Cancer Therapy: Preclinical

Targeting Activated Akt with GDC-0068, a Novel Selective Akt Inhibitor That Is Efficacious in Multiple Tumor Models

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Abstract

Purpose: We describe the preclinical pharmacology and antitumor activity of GDC-0068, a novel highly selective ATP-competitive pan-Akt inhibitor currently in clinical trials for the treatment of human cancers.

Experimental Design: The effect of GDC-0068 on Akt signaling was characterized using specific biomarkers of the Akt pathway, and response to GDC-0068 was evaluated in human cancer cell lines and xenograft models with various genetic backgrounds, either as a single agent or in combination with chemotherapeutic agents.

Results: GDC-0068 blocked Akt signaling both in cultured human cancer cell lines and in tumor xenograft models as evidenced by dose-dependent decrease in phosphorylation of downstream targets. Inhibition of Akt activity by GDC-0068 resulted in blockade of cell-cycle progression and reduced viability of cancer cell lines. Markers of Akt activation, including high-basal phospho-Akt levels, PTEN loss, and PIK3CA kinase domain mutations, correlate with sensitivity to GDC-0068. Isogenic PTEN knockout also sensitized MCF10A cells to GDC-0068. In multiple tumor xenograft models, oral administration of GDC-0068 resulted in antitumor activity ranging from tumor growth delay to regression. Consistent with the role of Akt in a survival pathway, GDC-0068 also enhanced antitumor activity of classic chemotherapeutic agents.

Conclusions: GDC-0068 is a highly selective, orally bioavailable Akt kinase inhibitor that shows pharmacodynamic inhibition of Akt signaling and robust antitumor activity in human cancer cells in vitro and in vivo. Our preclinical data provide a strong mechanistic rationale to evaluate GDC-0068 in cancers with activated Akt signaling. Clin Cancer Res; 19(7); 1760–72. ©2012 AACR.

Introduction

The serine/threonine kinase Akt (a.k.a. protein kinase B or PKB) is encoded by 3 closely related genes in humans, Akt1 (PKB-a), Akt2 (PKB-b), and Akt3 (PKB-g), that belong to the AGC family of kinases and share high homology with protein kinase A (PKA) and PKC. Akt is the central node of the PI3K–Akt–mTOR pathway and is negatively regulated by the tumor suppressor PTEN, a phospholipid phosphatase that counteracts the activity of phosphoinositide 3-kinase (PI3K). The products of PI3K activity, the lipid second messengers phosphatidylinositol (3,4,5) trisphosphate [PI(3,4,5)P3] and PI(3,4)P2, promote membrane association and activation of Akt. Akt is phosphorylated at 2 residues critical for its full activation: a threonine residue in the activation loop of the kinase domain (T308) by phosphoinositide-dependent kinase 1 (PDK1) and a serine residue within the hydrophobic motif of the regulatory domain (S473) that can be phosphorylated by a number of kinases, most prominently mTOR complex 2 (mTORC2; reviewed in ref. 1). Activated Akt phosphorylates and regulates the functions of numerous cellular proteins, including the FoxO proteins, mTOR complex 1 (mTORC1), and S6 kinase, thereby playing an essential role in cell proliferation, survival, growth, migration, and energy metabolism (2).

Activation of Akt constitutes a hallmark of a variety of human cancers (3, 4). Multiple mechanisms can lead to Akt activation in human cancers, among which the most frequent genetic alterations include loss of the tumor suppressor PTEN (5, 6), and mutational activation of the p110α catalytic subunit of PI3K (7, 8). Amplification of the genes encoding either Akt or PI3K has also been observed in a subset of human cancers (9, 10). In addition, mutations in...
Akt can result in its constitutive activation in diverse cancers (11, 12), and has recently been identified as the underlying genetic abnormality associated with the Proteus syndrome (13). Hyperactivation of Akt also occurs via deregulated signaling of many cell surface receptors, intracellular linkers, and signaling molecules, including amplification/mutation of the EGFR/ErbB growth factor receptor family members and oncogenic mutations in the Ras family (reviewed in ref. 14). Moreover, Akt activation has been associated with resistance to both chemotherapeutic agents and targeted agents (15). These observations make Akt an attractive target for anticancer drug discovery. The eventual success of drugs targeting the PI3K–Akt–mTOR pathway will depend on their therapeutic index and the ability to stratify patients likely to respond to these therapeutics.

The 3 isoforms of Akt have both overlapping and distinct functions and expression profiles (16, 17). Activation of all 3 Akt family members have been detected in a variety of human malignancies, and inducible short hairpin RNA (shRNA) knockdown studies suggest that inhibition of all 3 Akt isoforms is required for maximum efficacy in PTEN-deficient cancer xenograft models (18). Strategies for targeting Akt have included both ATP-competitive and allosteric compounds, several of which were or are being tested in clinical trials (19). Previously reported ATP-competitive inhibitors have significant off-target activity on other members of the AGC kinase family (20, 21), therefore challenges in inhibitors have significant off-target activity on other members of the AGC kinase family (20, 21), therefore challenges in describing preclinical pharmacology and antitumor activity of Akt inhibitors that are suitable as drug candidates. Here, we interrogate our hypotheses in the clinic. Consistent with its high selectivity and specific targeting of activated Akt, GDC-0068 not only shows dose-dependent inhibition of Akt signaling and robust antitumor activity, but also exhibits differential activity in cells with and without activated Akt signaling that is distinct from phosphoinositide 3-kinase (PI3K) inhibitors. These preclinical data suggest that a rational strategy can be applied to stratify patients likely to benefit in clinical trials with a potentially achievable therapeutic index.

Materials and Methods

Cell culture

Cell lines were originally obtained from the American Type Culture Collection or from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH cell bank and genotyped by Genentech’s cell banking facility. Lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI supplemented with 10% FBS at 37°C under 5% CO₂. MCF7-neo/HER2 ectopically expresses HER2 in the MCF7 parental cell line and was developed at Genentech (22). MCF10A isogenic cells were obtained from Horizon Discovery Ltd. (http://www.horizondiscovery.com) and maintained in MCF10A growth medium as described previously (23) in a 1:1 mixture of DMEM and F12 medium (DMEM–F12) supplemented with 5% horse serum, hydrocortisone (0.5 μg/mL), insulin (10 μg/mL), EGF (20 ng/mL), and 0.1 μg/mL choleater toxin. In cell viability assays, assay medium containing DMEM–F12 supplemented with 5% horse serum, hydrocortisone (0.5 μg/mL) was supplemented with either 20 or 0.2 ng/mL EGF.

Compounds and antibodies

GDC-0068, GDC-0941, and GDC-0980 were supplied by Genentech, Inc. Antibodies used include phospho-AktThr308, phospho-PI3KαSer473, Akt, phospho-PRAS40Ser246, phospho-FoxO1S32, phospho-S6Ser235/236, phospho-S6Ser240/242, phospho-4EBP1Ser65, S6, PARP, cleaved PARP, and PTEN from Cell Signaling, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Advanced ImmunoChemical and a β-actin antibody from Sigma.

Cell viability assays

The 384-well plates were seeded with 2,000 cells per well in a volume of 54 μL per well followed by incubation at 37°C under 5% CO₂ overnight (~16 hours). Compounds were diluted in dimethyl sulfoxide (DMSO) to generate the desired stock concentrations then added in a volume of 6 μL per well. All treatments were tested in quadruplicates. After 4 days incubation, relative numbers of viable cells were estimated using CellTiter-Glo (Promega) and total luminescence was measured on a Wallac Multilabel Reader (PerkinElmer). The concentration of drug resulting in IC₅₀ was calculated from a 4-parameter curve analysis (XMfit, IDBS software) and was determined from a...
minimum of 3 experiments. For cell lines that failed to achieve an IC_{50}, the highest concentration tested (10 μmol/L) is listed.

Protein assays
For immunoblots, cells were washed with cold PBS and lysed in 1 × Cell Extraction Buffer (Biosource) supplemented with protease inhibitors (Roche), 1 mM l-phenylmethylsulfonylfluoride (PMSF), and phosphatase inhibitor cocktails 1 and 2 from Sigma. Protein concentration was determined using the Bradford method (Bio-Rad). Equal protein amounts were separated by electrophoresis through Tris-glycine 4% to 20% gradient gels (Invitrogen) and proteins transferred onto nitrocellulose membranes. Primary antibodies were detected using IR Dye 800-conjugated (Rockland) and Alexa-Flouro 680-conjugated (Molecular Probes) species-selective secondary antibodies. Detection and quantification were conducted using an Odyssey infrared scanner (LI-COR) using the manufacturer’s software.

Quantitative measurements of phospho and total Akt were conducted using a Lumixin assay (Invitrogen) or a Meso Scale Discovery Multi-Spot Biomarker Detection System (Meso Scale Discovery). Phospho and total PRAS40 were quantified using a Lumixin assay (Invitrogen) or a human ELISA kit (Invitrogen). Phospho and total S6 were quantified using a Meso Scale Discovery Multi-Spot Biomarker Detection System (Meso Scale Discovery).

Flow cytometry assays
For cell-cycle analyses, cells were trypsinized from the plates, fixed, and permeabilized by slowly dropping into cold 70% ethanol, then incubated overnight at −20°C. The cells were then washed with PBS and incubated in PBS containing 50 μg/mL propidium iodide (PI; Invitrogen) and 50 μg/mL RNaseA (Novagen) on ice for 30 minutes, and analyzed by flow cytometry. Cell-cycle distribution was determined using the ModFit software (Verity Software House). To detect apoptosis, cells were resuspended in PBS containing 4 mM CaCl₂, 5% Annexin V-FITC (BD Pharmingen), and 5 μg/mL PI. The mixture was incubated on ice for 30 minutes and cells analyzed by flow cytometry (BD Biosciences).

Xenograft studies
In vivo efficacy was evaluated in multiple tumor cell line and patient-derived xenograft models. Cells or tumor fragments were implanted subcutaneously into the flank of immunocompromised mice. Female or male nude (nu/nu) or severe combined immunodeficient mice (SCID)/beige mice were obtained from Charles River Laboratories, Harlan Laboratories, or Taconic. For the MCF7-neo/HER2 model, 17β-estradiol pellets (0.36 mg/pellet, 60-day release, no. SE-121; Innovative Research of America) were implanted into the dorsal shoulder before cell inoculation. The LuCaP35V patient-derived primary tumors were obtained from Dr. Robert Vessella at University of Washington (Seattle, WA; ref. 24); male mice were castrated before implantation of tumor fragments. After implantation of tumor cells or fragments into mice, tumors were monitored until they reached mean tumor volumes of 180 to 350 mm³ and distributed into groups of 8 to 10 animals/group. GDC-0068 was formulated in 0.5% methylcellulose/0.2% Tween-80 (MCT) and administered daily (QD), via oral (per os; PO) gavage. Docetaxel (Sanofi Aventis) was formulated in 3% EtOH/97% saline and dosed intravenously (IV) every week (QW) at 2.5 or 7.5 mg/kg. Carboplatin (St. Mary’s Pharmacy) was formulated in saline and dosed intraperitoneally (IP) weekly at 50 mg/kg.

Tumor volumes were determined using digital calipers (Fred V. Fowler Company, Inc.) using the formula (L × W × W)/2. Percentage tumor growth inhibition (%TGI) was calculated as the percentage of the area under the fitted curve (AUC) for the respective dose group per day in relation to the vehicle, such that %TGI = 100 × [1 − (AUC_{treatment}/d)/(AUC_{vehicle}/d)]. Curve fitting was applied to log₂ transformed individual tumor volume data using a linear mixed-effects (LME) model with the R package nlme, version 3.1-97 in R v2.13.0 (R Development Core Team 2008; R Foundation for Statistical Computing; ref. 25). Tumor sizes and body weights were recorded twice weekly over the course of the study. Mice with tumor volumes ≥2,000 mm³ or with losses in body weight 20% or more from their weight at the start of treatment were euthanized per Institutional Animal Care and Use Committee (IACUC) guidelines.

For pharmacodynamic marker analysis, xenograft tumors were excised from animals and immediately snap frozen in dry ice and LN₂. Frozen tumors were weighed and processed using a pestle (Scienceware) in 1 × cell extraction buffer.

Immunohistochemistry
Immunohistochemistry (IHC) for cleaved caspase-3 (CC3) was carried out 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-CC3 primary antibody (Cell Signaling Technology) at 37°C. Bound antibody was detected using DABMap technology (VMSI) and sections were counterstained with hematoxylin.

Statistics
Significant differences (P values) comparing treatment data using cell lines with and without evaluated genetic abnormalities were determined by two-tailed Mann–Whitney test calculated using the JMP statistical software, version 5.1.2 (JMP Software).

Results
GDC-0068 blocked Akt signaling and showed antiproliferative and apoptosis-inducing effects in human cancer cell lines
GDC-0068 is an ATP-competitive inhibitor of Akt (Supplementary Fig. S1; ref. 26) and is equipotent against all 3 Akt isoforms, which share more than 95% sequence identity within the ATP-binding pocket, with potencies ranging from 5 to 18 nmol/L (Supplementary Table S1).
Because of the high degree of homology in the ATP-binding pockets among Akt, p70S6K, PKA and other members of the AGC family of kinases, selectivity against these kinases has been a challenge for the development of potent and specific Akt inhibitors. GDC-0068 showed more than 600 and more than 100-fold selectivity for Akt1 in IC50 against the closely related kinases PKA and p70S6K, respectively (Supplementary Table S1). When tested at 1 μmol/L in a panel of 230 protein kinases, which includes 36 human AGC family members, GDC-0068 inhibited only 3 other kinases by more than 70% at 1 μmol/L concentration (PRKG1α, PRKG1β, and p70S6K). IC50 values measured for these 3 kinases were 98, 69, and 860 nmol/L, respectively. Thus, with the exception of PKG1 (relative to which GDC-0068 is >10-fold more selective for Akt1), GDC-0068 displays a more than 100-fold selectivity for Akt1 over the next most potently inhibited non-Akt kinase, p70S6K, in the screening kinase panel (Supplementary Table S1; ref. 26).

The biologic activity of GDC-0068 was evaluated in cell-based assays in vitro. Similar to other ATP-competitive Akt inhibitors (21, 27, 28), GDC-0068 induced a dose-dependent increase in Akt phosphorylation at both Thr308 (T308) and Ser473 (S473) residues in all cell lines tested, including lines in which the PI3K/Akt pathway is activated, such as PC-3 (PTEN homozygous deletion mutant, prostate), BT474M1 (PIK3CA1105E mutant and HER2-amplified, breast), IGROV-1 (PTEN106X/1055C and PIK3CA106/96W, ovarian; Fig. 1A). As we and others have shown, binding of the ATP competitive inhibitors to the active site of Akt can protect these sites from phosphatases, leading to increased pAkt (29). Despite this increase in pAkt, downstream Akt signaling activity was inhibited in a dose-dependent manner as shown by the diminished phosphorylation of the proline-rich Akt Substrate of 40 kDa (PRAS40) within 15 minutes of treatment (Fig. 1A). Maximum inhibition of pPRAS40 was achieved within 1 hour, with IC50 values of approximately 200 nmol/L in multiple cancer cell lines (Supplementary Table S1). Phosphorylation of other downstream targets, such as FoxO1 and FoxO3a, 4EBP1 and S6, were also inhibited in a dose- and time-dependent manner (Fig. 1A and B). Inhibition of pS6, the substrate of p70S6K that is further downstream of Akt, exhibited a delayed response compared with the proximal Akt targets, such as pPRAS40 (Fig. 1B).

The effects of GDC-0068 on cell-cycle progression and cell death were also assessed in cancer cell lines in which the PI3K/Akt pathway is activated, including PC-3, BT474M1, and the MCF7-neo/HER2 (PIK3CA E545K mutant stably expressing a HER2 transgene) breast cancer cell lines (25). A dose-dependent increase in the G0–G1 phase population was observed in all cell lines tested (Fig. 2A and Supplementary Fig. S2A). This effect was apparent within 15 hours of treatment and persisted for at least 72 hours in the

![Figure 1. Dose-dependent effect of GDC-0068 on Akt pathway biomarkers. A, Western blot analysis of phospho biomarkers in response to increasing concentrations of GDC-0068 in PC-3, BT474M1, and IGROV-1 cell lines after 1 hour of treatment. The expected positions of pFoxO3T32 and pFoxO1T24, which were detected with the same antibody, were indicated with an asterisk and a dot, respectively. B, quantification of dose-dependent effect of GDC-0068 on pPRAS40 (by the Luminex assay) and pS6 (by the meso scales assay) levels in PC-3 cells after 15 minutes, 1 hour, or 4 hours of treatment. Ratios of each phosphorylated protein to total protein were expressed as percentage of the ratio obtained from cells treated with DMSO vehicle control. Error bars represent SEM from 3 experiments.](www.aacrjournals.org)
presence of GDC-0068. A dose-dependent increase in the sub-G₁ peak was also observed in BT474M1 and MCF7-neo/HER2 cells by 48 hours of treatment (Fig. 2A). Similarly, Annexin V/PI staining indicated that GDC-0068 treatment caused a dose- and time-dependent increase in apoptotic and necrotic populations in BT474M1 and MCF7-neo/HER2 cells, but not in PC-3 cells (Fig. 2B and Supplementary Fig. S2B).
High Akt activity predicts sensitivity to GDC-0068

Consistent with its effect on cell-cycle progression and apoptosis, GDC-0068 exhibited dose-dependent inhibition of overall viability in multiple cancer cell lines (Fig. 3A and Supplementary Table S2). In a panel of 100 cell lines, we determined IC_{50} values on cell viability in response to GDC-0068. To investigate potential biomarkers that may predict response to GDC-0068, we also determined baseline pAkt^{S473} levels in these cell lines, as well as mutational status of key components in cancer signaling pathways including loss or mutation of the tumor suppressor PTEN, mutations in PI3K, amplification of HER2, or mutations in the KRAS or BRAF oncogenes. Sensitivity to GDC-0068 was strongly associated with pAkt levels above the median value \((P = 1.8 \times 10^{-7})\), and cells with loss of PTEN protein or genetic mutations in PTEN were also significantly more sensitive to GDC-0068 than those without \((P = 7.1 \times 10^{-7})\). Cells with PIK3CA mutations did not show significantly increased overall sensitivity to GDC-0068 compared with PIK3CA wild-type (WT) cells \((P = 0.14)\); however, mutations in the kinase domain (e.g., H1047R; ref. 8) were significantly associated with increased sensitivity \((P = 0.002)\), whereas helical (e.g., E545K) and other domain (e.g., I391M) mutations did not show significant association \((P = 0.75)\). Conversely, mutations in KRAS or BRAF were associated with resistance to GDC-0068 \((P =

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**Figure 3.** Effect of GDC-0068 on cell viability in a panel of cancer cell lines and MCF10A cells isogenic at the PTEN locus. A, IC_{50} values of GDC-0068 on cell viability sorted from low to high in a panel of 100 cancer cell lines. pAkt levels, known genetic alterations in the indicated biomarkers, as well as tissue origins are indicated as a colored box under each cell line: pAkt levels are represented by a heatmap; PTEN (-), PTEN loss by Western blot analysis or mutated, green; PI3K mut, PIK3CA mutated, honeydew (those with kinase domain mutations are indicated with a bolded border); HER2 +, HER2-amplified or overexpressed breast cancer cell lines, blue; TN, triple-negative breast cancer lines, pink; BRAF mut, BRAF mutated, brown; KRAS mut, KRAS mutated, red. Tissue origins for each cell line are indicated in different colors with letters indicating breast (Br), cervical (Ce), colon (Co), endometrial (En), ovarian (Ov), pancreatic (Pa), and prostate (Pr). B, dose-response curves of 3 different PI3K pathway inhibitors on the viability of isogenic MCF10A cells with or without PTEN knockout (KO), in assay medium containing either 20 ng/mL EGF or 0.2 ng/mL EGF (low EGF). Error bars represent SEM from quadruplicates. Representative data from more than 3 independent experiments are shown.
Figure 4. Single agent efficacy of GDC-0068 in human tumor xenograft models. A, fitted tumor volume dose–response plots of GDC-0068 treatment in the following xenograft models: LNCaP (PTEN-null, androgen-sensitive AR⁺ prostate cancer cell line), LuCaP 35V (PTEN-low, primary patient-derived androgen-independent AR⁺ prostate cancer xenograft), HGC-27 (PTEN-null gastric cancer cell line), MCF7-neo/HER2 (PIK3CAE545K, HER2 overexpressed breast cancer cell line), TOV-21G.x1 (PTEN-null, PIK3CAH1047R, KRASG13C ovarian cancer cell line), and NCI-H2122 (KRASG12C NSCLC cell line). B, comparison of %TGI in multiple tumor xenograft models at day 21 after daily oral treatment with 100 mg/kg of GDC-0068. Dashed line demarcates tumor stasis, which is defined as 100% TGI. Tumor growth delay increases from 0% to 100% TGI, and more than 100% TGI indicates tumor regression. PTEN(-), PTEN-null or PTEN-low by Western blot analysis or IHC; PI3K mut/amp, PIK3CA mutated or amplified; HER2⁺, HER2-amplified or overexpressed; BRAF mut, BRAF mutated; KRAS mut, KRAS mutated. Tissue origins are also indicated.
1.0 \times 10^{-7})$; in cells with these mutations the correlation of sensitivity with high pAkt or PTEN-deficiency was often lost, such as in the pancreatic cell lines. In addition, triple-negative breast cancer cell lines tended to be less sensitive to GDC-0068 than HER2$^+$ or ER$^+$ breast cancer cell lines ($P = 0.018$), likely reflecting the enrichment of an activated RAS pathway gene expression signature in this subset of breast cancers (30).

These data are consistent with previous reports that loss of PTEN strongly correlates with Akt activation (31). Indeed, higher pAkt levels were significantly associated with PTEN loss or mutation in our panel (mean pAkt levels = 11.44 arbitrary units in PTEN deficient cells vs. 4.85 in other cell lines; $P = 0.00016$), and with PIK3CA kinase domain mutants (mean pAkt levels = 13.32 in kinase domain mutants vs. 6.32 in all other mutant or WT cell lines; $P = 0.013$) but not with other nonkinase domain PI3K mutants (mean pAkt levels = 7.63 vs. 6.02 in PIK3CA WT cells; $P = 0.45$). To further determine whether PTEN loss can result in increased sensitivity to GDC-0068, we evaluated nontransformed human mammary epithelial cells, MCF10A, with their isogenic PTEN knockout counterparts (23). Cells were grown in regular MCF10A growth medium, and assayed for dose–response to GDC-0068 in assay medium containing either 20 ng/mL EGF or 0.2 ng/mL EGF (low EGF; Fig. 3B). Although very little pAkt was detectable in the parental MCF10A cells, Akt phosphorylation at both S473 and T308 were markedly elevated in the PTEN knockout cells under both conditions, along with increased phosphorylation of Akt downstream targets such as PRAS40 and S6 (Supplementary Fig. S3). The parental nontransformed MCF10A cells were quite resistant to GDC-0068 under the high EGF condition, with only 39% maximum inhibition of viability at concentrations up to 20 μmol/L of GDC-0068 (Fig. 3B). Reducing EGF increased sensitivity to 55% maximum inhibition in the parental line. Loss of PTEN resulted in a leftward shift of the dose–response curves under both conditions, with maximum inhibitions of 67% and 79% under high and low EGF conditions, respectively. A greater than 4-fold decrease in IC$_{50}$ values was observed under low EGF conditions in PTEN knockout versus WT cells (1.8 vs. 7.3 μmol/L, respectively), and a greater than 3.5-fold decrease in IC$_{50}$ values was observed under high EGF conditions in PTEN knockout versus WT cells (5.7 vs. > 20 μmol/L, respectively; Fig. 3B).

The strong correlation of GDC-0068 sensitivity with PTEN loss is in contrast to the previous reports in which no significant correlation with PTEN loss was observed for sensitivity to other PI3K-targeting inhibitors including the class I PI3K inhibitor GDC-0941 (32, 33) and dual PI3K/mTOR inhibitor GDC-0980 (25) but is consistent with another ATP-competitive inhibitor AZD5363 reported recently (20). We therefore examined the sensitivity of the MCF10A PTEN isogenic cells to the PI3K inhibitor GDC-0941 and the PI3K/mTOR inhibitor GDC-0980. Both inhibitors were more potent at inhibiting the parental MCF10A cells than GDC-0068 (Fig. 3B). However, in direct contrast to GDC-0068, PTEN loss resulted in a 3-fold increase in IC$_{50}$ for GDC-0941 (0.78 vs. 2.3 μmol/L) under high EGF conditions and 2-fold increase (0.20 vs. 0.44 μmol/L) under low EGF conditions, respectively. Smaller increases in GDC-0980 IC$_{50}$ were also observed under both conditions. Together, these data indicate that PTEN loss in MCF10A cells increased sensitivity to the Akt inhibitor GDC-0068, whereas decreased sensitivity to PI3K inhibitors. This is consistent with the hypothesis that Akt activation becomes less dependent on PI3K activity upon PTEN-loss, and that activated Akt is preferentially targeted by ATP-competitive inhibitors such as GDC-0068 (29).

GDC-0068 is efficacious in a broad spectrum of human cancer xenograft models

The in vitro sensitivity profile of GDC-0068 was recapitulated in vivo in xenograft models representing a spectrum of cancer types including prostate, breast, ovarian, colorectal, non–small cell lung, glioblastoma, and melanoma (Fig. 4A and B). GDC-0068 was typically efficacious in xenograft models in which Akt was activated because of genetic alterations including PTEN loss, PIK3CA mutations/amplifications, or HER2 overexpression. In these models, tumor growth delay, stasis, or regression was achieved at or below 100 mg/kg daily oral dose, which was the maximum dose tested in immunocompromised mice that was well tolerated. In contrast, cancer cell lines and xenograft models that harbor mutations in KRAS or BRAF, such as the KRAS$^{G12C}$ mutant NCI-H2122 non–small cell lung carcinoma (NSCLC) or the Kras$^{G12D}$ and PIK3CA$^{H1047R}$ mutant HCT-116 colorectal cancer (CRC) model, were less sensitive to GDC-0068 both in vitro and in vivo, even in models with a coexisting PIK3CA mutation (Figs. 3A, 4A and B). Interestingly, an ovarian cancer cell line that contains both PTEN loss and the PIK3CA$^{H1047R}$ hot-spot mutation, TOV-21G and its in vivo selected subline TOV-21Gx1, remained exquisitely sensitive to GDC-0068 both in vitro and in vivo despite the presence of the KRAS$^{G12C}$ mutation (Figs. 3A, 4A and B and Supplementary Table S2), suggesting that Akt activity is indispensable for cell viability in this line. Single agent treatment of GDC-0068 was well tolerated with less than 10% body weight loss observed compared with vehicle controls in all models tested in vivo (Supplementary Table S3).

The relationship between pharmacokinetics (PK) and pharmacodynamics (PD) of GDC-0068 was investigated in 3 xenograft models that showed dose-dependent response to drug treatment: MCF7-neo/HER2, TOV-21G, x1, and LNCaP (Fig. 4A). The mean in vitro cell viability IC$_{50}$ of GDC-0068 in these 3 cell lines is 2.56, 0.44, and 0.11 μmol/L, respectively. The phosphorylation levels of Akt, PRAS40, and S6 in MCF7-neo/HER2 tumors, as well as plasma and tumor concentrations of GDC-0068, were evaluated over 24 hours following a single dose of GDC-0068 at 0, 12.5, 50, or 100 mg/kg (Fig. 5A). Within 0.5 hour, a dose-dependent increase in Akt phosphorylation at both T308 and S473 sites was observed, reaching maximal levels between 4 and 8 hours. The kinetics of pAkt levels correlated better with GDC-0068 drug kinetics in tumors than in plasma and tumor concentrations of GDC-0068.
mouse plasma, given the higher levels of GDC-0068 in tumors compared with plasma beyond 4 hours due to rapid distribution of GDC-0068 from plasma to the tumor (Fig. 5A). Correlating closely with the increase in pAkt levels, a dose- and time-dependent decrease in phosphorylated PRAS40 at Thr246 was observed, with more than 50% knockdown associated with 50 and 100 mg/kg of GDC-0068, which was sustained up to 8 hours.
of S6 at Ser\textsuperscript{235} and/or Ser\textsuperscript{236} was also inhibited in a dose- and time-dependent manner, albeit with delayed kinetics consistent with the distal nature of this biomarker downstream of Akt. The tumor pharmacodynamics effect of GDC-0068 was also analyzed in the TOV-21G.x1 ovarian cancer xenograft model. Similar to observations in the MCF7-neo/HER2 xenograft model, a dose-dependent reduction in pPRAS40\textsuperscript{T246} was observed in the TOV-21G.x1 tumors with 50% or more reduction sustained between 3 to 8 hours when animals were dosed with 25 and 50 mg/kg GDC-0068 (Fig. 5B).

GDC-0068 elicited tumor stasis at 25 mg/kg in the TOV-21G.x1 model, with partial regressions observed at 50 mg/kg or higher, suggesting a strong dependence on Akt signaling in this model (Fig. 4A). To further investigate the mechanism of GDC-0068-mediated TGI, the level of CC3 was determined by IHC in the TOV-21G.x1 tumors treated with GDC-0068. A significant increase in nuclear CC3 was observed within 1 hour, with maximum levels observed at 3 hour in the 50 mg/kg group (Fig. 5C and Supplementary Fig. S4). Substantial tumor regression was also observed in the PTEN-null prostate cancer model LNCaP with 50 mg/kg or higher doses of GDC-0068 (Fig. 4A). Induction of apoptosis was also evidenced by a dose-dependent increase in the cleavage of PARP within 4 hours postdose, correlating with inhibition of pPRAS40\textsuperscript{T246} and pS6\textsuperscript{S240/244} in this model (Fig. 5D and E). Thus, efficacy of GDC-0068 in xenograft models is associated with efficient inhibition of Akt signaling nodes and induction of cell death in the sensitive models.

**GDC-0068 enhanced the antitumor activity of chemotherapeutic agents**

Akt activation has been associated with resistance to chemotherapeutic agents (15). We hypothesized that inhibiting Akt activity with GDC-0068 would overcome resistance or enhance the antitumor activity of standard of care chemotherapeutic drugs. Indeed, combination of multiple chemotherapeutic agents with GDC-0068 resulted in combination index (CI) values below 0.8 in the majority of cancer cell lines tested (as determined by the Chou and Talalay method; ref. 34), suggesting synergism for most of the combinations (Supplementary Fig. S5A). Interestingly, MCF10A cells with isogenic knockin of the activating PIK3CA mutation H1047R exhibited decreased sensitivity to docetaxel compared with the WT parental cells (Supplementary Fig. S5B). Combination with GDC-0068 resulted in significantly increased inhibition of cell viability in both PIK3CA WT and H1047R mutants compared with each single agent alone, suggesting Akt activation contributes to the resistance to docetaxel (Supplementary Fig. S5C).

When tested in vivo, daily dosing of GDC-0068 in combination with docetaxel induced tumor regression and stasis in the PC-3 and MCF7-neo/HER2 xenograft models, at doses where each single agent was ineffective or only caused modest tumor growth delay (Fig. 6A and B). Similarly, increased TGI was observed in the OVCAR3 ovarian cancer xenograft model when GDC-0068 was combined with carboplatin (Fig. 6C). The combination of GDC-0068 with docetaxel or carboplatin was tolerated with less than 5% body weight loss when compared with treatment with each chemotherapeutic agent alone (Supplementary Table S3).

**Discussion**

Identified 2 decades ago, the serine/threonine kinase Akt has emerged as a promising target for drug development. Akt is critically involved in multiple signaling cascades, controlling cell growth and proliferation, and its activation is a prominent feature of many human cancers. On the basis of the strong rationale for targeting Akt for cancer therapy, multiple attempts to identify Akt inhibitors with acceptable pharmaceutical properties have been pursued (17). However, despite the significant progress in identifying Akt small-molecule inhibitors, selectivity has been a key issue for many previously reported ATP-competitive Akt inhibitors (relative to the kinome, especially within the AGC kinase family), raising concerns on safety and unclear mechanisms of action of these drugs. Even allosteric inhibitors, which held the promise of greater selectivity against the kinome, have been reported to exhibit unexpected nonkinase off-target effects (35).

GDC-0068 is a highly selective, orally available pan-Akt inhibitor discovered through a structure-based drug discovery approach guided by cocrystal structures of ATP-competitive inhibitors in complex with Akt1 and the closely related PKA (26). It exhibited unprecedented selectivity against the kinome, including AGC family members previously shown to be significantly inhibited by other ATP-competitive inhibitors (20, 21). Our preclinical work presented in this report shows that GDC-0068 effectively inhibited Akt signaling to downstream biomarkers and dose-dependently decreased tumor cell viability in a broad spectrum of tumor models in vitro and in vivo. Moreover, we observed a strong association between cellular sensitivity to GDC-0068 and baseline levels of Akt activation. Genetic alterations that lead to Akt activation, most notably PTEN loss and PIK3CA kinase domain mutations, also showed positive correlation with sensitivity to GDC-0068, whereas mutations in KRAS or BRAF were negative predictors of GDC-0068 sensitivity. Interestingly, while PTEN-loss decreased sensitivity to PI3K inhibitors GDC-0941 and GDC-0980 in PTEN knockout MCF10A cells compared with the isogenic parental cells, it increased the sensitivity of these cells to GDC-0068, which is consistent with the notion that PTEN-loss reduces the dependence of Akt activation on PI3K activity and promotes cell proliferation in an Akt-dependent manner. Lessons learned from clinical experiments have indicated that broad and potent preclinical activity does not necessarily translate into clinical success without acceptable therapeutic index. As an ATP-competitive inhibitor, selective targeting of activated Akt is expected to further increase GDC-0068’s effectiveness in cells with high pAkt levels (29), such as tumor cells with PTEN-loss (31), whereas decrease its potency in cells with low Akt activity, such as normal cells, thereby
potentially widen its therapeutic index. Interestingly, different safety profiles have been observed between allosteric and ATP-competitive inhibitors in the clinic, for example, rash is reported as a dose-limiting toxicity for the allosteric inhibitor MK2206 but not several ATP-competitive Akt inhibitors including GDC-0068 (36–38), raising the possibility that differences in on-target effects on normal cells could exist between the 2 classes of inhibitors due to their different mechanisms of action.

In xenograft models, GDC-0068 was efficacious as a single agent when administered orally in models with genetic alterations that are predicted to cause activation of Akt, consistent with our in vitro results. The maximum antitumor effect of GDC-0068 achieved in the PC-3 xenograft model was comparable with the TGI generated by inducible shRNA knockdown of all 3 Akt isoforms in this model (18), consistent with an on-target drug effect. We also confirmed the efficacy of GDC-0068 in more clinically relevant disease models such as the patient-derived androgen-independent prostate cancer primary xenograft model LuCaP35V, which expresses reduced levels of the PTEN protein. Collectively, GDC-0068 shows single agent and on-target activity in tumor models in which the Akt pathway is activated.

Analysis of GDC-0068 pharmacokinetics revealed dose-dependent plasma and tumor exposure. GDC-0068 caused an increase in pAkt levels even at sub efficacious doses and the kinetics of pAkt increase correlated with tumor drug levels. This is consistent with our finding that ATP-competitive inhibitors lock Akt in a hyperphosphorylated yet nonfunctional state by preventing the accessibility of phosphatases (29), and as such, pAkt increase is a direct indicator of GDC-0068 binding to Akt. At efficacious doses a 50% to 75% suppression of the Akt substrate pPRAS40 and the downstream target pS6 was observed, showing inhibition of Akt signaling is necessary for robust antitumor responses in vivo. However, the degree of TGI varies between different models, with responses ranging from tumor growth delay to tumor regression even in the presence of comparable pPRAS40 suppression. The latter suggests that genetic background of each tumor model determines the dependence of tumor growth on Akt signaling.

Akt is known to be critically involved in glucose metabolism (15), and indeed we observed a transient, completely reversible increase in glucose and insulin levels in preclinical models after GDC-0068 treatment (data not shown), consistent with an on-target effect on Akt. Details of these data will be discussed elsewhere.

In summary, GDC-0068 is a novel, highly selective ATP-competitive Akt inhibitor with compelling selectivity, efficacy, and oral pharmacokinetics that support its clinical development as an anticancer agent either singly or in combination with chemotherapeutic agents. The selective activity of GDC-0068 in cancer cells with activated Akt signaling allows for a rational strategy to identify patients who will potentially benefit in clinical trials, which are currently underway.
Disclosure of Potential Conflicts of Interest

J. Lin, D. Sampath, M.A. Nannini, B.B. Lee, M. Degtyarev, J. Oeh, H. Savage, Z. Guan, R. Hong, R. Kassees, L.B. Lee, B.M. Liederer, H. Koeppen, N. J. Skelton, J.J. Wallin, M. Belvin, E. Punnoose, L.S. Friedman, and K. Lin are employees of Genentech, a member of the Roche Group, and have ownership interest (including patents) in it. S. Gross has ownership interest (including patents) in Array Biopharma. T. Risom disclosed no potential conflicts of interest.

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Targeting Activated Akt with GDC-0068, a Novel Selective Akt Inhibitor That Is Efficacious in Multiple Tumor Models

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