Dual HER2 Targeting Impedes Growth of HER2 Gene–Amplified Uterine Serous Carcinoma Xenografts

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Abstract

Purpose: Uterine serous carcinoma (USC) is an aggressive subtype of endometrial cancer that commonly harbors HER2 gene amplification. We investigated the effectiveness of HER2 inhibition using lapatinib and trastuzumab in vitro and in xenografts derived from USC cell lines and USC patient-derived xenografts.

Experimental Design: Immunohistochemistry and FISH were performed to assess HER2 expression in 42 primary USC specimens. ARK1, ARK2, and SPEC2 cell lines were treated with trastuzumab or lapatinib. Cohorts of mice harboring xenografts derived from ARK2 and SPEC2 cell lines and EnCa1 and EnCa2 primary human USC samples were treated with either vehicle, trastuzumab, lapatinib, or the combination of trastuzumab and lapatinib. Acute and chronic posttreatment tumor samples were assessed for downstream signaling alterations and examined for apoptosis and proliferation.

Results: HER2 gene amplification (24%) correlated significantly with HER2 protein overexpression (55%). All models were impervious to single-agent trastuzumab treatment. Lapatinib decreased in vitro proliferation of all cell lines and in vivo growth of HER2-amplified xenografts (ARK2, EnCa1). In addition, dual therapy with trastuzumab and lapatinib resulted in significant antitumor activity only in ARK2 and EnCa1 tumors. Dual HER2 therapy induced on target alteration of downstream MAPK and PI3K pathway mediators only in HER2-amplified models, and was associated with increased apoptosis and decreased proliferation.

Conclusions: Although trastuzumab alone did not impact USC growth, dual anti-HER2 therapy with lapatinib led to improved inhibition of tumor growth in HER2-amplified USC and may be a promising avenue for future investigation. Clin Cancer Res; 20(24); 6517–28. ©2014 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy affecting nearly 50,000 women in the United States annually (1). Approximately 80% to 85% of women who undergo comprehensive surgical staging with or without postoperative radiotherapy will be cured of their disease with a low recurrence risk (2, 3). In contrast, the distinct subset of women who present with high-grade carcinomas of various histologic types, including high-grade endometrioid, uterine serous carcinoma (USC), and carcinosarcoma, are at increased risk for recurrence of aggressive disease and chemotherapy resistance (4, 5). These cases account for the majority of the 8,000 annual deaths from endometrial cancer (5–8). The limitations of conventional cytotoxic and radiotherapies to treat women with these aggressive tumors highlight the dire need for scientific investigation to understand molecular signatures that may confer sensitivity to targeted therapy.

HER2, also called HER2/neu or c-erbB2, is a well-characterized member of the HER superfamily that consists of three other tyrosine kinase receptors (HER1/EGFR, HER3, and HER4; ref. 9). The HER2 gene encodes a 185-kDa transmembrane tyrosine kinase receptor and is located on chromosome 17q21. When activated, HER2 can dimerize and induce signal transduction through the MAPK and PI3K signaling pathways (10). This downstream activation leads to induction of genes that can promote oncogenic transformation via cell survival, proliferation, angiogenesis, and metastasis. Unlike the other EGFRs, HER2 has no known ligand, highlighting the fact that it may be constitutively...
Translational Relevance

Biologic therapies targeting HER2 (ERBB2) have become a mainstay of effective therapy for breast tumors found to harbor HER2 gene amplification. Although a subset of uterine serous carcinoma (USC) exhibit HER2 gene amplification, single-agent anti–HER2-directed therapy has failed to produce responses in clinical trials, even those that selected for tumors overexpressing HER2. This investigation utilized nonimmortalized USC cell lines and USC patient-derived xenografts to test the efficacy of lapatinib and trastuzumab, as single agent and combination therapy. We have demonstrated that all HER2-amplified models exhibited trastuzumab resistance that was overcome with the addition of lapatinib. Antitumor responses were associated with significantly increased apoptosis and decreased proliferation as well as decreased Akt and Erk signaling. These data support a potential role for dual anti-HER2 therapy in women with HER2 gene–amplified USC.

Activated and could act independently to drive an invasive phenotype (9). Amplification of the HER2 (ERBB2) gene and overexpression of the HER2 protein have been described in many human malignancies, including breast, colon, gastric, esophageal, ovarian, and endometrial cancer. For some of these cancers, anti-HER2 therapies have become a mainstay of treatment (11, 12). HER2 protein overexpression or gene amplification has been utilized most successfully in breast cancer as a potent biomarker to select those women most likely to respond to anti-HER2 therapies, such as trastuzumab, a monoclonal antibody, or lapatinib, a small-molecule tyrosine kinase inhibitor. In breast cancer, nearly 30% of tumors have been found to harbor HER2 expression via gene amplification or protein overexpression, and are thus designated as HER2 "positive." Although HER2 overexpression was initially associated with the most guarded prognosis in breast cancer, the advent of targeted anti-HER2 therapy has resulted in women with HER2-positive tumors having one of the most favorable prognoses (12, 13). Currently, trastuzumab, pertuzumab (both humanized monoclonal antibodies to the HER2 extracellular domain), trastuzumab emtansine (anti–HER2 antibody conjugate to cytotoxic mertansine), as well as lapatinib (a dual HER1/HER2 small-molecule tyrosine kinase inhibitor) are FDA-approved agents for women with HER2-positive local and metastatic breast cancer to be used in concert with conventional cytotoxic chemotherapy (14–17).

Like breast cancer, USC has been shown to harbor a 10% to 30% rate of HER2 gene amplification, with up to 70% of tumors exhibiting HER2 protein overexpression (18–20). HER2-overexpressing USC has been associated with decreased overall survival (19). Preclinical in vitro data have suggested that cells derived from HER2 gene–amplified USC tumors are more responsive to anti-HER2 therapies compared with cells derived from nonamplified tumors (21). Despite promising preclinical data, the two published phase II trials of anti-HER2 therapy in recurrent endometrial cancer manifested poor responses. One trial evaluated the efficacy of lapatinib in patients with persistent or recurrent endometrial cancer regardless of histology and HER2 status, and found a 3% partial response rate (22, 23). Another recent phase II trial presel ected patients with HER2-positive recurrent endometrial tumors and administered the HER2 monoclonal antibody trastuzumab (24). Unlike an extensive body of breast and gastric cancer literature suggesting HER2 overexpression to be a biomarker for response to anti-HER2 therapy (25, 26), trastuzumab treatment revealed no responses in this trial with HER2-positive endometrial cancer patients (24). Although there is disagreement regarding why lapatinib and trastuzumab as single agents failed to demonstrate any significant durable clinical benefit in endometrial cancer, these trials suggest that single-agent anti-HER2 therapies have limited effect, possibly due to innate or drug-induced resistance pathways (27).

In breast cancer, investigators are propounding the concept of dual anti-HER2 therapy, where biologic therapeutics targeting different aspects of the HER2 protein may someday obviate the need for conventional chemotherapy for women with HER2-positive breast cancer (28). Dual anti-HER2 therapy remains untested in HER2-positive endometrial cancer and given the emerging breast cancer experiences, we explored HER2 expression in USC, and used in vitro and in vivo models to test single and dual anti-HER2 therapy in USC cell lines and patient-derived xenografts (PDxs) with and without HER2 gene amplification.

Materials and Methods

Tissue samples

Using an Institutional Review Board (IRB)-approved protocol, a retrospective cohort of 42 patients with USCs, who were surgically treated at our institution between 2000 and 2012, was established. Formalin-fixed, paraffin-embedded primary USC specimens of all patients were obtained from the pathology department.

Immunohistochemistry

Paraffin-embedded USC tissue sections of 5 μm in thickness were subjected to IHC for HER2 using the HercepTest (Dako), following the manufacturer’s recommendations. The intensity and pattern of the HER2 membrane immunostaining were evaluated, and all samples were scored by a pathologist on a 0–3+ scale with 0 representing no staining, 1+ representing weak staining in >10% of invasive tumor cells, 2+ representing moderate intensity in >10% of invasive carcinoma cells or intense staining in <10%, and 3+ staining defined as intense circumferential membranous staining in >10% of the invasive carcinoma.

USC xenograft sections (5 μm) were examined for Ki67 expression levels by IHC. Antigen retrieval was performed using a 10 mmol/L citrate buffer, and slides were treated with 3% hydrogen peroxide. The blocking reagent and
SNaPshot multiplex system was used to genotype 3 mm
USC model genotyping

Tuzumab was obtained from the clinical pharmacy of the
Drugs

Pyruvate, 2% MEM vitamin solution, and 1% MEM non-
tamine, supplemented with 10% FBS, 1 mmol/L sodium
with USC. We authenticated these cell lines by confirming
Haven, CT, USA) and have been characterized in previous
Houston, TX, USA) and Dr. A. Santin (Yale University, New
in 2011 by Dr. I. Fidler (MD Anderson Cancer Center,
lines (ARK1, ARK2, and SPEC2) were generously provided

To determine the HER2 gene copy number of primary
USC samples and USC cell line–derived xenografts, FISH
was performed on 5 μm thick tissue sections. A PathVysion
HER2 DNA Probe Kit (Abbott Laboratories) was used,
consisting of an LSI HER2 probe with SpectrumOrange
label and a CEP 17 control probe with a SpectrumGreen
tag directed against the centromere region of chromosome
17. Counting was carried out using Vectashield
mounting medium with 4',6-diamidino-2-phenylindole
(DAPI; Vector Laboratories). All samples were visualized
and scored using the CytoVision platform (Leica Biosys-
tems). For each specimen, the HER2 to CEP 17 ratio was
determined by counting the red (HER2) and green (CEP 17)
signals in a minimum of 50 nuclei. Samples with a HER2 to
CEP 17 ratio greater than 2.0 were considered amplified.

Cell culture

Three established human, nonimmortalized USC cell
lines (ARK1, ARK2, and SPEC2) were generously provided
in 2011 by Dr. I. Fidler (MD Anderson Cancer Center,
Houston, TX, USA) and Dr. A. Santin (Yale University, New
Haven, CT, USA) and have been characterized in previous
reports (29, 30). Each cell line was derived from a patient
with USC. We authenticated these cell lines by confirming
the HER2 protein and gene status as well as serum histology
via pathologic review. ARK1 and ARK2 cell lines were
cultured in RPMI1640 medium (Corning) supplemented
with 10% FBS, 1% penicillin, and streptomycin (Life
Technologies). SPEC2 cells were cultured in Eagle’s minimum
essential medium (MEM) containing Earle’s salts and l-glut-
tamine, supplemented with 10% FBS, 1 mmol/L sodium
pyruvate, 2% MEM vitamin solution, and 1% MEM non-
esential amino acids (Life Technologies). BT-474 breast
cancer cells were cultured in DMEM/F12 medium with
l-glutamine supplemented with 10% FBS. All cell lines were
maintained in an atmosphere containing 5% CO₂ at 37°C.

Drugs

Lapatinib was purchased from LC Laboratories. Tras-
tuzumab was obtained from the clinical pharmacy of the
institution.

USC model genotyping

An adapted version of the Applied Biosystems Prism
SNaPshot multiplex system was used to genotype 3 mm
core samples from the EnCa1 and EnCa2 primary tumors as
well as extracted genomic DNA from the three nonimmor-
talized human cell lines ARK1, ARK2, and SPEC2 as previ-
ously described (31). This clinical mutational profiling
platform screens for 130 well-characterized mutations that
are distributed across 15 cancer genes, including AKT1, APC,
BRAF, CTNNB1, EGFR, ERBB2, ID1H, KIT, KRAS, MAP2K1,
NOTCH1, NRAS, PIK3CA, PTEN, and TP53 (31).

In vitro treatment of USC cell lines

ARK1, ARK2, and SPEC2 were seeded on 6-well plates and
and serum starved using growth medium containing 1% FBS.
The next day, cells were treated in duplicate with increasing
concentrations of lapatinib or trastuzumab in growth medi-
num with 1% FBS. Treated cells were incubated for 2 days
(lapatinib) or 5 days (trastuzumab), then collected and
counted. With trastuzumab dose–response experiments,
the HER2 overexpressing breast cancer cell line BT-474 was
cotreated to serve as positive control for drug response. After
the IC₅₀ dose of lapatinib was determined for each cell line,
cells treated with this dose for 48 hours were used for
Western blotting analyses of members of the PI3K and
MAPK signaling pathways.

Generation and propagation of USC xenografts

All mouse studies were carried out in compliance with the
Institutional Animal Care and Use Committee guidelines.
Xenografts derived from ARK2 or SPEC2 cells were estab-
lished by subcutaneous injection of cultured cells into 6- to
8-week-old female NOD/SCID mice (Jackson Laboratory),
in a 1:1 suspension of PBS and Matrigel (BD Biosciences).
As previously described, PDXs were established from patients with USC (32). Briefly, primary human USC speci-
mens were obtained under an IRB-approved tissue collec-
tion protocol. They were enzymatically processed, followed by depletion of endothelial and hematopoietic cells. The
remaining purified USC cells were suspended in PBS with
Matrigel (1:1) and injected subcutaneously into 6- to 8-
week-old female NOD/SCID mice. Xenograft formation
was monitored regularly, and mice were euthanized by
CO₂ inhalation when tumors had reached a diameter of
15 to 20 mm. Tumors were then excised and enzymatically
processed, followed by depletion of H-2Kd–positive mouse
cells. The resulting single tumor cells were resuspended in
PBS/Matrigel (1:1) and injected subcutaneously into female
NOD/SCID mice. Serial transplantation of USC xenografts resulted in the generation of cohorts of mice
that each harbored a tumor derived from the same primary
USC. A total of 217 mice were euthanized to conduct the
described in vivo experiments.

Treatment of mice harboring USC xenografts

Mice bearing xenografts derived from ARK2 or SPEC2
cells were randomized into two groups of 5 (ARK2) or 6
(SPEC2) mice with equivalent average tumor volumes. The
formula (length in mm × width in mm × height in mm)/2
derived from caliper measurements was used to calculate
tumor volumes as has previously been described (32). One
at 4°C. Primary antibodies directed against phospho-Akt (p-Akt, Thr308), Akt, phospho-Erk1/2 (p-Erk1/2, Thr202/Tyr204), Erk1/2, phospho-PRAS40 (p-PRAS40, Thr246), PRAS40, phospho-p70S6K (p-p70S6K, Thr389), and p70S6K (all from Cell Signaling Technology) were used. The dilution for all primary antibodies was 1:1,000. The blots were then probed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) and developed using a chemiluminescent detection reagent (ECL Prime, GE Healthcare Life Sciences). Each blot was stripped with 1 mol/L glycine when the same membrane was used to reprobe with another primary antibody. Finally, all membranes were stripped and reprobed with either a mouse anti-Pan-actin antibody (1:10,000, NeoMarkers) or a rabbit anti-GAPDH antibody (1:1,000, Cell Signaling Technology) to verify equal protein loading.

**TUNEL assay**

Apoptosis was assessed in paraffin-embedded xenograft sections of 5 μm in thickness, collected at the end of each four-arm treatment experiment. The DeadEnd Fluorometric TUNEL System (Promega) was used, according to the manufacturer’s instructions with minor modifications. A negative control without addition of TdT enzyme was included. Detection of fragmented DNA was conducted on a fluorescence microscope (Nikon Eclipse). For each sample, the number of DAPI-stained nuclei and the number of TUNEL-positive nuclei were quantified in four randomly selected fields using Fiji software (ImageJ). The TUNEL index was calculated as the number of TUNEL-positive cells × 100/total number of nuclei.

**Statistical analysis**

Fisher exact testing was utilized for comparison of proportions. Survival analysis was done using the Kaplan-Meier method along with a Cox proportional hazards model incorporating age and stage of disease as continuous variables. Two-way ANOVA analysis was used to determine the statistical significance of the effects of lapatinib treatment on the different cell lines in vitro. Statistical significance of the observed differences in xenograft growth and mouse weights between the different treatment arms was assessed with nonparametric Wilcoxon rank sum tests. One-way ANOVA analyses were performed to determine significance of the observed differences in Ki67- and TUNEL-positive cell counts. Stata software version 11.1 (StataCorp, LP) and GraphPad Prism software version 6 (GraphPad Software, Inc.) was used, and a P < 0.05 was considered statistically significant.

**Results**

**Prevalence of HER2 protein overexpression and HER2 gene amplification in USC**

HER2 protein expression as well as HER2 gene copy number were analyzed in a cohort of 42 primary USC tissue blocks. Supplementary Table S1 summarizes the clinical characteristics of the corresponding patients. The mean age of the cohort was 68.7 years, and all stages of disease were represented with an average overall survival rate of 2.4 years from diagnosis. IHC for HER2 revealed 2+ or 3+ protein expression in 55% of the cohort (Fig. 1A and B). A lower rate of HER2 gene amplification (HER2 to CEP 17 ratio > 2.0) was observed by FISH, with 10 of 42 USC samples (24%) found to be amplified (Fig. 1A and B). HER2 gene amplification significantly associated with HER2 protein overexpression in our cohort (P < 0.02, Fig. 1B). When comparing clinical outcome of HER2-amplified USCs with nonamplified tumors, a worse overall survival rate was
observed in patients harboring HER2 amplification when controlling for age and stage (Fig. 1C; $P = 0.015$).

**Cell line genotyping**

Multiplex tumor analysis of both EnCa1 and EnCa2 revealed no gain-of-function mutations, although the testing confirmed the heightened HER2 gene dosage that was observed on FISH analysis. No gain-of-function mutation was detected in the ARK2 cell line, though a PIK3CA mutation (1624G to A, E542K) was confirmed in ARK1, as described in ref. (33), and SPEC2 was found to harbor an NRAS mutation (34G to T, G12C) of uncertain clinical significance.

**HER2 inhibition using lapatinib and trastuzumab in USC cell lines**

HER2 protein overexpression (3+ staining) and gene amplification were demonstrated in ARK1- and ARK2-derived xenografts by IHC and FISH, whereas SPEC2-derived tumors were shown to harbor low protein expression and normal HER2 gene status (Supplementary Fig. S1). In all three USC cell lines, treatment with trastuzumab showed no effect on cell proliferation (data not shown). However, a dose-dependent reduction in cell number was found in ARK1 and ARK2 cells after lapatinib treatment as compared with vehicle controls. ARK2 cells showed the strongest response to lapatinib treatment in vitro, with observed IC50 values of 0.05 μmol/L for ARK2 and 0.5 μmol/L for ARK1 (Fig. 2A; $P < 0.0001$). ARK1 was sensitive at higher doses compared with ARK2 likely as a result of the PIK3CA gene mutation that uncouples the HER2 inhibition.

We next studied the effect of lapatinib treatment on signaling molecules downstream of HER2, by assessing protein expression and phosphorylation of members of the PI3K and MAPK pathways. Western blotting analyses showed decreased levels of phosphorylated Akt (Thr308) and phosphorylated Erk1/2 in ARK2 xenografts treated with lapatinib, as compared with control xenografts (Supplementary Fig. S2). These observations were in agreement with the in vitro results, indicating that HER2 inhibition is associated with downregulation of the PI3K and MAPK pathways in ARK2 xenografts.

**Figure 1.** HER2 gene amplification was observed in 24% of USC specimens, which was significantly associated with HER2 protein overexpression and worse overall survival. A, representative images of FISH (top), 100×, and IHC, 10× (bottom) for HER2: top left HER2 nonamplified; top right HER2 amplification; bottom left HER2 immunostaining score 1+; bottom right HER2 immunostaining score 3+. B, overview of FISH and IHC findings. A significant association between HER2 gene amplification and 2+ or 3+ HER2 protein expression was found ($P < 0.02$). Low protein expression was defined as a score of 0 or 1+, and high HER2 protein expression as a score of 2+ or 3+. C, Kaplan–Meier curve showing the overall survival differences between patients with tumors harboring HER2 gene amplification and patients with tumors with normal HER2 gene status. In a Cox proportional hazards model incorporating age and stage, HER2 amplification independently correlated with overall survival, with a median of 1.2 years for patients with HER2 amplification, as opposed to 2.9 years for those with normal HER2 gene status ($P = 0.02$).
in ARK1 and ARK2 cells and reduced expression of phosphorylated Erk in ARK2 and SPEC2 cells after treatment with lapatinib, compared with untreated controls (Fig. 2B). The levels of total Akt and Erk proteins were not affected by treatment with lapatinib.

**HER2 inhibition with lapatinib and trastuzumab in USC xenografts**

HER2 protein overexpression and HER2 gene amplification were observed in EnCa1 xenografts, whereas low levels of the HER2 protein and gene were found in EnCa2 xenografts (Supplementary Fig. S1). The single-agent lapatinib experiments employed two cohorts of mice bearing xenografts derived from either ARK2 or SPEC2. A reduction of tumor growth was observed in ARK2 xenografts ($P = 0.02$), but not in SPEC2 xenografts, after treatment with lapatinib, as compared with vehicle-treated tumors (Supplementary Fig. S2).

To further explore the effects of anti-HER2 therapy, four mouse cohorts harboring tumors derived from ARK2 and SPEC2 as well as EnCa1 and EnCa2 were used to study the antitumor efficacy of trastuzumab alone, lapatinib alone, and the combination of trastuzumab and lapatinib (Fig. 3). Single-agent trastuzumab treatment induced significant regression of BT-474 xenografts ($P < 0.03$), confirming effective targeting of HER2 using trastuzumab in immunocompromised mice (data not shown). In all four USC xenograft cohorts, no significant inhibition of tumor growth was observed following treatment with trastuzumab alone as compared with vehicle-treated controls. However, significantly superior antitumor activity was observed with the combination of trastuzumab and lapatinib in ARK2 tumors, compared with lapatinib alone and trastuzumab alone ($P < 0.01$). Similarly, the strongest reduction of EnCa1 tumor growth was found in the dual therapy arm and while statistically different from vehicle, there was no statistical improvement over single-agent lapatinib treatment which also significantly inhibited EnCa1 tumor growth ($P < 0.03$). In both tumors lacking HER2 gene amplification (EnCa2 and SPEC2), no therapy induced antitumor activity. Importantly, mouse weights revealed no statistically significant alterations during the treatment period of all xenograft cohorts (Supplementary Fig. S3).

**Effects of HER2 inhibition in vivo on downstream signaling proteins**

Activity of the PI3K and MAPK pathways was assessed by studying the phosphorylated and total protein levels of Akt, PRAS40, p70S6K, and Erk1/2 in xenografts harvested 6 hours after administration of a single dose of trastuzumab, lapatinib, both agents, or vehicles only (Fig. 4). Reduced levels of p-Akt and p-Erk were observed in lapatinib-treated ARK1 cells, while a decrease in p-Erk but not p-Akt was seen in lapatinib-treated SPEC2 cells. Expression of Pan-actin or GAPDH was used as loading control.
Effects of HER2 inhibition on cell proliferation and apoptosis

To analyze the effect of treatment with trastuzumab, lapatinib, and dual therapy on proliferation and apoptosis, xenograft samples harvested at the end of each treatment experiment were subjected to Ki67 IHC as well as TUNEL analysis. Significantly decreased expression of Ki67 was observed in ARK2 and EnCa1 samples treated with lapatinib alone or the combination of trastuzumab and lapatinib, compared with vehicle-treated tumors. Treatment with trastuzumab alone led to a smaller though significant decrease in ARK2 Ki67 levels, whereas no significant difference was seen in EnCa1 tumors. Increased TUNEL staining was found in ARK2 and EnCa1 xenografts treated with lapatinib with and without trastuzumab, compared with vehicle controls. These differences in Ki67 and TUNEL staining were not seen in SPEC2- and EnCa2-treated samples.

Discussion

HER2 gene amplification has been shown to be a prevalent and prognostic signature that in other disease sites has been effectively targeted for significant clinical benefit (34). Echoing clinical trial experience, the data presented here support that USCs present with innate trastuzumab resistance, which is overcome with lapatinib, and that combination therapy can be effective in xenografts derived from ARK2 and EnCa1 primary USCs (35).

Figure 3. Anti-HER2 therapies of USC xenografts stratified by HER2 gene amplification. Significant antitumor activity of lapatinib and a synergistic effect of lapatinib with trastuzumab were observed in HER2-amplified USC xenografts (ARK2, EnCa1), whereas no effect was seen in nonamplified xenografts (SPEC2, EnCa2). Mice bearing xenografts derived from ARK2 or SPEC2 cell lines or EnCa1 or EnCa2 primary USCs were treated with either vehicle, lapatinib alone, trastuzumab alone, or the combination of lapatinib and trastuzumab. Tumors were measured twice weekly, and the percent change in tumor volume compared with baseline volume (100%) is shown. Error bars represent the SEM. While trastuzumab had no significant antitumor effect, dual therapy with lapatinib and trastuzumab impeded the growth of HER2-amplified ARK2 xenografts when compared to all other arms (P < 0.01). Dual therapy with lapatinib and trastuzumab delayed tumor growth in the EnCa1 xenografts compared to vehicle controls (P < 0.02), though the latter was not statistically different from single-agent trastuzumab or lapatinib. In both non-HER2-amplified models (SPEC2 and EnCa2), lapatinib and trastuzumab did not affect xenograft growth.
resistance that may be mitigated by administering concurrent lapatinib, an anti-HER2 therapy that targets the intracellular domain of the receptor. While in vitro models demonstrated a reduction in cell viability regardless of HER2 gene amplification status when treated with lapatinib, only HER2 gene-amplified xenografts (ARK2, EnCa1) responded to lapatinib in vivo, with the most robust responses observed with the use of combination lapatinib and trastuzumab. This is the first report to our knowledge that has utilized cell line–derived and patient-derived USC xenografts to suggest that HER2 gene amplification can be a biomarker associated with response to anti-HER2 therapies in USC. Despite unsuccessful efforts in the clinic to utilize HER2 inhibition in high-grade endometrial cancer, these data support a role for the use of dual anti-HER2 therapy in gene-amplified USC as has been propounded for the treatment of trastuzumab-resistant breast carcinoma (35).

This preclinical investigation confirms and extends the findings of numerous investigations that have sought to characterize HER2 expression in USC. Early reports of HER2 protein overexpression and gene amplification demonstrated this to be a prevalent signature associated with aggressive disease and a worse prognosis (19, 36, 37). The rates reported were in the 17% to 30% range for HER2 gene amplification, not dissimilar from that observed in breast and gastric cancers where trastuzumab was found to offer significant benefit (38). Santin and colleagues later refined this experience, showing that HER2 expression was over-represented in African American women compared with Caucasian women (39). This finding might lend insight into the increased mortality observed in this population despite similar medical access and care. Most investigators suggested that HER2 gene amplification could be a potent biomarker for the selection of women most likely to respond to trastuzumab, prompting the Gynecologic Oncology Group (GOG) to initiate a trial in recurrent endometrial cancer. Although the trial accrued more than 30 women, all with HER2 overexpressing recurrent endometrial tumors, no responses to trastuzumab were observed (24). While many authors suggested the trial was flawed as it lacked a high proportion of endometrial cancers known to harbor the highest HER2 expression (African American subjects and serous histology), the complete lack of signal may indicate that HER2 overexpressing endometrial cancer presents with innate trastuzumab resistance.

In this investigation, we tested trastuzumab and found that in both the USC cell line- and patient-derived USC xenografts studied, trastuzumab failed to induce any alterations in vitro or in vivo. We believe this effect was not due to failure of antibody therapies in our models, as trastuzumab...
induced significant antitumor activity in the HER2 overexpressing BT-474 cells. Interestingly, when lapatinib was utilized, all cell lines regardless of gene amplification status demonstrated a decrease in cell viability, with higher doses needed for this response in the nonamplified SPEC2 cells. The most robust response to lapatinib was observed in ARK2, likely because SPEC2 lacks HER2 gene amplification, and ARK1 was found to harbor a PIK3CA gene mutation both of which would act to render the tumor cells less sensitive to anti-HER2 therapies.

Unlike the in vitro experiments, however, the in vivo studies demonstrated that lapatinib as single agent and in combination with trastuzumab induced significant tumor-static effects only in those tumors harboring HER2 gene amplification (ARK2, EnCa1). In the nonamplified SPEC2 and EnCa2 xenografts, a complete lack of response to any administered therapy was seen. These models strongly support the hypothesis that HER2 gene amplification is a biomarker for response to HER2 inhibition in USC as has been shown in breast and gastric carcinomas (11, 34, 40).

The antitumor effects following HER2 blockade in ARK2 and EnCa1 xenografts were associated with increased apoptosis and decreased proliferation as has been shown in lung and head and neck carcinomas (41, 42), as well as reduced levels of signaling proteins of the PI3K and MAPK pathways. The most potent acute abrogation of p-Erk, p-Akt, p-PRAS40, and p-p70S6K was seen following dual treatment with lapatinib and trastuzumab, supporting a rationale for the synergistic inhibition of tumor growth observed. Unlike in breast carcinoma, where HER2 inhibition has been primarily associated with blockades in the PI3K signaling pathway, these findings in USC suggest downregulation of both MAPK and PI3K signaling with PI3K signaling pathway, these findings in USC suggest downregulation of both MAPK and PI3K signaling with dual therapy (43). In the two different xenograft examples lacking HER2 gene amplification (SPEC2, EnCa2), no downstream alterations could be discerned. This suggests that without the HER2 signature, both trastuzumab and lapatinib fail to produce meaningful changes in the target pathways.

It is unclear why HER2 gene–amplified USC is impervious to trastuzumab though this phenomenon has been noted in many other non-breast adenocarcinomas found to harbor HER2-positive tumors (34). In breast carcinoma, much clinical and preclinical effort has been focused on trastuzumab-resistant tumor cells. Pohlmann and colleagues (44) and others (45) have reviewed this topic extensively, generally in the setting of breast cancer where trastuzumab is a mainstay of therapy for HER2 overexpressing tumors. Many factors are likely to contribute to this observed preclinical and clinical resistance in endometrial cancer, including a high prevalence of PI3K pathway activation via PIK3CA mutation and PTEN loss-of-function. One potential mechanism of resistance implicates a
truncated variant of HER2, p95HER2, that lacks the extracellular domain (ECD) where trastuzumab binds, thus decreasing therapeutic efficacy (46). High expression of this variant has been associated with trastuzumab resistance and lapatinib sensitivity in breast cancer, and prospective trials are underway to validate p95HER2 expression as a biomarker associated with response to specific HER2 therapies (47, 48). Elevated ECD levels have been observed in USC (49), though the expression of p95HER2 in HER2 overexpressing USC has yet to be defined and would require prospective validation as a biomarker associated with resistance to trastuzumab.

Investigators have suggested that trastuzumab resistance may be overcome through the use of dual anti-HER2 therapies that interact with the receptor in different ways, either by prohibiting dimerization or tyrosine kinase function. Scaltriti and colleagues described how breast cancer cell line therapy with lapatinib led to a potent upregulation of HER2 protein expression which subsequently sensitized previously resistant cells to trastuzumab (50). In the USC xenografts used in this study, concurrent trastuzumab and lapatinib treatment led to the statistically most robust antitumor activity when compared with vehicle only in the HER2 gene—amplified tumors.

Figure 6. Increased apoptosis was observed in ARK2 and EnCa1 tumors following treatment with lapatinib or the combination of lapatinib and trastuzumab. Xenografts collected at the end of the in vivo treatment experiments were examined for apoptosis using TUNEL analysis. Representative images of TUNEL-stained (green) sections with DAPI counterstain (blue) of the differently treated ARK2 (A) and EnCa1 (B) tumors are shown. The percentage of TUNEL-positive cells was significantly higher in ARK2 (C) and EnCa1 (D) xenografts treated with lapatinib with or without trastuzumab, compared with vehicle controls. Trastuzumab alone had no effect on tumor cell apoptosis. Scale bars, 50 μm. Error bars, SEM. **, P < 0.01; *, P < 0.05; **, P < 0.001.
Interestingly, the ARK2 cell line–derived xenografts exhibited uniform HER2 gene amplification with a ratio of >15.0 in all nuclei counted, whereas the patient-derived EnCa1 xenografts demonstrated more heterogeneity regarding HER2 gene amplification. In the latter, a gene copy ratio of 9.0 was found though not all tumor cells demonstrated increased HER2 gene copy numbers. Intratumor heterogeneity of HER2 gene amplification was also observed in the retrospective cohort, and has been noted recently in the literature (20). In the current study, lower antimtor activity of dual HER2 inhibition was seen in the EnCa1 xenografts with lower prevalence of HER2-amplified cells when compared with the ARK2 model in which tumor regression was observed. Extrapolating from these pilot data, merging dual anti-HER2 therapy with conventional cytotoxics or an additional biologic could be a rational method to target the nonamplified tumor cells that commonly coexist in HER2-amplified tumors.

Currently, there are no clinical trials registered in the United States examining dual anti-HER2 therapies in HER2 overexpressing endometrial cancer. Two trials that are utilizing HER2 overexpression as a biomarker are a phase I trial of trastuzumab in concert with paclitaxel and carboplatin for upfront therapy of USC (NC101367002) and a phase I trial testing lapatinib with ixabepilone for recurrent uterine carcinoma and carcinosarcoma. The preclinical data described in this study underscore the potential for USC to harbor innate trastuzumab resistance and suggest that combination therapy with lapatinib induces significant cell death and in vivo antitumor activity. Further investigation of dual HER2 inhibition in HER2 gene–amplified USC may be warranted in a clinical trial setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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