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# EGFR trafficking: effect of dimerization, dynamics, and mutation

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Spontaneous dimerization of EGF receptors (EGFR) and dysregulation of EGFR signaling has been associated with the development of different cancers. Under normal physiological conditions and to maintain homeostatic cell growth, once EGFR signaling occurs, it needs to be attenuated. Activated EGFRs are rapidly internalized, sorted through early endosomes, and ultimately degraded in lysosomes by a process generally known as receptor down-regulation. Through alterations to EGFR trafficking, tumors develop resistance to current treatment strategies, thus highlighting the necessity for combination treatment strategies that target EGFR trafficking. This review covers EGFR structure, trafficking, and altered surface expression of EGFR receptors in cancer, with a focus on how therapy targeting EGFR trafficking may aid tyrosine kinase inhibitor treatment of cancer.

## KEYWORDS

EGFR trafficking, dimerization, cancer, clathrin-mediated endocytosis, lysosome

## Introduction

Epidermal growth factor receptor (EGFR) is largely considered to be the most well-studied receptor tyrosine kinase (RTK) (1–3). Following the initial discovery of epidermal growth factor (EGF) by Stanley Cohen in 1963, the EGFR family was identified as receptors for EGF (4, 5). Consisting of four members, ErbB1–4 (human epidermal growth factor receptors HER1–4), both homo- and hetero-dimerization of receptors within this family lead to downstream signaling. So far, eight EGFR (HER1) ligands have been reported. However, there are no known ligands for EGFR family member HER2(6–10) despite reports that HER2 undergoes dimerization with other EGF receptors and generates signals for cell growth (11). Importantly, HER2 overexpression and mutation have been observed in many human cancers and the presence of these abnormalities can determine clinical treatment [reviewed in (12)].

Signaling pathways engaged by the EGFR family regulate cell growth, differentiation, invasion, and wound healing. The signal transduction mechanism is tightly regulated by ligand binding to extracellular domains (ECD) of EGFRs, resulting in a change in

conformation and dimerization, passing the signal from outside the cell to the cytoplasmic side via the transmembrane domain and finally cross-phosphorylation by the cytosolic kinase domain (1–3). Adapter proteins help carry out the downstream signaling events leading to the activation of transcription factors for cell growth. Signaling is terminated by receptor downregulation, whereby active receptors undergo endocytosis and are sorted into the lysosome for degradation following ubiquitination. However, some of the receptor molecules are recycled back to the cell surface to maintain the number of EGF receptors on the surface in a process termed EGFR recycling (13–16) (Figure 1). The disruption of proper EGFR signaling and trafficking leads to diseases like cancer, where overexpression or activating mutations within EGFR promote tumor growth. Current treatments for these types of cancers include tyrosine kinase inhibitors (TKIs), which inhibit downstream signaling by directly impairing EGFR tyrosine kinase activity. However, many patients have tumors that become non-responsive to TKIs, thus drawing the need for new treatment strategies in EGFR-driven tumors (17–19).

In the present review, we summarize the role that EGFR structure, dimerization, and trafficking play in signal modulation (Figure 1). Further, we discuss how targeting of these processes in combination may be able to overcome current treatment limitations for tumors that overexpress EGFR or have an EGFR-activating mutation.

## Biochemical basis of activation

All four EGF receptors have a similar structure, with a nearly 620 amino acid extracellular domain (ECD), short transmembrane domain (TM), juxtamembrane domain (JM), a 540 amino acid intracellular region containing the kinase domain (KD), and a

carboxy-terminal tail made of 230 amino acids with multiple phosphorylation sites (Figure 2). The ECD consists of domains I to IV: I and III are involved in ligand binding, and domains II and IV are cysteine-rich domains and contain a string of disulfide bonds. Structures of EGFR “open” and “closed” conformations have been elucidated by X-ray crystallography (9, 20, 21). In the closed (tethered) conformation (Figure 3), domain II forms contact with domain IV, thereby blocking any other molecular contact with domain II. Domains I and III form a huge groove on one side of the molecule that can be occupied by EGFR ligands. Upon ligand binding, domains I and III come close, promoting the extended conformation in which domain IV moves away from domain II, thus opening domain II and IV for interaction of its dimerization partner (open conformation). Different possible dimers of EGFR (e.g., EGFR-EGFR, EGFR-HER2, HER2-HER3) have a similar extracellular dimer structure (Figure 3). Importantly, HER2, which is not known to interact with any EGF ligands, exists in an open conformation, thus allowing it to partner with other EGFR molecules that have bound ligand. Dimerization of the ECD then induces dimerization of the TM helical region through N-terminal GxxxG-like motifs. Further, the JM domain also contacts its partner EGFR molecule resulting in asymmetric dimerization of the kinase domain. EGFRs that are studied in detergent micelles suggest that the dimerization of ECD does not necessarily lead to the dimerization of the kinase domains (23, 24). The kinase domain contains C and N-lobes; upon dimerization, the C-lobe of one kinase interacts with the N-lobe of the dimerization partner’s kinase allowing for cross-phosphorylation. This results in downstream signaling through the recruitment of adaptor proteins to the phosphorylated tyrosine residues (25). Although the biochemical action of EGFR is described at the molecular level, most of the information about EGFR structure and mechanism of action is based on the structure of individual domains since full-length EGFR

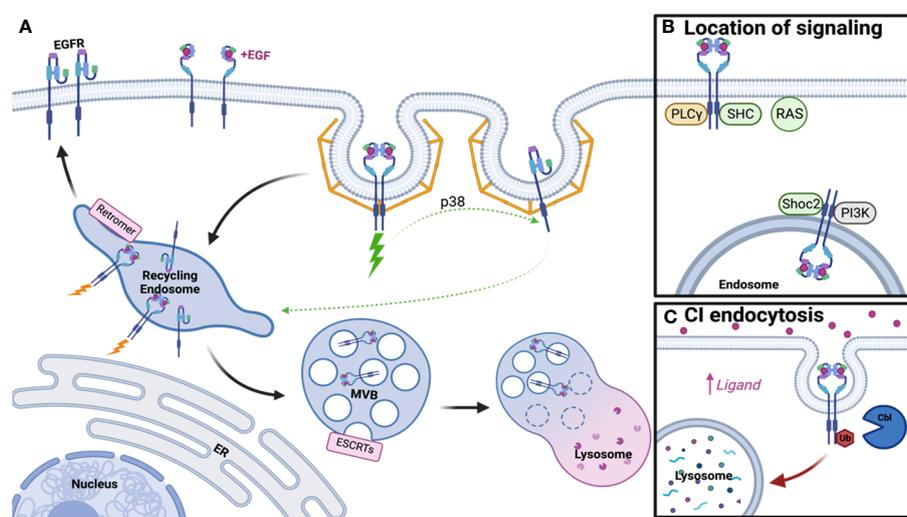
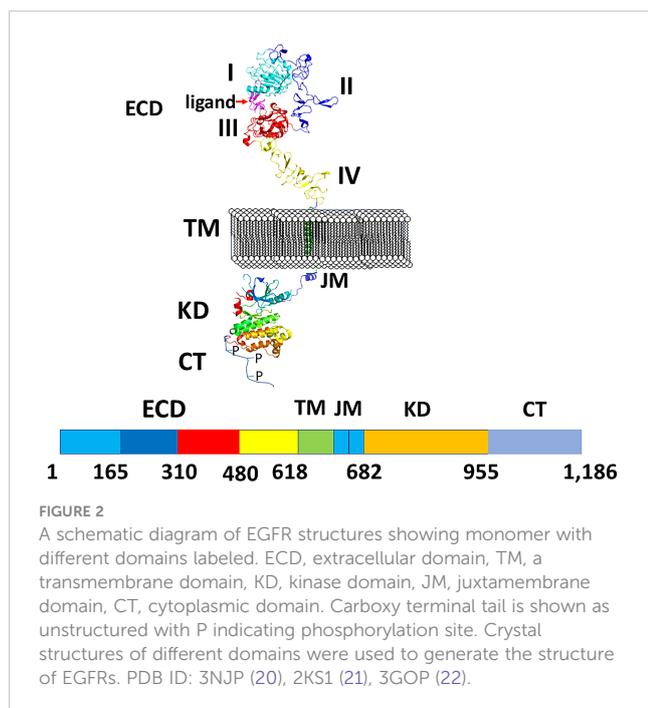


FIGURE 1

Schematic of EGFR Trafficking and Signaling. (A) Clathrin-mediated endocytosis of EGFR dimers and monomers under low ligand conditions promotes receptor recycling, while ubiquitinated dimers will be degraded. (B) Distinct signaling adaptors are associated with EGFR dimers at the plasma membrane and endosome. (C) Clathrin-independent endocytosis occurs under high ligand concentration, promotes receptor ubiquitination, and subsequent lysosomal degradation. Created with BioRender.com.



structure is difficult to elucidate with available experimental methods. Therefore, one has to put the available structural pieces together to get the purported overall structure of full-length EGFR family members, and to infer mechanisms contributing to their dimerization and signaling in cells.

The ECD of EGFRs is known to dimerize upon ligand addition and induce the dimerization of the intracellular kinase domain, however, there are reports that isolated kinase domains dimerize and activate signaling when JM segments are present (22, 26, 27). Therefore, it is proposed that ligand-free EGFRs can undergo dimerization and exist as active and inactive dimers. In support of this notion is the observation that overexpression of wild type EGFR leads to ligand-independent activation of IRF3, rather than the ERK or AKT pathways, which has been termed non-canonical EGFR signaling (28). This has been further supported by changes in distribution within the plasma membrane, with high EGFR expression promoting oligomerization and ligand-independent phosphorylation that appears to have no impact on ERK or AKT signaling (28, 29). In addition to EGF-induced and ligand-independent activation, EGFR activation can be induced by six other ligands (30). These EGFR ligands have a varying affinity, induce specific dimerization pairings of the four EGFRs, and lead to distinct cellular outcomes. Thus, activation of EGFR signaling can occur in scenarios outside of EGF addition, which leads to specific receptor dimerization/oligomerization and varying cellular response.

The plasma membrane also plays a role in modulating EGFR dimerization, activation, and autoinhibition. Within the plasma membrane, cholesterol-rich lipid microdomains have been found to promote quick movement of EGFR and HER2, which allows for rapid dimerization and signaling upon ligand addition (31–34). Conversely, depletion of membrane cholesterol through methyl- $\beta$ -cyclodextrin treatment leads to the accumulation of EGFR within confined regions of the membrane and therefore promotes ligand-

independent receptor activation (31). While these findings were in live cells using microscopic techniques, they have limitations in terms of what EGFR is doing at a structural level within the membrane that recent molecular dynamics (MD) simulations have started to fill in.

The EGFR kinase domain surface has many basic residues (27), which are shielded by the C-terminal domain in the active dimer. MD simulations suggest that the EGFR kinase domain is attached to the membrane by interaction of the basic residues with anionic lipids (35). Thus, the active site of the kinase domain is not exposed. On the other hand, asymmetric dimers of kinases have less interaction with anionic lipids, and the kinase domain is available for phosphorylation, making EGFR active (36). Although EGFR ECDs are assumed to be perpendicular to the plane formed by the cell membrane, MD simulations studies found that the ECD of EGFR molecules lie down on the membrane. This orientation brings the EGF-binding site adjacent to the membrane surface where it can interact with the membrane-bound ligand (37). This asymmetric nature of EGFR dimers, where one of the ligands is bound to the membrane as well as EGFR and the other only bound to EGFR, may lead to negative cooperativity. Although MD simulations provide some insight into the dynamic nature of EGFR receptors and their dimers, these structures are modeled based on X-ray crystal structure and solution structure using NMR and modeling methods. In reality, dimerization and activation of the kinase domain seem to depend on the microenvironment and charges of lipid head groups facing the cytoplasmic side of the membrane (24, 27, 35–37). Thus, signal transduction processes at the molecular level have yet to be elucidated in detail.

Moving forward, structural elucidation of full-length EGFRs using cryo-EM and molecular modeling may provide new insights into the signal transduction process of EGFR family members. Furthermore, EGFRs that lack part or full ECD have been found in clinical studies (38–40), calling into question the importance of ECD and conformational changes associated with ECD and TM for kinase activity. Thus, altered or mutated forms of EGFR exist in disease states of patients, which don't fit into the current models, and hence extensive structural and functional studies of EGF family receptors are needed to address those limitations.

## Regulation of EGFR trafficking and signaling

In 1976, Carpenter and Cohen published the first paper on EGFR trafficking, postulating that EGFR-bound growth factor could enter human fibroblasts and be degraded within the lysosome (41). However, little was known about the regulation of this pathway and whether it served a purpose for receptor signaling. It is now appreciated that along the pathway to the lysosome, several steps participate in the regulation of EGFR trafficking and signaling, including endocytosis, protein recycling, and protein degradation (Figure 1A).

At the plasma membrane, active EGFR dimers generate a conformation that leads to the trans-phosphorylation of tyrosine

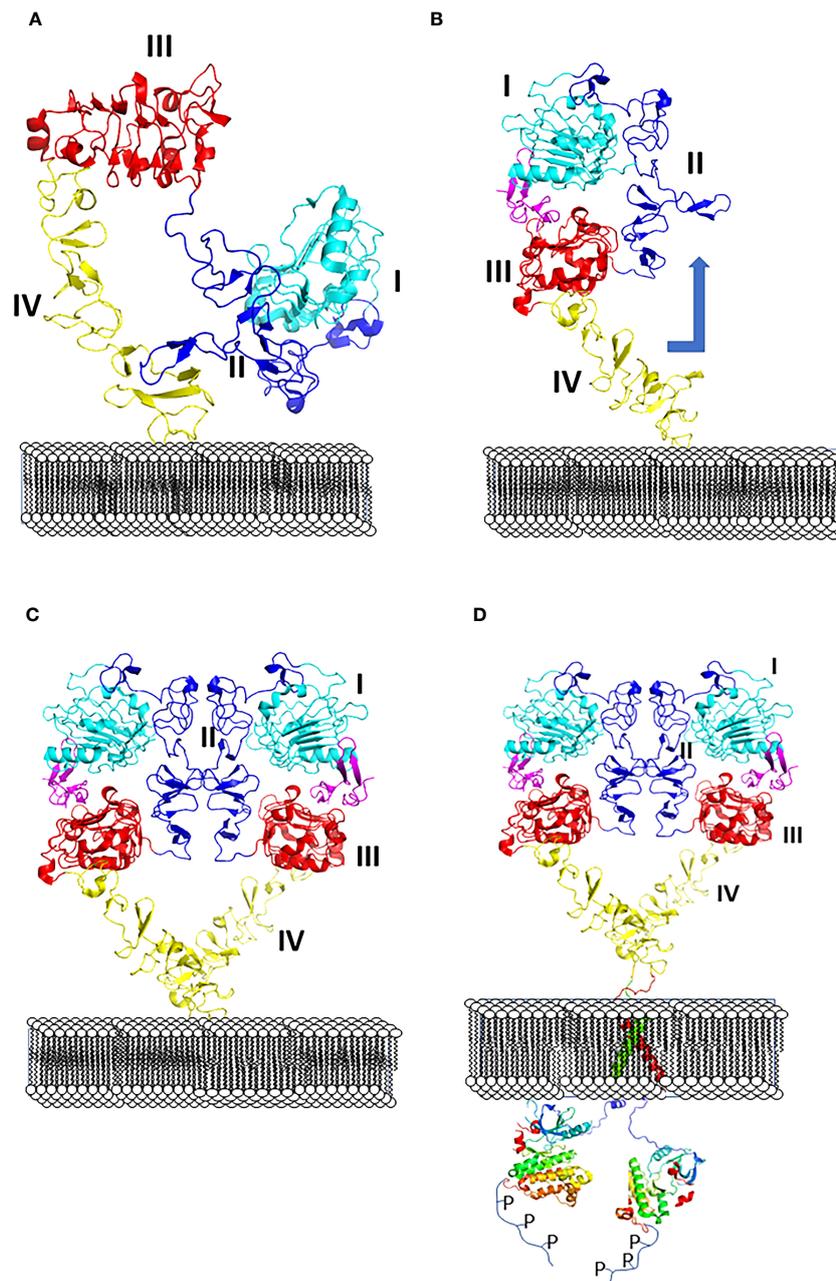


FIGURE 3

(A) Closed (left) and (B) open (right) conformation of EGFR extracellular domain and changes in conformation with respect to the membrane surface. (C) Upon change in conformation monomers of ECD of EGFR form dimers, dimer interface with domains I to IV are shown. (D) Dimerization of ECD results in phosphorylation of kinase domain by changes in transmembrane domain and kinase domain activation. PDB ID:1NQL (2) and 3NJP (20), 2KS1 (21), 3GOP (22).

residues in the cytoplasmic tail, promoting the recruitment of numerous signaling adaptor proteins that engage pathways such as the RAS-MAPK cascade and the phosphoinositide-3-kinase (PI3K) pathway. In addition to signaling adaptors, phosphorylation of EGFR at Tyrosine 1068 and 1086 leads to growth factor receptor-bound protein 2 (Grb2) binding, thus promoting the recruitment of adaptor protein complex-2 (AP-2) and allowing EGFR to undergo clathrin-mediated endocytosis (CME) (42, 43). CME is also regulated by ubiquitination and acetylation of EGFR (44), with clathrin acting as a signaling

scaffold for the AKT pathway (45). However, recent literature suggests endocytosis of EGFR is more complicated than the canonical CME model, with subsets of clathrin-coated pits that may not require AP-2, and instead rely on other endocytic adaptors, to promote endocytosis (46, 47). A further layer of complexity to EGFR endocytosis is added when ligand concentration is taken into consideration. In contrast to low concentration of ligand, which promotes CME, high concentration of ligand promotes ubiquitination of active EGFR by the E3 ubiquitin ligase Cbl, thus allowing it to undergo rapid clathrin-independent endocytosis

(CIE) and steering it toward eventual degradation (46). Thus, a simplified model of EGFR dimer activation and endocytosis has been generated, however this fails to address any further clustering of active dimers and endocytosis of EGFR monomers.

Unlike the simplified model, which only requires phosphorylation of a dimer for signaling to occur, a more complicated model emerges whereby this activation depends on ligand addition and further oligomerization of EGFR (48, 49). Under low ligand concentration, it's been thought that ligand-bound dimerized EGFR can trans-phosphorylate nearby dimers that are unbound, thus amplifying the signal (48). However, this model is apart from the traditional endocytosis model and more work is needed to elucidate if endocytosis of all receptors within the oligomer would occur in a CME manner. In contrast to oligomers, more work has been done on how inactive monomers internalize. Unbound EGFRs are internalized at a slower rate compared to ligand-bound EGFRs (50), which may in part be due to endocytic regulation based on signals from active dimers. Active EGFR triggers p38 activation, which phosphorylates EGFR monomers near Serine 1015, resulting in CME (51). Thus, ligand concentration and receptor activation are closely linked to the regulation of endocytosis for both active dimers and unbound monomers.

Following endocytosis, EGFR traffics to the early endosome, where decisions are then made for receptor recycling or degradation. Apart from these pathways, EGFRs are also known to be transported into the nucleus after early endosomal sorting to participate in transcriptional regulation (52, 53). While signaling is initiated at the plasma membrane by ligand binding and dimerization, whether the receptor continues to signal from the endosome remains highly debated. Early literature suggested active EGFR continued to signal from the endosome until incorporation into intraluminal vesicles (ILVs), thus inhibiting access of the EGFR C-terminus to cytosolic signaling effectors. The first study to assess the relationship between signaling and cellular location utilized mutant dynamin and proposed that phospholipase C gamma (PLC $\gamma$ ) and Shc signaling occur at the plasma membrane while further EGFR phosphorylation, ERK, and PI3K signaling derive from the endosome (54). Further literature suggested this may be in part due to which adaptors are associated with EGFR at these cellular locations, with some only interacting at the plasma membrane or intracellularly and others appearing in both populations (55). Some adaptors appeared to traffic from the plasma membrane to the endosome with EGFR upon EGF addition and sustain signaling throughout this process (56–58) and adaptors/scaffolds that aid in signaling at the endosome, such as Shoc2, have also been reported (59). Utilizing spatial proteomics, many signaling molecules have also been found in proximity to endosomal EGFR (60). However, the depletion of dynamin in mouse fibroblasts suggested signaling was unimpacted and therefore a majority of EGFR signaling may occur at the plasma membrane (61). Further, RAS primarily localizes to the plasma membrane and not the endosome, thus limiting where EGFR signaling mediated by RAS can occur (62, 63). These studies highlight that while some adaptors traffic with EGFR to the endosome, signaling may be limited by localization of other required components to the plasma membrane (Figure 1B). Thus,

the debate on where signaling occurs is still ongoing but may depend on factors like cell type, protein depletion, method of overexpression, or technical limitations.

While at the endosome, one potential fate for EGFR is recycling back to the plasma membrane, which is mediated by the Retromer, Retriever, COMMD/CCDC22/CCDC93 (CCC), and WASP and SCAR homologue (WASH) complexes (64). Depletion of these complexes promotes lysosomal degradation of cargo (65, 66) thus suggesting more of an active process for cargo selection in recycling than previously appreciated (59). For unbound EGFR monomers, this recycling is regulated by p38-mediated phosphorylation either downstream of low-concentration EGF addition (51) or phosphatidic acid signaling (67), though it remains unclear which recycling machinery is necessary for this process. On the way back to the plasma membrane, it's also thought that any spontaneously active monomers are shut off by protein tyrosine phosphatase 1B (PTP1B)-mediated dephosphorylation (68). In addition to active cargo selection, proteins that inhibit selection for degradation, like lipocalin-2, also induce recycling and sustained EGFR activity (69). Outside of recycling logistics for monomers, most research on EGFR recycling and signaling has been conducted in the context of benefitting cancer growth and progression, as discussed later.

Alternatively, endosomal EGFR may undergo selection for lysosomal degradation. Entrance into the degradation pathway is reliant upon EGFR ubiquitination, by ligases such as Cbl and ZNRF1, thus allowing recognition by the endosomal sorting complexes required for transport (ESCRTs) for incorporation into ILV (59, 70–73). Subsequent fusion of the multivesicular body (MVB) with the lysosome leads to EGFR degradation (74). Mutations in the ESCRT pathway have been associated with endosomal EGFR accumulation and enhanced signaling, but delayed receptor turnover, thus providing some support for the endosomal signaling model. Post-translational modification of the ESCRT machinery, such as glycosylation of HRS/HGS, has also been shown to play a role in regulation of EGFR degradation and signaling (75). It's been suggested that ligand concentration/method of internalization dictates receptor fate, with clathrin independent endocytosis under high ligand concentration leading to receptor degradation (Figure 1C). Interactions with the ER may provide some cues for determining EGFR fate, with ER resident proteins able to keep the endosome in the perinuclear region of the cell to promote degradation and signal termination (76, 77). Thus, degradation and signaling are not only mediated by the ESCRT machinery, but also by intracellular localization of the endosomal compartment.

Spatially, the recycling and degradation machinery reside in different microdomains within the endosome, and a present area of focus is how cargo moves into the proper microdomain. Thus far, there have been studies linking recycling machinery such as WASH and receptor-mediated endocytosis -8 (RME-8) to recruitment and activity of ESCRT-0 component HRS, though no direct interaction has been found (78, 79). Based on actin nucleation being regulated by WASH, the transition to ESCRT-0 and choice between cargo recycling and degradation may be partially dependent upon actin binding/recognition. Further, RME-8 interacts with Hsc70 to disassemble endosomal clathrin, which is thought to be critical

for assembly of HRS and transition to a degradative microdomain (80, 81). Thus, coats of either actin or clathrin play a role in determining the endosomal microdomain which cargo is incorporated into and therefore fate of the cargo. In addition to these coats providing a microdomain platform, studies in plants have shown a direct interaction between ESCRT-associated ALIX and retromer subunits (82), thus suggesting there may be additional processes/mechanisms by which these microdomains are regulated. Cumulatively, these microdomains are a continued area of study which may provide the ability to modulate trafficking and signaling of receptor cargo.

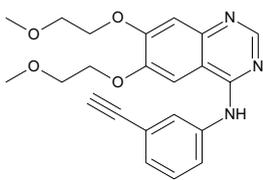
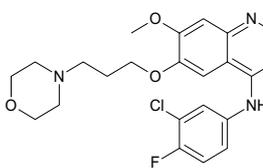
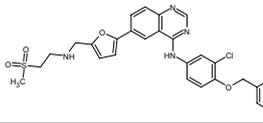
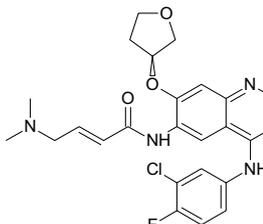
## Limitations of EGFR-mediated cancer treatment strategies

Tyrosine kinase inhibitors (TKIs) serve as the main therapy to target EGFR in cancer and have evolved through many generations. Imatinib was the first TKI approved for cancer therapy just over two decades ago (83) and was followed by first-generation EGFR-specific TKIs, gefitinib and erlotinib, thus revolutionizing targeted

therapy by kinase inhibitors (84). Presently, there are four generations of TKIs, with those up through the third being approved for clinical use (Table 1) (104, 105). However, TKIs only delay tumor growth, and most tumors develop resistance within two years due to intrinsic or acquired changes.

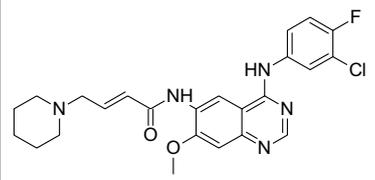
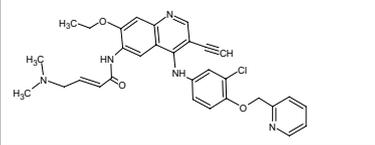
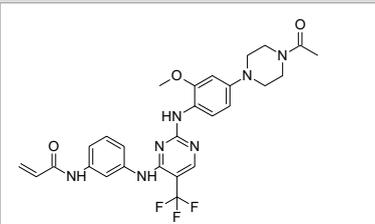
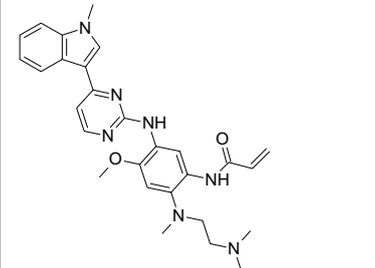
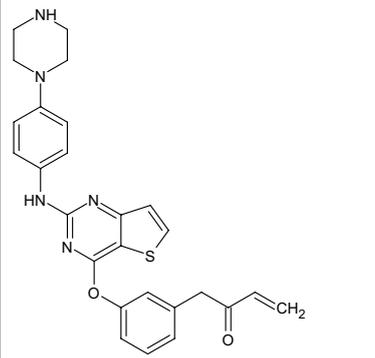
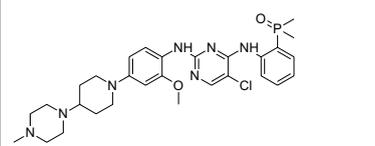
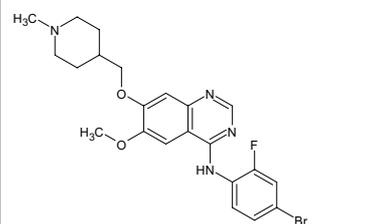
Intrinsic resistance can be caused by genetic aberrations in multiple cancer signaling pathways, as is the case non-small cell lung cancer (NSCLC) patients with EGFR T790M mutation who do not respond to gefitinib and instead maintain proliferative and cell survival signaling in the presence of TKIs using alternative pathways such as integrin signaling (106, 107). It was shown that interaction of EGFR and integrin  $\beta 4$  can affect the sensitivity of gefitinib treatment in gastric cancer (108). Third generation TKIs, such as Osimertinib, have been developed to treat these patients (109), however they develop drug resistance through unknown mechanisms (110). Fourth generation inhibitors or multi-targeting TKIs such as brigatinib and vandetanib are developed to overcome the resistance developed by third generation TKIs. However, brigatinib therapy seems to develop resistance due to NTRK rearrangement in some patients (99–103). A small-molecule EGFR inhibitor, ERAS-801 has received an orphan drug

TABLE 1 Representative EGFR tyrosine kinase inhibitors from each generation to overcome resistance and mutations of EGFR kinase domain.

TKI/year of approval	Structure	Targeted mutation/reversible, irreversible	Possible Resistance development	Reference
<b>First generation</b>				
Erlotinib 2004		Glu746-Ala750 deletion in exon 19 and the common p.Leu858Arg substitution in exon 21 Reversible	EGFR T790M HER2 amplification	(84)
Gefitinib 2003		Glu746-Ala750 deletion in exon 19 and the common p.Leu858Arg substitution in exon 21 Reversible	EGFR T790M HER2 amplification	(85, 86)
Lapatinib 2007		Targets both EGFR and HER2 kinase Reversible	Overexpression with activation of other tyrosine receptor kinases Ax1, MET, IGF-1R, VEGF	(87)
<b>Second generation</b>				
Afatinib 2013		Del19/L858R uncommon EGFR mutations (S768I/G719X/L861Q) Irreversible	EGFR T790M HER2 amplification MET amplification	(88)

(Continued)

TABLE 1 Continued

TKI/year of approval	Structure	Targeted mutation/ reversible, irreversible	Possible Resistance development	Reference
Dacomitinib 2018		EGFR exon 19 deletion or exon 21 L858R substitution mutations Irreversible	EGFR T790M HER2 amplification MET amplification	(89) (90)
Neratinib 2017		Targets EGFR, HER1, HER2, and HER4. Irreversible	HER2 alteration, alterations in the HER3/PI3K/protein kinase B, AKT (mTOR) and MAPK signaling.	(91–93)
<b>Third generation</b>				
Rociletinib 2022		EGFR Ex19del L858R, T790M Irreversible	EGFR C797S HER2 amplification, MET amplification KRAS mutation	(94) (95)
Osimertinib 2015		EGFR T790M and EGFR sensitive L858R and Del19 Irreversible	EGFR C797S MET amplification HER2 amplification/mutation PIK#CA Amplification/mutation	(96) (97)
Olmudinib 2016		EGFR Ex19del L858R, T790M Irreversible	EGFR C797S	(98, 99)
<b>Fourth generation or Multi-target TKIs</b>				
Brigatinib 2017		EGFR Ex19de L858R, C797S T790M Reversible	Possible NTRK rearrangement (L1PI-NTRK1)	(100) (99)
Vandetanib 2011		VEGFR, EGFR and RET tyrosine kinases. Reversible	Substitution at codon 904 in the activation loop of the RET kinase domain	(101–103)

Structure of TKIs were drawn based on structures in Selleckchem.com.

designation by the FDA for malignant glioma. It is an orally available molecule that has significant CNS penetration (111). Another small molecule tucatinib, a reversible TKI, is known to bind to HER2 with specificity. Tucatinib in combination with antibody trastuzumab has shown efficacy in breast cancer and this drug was recently approved by FDA for the treatment of HER2 positive metastatic breast cancer (112, 113). Acquired resistance is caused by on-target secondary mutations and new generations of TKIs were developed to overcome the resistance (86, 114), but these still lack the ability to address drug resistance associated with altered receptor trafficking (115–117). For example, in a subset of NSCLC the mutant EGFRs preferentially recycle rather than degrade, leading to enhanced signaling by EGFR and the proto-oncogene tyrosine kinase Src (14, 116, 118–121). Cumulatively, further understanding and modulation of EGFR trafficking in cancer may provide an additional treatment option to bypass resistance to TKI treatment.

One of the mechanisms utilized by cancer cells to develop resistance is to avoid ubiquitin-mediated degradation of EGFR. In tumors harboring EGFR variant VIII, signaling is independent of ligand addition and remains constitutively active, however ubiquitination by the ubiquitin ligase Cbl promotes lysosomal degradation and therefore signal termination (122, 123). However, further EGFR mutations, which impair the interaction with Cbl, limit incorporation into MVBs for lysosomal degradation and instead cause prolonged signaling (124). Another way to avoid ubiquitin-mediated degradation is relocation of EGFR to the nucleus mediated by the tumor suppressor protein TIP30 (125, 126) and nuclear translocation signals within the juxtamembrane region (127), thus mutation at this level would also render TKI therapy ineffective and promote resistance.

Another mechanism of resistance is inhibition of EGFR downregulation by HER2. HER2 can act as an inhibitor of the downregulation of other EGFR family members due to the ECD existing in an open conformation thereby promoting receptor heterodimerization (2, 128–130). Overexpression of HER2 is thought to impact EGFR trafficking in two ways: decrease downregulation/internalization from the plasma membrane (131–135), and/or reroute internalized EGFR from the degradation pathway to the recycling pathway (136). Multiple proposed mechanisms exist for how HER2 may inhibit downregulation including inducing a conformation change, interactions with lipid raft components (137), and inhibition of clathrin coated pits (138, 139). In contrast, HER2 may promote recycling of EGFR since it isn't contained in an endolysosomal compartment (138, 140–143) and is recycled back to the plasma membrane in a sortilin-related receptor 1 (SORLA)-dependent manner (144, 145). It's also been thought that at least some regulation of HER2 trafficking is through the kinase domain, with binding of Hsp90 allowing for sequestration and preventing catalytic activity of HER2 (71, 146–148). Lastly, it was recently shown in breast cancer cells that pharmacologic inhibition of endosomal recycling using primaquine or knockdown of the Rab coupling protein led to the lysosomal accumulation and degradation of HER2 (149). These manipulations were found to synergize with anti-HER2 therapies and overcome resistance to the TKI lapatinib, thus suggesting that manipulating endosomal recycling could be a

viable strategy to overcome resistance to therapies targeting the EGFR family of receptors.

Thus far, antibodies have been designed to target HER2 trafficking mechanisms, however these rely on HER2 being the main source of resistance for a tumor. One such antibody, Trastuzumab, has been found to inhibit ligand-independent activation, promote internalization, and prevent shedding of the HER2 ECD (150–153). In the case that a truncated form is present that lacks the ECD (p95HER2) (154–157), it's thought that resistance to trastuzumab can develop, but thus far clinical studies have found no significant difference in survival among patients with p95HER2 relative to those lacking it (40, 158–160). Pertuzumab has also been shown to prevent dimerization of HER2 (161) and a combination with trastuzumab produces synergistic effects (162–166). Additionally, polyclonal, and bispecific anti-HER2 antibodies have been shown to promote rapid internalization and subsequent degradation of HER2 (165, 167, 168). These data show that HER2 can be targeted and regulate EGFR in cancer, however they fail to address how trafficking of EGFR, HER2, and HER3 impacts other receptors. EGFR trafficking in the absence of other family members has been extensively studied, but EGFR trafficking in these conditions is an ongoing area of research.

## Combining TKIs with EGFR trafficking inhibitors for cancer therapy

Since the main mechanism of resistance to TKI therapy involve vesicular trafficking, either by avoiding ubiquitin-mediated degradation or through HER2 trafficking, it stands to reason that a combination therapy approach could be effective. Preliminary understanding of efficacy in part derives from HER2 antibody studies, as discussed in the previous section, but also from existing TKIs that can alter trafficking of HER2 at high concentration. An example of this is Neratinib, a pan-HER TKI (169), which inhibits kinase activity and induces the internalization of EGFR/HER2 receptors through CME leading to their degradation only at a high dose (170). While adjusting TKI concentrations can be easily done in the context of tumor cell lines, it poses a potential toxicity problem when translating to humans. As such, further development of either TKIs that alter trafficking at low concentration or of small molecules that can be combined with TKIs is necessary.

One of the trafficking steps often dysregulated during TKI resistance in endocytosis, so preliminary studies on combination therapy have utilized molecules that inhibit CME. Through *in vitro* work and mouse models, it's become appreciated that pairing the TKI gefitinib with endocytosis inhibitors decreases tumor cell survival (171, 172). Thus, a model has been formed that upon CME inhibition, EGFR undergoes macropinocytotic-dependent internalization, thereby promoting lysosomal degradation rather than receptor recycling (173). Depending on the molecule used to inhibit CME, mechanisms of internalization beyond macropinocytosis may also be utilized. This is the case with the small molecule DPBA, which mediates flotillin-dependent

internalization through lipid rafts rather than relying on post-translational modification of EGFR (174), thus allowing it to work for WT or TKI resistant tumors. However, CME is just one step of EGFR trafficking and several other steps exist before EGFR degradation that may also be beneficial to target for combination therapy.

After endocytosis, EGFR moves through the endocytic system where it may continue to signal until incorporation into a MVB for degradation. Studies have suggested that ability to sustain EGFR signaling from endosomes promotes apoptosis (175), however the cell lines utilized are known to undergo EGFR-mediated death. Additional studies have supported the notion by knockdown of Neuropilin-2 leading to entrapment of EGFR in early endosomes and subsequent cell death (176), though the definitive linkage between EGFR signaling and cell death remains unclear. Targeting this step of trafficking may prove to be the most controversial approach to TKI resistance due to the unknowns on whether EGFR actually signals from endosomes. Further questions arise when considering that most endocytic machinery have been historically considered tumor suppressors, and the sustained EGFR signaling thought to be advantageous for survival. Thus, advanced understanding of endosomal EGFR signaling and how it may impact tumor viability are necessary before this strategy is viable for combination therapy with TKIs.

Finally, nuclear translocation of EGFR is another mechanism of TKI resistance that may be a useful target for combination therapy. Like the strategies above for keeping EGFR at endosomes to modulate signaling, strategies at this step of trafficking aim to inhibit nuclear translocation and therefore keep EGFR at endosomes. Early studies in cell lines have shown that small molecules, such as Primaquine and 1,25-dihydroxyvitamin D, block nuclear translocation and may promote changes in EGFR signaling and cell death (177, 178). However, limitations of these studies are apparent in that both treatments may have unintended consequences for other cell processes that make it hard to discern EGFR-dependence. Additionally, combination of these therapies with TKIs has not yet been assessed, therefore highlighting additional understanding required before these approaches can be translated to patients. Though many steps in EGFR trafficking exist that can be utilized to overcome resistance to TKI therapy, research on combination of TKIs with endocytosis inhibitors is the most advanced but still has a way to go before advancing to the clinic. Thus, combination of EGFR trafficking and TKIs presents an understudied area that has potential opportunity to benefit cancer patients with TKI resistance.

## Concluding remarks

The EGFR family of growth factor receptors remains the most extensively studied receptor family due to its clear association with cancer development and progression. Indeed, overexpression of these receptors, mutation, evasion of degradation, enhanced

recycling, and/or altered signaling pathways of EGFR results in cancer development through enhancing downstream signaling. The generation of detailed structures of these receptors has provided important insight into the underlying molecular mechanisms contributing to receptor activation/dimerization and signal transduction. Moreover, they have provided atomic level detail on the mechanism of action of TKIs and monoclonal antibodies targeting the EGFR family of receptors. Many tyrosine kinase inhibitors have been developed as therapeutic agents for cancer. However, most EGFR-expressing tumors eventually become resistant to these inhibitors, thus requiring new treatment strategies. It is becoming clear that EGFR trafficking has implications in different types of cancer and that the development of resistance to TKIs is related to altered EGFR trafficking. Therefore, in addition to TKI therapy, EGFR/HER2 trafficking may be an additional target for cancer treatment. Findings in this area could increase efficacy and overcome resistance to TKI treatments that occur in the patient population.

## Author contributions

SJ: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing. DS: Writing – original draft, Writing – review & editing. DB: Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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