

# Co-Targeting Insulin-Like Growth Factor I Receptor and HER2: Dramatic Effects of HER2 Inhibitors on Nonoverexpressing Breast Cancer

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## Abstract

**The insulin-like growth factor I receptor (IGFIR) and HER2 display important signaling interactions in breast cancer. We examined the effect of combinations of antagonists of these receptors using two human breast cancer cell lines: BT474 (HER2+, IGFIR low) and MCF7 (HER2 low, IGFIR high). In BT474 cells, growth was inhibited by HER2 antagonists but not by IGFIR antagonists; however, IGFIR antagonists enhanced the effect of HER2 inhibitors. In MCF7 cells, growth was inhibited by IGFIR antagonists but not by HER2 antagonists; however, HER2 antagonism enhanced the effect of IGFIR inhibitors. Synergistic inhibition of soft agar growth was also observed. Although HER2 and IGFIR antagonists individually only minimally affected cell cycle, their combination gave a small enhancement of their effects. No single receptor-targeting drug was capable of inducing apoptosis, but combining antagonists of both receptors induced a dramatic degree of apoptosis in both cell lines. Induction of apoptosis was most striking in MCF7 cells using a Herceptin/IGFIR antagonist combination despite these cells being HER2 non-overexpressing. Toward understanding the mechanism of these effects, we detected coassociation IGFIR and HER2 in both cell lines. Specific inhibitors of one of these receptors could cross-inhibit the activity of the other. Targeting both receptors gave the maximal inhibition of their downstream extracellular signal-regulated kinase 1/2 and AKT signaling pathways. Hence, such drug combinations may be clinically useful and may be beneficial even in tumors in which single drugs are inactive, as exemplified by the effect of the HER2/IGFIR inhibitor combination in HER2 nonoverexpressing MCF7 cells. [Cancer Res 2008;68(5):1538–45]**

## Introduction

HER2, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, is overexpressed in 20% to 25% of breast cancers, and its overexpression is associated with poor prognosis (for review see refs. 1, 2). HER2 is a validated therapeutic target in breast cancer. Herceptin (trastuzumab) is a humanized monoclonal antibody (mAb) against the extracellular domain of HER2 that has significant therapeutic benefit in the treatment of patients with HER2-overexpressing breast cancer. Targeted therapies have taken center stage in treatment of malignancy. Further

progress in treating solid tumors is likely to come from the use of wisely chosen combinations of targeted therapeutics.

Recent studies have shown interactions between insulin-like growth factor I receptor (IGFIR) signaling and HER2. In addition, signaling by IGFIR has been linked to resistance to Herceptin. When SKBR3 cells, which possess low levels of IGFIR, are transfected with IGFIR and cultured with insulin-like growth factor I (IGF-I), they become resistant to growth inhibition by Herceptin (3). Using HER2-transfected MCF7 cells, this group showed synergistic inhibition of anchorage-independent growth when combining Herceptin with inducible dominant-negative IGFIR (4); effects on apoptosis or other aspects of the transformed phenotype were not examined. This group also showed that an IGFIR tyrosine kinase inhibitor (TKI), AG1024, and the EGFR TKI Iressa gave additive-to-synergistic growth inhibition of several breast carcinoma cell lines, and the combination gave greater apoptosis than either single agent (5). Other work has used SKBR3 cells cultured for resistance to Herceptin by continuous maintenance in the presence of Herceptin (6); this work used pools of such resistant cells, and comparisons were made to parental SKBR3 cells. An intimate IGFIR/HER2 interaction was noted exclusively in the resistant pools but not in the parental cells. IGFIR and HER2 could be coimmunoprecipitated from the resistant cells but not from the parentals. Other findings included IGF-I-induced stimulation of HER2 phosphorylation and decreased HER2 phosphorylation when IGFIR was inhibited but again only in the resistant cells. Inhibiting IGFIR restored sensitivity to Herceptin. Subsequent work by the same group showed that the EGFR/HER2 dual TKI lapatinib could induce killing of the Herceptin-resistant SKBR3 cells, and that an IGFIR antagonistic antibody could potentiate this effect (7). Association of IGFIR with clinical resistance to Herceptin has also been reported (8).

Evidence has shown that IGFIR signaling is necessary for cellular transformation by a variety of agents (9, 10). Such a critical role of IGF signaling in malignancy makes it a promising therapeutic target in treatment of cancer. Targeting the IGFIR is a promising strategy particularly for breast cancer because of its overactivation in this disease, its critical role in transformation in general (particularly its antiapoptotic function), and its intimate cross-talk with both estrogen receptor and HER2. As a solo therapy, IGFIR inhibition has not uniformly displayed *in vivo* antitumor activity (11). However, the critical antiapoptotic role in established tumors makes IGFIR targeting an especially promising strategy to combine with other therapies, including conventional chemotherapy, or, in what may be an even more appealing strategy, with other targeted therapies. Given the interactions of IGFIR with HER2 and the established success of targeting HER2 in breast cancer, we aimed to explore the effect of co-targeting IGFIR concomitantly with HER2.

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## Materials and Methods

**Drugs.** Herceptin was obtained from the Yale Cancer Center Medical Oncology pharmacy. A 20 mg/mL stock preparation was kept at 4°C and was further diluted in sterile PBS before addition to cells in culture.  $\alpha$ -IR3 (anti-IGF1R antagonistic antibody), AG1024 (IGF1R-specific TKI), and AG825 (HER2-specific TKI) were purchased from Calbiochem. All TKIs were dissolved in DMSO to make 10 mmol/L stock solutions.

**Antibodies.** Primary antibodies were purchased from the following sources: anti-HER2/neu polyclonal antibody sc-284 and anti-IGF1R polyclonal antibody (clone C-20) were from Santa Cruz Biotechnology, Inc.; anti-phospho-HER2/neu mAb (Tyr-1248; clone PN2A) and anti-phospho-IGF1R were from NeoMarkers and Biosource International, Inc., respectively; rabbit polyclonal antibody against phospho-AKT, AKT, phospho-extracellular signal-regulated kinase (ERK)1/2, ERK1/2, and poly(ADP-ribose) polymerase (PARP) were all from Cell Signaling Technology; and horseradish peroxidase (HRP)-conjugated Goat anti-rabbit IgG or goat anti-mouse IgG were purchased from Santa Cruz Biotechnology.

**Cell culture.** Two ER<sup>+</sup> human breast cancer cell lines that differ in their HER2 expression level were chosen for these studies. BT-474 highly overexpresses HER2 in association with gene amplification, whereas MCF7 expresses a low level (nonoverexpressed) of HER2. BT474 cells, obtained from American Type Culture Collection, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated), 2 mmol/L L-glutamine, 10  $\mu$ g/mL insulin, and penicillin at 37°C in 5% CO<sub>2</sub> humidified air. MCF7 cells, a gift from Marc Lippman (University of Michigan, Ann Arbor, MI), were maintained in phenol red-free Iscove's Modified Medium (IMEM) with 10% charcoal-stripped FCS (Biosource), 1 nmol/L estradiol added back, and penicillin, at 37°C in 5% CO<sub>2</sub> humidified air. To maintain the estrogen sensitivity of MCF7 cells, before each experiment cells are cultured for 5 days in estrogen-free medium (culture medium without the estradiol). Estradiol is then added back, with or without drugs, at the beginning of each experiment.

**WST-1 colorimetric growth assay.** BT-474 and MCF7 cells were plated in 96-well plates at a density of 10<sup>4</sup> and 10<sup>3</sup> cells per well, respectively. They were allowed to adhere overnight and then treated with drugs the following day (day 0). After a 5-day (BT474 cells) or 6-day (MCF7 cells) incubation period, the WST-1 tetrazolium salt colorimetric growth assay (Boehringer Mannheim Biochemicals) was performed by adding 1/10 volume of WST-1 solution and incubating for 2 h at 37°C. Absorbance at 450 nm was determined using a microplate reader (Molecular Devices Corp.). A reading was always taken at day 0 and subtracted from the 5- or 6-day reading. Results were expressed as a percentage of control (vehicle-treated cells). The mean and SD values of four or more wells in at least three experiments for each point were reported.

**Soft agar colony growth assay.** To prepare base plates, for BT474, 1 mL of RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 10  $\mu$ g/mL insulin, 10 mmol/L HEPES, and 0.8% agarose was added into each 35-mm dish. For MCF7, 1 mL IMEM containing 10% charcoal stripped FCS, 1 nmol/L estradiol, and 0.8% agarose was added into each 35-mm dish. The dishes were placed at 4°C to solidify the agarose and then transferred to a 37°C incubator. Cells were mixed with their respective medium in the presence of 10  $\mu$ mol/L AG1024, 1  $\mu$ g/mL Herceptin (for BT474) or 10  $\mu$ g/mL Herceptin (for MCF7), both drugs, no drug ("control"), or vehicle then plated on top of the base at a density of 2  $\times$  10<sup>4</sup> cells per 35-mm dish for BT474 and 1  $\times$  10<sup>2</sup> for MCF7, in triplicate. Dishes were incubated at 37°C for 21 days. Clusters of greater than or equal to six cells were scored as colonies, and single cells as well as clusters of fewer than six cells were scored as single cells. Five microscopic fields ( $\times$ 60) of each dish were scored, and the colony formation percentage of each dish was calculated by dividing the number of colonies by the total of colonies plus single cells. Final results were expressed as the percentage of untreated ("control") cells.

**Analysis of cell cycle by flow cytometry.** Cells in exponential growth were seeded at a confluency of ~50% in 100-mm dishes. After overnight incubation, cells were treated either with vehicle or drugs. At indicated time points, cells were trypsinized, washed with cold PBS, resuspended in 2 mL

of ice cold PBS, and fixed by three stepwise additions of 2 mL each of 95% ethanol (12). After fixation for at least 1 h, cells were resuspended in 1 mg/mL RNase (Sigma) in PBS and stained with 0.05 mg/mL propidium iodide (Sigma) for 1 h on ice. Flow cytometric analysis was performed with a FACS Vantage flow cytometer (Becton Dickinson). A minimum of 15,000 cells was analyzed for each sample. Data analysis was performed using Modfit 5.2 analysis software (Verity Software House). Each point represents duplicate samples from at least three experiments.

**Apoptosis determination by sub-G<sub>1</sub> analysis.** Cells in exponential growth were seeded at a confluency of 50% in 100-mm dishes. After incubation for an indicated period of time with drug, both adherent and nonadherent cells were harvested, washed twice with PBS, and fixed in ice-cold 70% ethanol at least for 1 h. Cells were centrifuged and washed twice with cold PBS, resuspended in 1 mg/mL RNase (Sigma Co.), and stained with 0.05 mg/mL propidium iodide (Sigma Co.) for 1 h on ice in the dark. Analysis of cellular DNA and hypodiploid DNA content indicative of apoptotic DNA fragmentation was determined by collecting 10,000 events using a FACS Calibur flow cytometer and Cell Quest Software (Becton Dickinson). Cell debris and small particles were excluded from the analysis by forward/sideward scatter criteria (13).

**Apoptosis determination by Annexin V assay.** After 5 to 6 days of drug treatment, both adherent and nonadherent cells were harvested and stained using Vybrant Apoptosis Assay kit #2 (Molecular Probes). Briefly, the cells were incubated with Alexa Fluor 488 Annexin V and propidium iodide in 1 $\times$  Annexin-Binding Buffer (provided with the kit) for 15 min at room temperature. The percentages of Annexin V- and propidium iodide-positive cells were determined by flow cytometry, and any necrotic cells (stained with both propidium iodide and FITC) were gated out so that an accurate determination of the percentage of apoptotic cells could be made.

**Apoptosis determination by PARP cleavage analysis.** MCF7 and BT474 cell lysates were prepared using radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L  $\beta$ -glycerol phosphate, 25 mmol/L NaF, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L phenylmethylsulfonyl fluoride, 5  $\mu$ g/mL Leupeptin, 10  $\mu$ g/mL Aprotinin, and 5  $\mu$ g/mL Pepstatin]. The lysates were cleared by microcentrifugation (13,000  $\times$  g; 20 min; 4°C). Extracts (40  $\mu$ g protein per lane) were immunoblotted with anti-PARP polyclonal antibody (1:1,000 in Tris-buffered saline/Tween) for 1 h followed by goat anti-rabbit IgG conjugated with HRP (Santa Cruz; 1:1,000 dilution; 60 min). Bands were visualized with the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) on X-ray film (Eastman Kodak).

**Immunoblot analysis for phospho-IGF1R, total IGF1R, phospho-HER2, total HER2, phospho-AKT, total AKT, phospho-ERK-1/2, and total ERK-1/2.** Cells lysates were prepared as above. MCF7 and BT474 cell extract (40  $\mu$ g protein per lane) were immunoblotted with the primary antibodies at a dilution of 1:1,000 in Tris-buffered saline/Tween for o/n at 4°C followed by 1 h with HRP-conjugated respective secondary antibody (1:1,000 dilution). Bands were visualized with the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) on X-ray film (Eastman Kodak).  $\beta$ -actin was used for loading control.

**Coimmunoprecipitation of IGF1R $\beta$  and HER2.** Cell lysates prepared as above were precleared with protein A/G agarose (Santa Cruz Biotechnology) then immunoprecipitated with either anti-HER2 (mouse mAb; 9G6; Santa Cruz Biotechnology) or anti-IGF1R $\beta$  (rabbit polyclonal antibody; C20; Santa Cruz Biotechnology) antibodies overnight at 4°C. In some experiments, rabbit or mouse IgG were used for immunoprecipitation instead as negative controls. Immunoprecipitates were washed thrice with lysis buffer, mixed with electrophoresis sample buffer, heated, and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels. Electrophoresed proteins were transferred to nitrocellulose membranes. The filters were immunoblotted with anti-IGF1R $\beta$  (rabbit polyclonal C20; Santa Cruz Biotechnology) or anti-HER2 antibodies (rabbit polyclonal; sc-284; Santa Cruz Biotechnology). Detection was with HRP-coupled antibodies to rabbit IgG using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

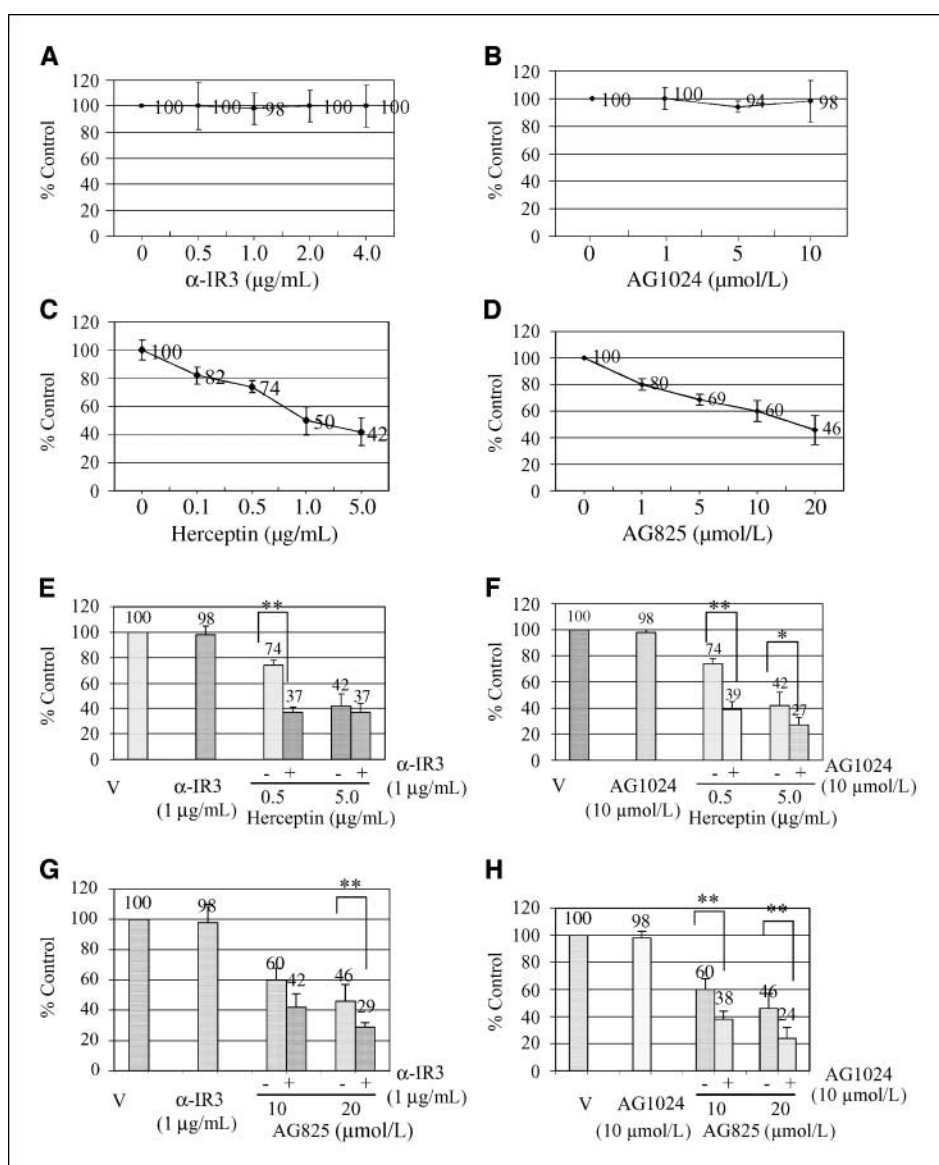
## Results

**Expression of IGFIR in BT474 and MCF7 cells.** A comparison of relative IGFIR expression levels between BT474 and MCF7 cells was performed by analyzing equivalent amounts of protein from cell lysates of each line in an anti-IGFIR immunoblot experiment as described in Materials and Methods. We found that both lines express IGFIR, with relatively high levels observed in MCF7 cells and comparably low levels in BT474 cells (data not shown). These results are consistent with previously published reports.

**Effect of IGFIR inhibitors alone and in combination with HER2 antagonists on cell growth.** Dose-response curves for the effect on cell proliferation were generated for antagonists of IGFIR and HER2. Using BT474 cells, we found little or no growth inhibition from the IGFIR antagonists  $\alpha$ -IR3 or AG1024, whereas growth was inhibited in a dose-dependent fashion by inhibitors of HER2 (Herceptin and AG825; Fig. 1A-D). However, the addition of IGFIR antagonists significantly enhanced the growth inhibition observed with Herceptin or AG825, although the IGFIR inhibitors were used at concentrations showing no growth inhibition at all as single agents (Fig. 1E-H).

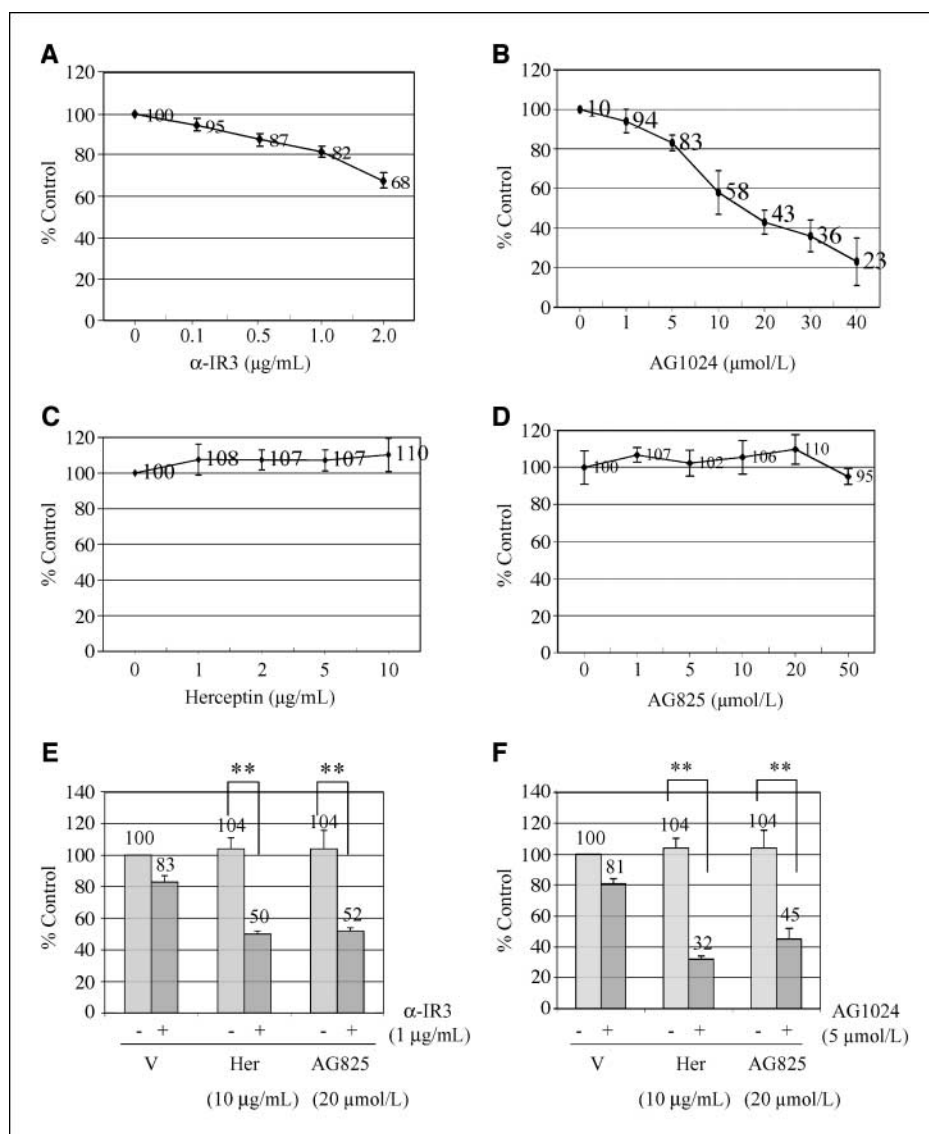
Using MCF7 cells, which do not have HER2 overexpression but express high levels of IGFIR, growth is inhibited by the IGFIR antagonists AG1024 and  $\alpha$ -IR3 but, as expected, not by the HER2 antagonists Herceptin or AG825 (Fig. 2A-D). When combinations were examined, although the single agents Herceptin and AG825 had no growth inhibitory activity, they enhanced the growth inhibition elicited by the IGFIR antagonists  $\alpha$ -IR3 or AG1024 (Fig. 2E and F).

**Effect on anchorage-independent clonogenicity.** The effect of the presence of AG1024, Herceptin, and the combination on the soft agar colony growth was analyzed. For these experiments, drug was present continuously during colony growth as described in Materials and Methods. Results are expressed as a percentage of control (no drugs or vehicles added). For BT474 cells, 10  $\mu$ mol/L AG1024 was without effect, whereas 1  $\mu$ mol/L Herceptin resulted in 68%  $\pm$  8% (mean  $\pm$  SD) of control number of colonies; the combination resulted in 37%  $\pm$  5% of control. For MCF7 cells, 10  $\mu$ g/mL Herceptin had no effect, whereas 10  $\mu$ mol/L AG1024 resulted in 82%  $\pm$  9% of control number of colonies, and the combination resulted in 41%  $\pm$  3% of control.



**Figure 1.** Effect of IGFIR and HER2 antagonists alone and in combination on BT474 cell proliferation. BT474 cells in exponential growth were seeded on day 1 in 96-well plates at  $1 \times 10^4$  cells per well. They were allowed to adhere overnight and then treated with drugs the following day (day 0) at the indicated doses (A-D). IGFIR antagonists  $\alpha$ -IR3 (1  $\mu$ g/mL) and AG1024 (10  $\mu$ mol/L) were combined with either Herceptin (E and F) or AG825 (G and H). After 5 d of incubation, the WST-1 proliferation assay was performed as described in Materials and Methods. Results were expressed as a percentage of control (V, vehicle-treated cells). Columns, mean of four or more wells from at least three experiments for each point; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Figure 2.** Effect of IGF1R and HER2 antagonists alone and in combination on MCF7 cell proliferation. MCF7 cells in exponential growth were seeded on day 1 in 96-well plates at  $1 \times 10^3$  cells per well. They were allowed to adhere overnight and then treated with drugs the following day (day 0) at the indicated doses (A–D). IGF1R antagonists  $\alpha$ -IR3 (1  $\mu$ M) and AG1024 (5  $\mu$ M) were combined with HER2 inhibitors, Herceptin (Her) or AG825 (E and F), as indicated. After 6 d of incubation, the WST-1 proliferation assay was performed as described in Materials and Methods. Results were expressed as a percentage of control (V, vehicle-treated cells). Columns, mean of four or more wells from at least three experiments for each point; bars, SD. \*,  $P < 0.01$ ; \*\*,  $P < 0.005$ .



**Effect on cell cycle.** In general, inhibition of growth factor receptor tyrosine kinases such as HER2 effects cell cycle by inducing  $G_1$  arrest and/or reducing percentage of cells in S phase. Effects on cell cycle were examined after 24 to 48 h of drug treatment with similar results at both time points. Results are shown for 48 h treatment. In BT474, we found that Herceptin increased the percentage of cells in  $G_1$  phase and decreased the percentage in S phase (Fig. 3A). IGF1R antagonists exert similar effects and slightly augment the effects of HER2 antagonists (Fig. 3A). In MCF7 cells, HER2 and IGF1R antagonists individually caused minimal increase in percentage of cells in  $G_1$  and decrease in percentage of cells in S phase, and their combinations caused a small enhancement of these effects (Fig. 3B).

**Induction of apoptosis.** Apoptosis was assayed after 5 to 6 days of drug treatment by analyzing the percentage of cells in the sub- $G_1$  peak (Fig. 4A and B), by the Annexin-V apoptosis assay (Fig. 4C and D), and by the PARP cleavage assay (Fig. 4E and F). Although antagonists of HER2 did not induce apoptosis in BT474 cells, they dramatically enhanced the otherwise minimal level of apoptosis induced by IGF1R antagonists (Fig. 4A and C). Similarly, in MCF7

cells, minimal apoptosis is induced by any single drug. However, massive induction of apoptosis was observed with the combination of IGF1R inhibitor plus HER2 inhibitor, especially with Herceptin (Fig. 4B and D). This is particularly remarkable because this cell line does not overexpress HER2 or respond at all to single agent Herceptin.

These results were confirmed by the PARP cleavage assay (Fig. 4E and F). For both cell lines [BT474 (Fig. 4E); MCF7 (Fig. 4F)], individual agents do not produce a significant amount of PARP cleavage product (89 kDa), but an increase in PARP cleavage is observed for the drug combinations.

**Cross talk between HER2 and IGF1R.** Using either BT474 or MCF7, a physical association between HER2 and IGF1R was detectable by coimmunoprecipitation experiments (Fig. 5A). In BT474, single drugs or combinations did not affect HER2 levels over a 2-day exposure. However, the inhibition of HER2 phosphorylation resulting from treatment with HER2 antagonists was significantly augmented by IGF1R antagonists (Fig. 5B). This experiment suggests that IGF1R contributes to HER2 signaling, and HER2 signaling can be more completely inhibited

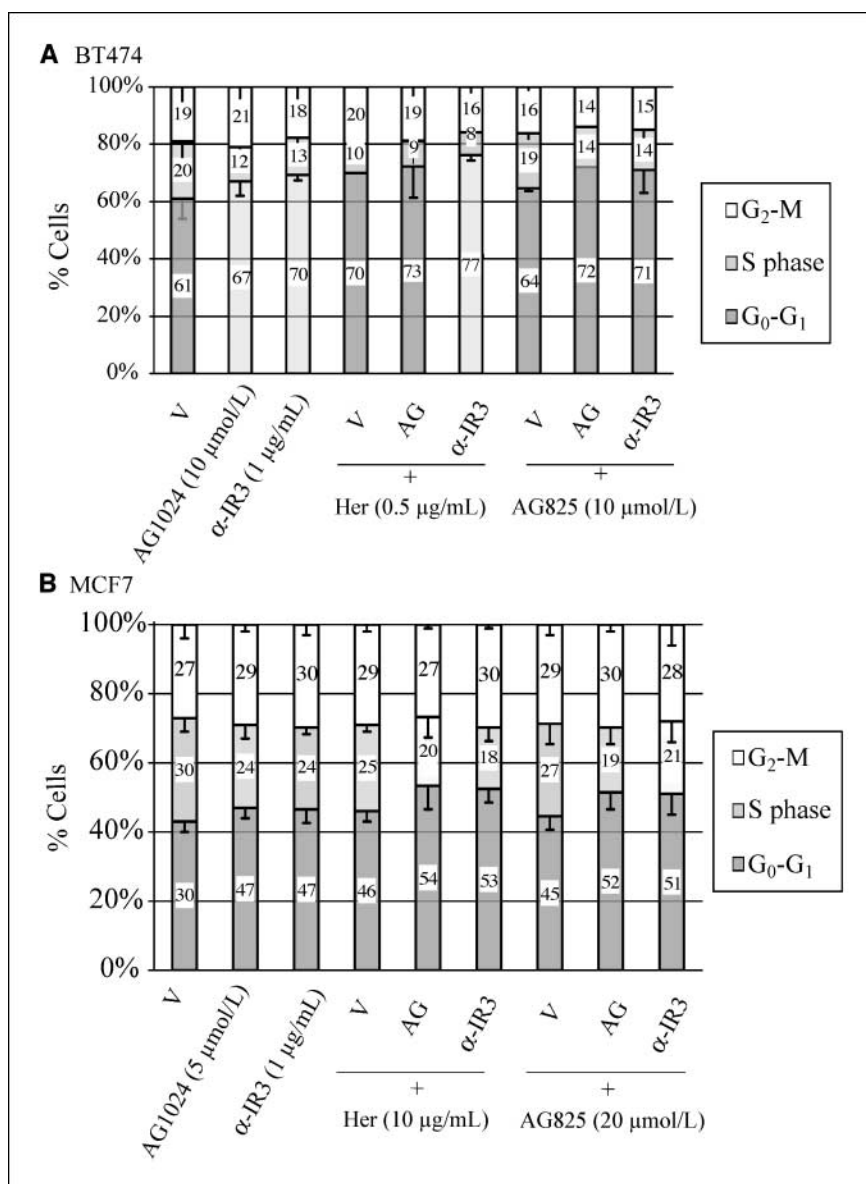
by combining Herceptin with an IGFIR inhibitor, even in breast carcinoma cells that have not been cultivated for acquired Herceptin resistance.

Similarly, although to a lesser degree, maximal inhibition of IGFIR phosphorylation was noted in MCF7 cells when Herceptin was added to IGFIR inhibitors (Fig. 5C). These data suggest that there exists cross-talk between HER2 and IGFIR in both cell lines.

**Effect of co-targeting on IGFIR and HER2 signal transduction.** We further investigated the effect of combination treatment on the downstream growth factor signaling pathways. In BT474 cells, maximal inhibition of ERK1/2 activity required the combination of both HER2 and IGFIR antagonists. Similar but less striking trends were observed for drug effects on AKT activity (Fig. 6A). In MCF7 cells, only AG1024 showed single agent partial inhibitory effect on ERK1/2 activity; however, combining Herceptin with either of the IGFIR inhibitors profoundly inhibited ERK1/2 activity (Fig. 6B). Although AKT was difficult to detect in this cell line, again, the trend was for the drug combinations to have the greatest inhibitory effect (Fig. 6B).

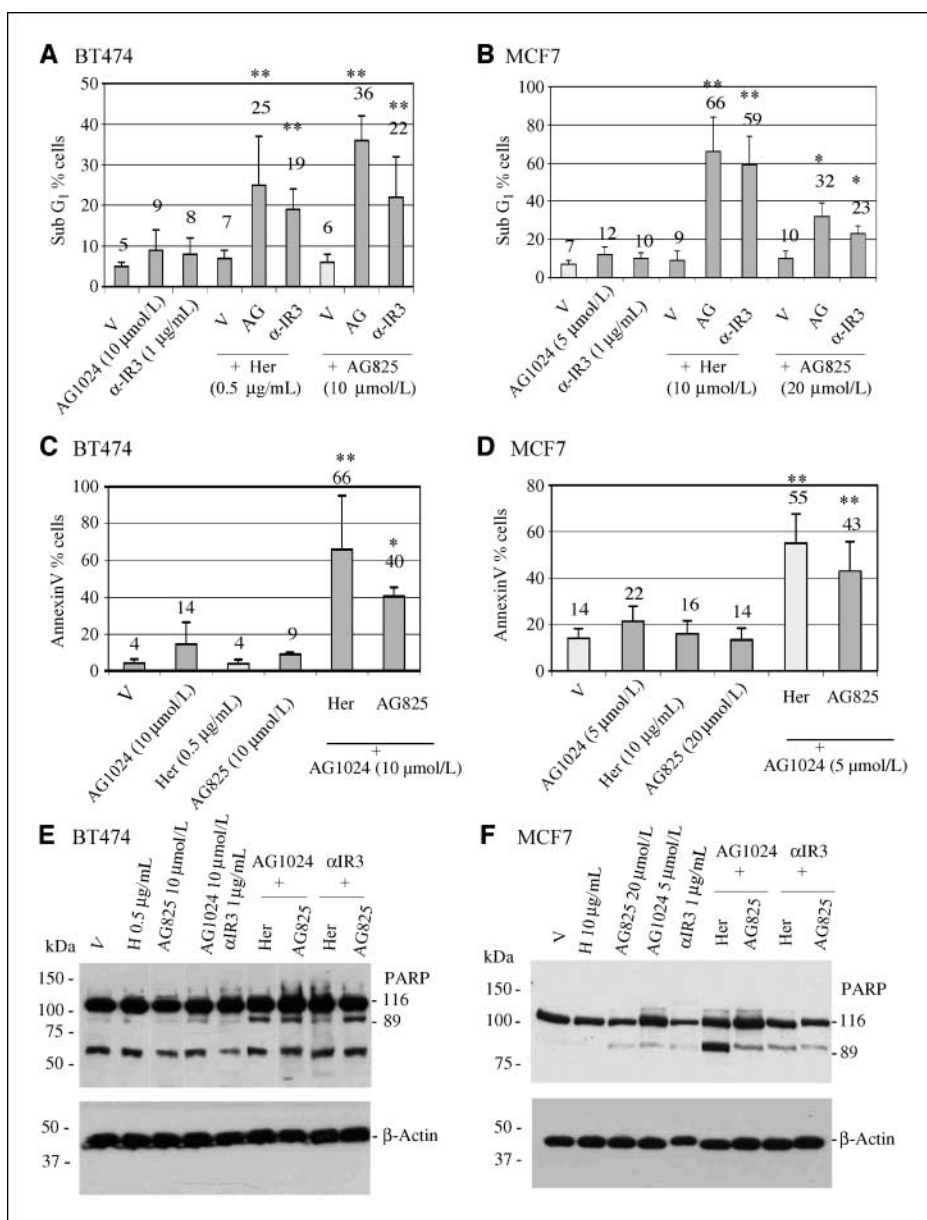
## Discussion

We have discovered dramatic effects of adding IGFIR inhibitors to agents that target HER2. Synergistic effects of these receptor inhibitors affect all aspects of the transformed phenotype: proliferation, anchorage independence, and survival. The most remarkable results are the effects of IGFIR/HER2 targeting in the HER2-nonoverexpressing MCF7 cells, for which Herceptin typically produces no effect as a single agent. Herceptin was developed as an agent that would inhibit HER2-overexpressing tumor cells without affecting HER2-nonoverexpressing cells. Recent preliminary reports suggest that clinical activity of Herceptin when combined with chemotherapy may be observed in subsets of patients not classically thought to be "HER2 positive" (14, 15). We believe that our results support that HER2 has an important function in breast cancer even when it is not overexpressed/amplified; we postulate that in nonoverexpressing cells, loss of that function can be compensated for by other signaling pathways. However, when such other pathways are themselves inhibited (IGFIR in the present case), the critical importance of HER2 becomes apparent.



**Figure 3.** Effect of drugs on cell cycle for BT474 (A) and MCF7 (B) cells. IGFIR antagonists  $\alpha$ -IR3 and AG1024, alone and in combination with Herceptin or AG825, were tested at the indicated concentrations for their effect on cell cycle using BT474 (A) and MCF7 (B) cells. After 2 d of drug treatment, cells were analyzed as described in Materials and Methods. Results are expressed as the mean  $\pm$  SD from three different data points and expressed as percentage of cells in G<sub>0</sub>-G<sub>1</sub> (darkly shaded bar), in S phase (lightly shaded bar), and in G<sub>2</sub>-M (blank bar).

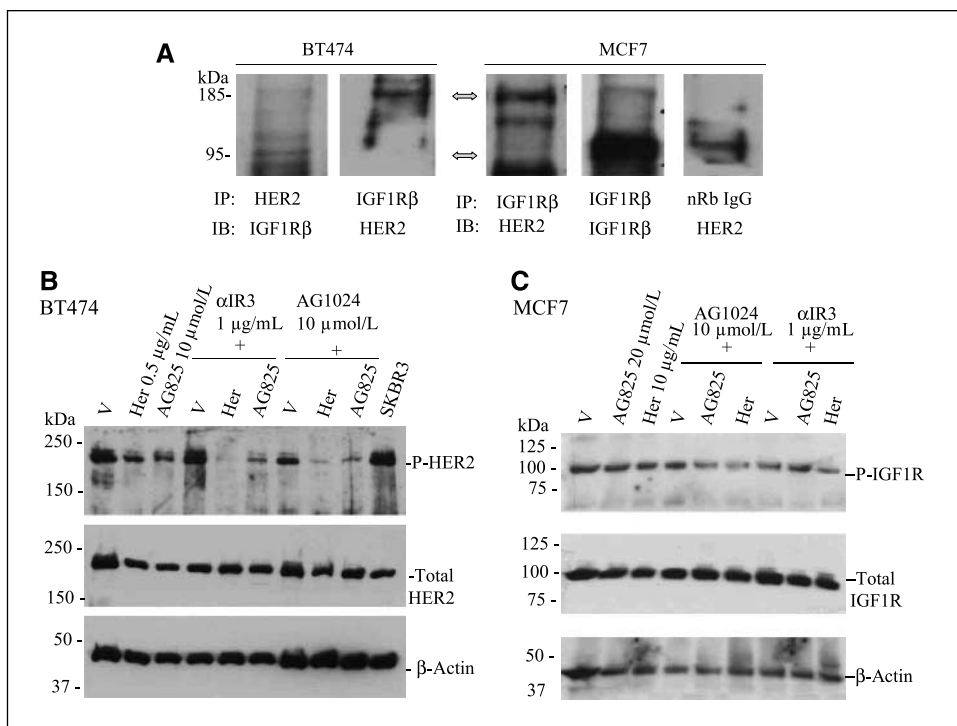
**Figure 4.** Influence of drugs alone and in combination on apoptosis of BT474 (A, C, and E) and MCF7 (B, D, and F) cells. A and B, percentage of cells in sub-G<sub>1</sub> phase of cell cycle. Sub-G<sub>1</sub> was detected after 5 d (for BT474; A) or 6 d (for MCF7; C) of drug treatment by fluorescence-activated cell sorting analysis as described in Materials and Methods. Data are expressed as the percentage of cells in sub-G<sub>1</sub> phase and calculated as the mean  $\pm$  SD from three independent experiments. C and D, results of the Annexin-V apoptosis assay. Columns, mean from three different experiments; bars, SD. E and F, apoptosis as assayed by PARP cleavage, in which cells were treated with single drugs or combinations as specified for 4 d, adherent and floating cells were harvested, and whole cell lysates were prepared as described in Materials and Methods. Immunoblotting was carried out using an anti-PARP antibody and also with  $\beta$ -actin as a loading control. Detection was carried out using ECL reagent. \*,  $P < 0.001$ ; \*\*,  $P < 0.05$ , when compared with vehicle control. H or Her, Herceptin; AG, AG1024.



Evidence of cooperation between IGFs and HER family ligands has been well-documented (16–18). Prolonged activation of ERK2 by epidermal growth factor (EGF) seen in wild-type cells was absent in cells from IGF1R knockout mice embryos but could be rescued by reintroduction of the IGF1R (19); IGF1R signaling was also required for EGF-induced 3T3 proliferation. Breast cancer cells cultured for resistance to Iressa had elevated levels of activated IGF1R and increased sensitivity to an IGF1R TKI (20). In one model, the mitogenic effect of neuregulin was inhibited by an antisense oligodeoxynucleotide to the IGF1R (21). This model also showed IGF1R/HER2 cross-talk and physical association (22). IGF1R and HER2 physically associated as shown by coimmunoprecipitation and confocal microscopy. In MCF7 cells, IGF1R/HER2 association was inducible with either neuregulin or IGF-I. A possible role for IGF1R in Herceptin resistance has been suggested by both preclinical (3–7) and clinical (8) studies.

Evidence suggests that the ras/mitogen-activated protein kinase (ERK1/2) pathway and PI3K/AKT pathway are the most important

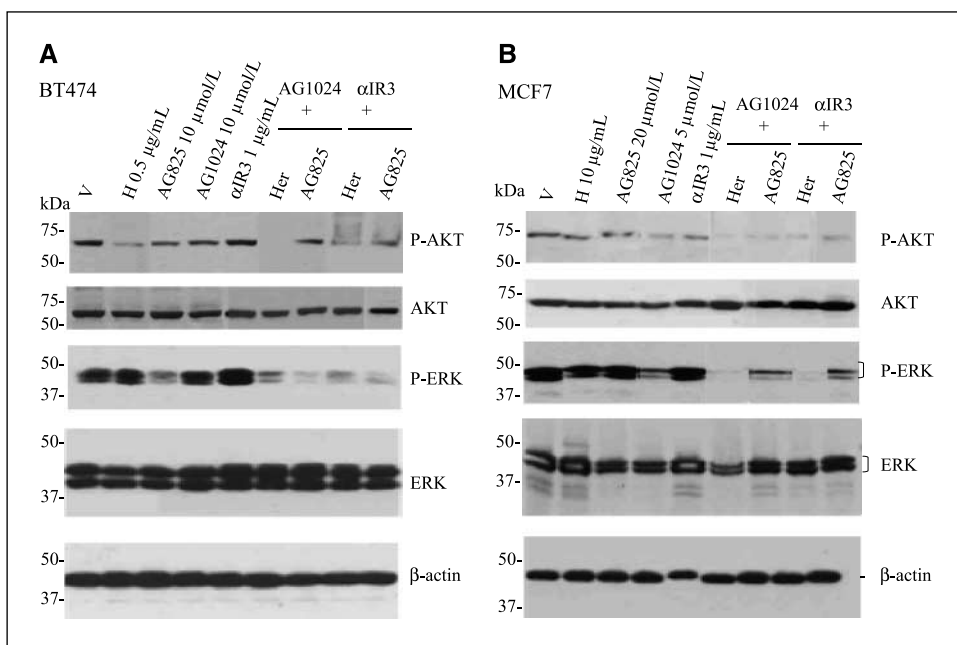
signal transduction outputs in the roles of both IGF1R and HER2 in transformed cells. Our mechanistic studies suggest cross-talk between HER2 and IGF1R at the level of receptor activities. This may be indirect; however, in our experiments a physical association of the two receptors in an immunoprecipitable complex was detected. In addition, we found that co-targeting both receptors resulted in the most efficient inhibition of ERK1/2 and AKT activity. We postulate that this more complete inhibition of these pathways underlies the observed synergy, although alternative mechanisms cannot be excluded. For example, we occasionally observed no effect of a given drug on a specific pathway, but potentiation of inhibition of that pathway when the same “inactive” drug was used in a combination. For example, in both cell lines,  $\alpha$ IR3 had no single agent effect on ERK1/2 activity, but the agent substantially potentiated the ability of HER2 antagonists to inhibit ERK activity. Such results suggest that the drug combination effects may not always be simply due to a dose-response to blocking common pathway(s) downstream of each receptor, but rather a more



**Figure 5.** Analyses of cross-talk between IGF1R and HER2 in BT474 and MCF7 cell lines. **A**, coimmunoprecipitation of IGF1R and HER2. Lysates from BT474 or MCF7 cells were subjected to HER2 immunoprecipitation (IP; Ab 9G6; mouse mAb), IGF1Rβ immunoprecipitation (Ab C-20; rabbit polyclonal), or normal rabbit IgG immunoprecipitation by immunoblot (IB) for HER2 (Ab sc-284; rabbit polyclonal) or IGF1R (Ab C-20). *Top arrow*, 185-kDa HER2 band; *bottom arrow*, 95-kDa IGF1Rβ band. **B** and **C**, effect of co-targeting on HER2 and IGF1R phosphorylation for BT474 and MCF7 cells, respectively. Cells were treated with single drug or combinations as specified for 2 d. Cell lysates were prepared, and equal amounts (40 μg) of protein per lane were loaded. Membranes were immunoblotted for the detection of phosphorylated HER2 (P-HER2) and total HER2 (in BT474; **B**), and phosphorylated IGF1R (P-IGF1R) and total IGF1R (in MCF7; **C**). Immunoblotting for β-actin was also done as a loading control.

complex phenomenon, such as an effect on receptor to receptor cross-talk, a threshold effect, or other indirect mechanisms. However, another recent study examined the antitumor potential of recombinant human IGF1R-3, an IGF-I antagonistic protein with high affinity for the IGF-I ligand (23). This study found that IGF1R-3 displayed a synergistic interaction with Herceptin in Herceptin-resistant breast cancer cell lines, and, such as our study, a suggestion that the ability to effectively inhibit activity of Akt and ERK1/2 was important mechanistically.

In summary, we have found that in HER2-overexpressing (BT474) or nonoverexpressing (MCF7) human breast carcinoma cells, the combination of HER2/IGF1R antagonists potentiate growth inhibition and also induce massive apoptosis of cells growing in monolayer. Furthermore, in both cell lines, our results show that HER2/IGF1R co-targeting results in maximal inhibition of ERK1/2 and AKT activity. The most striking result with potentially wide-ranging clinical implication is the ability of Herceptin to induce apoptosis in HER2-nonoverexpressing MCF7



**Figure 6.** Effect of drugs on total and phospho-AKT and phospho-ERK1/2 in BT474 (**A**) and MCF7 (**B**) cells. Cells were treated with single drugs or combinations as specified for 2 d. Cell lysates were prepared, and 40 μg of protein per lane were loaded. Membranes were immunoblotted for P-AKT, total AKT, P-ERK, total ERK, and β-actin (as a loading control). Detection of bands was carried out using ECL reagent.



cells when added to IGFIR antagonists. These compelling results imply that wisely chosen inhibitor combinations may have dramatic synergy, inducing cell death even when one signaling pathway in isolation is dispensable, and therefore, such combinations are potentially applicable to a much broader range of patients than one would predict based on receptor expression levels or effects of individual drugs. Until recently, Herceptin had only shown activity in patients with HER2 overexpression/amplification. Recent preliminary results reported in abstract form suggest that Herceptin may occasionally augment chemotherapy effect in patients having tumors without detectable HER2 overexpression or amplification. Our data provide a tantalizing suggestion that an IGFIR/HER2 antagonist combination may be applicable to treatment of a broad array of breast cancer patients regardless of HER2 status. Herceptin has not shown significant clinical activity in malignancies other than breast cancer. Of note, in osteosarcoma or Ewing sarcoma cell lines, Herceptin was

without effect unless it was combined with IGFIR inhibition (24). Hence, lessons learned from idealized model systems such as HER2-driven breast cancer may inform investigators as to how such targeted drug combinations may be used in other malignancies by potentially revealing a set of pathways crucial to the maintenance of the malignant phenotype of solid tumors in general.

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