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Invited Review

Small molecule inhibitors targeting the EGFR/ErbB family of protein-tyrosine kinases in human cancers

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ABSTRACT

The EGFR family is among the most investigated receptor protein-tyrosine kinase groups owing to its general role in signal transduction and in oncogenesis. This family consists of four members that belong to the ErbB lineage of proteins (ErbB1–4). The ErbB proteins function as homo and heterodimers. These receptors contain an extracellular domain that consists of four parts: domains I and III are leucine-rich segments that participate in growth factor binding (except for ErbB2) and domains II and IV contain multiple disulfide bonds. Moreover, domain II participates in both homo and heterodimer formation within the ErbB/HER family of proteins. Seven ligands bind to EGFR including epidermal growth factor and transforming growth factor-α, none bind to ErbB2, two bind to ErbB3, and seven ligands bind to ErbB4. The extracellular domain is followed by a single transmembrane segment of about 25 amino acid residues and an intracellular portion of about 550 amino acid residues that contains (i) a short juxtamembrane segment, (ii) a protein kinase domain, and (iii) a carboxyterminal tail. ErbB2 lacks a known activating ligand and ErbB3 is kinase impaired. Surprisingly, the ErbB2–ErbB3 heterodimer complex is the most active dimer in the family. These receptors are implicated in the pathogenesis of a large proportion of lung and breast cancers, which rank first and second, respectively, in the incidence of all types of cancers (excluding skin) worldwide. On the order of 20% of non-small cell lung cancers bear activating mutations in *EGFR*. More than 90% of these patients have exon-19 deletions (^{746}ELREA^{750}) or the exon-21 L858R substitution. Gefitinib and erlotinib are orally effective type I reversible *EGFR* mutant inhibitors; type I inhibitors bind to an active enzyme conformation. Unfortunately, secondary resistance to these drugs occurs within about one year owing to a T790M gatekeeper mutation. Osimertinib is an irreversible type VI inhibitor that forms a covalent bond with C797 of EGFR and is FDA-approved for the treatment of patients with this mutation; type VI inhibitors generally form a covalent adduct with their target protein. Resistance also develops to this and related type VI inhibitory drugs owing to a C797S mutation; the serine residue is unable to react with the drugs to form a covalent bond. Approximately 20% of breast cancer patients exhibit *ErbB2/HER2* gene amplification on chromosome 17q. One of the earliest targeted treatments in cancer involved the development of trastuzumab, a monoclonal antibody that interacts with the extracellular domain ErbB2/HER2 causing its down regulation. Surgery, radiation therapy, chemotherapy with cytotoxic drugs, and hormonal modulation are the mainstays in the treatment of breast cancer. Moreover, lapatinib and neratinib are FDA-approved small molecule ErbB2/HER2 antagonists used in the treatment of selected breast cancer patients. Of the approximate three dozen FDA-approved small molecule protein kinase inhibitors, five are type VI irreversible inhibitors and four of them including afatinib, osimertinib, dacomitinib, and neratinib are directed against the ErbB family of receptors (ibrutinib is the fifth and it targets Bruton tyrosine kinase). Avitinib, olmutinib, and pelitinib are additional type VI inhibitors in clinical trials for non-small cell lung cancer that target EGFR. Secondary resistance to both targeted and cytotoxic drugs is the norm, and devising and implementing strategies for minimizing or overcoming resistance is an important goal in cancer therapeutics.

Abbreviations: AS, activation segment; CS or C-spine, catalytic spine; CL, catalytic loop; EGFR, epidermal growth factor receptor; GK, gatekeeper; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; PKA, protein kinase A; pY or pTyr, phosphotyrosine; RS or R-spine, regulatory spine; Sh2, shell residue 2; VEGFR, vascular endothelial growth factor receptor

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1. Introduction

The ErbB/HER receptor protein-tyrosine kinases are among the most studied cell signaling families in biology [1]. Stanley Cohen started this line of investigation when he described the epidermal growth factor (EGF), its receptor (EGFR), and many of its biochemical actions [2]. He discovered that EGFR exhibited protein-tyrosine kinase activity and not protein-serine/threonine kinase activity, which was an unexpected finding at the time (see Ref. [3] for a historical review). Cohen et al. discovered that a solubilized 170-kDa polypeptide had both EGF binding activity as well as protein kinase activity [4]. EGFR was also the first receptor that provided evidence for a relationship between mutation, overexpression, and cancer [5]. The EGFR family is among the most investigated receptor protein-tyrosine kinase families owing to its general role in signal transduction and in oncogenesis.

The human protein kinase superfamily consists of more than 500 members thus making it one of the largest gene families [6]. These enzymes mediate the following reaction:

$$MgATP^{-1} + \text{protein–OH} \rightarrow \text{Protein–}OPO_3^{2-} + MgADP + H^+$$

Based upon the nature of the phosphorylated residue, these enzymes are classified as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90 members), and tyrosine-kinase like proteins (43 members). Of the 90 protein-tyrosine kinase members, 58 are receptors and 32 are nonreceptors. A small set of dual-specificity protein kinases such as MEK1 and MEK2 catalyze the phosphorylation of both tyrosine and then threonine in target proteins; dual-specificity kinases are evolutionarily related to the protein-serine/threonine kinase enzyme family. Protein phosphorylation is the most prevalent class of post-translational modification used in cell signaling. Moreover, families of phospho-protein phosphatases catalyze the dephosphorylation of proteins thereby making phosphorylation-dephosphorylation an overall reversible process that can be repeated numerous times [7].

Protein kinases play a major regulatory role in nearly every facet of cell biology [6]. These enzymes regulate apoptosis, cell cycle progression, cell division, cytoskeletal rearrangement, cell differentiation, development, the immune response, nervous system dynamics, transcription, and translation. Moreover, dysregulation of protein kinase activities occurs in various diseases such as cancer, diabetes, and autoimmune, cardiovascular, hematopoietic, inflammatory, and nervous disorders. Considerable work has been expended in an effort to determine the physiological as well as pathological functions of receptor protein-kinase signal transduction pathways over the past 35 years. The EGFR family has undergone extensive study owing to its general role in signal transduction and in the pathogenesis of a variety of malignancies including lung, breast, stomach, colorectal, head and neck, and pancreatic carcinomas [8].

The role of the ErbB family in these malignancies has led to the development of afatinib, dacomitinib, erlotinib, gefitinib, and osimertinib; these are FDA-approved EGFR/ErbB1 inhibitors used for the treatment of non-small cell lung cancer (NSCLC) (www.brimr.org/PKI/PKIs.htm) [8,9]. Lapatinib and neratinib target ErbB2 and are approved for the treatment of HER2-positive breast cancer. The number of newly diagnosed lung cancers in the United States and worldwide is estimated to be 234,000 and 2.09 million and the number of deaths is estimated to be 154,000 and 1.76 million, respectively [10,11]. About 85% of all lung cancers are of the non-small cell variety. The total number of newly diagnosed breast cancers in women in the United States and worldwide is estimated to be 266,000 and 2.08 million and the number of deaths is estimated to be 41,000 and 627,000, respectively. About 20% of the newly diagnosed breast cancers overexpress ErbB2. The incidence of lung and breast cancer rank first and second, respectively, among all types of cancers worldwide [11] indicating the practical importance of developing effective treatments for these disorders.

2. Overview of the ErbB/HER protein kinase family and their ligands

2.1. ErbB protein kinases

The human EGF receptor (HER) family consists of four members that belong to the ErbB pedigree of proteins (ErbB1–4) [8]. The *ERBB* gene symbol is derived from the avian viral erythroblastosis oncogene to which these receptors are related. Human gene symbols are generally designated in uppercase italics (*EGFR*). The four members of the human epidermal growth factor receptor gene family include: (i) *EGFR/ERBB1/HER1,* (ii) *ERBB2/HER2/NEU*, (iii) *ERBB3/HER3,* and (iv) *ERBB4/HER4.* Although there is a considerable crossover, the HER nomenclature is used more commonly in clinical papers and reports whereas the ErbB nomenclature is associated with the biological sciences. Schechter et al. discovered that a series of rat neuro/glioblastomas contains the *Neu* oncogene, which is related to the rat *Erbb2* gene of the EGFR family [12]. This discovery provided evidence for the possible role of the ErbB family of receptors in the pathogenesis of cancer and *NEU* is sometimes used in human gene nomenclature. This lineage of receptors is ubiquitously expressed in epithelial, mesenchymal, and neuronal cells as well as their undifferentiated precursors.

The four human *ERBB* genes are found on different chromosomes. Null mutations of each of the *Erbb* genes in mice produce embryonic or perinatal lethality [13–15]. Miettinen et al. reported that the *Erbb1* gene knockout results in gastrointestinal, lung, and skin defects [16]. Rajagopalan et al. discovered that selective disruption of adult cardiac ErbB1 receptor-mediated signaling in mice, along with diminished ErbB2 function, leads to compromised cardiac function [15]. Moreover, hearts of *Erbb2*- or *Erbb4*-mutant mice [17,18] fail to develop normally and the mice display an irregular cardiac rhythm. An inductive signal from Nrg-1 to the ErbB2 and ErbB4-expressing myocardium initiates ventricular differentiation. Additionally, *Erbb3* knockout mice exhibit dilated and thinned atrioventricular valves leading to death by embryonic day 13.5 [19]. The cardiotoxicity following ErbB-targeted treatment [20] is most likely related to the expression of these receptors in the heart.

Based upon the primary amino acid structure of EGFR as determined by cDNA analysis, Ulrich et al. reported that the receptor consisted of a single hydrophobic transmembrane segment that separates the extracellular ligand-binding domain and the intracellular protein kinase domain [21]. This hypothesis, which has stood the test of time, applies to nearly all receptor protein kinases. The ErbB/HER family of protein kinases consists of an extracellular domain that consists of four parts: domains I and III are related leucine-rich segments that participate in ligand binding and domains II and IV contain cysteine residues that participate in the formation of about a dozen disulfide bonds. Moreover, domain II participates in both homo and heterodimer formation with ErbB/HER family members. The extracellular domain is followed by a single transmembrane segment of about 25 amino acid residues and an intracellular portion of about 550 amino acid residues that contains (i) a short juxtamembrane segment, (ii) a protein kinase domain, and (iii) a long carboxyterminal tail (Fig. 1).

There are two commonly used residue numbering schemes for the ErbB/HER family amino acid residues. The format used in the UniProtKB knowledge base includes the signal peptide and corresponds to the nascent receptor. The format employed by Ullrich et al. [21] for ErbB1/HER1 excludes the 24-residue signal peptide and corresponds to the mature protein. Although the employment of the mature protein numbering system is ingrained in the literature, it is simpler to use the nascent protein numbers when going from DNA to RNA and then to protein. Accordingly, the numbering scheme including the signal peptide is used throughout this paper.

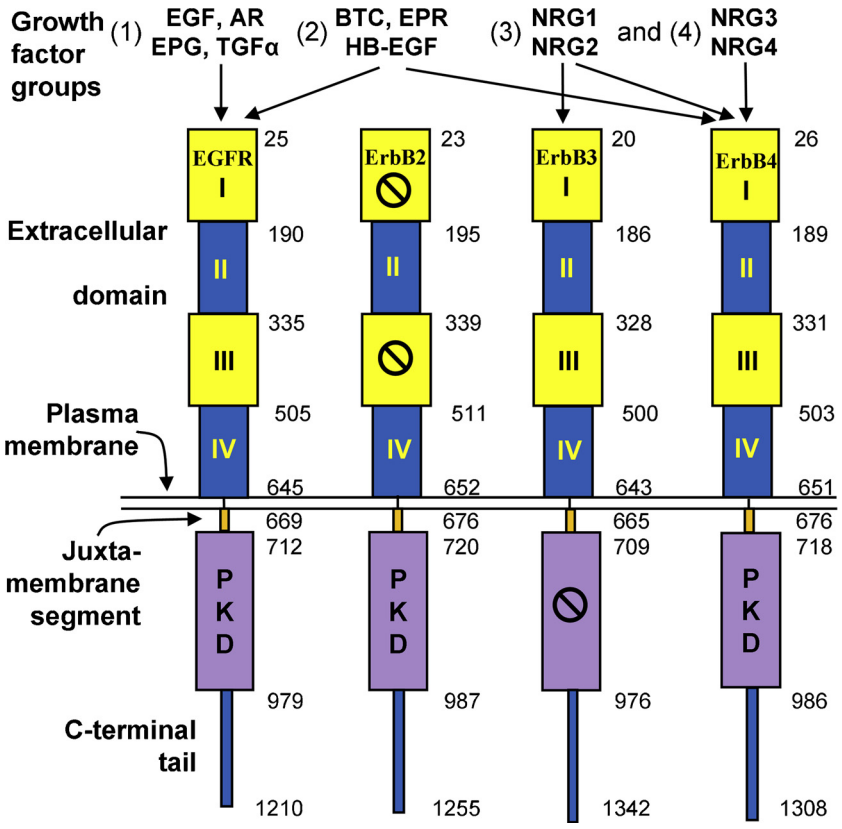

Fig. 1. Organization of the human epidermal growth factor receptor family members including EGFR/ErbB1/HER1, ErbB2/HER2/NEU, ErbB3/HER3 and ErbB4/HER4. The extracellular sector of each receptor consists of four domains (I–IV). Domains I and III take part in ligand binding (except for those of ErbB2/HER2, which are marked with the stop symbol), and domain II plays a part in dimer formation. The carboxyterminal tail contains several tyrosine phosphorylation sites. The protein kinase domain of ErbB3/HER3, which is marked with the stop symbol, is kinase-impaired. The numbers correspond to amino acid residues of the nascent protein including the signal peptide (which is not depicted); each number corresponds to the initial residue of the adjacent segment except for (i) the last residues of the extracellular domains and (ii) the end of the proteins. The growth factor groups (1–4) that bind to the receptors are indicated. EGF, epidermal growth factor; AR, amphiregulin; EPG, epigen; TGFα, transforming growth factor-α; BTC, betacellulin; EPR, epiregulin; HB-EGF, heparin-binding epidermal growth-like factor; Nrg-1/2/3/4, neuregulin-1/2/3/4; PKD, protein kinase domain.

2.2. ErbB family ligands

The ligands that bind to each of the monomeric receptors are listed in Fig. 1. The name neuregulin (Nrg) refers to the *NEU* gene and is synonymous with heregulin. Fig. 1 indicates that seven ligands bind to EGFR, none bind to ErbB2, two bind to ErbB3, and seven factors bind to ErbB4. The ErbB3 receptor is kinase impaired. The ErbB family, like all protein-tyrosine kinase receptors, functions as dimers or higher oligomers. There is one principal isoform of ErbB1, two full-length isoforms of ErbB2 that differ slightly owing to alternative mRNA splicing, and two full-length isoforms of ErbB3, one of which is missing residues 1–59. There are two different extracellular juxtamembrane versions (JMa and JMb) and two different versions of the carboxyterminal tail (CTa and CTb) of the ErbB4 receptor. Accordingly, there are four full-length transmembrane isoforms of ErbB4 that are produced by alternative pre-mRNA splicing: JMaCTa, JMaCTb, JMbCTa, and JMbCTb. Using reverse transcription polymerase chain reaction (RT-PCR) procedures in mice, Elenius et al. demonstrated that the JMa isoform is expressed in the kidney and the JMb isoform is expressed in the heart and adrenal [22]. Furthermore, both forms are expressed in the eye, cerebral cortex, cerebellum, and spinal cord. The functional significance of the four isoforms of ErbB4 is unclear.

Because ErbB2 fails to bind to any ligand, growth factor-induced homodimer formation is unlikely. However, Ghosh et al. reported that the nonphysiological overexpression of ErbB2 leads to the formation of a functional homodimer [23]. ErbB3 is kinase impaired so that induced homodimer formation would fail to stimulate protein kinase activity and downstream signaling. However, Shi et al. discovered that ErbB3 possesses 1/1000th of the autophosphorylation activity of ErbB1 [24] and the possibility exists that the ErbB3 homodimer is functional. Experiments indicate that ErbB2 is the favored dimerization partner for all of the other ErbB family members [25,26]. Moreover, early work by Pinkas-Kramarski et al. demonstrated that the ErbB2 heterodimer combinations with ErbB1 or ErbB3 exhibit robust signaling activity [27].

All of the ErbB receptor family ligands are initially expressed as single-pass integral membrane proteins [28]. These growth factor precursors have an extracellular component, a transmembrane segment, and a small intracellular component. The growth factor components occur in the extracellular portion and are liberated by proteolysis as catalyzed by members of the ADAMs (A Disintegrin And Metalloproteases) family [29]. This process is called protein ectodomain shedding and the proteolytic enzymes are sometimes called sheddases. The human ADAMs family is made up of more than two dozen members [30]. These membrane-bound catalysts require Zn^{2+} for their activity accounting for their classification as metalloproteases. The ADAMs family consists of an N-terminal signal sequence, a pro-domain, a metalloprotease catalytic domain, a disintegrin domain, a cysteine-rich segment, an EGF-like domain, a transmembrane segment, and an intracellular section [29]. The metalloprotease catalytic domain contains a conserved zinc-binding His-Glu-Xxx-Gly-His sequence that confers enzymatic activity. See Ref. [8] for a summary of the size of the activating EGFR family ligand precursors and mature active polypeptides.

3. Overall structure of the ErbB/HER protein kinase domains

3.1. Structures of the small and large lobes and the protein kinase fold

Like all other protein kinases, the ErbB protein kinase domains have a small N-terminal lobe and large C-terminal lobe (Fig. 2) that was first described by Knighton et al. for PKA (PDB ID: 2CPK) [31]. The two lobes form a crevice that serves as a binding site for ATP. The N-terminal lobe contains a conserved flexible glycine-rich ATP-phosphate–binding loop, which is sometimes called the P-loop because it is near the phosphates of the ATP substrate. The β1- and β2-strands of the N-lobe dock with the adenine moiety of ATP and they interact with ATP-competitive small molecule inhibitors. The β3-strand typically contains a conserved Ala-Xxx-Lys sequence, the lysine of which in human EGFR

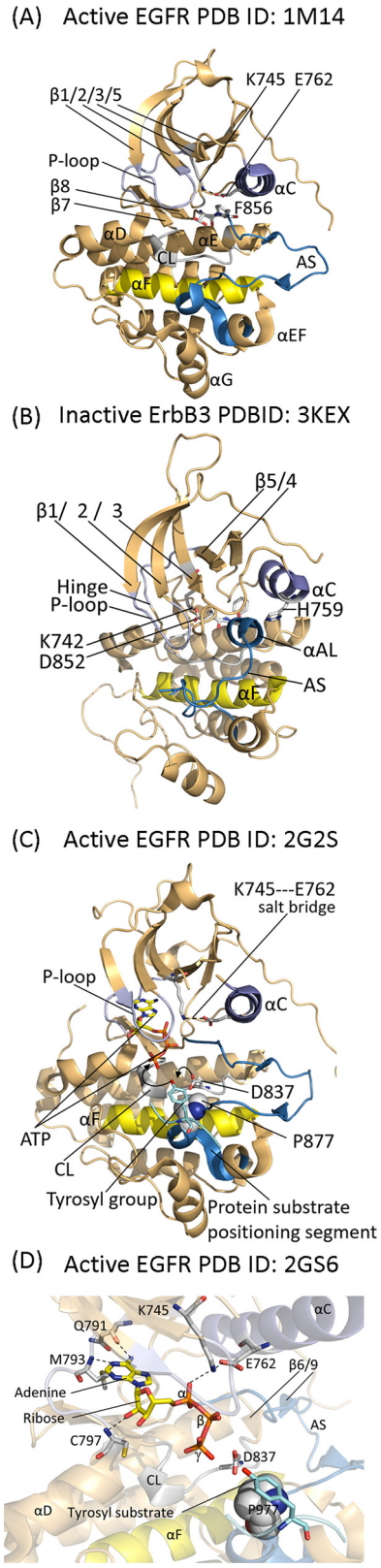

Fig. 2. (A) Active EGFR. (B) Kinase-impaired inactive ErbB3. (C) Active EGFR with the protein substrate positioning segment including P877. (D) EGFR ATP-binding site. AS, activation segment; CL, catalytic loop. Figs. 2, 3 and 6 were prepared using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

(K745) forms a salt bridge with a conserved glutamate near the center of the protein-kinase αC-helix (E762) (Table 1). The formation of an electrostatic bond between the β3-lysine and the αC-glutamate is required for the formation of the active enzyme state and corresponds to the "αC_{in}" conformation. In contrast, K745 and E762 of dormant EGFR fail to form a salt bridge and this structure corresponds to the displaced "αC_{out}" conformation. The αC_{in} conformation is necessary, but not sufficient, for the expression of full protein kinase catalytic activity.

The C-terminal lobe contains a mobile activation segment with an extended or open conformation in active enzymes and closed conformation in inactive enzymes. The first residues of the protein kinase activation segment consist of DFG (Asp-Phe-Gly). In various protein kinases, the DFG exists in two different conformations. In the dormant activation segment conformation of many protein kinases such Abl, the aspartate side chain of the DFG sequence extends away from the active site. This is called the "DFG-D_{out}" conformation. In the active state, the aspartate side chain extends toward the ATP-binding pocket and coordinates Mg^{2+}. This is called the "DFG-D_{in}" conformation. It is the ability of aspartate to bind (DFG-D_{in}) or not bind (DFG-D_{out}) to Mg^{2+} in the active site that is the crucial property. See Ref. [32] for details concerning the two activation segment conformations. However, the inactive conformations of the ErbB family kinases including kinase-impaired ErbB3 exist in the DGF-D_{in} conformation with a closed activation segment or with an αC_{out} conformation.

Although the activation segment of protein kinases typically ends with APE (Ala-Pro-Glu), it ends with ALE (Ala-Leu-Glu) in the ErbB family. The last eight residues of the activation segment in the four ErbB family members include PIKWMALE and this sequence makes up the protein-substrate positioning loop. The R-group of proline in this sequence functions as a platform that buttresses the tyrosyl residue of the protein substrate that is phosphorylated (Fig. 2C) [33]. In protein-serine/threonine kinases, the seryl or threonyl group interacts with peptidyl backbone residues near the end of the activation segment and not with an R-group. Although the activation segment of the ErbB family contains a phosphorylatable tyrosine, its phosphorylation is not required for enzyme activation [34].

ErbB1/2/4 are operative protein-tyrosine kinases that occur in similar active and inactive conformations. In contrast to these enzymes, ErbB3 lacks essential catalytic residues and is kinase impaired. Its structure is that of an inactive protein kinase. Although it possesses all of the α-helices and most of the β-sheets observed in all protein kinases, the αC-helix of ErbB3 is notably short (Fig. 2B). The C-terminal lobe of the ErbB family of protein kinases is mainly α-helical with seven conserved segments (αD–αI and αEF) that occur in all protein kinases [35]. The first X-ray crystallographic structure of PKA possessed a short helix proximal to the αF-helix, which was unnamed (PDB ID: 2CPK). However, this αEF helix is conserved in all active protein kinase structures and represents a seventh-conserved helix in the C-lobe (Fig. 2A). The initial portion of the Activation Loop of inactive ErbB3 contains an αAL-helix that abuts against the αC-helix that favors an inactive displaced conformation (Fig. 2B). The inactive enzyme forms of ErbB1/2/4 also contain this αAL-helix in the proximal portion of the activation loop. The activation segment of active EGFR extends outward while that of the less active ErbB3 is closed and more compact (Fig. 2A and B).

The carboxyterminal lobe of active protein kinases contains four short β-strands (β6-β9) (Fig. 2A). The β6-strand, the primary structure of which occurs before the catalytic loop, interacts with the activation segment β9-strand. The primary structure of the β7-strand is located between the catalytic loop and the activation segment and it interacts with the adjacent downstream β8-strand. The dormant forms of all four ErbB family members contain the β7- and β8-strands, but they all lack the β6- and β9-strands.

There are two general conformational motions associated with all protein kinases including those of the ErbB family. The first involves the interconversion of less active and more active enzyme forms. Activation typically involves changes in the orientation of the αC-helix in the N-

Table 1
Important residues in human ErbB receptors.

	EGFR	ErbB2	ErbB3	ErbB4
Number of residues	1210	1255	1342	1308
Signal peptide	1-24	1-22	1-19	1-25
Extracellular segment	25-645	23-652	20-643	26-651
Transmembrane segment	646-668	653-675	644-664	652-675
Intracellular segment	669-1210	676-1255	665-1342	676-1308
Protein kinase domain	712-979	720-987	709-966	718-985
Glycine-rich loop; GSGAFG	719-724	727-732	716-721	725-730
The K of K/E/D/D, or the β3-lysine	K745	K753	K742	K751
αC-E residue	E762	E770	H759	E769
Hinge residues	791-796; QLMPFG	799-804; QLMPYG	788-793; QYLPLG	797-802; QLMPHG
Gatekeeper residue	T790	T798	T787	T796
Catalytic HRD residue, the first D of K/E/D/D	837	845	N834	843
Catalytic loop N $(HRD(x)_4N$	842	850	839	848
Activation segment DFG, the second D of K/E/D/D	855	863	852	861
Activation segment tyrosine phosphorylation site	869	877	868	875
End of the activation segment, ALE	882-884	890-892	879-881	888-890
Molecular weight (kDa)	134	138	148	147
UniProtKB ID	P00533	P04626	P21860	Q15303

terminal lobe and the activation segment in the carboxyterminal lobe. The interconversion of the inactive and active forms of the ErbB kinases also involves an electrostatic switch. In the less active enzymes, the β3-lysine (K742) forms a salt bridge with the DFG-D (D852) residue, as indicated for ErbB3 (Fig. 2B). The conversion to the active enzyme involves an electrostatic switch where the β3-lysine (K745) forms a salt bridge with the αC-glutamate (E762) with the concomitant formation of the αC_{in} conformation as seen for active EGFR (Fig. 2A). The active forms of the ErbB1/4 possess the β3-lysine–αC-glutamate salt bridge (e.g., PDB ID 1M14 for EGFR and 3BCE for ErbB4) and all of the dormant ErbB1–4 enzymes can form the β3-lysine–DFG-D electrostatic bond (e.g., PDB ID 4HJ0 for EGFR, 3RCD for ErbB2, 3KEX for ErbB3, and 3BBW for ErbB4). The second class of conformational change occurs as the active kinase then toggles between open and closed conformations as it goes through the catalytic cycle. The more open form of the active protein kinase binds MgATP and the protein substrate; this is accompanied by the formation of the closed form as catalysis occurs. Following catalysis, phosphorylated protein and then MgADP are liberated as the enzyme is reconverted to the open form prior to the next catalytic cycle.

3.2. Structures of the hydrophobic spines in the active and in the dormant ErbB/HER protein kinase domains

3.2.1. The regulatory spine

Kornev et al. [36,37] investigated the tertiary structures of inactive and active conformations of about two dozen protein kinases and they established the identity of functionally significant residues by a local spatial pattern (LSP) alignment algorithm. The residues that constitute the regulatory and catalytic spines were identified by their three-dimensional location based upon a comparison of the X-ray crystallographic structures and not by an amino acid signature sequence such as DFG or HRD. The local spatial alignment analysis revealed a supporting skeleton of four nonconsecutive hydrophobic residues that form a regulatory or R-spine and eight hydrophobic residues that form a catalytic or C-spine (Fig. 3). These spines are made up of residues that are derived from both the N- and C-terminal lobes. The regulatory spine contains one residue from the activation segment and another from the αC-helix, whose configurations are important in defining inactive and active states. The C-spine mediates catalysis by promoting ATP binding. The proper alignment of the spines is necessary for the assembly of an active kinase.

EGFR/ErbB1, ErbB2, and ErbB4 have been observed in both inactive and active conformations by X-ray crystallography. The authors who determined the structure of ErbB2 (PDB ID:3PP0) bound to an inhibitor described it as an “active-like enzyme” [38]. The activation segment exhibits an open conformation that is typically found in active enzymes. However, the β3-strand K753 and the αC-helix E770 fail to form an electrostatic bond (the β3-strand K753 binds to DFG-D863) so that this enzyme lacks the characteristics of a fully active protein kinase (not shown). The structure of kinase-impaired ErbB3 is observed in an inactive state with a displaced αC-helix and a closed activation segment (Fig. 3B) (PDB ID: 3KEX).

The EGFR regulatory spine consists of a residue from the beginning of the β4-strand (L777), from the carboxyterminal end of the αC-helix (M766), DFG-F856, along with HRD-H835 of the catalytic loop. M766 and comparable residues from other protein kinases are four residues carboxyterminal to the conserved αC-glutamate. The backbone of H835 is anchored to the αF-helix by a hydrogen bond to a conserved aspartate residue (D872). The activation segment, the protein-substrate positioning loop, and the αHI loop of protein kinase domains, including the ErbB/HER family, interact hydrophobically with the αF-helix [36].

3.2.2. The catalytic spine

The protein kinase catalytic spine consists of residues from the amino-terminal and carboxyterminal lobes and is completed by the adenine base of ATP [37]. The two residues of the amino-terminal lobe of the EGFR that form contacts with the adenine group of ATP include V726 near the beginning of the β2-strand and A743 from the conserved Ala-Xxx-Lys of the β3-strand. Moreover, L844 from the middle of the β7-strand of the carboxyterminal lobe binds to the adenine base in the active enzyme. V726, A743, and L844 characteristically make hydrophobic contact with the scaffolds of ATP-competitive small molecule inhibitors. V843 and V845, hydrophobic residues that flank L844, bind to L798 at the beginning of the αD-helix. The αD-helix L798 interacts with T903 and the β7-strand V843 interacts with L907, both residues of which are in the αF-helix. Note that both the C-spine and R-spine are anchored to the αF-helix, which is a very hydrophobic structure that is entirely within the protein. The αF-helix supports the spines, which in turn anchor the protein kinase catalytic residues. See Table 2 for a list of the residues of the spines of human ErbB1–4. See Refs. [39,40] for a summary of the properties of the ALK receptor protein-tyrosine kinase spine residues, Ref. [41] for the cyclin-dependent protein-serine/threonine kinase spine residues, Ref. [42] for the ERK1/2 spine residues, Ref. [43] for the Janus kinase spine residues, Ref. [44] for the Kit receptor protein-tyrosine kinase spine residues, Ref. [45] for the MEK1/2 spine residues, Ref. [46] for the PDGFRα/β spine residues, Refs. [47,48] for the RAF spine residues, Ref. [49] for the RET protein-tyrosine kinase spine residues, Ref. [50] for the ROS1 protein-tyrosine kinase spine residues, Refs. [51,52] for the Src spine residues, and Ref.

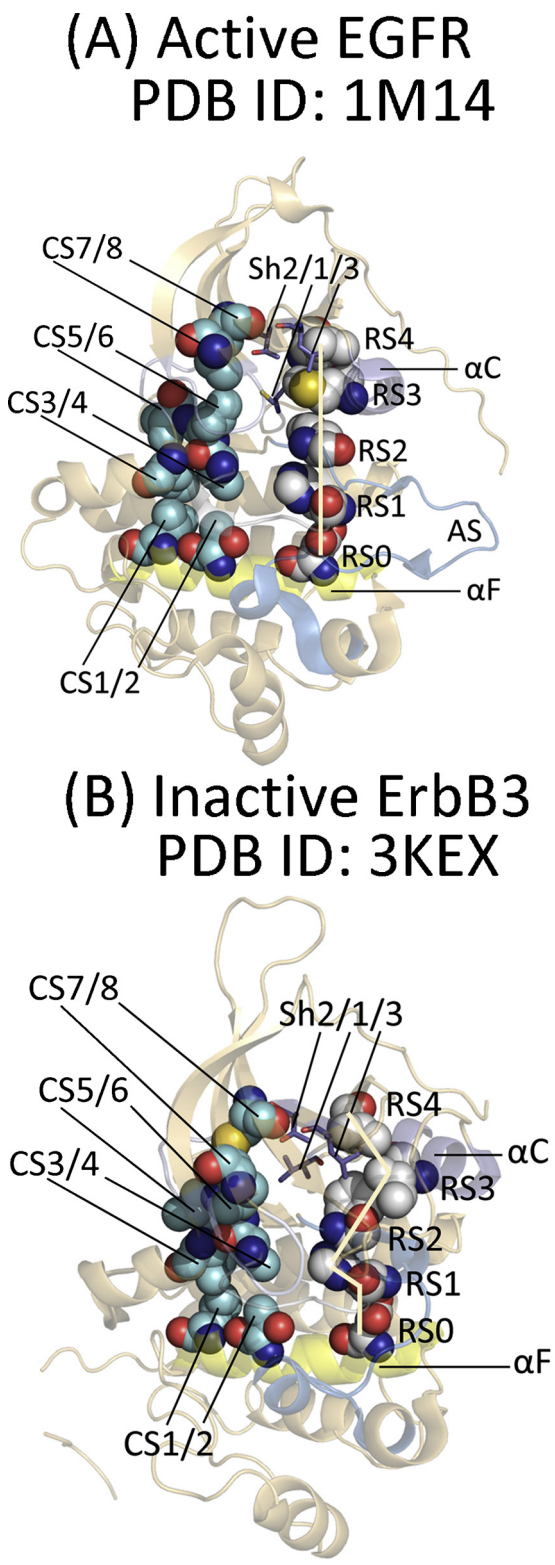

Fig. 3. Catalytic and regulatory spines of active EGFR (A) and kinase-impaired ErbB3. AS, activation segment; CS, catalytic spine; RS, regulatory spine.

[53] for the VEGFR1/2/3 spine residues.

3.2.3. The gatekeeper and other shell residues

Using site-directed mutagenesis, Meharena et al. identified three residues in PKA that stabilize the R-spine which they called shell residues [54]. Going from the aspartate in the αF-helix to the β4-strand residue at the top of the R-spine, these investigators labeled the R-spine residues RS0, RS1, RS2, RS3, and RS4 (Fig. 3A). The three shell residues are labeled Sh1, Sh2, and Sh3. Sh2 represents the classical gatekeeper residue. The term gatekeeper refers to the role of such residues in regulating access to a hydrophobic back pocket adjacent to the adenine binding site [55,56] that is occupied by portions of many small molecule inhibitors. Using local spatial pattern alignment data, Meharena et al. reported that only three of 14 amino acid residues in PKA surrounding RS3 and RS4 are conserved and these shell residues stabilize the protein kinase R-spine [54]. A comparison of the active and inactive EGFR R-spines shows that RS2, RS3, and RS4 of inactive EGFR are displaced when compared with active EGFR, a result that is consistent with the displaced αC-helix configuration of the inactive enzyme (Fig. 3B).

Table 2
Human ErbB1–4 residues that form the R-spine, C-spine and Shell residues.

	KLIFS No.[a]	EGFR	ErbB2	ErbB3	ErbB4
Regulatory spine					
β4-strand (N-lobe)	RS4	38	L777	L785	L774	L783
C-helix (N-lobe)	RS3	28	M766	M774	I763	M772
Activation loop (C-lobe) F of DFG	RS2	82	F856	F864	F853	F862
Catalytic loop His (C-lobe)	RS1	68	H835	H843	H832	H841
F-helix (C-lobe)	RS0	None	D896	D904	D893	D902
R-shell					
Two residues upstream from the gatekeeper	Sh3	43	L788	L796	L785	L784
Gatekeeper, end of β5-strand	Sh2	45	T790	T798	T787	T796
αC-β4 loop	Sh1	36	V774	V782	V772	V781
Catalytic spine					
β3-AxK motif (N-lobe)	CS8	15	A743	A751	C740	A749
β2-strand (N-lobe)	CS7	11	V726	V734	V723	V732
β7-strand (C-lobe)	CS6	77	L844	L852	L841	L850
β7-strand (C-lobe)	CS5	78	V845	V853	V842	V851
β7-strand (C-lobe)	CS4	76	V843	V851	V840	V849
D-helix (C-lobe)	CS3	53	L798	L806	L795	L804
F-helix (C-lobe)	CS2	None	L907	L915	L904	L913
F-helix (C-lobe)	CS1	None	T903	T911	T900	T909

[a] From Ref. [110].

4. Conserved catalytic and structural residues in the ErbB protein kinase domains

4.1. Binding pocket for ATP and small molecule inhibitors

The glycine-rich P-loop occurs universally in protein kinases and consists of a conserved GxGxΦG sequence where Φ refers to a hydrophobic residue. This sequence in the ErbB family consists of GSGAFG. The P-loop forms a lid above the ATP phosphates and is generally one of the most mobile portions of the protein kinase domain. Such mobility is necessary owing to the role that this part of the enzyme plays in binding ATP and then releasing ADP following catalysis. The exocyclic amino group of ATP characteristically interacts with the protein kinase peptide backbone of the first hinge residue. Hinge residues occur after the β5-strand and they connect the N-terminal and C-terminal lobes. Thus, the exocyclic 6-amino group of the adenine ring of ATP forms a hydrogen bond with the carbonyl oxygen of Q791 (PDB ID: 2GS6), which is the first hinge residue of EGFR. The adenine ring *N*1 forms a hydrogen bond with the main chain –NH group of the M793, the third hinge residue. The α-phosphate group binds to K745 of the β3-strand, which in turn forms an electrostatic bond with E762 of the αC-helix (Fig. 2D). The ATP γ-phosphate binds to Mg^{2+}, which coordinates with DFG-D855 (not shown). Notice that the adenine base only extends to the β2 strand, but not to the β3-strand. In contrast, most small molecule ATP-competitive inhibitors extend to the β3-strand and many extend even further toward the αC-helix.

4.2. Catalytic loop and activation segment

Hanks et al. identified 12 subdomains (I–VIa, VIb–XI) with conserved amino-acid-residue signatures that make up the core of protein kinases [57]. Of these, the following four amino acids define a K/E/D/D (Lys/Glu/Asp/Asp) signature and illustrate the catalytic properties of the EGFR family (Table 1). As noted earlier, the first residue of this signature in EGFR occurs as the β3-strand K745 and it forms an electrostatic bond with the αC-helix E762. The catalytic loop near the actual site of phosphoryl transfer consists of HRD(x)$_4$N. The catalytic loop HRD is the first D of K/E/D/D. This loop consists of an HRDLAARN sequence in receptor protein-tyrosine kinases including ErbB1/2/4. The catalytically impaired ErbB3 protein kinase contains HRNLAARN with an asparagine (N) substituting for aspartate (D). The catalytic aspartate (D837) of ErbB1 serves as a base that abstracts a proton from the tyrosyl –OH group (Fig. 2C). Zhou and Adams suggested that the catalytic aspartate of protein kinases positions the substrate hydroxyl for an in-line nucleophilic attack [58]. DFG-D855 of EGFR at the beginning of the activation segment binds Mg^{2+} (1) and the asparagine at the end of the catalytic loop (N842) coordinates a second Mg^{2+}(2). The activation loop DFG is the second D of K/E/D/D. The activation loop contains a tyrosine residue that may undergo phosphorylation, but unlike many other protein-tyrosine kinases, this phosphorylation is not required for ErbB receptor activation [34]. The last eight residues of the ErbB/HER activation segments (PIKWMALE) make up the protein-substrate positioning segment (Fig. 2C).

5. Therapeutic small molecule inhibitors of the ErbB/HER protein kinases

5.1. EGFR/ERBB1/HER1 activating oncogenic mutants in lung cancer

EGFR/ErbB1 plays a significant role in the pathogenesis of many lung cancers. Herbst et al. found that *EGFR* kinase-domain mutations occur in 10–40% of lung cancer samples [59]. The frequency of *EGFR* kinase-domain mutations is around 10% in Caucasians and around 30–40% in Asian patients. Early studies indicated that approximately 10% of unselected patients with NSCLC exhibited very good responses to gefitinib [60]. Three groups in 2004 compared the tumors of people who responded to gefitinib with those who did not [61–63]. These investigators reported that most of the responders exhibited mutations of the *EGFR* kinase domain while those of the nonresponders lacked such mutations. The most common mutations that these investigators found were (i) deletion of five exon-19 residues (^{746}ELREA^{750}) that occur immediately before the αC-helix and (ii) the exon-21 substitution of an arginine for leucine (L858R) in the activation segment. These two mutations account for more than 90% of the activating *EGFR* mutations found in NSCLC. Pao et al. also observed that patients who responded to erlotinib also possessed these *EGFR* mutations [62]. The ^{719}GCARDVS^{725} P-loop mutations account for about 3% of the activating *EGFR* gene mutations. All together, more than 200 *EGFR* mutations have been found in NSCLC [63]. The FDA approved gefitinib for the treatment of NSCLC in 2003 [64] and erlotinib in 2004 [65].

The gain-of-function mutations of oncokinases often occur in or near important regulatory regions such as the αC-helix, the activation loop, or the ATP-phosphate binding loop. A common mechanism for the oncogenic activation of the ErbB family of receptors involves the destabilization of the inactive state thereby promoting the conversion to a more active state. Yun et al. documented this destabilization as the mechanism responsible for the activation of EGFR for the L858R and G791S mutants [66]. The L858R mutation occurs in the N-terminal portion of the activation loop; it immediately follows the ^{855}DFG^{857} sequence that signifies the beginning of the activation loop. The substitution of the larger positively charged arginine R-group for the hydrophobic leucine R-group prohibits its occurrence in the inhibitory αAL loop in the proximal activation segment while it is readily accommodated in the open conformation of the active EGFR protein kinase domain (the analogous αAL helix in the activation loop of ErbB3 is depicted in Fig. 2B) [66]. These investigators hypothesized that the L860Q activation loop mutant that occurs in gefitinib and erlotinib-responsive NSCLCs is activated by a similar mechanism.

Red Brewer et al. characterized the interaction of the L858R-activated mutant and the L858R/T790M drug-resistant double mutant with wild type *EGFR* or wild type *ErbB2* [67]. Based upon co-immunoprecipitation studies, they found that the L858R mutant and drug-resistant double mutant enhance the strength of the donor/acceptor protein interaction that promotes EGFR activation. Zhang et al. found that ligand-activated EGFR kinase domains form an asymmetric homodimer [68]. One kinase domain plays the role of an activator/donor and the other kinase domain plays the role of a receiver/acceptor. The newly activated receiver kinase catalyzes the phosphorylation of tyrosine residues of the activator kinase, which then serve as docking sites for downstream signaling. A similar mechanism is responsible for the activation of the other homo and heterodimers of the ErbB family of enzymes. Red Brewer et al. studied the X-ray crystal structure of the L858R/T790M double mutant and found that the enzyme forms an asymmetric dimer like that seen with wild type *EGFR* [67]. Their experiments support the concept that these activated *EGFR* mutants preferentially function as receiver/acceptors in the asymmetric dimer resulting in *EGFR* mutant activation. These investigators noted that the L858R mutation or the L858R/T790M double mutation destabilizes the dormant conformation and the energetic cost of promoting the acceptor-kinase active conformation is lower in the mutants than in the wild type receptors.

5.2. Small molecule ErbB1/HER1 kinase domain inhibitors

Gefitinib and erlotinib are first-generation FDA-approved

Table 3
Properties of selected orally effective small molecule EGFR family inhibitors.

Name (code) trade name	Targets	PubChem CID[a]	Formula	MW (Da)	D/A[b]	FDA-approved indications (year) or clinical trial study
Gefitinib (ZD1839) Iressa	EGFR	123631	$C_{22}H_{24}ClFN_4O_3$	446.9	1/8	NSCLC (2003)
Erlotinib (OSI-774) Tarceva	EGFR	176870	$C_{22}H_{23}N_3O_4$	393.4	1/7	NSCLC (2004) and pancreatic cancer (2005)
Afatinib (BIBW2992) Tovok	ErbB1/2/4	10184653	$C_{24}H_{25}ClFN_5O_3$	485.9	2/8	NSCLC (2013)
Osimertinib (AZD-9291) Tagrisso	EGFR	71496458	$C_{23}H_{33}N_7O_2$	499.6	2/7	NSCLC (2015)
Dacomitinib (PF299804) Visimpro	Pan-HER	11511120	$C_{24}H_{25}ClFN_5O_2$	469.9	2/7	NSCLC (2018)
Lapatinib (GW572016) Tykerb	EGFR/ErbB2	208908	$C_{29}H_{26}ClFN_4O_4S$	581.1	2/9	Breast cancer (2007)
Neratinib (HKI-272) Nerlynx	ErbB2/HER2	9915743	$C_{30}H_{29}ClN_6O_3$	557.1	2/8	Breast cancer (2015)
Avitinib (AC0010MA)	EGFR	72734520	$C_{26}H_{26}FN_7O_2$	487.5	3/8	Phase I and II clinical trials for NSCLC
Olmutinib (HM61713)	EGFR	54758501	$C_{26}H_{26}N_6O_2S$	486.6	2/8	Phase II clinical trials for NSCLC
Pelitinib (EKB-569)	EGFR	6445562	$C_{24}H_{23}ClFN_5O_2$	467.9	2/7	Phase I clinical trials for NSCLC and colorectal cancer

[a] www.ncbi.nlm.nih.gov/pccompound.
[b] No. of hydrogen bond donors/acceptors.

quinazoline-based reversible EGFR inhibitors that are used in the treatment of NSCLC harboring *EGFR* exon-19 deletions and the exon-21 L858R mutation (Table 3) [64,65,69]. Essentially all NSCLC patients with *EGFR*-activating mutations develop resistance to these drugs with a median duration of 10–13 months [70]. The most common resistance mechanism, which occurs in 50–60% of patients, involves the development of the exon 20 T790M gatekeeper mutation [71]. This mutation results in the replacement of threonine with the larger methionine near the ATP-binding pocket.

Afatinib is a quinazoline derivative like gefitinib and erlotinib and it irreversibly inhibits the activated L858R gatekeeper mutant by forming a covalent bond with EGFR C797. Drugs such as afatinib with an αβ-unsaturated carbonyl group undergo a Michael reaction that involves the addition of a nucleophile (the –SH of C797) to the double bond to form a covalent Michael adduct. Noncovalent contacts place the drug in a suitable orientation within the ATP-binding pocket that facilitate the covalent modification. Four other FDA-approved drugs use this inhibitory mechanism including dacomitinib (targeting mutant *EGFR* in lung cancer), neratinib (targeting ErbB2 in HER2-positive lung cancer), osimertinib (targeting *EGFR* T970M mutants in NSCLC), and ibrutinib (targeting BTK in mantle cell lymphoma, chronic lymphocytic leukemia, marginal zone lymphoma, chronic graft vs. host disease, and Waldenström macroglobulinemia) (www.brimr.org/PKI/PKIs.htm).

Afatinib readily fits into the EGFR ATP-binding site and this finding suggests that the substitution of the larger methionine for the smaller threonine does not sterically block drug binding [72]. Furthermore, Yun et al. found that the activating L858R mutant, the T790M mutant, and the double mutant bind gefitinib with greater affinity than the wild type enzyme [73]. They also discovered that the K_m for ATP is increased in the L858R mutant when compared with the wild type enzyme, but the second T790M mutation decreases the K_m for ATP; the decrease in the K_m increases the ability of ATP to compete with gefitinib for binding and thereby decreases the inhibitory effect of the drug *in vivo*. A methionine gatekeeper may also stabilize the hydrophobic spine [39], which may lead to greater activity of the *EGFR* L858R/T790M double mutant. Engelman et al. found that up regulation of the hepatocyte growth factor receptor, or c-Met, represents another mechanism of resistance to gefitinib or erlotinib and this occurs in about 22% of patients [74]. Afatinib is FDA approved for the first-line treatment of NSCLC in patients harboring the activating (i) exon-19 deletions or (ii) the L858R mutation. Although this drug also inhibits the L858R/T790M double mutant in pre-clinical studies, this efficacy has not been demonstrated in the clinic owing to dose-limiting toxicities [75]. Pre-clinical studies generally refer to experiments performed with animals, animal cells, or human cells whereas clinical studies involve the direct observations of human subjects.

Osimertinib is a targeted small molecule anilino-pyrimidine protein kinase inhibitor that is FDA-approved for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* exon-19 deletions or exon-21 L858R mutations as detected by an FDA-approved test [76]. The drug is also approved for the second-line treatment of patients with metastatic *EGFR* T790M mutation-positive NSCLC whose disease has progressed on or after EGFR protein-tyrosine kinase inhibitor therapy. This drug is a third generation EGFR antagonist that irreversibly inhibits its target enzyme by forming a covalent bond with C797 and it was the first drug approved for the treatment of patients with the T790M gatekeeper mutation. The first-line treatment was associated with a median progression-free survival of 22.1 months and an overall response rate of 67% [77]. This compares with previous clinical trials of gefitinib and erlotinib in similar trials with a progression-free survival of 8.4–13.1 months [78]. Mechanisms of resistance to osimertinib include *KRAS* amplification and the *EGFR* C797S mutation [77]. The serine residue in the latter mutation is unable to form a covalent adduct with afatinib. Clinical trials comparing osimertinib vs. gefitinib, erlotinib, or afatinib are planned or are underway (www.clinicaltrials.gov).

Dacomitinib is an anilino-quinazoline derivative [79] that is FDA-approved for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* exon-19 deletions or exon-21 L858R mutations. Like afatinib and osimertinib, dacomitinib is an irreversible EGFR inhibitor that forms a covalent bond with C979. Although early clinical studies were not promising [80], more recently Mok et al. reported that the overall survival was 34.1 months in patients treated with dacomitinib vs. 26.8 months in patients treated with gefitinib [81]. This study suggests that dacomitinib should be considered as one of the standard treatment options for patients with NSCLC bearing these mutations. Studies performed with cells in culture not derived from patient samples tentatively indicate that resistance to this agent is related to either T790M or C979S *EGFR* mutations [82].

Avitinib is a pyrrolopyrimidine derivative that is in its early developmental stages for the treatment of T790M mutant NSCLC (Table 3) [83]. Olmutinib is an anilino-thienopyrimidine derivative that is in early developmental stages for the treatment of NSCLC harboring the L858R/T790M double mutation or the exon-19 deletions [84]. Based on positive activity data and a favorable safety profile, phase II and phase III trials are underway to assess the efficacy and safety of olmutinib as monotherapy or in combination with other therapies including afatinib, nintedanib, bevacizumab, and pembrolizumab (a monoclonal antibody that targets the PD-1 receptor of lymphocytes, an immune checkpoint inhibitor). Pembrolizumab in combination with carboplatin and paclitaxel was FDA-approved as a first-line therapy for metastatic squamous NSCLC in 2018. Pelitinib is a fluroanilino-quinoline derivative with a long half-life in the human circulation that was designed to inhibit EGFR and ErbB2/HER2 [85,86]. The drug has been in clinical trials for NSCLC and colorectal cancer, but it is unclear whether it will undergo further evaluation. Avitinib, olmutinib, and pelitinib bear an acrylamide group (an αβ-unsaturated carbonyl group) and are irreversible inhibitors that form a covalent bond with EGFR C979. See Ref. [87] for a comprehensive review of small molecule inhibitors that have been approved or that have been in previous or current clinical trials for the treatment of NSCLC.

5.3. Treatment of breast cancer

5.3.1. Classification and general treatment

Breast carcinoma is the leading cause of death from malignancies predominantly (breast) or exclusively (ovary, uterine corpus, uterine cervix) confined to women in the United States and worldwide [10,11]. For purposes of treatment, breast cancers are grouped into three categories, which are not mutually exclusive: these include (i) overexpression of *ERBB2/HER2/NEU*, (ii) hormone receptor-positive, and (iii) triple-negative breast cancer. Triple-negative breast cancer refers to those (i) without *ERBB2* amplification or overexpression and lacking (ii) estrogen and (iii) progesterone receptors. Wittliff reported that ErbB2 overexpression occurs in 20–30% of breast cancers while 10–20% of breast cancers are triple-negative and lack hormone receptors and fail to overexpress ErbB2/HER2 [88]. ErbB2 overexpression was correlated with a poor prognosis prior to the advent of ErbB2 targeted therapies. He also reported that receptors for estrogen, progesterone, or both occur in about 79% of all breast cancers. Moreover, he found that 56% of breast cancers contain both the estrogen and progesterone receptors while 14% contain only the estrogen receptor and 9% contain only the progesterone receptor while 21% lack both receptors [88].

Surgery is the principal treatment modality for localized breast cancer, followed by radiotherapy, chemotherapy, and adjuvant hormonal therapy (with tamoxifen or an aromatase inhibitor) for hormone receptor-positive tumors [8]. Many patients that are hormone receptor-positive benefit from treatment with anastrozole or letrozole. These are aromatase inhibitors that block the formation of the aromatic A ring of estradiol from androgenic precursors. Various cytotoxic drugs are used in the treatment of advanced breast cancers, especially those cancers

Table 4
Cytotoxic drugs, anti-estrogens, and monoclonal antibodies used in the treatment of EGFR-family–driven neoplasms.

Drug[a]	Mechanism of action
Cytotoxic	
Capecitabine	A prodrug that is metabolized to 5-flurorouracil, which inhibits thymidylate synthase, DNA synthesis and function, and RNA function.
Cyclophosphamide	An alkylating agent that forms both intrastrand and interstrand DNA cross links that alter DNA structure, base pairing, replication, and transcription.
Docetaxel	An anti-mitotic taxane that binds to and enhances polymerization of microtubules and inhibits their function.
Doxorubicin	An anthracycline antibiotic that intercalates with DNA, inhibits the progression of topoisomerase II, and produces oxygen-dependent single and double stranded DNA breaks with subsequent inhibition of DNA function.
5-Fluorouracil	An anti-metabolite that inhibits thymidylate synthase, DNA synthesis and function, and RNA function.
Gemcitabine	A cytidine analogue that inhibits (i) DNA synthesis, repair, and function, (ii) ribonucleotide reductase, and (iii) RNA function.
Paclitaxel	An anti-mitotic taxane whose mechanism is the same as docetaxel, which is noted above.
Pemetrexed	An anti-folate that inhibits dihydrofolate reductase, thymidylate synthase, and purine synthesis *de novo*.
Vinorelbine	An anti-mitotic that binds to tubulin to inhibit microtubule function and arrest mitosis.
Anti-estrogens	
Anastrozole	A non-steroidal anti-estrogen aromatase inhibitor.
Letrozole	A non-steroidal anti-estrogen aromatase inhibitor.
Tamoxifen	A selective estrogen receptor modulator (SERM) that produces estrogenic and anti-estrogenic effects depending upon the cell
Monoclonal antibodies[b]	
Bevacizumab	Bevacizumab is a humanized monoclonal antibody that blocks angiogenesis by binding to VEGF-A that is approved for the treatment of non-squamous NSCLC.
Cetuximab	Cetuximab is a human-mouse chimeric IgG1 monoclonal antibody that binds to domain III of the extracellular segment of the tethered inactive state of EGFR and directly blocks activating ligand binding.
Panitumumab	Panitumumab is a fully human monoclonal antibody that binds to the extracellular domain of EGFR and prevents its activation.
Pertuzumab	Pertuzumab is a monoclonal antibody directed against ErbB2/HER2 and prevents its dimerization with other ErbB family members.
Trastuzumab	Trastuzumab is a monoclonal antibody directed against the extracellular domain of ErbB2/HER2 that produces HER2 internalization and down-regulation and induces immune cells to kill the HER2-expressing cell.
Ado-trastuzumab emtansine	This is a trastuzumab-emtansine conjugate that delivers the microtubule inhibitor to ErbB2/HER-positive cells.

[a] www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm.
[b] Therapeutic antibody nomenclature conventions: -mab refers to a monoclonal antibody; -mumab refers to a human mab (e.g., panitumumab), –ximab refers to a chimeric mab (e.g., cetuximab), and zumab refers to a humanized mab (e.g., trastuzumab); -tuxxmab is directed toward the tumor (pertuzumab), -cixxmab is directed toward the cardiovascular system, (e.g., bevacizumab).

that are hormone receptor-negative or triple-negative [89]. These include doxorubicin, cyclophosphamide, docetaxel, and paclitaxel. One of the preferred chemotherapeutic regimens recommended by the National Comprehensive Cancer Network includes doxorubicin and cyclophosphamide followed by paclitaxel. Several other cytotoxic drugs are used in the treatment of breast carcinomas including capecitabine, gemcitabine, pemetrexed, and vinorelbine (Table 4) [8].

5.3.2. Activating ERBB2 mutations and breast cancer

In addition to the overexpression of wild type *ERBB2* in 20–30% of breast cancers, Bose et al. reported that about 1.6% of breast cancer patients possess an *ERBB2* mutation [90]. They suggest that the incidence of new cases of *ERBB2*-mutant breast cancer in the United States is approximately 4000 per year. Of 1499 patients that lacked *ERBB2* gene amplification, they found that 25 of these patients possessed *ERBB2* mutations. In their overall summary, they found two mutations in the extracellular domain at codon 309 and three at codon 310. Moreover, they found one mutation occurred at codon 1220 in the carboxyterminal tail and 12 different mutations within the protein kinase domain. The most common mutation, which was observed in six patients, was an L755S mutation that corresponds to the end of the β3-strand of ErbB2/HER2.

Bose et al. found that seven *ERBB2* mutations activated the receptor as determined by enzyme activity, activation of downstream ErbB2 signaling, or by the ability to enhance tumor formation in mouse xenografts [90]. The activated mutants include a G309 A mutation in the extracellular domain. This particular residue participates in the heterodimerization of ErbB2 with ErbB1 and the mutation may expedite heterodimer formation. The D769 H/Y mutations occur in the αC-helix and these mutations may destabilize the dormant enzyme state. The V777L mutant and the Pro780 insertion, which occurs immediately after the αC-helix, also result in receptor activation. The Pro780 insertion also occurs in patients with NSCLC while the other mutations are restricted to patients with breast cancer. The V842I and the R896C mutations occur in the C-terminal lobe and are removed from any of the classical regulatory sites so that the mechanism for this activation is unclear. Several of the *ERBB2* mutations fail to activate the receptor including L755S (the most common *ERBB2* mutant), R678Q, I767M, and the Y835F. The identification of these *ERBB2* mutants provides additional targets for drug discovery. Bose et al. found that the L755S mutant is resistant to lapatinib, a reversible ErbB2 inhibitor, but all of their other mutants are sensitive to neratinib, an irreversible ErbB2 inhibitor [90].

5.3.3. Targeted small molecule breast cancer treatments

Lapatinib is a reversible chlorophenyl-quinazoline ErbB2/HER2 inhibitor that is FDA-approved for the treatment of breast carcinoma in combination with (i) capecitabine or (ii) with letrozole in patients overexpressing this receptor [91,92]. The treatment of advanced breast cancer is intricate and involves trastuzumab, pertuzumab, and taxanes (docetaxel or paclitaxel) or trastuzumab-emtansine for first- and second-line treatments and trastuzumab or lapatinib along with cytotoxic chemotherapy as third line therapies. See Refs. [93,94] for a comprehensive discussion of the ErbB2/HER-positive breast cancer treatments.

Neratinib is an irreversible chloroanilino-quinazoline ErbB2/HER2 inhibitor that is FDA-approved for the extended adjuvant treatment of adult patients with early stage HER2-overexpressed/amplified breast cancer, to follow adjuvant trastuzumab-based therapy. Adjuvant therapy often refers to therapy given after localized therapy (surgery) and extended adjuvant therapy is given after adjuvant therapy. Neoadjuvant therapy is given prior to localized therapy with the idea of decreasing the tumor size prior to surgery. Neratinib possesses an acrylamide group and forms a covalent adduct with C805 near the ErbB2 ATP-binding site [95]. Canonici et al. reported that neratinib overcomes trastuzumab resistance in HER2-amplified breast carcinoma [96]. Several clinical trials have been designed to investigate the efficacy of neratinib in treating ErbB2/HER2-positive breast cancer alone or in combination with trastuzumab [97]. Neratinib was effective as a single agent or in combination with different chemotherapy drugs in the

treatment of ErbB2/HER2-positive metastatic breast cancer patients and patients with early disease. See Refs. [97–99] for a summary of the clinical trials that lead to the approval of this drug.

6. Classification of protein kinase-drug complexes

Dar and Shokat described three classes of small molecule protein kinase inhibitors and labeled them types I, II, and III [100]. Type I inhibitors bind within the adenine-binding pocket of an active protein kinase; type II inhibitors bind to a dormant protein kinase with the DFG-D of the activation segment pointing away from the active site (DFG-D_{out}); type III inhibitors bind to an allosteric site, which is outside of the adenine-binding pocket. Zuccotto later defined type I½ inhibitors as drugs that bind to a dormant protein kinase with the DFG-D directed inward (DFG-D_{in}) toward the active site (in contradistinction to the DFG-D_{out} conformation) [101]. The inactive enzyme may display an αC_{out} conformation, a closed activation segment, a nonlinear or broken regulatory spine, or various combinations thereof. Gavrin and Saiah later divided allosteric inhibitors into two types: III and IV [102]. Type III inhibitors bind within the cleft between the N-terminal and C-terminal lobes and next to, but independent of, the ATP binding site while type IV inhibitors bind elsewhere. Furthermore, Lamba and Gosh classified bivalent inhibitors as those antagonists that span two distinct parts of the protein kinase domain as type V inhibitors [103]. For example, an antagonist that bound to the adenine-binding site as well as the peptide substrate site would be classified as a type V inhibitor. To complete this classification, we labeled inhibitors that bind covalently with the target enzyme as type VI antagonists [104]. For example, afatinib is a type VI covalent FDA-approved inhibitor of EGFR that is used for the treatment of NSCLC. Mechanistically, this agent binds initially to an active EGFR conformation (like a type I inhibitor) and then the C797 –SH group of EGFR attacks the drug to form an irreversible covalent Michael adduct [104].

Owing to the variability of inactive conformations as compared with the conserved active protein kinase conformation, it was hypothesized that type II inhibitors would be more selective than type I inhibitors, which bind to the conserved active conformation. The evaluation of Vijayan et al. support this hypothesis [105] while those of Zhao et al. and Kwarcinski et al. do not [106,107]. Type III allosteric inhibitors bind adjacently to the adenine binding pocket [102]. Owing to the greater variation in this region when compared with the adenine-binding pocket, type III inhibitors have the potential to exhibit greater selectivity than type I, I½, or II inhibitors. Moreover, Kwarcinski et al. suggest that inhibitors that bind to the αC_{out} conformation (type I½ inhibitors) may be more selective than type I and II antagonists [107]. FDA-approved αC_{out} inhibitors include lapatinib (an EGFR/HER1 and ErbB2/HER2 antagonist) and neratinib (an ErbB2/HER2 antagonist), both drugs of which are used in the treatment of advanced breast cancer. However, Kwarcinski et al. suggest that not all kinases are able to assume the αC_{out} conformation while they propose that all protein kinases are able to adopt the DFG-D_{out} conformation [107].

We had divided the type I½ and type II inhibitors into A and B subtypes [104]. Drugs that bind to the DFG-D_{out} structure of the protein kinase domain and extend into the back cleft are classified as type IIA inhibitors. In contrast, drugs that (i) bind to the DFG-D_{out} conformation and (ii) do not extend into the back cleft as are classified as type IIB inhibitors. Based upon incomplete data, the potential significance of this difference is that type A inhibitors bind to their target enzyme with longer residence times when compared with type B inhibitors [104].

Ung et al. examined a variety of structural features based upon the location of the αC-helix and the DFG motif to define the conformation space of the catalytic domain of protein kinases [108]. They reported that the αC-helix can move from its active αC_{in} location to the αC_{out} position by rotation and tilting. Correspondingly, the DFG motif can move from its active DFG-D_{in} location to the dormant DFG-D_{out} location. These authors defined five different protein kinase configurations: αC_{in}-DFG-D_{in} (CIDI), αC_{in}-DGF-D_{out} (CIDO), αC_{out}-DFG-D_{in} (CODI), αC_{out}-DFG-D_{out} (CODO), and ωCD representing structures with variable locations of the αC-helix or DFG-D intermediate states. CIDI represents the catalytically active conformation with a linear R-spine. Type I protein kinase inhibitors compete with ATP for its binding site and they generally interact with the hinge region. CIDO has the DFG-D motif 180° flip that reshapes the ATP-binding site and displaces DFG-F thereby breaking the R-spine. CODI signifies the αC_{out} and DFG-D_{in} conformation. The folding of the activation loop deforms the protein-substrate binding site while also displacing the αC-helix to the αC_{out} position. Alternatively, a drug such as lapatinib may induce the outward movement of the αC-helix, which allows for its binding to ErbB2. CODO has both αC_{out} and DFG-D_{out} along with a distorted R-spine. There are limited structural data on CODO conformations. ωCD structures are highly heterogeneous with variable αC-helix positioning and diverse DFG-D intermediate states. Moreover, ωCD structures may represent transition states among the various primary configurations.

7. Drug-ligand binding pockets

Liao [109] and van Linden et al. [110] divided the section between the protein kinase N-terminal and C-terminal lobes into a front cleft or front pocket, a gate area, and a back cleft. The back pocket or hydrophobic pocket II (HPII) includes the gate area and back cleft (Fig. 4). The front cleft includes the adenine-binding pocket, the adenine-binding hinge residues, the glycine-rich P-loop, the segment connecting the hinge residues to the C-terminal lobe αD-helix, and the amino acid residues within the catalytic loop ($HRD(x)_4N$). The gate area includes the β3-strand of the N-terminal lobe and the proximal section of the activation segment including DFG. The back-cleft projects to the αC-helix, the αC-β4 back loop, to portions of the β4- and β5-strands of the small lobe, and to a section of the αE-helix within the large lobe. One of the hurdles in the development of protein kinase inhibitors is to increase selectivity to reduce unwanted side effects [111], a process that is facilitated by characterizing drug-kinase interactions [112–114].

van Linden et al. described several components that are found in these three regions [110]. For example, the front cleft includes an adenine-binding pocket (AP) together with two front pockets (FP-I and FP-II). FP-I occurs between the solvent-exposed segment that connects the hinge residues to the αD-helix and the xDFG-motif (where x is the amino acid immediately before the activation segment DFG) and FP-II is found between the glycine-rich P-loop and the β3-strand at the ceiling of the cleft. BP-I-A and BP-I-B are located in the gate area between the xDFG-motif, the β3- and β4-strands, the conserved β3-strand K of the AxK signature, and the αC-helix. The smaller BP-I-A is found at the top of the gate area and is bordered by residues of the β3- and adjacent β5-strands including the β3-AxK and the αC-helix. The larger BP-I-B occurs at the center of the gate area permitting access to the back cleft. Both BP-I-A and BP-I-B occur in the DFG-D_{in} and DFG-D_{out} conformations (Fig. 4).

BP-II-A-in and BP-II-in are found within the back cleft in the DFG-D_{in} conformation [109]. These sub-pockets are bordered by the C-terminal lobe DFG-motif and the N-terminal lobe αC-helix, the αC-β4 back loop, and the β4- and β5-strands. Major changes of BP-II-A-in and BP-II-in occur to generate BP-II-out as it occurs in the DFG-D_{out} configuration; this structural transformation occurs with a change in the location of DFG-F. The resulting compartment is called back pocket II-out (BP-II-out); it occurs where the DFG-F is found in the DFG-D_{in} configuration. BP-II-B is bordered by the αC-helix and the adjacent β4-strand in both the DFG-D_{in} and DFG-D_{out} conformations. Back pocket III (BP-III) occurs only in the DFG-D_{out} conformation. This compartment is found on the floor of BP-II-out between the activation segment DFG-D_{out} motif, the conserved catalytic loop HRD-H, the β6-strand, and the αE-helices of the large lobe along with the αC-β4 back loop and the αC-helices of the N-terminal lobe. Two pockets that are partially solvent exposed (BP-IV and BP-V) occur between the N-terminal lobe αC-helix

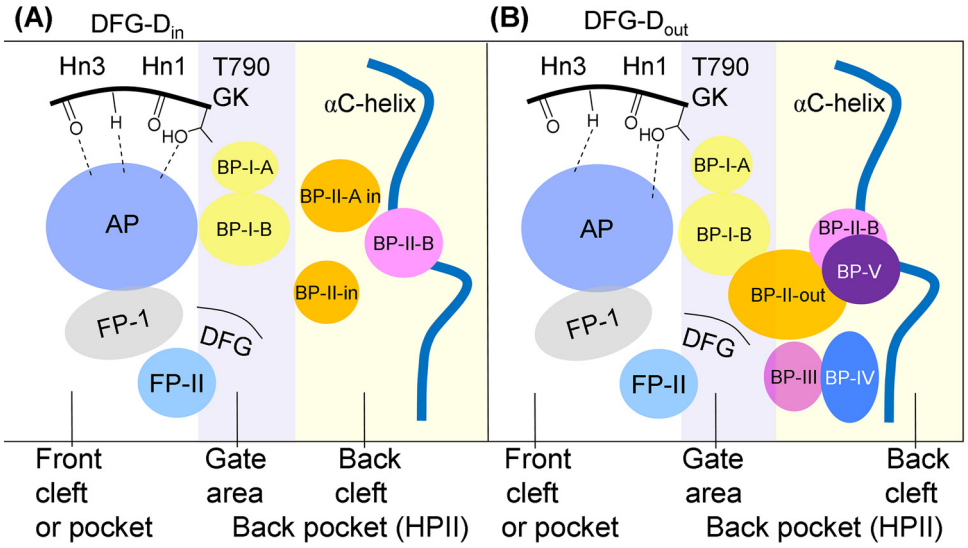

Fig. 4. Location of the protein kinase domain drug-binding pockets. AP, adenine pocket; BP, back pocket; FP, front pocket; Hn, hinge; HPII, hydrophobic pocket II; GK, gatekeeper. Adapted from Refs. [109,110].

and the C-terminal lobe DFG-D_{out} motif, the catalytic loop, the β6-strand, and the activation segment (Fig. 4).

van Linden et al. developed a comprehensive directory of drug and ligand binding to more than 1200 human and mouse protein kinase domains [110]. Their KLIFS (kinase–ligand interaction fingerprint and structure) catalog includes an alignment of 85 ligand binding-site residues occurring in both the small and large lobes; this catalog facilitates the classification of drugs and ligands based upon their binding characteristics and aids in the detection of related interactions. Moreover, these authors devised a standard amino acid residue numbering system that aids in the comparison of many protein kinases. Table 2 specifies the relationship between the KLIFS database numbering and the catalytic spine, shell, and regulatory spine amino acid residue nomenclature. Moreover, this group established an invaluable free and searchable web site that is regularly updated thereby providing comprehensive data on the interaction of protein kinases with drugs and ligands (klifs.vu-compmedchem.nl/). Moreover, Carles et al. have developed a comprehensive directory of protein kinase inhibitors in clinical trials [115]. They have established a free and searchable web site that is regularly updated which includes the inhibitor structures and physical properties, protein kinase targets, therapeutic indications, year of first approval (if applicable), and trade name (http://www.icoa.fr/pkidb/).

8. Structures of EGFR- and ErbB2-drug complexes

Gefitinib (Fig. 5A) is a reversible EGFR quinazoline inhibitor that is approved for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* exon 19 deletions or the exon 21 L858R substitution mutation [64,116]. The X-ray crystallographic structure shows that the *N*1 quinazoline nitrogen forms a hydrogen bond with the N—H group of M793, the third hinge residue. The 3-chloro-4-fluorophenyl group makes hydrophobic contact with residues in the gate area. The aniline ring makes a 45° angle with the plane of the quinazoline while the chlorine atom is directed upward (Fig. 6A). The 7-methoxy group of the quinazoline is in van der Waals contact with G796 at the end of the hinge while the 6-propylmorpholino group extends into the solvent. Overall the drug makes hydrophobic contact with the β1-strand L718 before the G-rich loop, the β2-strand V726 (CS7), the β3-strand A743 (CS8) and K745, the αC-helix M766 of the R-spine (RS3), the β5-strand L788, the gatekeeper T790, ^{791}QLMP^{794} of the hinge, and the β7-strand L844 (CS6). Moreover, the drug makes van der Waals contact with DFG-D855 of the activation segment (not shown). Gefitinib binds within the front pocket and gate area (BP-I-A, BP-I-B). The drug binds to an active protein kinase conformation with αC_{in}, DFG-D_{in}, and with an open activation segment and it is therefore classified as a type I inhibitor [104]. Gefitinib was initially approved by the United States FDA in 2003, but its approval was withdrawn in 2005 only to be reinstated in 2015.

Erlotinib (Fig. 5B) is approved for (i) the first-line treatment of metastatic NSCLC bearing *EGFR* exon 19 deletions or the exon 21 L858R substitution or for (ii) the second- or greater-line treatment of NSCLC after progression following at least one prior chemotherapy regimen. It is also approved for the first-line treatment in combination with gemcitabine of patients with locally advanced, unresectable, or metastatic pancreatic cancer. The structure shows that the *N*1 nitrogen of its quinazoline makes a hydrogen bond with the EGFR M793 N—H group of the hinge (Fig. 6B). The *N*3 nitrogen is not within hydrogen

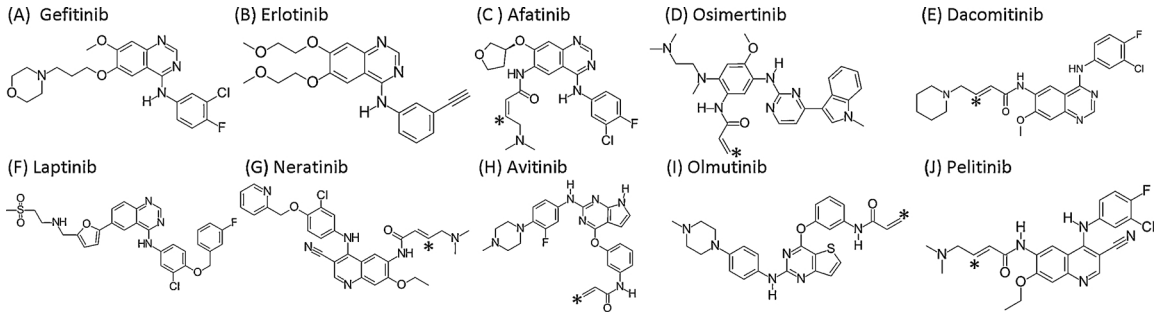

Fig. 5. Structures of selected EGFR (A–E, H–J) and ErbB2 (F, G) inhibitors. The asterisks indicate where covalent modification occurs.

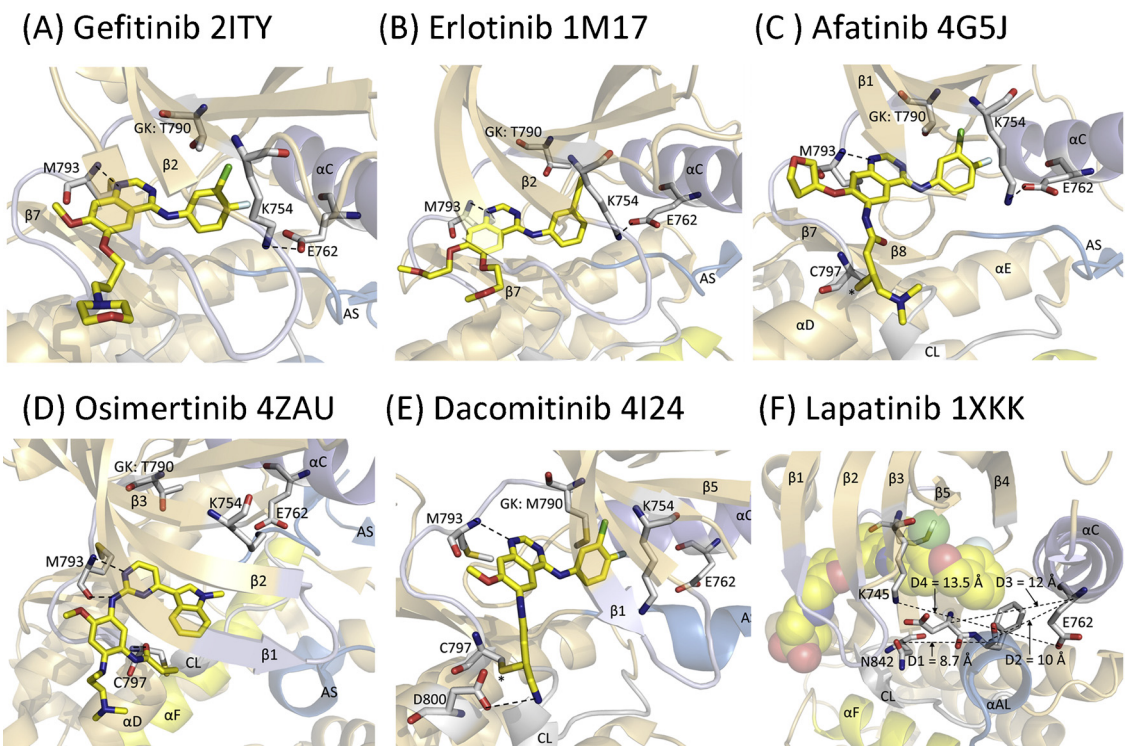

Fig. 6. (A – E) Structures of EGFR-drug complexes. (F) Measurements of D1/2/3/4 for the αC_{out}-EGFR-lapatinib complex. The protein data base ID numbers are given next to the name of the drug. The carbon atoms of the drugs are colored yellow. αAL, the proximal helix near the beginning of the activation loop. AS, activation segment; CL, catalytic loop; GK, gatekeeper.

bonding distance with the T790 gatekeeper (4.1 Å), but a water molecule bridges this gap (not shown). The 3-ethynylphenyl group of erlotinib makes hydrophobic contact with residues within the gatekeeper area. Overall the drug makes hydrophobic contact with the β1-strand L718, the β3-strand A743 (CS8) and K745, the β5-strand L788, the gatekeeper T790 (Sh2), ^{791}QLMPF^{795} of the hinge, the β7-strand L844 (CS6), and T854 before the activation segment (where T is the x residue of xDFG); the drug makes van der Waals contact with DFG-D855. Both methoxyethoxyl groups are directed toward the solvent. The drug-free protein kinase domain and the erlotinib-EGFR complex are superimposable with RMS deviations of only 0.4 Å. Erlotinib binds within the front pocket and gate area (BP-I-A, BP-I-B). The drug binds to an active protein kinase conformation with αC_{in}, DFG-D_{in}, and with an open activation segment and it is therefore classified as a type I inhibitor [104]. Erlotinib also binds to an inactive αC_{out} conformation of EGFR (PDB ID: 4HJO). This form of interaction is that of a type I½B inhibitor because the drug binds to an inactive enzyme with DFG-D_{in} and does not extend past the gate area [104].

Although erlotinib is approved for the treatment of pancreatic cancer (Table 3), this is one of the most lethal forms of cancer and its benefits are marginal. For example, Moore et al. found that the overall survival in patients with advanced pancreatic carcinoma was improved with erlotinib and gemcitabine (6.24 months) compared with placebo plus gemcitabine (5.91 months) [117]. Although the results were statistically significant, the data indicate that the ErbB1-targeted inhibitor increases patient life by only 10 days. Nevertheless, this study led to the FDA approval of this combination therapy. The effectiveness of treatment was the same in *EGFR*-mutant positive and negative tumors.

Afatinib (Fig. 5C) is an FDA-approved irreversible quinazoline inhibitor of EGFR harboring exon-19 deletions or the L858R activation segment mutation [118,119]. Although it has activity against the T790M gatekeeper resistance mutation in some studies, dose-limiting toxicities preclude its use in patients with the gatekeeper mutation [75]. The structure shows that the *C*3 carbon of the acrylamide group of afatinib forms a covalent Michael adduct with C797 within the hinge of EGFR and the *N*1 nitrogen of the quinoline group forms a hydrogen bond with the N–H group of M793, the third hinge residue (Fig. 6C) [72]. The drug also makes hydrophobic contact with the β1-strand L718 before the G-rich loop, K728 after the G-rich loop, the β3-strand A743 (CS8) and K745, the αC-helix M766 (RS3) of the R-spine, ^{791}QLMP^{794} of the hinge, R841 within the distal catalytic loop (HRD-LAARN), the β7-strand L844 (CS6), and T854 just before the activation segment (the x of xDFG). The tetrahydro-3-furanyl group is exposed to the solvent and the 3-chloro-4-fluorophenyl-amino group occurs in the gate area. Afatinib binds to the front cleft, gate area, and subpockets BP-I-A and BP-I-B in the active conformation of EGFR. As an irreversible covalent inhibitor of the target enzyme, it is classified as a type VI antagonist [104].

Osimertinib is an irreversible indole-pyrimidine derivative (Fig. 5D) [120] that is approved for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* exon-19 deletions or the exon 21 L858R mutation and for the second-line treatment of patients with metastatic *EGFR* T790M mutation-positive NSCLC, whose disease has progressed on or after EGFR protein-tyrosine kinase therapy. It was the first drug that was approved for patients with the T790M drug-resistant variant [121]. Moreover, osimertinib is somewhat more effective than gefitinib or erlotinib in treating patients with brain metastases from EGFR-positive NSCLC [122]. The X-ray crystallographic structure shows that the pyrimidine nitrogen forms a hydrogen bond with the N−H group of M793 and an amino N−H group forms a hydrogen bond with the carbonyl group of M793 (Fig. 6D) [123]. The acrylamide group is adjacent to C797 where it will form a covalent Michael adduct. The drug makes hydrophobic contact with the β1-strand L718 before the G-rich loop, the β2-strand V726 (CS7), the β3-strand A743 (CS8) and K745, ^{792}LMP^{794} of the hinge, and the β7-strand L844 (CS6). Osimertinib binds chiefly within the front pocket of a protein in an active conformation. Because it forms a covalent bond with its target, it is classified as a type VI inhibitor [104]. Drug resistance mutations occur in almost all patients treated with protein kinase inhibitors [32] and osimertinib is no exception. One of the

mechanisms of osimertinib resistance results from a C797S mutation that converts a cysteine to a serine, which is unable to form a covalent adduct with the drug [124].

Dacomitinib is an irreversible quinazoline-based EGFR/ErbB2/ErbB4 inhibitor (Fig. 5E) [125] that is approved for the first-line treatment of patients with metastatic NSCLC with *EGFR* exon 19 deletions or the exon 21 L858R substitution mutation [79–81,125]. The structure shows that the *C*3 carbon of the acrylamide group of the drug forms a covalent Michael adduct with C797 within the hinge of EGFR and the *N*1 nitrogen of the quinazoline group forms a hydrogen bond with the N—H group of M793, the third hinge residue (Fig. 6C). The drug also makes hydrophobic contact with the β1-strand L718 before the G-rich loop, the β3-strand A743 (CS8), I744, and K745, ^{788}LIMQLMPIGC^{797} including the hinge, the β7-strand L844 (CS6), and T854 or the x of xDFG. The drug is found in the front pocket, gate area, and subpocket BP-I-B (klifs.vu-compmedchem.nl/). The drug is bound to an inactive enzyme form with αC_{out} and a closed activation segment with an αAL-helix. However, because it is bound covalently with its target, it is classified as a type VI inhibitor [104]. As in the case of osimertinib, one of the mechanisms of resistance to dacomitinib is the result of an *EGFR* C797S mutation [82,124].

Lapatinib is a reversible FDA-approved EGFR and ErbB2 inhibitor that is used in combination with capecitabine for the treatment of patients with advanced or metastatic breast carcinomas whose tumors overexpress ErbB2 and who have received prior therapy including an anthracycline, a taxane, and trastuzumab. It is also approved in combination with letrozole for the treatment of postmenopausal women with hormone receptor-positive metastatic breast cancer that overexpresses ErbB2 for whom hormonal therapy is indicated [91,92].

We have no X-ray crystallographic structures of lapatinib bound to ErbB2, its presumed primary target in patients with breast cancer. However, we have structures of the drug bound to the αC_{out} forms of EGFR and to ErbB4. As noted in Section 3.1, the relative location of the β3-strand with respect to the αC-helix is an important structural parameter that has led to the αC_{in} and the αC_{out} classification. Moreover, Vijayan et al. surveyed the structures of about 200 hundred protein kinases and they divided the αC_{out} structures into (i) classical and (ii) nonclassical groups [105]. They formulated two measurements that differentiated between the two αC_{out} groups and called them D1 and D2. D1 is the distance between the α-carbon atom of the HRD(x)$_4$N-asparagine at the end of the catalytic loop and that of the DFG-F of the activation segment; D2 is the distance between the α-carbon atom of the DFG-F and that of the αC-helix–glutamate residue. The protein kinase exhibits a classical αC_{out} structure when D2 is greater than 9 Å; classical αC_{out} structures have long DFG-F and αC-helix–glutamate distances (D2 ≥ 9.0 Å) and short DFG-F and catalytic-loop asparagine distances (D1 ≤ 7.2 Å).

The D2 value in the structure of dormant αC_{out}/DFG-D_{in} EGFR (PDB ID: 1XKK, bound to lapatinib) equals 10 Å (Fig. 6F); accordingly, this is within the classical αC_{out} group that is characteristic of an inactive enzyme. These investigators measured the distance from the α-carbon atom of αC-helix glutamate and DFG-D, which we named D3 [44] and they found that a D3 measurement of less than 9 Å represents the αC_{in} structure while those with a D3 measurement greater than 10.5 Å represents an αC_{out} configuration while values within this range are classified as αC-dilated. The αC-dilated structure occurs commonly in type II inhibitors with DFG-D_{out} [105]. The D3 value in the EGFR-lapatinib structure is 12 Å, which also corresponds to the αC_{out} configuration. The electrostatic bond between the β3-strand K745 and the αC-helix E762 is broken in the inactive EGFR αC_{out} configuration. The distance between the β3-lysine ε-amino group and αC-glutamate–carboxyl group, which we called D4, is 13.5 Å in the αC_{out} /DFG-D_{in} structure. Lapatinib binds to the front pocket, gate area, and back pocket (BP-I-A, BP-I-B, BP-II-in, and BP-II-A-in) of EGFR (PDB ID: 1XKK) and the front pocket, gate area, and back pocket (BP-I-A, BP-I-B, and BP-II-A-in) of ErbB4 (PDB ID: 3BBT). Lapatinib is a type I½A inhibitor because it binds to an inactive DFG-D_{in} enzyme form and the drug extends into the back pocket [104].

Neratinib is an irreversible ErbB2 inhibitor that is approved for the extended adjuvant treatment of adult patients with early stage ErbB2-overexpressed/amplified breast carcinomas following adjuvant trastuzumab-based therapy [95–99]. We have no X-ray crystallographic structures of neratinib bound to ErbB2, its presumed primary target in patients with breast cancer. However, the structure of the drug bound to the *EGFR* L858R/T790M double mutant (PDB ID: 3W2Q) shows that it binds to an inactive form of the enzyme (αC_{out}) with DFG-D_{in}. The drug binds to the front pocket, gate area, and back pocket (BP-I-A and BP-I-B). Neratinib is an irreversible antagonist and is thereby classified as a type VI inhibitor [104]. It is likely that this drug binds to ErbB2 in a similar fashion.

Avitinib is an irreversible pyrrolo-pyrimidine inhibitor of EGFR that is in phase II clinical trials for the treatment of NSCLC (www.clinicaltrials.gov) [83,84,126]. Olmutinib is an irreversible thienylpyrimidine that is in phase II clinical trials for the treatment of NSCLC (www.clinicaltrials.gov) [84]. Pelitinib is an irreversible quinoline inhibitor of EGFR that is in phase I clinical trials for NSCLC and colorectal carcinomas (www.clinicaltrials.gov) [85,86]. No X-ray crystallographic structures of avitinib, olmutinib, or pelitinib are available, but they are classified as type VI inhibitors that covalently modify their target receptors [104].

To summarize this section, the drugs that bind to EGFR form a hydrogen bond with M793 (the third hinge residue) and they interact hydrophobically with the β1-strand L718, the β2-strand V726 (CS7), the β3-strand A743 (CS8), L792 (the second hinge residue), and the β7-strand L844 (CS6) on the floor of the adenine pocket. All of the drugs except for osimertinib and dacomitinib bind within BP-I-A and BP-I-B. Gefitinib and erlotinib are type I inhibitors that bind reversibly to the active form of EGFR and lapatinib is a type I½A inhibitor that binds reversibly to the inactive αC_{out} configuration. Afatinib, osimertinib, dacomitinib, neratinib, avitinib, olmutinib, and pelitinib are type VI irreversible inhibitors of their target enzyme. Of the approximate three dozen FDA-approved protein kinase inhibitors, five are type VI irreversible inhibitors and four are directed against the ErbB family of receptors (afatinib, dacomitinib, neratinib, osimertinib). The fifth inhibitor, ibrutinib, targets Bruton tyrosine kinase (BTK) (www.brimr.org/PKI/PKIs.htm).

9. Epilogue

The number of newly diagnosed lung cancers in the United States and worldwide and the total number of deaths is estimated to be 234,000 & 2.09 million and 154,000 & 1.76 million, respectively [10,11]. At the time of diagnosis, about 16% of patients have localized disease, 22% have disease that has spread only to regional lymph nodes, 57% have distant metastases, and 5% have unknown staging (https://seer.cancer.gov/statfacts/html/lungb.html). The overall five-year survival is 19% and patients with distant metastases at the time of diagnosis have less than a 5% chance of living five years; accordingly, lung cancer is one of the most serious human malignancies. The frequency of *EGFR* kinase-domain mutations is around 10% in Caucasians and around 30–40% in Asian patients with an overall geometric mean occurrence of about 20%. Based upon the estimate that 85% of all lung cancers are of the non-small cell type, the total number of people in the United States and worldwide with *EGFR* mutations is about 40,000 and 340,000 respectively. Exon 19 deletions and the exon-21 L858R mutation account for more than 90% of the oncogenic *EGFR* NSCLC mutations and these respond to gefitinib, erlotinib, or afatinib treatment. The most common resistance mutation to these therapies is that of the T790M gatekeeper and osimertinib is the only antagonist that is approved for the treatment of EGFR bearing the T790M resistance mutation. One advantage in the treatment of receptors harboring activating mutations vs. the overexpression of wild type proteins is that

drugs can be engineered to be more potent against mutant rather than wild type targets. For example, avitinib inhibits T790M mutants with 300-fold greater potency compared with wild type *EGFR* [126]. Such selectivity promises to minimize toxicity related to the inhibition of wild type targets.

The total number of newly diagnosed breast cancers in women in the United States and worldwide and the total number of deaths is estimated to be 266,000 & 2.08 million and 41,000 & 627,000 in 2018, respectively [10,11]. At the time of diagnosis, about 62% of patients have localized disease, 31% have disease that has spread only to regional lymph nodes, 6% have distant metastases, and 2% have unknown staging (https://seer.cancer.gov/statfacts/html/breast.html). The total five-year survival is about 90% making this one of the more manageable cancers. About 20% of the newly diagnosed breast cancers overexpress wild type ErbB2/HER2, which corresponds to about 50,000 in the United States and 400,000 worldwide. Systemic adjuvant treatment as the standard of care occurred in the 1980s and women with early stage breast carcinomas often receive systemic treatment lasting more than 10 years after diagnosis [127]. Typical first-line adjuvant treatments generally include trastuzumab, pertuzumab, and a taxane such as docetaxel [128]. The trastuzumab-emtansine antibody-drug conjugate is a second-line treatment. A variety of regimens beyond this second-line treatment are available, but the best ordering of these treatments has not been determined. The possibilities include (i) lapatinib with capecitabine, (ii) trastuzumab with cytotoxic chemotherapy, (iii) lapatinib with trastuzumab, and (iv) neratinib for extended adjuvant treatment. Immune checkpoint inhibitors are also being tested in the treatment of HER2-positive tumors [128]. The addition of pembrolizumab to (i) trastuzumab or (ii) paclitaxel with trastuzumab and pertuzumab is being tested in HER2-positive patients. See Ref. [128] for a review of other antibodies and small molecule inhibitors undergoing evaluation for the treatment of HER2-positive breast cancer. Orally effective small molecule CDK4/6 inhibitors that have been FDA-approved for the treatment of hormone receptor-positive and ErbB2-negative breast cancers include abemaciclib, palbociclib, and ribociclib [41].

The number of newly diagnosed colorectal cancers in the United States and worldwide in 2018 is estimated to be about 140,000 & 1,096,000 and the number of deaths is projected to be 51,000 & 430,000, respectively [10,11]. The five-year survival is about 65% (https://seer.cancer.gov/statfacts/html/colorect.html). At the time of diagnosis, about 39% have localized disease, 35% have spread to regional lymph nodes, 21% have distant metastases, and 4% have unstaged disease. Panitumumab is FDA-approved for the treatment of patients with colorectal cancer in patients with wild type *RAS* and it is contraindicated in patients with mutant *KRAS* or *NRAS*. The incidence of *RAS* mutations in colorectal cancers is about 50% [129]. Surprisingly, there is no correlation of effectiveness of panitumumab treatment with the EGFR status [130]. Amplification of ErbB2/HER2 occurs in 2–6% of colorectal cancer patients and treatments with trastuzumab and (i) lapatinib or (ii) pertuzumab have an overall response rate of about 33% [130].

The total number of newly diagnosed pancreatic cancers in the United States and worldwide in 2018 is estimated to be 55,000 & 460,000 and the number of deaths is projected to be 44,000 & 432,000, respectively (https://seer.cancer.gov/statfacts/html/pancreas.html) [10,11]. The five-year survival is about 8.5%. At the time of diagnosis, about 10% of patients have localized disease, 29% have spread to regional lymph nodes, 52% have distant metastases, and 8% are unstaged. As noted in Section 8, erlotinib is FDA-approved for the treatment of advanced pancreatic carcinomas in combination with gemcitabine, but the added beneficial effects of the combination therapy are minuscule when compared with gemcitabine monotherapy [117].

In addition to lung, breast, colorectal, and pancreatic cancers, the ErbB family has undergone extensive study owing to its potential role in the pathogenesis of glioblastoma and bladder, liver, ovarian, head and neck as well as stomach carcinomas. Cetuximab is FDA-approved as part of combination regimens for the treatment of colorectal as well as head and neck cancers. Owing to the central role of the ErbB family in the pathogenesis of many cancers, we can expect additional drugs for the treatment of these and other disorders in the future.

As noted by Winer et al. “Biologically, the cancer cell is notoriously wily; each time we throw an obstacle in its path, it finds an alternate route that then must be blocked” [131]. The development of resistance occurs with both targeted antagonists as well as cytotoxic drugs. The most common mechanism of resistance to gefitinib and erlotinib in EGFR-driven lung cancer involves the development of the T790M gatekeeper mutation [77]. Although the gatekeeper mutation was also the most common mechanism for the development of resistance to afatinib, C797S and L792 F mutations were also observed [132]. The acquired *EGFR* C797S mutation mediates resistance to osimertinib in NSCLC harboring the *EGFR* T790M mutation in addition to resistance because of *KRAS* amplification [77,87,123,124]. The C797S mutation also mediates resistance to dacomitinib [82,124]. Except for imatinib in the treatment of chronic myelogenous leukemia, the development of resistance to all protein kinase targeted drugs within months to one or two years is the norm [32].

Gefitinib, erlotinib, afatinib, dacomitinib, and osimertinib are all FDA approved as first-line therapies for the treatment of advanced NSCLC patients harboring exon-19 deletion or the L758R *EGFR* mutation. Afatinib and erlotinib are also approved as second-line treatments for patients who have received prior chemotherapy. Osimertinib is the only agent among these that is approved for the treatment of the T790M gatekeeper resistance mutation. It is currently unclear on how to optimally order of the administration of these drugs [133]. One possibility is to begin with a first-generation drug such as gefitinib or erlotinib until resistance is apparent owing to the secondary T790M gatekeeper mutation and then switch to osimertinib; this is the so-called sequential strategy. Alternatively, it has been suggested that one should begin initial therapy with osimertinib. The most common tertiary mutation, which occurs in 20–40% of patients, is the C797S mutation that affects the reactive target of osimertinib and the other irreversible inhibitors. Studies are underway to determine whether the sequential approach or the initial use of a latter-generation inhibitor such as osimertinib is better. Devising strategies for minimizing or overcoming resistance to cancer therapies is one of the most important problems faced by concerned biomedical scientists and oncologists.

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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