Characterization of an Akt Kinase Inhibitor with Potent Pharmacodynamic and Antitumor Activity

Nelson Rhodes,[1] Dirk A. Heerding,[2] Derek R. Duckett,[1] Derek J. Eberwein,[1] Victoria B. Knick,[1] Timothy J. Lansing,[1] Randy T. McConnell,[1] Tona M. Gilmer,[1] Shu-Yun Zhang,[1] Kimberly Robell,[1] Jason A. Kahana,[1] Robert S. Geske,[3] Elena V. Kleymenova,[3] Anthony E. Choudhry,[4] Zhihong Lai,[4] Jack D. Leber,[2] Elisabeth A. Minthorn,[5] Susan L. Strum,[6] Edgar R. Wood,[6] Pearl S. Huang,[1] Robert A. Copeland,[1] and Rakesh Kumar[1]

[1]Oncology Biology, [3]Medicinal Chemistry, [3]Discovery Technology Group, [4]Enzymology and Mechanistic Pharmacology, [5]Drug Metabolism and Pharmacokinetics, and [6]Assay Development, GlaxoSmithKline, Collegeville, Pennsylvania

Abstract

Akt kinases 1, 2, and 3 are important regulators of cell survival and have been shown to be constitutively active in a variety of human tumors. GSK690693 is a novel ATP-competitive, low-nanomolar pan-Akt kinase inhibitor. It is selective for the Akt isoforms versus the majority of kinases in other families; however, it does inhibit additional members of the AGC kinase family. It causes dose-dependent reductions in the phosphorylation state of multiple proteins downstream of Akt, including GSK3β, PRAS40, and Forkhead. GSK690693 inhibited proliferation and induced apoptosis in a subset of tumor cells with potency consistent with intracellular inhibition of Akt kinase activity. In immune-compromised mice implanted with human BT474 breast carcinoma xenografts, a single i.p. administration of GSK690693 inhibited GSK3β phosphorylation in a dose- and time-dependent manner. After a single dose of GSK690693, >3 μmol/L drug concentration in BT474 tumor xenografts correlated with a sustained decrease in GSK3β phosphorylation. Consistent with the role of Akt in insulin signaling, treatment with GSK690693 resulted in acute and transient increases in blood glucose level. Daily administration of GSK690693 produced significant antitumor activity in mice bearing established human SKOV-3 ovarian, LNCaP prostate, and BT474 and HCC-1954 breast carcinoma xenografts. Immunohistochemical analysis of tumor xenografts after repeat dosing with GSK690693 showed reductions in phosphorylated Akt substrates *in vivo*. These results support further evaluation of GSK690693 as an anticancer agent. [Cancer Res 2008;68(7):2366–74]

Introduction

Akt is a serine-threonine protein kinase that plays a critical role in cellular survival, metabolism, proliferation, and growth (1, 2). The Akt family consists of three isoforms (Akt1, 2, and 3) that are regulated in a similar fashion and have overlapping functions. However, knockout of individual isoforms in mice suggest a role of Akt1 in overall growth (3, 4), Akt2 in insulin signaling (5, 6), and Akt3 in brain development (7, 8). In adults, Akt1 expression is ubiquitous, expression of Akt2 is elevated in insulin-responsive tissues, and expression of Akt3 is also ubiquitous with low levels in skeletal muscle and liver.

Hyperactivation of Akt kinases is one of the most common molecular findings in human malignancies (9, 10). Many oncogenes and tumor suppressor genes result in activation of phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase activity. Amplification (ErbB2 and Met) and mutation [epidermal growth factor receptor (EGFR), PI3K, platelet-derived growth factor receptor, and c-Kit] of various receptor tyrosine kinases, mutation in downstream signaling molecules (ras, src, and PI3K), loss or mutation of tumor suppressor protein (PTEN), as well as mutation and amplification of Akt itself result in increased Akt signaling in tumor cells. Increased Akt1 activity has been observed in ~40% of breast and ovarian cancers and >50% of prostate carcinomas. Activation of Akt2 kinase has been observed in 30% to 40% of ovarian and pancreatic cancers (11, 12). Increased Akt3 enzymatic activity was found in estrogen receptor–deficient breast cancer and androgen-insensitive prostate cancer cell lines, suggesting that Akt3 may contribute to the aggressiveness of steroid hormone–insensitive cancers (2, 13). Recently, an activating mutation in the pleckstrin homology domain of Akt1 has been identified in human breast, ovarian, and colorectal cancers, suggesting a direct role of Akt1 in human cancers (14).

Akt signaling promotes cell survival and proliferation. Constitutively active Akt has been shown to protect cells from PTEN-mediated apoptosis and also to reduce the sensitivity of tumor cells to proapoptotic cytotoxic agents (15). A dominant-negative mutant of Akt inhibited tumor growth in a mouse model and selectively induced apoptosis of tumor cells expressing activated Akt (16). Genetic ablation of Akt1 inhibited mammary tumor growth in transgenic mouse models (17, 18). Simultaneous inhibition of Akt1 and Akt2 was shown to be superior to inhibition of a single isozyme for induction of caspase-3 activity in tumor cells (15). The antitumor activity of rapamycin and its analogues, which target mammalian target of rapamycin (mTOR) kinase, a downstream substrate of Akt signaling, provide evidence that targeting of the Akt pathway is a rational approach to cancer therapy (13, 19).

The present study describes a novel, ATP-competitive, pan-Akt kinase inhibitor with potent enzyme and cellular activity. GSK690693 also inhibits phosphorylation of Akt substrates in

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Present address for D.R. Duckett: Scripps Florida, Drug Discovery, 5353 Parkside Drive, Jupiter, FL 33458.

Present address for P.S. Huang: Oncology, Merck & Co., 351 North Sumneytown Pike, North Wales, PA 19454.

Requests for reprints: Rakesh Kumar, Oncology Biology, 1250 South Collegeville Road, UP1450, Collegeville, PA 19426. Phone: 610-917-4855; Fax: 610-917-4181; E-mail: rakesh.2.kumar@gsk.com.

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human tumor xenografts in mice and reduces the growth of various human tumor xenografts *in vivo*. Based on these results, GSK690693 is currently being investigated in a phase I clinical trial in cancer patients.

Materials and Methods

GSK690693 preparation. GSK690693 is a novel Akt kinase inhibitor derived from the aminofurazan chemical series (Table 1) synthesized at GlaxoSmithKline. The discovery and synthesis of GSK690693 will be described in a separate article.[7] For all *in vitro* studies, GSK690693 was dissolved in DMSO at a concentration of 10 mmol/L before use. For the tumor xenograft and pharmacodynamic studies, GSK690693 was formulated in either 4% DMSO/40% hydroxypropyl-β-cyclodextrin in water (pH 6.0) or 5% dextrose (pH 4.0).

Animals. Female CD1 Swiss nude mice were obtained from Taconic and C.B-17 severe combined immunodeficient (SCID) mice were obtained from Charles River. All animal studies were performed in compliance with federal requirements, GlaxoSmithKline policy on the Care and Use of Animals, and with related codes of practice.

Kinase assays. The ability of GSK690693 to inhibit the activity of a wide variety of kinases was tested *in vitro*. His-tagged full-length Akt1, 2, or 3 were expressed and purified from baculovirus. Activation was carried out with purified PDK1 to phosphorylate Thr^{308} and purified MK2 to phosphorylate Ser^{473}. To more accurately measure time-dependent inhibition of Akt, activated Akt enzymes were incubated with GSK690693 at various concentrations at room temperature for 30 min before the reaction was initiated with the addition of substrate. Final reaction contains 5 to 15 nmol/L Akt1, 2, and 3 enzymes; 2 μmol/L ATP; 0.15 μCi/μL [$\gamma-^{33}$P]ATP; 1 μmol/L Peptide (Biotin-aminohexanoicacid-ARKR-ERAYSFGHHA-amide); 10 mmol/L $MgCl_2$; 25 mmol/L MOPS (pH 7.5); 1 mmol/L DTT; 1 mmol/L CHAPS; and 50 mmol/L KCl. The reactions were incubated at room temperature for 45 min, followed by termination with Leadseeker beads in PBS containing EDTA (final concentration, 2 mg/mL beads and 75 mmol/L EDTA). The plates were then sealed and the beads allowed to settle for at least 5 h, and product formation was quantitated using a Viewlux Imager (PerkinElmer).

To determine the selectivity of GSK690693, we characterized the inhibition of 89 protein kinases at GlaxoSmithKline. GSK690693 was also tested at 10 μmol/L concentration in the IC_{50} Profiler Express panel (Upstate) against 209 protein kinases using filter-binding activity assays, and Ambit Biosciences panel against 180 protein kinases measuring binding with phage display technology. A subset of kinases that showed strong inhibition at 10 μmol/L were followed up with a full dose response and IC_{50} generation. As much as possible, the assays were configured so that the IC_{50} values approximate the intrinsic binding constant (K_i or K_d) of GSK690693 to each enzyme and can therefore be compared for selectivity against these kinases. However, the selectivity in cells may be different because inhibitor potency will be affected to differing degrees based on the ATP K_m values for each kinase.

The data for dose responses were plotted as % activity calculated with the data reduction formula in Eq. 1:

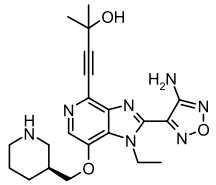
$$\%activity = \left(\frac{U - C1}{C2 - C1}\right) \cdot 100 \quad (1)$$

where U is the signal measured at indicated compound concentrations, $C1$ is the average of signal in the absence of compound (with 1% DMSO), and $C2$ is the average of signal in the no reaction control (defined by quenching the reaction with 75 mmol/L EDTA before adding enzyme). Inhibition curves were generated by plotting percentage control activity versus the concentration of inhibitor in a semilog fashion. The IC_{50} values were calculated by nonlinear regression fitting to Eq. 2:

$$y = \frac{y_{max} - y_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^h} + y_{min} \quad (2)$$

where y is the % activity, y_{min} is the minimum value of y, y_{max} is the maximum value of y, [I] is the concentration of inhibitor, and h is the Hill coefficient.

Table 1. GSK690693 structure and enzyme inhibition data



	IC_{50} (nmol/L)
Akt1	2
Akt2	13
Akt3	9
AMPK	50
DAPK3*	81
PAK4*	10
PAK5*	52
PAK6*	6
PKA	24
PKCη	2
PKCθ	2
PKCβ1	19
PKCδ	14
PKCε	21
PKG1β	33
PrkX	5

NOTE: Data for kinases with IC_{50} <100 nmol/L are shown in the table.
*Data are from a binding assay. All other data are using activity assays.

Inhibition of Akt substrate phosphorylation in cells. An ELISA was developed for the analysis of GSK3β phosphorylation in cells. Tumor cells were treated with GSK690693 at various concentrations in a 96-well plate for 1 h. Cell lysates were analyzed for phospho-GSK3β using anti-GSK3β antibody (BD Biosciences) for capture and antiphospho-GSK3α/β antibody (Cell Signaling) for detection. IC_{50} values were obtained by fitting data to Eq. 2.

For Western blot analysis of various substrates of Akt phosphorylation, BT474 cells were treated with GSK690693 at concentrations ranging from 10 μmol/L to 1 nmol/L. Five hours later, the cells were then lysed in radioimmunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors. The lysates were diluted with sample loading buffer (Invitrogen), and 10 μg total protein were run on 4% to 20% SDS-PAGE gels. The samples were then transferred to polyvinylidene difluoride membranes and probed with the antibodies for phospho-Forkhead (FKHR/FKHRL1), phospho-p70S6K, phospho-PRAS40, phospho-GSK3α/β, phospho-EGFR, phospho-ErbB2, phospho–extracellular signal-regulated kinase (ERK), and phospho-Akt at 1:1,000 dilution. Secondary, fluorescently labeled antibodies were used at 1:5,000 dilutions. Tubulin was used as a loading control. All phosphosite specific antibodies were obtained from Cell Signaling, except

[7] D.A. Heerding et al., in preparation.

phospho-PRAS40 (BioSource) and phospho-ERK (Santa Cruz Biotechnology). Antitubulin antibody was obtained from Sigma. All blots were imaged on a LiCor Odyssey instrument as directed by the manufacturer.

FOXO3A–green fluorescent protein translocation assay. FOXO3A was cloned from human lung and brain cDNA. The FOXO3A cDNA (amino acids 1–664) was subcloned into the vector pEGFP-N1 (Clontech). The resulting construct had the green fluorescent protein (GFP) fused to the carboxyl terminus of the FOXO3A sequence. U2OS cells stably transfected with pEGFP-N1 FOXO3a were plated in 6-well polystyrene plates at 250,000 cells per well and allowed to adhere overnight. GSK690693 was added using a 10-fold serial dilution at concentrations ranging from 10 μmol/L to 1 nmol/L (and untreated control). After 90 min in culture, the cells were analyzed by fluorescence microscopy.

Proliferation assay. Cell proliferation assays were performed for a number of cell lines as described earlier with some modifications (20). For these assays, cells were plated at densities that allowed untreated cells to grow logarithmically during the course of a 3-d assay. Briefly, cells were plated in 96- or 384-well plates in culture medium containing 10% fetal bovine serum and incubated overnight at 37° C in 5% CO_2. Cells were then treated with GSK690693 (ranging from 30 μmol/L–1.5 nmol/L) and incubated for 72 h. Cell proliferation was measured using the CellTiter Glo (Promega) reagent according to the manufacturer's protocol. Data were analyzed using the XLFit (IDBS Ltd.) curve-fitting tool for Microsoft Excel. IC_{50} values were obtained by fitting data to Eq. 2.

Reverse-phase protein microarray. Protein arrays were constructed as described previously (21, 22). Briefly, serially diluted protein lysates from cell growing logarithmically were printed in duplicate onto nitrocellulose-coated glass slides. Total protein was quantified in selected arrays that were stained with Sypro Ruby Protein Blot Stain (Molecular Probes) according to the manufacturer's instructions. The lysate arrays were incubated for at least 5 h in blocking solution [1g I-block (Tropix); 0.1% Tween 20 in 500 mL PBS] at room temperature with constant rocking. Blocked arrays were stained with phospho-Akt (Ser^{473}) antibody on an automated slide stainer (Dako Cytomation) using the Catalyzed Signal Amplification System kit according to the manufacturer's recommendation (CSA; Dako Cytomation). Stained slides were scanned individually on a UMAX PowerLook III scanner (UMAX) at 600 dpi and saved as TIF files in Photoshop 6.0 (Adobe). The TIF images for antibody-stained slides and Sypro-stained slide images were analyzed with MicroVigene image analysis software, version 2.200 (Vigenetech) and Microsoft Excel 2000 software. Images were imported into Microvigene, which performed spot finding, local background subtraction, replicate averaging, and total protein normalization, producing a single value for each sample at each end point.

Pharmacodynamic assay. Female SCID mice (3 mice per dosing group) with BT474 xenograft tumors (200–400 mm^3) were dosed i.p. with 10, 20, or 40 mg/kg GSK690693 in 4% DMSO/40% HP-β-CD in water (pH 6.0). Four hours after administration of GSK690693, tumors were harvested. Frozen tumor samples were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein lysate (50 μg per sample) were analyzed by Western blots using antibodies against total GSK3α/β (Invitrogen) and phospho-GSK3β (Ser^9; Cell Signaling). Time course of the pharmacodynamic effect was measured using a 20 mg/kg single i.p. dose of GSK690693. Blood glucose, plasma insulin, phospho-GSK3β in BT474 tumors, and drug concentration in blood and tumor were analyzed at baseline, 1, 2, 4, 8, and 24 h after compound administration. Blood glucose was measured from tail vein nicks using an Accu-Chek Compact glucometer (Roche Diagnostics). Circulating insulin levels were measured in plasma from mice using an ELISA kit (Crystal Chem Inc.).

Drug concentration analysis in blood and tumor tissue. Blood samples were hemolyzed with high performance liquid chromatography (HPLC) grade water before analysis. Tumor samples were mixed with HPLC grade water and homogenized using a probe-type homogenizer. Samples were assayed for GSK690693 using protein precipitation with acetonitrile followed by HPLC/MS/MS analysis using positive TurboIonSpray ionization or atmospheric pressure chemical ionization. This assay method was sufficiently accurate and precise for the determination of GSK690693 with a limit of detection as low as 10.0 ng/mL using 25 μL of whole blood or 50 μL of tumor homogenate. Tumor homogenate concentrations (ng/mL homogenate) were converted to tissue concentrations (ng/g tissue) by multiplying the homogenate concentrations with the dilution factor, which resulted from the addition of water during homogenization of the samples. This method assumes complete recovery of the analyte from the sample matrix during bioanalysis.

Tumor xenografts. Tumors were initiated by injection of tumor cell suspension (HCC1954, MDA-MB-453, and LNCaP) or tumor fragments (BT474, SKOV-3, and PANC1) s.c. in 8- to 12-wk-old CD1 Swiss Nude mice (LNCaP, SKOV-3, and PANC1) or SCID mice (HCC1954, MDA-MB-453, and BT474). When tumors reached a volume of 100 to 200 mm^3, mice were randomized and divided into groups of 8 to 12 mice per group. GSK690693 was administered once daily at 10, 20, and 30 mg/kg by i.p. administration. Animals were euthanized by inhalation of CO_2 at the completion of the study. Tumor volume was measured twice weekly by calipers, using the equation: tumor volume (mm^3) = (length × $width^2$)/2. Results are reported as % inhibition on day 21 of treatment = 100 × [1-(average growth of the drug-treated population/average growth of vehicle-treated control population)]. Statistical analysis was done using two-tailed t test.

Immunohistochemistry. Before immunohistochemically (IHC) staining the study samples, protocols were optimized and antibodies were validated histologically by reproducing Western blot data from BT474 cells treated with 1 μmol/L GSK690693. Antibody specificity was confirmed by pretreating tissue sections with λ phosphatase to remove phosphorylation sites before application of primary antibody. Four-micrometer paraffin sections were prepared from the human tumor xenograft samples and affixed to glass slides. After tissue deparaffinization, antigens were retrieved using citrate buffer, and endogenous biotin and peroxidase were blocked. Nonspecific antibody binding was addressed through application of appropriate sera. Tissues were incubated with antibody against pPRAS40 (Calbiochem) and pFKHR-L (Cell Signaling) for 1 or 4 h, respectively, followed by a biotinylated goat anti-rabbit IgG secondary antibody. A streptavidin reagent conjugated to a reporter enzyme (peroxidase) was applied to complete the multilayer sandwich method. Immunoreactive areas were visualized using 3,3′-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin before coverslipping with a permanent mounting medium. Areas of immunoreactivity are brown (DAB) and nuclei are blue (hematoxylin). Photomicrographs were taken using Leica DMRXA2 microscope (Leica Microsystems, Inc.) at ×20 magnification. Images were captured on Nuance spectral imaging camera (CRI) and a composite was made in Photoshop 7.01 (Adobe).

Results

Kinase activity of GSK690693. GSK690693 is an ATP-competitive, low nanomolar inhibitor of Akt kinases with IC_{50} values of 2, 13, and 9 nmol/L for Akt1, 2, and 3, respectively (Table 1). The apparent K_i^*s for full-length Akt1, 2, and 3 were determined as 1, 4, and 12 nmol/L, respectively.

To estimate the selectivity of GSK690693, we measured its ability to inhibit >250 *in vitro* expressed human protein kinases in either activity assays or binding assays. IC_{50} values were generated against 95 kinases. Kinases with IC_{50} values <100 nmol/L are shown in Table 1. GSK690693 is very selective for the Akt isoforms versus the majority of kinases in other families; however, it is less selective for members of the AGC kinase family including PKA, PrkX, and PKC isozymes. Other kinases inhibited by GSK690693 are AMPK and DAPK3 from the CAMK family, and PAK4, 5, and 6 from the STE family (Table 1).

Crystallography and biochemical mechanism of action analysis show that GSK690693 is an ATP competitive inhibitor for the Akt enzymes. We have also discovered that GSK690693 inhibits Akt1 and 2 in a time-dependent and reversible manner with a half life

Table 2. Cellular activity of GSK690693

Cell line	Cell type	pAkt*	Proliferation	pGSK3β
			IC_{50} (nmol/L) †	IC_{50} (nmol/L) †
T47D	Breast	3.975	72	—
ZR-75-1	Breast	9.777	79	—
BT474	Breast	6.753	86	160
HCC1954	Breast	16.610	119	—
MDA-MB-453	Breast	4.096	975	—
MDA-MB-468	Breast	8.248	3,208	—
SkBr3	Breast	8.248	>10,000	—
MDA-MB-231	Breast	0.427	>10,000	—
HCT116	Colon	0.427	5,836	—
HT29	Colon	3.706	>10,000	—
HFF	Fibroblast (normal)	0.458	7,615	—
HN5	Head and neck	3.633	>10,000	—
786-0	Kidney	10.074	4,009	150
H157	Lung	5.003	2,642	—
NCI-H460	Lung	0.571	>2,500	—
SKOV-3	Ovarian	10.074	2,126	—
OVCAR-3	Ovarian	2.718	2,918	—
BXPC3	Pancreas	2.638	3,141	—
MiaPaCa	Pancreas	0.071	5,433	—
PANC-1	Pancreas	1.632	8,681	—
LNCaP	Prostate	16.610	147	43
DU145	Prostate	1.284	3,812	—
PC3	Prostate	3.490	>10,000	49

*Basal phospho-Akt (Ser^{473}) levels measured using reverse phase protein array and represented as arbitrary units.
† Data represent average of ≥2 experiments.

for dissociation ($t_{1/2}$) at 38 and 30 minutes for Akt1 and Akt2, respectively. Details of these studies and structure activity relationship of the series will be published in a separate article.[7]

Cellular activity of GSK690693. To measure inhibition of Akt kinase activity in cells by GSK690693, the levels of phosphorylated GSK3β (Ser^9) from cell lysates were determined using an ELISA. GSK690693 inhibited the phosphorylation of GSK3β in tumor cells with average IC_{50}s ranging from 43 to 150 nmol/L (Table 2). Because GSK3β can be phosphorylated on Ser^9 by kinases other than Akt, notably PKA (23), other Akt substrates were evaluated as additional measures of intracellular Akt inhibition. The phosphorylation of FKHR/FKHRL1, p70S6K, GSK3α/β, and PRAS40 in BT474 breast tumor cells was also inhibited by GSK690693 in a dose-dependent manner (Fig. 1*A*). Decreases in phosphorylation are evident for these Akt substrates at concentrations >100 nmol/L. An increase in Akt (Ser^{473}) phosphorylation was observed at all GSK690693 concentrations except the highest (10 μmol/L), as has been described with an Akt inhibitor from a different chemical series (24). No effect on EGFR, ErbB2, and ERK phosphorylation was observed in BT474 cells, suggesting a lack of activity on upstream receptors or mitogen-activated protein kinase (MAPK) pathway. To evaluate the functional consequence of inhibiting Akt substrate phosphorylation in cells by GSK690693, cellular localization of a FOXO3A-GFP fusion protein was determined (Fig. 1*B*). It has been previously shown that phosphorylation of FOXO3A by Akt results in nuclear accumulation consistent with its role in apoptotic signaling (25). GSK690693 induced accumulation of the FOXO3A fusion protein in the nucleus at concentrations of 1 μmol/L or greater.

The effect of GSK690693 on cell proliferation was evaluated against normal fibroblasts and a variety of tumor cell lines (Table 2). Of the eight breast cancer cell lines tested, five of them had IC_{50} values <1 μmol/L. The LNCaP (prostate) tumor cell line, which has constitutively active Akt due to the loss of PTEN (26), also showed sensitivity to GSK690693 (IC_{50} = 147 nmol/L). None of the other cell lines tested was very sensitive to the Akt inhibitor (IC_{50} >1 μmol/L), including the PTEN-null or mutant tumor cell lines 786-O, H157, MDA-MB-468, and PC-3 (27–30). Normal human foreskin fibroblast (HFF) cells were also insensitive to GSK690693 (IC_{50} >7 μmol/L). Phospho-Akt levels were higher in cells that were sensitive to GSK690693, although all cell lines with high phospho-Akt levels were not very sensitive, suggesting that other signaling pathways are likely activated in these cells.

Because Akt is an important regulator of cell survival, apoptosis as a mechanism of growth inhibition by GSK690693 was evaluated in LNCaP and BT474 cells after treatment with various concentrations of GSK690693 for 24 or 48 hours, respectively. As measured by histone-complexed DNA fragments, GSK690693 was found to induce apoptosis at concentrations >100 nmol/L in both LNCaP and BT474 cells (Supplementary Fig. S1).

***In vivo* activity of GSK690693.** To investigate the pharmacodynamic effect of GSK690693 *in vivo*, phosphorylation of GSK3β was measured from tumor lysates. In immune-compromised mice

implanted with human breast carcinoma (BT474) xenografts, a single i.p. administration of GSK690693 inhibited GSK3β phosphorylation in a dose-dependent manner (Fig. 2). After a single 20-mg/kg i.p. dose of GSK690693, drug concentrations were measured in blood and BT474 tumor tissue (Fig. 2). Drug concentrations >3 μmol/L (~1,500 ng/g) in BT474 tumor xenografts correlated with a sustained decrease (>60%) in GSK3β phosphorylation up to 8 hours (Fig. 2).

Because Akt is a critical regulator of insulin signaling, blood glucose and insulin levels were measured in mice after administration of GSK690693. Treatment with GSK690693 resulted in an acute increase in blood glucose that returned to baseline levels as circulating drug concentration decreased (Fig. 2). A rapid elevation of plasma insulin levels was also observed, reaching a maximum of 643 ng/mL 4 hours postdosing compared with the baseline level of 0.73 ng/mL (Fig. 2). The increase in insulin preceded the increase in blood glucose and decreased in parallel with the decrease in circulating drug concentration. Both glucose and insulin levels had declined by 8 hours and returned to baseline by 24 hours (Fig. 2). The treated mice had no sign of clinical distress, and the compound was well-tolerated with once daily administration up to 3 weeks.

GSK690693 was evaluated for its ability to inhibit growth of various human tumor xenografts in immunocompromised mice. Repeated i.p. administration (once daily for 21 days) produced significant antitumor activity in mice bearing established SKOV-3 ovarian, LNCaP prostate, and BT474 and HCC-1954 breast carcinoma xenografts (Fig. 3). Maximal inhibition of 58% to 75% was observed at the end of dosing period with 30 mg/kg/day dose. Daily administration of GSK690693 for 21 days was well-tolerated in mice with <10% body weight change with no overt clinical sign of toxicity. Effect of GSK690693 treatment on phosphorylation of Akt substrates was evaluated in BT474 tumor xenografts. Similar to the reduction of GSK3β phosphorylation observed after a single dose of Akt inhibitor (Fig. 2), IHC analysis of BT474 tumor xenografts after repeat dosing with GSK690693 showed a reduction in

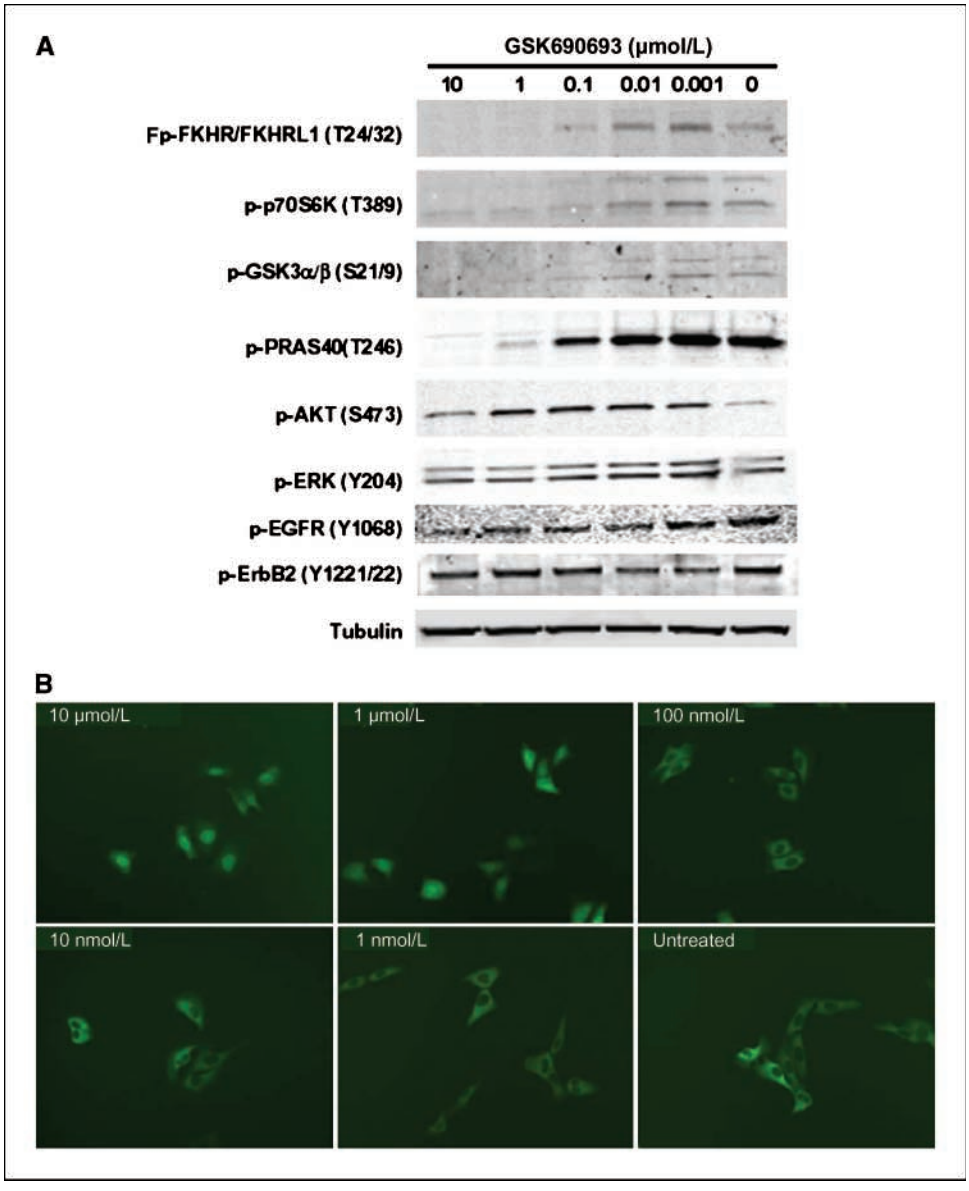


Figure 1. Inhibition of Akt pathway phosphorylation and nuclear translocation of FOXO3A induced by GSK690693. *A,* Western blots were performed to evaluate the phosphorylation state of FKHR/FKHRL1, p70S6K, GSK3α/β, PRAS40, and Akt (Ser^{473}) in BT474 cells treated for 5 h with GSK690693. Actin was used as a loading control. *B,* U2OS cells stably expressing FOXO3A-GFP were treated for 90 min with the indicated concentrations of GSK690693 and analyzed by fluorescence microscopy.

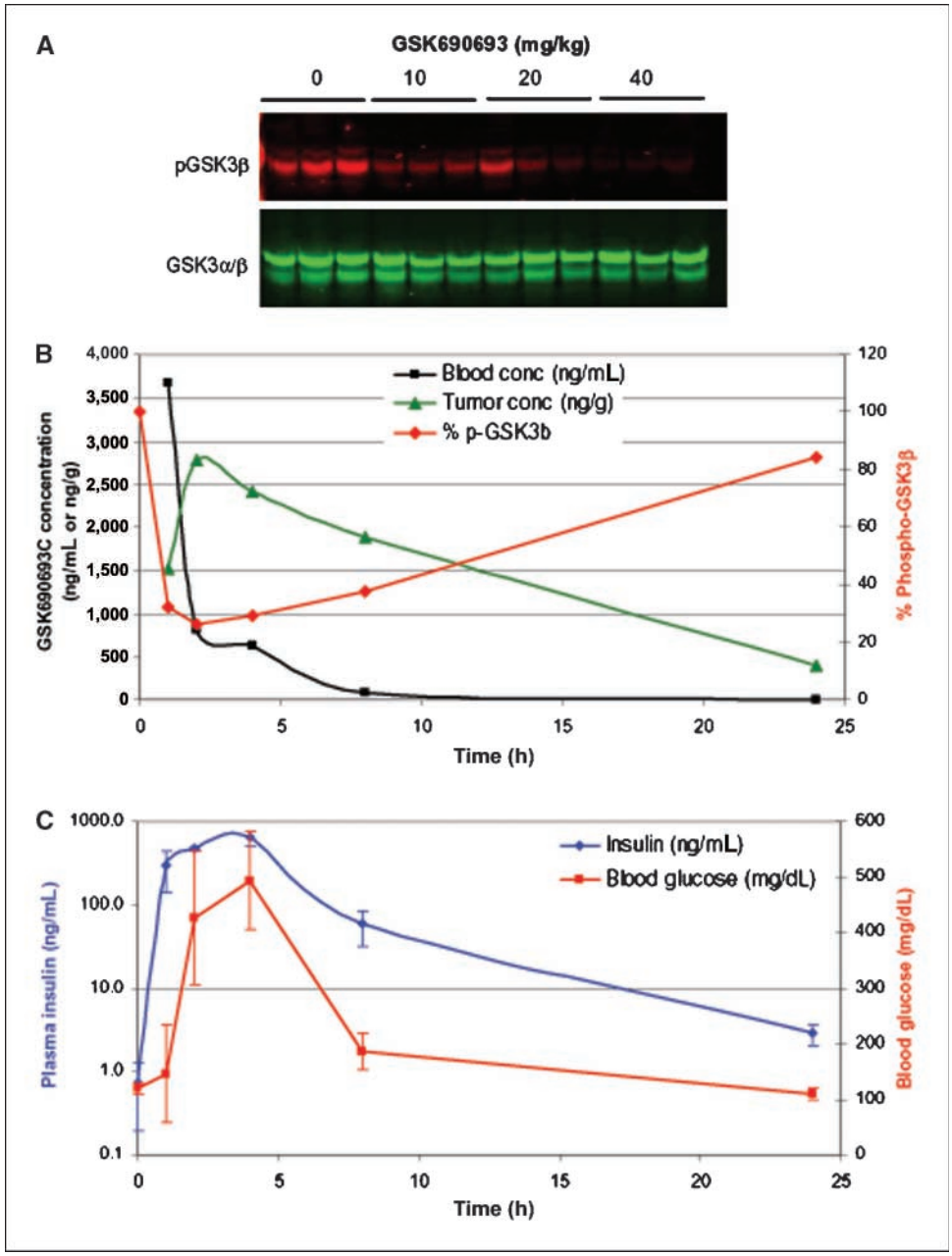


Figure 2. Pharmacokinetic, pharmacodynamic, and metabolic effects of GSK690693 in mice with BT474 xenografts. *A,* GSK690693 was evaluated in SCID mice (three mice per group) bearing BT474 tumor xenografts for its effect on GSK3β phosphorylation. Established tumors (200–400 mm^3) were collected 4 h after a single i.p. administration of GSK690693 given at 0 (vehicle), 10, 20, or 40 mg/kg. GSK690693 showed a dose-dependent inhibition of GSK3β phosphorylation in BT474 xenografts with a maximum of 87% inhibition at the highest dose. *B,* a time course of the PD effect was performed using a 20 mg/kg single i.p. dose of GSK690693 [in 4% DMSO/40% HP-β-CD in water (pH 6.0)]. Blood and tumor levels of GSK690693 were determined. *C,* blood glucose and plasma insulin levels were determined in the time course study using 20 mg/kg GSK690693.

phosphorylation of the Akt substrates, PRAS40, and FKHR/FKHRL1 (Fig. 4). No significant change in proliferation (Ki-67) or apoptosis (activated caspase-3) were observed between vehicle and drug-treated samples using IHC (data not shown).

Discussion

The present study describes the pharmacologic characterization of a novel pan Akt kinase inhibitor, GSK690693, which belongs to the aminofurazan compound class. GSK690693 is ATP competitive and exhibits time-dependent inhibition of the Akt isoforms. Selectivity of GSK690693 was assessed in a broad kinase panel. In addition to Akt, 13 kinases of the >250 unique kinases tested show IC_{50} values ≤100 nmol/L, suggesting a high degree of kinase selectivity. Most of the kinases inhibited by GSK690693 belong to the AGC kinase family that includes Akt enzymes. AMPK and DAPK3, members of the CAMK family, showed 25- and 40-fold lower potency compared with Akt1. GSK690693 also inhibited members of group II PAK kinase family (31).

Akt1, 2, and 3 is activated in tumor cells by a number of upstream stimuli, such as activation of various receptor tyrosine kinases, PI3K, Ras, or by ligand-induced cell signaling. Akt, in turn, mediates its effect via regulation of a variety of downstream substrates (2). We measured the phosphorylation status of several Akt substrates (Fig. 1; Table 2) as a way to evaluate inhibition of Akt kinase activity in cells. GSK690693 caused a dose-dependent reduction in the phosphorylation state of multiple proteins downstream of Akt such as GSK3β, PRAS40, p70S6K, and FKHR/FKHRL1 in tumor cells. Treatment of tumor cells with GSK690693 led to a dose-dependent increase in the nuclear accumulation of the

transcription factor FOXO3A, which shows a functional effect of Akt inhibition by a change in the phosphorylation status of FOXO3A protein (25).

Several recent studies have shown that mTOR inhibition results in an up-regulation of PI3K/Akt/mTOR signaling by relieving the negative p70S6K-mediated feedback inhibition on IRS-1 (32–34). Furthermore, Han et al. (24) described mTORC1-independent regulation of Akt phosphorylation upon treatment with the Akt kinase inhibitor, A-443654. Consistent with these previous studies, GSK690693 treatment resulted in a dose-dependent increase in the phosphorylation of Akt at both Ser^{473} (Fig. 1) and Thr^{308} (data not shown). Phosphorylation of proteins that are either upstream of Akt or involved in Akt-independent pathways, e.g., EGFR, ErbB2, MAPK, etc., was not altered on treatment with GSK690693,

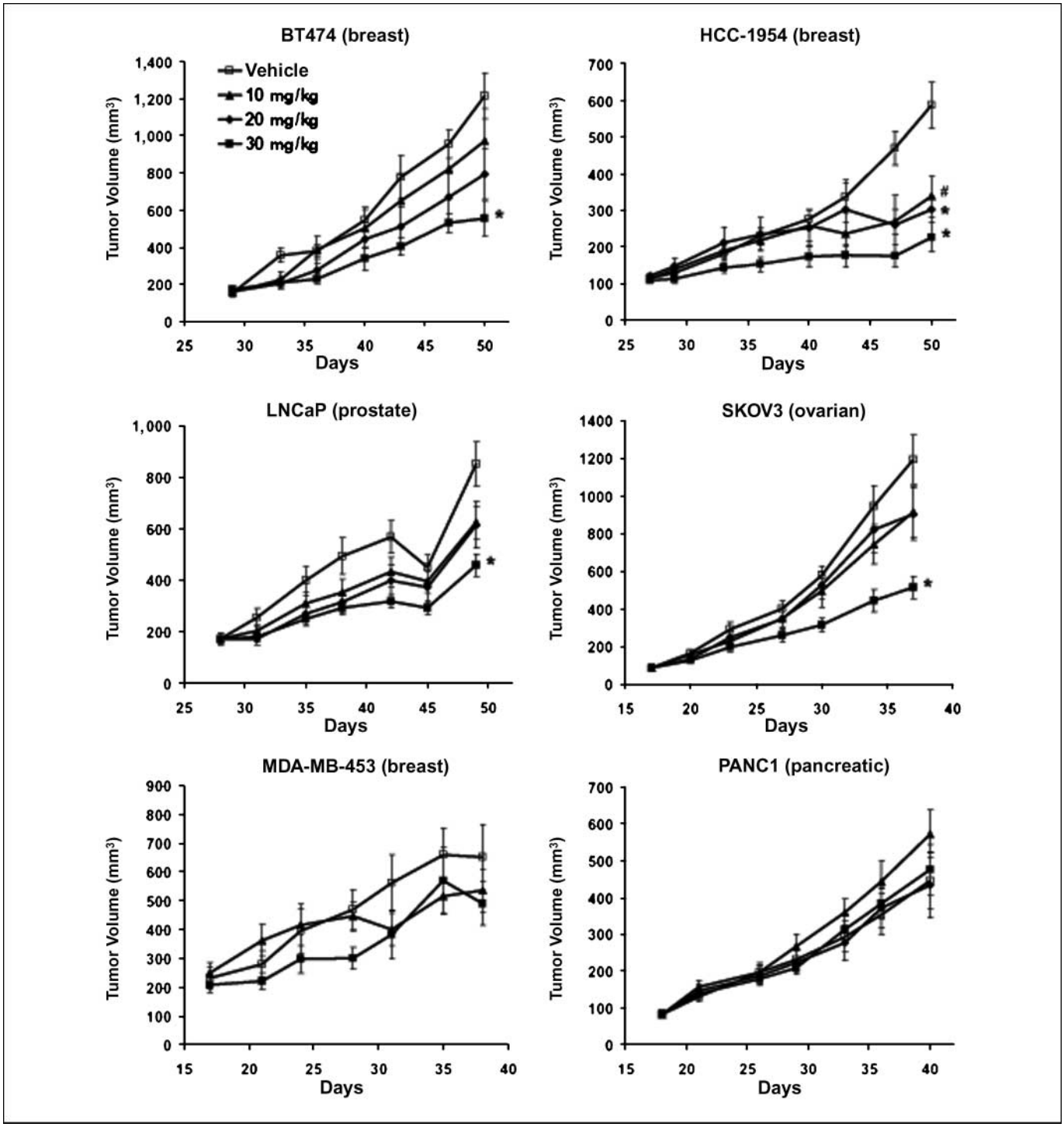


Figure 3. Antitumor activity of GSK690693 *in vivo*. Mice bearing established human tumor xenografts were given i.p. administration of vehicle 5% dextrose (pH 4.0) or GSK690693 (10, 20, or 30 mg/kg), once daily for 21 d. *Points,* mean (n = 8–12 mice per group); *bars,* SE. *, $P < 0.01$; #, $P < 0.05$, significantly different from vehicle-treated animals on day 21 of treatment.

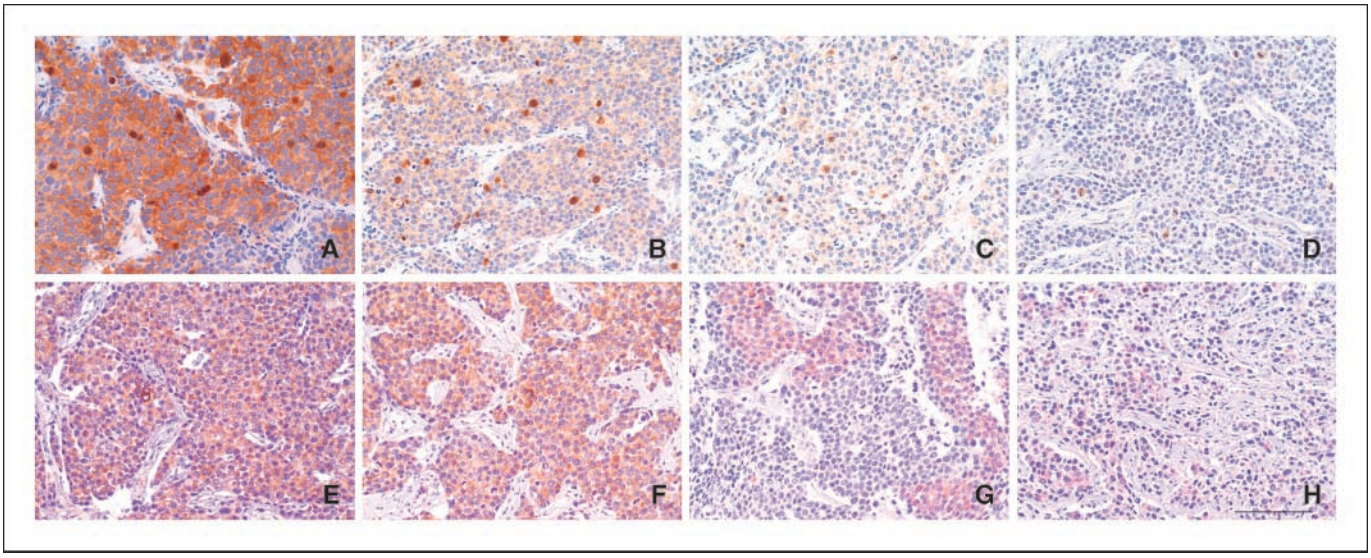


Figure 4. Inhibition of Akt substrates in tumor xenografts. Mice bearing BT474 tumor xenografts were treated with vehicle (*A* and *E*), 10 mg/kg (*B* and *F*), 20 mg/kg (*C* and *G*), or 30 mg/kg (*D* and *H*) GSK690693, once daily. Tumor sections were stained with antibodies for phospho-PRAS40 (Thr^{246}; *A–D*) on day 21 and phospho-FKHR/FKHRL1 ($Thr^{24/32}$; *E–H*) on day 3. *Bar,* 100 μ.

suggesting an Akt-specific feedback mechanism. Up-regulation of Akt activity after treatment with a mTOR inhibitor has been hypothesized to attenuate the antiproliferative effect of these agents due to mTOR-independent effects of Akt (32, 35). However, as shown by the reduction in the phosphorylation of multiple Akt substrates, GSK690693 effectively inhibited Akt kinase activity in cells (Fig. 1) regardless of any feedback hyperphosphorylation of Akt.

GSK690693 showed an antiproliferative effect in a subset of tumor cell lines tested, suggesting that Akt is one of several factors effecting the growth and survival of tumor cells. Our data show that the lack of antiproliferative effects were not due to a lack of Akt inhibition in the insensitive tumor cells, as the IC_{50} for inhibition of GSK3β phosphorylation was similar in both sensitive and insensitive cell lines (Table 2). This observation is not surprising given the abundance of genetic alterations associated with tumorigenicity and redundancy in cellular signaling. Akt1 and 2 siRNA inhibited the proliferation of BT474 and LNCaP cells, with no significant effect on SKOV-3 and OVCAR-3 cells (Supplementary Fig. S2). These results are consistent with the effects observed with GSK690693, although others have shown ~50% decrease in OVCAR-3 cell proliferation with Akt2 siRNA (36). The differences between our results and earlier reports can be due to differences in reagents and/or experimental conditions. Furthermore, potential off target effects of siRNAs have also been observed in some cases (37, 38). The correlation between *in vitro* sensitivity and activity in tumor xenografts with GSK690693 was reasonable with the exception of SKOV-3, which seems to be more sensitive as a xenograft. These differences can be attributed to differences in availability of growth factors and cellular signaling between cell culture and *in vivo* anchorage-independent growth as well as differences in the drug exposure. We are currently investigating the molecular markers of sensitivity and resistance to GSK690693. Combination of GSK690693 with other targeted agents and standard chemotherapeutic agents is ongoing to further explore the full therapeutic potential of Akt inhibition.

In addition to the potent inhibition of Akt kinases, GSK690693 also inhibit novel PKCs (η, θ, δ, and ε); PKCβ1; PAK-4,5,6; PKA; PKG1β; and PrkX, which can potentially contribute to the observed antitumor effect. Several PKC isozymes have been shown to play an important role in cell proliferation and tumor growth; however, certain isozymes are also involved in differentiation and inhibition of proliferation (39). PKCα, βII, θ,ι, and η are pro-oncogenic in various tissues, whereas PKCδ likely has tumor inhibitory potential in most tissues, except in the brain (40). Recent studies have elucidated the role of PAK kinases in cellular signaling through various oncogenes as well as altered expression of PAK1 and PAK4 in various cancers (31). Modulation of PKA levels by antisense has also been shown to effect cell proliferation and transformation; however, there are conflicting data in the literature on the role of PKA in human neoplasms (41, 42).

In addition to its role in cell survival and proliferation, the Akt pathway, particularly Akt2, is an integral part of the insulin signaling pathway (11, 13). *In vitro* experiments in tumor cells suggest that inhibition of Akt1 and Akt2 is necessary for antitumor effects (15). Therefore, it is expected that interruption of insulin signaling will result in hyperglycemia during treatment with drugs that are pan-Akt inhibitors. Treatment with GSK690693 resulted in acute hyperglycemia with blood glucose levels returning to baseline 8 to 10 hours after drug administration (Fig. 2). The concomitant-measured increase in circulating insulin observed after GSK690693 treatment is likely a homeostatic response to elevated glucose levels. Consistent with this is the observation that insulin level returned to baseline as the blood glucose levels declined. Daily administration of GSK690693 at the doses used in this study was well-tolerated and had no overt clinical effect in mice such as loss of body weight.

In vivo administration of GSK690693 resulted in decreased phosphorylation of Akt substrates in tumor xenografts (Figs. 2 and 4) and normal tissue (data not shown), indicating a clear pharmacodynamic effect in mice. Furthermore, daily administration of the compound inhibited growth of multiple human tumor

xenografts in mice (Fig. 3), consistent with the antiproliferative effects observed in tissue culture (Table 2). The overall pharmacologic profile of GSK690693 is consistent with a selective Akt kinase inhibitor and it is currently being evaluated in clinical trials on human cancer patients.

Acknowledgments

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References

1. Yang Z-Z, Tschopp O, Baudry A, Dümmler B, Hynx D, Hemmings BA. Physiological functions of protein kinase B/Akt. Biochem Soc Trans 2004;32:350–54.
2. Bellacosa A, Kumar CC, DiCristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res 2005;94:29–86.
3. Chen WS, Xu PZ, Gottlob K, et al. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev 2001;15: 2203–8.
4. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKB is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 2001;276:38349–52.
5. Cho H, Mu J, Kim JK, et al. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). Science 2001;292:1728–31.
6. Garofalo RS, Orena SJ, Rafidi K, et al. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKBβ. J Clin Invest 2003;112:197–208.
7. Easton RM, Cho H, Roovers K, et al. Role for Akt3/protein kinase B in attainment of normal brain size. Mol Cell Biol 2005;25:1869–78.
8. Tschopp O, Yang ZZ, Brodbeck D, et al. Essential role of protein kinase B (PKB/Akt3) in postnatal brain development but not in glucose homeostasis. Development 2005;132:2943–54.
9. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988–1004.
10. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signaling controls tumour cell growth. Nature 2006;441:424–30.
11. Bellacosa A, Testa JR, Moore R, Larue L. A portrait of AKT kinases. Cancer Biol Ther 2004;3:268–75.
12. Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene 2005;24:7482–92.
13. Altomare DA, Testa JR. Perturbations of the AKT signalling pathway in human cancer. Oncogene 2005;24: 7455–64.
14. Carpten JD, Faber AL, Horn C, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 2007;448:439–44.
15. DeFeo-Jones D, Barnett SF, Fu S, et al. Tumor cell sensitization to apoptotoic stimuli by selective inhibition of specific Akt/PKB family members. Mol Cancer Ther 2005;4:271–9.
16. Jetzt A, Howe JA, Horn MT, et al. Adenoviral-mediated expression of a kinase-dead mutant of Akt induces apoptosis selectively in tumor cells and suppresses tumor growth in mice. Cancer Res 2003;63: 6697–706.
17. Maroulakou IG, Oemler W, Naber SP, Tsichlis PN. Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. Cancer Res 2007;67:167–77.
18. Ju X, Katiyar S, Wang C, et al. Akt1 governs breast cancer progression *in vivo*. Proc Natl Acad Sci U S A 2007;104:7438–43.
19. Morgensztern D, McLeod HL. PI3K/Akt/Mtor pathway as a target for cancer therapy. Anti Cancer Drugs 2005;16:797–803.
20. Rusnak DW, Lackey K, Affleck K, et al. The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW572016, on the growth of human normal and tumor-derived cell lines *in vitro* and *in vivo*. Mol Cancer Ther 2001;1:85–94.
21. Petricoin EF, Espina V, Araujo RP, et al. Phosphoprotein signal pathway mapping: Akt/mTOR pathway activation association with childhood rhabdomyosarcoma survival. Cancer Res 2007;67:3431–4.
22. Liotta LA, Espina V, Mehta AI, et al. Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 2003;3:317–25.
23. Fang X, Yu SX, Lu Y, Bast RC, Woodgett JR, Mills GB. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc Natl Acad Sci U S A 2000;97:11960–5.
24. Han EK-H, Leverson JD, McGonigal T, et al. Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent on mTORC1 inhibition. Oncogene 2007;26:5655–61.
25. Takaishi H, Matsuzaki H., Ono Y, et al. Regulation of nuclear translocation of Forkhead transcription factor AFX by protein kinase B. Proc Natl Acad Sci U S A 1999; 96:11836–41.
26. Huang H, Cheville JC, Pan Y, Roche PC, Schmidt LJ, Tindall DJ. PTEN induces chemosensitivity in PTEN-mutated prostate cancer cells by suppression of Bcl-2 expression. J Biol Chem 2001;19:38830–6.
27. Frogacs E, Biesterveld EJ, Sekido Y, et al. Mutational analysis of the PTEN/MMAC1 gene in lung cancer. Oncogene 1998;17:1557–65.
28. Ramaswamy S, Nakamura N, Vazquez F, et al. Regulation of G_1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci U S A 1999;96:2110–5.
29. Nicholson KM, Streuli CH, Anderson NG. Autocrine signalling through erbB receptors promotes constitutive activation of protein kinase B/Akt in breast cancer cell lines. Br Cancer Res Treat 2003;81:117–28.
30. Zhao H, Dupont J, Yakar S, Karas M, LeRoith D. PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-1R expression in prostate cancer cells. Oncogene 2004;23:786–94.
31. Kumar R, Gururaj AE, Barnes CJ. P21-activated kinases in cancer. Nat Rev Cancer 2006;6:459–71.
32. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006;66:1500–8.
33. Sun SY, Rosenberg LM, Wang X, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 2005;65:7052–8.
34. Zitzmann K, De Toni EN, Brand S, et al. The novel mTOR inhibitor RAD001 (Everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. Neuroendocrinology 2007;85:54–60.
35. Abraham RT, Gibbons JJ. The mammalian target of rapamycin signaling pathway: twists and turns in the road to cancer therapy. Clin Cancer Res 2007;13:3109–14.
36. Noske A, Kaszubiak A, Weichert W, et al. Specific inhibition of AKT2 by RNA interference results in reduction of ovarian cancer cell proliferation: increase expression of AKT in advanced ovarian cancer. Cancer Lett 2007;246:190–200.
37. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotech 2003;21:635–7.
38. Fedorov Y, Anderson EM, Birmingham A, et al. Off-target effects of siRNA can induce toxic phenotype. RNA 2006;12:1188–96.
39. Hofmann J. Protein kinase C isozymes as potential targets for anticancer therapy. Curr Cancer Drugs Targets 2004;4:125–46.
40. Teicher BA. Protein kinase C as a therapeutic target. Clin cancer Res 2006;12:5336–45.
41. Cho-Chung YS, Nesterova MV. Tumor reversion: protein kinase A isozyme switching. Ann N Y Acad Sci 2005;1058:76–86.
42. Bossis I, Voutetakis A, Bei T, Sandrini F, Griffin KJ, Stratakis CA. Protein kinase A and its role in human neoplasia: the Carney complex paradigm. Endocr Relat Cancer 2004;11:265–80.