas: its expression on proliferating cells. Br J Cancer. 1999;81(3):404.
31. Bischoff J, Lodish HF. Two asialoglycoprotein receptor polypeptides in human hepatoma cells. J Biol Chem. 1987;262(24):11825–32.
32. Meier M, Bider MD, Malashkevich VN, Spiess M, Burkhard P. Crystal structure of the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor. J Mol Biol. 2000;300(4):857–65.
33. Spiess M, Lodish HF. Sequence of a second human asialoglycoprotein receptor: conservation of two receptor genes during evolution. Proc Natl Acad Sci. 1985;82(19):6465–9.
34. Harris RL, Van den Berg CW, Bowen DJ. ASGR1 and ASGR2, the genes that encode the asialoglycoprotein receptor (Ashwell receptor), are expressed in peripheral blood monocytes and show interindividual differences in transcript profile. Mol Biol Int. 2012;2012:1.
35. Henis YI, Katzir Z, Shia MA, Lodish HF. Oligomeric structure of the human asialoglycoprotein receptor: nature and stoichiometry of mutual complexes containing H1 and H2 polypeptides assessed by fluorescence photobleaching recovery. J Cell Biol. 1990;111(4):1409–18.
36. Braiterman LT, Chance SC, Porter WR, Lee YC, Townsend RR, Hubbard AL. The major subunit of the rat asialoglycoprotein receptor can function alone as a receptor. J Biol Chem. 1989;264(3):1682–8.
37. Ishibashi S, Hammer RE, Herz J. Asialoglycoprotein receptor deficiency in mice lacking the minor receptor subunit. J Biol Chem. 1994;269(45):27803–6.
38. Shia MA, Lodish HF. The two subunits of the human asialoglycoprotein receptor have different fates when expressed alone in fibroblasts. Proc Natl Acad Sci. 1989;86(4):1158–62.
39. Tozawa RI, Ishibashi S, Osuga JI, Yamamoto K, Yagyu H, Ohashi K, Tamura Y, Yahagi N, Iizuka Y, Okazaki H, Harada K. Asialoglycoprotein receptor deficiency in mice lacking the major receptor subunit its obligate requirement for the stable expression of oligomeric receptor. J Biol Chem. 2001;276(16):12624–8.

40. Valladeau J, Duvert-Frances V, Pin JJ, Kleijmeer MJ, Ait-Yahia S, Ravel O, Vincent C, Vega F, Helms A, Gorman D, Zurawski SM. Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. J Immunol. 2001;167(10):5767–74.
41. Massarelli I, Murgia L, Bianucci A, Chiellini F, Chiellini E. Understanding the selectivity mechanism of the human asialoglycoprotein receptor (ASGP-R) toward gal-and man-type ligands for predicting interactions with exogenous sugars. Int J Mol Sci. 2007;8(1):13–28.
42. Mcabee DD, Jiang X, WALSH KB. Lactoferrin binding to the rat asialoglycoprotein receptor requires the receptor's lectin properties. Biochem J. 2000;348(1):113–7.
43. Onizuka T, Shimizu H, Moriwaki Y, Nakano T, Kanai S, Shimada I, Takahashi H. NMR study of ligand release from asialoglycoprotein receptor under solution conditions in early endosomes. FEBS J. 2012;279(15):2645–56.
44. Weigel PH, Yik JH. Glycans as endocytosis signals: the cases of the asialoglycoprotein and hyaluronan/chondroitin sulfate receptors. Biochim Biophys Acta. 2002;1572(2–3):341–63.
45. Spiess M, Schwartz AL, Lodish HF. Sequence of human asialoglycoprotein receptor cDNA. An internal signal sequence for membrane insertion. J Biol Chem. 1985;260(4):1979–82.
46. Ramadugu SK, Chung YH, Fuentes EJ, Rice KG, Margulis CJ. In silico prediction of the 3D structure of trimeric asialoglycoprotein receptor bound to triantennary oligosaccharide. J Am Chem Soc. 2010;132(26):9087–95.
47. Bider MD, Wahlberg JM, Kammerer RA, Spiess M. The oligomerization domain of the asialoglycoprotein receptor preferentially forms 2: 2 heterotetramers in vitro. J Biol Chem. 1996;271(50):31996–2001.
48. D'Souza AA, Jain P, Galdhar CN, Samad A, Degani MS, Devarajan PV. Comparative in silico–in vivo evaluation of ASGP-R ligands for hepatic targeting of curcumin Gantrez nanoparticles. AAPS J. 2013;15(3):696–706.
49. Braun JR, Willnow TE, Ishibashi S, Ashwell G, Herz J. The major subunit of the asialoglycoprotein receptor is expressed on the hepatocellular surface in mice lacking the minor receptor subunit. J Biol Chem. 1996;271(35):21160–6.
50. Kolatkar AR, Weis WI. Structural basis of galactose recognition by C-type animal lectins. J Biol Chem. 1996;271(12):6679–85.
51. Huang X, Leroux JC, Castagner B. Well-defined multivalent ligands for hepatocytes targeting via asialoglycoprotein receptor. Bioconjug Chem. 2016;28(2):283–95.
52. Marth JD, Grewal PK. Mammalian glycosylation in immunity. Nat Rev Immunol. 2008;8(11):874.
53. Weis WI, Taylor ME, Drickamer K. The C-type lectin superfamily in the immune system. Immunol Rev. 1998;163(1):19–34.
54. Rohilla R, Garg T, Goyal AK, Rath G. Herbal and polymeric approaches for liver-targeting drug delivery: novel strategies and their significance. Drug Deliv. 2016;23(5):1645–61.
55. Maitani Y, Kawano K, Yamada K, Nagai T, Takayama K. Efficiency of liposomes surface-modified with soybean-derived sterylglucoside as a liver targeting carrier in HepG2 cells. J Control Release. 2001;75(3):381–9.
56. Selim KK, Ha YS, Kim SJ, Chang Y, Kim TJ, Lee GH, Kang IK. Surface modification of magnetite nanoparticles using lactobionic acid and their interaction with hepatocytes. Biomaterials. 2007;28(4):710–6.
57. Li M, Zhang W, Wang B, Gao Y, Song Z, Zheng QC. Ligand-based targeted therapy: a novel strategy for hepatocellular carcinoma. Int J Nanomedicine. 2016;11:5645.
58. Hu HM, Zhang X, Zhong NQ, Pan SR. Study on galactose–poly (ethylene glycol)–poly (L-lysine) as novel gene vector for targeting hepatocytes in vitro. J Biomater Sci Polym Ed. 2012;23(5):677–95.
59. Wu GY, Wu CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J Biol Chem. 1987;262(10):4429–32.
60. Robinson MA, Charlton ST, Garnier P, Wang XT, Davis SS, Perkins AC, Frier M, Duncan R, Savage TJ, Wyatt DA, Watson SA. LEAPT: lectin-directed enzyme-activated prodrug therapy. Proc Natl Acad Sci. 2004;101(40):14527–32.

61. Huang Y. Preclinical and clinical advances of GalNAc-decorated nucleic acid therapeutics. Mol Ther Nucleic Acids. 2017;6:116–32.
62. Ivanenkov YA, Maklakova SY, Beloglazkina EK, Zyk NV, Nazarenko AG, Tonevitsky AG, Kotelianski VE, Majouga AG. Development of liver cell-targeted drug delivery systems: experimental approaches. Russ Chem Rev. 2017;86(8):750.
63. Fiume L, Di Stefano G, inventors; UNIVERSITA'DI BOLOGNA DIPARTIMENTO DI PATOLOGIA SPERIMENTALE, assignee. Use of conjugates of doxorubicin with lactosaminated albumin. United States patent application US 12/067,211. 2008 Sep 18.
64. Singh Y, Palombo M, Sinko PJ. Recent trends in targeted anticancer prodrug and conjugate design. Curr Med Chem. 2008;15(18):1802–26.
65. Fiume L, Mattioli A, Balboni PG, Tognon M, Barbanti-Brodano G, De Vries J, Wieland T. Enhanced inhibition of virus DNA synthesis in hepatocytes by trifluorothymidine coupled to asialofetuin. FEBS Lett. 1979;103(1):47–51.
66. Fiume L, Mattioli A, Busi C, Accorsi C. Selective penetration and pharmacological activity of lactosaminated albumin conjugates of adenine arabinoside 5-monophosphate (ara-AMP) in mouse liver. Gut. 1984;25(12):1392–8.
67. Muro S. Challenges in design and characterization of ligand-targeted drug delivery systems. J Control Release. 2012;164(2):125–37.
68. Wu J, Yuan J, Ye B, Wu Y, Xu Z, Chen J, Chen J. Dual-responsive core crosslinking glycopolymer-drug conjugates nanoparticles for precise hepatocarcinoma therapy. Front Pharmacol. 2018;9
69. Ishihara T, Kano A, Obara K, Saito M, Chen X, Park TG, Akaike T, Maruyama A. Nuclear localization and antisense effect of PNA internalized by ASGP-R-mediated endocytosis with protein/DNA conjugates. J Control Release. 2011;155(1):34–9.
70. Ma Y, Chen H, Su S, Wang T, Zhang C, Fida G, Cui S, Zhao J, Gu Y. Galactose as Broad ligand for multiple tumor imaging and therapy. J Cancer. 2015;6(7):658.
71. Willoughby JL, Chan A, Sehgal A, Butler JS, Nair JK, Racie T, Shulga-Morskaya S, Nguyen T, Qian K, Yucius K, Charisse K. Evaluation of GalNAc-siRNA conjugate activity in preclinical animal models with reduced asialoglycoprotein receptor expression. Mol Ther. 2018;26(1):105–14.
72. Varshosaz J, Asheghali F. Chondroitin/doxorubicin nanoparticulate polyelectrolyte complex for targeted delivery to HepG2 cells. IET Nanobiotechnol. 2016;11(2):164–72.
73. Yu CY, Wang YM, Li NM, Liu GS, Yang S, Tang GT, He DX, Tan XW, Wei H. In vitro and in vivo evaluation of pectin-based nanoparticles for hepatocellular carcinoma drug chemotherapy. Mol Pharm. 2014;11(2):638–44.
74. Nair JK, Willoughby JL, Chan A, Charisse K, Alam MR, Wang Q, et al. Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc. 2014;136(49):16958–61.
75. Ong ZY, Yang C, Gao SJ, Ke XY, Hedrick JL, Yan YY. Galactose-functionalized cationic polycarbonate diblock copolymer for targeted gene delivery to hepatocytes. Macromol Rapid Commun. 2013;34(21):1714–20.
76. Yang XC, Niu YL, Zhao NN, Mao C, Xu FJ. A biocleavable pullulan-based vector via ATRP for liver cell-targeting gene delivery. Biomaterials. 2014;35(12):3873–84.
77. Dehshahri A, Kazemi Oskuee R, Thomas Shier W, Ramezani M. β-Galactosylated alkyl-oligoamine derivatives of polyethylenimine enhanced pDNA delivery into hepatic cells with reduced toxicity. Curr Nanosci. 2012;8(4):548–55.
78. Di Stefano G, Colonna FP, Bongini A, Busi C, Mattioli A, Fiume L. Ribavirin conjugated with lactosaminated poly-L-lysine: selective delivery to the liver and increased antiviral activity in mice with viral hepatitis. Biochem Pharmacol. 1997;54(3):357–63.
79. Ma J, Huang C, Yao X, Shi C, Sun L, Yuan L, Lei P, Zhu H, Liu H, Wu X, Ning Q. Inhibition of hepatitis B virus and induction of hepatoma cell apoptosis by ASGPR-directed delivery of shRNAs. PLoS One. 2012;7(10):e46096.
80. Tomiya N, Jardim JG, Hou J, Pastrana-Mena R, Dinglasan RR, Lee YC. Liver-targeting of primaquine-(poly-γ-glutamic acid) and its degradation in rat hepatocytes. Bioorg Med Chem. 2013;21(17):5275–81.

81. Hashida M, Hirabayashi H, Nishikawa M, Takakura Y. Targeted delivery of drugs and proteins to the liver via receptor-mediated endocytosis. J Control Release. 1997;46(1–2):129–37.
82. Nishikawa M, Kamijo A, Fujita T, Takakura Y, Sezaki H, Hashida M. Synthesis and pharmacokinetics of a new liver-specific carrier, glycosylated carboxymethyl-dextran, and its application to drug targeting. Pharm Res. 1993;10(9):1253–61.
83. McMahon A, O'neill MJ, Gomez E, Donohue R, Forde D, Darcy R, O'driscoll CM. Targeted gene delivery to hepatocytes with galactosylated amphiphilic cyclodextrins. J Pharm Pharmacol. 2012;64(8):1063–73.
84. Popielarski SR, Pun SH, Davis ME. A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 1. Synthesis and characterization. Bioconjug Chem. 2005;16(5):1063–70.
85. Biessen EA, Beuting DM, Vietsch H, Bijsterbosch MK, Van Berkel TJ. Specific targeting of the antiviral drug 5-iodo 2′-deoxyuridine to the parenchymal liver cell using lactosylated poly-L-lysine. J Hepatol. 1994;21(5):806–15.
86. Murao A, Nishikawa M, Managit C, Wong J, Kawakami S, Yamashita F, Hashida M. Targeting efficiency of galactosylated liposomes to hepatocytes in vivo: effect of lipid composition. Pharm Res. 2002;19(12):1808–14.
87. Naicker K, Ariatti M, Singh M. PEGylated galactosylated cationic liposomes for hepatocytic gene delivery. Colloids Surf B: Biointerfaces. 2014;122:482–90.
88. Nishikawa M, Ohtsubo Y, Ohno J, Fujita T, Koyama Y, Yamashita F, Hashida M, Sezaki H. Pharmacokinetics of receptor-mediated hepatic uptake of glycosylated albumin in mice. Int J Pharm. 1992;85(1–3):75–85.
89. Zhang YN, Poon W, Tavares AJ, McGilvray ID, Chan WC. Nanoparticle–liver interactions: cellular uptake and hepatobiliary elimination. J Control Release. 2016;240:332–48.
90. Arima H, Motoyama K, Higashi T. Potential use of cyclodextrins as drug carriers and active pharmaceutical ingredients. Chem Pharm Bull. 2017;65(4):341–8.
91. Quan G, Pan X, Wang Z, Wu Q, Li G, Dian L, Chen B, Wu C. Lactosaminated mesoporous silica nanoparticles for asialoglycoprotein receptor targeted anticancer drug delivery. J Nanobiotechnol. 2015;13(1):7.
92. Zheng G, Zhao R, Xu A, Shen Z, Chen X, Shao J. Co-delivery of sorafenib and siVEGF based on mesoporous silica nanoparticles for ASGPR mediated targeted HCC therapy. Eur J Pharm Sci. 2018;111:492–502.
93. Zhang C, An T, Wang D, Wan G, Zhang M, Wang H, Zhang S, Li R, Yang X, Wang Y. Stepwise pH-responsive nanoparticles containing charge-reversible pullulan-based shells and poly (β-amino ester)/poly (lactic-co-glycolic acid) cores as carriers of anticancer drugs for combination therapy on hepatocellular carcinoma. J Control Release. 2016;226:193–204.
94. Xue WJ, Feng Y, Wang F, Guo YB, Li P, Wang L, Liu YF, Wang ZW, Yang YM, Mao QS. Asialoglycoprotein receptor-magnetic dual targeting nanoparticles for delivery of RASSF1A to hepatocellular carcinoma. Sci Rep. 2016;6:22149.
95. Zhu D, Tao W, Zhang H, Liu G, Wang T, Zhang L, Zeng X, Mei L. Docetaxel (DTX)-loaded polydopamine-modified TPGS-PLA nanoparticles as a targeted drug delivery system for the treatment of liver cancer. Acta Biomater. 2016;30:144–54.
96. Tsend-Ayush A, Zhu X, Ding Y, Yao J, Yin L, Zhou J, Yao J. Lactobionic acid-conjugated TPGS nanoparticles for enhancing therapeutic efficacy of etoposide against hepatocellular carcinoma. Nanotechnology. 2017;28(19):195602.
97. Chen W, Zou Y, Meng F, Cheng R, Deng C, Feijen J, Zhong Z. Glyco-nanoparticles with sheddable saccharide shells: a unique and potent platform for hepatoma-targeting delivery of anticancer drugs. Biomacromolecules. 2014;15(3):900–7.
98. Guan M, Zhu QL, Liu Y, Bei YY, Gu ZL, Zhang XN, Zhang Q. Uptake and transport of a novel anticancer drug-delivery system: lactosyl-norcantharidin-associated N-trimethyl chitosan nanoparticles across intestinal Caco-2 cell monolayers. Int J Nanomedicine. 2012;7:1921.
99. Thapa B, Kumar P, Zeng H, Narain R. Asialoglycoprotein receptor-mediated gene delivery to hepatocytes using galactosylated polymers. Biomacromolecules. 2015;16(9):3008–20.

100. Liang HF, Chen CT, Chen SC, Kulkarni AR, Chiu YL, Chen MC, Sung HW. Paclitaxel-loaded poly (γ-glutamic acid)-poly (lactide) nanoparticles as a targeted drug delivery system for the treatment of liver cancer. Biomaterials. 2006;27(9):2051–9.
101. Zhu X, Du Y, Yu R, Liu P, Shi D, Chen Y, Wang Y, Huang F. Galactosylated chitosan oligosaccharide nanoparticles for hepatocellular carcinoma cell-targeted delivery of adenosine triphosphate. Int J Mol Sci. 2013;14(8):15755–66.
102. Ding J, Xiao C, Li Y, Cheng Y, Wang N, He C, Zhuang X, Zhu X, Chen X. Efficacious hepatoma-targeted nanomedicine self-assembled from galactopeptide and doxorubicin driven by two-stage physical interactions. J Control Release. 2013;169(3):193–203.
103. Shi L, Tang C, Yin C. Glycyrrhizin-modified O-carboxymethyl chitosan nanoparticles as drug vehicles targeting hepatocellular carcinoma. Biomaterials. 2012;33(30):7594–604.
104. Chittasupho C, Jaturanpinyo M, Mangmool S. Pectin nanoparticle enhances cytotoxicity of methotrexate against hepG2 cells. Drug Deliv. 2013;20(1):1–9.
105. Huang L, Wang Y, Ling X, Chaurasiya B, Yang C, Du Y, Tu J, Xiong Y, Sun C. Efficient delivery of paclitaxel into ASGPR over-expressed cancer cells using reversibly stabilized multifunctional pullulan nanoparticles. Carbohydr Polym. 2017;159:178–87.
106. Pranatharthiharan S, Patel MD, Malshe VC, Pujari V, Gorakshakar A, Madkaikar M, Ghosh K, Devarajan PV. Asialoglycoprotein receptor targeted delivery of doxorubicin nanoparticles for hepatocellular carcinoma. Drug Deliv. 2017;24(1):20–9.
107. Jain N, Rajoriya V, Jain PK, Jain AK. Lactosaminated-N-succinyl chitosan nanoparticles for hepatocyte-targeted delivery of acyclovir. J Nanopart Res. 2014;16(1):2136.
108. Li X, Wu Q, Chen Z, Gong X, Lin X. Preparation, characterization and controlled release of liver-targeting nanoparticles from the amphiphilic random copolymer. Polymer. 2008;49(22):4769–75.
109. Joshi VM, Devarajan PV. Receptor-mediated hepatocyte-targeted delivery of primaquine phosphate nanocarboplex using a carbohydrate ligand. Drug Deliv Transl Res. 2014;4(4):353–64.
110. Varshosaz J, Hassanzadeh F, Sadeghi H, Khadem M. Galactosylated nanostructured lipid carriers for delivery of 5-FU to hepatocellular carcinoma. J Liposome Res. 2012;22(3):224–36.
111. Xu Z, Chen L, Gu W, Gao Y, Lin L, Zhang Z, Xi Y, Li Y. The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. Biomaterials. 2009;30(2):226–32.
112. Chen S, Tam YY, Lin PJ, Leung AK, Tam YK, Cullis PR. Development of lipid nanoparticle formulations of siRNA for hepatocyte gene silencing following subcutaneous administration. J Control Release. 2014;196:106–12.
113. Akinc A, Querbes W, De S, Qin J, Frank-Kamenetsky M, Jayaprakash KN, Jayaraman M, Rajeev KG, Cantley WL, Dorkin JR, Butler JS. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. Mol Ther. 2010;18(7):1357–64.
114. Oh H, Jo HY, Park J, Kim DE, Cho JY, Kim PH, Kim KS. Galactosylated liposomes for targeted co-delivery of doxorubicin/vimentin siRNA to hepatocellular carcinoma. Nano. 2016;6(8):141.
115. Zhou X, Zhang M, Bryant Yung HL, Zhou C, Lee LJ, Lee RJ. Lactosylated liposomes for targeted delivery of doxorubicin to hepatocellular carcinoma. Int J Nanomedicine. 2012;7:5465.
116. Hai L, Zhang ZR, Wang S, Xiao X, Wu Y. Synthesis of multivalent galactosides as targeting ligand for gene delivery. Synth Commun. 2010;40(12):1802–11.
117. Sun X, Hai L, Wu Y, Hu HY, Zhang ZR. Targeted gene delivery to hepatoma cells using galactosylated liposome-polycation-DNA complexes (LPD). J Drug Target. 2005;13(2):121–8.
118. Zhang X, Guo S, Fan R, Yu M, Li F, Zhu C, Gan Y. Dual-functional liposome for tumor targeting and overcoming multidrug resistance in hepatocellular carcinoma cells. Biomaterials. 2012;33(29):7103–14.
119. Zhang Y, Qi XR, Gao Y, Wei L, Maitani Y, Nagai T. Mechanisms of co-modified liver-targeting liposomes as gene delivery carriers based on cellular uptake and antigens inhibition effect. J Control Release. 2007;117(2):281–90.

120. Cho HA, Park IS, Kim TW, Oh YK, Yang KS, Kim JS. Suppression of hepatitis B virus-derived human hepatocellular carcinoma by NF-κB-inducing kinase-specific siRNA using liver-targeting liposomes. Arch Pharm Res. 2009;32(7):1077–86.
121. Suriano F, Pratt R, Tan JP, Wiradharma N, Nelson A, Yang YY, Dubois P, Hedrick JL. Synthesis of a family of amphiphilic glycopolymers via controlled ring-opening polymerization of functionalized cyclic carbonates and their application in drug delivery. Biomaterials. 2010;31(9):2637–45.
122. Zhong Y, Yang W, Sun H, Cheng R, Meng F, Deng C, Zhong Z. Ligand-directed reduction-sensitive shell-sheddable biodegradable micelles actively deliver doxorubicin into the nuclei of target cancer cells. Biomacromolecules. 2013;14(10):3723–30.
123. Liu S, Huang Y, Chen X, Zhang L, Jing X. Lactose mediated liver-targeting effect observed by ex vivo imaging technology. Biomaterials. 2010;31(9):2646–54.
124. Oishi M, Nagatsugi F, Sasaki S, Nagasaki Y, Kataoka K. Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages. Chembiochem. 2005;6(4):718–25.
125. Zou Y, Song Y, Yang W, Meng F, Liu H, Zhong Z. Galactose-installed photo-crosslinked pH-sensitive degradable micelles for active targeting chemotherapy of hepatocellular carcinoma in mice. J Control Release. 2014;193:154–61.
126. Yang R, Meng F, Ma S, Huang F, Liu H, Zhong Z. Galactose-decorated cross-linked biodegradable poly (ethylene glycol)-b-poly (ε-caprolactone) block copolymer micelles for enhanced hepatoma-targeting delivery of paclitaxel. Biomacromolecules. 2011;12(8):3047–55.
127. Craparo EF, Triolo D, Pitarresi G, Giammona G, Cavallaro G. Galactosylated micelles for a ribavirin prodrug targeting to hepatocytes. Biomacromolecules. 2013;14(6):1838–49.
128. Villa R, Cerroni B, Viganò L, Margheritelli S, Abolafio G, Oddo L, Paradossi G, Zaffaroni N. Targeted doxorubicin delivery by chitosan-galactosylated modified polymer microbubbles to hepatocarcinoma cells. Colloids Surf B: Biointerfaces. 2013;110:434–42.
129. Wei W, Yue ZG, Qu JB, Yue H, Su ZG, Ma GH. Galactosylated nanocrystallites of insoluble anticancer drug for liver-targeting therapy: an in vitro evaluation. Nanomedicine. 2010;5(4):589–96.
130. Duan C, Gao J, Zhang D, Jia L, Liu Y, Zheng D, Liu G, Tian X, Wang F, Zhang Q. Galactose-decorated pH-responsive nanogels for hepatoma-targeted delivery of oridonin. Biomacromolecules. 2011;12(12):4335–43.
131. Managit C, Kawakami S, Yamashita F, Hashida M. Uptake characteristics of galactosylated emulsion by HepG2 hepatoma cells. Int J Pharm. 2005;301(1–2):255–61.
132. Liu D, Hu H, Zhang J, Zhao X, Tang X, Chen D. Drug pH-sensitive release in vitro and targeting ability of polyamidoamine dendrimer complexes for tumor cells. Chem Pharm Bull. 2011;59(1):63–71.
133. Kuruvilla SP, Tiruchinapally G, Crouch AC, ElSayed ME, Greve JM. Dendrimer-doxorubicin conjugates exhibit improved anticancer activity and reduce doxorubicin-induced cardiotoxicity in a murine hepatocellular carcinoma model. PLoS One. 2017;12(8):e0181944.
134. Nasr M, Nafee N, Saad H, Kazem A. Improved antitumor activity and reduced cardiotoxicity of epirubicin using hepatocyte-targeted nanoparticles combined with tocotrienols against hepatocellular carcinoma in mice. Eur J Pharm Biopharm. 2014;88(1):216–25.
135. Peng J, Wang K, Tan W, He X, He C, Wu P, Liu F. Identification of live liver cancer cells in a mixed cell system using galactose-conjugated fluorescent nanoparticles. Talanta. 2007;71(2):833–40.
136. Yang SH, Heo D, Lee E, Kim E, Lim EK, Lee YH, Haam S, Suh JS, Huh YM, Yang J, Park SW. Galactosylated manganese ferrite nanoparticles for targeted MR imaging of asialoglycoprotein receptor. Nanotechnology. 2013;24(47):475103.
137. Kikkeri R, Lepenies B, Adibekian A, Laurino P, Seeberger PH. In vitro imaging and in vivo liver targeting with carbohydrate capped quantum dots. J Am Chem Soc. 2009;131(6):2110–2.
138. Ketkar-Atre A, Struys T, Dresselaers T, Hodenius M, Mannaerts I, Ni Y, Lambrichts I, Van Grunsven LA, De Cuyper M, Himmelreich U. In vivo hepatocyte MR imaging using lactose functionalized magnetoliposomes. Biomaterials. 2014;35(3):1015–24.

139. Kim EM, Jeong HJ, Kim SL, Sohn MH, Nah JW, Bom HS, Park IK, Cho CS. Asialoglycoprotein-receptor-targeted hepatocyte imaging using 99mTc galactosylated chitosan. Nucl Med Biol. 2006;33(4):529–34.
140. Sasaki N, Shiomi S, Iwata Y, Nishiguchi S, Kuroki T, Kawabe J, Ochi H. Clinical usefulness of scintigraphy with^ 9^ 9^ mTc-galactosyl-human serum albumin for prognosis of cirrhosis of the liver. J Nucl Med. 1999;40(10):1652–6.
141. Stadalnik RC, Vera DR, Woodle ES, Trudeau WL, Porter BA, Ward RE, Krohn KA, O'Grady LF. Technetium-99m NGA functional hepatic imaging: preliminary clinical experience. J Nucl Med. 1985;26(11):1233–42.
142. Jeong JM, Hong MK, Lee J, Son M, So Y, Lee DS, Chung JK, Lee MC. 99mTc-neolactosylated human serum albumin for imaging the hepatic asialoglycoprotein receptor. Bioconjug Chem. 2004;15(4):850–5.
143. Liang J, Zhang X, Miao Y, Li J, Gan Y. Lipid-coated iron oxide nanoparticles for dual-modal imaging of hepatocellular carcinoma. Int J Nanomedicine. 2017;12:2033.
144. Kouda K, Kil Ha-Kawa S, Tanaka Y. Increased technetium-99m-GSA uptake per hepatocyte in rats with administration of dimethylnitrosamine or hepatocyte growth factor. J Nucl Med. 1998;39:1463–7.
145. Haubner R, Vera DR, Farshchi-Heydari S, Helbok A, Rangger C, Putzer D, Virgolini IJ. Development of 68 Ga-labelled DTPA galactosyl human serum albumin for liver function imaging. Eur J Nucl Med Mol Imaging. 2013;40(8):1245–55.
146. Song MG, Jang SA, Youn H, Lee YS, Jeong JM, Chung JK, Kratz F, Lee DS, Kang KW. Enhancement of targeting ability of 64Cu-lactosaminated HSA to asialoglycoprotein receptor positive cells by addition of lactosamine residues. J Nucl Med. 2014;55(supplement 1):1046.
147. Jung Y, Hwang HS, Na K. Galactosylated iodine-based small molecule IV CT contrast agent for bile duct imaging. Biomaterials. 2018;160:15–23.
148. Yoshinori M, Masaharu M, Yuri H, Sayoko N, Nao S, Makoto H. Molecular imaging contrast media for visualization of liver function. Magn Reson Imaging. 2010;28(5):708–15.
149. Zhang D, Guo Z, Zhang P, Li Y, Su X, You L, Gao M, Liu C, Wu H, Zhang X. Simplified quantification method for in vivo SPECT/CT imaging of asialoglycoprotein receptor with 99m Tc-p (VLA-co-VNI) to assess and stage hepatic fibrosis in mice. Sci Rep. 2016;6:25377.
150. Wu LJ, Pan YD, Pei XY, Chen H, Nguyen S, Kashyap A, Liu J, Wu J. Capturing circulating tumor cells of hepatocellular carcinoma. Cancer Lett. 2012;326(1):17–22.
151. Xu W, Cao L, Chen L, Li J, Zhang XF, Qian HH, Kang XY, Zhang Y, Liao J, Shi LH, Yang YF. Isolation of circulating tumor cells in patients with hepatocellular carcinoma using a novel cell separation strategy. Clin Cancer Res. 2011;17(11):3783–93.
152. Deal KA, Cristel ME, Welch MJ. Cellular distribution of 111In-LDTPA galactose BSA in normal and asialoglycoprotein receptor-deficient mouse liver. Nucl Med Biol. 1998;25(4):379–85.
153. Richard C, Chaumet-Riffaud P, Belland A, Parat A, Contino-Pepin C, Bessodes M, Scherman D, Pucci B, Mignet N. Amphiphilic perfluoroalkyl carbohydrates as new tools for liver imaging. Int J Pharm. 2009;379(2):301–8.
154. Iimuro Y, Kashiwagi T, Yamanaka J, Hirano T, Saito S, Sugimoto T, Watanabe S, Kuroda N, Okada T, Asano Y, Uyama N. Preoperative estimation of asialoglycoprotein receptor expression in the remnant liver from CT/99mTc-GSA SPECT fusion images correlates well with postoperative liver function parameters. J Hepatobiliary Pancreat Sci. 2010;17(5):673–81.
155. Ogawara KI, Nishikawa M, Takakura Y, Hashida M. Pharmacokinetic analysis of hepatic uptake of galactosylated bovine serum albumin in a perfused rat liver. J Control Release. 1998;50(1–3):309–17.
156. Ha-Kawa SK, Tanaka Y, Hasebe S, Kuniyasu Y, Koizumi K, Ishii Y, Yamamoto K, Kashiwagi T, Ito A, Kudo M, Ikekubo K. Compartmental analysis of asialoglycoprotein receptor scintigraphy for quantitative measurement of liver function: a multicentre study. Eur J Nucl Med. 1997;24(2):130–7.

157. Vera DR, Stadalnik RC, Metz CE, Pimstone NR. Diagnostic performance of a receptor-binding radiopharmacokinetic model. J Nucl Med. 1996;37(1):160–4.
158. Sharma A, Kim EJ, Shi H, Lee JY, Chung BG, Kim JS. Development of a theranostic prodrug for colon cancer therapy by combining ligand-targeted delivery and enzyme-stimulated activation. Biomaterials. 2018;155:145–51.
159. Zeng Y, Zhang D, Wu M, Liu Y, Zhang X, Li L, Li Z, Han X, Wei X, Liu X. Lipid-AuNPs@PDA nanohybrid for MRI/CT imaging and photothermal therapy of hepatocellular carcinoma. ACS Appl Mater Interfaces. 2014;6(16):14266–77.
160. Ji DK, Zhang Y, Zang Y, Liu W, Zhang X, Li J, Chen GR, James TD, He XP. Receptor-targeting fluorescence imaging and theranostics using a graphene oxide based supramolecular glycocomposite. J Mater Chem B. 2015;3(47):9182–5.
161. Quan S, Wang Y, Zhou A, Kumar P, Narain R. Galactose-based thermosensitive nanogels for targeted drug delivery of iodoazomycin arabinofuranoside (IAZA) for theranostic management of hypoxic hepatocellular carcinoma. Biomacromolecules. 2015;16(7):1978–86.
162. Lee MH, Sessler JL, Kim JS. Disulfide-based multifunctional conjugates for targeted theranostic drug delivery. Acc Chem Res. 2015;48(11):2935–46.
163. Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, Doran J, Young AM, Burtles S, Kerr DJ. Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin. J Clin Oncol. 2002;20(6):1668–76.
164. Roy M, Finley J, Coskran T, Shen A, Xia S, Thuma B, Mascitti V. Characterization of asialoglycoprotein receptor (ASGPR) directed hepatocellular delivery using a pfizer developed targeting ligand PF-06853291. FASEB J. 2017;31(1_supplement):938–7.
165. Rajeev KG, Nair JK, Jayaraman M, Charisse K, Taneja N, O'Shea J, Willoughby JL, Yucius K, Nguyen T, Shulga-Morskaya S, Milstein S. Hepatocyte-specific delivery of siRNAs conjugated to novel non-nucleosidic trivalent N-acetylgalactosamine elicits robust gene silencing in vivo. Chembiochem. 2015;16(6):903–8.
166. Prakash TP, Graham MJ, Yu J, Carty R, Low A, Chappell A, Schmidt K, Zhao C, Aghajan M, Murray HF, Riney S. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. Nucleic Acids Res. 2014;42(13):8796–807.
167. A study of ALN-HBV in healthy adult volunteers and non-cirrhotic patients with chronic hepatitis B virus (HBV) infection [Internet]. NIH. 2016 [cited June 16, 2019]. Available from: https://clinicaltrials.gov/ct2/show/NCT02826018?term=ALN-HBV&rank=1.
168. Terada T, Iwai M, Kawakami S, Yamashita F, Hashida M. Novel PEG-matrix metalloproteinase-2 cleavable peptide-lipid containing galactosylated liposomes for hepatocellular carcinoma-selective targeting. J Control Release. 2006;111(3):333–42.

Chapter 13
CD Receptor and Targeting Strategies

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Abstract The clusters of differentiation (CD) are cell-surface receptors involved in cellular functions like activation, adhesion, and inhibition. These ubiquitous receptors express elevated levels of CD on cells which can serve as key marker in several cancers and infectious diseases. We emphasize on CD receptors involved in cancer, infections, and immune response. In particular, we cover the physiology and pathophysiology of the CD receptor and track the latest developments in targeted delivery of therapeutics and diagnostics mediated via CD receptor.

Keywords Clusters of differentiation · CD44 · Receptor · Cancer · Infectious diseases · Immunology

Abbreviations

ADC	Antibody-conjugated drug
Anti-EpCAM	Human epithelial adhesion molecule
BCR	B-cell receptor
BP	Binding peptide
Ca^{2+}	Calcium
CD	Cluster of differentiation
Ce6	Chlorine 6
CLL	Chronic lymphocytic leukemia

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CmAbs	Chemotherapeutic monoclonal antibodies
CMC	Critical micelle concentration
CR3	Complement receptor
CS	Chondroitin sulfate
CTCL	Cutaneous T-cell lymphoma
CTLs	Cytotoxic T lymphocytes
DAF	Decay acceleration factor
DM4	Maytansine derivative 4
DOX	Doxorubicin
Fcgr	Fcgamma receptor
GEM	Gemcitabine
GO	Graphene oxide
GP120	Envelope glycoprotein
GPI	glycosylphosphatidylinositol
HA	Hyaluronic acid
HSA	Human serum albumin
HCCLM3	Human hepatocellular carcinoma cell line
HCT-116	Human colorectal carcinoma cell line
HepG2	Liver hepatocellular carcinoma
HIV-1	Human immunodeficiency virus type 1
HPMA	N- (2-hydroxypropyl) methacrylamide
HSV-ttk	Herpes simplex virus truncated thymidine kinase
IFN-γ	Interferon gamma
Ig	Immunoglobin
ITAMs	Immunoreceptor tyrosine-based activation motifs
Lck	Lymphocyte-specific protein tyrosine kinase
LNP	Indinavir-lipid nanoparticles
mAb	Monoclonal antibody
MDA-MB-231	M.D. Anderson and MB stands for Metastasis Breast 231
MDR	Multidrug resistance
MHC	Major histocompatibility complex
MS4A1	Membrane Spanning 4-Domains A1
MSNs	Mesoporous silica nanoparticles
MTX	Mitoxantrone
NeoR	Neomycin B-arginine conjugate
NHL	non-Hodgkin lymphoma
OPN	Osteopontin
PDAC	Pancreatic ductal adenocarcinoma
PEG-PLGA	Polyethylene glycol–poly lactic acid-co-glycolic acid
PEG–PLGA	Polyethylene glycol–poly lactic acid-co-glycolic acid
PIT	Photo-induced therapy
pMHC	Peptide-major histocompatibility complex
PNA	Peptide nucleic acids
PTKs	Protein tyrosine kinase
PTX	Paclitaxel

RFP	Red fluorescent protein
Rluc	Renilla luciferase
SCID	Significant toxicity immunodeficient
SH2	src homology 2
SLN	Solid lipid nanoparticle
TCR	T-cell receptor
TF	Triple fusion
TFIL	Tri-functional immunoliposome
Th	T helper
TNBC	Triple negative xenograft model of breast cancer
TNF-α	Tumor necrosis factor alpha
ZAP 70	Zeta-chain-associated protein kinase

1 Introduction

The immune system protects human body from being infected by invading foreign organisms by well-controlled feedback mechanisms [1]. The cluster of differentiation (CD), which is also termed as CD molecule, CD marker, or CD antigen, is a cell surface glycoprotein that plays significant role in a number of biological functions, a major role being mediating immune reactions. CD receptors are considered to be highly promising drug targets for cancer, inflammatory, immunological, and infectious diseases [2, 3]. This chapter discusses the major CD involved in enabling improved therapy. An important focus is targeted delivery by receptor-mediated endocytosis via CD44 which has been widely investigated.

2 The Receptor

The "clusters of differentiation" (CD) is a term used to denote cell surface molecules which serve as targets for immunophenotyping of cells. As far as physiology is concerned, CD antigens can behave in many ways, often acting as important receptors or ligands. Some CD antigens do not play a part in the signaling of cells, but have other features such as cell activation, cell adhesion, and cell inhibition [4, 5]. In immunophenotyping, CD antigens are widely used as cell markers, enabling cells to be characterized based on what molecules are present on their surface. These markers serve to associate different cells with specific immune functions. Over 350 unique and distinctive CD markers have been recognized by the official list of determinants [6]. A discussion on all is beyond the purview of this chapter. We focus on six receptors, namely, CD3, CD4, CD8, CD19, CD20, and CD44 which play important roles in cancer and infectious disease. Details on other CD antigens/receptors may be referred in [6].

3 The CD Antigens and Receptors

CD antigens and receptors engage in diverse roles, acting as either receptors on cells or ligands for receptors to initiate a signal cascade that could alter some cellular function.

3.1 CD3 Complex (T-Cell Receptor (TCR) Complex)

TCR complex is noncovalently coupled with a set of five immunoglobulins associated with transmembrane proteins. Five polypeptides involved are CD3γ (21–28 kDa), CD3δ (20–28 kDa), CD3ε (20–25 kDa), CD3ζ (16 kDa), and CD3η (22 kDa) shown in Fig. 13.1a. CD3 proteins have an extracellular region of the N-terminal, a transmembrane domain, and a cytoplasmic tail with immunoreceptor tyrosine activation motifs (ITAMs). There is also an immunoglobulin-like domain. The cytoplasmic region and domain of CD3 ε, CD3 γ, CD3 δ, and CD3 ζ comprise totally 10 ITAMs which make the complex extremely susceptible to antigen binding. Aspartic acid imparts a negative charge to CD3γ, CD3δ, and CD3ε. The side

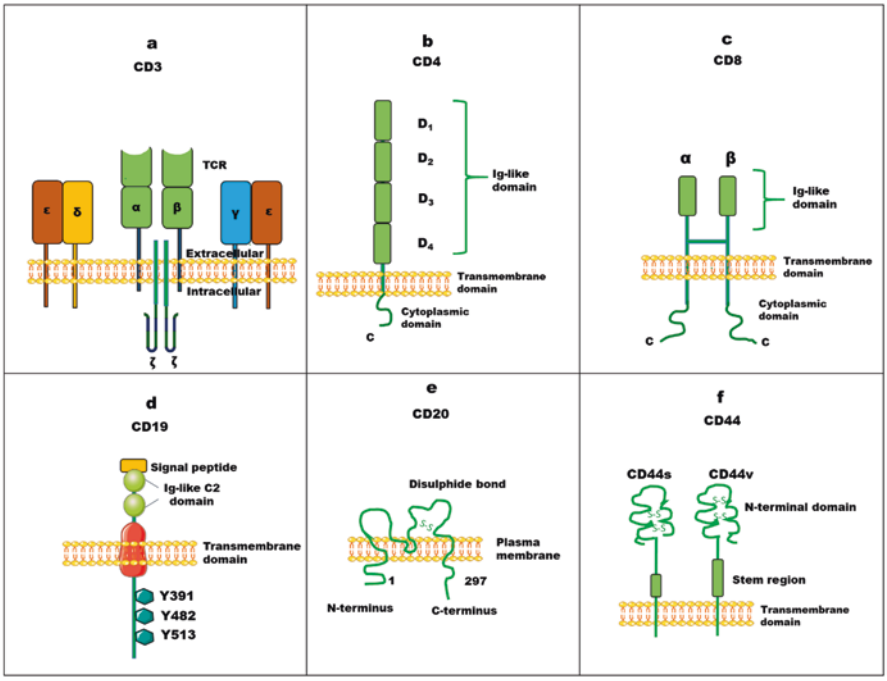

Fig. 13.1 Schematic representations of the structures of (**a**) CD3, (**b**) CD4, (**c**) CD8, (**d**) CD19, (**e**) CD20, and (**f**) CD44

chain of this negatively charged amino acid interacts with the strongly loaded side chains of proteins in the TCRα and β transmembrane areas, an interaction that can either add to complicated integrity or signaling function [7–15].

CD3 functions as T-cell coreceptor and enables activation of the cytotoxic $CD8^+$ and $CD4^+$ naive T cells. The CD3 chains serve two distinct functions for activating T cells, namely, facilitating the introduction of TCR into the plasma membrane and transduction of signals following involvement through the TCR binding site. Together, the TCR and the CD3 molecules form the TCR complex. To produce an activation signal in T lymphocytes, these CD3 chains are associated with the TCR and the ζ chain. The complex functions by transmitting intracellular signals during the recognition of antigen TCR. CD3 ε is one of at least three invariant proteins associated with the TCR variable antigen recognition chains and signal transduction function. Because CD3 is necessary for the activation of T cells, drugs especially monoclonal antibodies are explored for immunosuppressant therapies [7–9].

3.2 CD4

CD4, a transmembrane glycoprotein (Fig.13.1b), is expressed on the cell surface as a single polypeptide having four immunoglobin (Ig)-like extracellular domains, making it a superfamily member of the Ig. It has a hydrophobic transmembrane domain with 38 amino acids comprising an extremely fundamental cytoplasmic tail, together with phosphorylated serine residues. CD4's N-terminal Ig-like domains interact with the non-polymorphic α2 and β2 domains of MHC class II molecules, mediating functions of recognition and adhesion CD4's cytoplasmic tail has locations that make better physical linkage with cytotoxic T lymphocytes (CTLs, a lymphocyte-specific protein tyrosine, Lck) kinase [16].

CD4, the component of the T lymphocyte receptor and coreceptor of the human immunodeficiency virus, is downmodulated when antigen or phorbol esters activate cells. Amplification of CD4 by activated T cell as a response to an enzyme-based signaling cascade generates signal by the TCR which produces various kinds of T-helper cells. Using the extracellular domain, CD4 binds with MHC class II molecules which are present on the APC. Both the TCR complex and CD4 are designated for binding to distinct areas of the MHC II molecule during antigen presentation [17].

3.3 CD8

In both humans and mice, majority of CD8 molecules expressed on T-cell surface confined by MHC class I are CD8αβ heterodimers consisting of CD8α (38 kDa chain) and CD8β (30 kDa chain) joined by disulfide linkage which are depicted in Fig.13.1c. Some intestinal T cells on the cell surface, however, express a

CD8αα homodimer. The complete CD8 protein consists of one Ig-like extracellular hydrophobic domain and 26-residue cytoplasmic tail. Adherence of CD8 to the α3 MHC class I domain occurs following recognition of the TCR-domain-based recognition and adherence to TCRs. The CD8 cytoplasmic tail connection to Lck (lymphocyte-specific protein tyrosine kinase) is weak compared to CD4 [10–15, 18].

The CD8 cell surface marker protein helps to differentiate between the CTLs $CD8^+$ and T helper (Th) cells $CD4^+$. Involvement of CD8 in CTL coactivation has been associated with enhancing antigen sensitivity and stabilization of the TCR/MHC interaction. The CD8 coreceptor interacts with a distinct invariant region of the MHC I molecule compared to TCR, thereby enabling formation of a tripartite (TCR/peptide-major histocompatibility complex, pMHC)/CD8) complex.

3.4 CD19

CD19 antigen (95 kDa) is a transmembrane glycoprotein. It belongs to superfamily of Ig, the antigen which is categorized as a type I transmembrane protein. It consists of a single transmembrane domain, with C terminus in the cytoplasm and N terminus positioned extracellularly. It is encoded by the CD19 gene of 7.41 kilobyte situated on chromosome 16's short arm which codes for the CD19 molecule. The extracellular component includes two Ig-like domains of type C2 separated by a narrower prospective non-Ig-like domain associated with disulfide, and additional carbohydrate sites linked to N. There is no vital homology between CD19 and other recognized proteins [19]. The structure of CD19 receptor is shown in Fig.13.1d. CD19 modulates B-cell receptor (BCR)-dependent and independent signaling. Another important role is to establish intrinsic B-cell signaling thresholds. It supports growth of antigen-independent development. It also influences the activation of B cells induced by immunoglobulin that is crucial to generate an ideal immune response. In B-cell activation, CD19 as an adaptor protein helps to incorporate signaling proteins from the cytoplasm to the membrane. It is also considered important as a signal subunit for CD19/CD21 conjugation by linking with the BCR.

3.5 CD20

CD20 is a nonglycosylated phosphoprotein of 35 kDa which is embedded in the cell membrane. In humans, CD20 is encoded by the MS4A1gene, which belongs to the membrane-spanning 4A gene family. It possesses four domains spanning the membrane with a short extracellular segment of about 43 residues and the terminal amino and

carboxy N in the cytoplasm. This orientation prevents antigen shedding. It acts as a marker in lymphocytic leukemia and lymphocyte-predominant Hodgkin lymphoma and aids in targeting of monoclonal antibodies. The structure of CD20 receptor is shown in Fig.13.1e. CD20 is discovered in the pro-B stage of healthy mature B cells, in chronic lymphocytic leukemia and in Hodgkin lymphoma occurrences. CD20 is a component of cell surface complex which regulates the transfer of calcium (Ca^{2+}) channel-based intracellular signaling pathway. However, interruption of the encoding of the Ca^{2+} channel gene shows no effect on the growth of B-cells or on the execution of immune responses.

3.6 CD44

CD44 is 37-kDa protein with three domains: extracellular, intracellular, and transmembrane domains (Fig.13.1f). The extracellular domain comprising a globular protein with amino terminal (~180 aa) serves as a ligand-binding receptor mainly for hyaluronic acid (HA), a negatively charged glycosaminoglycan. It has six cysteine residues that form three disulfide bonds and sites for N glycosylation of five conserved residues in the amino acid terminal of CD44. The unconserved extracellular domain is primarily linked with carbohydrate alteration (O-glycosylation), while alternative splicing enables inclusion of additional aa sequence taken from the CD44 gene's variable exons. The transmembrane domain is useful in mediating HA binding.

Wide distribution of tissue and several isoforms enable CD44 to play a variety of biological functions as depicted in Fig.13.2.

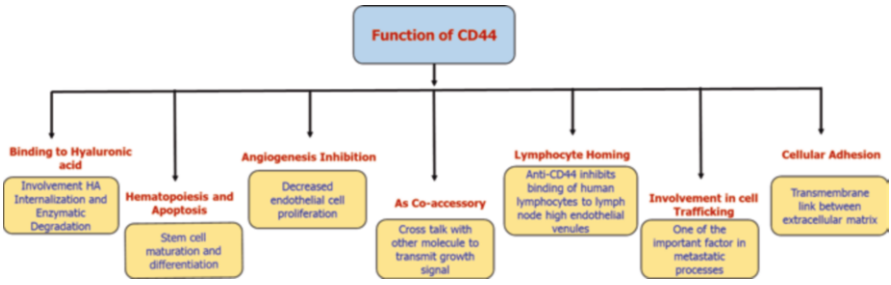

Fig. 13.2 Physiological function of CD44

4 Ligand Binding and Receptor-Mediated Signaling

4.1 CD3

Antigen-based stimulation results in conformational modifications in the CD3 polypeptide cytoplasmic tails triggered by stimulating MHC/TCR interaction with protein tyrosine kinase (PTKs). These PTKs, like Lck and Fyn, are members of the Src family. They phosphorylate conserved tyrosine residues that are present on the CD3 complex's ITAMs, to produce a protein-docking site (Zeta-chain-associated protein kinase, ZAP 70). PTKs trigger interaction between MHC/TCR which leads to modified conformation of the CD3 polypeptides in the cytoplasmic tails after antigen stimulation. CD3-integrated ZAP-70 initiates phosphorylation of transmembrane protein that serves as a linker of T cells which are activated subsequently by association with phospholipase Cγ1 (PLCγ1). Cγ-dependent pathways include signals for calcium and diacylglycerol (DAG)-initiated responses, rearrangements in cytoskeletal rearrangements and integrin activation pathways that mediate cell-to-cell and cell-to-matrix interactions [20, 21].

4.2 CD4 and CD8

TCR produce signals which are amplified by CD4 and CD8 by using their intracellular domains. MHC II recognizes CD4 and MHC I recognizes CD8 which results in phosphorylation of MHC-based engagement of TCRαβ. CD4 and CD8 are thought to function as coreceptors and are present near the TCR in the membrane which increases physical association of Lck and brings them in close proximity with the tails of the CD3 chains. The Lck causes phosphorylation of the ITAMs in the CD3 tails which results in generation of cascading event-associated intracellular signaling that leads to T-cell activation.

4.3 CD19

CD19 is mostly located on B cell and acts as BCR-associated coreceptor that enhances BCR signaling. Two signaling pathways are followed after activating the antigen which include B-cell receptor (BCR)-dependent signaling and BCR-independent signaling. First pathway includes BCR ligation through tyrosine-based CD19 phosphorylation and recruiting src homology 2 (SH2) domain. On the other hand, the CD19 receptor serves as a subunit for signal transduction for the CD19/CD21 cluster in the second pathway. These immune complexes possessing C3dg and antigen would bridge CD21, and thereby CD19, to activate the B cell.

4.4 CD20

Antibody binding to CD20 initiates movement of molecules on to lipid rafts (membrane microdomains) which are rich in cholesterol and sphingolipids (Fig.13.1e). Lipid rafts aided signal transduction, enabling receptors and signaling effectors to colocalize [22].

4.5 CD44

The involvement of CD44-associated signaling with tumorigenesis is established through HA- independent or dependent signaling pathways. Binding of low molecular weight HA can block the oncogenic pathways associated with CD44 thereby enabling tumor suppression [23–25].

5 Structure Activity Relationship (SAR)

The SAR data are reported only for CD44 which is among the most investigated CD receptor and is discussed in this section. Glycosaminoglycans are repeating amino-associated disaccharide units that connect the chain to key proteins in the receptor. The glycoaminoglycans heparan sulfate and chondroitin sulfate are large molecules with sulfate and carboxylate groups that are charged with a large number of anionic groups. These anionic side chains exhibit affinity for cations. Different isoforms of CD44 seem to have distinctive glycosylation patterns. CD44s have a tiny portion of chondroitin sulfate, whereas the CD44 keratinocyte molecule (CD44v3–v10) is primarily glycosylated by heparan sulfate. CD44 isoforms occur in both glycosylated and nonglycosylated forms, indicating that some of the activities of the molecule do not depend on the side chains of glycosaminoglycans. Due to their degree of glycosylation, some CD44 isoforms exceed 200 kDa. Intermittent size isoforms (110–160 kDa) are mainly expressed on epithelial cells [26, 27].

6 Pathophysiological Features of CD

CD receptor's expression in the pathogenesis of cancer and various infectious diseases are under investigation. Many studies have demonstrated CD expression-based regulation in various diseases. Some of the major diseases related to the pathophysiological significance of CD isoforms are discussed in this chapter.

6.1 Cancer

CD19 mediates establishment of intrinsic B-cell signaling thresholds by modifying B-cell-dependent and autonomous signaling. But CD19 is a less specific marker of B-cell lineage because CD20 is five times more expressed on B cell than CD19 antigen. CD20 is associated with chronic lymphocytic leukemia and in lymphocyte-predominant Hodgkin lymphoma. Likewise, researchers demonstrated the expression of CD20, a B-cell marker in thyroid cancers including classic papillary thyroid carcinomas and high-grade thyroid cancers [28]. Pancreatic ductal adenocarcinoma (PDAC) is the most common fatal cancer of pancreas. In PDAC, multiple immune responses and related pathways including phosphorylation of CD3 are associated with progression of pancreatic ductal adenocarcinoma [29, 30]. In pancreatic cancer, the CD44 variant isoform v6 (CD44v6) expression was found elevated, which is responsible for metastasis.

Conversion of expression from CD44v to CD44s was observed during breast cancer progression in murine model. Head and neck squamous cancer cell line was associated with overexpression of CD44v3 which lead to significant increase in cell migration. In gastrointestinal cancer, CD44v9 levels were found elevated in gastric adenocarcinoma, while the CD44v (v6–10, v7–10, v8–10) were primarily found in gastric tumors when studied in the transgenic mice model of gastric carcinogenesis [31].

6.2 HIV

The human immunodeficiency virus type 1 (HIV-1) encephalitis causes damage to the brain by longstanding HIV infection, in which predominant cells like monocytes/macrophages are available. Angiocentric $CD3^+$ T-cell-based endothelial injury resulted in infection of the child brain with HIV via the blood-brain barrier [32]. HIV-infection-based reduction in $CD4^+$ T cell gradually affects immune cell homeostasis which may cause death. HIV replicates by binding to the CD4 molecule on the outer body of Th cells which leads to destruction of $CD4^+$ T cells and gradual reduction in number of T cell [33]. While CD4 levels decline, the expansion in CD8 T cells, which occurs resulting in disturbances of the T-cell compartment, is attributed to the severe symptoms seen in HIV-infected patients. Dysregulation in the levels of CD4 and CD8 provide insights into the immunological response to acute HIV infection. CD4:CD8 ratios have profound effect on HIV disease progression which marks imbalance in CD4 T-cell regeneration and persistently elevated CD8 T-cell counts [34].

6.3 Tuberculosis

CD4 and CD8 T cell have significant role in protection from *Mycobacterium tuberculosis* [35–37]. Several reports suggest that $CD4^+$ T cells exert a profound role in protection against *Mycobacterium tuberculosis* than $CD8^+$ T cell. $CD4^+$ T cells have several subsets, like Th1, Th2, Th17, and regulatory T cells, and all these subsets come together to enable infection management. Some CD4 subsets may distinguish between active and dormant forms in *mycobacterium tuberculosis* infection. $CD4^+$ Th1 cell-based defense mechanism against infection leads to secretion of cytokines like interferon-gamma (IFN-γ) or tumor necrosis factor (TNF-α) which are involved in the recruitment and activation of monocytes and granulocytes [38].

7 Antagonist for Ligand Binding

The antagonists of CD are group of antibodies, antigen-binding portions of antibodies, small organic molecules, aptamers, or polypeptides. The efficacy of anti-CD antibodies against various cancers and HIV has been well studied. Studies with such antagonist are summarized in Table 13.1.

8 Ligands Explored

8.1 Osteopontin (OPN)

OPN binding induces CD44-mediated cell signaling which results in tumor suppression and metastasis. OPN and CD44 expression revealed positive correlation in 159 non-small cell lung cancers and 243 gastric cancer patients' tissues. CD44 knock-down or anti-CD44 antibody reduces the secretion of OPN which prevented OPN-activated c-jun-NH2-kinase signals leading to a reduction in colorectal cancer cells clonogenicity [51, 52].

8.2 Chondroitin Sulfate (CS)

CS potentiated efficacy of gemcitabine by enhancing inhibition of bladder cancer cells. CS-g-polymer-based camptothecin micelles exhibited dual receptor-mediated uptake mechanism through CD44 via clathrin-mediated endocytosis and exhibited potentiated effect in lung cancer cells compared to free drug [53, 54].

Table 13.1 Anti-CD antibodies effective against cancer and HIV

Antibody	CD specific	Comments	References
OKT3 (muromonab, murine)	CD3	Reduced acute rejection in organ transplantation procedures	[39]
Visilizumab (HuM291, humanized)	CD3	Efficacy against graft-versus-host disease in marrow transplantation (prevented rejection)	[40]
Ibalizumab (humanized)	CD4	Increased protection against HIV fusion and entry	[41]
TNX-355 (humanized)	CD4	Potent activity against HIV-1 by blocking HIV binding to the CCR5 and CXCR4 coreceptors	[42]
Zanolimumab (humanized)	CD4	Treatment of cutaneous T-cell lymphoma (CTCL) by inhibition of T-cell signaling via a dual action, namely, potent Fc-dependent lysis of $CD4^+$ T cells and downregulating CD4	[43]
AFM11 (humanized)	CD3, CD19	Promising for treatment of $CD19^+$ malignancies the advantage of improved safety risk profile	[44]
GBR401 (humanized)	CD19	Markedly, higher antibody-dependent cytotoxicity on primary malignant B cells at 500 times lower concentration compared to fucosylated similar mAb and to rituximab	[45]
SAR3419 (humanized)	CD19	Trigger cell-cycle arrest and apoptosis, leading to cell death and tumor regression	[46]
Combotox (humanized antibody huB4 conjugated to the maytansine derivative DM4)	CD19, $CD22^+$ diphtheria toxin	Enhanced specificity and potent activity in human pre-B-ALL blasts cells	[47]
DT2219ARL (humanized)	CD19, $CD22^+$ diphtheria toxin	Treatment of positive B-cell leukemia or lymphoma	[48]
Ofatumumab (Arzerra/ Genmab) (fully human)	CD20	Long-lasting depletion of B cells from peripheral blood and lymph nodes of cynomologus monkeys compared to rituximab	[49]
KM201, KM114, IM7 (Rat)	CD44	Reduced the clinical severity of arthritis in ameliorate murine model	[50]

9 Receptor-Mediated Targeting Strategies

Targeting to immune cell subsets such as CD receptor which are overexpressed in tumor conditions are more beneficial than attempts to target cancer cells directly because immune cells subsets can migrate actively into cancer cells [55].

Antibody-conjugated drugs (ADC) are considered as new therapeutics for cancer treatment and infectious diseases that comprise of CD-antibody which can specifically target disease-associated CD antigen conjugated with drug. The efficacy of ADC highly depends on intracellular-uptake-enabled apoptosis of diseased cells. Antibody-conjugated drug is internalized through receptor-mediated endocytosis, that is, clathrin/caveolae-mediated endocytosis [56–58].

9.1 Prodrugs

Targeted antibody-mediated prodrug therapy is an approach wherein the antibody recognizes the tumor site to enable localization following which enzymes at the site will hydrolyze the prodrug to release the anticancer agent in the tumor [59, 60]. Such antibody-directed enzyme prodrug therapy (ADEPT) provides many advantages such as amplification (increase the number of drug molecules at site of action), has bystander effect (ability of drug molecule to diffuse to neighboring cells), reduce systemic side effects and potential to overcome drug resistance [61].

Chimeric anti-CD20 antibody are highly effective in the therapy of B-cell malignancies and non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) either individually or as combinatorial approach with other chemotherapy treatments [62]. For example, Haisma et al. demonstrated antibody-mediated enzyme prodrug therapy by engineering monochain of fusion protein Anti-CD20 antibody and human β-Glucuronidase enzyme to convert a prodrug N-[4-doxorubicin (DOX)-N-carbonyl(-oxy ethyl) phenyl] O-β-glucuronyl carbamate into DOX at the tumor site [63].

9.2 Drug Conjugates

Conjugates can be prepared by reaction between polymer and targeting antibody/proteins or therapeutic cargoes like drug and antigens. Release of the therapeutic cargo from the conjugates at the targeted site is mediated by intracellular lysosomal enzymes after cell internalization. DOX conjugated to a copolymer of N-(2-hydroxypropyl) methacrylamide (HPMA) linked with targeting antibody such as anti-CD4, anti-CD71, reduced the MDR of DOX [64]. Targetable anti-CD3 antibody DOX-N-(2-hydroxypropyl) conjugate revealed inhibition of proliferation of human T cell and peripheral-blood lymphocyte [65]. Neomycin B-arginine conjugate (NeoR) showed 30-fold enhanced potency by inhibiting HIV particle binding to CD4 in HIV-1 strains R5 and X4 [66]. Human serum albumin (HSA)-CD4 hybrid revealed comparable in vitro binding and antiviral properties with 140-fold higher elimination half-life compared to soluble CD4 in rabbit model [67]. In another study, SAR3419 an anti-CD19 antibody was attached to the DM4 (maytansine derivative) via a cleavable disulfide linker. Mice xenograft-model-based preclinical

study of maytansine derivative–anti-CD19 conjugate followed by conduction of Phase I trials revealed antitumor efficacy with acceptable safety in human B-cell lymphoma models [68]. The conjugation of proteins and DNAs or peptide nucleic acids (PNAs) has also been explored. A tetramer of anti-CD20-Fab conjugated with PNAs showed in vitro potent and specific cytotoxicity followed by induction of apoptosis of $CD20^+$ Ramos cells [69].

9.3 Nanocarriers

9.3.1 Liposomes

Liposomes are lipid bilayer vesicles made up of natural or synthetic lipid which are used for active targeting by functionalizing with ligand. Targeted-liposome delivery of drugs enhances the interaction with the target cells by fusion with the cell membrane surface or internalization by endocytosis. Antibody-combined liposomes called immunoliposomes can be prepared by antibody lipid conjugates, covalently attaching antibody to liposomes or by adsorption on the liposomal surface.

Vaidya et al. developed tri-functional immunoliposome (TFIL) by conjugating Trastuzumab (anti-human epidermal growth factor receptor-2) and OKT-3 (anti-CD3) antibodies to DOX-loaded liposome using the micelle transfer method. TFIL revealed good cytotoxicity in breast cancer tumor cells by activating immune cells (T lymphocyte) [70]. Ishida et al. prepared sterically stabilized immunoliposomes (SIL) by attaching anti-CD19 antibody to PEG lipid derivatives which revealed stealth property enabled threefold enhancement in liposomal association efficiency with $CD19^+$ human B cell lymphoma cells [71]. Theresa et al. demonstrated that anti-CD19 antibody-conjugated liposomal DOX improved the therapeutic efficiency without significant toxicity in immunodeficient (SCID) mice inoculated with human B-cell lymphoma (Namalwa) cells [72]. Flasher et al. examined binding ability of soluble CD4-conjugated liposomes to glycoprotein gpl20 of HIV-infected cell and revealed high association efficiency compared to unconjugated liposomes [73]. Glycosylphosphatidylinositol (GPI)-anchored form of gp120 of HIV-1 which employed GPI signal of decay acceleration factor (DAF) revealed spontaneous insertion into liposome membrane using GPI anchor. This liposome exhibited specific binding to CD4. A succinct summary of other liposome-based CD44 receptor targeting for cancer is provided in Table 13.2.

9.3.2 Nanoparticles

The diversity and physical, chemical, and biological versatility of nanoparticles can be exploited for use in targeted cancer and infectious disease therapy. The specificity of antibody-conjugated nanoparticles can be exploited to focus the delivery of drug to targeted cells overexpressing the targeted surface antigen.

Table 13.2 Liposomes and nanoparticles for CD44 targeted delivery in cancer

Active/ targeting system	Ligand	Study outcome	References
Liposomes			
Timosaponin AIII	Anti-CD44 antibody	Enhanced circulation time and receptor- mediated targeting in HepG2 tumor-bearing mice models	[74]
DOX	Hyaluronic acid	Avid binding and temperature-dependent internalization in B16F10 cells (murine melanoma)	[75]
Arginine- and histidine-rich cell penetrating peptide	Hyaluronic acid	Enhanced liposomal uptake and improved efficacy in a murine hepatic carcinoma tumor xenograft model	[76]
Modified RNA Aptamer (APT)	–	Higher sensitivity and selectivity for APT liposome in human lung cancer cells (A549) and human breast cancer cells (MDA-MB-231)	[77]
Nanoparticles (NPs)			
Mitoxantrone (MTX)-conjugated HA-PEGylated magnetic nanoparticles	Hyaluronic acid (HA)	Significantly improved binding and internalization into MDA-MB-231 cell line	[81]
SN38-conjugated hyaluronic acid gold nanoparticles	Hyaluronic acid (HA)	LED radiation-induced increased cytotoxicity and retention up to 8 days in metastatic colon cancer	[82]
HA-coated mesoporous silica nanoparticles (MSNs)	Hyaluronic acid (HA)	Higher endocytosis HCT-116 cells	[83]
Camptothecin-loaded HA-decorated mesoporous silica nanoparticles	Hyaluronic acid (HA)	Rapid internalization in HeLa cells and enhanced antitumor activity compared to free drug	[84]
Hyaluronic acid-paclitaxel (PTX) -self-assembled nanoparticles	Hyaluronic acid (HA)	Fourfold reduction in tumor volume on day 14 in H22 tumor-bearing mice model	[85]
Hyaluronic acid deoxycholic acid conjugated NPs	Hyaluronic acid (HA)	Enhanced endocytosis in human breast adenocarcinoma cells with higher tumor targeting capacity in tumor-bearing mice	[86]

Biotinylated-anti-CD3 antibodies conjugated with gelatin nanoparticles are reported to achieve specific receptor-mediated endocytic cellular uptake into human T-cell leukemia cells [78]. The targeting ability of anti-human CD8 antibody coupled with PLGA (poly(lactic-co-glycolic acid) nanoparticles to CD8 expressing mammalian cells was confirmed by the presence of anti-CD8-conjugated nanoparticles in cells within 1 h while unconjugated nanoparticles took 48 h [79]. Immunonanoparticles obtained by surface functionalization of paclitaxel-loaded polymeric nanoparticles with anti-HER2 (Herceptin®) and anti-CD20 (Mabthera®) antibodies demonstrated improved anticancer drug efficacy due to active targeting using immune nanoparticles [80].

CD44 is among the more extensively evaluated receptors for targeted delivery in cancer therapy. Strategies employing CD44 targeted nanoparticles for drug delivery in cancer are enlisted in Table 13.2.

9.3.3 Micelles

Micelles are nanoscopic structures, less than 100 nm described by a hydrophobic core structure in the center and are formed by self-assembly of amphiphilic copolymers in aqueous media above a concentration called the critical micelle concentration (CMC). DOX-loaded micelles conjugated with hyaluronic acid (HA)-grafted with folic acid developed to target CD44-positive tumor, revealed significant enhancement in cellular uptake by CD44 receptor-mediated endocytosis, and promising tumor targeting in HCCLM3 (human hepatocellular carcinoma cell line) tumor-bearing nude mice [87]. In tumor-associated tissue, various glycans are often overexpressed as compared to normal tissues, which can be targeted for tumor therapy [88, 89]. Agrawal et al. conjugated hyaluronic acid with DOX-loaded PEG–PLGA (polyethylene glycol–poly lactic acid-co-glycolic acid) micelles to improve targeting in CD44 overexpressed in the Ehrlich ascites tumor-bearing mice. The developed system revealed high efficacy and also blood circulation longevity which could have facilitated high tumor accumulation [90].

9.3.4 Solid Lipid Nanoparticles (SLN)

Receptor-mediated targeting strategies for cancer or infected cells using SLN have specific applications for delivering hydrophobic cargos. For instance, Shen et al. developed paclitaxel- loaded HA-coated SLNs which provided effective intracellular delivery of paclitaxel and induced apoptosis in $CD44^{+}$ cells in vitro, while in vivo study demonstrated selectively enhanced targeting in CD44 overexpressed tumor-bearing lung tissues along with enhanced antitumor activity at low doses of paclitaxel [91]. Indinavir-lipid nanoparticles (LNP) were engineered for surface modification with two CD4 binding peptides (BP4 and BP2). These CD4-binding peptides coupled on LNPs led to blockage of CD4 with considerably enhanced anti-HIV activity [92].

9.3.5 Miscellaneous

Nanosheets

Li et al. have demonstrated HA-conjugated Graphene oxide (GO) sheets loaded with a photosensitizer Chlorine6 (Ce6). Nanohybrids of HA–GO/Ce6 revealed a significantly rapid and tenfold enhanced uptake in HeLa cancer cell line in comparison to free Ce6 which was ascribed to HA-facilitated CD44-receptor-mediated endocytosis [93].

Scaffolds

Tumor-specific, anti-CD20-bound paclitaxel-loaded albumin scaffold was engineered. In vivo biodistribution study showed greater therapeutic potential than only anti-CD20 and paclitaxel-loaded albumin scaffold. Preclinical testing of this formulation demonstrated increased deposition of antibody-mediated drug in the tumor, justifying translation for clinical evaluation of this nano-antibody-targeted chemotherapy [94].

Nanoparticles as Theranostics

Molecular imaging with targeted drug delivery in cancer has been extensively used for effective therapy and diagnosis [95]. For instance, Jiefu Jin et al. studied CD44 targeted mAb (monoclonal antibody) photosensitizer complex for simultaneous detection with photo-induced therapy (PIT) in the CD44-positive triple negative xenograft model of breast cancer (TNBC) and found it promising [95]. Liposomes were loaded with DOX or a triple fusion (TF) gene containing the truncated thymidine kinase (HSV-ttk) of herpes simplex virus and the red fluorescent protein (RFP) renilla luciferase (Rluc) for imaging. These Anti-CD44 antibody-conjugated liposomes ensured targeting of CD44 hepatocellular carcinoma cells with good cytotoxicity and also revealed delay in growth of hepatocellular tumor [96]. Capolla et al. developed anti-CD20-conjugated biodegradable nanoparticles for animal imaging with drug delivery to cancerous cells. The nanoparticles exhibited good binding specificity with MEC1 cells and chronic lymphocytic leukemia patient's cells. In vivo study proposed that the anti-CD20-conjugated nanoparticles improved tumor pharmacokinetic profiles and enhanced tumor imaging compared to nontargeted nanoparticles in human/mouse model of B-cell malignancy [97].

9.4 Clinical Studies

Monoclonal antibodies are considered as an emerging class of pharmaceuticals for targeted therapy of many human diseases such as cancer, HIV or other infectious diseases. Although many antibody-targeting strategies are developing and five antibodies are now approved for cancer or infectious diseases, more approvals are expected from among the 20 or so antibodies currently in clinical development. The efficacy of anticancer drug by targeting to site of action is being met by the exploration of a wide range CD-antigen-targeted strategies, however, there is clinical need to increase the safety [98]. In clinical trials, Besponsa® and Mylotarg™ are recently approved and >50 ADCs, which are in various phases of clinical trial as monotherapy or in combination with other anticancer drug for treatment of several types of cancer, show desirable results [99]. Clinical trial status with these receptor-mediated antibodies is listed in Table 13.3.

Table 13.3 List of CD targeting antibodies in clinical trials

Antibodies	CD Targeting moieties	Clinical trials	Indication	Outcome/ measures	References
BI-1206 with rituximab	Anti-CD32b monoclonal antibody (BI-1206) and anti-CD20 antibody	Phase 1 (ongoing)	B-cell lymphoma and leukemia	Study ongoing	[100]
Removab	Anti-EpCAM (human epithelial adhesion molecule) x Anti-CD3	Phase 1/2	Ovarian cancer	Study revealed decreased in EpCAM+ malignant cells in ascites by up to 5 log	[101]
Ibalizumab	Anti-CD4	Completed	Multidrug-resistant HIV-1 infection	Study revealed significant reduction in HIV. RNA was 1.1 log10 copies per ml	[102]
HuMax-CD4	Human monoclonal anti-CD4 antibody	Completed	Refractory cutaneous T-cell lymphoma	Well tolerated with no major toxicity	[103]
Rituximab	Anti-CD20 monoclonal antibody	Completed	Low-grade non-Hodgkin lymphoma	Well tolerated and extremely effective in patients	[104]

9.5 Advantages and Limitations

CD receptor-mediated treatments for infections and cancer revealed targeting based enhancement in efficacy which could result in long-term survival and eventual care of such diseases [105–106]. Anti-CD chemotherapeutic monoclonal antibodies (CmAbs) possess generally mild adverse effects compared to standard chemotherapy, while conjugated CmAbs precipitate serious adverse effects. These adverse effects are usually associated with the antigens which they are targeting and with the intravenous route of administration [106]. For example, bevacizumab targets tumor growth factor in the blood vessels and produces adverse effects such as high blood pressure and renal damage [107]. Rituximab treatment has infusion-related responses such as syndrome of cytokine release and syndrome of tumor lysis [108]. Other negative reactions prevalent with most CmAbs include chills, weakness, headache, nausea, vomiting, diarrhea, hypotension, and eruption.

Although excellent progress is evident in the field of antibody engineering and cancer therapy, manufacturing costs are approximately twice the price of conventional medicines [109]. There is no doubt that CmAbs engineering has marked a

major milestone in cancer therapy, and with the success rate of marketing these drugs better than that of small molecular drugs, pharmaceutical companies are anticipated to continue to move toward more specific, less harmful, and more cost-effective CmAb.

10 Conclusion

CD receptor-targeted drug delivery provides tremendous scope in clinical application. Targeting the overexpressed CD receptor represents an exciting novel approach for infectious diseases and cancer.

References

1. Zola H, editor. Medical applications of leukocyte surface molecules—the CD molecules. Molecular medicine. Springer; 2006. New York, USA.
2. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF. Leucocyte typing: human leucocyte differentiation antigens detected by monoclonal antibodies. Specification-classification-nomenclature/Typage leucocytaire Antigenes de differenciation leucocytaire humains reveles par lesanticorps monoclonaux: Rapports des etudes com. Springer Science & Business Media; 2013.
3. Erber WN. Human leucocyte differentiation antigens: review of the CD nomenclature. Pathology. 1990;22(2):61–9.
4. Zola H, Swart B, Nicholson I, Aasted B, Bensussan A, Boumsell L, et al. CD molecules 2005: human cell differentiation molecules. Blood. 2005;106(9):3123–6.
5. Zola H, Swart B, Banham A, Barry S, Beare A, Bensussan A, et al. CD molecules 2006—human cell differentiation molecules. J Immunol Methods. 2007;319(1–2):1–5.
6. Prchal J, Levi MM. Williams hematology. New York: The McGraw-Hill Companies; 2010.
7. Chetty R, Gatter K. CD3: structure, function, and role of immunostaining in clinical practice. J Pathol. 1994;173(4):303–7.
8. Alarcon B, Berkhout B, Breitmeyer J, Terhorst C. Assembly of the human T cell receptor-CD3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the CD3-gamma. delta. epsilon core and single T cell receptor alpha or beta chains. J Biol Chem. 1988;263(6):2953–61.
9. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu Rev Immunol. 1988;6(1):629–62.
10. Garcia KC, Adams JJ, Feng D, Ely LK. The molecular basis of TCR germline bias for MHC is surprisingly simple. Nat Immunol. 2009;10(2):143.
11. Godfrey DI, Rossjohn J, McCluskey J. The fidelity, occasional promiscuity, and versatility of T cell receptor recognition. Immunity. 2008;28(3):304–14.
12. Krangel MS. Mechanics of T cell receptor gene rearrangement. Curr Opin Immunol. 2009;21(2):133–9.
13. Marrack P, Scott-Browne JP, Dai S, Gapin L, Kappler JW. Evolutionarily conserved amino acids that control TCR-MHC interaction. Annu Rev Immunol. 2008;26:171–203.
14. Morris GP, Allen PM. How the TCR balances sensitivity and specificity for the recognition of self and pathogens. Nat Immunol. 2012;13(2):121.

15. Van Der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. Nat Rev Immunol. 2011;11(1):47.
16. Littman DR. The structure of the CD4 and CD8 genes. Annu Rev Immunol. 1987;5(1):561–84.
17. Ellmeier W, Sawada S, Littman DR. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu Rev Immunol. 1999;17(1):523–54.
18. Rudd CE. CD4, CD8 and the TCR-CD3 complex: a novel class of protein-tyrosine kinase receptor. Immunol Today. 1990;11:400–6.
19. Zhou L-J, Ord DC, Hughes AL, Tedder TF. Structure and domain organization of the CD19 antigen of human, mouse, and guinea pig B lymphocytes. Conservation of the extensive cytoplasmic domain. J Immunol. 1991;147(4):1424–32.
20. Pitcher LA, Van Oers NS. T-cell receptor signal transmission: who gives an ITAM? Trends Immunol. 2003;24(10):554–60.
21. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27:591–619.
22. Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. Blood. 2004;104(9):2635–42.
23. Louderbough JM, Schroeder JA. Understanding the dual nature of CD44 in breast cancer progression. Mol Cancer Res. 2011;9(12):1573–86.
24. Louderbough JM, Brown JA, Nagle RB, Schroeder JA. CD44 promotes epithelial mammary gland development and exhibits altered localization during cancer progression. Genes Cancer. 2011;2(8):771–81.
25. Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, et al. Hyaluronan–CD44 interactions as potential targets for cancer therapy. FEBS J. 2011;278(9):1429–43.
26. Naor D, Sionov RV, Ish-Shalom D. CD44: structure, function and association with the malignant process. Adv Cancer Res. 1997;71:241–319; Elsevier.
27. Sneath R, Mangham D. The normal structure and function of CD44 and its role in neoplasia. Mol Pathol. 1998;51(4):191.
28. Bychkov A, Jung CK. Aberrant expression of CD20 in thyroid cancer and its clinicopathologic significance. Hum Pathol. 2018;71:74–83.
29. Khatri I, Ganguly K, Sharma S, Carmicheal J, Kaur S, Batra SK, et al. Systems biology approach to identify novel genomic determinants for pancreatic cancer pathogenesis. Sci Rep. 2019;9(1):123.
30. Fedorchenko O, Stiefelhagen M, Peer-Zada AA, Barthel R, Mayer P, Eckei L, et al. CD44 regulates the apoptotic response and promotes disease development in chronic lymphocytic leukemia. Blood. 2013;121(20):4126–36.
31. Chen C, Zhao S, Karnad A, Freeman JW. The biology and role of CD44 in cancer progression: therapeutic implications. J Hematol Oncol. 2018;11(1):64.
32. Katsetos CD, Fincke JE, Legido A, Lischner HW, de Chadarevian J-P, Kaye EM, et al. Angiocentric CD3+ T-cell infiltrates in human immunodeficiency virus type 1-associated central nervous system disease in children. Clin Diagn Lab Immunol. 1999;6(1):105–14.
33. Vidya Vijayan K, Karthigeyan KP, Tripathi SP, Hanna LE. Pathophysiology of CD4+ T-cell depletion in HIV-1 and HIV-2 infections. Front Immunol. 2017;8:580.
34. Pahwa S, Read JS, Yin W, Matthews Y, Shearer W, Diaz C, et al. CD4/CD8 ratio for diagnosis of HIV-1 infection in infants: the Women and Infants Transmission Study. Pediatrics. 2008;122(2):331.
35. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc Natl Acad Sci. 1992;89(24):12013–7.
36. Tascon RE, Stavropoulos E, Lukacs KV, Colston MJ. Protection against Mycobacterium tuberculosis infection by CD8+ T cells requires the production of gamma interferon. Infect Immun. 1998;66(2):830–4.
37. Stenger S, Modlin RL. T cell mediated immunity to Mycobacterium tuberculosis. Curr Opin Microbiol. 1999;2(1):89–93.

38. Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F, Caccamo N. Functional signatures of human CD4 and CD8 T cell responses to Mycobacterium tuberculosis. Front Immunol. 2014;5:180.
39. Bolt S, Routledge E, Lloyd I, Chatenoud L, Pope H, Gorman SD, et al. The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties. Eur J Immunol. 1993;23(2):403–11.
40. Carpenter PA, Appelbaum FR, Corey L, Deeg HJ, Doney K, Gooley T, et al. A humanized non–FcR-binding anti-CD3 antibody, visilizumab, for treatment of steroid-refractory acute graft-versus-host disease. Blood. 2002;99(8):2712–9.
41. Bruno CJ, Jacobson JM. Ibalizumab: an anti-CD4 monoclonal antibody for the treatment of HIV-1 infection. J Antimicrob Chemother. 2010;65(9):1839–41.
42. Kuritzkes DR, Jacobson J, Powderly WG, Godofsky E, DeJesus E, Haas F, et al. Antiretroviral activity of the anti-CD4 monoclonal antibody TNX-355 in patients infected with HIV type 1. J Infect Dis. 2004;189(2):286–91.
43. Rider DA, Havenith CE, de Ridder R, Schuurman J, Favre C, Cooper JC, et al. A human CD4 monoclonal antibody for the treatment of T-cell lymphoma combines inhibition of T-cell signaling by a dual mechanism with potent Fc-dependent effector activity. Cancer Res. 2007;67(20):9945–53.
44. Reusch U, Duell J, Ellwanger K, Herbrecht C, Knackmuss SH, Fucek I, et al., editors. A tetravalent bispecific TandAb (CD19/CD3), AFM11, efficiently recruits T cells for the potent lysis of CD19+ tumor cells. MAbs; 2015: Taylor & Francis. Oxfordshire United Kingdom
45. Breton CS, Nahimana A, Aubry D, Macoin J, Moretti P, Bertschinger M, et al. A novel anti-CD19 monoclonal antibody (GBR 401) with high killing activity against B cell malignancies. J Hematol Oncol. 2014;7(1):33.
46. Blanc V, Bousseau A, Caron A, Carrez C, Lutz RJ, Lambert JM. SAR3419: an anti-CD19-Maytansinoid immunoconjugate for the treatment of B-cell malignancies. Clin Cancer Res. 2011;17(20):6448–58.
47. Sun X, Widdison W, Mayo M, Wilhelm S, Leece B, Chari R, et al. Design of antibody–maytansinoid conjugates allows for efficient detoxification via liver metabolism. Bioconjug Chem. 2011;22(4):728–35.
48. Kiprijanov SM. Bispecific antibodies and immune therapy targeting. Drug Deliv Oncol. 2012:441–82.
49. Lin TS. Ofatumumab: a novel monoclonal anti-CD20 antibody. Pharmgenomics Pers Med. 2010;3:51.
50. Mott PJ, Lazarus AH. CD44 antibodies and immune thrombocytopenia in the amelioration of murine inflammatory arthritis. PLoS One. 2013;8(6):e65805.
51. Rao G, Wang H, Li B, Huang L, Xue D, Wang X, et al. Reciprocal interactions between tumor-associated macrophages and CD44-positive cancer cells via osteopontin/CD44 promote tumorigenicity in colorectal cancer. Clin Cancer Res. 2013;19(4):785–97.
52. Pietras A, Katz AM, Ekström EJ, Wee B, Halliday JJ, Pitter KL, et al. Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. Cell Stem Cell. 2014;14(3):357–69.
53. Ferro M, Giuberti G, Zappavigna S, Perdonà S, Facchini G, Sperlongano P, et al. Chondroitin sulphate enhances the antitumor activity of gemcitabine and mitomycin-C in bladder cancer cells with different mechanisms. Oncol Rep. 2012;27(2):409–15.
54. Liu Y-S, Chiu C-C, Chen H-Y, Chen S-H, Wang L-F. Preparation of chondroitin sulfate-g-poly (ε-caprolactone) copolymers as a CD44-targeted vehicle for enhanced intracellular uptake. Mol Pharm. 2014;11(4):1164–75.
55. Schmid D, Park CG, Hartl CA, Subedi N, Cartwright AN, Puerto RB, et al. T cell-targeting nanoparticles focus delivery of immunotherapy to improve antitumor immunity. Nat Commun. 2017;8(1):1747.
56. Chalouni C, Doll S. Fate of antibody-drug conjugates in cancer cells. J Exp Clin Cancer Res. 2018;37(1):20.

57. Hellmann I, Waldmeier L, Bannwarth-Escher M-C, Maslova K, Wolter FI, Grawunder U, et al. Novel antibody drug conjugates targeting tumor-associated receptor tyrosine kinase ROR2 by functional screening of fully human antibody libraries using Transpo-mAb display on progenitor B cells. Front Immunol. 2018;9:2490.
58. Kalim M, Chen J, Wang S, Lin C, Ullah S, Liang K, et al. Intracellular trafficking of new anticancer therapeutics: antibody–drug conjugates. Drug Des Devel Ther. 2017;11:2265.
59. Niculescu-Duvaz I, Springer C. Antibody-directed enzyme prodrug therapy (ADEPT): a review. Adv Drug Deliv Rev. 1997;26(2–3):151–72.
60. Xu G, McLeod HL. Strategies for enzyme/prodrug cancer therapy. Clin Cancer Res. 2001;7(11):3314–24.
61. Bagshawe KD. Antibody-directed enzyme prodrug therapy. In: Prodrugs: Springer; 2007. p. 525–40. New York, USA
62. Hammer O, editor. CD19 as an attractive target for antibody-based therapy. MAbs; 2012: Taylor & Francis. Oxfordshire United Kingdom
63. Haisma HJ, Sernee MF, Hooijberg E, Brakenhoff RH, vd Meulen-Muileman IH, Pinedo HM, et al. Construction and characterization of a fusion protein of single-chain anti-CD20 antibody and human β-glucuronidase for antibody-directed enzyme prodrug therapy. Blood. 1998;92(1):184–90.
64. Šťastný M, Strohalm J, Plocova D, Ulbrich K, Říhová B. A possibility to overcome P-glycoprotein (PGP)-mediated multidrug resistance by antibody-targeted drugs conjugated to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer carrier. Eur J Cancer. 1999;35(3):459–66.
65. Ulbrich K, Strohalm J, Šubr V, Plocová D, Duncan R, Říhová B, editors. Polymeric conjugates of drugs and antibodies for site-specific drug delivery. Macromolecular Symposia; 1996: Wiley Online Library. New Jersey,USA
66. Cabrera C, Gutiérrez A, Barretina J, Blanco J, Litovchick A, Lapidot A, et al. Anti-HIV activity of a novel aminoglycoside-arginine conjugate. Antivir Res. 2002;53(1):1–8.
67. Yeh P, Landais D, Lemaitre M, Maury I, Crenne J-Y, Becquart J, et al. Design of yeast-secreted albumin derivatives for human therapy: biological and antiviral properties of a serum albumin-CD4 genetic conjugate. Proc Natl Acad Sci. 1992;89(5):1904–8.
68. Raufi A, Ebrahim AS, Al-Katib A. Targeting CD19 in B-cell lymphoma: emerging role of SAR3419. Cancer Manag Res. 2013;5:225.
69. Kazane SA, Axup JY, Kim CH, Ciobanu M, Wold ED, Barluenga S, et al. Self-assembled antibody multimers through peptide nucleic acid conjugation. J Am Chem Soc. 2012;135(1):340–6.
70. Vaidya T, Straubinger RM, Ait-Oudhia S. Development and evaluation of tri-functional immunoliposomes for the treatment of HER2 positive breast cancer. Pharm Res. 2018;35(5):95.
71. Ishida T, Iden DL, Allen TM. A combinatorial approach to producing sterically stabilized (stealth) immunoliposomal drugs. FEBS Lett. 1999;460(1):129–33.
72. Allen TM, Mumbengegwi DR, Charrois GJ. Anti-CD19-targeted liposomal doxorubicin improves the therapeutic efficacy in murine B-cell lymphoma and ameliorates the toxicity of liposomes with varying drug release rates. Clin Cancer Res. 2005;11(9):3567–73.
73. Flasher D, Konopka K, Chamow SM, Dazin P, Ashkenazi A, Pretzer E, et al. Liposome targeting to human immunodeficiency virus type 1-infected cells via recombinant soluble CD4 and CD4 immunoadhesin (CD4-IgG). Biochim Biophys Acta Biomembr. 1994;1194(1):185–96.
74. Lu L, Ding Y, Zhang Y, Ho RJ, Zhao Y, Zhang T, et al. Antibody-modified liposomes for tumor-targeting delivery of timosaponin AIII. Int J Nanomedicine. 2018;13:1927.
75. Eliaz RE, Szoka FC. Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells. Cancer Res. 2001;61(6):2592–601.
76. Jiang T, Zhang Z, Zhang Y, Lv H, Zhou J, Li C, et al. Dual-functional liposomes based on pH-responsive cell-penetrating peptide and hyaluronic acid for tumor-targeted anticancer drug delivery. Biomaterials. 2012;33(36):9246–58.
77. Alshaer W, Hillaireau H, Vergnaud J, Ismail S, Fattal E. Functionalizing liposomes with anti-CD44 aptamer for selective targeting of cancer cells. Bioconjug Chem. 2014;26(7):1307–13.

78. Dinauer N, Balthasar S, Weber C, Kreuter J, Langer K, von Briesen H. Selective targeting of antibody-conjugated nanoparticles to leukemic cells and primary T-lymphocytes. Biomaterials. 2005;26(29):5898–906.
79. Bicho A, Peça IN, Roque A, Cardoso MM. Anti-CD8 conjugated nanoparticles to target mammalian cells expressing CD8. Int J Pharm. 2010;399(1–2):80–6.
80. Cirstoiu-Hapca A, Bossy-Nobs L, Buchegger F, Gurny R, Delie F. Differential tumor cell targeting of anti-HER2 (Herceptin®) and anti-CD20 (Mabthera®) coupled nanoparticles. Int J Pharm. 2007;331(2):190–6.
81. Sargazi A, Shiri F, Keikha S, Majd MH. Hyaluronan magnetic nanoparticle for mitoxantrone delivery toward CD44-positive cancer cells. Colloids Surf B: Biointerfaces. 2018;171:150–8.
82. Hosseinzadeh H, Atyabi F, Varnamkhasti BS, Hosseinzadeh R, Ostad SN, Ghahremani MH, et al. SN38 conjugated hyaluronic acid gold nanoparticles as a novel system against metastatic colon cancer cells. Int J Pharm. 2017;526(1–2):339–52.
83. Yu M, Jambhrunkar S, Thorn P, Chen J, Gu W, Yu C. Hyaluronic acid modified mesoporous silica nanoparticles for targeted drug delivery to CD44-overexpressing cancer cells. Nanoscale. 2013;5(1):178–83.
84. Chen Z, Li Z, Lin Y, Yin M, Ren J, Qu X. Bioresponsive hyaluronic acid-capped mesoporous silica nanoparticles for targeted drug delivery. Chem Eur J. 2013;19(5):1778–83.
85. Xu C, He W, Lv Y, Qin C, Shen L, Yin L. Self-assembled nanoparticles from hyaluronic acid–paclitaxel prodrugs for direct cytosolic delivery and enhanced antitumor activity. Int J Pharm. 2015;493(1–2):172–81.
86. Li J, Huo M, Wang J, Zhou J, Mohammad JM, Zhang Y, et al. Redox-sensitive micelles self-assembled from amphiphilic hyaluronic acid-deoxycholic acid conjugates for targeted intracellular delivery of paclitaxel. Biomaterials. 2012;33(7):2310–20.
87. Yang Y, Zhao Y, Lan J, Kang Y, Zhang T, Ding Y, et al. Reduction-sensitive CD44 receptor-targeted hyaluronic acid derivative micelles for doxorubicin delivery. Int J Nanomedicine. 2018;13:4361.
88. Wu R-L, Sedlmeier G, Kyjacova L, Schmaus A, Philipp J, Thiele W, et al. Hyaluronic acid-CD44 interactions promote BMP4/7-dependent Id1/3 expression in melanoma cells. Sci Rep. 2018;8(1):14913.
89. Chen S, Yang K, Tuguntaev RG, Mozhi A, Zhang J, Wang PC, et al. Targeting tumor microenvironment with PEG-based amphiphilic nanoparticles to overcome chemoresistance. Nanomedicine. 2016;12(2):269–86.
90. Yadav AK, Mishra P, Mishra AK, Mishra P, Jain S, Agrawal GP. Development and characterization of hyaluronic acid–anchored PLGA nanoparticulate carriers of doxorubicin. Nanomedicine. 2007;3(4):246–57.
91. Shen H, Shi S, Zhang Z, Gong T, Sun X. Coating solid lipid nanoparticles with hyaluronic acid enhances antitumor activity against melanoma stem-like cells. Theranostics. 2015;5(7):755.
92. Endsley AN, Ho RJ. Enhanced anti-HIV efficacy of Indinavir after inclusion in CD4 targeted lipid nanoparticles. J Acquir Immune Defic Syndr (1999). 2012;61(4):417.
93. Li F, Park S-J, Ling D, Park W, Han JY, Na K, et al. Hyaluronic acid-conjugated graphene oxide/photosensitizer nanohybrids for cancer targeted photodynamic therapy. J Mater Chem B. 2013;1(12):1678–86.
94. Nevala WK, Butterfield JT, Sutor SL, Knauer DJ, Markovic SN. Antibody-targeted paclitaxel loaded nanoparticles for the treatment of CD20+ B-cell lymphoma. Sci Rep. 2017;7:45682.
95. Kim HS, Cho HR, Choi SH, Woo JS, Moon WK. In vivo imaging of tumor transduced with bimodal lentiviral vector encoding human ferritin and green fluorescent protein on a 1.5 T clinical magnetic resonance scanner. Cancer Res. 2010;70(18):7315–24.
96. Wang X, Yang L, Chen Z, Shin DM. Application of nanotechnology in cancer therapy and imaging. CA Cancer J Clin. 2008;58(2):97–110.
97. Capolla S, Garrovo C, Zorzet S, Lorenzon A, Rampazzo E, Spretz R, et al. Targeted tumor imaging of anti-CD20-polymeric nanoparticles developed for the diagnosis of B-cell malignancies. Int J Nanomedicine. 2015;10:4099.

98. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer. 2001;1(2):118.
99. Hoffmann RM, Coumbe BG, Josephs DH, Mele S, Ilieva KM, Cheung A, et al. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). Oncoimmunology. 2018;7(3):e1395127.
100. A study of BI-1206 in combination with rituximab in subjects with indolent B-cell non-Hodgkin lymphoma [updated April 1, 2019; cited 2019 June 1]. Available from: https://clinicaltrials.gov/ct2/show/NCT03571568?term=BI-1206+with+Rituximab&rank=2.
101. Burges A, Wimberger P, Kümper C, Gorbounova V, Sommer H, Schmalfeldt B, et al. Effective relief of malignant ascites in patients with advanced ovarian cancer by a trifunctional anti-EpCAM× anti-CD3 antibody: a phase I/II study. Clin Cancer Res. 2007;13(13):3899–905.
102. Dose-response study of Ibalizumab (monoclonal antibody) plus optimized background regimen in patients with HIV-1 (TMB-202) [updated May 5, 2014; cited 2019 June 1]. Available from: https://clinicaltrials.gov/ct2/show/NCT00784147.
103. HuMax-CD4 in non-cutaneous T-cell lymphoma [updated July 11, 2018; cited 2019 June 1]. Available from: https://clinicaltrials.gov/ct2/show/NCT00877656.
104. Rituximab in treating patients with non-Hodgkin's lymphoma or Hodgkin's disease [updated July 11, 2018; cited 2019 June 1]. Available from: https://clinicaltrials.gov/ct2/show/NCT00003849.
105. Porter DL, Hwang W-T, Frey NV, Lacey SF, Shaw PA, Loren AW, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci Transl Med. 2015;7(303):303ra139.
106. Hansel TT, Kropshofer H, Singer T, Mitchell JA, George AJ. The safety and side effects of monoclonal antibodies. Nat Rev Drug Discov. 2010;9(4):325.
107. Gressett SM, Shah SR. Intricacies of bevacizumab-induced toxicities and their management. Ann Pharmacother. 2009;43(3):490–501.
108. Coiffier B, Lepage E, Brière J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235–42.
109. Craik DJ, Fairlie DP, Liras S, Price D. The future of peptide-based drugs. Chem Biol Drug Des. 2013;81(1):136–47.

Chapter 14
Folate Receptor and Targeting Strategies

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Abstract The folate receptor (FR) is essential for intracellular transport of folic acid, a vital enzymatic cofactor required for cell survival and growth. FR exists in four isoforms termed as α, β, γ, and δ having variable affinity for folate ligand and exhibit differential expression in normal tissues. The receptors are known to amplify in a broad spectrum of cancers and therefore have been extensively explored to treat as well as to diagnose various cancers. This chapter presents an overview of the receptor family, ligands explored, pathophysiological features, importance of FR in therapeutics and diagnostics, different FR-mediated delivery systems, and clinical studies relying on FR targeting.

Keywords Folate · Cancer · Infectious disease · Macrophage · Targeting · Photodynamic therapy · Theranostics

Abbreviations

5-CH_3-THF	5 Methyl tetrahydrofolate
5-CHO-THF	5-Formyltetrahydrofolate
A549	human lung adenocarcinoma cell line
Ala	Alanine
ALL	Acute lymbhoblastic leukemia
AML	Acute myelogenous leukemias

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Arg	Arginine
CD	Clusters of differentiation
CDDP	Cis-diamminedichloroplatinum
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemias
CT	Computed tomography
DNA	Deoxyribonucleic acid
DPPE	Dipalmitoyl phosphatidylethanolamine
DSPC	1,2-Distearoyl-sn-glycero-3-phosphatidylcholine
DSPE	1,2-Distearoylphosphatidylethanolamine
EPR effect	Enhanced permeation and retention effect
FA	Folic acid
FA-PEG/PEO–PPO–PCL	Folic acid–polyethyleneglycol/polyethyleneoxide-poly(Ɛ-caprolactone)
FA-PEG-DOX	Folic acid–polyethylene glycol–doxorubicin
FA-PEG-PLA	Folic acid–polyethylene glycol–polylactic acid
FITC	Fluorescein isothiocyanate
FR	Folate receptor
Glu	Glutamic acid
Gly	Glycine
GPI	Glycosyl phosphatidylinositol
HDL	High density lipid
HeLa	Cervical cancer cell lines
HepG2	Hepatocellular carcinoma cell line
His	Histidine
HT 29	A human colorectal adenocarcinoma cell line
HT-1080	A human fibrosarcoma cell line
HuR	Human antigen R
IC50	Inhibitory concentration 50
IM	Intramuscular
JEG-3 and JAR	Placental choriocarcinoma cell lines
KB	Line KB is now known to be a subline of the ubiquitous KERATIN-forming tumor cell line HeLa
Leu	Leucine
mab 343	A monoclonal antibody to FR-α
mab 909	A monoclonal antibody to FR-β
met	Methionine
MKN28	Gastric cancer cell line
MRI	Magnetic resonance imaging
m-RNA	Messenger RNA
NIR	Near infra red
NLCs	nanostructured lipid carriers
PAMAM	Polyamidoamine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol

PEO–PPO–PCL	Polyethylene oxide-polypropylene oxide-poly (Ɛ-caprolactone)
PET	Positron emission tomography
P-gp	P-glycoprotein
Phe	Phenylalanine
PLGA	Poly lactic-co-glycolic acid
Pro	Proline
PTX	Paclitaxel
RFC	Reduced folate carrier
RNA	Ribonucleic acid
Ser	Serine
siRNA	Small interfering RNA
SKOV3	Ovarian cancer cell line
SLN	Solid lipid nanoparticles
TAM	Tumor-associated macrophages
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UVA	Ultraviolet A
Val	Valine

1 Introduction

The folate receptor (FR) family plays a crucial role in the uptake of folic acid, commonly known as vitamin B9, which plays a key role in the synthesis of RNA and DNA, aids epigenetic processes and is also involved in cellular proliferation and survival [1, 2]. It is overexpressed on proliferating cells which require large amounts of folic acid to satiate the need. The receptor is also expressed on activated macrophages [3]. Targeting the FR to prevent folic acid uptake or employing the FR to deliver therapeutics using nanocarrier strategies can be rewarding in improved therapy of cancer and perhaps infectious diseases.

2 The Folate Receptor

The FR family includes four FR isoforms, out of which FR-α, FR-β, and FR-δ are glycosylphosphatidylinositol (GPI) anchored receptors, whereas FR-γ is a soluble glycoprotein secreted by lymphoid cells [4, 5]. FR-α is widely expressed by epithelial linings, FR-β serves as a differential marker in macrophage maturation and FR-δ is expressed by regulatory T cells. Out of the four isoforms, FR-α and FR-β are considered to be major players for regulating the folic acid transport into the cell. FR-α appears on apical side of renal tubular epithelial cell and plays an important role in renal folate uptake, whereas FR-β is responsible for folate transport through pla-

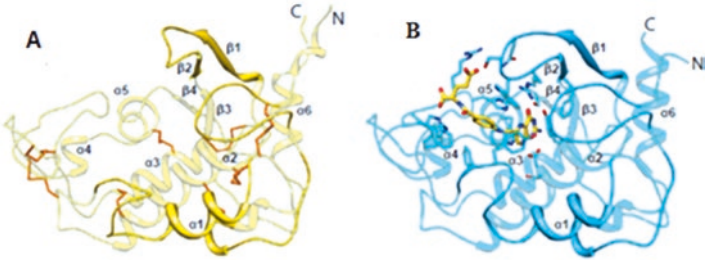

Fig. 14.1 Schematic representation of (**A**) folate receptor and (**B**) folic acid binding to folate receptor. (Reproduced from [8])

centa [6]. FR-α and FR-β are of prime importance in cancer and infectious diseases, therefore, the present chapter focuses only on FR-α and FR-β and their exploitation in receptor-mediated uptake.

FR-α and FR-β are cysteine-rich membrane-associated globular glycoproteins both consisting of 6 α-helices and 4 β-strands and many loop regions. These α helices and β strands together form a pocket for folate binding and the tertiary structure thus formed is stabilized due to the interaction between the cysteine residues which form disulfide bonds [7] (Fig. 14.1A) [8]. FR-α and FR-β have molecular weight in the range of 38–44 kDa and are encoded by two genes FOLR1 and FOLR2 located on chromosome 11 [5]. FR-α and FR-β contain around 229–236 amino acids having 68–76% similarity in amino acid structure with 3 and 2 sites for N-glycosylation respectively [6].

2.1 Ligand Binding and Internalization

FR has a deep negatively charged pocket for folate binding. Folic acid binding occurs in such a way that the basic pteroate moiety fits into the pocket, whereas glutamate residue hangs out of the positively charged entrance [7] (Fig. 14.1B) [8]. Interactions of pteroate with the FR pocket are mainly hydrogen bonding and hydrophobic interactions. The most prevalent physiological form of folic acid, 5-methyl tetrahydrofolic acid also follows the same pattern of binding to FR [7]. As glutamic acid is not involved in receptor binding, it can be utilized to conjugate chemotherapeutic agent without altering the high binding affinity of folic acid.

Once folate is bound to the receptor, it is carried into the cell via caveolae-mediated endocytosis. FRs are concentrated in small flask-like membrane invaginations known as caveolae; upon binding to folate ligand caveolae closes itself thereby sequestering FR inside the caveolae. Transient acidification occurs inside caveolae resulting in dissociation of FR complex and free apo-FRs are recycled to membrane. Released folates are then transported to cytoplasm through anion transporters (RFC) through process called as potocytosis [5, 9].

FRs have high affinity for folic acid with KD of approximately 1 nM and around 1–10 nM for reduced folate [10]. High-binding affinity between receptor and ligand forms the basis of targeted drug delivery system. There exists a significant difference in relative affinities toward FR-α for 6S (physiologic) and 6R (nonphysiologic) diastereomers of various folates such as 5-CH_3-THF and 5-CHO-THF. On the contrary, FR-β binds preferably to (6R) stereoisomer over (6S) stereoisomer of 5-CH_3-THF. Such differences in binding affinities are localized to Leu-49, Phe-104, and Gly-164 in FR-β as replacement of these residues with the corresponding amino acids from FR-α (Ala, Val, and Glu, respectively) effectively reconstitutes the FR-β-binding phenotype [6].

2.2 Natural and Synthetic Ligands

Folic acid and its prevalent physiological form 5-methyl tetrahydrofolate and 10-formyl tetrahydrofolate are naturally occurring ligands to FR. Synthetic ligands for FR-β such as [18F]fluoro-PEG-folate, [99mTc]folate, γ-[18F]-4-fluorobenzylamine-folate, [18F]-Click folate, and 2-[18F]fluorofolic acid have been reported as PET imaging agents for cancer and in inflammation diseases [11]. Lijun Xing et al. report a synthetic peptide C7 (Met-His-Thr-Ala-Pro-Gly-Trp-Gly-Tyr-Arg-Leu-Ser) which acts as a potent agonist selectively for FR-α. Docking study confirmed that C-terminal amino acid plays an important role in forming stable hydrogen bond with FR-α with affinity of around 0.3 μM. In cell line studies using SKOV3, FITC-conjugated C7 peptide exhibited improved internalization hence highlighting the possible potential of C7 peptide conjugate for receptor-mediated drug delivery [12].

2.3 Antifolates and Antifolate Receptor Antibodies

Antifolates are the inhibitors of dihydrofolate reductase enzyme and function by reducing dihydrofolic acid to tetrahydrofolic acid. Examples of such antifolates are methotrexate, pemetrexed, aminopterin, etc. Methotrexate is considered as a gold standard in the treatment of various cancers including lymphoma, acute lymbhoblastic leukemia (ALL), breast cancer, and osteogenic sarcomas [6]. Majority of antifolates have low affinity for FR with better affinity for FR-α as compared to FR-β. Affinity of methotrexate toward FR-α is 100 times lower as compared to affinity of folic acid [7].

Anti-FR antibodies are another important class of anticancer agent. Anti-FR antibodies are the antibodies against FR which elicit antibody-dependent and complement-dependent cytotoxicity upon binding to FRs thus inhibiting tumor growth. Such antibodies can be combined with chemotherapeutic agent to achieve receptor-mediated uptake for cancer therapy [13]. Two anti-FR antibodies are currently in the clinical trials, details of which are discussed in Sect. 6 of this chapter.

3 Pathophysiological Features in Cancer and Infection

3.1 Expression in Normal Tissue

FR-α is normally expressed on columnar epithelium of fallopian tube, kidney, lung (type I and II) pneumocytes [14], breast [15], and choroid plexus [16], whereas FR-β is majorly expressed on hematopoietic cells [17] and placenta [18]. FR-α is specifically expressed on the apical (luminal) side of epithelial cells of most of the human tissues [19], but on basolateral side of human retinal pigment endothelial cells [20]. It is noted that although FR is expressed in most of the normal tissues, it exhibits isoform tissue specificity and variability in extent of expression in different tissues.

3.2 Expression in Various Malignancies

FR overexpression in cancerous tissue suggests immense relevance in harnessing the receptor as a target for cancer therapy. Many cancers are known to express FRs at a level that is significantly higher as compared to normal human tissues; exploring this fact, various approaches can be developed for specific delivery of chemotherapeutic agents to cancerous tissue. FR is regulated in carcinoma at various levels such as translational level [21, 22] m-RNA level [23] or at gene amplification level.

3.2.1 Ovarian Cancer

FR overexpression is commonly observed in cancerous gynecological tissues including epithelial ovarian cancers, ovarian cancer originating from fallopian tube, fallopian adenocarcinoma and breast carcinomas [15]. FR is present in negligible amounts (<2.5 pmol/mg) in normal ovarian tissue and mucinous ovary cancers [16, 24] and in contrast, extensively expressed (16–34 pmol/mg) in nonmucinous ovarian cancers such as endometrioid, serous carcinoma, and in metastatic ovarian cancers. Therefore, ovarian cancer targeting via FR is a feasible approach and has been studied in great detail [24, 25].

3.2.2 Breast Cancer

Reports are published on folate distribution profile in normal and malignant breast tissue [18, 26]. Interestingly, focus has been shifted to establish the relation of FR expression with other hormone receptors, tumor grades, and clinical outcomes, and thus to utilize FR as a prognostic tool in breast cancer patients [27]. FR overexpression confers growth advantage in triple negative breast cancer cells in low folate environment [28]. In another study, significant FR-α expression was observed in

invasive ductal breast carcinoma as compared to nonneoplastic breast specimens. FR-α expression was inversely related to hormone receptors (estrogen and progesterone) expression [29].

3.2.3 Fallopian Tube Adenocarcinoma

FR-α is abundantly expressed specifically in columnar epithelium of normal fallopian tube. On the contrary, stromal compartment appears to be negative for both FR-α as well as for FR-β. Interestingly, in fallopian tube adenocarcinoma, FR-α was expressed significantly in epithelial cells, while mRNA for FR-β was found in stromal cells surrounding the fallopian tube adenocarcinoma which were associated with the tumor-associated macrophages (TAM) [15], proving the isoform specificity of FR. FR-α was also expressed in placental choriocarcinoma cell lines (JEG-3 and JAR) [18] and in 40% of the endometrial carcinoma samples [24].

3.2.4 Head and Neck Cancer

FR expression was studied in head and neck tumor explants and in carcinoma cell lines. Tumor tissue explants exhibited moderate levels of FR-β, but insignificant expression of FR-α [18].

3.2.5 Lung Cancer

A study of lung carcinoma samples revealed that although 73% samples were FR+, FR expression was not enhanced and was comparable to normal lung tissue [24]. However, in case of pleural mesothelioma, 72% samples exhibited 2–4 fold enhancement in FR-α mRNA expression as compared to normal lung tissue [14].

3.2.6 Brain Cancer

FR was found to be overexpressed in 4 out of 6 brain tumor biopsy samples using immunoblot technique with MOv l9 [16]. In primary pediatric malignancies involving the central nervous system, association of FR with ependymoma tumors was observed using western blot technique with MOv19 [30].

3.2.7 Renal Cancer

Significantly high levels of FR-β were observed in nonepithelial carcinomas of kidney in comparison to healthy kidney tissue, whereas epithelial carcinoma revealed overexpression of both FR-α and FR-β [18]. In one study, all renal carcinoma samples were observed to be highly positive for FR expression (12.42 pmol/mg) [16].

3.2.8 Leukemia

FR-mediated targeting is not limited to solid tumors but can be explored for leukemia cells. FR-β is specific for hematopoietic cells and carcinomas associated with hematopoietic tissue like bone marrow, spleen, and thymus [17, 31]. The presence of FR-β in normal spleen and bone marrow as well as in their malignancies including chronic (CML) and acute (AML) myelogenous leukemias, chronic lymphocytic leukemia (CLL) is demonstrated [31]. FR-β is specifically expressed by neutrophils suggesting that FR-β is restricted to neutrophil lineage. Thus, FR-β can act as potential marker for myeloid leukemia and also can be looked as a prognostic tool for mapping leukemia progression. This selective expression of FR-β isoforms in leukemic cells can be selectively targeted in chemotherapy without affecting normal cells [17].

3.2.9 Bladder Cancer

FR expression in 43% of human invasive urothelial carcinoma when analyzed using mab 343 (a monoclonal antibody to FR-α) and mab 909 (a monoclonal antibody to FR-β) revealed that both FR-α and FR-β are expressed in bladder carcinoma [32].

3.2.10 Colorectal Cancer

Although FR is not observed in normal colorectal mucosa and in adenoma, FR-α is overexpressed in primary and metastatic colorectal carcinoma [33, 34].

3.2.11 Metastatic Cancers

In case of metastatic cancers, it has been shown that some overexpressed FR-α, others overexpressed FR-β, while in case of certain cancers there was no considerable FR overexpression observed [24].

3.3 Expression in Inflammatory and Infectious Diseases

FR-β is induced on macrophages when activated by external stimuli such as infection. In activated macrophages, FR-β is coexpressed with other cell surface antigens such as CD 68, CD 80, and CD 11b. Macrophages that are not activated do not express FR. Thus, FR serves as a marker for macrophage activation. Such macrophage activation can occur due to opportunistic infections or in the case of autoimmune and inflammatory conditions. FR-β targeting provides a useful strategy for

targeting drugs selectively to activated macrophages. In rheumatoid arthritis patients, inflamed joints have been found to accumulate FR+ macrophages. Such expression of FR has been reported on a subset of peritoneal and synovial macrophages [3].

Interestingly, two reports put forth the hypothesis of FR being utilized for virus transport into the human cell. S Y Chan et al. showed that Ebola and Marburg viruses causing hemorrhagic fever in humans use FR-α as a cofactor for cellular entry [35]. His findings were challenged by Graham Simmons et al. who proved that FR-α is not required for Ebola entry into the cells [36].

4 Receptor Targeting Strategies

FR-targeted systems have shown great promise for the treatment of FR-expressing carcinomas including ovarian, cervical, breast cancer, small lung cell carcinoma, leukemia, etc. This strategy has majorly been explored for the purpose of targeting chemotherapeutic agents such as doxorubicin, paclitaxel, cisplatin, and daunorubicin to FR+ tumor tissue. Furthermore, focus is now shifting to utilize folic acid as a ligand for theranostic application that serves the dual function of noninvasive imaging cum therapy simultaneously. Progress has also been made in utilizing folate-anchored nanocarriers for relatively new cancer treatments including photodynamic therapy, neutron capture therapy, gene therapy, and immunotherapy [37].

There are two ways to target the FR, one is to encapsulate drug-imaging agent into carrier decorated with folic acid and second is through folic acid drug conjugates. Myriad of folate-conjugated nanocarriers including liposomes**,** micelles**,** nanoparticles, solid lipid nanoparticles (SLN), nanoemulsions have been evaluated for various cancers [37–39]. Moreover, the number of chemotherapeutic agents and macromolecules have been successfully conjugated to folic acid for chemotherapy, imaging and for immunotherapy in cancer. Major applications of FR targeting are directed toward cancer therapy and are discussed below.

4.1 Folate Conjugates

Folic acid is a small molecule having carboxylic acid functional group thus can be easily conjugated to drug molecule. Folate–drug conjugates have been reported for anticancer drugs such as vinblastin, doxorubicin, cis-diamminedichloro platinum, etc. In one report, folic acid and doxorubicin were separately attached at two ends of a PEG chain to obtain FA-PEG-DOX which forms nano aggregates in aqueous media [32]. In another study, vinblastin was linked to folic acid via a self-immolative linker which gets cleaved in the endosome to release vinblastin to treat invasive urothelial carcinoma [40]. Another report presents,

conjugation of cis-diamminedichloroplatinum (CDDP) and folic acid via Auricularia auricular polysaccharide aimed to reduce CDDP toxicity [41]. An aqueous nanosuspension of folate conjugated via PEG to Annonaceous acetogenins, an anticancer phytoconstituent demonstrated 76.45% tumor inhibition rate as against 25.29% for nontargeted nanosuspension in female Balb/c nude mice bearing HeLa tumor [42]. In one report, folic acid-ursolic acid conjugate was found to be more effective in cellular uptake and cytotoxicity in KB cell as compared to ursolic acid [43].

Folate can also be conjugated to haptens such as fluorescein isothiocyanate for immunotherapy against cancer and arthritis [44] and to macromolecular toxins such as momordin [45] and gelonin [46] to elucidate cytotoxic effect selectively to cancer cell.

4.2 Nanocarriers

4.2.1 Liposomes

Folate can be easily attached to liposome lipids via polyethylene glycol (PEG). Various liposomal phospholipids including 1,2-Distearoylphosphatidylethanolamine (DSPE), 1,2-Distearoyl-sn-glycero-3phosphatidylcholine (DSPC), dipalmitoyl phosphatidylethanolamine (DPPE), and Phosphatidylethanolamine (PE) have been commonly utilized for folate conjugation. A long chain PEG linker (M.W. $\approx$ 3350) has shown to enhance accessibility of attached folate to FR as compared to small molecular weight PEG (PEG 2000). Methods to incorporate FA-PEG-lipid into liposome include addition during manufacturing, or insertion post manufacturing by incubation of FA-PEG-lipid with preformed liposomes [47].

Among various chemotherapeutic agents, doxorubicin is the most evaluated anticancer drug for folic acid-based targeting using liposomes [47–52]. FR-targeting pH-sensitive liposomes were evaluated to deliver paclitaxel (PTX) to treat breast cancer metastasis in brain [53] and for coadministration of doxorubicin-cum-imatinib to overcome doxorubicin resistance in breast cancer [54]. Delivery of oligodeoxynucleotides (ODN) to FR+ KB cell line has been reported via cationic liposomes which showed 8–10-fold increment in uptake as compared to nonfolate-conjugated lipidic ODN and free ODN [55]. Folate-anchored lipopolyplex containing siRNA revealed improved survival in leukemia mice model when administered in combination with anticancer drug pretubulysin [56]. In another report, folate-targeted lipopolyplex loaded with pCMVLuc/pCMVIL-12 plasmid demonstrated 2.5-fold higher transfection efficiency of gene interleukin-12 as compared to non-targeted lipopolyplex in mouse melanoma cell line [57].

Folate-conjugated liposomes were tested as a carrier for boron neutron capture therapy to treat epithelial carcinoma. These targeted liposomes revealed improved boron uptake in KB cell line as compared to nontargeted ones [58].

4.2.2 Nanoparticles

Major advantage of nanoparticles for cancer treatment is their size in nanoscale due to which they exhibit enhanced permeation and retention effect (EPR) and allow improved biodistribution in tumor tissue. This passive targeting can be further coupled with active targeting by attaching folic acid to nanoparticle surface to target FR+ tumor. Nanoparticles can be multifunctionalized by coupling with more than one drug or imaging agent. Furthermore, coupling of folic acid as ligand to nanoparticles can be easily achieved through PEG linker [38]. Different folate-conjugated nanoparticles ranging from polymeric nanoparticles, inorganic nanoparticles to lipid-based nanoparticles have been reported in the art [39].

In one report, folic acid was attached to lysine which was conjugated to PEGylated hydroxyl-terminated hyperbranched polymer. Nanoparticles exhibited pH-dependent release of 5-fluorouracil at endosomal pH and showed enhanced uptake by HeLa cells [59]. Folic acid–chitosan nanoparticle platform was used to deliver tetramethylprazine aiming to reverse doxorubicin resistance in multidrug-resistant human breast cancer cell line (MCF-7/ADM). Stronger reversal of resistance was observed for FR-targeted nanoparticles as against nontargeted nanoparticles in FR+ MCF-7/ADM and MCF-7 cell lines, however, in FR-deficient cell lines K562/ADM and K562 no enhancement was observed [60]. Improved HeLa and HT 29 cell line uptake of curcumin-loaded folic acid linked O-carboxylmethyl chitosan nanoparticles was demonstrated [61]. The role of FR in mediating uptake was demonstrated using folate-conjugated chitosan nanoparticles wherein localization in MCF-7 cells was reduced in the presence of folate in culture media [62]. In one study, reduction-sensitive folic acid-conjugated polyphosphoester nanoparticles were observed to release encapsulated doxorubicin at high glutathione concentration in tumor site [63]. Disulfiram, an oral aldehyde dehydrogenase inhibitor encapsulated in FR-targeted PLGA-PEG nanoparticles revealed improved MCF-7 cell uptake, increased reactive oxygen species generation and reduced tumor weight in BALB/c mice with breast cancer [64].

Folate-conjugated silica and mesoporous silica have gained importance for cancer chemotherapy in recent years due their biocompatibility, chemical stability, high drug loading ability, controllable particle size, and tunable pore structure [65]. Folic acid decorated redox sensitive mesoporous silica nanoparticles loaded with cisplatin (IV)-prodrug were prepared aiming to improve localization and reduction-guided release thereby mitigating toxic side effects of cisplatin [66]. Paclitaxel-loaded folic acid-conjugated mesoporous silica nanoparticles revealed high cellular uptake and apoptosis in SMMC-7721 liver cancer cell line and also improved the accumulation of paclitaxel at the tumor site in nude mice bearing SMMC-7721 tumor [65]. Folic acid-conjugated mesoporous silica shell-core nanoparticles encapsulating rare earth metal complexes were fabricated for improved delivery to HepG2 cancer cells [67]. In another report, gold nanorods coated with folic acid-conjugated silica as a radiation sensitizer were administered along with iodine 125

radiotherapy. Enhanced apoptotic rate was seen in hepatocellular carcinoma cell line (HepG2) thus highlighting the potential of combination therapy to treat primary liver cancer [68].

Other examples of inorganic nanoparticles include hydroxyapatite nanoparticles, magnetic nanoparticles, boron nitride nanoparticles, and carbon nanoparticles, etc. In one study, 5-fluorouracil when encapsulated in nanoparticles having hydroxyapatite core and folic acid–gelatin-coated Al_2O_3 shell demonstrated improved cytotoxicity in SKOV3 ovarian cancer cell line as compared to free drug and free nanoparticles [69]. Glucose-decorated magnetic nanoparticles loaded with doxorubicin and encapsulated in erythrocyte membrane vesicles anchored with folic acid-PEG-DSPE ligand revealed 10.33 times less IC50 values for A2780 cell line and 3.93-fold IC50 reduction for OVCAR3 cell line compared to nonfolate-conjugated nanoparticles [70]. FR-targeted magnetic nanoparticles were studied for magnetic field-driven camptothecin delivery to tumor site. β-cyclodextrin-folate-anchored dextran polymer-coated camptothecin nickel–zinc ferrite nanoparticles demonstrated improved anticancer activity against HeLa cells [71]. Doxorubicin was encapsulated in folate-decorated boron nitride nanospheres which exhibited enhanced cytotoxicity against HeLa cell line in comparison with free doxorubicin and nonfolate-conjugated nanoparticles [72]. One study attempted to tackle toxicity issue of doxorubicin by encapsulating it into folate-anchored PEGylated polypyrrole nanocapsules. Enhanced tumor localization and reduced toxicity to normal organs was observed in vivo which can be attributed to FR targeting as well as pH-responsive release [73]. FR-targeted and magnetic field responsive carbon nanotube platform technology was fabricated in which folic acid is attached via PEG linker to multiwall carbon nanotubes decorated with Fe_3O_4 nanoparticles [74].

Folate–albumin nanoparticles containing cabazitaxel showed 1.87-fold increased uptake in HeLa cell line and enhanced tumor site accumulation as compared to nontargeted nanoparticles with reduction in organ toxicity compared to paclitaxel solution [75]. Paclitaxel-loaded folate–albumin nanoparticles showed improved delivery to human prostate cancer PC3 cells as compared to nontargeted nanoparticles [76]. Akt1 antisense oligonucleotide-loaded folate-decorated lipid albumin nanoparticles showed cell growth inhibition with IC 50 value of 11.9 μM as compared to 32 μM for nonfolate-conjugated nanoparticle in KB cell line [77].

4.2.3 Micelles

Micellar systems using various polymers such as polylactide co glycolide-PEG [78], pullulan-based copolymer [79], succinyl gelatin [80], folate-conjugated PEGylated *b*-copolycarbonates, and methoxy-PEG-*b*-copolycarbonates [81] are reported in the literature for delivering doxorubicin to FR+ tumors. Doxorubicin loaded into polylactide coglycolide-PEG micelles exhibited enhanced tumor accumulation and improved tumor regression in the nude mice xenograft model after systemic administration. Pullulan-based nanomicellar platform was used to coad-

minister doxorubicin along with short hairpin RNA of Beclin1 aiming to improve anticancer efficacy of doxorubicin. Beclin1 expression was found to be suppressed and apoptotic function was enhanced as a result of codelivery in FR+ HeLa cell line. Improved MCF-7 uptake was noted with folic acid-conjugated succinyl gelatin micelles in contrast to nonfolic acid-conjugated micelles. Mixed micelles of folate-conjugated PEGylated *b*-copolycarbonates and methoxy-PEG-*b*-copolycarbonates presented a reduction-sensitive system which demonstrated improved cytotoxicity in HeLa cells.

In another report on micellar system, enhanced docetaxel accumulation was noted in FR+ MCF-7 cell line when delivered via folate-conjugated PEGylated PEO–PPO–PCL as against PEO–PPO–PCL micelles [82]. Micellar system of polyethyleneimine-graft polycaprolactone-block-poly(ethylene glycol) fabricated to coadminister siRNA and paclitaxel was found to effectively deliver siRNA to SKOV-3 cells, improved cell apoptosis and could also reverse paclitaxel resistance in ovarian cancer [83]. In another report, tamoxifen-loaded folate–lysine–PEG–poly caprolactone micelles displayed enhanced cytotoxicity in the MCF-7 breast cancer cell line [84]. Dual targeting to CD44 and FR via pH-sensitive micelles was designed using a combination of the oligosaccharides of hyaluronan conjugates and folate for delivery of curcumin. Hyaluronic acid served as a ligand for CD44 targeting, while folic acid enabled FR targeting. Improved cytotoxicity of this dual targeting approach was confirmed in MCF-7 (FR+ and CD44+) and A549 (FR- and CD44+) cell lines [85].

4.2.4 Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC)

Folate-conjugated copolymer of PEG and trimethylated derivative of chitosan containing paclitaxel-loaded SLNs were developed to target FR-α overexpressed in lung cancer via inhalation chemotherapy. Improved pulmonary retention up to 6 h and reduced systemic exposure was observed with coated SLNs when administered via pulmonary route to mice [86]. In another report, SLN comprising of folate–chitosan and cholesterol served as a nonviral vector for gene delivery and demonstrated improved reporter gene expression in FR + SKOV3 cells than FR lacking A549 cells [87]. Enhanced anticancer activity of oxaliplatin-loaded folate-decorated SLN in colorectal cancer cell line HT-29 was also reported [88]. Brain targeting of docetaxel and a P-gp inhibitor ketoconazole studied via folate-anchored SLN showed improved brain concentration of docetaxel as compared to marketed formulation taxotere in a pharmacokinetic study [89].

Docetaxel-loaded NLCs composed of crodamol-solutol and soya lecithin were surface modified using DSPE-PEG2000-tumor microenvironment-sensitive polypeptides and DSPE-PEG5000-folate. Improved uptake and cytotoxicity was elicited by the NLCs in KB, HT-1080, MCF-7, and A549 cells. In athymic nude mice, the NLCs exhibited deep penetration into inner region of multicellular tumor spheroids and also demonstrated higher apoptosis in KB tumor model [90].

4.2.5 Miscellaneous Drug Carriers

Folate-conjugated polyamidoamine dendrimer was prepared to co-deliver cis-diamine platinum and siRNA against HuR mRNA for lung cancer treatment. Dendrimer system was found to be nontoxic against normal MRC9 lung fibroblast cells and enhanced cytotoxicity was seen against FR+ H1299 lung cancer cells in comparison with individual drugs. Further better efficacy was noted with the targeted folate-conjugated dendrimer system [91].

Other drug delivery carriers include DNA nanocages for doxorubicin which demonstrated 40-fold higher uptake in HeLa cell line as compared to FR-deficient cell lines [92]. Chitosan and hyaluronic acid-based injectable in situ thermosensitive hydrogel loaded with doxorubicin-anchored folate-conjugated graphene oxide showed great promise for intratumoral delivery in breast cancer. The targeted in situ gel showed significant reduction in MCF-7 tumor volume induced in BALB/c nude mice as compared to plain doxorubicin and nontargeted gel [93]. A dual complex of folate-methyl-β-cyclodextrin with adamantane–hyaluronic acid showed maximum cytotoxic effect in human colon cancer cell line HCT116 cells (FR-α [+], CD44 [+]) as compared to other treatment groups [94].

4.3 Other Applications

4.3.1 Photodynamic Therapy

Photodynamic therapy uses a photosensitizer which when irradiated with light generates cytotoxic reactive oxygen species. In one report, folate-decorated poly lactic-co-glycolic acid (PLGA) nanoparticles loaded with photosensitizer pheophorbide displayed enhanced cytotoxicity and also improved accumulation in the tumor in MKN28 tumor-bearing mice [95]. Some conjugated polymers are reported to generate reactive oxygen species when irradiated with light and thus by themselves can serve as a photosensitizer. Example of one such delivery system is nanoparticles prepared using folic acid-conjugated PEGylated poly(styrene–comaleic anhydride) which confirmed improved cytotoxicity in HeLa cell line [96]. Folate-conjugated photoresponsive gold-decorated polymer nanoparticles have been reported for brain cancer therapy [97]. Hypocrellin B (HB) as photosensitizer was loaded into biodegradable FA-PEG-PLA micelles for intraperitoneal delivery to ovarian cancer wherein 20-fold more accumulation of photosensitizer at tumor site than nontargeted micelles was demonstrated [98]. In another report, indocyanine green encapsulated mixed micellar system revealed enhanced growth suppression in HeLa cell line than in FR-deficient HT-29 cell line [99].

Reports also cite FR-targeted strategies with combined approach of photothermal/photodynamic therapy and photoresponsive chemotherapy. For example, core-shell type of nanoparticles consisting NIR fluorescent dye indocyanine green encapsulated poly(D,L-lactide-co-glycolic acid) as core and platinum (IV) prodrug

-DSPE-PEG-FA as coat were found to be more toxic to human ovarian carcinoma SKOV3 cells than non-FR-targeted and non-IR-activated systems [100]. Doxorubicin–thioacetal photocleavable linker–folic acid-conjugated dendrimer coated onto a core consisting of upconversion nanocrystal and photosensitizer protoporphyrin IX aided photoresponsive delivery of doxorubicin. Upon NIR emission, upconversion nanocrystal emitted UV and visible light which cleaved the linker thereby triggering doxorubicin release. Cytotoxicity against FR + KB carcinoma cells increased significantly when nanosystem was irradiated with UVA or NIR (980 nm) light [101]. Curcumin-loaded nanocarriers prepared by immobilizing gold nanoparticles onto folate-modified dendritic mesoporous silica-coated graphene oxide nanosheets for pH-sensitive photothermal therapy showed improved internalization and cytotoxicity as compared to free curcumin, blank nanocarriers, and nanocarriers without folate conjugation in MCF-7 cells [102].

4.3.2 Diagnostic Applications

FR-targeted imaging agents serve as a noninvasive tool to analyze the extent of FR expression in tumor which can serve as a basis for treatment selection. Folate can either be conjugated directly to inorganic nanoparticles which by themselves can act as imaging agent. Examples of this strategy include superparamagnetic iron oxide nanoparticle for MRI in breast cancer [103], amino-functionalized mesoporous silica nanoparticles as cytosensors to selectively detect MCF-7 breast cancer cells [104], upconversion luminescent nanoparticles as NIR imaging agent for imaging MCF-7 cells [105], folic acid–cysteamine- modified gold nanoparticles for computed tomography (CT) imaging [106] copper nanoclusters [107], and graphene quantum dots [108] for cancer fluorescent imaging. Folate conjugation to NIR chromophores [109, 110] and PET agent [111] to diagnose FR + tumors have been reported.

The other option is to use folic acid decorated nanocarriers for encapsulating imaging agent, for example, bioimaging of SKOV-3 ovarian cancer cells using squaraine dye (SQR23)-loaded PEGylated liposomes [112], NIR fluorescent dye-loaded HDL nanoparticles for metastatic ovarian cancer imaging [113], gadolinium-loaded dendrimer for imaging and characterization of ovarian tumor [114], and 99mTc conjugated oligomeric nanoparticles for liver cancer [115], etc.

4.3.3 Theranostic Application

Imaging agent and chemotherapeutic agent can also be simultaneously attached or encapsulated into nanocarriers rendering it suitable for theranostic purpose. Doxorubicin is the most commonly used chemotherapeutic agent for theranostic application. Iron oxide nanoparticles conjugated with doxorubicin and folic acid were developed for intratumor controlled delivery of doxorubicin with MRI capability [116]. Multifunctional polymeric nanoparticles loaded with doxorubicin and

superparamagnetic iron oxide were explored for liver cancer chemotherapy and imaging [117]. In another report, doxorubicin and superparamagnetic iron oxide nanocrystals were loaded in a core-shell type of nanocarrier [118]. Other reports on theranostic applications include combinations of fluorescent mesoporous silica and palladium complex [119] and gold nanoparticles containing ^{177}Lu-dendrimer (PAMAM-G4)-folate-bombesin as theranostic radiopharmaceuticals for breast cancer [120]. Nanoemulsion loaded with docetaxel and gadolinium was reported for targeting FR-α overexpression in ovarian cancer as a theranostic [121].

5 Folate Receptor Targeting for Infectious Diseases

FR-β is overexpressed on activated macrophages; hence, FR-β can be used to target macrophages in infectious and inflammatory diseases. Puligujja et al. reported nanosuspension of atazanavir coated with folate-decorated poloxamer 407 to target macrophage FR-β for enhanced uptake, retention, and antiviral activity of atazanavir. Targeted nano formulation showed fivefold enhanced plasma and tissue distribution as compared to nontargeted one when injected IM in Balb/cJ mice [122].

Our research group has developed FR-targeted polymeric nanoparticles to deliver rifampicin to the macrophages in the lungs for tuberculosis therapy. Date et al. synthesized folate-decorated rifampicin Gantrez AN-119 (poly methyl vinyl ether maleic anhydride copolymer) nanoparticles using simple ionic complexation method. Noncovalent approach of conjugating folic acid to Gantrez AN-119 was achieved using ionic interaction between protonated folic acid and carboxylic acid group of Gantrez AN-119. A 480% improvement in rifampicin uptake with FA-targeted nanoparticles and 300% with nontargeted nanoparticles compared to free drug in human macrophage cell line U-937 confirmed the role of FR in macrophage targeting [123]. Similarly, Patel and coworkers reported increased macrophage uptake of folate-anchored rifampicin poly(ethylene sebacate) naoparticles in comparison with the nonfolate-conjugated poly(ethylene sebacate) nanoparticles in U-937 cells [124].

6 Clinical Studies

Out of various FR-targeted delivery systems discussed earlier, only two major strategies have been translated to clinical trials; one of which is folate-conjugated drug/imaging agent (folate analog) and the second one is anti-FR antibody or its conjugate with chemotherapy agent [125]. Based on studies failing to meet desired outcomes; trials for vintafolide [126, 127], epofolate [128, 129], folate-FITC [130, 131] were suspended or even terminated. A phase 3 trial; wherein ^{99m}TC-conjugated etarfolatide evaluated as an imaging agent with vintafolide was suspended as the study failed to meet progression-free survival outcome measures and not because of the safety issues of ^{99m}Tc etarfolatide. This retains possibility for evaluation of this conjugate

Table 14.1 List of folate receptor targeting moieties in clinical trials

FR-targeting moieties	Type of cancer	Clinical trial status	Outcome	References
Folate–drug conjugate				
EC0489 Folate-conjugated to desacetyl vinblastine hydrazide via modified linker	Refractory or metastatic tumors	Phase 1 completed	Not reported	[133, 134]
EC0225 Folate conjugated to vinca alkaloid and mitomycin	Refractory or metastatic tumors	Phase 1 completed	Well tolerated at dose levels $\leq$2.3 mg/m^2	[135, 136]
EC1456 Folate conjugated to tubulysin B hydrazide	Solid tumors, nonsmall-cell lung carcinoma	Phase 1 completed	Well tolerated with no treatment-related deaths	[137, 138]
Folate-imaging agent conjugates				
OTL38 FR-targeted near infrared contrast agent	Ovarian cancer	Phase 3 recruiting	Not available	[133, 139]
Anti-FR antibody				
Farletuzumab (MORAb-003) Fully humanized antibody derived from the murine antibody LK26	Epithelial ovarian cancer	Phase 3 completed	Both doses (1.25 mg/kg and 2.5 mg/kg) tested failed to meet progression-free survival end point of the study	[13, 140]
Mirvetuximab soravtansine (IMGN853) Humanized anti-FRα antibody conjugated to tubulin-disrupting maytansinoid DM4	Ovarian cancer, primary peritoneal carcinoma, fallopian tube cancer	Phase 3 active, not recruiting	Not available	[141–143]

with other FR-targeted carriers [126, 127, 132]. Table 14.1 summarizes the clinical trials based on FR targeting which are ongoing or cite positive outcomes.

7 Advantages and Limitations

FR-mediated targeting presents a promising approach for cancer treatment and diagnosis particularly due to significant amplification in certain malignancies. FR-α can be targeted to treat carcinomas of epithelial origin, whereas hematopoietic carcinomas can be targeted through FR-β. Possible bypass of lysosomal destruction due to caveolin-mediated endocytosis, which can deliver drugs to the cytosol and cell organelles is yet another major advantage of FR-based targeting [123].

Folic acid, due to its high affinity for FR, small molecular weight, nonimmunogenic nature makes this a viable approach [37].

The major limitation of the FR-mediated targeting is its ubiquitous expression on majority of organs which can lead to toxicity due to nonspecific binding. Fine balance needs to be struck between treatment efficacy and side effects while considering FR for targeted delivery. FR targeting needs to be judiciously utilized unlike other receptors discussed in this book which are specifically expressed by certain organs.

8 Future Directions

Considering poor clinical outcomes and severe off site toxicity of current chemotherapy, personalized anticancer treatment seems to be the future of cancer therapy wherein FR can serve as a reliable diagnostic, prognostic tool and a medium for transporting cargo for active tumor targeting. Newer therapies such as vaccines, immunostimulants, gene therapy, and antisense oligonucleotides delivery can be further exploited using FR-mediated uptake to treat various cancers. Further reports on FR involvement in infectious diseases open up a great opportunity for researchers to understand the pathophysiological role of FR and its targeting potential to treat infectious diseases.

References

1. Kelemen LE. The role of folate receptor α in cancer development, progression and treatment: cause, consequence or innocent bystander? Int J Cancer. 2006;119(2):243–50.
2. Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab. 2000;71(1–2):121–38.
3. Xia W, Hilgenbrink AR, Matteson EL, Lockwood MB, Cheng JX, Low PS. A functional folate receptor is induced during macrophage activation and can be used to target drugs to activated macrophages. Blood. 2009;113(2):438–46.
4. Shen F, Wu M, Ross JF, Miller D, Ratnam M. Folate receptor type ϒ is primarily a secretory protein due to lack of an efficient signal for glycosylphosphatidylinositol modification: protein characterization and cell type specificity. Biochemistry. 1995;34(16):5660–5.
5. Antony AC. Folate receptors. Annu Rev Nutr. 1996;16(1):501–21.
6. Matherly LH, Goldman D. Membrane transport of folates. Vitamins and hormones. 2003 Jan 1;66:405–57. Academic Press, USA.
7. Chen C, Ke J, Zhou XE, Yi W, Brunzelle JS, Li J, Yong E-L, Xu HE, Melcher K. Structural basis for molecular recognition of folic acid by folate receptors. Nature. 2013;500(7463):486–9.
8. Wibowo AS, Singh M, Reeder KM, Carter JJ, Kovach AR, Meng W. Structures of human folate receptors reveal biological trafficking states and diversity in folate and antifolate recognition. PNAS. 2013;110(38):15180–8.
9. Sabharanjak S, Mayor S. Folate receptor endocytosis and trafficking. Adv Drug Deliv Rev. 2004;56(8):1099–109.
10. Shannessy DJO, Somers EB, Albone E, Cheng X, Park C, Tomkowicz BE, et al. Characterization of the human folate receptor alpha via novel antibody-based probes. Oncotarget. 2011;2(12):1227–43.

11. Pissarek M. Activated microglia in the brain: mitochondrial and cell membrane-associated targets for positron emission tomography. World J Neurosci. 2019;8:50–81.
12. Xing L, Xu Y, Sun K, Wang H, Zhang F, Zhou Z, et al. Identification of a peptide for folate receptor alpha by phage display and its tumor targeting activity in ovary cancer xenograft. Sci Rep. 2018;8(1):8426.
13. Walters CL, Arend RC, Armstrong DK, Naumann RW, Alvarez RD. Folate and folate receptor alpha antagonists mechanism of action in ovarian cancer. Gynecol Oncol. 2013;131(2):493–8.
14. Bueno R, Appasani K, Mercer H, Lester S, Sugarbaker D. The α folate receptor is highly activated in malignant pleural mesothelioma. J Thorac Cardiovasc Surg. 2001;121(2):225–33.
15. O'Shannessy DJ, Somers EB, Wang LC, Wang H, Hsu R. Expression of folate receptors alpha and beta in normal and cancerous gynecologic tissues: correlation of expression of the beta isoform with macrophage markers. J Ovarian Res. 2015;8(1):1–9.
16. Weitman SD, Lark RH, Coney LR, Fort DW, Frasca V, Zurawski VR, et al. Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. Cancer Res. 1992;52(12):3396–401.
17. Ross JF, Wang H, Behm FG, Mathew P, Wu M, Booth R, et al. Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. Cancer. 1999;85(2):348–57.
18. Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Cancer. 1994;73(9):2432–43.
19. Chancy CD, Kekuda R, Huang W, Prasad PD, Kuhnel JM, Sirotnak FM, et al. Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor α in mammalian retinal pigment epithelium. J Biol Chem. 2000;275(27):20676–84.
20. Wu M, Gunning W, Ratnam M. Expression of folate receptor type α in relation to cell type, malignancy, and differentiation in ovary, uterus, and cervix. Cancer Epidemiol Biomark Prev. 1999;8(9):775–82.
21. Sun X, Antony AC. Evidence that a specific interaction between an 18-base cis-element in the human folate receptor-alpha mRNA and a 46-kDa cystolic trans-factor is critical for translation. J Biol Chem. 1996;271(41):25539–47.
22. Xiao X, Tang YS, Mackins JY, Sun XL, Jayaram HN, Hansen DK, et al. Isolation and characterization of a folate receptor mRNA-binding trans-factor from human placenta. Evidence favoring identity with heterogeneous nuclear ribonucleoprotein E1. J Biol Chem. 2001;276(44):41510–7.
23. Chung-Tsen HDB. Altered folate-binding protein mRNA stability in KB cells grown infolate-deficient medium. Biochem Pharmacol. 1993;45(12):2537–45.
24. Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. Anal Biochem. 2005;338(2):284–93.
25. Li PY, Del Vecchio S, Fonti R, Carriero MV, Potena MI, Botti G, Miotti S, Lastoria S, Menard S, Colnaghi MISM. Local concentration of folate binding protein GP38 in sections of human ovarian carcinoma by in vitro quantitative autoradiography. J Nucl Med. 1996;37(4):665–72.
26. O'Shannessy DJ, Somers EB, Maltzman J, Smale R, Fu YS. Folate receptor alpha (FRA) expression in breast cancer: identification of a new molecular subtype and association with triple negative disease. Springerplus. 2012 Dec 1;1(1):22.
27. Zhang Z, Wang J, Tacha DE, Li P, Bremer RE, Chen H, et al. Folate receptor α associated with triple-negative breast cancer and poor prognosis. Arch Pathol Lab Med. 2014;138(7):890–5.
28. Necela BM, Crozier JA, Andorfer CA, Lewis-Tuffin L, Kachergus JM, Geiger XJ, et al. Folate receptor-α (FOLR1) expression and function in triple negative tumors. PLoS One. 2015;10(3):e0122209.
29. Aboulhagag NAER, Torky RF, Fadel SA. Folate receptor α is associated with poor clinicopathological perspectives in breast carcinoma. Pathophysiology. 2018;25(1):71–6.
30. Weitman SD, Frazier KM, Kamen BA. The folate receptor in central nervous system malignancies of childhood. J Neuro-Oncol. 1994;21(2):107–12.

31. Shen F, Ross JF, Wang X, Ratnam M. Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. Biochemistry. 1994;33(5):1209–15.
32. Dhawan D, Ramos-Vara JA, Naughton JF, Cheng L, Low PS, Rothenbuhler R, et al. Targeting folate receptors to treat invasive urinary bladder cancer. Cancer Res. 2013;73(2):875–84.
33. D'Angelica M, Ammori J, Gonen M, Klimstra DS, Low PS, Murphy L, et al. Folate receptor-α expression in resectable hepatic colorectal cancer metastases: patterns and significance. Mod Pathol. 2011;24(9):1221–8.
34. Shia J, Klimstra DS, Nitzkorski JR, Low PS, Gonen M, Landmann R, et al. Immunohistochemical expression of folate receptor α in colorectal carcinoma: patterns and biological significance. Hum Pathol. 2008;39(4):498–505.
35. Chan SY, Empig CJ, Welte FJ, Speck RF, Schmaljohn A, Kreisberg JF, et al. Folate receptor-α is a cofactor for cellular entry by Marburg and Ebola viruses. Cell. 2001;106(1):117–26.
36. Simmons G, Rennekamp AJ, Chai N, Vandenberghe LH, Riley JL, Bates P. Folate receptor alpha and caveolae are not required for Ebola virus glycoprotein-mediated viral infection. J Virol. 2003;77(24):13433–8.
37. Zhao X, Li H, Lee RJ. Targeted drug delivery via folate receptors. Expert Opin Drug Deliv. 2008;5(3):309–19.
38. Zwicke GL, Mansoori GA, Jeffery CJ. Utilizing the folate receptor for active targeting of cancer nanotherapeutics. Nano Rev. 2012;3(1):18496.
39. Reddy J, Allagadda VM, Leamon CP. Targeting therapeutic and imaging agents to folate receptor positive tumors. Curr Pharm Biotechnol. 2005;6(2):131–50.
40. Yoo HS, Park TG. Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate. J Control Release. 2004;100(2):247–56.
41. Qiu J, Zhang H, Wang Z, Liu D, Liu S, Han W, et al. The antitumor effect of folic acid conjugated-Auricularia auricular polysaccharide-cisplatin complex on cervical carcinoma cells in nude mice. Int J Biol Macromol. 2018;107:2180–9.
42. Li H, Li Y, Ao H, Bi D, Han M, Guo Y. Folate-targeting annonaceous acetogenins nanosuspensions: significantly enhanced antitumor efficacy in HeLa tumor-bearing mice. Drug Deliv. 2018;25(1):880–7.
43. Ahn GY, Kang R, Lee ES, Choi S. Electrosprayed folic acid-conjugated ursolic acid nanoparticles for tumor therapy. Macromol Res. 2018;26(7):573–6.
44. Lu Y, Low PS. Folate targeting of haptens to cancer cell surfaces mediates immunotherapy of syngeneic murine tumors. Cancer Immunol Immunother. 2002;51(3):153–62.
45. Leamon CP, Low PS. Cytotoxicity of momordin-folate conjugates in cultured human cells. J Biol Chem. 1992;267(35):24966–71.
46. Atkinson SF, Bettinger T, Seymour LW, Behr J, Ward CM. Conjugation of folate via gelonin carbohydrate residues retains ribosomal-inactivating properties of the toxin and permits targeting to folate receptor positive cells ∗. J Biol Chem. 2001;276(30):27930–5.
47. Gabizon A, Shmeeda H, Horowitz AT, Zalipsky S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. Adv Drug Deliv Rev. 2004;56(8):1177–92.
48. Goren D, Horowitz A, Tzemach D. Nuclear delivery of doxorubicin via folate targeted liposomes with bypass of multidrug resistance efflux pump 1. Clin Cancer Res. 2000;6(5):1949–57.
49. Guo W, Lee T, Sudimack J, Lee RJ. Receptor-specific delivery of liposomes via folate-Peg-Chol. J Liposome Res. 2000;10(2&3):179–95.
50. Saul JM, Annapragada A, Natarajan JV, Bellamkonda RV. Controlled targeting of liposomal doxorubicin via the folate receptor in vitro. J Control Release. 2003;92(1–2):49–67.
51. Shmeeda H, Mak L, Tzemach D, Astrahan P, Tarshish M, AG, Experimental. Intracellular uptake and intracavitary targeting of folate-conjugated liposomes in a mouse lymphoma model with up-regulated folate receptors. Mol Cancer Ther. 2006;5(4):818–24.
52. Pan XQ, Zheng X, Shi G, Wang H, Ratnam M, Lee RJ. Strategy for the treatment of acute myelogenous leukemia based on folate receptor β-targeted liposomal doxorubicin combined with receptor induction using all-trans retinoic acid. Blood. 2002;100(2):594–602.

53. Li M, Shi K, Tang X, Wei J, Cun X, Long Y, et al. Synergistic tumor microenvironment targeting and blood-brain barrier penetration via a pH-responsive dual-ligand strategy for enhanced breast cancer and brain metastasis therapy. Nanomedicine. 2018;14(6):1833–43.
54. Chen Y, Cheng Y, Zhao P, Zhang S, Li M, He C, et al. Co-delivery of doxorubicin and imatinib by pH sensitive cleavable PEGylated nanoliposomes with folate-mediated targeting to overcome multidrug resistance. Int J Pharm. 2018;542(1–2):266–79.
55. Zhou W, Yuan X, Wilson A, Yang L, Mokotoff M, Pitt B, et al. Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. Bioconjug Chem. 2002;13(6):1220–5.
56. Michael P, Kern S, Lee D, Schmaus J. Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing in vivo. Biomaterials. 2018;178:630–42.
57. Urbiola K, García L, Zalba S, Garrido MJ, Tros De Ilarduya C. Efficient serum-resistant lipopolyplexes targeted to the folate receptor. Eur J Pharm Biopharm. 2013;83(3):358–63.
58. Pan XQ, Wang H, Shukla S, Sekido M, Adams DM, Tjarks W, et al. Boron-containing folate receptor-targeted liposomes as potential delivery agents for neutron capture therapy. Bioconjug Chem. 2002;13(3):435–42.
59. Öztürk AB, Cevher E, Pabuccuoğlu S, Özgümüş S. pH sensitive functionalized hyperbranched polyester based nanoparticulate system for the receptor-mediated targeted cancer therapy. Int J Polym Mater Polym Biomater. 2018;68(8):417–32.
60. Ma H, Deng C, Zong X, He Y, Cheng L, Fan Q, et al. Reversal of doxorubicin-resistance by delivering tetramethylprazine via folate-chitosan nanoparticles in MCF-7 / ADM cells. Int J Clin Exp Med. 2016;9(3):5439–48.
61. Thu HP, Nam NH, Duong LQ, Tham NT, Quang BT, Thi HTM, et al. Targeting effect of folate on cancer cell through curcumin carrier nano-system. Int J Drug Deliv. 2014;6(4):351–8.
62. Cheng L, Ma H, Shao M, Fan Q, Lv H, Peng J, et al. Synthesis of folate-chitosan nanoparticles loaded with ligustrazine to target folate receptor positive cancer cells. Mol Med Rep. 2017;16(2):1101–8.
63. Cao Y, He J, Liu J, Zhang M, Ni P, Cao Y, et al. Folate-conjugated polyphosphoester with reversible cross-linkage and reduction-sensitivity for drug delivery folate-conjugated polyphosphoester with reversible cross- linkage and reduction-sensitivity for drug delivery. ACS Appl Mater Interfaces. 2018;10(9):7811–20.
64. Fasehee H, Dinarvand R, Ghavamzadeh A, Esfandyari-Manesh M, Moradian H, Faghihi S, et al. Delivery of disulfiram into breast cancer cells using folate-receptor-targeted PLGA-PEG nanoparticles: in vitro and in vivo investigations. J Nanobiotechnol. 2016;14(1):1–18.
65. Xu X, Wu C, Bai A, Liu X, Lv H, Liu Y. Folate-functionalized mesoporous silica nanoparticles as a liver tumor-targeted drug delivery system to improve the antitumor effect of paclitaxel. J Nanomater. 2017;2017:1–13.
66. Alvarez-Berríos MP, Vivero-Escoto JL. In vitro evaluation of folic acid-conjugated redox-responsive mesoporous silica nanoparticles for the delivery of cisplatin. Int J Nanomedicine. 2016;11:6251–65.
67. Xiuling Xu, Fan Hu, Qi Shuai. Facile synthesis of highly biocompatible folic acid-functionalized SiO2 encapsulated rare-earth metal complexes nanoparticles and its application on targeted metal-based complex delivery. Dalton Trans. 2017;46(44):15424–33.
68. Gao B, Shen L, He KW, Xiao WH. GNRs@SiO2-FA in combination with radiotherapy induces the apoptosis of HepG2 cells by modulating the expression of apoptosis-related proteins. Int J Mol Med. 2015;36(5):1282–90.
69. Ramesh I, Meena KS. Folic acid gelatin coated HAp @ Al_2O_3 core-shell NPs for receptor mediated targeted drug delivery system. Int J Curr Res. 2016;8(03):28000–6.
70. Ak G, Yilmaz H, Güneş A, Sanlier SH. In vitro and in vivo evaluation of folate receptor- targeted a novel magnetic drug delivery system for ovarian cancer therapy. Artif Cells Nanomed Biotechnol. 2018;46(Suppl 1):926–37.
71. Ramasamy S, Jeya R, Sam R, Enoch IVMV, Ramasamy S, Jeya R, et al. Folate-molecular encapsulator-tethered biocompatible polymer grafted with magnetic nanoparticles for augmented drug delivery. Artif Cells Nanomed Biotechnol. 2018;46(Suppl 2):675–82.

72. Feng S, Zhang H, Yan T, Huang D, Zhi C, Nakanishi H, Gao X-D. Folate-conjugated boron nitride nanospheres for targeted delivery of anticancer drugs. Int J Nanomedicine. 2016;11:4573–82.
73. Li X, Sun Y, Hu Y, Peng Y, Li Y, Yin G, et al. Synthesis of size-tunable hollow polypyrrole nanostructures and their assembly into folate targeting and pH-responsive anti- cancer drug delivery. Chem Eur J. 2017;23(68):17279–89.
74. Jafaria M, Heidaria D, Ebrahimnejad P. Synthesizing and characterizing functionalized short multiwall carbon nanotubes with folate, magnetite and polyethylene glycol as multi- targeted nanocarrier of anti-cancer drugs. Iran J Pharm Res. 2016;15(2):449–56.
75. Yao Y, Lee RJ. Folic acid receptor-targeted human serum albumin nanoparticle formulation of cabazitaxel for tumor therapy. Int J Nanomedicine. 2019;14:135–48.
76. Press D. Preparation, characterization, and in vitro targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. Int J Nanomedicine. 2010;5:669–77.
77. Li H, Liu Y, Chen L, Liu Q, Qi S, Cheng X, et al. Folate receptor-targeted lipid-albumin nanoparticles (F-LAN) for therapeutic delivery of an Akt1 antisense oligonucleotide. J Drug Target. 2018;26(5–6):466–73.
78. Yoo HS, Park TG. Folate receptor targeted biodegradable polymeric doxorubicin micelles. J Control Release. 2004;96(2):273–83.
79. Chen L, Qian M, Zhang L, Xia J, Bao Y. Co-delivery of doxorubicin and shRNA of Beclin1 by folate receptor targeted pullulan-based cancer therapy. RSC Adv. 2018;8(32):17710–22.
80. Wang Y, Ren J, Liu Y, Liu R, Wang L, Yuan Q, et al. Preparation and evaluation of folic acid modified succinylated gelatin micelles for targeted delivery of doxorubicin. J Drug Deliv Sci Technol. 2018;46:400–7.
81. Lv Y, Yang B, Li YM, He F, Zhuo RX. Folate-conjugated amphiphilic block copolymer micelle for targeted and redox-responsive delivery of doxorubicin. J Biomater Sci Polym Ed. 2018;29(1):92–106.
82. Shi C, Zhang Z, Wang F, Luan Y. Active-targeting docetaxel-loaded mixed micelles for enhancing antitumor ef fi cacy. J Mol Liq. 2018;264:172–8.
83. Jones SK, Lizzio V, Merkel OM. Folate receptor targeted delivery of siRNA and paclitaxel to ovarian cancer cells via folate conjugated triblock copolymer to overcome TLR4 driven chemotherapy resistance. Biomacromolecules. 2016;17(1):76–87.
84. Zamani M, Rostamizadeh K, Manjili HK, Danafar H. In vitro and in vivo biocompatibility study of folate-lysine- PEG-PCL as nanocarrier for targeted breast cancer drug delivery. Eur Polym J. 2018;103:260–70.
85. Chen D, Song X, Wang K. Design and evaluation of dual CD44 receptor and folate nanocarrier double-smart pH-response multifunctional nanocarrier. J Nanopart Res. 2017;19(12):400.
86. Rosière R, Van Woensel M, Gelbcke M, Mathieu V, Hecq J, Mathivet T, et al. New folate-grafted chitosan derivative to improve delivery of paclitaxel-loaded solid lipid nanoparticles for lung tumor therapy by inhalation. Mol Pharm. 2018;15(3):899–910.
87. Liu Z, Zhong Z, Peng G, Wang S, Du X, Yan D, et al. Folate receptor mediated intracellular gene delivery using the charge changing solid lipid nanoparticles. Drug Deliv. 2009;16(6):341–7.
88. Rajpoot K, Jain SK. Colorectal cancer-targeted delivery of oxaliplatin via folic acid-grafted solid lipid nanoparticles: preparation, optimization, and in vitro evaluation. Artif Cells Nanomed Biotechnol. 2018;46(6):1236–47.
89. Venishetty VK, Komuravelli R, Kuncha M, Sistla R, Diwan PV. Increased brain uptake of docetaxel and ketoconazole loaded folate-grafted solid lipid nanoparticles. Nanomedicine. 2013;9(1):111–21.
90. Gao W, Xiang B, Meng T, Liu F, Qi X. Chemotherapeutic drug delivery to cancer cells using a combination of folate targeting and tumor microenvironment-sensitive polypeptides. Biomaterials. 2013;34(16):4137–49.
91. Amreddy N, Babu A, Panneerselvam J, Srivastava A, Ms RM, Ms AC, et al. Chemo-biologic combinatorial drug delivery using folate receptor-targeted dendrimer nanoparticles for lung cancer treatment. Nanomedicine. 2017;14(2):373–84.

92. Raniolo S, Vindigni G, Ottaviani A, Unida V, Iacovelli F, Manetto A, et al. Selective targeting and degradation of doxorubicin-loaded folate- functionalized DNA nanocages. Nanomedicine. 2018;14(4):1181–90.
93. Fong Y, Chen C-H, Chen J. Intratumoral delivery of doxorubicin on folate-conjugated graphene oxide by in-situ forming thermo-sensitive hydrogel for breast cancer therapy. Nano. 2017;7(11):388.
94. Elamin KM, Motoyama K, Higashi T, Yamashita Y, Tokuda A, Arima H. Dual targeting system by supramolecular complex of folate-conjugated methyl-β-cyclodextrin with adamantane-grafted hyaluronic acid for the treatment of colorectal cancer. Int J Biol Macromol. 2018;113:386–94.
95. Son J, Yang SM, Yi G, Roh YJ, Park H, Park JM, et al. Folate-modified PLGA nanoparticles for tumor-targeted delivery of pheophorbide a in vivo. Biochem Biophys Res Commun. 2018;498(3):523–8.
96. Peng F, Qiu L, Chai R, Meng F, Yan C, Chen Y, et al. Conjugated polymer-based nanoparticles for cancer cell-targeted and image-guided photodynamic therapy. Macromol Chem Phys. 2018;219(4):1–6.
97. Keyvan Rad J, Mahdavian AR, Khoei S, Shirvalilou S. Enhanced photogeneration of reactive oxygen species and targeted photothermal therapy of C6 glioma brain cancer cells by folate-conjugated gold-photoactive polymer nanoparticles. ACS Appl Mater Interfaces. 2018;10(23):19483–93.
98. Li J, Yao S, Wang K, Lu Z, Su X, Li L, et al. Hypocrellin B-loaded, folate-conjugated polymeric micelle for intraperitoneal targeting of ovarian cancer in vitro and in vivo. Cancer Sci. 2018;109(6):1958–69.
99. Chien YY, Wang TY, Liao PW, Wu WC, Chen CY. Folate-conjugated and dual stimuli-responsive mixed micelles loading indocyanine green for photothermal and photodynamic therapy. Macromol Biosci. 2018;18(6):1–12.
100. Yu S, Tian-yi S, Ling-yun Z, Yu-yan Z, Bai-wang S, Xiao-ping L. Folate-decorated and NIR-activated nanoparticles based on platinum(IV) prodrugs for targeted therapy of ovarian cancer. J Microencapsul. 2017;34(7):675–86.
101. Wong PT, Tang S, Cannon J, Chen D, Sun R, Phan J, et al. Photocontrolled release of doxorubicin conjugated through a thioacetal photocage in folate-targeted nanodelivery systems. Bioconjug Chem. 2017;28(12):3016–28.
102. Malekmohammadi S, Hadadzadeh H, Hossein Farrokhpour ZA. Immobilization of gold nanoparticles on the folate-conjugated dendritic mesoporous silica-coated reduced graphene oxide nanosheets: a new nanoplatform for curcumin pH-controlled and targeted delivery. Soft Matter. 2018;14(12):2400–10.
103. Meier R, Henning TD, Boddington S, Piontek G, Rudelius M. Breast cancers: MR imaging of folate-receptor expression with the folate specific nanoparticle P1133. Radiology. 2010;255(2):527–35.
104. Soleymani J, Hasanzadeh M, Somi MH, Shadjou N, Jouyban A. Probing the specific binding of folic acid to folate receptor using amino-functionalized mesoporous silica nanoparticles for differentiation of MCF 7 tumoral cells from MCF 10A. Biosens Bioelectron. 2018;115:61–9.
105. Chávez-García D, Juárez-Moreno K, Campos CH, Alderete JB, Hirata GA. Upconversion rare earth nanoparticles functionalized with folic acid for bioimaging of MCF-7 breast cancer cells. J Mater Res. 2018;33(2):191–200.
106. Khademi S, Sarkar S, Shakeri-zadeh A, Attaran N. Folic acid-cysteamine modified gold nanoparticle as a nanoprobe for targeted computed tomography imaging of cancer cells. Mater Sci Eng C. 2018;89(2017):182–93.
107. Xia J, Wei X, Chen X, Shu Y. Folic acid modified copper nanoclusters for fluorescent imaging of cancer cells with over-expressed folate receptor. Microchim Acta. 2018;185(3):205.
108. Li R, Wang X, Li Z, Zhu H, Liu J. Folic acid-functionalized graphene quantum dots with tunable fluorescence emission for cancer cell imaging and optical detection of Hg2+. New J Chem. 2018;42(6):4352–60.
109. Moon WK, Lin Y, O'Loughlin T, Tang Y, Kim DE, Weissleder R, et al. Enhanced tumor detection using a folate receptor-targeted near-infrared fluorochrome conjugate. Bioconjug Chem. 2003;14(3):539–45.

110. Predina JD, Newton AD, Connolly C, Ashley Dunbar MB, Deshpande C, Cantu E III, Stadanlick J, Kularatne SA, Low PS, Singhal S. Identification of a folate receptor-targeted near-infrared molecular contrast agent to localize pulmonary adenocarcinomas. Mol Ther. 2018;26(2):390–403.
111. Chen Q, Meng X, McQuade P, Rubins D, Lin SA, Zeng Z, et al. Folate-PEG-NOTA-Al18F: a new folate based radiotracer for PET imaging of folate receptor-positive tumors. Mol Pharm. 2017;14(12):4353–61.
112. Dong S, Teo JDW, Chan LY, Lee CK, Sou K. Far-red fluorescent liposomes for folate receptor-targeted bioimaging. ACS Appl Nano Mater. 2018;1(3):1009–13.
113. Corbin IR, Ng KK, Ding L, Jurisicova AZG. Near-infrared fluorescent imaging of metastatic ovarian cancer using folate-receptor targeted high-density lipoprotein. Nanomedicine. 2013;8(6):875–90.
114. Konda SD, Aref M, Wang S, Brechbiel M, Wiener EC. Specific targeting of folate-dendrimer MRI contrast agents to the high affinity folate receptor expressed in ovarian tumor xenografts. MAGMA. 2001;12(01):104–13.
115. Liang L, Zhang X, Su X, Li J, Tian Y, Xue H, et al. 99m Tc-labeled oligomeric nanoparticles as potential agents for folate receptor-positive tumor targeting. J Label Compd Radiopharm. 2018;61(2):54–60.
116. Rajkumar S, Prabaharan M. Multi-functional nanocarriers based on iron oxide nanoparticles conjugated with doxorubicin, poly(ethylene glycol) and folic acid as theranostics for cancer therapy. Colloids Surf B Biointerfaces. 2018;170:529–37.
117. Maeng JH, Lee DH, Jung KH, Bae YH, Park IS, Jeong S, et al. Multifunctional doxorubicin loaded superparamagnetic iron oxide nanoparticles for chemotherapy and magnetic resonance imaging in liver cancer. Biomaterials. 2010;31(18):4995–5006.
118. Wang H, Wang S, Liao Z, Zhao P, Su W, Niu R, et al. Folate-targeting magnetic core – shell nanocarriers for selective drug release and imaging. Int J Pharm. 2012;430(1–2):342–9.
119. Poshteh Shirani M, Rezaei B, Khayamian T, Dinari M, Karami K, Mehri-Lighvan Z, et al. Folate receptor-targeted multimodal fluorescence mesosilica nanoparticles for imaging, delivery palladium complex and in vitro G-quadruplex DNA interaction. J Biomol Struct Dyn. 2018;36:1456–69.
120. Mendoza-nava H, Ferro-flores G, Ramírez FDM, Ocampo-garcía B, Santos-cuevas C, Aranda-lara L, et al. Lu-dendrimer conjugated to folate and bombesin with gold nanoparticles in the dendritic cavity: a potential theranostic radiopharmaceutical. J Nanomater. 2016;2016:1039258.
121. Patel NR, Piroyan A, Ganta S, Morse AB, Candiloro KM, Solon AL, et al. In vitro and in vivo evaluation of a novel folate-targeted theranostic nanoemulsion of docetaxel for imaging and improved anticancer activity against ovarian cancers. Cancer Biol Ther. 2018;19(7):554–64.
122. Puligujja P, McMillan JE, Kendrick L, Li T, Balkundi S, Smith N, et al. Macrophage folate receptor-targeted antiretroviral therapy facilitates drug entry, retention, antiretroviral activities and biodistribution for reduction of human immunodeficiency virus infections. Nanomedicine. 2013;9(8):1263–73.
123. Date PV, Patel MD, Majee SB, Samad A, Devarajan PV. Ionic complexation as a non-covalent approach for the design of folate anchored rifampicin gantrez nanoparticles. J Biomed Nanotechnol. 2013;9(5):765–75.
124. Patel MD, Date PV, Gaikwad RV, Samad A, Malshe VC, Devarajan PV. Comparative evaluation of polymeric nanoparticles of rifampicin comprising Gantrez and poly(ethylene sebacate) on pharmacokinetics, biodistribution and lung uptake following oral administration. J Biomed Nanotechnol. 2014;10(4):687–94.
125. Teng L, Xie J, Teng L, Lee RJ. Clinical translation of folate receptor-targeted therapeutics. Expert Opin Drug Deliv. 2012;9(8):901–8.
126. Vergote I, Leamon CP. Vintafolide: a novel targeted therapy for the treatment of folate receptor expressing tumors. Ther Adv Med Oncol. 2015;7(4):206–18.
127. Study for women with platinum resistant ovarian cancer evaluating EC145 in combination with Doxil® (PROCEED) (PROCEED) [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT01170650?cond=NCT01170650&rank=1.

128. Peethambaram PP, Hartmann LC, Jonker DJ, de Jonge M, Plummer ER, Martin L, Konner J, Marshall J, Goss GD, Teslenko V, Clemens PL, Cohen LJ, Ahlers CM, Alland L. A phase I pharmacokinetic and safety analysis of epothilone folate (BMS-753493), a folate receptor targeted chemotherapeutic agent in humans with advanced solid tumors. Invest New Drugs. 2015;33(2):321–31.
129. A phase 1/2 study of epofolate (BMS-753493) in subjects with advanced cancer (Schedule 2) [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT00550017?cond=NCT00550017&rank=1.
130. Messmann R, Amato R, Hernandez-McClain J, Conley B, Rogers H, Lu Y, Low P, Bever S, Morgenstern D. A phase II study of FolateImmune (EC90 with GP1-0100 adjuvant followed by EC17) with low dose cytokines interleukin-2 (IL-2) and interferon-{alpha} (IFN-{alpha}) in patients with refractory or metastatic cancer. J Clin Oncol. 2007;25(18_suppl):13516.
131. A phase II study of EC17 (Folate-hapten Conjugate) in patients with progressive metastatic renal cell carcinoma [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT00485563?cond=NCT00485563&rank=1.
132. Maurer AH, Elsinga P, Fanti S, Nguyen B, Oyen WJG, Weber WA. Imaging the folate receptor on cancer cells with 99mTc-Etarfolatide: properties, clinical use, and future potential of folate receptor imaging. J Nucl Med. 2014;55(5):701–4.
133. Cheung A, Bax HJ, Josephs DH, Ilieva KM, Pellizzari G, Opzoomer J, et al. Targeting folate receptor alpha for cancer treatment. Oncotarget. 2016;7(32):52553–74.
134. Study of EC0489 for the treatment of refractory or metastatic tumors [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT00852189?cond=NCT00852189&rank=1.
135. Sharma S, Sausville EA, LoRusso P, Vogelzang NJ, Samlowski WE, Carter J, Forman K, Bever S, Messmann RA. A phase I study of EC0225 administered weeks 1 and 2 of a 4-week cycle. J Clin Oncol. 2010;28(15 Suppl):3082.
136. Study of EC0225 for the treatment of refractory or metastatic tumors [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT00441870?cond=NCT00441870&rank=1.
137. Sachdev JC, Matei D, Harb WA, Clark R, Edelman MJ, Starodub A. A phase 1 dose-escalation study of the folic acid-tubulysin small molecule drug conjugate (SMDC) folate-tubulysin EC1456 in advanced cancer patients. J Oncol. 2016;34(15_suppl):2585.
138. Folic acid-tubulysin conjugate EC1456 in patients with advanced solid tumors [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT01999738?cond=NCT01999738&rank=1.
139. OTL38 for intra-operative imaging of folate receptor positive ovarian cancer [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT03180307?cond=NCT03180307&rank=1.
140. Vergote I, Armstrong D, Scambia G, Teneriello M, Sehouli J, Schweizer C, et al. A randomized, double-blind, placebo-controlled, phase 3 study to assess efficacy and safety of weekly farletuzumab in combination with carboplatin and taxane in patients with ovarian cancer in first platinum-sensitive relapse. J Clin Oncol. 2016;34(19):2271–8.
141. Ab O, Whiteman KR, Bartle LM, Sun X, Singh R, Tavares D, et al. IMGN853, a folate receptor-α (FRα) – targeting antibody – drug conjugate, exhibits potent targeted antitumor activity against FR a – expressing tumors. Mol Cancer Ther. 2015;17:1605–14.
142. Moore KN, Vergote I, Oaknin A, Colombo N, Oza A, Pautier P, et al. FORWARD I: a phase III study of mirvetuximab soravtansine versus chemotherapy in platinum-resistant ovarian cancer. Future Oncol. 2018;14(17):1669–78.
143. PH3 Study of Mirvetuximab Soravtansine vs Investigator's Choice of Chemotherapy in Women With FRa+ Adv. EOC, Primary Peritoneal or Fallopian Tube Cancer (FORWARD I) [Internet]. Available from: https://clinicaltrials.gov/ct2/show/NCT02631876?cond=NCT02631876&rank=1.

Chapter 15
Mannose Receptor and Targeting Strategies

Priyanka Jahagirdar, Amit S. Lokhande, Prajakta Dandekar, and Padma V. Devarajan

Abstract The mannose family receptors are unique multidomain, multifunctional endocytic receptors belonging to the C-type lectin family. These receptors, although structurally similar, exhibit differential binding to discrete ligands. This chapter discusses such similarities and differences between the structures, ligands, the expression, and molecular trafficking among the members of mannose receptor family. Further, targeted drug delivery strategies in infections and cancer to the most widely investigated receptor of the family, the mannose receptor, are comprehensively explained with examples.

Keywords Mannose receptor family · Mannose conjugates · Nanoparticles · Liposomes · Vaccines · Infections · Cancer

Abbreviations

CD	Cluster of differentiation
CTLD	C-type lectin domain
DCs	Dendritic cells
DRV	Dehydration–rehydration vesicle
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin

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LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NPs	Nanoparticles
PEG	Polyethylene glycol
PLA_2	Phospholipase A_2
PLGA	Poly (lactide-co-glycolide)
RES	Reticuloendothelial system
SLA	Soluble leishmanial antigen
SPIONs	Superparamagnetic iron oxide nanoparticles
TAM	Tumor-associated macrophages
TB	Tuberculosis

1 Introduction

The C-type lectin superfamily comprising of transmembrane and soluble proteins like selectins, collectins, and asialoglycoprotein receptor has garnered attention since eons [1]. The family of mannose receptors is an integral part of the C-type lectin family. Multiple lectin domains in a single polypeptide structure make this family an unconventional member of the lectin superfamily [2]. The mannose family receptors are involved in antigen capture, recognition of mannosylated structures of pathogenic cell walls and may be overexpressed in certain diseased states. Targeting the mannose receptor provides an attractive strategy to combat number of infections and certain cancers [3, 4]. A complete understanding of the receptors, ligands, and binding interactions is quintessential for successful targeting applications. This chapter focuses on the mannose receptor family and its physiology in normal state and in pathologies. Drug delivery approaches to harness targeting effectively in the therapy of infectious diseases and cancer are also discussed.

2 Mannose Receptor Family

The family of mannose receptors comprises of four endocytic glycoprotein receptors, namely mannose receptor, M-type receptor for phospholipases A_2 (PLA_2R), DEC-205/CD205/gp200-MR6, and Endo180/uPARAP [5–8]. Mannose receptor, the first member of the family, was identified in the late 1970s. Multiple C-type lectin domains (CTLDs) in a single polypeptide backbone constitute a distinct feature of this receptor family. The members of mannose receptor family share mutual structural features, namely cysteine-rich domain, fibronectin type II domain, and CTLDs which vary from eight to ten. However, C-type lectin activity is not exhibited by all members. The C-type lectin activity for interacting with mannosylated

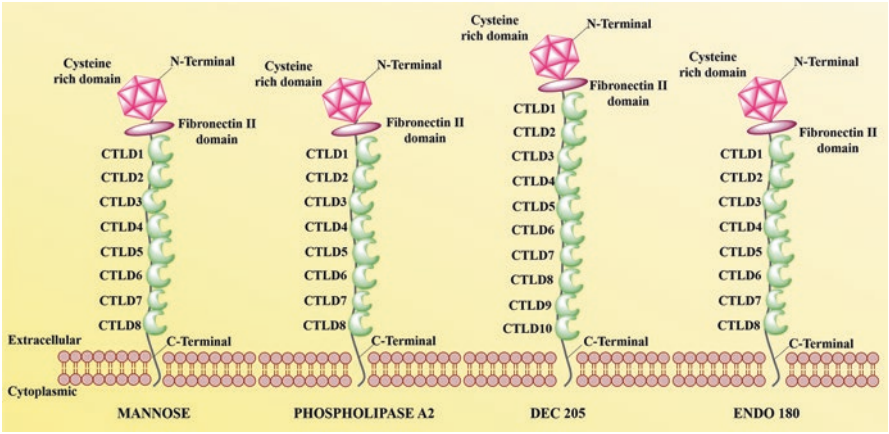

Fig. 15.1 The mannose receptor family

moieties is displayed only by mannose receptor and Endo180. CTLD5 of PLA_2R is involved in protein–protein interactions, a nonlectin activity. The cysteine-rich domain is involved in the recognition of sulfated carbohydrates whereas the fibronectin type II domain internalizes collagen. A functional cysteine-rich domain for binding to sulfated carbohydrates like galactose is present only in the mannose receptor. The receptors of mannose family terminate into short cytoplasmic domains. The receptors are rapidly internalized inside the cell and deliver the extracellular content to the intracellular compartments. Delivery to intracellular locations occurs via interactions between the motifs of terminal cytoplasmic domains and the endocytic machinery. The members of mannose family and their recognition domains are depicted in Fig. 15.1.

2.1 Receptor Recognition Domains

2.1.1 Cysteine-Rich Domain

Although present in all family members, the cysteine-rich domain lacks homology among the mannose receptor family. A 25–30% sequence identity is observed among the family members. Among the four receptors, only the mannose receptor has a functional N-terminal cysteine-rich domain which can exhibit binding to sulfated molecules. The receptor can bind to glycoproteins containing sulfated N-acetylglucosamine and sulfated galactose residues in hormones like lutropin and thyrotropin via this domain [9]. Binding to chondroitin sulfate A, chondroitin sulfate B, sulfated Lewis antigens, CD45, and sulfated transmembrane protein sialoadhesin is also reported [10, 11]. The binding is Ca^{2+} independent and occurs through a neutral binding site. The exact mechanism of binding is beyond the purview of this chapter and is well explained in the literature [2, 12].

2.1.2 Fibronectin Type II Domain

Fibronectin type II domain is the most conserved extracellular domain of mannose receptor family. This domain occurs in different proteins like matrix metalloproteinases (MMP) 2 and 9 [13]. This domain mainly binds to denatured collagen. The collagen binding may be a result of interaction between aromatic structures of the hydrophobic pocket exposed by the solvent with the nonpolar collagen residues leading to disruption of triple helix. The conserved amino acid (Arg^{34} and Asp^{36}) residues can play a role in stabilizing this interaction [2]. Another hypothesis suggests the fibronectin type II domain can bring about N-terminal extension to bring the N-terminal in the vicinity of the C-terminal leading to stabilized interaction with collagen [14]. The binding of fibronectin type II domain of mannose receptor to collagen has been studied. An extended conformation at physiological pH and a compact conformation at acidic pH was reported. At physiological pH, a calcium-dependent binding was observed whereas acidic pH calcium did not affect the collagen binding [15]. This behavior could play a critical role in the intracellular trafficking of cargo delivered through mannose receptor endocytosis.

Mannose receptor demonstrates the ability to bind to collagens I, II, III, and IV while exhibiting a weak binding to collagen V [16]. Fibronectin type II domain of M-type PLA_2 expressing cells binds to collagens I and IV, while Endo180 fibronectin type II domain preferentially binds to collagen V over collagens I and IV. No information is available regarding the ability of DEC-205 to recognize collagen, although this is a likely possibility.

2.1.3 C-Type Lectin Domains

CTLDs contain 120 amino acids. Noncovalent and covalent interactions between two antiparallel β sheets and two α helices lead to the formation of a hydrophobic fold. The carbohydrate interactions in functional CTLDs occur in the hydrophobic fold that imparts stability by hydrophobic core formation. Two disulfide bonds are also formed between cysteine residues. This hydrophobic fold of functional CTLDs permits interactions with sugars by facilitating contact with residues integral for coordination with Ca^{2+} and sugar moieties [2].

In the case of mannose receptor, the binding of terminal carbohydrate residues like mannose, fucose, and N-acetylglucosamine occurs in the presence of Ca^{2+}. A higher affinity is demonstrated by mannose receptor CTLDs toward mannose and fucose whereas the binding affinity to N-acetylglucosamine and glucose is lower. Only the mannose receptor CTLD4 is involved in sugar binding. Similar to mannose receptor, CTLD2 of Endo180 shows binding dependent on Ca^{2+} to glycoconjugates, while CTLD5 of PLA_2R is involved in binding to the nonglycosylated PLA_2 ligand via Ca^{2+}-independent pathways. Further, instead of lectin interactions, CTLD5 mediates protein–protein interactions. DEC-205 is devoid of C-type lectin activity.

2.2 Ligand Binding

In most multidomain receptors, domains which mediate ligand interaction are often stationed at a distance from the membrane. Surprisingly, among the mannose family receptors, CTLD4 and CTLD5 which exhibit a crucial role in binding are found in the central region of the mannose receptor and the PLA_2 receptor, respectively. An extensive study of mannose receptor revealed an extracellular domain with a rigid and extended conformation and close interactions between neighboring CTLDs (CTLDs 1 and 2, CTLDs 4 and 5, CTLDs 7 and 8) with exposed flexible linker regions on either side of CTLDs 3 and 6 [2, 17]. CTLD5 of mannose receptor demonstrates weak binding to sugars in addition to CTLD4, the principal sugar-binding domain. The association of these two CTLDs results in the formation of a protease-resistant core. Such domain disposition enables binding to multiple sugar moieties, enhanced binding of CTLDs, and/or modulates the rigidity of CTLDs.

Closeness of the N- and C-terminal of fibronectin type II domain brings it near to the other domains, as suggested by the sequence analysis. A close association of the cysteine-rich domain, fibronectin type II domain, and C-type lectin domains is seen by protease studies. Such an arrangement stabilizes the interaction with collagen and projects the cysteine-rich domain away from the membrane. This projection is desirable for interactions of cellular sulfated glycoproteins and the domain.

Further, the ligand–receptor binding in mannose receptor is highly pH dependent. Mannose receptor shows poor binding of ligands at pH 5 and optimal binding at pH 7. Such pH dependency is prominent in ligands dissociating in the acidic endosomal compartments. The pH-dependent binding enables separation of the ligand and receptor and recycling of the free receptors to the cell surface. Additionally, the Ca^{2+} dependency in binding may aid in the endosomal dissociation [18].

2.3 Intracellular Internalization

The rapid internalization of mannose receptor family members mainly occurs via clathrin-mediated endocytosis. Internalization by phagocytosis is another pathway mediated by mannose receptor expressed on the macrophages. Under steady state, the cell surface receptors constitute 10–30% whereas remaining 70–90% receptors are intracellular.

2.3.1 Clathrin-Mediated Endocytosis

During endocytic uptake, ligands packed in clathrin-coated vesicles are internalized from the plasma membrane and are delivered in the endosomal system. Smaller particles (<0.2 μm) are taken up by this pathway. The mannose family receptors recycle about 10 times an hour.

Two endocytic motifs, namely tyrosine residue-based motif and dihydrophobic motif, are present in the cytoplasmic domain. Although directed to the same intracellular compartment, the mannose receptor and Endo180 mediate the transport via different motifs. Mannose receptor and PLA_2R recruit tyrosine-based motif whereas Endo180 utilizes the dihydrophobic motif [19]. Internalization occurs from the clathrin-coated pits into the early endosomes. This is followed by transportation to late endosomes and fusion with lysosomes followed by release of cargo into the cytoplasm. A different destination of DEC-205 within the cells is reported. Whereas mannose receptor is located in the early endosomes, localization of DEC-205 is seen in the late endosomes [20].

2.3.2 Phagocytosis

The uptake of particles of >0.2 μm occurs via phagocytosis. Fc receptors and complement receptors, the opsonic receptors, initiate phagocytosis signaling resulting in extension of membrane around the particle via regulation of actin cytoskeleton [21]. A phagosome is formed which then fuses with endosomes/lysosomes leading to exposure of the cargo to hydrolytic enzymes. The direct role of mannose receptor in phagocytosis is questionable. Phagocytic pathway may proceed upon binding to a mannosylated residue which may in turn activate a classical phagocytosis receptor [2]. As PLA_2R and DEC-205 are mainly involved in uptake of macromolecules and are not expressed on phagocytic macrophages, their involvement in phagocytic machinery is unlikely. Although Endo180 is expressed on macrophages, an involvement in phagocytosis analogous to the mannose receptor is not observed in vitro [19].

3 Receptor Location and Expression

Mannose receptor, a 175-kDa type I membrane glycoprotein receptor, was originally isolated in liver and alveolar macrophages [22]. The receptor is predominantly found in most tissue macrophages and dendritic cells (DCs). It is also located in endothelial cells of liver and splenic sinusoids [23]. The receptor is also expressed on the microvascular endothelial cells of the dermis [24], cells of Kaposi's sarcoma [25], human keratinocytes [26], and retinal pigment epithelium [27]. Although initially termed as the macrophage mannose receptor, it is now designated as the mannose receptor, as the occurrence is not exclusively limited to macrophages. The involvement of mannose receptor in phagocytosis of mannosylated structures and pinocytosis of soluble molecules is reported. It also acts as pattern recognition receptor by recognizing the mannosylated ligands of microbes [28–30]. Other functions of this receptor constitute improved presentation of antigens, modulation of cellular trafficking, and maintaining homeostasis by scavenging nonessential mannoglycoproteins and circulating pituitary hormones like lutropin and thyrotropin. PLA_2R is expressed on muscle cell membranes and internalizes PLA_2, the lipolytic

Table 15.1 Mannose receptor family

	Mannose receptor	PLA_2R	DEC-205	Endo180
Occurrence	Macrophages, DCs, few lymphatic or endothelial cells	Muscle cell membranes	DCs, epithelia, B cells, bone marrow stroma, and endothelial cells	Fibroblastic cells, stromal cells, macrophages, and a subset of endothelial cells
Functions	Antigen presentation, phagocytosis of mannosylated structures, homeostasis regulation, modulation of cellular trafficking	Phospholipid digestion, cell proliferation, cell migration, and hormone release	Antigen uptake, presentation of cargo to T cells	Remodeling of cellular membranes
Domain				
Cysteine-rich	Active Enables binding to sulfated carbohydrates like galactose	Inactive	Inactive	Inactive
Fibronectin type II	Binds to collagens I, II, III, and IV and weakly to collagen V	Binds to collagens I and IV	Unknown	Binds to collagen V over collagens I and IV
CTLDs	8 C-type lectin activity	8 Non-lectin activity	10 No C-type lectin activity	8 C-type lectin activity

enzymes required for digestion of phospholipids [5]. DEC-205 which is expressed by dendritic cells has shown involvement in uptake of antigens and delivery of cargo to T cells whereas Endo180 is an endocytic receptor involved in remodeling of cellular membranes.

A comprehensive overview of the four mannose receptor family members is provided in Table 15.1.

4 Pathophysiological Features

The expression of mannose receptor is regulated by macrophage differentiation pattern. Consequently, differentiated macrophages reveal abundant receptor expression whereas circulating monocytes do not express mannose receptor [31]. The physiological status also affects the expression pattern. Anti-inflammatory molecules (corticosteroids, IL-10) [32, 33], Vitamin D3 [34], prostaglandin E [35], and Th2 cytokines (IL-4, IL-13) upregulate the mannose receptor expression by promoting synthesis whereas interferon ɣ (IFN ɣ) [32], lipopolysaccharide (LPS) [36], and immune complexes [37] downregulate the expression by restricting the synthesis.

Binding of pathogenic mannosylated ligands to mannose receptor may induce interleukin (IL)-10 and curb IL-12, thereby inhibiting pathways that could enable protective immune responses [30]. Mannose receptor recognizes the mannosylated

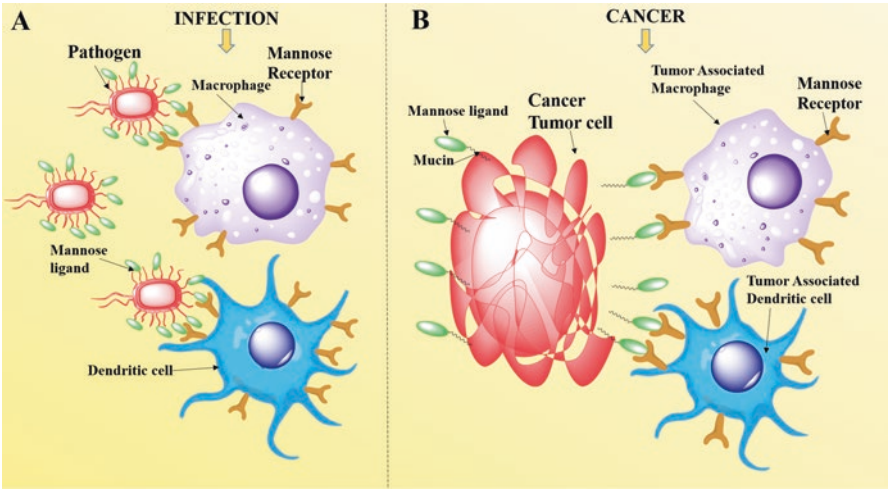

Fig. 15.2 Recognition of (**a**) mannosylated pathogenic cell walls by the mannose receptor present on macrophages and dendritic cells in infections and (**b**) mannose ligands of tumoral mucins by the mannose receptor present on tumor-associated macrophages (TAMs) and dendritic cells in cancers

cell walls of bacteria, fungi, viruses, or parasites, enabling their internalization in the cells (Fig. 15.2a). Pathogens entering the cellular environment using the mannose receptor portal do not evoke an immune reaction.

Although mainly associated with infections, mannose receptor also shows a peculiar expression pattern in cancers. The tumor site shows the presence of mannose receptor expressing macrophages accompanied by ligands for mannose receptor like tumoral mucins [38, 39]. Tumoral mucin MUC1, a ligand of mannose receptor positive cancer cells, comprises of mannose and galactose residues. The tumor cells express abnormal quantities or irregular forms of mucins compared to the healthy cells [40]. The tumoral mucins can invade the immune responses occurring in the tumor microenvironment by binding to mannose receptor on the DCs and tumor-associated macrophages (TAMs) (Fig. 15.2b) and lead to upregulation of IL-10 and suppression of IL-12, thus suppressing Th1-polarized responses, similar to that in infections.

5 Ligands

Mannose receptor binds to various endogenous ligands and acts as a homeostasis regulator by clearing the unwanted molecules from circulation [30, 41]. As discussed earlier, the cysteine region of mannose receptor binds to sulfated moieties whereas the CTLDs bind to glycoproteins rich in mannose oligosaccharides. The fibronectin type II domain shows collagen-specific binding. Classification of ligands

Table 15.2 Ligands for mannose receptor based on domain structure

Domain	Ligands	References
Cysteine-rich domain	Anterior pituitary hormone lutropin	[42]
	CD45	[11]
	Chondroitin sulfate A and chondroitin sulfate B	[10]
	Lewis antigen^{A}, Lewis antigen^{X}	[10]
	Sialoadhesin	[11]
	Sulfated D-galactose	[2]
	Sulfated N-acetyl-D-galactosamine	[43]
	Sulfated N-acetyl-D-glucosamine	[2]
Fibronectin type II domain	Collagen	[16]
CTLDs	Fucose	[2]
	Mannose	[2]
	N-acetyl-D-glucosamine	[2]

based on binding domains is presented in Table 15.2. Utilization of mannose, the most popular ligand, and other ligands like sulfated residues of N-acetyl-D-galactosamine and mannans, etc., for targeted intracellular delivery of therapeutics/antigens is discussed in Sect. 6.

The mannose receptor also binds to exogenous ligands from several microbes and enables their entry into the cell. Microbes may target the mannose receptor to provoke an anti-inflammatory/immune-suppressive response and cause a resistant infection. Mannose receptor lacks the ability to distinguish between pathogenic and nonpathogenic strains, thus internalizing both, unlike the Toll-like receptors [28]. Pathogens, such as *Mycobacterium tuberculosis* [44], *Leishmania donovani* [45], *Trypanosoma cruzi* [46], *Trichinella spiralis* [47], *Streptococcus pneumoniae* [48], HIV virus [49], and influenza virus [50], enter the intracellular environment aided by the carbohydrate ligands on their cell membranes. In the case of *Mycobacterium tuberculosis,* lipoarabinomannan (LAM), a glycolipid present in the mycobacterial cell wall, contains terminal mannose residues that can interact with the mannose receptors. The internalization of LAM-anchored polystyrene beads by mannose receptor mediated phagocytosis is reported. However, mannose receptors bind to virulent H_{37}Rv and Erdman strains but do not bind to the avirulent H_{37}Ra strain of *Mycobacterium tuberculosis* [44]. The biological responses attributed to LAM may be a result of interaction with mannose receptors or other receptors that recognize LAM-like CD14 receptors. In addition to bacterial and viral sugar residues, mannose receptor also recognizes several fungal ligands including glycoprotein A of *Pneumocystis carinii* [51] and mannan from *Candida albicans* [52].

The ligands for other mannose receptor family members are relatively few. Pancreatic $sPLA_2$IB is reported as the only ligand of PLA_2R; however, an interspecies variation in binding affinity was observed [53, 54]. Other potential ligands include $sPLA_2$-V, $sPLA_2$-IID, and $sPLA_2$-X [55, 56]. Specific ligands for DEC-205 are not reported. DEC-205 cysteine-rich domain does not interact with sulfated sugars

and also lacks the C-type lectin activity. Like the mannose receptor, Endo180 is also multifunctional and exhibits binding to a distinct set of ligands. Ca^{2+} - dependent binding of Endo180 to mannose, fucose, and N-acetylglucosamine is evident. Endo180 does not bind to galactose and sulfated sugars [57]. It exhibits binding to components of the extracellular protease systems (MMP13 and uPAR). An interaction of Endo180 with collagen via the fibronectin type II domain is reported.

6 Receptor Targeting Strategies

Among the family of mannose receptors, the most extensively exploited and studied receptor for targeted drug delivery is the mannose receptor. Hence, this section focuses mainly on mannose receptor enabled intracellular delivery. The endocytosis and phagocytosis of microbes in the macrophages occur by interaction of glycoproteins in the cell walls with the mannose receptor. Mannose conjugates and mannosylated nanocarriers target these intracellular pathogens by promoting uptake of the drug-loaded mannosylated constructs in the infected cells via mannose receptor. Nanocarrier-based strategies to target mannose receptor overexpression in tumor microenvironment are reported. Moreover, interaction of mannose ligands with mannose receptor expressed on macrophages/dendritic cells can lead to induction of immune signaling pathways, an approach of great importance in vaccine delivery [58]. Targeting desired cells via ligand-mediated approach can minimize systemic distribution and off-site toxicity. Decoration of surface of nanocarriers with ligands with high affinity to the mannose receptor is the strategy employed for targeting.

6.1 Mannose Conjugates

Mannose conjugates can be prepared by reaction between mannose derivatives and proteins or therapeutic agents like antigens. The stability of the conjugate within the body and release of the therapeutic agent at the site of action depend on the bond between the mannose derivative and the system. Most of the strategies studied involve use of endogenous mannose receptor ligands. Recent studies report utilization of synthetic ligands specific to mannose receptor expressed on macrophages or DCs. Polysaccharide from Bletilla striata (a glucomannan) having high affinity to mannose receptor expressing cells was conjugated to alendronate, a bisphosphonate. The conjugate revealed inhibition of angiogenesis and elimination of TAMs leading to suppressed tumor progression [59]. In some instances, mannose ligand has been employed to act as antigen and potentiate the immunogenicity of the conjugated protein/peptide molecule. A mannosylated vaccine formed by conjugation of glucuronoxylomannan, a polysaccharide found in *Cryptococcus neoformans* capsule and tetanus toxoid, elicited high levels of capsular antibodies [60]. Another study

reports coupling of heptasaccharide oligosaccharide, the immunodeterminant of glucuronoxylomannan with human serum albumin which resulted in induction of immunogenic responses [61].

6.2 *Mannosylated Nanocarriers*

Mannosylated nanocarriers can be prepared by coating/conjugation of mannose ligands to the surface of nanocarriers like liposomes or nanoparticles. Such mannosylated systems enable targeting to the mannose receptor and permit the delivery of cargo (antigen/drug) at the site of interest. Furthermore, particulate nature of the nanocarriers accompanied by mannose association significantly improves uptake by the endocytic and phagocytic pathways.

6.2.1 Mannosylated Liposomes

Liposomes have been extensively studied in the literature as carriers for drugs, proteins, and even fluorescent markers. Mannosylation of liposomes enables their application in treatment of intracellular infections like tuberculosis (TB) and leishmaniasis or as vaccine candidates in cancers or infections. Mannosylated liposomes can be prepared by using mannose lipid conjugates, covalently attaching mannose derivatives to liposomes, or by adsorbing the ligand on liposomal surface [3]. The click reaction was used for the preparation of cytotoxic mannose click conjugates by reaction with aminobenzoic acid derivatives [62]. In one study, mannose-cholesterol conjugates were synthesized by click reaction for liposomal drug delivery systems [63]. Wang et al. studied the effect of varying the chain length of the polyethylene glycol (PEG) linker and the optimal mannose-cholesterol conjugates were used for liposomal messenger RNA (mRNA) delivery [64].

Drug-related issues like toxicity and resistance in leishmaniasis have been tackled by treatment with mannosylated liposomes. Amphotericin B liposomes coated with palmitoyl mannose (Man-Lip) or 4-sulfated N-acetyl galactosamine (sulf-Lip) revealed rapid intracellular uptake of Sulf-Lip and higher liver and spleen Amphotericin B levels indicating specificity of 4-sulfated N-acetyl galactosamine to resident macrophages [65]. In a similar study, mannosylated Amphotericin B loaded liposomes demonstrated maximum reduction in parasite load (78.8 ± 3.9%) compared to Amphotericin B solution (42.5 ± 1.8%) and cationic Amphotericin B loaded liposomes (61.2 ± 3.2%) in *Leishmania donovani*-infected golden hamster model [66]. Among three sugar grafted liposomes (mannose, glucose, and galactose), mannose liposomes loaded with pentamidine isethionate revealed superior reduction in parasite loads [67]. Sinha et al. reported reduced spleen parasitic burden with mannosylated andrographolide loaded liposomes when tested in experimental hamster leishmaniasis model [68]. A succinct summary of other liposome-based mannose receptor targeting for intracellular infections and cancer is provided in Table 15.3.

Table 15.3 Mannosylated liposomes for targeted delivery

Disease	Active	Ligand/nanosystem	Study outcome	Reference
Infections				
Aspergillosis	Hamycin	Mannose	Reduced fungal load in infected organs	[69]
HIV	Stavudine	O-palmitoylmannose-coated liposomes	High uptake in reticuloendothelial system (RES) organs such as lung, liver, and spleen and high systemic clearance	[70]
Leishmaniasis	Benzyl derivative of *Penicillium nigricans* derived compound MT81 (Bz_2MT81)	p-aminophenyl-α-D-mannoside coupled liposomes	Lowering of splenic parasitic burden and reduction in effective dose to kill the splenic parasite	[71]
	CpG-containing oligodeoxynucleotide	p-aminophenyl-α-D-mannopyranoside coupled to liposomes	Inhibition of amastigote multiplication in macrophages and elimination of splenic parasite load in visceral leishmaniasis mouse model	[72]
	Doxorubicin and IFN ɣ	p-aminophenyl-α-D-mannopyranoside coupled to liposomes	Complete elimination of splenic parasites	[73]
Parasitic infection	Ciprofloxacin	Mannose	High uptake and antibacterial efficacy in vitro	[74]
Pneumococcal meningitis	Dichloromethylene diphosphonate	Mannose	Reduced migration of white blood cells into cerebrospinal fluid in experimental infection models	[75]
Cancer				
Drug-resistant colon cancer	Dihydroartemisinin and doxorubicin	Mannose was conjugated to the DSPE-PEG2000-NH2	Improved tumor inhibition and tackling of drug resistance	[76]

Mannosylated liposomes have been reported for vaccination against infections and cancers. Garcon et al. covalently coupled mannosylated albumin to the surface of dehydration–rehydration vesicles (DRVs). These mannosylated DRVs containing tetanus toxoid revealed selective binding to mouse peritoneal macrophages compared to nonmannosylated DRVs with an augmented immunoadjuvant activity in Balb/c mice [77]. In another study, liposomes coated with neoglycolipids (mannopentose or mannotriose) revealed high serum levels of soluble leishmanial antigen (SLA)-specific IgG2a antibody titer and low level of IgG1 antibody titers in comparison to uncoated liposomes along with a delayed footpad swelling progression [78]. In contrast to uncoated liposomes, subcutaneous immunization with oligomannose residue coated liposomes encapsulating peptides representing epitopes of gp120 (a HIV1 envelope glycoprotein) induced MHC class I-restricted CD8+ cytotoxic T-lymphocyte response [79].

A protective immune response against cancer can be elicited by association of immunostimulants or immunomodulators with mannosylated antigen loaded liposomes. Mannosylated liposome–plasmid DNA complex (Man-lipoplex), prepared as a potential DNA vaccine for melanoma, revealed greater pUb-M gene transfection into antigen-presenting cells than uncoated liposomes and demonstrated prolonged survival coupled with melanoma inhibition in mice model [80]. A similar study performed by White et al. revealed mannosylated liposomes of lipid core peptide with Quil A adjuvant acted as prophylactic anticancer vaccines and protected mice against tumors [81].

6.2.2 Mannosylated Nanoparticles (NPs)

Mannosylated NPs are widely investigated in infections and cancers akin to the mannosylated liposomes. Mannosylation of polyanhydride NPs can be performed by techniques such as desolvation or direct coating. Iron oxide NPs may be coated by precipitation of iron salts by incubation with D-mannose solution or by oxidation of NPs followed by addition of D-mannose solution. Chemical modification of polymers with mannosylated ligands is also reported [3].

The mannose receptor is profusely overexpressed on the macrophages, DCs, and foamy cells which constitute the TB granuloma. This permits utilization of mannosylated NPs for targeted intracellular delivery in TB. A multilayer mannosylated drug delivery system for intracellular delivery of first-line antibiotics Rifampicin and Isoniazid has been developed [82]. Isoniazid loaded mannosylated gelatin NPs reduced drug hepatotoxicity and significantly decreased bacterial burden in lungs and spleen of infected Balb/c mice [83]. In an analogous study, licorice loaded mannosylated gelatin NPs revealed enhanced uptake in RAW 264.7 cells and reduced spleen and lung bacterial loads in *Mycobacterium tuberculosis* H_{37}Rv-infected mice compared to untreated animals [84]. Other strategies employing mannosylated NPs for drug delivery in infections and cancer are enlisted in Table 15.4. A summary of mannosylated NPs employed as vaccine carriers is presented in Table 15.5.

Table 15.4 Mannosylated NPs in infections and cancer

Disease	Active	Ligand/nanosystem	Study outcome	Reference
Infections				
Helicobacter pylori infection	Acetohydroxamic acid	Fucose-specific (UEA-I) and mannose-specific (Conconavalin A) lectins conjugated to gliadin NPs	NPs inhibited binding of *Helicobacter pylori* to human stomach cells	[85]
HIV	Didanosine	Mannose conjugated to gelatin NPs	High macrophage uptake and RES localization	[86]
	Didanosine	Mannan conjugated to gelatin NPs	Fivefold higher intracellular uptake and greater localization in spleen, lymph nodes, and brain	[87]
	Stavudine	Mannose conjugated to gelatin NPs	High macrophage uptake and RES localization	[88]
Leishmaniasis	Amphotericin B	Gelatin conjugated to mannose via direct coupling or via PEG spacer	5.4-fold reduction in IC_{50} compared to free Amphotericin B solution in intracellular amastigote model	[89]
	Amphotericin B	4-sulfated Sulfated N-acetyl galactosamine-coated NPs	High RES localization and reduced splenic parasite burden	[90]
	Curcumin	D-mannose conjugated to chitosan NPs	Low in vitro cytotoxicity and reduced parasite loads in spleen	[91]
	Doxorubicin	4-sulfated Sulfated N-acetyl galactosamine-coated NPs	Enhanced intracellular uptake and high RES localization	[92]
	Rifampicin	D-mannose Mannose conjugated to chitosan NPs	High ex vivo uptake and high RES localization	[93]
TB	Isoniazid	Mannose-conjugated solid lipid NPs	High uptake and reduced cytotoxicity in vitro	[94]
	Rifabutin	Mannose-coated solid lipid NPs	Sixfold higher uptake ex vivo and low immunogenicity compared to uncoated formulation. Prolonged circulation and targeted delivery to alveolar tissues	[95]

(continued)

Table 15.4 (continued)

Disease	Active	Ligand/nanosystem	Study outcome	Reference
Cancers				
Lung adenocarcinoma	Gemcitabine	D-Mannose-conjugated solid lipid NPs	Improved uptake and high cytotoxicity in A549 cells with preferential lung accumulation	[96]
Lung cancer	DNA	Mannan-modified solid lipid NPs	Higher gene expressions compared to unmodified DNA loaded NPs suggesting applicability for nonviral vector gene delivery	[97]
Tumor	Doxorubicin	4-Aminophenyl α-D-mannopyranoside modified albumin NPs	Improved localization in brain glioma cells and reduction in tumor size	[98]
	Doxorubicin	Self-assembly of heptamannosylated β-cyclodextrin into NPs	Slow tumor growth in murine xenograft tumor models	[99]

Table 15.5 Mannosylated NPs-based vaccines

Antigen	Ligand/nanosystem	Study outcome	Reference
Ag85A	Mannose moiety of guar gum NPs	Strong systemic and mucosal immune response following oral administration, protecting the antigen from harsh gastric environment.	[100]
Nil	Mannan-coated PLGA NPs	Improved dendritic cells' maturation and stimulatory function.	[101]
Nil	Dimannose and lactose decorated polyanhydride NPs	Surface functionalized pathogen like NPs revealed enhanced expression of MHC II, CD86 and CD40, CIRE, and mannose receptor on the cell surface.	[102]
Ovalbumin	Mannan decorated polylactide-co-glycolide (PLGA) NPs	Enhanced CD4+ and CD8+ T-cell responses in comparison to nonconjugated NPs	[103]
Ovalbumin	Mannosamine-coated polyanhydride NPs	Single subcutaneous or oral dose demonstrated higher and balanced IgG1 and IgG2a antibody responses compared to uncoated NPs. Oral immunization elicited higher levels of intestinal secretory IgA levels than subcutaneous immunization.	[104]
Toll-like receptor 7 agonist, imiquimod (R837)	PLGA NPs coated with mannosylated cancer cell membrane	Enhanced uptake by DCs and delayed tumor development	[105]

6.3 Miscellaneous Applications

6.3.1 Mannosylated SPIONs as MRI Contrast Agents

Superparamagnetic iron oxide nanoparticles (SPIONs) are reported as promising magnetic resonance imaging (MRI) contrast agents. Surface modification of SPIONs becomes essential owing to their drawbacks such as aggregation in water, chemical instability, and nonspecific targeting. To overcome these issues, SPIONs were coated with mannan to enable recognition by mannose receptor present on macrophages [106]. Mannan-coated SPIONs of 28.4 ± 7.2 nm size demonstrated low cytotoxicity in RAW 264.7 cells. Surface coating with mannan prevented aggregation of SPIONs enabling selective delivery into antigen-presenting cells, suggesting applicability as macrophage-targeted MRI contrasting agent.

6.3.2 Two-Photon Photodynamic Therapy

Photodynamic therapy combined with two-photon excitation offers a noninvasive alternative approach to chemo- and radiotherapy to reduce small solid tumors. The photosensitizer was covalently attached to mesoporous silica NPs followed by mannose coating. A single injection aided targeting to tumor site by mannose receptor and two-photon photodynamic therapy led to reduction in tumor size [107].

6.3.3 Biomarker for Pulmonary TB Patients

The serum and pleural concentrations of mannose receptor (CD206) were monitored in pulmonary TB subjects. An increased CD206 level was observed in sera but not in pleura with a sensitivity of 77.3% and specificity of 86.5%. This presents a new application of mannose receptor as a biomarker of pulmonary TB [108].

6.3.4 Lysosomal Targeting in Storage Diseases

Therapeutic enzymes were conjugated to yeast cell wall, a natural source of mannose-6-phosphate (M6P) glycan for utilization in glycogen storage diseases like Pompe disease. Recombinant acid α-glucosidase, a therapeutic in Pompe disease when conjugated to M6P glycans from cell wall of glyco-engineered yeast, revealed efficient intracellular localization and improved accumulated glycogen digestion [109].

7 Clinical Studies

Although targeting to the mannose receptor has been widely investigated, very few mannosylated candidates have entered the clinical trials. Herein, we discuss a promising mannose-based targeted strategy DermaVir, a topical preparation for the treatment of HIV/AIDS and FDA-approved radiopharmaceutical ^{99m}Tc-tilmanocept for sentinel lymph node mapping.

DermaVir (Genetic Immunity) is currently enrolled for Phase III clinical trials, set to begin in 2019. It represents topical immunotherapy for the treatment of HIV/AIDS comprising of plasmid DNA-based mannosylated particles [110, 111]. The mannosylated particles are formed by complexation of DNA with a cationic polymer (PEIm) while glucose present in the formulation acts as an aggregation inhibitor and stabilizer. The formulation when applied on the epidermal layer penetrates the skin surface and triggers immune responses.

Staging of cancer progression relies on the mapping of lymph node metastases. Mapping of the sentinel lymph node requires an agent that quickly clears the injection site, rapidly enters, and retains in the sentinel lymph node, without entering the distal lymph nodes. FDA-approved ^{99m}Tc-tilmanocept by Navidea Biopharmaceuticals is a mannose-targeted radiopharmaceutical for the detection of sentinel lymph node and lymphatic mapping in tumors [112, 113]. The radiopharmaceutical has crossed several clinical trials [114–116] and is now employed for stage determination of cancers under the trade name "Lymphoseek."

Chemically, it is ^{99m}Tc-diethylenetriaminepentaacetic acid–mannosyl–dextran comprising of diethylenetriaminepentaacetic acid and mannose units covalently linked to a 10-kDa dextran backbone. The binding occurs via the mannose residues to the receptors expressed by the myeloid cells. Following injection, ^{99m}Tc-tilmanocept enters the lymphatic channels and localizes in the sentinel lymph node by binding to the mannose receptor, thus enabling the mapping of lymph.

8 Advantages and Limitations Related to Specific Targeting through Through Mannose Receptor

As mannose receptor is predominantly located on macrophages, the abode of intracellular infections, specific targeting via mannosylated conjugates and mannose decorated nanocarriers can improve the efficacy of therapeutics and vaccine candidates. Additionally, mannose receptor mediated targeting could provide a practical approach for development of intracellular vaccines. Nevertheless, mannose-targeted vaccines would need to be coupled with other agents to enhance immune response. Interestingly, vaccines based on mannose receptor endocytosis may not only enhance immune responses against cancer and infectious diseases but could also find application in autoimmune disease therapeutics [58]. Surface-modified

mannosylated constructs could provide the additional advantage of both phagocytic and endocytic uptake to augment intracellular drug concentrations. However, the ubiquitous presence of macrophages all over the body could provide challenges in targeting specific macrophages through mannosylated carriers.

9 Conclusion

Targeting the mannose receptors represents an exciting therapeutic strategy for infections and cancers overexpressing the receptors. Extrapolating this strategy to vaccines provides exciting opportunities in the design of targeted therapeutics.

References

1. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. FEBS J. 2005;272(24):6179–217.
2. East L, Isacke CM. The mannose receptor family. Biochimica et Biophysica Acta (BBA)-General Subjects. 2002;1572(2–3):364–86.
3. Irache JM, Salman HH, Gamazo C, Espuelas S. Mannose-targeted systems for the delivery of therapeutics. Expert Opin Drug Deliv. 2008;5(6):703–24.
4. Azad AK, Rajaram MVS, Schlesinger S. Exploitation of the macrophage mannose receptor (CD206) in infectious disease diagnostics and therapeutics. J Cytol Mol Biol. 2007;137(12):2696–700.
5. Ancian P, Lambeau G, Mattéi MG, Lzadunski M. The human 180-kDa receptor for secretory phospholipases A2. J Biol Chem. 1995;270(15):8963–70.
6. Behrendt N, Jensen ON, Engelholm LH, Mørtz E, Mann M, Danø K. A urokinase receptor-associated protein with specific collagen binding properties. J Biol Chem. 2002;275(3):1993–2002.
7. Sheikh H, Yarwood H, Ashworth A, Isacke CM. Endo180, an endocytic recycling glycoprotein related to the macrophage mannose receptor is expressed on fibroblasts, endothelial cells and macrophages and functions as a lectin receptor. J Cell Sci. 2000;113:1021–32.
8. Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Nussenzweig M, Steinman RM. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature. 1995;375:151–5.
9. Simpson DZ, Hitchen PG, Elmhirst EL, Taylor ME. Multiple interactions between pituitary hormones and the mannose receptor. Biochem J. 1999;343(2):403–11.
10. Leteux BC, Chai W, Loveless RW, Yuen C, Uhlin-hansen L, Combarnous Y, et al. The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and sulfated oligosaccharides of blood group Lewis a and Lewis x types in addition to the sulfated n-glycans of lutropin. J Exp Med. 2000;191(7):1117–26.
11. Crocker PR, Da Silva R, Holmes N, Colominas C, Rudd P, Dwek R, et al. Cell-specific glycoforms of sialoadhesin and CD45 are counter-receptors for the cysteine-rich domain of the mannose receptor. J Biol Chem. 1999;274(49):35211–8.
12. Liu BY, Chirino AJ, Misulovin Z, Leteux C, Feizi T, Nussenzweig MC, et al. Crystal structure of the cysteine-rich domain of mannose receptor complexed with a sulfated carbohydrate ligand. J Exp Med. 2000;191(7):1105–15.
13. Morgunova E, Tuuttila A, Bergmann U, Isupov M, Lindqvist Y, Schneider G, Tryggvason K. Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. Science. 1999;284:1667–70.

14. Pickford AR, Potts JR, Bright JR, Phan I, Campbell ID. Solution structure of a type 2 module from fibronectin : implications for the structure and function of the gelatin-binding domain. Structure. 1997;5(3):359–70.
15. Hu Z, Shi X, Yu B, Li N, Huang Y, He Y. Structural insights into the pH-dependent conformational change and collagen recognition of the human mannose receptor. Structure. 2018;26:60–71.
16. Napper CE, Drickamer K, Taylor ME. Collagen binding by the mannose receptor mediated through the fibronectin type II domain. Biochem J. 2006;395:579–86.
17. Napper CE, Dyson MH, Taylor ME. An extended conformation of the macrophage mannose receptor. J Biol Chem. 2001;276(18):14759–66.
18. Stahl PD. The macrophage mannose receptor: current status. Am J Respir Cell Mol Biol. 1990;2(4):317–8.
19. Howard MJ, Isacke CM. The C-type lectin receptor Endo180 displays internalization and recycling properties distinct from other members of the mannose receptor family. J Biol Chem. 2002;277(35):32320–31.
20. Mahnke K, Guo M, Lee S, Sepulveda H, Swain SL, Nussenzweig M, et al. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. J Cell Biol. 2000;151(3):673–83.
21. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol. 1999;17(1):593–623.
22. Wileman TE, Lennartz MR, Stahl PD. Identification of the macrophage mannose receptor as a 175-kDa membrane protein. Proc Natl Acad Sci. 1986;83(8):2501–5.
23. Linehan SA, Weber R, McKercher S, Ripley RM, Gordon S, Martin P. Enhanced expression of the mannose receptor by endothelial cells of the liver and spleen microvascular beds in the macrophage-deficient PU.1 null mouse. Histochem Cell Biol. 2005;123(4–5):365–76.
24. Groger M, Holnthoner W, Maurer D, Lechleitner S, Wolff K, Mayr BB, et al. Dermal microvascular endothelial cells express the 180-kDa macrophage mannose receptor in situ and in vitro. J Immunol. 2000;165(10):5428–34.
25. Mulay SR, Desai J, Kumar SV, Eberhard JN, Thomasova D, Romoli S, et al. Kaposi's sarcoma cells express the macrophage-associated antigen mannose receptor and develop in peripheral blood cultures of Kaposi's sarcoma patients. Am J Pathol. 1997;150(3):929–38.
26. Szolnoky G, Bata-Csörgö Z, Kenderessy AS, Kiss M, Pivarcsi A, Novák Z, et al. A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans. J Investig Dermatol. 2001;117(2):205–13.
27. Wilt ST, Greaton CJ, Lutz DA, McLaughin B. Mannose receptor is expressed in normal and dystrophic retinal pigment epithelium. Exp Eye Res. 1999;69(4):405–11.
28. Astarie-Dequeker C, N'Diaye EN, Le Cabec V, Rittig MG, Prandi J, Maridonneau-Parini I. The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. Infect Immun. 1999;67(2):469–77.
29. Stahl PD, Ezekowitz RAB. The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol. 1998;10(1):50–5.
30. Ailavena P, Chieppa MMP, Piemonti L. From pattern recognition receptor to regulator of homeostasis: the double-faced macrophage mannose receptor. Crit Rev Immunol. 2004;24(3):179–92.
31. Stahl PD. The mannose receptor and other macrophage lectins. Curr Opin Immunol. 1992;4(1):49–52.
32. Mokoena T, Gordon S. Modulation of mannosyl, fucosyl receptor activity in vitro by lymphokines, gamma and alpha interferons, and dexamethasone. J Clin Invest. 1985;75(2):624–31.
33. Shepherds VL, Konish G, Stahl P. Dexamethasone increases expression of mannose receptors and decreases extracellular lysosomal enzyme accumulation in macrophages. J Biol Chem. 1985;260(1):160–4.
34. Clohisy DR, Bar-shavitsb Z, Chappel JC, Teitelbaum LI. 1,25-dihydroxyvitamin D3 modulates bone marrow macrophage precursor proliferation and differentiation. J Biol Chem. 1987;262(33):15922–9.

35. Schreiber S, Blumt JS, Chappel JC, Stenson WF, Stahlt PD, Teitelbaum SL, et al. Prostaglandin E specifically upregulates the expression of the mannose-receptor on mouse. Cell Regul. 1990;1(5):403–13.
36. Shepherd VL, Abdolrasulnia RA, Garrett M, Cowan HB. Down-regulation of mannose receptor activity in macrophages after treatment with lipopolysaccharide and phorbol esters. J Immunol. 1990;145(5):1530–6.
37. Schreiber S, Stenson WF, MacDermott RP, Chappel JC, Teitelbaum SL, Perkins SL. Aggregated bovine IgG inhibits mannose receptor expression of murine bone marrow-derived macrophages via activation. J Immunol. 1991;147(4):1377–82.
38. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M. MUC1 and cancer. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 1999;1455(2–3):301–13.
39. Nath S, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. Trends Mol Medicine. 2017;20(6):332–42.
40. Lau SK, Weiss LM, Chu PG. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol. 2004;122(1):61–9.
41. Taylor PR, Gordon S, Martinez-Pomares L. The mannose receptor: linking homeostasis and immunity through sugar recognition. Trends Immunol. 2005;26(2):104–10.
42. Roseman DS, Baenziger JU. Molecular basis of lutropin recognition by the mannose/GalNAc-4-SO4 receptor. Proc Natl Acad Sci. 2000;97(18):9949–54.
43. Fiete DJ, Beranek MC, Baenziger JU. A cysteine-rich domain of the "mannose" receptor mediates GalNAc-4-SO4 binding. Proc Natl Acad Sci. 1998;95(5):2089–93.
44. Schlesinger LS, Hull SR, Kaufman TM. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of Mycobacterium tuberculosis to human macrophages. J Immunol. 1994;152(8):4070–9.
45. Wilson ME, Pearson RD. Evidence that Leishmania donovani utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. J Immunol. 1986;136(12):4681–8.
46. Kahn S, Wleklinski M, Aruffo A, Farr A, Coder D, Kahn M. Trypanosoma cruzi amastigote adhesion to macrophages is facilitated by the mannose receptor. J Exp Med. 2004;182(5):1243–58.
47. Gruden-Movsesijan A, Milosavljevic LS. The involvement of the macrophage mannose receptor in the innate immune response to infection with parasite Trichinella spiralis. Vet Immunol Immunopathol. 2006;109(1–2):57–67.
48. Macedo-Ramos H, Campos FSO, Carvalho LA, Ramos IB, Teixeira LM, De Souza W, et al. Olfactory ensheathing cells as putative host cells for Streptococcus pneumoniae: evidence of bacterial invasion via mannose receptor-mediated endocytosis. Neurosci Res. 2011;69(4):308–13.
49. Nguyen DG, Hildreth JE. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophage. Eur J Immunol. 2003;33(2):483–93.
50. Reading PC, Miller JL, Anders EM. Involvement of the mannose receptor in infection of macrophages by influenza virus. J Virol. 2000;74(11):5190–7.
51. O'Riordan DM, Standing JE, Limper AH. Pneumocystis carinii glycoprotein A binds macrophage mannose receptors. Infect Immun. 1995;63(3):779–84.
52. van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJ, Cheng SC, Joosten I, et al. The macrophage mannose receptor induces IL-17 in response to Candida albicans. Cell Host Microbe. 2009;5(4):329–40.
53. Lambeau G, Lazdunski M. Receptors for a growing family of secreted phospholipases A2. Trends Pharmacol Sci. 1999;20:162–70.
54. Cupillard L, Mulherkar R, Gomez N, Kadam S, Valentin E, Lazdunski E, Lambeau G. Both group IB and group IIA secreted phospholipases A2 are natural ligands of the mouse 180-kDa M-type receptor. J Biol Chem. 1999;274(11):7043–51
55. Hanasaki K, Ono T, Saiga A, Morioka Y, Ikeda M, Kawamoto K, Higashino KI, Nakano K, Yamada K, Ishizaki J, Arita H. Purified group X secretory phospholipase A2 induced prominent release of arachidonic acid from human myeloid leukemia cells. J Biol Chem. 1999;274(48):34203–11.

56. Hanasaki K, Arita H. Biological and pathological functions of phospholipase A2 receptor. Arch Biochem Biophys. 1999;372(2):215–23.
57. East L, Rushton S, Taylor ME, Isacke CM. Characterization of sugar binding by the mannose receptor family member, Endo180. J Biol Chem. 2002;277(52):50469–75.
58. Keler T, Ramakrishna V, Fanger MW. Mannose receptor-targeted vaccines. Expert Opin Biol Ther. 2004;4(12):1953–62.
59. Zhan X, Jia L, Niu Y, Qi H, Chen X, Zhang Q, et al. Targeted depletion of tumour-associated macrophages by an alendronate glucomannan conjugate for cancer immunotherapy. Biomaterials. 2014;35(38):10046–57.
60. Devi SJ. Preclinical efficacy of a glucuronoxylomannan-tetanus toxoid conjugate vaccine of Cryptococcus neoformans in a murine model. Vaccine. 1996;14(9):841–4.
61. Oscarson S, Alpe M, Svahnberg P, Nakouzi A, Casadevall A. Synthesis and immunological studies of glycoconjugates of Cryptococcus neoformans capsular glucuronoxylomannan oligosaccharide structures. Vaccine. 2005;23(30):3961–72.
62. Hradilová L, Poláková M, Dvořáková B, Hajdúch M, Petruš L. Synthesis and cytotoxicity of some D-mannose click conjugates with aminobenzoic acid derivatives. Carbohydr Res. 2012;361:1–6.
63. Nguyen H, Katavic P, Bashah NA, Ferro V. Synthesis of mannose-cholesterol conjugates for targeted liposomal drug delivery. ChemistrySelect. 2016;1(1):31–4.
64. Wang F, Xiao W, Elbahnasawy MA, Bao X, Zheng Q, Gong L, Zhou Y, Yang S, Fang A, Farag MM, Wu J. Optimization of the linker length of mannose-cholesterol conjugates for enhanced mRNA delivery to dendritic cells by liposomes. Front Pharmacol. 2018;9:1–14.
65. Singodia D, Verma A, Verma RK, Mishra PR. Investigations into an alternate approach to target mannose receptors on macrophages using 4-sulfated N-acetyl galactosamine more efficiently in comparison with mannose-decorated liposomes: an application in drug delivery. Nanomedicine. 2012;8(4):468–77.
66. Rathore A, Jain A, Gulbake A, Shilpi S, Khare P, Jain A, et al. Mannosylated liposomes bearing Amphotericin B for effective management of visceral Leishmaniasis. J Liposome Res. 2011;21(4):333–40.
67. Banerjee G, Nandi G, Mahato SB, Pakrashi A, Basu MK. Drug delivery system: targeting of pentamidines to specific sites using sugar grafted liposomes. J Antimicrobial Chemother. 1996;38(1):145–50.
68. Sinha J, Mukhopadhyay S, Das N, Basu MK. Targeting of liposomal andrographolide to L. donovani-infected macrophages in vivo. Drug Deliv. 2000;7(4):209–13.
69. Moonis M, Ahmad I, Bachhawat B, Moonis M, Ahmad I, Bachhawat BK. Mannosylated liposomes as carriers for hamycin in the treatment of experimental aspergillosis in Balb/C mice. J Drug Target. 1993;1(2):147–55.
70. Garg M, Asthana A, Agashe HB, Agrawal GP, Jain NK. Stavudine-loaded mannosylated liposomes: in-vitro anti-HIV-I activity, tissue distribution and pharmacokinetics. J Pharm Pharmacol. 2006;58(5):605–16.
71. Mitra M, Mandal AK, Chatterjee TK, Das N. Targeting of mannosylated liposome incorporated Benzyl derivative of Penicillium nigricans derived compound MT81 to reticuloendothelial systems for the treatment of visceral leishmaniasis. J Drug Target. 2005;13(5):285–93.
72. Datta N, Mukherjee S, Das L, Das PK. Targeting of immunostimulatory DNA cures experimental visceral leishmaniasis through nitric oxide up-regulation and T cell activation. Eur J Immunol. 2003;33(6):1508–18.
73. Kole L, Das L, Das PK. Synergistic effect of interferon-gamma and mannosylated liposome-incorporated doxorubicin in the therapy of experimental visceral leishmaniasis. J Infect Dis. 1999;180(3):811–20.
74. Chono S, Tanino T, Seki T, Morimoto K. Efficient drug targeting to rat alveolar macrophages by pulmonary administration of ciprofloxacin incorporated into mannosylated liposomes for treatment of respiratory intracellular parasitic infections. J Control Release. 2008;127(1):50–8.
75. Zysk G, Brück W, Huitinga I, Fischer FR, Flachsbarth F, Van Rooijen N, et al. Elimination of blood-derived macrophages inhibits the release of interleukin-1 and the entry of leukocytes

into the cerebrospinal fluid in experimental pneumococcal meningitis. J Neuroimmunol. 1997;73(1–2):77–80.
76. Kang XJ, Wang HY, Peng HG, Chen BF, Zhang WY, Wu AH, Xu Q, Huang YZ. Codelivery of dihydroartemisinin and doxorubicin in mannosylated liposomes for drug-resistant colon cancer therapy. Acta Pharmacol Sin. 2017;38(6):885–96.
77. Garcon N, Gregoriadis G, Taylor M, Summerfield J. Mannose-mediated targeted immunoadjuvant action of liposomes. Immunology. 1988;64:743–5.
78. Shimizu Y, Takagi H, Nakayama T, Yamakami K, Tadakuma T, Yokoyama N, Kojima N. Intraperitoneal immunization with oligomannose-coated liposome-entrapped soluble leishmanial antigen induces antigen-specific T-helper type immune response in BALB/c mice through uptake by peritoneal macrophages. Parasite Immunol. 2007;29(5):229–39.
79. Fukasawa M, Shimizu Y, Shikata K, Nakata M, Sakakibara R, Yamamoto N, Hatanaka M, Mizouchi T. Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. FEBS Lett. 1998;441(3):353–6.
80. Lu Y, Kawakami S, Yamashita F, Hashida M. Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. Biomaterials. 2007;28(21):3255–62.
81. White K, Rades T, Kearns P, Toth I, Hook S. Immunogenicity of liposomes containing lipid core peptides and the adjuvant Quil A. Pharm Res. 2006;23(7):1473–81.
82. Praphakar RA, Shakila H, Dusthackeer VN, Munusamy MA, Kumar S, Rajan M. A mannose-conjugated multi-layered polymeric nanocarrier system for controlled and targeted release on alveolar macrophages. Polym Chem. 2018;9(5):656–67.
83. Saraogi GK, Sharma B, Joshi B, Gupta P, Gupta UD, Jain NK, et al. Mannosylated gelatin nanoparticles bearing isoniazid for effective management of tuberculosis. J Drug Target. 2011;19(3):219–27.
84. Viswanathan V, Mehta H, Pharande R, Bannalikar A, Gupta P, Gupta U, Mukne A. Mannosylated gelatin nanoparticles of licorice for use in tuberculosis: formulation, in vitro evaluation, in vitro cell uptake, in vivo pharmacokinetics and in vivo anti-tubercular efficacy. J Drug Delivery Sci Technol. 2018;45:255–63.
85. Umamaheshwari RB, Jain NK. Receptor mediated targeting of lectin conjugated gliadin nanoparticles in the treatment of Helicobacter pylori. J Drug Target. 2003;11(7):415–24.
86. Jain SK, Gupta Y, Jain A, Saxena AR, Khare P, Jain A. Mannosylated gelatin nanoparticles bearing an anti-HIV drug didanosine for site-specific delivery. Nanomedicine. 2008;4(1):41–8.
87. Kaur A, Jain S, Tiwary AK. Mannan-coated gelatin nanoparticles for sustained and targeted delivery of didanosine: in vitro and in vivo evaluation. Acta Pharma. 2008;58(1):61–74.
88. Mahajan S, Prashant CK, Koul V, Choudhary V, Dinda AK. Receptor specific macrophage targeting by mannose-conjugated gelatin nanoparticles- an in vitro and in vivo study. Curr Nanosci. 2010;6(4):413–21.
89. Nahar M, Dubey V, Mishra D, Mishra PK, Dube A, Jain NK. In vitro evaluation of surface functionalized gelatin nanoparticles for macrophage targeting in the therapy of visceral leishmaniasis. J Drug Target. 2010;18(2):93–105.
90. Tripathi P, Dwivedi P, Khatik R, Jaiswal AK, Dube A, Shukla P, Mishra PR. Development of 4-sulfated N-acetyl galactosamine anchored chitosan nanoparticles: a dual strategy for effective management of Leishmaniasis. Colloids Surf B: Biointerfaces. 2015;136:150–9.
91. Chaubey P, Mishra B, Mudavath SL, Patel RR, Chaurasia S, Sundar S, Suvarna V, Monteiro M. Mannose-conjugated curcumin-chitosan nanoparticles: efficacy and toxicity assessments against Leishmania donovani. Int J Biol Macromol. 2018;111:109–20.
92. Dwivedi P, Kansal S, Sharma M, Shukla R, Verma A, Shukla P, Tripathi P, Gupta P, Saini D, Khandelwal K, Verma R. Exploiting 4-sulphate N-acetyl galactosamine decorated gelatin nanoparticles for effective targeting to professional phagocytes in vitro and in vivo. J Drug Target. 2012;20(10):883–96.
93. Chaubey P, Mishra B. Mannose-conjugated chitosan nanoparticles loaded with rifampicin for the treatment of visceral leishmaniasis. Carbohydr Polym. 2014;101(1):1101–8.

94. Costa A, Sarmento B, Seabra V. Mannose-functionalized solid lipid nanoparticles are effective in targeting alveolar macrophages. Eur J Pharm Sci. 2018;114:103–13.
95. Nimje N, Agarwal A, Saraogi GK, Lariya N, Rai G, Agrawal H, et al. Mannosylated nanoparticulate carriers of rifabutin for alveolar targeting. J Drug Target. 2009;17(10):777–87.
96. Soni N, Soni N, Pandey H, Maheshwari R, Kesharwani P, Tekade RK. Augmented delivery of gemcitabine in lung cancer cells exploring mannose anchored solid lipid nanoparticles. J Colloid Interface Sci. 2016;481:107–16.
97. Yu W, Liu C, Liu Y, Zhang N, Xu W. Mannan-modified solid lipid nanoparticles for targeted gene delivery to alveolar macrophages. Pharm Res. 2010;27(8):1584–96.
98. Byeon HJ, Thao LQ, Lee S, Min SY, Lee ES, Shin BS, et al. Doxorubicin-loaded nanoparticles consisted of cationic- and mannose-modified-albumins for dual-targeting in brain tumors. J Control Release. 2016;225:301–13.
99. Ye Z, Zhang Q, Wang S, Bharate P, Varela-Aramburu S, Lu M, et al. Tumour-targeted drug delivery with mannose-functionalized nanoparticles self-assembled from amphiphilic β-cyclodextrins. Chem Eur J. 2016;22(43):15216–21.
100. Kaur M, Malik B, Garg T, Rath G, Goyal AK. Development and characterization of guar gum nanoparticles for oral immunization against tuberculosis. Drug Delivery. 2015;22(3):328–34.
101. Haddadi A, Hamdy S, Ghotbi Z, Samuel J, Lavasanifar A. Immunoadjuvant activity of the nanoparticles' surface modified with mannan. Nanotechnology. 2014 Aug 13;25(35):355101.
102. Carrillo-Conde B, Song EH, Chavez-Santoscoy A, Phanse Y, Ramer-Tait AE, Pohl NLB, et al. Mannose-functionalized "pathogen-like" polyanhydride nanoparticles target C-type lectin receptors on dendritic cells. Mol Pharm. 2011;8(5):1877–86.
103. Hamdy S, Haddadi A, Shayeganpour A, Samuel J, Lavasanifar A. Activation of antigen-specific T cell-responses by mannan-decorated PLGA nanoparticles. Pharm Res. 2011;28(9):2288–301.
104. Salman HH, Irache JM, Gamazo C. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccine. 2009;27(35):4784–90.
105. Yang R, Xu J, Xu L, Sun X, Chen Q, Zhao Y, et al. Cancer cell membrane-coated adjuvant nanoparticles with mannose modification for effective anticancer vaccination. ACS Nano. 2018;12(6):5121–9.
106. Yoo MK, Kim IY, Kim EM, Jeong HJ, Lee CM, Jeong YY, et al. Superparamagnetic iron oxide nanoparticles coated with galactose-carrying polymer for hepatocyte targeting. Biomed Res Int. 2008;8:5196–202.
107. Gary-Bobo M, Mir Y, Rouxel C, Brevet D, Basile I, Maynadier M, et al. Mannose-functionalized mesoporous silica nanoparticles for efficient two-photon photodynamic therapy of solid tumors. Angew Chem Int Ed. 2011;50(48):11425–9.
108. Suzuki Y, Shirai M, Asada K, Yasui H, Karayama M, Hozumi H, et al. Macrophage mannose receptor, CD206, predict prognosis in patients with pulmonary tuberculosis. Sci Rep. 2018;8(1):1–9.
109. Kang JY, Shin KK, Kim HH, Min JK, Ji ES, Kim JY, Kwon O, Oh DB. Lysosomal targeting enhancement by conjugation of glycopeptides containing mannose-6-phosphate glycans derived from glyco-engineered yeast. Sci Rep. 2018;8(1):1–14.
110. Lisziewicz J, Trocio J, Whitman L, Varga G, Xu J, Bakare N, Erbacher P, Fox C, Woodward R, Markham P, Arya S. DermaVir: a novel topical vaccine for HIV/AIDS. J Investig Dermatol. 2005;124(1):160–9.
111. Lori F. DermaVir: a plasmid DNA-based nanomedicine therapeutic vaccine for the treatment of HIV/AIDS. Expert Rev Vaccines. 2011;10(10):1371–84.
112. Vera DR, Wallace AM, Hoh CK, Mattrey RF. A synthetic macromolecule for sentinel node detection: (99m)Tc-DTPA-mannosyl-dextran. J Nucl Med. 2001;42(6):951–9.
113. Surasi DS, O'Malley J, Bhambhvani P. 99mTc-Tilmanocept: a novel molecular agent for lymphatic mapping and sentinel lymph node localization. J Nucl Med Technol. 2015;43(2):87–92.
114. Leong SP, Kim J, Ross M, Faries M, Scoggins CR, Metz WL, Cope FO, Orahood RC. A phase 2 study of 99mTc-tilmanocept in the detection of sentinel lymph nodes in melanoma and breast cancer. Ann Surg Oncol. 2011;18(4):961–9.

115. Wallace AM, Han LK, Povoski SP, Deck K, Schneebaum S, Hall NC, Hoh CK, Limmer KK, Krontiras H, Frazier TG, Cox C. Comparative evaluation of [99m Tc]tilmanocept for sentinel lymph node mapping in breast cancer patients: results of two phase 3 trials. Ann Surg Oncol. 2013;20(8):2590–9.
116. Sondak VK, King DW, Zager JS, Schneebaum S, Kim J, Leong SP, Faries MB, Averbook BJ, Martinez SR, Puleo CA, Messina JL. Combined analysis of phase III trials evaluating [99mTc] tilmanocept and vital blue dye for identification of sentinel lymph nodes in clinically node-negative cutaneous melanoma. Ann Surg Oncol. 2013;20(2):680–8.

Chapter 16
Transferrin Receptor and Targeting Strategies

Harsh A. Joshi, Esha S. Attar, Prajakta Dandekar, and Padma V. Devarajan

Abstract Treatment of cancer is an extraordinary challenge using conventional therapy due to serious side effects. Targeted delivery of therapeutics has changed this paradigm. Among various approaches, receptor-mediated targeting presents great promise. Since transferrin receptor (TfR) is known to be overarticulated in various cancers, it is a lucrative target in cancer research. TfR-mediated drug delivery can deliver therapeutic cargo into tumor cells to enhance cytotoxicity with significant reduction in systemic toxicity. This has propelled the exploration of innovative targeting approaches using nanodrug delivery systems. This chapter discusses the TfR physiology and related pathophysiology, and also summarizes various drug-targeting strategies and theranostics, which rely on transferrin (Tf) as the targeting ligand for cell-directed delivery. Further forays into evaluating TfR-mediated targeting as a strategy to tackle brain infections are also proposed.

Keywords Transferrin · targeted drug delivery · cancer · infection · blood–brain barrier · theranostics

Abbreviations

AL	Acute leukemia
ALL	Acute lymphoblastic leukemia
CMC	Critical micelle concentration
Copper sulfate	CuS

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DA	Dodecylamine
DAB	Diaminobutyric poly(propylene imine)
Fe-Tf	Iron-carrier protein transferrin
HFE	Hereditary hemochromatosis protein
H-ferritin	Ferritin-H homopolymer
HIF	Hypoxia-inducible factors
hLf	Human lactoferrin
HRE	Hypoxia-responsive elements
hTf	Human serum transferrin
hTF/2N	Human serum transferrin
IgG1	Antihuman TfR antibody
IgG1	Monoclonal antibody 454A12 P
IgG1	Monoclonal antibody 5E9
IgG1	Monoclonal antibody HB21
IgG3	Chimeric human TfR antibody fused
IRE	Iron-responsive elements
IRP	Iron regulatory proteins
MrTf	Rabbit serum transferrin
MTF	Melanotransferrin
mTOR	Mammalian target of rapamycin
NIR	Near-infrared
NLCs	Nanostructured lipid carriers
oTf	Chicken ovotransferrin
PEG	Polyethylene glycol
PEG-PE	Polyethylene glycol-phosphatidylethanolamine
PHD	Prolyl hydroxylases
Reg1	Regnase-1
RGD	Arginine–glycine–aspartic acid
ROS	Reactive oxygen species
SA	Stearylamine
SLNs	Solid lipid nanoparticles
SP	Spermine
Tf	Transferrin
TfR	Transferrin receptor
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TPGS-COOH	D-α-tocopheryl polyethylene glycol 1000 succinate
TTP	Tristetraprolin

1 Introduction

An ongoing approach to increase the efficacy of therapeutic drugs is the intracellular delivery of actives to defined target cells, warding them off healthy cells to decrease overall toxicity, to enable effective treatment with superior resilience. Attempts to discover the potential of precise and target-oriented delivery systems mention

precedents such as polymeric nanoparticles [1], liposomes [2], and ligand-assisted delivery systems [3, 4]. In recent years, an important focus has been on ligand-based targeted delivery to receptors expressed/overexpressed on cells, as these also exhibit low immunogenicity. The well-described cellular uptake pathway of Tf is being successfully used for targeting various therapeutic agents into proliferating cancer cells which exhibit TfR overexpression [4]. As compared to the normal cells, the articulation of TfR is many times higher in cancerous cells and a direct relationship to cancer stage and tumor development is suggested [5]. Such overarticulation and the necessity of iron for tumor cell growth mark TfR a promising target for tumor therapy. This chapter details the TfR and its regulation and the expression in different cancers. Furthermore, various approaches of delivering therapeutics and theranostics mediated via TfR uptake in cancer therapy are summarized and newer opportunities proposed.

2 Transferrin Receptor

The TfRs regulate intracellular transport of iron facilitated by Tf, a nonheme protein that can bind to and transport iron in the systemic circulation [6, 7]. The primary structure of the receptor (Fig.16.1) comprises two similar glycosylated subunits of 95 kDa which are connected via two disulfide bonds at cysteines 89 and 98 to form a dimer

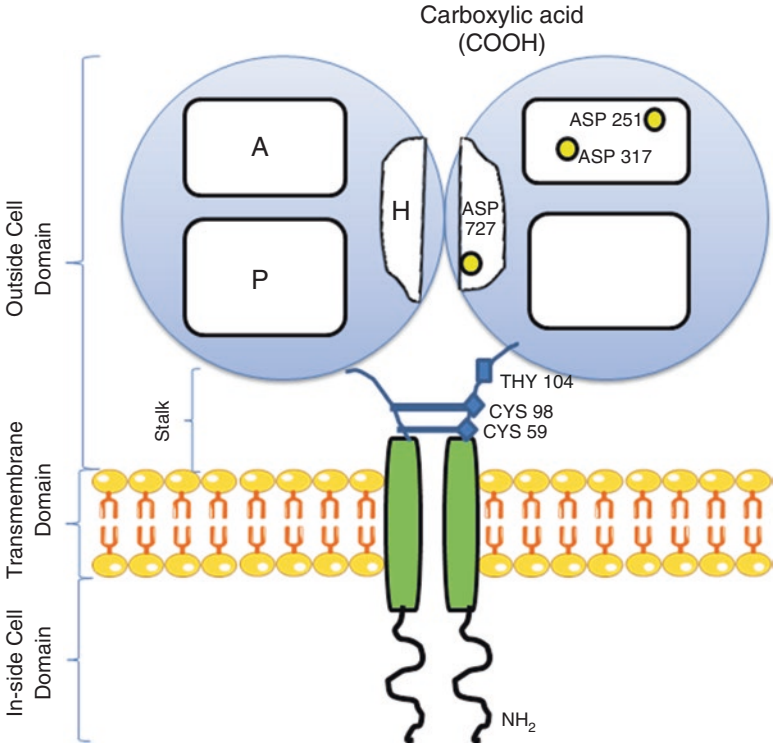

Fig. 16.1 Schematic representation of the transferrin receptor

[8]. Each subunit (760 amino acids) is made up of three domains. The extracellular C-terminal domain (671 amino acids) which is large, globular, and known as ectodomain has a Tf-binding site. Other domains are short N-terminal cytoplasmic (61 amino acids) and a hydrophobic transmembrane domain (28 amino acids) [9]. Crystallographic investigations revealed that the homodimer of transferrin receptor 1 (TfR1) was shaped like a butterfly. The ectodomain contains three glycosylation residues on the nitrogen of arginine at 727, 317, and 251, one glycosylation residue on the oxygen at 104 of threonine. Glycosylation on these positions is an important requirement for the receptor to function. Each TfR1 unit comprises of three different globular subdomains [10], recognized as helical domains (H), apical (A), and protease-like (P), which form a cleft-like structure laterally which is proposed to be in close association with the Tf molecules which are folded [10]. Tf peptide binds with both subunits each, and since two ferric ions can bind with one Tf peptide, four ferric ions can be internalized simultaneously during TfR-mediated endocytosis. Tf exhibits a very high binding affinity to TfR1 wherein two molecules of Tf can bind with one unit of TfR1, with binding affinity in the order of 10^5–10^{10} M^{-1} [11]. The di-ferric Tf always has a 1000-fold and 10- to 30-fold more affinity for the TfR1, as compared to apo-Tf and mono-ferric, respectively [12]. Although the exact binding mechanism is not yet elucidated, the C-lobe of Tf is thought to be the main recognition site for human TfR [13].

2.1 Physiological Functions of Transferrin Receptor

The TfR is a glycoprotein associated with the cell membrane that acts as a gatekeeper in regulating take-up of iron in cells from Tf, which is a plasma protein that transports iron in circulation [6]. High reactive oxygen species (ROS) can be generated due to iron redox activity, which leads to damage of biomolecules (proteins, lipids, nucleic acids) and results in the progression of oxidative stress [12]. Thus, the consequences of both iron overload and deficiency are to be considered as serious. Iron is an essential nutrient for cancer cells and intracellular iron delivery is facilitated by the TfR. Various control mechanisms are developed by organisms to keep the adequate and safe concentration of intracellular and systemic iron. Iron homeostasis is maintained by a matrix of proteins [14]. In this way, iron deficiency or iron overburden in tumor cells can restrain tumor development and cause tumor cell demise. Blocking this iron uptake pathway is, therefore, an important approach to treat cancer. Strategies that attempt to deplete intracellular iron using iron chelators or cytotoxic drug conjugates with TfR1 are also reported [15, 16].

2.2 Transferrin Receptor Location and Regulation of Expression

Higher levels of the receptor are expressed under conditions of cell proliferation in the blood–brain barrier (BBB), deep epidermis, and villi of the intestine [9]. High levels of TfR expression were also found in activated peripheral blood mononuclear

cells [17]. The placental trophoblasts, which express TfR, fulfill the iron requirements of mature erythroid cells and fetus for heme production [18]. In addition, though normal circulating lymphocytes do not express TfRs, mitogen-induced proliferation can result in their expression to meet the demands of high iron [9]. The TfR1 expression in monocytes, hepatocytes, cerebrum and the BBB, intestinal cells, red blood cells, and erythroid cells is low.

The expression of the TfR is governed and inversely proportional to the availability of iron in cells. While an iron chelator desferrioxamine can result in an up to fivefold enhancement of the TfR [9], exogenous iron presence as hemin or ferric ammonium citrate can significantly reduce the TfR expression [19]. Iron regulatory proteins (IRPs) also serve as specific stimuli, especially IRP1 and IRP2, and can modulate iron-dependent TfR1 expression at the posttranscriptional stage. The depletion of cellular iron activates IRPs which are stopped by iron overload [20]. Under conditions of low iron, the TfR1 transcript is protected by the binding of IRPs that neutralize degrading nucleases' enhancing levels of the TfR1 protein and ensuring iron uptake [12]. In general, cell iron shortage results in enhanced TfR1 synthesis and increases TfR1 receptors at the cell surface.

IRP activity is also regulated by other iron-dependent stimuli like inflammation [21], oxidative stress [22], hypoxia and xenobiotics, or other stimuli under physiological conditions [23]. In the case of hypoxia, TfR1 gene transcription is mediated by hypoxia-inducible factor (HIF) [24]. In cells with iron deficiency, HIF-mediated TfR1 transcription is also induced because the regulation of HIF stability is governed by ferrous iron [12]. The regulation of TfR2 articulation is markedly different from the regulation of TfR1 articulation and is characterized by the absence of elements responsive to iron. It is governed at the transcriptional and posttranslational levels, by the transcription factors C/EBPα, GATA-1, and hepatic HNF4α which enhance TfR2 levels. Further holo Tf can protect TfR2 from degradation by lysosomal enzymes, thereby increasing its half-life [25].

3 Recognition Domain of Transferrin Receptor

TfR exhibits binding to iron carrier serum protein Tf (Fe-Tf) and hereditary hemochromatosis protein (HFE). Two atoms of di-ferric Tf (Fe-Tf) bind in the cleft of the receptor formed by the two folded lobes namely the C- and N-lobes, with conformational changes occurring when Fe-Tf is converted to free iron [26]. Moreover, continuous repacking at the interface between the flaps results in dynamically occurring changes in the covered and uncovered residues. A TfR helical domain is recognized wherein the TfR homodimer binds to four Tf molecules [27]. An arginine–glycine–aspartic acid (RGD) sequence (residues 646–648) is also recognized as an essential component in the recognition site. Other domains are also recognized [28].

3.1 Structure–Activity Relationship of Substrate/Ligand for Selective Binding

TfR associates with Tf and HFE, both being proteins crucial for influencing the fate of iron [29]. A density map of the di-ferric Tf and receptor complex [13] demonstrates the C-lobe in the lateral region of TfR and N-lobe in the region representing the stalk of the receptor. The N-lobe consequently mounts in the space between the ectodomain of the TfR and the cell membrane. Binding of TfR homodimer occurs through N-lobes of both Tf molecules and two stalks of receptors. The apical region is not involved in association with iron and hence the function remains elusive.

Like the class I major histocompatibility complex HFE must assemble with β_2 microglobulin [17]. Similar to Fe-TF, as HFE binds to the TfR in endosomes, it may be subjected to lysosomal degradation. Although TF and HFE compete for receptor binding with TfR, iron release from Tf in the endosome is not affected by HFE [17]. However, HFE can hinder cellular uptake of iron by degradation of TfR, thereby preventing the recycling of TfR.

4 Pathophysiological Features in Cancer

The normal cells' transformation to malignant cells and tumor progression involve complex processes that are still largely obscure. Several investigations have demonstrated an upregulation of the TfR on drug-resistant and metastatic tumors, compared with typical tumors, for example, pancreas [30], liver [31], breast [32], lung [33], and colon [34]. The upregulation of TfR1 is triggered by iron deficiency induced by rapid cell proliferation justifying the higher TfR1 expression on cancer cells. Further, such overexpression seems to relate to the progression of cancer and the stage of the tumor. While malignant cells can express about 10^4–10^5 molecules per cell, the receptor is barely detectable in normal cells [7]. The expressions of TfR1 in different cancers are conflicting and the mechanisms by which TfR1 is involved in tumor progression remain elusive.

4.1 In Brain Cancer

TfR1 is highly expressed in brain cancer and involved in regulating the physiology of glioma cells and hence aids progression of brain cancer [35]. ROS generation and iron accumulation via TfR1 act as main effectors of respective transcription factors facilitating proliferation of glioma and glioma-induced death of neurons [36]. Dysregulation of IRPs affects iron which is required for cell division and cancer pathophysiology. The presence of TfRs on the blood–brain barrier provides additional opportunity for drug targeting to the brain.

4.2 In Breast Cancer

A correlation of expression of the TfR with the stage of malignancy also has been evaluated in breast cancer by studying a range of noncancerous and malignant breast tissue samples [37]. Premalignant lesions and invasive carcinoma revealed higher levels of the receptor compared to noncancerous tissues and benign lesions. Reverse transcription-polymerase chain reaction revealed more TfR expression in MCF-7 cells than in human mammary epithelial MCF-12A cells [5] and that IRP2 dominated in iron accumulation and increased TfR1 [38]. Thump down of IRP2 in triple negative breast cancer MDA-MB-231 cell line raised the expression of ferritin heavy chain with diminished TFR1, subsequently diminishing the labile iron pool and hindering proliferation of MDA-MB-231 cell line in the mammary fat cushion of mice [5].

4.3 In Colon Cancer

Okazaki et al. found that the circadian organization of molecular clock affected TfR1 expression in colon cancer cells of mice and the 24-h rhythm of IRP2 expression which modulates TFR1 expression, useful for targeting in colon cancer therapies [39]. Compared with the normal colon mucosa, IRP2 overexpression in colorectal cancer positively correlated with the expression of TfR1 [40].

4.4 In Leukemia

Numerous studies have discovered that TfR1 was upregulated in leukemia, hence TfR1 could be a potential marker in the determination of acute leukemia (AL) [41] and acute lymphoblastic leukemia (ALL). TfR1 articulation was significantly higher on T-cell leukemias while in the B-cell leukemia mature B-ALL revealed higher expression of TfR compared to precursor cells of B-ALL. A noteworthy change in TfR1 articulation existed between precursors and matured B acute lymphoblastic leukemia, which exhibited higher TfR1 articulation. TfR1 also exhibited good correlations for diagnosis with hemoglobin concentration [42].

5 Binding of Substrate and Ligand and Internalization Pathway

Cells internalize iron by four general pathways [43]. The most studied and understood pathway which is also most predominant is the uptake of iron bound to Tf [6, 25]. Two iron molecules bind to TfR, one to each lobe. Cellular internalization of

di-ferric Tf is enabled by a clathrin-mediated endocytic process. Fe^{3+} is liberated from Tf as conformational change occurs when the pH decreases to 5.5 within the vesicle. At this low pH, TfR1 remains associated with Apo-Tf, as it is reused back to cell surface and the two dissociate at cytosolic pH. Free ferric ion is reduced to ferrous in the endosomes and transported out into the cytosol, where it can be utilized in various cell functions. At physiologic pH, di-ferric Tf exhibits an affinity which is 10–110 times higher than Apo-Tf. Unlike most other receptors, TfR can be internalized even in the absence of a ligand.

5.1 Antagonist for Ligand Binding

The antagonist of TfRl is the group consisting of antibodies, antigen-binding portions of antibodies, small organic molecules, aptamers, or polypeptides. A small molecule like ferristatin has been reported as an antagonist which restrains iron uptake from Tf–TfR by promoting degradation of cell surface TfR [44]. Anti-TfR antibodies have displayed the ability to hinder the growth of cancerous cells by different mechanisms. Studies using murine antagonists are summarized in Table 16.1.

Table 16.1 Murine antagonist antibodies in tumor therapy

Antibody	Isotype	Outcome	References
Rat anti-mouse TfR			
R17 208	IgM	Blocked internalization of the Tf–TfR complex, did not interfere with Tf binding. Cells arrested in G2/M phase of the cell cycle	[45, 46]
REM17	IgM	Blocked Tf function. Inhibited cell growth of hematopoietic tumor cells in vitro and in vivo	[47]
R17 217	IgG2a	Did not inhibit cell growth of hematopoietic tumor cells. Did not block Tf–TfR internalization. Downregulated surface TfR expression	[46, 48]
C2	IgG2a	Did not inhibit cell growth of hematopoietic tumor cells, unless cross-linked or used in combination	[49]
Murine antihuman TfR			
43/31	IgG[a]	Inhibited binding of Tf to TfR. Cytotoxic to normal granulocyte/macrophage progenitor cells. Not cytotoxic to mitogen-stimulated mononuclear or CCRF-CEM cells	[50–52]
E2.3	IgG1	Cytotoxic to IL-6 dependent hematopoietic tumor cells	[53]
D65.30	IgG1	Inhibited growth of CCRF-CEM cells	[51]
A27.15	IgG1	Cytotoxic to IL-6 dependent hematopoietic tumor cells	[54]
A24	IgG2b	Competed with Tf for TfR binding and blocked iron uptake. Reduced TfR expression and impaired TfR recycling. Induced apoptosis of activated T cells	[55]

[a]IgG isotype not described

5.2 *Ligands Explored*

5.2.1 Transferrin

Tf is perceived as one of the essential ligands exploited for effective delivery of therapeutic agents to tumor site. Tf protein superfamily includes human serum Tf (hTf), chicken ovoTf (oTf), MelanoTf (MTF, tumor-bound antigen 97), human lactoferrin (hLf), and rabbit serum Tf (rTf). Researchers have revealed Fe coordination in binding sites with four protein ligands (one histidine, one aspartate, and two tyrosines) and two oxygens from the synergistic carbonate anion results in an octahedral arrangement similar to the octahedrally bound ferric ion (Fig. 16.2). In the N-lobe of Tf (hTF/2N), the ligands are Asp 63, Tyr 188, Tyr 95, and Asp 63, His 249, with NI domain, Tyr 188 on the NII domain, and His 249, Tyr 95 on the hinge strands. Tf can also attach to metal ions like di-, tri-, and tetravalent. Anions which possess a carboxylate group are capable of functioning as the "synergistic anion," which can coordinate with the metal center replacing carbonate [56]. The carboxylate group is an excellent ligand for Fe (III) and serves as such in many nonheme iron proteins. In Tf, it coordinates the metal through one carboxylate oxygen and hydrogen bonding between the two TfR domains with the second oxygen atom [57]. Such carbonate binding supports iron binding thereby exhibiting synergy.

5.2.2 Human Hemochromatosis Protein (HFE)

HFE which is a heterodimeric protein comprises of a heavy chain which is membrane bound in noncovalent association with microglobulin which represents a significantly lighter chain. With properties, similar to the major histocompatibility

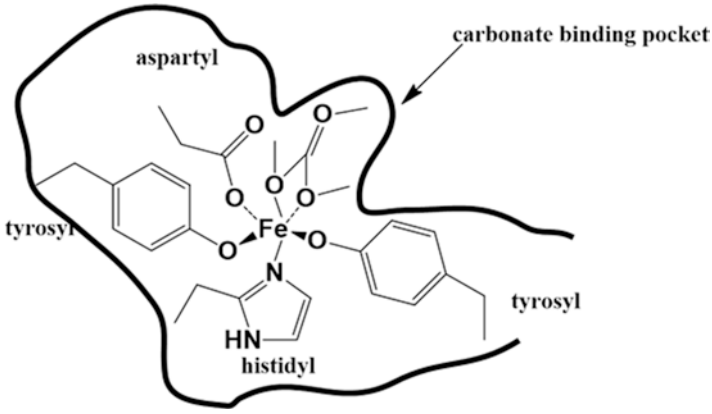

Fig. 16.2 Schematic diagram of the six-coordinate iron site; the coordination geometry is distorted octahedrally

complex proteins (class I), HFE can aid in the presentation of antigenic peptides to T lymphocytes. The finding that TfR1 can readily complex with HFE, wherein two HFE molecules could bind to TfR [26], has confirmed the implication of HFE in iron homeostasis.

5.2.3 Ferritin

Tf is the main iron transporter in circulation while ferritin is involved in the storage of iron. TfR1 which is responsible for TF uptake can also enable uptake of ferritin [58]. However, increased levels of TfR1 are required for uptake of ferritin. Hence, erythroblasts which express very high levels of TfR1 readily incorporate ferritin. Further only ferritin-H (homopolymer) exhibits such uptake which is not exhibited by ferritin. Uptake of ferritin is clathrin-mediated endocytosis and subsequent transfer to the lysosomes for degradation and release of iron.

5.2.4 Anti-TfR Antibody

Many of the anti-TfR antibodies function as ligands by conjugation with toxins to enable their cellular uptake mediated by TfR [9, 59]. Table 16.2 summarizes the same.

Table 16.2 Anti-transferrin receptor antibody

Conjugated compound	Targeting moiety	Outcomes	References
Gelonin	Monoclonal antibody 5E9 (IgG1)	High in vitro cytotoxicity in leukemia cell lines. Reduced tumor burden in lymphoma-bearing mice and prolonged survival	[60]
Ricin	Antihuman TfR antibody (IgG1)	Specifically induced cell death in tumor cells. Human melanoma growth was inhibited in nude mice	[61]
	Antihuman TfR antibody (IgG1)	Enhanced efficacy by killing brain tumor cell lines at lower IC_{50} values	[62]
	Monoclonal antibody 454A12 (IgG1)	No systemic toxicity detected in patients. More than 50% reduction of tumor cell counts	[63]
Saporin	Chimeric human TfR antibody fused to (IgG3)	Increased cytotoxicity in both sensitive and resistant lymphoblastoid cells	[64]
Pseudomonas exotoxin	Monoclonal antibody HB21 (IgG1)	Enhanced cytotoxicity in human ovarian carcinoma cell line and KB cells	[65]

5.2.5 Natural Tropism

Viruses like the new world arenavirus and canine parvovirus exhibit natural tropism for the TfRs [66]. These viral vectors are seen to predominantly localize either in the lysosomes or in the endosomes [67] and could therefore serve as a promising platform for tumor targeting. Canine parvovirus-like particles in particular demonstrated high uptake in all TfR-expressing cells.

5.2.6 Peptides

Peptides can exhibit malignant cell recognition and capture and hence provide a useful strategy for specific targeting of Y1 (VHWDFRQWWQPS) and Y2 (AWYSNLLPLARF) peptides which can enter into cancer cells via TfR. Peptide Y1 exhibits binding which is different from Tf or monoclonal antibody (mAb) and hence can possibly replace Tf to deliver drugs without competing with endogenous Tf [68]. Another peptide T7 (HAIYPRH) also displayed targeting capability for TfR with similar advantages [69].

6 Receptor-Mediated Targeting Strategies

Targeting mainly focuses on enhanced intracellular drug uptake to increase efficacy with simultaneous decrease in toxicity. The two fundamental approaches in drug targeting are active or passive targeting. Receptor-mediated targeting is considered as an active targeting strategy. Overexpression of TfR makes it especially attractive to explore this receptor-mediated strategy for intracellular drug delivery. Various approaches explored are detailed in the following text.

6.1 Drug Conjugates

Drug conjugates involve drugs covalently conjugated to ligands and thereby enable ligand-based targeting to receptors [70]. Conjugation of adriamycin to Tf by glutaraldehyde demonstrated cytotoxicity to adriamycin-resistant HL-60 cells [71]. Paclitaxel-Tf conjugate indicated slight decrease in cytotoxicity of paclitaxel in lung cell line [72], while paclitaxel-conjugated Fmoc-L-glutamic corrosive 5-tert-butyl ester (linker) and Tf demonstrated enhanced tumor-targeting capacity in the resistant breast cancer cell lines (MDA-MB-231), MCF-7 and lung cancer cell line A549 [73]. Cisplatin Tf conjugates increased cellular uptake and avoidance of drug resistance in A2780S and A2780CP70 cell lines and targeted delivery of drug in *A2780CP70 tumor-bearing mice* [74]. Similarly, antiproliferative activity and

prolonged circulation were observed in human epidermoid carcinoma cell line (A431) with the help of cisplatin Tf-binding conjugates [75]. Cisplatin Tf-binding conjugates exhibited ten-fold enhanced inhibition compared to free drug in HeLa and MCF-7 cell lines [76]. Doxorubicin thiolated Tf conjugates displayed inhibitory efficacy in breast cancer (MDA-MB-468), leukemia cell lines (U937), and lung carcinoma (LXFL 529) [77]. Doxorubicin Tf-binding conjugates prolonged the survival of mesothelioma tumor mice as compared to free doxorubicin [78]. Tirapazamine Tf conjugates coupled with cisplatin improved cytotoxic impacts in human colorectal adenocarcinoma cell line (SW1116) [79].

6.2 Liposomes

Liposomes are small phospholipid-based vesicles comprising bilayers encompassing an aqueous center. This makes it versatile to encapsulate both hydrophilic and lipophilic drugs [80]. Liposomes being highly compatible are among the preferred nanocarriers for targeted drug delivery. Tf-coupled liposomes established high brain uptake of 5-fluorouracil (5-FU) across the BBB [70]. Conjugating Tf to polyethylene glycol (PEG) or to other ligands using various chemical cross-linking strategies is also demonstrated. In such manner, docetaxel liposomes coupled with TPGS-COOH (D-α-tocopheryl polyethylene glycol 1000 succinate) and covalently conjugated to amino end groups of Tf that assembled on the surface of the liposomes revealed significantly increased transport across the BBB. Such liposomes may also serve to actively target the drug to brain tumor cells that overexpress Tf [78]. Dual targeted liposomes of paclitaxel using arginine–glycine–aspartic acid (RGD) and Tf revealed excellent apoptotic activity in glioma cell line and its spheroids [81]. However, conjugating liposomes with PEGylated Tf as ligand is among the more widely explored strategies. Stealth imparted to the liposomes by PEG enables their access to the BBB, to facilitate targeted delivery across the BBB. Outcomes of studies on PEGylated Tf-targeted liposomes are summarized in Table 16.3.

6.3 Polymeric Nanoparticles

Nanoparticles are colloidal carriers made from biodegradable polymeric carrier materials. A major advantage is their greater stability compared to liposomes. Tf-conjugated paclitaxel-loaded poly(lactic-*co*-glycolide) nanoparticle revealed five times lower IC_{50} as compared to unconjugated nanoparticles in human prostate cancer PC3 cell line and greater inhibition of tumor in a prostate cancer murine model [94]. Tf conjugation with PEGylated nanoparticle proved a promising strategy for doxorubicin–PLGA nanocarriers which showed threefold reduction in IC_{50}

Table 16.3 Transferrin-conjugated liposomes for targeted delivery

Active	In vitro/ in vivo model	Outcomes	Reference
Docetaxel	K562 and KB cells	Efficient uptake in K562 and 3.6-fold greater cytotoxicity in KB cells	[82]
Cisplatin	Nude mice with MKN45P human gastric tumor cells	Higher survival rate with Tf-PEG liposome	[83]
Resveratrol	U87MG cells and mouse model of glioblastoma	More cytotoxicity in GBM cells. Inhibited tumor growth and improved survival in mice	[84]
Doxorubicin	Lung cancer model in nude rat	More animals survived and have small and fewer tumors in tissues	[85]
Isoquinoline	HeLa and HEK-293T cells	Superior antitumor activity in vitro	[86]
Anti-*BCR-ABL* siRNA or asODN	K562 and LAMA-84 cells	Tf liposomes allowed cellular targeting	[87]
Cisplatin	C6 glioma cell line and immortalized mouse brain endothelial cell line bEnd3	Sequential distribution into the nucleus of C6 cells and higher acquisition in the lysosome of bEnd3	[88]
Doxorubicin	C6 glioma	Significantly increased in gliomal doxorubicin with Tf-PEG liposomes compared to other liposomes	[89]
Doxorubicin	Cell line HepG2 and tumor-bearing mice with HepG2 cells	Higher uptake in HepG2 cells and enhanced doxorubicin concentration in tumor	[90]
Monoclonal IgG antibody, 34A	Colon 26 tumor-bearing mice	Tf-PEG liposomes had the capabilities of specific receptor binding and receptor-mediated endocytosis to target cells after extravasation into solid tumors in vivo	[91]
Phosphorothioate antisense oligodeoxyribonucleotide (G3139)	K562 leukemia cells	Efficient uptake of Tf-conjugated G3139-containing liposomes in TfR positive K562 cells. Could be blocked by excess free Tf	[92]
Vincristine and tetrandrine	C6 glioma cell line and glioma-bearing mice	Strong efficacy in both in vitro and in vivo brain glioma models	[93]

values in prostate cancer PC3 cell line [95]. In another study, Tf attached PEGylated paclitaxel and vorinostat nanoparticles significantly enhanced the cytotoxicity in HepG2, MCF-7, and MDA-MB-23 cancer cells and displayed excellent antitumor efficacy in HepG2 tumor-bearing mice [96]. Enhanced targeting ability of doxorubicin-conjugated Tf nanoparticles with various polymers was showcased in many studies such as in 9L gliosarcoma cells [97], HCT119 [98], PC3 [99], HeLa, and K562 cells [100] and antitumor activity in A549 tumor-bearing mice [95]. Tf-mediated doxorubicin and curcumin nanoparticles with a pH-sensitive polymer showed efficient tumor-targeted delivery in xenograft mice bearing MCF-7 cells and increased cytotoxic effects in MCF-7 cells [101]. Also, OX26 (monoclonal antibody) functionalized temozolomide PLGA nanoparticles designed to target TfR revealed higher cellular internalization and enhanced antitumor activity in glioblastoma cells (U215 and U87) [102]. For more details, the readers are directed to the following reviews [36, 103].

6.4 Polymeric Micelles

Polymeric micelles comprise polymeric surfactants which self-assemble above critical micelle concentration to form micellar structures with central hydrophobic core. Tf-modified polymeric micelles of PEGylated-phosphatidylethanolamine (PEG-PE) co-loaded with curcumin and paclitaxel provided better intracellular localization with high cytotoxicity [104]. The same combination also showed clinical advantages for the treatment of paclitaxel-resistant ovarian cancer [105], and when loaded with selective ATP-competitive cyclin-dependent kinase inhibitor it was found promising for the treatment of tumors overexpressing TfR [106]. Micelles made from polylactic acid-D-α-tocopheryl polyethylene glycol 1000 succinate copolymer containing docetaxel revealed 70-fold more impact in A549 cells compared to nontargeted micelles [107]. Tariquidar and paclitaxel in Tf-anchored micelles showed enhanced cytotoxicity against the SKOV-3TR and A2780-Adr multidrug-resistant (MDR) cells [108].

6.5 Lipid-Based Nanoparticles

Lipid-based nanoparticles are categorized as solid lipid nanoparticles (SLNs) and when modified with liquid lipid to enable better drug loading, as nanostructured lipid carriers (NLCs) [109]. SLNs exhibit distinctive properties such as high drug loading of hydrophobic drugs, large surface area, and protection of the drug from the environment and enhanced bioavailability. They can be anchored with Tf to exhibit TfR-mediated targeted delivery [110]. Gene-loaded Tf polyethylene glycol-phosphatidylethanolamine SLN was synthesized which enhanced the efficacy of cancer therapy in A549 tumor-bearing mice [111]. Tf-conjugated etoposide-loaded

SLN showed significantly higher anticancer activity in the human nonsmall-cell lung cancer cell line (A549) [112]. Similarly, NLCs also demonstrated Tf-based targeted delivery and improved outcomes. Tf-modified doxorubicin and green fluorescence protein plasmid co-encapsulated NLCs demonstrated remarkably increased anticancer activity due to a combination of gene therapy and chemotherapy [111]. DNA and doxorubicin Tf-conjugated NLCs demonstrated noticeably enhanced antitumor activity in lung tumor-bearing C57BL/6 mice [113]. Tf-conjugated NLCs modified using fatty amines like spermine (SP), dodecylamine (DA), and stearylamine (SA) revealed enhanced cellular uptake in acute myelogenous leukemia cells (K562) [114]. Optimized paclitaxel NLC when coupled with Tf showed higher cytotoxic activity against U-87 brain cancer cell line [115].

6.6 Dendrimers

Dendrimers are hyperbranched polymeric structures which have a core with radial stents that enable a globular nanoparticle structure. Their special feature is the very small size of 2–10 nm, the possibility to modify the volume based on generations and hyperbranched structures which allow covalent conjugation of various functional residues including ligands. Tf-conjugated G-3 dendrimer with a basic diaminobutyric poly(propylene imine) enhanced efficacy of plasmid DNA in (T98G) human glioma cell line and (A431) human epithelial carcinoma cell line. Higher expression of the enzyme in the tumor of (A431) compared to other organs confirmed the efficacy of gene delivered in vivo. The same Tf-anchored dendrimer also revealed complete tumor reduction in 90% of the studies when conjugated with another plasmid. This was in contrast to only 40% reduction seen in the absence of Tf [116].

6.7 Antibodies Targeting Transferrin Receptor

The ability of antibodies to bind to TfR has been exploited for cancer therapy. Conjugation of the murine IgG1 antihuman TfR (5E9) and doxorubicin with a pH-sensitive linker demonstrated efficacy in vitro and in vivo against human hematopoietic malignant cell lines Daudi and Raji [117]. Conjugation of bovine pancreatic ribonuclease A enzyme to an anti-TfR1 antibody showed promising response in leptomeningeal neoplasia, B-cell lymphoma, epidermoid tumor, and glioblastoma, and when conjugated to adriamycin-loaded liposomes enhanced activity was seen in adriamycin-resistant human leukemia cell line (K562/ADM) [118]. A diagnostic application of complexing the anti-TfR2 antibody with the radionuclide ^{90}Y enabled determination of overexpressed TfR1 in a xenograft model of pancreatic tumor [119]. The single-chain variable fragment (scFv) component of the anti-TfR1 when genetically combined to a truncated mutant of Pseudomonas exotoxin revealed

cytotoxicity against various carcinomas including prostate carcinoma, human epidermoid carcinoma, breast carcinoma, and ovarian carcinoma and colon carcinoma [120]. Similarly, the scFv anti-TfR1 5E9 loaded in liposomes with docetaxel revealed delivery of the p53 tumor suppresser gene selectively in tumor cells to achieve prolonged survival in a metastasis model of human breast cancer [121].

7 Theranostic Application

Theranostics combine delivery of drugs with imaging agents which are coadministered to enable therapy while simultaneously monitoring efficacy. The ultimate objective is to ensure customized therapy and better control over the disease [122]. Anchoring suitable ligands is practiced to enhance the targeting potential of the delivery system. Among others, the TfR has been studied as one of the effective targets [78]. Tf nanoparticles attached with IR780, a near-infrared (NIR) dye, showed notable targeting and theranostic potential in CT26 tumor-bearing mice [123]. An artesunate Tf conjugated to mesoporous copper sulfide (CuS) system was designed. This relied on near-infrared (NIR) absorption coupled with photothermal conversion enabled by CuS to release the contrast agent for photoacoustic imaging to monitor chemo-phototherapy. High antitumor effect was evident in MCF-7 cell tumor-bearing mice [124], whereas Fe_3O_4@ZnO nanocomposites containing doxorubicin could mediate the theranostic strategy for hepatocellular carcinoma bearing murine and orthotopic models [125]. Doxorubicin Tf conjugated to copper nanoclusters showed superior targeting efficiency with tumor growth inhibition resulting in enhanced survival rates of HeLa, MCF-7, and Daltons and lymphoma ascites bearing mice [126].

8 Clinical Trials

TfR-targeted clinical trials are summarized in Table 16.4 [127]. Most clinical trials are in Phase I/II and are being evaluated in solid tumors, pancreatic cancer, gastrointestinal (GI) cancer, and glioblastoma.

9 Advantages and Limitations Related to the Specific Cancer Targeting Through the Receptor

Tf has various advantages for targeted tumor delivery. Without causing any toxicity, nonimmunogenic human Tf can be safely delivered. Tf-conjugated drugs prevent cardiotoxicity and drug resistance. Moreover, Tf binding to tumor cells is greater than nonmalignant cells since TfRs on tumor cells recycle to the surface more quickly. Tf can be obtained from human sources.

Table 16.4 Transferrin receptor targeted clinical trials

Clinical Trial Identifier	Condition	Intervention	Trial Phase
NCT00355888	Metastatic solid tumors	Oxaliplatin delivery through liposome-conjugated Tf (MBP-426)	Phase I
NCT02354547	Pediatric patients with solid tumors	scFv/liposome complex loaded with p53 DNA plasmid containing topotecan and cyclophosphamide	Phase I
NCT00470613	Solid tumors	Complex of scFv/liposome containing p53 DNA plasmid containing docetaxel	Phase Ib
NCT00964080	Gastric, gastroesophageal, or esophageal adenocarcinoma	MBP-426/leucovorin/5-FU	Phase Ib/II
NCT02340117	Metastatic pancreatic cancer	Complex of scFv/liposome made up of p53 DNA plasmid containing gemcitabine/nab-paclitaxel	Phase II
NCT02340156	Glioblastoma	Complex of scFv/liposome containing p53 DNA plasmid with temozolomide	Phase II
NCT00083447	Glioblastoma	TransMID (combination of transferrin and diphtheria toxin)	Phase III

However, there are some limitations of Tf as a targeting ligand. Tf-conjugated drug delivery in higher dose may result in damage to other cells that express low levels of TfR. Further, high levels of endogenic Tf can result in competitive binding to tumor cell receptors, limiting the uptake of carriers designed for receptor-mediated uptake therefore limiting efficacy [128]. A Phase I trial (NCT00689065) of siRNA delivery system based on cyclodextrin as carrier, adamantane polyethylene glycol for surface modification, and Tf as the ligand was terminated due to adverse events in 21% of the patients, which was attributed to instability specifically of the Tf-targeting ligand.

10 Future Perspectives

A focused effort in TfR-mediated targeting for brain cancer therapy is evident, further relying on the TfR to cross the BBB can augment therapeutics designed for other brain afflictions. Brain afflictions other than cancer include meningoencephalitis, neurosyphilis, tuberculous meningitis, neurocysticercosis, toxoplasmosis, and lifestyle CNS diseases like Alzheimer's and Parkinsonism. CNS diseases would require prolonged treatment, and hence oral administration may not be reliable for brain targeting. However, for severe brain infections involving parenteral administration, TfR-mediated high brain uptake could show immense promise for improved therapeutic outcomes and throws open a huge arena of possibilities.

11 Conclusion

Targeted delivery exploiting the TfR provides a number of opportunities for improved therapy specifically for brain cancer. Extending this strategy for the effective treatment of brain infections opens exciting possibilities.

References

1. Langer R. Drug delivery and targeting. Nature-London. 1998;30:5–10.
2. Maruyama K, Ishida O, Takizawa T, Moribe K. Possibility of active targeting to tumor tissues with liposomes. Adv Drug Deliv Rev. 1999;40(1–2):89–102.
3. Vyas SP, Sihorkar V. Endogenous carriers and ligands in non-immunogenic site-specific drug delivery. Adv Drug Deliv Rev. 2000;43(2–3):101–64.
4. Li H, Qian ZM. Transferrin/transferrin receptor-mediated drug delivery. Med Res Rev. 2002;22(3):225–50.
5. Shen Y, Li X, Dong D, Zhang B, Xue Y, Shang P. Transferrin receptor 1 in cancer: a new sight for cancer therapy. Am J Cancer Res. 2018;8(6):916–31.
6. Ponka P, Lok CN. The transferrin receptor: role in health and disease. Int J Biochem Cell Biol. 1999;31(10):1111–37.
7. Qian ZM, Li H, Sun H, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev. 2002;54(4):561–87.
8. McClelland A, Kühn LC, Ruddle FH. The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. Cell. 1984;39(2):267–74.
9. Tortorella S, Karagiannis TC. Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. J Membr Biol. 2014;247(4):291–307.
10. Lawrence CM, Ray S, Babyonyshev M, Galluser R, Borhani DW, Harrison SC. Crystal structure of the ectodomain of human transferrin receptor. Science. 1999;286(5440):779–82.
11. Sun H, Li H, Sadler PJ. Transferrin as a metal ion mediator. Chem Rev. 1999;99(9):2817–42.
12. Gammella E, Buratti P, Cairo G, Recalcati S. The transferrin receptor: the cellular iron gate. Metallomics. 2017;9(10):1367–75.
13. Cheng Y, Zak O, Aisen P, Harrison SC, Walz T. Structure of the human transferrin receptor-transferrin complex. Cell. 2004;116(4):565–76.
14. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A red carpet for iron metabolism. Cell. 2017;168(3):344–61.
15. Lui GY, Kovacevic Z, Richardson V, Merlot AM, Kalinowski DS, Richardson DR. Targeting cancer by binding iron: dissecting cellular signaling pathways. Oncotarget. 2015;6(22):18748–79.
16. Torti SV, Torti FM. Iron and cancer: more ore to be mined. Nat Rev Cancer. 2013;13(5):342–55.
17. Woith W, Nüsslein I, Antoni C, Dejica DI, Winkler TH, Herrmann M, Pirner K, Kalden JR, Manger B. A soluble form of the human transferrin receptor is released by activated lymphocytes in vitro. Clin Exp Immunol. 1993;92(3):537–42.
18. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML. The transferrin receptor part I: biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol. 2006;121(2):144–58.
19. Rouault T, Rao K, Harford J, Mattia E, Klausner RD. Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J Biol Chem. 1985;260(27):14862–6.
20. Kazan HH, Urfali-Mamatoglu C, Gunduz U. Iron metabolism and drug resistance in cancer. Biometals. 2017;30(5):629–41.

21. Lazaros G, Antonatou K, Vassilopoulos D. The therapeutic role of interleukin-1 inhibition in idiopathic recurrent pericarditis: current evidence and future challenges. Front Med. 2017;4:78.
22. Muto Y, Nishiyama M, Nita A, Moroishi T, Nakayama KI. Essential role of FBXL5-mediated cellular iron homeostasis in maintenance of hematopoietic stem cells. Nat Commun. 2017;8:16114.
23. Anderson SA, Nizzi CP, Chang YI, Deck KM, Schmidt PJ, Galy B, Damnernsawad A, Broman AT, Kendziorski C, Hentze MW, Fleming MD. The IRP1-HIF-2α axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. Cell Metab. 2013;17(2):282–90.
24. Udensi UK, Tackett AJ, Byrum S, Avaritt NL, Sengupta D, Moreland LW, Tchounwou PB, Isokpehi RD. Proteomics-based identification of differentially abundant proteins from human keratinocytes exposed to arsenic trioxide. J Proteomics Bioinform. 2014;7(7):166–78.
25. Chen J, Wang J, Meyers KR, Enns CA. Transferrin-directed internalization and cycling of transferrin receptor 2. Traffic. 2009;10(10):1488–501.
26. Giannetti AM, Snow PM, Zak O, Björkman PJ. Mechanism for multiple ligand recognition by the human transferrin receptor. PLoS Biol. 2003;1(3):e51.
27. Buchegger F, Trowbridge IS, Liu LF, White S, Collawn JF. Functional analysis of human/chicken transferrin receptor chimeras indicates that the carboxy-terminal region is important for ligand binding. Eur J Biochem. 1996;235(1–2):9–17.
28. Dubljevic V, Adnan SA, Goding JW. A conserved RGD (Arg-Gly-Asp) motif in the transferrin receptor is required for binding to transferrin. Biochem J. 1999;341(1):11–4.
29. Waheed A, Grubb JH, Zhou XY, Tomatsu S, Fleming RE, Costaldi ME, Britton RS, Bacon BR, Sly WS. Regulation of transferrin-mediated iron uptake by HFE, the protein defective in hereditary hemochromatosis. Proc Natl Acad Sci. 2002;99(5):3117–22.
30. Ryschich E, Huszty G, Knaebel HP, Hartel M, Büchler MW, Schmidt J. Transferrin receptor is a marker of malignant phenotype in human pancreatic cancer and in neuroendocrine carcinoma of the pancreas. Eur J Cancer. 2004;40(9):1418–22.
31. Kindrat I, Tryndyak V, de Conti A, Shpyleva S, Mudalige TK, Kobets T, Erstenyuk AM, Beland FA, Pogribny IP. MicroRNA-152-mediated dysregulation of hepatic transferrin receptor 1 in liver carcinogenesis. Oncotarget. 2016;7(2):1276–87.
32. Rychtarcikova Z, Lettlova S, Tomkova V, Korenkova V, Langerova L, Simonova E, Zjablovskaja P, Alberich-Jorda M, Neuzil J, Truksa J. Tumor-initiating cells of breast and prostate origin show alterations in the expression of genes related to iron metabolism. Oncotarget. 2017;8(4):6376–98.
33. Wang B, Zhang J, Song F, Tian M, Shi B, Jiang H, Xu W, Wang H, Zhou M, Pan X, Gu J. EGFR regulates iron homeostasis to promote cancer growth through redistribution of transferrin receptor 1. Cancer Lett. 2016;381(2):331–40.
34. Prutki M, Poljak-Blazi M, Jakopovic M, Tomas D, Stipancic I, Zarkovic N. Altered iron metabolism, transferrin receptor 1 and ferritin in patients with colon cancer. Cancer Lett. 2006;238(2):188–96.
35. Rosager AM, Sørensen MD, Dahlrot RH, Hansen S, Schonberg DL, Rich JN, Lathia JD, Kristensen BW. Transferrin receptor-1 and ferritin heavy and light chains in astrocytic brain tumors: expression and prognostic value. PLoS One. 2017;12(8):e0182954.
36. Chirasani SR, Markovic DS, Synowitz M, Eichler SA, Wisniewski P, Kaminska B, Otto A, Wanker E, Schäfer M, Chiarugi P, Meier JC. Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells. J Mol Med. 2009;87(2):153–67.
37. Singh M, Mugler K, Hailoo DW, Burke S, Nemesure B, Torkko K, Shroyer KR. Differential expression of transferrin receptor (TfR) in a spectrum of normal to malignant breast tissues: implications for in situ and invasive carcinoma. Appl Immunohistochem Mol Morphol. 2011;19(5):417–23.
38. Wang W, Deng Z, Hatcher H, Miller LD, Di X, Tesfay L, Sui G, D'Agostino RB, Torti FM, Torti SV. IRP2 regulates breast tumor growth. Cancer Res. 2014;74(2):497–507.
39. Okazaki F, Matsunaga N, Okazaki H, Azuma H, Hamamura K, Tsuruta A, Tsurudome Y, Ogino T, Hara Y, Suzuki T, Hyodo K. Circadian clock in a mouse colon tumor regulates intracellular iron levels to promote tumor progression. J Biol Chem. 2016;291(13):7017–28.

40. Horniblow RD, Bedford M, Hollingworth R, Evans S, Sutton E, Lal N, Beggs A, Iqbal TH, Tselepis C. BRAF mutations are associated with increased iron regulatory protein-2 expression in colorectal tumorigenesis. Cancer Sci. 2017;108(6):1135–43.
41. Liu Q, Wang M, Hu Y, Xing H, Chen X, Zhang Y, Zhu P. Significance of CD71 expression by flow cytometry in diagnosis of acute leukemia. Leuk Lymphoma. 2014;55(4):892–8.
42. Płoszyńska A, Ruckemann-Dziurdzińska K, Jóźwik A, Mikosik A, Lisowska K, Balcerska A, Witkowski JM. Cytometric evaluation of transferrin receptor 1 (CD71) in childhood acute lymphoblastic leukemia. Folia Histochem Cytobiol. 2012;50(2):304–11.
43. Crielaard BJ, Lammers T, Rivella S. Targeting iron metabolism in drug discovery and delivery. Nat Rev Drug Discov. 2017;16(6):400–23.
44. Horonchik L, Wessling-Resnick M. The small-molecule iron transport inhibitor ferristatin / NSC306711 promotes degradation of the transferrin receptor. Chem Biol. 2008;15(7):647–53.
45. Trowbridge IS, Lesley J, Schulte R. Murine cell surface transferrin receptor: studies with an anti-receptor monoclonal antibody. J Cell Physiol. 1982;112(3):403–10.
46. Lesley JF, Schulte RJ. Inhibition of cell growth by monoclonal anti-transferrin receptor antibodies. Mol Cell Biol. 1985;5(8):1814–21.
47. Sauvage CA, Mendelsohn JC, Lesley JF, Trowbridge IS. Effects of monoclonal antibodies that block transferrin receptor function on the in vivo growth of a syngeneic murine leukemia. Cancer Res. 1987;47(3):747–53.
48. Lesley J, Schulte R, Woods J. Modulation of transferrin receptor expression and function by anti-transferrin receptor antibodies and antibody fragments. Exp Cell Res. 1989;182(1):215–33.
49. Kemp JD, Cardillo T, Stewart BC, Kehrberg E, Weiner G, Hedlund B, Naumann PW. Inhibition of lymphoma growth in vivo by combined treatment with hydroxyethyl starch deferoxamine conjugate and IgG monoclonal antibodies against the transferrin receptor. Cancer Res. 1995;55(17):3817–24.
50. Taetle R, Honeysett JM, Trowbridge I. Effects of anti-transferrin receptor antibodies on growth of normal and malignant myeloid cells. Int J Cancer. 1983;32(3):343–9.
51. Petrini M, Pelosi-Testa E, Sposi NM, Mastroberardino G, Camagna A, Bottero L, Mavilio F, Testa U, Peschle C. Constitutive expression and abnormal glycosylation of transferrin receptor in acute T-cell leukemia. Cancer Res. 1989;49(24 Part 1):6989–96.
52. White S, Taetle R, Seligman PA, Rutherford M, Trowbridge IS. Combinations of anti-transferrin receptor monoclonal antibodies inhibit human tumor cell growth in vitro and in vivo: evidence for synergistic antiproliferative effects. Cancer Res. 1990;50(19):6295–301.
53. Taetle R, Santos BD, Ohsugi Y, Koihihara Y, Yamada Y, Messner H, Dalton W. Effects of combined antigrowth factor receptor treatment on in vitro growth of multiple myeloma. JNCI: J Nat Cancer Inst. 1994;86(6):450–5.
54. Jones DT, Trowbridge IS, Harris AL. Effects of transferrin receptor blockade on cancer cell proliferation and hypoxia-inducible factor function and their differential regulation by ascorbate. Cancer Res. 2006;66(5):2749–56.
55. Moura IC, Lepelletier Y, Arnulf B, England P, Baude C, Beaumont C, Bazarbachi A, Benhamou M, Monteiro RC, Hermine O. A neutralizing monoclonal antibody (mAb A24) directed against the transferrin receptor induces apoptosis of tumor T lymphocytes from ATL patients. Blood. 2004;103(5):1838–45.
56. Baker EN. Structure and reactivity of transferrins. Adv Inorg Chem. 1994;41(C):389–463.
57. Kawabata H, Nakamaki T, Ikonomi P, Smith RD, Germain RS, Koeffler HP. Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells. Blood. 2001;98(9):2714–9.
58. He QY, Mason AB, Woodworth RC, Tam BM, MacGillivray RT, Grady JK, Chasteen ND. Inequivalence of the two tyrosine ligands in the N-lobe of human serum transferrin. Biochemistry. 1997;36(48):14853–60.
59. Daniels-Wells TR, Penichet ML. Transferrin receptor 1: a target for antibody-mediated cancer therapy. Immunotherapy. 2016;8(9):991–4.
60. Scott CF Jr, Goldmacher VS, Lambert JM, Jackson JV, Mclntyre GD. An immunotoxin composed of a monoclonal antitransferrin receptor antibody linked by a disulfide bond to

the ribosome-inactivating protein gelonin: potent in vitro and in vivo effects against human tumors. J Natl Cancer Inst. 1987;79(5):1163–72.
61. Trowbridge IS, Domingo DL. Anti-transferrin receptor monoclonal antibody and toxin–antibody conjugates affect growth of human tumour cells. Nature. 1981;294(5837):171–3.
62. Martell LA, Agrawal A, Ross DA, Muraszko KM. Efficacy of transferrin receptor-targeted immunotoxins in brain tumor cell lines and pediatric brain tumors. Cancer Res. 1993;53(6):1348–53.
63. Laske DW, Muraszko KM, Oldfield EH, DeVroom HL, Sung C, Dedrick RL, Simon TR, Colandrea J, Copeland C, Katz D, Greenfield L. Intraventricular immunotoxin therapy for leptomeningeal neoplasia. Neurosurgery. 1997;41(5):1039–51.
64. Daniels TR, Ng PP, Delgado T, Lynch MR, Schiller G, Helguera G, Penichet ML. Conjugation of an anti-–transferrin receptor IgG3-avidin fusion protein with biotinylated saporin results in significant enhancement of its cytotoxicity against malignant hematopoietic cells. Mol Cancer Ther. 2007;6(11):2995–3008.
65. Pirker R, FitzGerald DJ, Willingham MC, Pastan I. Enhancement of the activity of immunotoxins made with either ricin A chain or Pseudomonas exotoxin in human ovarian and epidermoid carcinoma cell lines. Cancer Res. 1988;48(14):3919–23.
66. Daniels TR, Bernabeu E, Rodríguez JA, Patel S, Kozman M, Chiappetta DA, Holler E, Ljubimova JY, Helguera G, Penichet ML. The transferrin receptor and the targeted delivery of therapeutic agents against cancer. Biochimica et Biophysica Acta (BBA)-General Subjects. 2012;1820(3):291–317.
67. Singh P, Destito G, Schneemann A, Manchester M. Canine parvovirus-like particles, a novel nanomaterial for tumor targeting. J Nanobiotechnol. 2006;4(1):2.
68. Tan Y, Liu W, Zhu Z, Lang L, Wang J, Huang M, Zhang M, Yang C. Selection and identification of transferrin receptor-specific peptides as recognition probes for cancer cells. Anal Bioanal Chem. 2018;410(3):1071–7.
69. Riaz MK, Zhang X, Wong KH, Chen H, Liu Q, Chen X, Zhang G, Lu A, Yang Z. Pulmonary delivery of transferrin receptors targeting peptide surface-functionalized liposomes augments the chemotherapeutic effect of quercetin in lung cancer therapy. Int J Nanomedicine. 2019;14:2879–902.
70. Soni V, Kohli DV, Jain SK. Transferrin-conjugated liposomal system for improved delivery of 5-fluorouracil to brain. J Drug Target. 2008;16(1):73–8.
71. Barabas K, Sizensky JA, Faulk WP. Transferrin conjugates of adriamycin are cytotoxic without intercalating nuclear DNA. J Biol Chem. 1992;267(13):9437–42.
72. Bicamumpaka CY, Pagé MI. In vitro cytotoxicity of paclitaxel-transferrin conjugate on H69 cells. Oncol Rep. 1998;5(6):1381–4.
73. Shan L, Shan X, Zhang T, Zhai K, Gao G, Chen X, Gu Y. Transferrin-conjugated paclitaxel prodrugs for targeted cancer therapy. RSC Adv. 2016;6(81):77987–98.
74. Peng H, Jin H, Zhuo H, Huang H. Enhanced antitumor efficacy of cisplatin for treating ovarian cancer in vitro and in vivo via transferrin binding. Oncotarget. 2017;8(28):45597–611.
75. Hoshino T, Misaki M, Yamamoto M, Shimizu H, Ogawa Y, Toguchi H. Receptor-binding, in vitro cytotoxicity, and in vivo distribution of transferrin-bound cis-platinum (II) of differing molar ratios. J Control Release. 1995;37(1–2):75–81.
76. Head JF, Wang FE, Elliott RL. Antineoplastic drugs that interfere with iron metabolism in cancer cells. Adv Enzym Regul. 1997;37:147–69.
77. Kratz F. Drug conjugates with albumin and transferrin. Expert Opin Ther Pat. 2002;12(3):433–9.
78. Sonali, Singh RP, Singh N, Sharma G, Vijayakumar MR, Koch B, Singh S, Singh U, Dash D, Pandey BL, Muthu MS. Transferrin liposomes of docetaxel for brain-targeted cancer applications: formulation and brain theranostics. Drug Deliv. 2016;23(4):1261–71.
79. Yu X, Feng L, Liu D, Zhang L, Wu B, Jiang W, Han Z, Cheng S. Quantitative proteomics reveals the novel co-expression signatures in early brain development for prognosis of glioblastoma multiforme. Oncotarget. 2016;7(12):14161.

80. Mukherjee A. A review on liposomes and polymeric nanoparticles as drug delivery vehicles to the brain. J Mole Biol Tech. 2017;1(1):1–5.
81. Qin LI, Wang CZ, Fan HJ, Zhang CJ, Zhang HW, Lv MH, Cui SD. A dual--targeting liposome conjugated with transferrin and arginine-glycine-aspartic acid peptide for glioma-targeting therapy. Oncol Lett. 2014;8(5):2000–6.
82. Zhai G, Wu J, Yu B, Guo C, Yang X, Lee RJ. A transferrin receptor-targeted liposomal formulation for docetaxel. J Nanosci Nanotechnol. 2010;10(8):5129–36.
83. Iinuma H, Maruyama K, Okinaga K, Sasaki K, Sekine T, Ishida O, Ogiwara N, Johkura K, Yonemura Y. Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer. Int J Cancer. 2002;99(1):130–7.
84. Jhaveri A, Deshpande P, Pattni B, Torchilin V. Transferrin-targeted, resveratrol-loaded liposomes for the treatment of glioblastoma. J Control Release. 2018;277:89–101.
85. Gaspar MM, Radomska A, Gobbo OL, Bakowsky U, Radomski MW, Ehrhardt C. Targeted delivery of transferrin-conjugated liposomes to an orthotopic model of lung cancer in nude rats. J Aerosol Med Pulm Drug Deliv. 2012;25(6):310–8.
86. Yang X, Yang S, Chai H, Yang Z, Lee RJ, Liao W, Teng L. A novel isoquinoline derivative anticancer agent and its targeted delivery to tumor cells using transferrin-conjugated liposomes. PLoS One. 2015;10(8):e0136649.
87. Mendonça LS, Firmino F, Moreira JN, Pedroso de Lima MC, Simões S. Transferrin receptor-targeted liposomes encapsulating anti-BCR-ABL siRNA or asODN for chronic myeloid leukemia treatment. Bioconjug Chem. 2009;21(1):157–68.
88. Lv Q, Li LM, Han M, Tang XJ, Yao JN, Ying XY, Li FZ, Gao JQ. Characteristics of sequential targeting of brain glioma for transferrin-modified cisplatin liposome. Int J Pharm. 2013;444(1–2):1–9.
89. Savage DF, Egea PF, Robles-Colmenares Y, O'Connell JD III, Stroud RM. Architecture and selectivity in aquaporins: 2.5 Å X-ray structure of aquaporin Z. PLoS Biol. 2003;1(3):e72.
90. Li X, Ding L, Xu Y, Wang Y, Ping Q. Targeted delivery of doxorubicin using stealth liposomes modified with transferrin. Int J Pharm. 2009;373(1–2):116–23.
91. Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, Yanagie H. Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. Pharm Res. 2001;18(7):1042–8.
92. Chiu SJ, Liu S, Perrotti D, Marcucci G, Lee RJ. Efficient delivery of a Bcl-2-specific antisense oligodeoxyribonucleotide (G3139) via transferrin receptor-targeted liposomes. J Control Release. 2006;112(2):199–207.
93. Song XL, Liu S, Jiang Y, Gu LY, Xiao Y, Wang X, Cheng L, Li XT. Targeting vincristine plus tetrandrine liposomes modified with DSPE-PEG2000-transferrin in treatment of brain glioma. Eur J Pharm Sci. 2017;96:129–40.
94. Sahoo SK, Ma W, Labhasetwar V. Efficacy of transferrin-conjugated paclitaxel-loaded nanoparticles in a murine model of prostate cancer. Int J Cancer. 2004;112(2):335–40.
95. Guo Y, Wang L, Lv P, Zhang P. Transferrin-conjugated doxorubicin-loaded lipid-coated nanoparticles for the targeting and therapy of lung cancer. Oncol Lett. 2015;9(3):1065–72.
96. Ruttala HB, Ramasamy T, Poudal BK, Choi Y, Choi JY, Kim J, Ku SK, Choi HG, Yong CS, Kim JO. Molecularly targeted co-delivery of a histone deacetylase inhibitor and paclitaxel by lipid-protein hybrid nanoparticles for synergistic combinational chemotherapy. Oncotarget. 2017;8(9):14925–40.
97. Jeong YI, Kim YW, Jung S, Pei J, Wen M, Li SY, Ryu HH, Lim JC, Jang WY, Kim IY, Moon KS. Delivery of transferrin-conjugated polysaccharide nanoparticles in 9L gliosacoma cells. J Nanosci Nanotechnol. 2015;15(1):125–9.
98. Kim SS, Rait A, Rubab F, Rao AK, Kiritsy MC, Pirollo KF, Wang S, Weiner LM, Chang EH. The clinical potential of targeted nanomedicine: delivering to cancer stem-like cells. Mol Ther. 2014;22(2):278–91.
99. Lopes AM, Chen KY, Kamei DT. A transferrin variant as the targeting ligand for polymeric nanoparticles incorporated in 3-D PLGA porous scaffolds. Mater Sci Eng C. 2017;73:373–80.

100. Ding W, Guo L. Immobilized transferrin Fe3O4@ SiO2 nanoparticle with high doxorubicin loading for dual-targeted tumor drug delivery. Int J Nanomedicine. 2013;8:4631–9.
101. He YJ, Xing L, Cui PF, Zhang JL, Zhu Y, Qiao JB, Lyu JY, Zhang M, Luo CQ, Zhou YX, Lu N. Transferrin-inspired vehicles based on pH-responsive coordination bond to combat multidrug-resistant breast cancer. Biomaterials. 2017;113:266–78.
102. Ramalho MJ, Sevin E, Gosselet F, Lima J, Coelho MA, Loureiro JA, Pereira MC. Receptor-mediated PLGA nanoparticles for glioblastoma multiforme treatment. Int J Pharm. 2018;545(1–2):84–92.
103. Daniels TR, Delgado T, Helguera G, Penichet ML. The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin Immunol. 2006;121(2):159–76.
104. Yang Z, Sun N, Cheng R, Zhao C, Liu Z, Li X, Liu J, Tian Z. pH multistage responsive micellar system with charge-switch and PEG layer detachment for co-delivery of paclitaxel and curcumin to synergistically eliminate breast cancer stem cells. Biomaterials. 2017;147:53–67.
105. Abouzeid AH, Patel NR, Sarisozen C, Torchilin VP. Transferrin-targeted polymeric micelles co-loaded with curcumin and paclitaxel: efficient killing of paclitaxel-resistant cancer cells. Pharm Res. 2014;31(8):1938–45.
106. Sawant RR, Jhaveri AM, Koshkaryev A, Zhu L, Qureshi F, Torchilin VP. Targeted transferrin-modified polymeric micelles: enhanced efficacy in vitro and in vivo in ovarian carcinoma. Molecular pharmaceutics. 2013;11(2):375–81.
107. Singh RP, Sharma G, Agrawal P, Pandey BL, Koch B, Muthu MS. Transferrin receptor targeted PLA-TPGS micelles improved efficacy and safety in docetaxel delivery. Int J Biol Macromol. 2016;83:335–44.
108. Zou W, Sarisozen C, Torchilin VP. The reversal of multidrug resistance in ovarian carcinoma cells by co-application of tariquidar and paclitaxel in transferrin-targeted polymeric micelles. J Drug Target. 2017;25(3):225–34.
109. Naseri N, Valizadeh H, Zakeri-Milani P. Solid lipid nanoparticles and nanostructured lipid carriers: structure, preparation and application. Adv Pharmaceut Bull. 2015;5(3):305–13.
110. Dolatabadi JE, Valizadeh H, Hamishehkar H. Solid lipid nanoparticles as efficient drug and gene delivery systems: recent breakthroughs. Adv Pharmaceut Bull. 2015;5(2):151–9.
111. Han Y, Zhang Y, Li D, Chen Y, Sun J, Kong F. Transferrin-modified nanostructured lipid carriers as multifunctional nanomedicine for codelivery of DNA and doxorubicin. Int J Nanomedicine. 2014;9:4107–16.
112. Mulik RS, Mönkkönen J, Juvonen RO, Mahadik KR, Paradkar AR. Apoptosis-induced anticancer effect of transferrin-conjugated solid lipid nanoparticles of curcumin. Cancer Nanotechnol. 2012;3(1):65–81.
113. Han Y, Zhang P, Chen Y, Sun J, Kong F. Co-delivery of plasmid DNA and doxorubicin by solid lipid nanoparticles for lung cancer therapy. Int J Mol Med. 2014;34(1):191–6.
114. Khajavinia A, Varshosaz J, Dehkordi AJ. Targeting etoposide to acute myelogenous leukaemia cells using nanostructured lipid carriers coated with transferrin. Nanotechnology. 2012;23(40):405101.
115. Emami J, Yousefian H, Sadeghi H. Targeted nanostructured lipid carrier for brain delivery of artemisinin: design, preparation, characterization, optimization and cell toxicity. J Pharm Pharmaceut Sci: Publ Canadian Soc Pharmaceut Sci, Societe canadienne des sciences pharmaceutiques. 2018;21(1s):225s–41s.
116. Koppu S, Oh YJ, Edrada-Ebel R, Blatchford DR, Tetley L, Tate RJ, Dufès C. Tumor regression after systemic administration of a novel tumor-targeted gene delivery system carrying a therapeutic plasmid DNA. J Control Release. 2010;143(2):215–21.
117. Braslawsky GR, Kadow K, Knipe J, McGoff K, Edson M, Kaneko T, Greenfield RS. Adriamycin (hydrazone)-antibody conjugates require internalization and intracellular acid hydrolysis for antitumor activity. Cancer Immunol Immunother. 1991;33(6):367–74.
118. Suzuki S, Inoue K, Hongoh A, Hashimoto Y, Yamazoe Y. Modulation of doxorubicin resistance in a doxorubicin-resistant human leukaemia cell by an immunoliposome targeting transferring receptor. Br J Cancer. 1997;76(1):83.

119. Sugyo A, Tsuji AB, Sudo H, Okada M, Koizumi M, Satoh H, Kurosawa G, Kurosawa Y, Saga T. Evaluation of efficacy of radioimmunotherapy with 90Y-labeled fully human anti-transferrin receptor monoclonal antibody in pancreatic cancer mouse models. PLoS One. 2015;10(4):e0123761.
120. Batra JK, Fitzgerald DJ, Chaudhary VK, Pastan I. Single-chain immunotoxins directed at the human transferrin receptor containing Pseudomonas exotoxin A or diphtheria toxin: anti-TFR (Fv)-PE40 and DT388-anti-TFR (Fv). Mol Cell Biol. 1991;11(4):2200–5.
121. Xu L, Huang CC, Huang W, Tang WH, Rait A, Yin YZ, Cruz I, Xiang LM, Pirollo KF, Chang EH. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes 1. Mol Cancer Ther. 2002;1(5):337–46.
122. Kelkar SS, Reineke TM. Theranostics: combining imaging and therapy. Bioconjug Chem. 2011;22(10):1879–903.
123. Wang K, Zhang Y, Wang J, Yuan A, Sun M, Wu J, Hu Y. Self-assembled IR780-loaded transferrin nanoparticles as an imaging, targeting and PDT/PTT agent for cancer therapy. Sci Rep. 2016;6:27421.
124. Hou L, Shan X, Hao L, Feng Q, Zhang Z. Copper sulfide nanoparticle-based localized drug delivery system as an effective cancer synergistic treatment and theranostic platform. Acta Biomater. 2017;54:307–20.
125. Zhang H, Patel N, Ding S, Xiong J, Wu P. Theranostics for hepatocellular carcinoma with Fe 3 O 4@ ZnO nanocomposites. Biomater Sci. 2016;4(2):288–98.
126. Goswami U, Dutta A, Raza A, Kandimalla R, Kalita S, Ghosh SS, Chattopadhyay A. Transferrin–copper nanocluster–doxorubicin nanoparticles as targeted theranostic cancer Nanodrug. ACS Appl Mater Interfaces. 2018;10(4):3282–94.
127. Clinicaltrials.gov. Home – ClinicalTrials.gov. [online]. 2019. Available at: https://clinicaltrials.gov/. Accessed 16 Jun 2019.
128. Alexander-Bryant AA, Berg-Foels WS, Wen X. Bioengineering strategies for designing targeted cancer therapies. Adv Cancer Res. 2013;118:1–59. Academic Press.

Part V
Models for Evaluation of Targeted Delivery in Cancer and Infectious Diseases

Chapter 17
In Vitro and In Vivo Models for Cancer and Infectious Diseases

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Abstract Preclinical evaluation of therapeutic molecules and their formulations is vital during drug discovery and development. These are also obligatory as a regulatory requirement. In vitro and in vivo studies serve as a powerful tool to obtain preliminary data regarding preclinical safety and efficacy of novel drugs and formulations as a precursor to clinical studies. The first step in preclinical evaluation includes in vitro tests, wherein novel drugs and formulations are evaluated through cell-based assays or using specific microorganisms. These are used for high-throughput screening of several entities to identify and shortlist candidates with promising efficacy and safety. Inhibitors to block specific endocytic pathways and methods to elucidate the endocytic pathway are also described. Such potential candidates are further evaluated in vivo using animal models closely resembling the human physiology and pathogenesis of the disease. This chapter reviews various in vitro and in vivo models for the analysis of drugs and formulations targeting two diseases which are leading causes of death globally, namely, cancer and infectious diseases which are the major focus of this book.

Keywords Cancer · Infectious diseases · Preclinical studies · Nanoformulations · Models

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Abbreviations

3H-T	Tritiated thymidine
AMR	Amiloride
AUC	Area under the concentration–time curve
BrdU	Bromodeoxyuridine
CAM	Chorioallantoic membrane
CFU	Colony-forming unit
C_{max}	Peak concentration
Col	Colchicine
CPZ	Chlorpromazine
EGFR	Epidermal growth factor receptor
EIPA	5-(N-ethyl-N-isopropyi)-amiloride
EMT	Epithelial to mesenchymal transition
FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
GC	Gas chromatography
GEMMs	Genetically engineered mouse models
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LC-MS	Liquid chromatography–mass spectrometry
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
MβCD	Methyl-β-cyclodextrin
NY	Nystatin
PD_{50}	Protective dose
PDOX	Patient-derived orthotopic xenograft models
PDTC	Pyrrolidine dithiocarbamate
PDX	Patient-derived xenograft model
PK/PD	Pharmacokinetic/pharmacodynamic
S.C.	Subcutaneous injection

1 Introduction

Preclinical evaluation, comprising in vitro and in vivo studies, is an important aspect during drug discovery and development. These studies provide useful tools to assess the preclinical safety and efficacy of therapeutic molecules and their formulations before they may be certified as suitable for clinical studies. They furnish the researcher with a rationale for conducting clinical studies and are predictive of clinical outcomes. A good preclinical model, characteristically, should be relatively

inexpensive, amenable to high-throughput screening, easy to develop, have the capacity to recapitulate the pathogenicity of the disease, and grant insights into molecular mechanisms of action of test specimens for in depth understanding of their activity [1].

This chapter, in keeping with the main theme of the book, discusses models for in vitro and in vivo analyses of two important diseases, cancer and infectious diseases. These two diseases are the leading causes of death, globally. Thus, it is imperative to develop effective therapeutic strategies to combat and control these conditions and to develop tools to ascertain their safety and efficacy.

Cancer is a complex and multifactorial disorder involving complex pathogenesis. The cellular genome undergoes a number of successive changes that leads to abnormal growth, proliferation, and metastasis [2]. In vitro models for testing anticancer therapeutics have been the mainstay of evaluation from the time of discovery of the first human cancer cell line [1]. Since then, with increased understanding of the pathological mechanisms for initiation and progression of cancer, a number of different in vitro models have been developed to understand the complexity and diversity of cancers. Although in vitro models do not ideally resemble the physiology and simulate only a limited aspect of the tumor microenvironment, they allow control of most of the experimental variables and permit qualitative and quantitative analyses of tumor markers that serve as hallmarks of particular cancers [3].

An ideal in vivo model for the evaluation of anticancer therapeutics should closely resemble the exact stage of human tumorigenesis, reflect human tumor biology, disease progression, and the probable therapeutic or toxic response. The tumors developed in vivo should mimic the physiological and molecular features of the human tumors, including the tumor microenvironment and associated immune responses. The model should also ideally recapitulate the phenotypic heterogeneity, polymorphism, resilience, and capacity of the tumor cells to metastasize [4]. This chapter discusses the different cellular and animal models that are usually employed for evaluating novel anticancer compounds and formulations.

A number of microorganisms such as viruses, mycoplasmas, bacteria (including rickettsiae, chlamydiae, mycobacteria, and actinomycetes), fungi (including yeasts), and protozoa are responsible for causing infectious diseases in human. Each organism differs in characteristics such as its genetic composition, structure, site of multiplication (intra- or extracellular), and nutritional requirements. For example, the human pathogens of bacterial origin belong to two general categories, that is, those causing intracellular and extracellular infections [5]. Thus, the spectrum of an anti-infective molecule is governed by the classes of microorganisms responsible for diverse infections.

As in the case of anticancer drugs, the anti-infective drugs or their formulations also require extensive evaluation in appropriate in vitro systems and animal models, recapitulating the disease. These investigations reflect the biological spectrum of activity, pharmacological profile, and toxicity of the test samples.

The first step of preclinical evaluation involves tests that are designed to determine the activity of test samples against a specific target microorganism(s). Based on these results, the test systems can be shortlisted for determining their

mechanistic activity and toxicity. Further assessments include determining the development of resistance by the microorganisms toward the test moieties and understanding their interactions such as synergistic, additive, and antagonistic effects with other anti-infective drugs [6]. This stage may also involve understanding the biological interactions of molecules.

The next step in preclinical study is the evaluation in animal models. In vivo animal models are developed by artificial induction of infections, with an attempt to mimic the infectious process seen in human [7]. The basic screening models employed during in vivo evaluation provide information regarding the efficacy of a potential new drug or a novel formulation. It also helps in determining the route of administration, optimizing the dosing regimen, and identifying toxicity [8]. These screening models are suitable as they generally involve a single step, have short time duration, and are reflective of simple infections with the endpoint usually being lethality [9]. These are standardized and are thus reproducible and effective in studying particular disease aspects, which is almost impossible in clinical studies [8]. These screening models are followed by ex vivo models, monoparametric models, and discriminative models, which are associated with sophisticated and complicated techniques to demonstrate efficacy or toxicity of therapeutic molecules and their formulations.

2 In Vitro Models for the Evaluation of Anticancer Agents

2.1 Determination of Cell Viability by MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is relatively simple, high-throughput, and a routine technique for determining the cellular viability. The assay is based on mitochondrial activity of viable cells. Viable cells have constant mitochondrial activity and thus an increase or decrease in the number of viable cells results in a corresponding linear deviation in the mitochondrial activity. During this assay, the mitochondrial activity of the cells is measured by the conversion of the yellow colored tetrazolium salt MTT into blue formazan crystals, which are solubilized in DMSO for homogenous and reproducible measurement. Therefore, any increase or decrease in the number of viable cells can be detected by measuring formazan concentration by recording its optical density (OD) at 540 and 720 nm using a plate reader [10]. This is a cost-effective assay, which can be used for preliminary high-throughput screening of drugs and formulations. The assay can be conducted in various cell lines, for the evaluation of different targeted drug delivery systems, as stated in Table 17.1. This experiment can be used to determine IC_{10} or IC_{50} values, that is, the concentration of test substance that results in 10% or 50% of cell death, as well as the maximum nontoxic concentration of the test substance. Thus, it serves as an important tool for providing the preliminary indication regarding in vitro efficacy as well as safety [11].

Table 17.1 Cell lines used during MTT assay of targeted anticancer drug delivery systems

Name of cell line	Origin	Reference
SKVO-3	Human ovarian adenocarcinoma cell lines	[12]
A549 cells	Human adenocarcinoma alveolar-based lung cancer cell line	[12]
LoVo	Colon adenocarcinoma cells	[12]
HeLa	Cervical cancer cell line	[13]
MDA-MB 231	Human triple-negative breast cancer cell line	[14]
UMUC3	Human bladder cancer cell line	[14]
HepG2	Human liver adenocarcinoma cells	[15]
Huh7	Human hepatocellular carcinoma cells	[16]

The choice of the cell line depends on the type of cancer being targeted by the formulation under investigation. Table 17.1 provides a list of cancer cell lines used for the evaluation of targeted drug delivery systems. Table 17.2 enlists nano drug delivery systems whose cytotoxic effect has been evaluated using MTT assay.

2.2 Methods to Determine Cell Proliferation

These assays measure cellular proliferation based on their DNA content. In every cell, DNA synthesis and replication precede cell division; therefore, measurement of the newly synthesized DNA is proportional to cellular proliferation. Cell proliferation is generally measured by quantifying the amount of a labeled nucleoside inside the genomic DNA. The most widely used labeled nucleosides are 3H-labeled thymidine (3H-T) and BrdU (bromodeoxyuridine). While incorporation of 3H-T, a radiolabeled nucleoside, is determined by measuring the radioactivity, BrdU is quantified by using a labeled monoclonal antibody against BrdU [23].

The tritiated thymidine (3H-T) labeling index has been widely used for estimating the proportion of cells in the S phase. The procedure is very similar to MTT assay. Cell proliferation can also be measured by labeling cells with halogenated thymidine analogs, such as bromodeoxyuridine (BrdU), in either in vitro or in vivo assays. Both tritiated thymidine and BrdU are incorporated into the nuclear DNA during the S phase of the cell cycle [24]. However, it should be noted that both tritiated thymidine and BrdU are markers of only DNA synthesis and not cell division. This is because DNA synthesis can also occur independently of mitosis, for example, during gene replication, repair, or apoptosis [25]. Table 17.3 provides a list of drug delivery systems and formulations that have been evaluated for their effect on cellular proliferation using tritiated thymidine (3H-T) labeling assay or BrdU incorporation assay.

Table 17.2 Nano drug delivery systems evaluated by the MTT assay

No.	Delivery system	Cell line	Outcome	Reference
1.	Phospholipid-based emulsions, liposomes, and aqueous lecithin dispersions	HEK-293 (human embryonic kidney) and HL-60 (human promyelocytic leukemia) cells	Low cytotoxicity of all formulations, suggesting their safety as parenteral drug carriers	[17]
2	Fish gelatin methacryloyl-based nanogels	Rat fibroblasts (NIH3T3) cells	Cell viability decreased at the concentration of 5 mg/ml of the nanogels	[18]
3.	Superparamagnetic iron oxide nanoparticles loaded with doxorubicin	Breast cancer cells (MCF-7 and MDA-MB-231)	Nanoparticles (NPs) significantly nontoxic to noncancerous cells due to minimum internalization. Significantly cytotoxic to cancerous cells due to synergistic action, combined with hyperthermic effects	[19]
4.	Rh2-treated graphene oxide (GO-Rh2), lysine-treated highly porous graphene (Gr-Lys), arginine-treated Gr (Gr-Arg), Rh2-treated Gr-Lys (Gr-Lys-Rh2), and Rh2-treated Gr-Arg (Gr-Arg-Rh2) NPs	Ovarian cancer (OVCAR3), breast cancer (MDA-MB), human melanoma (A375), and human mesenchymal stem cells (MSCs)	Gr-Arg, Gr-Lys, Gr-Arg-Rh2, and Gr-Lys-Rh2 were more cytotoxic to the cancer cell lines in comparison with their activity against normal cell lines (MSCs). The IC_{50} values were higher than 100 μg/ml	[20]
5	Liposomes loaded with doxorubicin for targeting prostate cancer	Prostate cancer cell line (PC-3)	Concentration-dependent cytotoxicity with IC_{50} value of 36.71 ± 5.36 μg/ml	[21]
6	Docetaxel-loaded nanostructured lipid carriers	Human liver cancer cell line (HepG2), human ovarian cancer cell line (SKOV3), human alveolar basal epithelial cell line (A549), and murine melanoma cell line (B16)	Dose-dependent cytotoxicity against all the cell lines	[22]

Table 17.3 Nano drug delivery systems evaluated for effect on cell proliferation

No.	Delivery system	Cell line	Outcome	Cell proliferation assay	Reference
1.	Thymopentin, a synthetic polypeptide of human hormone thymopoietin, loaded into polybutylcyanoacrylate nanoparticles	Thymocytes and spleen cells isolated from mice	Human hormone thymopoietin-loaded nanoparticles increased cellular proliferation which was similar to free thymopoietin	Tritiated thymidine (3H-T) labeling	[26]
2	Gold nanoparticle (AuNP) consisting of folic acid (FA), mercaptopolyethylene glycol (PEG-SH), and cisplatin (CP) [Au-PSH-CP-FA]	HUVEC, ovarian surface epithelial cells (OSE), ovarian cancer cells (OVCAR-5, OV-167)	Cell proliferation was decreased by both free cisplatin and NPs, but free drug had lower efficiency to inhibit cell proliferation	Tritiated thymidine (3H-T) labeling	[27]
3.	Liposomes and immunoliposomes encapsulating doxorubicin	Squamous cell carcinoma cell line (KLN-205)	Doxorubicin-loaded liposomes tagged with antibody (immunoliposome) resulted in higher inhibition of cell proliferation as compared to doxorubicin-loaded liposomes and as free drug	Tritiated thymidine (3H-T) labeling	[28]
4.	Four quaternized chitosan formulations	CaCo-2 cells	Lower degree of substitution and low concentration had low effect on cell proliferation	BrdU incorporation assay	[29]
5	Pravastatin-containing long-circulating liposomes	Murine melanoma cells (B16.F10), human umbilical vein endothelial cells (HUVEC), and J774 cells (macrophage cell line)	Cell proliferation decreased by 50–60% in J774 cells incubated with liposomal pravastatin for 72 h at concentrations of 100–200 μg/ml. There was no significant effect on proliferation of HUVEC or B16.F10	BrdU incorporation assay	[30]
6	Doxorubicin-loaded calcium carbonate nanocrystals	MG63 osteosarcoma cancer cell line	Doxorubicin-loaded calcium carbonate nanocrystals suppressed proliferation of MG63 in a dose- and time-dependent manner	BrdU incorporation assay	[31]

2.3 Methods for Evaluation of Drugs and Formulations Inhibiting Angiogenesis

Angiogenesis is a physiological process of formation of new blood vessels from preexisting vessels and is tightly regulated by certain biomolecules. New blood vessels are generated from preexisting blood vessels by "sprouting" of endothelial cells, which results in expansion of vasculature [32]. Physiologically, angiogenesis is vital during embryo development, wound healing, and collateral formation of blood vessels for improved oxygen perfusion. However, aberrant angiogenesis is deleterious in cancer as it promotes the cancer cells to penetrate blood vessels or lymphatic vessels, circulate through the intravascular stream, and then proliferate at another site, resulting in "cancer metastasis." Therefore, cancer treatment strategies are aiming at combining antiangiogenic agents with the conventional anticancer regimens [33].

Chorioallantoic membrane (CAM) assay has been widely used to study angiogenesis, tumor cell invasion, and metastasis. It is a well-established model for the investigation of tumor angiogenesis and invasion for malignancies such as bowel cancer, glioma, prostate cancer, leukemia, osteosarcoma, and ovarian cancer [34]. The CAM matrix represents the physiological cancer environment because of a multilayer epithelium comprising the ectoderm at the air interface, mesoderm or stroma, and endoderm at the interface with allantoic sac along with extracellular matrix proteins such as fibronectin, laminin, and collagen type I [35, 36].

Zebrafish has emerged as an excellent vertebrate model system for studying blood and lymphatic development. The zebrafish is a small teleost fish, measuring about 3–4 cm in length and weighing about 2 g. It has the ability to regenerate many tissues, including the caudal (tail) fin, heart, and nervous system. Thus, this assay has emerged as a promising and highly reproducible model for time-efficient, quantitative evaluation of angiogenesis. The main advantage of this method is that the zebrafish tail fin is very thin and optically transparent and comprised of well-defined tissues and blood vessels. The procedure involves amputation of the caudal fin at the mid-fin level. The amputated blood vessels start healing within one day postamputation. The arteries and veins then reconnect via anastomosis to resume blood flow at the wound sites by second day postamputation. Then, by third day postamputation, the network of endothelial cells in regenerated tissue form a vascular plexus that is extended to the fin tip. The above mentioned process is interrupted in the presence of antiangiogenic drugs [37].

Endothelial cell migration assay is based on the chemotactic movement of endothelial cells in response to an angiogenic factor. The setup comprises of two compartments separated by a membrane having an accurately defined pore size. A potential chemotactic factor is placed in one compartment and a gradient is allowed to develop across the membrane. The chemotactic factors include angiogenic paracrine factors, which are normally released by tumor cells during angiogenesis, such as angiogenin, vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor β. These factors activate proliferation of endothelial cells; the endothelial cells placed in the other compartment migrate along this gradient. The number of cells that

Table 17.4 Nano drug delivery systems evaluated via in vitro angiogenesis assays

No.	Delivery system	Outcome	Assay	Reference
1.	Taxol-loaded microspheres composed of a blend of ethylene-vinyl acetate copolymer and poly(D,L-lactic acid)	The taxol released from microspheres was sufficient to produce vascular regression and inhibition of angiogenesis	Chick chorioallantoic membrane assay	[39]
2.	Doxorubicin encapsulated in stabilized immunoliposomes (Dox-SL) or NGR peptides that targets the angiogenic endothelial cell marker aminopeptidase N coupled to Dox-SL (NGR-SL) or nonspecific peptide-targeted formulation (ARA-SL)	NGR-SL inhibited neuroblastoma-induced angiogenic responses in the CAM assay model	Chick chorioallantoic membrane assay	[40]
3.	Polymer–alendronate–taxane conjugate	The proliferation of HUVEC cells was inhibited by the conjugates	Endothelial cell migration assay	[41]
4.	N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-aminohexylgeldanamycin-RGDfK (Arg-Gly-Asp) conjugates	RGDfK-targeted conjugates showed a significantly more potent inhibition of endothelial migration and tube formation as compared to nontargeted polymer–drug conjugates at the same dose	Endothelial cell migration assay	[42]
5.	Paclitaxel encapsulated into cationic liposomes (PTX-CL) followed by internalization in the neutrophils (NE) to obtain NE-based delivery vehicles (PTX-CL/NEs)	PTX-CL/NEs activated chemotaxis in response to fMLP, a chemotactic factor	Endothelial cell migration assay	[43]

have migrated through the membrane can be counted after adding the test substance. This test is used to determine the prevention of metastasis by the drugs or formulations [38]. Table 17.4 provides a list of nano drug delivery systems that have been evaluated via various in vitro angiogenesis assays.

2.4 Topoisomerase Inhibition Assay

Topoisomerases are family of enzymes that are present in the nuclei of cells and play a key role in DNA replication, transcription, chromosome segregation, and recombination. There are two major forms of topoisomerases, namely, topoisomerase I and topoisomerase II. Topoisomerase I makes single-stranded cuts in the

torsionally stressed DNA by recognizing the torque. This causes rotation of the nicked stand and reanneals the strand in a more relaxed state. Topoisomerase II cuts both strands of a double-stranded DNA molecule, passes another portion of the duplex through the cut, and reseals the cut in a process that utilizes ATP. After the process of DNA replication is complete, the strands are paired, followed by the coiling of DNA. Thus, inhibition of topoisomerase is detrimental to cells due to inhibition of DNA replication, uncoiling of DNA for protein synthesis, and DNA repair. Topoisomerases I and II inhibition assays have been described in detail by John et al. [44].

Barret et al. investigated the topoisomerase II inhibition activity of F12512, an antitumor agent vectored into cancer cells via the polyamine transport system. This system displayed a highly potent inhibition of topoisomerase II in vitro, which was attributed to its DNA binding capacity via the polyamine tail [45].

2.5 Cell Cycle Analysis

A detailed account of cell cycle analysis by flow cytometry has been described by Piotr Pozarowski et al. The protocol describes cell cycle analysis by the measurement of the DNA content using propidium iodide (PI) as the staining agent. Propidium iodide is a fluorogenic compound that binds stoichiometrically to DNA by intercalating between the base pairs. Therefore, its fluorescence emission is directly proportional to the DNA content of the cells. PI is a membrane impermeable dye which is excluded by viable cells; however, it easily permeates the damaged membranes of nonviable cells. This can be quantitatively measured due to the virtue of PI being excited at 488 nm and showing an emission maximum at 617 nm. This special characteristic also provides an advantage of using it in combination with other fluorochromes excited at 488 nm, such as fluorescein isothiocyanate (FITC). The most common and widely used method for evaluating the cell cycle involves measurement of the DNA content by flow cytometry. During this process, a DNA biding fluorescent dye is added to cell suspension of permeabilized or fixed cells. It is essential for the cells to be in a fixed state or permeabilized state to allow the dye to enter into the cells, which would otherwise be actively pumped out by the living cells. Table 17.5 provides a list of various nano drug delivery systems that have been evaluated using cell cycle analysis.

2.6 Apoptosis Assays

Cell death can be classified into two types, namely, accidental cell death termed as "necrosis" and programmed cell death termed as "apoptosis." Necrotic cells are characterized by cellular lysis, loss in membrane integrity, and acute inflammation. In contrast, apoptotic cells appear as small membrane-bound vesicles termed as

Table 17.5 Nano drug delivery systems evaluated using cell cycle analysis

No.	Delivery system	Cells	Outcomes	Reference
1.	Chitosan-coated doxorubicin nanoparticles	Liver cancer cells (HepG2 cells)	Chitosan-coated doxorubicin nanoparticles arrested the cells in G2/M phase, which was correlated with upregulation of p53	[46]
2.	Paclitaxel-loaded chitosan and polyethylene glycol-coated PLGA (PLGA–CS–PEG) nanoparticles	Human retinoblastoma cell line (Y79 and WERI-Rb-1), breast cancer cell line (MCF 7), and pancreatic cell line (MIA PaCa-2)	PLGA–CS–PEG induced arrest of greater fraction of cells in G2/M phase than plain paclitaxel	[47]
3.	pH-responsive nanocarriers for delivery of ursolic acid (UA)	Liver cancer cells (HeLa, HepG2 cells)	Free UA and nanodrugs of ursolic acid blocked cell cycle progression in the G0/G1 phase and the G2/M phase	[48]
4.	Nitrocamptothecin, a topoisomerase I inhibitor, loaded into liposomes	HepG2, Bel-7402, Hep3B, and L02 cells	A delay in the S phase was observed after exposure to liposomes for 24 h, while a delay in G2/M phase was observed after exposure to the liposomes for 72 h in all the test cell lines	[49]
5.	Nanodiamonds covalently linked to paclitaxel	Lung cancer cells (A459 cells)	Nanodiamond–paclitaxel dramatically decreased the fraction of cells in G1/G0 phase and increased the fraction of cells in G2/M phase in A549 cells	[22]

"apoptotic bodies," which are engulfed by macrophages. Most of the anticancer drugs induce cell death by apoptosis.

Apoptosis is characterized by specific morphological features, such as loss of plasma membrane symmetry, plasma membrane blebbing, condensation of cytoplasm and nucleus, and cleavage of DNA. One of the earliest events in apoptosis is plasma membrane asymmetry due to translocation of membrane phospholipid phosphatidyl serine (PS) from the inner leaflet to the outer leaflet of plasma membrane. This translocation exposes PS to the external cellular environment. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein, having molecular weight of 35–36 kDa, with a high affinity for PS. This protein binds to the exposed apoptotic cellular surface. Thus, conjugation of annexin V with fluorochromes, such as FITC, can be used for the determination of early apoptosis. Late apoptotic cells are characterized by the loss of membrane integrity and increased permeability to dyes such as propidium iodide. In conjunction with propidium iodide, this assay can be used for distinguishing between cells undergoing early and late apoptosis. Cells with

intact membrane will exclude PI, whereas dead and damaged cells will be permeable to PI. Therefore, viable cells will be both annexin V and PI negative, early apoptotic cells will be annexin V positive and PI negative, whereas late apoptotic cells will be both annexin V and PI positive [50].

Mitochondrial permeabilization and subsequent caspase activation are the other important steps in the process of apoptosis. In contrast, necrotic cell death lacks caspase activation, thus making caspase a more selective marker for determination of apoptotic cell death. Caspase activity can be determined using a "fluorescence imaging reporter" that detects caspase-3 activity by Förster resonance energy transfer (FRET). FRET occurs across very short distances between donor and acceptor fluorophores that overlap in their emission and absorption spectra. FRET-compatible green fluorescent protein (GFP) variants that have been fused with short peptide linkers containing caspase cleavage sites can be used to measure the caspase activity. In the intact probes, the donor and acceptor fluorophores are in proximity and FRET is highly efficient. Upon caspase activation, proteolysis of the linker occurs and the donor gets separated from the acceptor. This results in a disruption of resonance energy transfer and an increase in donor fluorescence quantum yield. Several highly sensitive FRET probes, based on the cyan fluorescent protein–yellow fluorescent protein (CFP-YFP) pair, or improved variants thereof have been developed to detect intracellular caspase activities. The measurement of caspase activity has been described in detail by Markus Rehm et al. [51]. Table 17.6 lists nano drug delivery systems evaluated for their apoptotic potential.

3 In Vivo Models for Evaluating Anticancer Action

The introduction of mouse models for preclinical evaluation of anticancer therapeutics was based on the need to simultaneously screen a plethora of chemicals identified as potential anticancer agents. This led to the development of the tumor models for high-throughput screening which employed serially propagated tumor cells in syngeneic hosts [56, 61]. Over the years, the tumor models have undergone evolution to recapitulate a number of stages in cancer progression, that is, from its initiation to its progression to aggressive tumors and to metastasis [56, 57].

The various types of human–mouse xenograft models that are used for screening anticancer therapeutics include the following:

- Ectopic tumor xenograft
- Orthotopic tumor xenograft
- Metastatic cancer model
- Patient-derived tumor xenograft model
- Germline transgenic and conditional transgenic models (GEMMs)

Xenograft Mouse Models

Xenograft mouse models are the most widely used animal models for the preclinical evaluation of anticancer therapeutics. These are used to assess the tumor development, tumor growth rate, malignant transformation, invasion, and metastasis [58].

Table 17.6 Nano drug delivery systems evaluated for their apoptotic activity

No.	Delivery system	Cells	Outcomes	Reference
1.	Nitrocamptothecin, a topoisomerase I inhibitor, loaded into liposomes	Liver cancer cells (HepG2, Bel-7402, Hep3B, and L02 cells)	Apoptosis was induced in dose-dependent manner in all cell lines. Hep3B cells were less sensitive, whereas HepG2 cells were more sensitive to apoptosis	[49]
2.	Alginate nanoparticles with methylene blue (a P-glycoprotein inhibitor) for delivery of doxorubicin	Drug-resistant tumor cells (NCI/ADR-RES)	Methylene blue doxorubicin nanoparticles resulted in a significant increase in the fraction of apoptotic and necrotic cells at the end of 12 h	[52]
3.	Paclitaxel (PTX) conjugated to folate-modified adenovirus (Ad) nanoparticles using succinic anhydride and Fmoc-Glu (OtBu)eOH as linkers	Breast cancer cell line (MDA-MB-231)	Paclitaxel-containing drug delivery system induced apoptosis and greatly enhanced the antitumor activity at low dose of paclitaxel	[53]
4.	PVP-coated silver nanoparticles and silver ions	Human monocytic cell line (THP-1 monocytes)	There was a significant reduction in the percentage of viable cells after 24 h of treatment with PVP-coated silver nanoparticles or silver ions	[54]
5.	Magnetic Fe_3O_4 nanoparticles coencapsulated with doxorubicin into biodegradable PLGA nanocarriers	LLC cells	The percentage of apoptotic cells was higher in the nanoparticle–doxorubicin system (80%) as compared to free doxorubicin (29%)	[55]

Xenograft models can be differentiated according to the source-implanted material into:

- *Cell line-derived xenograft model*: The implantation material is of cell line origin.
- *Patient-derived xenograft*: The implantation material is directly obtained from cancer patients in the form of tumor tissues or cells [58].

3.1 Cell Line–Derived Xenograft Model

The cell line-derived xenograft model employs cell lines derived from human and animal cancer tissues.

3.1.1 Ectopic Tumor Xenograft Model

Ectopic tumor xenograft model includes subcutaneous injection of established human or murine tumor cells from cell cultures, generally under the skin of the hind legs (flanks) or back. With human cancer cell lines, the reproducible take rate of the xenograft model is over 90% [59].

The ectopic xenograft implantation into syngeneic (genetically identical) and immunocompromised rodents is used to quantitatively monitor the tumorigenicity and tumor growth reproducibility during early screening of novel agents. This model is cost- and time-effective, with modest throughput capacity, which is feasible with a variety of cell lines. It is also valuable in studying the effect of immune responses in an immunocompetent host [4, 59]. Table 17.7 presents a list of nanosystems evaluated in ectopic tumor models.

3.1.2 Orthotopic Tumor Xenograft

Orthotopic xenograft model is an alternative in vivo cancer model which involves injecting or implanting cell lines or patient-derived tumor cells at the site of cancer origin. The orthotopic xenograft model provides a similar microenvironment to the cancer cells resembling the tumor cell phenotype. This includes the tumor–stromal microenvironment-based interactions important for tumor growth and metastasis, thus complimenting the natural tumorigenesis [4]. The orthotopic model has been therefore reported to result in a much higher metastatic rate as compared to the ectopic model as it recapitulates the pathological phenotype more closely to the human clinical course of metastatic disease [4]. Furthermore, the effect of immune response on tumorigenesis can also be studied by using immunocompetent hosts.

The orthotopic xenograft model can be utilized effectively to evaluate the tumor growth inhibition versus tumor regression in context of survival of tumor-bearing animals. It is usually employed for mid- to later stages of drug discovery screening, evaluation of antitumor efficacy against primary tumors and metastases, and acquired chemotherapy resistance [4, 59, 64]. Table 17.8 enlists the examples of nanosystems evaluated in orthotopic xenograft model.

3.1.3 Metastatic Cancer Model

Cancer metastasis is a stage of tumor progression caused due to the spread of cancerous cells from the primary tumor to develop a secondary tumor in a distant organ. This stage is characterized by genetic and proteomic changes in the cells as they transform in situ into the hyperplastic state, the carcinoma, through dysplasia, and ultimately to the invasive and metastatic stage.

Metastasis is a highly complex, multistep process encompassing mechanisms causing deregulation of interacting proteins and genes, leading to tumor cell invasion, intravasation, transmission through circulation, circumvention of the immune

Table 17.7 Nano drug delivery systems evaluated in ectopic xenograft tumor models

No.	Nano system	Animal model	Outcome	Reference
1.	pH-sensitive (EGFR) targeting immunoliposomes (PSL) encapsulating gemcitabine for epidermal growth factor receptor	Subcutaneous (S.C.) implantation of a human non–small cell lung cancer cell line (A549 cells; 8×10^6–1×10^7 cells) in hind flank region of BALB/c-nu/nu mouse	Ab-PSLs with gemcitabine showed significant antitumor efficacy with a higher regression of the established tumors in nude mouse models	[60]
2.	Albumin-bound paclitaxel nanoparticle nab-PTX, also called Abraxane®	S.C. implantation of C26 murine colon cancer (2×10^6) in back of C57BL6 mice	nab-PTX therapy combined with S-nitrosated human serum albumin dimer (SNO-HSA dimer) enhanced tumor selectivity of nab-PTX and attenuated myelosuppression	[61]
3.	Albumin-bound paclitaxel nanoparticle nab-PTX, also called Abraxane®	S.C. implantation of B16 murine melanoma cells (2×10^6) in the back of BALB/c mice	nab-PTX therapy combined with SNO-HAS dimer augmented the tumor growth inhibition of nab-PTX in low vascular permeability B16 murine melanoma subcutaneous model	[61]
4.	Nanopolymeric micelles (lodamin): TNP 470 conjugated to monomethoxy-polyethylene glycol–polylactic acid	S.C. implantation of murine LLC cells, B16/F10 melanoma cells (1×10^6) in the C57Bl/6 J mice. Effect of lodamin compared to free TNP 470	Lodamin was successful in significantly inhibiting the tumor growth without causing neurological impairment in tumor-bearing mice	[62]
5.	Folate-targeted ursolic acid stealth liposome (FTL-UA)	S.C. inoculation of human oral cancer KB cell line (3.6×10^6) in right hind flank of Balb/c nu/nu mouse. Treatment includes FTL-UA, nontargeted PEGylated liposome (PL-UA) and free UA	FTL-UA showed significantly higher inhibition of human epidermoid carcinoma (KB) in Balb/c nu/nu mice compared to PL-UA or free UA. The results indicated the great potential of FTL-UA against KB tumor	[63]

attack, extravasation, and growth at the secondary site [69]. The prerequisite of metastasizing cells is the epithelial to mesenchymal transition (EMT), which involves a switch in the gene expression profiles leading to cells with higher motility and invasiveness. The stage of metastasis, which can be studied in a particular model, depends on the mode of delivery of cells into the murine hosts. Experimental metastatic mouse models involve direct intravascular injection of tumor cells into the circulation of mice and monitoring the formation of metastatic tumors [70, 71].

Table 17.8 Nano drug delivery systems evaluated in orthotopic xenograft model

No.	Nano system	Animal model	Outcome	Reference
1.	Albumin-bound paclitaxel nanoparticle (nab-PTX, also called Abraxane®)	Orthotopic pancreatic cancer model was achieved by implanting 1×10^5 SUIT2-GLuc human pancreatic cancer cells in pancreas of BALB/c nu/nu mice	nab-PTX therapy combined with S-nitrosated human serum albumin dimer (SNO-HSA dimer) exhibited greater antitumor activity and improved survival rate in the SUIT2 human pancreatic cancer orthotopic model	[62]
2.	Poly(lactic-co-glycolic acid)-based curcumin nanoparticle formulation (nano-CUR)	CaSki cervical cancer cells (4×10^6 cells/per mouse) were injected directly into the cervix of the NOD SCID gamma (NSG) mice	Nano-CUR effectively reduced the tumor burden and reduced the expression of E6/E7 HPV oncoproteins.	[65]
3.	"Cellax": conjugate of docetaxel (DTX) and an acetylated and PEGylated carboxymethylcellulose polymer	Two orthotopic breast cancer models involving injection of mouse 4 T1 breast cancer cells in Balb/c mice and human MDA-MB-231 breast cancer cell line in NOD SCID mice	Cellax was reported to target tumor stroma and achieved better efficacy than docetaxel and Abraxane	[66]
4.	Curcumin–cyclodextrin (CD) complexes	100 μL of suspension containing 2×10^6 cells of murine Lewis lung carcinoma cells (LLC) inoculated orthotopically in C57Bl/6 mice	Only <45% of animals treated with curcumin–CD complexes and gemcitabine developed lung tumors and displayed reduction in the size of lung tumors	[67]
5.	Lipopepsomes: tumor-targeted delivery of doxorubicin hydrochloride	Orthotopic injection of 5×10^6 bioluminescent A549-Luc lung cancer cells suspended in 100 μL of PBS/Matrigel (4/1, v/v) into the left lung parenchyma of Balb/c mice	cRGD-decorated lipopepsomes of doxorubicin (cRGD-LPP-Dox) exhibited markedly enhanced toleration and tumor accumulation than liposomal doxorubicin. They effectively suppressed the orthotopic lung tumor which resulted in significantly improved survival rate	[68]

Currently, two broad approaches are described to develop metastatic cancer models, which are as follows:

A. *Transplant models*

(i) Spontaneous metastatic mouse model
(ii) Experimental metastatic mouse model

B. *Genetically engineered mouse models (GEMMs)*

A. *Transplant models*

The transplantation models are established by transplanting or inoculating the human or mouse tumor cells/tissues into the hosts. The mode of delivery dictates the stages of metastasis that can be assessed. It is a basic model to investigate the mechanisms and therapeutic interventions for various stages of the invasion–metastasis cascade [72].

(i) *Spontaneous metastatic mouse model*

Spontaneous metastatic mouse model involves the inoculation of cells into an ectopic or orthotopic site to develop a primary tumor which can subsequently metastasize. It assesses the ability of cells to disseminate from a primary tumor to generate a secondary tumor. Therefore, this model has been exploited to study the early as well as late stages of metastasis [70]. It is also possible to simultaneously evaluate the function of any carcinogenic gene on the tumor growth and metastasis by the use of inhibitors or genetic manipulation [70]. In some cases, there is a longer latency, lower predictability of the pattern of dissemination, and lower overall incidence of metastasis. Tumor resection approach is required to allow proper development of metastases, for example, in melanoma and mammary tumors [71].

(ii) *Experimental metastasis*

The site of colonization of the tumors in the experimental metastasis model depends on the site of vascular injection. The cells once transplanted bypass the processes involving formation of primary tumors and early stages of metastasis. The site of injection determines the metastatic potential and consequently the outcome. The natural/common metastatic distribution of the test tumor cells should be taken into consideration while determining the site of transplantation [70]. The sites for injection may be the lateral tail vein injection for lung metastases [71], intraportal injection for liver metastases [73–75], intracarotid injection for brain metastases [76], intracardiac injection for wider spread of cells when metastasis is observed in liver, ovary, adrenal glands, brain, and bone [70] and also commonly used for brain and bone metastasis [71], intraperitoneal injection for local dissemination for ovarian cancer [71], and intrasplenic injection of colon cancer cells for metastatic development within the liver [71].

Experimental metastasis models are rapid and are more reproducible. They are therefore widely utilized for testing therapies targeting the late stage

metastasis. These are frequently employed to model metastatic dissemination to sites that take longer to develop tumors, such as the central nervous system (CNS) metastases. They are particularly attractive models to investigate human tumor lines with limited metastatic potential from orthotopic sites [70–72]. Table 17.9 enlists the examples of nanosystems evaluated using spontaneous and metastatic models.

Table 17.9 Nano drug delivery systems evaluated in spontaneous and experimental metastatic models

No.	Nano system	Animal model	Outcome	Reference
1.	Nanopolymeric micelles: TNP 470 (lodamin) conjugated with monomethoxy-polyethylene glycol–polylactic acid	Liver metastasis generated by inoculating 5×10^5, 50 μL murine B16/F10 melanoma cells via spleen injection in C57Bl/6 J mice	TNP 470 micelles show selective uptake by tumors retaining antiangiogenic potential and prevents liver metastasis	[62]
2.	"Cellax": conjugate of docetaxel (DTX) and an acetylated and PEGylated carboxymethylcellulose polymer	Inoculation of mouse 4 T1 breast cancer cell line (1×10^6 cells/50 μL media) into the fat pad in BALB/c mice	Cellax treatment-controlled metastases. It reduced the incidence of lung metastasis to 40%	[76]
3.	Nanoformulated liposome encapsulating all transretinoic acid (ATRA) using a phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol (DSPC lipo-ATRA) for targeting receptor and tumor suppressor RAR-β in the lung	Induction of lung metastasis using B16F10 melanoma cells (1×10^6 cells in 1 mL) in C57BL/6 mice via tail vein. The effects are compared with free ATRA	Lipo-ATRA treatment exhibited significant induction compared to free ATRA treatment indicating DSPC liposome might be suitable as a targeted delivery system for ATRA in the treatment of lung cancer	[77]
4.	Long-circulating, sterically stabilized (Stealth) immunoliposomes (SIL) of vincristine (VCR) and doxorubicin (DXR) for targeting anti-CD19 or its Fab' fragment	Tail vein injection of human Burkitt's lymphoma cell line Namalwa cells (5×10^6 in 0.2 mL PBS) in SCID mice	SIL[CD19] or SIL[Fab] had higher association and cytotoxicity against the Namalwa cells than the nontargeted liposomes	[78]
5.	SP141FcNP: specific murine double minute 2 (MDM2) oncogene inhibitor (SP141) IgG Fc-conjugated maleimidyl-poly(ethylene glycol)-co-poly(ε-caprolactone) (Mal-PEG-PCL) nanoparticles	Human breast cancer cell lines MDA-MB-231 cells (1×10^6 in 20 μL) implanted into the second thoracic mammary fat pad of athymic nude mice	SP141FcNP was found to exert enhanced inhibitory effects on tumor growth and metastasis without any toxicity toward the host	[79]

3.2 *Patient-Derived Xenograft Models (PDX)*

Patient-derived xenograft model as the name suggests directly utilizes the fresh/cryopreserved human tumor tissue or cancerous cells from patients. These tissue/cells are engrafted into a secondary recipient host, such as immunodeficient mice or rat, either subcutaneously or orthotopically [80, 81]. The orthotopically implanted tissue or cell models are called as patient-derived orthotopic xenograft (PDOX) models [82].

PDX exhibits biologically stable tumor architecture, drug responsiveness, mutational status, and global gene expression patterns [83]. It provides an advanced preclinical model as it closely represents the human condition as compared to the other xenograft models or in vitro models. It recapitulates the heterogeneity of the patient tumors [83]. It provides a tumor microenvironment that is similar to the human situation, which helps in mimicking growth and metastasis. PDX acts as representative of individual tumors and has the ability to sufficiently mimic the patient drug response [80]. Table 17.10 enlists the examples of nanosystems evaluated in patient-derived xenograft models.

Table 17.10 Nano drug delivery systems evaluated in patient-derived xenograft models

No.	Nano system	Animal model	Outcome	Reference
1.	N-hydroxy-N′-(4-butyl-2 methylphenyl) formamidine (HET0016) with 2-hydroxypropyl beta cyclodextrin (HPßCD)	Human glioma U251 cells (400 k in 5 µL) were implanted orthotopically at 3 mm to the right and 1 mm anterior to bregma in nude rats (RNU nu/nu) to develop animal model of human and syngeneic glioblastoma (GBM)	HPßCD-HET0016 was found to be effective in inhibiting tumor growth by decreasing proliferation and neovascularization. It also significantly prolonged the animal survival	[84]
2.	Theranostic nanoporphyrins, PLZ4-nanoporphyrin (PNP) PLZ4, bladder cancer-specific ligand	Three different PDX models with BL269, BL440, BL645, and BL293 were injected S.C. in the flank or into bladder wall NOD SCID gamma (NSG) mice For local diagnosis, PNPs were administered intravesically	PNP eliminated orthotopic PDX bladder cancer after intravesical treatment and photodynamic therapy was significantly more potent than 5-aminolevulinic acid	[85]
3.	Daunorubicin-containing disulfide cross-linked C-type 46 lectin-like molecule-1 (CLL1)-targeting nanomicelles (DC-CTM-DNR)	Acute myeloid leukemia (AML) patient-derived xenograft model was achieved by transplanting patient AML samples into immunodeficient NSG (NOD-Scid-IL2Rgc^{null}) mice	DC-CTM-DNR-treatment showed significantly reduced AML engraftment as compared to the groups treated with free DNR	[86]

(continued)

Table 17.10 (continued)

No.	Nano system	Animal model	Outcome	Reference
4.	Cabazitaxel-loaded poly(2-ethylbutyl cyanoacrylate) nanoparticles	Basal-like breast cancer patient-derived xenograft model was achieved by implanting 1–2 mm^3 pieces of healthy tumor tissue specimen (MAS98.12) bilaterally into the mammary fat pad of female athymic mice	Cabazitaxel NPs had a much better efficacy than the similar concentrations of free drug in basal-like patient-derived xenografts with complete remission of six of eight tumors	[87]
5.	IRAK1/4 inhibitor and ABT-737 coencapsulated into polyethylene glycol-modified poly(lactic-co-glycolic acid) nanoparticles (IRAK/ABT-NP)	Human cell line xenograft T-ALL mouse model was achieved by injecting human T-cell leukemia cell line (Jurkat cells) (3×10^6) intravenously into female NPG mice	IRAK/ABT-NP exhibited greater cytotoxicity toward T-ALL cells, also significantly restored white blood cell number in peripheral blood and improved survival time of T-ALL mice	[88]

3.3 Germline Transgenic and Conditional Transgenic Models/ Genetically Engineered Mouse Models

Genetically modified or engineered mouse models are one of the recent most commonly used approaches to model human cancer studies. These models could be conceptualized due to the rapid advances in mouse and human genomics and development of sophisticated tools for manipulation of the mouse genome and gene targeting [89].

GEMMs are generated by either activating the oncogenes or inactivating the tumor suppressor genes (or both) in vivo via transgenic and gene-targeting approaches, such as knockouts and knockins [2]. The knockout or conditional knockout alleles are employed to study the loss of functions, while the transgenic, conditional transgenic, and knockin approaches are utilized to understand the gain of functions. Genetically modified mice are developed by microinjecting DNA in the pronuclei of fertilized zygotes and integrating the transgene into the genome [2]. These cancer-prone mouse strains can help to understand the role of individual genes and their mutated counterparts during tumorigenesis, as well as the cooperation of individual mutations during tumor development [90].

GEMMs present the phenotypic, histological, biochemical, proteomic, and genetic features specific to primary cancers. Thus, they mimic cancer initiating and promoting mutations. Tumor development is often similar to that of human cancer and is spontaneous [91]. GEMMs developed in immunocompetent animals can help to understand the influence of immune system during tumor development, growth, and invasion. GEMMs play an important role in deciphering the effect of gene functions on the development of anticancer therapeutics [91].

4 In Vitro Models for Evaluation of Anti-infective Agents

The potency and efficacy of a chemotherapeutic agent directed against intracellular infectious disease is largely governed by two factors, namely, the penetration of the drug inside the cells and its ability to reach the infected subcellular compartments to produce the desired therapeutic effect [92]. In vitro PK/PD modeling and simulation serve as vital tools during the drug development process. The pharmacodynamic effect of an anti-infective agent can be evaluated by measuring the bacterial growth and death following its administration. This is relatively difficult to quantify in human and animals; therefore, in vitro systems play a crucial role in understanding the concentration–effect relationship of the anti-infective agents. These allow direct interaction between the drug and bacteria, which enables high-throughput screening of the candidate compounds and enables determination of effective drug concentrations, which can be tested using in vivo animal models. Similarly, in vitro cell-based systems can be used for the evaluation of pharmacokinetic parameters, such as intracellular accumulation and release, as well as subcellular localization of the test agents [93]. Although in vitro PK/PD models cannot incorporate all the aspects observed in in vivo physiological systems, they are valuable because they assist in arriving at critical information regarding the safety and efficacy of anti-infective molecules for further preclinical and clinical studies [94].

Determination of minimum inhibitory concentration (MIC) is the primary evaluation criteria for any drug or formulation, which aims at anti-infective effect. The test utilizes liquid growth medium containing increasing concentrations, usually two-fold dilution series, of the test agent. The above mentioned mixture is incubated with a specific number of bacterial cells. If the total test volume is $\geq$2 mL, then the method is termed as "macrodilution." When the test is performed in microtiter plates with volume $\leq$ 500 μL, it is referred as microdilution technique. After incubation, the optical density is measured which is directly proportional to the growth of organism. The MIC is defined as the lowest concentration of the test agent that prevents the visible growth of the microorganism. Thus, lower the MIC, more is the potency of the test agent. Microdilution technique allows rapid screening of the anti-infective potential of the test agents [95, 96].

Time kill assays determine the rate of anti-infective activity, employing varying concentrations of the test agent, over specific time period. The time points used in this assay depend on the pharmacokinetic literature available for the drug to be tested. This assay also provides information regarding the bacteriostatic or bactericidal activity of the test agent. The postantibiotic effect provides information about the duration of action of the test agent after it has been removed from the culture [97]. The clinical significance of these tests is that a test agent that produces a long postantibiotic effect may require larger dosing intervals and less dosing frequency clinically, thus reducing the adverse effects, as well as the cost-associated limitation of the anti-infective therapies [98]. Table 17.11 provides the drug delivery systems evaluated for anti-infective activity using MIC, time kill assay, and post-antibiotic effect.

Table 17.11 Drug delivery systems evaluated for anti-infective activity using MIC, time kill assay, and postantibiotic effect

No.	Formulation	Organism	Outcomes	Reference
1.	Liposomal antibiotic formulation composed of 1,2-distearoyl-sn-glycero-3-phosphocholine and cholesterol with either gentamicin, tobramycin, or amikacin	*Burkholderia cenocepacia*	MIC of liposomal antibiotics was significantly lower than those of free drugs; time kill assay showed that the liposomal antibiotic formulations reduced killing doses of tobramycin and gentamicin compared with free drugs	[99]
2.	Liposome-encapsulated gentamicin	*Pseudomonas aeruginosa*	The MICs for liposomal gentamicin were significantly lower (32 mg/L) than those of corresponding free gentamicin (512 mg/L). The time kill values for liposomal gentamicin were either equivalent to or better than those of the free antibiotic	[100]
3.	Cationic and anionic liposomal meropenem formulations	*P. aeruginosa*	The MICs of cationic liposomal meropenem formulations were 2–4 times lower than free meropenem	[101]
4.	Ticarcillin-loaded nanoliposomes having positive, negative, and neutral surface charges	*P. aeruginosa*	The MICs of ticarcillin cationic nanoliposomes (3 mg/L) and neutral nanoliposomes (6 mg/L) were lower than free drug (24 mg/L). Time kill studies showed that the killing rates of cationic nanoliposomes were higher as compared to other drug forms	[102]
5.	Azithromycin-loaded nanospheres	*Escherichia coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, *S. pneumoniae*	The MIC values of azithromycin loaded nanospheres were eight times lower than the free drug	[103]

4.1 In Vitro Cellular Uptake and Release of Targeted Drug Delivery Systems

The cellular uptake and release of targeted drug delivery systems can be determined using semi-quantitative or quantitative techniques. In both the techniques, cells of interest are cultured in T-25 or T-75 flask, in an appropriate medium, and in an

atmosphere comprising of 5% CO_2 and 95% air at 37 °C. After being cultured in T-25 or T-75 flask, the cells are transferred in a 96-well plate and allowed to grow for 48 h.

Measurement of cellular uptake by quantitative techniques is conducted by growing the cells and incubating them for a desired period with free drug, delivery system without the drug, drug delivery system using at least five different drug concentrations. After incubation, the medium is removed and cells are washed thrice with PBS to remove any traces of free drug, delivery system without the drug, drug delivery systems that have not been internalized by the cells. Cells are then lyzed using 0.5% Triton X-100 in 0.2 N NaOH. The amount of drug present in each well is determined by HPLC, LC-MS, GC, etc., depending on physiochemical properties of the drug.

Fluorescence microscopy and flow cytometry are also used to measure the cellular uptake of the drug. The choice of the technique depends on the intended use. Flow cytometry is relatively more expensive as compared to microscopic evaluation. Therefore, fluorescence microscopy is recommended for routine screening purposes, while flow cytometry may be used for more advanced applications. Both the techniques involve conjugating the free drug or the drug delivery system with a fluorescent probe.

5 Competitive Inhibition Assay to Determine Receptor-Mediated Uptake of the Targeted Drug Delivery System

This assay is the most commonly used method to determine specificity and receptor-mediated endocytosis of the targeted drug delivery system. The general procedure is to competitively inhibit the receptor-mediated uptake of the targeted drug delivery system by the addition of an excess of free endogenous ligand for the receptor. The uptake of targeted drug delivery system will decrease proportionally with an increase in the concentration of the free endogenous ligand, establishing the specificity of targeted drug delivery system toward the receptor. Table 17.12 describes list of receptors along with their free endogenous ligands.

Table 17.12 Receptors and their free endogenous ligands

Receptor	Free endogenous ligand	Reference
Folate receptor	Folic acid	[104]
Asialoglycoprotein receptor	Lactose (1 μg/ml), galactose	[105, 106]
Mannose receptor	Mannose (10, 20, and 50 mM)	[107]
Transferrin receptor	Transferrin	[108]

6 Receptor Uptake Mechanism by Inhibitors for Various Uptake Pathways

Endocytosis is a fundamental feature of living cells which involves formation and inward cytosolic movement of plasma membrane vesicles to transport extracellular material and plasma membrane inside the cell [109, 110].

Endocytosis is generally classified into phagocytosis and pinocytosis. Pinocytosis is further classified into macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. Considering from the therapeutic point of view, endocytosis provides a means to actively transport compounds which are usually unable to be uptake by passive diffusion. It serves as an ideal mechanism for delivery of nanomedicines and is exploited for improving selectivity [111]. Careful adjustment of the physicochemical properties of NPs to optimize cellular targeting, uptake, and trafficking is an important task.

Evaluation of nanoparticles for biomedical application includes quantitative and qualitative studies on the cellular uptake of nanoparticles with respect to their size and shape. This is important to assess uptake kinetics, toxicity, and for designing multifunctional NPs [112]. Identifying uptake pathways also constitutes an important study. This can be evaluated by means of selective inhibition by pharmacological inhibitors, molecular probes, and organelle-specific dyes. Quantitative assessment of cellular uptake is generally carried out by labeling of NPs with fluorescent dyes or radioisotopes [113, 114].

6.1 Pharmacological Inhibitors

Pharmacological inhibitors for specific endocytic pathways are used to block that receptor/pathway in order to confirm its involvement in uptake of transporting carriers. They provide a simple, reliable, time, labor efficient, and affordable tool to analyze endocytosis both in vitro and in vivo. During analysis, inhibitors equally affect all cells in a population and can be easily titrated and quantified. As the cells are usually exposed to inhibitors over a short period of time it does not lead to development of delayed side effects or compensatory mechanisms. This makes pharmacological inhibitors the method of choice for in vivo studies as it provides a means for direct probing of endocytosis in living cells [109, 114]. The pharmacological inhibitors can be used along with molecular probes to confirm the endocytic mechanisms and intracellular fate of the nanoparticles and achieve a more convincing result. However, use of pharmacological inhibitors suffers from certain drawbacks with regard to their poor specificity. All endocytic pathways being energy-dependent processes can be inhibited by low temperature and ATPase inhibitors (like sodium azide) at the same time [109, 114]. Table 17.13 enlists commonly utilized inhibitors to study endocytosis pathways.

Table 17.13 Commonly used endocytic inhibitors for endocytosis pathways

No.	Endocytosis pathways	Inhibitors
1.	Clathrin-mediated endocytosis	(i) Hypertonic sucrose (0.4–0.5 M) (ii) Potassium depletion (iii) Cytosolic acidification with 10–30 mM NH_4Cl/acetic or succinic acids (iv) Chlorpromazine (50–100 μM) (v) Monodansylcadaverine (MDC) (vi) Phenylarsine oxide (1–20 μM)
2.	Lipid raft/caveolae-mediated endocytosis	(i) Statins 10–100 μM of lovastatin, simvastatin, and pravastatin (ii) Methyl-β-cyclodextrin (MβCD) 5–10 mM (iii) Filipin (∼1 μM) (iv) Nystatin (20–50 μM) (v) Cholesterol oxidase
3.	Macropinocytosis and phagocytosis	(i) Sodium–proton exchange inhibitors: amiloride and its derivatives 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and dimethyl amiloride (DMA) (ii) F-Actin-depolymerizing drugs: cytochalasin D and latrunculins (iii) Phosphoinositide metabolism inhibitors: wortmannin (100–200 nM)

6.2 Molecular Probes

Molecular probes are specific markers which label the NPs to assess their intracellular fate. The labeled NPs, when inside the intracellular compartments or organelles, can be evaluated by confocal imaging [114, 115]. The markers which are known to be internalized through specific endocytic pathway can be utilized in studying endocytic uptake.

Colocalization can be detected by certain organelle-specific dyes. LysoTracker is a widely used dye utilized for evaluating colocalization of labeled NPs with lysosomes by confocal microscopy [116].

6.3 Genetic Approaches

Genetic approaches dealing with altering the expression of specific proteins involved in a specific endocytosis pathway have been employed recently to avoid the non-specificity of pharmacological inhibitors. These include the use of knockout cell lines to exclude or verify specific endocytic pathways for nanoparticles [110, 114]. Table 17.14 enlists some examples of nanosystems evaluated using pharmacological inhibitors.

Table 17.14 Nanosystems evaluated using pharmacological inhibition of endocytosis pathways

No.	Nanosystems	Model	Outcome	Reference
1.	Gold nanoparticles (NPs), plain NPs compared with NPs coated with polyethylene glycol (PEG)	NPs exposed to human alveolar epithelial cells (A549). Endocytosis was studied by caveolin- and clathrin-mediated pathways using MβCD	A significant reduction of NPs is observed intracellularly indicating predominant uptake by endocytosis	[117]
2.	Epirubicin (EPI)-loaded folic acid-conjugated pullulan acetate (FPA/EPI) nanoparticles	Dose- and time-dependent cellular uptake effects of FPA/EPI nanoparticles were determined on rat Kupffer cells (KC), preincubated with the CPZ, NY, Col, AMR, and PDTC	PDTC + NY exhibited strongest inhibitory effect, indicating clathrin- and caveolae-mediated endocytosis as the main route of entry in KC	[118]
3.	Carboxyl- and amino-functionalized silica nanoparticles (SiNP)	5×10^4 Human breast adenocarcinoma cell line (MCF-7) and MCF-7-derived breast cancer stem cells (BCSCs) were pretreated with genistein; dynasore, cyto D, polyinosinic acid potassium salt (Poly-I), nocodazole, NY, CPZ	SiNPs with the same functionalization can be uptaken via different endocytic mechanisms in MCF-7 and BCSCs	[119]
4.	Negatively and positively charged model nanoparticles	Colon carcinoma Caco-2 cell monolayers were incubated with dynasore, genistein, EIPA, nocodazole, CPZ, and MβCD	Negatively charged NPs and positively charged NPs were found to be uptaken by different pathways	[120]
5.	DNA–chitosan nanoparticles: (a) Self-branched and trisaccharide substituted chitosan oligomers (SBTCO) (b) Linear chitosan (LCO)	HeLa cells preincubated with the endocytic inhibitors (10 μg/ml chlorpromazine, 30 μg/mL dynasore, and 70 μg/mL genistein) for 30 min prior to addition of YOYO-1-labeled DNA–chitosan polyplexes	(a) SBTCO: Clathrin-independent endocytosis. (b) LCO: Clathrin-dependent and clathrin-independent pathways.	[121]

7 In Vivo Studies for Evaluating Anti-Infective Effect

7.1 Primary Rodent Infection Models

An animal infection model is imperative to address the complex relationship between the drug, host, and pathogen in question. The goal of animal model is to closely recapitulate the infectious disease seen in human, including the virulence and resistance, and to allow for robust PK/PD evaluation. The animal model should be successful in determining the optimal drug exposures that could ultimately lead to therapeutic success. A number of variables are needed to be taken into

consideration while designing the animal model. These can include host-specific, pathogen-specific, and therapeutic variables. The host-specific variables include animal species, route of infection, infection site, immune status, end-organ/tissue sampling, and optimal endpoint measure [7, 9, 122]. The pathogen-specific variables include the genus/species, inoculum size, virulence, and drug susceptibility. The therapeutic variables include route of drug administration, timing of therapy, dose level, frequency of administration, penetration to the site of infection, metabolism and/or elimination, and duration of therapy [7, 9, 122].

The ultimate aim of considering these variables while designing an animal model is an accurate PK/PD evaluation to allow optimization of the dosing regimen, limiting drug-related toxicity, guiding therapeutic drug monitoring, and setting of drug susceptibility breakpoints [122].

The primary infection animal models are routinely utilized for screening of potential molecules to have rough estimates of their efficacy and toxicity potentials, to optimize their route of administration, and their dosage regimen. Basic screening of antibacterial efficacy is conducted using the three models described in the following text. Evaluation in these models enables determination of the protective dose (PD_{50}), that is, the dose that prevents mortality or inhibits thigh swelling in 50% animals [9].

7.1.1 Mice Bacterial Peritonitis Model

The mice peritonitis model was first used in 1935 to prove the efficacy of prontosil and derivative sulfonamides against *Streptococcus pyogenes*. Since then, it is an important in vivo model to evaluate the potential of novel molecules as antibiotics and to correlate the in vitro activity with in vivo efficacy of the test compound [9, 123]. This is the most commonly used acute systemic infection model. It is an important model for early demonstration of anti-infective potential and corelating the in vitro potency with the in vivo efficacy.

Mice bacterial peritonitis model involves inoculation of predetermined concentration or number of infecting agents by intraperitoneal injection to the mice. The animals are checked for the signs of infection. The test compounds can be administered to the animals by different routes depending on their solubility and clinical use, that is, prophylactic or preventive therapy. Endpoints include mortality (% survival), bacterial load (CFU) in the blood and different tissues, and protective dose (PD_{50}) [9]. Table 17.15 gives a list of nanosystems evaluated using mice bacterial peritonitis model.

7.1.2 Bacterial Respiratory Tract Infection Model/Lung Infection

The mouse respiratory tract infection model is utilized to evaluate the efficacy of antimicrobials against respiratory tract infections. The first step in pneumonia results from the defeat of the innate immune defense system by the infection as innate immunity is essential in host defense against virulent pathogens [129]. The respiratory tract infection (RTI) model considers bacterial virulence, the local host defense system, the course of disease, the rate at which bacteria are cleared from the

Table 17.15 Nanosystems evaluated by mice bacterial peritonitis model

No.	Nano system	Animal model	Outcome	Reference
1.	Exosome-encapsulated linezolid	Kun Ming mice infected with 5×10^7 CFU of MRSA WHO-2 by intraperitoneal (i.p.) injection	The exosome linezolid was found to be more effective than the free linezolid	[124]
2.	Pluronic-based nano-self-assemblies of bacitracin A with Pluronic® P85 (Nano-BA_{P85})	Kun Ming mice infected with 10^9 CFU/ml of *Staphylococcus aureus* and *Escherichia coli*. Nano-BA_{P85} is compared with (Nano-BA_{PLGA})	Nano-BA_{P85} was found to significantly suppress the bacterial growth and prolong survival time as compared to Nano-BA_{PLGA} with negligible toxicity	[125]
3.	Folic acid receptor-targeted poloxamer 407 nanocrystals containing ritonavir-boosted atazanavir (FA-P407-ATV/r)	Folate-deficient NSG mice infected with HIV-1_{ADA} after pretreatment with P407-ATV/r or FA-P407-ATV/r	FA-P407-ATV/r showed significantly enhanced antiretroviral responses as compared to the native drug or nontargeted formulations	[126]
4.	Nanoformulated myristoylated cabotegravir prodrug (NMCAB)	HIV-1_{ADA} challenged NSG mice after pretreatment with NMCAB or CAB LAP	NMCAB treatment showed significantly lower plasma and tissue viral loads as compared to CAB LAP treatment	[127]
5.	Gentamicin-loaded poly(lactide-co-glycolide) (PLGA) nanoparticles	CD-1 mice infected with 10^7 CFU/ml of *Pseudomonas aeruginosa* i.p.	Gentamicin nanoparticles exhibited significantly improved antimicrobial effects due to lower plasma and peritoneal lavage colony-forming units	[128]

lungs and/or disseminate to the bloodstream, and the penetration and disposition of the drug into the lung tissue [8, 9].

Several methods have been employed to induce lung infections which include intranasal inhalation [9], aspiration via the oropharyngeal route [130], aerosol exposure [122], and intratracheal inoculation [9]. Parameters studied as endpoints include bacterial burden in lungs, cytokine levels, protective dose (PD_{50}). Table 17.16 provides the list of nanosystems evaluated by bacterial respiratory tract infection model/lung infection model.

7.1.3 Thigh Lesion/Thigh Burden Model

The thigh lesion model is the most commonly used to determine the pharmacokinetic/pharmacodynamic (PK/PD) relationships of antibiotics [136]. It is simple to establish and is a reproducible model. The measurement of drug levels in both serum and tissues can be performed with this model [9, 137].

Table 17.16 Nanosystems evaluated via bacterial respiratory tract infection model/lung infection model

No.	Nano system	Animal model	Outcome	Reference
1.	Immunogene therapy fusogenic nanoparticles selectively targeting macrophages (*Irf5* gene) (F-siIRF5-CRV)	Intratracheal injection of ~1 × 10^7 CFU of *Staphylococcus aureus* in Balb/C mice	The CRV-targeted NP constructs localized significantly in infected and nonhealthy lungs as compared to sham NPs	[131]
2.	Cyclic 9-amino acid peptide CARGGLKSC (CARG)-conjugated vancomycin-loaded NPs (CARG-pSiNP-vancomycin)	Intratracheal inoculation of *S. aureus* (5 × 10^7 CFU) bacteria	CARG-pSiNP-vancomycin enhanced the efficacy by effectively suppressing the staphylococcal infections	[132]
3.	Peptide-loaded phosphonate pSiNPs (peptide-pSiNP)	Intratracheal inoculation of 2 × 10^5 CFU of *Pseudomonas aeruginosa* in neutropenic CD-1 mice	Peptide-pSiNP treatment brought about a large reduction in bacterial titers and markedly improved survival as compared to the untreated mice	[133]
4.	Shell cross-linked needle-like polymeric nanoparticles (SCK NPs)	Intranasal inoculation of (~1.2 × 10^6 CFU per mouse) *P. aeruginosa*	SCK NP SCC10-loaded core formulations exhibited excellent antimicrobial activity and efficacy	[134]
5.	Diphyllin-loaded polymeric nanoparticles comprised poly(ethylene glycol)-block-poly(lactide-coglycolide) (PEG-PLGA)	Intranasal inoculation with a sublethal dose of influenza H1N1 virus in BALB/c mice and further challenged with a lethal dose of influenza H1N1	Diphyllin nanoparticles curtailed the bodyweight loss and viral titer in the lungs. It improved the survival in animals after lethal influenza viral challenge	[135]

The infection is induced by an intramuscular injection of inoculum in the thigh muscle of a mouse. The test agent is administered to the animals. The virulence of the injected organism and the effect of the test agent are evaluated hereafter. If the infection is cleared within 24 h, the mice have to be rendered neutropenic to attain a robust infection [9].

Neutropenic mice are usually employed when the infection is observed to be cleared within 24 h of establishment. The mouse can be rendered neutropenic by treatment with cyclophosphamide on days −4 and −1, producing neutropenia by day 0 [136]. The model can be worked up with the help of immunocompetent mice to study the effect of leukocytes [122]. The infection develops in 2–4 days and is rarely fatal. It may spontaneously resolve after 6 days. An important consideration is the time difference between inoculation and the commencement of therapy [136]. A number of different infective agents can be evaluated with this model. Parameters to be studied as endpoints include, lesion measurement, bacterial burden [136], PK/PD indices (T > MIC, AUC/MIC, or C_{max}/MIC) [136]. Table 17.17 enlists the nanosystems evaluated by thigh lesion/thigh burden model.

Table 17.17 Nano drug delivery systems evaluated using thigh lesion/thigh burden model

No.	Nano system	Animal model	Outcome	Reference
1.	Ceftriaxone–loaded chitosan nanoparticles (Cef-CNPs)	Intramuscular injection of 0.1 ml (10^7 to 10^8 CFU/ml) MRSA and *Escherichia coli* inoculum in Swiss albino mice	Only Cef-CNPs treatment showed a significant decrease in microbial burden enabling a reduction in dose- and cost-effective treatment	[138]
2.	Accurin® colistin nanoparticles	Thigh infection model developed by intramuscular injection of *Klebsiella pneumoniae* 9 in CD-1 mice	Accurin® resulted in significant decrease in bacterial burden as compared to free colistin sulfate	[139]
3.	VRT001-C: stealth-targeted nanoparticles (STN) of ceftriaxone	Intramuscular injection of 1.6 × 106 CFU/thigh with *E. coli* in both thighs of neutropenic BALB/c mice	VRT001-C exhibited a potent efficacy against *E. coli* in the thigh infection model with ≈2-log10 reduction in bacterial burden	[140]
4.	PEGylated self-assembled nanobacitracin A	Intramuscular injection of 100 μL of 109 colony-forming units (CFU)/mL mixture of *E. coli* and *Staphylococcus aureus* into the thigh muscle of each hind leg of KM mice	PEGylated nano-BA exhibited strong antibacterial efficiency against both, gram-positiveGram-positive and gram-negative bacteria	[141]

Note: T > MIC: Residence time of the drug delivery system having cumulative percentage higher than the MIC at steady-state pharmacokinetic conditions of 24-h period. This indicates good anti-infective control by the drug delivery system

8 Conclusion

A relevant screening model for any novel drug delivery system forms the basis of preclinical development studies. An in vitro and in vivo model should be selected based on the targets and ability of the model to recapitulate the target features. It is also important to consider the degree of drawbacks associated with the particular model. Another significant aspect to be considered is the translational value of the preclinical outcomes into clinical efficacy. Hence, a standardized and validated preclinical model both in vitro and in vivo serves as a key attribute toward development of an anticancer and anti-infective agent and drug delivery system.

References

1. Dhandapani M, Goldman A. Preclinical cancer models and biomarkers for drug development: new technologies and emerging tools. J Mol Biomark Diagn. 2017;8(5):356.
2. Cheon DJ, Orsulic S. Mouse models of cancer. Annu Rev Pathol. 2011;6:95–119.

3. Katt ME, Placone AL, Wong AD, Xu ZS, Searson PC. In vitro tumor models: advantages, disadvantages, variables, and selecting the right platform. Front Bioeng Biotechnol. 2016;4:12.
4. Ruggeri BA, Camp F, Miknyoczki S. Animal models of disease: pre-clinical animal models of cancer and their applications and utility in drug discovery. Biochem Pharmacol. 2014;87(1):150–61.
5. Shevach EM. Animal models for infectious diseases. Curr Protoc Immunol. 2011;93:19.0.1–4.
6. Beam TR Jr, Gilbert DN, Kunin CM. General guidelines for the clinical evaluation of anti-infective drug products. Infectious Diseases Society of America and the Food and Drug Administration. Clin Infect Dis. 1992;15 Suppl 1:S5–32.
7. Knudsen JD, Frimodt-Møller N. Animal models in bacteriology. In: Schmidt A, Weber OF, editors. Animal testing in infectiology, Contributions to microbiology, vol. 9. Basel: Karger; 2001. p. 1–14.
8. Zak O, O'Reilly T. Animal models in the evaluation of antimicrobial agents. Antimicrob Agents Chemother. 1991;35:1527–31.
9. Marra A, Girard D. Primary rodent infection models for testing antibacterial compound efficacy in vivo. Curr Protoc Pharmacol. 2005;31:13A.4.1–13A.4.13.
10. van Meerloo J, Kaspers G, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237–45.
11. Tolosa L, Donato MT, Gomez-Lechon MJ. General cytotoxicity assessment by means of the MTT assay. Methods Mol Biol. 2015;1250:333–48.
12. Jones SK, Lizzio V, Merkel OM. Folate receptor targeted delivery of siRNA and paclitaxel to ovarian Cancer cells via Folate conjugated Triblock copolymer to overcome TLR4 driven chemotherapy resistance. Biomacromolecules. 2016;17(1):76–87.
13. Muller C, Schubiger PA, Schibli R. In vitro and in vivo targeting of different folate receptor-positive cancer cell lines with a novel 99mTc-radiofolate tracer. Eur J Nucl Med Mol Imaging. 2006;33(10):1162–70.
14. Godeshala S, Nitiyanandan R, Thompson B, Goklany S, Nielsen DR, Rege K. Folate receptor-targeted aminoglycoside-derived polymers for transgene expression in cancer cells. Bioeng Transl Med. 2016;1(2):220–31.
15. Chen G, Li D, Li J, Cao X, Wang J, Shi X, et al. Targeted doxorubicin delivery to hepatocarcinoma cells by lactobionic acid-modified laponite nanodisks. New J Chem. 2015;39(4):2847–55.
16. Oh HR, Jo HY, Park JS, Kim DE, Cho JY, Kim PH, et al. Galactosylated liposomes for targeted co-delivery of doxorubicin/vimentin siRNA to hepatocellular carcinoma. Nanomaterials (Basel). 2016;6(8):141.
17. Placzek M, Watrobska-Swietlikowska D, Stefanowicz-Hajduk J, Drechsler M, Ochocka JR, Sznitowska M. Comparison of the in vitro cytotoxicity among phospholipid-based parenteral drug delivery systems: emulsions, liposomes and aqueous lecithin dispersions (WLDs). Eur J Pharm Sci. 2019;127:92–101.
18. Kang MG, Lee MY, Cha JM, Lee JK, Lee SC, Kim J, et al. Nanogels derived from fish gelatin: application to drug delivery system. Mar Drugs. 2019;17(4):246.
19. Catalano E. Targeted tumor drug delivery and magnetic hyperthermia for cancer treatment by chemotherapeutic-conjugated magnetic nanoparticles. 2018.
20. Zare-Zardini H, Taheri-Kafrani A, Amiri A, Bordbar AK. New generation of drug delivery systems based on ginsenoside Rh2-, lysine- and arginine-treated highly porous graphene for improving anticancer activity. Sci Rep. 2018;8(1):586.
21. Wang L, Qu M, Huang S, Fu Y, Yang L, He S, et al. A novel alpha-enolase-targeted drug delivery system for high efficacy prostate cancer therapy. Nanoscale. 2018;10(28):13673–83.
22. Liu D, Liu Z, Wang L, Zhang C, Zhang N. Nanostructured lipid carriers as novel carrier for parenteral delivery of docetaxel. Colloids Surf B Biointerfaces. 2011;85(2):262–9.
23. Romar GA, Kupper TS, Divito SJ. Research techniques made simple: techniques to assess cell proliferation. J Invest Dermatol. 2016;136(1):e1–7.
24. Nowakowski RS, Lewin SB, Miller MW. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989;18(3):311–8.

25. Yang Y, Geldmacher DS, Herrup K. DNA replication precedes neuronal cell death in Alzheimer's disease. J Neurosci. 2001;21(8):2661–8.
26. He W, Jiang X, Zhang ZR. Preparation and evaluation of poly-butylcyanoacrylate nanoparticles for oral delivery of thymopentin. J Pharm Sci. 2008;97(6):2250–9.
27. Patra CR, Bhattacharya R, Mukherjee P. Fabrication and functional characterization of goldnanoconjugates for potential application in ovarian cancer. J Mater Chem. 2010;20(3):547–54.
28. Ahmad I, Allen TM. Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. Cancer Res. 1992;52(17):4817–20.
29. Wongwanakul R, Jianmongkol S, Gonil P, Sajomsang W, Maniratanachote R, Aueviriyavit S. Biocompatibility study of quaternized chitosan on the proliferation and differentiation of Caco-2 cells as an in vitro model of the intestinal barrier. J Bioact Compat Polym. 2016;32(1):92–107.
30. Coimbra M, Banciu M, Fens MH, de Smet L, Cabaj M, Metselaar JM, et al. Liposomal pravastatin inhibits tumor growth by targeting cancer-related inflammation. J Control Release. 2010;148(3):303–10.
31. Kamba SA, Ismail M, Hussein-Al-Ali SH, Ibrahim TA, Zakaria ZA. In vitro delivery and controlled release of doxorubicin for targeting osteosarcoma bone cancer. Molecules. 2013;18(9):10580–98.
32. Rajabi M, Mousa SA. The role of angiogenesis in cancer treatment. Biomedicines. 2017;5(2):34.
33. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. Vasc Health Risk Manag. 2006;2(3):213–9.
34. Lokman NA, Elder AS, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. Int J Mol Sci. 2012;13(8):9959–70.
35. Valdes TI, Kreutzer D, Moussy F. The chick chorioallantoic membrane as a novel in vivo model for the testing of biomaterials. J Biomed Mater Res. 2002;62(2):273–82.
36. Giannopoulou E, Katsoris P, Hatziapostolou M, Kardamakis D, Kotsaki E, Polytarchou C, et al. X-rays modulate extracellular matrix in vivo. Int J Cancer. 2001;94(5):690–8.
37. Hlushchuk R, Bronnimann D, Correa Shokiche C, Schaad L, Triet R, Jazwinska A, et al. Zebrafish caudal fin angiogenesis assay-advanced quantitative assessment including 3-way correlative microscopy. PLoS One. 2016;11(3):e0149281.
38. Cao Z, Zheng L, Zhao J, Zhuang Q, Hong Z, Lin W. Anti-angiogenic effect of *Livistona chinensis* seed extract in vitro and in vivo. Oncol Lett. 2017;14(6):7565–70.
39. Burt HM, Jackson JK, Bains SK, Liggins RT, Oktaba AM, Arsenault AL, et al. Controlled delivery of taxol from microspheres composed of a blend of ethylene-vinyl acetate copolymer and poly(D,L-lactic acid). Cancer Lett. 1995;88(1):73–9.
40. Pastorino F, Brignole C, Di Paolo D, Nico B, Pezzolo A, Marimpietri D, et al. Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy. Cancer Res. 2006;66(20):10073–82.
41. Miller K, Erez R, Segal E, Shabat D, Satchi-Fainaro R. Targeting bone metastases with a bispecific anticancer and antiangiogenic polymer-alendronate-taxane conjugate. Angew Chem Int Ed Engl. 2009;48(16):2949–54.
42. Greish K, Ray A, Bauer H, Larson N, Malugin A, Pike D, et al. Anticancer and antiangiogenic activity of HPMA copolymer-aminohexylgeldanamycin-RGDfK conjugates for prostate cancer therapy. J Control Release. 2011;151(3):263–70.
43. Xue J, Zhao Z, Zhang L, Xue L, Shen S, Wen Y, et al. Neutrophil-mediated anticancer drug delivery for suppression of postoperative malignant glioma recurrence. Nat Nanotechnol. 2017;12(7):692–700.
44. Nitiss JL, Soans E, Rogojina A, Seth A, Mishina M. Topoisomerase assays. Curr Protoc Pharmacol. 2012;Chapter 3:Unit 3.
45. Barret JM, Kruczynski A, Vispe S, Annereau JP, Brel V, Guminski Y, et al. F14512, a potent antitumor agent targeting topoisomerase II vectored into cancer cells via the polyamine transport system. Cancer Res. 2008;68(23):9845–53.

46. Ye BL, Zheng R, Ruan XJ, Zheng ZH, Cai HJ. Chitosan-coated doxorubicin nano-particles drug delivery system inhibits cell growth of liver cancer via p53/PRC1 pathway. Biochem Biophys Res Commun. 2018;495(1):414–20.
47. Parveen S, Sahoo SK. Long circulating chitosan/PEG blended PLGA nanoparticle for tumor drug delivery. Eur J Pharmacol. 2011;670(2–3):372–83.
48. Jiang K, Chi T, Li T, Zheng G, Fan L, Liu Y, et al. A smart pH-responsive nano-carrier as a drug delivery system for the targeted delivery of ursolic acid: suppresses cancer growth and metastasis by modulating P53/MMP-9/PTEN/CD44 mediated multiple signaling pathways. Nanoscale. 2017;9(27):9428–39.
49. Zheng S, Chang S, Lu J, Chen Z, Xie L, Nie Y, et al. Characterization of 9-nitrocamptothecin liposomes: anticancer properties and mechanisms on hepatocellular carcinoma in vitro and in vivo. PLoS One. 2011;6(6):e21064.
50. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. J Vis Exp. 2011; Apr 24 (50):pii: 2597.
51. Rehm M, Parsons MJ, Bouchier-Hayes L. Measuring caspase activity by Forster resonance energy transfer. Cold Spring Harb Protoc. 2015;2015(1):pdb prot082560.
52. Khdair A, Handa H, Mao G, Panyam J. Nanoparticle-mediated combination chemotherapy and photodynamic therapy overcomes tumor drug resistance in vitro. Eur J Pharm Biopharm. 2009;71(2):214–22.
53. Shan L, Cui S, Du C, Wan S, Qian Z, Achilefu S, et al. A paclitaxel-conjugated adenovirus vector for targeted drug delivery for tumor therapy. Biomaterials. 2012;33(1):146–62.
54. Foldbjerg R, Olesen P, Hougaard M, Dang DA, Hoffmann HJ, Autrup H. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol Lett. 2009;190(2):156–62.
55. Jia Y, Yuan M, Yuan H, Huang X, Sui X, Cui X, et al. Co-encapsulation of magnetic Fe3O4 nanoparticles and doxorubicin into biodegradable PLGA nanocarriers for intratumoral drug delivery. Int J Nanomedicine. 2012;7:1697–708.
56. Decker S, Sausville E. Perspectives on the history and evolution of tumor models. In: Teicher B, editor. Tumor models in cancer research. Cancer drug discovery and development. Totowa: Humana Press; 2011.
57. Porru M, Leonetti C. The role of mouse models in translational cancer research: present and future directions. Transl Med Rep. 2017;1:6598.
58. Khan AQ, Siveen KS, Prabhu KS, Kuttikrishnan S, Akhtar S, Shanmugakonar M, Al-Naemi HA, Haris M, Uddin S. Role of animal research in human malignancies. In: Azmi A, Mohammad RM, editors. Animal models in cancer drug discovery. London, UK/Cambridge, MA: Academic Press; 2019. p. 1–29.
59. Jung J. Human tumor xenograft models for preclinical assessment of anticancer drug development. Toxicol Res. 2014;30(1):1–5.
60. Kim IY, Kang YS, Lee DS, Park HJ, Choi EK, Oh YK, Son HJ, Kim JS. Antitumor activity of EGFR targeted pH-sensitive immunoliposomes encapsulating gemcitabine in A549 xenograft nude mice. J Control Release. 2009;140(1):55–60.
61. Kinoshita R, Ishima Y, Chuang VTG, Nakamura H, Fang J, Watanabe H, Shimizu T, Okuhira K, Ishida T, Maeda H, Otagiri M, Maruyama T. Improved anticancer effects of albumin-bound paclitaxel nanoparticle via augmentation of EPR effect and albumin-protein interactions using S-nitrosated human serum albumin dimer. Biomaterials. 2017;140:162–9.
62. Benny O, Fainaru O, Adini A, Cassiola F, Bazinet L, Adini I, Pravda E, Nahmias Y, Koirala S, Corfas G, D'Amato RJ, Folkman J. An orally delivered small-molecule formulation with antiangiogenic and anticancer activity. Nat Biotechnol. 2008;26(7):799–807.
63. Yang G, Yang T, Zhang W, Lu M, Ma X, Xiang G. In vitro and in vivo antitumor effects of folate-targeted ursolic acid stealth liposome. J Agric Food Chem. 2014;62(10): 2207–15.
64. Qiu W, Su GH. Development of orthotopic pancreatic tumor mouse models. Methods Mol Biol. 2013;980:215–23.

65. Zaman MS, Chauhan N, Yallapu MM, Gara RK, Maher DM, Kumari S, Sikander M, Khan S, Zafar N, Jaggi M, Chauhan SC. Curcumin nanoformulation for cervical cancer treatment. Sci Rep. 2016;6:20051.
66. Murakami M, Ernsting MJ, Undzys E, Holwell N, Foltz WD, Li SD. Docetaxel conjugate nanoparticles that target α-smooth muscle actin-expressing stromal cells suppress breast cancer metastasis. Cancer Res. 2013;73(15):4862–71.
67. Rocks N, Bekaert S, Coia I, Paulissen G, Gueders M, Evrard B, Van Heugen JC, Chiap P, Foidart JM, Noel A, Cataldo D. Curcumin-cyclodextrin complexes potentiate gemcitabine effects in an orthotopic mouse model of lung cancer. Br J Cancer. 2012;107(7):1083–92.
68. Qiu M, Sun H, Meng F, Cheng R, Zhang J, Deng C, Zhong Z. Lipopepsomes: a novel and robust family of nano-vesicles capable of highly efficient encapsulation and tumor-targeted delivery of doxorubicin hydrochloride in vivo. J Control Release. 2018;28(272):107–13.
69. Paweletz CP, Charboneau L, Liotta LA. Overview of metastasis assays. Curr Protoc Cell Biol. 2001;12:19.1.1–9.
70. Pearson HB, Pouliot N. Modeling metastasis in vivo. In: Madame curie bioscience database [internet]. Austin: Landes Bioscience; 2000–2013. https://www.ncbi.nlm.nih.gov/books/NBK100378/.
71. Gómez-Cuadrado L, Tracey N, Ma R, Qian B, Brunton VG. Mouse models of metastasis: progress and prospects. Dis Model Mech. 2017;10(9):1061–74.
72. Saxena M, Christofori G. Rebuilding cancer metastasis in the mouse. Mol Oncol. 2013;7(2):283–96.
73. Goddard ET, Fischer J, Schedin P. A portal vein injection model to study liver metastasis of breast cancer. J Vis Exp. 2016;118:e54903.
74. Thalheimer A, Otto C, Bueter M, Illert B, Gattenlohner S, Gasser M, Meyer D, Fein M, Germer CT, Waaga-Gasser AM. The intraportal injection model: a practical animal model for hepatic metastases and tumor cell dissemination in human colon cancer. BMC Cancer. 2009;9:29.
75. Zhang C, Lowery FJ, Yu D. Intracarotid cancer cell injection to produce mouse models of brain metastasis. J Vis Exp. 2017;120:e55085.
76. Ernsting MJ, Murakami M, Undzys E, Aman A, Press B, Li SD. A docetaxel-carboxymethylcellulose nanoparticle outperforms the approved taxane nanoformulation, Abraxane, in mouse tumor models with significant control of metastases. J Control Release. 2012;162(3):575–81.
77. Berlin Grace VM, Reji RM, Sundaram V. Enhanced expression of tumour suppressor RAR-β by DSPC nano-formulated lipo-ATRA in the lung of B16F10 cell-implanted C57BL6 mice and in A549 cells. Life Sci. 2017;184:10–7.
78. Sapra P, Moase EH, Ma J, Allen TM. Improved therapeutic responses in a xenograft model of human B lymphoma (Namalwa) for liposomal vincristine versus liposomal doxorubicin targeted via anti-CD19 IgG2a or Fab' fragments. Clin Cancer Res. 2004;10(3):1100–11.
79. Parhi P, Suklabaidya S, Kumar SS. Enhanced anti-metastatic and anti-tumorigenic efficacy of Berbamine loaded lipid nanoparticles in vivo. Sci Rep. 2017;7(1):5806.
80. Jung J, Seol HS, Chang S. The generation and application of patient-derived xenograft model for cancer research. Cancer Res Treat. 2018;50(1):1–10.
81. Adeegbe DO, Liu Y. Patient-derived xenografts in oncology. In: Aref A, Barbie D, editors. Ex vivo engineering of the tumor microenvironment, Cancer drug discovery and development. Cham: Humana Press; 2017.
82. Hoffman RM. Patient-derived orthotopic xenografts: better mimic of metastasis than subcutaneous xenografts. Nat Rev Cancer. 2015;15(8):451–2.
83. Ji X, Chen S, Guo Y, Li W, Qi X, Yang H, Xiao S, Fang G, Hu J, Wen C, Liu H, Han Z, Deng G, Yang Q, Yang X, Xu Y, Peng Z, Li F, Cai N, Li G, Huang R. Establishment and evaluation of four different types of patient-derived xenograft models. Cancer Cell Int. 2017;17:122.
84. Jain M, Gamage NH, Alsulami M, Shankar A, Achyut BR, Angara K, Rashid MH, Iskander A, Borin TF, Wenbo Z, Ara R, Ali MM, Lebedyeva I, Chwang WB, Guo A, Bagher-Ebadian H, Arbab AS. Intravenous formulation of HET0016 decreased human glioblastoma growth and implicated survival benefit in rat xenograft models. Sci Rep. 2017;7:41809.

85. Lin TY, Li Y, Liu Q, Chen JL, Zhang H, Lac D, Zhang H, Ferrara KW, Wachsmann-Hogiu S, Li T, Airhart S, deVere White R, Lam KS, Pan CX. Novel theranostic nanoporphyrins for photodynamic diagnosis and trimodal therapy for bladder cancer. Biomaterials. 2016;104:339–51.
86. Lin TY, Zhu Y, Li Y, Zhang H, Ma AH, Long Q, Keck J, Lam KS, Pan CX, Jonas BA. Daunorubicin-containing CLL1-targeting nanomicelles have anti-leukemia stem cell activity in acute myeloid leukemia. Nanomedicine. 2019;20:102004.
87. Fusser M, Øverbye A, Pandya AD, Mørch Ý, Borgos SE, Kildal W, Snipstad S, Sulheim E, Fleten KG, Askautrud HA, Engebraaten O, Flatmark K, Iversen TG, Sandvig K, Skotland T, Mælandsmo GM. Cabazitaxel-loaded poly(2-ethylbutyl cyanoacrylate) nanoparticles improve treatment efficacy in a patient derived breast cancer xenograft. J Control Release. 2019;293:183–92.
88. Wu X, Wang L, Qiu Y, Zhang B, Hu Z, Jin R. Cooperation of IRAK1/4 inhibitor and ABT-737 in nanoparticles for synergistic therapy of T cell acute lymphoblastic leukemia. Int J Nanomedicine. 2017;12:8025–34.
89. HogenEsch H, Nikitin AY. Challenges in pre-clinical testing of anti-cancer drugs in cell culture and in animal models. J Control Release. 2012;164(2):183–6.
90. Becher OJ, Holland EC. Genetically engineered models have advantages over xenografts for preclinical studies. Cancer Res. 2006;66(7):3355–8, discussion 3358–9.
91. Lum DH, Matsen C, Welm AL, Welm BE. Overview of human primary tumorgraft models: comparisons with traditional oncology preclinical models and the clinical relevance and utility of primary tumorgrafts in basic and translational oncology research. Curr Protoc Pharmacol. 2012;Chapter 14:Unit 14.22.
92. Meibohm B, Derendorf H. Pharmacokinetic/pharmacodynamic studies in drug product development. J Pharm Sci. 2002;91(1):18–31.
93. Katsube T, Yano Y, Yamano Y, Munekage T, Kuroda N, Takano M. Pharmacokinetic-pharmacodynamic modeling and simulation for bactericidal effect in an in vitro dynamic model. J Pharm Sci. 2008;97(9):4108–17.
94. White RL. What in vitro models of infection can and cannot do. Pharmacotherapy. 2001;21(11 Pt 2):292S–301S.
95. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.
96. EUCAST. European Committee for Antimicrobial Susceptibility Testing (EUCAST). Clin Microbiol Infect. 2003;9(8):9–15.
97. Motyl M, Dorso K, Barrett J, Giacobbe R. Basic microbiological techniques used in antibacterial drug discovery. Curr Protoc Pharmacol. 2006;Chapter 13:Unit13A 3.
98. Chen H, Li L, Liu Y, Wu M, Xu S, Zhang G, et al. In vitro activity and post-antibiotic effects of linezolid in combination with fosfomycin against clinical isolates of *Staphylococcus aureus*. Infect Drug Resist. 2018;11:2107–15.
99. Halwani M, Mugabe C, Azghani AO, Lafrenie RM, Kumar A, Omri A. Bactericidal efficacy of liposomal aminoglycosides against *Burkholderia cenocepacia*. J Antimicrob Chemother. 2007;60(4):760–9.
100. Rukholm G, Mugabe C, Azghani AO, Omri A. Antibacterial activity of liposomal gentamicin against *Pseudomonas aeruginosa*: a time-kill study. Int J Antimicrob Agents. 2006;27(3):247–52.
101. Drulis-Kawa Z, Gubernator J, Dorotkiewicz-Jach A, Doroszkiewicz W, Kozubek A. In vitro antimicrobial activity of liposomal meropenem against *Pseudomonas aeruginosa* strains. Int J Pharm. 2006;315(1–2):59–66.
102. Gharib A, Faezizadeh Z, Godarzee M. In vitro and in vivo activities of ticarcillin-loaded nanoliposomes with different surface charges against *Pseudomonas aeruginosa* (ATCC 29248). Daru. 2012;20(1):41.
103. Azhdarzadeh M, Lotfipour F, Zakeri-Milani P, Mohammadi G, Valizadeh H. Anti-bacterial performance of azithromycin nanoparticles as colloidal drug delivery system against different gram-negative and gram-positive bacteria. Adv Pharm Bull. 2012;2(1):17–24.

104. Barwal I, Kumar R, Kateriya S, Dinda AK, Yadav SC. Targeted delivery system for cancer cells consist of multiple ligands conjugated genetically modified CCMV capsid on doxorubicin GNPs complex. Sci Rep. 2016;6:37096.
105. Diaz-Galvez KR, Teran-Saavedra NG, Burgara-Estrella AJ, Fernandez-Quiroz D, Silva-Campa E, Acosta-Elias M, et al. Specific capture of glycosylated graphene oxide by an asialoglycoprotein receptor: a strategic approach for liver-targeting. RSC Adv. 2019;9(18):9899–906.
106. Huang G, Diakur J, Xu Z, Wiebe LI. Asialoglycoprotein receptor-targeted superparamagnetic iron oxide nanoparticles. Int J Pharm. 2008;360(1–2):197–203.
107. Asthana GS, Asthana A, Kohli DV, Vyas SP. Mannosylated chitosan nanoparticles for delivery of antisense oligonucleotides for macrophage targeting. Biomed Res Int. 2014;2014:526391.
108. Choi CH, Alabi CA, Webster P, Davis ME. Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. Proc Natl Acad Sci U S A. 2010;107(3):1235–40.
109. Pharmacological IAI. Inhibition of endocytic pathways: is it specific enough to be useful? In: Ivanov AI, editor. Exocytosis and endocytosis, Methods in molecular biology, vol. 440. Totowa: Humana Press; 2008.
110. Dutta D, Donaldson JG. Search for inhibitors of endocytosis: intended specificity and unintended consequences. Cell Logist. 2012;2(4):203–8.
111. Akinc A, Battaglia G. Exploiting endocytosis for nanomedicines. Cold Spring Harb Perspect Biol. 2013;5(11):a016980.
112. Chithrani BD, Ghazani AA, Chan WC. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett. 2006;6(4):662–8.
113. Salatin S, Yari Khosroushahi A. Overviews on the cellular uptake mechanism of polysaccharide colloidal nanoparticles. J Cell Mol Med. 2017;21(9):1668–86.
114. Kou L, Sun J, Zhai Y, He Z. The endocytosis and intracellular fate of nanomedicines: implication for rational design. Asian J Pharm Sci. 2013;8(1):1–10.
115. Li Z, Zhang Y, Zhu D, Li S, Yu X, Zhao Y, Ouyang X, Xie Z, Li L. Transporting carriers for intracellular targeting delivery via non-endocytic uptake pathways. Drug Deliv. 2017;24(sup1):45–55.
116. Collins TP, Bayliss R, Churchill GC, Galione A, Terrar DA. NAADP influences excitation-contraction coupling by releasing calcium from lysosomes in atrial myocytes. Cell Calcium. 2011;50(5):449–58.
117. Brandenberger C, Mühlfeld C, Ali Z, Lenz AG, Schmid O, Parak WJ, Gehr P, Rothen-Rutishauser B. Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles. Small. 2010;6(15):1669–78.
118. Tang H, Chen H, Jia Y, Liu X, Han Z, Wang A, Liu Q, Li X, Feng X. Effect of inhibitors of endocytosis and NF-kB signal pathway on folate-conjugated nanoparticle endocytosis by rat Kupffer cells. Int J Nanomedicine. 2017;12:6937–47.
119. Sun J, Liu Y, Ge M, Zhou G, Sun W, Liu D, Liang XJ, Zhang J. A distinct endocytic mechanism of functionalized-silica nanoparticles in breast cancer stem cells. Sci Rep. 2017;7(1):16236.
120. Bannunah AM, Vllasaliu D, Lord J, Stolnik S. Mechanisms of nanoparticle internalization and transport across an intestinal epithelial cell model: effect of size and surface charge. Mol Pharm. 2014;11(12):4363–73.
121. Garaiova Z, Strand SP, Reitan NK, Lélu S, Størset SØ, Berg K, Malmo J, Folasire O, Bjørkøy A, Davies CL. Cellular uptake of DNA-chitosan nanoparticles: the role of clathrin- and caveolae-mediated pathways. Int J Biol Macromol. 2012;51(5):1043–51.
122. Zhao M, Lepak AJ, Andes DR. Animal models in the pharmacokinetic/pharmacodynamic evaluation of antimicrobial agents. Bioorg Med Chem. 2016;24(24):6390–400.
123. Frimodt-meuer N. The mouse peritonitis model : present and future use. J Antimicrob Chemother. 1993;31 Suppl D:55–60.
124. Yang X, Shi G, Guo J, Wang C, He Y. Exosome-encapsulated antibiotic against intracellular infections of methicillin-resistant *Staphylococcus aureus*. Int J Nanomedicine. 2018;13:8095–104.
125. Hong W, Liu L, Zhao Y, Liu Y, Zhang D, Liu M. Pluronic-based nano-self-assemblies of bacitracin a with a new mechanism of action for an efficient in vivo therapeutic effect against bacterial peritonitis. J Nanobiotechnology. 2018;16(1):66.

126. Puligujja P, Balkundi SS, Kendrick LM, Baldridge HM, Hilaire JR, Bade AN, Dash PK, Zhang G, Poluektova LY, Gorantla S, Liu XM, Ying T, Feng Y, Wang Y, Dimitrov DS, McMillan JM, Gendelman HE. Pharmacodynamics of long-acting folic acid-receptor targeted ritonavir-boosted atazanavir nanoformulations. Biomaterials. 2015;41:141–50.
127. Zhou T, Su H, Dash P, Lin Z, Dyavar Shetty BL, Kocher T, Szlachetka A, Lamberty B, Fox HS, Poluektova L, Gorantla S, McMillan J, Gautam N, Mosley RL, Alnouti Y, Edagwa B, Gendelman HE. Creation of a nanoformulated cabotegravir prodrug with improved antiretroviral profiles. Biomaterials. 2018;151:53–65.
128. Abdelghany SM, Quinn DJ, Ingram RJ, Gilmore BF, Donnelly RF, Taggart CC, Scott CJ. Gentamicin-loaded nanoparticles show improved antimicrobial effects towards *Pseudomonas aeruginosa* infection. Int J Nanomedicine. 2012;7:4053–63.
129. Jacqueline C, Roquilly A, Desessard C, Boutoille D, Broquet A, Le Mabecque V, Amador G, Potel G, Caillon J, Asehnoune K. Efficacy of ceftolozane in a murine model of *Pseudomonas aeruginosa* acute pneumonia: in vivo antimicrobial activity and impact on host inflammatory response. J Antimicrob Chemother. 2013;68:177–83.
130. Robbe A, Tassin A, Carpentier J, Declèves AE, Mekinda Ngono ZL, Nonclercq D, Legrand A. Intratracheal bleomycin aerosolization: the best route of administration for a scalable and homogeneous pulmonary fibrosis rat model? Biomed Res Int. 2015;2015:198418.
131. Kim B, Pang HB, Kang J, Park JH, Ruoslahti E, Sailor MJ. Immunogene therapy with fusogenic nanoparticles modulates macrophage response to *Staphylococcus aureus*. Nat Commun. 2018;9(1):1969.
132. Hussain S, Joo J, Kang J, Kim B, Braun GB, She ZG, Kim D, Mann AP, Mölder T, Teesalu T, Carnazza S, Guglielmino S, Sailor MJ, Ruoslahti E. Antibiotic-loaded nanoparticles targeted to the site of infection enhance antibacterial efficacy. Nat Biomed Eng. 2018;2(2):95–103.
133. Kwon EJ, Skalak M, Bertucci A, Braun G, Ricci F, Ruoslahti E, Sailor MJ, Bhatia SN. Porous silicon nanoparticle delivery of tandem peptide anti-infectives for the treatment of *pseudomonas aeruginosa* lung infections. Adv Mater. 2017;(35). https://doi.org/10.1002/adma.201701527.
134. Shah PN, Lin LY, Smolen JA, Tagaev JA, Gunsten SP, Han DS, Heo GS, Li Y, Zhang F, Zhang S, Wright BD, Panzner MJ, Youngs WJ, Brody SL, Wooley KL, Cannon CL. Synthesis, characterization, and in vivo efficacy of shell cross-linked nanoparticle formulations carrying silver antimicrobials as aerosolized therapeutics. ACS Nano. 2013;7(6):4977–87.
135. Hu CJ, Chen YT, Fang ZS, Chang WS, Chen HW. Antiviral efficacy of nanoparticulate vacuolar ATPase inhibitors against influenza virus infection. Int J Nanomedicine. 2018;13:8579–93.
136. Velkov T, Bergen PJ, Lora-Tamayo J, Landersdorfer CB, Li J. PK/PD models in antibacterial development. Curr Opin Microbiol. 2013;16(5):573–9.
137. Zak O, Sande MA. Handbook of animal models of infection. Experimental models in antimicrobial chemotherapy. London: Academic Press; 1999.
138. Mushtaq S, Khan JA, Rabbani F, Latif U, Arfan M, Yameen MA. Biocompatible biodegradable polymeric antibacterial nanoparticles for enhancing the effects of a third-generation cephalosporin against resistant bacteria. J Med Microbiol. 2017;66(3):318–27.
139. Zaytseva-Zotova D, Zinchenko A, Levada T, Andreev D, Shilov Y, Safarova E, Troiano G, Zale S. Accurin(R) nanoparticles improve the therapeutic index of colistin in preclinical models of infection. Paper presented at 26th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam, 9–12 Apr 2016.
140. Payasi A, Ganguly K, Roy D, Sachdeva A, Chaudhary M, Chaudhary S, Aggarwal A. L0029 Pharmacodynamic assessment of VRT001-C (Oral ceftriaxone) vs. intravenous ceftriaxone against *Escherichia coli* in the neutropenic murine thigh infection model. Paper presented at 29th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam, 13–16 Apr 2019.
141. Hong W, Zhao Y, Guo Y, Huang C, Qiu P, Zhu J, Chu C, Shi H, Liu M. PEGylated self-assembled Nano-bacitracin a: probing the antibacterial mechanism and real-time tracing of target delivery in vivo. ACS Appl Mater Interfaces. 2018;10(13):10688–705.

Part VI
Cellular Assays

Chapter 18
Protocols for Cellular Evaluation of Targeted Drug Delivery Systems for Cancer and Infectious Diseases

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Abstract The most critical stage in evaluation of a novel drug or its delivery system is assessment of its safety and efficacy. Traditionally, in vivo animal models were used for this assessment. However, due to growing ethical concerns in animal usage, these in vivo animal models have largely been replaced by cell-based assays. Cell-based assays offer several advantages which have been described in this section. This chapter describes in detail the protocols along with critical parameters for various cell-based assays which can be used for evaluation of targeted drug delivery systems for cancer and infectious diseases.

Keywords Cell-based assays · In vitro · Cancer · Infectious diseases

Abbreviations

3H-T	3H-labeled thymidine
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
BrdU	5-Bromo-2′-deoxyuridine
CAM	Chick embryo chorioallantoic membrane

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cfu	Colony-forming units
CLSI	The Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBM	Endothelial basal media
EDTA	Ethylenediaminetetraacetic acid
EGTA	(Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
FITC	Fluorescein isothiocyanate
FRET	Forster resonance energy transfer
HPLC	High performance liquid chromatography
HUVECs	Human umbilical vein endothelial cells
LC-MS	Liquid chromatography-mass spectrometry
MHB	Mueller–Hinton broth
MIC	Minimum inhibitory concentration
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
NDDS	Novel drug delivery system
PAE	Post antibiotic effect
PI	Propidium iodide
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1 Introduction

Drug discovery and development are vital for providing novel treatments for diseases. However, this is a challenging process, which involves significant time and cost, and is often met with failures. It is estimated that developing a marketable drug formulation can cost over $1 billion and may require about 12–15 years [1]. The first step in evaluation of any drug and/or formulation is the assessment of its safety and efficacy. Traditionally, safety and efficacy were evaluated in in vivo animal models. However, in 1950, W. M. S. Russell and R. L. Burch described the "Three Rs," namely, replacement, reduction, and refinement due to the growing ethical concerns in animal usage [2]. The first principle, "replacement" suggests that "use of animals should be replaced with alternative techniques if available"; since then, animal testing has largely been replaced with cell-based assays. Although cell-based assays do not completely resemble the 3-D physiological environment of an animal model of disease, they have several advantages. Cell-based assays are associated with fewer ethical issues. Moreover, these can be used for high-throughput screening, to rapidly evaluate libraries of newer drugs and delivery systems. They also provide substantive data on various cellular responses that occur upon exposure to a drug candidate [3]. Cell-based assays can be combined with advanced microscopy techniques, which allow monitoring of cellular events in both, spatial and temporal resolution [4]. Thus, cell-based assays offer a promising approach to screen and evaluate the safety and efficacy of newer drugs and formulations.

This chapter discusses protocols for in vitro cell-based assays for evaluating the drugs and delivery systems against two of the most important classes of diseases, namely, cancer and infectious diseases.

2 Cell Viability by MTT Assay [5]

Requirements

- MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide).
- Class 2B biosafety cabinet suitable for drug experiments.
- Incubator with 5% CO_2 at 37 °C.
- 96-well microplates. It is advised that for suspension cells, round-bottom wells or flat-bottom wells can be used, while for adherent cells, only flat bottoms must be used.
- Cell line.

Methods

Solutions and Solvents

- MTT solution: Dissolve 500 mg MTT in 10-mL PBS. Stir with a magnetic stirrer for approximately 1 hour in the dark. Filter sterilize the solution with a 0.22-mm filter and store in 1-mL aliquots at −20 °C. Warning: MTT is toxic and harmful. MTT is light sensitive, and hence protect from light.
- DMSO: Use DMSO to dissolve formazan crystals.

Procedure

- Seed the cells in 96-well plates at density of 1×10^4 cells/well and incubate for 24 hours to allow cell attachment.
- Change the medium on alternate days.
- After cells are adhered, incubate with the formulation at various concentrations for 48 hours and/or 96 hours. Each concentration should be tested at least in triplicates to ensure reproducible results.
- Maintain appropriate solvent and media controls. Solvent control should be maintained when an organic solvent such as ethanol or DMSO is used to solubilize the drug/formulation. Solvent control should contain media along with the solvent at the concentration used to solubilize the drug/formulation.
- At designated time interval, add 20-µl MTT solution and incubate in dark for 4 hours.
- Remove MTT solution from culture wells after 4 hours.
- Dissolve the formazan crystals in 100-µl DMSO.
- Measure the absorbance measured at 540 nm.
- Calculate cell viability using the following equation:

$$\text{Cell viability } (\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3 Methods to Determine Cell Proliferation [6]

3.1 3H-Labeled Thymidine (3H-T) Assay

Procedure

- Seed the cells in 96-well plates at density of 1×10^4 cells/well and incubate for 24 hours to allow cell attachment.
- Change the medium on alternate days.
- After cells are adhered, incubate with the formulation at various concentrations for 48 hours and 96 hours.
- Maintain appropriate solvent and media controls.
- Add 3H-labeled thymidine (9.25 KBq; 0.25 mci/well) to each well and incubate for another 24 hours.
- Collect the cells on a glass filter using a cell harvester.
- Measure the radioactivity using liquid scintillation counter.

$$\text{Stimulation index} = \frac{\text{3H-T incorporation in test wells}}{\text{3H-T incorporation in control wells}}$$

3.2 BrdU-Labeled Assay to Determine Cell Proliferation [7]

Procedure

- Grow cells in a six-well plate. The seeding density depends on the cell type and should be optimized for each cell line. Incubate the cells for 24 hours to facilitate cell attachment for adherent cells. Change the medium on every alternate day.
- Incubate cells with test formulation at various concentrations for 48 and 96 hours. Maintain appropriate solvent and media controls.
- *Preparation of 10 μM BrdU labeling solution*: Dissolve 3 mg of BrdU in 1 ml of sterile autoclaved distilled water to yield a 10-mM stock solution. Dilute the 10-mM stock solution in cell culture medium to obtain 10-μM BrdU labeling solution. Filter the 10-μM BrdU labeling solution through a 0.2-μM filter under sterile conditions, for example, under laminar airflow hood.
- After incubation with the test formulation at desired time intervals, remove culture medium and replace with 10-μM BrdU labeling solution. Incubate the cells in 10-μM BrdU labeling solution for 1–24 hours at 37 °C in a CO_2 incubator. The incubation time varies for each cell line used and depends on cell growth rate. Thus, it is important to determine the population doubling time for each cell line by performing cell growth curve experiment.
- After incubation with 10-μM BrdU labeling solution, remove the medium from the cells and wash the cells twice with phosphate-buffered saline to remove excess labeling solution.

- *Fixation of cells*: Aspirate phosphate-buffered saline and add 1 ml of trypsin EDTA to each well. Incubate at 37 °C for 5 minutes in a CO_2 incubator, to facilitate cellular detachment. Add 2 ml of growth medium containing fetal bovine serum to neutralize the trypsin. Transfer the cell suspension to a 15-ml falcon tube and centrifuge for 4 minutes at 350*g* to obtain a cell pellet. Determine cell count and adjust the final cell concentration to 2×10^6 cells/2 ml for solution in 60% ice-cold ethanol (vol/vol) in phosphate-buffered saline. First, add 0.8 ml of ice-cold phosphate-buffered saline slowly while vortexing the pellet. Following this, add 1.2-ml ice-cold 100% ethanol. Continue to vortex for 15 seconds. Leave overnight at 4 °C to allow fixation of cells.
- *DNA hydrolysis step*: This step is carried to denature DNA to allow the anti-BrdU antibody access the BrdU incorporated within the DNA. Vortex the fixed cells for 15 seconds and centrifuge at 350*g* for 4 minutes, discard the supernatant. Add 1.5 ml of 2 N HCl per 2×10^6 cells while vortexing the tube at low speed. Incubate the cells for 20 minutes at 37 °C. Add 0.1-M sodium borate (twice the volume of HCl used) to each tube while vortexing. Centrifuge at 350*g* for 4 minutes and discard the supernatant. Add 6 ml of 0.5% Tween 20 + 0.5% Bovine serum albumin (vol/vol) in phosphate-buffered saline (PBTB) to each tube while vortexing, centrifuge for 4 minutes at 350*g*. Aspirate the supernatant.
- *Staining and analysis*: Add 0.2 ml of anti-BrdU monoclonal antibody in phosphate-buffered saline +0.5% Tween-20 (vol/vol) at 1:100 dilution, mix gently, and incubate for 60 minutes, at room temperature in the dark. Add 3-ml PBTB while vortexing, centrifuge for 4 minutes at 350*g*, and aspirate the supernatant. Add 0.2-ml secondary antibody in PBTB +1.0% (vol/vol) normal goat serum, at appropriate dilution (this should be referred from the product sheet.) and mix gently. Incubate in dark for 45 minutes at room temperature. Add 3-ml PBTB, mix gently and save an aliquot of 10 µl for counting nuclei. Centrifuge the cell suspension at 350*g* for 4 minutes. Aspirate the supernatant and add propidium iodide (10 µg/ml in PBTB) to adjust the final concentration to 1×10^6 nuclei/ml of suspension. Mix gently and incubate the suspension overnight at 4 °C in dark. On the following day, 30 minutes prior to analysis add 20 µg RNase per 1×10^6 nuclei/ml of suspension. Analyze the samples by flow cytometer. A detailed description regarding the procedures and critical parameters in flow cytometry analysis is given by Nicholas et al. [7].

4 Methods for Evaluation of Drugs Inhibiting Angiogenesis

4.1 Chick Chorioallantoic Membrane Assay

Quantification of antiangiogenic activity can be performed using chick embryo chorioallantoic membrane (CAM) using a method described by Mai Nguyen et al. [8]. The principle relies on the vertical growth of new capillary blood vessels from the

CAM into a collagen gel. The advantage of this method is that it eliminates the interference from background veins, arteries, and nongrowing capillaries lying in the horizontal plane of CAM.

Procedure

I. *Preparation of Shell-Less Chick Embryo*

1. Incubate the Leghorn chicken eggs by placing them horizontally in an incubator at 37 °C and 65–70% relative humidity.
2. On Day 3, crack the eggs and transfer the embryos individually in petri dishes. Transfer the petri dishes with the embryos into a CO_2 tissue culture incubator with 2–4% CO_2 maintained at 37 °C and 65–70% relative humidity. This step should be performed inside a tissue culture hood without the blower running. All the glassware and gloves used should be sterilized prior to use to avoid contamination.

II. *Preparation of Collagen-Based Gel Matrix*

This structure provides support for vertical growth of the newly formed blood capillaries.

1. Suspend recombinant basic fibroblast growth factor (bFGF) in sucralfate (aluminum sucrose octasulfate) and vitrogen, a Type I collagen. (0.1–10 µg bFGF prepared in 1 mg Bovine serum albumin/1 ml saline+20 mg sucralfate +400 µl Type I collagen (e.g., vitrogen) vitrogen). Sucralfate should be sterilized in 200-µl boiling double-distilled water for 1 minute, followed by centrifugation. Then remove supernatant double-distilled water. The pH of vitrogen should be adjusted to pH 7.4 prior to use by addition of 80% vitrogen +10% 10× phosphate-buffered saline +10% 0.1 M NaOH. It should be kept on ice at 0 °C throughout the sample preparation to prevent premature gelation. Angiogenesis is induced by bFGF, a most potent angiogenic factor which is slowly released by sucralfate. Sucralfate added to collagen protects bFGF from degradation and maintains its sustained release.
2. The test drug or formulation to be analyzed should be dissolved in double-distilled water or saline and then mixed with neutralized vitrogen at 1:2 ratio as in Step 1.
3. The Teflon mesh (e.g., Tetko, Bluehill Plastics, Cambridge) should be cut into desired dimensions, autoclaved for 10 minutes at 15 PSI, 121 °C followed by vacuum drying. A 20-µl aliquot of collagen-based gel matrix should be deposited on a mesh.
4. The sample is allowed to form a gel by placing it on the top of a flat end of a 1/8 inch diameter Teflon rod mounted within a 100-mm glass petri dish. Place 6 ml of water in petri dish to maintain constant humidity. Sixteen Teflon rods can be placed in 100-mm dish.
5. Seal the petri dish with a plastic cover and place in an incubator at 37 °C and 65–70% relative humidity for 15–20 minutes.

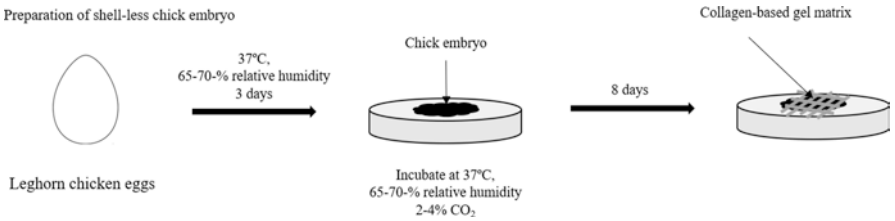

Fig. 18.1 Diagrammatic representation of Chick chorioallantoic membrane assay

III. *Measurement of Angiogenesis*

1. Transfer the collagen-based gel matrix onto the CAM (on the outer third region, approximately 2–3 mm away from the central vessel) of an eight-day-old chick embryo with sterile forceps. The procedure should be carried at temperature of 20 °C–24 °C and relative humidity of 40–50%.
2. Place a smaller piece of mesh (2 × 2 mm) on the top of collagen gel.
3. Place the embryos in the incubator and observe on Day 3 to Day 9 post implantation with a light microscope. Count the number of squares in the mesh containing new blood vessels.
4. Express the antiangiogenic activity of the test as percentage of control.

Figure 18.1 provides the representation of Chick chorioallantoic membrane assay.

4.2 Zebrafish Caudal Fin Regeneration Assay [9]

Procedure

1. Place the zebrafish (10 per each group) in tanks maintained 28 ± 0.5 °C on a 14-hour light/10-hour dark cycle.
2. Cryo-anesthetize the fish by placing them on ice for 2–3 minutes until gills stop moving with loss of body movement and balance and no response to stimuli. Promptly, transfer them to glass slide and amputate the fin at midfin level.
3. Place the fish into a recovery tank and ensure their recovery within 3 minutes. The recovery of fish occurs in three stages; Stage I: by immobility but opercular movement, Stage II: uncoordinated body movements with opercular movement, and Stage III: normal body movement.
4. Add the test and standard formulation to the tanks maintained 28 ± 0.5 °C for seven-day post amputation.
5. On Day 8, place each fish under light microscope to determine the length of fin regeneration and regenerative blood vessels.

Critical parameter The caudal fin will regenerate after amputation up to the level where the scales extend from the body. Therefore, for regeneration studies, it is important to cut the fin halfway between where the scales end and tip of the fin. If

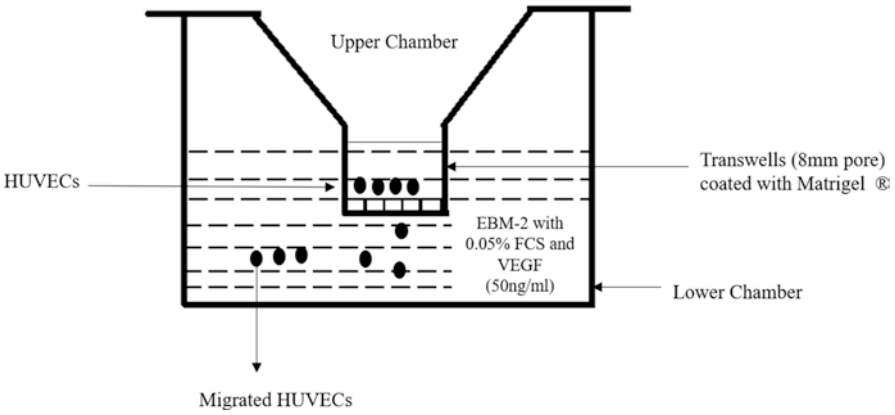

Fig. 18.2 Diagrammatic representation of endothelial cell migration assay

the fin is cut more anterior than the scales, regeneration becomes unreliable either failing completely or resulting in abnormally shaped regenerates.

4.3 Endothelial Cell Migration Assay [10]

Procedure

1. Transwells (8-mm pore) precoated with 200 mg/ml Matrigel® are used for assay.
2. Plate Human umbilical vein endothelial cells (HUVECs) (1.5×10^5) in EBM-2 containing 0.05% FCS in upper chamber of transwell in presence of various concentration of test/standard formulations for 30 minutes at 37 °C.
3. EBM-2 containing 0.05% FCS and VEGF (50 ng/ml) should be added to lower chamber.
4. After 5 hour of incubation, stain the cells. Remove the nonmigrated cells (on top side of membrane) by cotton swab.
5. The number of migrated cells is quantified by counting cells under ×40 objective.

Figure 18.2 provides a representation of endothelial cell migration assay.

5 Topoisomerase I Inhibition Assay [11]

5.1 Topoisomerase I Inhibition Assay

This assay can also be used for studying inhibition of relaxation catalyzed by topoisomerase I. Topoisomerase I introduces single-stranded cuts in DNA and makes it in a relaxed state so that it can replicate. Topoisomerase inhibitors inhibit the

enzyme and therefore inhibit the relaxation of DNA; thus, replication gets hindered and cells undergo apoptosis.

Requirements

- Purified Topoisomerase I or cell extract from eukaryotic cells
- 10× topoisomerase reaction buffer: 500-mM Tris-Cl, pH 7.5, 1-M KCl, 10-mM dithiothreitol, 100-mM EDTA, 50-μg/ml acetylated bovine serum albumin
- Substrate: Plasmid DNA
- 5× loading dye: 30% (v/v) glycerol, 0.25 mg/ml bromophenol blue
- Apparatus, reagents, and equipment for Gel electrophoresis: gel box, comb, power supply, ethidium bromide staining, and gel photography
- 0.8% agarose gel
- 1.5-ml microcentrifuge tubes
- UV transilluminator

Procedure

1. Add 2 μl of 10× topoisomerase I reaction buffer and 200-ng plasmid to each of a series of 1.5-ml microcentrifuge tubes on ice. Adjust volumes with distilled water so that the final reaction volume in each tube, including that of the protein or extract added in step 2, is 20 μl.
2. Dissolve the test formulation in an appropriate solvent (such as DMSO). It is important that the concentration of the solvent in the reaction mixture does not exceed 0.5%. An appropriate solvent control should be maintained.
3. Add purified topoisomerase I protein or cell extract to the tubes. Incubate the reaction mixture for 30 minutes at 37 °C. For crude extracts, it is important to use a range of concentrations and standardize the most appropriate concentration.
4. Add 5 μl of 5× loading dye to each tube and load contents on 0.8% agarose gel. Run the gel for 2–3 hours at 5–10 V/cm. Stain the gel with ethidium bromide, destain briefly with water, and photograph the gel with a UV transilluminator. Caution should be exercised while handling ethidium bromide, as it is a mutagen and potential carcinogen. It should be handled with gloves, and solutions should be disposed according to institutional guidelines.
5. For determination of quantitative inhibition of topoisomerase I by test sample, scan photographic negatives densitometrically using an imager (such as Alpha imager 2200, AlphaEase version 5.5). The percentage inhibition of topoisomerase I is calculated as follows:

$$\%\text{Inhibition} = \frac{\text{Intensity of sample treated DNA}}{\text{Intensity of vehicle treated control DNA}} \times 100$$

5.2 Topoisomerase II Inhibition Assay

Topoisomerase II has unique property to catalyze the decatenation of intact double-stranded DNA. Decatenation refers to separating the physical links between the two double strands of DNA. This property allows the enzyme to separate replicated DNA molecules at mitosis. This assay uses Topogen ®, a kinetoplast DNA from *Crithidia fasciculata*. This DNA is ideal for the assay, since it forms a large network of interlocked (termed as catenated) circles. Topoisomerase II decatenates the circles from the network, the catenated circles are unable to enter an agarose gel; however, upon decatenation the free circles are detected as a discrete band on the gel. Topoisomerase I cannot catalyze the decatenation; therefore, this assay is selective for topoisomerase II imbibition.

Requirements

- 10× topoisomerase II reaction buffer: 200-mM Tris-Cl, pH 7.5, 100-mM $MgCl_2$, 10-mM ATP, 10-mM EDTA, 10-mM dithiothreitol (DTT), 1.5-M KCl, 300-µg/ml acetylated bovine serum albumin
- Substrate: kinetoplast DNA (Topogen®)
- Purified topoisomerase II or cell extract
- 5× loading dye: 30% (v/v) glycerol, 0.25 mg/ml bromophenol blue
- 0.8% agarose gel
- Apparatus, reagents, and equipment for Gel electrophoresis: gel box, comb, power supply, ethidium bromide staining, and gel photography
- 1.5-ml microcentrifuge tubes
- UV transilluminator

Procedure

Relaxation by Topoisomerase II requires ATP and a divalent cation; therefore, the buffer for topoisomerases I and II are different. The evaluation method is the same as that for Topoisomerase I.

6 Protocol for Cell Cycle Analysis by DNA Content Measurement (using Propidium Iodide) [12]

This method is used to distinguish the cells in different phases of their cell cycle by flow cytometry. Prior to analysis, the cells are permeabilized and treated with PI which stains the DNA; and thus, the fluorescent intensity of stained cells is proportional to the cellular DNA content.

Procedure

Treatment of Cells and Fixation

- Culture the cells of interest at appropriate seeding density in six-well culture plates. The cells are then subjected to treatment for indicated time. It is recommended to test at least 3–5 graded doses of the formulation along with appropri-

ate solvent and media controls. A broader concentration range is advantageous, as it will provide a more comprehensive result.

- After treatment, collect the cells in a 1.5-ml microcentrifuge tube by trypsinization and centrifugation. Wash the pellet with phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) and fix the cells with ice-cold 70% ethanol at −20 °C overnight.

Staining with Propidium Iodide

- Centrifuge the fixed cells and wash with ice-cold phosphate-buffered saline (without Ca^{2+} and Mg^{2+}).
- Resuspend cells in 300–500 µl PI/Triton X-100 staining solution and incubate for 30 minutes at 37 °C in dark. The staining solution is prepared by addition of 10 ml of 0.1% (v/v) Triton X-100 in PBS to 2-mg DNAse-free RNAse and 0.40 ml of 500 µg/ml PI. This solution should be prepared freshly. It is important to incorporate RNAse in the staining solution to distinguish between RNA and DNA staining, as PI also binds to RNA.
- After incubation, transfer the tubes with cells on to ice or store at 4 °C in dark.
- Analyze the cell cycle distribution using FACS.

Analysis of Results and Interpretation

The analysis is performed by the software associated with the flow cytometer. Following are plots which are displayed by the cytometer:

- Forward scatter (FS) and side scatter (SS) are measured to identify single cells. Forward scatter gives an estimation of cell size, whereas side scatter gives an estimation of cellular granularity.
- Pulse processing (pulse shape analysis) is used to identify and exclude cell doublets from the analysis. This is achieved by using pulse area vs. pulse width/pulse height depending on the type of flow cytometer.
- PI histogram obtained by plotting forward scatter vs. PI signal.
- Flow cytometry data analysis is based on the principle of "gating." Gating refers to distinguishing populations of cells based on their forward and side-scatter properties. For analysis, gating is performed on the single-cell population using pulse width vs. pulse area. These data are used to gate the scatter plot to gate out the debris. The gates are combined and applied to PI histogram plot. This gives cell count vs. PI intensity.

Staining of Surface Antigens

This procedure can be used simultaneously to determine the presence of surface antigens. An antibody which shows excitation at 488 nm such as FITC-conjugated antibody may be used for this purpose.

Critical Parameters

- PI is a suspected carcinogen and should be handled with care. The dye must be disposed of safely and in accordance with applicable local regulations (e.g., Control of Substances Hazardous to Health (COSHH) regulations).
- Do not wash cells after the addition of the PI staining solution.

7 Apoptosis Assays

7.1 Annexin V-FITC/Propidium Iodide Assay

Apoptosis is characterized by specific morphological features, such as loss of plasma membrane symmetry, plasma membrane blebbing, condensation of cytoplasm and nucleus, and cleavage of DNA. One of the earliest events in apoptosis is plasma membrane asymmetry due to translocation of membrane phospholipid phosphatidyl serine (PS) from the inner leaflet to the outer leaflet of plasma membrane. This translocation exposes PS to the external cellular environment. Annexin V is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS, and it binds to the exposed apoptotic cellular surface. Thus, conjugation of annexin V with fluorochrome such as FITC can be used for determination of early apoptosis. Late apoptotic cells are characterized by loss of membrane integrity and become permeable to dyes such as propidium iodide. In conjunction with propidium iodide, this assay can be used for distinguishing between early and late apoptosis. Cells with intact membrane will exclude PI, whereas dead and damaged cells will be permeable to PI. Therefore, viable cells will be both annexin V and PI negative; early apoptotic cells will be annexin V positive and PI negative, whereas late apoptotic cells will be both annexin V and PI positive. The following protocol is described by Aja M. Rieger, for accurate assessment of cellular apoptosis [13].

Procedure

Treatment of Cells and Preparation

- Culture the cells of interest at appropriate seeding density in six-well culture plates. The cells are then subjected to treatment for indicated time. Appropriate solvent and media controls should be maintained. The optimum concentration for flow cytometry analysis is 2–4 × 10^6 cells per 200 µl volume. However, the procedure may result in loss of cells; therefore, it is recommended that each sample consist of at least 4 × 10^6 cells at the start of the procedure.
- Prepare phosphate-buffered saline without Ca^{2+} and Mg^{2+} using the indicated formula (Potassium chloride 0.2 g, Potassium phosphate monobasic 0.2 g, Sodium chloride 8 g, Sodium phosphate dibasic 1.15 g dissolved in 1 L of nuclease free water). After treatment, collect the cells by trypsinization and centrifugation. Wash the pellet with phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), centrifuge, and decant the supernatant. Resuspend cells in 100 µl × Annexin V binding buffer.

Staining the Cells with Annexin V-FITC and PI

- Add Annexin V-FITC (e.g., 5 µl Annexin V Alexa Fluor 488, Thermofisher Scientific Catalogue number: A13201) according to the manufacturer's instructions.
- Incubate tubes in the dark for 15 minutes at room temperature.

- Add 100 µl of 1× Annexin V binding buffer +100 µl of cell suspension (2–4 × 10^6 cells) to each reaction tube. There should be approximately 200 µl in each tube.
- Add 4 µl of PI (diluted 1:10 in 1 × Annexin V binding buffer).
- Incubate tubes in the dark for 15 minutes at room temperature.
- Add 500 µl of 1 × Annexin V binding buffer to wash the cells.
- Centrifuge samples at 335× *g* for 10 minutes and decant the supernatant.
- Resuspend cells in 500 µl of 1 × Annexin V binding buffer and 500 µl 2% formaldehyde to create a 1% formaldehyde (fixative) solution. Mix tubes by gentle flicking.
- Fix samples on ice for 10 minutes or store the samples overnight at 4 °C in the dark.
- Add 1-ml phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) to each sample and mix gently by flicking.
- Centrifuge tubes at 425× *g* for 8 min and decant the supernatant.
- Repeat washing twice.
- To the above pellet, add 16 µl of 1:100 diluted RNase A to give a final concentration of 50 µg/ml. Incubate for 15 minutes at 37 °C.
- Add 1-ml phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) and mix gently by flicking.
- Centrifuge tubes at 425× *g* for 8 minutes.
- Analyze the samples using flow cytometry.

Analysis of Results and Interpretation

The analysis is performed by the software associated with the flow cytometer. Following are plots which are displayed by the cytometer:

- Forward scatter (FS) and side scatter (SS) are measured to identify single cells. Forward scatter gives an estimation of cell size, whereas side scatter gives an estimation of cellular granularity.
- Pulse processing (pulse shape analysis) is used to identify and exclude cell doublets from the analysis. This is achieved by using pulse area vs. pulse width/pulse height depending on the type of flow cytometer.
- PI histogram obtained by plotting forward scatter vs. PI signal.
- Flow cytometry data analysis is based on the principle of "gating." Gating refers to distinguishing populations of cells based on their forward- and side-scatter properties. For analysis, gating is performed on the single-cell population using pulse width vs. pulse area. These data are used to gate the scatter plot to gate out the debris. The gates are combined and applied to PI histogram plot. This gives cell count vs. PI intensity. Similarly, obtain a plot of cell count vs. Annexin V-FITC intensity. The software can combine the two plots to give Annexin V-FITC vs. PI plot which provides the following information:

    1. Annexin V-FITC negative + PI negative cells, representing viable cell population

2. Annexin V-FITC positive + PI negative cells, representing early apoptotic cell population
3. Annexin V-FITC positive + PI positive cells, representing late apoptotic cell population

7.2 *Measurement of Caspase Activation Using Forster Resonance Energy Transfer (FRET) [14]*

Procedure

Cell Preparation for Time Lapse Measurements

- Plate 2×10^4 cells of interest expressing a FRET-based caspase reporter in cell growth medium in each well of a four-chamber glass-bottomed plate. Incubate the cells for 24 hours at 37 °C to allow the cells to adhere to the glass surface.
- Turn on the microscope and set the temperature to 37 °C at least 1 hour before imaging the cells. Maintain the cells at a constant temperature for the duration of the time lapse experiment using a temperature controller that sits on the microscope stage or a microscope with incubator enclosure.
- Remove the medium from the cells and replace it with cell growth medium (containing supplements for imaging) prewarmed to 37 °C. The supplements in the cell growth medium for imaging are HEPES (a buffer) and 2-mercaptoethanol which prevent unwanted accumulation of reactive oxygen species that can be toxic to the cells. HEPES buffer can be prepared by adding 119.15 g of HEPES to 400 ml of distilled water; adjust the pH to 7.0 by addition of 1M NaOH and make up the volume to 500 ml with distilled water.
- Place the dish (or chamber slide) on the microscope stage using the correct adaptor at least 30 minutes before imaging the cells.
- Locate and focus on the cells of interest. Adjust the instrument for automated acquisition of three channels, that is, donor excitation/emission, donor excitation/acceptor emission (FRET), and acceptor excitation/emission and capture images. It is also recommended to capture a transmission light image to document cellular morphology. Load the images for each fluorescence channels into a suitable software such as ImageJ. Select a background region and subtract the background intensity from each respective image. After background subtraction, compare the intensities between the channels for each test sample. Calculate donor and acceptor bleed through as follows:

$$\text{Donor bleed through} = \frac{\text{FRET}}{\text{Donor excitation}}$$

$$\text{Acceptor bleed through} = \frac{\text{FRET}}{\text{Acceptor excitation}}$$

Correct the FRET channel for donor and acceptor cross talk by multiplying donor channel images by the bleed through factor and subtract the resulting images from the FRET channel.

Save the corrected FRET images, subtract background from each of these images. Convert the acceptor images to a stack of binary masks. Multiply the binary masks with the $CFP_{Background\ corrected}$ and $FRET_{Donor\ corrected}$ image stacks. Divide the new CFP image stack by the new FRET image stack to obtain a ratiometric CFP/FRET image stack. Use the resulting ratiometric image stack to display FRET substrate cleavage as changes in signal intensity. Caspase activity in the sample is directly proportional to the signal intensity.

8 Determination of Minimum Inhibitory Concentration (MIC) by Broth Microdilution Technique

Minimum inhibitory concentration (MIC) of a chemotherapeutic agent is defined as the lowest concentration of the drug that will inhibit the visible growth of a microorganism after overnight incubation. Broth microdilution refers to the determination of MIC in microdilution plates with a capacity of ≤500 μl per well. The following procedure has been described by European Committee for Antimicrobial Testing, 2003 [15, 16].

Requirements

Medium: Cation-supplemented Mueller–Hinton broth. Mueller–Hinton broth (MHB) is a general-purpose medium which is used for wide variety of nonfastidious organisms. For cultivation of fastidious organisms, the broth needs to be supplemented depending on the type of organism. For example, for fastidious streptococci, defibrinated or lyzed blood (3–5% v/v) is added as supplement to MHB. The Clinical and Laboratory Standards Institute (CLSI) CLSI guidelines provide specific recommendations for the composition of medium and test conditions for each species of organism [17].

Test compound: Prepare a stock solution of test compound/drug delivery system solubilized appropriately (generally 1 mg/ml). Dilute the stock solution twofold serially to achieve the test concentrations. The range of concentrations to be tested depends on the antimicrobial agent and the organism to be tested. The MIC should be determined by using at least 12 dilutions.

Bacteria: The bacteria subjected to antimicrobial susceptibility testing must be isolated in pure culture. It is essential that they have been identified at the genus and species level. Most organisms are available from hospital laboratories or the American Type Culture Collection (ATCC).

Inoculum: The bacterial inoculum size (colony-forming units/ml) used for susceptibility testing is one of the most critical parameters governing the accuracy and reproducibility of results. The recommended final inoculum size for broth dilution is 5×10^5 colony-forming units/ml (range $3–7 \times 10^5$ colony-forming units/ml).

Procedure

Preparation of Bacterial Suspension

- Steak the bacterial isolate from hospital laboratories or ATCC on to a nutrient-rich agar plate to obtain single colonies. To verify that the test results are accurate, it is essential to include at least one quality control organism with every batch of MIC determination. The MICs of routinely used antibiotics are available in published literature [16, 17].
- Incubate the plate overnight at 37 °C.
- The inoculum can be prepared in various ways such as colony suspension method, growth method, or growth method using overnight cultures. These procedures have been described in detail by Irith [18]. In each of the procedure, it is important to adjust the turbidity of the bacterial suspension to McFarland Standard 0.5 by addition of sterile water, broth, or saline, if the turbidity is too high, or by addition of bacteria if turbidity is low. The turbidity of McFarland Standard 0.5 is equal to 1×10^8 colony-forming units/ml. These are commercially available from several manufacturers such as bioMerieux (Catalogue number: 70900), Thermofisher Scientific (Catalogue number: 10108582). After the adjustment of turbidity, the bacterial suspension should be used within 30 minutes to prevent the change in bacterial number.

Broth Microdilution

- Label the 96-well microtiter plate with the respective antibiotic/formulation concentrations to be tested.
- Add 50 µl of each antibiotic/formulation dilution in the respective well.
- Inoculate each well containing the antibiotic/formulation dilution with 50 µl of bacterial suspension.
- Maintain a growth control well containing 50 µl of bacterial suspension and a sterility control well containing 100 µl of broth.
- Remove 10-µl sample from growth control well immediately after inoculating the plate and pipette into a sterile Eppendorf tube containing 990 µl saline or broth. Vortex the tube and plate 100 µl of this suspension on a nutrient-rich agar plate. Dilute the suspension (1:10) and plate the diluted suspension on another nutrient-rich agar plate.
- Incubate the 96-well microtiter plate and the agar plates at 37 °C for 16–20 hours.
- Count the colonies on the next day.

Calculation and Analysis of the Data

The MIC is defined as the lowest concentration of the test compound that inhibits the growth of the bacteria which is directly proportional to the turbidity in the well. Turbidity is monitored by measuring the optical density at 630 nm. Plot a standard curve using McFarland Standard 0.5 (equal to 1×10^8 colony-forming units/ml). Dilute the McFarland Standard 0.5 with the growth medium serially to obtain various standards (1×10^7 to 1×10) and measure the optical densities of the standards.

Plot the optical densities against the colony-forming units to obtain standard curve. Determine the number of colonies by extrapolating the optical density on the standard curve [19, 20].

The method is validated when the MIC of quality control organism is within one twofold dilution of published values for routinely used antibiotics. In this context, one twofold means, for example, if published MIC is 2, then the MIC obtained should be between 1 and 4. The ESCMID guidelines specify the target MICs for routinely used antibiotics against quality control organism. The guidelines recommend that a relevant quality control organism should be tested each time the test is performed to ensure validity of the method. Therefore, the MIC should be within one twofold as compared to the values published in the given guideline, and this should be validated each time the test is performed [16].

9 Time-Kill Assay and Post Antibiotic Effect [20]

Requirements

- Microorganism against the formulation to be tested
- Nutrient agar (along with additional supplements specific for the organism used)
- Incubator, 35 °C ± 2 °C with orbital shaker (50 rpm)
- Sterile inoculating loops
- Sterile, disposable 25 × 150–mm glass culture tubes with caps
- Sterile Pyrex glass beads (~4-mm diameter) or spreading rod and turntable
- 2.0-ml cryovials

Procedure

Time-Kill and Postantibiotic Effect (PAE) Assay

- Label and setup three sterile and disposable 25 × 150-mm glass culture tubes in duplicates (one for PAE assay) as follows:
  (i) Tube 1 (kill curve tube): organism containing the medium with the drug/test formulation. It is recommended that various concentrations of drug/test formulation, for example, 1 × MIC, 2 × MIC, 3 × MIC, etc., should be tested.
  (ii) Tube 2 (growth control tube): containing the organism with growth medium (no drug).
  (iii) Tube 3 (sterility control tube): containing growth medium only.
- To Tubes 1 and 2, add bacterial colonies (1–5 × 10^5 colony-forming units/ml) from an overnight culture plate using a sterile inoculating loop. Tube 3 will contain only growth medium.
- At the first time point (0 minute), withdraw 100 µl of the cell suspension from each tube and dilute it tenfold using sterile saline. Culture 100 µl of this diluted suspension on the nutrient agar plate and incubate overnight at 37 °C to allow formation of colonies. The medium from sterility control tube should not form any colonies on incubation.

- Incubate all the tubes at 37 °C with orbital agitation at 50 rpm.
- At appropriate time intervals (1 hour, 2 hours, 4 hours, etc.), withdraw 100 µl of the cell suspension from each tube and dilute it tenfold using sterile saline. Culture a 100 µl of this diluted suspension on the nutrient agar plate and incubate overnight at 37 °C to allow formation of colonies. At each withdrawal, replace the media with 100 µl of fresh growth medium to maintain the volume uniformly.
- After overnight incubation, count the colonies. Plot data on a logarithmic scale with time (h) on the *x*-axis and colony-forming units per milliliter (CFU/ml) on the *y*-axis to obtain a kill curve.
- For determination of PAE, centrifuge the above tubes for 10 minutes at 2000 g at room temperature. Remove the drug, by decanting the supernatant and resuspend the pellet (organism) in fresh media at 37 °C. Repeat the washing for three times to remove any traces of drug.
- After the last wash, remove 500 µl of the cell suspension from each tube (time = 0 minute) and dilute it tenfold using sterile saline. Place 100 µl of the cell suspension on a nutrient agar plate and incubate for 24 hours at 37 °C. Repeat the process at hourly intervals.
- After overnight incubation, count the colonies. Plot data on a logarithmic scale with time (hour) on the *x*-axis and colony-forming units per milliliter (CFU/ml) on the *y*-axis to obtain a PAE curve.

Calculation and Interpretation of Results

1. Kill-curve data
    - A test agent is bactericidal if it causes a ≥3 log 10 reduction in CFU/ml after a 24-hour incubation with the culture.
    - A test agent is bacteriostatic if there is no significant increase in CFU/ml after a 24-hour incubation. It means that there is no significant difference in the number of colony-forming units inoculated at $t = 0$ and the counts after 24-hour incubation, indicating that they are almost equivalent. This indicates that the drug can only prevent the bacteria from growing but cannot kill it (i.e., no bactericidal action).
2. PAE curve

$$\text{PAE} = T - C$$

where T = time taken to observe a log 10 increase in inoculum (CFU/ml) vs. the inoculum (CFU/ml) observed immediately after drug removal.

C = time taken to observe a log 10 increase in inoculum (CFU/ml) in a similarly treated drug-free control.

The PAE of the antibiotics has a clinical relevance because it influences the antibacterial therapy dosing regimens. In theory, drugs with no PAE or low PAE may require more frequent administration than those with high PAE.

Toxicity to Human Cells
It is essential to evaluate the toxicity of a novel drug delivery system (NDDS) on the human cells to ensure selectivity against the infection and safety towards the host cells. This can be determined by a simple MTT assay which has been described in detail in the earlier section. The NDDS should be tested in cell lines for which the drug is intended to be used. The choice of cell line will be based on the indication for which the antiinfective agent is to be used.

10 In Vitro Cellular Uptake

The cellular uptake of targeted drug delivery system can be determined by semi-quantitative or quantitative techniques, with each technique having its own advantages and limitations. This section describes general procedures for determining the uptake of targeted drug delivery system by cells.

10.1 Culturing of Cells

- Select the cancer cell lines and culture them in T-25/T-75 flask in an appropriate medium under atmosphere of 5% CO_2 and 95% air at 37 °C.
- The media should be changed at least every 2 days to ensure sufficient nutrients and remove dead cells.
- After being cultured in T-25 or T-75 flasks, transfer the cells in a 96-well plate and allow to grow stable for 48 hours.

10.2 Measurement of Cellular Uptake by Quantitative Techniques

- Identify the cell line for the experiment and culture the cells according to the specifications until the cells reach confluency.
- Incubate the cells with the antiinfective agent/test sample at various concentrations at desired time intervals. The time intervals selected should be based on the pharmacokinetic data available for the test compound.
- At the appropriate time intervals, remove the cells from the culture media. For adherent cell cultures, the cells should be trypsinized followed by centrifugation to obtain the cell pellet. In case of suspension cell cultures, the culture media should be centrifuged to obtain a pellet.
- Wash the cell pellet thus obtained with ice-cold PBS followed by treatment with 100 µl of 0.01% triton to facilitate cell lysis. Use an aliquot of the cell suspension

to determine cell protein content by Folin-Ciocalteu/biuret method. Centrifuge the cell suspension and discard the cellular debris.
- The supernatant is assayed for the drug content by using analytical techniques appropriate for the sample to be analyzed.
- The cellular drug content is expressed in reference to the total cell protein content.

The selection of analytical technique for quantification depends on the physiochemical properties of the antiinfective agent. Various analytical techniques such as UV spectrometry, HPLC, and LC-MS can be used depending on the properties of the test agent.

10.3 Measurement of Cellular Uptake by Semiquantitative Techniques

10.3.1 Fluorescence Microscopy and Flow Cytometry Technique

Measurement of cellular uptake can be determined by fluorescence microscopy and flow cytometry by conjugating the free drug or the drug delivery system with a fluorescent probe. Tagging the drug with fluorescent probe is not required in cases where the drug shows fluorescence at wavelength (e.g., doxorubicin shows red fluorescence when excited at 480 nm).

Procedure for Confocal Microscopy
- Label the free drug or delivery system with a fluorescent probe if required.
- Culture the cells as mentioned above (In vitro cellular uptake culturing of cells).
- Sequentially treat coverslips measuring 14 mm in diameter (Thermofisher Scientific, Catalogue number: CB00140RA020MNT0) with 5% HCl, 30% HNO_3, and 75% alcohol for 10 minutes. Place the coverslips in a 24-well cell culture plate.
- Seed the appropriately identified cells for the purpose/expressing the receptor of interest into each well with 1 ml of fresh medium and culture at 37 °C, 5% CO_2, for 12 hours for cells to attach onto the coverslips.
- Discard the medium and add free drug, delivery system without the drug, drug delivery system (at least five different drug concentration).
- Incubate the cells for 4 hours 37 °C, 5% CO_2.
- After incubation, wash the cells thrice with PBS to remove any traces of free drug, delivery system without the drug, drug delivery system, not internalized by the cells.
- Fix the cells with 2.5% glutaraldehyde for 15 minutes at 4 °C. Glutaraldehyde is stronger protein cross-linker and preserves the cell ultrastructure as compared to other aldehyde fixatives such as formalin and paraformaldehyde.

- If fluorescent probe is not used (when drug itself shows fluorescence), counterstain the cells with Hoechst 33342 (1 mg/ml) for 15 minutes at 37 °C. Hoechst 33342 is a fluorescent dye which binds to double-stranded DNA, and it is used to counterstain the nucleus. Localization of the fluorescent dye is determined laser scanning confocal microscope.

Procedure for Flow Cytometry

- Label the free drug or delivery system with a fluorescent probe if required.
- Culture the cells as mentioned in above (In vitro cellular uptake culturing of cells).
- Seed the cells expressing the receptor of interest in 24-well cell culture plate 1 day prior the experiment to bring the cells to confluence. Culture at 37 °C, 5% CO_2.
- Discard the medium and replace with 1 ml of fresh medium containing the free drug, delivery system without the drug, and drug delivery system (at least five different drug concentration).
- Incubate the cells for 4 hours at 37 °C, 5% CO_2.
- After incubation, wash the cells thrice with PBS to remove any traces of free drug, delivery system without the drug, and drug delivery system, not internalized by the cells.
- Trypsinize the cells to facilitate cell detachment using 0.5% Trypsin-0.25% EDTA solution. Add double amount of growth media to neutralize trypsin followed by centrifugation. Resuspend the cell pellet in PBS and analyze the cellular uptake using FACS flow cytometer. The intensity of fluorescent signal in the cells is directly proportional to the cellular uptake of the formulation.

10.4 Important Considerations

- All the solutions should be prepared in media appropriate for the selected cell lines.
- A control should be maintained comprising of cells grown in the fluorescent probe without the drug and/or the delivery system.
- The abovementioned method can be modified to determine the subcellular distribution of the drug. The cell pellet obtained in the above method is washed with 0.25 M sucrose-1 mM EGTA-3 mM imidazole followed by homogenization with a Dounce tissue grinder. The homogenate is then subjected to fractional centrifugation for 10 minutes at 770, 625, and 500 g to obtain nuclear and cytoplasmic fractions. The cytoplasmic fraction thus obtained can be further fractionated by high-speed centrifugation at 145,000 g for 30 minutes into “granule fraction” comprising of bulk of cells, organelles, and membranes in form of a pellet and a supernatant fraction consisting of soluble proteins and free ribosomes.

References

1. Hughes JP, Rees S, Kalindjian SB, Philpott KL. Principles of early drug discovery. Br J Pharmacol. 2011;162(6):1239–49.
2. Goldberg AM. Animals and alternatives: societal expectations and scientific need. Altern Lab Anim. 2004;32(6):545–51.
3. Feng Y, Mitchison TJ, Bender A, Young DW, Tallarico JA. Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds. Nat Rev Drug Discov. 2009;8(7):567–78.
4. Clemons PA. Complex phenotypic assays in high-throughput screening. Curr Opin Chem Biol. 2004;8(3):334–8.
5. van Meerloo JKG, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237–45.
6. Romar GA, Kupper TS, Divito SJ. Research techniques made simple: techniques to assess cell proliferation. J Invest Dermatol. 2016;136(1):e1–7.
7. Terry NH, White RA. Flow cytometry after bromodeoxyuridine labeling to measure S and G2+M phase durations plus doubling times in vitro and in vivo. Nat Protoc. 2006;1(2):859–69.
8. Nguyen M, Shing Y, Folkman J. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. Microvasc Res. 1994;47(1):31–40.
9. Hlushchuk R, Bronnimann D, Correa Shokiche C, Schaad L, Triet R, Jazwinska A, et al. Zebrafish caudal fin angiogenesis assay-advanced quantitative assessment including 3-way correlative microscopy. PLoS One. 2016;11(3):e0149281.
10. Cao Z, Zheng L, Zhao J, Zhuang Q, Hong Z, Lin W. Anti-angiogenic effect of Livistona chinensis seed extract in vitro and in vivo. Oncol Lett. 2017;14(6):7565–70.
11. Nitiss JL, Soans E, Rogojina A, Seth A, Mishina M. Topoisomerase assays. Curr Protoc Pharmacol. 2012; Chapter 3:Unit 3; https://doi.org/10.1002/0471141755.ph0303s57.
12. Pozarowski P, Darzynkiewicz Z. Analysis of cell cycle by flow cytometry. Methods Mol Biol. 2004;281:301–11.
13. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. J Vis Exp. 2011;(50) https://doi.org/10.3791/2597.
14. Rehm M, Parsons MJ, Bouchier-Hayes L. Measuring caspase activity by Forster resonance energy transfer. Cold Spring Harb Protoc. 2015;2015(1):pdb prot082560.
15. Muller C, Schubiger PA, Schibli R. In vitro and in vivo targeting of different folate receptor-positive cancer cell lines with a novel 99mTc-radiofolate tracer. Eur J Nucl Med Mol Imaging. 2006;33(10):1162–70.
16. EUCAST. European Committee for Antimicrobial Susceptibility Testing (EUCAST). Clin Microbiol Infect. 2003;9(8):9–15.
17. Wayne P, CLSI. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. 29th ed. Wayne: Clinical and Laboratory Standards Institute; 2019.
18. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.
19. Forry SP, Madonna MC, Lopez-Perez D, Lin NJ, Pasco MD. Automation of antimicrobial activity screening. AMB Express. 2016;6(1):20.
20. Zhao DH, Yu Y, Zhou YF, Shi W, Deng H, Liu YH. Postantibiotic effect and postantibiotic submininum inhibitory concentration effect of valnemulin against Staphylococcus aureus isolates from swine and chickens. Lett Appl Microbiol. 2014;58(2):150–5.
21. Motyl M, Dorso K, Barrett J, Giacobbe R. Basic microbiological techniques used in antibacterial drug discovery. Curr Protoc Pharmacol. 2006; Chapter 13:Unit13A 3; https://doi.org/10.1002/0471141755.ph13a03s31.

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