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Review

Fighting tertiary mutations in EGFR-driven lung-cancers: Current advances and future perspectives in medicinal chemistry

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The corrections made in this section will be reviewed and approved by a journal production editor.

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Abstract

Third-generation inhibitors of the epidermal growth factor receptor (EGFR), best exemplified by osimertinib, have been developed to selectively target variants of EGFR bearing activating mutations and the mutation of gatekeeper T790 in patients with EGFR-mutated forms of Non-Small Cell Lung Cancer (NSCLC). While the application of third-generation inhibitors has represented an effective first- and second-line treatment, the efficacy of this class of inhibitors has been hampered by the novel, tertiary mutation C797S, which may occur after the treatment with osimertinib. More recently, other point mutations, including L718Q, G796D, G724S, L792 and G719, have emerged as mutations mediating resistance to third-generation inhibitors. The challenge of overcoming newly developed and recurrent resistances mediated by EGFR-mutations is thus driving the search of alternative strategies in the design of new therapeutic agents able to block EGFR-driven tumor growth. In this manuscript we review the recently emerged EGFR-dependent mechanisms of resistance to third-generation inhibitors, and the achievements lately obtained in the development of next-generation EGFR inhibitors.

Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor

Keywords:

EGFR, NSCLC, TK inhibitors, Osimertinib resistance

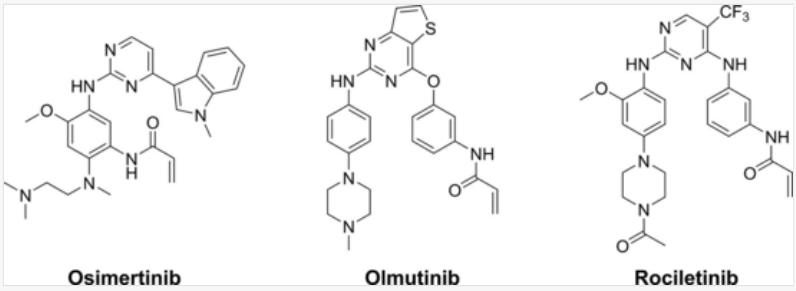
1 Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that mediates proliferation, differentiation and survival signals upon its ATP-dependent activation. EGFR mutations, overexpression and dysregulation are thus deeply linked to tumorigenesis [1-3]. Activating mutations within the intracellular tyrosine kinase (TK) domain of the protein, such as the L858R mutation and the exon-19 deletions [4], which destabilize the inactive conformation and promote a constitutively activated form of EGFR, even in absence of EGF [5], are recognized as a

major oncogenic driver in the 15% of Caucasian patients and in the 40% of Asian patients affected by Non-Small Cell Lung Cancer (NSCLC). The efficacy of first-generation EGFR inhibitors, such as gefitinib [6,7] and erlotinib [8], that have an anilino-quinazoline scaffold targeting the ATP binding site, is dampened by a threonine-to-methionine substitution, the T790M mutation. This mutation is sufficient to re-shape the architecture of the active site, hampering the binding of this class of inhibitors [9]. Second-generation inhibitors (such as afatinib and dacomitinib) share an acrylamide warhead that targets the residue C797 forming a covalent adduct, thus overcoming competition from ATP. They still bear an anilino-quinazoline scaffold designed to accommodate within the ATP-binding site, and approximately 40–50% of patients treated with these inhibitors in first line acquired T790M mutation, similarly to first-generation EGFR-TKI [10]. Moreover, this class of inhibitors indiscriminately targets the mutant and the wild-type (WT) form of EGFR, thus provoking severe side-effects [11-13]. Nonetheless, among the second-generation inhibitors, afatinib [14,15] is currently approved as first-line treatment of patients with metastatic NSCLC harboring also the uncommon EGFR mutations G719X, L861Q, and S768I G719X, L861Q, and S768I [16].

More recently, third-generation inhibitors have been developed to overcome the T790M-dependent clinical resistance. These inhibitors are generally characterized by an acrylamide which alkylates the C797 [17-19], and by an anilino-pyrimidine scaffold that accommodates within the active site without suffering the higher steric hindrance of M790, thus providing a good selectivity toward the T790M mutant over the WT EGFR [20-22]. Third-generation inhibitors (Fig. 1) are best exemplified by osimertinib [23,24] that has been lately approved as front-line treatment for EGFR mutated NSCLC patients [25,26] Despite the good efficacy exhibited by third-generation inhibitors, however, the occurrence of additional resistance mediated by several protein mutations, has emerged as an unavoidable issue that urges now the identification of novel strategies to target EGFR in NSCLC.

Fig. 1



Representative third-generation inhibitors. These compounds share an amino-pyrimidine scaffold and the presence of an acrylamide warhead that covalently modifies residue C797.

Unlike what observed for first- and second-generation inhibitors, which are mostly affected by the gatekeeper mutation T790M, the mechanisms that govern the resistance to third-generation inhibitors represent a more complex scenario [27,28].

In this review, we highlight the EGFR-dependent mechanisms, focusing on the primary mutations that affect the affinity of third-generation inhibitors to EGFR and mediate the acquired resistance to osimertinib. We also briefly discuss EGFR-independent mechanisms that operate through bypass signaling, aberrant downstream signaling and histologic transformation. Finally, we review the most promising medicinal chemistry strategies aimed at the design and optimization of novel active compounds, able to overcome resistance to third-generation inhibitors, and discuss the limitations that these strategies may encounter in the further development of fourth-generation EGFR inhibitors.

2 C797S mutation as the major EGFR-dependent mechanism

The C797S mutation has been reported as the most frequent tertiary mutation leading to acquired drug resistance [29-31]. The substitution of the cysteine residue with a less nucleophile serine is indeed sufficient to hamper the formation of a covalent adduct with the acrylamide fragment of the inhibitor. Consistently with *in vitro* studies, xenograft models showed the complete loss of sensitivity toward osimertinib, but also retained a slight sensitivity to first-generation inhibitors and to afatinib [32], that exert their inhibitory activity independently of the reactive cysteine 797. Noteworthy, in some cases, patients harboring the gate-keeper mutation treated with osimertinib and developing the C797S-dependent resistance to the treatment could convert from T790M^{+} to T790M^{-} [29], suggesting that patients harboring

the T790M and C797S mutations in *trans* might be efficaciously treated with a combination of both third- and first-generation inhibitors, such as osimertinib and gefitinib [33]. On the other hand, when C797S and T790M mutations are in *cis*, the cells acquire a complete resistance to any tyrosine kinase inhibitor.

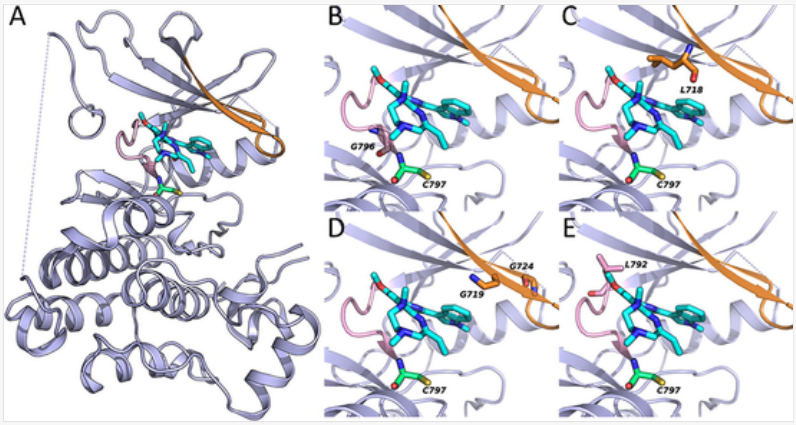
3 Other mutations responsible for additional EGFR-dependent resistance mechanism

While the mutation of residue C797 is expected to hamper the interaction with any covalent inhibitor and is recognized as the preeminent cause of EGFR-dependent osimertinib acquired resistance, additional resistance mechanisms have been correlated to other and less recurrent mutations.

3.1 Point mutation of residue G796

Additional mutations occurring at solvent-front of the protein, close to the nucleophilic cysteine targeted by the third-generation inhibitors, are represented by point mutations of G796, such as G796S, G796R or G796D [34,35]. The close proximity to C797 suggests that bulkier amino acids would clash with the scaffold of EGFR inhibitors (Fig. 2B).

Fig. 2



Panel A: X-ray structure of osimertinib (represented in sticks with cyan carbon atoms) in a non-covalent complex with EGFR^{T790M} (light blue cartoon; PDB ID: 4ZAU) [85]. The inhibitor assumes a bent conformation and occupies the outer edge of the ATP-binding pocket, delimited by the hinge region (pink cartoon) and the P-loop (orange cartoon). In this unbound complex, the acrylamide fragment of osimertinib is in close contact with the side chain of C797 (represented in sticks with green carbon atoms). Panel B: detail of binding pocket occupied by osimertinib. Residue G796 is represented in pink; the mutation of this residue would hamper the proper accommodation of the ligand in the binding site. Panel C: Residue L718 is represented in stick with orange carbon atoms; the hydrophobic side chain takes van der Waals contacts with the osimertinib phenyl ring, that are required for the stabilization of the ligand–protein complex. Panel D: residues G719 and G724 are represented in stick with orange carbon atoms; the substitution of glycines with bulkier residues would impede the binding of osimertinib, Panel E: Residue L792 is represented in pink; replacement of L792 with bulkier amino acids strongly reduces the affinity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2 Point mutation of residue L718

Mutations of the residue L718 represent an alternative and frequent trigger for osimertinib acquired resistance [36]. Residue L718 is situated within the ligand binding site and, together with residues V726 and L844 forms a hydrophobic clamp engaging the scaffold of third-generation inhibitors in non-polar interactions critical for the

stabilization of the ligand–protein complex [37]. The mutation of this residue into a glutamine or valine residue is thus sufficient to abolish these interactions and to impede a proper accommodation of the inhibitors within the active site, or to stabilize the protein in a non-reactive conformation (Fig. 2C) [38]. Notably, in most cases, patients harboring the L718Q mutation do not carry the C797S mutation [27]. Moreover, it is important to underline that in patients showing L718Q-mediated resistance upon first-line treatment with osimertinib, first- and second-generation inhibitors, such as afatinib, proved to be clinically effective. On the other hand, the L718Q mutation in *cis* with the secondary mutation T790M severely decreases the inhibitory activity of both second- and third-generation inhibitors

3.3 Point mutation of residue G719 and G724

Similarly to L718, residue G719 is situated in close proximity with the ligand binding site and is situated on the glycine-rich loop, a highly conserved, flexible structural element that concurs to the ATP binding.

While the mechanisms that lie behind the G719X-dependent drug acquired resistance are still unaddressed [39], it has been postulated that the mutation of this residue, that most commonly encompasses a mutation to serine, alanine or cysteine, would determine an increase of steric hindrance, thus impeding the proper binding of the inhibitors (Fig. 2D). Moreover, the mutation of residue G719 usually arises in *cis* with another uncommon mutation, L861Q. Notably, it has been observed that patients harboring mutations of residue G719 show a variable response to TKI treatment rather than a complete loss of sensitivity toward EGFR inhibitors [40]. More interestingly, different mutations of G719 determine different sensitivity profiles towards different EGFR inhibitors. For example, the G719S mutation is related to an augmented sensitivity to afatinib *in vitro*, but a reduced response to osimertinib [41,42]. The G719C mutant, similarly, was sensitive to both afatinib and erlotinib [43] and, in xenograft models, to osimertinib [32]. Finally, the mutation to alanine gave resistance to afatinib and erlotinib, but gained sensitivity toward osimertinib [32].

A further mutation within the glycine-rich loop involves the residue G724. G724S has been found to be an increasingly recurrent mutation, that is responsible for osimertinib acquired resistance in patients harboring the T790M mutation [44] , but also in patients negative for T790M [45].

3.4 Point mutation of residue L792

Residue L792 is situated on the loop connecting the N- and C-lobes, within the hinge region, where its hydrophobic side chain takes interactions with a methoxy group shared by third-generation inhibitors such as osimertinib and rociletinib. Multiple mutations of L792 have been identified in patients with acquired resistance to osimertinib, including L792F, L792Y and L792H, with L792H being the most abundant [46]. The replacement of L792 with bulkier amino acids strongly reduces the affinity of third-generation inhibitors, suggesting that modifications within the ligand binding site produce dramatic effects on the affinity of EGFR inhibitors (Fig. 2E). Notably, all these mutations were in *cis* with T790M, but in *trans* with C797S.

4 EGFR-independent mechanisms of resistance

Beside point mutations that alter the binding affinity of the inhibitors toward EGFR, tumor cells might escape the effects of the application of third-generation inhibitors by encompassing alternative bypass pathways, aberrant downstream signaling and histologic transformation.

For example, the *MET* amplification represents the most recurrent cause of osimertinib acquired resistance based on bypass pathway activation and is observed both in T790M^{+} and T790M^{-} and, in some cases co-exists with the C797S mutation [47,48]. Although to a lower extent, *HER2* amplification represents an additional pathway-based resistance mechanism, which can arise in patients harboring mutations of L792 and C797 and showing *PIK3CA* amplification, in patients with G796 mutation and *MET* amplification, or patients with *PIK3CA* amplifications [49]. *PIK3CA* mutations, that are responsible for aberrations of the PI3K pathway, frequently co-exist together with mutations in other oncogenic driver genes in patients developing osimertinib resistance [50]. Osimertinib resistance is also related to RAS-MAPK pathway aberrations, as a consequence of *NRAS* mutations [51], *KRAS* mutations [52,53], and *BRAF* mutations [54-56]. In addition, the Yes-associated protein (YAP) over-expression was found to be associated to resistance to TKIs and it was responsible for the phenotypic changes required to cause epithelial to mesenchymal cell transformation (EMT) after binding with its transcriptional co-activator TEAD [57,58]. Other relevant mechanisms of resistance are related to

alterations in the expression of the genes that encode cell-cycle proteins, including cyclins and cyclin-dependent kinases [59], as well as oncogenic fusions, such as *FGFR3, NTRK, RET, ALK,* and *BRAF* [60-62].

This evidence outlines a complex scenario, in which the drug resistance appears as a result of concomitant events that involve not only EGFR, but also the signaling cascade that follows the activation of the tyrosine kinase, and that allow the tumor cells to evade therapeutic approaches based on the administration of small molecules targeting EGFR. In this perspective, therapeutic approaches based on the combination of agents targeting both EGFR and other signaling pathways have been evaluated, as it has been recently reviewed by Leonetti A. and coworkers [63].

5 Discovery of fourth-generation inhibitors

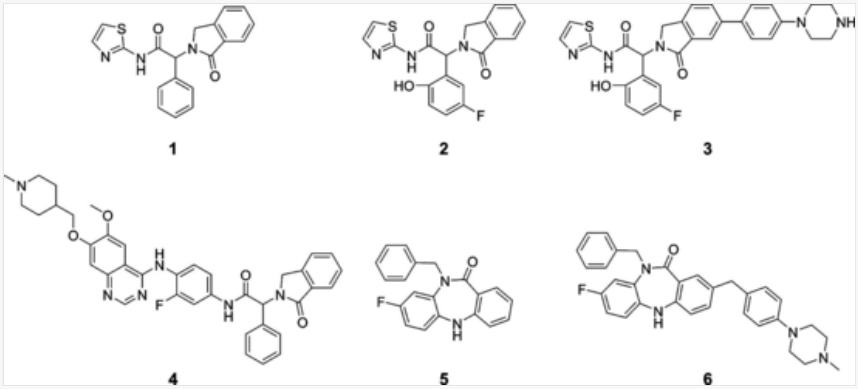
Since the discovery of the first cases of drug resistance limiting the efficacy of third-generation inhibitors, a great effort has been put into the search of a new generation of compounds able to efficiently target mutant forms of EGFR. Different strategies have been followed, in order to combine selectivity over the WT EGFR and other kinases, while overcoming the mutation of C797, which dampen the activity of covalent inhibitors. An approach that has been widely evaluated is based on the design of molecules targeting an allosteric cleft within the binding site of EGFR. This approach, however, relies on the binding of the ligand in pocket originated by the conformational reshaping that occurs upon the transition from the active to the inactive form and the protein dimerization. On the other hand, different scaffolds targeting the orthosteric site have been designed, in order to achieve a reversible inhibition of the enzyme, or the interaction with residues that are not affected by point mutations. In this light, the increasing number of crystallographic structures of different mutant forms of EGFR in complex with the available inhibitors represents an element of pivotal importance in the rational design of novel inhibitors.

5.1 Allosteric inhibitors of EGFR

5.1.1 EAi045

Starting from a screening campaign aimed at the identification of potential non-competitive and mutant selective EGFR inhibitors, Jia Y. and coworkers identified EAI001, an oxoisoindolinyl derivative showing a promising activity and selectivity profile (compound **1** in Fig. 3) [64]. A medicinal chemistry driven optimization study led to the identification of EAI045 (compound **2** in Fig. 3). EAI045 is a non-competitive inhibitor (IC_{50} = 3 nM against the L858R/T790M mutant in biochemical assays), and it is characterized by a 1000-fold selectivity versus the WT EGFR and by an optimal selectivity profile against other kinases and non-kinase targets. The X-ray structure of the parent compound EAI001 in complex with EGFR also revealed that these compounds occupy an allosteric pocket formed upon the outward displacement of the receptor C-helix during the transition from the active to the inactive form. The peculiar "three-bladed propeller" shape of these compounds allow them to occupy a hydrophobic cleft delimited by L777 and F856, situated at the back of the pocket, a region comprised between the mutant gatekeeper methionine and K745 and, finally, the aperture to solvent along the C-helix. In opposition to the promising biochemical data, cell-based studies revealed a weak anti-proliferative activity of EAI045 in H1975 cells, an L858R/T790M-mutant NSCLC cell line, and in NIH-3 T3 cells transfected with the L858R/T790M mutant. This inconsistent behavior, however, is attributed to an asymmetric ligand-induced dimerization, in which the C-lobe of the "activator" subunit, interacting with the ligand, would promote an active conformation by favoring the inward position of the C-helix of the "receiver" subunit, thus hampering the proper binding of a second molecule of EAI045 within the "receiver" allosteric site. To overcome this issue, EAI045 has been tested in combination with monoclonal antibody cetuximab, that should synergistically promote the inhibitory activity of EAI045 by blocking the dimerization process. Consistently with the expectations, the combination of cetuximab and EAI045 induced a significant tumor shrinkage in genetically engineered L858R/T790M/C797S mice, confirming EAI045 as the first inhibitor able to overcome both T790M and C797S mutations [58].

Fig. 3



Structures of EGFR inhibitors targeting the allosteric site.

5.1.2 Other allosteric inhibitors

Starting from the scaffold of EAI045, To C. and colleagues designed a novel derivative (JBJ-04−125-02; compound **3** in Fig. 3) by functionalizing the isoindolinone moiety of EAI045 with a phenylpiperazine fragment [65]. As supported by X-ray experiments, the decoration of the isoindolinone moiety allows a wider occupation of the binding cleft delimited by the regulatory C-helix, leaving the orthosteric ATP-binding pocket unoccupied. The compound showed a good inhibitory activity and, similarly to what observed for EAI045, the combination of JBJ-04−125-02 with cetuximab allowed a significant reduction of the proliferation in H3255GR cells harboring the L858R/T790M mutations. Notably, the combination of JBJ-04−125-02 with osimertinib [65,66] led to an increase in apoptosis, an enhanced inhibition of cellular growth, and an augmented efficacy both *in vitro* and *in vivo* compared with the either the single agents alone.

Li Q. and coworkers designed a series of hybrid inhibitors, able to target both the allosteric site occupied by EAI045 and the orthosteric ATP binding site, by combining the aminoquinazoline scaffold of the EGFR weak inhibitor vandetanib (IC_{50} = 369.2 nM on $EGFR^{L858R/T790M/C797S}$) and the oxoisoindolinyl-phenylacetamide moiety of EAI045 [67]. This strategy led to the identification a noncovalent reversible inhibitor showing a high inhibitory activity profile (IC_{50} = 2.2 nM on $EGFR^{L858R/T790M/C797S}$ in biochemical assays; compound **4** in Fig. 3) and a good selectivity profile. Furthermore, the compound selectively inhibited the growth of BaF3- $EGFR^{L858R/T790M/C797S}$ cells.

Noteworthy, the same screening campaign that led to the identification of EAI001, the parent compound of EAI045 [64], also outlined EAI002, a dibenzodiazepinone-based scaffold with a promising inhibitory activity (IC_{50} = 52 nM on $EGFR^{L858R/T790M}$; compound **5** in Fig. 3) and a good selectivity profile. Starting from this scaffold De Clercq D.J.H. and coworkers designed a compound (compound **6** in Fig. 3) bearing a phenylpiperazine moiety at the C2 position accommodated within the ATP-binding site and placing the dibenzodiazepinone fragment anchored in the same binding cleft occupied by EAI045 [68]. The compound inhibited $EGFR^{L858R/T790M}$ and $EGFR^{L858R/T790M/C797S}$ with an IC50 of ≈11 nM in biochemical assays and showed an optimal selectivity across the human kinome. Consistently with the biochemical assays results, the administration in combination with cetuximab exerted an anti-proliferative effect in Ba/F3 cells expressing the mutant $EGFR^{L858R/T790M/C797S}$.

While the development of allosteric inhibitors appears as an attractive strategy to counteract the resistance to third-generation inhibitors in $EGFR^{L858R/T790M/C797S}$ mutants, it is important to underline that the efficacy of these compounds is drastically dampened against EGFR triple mutants harboring the exon 19 deletion mutations. These mutations affect a protein strand near the C-helix, likely restraining and modifying the conformation of this region. Although all the attempts to obtain a crystallographic structure of del19 mutants have been unsuccessful to date, the shift of the conformational equilibrium toward the active form would likely impede the disclosure of the allosteric pocket and the proper accommodation of these compounds, providing a rational explanation of the drop of activity of allosteric inhibitors against the del19 variants. Finally, evidence showing that the antiproliferative activity exerted by allosteric inhibitors is significantly increased by the co-administration of EGFR antibodies [64] or of orthosteric TKIs [65,66] suggests that the development of these inhibitors may particularly boost a double hit approach, based on the combination of different compounds targeting EGFR.

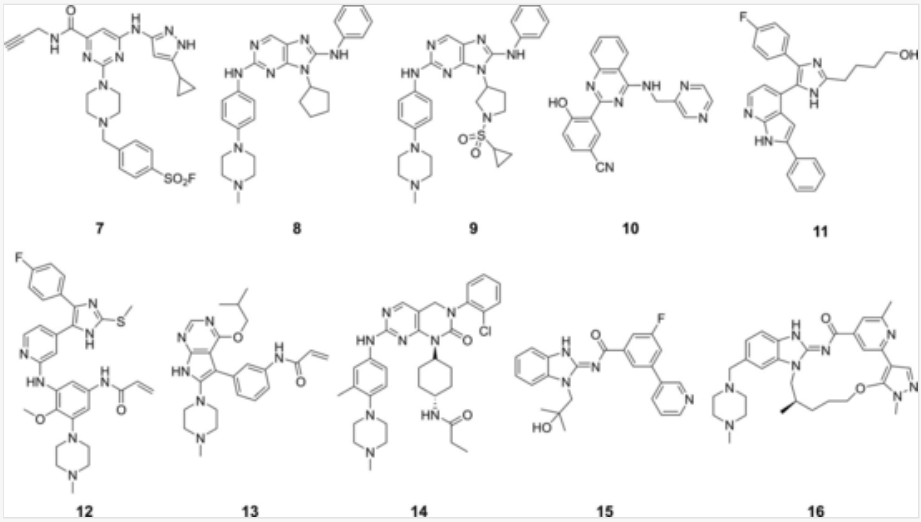
5.2 Inhibitors targeting the orthosteric site

While the administration of allosteric inhibitors of EGFR in combination with other inhibitors might represent a promising approach to the treatment of EGFR mutants, another strategy to overcome the new resistance mechanisms is represented by the design of novel compounds targeting the orthosteric site and able to overcome the C797S mutation.

The ALK-inhibitor brigatinib was found effective against triple-mutant cells *in vitro* [69], and while a combination with monoclonal antibodies was required to reach an effective inhibition also *in vivo*, the example of the repurposing brigatinib to target EGFR has prompted the search for novel compounds.

The results of a broad-spectrum kinase profiling using lysine-directed sulfonyl fluoride probes have outlined the possibility to overcome the drug resistance determined by the point mutation of C797 by targeting the catalytic lysine residue K745.70 The crystallographic structure of compound XO44 (compound **7** in Fig. 4) covalently bound to K745 revealed that the pyrazolopyrimidine scaffold of the inhibitor serves as anchoring fragment within the active site, where it takes polar interactions with hinge region, while the piperazinylbenzensulfpnyl fluoride moiety spans within the ATP binding pocket towards residue K745. Compound XO44 has been found to covalently modify approximately 130 diverse kinases, including EGFR. While the pyrazolopyrimidine scaffold is not optimized to achieve selectivity across the kinome [70] the results provided in this work prompted the foundation for the search of novel inhibitors able to overcome the C797S resistance by targeting the K745 residue.

Fig. 4



Structures of EGFR inhibitors targeting the orthosteric site.

However, the best results in the quest for selective EGFR inhibitors that conserve good affinity for the ATP binding site with C797X mutations have been achieved so far with non-covalent reversible inhibitors.

5.2.1 Purine derivatives.

Starting from the aminopyrimidine scaffold of the third-generation inhibitor WZ4002 [20], Zhu W. and coworkers identified a reversible class of purine derivatives [71] targeting the hydrophobic cleft delimited by the gatekeeper residue and a hydrophobic pocket in the back of the ATP-binding site [37]. More specifically, the most promising compound of this series is characterized by a cyclopentyl fragment that forms non-polar interactions with residues L718, V726 and L844, which is sufficient to gain affinity and to improve the inhibitory activity against EGFR (IC_{50} = 0.5 nM on $EGFR^{L858R/T790M}$ in biochemical assays; compound **8** in Fig. 4). Notably, while mutations of the residues of the hydrophobic cleft occupied by the cyclopentyl fragment were detrimental for the inhibitory activity, the compound was found to be 10-fold less potent on the $EGFR^{L858R/T790M/C797S}$.

Starting from previously reported 8-phenylamino-9*H*-purine derivatives [72], Lei H. and co-workers recently reported novel 9-heterocyclyl substituted 9*H*-purines as effective L858R/T790M/C797S mutant EGFR inhibitors (compound **9** in Fig. 4) [73]. The most promising compound inhibited the $EGFR^{L858R/T790M/C797S}$ with an IC_{50} value of 18 nM in biochemical assays and showed antiproliferative effects in HCC827 and H1975 cell lines (IC_{50} = 0.88 nM and 0.20 μM, respectively). Furthermore, compound 9 was able to inhibit the EGFR phosphorylation, induce the apoptosis, arrest cell cycle at G0/G1, and inhibit colony formation in HCC827 cell line in a dose-dependent manner.

5.2.2 2-aryl-4-aminoquinazoline derivatives

Further examples of reversible inhibitors comprise the 2-aryl-4-aminoquinazoline derivatives, identified through a virtual *de novo* design campaign (compound **10** in Fig. 4) [74]. Compounds of this class showed inhibitory activity in the nanomolar range, and good selectivity for the d746-750/T790M/C797S mutant (>1000-fold over the WT EGFR). Notably, molecular modelling studies suggested that these compounds may take polar interactions with the mutated residue S797, thus overcoming the resistance due to the reduced reactivity of serine compared to cysteine.

5.2.3 Tri-substituted imidazole derivatives

In an attempt to identify novel inhibitors able to overcome the C797S-dependent resistance, tri-substituted imidazole derivatives have been evaluated as potential EGFR inhibitors. Günther M. and colleagues first described derivatives bearing an aromatic or aliphatic alcohol group at position 2 of the imidazole (compound **11** in Fig. 4) [75]. More recently, the X-ray structure of these compounds in complex with EGFR reveals that the imidazole ring takes polar interactions with the side chain of residue K745, while the hydroxy group of the alcohol fragment is oriented towards the hydrophilic cleft of the protein, where it can interact with residues N842 and D855 [76]. While these compounds show a promising activity against $EGFR^{L858R/T790M}$ and $EGFR^{L858R/T790M/C797S}$, they lack selectivity over WT EGFR. In another recent work, a strategy based on the design of ligands maintaining the beneficial interactions mediated by the imidazole ring and allowing the covalent modification of residue C797. In this line, a 2-methylthio-imidazole scaffold has been decorated with an aniline ring bearing an acrylamide group, able to engage the residue C797 in a covalent interaction, leading to the identification of a compound (compound **12** Fig. 4) showing a high potency against $EGFR^{L858R/T790M}$, and maintaining a good activity against $EGFR^{L858R/T790M/C797S}$ (IC_{50} < 1 nM and = 11 nM, respectively, in enzymatic assays) [77]. Despite the promising inhibitory activity showed in biochemical assays, these first series of tri-substituted imidazole derivatives did not show a sufficient selectivity over WT EGFR. On the other hand, the structural data available and structure–activity relationships of these compounds suggest that imidazole scaffold may be exploited for the design of novel classes of inhibitors.

5.2.4 Pyrrolopyrimidine derivatives

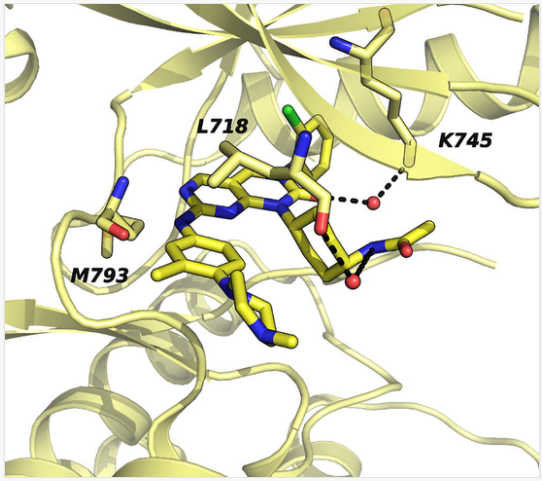
Starting from the analysis of X-ray structures of pyrrolopyrimidine ligands in complex with different kinases, Lategahn and colleagues recently developed novel derivatives able to bind and inhibit, through a reversible mechanism of action, the triple mutant form of EGFR. The initial compound, characterized by a 6-phenyl-pyrrolopyrimidin-5-yl-phenylacrylamide scaffold and showing promising inhibitory activity against $EGFR^{L858R/T790M}$ (IC_{50} = 176 nM), was the premise for a thorough medicinal chemistry study which led to the identification of a derivative bearing a 4-methylpiperazinyl-phenyl substituent at position 6 and an isobutyl group at position 4 (compound **13** in Fig. 4) [78]. Notably, while the design of this class of compounds was first aimed at the identification of covalent inhibitors targeting the gatekeeper mutant $EGFR^{L858R/T790M}$, biochemical assays revealed the highly reversible binding character of these ligands, which conferred to this class of compounds a lower propensity to lose activity towards the triple mutant $EGFR^{L858R/T790M/C797S}$. More specifically, compound **13** showed a high potency against $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ (IC_{50} < 0.1 nM in biochemical assays) and maintained a single digit nanomolar activity against the triple mutant (IC_{50} 8.5 nM in biochemical assays). Compound **13** also showed a high selectivity over the WT bearing cell line A431, and over mutant KRAS-bearing A459 and H358 cells and was characterized by significant metabolic stability and high plasma concentrations in mice. Furthermore, the crystal structures of the inhibitor in complex with the mutant form of EGFR provided important information for the rationalization the structure–activity relationships of these compounds.

5.2.5 Pyrimidopyrimidinone derivatives

A random screening of a kinase inhibitors library led to the discovery of a potent pyrimidopyrimidinone derivative that showed a high potency against $EGFR^{L858R/T790M/C797S}$ *in vitro* (IC_{50} = 5.8 nM) and a good selectivity profile (compound **14** in Fig. 4) [79]. The compound was also found to suppress the phosphorylation of $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$ in a dose-dependent manner, and to exert an antiproliferative effect on BaF3 cells transfected with $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$. The anticancer efficacy of this compound was finally evaluated *in vivo* in a xenograft mouse model. Notably, in mice bearing BaF3-$EGFR^{19D/T790M/C797S}$ mouse xenograft tumors, the compound produced a tumor growth inhibition value of 42.2%, thus showing higher efficacy than the combination of the allosteric inhibitor EAI045 and cetuximab (22.3%). The

crystallographic structure of the ligand in complex with the EGFR$^{T790M/C797S}$ mutant (PDB ID: 5ZTO.pdb [79], Fig. 5) showed that the ligand can adopt a "U-shaped" conformation, stabilized by a bidentate polar interaction between the pyridopyrimidinone scaffold and residue M793 within the hinge-region, and by water-mediated interaction between the carbonyl group of the ligand and K745. Following the bent conformation assumed by the ligand, the propionamide fragment occupies a solvent-exposed region of the binding site, while the phenylpiperazine moiety takes van der Waals contacts with residue G796.

Fig. 5

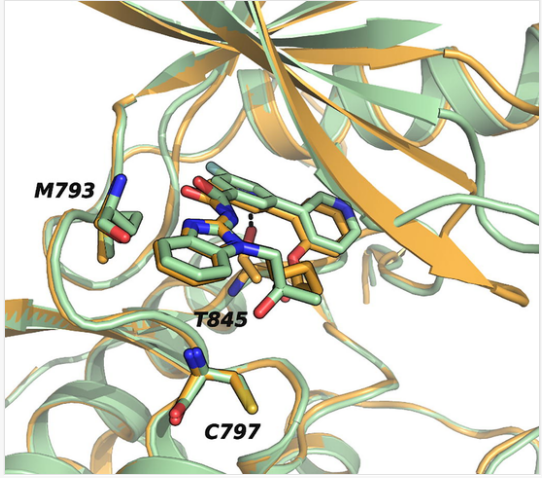


Crystallographic structure of compound **14** (represented in sticks with yellow carbon atoms) bound to EGFR (represented in light-yellow cartoon; PDB ID: 5ZTO.pdb) [79]. The pyridopyrimidinone group is oriented toward the backbone of residue M793 (represented in sticks with light-yellow carbon atoms), while water-mediated interactions with residues L718 and K745 stabilize the "U-shaped" conformation assumed by the ligand within the binding site.

5.2.6 Macrocyclic benzimidazole derivatives

More recently, Engelhardt H. and coworkers have identified a non-covalent WT EGFR sparing macrocyclic inhibitor [80]. A preliminary screening campaign was performed in order to identify scaffolds endowed with a high selectivity over WT EGFR, rather than with a high activity. The triple mutant EGFR$^{L858R/T790M/C797S}$ was used throughout the biochemical assay, which led to the identification of a benzimidazole derivative showing an optimal selectivity profile and an inhibitory activity of 2100 nM and 250 nM on EGFR$^{L858R/T790M/C797S}$ and EGFR$^{del19/T790M/C797S}$, respectively (compound **15** in Fig. 4). The X-ray structure of this first compound in complex with EGFR and molecular modelling studies were then used to instruct the optimization of the inhibitor, leading to the design of a macrocyclic derivative. The optimized compound showed a high potency on EGFR$^{del19/T790M/C797S}$ (IC_{50} = 0.2 nM), while maintaining an excellent selectivity profile (compound **16** in Fig. 4). Notably, the superposition of the X-ray structures of compounds **15** and **16** (PDB ID: 6S9B.pdb [80] and 6S9D.pdb [80], respectively; Fig. 6) show that the 1,3-dihydrobenzimidazole scaffold shared by the two ligands perfectly overlaps, taking polar contacts with the backbone of residue M793 within the hinge region. Compound **16** also showed sub-nanomolar (IC_{50} = 0.2 nM) antiproliferative effects on EGFR$^{del19/T790M/C797S}$ harboring BaF3 cells. Finally, the macrocyclic derivative also determined a strong regression, and produced a significant tumor growth inhibition in mice bearing subcutaneous PC-9 $^{del19/T790M/C797S}$ xenotransplants.

Fig. 6



Crystallographic structures of compound **15** (light-green carbon atoms and cartoon representation; PDB ID: 6S9B.pdb) [80] and of compound **16** (orange carbon atoms and cartoon representation; PDB ID: 6S9D.pdb) [80]. The 1,3-dihydrobenzimidazole scaffold of the two ligands perfectly overlaps, while the binding mode of compound **16** is stabilized by the additional interaction with the side chain of residue T845. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6 Conclusions

In the last decade, a great deal of effort has been put into the search of novel compounds able to selectively target $EGFR^{T790M}$ to overcome acquired resistance towards first- and second-generation inhibitors. This has led to the identification of a new generation of compounds, sharing an aminopyrimidine scaffold and covalently modifying the residue Cys797 through an acrylamide warhead. These mutant-selective inhibitors are best represented by osimertinib, showing a high efficacy and mild adverse effects, which has been proven to be effective as first-line treatment in patients with previously untreated EGFR mutation-positive advanced NSCLC [81], as well as in T790M positive patients progressing after first or second-generation TKI treatment [82]. Despite the great results obtained with third-generation inhibitors, however, the inevitable occurrence and progression of acquired resistance rapidly limited the clinical applications of this class of compounds and urged the search for novel strategies to target EGFR. While the tertiary point mutation C797S has emerged as a major event in the development of acquired resistance, other EGFR-dependent mechanisms have been described, showing that also point mutations occurring at different positions within the binding site of EGFR, and not only at the residue targeted by covalent inhibitors, severely compromise the ability of these ligands to bind and block the mutated protein. Moreover, other EGFR-independent mechanisms that determine resistance to third-generation inhibitors, involving bypass pathways and aberrant downstream signaling, have been elucidated.

The increased knowledge on the biochemistry of EGFR, resistance pathways and on the structural elements of the protein binding site architecture have provided a resource of critical importance for the design of novel inhibitors, and for the development of combination therapies. For example, a screening campaign have outlined the possibility to covalently target the residue K745 and have provided a new perspective for the design of potential novel inhibitors.

In the last years, different medicinal chemistry strategies have been attempted, in order to develop potential fourth-generation EGFR inhibitors. Allosteric inhibitors, such as EAI045, and other noncovalent ATP-competitive ligands have been evaluated for their anti-proliferative activity. While showing encouraging initial results, these strategies present nonetheless some issues and limitations. For example, allosteric inhibitors show a significant inhibitory activity only in combination with monoclonal antibodies, such as cetuximab, that counteract the protein dimerization. In other cases, poor efficacy and low selectivity in cellular and *in vivo* studies have discouraged the development of compounds showing promising results in enzymatic assays. On the other hand, a medicinal chemistry campaign based on the optimization of a highly selective scaffold have provided a single digit nanomolar compound, showing an excellent *in vitro* and *in vivo* profile.

As the landscape of the resistance to third-generation inhibitors is particularly complex and comprises also EGFR-independent mechanisms, an alternative strategy to counteract this issue relies on the combination of osimertinib with other compounds targeting different pathways [63]. In this respect, an allosteric inhibitor of the Src homology 2

domain-containing phosphatase (SHP2), which controls the activation of the MAPK pathway and regulates the signaling downstream of several tyrosine kinases, has been recently found to exert an antiproliferative effect in NSCLC models resistant to osimertinib and harboring both EGFR -dependent and -independent mechanisms of resistance [83].

The issue of drug resistance to third-generation inhibitors, with its complex background of driving mechanisms, represents an unprecedented challenge in the treatment of NSCLC. Despite the effort put into the search of novel compounds, no relevant advances have been achieved in the identification of inhibitors able to overcome the acquired drug resistance and to exert their activity both *in vitro* and *in vivo* and, to date, no clinical studies ongoing are reported. In this respect, Blueprint Medicines Corporation has recently identified BLU-945 [84], a novel potent and selective EGFR inhibitor, for which, however, neither the structure nor the mechanism of action have been disclosed. This compound has been found to exert a high inhibitory activity against $EGFR^{L858R/T790M/C797S}$ and $EGFR^{del19/T790M/C797S}$ triple mutants in biochemical and *in vitro* assays, and to significantly reduce the tumor mass in an osimertinib resistant $EFGR^{19del/T790M/C797S}$ patient-derived xenograft mouse model. More interestingly, BLU-945 showed an increased anti-tumor activity in combination with either gefitinib or osimertinib, suggesting that this inhibitor may be effective in monotherapy and in combination therapy in clinical settings. The preclinical data collected for this compound thus strongly encourage its further evaluation in clinical development.

A renewed effort in the search of the next generation EGFR inhibitors is compelling, and the complexity of the mechanisms that drive the acquired resistance to third generation TKIs hampers the path towards the successful identification of novel ligands. Nonetheless, the increasing amount of information on structural and biological data on EGFR and the pathways related to the insurgence and progression of NSCLC represents today a heritage of pivotal importance for the continuous search for novel EGFR inhibitors.

CRediT authorship contribution statement

Laura Scalvini: Conceptualization, Writing - original draft. **Riccardo Castelli:** Conceptualization, Writing - original draft. **Silvia La Monica:** Conceptualization, Writing - review & editing. **Marcello Tiseo:** Writing - review & editing, Funding acquisition. **Roberta Alfieri:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Q3 References

The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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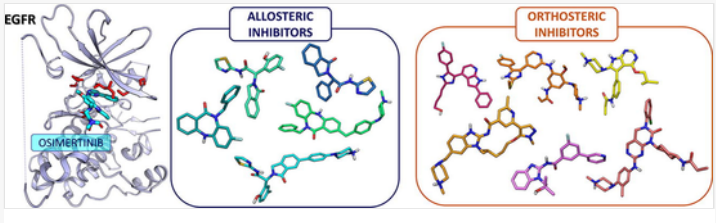

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