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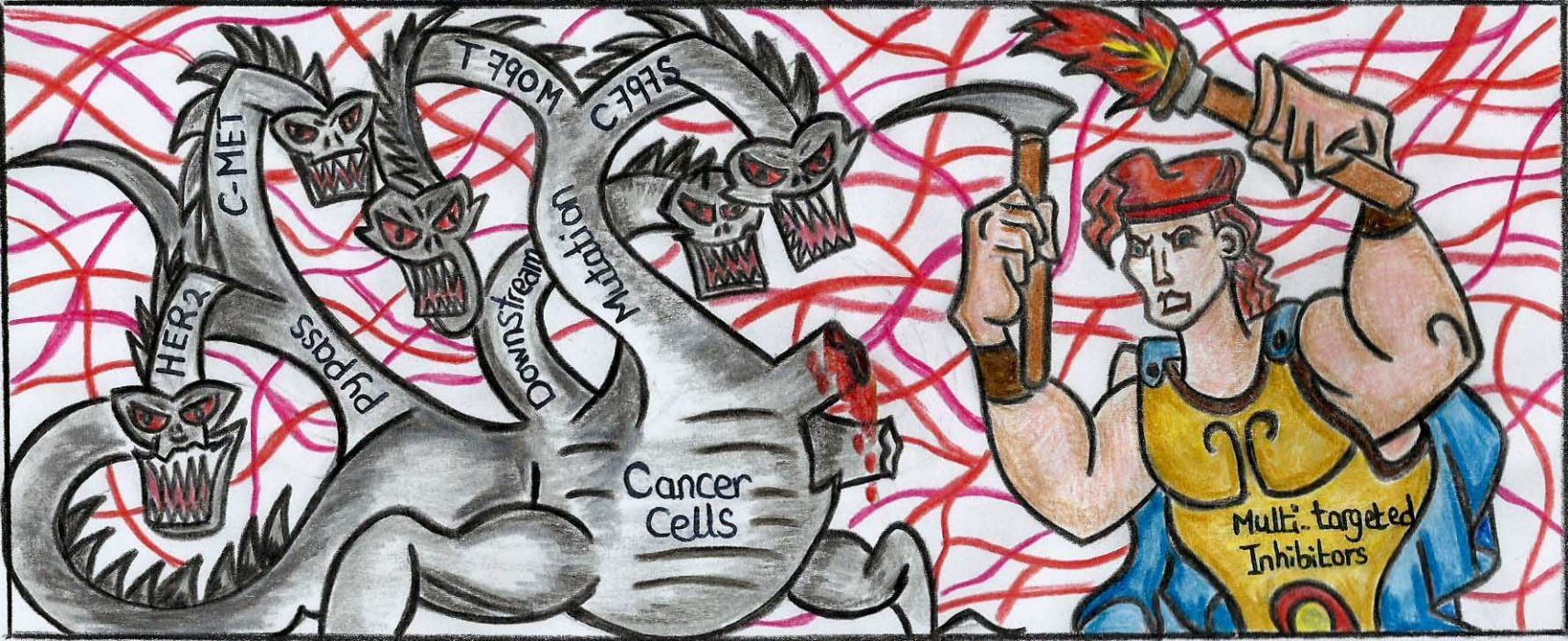
HER2
C-MET
PIPOSS
T790M
C797S
Downstream
Mutation
Cancer
Cells
Multi-targeted
Inhibitors

How to train your inhibitor: Design Strategies to Overcome Resistance to Epidermal Growth Factor Receptor Inhibitors

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ABSTRACT [1]

Epidermal Growth Factor Receptor (EGFR) stands out as a key player in the development of many cancers. Its dysregulation is associated with a vast number of tumors such as non-small-cell lung cancer, colon cancer, head-and-neck cancer, breast and ovarian cancer. Being implicated in the development of a number of the most lethal cancers worldwide, EGFR has long been considered as a focal target for cancer therapies, ever since the FDA approval of "Gefitinib" in 2003 and up to the last FDA approved small molecule EGFR kinase inhibitor "Osimertinib" in 2015. Studies are still going on to find more efficient EGFR inhibitors due to the continuous emergence of resistance to the current inhibitors. Cancerous cells resist EGFR tyrosine kinase inhibitors (TKIs) through various mechanisms, the most commonly reported ones are the T790M mutation and HER2 amplification. Therefore, tackling EGFR TKIs-resistant tumors through a multi-targeting approach comprising a dual EGFR/HER2 inhibitor that is also capable of inhibiting the mutant T790M EGFR is anticipated to overcome drug resistance. In this review, we will survey the structural aspects of EGFR family and the structure-activity relationship of representative dual EGFR/HER2 inhibitors. To follow, we will discuss the structural aspects of the mutation-driven resistance and various design strategies to overcome it. Finally, we will review the SAR of exemplary irreversible dual EGFR/HER2 inhibitors that can overcome the mutation-driven resistance.

Keywords: Dual EGFR/HER2 inhibitors, Resistance, T790M/L858R mutant EGFR, Irreversible Inhibitors

1. Introduction

Cancer is a multi-faceted disease resembling, metaphorically speaking, the Lernaean Hydra. According to the famous Greek mythology, the Hydra was a multi-headed serpentine monster that, upon the decapitation of one of its heads, would regrow more heads. In a very similar fashion, our Hydra "the Cancer" can resist the therapeutic agents, and for every survival pathway that we block for cancerous cells, cancer would find a way to resist and turn around the blockade.

According to Hanahan and Weinberg [1], cancer cells acquire certain capabilities in order to become malignant and to guarantee tumor development and dissemination. They defined these capabilities as the "Hallmarks of Cancer". One of the fundamental

Abbreviations: **ADMET**, Absorption/Distribution/Metabolism/Excretion/Toxicity; **Cbl**, Casitas B-lineage Lymphoma; **c-MET**, cellular mesenchymal to epithelial transition factor; **DM**, double mutant; **ErbB**, avian erythroblastosis oncogene B; **FDA**, Food and Drug Administration; **GI_{50}**, half maximal cell growth inhibitory concentration; **HB**, hydrogen bond; **HER2**, human epidermal growth factor receptor 2; **IC_{50}**, half maximal inhibitory concentration; **JAK3**, Janus kinase 3; **NSCLC**, non-small cell lung cancer; **PDB**, protein data bank; **PI**, Pseudo-irreversible; **PIK3CA**, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; **PK**, Pharmacokinetic; **RTK**, receptor tyrosine kinase; **SAR**, structure-activity relationship; **TKIs**, tyrosine kinase inhibitors; **TMLR**, T790M/L858R; **WT**, wild type.

hallmarks of cancer cells is their ability to sustain the proliferative signaling, accordingly they proliferate indefinitely, leading to the development of tumors. Cancer cells can sustain the proliferative signaling by manipulating the whole system of the receptor tyrosine kinases (RTK), their effector ligands and their associated downstream signaling pathways. Epidermal Growth Factor Receptor (EGFR) family, a family of four related receptor tyrosine kinases, is a key player in the development of many cancers. Its dysregulation is associated with a vast number of tumors such as non-small-cell lung cancer, colon cancer, head-and-neck cancer, breast and ovarian cancer [2]. When ligands (e.g., growth factors) bind to the receptor extracellular domain, homo- or heterodimerization of the family members occurs consequently, the now functionally active dimer will autophosphorylate certain tyrosine residues in the intracellular domain that triggers a cascade of downstream signaling pathways, those signals end up in the nucleus resulting in cell proliferation, decreased apoptosis, metastasis and neoangiogenesis [3–5].

Being implicated in the development of a number of the most lethal cancers worldwide [6], EGFR has long been considered as a focal target for cancer therapies, ever since the FDA approval of "Gefitinib" in 2003 [7] and up to the last FDA approved small molecule EGFR kinase inhibitor "Osimertinib" in 2015 [8]. Studies are still going on to find more efficient EGFR inhibitors due to the continuous emergence of resistance to the current inhibitors.

The major mechanisms of resistance developed by the cancerous cells against EGFR tyrosine kinase inhibitors are summarized in **Figure 1**, which can be classified into three categories; alterations in the primary drug target (the EGFR), alterations in the associated downstream signaling pathways or bypassing mechanisms that alter other RTKs as a compensation for EGFR blockade [9–12]. The prevalence rates of the different mechanisms vary greatly, numbers may vary from cohort to the other, but there are some mechanisms that are reported to occur more frequently than others (marked with stars in **Figure 1**). The most common resistance mechanism is the **T790M mutation**, which accounts for 50-60% of the acquired resistance cases [12]. The second most common acquired resistance mechanism is **HER2 amplification** accounting for 12% of the cases [13]. Then comes c-Met amplification (5%) and *PIK3CA* mutation (5%). While in almost 30% of the cases, the cause of the resistance is still not well understood [12].

So, is there no way to defeat the Lernaean Hydra? Would it eternally regrow each cut head rendering any efforts to kill it in vain? According to the mythology, Hercules managed to defeat the Hydra through cutting each of its heads and cauterizing the stumps making it impossible for the heads to regrow, then he eventually killed the one-headed vulnerable hydra. Copying Hercules way of defeating the Hydra, we can make use of a dual EGFR/HER2 inhibitor that is also capable of inhibiting the mutant T790M EGFR, thus blocking two major resistance mechanisms developed by the cancerous cells against EGFR inhibitors.

In this review, we will be surveying the Structure-Activity Relationship (SAR) of representative dual EGFR/HER2 inhibitors, also, the SAR of mutant EGFR inhibitors. Finally, we will discuss the possibility of combining the features of the two types of inhibitors into one inhibitor that is proposed to be active against many of the resistant cancers. Hopefully we can shed a light on a clue for a prepared mind.

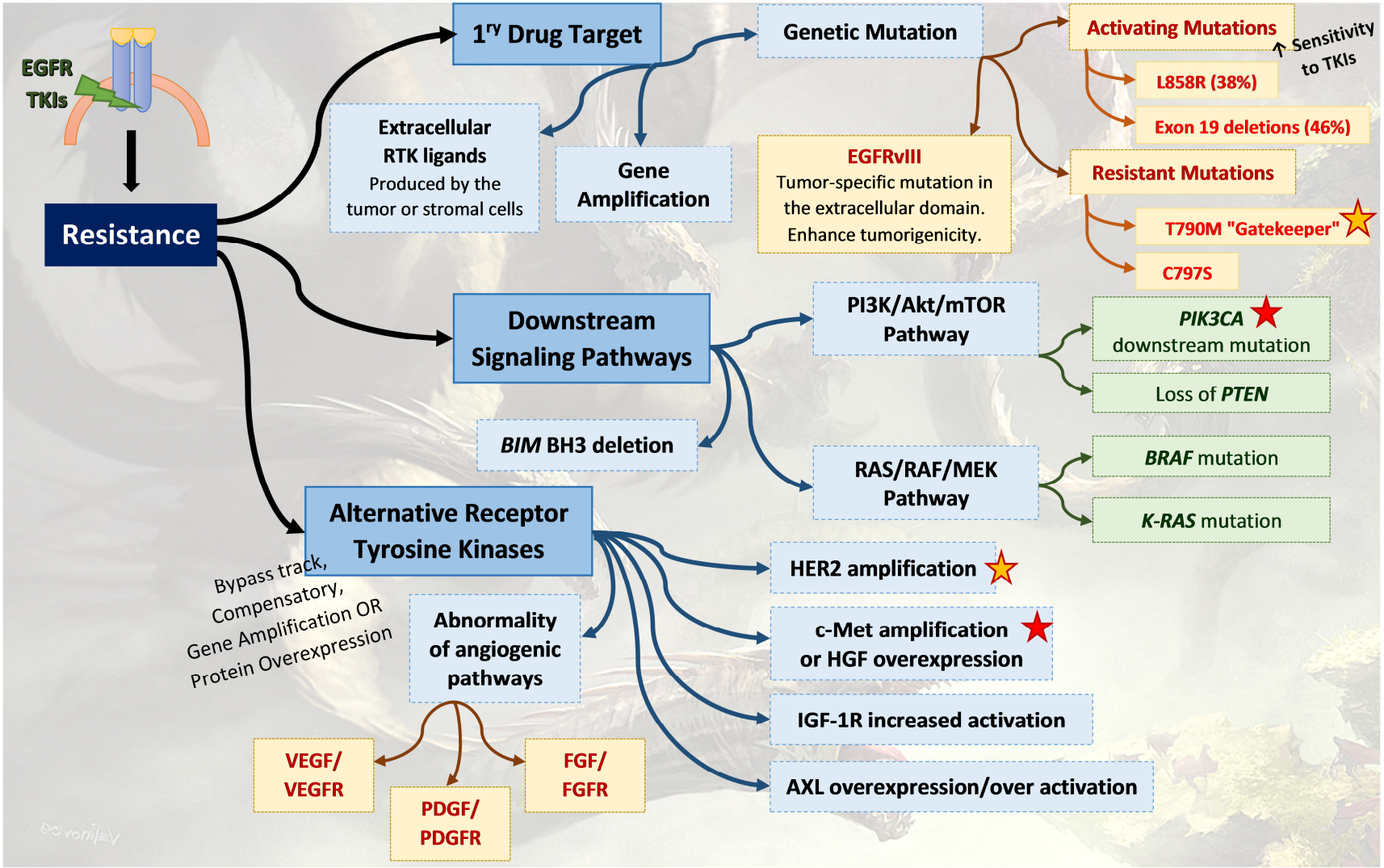


Figure 1 Summary of mechanisms of resistance to EGFR TKIs [9–12,88] (Yellow stars = most common resistance mechanisms, Red stars = second most common resistance mechanisms)

2. Dual EGFR/HER2 inhibitors

2.1. The Rationale behind using a dual EGFR/HER2 inhibitor

Potentiate EGFR signaling pathway blockade

Ligand binding to the extracellular domain of the EGFR receptors initiates their activation through receptor dimerization followed by intracellular phosphorylation. An exception is HER2 receptor that has a different extracellular domain. HER2 extracellular domain is mainly found in a single "open" conformation resembling the ligand-activated conformation of EGFR, and it binds none of the known EGF-related ligands. At the same time HER2 homodimers don't usually form due to strong repulsions between the dimerization arms [2], that led to the belief that HER2 mainly acts as a co-receptor that mediates a network of signaling pathways [14].

Having four members in the EGFR family, there are ten possible dimers (either homo- or heterodimers) that can form, however, not all ten dimers can equally elicit the downstream signaling pathways. Among the four receptors, it seems that HER2 is the preferred dimerization partner forming heterodimers of higher stability and more potent signaling potential. HER2-containing heterodimers are characterized by slow ligand dissociation, slow endocytosis, reduced EGFR degradation (inhibits EGFR binding to ubiquitin ligase Cbl [15]) and rapid receptor recycling, thus prolonging and enhancing downstream cellular signals, hence the theory that HER2 potentiates EGFR signaling activity [16–18]. Extrapolating those effects to the cancer setting, HER2 overexpression potentiates the effects of EGFR signaling such as increased tumor proliferation, progression and migration. HER2 overexpression is therefore considered as a predictor of poor prognosis [14,16].

So if HER2 enhances EGFR signaling, targeting both receptors simultaneously will presumably potentiate the signaling blockade, and there are some clinical evidences illustrating such effect [19]. The dual inhibition might block more signaling pathways depending on HER2-heterodimers, prevent heterodimerization, and generally decrease the downstream signals [19].

Overcome EGFR inhibitors resistance

Among the reported mechanisms of acquired resistance to EGFR TKIs is HER2 amplification as an alternative way to EGFR signaling blockade [13]. So we propose that by simultaneously blocking the two pathways, it might overcome the resistance arising from that bypass mechanism of HER2 amplification.

2.2. ATP binding sites in EGFR and HER2: similarities and differences

The general kinases ATP-binding site can be divided into a set of regions (**Figure 3a**); the hinge binder region (adenine pocket), ribose region and phosphate-binding region (solvent-accessible front pocket). In addition to these, there are other regions adjacent to the ATP-binding region that are not occupied by the ATP and act as targets for inhibitors binding, they are mainly hydrophobic regions that can be divided

into a hydrophobic region I (selectivity pocket), an allosteric binding site (also known as the back pocket) lying behind the gatekeeper residue, and a hydrophobic region II. The back pocket is only accessible in the inactive conformations of the kinase domain with DFG-Asp out and Activation segment closed displacing the αC-helix out and opening the back pocket region. (**Figure 3**) [20,21].

EGFR and HER2 share a very similar kinase domain sequence (≈80% homology) [22,23] (**Figure 2**), and consequently they have a very similar ATP-binding site architecture (**Figure 3c,d**), except for key amino acids differences that impart unique specific features to each binding site.

One of the main differences between EGFR and HER2 kinase domains is the degree of flexibility and the positioning of the αC-helix. The position of the αC-helix relative to the active site is one of the main determinants of the activation state of the kinase domain. In EGFR, an active conformation requires that the αC-helix is positioned in an "in conformation", forming a contact point between the αC-helix and αE-helix, where this positioning is stabilized by hydrogen bonding between the Ser^{768} and Asp^{770} of the sequence ^{768}SVDN^{771} of the αC-β4-loop and Arg^{831} and Tyr^{827} of the αE-helix. On the contrary, in the inactive conformation, with the αC-helix in an "out conformation", the activation segment forms a short α-helix and displaces the αC-helix away from the binding site opening a large back hydrophobic pocket (**Figure 4**). On the other hand in HER2, the corresponding sequence that is supposed to stabilize the αC-helix in an "in conformation" is found to be a glycine-rich region ^{776}GVGS^{779} (**Figure 2**) which is incapable of forming the same hydrogen bonds and so fails to stabilize the αC-helix. This explains the high flexibility of the αC-helix in HER2, the lower stability of its active conformation and the immediate accessibility of the back hydrophobic pocket. It is generally noted that dual EGFR/HER2 inhibitors tend to have hydrophobic back-pocket binders, and they usually bind the inactive EGFR conformation with the αC-helix out (**Figure 3**). [24]

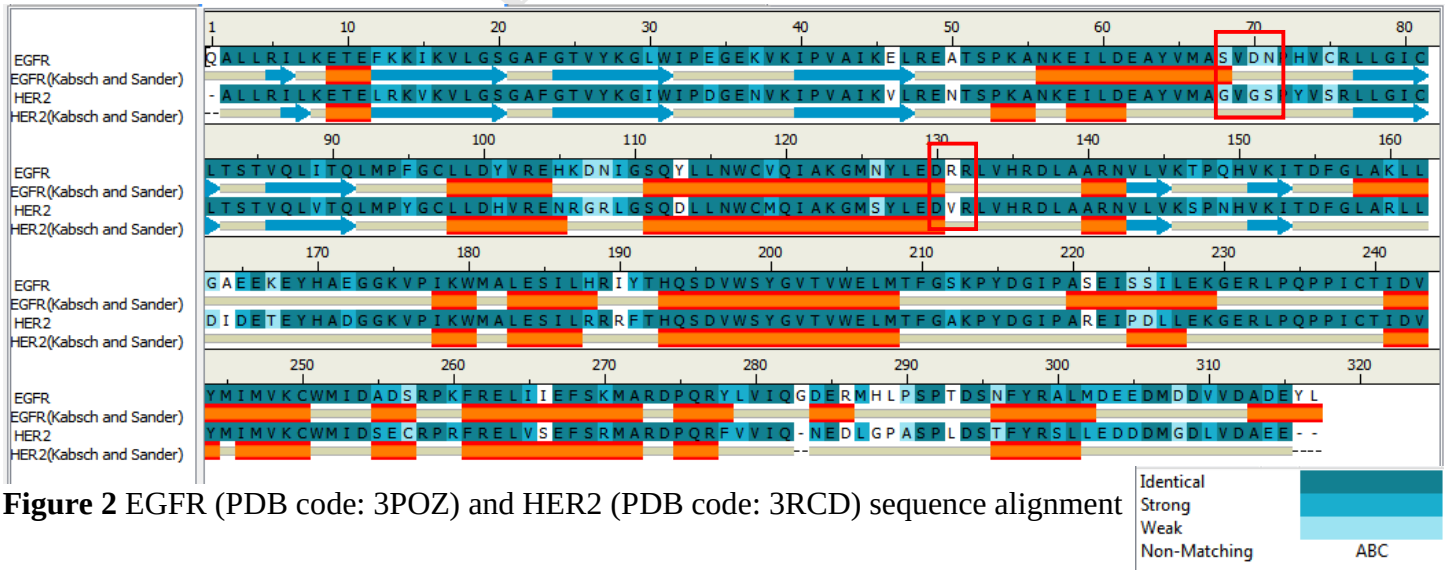


Figure 2 EGFR (PDB code: 3POZ) and HER2 (PDB code: 3RCD) sequence alignment

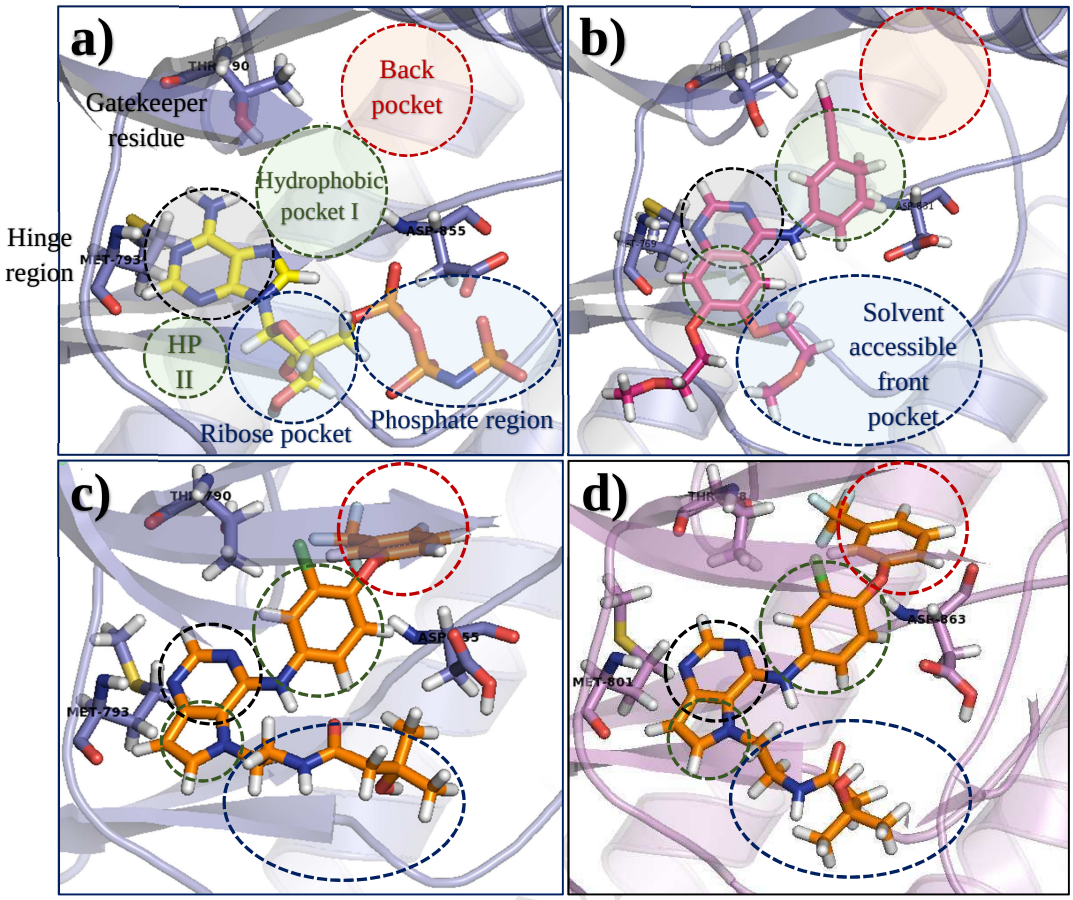


Figure 3 EGFR Kinase domain: ATP binding site. (a) EGFR (active conformation) complexed with AMP-PNP (PDB: 3VJO). (b) EGFR (active conformation) complexed with Erlotinib (PDB: 1M17). (c) EGFR (inactive conformation) complexed with TAK-285 (PDB: 3POZ). (d) HER2 complexed with TAK-285 (PDB: 3RCD).

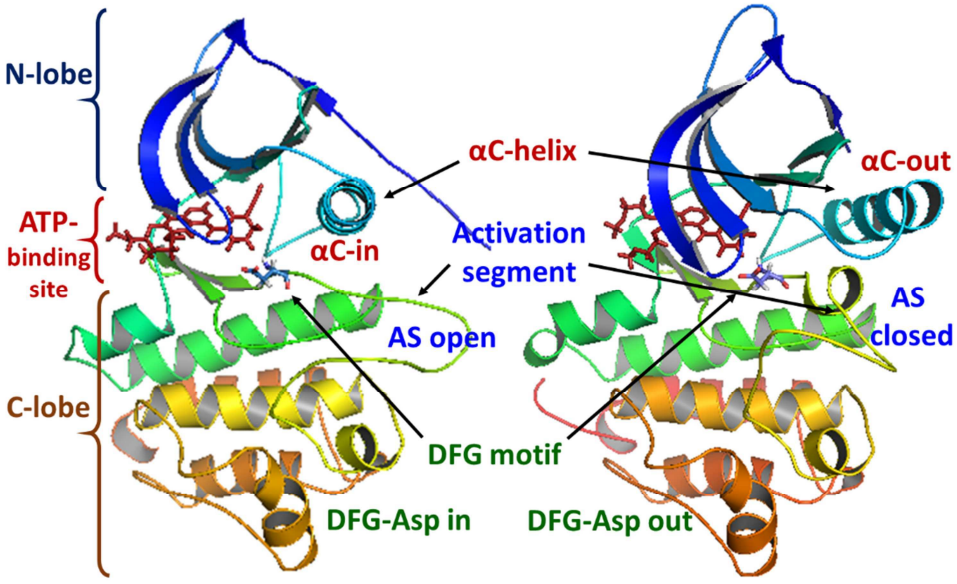


Figure 4 Active vs Inactive conformations of EGFR kinase domain

2.3. Dual EGFR/HER2 inhibitors: a literature review

Many studies categorized protein kinase inhibitors based on their binding modes and the relative conformations adopted by the kinase domain upon their binding. So far we have six classes of inhibitors; classes I-V are reversible inhibitors, and class VI comprises the irreversible covalent-binding inhibitors. Class I inhibitors bind to the active kinase conformation (DFG-Asp in and αC-helix in) (e.g. Gefitinib, Erlotinib in **Figure 3b**), while class II binds the inactive conformation (DFG-Asp out and αC-helix out). In between the two classes, lies a class that can be called "I½" in which the inhibitors bind an active-like conformation with the DFG-Asp in and the αC-helix out, allowing them to extend to the back pocket (e.g. Lapatinib, TAK-285 in **Figure 3c**). Classes III and IV are allosteric non-ATP competitive inhibitors, with class III binding in an allosteric binding site adjacent to the ATP-binding site and class IV binding away from this site. Class V are bivalent inhibitors spanning two distant sites in the kinase domain. The reported dual EGFR/HER2 inhibitors mostly belong to classes I½, II and VI, while class I inhibitors are usually EGFR selective. [25]

Reversible dual EGFR/HER2 inhibitors

The general layout of these compounds is composed of (1) a core scaffold - serving also as the hinge binder - that carries (2) a hydrophobic group - interacting with the selectivity pocket and usually extends into the back pocket - and (3) a solubilizing group that extends into the solvent-accessible region. (**Figure 5**)

While the kinase selectivity is mainly attributed to the hydrophobic group, the hydrophilic solubilizing group balances the LogP of the inhibitors and optimizes its drug-likeness. The solvent-accessible region can tolerate a wide range of solubilizing groups without affecting the inhibitor's enzymatic activity while modulating its cellular activity. Moreover, potency and selectivity profiles could be optimized upon introducing certain functionalities into the solubilizing group. [26]

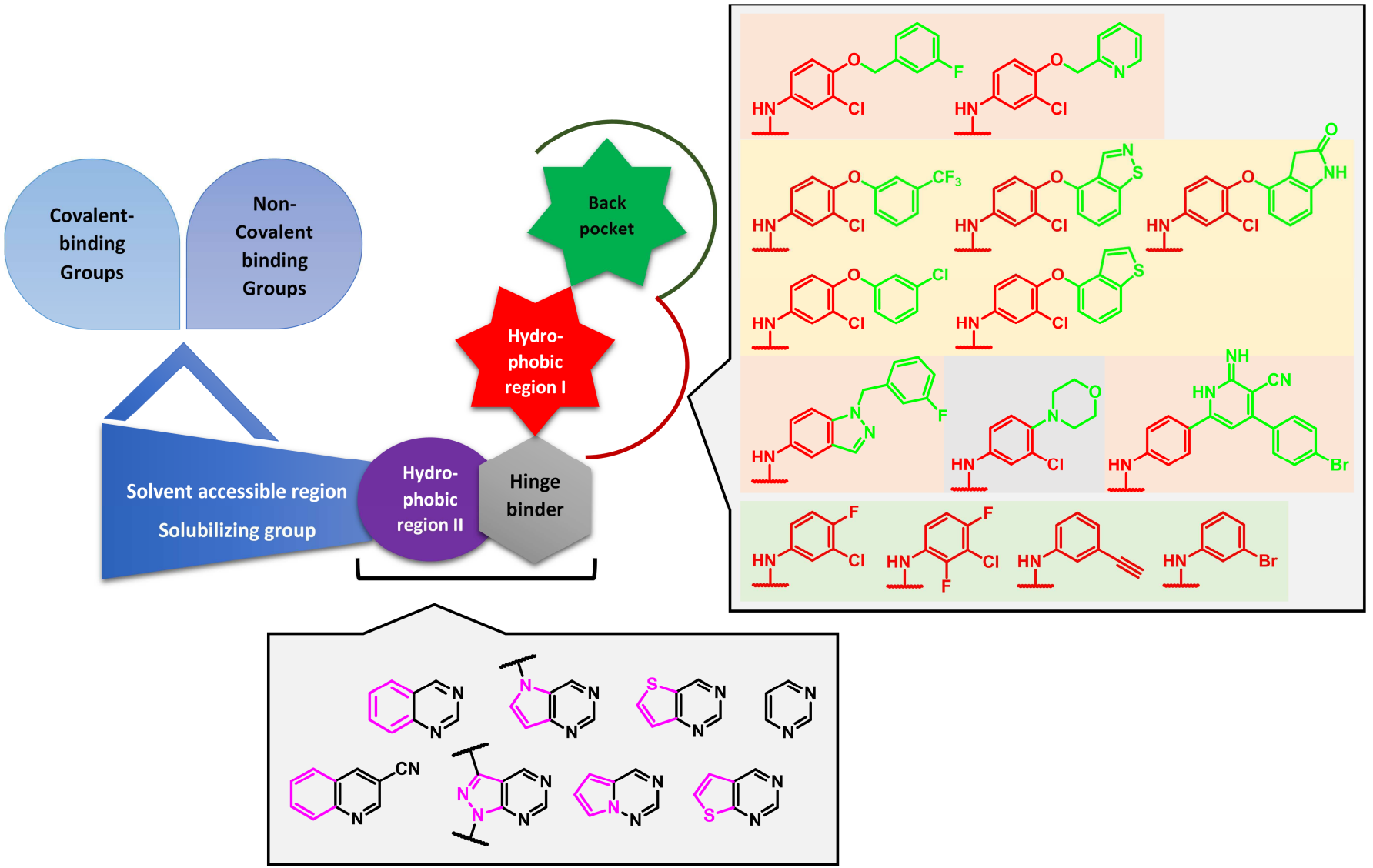
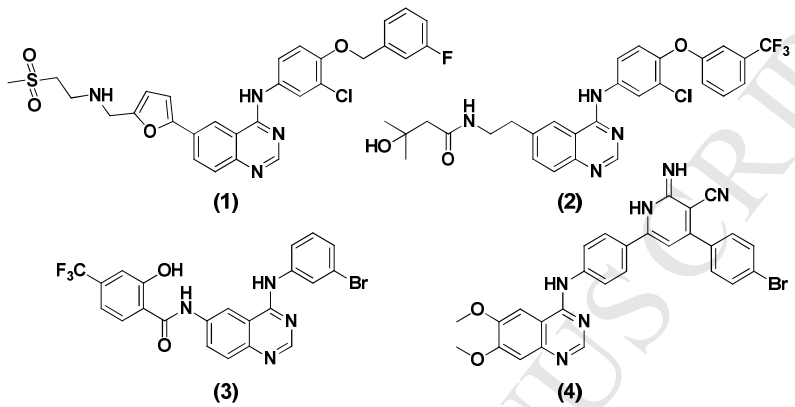


Figure 5 Binding mode and SAR of dual EGFR/HER2 inhibitors, and fragments used in the design of dual EGFR/HER2 inhibitors.

Based on their core scaffolds, dual EGFR/HER2 inhibitors can be classified into:

Quinazoline-based inhibitors

Quinazoline is one of the most commonly used hinge binder scaffolds for EGFR kinase inhibitors, evidently five out of the six FDA-approved EGFR inhibitors share a quinazoline scaffold [27]. The SAR of the quinazoline-based inhibitors is well-established [2].



Lapatinib (GW572016) (1) was the first dual EGFR/HER2 inhibitor to be approved by FDA in 2007 for breast cancer [28]. It has IC_{50} values of 10.8 nM and 9.2 nM against EGFR/HER2, respectively, and it showed high selectivity in a panel of kinases [29]. The studies published in the course of the development of Lapatinib (**1**) by GSK illustrated the types of 4-anilino substituents that favor a dual inhibitory activity, where their studies clearly demonstrated that a bulkier aniline derivative (such as N1-benzylindazolyl and 4-benzyloxyaniline) is required for dual inhibition, while smaller anilines (such as 3-ethynylaniline (Erlotinib), and 4-fluoro-3-chloroaniline (Gefitinib)) would rather inhibit EGFR only [30,31]. The fine-tuning of the 4-benzyloxyaniline led to the identification of the well-established 4-(3-fluorobenzyloxy)-3-chloroaniline side chain of Lapatinib (**1**) [32]. The crystal structure of EGFR kinase domain co-crystallized with Lapatinib (**1**) (**Figure 6**) illustrates that Lapatinib (**1**) is a class I½ inhibitor, binding to the inactive EGFR conformation with the activation segment in a closed conformation displacing the αC-helix to the "out" conformation, giving the 3-fluorobenzyloxy group access to the back pocket [33]. This extra binding in the back pocket contributes to the longer residence time observed for Lapatinib (300 min) compared to that of Gefitinib and Erlotinib (<10 min) which don't extend into the back pocket [25].

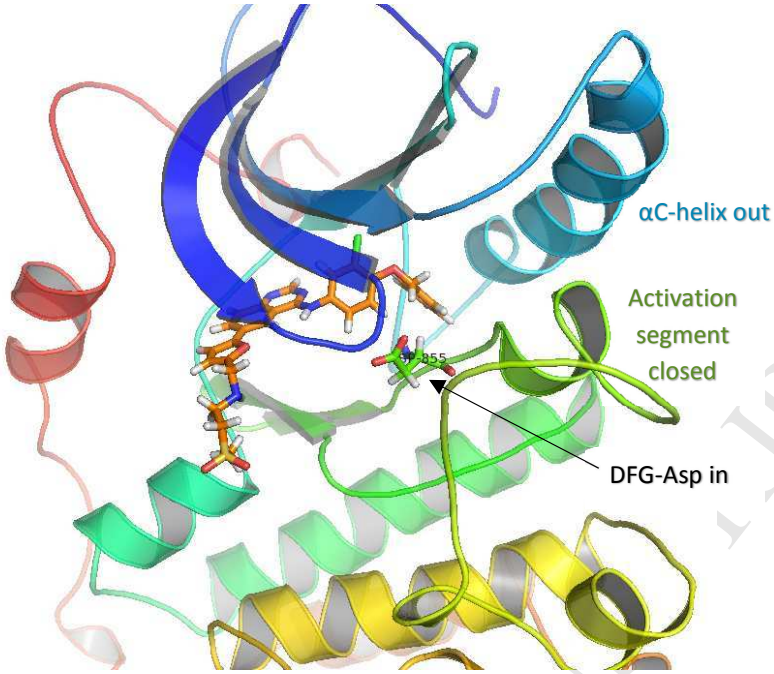


Figure 6 EGFR kinase domain co-crystallized with Lapatinib (orange) (PDB: 1XKK)

Compound A-10 (2) is a dual EGFR/HER2 inhibitor with IC_{50} values of 9.8 nM and 19 nM, respectively, that is also active against the double mutant T790M/L858R EGFR with IC_{50} of 33 nM. Its performance in the antiproliferative cellular assays was better than Gefitinib and Erlotinib with IC_{50} values of 41 nM against the cell line A431 (overexpressing wild-type EGFR), 127 nM against H1975 (overexpressing T790M/L858R EGFR) and 94 nM against MCF-7 (overexpressing HER2). A-10 (**2**) was developed through a fragment-based drug design protocol that was initially constructed to identify potent inhibitors active against the drug-resistant T790M EGFR. The detailed structural analysis of the available crystal structures of the EGFR family, followed by a fragment-based design and an extensive docking study identified the hydrophobic group 3-chloro-4-(3-(trifluoromethyl)phenoxy)aniline as a good fit for the activity against T790M EGFR and also against HER2. [34]

Compound (3) is a 6-salicyl-4-anilinoquinazoline derivative that was developed as a dual EGFR/HER2 inhibitor with IC_{50} values of 0.12 μM and 0.096 μM, respectively. It showed 100-fold selectivity in a panel of 9 closely similar kinases. It performed well in cell-based assays with IC_{50} values of 1.64 μM against A549 (human alveolar basal epithelial carcinoma), 0.49 μM against MCF-7 and 0.67 μM against A431. The unusual somewhat hydrophobic group (4-trifluoromethylsalicyl) that is in the position of the solubilizing group along with a less bulky aniline, and the dual inhibitory activity of their combination, is a noteworthy finding. The potency of the compound is attributed to the trifluoromethyl moiety, inasmuch as switching the trifluoromethyl into a chloro substituent results in 10-fold decrease in EGFR inhibition and 30-fold decrease in HER2 inhibition. The reported docking study suggests a different binding mode with the compound flipped and the 4-

trifluoromethylsalicyl group directed to the back pocket instead of the solvent accessible region. [35]

Compound (4) is a dual EGFR/HER2 inhibitor with moderate potency, IC_{50} values of 1.935 μM and 1.036 μM respectively. It has an unusual bulky aryl 2-imino-1,2-dihydropyridine derivative in the position of the back pocket binder, that illustrates the degree of flexibility in this region and adds to the dual inhibitors aniline fragments database. [36]

Pyrrolo[2,3-d]pyrimidine-based inhibitors

A series of pyrrolo[2,3-*d*]pyrimidine-based dual EGFR/HER2 inhibitors was developed by a research group in Takeda Pharmaceutical Co. Their focus was to find back-pocket binders with optimal dual inhibitory activity, while optimizing the cellular activity and the pharmacokinetic profiles through manipulating the solubilizing group (**Figure 7**).

They developed some potent dual inhibitors **(5-8)** with potent cell growth inhibitory activities (**Table 1**).

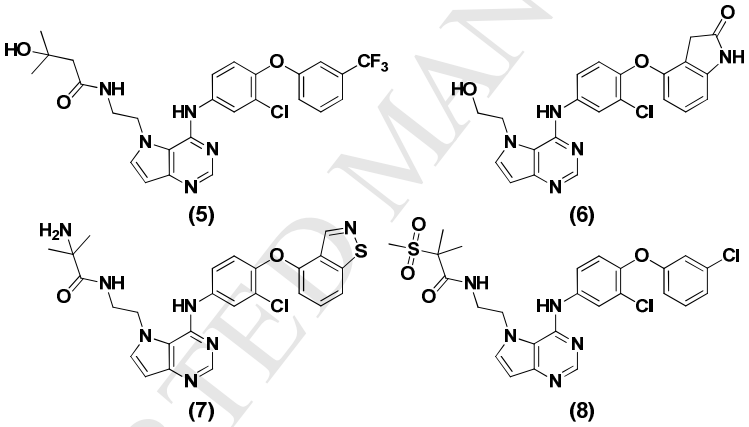


Table 1 Biological Data for Pyrrolo[2,3-*d*]pyrimidines (5-8)

Compound	EGFR IC_{50} (nM)	HER2 IC_{50} (nM)	BT-474* GI_{50} (nM)	Reference
TAK-285 (5)	23 nM	17 nM	17 nM	[37]
(6)	3 nM	1.6 nM	13 nM	[38]
(7)	2.6 nM	0.98 nM	2 nM	[38]
(8)	11 nM	11 nM	56 nM	[39]

*BT-474: HER2-overexpressing human breast cancer cell line

They managed to co-crystallize **TAK-285 (5)** (a selective dual EGFR/HER2 inhibitor) with both EGFR (PDB: 3POZ) [40] and HER2 (PDB: 3RCD) [37] to be the first dual inhibitor co-crystallized with both receptors. These crystal structures revealed much about the structural basis required for dual inhibition. The crystal structures **(Figure 3c,d)** confirmed the expected binding modes (as in **Figure 7**). The

3-trifluoromethylphenoxy moiety extends to the back pocket displacing the αC-helix into the inactive conformation, this extra back-pocket binding anchors the inhibitor firmly to the binding site, which explains the slow off-rate observed for this inhibitor or the "pseudo-irreversible" (PI) mode. A stronger "pseudo-irreversible" (PI) activity is correlated to higher cellular inhibition [37,38].

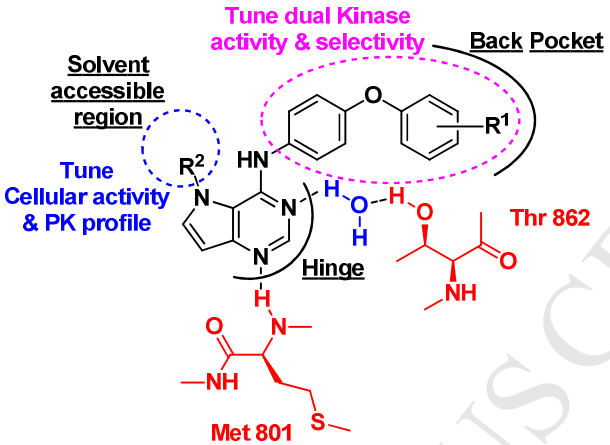


Figure 7 Design Strategy of Pyrrolo[2,3-*d*]pyrimidine dual inhibitors (Adapted from Ishikawa et al. [37])

Compounds (6) and **(7)** were developed with the aim of maximizing the PI activity. A docking study proposed that a carboxamide-containing compound (**Figure 8**) would have an enhanced PI profile through the extra hydrogen bonding of the carboxamide substituent with the amino acids in the back pocket; Ser783/Arg784/Thr798 in HER2 and likewise Cys775/Arg776/Thr790 in EGFR, and so they screened for a back-pocket binder with a fused ring system that can establish the same binding mode. The screening identified compounds **(6)** and **(7)** to be potent dual inhibitors with potent cellular activities (**Table 1**) and strong PI effects demonstrated by their remarkably slow off-rates, conforming to what was proposed by docking (**Figure 8**). [38]

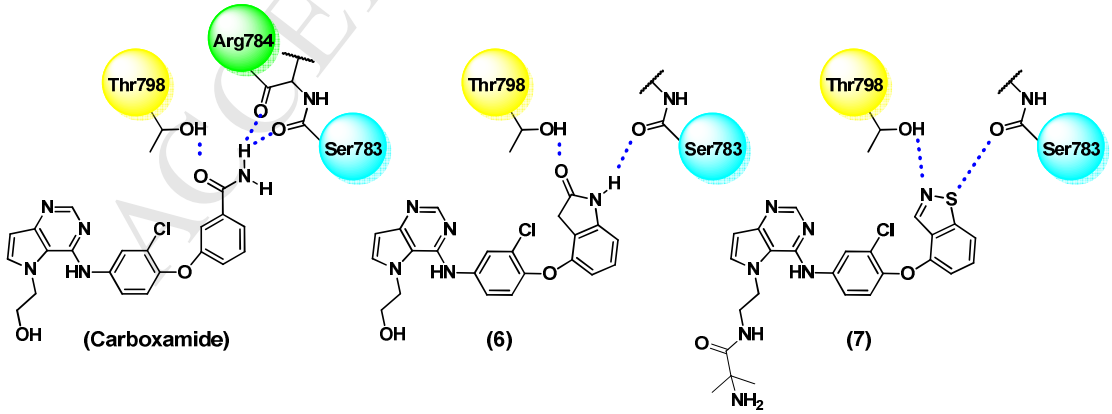
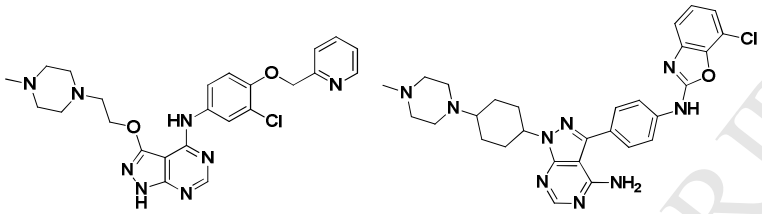


Figure 8 Proposed binding mode in HER2 back pocket (Adapted from Kawakita et al. [38])

Compound (8) was identified in an attempt to improve the physicochemical and pharmacokinetic profiles of TAK-285 (5) through optimizing the solubilizing group and the 3-substituent on the phenoxy ring. [39]

1H-pyrazolo[3,4-d]pyrimidine-based inhibitors



(9) **(10)**

The development of **Compound (9)** followed the classical kinase inhibitors design; the research group at AstraZeneca proposed the 1*H*-pyrazolo[3,4-*d*]pyrimidine scaffold as a hinge binder, and screened for a benzyloxy group providing optimum dual inhibition, and finally tuned the solubilizing group to identify **compound (9)** with IC_{50} values of 5 nM and 1 nM against EGFR/HER2, respectively, and IC_{50} of 17 nM against BT474 cell line. [41]

One of the mechanisms of EGFR inhibitors resistance is the activation of a compensatory alternative receptor tyrosine kinase pathway (**Figure 1**), one of which is the Insulin-like growth factor-1 receptor (IGF1R), such an observation led to the assumption that IGF1R inhibitors could hinder the development of acquired resistance [42] which encouraged a research group in Abbott Laboratories to develop multi-targeted kinase inhibitors targeting both EGFR/HER2 and IGF1R. A lead compound with the 4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine scaffold was identified in a high throughput screening. Subsequent optimization led to the development of **compound (10)** with IGF1R IC_{50} of 12.5 nM, EGFR (L858R) IC_{50} of 31.5 nM and HER2 IC_{50} of 7.5 nM. [43]

Pyrrolotriazine-based inhibitors

A research group at Bristol-Myers Squibb identified the pyrrolotriazine scaffold as a hinge binder alternative to the quinazoline [44]. They developed a series of pyrrolotriazine-based dual inhibitors (11-13) with potent inhibitory activities and high selectivity to EGFR-dependent cancer cell lines (**Table 2**). They screened for a bulky anilino group that could provide the optimum dual inhibition, to eventually identify the 1-(3-fluorobenzyl)-1*H*-indazol-5-amine as a back-pocket binder in **compounds (11)** and **(12)** [45]. The subsequent modifications in the region of the solubilizing group led to the development of **BMS-599626 (12)** with desirable pharmacodynamics and pharmacokinetic profiles that was advanced into clinical trials [46].

Compound (13) was developed with the aim of reducing the molecular weight and the LogP for a better pharmacokinetic profile while maintaining the potency and the selective dual inhibition if not improving it. Interestingly enough, **compound (13)** has a small aniline group that usually can't extend into the back pocket yet it maintains potent and selective dual inhibition, it is an unlikely finding since most of the reported dual inhibitors usually have a bulky back-binder anilino moiety. [47]

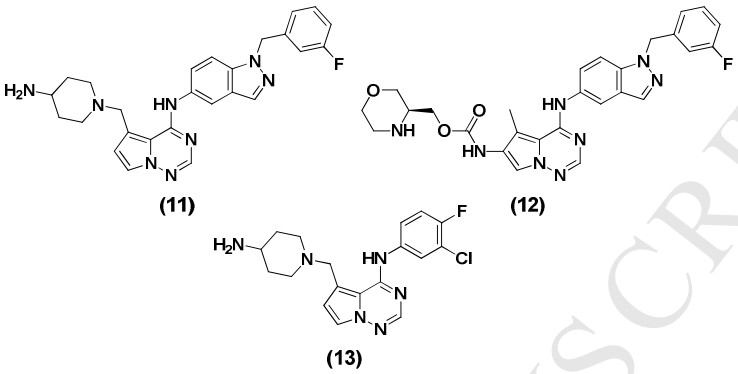


Table 2 Biological Data for Pyrrolo[2,1-*f*][1,2,4]triazines (11-13)

Compound	EGFR IC_{50} (nM)	HER2 IC_{50} (nM)	N87[a] IC_{50} (nM)	HT29[b] or A2780[c] IC_{50} (nM)	Reference
(11)	35	23	35	4300[b]	[45]
BMS-599626 (12)	22	32	450	>10,000[c]	[46]
(13)	6	10	120	>5000[c]	[47]

[a] N87: human gastric carcinoma overexpressing EGFR and HER2
[b] HT29: human colon carcinoma, independent on EGFR/HER2 signaling
[c] A2780: human ovarian carcinoma, independent on EGFR/HER2 signaling

Thienopyrimidine-based inhibitors

The thienopyrimidine core was found to be an effective bioisostere for the quinazoline as a hinge binder, where **compound (14)**, with the exact same aniline and solubilizing groups as Lapatinib (1), showed comparable potent dual inhibition to EGFR/HER2 with good cellular activity and low cytotoxicity (**Table 3**).

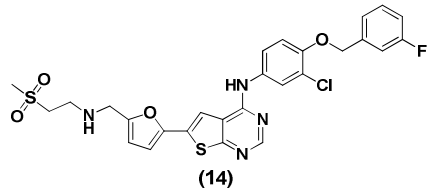


Table 3 Biological Data for Compound (14)

EGFR IC_{50}	HER2 IC_{50}	HN5[a] IC_{50}	BT474[b] IC_{50}	HFF[c] IC_{50}	Reference

12 nM	6 nM	0.42 µM	0.42 µM	19.45 µM	[48]

[a] HN5: human Head & Neck carcinoma overexpressing EGFR
[b] BT474: human breast carcinoma overexpressing HER2
[c] HFF: human foreskin fibroblast cell line, normal cells, control

Pyrimidine-based inhibitors

Not only that the quinazoline core serves as a hinge binder but it also positions the remaining pharmacophoric features properly towards their contact points. Based on that, a research group at Johnson & Johnson developed a series of 4-aminopyrimidines (**15-17**) as dual EGFR/HER2 inhibitors. They proposed that the 4-aminopyrimidine would form an intramolecular hydrogen bond (between the 4-amino group and an acceptor-containing side chain at C-5) and cyclize into a 6-membered ring resembling the quinazoline (**Figure 9**), and so it would direct the hydrophobic and the solubilizing groups to their positions and at the same time interact with Met793 in the hinge region. Attempts to methylate the 4-amino group abolished the EGFR/HER2 activity which supported the proposed model (**Figure 9**).

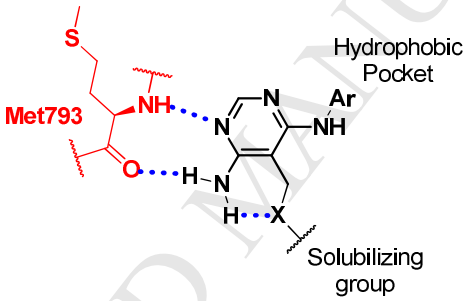


Figure 9 Pyrimidine-based dual inhibitors (15-17) binding mode [49].
X = N or O. Blue dotted lines represent hydrogen bonds.

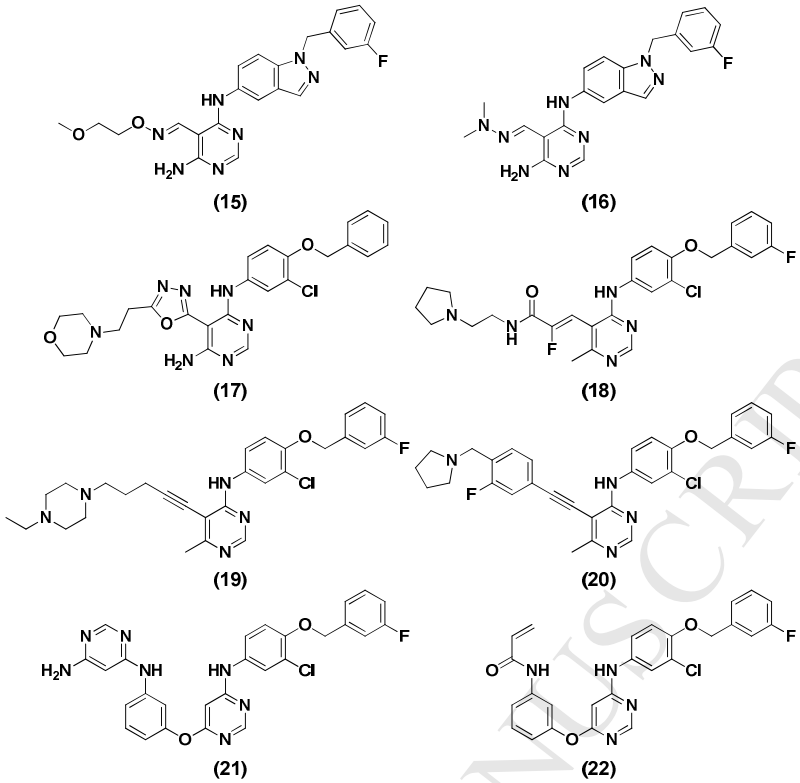


Table 4 Biological Data for Pyrimidine-based inhibitors (15-22)

Compound	EGFR IC_{50} (nM)	HER2 IC_{50} (nM)	Cellular activity IC_{50} (μM)	Reference
(15)	14	6	BT-474 [a] = 60% at 10 μM SK-BR-3 [b] = 84% at 10 μM	[49]
(16)	8	2	BT-474 [a] = 0.063 SK-BR-3 [b] = 0.148 N87 [c] = 0.131 HeLa [d] > 100	[50]
(17)	30	80	BT-474 [a] = 0.31 SK-BR-3 [b] = 0.33 N87 [c] = 0.41 HeLa [d] > 100	[51]
(18)	3	19	BT-474 [a] = 1.13	[52]
(19)	9	36		[52]
(20)	190	62	BT-474 [a] = 0.05 N87 [c] = 0.06	[53]
(21)	61	42	A431 [e] = 4.24 SK-OV-3 [f] = 0.71	[54]
(22)	37	29	A431 [e] = 3.25 SK-OV-3 [f] = 0.89	[54]

[a] BT-474 = human breast carcinoma overexpressing HER2

[b] SK-BR-3 = human breast carcinoma overexpressing HER2

[c] N87 = human gastric carcinoma overexpressing EGFR and HER2
[d] HeLa = human cervical adenocarcinoma, independent on EGFR/HER2 signaling
[e] A431 = human epidermoid carcinoma overexpressing WT-EGFR
[f] SK-OV-3 = human ovarian carcinoma overexpressing HER2

The design of **compound (15)** started with 4-aminopyrimidine-5-carboxaldehyde oxime to mimic the quinazoline core, then the group screened for an aniline that can afford a desirable dual inhibitory activity. They found that small anilines and simple fused anilines favored EGFR inhibition, while bulkier anilines with benzyl-substituted fused rings were inclined towards dual inhibition, conforming to the previously reported SAR. The 1-benzylindazole showed good dual inhibition, with the 3-fluoro substituted benzyl specifically favored, given that the addition of that 3-fluoro had a dramatic effect on the activity compared to the unsubstituted 1-benzylindazole. A final screening for a solubilizing group on the oxime side chain at C-5 afforded **compound (15)** with a methoxyethoxy moiety attached to the oxime, which demonstrated potent dual inhibition (**Table 4**). The crystal structure of (**15**) with EGFR (**Figure 10a**) proved the proposed conformation and binding mode (**Figure 9**). The 4-aminopyrimidine core forms bidentate hydrogen bonds (HB) with Met793 of the hinge region, the 1-(3-fluorobenzyl)indazole extends into the back pocket where the N-2 of the indazole forms a HB with Leu788 and the 3-fluoro forms HBs with Arg776 and Thr790, explaining the special effect the fluoro had on the activity. [49]

Further optimization by replacing the oxime with *N*,*N*-disubstituted hydrazone to restrict the rotation of the side chain and maintain the favorable conformation resulted in **compound (16)** with improved enzymatic and cellular activities (**Table 4**), and its crystal structure showed the same binding mode as (**15**) maintaining the same interactions with the binding site (**Figure 10b**). [50]

Compound (17) originated from a hit dual inhibitor that had a 5-methyl carboxylate ester group (**Figure 11**, left), but for metabolic stability issues the oxadiazole ring was put forth as a bioisostere for the ester, which worked out well giving compound (**17**) with potent dual inhibition and high selectivity to EGFR/HER2 (**Table 4**). It was hypothesized that the activities of the ester and the oxadiazole derivatives were attributed to their abilities to form 2 intramolecular hydrogen bonds and thus maintaining the favored conformation (**Figure 11**). [51]

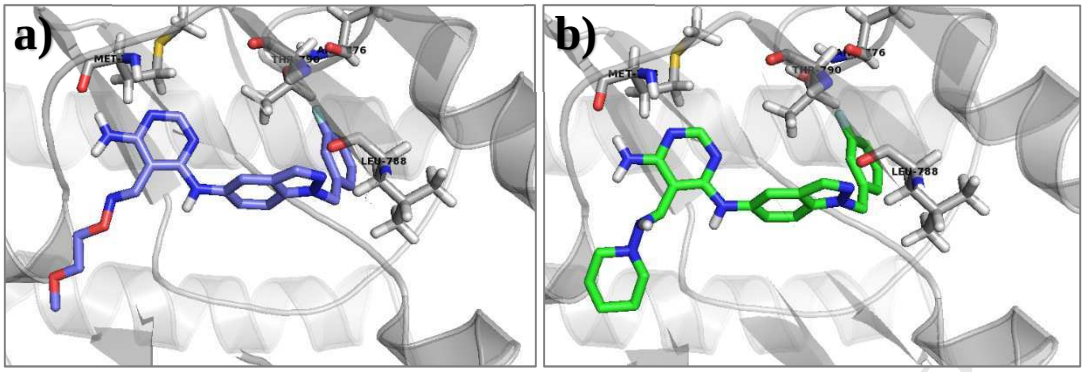


Figure 10 Pyrimidine-based inhibitors binding mode. (a) Crystal structure of EGFR complexed with compound (**15**) (PDB: 3BEL) [49]. (b) Crystal structure of EGFR complexed with compound (**16**) (PDB: 2RGP) [50].

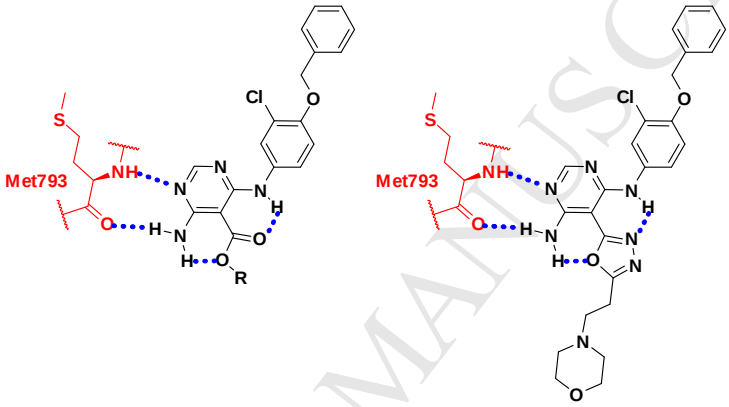


Figure 11 Proposed binding mode of compound (**17**) (right) [51]. Blue dotted lines represent hydrogen bonds.

Another research group at Shionogi Medicinal Research Laboratories reported the development of pyrimidine-based dual inhibitors (**18**-**20**) with the aim of mimicking the quinazoline hinge binder while improving the physicochemical properties of the compounds. They retained the 4-(3- fluorobenzyloxy)-3-chloroaniline group of Lapatinib to guarantee dual inhibition and focused their efforts on optimizing the solubilizing group. The SAR study showed that any further extension of the 6-methyl decreased the activity, which is logical as it will hinder the hinge binding, and the most potent solubilizing groups at C-5 were the groups that could maintain a coplanar conformation with the pyrimidine mimicking the quinazoline bicyclic system; namely the 5-alkenyl (**compound 18**) and the 5-alkynyl (**compound 19**) side chains (**Figure 12**). It was observed that the introduction of aromatic rings to the solubilizing group would improve cellular activity, which led to the development of **compound 20** with improved cellular activity (**Table 4**) and high selectivity to the HER family. [52,53]

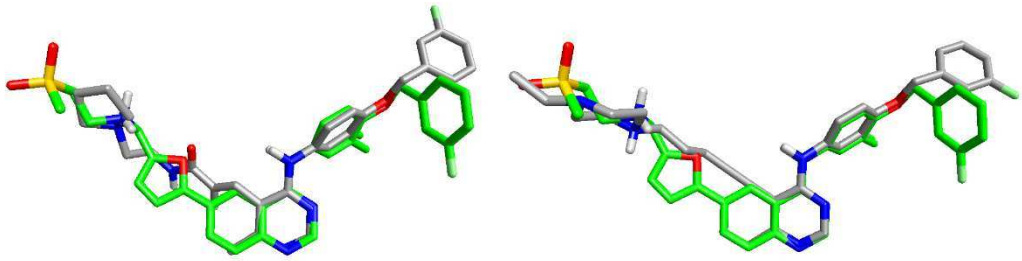


Figure 12 Compound (**18**) (left) and (**19**) (right) aligned to Lapatinib bioactive conformation (green) (PDB: 1XKK)

Likewise based on the design of Lapatinib, compounds (**21**-**22**) were developed with 6-(3-substituted phenoxy) side chain as a solubilizing group, which is assumed to adopt the U-shaped conformation (**Figure 13**) to mimic the quinazoline core, avoid bumping into the hinge region and direct the side chain into the solvent-accessible region. [54]

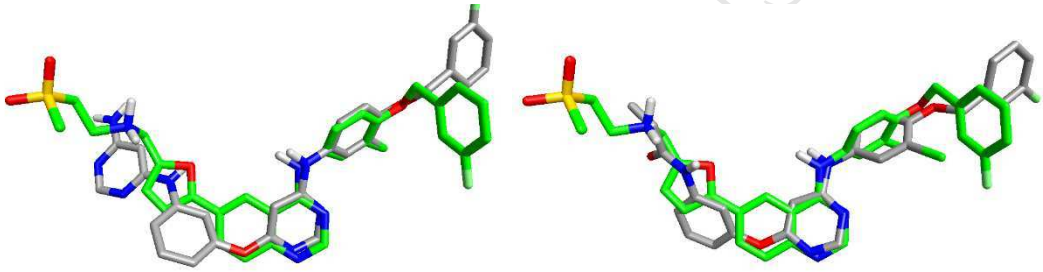
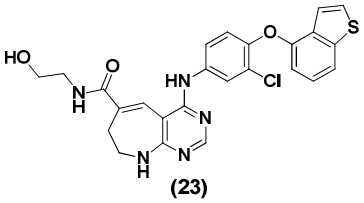


Figure 13 Compound (**21**) (left) and (**22**) (right) aligned to Lapatinib bioactive conformation (green) (PBD: 1XKK)

Pyrimido[4,5-b]azepine inhibitors

Takeda's research group, the developers of TAK-285, was investigating the employability of pyrimido[4,5-*b*]azepine as a scaffold and hinge binder. They anticipated that the N-9 of the azepine ring would form an extra hydrogen bond with the hinge region enhancing the inhibitor's binding affinity and so its potency. When they replaced the 3-chloro-4-(3-(trifluoromethyl)phenoxy)aniline (the aniline used in TAK-285) with the 4-(benzo[*b*]thiophen-4-yloxy)-3-chloroaniline in **compound (23)**, the dual inhibitory potency and the cellular activity increased (**Table 5**), in addition to an enhanced pseudo-irreversible activity compared to TAK-285. The crystal structure of (**23**) complexed with EGFR (**Figure 14**) shows that the N-1 and N-9 form bidentate hydrogen bonds with Met793 (hinge region) as anticipated, it also shows that the benzothiophene extends into and interacts with the back pocket without affecting its

conformation, which means it is better accommodated in that pocket, which might explain the distinctively slow off-rate observed for this compound. [55]



(23)

Table 5 Biological Data for Compound (**23**)

EGFR IC_{50}	HER2 IC_{50}	BT474[a] IC_{50}	Reference
36 nM	24 nM	18 nM	[55]

[a] BT474: human breast carcinoma overexpressing HER2

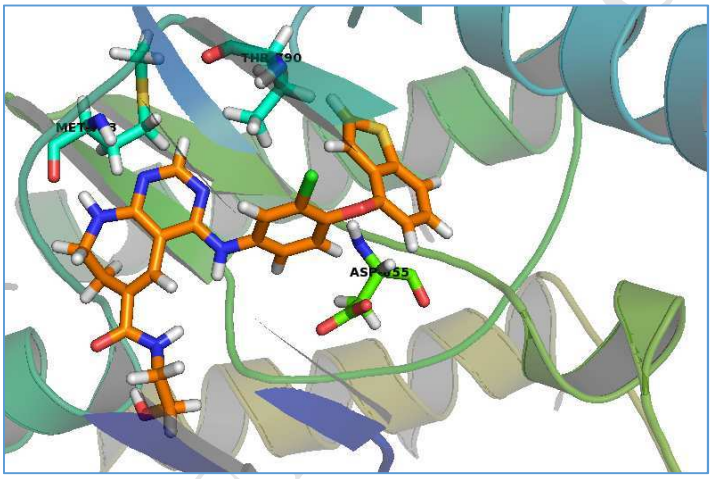


Figure 14 EGFR kinase domain complexed with Compound (**23**) (PDB: 3W33)

Miscellaneous

The discoveries of this group of structurally diverse dual inhibitors (**24-26**) just proved the immense chemical and structural space that can be employed to develop novel dual EGFR/HER2 inhibitors, which can provide various opportunities to enhance the pharmacokinetic profile of such inhibitors and to tailor inhibitors that can overcome drug resistances arising from receptor mutations.

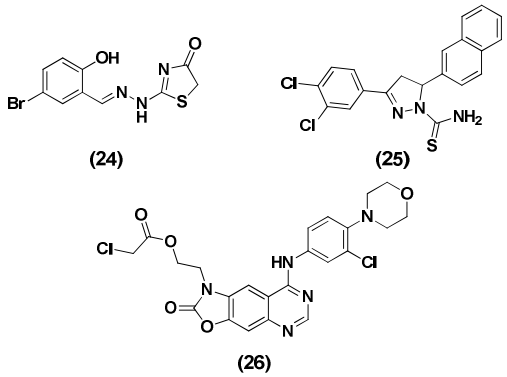


Table 6 Biological Data for miscellaneous dual inhibitors (24-26)

Compound	EGFR IC_{50}	HER2 IC_{50}	Cellular activity IC_{50}	Reference
(24)	0.09 μM	0.42 μM	MCF-7 [a] = 0.06 μM	[56]
(25)	0.66 μM	0.88 μM	MCF-7 [a] = 0.35 μM A549 [b] = 0.31 μM	[57]
(26)	0.019 μM	0.035 μM	A546 [b] = 4.49 μM SK-BR3 [c] = 0.47 μM HELF [d] > 100 μM	[58]

[a] MCF-7 = human breast carcinoma

[b] A549 = human lung carcinoma overexpressing EGFR

[c] SK-BR3 = human breast carcinoma overexpressing HER2

[d] HELF = human embryonic lung fibroblast cell line, normal cells, control

3. Mutant T790M/L858R EGFR inhibitors

L858R is a somatic activating mutation, it represents 40% of EGFR activating mutations which are usually associated with increased sensitivity to EGFR-selective TKIs. It is a missense mutation in the activation segment where Leu858 is substituted with Arg858 [59]. This results in an activating mutation since it stabilizes the enzyme's active conformation causing constitutive activation, promotes heterodimerization and decreases ATP binding affinity [12].

T790M is a secondary acquired resistant mutation, it is the most commonly reported EGFR-resistance mechanism accounting for 50-60% of the acquired resistance cases in patients treated with EGFR-selective small molecule inhibitors (e.g. Gefitinib and Erlotinib) [12]. It is a point mutation in which the gatekeeper residue Thr790 is substituted with a Met790.

3.1. Structural aspects of the T790M/L858R EGFR mutations

In the inactive conformation of EGFR kinase domain, the activation segment closes and turns into a short helix displacing the αC-helix outwards (**Figure 4**). The Leu858 in the activation segment forms hydrophobic interactions with nearby residues stabilizing this short helix and this inactive conformation (**Figure 15b**). In the L858R mutation, the Leu858 is substituted with the bulkier polar Arg858 that fails to form the short helix and so fails to adopt the inactive conformation locking the enzyme in the active conformation (**Figure 15a**), hence the constitutive activation. [60]

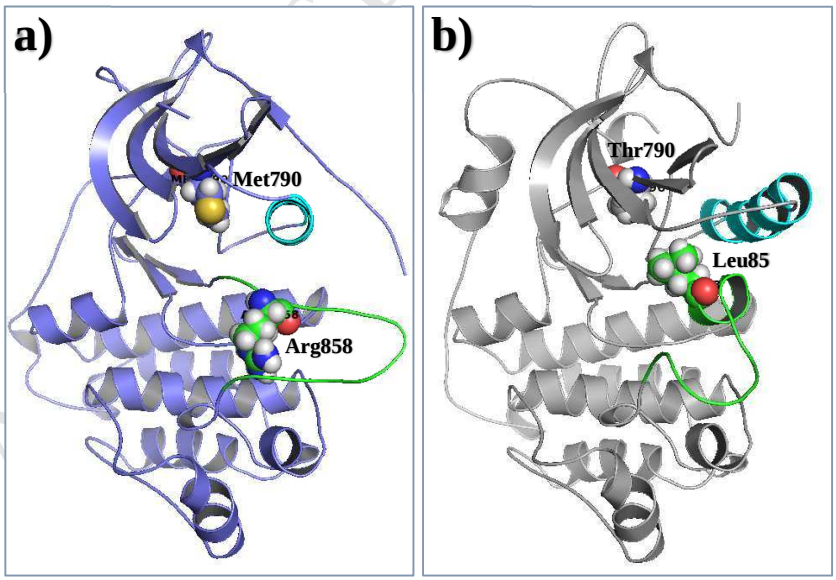


Figure 15 Structural aspects of EGFR mutations. (a) Double mutant T790M/L858R EGFR (PDB: 3W2O). (b) Wild-type EGFR in the inactive conformation (PDB: 3POZ). (αC-helix in cyan, activation segment in green)

T790M mutation also causes constitutive activation, however, there are other consequences to the alteration from Threonine to Methionine. T790M mutation renders the enzyme resistant to EGFR inhibitors for the following reasons (summarized in **Figure 16a**); [61]

(1) **The stabilized active-like αC-helix:** The more hydrophobic Met790 maintains a hydrophobic contact between the N-lobe and the αC-helix, thus stabilizing the αC-helix in the active-like "in conformation", hence the constitutive activation.

(2) **Energy penalty of displacing the αC-helix:** The binding of EGFR inhibitors into the active site requires the binding of the anilino group into the hydrophobic pocket between the gatekeeper and the αC-helix, displacing the αC-helix outwards, this tilting of the αC-helix imposes an energy penalty that decreases the binding affinity of the EGFR inhibitor to the mutant T790M.

(3) **The steric effect of Met790:** The bulkier Met790 imposes a steric challenge to the binding of EGFR inhibitors, since the binding of the anilino group near the gatekeeper forces the Met790 sidechain to rotate into a less favorable conformation, which also decreases the binding affinity of the EGFR inhibitor to the mutant T790M. Meanwhile, the binding affinity of ATP is not affected since it doesn't interact with the gatekeeper or the back pocket region, resulting in an overall increased sensitivity of the mutant T790M to ATP.

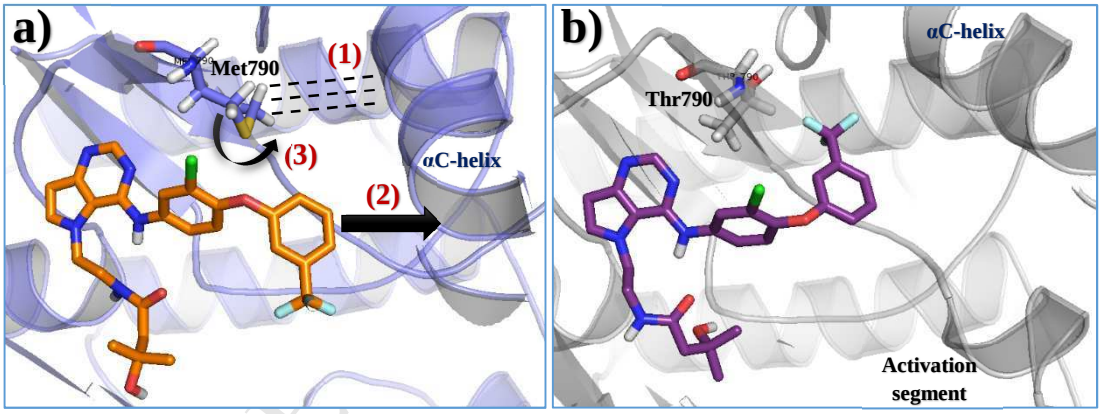


Figure 16 Structural basis of T790M resistance. (a) Double mutant T790M/L858R EGFR complexed with TAK-285 (orange) (PDB: 3W2O). (b) Wild-type EGFR complexed with TAK-285 (violet) (PDB: 3POZ).

In conclusion, the double mutant T790M/L858R is constitutively activated with increased affinity to ATP and decreased affinity to EGFR inhibitors, hence the resistance and the overactivation.

3.2. Design strategies to overcome T790M/L858R resistance

In order to design an inhibitor that can tackle the mutation-driven resistance, the previously mentioned structural basis of the mutation should be considered. The design strategies (summarized in **Figure 17**) proposed to overcome the double mutation will be enumerated in the same order as the corresponding mutant structural modification in the previous paragraph (summarized in **Figure 16a**):

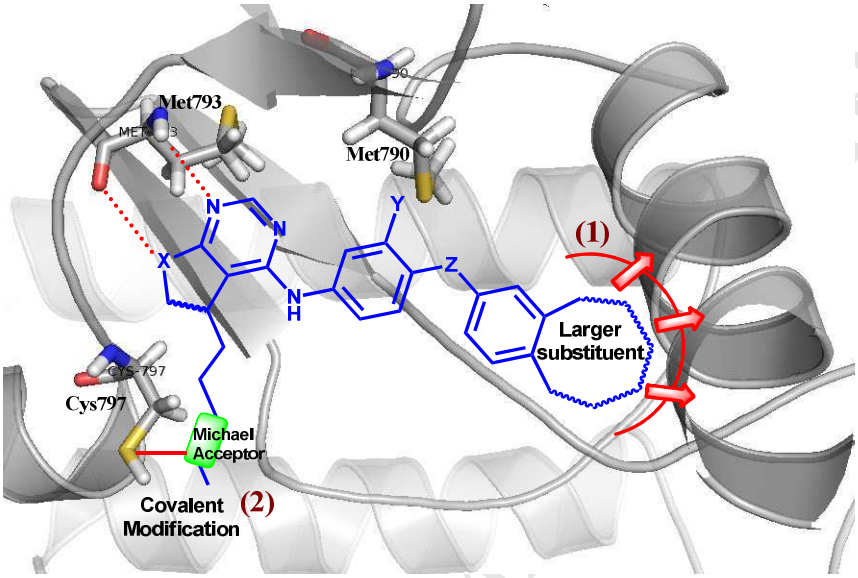
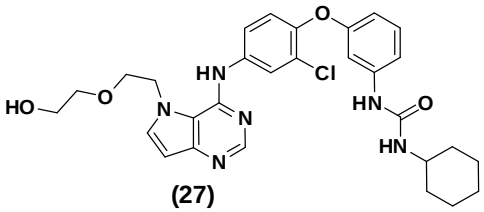


Figure 17 Design Strategies to overcome T790M/L858R resistant mutation. The protein model is for the double mutant T790M/L858R EGFR (PDB: 3W2O)

(1) **Overcoming the stabilized active-like αC-helix:** in order to attain the inactive conformation, the activation segment should be able to form a short helix that displaces the αC-helix and forms hydrophobic contacts to stabilize the inactive conformation, which is not attainable in the mutant T790M/L858R. Based on this concept, TAK-285 research group in Takeda developed **compound (27)**. They hypothesized that the hydrophobic group in TAK-285 (the 3-trifluoromethylphenoxy) is not bulky enough to displace the αC-helix in the mutant T790M/L858R (TMLR) in absence of the short helix of the activation segment, therefore TAK-285 is inactive against the TMLR. So they designed compound (**27**) with the extra-bulky cyclohexyl-urea substituent to interact with the activation segment, displace the αC-helix and establish the hydrophobic contacts to stabilize the inactive conformation. Their hypothesis was proven correct considering compound (**27**) ability to inhibit the TMLR with good potency, and the expected binding pattern was illustrated in the crystal structure of compound (**27**) with TMLR (**Figure 18a**). [62]



(27)

EGFR-WT IC_{50} = 6.9 nM

T790M/L858R IC_{50} = 19 nM

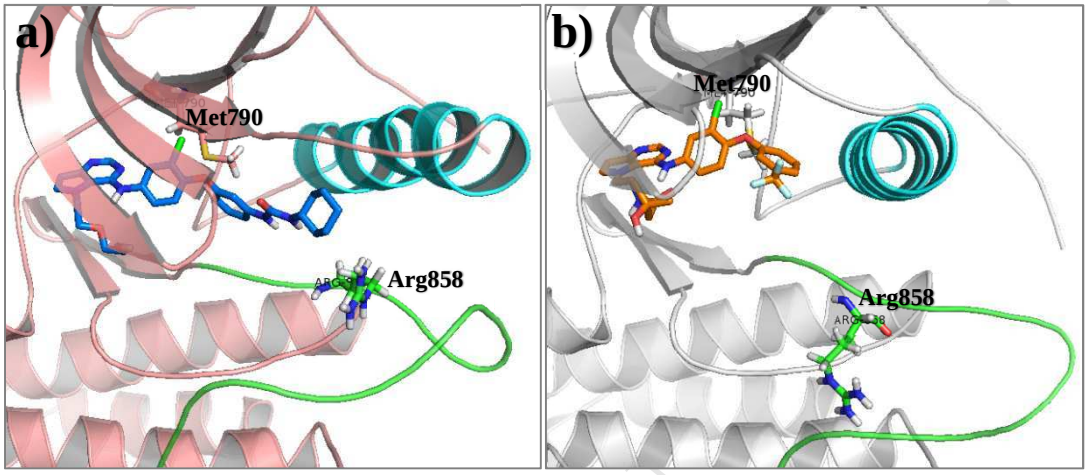


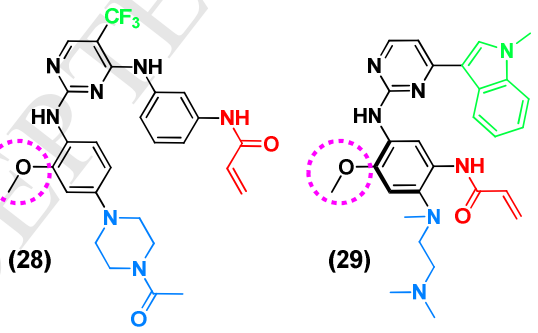
Figure 18 Overcoming the stabilized active-like αC-helix. (a) Double mutant T790M/L858R EGFR complexed with compound (**27**) (blue) (PDB: 3W2R). (b) Double mutant T790M/L858R EGFR complexed with TAK-285 (orange) (PDB: 3W2O). (αC-helix in cyan, activation segment in green)

(2) **Overcoming the energy penalty of displacing the αC-helix:** the binding of EGFR inhibitors to the TMLR is less favored as a result of the energy penalty of displacing the αC-helix and due to the steric effect of the Met790, however, it was found that the addition of a Michael acceptor to the inhibitor in a position that can form a covalent bond with Cys797 in the solvent accessible region will ultimately shift the binding to the final protein-inhibitor complex causing the inactivation of the TMLR [63]. Based on this strategy, numerous irreversible inhibitors were designed with the aim of overcoming resistant mutations (*vide infra*).

(3) **Overcoming the steric effect of Met790:** a so-called third generation of EGFR inhibitors was developed in response to the emergence of drug resistances to the previous EGFR inhibitors, the design strategy was focused on tailoring inhibitors selective to the mutant EGFR sparing the wild-type EGFR for enhanced safety profiles. **Rociletinib (CO-1686) (28)** was among the earliest mutant-selective inhibitors developed, its selectivity reaches 22-fold selectivity to the double mutant (DM) EGFR over the WT [64]. The design concept for these mutant-selective inhibitors targeted the Met790 as the key difference between the mutant and the WT EGFR, for instance, they tried to avoid the steric effect imposed by Met790 and to benefit from the hydrophobicity of Met790 as opposed to Thr790. The design of the third generation inhibitors (**Figure 19**) followed a U-shaped

structure, whose core scaffold (mostly pyrimidine) serves as a hinge binder, and orients the rest of the compound in that U-shaped conformation. At the end of the U-shape, attached a solubilizing hydrophilic group and a Michael acceptor group to form a covalent bond with Cys797 to impart all the advantages of irreversible inhibitors to the compounds (*vide infra*). Regarding the hydrophobic pocket and the back pocket, that are usually occupied with substituted anilines in the first and second generation EGFR inhibitors, they are not similarly occupied in the case of third generation inhibitors. Only the first hydrophobic pocket preceding the gatekeeper is occupied with a suitable hydrophobic group that doesn't bump into the gatekeeper Met790, and at the same time it establishes hydrophobic contact with Met790 sidechain, hence rendering these inhibitors to be rather mutant-selective. On the other hand, these inhibitors usually don't extend into the back pocket to avoid steric clash with Met790. The methoxy group (encircled in violet in the structures **28** and **29**) is frequently seen in the third generation inhibitors, it was found to be directed towards Leu792 and Pro794 in the hinge region, which is believed to contribute to the high selectivity of these inhibitors, since other enzymes such as JAK3 and TEC-family have bulkier amino acids in the position of Leu792 that would clash with the methoxy group. [65–67]

Based on this design strategy, **Osimertinib (AZD9291) (29)** was developed and approved by the FDA in 2015 for the treatment of metastatic T790M-positive NSCLC progressed after EGFR inhibitor therapy [8]. Numerous, structurally diverse third generation EGFR inhibitors were developed, and they are well-reviewed in the literature [67]. Continuing efforts are being exerted to develop novel inhibitors with improved selectivity and safety profiles [68].



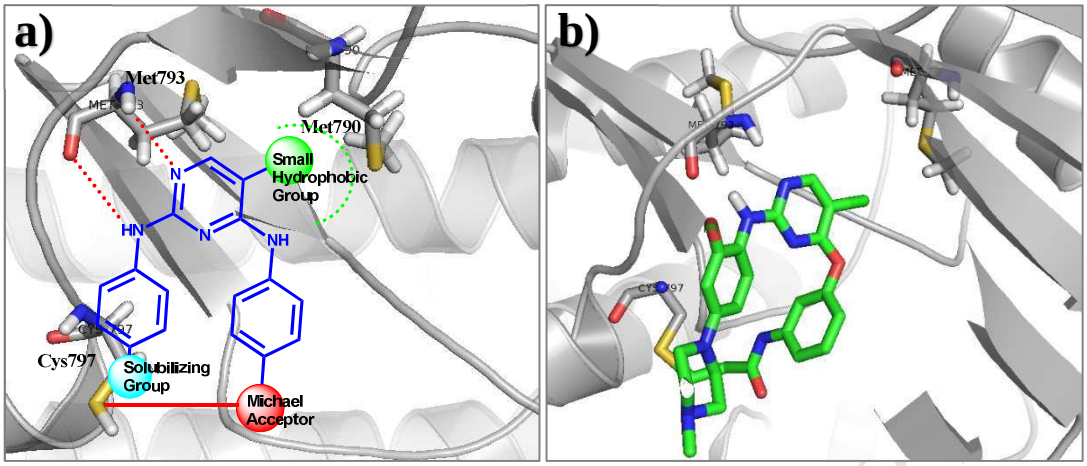


Figure 19 Design of third generation EGFR inhibitors. (a) SAR of third generation EGFR inhibitors. (b) Mutant T790M EGFR complexed with a T790M-selective inhibitor (PDB: 3IKA).

Irreversible dual EGFR/HER2 inhibitors

The presence of a conserved Cysteine residue close to the solvent-accessible region in the ATP binding site of the EGFR family (**Figure 20**) [Cys797 in EGFR and Cys805 in HER2] prompted the design of a new class of covalently-binding irreversible kinase inhibitors, known as class VI inhibitors [25]. These inhibitors function through the formation of a Michael adduct between the thiol of the Cysteine and a Michael acceptor functionality incorporated in the solubilizing group of the inhibitor (**Figure 21**).

The use of covalent-binding drugs in therapy is an established approach dating back to the introduction of Aspirin (1899) followed by Penicillin (1928), however, covalent EGFR inhibitors were only sought in the 1990s to combat the rising acquired drug resistance. The use of covalent irreversible EGFR inhibitors versus reversible inhibitors avails much in fighting resistant mutations (e.g. T790M); [69–71]

(i) Since the resistant mutation T790M increases EGFR affinity for ATP, therefore it decreases the potency of the conventional ATP-competitive inhibitors, hence the resistance [61]. However, irreversible inhibitors can thwart ATP competition by covalently modifying the binding site.

(ii) Since the target Cysteine residue is conserved in EGFR family members and not commonly found in other kinases in the same position, it can be an asset for tuning kinase selectivity.

(iii) Upon covalently binding to the enzyme, their biological effect will be sustained until the receptor is recycled, independent of the drug plasma concentration, i.e. they have a prolonged duration of action without the need for constantly elevated drug plasma concentration, which will reduce the drug administration frequency, diminish adverse toxicities and improve compliance.

(iv) Sustained receptors blockage diminishes the opportunity to develop resistances.

(v) Since the covalent binding increases the rate of inhibition (not the magnitude of inhibition) (*vide infra*), so even with rapid drug clearance they will still be able to inhibit the slower-reacting mutant receptors and attain full inhibition.

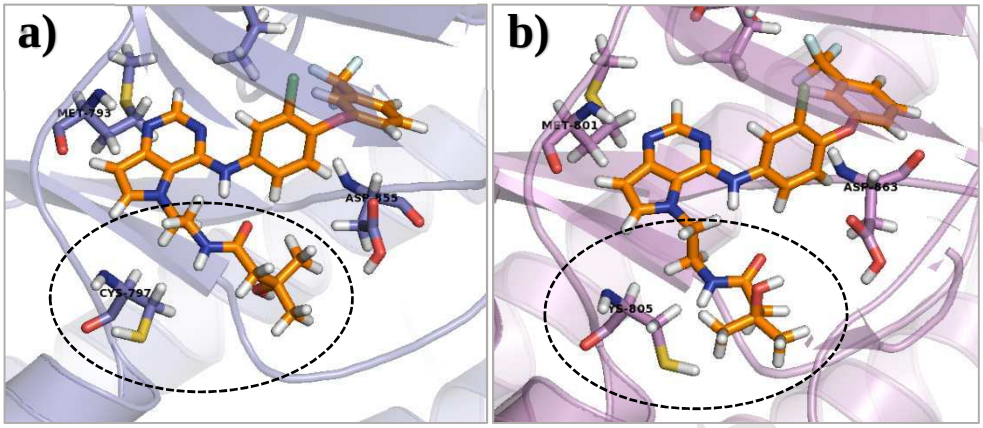


Figure 20 Conserved Cysteine in the solvent-accessible region in the ATP binding site of EGFR family: (a) EGFR complexed with TAK-285 (PDB: 3POZ). (b) HER2 complexed with TAK-285 (PDB: 3RCD)

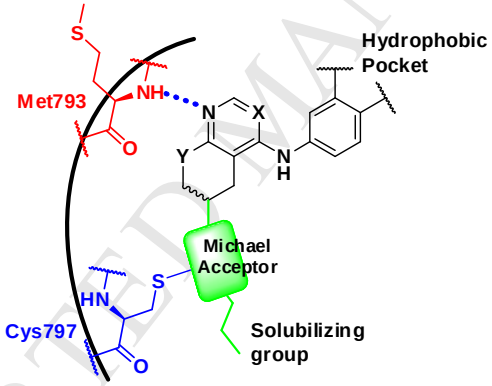


Figure 21 Design and Binding mode of covalent irreversible inhibitors

$$E + I \underset{K_i}{\rightleftharpoons} E.I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}I \qquad \text{(Equation1)}$$

Initial non-covalent complex

Final covalent complex

Adapted from Singh et al., 2011 [70]

Equation (1) illustrates the stages of enzyme inhibition by an irreversible inhibitor; the inhibitor has to bind non-covalently first into the binding site forming a reversible non-covalent enzyme-inhibitor complex, once formed the electrophilic Michael acceptor warhead group, now correctly positioned in proximity to the cysteine's nucleophilic thiol, will be able to bind covalently to the protein giving the final irreversible complex resulting in enzyme inhibition. For fully irreversible inhibitors, k_{-2} = zero, while the fully non-covalent inhibitors will have a k_2 = zero. There are reversible covalent inhibitors, with k_2 and k_{-2} having certain values, and the covalent bond formation positively affect the inhibitor's potency. Also, there are slow, strong-

binding non-covalent inhibitors that induce slow conformational changes in the protein, these will have a long residence time like the back-binders previously discussed (e.g. Lapatinib, TAK-285 and the related inhibitors). [70]

From equation (1) it is deducible that there are 2 components governing the potency of the irreversible inhibitor; the initial rate of formation of the non-covalent complex (K_i), and the rate of covalent bond formation (k_2). The formation of the initial non-covalent complex depends on the basis of molecular recognition, basically for EGFR inhibitors they would share the same SAR of the reversible inhibitors with a hinge binder and an aniline as a hydrophobic moiety that binds to the specificity pocket of EGFR/HER2. These features allow the inhibitor to bind non-covalently and selectively to the binding site of EGFR/HER2. Once this complex is formed, it should last long enough for the covalent bond to form. So these initial non-covalent interactions will influence both the affinity and the selectivity of the inhibitor, hence should be considered in the course of the design. The second component is the formation of the covalent bond, which should be fast enough to proceed to inhibition before the complex dissociates, and considering that the use of highly reactive Michael acceptors should be avoided, increasing the rate of the reaction must be achieved through optimizing other factors such as the optimum positioning of the electrophile in the solubilizing group to be in a favored proximity to the solvent-accessible Cysteine upon binding of the inhibitor. [69,70]

The two most commonly used Michael acceptors in the reported irreversible dual EGFR/HER2 inhibitors are the acrylamides and the alkynyl derivatives.

A sensible design of irreversible dual EGFR/HER2 inhibitors might result in an inhibitor that can overcome the two most reported resistance mechanisms; namely the T790M mutation and HER2 amplification.

Like the reversible dual inhibitors, the irreversible dual EGFR/HER2 inhibitors could also be classified based on their core scaffolds as follows:

Quinoline-3-carbonitriles-based irreversible inhibitors

A research group at Wyeth Research developed **EKB-569 (30)** from a previously reported irreversible inhibitor bearing a 6-acrylamide group. They found that the addition of the dialkylamine separated from the acrylamide by a methylene spacer improved the pharmacokinetics of the inhibitor (acting as a solubilizing group) and enhanced its biological properties. They proposed that the basic dialkylamine will serve as intramolecular catalyst for the Michael addition reaction abstracting the proton from the thiol of the Cysteine and then the reaction will proceed as illustrated in **Figure 22**. [72]

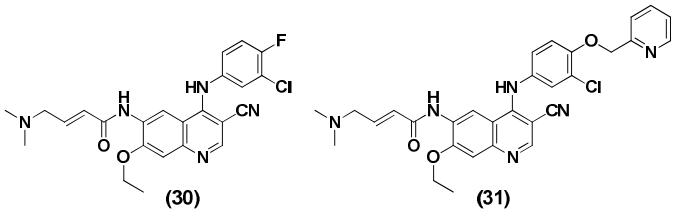


Table 7 Biological Data for Quinoline-3-carbonitriles (30-31)

Compound	EGFR IC_{50} (μM)	HER2 IC_{50} (μM)	A431[a] IC_{50} (μM)	SK-BR3[b] IC_{50} (μM)	SW620[c] IC_{50} (μM)	Reference
EKB-569 (30)	0.08	1.23	0.08	0.01	0.68	[72]
Neratinib (HKI-272) (31)	0.092	0.059	0.086	0.0018	0.73	[73]

[a] A431: human epidermoid carcinoma overexpressing WT-EGFR
[b] SK-BR3: human breast carcinoma overexpressing HER2
[c] SW620: human colon carcinoma, not overexpressing EGFR/HER2, control

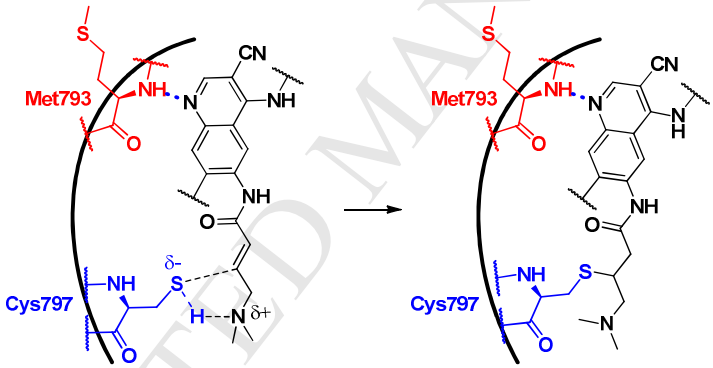


Figure 22 Mechanism of base-catalyzed Michael addition [72]

EKB-569 (30) was much less effective against HER2, so the group screened for larger back-pocket binding anilines to effect dual inhibition. This modification led to the development of **Neratinib (31)** which is a potent highly selective irreversible dual EGFR/HER2 inhibitor with improved cellular activity against HER2-overexpressing cell lines (**Table 7**) [73]. In vivo, Neratinib (**31**) was found to be effective in inhibiting the growth of HER2-dependant tumors [74]. Neratinib (**31**) is also active against the double mutant T790M/L858R EGFR [62]. As a dual inhibitor, Neratinib (**31**) was expected to have a binding mode similar to that of Lapatinib (**1**) (**Figure 23b**) with the bulky aniline extending into the back pocket, the activation segment is in and αC-helix is out, however, with the inability of DM EGFR to attain the inactive conformation, the bioactive conformer of Neratinib (**31**) co-crystallized with the DM (**Figure 23a**) seems to be in a less favorable conformation, yet it retains activity against the DM due to the inactivation of the enzyme by covalent modification [62].

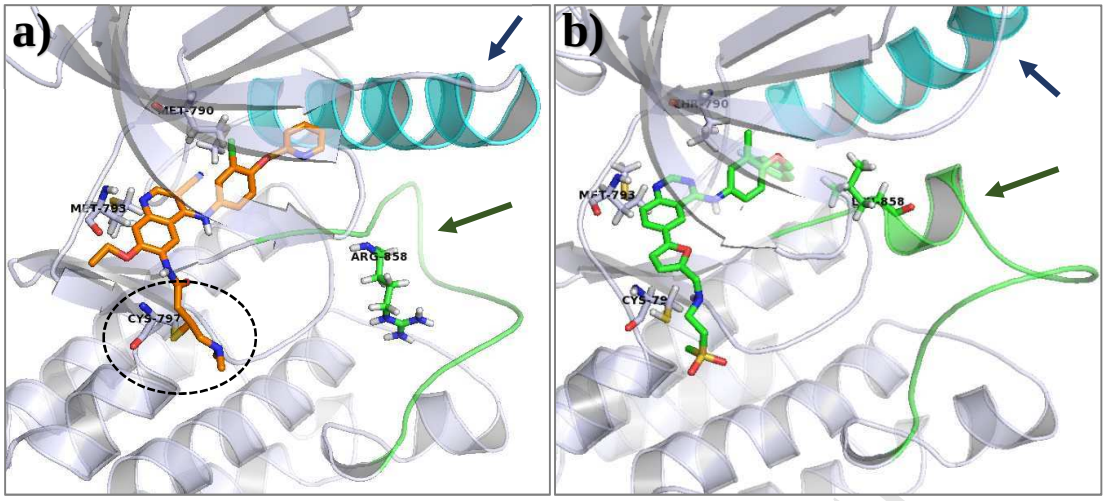


Figure 23 Mutant EGFR vs WT EGFR kinase domain: (a) EGFR T790M/L858R mutant kinase domain complexed with Neratinib (**31**) (PDB: 3W2Q). (b) EGFR WT kinase domain complexed with Lapatinib (**1**) (PDB: 1XKK).
(αC-helix in cyan, activation segment in green)

Quinazoline-based irreversible inhibitors

Dacomitinib (PF00299804) (32) is an irreversible pan-ErbB inhibitor, it potently inhibits EGFR and HER2 (**Table 8**) as well as HER4 (IC_{50} = 73.7 nM). It is selective to the ErbB family when tested against 38 other kinases. Owing to its Michael acceptor group and its ability to covalently modify the enzyme (**Figure 24**) [75], it effectively inhibits T790M-mediated Gefitinib-resistant tumors both in vitro and in vivo. **Dacomitinib (32)** is effective against Gefitinib-resistant NSCLC cell lines (such as H1975, **Table 8**) and T790M xenograft model. [76]

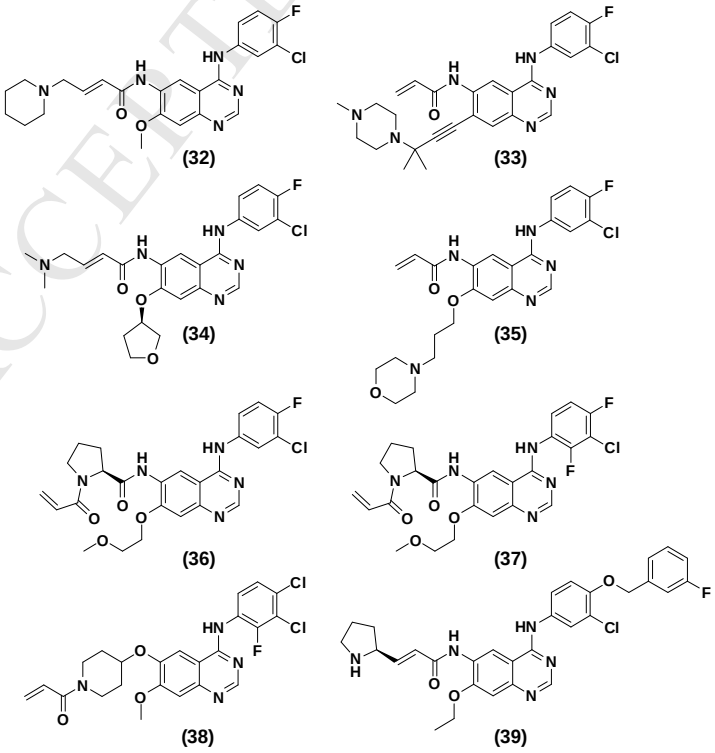


Table 8 Biological Data for Quinazoline irreversible dual inhibitors (32-39)

Compound	EGFR IC_{50}	HER2 IC_{50}	Activity against resistant mutations IC_{50}	Cellular activity IC_{50}	Reference
Dacomitinib (PF00299804) (32)	6 nM	45.7 nM	N/A	H1975[a] = 0.44 μM H1819[b] = 0.029 μM	[76]
AV-412 (MP-412) (33)	1.4 nM	19 nM	L858R = 0.51 nM T790M = 0.79 nM DM = 2.3 nM	A431[c] = 0.1 μM	[77]
Afatinib (BIBW2992) (34)	0.5 nM	14 nM	L858R = 0.4 nM DM = 10 nM	H1975[a] = 92 nM BT474[d] = 54 nM	[78,79]
Canertinib (35)	0.3 nM	30 nM	L858R = 0.4 nM DM = 26 nM	H1975[a] = 101 nM	[78]
(36)	13 nM	42 nM	T790M = 24 nM	A431[c] = 8 nM SK-BR3[e] = 8 nM H1975[a] = 213 nM	[80]
(37)	9 nM	18 nM	T790M = 11 nM	A431[c] = 7 nM SK-BR3[e] = 7 nM H1975[a] = 63 nM	[80]
Poziotinib (HM781-36B) (38)	3.2 nM	5.3 nM	T790M = 4.2 nM DM = 2.2 nM	A431[c] = 0.9 nM SK-BR3[e] = 1 nM H1975[a] = 5.7 nM	[81]
Transtinib (39)	N/A	N/A	N/A	A431[c] = 62 nM N87[f] = 47 nM H1975[a] = 34 nM	[82]

[a] H1975: human NSCLC cell line overexpressing DM (T790M/L858R)
[b] H1819: human NSCLC cell line overexpressing HER2
[c] A431: human epidermoid carcinoma overexpressing WT-EGFR
[d] BT474: human breast carcinoma overexpressing HER2
[e] SK-BR3: human breast carcinoma overexpressing HER2
[f] N87: human gastric carcinoma overexpressing EGFR and HER2

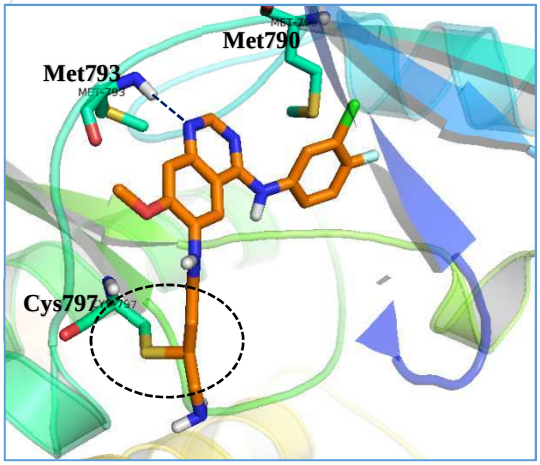


Figure 24 T790M EGFR kinase domain complexed with Dacomitinib (**32**) (PDB: 4I24)

AV-412 (MP-412) (33) is a potent dual EGFR/HER2 inhibitor that is also a potent inhibitor of different mutant EGFR forms (**Table 8**). Its potent activity is believed to be attributed to its Michael acceptor group, though unproven by a crystal structure. [77]

Afatinib (BIBW2992) (34) potently and selectively inhibits EGFR, HER2 and mutant EGFR forms in biochemical and cell-based assays (**Table 8**) [78]. Like Dacomitinib (32), it inhibits the T790M EGFR due to its ability to covalently modify the enzyme (**Figure 25**) [79]. The outstanding performance of Afatinib (**34**) led it to be the first irreversible EGFR inhibitor approved by the FDA in 2013 for the treatment of metastatic NSCLC with activating EGFR mutations [83].

Canertinib (35) is another irreversible dual EGFR/HER2 inhibitor that is also active against mutant EGFR forms with activity comparable to Afatinib (**34**) [78]. Canertinib (**35**) has its basic tertiary amine group attached to the 7 position instead of being attached to the acrylamide group like in Afatinib (**34**). The basic amine attached to the acrylamide is known to act as an intramolecular catalyst for the Michael addition reaction.

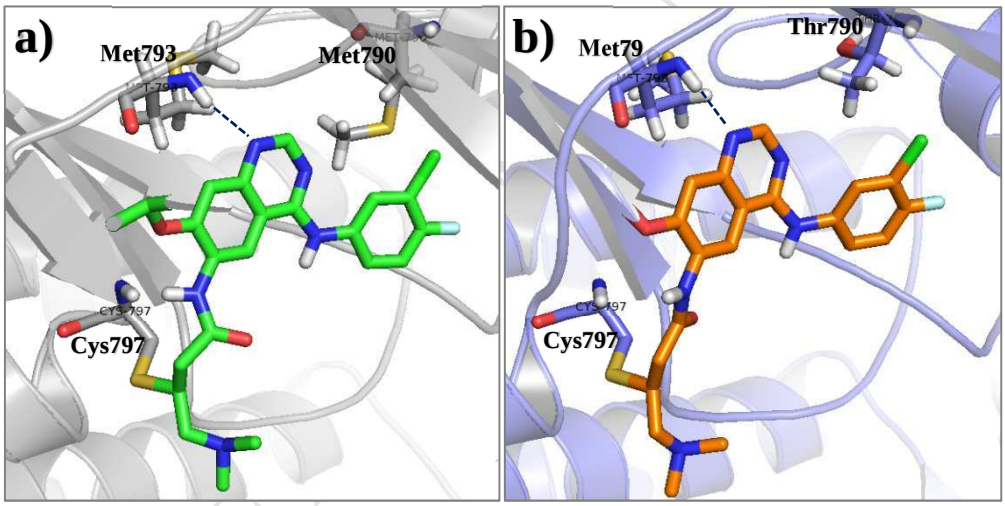


Figure 25 Afatinib (**34**) binding mode. (a) T790M EGFR kinase domain complexed with Afatinib (**34**) (PDB: 4G5P). (b) WT-EGFR kinase domain complexed with Afatinib (**34**) (PDB: 4G5J)

Compounds (36) and (37) are potent, selective irreversible EGFR/HER2 inhibitors. They were developed with the aim of achieving selective dual inhibition and overcoming mutation-driven resistance. Screening for the optimal linker - that can perfectly orient the acrylamide warhead to specifically react with Cys797/EGFR and Cys805/HER2 – identified the L-proline linker with the most potent dual inhibitory activity. Screening for 4-aniline derivatives identified the 3-chloro-4-fluoroaniline (**36**) and the 3-chloro-2,4-difluoroaniline (**37**) with the most potent inhibitory activities in biochemical and cellular assays. These compounds showed high selectivity in a panel of 25 kinases. Being irreversible inhibitors, these compounds potently inhibit the T790M EGFR and Gefitinib-resistant cell lines (**Table 8**). The

extra fluorine in compound (**37**) enhanced the potency against the T790M with 2-fold increase in enzyme inhibition and 3-fold increase in cellular inhibition, and enhanced the HER2 inhibition (**Table 8**). [80]

Poziotinib (HM781-36B) (38), which has the same aniline moiety as compound (**37**) but with a different linker, is also a potent irreversible pan-ErbB inhibitor (**Table 8**) (HER4 IC_{50} = 23.5 nM) with potent activity against mutant EGFR. It is selective to the ErbB family, and it can covalently modify EGFR, HER2 and EGFR T790M while sparing normal cells (Hs-27, human fibroblast, GI_{50} = 3830 nM). It showed outstanding performance in Erlotinib-resistant cell-based assays and in EGFR-dependent xenograft models. [81]

Transtinib (39) has a structure that is similar to Lapatinib (**1**) except for the solubilizing group, where it has instead a Michael acceptor acrylamide with an attached pyrrolidine to act as an intramolecular catalyst. Though it is similar to Lapatinib (**1**), yet it effectively inhibits the Lapatinib-resistant cell line (H1975) (**Table 8**). This might be attributed to its Michael acceptor group. It also shows a notable prolonged response in H1975 (DM) xenograft model, that is more significant than its response in A431 (WT EGFR) model, so it might be more selective to mutant EGFR tumors than to WT EGFR. [82]

Thienopyrimidine-based irreversible inhibitors

Alkynyl thienopyrimidines can act as irreversible dual EGFR/HER2 inhibitors since they can covalently modify the conserved Cys residue in the ErbB family. The attachment of a basic amine moiety to the ethynyl group is required in order to act as an intramolecular catalyst for the Michael addition reaction (**Figure 26**). [84]

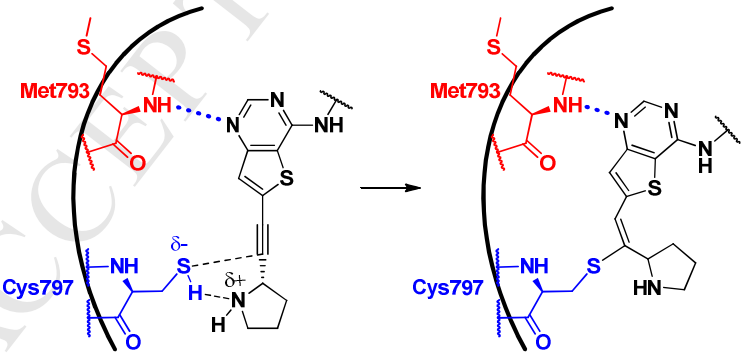


Figure 26 Covalent modification by alkynyl thienopyrimidines [84]

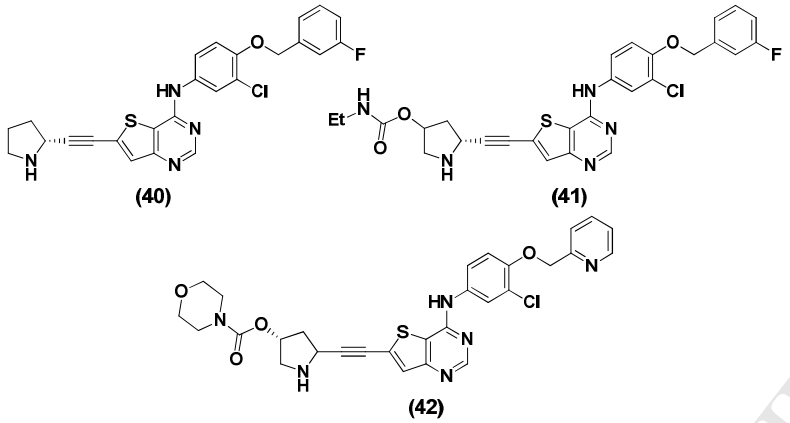


Table 9 Biological Data for Thienopyrimidine irreversible dual inhibitors (40-42)

Compound	EGFR IC_{50}	HER2 IC_{50}	Cellular activity IC_{50}	Reference
(40)	7 nM	13 nM	HN5[a] = 238 nM BT474[b] = 94 nM	[85]
(41)	32 nM	43 nM	HN5[a] = 95 nM BT474[b] = 30 nM	[85]
(42)	50 nM	30 nM	HN5[a] = 240 nM BT474[b] = 60 nM	[86]

[a] HN5: human Head & Neck carcinoma overexpressing EGFR
[b] BT474: human breast carcinoma overexpressing HER2

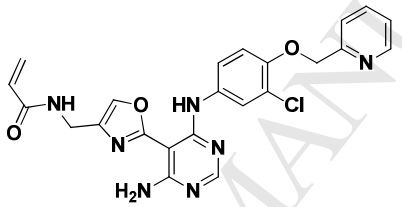
Compound (40) demonstrated the ability of the pyrrolidinylacetylenic thienopyrimidine scaffold to act as an irreversible covalent modifier of the HER family. With the Lapatinib-like anilino moiety, compound (**40**) showed good dual inhibitory activity in biochemical and cellular assays (**Table 9**). [85]

The addition of the carbamoyl moiety in **compound (41)** improved the pharmacokinetic profile of the inhibitor that was reflected on its improved cellular activity (**Table 9**). [85]

Screening for an aniline "headgroup" other than the Lapatinib-like one used in compounds (**40**) and (**41**) was sought with the aim of improving the selectivity (since the aniline headgroup binds in the specificity pocket) and the ADMET properties (decreased lipophilicity and protein binding, improved oral exposure and metabolic stability). The screening identified **compound** (**42**) with the pyridyl moiety. It showed improved ADMET properties like decreased lipophilicity and improved oral exposure. [86]

Pyrimidine-based irreversible inhibitors

Starting with a pyrimidine scaffold, screening for a suitable linker that can properly orient the Michael acceptor warhead in position for covalent modification identified the oxazolylmethyl group in **compound (43)**. The importance of the oxazole conformation for the proper warhead orientation was confirmed upon substituting the oxazole with an amide bioisostere that eliminated the antiproliferative activity. Following screening for an aniline headgroup that can provide the optimal dual potency and ADMET properties, led to the identification of compound (**43**) with the pyridin-2-ylmethoxy moiety. The same aniline moiety was found to maintain dual inhibitory activity and confer good ADMET properties to the inhibitor as in compound (**42**). Compound (**43**) showed potent irreversible pan-HER inhibition as well as potent inhibition of the mutant T790M EGFR (**Table 10**). It showed high selectivity against a panel of 20 kinases. It performed well in cell-based assays with potent activity against the Lapatinib-resistant cell lines (MDA-MB-453 and H1975). [87]



(43)

Table 10 Biological Data for Compound (43)

EGFR IC_{50}	T790M IC_{50}	HER2 IC_{50}	HER4 IC_{50}	N87[a] IC_{50}	MDA-MB-453[b] IC_{50}	H1975[c] IC_{50}	Reference
3.3 nM	3.2 nM	22.4 nM	4 nM	3 nM	5 nM	40 nM	[87]

[a] N87: human gastric carcinoma overexpressing EGFR and HER2

[b] MDA-MB-453: human breast carcinoma overexpressing HER2 with PTEN deficiency

[c] H1975: human NSCLC cell line overexpressing DM (T790M/L858R)

4. Conclusion

The most commonly reported resistance mechanisms against EGFR TKIs are HER2 amplification and the T790M mutation. HER2 potentiates EGFR signaling activity, and its overexpression predicts poor prognosis. Dual inhibition of EGFR and HER2 might potentiate the signaling blockade. Dual EGFR/HER2 inhibitors are usually composed of a hinge binding scaffold that carries a large hydrophobic group extending into the back pocket and a solubilizing group. Large hydrophobic back-binding groups favor dual inhibition, and modification of the solubilizing group can improve the PK and the selectivity profiles.

The T790M/L858R mutation leads to resistance through rendering the EGFR constitutively activated with increased affinity to ATP and decreased affinity to EGFR inhibitors. An inhibitor that can overcome the mutation-driven resistance should have one or more of the following structural features; (a) an extra-bulky hydrophobic back-pocket binder to displace the αC-helix into its inactive conformation, (b) a Michael acceptor group that can form a covalent bond with Cys797 causing the inactivation of the protein, or (c) a U-shaped conformation that avoids the steric clash with Met790. An irreversible dual EGFR/HER2 inhibitor might be able to overcome the two most common resistance mechanisms.

Declaration of interest

The authors have declared no conflict of interests.

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Author Contributions

S.N. Milik reviewed the literature and prepared the manuscript. D.S. Lasheen and R.A.T. Serya revised the manuscript. K.A.M. Abouzid supervised the preparation of the manuscript. All the authors reviewed the manuscript.

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Highlights

- T790M mutation and HER2 amplification are the most common resistance mechanisms.
- Dual EGFR/HER2 blockade outweighs selective EGFR inhibition.
- Inhibitors with bulky back-pocket binders are preferred for dual inhibition.
- The resistant $EGFR^{TMLR}$ is constitutively activated with increased affinity to ATP.
- Irreversible inhibitors can overcome mutation-driven resistance.