Chemical Scaffolds for the Clinical Development of Mutant-Selective and Reversible Fourth-Generation EGFR-TKIs in NSCLC

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Abstract

In non-small cell lung cancer (NSCLC), as well as in other tumours, the targeted therapy is mainly represented by tyrosine kinase inhibitors (TKIs), small molecules able to target oncogenic drivers' alterations affecting the gene encoding the Epidermal Growth Factor Receptor (EGFR). Up to now, several different TKIs have been developed. However, cancer cells showed an incredible adaptive tumour response to the inhibition of the sequentially mutated EGFR (EGFRM+) triggering the need of exploring novel pharmacochemical strategies. This review summarizes the recent efforts in the development of new reversible next generation EGFR's TKIs to fight the resistance against T790M and C797S mutations. Specifically, after giving an overview of the role of the EGFR's signaling pathways in cancer progression, we are going to discuss the most relevant approved drugs and drug candidates in terms of chemical structure, binding modalities, and their potency and selectivity against the mutated EGFR over the wild-type form. This could provide important guidelines and rationale for the discovery and iterative development of new drugs.

1. Introduction

The epidermal growth factor receptor (EGFR) represents the main druggable and strategic target in many cancer types, including non-small cell lung cancer (NSCLC). This receptor is frequently overexpressed and mutated, resulting in its aberrant activation. In detail, a single point mutation can induce the shift of the EGFR from its inactive state to the active one, leading to uncontrolled cell proliferation, angiogenesis, and invasion of cancer cells in healthy tissues. Therefore, in the last decades, the attempts of targeting cancer progression gradually shifted from the use of drugs against non-specific targets to more targeted ones. EGFR-targeted therapy is mainly represented by Tyrosine Kinase Inhibitors (TKIs), which enable the targeted treatment of patients harbouring activating mutations in the EGFR gene and provide better outcomes and lower toxicity compared to other treatment options. The development and design of effective and mutant-selective TKIs (ATP-competitive, allosteric, or covalent) is a clinically validated and effective strategy in the pharmaceutical industry. However, as for all the other targeted therapies, TKIs treatment induces the development of on-target drug resistance mechanisms, forcing researchers and companies to identify new chemical entities able to target the EGFR mutated forms, some of which are currently entering clinical trials. In this context, the chemical evolution of EGFR-TKIs represents a crucial aspect to be investigated when developing targeted cancer therapies. Indeed, the refinement and the optimization

of the already known TKIs' chemical structures might dramatically enhance their potency, selectivity and pharmacokinetic properties. Any new findings may eventually lead to changes in administration's guidelines or potential development of next-generation TKIs.

The purpose of this review is to provide an overview of the role of EGFR's signaling pathways in cancer progression followed by a comprehensive analysis of the evolution of the EGFR-TKIs with a particular focus on the main promising chemical scaffolds used for the design and development of the 4th generation TKIs, now entering phase II-III clinical trials for the treatment of metastatic NSCLC harbouring EGFR mutations.

2. Structure and EGFR signalling pathway in cancer

EGFR is one of the most known and described transmembrane tyrosine kinase receptors and it is part of the ERBB receptor family, which consists of: ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). All these receptors can form homodimers and heterodimers with one another, leading to a total of 28 different combinations. However, ERBB1/EGFR receptor is not present only in Humans. For instance, in *Caenorhabditis Elegans*, ErbB gene exists as a single receptor with a single ligand; in *Drosophila* ErbB consists also of a single receptor but it can interact with four different ligands. All the studies carried out in animals, such as *Drosophila*, have been extremely important and they have yielded a terrific amount of information, especially concerning embryogenesis, for which the expression of ErbB genes is critical in Vertebrates. It has been shown, indeed, that null mutations of any of the ErbB family members result in embryonic lethality in mice, with defects observed in organs, including the brain, heart, skin, lung, and gastrointestinal tract, depending on the receptor affected [1].

The EGFR-encoding gene is located on chromosome 7 short arm q22, spanning 110 Kb of DNA and consisting of 28 exons. It has been found that, in normal cells, the expression of EGFR corresponds roughly to 40,000-100,000 receptors per cell; in cancer cells, its overexpression leads to an accumulation of more than 1 million receptors per cell. The EGFR protein is translated as a 1210 residues precursor, consequently cleaved at the N-terminal region, and yielding the mature 1186 residues EGFR. As far as its structure is concerned, the receptor consists of: extracellular ligand-binding and dimerization domain (exons 1-16), transmembrane domain (exon 17), intracellular tyrosine kinase (TK) and C-terminal tail domains (exons 18-28) where important tyrosine autophosphorylation sites are located (**Figure 1**).

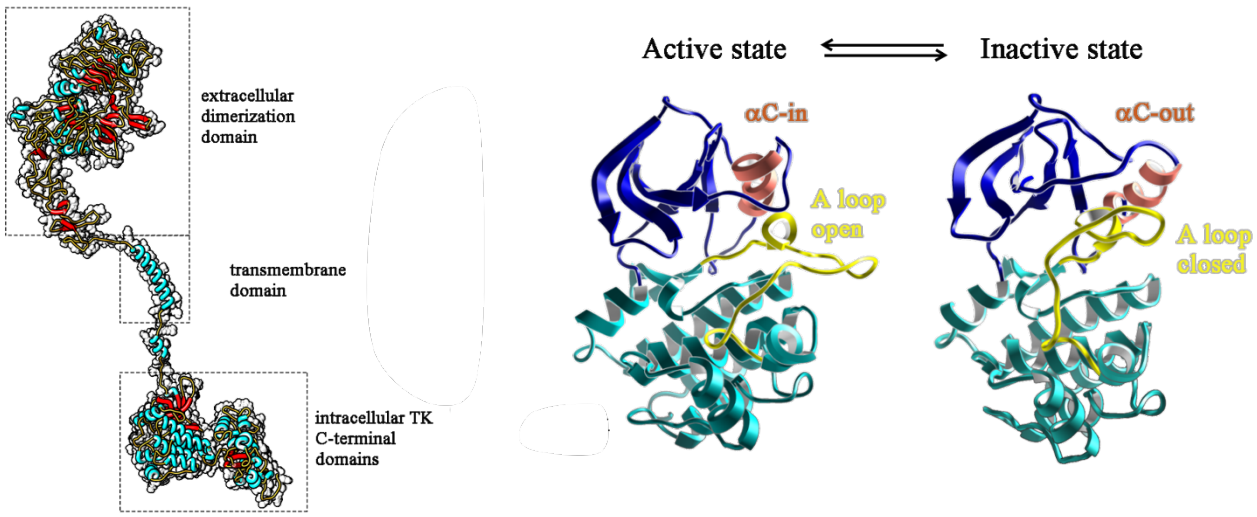


Figure 1. General structure of EGFR monomer and conformational features of active and inactive tyrosine kinase domain conformers. The α-helices, β-sheets, and coils are reported in cyan, red, and yellow respectively. The surface is reported in transparent Van der Walls spheres. The N- and the C- lobes are highlighted in blue and dark cyan respectively.

The TK domain has catalytic function and consists of an ATP-binding pocket located between the N-terminal and C-terminal lobes. Once the ATP binds this pocket, conformational changes are triggered in the EGFR structure, resulting in its activation. In the auto-inhibited inactive state, the short helix at the N-terminal of the activation loop is folded and the Glu762 residue of the αC-helix is rotated out of the catalytic site (αC-out) (**Figure 1**). The transition to the active state requires, therefore, the inward movement of the αC-helix (αC-in), which is kept in place by a salt bridge formed by Glu762 and Lys745 [2], while the short helix in the A loop undergoes unfolding.

Activating mutations in the EGFR kinase domain have been extensively described and they are considered as the main oncogenic driver mutations in NSCLC. Among the reported mutations, the most reoccurring ones in NSCLC are: exon 19 deletion (exDel19), L858R substitution at exon 21, T790M and C797S substitutions at exon 20. NSCLCs harboring EGFR L858R or exDel19 mutations are targeted by using 1st and 2nd generation TKIs, which are instead inactive against T790M and C797S mutations. To tackle these last two mutations, there was indeed a strong need to develop 3rd and even 4th generation TKIs, which will be discussed in more detail in this review later on. In any case, these mutations cause the hyperactivation of the kinase domain by inducing the stabilization of the active conformer [2–5] or the destabilization of the inactive one [6,7]. The following EGFR activation triggers downstream pro-survival signal pathways, which promote tumorigenesis in NSCLC cells, such as JAK-STAT, PKC-PLCγ1, MAPK/ERK and PI3K/Akt/mTOR signaling pathways. We will focus our attention mostly on the last two pathways, which are the ones that have been the most investigated and elucidated.

Historically, the first studies performed on animals and aimed at studying the epidermal growth factor (EGF) showed that EGF was able to trigger epidermal proliferation and keratinization [8]. Nowadays, we know that EGF can also stimulate cell proliferation, differentiation, migration and inhibit apoptosis. However, the EGF is only one of the ligands able to bind and activate EGFR, leading to its autophosphorylation at tyrosine residues located in the intracellular C-terminal tail (Y703, Y920, Y992, Y1045, Y1068, Y1086, Y1148, and Y1173). Once activated, the EGFR receptor is responsible for the propagation of a complex ramification of signaling pathways.

One of the most important pathways in mediating the biological response of the EGFR is without any doubt the **RAS-RAF-MEK-ERK MAPK** Pathway **(Figure 2)**. Following receptor activation, EGFR's Y1068 and Y1086 residues bind the SH2 domain of GRB2[9] (growth factor receptor binding protein 2), whereas EGFR's Y1148 and Y1173 residues bind the SH2 and PTB domains of SHC (Src homology and Collagen protein). The binding of EGFR to SHC leads to SHC's phosphorylation at Y317, which is the binding site for GRB2, therefore stabilizing GRB2/SHC interaction[10]. At this point, GRB2 binds the proline-rich C-terminal tail of SOS1 (son of sevenless 1 protein) through its SH3 domain. SOS plays an important role as guanine nucleotide exchange factor (GEF) for RAS guanosine triphosphate (GTPase), inducing the exchange of GDP to GTP and, therefore, activating RAS [11]. Activated RAS interacts with RAF-1 by its Ras-GTP-binding domain (RBD), triggering a series of events leading to RAF-1 phosphorylation at Ser338 and Tyr34[12], which are important binding sites for MEK1/2. RAF-1 can now activate MEK1/2 by phosphorylating it at Ser217 and Ser221 [13]. MEK 1/2 (mitogen-activated protein kinase kinase) consists of tyrosine and threonine/serine dual-specificity kinases and one of their main functions is activating ERK1/2. MEK1/2 phosphorylates, indeed, T202 and Y204 residues in the Thr-Glu-Tyr motif within ERK1/2's activation loop, leading to its activation [14]. Activated ERK1/2 phosphorylates multiple substrates to trigger a variety of physiological responses, including growth, differentiation, migration, and inhibition of apoptosis. In detail, it has been shown that activated ERK1/2 enhances, for instance, the transcription of MMP-2 through the upregulation of the transcription factor GATA-2, a DNA-binding protein able to specifically recognize the DNA sequence T/AGATAA/G. MMP-2 is part of the MMPs (matrix metalloproteinase proteins), a family of zinc- and calcium-dependent proteases known for their ability to cleave a wide subset of extracellular matrix proteins and, by doing so, able to regulate a variety of cellular processes, including attachment, migration, invasiveness, proliferation, and apoptosis [15].

Another crucial pathway in mediating the biological response of the EGFR is **PI3K-AKT-mTOR signaling** cascade (**Figure 2**). In cancer, the normal functionality of this pathway is often altered due to mutations affecting the EGFR family members and the down-modulation of the tumor suppressor

PTEN, responsible for dephosphorylating PIP3 to PIP2. As far as PI3K is concerned, it consists of two different domains: a regulatory p85 subunit, responsible for binding the EGFR receptor, and a catalytic p110 subunit, which is able to phosphorylate the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), a key secondary messenger acting as a linker between the lipid kinase activity of PI3K and an extensive network of downstream signaling pathways. More in detail, there are three classes of PI3K (I, II, and III), but class I PI3K seems to be the main downstream effector of EGFR.

The interaction between PI3K and EGFR is, however, indirect and precisely mediated by the adaptor protein GAB1 (GRB2-associated binder), that binds the SH3 domain of GRB2 protein through its proline-rich domain. This event triggers GAB1's phosphorylation at residues Y446, Y472, and Y589, important binding sites for the p85 subunit of PI3K [16]. Through the activation of PI3K-induced PIP3, activated EGFR stimulates the translocation towards the plasma membrane of AKT, which is then phosphorylated at T308 and S473 [17]. The phosphorylation of T308, a residue located in the kinase domain, would be sufficient for AKT activation; nevertheless, maximal activation is achieved by the concomitant phosphorylation at S473, located instead at the tail domain. More in detail, PDK1 is the kinase responsible for T308 phosphorylation, whereas mTORC2 is considered to be responsible for S473 phosphorylation. AKT mediates a variety of physiological responses, among which cell survival and it does so by phosphorylating components of cell death machinery. AKT phosphorylates the protein BAD (Bcl-2-associated death promoter) at residue S136, preventing its inhibitory activity towards the anti-apoptotic BCL-XL [18]. AKT also inhibits the apoptotic activity of caspase-9 by phosphorylating it at residue S196 [19], and the catalytic activity of FOXO1 by phosphorylating it at residues T32 and S253 [20]. Another important phosphorylation event mediated by AKT is MDM2's phosphorylation at residue S166, which enables MDM2 translocation to the nucleus, where it ubiquitinates and downregulates the tumor-suppressor p53 [21]. Moreover, AKT directly phosphorylates TSC2 protein (Tuberous Sclerosis Complex 2) on five residues (Ser939, Ser981, Ser1130, Ser1132 and Thr1462), therefore deactivating it and activating mTORC1, which is normally repressed by TSC2 [22]. mTORC1, mammalian target of rapamycin, can therefore phosphorylate 4E-BP (eukaryotic initiation factor 4E-binding protein) and S6K (p70 S6 kinase), which are, respectively, an inhibitor of translational inhibition and an activator of translation. Eventually, mTOR activation leads to the increased synthesis of proteins like cyclin D1 (CCND1). The dysregulation of CCND1 irremediably compromises the S-phase checkpoint, inducing forced progression of the cell cycle, disrupting DNA replication, and, therefore, promoting DNA damage and genomic instability, resulting in oncogenesis. Considering that D-type cyclins don't possess any enzymatic function, nowadays one of the most promising strategies to tackle cyclin D-based disorders involves targeting

the enzymatic activity of their partners (CDK4 and CDK6), even though this approach may lead to toxic side effects due to lack of selectivity of the currently available inhibitors [23].

Of note, in the literature it is also reported that EGFR activation promotes NSCLC cell migration through the secretion of cathepsin B by **NEDD4 E3 ubiquitin ligase**. Upon binding of its specific ligands, activated EGFR also stimulates the release of calcium from the ER pool to the cytoplasm and, as a result, NEDD4 protein is activated and recruited to the EGFR-loaded endosomes. On one hand, NEDD4 regulates the transport of the EGFR-loaded endosomes to the multivesicular bodies (MVBs) and lysosomes. On the other hand, the activated NEDD4 stimulates the secretion of cathepsin B, which degrades the extracellular matrix protein and cell-to-cell junction protein to initiate metastasis [24].

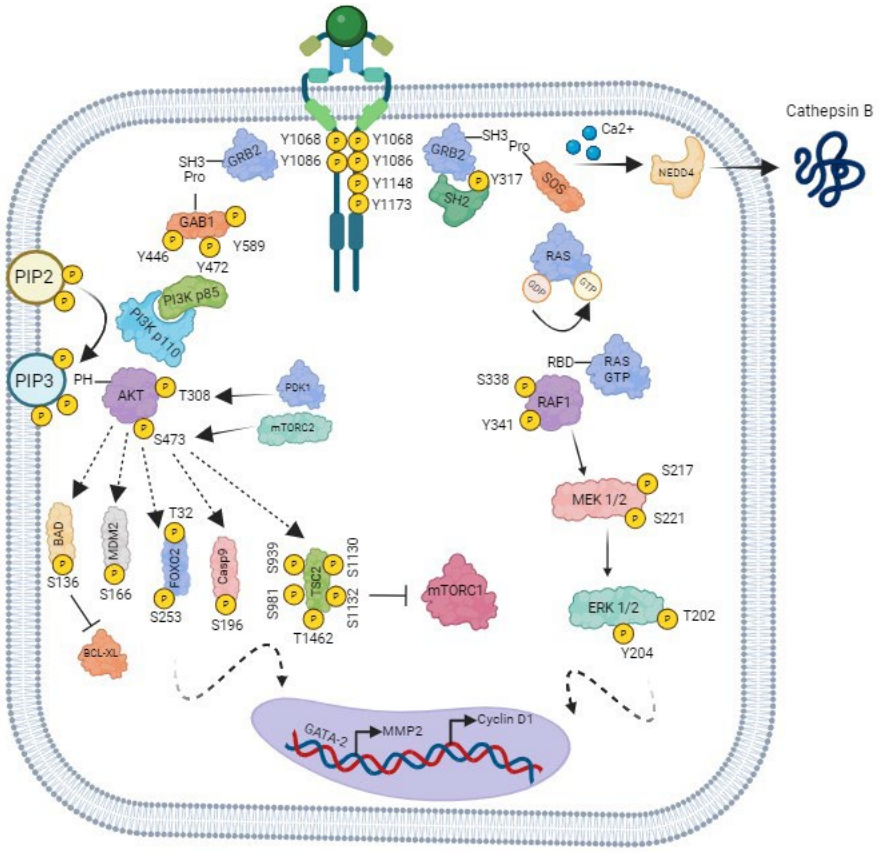


Figure 2. EGFR receptor and major downstream signaling pathways in non-small cell lung cancer. Upon ligand binding, the switch of EGFR to its active conformation triggers a series of complex and interconnected cellular pathways, including RAS-RAF-MEK-ERK MAPK, PI3K-AKT-mTOR and NEDD4/Cathepsin cascades, which are important in mediating physiological processes such as growth, differentiation, migration, and inhibition of apoptosis.

3. EGFR kinase ATP recognition and the role of mutations on the EGFR activation

It is well known that the catalytic domain of EGFR is located at the interface between the N- and the C- lobes and that the binding pocket is specific for the insertion of the ATP molecule. As shown in **Figure 3**, the adenine moiety anchors the ATP to the hinge region through the formation of two

specific hydrogen bonds with the kinase polar residues Gln791 and Met793, while establishing different interactions with the hydrophobic clamp motif of the ATP-binding site (Leu844, Val726, Ala743, Leu718, and Leu792) (**Figure 3A**). In addition, the hydrophilic phosphate moiety is kept in position by a specific interaction with the catalytic residues Lys745 and Asp855 in the Asp855-Phe856-Gly857 (DFG)[25].

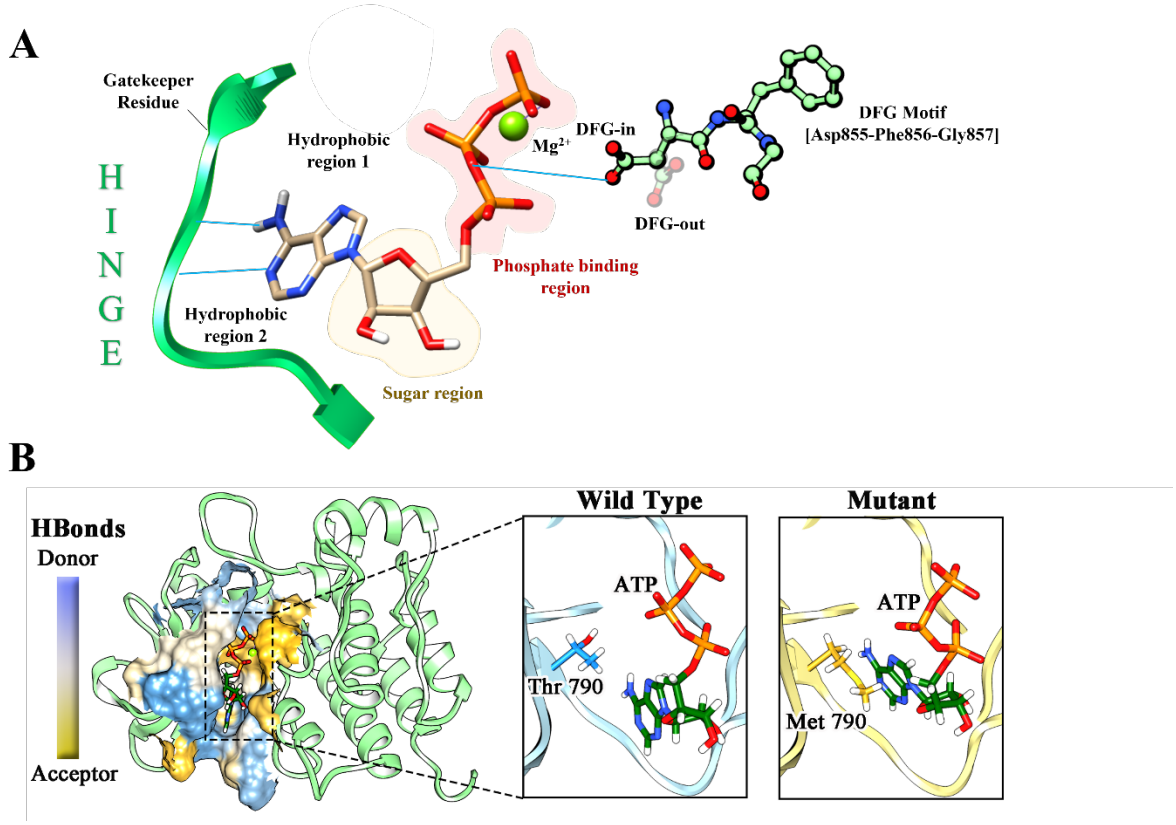


Figure 3. (A) Pharmacophore of ATP inside the ATP-binding pocket of EGFR. **(B)** H-Bond donor and acceptor maps of ATP inside wild type EGFR. Comparison of the close views between the structures of wild-type Thr790–ATP and mutant Met790–ATP in EGFR active site. No effective interaction of ATP is observed with Thr790 while a π-interaction can be observed between adenine and Met790.

This DFG motif shows the conformation of active kinases where Asp855 is oriented towards the ATP binding site, allowing the magnesium ion to bind to the β- and γ-phosphate groups of ATP, and leading to the DFG-in conformation [26]. However, this DFG motif flips outward in inactive kinases, causing the Asp855 not to be able anymore to coordinate the magnesium ion into the catalytic site, therefore promoting the DFG-out conformation [27]. An alteration in the orientations of Asp855 and Phe856 is found in the above-mentioned "out" form, in which phenylalanine occupies the in-position of aspartate[4]. Based on the mutagenesis studies, the so called "gatekeeper" residue can influence the affinity of kinases towards ATP and therefore the stabilization degree of the DFG-out conformation in the inactive EGFR conformer. This residue is the 790, and the name "gatekeeper" is due to its bulky size and to its position, that control the accessibility of the ATP or other molecules to the inner

hydrophobic binding pocket [28]. More in details, the substitution of the Thr790 with Met790 has been known to cause resistance to ATP-competitive TKIs inhibitors due to the enhanced affinity for ATP[29]. Our recently study confirms that the Met790 in EGFR T790M mutant stabilizes the ATP binding by a typical S-π interaction with the aromatic adenine moiety [2,30,31] (**Figure 3B**) and this correlates with the experimentally observed changes in Michaelis-Menten constant (K_m) with respect to wild-type EGFR[2,32]. Moreover, it is important to underline that the loss of efficacy of some TKIs in the EGFR mutants containing T790M mutations is also explained by the steric hindrance due to the presence of the bulky methionine which hinders an optimal binding inside the ATP binding pocket. Overall, the mutations in the ATP binding pocket directly affect the binding mode of a natural ligand: a higher ATP stabilization leads to a higher affinity of the EGFR to its own substrate, thus weakening the potency of ATP-competitive TKIs.

Some specific mutations can also affect the coordination degree of magnesium ions and the affinity towards ATP. Following our computational studies, we could demonstrate that ATP was able to form complexes with some EGFR mutated forms (EGFR L858R, EGFR T790M/L858R/L844F, and EGFR L858R/T790M/C797S) showing weak Mg^{2+} coordination[2]. However, ATP showed an optimal binding pose with the EGFR T790M, in line with the one observed in wild-type EGFR. The simultaneous presence of T790M and L858R restores indeed the optimal Mg^{2+} coordination, leading to an increase in the affinity towards ATP and a decrease in the K_m values, experimentally provided by kinetic studies[29,33]. Interestingly, the lower ATP affinity identified for the L858R and L858R/T790M/L844F mutations with respect to L858R/T790M or T790M mutations, could also depend on the effect of the hydration's degree on the ligand-protein stabilization. Moreover, molecular dynamics' simulations reveal higher ATP hydration only in EGFR L858R and EGFR L858R/T790M/L844F, showing, therefore, a negative correlation between the stability of ATP-EGFR complex and the number of water hydrogen bonds. On the other hand, both EGFR L858R/T790M and EGFR T790M make a water bridge with Cys797, highlighting its crucial role in the adenine moiety's orientation and in the observed higher K_m.

Finally, it has been demonstrated that the ATP can switch the inactive-active equilibrium, and the amplitude and direction of this trend is strictly correlated to the aminoacid pattern bound, *i.e.* mutations [2,34]. A perfect example of this phenomenon is the L858R mutation which seems to increase the stability of the active conformation through specific interactions involving the mutated residue (Arg858) and αC-helix aminoacids[2,35]. However, the direct interaction between Arg858 and the phosphate moiety of ATP could interfere with the natural tendency of EGFR L858R to switch to the active conformation increasing therefore the K_m value[2].

4. Chemical scaffold evolution from first to the third generation TKIs: a brief overview

The identification of ATP binding patterns could certainly be useful to pave the way for cancer therapy and to develop more personalized medicine, depending on the specific EGFR mutant. The mutant-specific changes in the EGFR structure and in the ATP affinity are most likely the key for the success of EGFR-directed TKIs in treating lung cancers driven by these mutations.

Historically, the evolution of the TKIs' chemical scaffolds begins with the use of erlotinib and gefitinib as the 1st generation of TKIs, which are able to target selectively exon 19 deletion [exDel19] or L858R point mutation at exon 21 [L858R][36]. These inhibitors consist of 4-anilinoquinazolines-based reversible TKIs and bind the ATP-binding pocket inside the TK domain of EGFR (**Figure 3**). However, during treatment, 60-70% of the patients naturally acquire a second mutation of the gatekeeper residue (T790M)[37]. Following our and other studies, it has been demonstrated that the substitution of the residue threonine to methionine sterically hinders the binding of erlotinib and gefitinib to the EGFR [37,38]; however, the main mechanism by which T790M confers drug resistance seem to be the enhancement of the ATP affinity[2,29]. The 2nd generation TKIs have been, therefore, designed and developed to address T790M resistance. These agents covalently bind to the EGFR and, irreversibly, inhibit the binding of the ATP to the TK domain, even in the presence of T790M point mutation. These TKIs showed higher efficacy compared to erlotinib and gefitinib, due to their ability of inhibiting EGFR activation for longer time. The two most effective compounds so far are dacomitinib (Pfizer)[39] and afatinib (Boehringer-Ingelheim)[40] (**Figure 4**).

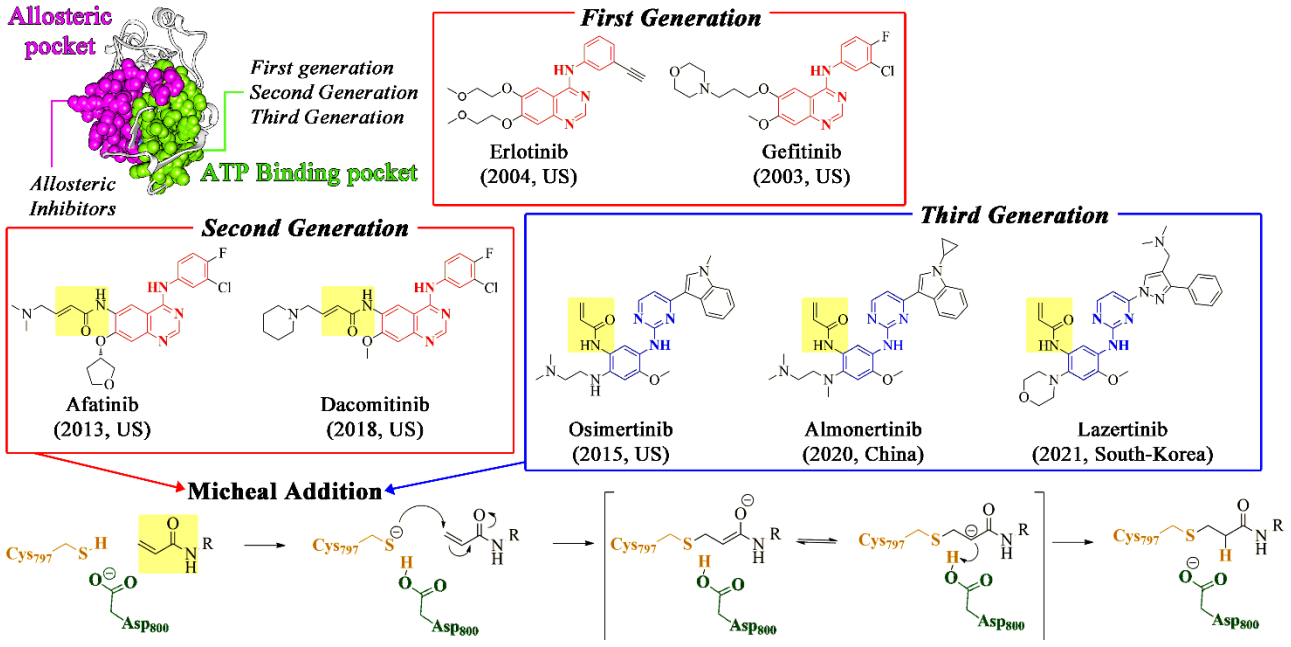


Figure 4. Chemical structures of the main globally launched EGFR-TKI, belonging to the first, second and third generations. In red and blue the 4-anilinoquinazoline and 2-anilinopyrimidine chemical scaffolds are respectively

highlighted, whereas in yellow the covalent warhead group is indicated. Micheal addition reaction mechanism proposed for the nucleophilic attack of Cys797 to the electrophilic β-carbon of an acrylamide moiety assisted by Asp800.

As for the first-generation TKIs, the 2nd generation TKIs have an anilino quinazoline core (highlighted in red in **Figure 4**) with the addition of the acrylamide warhead group, able to form a covalent bond with the sulfur atom of Cys797. However, 2nd generation TKIs' usage is limited, due to their lower selectivity against mutant EGFR and potential epithelium-based toxicities (e.g., skin rash). Moreover, the presence of a quinazoline moiety in their structure clashes with Met790's side chain, requiring therefore the administration of relatively high doses[41]. To overcome these issues, 3rd generation TKIs have been developed. They have as well an irreversible mode of action, but they show higher selectivity against T790M point mutation, due to their different structure; unlike the previous generation of TKIs, this class of compounds shares an anilinopyrimidine scaffold (highlighted in blue in **Figure 4**) which contributes to maximize and stabilize their interaction with the ATP binding pocket. One of the most recent 3rd generations TKIs, osimertinib, developed by AstraZeneca in the United States, dominated the global landscape of treatment of EGFR-positive NSCLC for several years and it is currently the standard of care for the 1st-line treatment of advanced EGFR-mutated NSCLC and EGFR T790M-positive NSCLC after 1st- or 2nd-generation EGFR-TKIs. During the last years, a 3rd generation of new TKIs were lunched, and their clinical development is extensively reviewed by recent publications[42,43]. Among them, the most promising active inhibitors include Almonertinib, developed by Jiangsu Hansoh Pharmaceutical Co., Ltd[44], and Lazertinib (Leclaza) which is under investigation by Johnson & Johnson and currently in Phase III for Non-Small Cell Lung Cancer[45] (**Figure 4**). Unfortunately, like other targeted therapies, new EGFR mutations emerged. The substitution of thiol by hydroxyl on residue 797 (C797S mutation), which prevents the formation of a covalent bond between EGFR and the Michael acceptor of 3rd generation TKI, so far represents the main on-target resistance mechanism to osimertinib (22-40% of all mutations)[46,47]. This discovery led the researchers to explore several strategies to overcome treatment resistance in patients with EGFR-mutated NSCLC such as the dual blockade of EGFR using anti-EGFR monoclonal antibody and EGFR-TKIs (reviewed in[48]) as well as the identification of lead compounds able to overcome EGFR activating mutations/T790M/C797S (triple mutants) with different mode of action, while retaining the specificity against mutant EGFR. After years of innovative research, a next generation of EGFR-TKIs has been developed and it is known as 4th generation TKIs. Some of these molecules have been already evaluated in pre-clinical stages and phase I-III clinical studies, and they are considered promising candidates to overcome the resistance to the previous FDA-approved TKIs.

5. The next 4th TKIs generation

In the last years, scientists have proposed several pharmacochemical strategies for the treatment of osimertinib-resistant NSCLC patients. As a result, groundbreaking 4th generation TKIs, able to target triple mutants, have been discovered. Although they are designed to target specifically C797S mutation, most of them are highly effective also against EGFR L858R/T790M and exDel19/T790M, paving the way for the use of these compounds both as alternative to osimertinib and as a 2nd-line treatment. In fact, considering their reversible binding, the occurrence of C797S mutation in T790M-positive NSCLC is expected to be highly unlikely.

Overall, this next generation of TKIs includes both allosteric and ATP-competitive EGFR inhibitors, whose structures are based on different chemical scaffolds. In this review, we are going to discuss mainly the 4th generation of TKIs, which are currently undergoing a thorough evaluation in clinical studies in patients with metastatic EGFR-mutant NSCLC (**Table 1**).

Table 1. 4th generation of TKIs evaluated in pre-clinical stages and phase I-III studies for NSCLC.

Drug	Company [49]	Target Mutation	Chemical Scaffold	Stage
EAI045	Novartis	T790M/C797S L858R/T790M L858R/T790M/C797S	Oxoisoindoline phenyl-acetamide	Pre-clinial
JBJ-04-125-02	Johnson & Johnson	T790M/C797S L858R/T790M L858R/T790M/C797S	Oxoisoindoline phenyl-acetamide-piperazine	Pre-clinical
EAI-432	Collaboration with Takeda Pharmaceuticals	T790M/C797S L858R/T790M L858R/T790M/C797S	Oxoisoindoline series	Pre-clinical IND-enabling studies
CH7233163	Roche Pharma	T790M/C797S L858R/T790M L858R/T790M/C797S exDel19/T790M/C797S	N-(pyridin-2-yl)pyrimidin-4-amine containing 1-(cyclopropylsulfonyl)-1H-pyrazole	Pre-clinical
Brigatinib	Takeda Pharmaceuticals	L858R/T790M/C797S exDel19/T790M/C797S T790M/C797S	2,4-bis anilinopyrimidine containing dimethylphosphine oxide	FDA-approved
TQB3804	Chia Tai Tianqing	L858R/T790M/C797S exDel19/T790M/C797S	2,4-bis anilinopyrimidine containing dimethylphosphine oxide	Phase III
BLU-945	Bluprint Medicines	L858R/T790M L858R/T790M/C797S	N-(pyridin-2-yl) pyrimidin-4-amine containing piperidine	Ph1/2 Symphony trial, but development being focused on 1st-line Osimertinib combo

5.1. *Allosteric EGFR inhibitors: the oxoisoindoline phenyl-acetamide derivatives*

The development of mutant-selective allosteric inhibitors represents a promising strategy to overcome the acquired resistance to the latest FDA-approved TKIs. Unlike the ATP-competitive TKIs, they specifically bind to the allosteric pocket located between the αC-helix and the A-loop (**Figures 1 and 4**). Interestingly, the allosteric pocket is not accessible either in the active or in the inactive state of EGFR, but it can be accessed only in the presence of specific mutations, such as

L858R, L858R/T790M and L861Q, which destabilize the inactive state of the EGFR in favour of the active one. During this conformational change, the unfolding of the short helix in the A loop induces, indeed, the formation of a transient intermediate state where the allosteric pocket is well exposed[5]. The consequent binding of these inhibitors stabilizes the inactive αC-out conformation preventing the activation of the kinase. Therefore, the mutant selectivity of allosteric inhibitors depends on the intrinsic flexibility of the mutant kinases. The early allosteric inhibitor EAI001 was identified by Jia Y and coworkers in 2016[50]. It is an oxoisoindoline phenyl-acetamide derivative displaying a promising mutant-selective inhibitory activity (**Figure 5A**). The X-ray structure of EAI001 bound to T790M-mutant EGFR showed that the compound binds the allosteric pocket with a "three-bladed propeller" shape. The aminothiazole moiety of EAI001 locates between the mutant gatekeeper Met790 and active site residue Lys745; the NH group of the formamide of the carboxamide forms a hydrogen bond with Asp855 in the DFG motif (**Figure 5B-C**); the 1-oxoisoindolinyl group extends along the C-helix, it makes a HB with ε-amine of the Lys745 side chain, and it is stabilized by several π-alkyl interactions. The π-sulfur interaction between the mutated Met790 and aminothiazole group plays a key role in the binding's stabilization and, consequently, in the mutant-selective activity of EAI001: the hydrophilic side chain of wt Thr790 hinders a stable interaction of thiazole group inside the pocket.

Afterwards, the introduction of ortho-hydroxyl and meta-fluorine atoms on the phenyl group of EAI001 led to the identification of EAI045 (**Figure 5A**) with an IC50 of 3 nM against the L858R/T790M mutant and a ~1000-fold selectivity over the wild-type EGFR[50]. The binding mode of EAI045 closely overlapped with the one from EAI001 and, additionally, a hydrogen bond was taking place between the hydroxyl group of the phenyl moiety and the backbone carbonyl group of the Phe856 residue of the DFG motif **Figure 5C** [51].

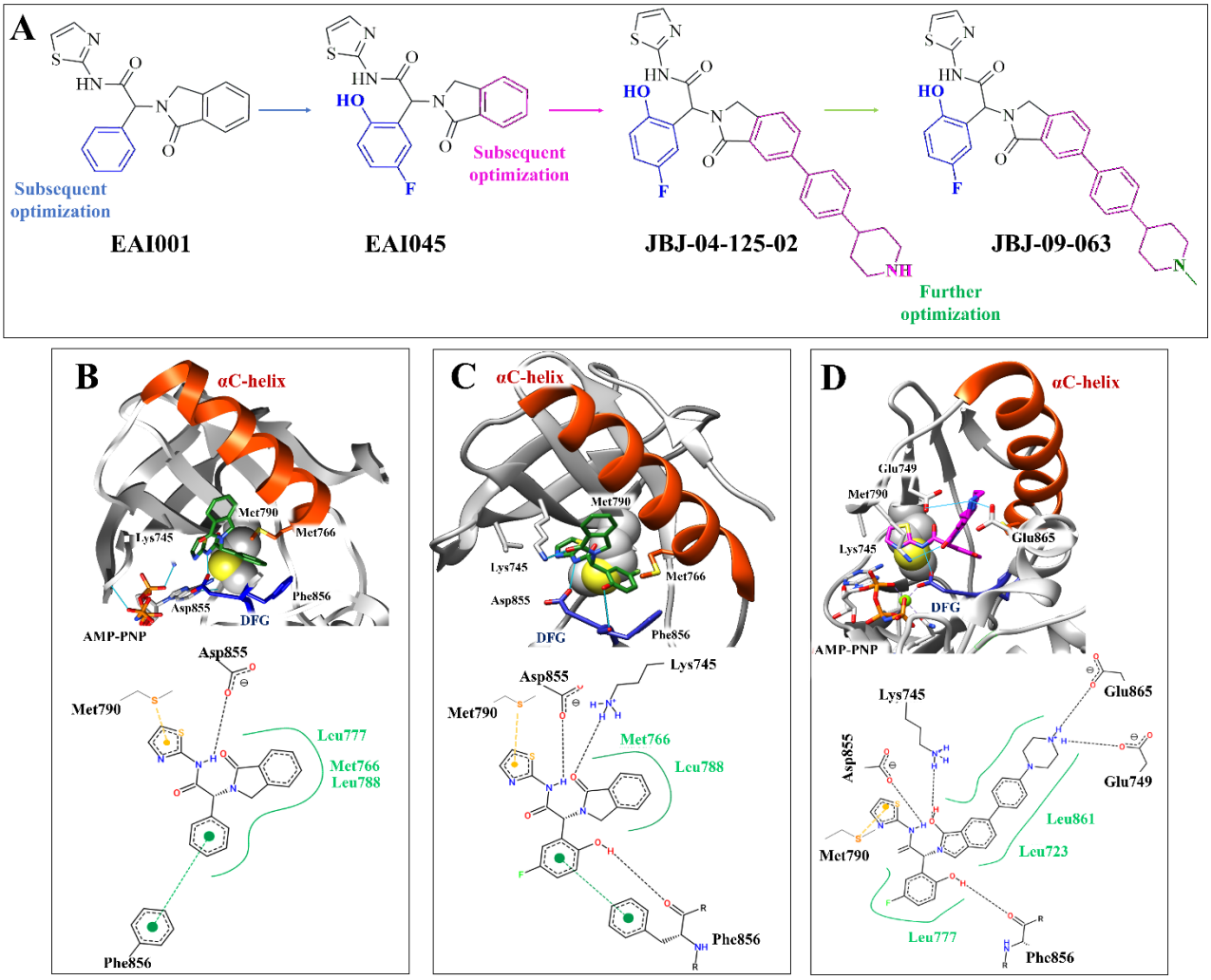


Figure 5. Structure, activity, and binding mode of allosteric EGFR inhibitors. **(A)** Optimization process from EAI001 to JBJ-04-125-02 and enzymatic inhibitory activity of compounds against wild type and mutants EGFR. **(B)** Overall view of the x-ray structure of EGFR (T790M/V948R) bound to EAI001 and AMP-PNP (PDB: 5D41), **(C)** EGFR T790M/C797S/V948R in complex with EAI045 (PDB: 5ZWJ) **(D)** and of EGFRT790M/V948R bound to JBJ-04-125-02 and AMP-PNP (PDB: 6DUK). EAI001, EAI045 and JBJ-04-125-02 are shown in CPK-coloured form with carbon atoms in light green, forest green and magenta, respectively, while hydrogen bonds are indicated as light blue lines. 2D interaction maps is obtained by means of poseview and discovery studio software.

EAI001 and EAI045 are, however, unable to inhibit EGFR in cells when administered as single agents. This behaviour is likely due to the asymmetric ligand-induced dimerization of EGFR, where the activator subunit, interacting with the ligand, promotes an active αC-in conformation in the receiver subunit, making the allosteric binding site inaccessible[50]. To promote the binding of the ligand to both subunits, it is therefore important to combine EAI045 with the monoclonal antibody cetuximab, which acts as an EGFR-dimer disrupting agent. Nevertheless, cetuximab is not EGFR-mutant specific, thereby resulting in on-target wild-type EGFR-mediated skin toxicities (reviewed in the manuscript[52]).

In order to develop a single-agent mutant-selective allosteric EGFR inhibitor, EAI001's and EAI045's scaffolds have been structurally modified, and other more effective allosteric inhibitors have been identified. To et al introduced a 5-indole substituent at the C-6 of the isoindolinone moiety of EAI001 obtaining an allosteric inhibitor (JBJ-02-112-05) with enhanced potency compared to EAI001 towards EGFR L858R/T790M but lower potency than to EAI045[53]. Further optimization led to JBJ-04-125-02 which incorporates the potency-enhancing 2-hydroxy-5-fluorophenyl group of EAI045 and a phenylpiperazine substituent on the C6 of isoindolinone (**Figure 5A**). JBJ-04-125-02 binds the allosteric pocket similarly to EAI001 (**Figure 5D**). In addition, the piperazine group makes a π-π stacking interaction with Phe723 and a bidentate HB with Glu865 and Glu749, thereby inducing a novel conformation of the A-loop. As a result, JBJ-04-125-02 shows higher potency compared to the EAI series. Moreover, the kinome profiling at 10 μM of JBJ-04-125-02 against a panel of 468 kinases reveals an excellent selectivity with S-Score of 0.02, only three off-targets not belonging to ERBB family (MAP4K5, TIE1 and TIE2) and no significant weight loss or toxicity is observed in the *in-vivo* efficacy studies. Even though this new compound seemed to be effective as single agent *in both vitro* and *in vivo* engineered models, unfortunately it did not work in patient-derived lung cancer cell lines or xenograft models[53]. This disconnect is likely attributed to the levels of EGFR dimers, whose increase is caused by the higher EGFR expression and/or EGFR ligands' concentration. Moreover, as for EAI001 and EAI045, JBJ-04-125-02 cannot overcome the resistance exhibited by the triple mutant containing EGFR exDel19. This mutation affects, indeed, the αC-helix conformation, reducing the volume of the allosteric pocket and, therefore, the proper accommodation of the ligand[50,53]. Since the allosteric EGFR's inhibition seems to be a promising approach to overcome T790M and/or CT797S resistance, several researchers and companies, such as Hoffmann-La Roche Inc. and Dana-Farber Cancer Institute, are ongoing designing and optimizing allosteric inhibitors for EGFR[53–57] in order to come up with compounds able to exhibit effective and selective single agent activity. In particular, in the last years, To, Gero, Scott et al identified a novel JBJ-04-125-02 analogue containing a terminal N-methypiperidine ring in place of the piperazine ring with improved enzymatic and cellular efficacy named **JBJ-09-063** (**Figure 5A**)[56,57]. The co-crystal structure of JBJ-09-063 in complex with EGFR T790M/V948R (PDB 7JXQ) confirms an allosteric binding mode with a similar network of interactions to that shown by JBJ-04-125-02. However, JBJ-09-063, in certain conditions, is susceptible to hydrolysis to the corresponding acid and therefore, the same authors chemically modified its structure in attempt to mitigate the hydrolysis risk. Very recently, starting from this isoindolinone series (**Figure 5A**), Dana-Farber Cancer Institute advances CNS-penetrant allosteric EGFR inhibitor **EAI-432** able to potently target L858R/T790M, L858R/C797S, and L858R/T790M/C797S while sparing wild type EGFR. It is currently progressing through

Investigational New Drug (IND)-enabling studies, to be completed in the second half of 2024. As for the JBJ-04-125-02[56,58] and JBJ-09-063[56], EAI-432 co-binds with osimertinib, allowing double-drugging of the mutant receptor[59].

5.2. ATP competitive reversible TKIs

The targeted treatment of mutation-driven NSCLC is evolving rapidly, and there are now at least five 4th-generation ATP competitive reversible EGFR-TKIs undergoing clinical trials. Overall, all these compounds share an anilinopyrimidine scaffold, thereby highlighting the important role played by this heterocycle moiety in the interaction with the ATP binding pocket.

5.2.1. 2,4 bis anilinopyrimidine containing dimethylphosphine oxide

Brigatinib (**Figure 6**) was discovered in 2016 by Huang et al, who successfully designed and synthesized several 2,4-diaminopyrimidine-based compounds containing a phosphine oxide (DMPO) moiety, in the attempt of searching for more effective anaplastic lymphoma kinase (ALK)–TKIs[60]. The kinome scan, performed to evaluate the kinase selectivity profile of Brigatinib, shows the already known ability to strongly inhibit ROS1, FLT3, and ALK with a high degree of selectivity against a broad panel of more than 250 kinases[60,61].

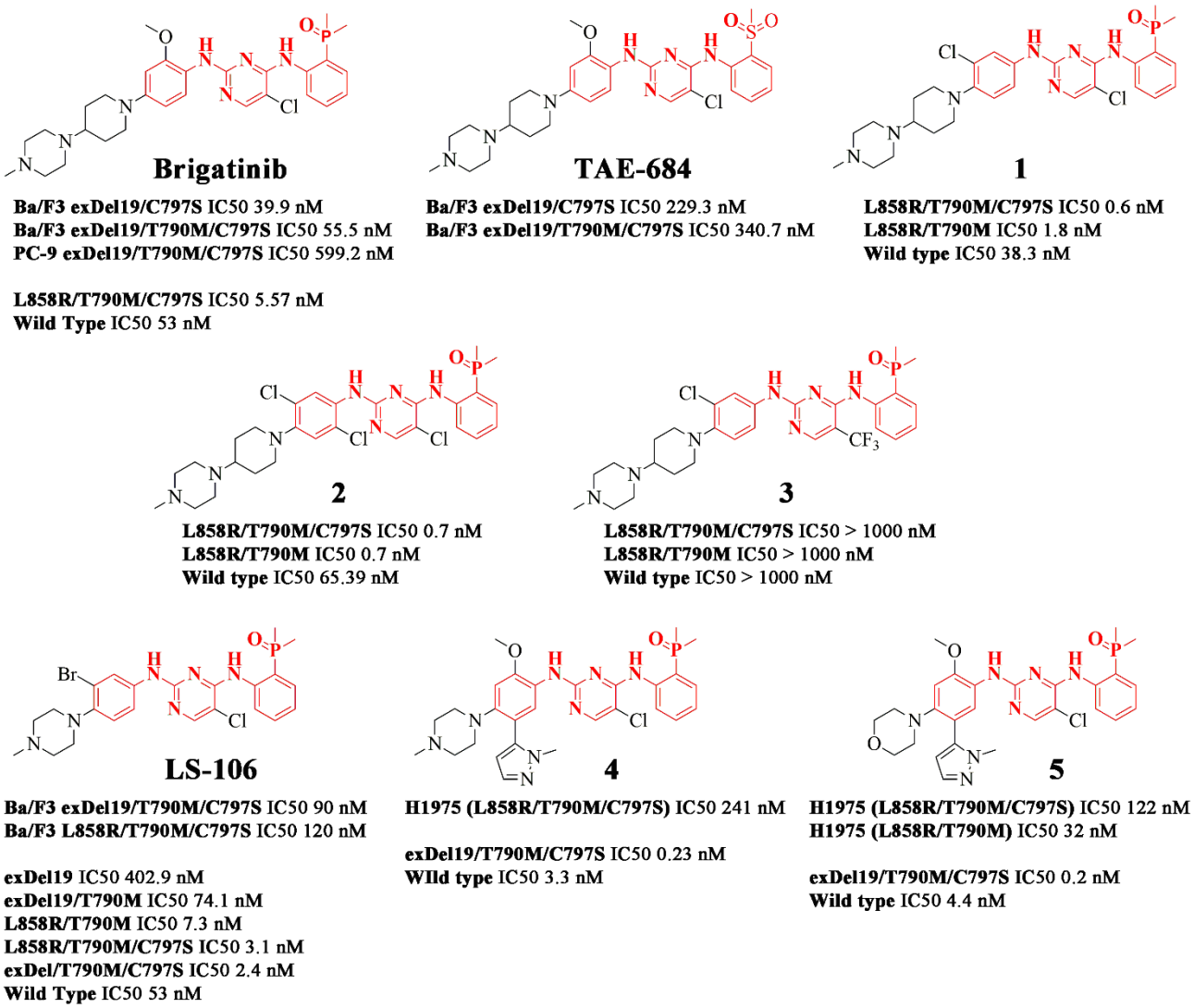


Figure 6. Comparison of Brigatinib and structural analogues in various EGFR mutation.

Moreover, in 2017, Uchibori et al performed an *in-silico* drug screening and found out that Brigatinib is a promising and selective TKI against triple mutant EGFR L858R/T790M/C797S (**Table 1**), especially when combined with Cetuximab [62]. The same compound demonstrated to be effective against other EGFR mutants as well, including EGFR T790M-cis-C797S and triple-exDel19[63,64]. To determine the mechanism by which brigatinib inhibits L858R/T790M/C797S mutant EGFR, *in silico* docking and molecular dynamic's (MD) simulations have been performed and showed that the compound is able to bind selectively inside the ATP-binding pocket without sterically hindering T790M or C797S[62]. Moreover, Brigatinib establishes a bidentate HB with the backbone amide of M793, which seems to be a key pharmacophoric feature for anchoring inhibitors to the hinge region[3,65]. The cocrystal structure of Brigatinib in complex with T790M/C797S-mutated EGFR (PDB 7ZYM) shows that the compound displays a binding mode identical to the one shown inside the ATP-binding pocket of the EGFR L858R/T790M/C797S (**Figure 7**).

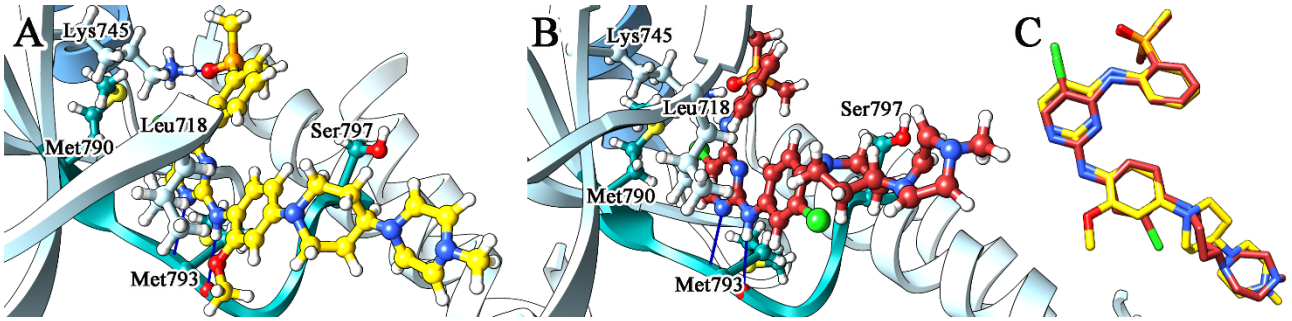


Figure 7. The X-ray crystal structure of Brigatinib **(A)** and compound 1 **(B)** complexed with the EGFR T790M/C797S protein (PDB: 7ZYM and 7ER2). **(C)** Docking poses of Brigatinib and compound 1 in the ATP binding pocket. The hinge region is highlighted in cyan.

Structure–activity-relationship (SAR) studies of 2,4-dianilinopyrimidine compounds as potent and selective TKIs targeting EGFR T790M/C797S have been studied to understand the chemical determinant for an optimal binding to EGFR mutants and, therefore, for an effective inhibition of the EGFR's phosphorylation (**Figure 6**)[62,66–68]. Replacing the phosphine oxide moiety with isopropylsulfonyl group (TAE684) leads to a lower activity against L858R/T790M/C797S EGFR[62]. However, the addition of 2-chloride on the aniline moiety (**1**) or the presence of a 2,5-dichloride (**2**) increases the EGFR inhibition activity of Brigatinib[66]. The X-ray crystal structure of compound **1** complexed with EGFR T790M/C797S (PDB 7ER2) (**Figure 5B**) shows a similar binding mode to the one of Brigatinib including the anchoring of the compound to the hinge region through a bidentate HB. Moreover, the phosphine oxide group is involved in a HB with Lys745 while the 5-chloride on the pyrimidine ring is directed towards the gatekeeper residue Met790, establishing a hydrophobic interaction. Therefore, the replacement of the 5-chloride with a larger hydrophobic group, such as trifluoromethyl (**3**), strongly reduces the ability of the compound to inhibit EGFR's activation. Liu et al.[67] removed the piperidine moiety and changed the methoxy group with bromine atom, ending up discovering the compound **LS-106** which dose-dependently inhibits several EGFR mutated forms. Molecular docking of LS-106 with EGFR L858R/T790M/C797S, reported in manuscript[67], showed a similar binding mode to those shown by Brigatinib and **1**. Additionally, Finlay et al designed and synthetised a series of Brigatinib derivatives, which had been modified through the addition at C-6 position of the aniline group of a pyrazole ring[68], and some of them showed a potent mutant-selective EGFR inhibition (**4-5**). In particular, the most promising Brigatinib-based compound is compound **5**, in which the piperidine moiety is replaced by a morpholine group, and it is able inhibit the tumour growth in a xenograft mouse model of NSCLC with double- and triple-mutant EGFR, showing good renal clearance and permeability. Although 5 shows a modest kinome selectivity, no significant body weight loss is evident in the *in vivo* studies[68].

Other SAR studies investigated how the addition of ring subunits, fused on the phenyl phosphine oxide moiety (Ring A in structure I, **Figure8A**), can affect tumour growth. In this context, Wu et al designed and tested a series of compounds with different substituents at different positions within Brigatinib's scaffold [69]. Among them, **TQB3804** stood out (compound 34 in[69]) as a potent and mutant-selective TKI, currently under clinical development by Chia Tai Tianqing Pharmaceutical Group and in Phase III for NSCLC[70]. Structurally, TQB3804 differs from Brigatinib's structure for *(i)* the presence of a pyrazine fused to the dimethyl(phenyl)phosphine oxide moiety; *(ii)* the addition of a 6-methyl on the anisole group and *(iii)* the replacement of chloride on the pyrimidine ring with a bromine atom (**Figure 8A**). This compound displays potent and selective enzymatic activity for both double- and triple-mutant EGFR with a higher mutant-selective antitumoral activity *in vitro*, as well as *in vivo,* in lung cancer models harbouring EGFR mutations.

In 2022, Fang et al[71] designed and synthesized a series of compounds which are structurally more similar to TQB3804's scaffold than Brigatinib. In particular, the replacement of the 6-methyl group with a 6-ethyl on the anisole moiety and the introduction of a sulfone side chain on the piperidine led to the identification of compound **6**, exhibiting a slightly higher potency than to TQB3804 (**Figure 8B**). Similarly, Guo et al have documented the optimization of several anilinopyrimidine derivatives, showing higher mutant selectivity than Brigatinib [72]. Compound **8** is a good representative of these derivatives, and it bears 2,3-dihydrobenzo[b][1,4] dioxine group, with a more potent inhibitory activity against EGFR triple mutants, as well as towards Ba/F3 cells expressing EGFR del19/T790M/C797S, compared to an identical compound differing only for the quinoxaline moiety **7**[72]. Moreover, compound **8** displays good pharmacokinetic properties, low toxicity profile and good anti-tumor efficacy *in vivo*. This class of compounds might surely be an optimal clinical candidate to overcome T790M/C797S resistance[70–72].

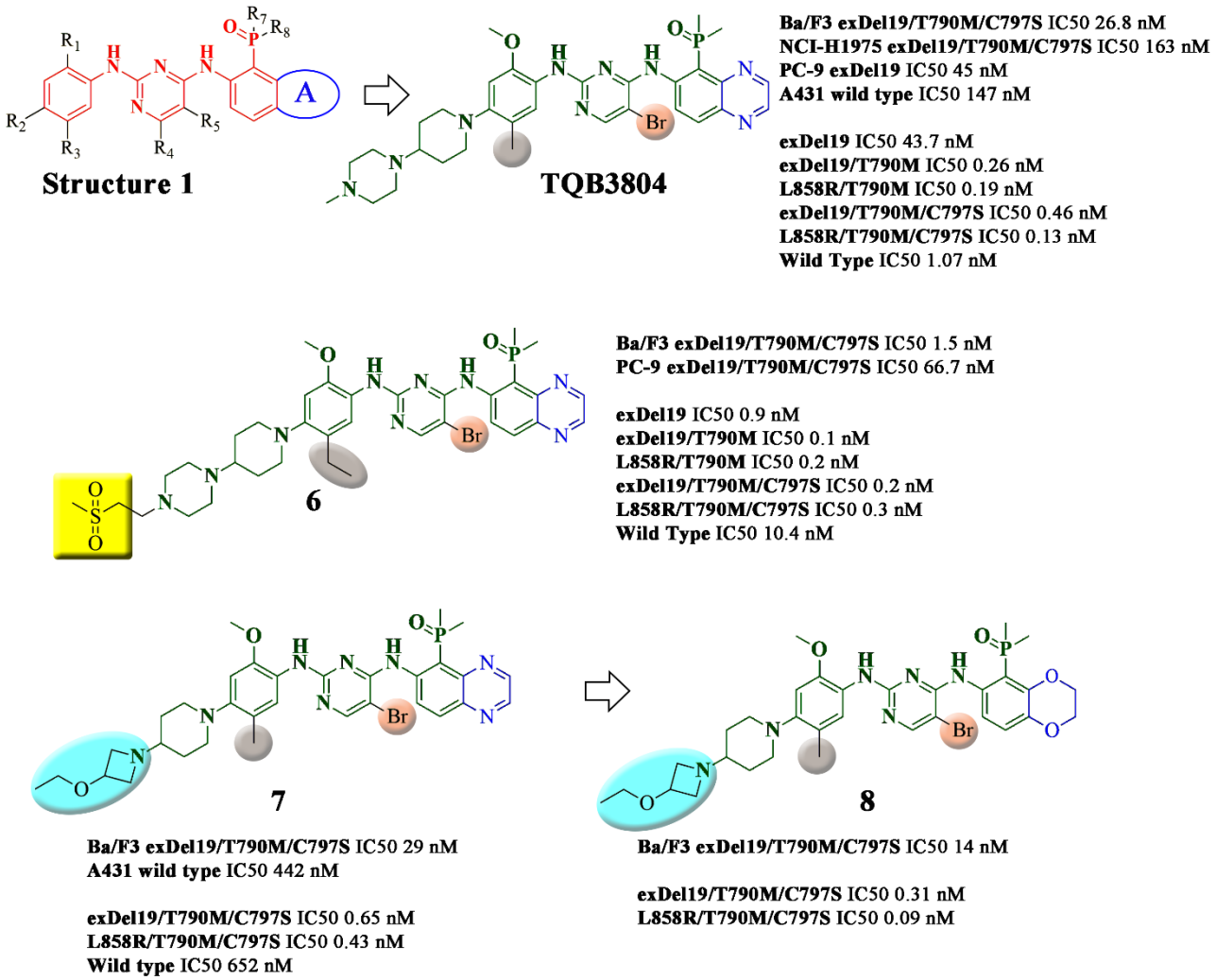


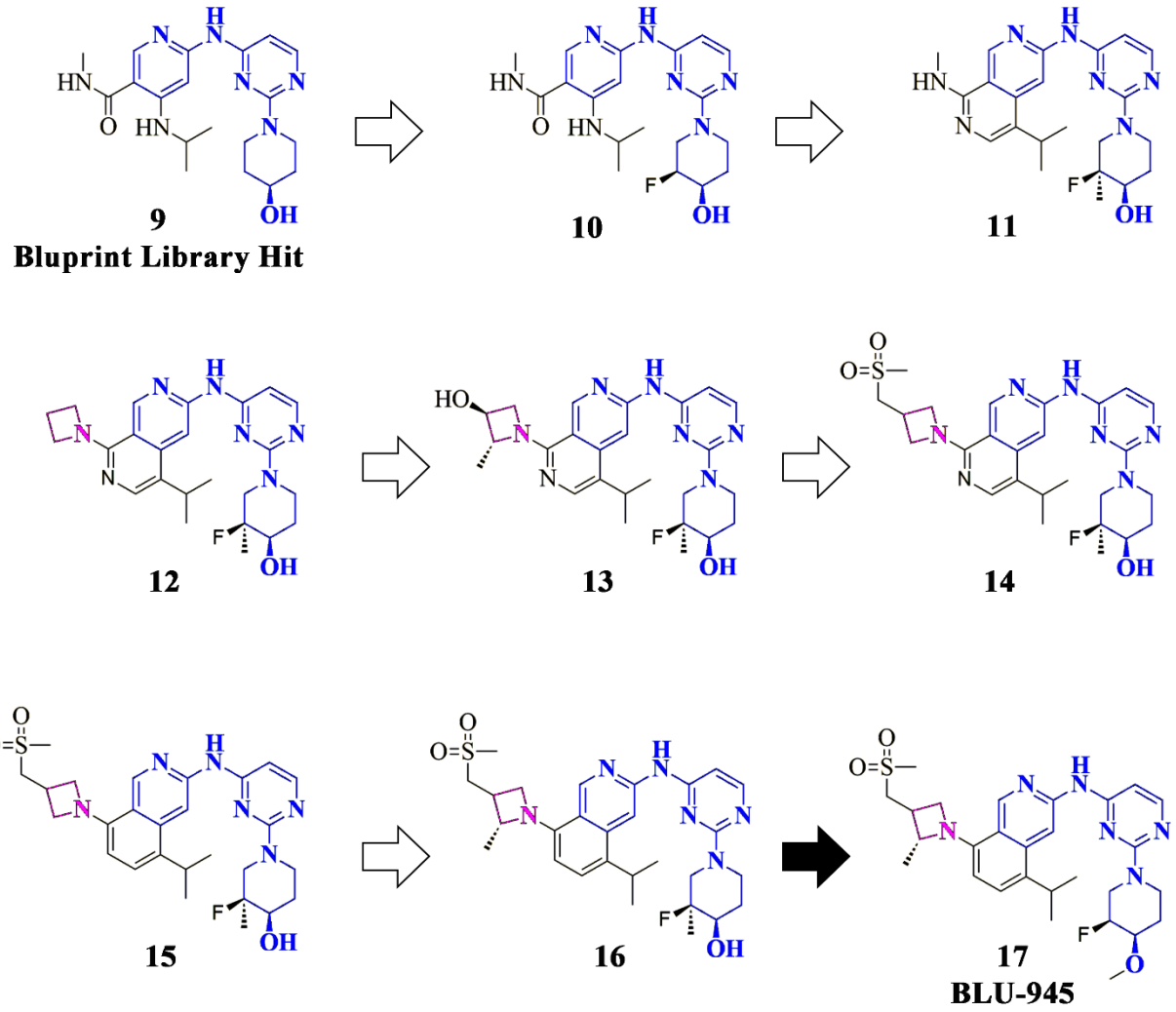
Figure 8. Identification and optimization of TQB3804-based compounds. Addition of ring subunits, indicated with A, fused on the phenyl phosphine oxide moiety of Brigatinib (Structure I) which led to the identification of the TQB3804 derivatives. All TQB3804's analogues structures and their TKI activity against EGFR mutants in enzyme-based assay and in cell models is showed.

5.2.2. N-(pyridin-2-yl)pyrimidin-4-amine derivatives

The US biotech Blueprint Medicines focused their research on the design of precision therapies for patients with cancer and other rare diseases. In this context, they have developed novel reversible and irreversible TKIs targeting EGFR resistance mutations in NSCLC. Among them, **BLU-945** is a reversible and orally available TKI, containing a N-(pyridin-2-yl)pyrimidin-4-amine scaffold functionalized with a piperidine moiety (highlighted in blue in **Table 2**) and showing activity against several EGFR mutated forms. BLU-945 was discovered by Eno and colleagues via a high-throughput virtual screening and a long process of structure optimization[73]. Starting from the virtual compounds' library of Blueprint Medicines, which contains more than 25,000 small-molecule kinase inhibitors, a

hit compound **9** was identified (**Table 2**). This compound had been structurally modified to improve EGFR's L858R/T790M and EGFR's L858R/T790M/C797S mutant potency. Among all the modifications carried out on the piperidinol ring, only the addition of a fluorine, next to the carbon bearing an alcohol group, led to a better inhibition against mutant EGFR (compound **10**). Additionally, the cyclization between the amide carbonyl and the aminopyridine NH and the addition of a methyl group at the fluorine-containing stereocenter gave rise to a novel and more potent early lead compound **11** containing 2,7-napthyridine moiety. However, this compound displayed only moderate kinome selectivity and metabolic stability; therefore, further optimization around 11 was required and performed by an extensive SAR investigation. Here, we show the main steps that led to the discovery of **BLU-945**. At first, in order to enhance the kinome selectivity, the chemists replaced the methylamine group with azetidine, obtaining **12** which was again modified by the addition of several polar substituents on the azetidine ring, resulting in a series of new compounds. Among them, 3-hydroxy-2-methyl-azetidine derivative **13**, was the most promising TKI with a higher mutant-selective activity, lipophilic efficiency and metabolic stability compared to 11 and 12. A further improvement was achieved with a sulfone azetidine-containing compound **14**, whose pharmacokinetic properties were thoroughly assessed and investigated by different approaches, including MDCK-MDR1 assay. Since 14 suffered of low oral bioavailability and high active efflux, its structure was optimized by replacing 2,7-napthyridine to give isoquinoline **15**, which gave a much better MDCK-MDR1 profile. The potency and wild type selectivity of 15 was consequently improved by the addition of a methyl group at C-2 of the azetidine ring, leading to compound **16**, which was subject to UGT-mediated glucuronidation in human hepatocytes, probably at the hydroxyl of the piperidinol. Protection of the -OH group with methyl was, thereby, required to lower the UGT-mediated clearance, and eventually led to the identification of **17**, namely **BLU-945** (compound 30 in [73]) also characterized by high level of kinome selectivity.

Table 2. Strategies to improve potency, kinome selectivity, and metabolic stability of 9.



	9	10	11	12	13	14	15	16	17
L858R/T790M IC50	290	99	8.1	2.1	0.3	0.1	0.2	0.3	0.4
L858R/T790M/CT797S IC50	266	105	6.1	12	0.4	0.1	0.2	0.2	0.5
exDel19/T790M/CT797S IC50	-	-	8.7	9.1	0.8	0.2	-	-	-
Wild type IC50	>10000	>10000	>10000	10000	4503	1023	385	505	683
pEGFR H1975 L858R/T790M IC50	914	420	67	20	7	4.8	2.7	1	1.1
pEGFR A541 wild type IC50	>25000	>25000	>25000	8 668	5290	1608	1362	1780	544
MDCK-MDR1 PA-B/efflux	-	-	-	-	-	-	17/4	9/3	8/1

A recently in-depth *in silico* study showed that the higher inhibitory activity showed by BLU-945's derivatives, compared to Erlotinib, is due to the formation of HBs with Lys728, Lys745 and Thr854

wherein the piperidinol and sulfone group, which seem to be the greatest contribution to the binding's energy observed in the EGFR mutants [74] (**Figure 9**). The X-ray structure of compound **14** in complex with EGFR L858R/T790M (PDB: 8D76) clearly shows the involvement of these amino acids in the binding's stabilization in addition to the classical anchoring of the TKIs to the hinge region through the aminopyridine moiety (**Figure 9A**). Moreover, the mutant selectivity could derive as well by the presence of a favourable hydrophobic interaction between the piperidine group and the mutated Met790 gatekeeper residue[75,76]. Although BLU-945 monotherapy demonstrated high *in vivo* antitumor activity in resistant NSCLC models, it has been shown even an enhanced activity in combination with osimertinib (SYMPHONY; NCT04862780)[77]. Unfortunately, BLU-945 exhibits poor efficacy against ex19Del mutation and, therefore, so far, its development has been narrowed down to a first-line combination with Osimertinib.

It is important to take into account that the N-(pyridin-2-yl)pyrimidin-4-amine scaffold, containing a piperidine moiety, was already used by Genentech Inc in 2015 to identify reversible TKIs, selectively targeting EGFR L858R/T790M [78]. More specifically, back then, Genentech Inc explored the effect of different azabenzimidazole substituents at the pyridine ring on TKI's potency, selectivity and metabolic stability and a representative structure of compound **18** is shown in **Figure 9B**.

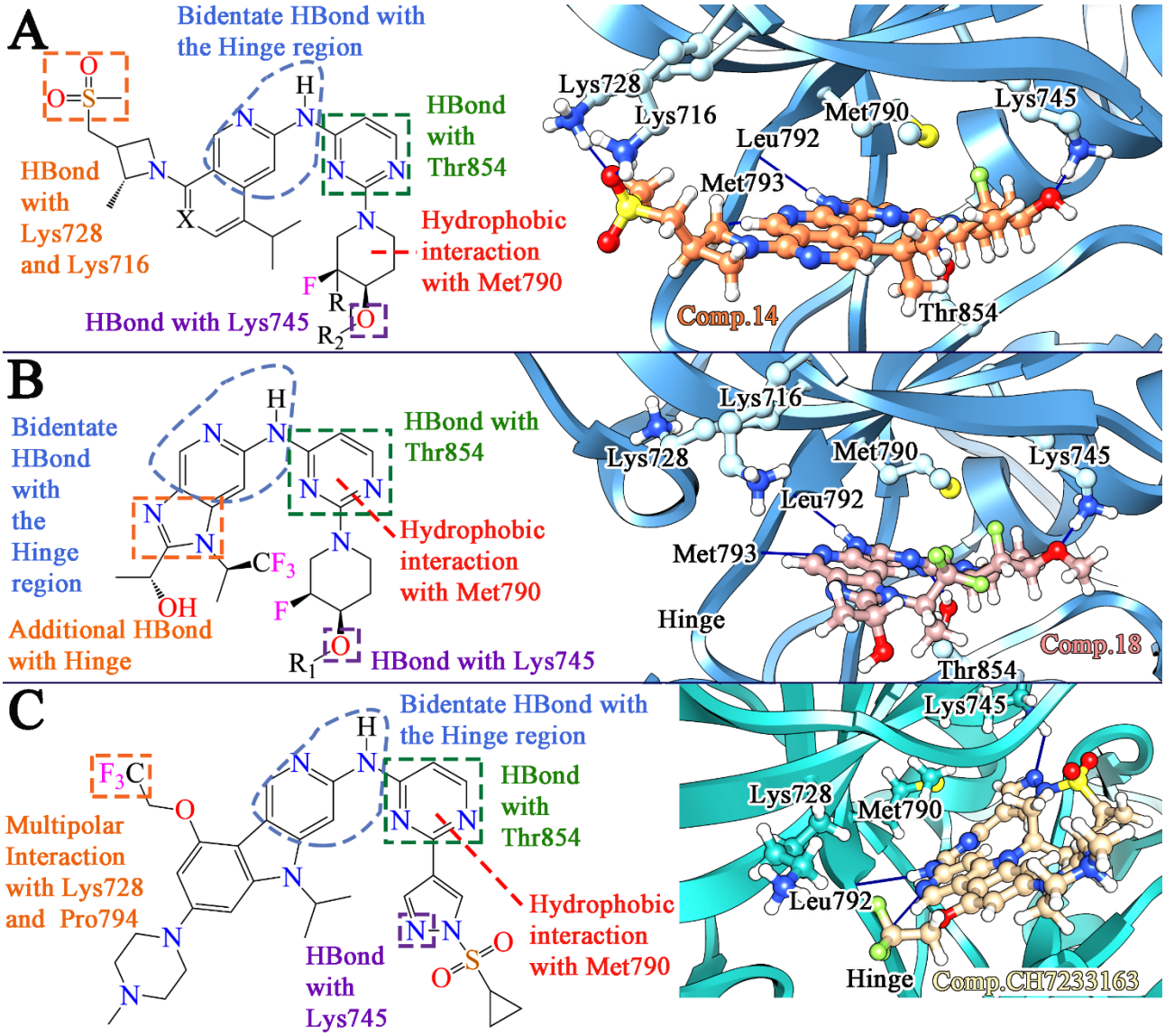


Figure 9. Pharmacophore of anilinopyrimidine-based TKIs inside mutated EGFR. Binding mode of compounds 14 (PDB: 8D76) **(A)** and 18 (PDB: 5CAU) **(B)** complexed with EGFR L858R/T790M, and **(C)** CH7233163 in complex with EGFR L858R/T790M/C797S (PDB: 6LUB). HBs are indicated as blue lines, while ligands and residues are represented with balls and sticks representation.

Although 18 is less active against EGFR L858R/T790M than compound 14, which belongs to the BLU-945 series, the comparison between their X-ray structures showed that both compounds interact with a similar binding mode with the EGFR L858R/T790M mutants, with the exception of the HBs made with Lys728 and Leu716 in the solvent-accessible region. Overall, these findings stress even more the crucial role of the aminopyridine, which promotes the formation of a bidentate HB with the hinge, and the important function of the pyrimidine moiety and the piperidinol in the efficient inhibition of the EGFR. The additional interaction with Leu716 and Lys728 could contribute as well to the higher TKI's potency and wild type selectivity, showed by BLU-945. These observations

provide a perfect example on how even small structural modifications can finely affect the ligand's binding and, consequently, the biological activity of a molecule.

The efficacy of EGFR-TKIs, whose structures are based on N-(pyridin-2-yl)pyrimidin-4-amine scaffold, is also demonstrated by the discovery of **CH7233163**, another potent EGFR mutant-selective TKI that, unlike BLU-945, strongly inhibits EGFR-Del19/T790M/C797S [79]. As for BLU945, CH7233163 was discovered via high-throughput screening, underlining once more the importance in the early stages of drug discovery of having available and easily-accessible chemicals' screening libraries in order to identify good chemical matter for effective *hit-to-lead* optimization. As we have previously mentioned above, the mutant-selective allosteric inhibitors, EAI-001/045 and their derivatives JBJ-04–125–02 and ATP-competitive TKI BLU-945, efficiently target L858R-triple mutant EGFR but they are unable to be effective against exDel19/T790M/C797S. For this reason, Kashima et al performed a virtual screening against exDel19-triple mutants with the aim to find new chemical matter targeting this EGFR mutated form. As shown in **Figure 9C,** excluding the presence of the of N-(pyridin-2-yl)pyrimidin-4-amine and the classical Y-shaped molecule, CH7233163's structure strongly differs from the one of BLU-945. The piperidinol moiety, linked to the pyrimidine, is replaced by 1-(cyclopropylsulfonyl)-1H-pyrazole group, while the pyridine is fused with a substituted indole. These modifications led to the identification of a compound that strongly inhibits not only the exDel19-triple EGFR mutants (IC50, 0.28 nM) but also able to target L858R/T790M/C797S mutations with a biochemical IC50 similar to the one for Del19/T790M/C797S. Additionally, CH7233163 shows greater ability to target EGFR's activating single mutations L858R and exDel19 as well as double EGFR mutated forms (L858R/T790M and exDel19/T790M) (IC50, 0.17-0.41 nM) with higher selectivity compared to the wild type EGFR[79].

Moreover, it is reported an excellent selectivity of CH7233163 across the human kinome (S-score = 0.015) with only five non-mutant and three mutant kinases inhibited other than EGFR (BUB1, CLK1, MELK, CLK4, MAP4K4, FLT3-D835V, FLT3-ITD and FLT3-ITD/ D835V). Also, in this case the high selectivity is consistent with the lack of major toxicity observed in the mice efficacy studies[79].

The X-rax co-crystal structure of CH7233163 and L858R-triple mutants showed as well the ability of the compound to interact with the key regions of the ATP-binding pocket with a binding mode, resembling the one of the compound 14. More in detail, the aminopyridine is involved in the formation of the bidentate HBs with the hinge and other interactions seem to be important, such as other HBs with Lys745 and Thr854 and a direct noncovalent interaction between the aminopyrimidine group and the mutated Met790. Taken together, these findings reinforce and emphasize that going after the aminopyrimidine scaffold would likely be the best and most effective direction to design potent and mutant-selective TKIs.

Finally, it is important to mention other promising 4th generation TKI which are in the early phase of clinical evaluation. In particular, Engelhardt et al. design and optimized a novel macrocyclic TKIs called BI-4020 which contains an aminobenzimidazole scaffold[80]. This is a noncovalent and ATP-competitive TKIs which is highly selective against EGFR Del19/T790M/C797S and L858R/T790M/cis-C797S with an EGFR Del19 background. In 2024, Suzuki et al. identify the brigatinib-resistant EGFR mutations by ENU mutagenesis screening and demonstrated the ability of BI-4020 to overcome the L718M mutation induced by brigatinib therapy[81].

KEYWORDS

Epidermal Growth Factor receptor (EGFR), a transmembrane tyrosine kinase receptor that represents one of the most potent oncogene kinases commonly altered in cancers including Non-Small Cell Lung Cancer (NSCLC); **EGFR targeted therapy**, which suppresses signal transduction pathways reducing tumor cell growth, proliferation, and metastasis; **Tyrosine Kinase Inhibitors (TKIs)**, organic compounds which block the phosphorylation of tyrosine residues, and therefore the activation of the EGFR downstream signalling pathways; **Resistance EGFR mutations**, is an adaptive tumour response to the TKIs which leads to a recovery of EGFR function even in the presence of the drug**; 1st generation TKIs**, which are able to reversible bind the EGFR at the ATP-binding pocket selectively targeting exon 19 deletion [exDel19] or L858R point mutation at exon 21 [L858R]; **2nd generation TKIs**, designed and developed to address T790M resistance which covalently bind to the EGFR and, irreversibly, inhibit the binding of the ATP; **3rd generation TKIs**, which have as well an irreversible mode of action, but they show higher selectivity against T790M point mutation; **4th generation TKIs**, able to target the resistance mutations induced by the treatment of 3rd generation TKIs.

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Credit Author Statement

Emiliano Laudadio: Conceptualization, Methodology, Investigation, Software, Visualization, Writing – original draft; **Luca Mangano**: Conceptualization, Formal Analysis, Investigation, Writing – original draft; **Cristina Minnelli**: Conceptualization, Formal Analysis, Investigation, Writing – original draft, Validation, Writing- Reviewing and Editing.

Declarations of interest

The authors declare no conflict of interest.

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