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Novel Technologies in Biosystems, Biomedical & Drug Delivery

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Preface

Novel technologies include precision systems which although cover entire gamut of activities extended to various walks of life. The need of such technologies is a must for advancing the quality of life of human beings. Biosystems or Biological systems involve a host of systems like organs, tissues to various earth support systems. The right and delicate balance among the components, preserving the identity of the biosystems is a must for healthy sustenance of the earth planet and to retain it as liveable. Coexistence and sustenance of living organisms is possible provided the environment is conducive although excessive human intervention has posed a threat to very survival and sustenance of biosystems. Although there are numerous biosystems still this book is confined to a few. It discusses and deliberates on how biosystems function, their role in our everyday life and applications thereof. The book further ponders upon various drug delivery systems, the way they work and how are they better placed as compared to conventional systems like chemotherapy. The precision and clinical way of functioning of these different types of drug delivery systems, the materials used therein and their architectures employed in delivering and release of drugs to targeted cancer cells in a reproducible manner without affecting the healthy cells so as to attain efficiency and efficacy of drugs. This edited volume also comprises different perspectives of expert academicians, researchers and professionals on various aspects and facets of biosystems, drug delivery systems and extensive applications in the field of biomedicine. The book will not only enlighten the students, academicians and researchers but will also enrich their knowledge base as well as it will make a sound value addition to the subject domain of medicine and pharmacy in particular. It will encourage researchers to get necessary inputs to kick-start their research as well as to further it to the next level and will pave the way for creating and advancing healthy biosystems for healthy life on earth.

This book is divided into four parts. First part is dedicated to biosystems. Second part is devoted to Biomedicine. Third Part is aimed to Drug delivery systems while fourth part is meant for Proteomics. First part contains three chapters. Chapter "Editorial: Bio-Systems: Relevance, Reflection and Impact" is an editorial on biosystems, their relevance, reflection and impact. Chapter "Potential of Biotechnology in Cancer Management" is aimed at exploring potential of biotechnology in cancer

management. Chapter "Biosimilars: Promising and Rapidly Emerging Biotherapeutics" sheds light on potential of bio-similars as bio-therapeutic agents. Second part has four topics in it. Chapter "Applications of Nanomaterials in Medicine: Current Status and Future Scope" discusses present and future scope of nanomaterials in the field of biomedicine. Chapter "Biomedical Applications of Nanofluids in Drug Delivery" throws light on applications of nanofluids in drug delivery systems in the field of biomedicine. Chapter "Metagenomics for Drug Discovery" deliberates on role of Metagenomics in Drug discovery application. Chapter "Potential of Heterocyclic Compounds as EGFR-TK Inhibitors in Cancer Therapy" promise of Heterocyclic compounds as EGFR-TK Inhibitors in Cancer Therapy. Third part contains two topics. Chapter "Potential of Nanocrystalline Drug Delivery Systems" entails Potential of Nanocrystalline Drug Delivery Systems. Chapter "Novel Techniques in Pulmonary Drug Delivery Systems" is dedicated to adoption of novel techniques in Pulmonary drug delivery Systems the fourth part is the concluding part of this volume containing two topics. Chapter "Proteomics in Oncology: Retrospect and Prospects" gives an account of the role of proteomics in Oncology: Retrospects & Prospects while the last chapter "Proteomics Novel Prospects in Target Therapy for Infectious Diseases" is aimed at explaining proteomics, their novel prospects in target therapy for Infectious Diseases. Thus the volume is a balanced, meaningful and productive learning material.

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Proteomics: Scope and Potential

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Abbreviations

ACE 2	Angiotensin-Converting Enzyme 2
ACI	Anderson cascade impactor
ADAs	Anti-Drug Antibodies
ADCs	Antibody-drug conjugates
ADH1A	Alcohol dehydrogenase 1A: ADH1A
ADME	Absorption, Distribution, Metabolism and Excretion
AgNPs	Silver Nanoparticles
AIDS	Acquired immunodeficiency syndrome
ALDOA	Aldolase fructose bisphosphate A: ALDOA
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
antiSMASH	Antibiotics and Secondary Metabolite Analysis SHell
API	Active Pharmaceutical Ingredient
ASC	Adult stem cells
ATP	Adenosine triphosphate
$Au@Fe_3O_4$	Gold core iron oxide coated nanoparticles
AUC	Area Under the Curve
AuNPs	Gold Nanoparticles
BAC	Bacterial artificial chromosome
BC	Breast cancer
Bcl-2	B-cell lymphoma-2
BGC	Biosynthetic Gene cluster
BMP-2	Bone Morphogenetic Protein 2
bp	Base pair
Caco	Cancer coli
CAF	Cancer associated fibroblasts
CART	Chimeric antigen receptor-T cells
CCHFV	Crimean-Congo Hemorrhagic Fever Virus
CCL	CC chemokine ligand
CDSCO	Central Drugs Standard Control Organization
CeO_2	Cerium oxide

CF	Cystic fibrosis
CFTR	CF transmembrane receptor
CINV	Chemotherapy Induced Nausea and Vomiting
circFARSA	A circRNA derived from exon 5–7 of the FARSA gene
circRNA	Circular RNA
cm	Centimeter
cmCFTR	Chemically modified CFTR mRNA
CMOS	Complementary metal-oxide-semiconductor
CO_2	Carbon dioxide
COAD	Chronic obstructive airway disease
COPD	Chronic obstructive pulmonary disease
COX-2	Cycloxygenase-2
CPC	Content per canister
CQA	Critical quality attributes
Cs	Saturation Solubility
CsrA	Carbon storage regulator
CT	Computed tomography
CTC	Circulating tumor cells
CTLA-4	Fusion protein of Cytotoxic T-lymphocyte-associated protein 4 and IgG1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTNNB1	Catenin Beta 1
CVL	Cervicovaginal Lavage
D10	10% of the particles are less than 10 μm
D50	50% of the particles are less than 50 μm
D90	90% of the particles are less than 90 μm
da	Aerodynamic particle diameter
DARPins	Designed ankyrin repeat proteins
DCGI	Drugs Controller General of India
DDU	Delivered dose uniformity
de	Diameter of predicted geometric particle
DENV	Dengue Virus
Derf2	Recombinant dermatophagosides farina mite group 2
Dichloromethane	Dichloromethane
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DPI	Dry power inhaler
DR	Death receptor
DSC	Differential Scanning Calorimetry
DTE	Dilution to extinction method
dv	Sphere shaped or equal particle diameter
EGFR	Epidermal Growth Factor Receptor
EGFR-TK	Epidermal Growth Factor Receptor-Tyrosine kinase
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency

EpCAM	Epithelial cell adhesion molecule
ePCR	Emulsion polymerase chain reaction
erbB1	Erythroblastic leukemia viral gene-1
ES	Embryonic stem
ESCs	Embryonic stem cells
ESI	Electrospray Ionization
eSNaPD	Environmental Surveyor of Natural Product Diversity
FARSA	Phenylalanyl-TRNA Synthetase Subunit Alpha
FC	Flow cytometry
FDA	Food and Drug Administration
Fe_3O_4	Magnetite nanoparticles
Fe_3O_4@Au	Gold coated Iron oxide nanoparticles
Fe_3O_4-Au	Iron oxide and gold hybrid nanoparticles
FePt	Iron-platinum
$FeSe_2$-Bi_2Se_3-^{64}Cu	Bis(selanylidene)Iron decorated bismuth selenide radiolabelled with copper-64
FPD	Fine particle dose
FPF	Fine particle fraction
FPM	Fine particle mass
FRET	Fluorescence resonance energy transfer
g	Gravitational constant
Gd^{3+}	Gadolinium
GDEPT	Gene directed enzyme prodrug therapy
GLOBOCAN	Global Cancer Observatory
GMP	Good manufacturing practice
GSD	Geometric standard deviation
GVHD	Graft-versus-host disease
h	Hour
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCT116	Human Colorectal Carcinoma 116
HDAC-1	Histone deacetylase 1
HDL	High density lipoproteins
HED	Human equivalent dose
HepG2	Hepatoma G2
HER-1	Human Epidermal Growth Factor Receptor-1
HER-2	Human Epidermal Growth Factor Receptor-2
HER-2, Erbb2	Erbb2 receptor tyrosine kinase
HER-3	Human Epidermal Growth Factor Receptor-3
HER-4	Human Epidermal Growth Factor Receptor-4
HFMC	Hollow-fiber membrane chamber
HIV	Human Immunodeficiency virus
HPLC	High Performance Liquid Chromatography

HPMC	Hydroxy Propyl Methyl Cellulose
HPV	Human papilloma virus
HSC	Hematopoietic stem cells
HSPPC96	HSP-gp96 complex
HSV	Herpes simplex virus
HUPO	Human Proteome Organization
I.V	Intravenous
IAEC	Institutional Animal Ethics Committee
IBD	Inflammatory Bowel Disease
IBSC	Institutional Bio-Safety Committee
IC_{50}	Half-maximal inhibitory concentration
ICAT	Isotope Coded Affinity Tag
ICH	International Council for Harmonization
iChip	Isolation chip
ICI	Immune checkpoint inhibitors
IEF	Isoelectric Focusing
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IHD	Ischemic heart disease
ILP	Isolated limb perfusion
IMG/ABC	Integrated Microbial Genomes Atlas of Biosynthetic gene Clusters
ION@Bi_2S_3	Bismuth sulfide coated Iron oxide nanoparticles
ION-^{89}Zr	Metallo Zirconium radionuclide iron oxide nanoparticles
IOP	Intraocular pressure
IP	Indian Pharmacopoeia
IR	Infrared
I-tip	In situ cultivation by tip
iTRAQ	Isobaric Tags for Relative and Absolute Quantification
ITS	Internal transcribed spacer ITS
JNK	c-Junction N-terminal Kinase
kb	Kilobase
KF	Karl fisher
KQA	Key Quality Attributes
KRAS	Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
K-ras	Kirsten rat sarcoma viral oncogene
L/min	Liter/min
L858R	Mutation that substitutes Arginine for Leucine at amino acid position 858
LC	Liquid Chromatography
LC	Lung Cancer
LC-MS	Liquid Chromatography- Mass Spectroscopy
LC-MS/MS	Liquid Chromatography with mass spectrometry
LDL	Low density lipoprotein
LGR	Leucine-rich repeat-containing G-protein-coupled receptor

LIGh	Laser-induced graphene
LIT	Linear Ion Trap
LKB1	Liver kinase B1
LMP1	Latent membrane protein
LNP	Lipid-based nanoparticles
LOX- 1	Lectin-like oxidized LDL receptor-1
m/z	Mass to Charge
mAb	Monoclonal antibody
MAGE-A3	Melanoma-associated antigen 3
MALDI	Matrix- Assisted Laser Desorption/Ionization
MALDI-MS	Matrix-assisted laser desorption/ionization Mass Spectrometry
MAPK	Mitogen Activated Protein Kinase
MCF7	Michigan Cancer Foundation 7
MDI	Metered dose inhaler
MERS CoV	Middle East Respiratory Syndrome coronavirus
Mgh	Mass Gravity Height
miRNA	Micro RNA
MMAD	Mass median aerodynamic diameter
MNFs	Magnetic nanofluids
MnO_2	Manganese Dioxide
MRI	Magnetic resonance imaging
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectroscopy
MSC	Mesenchymal stem cells
MSN	Mesoporous silica nanoparticles
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MVA	Modified vaccinia Ankara
NaGdF4	Sodium gadolinium fluoride
nanopore	Nanoscale protein pore
NCQD-HCS	N-doped quantum dot captured carbon nanospheres
NCs	Nanocrystals
NF	Nanofluid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGI	Next generation impactor
NHV	Normal Healthy Volunteers
NIR	Near infrared
NK	Neurokinin
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NP	Nanoparticles
NRPS	Non-ribosomal peptide synthetases
NSAIDs	Non-Steroidal Anti-inflammatory Drugs

NSC	Neural stem cells
NSCLC	Non-Small Cell Lung Cancer
O_2	Oxygen
ODGE	One-dimensional gel electrophoresis
OECM1	Human oral cavity squamous cell carcinoma cell line
OKT3	Murine anti-CD3 mAb Muromonab-CD3
OMV	Oncolytic measles virus
ORR	Objective Response Rate
PA	Photoacoustic imaging
PABA	Para amino benzoic acid
PaCa	Pancreatic Cancer
PC	Prostate cancer
pCO_2	Partial pressure for carbon dioxide
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PDDS	Pulmonary drug delivery system
PDL1	Programmed cell death protein ligand 1
PDT	Photo dynamic therapy
PEG	Poly ethylene glycol
PEG	Polyethylene Glycol
PEG- PLGA	Poly ethylene glycol poly lactic acid co-glycolic acid
PEG-b-PHSA	Poly ethylene glycol -block-poly(N-hexyl stearate l-aspartamide)
PEG-PLA	Poly ethylene glycol poly lactic acid
PELG-PEG-PELG	Poly(ethyl-l-glutamate)-poly (ethylene glycol)-poly(ethyl-l-glutamate)
PET	Positron emission tomography
pI	Isoelectric Point
PK	Pharmacokinetics
PKSs	Polyketide synthases
PLA	Polylactic acid
PLGA	Poly lactic acid co-glycolic acid
PMA	Permanent magnetic actuator
PMF	Peptide Mass Fingerprinting
pO_2	Partial pressure for oxygen
POPC-1	Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
P_p	Particle density
PPI	Protein- Protein Interactions
PRISM	PRediction Informatics for Secondary Metabolomes
PSURs	Periodic Safety update report
PTX-NPs	Paclitaxel-laden nanoparticles
PVA	Polyvinyl Alcohol
PVD	Physical vapor deposition
PVDF	Polyvinylidene fluoride
PXRD	Powder X-ray diffraction

PYCR2	Pyrroline-5-carboxylate reductase 2
QAs	Quality Attributes
RA	Rheumatoid arthritis
RF	Radiofrequency
RNA	Ribonucleic acid
ROR1	Receptor tyrosine kinase-like orphan receptor-1
ROS	Reactive oxygen species
RP	Reference Product
rRNA	Ribosomal Ribonucleic acid
SAR	Structure-Activity Relationship
SARS CoV	Severe Acute Respiratory Syndrome Coronavirus
SCM	Single-cell metagenomics
SDF-1	Stromal cell-derived factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI	Surface-Enhanced Laser Desorption/Ionization
SELDI-TOF	Surface-enhanced laser desorption/ionization-time of flight
SEM	Scanning Electron Microscopy
sEV	Small extracellular vesicles
SF	Subconjunctival fibrosis
SFL	Spray Freezing into Liquid
SH2	Src Homology 2
SiO_2	Silicic oxide
siRNA	Small interfering RNA
SMBGCs	Secondary metabolite biosynthetic gene clusters
$SnFe_2O_4$	Tin ferrite
$SOCl_2$	Thionyl chloride
SOD	Superoxide dismutase
SOMA	Slow-rate-modified aptamer
SPMNPs	Superparamagnetic nanoparticles
ssDNA	Single-stranded deoxyribonucleic acid
SSU	Small subunit
STAT	Signal transducer and activator of transcription
Syn-BNP	Synthetic Bioinformatic Natural Product approach
T790M	Mutation that substitutes Methionine for Threonine at amino acid position 790
TAA	Tumor associated antigens
TAT	Trans activator of transcription
TC	Thermal conductivity
TCA	Thermogravimetric analysis
TCGA	The Cancer Genome Atlas
TG	Triglycerides
TGF	Transforming Growth Factor
TiO_2	Titanium Oxide
TK	Tyrosine kinase
TKIs	Tyrosine kinase Inhibitors

TME	Tumor microenvironment
TMT	Tandem Mass Tag
TNF-α	Tumor necrosis factor alpha
TOF	Time of Flight
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TSA	Tumor specific antigens
U87MG	Malignant gliomas cell line
UNP	Upconversion nanoparticles
USFDA	United Stated Food and Drug Administration
UV	Ultra Violet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VLDL	Very low-density lipoprotein
V_{TS}	Square root of terminal velocity
W	The adhesion work
WGS	Whole metagenome sequencing
WHO	World Health Organization
x	Dynamic shape factor
ZIKV	Zika Virus
ZnO	Zinc Oxide
η	Gas viscosity
θ	The drug molecules interaction parameter
μm	Micrometer
ρ_0	Density
ρliq	Density of liquid
ρvap	Density of vapor
σ	Cumulative strength
σA	Adhesive strength interactions
Σc	Cohesive strength interactions
Ø	Ratio of the particle bulk density and particle true density
μm	Micrometer
2D-DIGE	Two Differential in Gel Electrophoresis
2DGE	Two-dimensional gel electrophoresis
2D-LC	Two-dimensional liquid chromatography
2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis

Biosystems: Nature, Relevance and Significance

Editorial: Bio-Systems: Relevance, Reflection and Impact

Shrikaant Kulkarni

Abstract Bio-systems (BSs) are the systems which are of pivotal importance because of their nature, cohesiveness with a delicate and right balance and sound interactions among their components. Each component has its well defined role and function in the smooth sailing and sustenance of the BSs. The very survival of the earth planet depends upon the preservation of the sanctity of the host of BSs. To preserve sustainability of the BSs a growing realization and awareness about their importance and understanding the way in which they go about in harmony is a prerequisite. BSs are within us and the outside world. Maintaining conducive environment for their sustenance is what is needed most or right ecosystems is most sought after. The excessive human intervention has threatened the very survival and sustenance of the plethora of BSs or biological systems. In fact, nature has bestowed upon us BSs in various forms, size and geometry, morphology for the better cause of blue planet. If we want the earth planet to be liveable BSs should be restored, maintained, strengthened and sustained. They are in the form of support systems for the spheres of the environment to survive and sustain. This chapter throws light upon introduction, relevance, reflection, impact and applications of BSs.

Keywords Ecosystems · Biological agents · Bio-imaging · Biomedicine · Drug delivery systems · Genetics · Computer simulation-based models etc.

1 Introduction

A system as such consists of an array of mutually reliant, inter-dependant parts in a cohesive manner as a unified whole. Every system has its own characteristic behaviour which is considered as a property in itself exhibited by the complete system rather than their isolated components. BS refers to an organism or a group of them that interact with the environment. Systems biology is evolving and is significantly

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expanding, but there is a lack of awareness about the retrospects of systems biology or behaviour which makes it interesting to explore. There have been transitions in perceptions about configuration and behaviour of systems as well as advancements too. The discussion here concentrates on system biology before an advent of genomics and biology with the sole purpose of gaining insights to present day systems biologists for developing and laying expansive foundations of the domain.

2 Relevance

There has been a meaningful and productive flow of concepts and ideas across various biological and artificial systems. Previous studies have been instrumental in furthering reinforcement learning (RL) algorithms for man-made systems and was driven by learning rules. Bush et al. developed such learning rules for the first time in biology. In recent past, temporal-difference RL, aimed at learning has laid down a framework as a foundation for making out the way dopamine neurons work. An emphasis is laid upon drawing knowledge from different subject domains and locate areas wherein futuristic research is influenced by the flow of information between such fields. Major chunk of work in bio-systems is devoted to easy learning exercises, governed by the flexibility in environmental conditions where dynamism and learning are very vital in semblance with the practical and global learning-based problems. While work dedicated to man-made systems emphasized upon learning a complex problem governed by particular environmental conditions. Further, advancement in every subject area takes place by the exchange of knowledge which is like a strength of it.

The comprehension of systems makes a significant effect on human sciences, covering social sciences and medicine. System biology brought about revolution in biological and evolutionary sciences made its ways in biochemistry, genetics, and Botany. Biology at molecule level too embraced a system approach recently. Rapid developments in genomics made it all possible. Present age is of systems, and system structure and system behaviour should always be at the centre stage. All bio-systems are primarily and necessarily systems embedded in other systems, as prescribed by Jacob. Gaining an understanding of the complex nature bio-systems in itself is quite challenging both intellectually and experimentally to biologist.

This chapter is structured in three parts. First, discussion on the way systems approach evolved followed by the way it replaced or refined. The hierarchy adopted in the structure of systems is pondered upon then as well as the premises of a definition. Since systems consist of inter-wined parts, the connect and interplay among the different parts and the inheritance followed is dwelt upon, and the chapter concludes with emphasis on aspects of systems behaviour which are have not been understood well and counterintuitive.

3 Reflection

BSs promote theoretical, computational and experimental studies that are instrumental in connecting biology, evolutionary, and information processing sciences. A study of BS will help in an understanding of the repercussions of the discoveries in biology and genomics for eliciting greater degree of knowledge about the way biological organization, mind and language originated and evolved, and became adaptable. System approach encompasses mechanism by virtue of which biological information is processed. It embodies quantum phenomena involved in the transfer of information, natural computing, coding, and complexity in biological systems, underlying biology-based theory. Currently, advances in the study of BSs is used to advantage in furthering the understanding and evolution of man-made life, computational and evolutionary modelling of vexed biology-based systems, use of conceptual understanding of biology in designing new systems of computation and application of bio-molecules for developing man-made systems that make use biology principles to process information.

The study of BSs is aimed at promoting the furtherance of biological computation, molecular recognition, biological coding systems, systems that organize by themselves and replicate, origin and evolution of mind and language as well as genetic mechanism, stochastic evolutionary algorithms, simulation of genetic and biological systems. Applications of it include neural networks, ML, robotics other than gaining novel biological insights from complex data using AI-driven language models etc.

A BS is a club of species or organs that operate in harmony for performing a given task. In vertebrates (including mammles), lymphatic, muscular, nervous, reproductive, respiratory, skeletal, immune, and urinary system etc. are the systems biological in nature.

A typical example is the cardiovascular system wherein the pumping of blood through the heart followed by circulation by virtue of blood vessels. In mammals, components include heart, blood vessels, and blood. Central Nervous system {CNS} is an another example. Human CNS comprises of spinal cord, brain, and peripheral NS.

4 Impact

Bio-systems ensure proper coordination through transmission of signals across the body. At micro-level (like bacteria), a system means macromolecular complexes or organelles in a cell.

Nature is also a complicated system with a host of components interwoven involving microbes, plants, animals, and holistic ecosystems with their well-defined and characteristic roles. Systems biology takes an approach which is holistic in studying living organisms. It peeps into the way numerous living organisms interact at varying degree of scales. e.g. An human is a system. A human system covers

organs, tissues, cells, and the molecules constituting them other than bacteria and other microbes living on skin and in our digestive track. Systems biology involves a study of all the parts and the way they work in tandem. Scientists can upscale or downscale a system biology approach based on the size of the system under examination. E.g. human organs are systems in themselves, and comprised of cells, proteins, and amino acids.

5 System Biology

Systems biology depends upon computational and mathematical analysis and modelling and is a confluence of disciplines drawing knowledge and data from biological sciences and technologies commonly known as "-omics." These "omics" include genomics (study of gene sequencing) and proteomics (study of proteins) in an organism. Such subjects emphasize upon analysing and quantitating the biological molecules instrumental in building, functioning and sustenance of organisms.

System biology studying the genomes of soil-borne microbes observed that microbes too are infested with many viruses that influence the way microbes metabolize carbonaceous organics.

Mapping and matching the decoded genomes of numerous plants helps in understanding the way plants sequester CO_2 and store carbon in cellulose and other polymeric materials of which plant body is constituted.

E.g. Baker's yeasts are employed in producing ethanol for beer as well as a biofuel. Systems biology study of them helps scientists to develop novel yeast strains that will help produce a product that can replace gasoline.

6 Applications

System biology possess innumerable applications. Bioenergy research is a notable application area. Scientists are exploring the potential of plants in their use as a biofuel, including the way they grow the microbes responsible for degrading organic matter, and the mechanism underlying the working of components in tandem. This kind of way of thinking helps scientists in improving the system underlying biofuels to produce highly efficient, cheap, and bio-fuels. System biology is very vital in gaining an insight in the smooth sailing of carbon cycle. Major chunk of the CO_2 at the global level is absorbed by forests as ecosystems Scientists are exploring the dynamic and complex interplay between the soil and plants which is instrumental in capturing carbon dioxide, microbes that degrade organic matter and release carbon as CO_2 to atmosphere.

Research that relies on a systems biology perspective includes application of system biology to issues concerning energy and environment. It further covers the

genetic information stored genomes of organisms, discovering the principles responsible for guiding the decoding of the genetic code. Further research id devoted to study the architectures for metabolism and regulation underlying the plant and microbe physiology on exposure to the environment. This approach should facilitate researchers in designing microbes and plants that are self-reliant and provide clean energy. E.g. systems biology should aim to improve biofuels, bio-products, enhanced carbon storage and proper regulation of nutrients and contaminants.

7 Regulation of BSs

Regulation of BSs involve systems modulated from cell to populations. It takes into cognizance mechanism involved in controlling negative feedback and the way sugar, water, and temperature is regulated in blood in human beings. It, covers control on population and ecosystem inheritance and many other controlling mechanisms in biological systems.

This is a must because there are implications of the biological discoveries and information derived as well as the genetic code in order to understand better the manner in which biological organizations, mind and language originated, and became adaptable to the environment.

The premises of BSs cover biological information processing, quantum phenomena responsible for information transfer, bio-computing, coding, complexity, artificial life, computational and evolutionary models for computation, biology principles in designing novel computing systems and application of biomolecules in producing man-made systems that leverage biological data.

8 Conclusion

This editorial emphasizes upon gaining an insight into the theoretical background, conceptual understanding, state-of-the-art developments and applications in the field of pharmaceuticals in general and drug design, discovery, BSs, Biomedical & Drug Delivery Technologies. Knowledge from interdisciplinary areas such as Chemistry, Biology, Material Science & Engineering, Statistics, Biomedicine, Genetics etc. is drawn as the topics subject domain identified is a confluence of a host of disciplines possessing plethora of applications like Bio imaging, Novel Biological Agents, testing, characterization and validation of drugs right from identification of right precursor to standard protocols followed in designing a drug using a right pathway, adoption of computer simulation-based models used in drug design, selection of right precursor and route, application of statistical tools in analysing and interpreting data, design, functionalization, and operation of drug delivery systems, their biocompatibility, capacity of carrying and release of drug and a host of ecosystems too will be

dealt with appropriate details etc. This chapter will certainly confirm to the requirements of the post graduate students, research scholars, academicians, scientists and researchers from the academia, pharmaceutical, Biotechnology and/or chemical engineering domain. The chapter covers conceptual understanding to exploration of drugs in tandem with intended uses, sound ecosystem development and carriers for drug and supplements delivery.

Potential of Biotechnology in Cancer Management

Alex George, Jinsu Varghese, and Hafiza Padinharayil

Abstract The application of biotechnology in cancer therapy is widespread, particularly when combined with traditional medicines. Immunotherapy the fifth pillar of cancer management is highly benefited with advancements in biotechnology over other methods such as surgery, radiation, chemotherapy, and targeted therapy. Engineered cytokines, designer vaccines, cell therapy, and gene therapy fall in immunology-based biotechnology approaches for cancer cure. These advancements along with combination therapy can be a potential remedy not only for cancer treatment but also for the current challenges of drug resistance, disease recurrence, and post treatment hazard effects.

Keywords Biotechnology · Cancer · mAb · CAR therapy · Stem cell therapy · Engineered cytokines · Vaccines · Combination therapy

1 Introduction

Over twenty million individuals globally were affected by cancer in 2020, and more than nine million people have died due to this malignancy(https://gco.iarc.fr/). The polyclonal growth tumor cells followed by stroma modification with the assistance of immune cells aid cancer progression (Fig. 1). Although current therapies including radiation treatment, and hormonal treatment are thought to be successful, their effectiveness is severely hampered by secondary resistance and hazard effects. In turn, this heightens the necessity for an alternate strategy in addition to traditional medications to treat the patients (Qiao et al. 2016). Recombinant DNA technology, an early kind of genetic engineering where scientists integrated genome data from many

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species in unique ways to aid patients in the treatment or management of specific ailments, gave rise to the older beginnings of biotechnology in the 1970s (Khan et al. 2016). Biotechnology approaches have been started to combine with conventional treatment options including chemotherapy and immunotherapy. Combining biotechnological approaches with traditional treatments improve patients' overall survival. The invention of inhibitors of the programmed cell death protein 1/programmed cell death protein ligand 1 (PD1/PDL1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) for cancer therapy was recognized with Nobel Prize (Tasuku Honjo and James P. Allison) (Sato et al. 2020). Biotechnology associated immunological approaches are highly competitive with other conventional therapies considering clinical outcome. For instance, over 20 recombinant products of available cytokines can not only function as important immune response signaling transmitters but also as powerful immunotherapy candidates. The efficient and safe injection of cytokines in therapy, however, faces a range of difficulties, from reduced half-life to post-hazard effects, and pleiotropic signaling to aggressive immune functions. Chimeric antigen receptor (CAR)-T cell therapy possess the capacity to develop into immunotherapeutic agents for the treatment of hematological disorders, which present emergency health demands. To overcome challenges like hazard effects corresponding to CAR-T, CAR-T/NK cell therapies have recently gained attention as innovative treatment interventions. Although vaccines possess the potential to benefit patients who are unresponsive to existing standard-of-care immunotherapies it has yet to be considered as an oncologic treatment.

In this chapter, current cancer epidemiology and the significance of biotechnology integrated immunotherapy studies using the data curated from The Cancer Genome Atlas (TCGA)-cBioportal is discussed. The clinical advancements of CAR T cell therapy, monoclonal antibody (mAb), stem cell therapy, engineered cytokines, and various types of designer vaccines are also discussed together with the necessity of biotechnology advancements to integrate with combinatorial approaches over other conventional therapies.

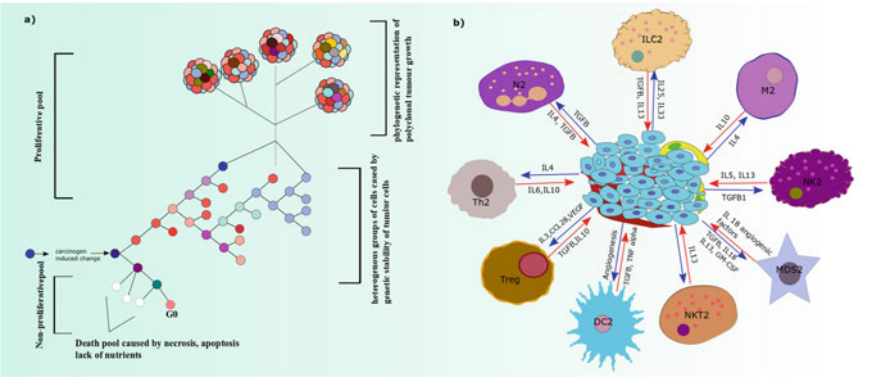

Fig. 1 **a** Polyclonal growth of cancer **b** immune cells and cytokines involved in immune evasion

2 Current Cancer Epidemiology

With over 8.8 million fatalities globally and 14 million new occurrences of cancer identified every year, cancer is among the most common diseases with high mortality. Monitoring epidemiologic data is essential because it offers crucial details on the cancer's current status statistical, biological, and geographical viewpoints, enabling the creation of suitable medical interventions (Montagnana and Lippi 2017). The most and least common cancers based on latest update of Global Cancer Observatory (GLOBOCAN)-2020 along with their percent increase or decrease comparing GLOBOCAN-2018 is as follows: prostate (9.7%), lung (4.6%) and colorectal cancer (3.7%) are the common cancer in male while Kaposi sarcoma (−3.4%), mesothelioma (−0.4%) and salivary gland tumors (1.4%) are the least. Females possess higher incidence of breast cancer (7.6%), lung cancer (5.8%) and colorectal cancer (4.8%), while it is lowest in Kaposi sarcoma (−24%) (https://gco.iarc.fr/).

A rise in cancer mortality rate even overtaking ischemic heart disease is predicted by WHO 2016–2060 projection data, and the prediction matches with GLOBOCAN 2020 update (https://gco.iarc.fr/). The immunogenomic studies reported in cBioportal based on their potential biotechnology approaches used was curated. Studies entitled Glioblastoma (Columbia: 42 samples), Metastatic melanoma (DFCI: 110 samples, MSKCC: 64 samples, UCLA: 38 samples), non-small cell lung cancer (NSCLC) (MSK: 75 and 16 samples), TMB and immunotherapy (MSKCC: 1661 samples), and Clear cell renal cell carcinoma (DFCI: 35 samples) were selected. Somatic mutations and copy number variations contributed to greater than 95% of genomic profiles in these studies. The KM plot for overall survival after pooling all the samples have shown a significant fall within a year after availing various cancer therapies. The five-year overall survival rate has shown to be less than 20%, which indicate the need for improved therapeutic approaches in cancer cure.

TCGA-cBioportal based 213 non-redundant studies were queried for *PDCD1, CD274,* and *CTLA4* gene (Table 1), which are greatly exploited in immunology-based biotechnology approaches including immune checkpoint inhibitors (ICIs), mAb, and vaccines (Fig. 2), and their mutual exclusivity were analyzed (Table 1). *PDCD1* and *CTLA4* genes were shown Log2 Odds ratio of greater than three ($p<0.001$), which indicate their combined roles in cancer progression.

3 Biotechnology in Cancer Therapy

3.1 Monoclonal Antibody

Although immune checkpoint inhibitors (ICIs) are thought of a type of mAb, they differ in their mode of action. mAbs can locate, bind, and interfere with neo-antigens present on cancer cells (Buchbinder and Desai 2016). CD38, CCR4, PDGFR,

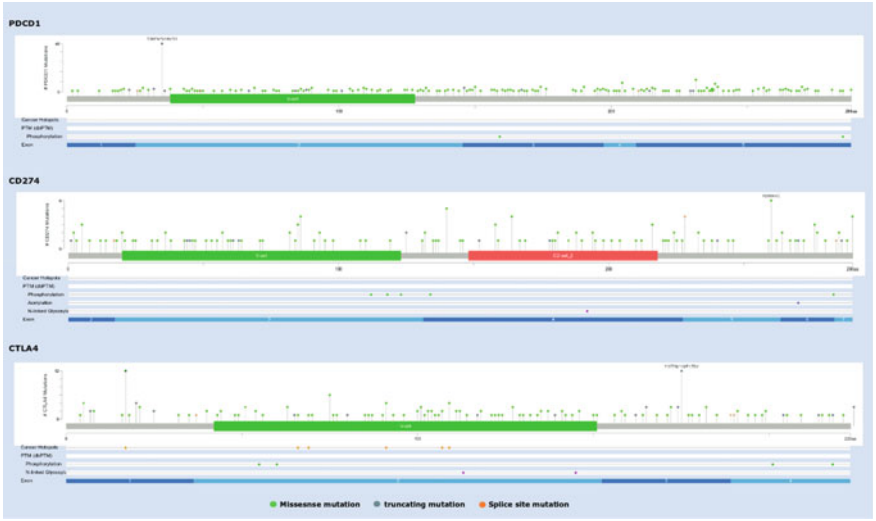

Fig. 2 TCGA cBioportal based data representing mutational burden of PDCD1, CD274, and CTLA4. *Data source* https://www.cbioportal.org/

Table 1 Mutual exclusivity of *PDCD1, CD274,* and *CTLA4* corresponding to the proteins PD1, PDL1, and CTLA4 (data curated from TCGA-cBioportal: https://www.cbioportal.org/)

A	B	Neither	A Not B	B Not A	Both	Log2 odds ratio	p-Value	Tendency
PDCD1	CTLA4	45,033	572	297	82	>3	<0.001	Co-occurrence
PDCD1	CD274	44,666	616	675	38	2.029	<0.001	Co-occurrence
CD274	CTLA4	44,917	688	354	25	2.205	<0.001	Co-occurrence

Nectin4, TROP2, CD3, CD20, CD79B, HER2, GD2 and SLAMF7 are few of neo-antigens mAbs target in malignant cells (Zahavi and Weiner 2020). They either hijack growth factor assisted signaling via receptor ligand complex inhibition, subsequently inhibiting tumor progression (Li et al. 2005). Cetuximab, an epidermal growth factor receptor (EGFR) mAb, inhibit receptor dimerization and ligand binding followed by neoplastic cell growth inhibition (Patel et al. 2009). Trastuzumab is the pioneer drug approved by Food and Drug Administration (FDA) against Erbb2 receptor tyrosine kinase (HER)-2 which in HER2 positive breast cancer inhibit receptor internalization (Wang and Xu n.d.; Chen et al. 2003). B cells corresponding to Non-lymphoma Hodgkin possess high level of CD20 expression on their surface, in contrast to normal, embryonic B cells. Consequently, a mAb therapy that specifically aim CD20 may kill the malignant cells while leaving behind embryonic B cells to restore the body's pool of normal tissues. As a result, CD20 was chosen as the primary target for mAb treatment, and rituximab (an anti-CD20 mAb) was the first mAb to get approval for

cancer therapy (Maloney et al. 1997). mAbs other than ICIs such as elotuzumab, mogamulizumab, ramucirumab, pertuzumab, enfortumab vedotin, sacituzumab, and govitecan are used to treat bladder cancer, multiple myeloma, sarcoma, gastric cancer, breast cancer, cutaneous T cell lymphoma, and triple negative cancer respectively (Anand 2019).

To determine the impact of mAbs in various cancers, multiple clinical trials are now being conducted. A phase II study (NCT04895137) combining mFOLFOX6 with PD1 and bevacizumab is being conducted to analyze tolerability and effectiveness of mAb conjunctions in people tested positive for colorectal cancer. The effectiveness of rituximab with ixazomib in treating mantle cell lymphoma has evaluated in a phase II study (NCT04047797). Rituximab possesses the capacity impair the competence of tumor cells and to alter the immune cells, therefore slowing the spread of the disease. The results indicates, patients with mantle cell lymphoma may benefit from using rituximab with ixazomib in addition to rituximab monotherapy (Vose et al. 2012).

3.2 Stem Cell Therapy

A promising approach in cancer therapeutics is stem cell treatment, which includes all conventional techniques using stem cells. Due to its greater focus on tumors and resulting reduction in target events, it can enhance the clinical effectiveness of other medicines. There are now several stem cells assisted cancer therapy methods being researched in preclinical studies, and they provide both enormous opportunities and concerns (Gomes et al.2017) (Table 2). A revolution in molecular genetics occurred in 2006 with the discovery of Yamanaka factors, which allowed transition of somatic cells to become pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). These iPSCs exhibit the same traits as embryonic stem cells (ESCs) while avoiding the moral dilemma of embryo killing. As of present, the creation of cancer vaccinations (Kooreman et al. 2018; Ouyang et al. 2019) and effector NK and T cells' activation rely on iPSCs and hESCs as significant sources of material. Many kinds of specialized cells for the tissue and organ may be produced by adult stem cells (ASCs). For cancer therapy, mesenchymal stem cells (MSCs), neural stem cells (NSCs), and hematopoietic stem cells (HSCs). Notch, hedgehog, PI3K/PTEN, NF-kB, Wnt/β-catenin, and JAK/STAT based regulation of typical stem cell growth is well known. Cancer cells and CSCs will develop as a result of the ongoing alteration in the signaling pathways, and crosstalks (Matsui 2016). CSCs possess a strong ability to differentiate and self-renew, which helps with tumor development, invasion and recurrence (Jordan et al. 2006; O'Brien et al. 2010). Additionally, such cells cause malignancies to resist standard treatment (Cojoc et al. 2015; Chang 2016; Batlle and Clevers 2017). To create an effective medicine for the cure of cancer, investigation into CSCs is crucial. Leukemia, gastrointestinal, lung, brain, and breast malignancies are just a few of the tumor types where CSCs have been shown to occur. These cells are routinely separated and recognized utilizing a number of techniques,

such as surface protein markers, signaling components, transcriptional candidates, and metabolic or functional characteristics (Codd et al. 2018; Toledo-Guzmán et al. 2018). Key traits and biochemical mechanisms of CSCs have some resemblance to regular stem cells, from whence they originated. Many surface markers, including CD133 HSC marker (Prominin-1), leucine-rich repeat-containing G-protein-coupled receptor (LGR)-5, CD24, CD44, epithelial cell adhesion molecule (EpCAM) are explored in distinguishing CSCs from extremely diverse cell types in malignancies, despite the fact that they may also be found in normal cells (Codd et al. 2018).

HSC transfusion has largely utilized as a primary therapy for leukemia, multiple myeloma, and lymphomas following multiple cycles of radiation or chemotherapy. Additionally, this method is now thoroughly explored in clinical trials of brain tumors (NCT00528437), breast cancer (NCT01807468), neuroblastoma, and sarcomas. Employing heterologous sources of HSCs, graft-versus-host disease (GVHD), which is frequently treated with immunoinhibitory medications with substantial adverse effects and reduced efficacy, is still a problem (Copelan 2006).

In individuals with persistent GVHD, MSCs with immune-educating properties may successfully lessen pronounced immunological reactions. Upon the injection of MSCs and HSCs together, clinical studies reported positive results without any associated negative effects. Mesenchymal-angioblast derived MSC transfusion is being evaluated for tolerability, durability, and effectiveness in people with steroid-resistant GVHD in active multi-center experiment (NCT02923375). MSCs can speed up the healing of damaged organs and may help the body tolerate high dosage chemotherapy, which will have a greater impact on tumor-destroying outcomes (Lee et al. 2011).

Stem cells as possible therapeutic carriers can enhance shelf life of enclosed cargo by protecting from degradation, enhance target specificity and reduce systemic hazard effects. Nanoparticle carrying stem cells being a long running candidate in the field of cancer therapeutics, their lack of proper targeting potential, uncontrollable cellular uptake, and fall in rapid secretion from body is still challenging (Rosenblum et al. 2018). Nanoparticles may be internally absorbed passively or actively by endocytosis, depending on their surface properties, size, processing period, and quantity (Behzadi et al. 2017). The key issues are medication dosage management and probable cell carrier hazard effects. Furthermore, the cells' quick nanoparticle exocytosis might result in the uncontrolled secretion of therapeutic medications into places that aren't intended for them. Despite affecting cell survival or functioning, Roger et al., demonstrated efficient MSC based internalization of PLA and lipid nanoparticles (Roger et al. 2010). Following their immediate tumoral infusion, MSCs transported these nanoparticles into brain tumors in the glioma mouse model. In a different investigation, intravenous injection of MSCs packed with paclitaxel-laden nanoparticles (PTX-NPs) produced drug deposits and increased nanoparticle localization in mice that established orthotopic lung tumors (Layek et al. 2018). Albeit the overall dosages of PTX-NPs used in these nanoparticle encapsulated MSCs were substantially lower than those used in PTX solution/PTX-NPs alone, it is worth noting that they greatly reduced tumor development and improved mouse longevity. MSCs confined in the lung parenchyma but later moved to tumor sites because of their cancer-tropic effect (Lee et al. 2009; Wang et al. 2019a, b). The method that promotes cellular nanoparticle

Table 2 Clinical trials considering various biotechnology approaches with specific regard to vaccines, CAR T cell therapy, and Stem cell therapy

	Disease	Target	Phase	Clinical outcome	NCT
Viral vaccine	Nasopharyngeal carcinoma	LMP1, LMP2	Ib	ELISPOT based determination of LMP2/EBNA1	NCT01800071
	Vulvar/anal squamous carcinoma	HPV E6/E7	IV	Recurrence of HSIL	NCT03051516
Mutant self, tumor specific antigen	Glioblastoma	EGFRvIII	II	Direct correlation between antibody response and treatment duration; high median PFS and OS	
	Glioblastoma	EGFRvIII	III	Antibody response without significant increase of PFS and OS	
Development specific, TAA	AML	Wilm's tumor 1	III	OS as a primary outcome	NCT04229979
	NSCLC	MAGE-A3	I/II	Safety and tolerability	NCT04908111
	Ovarian cancer	NY-ESO-1	I	Tumor inhibitory T cells with PFS of 19 months	
Tissue specific, TAA	Breast cancer	HER2	II	Expression of TIL and HER2 antibodies	NCT03632941
Tumor enriched, TAA	Epithelial ovarian cancer	P53	I	CD8+ T cell response Improved PFS	

(continued)

Table 2 (continued)

	Disease	Target	Phase	Clinical outcome	NCT
	Melanoma	IDO	I/II	80% ORR versus 41% ORR in control arm	
Personalized antigen cancer vaccines	Melanoma	RO7198457	II	Progression free survival	NCT03815058
	Melanoma	Lipo-MERIT	I	Adverse events	NCT02410733
	Glandular and epithelial cancers	GRT-C901/2	I/II	Four patients belong to GO-004 arm expressed CD8+ T cells	
Ex vivo vaccines	Melanoma	HSP	III	Subset of M1a/b stages reported DFS after ten vaccinations	
	Glioblastoma	HSP	II	1 year OS	NCT03018288
	Pancreatic cancer	GVAX	II	Significant difference in median OS was not noted	NCT02004262
	Glioblastoma	Autologous tumor-pulsed DCs	II	3-year overall survival	NCT03435952
In situ loaded vaccines	Melanoma	TVEC	III	50/295 patients in TVEC group availed CR	
	Pancreatic cancer	Parvovirus H1	I/II	Safety and tolerability, pharmacokinetics, humoral response,	NCT02653313
	Lymphoma	Flt3L	I/II	8/11 patients had disease regressions	NCT01976585

(continued)

Table 2 (continued)

	Disease	Target	Phase	Clinical outcome	NCT
CAR-T cell therapy	B-ALL	CD19	II	Progression free survival	NCT03876769
	DLBCL	CD19	III	Event free survival upto five years	NCT03391466
	B-NLH	CD20	I/II	Maximum tolerated dose and overall response rate determination	NCT03664635
Stem cell therapy	Advanced, recurrent or metastatic gastrointestinal adenocarcinoma	Ganciclovir encapsulated in HSV-TK-expressing MSCs	Phase I/II, completed		EudraCT 2012–003,741-15
	Recurrent high-grade gliomas	5-FC and Leucovorin encapsulated in CD-expressing NSCs	Phase I, ongoing	Number of doses limiting toxicities per dose level	NCT02015819

Data source https://gco.iarc.fr/

absorption may enhance clinical benefits. Trans activator of transcription (TAT), for instance, can increase the uptake of poly (lactic-co-glycolic acid) (PLGA) NPs into MSCs (Moku et al. 2019).

Immune cells are capable to quickly identifying and eliminating bare oncolytic viruses from the body. Interestingly, to preserve and transport oncolytic viruses to tumor locations, stem cells may be used as a potential carrier. For instance, hNSC line transfected with CRAd-Survivin-pk7 oncolytic virus, in conjunction using temozolomide and ionizing radiation, might boost cytotoxicity in vitro in glioma cells and prolong the lifespan of mice suffering glioblastoma multiforme (Tobias et al. 2013). Additionally, it was shown that MSCs could successfully carry modified oncolytic herpes simplex virus (HSV) and oncolytic measles virus (OMV) and to inhibit the formation of glioblastoma and hepatocellular carcinoma, respectively (Duebgen et al. 2014; Ong et al. 2013).

Furthermore, stem cell derived small extracellular vesicles (sEV) can be used to load cargo molecules such as miRNA, small drugs or proteins. Compared to other manufactured nanoparticles, these endogenous carriers have a number of advantages, such as exceptional intestinal absorption, durability, cargo loading efficiency, biocompatibility, and improved internalization into tumor cells (Fuhrmann et al. 2015). Additionally, they are readily functionalized by adding receptors or corresponding ligands to enhance targeted actions in tumor microenvironment (TME) (Smyth et al. 2014; Kooijmans et al. 2016; Wang et al. 2017). The conventional transfection method was effectively used to encapsulate genetic resources, such as anti-tumor siRNAs/miRNAs, into sEVs generated from stem cells. sEVs from bone stromal cells that expressed miR-146b have shown direct infusion into tumors in a brain tumor model resulted in a notable slowdown of the development of the glioma xenograft (Katakowski et al. 2013). Further research found that sorafenib's anticancer effects on a hepatocellular carcinoma tumor model were markedly improved by sEVs released by MSCs that contain the miR-122 gene (Lou et al. 2015). sEVs made from MSCs also successfully transferred siRNA in order to silence the polo-like kinase 1 gene in bladder cancer (Greco et al. 2016).

3.3 Gene Therapy

It was initially proposed in 1966, subsequently this approach used viruses as the carriers of transforming genetic material in 1968. Gene replacement therapy followed by gene therapy trials, X-SKIDS, oncolytic virus, a gene-based medicines was approved in the cancer therapeutic milieu. Numerous potential approaches are currently ongoing for using gene therapy to treat cancer. It include: (a) use of wild type tumor inhibiting gene; (b) preventing the oncogene expression using an antisense nucleotide approach; (c) expressing apoptosis inducing gene or increase tumor responsiveness to traditional therapy or drugs; and (d) improving the immunogenicity to promote immune cell recognition (Das et al. 2015).

Stem cell-based gene therapy is a recent advancement in cancer cure. Gene directed enzyme prodrug therapy (GDEPT) employ modification of soluble factors such as tumor associated cytokines or prodrug converting enzymes (Malekshah et al. 2016; Sage et al. 2016). A healthy stem cell's enzyme composition can convert prodrugs into bioactive components that are more harmful to tumor tissues. For instance, 5-fluorocytosine is successfully transformed into the tumor-toxic compound 5-fluororacil after being infused with MSCs/NSCs that display the cytosine deaminase enzyme (Malekshah et al. 2016; Lee et al. 2013). Similarly, irinotecan, relatively less powerful prodrug, can be converted into SN-38, a molecule that is thousand times more poisonous, when carboxylesterase is present. When carboxylesterase secreting NSCs and irinotecan were administered together rather than separately, neuroblastoma mouse xenograft model responded more effectively (Choi et al. 2016; Gutova et al. 2017).

3.4 CAR-T/NK Cell Therapy

Expression of CAR by genetically altering NK and T cells, may recognize TAA with precision. "Classical" CARs are made up of an extracellular domain (scFv) that is typically obtained from a mAb fragment and connected to ICD of T-cell receptor. T cell get activated upon tumor antigen coupling with scFv, in a way that is irrespective of MHC, followed by a cytotoxic effect (Hartmann et al. 2017). New CAR constructions have constantly been created, some of which may have modified cytosolic co-stimulatory sequences or targeting sequences. The targeting domain may be made up of other entities rather than scFvs, such as designed ankyrin repeat proteins (DARPins), nanobodies, or ligands (Balakrishnan et al. 2019; Duan et al. 2019; You et al. 2019; Zhylko et al. 2020). Separating antigen detection followed by CAR-cell stimulation has also led to the development of adapter CARs. Using CAR-cells that identify specific adapter molecules for tumor antigens, site specific and time-limited treatment is possible. Multiple antigens may be targeted simultaneously as a result, and the treatment can be modified if tumor types that lack antigens are discovered. This strategy also offers the opportunity to stop the immune reaction if serious adverse effects occur (Lee et al. 2019).

NK or T cells from peripheral blood are initially extracted for the CAR cell production. CAR-encoding nucleotides are then introduced into cells using viral vectors. To combat cancerous cells, CAR-modified cells are multiplied until an adequate cell count is reached and then administered by adoptive transfer to the recipient. Most treatment scenarios include lymphodepletion before the CAR-modified cells are infused to promote effective cell regeneration (Neelapu 2019). It is crucial that allogeneic CAR-T cell treatments are now being developed (Depil et al. 2020; Jamali et al. 2020; Müller et al. 2020; Reindl et al. 2020). FDA and the European Commission have approved three medications for the management of refractory or relapsed hematological malignancies using CAR-T cells during 2017–2021 (I; U.S; Detela and Lodge 2019). A new CART cell approach, which the FDA just authorized (U.S), is

now being evaluated in Europe. Following on such pinnacle results, other CAR-T cell treatments are presently being evaluated globally. Around 500 clinical studies looking into CAR-T cells for cancer care have reported. Of them, the most trials are being conducted in East Asia, followed by the US and Europe. In particular, CAR-NK cell therapies are increasingly being used in place of CAR-T cell therapies because they have promising benefits over CAR-T cells, including an inherent ability to destroy cancerous tissues and a limited number of hazard effects after infusion (Yoon et al. 2010; Rubnitz et al. 2010; Moretta et al. 2011). Although there are several CAR-T cell treatments available, only a small number of CAR-NK cell studies are being carried out globally. On clinicaltrials.gov, nineteen studies using CAR-NK cells for solid tumor treatment as well as hematological malignancies are officially enrolled. There are now three CAR-NK cell studies active in the China and US, and just one study being undertaken in Europe. Just few studies are also being conducted right now that focus on CAR-NK/T cell products and CAR-modified cytokine-based killer cells.

3.5 Engineered Cytokines

Cytokines impart various biological processes involved in cancer progression through acting as signal mediators in immune cells and cancer stroma. Structurally modified cytokines are potential immunotherapeutic candidates in cancer management, in which more than twenty modified cytokines are FDA approved for various diseases including cancer. Systematic hazards, off-target effects, poor circulation and reduced target specificity add to the limitations of cytokine-based immunotherapies. Modified cytokines regarding size, modifications in single amino acids (muteins), polymer conjugates and biomaterial implants correspond to engineering concepts of cytokines (Uricoli et al. 2021) (Table 3). The engineered immunostimulatory cytokines fall to size range of nanometer to millimeters for muteins to hydrogel implants respectively. IFNα was the first approved designer cytokine in 1986 which enhance the clinical efficacy of hairy cell leukemia by promoting apoptosis (Berraondo et al. 2019), followed by the approval of IL2 in 1992 for metastatic renal cancer. Both of these cytokines showed a favorable result in a small subset of sample groups while keeping a high degree of hazard effects (Golomb et al. 1986; Sleijfer et al. 2005) including cardiotoxicity, treatment related death and neurotoxicity (Rosenberg et al. 1989, 1998). Nonetheless, IL15 mediated NK cells and CD8+ T cells activation also had similar outcomes (Conlon et al. 2015).

Muteins, designed by single amino acid substitution, are relatively efficient in target based immune activation. IL2 can be considered as a potential pharmacological agent due to their pleotropic effects in vivo. IL2/IL2R complex is specific on CD8+T cells and NK cells than their homology counterpart (CD25) on Treg immunosuppressive cells, which substantiate tumor suppressive environment (Liao et al. 2013). For instance, IL2 superkine, mutated IL2 anywhere as R81D, I86V, I92F, L80F, and L85V, shows high binding affinity with IL2Rβ followed by activation of

Table 3 Comparison of various engineered cytokines and their delivery vehicles (Uricoli et al. 2021)

Engineered cytokines and delivery vehicles	Advantage	Disadvantage
Peptide conjugate or small molecule	Reduced rate of modification; relatively better tissue infiltration; specific targetability; better circulation half-life	Secondary resistance due to reduced expression of target molecule
Antibody-cytokine fusion	Recombinant product; scalability; target specificity; Receptor specificity can be modulated	Competitive affinity between cytokines and ligands
Nano/microparticles	Controllable release kinetics; target specificity; extended half-life	Regulatory considerations; costly production processes
Polymer conjugates	Receptor selectivity can be modified; extended half-life	Costly production processes; polymer specific immune responses
Hydrogels/implants	Scaffold for cell infiltration; controllable release kinetics	Costly encapsulation procedures; regulatory considerations

STAT5 and TGFβ signaling, which further promote tumor inhibiting crosstalk. Moreover, these muteins show competitive binding affinity comparing wildtype IL2/IL2R coupling, also IL2/mAb complex to an extent (Siegel and Puri 1991). Furthermore, two protein engineering method possess significant clinical potential by recombining IL2 superkine with EGFR for tumor cell targeting, and with Fc protein to prolong circulation and to enhance shelf life (Sun et al. 2019). This recombinant protein shows durable tumor suppressive effects both in monotherapy and in combination with chemotherapy. Muteins are also efficient in activating synthetic receptors on CAR-T cells (Sockolosky et al. 2018). IL2/IL2R complex crystal structures were used to create a double mutant IL2R (Y135F, H134D) which lack the ability to bind with wildtype cytokines, and then performed a yeast display-based evolution to enhance the affinity of IL2 to complex with double mutant receptor. Adoptively transferring CD4+/CD8+ T cells expressing adjuvant orthoIL2 or orthoIL2R therapy in immunocompetent mice showed enhanced expression of modified T cell population without any significant hazard effects. Similar effects were reported between wild type and engineered IL2 systems after transferring them into a syngeneic B16F10 mouse melanoma model, highlighting the possibilities of designer cytokine-based immunotherapies to work in conjunction with currently available cancer therapeutic options like CAR-T therapy (Sockolosky et al. 2018; June et al. 2018).

3.6 Strategies for Cytokine Engineering

PEGylation (protein modification using PEG) was first employed in 1970 contribute to efficient mode of cytokine chemical modification strategy (Alconcel et al. 2011; Ekladious et al. 2018). It enhances drug circulation, conformational flexibility, shelf life, drug activity, solubility and reduce nonspecific binding, enzymatic degradation, and opsonization (Naing et al. 2019). PEG modified on lysine residues of IL2 and IL2R on Treg cells improve sustained cytokine delivery in TME and peripheral blood (Charych et al. 2016). Bempegaldesleukin, a PEG modified cytokine, has improved antitumor immunity in syngeneic mouse models of colon and breast carcinoma when performed in conjunction with CAR-T cell therapy (Charych et al. 2016; Parisi et al. 2020) the drug gave promising result in clinical trial (phase I) with 35% tumor regression and 53.8% disease stabilization. Urotheial cancer, advanced melanoma, and muscle invasive bladder cancer is chosen as the disease arm (NCT04209114, NCT03635983, NCT03729245) (Bentebibel et al. 2019). Pegilodecakin, PEG modified IL10, promote oligoclonal T cell activation especially CD8+ Tc cells (Naing et al. 2019). Their phase I monotherapy trial showed 27% OS in renal cell carcinoma, but in combination with PDL1 antibodies showed 40% OS in renal ell carcinoma and 43% in NSCLC. Nonetheless, pegilodecakin has discontinued from phase II and III trials due to their challenging overall survival rates while comparing with FOLFOX therapy (Tsai et al. 2016) or antiPD1 therapy (Sun et al. 2020).

Photolabile polymers can enhance the target specificity of recombinant cytokines by masking them in conjugated form and restoring upon light exposure (Perdue et al. 2020). It can potentially enhance the activation strength, temporal control, and time scale of cytokine signals. PEG modified IL15, IL12, and IL2 linked with *o*-nitro benzyl linkers precisely retain protein activity upon the exposure of blue LED light. Modifications using photolabile molecules specifically enhanced IL12 half-life in C57BL/6 mice 16-fold by biasing IL2/IL2R binding, while monochromatic light exposure restored IL2 mediated T cell growth and JAK/STAT cascade.

Cytokine hybridization with ECM components is one of the potential strategies to target cytokine response in TME (Xu et al. 2019). Collagen can act as potential tumor agonistic-targeting candidate. IL2 and IL12 fused to collagen type I/IV-binding protein (lumican) significantly improved tolerability and clinical responses in melanoma mouse models, as compared to monotherapy. It further shows therapeutic potential while combining with checkpoint blockade immunotherapy and CAR-T cell therapy. A3 domain of collagen binding domain was fused with IL2 and immune checkpoint blocking antibodies like PDL1 and CTLA4. They reported total remission in 9 out of 13 animals getting combination treatment after systemic therapy in xenograft mouse models of melanoma. With regard to 1 out of 13 mice who received an unaltered combination treatment of collagen binding domain-checkpoint inhibitor and collagen binding domain-IL2, who achieved full remission, monotherapy with CBD-IL-2 did not show significant response (Ishihara et al. 2019).

Cytokines can also be combined with their corresponding receptor fragments to sterically hinder their binding affinity with specific immune cells. ALT-803 is

an agonist complex with N72D mutation in IL15. It possesses significant binding potential with IL15Rβ while fused with fragments of IgG1 Fc and IL15Rα. The IL15/IL15R binding affinity caused a 150-fold increase both in NK and T cells, promising enhanced survival in MOPC-315P and 5T33P multiple myeloma cancer models in an IFNγ independent mechanism. Moreover, CD44++ Tc cell proliferation was coupled with NKG2D upregulation without PD1 expression. This validates an innate-like nonspecific tumor suppression (Xu et al. 2013). ALT803 and IL15 in CT26 and B16F10 cancer models showed a competing advantage of anti-tumor activity achieved by 20 fold higher in vivo half-life. ALT803 phase I trial (NCT01885897) in lymphoma and leukemia cases showing relapse after hematopoietic cell transfer no hazard effects and 96h constant serum concentration. Furthermore, 19% of patients have shown measurable Tc cell and NK cell proliferation without Treg proliferation (Liu et al. 2016; Margolin et al. 2018). Combination of rituximab (anti-CD20) with ALT803 in preclinical studies has increased NK mediated granzyme secretion and IFNγ production in primary B-cell lymphoma and follicular lymphoma cells (Rosario et al. 2014). Nivolumab along with ALT803 is ongoing in a phase II trial focusing grade 3 tumors (NCT02523469) (Wrangle et al. 2018).

Antibody-cytokine complexes can further therapeutic potential of cytokine monotherapy. IgG recombined with IL21, IL10, IL12, IL4, IL2, and TNFα showed improved circulation and tissue specificity in clinical trials and murine models (Hutmacher et al. 2019). The whole antibodies or paratope fragments such as scFv, nanobody, Fab, and diabody domains can be linked with cytokines using the principles of affinity binding or using flexible peptide linkers. Antibody complexes can be used to bias cytokine site specific binding, for instance, IL2-S4B6 murine anti-IL2mAb complexes bind with IL2Rβ expressing Tc cells and NK cells but can bind with Treg cells while substituting this antibody with JES61 (Létourneau et al. 2010). The same effects were reported in vivo. Hu14.18-IL2 complex are made using antibody clone 14.18 binding Fc fragment of GD2, which is a disialoganglioside reported in NET like melanoma and neuroblastoma (Neal et al. 2004). Phase I/II clinical trials of Hu14.18-IL2 complex showed clinical outcomes when performed along with additional therapies. Hu14.18-IL2 along with GM-CSF showed 16.1% objective response, but 76% partial response with combination therapy (Shusterman et al. 2019). Clinical studies are now being conducted with Hu.14.18-IL2 for the treatment of Stage IV unresectable melanoma in conjunction with ipilimumab, nivolumab, and radiation therapy (NCT03958383), additionally for the ex vivo growth of functionalised NK cells in neuroblastoma (NCT03209869) (Albertini et al. 2012). Moreover, immunocytokines like L19, F8, F16 can also target neo vasculature or ECM constituents such as extra domain A/B of fibronectin (Villa et al. 2008). F8-IL2 complex specifically deliver IL2 to neo vasculatures, and showed improved clinical efficacy while treated with cytarabine in acute myeloid leukemia (Gutbrodt et al. 2013).

3.7 Methods of Cytokine Delivery

Genetic, particle and chemical-based approaches

IL10 is released by immune system cells, both adaptive and innate, to control the action of proinflammatory cytokines. Evidence to justify the use of autocrine IL10 signaling in cancer immunotherapy, particularly when combined with immune checkpoint suppression are reported. This signaling may extend CD8+ T cell activity (Trinchieri 2007). Utilizing IL-10, nanoparticle systems show tumor suppressive responses via the activation of Th17 cells in colon and lung cancer (Li et al. 2018). Polylactic acid (PLA) microspheres produced using phase inversion nanoencapsulation were used to administer IL-10 orally, and the colon cancer model showed improved survival after dosing. This remarkably effective treatment outcomes were linked to a decrease in pro-tumorigenic Tregs (Foxp3+ CD4+ RORγt-) and Th17 (RORγt+ CD4+ IL-17+) cells, as well as an increase in Tc cells (Gu et al. 2017). Intratracheal delivery of PLA IL10 in the LSL-K-rasG12D genetic model of NSCLC likewise resulted in the restoration of Th17 axis dysfunction and decreased tumor development, which further the potential of particle-based cytokine delivery (Li et al. 2018).

TRAIL can modulate innate immune systems. Lipopolysaccharide (LPS) and IFNβ stimulation induce TRAIL upregulation in monocytes and macrophages, as well IFNγ induce similar effects in dendritic cells and NK cells (Falschlehner et al. 2009). TRAIL/death receptor (DR)-4 and -5 coupling induce apoptotic effects in various cancers, and TRAIL-based immunotherapy demonstrated therapeutic benefits in at least six types of cancers. Their poor circulation and limited target specificity causes modest clinical responses (Soria et al. 2011). To manage, gold, lipid, DNA, and polymer-based structure formulations in nanoparticles are under consideration. TRAIL liposomes can effectively target the tissue sites and can benefit from membrane based presentations (de Miguel et al. 2015). For instance, TRAIL-functionalized liposomes in colon cancer xenograft models enhance ligand-mediated apoptosis through DR5 activation (de Miguel et al. 2015). More sophisticated method such as TRAIL and R8H3 based liposomes modified with hyaluronic acid gel (gel-lipid nanostructure) consisting of doxorubicin can deliver both TRAIL and doxorubicin with improved specificity achieving tumor suppressing potential in breast cancer xenograft model (Jiang et al. 2014). As well, PLA2 degradable POPC liposome shell surrounded by a DNA nanocore can efficiently transfer TRAIL to cancer sites (Sun et al. 2014). This complex achieved tumor cell apoptosis and leukocyte adherence, respectively, by using TRAIL and E-selectin dual conjugated nanoparticle. Xenograft mouse model corresponding to prostate cancer has shown enhanced apoptosis of circulating tumor cells (CTC) and inhibition of metastasis by integrating TRAIL based nanoparticles with leukocytes in vivo (Ming et al. 2017).

IFNγ can enhance effector activity of Tc cell, M1 macrophages, and dendritic cells. IFNγ-engineered delivery systems have the potency to improve the tumor site's localization prior to clearance to reduce the effects on systemic dosing. IFNγ encapsulated within liposomes can be a method in melanoma vaccination and has

experimented in melanoma induced mice models (van Slooten et al. 2000). External magnetic field directed IFNγ-encapsulated nanoparticle enhance tumor infiltration of macrophages and Tc cells in comparison to nondirected nanoparticles in Pan02 pancreatic cancer model (Mejías et al. 2011). IFNγ based nanoparticle consisting of poly lactic-co-glycolic acid (PLGA) and polyvinyl alcohol (PVA) layer for structural support and cytokine secretion, respectively, can adhere to macrophages in vitro while hindering phagocytosis (Wyatt Shields et al. 2020). Comparing naked IFNγ treatment, this complex enhanced M1 polarization and improved OS in 4T1 mammary tumor models.

TNFα assisted isolated limb perfusion (ILP) in soft tissue sarcoma can be competitively benefited with gold nanoparticles due to their potency to deliver rhTNFα in vivo. The particles can be stabilized using Au-S bond and surface conjugation of PEG-SH. Moreover, rhTNFα has three-fold tolerative dose comparing cytimmune (CYT-6091) with no dose-limiting hazard effects (Libutti et al. 2010). Zr/IFNγ and TNFα complexed colloidal gold (CYT-IFNg and CYT-Z-TNF respectively) are also considered (Jiang et al. 2014).

The poor circulation and notable hazard effects of engineered IL12 cause fall in phase III clinical trials (Berraondo et al. 2019). In hypoxic tumor microenvironments, Poly (β-amino ester) consisting of 2-(4-imidazolyl) ethylamine, 1,6-hexanediol diacrylate, and amino-terminal PEG disintegrate, therefore preferentially release IL-12 cargo. In a B16-F10 xenograft mouse model, this system has reported to be durable at physiological pH, significantly enhance intratumoral IL-12, suppress carcinogenesis, and alter TAM phenotype without exhibiting any harmful effects (Overwijk and Restifo 2001).

Hydrogel and implants-based approach

Cytokines can be encapsulated within macroscale matrices or implants to overcome systemic immunotherapy challenges (Chao et al. 2020). Degradable (lactate-) hydroxyethyl methacrylate dextran (dex-lactate-HEMA) hydrogels and nondegradable dextran methacrylated (dex-MA) hydrogels enable for the regulated and adjustable delivery of IL2 (Cadée et al. 2002). Similar clinical efficacy to exogenous IL2 administration and refusal of tumor cell reactivation was obtained after implanting IL2-based hydrogels in mice models of metastatic lymphosarcoma (Bos et al. 2004). Poly(ethyl-l-glutamate)-poly (ethylene glycol)-poly(ethyl-l-glutamate) hydrogels (PELG-PEG-PELG) were loaded with IL-2, IFN, and doxorubicin in B16-F10 melanoma mice model and has shown two-fold tumor inhibition capacity. Chemotherapy-immunotherapy combination approach has also shown similar results using cisplatin and IL15 encapsulated within mPEG-b-PELG assisted hydrogels (Wu et al. 2017). Moreover, IFNα loaded hydrogels hinder tumor proliferation in hepatic xenograft mice models. Hyaluronic acid-based hydrogel loaded with PEGylated TRAIL significantly reduce tumor burden in pancreatic xenograft mice models (Wang et al. 2022).

3.8 Cancer Vaccines

One of the biggest therapeutic breakthroughs of 20th century was the development of vaccinations to combat contagious illnesses, however the vaccination strategies go further than disease prevention (Lin et al. 2022). mAb trials was challenging two decades back before the success of nivolumab, rituximab, CAR-T cell and antiPD1 antibody (Berger 2008). The vaccines can generally mimic the structure of antigens such as tumor cells, neoantigens, long and short peptides, and RNA/DNA, while immune cells such as dendritic cells, carrier proteins, CD40L, and TLR agonists act as adjuvants (Melief et al. 2015). Antigens in vaccines may be either predetermined (known) or anonymous (unknown). The former contains predetermined common antigens (expressed in most patients) or predetermined individualized antigens (specific for individual patient). Ex vivo (in a lab) or in situ (in the tumor site) colocalization of anonymous antigen vaccines with APCs are under consideration (Lin et al. 2022).

3.9 Shared Vaccines

Shared antigens are displayed in a high enough percentage of cancer patients so that vaccine developers may focus on these patient populations. Shared antigens can target both tumor specific antigens (TSA) and tumor associated antigens (TAA), and can be tested using standard tests like IHC, flow cytometry and cytology. They are the major focus of preclinical and clinical studies since 1990 (Lin et al. 2022). Neo-epitope TSA EGFRvIII is exhibited in around 25% of EGFR-overexpressing glioblastomas (Katanasaka et al. 2013), while viral TSA HPV E7 and E6, and TAA Wilms' tumor protein are upregulated in most breast malignancies, Wilm's tumor, and acute myeloid leukemia (AML) (Maslak et al. 2018). These vaccines are beneficial in terms of time and resource comparing personalized vaccines. TSAs of EBV virus encode various neoantigens such as latent membrane protein (LMP)-1 and -2 in NKT cell lymphoma and nasopharyngeal carcinoma (Tsao et al. 2002).

Modified vaccinia Ankara (MVA) virus with positive for EBNA-LMP2 fusion protein enhanced T cell response (Taylor et al. 2014). P53 and RB proteins can be sequestrated by HPV E7 and E6 TSAs promoting squamous carcinoma progression. ISA101, a synthetic long peptide vaccine induces tumor regression through activating T cell response in intraepithelial neoplasia (Kenter et al. 2009). Moreover, LCMVi vectors with E7+ induce E7 specific T cell activation. Overexpressed EGFRvIII are mutant self-proteins widely reported in NSCLC and glioblastoma. Glioblastoma patients previously undertaken CAR-T cell therapy have shown promising anti-EGFRvIII outcomes (O'Rourke et al. 2017). Phase II clinical trial using rindopepimut, an EGFRvIII peptide vaccine, together with temozolomide and GM-CSF activate humoral responses (Schuster et al. 2015) albeit the inability of the trial to show significant clinical outcome (Weller et al. 2017).

TAAs are associated with either tumor site, tumor development, or tumor abundant proteins while comparing with TSAs. Wilm's tumor protein is a development-based TF that assist tumor progression (Cheever et al. 2009). WT1 peptide vaccines with higher HLA affinity enhance clinical responses in acute myeloid leukemia cases and prompted to phase III trials now (NCT04229979) (Maslak et al. 2018). Melanoma-associated antigen 3 (MAGE-A3) is a widely used biomarker in NSCLC, melanoma, and myeloma wherein it induces anti-apoptotic effect. TLR4-agonist MAGE-A3 vaccine, AS02B, as well show humoral tumor suppressive effects without significant clinical benefits (Vansteenkiste et al. 2013). However, AS15, another TLR4 agonist shows competing clinical benefits over AS02B, and is in phase II clinical trials now (Kruit et al. 2013).

HER2 is upregulated in 30% breast carcinoma but a relatively smaller percentage in ovarian and gastrointestinal tumors, and can get bound by anti-HER2 mAb. Nelipepimut-S, HLA-I-restricted peptide vaccine, can induce Tc cell response but with reduced clinical benefits (Mittendorf et al. 2016), and is similar to AE37 (HLA-II-restricted) (Mittendorf et al. 2019). Interestingly, a multi-epitope-based HLA-I or II restricted targeting showed Tc cell response durable for a year (Knutson et al. 2001).

3.10 Personalized Vaccines

Personalized vaccines are designed specific to cancer patients and are designed using high-throughput nucleic acid sequencing methods. Germline nucleic acid extraction, sequencing, and HLA typing are employed upstream to personalized vaccine designing. For instance, neo-epitopes of TSA *KRAS* G12D, which has reduced frequency in terms of oncogene, is employed. This method puts the responsibility on the vaccinator to repeatedly identify the significant epitopes, however it also enables the immune system to approach malignancies without recognized common antigens. The avidity between HLA and TCR ensures clinical efficiency of vaccine as a whole (Lin et al. 2022). When combined to checkpoint blockage, targeting customized antigens releases patients' broad T cell responsiveness is increased by T cells that evade thymic −ve determination(Sahin). Putative neo-epitopes are selected from somatic mutations using techniques like NetMHC algorithm based on their affinity for the patient's HLA alleles (Nielsen et al. 2003). After prioritizing neo-epitopes based on analysis of tumoral transcriptome data, It is customary to choose up to 20 neo-epitopes, and then produce RNA, neo-epitope viral vectors, or peptides that adhere to good manufacturing practice (GMP). Neo-epitopes may promote APC activation or keep APC uptake stable to help them be more immunogenic.

Patients diagnosed with advanced melanoma responded anecdotally to an earlier customized vaccination employing synthetic RNA vaccine to generate 10 neo-epitope potential targets, primarily CD8+ and CD4+ neo-epitope-specific T cell activity (Nielsen et al. 2003). These poly-specific processes, which might be made more potent by inhibiting PD-1 or rendered useless by reducing tumor cell HLA class

I, most certainly had a key role in the dramatic decline in long-term metastatic episodes. Accordingly, another study using 13–20 long fragments of projected NEO-PV-01 (neo-epitope) generated higher CD4+ over CD8+ T cells particular to altered sequence (Ott 2017).

Another tumor-specific alteration is the distinctive Ig or TCR idiotype, which results via somatic hypermutation and locus gene recombination. These mutations are often retained in tumor tissues and is reported in lymphomas, myelomas, and hematologic malignancies. In the Genitope and Favrille phase III studies, lymphoma patients who had received rituximab or chemotherapy but had an idiotype associated to KLH delivered with GM-CSF were given the vaccine. Neither research demonstrated a therapeutic advantage over a placebo. When given to patients in standard therapy following chemotherapy, a different NCI-Bioves trial (phase III), using the same vaccine approach showed a substantial DFS; however, the relevance of the consequence was obscured by the high rate of patient dropout prior to vaccination. Flt3L has shown advantage over GM-CSF based APC activation, substantially with more efficient adaptive immune response (2016).

3.11 Ex Vivo Vaccines

APC colocalized or ex vivo include tumor cell isolation and colocalization with APC in order to enhance their antigenic effects. Ex vivo vaccines in patient body can express antigens to T cells. HSP such as HSP70, gp96, and HSP110-based ex vivo vaccines has shown to cause tumor suppressive responses (Moseley 2000). HSP-gp96 complex (HSPPC-96) could not report significant survival benefits in renal cell carcinoma and melanoma (Testori et al. 2008; Wood et al. 2008). Glioblastoma patients receiving this vaccination showed inverse correlation between PDL1 expression and survival, suggesting combinatorial approaches using antiPDL1 and HSSPC-96(NCT03018288) (Bloch et al. 2017). TAAs can be enriched in ex vivo vaccines focusing KRAS, P53, and EGFR. Canvaxin, prostate GVAX, melacine, and lucaxin are few *ex-vivo* vaccines but with limited survival values (Kozłowska et al. 2013). Tumor cells expressed with GM-CSF (GVAX) in acute myeloid leukemia patients did not show clinical benefits (Ho et al. 2022), however their combination with anti-furin shRNA to inhibit TGFβ production shows clinical benefits in Ewing's sarcoma (Ghisoli 2016).

3.12 In Situ Vaccines

In situ vaccines present APC to stimulate tumor loading antigen, followed by T cell activation. They are benefited for their ability to present wide ranges of TSA while comparing with ex vivo. DCs which are usually inactivated in TME can be administered ex vivo to stabilize intratumoral cytokine levels including IL12, TNF

and IL8 (Subbiah et al. 2018). Despite minimal radiotherapy, embryonic dendritic cells with improved phagocytic capability can be infused with GMCSF and rituximab (Kolstad et al. 2015). The importance of vigilant immune surveillance is highlighted by periodic T cell regressions and activation at nearby and distant malignancies. Similar research without radiation produced lymphoma-specific Tc and Th activation as well as tumor regressions in untreated malignancies (Cox et al. 2019). CC chemokine ligand (CCL)-1 expressing DCs injected in NSCLC patients induced PDL1 expression and Tc cell infiltration, indicating their anti-tumoral effects (Lee et al. 2017).

Flt3L, cross-presenting fraction cDC1, is the main hematopoietic progenitor development and differentiation component that mobilizes dendritic cells. Therefore, administering Flt3L rather than their direct administration could be a more feasible way to replace tumor tissue dendritic cells. Nine out of 29 vaccinated NSCLC patients who received targeted irradiation with Flt3L infusion had corresponding responses (Cox et al. 2019). B cell lymphoma patients who received intratumoral Flt3L, low-dose radiation and poly-ICLC, as part of a phase I-II study had early signs of memory Tc cell infiltration to untreated tumor locations linked to overall tumor shrinkage, a few of them lasted from months to years (Hammerich et al. 2019), however their follow up trial targeting PD1 in situ vaccination in breast, head and neck cancer is ongoing (NCT03789097).

Dendritic cells like plasmacytoid (pDC) express TLR9 receptors but not in cDC1. TLR9 agonists include hypomethylated CpG islands especially CpG-A, -B, or -C that can cause IFN production that is pro-inflammatory. Phase III trial using CpG-B tilsotolimod and ipilimumab showed ORR of 9% (Haymaker), which is similar to ipilimumab monotherapy. CMP-001 (CpG-A) vaccine in melanoma patients 28% ORR while administered in combination with pembrolizumab albeit CMP-001 monotherapy causes systemic regression (Milhem et al. 2020). TLR3 expressed on cDC1 can activate nuclear factor (NF)-kB pathway. Hiltonol, a TLR3 agonist, can control tumor growth and prolonged survival in head and neck cancer, and liver cancer (Torre 2017; Kyi et al. 2018).

Oncolytic bacteria and viruses that are intratumorally injected can be a subtype of in situ vaccination. The possibility for systemic vaccination following intratumoral delivery of oncolytic viruses might lead to a variety of therapeutic strategies. The only oncogenic virus recognised by the FDA is talimogene laherparepvec (TVEC), a transgenic GM-CSF-releasing herpes simplex virus (HSV)-1 that has increased tumour shrinkage and survival in non-injected areas (Andtbacka 2015; Kaufman et al. 2016).

3.13 Vaccine Delivery Vehicles

Biotechnology has the potency to answer limitations of cancer vaccines especially regarding toxicity, shelf life, tumor penetration, opsonization, and off-target effects by designing vaccine delivery vehicles (Rosenblum et al. 2018; Kudling et al. 2022).

Nanoparticle based delivery systems can carry antagonists or agonists to modulate tumor immune system. It includes liposomes, extracellular vesicles, dendrimers, inorganic nanocarriers, and more (Li et al. 2022; Reda et al. 2022). It can be exploited well in combination therapy, for instance administration of cytokines along with chemotherapeutic drugs. Magnetic nanoparticles (Feridex, EndoremVR, GastromarkVR), virus derived, taxane based, and polymer nanoparticles can incorporate pharma compounds to the target sites (Carrasco-Esteban et al. 2021). Extracellular vesicles are nanosized membrane enclosed structures naturally derived from cells and consist of a wide spectrum of their parent cells (Wiklander et al. 2019). They are involved in normal physiology as well in pathology through transferring cargo (Yang et al. 2021). Engineered EVs derived from fibroblast like mesenchymal cells, for instance, target KRAS pathway with the help of their cargo such as siRNA and shRNA (Ma et al. 2021). Additionally, because they lack the carriers recommended for the treatment of cancer, pharmaceuticals themselves may function as nanoscale therapies. Antibody-drug conjugates (ADCs), drug nanocrystals, drug-drug conjugate nanoparticles, and prodrug self-assembled nanoparticles are examples of nanoparticles devoid of carrier molecules. For instance, the drug SN38 (7-ethyl-10-hydroxycamptothecin) has indeed been coupled with the PEG-CH=N-Doxorubicin precursor (pH-responsive) to increase drug release to tumors and destroy both CSC and non-CSCs (Carrasco-Esteban et al. 2021).

4 Biotechnology Approaches Over Traditional Therapy

Surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy are the five conventional cancer care modalities (Siamof et al. 2020). With unique shortcomings in these therapeutic approaches are now being addressed by designer, specially formulated drugs designed to increase survival rates and reduce adverse effects, clinical cancer treatment has undergone a fundamental revolution. Following surgery, the whole lung tumor and any nearby lymph nodes must be eliminated (Hoy et al. 2019). Radiation treatment cannot be performed in aggressive situations. It destroys malignant cells that are allowed for radiation. It also damages the normal tissue that are located in its exposure (Bogart et al. 2022). Chemotherapy stops malignant cells from growing and dividing, multiplying, and generating additional cells (Nagasaka and Gadgeel 2018). Immunotherapy aim to improve the body's natural defenses fight cancer. For the purpose of re-establishing immune response functionality, it uses substances that may be created *in vivo* or *in vitro*. Patients with NSCLC may get immunotherapy alone, or in conjunction with other chemotherapy medicines. When targeted treatment is not an option, immunotherapy or their combinational methods may be used (American Society of Clinical Oncology (ASCO) 2020).

ICIs, mAb, vaccines, and adoptive cancer therapy (ACT) are the recent biotechnology advancements in cancer immunotherapy. The biotechnology applications though ranges from nucleic acid-protein sequencing, drug discovery, vaccine designing, cancer model preparations, it is widely benefited in chemotherapy,

immunotherapy, and combination therapy. ICIs are generally designed to target PD1/PDL1, and CTLA4, and the former ones are FDA approved (Jain 2018; Sharon 2014) (Fig. 3). ICIs mainly function by inhibiting the immune suppressive signal crosstalk that is triggered by the interaction between PD1 and PDL1, regaining T lymphocytes' basic ability to eradicate tumor cells (Sławiński et al. 2020).

IgG1 and IgG4 antibodies, such as avelumab, atezolizumab, cemiplimab, durvalumab, pembrolizumab, and nivolumab, may hijack PD1/PDL1 axis in NSCLC in combinatorial first-line treatment. They can elicit tumor inhibiting effects via adaptive and innate immune system pathways (Chen 2017; Mezquita and Planchard 2018; Yuan et al. 2019; Akinleye and Rasool 2019). A range of disorders, particularly cancers with different origin, are now being treated using ICIs, especially blocking via mAbs. Integrated treatment, which employs mAbs as well as other drugs at a reasonable dosage, may increase patients' chances of surviving NSCLC (Rosenblatt and Avigan 2017).

The effectiveness of ipilimumab (CTLA-4 inhibitor) in ES-SCLC in conjunction with the first treatment was investigated in the CA184-041, ICE, and CA184-156 phase 2 and 3 investigations. 42 participants were included in the ICE study and administered ipilimumab along with etoposide and carboplatin. PFS was indeed the study's primary goal, however it wasn't effectively attained (PFS 6.9 months) (Antonia et al. 2016). CTLA4 based therapy require combinatorial approaches for better clinical benefits.

5 Combination Therapy Involving Biotechnology

Patients with NSCLC or aggressive melanoma respond well to ICI, but B cell acute lymphoblastic leukemia cases have reported with outstanding results in CAR T-cell therapy. Understanding why particular medicines have been extremely effective in treating certain cancers but less effective in treating others might aid in the more rational design of clinical trials to test therapy for additional malignancies (Khalil et al. 2016). Immune hijacking adopted by TME can suppress endogenous TILs and CAR-T cells from producing a potent antitumor response (John 2013; Moon et al. 2014). Immune modifying mAbs, including those that provide checkpoint blockage, would probably be required for the formation of immune based antitumor treatments for tumors with significant neoantigen expressing capability in a immunosensitive milieu. Evolutionarily conserved T cells may be oblivious to cancers with reduced neoantigen-presenting ability, like those with fewer possibly immunostimulatory genetic abnormalities such as B cell acute lymphoblastic leukemia (Alexandrov 2013) or those other ways, do not display neoantigens due to reduced antigen processing, demonstration, or HLA expression. It is possible that in an immunoinhibitory milieu, these cancer kinds did not face the urge to co-evolve. mAbs individually would be fewer likely to induce a potent antitumor activity in this circumstance since TIL and antigen presentation load are likely to be modest. In contrast, CAR-T

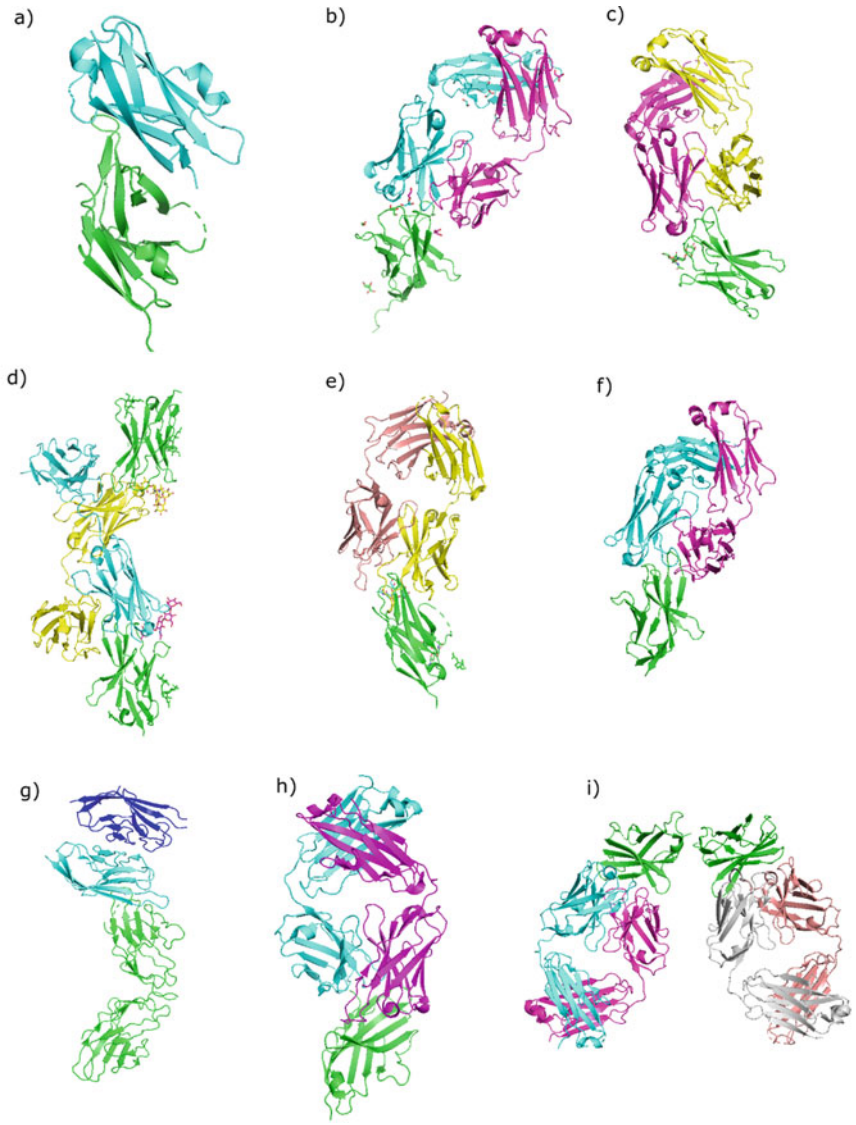

Fig. 3 RCSB-PDB structures of PD1 and CTLA4 complexed with various ligand or drug molecules. Green color represents PD1 and CTLA4, while further colors denote heavy and light chains of bound molecules. **a** 6UMT: PD1/PDL2 (Tang and Kim 2019) **b** 7VUX: PD1/609A Fab (Zhao et al. 2022) **c** 5WT9: PD1/nivolumab **d** 7CU5: PD1/camrelizumab (Liu et al. 2020) **e** 6JBT: PD1/toripalimab (Liu et al. 2019) **f** 7E9B: PD1/ HLX10 (anti-PD1 antibody) (Issafras et al. 2021) **g** 1I85: CTLA4/B7 (Schwartz et al. 2001) **h** 5GGV: CTLA4/tremelimumab (Lee et al. 2016) **i** 5TRU: CTLA4/ipilimumab (Ramagopal et al. 2017). *Data source* https://www.rcsb.org/

cells does not get inhibited by such inhibitors, and as shown for CD19 targeted CAR-T cell treatment for B-ALL, may cause fast full cures in approximately to 90% of participants with this cancer type, which has a modest mutational rate (Alexandrov 2013). Combination treatment will exert the efficient anticancer impact when PD1 axis and CTLA4 are blocked. Combined anti-PD1 t and anti-CTLA-4 treatment has reported a great deal of potential (Postow 2015; Chapman et al. 2015; Larkin 2015). For instance, individuals with metastatic melanoma (phase III) showed a remarkable 58% RR when treated using this strategy. Again, for treatment of malignancy, medicines hitherto believed to not work via immune regulation as well as combinations with other types of immune regulation are being extensively researched (Larkin 2015).

ISA101 (LSP vaccine) and anti-PD-1 medication were combined in research that showed improved therapeutic benefits compared to either treatment used alone, including in PD-L1 positive malignancies (Quezada et al. 2006). Higher humoral effects as well as an OS advantage as a supplementary, inadequate objective were seen in randomized phase II study of EGFRvIII vaccine along with bevacizumab. The findings imply that tumor inhibitory humoral mechanisms could not be enough and that selecting the best possible combination therapy may determine effectiveness of vaccines (Postow 2015). Neoantigen-based therapeutic benefits and T cell responses may be stronger comparing those anticipated with anti-PD1 monotherapy, according to a wider trial that included anti-PD-1 and neo-epitope vaccination in 60 melanoma cases, bladder cancer and NSCLC (Lozano et al. 2012). In a pilot investigation, anti-neo-epitope and tumor inhibitory effects by T cells connected to long-term survival were induced in 25 ovarian cancer cases when autologous dendritic cells with oxidized autologous tumor cell homogenate were injected alone or in combination with chemotherapy and anti-VEGF mAb (Wang et al. 2019a, b).

6 Conclusion and Future Perspectives

Although years of research in cancer have yielded many therapeutic strategies, disease resistance, recurrence, and post-medication hazard effects are still a major concern. The *PDCD1, CD274,* and *CTLA4* gene which are widely exploited in mAb designing and ICIs, were curated from TCGA-cBioportal and have shown significant co-occurrence and expression overlaps. This indicates the need for a combined approach in designing ICIs and mAbs. Moreover, the exact regulation or controlling engineered cytokines or their delivery vehicles' activity is a significant barrier to the efficiency of cancer immunotherapy. As an alternative approach, ongoing research examines the role of photolabile cytokines on adoptively transplanted T cell durability and specificity, and the application of photocages with enhanced local penetrance. The addition of cytokines with IgG motifs can significantly change target specificity as well as the eventual safety and effectiveness the otherwise poorly tolerated or

ineffective cytokine treatment. Continued studies on TME, novel antibody architectures, and cytokines in innate immunity may contribute to the designing of antibody-cytokine combinations and hybrids and, consequently, the therapeutic outcomes for patients receiving this potential category of immunotherapy. T cell responses corresponding to neo-antigens and clinical responses that may have exceeded those anticipated with anti-PD1 monoclonal therapy were found in larger research involving multiple solid tumors (Ott et al. 2020). Understanding the initial cancer mutational burden of each patient before the application of conventional therapeutic approaches and combining these advancements may be a potential strategy for cancer cure.

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Biosimilars: Promising and Rapidly Emerging Biotherapeutics

Sonali Manwatkar and Bimlesh Kumar

Abstract The fastest-growing category of biopharmaceuticals is known as a "biosimilar," which refers to a biological medication that is replicated and sold at a lower price than the original biological product. Treatment with such biologics has additional benefits over conventional medication due to the involvement of a specific target, high efficacy, and fewer adverse effects. In addition to preventive use of biologics being used to avoid the return of the illness condition, diseases like cancer, autoimmune diseases, and inflammatory ailments can be cured. However, their exorbitant price places a heavy load on health care. Biosimilars are created as a result of the biologics' patents expiring, with the intention of giving more patients access to cutting-edge treatment at a reasonable price. Biosimilars are not only identical to the reference standard used by the original creator, but also very identical in terms of efficacy and safety. The WHO sets internationally recognized norms and criteria that are widely accepted for the assessment of biotherapeutics as part of its obligation to confirm the global safety, efficacy and quality of products. The regulatory agencies have put a high priority on safety, and the development process follows a step-by-step methodology that is thoroughly explained in this chapter. Global regulations are contrasted, and suggestions are made for developing at the lowest possible expense. To accelerate the development process, the key components to establishing biosimilarity are outlined, including analytical and bioanalytical characterisation, nonclinical testing, clinical pharmacology testing, and clinical efficacy testing. There is also a summary of FDA-approved products. The goal of the current chapter is to deliver a brief compilation of biosimilars, their process of manufacturing, regulatory requirements, and to discuss both their current and prospective future roles in the field of medical sciences/biotherapeutics.

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1 Introduction

Biological therapeutic agents, also known as biologics, are a diverse group of substances that can be produced by cells or other living organisms through a number of different biological processes. Some examples of these processes include controlled transcription and translation process of protein synthesis, immunoglobulin technologies, and genetic engineering technologies (Humphreys 2022). Biologics have had a significant impact in the treatment of a variety of acute and chronic illnesses, including hormonal imbalance, a variety of inflammatory diseases, diabetes (Zhang et al. 2020) and autoimmune conditions, as well as cancer and haematological malignancies (Schiestl et al. 2020). In addition, the biologics sector of the pharmaceutical industry accounts for fifty percent of all products currently on the market for the administration and treatment of cancers. However, developing biological drugs requires a significant financial investment and a significant amount of time; as a result, the entire pharmaceutical industries is changing emphasis to the development of "Biosimilars." Biosimilars are a type of biopharmaceutical product comparable with its purity, efficacy, and safety, is very similar to other reference products that are already on the market. In spite of the fact that their amino acid sequences are comparable to those of their reference products, may still biosimilars possesses distinguishing characteristics (De Mora 2015). Some of these characteristics include their three-dimensional structures, protein aggregation isoform profiles and glycosylation sites. However, various parameters, such as their therapeutic indication, route of administration, mechanism of action, dosage form, and strength, required to be comparable with their reference product (Ruppach 2020). In the year 1980 marked the beginning of the treatment and management of cancer utilizing biological substances as the primary method. Interferon alfa-2b (INTRONATM, Schering Corporation, Kenilworth, USA) was the pioneer industry of biopharmaceutical received approval from the Food and Drug Administration (FDA) in June 1986. It is presently marketed under seven different brand names. After that, in October of 2005, EMA- European Medicines Agency initiate the guidelines for biosimilars was the very first regulatory authority. In 2015, the US-FDA (United States Food and Drug Administration) granted approval to Filgrastim under the brand name Zarxio®. In addition, FDA released the guidelines affiliated with biosimilars in the year 2015. These guidelines address the quality and scientific aspects of demonstrating biosimilarity to the original product. Both acquiring a license to sell on the market and providing direction regarding how the FDA will determine whether or not two products are biosimilar were primary goals of this project. In addition, the FDA issued guideline documents in the years 2016 and 2018 with the aim of addressing challenges such as evidences of clinical pharmacological to substantiate biosimilarity and labelling

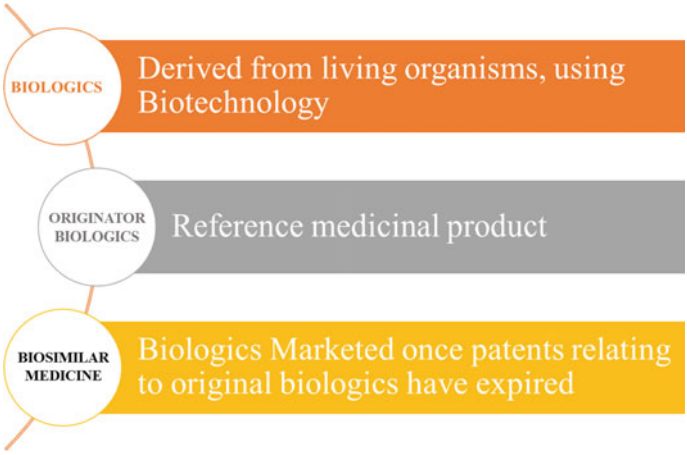

Fig. 1 Correlation between biologics, originator biologics and biosimilars

guidelines within the confines of Sect. 351(k) of the Public Health Service Act (42 U.S.C. 262(k)) (Lemery et al. 2017).

Till the date, due to the many bottlenecks which not allowing biosimilars fully accepted in the clinical practice. For instance, the most significant risk associated with biosimilars is known as immunogenicity. Immune reactions have a propensity to cause adverse effects, the majority of which have an influence on how effectively the product works (Joshi et al. 2022). As a result, it is necessary to conduct ongoing assessments of the product's efficacy and safety during both the clinical trials and the post-marketing stages. Lack of knowledge is another challenge that must be overcome in the case of biosimilars. Research has shown that implementation of biosimilars into clinical practice, there is a need to raise awareness of the concept of biosimilars among medical professionals (De Mora 2015) (Fig. 1).

This is necessary so that medical professionals should understand the biosimilar concepts based on trustworthy scientific data, generated from clinical trials. According to the findings of one research carried out by Cook and colleagues, around 26% of oncologists and approximate 21% of doctors only are aware with the concept of biosimilars. Although a number of regulatory authorities have established and published standardized guidelines along with approval procedures for biosimilars, the primary concern is still the transition from expensive biologics to less expensive biosimilars, particularly in terms of the safety of the treatment (Wiland et al. 2018).

2 Biosimilar Primer

An identical biological drug developed by the originator is developed as a low-cost competitor to the first biological product, and this is what is known as a biosimilar. The biosimilars category is the one within the biopharmaceuticals industry that is expanding at the fastest rate (Humphreys 2022). The regulatory agencies have placed an extreme emphasis on safety, and the process of development takes a stepwise strategy, stated in the chapter. The regulations that are in place all over the world are analysed, and recommendations are made. In the interest of accelerating the development process, the essential components necessary to establish biosimilarity, such as analytical and bioanalytical assessment, nonclinical testing, clinical pharmacology testing, and clinical efficacy testing, have been broken down and explained (Ishii-Watabe and Kuwabara 2019). In addition, a summary of FDA-licensed products along with additional details on the studies that were sent in and an update on the status of biosimilars provided here (Fig. 2).

Large and complicated pharmaceuticals known as biologic drugs have structures, physicochemical and biochemical properties, and manufacturing processes that directly affect their organic action. The development of biologics during the 1980s completely changed. How doctors handled their patients, particularly those who had diseases for which there was no effective treatment at the time (Iskit 2021). Ankylosing spondylitis, psoriasis, rheumatoid arthritis (Chadwick et al. 2018), and psoriatic arthritis are just a few of the chronic inflammatory illnesses that biologic medications have helped treat better, they also include some cancers (Joshi et al.

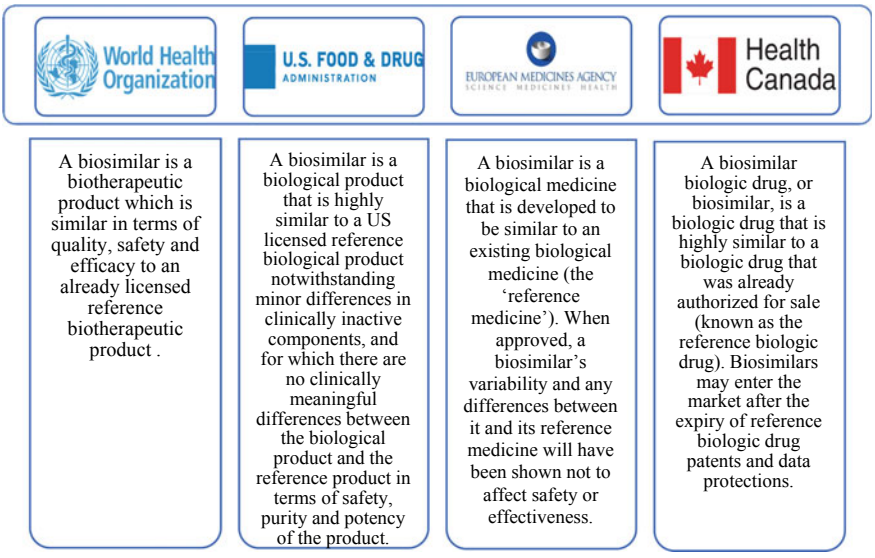

Fig. 2 Biosimilar official definitions

2022). Biologics have a high cost due to their complex manufacturing process, which places extra strain on the healthcare system. However, after their market-exclusivity patents have expired, Biosimilars have emerged as an alternative, cost-effective therapeutic option to reference product (the existing innovator biological therapies) and their active components. This is done to reduce healthcare spending and access to biological medicines promote greater. Notably, other words have also been used to refer to biosimilars, including follow-on biologics, similar biotherapeutic products, and biocomparables. The word "biosimilars" is now widely used in place of the latter. In comparison to generic versions of synthetic molecules, regulatory agencies around the globe demand a more and different involved procedure for the approval of biosimilars (Liu et al. 2022).

This is founded on a sophisticated set of tests for similarity called a biosimilarity exercise. Worldwide, a biosimilar must be comparably potent, pure, safe, and effective to the reference/ standard molecules based on a thorough process of comparability, with no clinically significant differences (Mysler et al. 2021). The regulatory pathway for proving biosimilarity is more stringent but shorter than that for an originator biologic in the Europe, United States, and globally based on WHO's standards. The regulation procedures are designed to determine if the new molecule is sufficiently comparable to the reference product in relation of purity, molecular structure, pharmacological characteristics, and clinical efficacy. It is common observation that slight variations can appear over time, even between batches of the same standard product (Lyu et al. 2022). Due to the potential for even minute differences to affect pharmacokinetics (PK), pharmacodynamics (PD), efficacy, and safety, a lot-to-lot assessment of biosimilars must be performed in comparison to the reference product as part of the similarity exercise. In most cases, a product can be considered a biosimilar only if all the criteria in the resemblance exercise are satisfied. Intended copies may exist when a molecule claims to be highly similar to an existing innovator molecule but fails to provide proof that it does so in accordance with the biosimilars regulatory pathway in its entirety. In addition to "biomimic" and "nonregulated biologic," other words have been used to describe these items (Niazi 2022).

The diagnosis, prevention, treatment, and management of many serious and chronic illnesses have been revolutionized by the development of biological products. They vary from more conventional, small-molecule medications like acetaminophen or acetylsalicylic acid [aspirin] in that biologic agents are substances that are naturally present in your body, such as sugars, proteins, nucleic acids, or particular cells or tissues. This is what sets them apart from more traditional, small-molecule drugs. In the process of treating illnesses such as cancer, various concentrations or formulations of these naturally occurring substances can be used, which ultimately results in the creation of biologic medicines. Since biosimilars are a relatively new entity, we are having this conversation at a very opportune moment because the very first biosimilar to be approved for use did so in Europe in 2006 and in the United States only very recently, in 2015 (Kang et al. 2023). The use of biologics is not something novel; in fact, decades have passed since the discovery of human growth hormone, insulin, and agents that stimulate red blood cell production. Patients with diabetes were required to use insulin that had been extracted and purified from the pancreas of

cows or swine prior to the year 1982. After that, researchers found a way to modify cells in the laboratory so that they would express insulin. With this new technique, insulin could be manufactured and distributed to patients. The disciplines of science known as genomics and proteomics, in addition to microarray, cell culture, and monoclonal antibody technology, are utilized in the process of developing biologics. As more genetic information and a deeper comprehension of disease processes have become available, the number of diseases that can be targeted by biologic therapies has expanded exponentially (Declerck et al. 2017). We are able to investigate the illness or condition more thoroughly and discover what is going on on the inside of each of our cells, in addition to the components that are responsible for making up each cell. Increasing our understanding of genetics and cellular processes has led to the discovery of potential new biologic (and drug) targets at each stage in the process of protein synthesis. This paves the way for brand new therapies that are extremely specific, which in turn leads to a better comprehension of diseases (Bachu et al. 2022).

The sequence of manufacturing biosimilars begins at the end, using a process that is referred to as "reverse engineering." This is done rather than starting from what could be considered the beginning of a traditional drug development practice (which would be the beginning of the process) (Wolff-Holz et al. 2019). Before beginning the manufacturing process, this method ensures that the biosimilar molecule will be very similar to the reference molecule in relation of safety, quality, and effectiveness. This method is called step-by-step manufacturing. Once the period of exclusivity for the innovator product has passed, the biosimilar is then reverse-engineered from the innovator product. This is done because the manufacturing specifics of the innovator product are proprietary information and a closely guarded secret. This indicates that the developer of the biosimilar must first acquire the product that was developed by the innovator, then work backward from the completed product using sophisticated analytical tools and previous clinical knowledge in order to design their own procedure/ process that will result in a molecule that is highly similar to the molecule that was originally developed. In the context of biologics and biosimilars, the product is extremely dependent on the process. The structure, function, and quality of these medications are all directly attributable to the manufacturing process that was used to produce them. Because of these reasons, regulatory agencies like the FDA acknowledge that a biosimilar cannot be structurally identical to the product that it is compared to (the reference product) because of differences in the manufacturing process that change the final product (Lemery et al. 2017). The FDA requires that a biosimilar not be "clinically different" from an originator biologic rather than requiring that a biosimilar be fundamentally identical to an originator biologic.

3 Biosimilars Approval and Regulatory Requirements

In general, the regulatory requirements for the approval of biosimilars are the same across all three of these organizations: the WHO, the EMA, and Health Canada, as well as the guidelines released by the FDA (Administration 2018). All of these regulatory bodies demand a methodical, step-by-step process in order to determine whether or not two products are biologically comparable, despite the fact that these guidelines might have some inconsequential, sometimes even terminological, differences. Comparative evaluations incorporating analytical, nonclinical, and clinical studies are a standard part of these well-established regulatory processes (Barbier et al. 2022). The European Medicines Agency (EMA) has assumed a leadership role in the global regulatory community by initiating the first legislation. This move has allowed the EMA to assume a position of authority within the global regulatory community. When it comes to requiring identical head-to-head comparison research, it was the EMA that paved the way for other agencies to follow suit (Jimenez and Brake 2011). When all of the evidence from each evaluation has been compiled, biosimilarity can be taken into consideration. However, each stage of this procedure needs to be supported by the stage that came before it. 1. The first step is ensuring the biosimilar's quality is grounded in its structural and functional similarities to the reference product using analytical analyses that employ numerous orthogonal approaches. The biosimilar must be compared to the standard product to establish its resemblance. Next, the biosimilar needs approval from the appropriate regulatory agency. 2. The second step, the biosimilar agent must be shown to have the same target or physiologic process as the reference product and to be equally as hazardous through nonclinical trials. In order for the biosimilar agent to be approved by authorities, certain studies are required. 3. The evaluation of a biosimilar product reaches its climax with the third step, which is also the most crucial stage of the procedure. It is a specialized clinical study program that evaluates biosimilar regard to pharmacokinetics, clinical effectiveness and safety, as well as immunogenicity (Anon 2022).

4 Biosimilar Manufacturing Process

The production of biosimilars involves a process that involves sequentially demonstrating that they are comparable to the Reference Biologic through comprehensive characterization studies that reveal the molecular and quality characteristics of the Reference molecule of biologics (Schiestl et al. 2020). In order to protect the general public's health and to adhere to the standards set forth by international organizations, biosimilar medications need to be able to demonstrate that they are secure, effective, and of high enough quality. The nonclinical and clinical assessment of the biosimilar is probably little less than that which is required for the reference product; however, it is absolutely necessary to sufficiently test biosimilars. These requirements

cannot be fulfilled for quality components because they require a demonstration of comparability, which is not possible. If the studies reveal that reference product and biosimilars not identical, they are significantly different in the aspect of efficacy, safety and purity, then the product will not be regarded as biosimilar. If the Reference product is used to treat more than one clinical condition, the biosimilar will only be eligible for all indications if it can be explained and if it satisfies the conditions that are outlined in the section that is titled "Extrapolation of Efficacy and Safety Data to other Indications." In the event that the Reference Biologic is used to treat more than one clinical condition, the biosimilar will only be eligible for all indications if it can be explained (McKinnon et al. 2018).

4.1 Choosing a Reference Biological

Reference Biologic is a product created by an innovator that has been authorized following review of the entire dossier, which is essential for the creation of biosimilar. Every comparability experiment involving quality, nonclinical, and clinical factors must use the Reference Biologic (Kang et al. 2023). The following considerations should be considered while choosing reference biological product:

- The Reference Biologic must be the creator's creation and must be licensed or authorized in India or one of the ICH nations. A complete set of safety, efficacy, and quality statistics should be used to license the Reference Biologic.
- The same Reference Biologic should be used throughout the research and development process of biosimilars to support the product's efficacy, safety, and quality.
- The Reference Biologic's dosage form, strength, and method should be used to administer the biosimilar. It must be demonstrated that the comparable biologic and the standard biologic's active medication component are equivalent.

4.2 Process of Manufacturing

The biosimilar producer must create an exact manufacturing process to create a product that is identical to the reference product in terms of identity, purity, and potency. The production of biosimilars must be verified in order to show that it is dependable and incredibly consistent. It is advised to use the same host primary cell line for the production of the biosimilar if the host primary cell line used to produce the Reference Biologic is made available. This prevents certain kinds of process-related impurities from being incorporated that might have a negative effect on clinical outcomes and immunogenicity, as well as the possibility of major changes in the product's quality attributes (QAs) (European Medicines Agency 1995; Galbraith 2017).

4.2.1 Upstream Process Development

- The upstream process needs to be fully described, till down to the elements of the media used for cell development.
- Data on reproducible fermentation from at least three batches during the pilot period. (Sufficient amount of purified product should be generated from the bache size, to generate nonclinical data).
- Carefully controlling and monitoring the main process is necessary. Information on pH, temperature, dissolved oxygen, cell growth, product formation, the pattern of primary nutrient consumption, and agitation rate are just a few examples of the specific details about upstream process kinetics that can be noticed from consistency batches.

Volumetric productivity and product per litre yield are the metrics that will be used to determine concentration. Information to demonstrate the consistency of the specific protein yield, or the quantity of protein produced for each unit of cell mass, across all upstream batches. Showcase ways to scale up replication and economic growth in general (Kesik-Brodacka 2018).

4.2.2 Construction of Downstream Processes

- Complete in-depth breakdown of the steps taken to gather the cells and remove the unwanted protein.
- The amount of protein per lot that needs to be purified.
- A precise breakdown of each stage that makes up a unit operation in the purification and recovery of proteins, as well as a quantitative evaluation of the quantity of protein recovered at each level (ICH 2010).
- The consistency of the recovery over three separate batches of cell culture or fermentation that were purified from three separate batches of those processes of manufacturing.
- Describe any variants that emerged following the translation.
- Information on how to remove impurities, such as host cells, impurities associated with the manufacturing process, and variations and impurities related to the product that are believed to pose an immunogenicity risk. (EMEA 1997), research to show that the pathogen has been eradicated.

4.3 Quality Control Consideration

4.3.1 Analytical Methods

The appropriate methods of analysis should be selected in accordance with the crucial product quality attributes in order to show product comparability. Various orthogonal

methods, like product aggregation, are frequently used to describe specific characteristics. Analytical method should be very sensitive even minor differences should be able to detect during the quality characterisation studies. If available Indian Pharmacopoeia should be referred for the quality attributes during the process of characterisations, properly qualified assays that are capable of reproducibility and dependability are needed. For batch release stability studies, in-process controls, and method validation in accordance with ICH standards (ICH Q27, Q5C8, and Q6B9) are required for quality attributes characteristics. The characterization studies should involve examples of the derived DNA product, control Reference Biologic, a known positive and negative control standard. Each quantitative experiment must be carried out a minimum of three times, and the results must be stated with mean and standard deviation, in order to give confidence in the statistical analysis' accuracy. The proper representation of the statistical significance in the appropriate formats must be included with all characterization data (Galbraith 2017; ICH 2010).

4.3.2 Characterization of Product

Functional assays, physicochemical properties, biological activity, immunological properties, purity (including impurities related to the manufacturing process and the product itself), contamination, strength, and substance are among the studies used to describe biosimilars. It is crucial to follow the guidelines outlined in the ICH Q6B rule.

Physicochemical and Structural Properties: The estimation of the structures of the biosimilar components and the finished product, as well as the measurement of any other important physicochemical properties, should be considered when analysing physicochemical characteristics (Kirchhoff et al. 2017). Biosimilar amino acid sequence must be confirmed because it is expected that sequence will be same as that of the Reference biologics. The analytical methods must be precise and accurate to a level that is appropriate. Identification and measurement of the post-translational modifications that have occurred are required to capture in cases if they are occurring. If any significant differences are found, it is essential that they are thoroughly examined in nonclinical studies as well as clinical experiments and supported by scientific data.

Biological Activities: Biological products may contain a variety of biological activities. In such situations, suitable biological assays will be used to illustrate the activities, ascertain the mechanism of action of the product, and identify the clinical activity (Kirchhoff et al. 2017). A national or international Reference standard should be used to validate biological assays when it is suitable and available. An internal Reference standard must be developed in accordance with ICH suggestions if there are no such standards. The methods of the bioassay(s) may be used for tests if they are mentioned in the specification.

Immunological Properties: It is well known that the production process has an impact on the number of process-related impurities and post-translational modifications in biosimilars. Such characteristics changes leads to effect of product sensitivity

or immunogenicity. Sufficient nonclinical studies need to perform to generate the data for affinity, specificity, binding, antibody products and different immunogenic parameters etc., and should be used in the evaluation process (Bielsky et al. 2020).

Purity and Impurities: The following must be assessed using a range of diagnostic methods when describing a biosimilar:

- Specific product variants (e.g., isomers etc.)
- Impurities related to products (e.g., oxidized, or aggregated)
- Contaminants connected to host cells (e.g., host cell protein and DNA etc.)
- Contaminants linked to processes (e.g., resin leachates or residual media components etc.).

Different nonclinical and clinical studies are chosen to conduct for assessment based on the existence of impurities in the biosimilar products (Galbraith 2017; ICH 2010; Jimenez and Brake 2011).

5 Specification

To guarantee a constant standard of product quality and that it is comparable to the Reference Biologic in accordance with the relevant guideline, the Specifications of Biosimilar (for drug substances and drug products) are organized around QAs (ICH Q6B). The analytical techniques used to characterize products and establish their comparability may or may not be the same as those employed to define product standards (Lemery et al. 2017).

6 Stability

The shelf life of drug substances and drug products, as well as the ideal storage conditions, should be determined using real-time stability tests, based on the relevant regulations (such as WHO TRS 822, ICH Q1 A(R2), and ICH Q5C). This is done to guarantee the validity of the stable study findings. Studies comparing the structure's accelerated and strained stability should be conducted side by side. The products similarity can be proven by showing degradation patterns that are similar to those of the reference biologic.

7 Comparison and Quality Analysis

It is absolutely necessary to compare the quality of Reference Biologic items to those of Biosimilar ones. Before beginning clinical trials, the applicant is required to first submit a complete quality dossier that is in accordance with the Central

Drug Standard Control Organization (CDSCO) guideline for industry, 2008. This dossier must include the outcomes of a comparability exercise that compares the biosimilar to the Reference Biologic. Generating and reporting the comparability data of biosimilars with reference is required. It is advised to use the first three standardized quantities that have been used in a row to show consistency in the process of manufacturing. It is essential to confirm that the active drug substance in the biosimilar has a chemical makeup identical to that of the active drug substance in the Reference Biologic. If it is found that the similarities and variations between the Reference Biologic and the biosimilar may affect the efficacy and safety of the biosimilar, further studies might be necessary to characterization similarities and differences.

Critical quality attributes (CQA) and Key quality attributes (KQA) are subcategories of quality attributes of a biosimilar.

(1) Critical quality attributes, or CQAs, are characteristics of a product that directly affect its therapeutic safety or efficacy. All the characteristics of the substance that directly affect the known mechanism(s) of action that it holds are included in this category. It is necessary to control CQAs within the parameters that must be established as appropriate based on the Reference Biologic.
(2) "Key Quality Attributes," or KQA for short, are Quality Attributes that are important from the standpoint of product and process consistency but are not known to have an impact on clinical safety and effectiveness. This category contains a molecule's characteristics that don't affect any of its known mechanisms of action. KQAs must be kept within allowable bounds, but these bounds must be carefully abided by the rules.

8 Nonclinical Studies Data Requirements

8.1 Prerequisite Before Conducting Nonclinical Studies

All of the RCGM's requirements, including proving that the process and product are consistent with one another, describing the product, and giving product specifications, must be met by the applicant. To obtain approval, the applicant must send the generated data to RCGM along with the following fundamental clinical data and nonclinical study protocols. After receiving approval from the RCGM, the toxicity research needs to start. The following details about the Reference Biologic and the Biosimilar may be regarded as some of the essential information:

Information on the fundamentals of Reference Biochemical.

- Information pertaining to the medication, including but not limited to dosage, delivery mechanism, route of administration, rate of absorption and excretion, therapeutic index, dose response, and so forth.

- The range of bioequivalence, if appropriate. if such knowledge can be obtained, tissue-specific localization.
- The latest toxicology information for the Reference Biologic. Details about the Biosimilars' core concepts
- The developer must send the application to RCGM with the permission of the Institutional BioSafety Committee (IBSC) and, if applicable, the Institutional Animal Ethics Committee (IAEC). Toxicity study detail protocol and the location of execution of toxicity study along with the detail of personals involved like principal investigator, study director, histopathologist, quality assurance person and researchers should be provided by the applicant.

8.2 Early-Stage Research Pharmacodynamic and Toxicology Studies

Nonclinical studies are required before starting a clinical trial. Non-clinical research comparing the Reference Biologic and the Biosimilar should identify any discrepancies between the two. Therapeutic index, indication spectrum, and other clinical factors can all influence the methodology of preclinical studies. The nonclinical overview needs to be 100% behind the method being used. Unless there is a good reason not to, nonclinical research should be done with both the Reference Biologic and the final version of the Biosimilar that will be used in the clinic.

Research that is not done in a clinical setting needs to be done before starting any kind of clinical investigation. These comparative nonclinical investigations should have as their primary objective the identification of any differences between the Reference Biologic and the Biosimilar. The design of the research that is not done in humans can change depending on the clinical parameters, which can include the therapeutic index as well as the type and quantity of indications that are applied. The nonclinical summary needs to provide complete backing for the approach that was selected. Unless there is a compelling reason not to, nonclinical studies ought to be carried out utilizing both the Reference Biologic and the final version of the Biosimilar that is designed for clinical use. The biosimilar medication should have the same dosage form, dose, strength, and method of administration as the reference biologic, and any differences between the two should be explained. In order to conduct a nonclinical evaluation, the following investigations are required:

Studies on Pharmacodynamics

i **Studies conducted in vitro**: To evaluate the comparability of biosimilar and reference biologics, cell-based in vitro bioassays (such as cell cytotoxicity, growth assays, neutralizing, and receptor binding assays) should be used.

ii **Studies conducted in vivo**: Clinically relevant efficacy and potency activity of reference biologic, if correctly reflect by invitro assay, in such condition in vivo evaluation of biological/pharmacodynamic activity may not be required. In cases

where in-vitro assays are unable to properly represent efficacy and potency, in vivo studies should be conducted.

9 Toxicological Studies

In vivo toxicity studies must include at least one repeat-dose toxicity trial in a pharmacologically relevant species using the intended route of administration. The applicant must provide a scientific justification for the choice of animal model(s) to be used, based on data from the scientific papers. Toxicology studies, with RCGM approval, must be performed using rodents or nonrodents. The only route of administration would be the one scheduled per schedule Y, regardless of whether the animal model used is pharmacologically relevant or not. Normally, the study would last at least 28 days, including a recovery period of 14 days. However, the time frame may change from case to case based on the dose and other factors (Table 1).

The procedures and study reports should include thorough descriptions of the following toxicity testing phases:

- Measures done prior to euthanasia, such as weighing the patient or drawing blood.
- The immediate aftermath of euthanasia, the necropsy, a thorough account, the weights of the organs, and the removal of organ samples for histopathology. Biochemical components, equipment, and English and metric phrases.
- Haematology test methodology and factors (automated or manual). The use of statistical methods.

Table 1 Treatment grouping for toxicological study

Group no.	Treatment	Dose
1	Control	
2	Control (Recovery Group)	
3	Protein-free dossier (for vaccines) if more than one additive is used, each one needs to be tested separately	
4	Biosimilar (Study duration)	1X of HED (Low dose)
5	Reference biologics	1X of HED (Low dose)
6	Biosimilar	2X of HED (Medium dose)
7	Biosimilar	5X of HED (High dose)

HED: Human equivalent dose, *Biosimilar groups and a recovery group continuing for 7–14 days after the conclusion of the study period

- Bone marrow was either extracted, subjected to a smear analysis, or subjected to histopathology examination. Candidates should consider the following things in instances of histopathological observations:
- It is necessary to note every observation that is believed to differ from the described normal histology, as well as how frequently it occurs in each group.
- The protocol should include the recommended course of action in the event of premature death or morbidity.
- If an animal's organs were not all examined, such as when only 4 of 5 livers were examined in 5 animals, the cause for this omission should be noted.

10 Immunogenicity

It is typically required to use a multi-tiered approach that includes immunoassays for screening and confirmatory purposes that identify binding ADAs (Anti-Drug Antibodies), followed by assays that estimate ADA magnitude and neutralization potential. Deviations from this approach must be justified.

The advantages and drawbacks of the assays and formats used today, as well as how to interpret the findings, have all been thoroughly reviewed. The method for testing antibodies and the assays chosen must be supported by the vendor. Assay control, validation and the establishment of cut-off lines for separating samples that contain antibodies from those that do not should both receive careful consideration. The pharmacological target and any remaining drug in the sample are two factors that could potentially interact with the matrix components. Corrective actions should be taken to lessen this influence. For instance, measures like providing time for the drug to be cleared from the circulation prior to sampling or incorporating steps for dissociating immune complexes and/or removing the drug can be used for drug interference (which frequently happens with samples taken from patients given mAbs). It is important to take precautions to make sure the application of such strategies does not jeopardize ADA diagnosis or patient care.

Comparative immunogenicity testing should be carried out when necessary using the same assay design and sampling frequency. Antibody testing is carried out using the therapeutic administered to the patient in order to evaluate immunogenicity in the development of novel drugs. Applying this idea to biosimilars makes it extremely difficult to create screening assays with comparable sensitivity for the two patient groups (biosimilar and RP) within the same trial. As a result, in the case of biosimilars, relative immunogenicity is frequently evaluated using a single assay that uses the biosimilar's drug component as the antigen for sample testing for both groups. All antibodies produced against the copycat can be found using this method. The manufacturer must show that the method(s) used are appropriate and provide evidence that they measure ADA to the RP and to the biosimilar to a comparable degree.

The potency assay of the product is typically the foundation for neutralization assays that represent the mechanism of action. In situations where the therapeutic binds to a soluble ligand and blocks its biological action, non-cell ligand-based assays

are pertinent. The use of functional cell-based bioassays is advised for products with a high risk (for instance, those with non-redundant endogenous homologs and those for which effector functions are crucial). Regulatory officials may be consulted when guidance is required regarding the need for a neutralization assay and the best format to use (cell-based, ligand-based, or based on enzyme activity).

If deemed clinically relevant or in unique circumstances (such as the occurrence of anaphylaxis or the use of specific assay formats), further characterization of antibodies (for example, isotype) should be carried out while taking into consideration the immunogenicity profile of the RP. It is doubtful that the biosimilar would elicit an IgE response, for instance, if the same expression system is used as in the RP. In instances where technical issues with the initial assay occurred, it will be necessary to store patient samples for later testing under the proper storage conditions.

11 Application Data Requirements for Clinical Trials

According to the CDSCO industry guidelines from 2008, the applicant must submit an application for the conduct of clinical trials in addition to the data that was given in the nonclinical application (Lemery et al. 2017). The provided quality data must show that all KQAs are tightly controlled and there is no variation in the CQAs in order to initiate with the clinical evaluation.

Pharmacokinetic (PK) profile studies

The PK findings should be able to support the ensuing Phase III clinical development since the purported biosimilar would be proven to be similar to the Reference product. A pharmacokinetic study of the biosimilar may be conducted after the thorough characterization and comparability checks on quality attributes have been completed in a suitable number of:

Individuals who are generally recognized as.

a. Healthy (NHV) and Normal/or
b. Unhealthy or Patients.

Priority should be given to the following factors when planning comparative pharmacokinetic studies: the condition and disease to be treated, the route of administration, and the indication. Pharmacokinetics primary and secondary parameters such as clearance, volume of distribution, half-life, and linearity of PK profile.

Design factors that are appropriate include:

I. Comparative, single-dose PK studies
II. Cross over
III. or parallel arm
IV. Multiple dose, Comparative parallel arm steady state.

The research on Normal Healthy Volunteers (NHV) is conducted before the study on the safety and effectiveness of Phase III in a sequential development strategy. A sound rationale should be used when choosing the dosage. On the basis of how perceptive is to differences, the administration's course should be selected. It is crucial to specify and support the comparability limits and have a statistically sound justification for the sample size before starting the pharmacokinetic study. Mostly ELISA, HPLC or LC–MS/MS methods employed for the bioanalysis of biologics. The sensitive bioanalytical method needs to be validated to have acceptable specificity, sensitivity, limit of detection, limit of quantification in addition to adequate an accuracy and precision. It should be able to recognize and monitor the evolution of Biosimilar in a complex biological matrix made up of numerous distinct proteins.

Pharmacodynamics (PD) Study

The pharmacokinetic (PK) studies that were part of the biosimilar demanded that the PD studies also place a priority on comparative analysis. In order to establish the differences between biosimilars and the Reference Biologic, comparative, parallel arm, or cross-over PD studies need to be carried out in the most relevant cohort possible (De Mora 2015) (patients or healthy volunteers). People who are otherwise healthy can develop Parkinson's disease (PD) if a PD marker is present, unless doing so would be unethical due to the possibility for adverse effects and toxicity, such as those caused by oncology drugs. Before beginning the demonstration of similarity in PD parameters, it is essential to establish acceptance ranges and to explain them in a way that is easy to understand. The clinical significance of the surrogate markers being used and their clinical validation are prerequisites for the PD research variables (Barbier et al. 2019). It is possible to combine pharmacodynamic and pharmacokinetic studies; in this situation, the PK/PD relationship needs to be described. A phase III clinical study can be combined with a PK study if there are no PD markers accessible but there is the possibility of conducting PK on patients.

Confirmatory safety and efficacy research

This is necessary in order to eliminate any potential risks that may have been overlooked. A further comparative safety and effectiveness trial is not required except in very specific circumstances, such as when comparing a biosimilar to a Reference Biologic at the analytical, non-clinical, and PK/PD levels and finding no residual uncertainties in the findings of those comparisons.

Lack of a study on efficacy and safety

If all of the following conditions are met, study can be excluded like, the confirmatory clinical safety and efficacy study:

i. Physicochemical and in vitro methodologies can be used with high confidence to characterize the structural and functional comparability of biosimilars and the Reference Biologic.
ii. The biosimilar is equivalent to the Reference Biologic in all preliminary evaluations.

iii. The applicant supported the efficacy/PD measurements and safety measurements, including meaningful immunogenicity assessments over an appropriate time period, in the PK/PD study, which demonstrated that clinically validated PD markers could be compared (Lemery et al. 2017).

The confirmatory clinical safety and efficacy study cannot be omitted, particularly for high-molecular-weight biologics such as monoclonal antibodies. If the safety and efficacy research is waived based on convincing PK/PD data and comparable quality non-clinical data, all of the approved indications for the reference product may be used.

Information on Safety and Immunogenicity

It is required that a clone go through both a pre-approval safety evaluation as well as a post-approval safety assessment. This provides details about the product's susceptibility to certain conditions. The primary goal of the safety statistics that are required for pre-approval is to guarantee that there will be no unforeseeable problems with the product's safety. In addition to the data that have already been published on the Reference Biologic, are necessary in order to guarantee that there are no unanticipated safety concerns. A comprehensive method must be supplied, in addition to the suggested non-comparative post-marketing study, in order to evaluate the biosimilar's level of safety.

The Extrapolation of Efficacy and Safety Information to Other Indications

The safety and efficacy statistics of a specific clinical indication of a biosimilar may be extrapolated to other clinical indications if the following criteria are met. This is due to the fact that clinical research has been done on the specific clinical indication in issue.

- Validation and demonstration of quality comparability to Reference Biologic.
- Comparability with regard to initial assessment in reference to the Reference Biologic has been shown.
- One indication has shown that the treatment is clinically safe and efficacious.
- For the treatment of other therapeutic indications, the mechanism of action is unaltered.
- The involved receptor(s) are identical to those found in other therapeutic applications.

12 Data Requirements for Market Authorization Applications

For market authorization the application must adhere to the guidelines outlined in the CDSCO industry advice document from 2008. Information on comparability of quality must also be provided with the proper justification in cases where commercial manufacturing is conducted at a different scale or using a different process than that

used to produce batches for phase III clinical trials. Each of these instances will be handled separately (Requirements and Authorization 2016).

13 Pharmacovigilance Strategy

It is unlikely that the rare adverse events will occur because the clinical studies on Biosimilars that were performed prior to market authorization were so small-scale. Post- market surveillance with comprehensive pharmacovigilance protocol should be designed by the applicant or manufacturer, to evaluate the safety of biosimilar (Baldo et al. 2018). Periodic Safety update report (PSURs), should include regular safety update report which is submitted every six months for the initial two years after the approval of biosimilar. For the later few years the PSURs, annually need to submitted to DCGI office as per the Schedule Y.

14 Post-marketing Analysis (Phase IV Study)

Data collected through a pre-defined single arm study with typically more than 200 evaluable patients and compared to historical data of the Reference Biologic after market approval for additional safety information is used to finally further reduce the residual risk of the biosimilar. If there are no exceptional circumstances, the research ought to be finished no later than two years after receiving either a manufacturing license or a marketing authorization, at the very latest. Because ensuring participant safety is the primary focus of the post-marketing phase IV research, the following factors need to be taken into account when designing the protocol:

- The top priority is safety.
- Secondary outcomes include immunogenicity and efficacy.

The findings of post-marketing studies should be submitted to DCGI due to the limited scope of clinical studies on Biosimilars conducted prior to market authorization. Post-market study plans should be included in pharmacovigilance plans, and CDSCO should be updated on the studies.

15 Exceptions

The size of the clinical trial community can be reduced in the case of a biosimilar that can be assessed for rare diseases depending on the rarity, severity, and limited availability of therapeutic alternatives (Table 2).

Table 2 Updated list of Biosimilars release by FDA

Biosimilar product information | FDA		
Biosimilar name	Approval date	Reference product
Idacio (adalimumab-aacf)	December 2022	Humira (adalimumab)
Vegzelma (bevacizumab-adcd)	September 2022	Avastin (bevacizumab)
Stimufend (pegfilgrastim-fpgk)	September 2022	Neulasta (pegfilgrastim)
Cimerli (ranibizumab-eqrn)	August 2022	Lucentis (ranibizumab)
Fylnetra (pegfilgrastim-pbbk)	May 2022	Neulasta (pegfilgrastim)
Alymsys (bevacizumab-maly)	April 2022	Avastin (bevacizumab)
Releuko (filgrastim-ayow)	February 2022	Neupogen (filgrastim)
Yusimry (adalimumab-aqvh)	December 2021	Humira (adalimumab)
Rezvoglar (insulin glargine-aglr)	December 2021	Lantus (insulin glargine)
Byooviz (ranibizumab-nuna)	September 2021	Lucentis (ranibizumab)
Semglee (Insulin glargine-yfgn)	July 2021	Lantus (Insulin glargine)
Riabni (rituximab-arrx)	December 2020	Rituxan (rituximab)
Hulio (adalimumab-fkjp)	July 2020	Humira (adalimumab)
Nyvepria (pegfilgrastim-apgf)	June 2020	Neulasta (pegfilgrastim)
Avsola (infliximab-axxq)	December 2019	Remicade (infliximab)
Abrilada (adalimumab-afzb)	November 2019	Humira (adalimumab)
Ziextenzo (pegfilgrastim-bmez)	November 2019	Neulasta (pegfilgrastim)
Hadlima (adalimumab-bwwd)	July 2019	Humira (adalimumab)
Ruxience (rituximab-pvvr)	July 2019	Rituxan (rituximab)
Zirabev (bevacizumab-bvzr)	June 2019	Avastin (bevacizumab)
Kanjinti (trastuzumab-anns)	June 2019	Herceptin (trastuzumab)
Eticovo (etanercept-ykro)	April 2019	Enbrel (etanercept)
Trazimera (trastuzumab-qyyp)	March 2019	Herceptin (trastuzumab)
Ontruzant (trastuzumab-dttb)	January 2019	Herceptin (trastuzumab)
Herzuma (trastuzumab-pkrb)	December 2018	Herceptin (trastuzumab)
Truxima (rituximab-abbs)	November 2018	Rituxan (rituximab)
Udenyca (pegfilgrastim-cbqv)	November 2018	Neulasta (pegfilgrastim)
Hyrimoz (adalimumab-adaz)	October 2018	Humira (adalimumab)
Nivestym (filgrastim-aafi)	July 2018	Neupogen (filgrastim)
Fulphila (pegfilgrastim-jmdb)	June 2018	Neluasta (pegfilgrastim)
Retacrit (epoetin alfa-epbx)	May 2018	Epogen (epoetin-alfa)
Ixifi (infliximab-qbtx)	December 2017	Remicade (infliximab)
Ogivri (trastuzumab-dkst)	December 2017	Herceptin (trastuzumab)
Mvasi (Bevacizumab-awwb)	September 2017	Avastin (bevacizumab)
Cyltezo (Adalimumab-adbm)	August 2017	Humira (adalimumab)
Renflexis (Infliximab-abda)	May 2017	Remicade (infliximab)

(continued)

Table 2 (continued)

Biosimilar product information | FDA		
Biosimilar name	Approval date	Reference product
Amjevita (Adalimumab -atto)	September 2016	Humira (adalimumab)
Erelzi (Etanercept-szzs)	August 2016	Enbrel (etanercept)
Inflectra (Infliximab-dyyb)	April 2016	Remicade (infliximab)
Zarxio (Filgrastim-sndz)	March 2015	Neupogen (filgrastim)

Conclusion

Despite the many challenges that must be overcome, research and development of biosimilars persist. Biosimilarity must be established using a personalized strategy across the whole product development process, beginning with the structural and functional assessment and progressing through nonclinical and clinical research. This must be completed before any inferences about biosimilarity may be made. It has become abundantly evident that a new approach is required for the creation of biosimilars. This is especially true when deciding upon CQAs and study outcomes for preclinical and clinical research. The healthcare business faces a difficulty with the launch of biosimilars because of the need to systematically understand and comprehend the scientific basis of resemblance to the reference product. The introduction of biosimilars raises this difficulty. When it comes to generating biosimilars, clinical studies are a blunt instrument, while analytical evaluation is a tool that is substantially more sensitive in determining similarity. This is something that should be made known to the widest possible audience.

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Biomedicine: Trends, Challenges, Prospects

Applications of Nanomaterials in Medicine: Current Status and Future Scope

Shruti Kakodkar, Pranjali Dhawal, and Janvi Kadam

Abstract Nanomaterials have proven to be advantageous in numerous frontier areas due to their use in fabrication of smarter products. Currently, the conventional approaches employed for drug release, imaging, as well as therapy warrant improvements. Nanomaterials offer exciting opportunities in medicine on account of their exceptional size-dependent characteristics. Research studies have revealed that nanomaterials can augment drug targeting capacities, bioavailability and biocompatibility. Moreover, nanomaterials have also been revealed to exhibit promising applications in imaging techniques as high contrast agents with improved permeability and tissue retention. Nanomaterials have also been vastly explored in detection and treatment of critical ailments viz. cancer and cardiovascular diseases among others. Considering the plethora of applications of nanomaterials in medicine, the current chapter aims to discuss two aspects of nanomaterials: first, we aim to highlight the scope of using nanomaterials in modern medicine as potent intervention tools in the treatment of varied diseases, and second, we review the possible environmental consequences of their medicinal application.

Keywords Nanomedicine · Nanoparticles · Treatment · Diagnosis · Imaging · Diseases

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1 Introduction

Nanotechnology based research studies have proven the diverse applications of nanomaterials in numerous areas of science and technology. What gives nanomaterials an added edge over their bulk equivalents is their small size and unique morphology. The classical definition of nanomaterials refers to a material which possesses a dimensional size range between 1 and 100 nm. This exceptional size dependent feature provides these nanoscale objects unique and improved optical, electrical, chemical and mechanical properties; making them promising agents in diagnostics, therapeutics, catalysis, sensing etc. Kadam et al. (2020). Nanomaterials have been characterized into inorganic, organic, carbon-based and composite nanomaterials (Jeevanandam et al. 2018). Vivid forms of these nanomaterials with varied sizes and shapes have displayed promising role in medicine.

Synthesis of nanomaterials need to be intricately designed for obtaining stable and pure nanoforms with desired applications in medicine. The pharmaceutical fabrication of desired sized nanomaterials is performed via bottom-up and top-down methods. The top-down processes breakdown of materials into smaller nanoforms using physical or chemical energy. Bottom-up approaches involves formation of nanoparticles by aggregation of atomic or molecular species. Physical method such as mechanical milling is a top-down approach which involved pulverization of bulk materials into small size particles. Other physical methods such as laser evaporation method, RF plasma method etc. are bottom-up approaches adopted for synthesizing nanomaterials from combination of smaller ions. Synthesis of nanomaterial with controlled particle size using reducing and stabilizing agents can be achieved by chemical synthesis. Biological method, on the other hand, consists of utilizing biological material such as plant extracts, microorganisms, DNA or protein as a source of reducing agents.

2 Nanomedicine-Shifting the Paradigm in Medicine

Nanomedicine signifies a sector of science that amalgamates the concepts of nanotechnology and medicine. The necessities in medical diagnosis and therapy warrants constant technological advancements. To meet these growing demands of addressing varied types of ailments, the medicinal sector has developed diverse pharmaceutical drugs and biomedical instruments. However, these advancements have been facing numerous challenges in terms of their selectivity, frequency of consumption, bioavailability and adverse reactions (Lu et al. 2021). Pharmaceutical industries have attempted to address these challenges through development of nanomaterials. From a therapeutic viewpoint, recent developments have shown that, nanomaterials hold an immense potential as valuable agents for diagnosis, monitoring and treatment of ailments.

Nanomaterials in medicine provide wide range of health benefits through comprehensive functional augmentation of human biological systems. They have been incorporated in engineered devices for enabling efficient detection, monitoring and treatment of diseases. Nanomaterials hold promising role in drug delivery and drug targeting. In recent years, nanomaterials have garnered immense attention as potential agents for treating tumours, infections, neurological diseases, immunotherapy, ophthalmic diseases etc. Presently, nanotechnology is proposed to further refine medicine and surgical procedures by allowing intricate tracking and addressal of physiological pathway-related issues at cellular level. The following sections comprehensively describe the diverse role of nanomaterials in various sectors of medicine.

3 Role of Nanomaterials in Treatment

3.1 Application of Nanomaterials as Antimicrobial and Antiviral Agents

Scientific exploration for identifying effective therapeutic agents against infectious disease-causing microbes is of paramount relevance in the current times. Conventionally, antibiotics are widely used for combating such microbes (Terreni et al. 2021). However, treatment using antibiotics and antifungal agents pose numerous challenges due to the increase in microbial resistance elicited by their rapid mutations (Terreni et al. 2021). The outer membrane of gram-negative bacteria is a key factor that confers strong antibiotic resistance (Breijyeh et al. 2020). The prospective applications of nanomaterials as antimicrobial agents are premised on their surface area, shape and reactivity (Sharmin et al. 2021). The underlying mechanisms of nanomaterials that cause microbial and viral death are postulated to be cell penetration, Reactive Oxygen Species (ROS), cellular malfunctioning, and DNA and protein impairment (Sharmin et al. 2021).

Nanomaterials exhibits strong antibacterial and antifungal activities. For instance, metal nanoparticles and metal oxide nanoparticles exhibit broad spectrum of antibacterial activities. As per a review, these particles also display promising activity against *Aspergillus niger*, *Fusarium. solani*, and *Aspergillus fumigatus* etc. (León-Buitimea et al. 2021). Nanomaterials have been shown to be effective against harmful fungi such as filamentous fungi *Aspergillus, Coccidioides, Mucorales*, and non-filamentous fungi such as *Candida auris* (León-Buitimea et al. 2020). A range of organic material-based nanomaterials and nanocomposites such as nanoferrites, nanosponge, AgNPs/chitosan biguanidine complexes etc. have displayed promising activity against *A. fumigatus.* A complex of 5-fluorouracil and AuNPs have been found to display enhanced antifungal activities against *Aspergillus niger* as compared to their individual forms.

Metal-based nanoparticles non-specifically bind to bacterial cells and inhibit their growth. This characteristic feature impairs the bacteria's ability to develop resistance against such nanoparticles. Carbon-based nanomaterials also display effective antibacterial agents. Their examples such as fullerenes and graphene sheets display promising application against gram-positive bacterial species (Dizaj et al. 2015). Suspensions of aggregated graphene sheets are capable of trapping and isolating bacterial cells from their microenvironment. This in turn obstructs their glucose consumption and causes bacterial inactivation (Zou et al. 2016).

Planktonic cells commonly cause infectious diseases such as sepsis and keratitis. Removing these bacterial cells, especially when they form biofilms, from the site of infection is a challenge (Sun et al. 2022a). When such bacteria develop drug resistance, they become less susceptible to conventional antimicrobial agents. Nanomaterials have been found to be efficient in combating harmful bacterial biofilm formation. Biosynthesized selenium and TiO_2 nanoparticles have been shown to impede *Escherichia coli*, *Bacillus subtilis, Pseudomonas aeruginosa* and fungal biofilms (Haghihi et al. 2013; Cremonini et al. 2016).

Previous research work has found that nanocomposite structures such as Ag/halloysite nanotubes/reduced graphene oxide, graphene/Ag, gold nanocomposites, TiO_2/ZnO display synergistic antibacterial activities. Tailoring of nanomaterials with antibiotics such as polymyxin B, ampicillin, vancomycin, ceftazidime, clindamycin, and ciprofloxacin have also been shown to be effective in boosting antimicrobial activity (Hemeg 2017). Gold and ZnO/antibiotics were shown to avert the growth of multi-drug resistant microorganisms (Hemeg 2017).

Nanomaterials have also been found to curb the action of infectious mycobacteria whose cell wall exhibit features of both gram positive and negative microbes (Fu and Fu-Liu 2002). Silver nanoparticles (AgNPs) have been shown to possess potential application as anti-tubercular agents. A research study showed that biogenically synthesized gold-silver bimetallic nanoparticles possess higher anti-tubercular activity as compared to gold nanoparticles (AuNPs) and AgNPs (Singh et al. 2016). Another interesting research reported that the efficacy of rifabutin and ciprofloxacin against *Mycobacterium abscessus* was improved when they were conjugated to nanoniosomes composites (Slavin et al. 2021). Considering these wide spectra of antimicrobial activities exhibited by nanomaterials, they are also being extensively explored as antibacterial coatings on implantable devices, bone cement, wound dressing, maxillofacial prostheses etc. for addressing the complex infections caused by the multidrug resistant bacteria harbouring in such biomedical devices (Wang et al. 2017).

Nanomaterials are considered to be a powerful agents for replacing anti-viral drugs that are prone to causing side-effects and require a high pill burden. There is extensive literature highlighting the excellent potency of nanomaterials against viral proliferation and viral attachment (Chakravarty and Vora 2021). The present retroviral immunotherapy serves as an effective treatment method to fight Human Immunodeficiency Viral (HIV) infection. However, the therapy fails to provide the infected patients a functional cure and introduces harmful comorbidities. Nanosystem-based drug targeting have been fabricated for HIV treatment (Singh et al. 2017). Recent

nanotechnology approaches have noted the remarkable potency of AgNPs and chitosan nanoparticles as a treatment strategy against hepatitis B virus infection (Boroumand et al. 2021). An interesting strategy for addressing influenza infection has been developed using thermoresponsive 'nanotrap' particles such as virus capturing sialylneolacto-N-tetraose c bearing liposomal decoys (Hendricks et al. 2013). An example is a commercially available poly-L-lysine dendrimer based nanoproduct called 'Vivagel®'. 'Vivagel®' is also a potent antiviral drug against sexually transmitted diseases caused by HIV, Human papillomavirus (HPV), Zika virus and Herpes simplex virus (HSV) (Singh et al. 2017). Human norovirus is one of the primary causes of gastroenteritis. A suitable and effective antiviral treatment against this virus is lacking. Recently, it has been reported that the virus is susceptible to treatment using gold/copper sulfide core/shell nanoparticles (Broglie et al. 2015). Nanovaccines based on liposome, lipid-based, ferritin and chitosan nanoparticles have been reported (Heng et al. 2022). Several other studies on nanotechnology-based vaccines are being conducted for targeting norovirus, Ebola virus, HPV, and rotavirus (Singh et al. 2017). In summary, the plethora of research literature available on the antiviral nature of nanomaterials against viruses suggests that nanomaterials might soon supersede the current conventional antiviral treatment methods.

4 Cancers

4.1 Breast Cancer

Breast cancer (BC), a life-threatening malignancy, impacts women all over the globe. According to Global cancer statistics, on December 15, 2020, BC officially became the world's most prevalent cancer. BC's high incidence and death rate may be linked to the disease's heterogeneity, metastasis, and lack of specificity in currently available chemotherapeutic medicines. Radiotherapy, chemotherapy, endocrine therapy, immunotherapy, and lastly combination therapy are common approaches used for the treatment of BC; however, these conventional treatments have their specific limitations, most common amongst them is non-specificity. Nanomaterials have been studied for determining their efficiency in delivering anticancer drugs directly to the tumours (Han et al. 2019). Organic nanoparticles have constituted the foundation of all approved nanoplatforms for BC treatment to date. Liposomal formulation of Eribulin mesylate, a cytoskeleton inhibitor, has been studied to treat metastatic BC, showing better efficacy and decreased off-target toxicity because of its controlled release (Yu et al. 2013). Organic nanoparticles in photodynamic therapy (PDT) are recognized as significant therapeutic enhancing technique for a more localized form of treatment. Here, these nanoparticles act as selective transporter of photosensitizers to the target cancerous cells, which are then destroyed due to ROS generation (Montaseri et al. 2020). In a different research endeavour, photochemotherapy of

breast cancer cells was performed using doxorubicin loaded onto polyethyleneimine-coated perfluorocarbon double polymeric nanoemulsions. This nanomaterial with attached homing ligand proved to be effective for intercellular uptake and elevated therapeutic effects (Lee and Ma 2017). Additionally, inorganic nanoparticles also have introduced promising new avenues for BC nanotherapeutics. Use of biogenic AuNPs and AgNPs to treat BC cells *in-vitro*, reveals a synergistic effect with standard anticancer therapies, and a larger lethal impact on malignant cells than healthy cells (Saravanan et al. 2020; Barabadi et al. 2019).

4.2 Lung Cancer

Currently, the choice of treatment for non-small cell lung cancer, a predominant form of lung cancer (LC), is radical surgery. However, only small percentage of diagnosed patients are allowed to be treated with this approach (Niloy et al. 2021). The induction of apoptosis in tumours is restricted. Iron oxide nanoparticles and $SnFe_2O_4$ nanocrystals, have been demonstrated in studies to trigger apoptosis of lung cancer cells by interacting with H_2O_2 through the Fenton reaction, resulting in the production of hydroxyl radicals that kill LC cells. In another study, composite nanomaterial of synthesized FePt nanoparticles on graphene oxide nanosheets increased the effectiveness of radiation therapy against LC cells. The proposed primary mechanism of such nanomaterials is based on the increment in oxidative stress and improved radiosensitivity of the cells (Zhao et al. 2021). Another type of mechanism for LC treatment can be attributed to nanotoxicity alone or as combination therapy with other chemotherapeutic drugs. Researchers are also working on nanoparticle-based delivery methods for small-interfering RNAs (siRNAs)and microRNAs (miRNAs), which destroy malignant cells by inhibiting oncogene and tumour-suppressor gene expressions.

4.3 Oral Cancer

Oral cancer is a major concern as it leads to high lymph node metastasis and lacks suitable biomarker for early diagnosis. Besides chemotherapy and radiotherapy, photodynamic therapy of oral cancers has gathered significant interest. As mentioned earlier, PDT works by the activation of photosensitizer by visible light thus leading to ROS generation and apoptosis of cancerous cells. As photosensitizer cannot penetrate deep into oral mucosa, recent use of Upconversion nanoparticles (UNPs) loaded with photosensitizer and excitation with near-infrared light, have shown great potential (Lucky et al. 2016). AuNPs have been evaluated as a nanodrug together with other therapies for treating oral cancer (Zhang et al. 2022). Further, a proposed mechanism for treating oral cancers is via modulation of autophagy. A study found

that iron core/gold shell nanoparticles promoted cytotoxicity in OECM1 oral malignant cells through mitochondria-mediated autophagy. Herein, only cancer cells, and not healthy ones, suffer an irreversible loss of mitochondrial potential and ROS generation due to the core–shell nanoparticles (Wu et al. 2011b). Additionally, E72-Chitosan-Ag_3AuS_2, mesoporous carbon nanospheres NCQD-HCS, etc. are few examples that have been studied for treatment against oral squamous cell carcinoma (Niu et al. 2022).

4.4 Pancreatic Cancer

In terms of cancer-related mortality, pancreatic cancer ranks fourth with 90% of the cases being malignant. Surgical resection remains the only hope for the cure of pancreatic cancer. Due to chemo-and radio resistance, the development of innovative and effective treatment options for pancreatic cancer is essential. The development of stellate cells for desmoplastic response is a characteristic feature of pancreatic cancer and plays a significant function in the initiation, development, and metastasis of tumours. Proteins expressed by these cells may also represent novel therapeutic targets (Ji et al. 2020). Yin et al. 2017 reported an increase in the inhibition of PaCa-2 pancreatic cancer cells by knocking down of *HDAC1* and *K-Ras* gene, *in-vivo*, using dual gene therapy with folic acid conjugated graphene oxide nanosheets and thermotherapy. Another strategy has included, targeting circular RNAs, which are abundantly expressed in tumour-derived extracellular vesicles in pancreatic cancer patients. Various nanomaterials like organic-based liposomes and porous silicon nanoparticles have been employed in the effective targeting of *circFARSA* gene knockdown using siRNA (Yuan et al. 2022).

Hypoxia is produced in tumours due to oxygen imbalance and consumption by fast-growing cancerous cells. Use of newly created nanocarriers as oxygen transporters is an effective way of alleviating hypoxia. For instance, in one study, hollow mesoporous organosilica nanoparticles functionalized with fluorocarbon chains were synthesized as oxygen reservoirs. Following treatment, a spontaneous increment in oxygen pressure was noted, which ultimately halted the progression of hypoxic pancreatic tumours (Chen et al. 2017).

4.5 Leukemia

Leukemia begins in the bone marrow and is indicated by the accumulation of WBCs. Chemotherapeutic agents are widely used in treatment plans, despite their high toxicity and negative side effects. Contemporary research has used fundamental nanotechnology to provide better diagnoses for leukemia. Biological markers and

genetic materials like siRNA and peptides labelled with nanoparticles are considered to be apt for imposing cytotoxicity in malignant cells. A study reported NF-κB targeted peptide-based siRNA nanotherapy for the management of adult T-cell leukemia (Rauch et al. 2021). Yet another study reported the role of carbon nanotubes in delivering siRNA for effective suppression of cyclin A2 that are responsible for tumour progression (Wang et al. 2008). Various inorganic nanomaterials such as ZnO, biosynthesized selenium, etc., exhibited cytotoxic effects against drug-resistant leukemia cell lines (Anu et al. 2020). According to another study, nanoliposomes-based drug delivery system 'Vyxeos' showed better effectiveness in phase 2 clinical trials in comparison to regular cytarabine and daunorubicin regimen as a therapeutic system against acute myeloid leukemia (AML). In 2017, the FDA authorized Vyxeos for the treatment of AML (Chen et al. 2018).

5 Respiratory Disorders

5.1 COPD

Chronic obstructive pulmonary disease (COPD) is marked by airflow obstruction with persistent breathing issues that can lead to death. Inflammatory cells infiltrate the surface epithelium of the central airways, which includes the trachea, bronchi, and bronchioles. Two primary events contribute to the aetiology of COPD: the excessive production of free radicals and subsequent progression of chronic inflammation. Anti-inflammatory, antioxidant, and corticosteroid medicines now utilized to treat these episodes are very risky because of their large dosage requirements and potentially fatal adverse effects. Due to this, nanotechnology based approach has gained attention for the development of new treatments (Saxena et al. 2022). Cerium oxide (CeO_2) nanoparticles can act as antioxidant and anti-genotoxic agents for treating COPD, according to a recent research. Supporting the pharmacological potential of CeO_2-nanoparticles, bronchial epithelial cells pre-treated with CeO_2 nanoparticles demonstrated reduced intracellular ROS and a decrease in DNA damage (Rubio et al. 2015).

Conventional inhalation therapy for COPD has limitations due to inadequate drug penetration through mucosal barriers. Drug bioavailability, transport, and administration can all be enhanced with the incorporation of diverse nanostructures. Dong et al. observed efficient mucus penetration of chitosan-coated polymeric nanoparticles via enhancement of baicalein distribution in airways (Dong et al. 2020a). Another studied has developed inhalable AuNPs that can adhere to the mouse alveolar epithelial cells suffering from COPD (Geiser et al. 2013). The advent of multifunctional nanomaterials, merging medicines and imaging procedures has enabled promising addressal of COPD. In one such study, dual purpose silk fibroin-based carrier nanocomposites effectively reduced oxidative stress while simultaneously imaging the cells (Passi et al. 2020).

5.2 Asthma

Multiple causes, both immunological and non-immune, may trigger or exacerbate asthma, making it a heterogeneous chronic airway inflammatory disease. Combination medication therapy based on glucocorticoids, which suppress pro-inflammatory factor expression, is the standard method of treatment. Asthma therapy research has focused on a wide range of inorganic nanomaterials because of their potential to scavenge ROS, and ease of coupling to the target. A research study found that macrophages carrying AuNPs could travel to the lungs of mice suffering from asthma and induce an anti-inflammatory effect (Kang et al. 2022). Further, Derf2 allergen-loaded hollow mesoporous silica nanoparticles served as an efficient vaccine carrier, thus preventing allergic asthma to a great extent with little adverse effects (Peng et al. 2018). In a study conducted on mast cells of rat models, nanocarriers loaded with organic- chitosan-cyclodextrin carrying heparin intracellularly inhibited IgE-independent histamine release (Oyarzun-Ampuero et al. 2011). Utilization of nanocarriers such as dendrimers for siRNA targeting displayed high efficiency in pulmonary therapeutics delivery (Khan et al. 2015). Furthermore, another study mentions the role of biocompatible fluorescent metal organic framework nanocomposites in detecting low levels of H_2S that is known to be a sign of asthma (Zhang et al. 2019).

5.3 Pneumonia

Over 80% of pneumonia cases are bacterial in origin, with the rest belonging to viral, fungal, and allergic categories. Antibiotics is the primary line of treatment for pneumonia. Nanoparticles based drug delivery systems may be used in systemic and topical medications. Polymeric nanoparticles such as non-toxic and biodegradable poly(lactic-co-glycolic acid (PLGA) are majorly used as nanoagents for mitigating bacterial lung infections. Certain nanometallic compounds are capable of reducing inflammation induced by bacterial infection via elimination of excess ROS. Depletion of H_2O_2 from lungs afflicted with bacteria have been analyzed using metal–organic framework nanosystem (Wu et al. 2021a). Nanotherapy and photothermal therapy combined have proved to be a promising strategy for treating pneumonia (Zhu et al. 2021). Furthermore, various carbon-based nanomaterials and nanocomposites have also proved to be effective against viral pneumonia (Serrano-Aroca et al. 2021).

5.4 Cystic Fibrosis

Cystic fibrosis (CF) is an inherited disorder consisting of a gene mutation coding for CF transmembrane receptor (CFTR) that leads to hypersecretion of a thick mucus in

the lungs and other organs. Airway blockage caused by an over secretion of mucus in the lungs and a change in the mucus's composition and consistency leaves the lungs vulnerable to endobronchial chronic inflammation and recurrent bacterial infections. Nanomedicines have been shown to be a viable therapy option for CF. In 2018, chemically modified CFTR mRNA (cmCFTR) was packaged and delivered using lipid-based NPs (LNPs) for restoring chloride secretion. LNP-delivered cmRNA efficiently translated into a protein that penetrated the cell membrane (Robinson et al. 2018). Inhalation of LNP encapsulating the drugs lumacaftor and ivacaftor, were found to effectively treat pulmonary symptoms of CF, according to another research. This effect was attributed to the nanomaterial that effectively penetrated through the mucosal barrier and delivered the drug effectively (Garbuzenko et al. 2019). Magnetic nanoparticles have been shown to function as nanoknives that convert magnetic energy into heat. This facilitates the piercing of the thick mucus, and delivery of medications to the intended sites (Tan et al. 2020).

6 Ocular Diseases

6.1 Glaucoma

Glaucoma is a series of disorders that cause permanent blindness due to optic nerve damage. Currently, intraocular pressure (IOP) reduction and ocular drug penetration using eye drops are the only proven treatment for glaucoma. However, insufficient reduction of IOP and dry eyes are the limitations of the aforementioned treatments. Recently, few clinical trials have investigated the potential of nanoliposomes in glaucoma treatment. Rabbits given a dorzolamide nanoliposome showed greater IOP decrease than those that were given a marketed formulation (Kouchak et al. 2016). Nanomaterials embedded in contact lenses can be utilized to deliver hydrophilic and hydrophobic drugs without any solubility issues. The possibility of contact lenses with incorporated nanoparticles as a treatment for glaucoma has been the subject of many investigations. Contact lenses with propoxylated glyceryl triacylate nanoparticles delivered timolol effectively for 1 month in beagle dogs (Jung et al. 2013). Until now, tonometers were employed for measuring IOP. In order to avoid visual obstruction and make the wearable sensors more adaptable to a range of eye shapes and curvatures, IOP sensors have been developed using transparent graphene (Zeng et al. 2016).

6.2 Cataract

Glycation, a non-enzymatic interaction between glucose and protein, is the primary driver of cataract development. The build-up of non-transparent aggregates (cataract)

results from misfolding of the crystallin protein assembly due to glycation or other post-translational changes that impair chaperon function. Thus, prevention of glycation of lens can be an effective approach for the reversal of cataracts. Azharuddin et al. demonstrated the role of curcumin nanoparticles in decreasing glycation activity in the presence of high fructose (Azharuddin et al. 2015). Secondary cataract is a common problem arising due to cataract surgery. A comparative study of silica-coated gold nanorods intraocular lens and commercially available intraocular lens revealed a significantly lower occurrence of secondary cataract in the former (Lin et al. 2017). The retina of the eye is exposed to ROS originating from oxidative reactions thus disturbing lens homeostasis and leading to lens opacification. A study reported nanoceria or cerium oxide nanoparticles coated with ethylene glycol with an excellent antioxidant potential that protects lens proteins against glucose-induced glycation (Hanafy et al. 2019). Another research found that freeze-dried liposomes with cytochrome c were more effective in delaying the development and progression of cataracts than only cytochrome c in rats (Zhang et al. 2009).

6.3 Pterygium

Pterygium is one of the most common and serious ocular illnesses. It involves the development of noncancerous fibrovascular conjunctiva on the cornea. Its treatment consists of surgery, argon laser treatment, and use of agents to prevent fibro-vascular growth. Recently, non-toxic curcumin nanoparticles loaded with chitosan grafted with deoxycholic acid treatment inhibited vascular endothelial growth factor (VEGF) mRNA gene expression in retinal pigment epithelial cells (Zheng et al. 2022). Similarly, it has been hypothesized that silicate nanoparticles can also possess anti-VEGF potential (Mohammadpour et al. 2014). Subconjunctival fibrosis (SF) is also an indication of various ocular diseases. Thus, reducing it may prevent the recurrence of pterygium after surgery. Literature has reported the potential of celastrol drug in inhibition of SF. However, its poor solubility in water has led to the use of nanofibrous membrane (Jin et al. 2022).

6.4 Conjunctivitis

Conjunctivitis is the inflammation or infection of the conjunctiva. There are 2 types of conjunctivitis- the infectious type caused by viral and bacterial infection, and the non-infectious type such as allergic conjunctivitis. Rapamycin is a standard therapy for allergic conjunctivitis. Poly (ethylene glycol)-b-poly (epsilon caprolactone) micelles are reported to deliver rapamycin (Forrest et al. 2006). A nanomaterial of polymannuronic acid modified with oleyl amine and containing tacrolimus has been investigated for treating allergic conjunctivitis (Cong et al. 2017).

7 Disease of Immune System

7.1 Arthritis

Arthritis such as osteoarthritis and rheumatoid arthritis is a disorder that causes joint pain, swelling, and stiffness. Due to its limited capacity to absorb systemically delivered medications and its quick clearance post intra-articular injection, the joint presents a formidable obstacle for drug delivery. Nanotechnology has overcome this drug delivery limitation. In a rat arthritis model, folic acid-chitosan-DNA nanoparticles expressing the interleukin-1 receptor antagonist gene prevented bone inflammation (Fernandes et al. 2008). Various nanomaterials have been studied as transporters that would increase the drug retention period within the knee cavity after intra-articular injection (Morgen et al. 2012). Literature suggests that these nanomaterials interact with biomolecules like collagen II, thus facilitating immobilization within the cartilage and prolonged joint retention. Epidermal growth factor receptor (EGFR) signalling is required for cartilage homeostasis and attenuating arthritis. Polymeric micellar nanoparticles conjugated with EGFR ligand have proven to be an effective strategy (Gui et al. 2021). Excess accumulation of ROS or imbalance in cellular redox can lead to cartilage damage, which is a characteristic condition during arthritis. Thus, scavenging ROS by exogenous superoxide dismutase (SOD) has been suggested as a viable therapeutic method. Studies have reported the use of nanomaterials including liposomes, silica nanospheres, polymerosomes, nanotubes, etc. for the effective delivery of SOD (Gui et al. 2022). Treatment of auto-inflammation in rheumatoid arthritis using selenium nanoparticles has demonstrated success in reducing oxidative stress-mediated inflammation (Rehman et al. 2021).

7.2 Aids

AIDS is a debilitating illness of the immune system. Despite a notable decrease in morbidity and death among AIDS patients as a consequence of highly active antiretroviral treatment, AIDS is still incurable since anti-AIDS medications cannot easily penetrate the blood–brain barrier or reach lymphoid organs. Therefore, a reservoir of virus remains unattended which can be linked to viral persistence. Different types of nanomaterials like organic, metallic, metallo-organic, etc. have been studied for their role in transporting anti-retroviral agents across the blood–brain barrier (Sagar et al. 2014). A study reported, excellent drug delivery potential of multi anti-HIV drug loaded lipid nanoparticles in adult male macaques. The nanocarrier not only increased the plasma's residence duration, but it also transported medications to lymph nodes, all throughout the body (Freeling et al. 2015).

7.3 Autoimmune Disease

When peripheral T-cell tolerance fails, it throws the body's immunoregulatory and inflammatory systems in disorder, leading to autoimmune disease. Autoimmune illnesses can be systemic or organ-/tissue-specific. Certain nanomaterials have been created to modify the antigen-presenting cells along with inhibiting the innate immune signals that are involved in adaptive autoimmune responses. Researchers showed that liposomes were efficacious in treating experimental autoimmune encephalomyelitis and multiple sclerosis (Chountoulesi et al. 2020). Furthermore, peptide ligated quantum dots have been shown to promote tolerance against self-reactive antibodies (Hess et al. 2017). A study reported that titanium oxide-based nanomaterials could significantly ameliorate experimental autoimmune encephalomyelitis and arthritis (Latha et al. 2016).

7.4 Disease of Thyroid Gland

The most common diseases of the thyroid gland include hypothyroidism and hyperthyroidism. Hypothyroidism is typically caused by autoimmune thyroiditis, in which the an individual's own immune cells attack the thyroid gland tissues. Thyroid autoimmunity has been linked to the *CTLA4Ig* gene, which codes for a protein that negatively regulates the immunological response of T lymphocytes. A study reported the role of silica nanoparticles coupled with *CTLA4Ig* in downregulating Th1 cytokine in a canine model (Choi et al. 2008). Moreover, nanomaterials have been studied in the detection of thyroid disease. A study reported the use of a biosensor of carbon-based metal–organic framework nanoparticles for determining T3 levels in biological fluids (Sheta et al. 2019).

8 Cardiovascular Diseases

8.1 Ischemic Heart Diseases

The main contributor to ischemic heart disease (IHD) is atherosclerosis, thrombosis, myocardial infarction, and arteriosclerosis. Nanoparticles have been shown to have a huge impact on treating ischemic heart diseases. Polyketal nanoparticles are polymers that can carry SOD 1 enzyme to enhance cardiac function in myocardial infarction (Seshadri et al. 2010). AuNPs have also been exploited for their cardioprotective properties by conjugating them with beta-blockers and other clinically important medicines. For instance, albumin-polyvinyl alcohol AuNPs and AuNPs conjugated with collagen-binding protein 35 were evaluated for improving cardiac restoration, and improving the quality of modality imaging of myocardial infarction (Haba et al.

2021). Many such targeted intramyocardial deliveries of cardioprotective agents such as angiogenic growth factors, thrombolytic agents, ATP-sensitive potassium channels, cardiac stem cell regeneration, atherosclerotic plaque stabilizers, antibodies, and LOX-1 inhibitors among others can be achieved by their conjugation with organic or inorganic nanomaterials with the cardioprotective ability and imaging modality (Galagudza et al. 2010). Further, atherosclerosis and arteriosclerosis are two major risk factors and contributors to IHD. The application of nanoparticles in addressing these effects is discussed in the next two sections.

8.2 Arteriosclerosis

The event of accumulation of LDL cholesterol and macrophages in the arteries is termed arteriosclerosis, and it can progress into lethal cardiovascular diseases causing infarction and stroke. Current approaches practiced for treating arteriosclerosis is the use of fibrinolytic drugs, macrophage remodeling, blood thinners, and drugs along with dietary interventions to control blood pressure, cholesterol, and sugar levels. However, treatment of arteriosclerotic plaques is crucial and is achieved by angioplasty, endarterectomy, stent placement, and in some cases coronary artery bypass graft surgery. However, such surgeries require intensive care and post-operative management. It has thus become important to manage and prevent such arteriosclerotic lesions with therapeutic agents of lesser side effects (Sun et al. 2022b). Nanomedicine is being explored to find biocompatible and therapeutically important nanomaterials that can improve cardiac health and be implicated in the strategic therapy of arteriosclerotic lesions. As mentioned earlier, nanomaterials can be combined with specific molecules, antibodies, and therapeutic drugs to regulate the function of endothelial cellular biomarkers such as integrins, growth factors, adhesion molecules, or macrophages involved in arteriosclerosis, or for targeting smooth muscle cells with pathological phenotype to achieve early detection of IHD. Such conjugated nanoparticles can be utilized to detect and treat vascular endothelial regions affected due to thrombosis, inflammation, hyperlipidemia, oxidative stress, and atherosclerotic plaques, among others (Gupta et al. 2017).

8.3 Atherosclerosis

Nanoparticles have been studied for treating the condition of atherosclerosis and in imaging modalities (Wu et al. 2021b). MRI techniques are mostly used in the diagnosis of atherosclerosis and activating nanoparticles can be used to alter the macrophages with the potential to increase the contrast for MRI imaging (Wickline et al. 2006). Such nanoparticles can transfer a high therapeutic dose to atherosclerotic plaques. They can inhibit the pro-inflammatory action of the macrophages present within the sclerotic lesions in turn stabilize the plaques by reducing inflammation.

Small and ultrasmall superparamagnetic iron oxide nanoparticles and ultra-small $NaGdF_4$ nanodots can be phagocytosed by macrophages, and they can deliver their therapeutic and diagnostic attributes to the inflammatory plaques (Chen et al. 2021). Other targets like the vascular cell adhesion protein 1, HDL, transferrin receptor-1, LyP-1 peptide, and nanoparticles are explored to deliver therapeutic drugs to treat lesions (Chen et al. 2020).

9 Metabolic Disorders

9.1 Diabetes

Chronic increases in blood glucose levels and an inability to maintain their levels in the body results in diabetes. Current treatment options available for diabetes are administration of metformin, insulin injectables, and dietary intervention. Type-1 diabetes is associated with the body's inability to synthesize insulin for glucose uptake from the bloodstream due to autoimmunity of the body towards β-cells of Langerhans. On the other hand, type-2 diabetes occurs because of reduced effectivity of insulin or insulin resistance. Hyperglycaemia can result in urinary tract infections, gangrene, and neurological and cardiac disorders. Three types of detection systems exist for detecting blood glucose levels such as electrical, optical, and magnetic. The incorporation of nanotechnology has increased efficiency, reproducibility, and consistency, and reduced the detection limits to measure minor fluctuation and time required to detect glucose in the bloodstream. Nanomaterials employed for the detection, monitoring, and treatment of diabetes include carbon nanotubes (multi-walled), platinum nanoparticles, gold nanoparticles, single-walled nanotubes, Ag/Au nanoshells, AgNPs, alginate-coated nanoparticles, polymersome nanovesicles, MnO_2 nanoparticles, mesoporous silica nanoparticles among others (DiSanto et al. 2015). Nanomaterials can be employed to provide prevention against pathophysiological complications associated with diabetes such as oxidative stress, inflammatory diseases, nephropathy, neuropathy, and increase in the levels of VEGF which can cause hepatocellular carcinoma, nephropathy, and retinopathy.

9.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is caused due to imbalance in the intestinal microflora. Several challenges are associated with conventional therapies (antibiotics, immunomodulators, antibodies, amino-salicylates) such as side effects including allergy, nausea, or colon delivery of specific drugs. With the advent of nanotechnology, the diagnosis, treatment, and management of IBD can be performed

with tools such as specialized drug (antibiotics, RNA, DNA, genes, vaccines) delivery nanomaterials and diagnosis with an increased resolution of imaging modalities.

Nano-based nutraceuticals can also prove effective for treating IBD, for instance, ginger-derived nanoparticles can show excellent anti-inflammatory activity by lowering pro-inflammatory cytokines along with increase in anti-inflammatory cytokines. Other examples of phytochemicals responsible for treating IBD include curcumin, embelin, zein protein, alkaloids, quercetin, resveratrol, silymarin, tannins, and thymoquinone which can be designed into protein-based nanovesicles, hydrogels, which can improve the drug bioavailability (Barani et al. 2021). Different nanoparticles are now being explored for the treatment of IBD, such as aminosalicylate conjugated nanoparticles (liposome, sodium alginate, silica, SiO_2, ZnO, and PLGA), corticosteroids-conjugated nanomaterials (lipid, carboxymethyl inulin, ascorbyl palmitate hydrogel), immunomodulator associated nanoparticles (PLGA, lipid), among others (Yang et al. 2020). Other reported nanoparticles for drug targeting and IBD treatment include, AuNPs, galactosylated trimethyl chitosan-cysteine nanoparticles, lipid nanospheres, polymeric nanoparticles, silicon dioxide nanoparticles, prohibitin 1-loaded nanoparticles, tripeptide lys-pro-val loaded-nanoparticles, lectin-decorated nanoparticles and magnetic nanoparticles (Nedelcu et al. 2021).

9.3 Hyperlipidemia

Ischemic heart diseases, lifestyle diseases such as obesity and diabetes, atherosclerosis, and arteriosclerosis can be attributed to excessive circulatory lipids in the bloodstream, a condition termed ‘hyperlipidemia’. Common treatment involves the use of statins to lower the blood lipid levels of VLDL and LDL (Zhang et al. 2018). Nanoparticles such as chitosan nanoparticles, solid-lipid nanoparticles with rosuvastatin calcium, lovastatin nanoparticles, oral estradiol nanoparticles, protein-nanoparticle conjugates, amorphous atorvastatin nanoparticles, etc. can be used to boost the efficiency of statin medicines (Sharma et al. 2014). Additionally, nanodots, quantum dots, AgNPs and AuNPs, magnetic nanoparticles, dendrimers, and ceramic nanoparticles have been reported to exhibit dual applications in diagnoses and therapy (Sharma et al. 2014).

10 Tissue Engineering

10.1 Periodontitis

Nanomaterials have revolutionized tissue engineering in dentistry by boosting the clinical potency involved in the partial or total restructuring of dental tissues. Several attributes of nanoparticles are implicated in tissue engineering such as antimicrobial activity, formation of nano-structured surfaces in dental implants, conjugation with therapeutically important materials such as growth factors and stem cells, promotion of remineralization, programmed drug delivery, and disease diagnosis. Nanoparticles studied in periodontitis include amorphous calcium phosphate nanoparticles in the prevention of dental caries, calcium phosphate nanoparticles as dental adhesives, AuNPs for the detection of malignant tumours, peptide amphiphile nanofibers for enamel restoration, and carbon nanotubes for dental bone repair and regeneration. The nanoparticles designed for periodontal tissue repair and regeneration include gelatin nanofibrous membranes, nano-hydroxyapatite, and chitosan composite membrane among others (Chieruzzi et al. 2016). Additionally, zinc oxide, silver and polyethyleneimine nanoparticles in tissue implants, calcium fluoride and calcium carbonate nanoparticles for remineralization, and nanorobotic mouthwashes and toothpaste for preventing calculus accumulation are being explored (Neel et al. 2015).

10.2 Bone Tissue

Due to the unavailability of organ donors, excessive use of immunosuppressive drugs, and high rate of rejection by the host, it has become imperative to find options such as tissue regenerative and engineering techniques to address these challenges. Nanotechnology can support the regeneration and engineering of human tissues due to its ability to increase diagnostic efficiency, targeted delivery, drug modulation, bioavailability, and reduced side effects and toxicity. Nanoceramics (hydroxyapatite, calcium carbonate, phosphate, calcium aluminate, etc.) hold promise in bone-regeneration research. AuNPs are known to induce bone regeneration. They have also been tested for their role in increasing the endothelialization and antithrombotic potential of stem cells (Fathi-Achachelouei et al. 2019). Bioactive glass nanoparticles have been studied in engineering of bone tissue due to their controlled delivery of therapeutic drugs, and increased biocompatibility with the bone tissues. Further carbon nanotubes conjugated with hydroxyapatite has been observed to trigger differentiation of bone stem cells to osteoprogenitors. Magnetic nanoparticles have been indicated to enhance drug delivery and differentiation to mesenchymal stem cells, collagen synthesis, and calcium deposition by osteocytes and osteoblasts, and increasing tensile strength (Walmsley et al. 2011). Thus, nanomaterials can regulate inflammatory responses, promote osteogenesis, and increase the mechanical

strength required for bone reconstruction and proper functioning (Bozorgi et al. 2021). Different types of nanomaterials tested for mesenchymal cell differentiations include dendrimers, chitosan-based nanoparticles, albumin nanoparticles, elastin-like nanoparticles, heparin-conjugated PLGA nanoparticles with BMP-2 plasmid DNA, silver nanoparticles miR-148b (micro RNA for osteogenesis), liposomal with dexamethasone or epidermal growth factors, and dendrimer with dexamethasone (Vieira et al. 2017).

10.3 Regeneration of Nerves

Nanoconduits are a result of tissue engineering and are involved in peripheral nerve regeneration, improvement in the rate of nerve degradation, and increasing mechanical properties of neurons. Due to the nanosize of neurons, nanoparticles can mimic the structures of extracellular matrix, allowing the exchange of gases and nutrients, removal of toxic materials, and promoting axonal growth of the neurons. The biomolecules involved in the engineering of peripheral nerve scaffolds are carbon nanotubes, graphene, and silk fibroin nanofibers. Inorganic nanoparticles are also employed in the regeneration of peripheral nervous system. Cerium oxide nanoparticles improved nerve regeneration and myelin sheath thickness (Javed and Ao 2022). PLGA with encapsulated SDF-1 (stromal cell-derived factor) have been noted to improve neuronal regeneration (Teleanu et al. 2019). Near-infrared irradiation of gold nanorods has been observed to stimulate electrical activity in the spiral ganglion neurons. The plasmon excitatory properties of AuNPs can trigger cell outgrowth in neural cells, and AgNPs can alter neuronal functions and hyper-excitability (Stoddart and Paviolo 2015).

11 Role of Nanomaterials in Diagnosis

Diagnostic tools modernization with nano-compounds has opened new avenues for real-time monitoring of diseases, decreased the time required, and increased the sensitivity of such diagnostic tools by multiple folds. Several such arenas of diagnostic tools have been revolutionized due to nanomaterials such as the detection of harmful pathogenic microorganisms or viruses, imaging tools such as MRI, PET, CT, and PA techniques, spectroscopic and infrared techniques, and DNA tagging or nanoELISA methods.

11.1 Detection of Pathogens

Fluorescence imaging technology, based on fluorescent materials, is used in real-time monitoring of biological processes and interactions (Chepurna et al. 2020). However, studies suggest that the organic photosensitizers used for detection are often inefficient due to their biological toxicity, photo instability, insufficient selectivity, and low absorptivity (Wang et al. 2018). Conjugated polymer nanomaterials show wide applications in photodynamic and photothermal therapy, biosensing, and imaging as they have stronger light absorption capacity, and photostability.

Inorganic nanomaterials are ideal candidates for the detection of pathogens by virtue of their unique optical properties including high photoluminescence quantum, high light absorption capacity, ability to increase Raman spectra, and photostability (Huang et al. 2022). AuNPs have been employed for identifying pathogens like *Listeria monocytogenes*, *E. coli*, and viruses that cause food poisoning. AuNPs coated with monoclonal antibodies can detect and attach to specific bacteria and can be detected via dynamic light scattering techniques. Vancomycin-coated AuNPs can bind and retain only live bacteria and detect antibiotic-resistant pathogens in the joint fluids. UV–visible spectroscopic revelations can be used to detect a shift in absorption pattern due to the interaction between AuNPs and sialic acid present on the influenza virus. (Giner-Casares et al. 2016).

Magnetic nanoparticles, derivatives of iron salts, have widespread applications in pathogen identification. Magnetic nanoparticles are involved in the detection of *S. aureus*, *S. saprophyticus, E. coli, B. cereus,* and *S. typhi* (Daramola et al. 2020). This interaction can be increased by conjugating magnetic nanoparticles to several components such as antibodies, antibiotics, oligonucleotide probes, and chitosan or increasing their peroxidase-like activity (Ali et al. 2021).

Titanium oxide nanoparticles display wide band gap semiconductor properties. Due to this, they have been employed for the detection of *Salmonella* antigens. These biosensors are quick, efficient, and have sensitivity as low as $10^{3–5}$ cells/ml (Viter et al. 2017). MALDI-MS detection of *Staphylococcus aureus* from direct nasal skin samples was facilitated by TiO_2 nanoparticles without any sample pre-treatment or initial culturing steps. This reliability is now being extended to differentiate between pathogenic and non-pathogenic strains with the aid of conjugates such as antibiotics particularly methicillin (Gopal et al. 2011).

SiO_2/Fe_3O_4 nanoparticles have been used in polymerase chain reaction (PCR) of DNA fragments of *S-gene* in HBV and nuclear antigen coding gene in EBV. The nanoparticles employed in the detection was found to increase the sensitivity and band intensity after the PCR reaction (Quy et al. 2013). Silica nanoparticles coupled with fluorescein isothiocyanate and ruthenium were found to efficiently detect *Salmonella species* and *Escherichia coli.* The samples required no former processing and a bacterial load as low as 10^5 cells/ml could be detected (Rajendran et al. 2014). Noble metal gold, silver, and platinum nanoparticle-hybrid nanocomposites were prepared by even distribution on the surface of laser-induced three-dimensional porous graphene (LIG). The AuNPs-LIG interdigitated array electrode

showed excellent performance as compared to others. No significant impendence was found against non-target bacteria such as *S. aureus, V. parahelminthis,* and *L. monocytogenes* suggesting the relatively higher sensitivity of the biosensor (You et al. 2020).

11.2 Role in Imaging

11.2.1 Magnetic Resonance Imaging

MRI, a minimally invasive, flexible visualization modality, allows exquisite spatial resolution (Blamire 2008). Their specificity can be amplified by using proper biochemical and cellular markers in combination with nanomaterials with excellent optical properties (Blasiak et al. 2013). For instance, iron-based nanoparticles can allow early detection of liver cancer by improving the sensitivity of MRI because of the accumulation of iron in the liver due to hepatobiliary action. MRI specificity can also be increased by conjugating the nanoparticles with antibodies specific to the overexpressed domains of the cancer cells such as growth factor receptors, secreted clustrins, or which are common receptors in the cancerous cells (Blasiak et al. 2013). Polymer-coated magnetic nanoparticles have been employed to provide an MRI contrast and effective delivery of the anticancer drug epirubicin for brain cancer (Liu et al. 2010). Iron oxide nanoparticles were amongst the very first to be applied in the MRI imaging for diagnosing atherosclerosis, tumors, and cardiovascular diseases (Rümenapp et al. 2012). Magnetic nanoparticles are also employed for theranostic purposes (Yoo et al. 2011). Carboxyl coated Fe_3O_4 nanoparticles are reported to enhance MRI imaging (Barick et al. 2014). Other reported nanoparticles like cobalt, gold, and platinum have found to display use in MRI imaging for the diagnosis of cancers (Blasiak et al. 2013). Gadolinium functionalized with carbon nanotubes also serves as an excellent contrasting agent for MRI (Marangon et al. 2014). Single or multiwalled carbon nanotubes serve as MRI contrast on account of their exceptional biocompatibility, magnetic and electronic properties, and cell-penetrating ability. Cobalt ferrite conjugates produce good MRI contrast and treat aggressive cancers (Wu et al. 2011a). Thus, MRI imaging could be optimized with the help of suitable nanomaterials to achieve high image resolution.

11.2.2 Computed Tomography

Nanoparticles with a high ability to absorb X-rays have been gaining attention in the field of diagnostics such as computed tomography (CT) scanning. It is of extreme importance to select a proper candidate for low toxicity, and good biocompatibility to improve CT imaging (Li et al. 2017). AuNPs have been analyzed for detecting hyperacute direct thrombus imaging using CT (Kim et al. 2013). Graphene oxide/AgNPs have been utilized as contrast agents in CT scan *in-vivo* for monitoring

kidney disease (Li et al. 2017). Dendrimer-entrapped AuNPs facilitate excellent CT scans of human lung adenocarcinoma cell line and xenografts post intratumoural and intraperitonial administrations (Wang et al. 2011). Metal hybrid nanomaterials such as $FeSe_2$–Bi_2Se_3–^{64}Cu, Fe_3O_4@Au, ION@Bi_2S_3, Au@Fe_3O_4, ION-^{89}Zr, Fe_3O_4–Au have also been utilized in CT scans along with MRI and PET imaging (Tian et al. 2018). AuNPs play an excellent role in the CT imaging of breast cancers conjugated to an anti-her2 antibody, prostate (bombesin), small-lung carcinoma (bombesin), head and neck cancer (anti-EFGR antibody), unstable macrophage plaques (HDL), among others. Passive targeting agents reach the site of diseases via reticuloendothelial systems, for e.g. bismuth sulfide, tantalum, and gold for lymph nodes, gold for liver tumours, iodine for lungs, iodine-liposome for breast cancer, among others. Nanoparticles with iodine, gold, tantalum, and bismuth can highlight blood vessels and increase the quality of CT images. This highlights the role of nanotechnology in CT in addition to its conventional imaging potential and delves into molecular targeting (Shilo et al. 2012).

11.2.3 Photoacoustic Imaging

In-vivo and *in-vitro* photoacoustic (PA) imaging is a far better technique than ultrasound imaging, that obtains images with high special resolution (5 μm and deep as 5–6 cm) (Li and Chen 2015). Additionally, associated techniques such as flow cytometry (FC) with PA allow the detection of circulating absorbing objects in direct subjects (Zharov et al. 2007). Since nanomaterials are known to have excellent physical properties including amplification and shifting tunable near-infrared pulses, they are regarded as important for detecting pathogens and diseases. Nanotechnology has increased the PA potential by enabling ultrasensitive detection and monitoring of stem circulatory tumour cells (CTCs) (Galanzha and Zharov 2013). Carbon nanotubes are utilized to get PA signals from strong tissues such as the tibia of mice and hence have applications in the early monitoring, progression, recurrence, and evaluation of therapy of bone-tissue cancer. Intravascular ultrasound and photoacoustic imaging of gold nanoshell-labeled inflammatory markers have been shown to facilitate the imaging of phagocytically active macrophages, E-selectin, and other biological compounds involved in atherosclerotic plaques (Li and Chen 2015). AuNP-based PA imaging also has applications in brain vasculature imaging. AuNPs along with PA imaging also have promising potential in real-time targeting and tracking the transport of recombinant human TNF-α and silicon phthalocyanine (Paciotti et al. 2004). Single-walled carbon nanotubes combined with polyethylene glycol in PA imaging enable early detection of glioblastoma tumours (Xiang et al. 2009).

11.2.4 Positron Emission Tomography (PET)

Radioactive nanoparticles may be used for molecular imaging for detecting cancer in early stages. PET-image technology is another important modulating imaging that

can be utilized in this decision-making strategy for treating cancer. The utilization of radioactive nanoparticles for PET is rather beneficial due to their electronic properties that can detect cancers in a target-specific manner. For instance, AuNPs coupled with ^{64}Cu can be utilized for the diagnosis of kidney diseases after conjugating with a specific protein target using PET imaging (Chen et al. 2016). Mesoporous silica nanoparticles display a promising role in the detection and photodynamic therapy of advanced cancers with monitoring using the PET technique (Xu et al. 2019). Different radioactive nanoparticles are used for detecting cancers *in-vitro* using the PET technique, for instance, quantum dots (prostate cancer PC3), AuNPs (glioblastoma U87MG), MSN (murine breast cancer 4T1), iron oxide nanoparticles (glioblastoma U87MG), upconversion nanoparticles (glioblastoma U87MG), CuS nanoparticles (keratin-forming tumour cells), and Au-iron oxide nanostructures (epidermoid cancer 4531) (Chakravarty et al. 2017). Human clinical trials for detecting melanoma metastasis have been conducted for determining the possibility of using nanoparticles (silica-based PEG-modified linked to a peptide moiety) as radioactive elements for PET scans. The radiolabelled nanoparticle displayed good compatibility, no effective toxicity, and produced reproducible pharmacokinetics, as well as renal excretion (Phillips et al. 2014).

12 Manipulation of Cells and DNA Tagging

Manipulation of macrophages with nanoparticles (Au, Ag, Fe, human serum albumin nanoparticles, polymer lipidoid hybrid nanoparticles, nanostructured lipid carriers, and magnetic fibrin nanoparticles, among others) can detect and treat RA (Li et al. 2021). Along with detection, these nanoparticles can be conjugated to herbal and conventional drugs that can regulate inflammation and can be employed in the treatment of RA. Quantum dots can interact with several clinically relevant materials such as proteases, thrombin, collagenase, caspase-1, and chymotrypsin, and can serve as intriguing diagnostic agents (Blum et al. 2015). DNA nanomachines have been used for identifying cancer biomarkers such as telomerase, circulating tumour DNA and adenosine triphosphate. Methylene-blue-tagged single stranded DNA immobilized on AuNPs have been used to detect targeted circulating tumour DNA to monitor the early progression of non-tumourigenic or tumourigenic cancer cells by employing a paper-based strip electrochemical detection system (Cinti et al. 2018). Fluorescence resonance energy transfer (FRET) is employed in the engineering of DNA nanomachines and certain nanoparticles such as upconversion, carbon dots, and quantum dots are being explored for enhancing the stability and brightness of the generated fluorescence (Dong et al. 2022b).

13 Dual Nanotools for Diagnostics and Therapeutics

Dual-applicability nanoparticles, also called theranostic nanoparticles, are designed for disease-specific therapeutic management with constant diagnostic monitoring. The pre-requisite of such nanoparticles is safety, non-toxic nature, specificity, and efficient drug without affecting organ functions. One way of developing theranostic nanoparticles is to attach nanomaterials to conjugates such as drugs, antibodies, HDL, growth factors, cytokines, and hormones of interest, and a contrasting agent for real-time monitoring of diseases and treatment. Even after facing challenges for its application in drug delivery, several theranostic nanoparticles such as AuNPs, silica nanoparticles, and gold–silica nanomaterials are now approved by FDA for conducting clinical trials. Two mechanisms govern the uptake of such theranostic nanoparticles by the body. First is through passive uptake, where permeability and leakages due to a diseased state allow the accumulation of such compounds, and second is active uptake which involves conjugation with biomolecules for targeted delivery. Active theranostic nanoparticles have a better chance of being utilized as therapeutic and diagnostic aids than passive ones since the physiology and behaviour of such passive nanoparticles may alter according to individuals (Chen et al. 2014). For instance, several nanoparticles-biosensors are now being tested based on their ability to detect blood glucose and alter the insulin dosage to be given (DiSanto et al. 2015). Similarly, carbon nanotubes, fullerenes, nanodots, graphene, and nanodiamonds are being tested for their dual applicability for the treatment of cancers since they can be tuned to optimize deep-tissue near infrared (NIR) penetrations, fluorescence emissions, and strong photoacoustic efficiency to give better diagnostic insights into drug delivery and progress of treatment. Iron oxide nanoparticles coated with chitosan, PLGA, dextran, and PVA provide an amphiphilic microenvironment to theranostic nanoparticles for enhancing their stability and bio-absorption (Sharma et al. 2021). Nanomaterials have also been used to deliver chemotherapeutic drugs viz. paclitaxel and doxorubicin. For instance, paclitaxel and Bcl-2 targeting siRNA in solid lipid nanoparticles for treating human lung carcinoma resulted in promising detection and anticancer activity (Barros and Soares 2014). Theranostic nanoparticles of cardiovascular diseases include protease-mediated theranostic nanoparticles for atherosclerotic lesions, gold nanorods for the removal of inflammatory macrophages, HDL-like magnetic nanostructures for treating hyperlipidemia, and solid-lipid nanoparticles against platelet aggregation. Nanotheranostic tools such as Mn-doped ZnO–NPs, paramagnetic gadolinium (Gd^{3+}), AuNPs, carbon nanodots, polymeric nanoparticles, nanocomposites and lipid-based nanoparticles are being explored for addressing myocardial infarcts and coronary artery blocks (Pala et al. 2021). Inorganic theranostic nanoparticles, mesoporous silica doped with gadolinium have been used in detection methods such as MRI, ultra-sound PA, and PA microscopy, fluorescence, and MRI (Vieira et al. 2017).

14 Nanomaterials-Drug Delivery and Bioavailability

Drug delivery to target organs and avoiding delivery and unintended harm to non-target organs requires lower drug dosages. Polymeric, liposomal, and solid-lipid carrier nanoparticles are currently utilized for delivering drugs and treating diseases such as cancer, cardiac, metabolic, microbial, and respiratory among others (Tewabe et al. 2021). Inorganic and organic nanoparticles can also be conjugated to drugs to track the treatment progress. Use of PEGlyated liposomes with doxorubicin for treating Kaposi's sarcoma and PEG-loaded with L-asparagine for treating acute lymphoblastic leukemia have been reported (Pardhiya and Paulraj 2016). Magnetic nanoparticles with natural and synthetic polymeric coating for targeting deliveries such as dextran, polyvinyl alcohol, and starch have been shown to facilitate drug transport to target site (McBain et al. 2008). Polymer–polymer micellar (PEG-PLGA, PEG-PLA), polymer-lipid micellar (PEG-b-PHSA), non-micellar polymeric (branched PEG, PLGA), and liposomal (PEG-liposome, transferrin-conjugated PEG liposome) nanoparticles have also been utilized as carriers of medicines such as paclitaxel, doxorubicin, vincristine, cisplatin, etc. in the treatment of breast cancers (Lee and Nan 2012). Nanoparticles enable targeted delivery of drugs and real-time monitoring of cardiovascular diseases such as atherosclerosis. For instance, drug deliveries through PLGA-coated polymeric nanosystem for decreased necrosis in the atherosclerotic plaques, liposomal nanoparticles to increase the size of the infarct, p-selectin conjugated liposomal nanoparticles for increasing the arterial density and reducing infarct size, and superparamagnetic iron oxide nanoparticles for treating infarction and atherosclerotic lesions are some of the applications (Mittal et al. 2018). Microbial infections have also been mitigated through nanodrug delivery, for e.g. use of liposomal nanoparticles conjugated with antimicrobial agents such as gentamicin for *Klebsiella pneumoniae,* streptomycin for *Mycobacterium ovium* and vancomycin and teicoplanin for methicillin-resistant *S. aureus* strains. Additional examples of nanomaterials designed for antimicrobial drug delivery include polymeric nanoparticles conjugated with rifampicin against *Mycobacterium tuberculosis*, PEG-PLA nanoparticles conjugated with halofantrine against *Plasmodium berghe*, glycerol palmitostearate with econazole nitrate for treating fungal infections (Zhang et al. 2010). Nanoparticles-based glucose delivery systems with alginate, chitosan, dextran, PLGA, and solid lipid nanoparticles conjugated to insulin have also been administered orally or through pulmonary routes for type 1- and, in extreme cases, type 2-diabetes (Sharma et al. 2015). Thus, based on the elaborate research studies on nanodrug delivery systems, it can be concluded that nanomaterials hold immense potential as next-generation treatment options (Ibarra-Sánchez et al. 2022).

15 Environmental Considerations

One vital aspect of nanomedicine that cannot be ignored is its impact on the environment. Presently, numerous monitoring studies have proven that the residues of conventional pharmaceutical products are eventually traced in the environment (Baun and Hensen 2008). There is a possibility of nanomaterials that offer an advantage over conventional medicines of having the same fate. As mentioned in the previous section, nanocarrier systems are an attractive prospect since they offer high bioavailability and targeted drug delivery. Nanomaterials can escape biological barriers and release into the environment. This in turn can produce undesirable effects on non-target organisms. Limited research studies have conducted ecotoxicological studies using actual nanomedicine formulations. Nanomaterials can non-specifically interact with environmental pollutants and subsequently enhance their bioaccumulation and toxicity (Baun and Hensen 2008). The environmental risk of AuNP-based nanomedicine on surface waters is reported to be minimal (Mahapatra and Sun 2015). The present preclinical research involves extensive *in-vitro* and *in-vivo* studies for eliminating any possible hazards of nanomedicines to humans. However, it is necessary to broaden these studies and include analysis that determines their environmental fate and effects. The Department of Biotechnology, Government of India, has laid down valuable guidelines for evaluating nanopharmaceuticals. These guidelines are applicable to finished nanomedicine formulations or active pharmaceutical ingredients of nanoscale dimensions. It is stated in the guidelines that the impact of non-biodegradable nanopharmaceuticals and their waste disposal on the environment should be declared. The established safety guidelines for nanomaterials in medicine are expected to set a precedent for other domains where nanomaterials hold immense promise. The evaluation of acute and chronic toxicity of nanomaterials in the ecosystem should be an integral part of nanopharmaceutical evaluation and, should be considered while regulating their commercial launch as a form of medicine.

16 Future Scope of Nanomedicine

Presently, there is a resurgent interest in the fabrication of new nanomedicines owing to the tremendous promise and scope they display in all branches of medicine. Nanomaterials have been exhaustively explored for discovering more effective substitutes for the currently used conventional pharmaceuticals. With the present advances, nanomaterials will soon become an integral part of day-to-day therapeutics. However, while we consider the positive effects of these nanomedicines, the flipside i.e. the possible negative repercussions of the same cannot be ignored. A cautious approach is warranted for ascertaining their safe application on humans. Based on the present studies, future research should evaluate the following possibilities:

- Development of reliable, rapid, and cost-effective multiplex diagnostic methods that will enable the treatment of broad spectrum of diseases.
- Improving the biocompatibility and specificity of nanoparticles for drug delivery.
- Study of interactions between nanoparticles and the immune system which will enable their use as immunostimulants and enhance their function in antigen presentation and processing of pathogens.
- Commercial manufacturing of nanomaterial-based formulations that will effectively attack and curb the infections caused by multi-drug resistant organisms.
- Refining the technology involved in nanorobotics. This will in turn aid in performing surgeries with high precision and specificity. It is proposed that such nanobots, in the future, will independently move, locate, remove obstructions and even kill cancer cells (Baudrit et al. 2017).
- Thorough evaluation of the toxicities of nanomaterials on humans.
- Investigation of any deleterious effects of nanomedicines on the environment.
- A more stringent framework of regulatory regimes for introducing nanomedicines into the pharmaceutical market.

Considering the rate of advent and applications of nanomaterials in medicine, it is quite evident that these nanosystems will revolutionize the field of medicine.

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Biomedical Applications of Nanofluids in Drug Delivery

Debojyoti Mandal, Keshav Anand, and Pranav Kumar Prabhakar

Abstract In the past few years, clinical studies and scientific research projects have found a wide range of applications for infusions of various nanoparticles or nanocomposites. Emulsions or dispersion of nanomaterials in liquid are known as nanofluids, and they can be utilized to improve heat exchange directly in a wide range of industrial purposes, including heat transfer, transit, semiconductors, healthcare, and the food sector. Nanofluids are these useful formulations that enable the dispersion and action of nanoparticles in uniform and stable environments. The benefits of nanofluids in biological techniques in various sectors have been covered in several research. Few review studies that provide an overview of the several biomedical uses for nanofluids, including both diagnosis and treatment, have been documented. In comparison to their solid counterparts, nanofluids' physicochemical qualities alter significantly due to the growing interest in using them in nanomedical applications. Nanofluids have recently been used in biomedical activities, including drug transport and antimicrobial treatments. The paper's primary focus is on nanosuspensions, which are nanofluids that contain solid particles. The primary class of nanofluids, nanosuspension, is the subject of most applications. Therefore, this study provides comprehensive information on the main biological uses of nanofluids in scanning, antimicrobial properties, and drug-delivery systems. Magnetic nanofluid systems are essential for targeted medication delivery, hyperthermia, and differential diagnosis. Additionally, the use of nanofluids as a potential antibacterial agent to combat antibiotic resistance is a possibility. This study might help outline cutting-edge, practical strategies for achieving success in modern medical practice.

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1 Introduction

Nanofluid, a suspension of nanoparticles, shows promise as a fluid used for enhancing heat transfer and finding numerous other uses because of its high ability to conduct heat and unique rheological properties (Mashali et al. 2019). To define this emerging area of nanotechnology-focused field thermal systems with thermodynamic properties superlative to that of conventional particle fluid suspensions, The term "nanofluids" (which refers to suspensions of fluid containing nanoparticles) was introduced by Choi in 1995 (Jawaid et al. 2019; Lee and Choi 1996). In the last few years, there has been notable advancement in the field of nanofluid technology, a new and highly significant interdisciplinary field at the intersection of nanotechnology, nanomaterials, and thermal engineering (Yu and Xie 2012). To create a nanofluid, a small quantity of nanoparticles are dispersed in a base fluid such as water, ethylene glycol, etc., either with or without methods to stabilize the mixture. Nanofluids aim to achieve high thermal properties at low levels (about 1%volume) by dispersing as well as suspending nanoparticles (around 10 nm in size) in host fluid (Ebrahimi et al. 2010).

Nanoparticles, or particles with size and shape on the nanometre scale, can be found in a fluid known as a nanofluid. These materials are composed of nanoparticles that are suspended in a base fluid (common base fluids include water, ethylene glycol, and oil) that have been engineered to have the properties of a colloidal suspension (Ebrahimi et al. 2010; Sharma et al. 2011). The most common types of nanoparticle materials that are utilized in nanofluids include metals, oxides, & carbon nanotubes. Earlier research has indicated that the thermo-physical characteristics of nanofluids, including electrical properties, thermal diffusivity, rheology, and convection thermal transfer coefficients, are drastically enhanced when compared to the base fluids like water or oil (Duangthongsuk and Wongwises 2008). Nanofluids offer numerous potential applications in heat transfer, which include: microfluidics, fuel cells, pharmaceutical operations, hybrid-powered engines, home refrigerators, cooling systems, convective heat transfer, nuclear reactor coolant, grinding, machine tools, space science, defence, vessels, as well as the reduction of boiler flue gas temperature (Choi 2009; Liu et al. 2005).

Dispersions of nano-sized particles are very different from nanofluids in several ways. Nanofluids have higher thermal conductivities than more common cooling liquids like water, kerosene, ethylene glycol, and microfluids (Kamel et al. 2016). So, nanofluids can be used in applications that involve heat exchange because they don't get in the way of flows and don't cause much of a pressure drop as they move. There is a wealth of information on the preparation, characterization, and stabilisation of nanofluids because this area has previously been part of colloidal science (Yu and Xie 2012).Nanofluids are much more stable than microfluids for implementations where

heat is transferred because of the robust Brownian motion of nanosized particles suspended in base fluids. This is one of the obvious benefits of using nanofluids over that of the microfluid (Arulprakasajothi et al. 2010). Nanofluids are promising coolants due to their high critical heat flux, high thermal conductivity, and high heat transfer capabilities. Research has shown that the addition of even a small quantity of nanoparticles to a base fluid significantly improves its thermal efficiency. Extreme stability as well as significant thermal conductivity are two crucial characteristics for heat transfer systems (Keblinski et al. 2005).

Very few review articles focus solely on the potential medical uses of nanofluids. Some papers have provided an overview of the many applications of nanofluids across many disciplines. On the topic of the medical uses of nanofluids, some authors have included a few paragraphs (Saidur et al. 2011). Another review article exists, but it does not focus on the medical uses (Choi 2009). In keeping with the earlier-discussed definition of a nanofluid, this article explores the use of nanofluids in delivery of drugs, antimicrobial therapeutic interventions as well as medical applications. In this following article, a comprehensive survey of the procedures as well as implementations of nanofluid in a wide variety of biomedical fields is presented. In these types of reviews, one of the topics that is discussed in a condensed and overarching manner is the various biomedical applications (Sheikhpour et al. 2020).

2 Nanofluid Preparation

Nanofluid preparation involves more than just adding nanoparticles to a solvent. Stabilization and adequate mixing are prerequisites for producing nanofluids with uniformly dispersed nanoparticles in specific environmental settings. There are many different ways to make nanofluids, but the two most common are:

2.1 One Step Method

To avoid the hassle of dehydration, storage, transporting, and distributing nanoparticles, this method uses a different technique. The Physical Vapor Deposition (PVD) method, which involves the specific condensation as well as humidification of nanoparticles within the base fluid, is used to create a stable nanofluid (Choi 2009; Rafiq et al. 2021). This technique yields nanoparticles that are both pure and uniform in size. As a result, less nanoparticle accumulation occurs. Two major drawbacks of the one-step approach are its high price and the fact that it leaves behind reactants in the nanofluids (Ali and Salam 2020; Rafiq et al. 2021). The particles, which can be broken down to agglomerations of nanomaterials, are created and dispersed inside the base fluids all in a single step (Fig. 1). Though the method's high price tag is a drawback, it does increase nanofluid stability (Fig. 1).

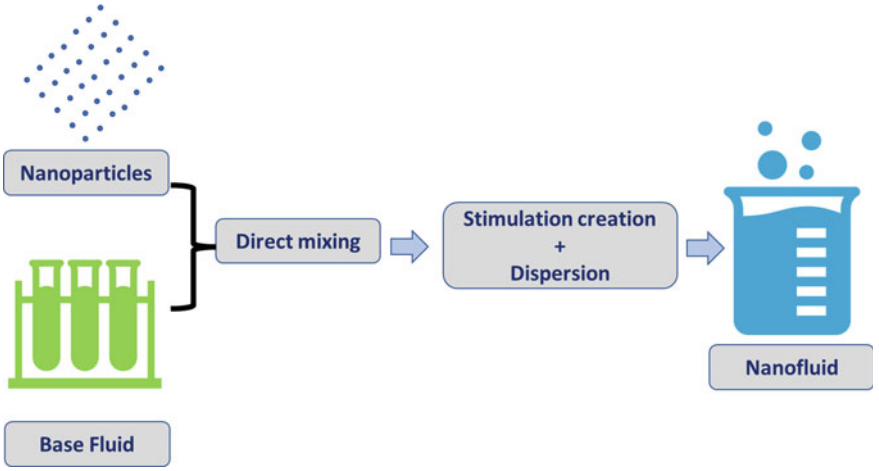

Fig. 1 Method of one-step technique to produce nanofluid

2.2 Two-Step Method

This method is the most efficient and economical way to get ready for nanofluid production on a large scale. In this method, the nanomaterials are first obtained through a variety of processes; once they have been obtained, the nanoparticles are then circulated throughout the main solvent i.e. base fluid to create the desired nanofluid (Sanjeevi and Loganathan 2020). This method of production is both low-cost and highly efficient. The aggregation of nanoparticles is the most significant drawback of the two-step method (Ali and Salam 2020). Surfactant is added because the substance is unstable. This is the standard procedure used in the commercial world to prepare nanofluid. When it comes to the preparation of nanofluid for research, the vast majority of scientists favour using this method. The most common approach to preparing nanofluids entails a two-stage process (Ali and Salam 2020; Yu and Xie 2012). Dry powders of nanoparticles, nanofibers, other nanomaterials are initially manufactured using chemical or physical processes and then used in this technique. The second stage of processing entails incorporating the nano powder into a fluid with the aid of high-shear mixing, homogenization, ball milling, and ultrasound agitation. Those are the five different types of agitation (Bairwa et al. 2015; Talaei et al. 2011).

The two-stage process, in contrast to the simpler one-stage method, is the standard in industry for mass-producing nanofluids (Paul et al. 2011; Sajid and Ali 2018).The first step of the two-stage procedure involves producing the nanoparticles through a number of synthetic processes; then, after being distributed into the base fluid, the nanoparticles are collected. Figure 2 is a schematic diagram that illustrates the two-stage approach for creating nanofluids. The vast majority of the researchers prepared the nanofluid using this technique (Esfe et al. 2015; Geng et al. 2020).

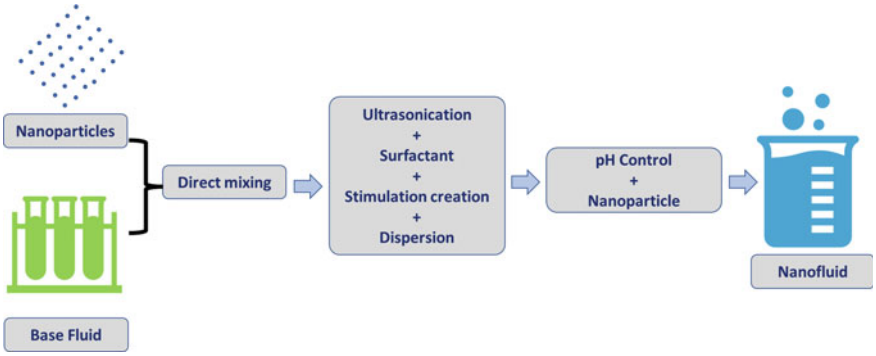

Fig. 2 Method of two-step technique to produce nanofluid

3 Characterization of Nanofluids

Scanning electron microscopy,"transmission electron microscopy, x-ray diffraction, dynamic light scattering, thermogravimetric analysis, and zeta potential"are among the techniques used for the assessment in the process of characterising nanofluids (Hernaiz et al. 2019; Kolappan et al. 2020; Otanicar et al. 2010; Vonarbourg et al. 2006).

- The DLS analysis calculates the average size of nanoparticles that are dispersed throughout the base liquid.
- The thermogravimetric analysis (TGA) is used to study how melting and heating affect the thermoelectric durability of nanoparticles.
- The value of the zeta potential is closely linked to the consistency with which nanoparticles are spread out in base fluid.

4 Advantages of Nanofluid

The incorporation of nanoparticles into the system even a minute volume fraction results in a significant improvement in heat transfer. Researchers are still looking into the benefits of using nanofluids and the factors that contribute to the improvement in heat transfer (Hernaiz et al. 2019; Sharma et al. 2022; Yu et al. 2010).

The following list provides examples of some of the benefits:

- The fluid's effective thermal conductivity is enhanced thanks to the nanomaterials which are distributed throughout it. The nanoparticle volume fraction has a direct bearing on the effective thermal conductivity of a material. It rises in proportion to the increase in the nanoparticles per volume that are present (Xie et al. 2011; Yu et al. 2010).

- More heat can be transferred from the particles to the fluid because of the larger specific surface area (Xie et al. 2011).
- When compared with traditional fluids, reduced particle clogging promotes system miniaturisation by reducing the likelihood of clogging (Otanicar et al. 2010).
- A higher degree of dispersion stability combined with a predominately Brownian motion of the particles. Dispersed nanoparticles, on the other hand, amplify the turbulence and the mixing fluctuation (Reddy and Murugesan 2017).
- Adjustable properties, such as heat capability and surface hydrophilicity, can be achieved by adjusting the fragment concentration in order to satisfy the requirements of a variety of applications (Arulprakasajothi et al. 2010).
- Power required to pump the mixture is less than that required for the pure liquid to achieve the same level of heat transfer intensification (Arulprakasajothi et al. 2010).

5 Application in the Biomedical Field (Fig. 3)

5.1 Cancer Treatment

In the arena of cancer treatment and drug carriers, there is a new effort that makes use of several attributes of specific nanofluids (Gavas et al. 2021). As part of this initiative, nanoparticles based on iron will be tested in cancer patients to see how well they work as drug carriers for drugs or radiation. The plan is to use magnetic nanofluids to transport the particles through the circulatory system and into the tumour (Tang et al. 2020). As a result, doctors will be able to treat cancer with relatively high doses of medication or rays without harming surrounding healthy cells, which is a common drawback of current treatments (Wong and Leon 2017).

Further, in human-safe alternating current magnetic fields, magnetic nanoparticle adheres more strongly to nanoparticles absorb more energy than microparticles and have a greater affinity for cancer cells than normal cells. When compared to other nanoparticles made of metal, magnetic ones have a unique property that makes them ideal for manipulating nanofluids using only magnetic fields. In an interchanging electromagnetic force, magnetic nanoparticle-laden nanofluid functions as a superparamagnetic fluid, absorbing energy to generate hyperthermia that can be modulated. Since hyperthermia selectively kills cancer cells, it can boost the efficacy of chemotherapy (Chiang et al. 2007).

Mekheimer et al. had designed nanofluid baes on gold nanoparticle with the intention of researching the consequences of heat transmission with blood circulation comprising gold nanoparticle in a space between the two coaxial tubes. In the end, they came to the conclusion that gold nanoparticles increase the temperature distribution, which makes it capable of destroying cancer cells (Mekheimer et al. 2018).

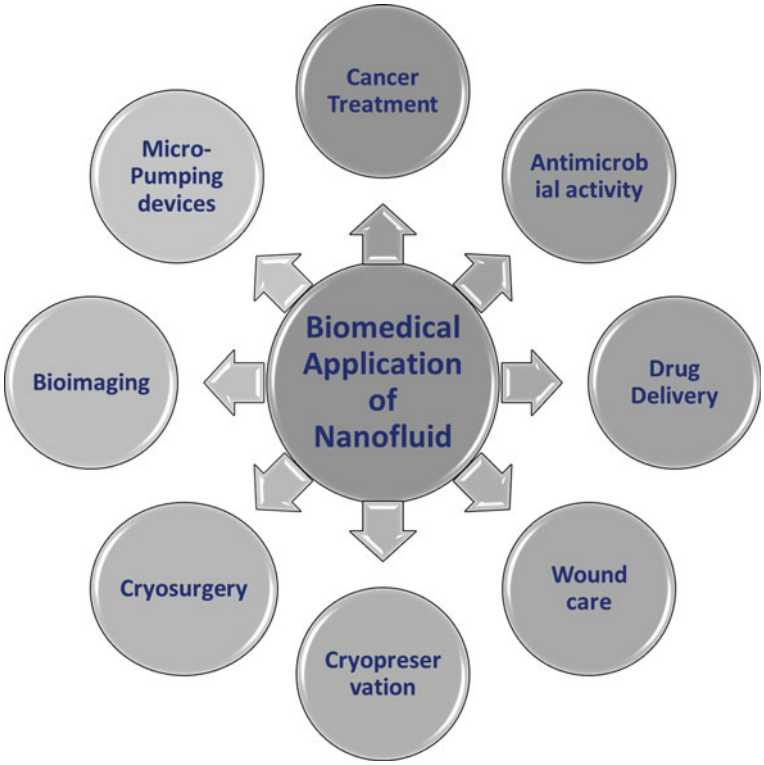

Fig. 3 Biomedical applications of nanofluids

Magnetic nanoparticles have been implanted into a bloodstream leading directly to the tumour's tissues. Peristaltic waves acting on the blood vessel's elastic walls cause the nanoparticles to move (Das et al. 2022). As a result, studying the flow of nanofluids under such conditions can notify the development of effective new therapies for cancerous tissues. Abdelhalim et al. mathematically modelled the slip peristaltic flow of nanofluid, and performed an analytical study of its properties. Their research provides some insight on the nano—fluids dynamics that are utilised in the clinical field that treat the cancerous tissues by using magnetic nanoparticles while the blood vessels are being peristaltic (Ebaid and Aly 2013).

The treatment of breast cancer with zinc oxide nanoparticles was the subject of research conducted by Abdolmohammadi and colleagues. The results of an investigation into cytotoxicity have been compiled. According to the findings of the in vitro study, ZnO NPs are non-toxic hold promise as potential cancer chemotherapy agents (Abdolmohammadi et al. 2017).

Sundar et al. (2019) report medical applications for cobalt oxide (Co_3O_4) and its composites. Cobalt compounds are safe to use in medicine because of its magnetic

properties. Magnetic particles can be used to track down tumours, and cobalt composites have proven to be the most effective material for this purpose.

There have been a number of other studies done on nanofluids for the treatment of cancer, and these studies show that the potential effect has room for further application (Ebaid and Aly 2013; Su et al. 2011).

5.2 Antimicrobial Activity

Organic antibacterial substances are typically less stable than their inorganic counterparts, particularly when exposed to extreme temperatures or increased pressure (Cloutier et al. 2015). As a direct result of this, inorganic materials like metals and metallic oxides have obtained a lot of focus over the course of the past decade as a direct result of their capability to withstand tough operating condition. Anti—bacterial NPs are of specific importance because of their capacity to endure severe procedure circumstances (Sirelkhatim et al. 2015). This is in contrast to organic natural antimicrobial agents, which lose their consistency under extreme temperature or pressures, so antibacterial NPs are able to withstand these conditions without being affected. The antibacterial property of nanofluids made of zinc oxide nanoparticles, which are non-toxic to human cells has been analysed primarily by contrasting it to the performance of other nanofluids (Vandebriel and Jong 2012).

ZnO nanofluids have been shown to exhibit antibacterial behaviour, which has been convincingly demonstrated (Zhang et al. 2007). Jalal et al. applied a sustainable technique for making ZnO nanoparticle. ZnO nanoparticle suspensions' antibacterial activity against Escherichia coli (*E. coli*) was determined by measuring the reduction ratio of bacteria after being exposed to the nanoparticles. Bacterial survival rates decrease with increasing ZnO nanofluid concentrations and exposure times (Jalal et al. 2010). Additional research has revealed that ZnO nanomaterials have broad-spectrum antibacterial effects against many different microorganisms. There is some speculation that the size of the ZnO particle as well as the existence of standard visible light are important factors in determining its antibacterial activity (Jones et al. 2008).

Copper and copper oxide nanoparticles have been shown to possess antibacterial properties against a diverse range of bacteria, including strains that are both gram-positive and gram-negative (Mahapatra et al. 2008; Usman et al. 2013). The size of the nanoparticles and the temperature at which they are produced can affect the antibacterial potency of the nanoparticles. Smaller CuO nanoparticles exhibit greater antibacterial activity. Additionally, CuO nanoparticles can penetrate the bacterial cell wall (Padil and Černík 2013). It is hypothesised that such NPs attach to the enzymes within the cell and impede the processes that are necessary for its survival (Akbar and Butt 2015).

Leung and colleagues' research (2014) revealed that the toxicity of MgO nanofluids towards E. coli may occur even in the absence of oxidative stress and the generation of reactive oxygen species (ROS). This led them to propose a new hypothesis regarding the mechanism behind MgO's toxic effects, which involves

damage to the cell membrane despite the lack of lipid peroxidation, which was previously thought to be responsible (Leung et al. 2014).

According to Dong et al., the antibacterial pathway of $Mg(OH)_2$ NPs of metal-based compounds. They had drawn the conclusion that MgO nanoparticle is an efficient antimicrobial entity against the foodborne pathogens which include E. coli and Salmonella sp. (Dong et al. 2010).

5.3 Drug Delivery

Over the past few decades, investigations have been carried out on nanofluids (NFs) for drug delivery purposes to enhance their efficacy and specificity. The nanoparticles' properties and traits in the nanofluid, including their small size, customisable surfaces, and multifunctionality, enable the NPs to interact with complex cellular functions in novel ways (Tripathi and Bég 2014; Wang et al. 2020).

NPs have been studied for their potential use as drug delivery systems. The use of gold nanoparticles is advantageous in drug and gene delivery applications due to their non-toxic nature as carriers (Ghosh et al. 2008). The monolayer in such systems allows for the optimizations of physicochemical characteristics like charge and hydrophobicity, while the gold core provides stability to the arrangement as a whole (Ghosh et al. 2008; Han et al. 2007). The main goal of any delivery of drugs system is to ensure the drug is released precisely where it needs to be. The first studies on drug delivery systems focused on the drug's dissolution pathway, its sensitivity to pH and temperature, polymeric materials, nasal delivery, as well as oral drug delivery (Gupta et al. 2002; Park 2014). Over the past few years, numerous researchers have contemplated the application of nanofluids is drug delivery systems because of the potential benefits they offer, including improved therapeutic properties and safety, reduced toxicity, and increased biocompatibility (Sawant et al. 2021). In the production of nanofluids for drug delivery, it is essential for the system to have the capability of producing enclosed drug and discharge properties, as well as having a prolonged storage life and compatibility with living organisms. Regarding nanofluidic medications, the electric charge of the nanoparticles that are dispersed in the fluid is one of the critical factors to take into account (Sheikhpour et al. 2020).

Madanipour et al. used the Moiré deflectometry methodology for investigating the absorption coefficient of gold NPs in water due to its importance in biomedical applications (Madanipour et al. 2015). Graphene-based nanofluid have been shown to have the potential to be utilised in anti—cancer medication delivery systems, according to a comprehensive study (Jampilek and Kralova 2021).

In the quest for more stable and effective drug release in medical nanofluids, thermal conductivity (TC) could perhaps play a significant role. Amin Kazemi-Beydokhti and his colleagues looked into the ways in which the TC values of a medical nanofluid changed. It was discovered that elevating the temperature increases the TC, while lowering the pH improves the thermophysical conditions for drug release. These results provide compelling evidence that TC may play a critical role

in the design of clinical nano—fluids for anticancer therapy (Kazemi-Beydokhti et al. 2015).

To improve drug specificity and efficiency, magnetic nanofluids (MNFs) are widely used in contemporary drug delivery methods (Hamad et al. 2021). It has been shown that MFs have an influence on nano-drug delivery methods. Das and colleagues examined the movement of conducting nanofluids through a porous tube under peristaltic flow while being exposed to a magnetic field (MF). The implementation of a magnetic field enables nanoparticles (NP)-based medications to be guided to a specific area inside the body. The multitude of advanced applications of this model makes it a valuable drug delivery system in the realm of biomedical engineering (Das et al. 2019). Shahzdi et al. examined the use of NFs as drug agents in copper and hybrid NFs by employing mathematical modelling. And had cluded NFs as potential agent in biomedical field (Shahzadi and Kousar 2019). In order to determine whether or not the cytotoxicity of ZnO quantum dots nanoparticles in the shape of ZnO nanofluids is evident, Fakhroueian et al. tested them on four different types of cancerous cell lines. ZnO quantum dots are suitable for use as drug agents due to their ability to destroy cancer cell lines (Fakhroueian et al. 2018).

5.4 Wound Care

In recent years, there has been a prevalence of cutting-edge treatment options for wounds. Wound healing at various stages has been aided by the use of nanostructured systems (Tottoli et al. 2020). Assessment of biomedical interoperability, assessment of anti-microbial efficacy, and assessment of in vivo efficacy utilising full-thickness skin models are all crucial steps in the creation of innovative systems for wound healing (Chakrabarti et al. 2019; Kalashnikova et al. 2015). During treatment, it's not uncommon for skin lesions to become infected, so finding a good dressing is crucial (Negut, Grumezescu, Grumezescu).

Anghel and colleagues carried out a study investigating the use of iron nanofluids in wound care with the aim of preventing the colonization of *Candida albicans* and the formation of biofilm. The dressing patches were soaked in a nanofluid containing the targeted nanoparticles, composed of a blend of active magnetic nanoparticles such as iron oxide. The primary outcome of this study was that combining the unique characteristics of iron oxide nanoparticles and Satureja hortensis oil could lead to the creation of a novel product that hindered the growth of biofilms and fungi (Anghel et al. 2013).

5.5 Cryopreservation

Nanofluids give the impression of having the ability to significantly enhance heat transfer rates in awide variety of uses, including those in the areas of instrumentation

as well as bio-medicine (Baby et al. 2011; Wong et al. 2017). When compared to base fluids like oil or water, the thermos-physical characteristics of nanofluids, which including heat capacity, temperature gradient, rheology, as well as heat transfer coefficients, have been found to be significantly improved (He et al. 2008; Krishna et al. 2020; Verma and Tiwari 2015).

Traditional methods of vitrification typically involve the application of cryoprotectants in extremely high concentrations (commonly greater than 4 M), which are frequently toxic. In order to bypass this obstacle, The concentration of cryoprotectant that he and his colleagues had used was only 2 M, which is the same concentration that is typically utilised for the conventional slow-freezing procedures. They had created an ultra-modern quartz micro-capillary system that was based on nanofluid for the novel cryopreservation of mammalian cells. According to the findings of their research, vitrification of murine embryonic stem cells in the presence of a small levels of intracellular cryoprotectants could be an approach that is both practical and successful for the purpose of cryopreserving these cells. This technique was used to vitrify murine embryonic stem (ES) cells, and the results were comparable to those obtained with non-frozen control cells in terms of the viability rate. The expression of three distinct proteins, which included a variety of transcription factors, served as a verification that the pluripotent murine ES cells derived from post-vitrification embryos maintained their undifferentiated properties (Ilyas et al. 2017).

5.6 Cryosurgery

Cryosurgery is considered as one of the most efficient methods for entire treatment of tumour cells and for maintaining control over those cells. Liquid nitrogen as well as solid carbon dioxide are used in this process (Cooper and Dawber 2001). The creation of ice crystals is a result of artificially creating a very cold environment; this decrease in liquid water could cause the cell wall of the intended cell to rupture (dehydration) (Singh and Bhargava 2014). However, there are drawbacks to using this method, including the potential for collateral damage to healthy tissues due to improper freezing. Highly conductive NPs are loaded into the tumour tissue to enhance its freezing capacity (Hou et al. 2018; Singh and Bhargava 2014). Using simulations of phase change bio-HT at the cellular level, Yan and Liu (2008) determined the temperature difference between traditional cryosurgery and nano cryosurgery. As a result of the encouraging findings, they reasoned that nanocyrosurgery concepts could pave the way for novel approaches to tumour treatment in the future.

5.7 Bioimaging

Because of their unique combination of magnetic and physicochemical parameters, nanofluid-based NPs have been proposed as a promising tool for biological imagining applications and cancer detection (Hosu et al. 2019). Because of their unique optical properties, adaptability in surface modification, and capacity to transport a wide range of sensing and therapeutic components, nanofluid-based nanoparticles have recently found application in bioimaging (Nune et al. 2009). Moreover, Nanomaterials should be nontoxic with the biomedical platform being tested, demonstrate exceptional optical characteristics, decent light absorption, and be able to report on the rapid fluctuations of the biological process. Nanoparticles used in bioimaging can be categorised as either self-emitting specific optical signals (such as quantum dots or carbon dots) or requiring fluorophore labelling to be visualised (Pratiwi et al. 2019).

The utilization of nanofluids based nanomaterials in electromagnetic resonance imaging is one of the most fascinating applications that can be found in the field of diagnostic medicine (MRI) (Sheikhpour et al. 2020; Wang et al. 2022). In MRI, the utilization of magnetic nanoparticles improves contrast as well as sensitivity. When it comes to increasing contrast in MRI scans, superparamagnetic nanoparticles (SPMNPs) are among the most effective options. Inorganic compounds, gold, and nanoparticles of gadolinium could all be components of these agents (Shokrollahi 2013).

In their study on magnetic particle imaging, Euting et al. looked into the possibility of using a biocompatible as well as reliable form of ferromagnetic carbon NF. The magnetization of superparamagnetic material was compared to that of ferromagnetic material. This study provided evidence that ferroMNFs could be useful like a contrast agent and demonstrated their practicability. Based on the research results, the use of ferromagnetic NPs leads to images that are less blurry and have more edge imaging interpretation (Euting et al. 2012).

The utilization of iron oxide nanoparticles within assessment as well as diagnosis of atherosclerosis was investigated and reviewed by Talev et al. They discussed the use of NPs for the purpose of molecular imaging of atherosclerosis (Talev and Kanwar 2020).

Through the use of nanosecond pulsed laser ablation, Yogesh et al. successfully synthesised graphene oxide NPs and studied their properties for use in bioimaging. And the results suggest that graphene oxide NPs are suitable for bioimaging because they are biocompatible and emit a photoluminescent signal (Yogesh et al. 2020).

5.8 Micro-Pumping Devices

Devices that utilise microfluidics and nanofluidic are used extensively in the field of biomedicine (Hamad et al. 2021). Nanofluids play an integral role in micro-pumps,

which can be either passive or active depending on its functionality (Attia and Alcock 2010). Traditional active pumps can't be used in NFs because the hydraulic resistance they'd cause in such a tiny space would be too great. active pumps are not appropriate for utilization with NFs due to the very high hydraulic obstruction they present in a nanochannel and the extremely small volume that can be accommodated by the channel (Attia and Alcock 2010; Liang et al. 2018). As a direct consequence of this, researchers started looking for different options. A nanofluidic high-pressure micropump along with incredible accuracy and the capability to provide up to 20 MPa was developed by Liang and colleagues (2018).

In their research, Serkan and colleagues proposed the application of a novel, mobile permanent magnetic actuator (PMA) configuration to manipulate magnetic nanofluids for driving chemical substances within circular micro-sized channels. The results indicate that the suggested PMA system meets the criteria for flow rates in analytical applications, such as administering low-flow drug delivery, sorting cells, and monitoring microorganisms (Doganay et al. 2020).

Electrokineti-cmicroperistaltic pumps are essential pieces of biomechanical equipment that assist in the localised administration of medication to diseased areas of the body. Mathematical modelling and analysis of various significant aspects of this kind of fluid flow inside a rectangular channel with wall properties had been the primary focus of Ahmed and co-workers' research. Further in their research, they model the transport of a CNT-water nanofluid and perform an analytical analysis of the resulting flow problem. In the context of pharmacological engineering, the application of their research involved the creation of a system for transporting or targeting the delivery of drugs using peristaltic micropumps and magnetic fields (Zeeshan et al. 2021).

6 Challenges

Over the course of the last few decades, scientists have uncovered a plethora of nanofluids' potential properties (Yang et al. 2020). However, there are a number of issues that could serve as roadblocks in the process of commercializing nanofluids. Therefore, the scientist ought to place a greater emphasis on finding solutions to these problems. It seems that nanofluids could be useful in a wide range of applications; however, the advancement of this field is being hampered by a number of factors like (Assael et al. 2019; Puliti et al. 2011; Saidur et al. 2011; Sreelakshmy et al. 2014).

- Discordance between findings from various labs
- The inaccurate labelling of suspensions
- As a result of not having a firm theoretical grasp on what causes the reported alterations in properties.

Most studies in the literature (Elias et al. 2014; Senthilraja et al. 2015) found nanofluids with an insufficient stability period, suggesting that the stability of these

materials is an issue. Notable is the fact that most research concentrates on thermo-physical properties like thermal conductivity, rheology, composition, temperature transfer coefficient, etc. Chen et al. (2008), Nabil et al. (2018), Timofeeva et al. (2009), Zeng and Xuan (2018). Further, more emphasis should be placed on stabilisation, which is a key issue in ensuring the credibility of the research findings, especially when they are put to use in the real world. There is a dearth of studies examining the properties and behaviour of nanofluids at extreme temperatures (Amiri et al. 2016; Esfe et al. 2017) in the existing literature. Understanding the mechanism of effectiveness of nanofluid at both low and high temperatures may benefit from research into these fluids at both extremes of temperature. Most interestingly, there are no findings regarding the total prices of the various kinds of nanofluids. Because nanofluids have novel properties, conventional fluids are really being phased out and replaced by various types of nanofluids (Assael et al. 2019; Sidik et al. 2014). In the event that a significant quantity of conventional fluids is changed out for nanofluids, the resulting heat transfer equipment can be made relatively more straightforward and of a more compact size, which can result in cost savings. In the meantime, the method of preparation, properties, etc., all contribute to the high cost of various nanoparticles (Esfe et al. 2017). The total cost of using various nanofluids alongside the required infrastructure in a wide range of real-world settings is an intriguing topic that could be explored in future research. The following are a few of the more notable points.

1. **High viscosity**—The higher the amount of particles that are suspended in the nanoparticle-water mixture, the higher the viscosity of the suspension. Therefore, there is a limit to how much the particulate mass fraction can increase and this could be considered as one of the drawbacks of nanofluid application (Sheikhpour et al. 2020; Yang et al. 2020).
2. **High cost**—One potential barrier to nanofluids' widespread industrial use is their more expensive production. One-step or two-step processes can be used to create nanofluids. Nonetheless, high-tech instruments are essential for either approach (Huaxu et al. 2020).
3. **Lower specific heat**—In comparison to base fluid, nanofluids have a lower specific heat, which is the most significant disadvantage of nanofluid (Lee and Mudawar 2007).
4. **Production challenges**—Nanoparticles are synthesised in their entirety through reduction reactions and ion exchange. Also, it is challenging, if not impossible, to purge the base fluids of their various other ions as well as reaction products. The advantages of high-surface-area nanoparticles are diminished because of the particles' propensity to clump together. In order to combat this issue, particulate displacement preservatives are typically added to the nanoparticle-carrying base fluid. However, doing so can alter particle surface properties, as well as nanofluid manufactured in this manner may have unacceptably high impurity levels (Saidur et al. 2011).

7 Future Scope

There is a wide variety of uses for nanofluids currently, and this number is expected to grow in the future. The development of nanofluidic systems that are capable of delivering energy in a manner that is both effective and efficient ought to be the primary focus of research. Concerns have been raised about the safety of the production and use of nanofluids in the present day, and these issues will also be the subject of research in the future. It's possible that in the future, research will focus on both the modification of nanoparticles in nanofluids and their applications in biomedical areas. Nanofluids have the potential to assist in the delivery of drugs in a targeted manner, which will allow the pharma industry to achieve significant advances.

8 Conclusions

Nanofluids have many potential uses, but one of the most important is in the medical and biomedical fields. In this article, a survey of the implementations of nanofluids is conducted. These applications include delivery of drug, medical care, disease diagnosis, antimicrobial implementations, wound care and so on. The papers about the above case were reviewed, and one's contents and results described and analysed, with a focus on these novel topics.

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Metagenomics for Drug Discovery

Amit Jethwa, Jayesh Bhagat, Jacinta Teresa George, and Sagar Shah

Abstract In the twenty-first century, approximately 100 years since the discovery and use of the first drug penicillin, antimicrobial resistance has become the major global threat to human health, thereby, demanding the need to discover new candidate metabolites exhibiting therapeutic potential to meet current medical treatment demands. Newer emerging diseases, relatively long duration of discovery and development process of drugs among others also necessitate identification of novel drug candidates. Till date thousands of drugs have been discovered, two thirds of which have their origins from the culturable microbes present in soil or diverse habitats around the globe. Since the advancements in high-throughput technologies in genomics, it has become possible to discover novel candidate metabolites having therapeutic potential from complete microbiomes chosen in a culture independent manner leading to an exponential increase in novel drug discoveries. The present chapter, thus, aims to bring to focus the role of metagenomics—a biotechnological tool that involves extraction of DNA from communities of microbial populations circumventing the need of culturing the organism followed subsequently by screening and/or sequencing of DNA—in drug discovery. The chapter will begin with a background of drugs including microbial-derived ones followed by a section on various approaches currently available for drug discovery from microbes. This will lead to the field of metagenomics, various techniques involved and how metagenomics of microbes can help bioprospect their potential to synthesize drugs as well as how

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metagenomics can assist in selection of microbes having the ability to synthesize drugs of higher therapeutic potential.

Keywords Bioprospect · Drug discovery · High-throughput technologies · Metagenomics · Therapeutic potential

1 Introduction to Drugs

1.1 What Are Drugs?

The words 'drugs', 'natural products' and 'pharmaceuticals' are being quite interchangeably used worldwide (Taylor 2015). The word 'drugs' can also be used to refer to an illegal substance banned for consumption. However, in the medical or pharmaceutical context, drugs refer to majorly secondary metabolites, but could also be primary metabolites, sourced from various types of living forms on earth including plants, animals (terrestrial and marine vertebrates or invertebrates) or microorganisms that have the potential to treat ailments or diseases (Demain and Sanchez 2009; Dias et al. 2012; Pham et al. 2019; Abdel-Razek et al. 2020). Primary metabolites refer to intermediates or terminal products of primary metabolic pathways that are crucial for growth and survival of the living organism. Contrastingly, secondary metabolites refer to compounds not crucial for development, reproduction and/or growth of a given organism. The obvious question that riddles our minds is, 'Why do these organisms then synthesize such compounds?' Majority of these compounds synthesized act as defence molecules for these organisms especially even more crucial for living systems such as plants that cannot evade predators or stresses that easily. Some are also produced because of an adaptation mechanism of the organism to its changing environment (Dias et al. 2012). This does seem logical since surviving under constant biotic and abiotic stresses would be a key housekeeping chore for organisms throughout the span of evolution. With the assistance of modern screening programs and analysis techniques, over 1 million natural compounds have been discovered till date with approximately 20–25% of the natural products showing some biological activity. An interesting fact is that about 10% of this established activity are from natural products obtained from microbes (Demain and Sanchez 2009).

However, not all drugs under survey for pharmaceutical application or those in use are strictly natural products. Many of the drug candidates and drugs in present times are derivatised molecules from natural products using semi-synthetic methods or are completely synthesised in a chemical fashion (Demain and Sanchez 2009; Mathur and Hoskins 2017). Drugs have been further categorised as natural products, biologics referring to usually large peptides (>50 amino acid residues) or proteins, natural products botanicals (NB) for those derived from plants, natural products mimics, totally synthetic drugs, synthetic drugs but having pharmacophore based on natural products and vaccines (Newman and Cragg 2020). Based on the latest

review by Newman and Cragg (2020), >50% of drugs that have obtained clearance for use from the United States Food and Drug Administration (USFDA) in the span of 1981–2019 have been procured from natural sources either in a derivative form or as inspired from natural structures.

Based on their pharmacological effects, drugs can further be categorized as antibiotic, antifungal, antiparasitic, antitumor/cancer, anti-inflammatory and immunosuppressive, biofilm inhibitory compounds among others. Antibiotics are drugs that combat bacterial infection where some are narrow-range antibiotics having limited or specific target bacteria whereas some are broad-spectrum that exert their effects against a large range of bacteria (examples: penicillin, erythromycin, streptomycin) (Pham et al. 2019). Antifungal and antiparasitic agents, likewise, are drugs that target fungal (examples: nystatin, amphotericin B) (Pham et al. 2019) and parasitic (examples: chloroquine, metronidazole and tinidazole) (Campbell and Soman-Faulkner 2022) infections respectively. Antitumor and anticancer drugs are those that inhibit the proliferation of cancer/tumour cells or those that target these cells for killing (examples: bleomycin, actinomycin D, rapamycin) (Pham et al. 2019). Anti-inflammatory drugs are those used to reduce inflammation found in infections as well as non-infectious cases (examples: rapamycin, strepsesquitriol, diclofenac, ibuprofen) (Pham et al. 2019; Ghlichloo and Gerriets 2022). Immunosuppressive agents are used for treating autoimmune and immune-mediated diseases as well as employed to prevent the rejection of grafts and/or organs during transplantation by suppressing the immune reactivity towards the transplant (examples: rapamycin, murine anti-CD3 mAb Muromonab-CD3 (OKT3), voclosporin) (Wiseman 2016; Pham et al. 2019). Yet another category of drugs is that of biofilm inhibitors which target biofilm formation especially crucial to prevent biofilm formation as well as disrupt biofilms that have been formed on hospital devices and prevent nosocomial infections (examples: aminoglycosides, quinolones, deacylated lipopolysaccharide, actinomycin D) (Pham et al. 2019; Ghosh et al. 2020). These are just a few that have been specified; the entire list of pharmacological classification is quite enormous and can be viewed at https://www.fda.gov/drugs/investigational-new-drug-ind-application/general-drug-categories.

1.2 Historical Background of Drugs

Drugs have been used by human beings for treatment of ailments and/or diseases since time immemorial with records of such use being reported in China, India, Greece, Egypt among other nations (Taylor 2015; Pham et al. 2019; Karmakar et al. 2020; Abdel-Razek et al. 2020). These drugs were primarily of plant and/or animal origin with little or no knowledge about the mechanics of the drug involved in the treatment being rendered; the entire treatment was primarily based on empiricism (Taylor 2015). This concept, however, underwent a dramatic change in the 18th century with the foundations of pharmacology being laid down wherein research into the mechanism of drug action slowly began to gain popularity. One of the first compounds

to be isolated and studied in detail was digitalis which was extracted from foxglove by William Withering in 1780s (Taylor 2015). Similar works continued in the next century too with the contribution of Sertüner towards chemical studies of opium and extraction of morphine from opium in 1815 (Pina et al. 2010) and the pioneering works of Oswald Schmiedeberg (1838–1921) who is considered the father of pharmacology (Taylor 2015). This paved way for interdisciplinary research to contribute to drug discovery and development. Majority of the drugs being discovered, however, were still primarily from plants up to this time point.

One of the hallmarks of drug discovery came as a happenstance event in 1928 with an extraordinary discovery by Alexander Fleming that a mold, *Penicillium notatum*, secreted a compound potent enough to kill a bacteria *Staphylococcus aureus* that he had grown in a petri-dish (Fleming 1929). The active ingredient was identified as being penicillin which following isolation was used widely as an antibiotic during World War II (Demain and Sanchez 2009). This became the first microbial drug to be recognized worldwide and initiated the hunt for more such antibiotics and pharmaceuticals from microbes. This was followed by several other success stories such as the discovery of streptomycin from *Streptomyces griseus* (Waksman et al. 1946), cephalosporin-C from *Cephalosporium acremonium* (Newton and Abraham 1955) and vancomycin from *Amycolatopsis orientalis* (Geraci et al. 1956). The age-old method of fermentation was employed for the production of these antibiotics in bulk quantity. Moyer and Coghill (1946) of Northern Regional Research Laboratory, Illinois developed a specific medium for growing *Penicillium* using corn steep liquor and lactose that increased yields of penicillin. Culturing methods employed included surface culture and submerged fermentation (Raper and Benedict 1950).

Post-World War II dawned the Golden era of pharmacology with accelerated research into the discovery of newer antibiotics, analgesics, oral contraceptives, β-adrenergic blockers, anti-cancer drugs among others (Taylor 2015). This was possible due to advancements in the fields of chemistry, biochemistry, molecular biology, and computational biology which helped identify how drugs interact with proteins in the human systems thereby mediating their effects, and also helped design drugs and identify scaffold structures involved in pharmacological effects (Pina et al. 2010). Towards the closure of the 20th century, further revolutionary advancements in the fields of cellular and molecular biology including hybridoma technology and recombinant DNA technology revolutionized the pharmaceutical industry. This paved way for the bulk production of monoclonal antibodies for use as therapeutics as well as enabled eukaryotic and other recombinant therapeutic proteins to be synthesized in large amounts in prokaryotic cells such as *Escherichia coli*, eukaryotic cells such as *Saccharomyces cerevisiae* and even animal cell lines, for example Chinese hamster ovary cells (Pina et al. 2010; Pham et al. 2019).

In the last few decades several high throughput screening technologies that screen various types of libraries inclusive of small metabolites and peptide libraries including combinatorial chemistry have emerged as the key technologies influencing drug discovery (Szymański et al. 2011). The field of omics including genomics, metagenomics, proteomics, transcriptomics, and metabolomics has also in more recent times contributed immensely towards drug discovery.

1.3 General Pipeline for Drug Discovery and Development

The multifaceted process of discovering drugs and developing them to the marketable stage is divided into several phases. The first phase is that of drug discovery by single or combinatorial approaches. A typical workflow for microbial-based drug discovery is illustrated in Fig. 1. Once a drug is discovered, the next phase involves target identification and validation where understanding the disease target or infectious agent is critical for effective evaluation of drug effectiveness. This is followed by hit identification involving estimating which of the various drug candidates interact with the target followed by validation. The hits are then converted to leads by employing secondary assays to assess off-target effects, estimate solubility properties, and assay for the "absorption, distribution, metabolism and excretion" (ADME) properties of the lead molecules. Further, lead optimization is performed where the lead compounds are chemically modified, or structural analogues are queried to improve drug efficacy. The next phase includes in vivo studies beginning with animal models. Once toxicity and dosage responses are established in animal models, the candidate drug is subjected to clinical trials in humans including Phase I (20–80 participants), Phase II (100–300 participants) and Phase III (1000–3000 participant) trials. Following this, the drug is reviewed by FDA for safety and effectiveness. The last step is Phase IV where following drug approval the drug is tested on a larger sample set with more than 1000 participants being involved (Taylor 2015; Sinha and Vohora 2018; Sun et al. 2022).

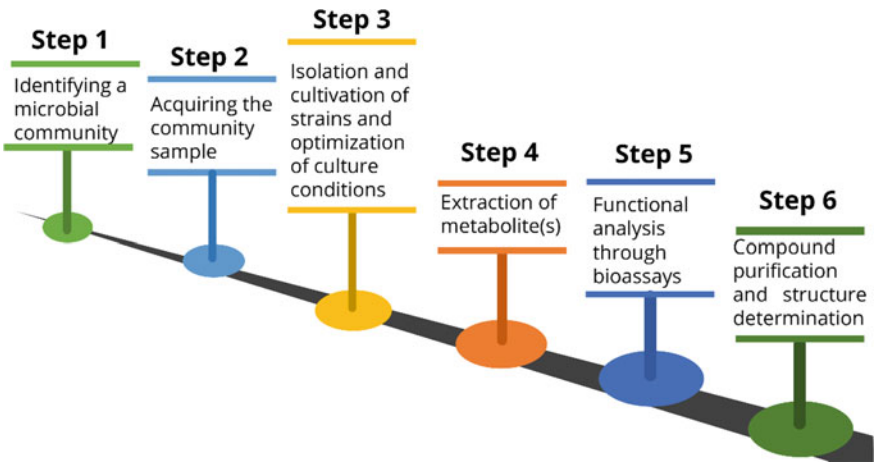

Fig. 1 Typical workflow followed during conventional drug discovery process

1.4 Need for Newer Drug Discoveries

"Two new drugs to fight superbugs available on NHS soon" (BBC news 2022), "Sunlenca® (lenacapavir) Receives FDA Approval as a First-in-Class, Twice-Yearly Treatment Option for People Living With Multi-Drug Resistant HIV" (Gilead Press Release 2022)—headlines such as these are not so common and capture our rapt attention. Indeed, as compared to the drug demand, there seems to be a large imbalance in the number of drugs emerging on the market. This surely makes one wonder regarding the reason for this large void. Some of the reasons for this void are mentioned below.

To begin with, the entire process of drug discovery and development including the various clinical trials takes several years to reach fructification. As outlined in the section above, there are several steps involved in this process. In the earlier years of drug development, these steps progressed in a linear fashion. However, considering the time consumed in the same, many pharmaceutical companies now have started running some of the phases in parallel to minimize the time taken till the final drug reaches the patient. For instance, modifications of lead drug molecules and conversion of possible drugs into more 'druggable' forms may be conducted in parallel (Taylor 2015).

Further, according to Sun et al. (2022), 90% of failure in drug development accounts from a lack of clinical efficacy, very high unmanageable toxicity, poor drug-like properties of the candidate molecules, and poor understanding of commercial and societal needs and strategic planning. Hingorani et al. (2019) also implicated a lack of efficiency in disease indication as the foremost cause of delayed drug development and high failures in the same. This necessitates the discovery of newer drug candidates with better potential especially those that are natural in origin.

The need for newer drug discoveries, especially for antibiotics and antitumor or anticancer drug, has also arisen due to the increased drug resistance found in bacterial and human cells to these agents respectively. In the case of antibiotic resistance, the problem is not restricted to just resistance to one drug but the observation of resistance to multiple drugs in bacteria. These superbugs are evolving at an alarming rate with majority of them being pathogenic in nature (Demain and Sanchez 2009). Examples of these include methicillin-resistant *Staphylococcus aureus* and multidrug resistant *Pseudomonas aeruginosa* among many others (van Duin and Paterson 2016). This resistance in bacteria to drugs has resulted from either natural resistance or acquired resistance that the bacteria have developed to combat the drug effects. These include reduction in outer membrane permeability, expression of efflux pumps that pump the drugs from the interior of the bacterial cell into the extracellular side of the cells, modifying the target of the drugs and inactivation of drugs (Nikaido 2009; Reygaert 2018). Similar evasion and tolerance mechanisms have also been exhibited by cancer cells while developing resistance to single and multiple drugs. These include increased drug efflux, reduced inflow of drug, drug compartmentalization, changes in drug metabolism, alterations in drug targets, increased resistance to apoptotic cell death and increased repair of DNA damage induced due to the drug (Mansoori et al. 2017; Emran et al. 2022).

Further, there is a need for novel classes of anti-infective compounds to be developed and released at the market level to combat newer emerging diseases. Many of these emerging infectious agents are those that the host has never encountered before, thereby, posing an even greater challenge to the host's immunity (Demain and Sanchez 2009). Furthermore, one cannot neglect the fact that majority of drugs available in the market today have side effects too such as vomiting, diarrhoea, stomach upsets, rashes, and allergic reactions among others.

Against this background, one can conclude that there is a monumental need for discoveries of newer drug molecules with better therapeutic potential and preferably lesser or absolutely no side effects. The promise of high throughput technologies and combinatorial compound libraries to deliver a diversity of new drugs within a short period of time has not been fulfilled majorly owing to unrealistic expectations (Abdelnasser et al. 2012). This has resulted in a rekindling of interest in nature-derived products for their therapeutic potential. Natural products offer special advantages with respect to features when compared with synthetic molecules. These features include exhibiting massive scaffold diversity, high molecular mass, structural complexity, higher molecular rigidity to name a few. Drugs derived from natural origin have the advantage of being optimized by evolution in terms of structure and function that permit and favour better interaction with biological molecules which is the major basis of drug action (Atanasov et al. 2021). Natural products have also been found to show lesser side effects as compared to their synthetic counterparts (Mathur and Hoskins 2017).

Amongst the various natural sources of drug candidates, microbes provide the largest contribution both in terms of numbers as well as diversity of drug molecules. Therefore, it is not surprising that a lot of present-day discovery programs are channelizing their resources back towards bioprospecting microbes for new drug molecules.

2 Current Approaches for Microbial-Derived Drug Discovery

2.1 Unique Habitats and Novel Metabolites

For the discovery of metabolites having unique and novel pharmaceutical properties and effects, the need of the hour is to identify and screen novel bio-resources or revisit the already explored habitats or the known resources. Exploration of existing known habitats by new cultivation methods combined with genomics has shown to yield more fruitful results. Further, expeditions to not so explored habitats such as deep ocean hydrothermal vents have hinted to us the existence of unique ecosystems which can offer novel drugs. This is possible due to robotic sampling vehicles, novel cultivation methods, or extensive use of recombinant DNA technology

combined with metagenomic technology. Various habitats such as soil, aquatic sediments, deserts, hot springs, marine habitats including abyssal plains, hydrothermal vents, marine organisms (corals and sponges), mangroves and coastal ecosystems have been tapped for novel metabolites by isolating associated microorganisms. Microorganisms useful for drug discovery can be free-living organisms, may exist as symbionts, or can be endophytic (Pereira 2019). Tapping the potential of these habitats for novel metabolites requires unique approaches. Discussed below are the conventional and emerging cultivation-dependent methods and their limitations.

2.2 Conventional and Emerging Cultivation Techniques

2.2.1 Conventional Cultivation Techniques

The conventional techniques for bioprospecting microbes for drugs include first enriching and culturing the microorganisms under investigation from varied sources. Enrichment culture techniques are routinely employed microbiology techniques which are used for increasing low counts of the targeted organisms to detectable and cultivable levels (Bari and Yeasmin 2022). Enrichment employs the principle of natural selection, in which a collected sample having a heterogenous population of microorganisms is transferred into a medium having well-defined chemical composition. This inoculated sample is then allowed to proliferate under controlled conditions which are either the primary requirements for growth or natural conditions from where the sampling was done. These include temperature, light, oxygen or carbon dioxide supply, pH, pressure, etc. Such naturally simulated conditions may only favour the growth of one or more particular type of microbes. The enrichment culturing can be done in submerged (liquid medium) or in solid-state fermentation where solid substrates are used as either raw or processed material.

Once the microorganisms' presence is detected, the sample can be subjected to isolation on agar plates using the same nutritional and environmental conditions provided during enrichment. Often to reach this stage, the enriched sample may need to be serially diluted multiple times before being plated onto agar medium. In the case of microalgae, serial dilution may itself suffice without isolation on a solid plate.

However, the conventional enrichment cultivation approach suffers major limitations due to one or multiple reasons leading to "the great plate count anomaly"—a term introduced in 1985 by Staley and Konopka. It refers to the anomaly in numbers of colonies successfully grown on media as against the numbers present in the environmental sample. The "great plate anomaly" phenomenon is widely observed in marine ecosystems where only 0.01–0.1% of marine oceanic bacterial cells could be isolated as colonies by standard bacterial isolation techniques (Connon and Giovannoni 2002).

This anomaly is attributed to the lack of knowledge on specific physiological needs, oligotrophic requirements, overgrowth of fast growers or too slow growth,

poor cell density in the sample, dormant stage of an organism, special chemical or physical needs, requirement of solid surface for attachments, loss of interactions between interdependent microorganisms and complexity of microbial communities. Together these contribute to "uncultivable" communities. It has been understood that of 40 known prokaryotic phyla, only 50% could be cultured in the laboratory. The remaining uncultured organisms are believed to offer possibility of novel metabolites of medical interest.

2.2.2 Emerging Cultivation Techniques

To harness this unexplored possibility from uncultivable microbes, novel cultivation techniques have emerged with the potential to recover the uncultivable organisms several folds greater than the conventional approaches. Emerging cultivation methods addresses the limitations of conventional cultivation strategies by permitting the growth of a wide range of microorganisms (Lozada and Dionisi 2015).

These novel cultivation methods are high throughput techniques able to handle several samples and having the ability to isolate a significant proportion of microorganisms from natural environments. However, these methods require basic biology knowledge along with information and understanding of the ecological background of the sample. Detailed below are some of the novel cultivation methods that have been developed and employed successfully.

Dilution to Extinction Method (DTE)

By this approach, ubiquitous, however, not so easy to cultivate or isolate bacteria and archaea can be successfully isolated. In one of the studies, Rappé et al. (2002) isolated marine bacterioplanktons by inoculating freshly obtained sample from Oregon coast seawater into microtiter wells by dilution (to achieve approximate 1–20 cells per well). The authors employed media prepared by adding necessary salts and a defined formulation of organic carbon compounds in sterile Oregon coast sea water and incubated the same at 15 °C for 23 days either under dark or 14 h light/10 h dark cycle. The technique is assisted with epifluorescence microscopy technique. Before cultivation it allows rapid detection of number of cells present in the sample whereas during cultivation it allows detection of growing cells (Rappé et al. 2002).

Benítez et al. (2021) have also reported the use of DTE methodology for isolating non-filamentous bacteria associated with marine sponges having the capacity for production of cytotoxic compounds that can be antineoplastic.

Diffusion Chambers

Diffusion chambers makes use of an apparatus in which microbial cells from a sample are combined with agar layer which in turn is bracketed on either side by semi-permeable membrane of porosity ranging from 0.2 μm to 0.3 μm. The assembled chamber is incubated in natural environment for one to several weeks. The membrane serves to isolate the caged cells from the environment; however, it allows the passage of growth factors and nutrients from the media. This favours the growth and thus isolation of otherwise uncultivable bacteria from diverse environments (Kaeberlein et al. 2002; Jung et al. 2021).

Microbial Trap Technique

Microbial trap technique is an alteration of the diffusion chamber technique to capture unusual and rare actinomycetes in situ. This is possible due to their ability to produce hyphae and the penetrability of these hyphae through solid environments. It involves a trap formed by sandwiching sterile agar between two semi-permeable membranes with the base having a pore size of 0.2 to 0.6 μm and the top membrane which has a size of 0.03 μm. The trap assembly is incubated on top of the microbial source where the hyphae of filamentous microbes selectively penetrate through the membranes and colonize the agar layer (Gavrish et al. 2008).

Microdroplet Encapsulation

This is yet another novel high-throughput cultivation method which combines encapsulation of single cell with flow cytometry. Sufficiently diluted sample is mixed with molten hydrogel such as agarose followed by emulsification to form microcapsules usually of size 50 and 80 μm. The microcapsule thus formed is expected to entrap a single cell, which when cultivated in natural like media and simulated growth conditions leads to the formation of microcolonies. The porous nature of agarose permits the diffusion of nutrients and signalling molecules into the capsule providing nutrition to the expanding colony while simultaneously permitting the waste metabolites to diffuse out. Growth of colonies can be detected using flow cytometry. Microcapsules can easily be separated, and the cultivated microbes can further be grown in rich media in microtiter plates and can thus be isolated with ease. Using the microdroplet encapsulation, several bacteria and fungi have been isolated from natural samples (Zengler et al. 2005).

In Situ Cultivation by Tip (I-tip)

Jung et al. in (2014) developed an in-situ cultivation method which targeted isolating symbionts from aquatic invertebrates. This approach uses a commercial device

comprised of a micropipette tip. The upper part of the tip is blocked with the help of an adhesive to prevent entry of contaminants. The pointed end of the tip is filled with agar layers and microbeads which is directly positioned onto the surface of the target environment. The microbead layer does not permit the entry of organisms larger in size while the layer of agar supports the growth of microbes due to the permeability of nutrients entering from the natural habitat. Using this approach uncultivated sponge-associated bacteria have been efficiently isolated (Jung et al. 2021).

Isolation Chip (iChip)

Automation of first-generation high throughput methods have further led to emergence of second-generation methods. Isolation chip (iChip) is one such example. iChip device is composed of numerous miniature diffusion chambers ranging in counts of hundreds. These chambers are inoculated with single cells, followed by incubation in an environment that mimics its natural niche conditions which can lead to several fold increased isolation of uncultivable microorganisms (Palma Esposito et al. 2018).

Hollow-Fibre Membrane Chamber (HFMC)

The HFMC approach is quite like iChip wherein a chamber for cultivating the microorganisms in isolation is created using a piece of hollow-fibre polyvinylidene fluoride (PVDF) porous membrane. A single HFMC system comprises of approximately 48- to 96-chamber units made up of porous hollow membranes of approximately around 30 cm in length, 67–70% porosity. Further, these chambers are equipped with syringes for injection and sampling. The environmental sample is first serially diluted, then injected into a chamber, which is then placed in a natural system or environment simulated in laboratory. Due to the porosity of the membrane, free exchange of nutrients, signalling molecules and metabolites can occur while preventing the movement of microbes thereby resulting in establishment of pure cultures of different types in each chamber (Aoi et al. 2009).

Once the enriched or unculturable microorganism is recovered, the organism is further transferred onto agar plates and multiplied. However, it may show the presence of mixed cultures or culture surviving only till limited division cycles. The cultivated microbes are then subjected to bioprospecting using analytical techniques or other appropriate methods to determine the presence of unique or significance secondary metabolites of medical importance.

3 Role of Metagenomics in Drug Discovery

3.1 Metagenomics Approach for Bioprospecting

Despite timely troubleshooting combined with recent advances, majority of the microorganisms from the natural world are still unculturable and marine microorganisms top this list. The hallmark studies of Woese and Pace combining molecular biology and molecular ecology concepts revolutionized the understanding of microbial diversity. The advent of metagenomics brought to limelight the potential of different ecosystems in harbouring a diversity of microorganisms with unknown functions which hitherto had remained masked and undiscovered due to the tag of uncultivability (Abdelnasser et al. 2012; Lozada and Dionisi 2015). This approach relies on the information obtained from DNA sequences regarding the genetic and, therefore, molecular diversity of a given organism or groups of organisms growing in a particular niche. This bypasses the need of cultivation by directly extracting the DNA from the natural niche (Lozada and Dionisi 2015).

Metagenomics is the study of community genetics based on next generation sequencing technology. This methodology aids researchers in learning about the variety, purposes, and evolution of uncultivated microorganisms found in a variety of habitats or ecosystems. This upcoming approach and its coexisting technology can reveal the capacities of microbial communities which drive the earth's energy and nutrition cycles, maintain the health of its people, and determine life's elaboration (Chopra et al. 2020). Metagenomics also attempts to improve our understanding of the interactions between microbes to enhance people's health, food production, and energy production. Although originally metagenomics was developed for understanding microbial ecology, it soon has become a tool for bioprospecting new habitats as well as earlier explored habitats for microbes that could produce novel drugs.

The metagenomics approach commences with genomics DNA extractions from the sample as community DNA, followed by construction of metagenomic libraries leading to library screening using either the function-driven or sequencing-based approach for identifying genes or gene clusters that will implicate biosynthetic pathways of novel drug molecules or drugs with better potential (Abdelnasser et al. 2012). Outlined below are each of these techniques in detail.

3.2 Techniques Involved in Metagenomics

3.2.1 Extraction of DNA From a Selected Environmental Niche

Selection of the environmental niche for bioprospecting is a prerequisite to ensure maximum bioprospecting for novel natural products. For instance, if the aim of the metagenomics approach is to screen for anti-tumour agents, it would be advisable to bioprospect in marine ecosystems as compared to terrestrial ones since it has been

shown that terrestrial systems offer low diversity of such agents (0.01%) as compared to marine ecosystems that show higher diversity (1%) (Abdelnasser et al. 2012). Microbial communities being targeted for drug discovery are no longer restricted to terrestrial and marine ecosystems. Metagenomics has made it possible to even bioprospect inaccessible and difficult to culture microbes from intestinal tract of humans and other animals.

Once the environmental niche has been carefully selected to yield maximum bioprospecting success, the next step is that of extracting the environmental DNA. Two approaches are employed for the same, viz. direct, and indirect lysis methods. In the direct lysis method, various lytic strategies such as freeze-thaw, ceramic bead beating, and use of lytic buffers aids the lysis of microbial cells resulting in extraction of DNA into the lysis buffer. This is followed by purification of extracted DNA from the remnant cellular components. However, this method faces a few limitations such as the inability to discriminate between genetic material of different origins and co-extraction of humic acid. This results in a high fraction of extracted environmental DNA containing unwanted sequences, thereby, complicating the analysis. Contrastingly, the indirect method employs separation of microbial cells from the remaining cells in the environmental sample first which is then subjected to lysis to achieve extraction of DNA. This offers the advantage of eliminating unwanted DNA sequences in the extracted DNA. Another way for circumventing low DNA yield in the sample and unwanted sequences is to employ amplification strategies for whole genome sequences (Abdelnasser et al. 2012; Wydro 2022). One cannot dispute the fact that this step is the most critical step in bioprospecting a niche for newer drugs and requires improved methods of DNA extraction to maximize recovery of diversity of genes and functions especially from high humic content soils, low nutrient content samples and extremophilic conditions.

3.2.2 Construction of Metagenomic Libraries

The extracted and isolated environmental DNA is subjected to fragmentation followed by creation of clones by inserting the fragmented DNA into vectors such as plasmids, cosmids and fosmids, thereby, generating metagenome libraries. The first choice of vectors is that of plasmids; however, plasmid-based vectors are riddled with the limitation of small size insert restriction. For bioprospecting, one may require querying with larger inserts. Hence, it is desirable to use cosmids (35–40 kbp), fosmids (35–40 kbp) and bacterial artificial chromosome (BAC) vectors (100 kbp) for library preparation. Once the libraries have been created, the next step involves maintaining them in a suitable host, namely *Escherichia coli*, *Pseudomonas* spp. and *Streptomyces* spp. among others (Abdelnasser et al. 2012; Danhorn et al. 2012; Lozada and Dionisi 2015).

3.2.3 Screening of Metagenomic Libraries

Mining of metagenomic libraries for biological data employ two key approaches, viz. function-driven and sequence-driven analyses. Function-driven approach initially screens the clones expressing a trait of interest following which the selected clones are subjected to sequencing and biochemical analyses. This approach swiftly locates clonal fragments with various commercial and medicinal uses by concentrating on natural compounds with relevant activity. In contrast, sequence-driven analysis searches the metagenomic libraries for clones of regions of interest by creating PCR primers for hybridization or probes from DNA sequences that are conserved (Schloss and Handelsman 2003).

Function-Driven Analysis

By using a function-based approach, it is possible to find novel medications whose properties cannot be predicted only by DNA sequence (Lam et al. 2015). It involves phenotypic-based detection techniques that make use of reagent dyes and enzyme substrates, which are frequently connected to chromophores. The products are detected with the help of spectrophotometric analysis following the reaction of compounds of the individual metagenomic clones with specific reagents (Quintero et al. 2022). Functional analysis also entails assaying for the anti-cancer, anti-proliferative, anti-inflammatory, antibiotic potentials among others of the extracts derived from the clones. These assays are performed in respective susceptible cells to understand the drug potential of the novel compounds being identified (Abdelnasser et al. 2012). Functional screening-based methodologies maximize the probability of discovering new classes of genes that encode for activities which have been characterized before or those that are novel in nature. This is possible since there is no bias involved in the screening process due to the absence of requirement of sequence-dependent information.

There are few drawbacks that are observed in this metagenomic approach of searching novel genes or gene products. Choice of host and selection of appropriate methodology for the screening of functions is one of the major limitation of metagenomic clone libraries as the expression of gene/s of interest in the host is affected owing to differential preference in codon usage, lack of gene expression due to failure of recognizing promoter region, reduced transcription rate of genes due to non-recognition of transcription factors and altered end product due to improper protein folding and failure in exporting gene products out of host cell. It was also observed in few cases that foreign gene products were toxic to host (Taylor 2015). Cloning of target genes is also affected due to presence of large quantity of target genes in the environmental samples and longer target gene and library size. Also, it is difficult to search an appropriate host that will be able to conduct correct post-translational modifications and expression of single Biosynthetic Gene cluster (BGC) (Alam et al. 2021).

Another problem with functional based approach is with respect to the intracellular accumulation of the desired products irrespective of the choice of host system. This problem can be resolved by enabling the release of the product from the cells with the aid of lysis compounds such as detergents which allows better secretion of compounds without affecting the native conformation (Johnson 2013). This strategy can be specifically employed to screen for novel compounds using the microtiter plate approach co-incubated with specific substrate/s. Additional aid of specific robots along with plate readers for the handling of liquids and microbial colonies enhances the efficiency of this process (Ngara and Zhang 2018).

Sequence-Driven Analysis

In sequence-based metagenomic approach, the detection of a particular gene encoding product of interest is achieved by using specific primers of the genes and its amplification by polymerase chain reaction or by hybridization techniques using specific DNA probes. Probe design is based on conserved sections of characterized genes or proteins. This method involves searching existing metagenomic data sets and/or nucleic acid databases for regions of interest instead of using heterologous expression of any genes or clones. This is followed by synthesis of target genes using chemical-based methods (Bayer et al. 2009).

Sequence-based metagenomics includes collection of genomic information of the microbes without culturing them. High throughput sequencing platforms like Sanger's sequencing, Illumina/Solexa, Roche 454 pyrosequencing, PacBio, Oxford Nanopore and others are used to generate high quality sequence reads which are analysed for the prediction of their putative functions using bioinformatic tools. The introduction of cloning-independent "next-generation sequencing technologies", for instance pyrosequencing, has made the need for metagenome library creation redundant especially if the sequence-based approach is to be employed (Danhorn et al. 2012). Table 1 summarizes the sequencing techniques available and their basic principles of working.

Vast number of datasets obtained from sequencing projects from diverse environments is now catalogued in various databases which are publicly available. Sequence-based approaches will be more insightful about microbial communities as trends in environmental sequences begin to appear. Some specific studies use a target gene as an anchor to identify the clones for the further generation of metagenomic libraries. With the help of PCR-based techniques, target genes are amplified and sequenced to construct and screen metagenomic libraries. Anchors used to construct such libraries are often an rRNA gene or a BGC so that researchers can easily identify the clone of interest.

The sequence-driven approach itself can be categorized into three types, viz. metabarcoding, whole metagenome analysis and single-cell genome analysis.

Table 1 Sequencing techniques used for metagenomic analysis

Technique	Principle	Read lengths	Reference
Sanger's sequencing method	DNA sequencing based on chain termination method due to inability of DNA polymerase to elongate a chain following incorporation of a dideoxynucleotide	400–900 bp	Heather and Chain (2016), Totomoch-Serra et al. (2017)
Illumina/Solexa	Sequencing by synthesis involving immobilization of DNA fragment on a surface followed by PCR amplification to generate clusters of amplified DNA which are sequenced by labelled reversible terminators	2 × 150 bp, 300 bp	Sharpton (2014), Escobar-Zepeda et al. (2015)
454/Roche Pyrosequencing	Application of emulsion polymerase chain reaction (ePCR) to generate clones of DNA fragments within microscopic beads which are individually subjected to pyrosequencing. All four nucleotide triphosphates are sequentially added; nucleotide incorporation causes pyrophosphate release, detection of which is coupled to sulfyrylase and luciferase systems that emit light	~100–150 bp	Escobar-Zepeda et al. (2015), Heather and Chain (2016)
Ion Torrent	Microscopic beads containing clonally amplified DNA are loaded into picowell plate, followed by nucleotide triphosphate incorporation. The release of protons results in change in pH which is then measured with the aid of a "complementary metal–oxide–semiconductor" (CMOS) technology	~400 bp	Sharpton (2014), Escobar-Zepeda et al. (2015), Heather and Chain (2016)
Oxford Nanopore	The technology employs a nanopore (nanoscale protein pore) embedded in an electrically resistant membrane which is then positioned in an electrolyte solution with constant current applied across the membrane. Single stranded DNA is allowed to pass through this pore. When a single nucleotide exits on the other side of the membrane the change in voltage is measured which is distinctive for each of the four nucleotides thus permitting accurate sequencing of a DNA strand	>100 kb	Singh and Roy (2020), Wang et al. (2022)
Single molecule real time (SMRT)/ PacBio sequencing	It involves detection of fluorescent signal emitted on incorporation of a fluorescently labelled nucleotide by a DNA polymerase which is bound to a Zero Mode Waveguide well	>10 kb	Escobar-Zepeda et al. (2015), Ardui et al. (2018)

Metabarcoding

Metabarcoding approach, also referred to as amplicon sequencing or targeted sequencing, entails targeting a particular region of the genome from the environmental community as an indicator of diversity and phylogenetic relationships. SSU rRNA genes such as 16S for prokaryotes and 18S for eukaryotes and internal transcribed spacer (ITS) regions for both prokaryotes and eukaryotes are most frequently used as markers for identification. Specific primers designed to target these regions are used to explicitly amplify only these regions followed by diversity analysis. Additionally, from bioprospecting angle, gene clusters that have been identified as being involved in the synthesis of potential drug molecules can also be used in the targeted approach. Following marker-based amplification and sequencing, the generated reads are subjected to basic bioinformatics pipeline including demultiplexing of barcoded samples, performing pair-end assembly, elimination of chimeric reads, checking quality of reads and performing read filters, clustering followed by sequence alignment with a reference database (Trindade et al. 2015; Maghembe et al. 2020; Francioli et al. 2021). However, this approach does have a limitation of low resolution of species identity due to relative short length of the markers.

Whole Metagenome Sequencing (WGS)

Whole metagenome sequencing (WGS) refers to sequencing the entire environmental DNA obtained from the niche under study. With the help of shotgun sequencing, WGS has become a faster tool as compared to earlier sequencing that required cloning as a prior step. The entire metagenome is subjected to random fragmentation to produce fragments ranging in size from 2 to 300 kb. These fragments are then sequenced followed by assembly and binning using computerized programs to identify overlaps and build contigs. Using gene annotation tools, putative gene functions can be identified that may correspond to novel metabolite synthesis (Sharpton 2014; Pereira 2019). Since the amount of DNA involved in whole metagenome analysis is enormous, it is but evident that it would contribute to greater insights into niche diversity (microbial and functional). It can also aid in elucidation of candidate pathways involved in novel secondary metabolite synthesis as well as improve the understanding of microbial interactions amongst themselves as well as with the surrounding environment that might influence improved and higher metabolite production (Maghembe et al. 2020).

Single-Cell Metagenomics (SCM)

SCM performs analysis on single cells separated from like cells as well as from their attachment to the surrounding natural matrix. Additionally, cell sorting techniques can be employed to identify and distinguish target cells from associated unwanted populations through cell-sorting techniques. It provides a new avenue for working with uncultured organisms and bioprospecting them at individual levels (Lozada and Dionisi 2015; Alam et al. 2021).

Overall, both the approaches of metagenomics (Fig. 2) highlight the potential of discovering essential genes from environmental samples and provides opportunities for developing novel drugs using these strategies.

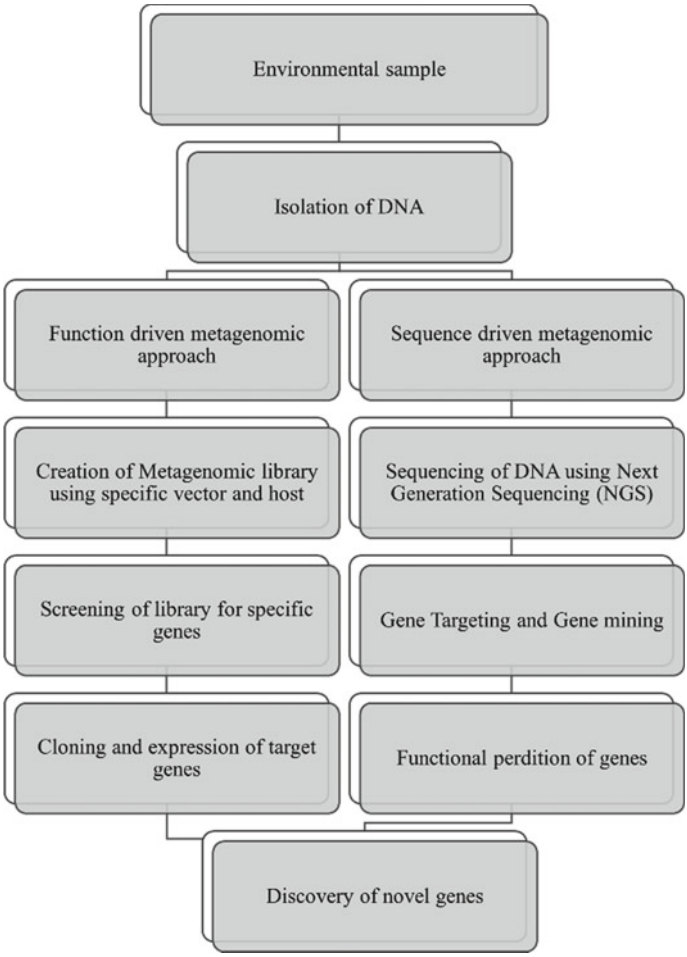

Fig. 2 Overview of gene discovery by metagenomic approach

3.3 *Metagenomics for Bioprospecting Drug Synthesizing Potential of Microbes*

3.3.1 Genome Mining

The process of querying and finding candidate genes that are active in the biosynthetic pathways for natural products or drugs from metagenome data is referred to as genome mining. This process involves the use of bioinformatics approaches to identify natural product gene clusters which encode for previously uncharacterized natural compounds from a known class of biosynthetic pathways or to predict an entirely new class of gene cluster/s involved in new biosynthetic pathways culminating in the identification of novel natural products. The process also involves analysing gene sequences that encode for enzymes encoded by genes and determination of gene products following experimental analysis (Albarano et al. 2020; Atanasov et al. 2021).

Identification of these biosynthetic genes is facilitated by screening for two characteristics namely,

(1) Presence of gene clusters and
(2) Presence of genes expressing enzymes (for instance, non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs)).

A group of genes found in the genome which are responsible for expression of proteins involved in the metabolic pathway producing products which are found to be chemically diverse is referred to as "biosynthetic gene clusters" (BGCs). These diverse chemical products are reported to exhibit varied bioactive properties ranging from antibacterial to cholesterol reducing properties to being an insecticide (Santana-Pereira et al. 2020).

There are many classes of BGCs which can be structurally classified. Bacteriocins, terpenes, β-lactam, NRPS, PKS, indole, and furans are some examples of the many (Belknap et al. 2020). Amongst these, NRPS and PKS are very widely used as targets to discover natural products since they are classically known to be involved in synthesis of antibiotics, immunosuppressants and molecules exhibiting excellent pharmaceutical value.

The polyketide synthases are enzyme complexes which catalyze the condensation of CoA fatty ester precursors to create the carbon skeleton of polyketide molecules (Gomes et al. 2013). Polyketides are metabolites found in nature that are characterized by numerous β-hydroxyketone or β-hydroxyaldehyde (–$H_2C(=O)CH_2CH(OH)CH_2C(=O)$–) functional groups. These compounds form the backbone of several anticancer, anticholesteremic, antifungal compounds as well as antibiotics, immunomodulators and antiparasite agents (Gomes et al. 2013). Streptomycin is a classic example of a polyketide.

Identification of gene clusters exhibiting enzyme activity comprises of finding the conserved domain sequence for putative function of the genes. Condensation domains from NRPS clusters and ketosynthase domains in PKS clusters are such

examples used as suitable targets for genomic analysis due to their conserved status. They may be exploited to differentiate between various NRPS/PKS pathways (Chen et al. 2020). An example of such a gene cluster was described by Qiao et al. (2011) found a BGS in the genome of *Aspergillus clavatus* that correlated to production of cytochalasin E and K. NaPDoS2, the second-generation Natural Product Domain Seeker, rapidly screens genomic, metagenomic or PCR amplicon-based sequences to identify and categorize condensation and ketosynthase domains.

Genome mining in the last decade has developed to the extent where it now facilitates better identification and prediction of SMBGCs "secondary metabolite biosynthetic gene clusters" through computational modes for in silico synthesis of putative predicted chemical drugs. Integration of numerous databases and web tools have been performed to improve the implementation of this approach. The following web tools are widely used in metagenome-based drug discovery.

(i) antiSMASH, "antibiotics and Secondary Metabolite Analysis SHell", (available at https://antismash.secondarymetabolites.org/) is a completely automated pipeline used for mining fungal and bacterial genome data for identification, annotation and SMBGCs analysis. It integrates and uses different tools for analysis such as NCBI BLAST+ , Muscle 3, HMMer 3, PySVG, FastTree and JQuery SVG. The most recent updated tool, antiSMASH v5, contains an elaborate group of manually curated and authenticated discovery guidelines for >50 types of BGCs (Medema et al. 2011, Palaniappan et al. 2020).

(ii) PRISM; "PRediction Informatics for Secondary Metabolomes", is an tool freely available on the web (available at https://bio.tools/prism_3), that performs prediction of metabolomes of secondary metabolites from the genomes/metagenomes. It predicts genes encoding natural product structures or BGCs from microbial genomes using BLAST for sequence homology, MUSCLE for Multiple Sequence alignment and HMMER tools for hidden Markov model generation and searches, and generates combinatorial libraries of structure predictions using Chemistry development kit and RDKit (Skinnider et al. 2015). This approach makes it possible to compare known natural drugs versus new ones. Most notable example is that of Malacidins and Humimycins which have been discovered using synthetic bioinformatics approach (Atanasov et al. 2021).

(iii) "Integrated Microbial Genomes Atlas of Biosynthetic gene Clusters" (IMG/ABC), is the world's biggest database which is publicly available and that contains experimentally determined as well as predicted SMBGCs. Since its launch in 2015, it has been updated in 2020 to incorporate the power of antiSMASH v5 for better prediction of BGCs within the IMG database. Its exceptional feature is that it incorporates both computationally predicted and empirically validated (through rigorous experimentation) SMBGCs in genomes and additionally also includes scaffold bins obtained from metagenome. It, thus, can reveal BGCs in populations and rare taxa which cannot be cultured (Palaniappan et al. 2020).

(iv) eSNaPD (environmental Surveyor of Natural Product Diversity) found at http://esnapd2.rockefeller.edu has been created to evaluate "Natural Product Sequence Tags" derived from metagenome using PCR. Profiles of diversity of BGCs hidden inside the (meta)genome is created by sequence comparison of sequence tags to referenced data of characterized BGCs in the eSNaPD data analysis pipeline (Reddy et al. 2014; Hover et al. 2018).

3.3.2 Novel Approaches: "Synthetic Bioinformatic Natural Product Approach" (Syn-BNP)

Once the genome of the microorganisms from the environmental samples is sequenced through high throughput techniques, the reads then are subjected to functional analysis pipeline. Steps in the pipeline may include BLAST+ sequence based searches for homology in gene cluster databases; further downstream analysis in this includes multiple sequence alignment, conserved domain analysis, phylogenetic tree construction, functional annotation which can be performed with the aid of numerous biological databases and bioinformatics tools (Araujo et al. 2018).

In Syn-BNP approach, functional annotation analysis (based on sequence homology) is used extensively to predict the putative function of the genes encoding enzymes in the SMBGCs. Further, metabolic pathway elucidation, bioproduction targets, biosynthetic routes and probable chemical reactions between substrates and the products formed may be performed manually or through computational prediction models like those used in biotransformation. Popular cheminformatic tools for the same are GEM-Path, BNICE.ch, ReactPRED, enviPath, NovoStoic, RetroPath2.0, Transform-MinER and novoPathFinder. These tools are used in recreating biosynthetic pathways using chemical reaction rules (Hafner et al. 2021).

In conventional natural product discovery approach, the natural product is purified through heterologous gene expression of the SMBGCs in a suitable host cell. However, in Syn-BNP the natural product is chemically synthesized using synthetic chemistry due to the silent nature of most natural product BGCs in the laboratory (Wang et al. 2022).

3.3.3 Success Stories of Drug Discoveries Using Metagenomics

Metagenomics has indeed revolutionized the process of drug discovery adding a new dimension to this field which desperately needed a boost. Table 2 provides a glimpse into some of the success stories of metagenomic-based drug discoveries.

The latest approach using Syn-BNP has also resulted in some monumental success stories. The study by Wang et al. (2022) stated the application of Syn-BNP to discover Lapcin (Fig. 3) which shows inhibitory effect on both topoisomerases I and II, exhibiting potent activity in distinct cancer cell lines. Their work entailed cloning of DNA from soil that would contain PABA-specific adenylation-domain sequences since they predicted that this sequence would result in a product

Table 2 Metagenomics-based drug discoveries

Drug	Source	Activity	Reference
Malacidin	Soil microbes probably unculturable *Myxobacterium* spp	Calcium dependent antibiotic	Hover et al. (2018)
Flouroquinolone	Polluted aquatic environment	–	Boulund et al. (2017)
Minimide	*Didemnum molle*	–	Donia et al. (2011)
Erdacin	Desert sand soil	Pentacyclic Polyketide activity not found	King et al. (2009)
Mycalamide And Pederin	Non culturable *Psuedomonans* linked with *Paederusfuscipes*	Anti-cancer	Singh and Roy (2020)
Bryostatin	Bacterial symbiont *Bugula neritina*	Anti-cancer	Hildebrand et al. (2004), Singh and Roy (2020)
Indirubin Violacein Deoxyviolacein Turbomycin A And B	Soil metagenome	Antibiotics	Lim et al. (2005), Singh and Macdonald (2010)
Diazepinomicin and Eco-7942	*Micromonospora* and *Streptomyces* spp	Anti-cancer	Singh and Macdonald (2010)
E-637 and E492	*Streptomyces* spp.	Anti-helminthic drug	Singh and Macdonald (2010)
Ecteinascidin ET-743, Polytheonamides Calyculin A	Bacterial endosymbiont of marine tunicates	Anti-cancer	Trindade et al. (2015), Singh and Roy (2020)
Psymberin	Different marine sponges	High cytotoxicity and selective antitumor polyketide	Trindade et al. (2015)
Divamides	Bacterial endosymbiont of marine tunicates	Anti-HIV	Smith et al. (2018)
Lapcin	Soil microbe	Dual topoisomerase inhibitor I/II	Wang et al. (2022)
Crocagins and Crocadepsins	*Chondromyces crocatus* Cm c5	Moderate inhibitory activity on the interaction between RNA and CsrA of *Yersinia pseudotuberculosis*	Surup et al. (2018)

(continued)

Table 2 (continued)

Drug	Source	Activity	Reference
Humimycin	Human gut Microbiome	Antibiotic active against MRSA	Chu et al. (2016, 2018)
Novel antitumor polyketides	Microbial symbionts of sponges *Discodermia dissolute, Pseudoceratina clavata* and *Theonella swinhoei*	Anti-tumor activity	Ferrer et al. (2009)

having N-acylated PABA and thiazole. Following cloning and identification, they used chemical synthesis to produce the final lapacin molecule. Their study also showed the advantage of combining metagenomics, bioinformatics prediction tools and databases, and total chemical synthesis of the compound to discover a novel metabolite from those BGCs which are uncharacterized. This opens opportunities to develop new pipelines and incorporate the use of Artificial Intelligence in discovering new drugs.

Another successful example of genome mining using the above approach was reported by Ueoka et al. (2022). Their study discovered structurally unprecedented polyketide alkaloids named janustatins with uncommon biological activity against cancer cells. Janustatins were found to kill the cells at sub-nanomolar concentrations but in a delayed and synchronized fashion. Their approach involved chemical prediction of PKS cluster from the genome of rhizhospheric marine bacteria *Gynuella sunshinyii* which had hitherto not been acknowledged with the aid of an *in silico* approach.

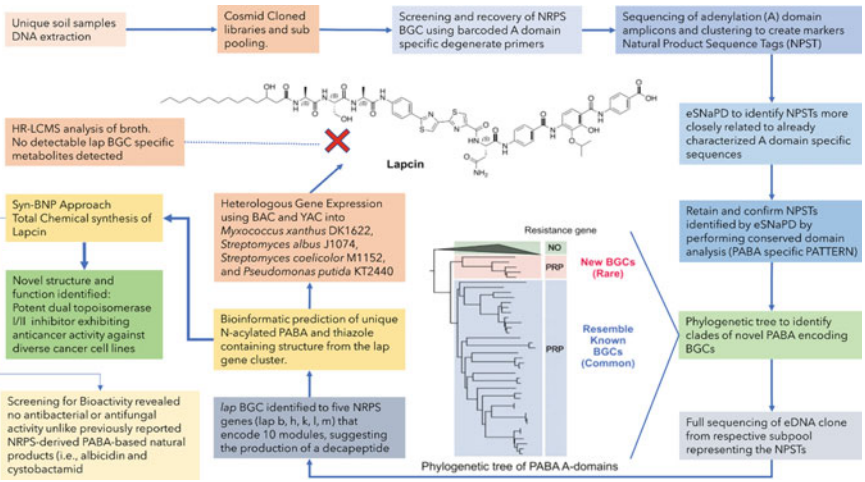

Fig. 3 Strategy used by Wang et al. (2022) to discover Lapcin using Syn-BNP approach

In one of the latest research findings, genome mining was successfully used for mining of anabolic pathways riddled with no identifiable enzymes resulting in identification of what is termed as "unknown-unknown natural products". The two unknowns refer to unidentified pathways and unidentified molecular structure of products. This approach recently identified a hypothetical fungal protein which in turn was found to be a novel arginine-containing cyclopeptidase which resulted in production of a novel natural product, cyclo-arginine-tyrosine-dipeptide (Yee et al. 2023).

4 Conclusion

The application of metagenomics for the identification and discovery of novel natural products has seen a revolutionizing enhancement in recent years due to the high speed and lesser expensive features of the newer sequencing technologies. Unculturable microbial symbionts and commensals of all life forms from different biomes are being studied using this approach and many have been reported to exhibit previously unknown metabolic pathways and SMBGCs leading to discovery of novel drugs. The success of naturally derived metabolite in drug discovery can be correlated to better understanding of the structural diversity of the molecules. Structural diversity in turn greatly influences how the drug interacts with biomolecules thereby mediating their mode of action (Mathur and Hoskins 2017). Metagenomics offers great potential in this regard and additionally shows great promise in identifying best lead candidates with low toxicity.

Despite the promising future that metagenomics portrays, microbial production of drugs still faces several challenges. These include low production titers, problems related to isolation of the natural products and/or structural identification. These, however, can be circumvented using approaches such as strain improvement, engineering precursor (primary metabolite) supply, pathway engineering, combinatorial biosynthesis using genetic engineering and mutasynthesis for novel product generation (Pham et al. 2019). Combining these approaches with metagenomics, proteomics, transcriptomics and metabolomics as well as the newer culturing techniques will surely enhance and enrich the manner in which drug discovery and development will proceed in the years to come.

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Potential of Heterocyclic Compounds as EGFR-TK Inhibitors in Cancer Therapy

Aniket P. Sarkate, Shashikant V. Bhandari, Poonam R. Inamdar, Pranali V. Kuthe, and Kalusing S. Padvi

Abstract Over the last few decades, cancer has become deadliest diseases worldwide. Vascular Endothelial Growth Factor Receptor (VEGFR), Human Epidermal Growth Factor Receptor-2 (HER-2) and Epidermal Growth Factor Receptor (EGFR) are among the many growth moieties available on the cell surface. Cell signaling pathways such as angiogenesis, metastasis, cell proliferation, and apoptosis depend on the EGFR. The heterocyclic compounds containing nitrogen, sulfur, and oxygen atoms have a remarkable ability to change their physiochemical and biological characteristics. There are many biological activities related with pyrazoline, pyridine, chromone, quinoline, quinazoline, coumarin, pyrazole, imidazole, indole, pyrimidine, and thiazole, including EGFR-TK inhibitors as anti-neoplastic drugs. This chapter provides an overview of potential heterocyclic EGFR-TK inhibitors with mechanistic and in silico investigations on structure activity relationship (SAR). It aids in creating novel EGFR-TK inhibitors with therapeutic promise. The creation of new potent and unique medication candidates with improved selectivity and efficacy will receive a boost from ongoing research and development.

Keywords Heterocycles · Epidermal Growth Factor Receptor (EGFR) · Quinoline · Pyrazole · Pyrimidine · Coumarin · Mutation · Non-Small Cell Lung Cancer (NSCLC) · Cytotoxicity

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1 Introduction

Cancer has risen to prominence throughout the past few decades as one of the world's deadliest diseases (Fitzmaurice et al. 2015; Roy and Saikia 2016). More than 10 million people worldwide died as a result of this major health issue caused by uncontrolled or abnormal cell growth (Cooper 2000). There is a growing demand for lead candidates with lower side effects and greater potency (Singh and Silakari 2019; Ayati et al. 2015) due to the severe side effects of chemotherapy and the acquired resistance to existing drug regimens caused by targets genetic mutations. Multiple growth factors are responsible for cell growth in cancer, which may be linked to an abnormality (Waris and Ahsan 2006). Growth factors including EGFR, VEGFR, and HER-2, among others, are available on cell surface and transmit signals from outside to cell inside (Lemmon et al. 2014). This mechanism is essential and required for healthy cellular functioning. Factors needed for growth are polypeptides that promote cell proliferation and/or differentiation in both healthy and malignant tissues (Wells 1999). Stanley Cohen, an American biologist, identified EGFR as the initial growth factor in 1968. Tyrosine Kinase (TK) activity is a characteristic of this group, which is also known as HER and c-erbB1. Three binding regions are present in the 1186 amino acid polypeptide chain that makes up the EGFR cavity: the hydrophobic region, the intracellular region that is capable of tyrosine kinase, and the extracellular ligand binding area (Nasser et al. 2020).

2 EGFR-TK Inhibitors as a Target for Tumors

The receptor has four subtypes, namely HER-1, HER-2, HER-3, and HER-4, which are all type-I tyrosine kinase receptors. The signaling system has several sub-families that causes tumour cells to proliferate, undergo apoptosis, spread throughout the body, migrate, and develop new blood vessels. Here, over-expression of EGFR and HER-2 kinase has different malignancies, counting breast, colon, bladder and lung (Li et al. 2008). Solid tumours with poor prognoses have strong EGFR and HER-2 expression (Nicholson et al. 2001).

2.1 Structural and Physiological Functions of EGFR

In EGFR kinase, EGFR/erbB-1 was most characterized (Sabbah et al. 2020). These receptors are single chain trans-membrane poly-peptide proteins with different domains such as:

1. Extra-cellular domains: responsible for the ligand binding that activates the receptors.

2. Trans-membrane domains: responsible for the dimerization's occurs in both receptors.
3. Intra-cellular tyrosine-kinase domains: which are phosphorylate to tyrosine moiety on substrates of the protein (Bogdan and Klambt 2001)

2.2 Extracellular Domains

The extracellular domain of EGFR, HER-2, HER-3, and has four sub-domains, according to their crystal structures (Ferguson 2004). The ligands bind to the EGFR structures, which can be seen on domains I through III and are in charge of ligand binding. Domain-projecting II's arms, which are each made of EGFR molecules, serve as points of interaction with additional EGFR monomers. The complexes of ligands and receptors that form the produced EGFR dimers are 1:1. The inactive versions of EGFR domain-I, domain-III, and domain-II interact with one another but are not in their closed conformations, which are changed to open conformations by ligand binding importance (Ferguson 2004).

2.3 Tyrosine-Kinase Domains

The EGFR's tyrosine-kinase domains are bilobate in form. The N-terminal lobe consists of five b-sheets and a helix, as opposed to C-terminal lobe, possessing helices that bind ATP in the cleft formation into two lobes. Hinge, catalytic site, activation loops, and kinases specificity pockets are crucial cleft structural components. Residues controlling the inhibitory actions and selectivity of ATP binding pocket antagonists interact with these binding pockets (Gotoh et al.1992; Zhang et al. 2006).

2.4 Activation and Role of EGFR

The most prevalent EGFR ligands include EGF, beta cellulin, TGF-a, epiregulin, amphiregulin, and heparin-binding EGF. When ligands are binding with extracellular domains then conformational changes occur in one EGFR members, which allowed to activation by both homo-dimerization and hetero-dimerization and other members of erbB (Sabbah et al. 2020). The EGFR transforms from a dormant monomer to an active homo-dimer in the presence of the ligands. The intrinsic intracellular protein tyrosine kinase activities of the EGFR trigger dimerization. The result shows, auto-phosphorylation of various tyrosine kinase residue in C-terminal domains of the EGFR. This auto-phosphorylation stimulates down-stream activations and signaling by several proteins that associates with phosphorylate tyrosine's

through them on phospho-tyrosine binding SH2 domain. The down-stream signaling proteins are initiates to different signal transduction cascades especially MAPK, JNK and Akt path way that leads to cell growth and DNA synthesis. The ligand initiates the receptor's conformational change by binding to the EGFR cell surface motif. The intrinsic tyrosine kinase of the cell, which is essential for cellular responses, may also be improved by the binding ligand (Hubbard 1999).

The receptor is homo- and heterodimerized when an EGFR ligand binds to it at the cell surface. The dimerized receptors are then internalized. Then internalization and dimerization of the intra-cytoplasmic EGFR-TK are auto-phosphorylated to each other (Franklin et al. 2002). An intracellular signal transduction cascade is initiated when phosphorylated tyrosine kinase residues attract with signal of transducer and activator of the intracellular substrates. These pathways control a number of physiological activities, including cellular growth, angiogenesis, apoptosis inhibition, gene expression, and cellular proliferation, all of which promote the development of cancer (Chan et al.1999). It facilitates the cancer cell metastasis, motility and adhesion. Healthy cells can be distinguished from tumour cells by the overexpression of the EGFR gene, enabling EGFR inhibitors to target cancer cells more accurately, stop their growth, and open out (Herbst et al. 2004).

The started receptor and ligand are either lysosomally degraded or eliminated, or via endocytosis and degradation procedures, they are recovered into the plasma membrane (French et al. 1994). Angiogenesis, metastasis, cell proliferation, and apoptosis are all EGFR-related processes that are supported by this pathway. Feedback and regulatory mechanisms can also be used to start the downstream regulation (both positive and negative regulation) (Seshacharyulu et al. 2012). Erlotinib, afatinib, gefitinib, osimertinib, and dacomitinib are just a few of the medications on the market that block the EGFR. However, they are linked to both acute and chronic side effects, such as dry skin, a rash on the skin, an infection of the finger or toenail, stomatitis, diarrhoea, and an inability to eat (Liu et al. 2017; Liang et al. 2017).

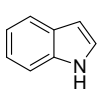
2.5 Different Strategies for Inhibition of EGFR-TK

In order to compete with natural ligands for a receptor's extracellular domain, monoclonal antibodies are utilised (Bennasroune et al. 2004). Small compounds that attach to intracellular tyrosine kinase domains, such as ATP binding sites, directly limit kinase activity. via targeting erbB receptors to deliver immune poisons in conjugate form. RNA interference (RNAi) or antisense oligo-nucleotides are lowering EGFR expression. The EGFR's downstream signalling cascade is inhibited by adaptor proteins, which blocks the signalling pathways. These are known type-I inhibitors that are reversible and bind to ATP binding sites to generate hydrogen bonds with kinase residues and hydrophobic contacts close to the area ATP binding sites. Small-molecule protein kinase inhibitors are classified according to their state of kinase activity. The type-II inhibitor works on enzymes that are stabilised in their inactive state. The Type-II and I enzymes functioned as ATP-competitive inhibitors that

were directed towards ATP binding sites (Zhang et al. 2009). Whose tumors that have additional copies of the EGFR genes. Allosteric binding sites rather than ATP binding sites are used by type-III inhibitors to bind to the protein, making them non-competitive ATP inhibitors. They bind to activation state enzymes without needing any conventional binding pockets.

3 Role of Heterocyclic Compounds as EGFR Inhibitor

Compounds, which are made up of nitrogen, sulfur, and oxygen atoms, have a variety of biological profiles and a remarkable ability to change their physiochemical and biological characteristics (Singh and Silakari 2018; Pathania et al. 2019). Due to the important significance in enhancement of distinctive lead motifs and considerable biological action in medicinal chemistry, heterocyclic congeners have helped medical chemists and researchers in drug design (Heravi et al. 2015).

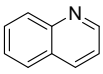

1H-indole


1,3,4-oxadiazole

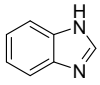

1,3,4-thiadiazole


isoxazole


triazole


quinoline


benzothiazole

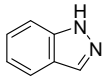

benzimidazole


imidazole


pyrazole


thiazine


oxanone


indazole

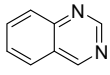

Morpholine

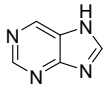

Tetrahydropyran


Pyridazine


Pyrimidine


Pyridine


Quinazoline


Purine


Indoline


Oxazole


Furan


Thiazole

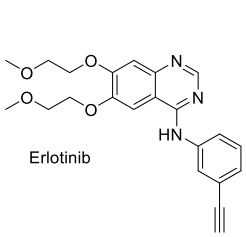

Oxirane

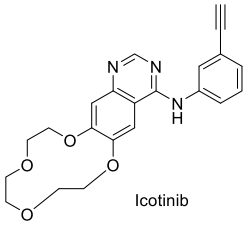
The molecules containing imidazole, pyrazole, quinazoline, pyrazoline, quinoline, coumarin, pyrimidine, and thiazole nucleus have significant biological activity

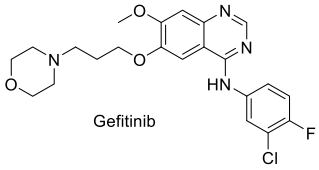
(Al-Mulla 2017; Sharma et al. 2020). EGFR plays an important role in the occurrence of human cancer (Luca et al. 2008).

1st generation EGFR-TKIs

EGFR 1st generation inhibitors like icotinib, gefitinib and erlotinib for cancer treatments, mutations of ATP binding pockets in EGFR exon 20 which leads from methionine and threonine substitutions on amino acids of T790M leads to drug inhibitions up to 50% of the cancer patients. Here, exon 20 was found to be inhibited by various first-generation EGFR-TK inhibitors at the same time as L858R mutations (Xiao et al. 2020).

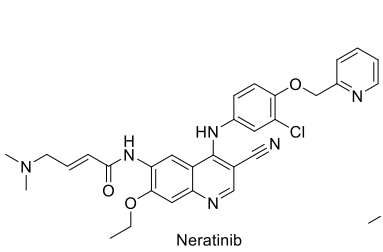


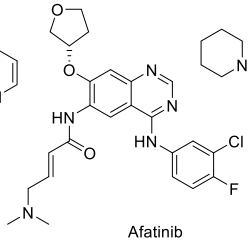


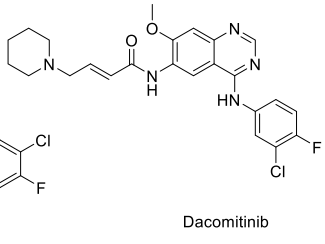


2nd Generation EGFR-TKIs

To combat tumour clones that are resistant to therapy, 2nd generation irreversible TK inhibitors containing Michael acceptor functional groups, many drugs, including as afatinib, neratinib, and dacomitinib, have been created. Poor kinase selectivity between the wild-type EGFR and the EGFR mutation T790M (Walter et al. 2013), resists the application of such inhibitors in clinical settings where side effects such as rashes on the skin and gastrointestinal damage have been recorded. In preclinical studies, it was discovered that second generation EGFR inhibitors efficiently block both T790M mutations and EGFR triggered mutations via interacting with Cys797 (Zhou et al. 2009).

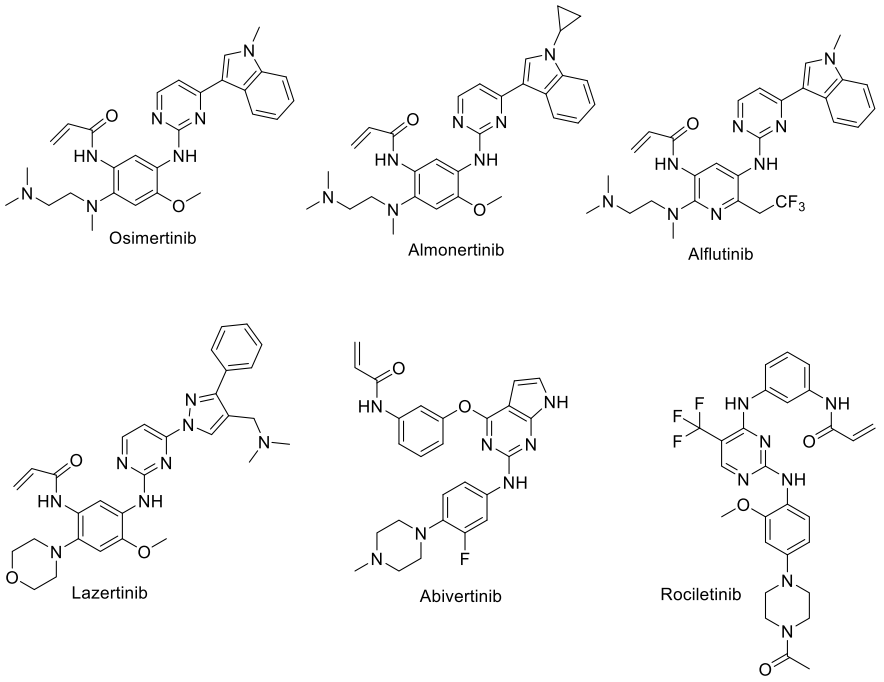






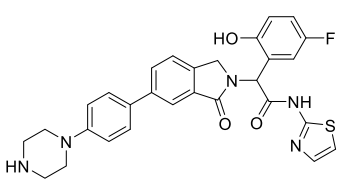
3rd Generation EGFR-TKIs

3rd generation TKIs spare the EGFR wild type and are mutant selective, targets EGFR mutations and EGFR-T790M, and include almonertinib, alflutinib, osimertinib (AZD9291), lazertinib, abivertinib, rociletinib, HM6171l3, ASP8273, and EGF816. They show less inhibitory effect on wild type EGFR so that overcome the toxicity limitation in 1st and 2nd generation EGFR TK inhibitors. WZ4002 was one of earliest drug candidate developed by in-vitro study (Niederst et al. 2015).



4th Generation EGFR-TKIs

4th generation EGFR-TK inhibitors were emerged like EAI045, CH7233163, TQB3804, BLU-945 and multi-targeted TK inhibitors like brigatinib that shows higher potency against targeted EGFR T790M/C797S mutations. EAI045 is an allosteric EGFR mutant selective compound and various allosteric EGFR inhibitors like DDC-01–163 and JBJ-04–125-02 are recently in trials (Jingyi et al. 2021). As ALK and ROS1 biomarkers, numerous kinase inhibitors such as birngatinib, crizotinib, lorlatinib, ceritinib, and ensartinib have been approved (Gettinger et al. 2016).



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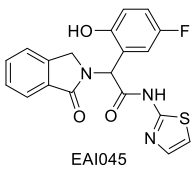
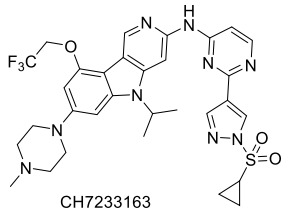


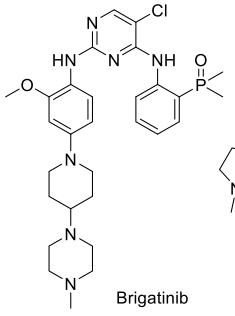
EAI045



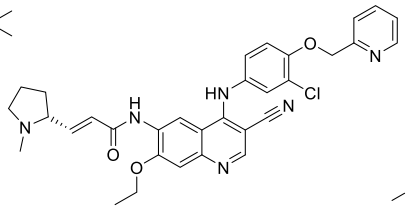
CH7233163

4 Multi-targets TK Inhibitors

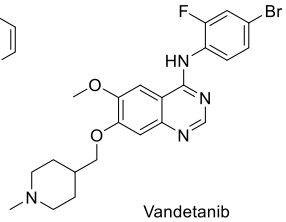
A second-generation ALK inhibitor called brigatinib is also used as a TK inhibitor with many targets. The brigatinib is highly potent ALK and EGFR kinases dual inhibitors like EGFR-T790M mutations and ALK-L1196M that found into pre-clinical and clinical trials. In comparison to crizotinib and crizotinib naive ALK rearranged NSCLC, brigatinib exhibits acceptable and improved clinical efficacy as well as acceptable safety profiles. The pyrotinib i.e., SHR1258 is a multi-targeted potent EGFR and HER-2 with good ADME qualities, two irreversible inhibitors are combined. The drug candidates were developing for the HER-2 positive in breast cancer. The pyrotinib shows that good safety and efficacy in chemotherapy for treatment of NSCLC with HER-2 exon 20 mutations with ORR of 30%. Multi-targeted inhibitors like ponatinib, cabozantinib, lenvatinib, sunitinib, sorafenib and vandetanib shows good efficacies in in-vivo studies (Drilon et al. 2013).



Brigatinib



Pyrotinib



Vandetanib

5 Quinoline

Numerous biologically active compounds are based on nitrogen-containing heterocycles (Afzal et al. 2015). Quinoline and its derivatives are among the most important heterocycles due to the numerous applications as anticancer agents (Solomon and Lee 2011; Thorngkham and Sirivat 2014). The quinoline moiety is a crucial structural component for many common medications, synthetic analogues with higher biological activity, and possible therapeutic effects. Many heterocycle-containing quinoline derivatives, inhibiting tubulin, VEGFR, protein kinases, topoisomerase I, etc., (Alonso et al. 2018) have shown significant anticancer action at a variety of locations. Camptothecin, irinotecan, topotecan and other quinolone-based cancer medications have received FDA approval. Quinoline, which has the chemical formula C9H7N, is an aromatic heterocyclic organic compound whose double-ring structure is produced when the benzene ring and two neighbouring carbon atoms in the pyridine ring ignite it, while others, for example Bosutinib, Cabozantinib, and Lenvatinib, and Tipifarnib, a farnesyltransferase inhibitor, are undergoing clinical trials (Moor et al. 2021).

Benzazine, 1-benzazine, and benzo[b]pyridine make up quinoline. In ether, alcohol, and other solvents, a yellowish greasy and hygroscopic liquid dissolves (Jain et al. 2019). Because of the nitrogen atom in quinoline, which attracts electrons via resonance, quinoline is important in medicinal chemistry. This aromatic bicyclic molecule has a moderate tertiary base structure and is an electron-deficient system. It has electrophilic and nucleophilic substitutions, which are likewise present in pyridine (Li et al. 2021).

5.1 Natural Sources of Quinoline

There are numerous distinct natural sources from which quinoline can be obtained. Cusparineine, Cusparine, Galipine, Echinopsine, Vasicine, Fabrifugine (Mathada 2022), Streptonigrin, Chelidonine (Santos and Adkilen 1932), Cinchona (Rao aet al. 1963), Lavendamycin (Balitz et al. 1982) and Nitidine (Douglas and Hughes 1991), Berberine derivatives (GuamaOrtiz et al. 2014) Chelerythrine, Camptothecin (Mao et al. 2020). These all alkaloids are effective in fighting cancer.

5.2 FDA Approved Quinoline Drugs

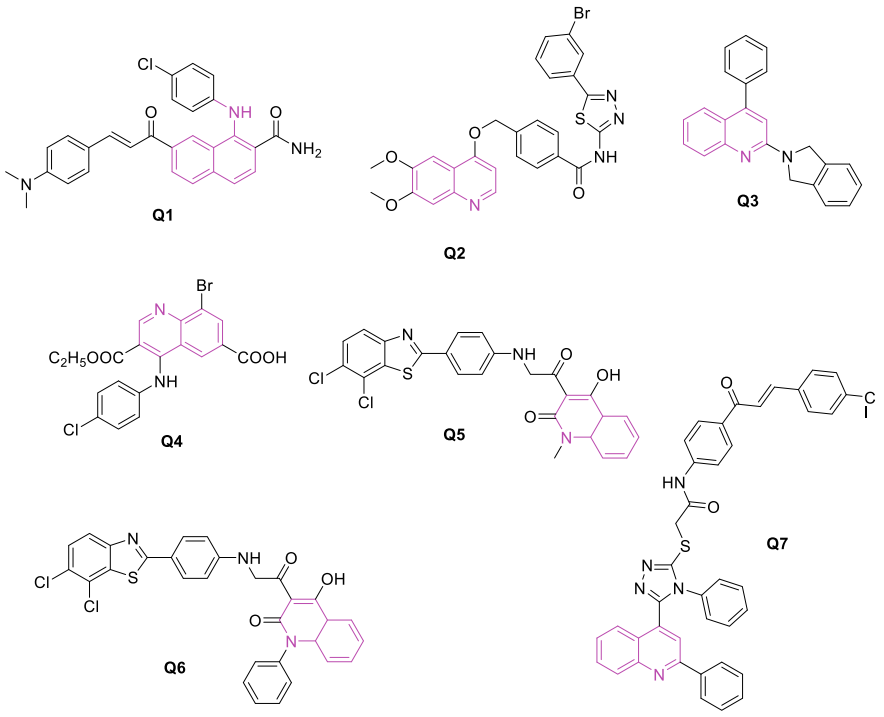
The quinoline moiety is an important core structure in many drugs having potent therapeutic activity for treatment of various diseases. The various anti-cancer drugs are developing for clinical use to treatment of various diseases (Dittrich et al. 2003; Singh et al. 2015; Wang and Liu 2016; Somagond et al. 2018). Neratinib,

Amsacrine, Irinotecan, Carbozantinib, Foretinib, Acridine carboxamide, AMG-208, Sitamaquine, Talazoparib which gives effect on blood cancer, breast cancer, acute leukemia, prostate cancer, thyroid cancer, central nervous system tumors, brain cancer, colorectal cancer, and lung cancer.

5.3 Quinoline as EGFR Inhibitor

Cancer cells' signalling pathway for cell proliferation. The family of tyrosine kinase receptors include of the receptor for epidermal growth factor. This enzyme can homodimerize with another EGFR or form a different dimer with another EGFR by changing the shape of the receptor with key similar ligands. Now that the crucial tyrosine groups have been autophosphorylated by EGFR, they are activated. The PI3K/AKT pathways, for example, are important signaling cascades triggered by these groups. The coordinated actions of these tracks help cells survive. Many activities, including cell death, cell growth, and cell multiplication, are frequently impacted by these receptor types, which are widely distributed in cell membranes (Guardiola et al. 2019). This protein has a substantial impact on a range of solid malignancies covering breast, lung, colorectal, neck, and many others, as well as head tumours.

By combining 4-substituted benzaldehyde with 6-acetyl quinoline-3-carboxamide under reflux conditions and catalysing the reaction using sodium hydroxide at 90°C, Ibrahim et al. developed and produced another family of 4-anilinoquinoline-3-carboxamide derivatives (Ibrahim et al. 2015). All derivatives were tested for breast cancer however, only Q1 molecule has shown reasonable effect. In comparison to ATP, the carboxamide based compounds shows strong inhibitory activity for anti-cancer drug development and having highly effective inhibitory property on the EGFR-TK, inhibiting growth by 67%, suggesting that it could be a potent antitumor drug. The quinoline compound based on methoxy was created by Xin-yang Li. Breast and lung cancer were significantly reduced in proliferation by the Q2 molecule. It works as a target inhibitor for both EGFR and HER-2, and thereby can decrease the growth of breast tumours and angiogenesis (Li et al. 2022).



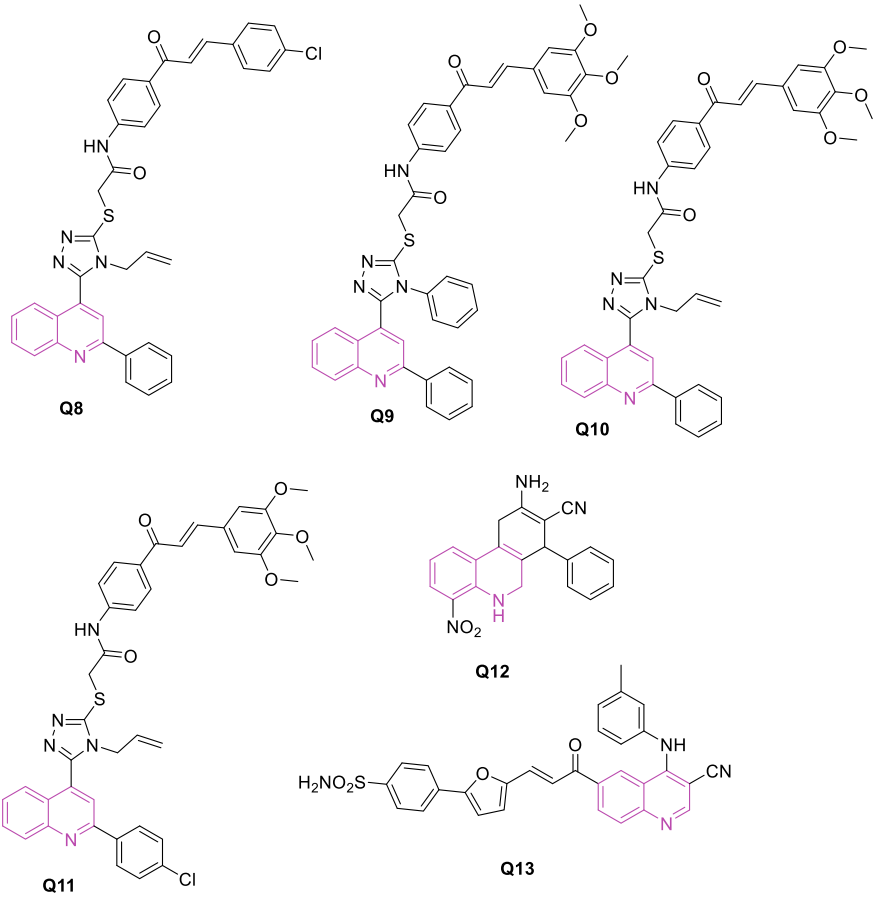
The aceto-acetanilide and polyphosphoric acid-derived quinoline-derived compounds were developed by Karnik and co-workers (Karnik et al. 2021a, b). 6-Chloro-2-(isoindolin-2-yl)-4-methylquinoline, a hydroxy substituted quinoline, has a significant inhibitory effect on the mutant EGFR kinase with an IC_{50} value of 0.91 M when aceto-acetanilide and polyphosphoric acid are combined to produce them.

Karnik and colleagues have created a potent anti-proliferative lung cancer treatment (Karnik et al. 2021a, b). The chloro substituted quinoline compound was created by chlorinating the quinoline compound with phosphorus oxychloride. The quinoline derivative (Q3), which has a potent anticancer effect on lung cancer, was subsequently produced in the presence of copper ion.

Khaled R. and his colleagues developed a special class of 4-(4-substituted-anilino) quinoline derivatives from their amine counterparts using the Gould-Jacob reaction (Abdellatif et al. 2017). The cytotoxicity of every chemical developed against breast and lung cancer was examined. The MCF7 and A549 activity of the substances under investigation was extremely varied. The IC_{50} values for the Q4 chemical against lung and breast tumour cell lines were 3.42μM and 5.97μM, respectively. Moreover, experiments on molecular docking were completed, and the outcomes matched the in-vitro cytotoxicity.

A novel series of quinoline-based compounds has been created by the research team of Bolakatti et al. (2021). These results allow for the conclusion that while the Q6 molecule exhibited a better anticancer effect than other derivative candidates, the

Q5 compound binds considerably. The cytotoxicity of each derivative was evaluated against the cancer cells WRL-68 and MCF7. Docking experiments were done to find out more about the way in which the chemicals in the title bind to the target enzyme's active site. Quinolones lay foundation for designing potential hybrids as precursors to develop future antibiotics and cancer formulations.

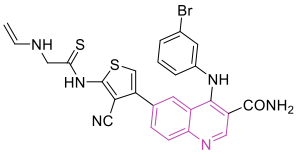


Mohassab et al. (2021) has demonstrated the synthesis of triazole-based quinoline derivatives from acetophenone and isatin under reflux conditions. On various cell lines, the compounds that were made have anti cancer properties that range from average to superior. The Q7–Q11 class of compounds has powerful anticancer activity against lung, breast, and colon cancer. The chemicals Q7, Q10, and Q11 each have a significant binding site, and docking studies showed as these substances had a substantial BRAF and EGFR kinase site centre. Erlotinib's pattern is similar to this one.

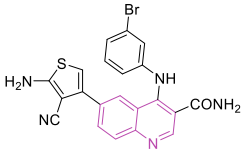
Thangaraj Arasa kumar and colleague shave developed innovative pyrazolo[4,3-c] derivatives likewise pyrano[3,2-c] quinoline (Arasakumar et al. 2017). Derivatives were created and synthesized under micro wave conditions with medium to high yields. Other pyrano quinoline compounds have been produced by a multicomponent, one-pot method. The cytotoxicity of the Q12 chemical towards breast and lung cancer cell lines was examined. Majority of the drugs were mild to severe in cytotoxicity to these cell lines. The pyrano[3,2-c] quinoline compounds were evaluated in this work and found to have significant activity. Moreover, they promoted apoptosis. Using molecular docking experiments, the EGFR inhibitor's molecular interactions were discovered.

Aly et al. (2017), they combined benzyl chloride 5-(3- or 4-substituted phenyl) furan-2-carbaldehydes, 4-(N, N-dimethylamino) benzaldehyde, and malononitrile to produce a 3,4,6-trisubstituted quinoline molecule. Q13–Q18 demonstrated a potent breast cancer anticancer agent with a high yield. Particularly, in EGFR, Q-13 has an IC_{50} of 2.61 μM for a furan derivative, Q15 has an IC_{50} of 0.49 μM for a thiophene derivative, and Q18 has an IC_{50} of 1.73 μM for a benzyloxy derivative. Due to their existence, quinoline-3-carboxamides are likely to exhibit a cytotoxic effect on breast cancer.

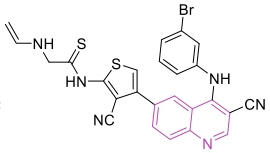
Esraa A et al. have written about how to make 1,3-diazanapthalene derivatives, 1-benzopyridine, and benzo-based iso-indazole (Abdelsalam et al. 2019). The anti-cancer characteristics of each of the generated compounds were assessed using the breast HCT116, HepG2, and Caco-2 carcinoma cell lines. Compounds Q19 and Q20 have strong anti-proliferative actions on breast cancer, with corresponding IC_{50} values of 7.70 0.39 and 7.21 0.43 M. ATP binding site on kinase domain of the enzyme was examined during docking procedure to determine the binding mechanism and any potential linkages. In accordance with (Kshipra et al. 2021), new substituted quinoline compounds are made in 2021 using substituted chloride and react with phenyl benzoate derivatives at room temperature in the presence of acetonitrile as a solvent and sodium methoxide to form CAC bonds. The said reaction is Suzuki coupling reaction.



Q15



Q14



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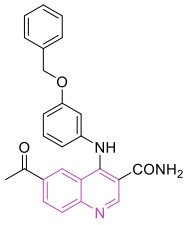
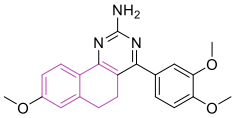
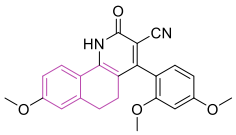


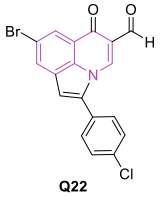
Q17



Q18



Q19



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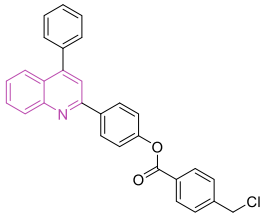
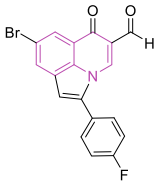


Q20



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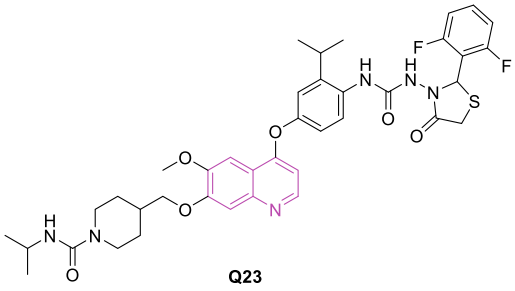
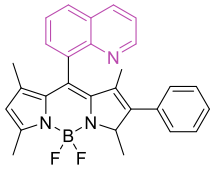
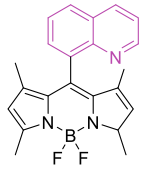


Q23



Q24



Q25

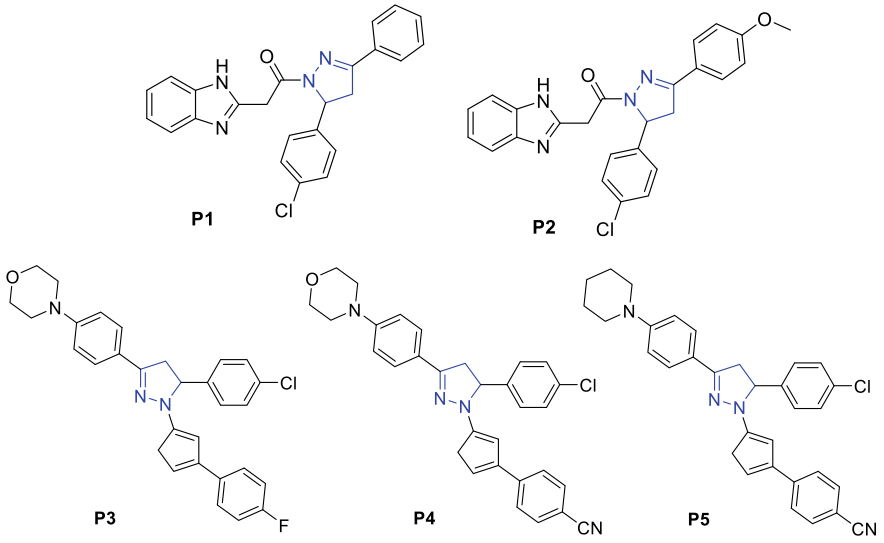
Malose J. et al. described novel sequence of pyrrolo-based quinoline compounds. They were examined using NMR, MS, and IR. The lattice pattern of it was confirmed using XRD on a single crystal. The samples were tested for in vitro cytotoxicity toward human embryonic breast and kidney cancer cell lines. These compounds are capable of destroying breast cell lines, according to an early apoptotic investigation utilising a DNA laddering assay. The Q22 and Q23 substances strongly inhibited the cyclooxygenase-2 and VEGFR-2 enzymes. Both in humans and in animals, the COX-2 inhibitory properties control breast cancer cells from spreading. The quinoline-based BODIPYs' anticancer effects on Hela cells have been shown to be potential EGFR inhibitors. Meso-substituted BODIPY can be easily produced through an acid-catalyzed condensation reaction using quinoline-based meso-substituted BODIPYs that were made by synthesizing 2-quinoline carboxylic acid in $SOCl_2$. The mixture was agitated in nitrogen at room temperature for 48 h. According to Gossauer et al., condensation to quinoline-8-carbonyl chloride and 2,4-dimethylpyrrole using DCM as solvent while being exposed to i-PrNEt2 is required for the non-oxidative synthesis of Q24. On Suzuki coupling of the 2-bromo BODIPY derivative, axial chirality is introduced by further de symmetrizing one of the two pyrrole units through bromination. These outcomes led to the formation of the asymmetric and axially chiral compound Q57 when combined with phenylboronic acid. The compounds Q24 and Q25 share some intriguing physicochemical properties with Lipinski's drug in terms of oral bioavailability. The findings of testing Q24 and Q25's in vitro anticancer activity on HeLa cells revealed significantly reduced cell viability than the control, with values of 18.51 and 6.52 m, respectively.

6 Pyrazole as EGFR Inhibitor

One endocyclic double bond, two nitrogen atoms nearby, and a plain five-member heterocyclic core are all present in pyrazoline. According to Akhtar et al., various pyrazoline compounds have been synthesised and have undergone extensive pharmacological testing as EGFR inhibitors over the years. A group of antiproliferative and EGFR inhibitory benzimidazole analogues coupled with pyrazoline structures were depicted by Akhtar et al. (2018). Studies on kinases are showing that the molecules of P1 and P2 had IC_{50} values 0.97 and 1.70 μM, respectively. The standard drugs erlotinib were shows greater inhibitory effects with $IC_{50} = 0.011$ μM against EGFR-TK. The cytotoxicity tests on a variety of tumour cell lines, including A549, HepG2, MDA-MB-231, MCF-7, and HaCaT, demonstrate a moderately weak effect against MCF-7 cell lines, with IC_{50} values of 9.60 M and 15.50 M. Cell cycle analysis also demonstrates that A549 cells can be made to undergo apoptosis by stopping the cell cycle at the G2/M phase. SAR analysis of compounds reveal that when chlorine atoms are substituted at "para" positions, the phenyl ring exhibits a high inhibitory effect. When chlorine atom is replaced by fluorine and bromine where activity is decreases. Molecular docking studies showed that with binding energies of DG = 34.581 kcal/mol and H-bonding and interaction greater binding contact to the EGFR

binding pocket of PDB: 1M17 was demonstrated. In the cardiomyopathy experiment, Compound P1 showed no necrosis or inflammation compared to the positive control medication doxorubicin (Akhtar et al. 2018).

Using erlotinib as the standard drug for biological evaluation as dual EGFR, i.e. HER-1/HER-2 inhibitors against A549, A375, and MCF-7 cell lines, Sever and coworkers developed pyrazoline congeners containing a thiazolyl moiety. Contrary to erlotinib, which had an IC_{50} of 22.35 2.84 M, compound P3 and P4 showed nearly identical inhibitory action against MCF-7 cells with IC_{50} of 9.59 1.95 and 8.05 1.47 M, respectively. Compounds P3 and P4 demonstrated higher inhibitory capacity in opposition to the A375, according to in-vitro cytotoxic tests. Moreover, compound P5 showed a relatively robust suppression of the A375 cell line with an $IC_{50} = 38.72$ and 5.47 M as compared with the positive control.

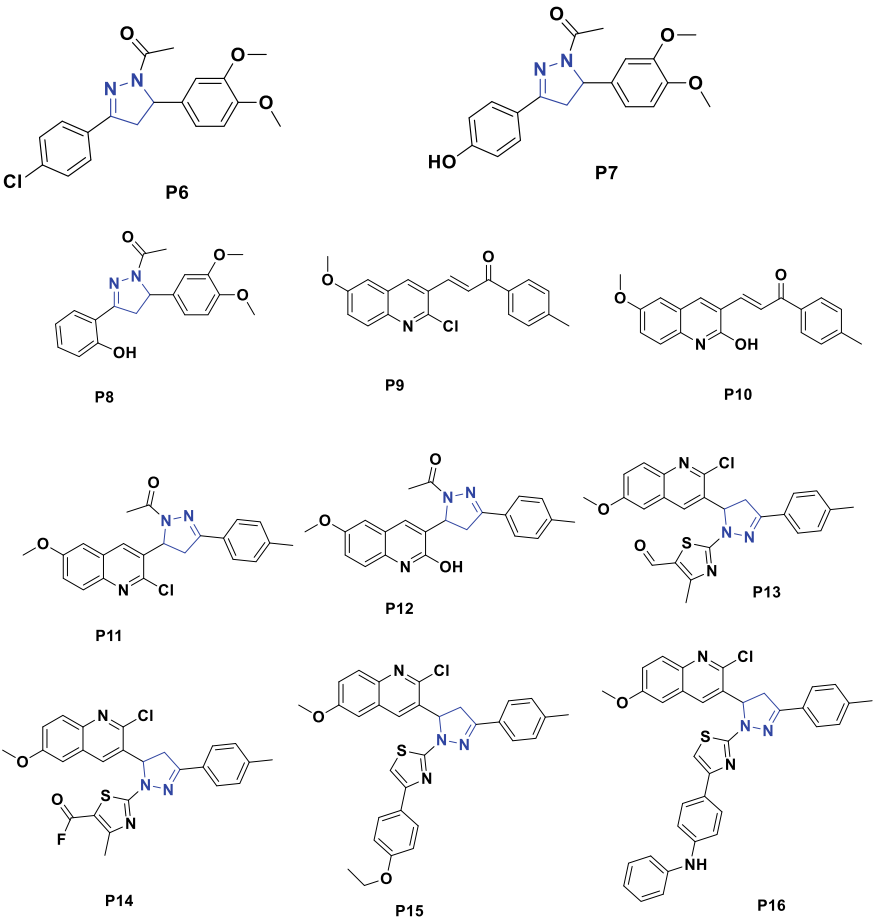


Moreover, highly aggressive candidates' efficacy against Jurkat and PBMC cell lines in blood cells indicates possible strong tumour selectivity. Apoptosis testing results indicated that compounds P3, P4, and P5 exhibited considerable apoptotic potential in the cancer cells A549 and MCF-7. The RTKs EGFR, HER-4, KDR, PDGFRb, IGF1R, PDGFRa, InsR, and HER-2 were also used in a kinase test, reveals that compound P4 is more responsive to multi-targeted EGFR and HER-2 inhibitors,

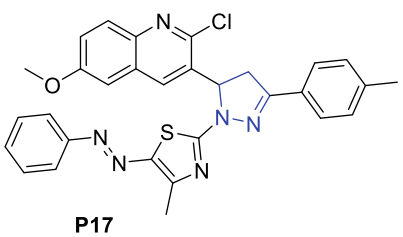
with IC_{50} values of 4.34 0.66 M and 2.28 0.53 M respectively. On the other hand, compound P5 exhibited significantly greater inhibition toward SAR studies revealed that piperidine ring had lower anti cancer activity than morpholine ring at R position. The activity was increased by substituting a cyanophenyl group at the R' position instead of nitrophenyl or methylphenyl groups. The anticancer activity of the piperidine ring at the R site was less potent than that of the morpholine ring, per SAR assays. A cyanophenyl group was substituted at the R' position in place of a nitrophenyl or methylphenyl group to increase activity. Compound P4 was precisely connected to the ATP-binding pocket of the EGFR and HER2 receptors, according to molecular docking studies, and it was identified as an amazing dual inhibitor (Sever et al. 2019).

Wahyuningsih and others reported some congeners of N-acetyl pyrazoline (P6-P8) as agents against proliferation. Compound P6 strongly inhibited T47D, HeLa, and MCF-7 cells with IC_{50} values of 26.51 g/mL, 40.47 g/mL, and 31.19 g/mL respectively. While testing compound P7 on cancer lines with IC_{50}values of 100 g/mL or higher, there was no observable cytotoxicity. Compound P8 substantially inhibited the MCF-7 cell line (IC_{50}= 94.02 g/mL) in the authors' in-vitro anticancer evaluation against vero (normal) and cervical cancer (HeLa), but it had no cytotoxicity against the T47D or HeLa cell lines. According to SAR studies, the most potent antitumour activity was obtained by substituting with a chlorine group at the R1 position. According to findings of molecular docking studies of the compound P6 is perfectly bind with EGFR kinase by H-bonding at Met 679 and interacting with Lys721 amino acids in the receptors binding pocket with a binding energy less than 7.24 kcal/mol (Wahyuningsih et al. 2019). The pyrazoline congeners (P9-P17) that George and his colleagues produced were pharmacologically evaluated as EGFR inhibitors and anti-tumor drugs with or without a thiazole scaffold. A number of P1, P6, and 5-tri substituted pyrazoline congeners (P18, P19, P20, P25, P26, P28, P29, and P30) were reported latter for cytotoxicity and EGFR inhibition of human breast tumor cells (MCF-7) and normal fibroblast cells (WI-38) using MTT method. The majority of compounds having average to strong anticancer activity against the breast cancer cell lines i.e. MCF-7 with IC_{50} values ranging between 3.79 and 37.73μM, compared to erlotinib and staurosporine as standard drugs, which had IC_{50} values of 4.74 μM and 10.61 μM respectively, according to cytotoxic studies. In addition, compounds P20, P25, P26, P28 and P29 with IC_{50} =4.46 0.11 μM, 4.33 0.06 μM, 4.53 0.28 μM, 3.93 0.07 μM and 3.79 0.04 μM. It exhibit the activities against MCF-7 cell lines to erlotinib with IC_{50} = 4.74 0.14 μM. Using erlotinib as the standard drug, the most potent compounds P20, P25, P26, P28, and P29 were analyzed for tyrosine kinase inhibitory activity. The findings showed that P20, P25, P26, and P29 had promising EGFR resistance with IC_{50} of 0.33, 0.46, 0.34 and 0.33 μ Min contrast with erlotinib with IC_{50} value of 0.23 M as the reference drug. However, P28 had moderate EGFR kinase inhibition potential with IC_{50} = 1.39 μM and superior anticancer activity with IC_{50} = 3.93 μM. SAR experiments demonstrated that the 4-methoxyphenyl group on 5th position of pyrazoline inhibited significantly more than un-substituted 4-chloro moiety. The carbothioamide moiety at the N1 position of the pyrazoline ring has a higher degree of lipophilicity than the carboxamide group. According to molecular docking studies P20, P25, P26, and P29, the EGFR

receptor's binding pocket contains hydrophobic interactions with Leu 764, Met 742, Val 702 and Ala 719 amino acids, as well as H-bonding interactions with Met769 (George et al. 2020).



Salum and co. pyrazoline congeners (P33–P42) and mono-chalcones (P31 and P32) as agents against proliferation. Congener P37 had a lower inhibitory potential than reference gefitinib ($IC_{50} = 0.75$ 1.8 M) against MCF-7 cells when tested against breast cancer cells MD-MB-231, MCF-7, and normal breast cells MCF-10A by using MTT assays. According to molecular docking studies, P37 occupied the active site P26, and P29 demonstrated EGFR kinase inhibitory activity with IC_{50} values of 0.33, 0.46, 0.34, and 0.33 M, compared to the standard drug erlotinib's IC_{50} value of 0.23 M. However, P28 had moderate EGFR kinase inhibition potential ($IC_{50} = 1.39$ M) and superior anticancer activity ($IC_{50} = 3.93$ M). SAR experiments demonstrated that, at 5th position of pyrazoline the 4-methoxyphenyl group are significantly inhibited more than unsubstituted 4-chloro moiety. The lipophilicity of the carbothioamide moiety at the N1 site of the pyrazoline ring was greater than that of the carboxamide group. According to molecular docking studies, P29, P25, P20 and P26, the EGFR receptor's binding pocket contains hydrophobic interactions with Leu764, Met742, Val702 and Ala719 amino acids, as well as H-bonding interactions with Met769 (George et al. 2020).



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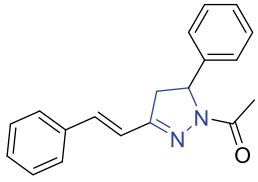

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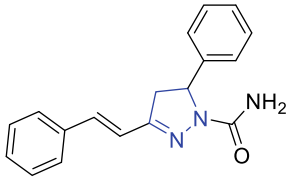

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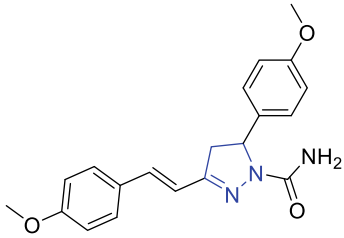

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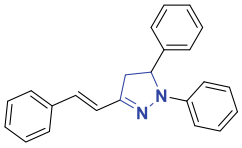

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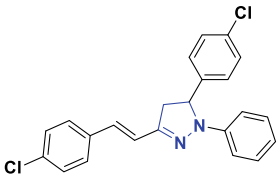

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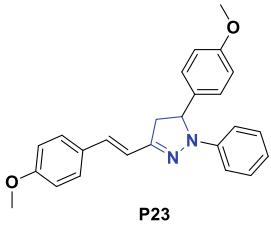

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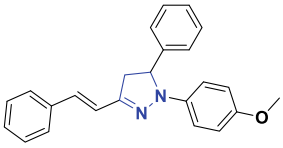

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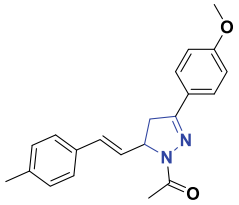

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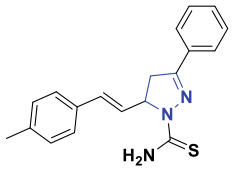

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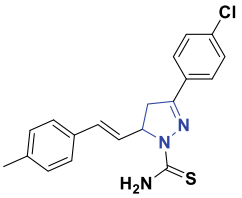

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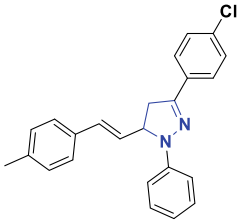

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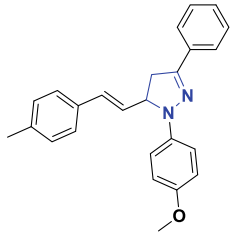

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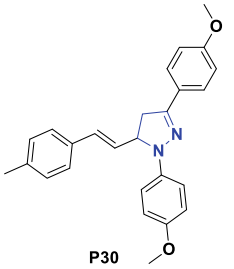

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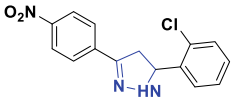

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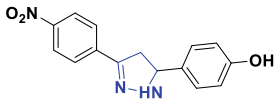

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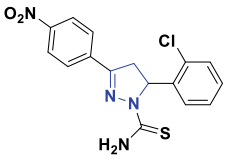

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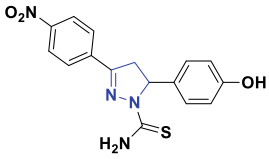

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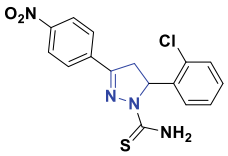

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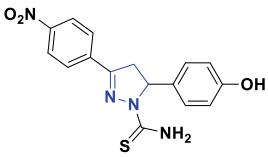

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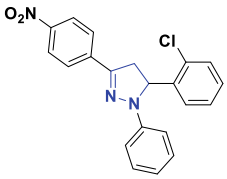

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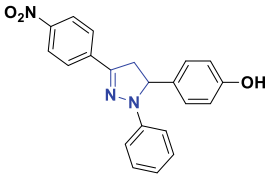

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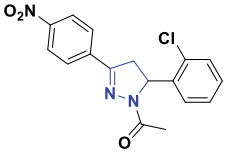

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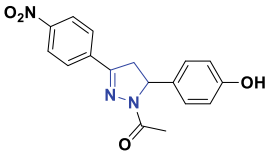

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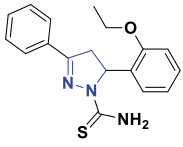

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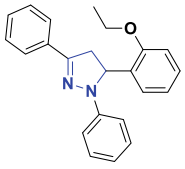


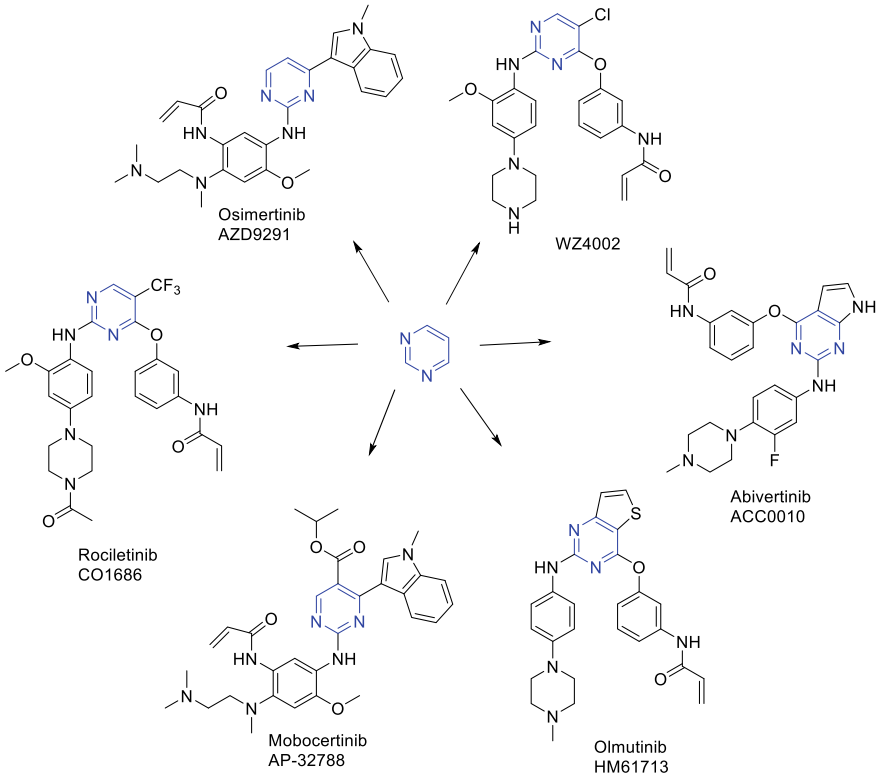
P42

7 Pyrimidine as EGFR Inhibitors

Pyrimidines are an alluring class of nitrogen-containing heterocyclic chemicals that serve a variety of biological purposes (Kumar et al. 2018). Tyrosine kinase inhibitors include EGFR. In this case, the ability of several pyrimidine derivatives to prevent a specific EGFR tyrosine kinase mutation was evaluated. Selective pyrimidine-based compounds are used as EGFR tyrosine kinase inhibitors, and numerous techniques are applied for improvement and optimisation. With improved EGFR inhibitory efficacy, the various forms of selective pyrimidine-containing derivatives have been assessed for therapeutic use. The pyrimidine derivative EGFR-TK inhibitors are identified and classified as 2-anilinopyrimidine, 2,4-diarylaminopyrimidine, 4,6-diarylpyrimidine, pyrrolopyrimidine, pyrazolopyrimidine, pyridopyrimidine, furopyrimidine, thiopyranopyrimidine, thienopyrimidine and miscellaneous (Ayati et al. 2020).

7.1 Pyrimidine Derivatives

First-generation irreversible kinase inhibitors like lapatinib, icotinib, gefitinib, and erlotinib are employed to cure patients afflicted with NSCLC vulnerable to EGFR mutation (Engelman et al. 2007). They differ structurally from EGFR-TK inhibitors based on irreversible pyrimidines, such as rociletinib (Sequist et al. 2015), osimertinib, and pyrrolo-pyrimidine-based abivertinib (AC0010) (Xu et al. 2016). When rociletinib and osimertinib's safety profiles by a variety of metabolic pathways and resistance mechanisms are compared with those of patients with EGFR-positive NSCLC, they offer a different therapy option for those who experience acquired inhibition to first-generation EGFR-TK inhibitors (Camidge et al. 2014). Afatinib, neratinib, and dacomitinib are examples of 2nd generation irreversible inhibitors whose clinical relevance is constrained by their narrower therapeutic window and consequently lower selectivity for mutant EGFR L858R/T790M. The shortcomings of inhibitors with decreased kinase selectivity, such as osimertinib (AZD9291), WZ4002, olmutinib (Kim 2016), CO1686, and EGF816 were addressed by the third generation EGFR-TK.

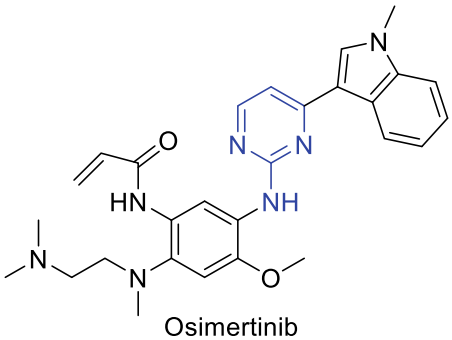


Mobocertinib (Alternative names: TAK 788, AP-32788, TAK-788 and TAK788) (Other names: TAK 788, AP-32788, TAK-788 and TAK788). The FDA approved osimertinib (AZD9291, TAGRISSOTM) in November 2015 as the first pyrimidine-containing third-generation EGFR inhibitor with exceptional clinical efficacy against NSCLC (Malapelle et al. 2018). With selectivity for EGFR wild type, EGFR-TK drugs are irreversible for both EGFR mutation and T790M resistant mutants. The compound abivertinib (AC0010), which contains pyrrolo-pyrimidines, was discovered and developed as a highly potential and selective irreversible EGFR inhibitor through molecular modelling approach and biological evaluation through biochemical and cellular assays, screenings, and in vivo animal model studies.

7.2 Amino-Pyrimidine Derivatives

To combat and reduce the negative effects, AZD9291 (Osimertinib, formerly known as mereletinib) was created by introducing a sulfoxide side chain at C-4 position of aniline (Cross et al. 2014). Several heterocycles were produced and tested against

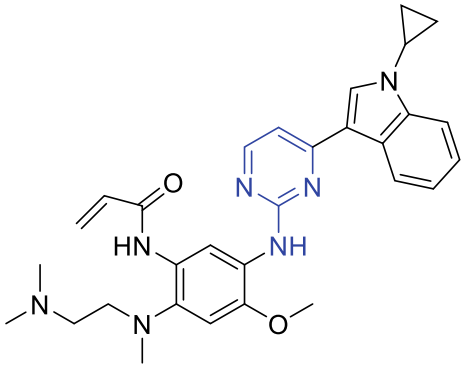
various protein kinases in the 2-amino pyrimidine-based derivatives. A mono-anilino-pyrimidine medication called osimertinib typically links with cysteine 797 residues in ATP binding sites on the covalent bond forms of EGFR kinases. Osimertinib inhibits EGFR phosphorylation in H3255 (L858R) and PC-9 (Del19) cell lines, with respective IC_{50} values of 13 to 54 nmol/l. IC_{50} potencies of 15 nmol/l for PC-9 VanR (Del19/T790M) and H1975 (L858R/T790M) resistant cell lines activities were reported in.



Osimertinib
AZD9291

PM1

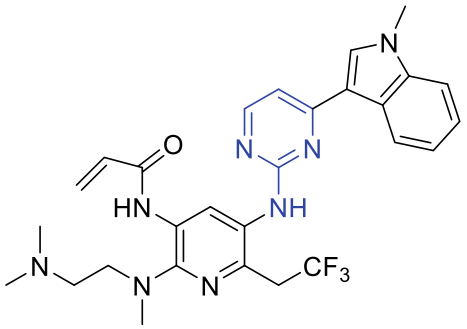
Osimertinib has an IC_{50} range of 480–1865 nmol/l, which suppresses the phosphorylation of the EGFR in both mutant and wild-type cell. It is extremely effective and selective for mutated receptors. Osimertinib is degraded to form the circulating metabolites AZD5104 and AZD7550. Although AZD5104 exhibits stronger potential activity against EGFR Del19, T790M mutant and wild type of EGFR, the biochemical assay of AZD7550 has good potency and selectivity that is equivalent with osimertinib. The potential application of osimertinib in conjunction with immune inhibitors and EGFR-TK inhibitors in treating NSCLC patients with the help of EGFR mutations. Osimertinib is a 3rd generation EGFR-TK inhibitor targets EGFR activating and EGFR T790M mutation. Osimertinib exhibits greater inhibition of EGFR-T790M mutations in NSCLC cell lines. Patients treated with EGFR-TK inhibitor for prolonged time because of the EGFR T790M mutation, the EMA and FDA have approved the drug osimertinib for first- and second-line therapy. The treatment efficacy and selectivity of osimertinib for the mutant EGFR have improved. Almonertinib is an osimertinib derivative that replaces the cyclo-propyl group on the indole ring with methyl groups. A metabolite of almonertinib called HAS-719 is less effective than almonertinib at treating various EGFR mutations. After progressions on earlier EGFR-TK inhibitors, almonertinib was prescribed for the treatment of EGFR T790M + NSCLC on March 19, 2020. The recommendation was attributed to the findings of APOLLO phase-II expansion section of the phase-I to phase-II studies.



Almonertinib

PM2

Alflutinib has a tri-fluoro-ethyl group in place of the methyl group and a pyridyl ring in place of the phenyl ring. The alflutinib pre-clinical trials have not been released. 130 individuals were identified in the phase-I, NCT02973763 and phase-I and phase-II study, NCT03127449 at five dose levels. On March 3, 2021, alflutinib was approved for usage in China to treat EGFR T790M + NSCLC. NCT03787992 is the identification for the phase-III trials contrasting alflutinib and gefitinib as initial therapies for patients with EGFR + NSCLC.

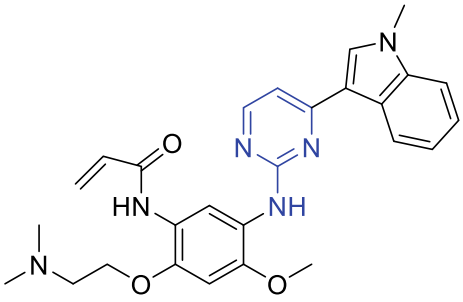


Alflutinib

PM3

Rezivertinib has an oxygen atom in place of the N-methyl group that served as the di-methyl amino-ethyl chain's connector at the phenyl ring of osimertinib. The EGFR mutation in different cells is being inhibited by rezivertinib in pre-clinical research. Phase I to phase II trials for the NCT03386955 study included 172 patients with EGFR T790M + NSCLC. Up to 52 of the 83 participants who were recruited in the 180 mg daily cohort studies were identified for evaluation. Rezivertinib with

gefitinib phase-II trials have totally accrued patients with EGFR T790M compared to phase-III trials.

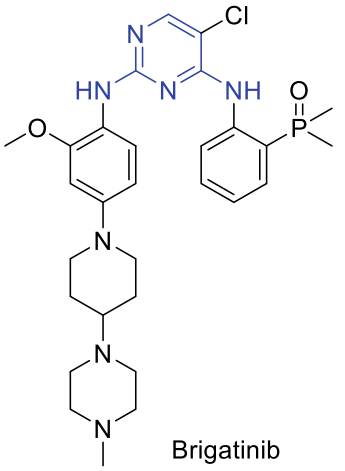


Rezivertinib

PM4

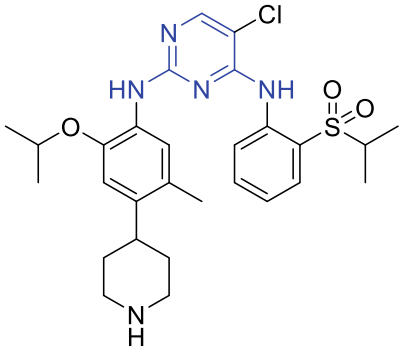
7.3 D2,4-Diamino Substituted Pyrimidine Derivatives

In cases of resistance to other ALK-inhibitors, brigatinib is a potent and innovative therapeutic candidate that is extremely effective in treating NSCLC. The chemical structure of brigatinib is attributed to various functionalities of phosphorus atom having better interaction and bind with targeted proteins that shows potent anti-cancer activity. Phase-II trials for brigatinib in those who have had second-generation ALK therapy are now being conducted. During the phase-II studies, it was determined that 67% of patients receiving a 90 mg dose of brigatinib and 42% of patients receiving a 180 mg dose had beaten lung cancer. In-vitro and in vivo, brigatinib inhibits a number of proteins, including AKT, STAT3, ERK-1/2, and S6. Moreover, it prevents phosphate groups or phosphorus atoms from adhering to ALK inhibitors (Jang et al. 2017). Brgatinib inhibits CD135, mutant EGFR variants L858R and FLT3 (D835Y), native EGFR, IGF-R1 and INSR, as well as to a lesser extent EGFR with T790M resistant mutations, with an IC_{50} of 1.5–2.1 nmol/L. The TK inhibitor brigatinib is 12 times more effective than crizotinib at inhibiting ALK kinases. It most effectively prevents the growth of tumours in ALK positive lung malignancies by suppressing mutations like L1152R and V1180L.



Brigatinib

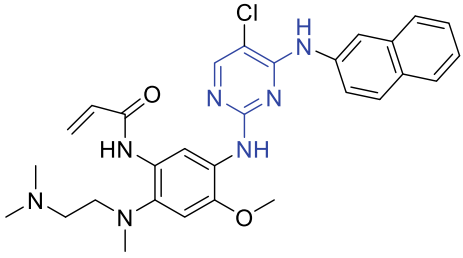
PM5



Ceritinib

PM6

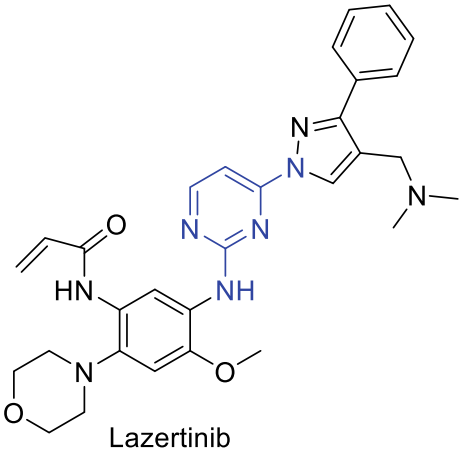
ASK120067 replaces the indole ring with a 2-amino-naphthyl ring and adds a chlorine atom to the pyrimidine ring. For EGFR del19, EGFR L858R, L858R/T790M, and EGFR WT, the biochemical enzyme $IC_{50}$for ASK120067 is 0.5, 0.5, 0.3, and 6.0 nM, respectively. EGFR del19 has a GI50 of 6 nM, L858R/T790M of 2 nM, and EGFR L858R of 12 nM (PC-9) (HCC827). First, second, and third stage trials have all been recorded and are currently being processed further.



ASK 120067

PM7

Lazertinib is differs from osimertinib with 4-phenyl-3-dimethyl-amino-methyl-1-pyrazoles groups are substituted the indole ring to pyrimidine ring and morpholine substituted dimethyl-aminoethyl-N-methyl chain to phenyl ring on 2nd positions. Lazertinib was approved for EGFR + T790M NSCLC on 18 January 2021 in Republic of Korea.

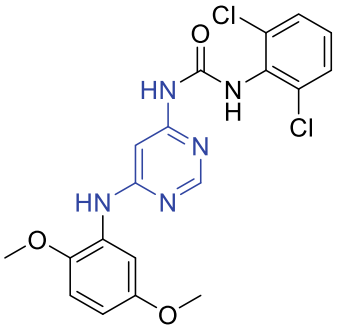


Lazertinib

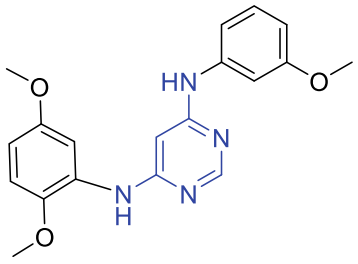
PM8

7.4 D4,6-Diamino Substituted Pyrimidine Derivatives

N4, N6-disubstituted pyrimidine was synthesised and biologically evaluated by Zhang et al. as an EGFR inhibitor for NSCLC. The most effective chemical was created and synthesised, and it had different substituents on the C-4 and C-6 side chains. In enzymatic and cellular testing of 4,6-diamino substituted pyrimidine derivatives, it demonstrated potential inhibitory action.



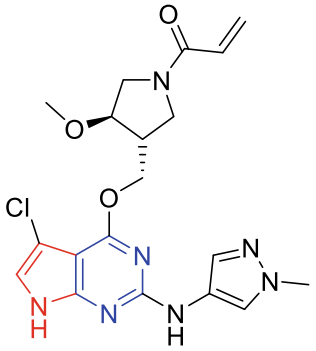
PM9



PM10

8 Pyrrolo-Pyrimidine Derivatives

The pyrrolo-pyrimidine derivatives represent the compound PF-06459988 that shows higher potential activity against NSCLC cells into different mutations like H1975 i.e., L858R/T790M, PC9-DRH i.e., Del/T790M, H3255 (L858R), HCC827 (Del), PC9 (Del) cancer cell lines with higher selectivity against to wild types of EGFR cell lines A549 i.e., EGFR-WT, with IC_{50}s ¼ 13, 7, 21, 140, 90, and 5100 nM, anti-proliferative results respectively. The hydrophobic interactions between pyrrolo pyrimidines and Met790 side chains are thought to increase the T790M inhibitory efficacy and selective activity, according to molecular docking studies.

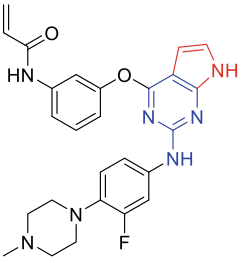


PF-06459988

PM11

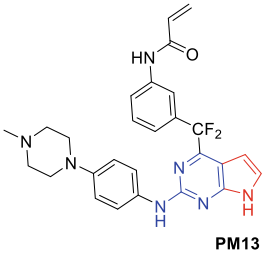
The medicines that contained pyrrolo[2,3-d]pyrimidine derivatives were tested for inhibition activity, EGFR-tyrosine kinase. Various substituents of synthetic derivatives were introduced into different places of the core structure to influence how many electron-withdrawing and electron-donating groups affected its inhibitory activity. The novel and powerful EGFR-TK inhibitors known as benzo-anellated pyrrolo-pyrimidine compounds are produced and tested. The affinities towards EGFR are depending on structures to discussed by the attachment of different substituents into both benzyl and residues on 4th positions of pyrrolopyrimidine. A series of heterocyclic derivative compounds are configured as potent and novel third generation EGFR-TK inhibitors. The SAR, study of the compounds by molecular modelling studies, the drug candidate abivertinib (AC0010) (Xu et al. 2016), as a potent and highly selective pyrrolo-pyrimidine based irreversible EGFR inhibitor was discovered. Formerly known as avitinib, abivertinib has a 43-fold greater potency toward T790M i.e., L858R/T790M, with IC_{50} ¼ 0.18 nM and then EGFR wild typewith IC_{50} ¼ 7.6 nM. In vitro studies demonstrated that it is a potent Brutons tyrosinekinase inhibitors(BTK) with IC_{50} ¼ 0.40 nM and JAK3 inhibitors with IC_{50} ¼ 0.09 nM. The abivertinib inhibited to EGFR phosphorylation's by the enzyme linked

immune-sorbent assays in NCIH1975 i.e., L858R/T790M cells with IC_{50} 7.3 nM and HCC827 i.e., EGFR del19 cell lines with IC_{50} 21.8 nM, where it is also barely inhibited to EGFR phosphorylation's in A-431 of wild type EGFR with IC_{50} 837 nM. With an IC_{50} of 8.3 nM, abivertinib exhibits strong reduction of cellular proliferation in the NCI-H1975 cell line. This is comparable to osimertinib's IC_{50} of 21.1 nM and rociletinib's IC_{50} of 44.6 nM. On May 21, 2020, Sorrento Therapeutics (San Diego, CA) obtained a licence for the ex-China rights of abivertinib.



Abivertinib

PM12



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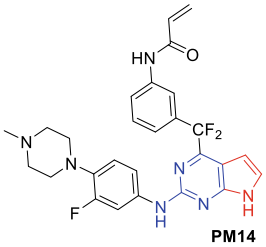

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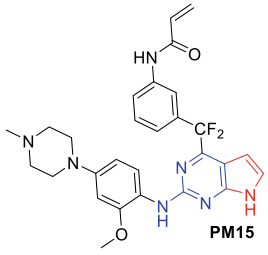

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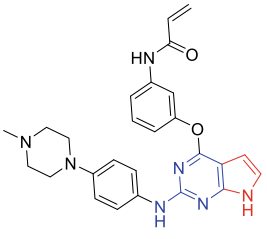

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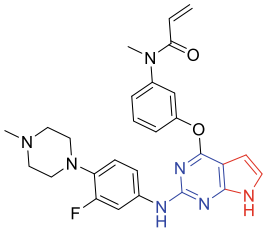
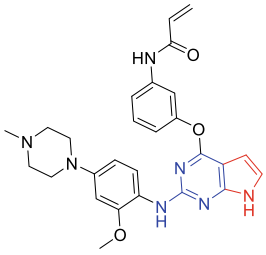


PM17

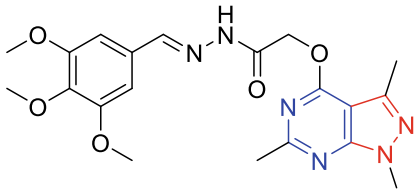


PM18

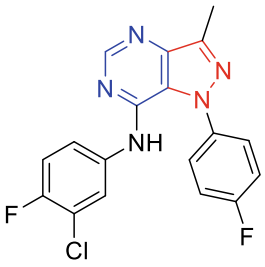
Selective inhibition of mutant EGFR and T790M-induced resistance over the wild type EGFR is amenable to the third generation of EGFR inhibitors. Abivertinib (AC0010) was created as a powerful and innovative irreversible inhibitor that is highly targeted to treat EGFR Mutated NSCLC and T790M-induced resistance over the wild type of EGFR (Mao et al. 2020).

8.1 Pyrazolo-Pyrimidine Containing Compounds

The molecule that provides good binding on the ATP binding sites of the EGFR kinases were identified for the core structure of the 4-Amino-pyrazolo-pyrimidine derivatives, which are substituted with N-acryl-amidopiperidines. On MCF7, HT29, and A549 cells, the various pyrazolo[3,4-d]pyrimidine derivative compounds are developed and examined (Gaber et al. 2018). The N-(3,4,5-tri-methoxy-benzylidene)-2-(3,6-dimethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yloxy)aceto-hydrazide shows higher potent activity against tumor with IC_{50} values of 6.14, 9.09 and 5.36 mM. In this work, a series of novel and potent pyrazolo[3,4-d]pyrimidines were created and tested against tumour cell lines (Abdelgawad et al. 2016). Compounds with 4-flourophenyl shows thatgood anti-tumor activities against ACHN, OVCAR4 and NCIH-460 cells with IC_{50}s ¼ 1.74, 5.53 and 4.44 mM (Engel et al. 2016). Several 2-bromophenyl pyrazolopyrimidine derivatives were tested for their ability to inhibit EGFR.



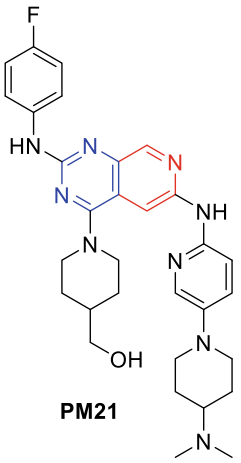
PM19



PM20

8.2 Pyrido-Pyrimidine Based Compounds

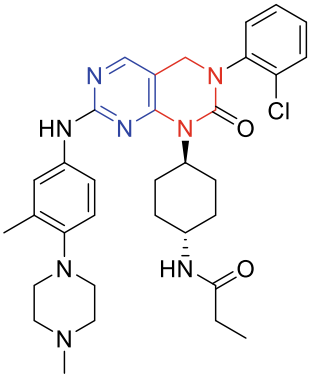
To reduce drug resistance caused by EGFR mutations, the pyrido[3,4-d] pyrimidines-based novel series was created and developed as an EGFR inhibitor. The 4th and 6th sites of pyrido [3,4 d] pyrimidine are linked with various linkers to create EGFR inhibitors. Using 2,4,6-tri-substituted pyrido[3,4-d] pyrimidine compounds, a novel class of mutant EGFR-TK inhibitors is created and tested. The most potent compounds are inhibiting to proliferations of H1975 and HCC827 tumor cell lines with IC_{50} 0.04 and 0.40 mM with potential inhibitory activities against mutations of EGFR-L858R with IC_{50} ¼ 1.1 nM and EGFR-L858R/T790M/C797S with IC_{50} ¼ 7.2 nM, kinase here observed for these compounds.



PM21

8.3 Pyrimido-Pyrimidine Derivatives

A hydrophobic group is added to pyrimido[4,5-d]pyrimidine molecules of various lengths and volumes at the N-6 position to produce the pyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dione derivatives. An original series of 2-oxo-3,4-dihydro-pyrimido[4,5-d]pyrimidine derivatives were prepared by using template structure of JND3229. In 2018, Hao Y et al. demonstrated that the JND3229 mutant was the most effective one against EGFR-C797S inhibitors (Hao et al. 2018).

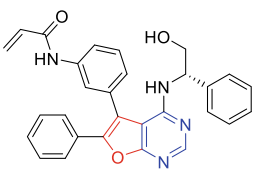


JND3229

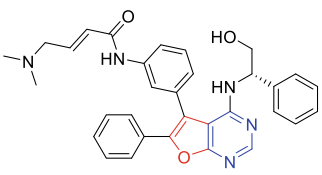
PM22

8.4 Furo-Pyrimidine Based Compounds

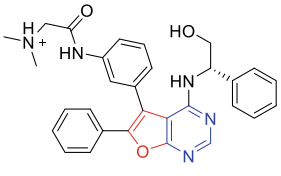
As EGFR-TK inhibitors, the 4-Anilinofuro[2,3-d] pyrimidine series was created. This series has functional groups at the fifth positions of acids, amides, esters, and alcohols, as well as by changes on 4-amino and 6-aryl functional groups. In this instance, derivatives of 6-arylfuro[2,3-d] pyrimidine-4-amine were also produced. By using acrylamide as a Michel acceptor at the third places on the furan ring, structural optimisation on the derivatives containing (S)-2-phenylglycinol was devised and synthesised. DBPR112 is a therapeutic candidate with excellent potential efficacy and selectivity for EGFR-L858R/T790M inhibitors. The majority of the HER-2 and EGFR inhibitory action is demonstrated by the chemicals in DBPR112. A new class of 4-substituted-5-methylfuro[2,3-d]pyrimidine derivatives was developed as an EGFR-TK inhibitor. The aryl group on the fourth position of the pyrimidine can be substituted to change the SAR of 5-methylfuro[2,3-d]pyrimidine derivatives.



PM23



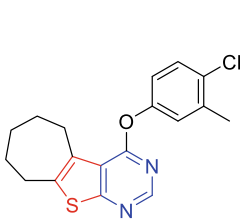
PM24



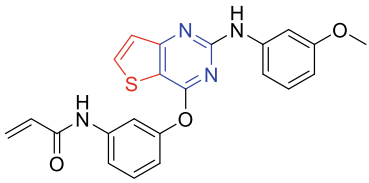
PM25

8.5 Thieno-Pyrimidine Derivatives

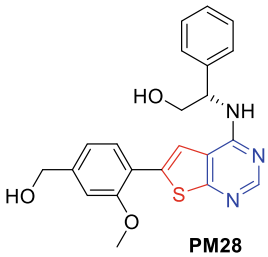
The novel dual EGFR inhibitors and microtubule polymerization inhibitors here proposed and produced are compounds based on 4-tri-methoxy-anilino thieno[3,2-d]-pyrimidines. Here, the 3,4,5-tri-methoxy-anilino group on the fourth position of the thieno[2,3-d] pyrimidine derivatives is used to screen for multi-targeted inhibitor compounds. We synthesised and evaluated the highly representative thieno[2,3-d]pyrimidine compounds for their action as EGFR kinase inhibitors. Main focus is on the enhancement of potency and ADME properties of the 4-amino and 6-aryl groups. Based on ADME and biological studies, the molecule containing benzylamine was demonstrated to be a superior aniline hybrid structure. Based on the binding mechanism of commercial dual HER-2 and EGFR inhibitors, a novel class of 6-phenyl-thieno[2,3-d]pyrimidines, including lapatinib, was designed and developed. As HER-2 and EGFR kinase inhibitors, a new series of 4-anilino-thieno[2,3-d]pyrimidine derivatives having aromatic and heteroaromatic sites on the benzyloxy-aniline moieties were made and evaluated (Milik et al. 2018).



PM26



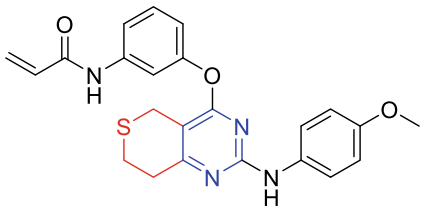
PM27



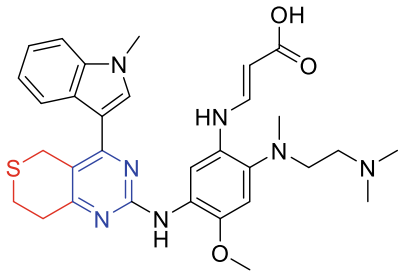
PM28

8.6 *Thiopyrano-Pyrimidine Derivatives*

New thiopyrano-pyrimidine-based compounds with acrylamide substitutions were developed and tested against the A549 and Hela cancer cell lines. The aliphatic heterocycle in the pyrimidine ring, which boosted activity and selectivity by occupying volume of proteins, is represented in the docking results of developed compounds. The Hela H1975, MCF7, and A549 cancer cell lines were targeted by a novel series of thiopyran-pyrimidine based compounds that were produced and tested as powerful and novel olmutinib derivatives. The docking results show that thiophene-pyrimidine was replaced with larger thiopyran-pyrimidine derivatives to modify the olmutinib structure, increasing the efficacy (Zhao et al. 2019).

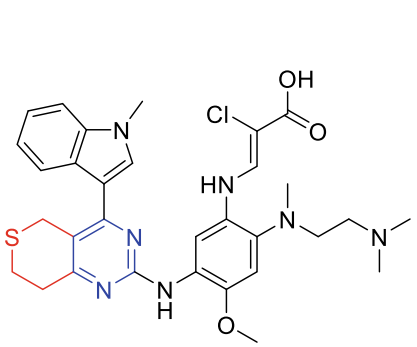


PM29

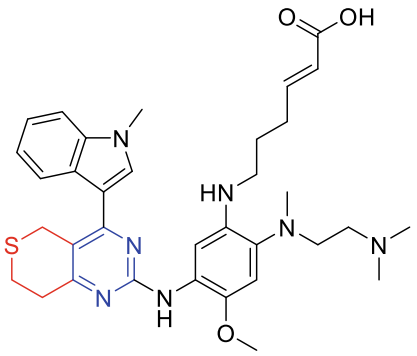


PM30

Also, methoxy-aniline were shows on 2nd position of thiopyran-pyrimidine.Were designed new derivatives by methoxy group of phenyl-piperazine side chain of WZ4002.



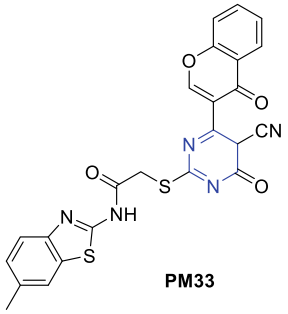
PM31



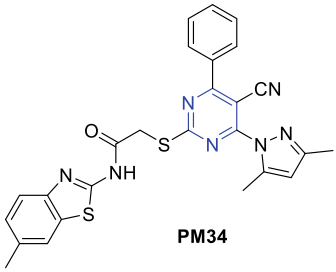
PM32

9 Miscellaneous Derivatives

As new multi-targeted potential anti-cancer medicines, various compounds including pyrimidine-benzothiazole hybrid, amide, and thiourea-containing derivatives were created. A brand-new class of 5-aminopyrimidine compounds with amide and thiourea groups, as well as their derivatives, were created as EGFR-TK inhibitors (Elkamhawy et al. 2017).



PM33



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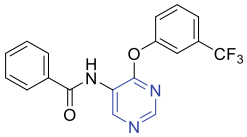
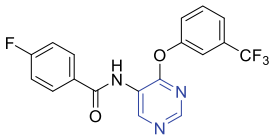
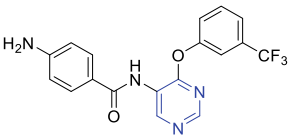


PM35



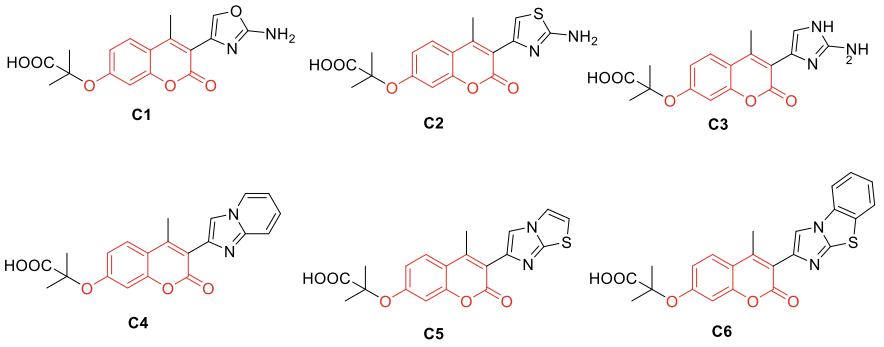
PM36



PM37

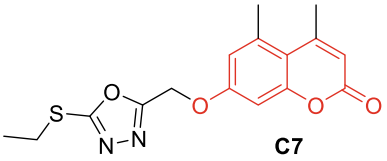
9.1 Coumarin Based EGFR-TK Inhibitors

The phenolic compounds known as coumarins have rings of the benzene and pyrone types connected. Kinase inhibitory and anti-cancer effects may be present in coumarin derivatives (Mudasir et al. 2021). A number of coumarin congeners have been altered, according to Sairam et al., to fight the HT29 (adenocarcinoma), MDA-231, and MCF-7 (breast cancer) cell lines. Compound C1, with an IC_{50} of 4.84, and Comparable with conventional drugs of "5-fluorouracil" with $IC_{50} = 1.96$, 1.86 mM, and "doxorubicin" with $IC_{50} = 3.76$, 3.24 mM, are shown to have moderate inhibitory effect against MCF-7 and MDA-231 cancer cell lines. Further it was reported that chemicals C1 and C2 are safe for use with healthy Vero and HepG2 cell lines. The in-silico analysis's findings also revealed that the synthesised compounds have topological polar surface areas (TPSA) of 140, lipophilic values ranging from 1.87 to 4.68, and absorption percentages between 64.6 and 76.5 percent. High antioxidant activity was also present in all of the generated congeners. The hydrophobic core of the EGFR kinase and the COX-2 enzyme were both engaged in binding interactions with all of the drugs (Sairam et al. 2016).

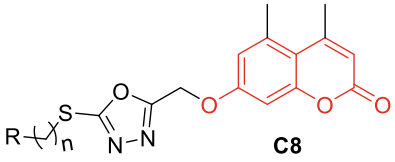


Using tamoxifen as a reference drug, Dhawan and colleagues developed the 1,3,4-oxadiazole derivatives with coumarin attached (C7, C8, C9, and C10) and evaluated their effects on the breast cancer cell lines. Congeners C9 and C10 were found to have potent inhibitory activity against MDA-MB-231 cancer cells in-vitro study with IC_{50} 7.07 μM, thatis seven times greater than that of tamoxifen (IC_{50} = 47.1 μM). In addition, compound C8 outperformed tamoxifen, which had an IC_{50} of 7.09 μM, in its activity against MCF-7 cells. Alkyl groups' anti-tumor efficacy was increased by more benzyl ring substitutions, the addition of propyl and benzyl moieties to the R-position, and SAR analysis using the MB-231 and MCF-7 breast cancer cell lines as a reference. Compounds C9 and C10 were found to have similar inhibitory activity against MDA-MB-231 cancer cell lines in-vitro with IC_{50} value of 7.07 μM, which are seven times higher than tamoxifen with IC_{50} = 47.1 μM. In addition, compound C8 outperformed tamoxifen, which had an IC_{50}

of 7.09 μM, in its activity against MCF-7 cells. According to SAR analysis, adding benzyl and propyl moieties to the R-position enhanced the anti-tumor impact more than adding alkyl groups, while adding a halogen group to the benzyl ring enhanced activity. Docking demonstrated that interactions with the amino acid residues in the active binding pocket of the EGFR-expressing MDA-MB-231 cells entail lipophilic interactions (Dhawan's et al. 2018).

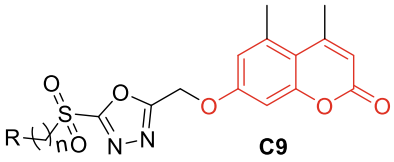


C7



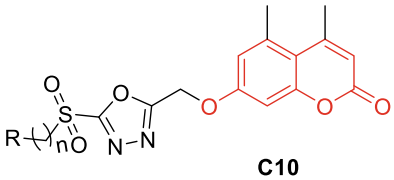
C8

n = 1
R = 2,4-diCl-Ph



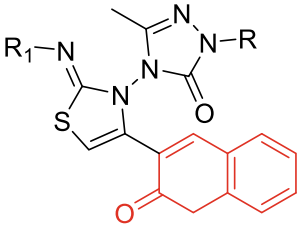
C9

n = 1
R = C_2H_5



C10

n = 1
R = Ph



C11 R= C_6H_5, R_1= 4-Cl-C_6H_4

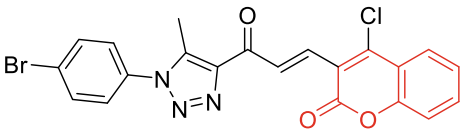
C12 R= 4-CH_3-C_6H_4, R_1= C_6H_5

C13 R= 4-OCH_3-C_6H_4, R_1= 4-Cl-C_6H_4

C14 R= 4-CH_3-C_6H_4, R_1= 4-OCH_3-C_6H_4

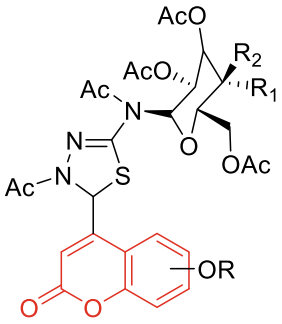
C15 R= 4-CH_3-C_6H_4, R_1= 4-Cl-C_6H_4

By employing MTT assays, Shaikh and colleagues developed a variety of "coumarin-3-yl-thiazol-3-yl-1,2,4-triazolin-3-one" analogues as anti-proliferative agents against HeLa, A549, MDA-MBA-231, and K562 cells. The IC_{50}values of C11, C12, C13, C14, and C15 demonstrated outstanding inhibitory effect against MDA-MBA-231 cells using doxorubicin as a reference. Compound 81c strongly inhibited the A549 cell line as well, with an IC_{50}value of 0.17 M. Contrarily, when using doxorubicin as standards, which has IC_{50}values of 0.54 M for A549 cell lines and 0.60 M for K562 cell lines, compound C12 and C13 exhibited a higher inhibitory effect on the K562 cell lines with values of 0.31 M. Moreover, doxorubicin did not inhibit the HeLa cell line as well as compound C11 did ($IC_{50} = 0.60$ M). According to SAR analysis, the phenyl ring's para-position can be replaced with an electron-withdrawing group to increase anticancer activity. According to molecular docking studies, the amino acid residues Lys721, Met769, and Thr830 in the binding pocket of the EGFR kinase domain interact with EGFR kinases through polar and hydrophobic interactions (Shaikh et al. 2018).



C16

According to Vagish and colleagues, a variety of coumarin-triazole analogues were created by using both traditional and microwave methods to manufacture them and testing them against the DU-145 and PC-3 prostate cancer cells. Compound C16's IC_{50} values for the PC-3 and DU-145 cell lines were 2.036 0.3 and 2.036 0.6 M, respectively, in cytotoxic experiments, although it still demonstrated a notable level of inhibitory potential. None of the produced congeners had a harmful effect on MCF-10A (normal mammary epithelial) cells. In the EGFR kinase active site, docking experiments also showed efficient binding interactions (Vagish et al. 2021).



	OR	R_1	R_2
C17	6-OC_2H_5	OAc	H
C18	6-OnC_4H_9	H	OAc
C19	6-OnC_5H_{11}	H	OAc
C20	6-OIC_5H_{11}	H	OAc
C21	6-OC_2H_5	OAc	H
C22	6-OIC_4H_9	H	OAc
C23	6-OIC_5H_{11}	H	OAc

Toan et al. recently discovered several coumarin congeners combined with 1,3,4-thiadiazoline (C17-C23) to be effective cancer-fighting compounds. In comparison to 5-fluorouracil, sorafenib and doxorubicin, diverse compounds indicates that inhibitory efficacy against MCF-7, HeLa, HepG2, LU-1 andSK-Mel-2 cancer cells with IC_{50} values of 1.18–11.81 μM, 1.72–9.43 μM, 1.98–13.16 μM, 1.82–11.25 μM, and 2.25–14 μM. Furthermore, the chemicals had no adverse effects on regular MRC-5 cells. Moreover, kinase experiments showed that all of the compounds beat the commonly used medicine sorafenib in terms of their capacity to inhibit EGFR kinases and HER-2 with IC_{50} values of 0.22–0.47 M and 0.13–0.35 M, respectively. In the binding pocket of the EGFR kinase, this chemical has a binding energy score of 6.687 kcal/mol, which is in harmony with the binding energy of −7.713 kcal/mol of sorafenib standard. Finally, SAR research demonstrated that having longer carbon chain and more terminal carbon branching enhanced anti-cancer activity (Toan et al. 2021).

10 Conclusion

The well-established pharmaceutical therapy of EGFR inhibition is used to treat a range of carcinomas, including prostatic cancer, breast cancer, non-small cell lung cancer, pancreatic cancer, colorectal cancer, ovarian malignancies, and glioblastomas. The compounds or molecules were obtained from various sources, including natural and synthetic sources as well as from rational designs for these receptors' activity as a therapeutic drug target. This chapter discussed the present state of drug discovery in medicinal chemistry, specifically for tyrosine kinase inhibitors, and provided a brief overview of heterocyclic compounds as EGFR-TK inhibitors. We also discussed various structural modifications of heterocyclic scaffolds, SAR, mechanisms of action, and in-silico studies. Based on the fundamental heterocyclic moieties, such as quinoline, quinazoline, pyrimidine, coumarin, and indole, among others, the numerous EGFR-TK inhibitors were studied. Researchers working on the design and development of novel drugs as well as formulating anti-cancer drugs will find this chapter useful.

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Drug Delivery Systems: Current Trends, and Advances

Potential of Nanocrystalline Drug Delivery Systems

Priyanka E. Doke-Bagade and Om M. Bagade

Abstract During the last decades, the field of drug delivery exploiting particle delivery techniques as conveyors for both large and small molecules has witnessed a substantial surge in scientific interest. Many atoms can be assembled into nanocrystals, which combine to form "clusters" that are crystalline forms of matter. They can be utilized to physically modify and choose the pharmacokinetic as well as pharmacodynamic characteristics of many groups of pharmaceutical drugs. They were utilized in vivo to protect the medication while it is moving through the blood. The preparation of nanocrystals covered spray drying, top down, bottom up, as well as other novel methods. These methods are opening the door to the fabrication of nanosized particles that can carry out a wide range of scientific functions. The primary benefits of the formulation of nanocrystal are increased bioavailability by buccal route, enhanced dose proportionality, decreased food consequences, appropriateness designed for administration via all routes, and potential for aseptic filtering because of a reduced particle size range. The choice is made centered on the locations and the capacity to transport the API with controlled, steady rate to the spot of act. We have gone through a number of fundamentals of nanocrystal production, characterization, the impact of these characteristics, and their use in pharmaceutical administration of therapeutic and pharmacological molecules. In this book chapter, along with the introduction, procedures, evaluation parameters, and extensive applications, the significance of nanocrystal treatments is covered.

Keywords Nanocrystals · Milling · Particle shape and sizes · Solubility · Bioavailability

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1 Introduction

Given the rise in chemicals with least aqueous solubility as well as dissolution that come as of discovery efforts. In the direction of achieve an adequate oral bioavailability, pharmaceutical scientists are always looking for innovative formulation strategies. The quickly developing subject of nanoscience currently offers unique opportunities. The formation of nanocrystals (Top-down technique), particle diameter reduction of bigger crystals, or precipitating of dissolved molecules can all be used to create the particles. High-pressure homogenizations as well as media milling are examples of top-down methods for producing drug nanocrystals. When compared to the former method, which involves mechanically attiring suspended drug particles with grinding media like glass, the latter method involves repeatedly driving a suspension across an incredibly narrow gap (approximately 25 m) at an extremely high velocity. Outlines the essential features of available pharmacological nanocrystal products. The table shows that the FDA has given its approval to the products. Second, all five of the items depend on top-down strategies, with one using high-pressure homogenization and the other four using media milling. No economic feasibility of these systems has yet been accomplished, despite the fact that bottom-up techniques have enormous potential for enhancing bioavailability by obtaining lower particle dimensions as far as size is considered (100 nm) and which must be a amorphous property. Crystals smaller than one millimeter are known as nanocrystals. The characteristics of a material drastically change as a crystal's particle size is reduced to roughly 100 nm. Drug manifolds' surface area and solubility increase due to their reduced size and the bioavailable of poorly soluble medications also rises proportionately. Comparing nanonization to micronization, an additional effect is present. It improves the solubility at saturation point of level while also escalating the area of the surface. The only variables that affect the solubility of ordinary-sized powders are temperature and solvent. However, the saturation solubility additionally acts as a consequence of particle size for crystals with particle sizes less than 1–2 m. Strong particle curvature causes a rise in the dissolution pressure, which raises the saturation solubility (Merisko-Liversidge 2002; Grant and Brittian 1995; Liversidge and Conzentino 1995; Cundy and Liversidge 1995; Lamprecht et al. 2001).

There are two outcomes of a rise in saturation solubility:

A. An elevation in solubility (saturation) that causes a rise in the dissolving rate.
B. The development of a solution of supersaturated level which heightens the slope of concentration between the blood and the gut lumen.

Diffusion of the medicine would be accelerated, aiding absorption. Because to their quicker dissolving and absorption rates, nanocrystals have a rapid onset of effect.

This is useful, especially for medications like naproxen where a rapid action is sought. Because of the larger van der Waals contact area, nanocrystals can exhibit high adhesion. Upon oral administration, the nanoparticles' adhesiveness to the gut wall improves absorption, raising the bioavailability. Due to their size and surface

characteristics, nanoparticles have inherited advantages such as better dissolution and solubility, enhanced bioavailability and uptake, avoidance of meal things, growth of safe dose, and superior safety, effectiveness, and tolerance profiles. NCs also increase the suppleness for collectively down scaling and scaling, which is incredibly beneficial when modifications to unit operating capabilities or formulation are required during the scaling up process. NCs stand out as a unique nanoparticle not just among pharmaceuticals but additionally among other nanoparticles due to their straightforward formulation and flexible production scaling capabilities, as well as their inherent tiny particle size and wide surface area. Six NC products have already been released on the market with regulatory approval and licensing (Gabor et al. 2004; Dong et al. 2010).

"Paxceed (paclitaxel), Theralux (thymectacin), Semapimod (guanylhydrazone), Nucryst (silver)", and are only a few of the drugs that are currently in the clinical stages; many more are in the preclinical phases. Every NC product that has been put on the market is an intelligent formulation design. To circumvent the unpleasant taste and constrained refrigerating circumstances of the original fabrication of rapamune, sirolimus was created as a prescribed amount form for tablets (rapamycin). The drawback of the aprepitant formulation at the time was that meal absorption had an impact on drug absorption. Emend was created utilizing a aprepitant nanosuspension, and it was designed as a spray coated concrete capsules form of dosage with improved bioavailability due to fewer fluctuations in the fast and fed states. Fenofibrate is available in the tablet forms TriCor and Triglide, which are intended to increase bioavailability and address fast and feed state dependent absorption differences that are present in other formulations of this medication. In comparison to other dosage forms of the medication, Megace ES is a liquid dispersion type of medication created to increase the bioavailability and dissolution of megestrol acetate. As a result, it offers a smaller dose. Invega Sustenna, Paliperidone palmitate (intramuscular suspension), which is an already when prolonged delayed sterile injectable liquid dispersion delivery system and is available in prefilled syringes, is notable for having a 2 years layer life. Due to the significant compliance issues that the tolerant population for which palipidone palmitate (antipsychotic) is recommended poses, the NC produce, which can be administered monthly receives points for its patient-friendly treatment. However, due to such rational formulation creation, "the Food plus Drug Administration (FDA)" considers an NC in the direction of be a new drug product rather than "generic" a previously approved product because the pharmacokinetic pattern is not significantly similar to that of other drug/s (Pandey et al. 2003; Shegokar et al. 2010).

For many years, the much more intelligent method that can be used with nonspecific formulation has been plummeting the dimensions of the particles. Drugs that have been micronized have more area of the surface, which raised the pace of diffusion and dissolution correspondingly (absorption). The micronization process does not, however, boost the drug's bioavailability or augment the solubility at saturation level of substances with very squat solubility. As a result, the technology to further shrink particle size to the nanoscale variety was able to develop. Particle reduction toward the inter—and intra range has recently come to be recognized as

a potent formulation strategy that can boost the availability of drug in the blood of inadequately aqua-soluble pharmaceuticals by increasing the dissolving rate and saturation solubility and perhaps reducing systemic side effects. Drug nanocrystals have expanded the consideration in excess of the around preceding decades as a cutting-edge technique for improving the dissolution of water hating pharmaceuticals since the process is quick and easy to implement. Beginning in the early 1990s, nanocrystals were developed, and starting in the year 2000, the first goods quickly hit the market. Nanometer range of the dimensions of the particles can be possible of any kind of drugs, drug nanocrystals are a results are comparable that is typically used to all poorly soluble drugs. Drug nanocrystals are crystals that are smaller than 1 mm in size and are made of their parent chemicals. In most cases, they are maintained with surfactants or polymeric electrostatic stabilizers and are made entirely of the medication without any carriers. The term "nanosuspensions" refers to drug nanocrystal dispersion in an outer liquid media that has been stabilized by interface active agents. The formulation of substances that are soluble neither in water nor oil, as well as the reformulation of current medications to eliminate toxicologically unfavorable excipients, can be done using nanosuspensions. Moreover, the poorly soluble medications can be created as nanosuspensions either by themselves or in conjunction with different pharmacological excipients (Liversidge et al. 1992; Müller et al. 2002; Bernard et al. 2008; Shaktish 2013; Neslihan and Levant 2009).

1.1 History

A material particle known as a nanocrystal, constructed using quantum dots (a type of nanoparticle), is one that has at a minimum one dimension below one hundred nanometers and is made up of atoms arranged either in a single crystal or in several crystals. Nanocrystals can be distinguished from bigger crystals by their size. For instance, silicon nanocrystals may be employed for memory components since they can emit light effectively but bulk silicon cannot. Nanocrystals may exhibit significantly more complex melting behavior when implanted in solids than typical solids, and they may even serve as the foundation for a unique class of solids. They can behave as single-domain systems, which can help put in plain words the actions of subatomic models of a analogous material without being complicated by the appearance of grain boundaries and some other defects. A single-domain system is a volume within a system that shares a single atomic or molecular configuration throughout. Quantum dots are another name for semiconductor nanocrystals with diameters lower than 10 nm (Dandagi et al. 2011; Keck and Muller 2009; Merisko-Liversidge et al. 2003; Kobierski and Keck 2008; Jens-Uwe and Junghanns 2008).

1.2 Properties of Nanocrystals

Rise in area results in fastest dissolution rate

With respect to the Whitney Noyes equation (NW equation), the size reduction increases surface area, which in turn increases dissolution velocity. Hence, in which the velocity of dissolution seems to be the velocity restrictive step, to make the particles in micron size; is an efficient method to successfully increase the drug availability in blood. The particle area is further enlarged as a result of the transition from micronization to nanonization, and the dissolving velocity also rises. Low saturation solubility is typically coupled with poor dissolving velocity. "NW equation is": dc by dt = DAh (Ca–Cb). "Where dc by dt is the pace of dissolution, D is the distribution coefficient, A is the area of API, h is the thickness of membrane, Ca is the drug's "solubility at saturation point", and Cb is the amount of drug's in the fluid around it at any given time" (Bushrab and Muller 2003; Rawat and Senthil 2011; Sharma and Aggarwal 2010).

- **Enhanced saturation solubility**

Based on the substance, the medium of dissolution, and the hotness, the saturation solubility Cs is constant. This is true for common powders that are at least one micrometer in size. However, the saturation solubility additionally acts as a purpose of size of particle lower a crucial dimension of one-two m. It rises as dimensions of the particles under 1000 nm decreases. Formulation of nanocrystals has higher solubility of a saturation state as a result. This offers two benefits (Sharma et al. 2002).

- **"The solubility of solid particles at saturation level"**

Depending on the "Ostwald as well as Freunlich expression (O–F expression)" and "model of Kelvin", varies on their radius of the particle and structure lattice. "In p (r1, r2) = P-γρvapour/(ρliq-ρvap) (1/r1 + 1/r2) Where P is the partial pressure at the flat interface, and p(r) is the evaporation at the curved contact with radius r ($r = \infty$) = peq, γ = surface tension, ρvap = Density of vapour, ρliq = Density of liquid, r1, r2 is equivalent to the radius of bending and along main axes of the bent area".

"According to the O-F expression, an API has a superior solubility when the radius of the molecule is reduced. Although not been noticeable for bigger molecules, this impact would be additionally noticeable for molecules between one-two m, above all those that are below 200 nano scale". The drug's crystalline structure is a significant aspect that affects solubility. The solubility decreases with increasing solid density and melting point. A polymorph form with much less packing, on the other hand, exhibits a bigger molar volume and a lower solid density. The association among the increasing solubility at saturation point and decreasing range of particle can also be expressed using the 'equation of Kelvin'. The refraction of fluid droplets in a phase of vapour is a function of the vapor pressure, as per the equation of Kelvin. When

curvature increases, the pressure of vapor rises (decreasing particle size) (Elaine et al. 2008; Lei et al. 2001; Banavath 2010).

1.3 Benefits of Nanocrystals

- Greater surface area;
- Improved solubility;
- Increased rate of dissolution;
- Increased oral bioavailability;
- Quicker onset of therapeutic effect;
- Lower dose necessary;
- Reduced fed fasted variability;
- Less patient to patient variability.

1.4 Drawback of Nanocrystals

- Physical stability, compaction and sedimentation are potential issues.
- It cannot be administered in a uniform and correct manner due to its weight,
- Handling and transportation must be done with care.

2 Methods/Technology (Rath et al. 2008; Ohara et al. 2008; Kwon et al. 2006; Felgner et al. 2004; Ren et al. 2011; Anuradha et al. 2001; Zeng and Li 1999; Sahoo et al. 2007)

2.1 Bottom-Up Technique

2.1.1 Nanoprecipitation

In this method, the API mix with the appropriate solvent which later on eliminated by precipitation. Expertise from "bottom up" depends on precipitation. This method's foundation is built on dissolving the active ingredient with the solvent prior mix in non-solvent. Then finally formulations precipitate with stabilizers (Rath et al. 2008; Ohara et al. 2008; Kwon et al. 2006; Felgner et al. 2004).

Advantages:

- It is easy and affordable.
- It's easy to scale up using this approach.

Disadvantage:

This method has the limitation that the drug molecules ought to be soluble with the solvent.

2.2 Top Down: (Milling and Homogenization) (Ohara et al. 2008; Kwon et al. 2006; Felgner et al. 2004; Ren et al. 2011; Anuradha et al. 2001; Zeng and Li 1999; Sahoo et al. 2007)

By utilizing various milling and homogenization technologies, "top-down" technology applies dispersion methods. Top-down technology, also referred to as "nanosizing," which is additionally general than bottom up tools. Moreover, it is a process that reduces massive crystalline particles to tiny fragments.

2.2.1 Pearl/Ball Milling

The APIs is put into the milling chamber in addition to the size reduction media, dispersion media, and some stabilizer. Small pearls or grinding balls are utilized. Particle size reduction results from the intense shear and impact forces produced by the action of milling media. There are two main milling principles in use. An agitator can be used to move the milling material, or a sophisticated movement can be used to move the entire container.

Advantage:

- Minimal cost
- straightforward technology, and capability of mass manufacturing.

Disadvantage:

- Product adhesion to the internal surfaces of the grinder and to the exterior of the milling pearl.
- Degradation from the grinding material resulting in product contamination.

2.2.2 High Pressure Homogenization System

This method can be used with water or a non-aqueous medium.

2.2.3 Micro Fluidizer Technology

- The concept behind this technique is the jet stream. High pressure and two fast-moving liquid streams meet frontally.

- Due to cavitations and strong shear force particle collisions, the size of the particles is lessened. To stabilize the appropriate particle size, surfactants or phospholipids are needed.
- The collision compartment may either be Z /Y kind in character.

2.2.4 'Piston Gap Homogenization in Water: (Dissocubes® Technology)'

- This method encompasses scattering a powdered medication in an aqueous solution of surfactant, which is subsequently pushed under intense pressure through a piston in the course of a small gap.
- Hydrophobic Piston gap homogenization in or reduced water mixtures: (Nanopure® technology).
- As a dispersion medium, this technology employs organic phases or phases with less aqua. It is preferable for drugs that hydrolyze in water to use non-aqueous mediums.
- Several media, such as oils, water-glycerol mixes, water-alcohol mixtures, etc. are employed for homogenization.
- The size reduction is caused by forces including colliding and trim forces that happen in a extremely disordered liquid in the gap.

2.2.5 'Technology as Per Bottom up and Top Down'

- 'Bottom up and top down' tools combines the two approaches. It requires combining a precipitation process with an annealing process, as well as a high energy homogenization step between the two.
- Several combination processes are performed using Smart Crystal® technology, depending on the physical properties of the medication.

Advantages:

- Improved physical stability;
- Combination technology can combat crystal development.

Disadvantage:

- Expensive

2.3 Spray Drying

- For drying liquids and suspensions, spray drying is frequently employed. Solution droplets are sprayed in a conical or cylindrical cyclone from top of roof to bottom and dried by hot air in the identical path to produce spherical particles.

- Scattering is done with spray can that fast rotates and scatters the monophasic fluid as a result of the centrifugal force.
- A peristaltic pump is used to deliver the fluid nearby inlet with a specific rate of flow, whereas air or nitrogen is delivered to the outer tube at a fixed pressure. Nozzles produce spraying which causes reduction in the dimensions of the droplets of the fluids which improves the region of surface either the agent which helps for drying or speeds up drying. It is possible to modify the solution's viscosity, temperature, concentration, spray rate, with its fluidity, particle dimensions and speed to make it dry.

3 Supplementary Technologies (Sahoo et al. 2007; Fang et al. 2008; Buscaglia et al. 2005; Hennings et al. 2001; Roy and Mohanta 2009; Abdelwahed et al. 2006; Ali et al. 2011)

3.1 'Rapid Expansion From a Liquefied-Gas Solution (RESS)'

The chemicals that are soluble in supercritical fluids can be treated using this method. In this procedure, the solid mass is initialy mixed in a fluid of supercritical phase before being forced at supersonic speed via a nozzle. Rapid expansion results from reducing the pressure of the fluid in the nozzle.

3.2 'Nanopure® XP Technology'

- It is the official business name cataloged by Pharma Sol GmbH/Berlin. Non-aqueous or water-reduced media can likewise create similar optimum particle size reduction.
- Direct formulation can be obtained extremely successfully by producing nanocrystals in non-homogenization media.
- The API containing nanocrystals suspended in fluid PEG (polyethylene glycol) or other oils directly put straight with capsules of HPMC.
- This approach has the benefit of not requiring removal of the dispersion media. Under more favorable conditions, evaporation occurs more quickly. For drugs that are thermolabile, this is helpful. Isotonic nanosuspension for intravenous injections is made by homogenizing in liquid solutions. The energy needed for various methods like fluidized along with spray drying.
- Pharmasol owns the intellectual property (IP) for water mixes and without water dispersion (e.g. oils, PEG). The major advantages of the 'NANOPURE® XP technology' are degree up with the capability to manufacture on a bigger scale

in moderate and usual circumstances. Pharmasol applied their NANOPURE® XP technology in conjunction with a pre-treatment stage of subsequent homogenization. Particles with a size below 100 nm result from this.

3.3 'Spray Freezing into Fluid (Liquid) (SFL)'

- Thois method created primarily and patented in 2003 by Texas University, (Austin).
- "The (Dow Chemical) Company was a first to commercialize this method (Midland, MI). Here, a drug-containing organic, aqueous, aqueous-organic emulsion, aqueous-organic co-solvent solution, or suspension is atomized directly into an acryogenic liquid or a compressed gas (such as, propane, helium or CO_2, ethane) (i.e. hydro fluoro ethers, argon, or nitrogen)".

3.4 "Solvent Evaporation"

- It basically content creating solutions of polymer in emulsions and solvents (volatile). However, currently, chloroform and dichloromethane were employed, which have superior toxicity report as compared to ethyl acetate.
- The obtained puffy powder of NPs after ultracentrifugation was lyophilized after being cleaned with distilled water in order to dispose of any additions like surfactants'.
- The polymer concentration, stabilizer concentration, and homogenizer speed all had an impact on particle size.

3.5 Sonocrystallization

- Sonocrystallization is a revolutionary method for reducing particle size based on crystallization through the use of ultrasound. Sonocrystallization uses ultrasonic power with a 20–100 kHz frequency range to cause crystallization.
- It increases the nucleation rate while also being a powerful tool for managing the active medicinal ingredient's size distribution and size reduction (API). The majority of applications utilised ultrasonic frequencies between 20 kHz and 5 MHz. It has also been investigated how to change the unfavorable properties of NSAIDs, namely their reduced dissolution solubility and rate and subsequently poor bioavailability.

3.6 'Melt Emulsification Technique'

- Melt emulsification is the primary process used to create solid lipid nanoparticles. Ibuprofen nanosuspensions are initially made by Kipp and colleagues using the melt emulsification process.
- The process consists of four steps. The stabilizer-containing aqueous solution is added before the drug.
- To create an emulsion, the solution is homogenized using a high-speed homogenizer after being exposing the APIs for the high temperature as an above its melting point.

4 Characterization of Nano Crystals (Cerdeira et al. 2010; Chan and Kwok 2011; Deschamps et al. 2009; Dong et al. 2010; Eerdenbrugh et al. 2008; Faure et al. 2001)

(1) Solubility
(2) Particle size analysis
(3) Determination of drug content
(4) Differential scanning calorimeter
(5) X-ray powder diffraction
(6) Microscopy by scanning electron technique
(7) Dissolution test
(8) Stability studies.

1. **Examining the size of the particle**

'The measurements of shape of particles which were dried and evaluated by scattering methods by dynamic light path using a granular dimension analyzer Nanotrac 150 (Japan) using this type of wet sampling system for redispersion in water consisting of 0.1 percentage aqueous stabilizer. The median particle size distribution was used to calculate the diameters provided'.

2. **Computing the drug content**

In order to verify the purity of the manufactured samples, the concentration of lyophilized specimen was examined by analytical tools. Aqueous formulations dispersions of preparations have been passed all the way through a 0.8 m filter in organize to quantitatively examine the amount of API content in formulations. The quantity of the API was assessed through spectroscopy at a wavelength of 291 nm using the filtrates that contained tiny particles <0.8 m mixed in 4% SLS. The nanocrystal yield was estimated as the proportion of the quantity of API in the deposit to the API concentration overall in the distribution.

3. **"Microscopy by scanning electron path"**

SEM was executed to inspect the samples (freeze-dried formulation) morphology of surface and commercial API powder. This, to arrange the specimen for analysis, they were accumulated and lying on metal discs with bi-adhesive carbon strip and covered with 80 nm Au/Pd using Blazers (120B) spluttering equipment.

4. **'X-ray Powder analysis by diffraction path (PXRD)'**

It is used to perform the PXRD at a inspection rate of 40/min 2 θ and a variety of 10 to other suitable ranges.

5. **"Differential scanning calorimetry (DSC)"**

The temperature related virtues of the marketed griseofulvin as a powder and the lyophilized samples was measured using this approach, which is fitted with a (liquid) nitrogen system as cooling one.

6. **Solubility**

Using a UV spectrophotometer, saturation solubility values were assessed by measuring ultraviolet absorption at 291 nm. In more than 100 ml of 4% (SLS) liquid, additional griseofulvin powder and compositions were added. The blend was then shaken mechanically for around a day at a temp of from 37 °C up to 50 °C using shaker. To confirm that there was extra sample in the solid state, a rigorous visual inspection was performed to determine when equilibrium attained.

7. **Dissolution test**

The produced specimen and the APIs were placed in 125 mg of cap and body and submitted to dissolving examination by using around 900 ml of a 4% SLS solution that was Heated and kept at 37.5 ± 0.5 °C as the dissolution average. A 75 rpm/min rotational speed was used for the baskets.

8. **Stability examination**

As indicated by the ICH recommendations, nano formulation was separated into 2 halves which stored at '30 ± 2 °C and 65% ± 5% RH and 40 ± 2 °C and 70% ± 5% RH', respectively. All of the formulations were then subjected to a stability analysis.

5 Comparison of the Benefits of Other Drug Delivery Systems and Nanocrystalline Drugs Delivery Systems (Ganta et al. 2009; Gao et al. 2008a, b)

1. Administration by Parental way
2. Administration by orally

3. Drug distribution via the lungs
4. Specific drug delivery
5. Drug administration via the skin
6. Boosting Bioavailability.

1. **Administration by Parental way**

Various parenteral administration routes, including intra articular injection, are available for administering drug nanocrystals in the state of nanosuspensions. An injection from the intraperitoneum into the vein. It has been discovered that nanosuspension improves the effectiveness of medications given intravenously. Poorly water soluble anti-leprosy APIs (clofazimine) nanosuspension shows an enhancement in stabilization and effectiveness above clofazimine containing liposome.

2. **Administration by orally**

Drugs are dramatically more orally absorbed and subsequently more bioavailable when they are nanosized (Sahoo et al. 2007; Shaktish 2013; Sharma and Aggarwal 2010).

3. **Drug distribution via the lungs**

Mechanical and ultrasonic inhalers can be used to nebulizer aqueous nanocrystals for lung administration. While treating lung infections with nebulization, budesonide, corticosteroid has completely transform in to the nanosuspension.

4. **Targeted/Specific drug delivery**

Target delivery is possible with nanocrystals. Bupravauone surface engineering mucoadhesive nanosuspension was used to target the cryptosporidium partum, the organism that causes cryptosporidiosis. Similarly, rather of using stealth liposomes, disorders like pulmonary related issues such as aspergillosis which may be effectively treated with the help of acceptable APIs.

5. **Drug administration via the skin**

The use of drug nanocrystals results in a greater gradient of concentration seen between formulation and the skin, making dermal nanosuspension only of relevance if conventional formulation procedures fail. The supersaturated formulations produced by the higher saturation solubility improve medication absorption through the pores. Positively charged polymers can be used as drug nanocrystal stabilizers to further increase this effect. The drug nanocrystals have a stronger affinity for the negatively charged stratum conium due to the opposite charge.

6. **Boosting Bioavailability**

Certain recently created compounds suffer from poor water solubility, which has an adverse effect on their permeability. However it is around 82.3% in nanosuspension Danazol. When measure up to the classic marketed product, Kayser et al. Nanosuspension's Amphotericin B demonstrated a noticeably improved oral absorption.

6 Examples (Marketed Formulation of Nanocrystals) (Ghosh et al. 2012; Hanafy et al. 2007)

1. **"Rapamune"**
   - Wyeth Pharmaceuticals introduced the first oral nanocrystals that the US FDA had approved in 2000 (Madison, NJ). It is made up of 'Sirolimus nanocrystals' mixed with physical mixture that is appropriate designed for immediate formation of edible tablets.
   - As comparison to Sirolimus solution, the oral BA of the nanocrystal tablet was found to be >21%. Rapamune was formerly only accessible as an oral solution that needed to be refrigerated for storage and combined with water and orange juice before delivery.
2. **'An Emend'**
   - This dosage was launched in the market by Merck in 2001 ('Winehouse Station, New Jersey') which contains aprepitant which often able to cure the emesis. Human substance P/neurokinin 1 (NK 1) receptors are specifically and highly affinitized to apepitant. The intentions of current treatments for chemotherapy persuaded vomiting and nausea include serotonin (5HT 3), dopamine, and corticosteroid receptors. Aprepitant has little to no affinity for these receptors (CINV)".
   - In animal models, aprepitant has been found to block central activities that cause emesis brought on by cytotoxic chemotherapy drugs like cisplatin.
3. **"A Tricor"**
   - "Which was marketed/ released by Abbott Laboratories, moreover its main component, fenofibrate, is offered in 48 and 145 in mg tablets".
   - "In order to raise decrease low density lipoprotein cholesterol (LDL), decrease apo lipoprotein B, decrease triglycerides (TG), decrease total cholesterol (Total C), and high density lipoprotein cholesterol (HDL) in grown person patients with crucial hypercholesterolemia or assorted dyslipidemia."
4. **Megace ES**
   - "Par Pharmaceutical Industries, Inc. ('Spring Valley NY'), which obtained the Megace name as of 'Bristol Myers Squibb', introduced Megace Es (megesterol acetate) (New York)". Megestrol, a synthetic progestin, exhibits similar physiological effects to those of progesterone in nature. Megestrol also inhibits the production of luteinizing hormone from the pituitary and directly causes cytotoxicity in breast tumor compartments in tissue culture.
   - It is mostly used to help patients who are receiving chemotherapy or who have HIV increase their weight and increase their appetite.

Table 1 Survey of some nanocrystal formulation as marketed product

Trade Name	Drug	Applied Technology	Status	Indication	Company
Emend	Aprepitant	Nanocrystal élan	Marketed	Anti emetic	Merck
Megace ES	Megestrol	Nanocrystal élan	Marketed	Anti anorexic	Par Pharmaceutical Companies
Nucryst	Silver	Own	Phase II	Anti bacterial	Nucryst Pharmaceuticals
Paxceed	Paclitaxel	Unknown	Phase III	Anti inflammatory	Angiotech
Rapamune	Rapamycin	Nanocrystals élan	Marketed	Immunosuppressive	Wyeth
Semapimod	Guanylhydrazone	Own	Phase II	TNF-α inhibitor	Cytokine Pharmasciences
Theralux	Thymectacin	Nanocrystal Élan	Phase II	Anti cancer	Celmed
Tricor	Fenofibrate	Nanocrystal élan	Marketed	Hypercholesterolemia	Abbott
Triglide	Fenofibrate	IDDP Skye Pharma	Maketed	Hypercholesterolemia	Sciele Pharma Inc.

Due to the capability of adjustable dosing to effectively stimulate hunger and promote weight gain, the lower volume and improved bioavailability result in better patient compliance (Table 1).

7 Conclusions

Drug that are not readily soluble in water, nanocrystals are considered to be the most important formulation strategy. To solve the solubility and bioavailability issues, this technology can be used with any medications that aren't very water soluble. The reduction in nanoscale particle size. In certain instances, the elevated solubility as well as dissolution pace which result into a favorable bioavailability. This method makes it possible to create formulations without the use of troublesome surfactants (like Cremophor EL), which could lead to more severe side effects or negative responses. Due to the drug's quick absorption and the nanoparticles' quick breakdown, nanocrystals also enable a rapid start to the action. This is advantageous, especially for drugs that must act quickly (e.g., naproxen for headache relief). Since drug nanocrystals can administer fewer doses to attain a moderate blood level, they can lessen the negative effects associated with giving greater dosages. It can be used for a variety of delivery methods, including cutaneous, ophthalmic, pulmonary, and parenteral administration. The solid dry powder can then be created from the liquid Nanosuspensions to produce tablets, capsules, or pellet dosage forms. With these

tools we used to create finished dosage forms that have better therapeutic targeting, superior redispersibility at sites of action, as well as higher drug loading capacities.

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Novel Techniques in Pulmonary Drug Delivery Systems

Priyanka Gondhale-Karpe

Abstract The Asthma, Bronchitis, Chronic obstructive pulmonary diseases (COPD) and Cystic fibrosis (CF) are observed now a day as the foremost cause for mortality worldwide. The lung organ having larger surface area and abundance vascular structure therefore the best site of choice for the various lung diseases treatment. The Anti-asthmatic and COPD drugs are developed for the treatment of COPD in variety of dosage forms namely Dry power inhaler, Metered dose inhaler and Nebulizers. To achieve the optimum distribution and absorption of the drug(s) its particle size must remain in the range of 1 to 5 μm. DPI dosage form formulated by various mixing techniques of drugs with the excipients preferably respiratory grade lactose. The MDI's are the solution or suspensions filled in canisters with propellant such as Hydrofluoroalkanes. For both DPI and MDI dosage form inhaler devices are utilized for the proper administration of drugs. For paediatric patients nebulization therapy is extensively utilized for the asthma and COPD management. DPI and MDI product development is challenging process as the optimum deposition and specific target site of action is achieved with narrow particle size distribution. The analytical tests such as Assay, Impurities and degradants, delivered dose uniformity, aerodynamic particle size distribution, extraneous particles, net content, moisture content, microbial-load, leachable content, and inhaler device constituent part characteristics are performed for all the three dosage forms. The spray pattern and plume geometry are the additional analytical tests performed for MDI's.

Keywords Dry powder inhaler · Metered dose inhaler · Nebulizers · Asthma · COPD · Analytical tests · Spray pattern · Plume geometry

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1 Introduction to Pulmonary Drug Delivery Systems (PDDS)

Asthma, COPD and cystic fibrosis treatment by inhaler treatment is used since last many years. The drug or combination of drugs is directly reaches in the lung region with quick action, higher efficacy and lesser side effects when compared with the oral or parenteral formulations (Patton et al. 2004).

The DPI, MDI and nebulizer formulations are extensively prescribed for prophylaxis and managing of lung diseases, asthma and complication related with it for localized and systemic effects (Patton et al. 2004). The lung provides an optimum alveoli surface area which is rich in capillaries and acts as an excellent absorbing surface for drug administration (Newhouse and Corkery 2001).

Because of the treatment efficacy in the severe diseases, suggested twenty seven multidisciplinary approaches to delivery of drug to the targeting organ or tissues. The efficacy of drug is optimized with controlled pharmacokinetics, pharmacodynamics, immunogenicity and pattern of bio-recognition (Newhouse and Corkery 2001). The newer research and development studies are based on the polymeric sciences, advances pharmaceutical technologies; bio-conjugate chemistry and molecular biology are some of the strategies carrying out in PDDS. PDDS research and development is carried out scientific and biomedical importance to reduce the loss and degradation of drug, and to improve drug bioavailability (Corkery 2000). Since last 2 decades, to achieve optimum drug targeting and drug delivery the researchers mainly focusing on the nanotechnology (Georgitis 1999).

The PDDS is found to be advantageous because; only respiratory system is exposed to drugs therefore very few adverse effects are observed with quick onset of action and rapid relief (Georgitis 1999; Bennett et al. 2002). The viscous pharmaceutical formulation combinations with carrier molecules like lipid, water or lipid/water administration is possible in PDDS in the form of nebulizer dosage forms. The drug formulations containing liposomes are found to be stable if given in the form of nebulization (Georgitis 1999; Bennett et al. 2002). The drug particles with the help of respiratory grade carrier lactose as a carrier molecule reach the lungs and size having 1 to 5 μ is deposited at the site of action. Similarly, In MDI dosage form the particles of drug are solubilized or dispersed in the propellant (Bennett et al. 2002).

Limitations of PDDS include the high oropharyngeal deposition due to improper handling of inhaler devices by the patients (Dhand 2001). The delivered drug reproducibility is mainly dependant on the variables such as physiological and pharmaceutical variables. Drug retention in the lungs is reduced by the lungs clearance system, which may improve drug absorption (Beck-Broichsitter et al. 2009). The lungs are not an easy and accessible organ for delivery of drug; hence complicated delivery systems are needed for targeted delivery of drug, such as inhaler devices or other ancillaries. Respiratory Deposition Mechanisms Inhaled aerosol particle deposition in the respiratory system is caused by three processes: Inertial impaction, Brownian diffusion and Gravitational settling. For any breathing condition, a theory is established to estimate deposition and distribution of drug particle in the airway. The particles

may deposit in several sites along the respiratory tract once it enters by the mouth or nose (Timsina et al. 994). The direction of airflow varies frequently when breathing in the nasal/mouth, pharynx, larynx, and airway bifurcations. In these areas, large particles (>0.5 μm) might be depositing through impaction because they are unable to monitor the air streamline. Infact, a significant percentage of the dose emitted by pMDI and DPI devices continues to be deposited by impaction in the oro-pharyngeal region (Beck-Broichsitter et al. 2010). The primary method of inhaled tiny particle deposition in the alveolar region and small airways is achieved by sedimentation.

2 Respiratory Tract as a Target for PDDS

Human respiratory system is divided into 2 parts; nose, nasal cavity, pharynx and associated structures which known as upper respiratory tract and larynx, trachea, bronchi and the lungs which known as lower respiratory tract (Nyström and Fadeel 2012).

Nose divided into two; external nose comprises of bones, hyaline cartilage enclosed with muscle, skin and mucus membrane and two opening termed as nostrils. The internal Nose also called as nasal cavity it connects pharynx posteriorly through two openings known as internal choncae. Nose provides airways for respiration. Due to the high mucosal vascularity, the nose enables the breath to become warmed. The thick mucosa in the nose allows it to warm both the air being inhaled and released. The olfactory sensors are situated in this structure, which also helps as a resonating chamber for speech and aids in filtering inhaled air (Sharifi et al. 2012).

The pharynx is a funnel shaped tube like structure approximately 13 cm and divided into the three parts namely; the nasal cavity supplies air to the nasal cavity's apical section, called as nasopharynx. To balance the air pressure between the pharynx and middle ear, it also exchanges a moderate amount of air with auditory tubes (Schleh et al. 2011). Since it serves as a common pathway for air, food, and liquids, the oropharynx is the middle section that has both respiratory and digestive functions. The oropharynx has two pairs of tonsils; the palatine and lingual tonsils. The inferior part of the pharynx is called the laryngopharynx (Schleh et al. 2011).

Larynx creates sound with pitch, volume, tone and joins the laryngopharynx and trachea. It safeguards the lower respiratory system. Larynx upward motion occurs during swallowing. The epiglottis supports in larynx closure and ensures that food enters the oesophagus instead of into the respiratory airway (Possmayer et al. 2010). The larynx assists in keeping the pharynx and trachea's airway passage. It's a tube-shaped airway. It has a diameter of 2.5 cm and a length of 12 cm. It runs from larynx to fifth thoracic vertebra, and it is situated anterior to the oesophagus. It is made up of 16 to 20 hyaline cartilage rings that are incomplete and horizontal, resembling the letter C (Todo et al. 2004). One after the other, they are appearing as a sack like structure. Each C-shaped cartilage has an open portion that faces the oesophagus. The C-shaped cartilage supports the trachea semi-rigidly, helping to minimise airway blockage and the collapse of the tracheal wall during inhalation (Schleh et al. 2011).

The trachea or windpipe has tubular branches called bronchi that carry inhaled air to the lungs divided in the left and right primary bronchus at the fifth thoracic vertebra. The bronchi then split into bronchioles, which are progressively smaller branching (Labiris and Dolovich 2003).

On either side of the chest are two air-filled, spongy organs called as lungs. The pleura are a thin tissue layer that covers the lungs. The lungs slide easily as they expand and collapse with each breath due to a thin coating of fluid acting as a lubricant (Dhand 2001; Possmayer et al. 2010). The thoracic wall and superior face of the diaphragm are protected by a parietal pleura. The visceral pleura are a layer that spreads to cover the exterior lung surface and lining the fissures (Beck-Broichsitter et al. 2009).

Gas exchange takes place in the moist, thin-walled compartments known as alveoli. The alveolar walls are kept from adhering together and collapsing by a slightly oily surfactant. Alveoli and capillary walls make up the alveolus, which is the respiratory surface. The membrane lining the alveoli and capillary walls are found to be similar. Lungs along with muscle of respiration carry out the process of breathing, i.e. inhalation and exhalation (Todo et al. 2004; Labiris and Dolovich 2003). Principle muscle of respiration is diaphragm and intercostal muscles. The 12 pairs of ribs gaps are occupied by the 11 pairs of intercostal muscles. The external and internal intercostal muscles are arranged in two layers. Both muscles help in breathing by elevating the ribcage. A narrow dome shaped muscle called the diaphragm divides the thoracic compartment, which consists of heart and lungs, from the intestine, stomach, liver etc. for breathing and respiration, the diaphragm is essential (Schleh et al. 2011). It is engaged in breathing, pulling the chest inward during inhale and pushing it outward during exhalation. It also involved in non respiratory functions, facilitating to expelling faeces, urine and vomit, from the body by increase in the intra-abdominal pressure. The diaphragm and intercostal muscles contract during inspiration or inhalation, and they relax during exhale (Possmayer et al. 2010).

2.1 Respiration Physiology

Respiration is the term used to describe the gases exchange O_2 and CO_2 within the body. The pulmonary ventilation, external and internal respiration are the three fundamental stages of respiration. As part of breathing or pulmonary ventilation, ambient air is inhaled and carbon dioxide rich alveolar air is exhaled. Gases O_2 and CO_2 are then diffused across the alveolar membrane. The main sites of gas exchange are alveoli, where carbon dioxide is released as a result of oxygen use by cells during catabolic reactions, diffusion of oxygen and carbon dioxide takes place (Possmayer et al. 2010). Additionally, the blood and tissues gas exchange occurs. In these sites, oxygen and carbon dioxide are exchanged primarily based on pressure or concentration gradients by simple diffusion. Other significant variables that can impact the rate of diffusion include the solubility of the gases and the thickness of the membranes engaged in diffusion. Partial pressure, denoted as pO_2 for oxygen

and pCO_2 for carbon dioxide is the pressure that each gas contributes to a mixture of gases. These two gases partial pressures in the atmospheric air were measured at their two sites of diffusion (Todo et al. 2004).

2.2 Classification of Anti Asthmatic and COPD Drugs

2.2.1 Bronchodilators

- B2 sympathomimetics- Salbutamol, Salmeterol, Formoterol, Terbutaline, Bambuterol
- Methylxanthines- Theophylline, Aminophylline, Doxophylline
- Anticholinergics- Tiotropium, Ipratropium
- Leukotriene antagonists- Montelukast, Zafirlukast
- Mast cell stabilizers- Sodium cromoglycate, Ketotifen

2.2.2 Corticosteroids

- Systemic- Hydrocortisone, Prednisolone
- Inhalation- Budesonide, Fluticasone propionate, Ciclesonide, Flunisolide
- Anti-IgE antibody- Omalizumab

2.2.3 Fundamental Aspects of PDDS

Two main independent risk factors, which have an im pact on the drug deposition profile, are as follows:

(a) Patient related factors

   a. Respiratory tract's anatomy and physiology
   b. Inhalation air flow rate
   c. Inhalation mode

(b) Physical properties

   a. Drug properties
   b. Excipient properties carrier systems
   c. Formulation related factors

3 Patient Related Factors

3.1 Respiratory Tract's Anatomy and Physiology

To supply oxygen from lung to organ cell and removal of CO_2 from the lungs are the main functions of respiratory system. There are two components in the respiratory tract consist of the upper respiratory tract consists of nose, nasal cavity and pharynx and the lower respiratory tract consists of larynx, trachea, bronchi and lungs. The trachea constitutes the essential respiratory path which connects the larynx, bronchi and at last to the lungs. The alveoli are the functional component for gas exchange. In respiratory system a steady reduction in diameter is observed in the direction of the alveoli. The alveolar deposition is therapeutically important as the alveoli have extensive surface area which is responsible for the rapid absorption of drugs. The drug deposition is considerably affected by the respiratory system geometry (Labiris and Dolovich 2003).

3.2 Inhalation Flow Rate

In DPI's, the patients inspiration efforts is the driving force in the respiratory airway for drug deposition, the rate of inhalation flow is essential to attain standard particle disaggregation. Nevertheless the patient's rate of inhalation flow cannot be controlled. The overall lung deposition is observed to be similar in both male and female patients. (Labiris and Dolovich 2003; Laube 2009) Female patients show high aerosol deposition in the trachea bronchial region and upper respiratory tract this might be due to the variation in airways in male and female patients; also it dependent on patients disease condition, gender, age, and height. In general in healthy human, the peak average inspiration flow rate found to be 300 L/min and patients suffering from asthma the average inspiration flow rate found to be 200 L/min or even less. Nature of airflow created inside the inhaler device that is laminar or turbulent also important in DPI dispersion. The turbulence in the air flow rate is optimum for particle dispersion of the DPI as compared to laminar airflow is proved (Laube 2009).

3.3 Inhalation Mode

Aerosol inhalation mode is responsible for the particle deposition in the airway. The inhalation mode comprises of airflow rate, air inhalation volume, and breath holding period (Lavorini et al. 2008).

4 Physical Properties

4.1 Properties of Pure Drug

The particle size reduction should be performed to produce the particles in the respirable fraction (i.e. >5 μm in diameter) therefore; milling, supercritical fluid and spray drying methods are the some methods to obtain the desired particle size for the drug products that we will discuss in detail in formulation aspects (Mishima 2008).

4.2 Properties of Excipient Carrier System

Excipients are used to enhance physicochemical stability of the active drug(s), also it enhance pharmaceutical and/or mechanical properties like permeation, dissolution etc. Newman and Busse (2002). In DPI formulations excipient used with main objective is carrier particles. In DPI few micrograms of active drug moiety needs to be delivered hence excipient provides bulk which is responsible for improved drug administration, drug distribution and quantification (Newman and Busse 2002, Nazrul and Ellen 2008). Excipient should also decrease the cohesiveness of drug by utilizing the higher energy sites of the drug particle to reach at pathological site in the lung (Nazrul and Ellen 2008). At present only lactose is the additive utilized in DPIs as it is safe and stable and easily available in numerous particle size and structural appearances. Its manufacturing process with controlled operating procedure can be estimated for purity and physical properties (Ren et al. 2008). Lactose is highly crystalline and has smooth surface morphology which results in improved flow ability which is necessary factor as a DPI carrier. Lactose is highly versatile and less hydroscopic when compared to other sugars but it is incompatible with drugs having primary amine moieties as lactose is a reducing sugar (Ren et al. 2008). In general, excipient's can make upto 99% of the DPI drug product w/w; this makes them vital elements for complete performance of dry powder inhalers (Ren et al. 2008). No excipients are every time mandatory, Plumicort (budesonide), Terbuhaler (AstraZeneca) are examples of only drug formulation (free from excipient) (Sanjar and Matthews 2015).

4.3 Formulation Related Factors

Particle size: Appropriate particle size is found to be better for the pulmonary delivery and finally to the pharmacological effects. There are significant literatures in the field of Inhalation drug and pharmaceutical aerosol sciences which connects aerodynamic

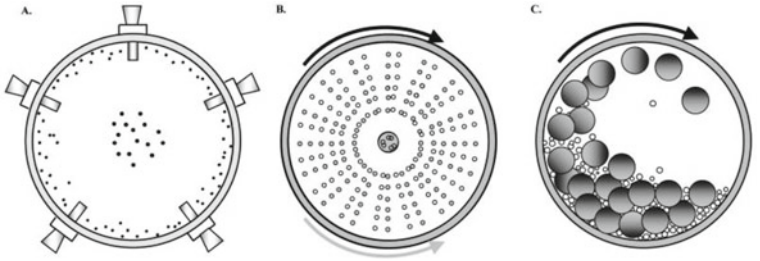

Fig. 1 Cross section view of A. Jet mill B. Pin mill C. Ball mill

size and distribution of particle size to the deposition possibility in definite pathological site into the lungs (Sermet-Gaudelus et al. 2002). The desired particle size range less than 5 μm in diameter can be achieved by milling procedure. There are numerous mills however only limited are capable to mill powder size to the necessary size of particle ranging from 2 to 5 μm. the important three mills are as follows:-

- Fluidized energy mill-Jet mill
- High speed peripheral mill-Pin mill
- Ball mill.

The basic designs (Refer Fig. 1).

The Jet milling or air attribution milling: It's a most convenient technique; which utilizes high velocity particle–particle collision mechanism to reduce particle size. Particle size less than 1 μm in diameter can be achieved by jet milling (Hue et al. 2001). The pin mill utilizes the Phenomenon of mechanical impaction to grind material both by particle–particle collision as well as particle-solid collision. The pin mill yields 1 μm particle size but not as small as when compared to jet mill also, pin mills requires less power consumption than other mill (Hue et al. 2001). The ball mill is fundamentally a rolling cylindrical container consists of powder blend to be milled; ball like structure crush the drug amongst each other and tumbled into the milling chamber. Ball milling is very slow as compared to all other mills; hence ball mills are often used in trial scale only (Hue et al. 2001). Spray drying and supercritical fluid crystallization can also be utilized to obtain desired particle size (Hue et al. 2001).

Particle morphology: Particle Morphology may be defined as the shape, structure and exterior properties of a particle (Shoyele and Cawthorne 2006).

Aerodynamic diameter of the particle: The sphere particles, a constant density and a determined velocity that is similar to that of a no spherical shape particle (Shaikh et al. 2010). To describe the aerodynamic properties of a particle and deposition pattern of the drug in the respiratory system, the term Aerodynamic diameter frequently employed. The terminal velocity square root (V_{TS}) is proportional to the particle aerodynamic diameter.

$$V_{TS} = \frac{p_0 d^2 ag}{18\eta}$$

$$da = de\sqrt{\frac{P_p}{xPo}}$$

where de = diameter of predicted geometric particle, P_p = particle density, x = dynamic shape factor, ρ_0 = density, da = aerodynamic particle diameter, η = gas viscosity, g = gravitational constant.

Mass median aerodynamic diameter: Some methods are used to measure invitro aerosol deposition, including ACI and NGI. The mainspring of all of this equipment is the cut off diameter on the individual collection stage, where fractional drug deposition takes place. MMAD is a form of sieve analysis, to put it simply. The cumulative mass of the collected powder quantity that is smaller than the specified dimensions of each individual impactor stage is used to compute the total quantity of drug recovered in the impactor (Shaikh et al. 2010). The MMAD can be determined by plotting the graph of cumulative quantity of powder against the effective cutoff diameter on a log scale. The key statistical variables are the geometric standard deviation (GSD) and the mass median aerodynamic diameter (MMAD) (Selroos et al. 2006). Particle size correlation with a cumulative sum of 50% can be used to determine the MMAD and the below formula can be utilized to determine the GSD.

$$GSD = \sqrt{X/Y}$$

where, X = the particle diameters related to 84% deposition of cumulative quantity and Y = the particle diameters related to 16% deposition of cumulative quantity (Selroos et al. 2006). Fine particle dose (FPD): It is the drug quantity in the prescribed dosage which is typically examined for the quantity required for entering into the pathological site in the lung i.e. respirable fraction; it is generally of 5 microns or less. The FPD is many times gets mislead by the term fine particle mass i.e. FPM. FPM is the drug quantity found after a single actuation of DPI, although the recommended drug dose may require several actuations. The FPD when expressed in terms of percentage of the drug dose delivered from an inhaler is termed as fine particle fraction (FPF) (Sermet-Gaudelus et al. 2002)

Inter-particle interactions: The interactions of particles, surface energy and the dispersion of powder are the three crucial factors affect the target of particle deposition in the respiratory system (Shoyele and Cawthorne 2006). The aggregate structure of particles affects the aerodynamic forces. The corresponding motion between the particle and the air stream is responsible for the generation of the aerodynamic forces (Shaikh et al. 2010). The powder dispersion in an airstream is carried out by the equilibrium within the aerodynamic force and the cumulative strength. When these forces are greater than the cumulative strength, deep lung deposition is attained and the powder particles are evenly disperse in an air stream (Shoyele and Cawthorne 2006). The cumulative strength determined as follows;

$$\sigma = 15.6\frac{\emptyset}{dv}$$

where Ø = ratio of the particle bulk density and particle true density; W (J/m^2) = the adhesion work and dv = sphere shaped or equal particle diameter. The aggregate strength is affected by the adhesion. It is expected that non spherical particles can also achieve improved drug distribution profile than sphere-shaped particles; because of the lower average non-spherical particle curve (Dolovich et al. 2005) this is directly proportional to the spherical or equal particle diameter (dv).

Vander Waals forces: the deposition of particle and its profile of dispersion are both influenced by the interparticle forces; these complex forces include electrostatic forces and Vander Waals forces etc. The most powerful force influencing particle deposition and drug dispersion behavior is the Van der Waals force. All aerosol formulations experience the Vander Waals forces caused by the induced dipole dipole interactions (Hue et al. 2001).

Work of adhesion or cohesion: In DPI's the cumulative strength is relying on the cohesive forces (originating from the interactions between drug-drug particles) or the adhesive forces (originating from the interactions between drug-excipient particles); also the induced dipole–dipole interactions are also dependent on Work of adhesion and cohesion. The effective interaction diameter (dv) is the difference between diameters of particles of drug(s) and excipient(s). Dv is determined as drug and additives mean of arithmetic diameters d1 and d2 respectively (Frijlink and De Boer 2004)

$$\mathrm{dv} = \frac{1\mathrm{d}2}{(\mathrm{d}1 + \mathrm{d}2)}$$

Electrostatic Interactions: The particle dispersion can also affected by electrostatic force and the associated Columbian forces. These interactions exhibit equally effective as Vander Waals force (Nolan et al. 2011). The electrostatic interactions between drug-drug and drug-excipient particles affect the quantity of particles of aerosol escaping the from the capsule or inhaler device. Agglomerate formation leads to the generation of differently charged particles (Nolan et al. 2011).

Aggregate strength: To determine the cumulative strength many theoretical procedures developed till date; and these methods are surely established on the particle surface dynamics. Nonetheless, in the estimation of the aggregate strength; the sum of interaction energies of drug particles must be taken into consideration. The particle dispersion, dipole–dipole and H_2 bonding; these forces are the main reason for the drug particle interactions (Copley scientific manual Edition 2019). These forces depend on the parameter of solubility of hildebrand i.e. δA and δC (Pai and Nahata 2001). The correlation amongst the solubilization parameter, the cohesive strength σC and adhesive strength σA interactions are

$$\sigma\mathrm{C} = 0.25\delta\mathrm{c}^2$$

$$\sigma\mathrm{A} = 0.25\theta\delta\mathrm{c}\delta\mathrm{A}$$

where θ = the drug molecules interaction parameter. Hence, it can be predicted that the work of adhesion and cohesion are proportionate to the drug-drug interactions and drug-excipient interactions strength. According to mentioned theoretical procedures, the conclusions made as follows:

- The reduction in aggregate strength takes place with subsequent particle diameter (dv) increases (Sermet-Gaudelus et al. 2002).
- Loose and weak aggregate structure is often produced by low bulk density powders.
- The reduction in the particle surface energy is associated with the enhance powder dispersion behavior hence; in case of DPI's coating is immensely suggested for precise cohesive strength (σC) and adhesive strength (σA) interaction (Shekunov et al. 2007).
- Hollow porous micro particle formulations produce a comparatively higher arithmetic volume diameter when correlated to the aerodynamic diameter.

Span index: An ideal aerosol drug particle must have a small range distribution of particle size and quick dispersibility at comparatively lower aerodynamic force (Roberto and Dal Negro 2015a, b). The constricted span index shows narrow particle size distribution and is desired requirement for the DPI (Sanjar and Matthews 2001) The span index can be defined as the measures of distribution of particle size associated to its median diameter. Span index can be determined as follows:

$$\text{SpanIndex} = \frac{\text{D90} - \text{D10}}{\text{D50}}$$

where, D10 = 10% of the particles are less than 10 μm, D50 = 50% of the particles are less than 50 μm, and D90 = 90% of the particles are less than 90 μm.

The aerosol particles fraction settled in the upper respiratory tract i.e. mouth and throat; is termed as impaction loss; and it is high for powdered formulations having larger span index. The loss in the impaction is directly proportionate to the rate of airflow and drug particles diameter square and is affected by the poly-dispersity of particles (Roberto and Dal Negro 2015a, b). Large particles are governed by its mass and impacts upper respiratory tract because of its inertia. Oropharyngeal deposition indicates high inter and intra variation. Important force mechanisms in deposition of particles into the lungs are as follows (Refer Fig. 2).

- Inertial impaction: The particle's deposition into the exterior of respiratory airway. Inertial impaction takes place near to the respiratory tract at the branching of the conducting airways. In this high flow velocity and variations in airflow directions are observed (Roberto and Dal Negro 2015a, b).
- Gravitational sedimentation: Gravitational sedimentation observed in the minor conducting airway in which the air velocity is relatively small and size of particle is <5 μm (Roberto and Dal Negro 2015a, b).

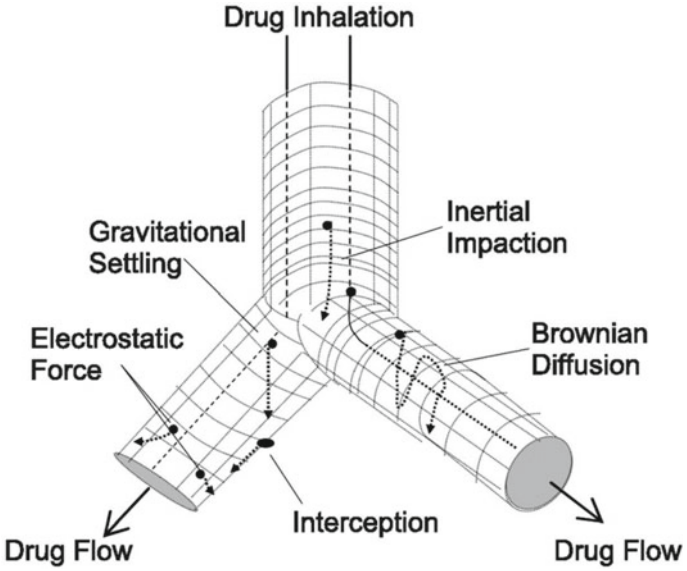

Fig. 2 Schematic diagram of forces involved in drug delivery of inhalation products

- Diffusion: In small airway and alveoli at which low velocity of air and particle size <0.5 μm; diffusion mechanism takes place (Roberto and Dal Negro 2015a, b; Sanjar and Matthews 2015).
- Interception: In Interception, deposition phenomenon may occur when a particle comes in contact with epithelial wall of respiratory tract; nevertheless its center of quantity may persist on the array of fluid. It is important only for particle aggregates (Roberto and Dal Negro 2015a, b).
- Electrostatic attraction: Improved drug deposition in consequences of electrostatic charges by expanding the attractive forces toward the airway (Roberto and Dal Negro 2015a, b).

5 Lung Diseases and Its Treatment by Various Dosage Forms Such as DPI's, MDI's and Nebulizers

5.1 Asthma

The chronic inflammatory disorder of the airways is called as asthma. Chronic inflammation increases the airways hyper responsiveness, which frequently results in episodes of coughing, chest tightness, wheezing and dyspnea, especially in the morning or at night. Their signs and indications are comparable to those of other

illnesses with restricted airflow, such as chronic bronchitis (Corkery 2000; Beck-Broichsitter et al. 2009). In wheezing a whistling sound made while breathing is known as wheezing. It happens as a result of air being pushed through the restricted and smaller air channel. One of the most typical symptoms of asthma is coughing. The cough can be dry or wet, and it may get worse at night, in the early morning or after activity. Breathlessness is the sensation that one breath is almost about to expire when another one is essential. It is referred as "air hunger." Due to mucus production many asthmatics produce an excessive amount of mucus, which can clog the airways and cause coughing. Chest constriction; this may feel as if something is pressing against or resting on the chest. The patient may experience a feeling of chest tightening because of the muscles surrounding the airway gets constrict.

5.2 Types of Asthma

Extrinsic asthma often known as allergic asthma: It occurs when an allergen specific immune reaction causes the symptoms. The IgE based type I immediate hypersensitivity reactions.

Intrinsic asthma often known as non-allergic asthma: It is brought on by airborne irritants that are not connected to allergens. These irritants cause inflammation and bronchoconstriction by stimulating parasympathetic nerve cells in these irritants cause inflammation and bronchoconstriction by stimulating parasympathetic nerve cells in the airways. These irritants cause inflammation and bronchoconstriction by stimulating parasympathetic nerve cells in the airways.

- Mixed asthma is occurring by both allergic and non-allergic symptoms are known as "mixed asthma" It's a most typical type of asthma.
- Cough variant asthma prolonged dry cough is the only symptom observed; the typical asthmatic symptoms such as wheezing and breathlessness are not observed.
- Physical activity triggered asthma affects the human during or after exercise.
- Nocturnal asthma is gets worsen at night is known as nocturnal asthma. Anyone with nocturnal asthma may feel indications at any time.
- Occupational asthma is triggered by stimuli present at person's place of employment, such as farming, woodworking, textile industry and chemical industry etc.

5.3 Pathophysiology of Asthma

See Fig. 3.

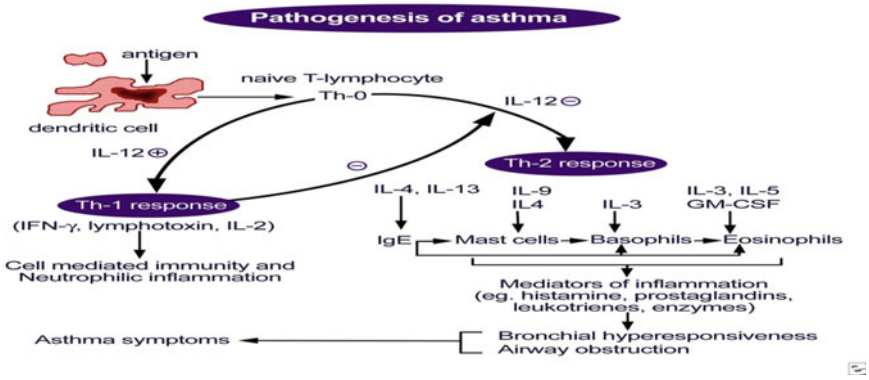

Fig. 3 Schematic representation of pathogenesis of Asthma

5.4 Diagnosis

- Spirometer—This instrument measures the amount of air we can exhale and the force with which we can do so. One could take the test both before and after inhaling the medication. An effective technique to measure how much breathing is affected during an asthmatic episode.
- Peak Expiratory Flow Determination—Measure the capacity to force air out of the lungs or the flowrate at air is expelled using the peak expiratory flow method. A peak flow meter is used in this test, which the patient can carry out at home to check lung function.
- Blood tests for allergies and chest X-rays can also be useful for diagnosis of asthma.

5.5 Chronic Obstructive Pulmonary Disease

The terms chronic obstructive pulmonary disease (COPD) or chronic obstructive airway disease (COAD) refer to the pathological conditions in which there is a persistent, incomplete or total airflow hindrance at several stages, from the trachea to the smallest airway, which results in the functional impairment of lungs. Smoking is one causative factor that is present in all kinds of COPD (Timsina et al. 1994; Beck-Broichsitter et al. 2009). The clinical complications falls under the COPD are emphysema, chronic bronchitis, bronchial asthma, bronchiectasis, and bronchiolitis etc. A group of respiratory disorders is known as COPD. It is challenging to breath and get worst over the period of time. The lung airway passage and air sacs are often elastic. The airways transport air to the air sacs during inhalation. Like a little balloon, the air sacs expand as they fill with air. The air sacs collapse as a person exhales, letting the air out. People with COPD have less airflow into and out of their

airways due to a several problems; the walls separating the air sacs are damaged, the airway walls get thicken, enlarged and produce more mucus than normal. The lung airways and air sacs also turn into less elastic (Timsina et al. 1994).

5.6 Types of COPD

There are two types of COPD.

- Emphysema—Lung air sacs and their interstitial walls are both impacted by emphysema. They deteriorate and lose their elasticity.
- Chronic bronchitis—the airway lining is continuously inflamed and irritated in chronic bronchitis. The lining swells and produces mucus.

These are some of the COPD risk factors. The biggest risk factor is smoking upto 75% of patients with COPD smoking habits. Exposure for a long period of time to irritants like chemical fumes, dusts and air pollution causes COPD risks. Age is the majority of COPD patients are at least 40 years old when their symptoms first appear. A genetic condition that comes into this class is alpha-1 antitrypsin deficiency. Also, if there is a family history of COPD and smokers who develop COPD are more likely to do so. Chest tightness and short breath, especially after intense physical activity, wheezing or noisy sound when patient breath, recurrent dry cough or wet coughing is observed commonly.

5.7 Treatment of COPD

Adopt lifestyle adjustments, such as giving up smoking and adopting a nutritious diet. Bronchodilator, inhalation steroids helps to expand airways and ease breathing by relaxing the muscles around them.

Since COPD patients are more likely to acquire the flu and pneumococcal pneumonia, vaccination is vital for the management of both illnesses. If you get a viral or bacterial lung infection, antibiotics are prescribed along with oxygen therapy to prevent the condition from getting severe. Exercise may be a part of a pulmonary rehabilitation programme, which is carried out to improve the patient's health and treat chronic respiratory problems.

Exercise may be a part of a pulmonary rehabilitation programme, which is carried out to improve the patient's health and treat chronic breathing issues. Surgery is typically used as a last option for patients with severe symptoms that have not responded to medical treatment. Emphysema is the main cause of COPD, which can be treated surgically by removing damaged lung tissue and the big air spaces (bullae) which develop when air sacs are damaged. The bullae could make difficulty in breathing; severe COPD could need to have their lungs transplant.

Fig. 4 DPI, MDI and nebulizer devices

6 Treatment of Asthma and Other Lung Diseases with DPI's, MDI's and Nebulizers

See Fig. 4 and Table 1.

7 Analytical Testing of DPI's, MDI's and Nebulizers

Common tests of DPI, MDI and Nebulizers include the identification test and other physicochemical tests such as Assay, related substances, fill weight or net content or average weight of the content, DDU and APSD etc. Tong et al. (2006). Valve Delivery per Actuation (Shot weight), Number of delivery per Canister, Leak Rate, Moisture Content by KF Coulometric Titration, content per canister (CPC), fine Particle Dose are other tests of MDI dosage forms (Tong et al. 2006; Usmani 2012). The SP and PG are the additional analytical tests performed for MDI's in which the tests are carried out on the basis of laser diffraction techniques (Usmani 2012; Copley scientific manual Edition 2019).

For generic product submission into the regulatory market it is foremost that the respiratory dosage forms needs to comply with the innovator sample for an approval.

Table 1 DPI, MDI and nebulizer dosage form their advantages and disadvantages

Dosage form	Advantages	Disadvantages
Nebulizers- air is passed through the drugs in the form of solution which produces a fine mist and inhaled with the help of face mask	• High doses of drugs can be delivered • Does not require patient's breathing coordination • Drug delivery is consistent and deposition is better than inhalers	• Very expensive • Not portable • Patient needs to wear mask • Requires supervision
Metered dose inhalers (MDI)-Drug(s) are in solution or suspension form used with propellant which delivered the drug as a fine mist or spray to the lungs	• Convenient and portable • Delivers fixed amount of dose to the patient	• Expensive when compared with DPI's • Not all the asthma and COPD drugs are available in DPI dosage forms • Requires vigorous shaking for 5–6 s prior to administration • Improper device use can leads to high drug deposition in the upper respiratory tract
Dry powder inhalers (DPI)-utilized with the breath activated inhaler devices for the drug delivery to the lungs	• High patient compliance • Convenient and portable • Economical • Mostly all the asthma and COPD drugs are available in DPI dosage forms	• Patient needs to breathe deeply to activate the device and to facilitate release of the medicament • Improper device use can leads to high drug settling in the upper respiratory tract

That's why the respiratory dosage forms are termed as complex generics (Usmani 2012; Copley scientific manual Edition 2019).

8 Conclusion

The chapter explains about inhalation therapy is utilized for the treatment of Asthma, Cystic fibrosis and COPD. The advantages of PDDS with anatomy and physiology of respiratory tract, physiology of respiration, mechanisms of respiratory deposition for PDDS is summarized. The detailed classification of antiasthmatic and COPD drugs is illustrated. The fundamental aspects of PDDS including patient related factors and physical properties of drug and carrier or vehicle molecule is taken into consideration.

The chapter also illustrated the important force mechanisms which are involved in drug deposition into the lungs. Lung diseases and its treatment by various dosage forms such as DPI's, MDI's and Nebulizers is narrated. The asthma and COPD its types, diagnosis and problems associated with it are explained in detail. Treatment of Asthma and other lung diseases with DPI's, MDI's and Nebulizers including the advantages and disadvantages of particular dosage forms with the analytical testing

associated with the DPI's, MDI's and Nebulizers are summarized. The chapter is useful to have a quick glance of pulmonary drug delivery system with its anatomy physiology, its formulation and analytical aspects.

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Proteomics: Scope and Potential

Proteomics in Oncology: Retrospect and Prospects

Amol D. Gholap, Navnath T. Hatvate, Pankaj R. Khuspe, Trushali A. Mandhare, Pooja Kashid, and Vinay D. Gaikwad

Abstract The main role for cancer disease treatment is to take care of mortality and morbidity rate by implementing all sorts of available options for health benefits of all patients with cancer including chemotherapy and or radiotherapy. But main obstacles are resistance and cancer patients' compliance towards to these mentioned therapies depending upon altered cellular pathways. The type of therapy implemented for the cancer is majorly governed through typical biomarkers with its respected mutated genes. The proteomic approach is a useful technique for determining the reasons why anticancer drug resistance emerges. The proteomics deals with analysis of whole proteome simultaneously and involved with certain approaches which help physician to identify particular biomarkers utilized for clinical response evaluation during the cancer treatment. The tissue biomarker and serum biomarker analysis are one of main key step in selection of cancer treatment and proteomics are implemented for the same. Proteomics are also helpful for analysis of protein expression and given exploration of activity for signaling pathway. Proteomics are also used to identify illness signs in the body. But there are still need to explore more detail perfection into sample preparation before analysis to get accurate results. The involvement of artificial learning methods for generation of data for the expression of protein along with proteomics can give new horizons for treatment selection and fruitful options to patient for the same.

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1 Introduction

The Human Genome Project completed the mapping of nearly all human genes at the beginning of the twenty-first century. Simultaneously, analytical tools for protein sequencing and further research are being developed and refined. These advancements gave rise to a novel area of proteomics in 1994. Proteomics is a subtype of multidimensional biological sciences called "omics" that has rapidly evolved and developed in various fields, mainly in the diagnosis and therapeutics fields. Marc Wilkins coined the term proteome for the first time in 1995 by combining the words protein and genome. Proteomics involves the organized study of various characteristics related to proteins with the objective of studying the composition, structure, function, and interactions of proteins with biological and cellular systems in various human health, disease, and disorder situations (Patterson and Aebersold 2003; Hood and Rowen 2013; Agrawal et al. 2013). If a comparison is made between proteomics and genomics, the proteomics technology provides superior data and information about the structure and function of the proteins. One of the limitations of proteomics is the complex and complicated prediction of expression proteins, which changes with time and environment. The human genome has between 26,000 and 31,000 protein codes. The human protein count estimated till date is near one million, with most of them containing modified form-like proteins with post-translational modifications (Wilkins et al. 1996; Holman et al. 2013). Proteomics technology provides the facility to detect post-translational modifications, protein isoforms, and protein interactions in addition to the several aspects of the composition, regulation, along with the activity of proteins. Proteomics can be implemented to analyse proteins in cells, tissues, and organs (Chandramouli and Qian 2009). Non-gel analytical techniques such as protein microarrays, shotgun Proteomics, western blot tests, and quantification by liquid chromatography–mass spectrometry can be implemented to effectively analyze the given samples. Proteomics technology has two major sections or two primary categories with respect to the mapping goal: one category maps protein expression, and the other maps protein interaction. The two-dimensional gel electrophoresis along with the mass spectroscopy are used for the quantitative mapping of the proteome, which is linked to the expression mapping of proteins in cells or bodily fluids (Florens and Washburn n.d.; Klose et al. 2002; Ong et al. 2002; Vercauteren et al. 2004). Understanding post-translational changes of proteins caused by diseases or various environmental factors is made possible by the expression mapping of proteins. The mapping of one protein's interactions with another protein makes use of both mass spectrometry and the yeast two-hybrid system as integrated technologies (Wolters et al. 2001; Cutler 2003; Schulz et al. 2007). Proteomics is a series of processes that must be meticulously controlled at each stage to prevent non-biological influences from interfering to that of the protein expression along with interactions. One of the crucial stage is sample preparation, since it solubilizes all the

sample's proteins and gets rid of any interfering inhibitory substances like lipids. To get trustworthy, correct, and reproducible results, enough of the sample is essential. The polyacrylamide gel electrophoresis technique is vital for protein separation and isolation. Dimensional gel electrophoresis related with one-dimensional gel electrophoresis along with two-dimensional gel electrophoresis and high-performance liquid chromatography are other techniques for protein separation. The Human Genome Project completed the mapping of nearly all human genes at the beginning of the twenty-first century. Simultaneously, analytical tools for protein sequencing and further research are being developed and refined. These advancements gave rise to a novel area of proteomics in 1994. Proteomics is a subtype of multidimensional biological sciences called "omics" that has rapidly evolved and developed in various fields, mainly in the diagnosis and therapeutics fields. Marc Wilkins coined the term proteome for the first time in 1995 by combining the words protein and genome. Proteomics involves the organized study of various characteristics related to proteins with the objective of studying the composition, structure, function, and interactions of proteins with biological and cellular systems in various human health, disease, and disorder situations (Pandey and Mann 2000; Rabilloud and Lelong 2011; Yoithapprabhunath et al. 2015). If a comparison is made between proteomics and genomics, the proteomics technology provides superior data and information about the structure and function of the proteins. One of the limitations of proteomics is the complex and complicated prediction of expression proteins, which changes with time and environment. The human genome has between 26,000 and 31,000 protein codes. The human protein count estimated till date is near one million, with most of them containing modified form-like proteins with post-translational modifications. Proteomics technology provides the facility to detect post-translational modifications, protein isoforms, and protein interactions in addition to the study of the composition, regulation, and activity of proteins. Proteomics can be implemented to analyze proteins in cells, tissues, and organs. Non-gel analytical techniques such as protein microarrays, shotgun Proteomics, western blot tests, and quantification by liquid chromatography–mass spectrometry can be implemented to effectively analyze the given samples. Proteomics technology has two major sections or two primary categories with respect to the mapping goal: one category maps protein expression, and the other maps protein interaction. Both two-dimensional gel electrophoresis and mass spectroscopy are used in the quantitative mapping of the proteome, which is linked to the expression mapping of proteins in cells or bodily fluids. Understanding post-translational changes of proteins caused by diseases or various environmental factors is made possible by the expression mapping of proteins. The mapping of one protein's interactions with another protein makes use of both mass spectrometry and the yeast two-hybrid system as integrated technologies. Proteomics is a series of processes that must be meticulously controlled at each stage to prevent non-biological influences from interfering with protein expression and interaction. The most crucial stage is sample preparation, since it solubilizes all the sample's proteins and gets rid of any interfering inhibitory substances like lipids. To get trustworthy, correct, and reproducible results, enough of the sample is essential. The polyacrylamide gel electrophoresis technique is vital for protein separation and isolation. Dimensional gel

electrophoresis related with one-dimensional gel electrophoresis along with two-dimensional gel electrophoresis and high-performance liquid chromatography are other techniques for protein separation (Rappsilber et al. 2000; Graves and Haystead 2002; Verrills 2006).

The idea behind one-dimensional gel electrophoresis is that proteins are get separated depending upon the molecular weight. While the dissolution of protein into sodium dodecyl sulphate is important prior to the isolation of the same. Since sodium dodecyl sulphate is a solvent for nearly all proteins, the solubility problem has not arisen. The benefits of one-dimensional gel electrophoresis include its ease of use, ability to isolate proteins along with molecular masses ranging from that of the 10–300 kDa. In the case of proteins with large molecular masses and complex natures the choice of the separation depends on the protein's net charge along with its molecular mass.

Proteomics can examine a protein's expression at various levels, enabling the evaluation of certain quantitative and qualitative biological reactions connected to that protein. At the post-transcriptional, transcriptomic, and genomic levels, proteomes are evaluated both qualitatively and quantitatively. Tracking changes in protein mixture composition and protein expression can be done using several types of approaches. Proteomics can compare protein expression between patients and healthy volunteers as controls and give information on the molecular mechanisms of disorders or diseases. Additionally, quantitative proteomics can offer in-depth understandings of cellular processes, disease or disorder mechanisms, and biomarker discoveries. Quantitative proteomics employs several novel techniques, including affinity labelling in addition to post-extraction or labelling of metabolic stable isotopes. The research done by using proteomics technology is helpful in various branches of science, like disease diagnosis like cancer, medicine, microbiology, and many more. This chapter will provide information about proteomics, its methods, some of its applications, and the difficulties this discipline is currently facing (Grønborg et al. 2006; Wang et al. 2007; Agnetti et al. 2007; Xiao et al. 2008; Low et al. 2013; Chandrasekhar et al. 2014).

1.1 Genomic Approaches

One of the processes related to the marking of DNA markers on specific chromosomes or the location of genes related to each other is known as "genome mapping." This mapping generates data that aids in the assembly of large amounts of DNA and the sequencing of genes. Physical mapping and linkage, or genetic mapping, are two major types of genome mapping. The utilization of a set of genetic markers with a suitable method to determine their place on the genome is necessary for proper and correct marking. Genetic refers to the sequencing and genotyping of a specific region of the genome (Table 1).

Cytogenetic technology is the study of the full chromosomal complement. Cytogenetic methods are very helpful to pinpoint the genomic abnormalities that are linked

Table 1 The comparisons between the genetic and genomic approaches

Genetic data approaches	Genomic data approaches
It is standardized analyses It contains less prejudice dependent on researcher It is simple to interpret It is cheaper since fewer markers were sequenced *Markers characteristics* Bi-parentally or Maternally inherited Co-dominant or dominant One or a more than one loci	It potentially provide greater resolution of population Stricture, demographic processes etc. *Markers characteristics* Only inherited Bi-parentally Only Co-dominant Thousands of loci
Markers Used Microsatellites Amplified Fragment Length Polymorphisms Mitochondrial DNA Chlomplast DNA	*Markers Used* Genome-wide single nucleotide polymorphisms Outlier genome-wide single nucleotide polymorphisms

to radiation and chemotherapy resistance. Many complex disease-related processes are investigated by using this technology. According to a recent study, the occurrence of resistance to some anticancer drugs like cisplatin was connected to the overexpression of chromosome 16 q on genes. A deeper comprehension for that of genes located in this type of chromosomal region could lead to the discovery of new potential therapeutic targets and provide insight into the important mechanisms of many anticancer agents like development of cisplatin resistance (Singh et al. 2000; Struski et al. 2003; Albertson and Pinkel 2003; Wilson et al. 2005; Saraswathy and Ramalingam 2011; Lobo et al. 2020).

1.2 Transcriptomic Approaches

Some of the processes, like profiling of gene expression, are increasingly frequently done using microarray-based technology. Finding gene expression alterations linked to the disease phenotype is the goal. Chemotherapy and radiation therapy resistance have both been studied in various types of tumours using gene-expression profiling. The Affymetrix HG-U113A microarray was used by Kang et al. has investigated acquired type of drug resistance in cell lines of carcinoma of gastric cancer. Four distinct cell lines of gastric cancer were used to create four 5-fluorouracil-, three-doxorubicin-, and three-cisplatin-resistant cell lines. In addition, eight potential genes for multidrug resistance, including a growth factor that binds to heparin, i.e., midkine, were linked to resistance to several other chemotherapeutic drugs. The depth knowledge related with biological system is depended on the knowledge of the proteins themselves must be added to the data gathered through genomic studies. The analysis

and research of the human proteome have improved with the advent and accessibility of new technological breakthroughs (Wu et al. 2002; Plebani 2005; Mena and Albar 2013).

1.3 Various Areas of Proteomics

In general, there are three main parts for the field of proteomics including expression proteomics, structural proteomics along with the functional proteomics as presented into Fig. 1. With the aim of discovering novel proteins through quantitatively assessing protein expression, expression proteomics focuses on the detection of total and differentially expressed proteins. Functional proteomics generally focuses on protein interactions by directly studying protein complexes, providing an understanding of functional organisation levels inside a cell. Structural proteomics is concerned with the investigation of structural protein complexes in relation to their cellular localization. The two main strategies in cancer proteomics are functional and expression proteomics. As an illustration, the identification of disease indicators, which is essential not only for early detection but also for accurate diagnosis, may be achieved by utilising expression proteomics to investigate the differential expression of various proteins in tissues or blood. On the other hand, by providing essential details about these interactions, functional proteomics can help us better understand how protein networks and complexes react to internal and external signals.

1.4 Protein Discovery

From the last ten years several different analytical tools like mass spectrometry with the help of genome sequence data are used for the advancement and development of proteomics. In the analysis several tools are implemented for different functions to give precise data related with the proteomics. The mass spectrometer basically contains ion generator, mass analyzer along wih the detector. The ion generator is implemented for the generation of ionize species through the ionization process while the mass analyzer is used for the determination of mass to charge ratio. The detector is utilized for the counting of the ions at each m/z value. Initially the mass spectrometer is only implemented for the small quantity and thermostable compounds. The same was not used for the analysis of high molecular compounds like proteins, peptides along with other biomolecules. The application of mass spectrometer for these is happened due to implementation of special ionization techniques like matrix assisted laser desorption along with that of electrospray ionization. The electrospray ionization method is implemented for the ionization of the sample out from that of the solution. It can be used along with liquid chromatography technique for better separation of the given compounds. The specific dry crystalline matrix is required to undergoes sublimation and further ionization and that can be achieved by using the

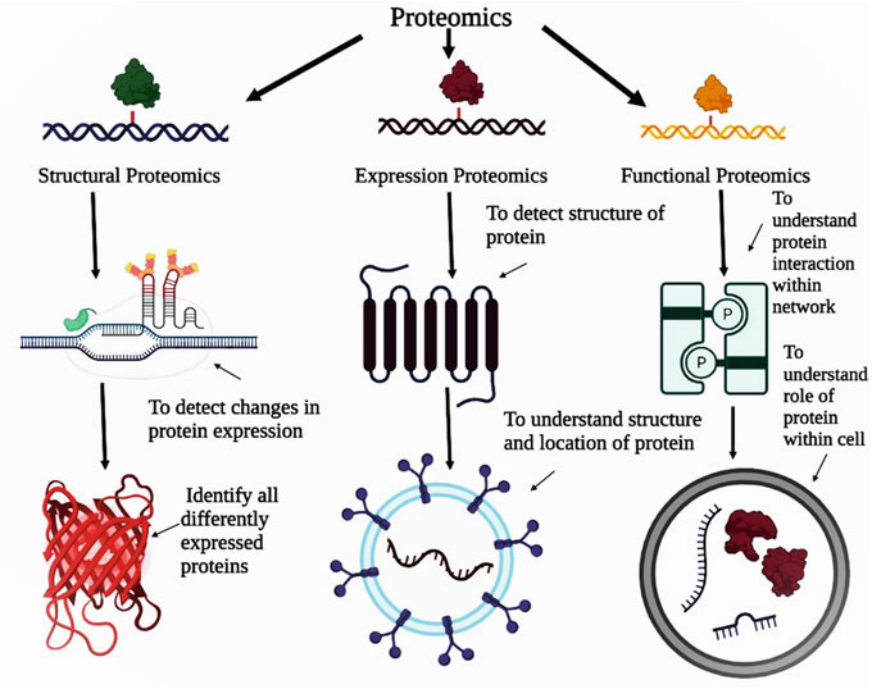

Fig. 1 Various areas of proteomics

matrix assisted desorption ionization method. The mass of ionize species is precisely calculated by this method. There are four different categories of mass analyzer technology used for the same. To get the proper accuracy, sensitivity for the determination of ionized species by using Fourier transform ion cyclotron resonance along with time of flight. The quadrupole and ion trap are also acts as a mass analyzer for the same. The large molecular weight of proteins makes them cumbersome for direct analysis on the mass spectrometer so, the initial polypeptides of the smaller sizes are converted from that of higher molecular weight proteins. For the preparation of smaller polypeptides the compounds like trypsin which used to degrade proteins into the arginine along with lysine residues. The tryptic peptides are getting release long with this which is specific for protein. This makes protein specific analysis for given pool of protein samples and helps into the identification for the same. The specific spectrum is produced from the mass spectrometer for protein so it's possible to identify the protein by suing measured masses. The more precise way used for the identification for the protein by using its masses is that from peptide mapping or peptide mass fingerprinting techniques. These techniques used the comparison of peptide spectrum measured mass to that of computed peptide masses available on the comprehensive genomic or that of protein database. The more accurate information can be got by using information about the amino acid sequence along with the

position or kind of the posttranslational modifications (Fenn et al. 1989; Keller et al. 2002; Aebersold and Mann 2003; Shiio and Aebersold 2006).

1.5 The Comparison and Profiling Method for Proteomes

The variation of protein expression is identified by using proteomics technology rather than study of general compiled protein data. Some of the protein has showed some changes in the course of disease especially into the early stages for the disease, so with the help of this protein change one can able to identify the disease prognosis, therapy response much earlier as compared to other type of methods. The protein subsets are also identified easily by using the monitoring of the changes associated with that of proteomics. Such type of protein subsets which are related to that of the biological conditions from which one can go for clinical significance like hypoxia condition. The difference between the protein expression into the samples are very important for the analysis purpose. So for better result the quantification is usually required which can be completed by using several other method like two dimensional gel electrophoresis. The quantification can be done by using the intensity of protein spot. This steps are important prior to that of the application of mass spectrometry where the sample get excised and get identified. But this overall method is suffering from some limitations like the use of computational complexity. The minute changes into the peptide abundance are not get detected by using mass spectrometer (Karpievitch et al. 2010; Kwon et al. 2021; Al-Amrani et al. 2021).

2 Proteomics Type

There are three different subtypes of proteomics involving expression proteomics along with functional proteomics. Cancer proteomics makes use of both expression and functional proteomics.

3 Proteomics of Expression

Expression proteomics makes use of the idea of protein expression to distinguish between healthy or control proteins and diseased or patient proteins. It has qualitative and quantitative methods that can find novel proteins and proteins that are particular to certain diseases. Studies on protein expression patterns in healthy and sick cells are part of the field of expression proteomics. By utilizing the two-dimensional electrophoresis along with mass spectrometry techniques, it is possible to identify differences present into the protein expression related with the tumour tissue compared

to normal tissue that are either present or absent. We can find illness markers using expression proteomics, which enables early identification and diagnosis.

4 Structural Proteomics

Structural proteomics takes advantage of NMR spectroscopy. They also get evaluated by using X-ray crystallography for the determination of functional proteins. These types of analytical tools are used for in-depth analysis of three-dimensional structure along with the complexities related to functional proteins. It involves several different types of interactions, like those between membranes and cell organelles. The interactions are also involved with ribosomes in the mixture. One example of structural proteomics is the study of the nuclear pore complex. Additionally, it provides details on how protein networks and complexes relate to internal and external signals so that one can better understand the molecular mechanisms at play in cancer development (Ifandi et al. 2006; Van Gool et al. 2020).

5 Multiplexed Proteomic Tools: Proteomic Pattern Diagnostic

Proteomics based on mass spectrometry can identify and count hundreds of proteins. The advanced quantification in this regard can be achieved by using isobaric tags. In this method with the help of single experiment the multiple samples global quantification can be done by for advanced formulations. These techniques are called as a multiplexed proteomics. Proteomics can be conducted using top-down or bottom-up procedures, respectively. Trypsin is used in the bottom-up workflow to breakdown proteins. Later, they get separated by specific columns before analysing the peptides by MS. It is further divided to that of the two groups depending on the specific fractionation step. The second method assisted with the digestion of the protein without fractionation, liquid chromatography is implemented for the separation of the peptides. Other analytical tools, such as mass spectrometry, are used for identification, whereas the first method isolates proteins from gel using two-dimensional gel electrophoresis. This approach is known as “shotgun proteomics.”

6 Methods for Quantitating and Identifying Proteins

The multiplexed proteomics technology has emerged earlier to that of isobaric tagging technology involving the some basic steps of the bottom up proteomics. In this approach the selected proteins are first get broken down into the peptides and

later on the newly generated peptides are evaluated. Isobaric labelling is a component of multiplexed proteomics techniques, which are used to analyses proteins in a variety of ways. Shotgun includes the following steps: a. Identification of Peptides in Shotgun Proteomics b. Absolute and Relative Quantification c. label-free, MS1-based quantification d. Labeling with heavy isotopes and MS1 as a basis for quantification e. data-independent acquisition.

7 Isobaric Labelling for Multiplexed Proteomics

The techniques that have been explored thus far have significant drawbacks. Measurement precision is relatively low with label-free quantification. Furthermore, it can be difficult to interpret qualitative data for missing values to that of the peptides which are only found in select samples. MSI based labelling is utilized to gen excellent quantification for the selected multiple peptides but the same method is suffer from some issues. This method can not be implemented for complex pattern study of proteomics as the complexity related to that mass spectrum get increased to that of increase in sample numbers which are inducing less accuracy into it. Some of the technology like DIA is largely implemented for the resolution of the issues associated with it by using the sample analysis step by step at one time with lesser accuracy and it take longer duration for the analysis.

8 Principles for Quantitative Multiplexed Proteomics

The isobaric tags involved multiplexed proteomics has great potential for protein analysis. Some of the examples like TMT and iTRAQ are the most popular types of isobaric tags available commercially. Isobaric tags are said to be agents who will make covalent modifications in peptides; they are typically introduced following digestion and use the tag's heavy isotope distribution to encode various conditions. The only distinction for the heavy isotopes which are spread throughout the tag. Every tag including a spot that breaks, generating reporter ions as well as a reactive group that interacts with the peptide and contains a variety of heavy isotopes.

The overall tags constant mass is kept by using the heavy isotopes on that of the mass balancer group with change. The process of single peak generation into the mass spectrum one is associated by simultaneous elution for related peptides from that of diverse samples. The greater number of additions of the samples are not increasing the complexity of the spectrum. The weak link is used for the distribution of the number of heavy isotopes associated with that of each tag. The broken pattern of isobaric tag gives low m/z reporter ions having several other masses based on their origin and these can be implanted for the relative quantification. The isobaric tags is used to induce the complementary reporter ions and these ions are generated from that of balanced part of the isobaric tags. When the isobaric tag along with

peptide backbone are get destroyed then the fragment ions get produced. As the experimental circumstances are also encoded by the isobaric tag's balance group, the complementary reporter ions can be exploited in a manner like quantification. May researcher groups became aware of the complementing reporter ions, although they did not at first use them for quantification. Instead, to improve the success rate of peptide identification, these peaks were deleted. More of the samples that may be labelled by utilizing the particular isobaric tag system is limited, and the labelling step that comes after protein digestion may introduce some unpredictability, but the benefits of isobaric tags more than outweigh these shortcomings. The majority of the "missing value" issue is resolved by the ability to evaluate numerous samples at once. The less quantity of the sample will produces issues of peptide signal generation. As a result, either all a peptide's labelled variants will be extracted at once or none at all. The inherent high repeatability between samples is another key benefit of merging the samples after labelling and co-analyzing. Data quality is even greater compared to MS1 quantification approaches like stable isotope labelling with the aid of amino acids because each study greatly improves the target peptides into that of the MS2 spectrum which is used for quantification for selected samples. As a result, even for low-abundance peptides, peptide ion statistics are generated. CVs are typically less than 5% because few peptides have CVs greater than 10% and multiplexed proteomics has a very high repeatability. It can be utilized for pre-fractionated sample analysis, and costs are kept to a minimum as a result. This produces a substantially higher quantity of proteins that can be quantified in the same amount of machine time as label-free techniques. Because of these advantages, the multiplexed proteomics very interesting option available for the relative quantification.

9 Innovative Proteomics Technologies

Multiplexed proteomics is currently quite intriguing and useful for a wide range of application research. But there are still significant issues. One of the major challenges associated with this is the detection of the low amount of protein. While some of the challenges associated with the proteins are related with their transcription factors along with signaling molecules. To address these issues the tailored proteomics and multiplexing approaches have been developed to consistently access low-abundance proteins. The maximum multiplexing capacity is yet another significant restriction on multiplexed proteomics. The current available TMT tag is 11 plex. The better accuracy is obtained in this field by doing the comparison between the hundreds or more than thousands several other samples. In the label free techniques, some of the peptides are searched and investigated in the several types of the tests. These tests theoretically classified into the 11 plex trials but the quantification analysis into these is quite challenging part. As shown in Fig. 2, some of the researchers suggested combining DIA techniques with the complement reporter ion quantification procedure which will helpful for results measurement with good quality.

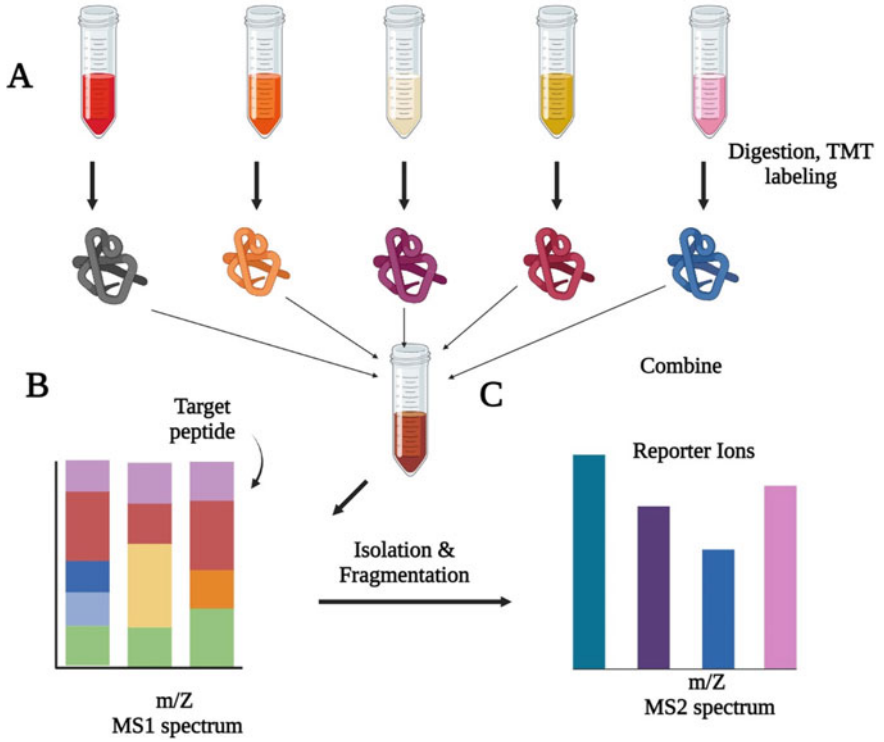

Fig. 2 The concept of multiplexed proteomics **a** Proteins are digested and given isobaric tags (such as TMT) in a variety of situations (replicates, time points, etc.). Different tags, symbolising various colours, serve as barcodes to identify the source of the sample. The peptide peaks from different situations are indistinguishable as presented into the MS1 spectrum because the isobaric tags in the tagging process are blended and simultaneously ionised onto a mass spectrometer (**b**). **c** The generation of reporter ion from that of isolated peptides are often condition specific. The relative amount of protein into them will help to get information about the intensities of the reporter ions. Once the peptide backbone gets damaged then it generates b and y ions which are used for further quantification studies (Reproduced/Adapted with permission)

9.1 Targeted Multiplexed Proteomics

For a better analysis of as many proteins as is feasible, the approaches we have so far discussed require performing a global analysis on protein samples. It is also possible to skip thorough coverage and focus the few available ion injection times on the peptides of 100 selected proteins. Such tactics are referred to as "targeted proteomics." Targeting, at least in theory, allows for the identification and quantification of specific ions by pre-directing data collection toward those ions that elute during those times. quantitation of significantly less frequent peptides that might otherwise go unnoticed. Despite the extensive preparation required, this method can be used to analyse low-abundance peptides that a shotgun approach would overlook.

9.2 Multiplexed Proteomics Implementation with Data Independent Acquisition

Multiplexed proteomics is important for the analysis by doing the comparison up to the eleven samples into the single experiment of analysis. But sometime for better accuracy one may required to have more than hundreds of samples in one experiment for better comparison of the results. The label free approaches are having missing value issues which are found in the multiplexed proteomics methods. But it is challenging to have a comparison between the protein's measures in 11 plexes. All these types of tests are called as bridging channels. Sometimes it is possible during the analysis that the required protein subsets or the anticipated amount may be not present into the selected protein samples. The quantification between the selected 11 plexes in such situations is depended on the unreliable ratios for the same.

9.3 Proteomics Pattern Diagnostics

Proteomic pattern diagnostics is a cutting-edge approach to disease diagnosis. In several publications, the actual diagnostic analysis for the ovarian cancer detection was a serum-based pattern made up of numerous unique types of the proteins, none of which could, on their own, distinguish between the diseased and healthy populations. Such patterns are able to giove information about the seroma proteome related to that of the disorder and in this type of cases one may not have disease related protein guidance for the same. There is significant role of the protein s which is played into the such disease conditions involving overexpression of the proteins, or they get wrongly shed, altered or clipped during the disease process. Some of the proteins may get eliminated from that of the proteome due to degradation for them during the disease. The change into the serum proteins happened due to protein–protein interactions related to the disease, bound complexes or specialized type of aggregations involved into the same. The kidney has not been able to get rid of the little molecular mass proteins in the serum because they are bound to bigger proteins. A plethora of physiologic knowledge may be found in the serum proteome's understudied low-molecular-weight region. Mass spectrometry can quickly profile this region of the proteome. Since this original report for ovarian cancer detection, this analytical technique has been used to detect prostate cancer and breast cancer, and it has confirmed the possibility of detecting cancer in general using this novel diagnostic technique. It is difficult to identify the critical discriminatory components in this massive data stream that cannot be seen physically or explained by simple linear regression methods. Although there are numerous types of bioinformatics data mining systems, most of them can be divided into two categories: unsupervised pattern recognition systems and supervised pattern recognition systems. Training sets for supervised systems require data with a predetermined outcome or classification. of these illness processes in humans have inherent variability. Any strategy

for identifying the ions must consider two crucial factors. The amplitude of specific ion peak is determined by several factors like desorption or ionization for the flight along with the protein population present in the same. It also gets influenced from the protein binding partners present into the sample which actual have a impact on the mutual ionization. The peak amplitude value is not able to determined from that of concentration sample or size. In the same spectral picture, a peak with a higher amplitude does not always indicate a protein in more amount as compared to that of correspond to lower amplitude. Electronic noise, chemical noise caused by impurities, and other noises can be found in the unfiltered mass spectra data (Conrads et al. 2003; Bakalarski and Kirkpatrick 2016; Pappireddi et al. 2019; Van Gool et al. 2020).

10 Methods of Proteomics

Proteomics thus focuses on identifying, quantifying, and understanding the role of proteins at the genome-wide level. There are so many challenges associated with these proteomics biomarkers, but they still lead to revolutionising new technologies and methodologies. For the identification of disease markers, proteome technologies can be used.

10.1 Two-Dimensional Gel Electrophoresis (2DGE) and Mass Spectrometry

High-resolution two-dimensional gel electrophoresis has been a useful method for expression proteomics for the past 30 years by separating individual proteins. When using two-dimensional gel electrophoresis (2DGE), the strips of immobilized pH gradient gen are used for the determination of first dimension and protein mixtures are resolved based on the isoelectric point (pI) or charge of each protein (IEF). With the help of sodium dodecyl sulphate polyacrylamide gel electrophoresis, these proteins are separated with the help of second dimension which is based on their molecular size (SDS-PAGE). The efficiency of analysis is heavily based on the sample preparation technique. It is important to choose the right combination of buffers when working with heterogeneous solutions that contain proteins with different hydrophobicity and agglomerate as a result of cell lysis. These buffers must comprise protease inhibitors, detergents, reductants, and denaturing agents. The pI, size, and solubility of the protein should all be considered when choosing the sample buffer. Gel-to-gel repeatability is considerably increased by properly prepping samples to guarantee optimum protein solubility and starting material uniformity. For the better visualization of the protein several techniques are implemented including staining techniques along with some protein binding dyes. There is preference given to that of the staining

technique as compared to other methodologies like mass spectrometry. Because of improvements in image analysis, data mining, and picture storage, researchers are now adopting 2-DE for the analysis of complicated samples. Under ideal circumstances, a single 2D gel can simultaneously characterize the expression pattern of thousands of different protein species. As shown in Fig. 3, advanced computational tools are used to compare protein profiles to determine the type of protein expression with the respective samples. The mass spectrometer utilized the ratio of mass to charge (m/z) to enable the creation, separation, and detection of molecular ions (Hood and Rowen 2013; Huang et al. 2021; Karpievitch et al. 2010; Keller et al. 2002; Klose et al. 2002; Kottakis et al. 2016; Kuruvilla et al. 2002; Kwon et al. 2021; Large et al. 2019; Le et al. 2017).

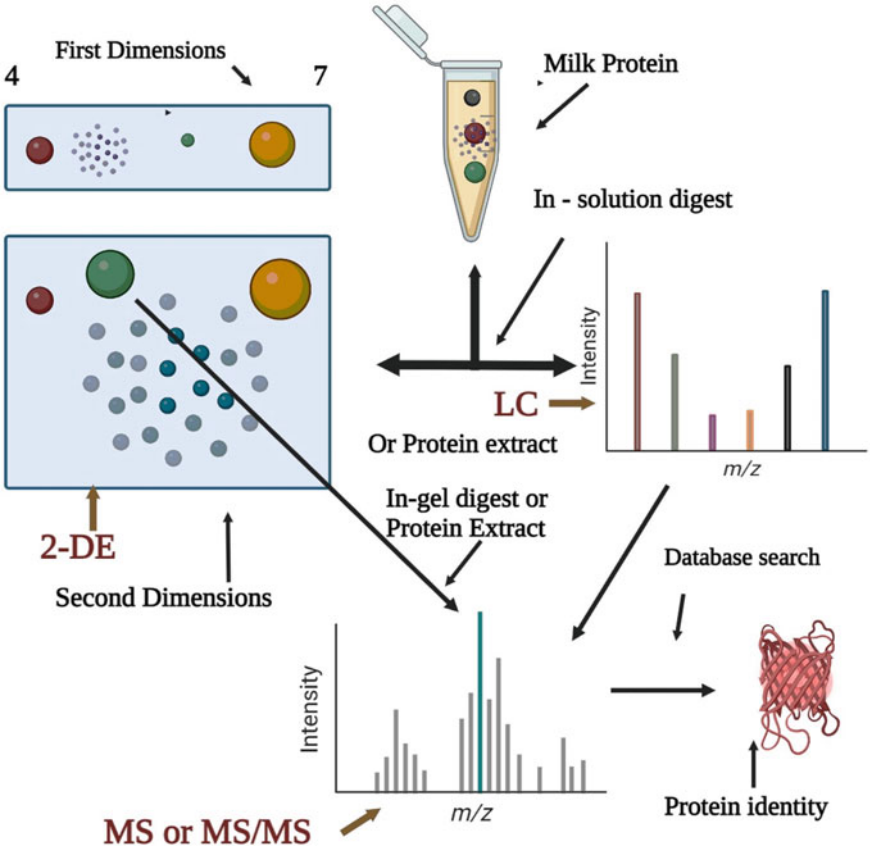

Fig. 3 Two-dimensional Gel Electrophoresis (2DGE) and Mass Spectrometry Reproduced/ Adapted with permission

10.2 Matrix-Assisted Laser Desorption/Ionization (MALDI)

Based on peptide ionization, this technique The measurement of the m/z value is possible thanks to singly charged ions generated by matrix-assisted laser desorption and ionization. 2,5-dihydroxybenzoic acid or cyano-4-hydroxycinnamic acid, which absorbs light of a particular wavelength that is suitable for the laser source, is mixed with the sample under inquiry (for an N2 laser source at 337 nm). The sample-matrix combination is then spotted onto a metal multiwell microtiter plate and left to air-dry until it forms a crystal lattice. The integrated peptide sample is in the crystal lattice. A laser is then used to gradually expose the plate, changing the solid crystalline form into a gaseous phase. The sample peptides receive the energy that is absorbed by the matrix molecules. Excited peptide ions are launched from the target surface and guided into a mass spectrometer, where they are measured. Laser desorption ionization time-of-flight is helped by a matrix (MALDI-TOF). MS is a frequently used technique for high-throughput protein identification due to its dependability and user-friendliness, as presented in Fig. 4.

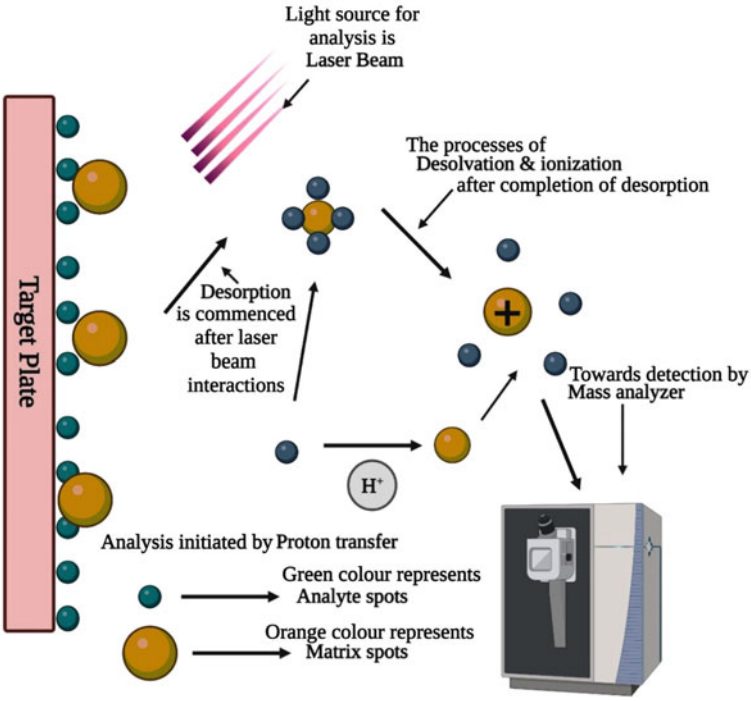

Fig. 4 The mechanism for Matrix-Assisted Laser Desorption/Ionization (MALDI)

10.3 Electrospray Ionization (ESI)

Using a mass spectrometry method known as "peptide ionization," the material is introduced into the mass spectrometer as ionised droplets. Through a microcapillary tube that is maintained at a high voltage, the liquid phase sample is introduced into the device (often from an HPLC system). A thin mist of charged droplets, including peptide ions as well as other elements of the HPLC mobile phase, is released as the flow stream leaves the tube (e.g., water and acetonitrile). Both passing the droplets through a heated capillary and spraying a stream of nitrogen across them are effective methods for peptide ion separation.

10.4 Aptamer-Based Assays

The cancer detection into the early stage is quite difficult to trace by suing biomarkers and these results are not that of reliable and accurate. So, there is need to develop special affinity reagents which given information and helps them for further investigation into the same. The aptamers consist of single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) molecules. Such compound is used to solve these problems. Studies show that aptamers have a dissociation constant between picomolar and nanomolar and can target almost any protein. After folding into tertiary structures, short single-stranded DNA, RNA, or peptide aptamers shows binding with protein. The binding with natural state of cognate protein. These effects will induce high affinity along with specificity. The slow-rate-modified aptamer (SOMA) scan assay is employed in this procedure. This process uses unique binding molecules known as SOMAers. A number of other characteristics that improve the aptamers' binding capacities may be present in those with different side chains (Rabilloud 1996; Yanagida 2002; Colantonio and Chan 2005; Alaiya et al. 2005; Smith et al. 2006; Wouters 2008; Deracinois et al. 2013; Le et al. 2017; Huang et al. 2021; Ding et al. 2022).

11 Bioinformatics in Proteomics

Proteomics, a scientific technique, allows one to determine the complete protein species present in a cell or tissue. During sample processing, it is crucial to solubilize the proteins effectively and efficiently from biological materials, such as blood or tissue. There are several types of interactions like protein with other protein, protein and lipid. Such type of interactions induced sample manipulation so these types of interactions must be disrupted without any changes into the protein modification. The buffers are used for the preparation of the sample. Just like every new technology, proteomics was greeted with enormous hope and promise when it was

first discovered. It is very important to understand the benefits and drawbacks of the typical technologies used in proteome research. Although proteomic technologies are usually used to characterise every protein in a particular cell, many researchers are also using them to comprehend protein changes between the sick and healthy states. As with any new technology, it takes time to work out every bug. Sample preparation, protein separation, and protein identification are major steps involved in it. Use staining techniques that work well with other approaches, such as mass spectrometry, if protein identification is the final aim. A thorough examination of a particular cellular metabolic system pertinent to a given research issue is possible through sub-proteomic analysis. For specific protein groups with similar biochemical features, it is vital to determine the ideal solubilization conditions for each protein individually. A subset of proteins that have been extracted from a complex protein mixture and that share a characteristic are subjected to sub-proteomic analysis, which is a proteomic analysis. Utilizing enrichment techniques, proteins having the same type of characteristics or biochemical along with the physical properties are identified using the sub-proteomic approach. Other methods for separating proteins include two-dimensional liquid chromatography and high-performance liquid chromatography (HPLC) (2D-LC). Following their capture, SELDI-TOF uses the masses of these complete proteins to induce a protein profile spectrum. One disadvantage of using SELDI-TOF MS for proteome analysis is the high abundance of a small number of the specific type of protein in the serum. Prior to MS analysis, SELDI has the benefit of being able to quickly and efficiently separate complicated protein mixtures with minimal sample modification. This is achieved by selecting proteins for examination while they are still intact and have not been digested, utilising chromatographic chip surface technology (ProteinChipR) (Ellington and Szostak 1990; Tuerk and Gold 1990; Shevchenko et al. 1996; Merchant and Weinberger 2000; Huber 2003; Tang et al. 2004; Stasyk and Huber 2004; Aslam et al. 2017).

In total, 192 studies on clinical proteomics were published, 71 of which looked at the biomarker D. A. There were much more articles published this year about clinical proteomics than there were in 2002, when 89 publications on the subject were released. Clinical proteomics is deals with the utilization of the proteome technique along with strategies into the medicine field. The many proteins in plasma have also been unsuccessfully removed using centrifugal filtration methods that use size exclusion. Whether a serum protein pool is high or low in abundance, it should be investigated for potential novel disease indicators. These two very abundant proteins can be diminished or deleted, which will remove around some percentage of the sample around 60% to that of the total serum proteins and simplify the study related to the less abundant proteins. The researchers compared the performance of three potential biomarkers to the tumour marker cancer antigen 125 (CA 125) in detecting ovarian cancer in healthy people. In order to properly perform a proteomic analysis of low abundance proteins, several research institutions are putting a lot of effort into removing high abundance proteins from serum. These findings highlight the critical need to confirm potential disease indicators using a variety of patient populations from various locations. The three putative biomarkers didn't perform any better than CA 125 when used alone, but when combined with CA 125, they

performed much better. The three biomarkers showed greater sensitivity for ovarian cancer identification when combined with CA 125 than CA 125. In general, it is vital to identify biomarkers discovered through proteome profiling. The identification of the biomarkers has considerable promise for clinical proteomics, but progress requires resolving issues related with the pre-analytical variables, along with analytical variability, and biological variation. Even though these variations might not have any bearing on a patient's condition, they do raise the difficulty of distinguishing between protein alterations brought on by illness and those caused by biorhythmic oscillations. When complicated protein samples, like serum, are contrasted between healthy and sick states, it becomes significantly more challenging to comprehend illness-induced protein modifications. Understanding the impact of pre-analytical factors on the test results are vital in any clinical laboratory as well as when investigating biomarkers. A Specimen Committee was established by the Human Proteome Organization (HUPO) in 2002 in order to investigate and address several of these problems (Georgiou et al. 2001; Rai et al. 2002; Govorukhina et al. 2003; Marshall et al. 2003; Zhang et al. 2004; Drake et al. 2004; Colantonio et al. 2005).

12 Application of Proteomics in Cancer

Cancer is characterised by abnormal cell proliferation. In this process, the normal cell's cell cycle is disturbed through several different genetic changes. The specific feature of cancer is that it can develop in any body tissue. The cancer cell is distinguished from the normal cell by its tendency for invasion and spreading to other organs or tissues. Malignant tumours are able to induce resistance to the treatments utilised in therapy, endangering the lives of patients in addition to growing quickly and metastasizing to numerous other tissues. A crucial scientific method for examining the biochemical alterations in cancer is proteomics. Key details, including protein targets and signalling pathways connected to those of growth and spread for the cancer cells, This type of process has been discovered using proteomics techniques.

12.1 Cancer Growth

Given that the main objective of cancer therapy is to stop abnormal cell proliferation, proteomics-based approaches can substantially contribute to the finding of biomarkers for the progression of cancer. With the help of patient samples for liver tumours and surrounding healthy tissue, the implementation of isobaric labelling for TMT proteomics has been done to analyse typical carcinoma cases of hepatocellular carcinoma (HCC). Such cases of carcinoma are related to those of hepatitis B virus-related hepatocellular carcinoma. According to phosphoproteomic methods, PYCR2 and ADH1A are connected to metabolic reprogramming for the HCC, along

with the phosphorylation of ALDOA, which assists in glycolysis as well as proliferation into the HCC cells with the CTNNB1 mutation. The mechanistic understanding gained from this study will assist in the advancement of efficient treatments for the clinical treatment of HCC. Several types of studies, including transcriptomics and proteomics, along with metabolic analyses in pancreatic cancer, have been created by utilising primary pancreatic epithelial cells and genetically modified mouse models. Using TMT labelling proteomics and a proteomic dataset, the work demonstrates that LKB1 is playing a major role in the primary version of pancreatic epithelial cells. Pathway modulation is correlative to glycolysis, and serine metabolism completes the cycle. This type of process suggests that the aforementioned LKB1 is important in the proliferation of pancreatic cancer cells. It also discovered a method by which the mTOR-dependent pathway facilitates the loss of LKB1 as well as the activation of KRAS, which is normally present in cancer cells. These findings highlight the importance of modern proteomics techniques in completing the process of evaluating genes and proteins in order to discover new cancer therapies and understand the molecular mechanisms that control cancer progression. Knowledge of the tumour and stromal compartments is required for better cancer cell treatment. Cancer-associated fibroblasts and other cancer cells in tumour tissues engage in interactions with the microenvironment (CAF). A significant metabolic modulator of CAF has been found using the proteomics method. Through the discovery of proteins and pathways, some of these investigations may result in the creation of potent therapies as well as bioinformatic tools to help fundamental research (Armstrong and Armstrong 2000; Brabletz et al. 2001; Hahn and Weinberg 2002; Soto and Sonnenschein 2004; Gupta and Massagué 2006; Kottakis et al. 2016; Lee et al. 2017; Eckert et al. 2019; Gao et al. 2019).

12.2 Metastasis

The variety of malignancies and the metastasis that takes place as cancer progresses are significant barriers to the effective development of therapies. Malignant malignancies frequently exhibit metastasis. Numerous studies of several types of proteomics have recently been carried out for better identification of the reason, which will clearly state the metastatic potential role of the cancer. Furthermore, it was discovered that elevated ROR1 expression levels and numerous metastasis-related processes were both influenced by increased GR activity, both of which were associated with decreased survival. Depletion of ROR1 in preclinical mice decreased metastatic development and increased survival, supporting these findings (Corso et al. 2008; Cleary et al. 2014; Brandi et al. 2017; Obradović et al. 2019; Harel et al. 2019; Chae et al. 2020; Zhang and Zhang 2020).

13 Drug Resistance

Cancer can come back despite therapies like chemotherapy and surgery. This type of condition states that recurrent cancer is more dangerous as it has cells that are resistant to anti-cancer drugs. According to numerous studies, cells that resist anti-cancer treatment in tumours in different parts of the body demonstrate particular protein expression. It also involves molecular pathways, which are associated with a low patient survival rate. These studies might make it possible to increase the effectiveness of chemotherapy by adding additional medications that regulate important proteins implicated in drug resistance. Drug-resistant cancer cells have traits that contribute to their stemness during formation, progression, recurrence, and metastasis (Görg et al. 2000; Wang et al. 2019; Le Large et al. 2019; Lignitto et al. 2019; Jeon et al. 2020; Shenoy et al. 2020; Zhang et al. 2021).

14 Clinical Proteomic Tools for Patient Tailored Therapeutics

The countless potential protein are available as a target and from these options one need to select the best group of proteins which are responding faster to that of the selected medicine in the disease condition. The most effective way to do this would be to analyse a small sample of patient-provided tissue, like a biopsy or serum. Utilizing proteomic technologies with a limited number of tissue samples typically comes with significant restrictions. The protein multiplexed analysis exemplifies a second type of limitation. The inability to analyse native protein samples is one of the limitations of many existing multiplexed proteomic techniques. Due to the disassembly of protein complexes and proteins and the destruction of 3-D protein structure after denaturation, these approaches would not be able to adequately assess the condition. Protein microarrays are the first innovative technology that can profile the state of a signalling pathway target even after the cell has been lysed and its contents have been denatured. A unique protein array called a “reverse phase protein array,” in which a protein lysate made via laser capture microdissection is arranged onto nitrocellulose slides, can be made using the sparse human cells present in human biopsy specimens. This method differs from tissue arrays and other types of protein arrays in several ways, one of which is its use of denatured lysates.

Due to their critical roles in cancer and biology, tiny molecular-weight chemicals and biologics with the ability to decrease and control particular kinase activity are a crucial area of research for the industry. A rational foundation for patient-tailored therapy will be created by the ongoing and upcoming development of new clinical proteomic tools, which will have significant translational applications for early detection in addition to the ongoing and concurrent technological advancements in diagnostic imaging. Oncologists will one day benefit from this proteomic toolbox.

Using mass spectrometry as the main clinical tool along with artificial intelligence-based pattern recognition algorithms, cancer could be detected early with improved specificity and sensitivity. By thoroughly analysing LMW biomarker fragments, the merging of nanotechnology and proteomics may produce instruments that can identify early changes in the underlying pathophysiology. Once it is known that a patient has cancer, their malignancy will be profiled utilising microscopic amounts of tissue samples, serum proteome pattern analysis, phosphoproteomic and protein network analysis, as well as genomic analysis (Li et al. 2000; Hanash 2000; Cheng et al. 2000; Druker et al. 2001; Paweletz et al. 2001; Traxler et al. 2001; Liotta et al. 2001, 2003; Zwick et al. 2002; Kuruvilla et al. 2002; Walter et al. 2002; Petricoin et al. 2002; Bray 2003; Nishizuka et al. 2003; Min and Lee 2022).

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Proteomics Novel Prospects in Target Therapy for Infectious Diseases

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Abstract RNA viruses that undergo continuous evolution can cause infectious diseases, leading to considerable harm to public health, individual well-being, and economic growth. The virus transmission can be through human-to-human, animals to humans and insects or vectors. The viruses involved are Middle East respiratory syndrome coronavirus, human immunodeficiency virus, severe acute respiratory syndrome coronavirus, Crimean Congo virus, flaviviruses such as zika and dengue Virus. The viral protein involves with host organism proteins plays a significant part in pathogenesis and infection by avoiding the host defense mechanisms. As proteins have a huge range of functions, proteomics approaches can offer significant prudence in analyzing disease pathogenesis, etiology and discovery of potential antiretroviral pharmaceutical drug target sites and improving diagnostics. Two-dimensional gel electrophoresis, liquid chromatography, Isotope-coded affinity tag labeling-based protein profiling and Mass spectrometry are utilized for defining the structural arrangement of viral pathogens, virus and host protein interactions, conduct sensitive, high-throughput analyses of them enormously and the development of therapy strategies and improving the diagnostics. This approach also made considerable contributions to the finding of signaling cascades, the information of disease mechanisms regarding proteome changes and posttranslational modifications at some point in infection, the unfolding of viral–host protein interactions and its novel mechanisms against viral proteins. This chapter is centered on describing the usefulness of proteomics techniques that provides large set of information regarding protein interaction and new drug target could be developed for infectious disease.

Keywords Proteomics approach · Infectious disease · Protein interaction · Mass spectroscopy · Coronavirus · Biosystem

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1 Introduction

An infectious disease is a caused because of a definite contagious agent, which spreads through direct communication of an agent from a diseased animals, humans or reservoir to a vulnerable host cell and indirectly through vector, animal or plant host, or the non-living surroundings. Pathogens such as fungi, viruses, parasites, bacteria cause an infectious disease (Porta et al. 2008). Infectious diseases are a main reason of individual agony in regards of sickness and death in the course of human. The development of human civilization has had an impact on the transmission of infectious diseases. For instance, Parasite and zoonotic infections caused by air and bacteria have increased in frequency because of residence and urbanization (Rose and Besdine 1982). According to WHO, globally over 659 million confirmed severe acute respiratory syndrome coronavirus cases and more than 6.6 million mortalities had been accounted until January 2023 (WHO 2023). In 2021, according to WHO globally around 38.4 million population were having HIV and 1.5 million are recently infected (WHO 2021). Crimean-Congo haemorrhagic fever virus produces viral haemorrhagic fever epidemics. Case mortality rates in CCHF epidemics can reach 40% (Spengler et al. 2018). 2.8 million Dengue cases, 0.27 million Chikungunya cases, and 37,869 were Zika cases worldwide were reported (Pierson and Diamond 2020).

The infection progression might be divided into the subsequent steps. If the contagious agent does not achieve the support, the individual was exposed only then the infection process may end. However, if the infectious agent achieves the stronghold with no symptomatic reactions, then the person would be colonized and with no infection. The infection would show its action only when the infectious agent affixes to host epithelium and multiplies. The agent releases the toxin that causes inflammation, cell or tissue injury and inhibition of protein synthesis, spreads the disease throughout the human body. The individual is considered asymptomatic when no clinical signs and symptoms of the disease are observed and if they show, any symptoms of the disease then are considered symptomatic (Cohen et al. 2016).

The source of infectious material refers to the origin or source of the microorganism that causes an infection. The source of infection can be divided into two primary classifications: endogenous and exogenous. Endogenous sources of infection are those that originate within the host's body, such as from the host's own normal flora. These sources can include bacteria that normally live in the gut, skin, or other parts of the body. Exogenous sources of infection are those that originate outside of the host's body, such as from other animals, plants, or the environment. Examples of exogenous sources of infection include bacteria or viruses that are transmitted through contact with contaminated surfaces, food, or water, or through bites from infected animals. Some infectious agents are also transmitted through human-to-human, from respiratory droplets, breast milk, blood, vaginal secretions, semen or skin contact (Wolfe et al. 2007).

2 Drawbacks of Current Strategies

Current strategies for infectious disease treatment and detection have several limitations and loopholes. Some of the main issues include:

(a) Lack of early detection: Many infectious diseases, such as tuberculosis and HIV, do not present with clear symptoms until the disease has progressed to a later stage. This makes early detection difficult, and by the time the disease is diagnosed, it may be more difficult to treat.
(b) Limited diagnostic options: Many diagnostic methods currently used for infectious disease detection are limited in their sensitivity and specificity. For example, some diagnostic tests may produce false-positive results, while others may not detect the disease at all. This can lead to misdiagnosis and delayed treatment.
(c) Antimicrobial resistance: The overuse and abuse of antimicrobial drugs has caused the appearance of antimicrobial drug-resistant strain of bacterias, viruses, and other pathogens. This makes it difficult to treat infections with traditional antimicrobial agents, and new drugs must be developed to combat these resistant strains.
(d) Limited treatment options: For many infectious diseases, treatment options are limited. For example, there are few effective treatments for viral infections such as HIV and hepatitis C. Additionally, many infectious diseases, such as tuberculosis, require prolonged treatment regimens that can be difficult for patients to adhere to.
(e) Limited understanding of disease mechanisms: A lack of understanding of the mechanisms of infectious diseases can make it difficult to develop new treatments and diagnostic methods. For example, the complex interactions between pathogens and host cells are not yet fully understood, which makes it difficult to develop new therapies that target specific pathogens or host-response pathways.
(f) Limited access to healthcare: Many individuals, particularly in resource-limited settings, do not have access to adequate healthcare. This can make it difficult to detect and treat infectious diseases in these populations.
(g) Diagnosis and treatment costs: The cost of diagnosis and treatment can be prohibitively expensive for many individuals, particularly in resource-limited settings. The difficulty in accessing the necessary care can pose a challenge for individuals.
(h) Limited data sharing: The lack of data sharing and collaboration among researchers and healthcare providers can make it difficult to track outbreaks and develop effective treatments and diagnostic methods.
(i) Limited public awareness: Limited public awareness about infectious diseases, including how to prevent and treat them, can make it difficult to control outbreaks and protect public health.

To improve the current strategies, it is important to continue to develop new diagnostic methods that are sensitive, specific, and affordable, to establish new treatment

options that are effective and easy to administer, to improve data sharing and collaboration among healthcare providers and researchers (Klepac et al. 2015; Cloeckaert and Kuchler 2020; Excler et al. 2021; WHO 2005).

3 Proteomics

Pathogens can cause a significant risk to animal and human. They can disrupt immune systems and cellular pathways of host, creating equilibrium among the pathogen and host. Despite the genetic information being encoded in DNA, the proteins carry out the functions of life. Therefore, biological diversity is protein-based. The proteins also known as proteome, formed through tissues or cells, play a crucial role in responding to environmental signals. The cyto-sensorium includes cell pathway proteins that work as receptors, sensors and message-transferring component and cyto-effectorium includes single or group of proteins in response to surrounding signal (Figeys 2004; Karr 2008; Anderson and Anderson 1996). Proteomics, the study of the proteome, can provide insight into the specific cellular behavior and proteomes interaction of the pathogen and host. This can help understand the host–pathogen equilibrium and the methods of viral infection and host immune defense way of action (Biron et al. 2005).

Proteomics is a powerful approach for infectious disease detection because it permits for the simultaneous multiple proteins analysis, which can offer complete knowledge of the disease state. One of the key advantages of proteomics for infectious disease detection is its ability to identify both pathogen-specific and host-response proteins. Pathogen-specific proteins can be used to directly identify the presence of a specific pathogen, while host-response proteins can indicate the presence of an infection even before the pathogen can be directly detected. This can be particularly useful in cases where the pathogen is difficult to culture or detect by other methods. Another advantage of proteomics is its ability to detect low-abundance or post-translational modification proteins which may not be detected by other methods. For example, other methods are incapable of protein quantification or post-translational modifications, but shotgun proteomics and targeted proteomics can be utilized for their identification. This can be particularly useful in cases where the pathogen is difficult to culture or detect by other methods (Stebbins and Galán 2001).

4 Unraveling Proteins: Proteomics Techniques for Separation and Identification and Viral Host Interaction Analysis

Proteomics include the proteome conformation, role and interactions. Proteins are essential biomolecules that perform a wide variety of functions in the cell like transportation of molecules across membranes, DNA replication, stimuli responsive and enhancement of the metabolism reaction. Understanding the proteome or the complete set of protein expression is crucial for understanding biology of cells and organisms. Several methods can be used in proteomic studies to analyze and identify proteins. Proteins are separated and identified using techniques like mass spectrometry and gel electrophoresis, which rely on their physical and chemical properties. Additionally, protein–protein interactions can be studied using yeast two-hybrid and immunoassays. In addition, bioinformatics tools and databases are used to analyze and interpret proteomic data. Proteomic techniques are now widely used in many research fields such as in the detection of new drug target sites, biomarkers identification for disease diagnosis, recognizing the disease molecular mechanisms and in the formulation of new therapeutics. Proteomics research offers new perspectives into the complex and varied composition of the proteome, and its relevance in the contexts of both illness and health (Bischoff and Luider 2004).

4.1 Two-Dimensional Polyacrylamide Gel Electrophoresis and 2D-DIGE Differential In-Gel Electrophoresis

2D-PAGE isolates and characterizes proteins by separating them according to their physical and chemical properties. The technique combines two separation dimensions: (a) the first element of 2D-PAGE differentiates protein according to the charge and size using polyacrylamide gel electrophoresis and (b) the second element of 2D-PAGE differentiates protein based on the isoelectric point (pI) where protein carries no net charge, using isoelectric focusing (IEF). Application of gel to the sample containing proteins of interest and later the electric current is provided to sample gel. Migration of proteins in the gel depends on their charge and size, with smaller, more positively charged proteins migrating faster than larger, more negatively charged proteins. This results in the separation of the proteins into distinct “bands” on the gel.

After separating the proteins in the first dimension gel, it is transferred onto an IEF gel and an electric current is applied to facilitate further separation of the proteins. Migration of proteins in the gel depends on their pI. The combination of these two dimensions of separation results in the separation of a wide range of proteins on the 2D PAGE.

Afterwards, proteins are checked by staining method using Coomassie Brilliant Blue stain or a fluorescent dye. 2D PAGE has many advantages like the capability

to separate a wide range of proteins, high resolution and the capability to detect small alteration in protein expression. However, it also has some limitations, such as the difficulty in resolving low-abundance proteins and the complex data analysis required to interpret the results.

Integration of 2D gel electrophoresis with other techniques like mass spectrometry (MS), which is known as 2D-DIGE to identify widely used in proteomics research to study post-translational modifications and alterations in expression of proteins in various biological systems. DIGE is similar to 2D PAGE, but it uses a fluorescent dye to label the proteins before separation. When investigating virus-host interactions, differential in-gel electrophoresis (DIGE) is a valuable tool that can be used to assess the proteome of both infected and uninfected cells. Additionally, it can be employed to judge against protein profiles between cells infected with varying viral strains. Both 2D PAGE and DIGE are useful techniques for detecting and characterizing virus-host protein interactions, but DIGE is more commonly used as it allows the simultaneous comparison of multiple samples, thus reducing sample-to-sample variation.

Peptide mass fingerprinting (PMF) or mass spectrometry techniques are commonly employed for protein identification following separation by 2D PAGE or DIGE. Protein digestion is done with protease enzyme for allowing the formation of peptides. Generated peptides are examined by MS or PMF to determine their mass and sequence, which can then be used to identify the corresponding proteins (Saraswathy and Ramalingam 2011; Coombs 2020).

2D PAGE or DIGE is combined with MS and LC/MS permits virus-host protein interaction analysis. 2D PAGE or DIGE is used to separate and visualize the proteins of interest. The resulting protein spots are cut and allowed digestion with a specific protease for generation of peptide mixtures. This is called in-gel digestion. MS or LC–MS analyzes the resulting peptides for identification of the protein interaction sequence and virulence determination pathway. When MS is used, the peptides are ionized using either MALDI or ES ionization. By matching observed mass-to-charge ratios to a database of known peptide sequences, the proteins can be identified. In tandem mass spectrometry, peptides break in to small fragments in the MS and the resulting fragments are analyzed. In LC–MS before ionization, peptide separation is done by LC and evaluate by MS. This allows for more efficient separation and better identification of low-abundance peptides. In the context of virus-host protein interactions, LC–MS or MS can be utilized for discovering the host proteins involved with the virus and their virulence determination. This information can be apply to know viral replication mechanisms, pathogenesis and to identify probable targets for antiretroviral drug development (Lauber et al. 2001; Nilsson et al. 2000).

4.2 Liquid Chromatography

LC in proteomics is used to separate out and purify proteins depending on their physical and chemical properties. LC separates proteins by pumping a mixture of proteins through a column filled with a stationary phase material, such as a resin or

gel. Different proteins will interact differently with the stationary phase, resulting in their separation based on their size, charge, and hydrophobicity. In virus-host protein interaction studies, LC/MS could be utilized for determining the relative abundance of host and virus proteins, as well as their interactions. LC/MS can be combined with peptide mass fingerprinting and protein identification technologies to further enhance their ability to separate and identify virus-host protein interactions. Peptide mass fingerprinting is a method that uses MS to identify proteins depending on their unique peptide patterns. The mixture of protein is digested into smaller peptides, separated and analyzed by MS. The peptide fragments are run on record of identified sequence of protein to recognize the proteins present in the sample. Protein identification technologies, such as database searching or de novo sequencing, can be utilized to authenticate the protein identities. To perform database searching, the peptide masses obtained from MS analysis are judged against a database of identified protein sequences in order to identify the best matches. De novo sequencing, on the other hand, involves generating a sequence of the peptides without reference to database, allowing for the recognition of previously uncharacterized proteins. This approach enables the acquisition of a better understanding of viral replication, pathogenesis, and post-translational modifications by providing valuable insights into these mechanisms (Viswanathan and Früh 2007; He et al. 2010; Sperk et al. 2020).

4.3 Isotope-Coded Affinity Tag Labeling

Quantitative proteomics offer a complementary approach to mRNA profiling for a comprehensive characterization of protein species. It involves the measurement of the abundance and distribution of proteins in biological samples. The ionization variability of different peptides presents a challenge in protein quantitation, but stable isotope labeling techniques have emerged as an effective solution. Among these techniques, the isotope-coded affinity tag (ICAT) labeling method has gained widespread popularity due to its high accuracy in relative quantification. This method involves covalently attaching mass-tagged reagents to specific cysteine residues in the particular protein. Labeled proteins are mixed with unlabeled proteins from other samples, subjugated to analysis. The mass-tagged reagents enable the differentiation and quantitation of the labeled proteins, providing a highly accurate relative measure of protein expression levels (Petriz and Franco 2014).

The ICAT labeling technique is widely adopted in proteomics research due to its compatibility with reagents. Though, it has limitations, such as the requirement for cysteine-containing proteins and its ability to provide only relative quantitation. The introductions of isobaric tags for relative and absolute quantification (iTRAQ) have been a solution to surpassing these limitations. It enables the simultaneous quantitation and identification of multiple samples using a set of isobaric reagents. This multiplexing capacity also presents statistical validation in a specific testing, making this

analysis method highly valuable for proteomics research. Moreover, iTRAQ facilitates the detection of a broad range of proteome and enhances sensitivity, enabling the investigation of post-translational modifications. Even though iTRAQ quantitation takes place in MS/MS, which demands higher performance instrumentation, the technique streamlines the analysis process (Trinh et al. 2013). In conclusion, both ICAT labeling and iTRAQ are widely adopted in proteomics research, offering valuable insights into the study of biological systems and the formulation of new therapeutic and its targets.

4.4 Mass Spectroscopy

In proteomics research, MS is extensively utilized for analysis and identification of protein. MS operates by separating proteins rely on its mass/charge ratio (m/z). Subsequently ionization, ions of analyte are propelled and separated into mass analyzer. The information is then recorded and analyzed by a computer. In proteomics, there are several ionization methods that are commonly employed, including electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI) because they preserve the molecular structure and cause minimal fragmentation during the ionization process (Greco and Cristea 2017).

4.4.1 Electrospray Ionization

Electrospray Ionization is a mass spectrometry technique that generates ions from a liquid sample by applying an electric potential. Small droplets spray is then formed containing the solvent and analyte. The solvent removal from droplets is done through collision with a gas or heat. Multiple ions carrying an electric charge $(M^{+n}H)^{n+}$ are produced as a result of the process. Then, analyte ions are passed through a MS where it gets separate depending on the m/z ratio. The resulting data could be converted in a spectrum that demonstrates the fragments molecular weight. Quadrupole analyzer is commonly utilized along with this technique. This method is often used in proteomics research as it allows for the use of liquid chromatography columns to feed the ESI mass spectrometer, enabling automation. This method could be automated with a LC column as the protein sources that are to be analyzed by the mass spectrometer. Additionally, linear ion trap (LIT) is an another type of spectrometer that has both MALDI and ESI characteristics and gives two separate ion optical channel and source that are linked to single mass analyzer opposite ends (Smith et al. 2007).

4.4.2 Matrix-Assisted Laser Desorption/Ionization

Matrix-Assisted Laser Desorption/Ionization involves ionizing proteins by suspending or dissolving them in a crystal matrix made of organic, small and UV-absorbing molecules. Crystal matrix takes up laser energy at the same wavelength utilized to ionize protein. The application of laser energy induces excitement in the matrix, leading to the conversion of both the matrix and analyte ions into a gaseous state. Main ion (M^+H^+) is detected using MALDI with laser intensity. Larger proteins may also result in signals for multiple charged ions and oligomeric forms. An electric field accelerates the ionized protein and protein travels into time of flight analyzer (TOF). The speed an ion attains at the detector, upon acceleration by a fixed voltage, is analyzed by their mass. MALDI is capable of analyzing protein in femtomole quantity and small amount of impurities. The results can be analyzed and compared with the record. It is believed that combining protein profiles with histological data will aid medical professionals in difficult diagnoses (Evangelou et al. 2007).

4.4.3 Surface Enhanced Laser Desorption/ Ionization

SELDI approach is utilized for assessing protein in mixtures that remain on the surfaces of chromatographic chips. The method can be used to identify and separate differentially expressed proteins by comparing peak intensity in mass spectrum. Chromatographic separation can be performed with one micro liter of sample. Various substances can be confined to the protein arrays like carbohydrates, receptors, antibodies and nucleic acids. Some surfaces have broad specificity, whereas other surfaces are extremely specific, capturing hardly any proteins from mixture. Afterwards, the protein array is washed to decrease non-specific binding. Upon exposed to laser, the attached proteins detached from surface of array and analyzed. A few of protein arrays contain covalently attached antibodies and capture its antigen from a complex mixture. The data obtained from the MS analysis is utilized to identify protein expressions by comparing peak intensity and to perform protein purification and protein interaction profiling. This technology enables the selective capture, quantification and evaluation of proteins from minute quantity of sample (Ndao 2012).

4.4.4 Linear Ion Trap Quadrupole-Orbitrap Mass Spectrometry

Linear ion trap quadrupole-orbitrap mass spectrometry is a combined spectrometric technique that merges the advantages of linear ion traps and Orbitrap mass analyzers. This technique is used in proteomic analysis for protein separation and identification, including analysis of protein-virus interactions. The process begins with sample preparation, which involves extracting proteins from the biological sample, and subsequently digesting the proteins into smaller peptides using proteases. These peptide mixtures are subjected to LC for separation. Separated peptides are then

introduced into spectrometer, where they are first trapped in the LIT and then moved to the Orbitrap mass analyzer for mass analysis. The peptides are trapped in LIT and manipulated through the application of radio frequency and DC voltage fields. This permits selection and definite ion depot for further analysis. After being confined, the ions are transported to the mass analyzer called Orbitrap, where they undergo analysis based on their m/z ratio. The Orbitrap mass analyzer operates on the principle of ion cyclotron motion, where the ions are trapped and analyzed based on their motion in a strong magnetic field. In the final step, the mass spectrometer software is used the obtained data to discover the peptide and their protein. The software matches the observed mass and charge state of the peptides with those of known proteins in databases, to determine the identity of the peptides and proteins. This proteomic approach permits identification and characterization of a wide range of proteins and its interaction, as well as those between host proteins and viruses. It is widely used in various fields, including drug discovery, disease diagnosis, and basic biological research (Cifani and kentsis 2017).

5 Host-Pathogen Interactions

Host-Pathogen interaction refers to the relationship between a pathogen (such as a virus, bacterium, or fungus) and the organism it infects (the host). This interaction can include physiological, molecular and cellular processes, and can result in a variety of outcomes, such as the pathogen establishing a successful infection, the host mounting an effective immune response, or the host and pathogen reaching a state of mutual tolerance. During the interaction, pathogens can use various mechanisms to elude the host immune system, while host can use different mechanisms such as innate and adaptive immunity to defend itself against the pathogen. Studying pathogen-host interactions can provide understanding of the infection and disease mechanisms, as well as the evolution of both pathogens and host immunity. This comprehension can be used to fabricate new strategies for treating and preventing infection (Malone et al. 2016).

Pathogen-host interactions at the molecular level involve a complex interplay between the pathogen and host cell during the pathogen's replication cycle. Intracellular pathogen, such as cytosolic bacteria and virus, need to enter the host cell, utilize components of cell (e.g., metabolites, lipids and proteins) for multiplication and reach the neighboring cells to establish an infection. Additionally, pathogens must counteract host immune system to survive and replicate within the host. However, despite this progress, there are still many unanswered questions and challenges in the field. One of these challenges is the appearance of new pathogen strains and its diseases require identification of new host-viral targets for diagnosis purpose and new drug development. This can be challenging because of complexity, variability of pathogen-host interactions, as well as the need for rapid and efficient diagnostic methods. Additionally, the appearance of antiviral drug-resistant pathogens calls for new strategies to block the spread of these organisms (Morens and Fauci 2012). These challenges

can be addressed by using contemporary methods such as genomics, proteomics, and metabolomics to study host–pathogen interactions at a large scale and discover new protein targets for diagnosis and therapeutic development.

Another important challenge in host–pathogen interactions is the appearance of drug-resistant virus strains call for the discovery of new host pathways for blocking the infection. This is particularly critical as many existing drugs and vaccines are becoming less effective due to resistance. In order to address this problem, researchers are currently exploring new strategies such as host-directed therapies, which aim to target host pathways that are essential for pathogen replication rather than the pathogen itself. Additionally, researches and studies are also centered on the advancement of novel drugs and vaccines that can overcome resistance mechanisms. There are also deadly viruses that have been studied for long time but goes on with no suitable vaccines or treatments. This is a ongoing challenge and a major concern for public health. Research in this field is ongoing and continues to be essential in the development of new treatments and vaccines for these viruses (Rieder and Steininger 2014).

In recent years, Omic approaches like proteomics, transcriptomics, genomics and metabolomics have become valuable tools in studying the biological pathways involved in host response, pathogen spread and multiplication and progression of disease. Proteomics, happen to be key approach in understanding pathogen-host interactions by allowing for protein identification, quantification and detection. The combination of proteomics with other omic techniques has been employed to study pathogen infections (Lum and Cristea 2016). By using a combination of these techniques, researchers can achieve knowledge of the infection process. In this field, researchers are currently focused on using proteomics to:

I. Elucidate pathogen-host protein interaction networks
II. Understanding the alteration in the host proteome structure and organization during the course of infection
III. Investigate infection-induced protein post-translational modulation.

Bioinformatics approaches also play a crucial role in proteomics research, as they help researchers to interpret large datasets and increase the power of discovery. As more data becomes available from other omic fields, multi-omic technologies are also being used to gain further insight into the mechanics of pathogen-host interactions (Lum and Cristea 2016).

6 Elucidating Pathogen-Host Protein Interaction Networks Through Proteomics Techniques

A chain of pathogen-host interaction determines the progression of an infection after entry in host cells. These interactions can be both detrimental and beneficial to the host. Pathogen overcomes host immune systems and reproduce, to achieve this, they interact with host proteins through various mechanisms such as suppressing or

hijacking regular protein functions of host. Identification of protein–protein interactions (PPIs) helps point to potential new drug targets for treatments against infectious disease.

Beyond identifying a protein–protein interaction, it is significant to recognize the nature of the interaction, whether they are direct or indirect. Direct interactions involve one protein interacts with another physically, while in indirect interactions there is interaction of proteins with other intermediate molecules. There are various proteomics approaches available for discovering pathogen-host interaction networks, direct interactions or intact protein complexes. These methods have advantages and disadvantages and have potential for upcoming developments in the studying infectious diseases. The understanding of interactions can give potential insights on how the pathogens interact with host proteins, which can be useful in developing new treatments against infectious diseases (Lum and Cristea 2016).

7 Characterizing Virus—Host Protein Interactions of Middle East Respiratory Syndrome Coronavirus (MERS CoV) and Severe Acute Respiratory Syndrome Coronavirus (SARS CoV)

The latest variants of Coronaviridae family have been discovered to be more virulent compared to the other strains. One potential reason for this is that these new strains can cause infections inside the upper respiratory tract and lower respiratory tract. To understand the increased pathogenicity of these new strains, it is important to study about the virus infecting the target cells and takes over the cell mechanism of host for their multiplication process. One main aspect of this course of action is the COV spike protein roles, which is greatly glycosylated and take part in the entry and attachment of virus into host cells. In Canada, MS analysis were conducted to understand the properties of the SARS–CoV proteins. where a large occurrence of SARS outside of Asia occurred. MS also allows the study of post-translational modifications. A Canadian study utilized MALDI TOF, to recognize 12 different glycosylation sites and sugars of the spike glycoprotein. In addition, scientists utilized high-resolution LC–MS/MS to chart the N– and O–glycosylation patterns on SARS–CoV-2 spike protein (Krokhin et al. 2003; Shajahan et al. 2020).

Through combination of immunoprecipitation and mass spectrometry analysis, experts were able to pinpoint the receptors utilized by MERS CoV and SARS-CoV for gaining entry into host. Dipeptidyl peptidase 4 and Angiotensin-converting enzyme 2 were recognized like the penetrating receptors for MERS–CoV and SARS–CoV, respectively (Li et al. 2003; Raj et al. 2013). Due to structural resemblance between SARS–CoV-2 and SARS–CoV, it was verified through targeted Western blot analysis which SARS–CoV-2 also employs the identical receptor for penetrating host cells (Letko et al. 2020). This discovery explicates why the viruses can infect the lower respiratory tract (Jia et al. 2005; Meyerholz et al. 2016). Information of the spike

protein's glycan repertoire and its entry receptors can be utilized to fabricate the entry receptor inhibitors. For instance, One promising strategy for blocking early stages of infection is using a human recombinant soluble form of ACE2 (Monteil et al. 2020).

Other research has employed proteomic techniques to achieve knowledge of the various virus proteins involved in coronavirus replication and host proteins interaction. For instance, a study conducted in Canada, which characterized viral proteins, discovered a novel nucleocapsid protein that shared 32% characteristics with previously known coronavirus nucleocapsid proteins. Notably, the examined SARS-CoV nucleocapsid lacked a caspase cleavage motif, which is present in several other coronaviruses and which helps in the removal of virus by infected cells. Another research employed LC–MS/MS and protein kinase analysis to examine the cellular mechanisms responsible for the formation of coronavirus on purified SARS-CoV particles. This technique revealed eight viral proteins and 172 host proteins, and it was found that nonstructural protein 3 is a consistent element of viral protein processing (Krokhin et al. 2003; Neuman et al. 2008).

The research discovered a connection between the host cyclophilin A and viral nucleocapsid protein, which had been forecasted by bioinformatics techniques and afterward it also identified interactions between different cyclophilins and nonstructural SARS-CoV protein 1 (Luo et al. 2004). A biological approach, specifically yeast two-hybrid screen, was applied for evaluation of the PPI (Pfefferle et al. 2011). To investigate the interaction of α-karyopherin proteins and MERS-CoV viral accessory protein 4b during infection of Huh 7 cells, a Co-IP MS screen was employed. According to the study, this interaction results in the hindrance of the NK-κB-dependent antiviral reaction by the viral accessory protein 4b (Canton et al. 2018). To understand SARS–CoV-2 interactions, a PPI map in the human embryonic kidney 293 T cell line was generated during the initial stages of the outbreak. The method involved tagging, cloning and expressing different 26 SARS–CoV-2 proteins, followed by utilization of affinity-purification MS to detect 332 interactions between virus and host proteins (Gordon et al. 2020). One approach involves using HEK293 cells that have been genetically modified to produce excessive amounts of SARS–CoV-2 genes fused with FLAG-epitopes. The viral protein complexes can then be extracted using affinity purification techniques, and subsequently analyzed using LCMS/MS methods. The results revealed the viral- host proteins interaction engaged in the protein synthesis, protein conformation, and proteolysis processes, resulting in advantages for viral expansion and multiplication (Li et al. 2021). In current proteo-transcriptomic analysis, SARS–CoV-2 infection in Huh7 cells was examined where disorganization of PI3K/AKT, MAPK and mTOR signaling pathways was observed (Appelberg et al. 2020). The proteins were estimated with RPLC tandem mass spectrometry and TMT labeling. MS-based quantitative proteomics studies can be utilized to identify changes in cell pathways, such as protein synthesis, splicing, carbon metabolism and cholesterol metabolism during viral infection.

7.1 Characterizing Virus—Host Protein Interactions of Human Immunodeficiency Virus

HIV-1 and HIV-2 are AIDS-causing virus belongs to Retroviridae family. Despite the ongoing efforts to understand and address the HIV-1 and HIV-2 virus, no cure has yet been found to completely eradicate these viruses. However, the formulation and administration of antiretroviral drugs has made HIV/AIDS a controllable disease. However, HIV/AIDS remains a virulent disease and a significant worldwide health issue. In current years, studies have been executed using proteomics to recognize the virus protein conformation, host immune responses and interaction. The studies have helped to improve our understanding of the origination and development of HIV-1. Recent studies have turned their attention to understanding the non-AIDS-related comorbidities by investigating non-plasma/serum body fluids and long-term antiretroviral treatment. These studies have used mass spectrometry to investigate the protein profile of cerebrospinal fluids and its extracellular vesicles in infected patients. Results have shown a rise in inflammatory markers and complement proteins in CSF, may contribute to development of neurocognitive disorders in people living with HIV (Landi et al. 2020; Zicari et al. 2019).

An investigation was subjected to MS analyses where semen and blood sample of infected male were collected and analyzed. Despite the fact that HIV-induced alterations were less common in semen, 43 unique proteins were identified in HIV-infected male semen compared to uninfected semen. These proteins were in abundance in interleukin signaling pathway and coagulation /complement cascades. To know HIV vulnerability and infection possibility, the HIV-negative female cervicovaginal lavage (CVL) had been investigated. Investigation had revealed that pregnant female have a distinct mucosal protein profile, including modifications in inflammatory pathways and enhanced mucosal cytokines linked to high risk of HIV infection compared to nonpregnant female (Kaddour et al. 2020; Farr Zuend et al. 2020).

7.2 Characterizing Virus—Host Protein Interactions of Crimean-Congo Hemorrhagic Fever Virus (CCHFV)

Crimean-Congo Virus produces haemorrhagic fever epidemics. Case mortality rates in CCHF epidemics can reach 40% (Spengler et al. 2018). In the discovery for novel antiviral drugs targeting CCHFV, various research employed proteomic techniques to discover host protein that associate with the viral glycoprotein that assist entrance into nucleocapsid protein, which facilitates viral multiplication. Co-immunoprecipitation coupled with MS peptide sequencing revealed an association of the receptor glycoproteins and cell specific nucleolin protein (Xiao et al. 2011).

A study used two proteomics approaches, 2D-DIGE and iTRAQ labeling, along with MS to investigate changes in protein levels in the host liver during infection

utilizing the samples of both mock-infected and CCHFV-infected HepG2 liver carcinoma cells. The results revealed that the large numbers of the 240 different proteins identified were related to proliferation, cell death, cell movement and cell growth. The protein expression levels in the clathrin-mediated endocytosis pathway were altered that take part in virus penetration and the Gc-interacting protein nucleolin. The intention of this study was to augment our perception of how viral fever pathogenesis impacts the liver, which, when infected, may result in liver failure which ultimately lead to mortality (Fraisier et al. 2014).

7.3 Characterizing Flaviviruses Host Protein Interactions

Flavivirus is a group of viruses, primarily transmitted by arthropods and can cause illness in humans. Disease spread by flavivirus, such as dengue and yellow fever, have been documented for centuries and are known to be infectious. Some of the most significant flaviviruses include Dengue Virus (DENV) (which has four different serotypes), yellow fever, Japanese encephalitis, tick-borne encephalitis, West Nile, chikungunya virus and more recently, Zika virus (ZIKV). Most of the proteomic research on DENV and ZIKV has aimed on viral-host proteins interaction, changes in host proteins after the attack of infection and development of diagnostic methods (Holbrook 2017; Rabelo et al. 2017).

In HepG2 cell line, dengue nonstructural protein NS1 expression was studied using Label free quantification analysis, which revealed that 107 host proteins were differentially expressed. All of these proteins were engaged in protein cell stress, apoptosis and degradation. Other study used a combination of different three infected cell lines with a DENV replicon recognizing only NS1 protein and MS investigation to create a complete host- NS1 protein interaction map. The scientists employed a combination of methods to investigate the host factors that take part in the dengue virus replication. They used a functional RNAi screen to detect host 58 dependency factors and 34 restriction factors, which interact with the viral protein NS1. To validate these factors, they performed immunoprecipitation experiments. The proteomic approaches in the dengue virus replication revealed crucial part for certain host proteins, such as RACK1, involved in RNA translation, the cytosolic chaperonin-containing T complex and oligosaccharyltransferase complex components through their interaction with NS1 viral protein (Rabelo et al. 2017; Hafirassou et al. 2017).

Proteomic methods have been employed in the diagnostic of flavivirus infections like dengue fever. One study used iTRAQ labeling with mass spectrometry analysis to analyze pediatric patient plasma samples for development of severe dengue. The patient results showed that before development of dengue, antithrombin III and level of angiotensinogen proteins were considerably augmented. Another study employed a quantitative TMT-based proteomic method to investigate dengue fever patient plasma samples. This study identified total seven proteins that could serve as biomarkers to predict severe dengue disease, allowing early recognition of patients at peril for severe disease (Nhi et al. 2016; Han et al. 2019). A new diagnostic assay has

been developed that can distinguish between different types of flaviviruses, including the different serotypes of dengue virus and Zika virus, using plasma samples. The assay employs a targeted quantitative mass spectrometry approach, using a precursor ion-based method that targets the viral protein NS1 present in all these flavivirus strains. This method has been shown to have high sensitivity and specificity when analyzing samples of patient between 1 and 8 days after the start of symptoms, and it performs similarly well in samples from patients having secondary infections (Wee et al. 2019).

8 Future Outlooks

The field of proteomics is rapidly growing and has the probable to develop the study of pathogen-host interactions in infectious diseases. With advancements in mass spectrometry and computational analysis, the sensitivity and accuracy of protein identification is increasing. This will allow for the detection of even more complex protein interactions, including those between viruses and host proteins. Additionally, the development of new sample preparation techniques, such as multiplexed immunoaffinity purification and targeted proteomics, will increase the specificity and throughput of proteomic analysis.

9 Conclusion

Proteomics is a valuable tool for pathogen-host interactions studies in infectious diseases. It allows for the characterization and detection of different and wide range of proteins and their interactions, providing knowledge of the complex mechanisms underlying disease progression. As the proteomics continues to progress, it is likely that new and more techniques that are sophisticated will be developed, leading to a greater knowledge of the recognition of pharmacological targets and underlying causes of infectious diseases. Overall, the future of proteomics in infectious disease research is promising, in addition to it is expected to act progressively more in the fighting against infectious diseases.

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