Inhibition of functional HER family members increases the sensitivity to docetaxel in human ovarian cancer cell lines

Marcel N.A. Bijman, Maria P.A. van Berkel, Mirjam Kok, Maarten L. Janmaat and Epie Boven

Human epidermal growth factor (HER) family-targeted therapy combined with standard cytotoxic agents might improve the treatment of ovarian cancer. Human ovarian cancer cell lines OVCAR-3, IGROV-1, and SKOV-3 with differential EGFR, HER2, and HER3 expression levels were used to study whether EGFR-directed (cetuximab) or HER2-directed (trastuzumab, pertuzumab) monoclonal antibodies inhibited cell growth and abrogated activated receptor signaling routes. Possible increase of antiproliferative effects and further activation of caspase-3 as a read-out for apoptosis were analyzed when monoclonal antibodies were combined with docetaxel. Cetuximab alone inhibited cell growth in OVCAR-3 and IGROV-1, which was more pronounced when combined with pertuzumab in OVCAR-3. SKOV-3 cell growth was not significantly affected by any of the antibodies. Cetuximab increased the 50% growth-inhibiting effects of docetaxel in OVCAR-3 and IGROV-1, but not in SKOV-3. Coaddition of pertuzumab to cetuximab plus docetaxel in OVCAR-3 and IGROV-1, and, to a lesser extent trastuzumab in OVCAR-3, inhibited cell growth even further. Caspase-3 activation by docetaxel was enhanced after addition of cetuximab in OVCAR-3 and after addition of cetuximab plus pertuzumab in IGROV-1 and SKOV-3. Functional EGFR-signaling, HER2-signaling, and HER3-signaling routes as shown from abrogation of EGF-stimulated and heregulin-stimulated phosphorylated ERK1/2 by cetuximab, trastuzumab, and pertuzumab, respectively, were shown in OVCAR-3 and IGROV-1, but hardly in SKOV-3. Pertuzumab was able to abrogate phosphorylated HER2 by EGF and heregulin, except in SKOV-3. In conclusion, a combination of docetaxel with inhibitors of HER family members, such as cetuximab plus pertuzumab, may be considered for a clinical trial in ovarian carcinomas with functional receptors. *Anti-Cancer Drugs* 20:450–460 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

Correspondence to Professor Epie Boven, MD, PhD, Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
Tel: +31 20 4444336; fax: +31 20 4444079; e-mail: e.boven@vumc.nl

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Introduction

Carboplatin plus paclitaxel chemotherapy in combination with optimal debulking surgery is considered as the most effective treatment approach for advanced ovarian cancer. Overall, the 5-year survival rate in these patients is approximately 50% [1]. In the current chemotherapy schedule paclitaxel may also be replaced by docetaxel, but the clinical outcome has not changed importantly [2]. Therefore, new drugs are needed to improve the survival of advanced ovarian cancer patients. In this respect, targeting of EGFR and/or HER2 in combination with standard cytotoxic agents might provide a new treatment strategy.

EGFR (HER1, ErbB1) and HER2 (ErbB2) are members of the human epidermal growth factor receptors (HER) family. HER3 (ErbB3) and HER4 (ErbB4) also belong to this family. EGFR and HER2 are expressed on a wide variety of normal cells and are involved in transmission of signals, mainly through the MAPK ERK1/2 and PI3K/Akt pathways, which control cell growth and differentiation. Various ligands, such as epidermal growth factor and the heregulins (HRGs), are known to bind to their specific receptor (epidermal growth factor binds to EGFR, HRGs bind to HER3 or HER4) and will stimulate receptor homodimerization or heterodimerization for initiation of signal transduction. No specific ligand has been identified for HER2 and, therefore, HER2 is thought to be the favored dimerization partner for the other receptors and is considered a key regulator in HER signaling [3,4]. HER3 lacks intrinsic tyrosine kinase activity and can initiate signaling by another HER family member, such as HER2 or EGFR [3,5]. The principal mechanism that drives EGFR-dependent PI3K/Akt activation is dimerization of EGFR with HER3 [5].

Amplification of the EGFR and HER2 genes induces receptor overexpression and, as a consequence, disruption of normal cellular control mechanisms resulting in cell proliferation, migration, invasion and stimulation of angiogenesis [3–5]. Overexpression of EGFR can be found in many cancer types, such as colorectal, pancreatic,

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head-and-neck, and lung cancer. HER2 is overexpressed in 25–30% of breast carcinomas in which amplification is usually linked with poor clinical outcome. In ovarian cancer, immunohistochemical staining has shown the presence of EGFR in 28–62% of samples [6–13], whereas approximately 13–35% of newly diagnosed ovarian cancer patients will express HER2 to some degree in their tumors [6–9,12–17]. The prognostic significance of EGFR and HER2 for survival in ovarian cancer, however, is not clear [6–17].

Monoclonal antibodies directed against HER family members may inhibit tumor growth. The EGFR-targeting monoclonal antibody cetuximab added to other treatment modalities is registered for patients with advanced colorectal cancer and head-and-neck cancer [18]. Trastuzumab is in standard use for treatment of HER2-overexpressing breast cancer patients. Addition of trastuzumab to taxane-based chemotherapy in these patients results in an improved response rate and overall survival in advanced disease as well as an improved recurrence-free survival and overall survival in primary breast cancer [19]. Trastuzumab has high affinity for the extracellular domain of HER2. Upon binding, it will induce receptor internalization and degradation. Pertuzumab, another monoclonal antibody directed against HER2, binds near the center of domain II and sterically blocks the region required for HER2 dimerization with other HER family members, such as HER3 [20]. Pertuzumab given alone has already been shown to be well tolerated in ovarian cancer patients with recurrent disease and has achieved a response rate of 4.3% [21]. Combination of pertuzumab with cytotoxic agents, among which was docetaxel, in experimental tumor models has shown that there are at least additive antitumor effects without additional toxicity [22]. Moreover, treatment with pertuzumab in combination with 3-weekly docetaxel in a phase I clinical trial has shown that more than 50% of the patients reached stable disease for a duration of ≥ 4 cycles [22]. Among these patients was one ovarian cancer patient with a 47% decrease in CA-125 tumor marker level.

Given the fact that human ovarian carcinomas express EGFR and HER2 to varying degrees, insight is required into whether inhibitors of these receptors will increase the efficacy of chemotherapy. Experiments in human ovarian cancer cell lines were designed to test this question. We selected docetaxel because of its activity in ovarian cancer [23] as well as its promising antitumor effects upon combination with pertuzumab in patients with solid tumors refractory to standard therapy [22]. Moreover, a recent phase Ib trial with first-line docetaxel and carboplatin in combination with the EGFR tyrosine kinase inhibitor erlotinib has shown a 52% response rate in 23 evaluable advanced ovarian cancer patients [24]. We used human ovarian cancer cell lines with differential expression levels of HER family members and first explored the antiproliferative effects of pertuzumab, trastuzumab, and cetuximab alone or combined by measuring the inhibition of cell growth *in vitro*. We then investigated whether the antitumor effects of docetaxel in the cell lines were increased upon addition of the various antibodies alone or combined. To clarify our findings, we assessed whether monoclonal antibodies could abrogate phosphorylation of activated receptors and downstream target proteins.

Materials and methods

Cell culture

Five human ovarian cancer cell lines: A2780, H134, OVCAR-3, IGROV-1, and SKOV-3 [25,26] were used in the experiments. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS, Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Bio-Whittaker, Verviers, Belgium) in a humidified atmosphere at 37°C in 5% CO_2.

In-vitro antiproliferative assay

The antiproliferative effects of docetaxel (Taxotere, kindly provided by Sanofi-Aventis, Antony, France), the antibodies cetuximab (Erbitux, anti-EGFR recombinant human/mouse chimeric monoclonal antibody, kindly provided by Merck Pharma, Darmstadt, Germany), trastuzumab (Herceptin, anti-HER2 humanized monoclonal antibody, kindly provided by Roche Molecular Systems, Belleville, New Jersey, USA), pertuzumab (Omnitarg, anti-HER2 recombinant humanized monoclonal antibody, kindly provided by Roche Diagnostics, Penzelberg, Germany), and combinations of drugs were measured in an antiproliferative assay. Cells were plated in quadruplicate in culture medium in a 96-well plate at 3000 cells/well. After 24 h, the cells were exposed to the various drugs and the assay was terminated after 96 h. The number of viable cells was then determined by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, Zwijndrecht, The Netherlands). The extinction of the formazan product was measured at 540 nm on a Multiscan plate reader (Thermo Biosciences, Breda, The Netherlands). Results were expressed as the percentage of cell growth of treated cells as compared with that of control cells after 96 h.

For docetaxel, cells were treated with a range of drug concentrations for 1 h, followed by a drug-free period of 96 h to calculate the IC_{50} values. The IC_{50} value was defined as the concentration of docetaxel inhibiting 50% of cell growth as compared with control cell growth. Thereafter, cells were exposed to docetaxel (IC_{50} value, 1-h exposure), cetuximab (5 µg/ml, 96 h), trastuzumab (2.9 µg/ml, 96 h), pertuzumab (20 µg/ml, 96 h), or combinations in which antibodies were added 2 h before

docetaxel exposure. Concentrations of antibodies were derived from experiments as described before [27–29]. Docetaxel treatment was followed by a wash step and addition of fresh culture medium enriched or not with antibodies.

Caspase-3 activity assay

The activity of caspase-3 was measured as described previously [26]. OVCAR-3, IGROV-1, and SKOV-3 cells were pretreated or not (2 h) with cetuximab, trastuzumab, pertuzumab, or combinations of cetuximab plus trastuzumab or pertuzumab followed by a 1-h treatment with docetaxel. After washing, a fresh culture medium was added without or with antibodies. After 48 h, adherent and nonadherent cells were recovered and washed with phosphate-buffered saline (Bio-Whittaker). The pellet was resuspended in lysis buffer (10 mmol/l Tris–HCl pH 7.6, 150 nmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100). After three freeze–thaw cycles, samples were centrifuged at 14 000 rpm for 10 min. Protein concentrations were determined by the Bradford protein assay [30].

In a 96-well plate, 10 μg of protein in a total volume of 20 μl was added to 80 μl of reaction buffer (100 mmol/l HEPES pH 7.3, 10% sucrose, 0.1% Nonidet-P40, 10 mmol/l dithiothreitol) complemented with 25 μmol/l of DEVD-7-amino-4-methylcoumarin (DEVD-AMC; Sigma-Aldrich), which is a substrate for active caspase-3. After 60 min, the fluorescence generated by the cleaved substrate was measured at λ^{exc} 360 nm and λ^{em} 446 nm in a spectrafluor multiplate reader (Tecan, Gorinchem, The Netherlands). To calculate caspase-3 activity in the experimental samples an extrapolation curve was constructed using known concentrations of free AMC (Sigma-Aldrich). The experiment was set-up in duplicate wells to generate mean fluorescence values.

Western blot

Cells were grown to 80% confluence in cell culture dishes. All individual cell lines were collected at this point for the measurement of basal EGFR, HER2, HER3, and HER4 expression levels. To analyze receptor-signaling activation in OVCAR-3, IGROV-1, and SKOV-3 cells, complete medium was replaced by serum-free medium with addition of antibodies blocking growth factor receptors. Cells were treated for 2 h with trastuzumab, pertuzumab, cetuximab or a combination of cetuximab with trastuzumab or pertuzumab followed by (i) immediate collection of a serum-free cell sample, (ii) collection of a cell sample 15 min after addition of 10% FCS-containing culture medium, (iii) cell sample collection 15 min after addition of 10 ng/ml EGF (Sigma-Aldrich) to serum-free medium, and (iv) sample collection 15 min after addition of 10 ng/ml neuregulin/HRG 1β (Sigma Aldrich) to serum-free medium. Adherent and non-adherent cells were collected, washed with ice-cold phosphate-buffered saline and resuspended in lysis buffer (1% sodium desoxycholate, 10 mmol/l Tris–HCl pH 7.5, 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate, and 1% NP-40) supplemented with protease inhibitors [1 mmol/l phenylmethylsulfonyl (Merck, Amsterdam, The Netherlands), 10 μmol/l leupeptin (Sigma-Aldrich), 0.5 mmol/l trypsin inhibitor (Sigma-Aldrich)] and phosphatase inhibitors [0.5 mol/l sodium orthovanedate (Sigma-Aldrich), 1 mol/l sodium fluoride (Merck)]. Protein concentrations were determined by the Bradford protein assay [27].

Protein samples of 50 μg were subjected to 10% polyacrylamide gel electrophoresis (130 V, 2 h). The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Etten-Leur, The Netherlands) by electrotransfer (400 mA, 2 h). The blots were blocked with 10% milk (Protifar; Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline–Tween 20 (TBS–T: 10 mmol/l Tris pH 8.0, 150 mmol/l NaCl, 0.0025% Tween 20) at room temperature for 1 h and incubated overnight at 4°C with specific antibodies against EGFR (rabbit polyclonal 1005; SantaCruz Biotechnology, Heerhugowaard, The Netherlands), HER2 (rabbit polyclonal Neu C-18; SantaCruz), HER3 (mouse monoclonal Ab-2; Neomarkers, Duiven, The Netherlands), HER4 (rabbit polyclonal Ab-2; Neomarkers), β-actin (mouse monoclonal; Sigma-Aldrich), p-EGFR (rabbit polyclonal Tyr1068; Cell Signaling Technologies, Leiden, The Netherlands), p-ERK1/2 (rabbit polyclonal p44/42 Thr202/Tyr204; Cell Signaling Technologies), p-Akt (rabbit polyclonal Ser473; Cell Signaling Technologies) or p-Tyr100 (mouse monoclonal # 9411; Cell Signaling Technologies) diluted in 5% BSA/TBS–T. After the membrane had been washed three times with TBS–T, it was incubated with 5% milk/TBS–T containing horseradish peroxidase-linked anti-mouse (DAKO, Amsterdam, The Netherlands) or anti-rabbit IgG secondary antibody (Cell Signaling Technologies) for 1 h at room temperature. After three TBS–T wash steps of 15 min, protein was visualized on photography film (Pharmacia, Uppsala, Sweden) by enhanced chemiluminescence.

Immunoprecipitation

Cells were grown to 80% confluence in Petri dishes. For determination of HER2 phosphorylation, complete medium was replaced by serum-free medium without or with the addition of pertuzumab at 20 μg/ml 2 h before (i) immediate collection of a serum-free cell sample, (ii) collection of a cell sample 15 min after addition of 10% FCS-containing culture medium, (iii) cell sample collection 15 min after addition of 10 ng/ml EGF to serum-free medium, and (iv) cell sample collection 15 min after addition of 10 ng/ml HRG to serum-free medium. After washing, cells were lysed in 1000 μl lysis buffer (supplemented with protease and phosphatase

inhibitors) and centrifuged to remove cell debris. To determine the total HER2 and β-actin levels, 40 µl of each sample was stored at −80°C for separate analysis. The remaining 960 µl of the supernatant was incubated with 50 µl protein-A/G-Plus agarose bead slurry (Santa Cruz) at 4°C while tumbling to loose aspecific binding of sample components to the beads. After 1 h cells were centrifuged and half of the supernatant was carefully transferred to a tube containing 50 µl protein-A/G-Plus agarose slurry to which 1 µg HER2 antibodies (Santa Cruz) were allowed to attach for 1 h. The other half of the supernatant was transferred to beads without bound HER2 antibodies to control for nonspecific binding to the agarose beads. The tubes were incubated overnight at 4°C while tumbling. Agarose beads were washed four times in lysis buffer, resuspended in 30 µl SDS buffer, boiled for 10 min to release bound HER2 and the supernatant was subjected to western blot (7.5% gel).

Statistics

The differences in the effects on cell proliferation and caspase-3 activity between docetaxel and docetaxel combined with trastuzumab, pertuzumab, cetuximab were analyzed by one-way analysis of variance followed by the Bonferroni's adjustment, using SPSS software (SPSS Inc., Chicago, Illinois, USA). The level of significance was set at a P value of less than 0.05.

Results

Differential expression levels of HER family members in human ovarian cancer cell lines

For detection of EGFR, HER2, HER3, and HER4, 50 µg of isolated protein from five unselected human ovarian cancer cell lines was subjected to western blot (Fig. 1). The cell lines displayed different receptor expression patterns. A2780 expressed HER2 and HER4, whereas it lacked EGFR and HER3. Both OVCAR-3 and IGROV-1 showed the same receptor pattern: EGFR, HER2, and HER3, whereas HER4 was absent. In H134, only HER2 was identified and EGFR, HER3, HER4 were not. In SKOV-3, EGFR and HER2 showed high expression levels, whereas HER3 and HER4 were not identified.

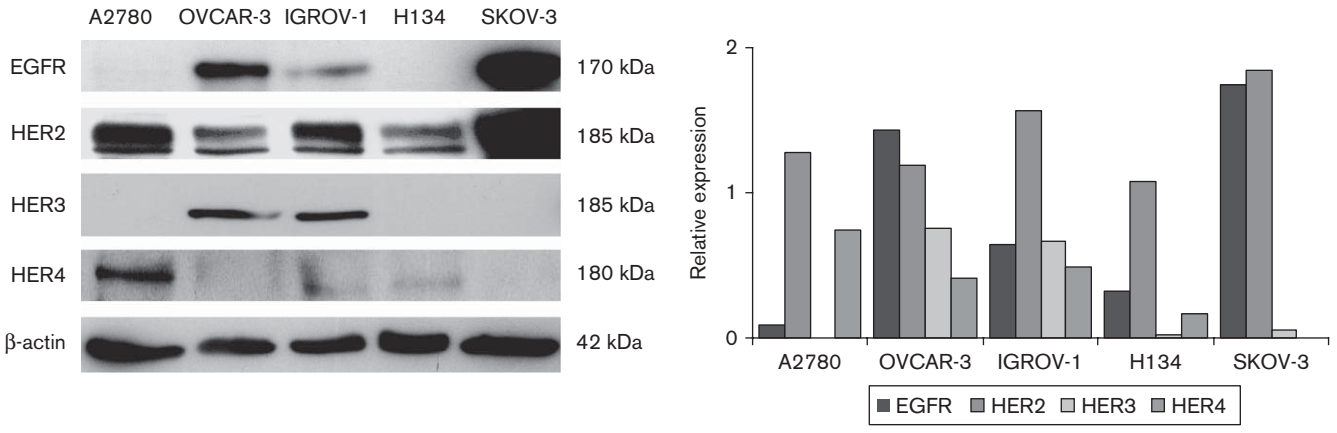
Cell lines with differential expression of HER family members were chosen for further experiments: OVCAR-3 (high EGFR, low HER2, presence of HER3), IGROV-1 (low EGFR, moderate HER2, presence of HER3), and SKOV-3 (high EGFR, high HER2, absence of HER3).

Anti-EGFR and anti-HER2 antibodies increase the antiproliferative effects of docetaxel in OVCAR-3 and IGROV-1, but not in SKOV-3

The mean IC_{50} values (± SD) of docetaxel after an exposure time of 1 h were (in nmol/l): in OVCAR-3, 23.8 (± 5.3); in IGROV-1, 7.8 ± (5.2); and in SKOV-3, 6.4 (± 2.7) as measured in at least three independent experiments. In the next experiments, cells were treated with antibodies either alone or combined for a period of 96 h. After 2 h of preincubation with antibodies, cells were exposed or not to docetaxel at the IC_{50} value for 1 h to assess the antiproliferative effects of the drugs.

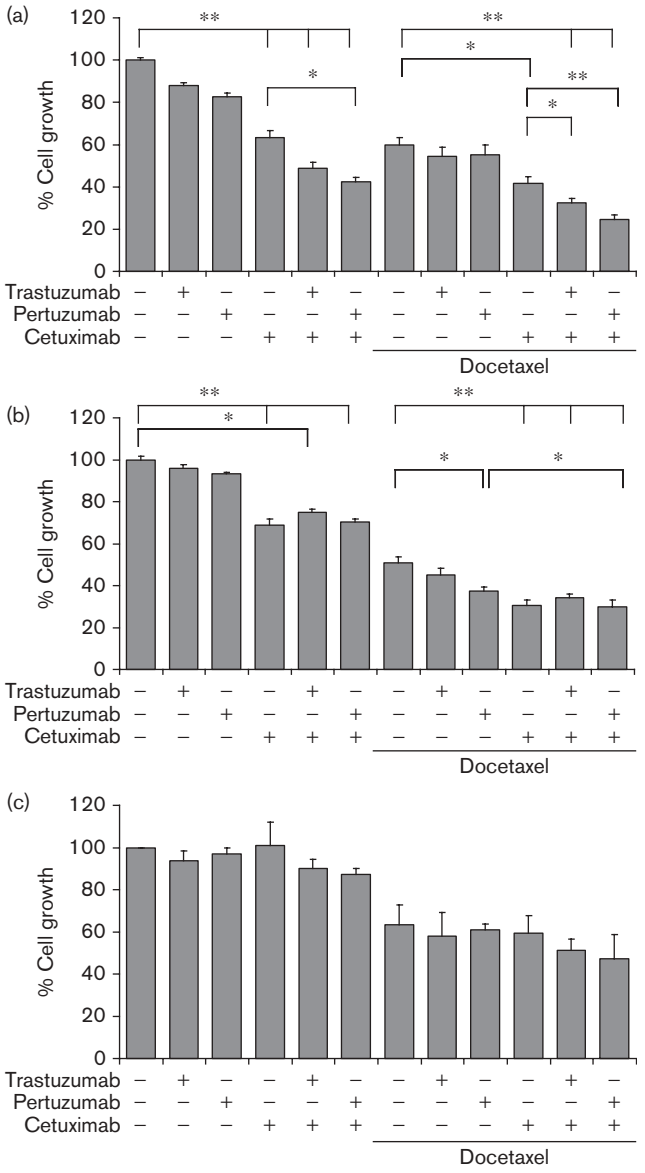
In OVCAR-3, exposure to cetuximab already resulted in significant growth inhibition ($P < 0.01$), whereas combination of cetuximab with trastuzumab ($P = NS$) and pertuzumab ($P < 0.05$, with reference to cetuximab alone) decreased cell proliferation even further (Fig. 2a, left panel). Cells treated with docetaxel showed a mean cell growth of 59.8% as compared with control growth

Fig. 1



Characterization of human epidermal growth factor (HER) family member expression in a panel of unselected human ovarian cancer cell lines; β-actin was used as a control for loading. Protein expression in lysates from freshly cultured cell lines (left panel). Quantification of band intensities after normalization for β-actin expression (right panel).

Fig. 2



Inhibition of human ovarian cancer cell proliferation by trastuzumab (2.9 μg/ml), pertuzumab (20 μg/ml), cetuximab (5 μg/ml), docetaxel (IC_{50}, 50% inhibitory concentration) or a combination in OVCAR-3 (a), IGROV-1 (b), and SKOV-3 (c). Cells were plated in a 96-well plate and exposed to drugs (docetaxel for 1 h, antibodies for 96 h and added 2 h before docetaxel). The number of viable cells was determined by addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 96 h. Results are expressed as the percentage cell growth of treated cells as compared with that of control cells (set at 100%). Mean values are the result of three independent experiments. Bars, SD; *$P<0.05$; **$P<0.01$.

(Fig. 2b, right panel). HER2-targeting antibodies alone did not affect growth inhibition by docetaxel. In contrast, cetuximab increased the antiproliferative effects of docetaxel; cell growth was 41.7% ($P < 0.05$). Coaddition of trastuzumab reduced cell growth further to 32.3% ($P < 0.05$, with reference to docetaxel combined with cetuximab), whereas this was 24.5% upon coaddition of pertuzumab ($P < 0.01$, with reference to docetaxel combined with cetuximab).

In IGROV-1, treatment with cetuximab alone also reduced cell growth, but coaddition of trastuzumab or pertuzumab did not further increase the antiproliferative effects of cetuximab (Fig. 2b, left panel). Docetaxel resulted in a mean cell growth of 50.9% (Fig. 2b, right panel). Docetaxel-induced antiproliferative effects were significantly increased by the addition of pertuzumab or cetuximab, resulting in 37.5% ($P < 0.05$) and 30.8% ($P < 0.01$) cell growth, respectively. Coaddition of pertuzumab and cetuximab to docetaxel decreased IGROV-1 proliferation to 29.9% ($P < 0.05$) when compared with the 37.5% cell growth upon addition of pertuzumab alone.

In SKOV-3, neither cetuximab, trastuzumab, and pertuzumab nor combinations significantly inhibited cell proliferation (Fig. 2c, left panel). The growth-inhibiting effects induced by docetaxel resulting in a mean cell growth of 63.3% were not further increased upon addition of any of the antibodies or a combination of them (Fig. 2c, right panel). Nevertheless, a trend was visible that cetuximab in combination with trastuzumab (51.2%) or pertuzumab (41.1%) added to docetaxel further reduced cell growth inhibition in SKOV-3.

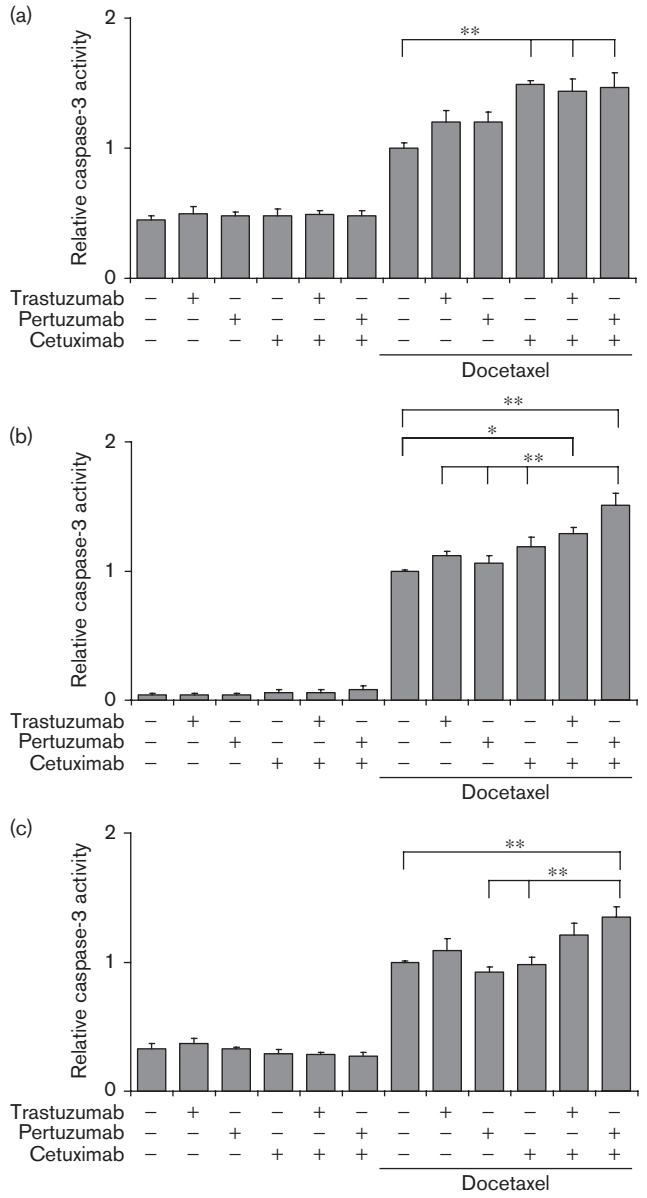
Altogether, cetuximab alone inhibited cell growth in OVCAR-3 and IGROV-1, which was more pronounced when combined with pertuzumab in OVCAR-3. Cetuximab also increased the antiproliferative effects of docetaxel in OVCAR-3 and IGROV-1. Coaddition of pertuzumab in OVCAR-3 and IGROV-1, and, to a lesser extent, trastuzumab in OVCAR-3 reduced cell growth even further. SKOV-3 cell growth was not significantly affected by any of the antibodies.

Anti-EGFR and anti-HER2 antibodies differentially enhance the docetaxel-induced activation of caspase-3

To determine whether EGFR-targeting and HER2-targeting antibodies were able to enhance the docetaxel-induced apoptotic cell death, caspase-3 activity was assessed 48 h after treatment with docetaxel (IC_{50}, 1-h exposure) alone or in combination with antibodies (continuous exposure). Antibodies alone did not significantly affect caspase-3 activity (Fig. 3, left panels).

In OVCAR-3 cells, treatment with docetaxel alone resulted in a 2.2-fold higher caspase-3 activity than basal levels in control cells. Addition of cetuximab significantly enhanced the caspase-3 activation; levels were 1.5-fold higher than those in cells treated with docetaxel alone ($P < 0.05$; Fig. 3a, right panel). The HER2-targeting antibodies slightly increased the docetaxel-induced caspase-3 activation when added alone ($P = \text{NS}$) and

Fig. 3



Induction of caspase-3 activity by trastuzumab (2.9 μg/ml), pertuzumab (20 μg/ml), cetuximab (5 μg/ml), docetaxel (IC_{50}, 50% inhibitory concentration) or a combination in OVCAR-3 (a), IGROV-1 (b), and SKOV-3 (c). Cells were plated in culture dishes and exposed to drugs (docetaxel for 1 h, antibodies for 48 h and added 2 h before docetaxel). Adherent and non-adherent cells were collected at 48 h. Results are expressed as the ratio of caspase-3 activity as compared with that of docetaxel-treated cells (set at 1). Mean values are the result of three independent experiments. Bars, SD; *$P<0.05$, **$P<0.01$.

their combination with cetuximab did not further enhance the activation levels as compared with addition of cetuximab alone.

In IGROV-1, docetaxel treatment resulted in a 34.6-fold higher caspase-3 activity than the control cell level. The docetaxel-induced caspase-3 activation was slightly enhanced when cetuximab or anti-HER2 antibodies were added (P = NS; Fig. 3b, right panel). The combination of cetuximab plus trastuzumab or pertuzumab resulted in a 1.3-fold ($P < 0.05$) and a 1.5-fold ($P < 0.01$) enhancement of caspase-3 activity, respectively, as compared with that of cells treated with docetaxel alone.

In SKOV-3, the docetaxel-induced caspase-3 activation of 3.2-fold did not clearly increase when cetuximab, trastuzumab, or pertuzumab were added (Fig. 3c, right panel). The docetaxel-induced caspase-3 activity, however, was enhanced when cetuximab was added together with either trastuzumab to 1.2-fold (P = NS) or pertuzumab to 1.4-fold ($P < 0.01$).

These experiments showed that antibodies by themselves could not activate caspase-3. In line with the antiproliferative effects shown in Fig. 2, caspase-3 activation by docetaxel enhanced when combined with cetuximab in OVCAR-3 and in combination with cetuximab plus trastuzumab or pertuzumab in IGROV-1. Of interest, coaddition of cetuximab and pertuzumab could also increase the docetaxel-induced activation of caspase-3 in SKOV-3.

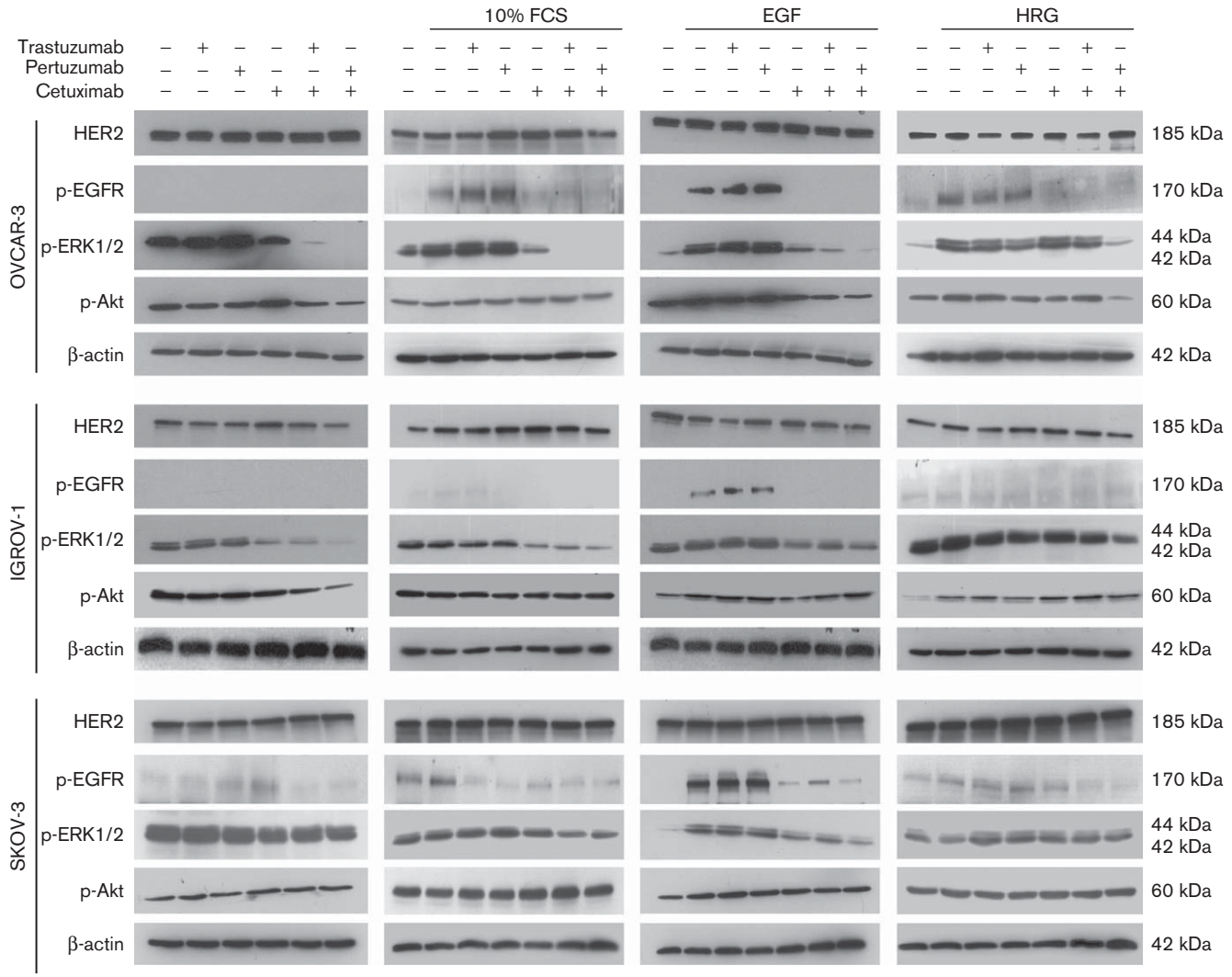
Functional HER-signaling routes are most pronounced in OVCAR-3 and IGROV-1

We assessed the inhibitory capacity of anti-EGFR and/or anti-HER2 treatment on receptor activation and downstream signals in protein samples of OVCAR-3, IGROV-1, and SKOV-3 by 10% FCS-containing culture medium, serum-free medium containing EGF or HRG, while serum-free medium only was included to measure baseline levels. Cells were exposed to trastuzumab, pertuzumab, cetuximab, or a combination for a period of 2 h before stimulation with 10% FCS, EGF, or HRG for 15 min (Fig. 4). Total EGFR, ERK1/2 and Akt levels remained unchanged during all experiments (data not shown). Total HER2 and β-actin protein levels served as loading controls.

In OVCAR-3, EGFR and correspondingly ERK1/2 was phosphorylated upon stimulation with 10% FCS, EGF, or HRG. EGF and HRG also slightly induced p-Akt levels. Neither trastuzumab nor pertuzumab could inhibit p-ERK1/2 or p-Akt. In contrast, cetuximab alone was able to inhibit p-EGFR and p-ERK1/2. Cetuximab did not decrease p-ERK1/2 in HRG-stimulated cells. The inhibitory effect of cetuximab on p-ERK1/2 was increased by the addition of trastuzumab and even more by the pertuzumab, in which the latter combination was also able to abrogate HRG-induced p-ERK1/2. The p-Akt was inhibited by cetuximab in EGF-stimulated cells and by pertuzumab added to cetuximab in HRG-stimulated cells.

In IGROV-1, EGFR was only phosphorylated upon addition of EGF. Basal p-ERK1/2 levels were not further

Fig. 4



Protein levels of total HER2, phosphorylated EGFR, phosphorylated ERK1/2, phosphorylated Akt and total β-actin in OVCAR-3, IGROV-1, and SKOV-3 in serum-free medium without or with 10% FCS, 10 ng/ml EGF or 10 ng/ml HRG in the presence or absence of trastuzumab (2.9 μg/ml), pertuzumab (20 μg/ml), or cetuximab (5 μg/ml). Antibodies were added 2 h before stimulation and samples were collected 15 min after stimulation. Protein samples (50 μg) were analyzed by western blot. Representative results of at least three independent experiments are shown. Note: lane 1 shows protein levels under serum-free conditions, but different exposure periods were used to visualize changes in phosphorylation levels. EGF, epidermal growth factor; FCS, fetal calf serum; HRG, heregulin.

increased when 10% FCS, EGF, or HRG was added. ERK1/2 activation was partially inhibited by cetuximab, except in cells stimulated by HRG. Pertuzumab, but not trastuzumab, could further downregulate p-ERK1/2 when added to cetuximab. Akt was already phosphorylated under serum-free conditions, which was further upregulated by addition of EGF or HRG. The p-Akt was only inhibited by a combination of cetuximab with either trastuzumab or pertuzumab under serum-free conditions.

In SKOV-3, EGFR was activated by the addition of EGF and to a lesser extent by 10% FCS or HRG. The p-EGFR was partially abrogated by cetuximab. ERK1/2 was already highly phosphorylated under serum-free conditions. Only EGF addition resulted in upregulation of p-ERK1/2 and only cetuximab was able to inhibit phosphorylation. Phosphorylation of Akt was already present in cells grown in serum-free medium and neither stimulation nor any of the antibodies affected p-Akt levels.

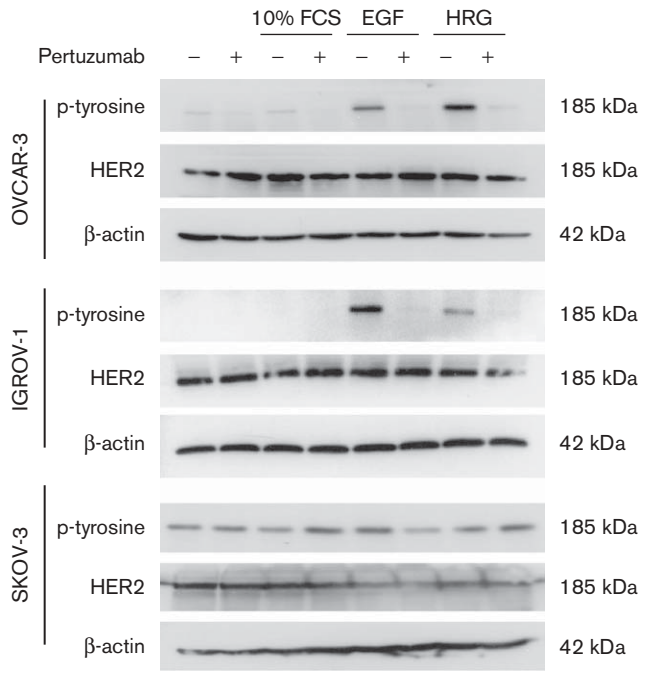
In general, when ovarian cancer cells were stimulated with EGF, phosphorylation of EGFR was further increased after exposure to trastuzumab and pertuzumab. This observation suggests that inhibition of HER2 or HER3 may activate another HER family member.

Altogether, functional EGFR-signaling, HER2-signaling, and HER3-signaling routes as shown by abrogation of EGF-stimulated and HRG-stimulated phosphorylated ERK1/2 by cetuximab, trastuzumab and pertuzumab, respectively, were present in OVCAR-3 and IGROV-1, but hardly in SKOV-3.

Functionally active HER2 can be inhibited in OVCAR-3 and IGROV-1, but not in SKOV-3

For a correct interpretation of the inhibitory capacity of anti-HER2 antibody treatment, immunoprecipitation experiments were carried out on protein samples to detect phosphorylated HER2 in OVCAR-3, IGROV-1, and SKOV-3. Cells were treated with or without pertuzumab at 20 µg/ml under serum-free conditions 2 h before addition of culture medium containing 10% FCS, before stimulation with EGF at 10 ng/ml or HRG at 10 ng/ml for a period of 15 min. The degree of tyrosine phosphorylation as a measure of HER2 activation in the acquired protein samples was visualized by western blot (Fig. 5).

Fig. 5



Detection of phosphorylated HER2 by immunoprecipitation. OVCAR-3, IGROV-1 and SKOV-3 were grown to 80% confluence and fresh serum-free medium was added without or with 10% FCS, 10 ng/ml EGF or 10 ng/ml HRG. Pertuzumab, when added, was administered 2 h prior to the medium change. Samples were collected 15 min thereafter. Cell lysates were divided and in one half used for HER2 and β-actin detection and the other half for HER2 precipitation. All samples were separated by gel electrophoresis. Total HER2 and β-actin in one sample and phosphorylated tyrosine in the precipitation sample were detected by western blot and enhanced chemoluminescence. Representative results of at least three independent experiments are shown. EGF, epidermal growth factor; FCS, fetal calf serum; HRG, heregulin.

In OVCAR-3 and IGROV-1, no phosphorylated HER2 was detected under serum-free conditions. Most specifically, after EGF or HRG stimulation in OVCAR-3 and IGROV-1, the receptor was clearly phosphorylated. Pretreatment with pertuzumab abrogated HER2 activation. In SKOV-3 cells, in which the receptor was already phosphorylated under serum-free conditions, pretreatment with pertuzumab followed by stimulation with 10% FCS, EGF, or HRG had no significant effects on the HER2 phosphorylation status.

Thus, pertuzumab was able to abrogate EGF-stimulated and HRG-stimulated phosphorylation of HER2 in OVCAR-3 and IGROV-1. Basal p-HER2 was present in SKOV-3, which could neither be stimulated nor inhibited, suggesting the absence of a functional HER2-signaling route.

Discussion

Here we report that human ovarian cancer cell lines show differential expression levels of HER family members. We showed that EGFR-targeting and HER2-targeting monoclonal antibodies may increase the antitumor effects of docetaxel by blocking functional receptors. Docetaxel exposure periods of 1 h were selected, because the drug is administered by a 1-h infusion in cancer patients. The IC_{50} values of docetaxel of 6.4–23.8 nmol/l *in vitro* were well below achievable plasma levels in patients, being 4 µmol/l at doses of 75–100 mg/m^2 [31]. Concentrations of the antibodies selected from studies reported previously [27–29] were sufficient for abrogating phosphorylation of activated target receptors and downstream functional HER-signaling routes. In addition, these concentrations *in vitro* were well below trough plasma levels measured in patients being 49 µg/ml at 3-weekly 6 mg/kg for trastuzumab [32], being 52 µg/ml at 3-weekly 420 mg for pertuzumab [33], and being 55 µg/ml at weekly 250 mg/m^2 for cetuximab [34] intravenous schedules. As our data were acquired *in vitro*, further testing in ovarian cancer patients should be done for proof of concept.

Although all selected ovarian cancer cell lines contained EGFR and HER2, differential antiproliferative effects were observed when monoclonal antibodies were given alone or combined with docetaxel. In OVCAR-3 and IGROV-1 cells, cetuximab could already significantly inhibit the cell proliferation, which was also the case when combined with docetaxel. Pertuzumab alone did not affect cell growth, but increased the inhibitory effects of cetuximab in OVCAR-3. In IGROV-1, pertuzumab increased the antiproliferative effects of docetaxel, which was more pronounced when combined with cetuximab. Trastuzumab could only enhance the antiproliferative effects in OVCAR-3 when these cells were also treated with docetaxel and cetuximab. None of the antibodies significantly affected the cell growth in SKOV-3, although

a trend was observed in further cell growth inhibition when docetaxel was combined with either cetuximab and trastuzumab or with cetuximab and pertuzumab. Although antibodies alone did not activate caspase-3, cetuximab could enhance the caspase-3 activation when added to docetaxel in IGROV-1. Of interest, caspase-3 activation was most pronounced in IGROV-1 and SKOV-3, when both cetuximab and pertuzumab were added to docetaxel. On the one hand, these data indicate that expression levels of HER family members in ovarian cancer cells are not predictive for antiproliferative effects of receptor inhibitors. Further, inhibition of cell growth by monoclonal antibodies alone does not result in the activation of caspase-3. On the other hand, when monoclonal antibodies alone induce antiproliferative effects, further reduction of cell growth and a concomitant increase in activated caspase-3 are shown upon their combination with docetaxel.

Our findings indicate that cetuximab may play a role in the treatment of ovarian cancer, but the presence of EGFR will not always predict cell sensitivity. The EGFR-signaling route as tested by stimulation with EGF was clearly functional in the three cell lines as visualized from the increase in p-EGFR, p-ERK1/2, and p-Akt. Constitutively activated p-Akt in SKOV-3, however, was not further stimulated by EGF. In line with the antiproliferative effects of cetuximab in OVCAR-3 and IGROV-1 cells, phosphorylation levels of EGFR and ERK1/2 were inhibited by blocking receptor function. In OVCAR-3 cells stimulated with EGF, increased p-Akt was also abrogated by cetuximab. Cetuximab combined with trastuzumab or pertuzumab could inhibit p-Akt in IGROV-1 cells under serum-free conditions. Although the EGF-stimulated increase in phosphorylation of EGFR and ERK1/2 in SKOV-3 cells could be abrogated by cetuximab, this did not result in the reduction of cell growth. Constitutively activated Akt in SKOV-3 could not be inhibited by any of the antibodies. The activation of the PI3K/Akt-signaling route downstream of EGFR might therefore be responsible for cetuximab resistance in SKOV-3.

Cetuximab resistance in EGFR-positive ovarian carcinomas has hardly been explored. Thus far, EGFR gene mutations are not common in ovarian carcinomas [13,35]. Recently, it has been found in colorectal cancer, a tumor type known for high EGFR expression, that patients on cetuximab monotherapy whose tumors contained wild-type *K-ras* had a significantly higher disease control rate than patients with *K-ras* mutations [36]. Further, EGFR-overexpressing colorectal cancer cell lines with highest cetuximab resistance contained both *K-ras*/*BRAF* and *PTEN*/*PIK3CA* mutations [37]. Of interest, EGFR inhibitors induced only limited antiproliferative effects in non-small-cell lung cancer cell lines with persistent activity of Akt [29]. Earlier, it has been shown in SKOV-3 that PTEN levels are low, which possibly accounts for increased levels of constitutive Akt activity [38]. The role of constitutive activation of signaling mediators downstream of EGFR in ovarian cancer needs to be explored.

Although ovarian carcinomas may express HER2 to some degree, the frequency of amplification and overexpression is less than 10% [13,16,17]. In a phase II trial in HER 2-overexpressing ovarian cancer patients, trastuzumab induced an overall response rate of only 7.3% [39]. Recently, the role of HER3 has gained interest in ovarian cancer. Positive HER3 expression was observed in 53.4% of ovarian cancer patients and was associated with decreased survival [40]. HER2, being the favored dimerization partner of EGFR and HER3, could be phosphorylated after stimulation with EGF or HRG in OVCAR-3 and IGROV-1, whereas constitutively phosphorylated HER2 in SKOV-3 was not affected (Fig. 5). HRG stimulation also induced phosphorylation of EGFR in OVCAR-3 cells. We could not detect HER3 in SKOV-3. HRG, however, induced low phosphorylation of EGFR in our SKOV-3 cells, which may indicate low expression of HER3. Gilmour *et al.* [41] have shown in ovarian cancer cells that the magnitude of HRG stimulation largely depends on HER2 expression levels. Although they found low HER3 expression levels in SKOV-3, HRG stimulation did not stimulate cell growth, and, in line with our data, p-HER2 and p-ERK1/2 responses were absent. A central role for HER3 as a co-receptor for HER2 has recently been reported by Lee-Hoeflich *et al.* [42]. This group has shown that HER3 knockdown caused tumor regression in an HRG-dependent *HER2*-amplified breast cancer model. In addition, pertuzumab, in contrast to trastuzumab, could efficiently inhibit in-vivo tumor growth. These and our data point toward the finding that HER3 may be a useful target for treatment, if part of functional dimerization partners EGFR and HER2.

As HER3 can play a functional role in ovarian cancer, pertuzumab may be a useful adjunct to chemotherapy. Jackson *et al.* [28] have shown that pertuzumab blocked not only HRG-mediated phosphorylation of HER2 and HER3, but also EGF-mediated phosphorylation of HER2. We obtained similar findings in OVCAR-3 and IGROV-1 in which pertuzumab could inhibit phosphorylation of HER2 upon stimulation with HRG as well as with EGF (Fig. 5). Earlier, pertuzumab treatment alone has been shown to moderately inhibit growth in two out of eight ovarian cancer cell lines with high expression of HER2, among which was SKOV-3 [28]. In ovarian cancer patients, the antibody alone had only modest activity [21]. We did not clearly detect antiproliferative effects from pertuzumab alone, but the drug could significantly enhance the inhibition of cell growth by docetaxel in IGROV-1. Combination of pertuzumab with chemotherapy is currently being investigated in a randomized phase II

trial in patients with advanced ovarian cancer to assess a possible advantage with reference to chemotherapy alone.

Monoclonal antibodies directed against growth factor receptors seem to be most effective when combined with other treatment modalities. In our experiments on ovarian cancer cell lines, comparing three monoclonal antibodies added to docetaxel, we found that cetuximab was superior in the increase of cell growth inhibition and the enhancement of activation of caspase-3. Coaddition of HER2 receptor inhibitors hardly increased the antiproliferative effects, but if present, pertuzumab seemed to be more potent than trastuzumab. Kawaguchi *et al.* [43] have already shown that the combination of cetuximab and trastuzumab could induce synergistic antiproliferative effects in several human esophageal squamous cell carcinoma cell lines expressing both receptors. Similarly, Friess *et al.* [44] have shown in human tumor xenografts of non-small-cell lung cancer and breast cancer expressing various degrees of EGFR and HER2, that combination of the EGFR tyrosine kinase inhibitor erlotinib with pertuzumab resulted in additive or greater than additive antitumor effects. Currently, it has become clear that HER family members interact and that ligands are involved in allowing tumor cells to evade the proapoptotic effects from targeted therapy [45,46]. As we observed further upregulation of p-EGFR in EGF-activated ovarian cancer cells treated with HER 2-targeting monoclonal antibodies, dual inhibition may thus prevent possible escape from inhibition of only one HER family member. Considering the adverse prognostic role of HER3 in ovarian cancer, pertuzumab plus cetuximab added to taxane-based chemotherapy would be the combination of choice to further study in patients with ovarian cancer with functional EGFR/HER2/HER3-signaling routes.

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