Sensitivity to pertuzumab (2C4) in ovarian cancer models: cross-talk with estrogen receptor signaling

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Abstract

Pertuzumab (Omnitarg, rhuMab 2C4) is a humanized monoclonal antibody, which inhibits HER2 dimerization. Because it has shown some clinical activity in ovarian cancer, this study sought to identify predictors of response to this agent in a model of ovarian cancer. A panel of 13 ovarian cancer cell lines was treated with heregulin β1 (HRGβ1) or transforming growth factor-α, and cell proliferation was assessed. Both agents increased cell number in the majority of cell lines studied, the response to both being similar ($r = 0.83$; $P = 0.0004$, Pearson test). HRGβ1 stimulation could be partially reversed by pertuzumab in 6 of 13 cell lines, with complete reversal in PE04 and PE06 cells. Addition of pertuzumab to transforming growth factor-α–stimulated cells produced growth inhibition in 3 of 13 cell lines (PE01, PE04, and PE06). The magnitude of HRGβ1-driven growth stimulation correlated significantly with an increase in extracellular signal-regulated kinase 2 ($P = 0.037$) but not Akt ($P = 0.99$) phosphorylation. Such HRGβ1-driven phosphorylation of extracellular signal-regulated kinase 1/2 and Akt could be reduced with pertuzumab, accompanied by changes in cell cycle distribution. In cell lines responsive to pertuzumab, HRGβ1-enhanced phosphorylation of HER2 (Tyr^{877}) was reduced. Estrogen-stimulated changes in growth, cell cycle distribution, and signaling were reversed by pertuzumab, indicating cross-talk between HER2 and estrogen signaling. These data indicate that there is a subset of ovarian cancer cell lines sensitive to pertuzumab and suggest possible predictors of response to identify patients who could benefit from this therapy. Furthermore, we have identified an interaction between HER2 and estrogen signaling in this disease. [Mol Cancer Ther 2007;6(1):93–100]

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Introduction

The growth and progression of many ovarian cancers is regulated by the HER/epidermal growth factor (EGF) receptor family of receptor tyrosine kinases, consisting of HER1 (EGF receptor), HER2, HER3, and HER4 (1–6). These receptors are activated by ligands of the EGF family, including transforming growth factor-α (TGF-α; which activates HER1) and heregulin (which activates HER3 and HER4; refs. 7–9). Binding of a given ligand to its receptor drives dimerization with either a receptor of the same type (homodimerization) or with another receptor type within the HER family (heterodimerization). HER2 itself has no direct ligand and is activated via either homodimerization (with itself) or heterodimerization with HER1, HER3, or HER4 (7–9). Through heterodimerization with other members of the HER family, TGF-α and heregulin β1 (HRGβ1) have been shown to stimulate the growth of ovarian cancer cells (1–3). Dimerization elicits phosphorylation of specific tyrosine residues on the COOH-terminal domain of the receptors, which act as docking sites for multiple signaling proteins. Intracellular signaling is then transmitted to the cell nucleus via pathways, which include (*a*) the Ras/Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK pathway and (*b*) the phosphatidylinositol 3-kinase/Akt pathway (ultimately regulating cell proliferation, differentiation, and apoptosis as well as tumor transformation and tumorigenesis; ref. 7). Because high levels of HER2 expression have been identified in 10% to 40% of ovarian cancers and this has subsequently been associated with poor survival (4–6), ovarian cancer patients are prospective candidates for HER2-targeted therapies. Trastuzumab has been studied previously against high HER2 ovarian cancer but showed a relatively low response rate (7.3%) in this group of ovarian cancer patients (10); furthermore, high HER2-overexpressing tumors only represent 11.4% of ovarian cancers compared with 20% to 30% of breast cancers. As such, the results of targeting HER2 overexpression with trastuzumab in this disease have been described as nonviable (11).

Pertuzumab (Omnitarg, rhuMAb 2C4) is the first of a new class of agents known as HER dimerization inhibitors, which act by binding to HER2, thus preventing dimerization with other HER partners (12). This antibody is active in low to moderate HER2-expressing breast cancer cell line models (in contrast to the activity of trastuzumab, which is restricted to high HER2-expressing tumors). Preclinical activity has been identified in several cancer types, including breast, colon, prostate and ovarian cancer (12–14). In a recent phase I clinical trial, antitumor activity has been shown in one of three ovarian cancer patients (15), with the drug subsequently moving into phase II trials in ovarian, breast, lung, and prostate cancer patients. A report

of the phase II ovarian cancer trial has described clinical activity as shown by partial response (4.3%), stable disease for >6 months, and CA125 reductions of >50% in 14.5% of patients (16).

The purpose of this study was therefore to explore sensitivity to pertuzumab in a panel of ovarian cancer cell lines and subsequently identify indicators of sensitivity to this agent.

Materials and Methods

Cell Lines

The ovarian cancer cell lines PEO1, PEO4, PEO6, PEO14, and PEA2 were established within the Edinburgh Cancer Research Centre (17); SKOV3 and CaOV3 cells were obtained from the American Type Culture Collection (Manassas, VA); OVCAR3, OVCAR4, and OVCAR5 were obtained from Dr. T.C. Hamilton (Fox Chase Institute, Philadelphia, PA); 41M, 59M, and OAW42 cells were obtained from the European Collection of Cell Cultures (Porton Down, United Kingdom). All cells were grown routinely as monolayer cultures in RPMI 1640 supplemented with 10% heat-inactivated FCS and 100 IU/mL penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Growth inhibition experiments were set up using log-phase cells seeded into 96-well flat-bottomed tissue culture plates (optimized around 1×10^4 in 1 mL) and incubated to reach 40% to 60% confluence before treatment.

Western Analysis

Cell lysates were prepared for Western blotting as described previously (18). In experiments using TGF-α or HRGβ1 (1 nmol/L, 15 min), cells were preincubated in medium containing 5% double charcoal-stripped serum for 48 h before treatment. Protein lysates (80 μg) were electrophoretically resolved on 10% SDS-PAGE and transferred to Immobilon-P membranes. After transfer, membranes were blocked with 1% blocking agent (Roche Diagnostics, Mannheim, Germany) in TBS before probing overnight at 4°C with the appropriate primary antibody. Antibodies used for Western blotting were as follows: anti–total HER2 (Cell Signaling Technology, Beverly, MA) at 1:1,000; anti–phosphorylated HER (pHER) 2 (Tyr^{877}; Cell Signaling Technology) at 1:1,000; anti–total EGF receptor (Cell Signaling Technology) at 1:1,000; anti–phosphorylated EGF receptor (Tyr^{845}; Cell Signaling Technology) at 1:1,000; anti-phosphorylated EGF receptor (Tyr^{1045}; Cell Signaling Technology) at 1:1,000; anti–total ERK 1/2 (Cell Signaling Technology) at 1:1,000; anti–phosphorylated ERK (pERK) 1/2 (Cell Signaling Technology) at 1:1,000; anti–total Akt (Cell Signaling Technology) at 1:1,000; anti–phosphorylated Akt (pAkt; Ser^{473}; Cell Signaling Technology) at 1:1,000; anti–total estrogen receptor α (ERα; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000; anti–phosphorylated ERα (Ser^{118}; Cell Signaling Technology) at 1:1,000; and anti-actin (Merck, Beeston, Nottingham, United Kingdom). Immunoreactive bands were detected using enhanced chemiluminescent reagents (Roche) and Hyperfilm enhanced chemiluminescence film (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Integrated absorbance values were obtained by densitometric analysis using a gel scanner and analyzed by 'Labworks' gel analysis software (UVP Life Sciences, Cambridge, United Kingdom).

Sulforhodamine B Growth Assays

Log-phase cells were seeded into 96-well flat-bottomed tissue culture plates (optimized between 1,000 and 4,000 cells per well). The following day, cells were washed in PBS and transferred to RPMI 1640 containing 5% double charcoal-stripped serum for 48 h before treatment. Cells were treated with either TGF-α (1 nmol/L; Sigma, Poole, Dorset, United Kingdom), HRGβ1 (1 nmol/L; Sigma), 17β-estradiol (E2; 1 nmol/L; Sigma), or tamoxifen (1 μmol/L; Sigma) ± pertuzumab (100 nmol/L; Roche Diagnostics GmbH, Penzberg, Germany). Cells were removed from the incubator after 72 h, and ice-cold 25% TCA solution (50 μL) was added to each well. All plates were placed on ice for 60 min after which the TCA solution was removed. Plates were washed under running tap water (10 times) and dried before staining with sulforhodamine B dye solution for 30 min at room temperature. Trays were again washed with 1% glacial acetic acid (four times) at room temperature, air dried, and resuspended in 10 mmol/L Tris buffer (pH 10.5; 150 μL) before reading at 540 nm.

Cell Cycle Assay

Flow cytometric DNA analysis of treated cells was carried out using methodology described by Levack et al. (19). After trypsinization, cells were resuspended in 100 μL citrate buffer and stored at −20°C before flow cytometric DNA analysis was carried out using a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ).

Annexin V Assay

Annexin V levels were measured in PE04 and SKOV3 cells treated as described above, and apoptosis was measured using the TACS Annexin V-FITC kit (R&D Systems, Minneapolis, MN) following the prescribed protocol.

Statistics

Relationships between variables were analyzed by the Student's *t* test and Mann-Whitney test where appropriate. Correlations were analyzed by the Pearson test.

Results

Pertuzumab Reverses HRGβ1- and TGF-α–Stimulated Growth in Selected Ovarian Cancer Cell Lines

To help identify cell line models sensitive to pertuzumab, a panel of 13 ovarian cancer cell lines, which expressed a range of HER receptor levels (Table 1; ref. 20), were initially treated with either HRGβ1 (1 nmol/L) or TGF-α (1 nmol/L) for 72 h, and cell proliferation was assessed by the sulforhodamine B assay (Fig. 1A and B). PE01, 41M, CAOV3, PE04, and PE06 cell lines were the most responsive to HRGβ1, whereas PEA2, OVCAR4, 59M, and SKOV3 cell lines showed no growth change. In general, the response to either HRGβ1 or TGF-α was similar ($r = 0.83$; $P = 0.0004$, Pearson test; Fig. 1C), although

Table 1. Expression levels of the HER receptor family and ERα in the panel of ovarian cancer cell lines

Cell line	HER1*	HER2	HER3	HER4	ERα†
PE01	+‡	++	++	−	+++
PE04	+	++	+	++	+++
PE06	+/−	++	+	++	+++
PE014	+	+	+	+	−
41M	+++	++	+	−	+/
59M	++	+	+/−	−	−
OAW42	+	+	+	+	−
CAOV3	++	+	+++	−	−
OVCAR3	++	+	+++	++	+/
OVCAR4	++	+	++	++	−
OVCAR5	+++	++	++	−	+/
SKOV3	++	+++	++	+	+++

*Adapted from Gilmour et al. (20).
†Adapted from O'Donnell et al. (21).
‡Expression levels based on Western analyses: +++, high; ++, moderate; +, low; +/−, borderline; and −, negative.

there was no simple relationship between growth factor stimulation and expression levels of any individual HER receptor (Table 1). Addition of pertuzumab (100 nmol/L) produced varying degrees of growth inhibition in all cell lines, which were stimulated by HRGβ1 (with the exception of OAW42). In some cell lines, HRGβ1 stimulation was completely reversed (PE04 and PE06), whereas in most cases, it produced a 40% to 60% reversal (Fig. 1A). Whereas pertuzumab also produced a marked reversal of TGF-α–driven growth in PE04 and PE06 cells (and partial reversal in PE01 cells), it was generally ineffective in the remaining cell lines (Fig. 1B). It should be noted that the PE01, PE04, and PE06 cell lines were derived from a single patient at different stages of treatment with PE04 and PE06 cells being chemoresistant, whereas PE01 cells are chemosensitive (17).

Pertuzumab alone produced only a small degree of growth inhibition (2–25%) throughout the panel of lines (Fig. 1D).

Pertuzumab Reversal of HRGβ1- and TGF-α–Stimulated Growth in PE04 Cells Is Accompanied by Changes in Cell Cycle Distribution but not Apoptosis

To help assess the functional changes associated with the growth inhibition of pertuzumab, the pertuzumab-sensitive PE04 cell line was compared with the relatively insensitive SKOV3 cell line. PE04 and SKOV3 cells were treated with HRGβ1 (1 nmol/L) or TGF-α (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L). Addition of pertuzumab to PE04 cells resulted in a reduction of both HRGβ1- and TGF-α–induced proliferation (Fig. 2A). No significant effects of pertuzumab on growth were seen in SKOV3 cells. Analysis of the cell cycle distribution showed that an increase in S-phase cells induced by both HRGβ1 and TGF-α in PE04 cells was reduced by addition of pertuzumab (Fig. 2B). No such changes on cell cycle distribution were seen in SKOV3 cells. Annexin V expression, indicative of early-stage apoptosis, was unaffected in both PE04 and SKOV3 cell lines (Fig. 2C), suggesting that apoptosis was not of significant importance in determining response.

Pertuzumab Inhibits HRGβ1-Activated but not TGF-α–Activated HER2 (Tyr^{877}) Phosphorylation

Because pertuzumab acts as a HER2 dimerization inhibitor, the activation of several phosphorylation sites on both HER2 and HER1 was explored to address whether

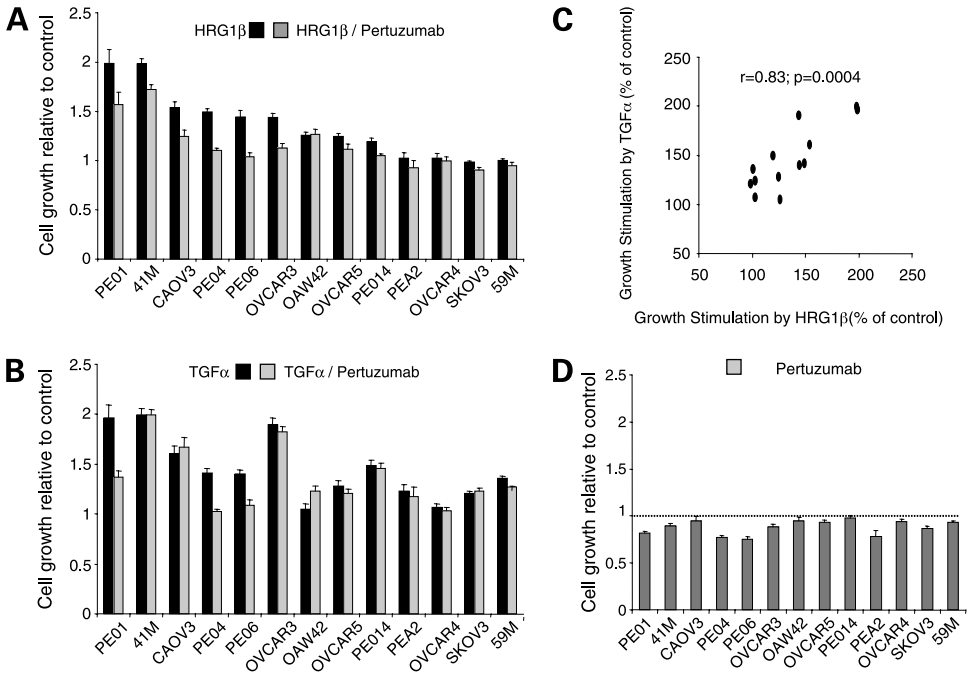

Figure 1. Growth of HRGβ1- and TGF-α–stimulated ovarian cancer cell lines treated with pertuzumab. **A,** 13 cell lines were stimulated with HRGβ1 (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L), and cell number was assessed after 72 h. *Columns,* mean; *bars,* SE. Cell proliferation was expressed relative to untreated control cells (charcoal-stripped serum in medium). **B,** cell lines were also stimulated with TGF-α (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L), and cell number was similarly assessed after 72 h. *Columns,* mean; *bars,* SE. **C,** relative growth stimulation by HRGβ1 and TGF-α was plotted against each other and showed a statistically significant correlation. **D,** the effect of pertuzumab alone (100 nmol/L) on cell growth was also investigated in the panel of unstimulated cell lines as described above. *Columns,* mean; *bars,* SE. Mean values of four independent experiments.

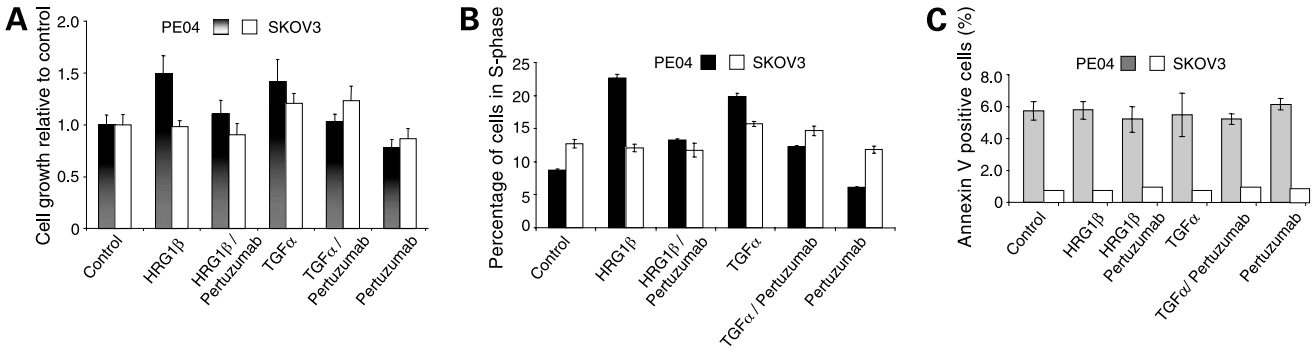

Figure 2. Effect of HRGβ1 and TGF-α on cell cycle distribution and apoptosis in PE04 and SKOV3 ovarian cancer cell lines. **A,** PE04 and SKOV3 cells were stimulated with HRGβ1 (1 nmol/L) and TGF-α (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L), and cell number was assessed after 72 h. *Columns,* mean; *bars,* SE. Cell proliferation was expressed relative to untreated control cells. **B,** cell cycle analysis was carried out in similarly treated PE04 and SKOV3 cells as described in Materials and Methods. The percentage of cells in S phase of the cell cycle is shown because this is where the most pronounced changes were shown. *Columns,* mean; *bars,* SD. **C,** early-stage apoptosis was assessed in PE04 and SKOV3 cells using an Annexin V assay where the proportion of apoptotic cells is expressed as a percentage of 20,000 events collected. *Columns,* mean; *bars,* SD.

there was any association between their phosphorylation status and growth response. Three antibodies targeting specific phosphorylated tyrosine residues were used; these were directed against the phosphorylated Tyr^{877} residue on HER2 and phosphorylated Tyr^{992} and Tyr^{1045} residues on HER1. Because these HER2/HER1 antibodies are known to cross-react, respective information on the homologous site of the other receptor was also obtained. Data on the Tyr^{845} residue of HER1, as well as Tyr^{1023} and Tyr^{1112} residues of HER2, were therefore obtained by virtue of their separation on Western blots.

Seven cell lines were selected for signaling studies; three of which were relatively sensitive to the effects of pertuzumab on growth factor stimulation (PE04, PE06, and CAOV3) and four of which were relatively insensitive (OVCAR4, OVCAR5, 41M, and SKOV3). HRGβ1 was able to stimulate phosphorylation of HER2 (Tyr^{877}) in all three pertuzumab-sensitive lines but not in the four insensitive cell lines (Fig. 3A). Interestingly, basal levels of HER2 (Tyr^{877}) were notably higher in SKOV3 cells and remained unchanged by the addition of HRGβ1. Furthermore, reduced phosphorylation of HER2 (Tyr^{877}) was shown in the three HRGβ1-driven cell lines, where growth was subsequently reversed by pertuzumab (PE04, PE06, and CAOV3). In the remaining cell lines where no increased HER2 phosphorylation was seen on addition of HRGβ1, pertuzumab paradoxically increased phosphorylation at several residues, including HER2 (Tyr^{877}).

Whereas HRGβ1 stimulated phosphorylation of HER2 (Tyr^{877}) in only a subset of cell lines, TGF-α stimulated HER1 (Tyr^{845}) in all seven cell lines studied, regardless of their growth response to pertuzumab. Unlike HRGβ1-driven phosphorylation of HER2 (Tyr^{877}), this TGF-α–driven HER1 (Tyr^{845}) phosphorylation could not be reversed by addition of pertuzumab. TGF-α was also shown to increase phosphorylation of HER1 (Tyr^{992}) in all seven cell lines (Fig. 3B) as well as pHER1 (Tyr^{1045}) and pHER1 (Tyr^{1173}) in OVCAR5, 41M (the two highest HER1-expressing cell lines), and CAOV3 cells (Fig. 3C).

Pertuzumab Inhibits HRGβ1-Activated ERK Phosphorylation and TGF-α–Activated Akt Phosphorylation

HRGβ1 and TGF-α stimulated both the ERK and the Akt pathways in all cell lines studied, with the exception of the SKOV3 cell line, in which only ERK was stimulated by TGF-α (Fig. 4A). In the sensitive cell lines (PE04, PE06, and CAOV3), such increased phosphorylation of ERK by HRGβ1 was partially or wholly reversed by pertuzumab, whereas TGF-α–stimulated cells showed no reductions. In contrast, only one of four of the less sensitive cell lines (OVCAR5) showed reversal of HRGβ1-stimulated ERK phosphorylation and again no reversal was seen in cells when ERK phosphorylation was stimulated by TGF-α. As with ERK phosphorylation, all cell lines (with the exception of SKOV3) showed increased Akt phosphorylation with both HRGβ1 and TGF-α. Pertuzumab could reduce this HRGβ1 drive in all but the SKOV3 cells. Despite phosphorylation of Akt being stimulated by TGF-α in all seven cell lines, reduction with pertuzumab was evident only for PE04 and PE06, again correlating with growth changes. Further analysis of pERK1/pERK2 signals showed that growth stimulation seen with HRGβ1 was significantly associated ($P = 0.037$, Pearson test) with a change in the level of pERK2 (relative to untreated control cells; Fig. 4B). No comparable association was found with pAkt ($P = 0.99$, Pearson test; Fig. 4C).

Pertuzumab Inhibits Growth, Signaling, and Cell Cycle Distribution in Estrogen-Stimulated Cells

The cell lines that were most effectively growth inhibited by pertuzumab after HRGβ1 and TGF-α stimulation were also estrogen responsive. In the panel of cell lines chosen for this study, we showed previously that only PE01, PE04, PE06, and SKOV3 cell lines have moderate to high levels of ERα, whereas the other lines have low or negligible levels (Table 1; ref. 21). Of these cell lines, only PE01, PE04, and PE06 (but not SKOV3) are growth modulated by estrogen (21). To explore potential interactions between estrogen signaling and pertuzumab

action, we assessed the effects of pertuzumab on E2-modulated growth, cell signaling, cell cycle distribution, and apoptosis in PE04 cells and compared these actions with effects in the estrogen-unresponsive SKOV3 (ERα positive) cell line.

E2 is known to stimulate the growth of PE04 cells in a dose-dependent manner (21) and this stimulation could be reversed with either tamoxifen or pertuzumab (Fig. 5A). In contrast, no growth effects were seen in SKOV3 cells. Growth stimulation of PE04 cells by E2 was also supported by alterations in the cell cycle distribution, notably in S phase where E2 caused an ~3-fold increase following exposure (Fig. 5B). This increase in S-phase fraction was significantly reduced with either tamoxifen or pertuzumab (although tamoxifen and pertuzumab had no significant effect on their own). In contrast to PE04 cells, no changes were shown in SKOV3 cells despite high ERα levels. Further analysis showed that Annexin V levels in PE04 cells were reduced slightly with E2 and similarly increased with tamoxifen (Fig. 5C). Pertuzumab alone had no significant effect in PE04 cells and again no changes were shown in SKOV3 cells.

Growth stimulation by E2 in PE04 cells was also accompanied by increased phosphorylation of HER2 (Tyr^{877}) together with both ERK and Akt activation (Fig. 6A). E2 increased expression of pHER2 (Tyr^{877}) by 7.2 (±2.0)–fold, pERK by 2.9 (±0.2)–fold, and pAkt by 0.6 (±0.7)–fold. Tamoxifen (acting like a weak estrogen) produced a similar but smaller effect for pHER2 (Tyr^{877}) and pERK (2.8-fold and 1.6-fold increases, respectively). These increases in pERK and pAkt were reduced in the presence of pertuzumab. Total HER2 and total ERK levels remained unchanged throughout. Furthermore, estrogen-stimulated phosphorylation of ERα at the Ser^{118} residue could be reversed with addition of pertuzumab (Fig. 6B). Treatment with tamoxifen similarly resulted in increased phosphorylation of ERα (Ser^{118}), which could again be reversed with pertuzumab. These results provide evidence for cross-talk between HER2 receptor and estrogen signaling.

Discussion

Pertuzumab has shown clinical activity in advanced ovarian cancer, albeit in a small subset of patients. In this study, we sought to identify signaling components associated with this growth inhibition. Our analysis of 13 ovarian cancer cell lines shows that pertuzumab can inhibit both HRGβ1- and TGF-α–stimulated growth and associated signaling in selected cell lines. Addition of pertuzumab to cells produced varying degrees of growth inhibition in the majority of cell lines, which were stimulated by HRGβ1; in some cell lines, such as PE04 and PE06, this HRGβ1 stimulation was completely reversed, although in most cells, it produced a 40% to 60% reversal. Whereas addition of pertuzumab to cells stimulated by TGF-α also produced complete growth inhibition in PE04 and PE06 (along with PE01) cells, it was generally ineffective in the remaining 10 cell lines. In those cell lines that were pertuzumab 'sensitive', HRGβ1 was also shown to stimulate HER2 (Tyr^{877}) expression. Furthermore, this stimulation was subsequently reduced

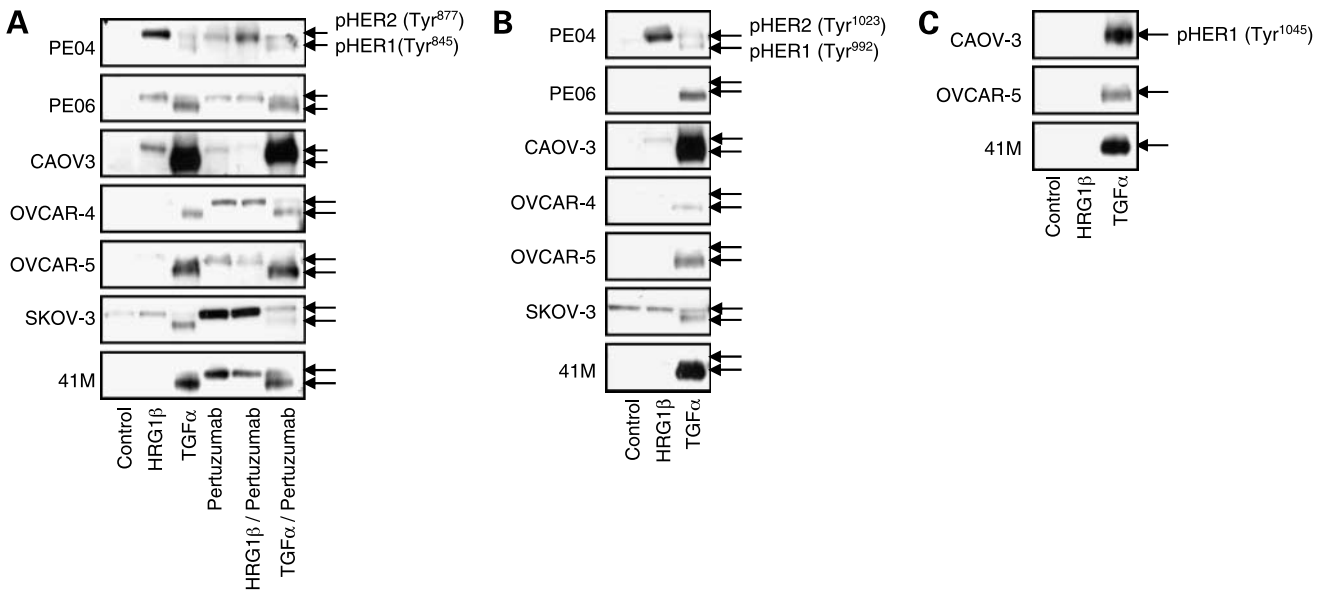

Figure 3. Effect of HRGβ1 and TGF-α on HER1 and HER2 phosphorylation in ovarian cancer cell lines. **A,** seven ovarian cancer cell lines were exposed (15 min) to HRGβ1 (1 nmol/L) or TGF-α (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L), and Western blots were carried out to detect relative expression levels of pHER2 (Tyr^{877}) as described in Materials and Methods. Data were also obtained for pHER1 (Tyr^{845}) on account of antibody cross-reactivity. **B,** similar data were also obtained in the same cell lines for pHER2 (Tyr^{1023}) expression, along with the associated pHER1 (Tyr^{992}). **C,** in certain cell lines, pHER1 (Tyr^{1045}) was also present.

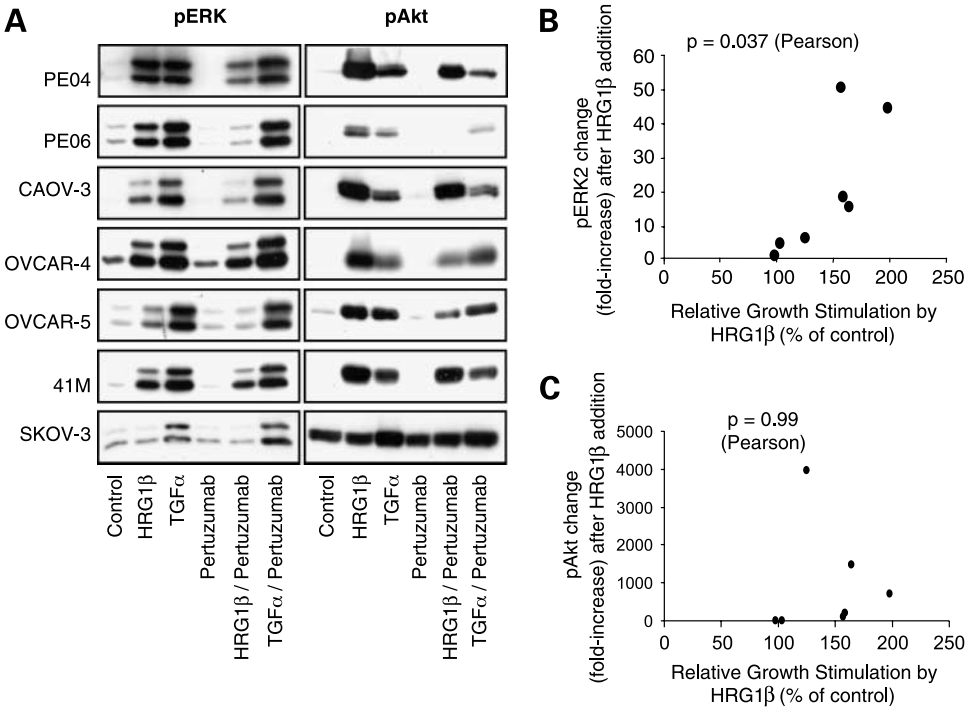

Figure 4. Cell signaling in ovarian cancer cell lines treated with HRGβ1 and TGF-α in the absence or presence of pertuzumab. **A,** ovarian cancer cell lines were exposed (15 min) to HRGβ1 (1 nmol/L) or TGF-α (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L). Western blots were carried out to detect relative expression levels of both pERK (*top band*, ERK1; *bottom band*, ERK2) and pAkt as described in Materials and Methods. Following HRGβ1 (1 nmol/L) treatment, growth stimulation was plotted against change in pERK2 (**B**) or pAkt (**C**) levels showing a statistically significant correlation. pERK2 change was obtained by dividing the pERK level (integrated absorbance) in the presence of HRGβ1 by the respective level in the absence of treatment.

in the presence of pertuzumab. Whereas the significance and functionality of HER2 (Tyr^{877}) phosphorylation is poorly defined (22), phosphorylation of the homologous HER1 (Tyr^{845}) site is nevertheless known to be src mediated (23). Because the homologous site to HER2 (Tyr^{877}) in the activated rat $p185^{neu}$ protein (HER2 Tyr^{882}) is an autophosphorylation site and mutation of this residue reduces the intrinsic kinase activity of the protein and its transforming potential, this suggests that this tyrosine residue has an important functional role (24). Other phosphorylation sites on HER1 and HER2 were less informative, although the increased phosphorylation status of HER1 (Tyr^{1045}) in OVCAR5 and 41M cells (Fig. 3C), which do not respond to pertuzumab, suggests that HER1 homodimerization is likely to be the preferred option where HER1 is present in excess relative to HER2. The observation of HRGβ1- and TGF-α–induced phosphorylation of HER2 is consistent with heterodimerization because these growth factors can only activate HER2 in this manner. Direct evidence of heterodimerization of HER2 by immunoprecipitation has been obtained in other studies and shown to relate to pertuzumab response in selected cell lines (12, 14). In the same study (12), pertuzumab was also shown to inhibit heterodimer

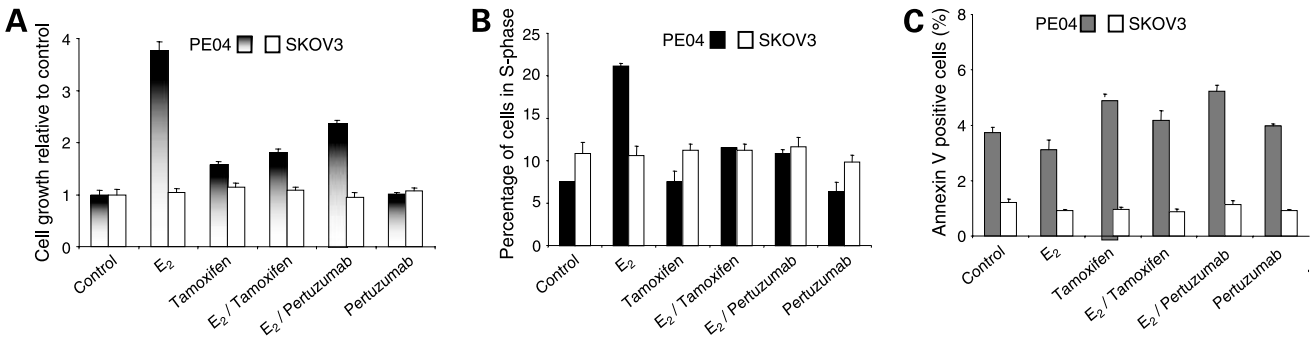

Figure 5. Growth, cell cycle distribution, and apoptosis in ovarian cancer cell lines treated with E2 in the absence or presence of tamoxifen or pertuzumab. **A,** PE04 and SKOV3 cells were incubated with E2 (1 nmol/L) in the absence or presence of tamoxifen (1 μmol/L) or pertuzumab (100 nmol/L), and cell number was assessed after 5 d by sulforhodamine B assay. *Columns,* mean; *bars,* SE. Cell proliferation was expressed relative to untreated control cells. **B,** cell cycle distribution was assessed after 3 d in similarly treated PE04 and SKOV3 cells, and the percentage of cells in S phase was selected because it showed the greatest changes. *Columns,* mean; *bars,* SD. The proportion of cells in S phase is expressed as a percentage of the total number of cells distributed throughout the cell cycle. **C,** the proportion of apoptotic cells was also assessed after 3 d using an Annexin V assay. *Columns,* mean; *bars,* SD.

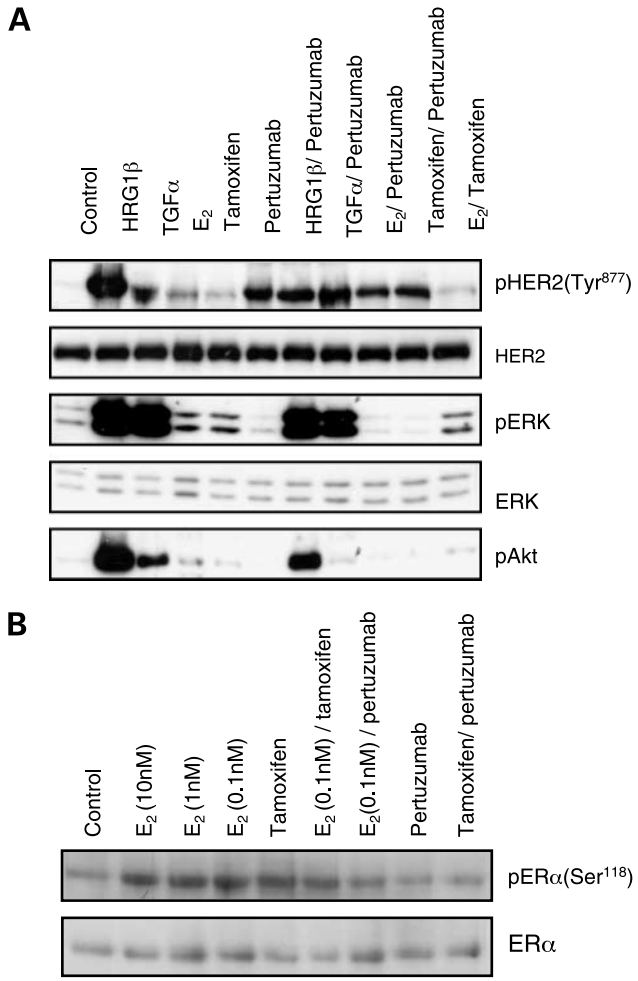

Figure 6. Effect of HRGβ1, TGF-α, E2, and tamoxifen on cell signaling in the absence or presence of pertuzumab in PE04 cells. **A,** PE04 cells were treated for 30 min with HRGβ1 (1 nmol/L), TGF-α (1 nmol/L), E2 (0.1 nmol/L), or tamoxifen (1 μmol/L) alone or in combination with pertuzumab (100 nmol/L). Western blots were carried out to detect relative expression levels of pHER2 (Tyr^{877}), total HER2, pERK (*top band*, ERK1; *bottom band*, ERK2), total ERK, and pAkt as described in Materials and Methods. **B,** PE04 cells were treated with varying doses of E2 (10, 1, and 0.1 nmol/L) or tamoxifen (1 μmol/L) alone or in combination with pertuzumab (100 nmol/L). Western blots were carried out to detect relative expression levels of phosphorylated ERα (*pERα*; Ser^{118}) or total ERα as described in Materials and Methods.

formation between HER2 and HER1 for one of the cell lines, in which pertuzumab was active (OVCA433), but not in a cell line where pertuzumab was ineffective (OVCA432). In the recent phase II ovarian cancer study (16), pHER2 status was shown to be associated with response to pertuzumab. This was based on the observation that in evaluable biopsies, overall median time to progression was 20.9 weeks for pHER2-positive patients (n = 8) but only 6.0 weeks for pHER2-negative patients (n = 23). Our data using multiple pHER2 antibodies suggest that certain phosphorylation sites are likely to be more informative than others and that HER2 (Tyr^{877}) seems to be a promising marker. An unexpected result in our study was the observation that pertuzumab treatment alone produced increased (rather than reduced) phosphorylation of HER2 (Tyr^{877}) in several cell lines but this was not associated with enhanced growth. However, this was not associated with either ERK or Akt activation consistent with a lack of growth response.

Both HRGβ1 and TGF-α were shown to stimulate phosphorylation of ERK and Akt in six of seven cell lines (the exception being SKOV3). The magnitude of HRGβ1-driven growth stimulation was significantly associated with the fold increase in pERK2 activation (P = 0.037, Pearson test) but not pAkt stimulation (P = 0.99, Pearson test). Pertuzumab inhibited HRGβ1-driven phosphorylation of both ERK and Akt in most cell lines but only inhibited TGF-α–stimulated phosphorylation of Akt in the growth-sensitive PE04 and PE06 cell lines. These data suggest that growth inhibition was more closely linked to the ERK pathway for HRGβ1-driven growth and to the Akt pathway for TGF-α–stimulated growth. The functional consequences were explored in the pertuzumab-sensitive PE04 cell line and the results suggest that increased cell growth seems to result more from cell proliferation than enhanced survival when either HRGβ1 or TGF-α was added. Furthermore, pertuzumab would seem to reverse this effect rather than enhance apoptosis. In other model systems, activation of both ERK and Akt by either HRGβ1 or TGF-α has been reported to be reversed by pertuzumab in MCF-7 breast cancer cells, although the reversal of HRGβ1 is generally more complete (12). Similarly, HRGβ1 activation of these pathways in two prostate cancer lines was fully reversed (12). In GEO colon cancer cells, pertuzumab inhibited both EGF and HRGβ1 stimulation of Akt but could only inhibit HRGβ1 stimulation of ERK (13).

There have been multiple reports of the interplay between HER receptor and ER signaling in breast cancer (reviewed in refs. 25, 26), but we are unaware of any comparable published data in ovarian cancer. The estrogen-induced phosphorylation of HER2, Akt, and ERK is however consistent with the nongenomic signaling reported in other cancers, including breast cancer (27). The increased phosphorylation of ERK and Akt was blocked by the addition of pertuzumab, consistent with HER2 involvement. Furthermore, both the estrogen and the tamoxifen activation of ERα (Ser^{118}) were reversed by pertuzumab, conceivably via the upstream blockades (via ERK) mentioned above. Together, these results indicate cross-talk between the HER receptor growth factor pathways and estrogen signaling and suggest an explanation of the reversal of estrogen-stimulated growth and cell cycle drive by pertuzumab.

In conclusion, these results using ovarian cancer cell lines support the clinical observations that a subgroup of ovarian cancers are growth responsive to pertuzumab. Characteristics of cells sensitive to pertuzumab include phosphorylation of HER (Tyr^{877}), expression of ERα, and growth responsiveness to E2. Sensitive tumors are also

more likely to have activated ERK and Akt, which are reversible on pertuzumab treatment. These results provide markers that could help select patients for future clinical trials of pertuzumab in ovarian cancer.

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