Signaling-inactive Epidermal Growth Factor Receptor/Ligand Complexes in Intact Carcinoma Cells by Quinazoline Tyrosine Kinase Inhibitors

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ABSTRACT

Several inhibitors of EGFR receptor (EGFR) tyrosine kinase activity have been developed that compete with ATP at its binding site such as the quinazolines PD 153035 and ZD 1839 or the 4,5-dianilino-phthalimides DAPH1 and DAPH2. When tested on human A431 cells, the quinazolines completely blocked EGF-induced receptor phosphorylation at 100 nM, whereas it was inhibited by DAPPH1 and DAPPH2 by only 20% at 3 μM. Quinazoline-treated A431 as well as tumor cells expressing less EGFR (A549, MDA MB 231, and T47D) bound 3- to 6-fold more 125I-labeled EGF than untreated intact control cells. Scatchard analysis revealed the disappearance of low- and high-affinity EGFR on A431 cells upon PD 153035 treatment. A single receptor class of intermediate ligand binding affinity emerged and its number corresponded to the sum of the two classes. DAPPH1 and DAPPH2 did not change ligand binding properties of EGFR. PD 153035 exerted the most potent effects on EGFR binding to A431 cells or in inhibiting EGF-stimulated growth of rat MTLn3 cells at low ligand concentrations. Cross-linking of EGFR on PD 153035-treated A431 cells indicated the formation of inactive dimers that further increased upon addition of EGFR. Chemical cross-linking of 125I-labeled EGF to PD 153035-treated A431 cells revealed increased binding to monomeric and dimeric EGFR. Thus, the quinazolines sequestered EGFR plus the ligand into inactive receptor/ligand complexes. This novel mode of action of quinazoline tyrosine kinase inhibitors may be the basis for their extraordinary potency especially in conditions when the ligand is present in limiting amounts.

INTRODUCTION

Analysis of clinical data indicates that expression of EGFR could play an important role in tumor development and progression. The EGFR is overexpressed in many tumors of epithelial origin, including glioblastoma and cancers of the lung, breast, head and neck, and bladder (1, 2). Patients with tumors overexpressing EGFR have a poor prognosis (3, 4).

The receptor can be stimulated upon autocrine or paracrine interaction with corresponding ligands such as EGF or transforming growth factor-α (5). The binding of ligand to EGFR induces receptor dimerization, followed by conformational changes activating the intrinsic tyrosine kinase, leading to receptor autophosphorylation and the phosphorylation of numerous cellular substrates. Several strategies have been developed to prevent EGFR activation, such as inhibition of ligand binding to EGFR (reviewed in Ref. 6) or blockade of the tyrosine kinase activity (reviewed in Refs. 7–11). Phosphotyrosine kinase inhibitors have been studied extensively in tissue culture systems of transformed cells and in animal models. It could be demonstrated that EGFR TKIs of different classes inhibited receptor phosphorylation and subsequent events, such as tumor cell adhesion and invasion and growth of tumor cells in tissue culture or in animals (reviewed in Refs. 8, 11), and even revert tumor cells to a phenotypically differentiated and nontransformed phenotype (12).

During the last years, extraordinary advances have been made in the area of EGFR TKIs. Most of these newer generation inhibitors are competitive with respect to ATP. Within this group of compounds, the quinazoline derivatives PD 153035 (13), ZD 1839 (14), and CP-358774 (15) showed outstanding potencies on the isolated receptor and in cellular assays. Recently, it was shown that the EGFR-specific quinazoline derivatives AG-1478 and AG-1517 not only compete with ATP in the classical mode of action but in addition induce the formation of inactive, unphosphorylated EGFR dimers, even in the absence of ligand (16). Receptor dimers are considered to have high affinity for the ligand. Therefore, in this study the effects of several reversible, ATP-competing TKIs were tested on ligand binding to the surface of intact tumor cells. Tyrosine kinase inhibitors of the quinazoline class (PD 153035 and ZD 1839) were compared with the 4,5-dianilinophthalimides DAPPH1 (17) and DAPPH2 (18) on a panel of human EGFR-positive carcinoma cell lines (A431, A549, MDA MB 231, and T47D). Furthermore, interference of PD 153035 with growth of rat mammary carcinoma MTLn3 cells was evaluated at escalating ligand and constant compound concentrations.

MATERIALS AND METHODS

Materials. 125I-Labeled EGF (100 μCi/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). EGF was supplied by Sigma Chemical Co. (Munich, Germany). The cross-linking agent BS3 was purchased from Pierce Chemical Co. (Heidelberg, Germany). The rabbit serum recognizing human EGFR (1005) was obtained from Santa Cruz Biotechnology. Generation and properties of mAb 14E1 interfering with EGF binding to human EGFR have been described previously (19). Precast SDS-PAGE gels (3–8%) were obtained from Invitrogen. The EGFR-specific quinazoline TKIs PD 153035 (13), ZD 1839 (14), the 4,5-dianilinophthalimides DAPPH1 (17) and DAPPH2 (18), and estradiol were synthesized accordingly, solubilized, and stored in DMSO.

Cell Lines and Culture Conditions. Human epidermoid A431, mammary MDA MB 231, lung A549 carcinoma, and rat mammary carcinoma cell clone MTLn3 (20) cells were cultured in a 1:1 mixture of DMEM and F12 (Biochrom, Berlin, Germany) supplemented with 10% FCS. Human mammary T47D cells were cultivated in RPMI 1640 without phenol red, supplemented with 10% FCS, 200 milliunits/ml insulin, and 0.1 nM estradiol.

EGFR-Phosphotyrosine ELISA. Tumor cells growing in 1% serum were incubated with the compounds for 15 min, then stimulated with 10 ng/ml EGF for 10 min and lysed, and the EGFR-phosphotyrosine content was determined by ELISA using mAb 14E1 as described (21).

Binding of 125I-Labeled EGF to Tumor Cells. Tumor cells were plated at 1 × 105 cells in 1 ml of medium with 1% FCS in 24-well plates and allowed to attach for 18–20 h. Cell monolayers were placed on ice and washed once with wash buffer (PBS containing 0.01% Mg2+ and 0.005% Ca2+ plus 1 mg/ml BSA), precooled to 4°C. Subsequently, cells were incubated in wash buffer with the TKIs, cold EGF or EGF-binding blocking antibody for 1 h on ice, followed by addition of 125I-labeled EGF (∼4 × 105 cpm) for 2 h at 4°C. For Scatchard analysis, increasing concentrations of unlabeled EGF (0.5–150 ng/ml) were added for 2 h at 4°C (22). After this time, monolayers were washed three times with wash buffer, lysed with 0.1 M NaOH, and counted in a counter. Nonspecific binding to untreated cells was determined by addition...
of either excess unlabeled EGF (250 ng/ml) or 500 ng/ml 14E1 antibody and was always <10% of total binding.

**Growth Studies.** MTLn3 cells were seeded in 100 μl of medium plus 0.5% of serum at 1500 cells/well in 96-well plates. Cells were allowed to adhere for 2 h, and then EGF plus or minus the TKI was added in 100 μl of medium. On day 3, cells were stained with crystal violet, and the absorbance was recorded (23). Values were normalized to the absorbance of untreated cells. Experiments have been performed at least twice and a representative experiment from six wells is shown.

**Cross-Linking Experiments.** For cross-linking of ligand to EGFR, A431 cells were plated at 1 × 10^5 cells in 2 ml of medium with 1% FCS in six-well plates and allowed to attach for 18–20 h. Cell monolayers were placed on ice and washed once with wash buffer (PBS containing 0.01% MgCl₂ and 0.005% CaCl₂). Cells were preincubated at 37°C for 15 min, and washed once with wash buffer. The TKIs or EGF-binding blocking antibody for 1 h, followed by addition of 125I-labeled EGF (~4 × 10⁶ cpm) for 2 h at 4°C. Cells were washed three times with ice-cold PBS plus MgCl₂ and CaCl₂, and incubated for an additional 15 min at 4°C in a 1 mM solution of BS3 in PBS. The reaction was stopped by adding 1 mL Tris to a final concentration of 50 mM and 60 mM at 4°C. After this, the cells were washed with PBS, scraped with a rubber policeman into 100 μl of solubilization buffer (150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 μM NaF, and 20 mM Tris-HCl, pH 7.6), and lysed end over end for 30 min at 4°C. Samples were centrifuged at 14,000 rpm for 15 min at 4°C. Lysate sample buffer was added to the supernatants, the samples were heated at 95°C for 5 min, and equal volumes were loaded onto the gel. In parallel aliquots, the radioactivity was measured in a gamma counter and found to correspond to the binding studies described above. Proteins were separated by SDS-PAGE on a conventional 4–15% gel, and labeled bands were visualized by autoradiography. For cross-linking of EGF, A431 cells were plated at 5 × 10³ cells in 2 ml of medium with 1% FCS in six-well plates and allowed to attach for 18–20 h. The TKIs were added for 30 min at 37°C, followed by EGF for 5 min. Subsequently, cells were washed three times with ice-cold PBS plus MgCl₂ and CaCl₂, and incubated for 60 min at 4°C in a 2 mM solution of BS3 in PBS. The reaction was stopped by adding 200 mM glycine in PBS to a final concentration of 100 mM and incubated for an additional 15 min at 4°C. After this, the cells were washed with PBS and 100 μl of 2× NuPAGE sample buffer plus 200 mM DTT added, the samples were heated at 70°C for 10 min, and equal volumes were loaded onto the gel. Proteins were separated by SDS-PAGE on a 3–8% NuPAGE gel and blotted onto nitrocellulose, and Western blot analysis was performed as described below.

**Western Blot Techniques.** Transfer of electrophoretically separated polypeptides onto nitrocellulose was carried out in 25 mM Tris-base, 192 mM glycine (pH 8.3) at 50 V for 24 h at 4°C. The transfer was blocked with 5% skimmed milk in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.9) for 2 h at room temperature and then washed with TBS plus 0.5% Tween 20 (TBS-T). The transfer was probed with rabbit anti-EGFR antibody 1005 (1:1000 dilution) for 1 h at room temperature, followed by a streptavidin-horseradish-peroxidase conjugate (1:3000 dilution in TBS-T) for 1 h at room temperature. After rinsing with TBS, labeled proteins were identified with the ECL detection system (Amersham Pharmacia) with exposure times between 3 and 10 s.

**RESULTS**

Arteaga et al. (16) had shown recently that in the absence of ligand EGFR dimerization is induced in A431 cells by the EGFR-specific quinazoline derivatives AG-1478 and AG-1517 with no alteration of ligand binding. We followed up on this study by comparing the effects of the extremely potent quinazoline derivatives PD 153035 (13) and ZD 1839 (14) with the two 4,5-bis-phthalimides DAPH1 and DAPH2 (17, 18) on ligand binding to tumor cells. To exclude cell type-specific effects only applicable to human epidermoid carcinoma A431 cells, other human EGFR-positive carcinoma cell lines were included, e.g., lung carcinoma A549 and mammary breast carcinomas MDA MB 231 and T47D, which vary in their levels of EGFR expression (24).

**Increased EGF Binding to Quinazoline-treated A431 Cells.** The potential of all four compounds in an EGFR-phosphotyrosine ELISA using EGFR-high expressing A431 cells was established first. Fig. 1 demonstrates a dramatic increase (18-fold) in the chemiluminescence signal upon addition of 10 ng/ml EGF within 10 min, which decreased dose dependently by the quinazoline derivatives PD 153035 and ZD 1839 with IC₅₀ values between 10 and 30 nM, respectively. PD 153035 was more potent than ZD 1839. In contrast, DAPH1 and DAPH2 showed only marginal effects at the highest concentration tested, with 20–30% inhibition at 3 μM.

![Fig. 1. Increase in chemiluminescence in A431 cells pretreated with PD 153035 or ZD 1839](image-url)

**Inhibition of EGFR Tyrosine Kinase in A431 Cells.** The potencies of all four compounds in an EGFR-phosphotyrosine ELISA using EGFR-high expressing A431 cells were established first. Fig. 1 demonstrates a dramatic increase (18-fold) in the chemiluminescence signal upon addition of 10 ng/ml EGF within 10 min, which decreased dose dependently by the quinazoline derivatives PD 153035 and ZD 1839 with IC₅₀ values between 10 and 30 nM, respectively. PD 153035 was more potent than ZD 1839. In contrast, DAPH1 and DAPH2 showed only marginal effects at the highest concentration tested, with 20–30% inhibition at 3 μM.

![Fig. 2. Binding of 125I-labeled EGF to intact A431 cells pretreated for 1 h with the compounds](image-url)
subsequently the 125I-labeled EGF binding was determined over a range of 0.25–150 ng/ml EGF by incubation for an additional 1 h at 4°C, and analysis of 125I-labeled EGF binding to untreated A431 cells resulted in a best fit according to the two-affinity model with two classes of binding sites (25) with 10–20% of sites in the high-affinity state (K_D (low)) and ligand dissociation constants (K_D) (Table 1). The binding experiments were also performed with DAPH1 and DAPH2 (range, 0.03–3 μM) and 80–90% of sites in the low-affinity state (K_D (high)).

Concentrations of PD 153035. This suggests conformational changes of EGFR attributable to the compound such that 14E1 affects ligand binding less efficiently. When the 14E1 concentration was raised to 5 μg/ml, binding of 125I-labeled EGF to PD 153035-treated cells was prevented completely (data not shown). The binding experiments were also performed with DAPH1 and DAPH2 (range, 0.03–3 μM) with absolutely no change in binding of radiolabeled ligand to A431 cells (data not shown).

Scatchard Analysis in the Presence of Quinazoline. Because the quinazolines affected binding characteristics of 125I-labeled EGF, A431 cells were subjected to Scatchard analysis in the absence and presence of 100 or 200 nM PD 153035 (Fig. 3 and Table 1). Scatchard analysis of 125I-labeled EGF binding to untreated A431 cells resulted in a best fit according to the two-affinity model with two classes of binding sites (25) with 10–20% of sites in the high-affinity state (K_D (low)) and 80–90% of sites in the low-affinity state (K_D (high)). In contrast, on PD 153035-treated A431 cells, only one class of EGFR was exhibited with intermediate binding affinities (0.13–0.26 nM) and the number representing the sum of the two classes displayed on untreated cells. Table 1 summarizes the data from three independent experiments. PD 153035 was most efficient in increasing 125I-labeled EGF binding to A431 cells when the concentration of free EGF was low (<2 nM), and the compound was without effect on EGF binding characteristics at higher ligand concentrations.

Growth Studies in the Presence of PD 153035. To further explore the phenomenon that PD 153035 exerts fewer effects on binding at high ligand concentrations, proliferation assays were performed at increasing ligand and constant PD 153035 concentrations. The human tumor cell lines used in this study are either growth inhibited by EGF (A431 and A549) or demonstrate very marginal responses to EGF in tissue culture (MDA MB 231 and T47D; Ref. 24). Thus, we used the rat mammary carcinoma MTLn3 clone, which is growth stimulated by EGF (26) and responds in adhesion assays very sensitively to PD 153035 (27). Fig. 4 shows that MTLn3 cells were dose dependently growth stimulated by EGF, reaching maximal levels (3-fold) at 0.1 nM EGF. Addition of PD 153035 reduced growth of cells already in the absence of exogenous ligand, which is in line with the finding that MTLn3 cells produce transforming growth factor-α (28). When given in combination with EGF, PD 153035 exerted the best inhibition of EGF-stimulated growth at low (0.01–0.3 nM) and the least potent at high (1–30 nM) ligand concentrations.

Cross-Linking of EGFR or Labeled Ligand to EGFR on A431 Cells Treated with TKIs. Arteaga et al. (16) had shown increased formation of EGFR dimers in the absence of ligand after treatment of cells with the quinazoline derivatives AG-1478 and AG-1517. We tested whether this applies also to the quinazoline PD 153035 in combination with DAPH1. Fig. 5A demonstrates dose-dependent EGFR dimer formation by 1–10 μM of PD 153035 but not of DAPH1 with no tyrosine phosphorylation of monomeric or dimeric EGFR (data not shown). Induction of dimers by 100 ng/ml EGF or 200 nM PD 153035, respectively, was barely visible, whereas concomitant administration resulted in pronounced formation of EGFR dimers (Fig. 5B). Having established a similar mechanism for PD 153035, we tested the effects of the four compounds on ligand binding to monomeric versus dimeric EGFR. Fig. 5C demonstrates that in control cells, most of the 125I-labeled EGF was bound to the monomeric receptor with a molecular mass of 175 kDa, whereas a faint staining in the higher molecular weight range indicated binding to receptor dimers (360 kDa). Addition of cold EGF in excess (250 ng/ml) or ligand blocking antibody 14E1 (500 ng/ml), respectively, efficiently reduced 125I-labeled EGF binding. Both quinazoline derivatives dramatically increased binding of 125I-labeled EGF to monomeric or dimeric EGFR 2–3-fold, respectively, as was shown by densitometry (data not shown), and this could be reduced by 14E1. In contrast, DAPH1 and DAPH2 did not affect binding of the ligand to either receptor class (shown in three independent experiments), whereas 14E1 again interfered with the labeling. These experiments confirmed that the quinazolines increase ligand binding to the receptor concomitantly with inducing the formation of inactive EGFR dimers.

Table 1: Scatchard analysis of 125I-labeled EGF binding to intact A431 cells in the presence of PD 153035

<table>
<thead>
<tr>
<th>Experiment</th>
<th>100 nM</th>
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* Binding parameters were determined from Scatchard plots to obtain receptor number (a) and ligand dissociation constants (K_D) 125I-labeled EGF binding was determined at 4°C for 1 h as described in “Materials and Methods.”

Fig. 3. Scatchard analysis of 125I-labeled EGF binding to control and PD 153035-pretreated A431 cells. Cells were incubated with 100 nM PD 153035 for 1 h at 4°C, and subsequently the 125I-labeled EGF binding was determined over a range of 0.25–150 ng/ml EGF by incubation for an additional 1 h at 4°C. Inset, amount of bound versus free ligand.

Fig. 4. Growth of MTLn3 cells in the presence of EGF and PD 153035. MTLn3 cells were seeded at 1500 per 96-well plate. After 2 h, EGF was added at increasing concentrations plus or minus 100 nM PD 153035. After 3 days, cells were fixed and stained with crystal violet. Bars, SD.
Tumor Cell Lines of Different Cellular Origins and Differential EGFR Expression. A431 cells are derived from a human vulva carcinoma and express an extremely high number of EGFR. To exclude receptor number as well as cell type-specific effects, tumor cells of different origins and different levels of EGFR expression (A549, MDA MB 231, and T47D) were included in the EGFR-phosphotyrosine ELISA (Fig. 6) and binding studies (Fig. 7). EGF addition resulted in a chemiluminescence signal with a 14-fold (A549), 6-fold (MDA MB 231), or 7-fold (T47D) increase compared with untreated cells (Fig. 6). Again, this increase was dose dependently inhibited by PD 153035 with maximal inhibition at 300 nM. Cells differed in their sensitivity for PD 153035, with A549 being the most sensitive cell line and MDA MB 231 the least sensitive cell line. A 4–6-fold increase was demonstrated in 125I-labeled EGF-binding on A549, MDA MB 231, and T47D cells with a maximum at 300 nM PD 153035 (Fig. 7). Radiolabeled ligand binding was efficiently prevented by EGF (250 ng/ml) or 14E1 (500 ng/ml) on cells treated with 1000 nM of the compound.

DISCUSSION

The synthesis of small molecular inhibitors of receptor tyrosine kinases was initially based on the nucleus of tyrosine itself. These classical TKIs are represented by the tyrphostins, which are potent inhibitors of tyrosine kinase activity of EGFR and cell proliferation in a variety of target cells in vitro and in vivo but showed no effects in our system (data not shown).

Recently, new classes of compounds interfering with EGFR tyrosine kinase activity by competing reversibly with ATP have been reported, such as 4,5-bis(anilino)-phthalamide derivatives (17, 18) and three 4-anilino-quinazoline derivatives (2-thioindole dimers) PD 153035 (13), ZD 1839 (14), and CP-358744 (15). The first report of a potent and selective quinazoline EGFR TKI was by Fry et al. (13). PD 153035 exhibited an IC50 of 29 pM for the inhibition of EGFR and blocked the autophosphorylation of EGFR at nM concentrations in a variety of cell types. However, its poor water solubility complicated the in vivo evaluation in tumor models (29). Thus, it did not enter clinical trials for the tumor indication.

The quinazolines ZD 1839 and CP-358744 exhibiting equal in vitro...
potencies were considered to have sufficient in vivo antitumor efficacy and desirable pharmacokinetic properties to be selected for clinical trials. ZD 1839 was investigated in a Phase I clinical trial and showed an oral pharmacokinetic and safety profile in humans compatible with its development for the treatment of a range of human tumors (11). CP-358744 also exhibited good efficacy in xenografts after oral administration to the animals (30). Both compounds are currently in Phase II clinical trials.

In this study, the effects of the two quinazoline derivatives PD 153035 and ZD 1839 are compared with the two 4,5-bis-anilino-phthalimides DAPH1 and DAPH2. PD 153035 and ZD 1839 were very potent at 10–300 nM in completely blocking EGF-induced receptor phosphorylation, whereas DAPH1 and DAPH2 inhibited EGF-induced receptor phosphorylation at the maximal concentration used (3 μM) by only 20%. Both compounds were completely ineffective in the other assays used.

Surprisingly, binding of 125I-labeled EGF to cells indicated a 2–3-fold increased ligand binding to quinazoline-treated intact A431 cells, whereas DAPH1 and DAPH2 showed no effect. This increased ligand binding was independent of receptor numbers or the cell type-specific origin of the tumor cells. Increased ligand binding was most pronounced at low EGF concentrations, and consequently the potency of PD 153035 was markedly reduced in cell proliferation assays on addition of excess EGF. Cross-linking of EGFR or of 125I-labeled EGF to EGFR on drug-treated A431 cells showed dimer formation in the absence of ligand, increase by ligand, and binding of radiolabeled ligand to monomeric and dimeric EGFR. Concomitantly, only one single class of EGFR was identified by Scatchard analysis. Both classes of EGFR with low- and high-affinity for the ligand had disappeared after treatment with PD 153035, and a single receptor class of intermediate ligand binding affinity emerged. The total receptor number remained constant and was the sum of the two classes. These results extend the data of Arteaga et al. (16) to other quinazoline derivatives, but they show in contrast that ligand binding to quinazoline-treated cells was increased. This might be attributable to different efficiencies of the compounds. However, it is more likely that ligand concentrations used by Arteaga et al. (16) were too high to see further enhancement by the quinazolines.

Similar however not identical changes of EGFR ligand binding characteristics upon exposure of cells to PD 153035 were reported by Nelson and Fry (12). In this study, A431 cells adapted to a prolonged suppression of EGFR tyrosine kinase were generated from parental A431 cells by prolonged growth in the presence of escalating concentrations of PD 153035 until 1 μM had been reached. These cells showed a dramatic change in cell morphology and growth characteristics. Although the parent cells continued to grow when confluency was reached, the resistant subline stopped growing and seemed to exhibit contact inhibition similar to nontransformed cells. There was no defect in EGFR kinase activity such as receptor autophosphorylation or receptor internalization in the resistant A431 subline. The most prominent change was the selective disappearance of the high-affinity EGFR class with equivalent receptor numbers compared with untreated cells. This seems to be in contrast to our study, because high- as well as low-affinity EGFR disappeared and a single receptor class of intermediate ligand binding affinity emerged. However, the different time periods of drug exposure (several days versus 3 h in this study) have to be considered.

Interestingly, in the PD 153035-resistant cells, profound changes in the actin filament system were observed with fewer EGFRs associated with the cytoskeleton (12). This is compatible with previous reports indicating that EGFR associates with the cytoskeleton, possibly via actin (31). This EGFR subpopulation represents mainly the high-affinity type (32) and can be linked to morphological changes (33). In our previous studies, PD 153035 very efficiently blocked the modulating effects of EGF on adhesion of rat (MTLn3) and human (A431, MDA MB 231, and MDA MB 468) tumor cells to extracellular matrix proteins at concentrations between 15 and 75 nM (27). We hypothesized that in the tumor cell adhesion assay, the TKI had efficiently blocked function of the high-affinity EGFR. The present study confirms our previous hypothesis of inhibition of high-affinity EGFR, because this receptor class disappeared in quinazoline-treated cells.

In summary, our data indicate that the investigated quinazolines not only cause the sequestration of EGFR into inactive dimers (16) but also trap the ligand into these complexes, thus reducing available levels of ligand. This novel mode of action of quinazoline TKIs is depicted in Fig. 8 and may contribute to their extraordinary potency in cellular assays and tumor xenografts where the ligand is present in limiting amounts. It remains to be determined whether this mode of action is restricted to EGFR or may also apply to other ligand-stimulated growth factor receptor tyrosine kinases.

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