Effect of Simultaneous Inhibition of Epidermal Growth Factor Receptor and Cyclooxygenase-2 in HER-2/Neu-Positive Breast Cancer

Susan Lanza-Jacoby, Randy Burd, Francis E. Rosato, Jr., Kandace McGuire, James Little, Noel Nougibilly, and Sheldon Miller

Abstract
Purpose: HER-2/erbB2/neu is overexpressed in 25% to 30% of all invasive breast cancers and is associated with an aggressive course and reduced survival. HER-2/erbB2/neu breast tumors are frequently associated with up-regulation of cyclooxygenase (COX)-2 and activation of the epidermal growth factor receptor (EGFR) pathway, which promote enhanced cell growth and resistance to apoptosis. This study investigated whether simultaneously blocking both EGFR and COX-2 pathways with ZD1839 and celecoxib, respectively, would be more effective in inhibiting cell growth and inducing apoptosis than either agent alone.

Experimental Design: The effects of ZD1839, celecoxib, and their combination on cell survival, cell cycle progression, and apoptosis were determined in NMF11.2 mouse mammary tumor cells. We also investigated the potential downstream EGFR-activated pathways that are altered by simultaneous inhibition of COX-2 and EGFR.

Results: Celecoxib alone and ZD1839 alone produced a concentration- and time-dependent inhibition of cell survival. Both agents combined produced supra-additive inhibitory effects on cell survival and apoptosis compared with either agent alone. This effect was associated with increased cleaved poly(ADP-ribose)polymerase and reduced protein expression of bcl-2. Phosphorylation of extracellular signal-regulated kinase 1/2 was partially blocked by ZD1839 and celecoxib alone and was completely blocked by the combination of both agents. The enhanced proapoptotic effects of the combined agents were also associated with decreased phosphorylation of Akt and increased phosphorylation of p38.

Conclusions: These findings show that both COX-2 and EGFR are important targets for inhibiting survival and inducing apoptosis in breast cancer. The data suggest a potential cross-talk between COX-2 and EGFR signaling in breast cancer cells overexpressing HER-2/erbB2/neu.
poor survival (14). Increased expression of COX-2 was observed in ductal carcinoma in situ compared with normal breast tissue, a fact suggesting that COX-2 is an early event in the development of breast cancer and is an appropriate target in breast cancer prevention (15). The most direct evidence for the link between COX-2 and tumorigenesis was shown in a study showing that overexpression of COX-2 in the mammary gland induces mammary tumors in transgenic mice (16).

Pharmacologic studies indicate that COX-2 and EGFR may be targets for breast cancer prevention. Dietary administration of celecoxib had a significant chemopreventive effect on the development of 7,12-dimethylbenzanthracene-formed rat mammary tumors. Recent findings (12, 13) show that celecoxib prevented the development of spontaneous mammary tumors in MMTV-neu202 transgenic mice. Another study showed that an EGFR tyrosine kinase inhibitor, ZD1839 (gefitinib, Iressa), significantly delayed mammary tumor development and tumor multiplicity in MMTV-neu202 transgenic mice (17).

Recent studies have suggested that there is cross-talk between the EGFR and the COX-2 pathways (18, 19). Because there also seems to be an interaction between HER-2 and COX-2, and EGFR preferentially forms heterodimers with HER-2, we investigated whether simultaneous inhibition EGFR and COX-2 will have greater growth inhibitory and proapoptotic effects than blocking each pathway alone. In this study, we showed that the combination of the EGFR tyrosine inhibitor ZD1839 and the COX-2 inhibitor celecoxib led to a supra-additive apoptotic effect in HER-2/neu mouse mammary tumor cells. The effectiveness of the combined treatment was also associated with greater inhibition of EGF-activated EGFR and extracellular signal-regulated kinase (ERK)-1/2 than either agent alone.

Materials and Methods

Cell cultures, inhibitors, and antibodies. The MMTV-HER-2/neu cell line NMF11.2 (provided by Sandra J. Gendler, Mayo Clinic, Scottsdale, AZ) was derived from mammary gland tumors from MMTV-HER-2/neu mice. Cells were cultured in Improved MEM Zinc Option Medium without phenol red (Life Technologies, Inc., Grand Island, NY). The medium was supplemented with 5% fetal bovine serum, which had been pretreated with charcoal and dextran (HyClone, Logan, UT) and 1% GlutaMax-1 supplement solution (Life Technologies) as an additional source of glutamine. Cells were maintained at 37°C in an atmosphere of 5% CO2. To remove the adherent cells from the flasks forpassaging or counting, cells were washed with HBSS without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA (Sigma Chemical Co., St. Louis, MO) for 5 to 10 minutes, and washed with culture medium by centrifugation.

The human breast cancer cell line BT474 was obtained from the American Type Culture Collection (Manassas, VA). BT474 cells were maintained in DMEM/Ham’s F-12 containing 0.1% fetal bovine serum without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA (Sigma Chemical Co., St. Louis, MO) for 5 to 10 minutes, and washed with culture medium by centrifugation.

Running Buffers. Then, proteins were electrophoresed under reducing conditions on a NuPAGE Invitrogen gel (3-8% Tris-acetate, 10% and 12% Bis-Tris; NuPAGE Novex, Invitrogen, Carlsbad, CA) at a constant voltage (150 or 200 V depending on the gel) using NuPAGE MOPS Running Buffers. Then, proteins were transferred to a polyvinylidene difluoride membrane and blocked with specific antibodies in 5% dry milk (Vector Laboratories, Burlingame, CA) for 30 minutes before being washed three times with washing buffer. Blots were incubated for 1 hour at room temperature, washed, and developed with a chemiluminescent reagent.
antirabbit secondary antibodies. The blots were given a final wash (3 × C2) before bands were visualized by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences Corp., Piscataway, NJ) and scanned with a densitometer (Sharp JX-330), quantitated by Image Master 1D, and expressed as arbitrary units relative to h-actin or the specific unphosphorylated protein.

Production of PGE2.

NMF11.2 cells were treated with 0.5 μmol/L ZD1839, 20 μmol/L celecoxib, or their combination for 48 hours. The medium was harvested by centrifugation at 5,000 g at 4°C. The amount of PGE2 released in the medium was measured with enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

Statistical analysis. The data shown are mean values of at least three replicate experiments and expressed as means ± SE. Cell survival data were analyzed using simple linear regression equivalent to two-way ANOVA to compare the four groups. ANOVA and Bonferroni correction were used to compare data for cell cycle, apoptosis, and protein expression. Statistical analyses were conducted using SAS 8.2 software package (SAS Institute, Inc., Cary, NC).

Results

Celecoxib plus ZD1839 inhibited cell growth in NMF11.2 cells in a supra-additive manner. NMF11.2 cells were incubated with different concentrations of celecoxib (0-40 μmol/L) for 24, 48, and 72 hours or ZD1839 (0-5 μmol/L) for 24, 48, and 72 hours. Analysis of cell growth using the CellTiter assay showed that celecoxib and ZD1839 produced a concentration-dependent and time-dependent inhibition of cell growth. The dose of 20 μmol/L celecoxib alone and 0.5 μmol/L ZD1839 alone inhibited cell growth by ≈ 50% (Fig. 1A and B). These concentrations were used as single or combined agents in the following experiments. Figure 1C shows that after 72 hours of incubation, cell growth was inhibited by 50%, 65%, and 89% with 20 μmol/L celecoxib, 0.5 μmol/L ZD1839, and the combination of the two agents, respectively.

The combination of celecoxib plus ZD1839 enhanced apoptosis. To determine whether the growth inhibitory effects of the combined treatment could be attributed to a delay in the cell cycle or increased apoptosis, we evaluated cell cycle progression and apoptosis with flow cytometric assays. NMF11.2 cells were treated with celecoxib, ZD1839, and the combination of these agents. Table 1 shows that celecoxib induced a G2 block; adding ZD1839 negated this effect. Celecoxib in combination with ZD1839 led to an accumulation of cells in the G0-G1 phase of the cell cycle in comparison with each of the single agents. Accordingly, a smaller fraction of NMF11.2 cells entered the DNA/synthesis phase.

Cytokeratin-18 degradation, identified with the M30 monoclonal antibody, was used to determine whether celecoxib and ZD1839 altered the early phase of apoptosis. Figure 2A shows that ZD1839, celecoxib, and the combination produced a 5-, 2-, and 16-fold increase, respectively, in the level of M30 antigen. We also determined cleavage of poly(ADP-ribose)polymerase as a marker of early apoptosis with Western blot

Table 1. Effect of ZD1839 and celecoxib on cell cycle distribution of NMF11.2 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2-M (%)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>43.3 ± 1.19</td>
<td>39.7 ± 0.25</td>
<td>16.8 ± 1.33</td>
</tr>
<tr>
<td>ZD1839</td>
<td>56.0 ± 0.56*</td>
<td>30.2 ± 0.58*</td>
<td>13.8 ± 1.16</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>38.8 ± 1.35*</td>
<td>25.7 ± 0.56*</td>
<td>35.5 ± 1.52*</td>
</tr>
<tr>
<td>ZD1839 + celecoxib</td>
<td>79.6 ± 1.58*</td>
<td>12.2 ± 0.99*</td>
<td>8.2 ± 1.05*</td>
</tr>
</tbody>
</table>

NOTE: NMF11.2 cells were incubated for 48 hours with vehicle or with 0.5 μmol/L ZD1839, 20 μmol/L celecoxib, or 0.5 μmol/L ZD1839 + 20 μmol/L celecoxib. Cells were harvested and stained with propidium iodide; DNA content was analyzed by flow cytometry as described in Materials and Methods. Values are means of three independent experiments ± SE.

*P < 0.05, compared with vehicle, celecoxib, or ZD1839.
Analysis. Poly(ADP-ribose)polymerase cleavage was increased by the combination of ZD1839 and celecoxib (Fig. 2B). Apoptosis was confirmed by the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay. Figure 2C illustrates that the apoptotic population of cells increased in a supra-additive manner with the combined treatment as compared with either agent alone (control, 2.4%; celecoxib, 7.6%; ZD1839, 8.0%; and celecoxib + ZD1839, 20.9%). These three methods of evaluating apoptosis provide strong evidence that combining ZD1839 and celecoxib enhances the apoptotic effects of each agent alone.

The combination ZD1839 plus celecoxib decreased bcl-2. Both bcl-2 and bax are involved in the balance between antiapoptotic and proapoptotic signals; bcl-2 prevents apoptosis and bax induces cell death. We determined the effects of ZD1839, celecoxib, and the combination of both agents on the protein expression of bcl-2 and bax. Quantification of the Western blots by densitometry is shown in Fig. 3. The combined treatments significantly decreased the relative density of bcl-2 more than the individual treatments ($P = 0.004$). There was no change in the protein levels of bax. The ratio of the relative densities of bax/bcl-2 (vehicle, 1.8; ZD1839, 2.5; celecoxib, 2.4; and the combination, 10.4) was increased by the combination of ZD1839 and celecoxib in favor of apoptosis.

ZD1839 decreased COX-2 protein expression. NMF11.2 cells were incubated for 48 hours with ZD1839 and celecoxib. The effect of the treatments on COX-2 protein expression was evaluated by Western blot. Treating the cells with ZD1839 significantly decreased protein expression of COX-2 in comparison with the vehicle-treated and celecoxib-treated samples ($P < 0.04$), which suggests that EGFR signaling may be involved in the regulation of COX-2 (Fig. 4). Celecoxib treatment produced a small increase in the level of COX-2 protein. The observed inhibition of COX-2 protein with the combination treatment seemed to be due to the effect of ZD1839.

Celecoxib reduced PGE2 production. To determine the effect of the combined treatments on COX-2 activity, the NMF11.2 cells were treated with ZD1839, celecoxib, and the combined agents for 48 hours; the medium was obtained by centrifugation and analyzed for PGE2 by an ELISA. Celecoxib alone significantly ($P < 0.01$) decreased the production of PGE2; the decrease in PGE2 production observed with the combination treatment seems to be due to celecoxib as ZD1839 had a minimal effect on PGE2 production (Table 2).

ZD1839 and celecoxib decreased phosphorylation of EGFR and HER-2/neu. To investigate the potential interaction of the COX-2 and EGFR signaling pathways in HER-2-overexpressing mammary tumors, we determined whether the combined treatment with ZD1839 and celecoxib decreased EGF-induced phosphorylation of EGFR and HER-2 to a greater extent than either agent alone. NMF11.2 cells were serum starved overnight and pretreated with ZD1839 and celecoxib followed by EGF.
The stress-activated p38 mitogen-activated protein kinases are associated with both cell survival (20) and apoptosis (21). The response may vary with different cell types, the external stimulus, or the presence of different isoforms (22). For example, it has been reported that the p38α and p38β isoforms are involved in apoptosis (23). When NMF11.2 cells were treated with ZD1839, there was no change in p38 phosphorylation (Fig. 6C). In contrast, celecoxib treatment decreased p38 phosphorylation ($P < 0.01$) compared with vehicle, ZD1839, or ZD1839 + celecoxib treatments. However, the combination of ZD1839 and celecoxib increased p38 phosphorylation 3-fold ($P < 0.001$) compared with the other treatments.

### Discussion

There is recent evidence to suggest a cross-talk between the EGFR and COX-2 signaling pathways in a number of tumor cells. Studies have found that COX-2-derived PGE$_2$ can transactivate EGFR in colon cancer cells (18, 24) and in cholangiocarcinoma cancer cells (25). In turn, activation of EGFR increased COX-2 expression and PGE$_2$ production in squamous carcinoma cells (26) and colon cancer cells (27). Inhibition of EGFR tyrosine kinase in squamous carcinoma cells has been shown to decrease COX-2 expression (26).

In the present study, we provide the first evidence in breast cancer cells that the combination of the EGFR tyrosine inhibitor ZD1839 and the COX-2 inhibitor celecoxib leads to a supra-additive induction of apoptosis and inhibition of EGFR phosphorylation. Our findings also show that ZD1839 in combination with celecoxib reduces downstream EGF-induced phosphorylation of ERK and Akt and stimulates p38 phosphorylation in breast cancer cells.

Both the EGFR and the PG signaling pathways promote cell survival through activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. Overexpression of HER-2/neu enhances the EGFR signaling response (5, 6), resulting in activation of the Ras/Raf/mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase C pathways, and up-regulates COX-2 expression (9, 10). In colon and cholangiocarcinoma cells, PGE$_2$ transactivates EGFR leading to downstream signaling through four PGE (EP)$_{1-4}$ receptors (18, 24, 25). We have also observed that PGE$_2$ transactivated EGFR in NMF11.2 cells (data not shown). This evidence provides a strong rational for targeting both EGFR and COX-2 in HER-2-overexpressing breast cancer.

### Table 2. Effect of celecoxib, ZD1839, and celecoxib + ZD1839 on COX-2 activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE$_2$ (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>595.1 ± 61.4</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>26.3 ± 1.4*</td>
</tr>
<tr>
<td>ZD1839</td>
<td>410 ± 83.7</td>
</tr>
<tr>
<td>Celecoxib + ZD1839</td>
<td>29.6 ± 0.9*</td>
</tr>
</tbody>
</table>

**NOTE:** NMF11.2 cells were treated with vehicle, 20 μmol/L celecoxib, 0.5 μmol/L ZD1839, or their combination for 48 hours. COX-2 activity was measured in the medium by determining PGE$_2$ levels with ELISA. Values are means ± SD ($n = 4$).

* $P < 0.01$, compared with vehicle or ZD1839.
As single agents, COX-2 and EGFR tyrosine kinase inhibitors have had limited success in preventing the development of spontaneous mammary tumors in mice. We observed that diets containing 900 ppm of celecoxib reduced the incidence of mammary tumors by 29% in HER-2/neu transgenic mice at 14 months of age (13). Another study by Howe et al. (12) found that celecoxib fed at 500 ppm reduced mammary tumor incidence by ~15% at 12 months of age. When HER-2/neu transgenic mice were fed a high dose of ZD1839 (100 mg/kg), tumor incidence was reduced by 75% at 10 months of age (17). The improved efficiency of combining these agents was shown in a study where the combination of an EGFR tyrosine inhibitor with a non-specific nonsteroidal anti-inflammatory drug sulindac protected APCMin/+ mice from intestinal neoplasia (28). More recent studies have shown that the combination of EGFR and COX-2 inhibitors has a greater antitumor effect in colon cancer (29) and in squamous cell carcinomas of the head and neck (30) than each agent alone.

In this study, we observed a greater apoptotic effect when treating NMF11.2 cells for 48 hours with the combination of ZD1839 plus celecoxib (30%) than ZD1839 treatment alone (8%) or celecoxib alone (7.8%). Consistent with our previous observation, inhibition of COX-2 led to a G2-M block in the cell cycle (31). Combining ZD1839 with celecoxib abrogated the G2-M block, forcing the cells into the cell cycle before repair was completed, thus inducing apoptosis. Several pathways leading to apoptosis converge at the mitochondrial membrane; the breakdown of the mitochondrial membrane is controlled, in part, by the balance of bax-2 and bax in cells (32). The protein expression of bcl-2 decreased in cells treated with celecoxib alone or the combination of ZD1839 + celecoxib, whereas there was no change in bax protein expression. Thus, the ratio of bax to bcl-2 was altered in favor of apoptosis. The role of bcl-2 in bcl-2-mediated apoptosis may be cell type specific. In prostate (33) and colon cancer cells (34), decreased bcl-2 expression was associated with COX-2 inhibition. In contrast, induction of apoptosis by celecoxib in cholangiocarcinoma cells was independent of bcl-2 (35). The enhanced apoptotic effect of celecoxib treatment alone seemed to be associated with a COX-2-independent effect; the concentration of celecoxib (20 μmol/L) that induced apoptosis was higher than the concentration (5 μmol/L) found to inhibit PGE2 production from NMF11.2 cells (31). Celecoxib has been shown to inhibit tumor cell growth and induce apoptosis by targeting non-COX-2 pathways such as Akt (36), 3-phosphoinositide-dependent kinase-1 activity (37), nuclear factor-κB (38), the mitochondrial membrane potential, activation of caspase-9 (39), peroxisome proliferator–activated receptor-γ (40), and β-catenin/adenomatous polyposis coli signaling (41).

In the NMF11.2 cells, celecoxib treatment increased COX-2 whereas ZD1839 decreased COX-2 expression (Fig. 4). Although the COX-2 protein increased, celecoxib significantly reduced the production of PGE2. The mechanism for the celecoxib-induced increase in COX-2 expression is not known but may be due to the removal of a negative feedback regulator(s) that controls COX-2 protein synthesis and/or breakdown. Other studies also reported that celecoxib increased COX-2 protein expression in squamous cell carcinoma (42) and MDA-MB231 breast cancer cells (43).

Previous studies have found that breast cancer cells with HER-2 overexpression are particularly sensitive to ZD1839 (44, 45). EGF, transforming growth factor-α, and amphiregulin stimulate phosphorylation of EGFR, which activates...
downstream mitogen-activated protein kinase/ERK, phosphatidylinositol 3-kinase/Akt, and phospholipase C pathways. We have observed that treating NMF11.2 cells with 0.5 μmol/L ZD1839 before EGF stimulation reduced phosphorylation of both EGFR and HER-2, which was accompanied by a decrease in cell survival. NMF11.2 cells express both EGFR and HER-2 and have very low levels of HER-3 and HER-4, which suggests that ZD1839 disrupts cell survival by targeting EGF/HER-2 heterodimers. Selective inhibition of COX-2 with celecoxib also reduced EGF-induced phosphorylation of EGFR, although not to the same extent as ZD1839. Recent reports have documented in colon cancer cells (18, 24) and in cholangiocarcinoma cancer cells (19) that COX-2-derived PGE2 trans-activated EGFR through the G protein–coupled receptor EP1. PGs mediate their signaling effects by interacting with specific EP1-4 receptors (46). Because EGF has been shown to stimulate COX-2 expression and PGE2 production, the reduction in EGF-induced phosphorylation of EGFR observed with celecoxib treatment suggests that the increase in EGF-induced PGE2 contributes to transactivation of EGFR in these cells.

Studies have reported that ZD1839 decreased HER-2 phosphorylation in BT474 (47) and SkBr3 (44) breast cancer cells with HER-2 overexpression. ZD1839 at 0.5 μmol/L also reduced HER-2 phosphorylation in NMF11.2 cells, whereas our laboratory and others (47) have found that a higher concentration of ZD1839 (10 μmol/L) was required to inhibit HER-2 phosphorylation in BT474 cells. In NMF11.2 and BT474 cells, celecoxib partially reduced HER-2/neu phosphorylation and the combination of ZD1839 with celecoxib completely blocked phosphorylation of HER-2/neu. These observations support the premise that there is interaction of COX-2 and EGFR signaling in breast cancer cells with overexpression of HER-2/neu.

Blocking EGF alone or COX-2 alone has been shown to reduce EGF-induced activation of the mitogen-activated protein kinase and Akt pathways and induce apoptosis in cancer cells (36, 37, 44, 47). In this study, we observed a supra-additive inhibitory effect on EGF-induced phosphorylation of ERK1/2 and Akt when ZD1839 and celecoxib were combined.

We next examined the effect of inhibiting EGF and COX-2 on p38 phosphorylation because of the importance of p38 in mediating stress response, which may occur when receptor tyrosine kinase survival pathway is disrupted. Treatment with ZD1839 alone induced a modest increase in p38 phosphorylation whereas celecoxib treatment decreased phosphorylated p38. Previous studies have shown that EGF and HER-2/neu tyrosine kinase inhibitors induced p38-dependent apoptosis in prostate cancer cells (48) and in HER-2-overexpressing breast cancer cells (49). This is the first study to show that the combined treatments synergistically increased p38 phosphorylation, creating an imbalance between ERK and p38 activation. These events correlate with the greater apoptotic effect observed with the combined treatment. Studies are ongoing to determine the role of these signaling molecules in mediating the enhanced apoptotic effects of the combined treatments.

Previous studies have shown that long-term treatment of squamous cell carcinoma of the head and neck with ZD1839 and celecoxib produced additive/synergistic growth inhibitory
effects and reduced basal levels of phosphorylated EGFR, ERK1/2, and Akt in comparison with the single agents (30). In contrast to our study, these cell culture experiments were conducted with 10% fetal bovine serum. In this study, the effectiveness of the treatments in blocking EGFR, ERK1/2, and Akt phosphorylation was determined in serum-starved cells that were stimulated with EGF. We found that treatment with celecoxib alone reduced expression of EGF-stimulated phosphorylated ERK and EGFR/2 in HER-2-overexpressing NMF11.2 cells. In squamous cell carcinoma of the head and neck that were cultured in serum, celecoxib did not have an effect on EGFR and ERK2 phosphorylation (30, 42). Serum-containing medium has been shown to block the growth inhibitory effects of celecoxib in pancreatic (50) and prostate cancer cells (51).

In summary, this is the first evidence that ZD1839 in combination with celecoxib promotes a supra-additive apoptotic response in breast cancer cell lines with overexpression of HER-2/neu. The enhanced growth inhibitory effect exhibited by the combined treatment was related to inhibition of EGF-dependent activation of EGFR, ERK1/2, and Akt and induction of p38. Our study provides an experimental basis to suggest that the combination of COX-2 and EGFR tyrosine kinase inhibitors may be useful for protection against HER-2-overexpressing breast cancer.

Acknowledgments

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