Combined inhibition of epidermal growth factor receptor and JAK/STAT pathways results in greater growth inhibition \textit{in vitro} than single agent therapy

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Abstract

Epidermal growth factor receptor (EGFR) inhibition with small molecule tyrosine kinase inhibitors results in antitumor activity in only a minority of patients whose tumors express EGFR. One hypothesis to explain this suboptimal clinical activity is that multiple growth regulatory pathways are abnormal in most EGFR-expressing cancers. Given the importance of Stat-3 signaling pathway in epidermoid tumors, we hypothesized that blocking complementary pathways in an epidermal growth factor (EGF)-driven model of proliferation in the A431 cell line would demonstrate improved antiproliferative activity. Exposure of A431 cells to the EGF results in a significant increase in EGFR and Stat-3 phosphorylation. However, inhibition of EGF by AG1478 fails to decrease EGF-induced Stat-3 phosphorylation. This suggests that EGF continues to drive Stat-3 phosphorylation through other receptors. Our study suggests that residual ErbB2 activation by EGF, despite EGFR blockade, is responsible for persistent downstream activation of Stat-3. In this setting, combined exposure to an EGFR blocker and Stat-3 blocker (AG490) results in significantly greater tumor growth inhibition than either agent alone. We conclude that targeting multiple pathways (EGFR and JAK/STAT pathways) in EGF-driven tumors may result in greater antiproliferative activity than blocking EGFR alone. [Mol Cancer Ther. 2004;3(4):459–463]

Introduction

The epidermal growth factor receptor (EGFR) is expressed in the majority of epidermoid tumors (1). Epidermal growth factor (EGF)/EGFR autocrine and paracrine processes driving proliferation have been found and are the basis for chemotherapeutic drugs targeting EGFR. However, EGFR inhibition with small molecule tyrosine kinase inhibitors results in antitumor activity in only a minority of patients whose tumors express EGFR. Trials of single agent small molecules targeting EGFR in EGFR-expressing non-small cell lung cancer (NSCLC) have demonstrated low response rates to EGFR tyrosine kinase inhibitors. In a study of OSI-774, a response rate of only 11% was seen in patients that had received prior therapy (2). Similar results have been seen with ZD1839 in patients with advanced NSCLC where a response rate of 11% was observed (3). It is unclear why despite expression and overexpression of EGFR in these tumor types low response rates are seen. One hypothesis to explain this suboptimal clinical activity is that multiple growth regulatory pathways are abnormal in most EGFR-expressing cancers. Thus, inhibition of EGFR alone may not affect other signal transduction pathways, which remain active and continue to promote proliferation. Indeed, there is evidence that multiple intracellular signal pathways are active in NSCLC including MAPK, AKT, and STAT (4–6). In addition, pathways downstream of EGFR may remain activated despite total EGFR blockade. In a study by Albanell et al. (7), EGFR inactivation (as defined by decreased tyrosine phosphorylation) was seen in all skin biopsies from patients receiving ZD1839; however, not all patients had a consistent decrease in MAPK phosphorylation on paired skin biopsies. In the same study, most patients actually demonstrated an increase in Stat-3 phosphorylation after EGFR blockade on serial skin biopsies (7). In \textit{in vitro} data further support this as blocking TGF-\(\alpha\)-mediated activation of EGFR did not decrease Stat-3 activation (8). Furthermore, there is evidence in NSCLC cells that AKT/Protein Kinase B complex downstream from EGFR may be constitutively activated. Thus, it may not be affected by anti-EGFR therapy, theoretically diminishing its therapeutic effects (4).

These data together suggest that multiple growth regulatory pathways downstream of EGFR may remain activated despite EGFR blockade. One strategy to improve the efficacy of anti-EGFR agents is to combine it with agents with different mechanisms or sites of action, such as those targeting downstream activated signal pathways. Traditionally, combination therapies of molecular-targeting agents have included cytotoxic drugs as the second agent. Additive effects have been demonstrated with the addition of several cytotoxic agents to EGFR inhibitors (9, 10). Alternatively, one could use molecular targeted agents that act on different pathways in combination to achieve improved antiproliferative activity. Given the importance of Stat-3 signaling pathway in driving proliferation in epidermoid tumors (11), we hypothesized that blocking complementary pathways in an EGF-driven model of proliferation in the A431 cell line would demonstrate improved antiproliferative activity.
Materials and Methods

Cells
A431 human cervical epidermoid carcinoma cells (over-expressing EGF receptor, 2–3 × 10^6 receptors/cell) were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamate, penicillin, and streptomycin. They were maintained in 37°C, 5% CO₂, fully humidified incubator, passed twice weekly, and prepared for experimental procedure after reaching 90% confluence.

Reagents
Tyrophostin AG1478, a specific inhibitor of the tyrosine kinase activity of the EGF receptor (12), was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 100% ethanol as a 630 mM stock solution before use. AG490 [α-cyano-(3,4-dihydroxy)-N-benzylcinnamid] was purchased from Calbiochem Biochemicals (La Jolla, CA) and dissolved in DMSO. AG490 inhibits the activation of Stat-3 by selectively blocking JAK2 (13). The doses of AG490 used in these experiments do not affect ERK1/2 or AKT activation status.3 Stock solutions were then diluted in DMEM/HAM’s F12 to achieve the final desired concentration. The final DMSO and ethanol concentration were less than 0.04%. The HER2 antibody rhuMAb 2C4 was provided graciously by Dr. Mark Sliwkowski (Genentech, Inc., South San Francisco, CA). 2C4 binds to a different epitope of HER2 ectodomain than trastuzumab and sterically hinders HER2 recruitment in heterodimers with other HER receptors. This results in the inhibition of signaling by HER2-based heterodimers both in cells with low and high HER2 expression.

Western Blotting
A431 cells were grown to 90% confluence. Cells were incubated in serum-free media for 16 h before use. The specific inhibitors were then added to the desired concentration and after 3 h, EGF was added (final concentration of 10 ng/ml) for 10 min. Cells were lysed [0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 5 mM EDTA, 10 μg/ml aprotenin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, all reagents were from Sigma] and sonicated. Samples containing 30–70 μg protein measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) were separated by SDS-PAGE consisting of a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) separating gel containing 0.1% SDS (5). The running buffer was comprised of 0.1% SDS, 25 mM Tris, and 250 mM glycine (pH 8.3). Electrophoretic fractionation was carried out at a constant current of 100 mA for 90 min. Proteins were then electrotransferred onto an Immobilon P15 membrane (Millipore Corp., Bedford, MA). The filters were blocked with 5% bovine serum albumin in PBS containing 0.1% Tween 20 (PBS-Tween) for 5 min and then incubated at room temperature with primary antibody (1:200 dilution) in blocking solution for 1 h. After washing in PBS-

3 Unpublished data.

Tween, the filters were incubated for 1 h in horseradish peroxidase-conjugated anti-immunoglobulin (1:5000). Following three washes in PBS-Tween (5 min each wash), bands were visualized by chemiluminescence and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham Life Science Inc., Arlington Heights, IL). The antibody to Stat-3 is a rabbit polyclonal antibody raised against a recombinant protein mapping to amino acids 50–240. The antibody against phosphorylated Stat-3 (p-Stat-3) is a mouse monoclonal IgG2b raised against a peptide corresponding to amino acid sequence containing phosphorylated Tyr-705 of Stat-3 of human origin. The antibody to EGF is an affinity-purified rabbit antibody raised against a peptide mapping to the carboxy terminus of the EGF receptor of human origin. The phospho-specific EGF antibody is a rabbit affinity-purified polyclonal antibody raised against a peptide corresponding to a short amino acid sequence containing phosphorylated Tyr-1173 of EGF of human origin. All antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Colorimetric Cell Proliferation Assay and Isobologram Analysis
A colorimetric cell proliferation assay was performed using the CellTiter 96 kit (Promega Corp., Madison, WI). Briefly, A431 cells were plated in 96-well plates (2000 cells/well) and cultured in DMEM/HAM’s F-12 supplemented with 10% FCS for 24 h. Cells were incubated in serum-free media for 24 h. EGF (10 ng/ml) was added to all wells. Tyrophostin AG1478 (0.25 mM) and AG490 (10 mM) were added alone or in combination and the culture was incubated for the appropriate time. Medium was aspirated and CellTiter 96 Aqueous One Solution Reagent (20 μl) was added to each well. The plates were incubated at 37°C for up to 1 h and absorbance recorded at 490 nm using a 96-well plate reader. Data were derived from at least three independent experiments (in triplicate) for the both single agents and combination studies. IC₅₀ values for Tyrophostin AG1478 (EGFR inhibitor) and AG490 (JAK/STAT inhibitor) were determined as described by Tsai et al. (14). The growth inhibitory effects of the combination were quantified according to the method of Chou et al. (15) using the CalcuSyn software program (Biosoft, Cambridge, United Kingdom).

Cell Counts and Viability
To assess cell viability, cells were assessed for trypan blue exclusion. In all cases, cell viability was >95%. Cell numbers were determined at each time point by manual inspection and counting using a hemocytometer. The number of cells per well was averaged from counting total cells from three wells. Cell numbers were expressed as total number of cells per well.

Results
Our first study sought to determine the total mass and relative activation states of EGFR and Stat-3 in the A431 cell line. As shown in Fig. 1A, A431 cells clearly express EGFR. They also have constitutive EGFR activation, as defined by tyrosine phosphorylation, which further

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increases (10-fold) on exposure to EGF. In examining downstream signal pathways activated by EGFR, there was no active Stat-3 at rest as determined by lack of Stat-3 tyrosine phosphorylation (Fig. 1B). On EGF stimulation, there is a significant increase in Stat-3 tyrosine phosphorylation. In this system, EGFR blockade should theoretically inhibit EGF-driven EGFR and Stat-3 activation and proliferation.

To determine the effect of selectively inhibiting EGFR, we determined the optimal dose of AG1478 to inhibit EGFR activation. As shown in Fig. 2A, increasing doses of AG1478 resulted in a progressive decrease in EGFR phosphorylation to >95% at 0.25 μM with no effect on cell viability. However, AG1478 blockade did not inhibit EGF-induced Stat-3 activation. As shown in Fig. 2B, in spite of a >95% decrease in p-EGFR, AG1478 did not decrease EGF-induced Stat-3 phosphorylation at any concentration. This suggests that only low levels of EGFR activation are required for Stat-3 phosphorylation or EGF can induce Stat-3 activation through pathways other than EGFR. These findings support our hypothesis that blockade of a pathway with a single agent may allow other pathways to remain active.

To elucidate the mechanism by which EGF continued to result in Stat-3 phosphorylation despite EGFR blockade, we hypothesized that other members of the EGFR superfamily may be activated by EGF. Specifically, we postulated that ErbB2 may activate Stat-3 in spite of near complete inhibition of EGF-induced EGFR activation with AG1478. Figure 3A demonstrates that there was incomplete blockade of ErbB2 activation by AG1478 with persistent phosphorylation of ErbB2. Thus, residual EGF-induced ErbB2 kinase activity may drive Stat-3 phosphorylation and allow continued proliferation after EGFR blockade. To test this hypothesis, we used the ErbB2 antibody, 2C4 (50 μg/ml) to determine its role in EGF-driven Stat-3 activation. 2C4 inhibits ErbB2 receptor dimerization, blocking all signaling through the receptor. As shown in Fig. 3B, AG1478 or 2C4 alone would not inhibit Stat-3 activation in spite of blocking EGFR or ErbB2 phosphorylation. However, when used in combination, these two agents resulted in complete abolition of Stat-3 phosphorylation. These data suggest that multiple signal pathways are activated by EGF exposure that will require concurrent blockade to inhibit proliferation.

To test the effect of inhibitors of EGFR, ErbB2, or the STAT pathway individually or in combination on cell proliferation, the A431 cell line was cultured with the inhibitors over 96 h. AG490 was used to selectively inhibit JAK/Stat-3 activation. At a dose of 10 μM, Stat-3 phosphorylation was decreased by >95% and cell viability was maintained (Fig. 4). Figure 5A demonstrates MTT absorbance results 72 h after EGF stimulation. By day 3, AG1478 alone resulted in a 36% (±12 SD) growth inhibition. The JAK/Stat-3 inhibitor, AG490 alone demonstrated a 53% (±18 SD) growth inhibition. When AG490 and AG1478 were combined 83% (±4.3 SD), growth inhibition was observed. The combination of AG1478 and 2C4 resulted in
a 63% (±11 SD) growth inhibition. Differences between all groups were statistically significant (P < 0.05) except for the JAK/Stat-3 inhibitor alone (AG490) versus the combination of EGFR inhibitor (AG1478) and the ErbB2 inhibitor (2C4) (P = 0.31). Using the trypan blue dye exclusion, cell viability was seen in >95% of cells in all groups. Daily cell counting confirmed the MTT results (Fig. 5B). Complete inhibition of cell line proliferation was only seen with a combination of AG1479 and AG490, while single agents yielded intermediate results.

When the IC50 of AG490 and AG1478 are used, the combination therapy is additive in its effects (combination index 1.1) (Fig. 6). However, progressive increase in the doses of each agent results in increasing synergism. At the IC50 and IC90 of each drug, the combination index is 0.7 and 0.2, respectively, indicating significant synergism by using both agents at higher doses.

**Discussion**

All clinical studies to date have demonstrated a low therapeutic efficacy for treating EGFR-expressing carcinomas with agents targeting EGFR. As most common solid cancers have accumulated multiple genetic abnormalities by the time they are clinically detected, it is unlikely that targeting one abnormality will result in significant clinical benefit. In addition, EGF or other EGFR ligands have the ability to activate other receptor systems that would be unaffected by EGFR antagonism. This is in contrast to tumors presumed to have single genetic events such as chronic myelogenous leukemia where there is constitutive Bcr/Abl kinase activity resulting in tumor growth or gastrointestinal stromal tumors (GIST) where the c-kit receptor is mutated and constitutively active (16). Investigators have traditionally combined molecular targeted agents with cytotoxic chemotherapeutic agents to increase their efficacy. Although some additive or synergistic activity can be demonstrated in vitro and in vivo, clinical studies have only shown modest efficacy in the case of HER-2-directed therapy added to chemotherapy (17) or not effective in the case of EGFR-directed treatments when combined with chemotherapy (18). We hypothesized that targeting multiple sites in the signal transduction pathway should be evaluated. EGFR, other members of the EGFR superfamily, and STAT represent important growth regulation pathways in epidermoid tumors (1, 6).

Although EGF-induced EGFR activation clearly leads to STAT pathway activation, EGFR blockade alone fails to decrease Stat-3 activation in the A431 cell line. Thus, low level EGFR activation or EGF signaling through other receptors continues to result in Stat-3 activation. In our model system, activation of ErbB2 appears to play a significant role in this regard. Indeed, studies have demonstrated the importance of ErbB2 kinase activity for constitutive Stat-3 activation. Fernandes et al. have demonstrated that STAT activation by TGF-α, another EGFR ligand, is mediated through ErbB2 kinase activity (8). On stimulation of lung epithelial cell lines with TGF-α, there is both homodimers of EGFR and heterodimers of EGFR/Erbb-2. EGFR was required, but not sufficient for the TGF-α-induced activation of Stat-3. Others have also demonstrated the importance of ErbB-2 activity for STAT pathway activation (19, 20). The rhuMAb 2C4 has been shown to block ErbB2 activation (21). In our study, the addition of 2C4 to AG1478 completely blocked Stat-3 phosphorylation. This may have important clinical implications as dual EGFR/ErbB2 blockade, in preclinical models, has recently been demonstrated to have superior antitumor activity as compared to EGFR blockade alone (22). In addition,
new small tyrosine kinase molecules have been developed with dual EGFR/ErbB2 inhibitory activities (23). Multiple clinical studies are ongoing using a combination of EGFR inhibitors and agents targeting ErbB-2 such as Herceptin. Given the lack of effect of EGFR blockade on Stat-3 activation in the presence of EGF, we hypothesized that blocking EGFR and STAT pathways will provide superior antiproliferative activity as compared to single pathway blockade. However, the doses we have employed do not affect the activation status of the AKT or MAPK pathways. Our study demonstrates significantly improved antiproliferative effects of the combined agents as compared to either agent alone in the A431 cell line, a model of EGF-driven proliferation. Although multiple agents have been developed to target EGFR, only recently has the importance of the STAT pathway been demonstrated in solid tumors with efforts brought forward to develop agents targeting these pathways (6). Nevertheless, we have proven the concept that targeting multiple pathways with small molecules may be a therapeutic strategy and in particular combined blockade of EGFR and STAT pathways may improve the therapeutic efficacy of EGFR inhibitors.

References


Figure 6. Classic isobologram for IC50, IC75, and IC90 of the EGFR inhibitor and JAK/STAT inhibitor when used in combination. Combination index is 1.1 when both drugs are used at IC50, 0.7 at IC75, and 0.2 at their IC90.
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