Dual HER2/PIK3CA Targeting Overcomes Single-Agent Acquired Resistance in HER2-Amplified Uterine Serous Carcinoma Cell Lines In Vitro and In Vivo

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Abstract

HER2/neu gene amplification and PIK3CA driver mutations are common in uterine serous carcinoma (USC) and may represent ideal therapeutic targets against this aggressive variant of endometrial cancer. We examined the sensitivity to neratinib, taselisib, and the combination of the two compounds in in vitro and in vivo experiments using PIK3CA-mutated and PIK3CA wild-type HER2/neu–amplified USC cell lines. Cell viability and cell-cycle distribution were assessed using flow-cytometry assays. Downstream signaling was assessed by immunoblotting. Preclinical efficacy of single versus dual inhibition was evaluated in vivo using two USC xenografts. We found both single-agent neratinib and taselisib to be active but only transiently effective in controlling the in vivo growth of USC xenografts harboring HER2/neu gene amplification with or without oncogenic PIK3CA mutations. In contrast, the combination of the two inhibitors caused a stronger and long-lasting growth inhibition in both USC xenografts when compared with single-agent therapy. Combined targeting of HER2 and PIK3CA was associated with a significant and dose-dependent increase in the percentage of cells in the G0–G1 phase of the cell cycle and a dose-dependent decline in the phosphorylation of S6. Importantly, dual inhibition therapy initiated after tumor progression in single-agent–treated mice was still remarkably effective at inducing tumor regression in both large PIK3CA and pan-ErbB inhibitor–resistant USC xenografts. Dual HER2/PIK3CA blockade may represent a novel therapeutic option for USC patients harboring tumors with HER2/neu gene amplification and mutated or wild-type PIK3CA resistant to chemotherapy.

Introduction

Endometrial cancer is the most common gynecologic malignancy with approximately 54,870 new cases and 10,170 estimated deaths related to the disease in the United States annually (1). Recently, using a comprehensive genetic investigation, The Cancer Genome Atlas (TCGA) Research Network provided compelling evidence that endometrial cancers result from heterogeneous somatic mutations and classified endometrial cancers into four categories: (i) polymerase epsilon (POLE)-ultramutated, (ii) microsatellite instability hypermutated, (ii) copy-number low, serous-like (2). In this landmark study, patients harboring uterine serous carcinoma (USC) were found to have the worst prognosis as compared with all the other groups of endometrial cancer (2). USC is high grade by definition. Because of its biologic aggressiveness, early-stage USC (i.e., stage I) is treated after surgical staging with systemic cytotoxic chemotherapy with or without localized radiotherapy (3–5). Unfortunately, about 70% of patients have extraterine metastases at the time of initial staging and up to 50% of patients treated with surgery will develop recurrent disease, which is fatal in the majority of the cases (5, 6). The development of novel and more effective treatment modalities remains an unmet medical need in USC patients.

HER2 is a member of the HER superfamily that consists of three additional tyrosine kinase receptors (HER1/EGFR, HER3, and HER4; ref. 7). Unlike the other EGFRs, HER2 has no known ligand and functions as a preferred partner for heterodimerization with any of the other members of the EGFR family and thus plays an important role in the coordination of the complex HER2/neu signaling network that is responsible for regulating cell growth and differentiation (7). Amplification of the HER2 (ERBB2) gene has been described in many human malignancies, including but not limited to breast, colon, and gastric cancer, and it has been reported in up to 35% of USC (8–11). Moreover, early reports have demonstrated that HER2 protein overexpression and gene amplification is associated with more aggressive disease, worse
progression and resistance to therapy in multiple human tumors, including USC (11–13).

The PI3KCA gene encodes for a heterodimeric protein with an 85-kDa regulatory subunit (PI3KR1) and a 110-kDa catalytic subunit (PI3KCA). PI3K pathway is known to play a fundamental role in cellular functions, including proliferation, survival, and growth in normal as well as neoplastic cells. Importantly, the catalytic subunit of the PI3KCA gene is frequently mutated or amplified in the different types of endometrial cancers and may therefore represent an attractive target for the development of novel, potentially effective therapies against biologically aggressive tumors such as USC (14–21).

Neratinib (HKI-272, Puma Biotechnology) is an oral, potent, and irreversible inhibitor of EGFR, HER2, and HER4 tyrosine kinases with promising preclinical activity against HER2-overexpressing cell lines (22). Importantly, neratinib has been demonstrated to be significantly more effective when compared with the first-generation (i.e., reversible) EGFR and HER2 inhibitors (22–25), and it is currently in phase III trials in breast cancer patients (NCT01808573). Taselisib, (GDC-0032, Genentech), is a novel, oral, selective inhibitor of PIK3CA. Taselisib binds the ATP-binding pocket of PI3K with selective preference for the mutated form of PIK3CA (26) and it is currently tested in phase II/III clinical trials against multiple human tumors (i.e., NCT02154490).

In this study, we have evaluated the effect of single versus dual HER2/PI3K inhibition in multiple FISH+/PIK3CA wild-type and FISH+/PIK3CA-mutated primary USC cell lines fully characterized by whole exome sequencing (WES; ref. 20). We demonstrate for the first time that the dual targeting of HER2 and PIK3CA with neratinib and taselisib is highly synergistic and an additive effect is considered as CI = 1, while antagonism is CI > 1, and an additive effect is considered as CI = 1 [at affected fraction (Fa) = 0.50, 0.75, 0.90, and 0.95]. The CI values were calculated using CompuSyn (CompuSyn, Inc.) as previously described (28).

Flow-cytometry analysis of cell cycle in primary USC cell lines

After 24-hour exposure to neratinib (0.01 μmol/L), taselisib (0.01 μmol/L), and the combination of both drugs, treated, and control cells were permeabilized with ice-cold 70% ethanol and fixed for 30 minutes at 4°C. After staining with 2,000 rpm for 5 minutes and discarding supernatant, cells were suspended in 1 mL of PBS. After additional spinning at 2,000 rpm for 5 minutes, 100 μL of ribonuclease (100 μg/mL, DNsase free, Sigma) was added for 5 minutes incubation at room temperature, before exposure to 400 μL of propidium iodide (50 μg/mL in PBS). Treated and untreated control cells were acquired with FACS Calibur; using Cell Quest software (BD Biosciences) and were analyzed using Flowjo software.

Immunoblotting

Cells were seeded in Petri tissue culture plates (100,000 cells) and left to adhere overnight. Cells were then treated with 0.01 μmol/L of neratinib, taselisib, and the combination of both for 24 to 48 hours. After incubation, cells were washed once with PBS, mechanically scraped, and lysed for 30 minutes on ice with 75 μL of radioimmunoprecipitation assay buffer (50 mmol/L Tris–HCl, pH 8, 150 mmol/L NaCl, Triton X-100 1%, Na deoxicolate 0.5%, SDS 0.1%, MgCl 5 mmol/L in H2O) supplemented with protease inhibitor cocktail (#78441, Thermo Scientific). Lysates were

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Abbreviations: AA, African-American; C, Caucasian; FIGO, International Federation of Gynecology and Obstetrics stage.
prepared by collecting supernatants after centrifugation for 10 minutes at 10,000 × g at 4°C. Protein levels were quantified with BCA Protein Assay Kit (#23225, Thermo Scientific), and equal amounts were loaded for SDS-PAGE using 4% to 20% acrylamide gel electrophoresis (Bio-Rad). Antibodies used for immune detection of proteins were pHER2 (#2243, Cell Signaling Technology, Inc.), pEGFR (#2234, Cell Signaling Technology, Inc.), pS6 (#4856, Cell Signaling Technology, Inc.), pAKT (#4060, Cell Signaling Technology, Inc.), pHER2 (#2243, Cell Signaling Technology, Inc.), and GAPDH (#2118, Cell Signaling Technology, Inc.). Incubation of the membranes with primary antibodies was carried overnight in 3% BSA in PBS-Tween at 4°C. After incubation, membranes were washed three times with 1% milk in PBS-Tween (0.2%) at room temperature and incubated with an HRP-linked secondary antibody (#7074, Cell Signaling Technology, Inc.) in 5% milk PBS-Tween (0.2%) for one hour before washing four times in 1% milk PBS-Tween. Signals were detected with Western blotting detection reagents (Thermo Scientific). Bands were then visualized and the blots developed using an enhanced chemiluminescent system (GEL Logic 1500, Carestream Health).

Flow-cytometry analysis of phosphorylated S6 intracellular levels

Next, we evaluated pS6 expression levels by flow cytometry. Cells were plated in a 6-well plate (40,000 cells per well). After 24-hour exposure to 0.01 μmol/L of taselisib, 0.01 μmol/L of neratinib, and the combination of both agents, treated and untreated control cells were fixed in 4% formaldehyde and permeabilized with ice-cold 90% methanol. Treated and untreated control cells were incubated with primary rabbit monoclonal antibody against pS6 (# 4856, Cell Signaling Technology, Inc.) following the protocol provided by the manufacturers and stained with a fluorescein isothiocyanate-conjugated goat anti-rabbit F(ab')2 immunoglobulin as a secondary reagent (Chemicon International, Inc.). Cells (i.e., 5,000 events per sample) were analyzed on FACScalibur, using Cell Quest software.

In vitro assay of drug effect

A representative FISH+/PIK3CA-mutated cell line (USPC-ARK-1) were injected into the subcutaneous region of 5- to 8-week-old SCID mice (Harlan Laboratories). After implantation of cells, tumors were monitored until they reached a tumor volume of 0.1 cm³ prior to initiating the treatment. Mice were then randomized into the following 4 groups: vehicle (0.5% methylcellulose-0.2% Tween 80), taselisib (10 mg/kg), neratinib (40 mg/kg), and the combination of taselisib (10 mg/kg) plus neratinib (40 mg/kg). Each group consisted of 5 mice. Treatments were given orally once a day, 5 days a week for 60 days, after which mice were euthanized. Each group consisted of 5 mice. Treatments were given orally once a day, 5 days a week for 60 days, after which mice were euthanized. Cells were plated in a 6-well plate (40,000 cells per well). After 24-hour exposure to different dose levels were normalized to the mean of the control group receiving no drug, so that all data were expressed as a proportion of the control. Normalized data were then fit via nonlinear regression to a normalized logistic response curve against the base-10 logarithms of dose in Mₚ and the resulting parameter estimates were used to calculate the value of the IC₅₀ (in log₁₀ units) for that experiment. One-way ANOVA was used to determine the statistical significance of the effects of combination treatment on the different cell lines in vitro when compared with the control and to the single-agent treatment. For the flow-cytometry experiments, changes in the phosphorylated S6 protein levels were analyzed comparing the mean intensity of fluorescence (MFI) before and after the exposure to the inhibitors. The measures of the MFIs under different dose levels were normalized to the mean of the control group receiving no drug, so that all data were expressed as a proportion of the control. An unpaired t test was used to assess pS6 changes and cell-cycle changes in the HER2/neu-amplified PIK3CA-mutated or wild-type cell lines. Statistical differences between mean tumor volumes at specific time points were performed using an unpaired t test. Error bars represent SEM. Differences in all comparisons were considered statistically significant at P values < 0.05.

Results

In vitro activity of neratinib and taselisib in HER2/neu gene-amplified cell lines

First, we evaluated the sensitivity of the USPC-ARK-1 cell line to neratinib. We found strong growth inhibition using increasing concentrations of neratinib with a mean inhibitory concentration (IC₅₀ ± SEM) of 0.023 ± 0.008 μmol/L (Supplementary Fig. S1). We next evaluated the effect of neratinib, taselisib, and the combination of both in all four cell lines at the IC₅₀ concentrations identified in previous studies (23, 27). As shown in Fig. 1, the combination of the two inhibitors was able to induce a more potent and highly significant cell growth inhibition when compared with single-agent therapy in all the cell line tested. To further confirm these results we evaluated the synergism between the two compounds in all cell lines tested following combination treatment at multiple paired concentrations. Data were evaluated for potential synergistic activity (CI values) using the software CompuSyn. In all four HER2/neu gene-amplified cell lines tested, the combination of neratinib and taselisib showed synergistic activity. Results are shown in Table 2. For the USPC-ARK-1 cell line, the following CI values were obtained: 0.48753 (Fa = 0.50), 0.24043 (Fa = 0.75), 0.11857 (Fa = 0.90), and 0.07332 (Fa = 0.95). For the USPC-ARK-2 cell line, the CI values were as follows: 0.46474 (Fa = 0.50), 0.32323 (Fa = 0.75), 0.25874 (Fa = 0.90), and 0.23124 (Fa = 0.95). For the USPC-ARK-20 cell line, the CI values were as follows: 0.72491 (Fa = 0.50), 0.60347 (Fa = 0.75), 0.64375 (Fa = 0.90), and 0.67345 (Fa = 0.95). For the USPC-ARK-21 cell line, the CI values were as follows: 0.70324 (Fa = 0.50), 0.65658 (Fa = 0.75), 0.59501 (Fa = 0.90), and 0.55665 (Fa = 0.95).

Cell-cycle analysis

Next, we investigate cell-cycle progression after treatment with neratinib, taselisib, and the combination of both inhibitors in two representative FISH+/PIK3CA-mutated or wild-type cell lines (USPC-ARK-2 and USPC-ARK-1). In USPC-ARK-2 (i.e., PIK3CA wild-type) taselisib as single agent, was unable to significantly delay cell cycle (increasing the G1 phase) after 24-hour exposure.
In contrast, neratinib was effective in inducing a significant increase in the percentage of the cells blocked in the G0–G1 phase (P = 0.01, Fig. 2). However, when we analyzed the effect of the combination of the two inhibitors, we found a significant increase in the percentage of cells blocked in the G1 phase of the cell cycle when compared with untreated controls or cells treated with the single agent neratinib and taselisib (P = 0.002, P = 0.03, and P = 0.003 respectively, Fig. 2). Next, we evaluated cell-cycle analysis in USPC-ARK-1 (i.e., PIK3CA mutated). We found both inhibitors, (i.e., neratinib and taselisib), to be able to increase the number of cells blocked in G0–G1 phase when compared with control cells (P = 0.007 and P = 0.03, respectively, Fig. 2). Furthermore, we found the combination to induce a significant increase in the percentage of the cells blocked in the G1–G0 phase of the cell cycle when compared with the untreated control, to neratinib and taselisib used as single agents (P = 0.0006, P = 0.01, and P = 0.001 respectively, Fig. 2).

**Immunoblotting**

Western blotting analyses of phosphorylated HER2, EGFR, AKT, and S6 were performed after 24 to 48 hours of treatment at the selected drug concentrations described in Materials and Methods section in USPC-ARK-2 and USPC-ARK-1. We found neratinib to be able to reduce the levels of p-HER2 and p-EGFR in both cell lines tested (Fig. 3A). Taselisib single agent was active in reducing pAKT in USPC-ARK-1 but not USPC-ARK-2 cell lines while combination treatment led to reduced levels of p-AKT and p-S6 in USPC-ARK-1 cells, and a decrease in p-S6 but not p-AKT in USPC-ARK-2-treated cells (Fig. 3A). Data presented in Fig. 3B (i.e., 48 hours after exposure to drugs) and S2 further confirm that the combination of the two inhibitors was able to induce a significant dephosphorylation of S6 when compared with control cells in both cell lines tested. In additional experiments, we performed Western blot analyses of phosphorylated HER2, EGFR, AKT, and ERK in the USPC-ARK-1 primary cell line after 2 weeks exposure to single-agent neratinib or taselisib (i.e., single-agent–resistant cell lines). These cells were confirmed to be resistant in vitro in proliferation assays to both single-agent taselisib and neratinib but highly responsive to the drug combination (Supplementary Fig. S3). We found a significant increase in phosphorylated AKT and a consistent reduction in the levels of p-HER2 and p-EGFR after prolonged exposure to neratinib (Fig. 3C). In contrast, we...
found a significant increase in phosphorylated HER2, EGFR, and ERK after two weeks exposure to taselisib.

**Antitumor activity of neratinib and taselisib in xenograft models**

To validate our *in vitro* results, we next determined the *in vivo* activity of neratinib, taselisib, and the combination of the two drugs in two different animal models. First, we evaluated the tumor growth of USPC-ARK-1 (FISH+/PIK3CA mutated) xenografts under the therapeutic conditions described above. Consistent with the *in vitro* data, both single-agent neratinib and taselisib were able to induce a significant tumor growth inhibition (after 4 and 14 days of treatment, respectively) when compared with the vehicle group (*P* = 0.01 and *P* = 0.03 respectively, Fig. 4, top). Furthermore, the combination of the compounds was able to induce tumor regression after 4 days of treatment when compared with the control (*P* < 0.0001) and to taselisib (*P* = 0.01). Starting at 11 days after the beginning of treatment, the combination of the two inhibitors was able to induce a remarkable tumor growth inhibition when compared with single-agent neratinib (*P* = 0.01). This effect lasted for the entire treatment period (i.e., 60 days), after which the animals were euthanized. To determine whether the *in vivo* acquired resistance to single-agent therapy was potentially reversible in the animal model, we also started a combination treatment adding the missing inhibitor to the neratinib or the taselisib group when the mean tumor volume reached 0.9 cm^3^. As shown in Fig. 4, the combination was highly effective in inducing tumor regression in both groups of animals on single-agent therapy. Next, we evaluated the *in vivo* activity of neratinib, taselisib, and the combination of the two drugs in the USPC-ARK-2 xenografts (FISH+/PIK3CA wild-type) under the same therapeutic conditions. Once again, both single-agent neratinib and taselisib were able to induce a significant tumor growth inhibition (after 4 and 11 days of treatment, respectively) when compared with the vehicle group (*P* = 0.005 and *P* = 0.02 respectively, Fig. 4). The combination of the compounds was able to induce tumor regression after 4 days of treatment when compared with the control (*P* = 0.01). Importantly, similarly to USPC-ARK-1 cell line, the combination of the two inhibitors was also able to overcome single-agent–acquired resistance and...
induced remarkable tumor regression in both group of animals in progression during neratinib or taselisib single-agent treatment (Fig. 4, bottom).

Discussion

Patients diagnosed with advanced or recurrent biologically aggressive endometrial cancers such as USC have an extremely poor prognosis. The development of novel, effective therapies against USC resistant to chemotherapy remain desperately needed. In the last few years, multiple comprehensive genetic studies from our group, the TCGA network as well as others have reported the mutational landscape of USC, giving the opportunity for identification of multiple deranged pathways as potential novel targets for the treatment of this highly lethal tumor (2, 20, 21, 29, 30). Because these comprehensive studies found that a large number of USC patients harbor alterations in the HER2/neu and/or the PIK3CA gene (20, 31, 32), the HER2/PI3K/AKT/mTOR pathway may represent a highly attractive therapeutic target against these rare tumors (31).

Consistent with this view, in the last few years several potent and highly selective compounds against the HER2/neu, PI3K, AKT, or mTOR pathways have been generated. However, only limited amounts of preclinical data are currently available about the use of HER2-targeted agents or PI3K TKIs against USC (23, 27, 31). In addition, while most of the preclinical studies showed promising result using pan-ErbB or PI3K/mTOR inhibitors as single agents in mice engrafted with HER2 FISH+ or HER2 FISH+/PIK3CA–mutated cell lines, none of these studies was able to demonstrate durable tumor growth inhibition in vivo (23, 27, 33). Consistent with these preclinical results, emerging clinical data have so far shown limited single-agent activity of these inhibitors at tolerated doses in endometrial cancer (34–36).

The high genomic instability and heterogeneity of USC (2, 10, 20), combined with the improved understanding of the mechanism of USC carcinogenesis have recently provided a new alternative of using targeted agents against HER2, PI3K, AKT, or mTOR in combination. Accordingly, in this study we have evaluated the efficacy of neratinib and taselisib as single agents and in combination against multiple genetically well characterized (i.e., whole exome sequenced) USC cell lines in vitro and in vivo. Similar to our previously reported findings (23, 27, 33), we found both single-agent neratinib and taselisib to be highly active in vitro but only transiently effective in vivo in controlling the growth of both USC xenograft models harboring HER2/neu gene amplification with or without oncogenic PIK3CA mutations. These in vivo results in USC xenografts using an irreversible pan-ErbB or PI3K inhibitor are therefore consistent with the results of clinical
studies in B-RAF–mutated melanoma or EGFR mutated lung cancer patients treated with vemurafenib or gefitinib, respectively.

In these studies, treatment with highly targeted agents resulted in an initial tumor shrinkage in the short-term (37, 38). Long-term follow-up suggested that patients who initially responded, tended to have progression typically only few months after starting treatment (39–41). Taken together these clinical results combined with our preclinical data in USC suggest that even in molecularly selected patients with well documented oncogenic “driver” gene mutations, the rapid acquisition of resistance may represent a significant barrier to the long-term survival of cancer patients.

Preclinical studies in both breast and lung cancer cell lines and mouse models suggest that small-molecule–mediated inhibition of the HER2 alone is insufficient for complete inhibition of PI3K/mTOR activity, which may contribute to both primary and acquired resistance via persistent overactivation of downstream signaling (42–44). Consistent with this view, previous in vivo models suggest the addition of a mTOR inhibitor to a HER2 inhibitor may result in synergistic tumor growth inhibition and regression (45, 46). The combination of these two drug classes was similarly synergistic in vitro, with combinatorial suppression of the PI3K–mTOR–S6 axis. Furthermore, the addition of HER2 inhibition to PI3K/mTOR inhibition may potentially block feedback activation of PI3K–AKT/protein kinase B and ERKs that occur after mTOR inhibition alone (45, 46). Accordingly, when the efficacy of the combination of PI3K and HER2 inhibition was tested in HER2/neu–amplified cell lines, we found a synergistic effect of the dual inhibition in all USC cell lines tested (i.e., 4/4) in vitro, with a more potent delay of the cell cycle and a more potent dephosphorylation of several downstream elements of the HER2/PI3K/mTOR pathway when compared with single-agent therapy (Fig. 3 and Supplementary Fig. S2). More strikingly, the association of neratinib and taselisib in vivo in both USC xenograft model available was highly synergistic and effective in preventing tumor outgrowth for the entire duration of the in vivo study (i.e., 2 months). Moreover, the dual targeting of this pathway was also able to overcome the in vitro (Supplementary Fig. S3) and in vivo acquired resistance to single-agent neratinib or taselisib in both tumor models. These latter results showing high sensitivity to taselisib (i.e., PI3K inhibitor) or neratinib (i.e., pan-HER inhibitor) in single-agent–resistant tumors may potentially be explained by the results of our Western blot experiments showing a significant increase in the phosphorylation of AKT (i.e., a downstream effector of PI3K) after prolonged exposure to neratinib as well as a significant increase in the phosphorylation of HER2, and EGFR (i.e., neratinib targets) after prolonged exposure to taselisib. Our molecular results in USC are therefore in agreement with the results of Serra and colleagues, in breast cancer cell lines overexpressing HER2 who also found that combined administration of PI3K inhibitors with HER2 inhibitors resulted in superior antitumor activity when compared with single-agent PI3K inhibitors as well as a compensatory activation of the downstream signaling pathways (47).

One possible limitation of dual therapy in the clinical setting is the potential increased toxicity as a result of combining two highly targeted agents. It is therefore worth noting that no evidence of increased in vivo acute or chronic toxicity was detected in our study. Indeed, no significant variation in behavior or body weights was found in mice receiving combination treatment when compared with the mice in the control groups (Supplementary Fig. S4). These data suggest that HER2-amplified USC resistant to neratinib or taselisib single-agent therapy may be responsive to combinatorial treatment. These results could have important implications for patients currently being treated.

In conclusion, while treatment with targeted therapeutics may initially lead to dramatic tumor regression, cancers seem to invariably acquire resistance to these drugs. Our study represents the first preclinical demonstration that synergistic dual targeting HER2/PI3KCA with neratinib and taselisib is able to achieve durable regression of established USC xenografts in vivo. Daily oral administration of the two compounds may represent a novel, potentially highly effective therapeutic strategy against HER2/neu-amplified USC harboring mutated or wild-type PI3KCA genes unresponsive to chemotherapy.

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

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*In Vitro* and *In Vivo*


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