GDC-0941, A Novel Class I Selective PI3K Inhibitor, Enhances the Efficacy of Docetaxel in Human Breast Cancer Models by Increasing Cell Death In Vitro and In Vivo

Jeffrey J. Wallin¹, Jane Guan¹, Wei Wei Prior¹, Leslie B. Lee¹, Leanne Berry¹, Lisa D. Belmont², Hartmut Koeppen³, Marcia Belvin¹, Lori S. Friedman¹ and Deepak Sampath¹*

¹Departments of Translational Oncology, ²Research Diagnostics and ³Pathology Genentech, Inc.

*Address correspondence to Deepak Sampath, Genentech Inc, 1 DNA Way, South San Francisco, CA. 94080. Phone: 650-225-7786; Fax: 650-225-5770; e-mail: dsampath@gene.com.

Disclosure of Potential Conflict of Interest: All authors are employees of Genentech, Inc.

Keywords: Breast Cancer, Docetaxel, PI3K

Running Title: GDC-0941 Enhances Docetaxel Anti-Tumor Activity
Statement of Translational Relevance

Docetaxel is commonly used as a front-line treatment option for breast cancer but patients ultimately relapse and succumb to disease progression. Therefore, combination therapies that enhance the anti-tumor activity of docetaxel may provide greater clinical benefit and outcomes. Given that phosphatidylinositol 3-kinase (PI3K) is frequently activated in breast cancer and induces chemoresistance, it is an attractive target for combination therapy with standard of care drugs such as docetaxel. We report that the combination of a class I selective PI3K inhibitor, GDC-0941, enhances anti-tumor activity of docetaxel in human breast cancer models in vitro and in vivo by rapid induction of pro-apoptotic mechanisms. Our data provides a preclinical rationale for evaluating GDC-0941 in combination with docetaxel for breast cancer treatment.
ABSTRACT

Purpose: Docetaxel (DTX) is a front-line standard of care chemotherapeutic drug for the treatment of breast cancer. Phosphatidylinositol 3-kinases (PI3K) are lipid kinases that regulate breast tumor cell growth, migration and survival. The current study was intended to determine if GDC-0941, an orally bioavailable class I selective PI3K inhibitor, enhances the anti-tumor activity of DTX in human breast cancer models in vitro and in vivo.

Experimental Design: A panel of 25 breast tumor cell lines representing HER2+, luminal and basal subtypes were treated with GDC-0941, DTX or the combination of both drugs and assayed for cellular viability, modulation of PI3K pathway markers and apoptosis induction. Drug combination effects on cellular viability were also assessed in non-transformed MCF10A human mammary epithelial cells. Human xenografts of breast cancer cell lines and patient-derived tumors were utilized to assess efficacy of GDC-0941 and DTX in vivo.

Results: Combination of GDC-0941 and DTX decreased the cellular viability of breast tumor cell lines in vitro but to variable degrees of drug synergy. Compared to non-transformed MCF10A cells, the addition of both drugs resulted in stronger synergistic effects in a sub-set of tumor cell lines that were not predicted by breast cancer subtype. In xenograft models, GDC-0941 enhanced the anti-tumor activity of DTX with maximum combination efficacy observed within 1 hour of administering both drugs. GDC-0941 increased the rate of apoptosis in cells arrested in mitosis upon co-treatment with DTX.
Conclusion: GDC-0941 augments the efficacy of DTX by increasing drug-induced apoptosis in breast cancer models.

INTRODUCTION

Breast cancer is the leading cause of non-smoking cancer related death in women and continues to be a major health concern globally (1). Although a number of genetic and environmental factors contribute to the development of mammary epithelial neoplasia and malignancy, tumorigenesis is initially controlled by estrogen-mediated induction of growth factors, transcription factors, cell cycle regulators and anti-apoptotic factors (2, 3). In addition, genetic alterations such as HER2 amplification can transform mammary epithelial cells in pre-clinical models and is clinically validated in a sub-set of breast cancer patients that overexpress HER2 and respond to biological therapeutics such as trastuzumab (4, 5).

Taxanes, such as paclitaxel (PTX) and docetaxel (DTX), inhibit microtubule function by altering their dynamic equilibrium and are used as standard of care treatment for breast cancer in combination with chemotherapeutics and targeted agents (6, 7). However, combination therapy with drugs that target additional growth factor signaling pathways may increase the therapeutic index and thereby provide greater clinical benefit (8-12). The phosphatidylinositol 3-kinase (PI3K) pathway plays an essential role in regulating tumor cell growth, migration, and survival upon growth-factor receptor or integrin activation (13). Activating and transforming mutations in the PIK3CA gene of the
p110α subunit are commonly found in HER2+ and ER+ breast tumors (14-17). In addition, genetic deletion or loss of function mutations within the tumor suppressor PTEN, a phosphatase with opposing function to PI3K, also results in aberrant activation of PI3K pathway signaling (18). As such, PI3K may be an ideal target for combination therapy in breast cancer.

PI3K inhibitors such as wortmannin and LY2942004 have been shown to increase efficacy of PTX and DTX in pre-clinical tumor models (19-21). However, their lack of selectivity confounds the ability to specifically define the contribution of PI3K inhibition to the anti-tumor effects observed when combined with taxanes (22). GDC-0941 is an orally bioavailable inhibitor of class I PI3K that is 100-fold more potent against class I compared to class II, III and IV family members and is in clinical development for solid tumor indications including breast cancer (23-26). Initial evaluation of the combination effects of GDC-0941 and DTX demonstrated increased anti-tumor activity in the HER2+ BT474M1 breast cancer model in vitro and in vivo (27). We aimed to expand upon these results by evaluating a broader panel of human tumor cell lines representing the major breast cancer molecular subtypes (HER2+, luminal and basal) in response to the combination of GDC-0941 and DTX. An additional goal was to ascertain the mechanism of action of the drug combination in sensitive breast tumor models.
MATERIALS AND METHODS

Materials. GDC-0941 was synthesized at Genentech, Inc as described before (23) and DTX was purchased from ChemShuttle (Wuxi City, China). Caspase inhibitor, Z-VAD-FMK, was purchased from Promega. Primary antibodies utilized: pAkt, Akt, pPRAS40, PRAS40, pS6, S6, pGSK3β, pP70S6K, pFOXO1, PTEN, Cyclin D1, pHistone H3, cleaved caspase 3, cleaved PARP (Cell Signaling Technology); anti-HER2 clone 4D5 (Genentech); β-actin (Sigma)

Cell Culture. Human breast tumor cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI or DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 2 mM L-glutamine, and 100 mg/ml streptomycin at 37°C under 5% CO2. MCF10A mammary epithelial cells were also obtained from ATCC and cultured in F12: DMEM (50:50) medium supplemented with 20 ng/ml human EGF and 10 µg/ml insulin. Human breast cancer MCF7-neo/HER2 cells were generated at Genentech by ectopically over-expressing HER2 in MCF7 cells. HER2 expression was confirmed by FACS analysis using anti-HER2 primary antibodies (clone 4D5). The level of HER2 expression in the MCF7-neo/HER2 cell line is comparable to breast cell lines with known HER2 amplification such as SK-BR-3 (Supplementary Fig. S1). However, based on gene expression profiling (including estrogen and progesterone receptor positivity) MCF7-neo/HER2 cells are phenotypically luminal.
Cell Viability Assay. All drug treatments were tested in quadruplicate during a 4-day incubation period and the relative number of viable cells was estimated using CellTiter-Glo (Promega). Total luminescence was measured on a Wallac Multilabel Reader (PerkinElmer). Cells were treated simultaneously with DTX (dose range = 0.0003-0.020 µM) or GDC-0941 (dose range = 0.083-5 µM) in an 8 X 10 matrix of concentrations chosen to encompass clinically relevant doses (24). The concentration of drug resulting in 50% maximal effective concentration (EC$_{50}$) was determined using Prism software (GraphPad). Combination synergy of GDC-0941 and DTX was determined by Bliss independence analyses (28). A Bliss expectation for a combined response (C) was calculated by the equation:

C = (A + B) – (A X B) where A and B are the fractional growth inhibitions of drug A and B at a given dose. The difference between the Bliss expectation and the observed growth inhibition of the combination of drugs A and B at the same dose is the “Delta.Bliss”. Delta.Bliss scores were summed across the dose matrix to generate a Bliss sum. Bliss sum = 0 indicates that the combination treatment is additive (as expected for independent pathway effects); Bliss sum > 0 indicates activity greater than additive (synergy); and Bliss sum < 0 indicates the combination is less than additive (antagonism). Statistical analysis comparing the Bliss sums for each cell line were performed by Student’s t-test.

Western Blotting. Cells were treated at EC$_{50}$ concentrations of GDC-0941, DTX or both for 4 or 24 hours and lysed in 1X Cell Extraction Buffer (Biosource) supplemented with protease inhibitors and Phosphatase Inhibitor Cocktails 1 and 2.
2 (Sigma). Protein concentrations were determined using the Pierce BCA Protein Assay Kit. For immunoblots, equal amounts of protein were separated by electrophoresis through NuPage Bis-Tris 10% gradient gels (Invitrogen), transferred onto PVDF membranes using the Criterion system and probed with mono-specific primary antibodies. Specific antigen-antibody interactions were detected with IRDye 680 or IRDye 800 infrared secondary antibodies using a Li-Cor imaging system.

**FACS Analysis.** For cell cycle analysis, cells were treated with EC$_{50}$ concentrations of GDC-0941 and/or DTX for 24 hours, fixed in 100% ice-cold ethanol and incubated in propidium iodide (PI) solution for 30 minutes and analyzed with a FACscan flow cytometer (Becton Dickinson). For cell death analyses, cells were incubated with GDC-0941, DTX or both drugs for 48 hours, stained with Annexin V-FITC (BD Biosciences) and propidium iodide (PI) solution (Sigma) according to the manufacturer's instructions and analyzed with a FACscan flow cytometer (Becton Dickinson).

**Time Lapse Microscopy Imaging.** Cells were seeded onto glass-bottom 24-well plates (Greiner Bio-one) and, 24 hours later, incubated with drug-containing media. Cells were treated with EC$_{50}$ concentrations of GDC-0941 and/or DTX, multiple fields per condition were selected, and fluorescent and phase-contrast images were recorded with a 10X objective every 15 minutes for 72 hours on an AxioObserver inverted microscope (Carl Zeiss), equipped with an environmental...
chamber (Okolab), MS2000 XY stage (Applied Scientific Instruments) and a CoolSnap CCD camera (Hewlett Packard). Mitotic events and cell death were scored as previously described (29). Cells were also co-treated with caspase inhibitor Z-VAD-FMK at a final concentration of 2 μM. Statistical analysis were performed by Student’s t-test.

**Cell Synchronization.** Cells were synchronized with a double thymidine block and released into drug-containing media. Adherent cells were washed twice with cold PBS and lysed in 1X Cell Extraction Buffer (Biosource) supplemented with protease inhibitors (Roche), 1 mmol/L PMSF, and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma). Protein concentration was determined using the Pierce BCA Protein Assay Kit.

**In Vivo Xenograft Models.** Female nu/nu mice were inoculated subcutaneously with MCF7-neo/HER2 or MX-1 breast cancer cells. When tumors reached a mean volume of 200 to 250 mm³, animals were size matched and distributed into groups consisting of 10 animals/group. DTX, formulated in 3% EtOH, 97% saline was administered intravenously once weekly. GDC-0941, formulated in MCT (0.5% methylcellulose, 0.2% Tween-80) was dosed orally and daily. MAXF1162 is a HER2+/ER+/PR+ patient-derived breast cancer tumor xenograft model established at Oncotest, Inc. by directly implanting tumors subcutaneously from patient to NMRI nu/nu mice. Tumor volume was calculated as follows: tumor size (mm³) = (longer measurement × shorter measurement²) × 0.5. Tumor sizes
were recorded twice weekly over the course of a study. Following data analysis, p-values were determined using Dunnett’s t-test.

For pharmacodynamic studies, tumor samples (n=4) were immediately frozen or fixed in 10% neutral buffered formalin. Tumors were dissociated in cell extraction buffer and lysates analyzed by Western blotting as described above. Immunohistochemistry was performed using 5 μm paraffin sections of formalin fixed tissue on a Ventana Benchmark XT instrument (VMSI) by deparaffinization, treatment with antigen retrieval buffer (VMSI) and incubation with anti-cleaved caspase 3 primary antibody (Cell Signaling Technology, Beverly MA) at 37°C. Bound antibody was detected using DABMap technology (VMSI) and sections were counterstained with hematoxylin.

RESULTS

Combination effects of GDC-0941 and DTX on human breast cancer cell viability in vitro. We previously observed that the combination of GDC-0941 and DTX increased anti-tumor activity in the HER2+ BT474M1 breast cancer model (27). Therefore, our aim was to expand upon these preliminary results by evaluating the combination effects of GDC-0941 and DTX in a panel of 25 human breast cancer cell lines representing the major breast cancer molecular subtypes (HER2+, luminal and basal) and were either PI3Kα wild-type, PI3Kα mutant (E545K or H1047R), or PTEN null (30).

Breast tumor lines were treated with DTX and GDC-0941 in an 8 x 10 dose matrix and the combination effects on cellular viability was evaluated using
the Bliss independence model as described in Methods. In comparison to single agent treatments the combination of GDC-0941 and DTX reduced tumor cell viability by 80% or greater in the breast cancer cell lines tested in vitro (Supplementary Table S1). A Bliss sum of 0 was determined in the MDA-MB-453 cell line indicating an additive combination effect while Bliss sums > 0 were calculated in the other tumor cell lines indicating a synergistic effect (Supplementary Table S1). However, the degree of synergy varied across the tumor lines tested as a range of Bliss sums from 1 to 451 were noted (Supplementary Table S1). For example, in the Hs578T1.2 line a Bliss sum of 73 indicates synergy between both drugs while stronger synergistic effects were observed in the MCF7-neo/HER2 and MX-1 models in which Bliss sums equaled 250 and 375, respectively, and illustrated as representative “heat maps” in Fig. 1A. A drug combination effect (Bliss sum=61) was also observed in the spontaneously immortalized MCF10A human mammary epithelial cell line (Fig. 1A). As a result of the latter, we focused our analysis on those breast tumor cell lines in which a stronger combination effect was observed relative to the non-transformed MCF10A cell line (i.e. Bliss sums > 61). A sub-set of breast tumor lines (11 out of 25) met the latter criteria (Supplementary Table S1). Within this sub-set, there was no significant difference in drug synergy across subtypes, PI3K<sub>α</sub> mutational or PTEN expression status (Fig. 1B and 1C).

**Modulation of PI3K pathway markers upon treatment with GDC-0941 and DTX in breast cancer cell lines.** To further analyze the combination effects of
GDC-0941 and DTX, we focused our attention on those tumor lines in which strong synergy was observed relative to the MCF10A line by evaluating their effects on modulation of the PI3K pathway biomarkers. As representative models we utilized Hs578T1.2 (PI3Kα wild-type), MCF7-neo/HER2 (PI3Kα mutant), and MX-1 (PTEN null). In all 3 models, GDC-0941 inhibited Akt phosphorylation and downstream targets of Akt signaling such as pPRAS40 and pS6 within 4 hours of treatment and this effect was sustained for 24 hours (Fig. 2). The phosphorylation of Akt substrates FOXO1 and GSK3β as well as phosphorylation of the downstream target of Akt-TORC1 signaling, p70S6K, were also inhibited through 24 hours after GDC-0941 treatment (Fig. 2). Treatment with DTX alone for 24 hours did not alter the PI3K pathway markers evaluated but an increase in phospho-histone H3 was detected confirming that the drug was pharmacologically active (Fig. 2).

**GDC-0941 decreases the time of DTX-induced mitotic arrest prior to apoptosis.** Given that DTX induces cell death in sensitive tumor cell lines (31), we determined if the increase in tumor growth inhibition observed in combination with GDC-0941 was due to increased apoptosis. Within 48 hours of combination treatment with GDC-0941 and DTX there was an increase in the percentage of apoptotic cells compared to cells treated with either drug alone (Table 1). The fold increases in the percentage of apoptotic cells after combination treatment was similar for Hs578T1.2, MCF7-neo/HER2, and MX-1 breast tumor cells (Table 1).
To investigate the mechanism of apoptosis that was induced by combination of GDC-0941 and DTX we performed time-lapse microscopy in the HCC-1954 (Bliss sum=212) and MCF7-neo/HER2 tumor lines to determine the timing and extent of cell cycle arrest and appearance of apoptotic markers following drug treatment. We detected apoptotic cells (based on fragmented morphology) with GDC-0941 treatment in HCC-1954 breast tumor cells (Supplementary Fig. S2). However, the majority of cells treated with DTX or the combination of GDC-0941 and DTX (82% and 100%, respectively) became fragmented after a prolonged mitotic arrest (Supplementary Fig. S2 and Fig. 3A). Interestingly, the time in mitotic arrest prior to fragmentation was significantly reduced when DTX was combined with GDC-0941 (p < 0.001). In addition, similar results were obtained in the MCF7-neo/HER2 cell line, albeit to a lesser extent, thereby confirming acceleration of cell death induced by the combination of GDC-0941 and DTX (Fig. 3A). The pro-apoptotic effect of combination drug treatment was caspase dependent since co-treatment with a caspase inhibitor, Z-VAD-FMK, reduced the number of fragmented cells and increased the duration of mitotic arrest (Fig. 3A).

Modulation of apoptosis pathway markers by the drug combinations during mitosis was assessed in HCC-1954 cells 12 hours after synchronization release which allowed the cells to progress through S phase prior to any treatments. Cells were synchronized by double-thymidine block and after release from synchronization, the cells advanced through the cell cycle and reached mitosis within approximately 24 hours based on increased levels of
phospho-histone H3 and Cyclin B1 (Supplementary Fig. S3). Consistent with our observations made with time-lapsed microscopy, we detected increased cleaved PARP to a greater extent in cells treated with the combination of GDC-0941 and DTX 24 hours after synchronization release (Fig. 3B). An increase in pBcl-xL$^{\text{Ser62}}$ after DTX treatment was also observed but Mcl-1 protein levels were not affected by either drug treatment (Fig. 3B). The levels of pAkt$^{\text{Ser473}}$ and pBad$^{\text{Ser136}}$ were decreased after GDC-0941 treatment confirming inhibition of PI3K pathway signaling and pro-survival activity (Fig. 3B). Therefore, selective modulation of Bcl-2 family proteins such as Bcl-xL and Bad by DTX and GDC-0941, respectively, may combine to drive increased cell death during mitotic arrest.

**Combination of GDC-0941 and DTX leads to enhanced anti-tumor efficacy and apoptosis in vivo.** To confirm our *in vitro* observations that the combination of GDC-0941 with DTX led to increased tumor growth inhibition we evaluated tumor xenograft models that were either PI3Kα mutant (MCF7-neo/HER2), PTEN null (MX-1) or PI3Kα wild-type (MAXF1162). Treatment of animals bearing MCF7-neo/HER2 breast cancer xenografts with 7.5 mg/kg DTX or 150 mg/kg GDC-0941 led to tumor growth delay and tumor stasis, respectively (Fig. 4A). In contrast, the combination of GDC-0941 and DTX resulted in tumor regressions during the treatment period leading to enhanced anti-tumor responses (Fig. 4A). Single agent and combination treatments were at maximum tolerated doses based on minimal changes in animal body weights (Fig. 4B). Similar to the MCF7-neo/HER2 xenograft model, we observed greater than additive effects
when GDC-0941 was administered in combination with DTX in the MX-1 xenograft model resulting in increased tumor regressions during the treatment period (Fig. 4C). Since the Hs578T1.2 tumor cell line is non-tumorigenic in vivo, we evaluated the MAXF1162 patient-derived breast tumor xenograft model, which is HER2+/ER+/PR+, PI3Kα wild-type and PTEN positive (Oncotest Inc; personal communication). Treatment of MAXF1162 primary breast tumor xenografts with 15 mg/kg DTX in vivo as a single agent resulted in tumor growth delay (Fig. 4D). However, the combination of 100 mg/kg GDC-0941 and DTX resulted in tumor stasis during the treatment period that was sustained after dosing ended (Fig. 4D). GDC-0941 and DTX were administered at maximum tolerated doses was and no additional change in animal body weights were noted when both drugs were combined (data not shown).

To confirm that the mechanism of action was primarily due to induction of apoptosis, we evaluated the pharmacodynamic activity of both drugs as single agents and in combination. Treatment with 150 mg/kg GDC-0941 alone resulted in decreased levels of pAkt, pPRAS40 and pS6 within 1 hour of administration demonstrating that the drug was pharmacologically active (Fig. 4E). Treatment with 7.5 mg/kg DTX had no effect on PI3K pathway suppression but increased phospho-histone H3 levels were observed with combination treatment indicative of G2/M cell-cycle arrest (Fig. 4E). Compared to each agent alone, a concomitant increase in apoptotic markers cleaved PARP and cleaved caspase 3 was observed in mice treated with GDC-0941 and DTX (Fig. 4E and 4F).
Dosing schedule of GDC-0941 in combination with DTX in vivo. Given that PI3K activity has been described to be necessary for progression through G₁, S and G₂ phases of the cell cycle (32, 33), we determined if the combination effects of GDC-0941 and DTX were dependent on the order of drug treatment. Increased apoptosis was detected when DTX was dosed 1 to 4 hours prior to GDC-0941 compared to DTX or GDC-0941 alone (Fig. 5A). Similarly, when DTX was dosed 4 hours prior to GDC-0941, an increase in the sub-G₁ cell population occurred indicative of increased cell death (Supplementary Fig. S4). However, increased apoptosis compared to DTX alone was not observed when GDC-0941 was dosed 4 hours before DTX (Fig. 5A).

We next investigated whether the drug combination efficacy observed in vivo was dependent on the order of administration. Maximum combination efficacy was observed in the MCF7-neo/HER2 model when GDC-0941 was administered 1 hour before or after DTX (Fig. 5B). Interestingly, maximum combination efficacy was also observed when GDC-0941 was dosed 4 hours after DTX administration but was abolished when GDC-0941 was dosed 4 hours before or 24 hours after DTX (Fig. 5C and 5D). We have previously reported that GDC-0941 has a half-life in immunocompromised mice of approximately 2.5 hours (34). In addition, DTX reaches peak plasma concentrations within 1 hour of dosing, which is within the window of time that maximum GDC-0941 drug concentrations are achieved (Supplementary Fig. S5A and B). Therefore, given the short half-lives of both DTX and GDC-0941 in vivo, maximum combination
efficacy in the MCF7-neo/HER2 xenograft model is achieved within 1 hour of
dosing both drugs.

DISCUSSION

DTX is frequently used as front-line standard of care therapy for breast
cancer and has proven to be effective but patients often become resistant to
treatment or are initially refractory (7). As a result, there remains an unmet
medical need to identify novel therapeutic agents in order to provide greater
clinical benefit. The PI3K pathway is frequently activated in breast cancer either
as a result of growth factor receptor activation, HER2 amplification, gain of
function mutations in PI3K\(\alpha\) or loss of PTEN (13-18). In addition, activation of
the PI3K pathway has been shown to confer resistance to targeted agents,
conventional chemotherapeutics, including taxanes, and radiation therapy (8, 35-
38). Thus, targeting PI3K in combination with DTX may provide greater
therapeutic benefit in the treatment of breast cancer. In support of the latter
hypothesis, inhibition of PI3K with non-selective PI3K inhibitors such as
LY294002 or wortmannin in combination with PTX enhanced anti-tumor activity
and increased PTX-induced apoptosis in preclinical ovarian cancer models (19,
20). Additionally, treatment of oral squamous carcinoma cells with wortmannin or
LY294002 enhanced DTX-induced apoptosis by regulating the expression and
post-translational modification of both pro-apoptotic (Bax) and anti-apoptotic (Bcl-
2, cIAP-1 and XIAP) factors, respectively (21). However, given that wortmannin
and LY294002 are known to have off-target activities the specific role of PI3K inhibition in taxane potentiation is not well defined.

The discovery of potent and selective inhibitors confers an opportunity to pharmacologically dissect the role of PI3K itself in combination with chemotherapeutics. In comparison to single agent treatments, the combination of a selective class I PI3K inhibitor, GDC-0941, and DTX decreased cellular viability in a panel of breast tumor cell lines \textit{in vitro} but to variable degrees of drug synergy. In addition, diminished cellular viability was also observed in the spontaneously immortalized non-transformed MCF10A human mammary epithelial cells when treated with the combination of both drugs. The latter may be due to a dependence on PI3K given the cellular growth requirement for insulin and EGF \textit{in vitro} (39). When benchmarked to the MCF10A cell line, stronger synergy was observed in a sub-set of tumor cell lines (11 out of 25) that was not predicted by breast cancer subtype, PTEN expression or PI3K$\alpha$ mutational (E545K or H1047R) status. The variability in drug synergy suggests that differential biological responses to treatment may be a reflection of the genetic heterogeneity in each model. In addition, the sensitivity of PI3K$\alpha$ wild-type and PTEN positive breast tumor lines to the drug combinations may be due to other mechanisms of PI3K pathway activation such as those driven by Akt1 mutations, RAS activation or estrogen receptor down-modulation (40, 41, 42, 43). The heterogeneity in drug synergy suggests that identification of additional biomarkers of PI3K pathway activation may improve the predictability of responses to GDC-0941 in combination with DTX.
The robust drug combination effects observed in a sub-set of breast tumor lines *in vitro* translated *in vivo* in human xenograft models. For example, we observed increased tumor regressions when GDC-0941 was combined with DTX in the MCF7-neo/HER2 and MX-1 xenograft model. A combination effect was also observed in a patient-derived breast cancer model (MAXF1162) that was HER2+/ER+/PR+ but PI3Kα wild-type and PTEN positive. The MCF7-neo/HER2 and MAXF1162 xenograft models are resistant to trastuzumab (25 and Oncotest Inc; personal communication), which suggests that combination therapy of GDC-0941 with DTX, may be effective in treating tumors that are refractory to anti-HER2 therapy.

Potentiation of DTX by GDC-0941 is primarily due to increased apoptosis as a consequence of inefficient escape from mitotic arrest when both drugs are combined. This results in acceleration of the time to mitotic catastrophe and a lowering of the cell death threshold, which may occur when the PI3K survival pathway is inhibited upon treatment with anti-mitotic drugs as cells attempt to escape mitotic arrest (44). Specific members of the Bcl-2 family that regulate intrinsic apoptosis may govern the cell death mechanisms that are activated by the combination of DTX and GDC-0941 during mitosis. For example, phosphorylation of Bcl-xL, which we observed in HCC-1954 breast tumor cells, has been reported to inhibit its pro-survival function after treatment with microtubule-targeted agents including PTX (45, 46). Consistent with Akt’s role in phosphorylating Bad and promoting cell survival (47, 48) we observed decreased levels of pAktSer473 and pBadSer136 after GDC-0941 treatment. Therefore,
concomitant activation of the pro-apoptotic BH3 only protein BAD by inhibition of Akt and inactivation of Bcl-xL by DTX may rapidly increase cell death during mitosis after combination treatment with both drugs. Interestingly, similar results were obtained with ABT-263 (navitoclax), a BAD mimetic that antagonizes Bcl-xL, in which combination with PTX or DTX accelerated apoptosis in epithelial cells during drug-induced mitotic arrest (49, 50).

Our findings with GDC-0941 confirm that selectively inhibiting class I PI3K is sufficient to augment the anti-tumor effects of DTX in breast cancer models in vitro and in vivo. The primary mechanism of action for the combination effect is shortening the duration in mitosis resulting in increased cell death. Our preclinical data provides a rationale to evaluate GDC-0941 in combination with DTX for the treatment of breast cancer.

**ACKNOWLEDGEMENTS:** We thank our Genentech colleagues Laurent Salphati for kindly providing exposure data for GDC-0941 and DTX in immuno-compromised mice and Gail Phillips for HER2 expression data in the MCF7-neo/HER2 cell line.
TABLE AND FIGURE LEGENDS

Table 1. Quantification of apoptotic cells after combination drug treatment. Fold increase in % apoptotic cells based on Annexin V positivity relative to vehicle (DMSO) control after treatment for 48 hours with GDC-0941, DTX or the combination of both drugs in Hs578T1.2, MCF7-neo/HER2 and MX-1 tumor cell lines.

Figure 1. Combination effects of GDC-0941 and DTX on cellular viability in breast cancer and MCF10A mammary epithelial cell lines. A, Hs578T1.2, MCF7-neo/HER2 and MX-1 breast cancer cell lines and MCF10A cells were treated with GDC-0941, DTX, or the combination of both drugs and evaluated in a 4-day cell viability assay. Percent inhibition and Delta.Bliss data were determined as described in Methods and plotted as a dose range for both drugs. B and C, Bliss sum scores of a sub-set of tumor cell lines treated with the combination of GDC-0941 and DTX were grouped into breast cancer subtypes (B); PI3Kα wild-type, mutational or PTEN expression status (C); and analyzed for pair-wise comparisons by Student’s t-test.

Figure 2. PI3K pathway modulation after treatment with GDC-0941 and DTX in breast cancer cell lines. Hs578T1.2, MCF7-neo/HER2, and MX-1 cell lines were treated with GDC-0941 (0.4, 0.5, and 0.8 μmol/L, respectively) and/or DTX (0.7, 1.6, 0.8 nmol/L, respectively) for 4 and 24 hours and cell lysates were
immunoblotted with antibodies to PI3K pathway markers indicated and phospho-histone H3 (pH3) as described in Methods.

**Figure 3.** Quantification of duration in mitosis after treatment of HCC-1954 and MCF7-neo/HER2 breast tumor cells with GDC-0941 and DTX. A, HCC-1954 cells were treated with 0.8 μmol/L GDC-0941 and/or 1.5 nmol/L DTX. MCF7-neo/HER2 cells were treated with 0.5 μmol/L GDC-0941 and/or 1.6 nmol/L DTX and measured for induction of apoptosis by time-lapsed microscopy as described in Methods. Each symbol represents a single cell and numbers in parenthesis indicate the percentage of cells that fragmented (indicative of apoptosis) during the course of treatment. Cells were also co-treated with caspase inhibitor Z-VAD-FMK at a final concentration of 2 μM. Statistical significance of the apoptotic effects in cells by GDC-0941 and DTX vs. GDC-0941 or DTX alone was determined by Student’s t-test (**p<0.001). B, Detection of apoptosis markers in mitosis after drug combination treatments. HCC-1954 cells were treated with GDC-0941 and DTX at the concentrations shown 12 hours (hrs) after release from synchronization. Cell extracts were isolated after an additional 12 hrs of treatment and evaluated for apoptosis pathway markers (pBad, BAD, pBcl-xL, Bcl-xL, Mcl-1 and cleaved PARP) as described in Methods. + 24 hrs; total time after synchronization release.
Figure 4. Combination efficacy of GDC-0941 and DTX in xenograft models.

Tumor-bearing mice were treated with orally and daily with GDC-0941 or vehicle (0.5% methycellulose, 0.2% Tween-80) and once weekly with DTX (intravenously). A, MCF7-neo/HER2 tumor-bearing mice were treated for 21 days. B, Mean animal body weights (grams) of MCF7-neo/HER2 tumor bearing mice measured twice weekly for 21 days. C, MX-1 tumor-bearing mice were treated with vehicle and drugs as indicated for 18 days. D, MAXF1162 tumor-bearing mice were treated for 21 days and monitored for tumor growth for an additional 21 days after dosing ended. E, PI3K pathway (pAkt, pPRAS40, pS6), cell cycle (pH3; phospho-histone H3) and apoptotic [cleaved (Cl) PARP] markers were assessed in MCF7-neo/HER2 tumor xenografts (n=4) after 21 days of treatment with vehicle, 150 mg/kg GDC-0941, 7.5 mg/kg DTX or the combination of both drugs by Western blotting as described in Methods. F, Representative photomicrographs of MCF7-neo/HER2 xenografts (n=4) treated with vehicle or the drugs indicated and stained for cleaved caspase 3 by immunohistochemistry as described in Methods. Statistical significance for combination efficacy of GDC-0941 and DTX vs. GDC-0941 or DTX alone was determined by Dunnetts t-test (*p<0.001; **p<0.0001).

Figure 5. Dosing schedule of GDC-0941 and DTX in vitro and in vivo. A, Fold increase in MCF7-neo/HER2 apoptotic cells (based on Annexin V positivity) relative to DMSO vehicle treated cells are shown for GDC-0941 and DTX alone or the combination of both drugs where GDC-0941 was added 1 or 4 hours (hrs)
before (GDC-0941 + DTX) or after DTX (DTX + GDC-0941). GDC-0941 and DTX were dosed at EC$_{50}$ concentrations (based on viability) and cells were analyzed 48 hrs after drug treatment. Statistical significance was determined by pair-wise comparisons using the Student's t-test. B-D, MCF7-neo/HER2 tumor-bearing mice were dosed daily and orally with vehicle (0.5% methycellulose/0.2% Tween-80) or GDC-0941 and weekly with DTX (intravenously) or the combination of both drugs for 21 days. B and C, Combination groups: GDC-0941 was dosed 1 before or after DTX (B) or 4 hours (hr) before or after DTX (C) as indicated. D, Combination groups: GDC-0941 was dosed 24 hr after DTX as indicated. Statistical significance for combination efficacy of GDC-0941 and DTX vs. GDC-0941 or DTX alone was determined by Dunnetts t-test (**p<0.001).
REFERENCES


Table 1

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Hs578T1.2 % Cell Death</th>
<th>Fold Increase</th>
<th>MCF7-neo/HER2 % Cell Death</th>
<th>Fold Increase</th>
<th>MX-1 % Cell Death</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0941</td>
<td>6.6 (± 3.0)</td>
<td>1.4</td>
<td>5.8 (± 2.2)</td>
<td>1.4</td>
<td>5.0 (± 2.4)</td>
<td>1.4</td>
</tr>
<tr>
<td>DTX</td>
<td>6.7 (± 2.3)</td>
<td>1.4</td>
<td>8.2 (± 3.8)</td>
<td>2.0</td>
<td>4.3 (± 1.3)</td>
<td>1.2</td>
</tr>
<tr>
<td>GDC-0941 + DTX</td>
<td>12.2 (± 4.1)</td>
<td>2.5</td>
<td>11.4 (± 2.6)</td>
<td>2.7</td>
<td>7.0 (± 1.9)</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Figure 1

A

**Hs578T1.2**

<table>
<thead>
<tr>
<th>Percent Inhibition</th>
<th>Delta.Bliss</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX (nmol/L)</td>
<td>GDC-0941 (μmol/L)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

**MCF7-neo/HER2**

<table>
<thead>
<tr>
<th>Percent Inhibition</th>
<th>Delta.Bliss</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX (nmol/L)</td>
<td>GDC-0941 (μmol/L)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

**MX-1**

<table>
<thead>
<tr>
<th>Percent Inhibition</th>
<th>Delta.Bliss</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX (nmol/L)</td>
<td>GDC-0941 (μmol/L)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

**MCF10A**

<table>
<thead>
<tr>
<th>Percent Inhibition</th>
<th>Delta.Bliss</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTX (nmol/L)</td>
<td>GDC-0941 (μmol/L)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

B

**Breast Cancer Subtype**

- Luminal
- HER2
- Basal

C

**PI3Kα/PTEN Status**

- PI3Kα Wild-type
- PI3Kα Mutant
- PTEN Null

*p = 0.822, p = 0.544, p = 0.590*

*p = 0.249, p = 0.361, p = 0.473*
A

HCC-1954

MCF7-neo/HER2

Mitosis Duration (hr)

DMSO  GDC-0941  DTX  DTX+0941  DMSO  GDC-0941  DTX  DTX+0941

+ Caspase Inhibitor

Synchronization Release:

DTX (8 nmol/L):

GDC-0941 (0.8 mmol/L):

B

Synchronization Release:

+24 hrs

DTX (8 nmol/L):

GDC-0941 (0.8 mmol/L):

pAkt_{\text{Ser}473}
pBad_{\text{Ser}136}
Bad
pBcl-x_{\text{Ser}62}
Bcl-x_L
Mcl1
Cleaved PARP
Cyclin B1
\beta\text{Actin}
Figure 4

A

Vehicle
7.5 mg/kg docetaxel
150 mg/kg GDC-0941
docetaxel + GDC-0941

Mean tumor volume (mm$^3$) ± SEM

Day

0 5 10 15 20 25

B

Vehicle
7.5 mg/kg docetaxel
150 mg/kg GDC-0941
docetaxel + GDC-0941

Mean body weight (g) ± SEM

Day

0 5 10 15 20 25

C

Vehicle
7.5 mg/kg docetaxel
150 mg/kg GDC-0941
docetaxel + GDC-0941

Mean tumor volume (mm$^3$) ± SEM

Day

0 5 10 15 20 25

D

Vehicle
7.5 mg/kg docetaxel
150 mg/kg GDC-0941
docetaxel + GDC-0941

Mean tumor volume (mm$^3$) ± SEM

Day

0 10 20 30 40 50

Treatment Period

E

Vehicle
GDC-0941
DTX
0941+DTX

pAktS473
pPRAS40T246
pS6S235/236
pH3S10
Cl PARP
Actin

F

Vehicle
GDC-0941

DTX
GDC-0941+DTX
Figure 5

(A) Fold increase in apoptotic cells (relative to DMSO control).

(B) Mean tumor volume (mm$^3$) ± SEM.

(C) Mean tumor volume (mm$^3$) ± SEM.

(D) Mean tumor volume (mm$^3$) ± SEM.
Clinical Cancer Research

GDC-0941, A Novel Class I Selective PI3K Inhibitor, Enhances the Efficacy of Docetaxel in Human Breast Cancer Models by Increasing Cell Death In Vitro and In Vivo

Jeffrey J. Wallin, Jane Guan, Wei Wei Prior, et al.

Clin Cancer Res  Published OnlineFirst May 14, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-2088

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/05/14/1078-0432.CCR-11-2088.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.