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HER receptor signaling confers resistance to the insulin-like growth factor-I receptor inhibitor, BMS-536924

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Abstract

We have reported previously the activity of the insulin-like growth factor-I (IGF-IR)/insulin receptor (InsR) inhibitor, BMS-554417, in breast and ovarian cancer cell lines. Further studies indicated treatment of OV202 ovarian cancer cells with BMS-554417 increased phosphorylation of HER-2. In addition, treatment with the pan-HER inhibitor, BMS-599626, resulted in increased phosphorylation of IGF-IR, suggesting a reciprocal cross-talk mechanism. In a panel of five ovarian cancer cell lines, simultaneous treatment with the IGF-IR/InsR inhibitor, BMS-536924 and BMS-599626, resulted in a synergistic antiproliferative effect. Furthermore, combination therapy decreased AKT and extracellular signal-regulated kinase activation and increased biochemical and nuclear morphologic changes consistent with apoptosis compared with either agent alone. In response to treatment with BMS-536924, increased expression and activation of various members of the HER family of receptors were seen in all five ovarian cancer cell lines, suggesting that inhibition of IGF-IR/InsR results in adaptive up-regulation of the HER pathway. Using MCF-7 breast cancer cell variants that overexpressed HER-1 or HER-2, we then tested the hypothesis that HER receptor expression is sufficient to confer resistance to IGF-IR-targeted therapy. In the presence of activating ligands epidermal growth factor or heregulin, respectively, MCF-7 cells expressing HER-1 or HER-2 were resistant to BMS-536924 as determined in a proliferation and clonogenic assay. These data suggested that simultaneous treatment with inhibitors of the IGF-I and HER family of receptors may be an effective strategy for clinical investigations of IGF-IR inhibitors in breast and ovarian cancer and that targeting HER-1 and HER-2 may overcome clinical resistance to IGF-IR inhibitors. [Mol Cancer Ther 2008;7(9):2589–98]

Introduction

The insulin-like growth factor-I (IGF-I) pathway is a complex and highly regulated system that is important in human growth and development (1). In human cancers, multiple components of this system become dysregulated and provide growth and survival advantages to tumor cells (2). In particular, the IGF-I system has been implicated in the development and growth of several cancers, including breast, prostate, and colon (3–5). It has also been identified as a mechanism by which the tumors evade death by several important anticancer therapies including cytotoxic chemotherapy, hormonal therapy, receptor tyrosine kinase inhibitor (TKI) therapy, and radiation therapy (6–14). Because the IGF-I pathway is active in the majority of solid and hematologic malignancies, targeting this system has been an area of increasing drug development interest.

In targeting the IGF-I system, there are multiple key components that must be considered (2, 15, 16). Central to the system are its two stimulatory ligands, IGF-I and IGF-II. These circulating ligands provide proliferative and prosurvival signaling through their binding to the receptor tyrosine kinases, IGF-I receptor (IGF-IR) and the insulin receptor (InsR). The affinity of IGF-IR and InsR for the binding of IGF-I and IGF-II, as well as the metabolic counterpart, insulin, is dependent on the presence hybrid IGF-IR/InsR pairs as well as the isoform of InsR. Specifically, the fetal form or isoform A of the InsR has proliferative and prosurvival effects on binding IGF-II, whereas the metabolic InsR isoform B has subphysiologic binding affinity for any ligand, except insulin (17, 18). Additionally, a nonsignaling membrane receptor, IGF-II receptor, binds and internalizes IGF-II, serving as a regulatory ''sink'' for this stimulatory ligand (19). Furthermore, the stimulatory effects of IGF-I and IGF-II are further regulated by circulating IGF-binding proteins (IGFBP) 1 to 6 (20). IGF-binding proteins, which vary in the binding affinities for IGF-I and IGF-II, limit the bioavailability of these ligands for receptor binding.

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There are several potential strategies by which to target and inhibit the IGF-I system, which have been reviewed elsewhere (21). However, a few strategies have emerged that are clinically feasible and are under early preclinical and clinical investigations. Monoclonal antibodies targeting the IGF-IR (IGF-IRmAb) are currently being investigated in phase I and II clinical trials. IGF-IRmAb is an attractive strategy, as it targets the major proliferative kinase in the IGF-I system and has little affinity for the InsR. Early clinical investigations with IGF-IRmAbs suggest that IGF-IRmAbs are very well tolerated and have shown early evidence of clinical activity (22). A potential liability to this strategy is that the mitogenic InsR isoform A is not targeted. TKIs of the IGF-I system are also in preclinical and clinical development. Due to the nearly identical kinase domain of the IGF-IR and InsR, small molecules inhibitors have been developed that can completely block IGF-I signaling through IGF-IR and InsR (23–26). However, the potential liability with this strategy is that TKIs may lead to hyperglycemia by blocking the InsR isoform B. The first clinical report of the phase I trial with the IGF-IRmAb, CP-751,871, in fact, reported hyperglycemia as this most common adverse event (22), suggesting some interference with the function of InsR.

As these agents are developed clinically, the mechanisms of resistance to IGF-IR targeting by TKIs or IGF-IRmAb will be important to understand as it can open new therapeutic strategies for the treatment of patients with cancer. Previous data suggested that IGF-IR signaling can provide a mechanism of resistance to HER receptor-targeted therapy (9, 27–31). To determine if this apparent cross-talk was bidirectional, we undertook the studies described herein. We investigated the role of IGF-IR cross-talk with HER in IGF-IR and HER resistance. We show that overexpression of activated HER receptors will confer resistance to IGF-IR/InsR inhibition by the TKI BMS-536924 and that, by simultaneously targeting HER and IGF-IR, synergistic antitumor effects occur in a panel of ovarian cancer cell lines. These results suggest strategies to overcome resistance to IGF-IR targeting and support the early clinical testing of a dual-pathway targeting approach.

Materials and Methods

Cell Lines and Reagents

OV167 and OV202 are ovarian cancer cell lines derived from a primary tumor specimen as described previously (32). A2780 and OVCAR3 ovarian cancer cells lines and MCF-7 breast cancer cell line were purchased from the American Type Culture Collection. SKOV3.ip1 cells were a kind gift from Dr. Ellen Vitetta. MCF-7 and OV202 cells were grown as described previously (23). Medium conditions for the remaining cell lines were as follows: A2780-RPMI with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, sodium bicarbonate, L-glutamine, penicillin, and streptomycin; OV167 and OVCAR3- RPMI with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. All cultures were grown in 5% CO_2 at 37°C. With the exception of fetal bovine serum, all medium and supplements were purchased from Cellgro/Mediatech.

Reagents were purchased as follows: bovine serum albumin, ampicillin, Tris-HCl, 4′,6-diamidino-2-phenylindole, Hoechst 33258, SDS, bromphenol blue, and glycerol from Sigma; CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit, Wizard Plus SV DNA minipreps, and 10× PBS solution from Promega; T4 DNA ligase, *Hin*dIII and *Xba*I from New England Biolabs; LongR3 IGF-I (Gro Pep) from Thebarton; fetal bovine serum, epidermal growth factor (EGF), Opti-fect, chemically competent *Escherichia coli* DH5α, and geneticin/G418 from Invitrogen/Biosource; heregulin 1 (NRG1-β1) from R&D Systems; and Plasmid Midi Kit from Qiagen.

Antibodies were purchased from the following vendors: poly(ADP-ribose) polymerase (mouse monoclonal), Bax (rabbit polyclonal), XIAP (rabbit polyclonal), phospho-AKT (Ser^{473} and Thr^{308}; rabbit polyclonal), phospho-extracellular signal-regulated kinase (ERK) 1/2 (Thr^{202}/Tyr^{204}; rabbit polyclonal), ERK (rabbit polyclonal), phospho-p70 S6 kinase (Thr^{421}/Ser^{424}; rabbit polyclonal), p70 S6 kinase (rabbit polyclonal), phospho-GSK3b (Ser^{21}/Ser^{9}; rabbit polyclonal), GSK3 (rabbit polyclonal), HER-2 (mouse monoclonal), phospho-HER-3 (Tyr^{1289}; rabbit monoclonal), and phospho-IGF-IR/InsR (Tyr^{1131}/Tyr^{1146}; rabbit polyclonal) from Cell Signaling Technology; Bcl-2 (mouse monoclonal), Bcl-xL (rabbit polyclonal), AKT (goat polyclonal), actin (goat polyclonal and mouse monoclonal), Raf-1 (mouse monoclonal), HER-4 (mouse monoclonal), and IGF-IR (rabbit polyclonal) from Santa Cruz Biotechnology; phospho-HER-2 (Tyr^{1248}; rabbit polyclonal), phospho-EGF receptor (EFGR; Tyr^{1173}; mouse monoclonal), and EGFR (sheep polyclonal) with A431-positive control lysate from Upstate Biotechnology; HER-3 (mouse monoclonal) from Lab Vision/Neomarkers; and actin (chicken polyclonal) from Novus Biologicals. Peroxidase-coupled secondary antibodies were supplied by KPL.

Construction of a Stable Cell Line

The pcDNA 3.1 mammalian expression vector was purchased from Invitrogen. pcDNA 3.1 vector containing wild-type EGFR was a gift from C. David James. pcDNA 3.1 vector containing wild-type HER-2 was a gift from Tai Wong. The vectors were amplified transforming chemically competent *E. coli* and selecting on LB + ampicillin culture plates. The appropriate vector clones were then verified by diagnostic (*Hin*dIII/*Xba*I) digesting small-scale mini preparations (Promega) and then verified by DNA sequencing. Midi-scale DNA preparations were then made (Qiagen) and used for mammalian cell transfection of MCF-7 cells using Opti-fect reagent per the product instructions. Stable transfectants were selected in 800 μg/mL G418 and clonal isolates were confirmed by Western blotting.

MTS proliferation assay was done as described previously (23). Briefly, 5,000 cells per well of a 96-well plate were plated in serum-containing conditions and allowed to attach overnight. The following day, the medium was

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changed to serum-free conditions in the presence of drug and/or ligands as noted in the text. After 72 h treatment, the MTS dye reduction was assessed as per the product information label. Proliferation was calculated as a percentage of the non-drug-treated controls. Experiments were done in at least triplicate. The method of Chou and Talalay was used to determine synergy as described previously (33, 34). Median effect analysis was done using Calcusyn software (Biosoft). With this method, a combination index > 1 is deemed antagonistic, a combination index < 1 is synergistic, and combination index = 1 is considered additive.

Clonogenic assays were done as described previously (35). Briefly, MCF-7 cell variants were trypsinized and plated in 60 mm tissue culture plates to a density of 500 to 1,000 per plate, respectively. Cells were allowed to adhere for 22 to 24 h, and drugs were added as indicated to final concentrations from 1,000-fold concentrated stocks. After 72 h incubation, plates were washed twice with serum-free medium, fresh medium was added, and cells were incubated until colonies were visible. The plates were washed once with PBS and stained with Coomassie brilliant blue. Visible colonies were counted and reported as percent of control (DMSO-treated) cells. Experiments were done in triplicate.

Western Blotting

Protein expression and activation was assessed by Western blotting as described previously (23). Briefly, after conditions/treatments were done as described in the text, cells were washed twice with ice-cold PBS. The PBS was then removed as completely as possible and the cells were then immediately lysed by adding 4× sample buffer [250 mmol/L Tris-HCl (pH 6.8), 8% SDS, 20% glycerol, 0.0075% bromphenol blue]. Lysates were then sonicated and frozen immediately at 20°C or assayed for total protein by the bicinchoninic acid method (29). Samples were boiled at 95°C for 15 min with 100 mmol/L DTT and separated by SDS-PAGE. After proteins were transferred to nitrocellulose or polyvinylidene difluoride membranes, they were blocked for 1 h in PBS-Tween 20/5% nonfat milk or bovine serum albumin and probed overnight at 4°C with primary antibodies. After three washes in PBS-Tween 20, blots were probed with horseradish peroxidase-conjugated secondary antibody for 1 h. After three additional washes, bands were visualized with enhanced chemiluminescence reagent (Amersham) on XoMAT film (Kodak). Experiments were done in triplicate.

Apoptosis Assay

Apoptosis was quantitated by assessing nuclear changes indicative of apoptosis (chromatin condensation and nuclear fragmentation) using the DNA-binding dye 4′,6-diamidino-2-phenylindole as described previously (36). Cells were seeded in 35 mm plates at 2×10^5 per well. After incubation for 24 h, the plates were washed and changed to serum-free medium containing drug at concentrations and for durations listed in the text. The cells were then stained with 4′,6-diamidino-2-phenylindole and counted by fluorescence microscopy (Nikon Eclipse TE200). For each treatment, at least 300 cells in six different high-power fields were counted. Experiments were done in triplicate.

Apoptotic Morphology Imaging

OV202 and SKOV3.ip1 cells were grown to approximately 70% confluency in serum-containing medium in six-well plates. Cells were then treated with BMS-536924, BMS-599626, or the combination in serum-free medium for 72 h as described in the text. Cells were then fixed within the six-well plates with 70% ethanol for 15 min and washed twice with PBS. Cells were then stained with Hoechst 33258 at 0.5 μg/mL in PBS for 60 min and immediately visualized by fluorescent microscopy as described previously (23). Experiments were done in triplicate. Images represent typical fields.

Results

IGF-IR/InsR or HER Receptor Inhibition Stimulates Reciprocal Receptor Phosphorylation

OV202 cells are an epithelial ovarian cancer cell line that express IGF-IR, HER-2, and low levels of the InsR and have

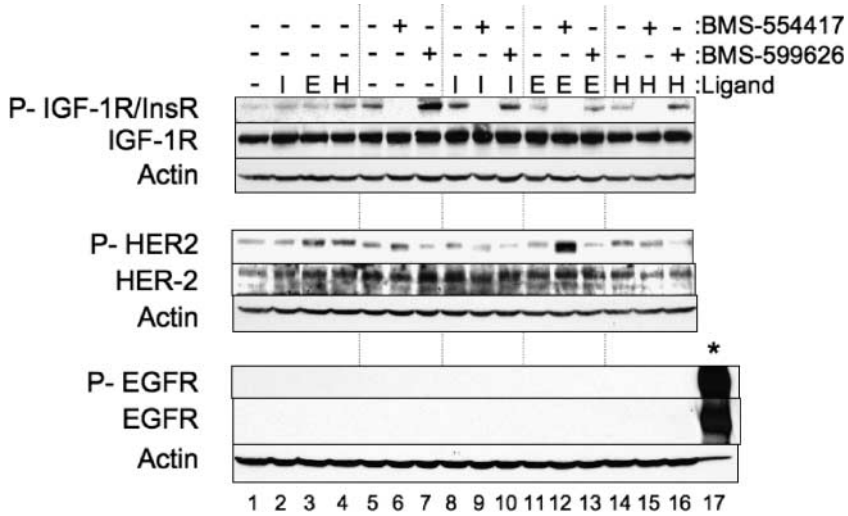

Figure 1. Bidirectional cross-talk signaling occurs in ovarian cancer cells. Subconfluent OV202 cells were treated with either DMSO, BMS-554417 (10 μmol/L), or BMS-599626 (10 μmol/L) for 1 h in serum-free conditions. For the final 15 min drug treatment, 10 nmol/L LongR3 IGF-I (*I*), 100 ng/mL EGF (*E*), or 5 ng/mL heregulin (*H*) were added to the medium. Lysates were then prepared and analyzed by Western blotting. EGF-stimulated A431 lysates (20 μg; *asterisk*) were loaded as positive control for total and activated EGFR.

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been described elsewhere (23, 37). These cells were used initially as a proof of concept for targeting the IGF-IR/InsR and confirming specificity. Phosphorylation of IGF-IR and InsR was completely inhibited (Fig. 1, *lanes 6, 9, 12*, and *15*) on treating cells with BMS-554417 at doses that resulted in antiproliferative activity (Fig. 2A). In a reciprocal manner, HER-2 phosphorylation increased (Fig. 1, *lane 6*) in response to BMS-554417 when compared with DMSO-treated controls (Fig. 1, *lane 5*). Despite the lack of detectable EGFR expression in OV202 cells, the increase in HER-2 phosphorylation with BMS-554417 treatment was further enhanced by the addition of EGF (Fig. 1, *lane 12*). To investigate whether this apparent cross-signaling was reciprocal in nature, we treated OV202 cells with a specific pan-HER inhibitor, BMS-599626 (Fig. 1, *lanes 7, 10, 13*, and *16*; ref. 38). At doses that caused reduction of HER-2 phosphorylation, IGF-IR/InsR phosphorylation increased compared with DMSO-treated controls (Fig. 1, *lane 7*). This increase in phosphorylation of IGF-IR/InsR was not enhanced further in the presence of IGF-I, EGF, or heregulin.

Dual Inhibition of IGF-IR/InsR and HER Receptors Causes Synergistic Cell Killing in Multiple Ovarian Cancer Cell Lines

Based on the findings above, OV202 cell proliferation was assessed on treatment with various concentrations of BMS-554417 alone, BMS-599626 alone, or in combination at a fixed ratio. At doses of the single agents that had modest antiproliferative effects, the combination treatment appeared to have a significant antiproliferative effect (Fig. 2A and B). Median effect analysis showed a marked degree of synergy as reflected by combination index values less than 0.4 (Fig. 2C; ref. 33). The degree of synergy in the absence or presence of insulin, IGF-I, EGF (data not

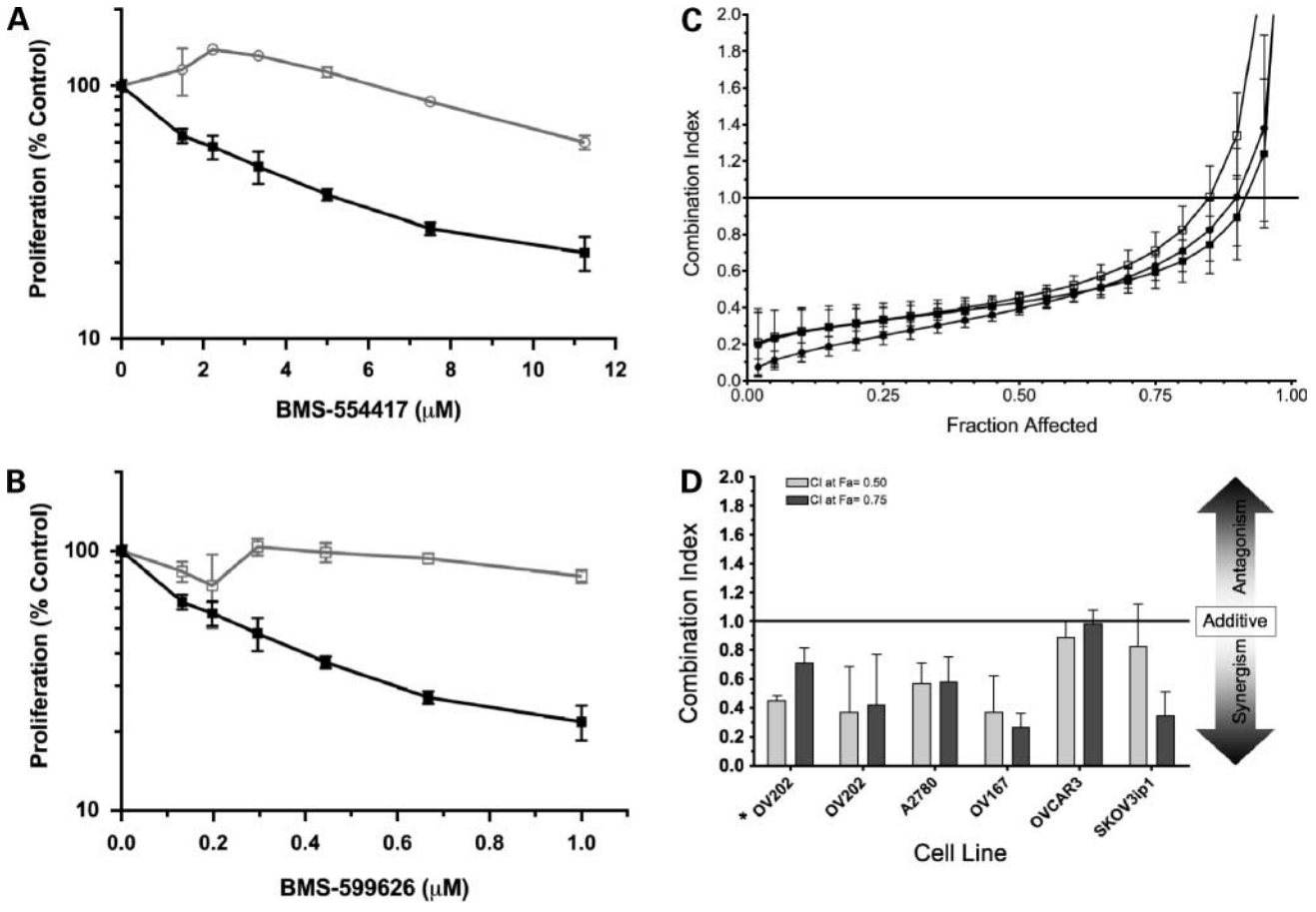

Figure 2. Simultaneous treatment with BMS-536924 and BMS-599626 has synergistic antiproliferative effects. **A** and **B,** OV202 cells were treated with increasing concentrations of BMS-554417 (-○-), BMS-599626 (-□-), or the combination (-■-) at a fixed ratio (11.25:1). Proliferation was assessed as a function of DMSO-treated controls by MTS proliferation assays and reported as a function of either BMS-554417 (**A**) or BMS-599626 (**B**) concentrations as described in Materials and Methods. **C,** synergy combination index plots were generated by median effect analysis of OV202 cells treated with the combination of BMS-554417, BMS-599626, or the combination in the absence (-□-) or presence of LongR3 IGF-I (10 nmol/L, -■-) or insulin (10 nmol/L, -●-). *Bars,* SD of four replicates. **D,** cells were treated with either BMS-536924, BMS-599626, or the combination at various doses. The antiproliferative effects were then assessed and median effect analysis was done. The combination index values at the 50% (*light gray columns*) and 75% (*dark gray columns*) fraction affected are shown. For comparison, OV202 cells treated with BMS-554417 and BMS-599626 (*asterisk*) were included.

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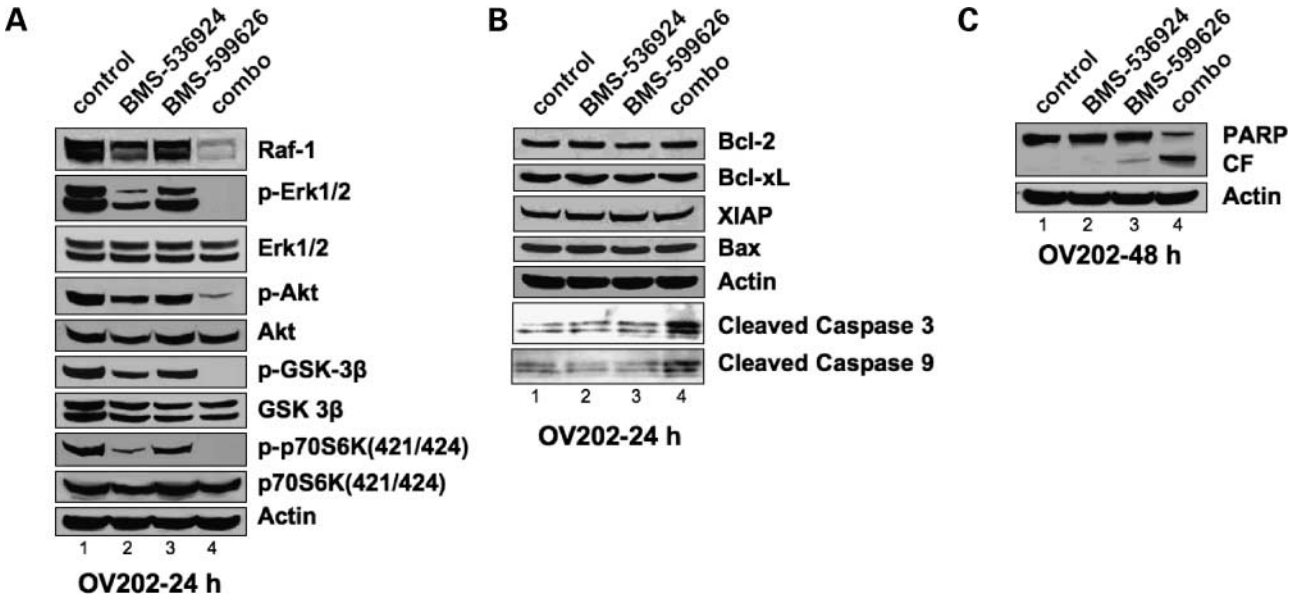

Figure 3. Combined Inhibition of IGF-I/HER signaling decreases activation of AKT/ERK activation and increases caspase/poly(ADP-ribose) polymerase cleavage. **A** to **C,** subconfluent OV202 cells were treated with DMSO (control), BMS-536924 (IC_{50} = 5 μmol/L), BMS-599626 (IC_{50} = 10 μmol/L), or the combination (*combo*) in serum-free conditions. After either 24 or 48 h treatment, cells were washed and 40 μg lysates were prepared and analyzed by Western blotting.

shown), and heregulin (data not shown) was similar. Further investigations with the 2-(4-substituted-2-oxo-1,2-dihydropyridin-3-yl)-benzimidazole derivative small-molecule inhibitors of the IGF-IR focused on the related analogue, BMS-536924, which has improved oral exposure and *in vivo* activity compared with BMS-554417 (26, 39). Repeat experiments to assess synergy confirmed that the antiproliferative effect of BMS-536924 in combination with BMS-599626 was synergistic when compared with the effects of the single agents alone (Fig. 2D).

To investigate whether this synergistic antiproliferative activity was specific to OV202 cancer cells, we evaluated the antiproliferative effects of BMS-536924 in combination with BMS-599626 with four other ovarian cancer cell lines. The combination of BMS-536924 showed synergistic antiproliferative activity in all ovarian cancer cell lines as determined by median effect analysis (Fig. 2D). Synergy was observed in OV202, A2780, and OV167 cells at both 50% and 75% fraction affected, whereas synergism in OVCAR3 and SKOV3ip1 cells approached additivity at the 75% and 50% fraction affected, respectively.

Simultaneous Inhibition of IGF-IR/InsR and HER Receptors Inhibited AKT/ERK Activation

To understand the molecular mechanism by which antiproliferative synergy was observed in ovarian cancer cells treated with BMS-536924 and BMS599626, Western blotting for the total and activated forms of key signaling intermediates of the IGF- and HER and intrinsic apoptotic pathways was done on OV202 lysates (Fig. 3A). In OV202 cells, treatment with the combination of BMS-536924 and BMS-599626 resulted in decreased phosphorylation of ERK, AKT, GSK-3β, and p70 S6 kinase. Whereas total protein expression of these proteins were unchanged, total Raf-1 protein levels were greatly reduced in OV202 cells treated with the combination compared with the single agent. Thus, proliferative and prosurvival signaling through the AKT and ERK pathways in OV202 cells was dramatically reduced in response to combination treatment with BMS-536924 and BMS-599626 compared with single-agent treatment.

Mechanism of Synergism of Combining BMS-536924 and BMS-599626 Is Enhanced Apoptosis

As we have shown previously that single-agent IGF-IR/InsR inhibition can induce apoptosis through the intrinsic pathway in OV2020 cells, we hypothesized that the synergistic activity of BMS-536924 and BMS-599626 in OV202 cells was due to enhanced apoptosis. To test this hypothesis, we assessed changes in biochemical and morphologic markers of apoptosis in OV202 cells in the presence of BMS-536924, BMS-599626, and the combination. There were no apparent changes in patterns of expression in the apoptotic proteins Bcl-2, Bcl-XL, XIAP, or Bax in response to treatment with BMS-536924 and/or BMS-599626 (Fig. 3B). Despite this, the combination treatment was associated with increased cleavage of caspase-3, caspase-9, and poly(ADP-ribose) polymerase when compared with the single agents, consistent with an enhanced apoptotic death on treatment with the combination BMS-536924 and BMS-599626 (Fig. 4C). To confirm the biochemical evidence of enhanced apoptosis with combination treatment, the extent of apoptotic changes in OV202 cells in the presence to BMS-536924, BMS-599626, and the combination was assessed by nuclear morphology in blinded fashion. At doses of BMS-536924 or BMS-599626

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that lead to relatively small amounts of apoptosis alone, the combination generated a large degree of apoptosis. The enhancement of apoptosis was apparent at 24 h (Fig. 4A). Evaluation of apoptosis using nuclear morphology was repeated in SKOV3.ip1 cells, which also showed a profound antiproliferative effect of combination treatment compared with the single agents at the 75% fraction affected (Fig. 4B). Similar to OV202 cells, substantial nuclear apoptotic morphology was seen in SKOV3.ip1 cells treated with the combination of BMS-536924 and BMS-599626 (Fig. 4C and D). Although the single-agent exposures to BMS-536924 and BMS-599626 were antiproliferative, only modest apoptotic effect was observed on treatment for up to 5 days (Fig. 4A, C and D).

Reciprocal HER Receptor Activation with IGF-IR Inhibition Can Be Inhibited by HER TKI

Based on our initial observation of reciprocal receptor phosphorylation in OV202 cells, we hypothesized that receptor expression and/or phosphorylation modulation occurred in the five ovarian cancer cell lines that showed synergistic antiproliferative activity with BMS-536924 and BMS-599626. The five ovarian cancer cell lines were treated with either DMSO, BMS-536924 at the IC_{50} concentration, BMS-599626 at the IC_{50} concentration, or the combination for 24 h (Fig. 5). Following treatment, cellular lysates were analyzed by Western blotting for total and phosphorylation forms of the HER and IGF-I system receptors. On treatment of cells with BMS-536924, all five ovarian cancer cells lines showed evidence of increased HER receptor signaling activity (Fig. 5, *lanes 2, 6, 10, 14*, and *18*). The specific HER receptor signaling changes that occurred varied by cell type. In all ovarian cell lines, this HER receptor signaling increase was blocked by BMS-599626 (Fig. 5, *lanes 3, 7, 11, 15*, and *19*). All ovarian cell lines had very low expression and no detectable activation of HER-4/erbB4 by Western blotting (data not shown).

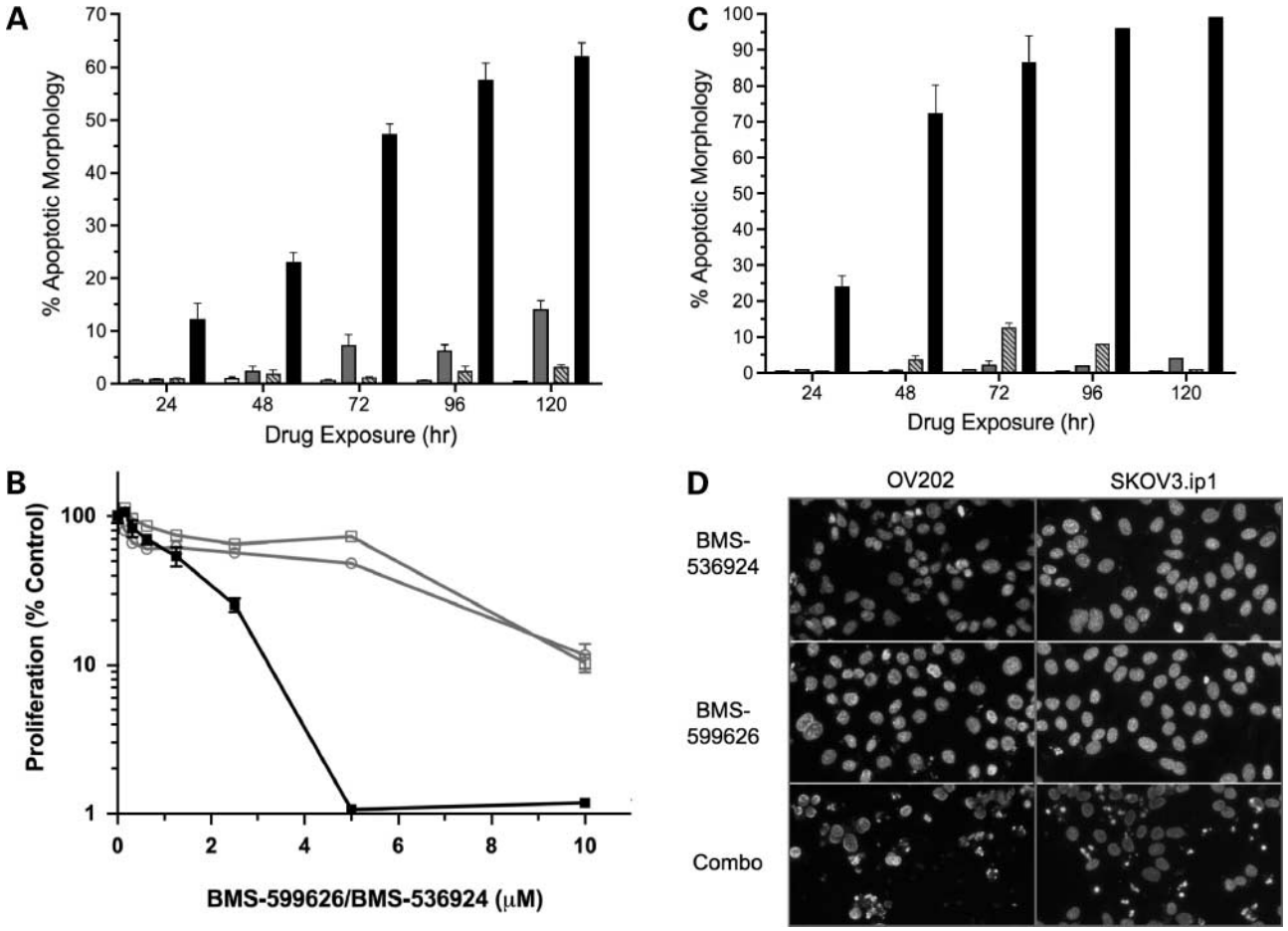

Figure 4. Combined treatment with BMS-536924 and BMS-599626 induce apoptosis in ovarian cancer cells. OV202 (**A**) or SKOV3.ip1 (**C**) cells were treated with either DMSO (*white columns*), BMS-536924 (*light gray columns*), BMS-599626 (*striped columns*), or the combination (*black columns*) at IC_{50} concentrations (Supplementary Table S1) at time points indicated. Apoptosis was assessed as described in Materials and Methods. **B,** antiproliferative effects of BMS-536924 (⊖), BMS-599626 (⊟), and the combination (■) in SKOV3.ip1 cells. *Bars,* SD (n = 3). **D,** OV202 and SKOV3.ip1 cells were stained with Hoechst 33258 at 0.5 μg/mL after treatment with either BMS-536924, BMS-599626, or the combination at the IC_{50} concentrations (Supplementary Table S1). Representative fields of three replicate experiments.

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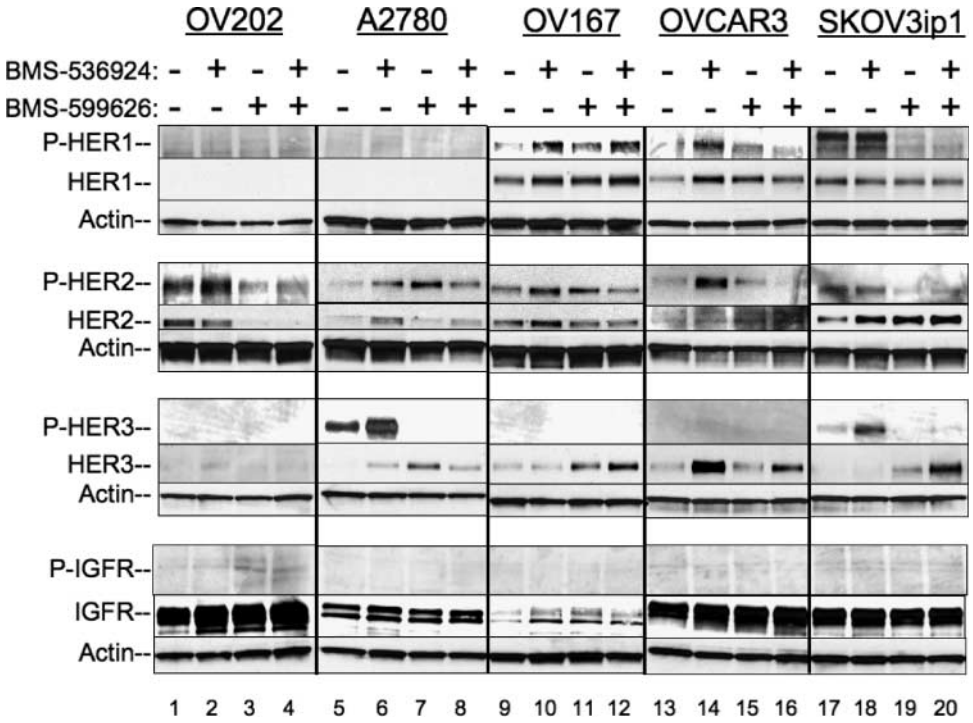

Figure 5. HER receptor modulation in ovarian cancer cells in response to BMS-536924 and BMS-599626 treatment. Ovarian cancer cells were treated with either DMSO or the IC_{50} concentrations (Supplementary Table S1) of BMS-536924, BMS-599626, or the combination for 24 h. Cells were then harvested for lysates and analyzed by Western blotting for total and activated HER receptors or IGF-IR as described in Materials and Methods.

Of note, in contrast to changes seen in 1 h with BMS-599626 treatment in OV202 cells (Fig. 1, *lane 7*), there were no changes in IGF-IR total or activated receptors in the ovarian cancer cell lines tested in response to treatment with BMS-599626 for 24 h, with the exception of OV202 cells (Fig. 5, *lane 3*). These data suggest that IGF-IR/InsR inhibition can stimulate increased HER receptor signaling in ovarian cancer cells.

Activated HER Receptor Expression Is Sufficient to Cause Resistance to BMS-536924

Based on the above observations of functional IGF-IR and HER receptor cross-talk and data suggesting that IGF-IR can confer resistance to HER-targeted therapy, we hypothesized that HER receptors could confer resistance to IGF-IR-targeted therapy. As our ovarian cancer cell lines had detectable expression of HER protein receptors and only relatively moderate sensitivity to IGF-IR inhibition as a single agent, we investigated the activity of BMS-536924 in the breast cancer cell line, MCF-7. MCF-7 parental cells were relatively sensitive to BMS-536924 and have no detectable expression of HER-1 or HER-2 (Fig. 6A). MCF-7 variants were constructed, which contained either an empty mammalian expression vector (MCF-7/pcDNA), the vector containing the full-length, wild-type EGFR (MCF-7/EGFR), or the vector containing the full-length, wild-type HER-2 receptor (MCF-7/HER-2). Western blot analysis of whole-cell lysate from untransfected MCF-7 and stably transfected MCF-7/pcDNA, MCF-7/EGFR, and MCF-7/HER-2 was done (Fig. 6A). MCF-7 and MCF-7/pcDNA cells had no detectable expression of EGFR or HER-2. However, MCF-7/EGFR cells contained high levels of EGFR and MCF-7/HER-2 cells contained high levels of HER-2. HER-2 was activated in MCF-7/HER-2 cells as shown by constitutive phosphorylation. Transfection of MCF-7 cells with vectors expressing either EGFR or HER-2 has no apparent effect of the expression levels of total or activated IGF-IR. Additionally, there were no apparent differences in total or activated AKT or ERK expression in all four cell lines.

To determine whether HER receptor expression was sufficient for conferring resistance to IGF-IR-targeted therapy, we did a proliferation assay on MCF-7 cells stably transfected with pcDNA, EGFR, or HER-2. In the absence of ligands, the antiproliferative effects of BMS-536924 were similar in all transfected cell lines (Fig. 6B). However, the addition of EGF or heregulin, which have little proliferative effects on MCF-7 cells (data not shown), was sufficient to confer a high level of resistance in MCF-7/EGFR and MCF-7/HER-2 cells, respectively. Furthermore, the addition of EGF to MCF-7/EGFR cells and heregulin to MCF-7/HER-2 cells greatly reduced the ability of BMS-536924 to prevent MCF-7 variant colony formation (Fig. 6C and D). These data suggest that activated EGFR and HER-2 heterodimer signaling, but not unstimulated EGFR or constitutively activated HER-2 alone, can confer high levels of resistance to IGF-IR/InsR inhibition.

Discussion

We have shown for the first time that blockade of the IGF-IR and InsR with a small-molecule TKI stimulates cross-talk signaling through the activation of the HER family of receptors. In a reciprocal fashion, inhibition of HER-2 stimulates phosphorylation of IGF-IR/InsR. Others

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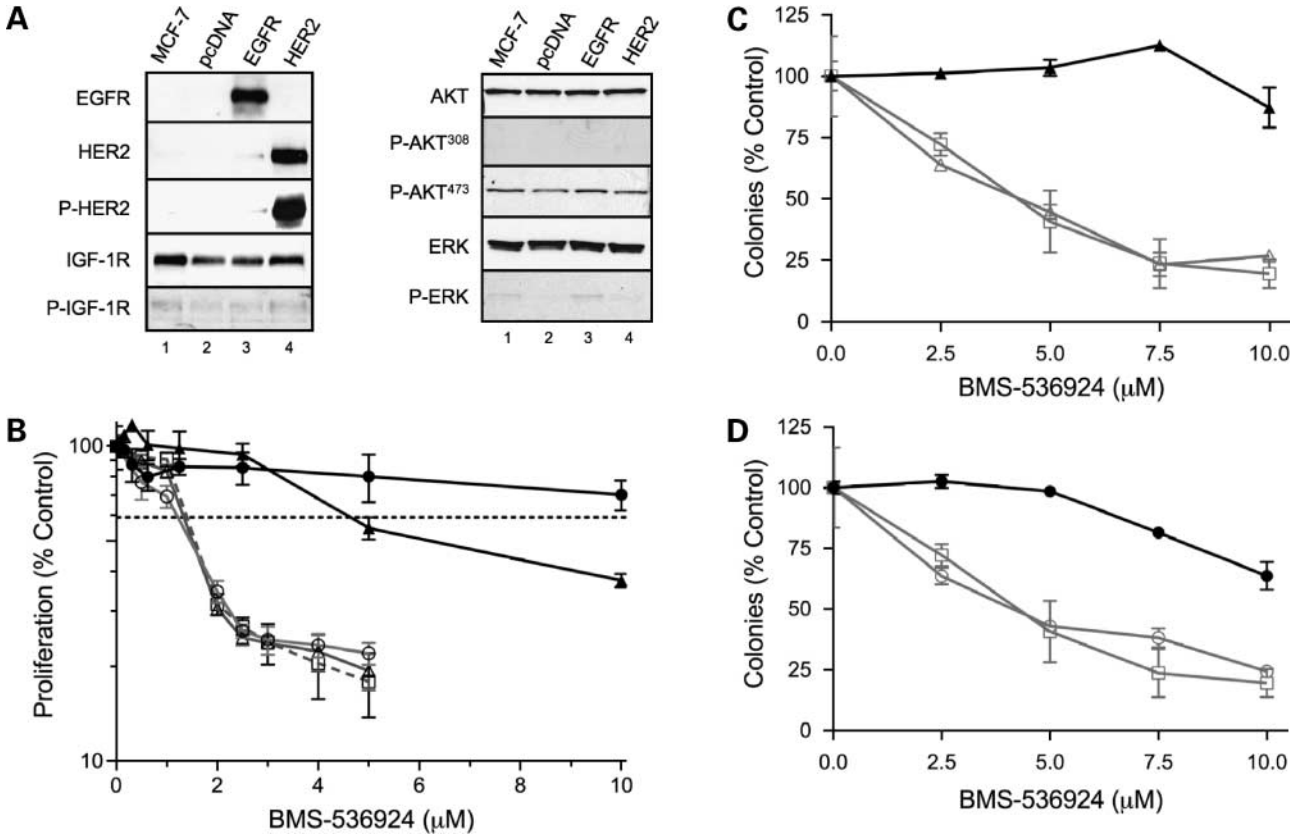

Figure 6. Activated HER receptors are sufficient for resistance to IGF-IR/InsR-targeted therapy. **A,** parental MCF-7 cells (MCF-7) and stable transfectants expressing either empty vector (pcDNA) or vector containing wild-type HER-1 (EGFR) or wild-type HER-2 (HER-2) were grown to near confluency and analyzed by Western blotting as described in Materials and Methods. The variants (pcDNA, ⊟; EGFR, △; HER-2, ⊖) were then treated with various doses of BMS-536924 for 72 h in the absence or presence of EGF (100 ng/mL, ▲) or heregulin (10 ng/mL, ●) as indicated. Proliferation (**B**) and clonogenicity (**C** and **D**) were assessed by MTS and clonogenic assays as described in Materials and Methods. *Bars,* SD.

have shown previously that the IGF-IR can potentially provide a mechanism of resistance to therapy targeting the HER family members, EGFR and HER-2 (8, 9, 27–31). These findings have supported the clinical development of therapies targeting the IGF-IR as a potential therapeutic strategy for overcoming or blocking IGF-IR-dependent resistance. Our data indicate that the signaling cross-talk is bidirectional and can occur through the various members of the HER receptor family.

The finding that activated HER signaling is sufficient to confer resistance to BMS-536924 has clear clinical implications. HER-2 overexpression is present and drives tumor proliferation and prosurvival signaling in 25% of breast cancers and confers a poor prognosis (40). EGFR overexpression and activating mutations are present in a significant number of non-small cell lung, head and neck, colon, and pancreatic cancers, which contributes to their tumorigenicity (41). The EGFR/HER-2 status of these tumors may be critical to determining their sensitivity to IGF-IR inhibition. Because HER-2 autophosphorylation is ligand independent, it was somewhat surprising that MCF-7/HER-2 cells alone were not resistant to BMS-536924. However, HER-2 homodimers in the absence of stimulatory ligands, such as heregulin, do not have access to the increase repertoire of adapter and intrasignaling molecules that heterodimers, such as HER-2/HER-3 (42, 43), do. Although HER-3 does not have a kinase domain, its cross-phosphorylation by other member of the HER family of receptors at residues with the YXXM motif, including Tyr^{1289}, stimulates phosphatidylinositol 3-kinase signaling (44). It is believed that the enhanced networking potential of HER-2-containing heterodimers explains their increased tumorigenicity compared with HER-2 homodimers (42). In our model, it appears that it is this level of HER receptor signaling that is required to overcome sensitivity to BMS-536924. Indeed, it may be that evaluation of HER-3 or heregulin in the presence of HER-2 may be important for predicting sensitivity.

The combined effects of IGF-IR/InsR and pan-HER inhibition show that cotargeting both pathways is sufficient to cause a large degree of apoptotic cell death. These finding would suggest that either IGF-I or HER family pathway is critical for ovarian cancer survival. Although BMS-536924 and BMS-599626 had antiproliferative activity in the ovarian cancer cell lines tested, they had no substantial apoptotic activity as single agents compared

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with DMSO-treated controls. Additionally, up-regulation of the HER family of receptor signaling shows the dynamic nature of receptor expression and how they may be modulated by targeted therapy, such as BMS-536924. Given these data, it not surprising that agents targeting single members the HER family of receptors have shown disappointing clinical activity in ovarian cancer (45, 46). In the ovarian cancer models we have tested, substantial apoptosis was only seen after complete blockade of the IGF and HER signaling pathways.

In summary, these data, as well as data from others, suggest bidirectional functional cross-talk between the IGF and HER family of receptors. Our findings support the hypothesis that HER receptor family signaling can provide a resistance mechanism for agents targeting the IGF-IR that are currently in phase I/II and III. As simultaneous inhibition of both IGF-I and HER pathways disrupts this adaptive cross-talk mechanism, our data suggest that simultaneous treatment with HER and IGF-IR inhibitors may be more effective than either alone. Additionally, our results would support the notion that patients developing resistance to HER-1- or HER-2-targeted therapy may become resensitized by continuing the HER-targeted agents in combination with IGF-IR inhibitors.

Disclosure of Potential Conflicts of Interest

J.M. Carboni, R.M. Attar, T.W. Wong, and M.M. Gottardis, employees of Bristol Meyers Squibb Pharmaceutical Research Institute. No other potential conflicts of interest were disclosed.

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