The Role of Cryo-Electron Microscopy in Anticancer Drug Discovery and Development

By

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

The thesis titled “The Role of Cryo-Electron Microscopy in Anticancer Drug Discovery and Development” submitted by Jemima Alam (17146005) of Summer, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 24.04.2021.

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Ethics Statement

The study does not involve any kind of animal trial or human trial.

Abstract

Multidrug resistance in cancer treatment is one of the major causes of the increasing need for newer, potent, and safer anticancer drugs. The discovery and development of novel anticancer drugs are limited by the limitations of the older imaging techniques like X-ray crystallography and NMR spectroscopy. Cryo-electron microscopy is a new imaging technology for developing a near-atomic level structural image of biological-macromolecules, small drug-molecules, and drug-receptor binding complexes. This review paper aims to study the role of cryo-EM in the discovery and development of newer anticancer drugs. It also highlights the impactful findings in the oncology field having significance in the anticancer drug discovery process. These include structural analysis of 80S ribosome, microtubule, DNA-PK holoenzyme, HER2-mAbs complex, CD20-antibody complex, human p97, etc. The observation suggests that further extensive analysis and studies of these structures can lead to a breakthrough in cancer research and the development of novel anticancer drugs.

Keywords: Cancer; Cryo-EM; Anticancer drug; Drug discovery; Macromolecular imaging.

Dedication

Dedicated to the innocent cancer sufferers

Acknowledgment

To begin with, I am immensely grateful to the Almighty Allah for blessing me with strength, knowledge, wisdom, and patience without which I would never have made it this far. It helped me to pass through all the obstacles, making me capable of handling situations towards reaching my goal and following my dream.

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List of Acronyms

EM	Electron Microscopy
ATP	Adenosine Triphosphate
CML	Chronic Myeloid Leukemia
NHL	Non-Hodgkin’s Leukemia
CLL	Chronic Lymphocytic Leukemia
EGFR	Epithelial Growth Factor Receptor
NMR	Nuclear Magnetic Resonance
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ET	Electron Tomography
MicroED	Microcrystal Electron Diffraction
SPA	Single Particle Analysis
TEM	Transmission Electron Microscope
CR	Continuous Rotation
RED	Rotation Electron Diffraction
SNR	Signal-to-Noise Ratio
SBDD	Structure-Based Drug Design
FBDD	Fragment-Based Drug Design
EMDB	Electron Microscopy Data Bank

PDB	Protein Data Bank
CADD	Computer-Aided Drug Design
MAO	Mode Of Action
MT	Microtubule
PF	Protofilament
PRC1	Protein Regulator of Cytokinesis 1
HER	Human Epidermal growth factor Receptors
PIKK	Phosphatidylinositol 3-kinase-related kinases
DNA-PK	DNA-Dependent Protein Kinase
DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
DNA	Deoxyribonucleic acid
DBS	Double Strand Break
NHEJ	Non-Homologous End Joining
ADP	Adenosine Diphosphate
UPS	Ubiquitin Proteasome System
ERAD	Endoplasmic Reticulum Associated Degradation
MAD	Mitochondria Associated Degradation
CD20	Cluster of Differentiation 20
mAb	Monoclonal Antibody

Fab	Fragment Antigen-Binding
Fc	Fragment Crystallizable
RTX	Rituximab
OBZ	Obinutuzumab
OFA	Ofatumumab
TKI	Tyrosine Kinase Inhibitor
STEAP	Six-Transmembrane Epithelial Antigen of Prostate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
FAD	Flavin Adenine Dinucleotide
ACLY	ATP-Citrate Lyase
DED	Death Effector Domain
ABC	ATP-Binding Cassette
MDR	Multidrug-Resistant
mTORC	Mammalian Target of Rapamycin Complex
LDH	Lactate Dehydrogenase
rRNA	Ribosomal Ribonucleic Acid
FDA	Food and Drug Administration
SAR	Structure Activity Relationship
GTP	Guanosine Triphosphate

HR	Homologous Recombination
ROS	Reactive Oxygen Species

Chapter 1

Introduction

1.1 Cancer

Cancer is the second most common cause of death worldwide. It is not a single disease rather a group of diseases, characterized by abnormal cell growth. Treatment options for cancer include surgery, radiotherapy, and chemotherapy, used either alone or in combination. Chemotherapy is an important approach to cancer management. Chemotherapeutic agents alone can cure 10 % of all the advanced stage cancer patient (Katzung, 2017). However, the need for discovering newer anticancer drugs is still an issue in search of new molecular targets, innovative mechanisms of action, and to combat the increasing incidences of anticancer drug resistance developments. The discovery and development of novel anticancer drugs are highly challenging due to tumor heterogeneity (Magalhaes et al., 2018). Drug treatment of cancer requires a clear understanding of tumor physiology and its genetic machinery.

Cancer is characterized by a defect in the normal control mechanism governing cell proliferation, survival, and differentiation. The cells exhibit chromosomal abnormalities including various translocations, genetic instability, and the appearance of amplified gene sequences. These tumor masses can undergo repeated proliferation and migrate to distant sites in the body by metastasis. Abnormal expressions of an oncogene, tumor suppressor gene, and DNA repair gene contribute to the formation of a tumor (Katzung, 2017). The Human p53 gene is one of the major tumor suppressor genes that gets mutated in most cases of cancer. The p53 gene induces p21 protein to complex with cdk2, a cell division stimulating protein. As a result, cell division can no longer take place (Cho, 1998). On the other hand, the protein of the bcl2 family is an oncogene that promotes cell survival inhibiting apoptosis. The over-expression of this gene contributes to tumor formation (Hardwick et al., 2013). Various cancer

chemotherapeutic agents act using a different mechanism of action including targeting at different genes, proteins, or enzyme specifically which has involvement in the growth and survival of the tumor cells (Kumar et al., 2015).

1.2 Target Sites for Different Anticancer Drugs

Currently available anticancer agents involve acting on diversified target sites. Cytotoxic drugs such as antimetabolites, alkylating agents, and antimicrotubule drugs work by interfering with the DNA synthesis of tumor cells. Targeted therapy drugs such as mAbs interfere with the protein activity involved in tumorigenesis and cancer progression (Magalhaes et al., 2018).

BCR-ABL tyrosine kinase is an oncoprotein overexpressed in chronic myeloid leukemia (CML). Drugs that selectively inhibit the protein, works to treat CML. STI-571 such as imatinib is a BCR-ABL tyrosine kinase inhibitor (TKI) (Rian et al., 2001).

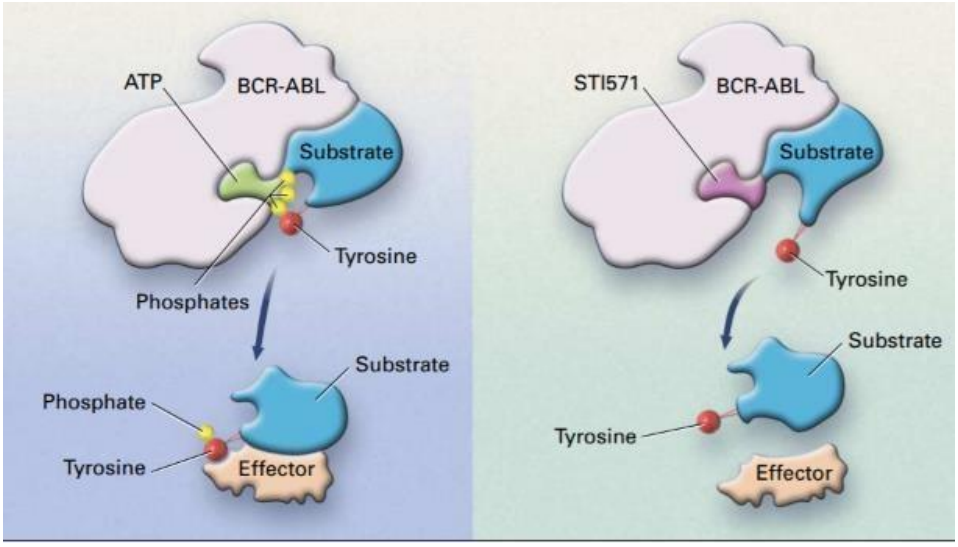


Figure 1: Mechanism of action of STI-571 (John M. Goldman, 2001)

Figure 1 shows how STI-571 binds to BCR-ABL to compete with ATP resulting in inhibition of phosphorylation of tyrosine. The phosphorylated tyrosine can interact with the effector molecule. The blocking of the phosphorylation prevents this interaction.

CD20 is a transmembrane protein that takes part in B-cell development. It is overexpressed in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). Anti-CD20 drug such as rituximab is used in the treatment of NHL and CLL. These drugs enhance the antitumor effect of cytotoxic chemotherapy as well as effective in chemotherapy-resistance (Smith, 2003). The drug binds to CD20 protein which is present on the surface of the CLL cell. It causes direct cell death and induces a complement pathway to produce cytotoxicity. It also has a binding affinity towards the FcγRIII receptor. This binding results in antibody-dependent phagocytosis as shown in Figure 2.

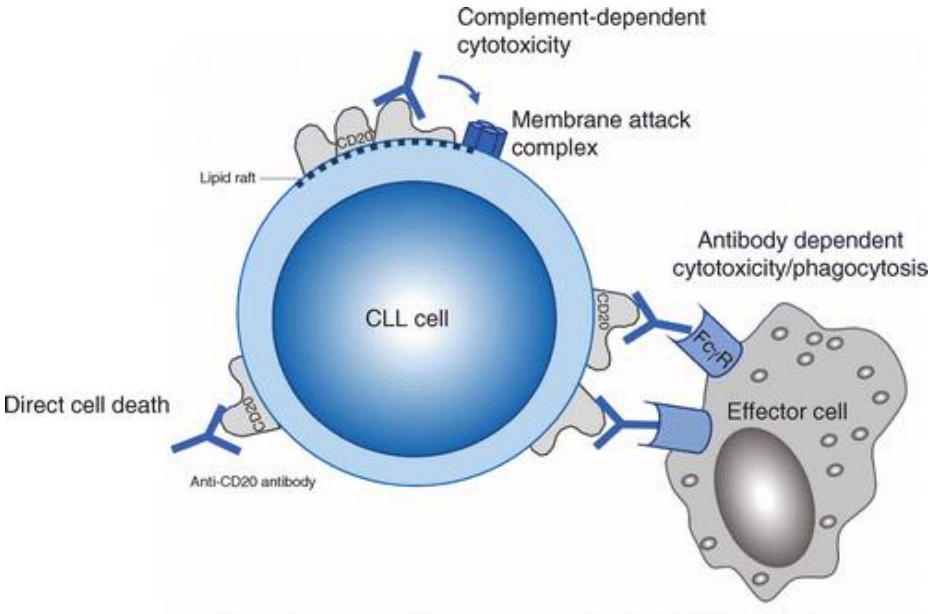


Figure 2: Mechanism of action anti-CD20 drug (Salvaris & Opat, 2018)

HER-2 is a receptor protein that regulates breast cell growth and repair. This protein is over-expressed in the case of breast cancer. Trastuzumab is an anticancer monoclonal antibody that acts on HER-2 expressing cancer cells. The drug bind to the HER-2 receptor resulting in its downregulation. Thus, it clinically benefits HER-2 positive breast cancer patients (Gajria & Chandarlapaty, 2011).

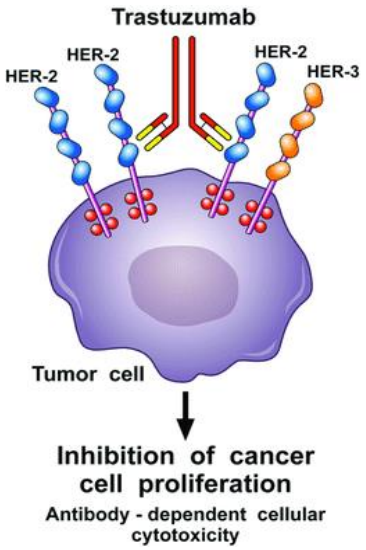


Figure 3: Mechanism of action of Trastuzumab (Varricchi et al., 2018)

A certain antitumor drug such as paclitaxel and, vincristine interacts with microtubule to inhibit its action resulting in inhibition of mitosis in a cancer cell. Another class of drugs inhibits the EGFR signaling pathway. EGFR is an epidermal growth factor receptor that is overexpressed in several solid tumors including colorectal cancer, head and neck cancer. Examples of such drugs are cetuximab, panitumumab, and necitumumab (Katzung, 2017). Understanding of structures of these proteins is vital for the development of drugs that act on these sites.

Table 1: Selected examples of anticancer drugs and their target sites

Drug Name	**Target site**	**Reference**
Irinotecan	DNA Topoisomerase I	(Haddad et al., 2018)
Trichostatin A	Histone Deacetylase	(Lin et al., 2018)
5-Fluorouracil	Thymidylate Synthatase	(Katzung, 2017)
Curcumin	Protein Kinase C, EGFR, Tyrosine Kinase and IκB Kinase	(Zhou et al., 2012)
Formestane	Aromatase	(Brown, 2012)
Rhizoxin	β Tubulin	(Rath et al., 2010)
Deguelin	PI3K/AKT	(Bortul et al., 2005)

Etoposide	DNA Topoisomerase II	(Baldwin & Osheroff, 2005)

1.3 Anticancer Drug Discovery Approaches and Challenges

From the year 2013 to 2016, a total of 33 new anticancer drugs have been approved to be marketed among which 36% are mAbs, and 61% are small molecules. Developing a drug from the preclinical phase to marketing takes around 11 years (Magalhaes et al., 2018). The steps in the preclinical phase are-

(i) Molecular target identification and validation

(ii) Hit identification and hit-to-lead optimization

(iii) Lead optimization to a clinical candidate.

The molecular target is a protein or gene to which a drug will bind and produce its effect. The first step of discovering a novel drug is to find the therapeutic target by identifying the biological origin of the tumor and its function. The molecular mechanism of the identified target is then characterized as part of validation. Hit is a suitable molecule that has an affinity to binds to the target. A promising compound is optimized from the hit molecule known as a lead molecule. It shows therapeutically useful pharmacological effects by a series of synthesis and characterization. Then the molecule is selected as a clinical candidate to perform clinical tests (Luxminarayan et al., 2019). To accelerate the process, researchers need to find a rapid method of narrowing down the promising drug candidates.

Drug discovery and development is a lengthy process. After passing through the preclinical and clinical phases, the new drug undergoes a process of market approval. Finally, the drug becomes available in the market for the consumers.

1.4 Cryo-Electron Microscopy in Drug Discovery

Cryo-EM is a revolutionizing imaging technique for developing high-resolution 3-dimensional image structures of large macromolecules (Renaud et al., 2018). Before the invention of this technology, most structures were identified using X-ray crystallography and NMR spectroscopy. Structures that were difficult to obtain using X-ray crystallography, can now be determined using cryo-EM. Cryo-EM has several advantages over other techniques including high-resolution structural information, high sensitivity since a very small amount of sample is required, no need for protein crystallization, and relatively short time is required to obtain the image (Wang, 2015). Thus, it can play an important role in all three stages of anticancer drug development.

1.5 Rationale of The Study

Cancer, being one of the major health problems worldwide, attracts more and more researchers to devote themselves to cancer research. Available anti-cancer drugs alone are not efficient enough to solve this issue. People are increasingly becoming resistant to available chemotherapeutic agents. The world requires newer anticancer drugs. Scientists are trying to find new molecular targets, better and safer mechanisms of action that can fight the increasing incidences of anticancer drug resistance developments. Tumor heterogeneity makes drug discovery and development more challenging. The major difficulties in anticancer drug discovery include limited understanding of tumorigenesis, restricted size of the structures, and resolution of the image. The researchers have to utilize genomics and system biology more efficiently. Cryo-EM is a newly developed imaging technique that is a powerful tool to depict the molecular structure of both drugs and biological macromolecules at near-atomic resolution. The world’s major pharmaceutical companies and academia are now investing in cryo-EM to facilitate novel drug discovery.

1.6 Aim of The Study

The purpose of this study is to find out whether and how cryo-EM can serve for the discovery and development of new anticancer drugs. Furthermore, the study aims to determine the role of cryo-EM in cancer research. In this study, cryo-EM has been chosen because it is the most recent imaging technology winning the Nobel prize of chemistry in 2017. The utility of cryo-EM is still under extensive research to improve its current status and overcome its shortcomings.

Chapter 2

Methodology

This review paper has been conducted based on recent and relevant research papers and articles from high-impact factor journals. A comprehensive search has been performed through peer-reviewed journals, official reports, and articles. To enrich the review paper, basic and additional information have been collected from different books. Following search engines have been used to collect data for this paper- ResearchGate, Google Scholar, Science Direct, PubMed, Cell Press, Elsevier, etc. in which the major publications include- Nature, ACS (American Chemistry Society), AACR (American Association for Cancer Research), Molecular Cell, Cancer Cell, Journal of Molecular Biology, Journal of Medicine, Science, etc. In-depth screening of the journals followed by narrowing down to the most recent (within the last 5 years) and relevant ones was done to create an ideal quality review on the role of cryo-EM in anticancer drug discovery and development.

Chapter 3

Cryo-Electron Microscope Technique

3.1 Cryo-Electron Microscope

Cryogenic electron microscope (cryo-EM) is an imaging technique of a new generation electron microscope that determines macromolecular structures (Rodriguez & Gonen, 2016). The imaging technique is vital for understanding and treating diseases and disorders. It has become a preferred method to gather structural information of drug receptors. It is a Nobel prize-winning technology for chemistry in 2017 (Renaud et al., 2018).

Determination of biomolecular structures is necessary to understand their biological function. Once the structural information is known, it helps researchers design drugs that fit into them. Isolated assemblies, molecular mass over 100 kDa, tissue section, and even whole-cell can be visualized in a cryo-EM if they are sufficiently thin to allow electron beam transmission. The thickness of the cell must be within 1 µM. The electrons interact with the specimen to produce an image containing the structural information (Carroni & Saibil, 2016). The produced image has a high noise ratio. To improve the signal-to-noise ratio, multiple copies of the same view of the molecule are averaged using the computational method. As a result, an image of high resolution with 3D structure is produced (Renaud et al., 2018).

The number of cryo-EM-based structures being published is rapidly increasing including membrane protein complex, large dynamic assemblies, and complexes with small molecules (Renaud et al., 2018). The first 3D structure using cryo-EM revealed was the TRPV1 membrane ion channel of 300 kDa with a resolution of 3.4 Å (Figure 4). Figure 4 shows the 3D structure of a selected region of TPRV1 at an atomic level. Many other structural models were resolved at the atomic level including several viruses, proteasomes, and ribosomes (Wang, 2015). Being able to visualize proteins at this level of detail allows us to better

understand how the proteins function. It opens up the possibility to design drugs that precisely fit proteins enhancing opportunities to treat diseases.

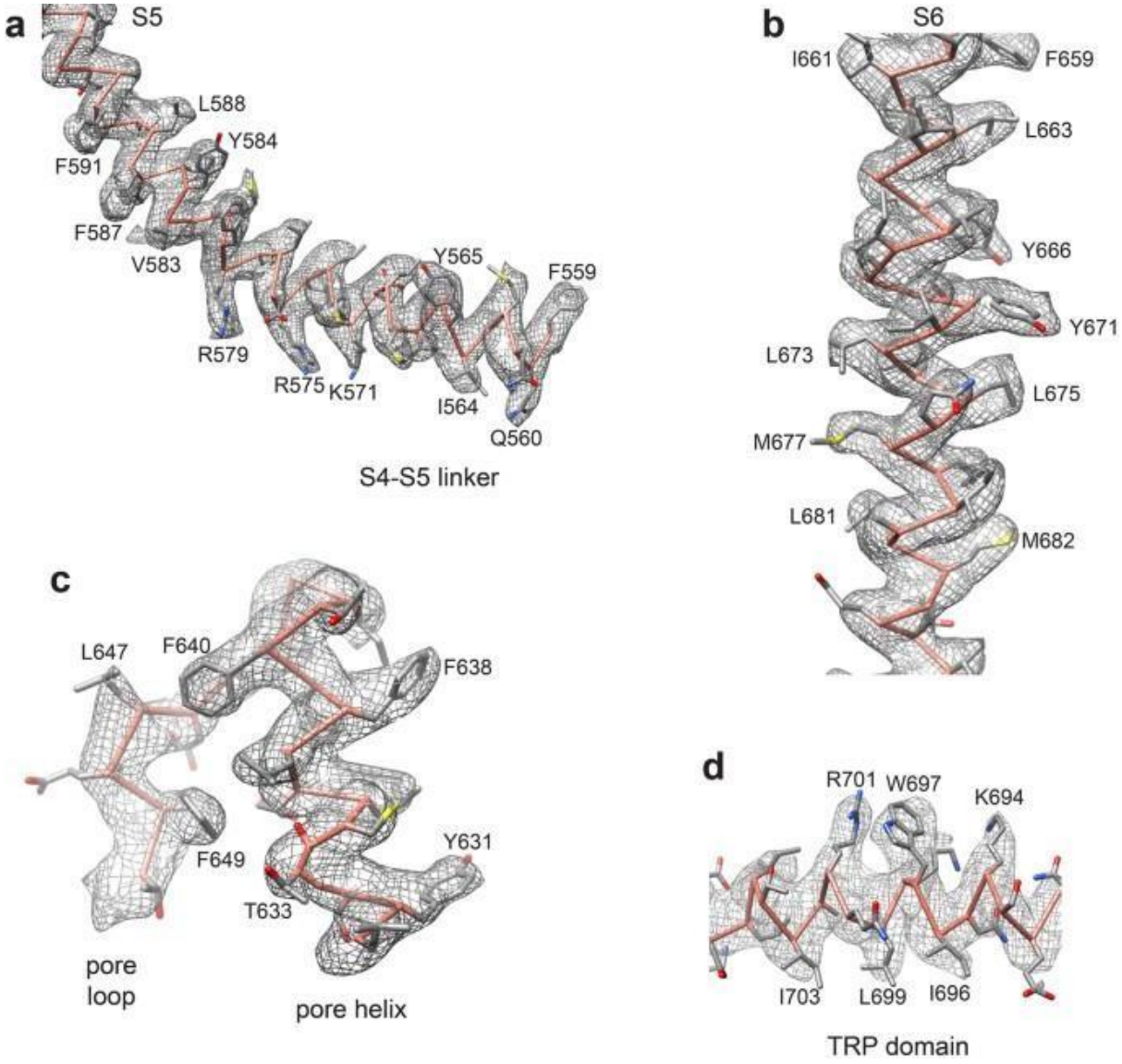


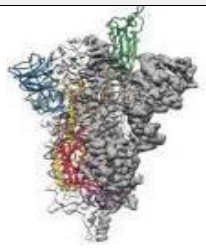
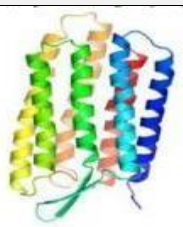
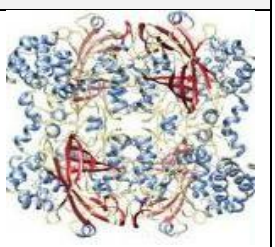
Figure 4: Cryo-EM image of TRPV1 membrane channel (Liao et al., 2013)

3.2 Classification of Cryo-EM Based Technique

The major techniques included in the field of cryo-EM are single particle analysis (SPA), microcrystal electron diffraction (MicroED), cryo-electron tomography (cryoET) and, 2D electron crystallography. (Nguyen & Gonen, 2020). All four techniques use strong electron interactions with material to generate the image. Among these, the SPA and microED are the

most used and advantageous variants. Table 1 shows a comparison between them. SPA does not require the sample to be crystallized, unlike microED (Pal, 2020). Recently, COVID-19 viral spike protein has been determined using SPA (Table 1). However, microED can generate a 3D structural image from amorphous powder with minimal sample preparation which is not possible using SPA. An example is shown in Figure 5 where microED is used to determine molecular structures of small compounds and drugs from a mixture of compounds (Jones et al., 2018).

Table 2: Comparison between different cryo-EM techniques (Nguyen & Gonen, 2020)

Cryo-EM Technique	**Cryo-ET**	**SPA**	**2D electron crystallography**	**MicroED**
Image				
Microscope mode	Imaging	Imaging	Imgaing/ Diffraction	Diffraction
Object	Whole cells, organelles	Isolates single particles	2D crystals	3D microcrystals
Advantage	Samples in near native states	High-resolution	Samples in lipid bilayer	High-resolution from small crystals
Challenge	Generally low resolution (~10 Å)	Molecular weight limit >40 kDa	Requires 2D crystal growth	Requires 3D crystal growth

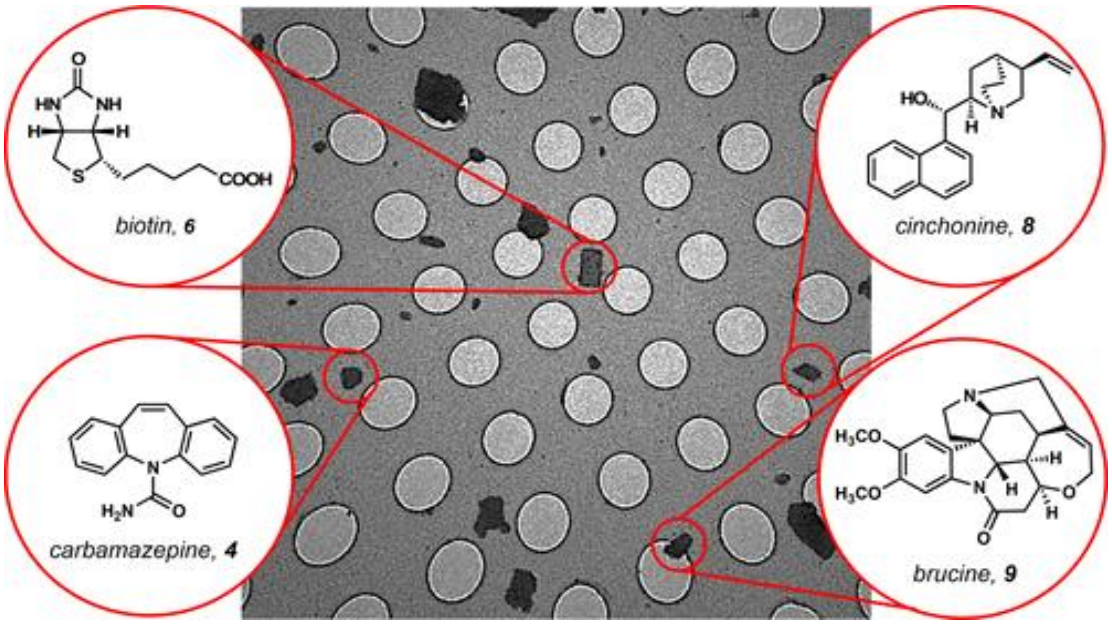


Figure 5: MicroED structures of different compounds from a mixture (Jones et al., 2018)

3.3 Working Principle

3.3.1 Microcrystal Electron Diffraction

Cryo-EM starts with sample preparation for both microED and SPA techniques. In the microED technique, the sample needs to be micro-crystallized upon removal of the precipitant solution. Crystals can be grown in any condition by hanging drops or sitting drops. If the obtained crystals er larger, they can be fragmented by crushing, vortexing, or sonicating (Kunde & Schmidt, 2019). Next is grid preparation. Approximately 4 µL of nanocrystal solution is applied to the grid. Excess liquid is removed from the grid using Vitrobot and the grid is then frozen in liquid ethane followed by storage in liquid nitrogen (Jones et al., 2018). After freezing the grid is transferred into a cryo-TEM under cryogenic condition ensuring sample integrity. Each nanocrystal can be observed individually using diffraction more. The data is collected by continuous rotation (CR) or rotation electron diffraction (RED) method as a video on a fast camera while the frozen crystals are continuously rotated (Nannenga & Gonen, 2019). As a result, the video is composed of many discrete frames, each containing hundreds

of diffraction peaks. The peaks reveal information about the underlying structures of the sample (Carroni et al., 2018).

The collected data is then processed by indexing, integration, and merging. Data processing software including XDS, MOSFLM, and DIALS identifies separate diffraction peaks since each of them has a different intensity (Carroni et al., 2018). This process is repeated for all frames in the video and the data is combined by scaling and merging. A complete 3D reciprocal representation has then obtained (Kunde & Schmidt, 2019). The process is represented in Figure 6.

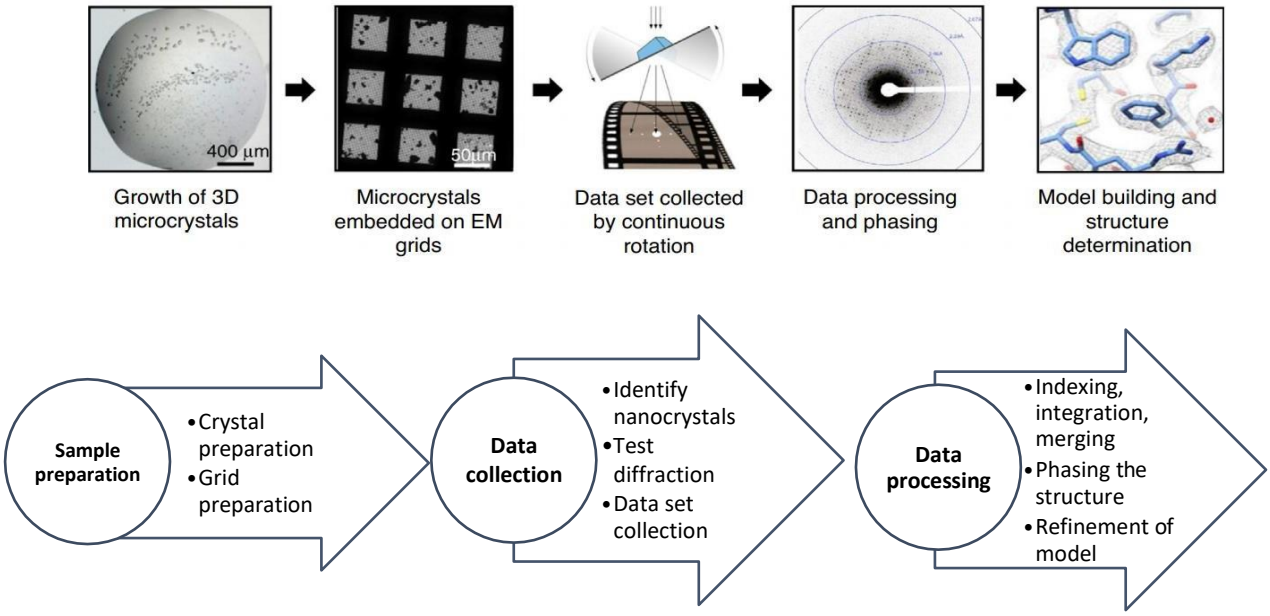


Figure 6: Workflow of cryo-EM microED (Nguyen & Gonen, 2020)

3.3.1 Single Particle Analysis

In SPA, an aqueous sample of biological material is collected. It should be purified using various techniques including SDS-PAGE and different types of chromatography. The sample solution for SPA is prepared at a concentration of 0.05 – 5 μM (Passmore & Russo, 2016).

The sample solution contains freely mobile nanoparticles. To capture an image, it must be frozen. A tiny drop of sample is placed onto a copper grid. The sample is loaded into a Vitrobot machine maintaining humidity and temperature of 4^{o} C. There are two blotting papers on each side of the sample. They come closer on the grid making a very thin layer of molecules in the solution. The sample is then quickly placed into liquid ethane which is surrounded by liquid nitrogen. The sample freezes so fast that the ice crystals cannot form. Therefore, it is called vitreous ice. In this process, a sample in solution is converted to molecules frozen in thin ice (Chung & Kim, 2017).

The grid containing the frozen sample is loaded into an electron microscope to reconstruct a 3D structure from the frozen molecule. The molecules are trapped in different orientations underneath a photographic film. An electron gun shoots electrons at the speed of light passing through the sample. Then specially designed high-tech camera captures the electrons to form an image (White et al., 2017).

Data collection can be performed manually or by using automated tools such as Titan Krios and CRYO ARM (de Carlo & Rémigy, 2020). The orientation of each molecule generates a unique shadow. These shadows contain all the 3-dimensional information of the molecule compressed into a 2D image. A computer algorithm collects and sorts the images to find pictures of the protein in the same orientation. Then the software processes the data to build a composite by adding the images together. The summed image provides a more detailed view of the molecule in this orientation (White et al., 2017). A suitable detector is needed to improve the image quality by improving the signal-to-noise ratio (SNR). For this purpose, a direct electron detector (DED) or charge-couple device (CCD) is used (de Carlo & Rémigy, 2020).

The same process is performed for the same molecule for different orientations. All the views are gathered and combined computationally. As a result, it acquires a high-resolution 3D image

of the molecule. Zooming in the image reveals the structural feature of the molecule, generating information about how it functions in the cell (White et al., 2017).

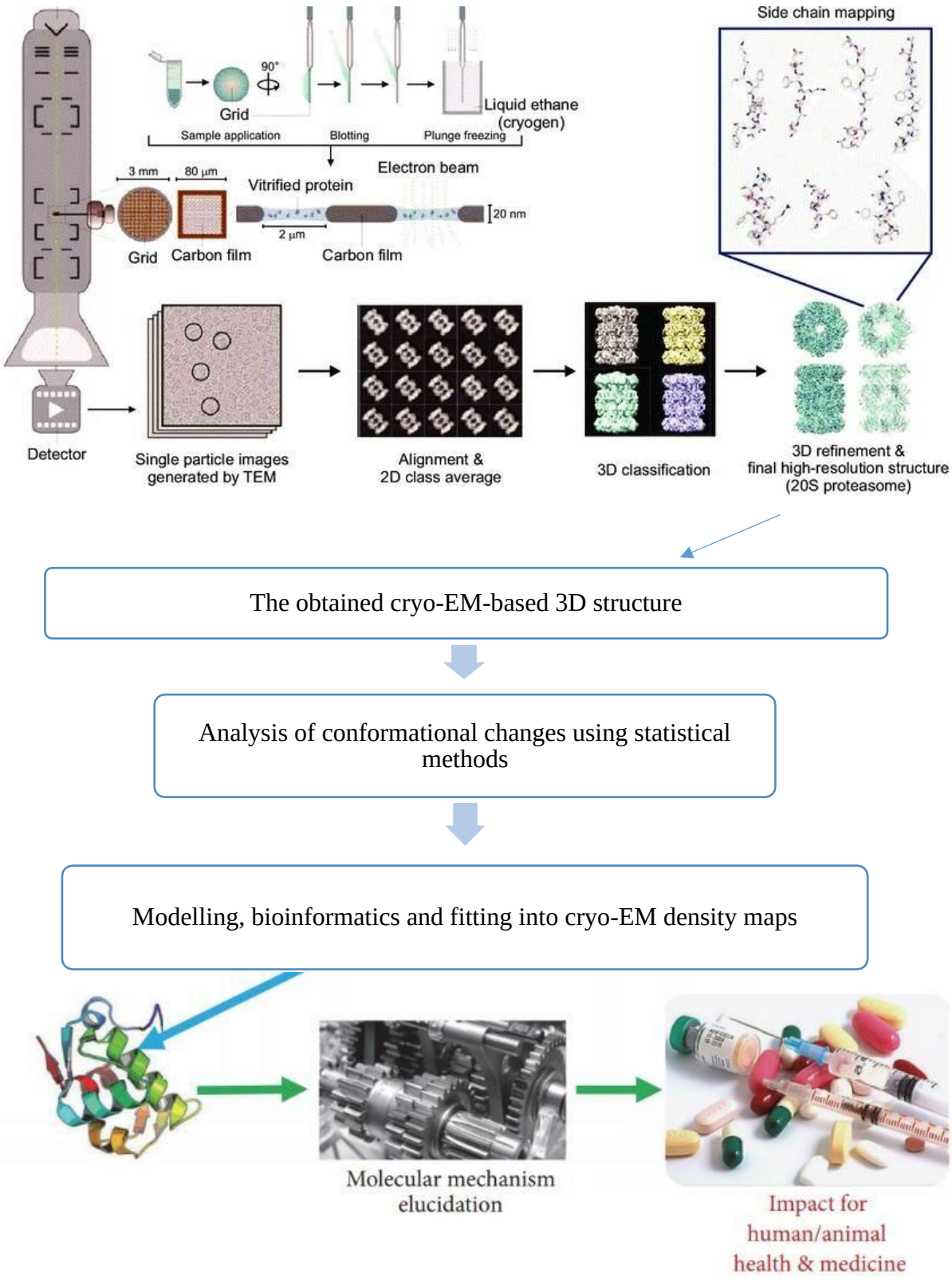


Figure 7: Workflow of cryo-EM SPA (Chung & Kim, 2017; White et al., 2017)

3.4 Role of Cryo-EM in Drug Discovery

Protein structure determination is an integral part of drug discovery. All parts of the drug development process require a broad understanding of the biological system, starting from initial target identification, hit identification, lead optimization to final clinical candidate determination.

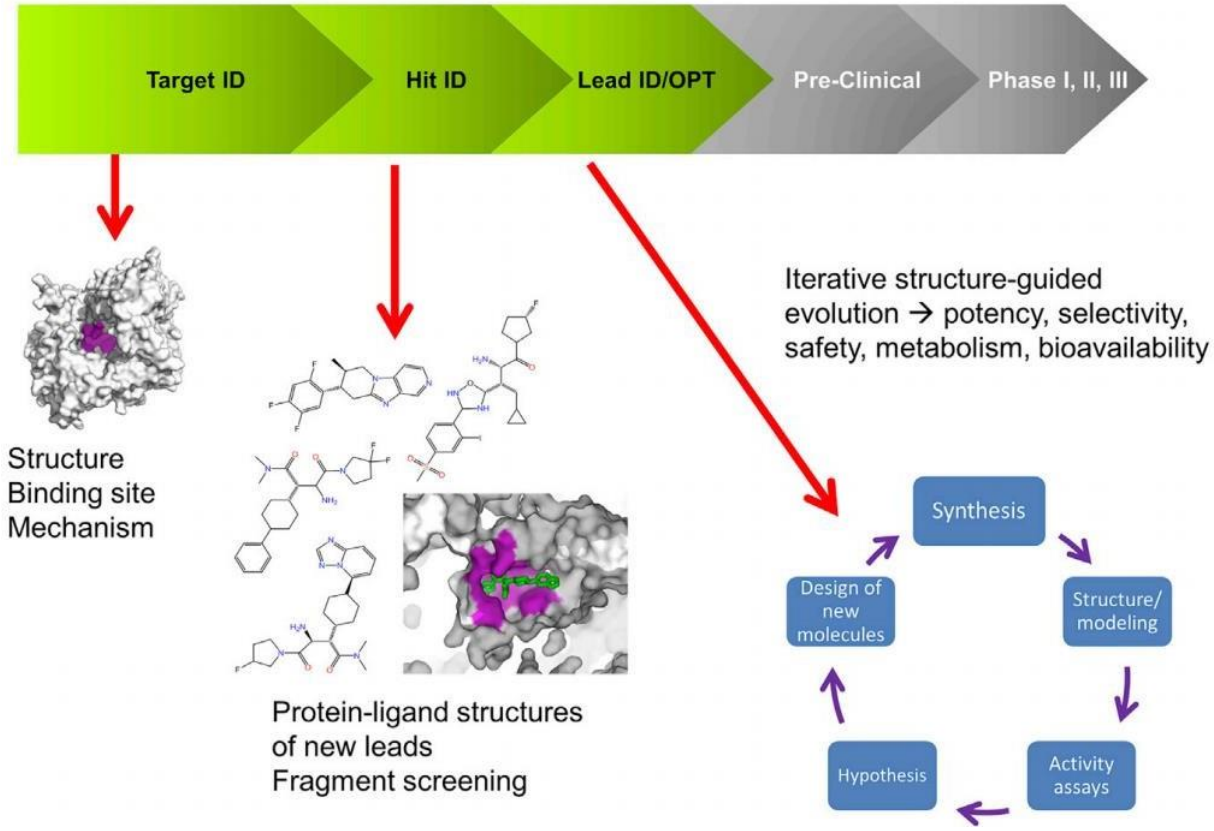


Figure 8: Role of cryo-EM in different stages of drug discovery (Scapin et al., 2018)

For target identification, the behavior of the protein to be targeted at an atomic level is important to understand. Cryo-EM can obtain structural information of a drug-receptor, disease association, and druggability assessment. This information helps in the prediction and proposal of the mechanism of action (MOA) of a new drug entity (Ceska et al., 2019; García-Nafría & Tate, 2020). One of the first drugs discovers using microED is bevirimat, an antiviral drug to fight against HIV. Cryo-EM revealed the structure of the HIV-GAG-bevirimat complex to

propose the mechanism of action of the drug. Here, HIV-GAG is an essential protein contributing to the HIV viral life cycle (Nguyen & Gonen, 2020).

Hit identification and optimization are facilitated using cryo-EM structures of the protein-drug complex. Cryo-EM can directly visualize conformational changes and binding of small molecules to the target protein. Thereby, it becomes possible to assess their binding affinity. EM maps are used to collect high-resolution data on close analogs. Using these data, binding modes of a different compound are identified and the potential optimized hit compound is passed over for computer-aided drug design (CADD) analysis. As a result, the affinity and selectivity of the compound can be improved (Ceska et al., 2019). The structure of the β-galactosidase-inhibitor complex is resolved at high-resolution using cryo-EM. Cryo-EM also resolved the structure of TRPV1 complexed with vanilloid antagonist capsazepine (Gao et al., 2016). This helps to propose a hypothesis for MOA of the hit compound resulting in hit-to-lead optimization.

Structure characterization of not only protein assembles but also small molecules and natural products are possible using cryo-EM. Sodium channel blocker carbamazepine, several small organic molecules, and recently developed antiviral drug Grippostad are some of the examples of cryo-EM microED derived structures. Cryo-EM revealed the anticancer property of brucine which was known as an alkaloid toxin. Its microED structure identifies two chiral centers of the molecule which is essential to distinguish between its toxicity and anticancer property (Nguyen & Gonen, 2020).

Cryo-EM has been found to be useful for both fragment-based drug design (FBDD) and structure-based drug design (SBDD). In SBDD, the resolution of the map is an important consideration. We need to understand the atom-atom interactions, atomic positions, and motion of the main chain or side chain in closer details. For this purpose, a high-resolution structural

image, at least 3 Å or higher must be used. Cryo-EM provides a high-resolution 3D characterization of molecules. (Scapin et al., 2018) An example of a cryo-EM-based SBDD approach is the visualization of the complex of antimalarial drug mefloquine bound plasmodium falciparum 80S ribosome to improve its potency. For this drug, the binding site was previously unknown (Renaud et al., 2018).

FBDD is an attractive method of drug discovery. In this strategy, potent small low-molecular-weight ligands are developed initiating from fragment to target binding (Bissaro et al., 2020). Besides SBDD, cryo-EM mediated FBDD is also feasible. Fragmented ligands have a binding mode that can be identified using cryo-EM. It can easily differentiate between fragments and noise (Saur et al., 2020).

Pharmaceutical companies including Astex, UCB, AstraZeneca, GlaxoSmithKline, Thermo Fisher, etc., and academia including the University of Cambridge and the MRC LMB are becoming increasingly interested in investing in cryo-EM based drug development (Ceska et al., 2019). The number of Electron Microscopy Database (EMDB) entries and Protein Data Bank (PDB) entries ensures that cryo-EM is rapidly increasing. In 2013, there were 28 EMDB entries of cryo-EM SPA structures including TRPV1 channel, 20S proteasome, and 80S ribosome. In 2014, 2015, 2016, 2017, and 2018, the rise was observed as 43, 140, 340, 435, and 672 respectively (Renaud et al., 2018; Scapin et al., 2018).

Table 3: Examples of selected medically relevant cryo-EM determined structures having the potential to facilitate novel drug discovery

Compound	**Resolution (Å)**	**Medical relevance**	**Reference**
Gripposad	0.81	Determine the efficacy of antiviral activity	(Gruene et al., 2018)
Mefloquine-80S ribosome of Plasmodium falciparum	3.2	Reveal MOA Develop mefloquine derivative	(Wong et al., 2017)
Antiepileptic drugs-Gabapentin & Pregabalin	3.6	Develop derivatives with increased selectivity and potency	(Kotev et al., 2018)
Yeast mitoribosomal large subunit	3.2	Propose new active site for antifungal drugs	(Amunts et al., 2014)
GPCR complexes	3-4	Facilitate membrane-protein bound drug design and assess binding affinity	(García-Nafría & Tate, 2020)
MBBF4 methylene blue derivative	0.9	Assess antimicrobial activity Promising API	(Gruene et al., 2018)
HIV1 gag CTD-SP1 - bevirimat complex	2.9	Reveal antiviral drug mechanism Improve potency	(Purdy D. et al., 2018)
Leishmania ribosome – Paromomycin complex	2.2	Reveal antiparasitic drug mechanism Reveal PAR-binding site to enhance the development of novel drugs	(Shalev-Benami et al., 2017)
TRPV1 - Capsazepine	3.4	Reveal MOA of ligand-protein interaction	(Gao et al., 2016)

Chapter 4

Cryo-EM Protein-Structures in Cancer Research

Different genes, enzymes, and proteins from the biological system having an impact on oncogenesis have been visualized under cryo-EM. The impactful protein-related findings aimed to facilitate cancer research and anticancer drug discovery and developments are demonstrated in this chapter of the review paper. Moreover, the assessment of the application and relevance of these structural analyses to anticancer drug development will be discussed here.

4.1 Human 80S Ribosome

Cryo-EM was used to visualize human cytosolic ribosomes. The human 80S ribosome consists of 2 subunits. The larger one is the 60S ribosomal subunit and the smaller one is the 40S subunit which in turn is divided into body and head region (Natchiar et al., 2017).

Figure 9 shows a detailed cryo-EM structural analysis of both ribosomal subunits in which A and B represent ribosomal subunits 40S and 60S respectively. C represents a structure of a functional site to which inhibitors most preferably bind. Examples of such inhibitors include homoharringtonine (HHT), cycloheximide (CHX), and edeine (EDE) (Gilles et al., 2020). D shows chemical modification, E and F show human 80S ribosome complexed with HHT and CHX respectively.

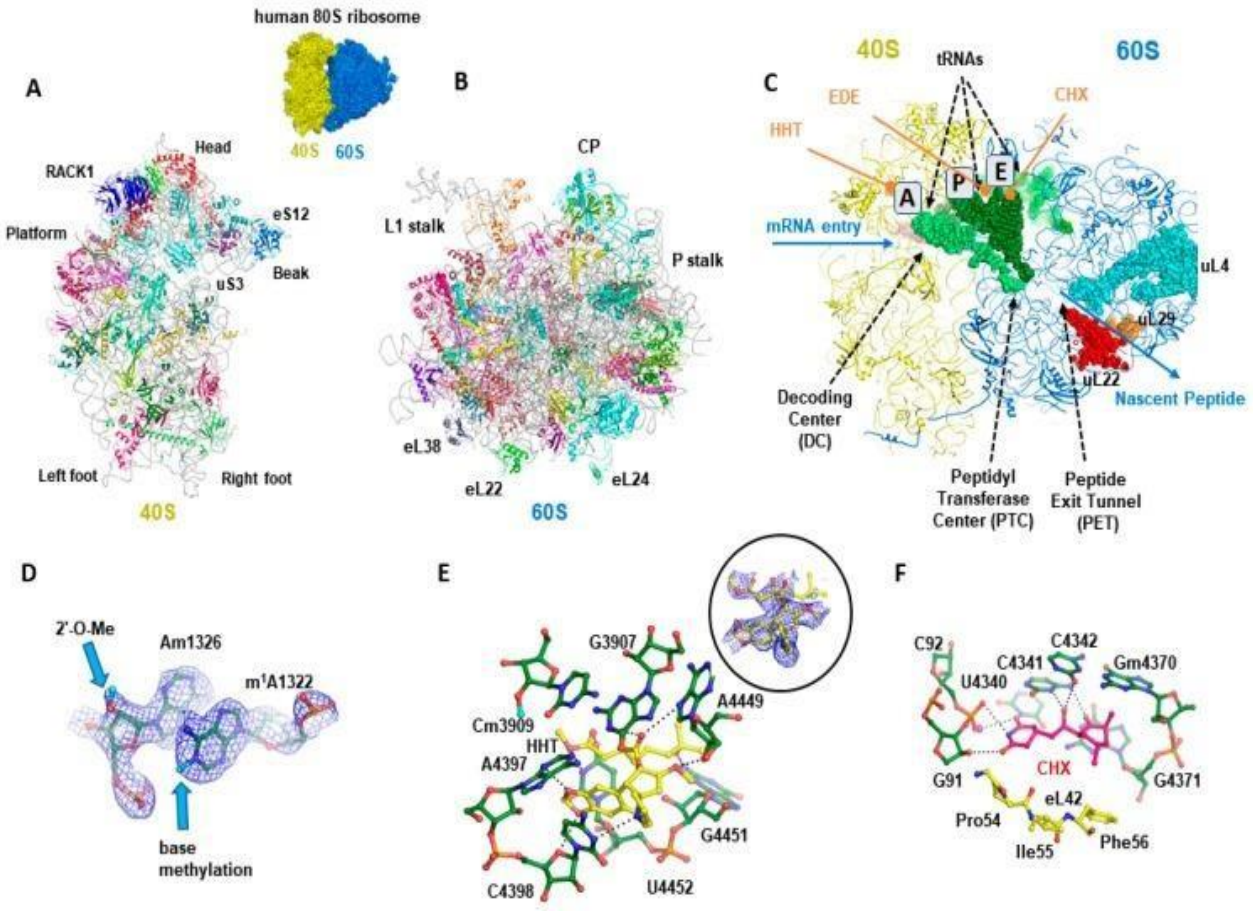


Figure 9: Cryo-EM Structure of Human 80S Ribosome (Gilles et al., 2020)

4.1.1 Significance of Human 80S Ribosome Structure

The human ribosome is involved in the biosynthesis of protein. It consists of four rRNA chains, three in 60S subunit and one in 80S subunit. Numerous chemical modifications take place in rRNA chains including methylation of 2′-OH ribose, Uridine (U) to pseudouridine (Ψ) conversion, and base methylation (Figure 9 D) (Natchiar et al., 2017). This chemical modification is necessary for translation. It is a part of the transcription and maturation process of rRNA in the nucleus and cytoplasm (Sloan et al., 2016). Earlier, rRNA modifications were observed and analyzed in bacteria (prokaryotic) and parasite (eukaryotic) ribosomes (Decatur & Fournier, 2002). Nevertheless, the visualization of modifications of the human 80S ribosome was not conducted. Therefore, the mechanism and functions of these modifications were mostly unidentified before analyzing it under cryo-EM. Structural analysis of a complete RNA

chemical modification has been carried out in 2018 (Taoka et al., 2018). During different stages of ribosome biogenesis, the rRNA modifications occur which assist in the stabilization of the rRNA secondary and tertiary structure. By this means, it ensures translational accuracy and efficiency (Sloan et al., 2016). It also signifies a vital basis of ribosome heterogeneity (Taoka et al., 2018). Accordingly, alteration in modification can impact both translation efficacy and accuracy undesirably. Cancer is associated with altered rRNA modification. Since modification dysregulation leads to proteome changes resulting in cancer. Therefore, the structural visualization of the ribosomal modification and their sites plays a major role in understanding cancer pathology and physiology. This in turn helps in the development of anticancer therapy.

Human 80S ribosomes are affected during cancer development by oncogenic proteins. Since. ribosomes provide proteins to the cancerous proliferating cells. Thus, cancer leads to an increased level of ribosome-biogenesis resulting from mutations in numerous ribosomal genes and proteins (Gilles et al., 2020). Hence, ribosome represents an attractive target for anticancer drugs. Structural study of the ribosome and their binding pockets for inhibitor provides crucial information for new drug design (Figure 9 C).

HHT is one of the currently used direct ribosome inhibitors which has been approved in 2012 by the FDA mainly for CML, indicated to patients having developed resistance for TKI. However, its anticancer mechanism was not properly identified (Kantarjian et al., 2013). Nevertheless, now the researchers have resolved the structural features of HHT bound 80S ribosome (Figure 9 E) making it possible to chemically modify the HHT structure on the way to increase its bioactivity, as well as metabolic stability. It provides valuable proof for using ribosome blockers alone or in combination with other chemotherapeutic drugs to affect cancer growth.

CHX is a result of cryo-EM-based drug design which has provided valuable insights for ribosomal inhibition (Figure 9 F). However, because of its toxicity researchers are trying to modify it in order to develop new potent CHX derivatives. Hopefully, new derivatives will be developed and enter the market soon with the help of cryo-EM-based structure-activity relationship (SAR) analysis.

For a long time, the 80S ribosomes have been under consideration as a promising target for anticancer moieties and now it is evident. The cryo-EM structure of the human 80S ribosome can be used for target identification and hit optimization in an anticancer drug development process. Visualization of the inhibitor binding pocket leads us to the target identification and analyzing the inhibitor bound complex of ribosome serves for hit optimization. Targeting and inhibition of ribosome interfere with protein synthesis of cancer cell, reduces oncogenic proteins level leading to the induction of death in a cancerous cell. The cryo-EM structure of the human 80S ribosome and its complexes with inhibitors improves our knowledge and understanding regarding their functioning mechanisms. Cryo-EM structural analyses serve greatly as the basis of a novel anticancer drug-design strategy. Using these structures, one can identify new active inhibitor molecules targeting the cancerous ribosome specifically. One approach for this can be, to search for derivatives of well-known ribosome inhibitors such as CHX, HHT, etc.

4.2 Human DNA-PK Holoenzyme

Human DNA-dependent protein kinase (DNA-PK) is a complex taking part in DNA repair by non-homologous end-joining (NHEJ) pathway. It is a part of the phosphoinositide-3-kinase-related kinase (PIKK) family. The complex is composed of DNA, KU70 and KU80 heterodimer, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). This complex initiates NHEJ when there is a Double-strand break (DSB) (Galli et al., 2019). Recently, the

cryo-EM structure of this complex has been determined at 6.6 Å resolution to expose its detailed structure and mechanism of activation. The structure has been deposited to EMDB with the number EMD-6803 (Yin et al., 2017). Figure 10 shows the cryo-EM structure of the DNA-PK complex where two of the complexes containing broken DNA ends are brought close together by NHEJ factors.

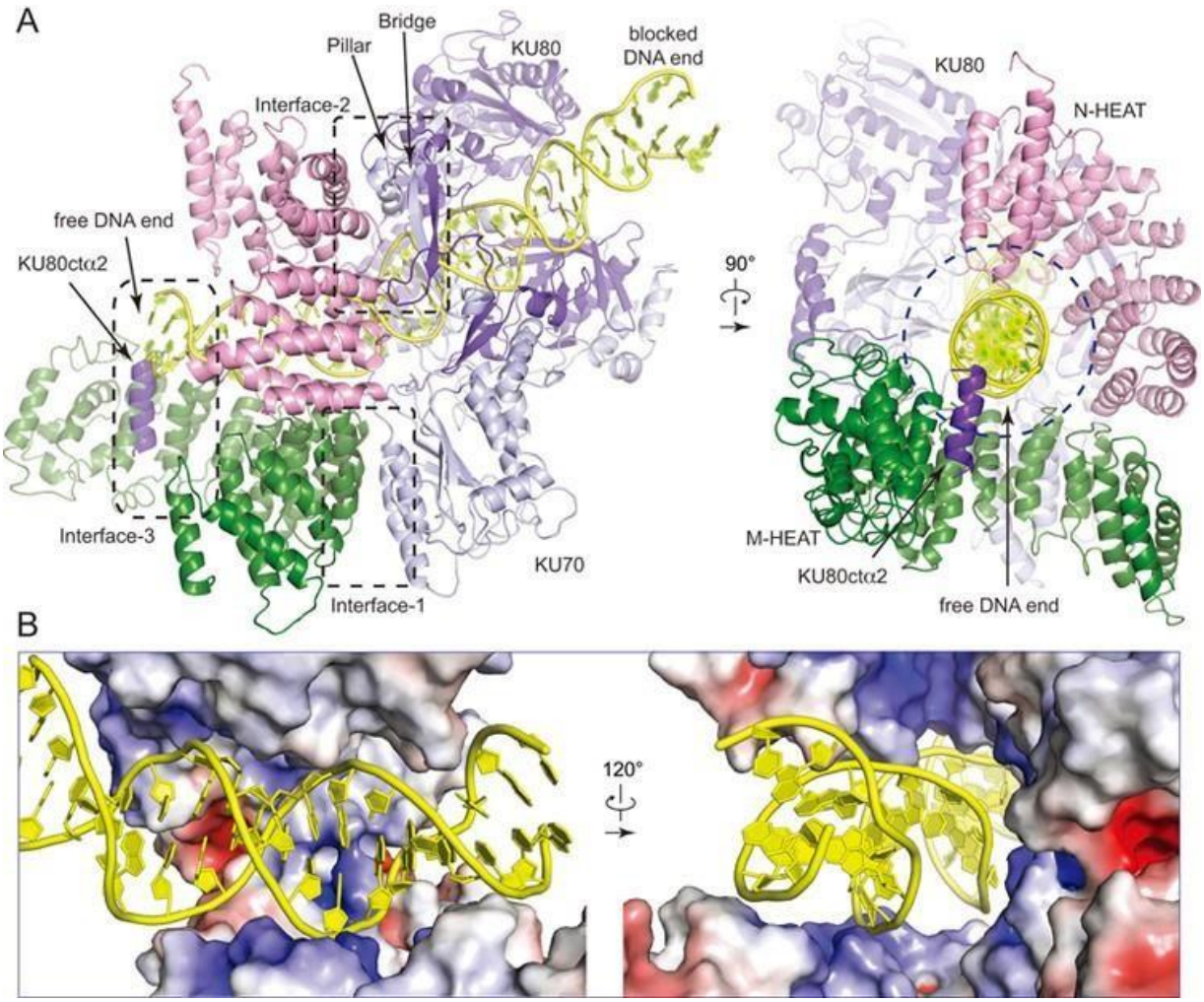


Figure 10: Cryo-EM Structure of DNA-PK Holoenzyme (Yin et al., 2017)

4.2.1 Significance of Human DNA-PK Holoenzyme Structure

Every organism protects its genome and repair damaged DNA. DSB is one of the major types of injury that can occur to DNA. DSB can be repaired using the DNA-PK complex via the NHEJ pathway (Davis et al., 2014). Besides DSB repairing, the DNA-PK complex also

functions in transcription, cell cycle progression, and telomerase maintenance (Galli et al., 2019). Hence, researchers are trying to utilize its relationship with DNA damage to develop new anti-tumor therapeutics targeting DNA-PK holoenzyme.

To develop a DNA-PK targeting molecule, understanding its role and function is the first and foremost necessity. DNA-PK holoenzyme is a complex of a variety of proteins including KU70/80 heterodimer, and DNA-PKcs, (Yin et al., 2017). DNA-PK holoenzyme takes part in DNA repair by both the NHEJ pathway and homologous recombination (HR) pathway. DNA repairs with HR are the most reliable pathway since it makes use of sister chromatids for DSB repairing. However, this occurs only at the cell cycle's S and G2 phases (Mohiuddin & Kang, 2019). The general mechanism of DSB repairing by NHEJ is initiated with recognition of the DSB by the Ku70 and KU80 heterodimer and gathering of the NHEJ factors at the site of DNA damage and stabilizing it (Figure 11). Consequently, it forms a bridge between the DNA ends and activates DNAPKcs kinase activity. Eventually, the broken DNA ends are ligated with the help of DNA ligase proteins namely XRCC4 and XLF. As a result, the DSB repair process is accomplished and the DNA-PK complex dissolves (Davis et al., 2014).

Inhibition of DNA-PK holoenzyme activity has an important role in cancer therapy. DNA-PKcs stimulates angiogenesis, and invasion and migration of melanoma primary tumor as well as take part in metastasis development. DNA-PK has a role in the pro-metastatic activity which is regulated by tumor modification. A direct link has been found between cancer metastasis and DNA damage repair (Kotula et al., 2015). Increased expression of DNA-PK holoenzyme activity has been seen in hepatocellular carcinoma. Blockade of DSB repair in patients with multiple myeloma results in genome instability and thus can be a therapeutic target for anticancer agents (Herrero et al., 2015). Therefore, the DNA-PK complex remains in the prime focus as a target for anti-tumor agents. DNA-PK blockers inhibit its function to downregulate phosphorylation of enzymes taking part in DNA repair, and thereby potentiates DNA damage.

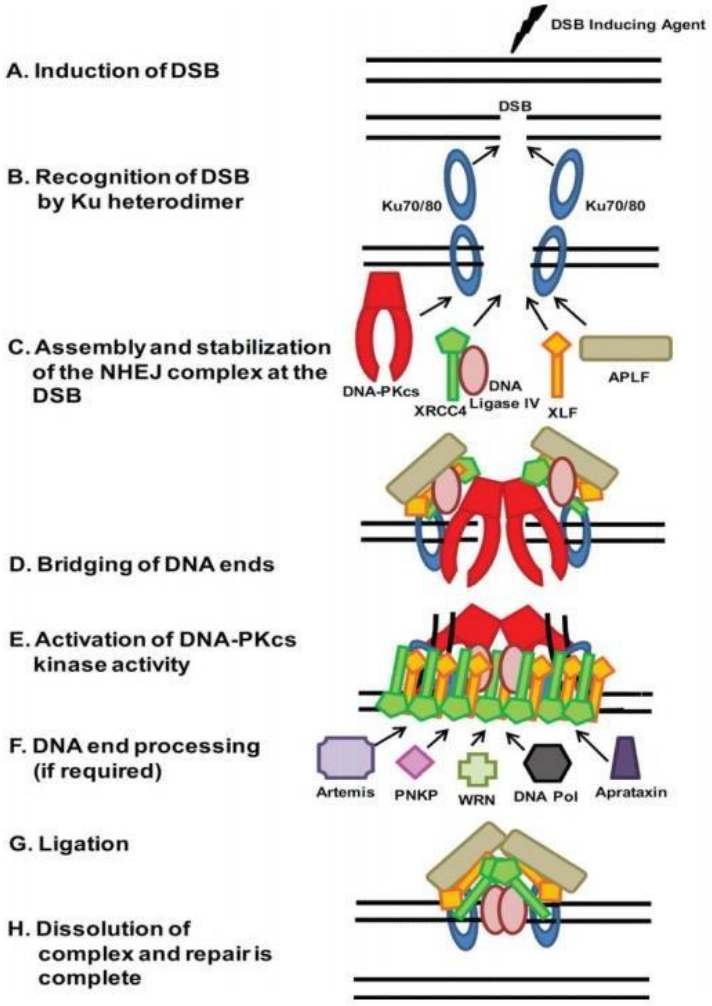


Figure 11: Mechanism of DSB repair by NHEJ pathway (Davis et al., 2014)

The cryo-EM study of DNA-PK holoenzyme (Figure 10) reveals the structural assembly of the complex and the mechanism of its activation. Moreover, the structure suggests the mechanism for how the broken DNA ends are recognized. The high-resolution cryo-EM data also provides the structural basis for designing future allosteric inhibitors targeting DNA-PKcs as promising anticancer drugs (Yin et al., 2017). In this way, cryo-EM anticancer drug development process during both hit identification and lead optimization. It contributes greatly to the novel inhibitor development that is more potent and more specifically targets DNA-PK holoenzyme.

4.3 Human p97

Human p97 is a mammalian protein, a hexameric AAA+ ATPase which is mutated in different neurodegenerative diseases as well as in cancer. Hexameric means that the protein is composed of six protomers. It performs various cellular activities including ubiquitin-proteasome system (UPS) mediated protein degradation, DNA replication, DNA repair, cell cycle regulation, NF-κB activation, mitochondria-associated degradation (MAD), endoplasmic reticulum-associated degradation (ERAD), Golgi formation, and autophagy (Stach & Freemont, 2017). The p97 structure has been identified at 2.3Å resolution using cryo-EM (Figure. 12).

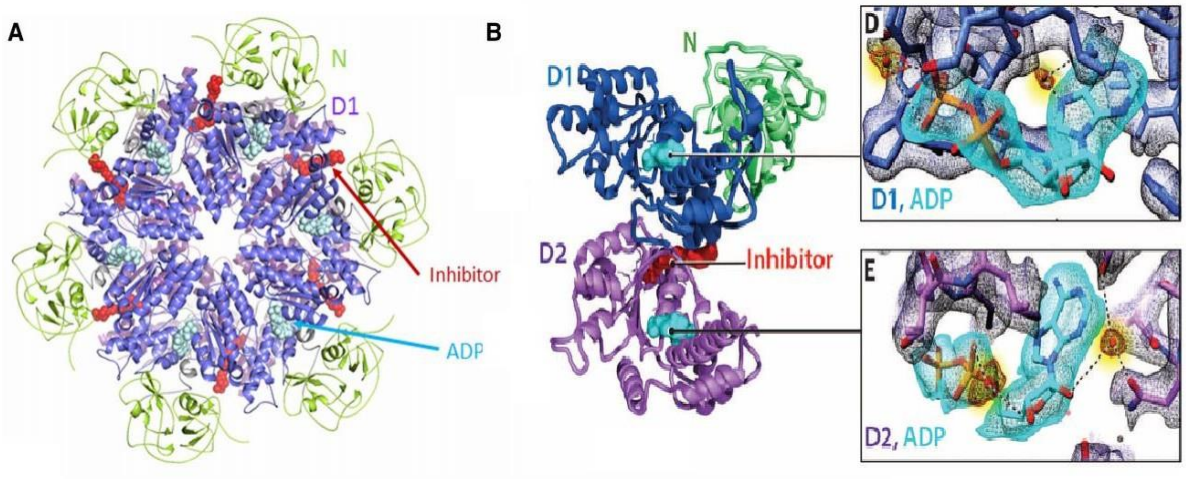


Figure 12: Cryo-EM structure of human p97 (Banerjee et al., 2016; Ceska et al., 2019)

Figure. 12, (A) demonstrates the cryo-EM structure of p97 bound to inhibitor and ADP (Adenosine Diphosphate). (B) demonstrates a single protomer of p97 with a closer view of ADP binding site in the domains D1 and D2 which alters the protein function (Banerjee et al., 2016; Ceska et al., 2019). The mutation occurs at the borderline between the D1 and D2 domains. The binding affinity of ADP to p97 is also altered in the case of mutation (Macario et al., 2016).

4.3.1 Significance of Human p97 Structure

The human p97 has an important function in cellular homeostasis, protein quality control UPS mediated protein degradation. Moreover, it regulates the liberation of sequestered proteins including transcription factors leading to functional proteins release (Huryn et al., 2020). It has been proven to regulate cellular metabolism (Parzych et al., 2019). The human p97 recognizes and translocates ubiquitinated proteins to relax cellular stress via ERAD, extracts proteins that are misfolded via MAD, and regulates degradation via autophagy and lysosomal systems, etc. (Figure 13) (Huryn et al., 2020).

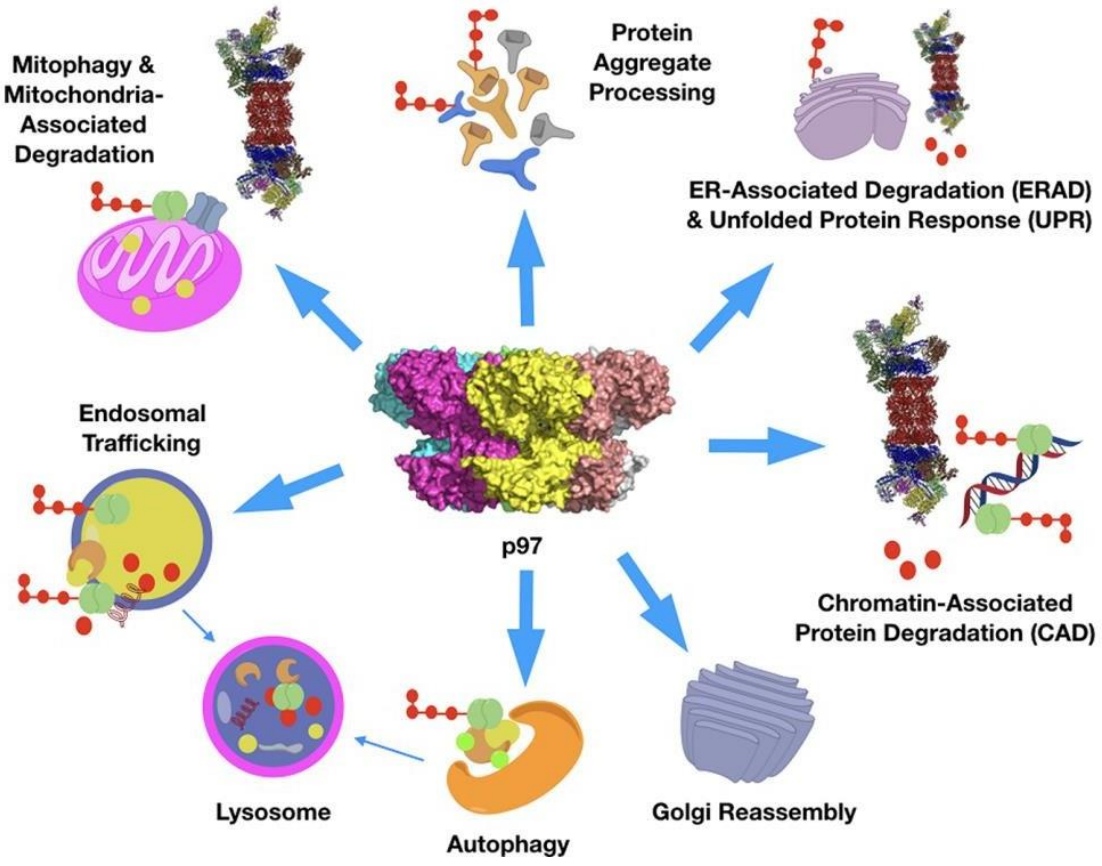


Figure 13: Various functions of the p97 in mammalian cell (Huryn et al., 2020)

However, the exact mechanism by which these functions are performed was still unknown. Therefore, the structural features of p97, its multiple functions, and the mechanism to perform these functions remain the key areas of investigation.

In cancer, Cellular dependency of p97 increases due to glutamine and glucose limitation to sustain proteostasis. The p97 is overexpressed due to glutamine depletion (Parzych et al., 2019). Tumorigenesis is associated with elevated stress which is managed by the increasing function of the p97. Several cancers are associated with mutation of the p97 and its upregulated including melanoma and breast carcinomas. The protein mutation may occur in cancer cells due to aneuploidy, an overload of protein degradation, and increasing dependability of cancer cells on wild-type p97 (Huryn et al., 2020).

The p97 has been a promising target for an anticancer drug to induce apoptosis in different types of cancer cells and solid tumors. Also, the investigation of p97 blockers shows to have effective in treating multiple myeloma (Rycenga et al., 2019). Inhibition of the p97 results in proteotoxic crisis, thereby apoptosis in tumor cells leaving the normal cells safe (Huryn et al., 2020). The p97 inhibition alters amino-acid turnover and metabolic processes. An amino acid-sensing kinase, GCN2 regulating stress signaling, and cell death are triggered by p97 inhibitors. It also modulates nutrient shortages, glycolytic metabolite turnover, and autophagy (Parzych et al., 2019).

CB5083 is an appealing anticancer drug targeting the p97 at the D2 domain (Anderson et al., 2016). However, it is showing an unwanted off-target effect. To improve its potency and site-selective activity, understanding its binding interaction is vital (Tang et al., 2019). Other recently found p97 inhibitors include NMS-873, MSC1094308, and UPCDC30245 producing allosteric inhibition at D1-D2 interface, 2-Aminopyridine indole amide producing allosteric inhibition at D2 domain, Withaferin A 27-Acetate and NMS-859 producing covalent inhibition at D2 ATP site, and Oxaspirol B and Clotrimazole having an unknown mechanism of action (Huryn et al., 2020). To improve selectivity and develop more potent and effective p97 blockers, understanding of p97's structural details at an atomic level and its binding mechanism to inhibitor are crucial.

The cryo-EM structure of human p97 reveals its structural details along with the inhibitor binding mode. Each protomer of the hexamer contains AAA+ ATPase cassettes. It is composed of an N-terminal domain (N domain) and two ATPase domains (D1 and D2) (Vekaria et al., 2016). The structure shows an inhibitor binding site located at the D1 and D2 domains interface (Figure 12). The mechanism of its inhibition involves the prevention of conformational change propagation which is necessary for p97 functioning (Banerjee et al., 2016). This high-resolution structure provides details for specific intermolecular hydrogen bonds and van-der-Waals interactions found between the inhibitor and p97 with accuracy (Boland et al., 2017). This structure helps to optimize the potency and specificity of the inhibitor, thereby facilitates hit and lead optimization of the anticancer drug discovery process.

4.4 Human STEAP

The human six-transmembrane epithelial antigen of the prostate (STEAP) family is a type of membrane antigen that functions in iron reduction and uptake, copper uptake, apoptosis, inflammation, and oxidative stress response. They work as a prostate tumor marker and are highly over-expressed in such cancer. STEAP1-STEAP4 are the members of the STEAP family (Burnell Id et al., 2019).

Among them, STEAP1 and STEAP4 have been analyzed under cryo-EM recently. Figure 15 shows the cryo-EM mediated structural analysis outcome of STEAP1 and STEAP4 (Oosterheert et al., 2018; Oosterheert & Gros, 2020). STEAP1 when fused to the STEAP4 NADPH-binding domain, it promotes iron reduction. STEAP1 itself lacks the NADPH-binding domain (Oosterheert & Gros, 2020). A study suggested that mAb 120.545 has a binding affinity for STEAP1 (Challita-Eid et al., 2007). Therefore, this complex of binding interaction was reviewed under cryo-EM at 3Å resolution (Figure 14 (A)) (Oosterheert & Gros, 2020).

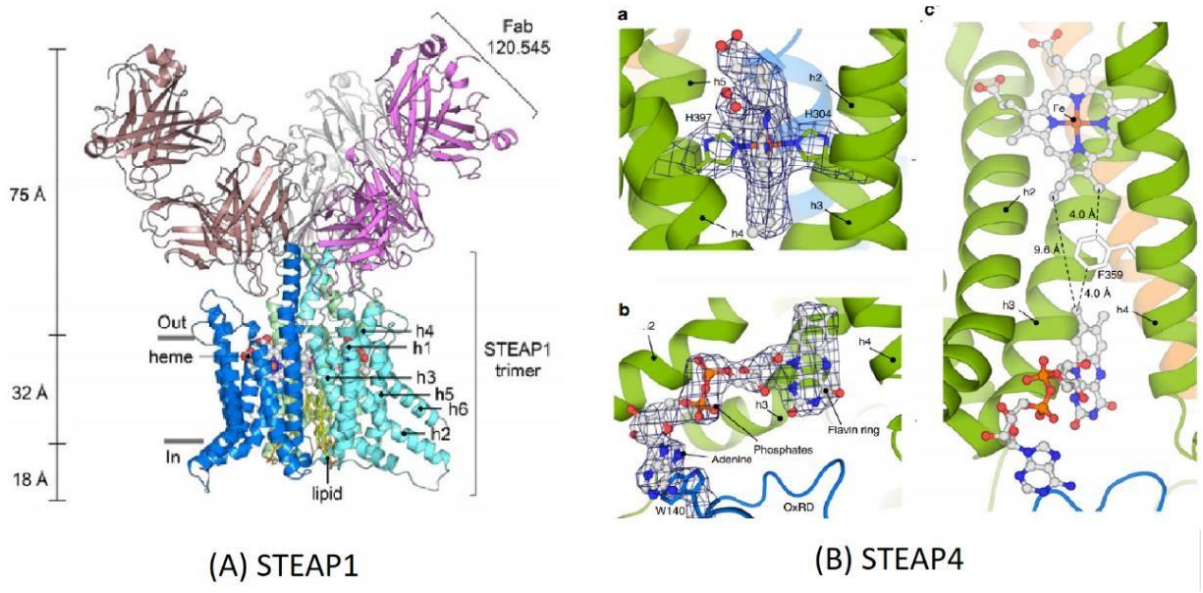


Figure 14: Cryo-EM structure of STEAP1 and STEAP4 (Oosterheert et al., 2018; Oosterheert & Gros, 2020)

On the other hand, STEAT4 has a trimeric structure containing NADPH-FAD-heme arrangement to which chelated iron binds. Figure 14 (B) shows the cryo-EM structure of STEAP4 at 3.1 Å resolution in which (a) demonstrates heme-binding pocket, (b) demonstrates FAD-binding site, and (c) shows the FAD-heme-STEAP4 complex (Oosterheert et al., 2018).

4.4.1 Significance of Human STEAP Structure

STEAP1, STEAP2, STEAP3, and STEAP4 are the members of the STEAP family, important for metal metabolism. It is a potential biomarker for cancer since STEAP1 is overexpressed in prostate cancer as well as in other cancers including lung, colon, breast, and bladder cancers (Elm et al., 2017). In breast cancer, STEAP1 and STEAP4 is a potential prognostic biomarker since it is found that low expression of this protein results in high overall mortality, whereas high expression shows a good prognosis (Wu et al., 2020).

The structure of STEAP is composed of a C-terminal, an N-terminal, and a 6-transmembrane domain. STEAP 1 and 2 genes are most abundant in the plasma membrane of both normal and malignant prostates. In contrast, STEAP 3 and 4 are mostly located in hematopoietic tissue and

adipose tissue respectively. Both STEAP 3 and 4 are found in bone marrow, skeletal muscle, placenta, and heart (Gomes et al., 2012).

STEAP1 is an antigen that acts as a transport protein or channel to promote intracellular communication in tumor cells (Elm et al., 2017). The channel has the potential to facilitate the invasion and proliferation of cancer cells by controlling Na^+, K^+, Ca^{2+} ions, and small molecular concentrations (Figure 15) (Gomes et al., 2012).

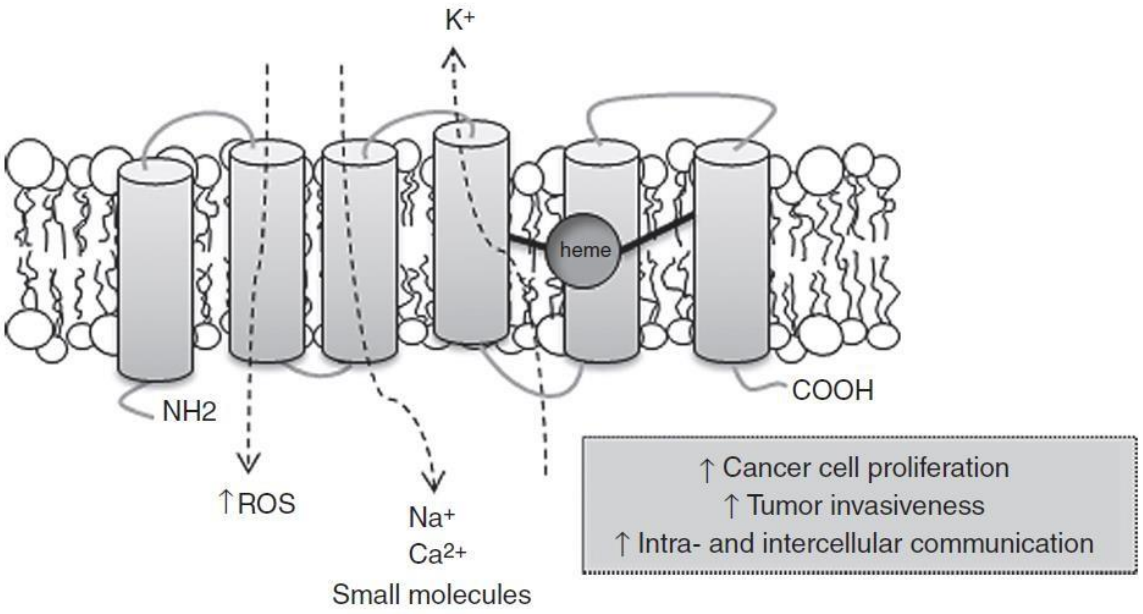


Figure 15: Role of STEAP1 in cancer progression (Gomes et al., 2012)

High levels of Na^+ are related to the invasiveness of prostate cancer cells. Besides, the regulation of K^+ and Ca^{2+} levels are related to apoptosis prevention, leading to prostate tumor progression. Furthermore, STEAP1 raises the levels of reactive oxygen species (ROS) to facilitate cell growth (Gomes et al., 2012)

STEAP4 is a metalloreductase having a role in copper and iron metal uptake as well as tumor and inflammation progression. Inflamed and malignant cells have higher levels of copper driven by STEAP4-depended IL-17 leading to E3-ligase, XIAP, and NFκB activation as well as suppression of caspase 3 activity. As a result, tumorigenesis is promoted by an inflammatory

response induced by copper uptake (Y. Liao et al., 2020). It is up-regulated in tumor cells to maintain iron homeostasis. STEAR4 helps in iron reduction from ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion since cellular iron uptake occurs only with Fe^{2+} (Oosterheert et al., 2018). The role of STEAP4 in mammalian cells is summarized in Figure 16.

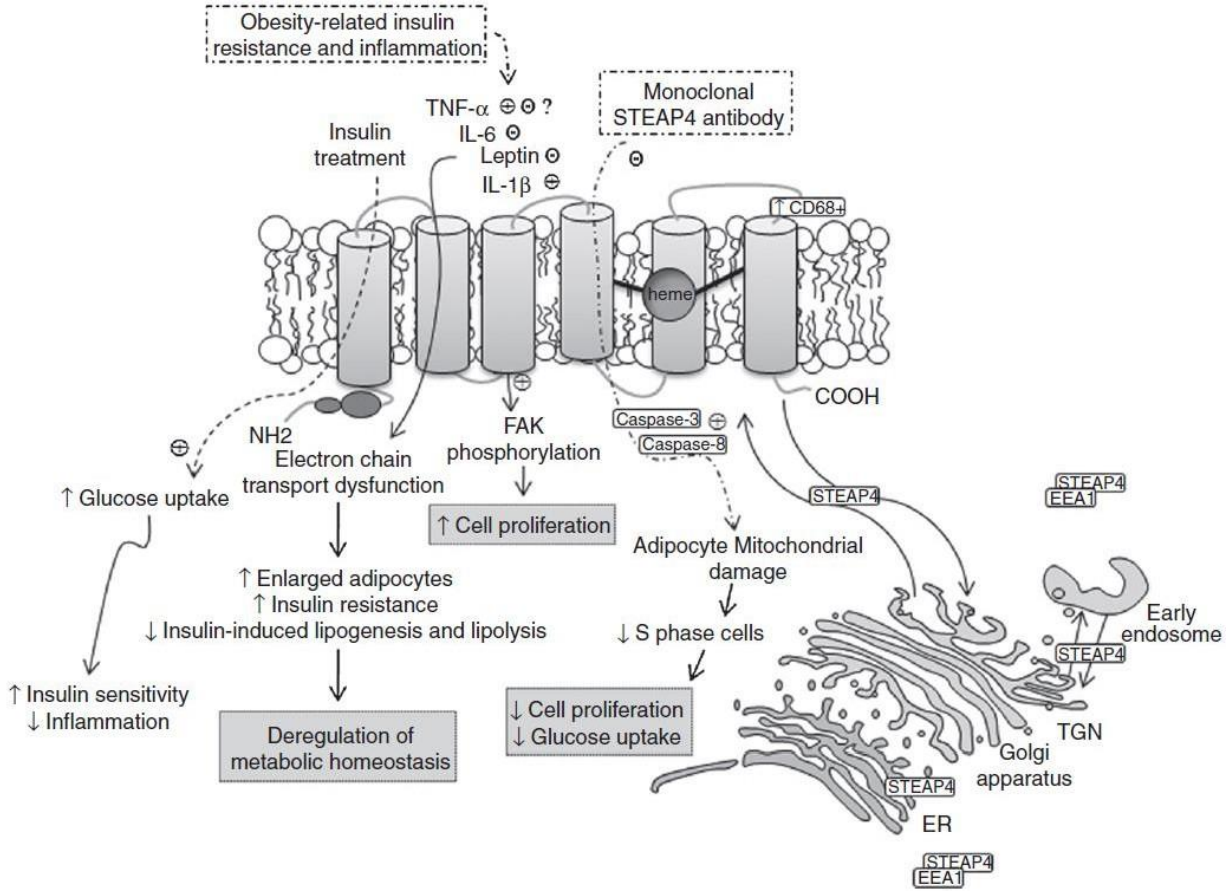


Figure 16: Role of STEAP4 in mammalian cell (Gomes et al., 2012)

STEAP was thought to be a transport channel before its cryo-EM structural analysis was performed. The cryo-EM structure of the trimeric STEAP1 fused to STEAP4 NADPH-binding domain (Figure 14A) reveals its reductase conformation and the mechanism by which STEAP1 can promote iron reduction. The high-resolution structure shows the binding mode of mAb120.545, an inhibitor. It reveals that mAb120.545 has a binding affinity specifically for STEAP1 and not for STEAP4. The inhibitor Fab can recognize ECL1 and ECL2 of the STEAP1 epitope and three Fabs can bind to this trimer. So, the ferric reductase activity is

inhibited only when STEAP1 is fused to STEAP4 and STEAP4 activity in a non-fused form remains unaltered. The structure also confirmed the ability of STEAP1 to conduct transmembrane electron transport (Oosterheert & Gros, 2020).

The cryo-EM structure of STEAP4 (Figure 14B) reveals the binding mode of FAD, NADPH, and heme to STEAP4. It also reveals the mechanism by which STEAP4 performs its iron reduction activity. In the STEAP4 – NADPH – FAD complex, FAD receives H^- ion from NADPH to form $FADH_2$. $FADH_2$ then loses electrons to heme and heme releases the electron to a Cu^{2+} or Fe^{3+} ion resulting in metal ion reduction and uptake into the cell (Oosterheert et al., 2018).

Cancer cell proliferation is linked to overexpression of STEAP and iron overload. The inhibition of iron reduction can prevent iron overload in cancer tissues. Thus, STEAP becomes an appealing target for an anticancer drug. The molecular principles concerning the function of STEAP are important to understand for designing novel anti-STEAP drugs. Hit identification and lead optimization for designing anti-STEAP drugs are possible using cryo-EM structures of STEAP protein.

4.5 Human ACLY

Human ATP-citrate lyase is an enzyme essential to catalyze the conversion of citrate to oxalate and coenzyme A to acetyl-CoA in presence of ATP. Inhibition of ACLY leads to the limiting of tumor cell proliferation as well as a reduction in tumor growth (Hatzivassiliou et al., 2005). The structure of ACLY at the atomic level was unknown. Thus, no precise inhibitor molecule could be developed. However, in 2019 a study regarding cryo-EM structural analysis of ACLY was published. This study not only revealed ACLY structure but also proposed potent inhibitor molecules. NDI-091143 is one of the suggested inhibitor molecules that showed potent binding affinity to ACLY under cryo-EM (Wei et al., 2019).

Figure 17 shows the cryo-EM structure of the ACLY- NDI-091143 complex. (a) and (b) show the ACLY tetramer complexed with ADP, acetyl CoA, and citrate. The inhibitor NDI-091143 is shown with black spheres. (c) represents only a protomer of the ACLY tetramer.

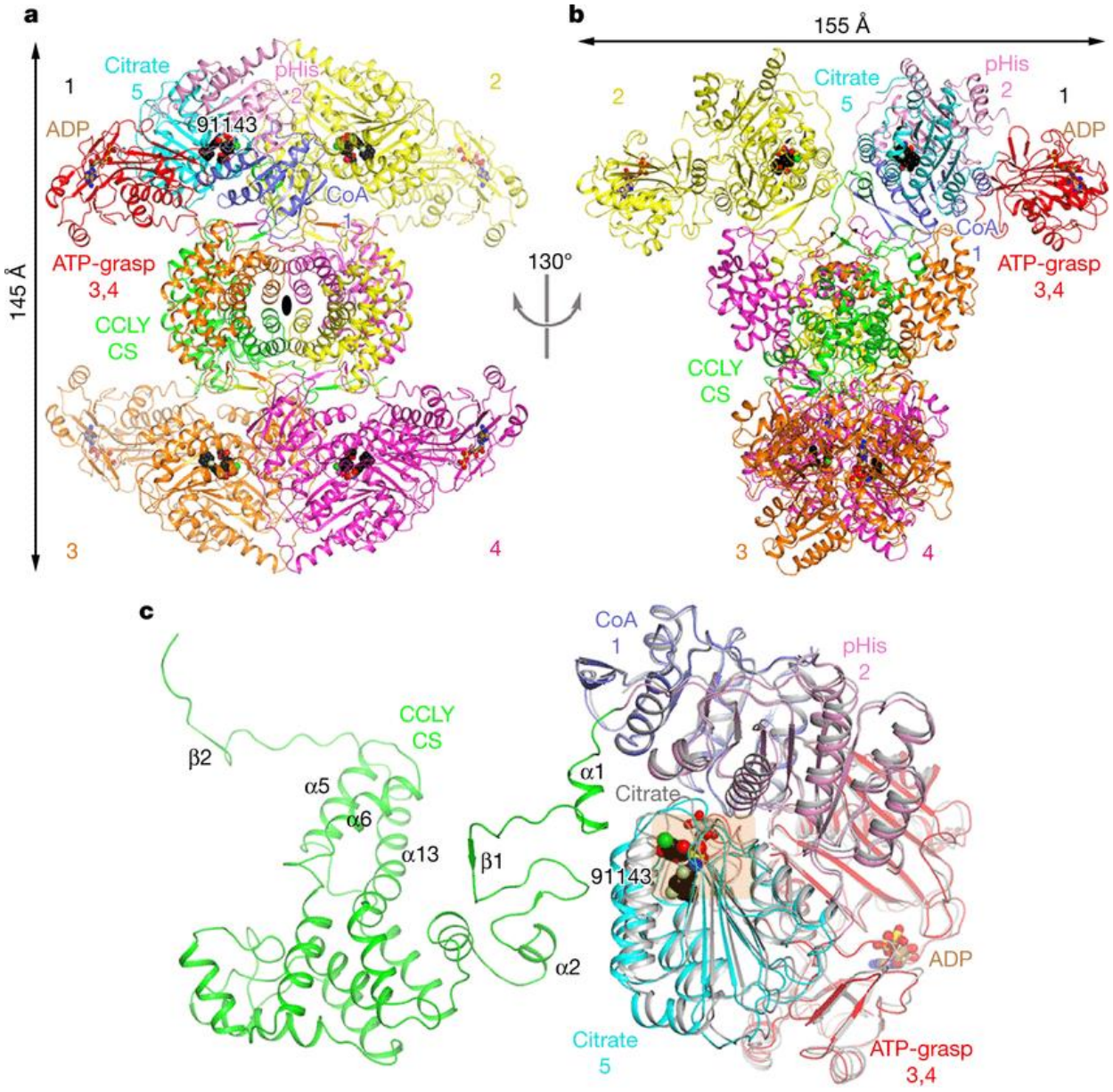


Figure 17: Cryo-EM structure of ACLY- NDI-091143 complex (Wei et al., 2019)

4.5.1 Significance of Human ACLY Structure

Human ACLY is an enzyme, overexpressed in several types of cancers including breast, colorectal, glioblastoma, and lung cancer. It catalyzes the formation of acetyl CoA from citrate

(Zaidi et al., 2012). Acetyl CoA is involved in protein modification, glucose metabolism, histone acetylation, and biosynthesis of cholesterol and fatty acid. ACLY has a role in the tumorigenesis of a cancer cell. (Khwairakpam et al., 2015).

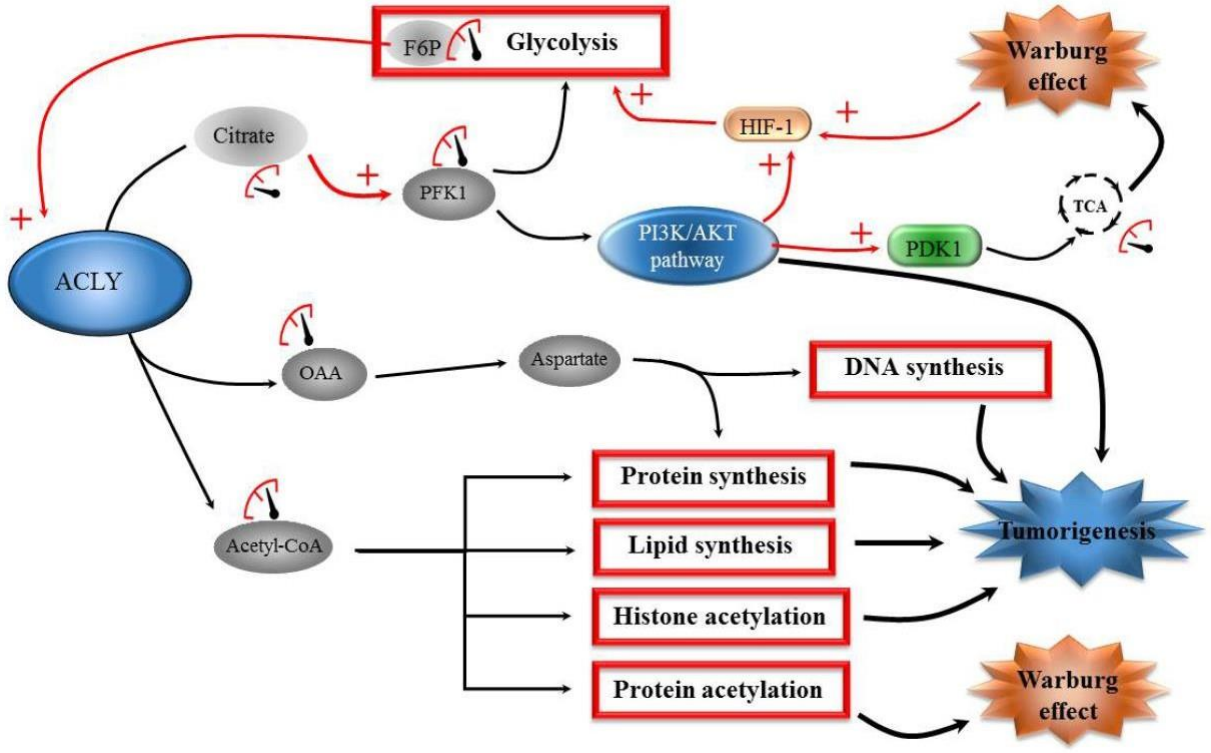


Figure 18: Role of ACLY in tumorigenesis (Icard et al., 2019)

ACLY plays a significant role in cancer cell proliferation and progression (Figure 18) via promoting lipid biogenesis. Cancer cells have a high rate of proliferation which demands an increased level of energy. As a result, ACLY is up-regulated to meet the increased enzyme requirement for increased metabolic activity (Zaidi et al., 2012). The ACLY up-regulation also decreases the citrate level in cytosol leading to the promotion of glycolysis. Lactate-mediated aerobic glycolysis results in the Warburg effect, a cancer marker (Icard et al., 2019).

Chemical inhibitors can be used to knock down the ACLY gene and downregulate the expression of ACLY to inhibit cancer cell growth. ACLY inhibition inhibits cancer cell metabolism which in turn inhibits tumor growth (Khwairakpam et al., 2015). Moreover,

research shows that inhibition of ACLY blocks triglyceride chain elongation along with C16 to C18. Thereby, fatty acid elongation in the endoplasmic reticulum and fatty acid oxidation in mitochondria are affected (Migita et al., 2014). Therefore, ACLY is an attractive target for developing an anticancer drug.

The cryo-EM structure of ACLY (Figure 17) demonstrates the detailed structure of ACLY tetramer bound to its inhibitor NDI-091143. The structure reveals a surprising mechanism of inhibition of the ACLY-blocker. Many ACLY-blockers have been found to date. However, most of them show weak activity. To develop newer potent ACLY inhibitors, an understanding of their binding mechanism is important. The cryo-EM structural analysis reveals an allosteric binding site for ACLY inhibitor for potent inhibition, which is located at a hydrophobic cavity near the citrate-binding site. The allosteric site changes its conformation upon binding to the inhibitor leading to disrupt binding of citrate indirectly. Structure-activity relationship analysis confirms the observed binding mode of these compounds (Wei et al., 2019). This allosteric site proposes an appealing target for developing new potent ACLY inhibitors. Thereby, anti-cancer drug development is facilitated by the cryo-EM structure of ACLY. The discovery of the allosteric binding site of ACLY facilitated the target identification stage of the anticancer drug development process.

Chapter 5

Cryo-EM Structures of Drug Bound Complexes in Cancer Research

It has been proven that the visualization of different human genes, proteins, and enzymes is possible using cryo-EM. Nevertheless, structural features of compounds having chemotherapeutic activity, drug-ligand interactions, and binding complexes are also visible using cryo-EM. Their significance in cancer research and how these research data contribute to the development of anticancer drugs will be discussed in this chapter.

5.1 HER2-Trastuzumab-Pertuzumab Complex

Trastuzumab and Pertuzumab are monoclonal antibodies targeting human epidermal growth factor receptors 2 (HER2) which is upregulated in breast cancer. The HER2 is composed of four subdomains (Montemurro et al., 2013). However, the binding sites for trastuzumab (on subdomain IV) and pertuzumab (on subdomain II) are not on the same subdomain. They bind to different subdomains of HER2. The cryo-EM structure of the HER2-trastuzumab-pertuzumab complex has been determined at resolution 4.36 Å as shown in Figure 19. This structure has been submitted in EMDB having the access number EMD-7137 (Hao et al., 2019).

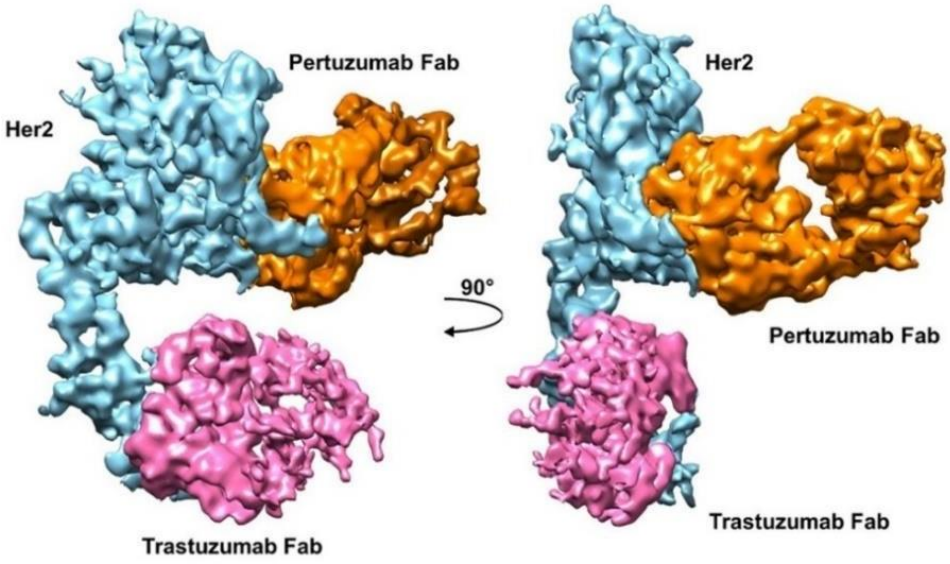


Figure 19: HER2-Trastuzumab-Pertuzumab Complex (Hao et al., 2019)

5.1.1 Contribution of HER2-Trastuzumab-Pertuzumab Complex Structure

HER2 is a protein having tyrosine kinase activity that helps the growth of cancer cells (Iqbal & Iqbal, 2014). In some breast cancer patients, the HER2 gene is over-expressed promoting the production of the HER2 protein. This type of cancer is called HER2-positive breast cancer. These tumor cells spread and grow faster than HER2-negative breast cancers (Mitri et al., 2012). Patients with HER2-negative breast cancer do not respond to HER2-targeted therapeutics, whereas HER2-positive breast cancers can be treated with HER2 targeting drugs like trastuzumab and pertuzumab. HER2 blockers are also indicated for gastric cancer. HER2 over-expression is also evident in other types of cancers including ovary, bladder, lung, colon, etc. (Iqbal & Iqbal, 2014). The HER family consists of four member domains, HER1, HER2, HER3, and HER4. HER2 undergoes dimerization with the other domains to serve as a ligand-binding active site since, HER2 itself does not have any identifiable ligand (Mitri et al., 2012).

Trastuzumab is a HER2 blocker, a monoclonal antibody that targets the extracellular domain (IV) on the HER2 receptor (Hao et al., 2019). Interaction of trastuzumab to HER2 receptor leads to inhibition of domain cleavage and its dimerization, resulting in antibody-dependent cell-mediated cytotoxicity (ADCC) and cell-proliferation reduction leading to tumor-cell lysis (Shuch, Brian; Linehan, B. W. M.L.; Srivasan, 2012). Pertuzumab is another monoclonal antibody that targets domain (II) of HER2. It hinders HER2 dimerization, blocking both heterodimerization and homodimerization leading to a complete HER2-signaling inhibition (Hao et al., 2019).

Even though trastuzumab and pertuzumab bind on distinct sites of the HER2 receptor, they produce a synergistic effect when used in combination (Richard et al., 2016). The combination of trastuzumab, pertuzumab, and docetaxel in patients with HER2-positive breast cancer has been approved by the FDA as first-line therapy (Kawajiri et al., 2014). The mechanism of their synergism was not clearly known. The cryo-EM study of the HER2-Trastuzumab-Pertuzumab

complex was performed to understand this mechanism to design novel HER2-blockers. The hypothesis suggested that the synergy is due to improved binding affinity resulting from the interaction between the two mAbs. The cryo-EM structure demonstrates the binding of pertuzumab and trastuzumab to domains (II) and (IV) of the HER2 receptor respectively.

Cryo-EM structure of HER2-trastuzumab-pertuzumab complex reveals that pertuzumab and trastuzumab both can simultaneously bind to the HER2 receptor with slight conformational change. Thus, the interaction between the two antibodies does not enhance their binding affinity towards HER2 (Hao et al., 2019). This suggests that the researchers can take advantage of the cryo-EM structure complex to build a novel bispecific antibody design targeting both domains. In this case, cryo-EM data helps to facilitate both hit identification and lead optimization stages of the drug development process.

To design new bispecific molecules targeting both domains different measures have been taken. It can be done through various combinations of engineered light and heavy chains of both trastuzumab and pertuzumab variable regions linked to their constant regions. So that, they recognize the same epitopes as pertuzumab and trastuzumab (Hao et al., 2019). After designing such molecules, they can be evaluated under cryo-EM for their binding affinity. Examples of such engineered bispecific molecules targeting the HER2 receptor are shown in Figure 20. The development of such HER2 blockers is necessary to produce a better anti-tumor effect and higher binding affinity than the combination of these drugs, which has been made possible using cryo-EM.

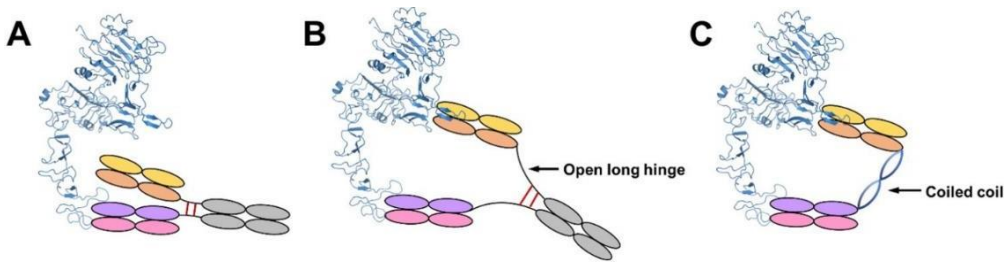


Figure 20: Bispecific HER2 blocker drug design (Hao et al., 2019)

5.2 Antibody-CD20 Binding Complex

The protein CD20 (Cluster of Differentiation 20) is a human antigen found on B cell, characterized as a tumor marker. It is over-expressed in certain types of cancer including leukemia and B-cell lymphomas (*Definition of CD20 Antigen - NCI Dictionary of Cancer Terms - National Cancer Institute,* n.d.). CD20 involves in cell growth and cell differentiation. Anti-CD20 is a type of mAb (Monoclonal antibody) that is used as a therapeutic agent for B-cell malignancy. Different mAb (type I, type II) acts in a different phase of cell cycle progression (Middleton et al., 2016). Structural analysis of the complex of CD20 with mAbs Rituximab (RTX), Ofatumumab (OFA), and Obinutuzumab (OBZ) has been done using cryo-EM at resolution 3.3 Å.

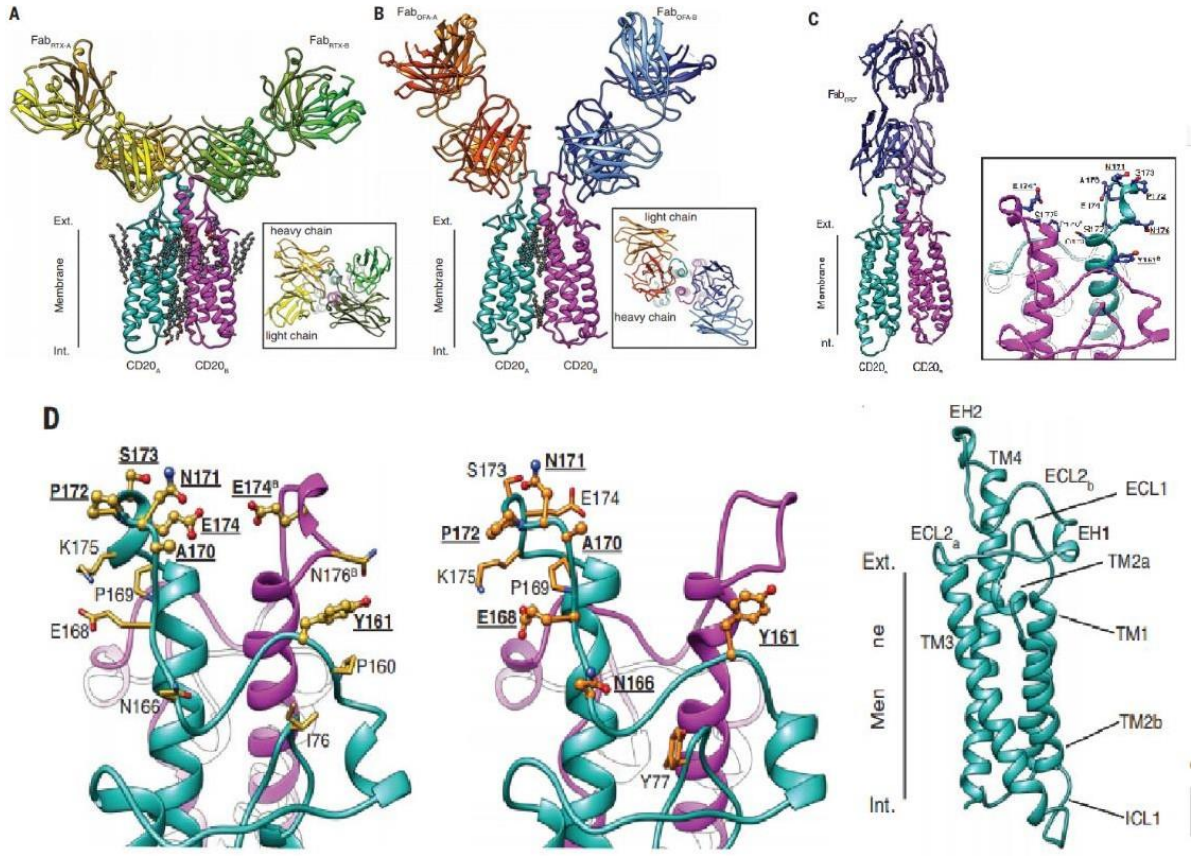


Figure 21: Cryo-EM structure of CD20-Antibody complexes (A. Kumar et al., 2020)

In Figure 21, A, B, and C represents complexes of CD20 bound to RTX, OFA, and OBZ respectively. D shows the major amino acid residues to which the mAbs bind along with their membrane views. CD20 is a dimer composed of $CD20_A$ and $CD20_B$. RTX binds to both dimer subunits while OFA recognizes only one epitope. Both of them are type I mAb. On the other hand, OBZ is a type II mAb. These structures reveal the interacting proteins to which each of the mAb binds. OFA mainly interacts with amino acids on ECL2 (Extracellular loop 2) and RTX interacts with residue on EH2 (Extracellular helices). OBZ binds to the tip of the CD20, mostly to the $CD20_A$ subunit, and also a little interaction with $CD20_B$ is found (A. Kumar et al., 2020).

5.2.1 Significance of Antibody-CD20 Binding Complex Structure

CD20 human antigen is found on B cell membrane and is a target for many mAbs for treating B cell lymphomas and autoimmune disease (Rougé et al., 2020). The CD20 expression varies in different types of B-cell malignancies and different patients having the same malignancy. Anti-CD20 drugs can be used against CLL, diffuse large B-cell lymphomas, and follicular lymphomas. Rituximab is the first developed anti-CD20 drug that was approved by the FDA in 1997. Obinutuzumab and Ofatumumab were engineered based on the action of RTX (Pavlasova & Mraz, 2020). Different anti-CD20 depletes B cells by acting via mechanisms including complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis, antibody-dependent cellular cytotoxicity, and direct cell death (Rougé et al., 2020).

RTX is therapeutically useful in CD20-positive NHL. It is composed of variable regions having light and heavy chains of human CD20 immunoglobulin fused to constant regions of kappa light-chain and IgG1 heavy-chain (Smith, 2003). RTX binds to the Fc region of CD20 located on immune cells including macrophage and natural killer cells (Tobinai et al., 2017). The binding initiates a series of events including induction of apoptosis via caspase 9 and caspase 3 pathways, antibody-dependent cellular cytotoxicity, and complement lysis. However, it has

the potential for producing resistance, and not all NHL patients respond in a similar manner (Smith, 2003). To improve patient response and reduce the resistance potential of mAbs, a proper understanding of the RTX-CD20 complex and its mechanism is important.

OFA is also used to treat CLL and NHL. Like RTX, OFA is also a type I antibody (A. Kumar et al., 2020). OFA binds to a novel membrane-proximal antigen epitope located closer to the CD20 N-terminal compared to RTX, resulting in more precise binding. Moreover, it dissociates at a slower rate than RTX from the target. Therefore, OFA is more potent and effective at killing cancerous cells at the same concentration (Zhang, 2009).

OBZ is a type II anti-CD20 mAb, whose action involves a different MOA (A. Kumar et al., 2020). Due to potential resistance growth in type, I anti-CD20 mAbs, the development of type II mAb was essential, which has enhanced binding affinity towards the epitope produced by glycoengineering Fc region. The mechanisms of apoptosis evoked by type II anti-CD20 mAbs are still poorly known and have been actively investigated (Tobinai et al., 2017).

CD20 plays an important role in B-cell differentiation leading to its activation whose exact function and structure are poorly understood (Smith, 2003). CD20 is a non-glycosylated protein located on the B lymphocyte cell surface of both normal and malignant forms. Three isoforms of CD20 have been identified which results from different phosphorylation. This phosphorylation is higher in malignant B cells that are actively proliferating compared to normal B cells (Pavlasova & Mraz, 2020).

The cryo-EM structure of the CD20 complex (Figure 21) reveals a distinct form of protein fold of CD20 that was previously unknown. It also reveals the assembly of CD20 to be found as a compact double-barrel dimer, which was previously viewed as a tetramer. The hydrophobic and van der Waals interactions within the protein are now clearly understood (Rougé et al.,

2020). The data shows that two RTX binds to each CD20 antigen, while only one OBZ Fab binds to it (Figure 22).

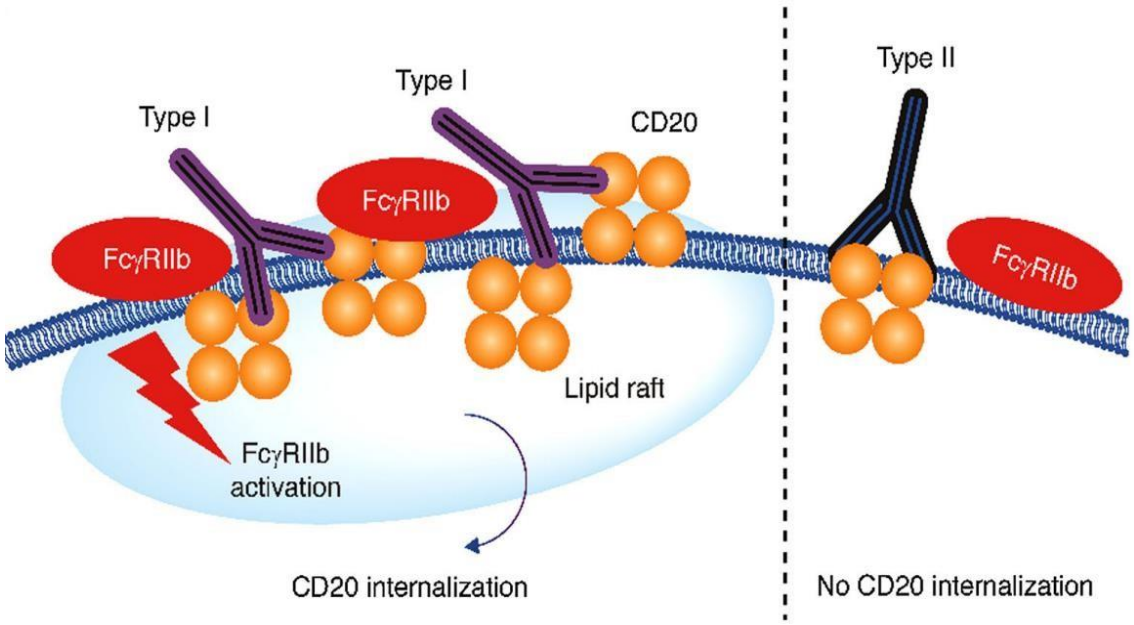


Figure 22: Binding difference between type I and type II anti-CD20 mAbs (Tobinai et al., 2017)

Type I mAb acts to increase local mAb concentration resulting in complement activation, type II forms a terminal complex and thereby prevents recruiting complement components and additional mAbs. OFA complex shows optimal geometry for recruiting complement components (Rougé et al., 2020). OFA and RTX bind to the overlapping epitopes on the extracellular surface of CD20, mostly through amino acid interactions with the heavy-chain regions of the complementary determinant. OFA arranges its antigen-binding fragments closer to one another than RTX. In contrast, OBZ binds the constant domain to the membrane plane forming a wide binding pocket involving both $CD20_A$ and $CD20_B$ monomers of the same antigen (A. Kumar et al., 2020).

The result of the cryo-EM data concludes that mAb potency is affected by its binding stoichiometry to the CD20 epitope and recruited complement. The molecular mechanisms found using cryo-EM can be used to facilitate the designing of new generation, more potent

anti-CD20 mAbs with reduced potential to develop resistance that shall help the patients suffering from B cell malignancies. Therefore, cryo-EM is a useful tool to facilitate the anticancer drug development process.

5.3 Microtubule-Antimitotic Drug Complex

The microtubule (MT) is a major element of the cytoskeleton. It takes part in cellular mitosis and consists of two polypeptide subunits α- and β-tubulin assembled in a cylindrical shape. Each forms a strand called protofilament (PF) (Flynn & Bradke, 2020). The high-resolution structure of MT at an atomic level has been determined using cryo-EM. It could identify the structural characteristics of MT in complexes with the cellular element, factors, and small ligands (Nogales & Kellogg, 2017).

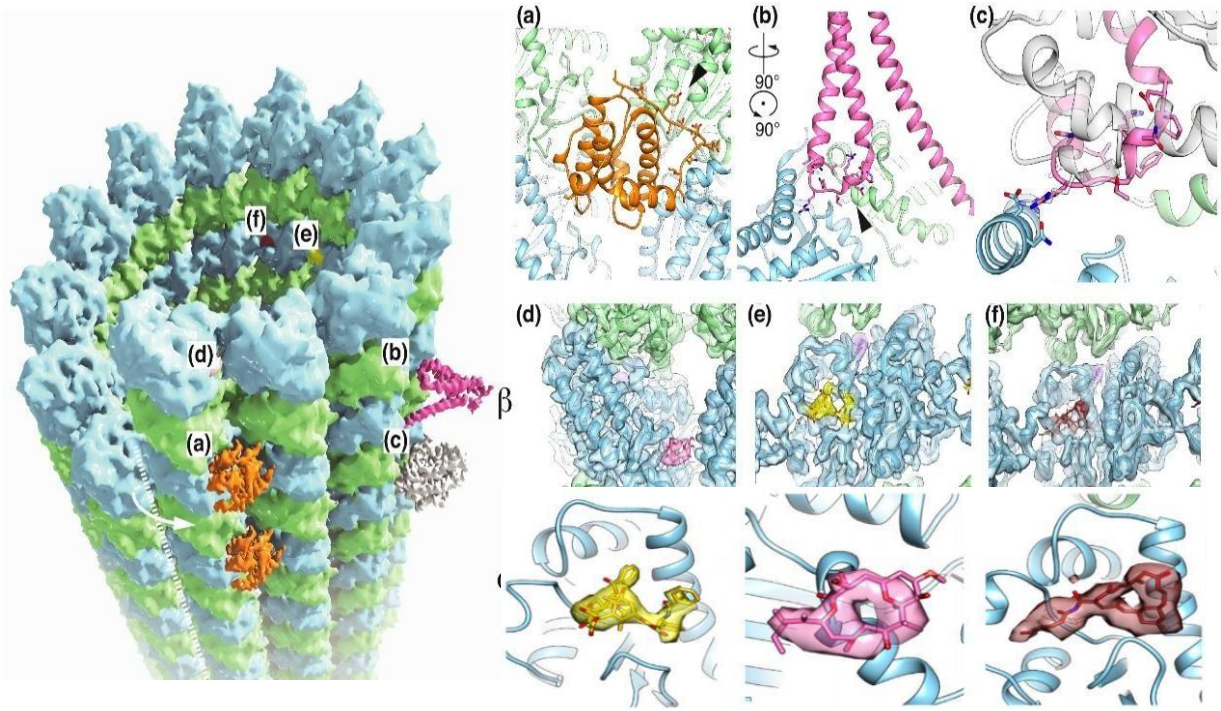


Figure 23: Cryo-EM Structure of Drug Bound Microtubule (Nogales & Kellogg, 2017)

In Figure 23, (a) represents the MT-PRC1 complex. PRC1 (Protein Regulator of Cytokines 1) is a human protein that regulates cytokines (Jiang et al., 1998). (b) shows a complex of MT with kinesin, a motor protein that disassembles MT (Ohi & Wordeman, 2013). (c) demonstrates

MT bound small molecule and (d), (e) & (f) demonstrate MT complexes with Taxol, Peloruside, and Zampanolide respectively along with a closer view of their binding pockets. Peloruside, Taxol (Paclitaxel), and Zampanolide are antimitotic drugs (Field et al., 2017; Ganguly et al., 2015.; Tew, 2007).

5.3.1 Contribution of Microtubule Complex Structure

MT undergoes rapid series of assembly and disassembly as tubulin dimers polymerize and depolymerize. This polymerization is regulated by binding of GTP to α- and β-tubulin leading to its hydrolysis to GDP. Consequently, the tubulin-binding affinity weakens for adjacent molecules, thereby facilitating depolymerization leading to the dynamic instability of MT (Cooper, 2000). MTs help in the formation of the mitotic spindle. They are involved in mitosis, movements of eukaryotic organelles, organelle distribution, chromosome segregation, cell division, intracellular transport, and maintaining motility, and cell shape (Hadfield et al., 2003). Upon destruction of the spindle, loss of chromosome segregation occurs. As a result, cell division is inhibited inducing cell death. This is how an antimitotic agent works. Drugs blocking mitosis works by suppressing the dynamic MTs, thereby killing the tumor cells (Mukhtar et al., 2014). Although MT is an obvious target for anticancer agents, a comprehensive structural analysis to serve for the basis of their mechanism of action was still missing. The cryo-EM study reveals the atomic model of MT and inhibitors bound to MT complexes (Figure 23).

The cryo-EM visualization of the microtubule revealed the binding of PRC1 to MT (Figure 23 a). PRC1 is an MT binding protein essential to induce cell cleavage, maintaining the spindle midzone (Mollinari et al., 2002). Likewise, the cryo-EM structure also revealed the binding of kinesin to MT (Figure 23 b). Kinesin gives MT its movement and monitors its direction (Berg et al., 2002). Moreover, the cryo-EM analysis of MT revealed the binding mode of inhibitors to MT (Figure 23 d, e, f). The inhibitors include taxol, peloruside, and zampanolide among

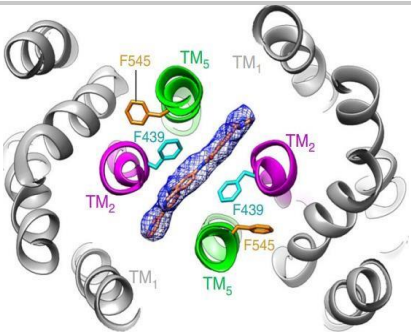
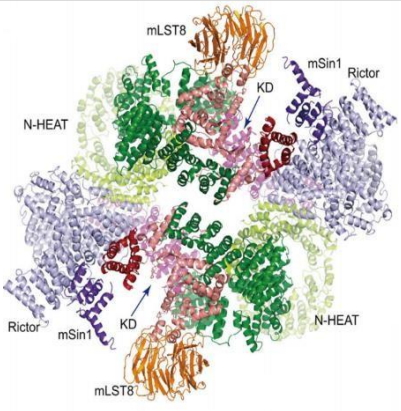
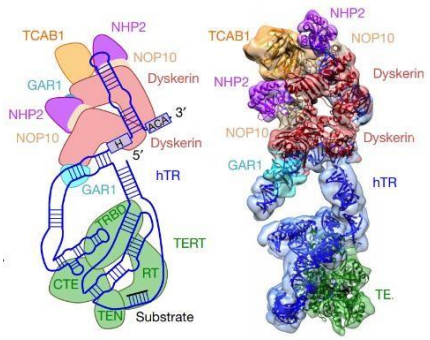
which zampanolide and taxol bind to taxane-site, and peloruside acts on non-taxane site on β-tubulin. Therefore, zampanolide and taxol target the same binding pocket on the MT lumen, whereas peloruside binds to a different pocket positioned on the MT exterior (Kellogg et al., 2017). Taxol is one of the first anti-mitotic agents that was discovered. It inhibits cancer cell proliferation. Cancer cells divide more rapidly compared to normal cells. Therefore, they are sensitive to mitotic inhibitors and the normal cells remain unaffected (Mukhtar et al., 2014).

The understanding of MT, its composition, assembly, and disassembly are significant to develop new mitotic inhibitors. The complexity of MT composition and function left many questions unanswered which now can be answered using cryo-EM high-resolution data. It reveals the details of inhibitor binding pockets on MT as well as states the differences in the taxane-binding pocket and non-taxoid pockets. This difference suggests that taxol and zampanolide inhibit MTs via a different mechanism than that of peloruside. The closer view of the binding pockets plays an important role in understanding their mechanism of action (Figure 23 d, e, f). It favors both the hit identification and lead optimization stages of the drug development process by analyzing the inhibitor-binding affinity to MT. Cryo-EM studies of MT and its accompanying cellular factors or drugs bound to MTs are now generating high-resolution atomic models, leading to prosper new information for the biological cytoskeletal system. This information carries significant medical value for the development of new antimitotic anticancer drugs.

5.4 Additional Recent Advances

There are other recent advances in the oncology field as summarized in Table 4. These can have an impactful contribution to the discovery of anticancer drugs.

Table 4: Recent advances in cancer research using cryo-EM

Component under investigation	Cryo-EM structure	Significance in cancer research	Reference
ABCG2 Transporter The study reveals the binding mode of the ABCG2-Anticancer drugs complex to develop a stabilizer.	 *Imatinib binding pocket in ABCG2 showing the effective inhibitory outcome.*	Chemotherapeutics are transports via ABCG2, resulting in limited accumulation in target sites. ABCG2 inhibitor can improve MDR. Imatinib was found to be an effective inhibitor.	(Orlando & Liao, 2020)
mTORC2 The study reveals its signaling pathway and promotes the development of mTORC2-specific inhibitors.	 *Cryo-EM structure of mTORC2 showing subunit-interactions.*	A promising binding site for anticancer drugs. Structural analysis of mTORC2 subunits Rictor, mLST8, and SIN1 and their interactions provide the basis for developing new targets.	(Chen et al., n.d.; Scaiola et al., 2020)
Substrate-bound human telomerase complex The study reveals the subunit		Takes part in cell division. Aberrant activation of telomerase results in tumorigenesis. The study facilitates	(T. H. D. Nguyen et al., 2018)

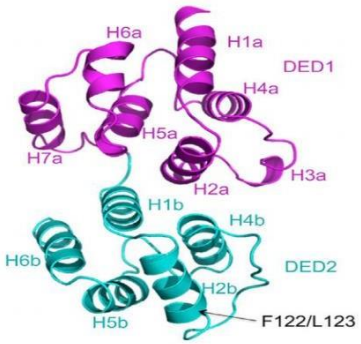
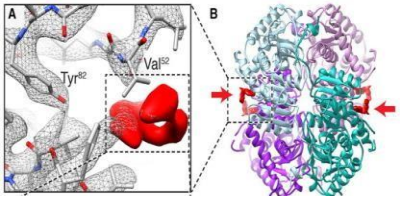
composition of telomerase and its binding to substrates.	*Cryo-EM structure of substrate-telomerase holoenzyme showing all the subunits.*	telomerase targeting drug design.	
DED in caspase-8 activation The study reveals the mechanism of DED filament formation and subsequently caspase-8 activation.	 *Cryo-EM structure of caspase-8 tandem DED where F122/L123 is pointing to the location of the mutation.*	DED activates caspase-8 to induce apoptosis which is inactivated in cancer due to mutation. Thus, it can be a promising therapeutic target for anticancer drugs.	(Fu et al., 2016)
Lactate dehydrogenase Study reveals LDH inhibitor binding pocket at 2.8 Å resolution to promote discovery of new inhibitor molecules.	 *Cryo-EM structure of isoform LDHB bound to inhibitor GSK2837808A shown in red.*	LDH levels in blood increase in cancer. Glycolysis produces lactate in cancer cells via the use of LDH. Therefore, inhibition of LDH is a potential target for anticancer drugs.	(Boland et al., 2017; Feng et al., 2018) (Merk et al., 2016)

Table 4 shows the cryo-EM structure of the ABCG2 transporter which reveals the mechanism of formation of multidrug resistance. It involves the transportation of chemotherapeutic drugs to limit its accumulation at the target site, which suggests that the inhibition of the ABCG2 transporter will produce an enhanced therapeutic effect of anticancer agents. In contrast, cryo-EM visualization of mTOR2 proposes it to be a promising anticancer target. Moreover, the cryo-EM structure of substrate-bound telomeres complex helps to facilitate telomeres targeting drug-design which has shown to be a potential anticancer target. Since telomeres support cell division in normal and its aberrant activation leads to tumor progression. Cryo-EM structure also helps to understand the role of DED in caspase-8 activation and suggests new potential for the anticancer target. Furthermore, it also suggests that LDH inhibitors can produce an anti-cancer effect.

5.5 Limitation and Challenges of Cryo-Electron Microscope Technique

Data collection requires several hours or days, resulting in a much slower throughput for cryo-EM than crystallography. Besides, cryo-EM sample preparation requires a lot of optimization. Sample screening is necessary before collecting the data. Another limitation is its high expense (Renaud et al., 2018). Many more pharmaceutical companies will be encouraged to use cryo-EM if the equipment is made cost-effective and more reliable.

Chapter 6

Discussion

Considering the cryo-EM research findings and their relevancy in cancer research, it is evident that cryo-EM structural analysis expands our understanding and knowledge of mechanisms regarding factors involved in cancer progression. This knowledge can be utilized to design novel anticancer drugs. Visualization of inhibitor-bound complexes provides an opportunity to assess their binding affinity. Utilizing this knowledge, known inhibitors can be modified to have better potency. A close structural view of the binding pocket helps to develop new molecules targeting the same site. As a result, previously unknown mechanisms of some drugs having anti-tumor property can now be determined and new drug molecules following the same signaling pathway can be now discovered.

Chapter 7

Conclusion

This report attempted to evaluate the role of cryo-EM in anticancer drug development. The result highlighted the most valuable cryo-EM-based research findings concerning oncology. In this review, we have studied cryo-EM structures of human 80S ribosome and its interaction with chemotherapeutics, microtubule bound to antimitotic drugs, interactions of mAbs trastuzumab and pertuzumab with HER2 receptor, human p97 protein, human STEAP, the interaction of antibodies with CD20 antigen, ACLY-ligand interaction, etc. which were considered to be most impactful and significant findings. They may contribute to the breakthrough of cancer research. Moreover, this report also discussed the usefulness of cryo-EM at different stages of the drug development process initiating from target identification up to preclinical trials to explain how it helps to facilitate anticancer drug discovery. Visualization of these structures at near-atomic resolution clarifies our understandings of their functions. Older imaging techniques such as X-ray crystallography and NMR spectroscopy could not identify many macromolecular structures at this level of detail. Cryo-EM can reveal previously unknown structural features. The result of this study concludes that cryo-EM structures play a significant role in chemotherapeutic drug discovery. Structural analysis of ligand-receptor complex and characterization of the binding pockets contributes to the development of new molecules to fit the pockets. The report also suggests that determination of binding affinity towards target site and mechanism of action different drugs and biological protein is also feasible through cryo-EM structural analysis.

7.1 Future Prospect

Cryo-EM is a recent technology that has come a long way in a very short time. It may replace traditional imaging methods. It is enhancing the opportunities to uncover the complexity of

previously inconceivable macromolecular structural biology. Pharmaceutical companies are already using cryo-EM to facilitate the drug discovery process. Thus, it is possible to speculate about the future of cryo-EM in anticancer drug development. It is expected that further investigation will be conducted regarding the potential target sites and promising anticancer molecules that were reviewed in this paper. Consequently, these new molecules may pass through the pre-clinical trials to the clinical trial to get FDA approval. So, cancer patients in the future get benefited to fight the disease reducing their sufferings.

Chapter 8

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