Vijay L. Maheshwari
Ravindra H. Patil *Editors*

Natural Products as Enzyme Inhibitors

An Industrial Perspective

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Preface

The history of inventions of enzymes and their applications (enzyme technology) is probably as old as the history of research in modern biochemical sciences. Initially, the term enzyme is coined by Kuhne in 1876 and the commercial uses of it were established after the discovery of uses of enzyme in the removal of stains from cloth in 1913 by Otto Rohm. Enzymes are biological catalysts that serve as a key unit in speeding up all biochemical reactions and are crucial for cellular metabolism in living cells. They are known for their amazing catalytic efficiencies and their high level of specificity for their substrate interactions in all types of biological environments. Therefore, enzymes are currently used in varied applications in many aspects of everyday life including aiding health care, food processing, agriculture, and other industrial applications. However, the kinetic regulation of enzymes is an important criterion when they are used in healthcare, commercial, and industrial processes. The regulation is possible with the help of enzyme-specific inhibitory molecules which may be from living cells (plants, microbes, animals, and algae) or synthetically prepared chemicals.

Enzyme inhibitors are the molecules that can form requisite interaction with the binding site of enzymes to suppress and regulate the rate of reaction of enzymes. These enzyme inhibitors are classified based on their binding potential and mode of action in two types—irreversible enzyme inhibitors and reversible enzyme inhibitors. Currently, the vast majority of enzyme inhibitors are reported to inhibit the various classes of enzymes. These enzyme inhibitors are the focus of the scientific community because they may answer an increasing array of questions in the research area of biological sciences (biochemistry, medicine, physiology, pharmacy, agriculture, ecology) as well as serve as useful tools in the study of enzyme structures and reaction mechanisms and the development of technologies useful in agriculture, food processing, health management, etc. There is tremendous diversity in enzyme inhibitors due to their varying mode of action, target enzymes, and sources (mostly from plants and microbes). While the major constraints found to be associated with the inhibition of enzyme activity are (1) inhibitors have strict enzyme specificity for their interaction with one out of several closely related isoenzymes or enzymes of

different species, (2) some of the enzyme inhibitory molecules form irreversible binding with enzymes and degrade the biochemically important enzymes, (3) some of the inhibitory molecules are associated with their toxicity towards nontarget organisms and mammals, (4) adaptation and advantageous mutations like responses of inhibitor-attached enzymes in living cells, and (5) the degradations of proteinaceous inhibitors due to excessive secretary proteases activities. In light of these facts, it will be really interesting to explore this area and update it. Three major areas of applications of enzymes covered in this book are (1) human health management, (2) agriculture and food processing and research (leading to drug discovery or enzyme activity mechanisms). It contains many interesting articles highlighting the plant-derived inhibitors of serine proteases, pancreatic lipase (PL) inhibitors from indigenous medicinal plants, amylase inhibitors and their applications in agriculture and food processing industries, advances in in silico techniques used in the study of enzyme inhibitors, etc.

Non-small cell lung cancer (NSCLC) is the leading cause of mortality in oncology, and EGFR-TK plays a critical role in this disease. Third-generation drugs (Osimertinib) can overcome the EGFR T790M mutation; a recent C797S mutation makes these agents ineffective against it. To overcome EGFR T790M/C797S resistance, allosteric mutant-selective fourth-generation EGFR inhibitors look to be a promising treatment approach. In *Chap. 1, Ahmad et al.* describe the advantages of blocking allosteric sites in the EGFR-TK receptor domains and compare novel fourth-generation EGFR TKIs for overcoming drug treatment resistance. Protease inhibitors from plants are proteinaceous molecules which form complex with proteases and inhibit their activity. Plants utilize these inhibitors to regulate different physiological processes as well as to protect themselves against insect pests or pathogens. In *Chap. 2, Barbole et al.* survey the different classes of protease inhibitory proteins and peptides from plant origin, with their agronomical and pharmaceutical applications, with emphasis on short peptides. The chapter also highlights various biological and synthetic approaches for production of peptides and methods for improving activity and specificity. *Chapter 3, by Kasar et al.*, systematically reviews different strategies of purification, biochemical characteristics, biological applications of α-AIs, and bottlenecks in commercial utilization of α-AIs. The challenges in the safe marketable utilization of α-AIs are discussed in detail, and alternative approaches and various efficient solutions based on recent advancements in biotechnological research which could be helpful to broaden the scope of α-AIs are also elaborated in detail.

Patil et al. in Chap. 4 highlight the recent developments in the virtual screening of enzyme inhibitors using various docking tools and their significant applications in designing potent inhibitors for the management of various metabolic and infectious diseases. Molecules like acetylcholine, histamine, and gastrin stimulate gastric acid secretions. H^+/K^+-ATPase, also referred to as the proton pump, plays a central role in controlling gastric secretions. The use of synthetic proton pump inhibitors (PPIs) has revolutionized the management of peptic ulcers; nevertheless, there are still various challenges associated with long-term usage that calls for pharmacotherapeutic alternatives. Adinortey and N'guessan present an overview of the structure of H^+/K^+-

ATPase highlighting its central role as one of the most appropriate drug targets necessary for the control of hyperacidity in *Chap. 5*. In addition, the role of plant natural nutraceutical products as inhibitors of H^+/K^+-ATPase is also discussed.

In Chap. 6, Rodrigues Silva et al. highlight an updated overview of advances in protease inhibitors in the field of insect pest control. The chapter also includes some interesting findings on the work carried out in their laboratory particularly on the biochemical mechanisms involved in plant-pest interaction from an enzymatic, proteomic, and molecular biology point of view.

Obesity is increasingly recognized as a global issue, and its prevalence is rising at an alarming rate around the globe. One of the investigated targets for the treatment of obesity is pancreatic lipase (PL) suppression. Orlistat is the only clinically approved drug as a lipase inhibitor and is currently available for long-term obesity treatment. However, various side effects are associated with the long-term usage of Orlistat, which warrants discovery of safe and effective treatment methods for obesity. *Chapter 7 by Patil et al.* thoroughly reviews the importance and PL inhibitory activity of different phytoconstituents from medicinal plants.

Chapter 8 by Shafie et al. presents the most recent data on sources, extraction/identification techniques, and mechanism of action of inhibiting angiotensin-converting enzyme (ACE) inhibitors. The chapter also highlights the commercialization potential and the effectiveness of ACE inhibitors after the oral consumption.

Proteases are the potential targets for the treatment of viral diseases. Since December 2019, the world has observed the emergence of SARS-CoV-2 that resulted in the COVID-19 pandemic and brought the world to a standstill. In silico approaches such as structure and ligand-based virtual screening, docking, and molecular dynamics were extensively used to search for the promising inhibitor of M^{pro} from the existing library of natural molecules. *Chapter 9 by Vadnere and Patil* summarizes the potential inhibitors of M^{pro} from the natural sources such as plant and microorganisms. *Srivastava et al. in Chap. 10* review the recent approaches to telomerase-directed therapy, deliberate the aids, their shortcomings, and speculate on the forthcoming perspective of inhibitors that target telomerase as cancer therapeutics. Modern agriculture practices use of enzyme inhibitors to control pests and to regulate the soil microbial activity. The use of enzyme inhibitors has several drawbacks like sensitivity to temperature, pH, development of resistance and phytotoxicity, etc. Use of nanotechnology in agriculture and human health is well appreciated by the scientific community. *Patil et al. in Chap. 11* highlight the applications of nanomaterial as enzyme inhibitors with its special application as pest control agents and fertilizers additives. Diabetes type 2 has become one of the ten leading causes of death worldwide. Alpha-glucosidase inhibitors are a class of oral medication used in treating diabetes mellitus type 2. However, the side effects of chemical inhibitors of alpha-glucosidase are well known warranting the discovery of new and safer molecules as alpha-glucosidase inhibitors. *Mishra and Bhatnagar in Chap. 12* review the use of plant metabolites as inhibitors of alpha-glucosidase and their potential in the management of type 2 diabetes.

We appreciate the patience and cooperation extended by all the contributors of this book. Thanks are also due to reviewers who took their time to review the

manuscripts in due time. We are also thankful to the entire team of Springer Nature Singapore Pvt. Ltd., Singapore, for giving us this opportunity. We hope that the contents of the book will serve as a useful resource for all those working in the area and will be received with enthusiasm and interest.

Jalgaon, Maharashtra, India — Vijay L. Maheshwari
Shirpur, Maharashtra, India — Ravindra H. Patil
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Contents

About the Editors

Vijay L. Maheshwari is currently serving as Hon. Vice-chancellor at Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India. He has a teaching experience of over 27 years. His areas of research/expertise include protein biochemistry, plant tissue culture and secondary metabolites and their biological activities. Prof. Maheshwari has received the "Young Scientist" award by IUBMB, India, in 1994. He is also a recipient of the BOYSCAST fellowship of the Government of India, Department of Science and Technology, for postdoctoral research at the University of Virginia, USA. He was Norman E. Borlaug fellow of the US Department of Agriculture, USA, and undertook advanced training in Risk Assessment in Genetically Modified Food. He has published around 100 research papers and review articles in journals of national and international repute. He has authored three books and successfully handled several research projects and supervised 22 students for doctoral degrees. He has also served as editor for a book published by Springer Nature Singapore Pvt. Ltd., Singapore.
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Chapter 1
Fourth-Generation Allosteric EGFR Tyrosine Kinase Inhibitors to Combat the Drug Resistance Associated with Non-small Cell Lung Cancer (NSCLC)

Iqrar Ahmad, Rahul Pawara, Asama Pathan, and Harun Patel

Abstract Non-small cell lung cancer (NSCLC) is the leading cause of mortality in oncology, and EGFR-TK plays a critical role in this disease. As a result, EGFR-TK is a viable target for therapeutic development in NSCLC. The T790M EGFR TK mutation was resistant to both first-generation and second-generation (selectivity issue) EGFR-TK inhibitors. Although third-generation drugs (Osimertinib) can overcome the EGFR T790M mutation, a recent C797S mutation makes these agents ineffective against it. All of the currently available EGFR kinase inhibitors target the kinase's highly conserved ATP site, underlining the need for therapeutics with a different mechanism of action (allosteric binding). EAI001, EAI045, JBJ-04-125-02, DDC4002 and a series of small compounds (fourth generation) having an affinity for the EGFR allosteric site have been discovered and are currently being investigated. To overcome EGFR T790M/C797S resistance, allosteric mutant-selective fourth-generation EGFR inhibitors look to be a promising treatment approach. This chapter discusses the advantages of blocking allosteric sites in the EGFR-TK receptor domains and compares novel fourth-generation EGFR-TKIs for overcoming drug treatment resistance.

Keywords EGFR · Non-small cell lung cancer · T790M/C797S · Allosteric inhibitors

Abbreviations

Ba/F3 cell	A murine Interleukin-3-dependent Pro-B cell line
C797S	Cystein797 to Serine790
EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor tyrosine kinase inhibitors

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PROTAC	Proteolysis-targeting chimera
PROTACs	Proteolysis-targeting chimeric molecules
RTKs	Receptor tyrosine kinases
T790M-C797S	Threonine 790 methionine-cysteine 797 serine
TKIs	Tyrosine kinase inhibitors
WT EGFR	Wild-type epidermal growth factor receptor

1.1 Introduction

Lung cancer continues to be one of the leading causes of cancer-related death, owing to the ineffectiveness of conventional chemotherapeutics (Sharma et al. 2007). Based on cellular morphology, lung cancer is further divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). According to the American Cancer Society, 85% of lung cancer patients have NSCLC, and 15% have SCLC (Fig. 1.1) (Molina et al. 2008; https://www.cancer.org/cancer/lung-cancer.html). Adenocarcinoma (AC) (40%), squamous cell carcinoma (SQCC) (25%), and large cell carcinoma (LCLC) (15%) are the three sub-types of NSCLC (Molina et al. 2008).

The involvement of the epidermal growth factor receptor (EGFR) in NSCLC is well recognized, and significant therapeutic progress has been made in the treatment of this disease in the last decade (Maulik et al. 2003; Wee and Wang 2017; Patel et al. 2018; Ahmad et al. 2020). The USFDA has approved gefitinib and erlotinib, two small molecule EGFR inhibitors, for the treatment of NSCLC. As first-generation, ATP-competitive, and reversible EGFR inhibitors, gefitinib and erlotinib were found to be efficacious in NSCLC patients with somatic EGFR mutations L858R and delE746 A750, which account for 90% of all EGFR mutations in NSCLC (Vansteenkiste 2004; Kobayashi et al. 2005a, b; Li et al. 2008; Kawahara et al. 2010; Pao et al. 2005; Patel et al. 2019). Unfortunately, the ability of these EGFR inhibitors to effectively treat NSCLC patients is to 10–12 months due to acquired secondary mutations such as the T790M mutation in EGFR, which leads to drug resistance in around half of NSCLC patients (Yun et al. 2008; Murakami et al. 2012). The EGFR T790M mutation recovers EGFR's affinity for ATP similar to wild-type (WT) EGFR and inhibits reversible inhibitors from binding at higher ATP concentrations (Singh et al. 2010; Ramalingam et al. 2012; Song et al. 2016).

The second-generation EGFR inhibitors, such as Afatinib and Dacomitinib, irreversibly inhibit the EGFR-TK by covalently binding with Cys797 residue. These inhibitors have effectively inhibited the activating (Exon 19 deletion or L858R) mutation and T790M mutation (Kobayashi et al. 2005a, b; Balak et al. 2006; Kosaka et al. 2006; Patel et al. 2017, 2020). Because of their low selectivity between the WT EGFR and the T790M EGFR, these inhibitors caused skin rash and diarrhoea due to WT EGFR inhibition (Michalczyk et al. 2008).

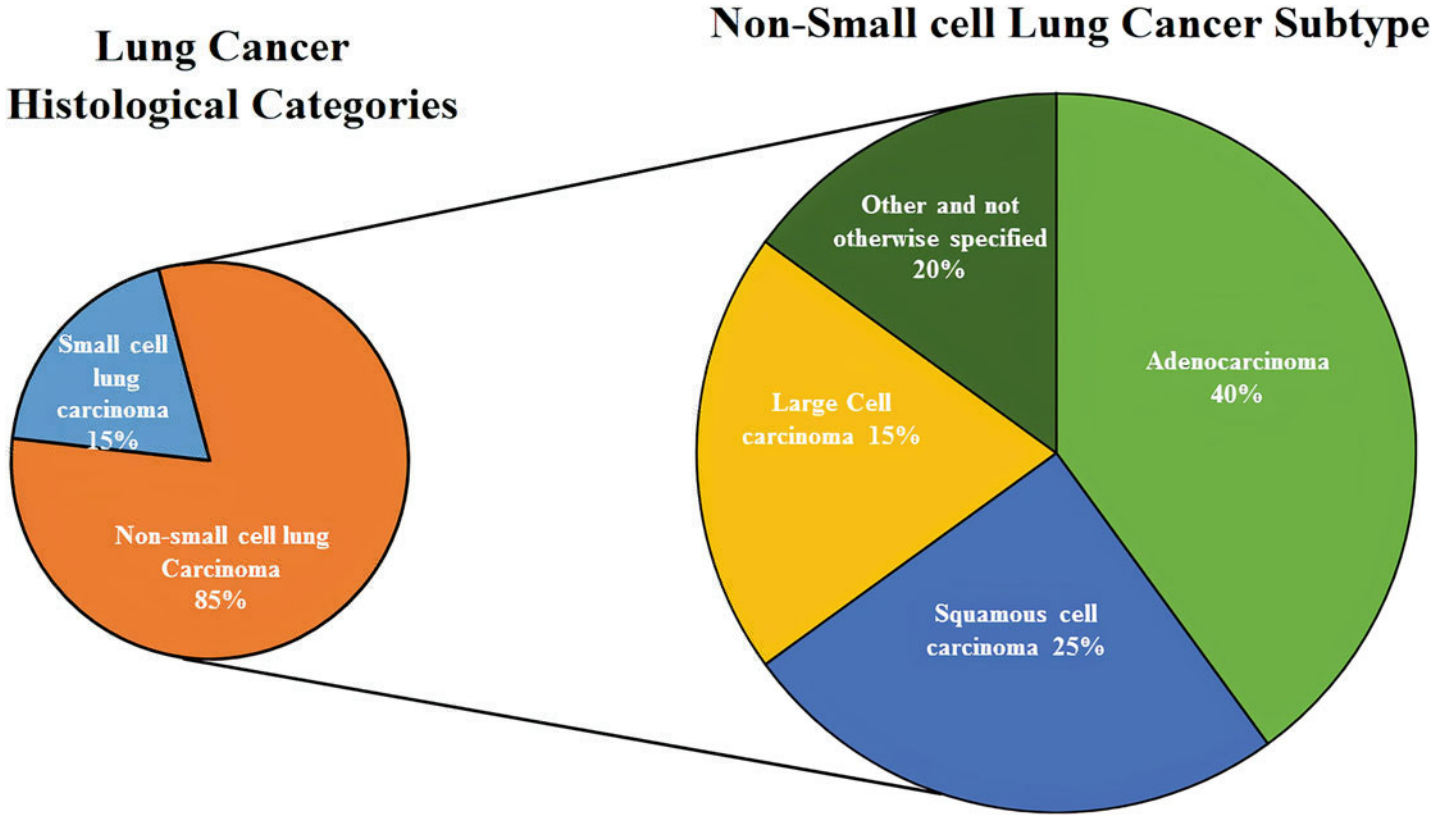


Fig. 1.1 Types of lung cancer

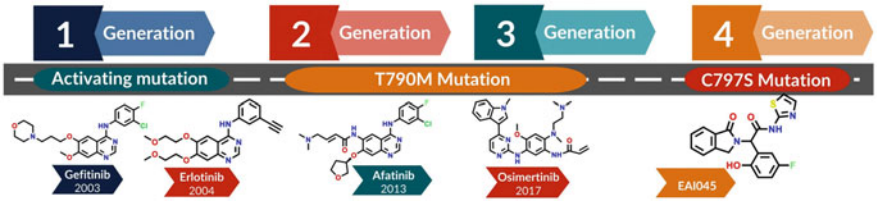


Fig. 1.2 First-, second-, third-, and fourth-generation EGFR-TK inhibitors

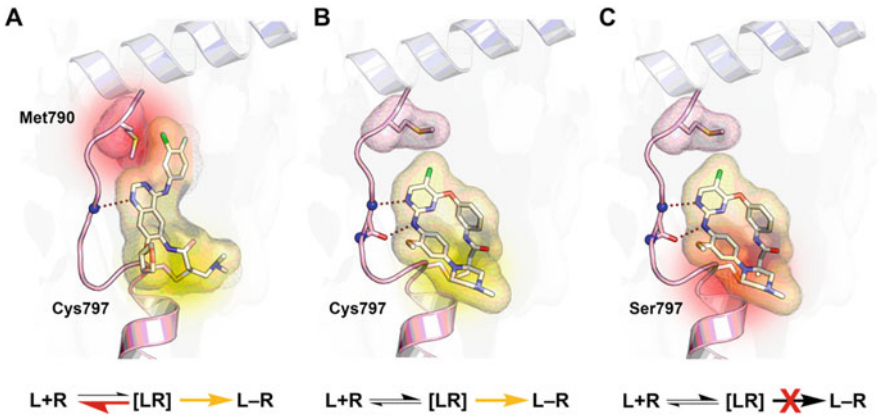


Fig. 1.3 (**a**, **b**) Covalent binding of second- and third-generation EGFR inhibitors with C797 residue of T790M EGFR-TK; (**c**) C797S resistance to the third-generation EGFR inhibitors, rendering them ineffective. (Reprinted (adapted) with permission from ref. Engel et al. 2015. Copyright 2016, American Chemical Society)

Several irreversible third-generation EGFR inhibitors with mutant selectivity have been developed to alleviate the toxicity induced by inhibition of WT EGFR (second-generation EGFR inhibitors), such as WZ4002 (Zhou et al. 2009), Rociletinib (Walter et al. 2013), Osimertinib (Finlay et al. 2014), and Olmutinib (Lee et al. 2014) (Fig. 1.2). Of these inhibitors, AstraZeneca's Osimertinib (Tagrisso™) got FDA approval in 2017 for the treatment of NSCLC patients with the T790M EGFR-TK mutation (AstraZeneca (n.d.); US FDA (n.d.); Ahmad et al. 2020). Despite its good clinical efficacy in treating EGFR mutant NSCLC patients, osimertinib resistance (tertiary mutation C797S) develops after 14–20 months of treatment (Fig. 1.3) (Thress et al. 2015; Grabe et al. 2018; Nie et al. 2018; Du et al. 2021; Patel et al. 2021a, b). However, osimertinib's selectivity for mutant kinases has not yielded the intended results.

In vivo, Osimertinib's binding to non-target EGFR receptors has resulted in serious side effects such as diarrhoea, rash, nausea, decreased appetite, hyperglycaemia, prolongation of the corrected QT interval, pneumonia (Jänne et al. 2015), and Vortex keratopathy (Chia and John 2015), all of which severely

limit Osimertinib's clinical application. As a result, a more effective mutant-selective EGFR L858R/T790M inhibitor that targets both the sensitizing and resistant T790M mutations while sparing the wild type of the receptor is desperately needed.

All currently available EGFR kinase inhibitors target the kinase's highly conserved ATP site, emphasizing the need for inhibitors with a different mechanism of action (Wan et al. 2019). The allosteric site's low sequence homology offers a unique potential for more targeted inhibition with less off-target pharmacology (Wan et al. 2019). Other benefits of allosteric inhibitors over traditional ATP-competitive inhibitors include the ability to overcome mutation-associated drug resistance, particularly mutations in the ATP-binding site, which confer resistance to almost all related ATP-competitive inhibitors (Zhang et al. 2009). Furthermore, allosteric inhibitors may not need to have nanomolar affinity to compete with high intracellular ATP concentrations, making it easier to identify weak binding inhibitors to overcome the drug resistance problem (Engel et al. 2015; Abe et al. 2011). The T790M and C797S mutations do not affect the efficiency of the allosteric inhibitors as the allosteric site is far away from the EGFR ATP-binding site (Tripathi and Biswal 2021). The purpose of this chapter is to provide a complete summary of fourth-generation allosteric EGFR inhibitors and their recent development (Fig. 1.3).

1.2 EGFR Tyrosine Kinase Allosteric Binding Site

For the treatment of NSCLC, EGFR targeting therapeutics are widely accepted as the treatment of choice. When a ligand binds with TK, the receptor undergoes conformational changes, which leads to autophosphorylation of the complex, which commences the further signal transduction cascade (Zang et al. 2017; Maity et al. 2020).

The activation of the cell proliferation cycle in NSCLC is triggered by this cascade, which activates the RAS/RAF/MAPK pathway, which is critical for cell survival regulation and cell proliferation. The cascade triggers the PI3K/AKT/mTOR pathway, which inhibits apoptosis and promotes malignant cell development (Zang et al. 2017; Maity et al. 2020).

The N-lobe, C-lobe, ATP site, hinge region, and allosteric site are the five key sections of the kinase structure (Fig. 1.4). The amino acid hand of N-lobe is short, with five anti-parallel β-sheets and one helix. The ATP-binding site, which is a highly reactive region of the kinase domain, connects the N-lobe to the C-lobe (Zhao et al. 2018; Li and Guo 2021). Binding of the ligand to the N-lobe results in an extension of the α-helix hand. The ligand interaction causes conformational changes in the kinase structure, allowing the ATP site to be activated for autophosphorylation. The C-lobe shrinks towards the N-lobe and the kinase receptor is activated once phosphorylation begins (Mitchell et al. 2018). Due to continual autophosphorylation, the ATP site is a highly reactive and unstable site. Epidermal growth factor (EGF), transforming growth factor (TGF-α), tumour necrosis factor

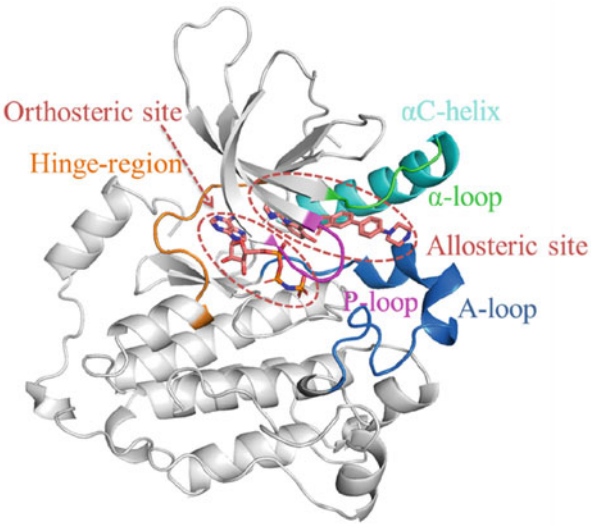


Fig. 1.4 Allosteric binding site of the EGFR Tyrosine Kinase. (Reprinted (adapted) with permission from Li and Guo 2021. Copyright 2021, American Chemical Society)

(TNF), epiregulin, and amphiregulin are some of the major ligands that interact with TK and cause dimerization of the TK complex (Maity et al. 2020).

There are three binding sites on a TK receptor: an ATP-competitive site, an inactive site, and an allosteric site. The majority of available drugs target the ATP-binding site, whereas no drugs bind at the inactive site (Gao et al. 2016; Santarpia et al. 2017; Zhou et al. 2018; Maity et al. 2020; Patel et al. 2021a, b). Targeting the allosteric region results in a stable complex formation with protein, which changes both the effectiveness and binding of the primary ligand. This renders the protein less active to the ligand-complex formation or exhibits the neutral functionality during ligand interaction (Maity et al. 2020). When allosteric site inhibitors bind to the ATP site but not the hinge region, kinase activity is blocked without ATP being displaced. Furthermore, autophosphorylation is blocked, and the conformational change in the kinase domain is also inhibited, making it easier to achieve equilibrium (Purba et al. 2017). As a result, allosteric sites can inhibit or stop the signal induction cascade completely. Thus, inhibiting the allosteric site can help to overcome the problem of "undruggable" resistance (C797S) with minimal adverse effects (Fig. 1.5) (Maity et al. 2020).

1.3 Allosteric Inhibitors

Jia et al. (2016) reported EAI001 (1), a new EGFR allosteric inhibitor hit molecule with a thiazole amide structure, which was identified using high-throughput screening (HTS) from a library of 2.5 million compounds with a specific selectivity for mutant EGFR. **EAI001** (**1**) had an IC_{50} of 24 nm against the L858R/T790M mutant

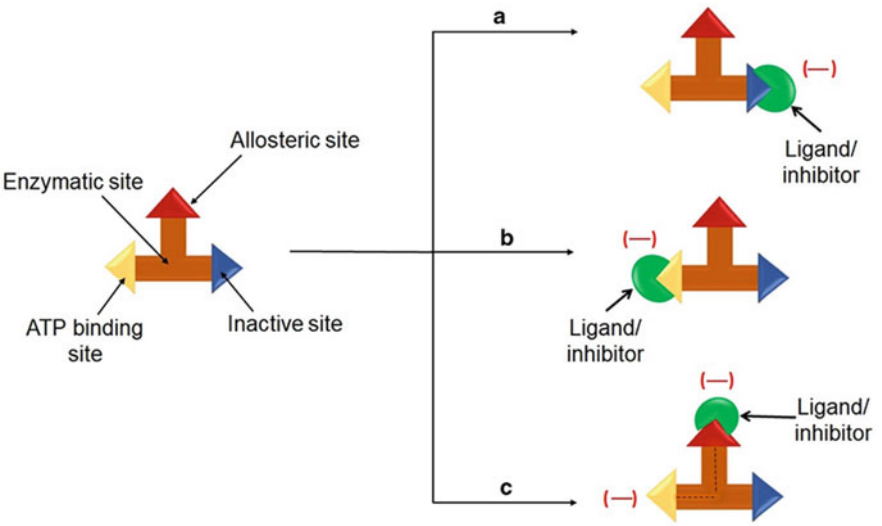


Fig. 1.5 Binding mechanism of the allosteric inhibitors. (Reprinted (adapted) with permission from Maity et al. 2020 under Creative Commons CC BY 4.0. Copyright 2020, Springer)

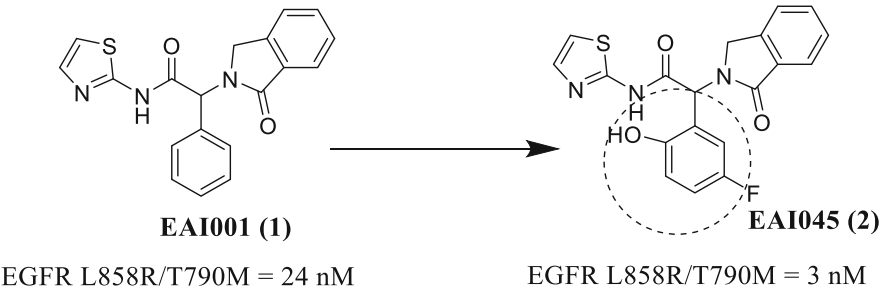


Fig. 1.6 Structural optimization of **EAI045** (**1**) from **EAI001**(**2**)

EGFR kinase, whereas the IC_{50} value for the WT EGFR was more than 50 μM (Fig. 1.6). According to X-ray crystal structure (PDB Id: 5D41), EAI001 binds to the allosteric region of EGFRT790M generated by displacement of the C-helix in the active conformation. EAI001 aminothiazole group makes direct interaction with gatekeeper Met790, while the carboxamide's NH group serves as a hydrogen bond donor with Asp-Phe-Gly (DFG) motif residue Asp855. The direct interaction with the gatekeeper residue and the inability to bind the inactive conformation of WT EGFR explain the selectivity for T790M EGFR. The 1-oxoisoindolinyl reaches to the solvent accessible area, while the phenyl group enters hydrophobic pocket formed by Met766, Leu777, and Phe856 (Fig. 1.7).

Further medicinal chemistry optimization of EAI001 (**1**) at phenyl ring provided a more potent analogue EAI045 that has proven to exhibit high mutant inhibitory

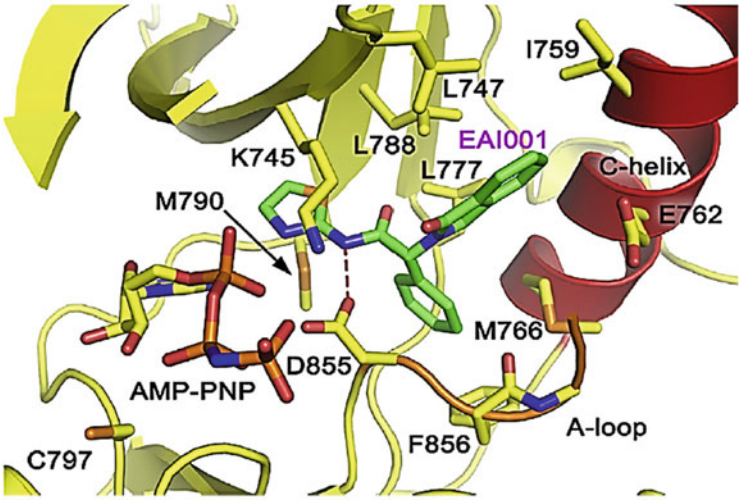


Fig. 1.7 Binding approach of **EAI001 (1)** in EGFR T790M/V948R kinase (PDB code 5D41). (Reprinted (adapted) with permission from Zhao et al. 2018. Copyright 2018, Elsevier)

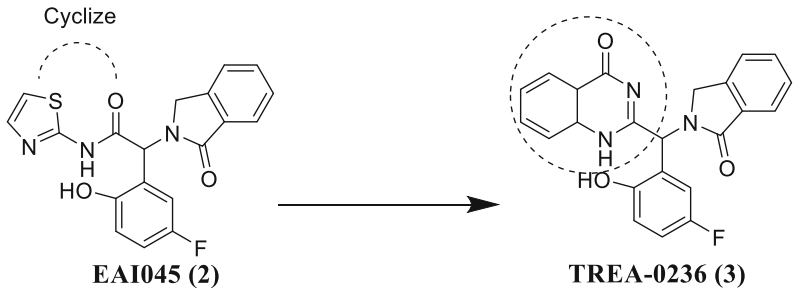


Fig. 1.8 Development of non-peptidic analogue of **EAI001 (1)**

potency (EGFR L858R/T790M IC_{50} = 3 nM) and 1000-fold selectivity (WT EGFR IC_{50} = 4.3 μM). On the other hand, EAI045 partially inhibited EGFR phosphorylation in cells and demonstrated weak antiproliferative activity in L858R/T790M mutant cells. The combination of EAI045 with EGFR dimer-disrupting antibody Cetuximab can stymie EGFR dimerization and cause significant tumour antiproliferative response in both genetically engineered mice carrying the L858R/T790M EGFR mutation and the L858R/T790M/C797S EGFR mutation. This is the first inhibitor reported that can overcome EGFR T790M and EGFR C797S mutations. However, human clinical trials are now required to validate EAI045's clinical efficacy.

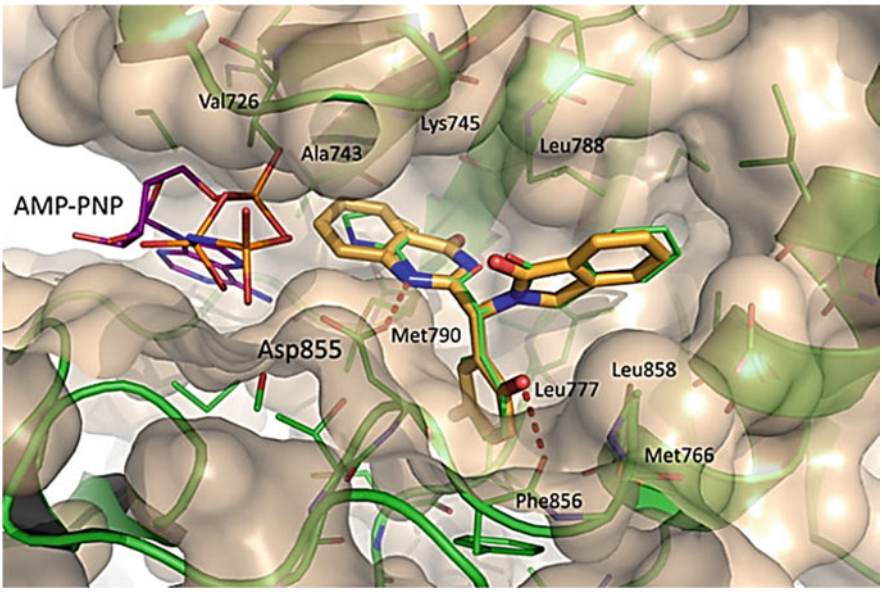
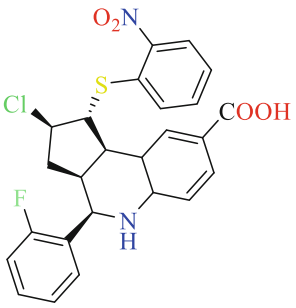


Fig. 1.9 Binding mode of **TREA-0236** (**3**) (*thick yellow sticks*) and **EAI001** (**1**) (*thin green lines*). (Reprinted (adapted) with permission from Lee et al. 2018. Copyright 2018, Korean Chemical Society)

Lee et al. (2018) synthesized TREA-0236 (3), a new EGFR allosteric inhibitor by substituting non-hydrolysable quinazoline-4-one for aminothiazole on EAI045 (Fig. 1.8). EAI045 has a di-peptide core with an aminothiazole at the *N*-terminus; peptides are metabolically unstable, yielding 2-aminothiazole metabolites; these metabolites found promiscuous hitting scaffold, causing methemoglobinemia toxicity; the reactive intermediate formation leads to extensive covalent protein binding, as seen in high-throughput screening (HTS).

To avoid haematological toxicities, they modified the structure of EAI045 (**2**) by cyclization, converting 2-aminothiazole amide to quinazoline-4-one for improved safety and pharmacokinetics. However, TREA-0236 (**3**) (EGFR L858R/T790M/C797S IC_{50} = 5.3 μM) was less potent in contrast to parent EAI045 (EGFR L858R/T790M/C797S IC_{50} = 4 nM) (Fig. 1.8). Further molecular modelling studies revealed that TREA-0236 (**3**) anticipated binding pose is nearly similar to EAI001 (**1**) crystal structure, which is close to the ATP-binding site. Their structure-activity relationship study suggests that the 5-fluoro-2-hydroxylphenyl substitution on the quinazoline-4-one scaffold is required for the EGFR L858R/T790M/C797S mutant activity and when it is replaced with alkyl group the activity of the compound is considerably diminished or even completely inactivated (Fig. 1.9).

Carlino et al. (2018) synthesized and reported SAR of hexahydrocyclopenta[*c*] quinoline derivatives as allosteric inhibitors of cyclin-dependent kinase-2 (CDK2) and EGFR (Fig. 1.10). Among the synthesized compound, **4** was a selective



Compound 4

WT EGFR (10μm)= 0% inhibition
WT EGFR (50μm)= 23% inhibition
L858R/T790M EGFR (10μm)= 28% inhibition
L858R/T790M EGFR (50μm)= 70% inhibition

Fig. 1.10 Chemical structure and reported as percent inhibition at 10 μm and 50 μm of compound **4**

allosteric inhibitor of the double mutant L858R/T790M EGFR with respect to wild-type EGFR/CDK2. Dose–response curves revealed that **4** was able to inhibit the T790M/L858R EGFR with an EC_{50} of 44 ± 4 μM. Compound **4** was further evaluated in the presence of a higher concentration of ATP (1 mM) to demonstrate its allosteric activity on the T790M/L858R EGFR.

The dose–response curves clearly showed that compound **4** retained its inhibitory profile, indicating that it had an allosteric mechanism of action. Induced-fit docking of the compound **4** to the T790M/L858R EGFR-KD crystal structure (PDB ID: 3W2R) revealed that the compound binds to the allosteric pocket by fitting the 2-phenyl-substituted ring into the deeper hydrophobic pocket lined by Leu788, Met766, Met790, and Phe856. The carboxylate group of the hexahydrocyclopenta [*c*]-quinoline forms a salt bridge with the polar heads of Arg858 and Arg748. Particularly, the interaction of compound **4** carboxylate with the primary mutated Arg858 residue may help to explain the compounds' selectivity for the double mutant EGFR over the wild-type EGFR. Molecular dynamics simulations of compound **4** within the T790M/L858R EGFR-KD revealed that the proposed binding mode was stable and that the key interactions within the allosteric pocket were remained throughout the 100 ns simulation time.

Their SAR study suggested that all the compounds in which the carboxylate group was removed or modified were inactive, when tested on the T790M/L858R protein. It was also observed that the various substituents of the 2-phenyl ring, which is suited to the deeper hydrophobic pocket lined by the side chains of Phe856, Leu788, Met766, Met790, Phe723, and Lys745 amino acid residues, played a crucial role in modulating the selectivity for the double mutant EGFR (Fig. 1.11).

Caporuscio et al. (2018) reported a low-cost high-throughput docking protocol for the fast identification of EGFR allosteric inhibitors using the crystal structure of the EGFR in complex with an allosteric inhibitor (PDB code 5D41). Commercially available drugs were employed for virtual screening by high-throughput docking

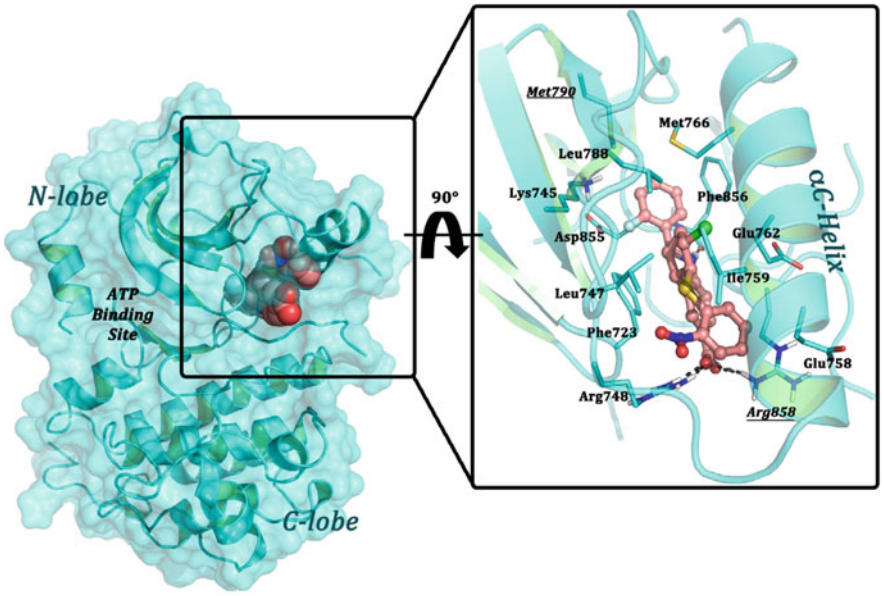
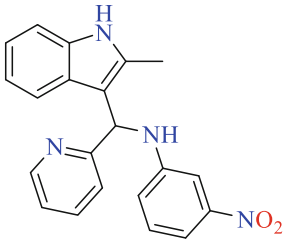


Fig. 1.11 Induced-fit docking binding approach of compound **4** within the cavity of L858R/T790M EGFR structure (PDB ID: 3W2R). (Reprinted (adapted) with permission from Carlino et al. 2018. Copyright 2018, Wiley-VCH Verlag GmbH and Co.)

Fig. 1.12 EGFR inhibition profile of compound **5**



Compound 5

WT EGFR (10μm)=70% inhibition

WT EGFR (50μm)=91% inhibition

L858R/T790M EGFR (10μm)=71% inhibition

L858R/T790M EGFR (50μm)=90% inhibition

using the structures of wild-type and T790M/L858R EGFRs, yielding 92 compounds for biological evaluation. In at least two fixed concentration tests with the same or different protein structures (WT, T790M, T790M/L858R), 9 compounds were discovered whose percentage of inhibition was higher than or about 50% at 50 μM and higher than or about 20% at 10 μM.

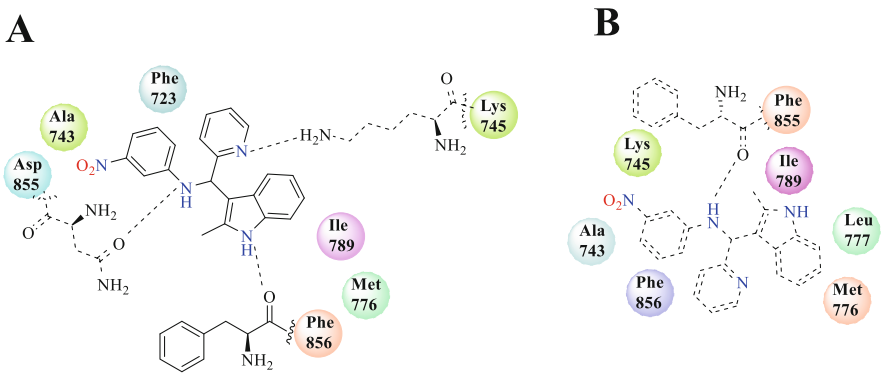


Fig. 1.13 Putative binding modes of compound **5**; (**a**) Binding mode of the (R)-enantiomer of compound **5**; (**b**) Binding mode of the (S)-enantiomer of compound **5**

The dose–response curve of promising compound **5** demonstrated that the compound was able to bind the WT EGFR and T790M/L858R double mutant EGFR with EC_{50} values of 4.7 μM ± 0.7 μM and 6.2 μM ± 0.5 μM, respectively (Fig. 1.12). Importantly, dose–response curve was unaffected by changing concentration of ATP (1 μM and 1000 μM), indicating that the compound inhibited the T790M/L858R EGFR with an allosteric mechanism. Moreover, compound **5** was able to inhibit the growth of non-small cell lung cancer cell lines (H1299, H1650, and H1975) with micromolar potency. Binding mode analysis revealed that compound **5** (R)-enantiomer's pyridine nitrogen forms H-bond with the Lys745 side chain, NH of indole forms hydrogen bonds with the Phe856 carbonyl group, and NH of nitrophenylaniline forms H-bond with the Asp855 carboxyl group. Polar moiety of compound **5** established good van der Waals interaction with Phe723, Ala743, the alkyl chain of Lys745, Leu747, Met766, Leu777, Leu788, Ile789, Met790, and Phe856. The (S)-enantiomer shows a hydrogen bond contact with the carboxyl group of Asp855 through its nitrophenylaniline NH and contacts with the Phe723, Val 726, Ala743, the alkyl chain of Lys745, Leu747, Met766, Leu777, Leu788, Ile789, Met790, and Phe856 (Fig. 1.13).

Surprisingly, the indole and pyridine rings are inverted in relation to the (R)-enantiomer's pose. According to the putative binding modes both enantiomers can form favourable interactions within the allosteric region.

To et al. (2019) and colleagues developed the JBJ-02-112-05 (**6**) mutant-selective allosteric EGFR inhibitor by linking 5-indole substituent on isoindolinone moiety of EAI001 (**1**).

For the EGFR L858R/T790M mutant variant, the compound JBJ-02-112-05 (**6**) exhibited an IC_{50} of 15 nM. Further medicinal chemistry optimization of JBJ-02-112-05 (**6**) by incorporating 2-hydroxy-5-fluorophenyl group of EAI045 (**1**) and phenyl-piperazine on isoindolinone substituents delivers a more potent EGFR allosteric inhibitor, JBJ-04-125-02 (**7**), with an IC_{50} of 0.26 nM for T790M/L858R mutant EGFR (Fig. 1.14). JBJ-04-125-02 (**7**) shown anticancer effect on L858R,

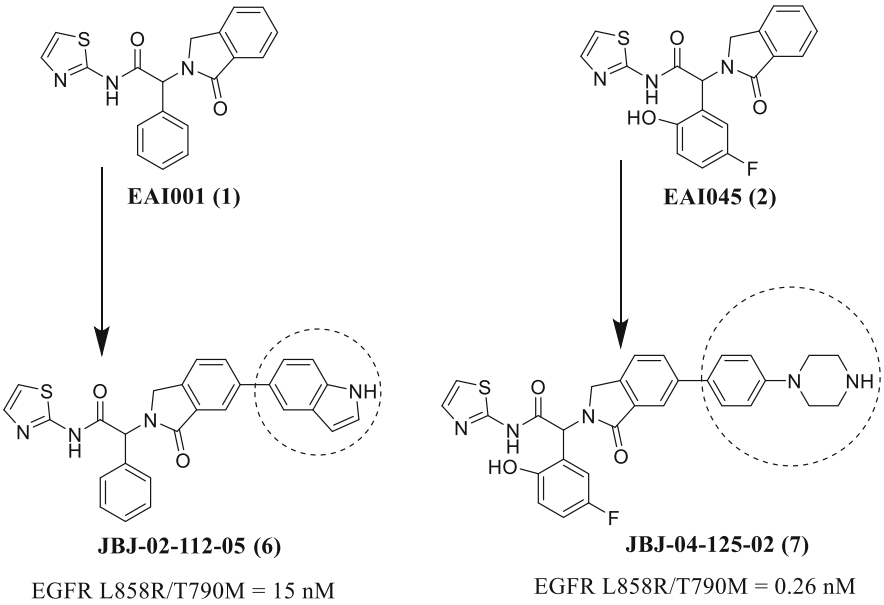


Fig. 1.14 Molecular structures of **JBJ-02-112-05 (6)** and **JBJ-04-125-02 (7)**, functionalized from **EAI001** (**1**) and **EAI054** (**2**)

L858R/T790M, or L858R/T790M/C797S mutations EGFR variant without co-administration of Cetuximab. JBJ-04-125-02 (**5**) has also no affinity for WT EGFR or the Ex19del mutant. Further investigation revealed that osimertinib may increase **JBJ-04-125-02** (**7**) binding affinity to EGFR, resulting in more potent cytotoxic activity. These results suggest that combining a covalent mutant-selective allosteric EGFR-TKI with a third-generation EGFR-TKI could be a useful treatment approach for some lung cancer patients who are resistant to third-generation EGFR-TKIs. On the other hand, both **EAI045** (**2**) and **JBJ-04-125-02** (**7**) are unable to overcome the resistance mediated by the triple mutant EGFR del19/T790M/C797S.

According to crystal structure (PDB code: 6DUK) **JBJ-04-125-02** (**7**) also binds to the allosteric pockets in the C-helix-out conformation of EGFR, like EAI045. **JBJ-04-125-02** (**7**) binding generated a unique A-loop conformation, which appears to be stabilized by a hydrogen bond between the compound's piperazine group and Glu865 in the A-loop (Fig. 1.15).

Dries and colleagues reported a second series of allosteric mutant-selective EGFR inhibitors. They previously reported the discovery of **EAI001** (**1**) through selective screening of mutant L858R/T790M EGFR inhibitors (Fig. 1.16) (De Clercq et al. 2019). The same screen also yielded EAI002 (**8**), which was composed of a 5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one scaffold that selectively inhibited L858R/T790M with a biochemical IC_{50} of 52 nM compared to >1000 nM for WT. Subtle fluorine shift optimization of EAI002 (**8**) resulted in DDC4002 (**9**),

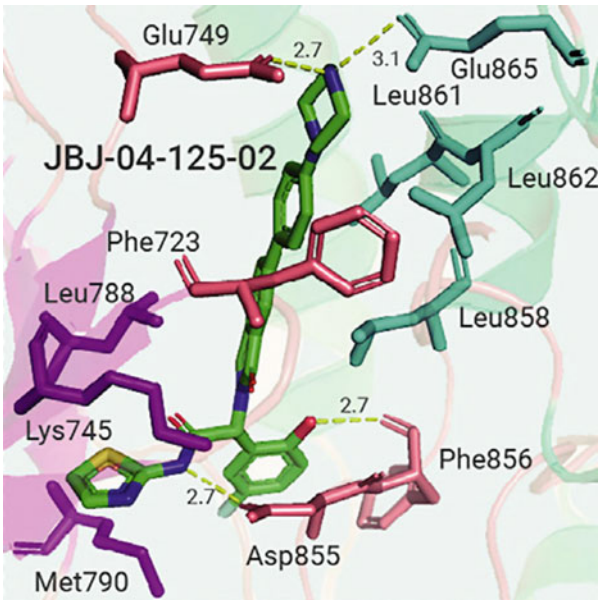


Fig. 1.15 Binding approach of **JBJ-04-125-02** (**7**). (Reprinted (adapted) with permission from Du et al. 2021 under Creative Commons CC BY 4.0. Copyright 2021, Elsevier)

which had mutant-selective nanomolar biochemical IC_{50} values towards the mutant L858R/T790M and L858R/T790M/C797S EGFR variant compared to WT EGFR.

C-2-functionalized EAI045 (**2**) and JBJ-04-125-02 (**7**) led to an increase in biochemical potency; inspired by this observation, the 4-(piperazinyl)-phenyl substituent was linked at C-2 to increase potency (**10**). Furthermore, the SAR study revealed that increasing the flexibility of C-2 site via the Ullmann biaryl ether linkage moderately improved the effectiveness of these inhibitors (**11** and **12**). They discovered that the biochemical potencies of the three compounds (**10–12**) obtained for L858R/T790M and L858R/T790M/C797S were equivalent to EAI045. Compound **12** exhibited the highest potency against EGFR L858R/T790M/C797S in a biochemical assay ($IC_{50} = 13$ nM) and significant antiproliferative action when co-administered with Cetuximab.

Binding analysis showed that the DDC4002 (**9**) diazo ring bends inward towards the C-helix, whereas the 8-fluorobenzene ring interacts in the hydrophobic pocket and the unsubstituted benzene ring accompanies the C-helix outward towards the solvent. A hydrogen bond is observed in between the diazepine NH and the DFG motif residue Asp856 backbone carbonyl. The benzyl substitution propagates to the *N*-lobe, which is wedged between AMP-PNP and the residues L788, K745, and T790M (Fig. 1.17).

Hoffmann La Roche disclosed a series of oxo-indole compounds as potent and selective allosteric EGFR inhibitors in the WO2020002487A1 patent (Duplessis et al. 2020). Among the synthesized compound, **13** and **14** were very effective inhibitors of the EGFR L858R/T790M/C797S kinase, with IC_{50} values of 5 nM

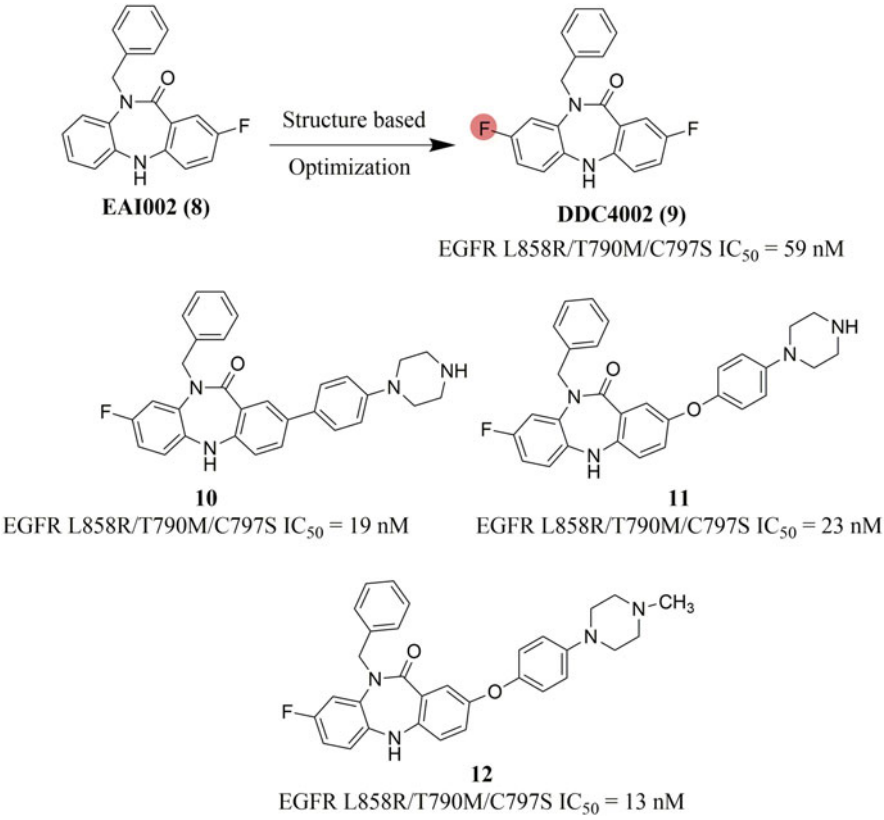


Fig. 1.16 Structure-based optimization of **EAI002 (8)** afforded **DDC4002 (9)** and chemical structure of C-2 functionalized compounds (**10–12**)

and 4 nM, respectively, against the Ba/F3 cell line harbouring EGFR L858R/T790M/C797S mutation (Fig. 1.18).

Jang et al. (2020) discovered mutant-selective allosteric EGFR degraders that are effective against a wide range of drug-resistant mutations. They used EAI001 (**1**) (allosteric inhibitor) as a starting point for their design of a degrader because the binding approach of the first allosteric inhibitor, EAI001 (**1**), revealed that it is in a deep allosteric pocket. They prepared the JBJ-07-149 (**15**) by introducing 1-(pyridin-2-yl) piperazine at the 6-position of isoindolinone, which resulted in the expansion of EAI001 (**1**) to a solvent-exposed area. In vitro catalytic activity of JBJ07–149 in EGFR L858R/T790M found that it inhibited the enzyme with an IC_{50} of 1.1 nM. JBJ07–149 (**15**) had a significant antiproliferative activity in the presence of Cetuximab ($EC_{50} = 0.148$ μM) but was unsuccessful as a single agent in proliferation tests ($EC_{50} = 4.9$ μM). They also developed a bifunctional degrader molecule, DDC-01-163 (**16**), by modifying JBJ-07-149 (**15**) and coupling it to the CRBN ligand pomalidomide via a linker conjugated to the piperazine moiety of JBJ-07-149

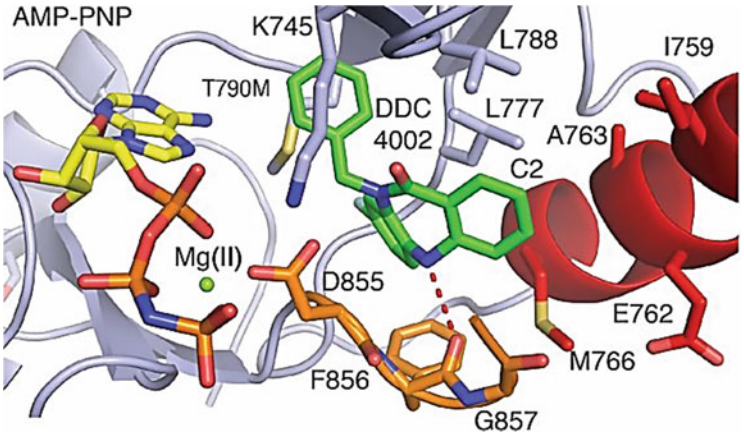
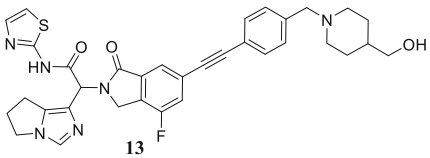
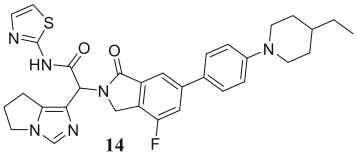


Fig. 1.17 Binding of **DDC4002 (9)** at the allosteric pocket. (Reprinted (adapted) with permission from De Clercq et al. 2019. Copyright 2019, American Chemical Society)



13

EGFR L858R/T790M/C797S IC_{50} = 3 nM



14

EGFR L858R/T790M/C797S IC_{50} = 4 nM

Fig. 1.18 Chemical structure and biological activity of **13** and **14**

(**15**) and orientated towards the solvent-exposed exterior (Fig. 1.19). DDC-01-163 (**16**) demonstrated reasonable potency in an in vitro biochemical experiment against EGFR L858R/T790M, with an IC_{50} value of 45 nM, comparable to its parent allosteric EGFR inhibitor JBJ-07-149 (**15**) (IC_{50} = 1.1 nM). DDC-01-163 (**16**) was further examined for its ability to inhibit mutant cell proliferation in Ba/F3 cell lines that had been stably transfected with either the EGFR L858R/T790M mutant or the wild type of EGFR. DDC-01-163 (**16**), which is 51-fold more powerful than its parent allosteric EGFR inhibitor (0.096 μM vs. 4.9 μM), substantially inhibited the growth of L858R/T790M mutant EGFR Ba/F3 cells (EC_{50} = 0.096 μM) while preserving wild-type EGFR Ba/F3 cells (EC_{50} > 10 μM). They performed competitive in vitro tests in which they treated L858R/T790M Ba/F3 cells with the CRBN ligand pomalidomide or the parent allosteric EGFR inhibitor JBJ-07-149 (**15**) in the presence or absence of 0.1 μM DDC-01-163 (**16**) to further establish the mechanism of action of DDC-01-163 (**16**).

Densitometry examination of western blotting revealed a significant reduction of EGFR protein to 44% in cells treated with DDC-01-163 (**16**) alone. Furthermore, even in the presence of DDC-01-163 (**16**), pomalidomide and JBJ-07-149 (**15**) inhibited the EGFR degradation, suggesting that EGFR degradation needed binding

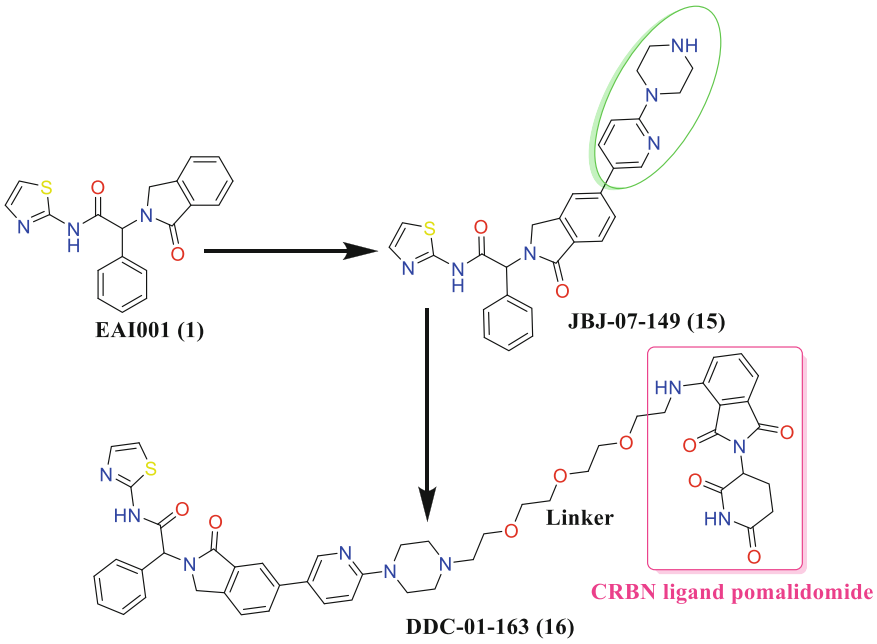


Fig. 1.19 Structure functionalization of EAI001 (**1**) yielded EGFR degrader DDC-01-163 (**16**)

to both EGFR and CRBN and confirmed that DDC-01-163 (**16**) antiproliferative action was substantially dependent on EGFR degradation. The researchers also showed that combining DDC-01-163 (**16**) with osimertinib dramatically shifted the dose–response curve, suggesting that the combination treatment was more effective at reducing cell proliferation than the single-agent treatment. DDC-01-163 (**16**) is a potential allosteric EGFR degrader with selective efficacy against several therapeutically relevant EGFR mutations whether used alone or in conjunction with an ATP site inhibitor.

Patel et al. (2021a, b) reported virtual screening of zinc compound library to search allosteric T790M/C797S EGFR inhibitor. From complete sequential filter of molecular docking, Lipinski's Rule of Five, ADMET prediction, and molecular dynamic simulation (MD) study, they found most promising compound ZINC20531199 (**17**).

Molecular docking study showed that ZINC20531199 binds to the Asp855 amino acid of the DFG motif via the amidal hydrogen of the ligands, which (DFG motif) is critical for regulating kinase activity in T790M/C797S EGFR. In MD study ZINC20531199 (**17**) exhibited the favourable interaction with the Asp855, Phe856, Met790, Leu754, and Lys745. These compounds demonstrated H-bond interactions with the Asp855 and Lys745 residues, which contributed to the ligand's binding affinity. The π–π stacking has been observed between Phe856 and the ZINC20531199 (**17**) phenyl ring, while Met790 and Leu747 interacted with