Epidermal growth factor receptor (EGFR)–related protein inhibits multiple members of the EGFR family in colon and breast cancer cells

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Abstract

Inactivation of epidermal growth factor receptor (EGFR) family members represents a promising strategy for the development of selective therapies against epithelial cancers. Current anti-EGFR therapies, such as cetuximab (Erbitux), gefitinib (Iressa), or trastuzumab (Herceptin), target EGFR or HER-2 but not both. Because solid tumors express different EGFRs, identification of inhibitor(s) targeting multiple EGFR family members may provide a therapeutic benefit to a broader patient population. We have identified a natural inhibitor of EGFRs called EGFR-related protein (ERRP), a 53 to 55 kDa protein that is present in most, if not all, normal human epithelial cells. The growth of colon (HCT-116, Caco2, and HT-29) and breast (MDA-MB-468 and SKBR-3) cancer cells expressing varying levels of EGFR, HER-2, and/or HER-4 was inhibited by recombinant ERRP in a dose-dependent manner. In contrast, ERRP caused no inhibition of growth of normal mouse fibroblast cell lines (NIH-3T3, NIH-3T3/PT-67), and the growth of nontransformed rat small intestinal IEC-6 cells expressing relatively low levels of EGFRs was inhibited only at high doses of ERRP. Transforming growth factor-α or heparin-binding epidermal growth factor–induced activation of EGFR and HER-2 was inhibited by ERRP in colon and breast cancer cells expressing high levels of EGFR or HER-2. In contrast, cetuximab inhibited the growth- and ligand-induced activation of EGFR in cell lines expressing high levels of EGFR, whereas trastuzumab was effective only in HER-2–overexpressing cells. ERRP and trastuzumab, but not cetuximab, attenuated heregulin-α–induced activation of colon and breast cancer cells that expressed high levels of HER-2. Furthermore, ERRP, but not cetuximab or trastuzumab, significantly induced apoptosis of colon and breast cancer cells. None of these agents induced apoptosis of either NIH-3T3 mouse fibroblast or normal rat small intestinal IEC cells. Our results suggest that ERRP is an effective pan-erbB inhibitor and, thus, may be a potential therapeutic agent for a wide variety of epithelial cancers expressing different levels and subclasses of EGFRs. [Mol Cancer Ther 2005;4(3):435–42]

Introduction

Members of the receptor tyrosine kinase family, which include epidermal growth factor receptor (EGFR), ErbB-2/HER-2, ErbB-3/HER-3, and ErbB-4/HER-4, are frequently implicated in experimental models of epithelial cell neoplasia in animals as well as in human cancers (1–4). Interference with growth factor receptor activation and/or with intracellular growth factor–activated signal transduction pathways represents a promising strategy for the development of novel and selective anticancer therapies (5–7). A large body of experimental evidence supports a key role for EGFR and HER-2 in a wide variety of human epithelial cancers (1, 3, 5, 7). Overexpression and/or activation of EGFR have been associated with resistance to cytotoxic drugs, and are generally considered indicators of poor prognosis (7–10). Indeed, small molecule inhibitors of EGFR, such as gefitinib (Iressa) and erlotinib (Tarceva, OSI-774), have progressed to large-scale randomized clinical trials, but with limited success (5). With respect to gefitinib, it has recently been approved for clinical use and showed that it is effective only in a subgroup of non–small cell lung cancer patients who have specific mutations in the *EGFR* gene (11, 12).

Monoclonal antibodies to EGFR (cetuximab/IMC-C225/Erbitux) and HER-2 (trastuzumab/Herceptin) have been approved by the Federal Drug Administration for treatment of tumors that express high levels of EGFR and HER-2, respectively. Although Erbitux and Herceptin treatments showed signs of success, failure in some patients may partly be due to coexpression of multiple EGFR family members, which may lead to an enhanced transforming potential and worsened prognosis (7–10, 13, 14). Therefore, identification of inhibitor(s), targeting multiple members of the EGFR family, may provide a greater therapeutic benefit to a broader range of patient population.

To this end, we investigated the effectiveness of EGFR-related peptide (ERRP) as a pan-erbB inhibitor that targets multiple members of the EGFR family. ERRP, a recently isolated negative regulator of EGFR, is a 53 to 55 kDa

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protein that possesses substantial homology to the extracellular ligand–binding domain of EGFR and its family members (15). ERRP expression has been found to be high in benign colonic and gastric mucosa as well as in the liver and pancreas, but low in the respective carcinomas of those tissues (16–21). Recent data from this laboratory further suggest that ERRP could be a potential therapeutic agent for colorectal and prostate cancers (16, 22, 23). This is supported by the observation that i.t. or s.c. injections of recombinant ERRP inhibits colon cancer HCT-116 cells xenograft tumors in severe combined immunodeficiency mice without any sign of toxicity (16, 22). *In vitro* studies have shown that ERRP inhibits proliferation of colon and prostate cancer cells (15, 16, 23). These changes have been attributed to ERRP-induced attenuation of basal and ligand-induced activation of EGFR (15, 16, 23). The present investigation was undertaken to determine whether ERRP could be a pan-erbB inhibitor. We examined the effects of recombinant ERRP on the growth and ligand-induced activation of EGFR and HER-2 in several colon and breast cancer cell lines that express varying levels of EGFR and other member(s) of its family, specifically HER-2. Additionally, we compared the growth inhibitory effect of ERRP with that of cetuximab or trastuzumab, monoclonal antibodies to EGFR and HER-2, respectively.

Materials and Methods

Cell Lines

In the present investigation, three colon cancer cell lines, HCT-116, Caco2, and HT-29, and two breast cancer cell lines, MDA-MB-468 (also called MDA-468) and SKBR-3, were utilized. In addition, nontransformed IEC-6 cells (derived from rat small intestinal crypt) and mouse fibroblast cell lines (NIH-3T3 and NIH-3T3-PT-67) were included. All cell lines, with the exception of NIH-3T3-PT-67, were purchased from the American Type Culture Collection (Rockville, MD). Mouse fibroblast cell line PT-67 was obtained from Clontech (Palo Alto, CA). The cell lines were maintained in RPMI or DMEM medium supplemented with 10% fetal bovine serum, 100 units/mL streptomycin-penicillin, and incubated at 37°C in an atmosphere of 95% air and 5% CO_2. The reason for choosing these cell lines is that they express varying levels of EGFR and/or other member(s) of its family as depicted later in Fig. 1A.

Generation of ERRP Fusion Protein

ERRP fusion protein was generated using the *Drosophila* expression system (Invitrogen, Carlsbad, CA) as previously described (16). The stable Schneider 2 *Drosophila* cell lines containing pMT/ERRP-V5-His plasmid were incubated in the presence of 0.5 mmol/L $CuSO_4$ to stimulate the expression of V5-His–tagged ERRP fusion protein, and subsequently lysed in lysis buffer (50 mmol/L Tris; 100 mmol/L NaCl; 2.5 mmol/L EDTA; 1% Triton X-100; 1% Nonidet P-40; 2.5 mmol/L Na_3VO_4; 25 μg/μL aprotinin; 25 μg/μL leupeptin; 25 μg/L pepstatin A; 1 mmol/L phenylmethylsulfonyl fluoride). ERRP was then purified from Schneider 2 cell lysate by two sequential immunoaffinity columns: Anti-ERRP antibodies followed by a second column of anti-polyhistidine antibodies as previously described (16). Silver staining of the SDS-PAGE showed a predominant protein band of M_r 53 to 55 following purification, a molecular weight that corresponded well with the expected value of ERRP, which is composed of 479 amino acids (data not shown).

Growth Inhibition Assay

Inhibition of cell growth in response to recombinant ERRP, cetuximab, or trastuzumab was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (16). Briefly, aliquots of cells (3×10^4 cells/mL) in DMEM/10% fetal bovine serum were plated in a 96-well culture plate with five replicates per treatment. After 24 hours of plating, the medium was

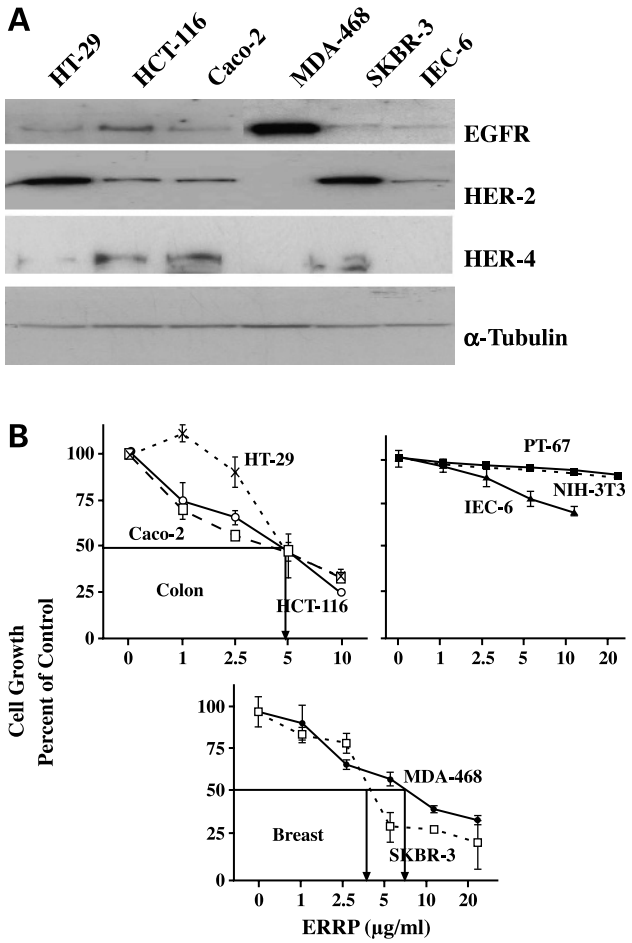

Figure 1. **A,** Western blot showing EGFR, HER-2, and HER-4 levels in different colon (HT-29, HCT-116, and Caco2) and breast (MDA-468 and SKBR-3) cell lines, and nontransformed rat small intestinal cell line IEC-6. Each lane contained 25 μg protein. **B,** effect of increasing concentrations of recombinant ERRP on the growth [determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] of colon and breast cancer cell lines as well as on cell lines derived from fibroblasts (NIH-3T3 and NIH-3T3/PT-67) and nontransformed rat small intestine (IEC-6). *Points,* mean of six to seven independent determinations; *bars,* SE.

replaced with the medium containing 2.5% fetal bovine serum, and the incubation at 37°C continued in the absence (control) or presence of recombinant ERRP, cetuximab, or trastuzumab for 48 hours as stated in the legends to figures. All incubations were terminated by adding 20 μL of 0.5 g/mL stock of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to each well. The reaction was allowed to proceed for 3 to 4 hours at 37°C. The culture medium was removed and formazan crystals were dissolved by adding 0.2 mL DMSO, and the intensity of color was measured at 570 nm.

Assessment of Apoptosis

The cell lines ($\sim 1 \times 10^5$/well) were plated in DMEM/10% fetal bovine serum. After 24 hours of plating, the medium changed to contain 2.5% fetal bovine serum to minimize the contribution of serum-derived growth factors and subsequently incubated for 24 hours in the absence (control; vehicle added) or presence of ERRP (5 μg/mL), cetuximab (20 μg/mL), or trastuzumab (20 μg/mL). At the end of the incubation period, the cells were lysed and changes in apoptosis were determined using the Cell Death Detection ELISA^{PLUS} kit from Roche Diagnostics GmbH (Penzberg, Germany), which measures the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). Briefly, cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Then, samples were incubated with anti–DNA peroxidase followed by color development with ABTS substrate. The optical densities of the samples were determined by the Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 405 nm.

Ligand-Induced Activation of EGFR and HER-2 and Western Blot Analysis

To determine whether and to what extent recombinant ERRP, cetuximab, or trastuzumab will affect the ligand-induced activation of EGFR or HER-2, aliquots of ($\sim 1 \times 10^5$ cells/well) colon (HCT-116 and HT-29) and breast cancer cell lines (MDA-MB-468 and SKBR-3) were plated in DMEM/10% fetal bovine serum in six-well culture plates. After 24 hours of plating, they were serum starved for 48 hours and subsequently incubated for another 24 hours in the absence (controls) or presence of recombinant ERRP (10 μg/mL), cetuximab (20 μg/mL), or trastuzumab (20 μg/mL), and then exposed to 5 nmol/L TGF-α or heparin-binding EGF (HB-EGF) or heregulin-α for 7 minutes. Parallel incubations were also done with TGF-α, HB-EGF, or heregulin-α in the absence of ERRP, cetuximab, or trastuzumab. Incubations were terminated by adding lysis buffer [50 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 2.5 mmol/L EDTA, 1% Triton X-100, 0.5% NP40, 2.5 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL aprotinin and leupeptin, 50 μg/mL soybean trypsin inhibitor]. The lysate was rotated for 30 minutes at 4°C and subsequently centrifuged at 11,000 × *g* for 15 minutes at 4°C. The supernatant was used for Western blot analysis after determination of protein concentration by the protein assay kit from Bio-Rad Laboratories (Hercules, CA).

Aliquots containing 50 μg protein were separated on 7.5% SDS-PAGE and then electroblotted to a nitrocellulose membrane. The membrane was blocked overnight with 5% nonfat dried milk in TBS-T buffer [20 mmol/L Tris (pH 7.6), 100 mmol/L NaCl, 0.1% Tween 20], followed by 3-hour incubation with the primary antibodies, phospho-EGFR or phospho-HER-2 (Upstate Biotechnology, Lake Placid, NY) at a final dilution of 1:1,000 in TBS-T buffer containing 5% nonfat dried milk at room temperature. After washing thrice with TBS-T buffer, the membranes were incubated with a horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5,000 dilutions) for 1 hour at room temperature. Proteins were visualized using enzyme-linked enhanced chemiluminescence detection system (ECL, Amersham, Arlington Heights, IL). Membranes were stripped (twice for 15 minutes at 55°C) in stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7). The membranes were then reprobed with β-actin antibodies (Boehringer Mannheim, Indianapolis, IN) as an internal control. Signals on the blots were visualized by autoradiography.

In experiments where total EGFR, HER-2, HER-3, or HER-4 levels were determined, 25 and 50 μg protein from different cell lines were subjected to Western blot analysis as described above using corresponding antibodies. Polyclonal antibodies to EGFR, HER-2, and monoclonal antibodies to HER-3 were purchased from Upstate Biotechnology, whereas anti-HER-4 polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis

Unless otherwise stated, data are expressed as mean ± SD. Where applicable, the results were compared by using the unpaired Student *t* test taking $P < 0.05$ as the level of significance.

Results

To determine whether and to what extent ERRP will be effective in inhibiting growth of epithelial cancers that express varying levels of EGFR and/or other member(s) of its family, several colon (HCT-116, Caco2, and HT-29) and breast (MDA-MB-468 and SKBR-3) cancer cell lines were utilized. In addition, nontransformed IEC-6 cells, derived from rat small intestinal crypt and mouse fibroblast cell lines NIH-3T3 and NIH-3T3-PT-67, were included as controls.

The relative levels of EGFR and its family members in colon and breast cancer cell lines as well in IEC-6 cells were determined by Western blot. The breast cancer cell lines MDA-468 and SKBR-3 expressed high levels of EGFR and HER-2, respectively, whereas the colon cancer cell lines HCT-116 and Caco2 as well as the nontransformed rat small intestinal cell line IEC-6 expressed moderate to low levels of EGFR and HER-2 (Fig. 1A). The colon cancer cell line HT-29 expressed high levels of HER-2, which were similar to that observed in SKBR-2 cells. However, the

levels of EGFR in HT-29 were relatively low (Fig. 1A). All cell lines expressed relatively low levels of HER-4, but HER-3 could not be detected in any of these cell lines (Fig. 1A). The latter could be due to the lack of sensitivity of the commercial antibodies because neither 25 nor 50 μg of total cellular protein provided any detectable levels of HER-3.

However, in spite of different levels of EGFR and/or other member(s) of its family, recombinant ERRP inhibited growth of the colon and breast cancer cell lines in a dose-dependent manner with IC_{50} varying between 4 and 7 μg/mL (Fig. 1B). In contrast to what we have observed in colon and breast cancer cell lines, ERRP caused no apparent change in growth of mouse fibroblast cell lines, NIH-3T3 and NIH-3T3/PT-67 (Fig. 1B), but the growth of the nontransformed rat small intestinal cell line IEC-6 was inhibited only at high doses of ERRP (Fig. 1B). The observed differences in IC_{50} of ERRP among the cell lines could be due to our use of different preparations of recombinant ERRP.

In recent years, a number of pharmacologic inhibitors of EGFR tyrosine kinases as well as antibodies directed against the EGFR and HER-2 have been developed (5). A monoclonal antibody to EGFR, cetuximab (IMC-C225, Erbitux), which has earlier been shown to inhibit EGF binding and anchorage-dependent and anchorage-independent growth and EGF-induced tyrosine kinase–dependent EGFR phosphorylation as well as growth of established tumor xenografts (24, 25), has recently been approved by the Federal Drug Administration for treatment of colorectal cancer expressing high levels of EGFR. Trastuzumab (Herceptin), a monoclonal antibody against HER-2, was approved earlier by the Federal Drug Administration for treatment of breast cancer expressing high levels of HER-2.

We postulate that ERRP may be a pan-erbB inhibitor that targets multiple members of the EGFR family. To test our hypothesis, we compared the effects of recombinant ERRP with cetuximab (Erbitux) and trastuzumab (Herceptin) on the growth of colon (HCT-116 and HT-29) and breast (MDA-468 and SKBR-3) cancer cells that express varying levels of EGFR and/or other member(s) of its family. As observed earlier, irrespective of the levels of EGFR, HER-2, and/or HER-4, the growth of all four cell lines was inhibited by recombinant ERRP in a dose-dependent manner (Fig. 2A and B). On the other hand, cetuximab inhibited the growth of HCT-116 and MDA-468 cells, but had no effect on SKBR-3 cells that expressed high levels of HER-2 (Fig. 2A). Moreover, although cetuximab caused inhibition of the growth of HT-29 cells that expressed high levels of HER-2, this inhibition was achieved only at high doses of the agent (Fig. 2A). Conversely, trastuzumab was effective in inhibiting the growth of HT-29 and SKBR-3 cells, but had no effect on MDA-468 cells, which expressed very high levels of EGFR (Fig. 2B). In fact, trastuzumab, at high doses, slightly stimulated the growth of MDA-468 cells. Growth of HCT-116 cells that expressed moderate to

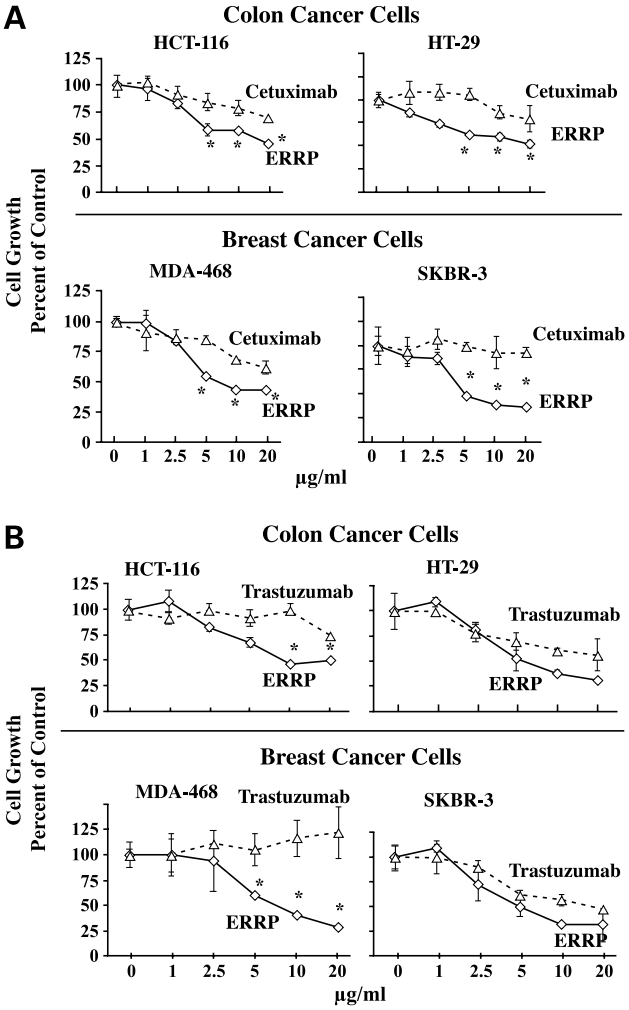

Figure 2. Effects of increasing concentrations of recombinant ERRP, cetuximab (**A**), or trastuzumab (**B**) on the growth of colon (*HCT-116* and *HT-29*) and breast (*MDA-468* and *SKBR-3*) cancer cell lines. Cells were exposed to recombinant ERRP, cetuximab, or trastuzumab for 48 h. *Points,* mean of six to seven determinations; *bars,* SE. **P* < 0.01, compared with the corresponding values of cetuximab- or trastuzumab-treated cells.

low levels of EGFR, HER-2, and HER-4 was inhibited only at high doses of trastuzumab (Fig. 2B). Additionally, we observed that magnitude of inhibition of the growth of both colon and breast cancer cells induced by ERRP was significantly or tended to be higher than that was achieved in response to either cetuximab or trastuzumab (Fig. 2A). This suggests a greater responsiveness of these cell lines to ERRP, compared with either cetuximab or trastuzumab.

Results of the foregoing study suggest that whereas cetuximab and trastuzumab are effective in inhibiting the growth of cells that express high levels of EGFR and HER-2, respectively, ERRP is effective in cells that express high levels of EGFR or other member(s) of its family. Ligand binding to EGFR and/or its family members results in

activation of the receptors and the subsequent signal transduction pathways leading to stimulation of growth. Although HER-2 has no known ligand, it is a heterodimerization partner for EGFR and other members of the EGFR family, with transactivation of HER-2 occurring following heterodimerization (3).

Because activation of EGFR and/or its family member(s) is one of the early events in the induction of signal transduction pathways, we compared the effects of ERRP with cetuximab or trastuzumab on ligand-induced activation (tyrosine phosphorylation) of EGFR and HER-2 in colon and breast cell lines that express varying levels of EGFR and/or other member(s) of its family. As expected, in the absence of ERRP, cetuximab, or trastuzumab, there was a marked induction of activation of EGFR and HER-2 by TGF-α and HB-EGF in colon (HCT-116 and/or HT-29) and breast (MDA-468 and/or SKBR-3) cancer cells, which was evidenced by increased levels of tyrosine phosphorylated form of EGFR and HER-2, when compared with the corresponding vehicle-treated controls (Fig. 3A and B).

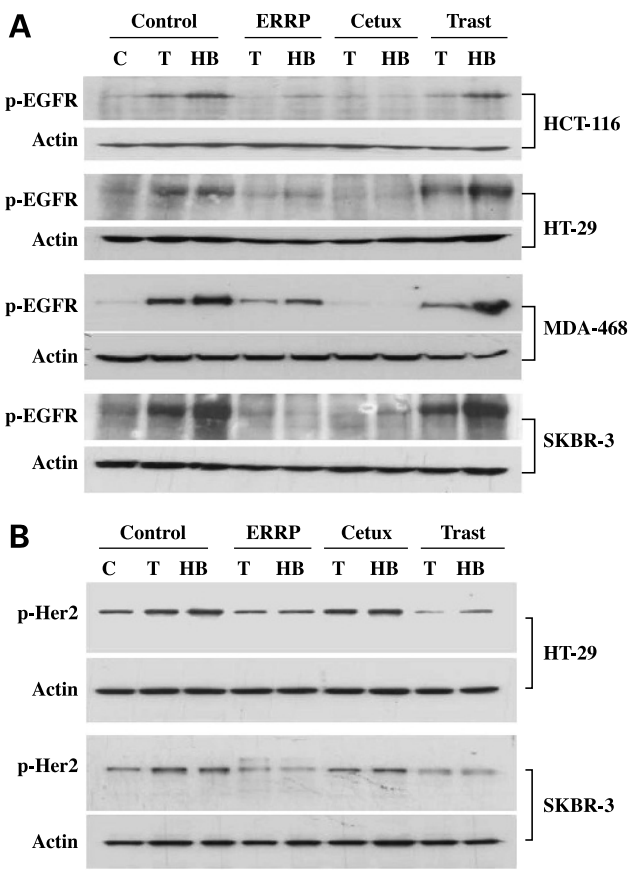

Figure 3. Attenuation of TGF-α (*T*) and HB-EGF (*HB*) induced tyrosine phosphorylation of EGFR (*p-EGFR*; **A**) and Her-2 (*p-Her2*; **B**) in the presence of recombinant ERRP, cetuximab (*Cetux*), or trastuzumab (*Trast*) in colon (*HCT-116* and *HT-29*) and breast (*MDA-468* and *SKBR-3*) cancer cells. Actin served as the loading control.

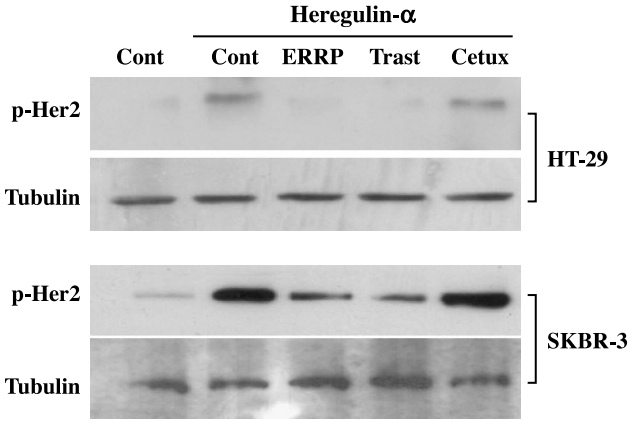

Figure 4. Attenuation of heregulin-α – induced tyrosine phosphorylation of HER-2 (*p-Her2*) in the presence of recombinant ERRP, cetuximab (*Cetux*), or trastuzumab (*Trast*) in colon (*HT-29*) and breast (*SKBR-3*) cancer cells. α-Tubulin served as the loading control.

In all cell lines, the ligand-induced activation of EGFR and HER-2 was inhibited by recombinant ERRP (Fig. 3A and B). In contrast, cetuximab inhibited the ligand-induced activation of EGFR, but not HER-2, whereas trastuzumab was effective against the induction of HER-2, but had no effect on EGFR activation (Fig. 3A and B).

Whereas TGF-α and HB-EGF primarily bind to EGFR, heregulin or Neu differentiation factor binds to ErbB3/HER-3 and ErbB-4/HER-4 resulting in activation of not only HER-3 and HER-4, but also HER-2 through heterodimerization with HER-3 or HER-4 (26). This offers an excellent opportunity to compare the effect of ERRP with cetuximab or trastuzumab on HER-3 or HER-4 activation by examining the levels of phosphorylated form of HER-2 in colon and breast cancer cells in response to heregulins. Although there are at least 10 identified isoforms of heregulins, we used the α form because it binds to both HER-3 and HER-4 (27). In HT-29 colon cancer and SKBR-3 breast cancer cells, which exhibit high levels of HER-2 and also possess HER-4, heregulin-α caused a marked increase in activation (tyrosine phosphorylation) of HER-2, when compared with the controls (Fig. 4). This increase was greatly abrogated by ERRP and trastuzumab, but not by cetuximab (Fig. 4).

In addition to regulating proliferation, EGFR and its family of receptors are also involved in mediating cell survival. The last set of experiment was, therefore, undertaken to examine whether inhibition of colon and breast cancer cell growth by ERRP, cetuximab, and trastuzumab involves apoptosis. We observed that whereas ERRP caused a marked 2- to 4-fold increase in apoptosis, neither cetuximab nor trastuzumab caused a significant increase in apoptosis in any of the cell lines, when compared with the corresponding controls (Fig. 5). None of these agents induced apoptosis of either NIH-3T3 mouse fibroblast or normal rat small intestinal IEC-6 cells (Fig. 5).

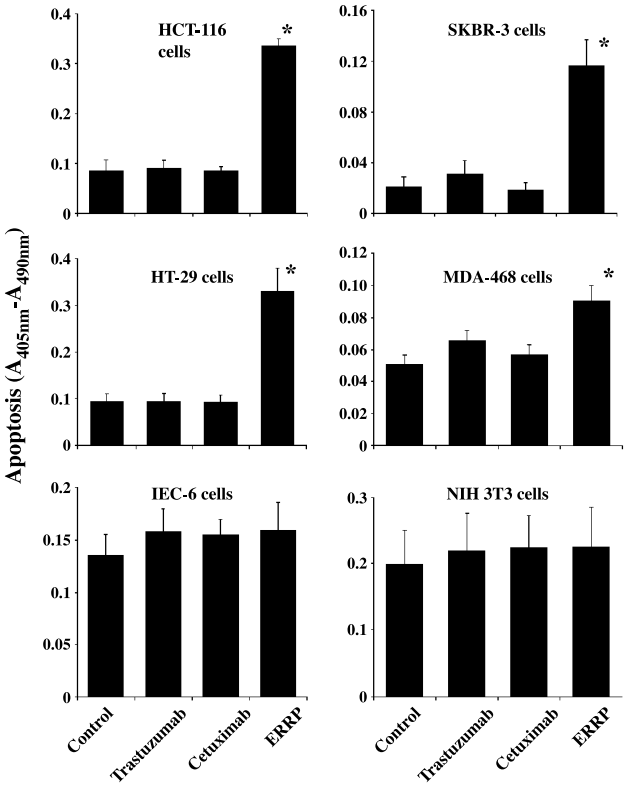

Figure 5. Effects of recombinant ERRP, cetuximab, or trastuzumab on apoptosis of colon (*HCT-116* and *HT-29*) and breast (*MDA-468* and *SKBR-3*) cancer cells as well as the mouse fibroblast (*NIH-3T3*) and nontransformed rat small intestinal IEC-6 cells. Cells were exposed to recombinant ERRP, cetuximab, or trastuzumab for 24 h. *Columns,* mean of four to five determinations; *bars,* SE. $*P < 0.01$, compared with the corresponding controls.

Discussion

Interference with activation of EGFR and/or its family members (ErbB-2/HER-2, ErbB-3/HER-3, and ErbB-4/HER-4) represents a promising strategy for the development of targeted therapies against a wide variety of epithelial cancers because of their preponderance in a variety of neoplastic cells. The rationale for this strategy comes from the observation that EGFR and/or other member(s) of its family, especially HER-2, are overexpressed in a wide variety of tumors (15, 28–30). For example, overexpression of EGFR has been implicated in the development and progression of head and neck, lung, pancreas, bladder, and breast cancers (8, 9). HER-2 overexpression has been observed in ~30% of breast cancer and has been related to the development of metastasis and shorter survival time (29–32). In addition, HER-2 expression has been related to poor prognosis in cancers of the ovary, colon, and bladder (33–35). Although the role of HER-3 and HER-4 in the development and progression of cancer is less well defined, increased expression of these receptors has been observed in breast and gastrointestinal cancers (1).

Preponderance of EGFR and HER-2 in a wide variety of solid tumors has prompted extensive drug development effort to design pharmacologic inhibitors of EGFR and HER-2. Indeed, several small molecule, quinazoline- or pyrimidine-based inhibitors of EGFR have been developed to interrupt the intracellular signaling cascade of EGFR induced by ligand binding of the receptor (5). Small molecule inhibitors to EGFR, gefitinib (Iressa, AstraZeneca Pharmaceuticals, Wilmington, DE), and erlonitib [Tarceva, OSI Pharmaceuticals (Melville, NY) and Genentech (South San Francisco, CA)] have progressed to large-scale randomized clinical trials and clinical use, but with limited success. Moreover, with respect to gefitinib, it has recently been shown that its efficacy is restricted to a subgroup of non–small cell lung cancer patients that have specific mutations in the *EGFR* gene (11, 12). In addition to small-molecule inhibitors of EGFR, monoclonal antibodies to EGFR, such as cetuximab (Erbitux, ImClone Systems/Bristol-Myers Squibb, Somerville, NJ), which competitively inhibit ligand binding to the receptor, resulting in attenuation of EGFR signaling leading to inhibition of tumor growth (5, 36–38). Although no small-molecule inhibitors of HER-2 have been developed, monoclonal antibodies to HER-2, such as trastuzumab (Herceptin, Genentech), have been used for the treatment of breast cancers that express high levels of HER-2. Although cetuximab and trastuzumab treatments showed signs of success in a limited number of patients with tumors showing increased expression of EGFR or HER-2, failure in others may partly be due to coexpression of multiple EGFR family members leading to an enhanced transforming potential and poor prognosis (13, 14). Therefore, identification of inhibitor(s), targeting multiple members of the EGFR family, is likely to provide a therapeutic benefit to a broad range of patient population.

Our current data suggest that ERRP could be a pan-erbB inhibitor that targets multiple members of the EGFR family. This is supported by the observation that ERRP inhibits the growth and stimulates apoptosis of a number of colon and breast cancer lines that express varying levels of EGFR, HER-2, and/or HER-4. The findings that ERRP causes no inhibition in growth of normal mouse fibroblast cell lines suggests that the growth inhibitory effect of ERRP is specific for malignant cells whose growth is primarily dependent on the signaling induced by EGFR and/or its family member(s). This inference is further supported by the observations that the growth of nontransformed rat small intestinal cell line IEC-6, which expresses relatively low levels of EGFR and HER-2, is inhibited only slightly at high doses of ERRP, and that ERRP causes no significant stimulation in apoptosis of this nontransformed cell line. Additional support comes from the observation that ERRP produces no significant stimulation in apoptosis of the NIH-3T3 mouse fibroblast cell line. Taken together, the results suggest a selective inhibition of growth of only malignant cells by the natural EGFR inhibitor, ERRP.

Further support for ERRP being a pan-erbB inhibitor is provided by the observation that neither cetuximab nor trastuzumab, the specific inhibitors of EGFR and HER-2 signaling pathways, respectively, caused significant apoptosis

of the colon and breast cell lines. Cetuximab or trastuzumab was only effective in inhibiting the growth of cell lines that expressed high levels of EGFR or HER-2, respectively. In contrast, ERRP inhibited the growth of cells that expressed high levels of either EGFR or other member(s) of its family, including HER-2. The same phenomenon was also noted when ERRP was compared with cetuximab or trastuzumab for TGF-α- and HB-EGF–induced stimulation of phosphorylation of EGFR and HER-2, one of the initial events of the signal transduction pathways of EGFR and its family members. Furthermore, the fact that ERRP inhibits heregulin-α–induced activation of HER-2 suggests that ERRP may also be effective against HER-3 and HER-4–induced signaling pathways. This inference is based on the notion that heregulin, which binds specifically to HER-3 and HER-4, leads to the formation of heterodimers of not only HER-3 and HER-4, but also activates HER-2 through heterodimerization with HER-3 or HER-4. Moreover, whereas ERRP inhibits ligand-induced activation of both EGFR and HER-2 in colon and breast cancer cell lines, cetuximab attenuated only EGFR activation, and trastuzumab was effective only against activation of HER-2. Whereas cetuximab and trastuzumab inhibited cell growth and ligand-induced activation of EGFR and HER-2 in cells that express high levels of EGFR and HER-2, respectively, they were ineffective in stimulating apoptosis in cell lines used in this investigation.

The precise mechanisms by which ERRP attenuates activation of EGFR and its family member(s) and in turn inhibit cellular growth remain to be fully delineated. However, we reported that in HCT-116 colon cancer cells, ERRP that possesses ~90% homology to the extracellular ligand–binding domain of EGFR binds/sequesters the EGFR ligand TGF-α and forms inactive heterodimers with EGFR, resulting in inhibition of EGFR activation and its signaling (16). We also reported that incubation of PC-3 prostate cells with recombinant ERRP leads to increased sequestration of EGFR ligands, TGF-α and HB-EGF, by ERRP (23). Blast search of database for interacting proteins revealed significant homologies of ERRP with HER-2, HER-3, and HER-4. This is likely to cause sequestration of ligands of HER-3 and HER-4 by ERRP as well. Support for this postulation comes from the observation that ERRP inhibits heregulin-induced activation of HER-2 in colon cancer HT-29 and breast cancer SKBR-3 cells that express high levels of HER-2 and possess HER-4.

Given the fact that ligand binding and subsequent homodimerization or heterodimerization of EGFRs are essential for activation of the receptors, any intervention that affects these processes is likely to have a profound effect on the signal transduction pathways induced by EGFR and/or its family member(s). Such a possibility was suggested, but not shown, for the 2.7 kb truncated rat liver EGFR, and the 3 kb cell surface–associated and the 1.8 kb alternate transcript for human EGFR (39–41). However, Matsuda et al. (42) showed that infection of pancreatic cancer cells lines with an adenoviral vector encoding a truncated EGFR markedly attenuates EGF- and HB-EGF–dependent cell growth and activation of EGFR family and the downstream signaling events, underscoring a therapeutic potential for the ectodomain of EGFRs. In support of this postulation, we have shown that ERRP inhibits the growth of colon cancer cells *in vitro* and *in vivo* and prostate cancer cells *in vitro* (15, 16, 22, 23). Moreover, our current observation that ERRP is also effective in inhibiting growth of cells expressing varying levels of EGFR and/or other member(s) of its family suggests that ERRP may be pan-erbB inhibitor, which could be utilized to target multiple members of the EGFR family.

In summary, our data show that ERRP attenuate ligand-induced activation of EGFR and HER-2 and inhibits cell growth and stimulates apoptosis in a number of epithelial cancer cell lines expressing varying levels of EGFRs. In contrast, cetuximab or trastuzumab are only effective against cell lines that express high levels of either EGFR or HER-2, respectively, but not both. These results suggest that ERRP may be a pan-erbB inhibitor that targets multiple members of the EGFR family and, thus, may be a potential therapeutic agent for a wide variety of epithelial cancers expressing different levels and subclasses EGFRs.

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