Synergy of Epidermal Growth Factor Receptor Kinase Inhibitor AG1478 and ErbB2 Kinase Inhibitor AG879 in Human Colon Carcinoma Cells Is Associated with Induction of Apoptosis

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Abstract
Previous studies have shown that constitutive activation of epidermal growth factor receptor (EGFR) and ErbB2 by elevated autocrine transforming growth factor-α (TGF-α) expression plays an important role in colon cancer progression. Coexpression of EGFR and ErbB2 is found in a subset of colon cancers and may cooperatively promote cancer cell growth and survival, as heterodimerization is known to provide for diversification of signal transduction. In this study, the EGFR-selective tyrosine kinase inhibitor (TKI) AG1478 inhibited cell growth of an aggressive human colon carcinoma cell line, FET6xS26X, which harbors constitutively activated EGFR after stable transfection with TGF-α cDNA. However, AG1478 failed to induce apoptosis in FET6xS26X cells at concentrations sufficient for cell growth inhibition and complete suppression of EGFR phosphorylation. Similarly, AG879, a selective ErbB2 TKI, was incapable of inducing apoptosis in FET6xS26X cells at concentrations sufficient to inhibit cell growth and ErbB2 phosphorylation. To test the hypothesis that targeting both ErbB family members would show better efficacy than targeting the single receptors, combinations of inhibitors at fixed ratios of 1:1, 5:1, and 10:1 of AG1478 and AG879, respectively, were compared with single drugs for inhibition of cell growth. All combinations resulted in synergistic effects as indicated by combination index analysis. Synergistic inhibition was associated with induction of apoptosis as reflected by poly(ADP-ribose) polymerase cleavage, caspase-3 activation, and Annexin V staining. Finally, Western blot analysis showed significant inhibition of phosphorylation of both EGFR and ErbB2 by the combination treatment. These data suggest that the strategy to target both EGFR and ErbB2 simultaneously might result in more efficient inhibition of tumor growth than to target single receptor alone. (Cancer Res 2005; 65(13): 5848-56)

Introduction
The four closely related members of the ErbB family, epidermal growth factor (EGF) receptor (EGFR)/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4, and their ligands trigger a series of intracellular signaling events regulating cell proliferation, migration, differentiation, and cell survival (1). The intrinsic tyrosine kinase activities of ErbB receptors are activated on ligand-induced receptor homodimerization or heterodimerization and subsequent conformational change. Activated ErbB receptors will autophosphorylate or transphosphorylate their COOH-terminal tyrosine residues, which serve as high-affinity binding sites for Src homology 2 (SH2) or phosphotyrosine homology-containing proteins (2). The ErbB network propagates signaling to a broad scope of possibilities through its complexity at several levels. First, a large family of growth factors, the EGF-related peptides, serves to provide ligands with differential binding affinities to ErbB receptors, a characteristic that influences signal strength and duration (3, 4). Secondly, there is a hierarchy of ErbB dimer formation dictated by the nature of the ligand and the cell’s complement of ErbB receptors. ErbB2 is an orphan receptor without a physiologic ligand and is the preferred dimerization partner for other ErbB members (2, 5). Thirdly, a variety of downstream signaling proteins containing SH2 domains, including Ras, Sbc, Grb2, Grb7, phosphatidylinositol 3-kinase, and phospholipase C-γ, can couple to ErbB dimers with differing efficiencies (6). The diversified cellular functions mediated by the ErbB network make the ErbB family a frequent target of oncogenic processes. Amplification of EGFR and ErbB2 genes are observed in many types of tumors, such as breast, stomach, bladder, head and neck, and ovary cancers (7). Activating mutations have also been identified in lung cancer, glioblastoma, and other tumors (8).

Besides gene amplification and receptor overexpression, hyperactivation of the ErbB network can occur via autocrine or paracrine activities involving overproduction of ligands by the tumor cells or by adjacent stromal cells in the latter case (7). Accumulating evidence suggests that constitutive activation of ErbB receptors at normal expression level contributes to colon cancer tumorigenesis and progression in a ligand-dependent manner (9–11). Although up to 77% of colon cancers express EGFR, overexpression of the ErbB family proteins is a rare event for colon cancers (7, 12). The typical EGFR density in colon carcinoma cells is ~0.4 × 10^6 to 2 × 10^6 per cell, several orders of magnitude lower than EGFR-amplified breast cancer cells (13). On the other hand, coexpression of EGFR and its ligand transforming growth factor-α (TGF-α) in colon carcinomas was seen in early-stage disease. The expression level of TGF-α correlated well with more aggressive disease, poor prognosis, decreased disease-free survival, overall survival, and increased risk of metastasis (14). Intriguingly, transgenic mouse models with TGF-α overexpression caused gastrointestinal tract tumors (15). Our previous work elucidated the important role of deregulated TGF-α/EGFR autocrine activity in colon cancer progression. We showed that the aberrant expression of TGF-α was associated with a highly aggressive phenotype in human colon carcinoma cell lines (16). In addition, autocrine TGF-α conveys a growth advantage to colon cancer cells in the form of enabling reentry into the cell cycle independent of exogenous growth factors (17). Furthermore, overexpression of
TGF-α through stable transfection of a TGF-α cDNA converted unaggressive colon cancer cells to an aggressive phenotype (18, 19).

The observation of deregulated EGFR signaling in colon cancer cells suggests that targeting EGFR is a potential strategy for colon cancer therapy. Two general strategies have been employed to inhibit EGFR: antagonistic antibodies targeting the extracellular domains and small soluble tyrosine kinase inhibitors (TKI) competing with ATP for binding to the catalytic domain (20). However, single-agent EGFR antibody or TKI clinical trials for colon cancer treatment have generated at best a 10% response rate, although 80% of colon cancers potentially use EGFR for pathogenesis (21, 22). One possible reason for the limited efficacy of EGFR TKIs is due to ErbB2 activation arising from heterodimerization when EGFR and ErbB2 are coexpressed in colon cancer cells (9). Cooperation between ErbB receptors has been observed in oncogenic transformation (23). In this context, ErbB2 signaling is of particular interest because more potent and/or diversified signaling networks are propagated through heterodimers of ErbB members than homodimers, because ErbB2 is the preferred dimerization partner of ErbB members (5). It is believed that ErbB2 serves as not only a substrate of EGFR on heterodimerization but also a lateral signal transducer to other ErbB members (24). Specific EGFR TKIs do not interfere with ligand binding to the receptor and subsequent activation of ErbB kinase activity or ErbB2 dimerizing to other ErbB family members, such as ErbB3 (9). Thus, targeting only one component of a complex of different receptors may not be sufficient.

Consequently, we hypothesized that targeting both EGFR and ErbB2 by combination therapy of EGFR and ErbB2 TKIs would be superior to targeting single receptors in human colon cancer cells. To test this hypothesis, we used the FET6xS26X human colon adenocarcinoma cell model that is independent of exogenous growth factors for optimal growth. After TGF-α cDNA stable transfection, the parental nontumorigenic FET cell line was converted into a highly tumorigenic, aggressive colon carcinoma cell line when grown as a xenograft, designated as FET6xS26X (18). In this study, we showed that combination of the EGFR TKI AG1478 and the ErbB2 TKI AG879 resulted in a synergistic effect of growth inhibition on FET6xS26X cells. Significantly, combination treatment induced apoptosis at concentrations that were incapable of inducing apoptosis as single drugs. Furthermore, the synergy of growth inhibition was associated with synergy of apoptosis. Finally, analysis of EGFR and ErbB2 phosphorylation by Western blot showed the combination of AG1478 and AG879 inhibited the phosphorylation of both receptors in a synergistic manner as well.

Materials and Methods

Cell culture. The FET human colon adenocarcinoma cell line was originally established in vitro from a primary human tumor and has been well characterized with respect to its growth factor dependence, autocrine TGF-α status, and tumorigenicity (25). The experimental model system used for this study was a full-length TGF-α cDNA-transfected clone of the parental FET cell line. Transfection and characterization of these cells has been described elsewhere (18). Both FET and FET6xS26X cells were maintained at 37°C in a humidified atmosphere of 6% CO₂. Cells were grown continuously in a chemically defined McCoy’s 5A (Cellgro, Herndon, VA) serum-free medium supplemented with pyruvate, vitamins, amino acids, and antibiotics plus epidermal growth factor (10 ng/mL, R&D Systems, Minneapolis, MN), insulin (20 μg/mL, Sigma, St. Louis, MO), and transferrin (4 μg/mL, Sigma). SM medium is the basal medium lacking transferrin, insulin, and EGF.

Kinase inhibitor treatment. The 4-anilinoquinazoline derivatives, AG1478 and AG879, which have high selectivity for inhibiting EGFR and ErbB2 tyrosine kinase activity, respectively (26), were purchased from Calbiochem (La Jolla, CA). AG1478 and AG879 were dissolved in DMSO to generate 20 mmol/L stock solutions. For 96-well format assays, a mixture of AG1478 and AG879 was prepared at different ratios (1:1, 5:1, or 10:1) and serially diluted in serum-free medium to working concentrations. An equivalent serial dilution of DMSO was used as the control treatment. When cells were seeded in 10 cm Petri dish for harvesting whole lysates or for flow cytometry analysis, drug solutions of various concentrations were prepared directly from the stocks by DMSO dilution and then further diluted with SM medium at 1:1,000 ratio to reach the final concentration of 0.1% DMSO.

Cell proliferation inhibition assay. Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Briefly, cells were seeded in 96-well tissue culture plates (Costar, Corning, NY) at 3,000 cells per well per 150 μL. At 24 hours after seeding, cells were treated with 50 μL serial concentrations of AG1478, AG879, or the combination of AG1478 plus AG879 at ratios of 1:1, 5:1, or 10:1. After 72 hours of drug exposure, MTT (100 μL) solution (2 mg/mL) was added to each well to incubate for 2 hours. The reaction was stopped by removal of MTT. Thereafter, DMSO (200 μL) was added to solubilize formazan crystals. Absorbance at 570 nm was recorded using a 96-well plate reader.

Combination index value analysis. The Chou and Talalay combination index (CI) analysis is a well-established index to determine the pharmacodynamic interactions of two drugs (27). The CI value of mononemocline drugs is calculated by the formula: CI = (Da + Db) / (Dxa + Dxb) + DaDb / DxaDxb. Da and Db are the doses of drugs A and B to inhibit X% of cell proliferation as single drugs, and Dxa and Dxb are the doses of drugs A and B to inhibit X% of cell growth in a combination regimen. When CI = 1 (represents an additive effect of the two drugs), the CI formula is in the same form as a traditional isobologram equation. Synergism is defined as more than the expected additive effect with CI < 1 and antagonism is defined as CI > 1. Nonexclusive competitors are defined as inhibitors binding to different targets or different sites of the same target. CI values were analyzed using the CalcuSyn software (Biosoft, Ferguson, MO). The inputs are the concentrations of single inhibitors, the combination doses at different ratios, and the fractional inhibition [fraction affected (Fa)] of single drugs and combinations. Fa = (A1glycos control – A3glycos treated) / A1glycos control. Fraction of unaffected cells (Fu) = 1 – Fa. A complete CI effect plot is generated from fixed ratio combination data by Monte Carlo simulation algebra of the software.

Antibodies. The polyclonal EGFR protein (SC-03) and ErbB2 protein (SC-284) antibodies and the monoclonal poly(ADP-ribose)polymerase (PARP; SC-8007) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody specific to EGFR phosphorysine Y1173 was purchased from Calbiochem. The monoclonal antibody to ErbB2 phosphorysine Y1248 was purchased from Upstate (Charlottesville, VA) and the phosphorysine Y1139 antibody was purchased from Biosource (Camarillo, CA). The caspase-3 antibody was purchased from Cell Signaling (Beverly, MA). Blocking buffer and working concentrations of the above antibodies were prepared according to the data sheets for the products.

Immunoblotting. Cells were washed twice with cold PBS and then lysed with TNEV lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 50 mmol/L NaF, 1 mmol/L NaVO₃, 25 μg/mL β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, one protease inhibitor cocktail tablet Roche, Indianapolis, IN)] per 10 mL for 30 minutes on ice. Protein estimation was determined by the Bio-Rad (Hercules, CA) method. After resolving the protein on SDS-PAGE gel, proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). The membranes were blocked in 5% nonfat dry milk in TBS-Tween 20 (TBST; 0.15 mol/L NaCl, 0.01 mol/L Tris-HCl [pH 7.4], 0.05% Tween 20) at room temperature for 1 hour. Primary antibodies at recommended dilutions were incubated overnight at 4°C in 5% TBST or 5% bovine serum albumin according to the data sheets. After washing, blots were then incubated with a 1:5,000 dilution of horseradish peroxidase–linked secondary antibody in blocking buffer for 1 hour followed by further
Flow cytometry analysis for Annexin V staining. Apoptosis was analyzed by an Annexin V assay kit (BD Biosciences PharMingen, San Jose, CA). Briefly, cells grown in 10 cm Petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM KCl, 1 mM CaCl$_2$, and 1 mM MgCl$_2$] and stained with Annexin V-FITC and propidium iodide (PI) provided from the kit at room temperature for at least 15 minutes in the dark. Cells were then analyzed in a FACScan flow cytometer (Becton, Franklin Lakes, NJ) within 1 hour after staining. Data were analyzed by WinList 5.0 software (Verity Software House, Topsham, ME).

**Results**

Concentration-dependent growth inhibition of FET6×S26X cells by AG1478 and AG879 alone or in combination. The EGFR kinase inhibitor, AG1478, has 4 orders of magnitude higher selectivity to EGFR (IC$_{50}$ 3 nmol/L) than ErbB2 (IC$_{50}$ >100 μmol/L) in cell-free systems (26). The EGFR kinase inhibitor, AG879, has at least 500-fold higher selectivity to ErbB2 (IC$_{50}$ 1 μmol/L) than EGFR (IC$_{50}$ >500 μmol/L) in cell-free systems (26). The concentration of AG1478 was 15.35 and 1.94 μmol/L, respectively, as determined by 72-hour drug exposure in MTT assays (Table 1). Because the IC$_{50}$ value of AG1478 is ~10-fold higher than AG879, a fixed ratio of concentrations of AG1478/AG879 at 10:1 was tested for combination treatments. The concentrations of AG1478 in combination experiments were 20, 15, 10, 7.5, 5, 3.56, 1.58, and 1 μmol/L (4/3, 1, 2/3, 1/2, 1/3, 1/4, 1/8, and 1/15 of the IC$_{50}$ of AG1478), whereas the concentrations of AG879 were correspondingly 10-fold lower. Growth inhibition effects were measured after 72-hour drug exposure and plotted as a dose-effect curve and the median-effect plot of AG1478. The shapes of the curves (sigmoid) were the same for AG1478, AG879, or AG1478 + AG879. Growth inhibition of FET6×S26X cells was 15.35 and 1.94 μmol/L, respectively, as determined by 72-hour drug exposure in MTT assays. The Fa for AG1478 + AG879 was shown in comparison with the single drugs when the inhibitory effect of combinations was plotted against the concentrations of AG1478 (Fig. 1A) or AG879 (Fig. 1B), respectively. This indicated higher sensitivity of FET6×S26X cells to AG1478 plus AG879 treatment than to single drugs. Significantly, 4- and 5-fold decrease in the concentration of AG1478 and AG879, respectively, was observed to inhibit 50% tumor cell proliferation in a 10:1 ratio combination compared with IC$_{50}$ concentrations of either drug alone (Table 1). Furthermore, the isobologram showed that the combination of AG1478 plus AG879 at a 10:1 ratio achieved more than an additive effect, as the data points of combinations all fell below the line defined from the concentrations of single agents at 90% (ED$_{90}$), 75% (ED$_{75}$), and 50% (ED$_{50}$) inhibition, respectively (Fig. 1C).

![Figure 1. Dose-effect curve, median-effect plot, and isobologram of AG1478, AG879, and AG1478 + AG879.](image)

**Table 1.** IC$_{50}$ values of AG1478, AG879, and AG1478 + AG879 at different ratio in FET6×S26X cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AG1478 (μmol/L)</th>
<th>AG879 (μmol/L)</th>
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<tbody>
<tr>
<td>AG1478 single drug</td>
<td>15.35</td>
<td>NA</td>
</tr>
<tr>
<td>AG879 single drug</td>
<td>NA</td>
<td>1.94</td>
</tr>
<tr>
<td>AG1478 + AG879 (1:1)</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>AG1478 + AG879 (5:1)</td>
<td>2.29</td>
<td>0.46</td>
</tr>
<tr>
<td>AG1478 + AG879 (10:1)</td>
<td>3.9</td>
<td>0.39</td>
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NOTE: IC$_{50}$ values were determined by MTT assay after 72-hour drug treatment.
Synergy between AG1478 and AG879 at different ratios by combination index analysis. Targeting both EGFR and ErbB2 kinase activity by the combination of AG1478 plus AG879 achieved more than an additive effect in growth inhibition on FET6sS26X cells as shown above. To further determine whether the inhibitory effects were synergistic, we analyzed CI values of AG1478 plus AG879 using CalcuSyn software (Materials and Methods). Moderate synergism (0.3 < CI < 0.7) was observed at the combination concentrations used with an AG1478/AG879 ratio of 10:1 (Fig. 2A). Notably, AG879 has a "flatter" dose-effect curve than AG1478 (Fig. 1A). This means that, at low doses, cells were more sensitive to AG879 than AG1478. This raised whether different ratios of AG1478/AG879 would generate different effects when analyzed by CI. To further confirm that synergy is independent of the ratios of AG1478/AG879, combinations of AG1478 and AG879 at 1:1 and 5:1 ratios were also tested. The CI values of both combination regimens were analyzed and synergism was indicated at both ratios with CI < 1 (Fig. 2B and C). Of note, although synergy was observed at all three ratios of combination treatment, different patterns of synergy were observed. In combination regimens with a 10:1 ratio of AG1478/AG879, the combination had stronger synergy at lower doses than higher doses. A similar trend was observed at a 24-hour treatment schedule (data not shown). In contrast, at combination doses with a 1:1 ratio, the strongest synergy was observed at 60% growth inhibition, whereas moderate synergy was observed for all the doses tested at a 5:1 ratio.

Synergy of growth inhibition of AG1478 and AG879 is associated with apoptosis. TGF-α and EGFR have been implicated in the regulation of apoptosis (28, 29). As a result of stable TGF-α overexpression, FET6sS26X cells are resistant to apoptosis for up to 72 hours of growth factor deprivation. FET6sS26X cells are resistant to apoptosis for up to 36 hours of treatment with the EGFR kinase inhibitor AG1478 at 20 μmol/L, a concentration well above the IC₅₀. We used concentrations of AG1478 and AG879 below their IC₅₀ concentrations as single agents for apoptosis analysis. PARP cleavage was used as an indicator of apoptosis. FET6sS26X cells (80% confluent) were incubated with 5, 10, and 15 μmol/L AG1478 alone or in combination with 1 μmol/L AG879 for 24 hours and subjected to growth factor deprivation. Higher concentrations of AG879 alone were used as single-agent controls (Fig. 3). Consistent with previous observations, AG1478 at 5, 10, and 15 μmol/L did not sensitize FET6sS26X cells to apoptosis after 24-hour growth factor deprivation. In contrast, after incubation of AG1478 in combination with 1 μmol/L AG879 (a concentration incapable of inducing apoptosis alone), PARP cleavage was induced in an AG1478 concentration-dependent manner (Fig. 3). Of note, PARP cleavage could be detected at 10 μmol/L AG879, which is 5-fold higher than the IC₅₀ of AG879. However, at this concentration, AG879 selectivity to ErbB2 is diminished (data not shown).

To further confirm apoptosis induction by the combination of AG1478 and AG879 treatment, caspase-3 activation was analyzed by Western blot (Fig. 4). Caspase-3 is one of the executioner caspases in response to the activation of the intrinsic mitochondrial apoptotic pathway, which can be triggered by blockade of ErbB signaling (30, 31). When caspase-3 is activated, the intact form of 33 kDa is cleaved into an activated form of 17/19 kDa, which in turn cleaves PARP (31). Consistent with the PARP cleavage results, AG1478 and AG879 failed to activate caspase-3 after 24-hour treatment as single drugs. However, activated caspase-3 was generated in a concentration-dependent manner after combination treatment by AG1478 and AG879. AG879 at 10 μmol/L was able to induce caspase-3 activation as expected from the PARP cleavage data. Furthermore, the apoptotic rates of FET6sS26X cells after AG1478 and AG879 treatment were quantified by Annexin V and PI double staining followed by flow cytometry analysis. Annexin V–positive and PI-negative cells were interpreted as early apoptotic cells. With 15 μmol/L AG1478 or 1 μmol/L AG879 treatments for 24 hours, the percentages of early apoptotic cells were similar to those of DMSO control cells. The AG1478 and AG879 combination treatment showed 10.72% of the cells undergoing apoptosis, indicating that the simultaneous inhibition of EGFR and ErbB2 induced apoptosis in a synergistic manner (Fig. 5).
AG1478 and AG879 combinations inhibit EGFR and ErbB2 phosphorylation. In human colorectal adenocarcinoma, only 1% of cancer cells is typically in an active cycling state, whereas the rest of the cells are in a quiescent state due to limited nutrients (32). We found that quiescent-state FET6oS26X cells were resistant to apoptosis after growth factor deprivation for 72 hours. We reported previously that enhanced TGF-α expression resulting in aberrant EGFR and ErbB2 activation in the quiescent state conveys a growth advantage for growth factor–independent cell cycle reentry by these cells (16, 18). This suggested a potential link between resistance to advantage for growth factor–independent cell cycle reentry by these phosphotyrosine.

In human colorectal adenocarcinoma, only 1% of a found that quiescent-state FET6S cells are in a quiescent state due to limited nutrients (32). We cancer cells is typically in an active cycling state, whereas the rest of the cells are in a quiescent state resulting in aberrant EGFR and ErbB2 under treatment with AG1478 and AG879 using phospho-EGFR and ErbB2-specific antibodies. FET6oS26X cells were subjected to growth factor deprivation for 72 hours to generate quiescence. Thereafter, the cells were replenished with nutrients in fresh SM medium (without growth factors) containing similar concentrations of AG1478, AG879, or AG1478 plus AG879 as shown previously. After 6-hour treatments, cells were lysed and the whole cell lysate was analyzed for EGFR and ErbB2 phosphorylation using a specific antibody to phosphotyrosine site Y1173 of EGFR and specific antibodies to phosphotyrosine sites Y1139 or Y1248 of ErbB2 (Fig. 6). EGFR Y1173 is a major tyrosine site activated by autophosphorylation after receptor dimerization (33), and Y1139 and Y1248 were reported previously as autophosphorylation sites of ErbB2 (34). Quantification of the Western blot by densitometry and analysis of ImageQuant software (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) are also shown. Interestingly, AG1478 (5 μM) was sufficient to inhibit EGFR phosphorylation up to 80% as shown in Fig. 6d; however, at this concentration, AG1478 was not sufficient to induce apoptosis (Fig. 4). This suggests that inhibition of EGFR phosphorylation is not sufficient to induce apoptosis in FET6oS26X cells. In contrast, AG879 had only a limited inhibitory effect (10%) on EGFR Y1173 phosphorylation at 5 μM, whereas the same concentration inhibited 60% and 40% phosphorylation of ErbB2 Y1139 and Y1248, respectively, which is consistent with the selectivity of this compound for ErbB2. Notably, the combination treatments inhibited the activation of EGFR on Tyr1173 to a similar extent as AG1478 single drug alone, suggesting that phosphorylation of this site is mainly through autophosphorylation and inhibition of EGFR was saturated by AG1478. On the other hand, both AG1478 and AG879 inhibited ErbB2 Y1139 phosphorylation in a concentration-dependent manner (Fig. 6b). This suggests that this site may be subject to both transphosphorylation by the EGFR and autophosphorylation by ErbB2. In support of this scenario, we observed a suboptimal concentration of AG879 (1 μM) with 20% inhibitory effect on Y1139 phosphorylation synergized with AG1478 at 5, 10, and 15 μM in inhibiting ErbB2 Y1139 phosphorylation, resulting in 50%, 70%, and 90% inhibitory effect at 5 + 1, 10 + 1, and 15 + 1 μM/combination treatments as opposed to the limited effect of AG1478 alone (10%, 30%, and 30% inhibitory effect, respectively). In contrast to Y1139, the ErbB2 Y1248 site was not affected by AG1478 at concentrations below 15 μM/L (Fig. 6c). However, similar to the Y1139 site (but to a lesser degree), phosphorylation of ErbB2 Y1248 was synergistically down-regulated by the combination of AG1478 and AG879 as shown by the quantification of Western blots (Fig. 6c). Taken together, our data suggested that ErbB2 Y1248 and Y1139 sites had differential responses toward EGFR and ErbB2 inhibitor treatment. Importantly, the correlation of combination drug treatments inhibiting both EGFR and ErbB2 phosphorylation with the concentrations necessary to induce apoptosis indicated that cell survival in FET6oS26X cells is dependent on activation of both receptors.

Discussion

Colon cancer is one of the most common human malignancies and the second leading cause of cancer death in North America just behind lung cancer. Advanced disease is associated with a poor prognosis and a 5-year survival rate of <5% (35). 5-Fluorouracil with leucovorin has been the primary chemotherapy regimen for

Figure 3. Effect of AG1478 + AG879 on PARP cleavage. Subconfluent FET6oS26X cells were incubated in SM medium with either 5, 10, or 15 μM/L AG1478 alone or in combination with 1 μM/L AG879 for 24 hours. Cell lysates were harvested and PARP cleavage was detected using a monoclonal PARP antibody. AG879 concentrations of 5 and 10 μM/L were used as controls.

Figure 4. Caspase-3 activation by AG1478 + AG879. Subconfluent FET6oS26X cells were incubated in SM medium with 5, 10, or 15 μM/L AG1478 alone or in combination with 1 μM/L AG879 for 24 hours. Cells were also incubated in SM medium with 1 and 10 μM/L AG879 alone for 24 hours. Cell lysates were harvested and caspase-3 was detected by Western blot using a polyclonal antibody specific for both intact and cleaved caspase-3. Actin was used as a loading control.
advanced colorectal cancer patients. New cytotoxic compounds are now available, including oral fluoropyrimidines, the topoisomerase I inhibitor, irinotecan (CPT-11), and the third-generation platinum compound oxaliplatin. However, these agents have not had dramatic effects on advanced disease. Novel approaches that selectively target deregulated molecular events in colon cancer cells are under development, such as targeting EGFR by monoclonal antibodies or small-molecule TKIs.

The currently available EGFR TKIs are structurally related to quinazoline, pyridopyrimidine, and pyrrolopyrimidine developed during the past decade (36). Quinazolines, such as AG1478, are potent EGFR inhibitors. The amount of quinazoline required to inhibit cell-free enzyme activity by 50% (IC_{50}) is in the nanomolar range (37). However, the typical IC_{50} values for cell growth inhibition of the clinically used quinazoline analogues in a variety of human carcinoma cell lines with moderate EGFR expression levels are in the micromolar range (38, 39). Indeed, we observed in this study that the IC_{50} value of AG1478 for the FET6αS26X cell line is 15.35 μmol/L (Table 1). The seemingly dramatic drop of the potency of these compounds in intact cells can be explained by high intracellular ATP concentrations. Typically, the ATP concentrations are at the millimolar range in intact cells but at the micromolar range in purified enzyme systems used to test potencies of EGFR TKIs (26, 40). The potency of ATP competitive inhibitors decreases in circumstances of increased ATP concentrations as observed in intact cells.

The mechanisms of the anticancer effects of the EGFR antagonists have been intensively studied. Both cytostatic and cytotoxic effects have been reported in response to EGFR and ErbB2 antagonists in many cancer cell types, including colon cancer. It is well documented that targeting EGFR or ErbB2 with TKIs or monoclonal antibodies generates G_{0}-G_{1} phase cell cycle arrest effects through up-regulation of p27 and suppression of cyclin-dependent kinase activity in several cancer cell types (41–44). On the other hand, reports have shown that EGFR TKIs induced apoptosis in breast cancer, non–small cell lung, ovarian, and colon cancer cells (45–48). However, the concentrations used are much higher than the concentrations sufficient to inhibit EGFR; thus, the resulting apoptosis likely involved nonelective effects. Moreover, these concentrations used in cell culture studies may not reflect achievable concentrations of EGFR TKIs used in the clinic. For example, it was reported that AG1478 induced apoptosis in several colon cancer cell lines at 25 μmol/L, a concentration much higher than what we used in this study, and it is questionable whether the concentration is achievable \textit{in vivo} (48). Intriguingly, EGFR antagonists readily induce apoptosis in those cells dependent solely on EGFR signaling for cell growth and survival. For example, the EGFR monoclonal antibody C225 and the EGFR kinase inhibitor Tarceva (OSI-774) induced apoptosis in the DiFi colon carcinoma cells (49, 50), which show amplification of wild-type EGFR (51). In addition, a glioblastoma cell line with overexpression of a mutated constitutive activated EGFR was shown to be sensitive to AG1478 and EGFR antagonistic antibody-induced apoptosis (52). Although it remains controversial whether the sensitivity of EGFR TKIs is dependent on EGFR expression level (53, 54), the potency of EGFR TKIs in cell culture seems to be determined by receptor expression level and whether EGFR signaling plays a dominant role for cell proliferation and survival in that particular cell line.

Figure 5. Annexin V and PI double staining for apoptosis. Subconfluent FET6αS26X cells were incubated in SM medium with 15 μmol/L AG1478 alone or in combination with 1 μmol/L AG879 for 24 hours. Cells were trypsinized and stained with Annexin V and PI for flow cytometry analysis. Early apoptotic cells were defined as Annexin V–positive, PI-negative cells.
Figure 6. Effect of AG1478 + AG879 on inhibition of EGFR and ErbB2 phosphorylation. Subconfluent FET6/S26X cells were incubated with SM medium for 72 hours and then released in SM medium with 5, 10, or 15 μmol/L AG1478 alone or in combination with 1 μmol/L AG879 for 6 hours. As controls, cells were also treated with 1 or 5 μmol/L AG879 alone. Cell lysates were analyzed for EGFR phosphorylation by Western blot analysis using a specific monoclonal antibody to phospho-Y1173 EGFR (A) or analyzed for ErbB2 phosphorylation by specific antibody to ErbB2 phospho-Y1139 (B) and Y1248 (C). The membranes were stripped and blotted with anti-EGFR or anti-ErbB2 antibody for loading control. Quantification of Western blot was done by densitometry and analyzed by ImageQuant software. The inhibitory effect of each treatment condition was plotted as bar graph.
The EGFR TKIs have shown promise as a novel strategy of anticancer therapy for several types of cancers (14, 55); however, EGFR TKIs have not had dramatic effects on colon cancer as single agents (21). Colon cancer is characterized by enhanced expression of ligands instead of the receptors, which may contribute to the EGFR TKIs having not shown robust success in the clinic as single agents. Given the clear role of EGFR in colon cancer tumorigenesis, the EGFR TKIs may not be at fault, but rather the strategy to optimize the regimens for using them. Therefore, it is reasonable to propose that inhibition of EGFR may not be sufficient to induce apoptosis in some cancers, such as colon cancer, which are dependent on both EGFR and ErbB2 activation for aberrant growth. Additional inhibition of other membrane tyrosine kinase receptors or intracellular kinase may be critical for successful targeted therapy. For example, ErbB2 could be one of them, because it plays a very important role for more potent and diversified signaling propagated through heterodimers.

This study is the first to show that a combination of EGFR and ErbB2 kinase inhibitors achieved synergy of growth inhibition in human colon cancer cells. In addition, the synergy of growth inhibition is associated with apoptosis. We tested AG1478 at concentrations ranging from 0.5 to 30 μmol/L and observed a concentration-dependent inhibition of EGFR phosphorylation by AG1478 treatment in FET6αS26X cells (data not shown). For the combination study, we chose concentrations of AG1478 sufficient to inhibit EGFR phosphorylation but low enough to retain selectivity to EGFR. At 15 μmol/L, a concentration sufficient for almost complete suppression of EGFR phosphorylation, AG1478 did not sensitize FET6αS26X cells to apoptosis (Fig. 6A). This indicated that an alternative signaling pathway(s) important for cell survival mediated by another ErbB member(s) was activated after TGF-α stable transfection, because reversal of TGF-α expression in these cells eliminates aberrant growth control and resistance to apoptosis (18). Similarly, AG879 inhibited ErbB2 phosphorylation in a concentration-dependent manner (data not shown). However, AG879 as a single drug is incapable of inducing apoptosis at a concentration (5 μmol/L) sufficient to inhibit ErbB2 activation (Fig. 6A). This suggested that EGFR activation also plays a role for cell survival. Our data have proven further that ErbB2 signaling is critical for enhanced survival of FET6αS26X cells, because a suboptimal concentration (1 μmol/L) of the ErbB2 inhibitor AG879 could synergize with EGFR inhibitor AG1478 to induce apoptosis.

Quinazoline EGFR TKIs are highly selective to EGFR compared with other tyrosine kinases (e.g., platelet-derived growth factor receptor; ref. 26). Although EGFR and ErbB2 share 80% homology in their catalytic domain, quinazoline EGFR TKIs have 104-fold higher selectivity toward isolated EGFR than ErbB2 due to the significant difference in the structure of the ATP binding sites of the two receptors (26). Interestingly, EGFR TKIs have been shown to be capable of inhibiting ErbB2 phosphorylation in cell culture without a direct effect on the receptor (56). In this study, we observed an inhibitory effect of AG1478 on ErbB2 phosphorylation. Considering our previous finding of the transactivation of ErbB2 by EGFR signaling in the cell model tested here, we propose that the transphosphorylation of ErbB2 through EGFR was inhibited by AG1478. Previously, we reported that aberrant heterodimerization between EGFR and ErbB2 was induced by overexpression of TGF-α in FET6αS26X cells (18). In this study, our data indicate that both autoactivation and transactivation between EGFR and ErbB2 exist during heterodimer formation. Both Y1139 and Y1248 of ErbB2 were shown to be autophosphorylated in an immunopurified system (34). Our data suggest that Y1139 of ErbB2 could be transphosphorylated in intact colon cancer cells. This is based on the observation that EGFR inhibitor AG1478 inhibited ErbB2 Y1139 phosphorylation in a concentration-dependent manner; meanwhile, this site was also sensitive to AG879 treatment. One explanation to this observation is that the Y1139 site on ErbB2 can be transactivated by EGFR kinase as a result of ErbB2 heterodimerization as well as autophosphorylation by ErbB2 itself. Furthermore, the Y1248 site is not sensitive to EGFR inhibitor treatment at concentrations below 15 μmol/L and less sensitive to ErbB2 kinase inhibitor as well, perhaps due to a higher affinity of this site to both kinase domains for its SH2 phosphorylation. Based on the differential sensitivities of these two sites to the ErbB inhibitors, we propose that activation of different tyrosine sites of ErbB receptors also follows a hierarchy of phosphorylation. The synergistic effect of the combination of AG1478 plus AG879 for inhibiting ErbB2 phosphorylation at both Y1139 and Y1248 sites, but to a lesser extent in the latter (Fig. 6), can be explained by this model. Thus, compared with ErbB2 Y1139 site, Y1248 may be a “priority” activation site by both EGFR and ErbB2 on heterodimerization. To inhibit phosphorylation of this site, a “threshold” would need to be passed for inhibition of both EGFR and ErbB2 tyrosine kinase activity, which can be overcome by combination treatment. A reasonable scenario is that sensitization of FET6αS26X to apoptosis by the combination of EGFR and ErbB2 inhibitors was due to the significant suppression of both EGFR and ErbB2 activation. Taken together, our studies suggest that targeting both EGFR and ErbB2 tyrosine kinase activities by a combination of TKIs could be a successful strategy in human colon cancer cells characterized by a phenotype with enhanced survival signaling that is dependent on heterodimerization.

Acknowledgments

Grant support: NIH grants CA44342, CA4807, and CA16056.

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We thank Lisa Humphrey, Karen K. Kuropatwinski, Jennie Hauser, Alexis N. Conway, and Michelle A. Vinci for their technical assistance and Weidong Yu for his guidance with CI analyses.

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Synergy of Epidermal Growth Factor Receptor Kinase Inhibitor AG1478 and ErbB2 Kinase Inhibitor AG879 in Human Colon Carcinoma Cells Is Associated with Induction of Apoptosis

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