Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo

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Abstract
The Akt kinases are central nodes in signal transduction pathways that are important for cellular transformation and tumor progression. We report the development of a series of potent and selective indazole-pyridine based Akt inhibitors. These compounds, exemplified by A-443654 (Ki = 160 pmol/L versus Akt1), inhibit Akt-dependent signal transduction in cells and in vivo in a dose-responsive manner. In vivo, the Akt inhibitors slow the progression of tumors when used as monotherapy or in combination with paclitaxel or rapamycin. Tumor growth inhibition was observed during the dosing interval, and the tumors regrew when compound administration was ceased. The therapeutic window for these compounds is narrow. Efficacy is achieved at doses ~2-fold lower than the maximally tolerated doses. Consistent with data from knockout animals, the Akt inhibitors induce an increase in insulin secretion. They also induce a reactive increase in Akt phosphorylation. Other toxicities observed, including malaise and weight loss, are consistent with abnormalities in glucose metabolism. These data show that direct Akt inhibition may be useful in cancer therapy, but significant metabolic toxicities are likely dose limiting. [Mol Cancer Ther 2005;4(6):977–86]

Introduction
Akt activity is elevated in a large proportion of human malignancies, where it plays a central role in inducing a malignant phenotype by both promoting cell growth and decreasing apoptosis (1, 2). Akt1 is a serine/threonine protein kinase that was first discovered as the human homologue of the transforming gene in the AKT-8 oncogenic virus, which was isolated from a spontaneous thymoma in the AKR mouse (3, 4). Since the discovery of human Akt1 (also called protein kinase B), two additional mammalian Akt isoforms, Akt2 and Akt3, have been identified (5–8).

Akt is downstream of phosphatidylinositol 3-kinase (PI3K) and is a critical node in this signal transduction pathway. The activation of Akt by PI3K is antagonized by the tumor suppressor PTEN (9). Thus, the increased Akt activity that is observed in most human malignancies could be the result of (a) an increased Akt expression, (b) increased PI3K activity, or (c) decreased PTEN activity (10). Correlative evidence for all three of these mechanisms has been found in human tumors. Akt are overexpressed in a variety of human tumors (7, 11–13), and at the genomic level, AKT1 and AKT2 have been shown to be amplified in a number of cancer types (7). PI3K activity is increased by numerous growth factors, many of which are themselves targets for cancer therapy (e.g., KDR and HER2; refs. 14–16). PTEN mutations that result in increased Akt activity have likewise been described in a wide variety of malignancies (17–19). Alterations that increase Akt activity (e.g., PTEN loss, PI3K up-regulation, and increased Akt expression) rival alterations in the p53/p16 pathway as the most common changes found in malignant tumor cells (10, 20).

In addition to the correlative data, there is also considerable experimental evidence for the importance of PI3K, Akt, and PTEN in neoplasia. The constitutive expression of active Akts promotes tumorigenesis when these Akt-expressing clones are inoculated into nude mice (21–23). Mice heterozygous for Pten deletions, where Akt activity is dramatically increased, develop neoplasms in colon, testis, thyroid, prostate, endometrium, and liver. These animals also have an increased incidence of lymphoma and leukemia (24, 25). In humans, there are three closely related and inherited cancer syndromes caused by mutations in the PTEN locus: Cowden disease (26), Lhermitte-Duclos disease (26), and Bannayan-Zonana syndrome (27). Patients with these disorders have multiple benign tumors and greatly increased incidence of carcinomas.
In Drosophila, a germ line deletion of dAkt reverses the large cell phenotype in dPen−/− flies (28). More recently, deletion of Akt1 has been shown to reverse the aggressive growth phenotype of Pen−/− mouse embryonic stem cells (29). These data suggest the tumorigenic phenotype caused by loss of PTEN can be largely reversed by inhibition of Akt.

The prevalence of Akt activation in human tumors, coupled with the genetic experiments showing that PTEN deletions or active Akt can induce tumor formation, suggests that inhibition of Akt may be useful in the treatment of neoplastic diseases. Indeed, there have been attempts to modulate the activity of this pathway. Inhibition of PI3K directly has been reported to decrease the progression of tumors in rodents (30, 31). Likewise, using rapamycin or one of its analogues to inhibit the PI3K pathway downstream at mammalian target of rapamycin (mTOR) has been shown to reduce tumor growth in mouse models (several compounds are currently in human clinical trials; refs. 32, 33). There have also been reports of compounds that inhibit the activation of Akt [e.g., heat shock protein 90 inhibitors (34), pleckstrin homology domain inhibitors (35, 36), and others (37)]. Here we report on the discovery of potent compounds that directly inhibit the kinase activity of Akt. We also report the effects of these compounds on transformed cells in vitro as well as on tumor progression in vivo.

Materials and Methods

Crystallization and X-ray Analysis

Protein kinase A (PKA) was purified (38), concentrated to 20 mg/mL, and complexed with peptide inhibitor of PKA for 1 hour and complexed with A-443654 (39). Crystals were transferred to cryosolutions that contained well solution plus increasing amounts of glycerol, soaking for 1 hour and complexed with peptide inhibitor of PKA (mTOR) has been shown to reduce tumor growth in mouse models (several compounds are currently in human clinical trials; refs. 32, 33). There have also been reports of compounds that inhibit the activation of Akt [e.g., heat shock protein 90 inhibitors (34), pleckstrin homology domain inhibitors (35, 36), and others (37)]. Here we report on the discovery of potent compounds that directly inhibit the kinase activity of Akt. We also report the effects of these compounds on transformed cells in vitro as well as on tumor progression in vivo.

Materials and Methods

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In Vitro Kinase Assays

Recombinant CK2, PKCα, and PKCθ (Calbiochem, San Diego, CA); PKA (Panvera, Madison, WI); cyclin-dependent kinase 2 (Cambridge Research, Beverly, MA); extracellular signal–regulated kinase 2 (New England Biolabs, Beverly, MA), cKit (544-976; ProQinase, Freiburg, Germany) were commercial obtained. His-tagged Akt1 (S378A, S381A, T450D, S473D; 139-480), Chk1 (1-269), KDR (789-1354), Flt-1 (786-1338), and phosphatidylinositol-dependent kinase 1 (1-396), were expressed using the FastBac baculovirus expression system (Life Technologies, Gaithersburg, MD) and purified using either nickel (his-tag) or glutathione S-transferase affinity chromatography. Peptides substrates had the general structure biotin-Ahx-peptide with the following sequences: Akt, EELSPFRGRSRSAPPNLWA-AQR; PKA, LRRASLG; PKCα, and PKCθ, ERMRRPK-RGQSVRRRV; CK2, RRADSDDDDD; cyclin-dependent kinase 2, LPPCSPKQKENGPSHTKLRRAAFDNQL; GSK3β, YRRAAVPSPSLSRHSPHS(p)EDEEE; MAPK-AP2, KKLNRITLSVA; RSK2, KKKNRTLSVA; extra-cellular signal–regulated kinase 2, KRELVEPTSGE-APNQALLR; Chk1, AKVSRSGLYRSPMPENLRPR; phosphatidylinositol-dependent kinase 1, KTFCGTPEY-LAEPYRPRILSEEQEMRFDYIADWC; KDR, Flt-1, and cKit, AEEYFLFLFA-amide. For Src assays, the biotinylated substrate PTK-2 (Promega, Madison, WI) was used. Inhibition of kinase activity was assessed using a radioactive FlashPlate-based assay platform as previously described (40).

Alamar Blue Cell Viability Assay

The cells on 96-well plates were gently washed with 200 μL of PBS. Alamar Blue reagent (BioSource International, Carmaillo, CA) was diluted 1:10 in normal growth media. The diluted Alamar Blue reagent (100 μL) was added to each well on the 96-well plates and incubated until the reaction was complete as per manufacturer’s instructions. Analysis was done using an fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA), set at the excitation wavelength of 544 nm and emission wavelength of 595 nm. Data were analyzed using SOFTmax PRO software provided by the manufacturer.

Protein Extraction and Western Blot Analysis

Cells were treated with 0 to 30 μmol/L of Akt inhibitors for 2 hours. All drug samples were adjusted to contain equal volumes of the vehicle (DMSO). Cells were harvested, sonicated for 5 minutes in ice-cold insect cell lysis buffer (BD Pharmingen, San Diego, CA; 10 mmol/L Tris-HCl (pH 7.5), 130 mmol/L NaCl, 1% Triton X-100, 10 mmol/L NaF, 10 mmol/L NaPi, 10 mmol/L NaPPi and 1 μg/mL microcystin LR plus the protease inhibitors aprotonin (10 μg/mL), leupeptin (10 μg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L)), and centrifuged at 15,300 rpm for 10 minutes. The concentrations of the total lysate protein were determined using a standard Bradford assay (Bio-Rad, San Diego, CA).

MiaPaCa-2 pancreatic tumor–bearing mice were treated with control vehicle or A-443654 at 7.5 or 75 mg/kg for 2 hours. Tumors were isolated and immediately frozen in liquid nitrogen. Frozen tumor tissues were sliced in thin sections and resuspended in 500 μL of lysis buffer. This suspension was homogenized with a Polytron PT 1200C (Kinematica AG, Luzern, Switzerland) for

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2 minutes. The homogenized tissues were centrifuged at 15,300 rpm for 10 minutes, supernatants were collected, and the protein concentrations were determined with the Bradford method.

For Western blot analysis, 40 μg of protein from the total cell lysate were electrophoresed by SDS-PAGE. The proteins were then electrotransfered onto immobilin-P membranes (Millipore, Bedford, MA), using transfer buffer (25 mmol/L Tris, 190 mmol/L glycerine, and 10% methanol). Membranes were treated with blocking buffer (50 mmol/L Tris, 200 mmol/L NaCl, 0.2% Tween 20, 5% nonfat dry milk) for 1 hour at room temperature and incubated with 1:1,000 dilution of antibodies (phospho-GSK3 α/β Ser21/9, phospho-TSC2 Thr1462, phospho-FOXO1A/3A Thr24/32, phospho-ribosomal protein S6 Ser235/236, phospho-Akt1, Akt2, or Akt3-Overexpressing Cell Lines)

Stable transfectants of FL5.12 cells expressing full-length human Akt1,Akt2, or Akt3 were generated by electroporation with a pCIneo plasmid (Promega) containing Akt cDNAs with an NH2-terminal HA-tag (41). Single clones were isolated by limiting dilution and selection for G418 resistance (Invitrogen, Carlsbad, CA). Expression was confirmed by immunoblotting with Akt isoform-specific antibodies. FL5.12/Akt cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% WEHI-3B conditioned medium as a source of IL-3, 20 mmol/L sodium pyruvate, 2 mmol/L glutamine, 50 μmol/L β-mercaptoethanol, 50 μg/mL G418, and 2.5 mmol/L HEPEs (pH 7.5) at 37°C in a humidified atmosphere containing 5% CO2.

FOXO3A Translocation Assay

The pFOXO3A-hrGFP construct was engineered by inserting a FOXO3A fragment encoding NH2-terminal amino acids 1 to 400 into plasmid phrGFP (Stratagene, La Jolla, CA) using standard PCR and DNA recombinant techniques. This sequence includes the nuclear import and export signals and Akt phosphorylation sites (42). HeLa cells were plated into 6-well plates at a density of 0.3 × 106 cells per well and incubated at 37°C at 5% CO2 over night. Cells were transiently transfected with pFOXO3A-hrGFP plasmid using Effectene (Qiagen, Valencia, CA). At 40 hours post-transfection, cells were treated with compounds added to culture medium lacking phenol red. Equal amounts of DMSO were added to cells to a final concentration of 0.5%. After 6 hours, images of fluorescent live cells were captured by microscopy.

Tumor Efficacy Studies

Animal studies were conducted following the guidelines of the internal Institutional Animal Care and Use Committee. Immunocompromised male scid mice (C.B-17-Prkdcscid) were obtained from Charles River Laboratories (Wilmington, MA) at 6 to 8 weeks of age. MiaPaCa-2 and PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). The 3T3-Akt1 cell line was developed and characterized in our laboratory (23). The 1 × 106 3T3-Akt1 or 2 × 106 MiaPaCa-2 and PC-3 cells in 50% Matrigel (BD Biosciences, Bedford, MA) were inoculated s.c. into the flank. For early treatment studies, mice were randomly assigned to treatment groups and therapy was initiated the day after inoculation. Ten animals were assigned to each group, including controls. For established tumor studies, tumors were allowed to reach a designated size and mice were assigned to treatment groups of equal tumor size (n = 10 mice per group). Tumor size was evaluated by twice weekly measurements with digital calipers. Tumor volume was estimated using the formula: 

\[ V = \frac{L \times W^2}{2} \]

A-443654 was given s.c. in a vehicle of 0.2% HPMC. A-674563 was given orally in a vehicle of 5% dextrose. Gemcitabine and paclitaxel were obtained from Eli Lilly and Company (Indianapolis, IN) and Bristol-Myers Squibb Co. (Princeton, NJ), respectively, and given according to the manufacturers’ guidelines.

For the determination of plasma drug concentrations, proteins were precipitated with two volumes of acidified methanol. Tissue samples were taken from the same animals and homogenized with 2 volumes of saline, then precipitated with 2 volumes of acetonitrile. Precipitated proteins were removed by centrifugation and supernatants were stored at −20°C until analysis by UV-liquid chromatography or liquid chromatography-mass spectrometry. Plasma and tissue extracts were injected directly on a C18 reversed phase column (YMC-AQ 5 μ; Waters Co., Milford, MA) and eluted using combinations of acetonitrile, methanol and 8 mmol/L triethylamine acetate buffer (pH 4) with UV detection (Shimadzu 10A/VP, Shimadzu Scientific, Columbia, MD) or with acetonitrile and 0.1% acetic acid in water for mass spectrometer analysis (LCQ Duo, Thermoquest, San Jose, CA). Pharmacokinetic variables were calculated using WinNonlin software (Pharsight Co., Mountain View, CA).

Immunohistochemistry

3T3-Akt1 and MiaPaCa-2 tumors were fixed for 48 hours in Streak Tissue Fixative (Streak Laboratories, Omaha, NE), processed, and embedded in paraffin; 5-μm tissue sections were blocked with streptavidin/biotin complex followed by 0.3% bovine serum albumin before incubation with primary antibody (caspase-3: rabbit anti-active Caspase-3 at 1:400, BD PharMingen; phospho-Akt: rabbit anti-phospho-Akt at 1:200, Cell Signaling Technology) for 1 hour at room temperature. Secondary antibody was used at 1:250 to 300 followed by streptavidin-biotin horseradish peroxidase and 3,3′-diaminobenzidine color development.
Results

Discovery of Indazole-Pyridine Series of Akt Inhibitors

The initial lead in this series was obtained from a high-throughput screen (Fig. 1A, compound 1). This compound weakly inhibited Akt \( (K_i = 5 \mu mol/L) \). The potency was improved by addition of an indole ring to the aliphatic side chain (Fig. 1A, compound 2), and still further improved by constraining rotatable bonds in the linker between the central and distal pyridine rings (Fig. 1A, compound 3). Addition of a second hydrogen bonding interaction to the hinge-binding region resulted in A-443654, that exhibited a \( K_i \) of 160 pmol/L, a 30,000-fold improvement in potency versus the initial lead molecule. Finally, a compound with oral activity was achieved by replacing the indole with a phenyl moiety (Fig. 1A, A-674563).

The series of indazole-pyridine compounds bind to the ATP site of Akt. These compounds are potent, ATP competitive, and reversible inhibitors of the Akt catalyzed phosphorylation activity. The three-dimensional structures of these compounds complexed with a closely related protein kinase, PKA (Fig. 1B-C), were determined by X-ray crystallography. Three hydrogen bond sets are critical to maintain potency. The first is a canonical hydrogen bond to the hinge region backbone found with most ATP-competitive kinase inhibitors. The second is formed between the inhibitor’s central pyridine ring and a conserved lysine (Lys\(^72\) in PKA and Lys\(^181\) in Akt1). The third set of important hydrogen bonding interactions form between the primary amino group of the inhibitor’s aliphatic side chain and the PKA residues Asn\(^171\) and Asp\(^284\) (Asn\(^279\) and Asp\(^292\) in Akt1). This position is normally occupied by a divalent cation of Mg\(^{2+}\) ATP.

In vitro Activity

A-443654 is a pan Akt inhibitor and has equal potency against Akt1, Akt2, or Akt3 within cells (Fig. 2). A-443654 is, however, very selective for Akt versus other kinases (Table 1). It is the least selective towards several closely related kinases in the AGC family, including PKA and protein kinase C (PKC). Even so, A-443654 is 40-fold selective for Akt over PKA. A-674563 is somewhat less selective, particularly against the cyclin-dependent kinases in the CMGC family. However, in spite of the selectivity differences between these two classes of Akt inhibitors, their behavior is similar in cells and in vivo (see below).

The phosphorylation of Akt downstream targets such as GSK3\(\alpha/\beta\), FOXO3, TSC2, and mTOR were measured as an indication of Akt activity within cells. The Akt inhibitors reduced the phosphorylation of all of these proteins in a dose-dependent fashion (Fig. 3A). As expected, we also observed that FOXO3 translocates into the nucleus upon the reversal of its phosphorylation induced by Akt inhibition (Fig. 3B). Phosphorylation of signaling molecules further downstream in the Akt pathway, such as the S6 protein, was also reduced by the Akt inhibitors.

Together with the decrease in phosphorylation of Akt targets, we observed a concomitant increase in the Thr\(^308\) and Ser\(^473\) phosphorylation of Akt. This increase has been observed with all of the Akt inhibitors tested and seems a sensitive marker of Akt inhibition. However, in spite of the increased Akt activation, the ability of Akt to phosphorylate its downstream targets was markedly decreased in the presence of the inhibitors (Fig. 3A). The addition
Figure 2. A-443654 inhibits Akt1, Akt2, or Akt3 equally within cells. Murine FL5.12 cells were stably transduced with constitutively active myristoylated human Akt1 (●), Akt2 (▲), or Akt3 (■). There is no appreciable phosphorylation of GSK3 in the parental cell lines, whereas there are high levels of phospho-GSK3 (P-GSK3) observed in all three Akt overexpressing lines. A-443654 reduces the P-GSK3 in a dose-responsive manner in all three cell lines. The broad-spectrum kinase inhibitor staurosporine was added at 1 μM/L as a positive control. The P-GSK3 was quantitated and normalized to the total GSK3.

Table 1. Selectivity of Akt inhibitors for selected kinases

<table>
<thead>
<tr>
<th>Family</th>
<th>Kinase</th>
<th>A-443654 fold (Ki, μmol/L)</th>
<th>A-674563 fold (Ki, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGC</td>
<td>Akt1</td>
<td>1 (0.00016)</td>
<td>1 (0.011)</td>
</tr>
<tr>
<td></td>
<td>PKA</td>
<td>40 (0.0063)</td>
<td>14 (0.016)</td>
</tr>
<tr>
<td></td>
<td>PKCγ</td>
<td>150 (0.024)</td>
<td>110 (1.2)</td>
</tr>
<tr>
<td></td>
<td>PKCδ</td>
<td>200 (0.033)</td>
<td>32 (0.36)</td>
</tr>
<tr>
<td></td>
<td>PDK1</td>
<td>&gt;120,000 (&gt;20)</td>
<td>&gt;1,800 (&gt;20)</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>19,000 (3.1)</td>
<td>&gt;330 (&gt;3.7)</td>
</tr>
<tr>
<td></td>
<td>cKIT</td>
<td>7,300 (1.2)</td>
<td>&gt;350 (&gt;5.8)</td>
</tr>
<tr>
<td></td>
<td>SRC</td>
<td>16,000 (2.6)</td>
<td>1,200 (13)</td>
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<td></td>
<td>FLI1</td>
<td>22,000 (3.6)</td>
<td>&gt;200 (&gt;2.2)</td>
</tr>
<tr>
<td></td>
<td>KDR</td>
<td>30,000 (3.1)</td>
<td>&gt;330 (&gt;3.7)</td>
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<td>cKIT</td>
<td>7,300 (1.2)</td>
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<td></td>
<td>FLI1</td>
<td>22,000 (3.6)</td>
<td>&gt;200 (&gt;2.2)</td>
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<td></td>
<td>CMGC</td>
<td>CDK2</td>
<td>4.2 (0.046)</td>
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<td></td>
<td>ERK2</td>
<td>2,100 (0.34)</td>
<td>24 (0.26)</td>
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<tr>
<td></td>
<td>GSK3β</td>
<td>260 (0.041)</td>
<td>10 (0.11)</td>
</tr>
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<td></td>
<td>CK2</td>
<td>15,000 (2.4)</td>
<td>490 (5.4)</td>
</tr>
<tr>
<td></td>
<td>CK2</td>
<td>15,000 (2.3)</td>
<td>235 (2.6)</td>
</tr>
<tr>
<td></td>
<td>MAPK-AP2</td>
<td>21,000 (3.3)</td>
<td>100 (1.1)</td>
</tr>
</tbody>
</table>

NOTE: The fold selectivity versus Akt1 is shown for each kinase and the Ki is in parentheses.

Abbreviations: PDK1, phosphatidylinositol-dependent kinase 1; MAPK, mitogen-activated protein kinase; GSK3, glycogen synthase kinase 3; ERK2, extracellular signal–regulated kinase 2.

can be given is limited to 14 days due to severe injection site irritation. Nevertheless, in spite of the differences between these compounds, both compounds behave similarly in vivo.

The effect of the inhibitors was examined in several Akt-dependent models. We tested a genetically engineered model, where 3T3 murine fibroblasts stably express a constitutively active form of Akt1. In contrast to the parental 3T3 cell line, the 3T3-Akt1 cells have a dramatically increased ability to form colonies in soft agar as well as tumors in immunocompromised mice, suggesting the active Akt1 is responsible for the transformed phenotype (23). In this model, A-443654 inhibited the growth of the tumors (Fig. 4A); whereas the 2-methyl analogue of A-443654, which is 5,000-fold less active versus Akt1, showed no effect in this model. We also tested the compounds in a MiaPaCa-2 human pancreatic cell xenograft model, where we compared the efficacy with gemcitabine. MiaPaCa-2 is a human pancreatic carcinoma line with constitutively active Akt (43). Here again, the Akt inhibitor significantly slowed the growth of the tumors (Fig. 4B). A-443654 also showed antitumor activity in two rat orthotopic syngeneic models of tumor growth, a MatLyLu prostate carcinoma and a 9L glioblastoma (data not shown).

In all models tested, the tumors rapidly regrew after compound administration was stopped. The cytostatic behavior of the inhibitors suggests that continuous dosing may lead to increased efficacy. The number of consecutive days the orally available compounds could be dosed, unlike the parenteral compounds, was not limited by injection site toxicity. Nevertheless, the oral compounds could only be given for 15 to 25 days, after which time the animals became moribund. In all cases, the compounds were given at their respective maximally tolerated doses.

Activation of the Akt pathway is thought to inhibit apoptosis induced by a variety of cytotoxic agents and has been reported to be an effective survival mechanism in many tumor cells (44–47). Therefore, we attempted to combine the Akt inhibitors with the antimitotic agent paclitaxel in a PC-3 xenograft model (48, 49). PC-3 is a
PTEN-deficient human prostate carcinoma cell line, where resistance to chemotherapeutic agents is at least partially dependent on Akt activity (50). When given in combination, A-674563 increased the efficacy of paclitaxel in a PC-3 xenograft model (Fig. 4C).

We tested the pharmacokinetics and pharmacodynamics to insure that we were inhibiting Akt in vivo (Fig. 5). When the inhibitors were given at their maximally tolerated doses on a twice daily (bid) schedule, their concentration in plasma remained above the cellular EC₅₀ for ~5 hours (Fig. 5A). Concentrations in tumors were significantly higher than those in plasma, and drug concentrations in tumor remained above the cellular EC₅₀ for the entire 12-hour dosing interval (Fig. 5B). In the tumors, as we have seen in the cellular assays, the inhibition of GSK3 and S6 phosphorylation are inhibited in a dose responsive manner by the Akt inhibitors (Fig. 5C). As also seen in the cellular studies, the increase in Akt phosphorylation seems the most sensitive marker for Akt inhibition. This suggests that the cells, in vivo, are also sensitive to Akt inhibition, and attempt to compensate for the loss of the activity.

We also examined caspase-3 activation (a measure of apoptosis) and Akt phosphorylation by immunohistochemistry. As expected, at 8 hours after a single dose of A-443654, we observed an increase in apoptotic cells (Fig. 5D), suggesting that at least part of the antitumor activity of the Akt inhibitors is due to apoptosis induction. Normal liver and colon tissues showed no increase in apoptosis following treatment (data not shown). Corroborating the Western blot analysis, we also observed that the phosphorylation of Akt increased when inhibitors are given (Fig. 5E).

As seen in Fig. 5C, doses near the MTD do not maximally inhibit Akt within tumors. Furthermore, drug washout studies in cells suggested that compounds were more effective at inducing apoptosis when maintained above their cellular EC₅₀ for at least 12 hours (data not shown). As stated above, the plasma concentrations of A-443654 fall below this EC₅₀ after about 5 hours. In an attempt to increase the efficacy of A-443654 by intensifying the dosing regime, the compound was given thrice in a single day, 3 hours apart, for a total of 50 mg/kg/d. The animals could not tolerate 50 mg/kg/d every day; thus, compound was given every fourth day. This regime maintains the plasma concentrations of A-443654 above its cellular EC₅₀ for >12 hours. This thrice daily (tid)/q4d schedule proved to be more efficacious than the bid/daily schedule (Fig. 4D). Note that for the tid schedule, dosing was started on established tumors...
whereas for the bid schedule dosing was started immediately after tumor cell inoculation. Similar to what was observed with the bid/daily schedule, the tumors regrew soon after dosing was stopped.

Akt inhibition was compared with inhibition further downstream in the PI3K pathway at mTOR, using rapamycin (Fig. 4D). When dosed, both the Akt inhibitors and rapamycin are efficacious in the MiaPaCa-2 xenograft tumor model. scid mice were inoculated with cells on day 0 and therapy was begun on day 1. A-443654 at 7.5 mg/kg/d (▲) or vehicle (●) was given s.c., bid for 14 d. Gemcitabine at 120 mg/kg/d (■) or vehicle (●) was given i.p., qd on days 3, 6, 9, and 12. A-443654 and gemcitabine both significantly inhibited tumor growth (P < 0.03). C, A-674563/paclitaxel combination therapy in the PC-3 prostate cancer xenograft model. scid mice bearing established PC-3 tumors were treated with A-674563 (▲) p.o., bid at 40 mg/kg/d for 21 d; paclitaxel (▲) i.p., qd at 15 mg/kg/d on days 20, 24, and 28; the combination (●); or the combination vehicle (●). Therapy was initiated 20 d after tumor inoculation when the mean tumor volume for each group was ~270 mm³. Although A-674563 showed no significant monotherapy activity, the efficacy of the combination therapy was significantly improved compared to paclitaxel monotherapy (P < 0.002). D, A-443654 and rapamycin efficacy in the MiaPaCa-2 pancreatic cancer xenograft model. scid mice bearing established tumors were treated with A-443654 (■) s.c., tid at 50 mg/kg/d on days 16, 20, and 24; rapamycin (●) i.p., qd at 20 mg/kg/d for 15 d; the combination of rapamycin plus A-443654 (▲); s.c. vehicle (●); or combination vehicle (●). Therapy was initiated 16 d after tumor inoculation when the mean tumor volume for each group was ~255 mm³. Each monotherapy showed significant activity throughout the study (P < 0.01) and the combination was statistically better than either monotherapy from day 23 onward (P < 0.01).

Metabolic Consequences of Akt Inhibition
Akt is downstream of the insulin receptor and transduces the insulin response in vivo. We therefore measured blood sugar and insulin responses related to the inhibition of Akt. We examined random blood sugars during multiple tumor studies and have never observed any differences between the treated and control groups. We also measured both blood sugar and plasma insulin levels in glucose challenge tests. Blood sugars were not changed upon administration of Akt inhibitors, even when supertherapeutic doses were given (data not shown). We did, however, observe significant increases in plasma insulin following administration of a single dose of Akt inhibitors at therapeutic doses (Fig. 5F). This data is consistent with a homeostatic response, where the animals increase insulin secretion to maintain blood glucose concentrations. This is also what has been observed in Akt2
Figure 5. The effects of Akt inhibitors after in vivo administration. Plasma (A) and tumor (B) concentrations of A-443654 are plotted versus the time after a final dose of A-443654 was given. Animals were treated at (●) 3.8, (■) 7.5, or (▲) 15 mg/kg bid for 3 d before measurement. C, 2 h after A-443654 administration, the phosphorylation of Akt (solid columns), S6 Protein (open columns), and GSK3 (cross-hatched columns) were measured via Western blotting (bottom). Akt phosphorylation increases in response to the Akt inhibitor. In spite of this increase in P-Akt, phosphorylation of targets downstream from Akt is inhibited in a dose-responsive manner. D, A-443654 induces apoptosis in 3T3-Akt1 flank tumors. The scid mice bearing established 3T3-Akt1 tumors were given a single s.c. dose of A-443654 at 50 mg/kg or vehicle. Tumors were removed 8 h after treatment and apoptosis was examined by immunohistochemical staining with an antibody specific for the activated form of caspase-3. E, A-443654 treatment leads to increased levels of phosphorylated Akt1 in MiaPaCa-2 tumors. The scid mice bearing established MiaPaCa-2 flank tumors were given a single s.c. 30 mg/kg dose of A-443654. One hour after treatment, tumors were removed and the level of phospho-Akt was examined by immunohistochemical staining. F, A-674563 and A-443654 increase plasma insulin in an oral glucose tolerance test. Fasted animals administered vehicle (●), 20 mg/kg A-674563 (■), 100 mg/kg A-674563 (▲), or 20 mg/kg A-443654 (▲) 30 min before the 1 g/kg glucose challenge (time 0). Insulin levels were elevated in all A-674563- and A-443654-treated animals. Similar increases in insulin responses were seen in the sham-challenged (no glucose challenge) animals after treating with Akt inhibitors (data not shown).
knockout animals (51, 52). When young, these animals have normal blood sugars but increased plasma insulin. Only when older, after islet cell failure, do the animals lose their ability to maintain normal blood glucose concentrations.

While determining the maximum tolerated dose, we found that the Akt inhibitors induce a dose- and time-dependent weight loss in treated animals. This weight loss is typically >10% of total body mass when compounds are given at thrice their maximum tolerated dose (data not shown). Whereas weight loss is a general toxicity, it is consistent with the expectation that the compounds may interfere with the utilization of glucose by interfering with the insulin pathway.

Discussion

We have shown that inhibitors of Akt, when dosed to levels that inhibit Akt in vivo, can slow the tumor growth in laboratory models of cancer. Several compounds have been tested and have shown efficacy in a number of tumor models. In all of these models, the dosing period is limited due to toxicity of the therapy. In addition, in all models, the tumors regrew rapidly when dosing is stopped. The Akt inhibitors show efficacy in the same models where activity is observed with rapamycin. However, the Akt inhibitors have a narrower therapeutic window than does rapamycin.

The dose-limiting toxicities for the oral compounds, malaise and weight loss, are consistent with a mechanism-based toxicity, where the inhibitors interfere with the metabolism of glucose (the limiting toxicity for parenteral compounds is an injection site irritation). However, weight loss is a general toxicity seen with many anticancer agents that do not inhibit Akt. Also consistent with mechanism-based toxicity is the observation that compounds with substantially different selectivity profiles, but similar Akt inhibitory activity, elicit the same types of toxicity. The increase in plasma insulin attendant with Akt inhibition also suggests metabolic toxicities may be dose limiting. Further studies with more widely divergent Akt inhibitors will be required to ascertain if Akt inhibition as cancer therapy will be limited by mechanism-based metabolic toxicities.

Whereas the efficacy observed in the tumor models is promising, it is clear that inhibition of Akt as a therapy for cancer would benefit from a widening of the therapeutic window. A number of active investigations are under way to further improve the therapeutic window. The inhibitors described here are pan-Akt inhibitors, and in cells have equal potency inhibiting Akt1, Akt2, or Akt3 within cells. Akt2 loss seems to predominate in toxicity related to insulin signaling (51, 52), whereas Akt1 has been reported to be more important in cancer cell survival. Thus, generation of inhibitors more selective for Akt1 over Akt2 might improve therapeutic window. Furthermore, it is clear from the paclitaxel study that Akt inhibitors may collaborate with chemotherapeutics that induce apoptosis. Akt has recently been shown to collaborate with Bcl2/BclXL in several cell lines to inhibit apoptosis in cancer cells (50). Thus, using these Akt inhibitors with other agents may produce the desired improvement in therapeutic window by improving efficacy without increasing toxicity.

Finally, the Akt inhibitors, along with rapamycin, illustrate that different nodes in the PI3K signal transduction pathway can be inhibited to slow tumor progression. The efficacy of the Akt and mTOR inhibitors suggests that, as monotherapy, other inhibitors of the PI3K pathway will also be efficacious. However, the differences in toxicity suggest that inhibiting at different nodes in the PI3K pathway might yield very different therapeutic windows. There are a number of potential targets in this pathway, including PI3K, phosphatidylinositol-dependent kinase 1, ILK, Akt1, Akt2, Akt3, mTOR, p70S6K, and 4E-BP1, all of which are under investigation for utility in human cancer therapy. The results of these investigations will ultimately determine where to target the PI3K pathway to provide the best efficacy and therapeutic window for the treatment of cancer.

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References

Novel, Potent, and Selective Akt Inhibitors


Molecular Cancer Therapeutics

Potent and selective inhibitors of Akt kinases slow the progress of tumors *in vivo*

Yan Luo, Alexander R. Shoemaker, Xuesong Liu, et al.


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