Chapter 2
Recent Advances in Tumor Targeting Approaches

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Abbreviations

17 AAG	17-Allylamino-17-Demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamine-17-demethoxy-geldanamycin
ADCC	Antibody dependent cellular cytotoxicity
ADEPT	Antibody directed enzyme prodrug therapy
ANA	Monoclonal antinuclear autoantibody
ASM	Acid sphingomyelinase
ATP	Adenosine triphosphate
BCS	Biopharmaceutical classification system
BET	Bromodomain and extra-terminal
BP	Binding protein
BRCA	Breast cancer gene
CDC	Complement-activation dependent cytotoxicity
CDKs	Cyclin dependent kinases
CNS	Central nervous system
CTLA	Cytotoxic T-lymphocyte antigen
dgRTA	Deglycosylated ricin A chain
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
DNA	Deoxy ribose nucleic acid
DOTA	d-Tyr-d-Lys(HSG)-d-Glu-d-Lys(HSG)-NH_2
EBRT	External-beam radiation therapy
ECM	Extracellular matrix
EPCs	Endothelial precursor cells

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EZH2	Enhancer of Zeste homolog 2
FAK	Focal adhesion kinase
FDG	2-Deoxy-2-(^{18}F) fluoro-D-glucose
GMCSF	Granulocyte-macrophage colony-stimulating factor
GRP78	78 kDa glucose-regulated protein
H3K4me3/2	Trimethylation and dimethylation of histone H3 at lysine 4
HAMA	Human anti-mouse antibody
HARA	Human anti-ricin antibody
hCG	Human chorionic gonadotrophin
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HOP	HSP90 organizing protein
HPMA	N-(2-hydroxy propyl) methacrylamide
HSP	Heat shock proteins
IFP	Interstitial fluid pressure
IL	Interleukin
IMP-288	1,4,7,10-Tetraazacyclododecane-N,N′,N″,N′″-tetraacetic acid
JAG1	Jagged 1 protein
LECs	Lymphatic endothelial cells
LLC	Lewis lung carcinoma
LTTs	Ligand-targeted therapeutics
mAbs	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporters
MMPs	Matrix metalloproteinases
MPS	Macrophagocytosis systems
mTOR	Mechanistic target of rapamycin
Myc	Myelocytomatosis oncogene
p14^{ARF}	Alternate reading frame
PI-3	Phosphoinositide 3-kinase
PDEPT	Polymer directed enzyme prodrug therapy
PE	Phosphatidyl ethanolamine
PKB	Protein kinase B
PSMC2	26S protease regulatory subunit 7 gene
pRb	Retinoblastoma tumor suppressor protein
RAIT	Radioimmunotherapy
Rb	Retinoblastoma
RBC	Red blood cell
RES	Reticuloendothelial system
SAR	Structure–activity relationship
STA	3-(2,4-dihydroxy-5-isopropyl-phenyl)-4-(1-methyl-indol-5-yl)-5-hydroxy-[1,2,4]triazole
TCMC	2-(4-isothiocyanotobenzyl)-1, 4, 7, 10-tetraaza-1, 4, 7, 10-tetra-(2-carbamonyl methyl)-cyclododecane

TNF	Tumor necrosis factors
TP53/p53	Tumor protein P53
VEGF	Vascular endothelial growth factor
vSMCs	Vascular smooth muscle cells
WHO	World Health Organization

2.1 Introduction

Tremendous technological developments in the field of cancer therapy have been observed in the past few decades to combat the ever-increasing mortality rate and its peculiar pathophysiology, referred to as carcinogenesis. As per WHO records, cancer is a leading cause of death across the globe accounting 8.2 million and 14 million new cases in 2012 with almost twofold rise in next couple of decades [1]. By definition, cancer is referred to as a generic terminology covering over 200 different types of cancers. Crudely, it is a pathophysiological condition in which the normal cells transform into immortal cells that grow without any control, often referred to as carcinogenesis. Principally, persistent tissue injury and/or genetic factors such as mutations, epigenetic and global transcriptome changes contribute to carcinogenesis (Fig. 2.1). Cumulatively, it could be considered as multistep (comprising a variety of genetic alterations), multipath (including various apoptotic and angiogenesis pathways), and multifocal (constitutive of both field carcinogenesis and clonal expansion) [2]. Subsequently these changes lead to distinct tumor

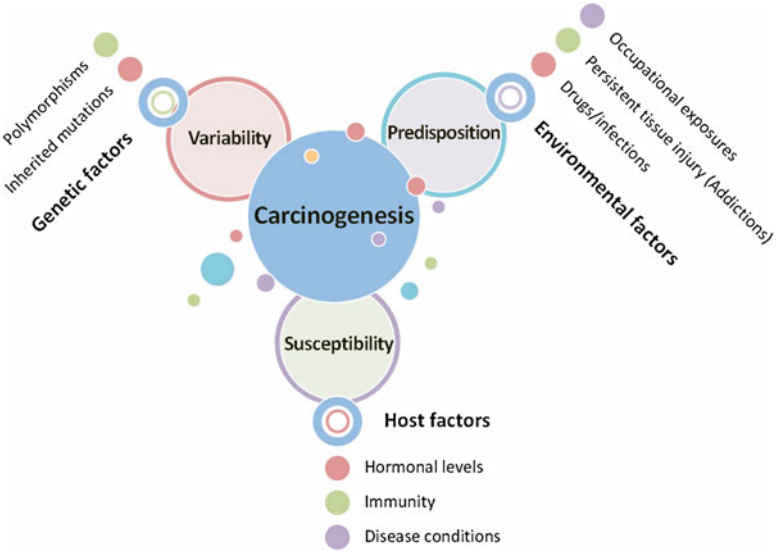

Fig. 2.1 Key contributing factors of carcinogenesis

microenvironment as compared to normal cells. This discriminatory microenvironment and altered pathophysiological signaling pathways have been classically used in the recent drug discovery approaches and set the genesis of molecularly targeted therapies.

Unfortunately, most of the anticancer drugs till time target the DNA or other biologicals actively involved in cell division and thereby control the rapidly diving cancer cells. However, in the course of that, the normal host tissues are also not spared and nonspecific generalized toxicity is noted which may be severely intense at times and may lead to either early termination of therapy or other secondary complications. The host tissues main targets include rapidly dividing lympho-hematopoietic cells, epithelial linings and other mucus secreting regions of gut, hair follicular regions, etc. These complexities lead to low chemotherapeutic index of the anticancer drugs. Secondly, rapid emergence of drug resistance also contributes majorly to the poor cancer chemotherapeutics [3]. Hence, there lies a strong need to develop selective anticancer therapeutics which would principally act to cancer cells without affecting the normal tissues. The materialization of the concept "magic bullets" seems to be mandatory considering the widespread prevalence of cancer.

Tumor targeting is defined as the improving the drug's chemotherapeutic index by (a) preferentially localizing its pharmacological activities at the site of action, (b) recognition and interaction with target cells, and (c) achieving cellular concentrations so as to exhibit therapeutic response [4]. Very often a variety of homing devices are being employed to direct the drug and/or carriers to the particular site of action. Mechanistically, these homing devices are the special molecular signatures that are expressed to a greater extent at the tumor tissues such as folic acid, etc. The principal need for tumor targeting is required due to limited accessibility of drugs to tumor tissues, requirement of high doses, intolerable cytotoxicity, development of multidrug resistance and nonspecific targeting [5]. However, although fascinating, the tumor targeting is often exposed to a variety of barriers mediated by peculiar tumor microenvironment.

2.2 Normal Vs Tumor Vasculature

Classically, there lies a prominent homeostasis among the proangiogenic and anti-angiogenic molecules in the normal tissues which are responsible for balanced organization of the blood vessels for meeting the metabolic demands. This system works in tandem with lymphatics for clearance of the cellular by-products. Carcinogenesis leads to imbalances in these systems leading to a variety of alterations. The chaos starts with alterations in the normal vasculature (abnormalities in the functional and structural aspects) leading to diminished nutrients supply and clearance of cellular waste products. Subsequently, compromised basal membrane, disorganized pericyte layer, downregulation of the adhesion molecules and endothelial linings contribute to high permeability of the tumor tissues.

Tumor vessels usually grow abnormally with unusual vessel diameter, lower vascular density and longer tortuous paths for diffusion of molecules. Further, fluctuant flow, poor plasma channels, and arteriolar-venous shunts for efficient RBCs supply are responsible for two types of hypoxic conditions, i.e., chronic due to compromised diffusion and acute due to lower exchanges, ultimately leading to heterogeneous tumor oxygenation supply [6]. Cumulatively, the principal barriers for the conventional chemotherapeutic agents to access the tumor tissues are malformed tumor vasculature which leads to altered vascular permeability, high interstitial pressure, extracellular acidosis, and hypoxia (due to high mitochondrial oxygen consumption) [7].

Secondly, the interstitial space is regarded as one of the important component for maintaining homeostasis in tissues. It is responsible for exchange of primary requirements of cells such as oxygen and nutrients along with clearance of waste products. In combination with hydrostatic pressure and colloidal osmotic pressure, the transcapillary flow maintains the hydraulic conductivity and plasma protein reflection coefficient among the cells and capillaries in the adjacent to the tissues (Fig. 2.2) [8]. Usually, this transcapillary pressure is slightly higher in capillary bed in the order of 1–3 mmHg so as to maintain the flow of solutes and water from capillaries to cells via interstitial spaces. However, in case of tumors interstitial pressure shoots up to 100 mmHg owing to three principal reasons. These include (a) compromised functionalities of the blood vessel and lymphatics, (b) osmotic pressure generated by drainage of solutes from tissues, and (c) high contractile characteristics of tumor tissues [7]. Interestingly, these are essential targets in the current drug discovery strategies for molecularly targeted therapies.

Thirdly, the pH of the tumor microenvironment is usually dropped relatively towards acidic, as evident from the direct measurement by placing sensitive electrodes into the solid tumors [9]. Classically, it has been widely accepted that hydrolysis of ATP via energy deficient pathways and anaerobic conditions leads to formation of acidic lactate moieties within tumor tissues [10]. Warburg studied the greater production of lactate within the tumor tissues as compared to that of normal tissues and was attributed to the respiratory impairment; however, exact biologics of reduction in tumor pH is not yet identified [11]. The recent advances using genetically modified tumor models revealed that existence of non-lactate mediated acidic microenvironments [12]. It further identified that there exists balance among the intracellular and extracellular pH mediated by proton pumps, which actually regulate the overall tumor pH [13]. Additionally, the uncleared cellular waste products also drastically contribute to the acidic tumor microenvironments [14].

2.3 Barriers to Tumor Targeting

The principal barriers associated with tumor targeting comprise peculiar tumor vasculature which principally comprises heterogeneous blood flow and vascular resistance [15]. In the purview of unregulated growth of tumor vasculature and there

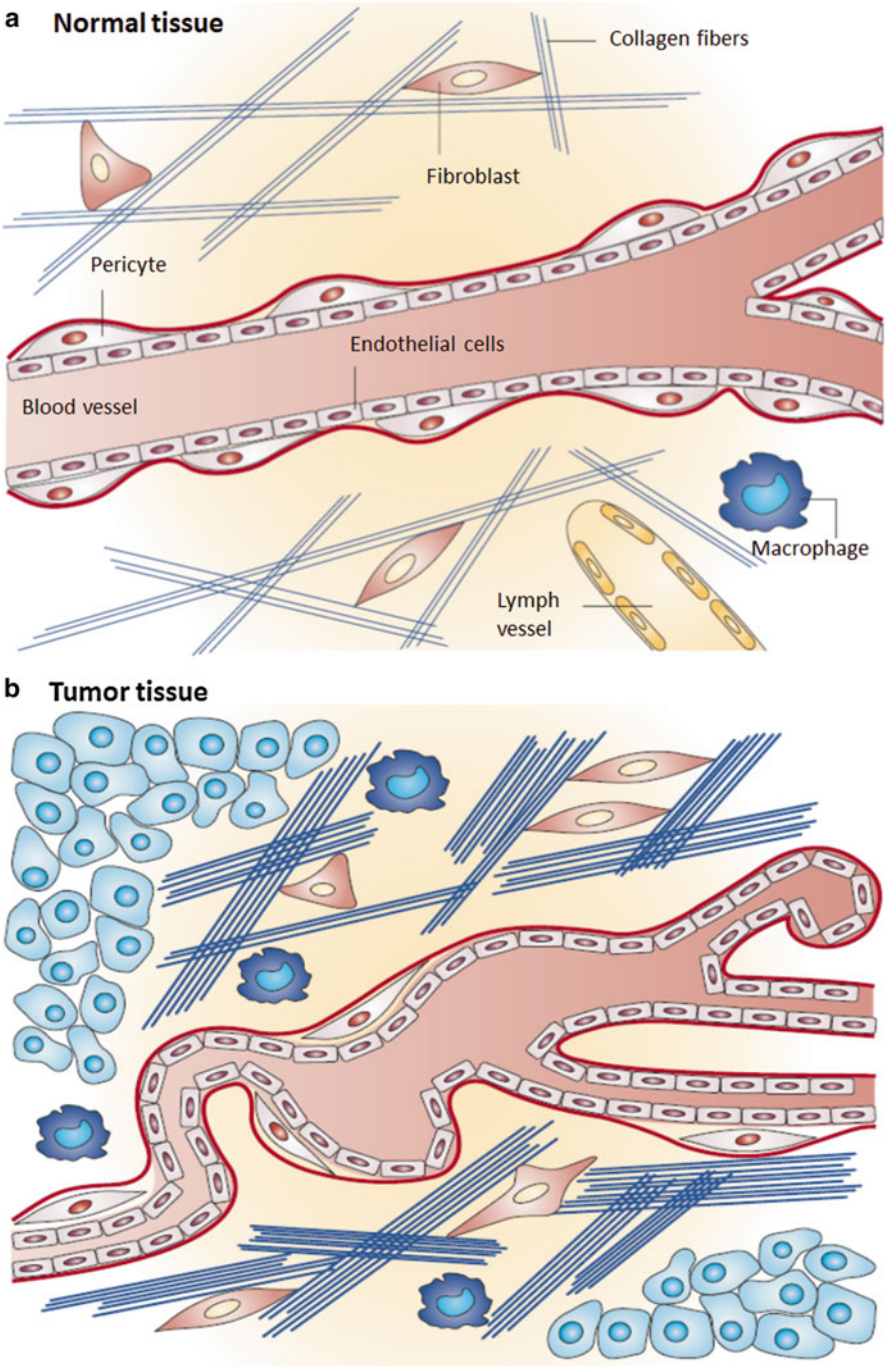

Fig. 2.2 Structural differences between normal and tumor tissues that affect interstitial fluid pressure. (**a**) Normal tissues contain linear blood vessels lined by a smooth layer of endothelial cells with pericytes maintaining the integrity of the vessel on its outside. The extracellular matrix

occurs nonuniform distribution of blood vessels across the tumor leading to patches of very high blood supply to almost negligible supply. This heterogeneity leads to uneven distribution of administered therapeutics often leading to poor therapeutic response. Such altered distribution also usually ends up in partial exposure of drug to the cells, thereby drastically increasing the multiple drug resistance with the tumor cells. Along with these, achievement of therapeutic responses of drug with cancer cells is further challenged by overexpression of efflux transporters, often referred to as ATP binding cassette (ABC) transporters such as P-glycoprotein, multidrug resistance proteins (MRP-1, -2), etc. Most of the anticancer drugs are substrates of such efflux transporters. This piece of information has been exhaustively reviewed by our group previously and is already available in scientific domain hence kept out of scope of this chapter [16]. Subsequently, other factors such as diffusional barrier due to high intercapillary distance, cell density, and extracellular matrix components also pose potential barrier to tumor delivery of therapeutics.

2.4 Conventional Strategies for Tumor Targeting

The principal goals of the targeted drug delivery system is aimed at protection of the drug in concern to the site of action from the metabolic degradation/inactivation during transit, particularity for specific target devoid of any nonspecific interactions with the host tissues and penetration of relevant concentrations of drug within the tumor tissues for therapeutic responses.

In this regard, selective accumulation of the drug at preferred site is also majorly affected by its physicochemical properties. Most of the anticancer drugs fall in the category II/IV of Biopharmaceutical Classification Systems (BCS), thereby posing pharmaceutical problems while water soluble drugs pose problems related to permeability across various biological barriers [16]. Classically to address these concerns, three major approaches could be employed which include (a) subtle structural modifications for improving the physicochemical properties in accordance with structure–activity relationships (SAR), (b) conjugating homing ligands for predetermined bio-distribution patterns, and (c) involvement of carrier based approaches

Fig. 2.2 (continued) consists of a loose network of collagen and other fibers, and contains a few fibroblasts and macrophages. Lymph vessels are also present in normal tissues. (**b**) Tumor tissues contain defective blood vessels that are leaky and irregularly shaped, with many sac-like formations, dead-ends and highly activated endothelia. Blood flow is therefore inefficient. These blood vessels are also covered by fewer pericytes than in normal tissues, resulting in decreased vessel stability. Furthermore, many tumors lack lymph vessels, so interstitial fluid and soluble proteins are inefficiently removed. The extracellular matrix of tumors contains a much denser network of collagen fibers, which are thicker than in normal tissues. Therefore, the tumor tissue is more rigid than normal loose connective tissue. Tumors also contain an increased number of fibroblasts, which bind to the collagen fibers in an integrin-dependent manner and exert an increased tension between the fibers, as well as an increased number of macrophages and other inflammatory cells; these cells release cytokines and growth factors that act on cells of blood vessels and stroma fibroblasts to increase interstitial fluid pressure. Reproduced from ref. [8]

for targeting the therapeutics at site of action [17]. Alternatively, targeting could be categorized as either passive or active depending upon the approach employed.

2.4.1 Passive Targeting

The natural biodistribution pattern of the drug delivery carrier is exploited for its preferential localization in the vicinity of the tumors such as enhanced permeation and retention effects, phagocytosis of particulate carrier by mononuclear phagocytosis systems (MPS) and preferential localization in the organs of reticuloendothelial system (RES). In addition, other typical properties of the tumor microenvironment such as low extracellular pH, relative micro-acidosis, mild hyperthermia, etc. could also be employed for availing passive targeting of therapeutics. However, the targeting potential of such a strategy is relatively low and often associated with partial nonspecific localization of therapeutics in the normal tissues which needs to be considered while employing such therapies.

2.4.1.1 Enhanced Permeation and Retention (EPR) Effect

The EPR effect was first noted three decades ago for the preferential localization of protein macromolecules in the vicinity of the tumor and since then it has been widely explored for the alteration in biodistribution patterns of most of the colloidal drug delivery systems such as liposomes, polymeric nanoparticles, polymer drug conjugates, etc. However, with the advent of the increased research in this field, EPR effect has been regarded as blanket terminology for increased efficacy of any cancer therapeutics. Aggressive studies in this direction suggested EPR effect as complex association of various processes such as angiogenesis, vascular permeability, hemodynamic regulation, genetic heterogeneities among tumors, lymphangiogenesis, and heterogeneous tumor microenvironment [18].

Classically, the cell proliferation leads to formation of solid mass and upon reaching a specific size, cells in the interior starts getting deprived of the nutrients which leads to cell death and release of growth mediators signaling the development of the blood vessels within tumor. However, the formed blood vessels are often leaky owing to absence of basal membrane leading to fenestrations within the size of 200–2,000 nm [19]. The presence of fenestrations results in poor resistance to the extravasation of macromolecules to the tumor microenvironment and contributes to the enhanced permeation part of EPR. Simultaneously, it has also been found that tumor mass is associated with nonuniform lymphatic drainage and experience a huge physical stress owing to rapid growth in the dimensions of the tumor mass [20]. This leads to the severe compromise in the drainage functionality of the vessels and contributes to the retention part of EPR effect [21].

Principally, the EPR effect is mediated by extravasation of the macromolecules from the blood vessels followed by the subsequent movement in the tumor

microenvironment via diffusion and convection. The principal factors affecting EPR effect includes vessel architecture, interstitial fluid composition, extracellular matrix composition, phagocyte infiltration, presence of necrotic domains, factors pertaining to the colloidal carriers such as blood circulation time, particle size, particle shape, surface charge, and surface functionalization, if any, (e.g., stealth characteristics by PEGylation). Exhaustive review on the factors influencing EPR effect and mobility of the colloidal carriers has been recently compiled and hence kept out of the scope of this chapter [18].

2.4.1.2 Surface Engineering of Colloidal Carriers for Stealth Characteristics

The colloidal carriers by virtue of their inherent properties are rapidly taken up by the mononuclear phagocyte system (MPS) via process of opsonization. However, drastic reduction in RES uptake and significant appreciation in the EPR effect of the colloidal carriers can be achieved by surface engineering [22]. Usually, the opsonins interact with the colloidal carriers via forces such as van der Waal's forces, weak electrostatic forces, ionic forces, and hydrophobic/hydrophilic forces. In purview of this, hydrophobic and charged particles are rapidly processed by RES and significant prolongation in the circulation half-life can be achieved by surface functionalizing PEG chains forming "stealth" systems [23].

A variety of natural materials such as dextrans, pullulans, gangliosides, etc. have been employed for proving stealth characteristics to the colloidal carriers. Of note, gangliosides represent the class of glycosphingolipids containing sialic acid and are regarded as integral component of plasma membrane, particularly red blood cells. The derivatives GM1 and GM type III have been exclusively explored for their potential in imparting stealth characteristics and appreciation in circulation half-life, while reduction in uptake by spleen and liver has been noted at numerous instances [24, 25]. Mechanistically, the stealth characteristics are imparted by steric barrier, shielding of anionic charge, and binding with dysopsonins [26].

The synthetic alternative of the natural polymers for imparting "stealthness" includes polyethylene glycol and their derivatives which have been widely explored and are often associated with numerous advantages such as simple anchoring process, biocompatibility, high solubility, stability, ease of availability at relatively inexpensive cost, flexibility in functionalization, etc. [22]. Although fascinating, the PEGylation of colloidal carriers is also associated with a variety of drawbacks such as significantly higher hydrophilicity hinders the efficient hydration of polar head groups of phospholipids leading to poor stability and problems of drug leaching [27], often necessitating higher levels of cholesterol to prevent aggregation and phase separation [28]. Secondly, there have been some instances of immunogenicity by PEGylated colloidal carriers resulting in hypersensitivity reactions [29]. The activation of complement system and induction of anti-PEG antibodies (IgM) has been observed to rapidly clear off the circulating PEGylated colloidal carrier by a mechanism called ABC phenomenon and is highly detrimental on appreciation in

bioavailability, passive targeting, and ultimately efficacy of the system, per se [30]. Further the long term safety of the PEGs is also scarcely established particularly the biological fate. In purview of this, physiological metabolism of PEGs (<400 Da) includes alcohol dehydrogenase mediated oxidation leading to formation of toxic diacid and hydroxyl acid metabolites [31]. On the other hand, the renal clearance cutoff for PEGs is 30–50 kDa, further narrowing the limits for its clinical use [32]. Hence, a series of alternative synthetic derivatives are currently being explored which include vinyl based lipopolymers, polyoxazolines based lipopolymers, polyamino based lipopolymers, zwitterionic lipopolymers, etc.

2.4.2 Active Targeting

Active targeting refers to the attachment of marker component to the colloidal carrier system which is specifically recognized by the target in concern may it be either from organelle or organ. Usually molecular targets are employed such as overexpression of surface receptors on tumor cells for site specific delivery of therapeutics such as dietary ligands (carbohydrate based, folate, etc.), monoclonal antibodies and their fragments, non-antibody ligands (peptidic ligands), etc. The active targeting could be divided into various levels depending upon extent of penetration, i.e., organ level, cellular level, and subcellular level. However, independent of the target location, the preliminary characteristic of the targeting ligand is its specificity which should be neither upregulated nor downregulated upon exposure to physiological conditions [33]. Concomitantly, the binding affinity of the targeting ligands should also remain unchanged which indirectly is affected by the binding site barrier leading to altered tumor penetration. At times very high binding affinities are required considering the higher mobility of the colloidal carrier systems in the physiological conditions.

2.4.2.1 Albumin Based Targeting

Albumin plays a critical role in maintaining the homeostasis by mobilizing key endogenous hydrophobic molecules. It specially binds via non-covalent interactions and executes the transport of molecules in concern by transcytosis across the endothelial cells into interstitial space. Paclitaxel bound albumin nanoparticle represents the classical example for establishing the potential of albumin based delivery of anticancer drugs [34]. Mechanistically, it binds to the gp60 receptor present at the cell surface and leads to the activation of the caveolin-1 mediated transcytosis which also unintentionally transports some of the unbound plasma constituents [35, 36]. Concomitantly, tumor cells also secrete albumin binding proteins, SPARC, also referred to as BM-40, which are acidic in nature and rich in cysteine, binding to the albumin tagged colloidal carrier systems. Such a system could fruitfully be exploited

for targeting the therapeutics to the brain via adsorptive mediated transcytosis. Cationized albumin significantly increased the uptake of β endorphin in isolated brain endothelial cells as compared to its native form [37]. Furthermore, ~4-fold increase in the cellular uptake of albumin bound paclitaxel by endothelial cells has been noted as compared to the clinical formulation, Taxol® which was completely inhibited upon coadministration with β cyclodextrin, the known inhibitor of gp60 suggestive of the active transport as predominant uptake mechanism for albumin based nanoparticles [38]. The principal advantages associated with albumin based targeting include superior stability over a wide range of pH (4–9) and temperature (10–60 °C), biodegradation, non-immunogenic, and nontoxic. A striking advantage includes its additional cryoprotectant effect which makes the lyophilization of formulation in concern quite easier than other systems in race.

2.4.2.2 Vitamin Based Targeting

The vitamins employed for targeting potential includes folate, vitamin B_{12}, thiamine, and biotin [39]. The principal advantages associated with vitamins, particularly folic acid, includes stability over shelf and physiological conditions, relatively inexpensive, nontoxic, non-immunogenic, endogenous homing ligand, wide flexibility for diverse chemical reactions, and relatively higher overexpression of folate receptors on most of the cancers [40]. It has been noted that folate functionalized colloidal carrier systems are preferably absorbed by receptor mediated endocytosis. Folate functionalized nanoparticles have been widely explored by numerous research groups including ours for its potential in preferentially localizing the therapeutics in the vicinity of the tumor tissues. Our group has developed methotrexate loaded folate functionalized albumin nanoparticles for significantly improving its antitumor efficacy and reducing the toxic side effects by virtue of altered biodistribution pattern to target tumor tissues as evident by pharmacoscintigraphic evaluation [41]. In a separate set of experiments functional magnetite nanoparticles have also been explored for active targeting potential which were found to selectively target and induce apoptosis in folate receptor overexpressing cancer cells, thereby imparting significantly higher anticancer properties as compared to parent drug [42].

Furthermore, folic acid functionalized carbon nanotubes have also been explored to a greater extent to assess its potential for cancer theranostic applications which comprised fluorochrome (Alexa Fluor 488/647), radionuclide (Technitium-^{99}m), tumor-targeting module (folic acid), and anticancer agent (methotrexate) [43]. The developed system exhibited significantly higher internalization within lung cancer cell lines (A549) and breast cancer cell lines (MCF-7) as evident by the lysosomal trafficking and resulting in higher anticancer activity. Subsequently in vivo experiments revealed ~19-fold increase in the tumor localization for the targeted formulation as compared to free drug. Table 2.1 reveals the representative list of formulation approaches employed for improving the tumor delivery of therapeutics using folate as targeting ligand.

Table 2.1 Folate conjugated nanoparticles for improved tumor delivery of therapeutics

Delivery system	Drug	Outcomes	Ref.
Magnetic multi-walled carbon nanotubes	Doxorubicin	Efficient uptake by U87 cells and higher intracellular release of DOX	[44]
HPMA copolymer conjugate	Doxorubicin	Higher apoptosis and greater tumor spheroid inhibition against Hela cells	[45]
High-density lipoprotein nanoparticles	–	Enhanced selectively towards ovarian cancer cells	[46]
Superparamagnetic iron oxide (Fe_3O_4)	Doxorubicin	~2.5-fold higher than that for the non-targeting group.	[47]
Folate-tagged liposomes	Ricin	Significant increase in the cytotoxicity up to 557.7-fold was demonstrated by monensin intercalated folate liposomes	[48]
pH responsive polymeric nanoparticle	Doxorubicin	Increased targeting efficiency of polymeric nanoparticles, resulted in enhanced cellular uptake by 100-fold	[49]
PLGA nanocapsules	Quercetin	Folate modified PLGA nanocapsules showed selective uptake and cytotoxicity to folate expressing Hela cells	[50]
Poly(L-γ-glutamyl glutamine) (PGG) nanoparticle	Docetaxel	Folate targeted PGG nanoparticle system was found to be highly effective against tumor cells and successfully localized in the tumor site	[51]
Polyhedral oligomeric silsesquioxane-F68 hybrid vesicles	Doxorubicin	Significantly enhanced the uptake in Hela and HOS cells	[52]
Polymersome	Doxorubicin	Higher anti-glioma effect compared to the treatments with free doxorubicin	[53]

2.4.2.3 Transferrin Based Targeting

Transferrin receptors are also exclusively overexpressed in most of major types of tumors including lung, lymphomas and breast cancers in the order of ~10-fold [54]. The important feature of employing transferrin as targeting ligand is its capability for enabling the transcytosis across blood brain barrier [55]. Sahoo et al. exhaustively explored the potential of transferrin conjugated paclitaxel loaded nanoparticles for variety types of cancer including breast cancer and prostate cancer [39]. Significantly higher levels of paclitaxel were noted in the case of transferrin conjugated nanoparticles during cell uptake studies as compared to that of free drug and non-targeted formulation counterparts [56]. Furthermore, in separate set of experiments, transferrin conjugated nanoparticles revealed about threefold higher uptake in PC-3 cell lines and concomitantly significant increase in therapeutic efficacy was noted for the developed formulation in in vivo murine model as compared to that of free drug and non-targeted formulations [57]. Table 2.2 represents the representative list of transferrin conjugated nanoparticles employed for improving the tumor delivery of therapeutics.

Table 2.2 Transferrin conjugated nanoparticles for improved tumor delivery of therapeutics

Delivery system	Drug	Outcomes	Ref.
Mesoporous silica nanoparticles	Camptothecin	Enhanced uptake by Panc-1 cancer cells and toxicity of cancer cells as compared to normal cells	[58]
TRAIL (TNF-related apoptosis-inducing ligand) nanoparticles	–	5.2-fold higher tumor accumulation	[59]
Liposomes	Doxorubicin	Significant improvement in survival time	[60]
Pegylated nanoscaled graphene oxide (GO)	Doxorubicin	Enhanced intracellular delivery, efficiency and stronger cytotoxicity	[61]
Lipoplex	Cytosine deaminase	Significant tumor reduction and enhanced apoptosis	[62]
DQAsomes	Paclitaxel	Higher uptake and tumor cytotoxicity	[63]
Polymeric micelles	Curcumin and Paclitaxel	Improved cytotoxic effect against the SK-OV-3 cells	[64]
Polymeric nanoparticles	si-RNA	Marked tumor accumulation	[65]
Selenium nanoparticles	Doxorubicin	Significantly enhanced cellular uptake	[66]

2.4.2.4 Lectin Based Targeting

Lectins represent a class of cyto-adhesive targeting ligands which is moderately recognized by glycans on the glycosylated cell surface proteins and lipids. Most of the cell surface expresses peculiar glycan arrays which can be sensed differentially and hence this could be a viable strategy as regards targeting perspectives [67]. The targeting potential of lectins has been explored in a wide field of applications including gastrointestinal targeting, nasal delivery, pulmonary delivery, buccal cavity, ocular drug delivery, and brain delivery. Of note, targeting of liver targeting has also been quite possible using lectins for delivering drugs and genes. The asialoglycoprotein receptors are specifically overexpressed on liver which recognizes either β-galactose or *N*-acetyl galactosamine residues [68]. Interestingly, this approach could also be employed using polymer drug conjugates wherein drug and galactose residues can be covalently linked to polymer backbone [69]. On similar line of action, asialofetuin tagged liposomes have also been explored to improve the hepatic delivery of hydrophilic molecules [70].

2.4.2.5 Peptide Based Targeting

Peptide based tumor targeting strategy is considered as most promising because relatively higher stability and smaller size of tumor specific peptides. The peptides

employed for tumor targeting could be either monomeric, homodimeric, heterodimeric oligomeric or tetrameric in nature. Cyclic RGD peptide anchored liposomes were previously prepared preferentially targeting anticancer drug 5-fluoro uracil to tumor vasculature. In vitro endothelial cell uptake studies revealed significantly higher uptake of RGD labeled liposomes as compared to non-targeted counterparts leading efficient prevention of spontaneous lung metastasis and angiogenesis[71]. The tumor specific peptides could be broadly categorized into two categories, one targeting tumor cell surface while other targeting tumor vasculature. The cell surface targets could be either lymphomas, myelomas, neuroblastomas, breast cancer, head cancer, neck cancer, prostrate cancer, endothelial cells, or human laryngeal carcinomas whereas the tumor vasculature targets could be $\alpha_v\beta_3$, $\alpha_v\beta_5$, aminopeptidases, proteoglycans, gelatinases, and vascular endothelial growth factors [72].

2.4.3 Physical Targeting

A variety of physical approaches have also been explored for their potential to preferentially localize anticancer medicaments in the vicinity of tumors. The physical stimuli for drug targeting may either be endogenous such as pH, temperature, redox potentials, etc., or be exogenous, i.e., employment of external forces such as magnetic, ultrasound, etc. [73]. As discussed earlier, the tumor microenvironment is slightly acidic and exhibits mild hyperthermia which could be specifically exploited as a stimulus for physical targeting. Stimuli responsive colloidal systems have been designed and developed that tend to degrade at acidic pH and/or elevated temperatures. On the other hand, magnet assisted tumor targeting approaches have also widely been explored considering its immense potential. In this particular system, the drug in concern is immobilized on ferromagnetic colloidal carriers and allowed to circulate in body. The external magnetic field is applied at the site of action which localizes the circulating carriers leading to exceptional tumor levels of drugs. Similarly, the circulating colloidal carrier may be accumulated at the desired site of action using ultrasound energy. Significantly higher tumor levels of doxorubicin were noted from polymeric micelles upon imparting external ultrasound as compared to that of free drug counterpart [74]. The driving force for preferential localization herein is the destabilization of colloidal carrier upon exposure of high energy external force.

2.5 Recent Advances in Tumor Targeting Approaches

2.5.1 Molecular Targeted Therapies

Persistent tissue injuries to the cells and/or factors generally tend to dysregulate the well organized signaling systems of cell cycle and ultimately leading to tumorigenesis. Further, understanding of the fact that either a particular site of molecule or

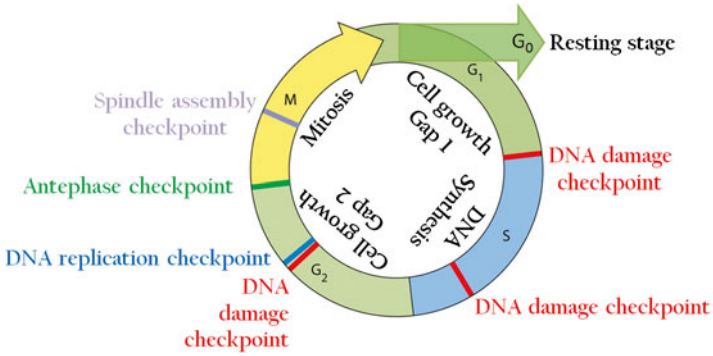

Fig. 2.3 Cell cycle checkpoint pathways

whole molecule itself can play very diverse role in the normal cells and cancer cells, makes the things quite complicated yet interesting. In this regard, molecular targeted therapies are sought and principally include agents which act on aberrant functions and expression of cell cycle involved in the pathophysiology of cancer such as interference with the pathways exclusively expressed in tumor cells. The cell cycle comprises four phases, viz., G_1, S, G_2, and M phase. Depending upon the cell signaling, the cells in G1 phase determines whether to proceed with S phase, apoptosis, or G_0 phase. Upon entering the S phase, DNA synthesis takes place which is followed by G_2 phase ultimately enabling the cell to enter M phase where cell division occurs and cell cycle continues. Concomitantly, the cell cycle processes are also regulated by a variety of kinases referred to as cyclin dependent kinases (CDKs) [75]. A series of CDKs and CDKs inhibitors have been known which are constantly employed for ensuring correct cell division processes [76]. Some of these CDKs also monitor checkpoints that cover DNA damage, antephase, and spindle assembly (Fig. 2.3).

In contrast, tumorigenesis involves multiple complex set of conditions wherein the genetic aberrations and dysregulation of cell cycle occur. Apart from downregulation of tumor suppressor genes such as TP53, BRCA1, BRCA2, alterations in the cell cycle also contribute equally to tumor progression. A variety of mediators have been known which actually bridge the gap between dysregulation of cell cycle and genomic instability such as telomere crisis [77]. Secondly, downregulation of retinoblastoma tumor suppressor protein (pRb2) is regarded as the hallmark of tumor cells and mediates by overcoming the S-phase checkpoint and is referred to as CDK/$p16^{INK4A}$/pRb pathway. Thirdly, p53/HDM2/$p14^{ARF}$ pathway is also considered one of the major cell cycle surveillance pathway operated by HDM2 gene amplification or p53 gene alterations [75]. It affects G1 checkpoint and is sensitive to a variety of stress signals such as DNA damage, hypoxia, etc. Of note, E3 ubiquitin ligase is the key enzyme responsible for p53 ubiquitylation and proteasome inhibition resulting in transcriptional changes and completes negative feedback loop. Inhibition of the CDKs is an important target and is explored to a greater extent. These strategic inhibitors could be designed to compete via inhibition of the ATP binding sites or upregulation of the native CDK inhibitors. Among various

cyclins known till date, cyclin D and E are often found to be overexpressed in a variety of malignancies [78, 79].

In the purview of molecular targeted therapies, the available bioactives could be crudely categorized among three broad generations, viz., first generation comprising ones that act predominantly via DNA damage, synthesis and/or other linked processes such as tubulins, second generation comprising agents that target cancer growth signaling mechanisms such as kinases, etc., and third generation, which is actively updated and is regarded as most recent and under development, comprising agents which act on cellular pathways indirectly related to cancer growth such as chromatin modifiers, protein chaperones, proteasome inhibitors, etc. (Fig. 2.4) [80].

The classical problems associated with first generation anticancer agents include genesis of secondary cancer such as multiple neoplasms in the pediatric patients survived with childhood cancers [81], long terms survivors of patients suffering from testicular carcinomas [82], etc. Furthermore, it also severely affects rapidly proliferating normal cells such as hair follicles, cells of hematopoietic system, gastrointestinal tract lining, and so on. In addition, detrimental effects on post mitotic tissues such as cardiac muscles and that of peripheral nervous systems have also been observed [83, 84].

Second generation molecularly targeted anticancer therapeutics, to some extent addressed the complications associated with classical drugs. Oncogene addiction and non-oncogene addiction targets have been identified which are either direct gene alterations or indirect alterations, respectively [85]. These alterations could be either due to gain- or loss-of-function mutations, amplification and/or overexpression of oncogenes such as MYC, Rb, p53, etc. Recently, it was identified that close to 20 % of kinases play critical role in tumorigenesis[86]. In this regard, Gleevac (imatinib), a potent inhibitor of tyrosine kinase, was the first product approved clinically for chronic myeloid leukemia [87]. Subsequently, a series of drug compounds were approved which includes Lapatinib (HER2 and epidermal growth factor receptor), vemurafenib (B-Raf), vismodegib (Hedgehog signalling pathway), ruxolitinib (Janus kinases), gefitinib (epidermal growth factor receptor), Sunitinib, sorafenib, and pazopanib (multiple tyrosine kinases), and tivatinib (hepatocyte growth factor receptor) [80]. In addition, the monoclonal antibodies, discussed in latter part of this chapter are also considered as second generation therapeutics. The other miscellaneous agents included under this category are non-oncogene addition targets such as checkpoint kinases [88], mTOR[89], etc. Although these agents act predominantly at oncoprotein targets and are less prone to toxic side effects, acquired resistance has been observed quite often with most of the drugs.

Third generation of molecular targeted therapies is further in move considering the complications associated with available drugs which focusses mainly on DNA synthesis, replication, repair, and cell division. These agents include:

2.5.1.1 Agents Acting on Protein Folding and Proteotoxic Stresses

Considering the typical microenvironment in the vicinity of tumor, cancer cells constantly experience a variety of stresses, especially permanent proteotoxic stress

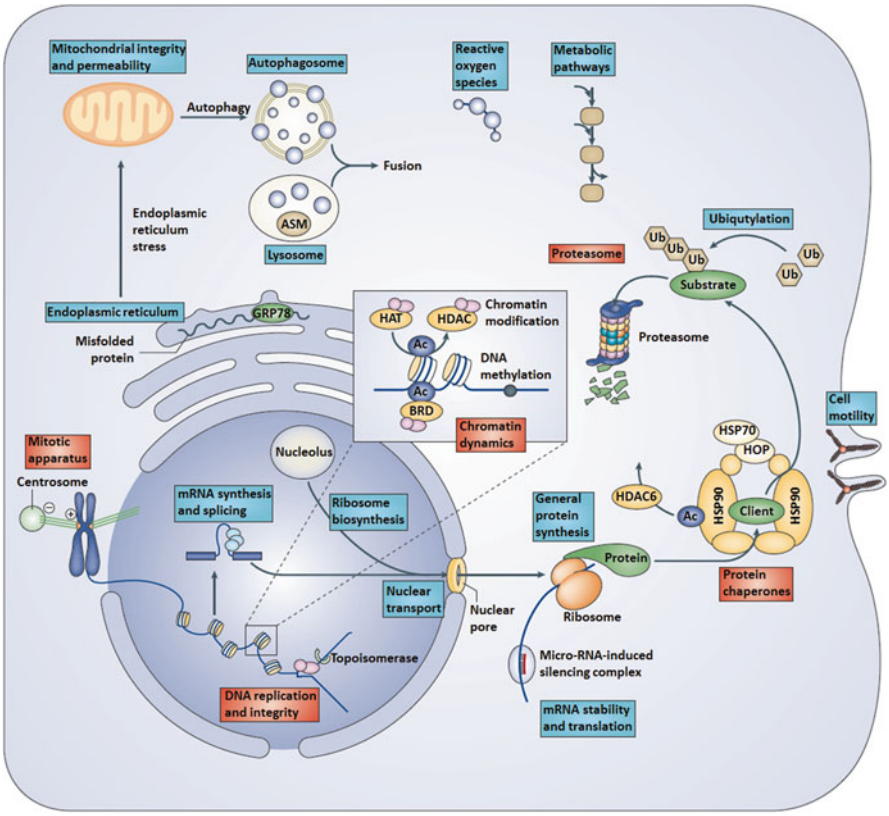

Fig. 2.4 Cellular multicomponent machineries as current and future targets for anticancer drugs. Current targets (shown in *red boxes*) include: DNA replication and integrity; the mitotic apparatus; chromatin; protein chaperones; and the protein degradation apparatus (the proteasome). Drugs that target DNA replication and integrity act via the following mechanisms: by crosslinking nucleobases in DNA and blocking DNA replication; by inhibiting DNA repair; by inserting planar polyaromatic molecules between DNA base pairs and stabilizing the DNA–intercalator–topoisomerase II ternary complex; by interfering with the polymerization of DNA (e.g., via the incorporation of nucleoside analogues); and by inhibiting nucleotide synthesis, typically using antagonists of ribonucleotide reductases or thymidine synthetase. Drugs that target the mitotic apparatus act by binding to the inner portion of microtubules (the "–" end; e.g., taxanes and epothilones), presumably leading to stabilization and enhanced rigidity of the spindle. Vinca alkaloids bind to the "+" end of microtubules—that is, the end that usually elongates the microtubule by adding subunits of α- and β-tubulin—thereby destabilizing the microtubule. Chromatin modification can be targeted by drugs that act on cellular enzyme complexes such as histone deacetylases (HDACs), bromodomain-containing proteins (BRDs) and DNA methyltransferases. Protein chaperones assist in refolding mutated or stress-misfolded proteins. Complexes consist of the heat shock proteins HSP90 and HSP70 (both of which are ATPases), as well as HSP90 organizing protein (HOP; also known as STIP1), multiple co-chaperones, adaptor proteins, the ubiquitin E3 ligase CHIP (carboxy terminus of HSP70 interacting protein) and the associated HDAC6 (a positive regulator and a cytoplasmic deacetylase that keeps HSP90 deacetylated and active). Drugs can inhibit HSP90, HSP70, or HDAC6. Drugs can inhibit different protease activities—e.g., chymotrypsin-like activity, trypsin-like activity, and/or caspase-like activity—within the 26S proteasome to disrupt the protein degradation apparatus. The ubiquitylation machinery and ubiquitin retrieval can also be manipulated by small molecules, providing additional opportunities for interfering with proteasomal degradation. Future targets for third-wave anticancer drugs are illustrated in *blue boxes*. *ASM* Acid sphingomyelinase, *GRP78* 78 kDa glucose-regulated protein. Reproduced from [80]

which is generally caused by the misfolding and aggregation of proteins. The latter effect is predominantly observed in cancer cells owing to molecular crowding of the cellular milieu [90]. Usually such stresses are counterfeited by a group of molecules inclusive of chaperones and protein remodeling factors. Many of these are responsive to heat and hence referred to as heat shock proteins (HSPs). HSPs interact with their client protein with the help of co-chaperones; however, under extremities of cellular stresses owing to oncogene alterations, chaperone pool vanishes quickly and chaos originates. In complementarily reactive oxygen species further exaggerates the situation and lead to even higher proteotoxic stress [91]. Taking HSP90 as potential target, a variety of therapeutics have been designed for molecular targeting of cancer which includes 17-allylamino-17-demethoxygeldanamycin (17 AAG), ganetespib, STA-9090, IPI-493, retaspimycin, tanespimycin, geldanamycin, radicicol, AT-13387, NPV-AUY922, KW-2478, BIIB-021, MPC-3100, NVP-HSP990, PU-H71, etc. [92] (Table 2.3).

2.5.1.2 Proteasome Inhibitors

Ubiquitin-proteasome system is yet another approach to address the proteotoxic stress covering almost 90 % of the total protein clients [93]. Mechanistically, it follows two steps essentially comprising ubiquitin conjugation mediated by a series of enzymes such as E1 (Ub-activating), E2 (Ub conjugating), and E3 (Ub ligating) to yield Lys48 linked proteins which are then processed for proteasomal degradation via 26S proteasome complex (Fig. 2.5). Herein tumor cells, the proteasome functions and its need is always unmet and hence pose a potential target in anticancer therapy. Concomitantly, the PSMC2 gene alterations further adds complexity to the overall situation. The classical proteasome inhibitors were designed to interact with proteasomal components such as 20S core subunit (Bortezomib, carfilzomib, etc.) [94], interference in the deconjugation of ubiquitin and substrates [95], inhibition of ubiquitin specific peptidase-14 [96], allosteric inhibition of E2 enzyme [97], neddylation[98], etc.

2.5.1.3 Targeting Chromatin Modifications

Chromatin is crudely regulated by three categories of enzyme systems, viz., epigenetic writers, epigenetic erasers, and epigenetic readers [99]. The first category includes enzymes which are responsible for adding chemical moieties to histones or DNA such as acetyltransferases, methyltransferases, etc. The second set of enzymes is primarily responsible for deletion of groups and includes deacetylases, demethylases, etc. The third group comprises the protein modules that are responsible for chromatin binding leading to either upregulation or downregulation of transcription processes, e.g., H3K4me3/2-specific histone demethylase. H3K4me3/2 is a ligand specific for plant homeodomain finger (PHD), zinc finger-like domain, with Cys4-His-Cys3 signature motif [100]. The principal chromatin modification targets

Table 2.3 List of Hsp 70 target based drug delivery system for cancer immunotherapy

Hsp 70 inhibitor	Source	Remarks
Geldanamycin (GA)	Natural benzoquinone ansamycin antibiotic	Compete at the ATP-binding site to induce the degradation of Hsp90 via the proteasome machinery of ubiquitin ligase
Radicicol (RD)	Natural macrocyclic lactone antibiotic	Inhibitory effect against tyrosine kinases countered by reducing agents such as dithiothreitol
17-DMAG	Derivatives of GA	Increased water solubility and better oral bioavailability
IPI-504	Hydroquinone derivative of 17-AAG	IPI-504 shows higher water solubility and also high mortality rate
IPI-493 (17-AG)	Metabolite of 17-AAG	Longer circulation time
KF25706	Oxime derivatives of RD	Stable in the presence of dithiothreitol (DTT)
Herbimycin A (HA)	Benzoquinoid ansamycin antibiotic	Tyrosine kinase inhibitor and exhibits severe hepatotoxicity
KW-2478	Non-ansamycin resorcinol derivatives	KW-2478 caused degradation of FGFR3 as well as Hsp90 proteins, i.e., IGF-igβ and c-Raf-1, which resulted in cleavages of PARP and activation of intrinsic apoptotic pathway
NVP-AUY922	Resorcinol derivatives	Evaluated in Phase I/II clinical trial for NSCLC, breast cancer, colorectal cancer, and advanced gastric cancer and visual toxicity, i.e., night blindness and blurred vision, was reported
HSP990	Resorcinol derivatives	Acts via proteasomal degradation of oncogenic client proteins
AT13387	Resorcinol derivatives	Evaluated in Phase II clinical trial for gastrointestinal stromal tumor in combination with imatinib
Gatenespib (STA-9090)	Resorcinol derivatives	Evaluated in multiple clinical trials for both advanced solid
		Tumors (NSCLC, colorectal, stomach, ocular melanoma, pancreas, prostate, breast) and hematological malignancies
BIIB-021 (CNF2024)	Purine-scaffold based rational drug	Evaluated in phase I clinical trials for advanced solid tumor, B-cell chronic lymphocytic leukemia
SNX-5422/ PF-04929113	Pyrazole containing scaffolds	SNX-5422/PF-04929113 is water-soluble prodrug of SNX-2112/PF-04928473 and is discontinued in Phase I study due to ocular toxicity

include methylation of DNA and a variety of other histone modifications such as acetylation, ubiquitylation, phosphorylation, etc. Two drugs, 5 azacytidine and decitabine represent the pioneer drugs approved for myelodysplastic syndrome. Recent advances in this area of molecular targeted therapeutics include design of novel agents affecting chromatin modifications such as histone deacetylase (HDAC)

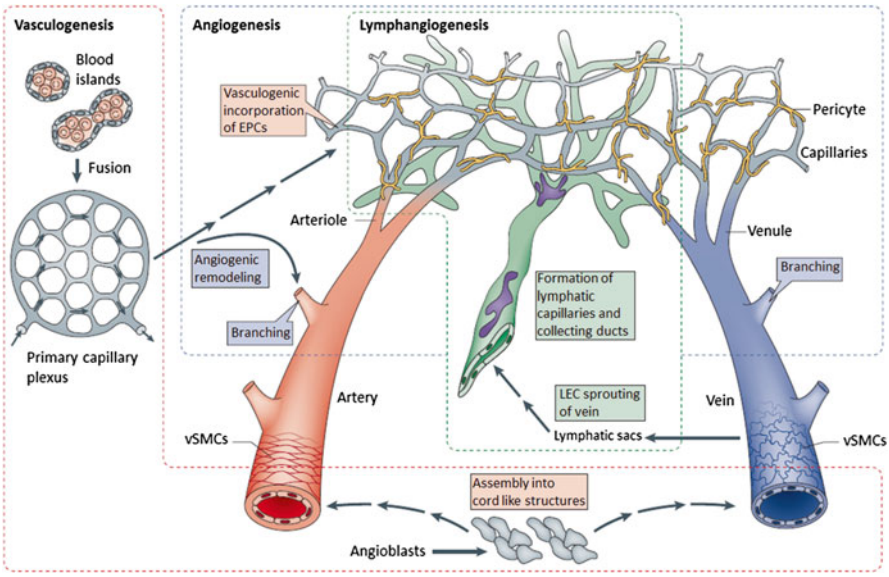

Fig. 2.5 Origin of endothelial cells and assembly of the vasculature. Mesodermal cells in the early embryo differentiate into endothelial precursor cells (EPCs, angioblasts) and form aggregates, known as blood islands (*left*). Fusion of blood islands leads to the vasculogenic formation of honeycomb-shaped primary capillary plexi in the yolk sac and embryo itself. Blood circulation is established and primary plexi are remodelled into a hierarchical network of arterioles and arteries (*red*), capillaries (*grey*), and venules and veins (*blue*). The dorsal aorta and cardinal vein are directly formed through the assembly of angioblasts. The vasculogenic incorporation of circulating EPCs into growing blood vessels may contribute to regenerative or pathological neovascularization in the adult. Vascular smooth-muscle cells (vSMCs) are associated with arteries and veins, whereas capillaries are covered by pericytes (*yellow*). The first lymphatic endothelial cells (LECs) sprout from the embryonic veins, then migrate and form lymphatic sacs. Further steps of lymphangiogenic growth involve sprouting, branching, proliferation, differentiation and remodeling processes. The recruitment of lymphangioblasts from the adjacent mesenchyme has been speculated to be a further source of LECs. Blind-ending lymphatic capillaries (*green*) feed into collecting vessels and ducts. These larger lymphatics are sparsely covered by SMCs (*purple*) and contain valves that prevent backflow. Reproduced from [108]

inhibitors, bromodomain and extra-terminal (BET) proteins downregulators, EZH2 inhibitors affecting histone methyltransferases, etc. [80].

2.5.2 *Tumor Angiogenesis*

In routine physiological conditions, the vascular network develops in three phases, viz., vasculogenesis, angiogenesis, and vascular remodeling. The first step comprises migration of angioblasts to the desired site followed by differential into endothelial cells and subsequent formation of initial vascular plexus [101]. The primary

vascular plexus then undergoes angiogenesis either sprouting or non-sprouting in the presence of various endothelial growth factors and is subjected to remodeling (Fig. 2.5) [102]. Classical long held view on the tumor angiogenesis was restricted to the fact that blood vessels in the tumor microenvironment grow only from the preexisting vessels. However, the recent advances in the field of vascular pharmacology revealed that vasculogenesis contributes majorly in tumor progression and share of endothelial cells derived from endothelial progenitor cells goes shoots beyond 40 % [103]. In purview of this, a variety of factors drives angiogenesis and includes vascular endothelial growth factors (VEGFs), fibroblast growth factors, angiopoeitins, netrins, semaphorins (class 3), SLIT proteins, JAG1, DLL4, ephrins, etc. The process of neovascularization is of utmost importance and starts as early as embryogenesis. However, angiogenesis has also been classically associated with a list of pathological conditions including cancer. Although the developmental and pathological angiogenesis operates on similar line of action, the major difference among both is that the latter remains unresolved and principally driven by pathological condition to address the unmet demands of nutrients and oxygen for cells in concern [104].

Crudely, angiogenesis is initiated in tumor upon reaching the size 1–2 mm and is typically coordinated by the hypoxic microenvironment of the tumor. Hypoxia inducible factors (HIFs), heterodimeric transcription factors, direct the expression of VEGF-A for angiogenic sequences [105]. Interestingly, since a series of physiological mediators are required for developing a fully functional vascular system, mere overexpression of VEGF renders aberrantly formed vessels that are often tortuous, fragile, pericyte deficient and leakier to raise the interstitial hypertension and often poor delivery of therapeutics to tumors [106]. Yet another complexity associated with tumor angiogenesis is *vasculogenic mimicry* which is actually a dedifferentiation program wherein the stem-like cells assist in formation of vascular system [107]. Cumulatively, these factors needs to be considered while employing and designing the antiangiogenesis based cancer chemotherapy.

Angiogenesis is considered as very dynamic process and is often regulated by a variety of indigenous angiogenic inducers and inhibitors. The former category includes principally VEGF-A, matrix metalloproteinases, fibroblast growth factors, placental growth factors, hepatocyte growth factors, etc., whereas the latter comprises thrombospondins, endostatin, angiostatin, and cytokines [109]. In the purview of antiangiogenic therapy, Fig. 2.6 depicts the possible targets for efficient management of tumor angiogenesis[103]. Classically, two approaches have been employed for targeting key regulators, i.e., VEGFs. Physiologically, VEGFs signaling system comprises five targeting ligands, VEGF-A to D and placental growth factor and three receptors, viz., VEGFR1-3 tyrosine kinases. The first approach comprises the use of antibodies for VEGF or its receptors and a series of drugs have already been approved clinically such as Bevacizumab (humanized variant, VEGF) and some others are under investigation such as VEGF-$Trap_{R1R2}$. However, the use of such antibodies is associated with side effects, pharmacoeconomic complications, etc. and hence second approach of VEGF receptor kinases inhibitor could also be sought for. Sorafenib was the pioneer candidate in this category to be

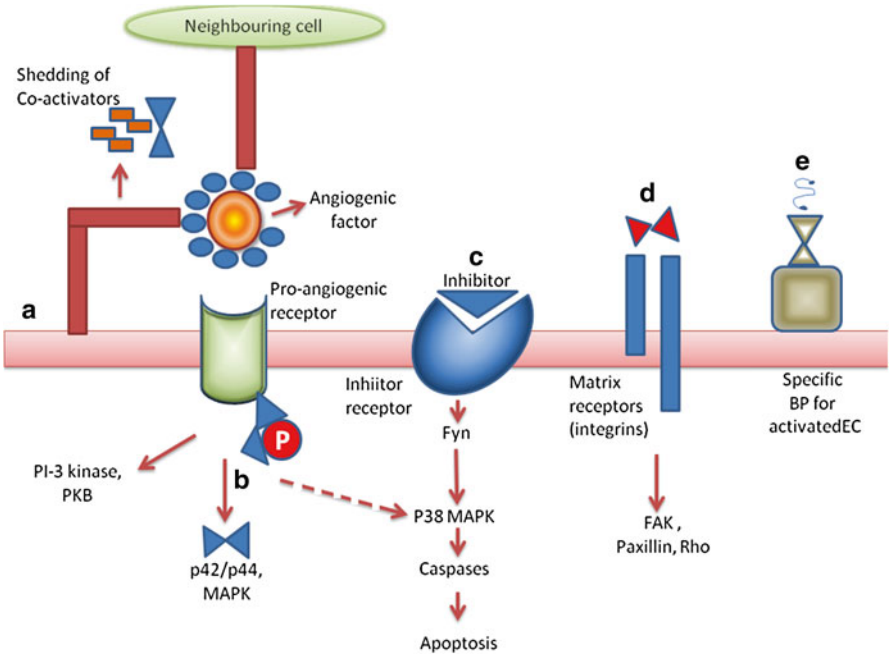

Fig. 2.6 Targeting tumor vasculature to inhibit angiogenesis. (**a**) Inhibition of binding to pro-angiogenic receptors and/or altering the interaction of angiogenic factors with co-receptors. (**b**) Penetration into the cells followed by binding with tyrosine kinase receptors (**c**) Direct activation of receptors, e.g., thrombospondin peptide mimetics (**d**) Extracellular matrix receptors, e.g., αvβ3 integrins. (**e**) Nonspecific inhibitors of proliferation

approved clinically and was followed by sunitinib, pazopanib, axitinib, etc. Interestingly, significantly higher therapeutic efficacy was noted upon combination of first approach with conventional chemotherapeutics which could be attributed to the *vascular normalization* capabilities of monoclonal antibodies leading to increased delivery of anticancer drugs to tumors. Bevacizumab when combined with irinotecan, fluorouracil, and leucovorin, improved therapeutic efficacy against metastatic colorectal cancer [110]. On similar line of action, combination of aflibercept with fluorouracil, leucovorin, and irinotecan drastically improves the survival in patients with metastatic colorectal cancer [111]. In contrast the actives in the second approach usually work best as single agent and the reasons for discrepancy is in part attributed to the tumor stromal architecture, intrinsic sensitivity, and resistance [112]; however, exact mechanisms are still under investigation [113].

Secondly, thrombospondin-1 represents the naturally occurring secretory angiogenic inhibitors and is principally responsible for organization of the perivascular matrix, endothelial cell adhesion and other process to counterfeit angiogenesis[114]. Numerous therapies have been employed for upregulation of thrombospondins such as metronomic dosing of antiangiogenic agents [115]. Cyclophosphamide has been found to upregulate circulating TSP-1 and not TSP-2 [116]. Notably, said approach

is also reported to sensitize the endothelial cells for TSP induced apoptosis mediated by Fas receptor overexpression [117]. In separate set of experiments, polymer implants containing TSP-2 overexpressed fibroblasts significantly increased therapeutic efficacy in the ovarian carcinoma and drastically higher levels of circulating TSP-2 were noted even after 5 weeks [118].

Thirdly, matrix metalloproteinases (MMPs) are the class of proteolytic enzymes primarily involved in the degradation of extracellular matrix and are part of well coordinated system of growth factors, inflammatory mediators and cell receptors [119]. These macromolecules are important for a variety of physiological functions including angiogenesis and hence, also explored as potential target in design antiangiogenic therapeutics [120]. Although dedicated MMPs inhibitors (MMPI) have not been successfully clinically, research is at advance stages to identify the specific properties which could be sought for rationalized development of MMPI leads.

Further, apart from VEGF based antiangiogenics which primarily block neovascularization, vascular disrupting agents represents a class of bioactives that selectively destroys the already formed tumor vessels by targeting dysmorphic endothelial cells [121]. Combretastatin A4 phosphate is a potent naturally occurring tubulin inhibitor and leads to vascular collapse and shut down of developed vessels and thus impart tumor regression [122]. Mechanistically, these are also reported to interfere with the functions of cadherins, thereby resulting in tumor necrosis. The other vascular disrupting agents include 5,6-dimethylxanthenone-4-acetic acid (DMXAA) that tends to increase nitric oxide, serotonin and tumor necrosis factor-α [123]; TZT-1027: dolastatin-10 analog; ZD6126: interferes with microtubules, Exherin®: cyclic pentapeptide; AVE8062A, ASA404, and MN-029.

2.5.3 Cancer Immunotherapy

The cancer immunotherapy seems to have a great potential in terms of clinical cancer therapy considering the remarkable progress in the field of molecular identifications of tumor antigens and increased understanding of various immunoregulatory pathways operative in the tumor microenvironment [124]. It is now well established that the tumor cells pose antigenicity and can be recognized by a variety of immune cells. Recently, the implementation of the shared tumor antigens has been largely replaced with the neoantigens that are generated by point mutations of genes specific to particular types of tumors. This has obvious advantage of improving the antitumor efficacy of the T cells by reducing the nonspecific interactions with that of normal host tissues and increasing the avidity of interactions among antigenic peptide and MHC molecule [125]. Exsome sequencing has been recently employed for defining the mutant antigens for a variety of cancers [126]. Furthermore, more than 20 antibodies are already approved for a series of disease conditions and a large number of them are under exhaustive investigation. Principally, these act through antibody dependent cellular cytotoxicity (ADCC) and complement-activation dependent cytotoxicity (CDC) for imparting cytotoxicity

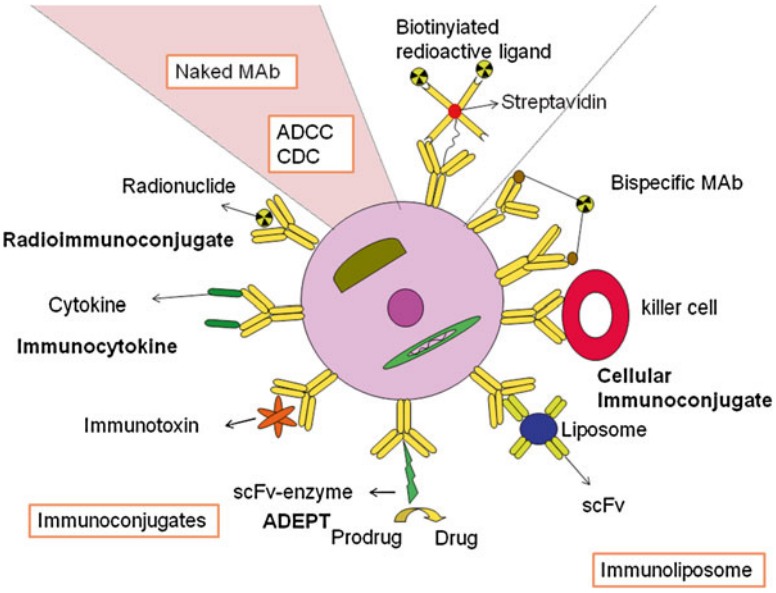

Fig. 2.7 Strategies for immunotherapy of cancer

and play a crucial role in efficient management of a variety of cancers [127]. In contrast to monotherapies, these antibodies are recently explored as adjuvants in drastically improving the therapeutic effectiveness of the conventional chemotherapies. Recently, antibody derived fragments have been explored to a greater extent as compared to that of parent whole antibodies considering the advantages such as manageable small size, relative ease in production, economic factors, and feasibility in antibody engineering for tailor-made applications. Figure 2.7 depicts various types of strategies that are currently employed for cancer immunotherapies [99, 128, 129].

2.5.3.1 Radioimmunotherapy (RAIT)

RAIT or Radioimmunoconjugates refer to the macromolecular entities where monoclonal antibodies (mAbs) are covalently attached to high linear energy transfer (LET) radionuclides. The radionuclides could either be alpha emitters such as ^{213}bismuth, ^{211}astatine, ^{223}radium, ^{149}terbium, ^{225}actinium, ^{227}thorium, ^{230}uranium or beta emitters such as ^{131}iodine, ^{90}yttrium, ^{60}cobalt, ^{99m}technetium capable of damaging DNA either by strand breakage or by other effects ultimately resulting in cell death. Concomitantly, the antibodies itself could also be detrimental to the cancerous cells by altering the signal transduction pathways such as alterations of signaling pathways and depression in gene expression by anti-CD20. Further, the conjugation of antibody with radiommunoconjugate leads to more *favorable biodistribution* [130]. Considering the huge potential of RAIT, a couple of products (Zevalin™,

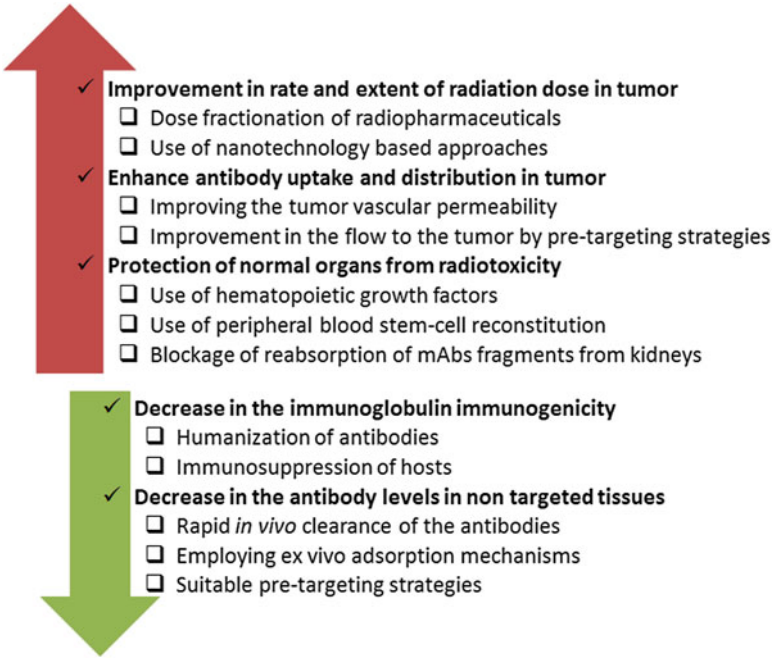

Fig. 2.8 Strategies to improve the therapeutic effectiveness of tumor radioimmunotherapy

Bexxar®, etc.) have already paved their way in clinical segment for treatment of non-Hodgkin lymphomas and many more are under clinical trials.

Since its genesis in early 1950s, the major applicability of RAIT has been hematological malignancies and few decades of research established preferential localization within tumor and subsequent therapeutic potential [131]. Drastic reduction in the bulky masses was noted in patients non-Hodgkin lymphomas treated with ^{131}I Lym-1 monoclonal antibodies [132]. Similar results were also observed with other antibodies such as anti-CD37 [133] and anti-CD20 [134]. The principal factors affecting RAIT includes type of mAbs, nature of radionuclide, and targeted host and tumor. The overall effectiveness of the RAIT further depends on the other factors such as rate and extent of dose administration, tissue penetration and sensitivity, location of target antigens (on tumor or within tumor), bone marrow toxicities, and tumor microenvironment [135]. Figure 2.8 depicts probable strategies that could be employed for improving the efficacy of RAIT whereas Table 2.4 reflects the exhaustive list of radionuclide and monoclonal antibodies employed for efficient management of cancer.

Recently, combination of neoadjuvant radiation therapy and chemotherapy are concomitantly employed for efficient management of cancers. Numerous clinical trials are presently undertaken in the direction of assessing the safety and antineoplastic potential of such RAIT, among which majority of trials encompasses combination of external-beam radiation therapy (EBRT), chemotherapeutics, and

Table 2.4 Exhaustive list of radionuclides and monoclonal antibodies employed in combination for efficient management of cancer

Radionuclide	Monoclonal antibody	Outcomes	Ref.
^{211}At	A33	Potential for treatment of micrometastases originating from colorectal carcinoma	[138]
^{213}Bi	Anti-EGFR	Improved therapeutic efficacy of radiation mediated by enhanced DNA damage	[139]
^{213}Bi	C595	Useful tool for the treatment of micro metastases or minimal residual disease (MRD)	[140]
^{213}Bi	Herceptin	Improved cell cytotoxicity against BT-474, SK-BR-3, and MDA-231 cell lines	[141]
^{213}Bi	MTAT	Inhibits lymph node micrometastases by induction of apoptosis	[142]
^{131}I	CC49 $(scFv)_2$	Provides a promising delivery vehicle for therapeutic applications	[143]
^{123}I	Tat-peptide	Significant G1–S phase arrest and efficient targeting of nuclear epitopes	[144]
^{131}I, ^{88}Y, ^{177}Lu, ^{186}Re	cG250	Improved stability and specific activity of the radionuclide conjugates	[145]
^{131}I, ^{88}Y, ^{188}Re	Mu-9 anti-CSAp	Promising results in the treatment of the GW-39 human colonic carcinoma	[146]
^{111}In	Anti-γH2AX	Significant increase in in vitro and in vivo anticancer efficacy	[147]
^{111}In	HuCC49ΔCH2/ cCC49	~4-fold appreciation in tumor to blood localization ratio of antibody conjugate	[148]
^{111}In	Mouse IgG (mIgG)	Tumor targeting was found to increase up to 15-fold	[149]
^{111}In	Trastuzumab	Shown potential HER2 specific targeting and radionuclide delivery ability	[150]
^{111}In	U36	Significantly higher uptake in tumor with a favorable biodistribution	[151]
^{111}In and ^{177}Lu	HER2/trastuzumab	Improved in vivo biodistribution profiles, tumor uptake and tumor-to-tissue activity	[152]
^{111}In and ^{90}Y	CC49	Significant reduction of extrahematopoietic toxicity	[153]
^{177}Lu	chCE7/ ChCE7agl	High and specific accumulation of radioactivity with enhanced antitumor efficacy	[154]

(continued)

Table 2.4 (continued)

Radionuclide	Monoclonal antibody	Outcomes	Ref.
^{177}Lu	M-BR96	Increased efficacy without significant increase in toxicity	[155]
^{212}Pb	Trastuzumab	Increased uptake rate in the tumor over a 72-h period with reduced systemic toxicity	[156]
^{149}Pm, ^{166}Ho, ^{177}Lu	CC49	Significant increase in the tumor uptake	[157]
^{99m}Tc	BIWA 1	Increased selectivity and tumor uptake with lower toxicity	[158]
^{227}Th	Rituximab	Novel approach for targeted delivery system	[159]
^{227}Th	Trastuzumab	Increased cumulative absorbed radiation dose to tumor by fractionation of the dosage	[160]
^{90}Y	HMFG1	Shown potential treatment efficacy at a dose of 18.5 mCi/m^2 with reduced toxicity	[161]

immunotherapy [136]. The principal component of the immunotherapy is usually a vascular endothelial growth factor (VEGF)-blocking antibody, whereas preferred chemotherapeutics is a platinum derivative such as cisplatin, oxaliplatin, etc. Table 2.5 lists the clinical trials presently undergoing on cancer RAIT [137].

With the advent of nanotechnology based approaches, the therapeutic effectiveness of the RAIT has been drastically improved. The said approach has been exclusively exploited in a variety of preclinical and clinical conditions and its potential has now been widely accepted. The principal amenities associated with these nanoparticles are their diverse functionalization potentials which help is improving the therapeutic efficacy and reducing the unwanted and toxic side effects to normal tissues. Figure 2.9 depicts the general architecture of these multifunctional nanoparticles and various advantages associated with said approach. In this context, a variety of nanocarriers have been fabricated till date for management of cancer and could broadly be categorized under the banner of either organic or inorganic nanocarriers [162]. Furthermore, employing the radiation chemistry, a radionuclide is conjugated to the antibody to form radionuclide–antibody conjugates which could be either conjugation of peptide ligands and antibodies or tumor targeted antibodies and radioisotopes [163]. The latter is then in incorporated into nanoparticles to yield targeted radioisotope labeled nanoparticles [164]. Table 2.6 list various nanocarrier based approaches employed for RAIT. The principal disadvantages with either plain radiation therapies or radio antibody conjugates are longer half lives, higher ϒ-emissions leading to undesirable side effects, inefficient internalization within lysosomes, mandate chelator requirements which often is associated with poor targeting or nonuniform dosimetry, etc. [165].

Table 2.5 Representative list of clinical trials comprising of radioimmunotherapy

Neoplasms	Phase	Radiation/antibody	Drug
Abdominal	I-III	^{90}Y-HMFG-1, ^{212}Pb-TCMC-Trastuzumab, ^{131}I-8H9	-
Adenocarcinoma	0-II	^{90}Y-m170, ^{90}Y-MN14, ^{18}F-FDG, cetuximab, filgrastim	Paclitaxel, docetaxel, cisplatin
Bronchial	0-II	^{131}I-L19SIP, ^{177}Lu-IMP-288, ^{111}In-IMP-288, antibody TF2, Pre-targeted radioimmuno-therapy, ^{90}Y-antiCEA cT84.66, ^{18}F-FDG, cetuximab	Cisplatin, docetaxel
Carcinoma	0-III	^{131}I-di-DTPA, carbon ion boost, ^{177}Lu-IMP-288, ^{111}In-IMP-288, ^{131}I-L19SIP, ^{90}Y-antiCEA cT84.66, ^{90}Y-DOTA anti-CEA M5A, ^{90}Y-HMFG-1, ^{111}In-MN14, antibody TF2, hMN14 (labetuzumab), cetuximab, bevacizumab, filgrastim	Doxorubicin hydrochloride, docetaxel, cisplatin, 5-fluorouracil, irinotecan hydrochloride, leucovorin calcium
CNS	I-II	^{131}I-3 F8, ^{131}I-8H9, ^{131}I-L19SIP	Cisplatin, lomustine, vincristine sulfate
Colorectal	I-II	^{177}Lu-IMP-288, ^{111}In-IMP-288, ^{90}Y-DOTA anti-CEA M5A, ^{90}Y-antiCEA cT84.66, ^{111}In-IMP-205xm734, ^{111}In-MN14, ^{90}Y-MN14, TF2, hMN14 (labetuzumab), filgrastim, bevacizumab	Oxaliplatin, leucovorin calcium, fluorouracil, gemcitabine, floxuridine, irinotecan, hydrochloride
Leukemia	I-II	^{90}Y-Epratuzumab, ^{111}In-LL2 IgG, ^{111}In-MN14, ^{131}I-Anti-B1, ^{131}I-BC8, ^{111}In-ibritumomab tiuxetan, ^{90}Y-ibritumomab tiuxetan, ^{131}I-tositumomab, rituximab, filgrastim, oprelvekin	Busulfan, cyclosporine, cyclophosphamide, etoposide, fludarabine phosphate, methotrexate, mycophenolate mofetil, melphalan, sirolimus, tacrolimus
Liver	I-II	^{90}Y-antiCEA cT84.66, ^{90}Y-DOTA anti-CEA M5A	Gemcitabine hydrochloride, floxuridine, fluorouracil, leucovorin calcium, oxaliplatin
Lymphomas	I-II	^{111}In-ibritumomab tiuxetan, ^{90}Y-ibritumomab tiuxetan, ^{131}I-tositumomab, ^{111}In-LL2 IgG, ^{90}Y-epratuzumab, ^{111}In-Lym-1, ^{90}Y-Lym-1, ^{111}In-MN14, rituximab, filgrastim	Cyclophosphamide, cisplatin, cytarabine, methylprednisolone, fludarabine phosphate, etoposide
Ovarian	I-III	^{212}Pb-TCMC-Trastuzumab, ^{90}Y-HMFG-1, ^{111}In-MN14, ^{90}Y-MN14, filgrastim	–
Prostrate	I	^{117}Lu-J591, ^{90}Y-m170, filgrastim	Cyclosporine, paclitaxel

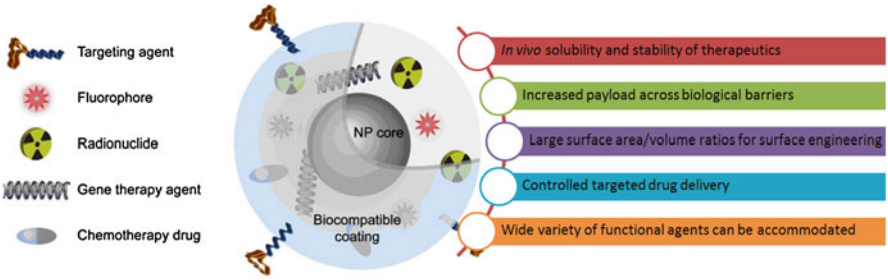

Fig. 2.9 General architecture of multifunctional nanoparticles and associated principal advantages. Adapted and modified with permission from [166]

2.5.3.2 Immunotoxins

Immunotoxins represents a group of macromolecular species essentially comprising toxin conjugates and targeting antibodies linked via gene fusions, peptide bonds, disulfide bonds, thioether bonds, etc. [180]. Upon successful internalization within target cells, the immunotoxins releases toxin by a variety of mechanisms such as degradation by proteases, reduction of disulfide bonds, or acid hydrolysis (Fig. 2.10). Primarily bacterial toxins such as diphtheria toxins, *Pseudomonas* exotoxin, and plant toxins such as ricin, modeccin, abrin, etc. are employed as toxin conjugates. These are usually proteinaceous in nature and are considered as highly potent, owing to which even limited availability at site of action is sufficient for execution of cellular responses [181]. Once inside the cells, these tend to inhibit the protein synthesis pathways and result in cell cytotoxicity. Although fascinating, the clinical intervention of immunotoxins is a great challenge owing to associated limitations such as poor antigen specificity, lower cytotoxicity potential, nonspecific side effects, immunogenicity, and manufacturing complications. Recently, in the last couple of decades, strategic developments in the field of immunotoxins have been noticed. Started with its genesis from isolation of potential toxins in early 1970s, a variety of immunotoxins have made their way to clinical trials successfully clearing in vitro tissue culture experiments and in vivo preclinical testing. One product, DT-IL2 (denileukin diftitox, Ontak™), has been clinically approved for human use and specifically targets IL-2 receptors [182].

The targeting antibodies could either be whole antibody or small fragments. In the latter case, A chain subunit of the toxin is linked to the monoclonal antibody and are referred to as A chain immunotoxins. A principal advantage associated with such A chain immunotoxins is lower nonspecific side effects in in vivo setting; however, in contrast some significant compromise in overall targeting potential and cell cytotoxicity was noted as compared to whole antibody immunotoxins [183]. The results are indicative of the crucial role of B chain in improving the interactions of immunotoxins with target cells and preferential assistance in entry to cytosol. Furthermore, considering the pharmacokinetic perspectives, the larger the constructs, the longer the circulation half-life. Furthermore, classical to any anticancer

Table 2.6 List of nanocarrier based approaches employed for RAIT

Radionuclide	Nanocarrier approach	Functionalization	Outcomes	Ref.
^{90}Y	Polymerized liposomes	Integrin antagonist; anti-Flk-1 mAb	Significant tumor growth delay in K1735-M2 and CT-26 tumors	[167]
^{99m}Tc	PEGylated liposomes	MIBI	Twofold higher uptake in MCF-7 ras tumor bearing mice	[168]
^{111}In, ^{88}Y	PAMAM dendrimer	Humanized anti-TacIgG (HuTac)	Significant differences in the biodistribution patterns of the saturated and unsaturated dendrimers were noted	[169]
^{111}In	PEGylated PE micelles	2C5	Significantly higher tumor accumulation in murine LLC	[170]
^{111}In	Albumin nanoparticles	RGDGSSV peptide, fibrinogen	Significant retardation in tumor growth and tumor specific reduction blood flow to B16F0 hind limb tumors	[171]
^{111}In	Perfluorocarbon nanoparticles	$\alpha\nu\beta_3$-integrin binding[a]	Fourfold higher mean tumor activity in Vx-2 tumor bearing rabbits as compared to nontargeted controls	[172]
^{188}Re	Liposomes	Doxorubicin	Significant increase in the therapeutic efficacy against C26 murine solid tumor animal model	[173]
^{111}In	Nanocapsules	Polysaccharides	Significant retardation in the clearance rate and preferential biodistribution within lymphatic system	[174]
^{131}I	Dextran magnetic nanoparticles	Sc-7269	Significant tumor growth delay and tumor inhibition rate were noted without any compromise in safety profile	[175]
^{90}Y	Apoferritin	Biotin	Significant pre-targeting capabilities	[176]
^{125m}Te	ZnS nanoparticles	mAb 201B	Significantly higher localization within lungs	[177]
^{64}Cu	Carbon nanotubes	RGD	Significantly higher tumor uptake with minimal renal clearance	[178]
^{225}Ac	PEGylated liposomes	PSMA J591 antibody, A10 PSMA aptamer	Significantly higher cytotoxicity against PSMA overexpressing human LNCaP cells, rat Mat-Lu cells and HUVEC cells	[179]

[a]Perfluorocarbon nanoparticles bind to $\alpha_v\beta_3$-integrin receptors

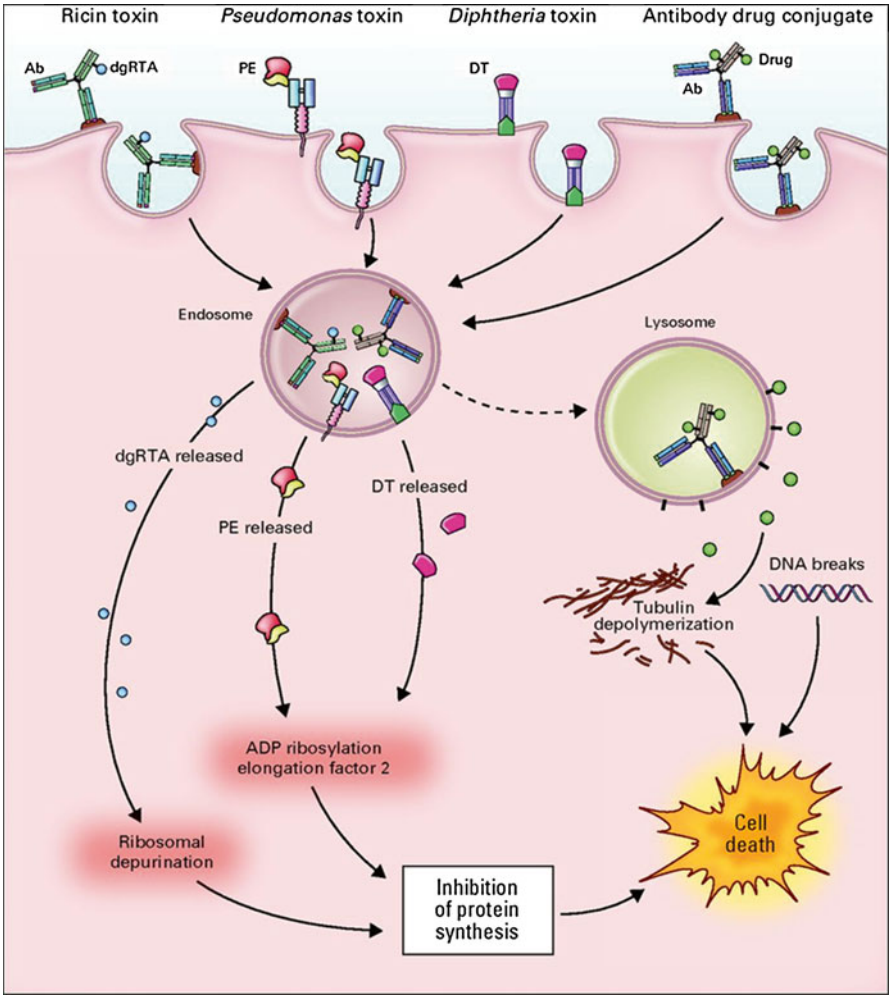

Fig. 2.10 Mechanisms of action of monoclonal antibody (Ab) conjugates. Monoclonal antibodies and their fragments can be conjugated or linked to cytotoxic agents. Chemotherapy and toxin conjugates must be internalized via receptor-mediated endocytosis, whereas internalization is not required for radioisotope conjugates. After internalization, the active cytotoxic component is released and mediates cell death. Ricin-based immunotoxins depurinate ribosomal RNA and inhibit protein synthesis. Pseudomonas (PE)- and diphtheria (DT)-derived immunotoxins ADP ribosylate elongation factor-2 and inhibit protein synthesis. Antibody drug conjugates mediate cytotoxicity by drug-specific actions (e.g., targeting tubulin by maytansin and auristatin, and induction of DNA breaks by calicheamicin). *dgRTA* deglycosylated ricin A chain. Reproduced from ref. [186]

chemotherapy, the potency of immunotoxins could be drastically improved by coadministration with *enhancing agents*. Such improvements in the entry of immunotoxins within the cells could be facilitated by understanding the vesicular entry systems classical to proteins, employing the pathways adapted by natural toxins

during pathological conditions, and exploiting the structure–function relationships of natural toxins in designing the entry systems [184]. Classically, pharmacologically active molecules such as lysosomotropic amines or carboxylic ionophores have also been employed as enhancing agents [185].

Concomitant with high therapeutic potency, there also lie numerous side effects associated with immunotoxins. These includes flu-like syndrome, vascular leak syndrome, infusion related hypersensitive reactions, and transient increase in the levels of hepatic toxicity markers such as transaminases owing to preferential processing of immunotoxins in liver [33]. Furthermore, immunotoxicity can also occur either due to monoclonal antibodies or toxins which could be marginally circumvented by employing antibody alterations or humanization.

Table 2.7 list various immunotoxins employed for management of cancer. Particularly, deglycosylated ricin A (dgA) chains have been particularly explored in a wide array of malignancies considering diminished hepatotoxicity (owing overexpression of mannose receptors in liver) classically observed with anti-B4 blocked ricin [187]. The lateris also associated with significant human anti-mouse antibody (HAMA), anti-ricin (HARA) immune responses and vascular leak syndrome [188]. In purview of this, the particular amino acid sequences in toxins responsible for both therapeutic effects and detrimental side effects have been identified and employed [189, 190]. The reduction in HARA immune responses could be mediated by employing the PEGylated ricin as compared to plain ricin [191]. Interestingly, PEGylation does not affect the inhibition of protein synthesis pathways by ricin.

Notably, immunotoxins usually lacks any bystander effects. However, considering the very therapeutic potency, this property could be fruitfully exploited in combination therapeutic regimen to combat minimal residual diseases, particularly hematological malignancies. Immunotoxins along with RAIT have been successfully employed for treating disseminated human B-cell lymphoma in immunodeficient mice model and curative therapeutic regimen was observed by optimizing the temporal order of administration without any life threatening vascular leak syndrome (Fig. 2.11) [209].

2.5.3.3 Immunocytokines

Immunocytokines represents yet another important type of macromolecular species employed for efficient management of cancer. These are fusion proteins essentially comprise monoclonal antibody and cytokine. Cytokines are referred to as cell signaling molecules responsible for cell–cell communication and mediate a variety of humoral and cellular immune responses to maintain homeostasis [210]. These include interleukins, interferons, chemokines, colony stimulating factors, lymphokines, and tumor necrosis factors, to name a few. Mechanistically, immunocytokines tend to accelerate the compromised tumor immune responses and hence the immunocytokines therapy seems to be most promising as compared to any other immunotherapies (Fig. 2.12). Considering such potential, IL-2 based immunocytokines therapy has been clinically approved for treatment of advanced stages of

Table 2.7 Immunotoxins employed for efficient management of cancer

Toxin conjugate	Monoclonal antibody	Remarks	Ref.
Diphtheria toxin (DT)	Trastuzumab (Herceptin)	Trastuzumab-DT conjugates exhibited significant killing of SK-BR-3 cells	[192]
Diphtheria toxin mutant (CRM9)	Anti-vascular endothelial growth factor (VEGF)	Markedly higher cell cytotoxicity than control groups	[193]
Gelonin	Monoclonal antibody 31 (MOC31)	MOC31-gelonin and 5-aminolevulinic acid (5-ALA) combination induced synergistic cytotoxic effect against the WiDr cells via enhanced photo chemical internalization as a result of protoporphyrin IX (PpIX)	[194]
Granzyme M	Humanized single-chain antibody fragment (scFv) H22	Specific and efficient toxicity upon binding to CD64, an FcγRI receptor overexpressed on activated myeloid cells and leukemic cells	[195]
Listeriolysin O (LLO)	B3	Specific elimination of antigen positive MCF7 cells with up to 80–250-fold less sensitivity towards antigen negative cell lines	[196]
LysPE38QQR (truncated form of Pseudomonas exotoxin)	K1 (murine IgG1)	Exhibits higher toxicity against mesothelin positive A431-K5 cells	[197]
Mutant Pseudomonas exotoxin A (ETA')	anti-EGFR 425(scFv)	Higher binding activity and specificity of towards EGFR-positive pancreatic carcinoma cell line L3.6p1	[198]
Mutant Pseudomonas exotoxin 38 (PE38)	B3	About 12-fold higher cytotoxicity on CRL1739 cell lines	[199]
Pseudomonas exotoxin (PE38)	Mutant MR1(Fv)	Increased affinity and cytotoxic activity	[200]
Pseudomonas exotoxin 38 (PE38)	RFB4	Exhibited fivefold to tenfold increase in activity on various CD22-positive cell lines and up to 50 times more cytotoxic to cells from patients with chronic lymphocytic leukemia and hairy-cell leukemia	[201]
Pseudomonas exotoxin 40, (PE40)	Humanized anti-CEA antibody (hMN14)	hMN14(Fv)-PE40 showed specific growth suppression of CEA expressing cell lines MIP-CEA (high CEA) and LS174T (moderate CEA) with IC_{50s} of 12 ng/mL (0.2 nM) and 69 ng/mL (1.1 nM) respectively with reduced toxicity towards normal tissues	[202]
Pseudomonas exotoxin A	anti-CD22	Remarkable increase in thermal stability and an enhanced resistance to trypsin degradation	[203]

(continued)

Table 2.7 (continued)

Toxin conjugate	Monoclonal antibody	Remarks	Ref.
Pseudomonas exotoxin A ETA	HER2-specific single-chain antibody scFv(FRP5)	scFv(FRP5)-ETA showed specifically higher cytotoxicity towards HER2 positive cell lines LNCaP	[204]
rAbrin	mAb F1G4	Immunotoxin mAb F1G4-rABRa-A, inhibits protein synthesis specifically on cells expressing the gonadotropin releasing hormone receptor and also it exhibited differences in the kinetics of inhibition of protein synthesis, in comparison to abrin, which was attributed to differences in internalization and trafficking of F1G4-rABRa-A within the conjugate	[205]
Recombinant gelonin toxin (rGel)	FGFR3-specific Fv fragments (3C)	3C/rGel fusion showed an significant reduction of IC50 value up to 200 nmol/L against cells compared with 1, 500 nmol/L for free rGel	[206]
Ricin A	(anti-PSMA) monoclonals (J591, PEQ226.5, and PM2P079.1	Various immunotoxins showed an significant reduction PSMA + cells with IC50 value in nanomolar range (IC_{50s} of 1.6–99 ng/mL) and complete eradication with J591-smpt-nRTA with IC_{50} of 0.35–31.7 ng/mL	[207]
Saporin	Trastuzumab (Herceptin) and cetuximab (Erbitux)	Trastuzumab (Herceptin) and cetuximab (Erbitux) were conjugated via cleavable disulfide bonds to the plant derived toxin saporin shown to have overcome the present limitations of therapeutic antibodies with a higher antitumoral efficacy via endosomal/lysosomal release of the toxin moiety	[208]

melanoma and renal carcinoma [211]. In addition, some allied pharmacological activities of cytokines have also been noted such as inhibition of tumor vasculature by tumor necrosis factors [212]. Considering the physiological functions of cytokines as either auto or paracrine factors, pretty high concentrations needs to achieved in the close proximities of site of action, i.e., producing cells. However, the clinical limitations does not permit such high dose administrations systemically [213] and hence the classical approaches includes either direct injection within solid tumor [214] or localized treatment such as isolated limb perfusion of tumor necrosis factor [215]. Unfortunately, the said approaches does not comply in most of the malignancies at the advanced stages and hence the concept of immunocytokines emerged wherein a tumor antibody is attached to the cytokine to achieve preferential higher tumor concentrations and hence drastically reduce toxic side effects to normal tissues. Furthermore, drastic improvements in the pharmacokinetics of cytokines could be achieved by said strategy [210].

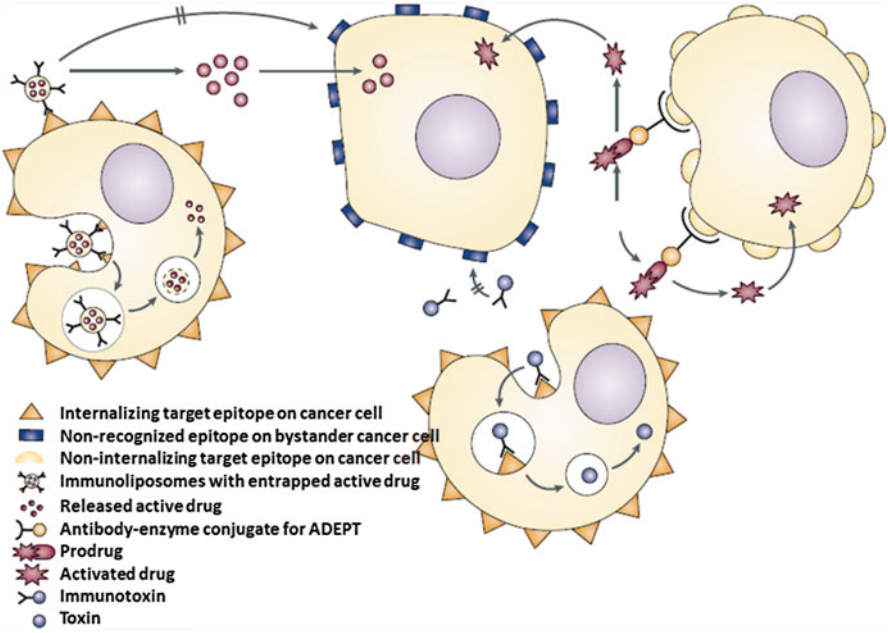

Fig. 2.11 Internalization of ligand targeted therapeutics and the "bystander effect." (**a**) Binding of the ligand-targeted therapeutics (LTTs) to their target epitopes will, in the case of some antibodies, promote receptor-mediated internalization of the LTT and, following release of the therapeutic intracellularly, lead to cytotoxicity (e.g., immunoliposomes and immunotoxins). (**b**) Binding of LTTs linked to noninternalizing antibodies will result in the LTT remaining attached at the target-cell surface (e.g., ADEPT (antibody-directed enzyme–prodrug therapy)). (**c**) All the cancer cells will preferably express the target epitope; however, some of the cancer cells might not. Drug that is released into the tumour interstitial space might be taken up non-selectively by cancer cells that do not express the target epitope; this results in cytotoxicity by the "bystander effect" (e.g., immunoliposomes and ADEPT). (**d**) Immunotoxins must be internalized to show cytotoxicity, so no opportunity for a bystander effects exists. Reproduced with permission from ref. [33]

Interleukins, especially IL-2, have been exhaustively studied for tumor immunotherapy. High dose IL-2 was the pioneer immunotherapy approved for treatment of melanomas and had complete response of ~6 % and partial response of ~10 % [216]. However, it is associated with severe side effects such as vascular leak syndrome, hypersensitivity reactions, etc. In contrast, the immunocytokines therapy demonstrated remarkably higher tumor accumulation of cytokines without notable compromise in safety profile [217]. In another set of experiments, 20-fold higher potency was noted for antibody targeted IL-12 in contrast to that of naked IL-12 [218]. On these grounds, a variety of immunocytokines based products such as Proleukin™, Aldesleukin™ comprising IL-2; Beromun™ comprising TNF-α; Roferon-A™ and Intron-A™ comprising interferon-α2; and Leukine™ and Leucomax™ comprising granulocyte-macrophage colony-stimulating factor (GMCSF) have been approved clinically [219]. In parallel, the exploration of

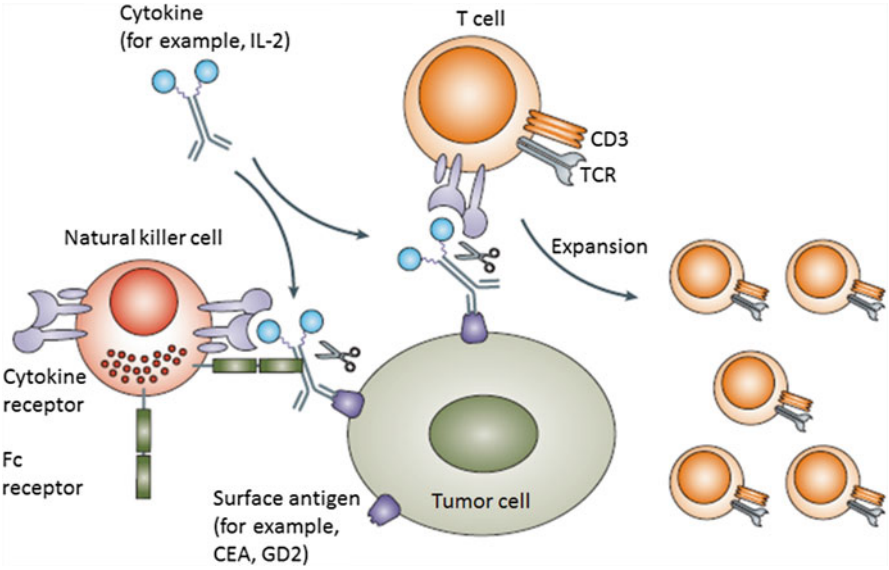

Fig. 2.12 Working mechanism of immunocytokines exemplified for tumor targeted IL-2. A monoclonal antibody specific for a tumor-associated antigen allows the enrichment of cytokines in the tumor microenvironment. In the case of interleukin-2 (IL-2) it enhances antibody-dependent cellular cytotoxicity mediated by Fc-receptor positive effector cells such as natural killer cells. In addition, tumor-targeted IL-2 stimulates T cells to expand and attack the tumor. High concentrations of plasmin at the tumor site enable the cleavage of IL-2 from the fusion protein through the plasmin cutting site within the linker (depicted in the figure by scissors). Reproduced from ref. [211]

cytokines as vaccine adjuvants is also in move. Upon combination with dendritic cells interesting memory immune responses were noted in the sense that site specific memory response mediated by effector memory T cells was executed whereas the central memory T cells were not formed [220]. The hypothesis was based on the fact that in rechallenge test the no tumor relapse was observed in organ in concern whereas tumor was induced in other organs. Recently, complete eradication in the B-cell lymphoma xenograft was observed upon treatment with combination of rituximab and L19-IL2 [221]. Similar results were also noted when L19-IL2 combined with (Cytotoxic T-Lymphocyte antigen 4) CTLA-4 blockade or L19-TNF [222]. Of note, not all cytokines exhibit antitumor effects, lymphotoxins represent a class of cytokines leading to physiology of tumorigenesis mediated by inflammatory pathways [223]. The said discrepancy could possibly be attributed to the typical structural motif of lymphotoxins which is trimeric in contrast to that of heterodimeric structure of potent antitumor cytokines such as IL-12 [185]. Hence, while employing immunocytokines based cancer therapy, the alterations in allied pathways and its clinical implications should be considered. Table 2.8 depicts various immunocytokines employed for cancer immunotherapy.

Table 2.8 List of immunocytokines employed for cancer immunotherapy

Cytokine	Monoclonal antibody	Outcomes	Ref.
IL-2	2aG4, PS targeting antibody	80 % of mice inoculated with 2aG4-IL2/4 T1 vaccine survived free of tumor and significantly increased 4 T1 specific cytotoxicity and ability to secrete interferon gamma (IFNγ)	[224]
IL-2	14G2A antibody	Uterine leiomyosarcoma diffusely expressed GD2 and binds the therapeutic immunocytokine14.18-IL2 and shown to be a potential target for effective management of aggressive tumors	[225]
IL-2	F8 antibody	F8-IL2 effectively inhibited the growth of EDA-Fn-expressing melanomas in combination with paclitaxel as a result of recruitment of F8-IL2-induced natural killer (NK) cells to the tumor via paclitaxel mediated enhanced tumor perfusion and permeability.	[226]
IL-2	F16 antibody	Selective tumor staining of F16 and preferential tumor accumulation of radiolabeled F16-IL2	[227]
IL-2	L19 and anti-CTLA-4 antibody	L19-IL2 exhibited complete tumor eradications when used in combination with CTLA-4 blockade	[222]
IL-2	F8 antibody	28 % cure rate and substantial tumor growth retardation were observed for the combination of sunitinib with F8-IL2 immunocytokine	[228]
IL-2	hu14.18 antibody	Patients treated with hu14.18-IL2 immunocytokine developed anti-idiotypic antibodies and anti-Fc-IL2 antibodies	[229]
IL2	huKS antibody	Paclitaxel and cyclophosphamide followed by huKS-IL2 resulted in enhanced antitumor responses against CT26/KSA colon, 4 T1/KSA mammary and LLCKSA Lewis lung carcinomas due to increased uptake of the huKS-IL2 immunocytokine into the tumor microenvironment by the virtue of reduced diffusion barrier by drug therapy	[230]
IL2	huKS antibody	Significantly increased (complete tumor resolution in 50 % of mice) the antitumor effect of RFA (Radiofrequency ablation) by combining it with huKS-IL2. Immunocytokine also showed antitumor effects against distant untreated tumor and greater proportion of cytokine-producing CD4 T cells and CD8 T cells	[231]
IL-7	F8 antibody	Improved tumor targeting performance with tumor: blood ratio = 16:1	[232]
IL-12	NHS76 antibody	Significant increase in the toxicity profile of NHS-IL12 was achieved as a result of attenuated IFN gamma production, selective targeting and longer half-life	[233]

(continued)

Table 2.8 (continued)

Cytokine	Monoclonal antibody	Outcomes	Ref.
IL-12	SS1 Fv	IL12-SS1 (Fv) immunocytokine significantly inhibited human tumor expressing mesothelin proteins, i.e., malignant mesothelioma (NCI-H226) and ovarian (OVCAR-3) cells as well as recombinant mesothelin on A431/H9 cells	[234]
IL-12	Recombinant human antibody fragment L19	Antitumor activity of EMD 521873 (Selectikine immunocytokine) was reported in heterogeneous patient population as prolonged disease stabilisation and a transient drop in tumor markers and also it was found that at all dose-levels there were transient increase in total lymphocyte, eosinophil, and monocyte counts	[235]
IL-15, GMCSF	L19 antibody	L19-IL-15 and L19-GM-CSF displayed a potent antitumor activity via CD8+ T cells	[236]
IFN-α	hRS7, hMN 15, hL243, and c225 antibodies	Up to 1,000-fold improved anti-proliferative potency of IFN-λ1, when tethered with antibodies hRS7, hMN 15, hL243, and c225, was demonstrated against targeted cancer cell lines along with increased antiviral activity against encephalomyocarditis virus and hepatitis C virus IFN-λ1 via (15)-λ1 or (c225)-λ1 respectively	[237]
IFN-γ	Anti-CD70 antibody	Anti-CD70 IFN-γ immunocytokines displayed high levels of species specific IFN-γ activity and selective binding to CD70 on human RCC cells and higher tumoricidal activity by the virtue of RIP1-dependent necrosis in RCC cells in the presence of bortezomib	[238]
TNF	scFv23	scFv23/TNF was found to be highly cytotoxic to TNF-resistant HER-2/neu-expressing pancreatic cancer cell lines and demonstrated a synergistic cytotoxic effect with 5-fluorouracil (5-FU) by the virtue of downregulation of HER-2/neu, p-Akt, Bcl-2 and upregulation of TNF-R1, caspase-8, and caspase-3	[239]

2.5.3.4 Antibody Directed Enzyme Prodrug Therapy (ADEPT)

ADEPT represents an improved version of cancer immunotherapy among the above discussed strategies for efficient management of advanced malignancies and solid tumors. Classical immunotherapies face challenges such as limited penetrating capabilities, need for internalization, antigen heterogeneity, nonuniform drug release and subsequent altered potency levels. Principally, ADEPT operates via usage of antibodies specific for tumor agents that are covalently linked to enzyme in the concern. In the first step, such antibody–enzyme conjugate is administered to the patients and allowed to preferentially localize in the vicinities of tumor. Once the unbound conjugates are cleared of the body by a variety of means, a prodrug

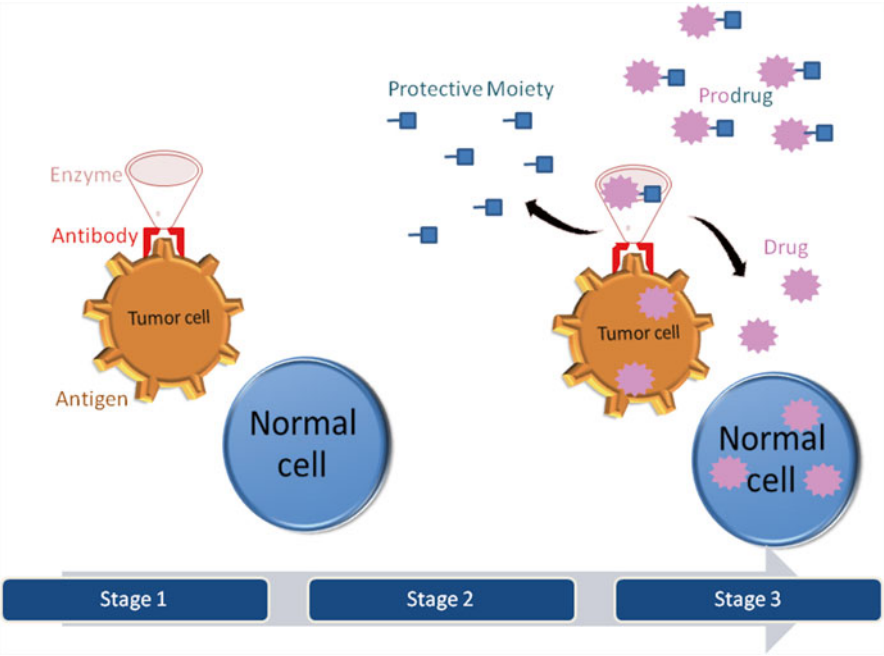

Fig. 2.13 Basic principle of ADEPT. *Stage 1*, administered AEC is allowed to localize at site of action. *Stage 2* the excess circulating prodrug is cleared of the blood. *Stage 3* nontoxic prodrug is administered that specifically generates active at site of action

sensitive to pre-targeted enzyme is administered which will generate very high local concentrations of active drug in the extracellular regions of tumor. The active drug can then be passively internalized within cells and impart cytotoxicity (Fig. 2.13) [240]. One of the promising advantages is bystander effects which also impart cytotoxicity to antigen negative cells (Fig. 2.11) [241].

The principal components of ADEPT include target, antibody, enzyme, abenzymes/catalytic antibodies, and prodrugs [242]. Firstly, target should usually be native to the tumor and should have minimal expression over normal tissues. However, it is practically difficult to find such targets and hence care should be taken that normal expression of target is at minimal at least on the vital organs so to avoid any irreparable damages to the critical body organs. However, heterogeneity among in antigen expression in epithelial tumors is quite common. Furthermore, the internalization of the antibody–enzyme conjugate should be avoided so as to avoid failure of therapy. The additional advantage of the surface linked enzyme is rapid conversion of many prodrug molecules and higher tumoral concentrations. While selecting target antigen, care should also be taken that it should not be secretory in nature; however, there have been some exceptions such as ADEPT in the case of human chorionic gonadotrophin (hCG) producing tumors was successful in spite of higher concentrations of hCG in central compartment [243].

Secondly, antibody plays an important role in preferentially localizing the enzymes and hence the therapeutics at the target location so as to achieve the maximum benefits, per se. This retention of enzymes at the target site may extend up to several days. In this regard, considering the binding affinity of the employed antibody, interplay of antibody–enzyme conjugate in the vicinity of tumor and in central compartment could be modulated with ideal situation being nil antibody–enzyme conjugate in central compartment for achieving maximum therapeutic efficacy. Such highly stable antibody complex could be achieved by employing class I or II IgG monoclonal antibodies. Furthermore, this balance could be modulated in a better way by usage of antibody fragments such as F(ab)′2 which are associated with principal advantages of rapid clearance from the central compartment imparting better targeting potential to the tumors; however, the binding affinity, size of fragment, renal clearance and hence total available enzymatic concentrations at the site of action, etc. should be taken into account while comparing it with whole antibodies [244].

Thirdly, enzyme has principal role for success of ADEPT. However, there lies certain restriction on type of enzymes which could be used such as human enzymes exhibit wide normal tissue distribution while nonhuman enzymes face problems of immunogenicity and possible human isoforms in physiological conditions. Among the available options, bacterial enzymes are explored considering their potential efficiency to a greater extent; however, their immunogenicity is debatable. In purview of decreasing the immunogenicity, efforts are being to identify the responsible amino acid sequences and their suitable replacement in modified enzymes [245, 246]. In this regard, yet another interesting concept of preparing mutant human enzymes is prevailing which will enable the prodrug processing only by mutated enzymes, whereas original human forms of these enzymes remain inactive, e.g., T268G mutant of human carboxypeptidase A1 for prodrugs of methotrexate [247]. Table 2.9 lists various enzymes employed for ADEPT.

Fourthly, the concept of catalytic antibodies is also emerging as promising component of ADEPT with the principal advantage of overcoming immunogenicity related problems [267]. Conceptually these are improved version of therapeutic antibodies which are equipped with additional enzymatic catalytic power (Fig. 2.14). The most classical methods to prepare such catalytic antibodies are transition state analogue approach, hapten substrate approach and reactive immunization approach. Their immense clinical relevance arises from the promising advantages such as improved circulation half-life in central compartment, increased specificity and affinity for the target and prodrugs, and feasibility to exploit antibody/genetic engineering along with the classical advantage of low immunogenicity. Some examples of catalytic antibodies include 38C2 for aldolase activity; 84G3, 85H6, 90G8, and VHHC10 for aliinase activity; 3D8-VL for mRNA of HER2 hydrolysis; A17 for degradation of organophosphate compounds, ETNF-6-H for hydrolysis of TNF-α, etc. Recently, cell targeting and prodrug activation capabilities of the next generation therapeutic antibody, catalytic Ab 38C2, have been established in the case of selective tumor immunotherapy with doxorubicin prodrugs [268].

Table 2.9 List of enzymes employed for ADEPT based cancer immunotherapy

Enzyme	Characteristics	Ref.
β-glucosidase	β-glucosidase conjugated with tumor associated monoclonal antibody HMFG1 enhances the cytotoxic effect of amygdalin by 36-fold while increased rate of survival of U87MG (HMFG1-negative) cells	[248]
β-glucuronidase	After fusion with a humanized single-chain antibody (scFv) of mAb CC49 to S2 (a human β-glucuronidase (hβG) variant) displays enhanced human enzyme catalytic activity and more effective in killing antigen-positive cancer cells	[249]
β-Lactamase	Fusion protein TAB2.5 (conjugation of CC49 and β-lactamase) showed prolonged retention (T1/2 = 36.9 h) with tumor to plasma ratios of up to 1,000	[250]
β-Lactamases	When fused with ACDCRGDCFCG peptide (RGD4C), it found to be active for specificity in MCF-7 cell lines	[251]
Carboxypeptidase-A	Anti-seminoprotein (SM) single-chain antibody/human carboxy peptidase A fusion protein (scFv/hCPA) mediated hydrolysis of methotrexate prodrug increased cytotoxicity up to 1,000-fold with no systemic toxicity of the prodrug	[252]
Carboxypeptidase A	HuA33 antibody–carboxy peptidase A (acts on methotrexate phenylalanine prodrug) demonstrates higher specificity of tumor uptake with eightfold reduced cytotoxicity (LD50 of MTX) to non-tumor sites	[253]
Carboxypeptidase G2 (CPG2)	Conjugation of CPG2 with SB43 anti-carcinoembryonic antigen antibody fragment A5B7-F(ab′)$_2$ shown to reduce the percentage of injected dose per gram in blood and a tumor-to-blood ratio of 45: 1 at 24 h, which increased to 100: 1 at 72 h	[254]
Carboxypeptidase G2 (CPG2)	F(ab′) [SUB2] anti-CEA antibody A5B7 and the bacterial enzyme CPG2 conjugates converts ZD2767P (4-[N,N-bis(2-iodoethyl) amino] phenoxycarbonyl L-glutamic acid) to ZD2767 (bifunctional alkylating agent) at the tumor site which resulted in 229-fold increase in activity.	[255]
Carboxypeptidase G2 (CPG2)	Anti-carcinoembryonic A5B7 conjugated to the bacterial enzyme carboxypeptidase G2 acts on the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid resulted in significantly enhanced tumor growth with no concomitant increase in systemic toxicity	[256]
Carboxypeptidase G2 (CPG2)	MFECP, a recombinant fusion protein of CP with MFE-23, a single chain Fv (scFv) antibody, modified to hexahistidine tag (His-tag) MFECP found to have significantly reduced human antibody response	[257]
Cytosine deaminase (CD)	Fused LinkCD, hyaluronan binding domain of TSG-6 (Link) and yeast cytosine deaminase (CD) with an N-terminal His(×6) tag shown to have increased duration of the enzyme activity and significant tumor size reduction in animals that received LinkCD/5-FC treatment	[258]

(continued)

Table 2.9 (continued)

Enzyme	Characteristics	Ref.
Cytosine deaminase (CD)	Fusion of anti-gpA33 single chain fragment, A33scFv, with cytosine deaminase from yeast (CDy), which converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), results demonstrate bifunctional activity of A33scFv::CDy fusion protein which is equally cytotoxic to equimolar amounts of 5-FU	[259]
Human β-glucuronidase (βG)	chTNT-3 and β-glucuronidase enzyme fusion protein (acts on doxorubicin glucuronide prodrug), specifically localize to tumor sites with rapid clearance from blood and normal tissues	[260]
Human β-glucuronidase (βG)	It was found that ortho-substituted phenyl carbamates (i.e., N-[4-O-(Methyl-βD-glucopyranosyluronate)-3 nitrobenzyloxycarbonyl] doxorubicin) are better substrates for fusion protein human β-glucuronidase-humanized CEA than the corresponding para-substituted analogues	[261]
Human β-glucuronidase (βG)	Anti-CD20 mouse monoclonal antibody (MoAb) 1H4 and human β-glucuronidase activates nontoxic prodrug *N*-[4-doxorubicin-*N*-carbonyl(-oxymethyl) phenyl] O-β-glucuronylcarbamate to doxorubicin at the tumor site	[262]
Mutant human purine nucleoside phosphorylase	Double mutant with amino acid substitutions E201Q:N243D (hDM) is the most efficient in cleaving (deoxy) adenosine-based prodrugs, i.e., 2-fluoro-2′-deoxyadenosine to a cytotoxic drug	[263]
Mutant human carboxypeptidase A1, changed at position 268 from the wild type threonine to a glycine (hCPA1-T268G)	hCPA1-T268G was conjugated to ING-1 (antibody that binds to the tumor antigen Ep-Cam) or to Campath-1H (an antibody that binds to the T and B cell antigen CDw52) which acts on MTX-α-3-cyclobutylphenylalanine and MTX-α-3-cyclopentyltyrosine prodrugs releasing free methotrexate. It is found that ING-1:hCPA1-T268G conjugate produced excellent activation of the MTX prodrugs to kill HT-29 cells as efficiently as MTX itself	[247]
Mutant prolylendopeptidase (Glu289 → Gly)	Mutant human prolyl endopeptidase was chemically conjugated with L19 human antibody, a marker of angiogenesis which acts on the prodrug N-protected glycine-proline dipeptide doxorubicin or melphalan, i.e., Benzyloxycarbonyl) glycylprolyl melphalan producing free melphalan	[264]
Nitroreductase enzyme	It bioactivates CB 1954 (alkylating agent) much more rapidly than Walker DT diaphorase as a result it overcomes the intrinsic resistance of human cell lines towards CB 1954	[265]
Penicillin-V amidase (PVA)	Folate-PVA-^{125}I (PVA covalently labeled with three molecules of folic acid) which converts doxorubicin-*N*-*p*-hydroxy phenoxyacetamide prodrug (DPO) into its potent parent drug, doxorubicin, bind specifically to KB cells (FR-positive tumor cells) but not to A549 cells (FR-negative tumor cells) with higher clearance from the blood	[266]

Fifthly, prodrugs remains the fundamental and rate limiting component of ADEPT. The ratio of activity of therapeutically active form to the inactive form majorly determines the potency of the ADEPT in concern and this ratio usually ranges from 100 to 1,000 s. Classically it is anticipated that the dose response of the

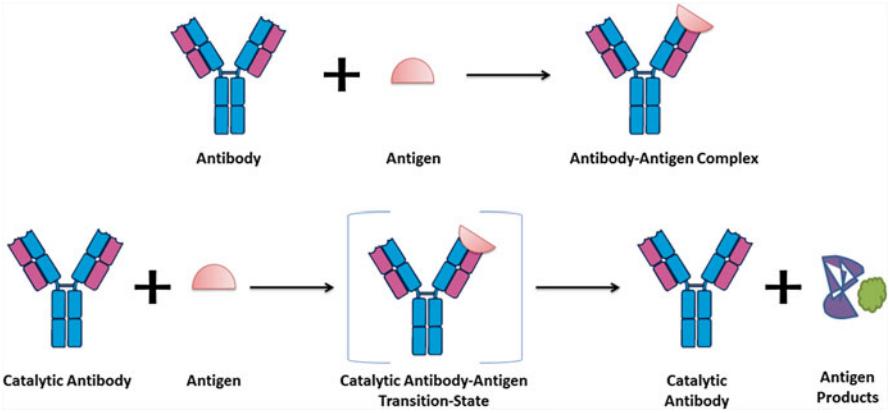

Fig. 2.14 Conceptual understanding of catalytic antibody

active drugs should be concentration dependent and should ideally have very low decay half-life so as to avoid any detrimental effects to the normal tissues from the drug that leaked into central compartment.

Recent trend in the field of ADEPT include design and developments in the field of two and three phase systems. Apart from activation of prodrug at tumor site, efforts are being made to rapidly clear the antibody–enzyme conjugates from the blood compartment in the view to avoid any possible toxicity to the normal tissues. The hypothesis has been classically tested in LS174T xenografted mice administered with monoclonal anti-carcinoembryonic antigen antibody fragment A5B7-$F(ab')_2$ conjugated to a bacterial enzyme, carboxypeptidase G2 (CPG2) [254]. Among the tested approaches, the first comprised inactivator of CPG2, SB43, which was galactosylated and administered to clear out plasma antibody–enzyme conjugates mediated by carbohydrate receptor in liver. Interestingly, upon optimizing dose schedule, minimal compromise in therapeutic efficacy could be achieved. The second approach in contrast includes direct galactosylation of the antibody–enzyme conjugate in a manner such that binding affinity with the tumor antigen is altered. The said conjugate acts on receptor saturation mechanism and either rapidly bound to tumor tissues or cleared from the plasma cumulatively leading to almost 100-fold tumor to blood ratio of antibody–enzyme conjugates.

Furthermore, on similar line of ADEPT, Gene directed enzyme prodrug therapy (GDEPT) has also been widely explored for its potential in improving the tumor delivery of anticancer therapeutics. It is also referred to as suicide gene therapy (SGT), virus directed enzyme prodrug therapy (VDEPT), and gene prodrug activation therapy (GPAT). The principal difference herein is the activation of drug at intracellular level in contrast to ADEPT which releases the drug in extracellular matrices. The principal examples include activation of cyclophosphamide and ifosfamide using cytochrome p450, 5-fluorocytidine and 5-fluorouridine using cytosine deaminase, etc. Primarily, the tumor specific regulatory element and gene encoding enzyme is loaded in a suitable colloidal carrier system which upon reaching at

intracellular levels results in transgene expression and leads to execution of prodrug activation system, thereby imparting cell cytotoxicity [269].

2.5.3.5 Immunoliposomes

Liposomes represent an important class of nanocarriers with established applicability in a variety of disease conditions and difficult-to-deliver therapeutics. Though concomitant with widespread applicability, there also lie some negative points such as rapid clearance from the plasma process of opsonization of proteins on the surface of plain phospholipid vesicles. However, with the advent of PEGylation, the problem has been addressed to some extent and some quality products have made their way to clinical practice such as Doxil®. This stealth property of hydrophilic coatings enable liposomal drug delivery system to evade the reticuloendothelial systems and lead to preferential localization in the vicinity of extracellular tumor matrices by so-called enhanced permeation and retention effect [270]. However, in the purview of further increasing the therapeutic potential of these sophisticated colloidal drug delivery systems to tumor tissues, antibody targeting could be sought for. Structurally, these comprise stealth liposomes end functionalized with antibody, usually referred to as immunoliposomes [271]. Both whole antibody and antibody fractions specific to tumor antigens have been exhaustively used for exploring the potential of immunoliposomes for cancer therapy.

In the purview of formulation development, numerous methods have been proposed till date for linking antibodies to liposomal surface; however, post insertion technique remains the most viable and widely accepted [272]. In some instances, even the micelles of PEG linked antibodies have been incubated with marketed liposomal products to form immunoliposomes [273]. The classical chemical strategies include linking hydrazine group of PEG chains with oxidized carbohydrate group of oligosaccharide portion of antibody, maleimide group linked with thiolated antibodies, pyridyldithio propionate method, and biotin–avidin method [274]. Employing these chemistries numerous antigen targeted ligands have been linked to liposomes; however, the fruitful alteration in the various physicochemical properties of immunoliposomes and its optimization is still underway. Primarily, internalizing antibodies are employed for achieving significant appreciation in the therapeutic efficacy such as CD19 epitopes for B-cell lymphomas [275]. Figure 2.15 depicts various internalization mechanisms with liposomal drug delivery systems. Recent trends include combining immunoliposomes with endosome disruptive peptides for improved cytosolic delivery of therapeutics such as fusogenic peptide diINF-7 linked liposomes targeted to ovarian carcinoma [276].

The principal advantage associated with immunoliposomes is the very high drug payload as compared to any other available immunotherapies. Secondly, liposomal surface pose maximum capability to link the antibodies than classical immunotherapeutic approaches. This feature could be fruitfully exploited in employing monovalent antibody fragments, thereby eliminating the need of formation of scFv fragments by excessive antibody engineering to improve upon their valences and

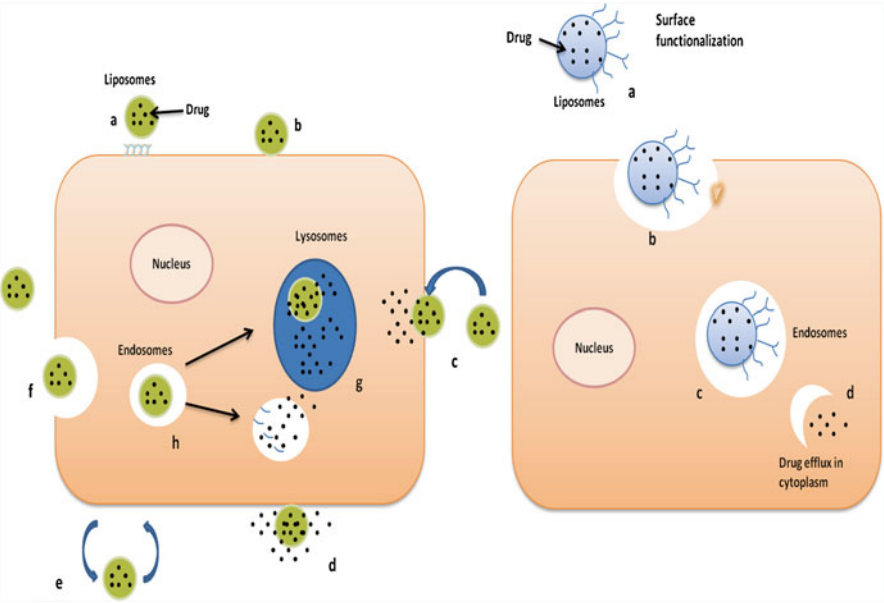

Fig. 2.15 Internalization of liposomal drug delivery systems. (A) Plain liposomes are entitled for either specific adsorption (**a**), nonspecific adsorption at cell surface (**b**) fusion with cell membrane (**c**), destabilized of liposomes upon adsorption at cell surface by internal components (**d**), direct or transfer-protein-mediated exchange of lipid component (**e**) and endocytosis (**f**). The endocytosed liposomes either fuses with lysosomes (**g**) or leads to endosomal escape and drug delivery to cytoplasm (**h**). (B) Surface functionalization of the liposomes with appropriate ligands can be performed (**a**) to interact with target receptors (**b**), followed by endocytosis (**c**), and drug release (**d**)

hence binding affinity [271]. The concept of immunoliposomes has emerged since old times and in numerous instances, potential has been established. Table 2.10 lists various approaches for immunoliposomes based drug delivery for efficient management of cancer [277].

2.5.3.6 Immunopolymers

Blending polymer engineering with antibody engineering is probably the most recent era of immunotherapy. Polymer based drug delivery is widely accepted as one stop solution for almost all kinds of therapeutics ranging from small molecules, nucleic acid, proteins, peptides, hormones, etc. The barriers of physicochemical properties of said therapeutics can be efficiently circumvented by designing apt polymeric drug delivery system. With the advent of antibody directed polymers, plethora of chemically and functionally diverse synthetic yet biomimetic systems have been explored in last couple of decades [294]. The principal advantages associated with antibody conjugation with polymers includes significant appreciation in solubility, immunocompatibility, favorable pharmacokinetics, improved stability,

Table 2.10 List of immunoliposomes based drug delivery system for cancer immunotherapy

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Anti-EGFR (epidermal growth factor receptor) Fab	Adriamycin and ribonucleotide Reductase M2 siRNA	EGFR positive hepatocellular carcinoma(HCC)	Targeted LPD (liposome–polycation–DNA complex) conjugated with anti-EGFR (epidermal growth factor receptor) Fab′ co-delivering adriamycin (ADR) and ribonucleotide reductase M2 (RRM2) siRNA (ADR-RRM2-TLPD) was prepared with enhanced therapeutic effects	[278]
Anti-EGFR-Fab	siRNA	MDA-MB-231 breast cancer cells	TLPD-FCC (Targeted liposome–polycation–DNA complex conjugated with anti-EGFR Fab′ by conventional conjugation) showed significantly enhanced binding affinity and luciferase gene silencing activity by ~20 % in EGFR	[279]
Anti-EGFR-Fab	siRNA	SMMC-7721 hepatocellular carcinoma cells	Two PEG derivative linkers (DSPE-PEG-COOH and DSPE-PEG-MAL) were used to develop immunoliposomes. Immunoliposomes derived from DSPE-PEG-MAL (TLPD-FPM) shown to have significantly greater cellular uptake and up to threefold higher luciferase gene silencing efficiency than that of TLPD-FPC	[280]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Anti-EGFR and anti-HER2	Topotecan (TPT)	HER2 overexpressing human breast cancer (BT474)	Stabilization of topotecan in nanoliposomes significantly improves the targetability and pharmacokinetic profile of topotecan	[281]
Anti-HB-EGF	Doxorubicin	HB-EGF positive breast cancer	Results showed selective binding and uptake of the immunoliposomes in HB-EGF-expressing cells	[282]
Antinucleosome monoclonal antibody 2C5	Doxorubicin	Nucleosome	Significant reduction in tumor growth and enhanced therapeutic efficacy of the drug in both drug resistant and drug sensitive mice was obtained	[283]
Anti-VEGFR2	Doxorubicin	HT-29 human colon cancer/MMTV-PyMT mouse model of breast cancer	Histopathological and molecular analysis revealed strong antiangiogenic effect of anti-VEGFR2-ILs-dox (Anti-VEGFR2-targeted immunoliposomes (ILs) loaded with doxorubicin)	[284]
Anti-RON antibody Zt/c9	Doxorubicin	CD24+, CD44+, ESA + (triple positive) pancreatic L3.6pl cancer cells	Anti-RON antibody Zt/c9-directing doxorubicin-immunoliposomes (Zt/c9-Dox-IL) was developed which specifically interacted with CSCs $^{+24/44/ESA}$	[285]
Anti-RON antibody Zt/g4	Doxorubicin	Hypoxic colon HCT116 and SW620 cells	Zt/g4-Dox-IL was found to be effective in killing hypoxic HCT116 and SW620 cells with reduced IC 50 values compared to Dox and pegylated-liposomal Dox	[286]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Cetuximab (α-hEGFR)	–	Glioblastoma multiforme	In vitro studies revealed significantly higher binding of α-hEGFR-ILs (PEGylated immunoliposomes conjugated with anti-human epidermal growth factor receptor (EGFR) antibodies) when compared with liposomes conjugated with isotypic nonimmune Ig resulting in enhanced uptake and accumulation of liposomes	[287]
Cetuximab	Doxorubicin	Human ovarian adenocarcinoma (SKOV3, SKOV3.i.p.1) cells	Showed efficient and specific receptor-mediated binding to ovarian carcinoma cells	[288]
IGFI-R antagonistic antibody (1H7)	Doxorubicin	Neuroendocrine tumors of the gastroenteropancreatic system (GEP-NETs)	Anti-IGFI-R immunoliposomes displayed specific tumor cell and internalization in human neuroendocrine tumor cells in vitro and superior antitumor efficacy in vivo	[289]
mAb 2C5	Doxorubicin	B16-F10, HeLa, and MCF-7 cell lines	Multifunctional immunoliposomal nanocarrier with pH-sensitive PEG-PE component, TATp and the cancer cell specific mAb 2C5 showed enhanced cytotoxicity and internalization by cancer cells	[290]
Trastuzumab	Bleomycin	HER2 positive human breast cancer	Immunoliposomes showed enhanced cytotoxicity towards HER2 positive MCF-7/Her18 cells and also affect trastuzumab-resistant MDA-453 cell	[291]

(continued)

Table 2.10 (continued)

Antibody	Drug(s)	Indication/target	Outcomes	Ref.
Trastuzumab	Curcumin and resveratrol	HER2 positive human breast cancer	Significant increase in the antiproliferative effects of curcumin and resveratrol in HER2 positive human breast cancer cells as a result of enhanced uptake of curcumin and resveratrol at intracellular level	[292]
Trastuzumab	Docetaxel	Her2/neu positive gastric tumor	Docetaxel-loaded immuno (trastuzumab) liposomes (IDL) showed a significantly higher distribution of docetaxel in the N87 xenograft tumor tissues and superior antitumor efficacy	[293]

relatively higher cellular uptake, reduced aggregation, relatively higher antibody payload, feasibility to modulate intracellular trafficking, etc. Recent advances in the field of recombinant technology has enable the use of a variety of products such as isolated antibodies and their fragments including humanized, tumor biomarkers, structural components of microbes, natural and synthetic sources of immunostimulators.

Immunopolymers primarily comprise antibody (either whole antibody or antibody fragments) linked to polymers (may be functionalized for specific applications). Recent advances in the field of antibody engineering enable the de novo design and development of antibody fragments for a variety of end applications [295]. These antibody fragments when linked to polymeric counterparts avail additional advantage of relatively higher binding density owing to smaller size [296]. PEGylation of antibody fragments has been explored to a greater extent in improving the circulation half-life, per se. A variety of approaches employed for linking these antibody fragments to polymer backbone include thiol modifications, linking via sugar portions, fusion proteins, etc. Generally, either N- or C- terminal end of the antibody fragments is opted for modifications; however, in some cases such as in scFvs inter-domain peptide linker may be sought for without any compromise in binding affinity [297]. Recently, novel functionalities in the scFvs could be added employing a variety of protein and chemical engineering approaches such as tagging with hexahistidine or streptavidin for site specific conjugation or delivery [298]. Similarly, C-terminal cysteine modification has also been greatly explored and could lead to ~100-fold appreciation in circulation half-life [299]. Furthermore, PEGylation of scFvs also render the resulting immunopolymers resistant to proteolytic enzymes [300]. Owing to these functionalities, PEGylated antibody fragments have been widely explored from tumor targeting perspectives. In a representative

study, PEGylated di(Fab′) exhibited significantly higher antitumor efficacy as compared to PEGylated IgG and the latter being comparable to that of plain IgG in xenograft tumor bearing thymic mice [301]. Similar results were also noted in case of PEGylated anti-CEA $F(ab')_2$ exhibiting significant increase in circulation half-life and tumor accumulation [302]. In separate set of experiments effect of molecular weight of PEG was also studied and it was found that higher molecular weights in the order of 25 kD tend to localize equally within normal tissues also in contrast to that of low molecular weights (~5 kD) [303]. In an interesting study, ^{111}In-cysteinyl-DOTA-PEG3400-diabody conjugate and ^{125}I-PEG3400-diabody were explored for its potential in imaging liver metastasis in a nude mouse xenograft model (Fig. 2.16) [304].

Apart from PEGylated antibodies, stimuli responsive polymers represent yet another area where antibodies are being explored to a greater extent. Classically, these stimuli responsive polymers are sensitive to pH, temperature, presence of

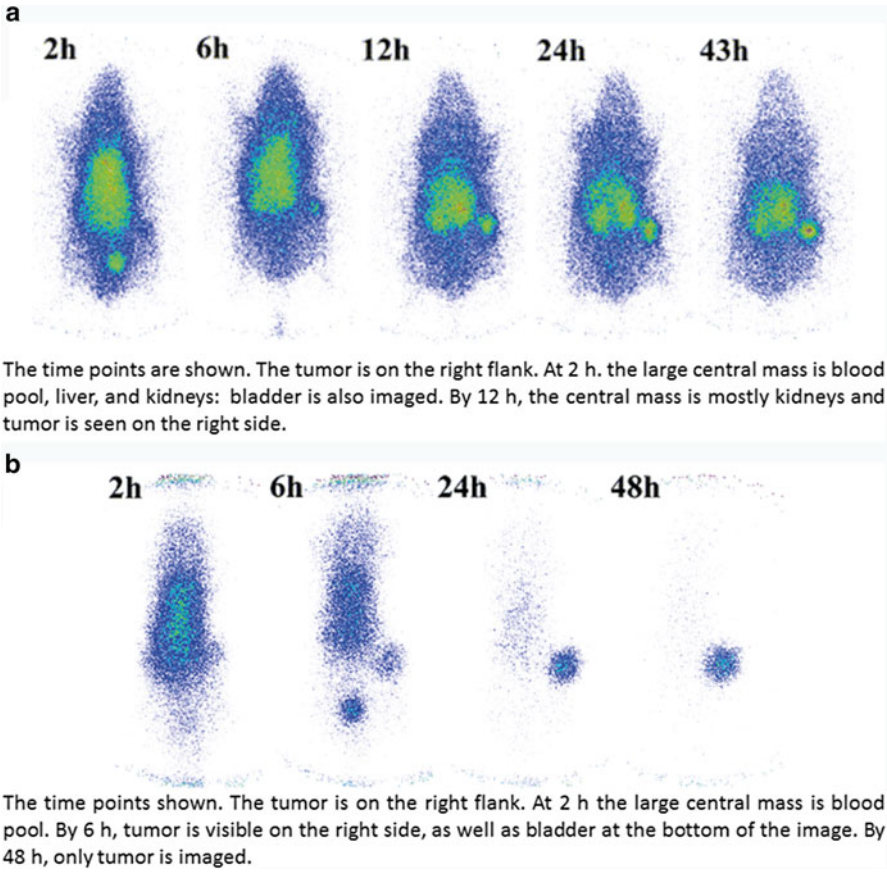

Fig. 2.16 Radioimmunoimaging of (**a**) ^{111}In-cys-DOTA-PEG3400-diabody and (**b**) ^{125}I-PEG3400-diabody in nude mice bearing LS174T xenografts. Reproduced from ref. [304]

small molecules such as amino acids, external energies such as electrical and magnetic, etc. [294]. In this domain, *N*- (2 hydroxy propyl) methacrylamide (HPMA) has been exhaustively explored. Significant increase in the cell cytotoxicity of Fab′ was noted against ovarian carcinoma when co-polymerized with HPMA [305]. On similar line of action, galactosylated HPMA conjugate comprising doxorubicin with Gly-Phe-Leu-Gly spacer revealed great clinical potential with notable responses in hepatoma patients [306]. Mechanistic studies with anti-Thy1.2 targeted or CD71 targeted HPMA polymers further revealed preferential nuclear localization tendencies intracellularly [307]. Recent advances in the field of polymer drug conjugates include its combination with ADEPT and the resulting therapy is referred to as polymer directed enzyme prodrug therapy (PDEPT) where combination of polymeric prodrug and polymer enzyme conjugate is employed to impart cytotoxicity at the site of action. PK1-HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate in combination with HPMA copolymer-cathepsin B led to 4.2-fold higher tumor accumulation in B16F10 tumor bearing mice as compared to plain free enzyme [308]. Table 2.11 lists immunopolymers employed for improving the deliverability of antibodies.

2.5.4 Targeting Multidrug Resistant Tumors

The principal problem associated with the advanced anticancer therapeutics, i.e., molecular targeted therapies and immunotherapy are increased chances of resistance and inter-, intra-tumor variability, often leading to poor therapeutic responses. Broadly, two factors have been considered responsible for multidrug resistance, viz., cellular and physiological factors [319]. The former includes a variety of genetic alterations at cellular levels such as efflux transporters, whereas the latter is more focused on the physicochemical changes at tissue levels such as pH, extracellular interactions and peculiar tumor microenvironment (Fig. 2.17). Based on these factors, efflux of the bioactives is regarded as most common mechanism of drug resistance in cancer therapeutics [320]. A variety of approaches could be employed to counterfeit drug efflux systems and these include pharmacologically active P-gp inhibitors, functional excipients such as natural polymers, surfactants, lipids, cyclodextrins, polyethylene glycol and derivatives, thiolated polymers, etc. [16].

Furthermore, in purview of increasing therapeutic responses in cancer chemotherapy and sensitize the multidrug-resistant tumors, nanotechnology seems to be most efficient approach. Recent advances in the field of nanocarrier based approaches have paved the way to efficiently deliver therapeutics to multidrug resistant cancer therapy and includes polymeric nanoparticles, lipid nanocarriers, dendrimers, carbon nanotubes and inorganic nanocarriers, to name a few. The principal focus of employing nanocarriers relies on improving the interactions with target cells, enhanced internalization mechanisms, tumor specific biodistribution pattern, availing benefits of “click chemistry,” reducing the nonspecific binding, tailoring the ligand properties such as choice of ligand and its density, charge, orientation, etc. [322].

Table 2.11 List of immunopolymers employed for improving the deliverability of antibodies

Antibody	Polymer	Outcomes	Ref.
Anti-CD20 monoclonal antibody	HPMA	Cytostatic activity of the anti-CD20 monoclonal Ab-targeted conjugates tested on several CD20-positive or negative human and mouse cancer cell lines confirmed considerable targeting capacity of the monoclonal Ab after its binding to the polymer carrier	[309]
Anti-EGF receptor antibody	DSPE-PEG lipid polymer complex	In vivo accumulation of PLNP-Mal-EGFR was found to be higher than that of nontargeted nanoparticles in SMMC-7721 HCC cells overexpressing EGFR with enhanced antitumor activity against HCC compared with nontargeted nanoparticles and free adriamycin	[310]
Anti-H*ER2*-affibody-anti-DTPA-Fab complexes (BAAC), anti-DTPA-Fab	Polyglutamic acid	There was no total body weight (TBW) loss at three times the doxorubicin equivalent maximum tolerated dose (MTD) with D-Dox-PGA. Therapeutic efficacy was equivalent in mice pre-targeted with BAAC/targeted with D-Dox-PGA to mice treated only with doxorubicin	[311]
Anti-PSMA antibody	HPMA	Rate of endocytosis of P-anti-PSMA was much faster than that of control HPMA copolymer conjugates containing nonspecific IgGvia clathrin-mediated endocytosis, macropinocytosis, and clathrin-, caveolae-independent endocytosis	[312]
Catalytic antibody 38C2	HPMA	Catalytic antibody–HPMA copolymer conjugate was evaluated in vitro for its ability to activate an etoposide prodrug and it was found that the inhibition using the prodrug and the conjugate was almost identical to inhibition by the free antibody and the prodrug	[313]
HD39 monoclonal antibody	Poly(propylacrylic acid) (PPAA)	Subcellular fractionation studies of HD39/SA-PPAA conjugates showed 89 % of HD39/SA was associated with endosomes (Rab5+) and lysosomes (Lamp2+), while 45 % of HD39/SA-PPAA was translocated to the cytosol (lactate dehydrogenase+) which demonstrate the endosomal releasing properties of PPAA with antibody–polymer conjugates	[314]
Monoclonal anti-RAGE and polyclonal human Ig (huIgG)	Poly(*N*-(2-hydroxypropyl)-methacrylamide) (poly-HPMA)	Antibody polymer conjugate, two different model antibodies, monoclonal anti-RAGE and polyclonal human Ig (huIgG) antibodies, were attached to maleimide functionalized poly(N-(2-hydroxypropyl)-methacrylamide) (poly-HPMA) through reversible addition -fragmentation chain transfer (RAFT) polymerization of pentafluorophenyl methacrylate via the intermediate step of an activated ester polymer was developed with preserved affinity	[315]

(continued)

Table 2.11 (continued)

Antibody	Polymer	Outcomes	Ref.
HPMA	Polyclonal and monoclonal anti-Thy 1.2 or anti-Ia^k antibody	Daunomycin toxicity of daunomycin-antibody-copolymer conjugate against hematopoietic precursors in bone marrow colony forming unit spleen was found to be decreased up to 80-fold and with no significant irritation of Kupffer cells in liver	[316]
OV-TL16 antibody	Pegylated polyethylenimine (PEG-PEI)	Sixfold higher degree of binding of PEG-PEI-Fab′/DNA complexes to OA3 positive human ovarian carcinoma cell lines compared to unmodified PEG-PEI/DNA complexes and up to 80-fold increase in luciferase reporter gene expression compared to PEG-PEI	[317]
Polyclonal rabbit anti-mouse thymocyte globulin (ATG)	Poly(ethylene glycol) (PEG)	Antibody polymer drug conjugates exhibited significant antitumor efficiency against murine T-cell EL 4 lymphoma in vivo	[318]

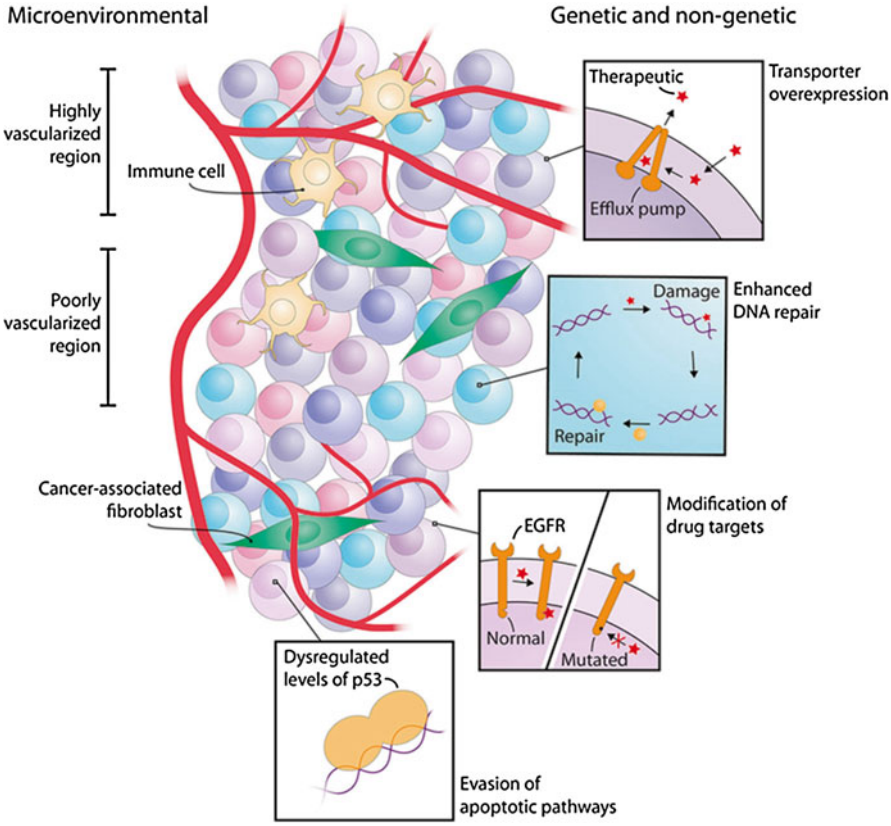

Fig. 2.17 Factors influencing tumor heterogeneity and drug resistance. Genetic, nongenetic, and microenvironmental factors give rise to tumor heterogeneity, which significantly influences the drug sensitivity of cancer cells through an array of cellular mechanisms, such as transporter overexpression. Reproduced from ref. [321]

2.6 Conclusion and Future Prospects

Recent advances in the field of tumor targeting focus on the design and development of highly sophisticated molecules with high tumor specificity. Molecular targeted therapies are rapidly changing its paradigm towards tumor specific antigen and have reached quite near to the original concept of *magic bullet*. Furthermore, in combination with antiangiogenics, immunotherapy and nanotechnology based approaches, the efficient management of even multidrug resistant tumors is also quite possible and several studies are currently under exhaustive clinical trials. The future work in the field of cancer therapy includes clearing the clinical trial pipeline into approved products followed by dedicated cancer prevention programs such as cancer vaccines.

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