Unliganded Epidermal Growth Factor Receptor Dimerization Induced by Direct Interaction of Quinazolines with the ATP Binding Site*

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Receptor dimerization is critical for signaling by the epidermal growth factor receptor (EGFR) tyrosine kinase. This occurs after binding of the receptor's extracellular domain by ligand or bivalent antibodies. The role of other receptor domains in dimerization is less clear, and there are no examples of dimers induced by direct perturbation of the EGFR kinase domain. Submicromolar concentrations of AG-1478 and AG-1517, quinazolines specific for inhibition of the EGFR kinase, induced reversible receptor dimerization in vitro and in intact A431 cells. Consistent with the inhibitory effect of quinazolines on receptor kinase activity, the dimers formed lacked a detectable Tyr(P) signal. Quinazoline-inhibited EGFR dimerization was abrogated in vitro by ATP and the ATP analog adenyl-5'-yl imidodiphosphate. Receptors with a single-point mutation in the ATP binding site as well as wild-type EGFR with a covalent modification of the ATP site failed to dimerize in response to AG-1478 and AG-1517. These data suggest that EGFR dimerization can be induced by the interaction of quinazolines at the ATP site in the absence of receptor ligand binding. In SKBR-3 cells, the quinazolines induced the formation of inactive EGFR/ErbB-2 heterodimers, potentially sequestering ErbB-2 from interacting with other coreceptors of the ErbB family. Structural studies of the quinazoline interaction with the EGFR tyrosine kinase domain should allow for an analysis of receptor-specific chemical features required for binding to the ATP site and disruption of signaling, a strategy that can be perhaps applied to other tumor cell receptor systems.

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular (EC) ligand-binding domain, a single transmembrane (TM) region, and an intracellular (IC) tyrosine kinase and regulatory domain (Ref. 1 and references therein). Ligand binding to the EC domain results in receptor dimerization and stimulation of the intrinsic tyrosine kinase, leading to receptor autophosphorylation and the phosphorylation of numerous cellular substrates (2–4). The EGFR interaction with a variety of signal transduction proteins ultimately transmits growth and differentiation signals (2–4). One of the earliest events leading to receptor activation is ligand-induced conformational changes in the EC domain followed by receptor dimerization (5–7). The EGFR domains mediating protein-protein interactions critical for dimerization are still under investigation. Studies using chemical crosslinkers to trap dimer formation demonstrate that soluble EGFR EC domains undergo ligand-mediated dimerization (8–10), suggesting that the IC domain may not be essential for EGFR oligomerization. However, expressed EGFR IC domains are capable of undergoing autophosphorylation as well as associating with full-length receptors and phosphorylating kinase-negative receptors (11, 12), suggesting a more than passive role of the IC domain in dimerization.

The requirement for the EGFR tyrosine kinase activity in cellular signaling is based upon observations that receptors in which Lys-721 within the ATP binding site has been mutated and, hence, lack detectable kinase activity, do not display the full range of biochemical responses (13–15). This apparent requirement for kinase activity has focused attention on the development of drugs capable of blocking kinase activity specifically. Quinazoline inhibitors of the EGFR kinase are competitive with ATP; in the 1–10 nm range, they block EGFR phosphorylation and Src kinase activity in vivo but do not inhibit the platelet-derived growth factor receptor, p210Bcr-Abl, insulin receptor, CSF-1 receptor, and bFGF receptor tyrosine kinases (16–19). In studying the ability of the EGFR kinase quinazoline inhibitors AG-1478 and AG-1517 to block TGFα-induced signaling, their effect on receptor dimerization was measured. These studies demonstrated that quinazoline inhibitors per se induce inactive EGFR homodimers in EGFR-overexpressing cells or EGFR/ErbB-2 heterodimers in cells overexpressing ErbB-2 and containing lower levels of EGFR. The ability of the quinazolines to inhibit kinase function by sequestering receptors into inactive dimers appears related to their interaction with the receptor ATP binding site. These data suggest a novel biochemical mechanism of (inactive) receptor dimerization in which the initial monomer interactions are

[Note: The rest of the text contains detailed scientific findings and discussion related to the interaction of quinazolines with the EGFR tyrosine kinase, including binding site analysis, dimerization mechanisms, and implications for drug development.]
mediated via the EGFR IC domain and point to the variety of mechanisms by which the activities of ErbB receptor kinases can be inhibited by such compounds.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The A431 human squamous carcinoma and SKBR-3 human breast cancer cell lines were purchased from the American Type Culture Collection (Rockland, MD). A431 cells were maintained in IMEM (Life Technologies, Inc.) supplemented with 10% FCS (JRH Biosciences, Lenexa, KS), while the SKBR-3 line was passaged in McCoy 5A medium (Life Technologies, Inc.) supplemented with 15% FCS. Chinese hamster ovary cells expressing either wild-type EGFR or one of two kinase-negative receptors; D813A, in which Asp-813, the catalytic base in phosphoryl transfer, is mutated to Ala, and K721R, in which Lys-721 within the ATP binding site is mutated to Arg, have been described previously (20, 21). These cell lines were maintained in minimum essential medium α medium (Life Technologies, Inc.) supplemented with 10% dialyzed FCS (Sigma). Genistein, ATP, and AMP-PNP were purchased from Sigma. AG-1478 and AG-1517 are quinazolines that display an IC50 for inhibition of EGFR kinase activity in vitro of 3 nM and 0.9 nM, respectively (18, 19). AG-1296 is a quinazoline inhibitor of the platelet-derived growth factor receptor kinase (16). SU-4231 is a tyrosphan inhibitor of the HER2/ErbB-2 kinase which exhibits an IC50 of 50 nM in vitro.2 Recombinant heregulin-β was kindly provided by Dr. Mark Sliwkowski (Genentech, Inc., South San Francisco, CA).

**EGFR and Tyr(P) Precipitation and Immunoblot Procedures**—Subconfluent A431 cells in IMEM, 10% FCS were treated with TGF-α at 37 °C, washed twice with ice-cold phosphate-buffered saline (PBS) on ice, and solubilized in TGP buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.4, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). After removing Triton-insoluble material by centrifugation at 14,000 × g for 10 min at 4 °C, lysates were precipitated at 4 °C using the 986 polyclonal EGFR antiserum (Ref. 23; kindly provided by Dr. John Mendolsohn, M. D. Anderson Cancer Center, Houston, TX) followed by protein A-Sepharose CL-4B (Sigma). For each washes with TGP buffer, the EGFR immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblot analyses of EGFR or Tyr(P) employed an anti-EGFR monoclonal IgG raised against a cytoplasmic receptor sequence (Zymed, South San Francisco, CA) or an anti-Tyr(P) IgG2 (Upstate Biotechnology, Inc, Lake Placid, NY), respectively. Bound antibodies were detected with horseradish peroxidase-linked anti-mouse Ig followed by ECL (Amersham Corp.).

**Chemical Cross-linking of EGFR in Intact Cells and Soluble EGFR**—Chemical cross-linking in intact cells was performed as described by Sorkin and Carpenter (25). After treatment with ligand or kinase inhibitors, cell monolayers were washed twice with ice-cold PBS on ice and solubilized for 20 min at 4 °C with TGP buffer containing freshly prepared 1 mM bis(sulfosuccinimidyl) suberate (BS3, Pierce) (26). To terminate the cross-linking reaction, a final concentration of 250 mM for an additional 5 min. Samples were then subjected to 5–7% gradient SDS-PAGE and EGFR immunoblot analysis.

**Modification of A431 Membrane Vesicles by FSBA—**A431 cell membrane vesicles were prepared by published procedures (27). To cross-link reactions were terminated by the addition of 2.5 mM glycerol to a final concentration of 250 mM for an additional 5 min. Samples were then subjected to 5–7% gradient SDS-PAGE and EGFR immunoblot analysis.

**In Vitro Kinase Assay of the EGFR IC Domain**—Aliquots of the recombinant TKD61 (provided by John Koland, University of Iowa), which corresponds to the entire cytoplasmic portion of the EGFR (residues 646–1186) in 20 mM Hepes, pH 7.4, 10 mM MnCl2, 0.1% Triton X-100 were incubated for 5 min at room temperature in the presence or absence of 10–50 μM ATP (Sigma) as described previously (28). Phosphorylation reactions were quenched by the addition of SDS-PAGE sample buffer and boiling for 5 min. Equal volumes from each sample were then resolved by 5–10% gradient SDS-PAGE and subjected to EGFR and Tyr(P) immunoblot procedures.

**Anchorage-independent Growth Assay—**For testing the growth effects of quinazolines, a 1-ml top layer containing a single-cell suspension of 3 × 104 cells, 0.8% agarose (Sea-Plaque, FMC BioProducts, Rockland, ME), IMEM, 10% FCS, and 10 mM Hepes, with or without different concentrations of inhibitors, was added to a 1-ml bottom layer of 0.8% agarose, 10% FCS in triplicate 35-mm dishes. In some cases, haptotaxis was added the following day in a 100-μl volume to the top layer. Dishes were incubated in a humidified 5% CO2 incubator at 37 °C, and colonies measuring ≥50 μm were counted after 7 days using an Omnicom Stem model II image analyzer (Bausch & Lomb, Rochester, NY).

**RESULTS**

**Blockade of EGFR Phosphorylation**—We initially studied the inhibitory potency of AG-1478 on basal and ligand-induced EGFR phosphorylation in intact A431 cells. A 30-min preincubation with AG-1478 inhibited the basal and TGF-α-stimulated tyrosine phosphorylation of the receptor without a detectable change in immunoprecipitable EGFR (Fig. 1). Similar results were obtained with AG-1517 (not shown). These compounds reversibly inhibit the growth of cultured EGFR-overexpressing human tumor cells in the submicromolar range without altering EGF binding (Refs. 16 and 19 and data below).
Quinazoline-mediated Dimerization of Wild-type and Mutant EGFR in Intact Cells—The effect of various inhibitors on EGFR dimerization in vivo was assessed by chemical cross-linking. TGF-α, AG-1478, and AG-1517 induced EGFR homodimers in A431 cells, whereas AG-1296, a quinoxaline inhibitor of the platelet-derived growth factor receptor, SU-4231, a quinazoline inhibitor of the HER2/ErbB-2 kinase, or genistein, a nonspecific competitive ATP inhibitor, had no effect on dimer formation (Fig. 2A). These cross-linking conditions would be expected to trap both internalized and cell surface EGFR dimers, since BS3 was added to cells simultaneously with detergent (25). Antiphosphotyrosine immunoblot analysis indicated that TGF-α induced the formation of active dimers as evidenced by the high levels of phosphorylation in the dimer band, whereas receptors treated with AG-1478 or AG-1517 exhibited no Tyr(P) signal (Fig. 2B), confirming that AG-1478 and AG-1517 specifically inhibit kinase activity. Genistein at 100 μg/ml blocked phosphorylation of the EGFR. EGFR dimerization by AG-1478 was dose-dependent and detectable at concentrations as low as 100 nM, occurred as early as 5 min after quinazoline treatment, and was reversible upon removal of the kinase inhibitor (not shown). To distinguish internalized from cell surface EGFR dimers and confirm that the quinazolines were inducing receptor dimerization in intact cells, we performed experiments in which BS3 was added in the absence of detergent to A431 cells pretreated with TGF-α, AG-1478, or AG-1517. Although at a lower level than when the cross-linking was performed in TGP buffer, EGFR dimers were induced by TGF-α and both kinase inhibitors in intact A431 cells (Fig. 2C). The increased amount of dimers detected after solubilization in BS3-containing TGP buffer could be due to availability of the EGFR cytoplasmic domain to the cross-linking agent in detergent-permeabilized/solubilized cell lysates.

We next studied the biochemical mechanisms of quinazoline-induced dimerization using Chinese hamster ovary cells expressing either wild-type EGFR or one of two kinase-negative receptors: D813A, in which Asp-813, the catalytic base in phosphoryl transfer, is mutated to Ala (20), or K721R, in which Lys-721 within the ATP binding site is mutated to Arg (21). Like TGF-α, which induced dimers of wild type and each EGFR mutant in intact cells, the treatment of cells expressing wild-type receptor or D813A with AG-1478 and AG-1517 also resulted in receptor dimerization. However, no detectable dimers were observed upon treatment of cells expressing K721R (Fig. 3). These results suggest that the direct interaction of quinazolines with residues within the ATP binding site, including Lys-721, is critical for the observed dimerization. Alternatively, the binding of the quinazolines at another site within the kinase domain allosterically induces a conformational change of the ATP binding site that precludes kinase activation but facilitates dimerization.

Effect of AMP-PNP and Modification of the ATP Site on Quinazoline-mediated EGFR Dimerization—To test whether a quinazoline-ATP site interaction is necessary for dimer for-
mation, we examined the competitive effect of the slowly hydrolyzable ATP analog, AMP-PNP, on EGFR dimerization induced by AG-1478. Initial experiments showed that like TGF-

α, AG-1478 and AG-1517 can dimerize Triton-solubilized EGFR at room temperature or at 37 °C, indicating that results in vitro paralleled those obtained with intact cells (data not shown). To facilitate binding of AMP-PNP to the ATP site, the divalent cations Mg2+ and Mn2+ were added to soluble EGFR preparations prior to quinazoline treatment. As shown in Fig. 4A, preincubation of soluble EGFR with 1 mM AMP-PNP drastically reduced EGFR dimerization induced by 0.1–10 μM AG-1478. Similar results were obtained using 200 μM ATP (data not shown). Further mechanistic evidence linking quinazoline binding to the ATP site and EGFR dimerization was obtained using A431 membrane vesicles modified with the ATP analog FSBA, which covalently reacts with Lys-721 within the ATP binding site of the EGFR, thus inhibiting kinase activity (28, 31, 32). Minimal dimerization of FSBA-modified EGFR occurred in the presence of 1–10 μM AG-1478, whereas AG-1478-mediated dimerization of unmodified EGFR was prominent at concentrations ≥0.1 μM (Fig. 4B). Under the conditions utilized, FSBA treatment results in the modification of approximately 80% of the EGFR present as assessed by inhibition of receptor kinase activity (28). Therefore, the residual dimeric signal probably corresponds to AG-1478-mediated dimerization of the remaining unmodified EGFR.

A number of tyrosine kinase inhibitors in the tyrphostin class display competition with both ATP and the protein substrate (16); however, the ability of the quinazolines to compete with an EGFR protein substrate is unknown. To investigate whether a peptide substrate would impair quinazoline-induced EGFR dimer formation, we used a previously characterized high affinity tyrosine-containing substrate (Glu-Glu-Leu-Glu-Asp-Asp-Tyr-Glu-Asp-Asp-Nle-Glu-Glu; tyrsub), which exhibits a K_m(app) of 32 μM for phosphorylation by the EGFR (33). Preincubation with 200 μM peptide substrate did not alter EGFR dimerization, nor did it enhance the inhibition of AG-1478-mediated dimerization by AMP-PNP (Fig. 5), suggesting that EGFR dimerization does not result from quinazoline interaction with the peptide substrate binding site but rather results predominantly from their effect on altering the conformation of the ATP binding site, either directly or allosterically.

Studies with EGFR IC Domain—We examined whether quinazoline-mediated EGFR dimerization required the EC and TM receptor domains by utilizing recombinant TKD61 protein, which corresponds to the entire cytoplasmic portion of the EGFR (29). As shown in Fig. 6, soluble holoreceptors from A431 cells but not TKD61 form dimers in the presence of AG-1478, suggesting that the EC and/or TM domains are required for stabilization of homodimer formation by the quinazoline. The functional integrity of the ATP site was tested by an in vitro kinase reaction in the presence of divalent cations (12, 29). In the presence of 10 mM MnCl2 and 10–50 μM ATP, TKD61 underwent phosphorylation on tyrosine and exhibited slower
migration on SDS-PAGE as determined by immunoblot analysis (data not shown).

Quinazoline-mediated EGFR/ErbB-2 Heterodimerization and Growth Effects—The effect of quinazolines on dimerization of the homologous receptor ErbB-2 (HER2/neu) was examined in SKBR-3 human breast cancer cells. These cells exhibit gene amplification for ErbB-2 (34), approximately 10^5 EGF binding sites/cell (35, 36), and constitutive tyrosine phosphorylation and association of both ErbB-2 and ErbB-3 (37). TGF-α treatment had little effect on the appearance of ErbB-2 on the region corresponding to dimeric receptors. The Tyr(P) signal in the dimer band resulting from TGF-α stimulation probably corresponds to cross-linked EGF or EGF/ErbB-2 heterodimers. However, AG-1517 effectively induced ErbB-2 dimers in SKBR-3 cells; these as well as the ErbB-2 monomers were not detected by Tyr(P) immunoblotting (Fig. 7, left and middle panels). These results suggest that in the presence of AG-1517, the elevated basal activity of the ErbB-2 kinase is inhibited, leading to a reduction in Tyr(P) content perhaps due to the action of cellular phosphatases. To investigate the composition of the dimers induced by TGF-α versus AG-1517, cross-linked lysates were precipitated using either anti-EGFR or anti-ErbB-2 monoclonal antibodies that recognize each respective receptor's EC domain. ErbB-2 immunoblot analysis revealed that the dimers induced by AG-1517 are a combination of EGFR/ErbB-2 heterodimers and ErbB-2 homodimers (Fig. 7, right panel). Similar results were obtained with 10 μM AG-1478. Of note, the Tyr(P) dimeric band enhanced by TGF-α treatment (Fig. 7, middle panel) was not precipitated at a detectable level by anti-EGFR antibodies (Fig. 7, right panel) suggesting that, given the relative amounts of each receptor present in SKBR-3 cells (ErbB-2[wt]/EGFR), TGF-α may not be as effective as EGF in inducing EGFR/ErbB-2 heterodimerization (38, 39) and/or that quinazolines are more effective in mediating stable association, albeit inactive, of the co-receptor tyrosine kinases.

We next examined the effect of incubation with quinazolines on anchorage-independent growth of A431 and SKBR-3 cells. These cell lines exhibit an EGFR- and ErbB-2-dependent phenotype in culture, respectively, as supported by studies with specific antibodies that block each respective receptor's EC domain and markedly inhibit tumor cell proliferation (40, 41). Both lines were growth-inhibited by AG-1517 in a dose-dependent manner (Fig. 8A). The IC50 for growth in A431 cells was <0.1 μM, consistent with the concentration of quinazoline required for inhibition of basal EGFR tyrosine phosphorylation (Fig. 1) and induction of inactive receptor homodimerization in vivo (Fig. 4) as well as with the published (nanomolar) concentrations required for inhibition of the EGFR kinase in vitro (16, 18). On the other hand, the IC50 for growth in the ErbB-2-overexpressing SKBR-3 cells was ≥1 μM (Fig. 8). This concentration corresponded to those required to block ErbB-2 phosphorylation and inducing inactive ErbB-2 dimerization in vivo (Fig. 7); however, it was markedly lower than the published quinazoline IC50 (>100 μM) for the ErbB-2 kinase in vitro (16). Finally, we tested the effect of AG-1517 on heregulin action in SKBR-3 cells. This ligand can induce ErbB-2 phosphorylation and cell proliferation via binding to ErbB-3 (42). As shown in Fig. 8B, AG-1517 blocked heregulin-β-stimulated colony growth. Examination of tyrosine phosphorylation of ErbB-2 in response to exogenous heregulin-β treatment of SKBR-3 cells was also inhibited by AG-1517.3 These results suggest that the quinazolines can alter growth factor responses by the formation of inactive ErbB-2 homodimers and/or heterodimers with other ErbB family members.

DISCUSSION

Previous studies have demonstrated that ligand- or bivalent antibody-mediated perturbation of the EGFR EC domain either in intact cells or in vitro results in receptor dimerization. Our studies suggest that the interaction of quinazoline inhibitors with the receptor’s kinase domain can also lead to receptor dimerization. This quinazoline-mediated EGFR dimerization is

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related to the inhibitors’ effects on the ATP binding site as indicated by 1) the absence of dimers in AG-1478-treated Chinese hamster ovary cells expressing a kinase-negative receptor mutated at the ATP site (K721R) versus another kinase-negative receptor mutated at the catalytic base (D813A); 2) the inhibition of quinazoline-induced EGFR dimer formation by AMP-PNP and ATP; 3) the inhibition by FSBA of AG-1478-induced dimerization of EGFR from A431 membrane vesicles. In addition, the lack of inhibition of homodimer formation by a high affinity peptide substrate for the EGFR suggests that quinazoline effects on the ATP site rather than on the substrate binding site mediate EGFR oligomerization. Taken together with previously published experiments in which quinazolines show competitive kinetics with respect to ATP (16, 18), these results provide support for a direct interaction of quinazolines within the ATP binding site rather than at an allosteric site.

Aside from inhibiting kinase activation by directly competing with ATP, quinazoline binding to the ATP pocket in the EGFR appears to perturb the three-dimensional structure of the receptor and hence stabilize interactions involved in receptor dimerization. The ability to induce dimerization was somewhat surprising, given that under physiological conditions of high ATP concentrations in intact cells or in the presence of 1 mM AMP-PNP (Figs. 4A and 5) or 200 μM ATP in vitro, little or no dimerization is observed. These results suggest that the affinity of the receptor for ATP is insufficient for ATP binding to stably induce and/or transduce the desired conformational changes required for dimerization, whereas the higher affinity interactions of quinazoline inhibitors with the ATP site are of adequate stability to induce a conformational change leading to dimerization. Our results further indicate that the presence of the EC and/or TM domains is required for the ability of quinazolines to cause EGFR dimerization by an induced fit mechanism, since the treatment of the TKD61 cytoplasmic EGFR domain with AG-1478 did not result in its dimerization. These results support previous studies suggesting that multiple receptor domains play a role in EGFR dimerization.

In terms of involvement of the EC domain in dimerization, the v-ErbB oncoprotein, a naturally occurring altered form of the EGFR that lacks the majority of the EC domain, did not display oligomerization as assayed by nondenaturing gel electrophoresis (43). In addition, EGFR can induce the dimerization of a soluble EGFR EC domain (8). Sorokin et al. (44) reported that insertion of an extra cysteine in the EC juxtamembrane region of the EGFR led to the formation, albeit ligand-induced, of a covalently linked dimeric receptor that exhibited constitutive activity upon ligand removal. In the case of the TM domain, an activating single-point mutation in the TM domain of the homologous receptor HER2/neu results in constitutive dimerization and kinase autophosphorylation (45). An equivalent Glu-627 → Val mutation in the EGFR TM domain expressed in NIH 3T3 cells resulted in constitutive receptor phosphorylation, hypersensitivity to exogenous ligand, and physical association with cellular substrates, although constitutive homodimers were not detected (46). These studies suggest that the TM domain plays more than a passive role in receptor dimerization.

The specific region(s) of the EC and TM domains required for quinazoline-mediated EGFR dimerization will require additional studies with site-directed mutants. In the context of other studies, however, our results suggest a possible role for the IC and/or kinase domains in EGFR dimerization. Supporting the possible involvement of these domains, a 110-kDa EC domain peptide generated by proteolytic cleavage of A431 cell-purified EGFR, dimerized less efficiently than full-length receptors as assayed by nondenaturing gel electrophoresis (43). In addition, EGF can induce the dimerization of a covalently linked dimeric receptor that exhibited constitutive activity upon ligand removal. In the case of the TM domain, an activating single-point mutation in the TM domain of the homologous receptor HER2/neu results in constitutive dimerization and kinase autophosphorylation (45). An equivalent Glu-627 → Val mutation in the EGFR TM domain expressed in NIH 3T3 cells resulted in constitutive receptor phosphorylation, hypersensitivity to exogenous ligand, and physical association with cellular substrates, although constitutive homodimers were not detected (46). These studies suggest that the TM domain plays more than a passive role in receptor dimerization.

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full-length EGFR, HER-2, and platelet-derived growth factor receptor in vivo, suggesting that the conserved regions in the kinase domain of these molecules (48) may be involved in receptor/receptor interactions. Finally, v-ErbB mutants exhibiting truncation of the majority of the ligand-binding domain heterodimerize covalently with native c-ErbB1 in chicken embryo fibroblasts in the absence of ligand (49). Recently, an EGFR mutant highly homologous to v-ErbB was isolated from human glioblastoma cells (50). Sucrose gradient centrifugation analysis of this receptor suggested the ligand-independent presence of a dimeric autophosphorylated truncated receptor population (51).

An obvious question is whether the quinazolines would also dimerize other receptors of the ErbB family (ErbB-2, ErbB-3, and ErbB-4), which exhibit 53–80% homology with the EGFR in their kinase domain (52). Since A431 cells contain low levels of these other tyrosine kinases, we examined this question in SKBR-3 cells, which, in turn, might dictate the net effect of quinazolines, not only high intracellular concentrations and acting very much like dominant-negative receptor mutants. Therefore, one could infer that for the antitumor effect of quinazolines, not only high intracellular concentrations in SKBR-3 cells. This resulted in inhibition of basal and heregulin-stimulated anchorage-independent growth (Fig. 8). The formation of inactive EGFR/ErbB-2 heterodimers as well as ErbB-2 homodimers in SKBR-3 cells. This indicated that quinazolines decrease the population of ErbB-2 available for constitutive and ligand-induced association with the kinase-weak ErbB-3 coreceptor, leading to the observed growth inhibition.

Taken together, these data imply that in addition to their affinity to and competition for the ATP site, these compounds can inhibit tyrosine kinase activity by causing receptor homo- and heterodimerization in an inactive state, thus sequestering EGFR and/or related ErbB receptors from signaling interactions and acting very much like dominant-negative receptor mutants. Therefore, one could infer that for the antitumor effect of quinazolines, not only high intracellular concentrations of ATP need to be considered but also the functional coexpression and content of ErbB family members within different cell backgrounds, which, in turn, might dictate the net impact of drug action. The latter is underscored by the large difference between the in vitro and in vivo IC_{50} for quinazolines against the ErbB-2 kinase (>10 μM versus <10 μM (Fig. 8), the latter in SKBR-3 cells). Although these compounds exhibit relatively higher specificity for EGFR at low concentrations, the heterodimerization between EGFR and ErbB-2 would be attractive in tumor cells co-overexpressing both receptors, a condition associated with cell transformation and a more malignant phenotype in human carcinomas (56–58). In addition, ErbB-2 inactivation per se has been shown to impair EGFR-mediated transformation (59, 60). In cells such as A431 that contain very high levels of EGFR, the preferred and perhaps only detectable mechanism of antitumor action would be the formation of inactive EGFR homodimers.

It should be pointed out that the proportion of receptors trapped by cross-linker during cell solubilization may indeed underestimate the total EGFR pool dimerized at the moment of BS³ addition. Cell solubilization could result in dissociation of EGFR from EGFR (25), suggesting a similar possibility for receptors bound by quinazolines. In addition, the efficiency of receptor cross-linking is limited due to the rapid hydrolysis of BS³ in aqueous solution. Since A431 cells secrete autocrine TGF-α (61) and exhibit a very high EGFR density, one could assume that in these cells, receptors are in a dynamic monomer-dimer equilibrium. All these considerations raise the possibility that, in cells with EGFR overexpression, most of the EGFRs could potentially be shifted into an inactive dimer state by quinazoline inhibitors as long as stable concentrations of these are maintained inside the cell. In tumor cells that are dependent on endogenous EGFR signaling for viability and/or the maintenance of their transformed phenotype, this scenario would have important clinico-biological implications.

In summary, the data presented indicate that the interaction of quinazolines with the ATP binding site can induce homodimerization of the EGFR as well as eliminate effective signal transduction. Binding to the ATP site of the EGFR by submicromolar concentrations of quinazolines results in marked growth inhibition of tumor cells with EGFR overexpression and/or gene amplification. At higher concentrations, this interaction with the EGFR can also induce detectable inactive heterodimers of EGFR with ErbB-2, thus preventing functional interactions with other preferred ErbB receptors. Structural three-dimensional studies of the quinazoline interactions with the receptor’s ATP site will shed light on the molecular basis for this novel mechanism of dimerization and should allow for further development of more specific and effective inhibitors of the EGFR kinase and related ErbB receptors.

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