Trastuzumab and pertuzumab produce changes in morphology and estrogen receptor signaling in ovarian cancer xenografts revealing new treatment strategies

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Statement of Translational Relevance

There is currently interest in identifying targeted therapies for the treatment of ovarian cancer. HER2 and ERα targeted therapies would be readily translatable options, with well-established use in other cancers. The complex interplay between ERα and HER2 signaling means that detailed understanding of these pathways is required in order to personalise therapy. We evaluated the use of HER2-targeted approaches in preclinical xenograft models of ovarian cancer and demonstrate that the combination of trastuzumab and pertuzumab are effective in high HER2 expressing xenografts. In addition, trastuzumab treatment results in increased expression of ERα, which in turn resulted in enhanced sensitivity to the anti-estrogen letrozole. These data indicate that treatment can change target receptor expression which may lead to increased sensitivity to alternative therapies. These results support the consideration of combination HER2 treatment in ovarian cancer and also combined anti-HER2 and anti-ERα therapy, perhaps as part of sequential therapy.
Abstract

Purpose
The aim of this study was to investigate the antitumor effects of HER2-directed combination therapy in ovarian cancer xenograft models to evaluate their potential. The combination of trastuzumab and pertuzumab and trastuzumab and aromatase inhibitor therapy were investigated.

Experimental Design
The effects of trastuzumab, pertuzumab and letrozole on growth response, apoptosis, morphology, gene and protein expression were evaluated in the SKOV3 ovarian cancer cell line xenograft and a panel of 5 human ovarian xenografts derived directly from clinical specimens.

Results
The combination of HER2-directed antibodies showed enhanced anti-tumor activity, compared with single antibody therapy in the SKOV3 xenograft model. Apoptosis, morphology and estrogen-regulated gene expression were modulated by these antibodies in both a spatial and temporal manner. A panel of ovarian cancer xenografts showed differential growth responses to the combination of trastuzumab and pertuzumab. High HER2 and increasing HER3 protein expression on treatment were associated with growth response. In trastuzumab-treated SKOV3 tumors, there was a change in tumor morphology, with a reduction in frequency of estrogen receptor alpha (ERα)-negative clear cell areas. Trastuzumab, but not pertuzumab, increased expression of ERα in SKOV3 xenografts when analysed by quantitative immunofluorescence. ERα and downstream signaling targets were modulated by trastuzumab alone and in combination. Trastuzumab enhanced the responsiveness of SKOV3 xenografts to letrozole when given in combination.

Conclusions
These data suggest that trastuzumab in combination with pertuzumab could be an effective approach in high HER2-expressing ovarian cancers and could also enhance sensitivity to endocrine therapy in ERα-positive ovarian cancer.
Introduction

Survival figures for epithelial ovarian cancer remain poor, with 65% of women dying of their disease within five years of diagnosis (1), in spite of generally good initial response rates to chemotherapy (2,3). This is partly because women with ovarian cancer present with late-stage disease, but also because advances in targeted therapy in ovarian cancer have not matched developments in other solid-organ tumors, such as the use of tamoxifen/aromatase inhibitors in breast cancer (4), or cetuximab in colorectal cancer (5). There is good evidence that platinum-taxane first-line chemotherapy is superior to other chemotherapy regimens for ovarian cancer (2, 6-9), but 20-30% of patients do not respond to this therapy and chemoresistance develops in the majority of tumors. Targeted agents with lower toxicity, such as trastuzumab (Herceptin) and pertuzumab, directed against the HER2 receptor, are not routinely used in clinical practice for ovarian cancer but may benefit some women (3, 10). There is therefore a clinical need to identify patients who will benefit from targeted therapy, and to establish which combinations are likely to show greatest efficacy.

HER2 (erbB2) expression in ovarian cancer is associated with advanced stage, higher recurrence frequency, shorter survival time and lower sensitivity to platinum-based chemotherapy (11, 12). Trastuzumab and pertuzumab are humanised anti-HER2 targeted antibodies with different modes of action (13, 14). Whilst trastuzumab binds to the juxta-membrane region (subdomain IV) of the extracellular domain of HER2, pertuzumab binds to HER2 near the center of domain II, the dimerization domain, inhibiting HER dimerization (15). Although both drugs result in changes in downstream signalling pathways, the mechanisms by which trastuzumab and pertuzumab induce regression of tumors are still not fully elucidated, although several processes have been proposed (14, 16). The combination of antibodies was shown to synergistically inhibit breast cancer cells (17). In phase II monotherapy clinical studies, trastuzumab has shown activity in certain HER2-overexpressed ovarian cancer, (response rate of approximately 7.5% with disease stabilization in 39%) (18), while pertuzumab has shown clinical benefit in 14.5% of unselected patients (19). Pertuzumab is currently undergoing ovarian cancer trials in combination with cytotoxic agents including gemcitabine (20) and carboplatin (21). A recent trial in breast cancer suggests that the combination of trastuzumab and pertuzumab can produce clinical benefit in 50% of trastuzumab-resistant disease (22), despite
pertuzumab having very limited activity as a single agent. The modest single agent response rate of PMAb was reported for breast cancer patients whose tumors did not overexpress HER2 (23). It is therefore feasible that the combination of trastuzumab and pertuzumab might be of clinical value in a disease such as ovarian cancer that is inherently relatively trastuzumab-resistant. Another target that may have clinical utility in ovarian cancer is the estrogen receptor α (ERα), Phase II trials of aromatase inhibitors (AIs) in ovarian cancer have shown modest response but rather better disease stabilization rates, especially when patients are selected on the basis of ERα expression (24). These results suggest that targeted therapies are feasible in ovarian cancer, but rational selection of both targets and patients is required in order to maximize patient benefit and reduce overtreatment.

During our investigation of tumors treated with combination anti-HER2 antibody therapy (trastuzumab and pertuzumab), we observed that the morphology of xenografts changed over time. Since different histological subtypes of ovarian cancer have different levels of receptor expression (25), we hypothesized that morphological changes might be useful surrogates for the underlying molecular pathology, and reflect a change in therapeutic responsiveness. This prompted us to study this phenomenon further and bridge this ‘phenotype-genotype’ gap for ovarian cancer. We investigated the effect of inhibition of HER2 signaling on ERα expression and endocrine responsiveness in ovarian cancer in vivo. HER2 inhibition with trastuzumab decreased the proportion of ER-negative clear cell areas in a SKOV3 xenograft model of ovarian cancer, and HER2 inhibition with trastuzumab (alone or in combination), but not pertuzumab, acted to up-regulate ERα expression in cell line and primary tumor xenografts. Accordingly, increased responsiveness to letrozole was observed when used in combination with trastuzumab, and therefore the dual combination of an endocrine and HER2 inhibitor could be an effective therapeutic strategy in ovarian cancer.
Materials and methods

**In vivo tumor models.** For xenograft studies, adult female CD-1 nude mice were implanted subcutaneously in the flank with SKOV3 tumor fragments (previously established from the cell line) or fragments from xenografts established from resected primary ovarian cancers or ascites. Tumors were allowed to grow to 4–6 mm in diameter (over a period of approximately 1 month). Animals were then allocated to treatment (5 mice/group) or control (10 mice/group) groups and treatment was commenced (defined as day 0). Mice were treated with trastuzumab (20 mg/kg), pertuzumab (20 mg/kg) or trastuzumab + pertuzumab (20 mg/kg each). Drugs were given via the intraperitoneal (i.p.) route in saline on days 0, 3, 7 and 10. In experiments with letrozole, the drug was given i.p. at a dose of 10 ug/kg/day for 14 days. Tumor size was measured twice weekly using calipers and the volume calculated according to the formula \( \pi/6 \times \text{length} \times \text{width}^2 \). Relative tumor volumes (%) were then calculated for each individual tumor by dividing the tumor volume on day t (V_t) by the tumor volume on day 0 (V_0) and multiplying by 100. Tumors were excised at the end of the experiment and divided with half of the material fixed in 10% buffered formalin and half snap frozen in liquid nitrogen.

**RNA extraction.** Total RNA was prepared from 10-50 mg of frozen tissue from SKOV3 xenografts after 4 days of treatment, preincubated with RNAlater-ICE (Ambion, Austin, TX) using the miRNeasy Mini kit (Qiagen) and TissueRuptor (Qiagen) following the manufacturers’ instructions. The RNA quality was checked by the RNA 6000 Nano assay on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA Integrity numbers were > 8.5.

**Tissue microarray construction.** Tissue microarrays (TMAs) were constructed for both tumor xenografts and primary ovarian tumors. For xenografts, two replicate TMAs were constructed and each TMA contained two 0.6mm cores of each primary tumor xenograft under different treatment conditions. The construction and clinicopathological details of the primary tumor TMA have already been described (26). Four replicate TMAs were constructed using established techniques (27), and three replicates were used for analysis within this study. Each TMA contained a
representative 0.6mm core from each of the 122 cases included within the study. Where primary lesions showed a mixed histological pattern, representative areas of each histological type were targeted, so that all types were adequately represented within the analysis.

**Immunohistochemistry.** Immunohistochemistry was performed on TMAs using a standard immunoperoxidase procedure. Antigen retrieval was performed by microwaving the slides under pressure for 5 min in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9), and slides were treated with 3% H₂O₂ for 10 min in order to quench peroxidase activity. Slides were then incubated in serum-free block solution (Dako) for 20 min to eliminate any non-specific background staining. Primary antibody incubations were Ki67 (#M7240 DAKO; 1:50), ERα ( #VP-E613 Vector Laboratories: 1:50), HER2 (#A0485 Dako; 1:400), HER3 (MS-201-PABX Labvision ; 1:500) for 1h at room temperature and cleaved caspase 3, (#9661 Cell Signaling Technology; 1:400) overnight at 4C. EnVision (HRP Rabbit/Mouse, Dako) was used as secondary antibody and positive staining was detected by incubation with 3, 3-diaminobenzidine solution (DAB+ chromogen, Dako) with hematoxylin counterstain. Quantification of proliferation (using Ki67) or apoptosis (cleaved caspase 3) (0–100%) was based on the number of immunoreactive nuclei divided by the total number of tumor cell nuclei in each TMA core.

**Immunofluorescence.** Immunofluorescence for ERα, was performed using methods previously described (28). For slide staining, 3μm tissue microarray slides were deparaffinized and antigen-retrieved by pressure-cooking in antigen retrieval buffer (sodium citrate pH 6.0). Endogenous peroxidases were blocked with 3% hydrogen peroxide for 15 min and non-specific binding blocked with serum-free protein block for 15 min. Slides were then incubated with primary antibodies (ERα, 1:50; Vector Laboratories) diluted in 0.05% PBST for 1 h at room temperature diluted in DAKO antibody diluent. After washing in 0.05% PBST sections were incubated for 1 h at room temperature with secondary antibodies, which included an Alexa 555–conjugated goat anti-mouse antibody diluted 1:100 in 0.1M TBS, and prediluted goat anti-rabbit antibody conjugated to a horseradish peroxidase–decorated dextran-polymer backbone (EnVision, Dako). Slides were then incubated for 10 min with
Cy5-tyramide, which is activated by horseradish peroxidase, to visualize target phosphorylation protein expression. 4′, 6-Diamidino-2-phenylindole (Invitrogen) was used to stain the nuclear compartment.

**Image analysis for morphological analysis.** For image analysis H&E stained sections of tumors were scanned onto the Ariol SL-50 image analysis system (Applied Imaging Genetix, UK). Briefly, three-colour filtered images were captured on a black-and-white charge-coupled device camera and reconstructed by the supplied proprietary software. Total tumor areas and clear cell areas were marked and the areas automatically calculated by the proprietary analysis software.

**AQUA automated image analysis.** A detailed description of the AQUA HistoRx methodology is available elsewhere (28,29). Pan-cytokeratin antibody was used to identify infiltrating tumor cells, DAPI-counterstain to identify nuclei, and Cy-5-tyramide detection for target for compartmentalized (tissue and subcellular) analysis of tissue sections. Monochromatic images of each TMA core were captured at x20 objective using an Olympus AX-51 epifluorescence microscope, and high-resolution digital images analyzed by the AQUAanalysis™ software. When whole tissue sections of xenograft tumors were analyzed, the whole section was divided into x20 objective magnification images and an AQUA score generated for each frame for heterogeneity analysis. Briefly, a binary epithelial mask was created from the cytokeratin image of each TMA core. If the epithelium comprised <5% of total core area, the core was excluded from analysis. Similar binary masks were created for cytoplasmic and nuclear compartments based on DAPI staining of nuclei. Target expression was quantified by calculating the Cy5 fluorescent signal intensity on a scale of 0-255 within each image pixel, and an AQUA score was generated by dividing the sum of Cy5 signal within the epithelial mask by the area of the cytoplasmic compartment. The same primary antibodies against HER2 and HER3 were used under the same conditions as for the immunohistochemistry.

**Gene Expression Profiling.** A full account of the microarray analysis is given in the parallel study (Sims et al. in preparation). Briefly, total RNA (0.5 µg) was amplified and biotinylated using the Illumina TotalPrep RNA Amplification Kit (Ambion)
according to manufacturers’ standard procedure as two identical aliquots for independent labeling and hybridization. The quality and quantity of cRNA in the samples was checked with an Agilent Bioanalyzer 2100 (Agilent) and samples were diluted to 150 ng/µL before hybridizing to Illumina HT-12 BeadChips. This was undertaken in the Wellcome Trust Clinical Research Facility (Edinburgh). Microarray data analysis was performed using the statistical programming language R and Bioconductor packages (30). Heatmaps were generated using the TreeView program, as described previously (31). All raw gene expression files and clinical annotation are publicly available from the caBIG database in the Edinburgh Experimental Cancer Medicine Centre (https://catissuesuite.ecmc.ed.ac.uk/caarray/).

**Quantitative RT-PCR**

RNA (1µg) was reverse transcribed using a QuantiTect Reverse Transcription kit (Qiagen). The amount of cDNA was quantified using Rotorgene (Corbett Research, San Francisco, CA) and the QuantiTect SYBR Green system (Qiagen). Primers were from Qiagen. The housekeeping gene GUSB was used as a reference gene and its level was constant across the microarray results.

**Western Analysis**  Xenografts were lysed in 50 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 10 µg/ml aprotonin (Sigma), Complete Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma) and spun for 10 min at 16000xg at 4°C. The protein content of the resulting supernatant was determined by the Bicinchoninic acid protein assay (Sigma). Protein lysates were electrophoretically resolved on 7.5-10% SDS-PAGE and transferred to Immobilon-P membranes. After transfer, membranes were blocked with 1% blocking agent (Roche) in TBS before probing overnight at 4°C with the appropriate primary antibody. Antibodies used for Western blotting were ERα (F-10; Santa Cruz biotechnology (sc-8002)) and anti-actin (Merck). 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).
**Statistics.** Relationships between variables were analyzed by one-way ANOVA. Analysis of the microarray data was performed using the statistical program R and the lumi package of the Bioconductor software. The Simpson’s diversity index for heterogeneity (32) was calculated as the sum of the squares of the proportion of AQUA scores belonging to each AQUA score range (0-50, 51-100, 101-150 etc). A Simpson’s index of 1 indicates total homogeneity, and 0 total heterogeneity.
Results

Anti-HER2 receptor antibodies inhibit the growth of SKOV3 xenografts and have differential effects on proliferation and apoptosis

To determine whether treatment with anti-HER2 monoclonal antibody therapy was more effective when administered alone or in combination, HER2-amplified SKOV3 xenografts were treated with trastuzumab, pertuzumab, or a combination of both (Fig 1A). In the first experiment, treatment was initiated when mean tumor volume was 44 mm³. Pertuzumab slowed xenograft tumor growth markedly (Fig 1A; left panel), but did not result in complete tumor regression in any of the xenografts. Trastuzumab alone caused a marked regression and combined treatment with trastuzumab and pertuzumab resulted in complete regression in all tumors (10/10). In a second experiment using larger SKOV3 xenograft tumors (Fig 1A; right panel) (mean tumor volume = 150 mm³), similar differential results were obtained. Pertuzumab slowed tumor growth, while trastuzumab alone or combination therapy resulted in stable disease. Combination treatment produced a more rapid cytostatic effect but trastuzumab alone produced comparable activity after 10 days (Fig 1A).

We next investigated the effects of single- or combined-agent therapy on xenograft proliferation and apoptosis. Proliferation and apoptosis were estimated by calculating a Ki67 and activated caspase 3 index on tissue microarrays of xenograft tissue taken from each time-point (Fig 1B). No significant differences in Ki67 proliferation were noted between treatment groups or over time. In contrast, the apoptotic index differed significantly (p<0.05, one-way ANOVA) between treatment groups and over time. The apoptotic index was significantly higher in the combination group 2 days after the initial treatment. These results suggest that tumor inhibition in the combination group is associated with induction of apoptosis rather than inhibition of proliferation.

Anti-HER2 therapy against a panel of human ovarian cancer xenografts

To extend these findings beyond a single cell line model, we assessed the effect of the trastuzumab / pertuzumab combination on 5 xenografts established directly from ovarian cancer primary tumors or ascites. HOX424 demonstrated a complete response to these drugs, HOX516 and HOX486 showed a partial response, while OV1003 and HOX493 were unaffected by the treatment (Fig 2A). Despite HOX 424 being the most cisplatin-resistant of this series (Supplementary Fig 1), it was the most sensitive
to this HER2-directed combination. HOX 424 was also derived from a mixed clear cell / endometrioid ovarian cancer while the other 4 xenografts were derived from serous (HOX493 and OV1002) or mixed serous / endometrioid ovarian cancers (HOX 516 and HOX 493). In an evaluation of biomarkers of response, expression of HER2 and HER3 were assessed by semi-quantitative immunohistochemistry. HER2 protein expression was highest in HOX424 and SKOV3 xenografts consistent with best response to the antibody combination while HER3 expression increased by the largest degree in HOX424 xenografts (Fig 2B). Quantitation of these markers using AQUA quantitative immunofluorescence confirmed these observations (Supplementary Fig 2).

**SKOV3 xenografts show both morphologic and molecular heterogeneity after treatment with HER2 inhibitors, suggesting that therapy changes the phenotype of constituent cell populations**

Histological examination of SKOV3 xenograft tumors demonstrated distinct differences in morphological appearance, both within the same tumor and between treatment groups. Xenografts showed a range of ovarian histological patterns, including endometrioid and clear cell patterns, similar to mixed ovarian tumors seen in clinical tumor samples (Fig 3A). Quantitative image analysis showed that clear cell areas were markedly reduced following treatment with trastuzumab and significantly reduced after combination therapy treated tumors (Fig 3B), suggesting that trastuzumab can selectively target different populations of cells within morphologically heterogeneous tumors.

In order to verify the change in morphology at the molecular level, we examined changes in expression of genes that had been identified as the most differentially expressed in clear cell versus non clear cell carcinoma from a previous study (33) following treatment with HER2 inhibitors using data from a parallel microarray study (Sims et al -in preparation). The majority of genes that were more highly expressed in clear cell tumors compared to other ovarian histological subtypes were reduced following treatment and those that were lower in clear cell tumors were predominantly higher following treatment (Fig 3C). These changes were of greater magnitude and more significant for trastuzumab alone or in combination, than with pertuzumab treatment alone, consistent with the changes in morphology. Two of the top three genes which have previously been shown to be more highly expressed in
ovarian clear cell carcinomas compared with other ovarian epithelial carcinomas (33); SPP1 and NNMT, were significantly down-regulated in the trastuzumab and combination therapy groups (p<0.002, p<0.0001, Fig 3C). Quantitative RT-PCR (qRT-PCR) measurement of these markers confirmed these changes (Supplementary Fig 3A). These genes were also identified in another study (34) comparing clear cell and other ovarian tumors; very few other genes overlap between these studies, which may also be due to the morphological heterogeneity of tumors observed.

*Estrogen receptor expression and downstream gene expression is modulated by trastuzumab, alone or in combination, in SKOV3 xenografts*

We reasoned that since HER2 receptor expression had been assessed and was constant both across the tumors and different histological subtypes, trastuzumab sensitivity must be mediated by differential activation of other pathways which are known to crosstalk with HER2 signaling and impact on therapeutic response. We therefore assessed the expression of ER\(\alpha\) across the tumors, since crosstalk with HER2 signaling is known to mediate sensitivity to endocrine therapies in breast cancer, and more recently it has been shown that ER\(\alpha\) may be upregulated by trastuzumab (35). Clear cell areas, but not other morphological patterns, were ER\(\alpha\) negative/low by both immunohistochemistry (Fig 4A), consistent with previous reports (25).

We next investigated whether ER\(\alpha\) gene and protein expression levels change in response to HER2-directed therapy in ovarian cancer. We used quantitative immunofluorescence to measure ER\(\alpha\) protein expression on tissue sections in order to establish both the magnitude and distribution of ER expression (Figs 4A and B and Supplementary Fig 4). ER\(\alpha\) nuclear protein expression was significantly increased in SKOV3 xenografts treated with either trastuzumab or trastuzumab and pertuzumab in combination, but not in tumors treated with pertuzumab alone (ANOVA p<0.05; Fig 4A). Western analysis indicated similar changes (Fig 4B). Since ER\(\alpha\)-negative clear cell areas were reduced in trastuzumab-treated tumors, we reasoned that this could be responsible for an average increase in ER\(\alpha\) expression in these tumors. Immunofluorescence confirmed that clear cell areas were negative for ER\(\alpha\) but also that the intensity of fluorescence was increased after treatment with trastuzumab in non-clear cell areas (Fig 4A). When the distribution of ER\(\alpha\) expression was assessed
using an index of heterogeneity (Simpson’s index), both the trastuzumab-treated and combination groups were more heterogeneous than control or pertuzumab-treated groups (Fig 4A), suggesting that in spite of a reduction in clear cell areas, these tumors became more variable in ERα expression, as well as showing an overall increase in receptor expression.

We confirmed that changes in ERα expression resulted in ERα-dependent gene expression, by analyzing changes in expression of ERα-target genes compared to untreated controls (Fig 4C). Genes that have previously been identified as being estrogen-regulated in ovarian cancer (36) or known classic estrogen-regulated genes were investigated (37). Trastuzumab-treated and combination groups showed increases or decreases in expression of ERα-target genes, appropriate to the expected activating or repressing action of ERα. Examples of up-regulated genes include TFF3, FBLN1, AGR2 and PDZK1 while down-regulated genes included TGFBI, LCN2, VIM, AKAP12, PLAU and CDH6 (Fig 4C). Quantitative RT-PCR measurement of expression of selected examples of these genes (TFF3, TGFBI and LCN2) confirmed these changes (Supplementary Fig 3B), which were of greatest magnitude and most significant for the combination of trastuzumab and pertuzumab. Less effect was noted for trastuzumab alone and even less effect with pertuzumab alone. Again, these molecular changes are consistent with the changes in morphology. In order to exclude the possibility that this was a cell line specific phenomenon, we measured ERα expression in the primary ovarian carcinoma xenografts treated with trastuzumab and pertuzumab. Individual tumors showed an increase in ERα expression in 4/5 cases (Fig 2B), suggesting that ERα regulation by HER2-directed therapy could be exploited as a therapeutic strategy in a proportion of ovarian cancers.

**Estrogen receptor expression is lower in clear cell than serous and endometrioid tumors**

Assessment of ERα expression using quantitative immunofluorescence in 122 ovarian tumors of mixed histological types confirmed lower expression of ERα in clear cell ovarian carcinomas compared to serous and endometrioid subtypes (Fig 5A). This suggests that in ovarian cancer, where mixed histology is frequently observed, different populations of cells should be targeted with different agents in order to achieve therapeutic success.
Combination therapy with an aromatase inhibitor and trastuzumab results in an increased rate of complete tumor response

The response of SKOV3 tumor xenografts to combination therapy with the aromatase inhibitor letrozole and trastuzumab was assessed, in order to establish whether the clinically-relevant combination of two targeted agents might be a useful therapeutic strategy in ovarian cancer. SKOV3 xenografts treated with a combination of trastuzumab and letrozole achieved complete tumor response in 44% (4 of 9) mice, compared to 10% (1 of 10) complete response in mice treated with trastuzumab alone (Fig 5B). None of the letrozole only-treated tumors responded completely to therapy. These data suggest that combining endocrine inhibition with trastuzumab therapy might be an effective therapeutic strategy in HER2- and ERα-positive ovarian cancer.

Discussion

Although the therapeutic effects of endocrine agents and HER2-targeted antibodies, and their interactions, are relatively well-characterized for breast cancer, there is little information on the action or interaction of these agents in ovarian cancer, even though they have shown promise for some patients in the clinic (19, 20, 24). When combined, trastuzumab and pertuzumab show an enhanced antitumor activity in ovarian cancer xenografts, as they do in breast (38,39) and non-small cell lung cancer xenografts (39). Since there is crosstalk between pathways downstream of RTKs (such as the MAPK pathway) and endocrine signaling pathways, understanding the molecular interactions between these agents is crucial to accurate selection of patients and maximizing clinical response rates. It is feasible that the combination of antibodies act to produce vertical inhibition of the pathway further modifying phenotype. Alternatively, they may act separately to activate apoptosis in different populations of tumor cells allowing clonal selection. To help identify ovarian cancers which might be sensitive to combined HER2-directed therapy, HER receptor biomarkers may be of value. High HER2 expression is predictive in breast cancer for response to trastuzumab and consistent with this, the two responsive xenografts, SKOV3 and HOX424 showed high expression of HER2 protein which did not apparently diminish on treatment in contrast to unresponsive xenografts which expressed reduced HER2 and wherein expression was reduced on treatment. HER3 has been proposed as a biomarker of...
pertuzumab response in ovarian cancer. Consistent with this, HER3 protein expression was increased by treatment with pertuzumab (20, 40).

Having observed that ovarian xenografts treated with trastuzumab and pertuzumab undergo morphological and gene expression changes in response to therapy, we investigated whether this corresponded to a change in molecular phenotype, and therefore responsiveness to other therapies. ERα was upregulated in response to treatment with trastuzumab (alone or in combination with pertuzumab), but not pertuzumab alone. A similar phenomenon has been reported in breast cancer (35). This was not simply a magnitude effect as the quantitative immunofluorescence is exquisitely sensitive. Although a decrease in ERα-negative clear cell areas was observed in trastuzumab-treated tumors, ERα was also upregulated after treatment, consistent with activation and repression of ERα-target genes. This effect was not cell line-dependent, with similar increases also seen in primary ovarian cancer xenografts. Finally, combining trastuzumab with the aromatase inhibitor letrozole resulted in an increased response compared with either agent alone. These data suggest that the therapeutic efficacy of endocrine therapy may be enhanced by dual endocrine/HER2-directed inhibitor therapy with trastuzumab, and that the choice of therapy and timing of administration may be important factors in determining ultimate therapeutic response. These observations parallel those obtained in breast cancer models wherein breast cancer cells which are endocrine-independent can develop HER2/MAPK activation (35). Blockade of HER2 with trastuzumab, upregulates ERα in these cells leading to estrogen dependence and sensitivity to letrozole and the combination of letrozole plus trastuzumab is more potent than single agent therapy in these endocrine-insensitive HER2-high / ERα-positive cells (35).

Mixed and heterogeneous histological patterns (for example serous and clear cell patterns) are frequently observed in epithelial ovarian cancers, but the observation that SKOV3 xenografts also represent a model for mixed histological types was unexpected, and has not previously been reported. The low expression of ERα in the clear cell areas of the xenografts is consistent with low ERα expression in clear cell carcinomas in surgical resection specimens both within our data and other published studies (25). It is intriguing that the ovarian cancer xenograft (HOX 424) that showed best response to the antibody combination contained a clear cell element. Ovarian clear cell cancers are frequently platinum-resistant (41, 42) so HER2-directed
therapies may provide an option for high HER2 expressing clear cell cancers. Recently, it has been reported that 14% of ovarian clear cell cancers possess HER2 gene amplification and protein overexpression and these represent a promising target group (43).

The morphology of the tumor may therefore be a useful surrogate for the underlying molecular phenotype, but these data also suggest that effective therapy in complex epithelial cancers requires assessment of the heterogeneity of the tumor at a phenotypic and molecular level, and that different components of tumors may require therapy with different agents in order to achieve complete therapeutic response. In this case, clear cell areas appear to be more sensitive to the effects of trastuzumab, while residual disease appears to be sensitized to the effects of endocrine therapy through upregulation of ERα. Consideration of both spatial and temporal heterogeneity is a challenge in the clinical setting since diagnostic biopsies are frequently small and may not be representative of the tumor as a whole, and multiple biopsies during the course of treatment may carry unacceptable clinical risk or be impossible; nevertheless, multiple tissue samples are often taken in cancer therapy in the neoadjuvant (that is systemic treatment before surgery) setting or in ‘window-of-opportunity’ studies in clinical trials (44). Our data suggest that molecular analysis of tumor samples after therapy may be more informative for driving future treatment decisions than tests performed on treatment-naive biopsies alone, since the cancer changes in response to therapy. Personalized cancer care may have to become more reactive to the changing disease in the future in order to improve patient outcomes.

In summary, our studies demonstrated that trastuzumab and pertuzumab showed enhanced antitumor activity in ovarian cancer xenografts than either agent alone. In addition, HER2 antibody-targeted therapy had a spatial and temporal effect on tissue morphology and the expression of ERα, which might be exploited for therapeutic benefit.

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References


**Figure legends**

**Figure 1.** (A) Growth of SKOV3 xenograft tumors in mice treated with the HER2 inhibitors trastuzumab, pertuzumab and combination of trastuzumab and pertuzumab. Both trastuzumab and pertuzumab show growth inhibitory activity, and combination therapy enhanced antitumor activity resulting in complete tumor regression in tumors with small starting volumes (left panel). Mean initial volume = 44 mm$^3$. When larger tumor volumes are implanted, sufficient tumor material remains for biological analysis (right panel). Mean initial volume = 150 mm$^3$. (B) Cell proliferation and apoptosis of tumors treated with trastuzumab, pertuzumab and combination. Cell proliferation (Ki67) and apoptosis (activated caspase 3) were measured in tissue microarrays. There was no significant anti-proliferative activity, but combination therapy induces significant apoptosis (asterisks indicate p<0.05, one-way ANOVA).

**Figure 2.** Panel of human ovarian cancer xenografs grown from material obtained from five different ovarian cancer patients were treated with the trastuzumab plus pertuzumab combination. (A) HOX424 displayed a complete growth response, HOX516 and HOX486 show a minor response and HOX493 and OV1003 were unresponsive. (B) HER2, HER3 and ER$\alpha$ immunoscores on xenografs collected on the final day of measurement. Mean values (+/- S.D.) of at least 4 tumors / group are shown. *p < 0.05 (t-test) shown.

**Figure 3.** Morphological and molecular heterogeneity in SKOV3 xenografts. (A) SKOV3 xenografts show a mixture of histological patterns reminiscent of histological patterns seen in clinical specimens including endometrioid (bottom left) and clear cell (bottom right) subtypes (H&E, x20 magnification). (B) After 14 days of treatment there is a significant decrease in clear cell areas in SKOV3 xenograft tumors treated with combination therapy (p<0.05, one-way ANOVA). (C) Genes that are high in clear cell carcinoma compared to other histological subtypes of ovarian cancer were decreased following trastuzumab and combination therapy and genes lowly expressed in clear cell carcinoma were increased upon trastuzumab and combination therapy, consistent with the morphological changes observed. Red = up-regulated and green = down-regulated relative to mean of controls. Asterisks show significance, *p<0.05, **p<0.001 (t-test), bold numbers are genes whose direction of change is consistent with morphological changes, C=control, T= trastuzumab, P= pertuzumab.
**Figure 4.** Changes in ERα expression in response to HER2-directed therapy in the SKOV3 xenograft. (A) Quantitative immunofluorescence of SKOV3 xenografts treated with trastuzumab, pertuzumab, or combination. The Simpson’s index (SI) for heterogeneity shows that tumors treated with trastuzumab become more, rather than less heterogeneous in ERα staining after therapy with trastuzumab of combination. (B) Western analysis of ERα expression in SKOV3 xenografts. Ratio of ERα vs actin expression is shown obtained by densitometric analysis from Western blots as described in Materials and Methods. (C) ERα-target gene expression in response to therapy. Red = up-regulated and green = down-regulated relative to mean of controls. Values are mean fold changes. asterisks show significance *p<0.05, **p<0.001 (t-test), , C=control, T= trastuzumab, P= pertuzumab, bold numbers are genes whose direction of change is consistent with morphological changes. (D) Immunoperoxidase staining for ERα, (x20 magnification) Clear cell areas show low expression of ERα (left of figure).

**Figure 5.** (A) Clear cell carcinomas show significantly less ERα staining than other histological subtypes in a panel of ovarian carcinomas stained by quantitative immunofluorescence (p=0.037, one-way ANOVA). Numbers of tumors in each group are indicated (B) Growth of SKOV3 xenograft tumors in mice treated with trastuzumab, letrozole or combination of trastuzumab and letrozole. Numbers of tumors demonstrating complete regression are shown in brackets.
Figure 1
Figure 2

A

Mean relative tumor volumes

- HOX 424
- HOX 516
- HOX 486
- HOX 493
- OV 1002

B

HER2

- HOX 424
- HOX 486
- OV 1002
- HOX 493
- HOX 516
- SKOV3

HER3

HER3 histoscore

- Control
- Trastuzumab + Pertuzumab

ERα

ERα histoscore

- Control
- T + P
Figure 3
Figure 4

A. ERa AQUA score

B. ERα / Actin

C. Trastuzumab Pertuzumab Combined

D. ERα
A

Figure 5

ERα AQUA score

n

35

Serous

35

Clear Cell

27

Endometrioid

20

Mixed

9

 MMM

9

Mucinous

B

Mean relative tumor volume

Day

Control

Trastuzumab

Letrozole

Combination

14

21

28

0

7

14

21

28

4/9

1/10

0/10

0/10

0/10
Trastuzumab and pertuzumab produce changes in morphology and estrogen receptor signaling in ovarian cancer xenografts revealing new treatment strategies

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