Predictive and Pharmacodynamic Biomarkers of Response to the Phosphatidylinositol 3-Kinase Inhibitor Taselisib in Breast Cancer Preclinical Models

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ABSTRACT

The PI3K signaling pathway serves as a central node in regulating cell survival, proliferation, and metabolism. PIK3CA, the gene encoding the PI3K catalytic subunit p110-alpha, is commonly altered in breast cancer resulting in the constitutive activation of the PI3K pathway. Using an unbiased cell line screening approach, we tested the sensitivity of breast cancer cell lines to taselisib, a potent PI3K inhibitor, and correlated sensitivity with key biomarkers (PIK3CA, HER2, PTEN, and ESR1). We further assessed how taselisib modulates downstream signaling in the different genomic backgrounds that occur within breast cancer. We found that sensitivity to taselisib correlated with the presence of PIK3CA mutations, but was independent of HER2 status. We further showed that HER2-amplified/PIK3CA wild-type cell lines are not as sensitive to taselisib when compared with HER2-amplified/PIK3CA-mutant cell lines. In a PIK3CA-mutant/PTEN null background, PI3K downstream signaling rebounded in the presence of taselisib correlating with decreased sensitivity at later time points. Finally, we observed that PIK3CA mutations cooccurred with mutations in the estrogen receptor (ER; ESR1) in metastatic tumors from patients with ER+ breast cancer. However, the cooccurrence of an ESR1 mutation with a PIK3CA mutation did not affect response to taselisib in a single agent setting or in combination with fulvestrant.

Introduction

The PI3K signaling pathway plays a critical role as a central prosurvival node controlling key regulators of cell homeostasis (1). Class I PI3K enzymes exist as heterodimers composed of a p110 catalytic subunit stabilized in a low activity state by being bound to a p85 regulatory subunit. Following ligand binding and stimulation of receptor tyrosine kinases (RTK), such as HER2, the p85 subunit mediates translocation of the complex to the cell membrane resulting in a conformational change in p110 causing its activation. A phosphorylation cascade commences, in which the p110–p85 complex phosphorylates phosphatidylinositol (4, 5)-bisphosphate (PIP2) producing phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that in turn leads to phosphorylation and activation of the serine-threonine kinase AKT. Activated AKT, a downstream mediator of the PI3K pathway, subsequently phosphorylates a wide range of substrates that control metabolism, survival, and proliferation. As with other prosurvival pathways, PI3K signaling is frequently dysregulated in human tumors.

For example, overexpression of RTKs such as HER2 can drive the pathway irrespective of ligand binding (2). Additional alterations that can occur within the PI3K pathway include activating somatic mutations in p110 or AKT1, and loss of the tumor suppressor PTEN (3, 4). Of the four isoforms of the p110 subunit (p110-alpha, beta, delta, and gamma), the gene that encodes for p110-alpha, PIK3CA, is mutated or amplified at relatively high frequencies in solid tumor malignancies, including lung, breast, and colorectal (3, 5–7). Oncogenic PIK3CA mutations are frequently found throughout the protein and include key hotspot mutations in the helical (E542 and E545) and kinase domains (H1047; refs. 6–8). Helical domain mutations are characterized to reduce the inhibitory effects of the p85 subunit on catalytic activity, whereas kinase domain mutations result in increased membrane binding (9). Other low frequency mutations have also been detected in tumors, such as mutations in the p85-binding, Ras-membrane binding (9). Other low frequency mutations have also been detected in tumors, such as mutations in the p85-binding, Ras-membrane binding, and C2 domains conferring PI3K pathway activation (5, 7).

Breast cancer is largely classified into three main subtypes, each of which directs different treatment regimens (10). The luminal subtype is the most prevalent and is comprised of tumors that express hormone receptors [estrogen receptor (ER) and/or progesterone receptor (PR)] and are treated with antiendocrine therapies. The next most frequent subtype is the HER2-positive subtype comprised of tumors that are HER2 amplified (HER2+) and may also express hormone receptors. Breast cancers in this subtype tend to have a worse prognosis than luminal cancers, but are often successfully treated with regimens containing anti-HER2 therapies such as trastuzumab and pertuzumab (11). Finally, tumors in the triple-negative subtype are negative for hormone receptors and HER2 overexpression, have the worst prognosis, and are commonly treated with chemotherapy. Alterations within the PI3K pathway occur at different frequencies within the different breast cancer subtypes. For example, luminal cancers have the highest frequency of PIK3CA mutations at approximately
Materials and Methods

Cell culture and reagents

Breast cancer cell lines were obtained from the ATCC, the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), Cell Line Service GmbH, Asterand, the Kawasaki Medical School (Kurashiki, Japan), and the University of Texas Southwestern (Dallas, TX) and were cultured according to the manufacturer's specifications and maintained at 37°C with 5% CO2 (Supplementary Table S1). Unless otherwise indicated by manufacturer, cell line media was supplemented with 10% heat-inactivated FBS, 1% MEM nonessential amino acids (Gibco, 11140-050), 2 mmol/L L-glutamine (Gibco, 15030-081), and 100 U/mL penicillin-streptomycin (Gibco, 15140-122). Cell line authentication and quality control was conducted by the Genentech centralized cell repository as previously described using short tandem repeat profiling, SNP fingerprinting, and Mycoplasma testing (17). The time between thawing and use of cell lines did not exceed 15 passages. Characterization of PI3KCA genomic alterations have been described previously (18) and PIK3CA mutation status of all cell lines is summarized in Supplementary Table S1. The ER, PR, HER2, and PTEN protein status of all cell lines as determined by immunoblot are shown in Supplementary Fig. S1A and summarized in Supplementary Table S1. The doxycycline-inducible ER-overexpressing MCF-7 (ERWT, ERβ1-β3, and ERδ) cells were engineered as described previously (19). Doxycycline-inducible cells were treated with 50 ng/ml doxycycline (Takara Bio USA, 631311) for 48 hours prior to drug dosing, and doxycycline was maintained for the duration of drug exposures. Taselisib (GDC-0032) was a beta-sparing PI3K inhibitor that potently inhibits p110-alpha (0.29 nmol/L), delta (0.12 nmol/L), and gamma (0.97 nmol/L), but has 31-fold less inhibition of p110-beta (9.1 nmol/L) when compared with p110-alpha (16). In this study, we sought to identify biomarkers of sensitivity to taselisib in preclinical breast cancer models to aid in the clinical development of next-generation PI3K molecules.

Cell viability assays

Cells were plated in quadruplicate in 384-well tissue culture–treated plates (Corning, 353962) in normal growth medium at cell line–specific densities to be a target confluency of 70%–80% upon lyase collection and treated as described in the figure legends. Cells were washed with PBS and scraped into ice-cold tissue protein extraction reagent (T-PER, Thermo Fisher Scientific, 78510) supplemented with protease (Roche, 1183617001) and phosphatase (Sigma-Aldrich, P0844) inhibitors. Cell lines that failed to achieve IC50 and the software was unable to calculate a value, the IC50 was nominally assigned as the highest concentration screened (10 μmol/L). Mean absolute taselisib IC50 values and SD are shown in Supplementary Table S1.

Immunoblot and phospho-RTK array analyses

For immunoblot analyses, cells were plated in 6-well tissue culture–treated plates (Corning, 3516) at cell line–specific densities to be at a target confluence of 70%–80% upon lyase collection and treated as described in the figure legends. Cells were washed with PBS and scraped into ice-cold tissue protein extraction reagent (T-PER, Thermo Fisher Scientific, 78510) supplemented with protease (Roche, 1183617001) and phosphatase (Sigma-Aldrich, P0844) inhibitors. Cell debris was pelleted by centrifugation at 13,000 rpm for 15 minutes at 4°C, and supernatants were removed and assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227). Equal amounts of protein prepared in 1× sample reducing agent (Invitrogen, NP0009) and 1× LDS sample buffer (Invitrogen, NP0007) were loaded onto 20- or 26-well NuPAGE 4%–12 Bis-Tris Gels (Invitrogen, WG1402 and WG1403), separated by electrophoresis in 1× MOPS SDS running buffer (Invitrogen, NP0001) with added antioxidant (Invitrogen, NP0005) at 170–180 V for 1 hour, and transferred to nitrocellulose membranes using the iBlot 2 Gel Transfer Device and Transfer Stacks (Invitrogen, IB23001). Efficient protein transfer and even loading were confirmed using the reversible ATX Ponceau S Red Staining Solution (Sigma-Aldrich, 09189). Membranes were blocked for 30 minutes to 1 hour in 5% BSA (Sigma, A7030) in tris-buffered saline-Tween (TBST) at room temperature with agitation, and subsequently incubated with primary antibodies diluted in 5% BSA-TBST overnight at 4°C with agitation. Anti-estrogen receptor alpha (MA1-39539, RRID_AB_10975344, 1:150) was purchased from Invitrogen; anti-actin HRP-conjugated antibody (sc-47778 HRP, RRID_AB_2714189, 1:20,000) was purchased from Santa Cruz Biotechnology. Anti-AKT (9272, RRID_AB_329827, 1:4,000), anti-phospho-AKT (4060, RRID_AB_2315049, 1:4,000), anti-cleaved caspase 3 (9664, RRID_AB_2070042, 1:1,000), anti-cleaved PARP (9541, RRID_AB_331426, 1:2,000), anti-EGF receptor (4267, RRID_AB_331015, 1:20,000), anti-phospho-HER (2247, RRID_AB_331725, 1:750), anti-progesterone receptor A/B (8757, RRID_AB_2797144, 1:1,000), anti-PTEN (9552, RRID_AB_10694066, 1:3,000), anti-S6 ribosomal protein (2217, RRID_AB_331335, 1:2,000), anti-phospho-S6 ribosomal protein (4858, RRID_AB_916156, 1:2,000), and anti-alpha-tubulin (3873, RRID_AB_1904178, 1:2,000) were purchased from Cell Signaling Technology. Primary antibody research resource identifiers (RRID) obtained from the RRID Portal at https://scicrunch.org/resources. Membranes were actively washed three times in TBST for 10 minutes/wash and then incubated with an anti-rabbit IgG (Cell Signaling Technology, 7074, RRID AB_2099233, 1:2,000) or anti-mouse IgG (Cell Signaling Technology, 7076, RRID_AB_330924, 1:2,000) horseradish peroxidase (HRP)–linked secondary antibody diluted in 5% BSA-TBST for 1 hour at room temperature with agitation. TBST washes were repeated as before and protein–antibody complexes were detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, 34075) on film or using the Azure Biosystems c600. To detect β-actin or α-tubulin as loading controls, membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, 46430) for 25

The concentration of drug resulting in 50% inhibition of cell viability (IC50) was calculated from a four-parameter curve analysis (XLfit, IDBS Software) from a minimum of three independent experiments. For cell lines that failed to achieve IC50 and the software was unable to calculate a value, the IC50 was nominally assigned as the highest concentration screened (10 μmol/L). Mean absolute taselisib IC50 values and SD are shown in Supplementary Table S1.
minutes at room temperature with agitation, washed with TBST, reblocked in 5% BSA-TBST, and then incubated with antibody for 1 hour at room temperature with agitation. TBST washes, secondary antibody incubation (if necessary), and chemiluminescent detection of protein–antibody complexes were repeated as before.

For the phospho-RTK array analyses, cells were plated in 100 mm tissue culture–treated plates (Corning, 430167) at cell line–specific densities to be at a target confluency of 70%–80% upon lysis collection and treated as described in Fig. 2. Lysate collections and assay procedures were followed as directed by the manufacturer using 300 μg of sample input on the Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, ARY001B).

PTEN siRNA-induced knockdown

Transient PTEN knockdown was achieved through reverse transfections of target cells seeded in 6-well (Corning, 3516) or 96-well (Perkin Elmer, 6005182) tissue culture–treated plates using siRNAs at a final concentration of 20 nmol/L and Lipofectamine RNAiMAX (Invitrogen, 13778-150) following the manufacturer’s instructions. The siGENOME nontargeting siRNA control pool #2 (Dharmacon, 001206-14-05) was used as a negative control. PTEN-targeted siRNAs were synthesized at Genetech and target sequences used were as follows: PTEN siRNA-1: GATCAGCATACAAATTA, and PTEN siRNA-2: GGCCTATGTGTATTATTA. All subsequent cell treatments as described in the figure legends were initiated at 48 hours post reverse transfection.

qRT-PCR

Cells were plated in duplicate in 6-well tissue culture–treated plates (Corning, 3516) at cell line–specific densities to be at a target confluency of 70%–80% upon lysis collection and treated as described in the figure legends. Total RNA was isolated as isolated using a RNase Mini QIAcube Kit (Qiagen, 74116) with added QIAshredder (Qiagen, 79654) and DNase I (Qiagen, 79254) digestion steps. RNA was quantified using either NanoDrop or QIAxpert systems and cDNA samples were amplified and analyzed using TaqMan Universal PCR Master Mix (Applied Biosystems, 4304437) and the Via 7 Real-Time PCR System (Applied Biosystems). All TaqMan gene expression assays were purchased from Thermo Fisher Scientific and included the following: GAPDH (Hs09999905_m1), ACTB (Hs99999903_m1), PTEN (Hs02621230_s1), ESRI (Hs00174860_m1), PGR (Hs01556702_m1), GREB1 (Hs00536409_m1), and IGFBP4 (Hs01057900_m1). Relative quantification of mRNA levels was calculated after normalization to GAPDH or ACTB mRNA using the 2–ΔΔCt method.

Tumor samples and sequencing

Samples were submitted to a Clinical Laboratory Improvement Amendments–certified, New York State–accredited, and CAP–accredited laboratory (Foundation Medicine) for hybrid capture–based next-generation sequencing–based genomic profiling. Approval for this study, including a waiver of informed consent and a HIPAA waiver of authorization, was obtained from the Western Institutional Review Board (protocol no. 20152817). The pathologic diagnosis of each case was confirmed by review of hematoxylin and eosin–stained slides and all samples that advanced to nucleic acid extraction contained a minimum of 20% tumor cells. The samples used in this study were not selected and represent an “all comers” patient population to Foundation Medicine genomic profiling. Samples were processed in the protocol developed for solid tumors as described previously (20). For solid tumors, DNA was extracted from 40 μm of unstained formalin-fixed, paraffin-embedded sections, typically 4 × 10 μm sections. Adapter-ligated DNA underwent hybrid capture for all coding exons of 287 or 395 cancer-related genes plus select introns from 19 or 31 genes frequently rearranged in cancer. Captured libraries were sequenced to a median exon coverage depth of >500× (DNA) using Illumina sequencing, and resultant sequences were analyzed for base substitutions, small insertions and deletions (indels), copy-number alterations (focal amplifications and homozygous deletions), and gene fusions/rearrangements, as described previously (20). Frequent germline variants from the 1000 Genomes Project (dbSNP142) were removed. To maximize mutation-detection accuracy (sensitivity and specificity) in impure clinical specimens, the test was previously optimized and validated to detect base substitutions at a ≥5% mutant allele frequency (MAF), indels with a ≥10% MAF with >99% sensitivity, and fusions occurring within baited introns/exons with >99% sensitivity (20). Known confirmed somatic alterations deposited in the Catalog of Somatic Mutations in Cancer (COSMIC v62) are called at allele frequencies ≥1% (21). Patients were not consented for raw data release. Therefore, associated raw sequence data is not shared. However, variants from a subset of the samples used in this analysis (>18,000) have been deposited in the Genomic Data Commons (accession #phs001179). ER status was automatically parsed from pathology reports using optical character recognition (Abby) and text mining, validated to 96% accuracy.

Results


taselisib sensitivity correlates with PIK3CA mutations in preclinical breast cancer models

To study which predictive biomarkers correlate with sensitivity to taselisib, we screened 50 breast cancer cell lines with differing genomic backgrounds using a cell viability assay based on ATP quantitation (Fig. 1A; Supplementary Table S1). ER–α, PR, HER2, and PTEN protein expression were verified by immunoblot and summarized along with PIK3CA mutation status (Supplementary Fig. S1; Supplementary Table S1). At 3 days following taselisib treatment, we observed a wide range of responses with IC50 ranging from 0.012 μmol/L to 10 μmol/L, the highest dose tested. Numerous cell lines displayed sensitivity to taselisib with 34% (17/50) of those tested having an IC50 below 0.2 μmol/L. A significant increase in taselisib sensitivity was observed in cell lines harboring oncogenic mutations in PIK3CA (PIK3CAmut) with a 26-fold difference between the mean IC50 values of PIK3CA wild-type (PIK3CAwt) and PIK3CAmut cells (Fig. 1B). We next assessed whether the site of a PIK3CA mutation associated with response to taselisib. We observed that cell lines with PIK3CA mutations in exon 9 (helical domain), exon 20 (kinase domain) and rarer activating mutations in exons 1 and 7 (p85-binding and C2 domains, respectively) were all sensitive to taselisib inhibition (Fig. 1C).

Next we used immunoblot analysis to investigate the pharmacodynamic effects of taselisib on downstream P38 signaling as direct analysis of p110-alpha phosphorylation was not feasible due to lack of available, specific antibodies (24). Taselisib caused a dose-dependent sensitivity to taselisib.
Figure 1.

In vitro response to taselisib and relationship to PIK3CA mutations. A, Absolute taselisib IC50 for 50 breast cancer cell lines at 3 days posttreatment determined from an ATP-based cell viability assay and ordered from lowest to highest. IC50 values were determined by calculating the mean from at least three independent experiments with quadruplicate technical replicates each; error bars denote SD. Boxes indicate activating mutations in PIK3CA (PIK3CAmut, blue), HER2 amplification (HER2+, red), loss of PTEN expression (PTENnull, green), ER-alpha expression (ER+, orange), and PR expression (PR+, purple). Relationship between absolute taselisib IC50 values from a 3-day cell viability assay and PIK3CA mutation status [n = 36 (WT) and n = 14 (PIK3CAmut); B] or PIK3CA mutation location [n = 36 (WT), n = 3 (Exon 9), n = 8 (Exon 20), and n = 3 (Other); C] in 50 breast cancer cell lines; error bars denote SEM. Welch t test used for statistical analysis. D, Immunoblots of the downstream PI3K signaling response (pAKT and pS6) in PIK3CAmut (EFM-19 and T47D) and PIK3CAwt (CAL-120 and MDA-MB-231) cell lines treated with 200 nmol/L taselisib for indicated time points. β-Actin was used as a protein loading control.
decrease in phosphorylated AKT (pAKT) and phosphorylated S6 (pS6) levels in the PIK3CAmut EFM-19 and T47D cell lines resulting in complete loss of phospho-signals at multiple time points (Supplementary Fig. S1B). At a taselisib concentration of 200 nmol/L, loss of phospho-signals occurred by the earliest timepoint assessed (1 hour) and was maintained out to 48 hours in PIK3CAmut cells (Fig. 1D).

Conversely, in PIK3CAWT CAL-120 and MDA-MB-231 cells, taselisib had less impact on pAKT and no observable effect on pS6. Notably, in CAL-120 cells, pAKT was lost at 1 hour posttreatment, and began to rebound by 4 hours posttreatment. These data demonstrate that differing sensitivities to taselisib in cell viability as seen in PIK3CAWT and PIK3CAmut cells correlate with differences in PI3K signaling at the phospho-protein level.

**Taselisib induces apoptosis and HER2 feedback in PIK3CAWT/HER2mut cell lines**

In HER2+ disease, PIK3CA mutation prevalence is reported at approximately 20%–32% (25–27). As HER2 amplification has been reported to activate the PI3K pathway (22), we investigated the preclinical effects of taselisib in HER2+ cell lines with and without concurrent activating PIK3CA mutations. When assessing cell viability, we found that HER2+/PIK3CAmut cells displayed a comparable mean IC50 to taselisib as observed in HER2 nonamplified (HER2WT)/PIK3CAmut cells (Fig. 2A). When compared with HER2WT/PIK3CAWT cells, HER2+/PIK3CAmut cells showed a significant increase in taselisib sensitivity with a 43-fold decrease in mean IC50. Interestingly, HER2+/PIK3CAWT cell lines had a lower mean IC50 compared with HER2WT/PIK3CAWT cell lines, but this did not reach statistical significance. Notably, the HER2+/PIK3CAWT cell lines had a wider range of taselisib sensitivity, some of which were as sensitive as the PIK3CAmut cell lines regardless of HER2 status.

Pharmacodynamic immunoblot analysis showed that HER2+/PIK3CAWT (AU565 and HCC1419) and HER2+/PIK3CAmut (KPL-4 and MDA-MB-361) cell lines initially responded to taselisib similarly with decreased pAKT at 1 hour following treatment (Fig. 2B; Supplementary Fig. S2A). Taselisib suppressed pAKT out to 48 hours in HER2+/PIK3CAmut cells comparable with HER2WT/PIK3CAWT cells, whereas pAKT rebounded in the HER2+/PIK3CAWT cells 24 hours posttreatment (Figs. 1D and 2B; Supplementary Fig. S2A). While both HER2+/PIK3CAWT cell lines responded to taselisib treatment with a complete loss of pS6 between 4 and 24 hours, little to no suppression was observed in the HER2+/PIK3CAWT cell lines.

As RTKs have been described to be upregulated following PI3K-AKT-mTOR inhibition (28, 29), we assessed feedback in HER2+ cell lines utilizing a phospho-RTK array to detect changes in the phosphorylation of 49 different RTKs. We observed that phosphorylated HER2 (pHER2), along with phosphorylated HER3 and phosphorylated EGFR, were induced following taselisib treatment in both the HER2+/PIK3CAWT AU565 and the HER2+/PIK3CAmut KPL-4 cells (Fig. 2C). Immunoblot analysis of a larger subset of HER2+ cell lines confirmed a consistent pattern of phospho-HER2 upregulation following taselisib treatment in HER2+/PIK3CAmut cells, but this was not seen in HER2+/PIK3CAWT cells (Fig. 2D). P38 pathway suppression and induction of apoptosis, as assessed by cleaved PARP and cleaved caspase 3, was induced by inhibition with the dual EGFR and HER2 inhibitor lapatinib in the HER2+/PIK3CAWT cell lines, whereas these effects were primarily induced by PI3K inhibition in the HER2+/PIK3CAmut cell lines (Fig. 2D; Supplementary Fig. S2B). Finally, treating HER2+ cells with a combination of taselisib and lapatinib further suppressed downstream phospho-substrates and induced a higher degree of apoptosis regardless of PIK3CA mutation status in the majority of preclinical models tested, which was not associated with EGFR expression (Supplementary Fig. S2C). Similar results were observed for pAKT and cleaved PARP with the HER2-targeted antibody trastuzumab (Supplementary Fig. S2B and S2D).

**PTEN loss partially compensates for the effects of taselisib treatment in PIK3CAmut cell lines**

Loss of the tumor suppressor PTEN, a phosphatase that dephosphorylates PIP3 to generate PIP2, has been shown to be an acquired resistance mechanism to the p110-alpha–specific inhibitor alpelisib (30). We therefore investigated whether PTEN loss would have an effect on sensitivity to taselisib. In cell viability assays, we found that the cooccurrence of PTEN loss and a PIK3CA mutation (PTENmut/PIK3CAmut) did not result in a significant shift in sensitivity to taselisib when compared with PIK3CAmut cell lines at 3 days following treatment (Fig. 3A). However, when assayed at 6 days, PTENmut/PIK3CAmut cells had a mean taselisib IC50 that was greater than that of PIK3CAmut cells (Fig. 3B; Supplementary Fig. S3A).

Pharmacodynamic immunoblot analysis showed that taselisib potently inhibited PI3K downstream substrates in the PIK3CAmut cell lines (EFM-19 and T47D), but this effect was much reduced in the PTENmut/PIK3CAmut cell lines (CAL-148 and CAL-51) after 4 hours of taselisib treatment (Fig. 3C). In addition, we observed that phospho-signals rebounded over time in the PTENmut/PIK3CAmut cells when compared with the PIK3CAmut cells (Fig. 1D; Supplementary Fig. S3B). As the assayed cell lines harbor different genomic backgrounds that may influence taselisib response beyond the effects of PTEN or PIK3CA status, we next assessed the role of PTEN loss in PIK3CAmut cell lines using an RNA interference approach. Transient knockdown of PTEN was achieved at ≥85% using two independent siRNAs and was maintained at 8 days post transfection at both the mRNA and protein levels in PIK3CAmut T47D cell lines (Supplementary Fig. S3C and S3D). PTEN knockdown in both the PIK3CAmut T47D and EFM-19 cell lines increased cell proliferation and induced pAKT signaling in the absence of any treatment (Fig. 3D and E; Supplementary Fig. S3E). Upon drug treatment, PTEN knockdown induced a modest increase in taselisib IC50 in both cell lines (Fig. 3D; Supplementary Fig. S3E), which was reversed with the combination of taselisib with the p110-beta–specific inhibitor AZD-6482 (Supplementary Fig. S3F). This reversal is consistent with previous studies reporting that in the absence of PTEN, cancer cells propagate PI3K signaling primarily through the activity of p110-beta (15, 30, 31). Loss of PTEN prevented the full suppression of pAKT, but not pS6, in cells treated with taselisib, an effect that was more pronounced in the T47D cell line (Fig. 3E).

**Taselisib induces ER expression and transcriptional activity in PIK3CAmut cell lines**

Evidence of cross-talk has been established between the ER and PI3K pathways in preclinical models (32). As treatment with alpelisib has been shown to promote ER transcriptional activity in PIK3CAmut cells (33), we next investigated the effects of taselisib in ER+ PIK3CAmut and PIK3CAWT cell lines. We found that taselisib significantly induced expression of the ER-alpha gene (ESR1) and promoted ER transcriptional activity, as assessed by expression of key ER-regulated genes, progesterone receptor (PGR), growth regulation by estrogen in breast cancer 1 (GREB1), and insulin-like growth factor-binding protein 4 (IGFBP4), in a time-dependent manner in the PIK3CAmut MCF-7 and T47D cell lines, but not in the PIK3CAWT HCC1500 and HCC1428 cell lines (Fig. 4A; Supplementary Fig. S4). As alpelisib-induced ER activity was previously shown to be enhanced by the...
presence of ligand (33), we addressed whether taselisib-induced ER activity also required the presence of beta-estradiol (E2). In MCF-7 and T47D cells, deprived of steroidal hormones using charcoal-stripped serum, taselisib treatment did not induce ER target genes (Supplementary Fig. S5A). However, the combination of taselisib and E2 promoted ER transcriptional activity in both cell lines to a greater degree than with E2 treatment alone. As PI3K inhibitors are often clinically developed in combination with antiendocrine therapies in hormone receptor–positive breast cancers (13, 14, 34–36), we next assessed the ability of taselisib to induce ER transcriptional activity in the presence of the selective ER degrader (SERD) fulvestrant. Cotreatment of taselisib with fulvestrant resulted in the inhibition of taselisib-induced ER transcriptional activity in both MCF-7 and T47D cells, with ER target gene expression suppressed to levels observed with fulvestrant alone (Fig. 4B; Supplementary Fig. S5B).

**Biomarkers of Response to Taselisib in Breast Cancer Models**

**Figure 2.**

In vitro effects of taselisib in HER2+ cell lines in relation to PIK3CA mutation status. A, Relationship between absolute taselisib IC50 values, HER2 status, and PIK3CA mutation status \( [n = 30 \text{ (WT)}, n = 6 \text{ (HER2+)}], n = 6 \text{ (HER2+/PIK3CAwt)} \) and \( n = 6 \text{ (HER2+/PIK3CAmut)} \) in a panel of 50 breast cancer cell lines. IC50 values from a 3-day cell viability assay were determined by calculating the mean from at least three independent experiments with quadruplicate technical replicates each; error bars denote SEM. Welch t test used for statistical analysis. B, Immunoblots of the downstream PI3K signaling response (pAKT and pS6) in HER2+/PIK3CAwt (AU565) and HER2+/PIK3CAmut (KPL-4) cells treated with 200 nmol/L taselisib for indicated time points. β-Actin was used as a protein loading control. C, Differences in RTK phosphorylation were assessed using a human phospho-RTK array in HER2+/PIK3CAwt (AU565) and HER2+/PIK3CAmut (KPL-4) cells treated with 200 nmol/L taselisib, 500 nmol/L lapatinib, or the combination for 48 hours. D, Immunoblots of the downstream PI3K signaling response and apoptotic marker expression (cleaved PARP and cleaved caspase 3) in HER2+/PIK3CAwt (AU565, HCC2218, and UACC812) and HER2+/PIK3CAmut (HCC1954, KPL-4, and MDA-MB361) cells treated with 200 nmol/L taselisib, 500 nmol/L lapatinib, or the combination for 48 hours. β-Actin was used as a protein loading control.

**Taselisib in combination with fulvestrant suppresses ER transcriptional activity in ESR1-mutant cell lines**

Activating mutations within the ligand-binding domain of ESR1, such as Y537S and D538G, have been described to arise in the metastatic setting in ER+ breast cancer and promote resistance to certain endocrine therapies (4, 12, 37–40). We therefore investigated whether PIK3CA mutations cooccurred with ESR1 mutations within the same tumor samples. Using the Foundation Medicine cancer genomic database, we identified 338 unique tumor samples from confirmed patients with ER+ metastatic breast cancer. Of the 338 metastatic tumors, 138 (41%) harbored a PIK3CA hotspot mutation(s) and 89 (26%) harbored an ESR1 hotspot mutation(s) (Fig. 5A; Supplementary Fig. S6A and S6B). Of the 138 PIK3CA-mutant tumors, 33 (24%) were found to also contain an ESR1 mutation, representing 10% of...
Using PIK3CA mutant MCF-7 cells with doxycycline-inducible WT (ER WT) or mutant ER-alpha (ERY537S and ERD538G), we examined whether ESR1 mutations could promote resistance to taselisib. Upon doxycycline treatment, upregulation of ER-alpha was observed at 4 hours and was maximal between 24 and 48 hours (Supplementary Fig. S7A). We observed no differences in sensitivity between MCF-7 parental, ERWT, ERY537S, and ERD538G cell lines when exposed to increasing concentrations of taselisib in cell viability assays (Supplementary Fig. S7B). Importantly, combining taselisib with fulvestrant was able to reverse taselisib-induced ER transcriptional activity in both parental and non-doxycycline-induced cells (Fig. 5D; Supplementary Fig. S7C).

Figure 3.

**In vitro effects of PTEN loss on taselisib sensitivity in PIK3CA mutant cancer cells.**

A, Relationship between absolute taselisib IC50 values, PTEN protein status, and PIK3CA mutation status [n = 22 (WT), n = 14 (PTENmut), n = 11 (PIK3CAmut), and n = 3 (PTENmut/PIK3CAmut)] in 50 breast cancer cell lines. IC50 values from a 3-day cell viability assay were determined by calculating the mean from at least three independent experiments with quadruplicate technical replicates each; error bars denote SEM. Welch t-test used for statistical analysis. B, Four-parameter dose-response survival curves in WT (CAL-120 and MDA-MB-231), PTENmut (BT-549 and MDA-MB-468), PIK3CAmut (EFM-19 and T47D), and PTENmut/PIK3CAmut (CAL-148 and MDA-MB-231) cell lines exposed to taselisib for 6 days. Curves are representative of data from at least three independent experiments with quadruplicate technical replicates each; error bars denote SD. C, Immunoblots of the downstream PI3K signaling response (pAKT and pS6) and PTEN expression in WT (CAL-120 and MDA-MB-231), PTENmut (BT-549 and MDA-MB-468), PIK3CAmut (EFM-19 and T47D), and PTENmut/PIK3CAmut (CAL-148 and MDA-MB-231) cell lines treated with 200 nmol/L taselisib for 4 hours. β-Actin was used as a protein loading control. D, Four-parameter dose-response survival curves in PIK3CAmut EFM-19 cells transfected with control or PTEN-targeted siRNA for 48 hours and then exposed to taselisib for 6 days. Curves are representative of data from three independent experiments with quadruplicate technical replicates each; error bars denote SD. E, Immunoblots of the downstream PI3K signaling response and PTEN knockdown in PIK3CAmut (EFM-19 and T47D) cells transfected with control or PTEN-targeted siRNA for 48 hours and then treated with 100 nmol/L taselisib for 24 hours. β-Actin was used as a protein loading control.

the total population (Fig. 5B). Using PIK3CA mutant MCF-7 cells with doxycycline-inducible WT (ERWT) or mutant ER-alpha (ERY537S and ERD538G), we examined whether ESR1 mutations could promote resistance to taselisib. Upon doxycycline treatment, upregulation of ER-alpha was observed at 4 hours and was maximal between 24 and 48 hours (Supplementary Fig. S7A). We observed no differences in sensitivity between MCF-7 parental, ERWT, ERY537S or ERD538G cell lines when exposed to increasing concentrations of taselisib in cell viability assays (Fig. 5C; Supplementary Fig. S7B). As the clinical development of PI3K inhibitors in the metastatic setting often occurs in combination with fulvestrant after patients have progressed on an aromatase inhibitor containing regimen (13, 14), we tested whether taselisib in combination with fulvestrant could suppress ER transcriptional activity in an ESR1-mutant setting. We found that taselisib treatment alone induced ER transcriptional activity in all of the doxycycline-induced MCF-7 ER WT, ERY537S, and ERD538G models at levels similar to those observed in both parental and non-doxycycline-induced cells (Fig. 5D; Supplementary Fig. S7C). Importantly, combining taselisib with fulvestrant was able to reverse taselisib-induced ER transcriptional activity.
transcriptional activity in the MCF-7 ERWT, ER^T537S, and ER^D538G cells to levels seen with fulvestrant treatment alone.

**Discussion**

Clinical development of kinase inhibitors, particularly those that target normal physiologic processes, can be challenging. The need to accurately identify the patient subsets most likely to derive optimal clinical benefit is critical to the success of these inhibitors. Despite the disappointing clinical results of pan-PI3K inhibitors over the past several years, important findings were made that resulted in the generation of isoform-selective PI3K inhibitors (13, 14). Two of the next-generation PI3K inhibitors, the p110-alpha-specific inhibitor alpelisib (BYL-719) and the beta-sparing inhibitor taselisib (GDC-0032), have shown single-agent activity in advanced stage patients with PIK3CA-mutant tumors, and with a seemingly much higher frequency than that observed with their predecessors (41, 42). These promising clinical results suggest that the next-generation PI3K inhibitors may have a more favorable safety profile that allows for optimal dosing. Recently, results from two phase III clinical trials were announced...
showing that the addition of either taselisib (SANDPIPER) or alpelisib (SOLAR-1) to fulvestrant increased progression-free survival in patients with ER⁺/HER2⁻ metastatic breast cancers harboring PIK3CA mutations (34, 35), a treatment effect that was more pronounced in SOLAR-1. These results suggest that further development of treatments targeting the PI3K signaling pathway is warranted and that in-depth understanding of biomarkers predicting activity is needed.

Using an unbiased cell line-screening approach, we found that sensitivity to taselisib highly correlated with PIK3CA mutation status in breast cancer cell lines. This finding corroborated previous reports that sensitivity to taselisib correlates with PIK3CA activation in head and neck squamous carcinoma and uterine serous carcinoma preclinical models (43, 44). In our study, the difference in mean IC₅₀ of taselisib following 3 days of treatment in PIK3CA mut cell lines is much more pronounced than that reported for the pan-PI3K inhibitor pictilisib (45), indicating that PIK3CA mutations may serve as a predictive biomarker for taselisib. Similarly, Morgillo and colleagues showed a synergistic combination of taselisib with antimicrotubule inhibitors in PIK3CA mut, but not wild-type cell lines (46). Of note, clinical responses to taselisib as a single agent have been seen primarily in patients with tumors harboring activating PIK3CA mutations (41).

It is interesting that the HER2⁺/PIK3CA mut and HER2⁺/PIK3CA WT cell lines showed marked differences in sensitivity to taselisib, as HER2 signaling can activate the PI3K pathway. We observed that within the HER2⁺/PIK3CA mut cell lines PIK3CA

Figure 5.
Effects of ER1 mutations on taselisib sensitivity in PIK3CA mut breast cancer. A, PIK3CA and ESRI mutation analysis in 338 patients with ER⁺ metastatic breast cancer as detected in tissue by the FoundationOne comprehensive genomic profile test. Mutations are colored according to exon (PIK3CA) or amino acid (ESR1); gray indicates no mutation detected. ER status determined from staining as disclosed in patient pathology report; metastatic status based on site of biopsy. Qualifying PIK3CA mutations limited to missense mutations at R88, N345, C420, E542, E545, Q546, M1043, H1047, and G1049; qualifying ESRI mutations limited to missense mutations at E380, S463, L469, V534, P535, L536, Y537, and D538, and the indel V422del. B, PIK3CA and ESRI cooccurrence in the patient population described in A. Numbers indicate count of patients with mutation(s) detected and percentages are relative to total population of patients queried (N = 338). C, Four-parameter dose–response survival curves in MCF-7 parental and doxycycline-inducible ER-alpha (ERWT, ERV537S, and ERD538G) overexpressing cells exposed to taselisib for 3 days. Doxycycline-inducible cells were treated with 50 ng/mL doxycycline for 48 hours prior to the addition of taselisib, and doxycycline treatment was maintained during taselisib exposure. Curves are representative of data from three independent experiments with quadruplicate technical replicates each; error bars denote SD. D, Relative PGR, GREB1, and IGFBP4 mRNA levels as detected by qPCR in MCF-7 parental and doxycycline-inducible ER-alpha–overexpressing cells treated with 200 nmol/L taselisib, 100 nmol/L fulvestrant, or the combination for 24 hours. Doxycycline-inducible cells were treated with 50 ng/mL doxycycline for 48 hours prior to the addition of drug(s), and doxycycline treatment was maintained during drug exposure(s). Data presented are relative to ACTB mRNA levels and DMSO treatment. Error bars denote SD of three independent experiments with duplicate technical replicates each. Welch t test used for statistical analysis (*, P < 0.001).
functioned as the main driver of cell survival, as inhibiting HER2 kinase activity with lapatinib failed to induce apoptosis that was seen with taselisib. However, in the HER2+/PIK3CAmut cell lines, HER2 appears to be the main driver of survival, as the EGF-HER2 inhibitor, lapatinib, induced apoptosis while taselisib did not. In addition, we observed a time-dependent increase in pHER2 following taselisib treatment in the HER2+/PIK3CAmut cell lines, reminiscent of the induced expression and phosphorylation of multiple RTKs in response to AKT inhibition as previously reported by Chandarlapaty and colleagues (28). Another study by Serra and colleagues demonstrated that PI3K-mTOR inhibition resulted in increased HER2 expression and phosphorylation, as well as induced ERK signaling (29). These findings suggest that PI3K inhibition in a HER2+/PIK3CAmut background may result in cells actively attempting to upregulate the PI3K pathway through increased pHER2 and pHER3, thus identifying a pathway reactivation mechanism. Interestingly, PIK3CA mutations have been associated with worse outcomes in patients with early and metastatic HER2þ breast cancer (25, 27). As such, when contemplating clinical development of next-generation PI3K inhibitors in HER2þ breast cancer, coadministration of HER2-targeted therapies with PI3K inhibitors is warranted to suppress HER2 kinase activity. Loss of the tumor suppressor PTEN has been described as a clinical resistance mechanism to alpelisib (30). As reported by Juric and colleagues, genomic alterations that resulted in the loss of PTEN protein expression were identified in the metastatic lesions obtained from a patient with PIK3CA-mutant breast cancer who initially responded, but then progressed on alpelisib. In this study, we demonstrated that taselisib transiently downregulated the PI3K pathway in PTENlow/PIK3CAmut cells, but pathway rebound was observed by 24 hours posttreatment. These findings suggest that loss of PTEN may partially compensate for taselisib-induced p110alpha suppression through a dependency on p110-beta signaling (15, 30, 31), which may explain the differences in sensitivity observed between 3 and 6 days of treatment. Further supporting this hypothesis, the addition of a PI3K-beta inhibitor in PTENmut/PIK3CAmut cells reverted the PTENmut-induced resistance observed with taselisib treatment alone. Studies of tumors from patients that respond and subsequently progress on taselisib are justified to determine whether PTEN alterations are observed posttreatment, as has been described for alpelisib.

Long term estrogen deprivation in ERþ cell lines has been shown to result in PI3K pathway upregulation in several breast cancer models, a phenotype that was reversed with the addition of an mTOR inhibitor (47). This discovery provided the first evidence that cotargeting the PI3K–mTOR and ER pathways may be an efficacious treatment option in patients with ERþ metastatic breast cancer who have progressed on antiendocrine therapies. Furthermore, Bosch and colleagues showed that alpelisib induced ER expression and activity in PIK3CAmut breast cancer cell lines, which was reversed by combination with fulvestrant (33). We demonstrate in our study that taselisib induced ER activity in PIK3CAmut but not PIK3CAWT cell lines, providing additional evidence that the next-generation PI3K inhibitors are likely to be most active in PIK3CAmut breast cancer. This is in contrast to mTOR inhibitors, which inhibit the PI3K pathway further downstream of the PI3K node, and upregulate ER activity in both PIK3CAmut and PIK3CAWT cell lines (32). Clinically, the mTOR inhibitor, everolimus, is approved for use in combination with exemestane in metastatic ERþ breast cancers regardless of PIK3CA mutation status (36).

ESR1 mutations have been described to evolve over the course of aromatase inhibitor therapies, with a prevalence ranging up to 39% in heavily pretreated ERþ patients with metastatic breast cancer (4, 12, 37–40). Moreover, ESR1 mutations in the ligand-binding domain have been shown to promote resistance to aromatase inhibitors, but to lesser degrees to SERDs such as fulvestrant (12, 37–39, 48). In this study, we found that PIK3CA mutations cooccurred with ESR1 mutations at a similar prevalence as the PIK3CAWT tumors in our cohort of metastatic ERþ breast cancer tissues. Our preclinical data suggest that taselisib, as either a single agent or in combination with fulvestrant, would have similar efficacy in PIK3CAmut/ESR1mut tumors as in PIK3CAWT/ESR1WT tumors suggesting that no selection for ESR1WT patients is needed in trials of PI3K inhibitors. However, further evidence from the analysis of samples from SANDPIPER or SOLAR-1 is required to test this hypothesis.

In recent years, cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors, such as palbociclib, have dramatically changed the treatment landscape of patients with metastatic ERþ breast cancer irrespective of PI3KCA mutation status (49). Interestingly, O’Leary and colleagues recently reported that PIK3CA mutations were significantly enriched during the course of fulvestrant with or without palbociclib, as assessed by circulating tumor DNA (ctDNA) analysis (49). These data suggest that PIK3CA mutations may emerge as a resistance mechanism to CDK4/6 inhibitors presenting two implications. First, a higher prevalence of patients may have a PIK3CA mutation following CDK4/6-targeted therapies and that PIK3CA mutation status should be assessed during treatment using ctDNA-based approaches rather than testing primary tissue. Second, combination of PI3K inhibitors with CDK4/6-targeted therapies may be a viable option to prevent the outgrowth of ESR1mut subclones. The reciprocal has also been observed by Vora and colleagues, who showed that PI3K-resistant PIK3CAmut breast cancer cell lines could be sensitized to PI3K inhibitors by cotreatment with a CDK4/6 inhibitor (50). Further data supporting the combination of next-generation PI3K inhibitors with CDK4/6-targeted therapies, especially in patients who have progressed on CDK4/6-targeted therapies, are warranted.

In summary, using an unbiased cell line–screening approach, we show that sensitivity to taselisib strongly correlates with the predictive biomarker of activating PIK3CA mutations in preclinical breast cancer models. We show that taselisib pharmacodynamically modulates the PI3K signaling pathway through analyses of downstream phospho-substrates in PIK3CAmut but not PIK3CAWT cells. We finally show how key cooccurring biomarkers, such as PTEN and HER2 expression and ESR1 mutations, may alter the ability of taselisib to modulate cell proliferation and signal transduction pathways in the different genomic backgrounds most relevant in breast cancer, each of which may have clinical implications.

Disclosure of Potential Conflicts of Interest

H.M. Moore is a scientific manager (paid consultant) at Genentech and has ownership interest (including patents) in Roche. H.M. Savage is a principal scientific researcher (paid consultant) at Genentech. E.S. Sokol is an employee (scientist; paid consultant) at, and has ownership interest (including patents) in, Foundation Medicine. M.E. Goldberg is a clinical data analyst (paid consultant) at and has ownership interest (including patents) in, Foundation Medicine. M. Leary is a principal scientist (paid consultant) at Genentech and has ownership interest (including patents) in Roche. M.R. Lackner is a director (paid consultant) at, and has ownership interest (including patents) in, Roche. T.R. Wilson is a principal scientist (paid consultant) at Genentech. Inc. and has ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.
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