GSK690693 Delays Tumor Onset and Progression in Genetically Defined Mouse Models Expressing Activated Akt

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Abstract

Purpose: Akt plays a central role in regulating tumor cell survival and cell cycle progression and is regarded as a promising therapeutic target. We used genetically defined mouse models that develop spontaneous tumors exhibiting activated Akt to determine if Akt inhibition by GSK690693 is effective in the treatment of cancer. The broad long-term objective of this project was to use preclinical cancer models with precisely defined genetic lesions to elucidate the efficacy of targeting Akt with GSK690693.

Experimental Design: We tested the in vivo effects of GSK690693 in Lck-MyrAkt2 transgenic mice that develop lymphomas, heterozygous Pten−/− knockout mice that exhibit endometrial tumors, and TgMI-SHR-TAg-DR26 mice that develop ovarian carcinomas, all of which exhibit hyperactivation of Akt. In addition to standard disease onset and histology, tumors arising in treated animals were examined by immunohistochemistry to verify downregulated Akt signaling relative to placebo-treated mice. When possible, drug response was evaluated in tumor cell cultures by standard proliferation and apoptosis assays and by immunoblotting with various phosphospecific antibodies.

Results: GSK690693 exhibited efficacy irrespective of the mechanism of Akt activation involved. Interestingly, GSK690693 was most effective in delaying tumor progression in Lck-MyrAkt2 mice expressing a membrane-bound, constitutively active form of Akt. Both tumors and primary cell cultures displayed downregulation of the Akt pathway, increased apoptosis, and primarily decreased cell proliferation.

Conclusion: These results suggest that GSK690693 or other Akt inhibitors might have therapeutic efficacy in human cancers with hyperactivated Akt and/or a dependence on Akt signaling for tumor progression.

Akt/protein kinase B kinases regulate a plethora of diverse cellular processes that contribute to tumor progression, including cell proliferation and survival, cell size and response to nutrient availability, tissue invasion, and angiogenesis. Hyperactivation of Akt signaling in numerous sporadic human cancers, as well as in several dominantly inherited cancer syndromes, is commonly attributed to activation or overexpression of tyrosine kinase growth factor receptors, mutation/overexpression of phosphoinositide 3-kinase (PI3K), or inactivation/downregulation of the PTEN tumor suppressor, a negative regulator of Akt signaling (reviewed in ref. 1). Consequently, there is considerable interest in targeting the PI3K/Akt pathway as a therapeutic strategy to combat human cancer.

GSK690393 is a novel ATP-competitive, pan-Akt inhibitor with strong selectivity for the Akt kinases (2). Initial studies showed potent pharmacodynamic and antitumor activity in several human tumor cell lines and xenografts (3) and more recently was shown to induce growth inhibition and apoptosis in acute lymphoblastic leukemia cell lines (4). To complement these studies, we used several genetically defined mouse models of cancer to assess the efficacy of GSK690693 in a preclinical setting. GSK690693 has been evaluated in a dose escalation phase I clinical trial in patients with lymphoma or solid tumors (5, 6).

Frequent hyperactivation of Akt kinases has been identified in a wide assortment of human solid tumors and hematologic malignancies (reviewed in ref. 1). Among the various mechanisms that contribute to activation of the Akt pathway in human tumors are perturbations of upstream PTEN and PI3K through somatic genetic and/or epigenetic changes, activation of PI3K due to autocrine or paracrine stimulation of receptor tyrosine kinases, overexpression of growth factor receptors such as the epidermal growth factor receptor, and/or Ras activation. Because the Akt signaling cascade is frequently deregulated...
Translational Relevance

We hypothesized that targeted inhibition of Akt, an important mediator of cell signaling pathways considered to be hallmarks of cancer, can impede tumor growth and progression. Three different genetically defined mouse models that develop spontaneous thymic lymphoma, endometrial carcinoma, or ovarian cancer showing hyperactivation of Akt were used to test a novel pharmaceutical agent, GSK690693, which directly targets Akt activity. GSK690693 was found to have efficacy in each of these preclinical models. Moreover, immunohistochemical analysis of tumor specimens and experiments on tumor-derived cell cultures showed that phosphorylation of downstream effectors of Akt were inhibited in vivo and in vitro, respectively, in response to GSK690693. These results are translational because they indicate that GSK690693 or other inhibitors of this class of drug might have therapeutic efficacy in human cancers with hyperactivated Akt and/or a dependence on Akt signaling for tumor progression.

in many types of cancer and has implications with regard to tumor aggressiveness and chemoresistance, there is potential utility in targeting components of the Akt pathway for cancer therapy and, possibly, cancer prevention.

Transgenic and knockout mouse models are valuable for delineating the role of Akt kinases in vivo. As preclinical models for testing the potential therapeutic efficacy of targeting Akt signaling with GSK690693, we used transgenic mice in which the Lck promoter drives expression of membrane-bound, myristylated Akt (MyrAkt) in early thymocyte development. The Lck-MyrAkt2 transgenic mice develop spontaneous, aggressive thymic lymphomas within 10 to 20 weeks (7–9), with the added advantage that the mutant transgene bypasses the need for activation of phosphoinositide 3,4,5-trisphosphate and phosphoinositide 4,5-bisphosphate generated by PI3K and thus cannot be inhibited by Pten. The Lck-MyrAkt2 model exhibits recurrent chromosomal rearrangements that result in overexpression of c-Myc, which is frequently observed in human lymphomas and postulated to cooperate with activated Akt to drive tumor formation (10, 11).

To further test the efficacy of drug treatment with GSK690693, we employed a Pten<sup>−/−</sup> knockout model that is susceptible to endometrial neoplastic lesions with full penetrance and characterized by activation of Akt in the endometrium (12). The Pten<sup>−/−</sup> model has relevance to human cancer in that loss of PTEN is one of the earliest detectable abnormalities in the endometrioid subtype of human endometrial cancer, and loss of PTEN results in deregulation and subsequent constitutive activation of Akt kinase (12). In addition, we also employed a transgenic mouse model of spontaneous epithelial ovarian cancer with expression of SV40 Tag tag under transcriptional control of the MISIIR promoter (13), which we used previously to test a chemoprevention strategy for targeting Akt/mTOR signaling with RAD001 (everolimus; Novartis Pharma; ref. 14). SV40 tag binds protein phosphatase PP2A and inhibits its activity, resulting in activation of PI3K/Akt and mitogen-activated protein kinase signaling (15), and SV40 Tag binds to and functionally inactivates products of the Tp53 and Rb1 genes, which are frequently mutated in human ovarian cancer (16).

Overall, we found that genetically defined murine tumor models known to be strongly dependent on Akt activity for tumor development exhibited marked response to GSK690693 in terms of delayed tumor progression, decreased phosphorylation of downstream targets of Akt, and decreased cell proliferation and/or increased apoptosis. Collectively, the pharmacologic profile of GSK690693 is consistent with a selective Akt kinase inhibitor, and elevated Akt phosphorylation in tumors may be considered a useful indicator of patients who may benefit from the use of an Akt kinase inhibitor.

Materials and Methods

Reagents. GSK690693 is an Akt kinase inhibitor derived from the aminofurazan chemical series synthesized at GlaxoSmithKline. For all in vitro studies, GSK690693 was dissolved in DMSO at a concentration of 10 mmol/L before use. For the tumor xenograft studies, GSK690693 was formulated in 5% dextrose (pH 4.0).

Anti-P-Akt (Ser<sup>473</sup>), anti-Akt, P-Akt blocking peptide, anti-P-mTOR (Ser<sup>2444</sup>), anti-mTOR, anti-P-p70S6K (Thr<sup>389</sup>), anti-p70S6K, anti-P-GSK3α/β (Ser<sup>21/9</sup>), anti-GSK3α/β, anti-P-FOXO1/3 (Thr<sup>24/32</sup>), P-FOXO1/3 blocking peptide, anti-FOXO, anti-P-PRAS40/Akt1s1 (Thr<sup>465</sup>), anti-PRAS40/Akt1s1, and anti–cleaved caspase-3 antibodies were from Cell Signaling. Anti–β-actin was from Sigma and anti–Ki-67 was obtained from Vector Laboratories. Anti-mouse Ki-67 rat monoclonal antibody was from Dako.

Transgenic mice and treatments. Animal experiments were approved by our Institutional Animal Care and Use Committee in accordance with NIH guidelines. Genetically defined mouse models were genotyped by PCR using previously described methodology (9, 12, 13). Treatment regimens for each mouse model were customized based on previously reported tumor latency of untreated mice. For each study, mice were assigned to two groups receiving either GSK690693 or placebo. For in vitro drug studies of the Lck-MyrAkt2 transgenic mouse model, GSK690693 was injected intraperitoneally at a dose of 30 mg/kg/d for 5 days/wk. Treatment was begun at age 8 weeks and continued for 4 weeks' duration, at which point all mice were euthanized. Treatment of Pten<sup>−/−</sup> knockout mice with 30 mg/kg GSK690693 was initiated at age 5 months and consisted of three cycles of 3 weeks of intraperitoneal injections, followed by 1-week rest, for a total treatment duration of 12 weeks. Treatment of TgMISIIR-Tag-DR26 mice with 30 mg/kg GSK690693 was initiated at 14 weeks and continued for 4 weeks' duration. For all
preclinical studies, mice were weighed weekly, and dosage was adjusted accordingly, so that the dose could be decreased if there was weight loss. No significant weight loss of >10% of the initial body weight was observed in the GSK690693- or placebo-treated groups. Tumor volumes in Lck-MyrAkt2 and TgMISIIR-TAg-DR26 mice were calculated as follows: 

\[ V = L \times W \times D / 6, \]

where \( V \) is volume, \( L \) is length, \( W \) is width, and \( D \) is depth. \( Pten^{-/-} \) mice were

**Fig. 1.** GSK690693 delays thymic tumor development in Lck-MyrAkt2 mice. A, percentage of mice that retained normal health (10% in placebo group versus 48% in treated group) or that developed hyperplasia or thymic lymphoma (90% in placebo group versus 52% in the treated group). B, tumors that developed in GSK690693-treated mice were diminished in size (112 mm\(^3\)) compared with those that developed in the placebo-treated group (231 mm\(^3\)). \( P = 0.026, \) Student's \( t \) test. C, a significant difference between Ki-67 staining in placebo- and GSK690693-treated mice (\( P = 0.001, \) \( t \) test). The number of positively staining tumor cells from five fields of five placebo-treated mice and five GSK690693-treated mice were scored using a \( \times 40 \) objective (0.075 mm\(^2\) field), and values were normalized relative to staining in placebo-treated mice. Bars, SE. D, immunohistochemical staining of thymic lymphomas arising in Lck-MyrAkt2 transgenic mice. Pictures were taken using a \( \times 20 \) objective. P-Akt staining was strongly localized to the cytoplasm and plasma membrane (indicative of MyrAkt2 expression) in tumors from placebo-treated mice. P-Akt staining was relatively unchanged in GSK690693-treated mice. P-FOXO1/3 generally exhibited cytoplasmic staining of ++ to + in placebo-treated mice and + to +/- in GSK690693-treated mice, with staining in the latter sometimes nuclear.
examined histologically for lesions. Tumors from Lck-Myr-Akt2 and TgMISIIR-TAg-DR26 mice also were examined histologically.

Immunohistochemistry. Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. Slides containing formalin-fixed, paraffin-embedded samples were deparaffinized, hydrated in water, and subjected to antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). Anti–P-Akt (Ser473), anti–P-FOXO1/3 (Thr24/32), anti–Ki-67, and anti–cleaved caspase-3 (each defined above) were detected with biotinylated secondary antibodies. Specificity for anti-Akt and anti-FOXO1/3 antibodies was confirmed by preincubation with antigen-specific blocking peptide. Tissue sections were stained with 3,3′-diaminobenzidine chromogen and counterstained with hematoxylin.

Photographs of endometrium and ovarian tumors were captured with an Eclipse E600 microscope (Nikon Instruments) fitted with a Nikon DXM1200 digital camera. Nikon ACT-1 version 2 software was used for acquisition of digital pictures using a ×40 objective. Photographs of thymic lymphomas were captured with an Arcturus PixCell IIe microscope (Molecular Devices) using a ×20 objective and version 2.0.0 software.

Ki-67 stain was scored counting either the percent of stained nuclei (endometrial lesions; n = 400-500 per slide, five slides per lesion and treatment group) or the number of stained nuclei per high magnification field (lymphomas and ovarian tumors; at least five fields per tumor and treatment group). The other immunohistochemical stains were scored using a semiquantitative scale based on stain intensity: 0, negative; +/−, marginal; 1+, low intensity stain; 2+, moderate intensity stain; and 3+, very intense stain.

Cell culture. T cells were isolated from thymic lymphomas of Lck-MyrAkt2 mice by passing tumor tissue through a 100 μm nylon mesh (BD Falcon) and culturing in Iscove’s modified Dulbecco’s medium containing 20% fetal bovine serum as described previously (9). Mouse ovarian carcinoma (MOVCAR) cell lines were obtained from ascites of TgMISIIR-TAg mice (13). Cell lines were cultured at 37°C in DMEM containing 4% fetal bovine serum, 1% 1× ITS, penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), and 2 mmol/L glutamine in a humidified atmosphere of 5% CO2. Primary mouse cell cultures were derived from different mice before this study. SKOV3 cells from the American Type Culture Collection were cultured in McCoy’s 5A with 10% fetal bovine serum. Experiments were done with growth medium containing 10% serum.

Cell viability assay. A MTT assay was used to analyze the effect of GSK690693 on cell viability. Cells were cultured overnight in 96-well plates (5 × 103 per well). Cell viability was assessed after addition of GSK690693 at the indicated concentrations for 72 h. In brief, the tetrazolium compound MTT was added to the wells, and the cells were incubated 2 h at 37°C. Detergent was added to the wells to solubilize the formazan dye crystals for 4 h at 37°C, and the absorbance at 595 nm was determined. Cell viability is expressed as follows: $A_{\text{experimental group}} / A_{\text{control}} \times 100$.

In vitro detection of apoptosis or cell cycle arrest. Cells were treated for 72 h with 0, 10, or 20 μmol/L GSK690693 and then double-labeled with propidium iodide and Annexin V for flow cytometry analysis. Analysis of apoptosis was done with a FACScan (Becton Dickinson) using CellQuest software (Becton Dickinson). Alternatively, cells were lysed, and DNA fragmentation was detected using a Cell Death Detection ELISA Kit (Roche) per the manufacturer’s instructions.

For cell cycle analysis, cells were treated for 72 h with 0, 10, or 20 μmol/L GSK690693, fixed in 70% ethanol at ~20°C, and then washed and stained with 10 μg/mL propidium iodide (Sigma). Cell cycle analyses were done with fluorescence-activated cell sorting using FlowJo software (Tree Star).

Western blot analysis. Cells were treated with either DMSO (vehicle) or 10 μmol/L GSK690693 for 8 h (thymic lymphoma cells) or overnight (ovarian cells). Cells were washed twice with ice-cold PBS and transferred to lysis buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L 4-(2-aminoethyl)benzenesulfonfluoride hydrochloride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1% Triton X-100] for 10 min at 4°C. Lysates were centrifuged at 12,000 × g at 4°C for 15 min, and protein concentrations of the supernatants were determined using Bio-Rad protein assay reagent. Equal amounts of proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. Blocking was done with 5% nonfat milk in 1× TBS. Western blot analyses were carried out with various specific primary antibodies. Immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system (Perkin-Elmer). Western blot results were confirmed in at least duplicate or triplicate runs.

Results

We recently described independently derived founder lines in which the Lck promoter was used to direct expression of myristylated, constitutively active Akt2 in immature T lymphocytes (9, 10). Tumors from Lck-MyrAkt2 founder line 55 exhibited a median tumor latency of 16.5 weeks (10) and activated Akt was found in histologically normal thymus from 4-week-old transgenic mice as well as in thymic lymphomas (9, 10).

Overall, GSK690693 delayed tumor development and reduced the size of tumors in Lck-MyrAkt2 transgenic mice. Nearly 50% of the 31 GSK690693-treated mice had normal thymic histology, whereas 90% of the 31 placebo-treated mice developed thymic lymphomas or hyperplasia (Fig. 1A). Analysis of the resulting tumors from each group revealed that the average size of the 22 thymic lymphomas from the placebo-treated group was ~2-fold larger than the 11 thymic lymphomas found in the treated group (Fig. 1B). Thus, GSK690693 was efficacious in delaying
tumor development in a mouse model genetically engineered to express constitutively active Akt. Moreover, immunohistochemical analysis of the thymic lymphomas derived from GSK690693-treated Lck-MyrAkt2 mice showed decreased staining for Ki-67, a marker of cell proliferation (Fig. 1C). Tumors also exhibited altered expression of phosphoproteins in the Akt signaling pathway in response to GSK690693 treatment (Fig. 1D). Specifically, as shown previously (3), diminished cytoplasmic P-FOXO1/3 and/or presence of nuclear P-FOXO1/3 was indicative of GSK690693 efficacy.

Because various human tumor cell lines have been tested previously for response to GSK690693 (3), we tested tumor cell cultures derived from the preclinical models for in vitro response to GSK690693. Dissociated thymic tumor cells were prepared from Lck-MyrAkt2 mice and shown by MTT assays to be highly sensitive to GSK690693 treatment (Fig. 2A). Primary tumor cells from mice 55-1143 and 55-2180 were found to have a lower IC₅₀ (IC₅₀ = 0.3 μmol/L) compared with 55-228 (IC₅₀ = 5 μmol/L) after 72 h of treatment. We then analyzed primary tumor cells for the effects of GSK690693 on the phosphorylation of Akt and downstream targets by immunoblotting with various phosphospecific antibodies (Fig. 2B). Thymic lymphoma cells exhibited downregulation of P-GSK3α/β, P-mTOR, P-p70S6K, and P-Akt1s1 and upregulation of P-Akt following an 8-h treatment with 10 μmol/L GSK690693. The upregulation of Akt phosphorylation is indicative of a feedback loop to Akt (3). However, the collective decreased phosphorylation of downstream effectors of Akt, decreased Ki-67 staining (Fig. 1C), and increased cleavage of caspase-3 are consistent with in vivo activity of GSK690693 seen in Lck-MyrAkt2 mice.

Moreover, the effectiveness of GSK690693 in inducing apoptosis also was assessed by flow cytometry analysis of tumor cells stained with propidium iodide and Annexin

![Fig. 2. GSK690693 inhibits cell viability and Akt signaling in primary tumor cells from Lck-MyrAkt2 mice.](image-url)

A lymphoma cell line was treated with various concentrations of GSK690693 for 72 h and assessed by MTT cell viability assay. Graphs represent percent viability of GSK690693-treated cells compared with corresponding untreated cells. Bars, total SD among replicate samples. **B**, Western blot analysis of thymic lymphoma cells treated with 10 μmol/L GSK690693 for 8 h. Cell lysates were screened with antibodies against components of the Akt signaling cascade to detect diminished phosphorylation of downstream targets.
Thymic lymphoma cells from Lck-MyrAkt2 mice responded rapidly to treatment with GSK690693, with a 2- to 3-fold increase in apoptotic cells observed within 24 h (Fig. 3).

To further investigate the efficacy of GSK690693 in another preclinical model, we used heterozygous Pten knock-out mice in a pure 129/Sv strain (12). In this model, all Pten−/+ female mice develop multiple hyperplastic lesions in the endometrium starting at age 3 months. Complete loss of Pten expression is common in small early lesions, with consequent activation of Akt. At ages 5 to 9 months, lesions progress from extensive atypical hyperplasia to in situ carcinomas. Locally invasive carcinomas, defined by clear myometrial/serosal invasion, occur in 30% of the mice, and most animals succumb by age 10 months. Overall, characteristics of the tumors closely mimic the natural progression and the pathologic features of the endometrioid subtype of endometrial cancer in humans.

As summarized in Fig. 4A, 80% of placebo-treated mice progressed to atypical endometrial hyperplasia compared with 30% of the GSK690693-treated mice. The group of atypical hyperplasias also contained more advanced cases that were classified as complex atypical hyperplasias. Further histopathologic assessment revealed that four mice in the placebo-treated group had possible carcinomas in contrast to no mice in the treated group. Thus, as in Lck-MyrAkt2 mice, GSK690693 was found to be efficacious in a second mouse model genetically engineered to express activated Akt. Because no primary cell cultures were available from the Pten−/+ mouse model, immunohistochemical staining was used to confirm reduced Ki-67 staining indicative of diminished cell proliferation (Fig. 4B) and downregulation of signaling downstream of Akt (Fig. 4C).

Additionally, we employed a transgenic mouse model of spontaneous epithelial ovarian cancer, in which tumors are initiated by expression of the early region of SV40 under transcriptional control of the 5’ upstream regulatory region of the Müllerian inhibiting substance type II receptor gene (MISIIIR). Female TgMISIIIR-TAg-DR26 transgenic mice develop bilateral ovarian tumors with variable latency and survive an average of 22 weeks, and the tumors recapitulate the phenotype of human ovarian serous cystadenocarcinoma (13). At ages 9 to 14 weeks, some tumors are still confined within the ovary, and strong immunoreactivity for Tag, P-Akt, P-mTOR, and P-p70S6K are detected in both early ovarian lesions and more advanced tumors, consistent with activation of the Akt pathway (14).
Overall, there was a tendency for GSK690693 to delay ovarian tumor progression in TgMISIIR-TAg-DR26 mice (Fig. 5A). All mice developed poorly differentiated ovarian carcinomas, although only 2 of 25 (8%) placebo-treated mice were interpreted to have early disease, whereas 6 of 23 (26%) GSK690693-treated mice had early or very early disease. Although all mice exhibited histologic evidence of bilateral disease, significantly more GSK690693-treated mice needed microscopic evaluation for detection of the tumors (16 of 46 ovarian tumors, 35%) compared with the placebo-treated mice (4 of 50 ovarian tumors, 8%; Supplementary Table S1; $P = 0.003$, $\chi^2$ test). Immunohistochemical staining showed diminished staining for Ki-67 and downstream P-FOXO1/3 (Fig. 5B and C).

Thus, GSK690693 had a modest effect in delaying tumor development in the TgMISIIR-TAg-DR26 mice, perhaps due to the fact that tumor progression in this mouse model is compounded by deregulation of p53 and Rb due to the expression of large T antigen, in addition to activation of Akt via deregulation of the phosphatase PP2A by small T antigen (reviewed in ref. 15). Moreover, compared with control human SKOV3 ovarian carcinoma cells (which are characterized by constitutive activation of Akt), one isolate of mouse ovarian carcinoma (MOVCAR5) cells was sensitive to GSK690693 and another isolate (MOVCAR6) was only marginally responsive to GSK690693 treatment as assessed by MTT assay (Fig. 6A). Even after 72 h of treatment, MOVCAR5 and MOVCAR6 cells did not exhibit a significant amount of apoptosis, although MOVCAR5 cells did exhibit ~50% increase in cell cycle arrest in G1 phase (Fig. 6C). These findings, along with the diminished Ki-67 staining in the GSK690693-treated tumors, suggest that response to the drug in ovarian tumor cells from TgMISIIR-TAg mice occurs via inhibition of cell cycle progression. Immunoblotting of ovarian tumor cells with phosphospecific antibodies following overnight treatment of tumor cells with 10 $\mu$mol/L GSK690693 showed that MOVCAR cells and control SKOV3 cells exhibited decreased expression of P-GSK3, P-FOXO1, and P-p70S6K with a lesser effect on P-FOXO3a and P-mTOR (Fig. 6B).

**Discussion**

**Akt** was first identified as an oncogene transduced by a murine retrovirus that induced thymic lymphomas (17),
and Akt kinases are frequently hyperactivated in human solid tumors and hematologic malignancies (reviewed in ref. 18). Our data show that Akt inhibition with GSK690693 delays tumorigenesis in several preclinical models of spontaneously arising tumors in genetically defined mice. Although GSK690693 treatment did not reduce tumor incidence, it did result in fewer mice with advanced disease.

Importantly, we did not use the same treatment regimen across all of the genetically defined mouse models, because each was known to develop spontaneous tumors with different latencies. For example, Lck-MyrAkt2 mice from founder line 55 develop aggressive thymic lymphomas with a median latency of 16 weeks (10). Consequently, we initiated treatments at age 8 weeks and continued for 4 weeks' duration, when a significant subset of untreated mice started exhibiting difficulty in breathing due to the presence of large thymic lymphomas that could constrict the heart and lungs. Interestingly, GSK690693 was most effective in delaying tumor progression in this mouse model, although the thymic lymphocytes expressed a membrane-bound, constitutively active form of Akt2 that is not dependent on upstream signaling by PI3K or Pten functionality. Previously, we have proposed that thymic lymphomas arising in these mice have a strong dependence on Akt2 for survival of the tumor cells (10). Treatment with GSK690693 delayed tumor progression as evidenced by a dramatic change in histopathology from the presence of thymic lymphomas in ~90% of the placebo-treated mice to a prevalence for hyperplasia or normal health in ~60% of the GSK690693-treated mice. Caliper measurements of thymic lymphomas arising in the remaining ~40% of the GSK690693-treated mice revealed that tumor volume was decreased by >2-fold compared with thymic lymphomas arising in placebo-treated mice.

A 30 mg/kg dose was used for all three models, although the schedule varied. We have reported previously the pharmacokinetic/pharmacodynamic relationship of GSK690693 in mice bearing subcutaneous xenograft models (3). As reported, the maximum concentration (C_{max}) was ~1 h or earlier with a very short half-life in mice, and almost all the drug is gone from circulation ~8 h after the intraperitoneal administration. No accumulation of drug was observed after repeat daily administration, and as such, the pharmacokinetic/pharmacodynamic relationship shown after a single dose is a fair reflection of events after repeat dosing. As reported before, >3 μmol/L concentration of GSK690693 in vivo was associated with sustained decrease in GSK3β phosphorylation in vivo for up to 8 h (3).
Pten+/- mice required the longest duration of treatment with GSK690693 relative to other preclinical models tested here. Because the hyperplastic lesions in the endometrium could be observed starting at age 3 months, with progression to atypical hyperplasia and in situ carcinomas at ages 5 to 9 months (12), treatment of Pten+/- knockout mice with GSK690693 was started at age 5 months and consisted of three cycles of 3 weeks of intraperitoneal injections followed by 1-week rest. Like the Lck-MyrAkt2 model, immunohistochemical evidence supports the fact that Akt is strongly activated in this knockout model as shown by the strong staining for P-Akt in the cytosol and plasma membrane. This preclinical model also exhibited a strong response to GSK690693 in that there was a shift in histology from the atypical hyperplasias that were observed in 80% of the placebo-treated mice to an earlier hyperplastic phenotype in the tumor progression cascade that was detected in 70% of the GSK690693-treated mice. Although increased apoptosis was not observed in tumors from GSK690693-treated Pten+/- mice, Ki-67 staining was significantly diminished, indicating that inhibition of cell proliferation is a primary response to GSK690693 in this mouse model.

In TgMISIIR-TAg-DR26 mice, 50% of females show ovarian tumors by ages 6 to 13 weeks (13), and in the present study, we wanted to determine the efficacy of GSK690693 as a therapeutic agent. Thus, treatment of TgMISIIR-TAg-DR26 mice with GSK690693 was initiated at age 14 weeks and continued for 4 weeks’ duration. In contrast, in our previous chemoprevention strategy with RAD001, treatment of TgMISIIR-TAg-DR26 mice was initiated at age 5 weeks and continued for 20 weeks (14). Tumor delay in our previous chemoprevention study was quite striking using this preclinical model. However, a therapeutic strategy is more relevant to the current situation in the clinic, because a chemoprevention approach...
has yet to be established for ovarian cancer. Overall, all placebo- and GSK690693-treated mice developed bilateral poorly differentiated ovarian carcinomas, although more early tumors were observed in the GSK690693-treated mice. In fact, unlike the situation in placebo-treated mice, a significant subset of tumors in GSK690693-treated mice required microscopic evaluation to be detected. This is consistent with our findings of decreased Ki-67 staining in tumors from GSK690693-treated mice and the induction of G₁ arrest in MOVCA5 cells treated in vitro with GSK690693. Thus, as in the other mouse models tested, decreased cell cycle progression appears to be the predominant effect of GSK690693 in TgMISIIR-TAg mice.

Measurement of downstream substrate phosphorylation represents an important means of assessing drug response on Akt activity. Our findings that P-FOXO1/3 cytoplasmic staining is reduced and that nuclear staining is sometimes observed in GSK690693-treated tumors from all three mouse models are consistent with previous reports showing that treatment of U2OS cells led to nuclear accumulation of FOXO (3). Moreover, we observed nuclear translocation of P-FOXO1/3 signal in normal ovarian tissue in response to GSK690693 in mice. Similarly, the effect of GSK690693 on GSKβ phosphorylation, another downstream readout of Akt activity, in normal liver was described in an earlier publication (6).

In fact, GSK690693 caused a dose-dependent reduction in the phosphorylation state of multiple proteins downstream of Akt such as P-GSK3α/β, P-mTOR, and P-p70S6K in tumor cells, in accordance with previous reports (3). However, GSK690693 treatment also resulted in a dose-dependent increase in the phosphorylation of Akt (3). An increase in Akt phosphorylation at both Ser473 and Thr³⁰⁸ sites was observed with GSK690693, consistent with the feedback mechanism observed previously with this and other Akt kinase inhibitors (3, 19). Upregulation of P-Akt levels is not unique to GSK690693, in that rapamycin and related mTORC1 inhibitors (20), as well as another Akt inhibitor, A-443654 (19), have been shown to enhance the activation of Akt via a feedback mechanism. It has been suggested that S6K-induced IRS-1 phosphorylation and mTORC2 are involved in this feedback mechanism. The upregulation of P-Akt by GSK690693 was not sufficient to rescue the downstream substrate phosphorylation.

As reported previously, GSK690693 treatment in cell culture results in some increase in apoptosis in LNCaP and BT474 cells at 24 to 48 h (3, 4). GSK690693 treatment also inhibited proliferation of a subset of tumor cell lines in vitro and inhibited growth of tumor xenografts in mice (3). Further analysis of the molecular mechanisms of GSK690693 action in cells is being investigated using phosphoproteomics and transcriptomics. Preliminary results show a predominant activation of cell cycle arrest genes with weak evidence for upregulation of proapoptotic pathways. These studies are being extended to multiple cell lines and xenografts to better understand the heterogeneity of responses (21). Collectively, our data with tumor-prone genetically engineered mice and derived tumor cells confirm previous studies that suggest that inhibition of Akt kinases regulates both cell proliferation and apoptotic pathways, although the primary effect appears to be antiproliferative in the cell lines and xenografts evaluated thus far.

The testing of GSK690693 in various models is important to address its overall potential utility in different tumors and tumor cell contexts. One cautionary note is that, in addition to the potent inhibition of Akt kinases, GSK690693 at higher concentrations can inhibit novel PKCs, PKCβ1, PAK-4, 5, 6, PKA, PKG1β, and PrkX, which can potentially contribute to the overall antitumor effect through inhibition of cell proliferation and transformation (3).

Overall, GSK690693 exhibited its greatest efficacy in tumors and tumor-derived cell lines in which there was specific, targeted deregulation of the Akt kinase, that is, those expressing myristylated, constitutively active Akt or loss of the Pen tumor suppressor protein. It is postulated that tumor cells that have an addiction for strong constitutive activation of Akt may be more sensitive to inhibition of the pathway. For example, early evidence from our laboratory suggests that thymic lymphoma cells derived from Lck-MyrAkt2 mice also are sensitive to downstream mTOR inhibition by rapamycin as shown by strong downregulation of P-p70S6K and P-4EBP1. As with GSK690693, rapamycin and its derivatives may be able to induce apoptosis under certain conditions, but the primary mechanism is to induce cell cycle arrest. Side-by-side preclinical comparisons between an Akt inhibitor such as GSK690693 and a mTOR inhibitor such as RAD001 are needed to address whether these inhibitors exhibit equivalent efficacy.

In vitro studies of primary tumor cell cultures were consistent with the in vivo findings in the corresponding mouse models and may help to explain the responsiveness of one model versus another. For example, in thymic lymphoma cell cultures from Lck-MyrAkt2 mice, treatment with GSK690693 was efficacious, and it is noteworthy that tumor cell cultures from multiple Lck-MyrAkt2 founder 55 mice have identical karyotypic findings, that is, a specific recurrent chromosomal translocation (10). On the other hand, variable responses to GSK690693 were observed in MOVCA5 cell lines from TgMISIIR-TAg mice, perhaps complicated by the fact that we have found that these cell cultures have variable cytogenetic findings, most likely attributed to the potential genetic instability that would be

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**Table 1. MOVCA5 cells treated with GSK690693**

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<tr>
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<th>% G₁</th>
<th>% S</th>
<th>% G₂</th>
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*Note: The table above shows the percentage of cells in G₁, S, and G₂ phases of the cell cycle in MOVCA5 cells treated with different concentrations of GSK690693. The data is based on in vitro studies and indicates a dose-dependent increase in G₁ arrest with increasing concentration of the inhibitor.*
expected as a result of perturbation of the p53 and p73 pathways due to the expression of SV40 large T antigen (reviewed in ref. 22). Variable responses to drug treatments in independent tumor cell lines are derived from the same animal model, either Lck-MyrAkt2 or TgMISIIR-TAg. Thus, the predisposing genetic modification in these tumor-prone mouse models may be accompanied by additional variable genetic and expression changes that influence tumor cell growth and chemosensitivity.

Of importance to this study, we have shown the utility of different genetically tumor-prone mouse models for the preclinical evaluation of a small-molecule inhibitor that targets the Akt signaling. A side-by-side comparison of GSK690693 in three different mouse models and in independently derived tumor cells derived from these mice showed that this class of inhibitor may have efficacy in delaying tumor growth and/or progression. Through preclinical testing of thymic lymphomas and endometrial and ovarian tumor models, our results provide rationale for the potential usefulness of GSK690693 in therapeutic trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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GSK690693 Delays Tumor Onset and Progression in Genetically Defined Mouse Models Expressing Activated Akt

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