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***In vitro* and *In vivo* Activity of Novel Small-Molecule Inhibitors Targeting the Pleckstrin Homology Domain of Protein Kinase B/AKT**

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Abstract

The phosphatidylinositol 3-kinase/AKT signaling pathway plays a critical role in activating survival and antiapoptotic pathways within cancer cells. Several studies have shown that this pathway is constitutively activated in many different cancer types. The goal of this study was to discover novel compounds that bind to the pleckstrin homology (PH) domain of AKT, thereby inhibiting AKT activation. Using proprietary docking software, 22 potential PH domain inhibitors were identified. Surface plasmon resonance spectroscopy was used to measure the binding of the compounds to the expressed PH domain of AKT followed by an *in vitro* activity screen in Panc-1 and MiaPaCa-2 pancreatic cancer cell lines. We identified a novel chemical scaffold in several of the compounds that binds selectively to the PH domain of AKT, inducing a decrease in AKT activation and causing apoptosis at low micromolar concentrations. Structural modifications of the scaffold led to compounds with enhanced inhibitory activity in cells. One compound, 4-dodecyl-N-(1,3,4-thiadiazol-2-yl) benzenesulfonamide, inhibited AKT and its downstream targets in cells as well as in pancreatic cancer cell xenografts in immunocompromised mice; it also exhibited good antitumor activity. In summary, a pharmacophore for PH domain inhibitors targeting AKT function was developed. Computer-aided modeling, synthesis, and testing produced novel AKT PH domain inhibitors that exhibit promising preclinical properties.

Introduction

The pleckstrin homology (PH) domain is a region containing 100 to 120 amino acids found in more than 250 human proteins (1). Although the amino acid sequence of PH domains is not universally conserved, the tertiary structure is remarkably conserved. Although PH

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domains bind to a variety of different targets, a unique property of a limited number of ~40 PH domains is the specificity with which they bind phosphorylated phosphatidylinositide (PtdIns) lipids within the biological cell membrane. PtdIns phosphorylation and the subsequent binding of PH domain-containing proteins are vital components of signal transduction pathways that regulate cell growth and survival and, thus, are opportunistic targets for up-regulation and oncogenic determinism (2,3).

The PtdIns 3-kinase (PtdIns 3-K)/AKT pathway is critical for cell proliferation and survival (4). Phosphorylation of PtdIns(4,5)P₂ to produce PtdIns(3,4,5)P₃ by PtdIns 3-K signals the recruitment and binding of AKT to the inner leaflet of the plasma membrane via the PH domain (5,6). This translocation of AKT from the cytosol to the membrane allows its phosphorylation on Thr³⁰⁸ by the plasma membrane-bound PtdIns-dependent kinase 1 (PDK1; ref. 7). Phosphorylation on the Ser⁴⁷³ residue occurs either by integrin-linked kinase, by the kinase activity of AKT itself, or by rictor-mammalian target of rapamycin (mTOR; ref. 8). Once fully phosphorylated, AKT translocates back to the cytosol and to the nucleus where it phosphorylates a variety of downstream targets, including proapoptotic promoters such as forkhead transcription factors and AFX, as well as the Bcl-2 family member Bad (4). AKT also promotes cell survival by activating cyclic AMP-responsive element binding protein (9) and promotes proliferation by activating p70S6 kinase (p70S6K; ref. 10) and glycogen synthase kinase 3 β (GSK3 β ; ref. 11), which contribute to cyclin D accumulation of cell cycle entry. Furthermore, AKT acts as a mediator for both vascular endothelial growth factor production and angiogenesis by phosphorylation of mTOR (12). Given the importance of AKT in proliferation and survival signaling, it has the potential to be an important target for cancer drug discovery (13).

Three genes encode for AKT within mammalian species giving AKT-1/ α , AKT-2/ β , and AKT-3/ γ (5). The different isoforms of AKT share a high degree of sequence homology within their PH domains but diverge within other regions (14). Although AKT-1 and AKT-2 are expressed throughout the organism, AKT-3 is predominantly found within the brain, heart, and kidney (15,16). AKT-1 is more important for overall growth, AKT-3 is more critical for brain development, and AKT-2 is mainly involved in glucose metabolism. However, knockout studies in mice have strongly suggested that there are functional overlaps among the AKT isoforms (17,18). Thus, because of the highly homologous PH domains, the AKT isoforms provide a fortuitous advantage in designing drugs that inhibit all AKT activity in cells.

Defects in the PtdIns 3-K/AKT pathway are found in a variety of cancers, with most abnormalities occurring through mutations or loss of the mixed function lipid phosphatase PTEN (19) or through mutations of PtdIns 3-K (20). Although overexpression of AKT itself is not a determinant for cancer, AKT can be oncogenic if the PH domain is mutated such that its affinity for PtdIns 3-phosphates increases, leading to its translocation and increased phosphorylation at the cellular membrane (21). In pancreatic cancers, >90% of the tumors contain an activating upstream ras mutation and about half of the surgically resected pancreatic cancer specimens have mTOR activation, a downstream target of AKT (22–24).

Most attempts to develop AKT inhibitors have focused on compounds that bind to the kinase ATP-binding pocket. Due to the similarity of the ATP pocket among serine/threonine kinases, particularly ACG family kinases to which AKT belongs, achieving target specificity has been extremely difficult. All the reported AKT ATP pocket inhibitors also inhibit PKA, which may account for the relatively high toxicity of this type of inhibitor observed in animals and in patients (13). Previous studies involving D-3-deoxyphosphatidylinositol ether lipid (DPIEL) have provided proof of principle for using PH domains as drug targets (25). DPIEL exhibits a high binding affinity and selectivity for the PH domain of AKT (25).

In addition, previous studies have shown that DPIEL does not inhibit other PH domain-containing proteins, including PDK1, IRS-1, mSOS, and β ARK (25). Unfortunately, DPIEL is not a useful drug candidate because of its pharmacokinetic and pharmacodynamic properties (26). In the present study, we have used *in silico* screening to identify compounds that were expected to bind the PH domain of AKT. The compounds were tested for direct binding and competitive inhibitory activities for the PH domain of AKT and for the inhibition of AKT function in pancreatic cancer cell lines. A lead compound was identified, and modeling was used to design analogues. On testing, it was found that several analogues exhibited good AKT PH domain-binding properties. The best analogue was tested *in vivo* and exhibited antitumor activity in a mouse xenograft model of pancreatic cancer cells in immunocompromised mice. This work provides proof of principle for the design of small-molecule inhibitors that bind to the PH domain of AKT and that exhibit antitumor activity.

Materials and Methods

Virtual screening and structure-activity relationship of available compounds

A three-dimensional pharmacophore search was carried out based on the hydrogen-bonding pattern between the inositol(1,3,4,5)-tetrakisphosphate ligand and the PH domain of AKT (1H10) using UNITY (Tripos, L.P.; ref. 27). A virtual library of ~300,000 compounds generated from databases (the National Cancer Institute Chemical and Natural Products Library, the Maybridge Available Chemicals Directory, and the Lead-Quest Chemical Library) was searched. Twenty compounds from each database were selected, the compounds were pooled, and duplicates were removed. This process led to the identification of the initial four hits (Supplementary Table S1, located in Supplementary Materials); each of the hits was examined in the active site using hand modeling and structure-based design. Unfortunately, because each of the four hits was negatively charged, the binding site had several formal charges, and the protein-ligand interactions were solvent exposed, we determined that additional structure-activity relationship (SAR) would be necessary to develop reliable binding models in this challenging system. Therefore, we sought additional available analogues of the initial four hits. A database of ~2.3 million unique available compounds was assembled from vendor databases. After an initial collection of several hundred analogues was identified, a subset of 46 compounds was selected manually using the following criteria: conservative analogues of the known hits, explore a range of new SAR, challenge the need for an anion in the hits, and avoid nonmedicinal, toxic, reactive, and unstable functional groups. Of these 46 compounds, 22 compounds were commercially obtainable.

PH domain structure and small-molecule preparation for docking

Computational docking was used to study the interactions between the AKT-1 PH domain and its inhibitors. One of the high-resolution (0.98 Å) complex AKT PH domain crystal structures (1UNQ; ref. 28) was retrieved from Protein Data Bank for docking simulations. Based on structural analysis and literature (28–30), residues Lys¹⁴, Glu¹⁷, Arg²³, and Arg⁸⁶ around the inositol-(1,3,4,5)-tetrakisphosphate [Ins(1,3,4,5)P₄] ligand were found to be essential for the protein-ligand interactions because they are involved in hydrogen bonds and responsible for the protein conformational change induced by the ligand binding. The detailed procedures for the PH domain structure and small-molecule preparation for docking are located in Supplementary Materials that accompany this article.

Molecular docking

Three commercially available docking packages, FlexX (FlexX 1.20.1, BioSolveIT GmbH), GOLD (GOLD 3.2), and Glide (Glide 4.5, Schrodinger), were used to dock the original ligand Ins(1,3,4,5)P₄ into the binding pocket to evaluate the applicability of each docking

package to this target. Details on the molecular modeling software and the meaning of the related scores are provided in Supplementary Materials that accompany this article.

Absorption, distribution, metabolism, and toxicologic modeling

The absorption, distribution, metabolism, and toxicologic (ADMET) properties, such as Caco-2 permeability and log *P* values, were calculated using our own ADMET predictors and ADME Boxes (ADME Boxes 4.0, Pharma Algorithms).

Expression of recombinant AKT PH and PDK PH domains

Expression and purification of the proteins were performed as already described (27). More details for the sequence and expression for the PH domains are given in Supplementary Materials that accompany this article.

Chemical compounds

Compounds **1** to **22** were obtained and the syntheses of compounds **2** and **24** to **28** are outlined in schemes 1 to 3 found in Supplementary Materials. The detailed synthetic procedures and the characterization data for these compounds are given in Supplementary Materials that accompany this article.

Surface plasmon resonance spectroscopy binding assays

All interaction analyses were performed with a Biacore 2000 using Biacore 2000 Control Software v3.2 and BIAevaluation v4.1 analysis software (Biacore) as already described (27). For the competitive binding assays and the *K_i* determination, PtdIns(3,4,5)phosphate biotin-labeled liposomes (Echelon Biosciences) and SA chips were used with increasing concentrations of the compound tested.

Cell culture and drug treatments

Human MiaPaCa-2, BxPC-3, and Panc-1 pancreatic cancer cells were obtained from the American Type Culture Collection. Cells were maintained and drug treated as described previously (27).

Confocal microscopy

BxPC-3 cells were grown on coverslips in DMEM plus 10% fetal bovine serum medium. After 4 h of incubation with 10 μ mol/L of compound **28**, the fluorescent NBD-labeled analogue of **27**, or a DMSO control, cells were washed twice in PBS and fixed using 4% paraformaldehyde. Coverslips were washed four times in PBS and mounted using mounting medium containing 4',6-diamidino-2-phenylindole obtained from Molecular Probes Invitrogen. Slides were then visualized using a Nikon PCM2000 confocal microscope (Nikon Instruments, Inc.).

Western analysis

Inhibition of the phosphorylation of AKT and its downstream targets was measured by Western blotting using rabbit polyclonal antibodies to phospho-Ser⁴⁷³-AKT, phospho-Thr³⁰⁸-AKT, total AKT, phospho-Ser⁹-GSK3 β , phospho-Ser²¹-GSK3 β , phospho-Ser²⁴¹-PDK1, and phospho-Thr³⁸⁹-p70S6K (New England Biolabs/Cell Signaling Technology, Inc.) as described previously (27). Bands corresponding to phospho-Ser⁴⁷³-AKT and total AKT were quantified using Eagle Eye software (Bio-Rad) and Kodak X-Omat Blue XB (NEN Life Science Products). β -Actin was used as a loading control.

Cell assays

Cell growth inhibition was determined using a microcyto-toxicity assay and apoptosis was measured as described previously (27,31).

Antitumor activity

Approximately 1×10^7 BxPC-3 pancreatic cancer cells in log cell growth suspended in 0.1 mL PBS were injected s.c. into the flanks of female severe combined immunodeficient (*scid*) mice. When the tumors reached volumes of $\sim 150 \text{ mm}^3$, the mice were stratified into groups of eight animals having approximately equal mean tumor volumes. Compound **27** was suspended in 0.2 mL of an aqueous solution containing 2.5% ethanol and 20% Trappsol (Cyclodextrin Technologies Development, Inc.) by i.p. injection at a dose of 125 mg/kg twice a day for 5 d. The animals were weighed weekly. Tumor diameters, measured twice weekly at right angles (d_{short} and d_{long}) using electronic calipers, were converted to volume by the following formula: $\text{volume} = (d_{\text{short}})^2 \times (d_{\text{long}})/2$ (32).

Pharmacodynamic and pharmacokinetic studies

Pancreatic cancer cells (1×10^7 BxPC-3) were injected s.c. into the flanks of female *scid* mice and allowed to grow to $\sim 300 \text{ mm}^3$. Mice received a single i.p. dose of compound **27** of 125 mg/kg suspended in 0.2 mL of 0.25% ethanol/20% Trappsol in water. Mice were killed after 1, 4, 6, 12, or 24 h; blood was collected into heparinized tubes; and plasma was stored frozen. The tumors were removed and immediately frozen in liquid N_2 . The tumors were then homogenized in 50 mmol/L HEPES buffer (pH 7.5), 50 mmol/L NaCl, 1% NP40, and 0.25% sodium deoxycholate. Western blotting was performed as described above. Plasma levels of compound **27** were measured by reverse-phase high-pressure liquid chromatography as previously described (27).

Results

Virtual screening

Four active compounds were identified from among the 60 hits identified by the pharmacophore screen (7% hit rate). The IC_{50} of these compounds ranged from 1 to 50 $\mu\text{mol/L}$ in a cellular AKT inhibition assay (Supplementary Table S1, located in Supplementary Materials). It is noteworthy that for all of the compounds except the third, cellular AKT inhibition and cell cytotoxicity track one another. Each of the four compounds identified represents a unique structural class with potential for further development. Although the first compound contains the undesirable alkyl, aryl azo moiety, and the third compound has a fairly high calculated log P (4.4), these four compounds provided a rich basis for further development. The first compound was further developed but did not exhibit antitumor activity (27). Each compound represents a unique molecular framework, yet they share some striking similarities. Each of the four compounds is a weak acid and will be an anion in typical intracellular compartments. This is perhaps not surprising given that the natural ligand is a polyanionic strong acid and that the binding site of the PH domain is strongly basic. These four molecules each contain a series of ring structures connected by short flexible linker regions.

Identification of compounds that bind to the AKT PH domain

An *in silico* screen was then conducted to identify small molecules that would be expected to bind to the PH domain of AKT. Twenty-two compounds were identified, obtained from commercial sources, and tested for their ability to inhibit phospho-Ser⁴⁷³-AKT in Panc-1 (Supplementary Fig. S1, *black columns*, located in Supplementary Materials) and MiaPaCa-2 (Supplementary Fig. S1, *gray columns*) pancreatic cancer cells. Two compounds, **2**

and **21** (9% hit rate), were found to be active against AKT in MiaPaCa-2 cells with IC₅₀ values of 20 and 25 $\mu\text{mol/L}$, respectively. Interestingly, these two compounds presented a similar chemical scaffold. Furthermore, they did not exhibit cytotoxicity in either cell line tested (Supplementary Materials; Supplementary Table S2). To further improve the potency of these two compounds, several computational approaches were used to study their binding to the PH domain of AKT as well as their ADMET properties. According to the docking studies using the GOLD algorithm, the sulfonyl moiety of compound **2** acts as a hydrogen bond acceptor interacting with residues Arg²³, Arg²⁵, and Lys¹⁴, whereas hydrogen-bonding interactions were observed between the nitrogen atoms in the thiadiazolyl group and residue Glu¹⁷ (Fig. 1A). The hydrogen-bonding interactions between compound **2** and the protein are similar to those in the original 1UNQ complex (Fig. 1B; ref. 29). In particular, the sulfonyl group interacts with the protein by mimicking the 3-position phosphate of the Ins(1,3,4,5)P₄ ligand (Fig. 1B). In contrast to **2**, compound **21** possesses 2 sulfonyl fragments that mimic the 1- and 3-position phosphate groups on the inositol ring and interact with Arg²³, Arg²⁵, and Lys¹⁴ (data not shown). The positively charged guanidinium cation of Arg²³ interacts with one of the benzyl rings of **2** by charge-charge interaction. Stacking interactions were observed between the thiadiazole ring of **21** and the phenyl ring of Tyr¹⁸.

Optimization of the aniline moiety: hit-to-lead discovery

Experimental cellular AKT inhibition analysis showed that compounds **2**, **7**, and **21** had approximately the same affinity, yet **2** had significantly better ligand efficiency (Supplementary Table S2; Supplementary Figs. S1 and S2; ref. 33). The smaller size of **2** affords greater freedom for structural modification and optimization and therefore was selected for hit-to-lead optimization. Analysis of docking poses showed that the phenyl ring of **2** points away from the binding site, and so modifications of the *para*-amino group were not predicted to affect the binding (Fig. 1C). Our docking results indicated that **21** was a stronger binder than **2**, so it was thought that appropriate modification (e.g., attachment of a flexible hydrophobic group) would enhance the Caco-2 cell permeability of the molecule based on the ADMET predictions (Table 1). Three analogues, **24** to **26**, were suggested, computationally docked into the PH domain of AKT, synthesized, and experimentally tested for AKT binding and inhibitory activity (Table 1). The docking results and calculated ADMET properties for compounds **24** to **26** are summarized in Table 1; synthesis schemes 1 and 2, experimental details, and compound characterization data are given in Supplementary Materials. The docking studies suggested that compound **24** would be a better inhibitor than **2** with a higher log *P* and Caco-2 permeability. The predictions were verified in cellular assays of AKT inhibition (Table 2). Compound **24** inhibits AKT at lower concentration than compound **2**.

The measured K_D using surface plasmon resonance (SPR) technology was 0.45 $\mu\text{mol/L}$ for compound **2** and was 19.6 $\mu\text{mol/L}$ for compound **24**. In comparison, we have previously shown that PtdIns(3,4,5)P₃ binds the PH domain of AKT with a K_D of $3.08 \pm 0.49 \mu\text{mol/L}$ (27). Compound **24** was predicted to have better Caco-2 permeability than compound **2**, which could explain its low IC₅₀ value in the cellular AKT inhibition assay. Interestingly, using a liposome displacement assay and SPR spectroscopy, which allowed calculation of a K_i , compound **24** can displace PtdIns(3,4,5)phosphate liposomes at lower concentrations than compound **2** (Fig. 2B; Table 2). To find out whether compound **24** is a prodrug of **2**, a nonamide analogue, compound **27**, was synthesized and experimentally evaluated. The docking studies indicated that the modification did not change the binding mode (Fig. 1C), and **27** showed a higher GOLD fitness of the binding to the PH domain. A lower IC₅₀ of $6.3 \pm 0.9 \mu\text{mol/L}$ for AKT inhibition was observed for this compound in Panc-1 cells (Table 2). However, low Caco-2 cell permeability was predicted for **27** with a high log *P* value compared with **24**. Consistent with the prediction, the K_i for compound **27** was significantly

lower than those of compounds **2** and **24**. For comparison, the displacement of diC8-PtdIns(3,4,5)P₃ exhibited a K_i of ~0.3 $\mu\text{mol/L}$.

Biological activities of the lead compound and its derivatives

AKT inhibition leads to cellular apoptosis (4). We therefore measured the ability of compounds **2** and **24** to **27** to induce cellular apoptosis and correlated this with the inhibition of AKT phosphorylation measured by Western blot analysis of phospho-Ser⁴⁷³-AKT (Supplementary Fig. S2; Fig. 2). Morphologic assays for the detection of apoptosis were also performed with compounds **2** and **21** (data not shown; Supplementary Fig. S2). Apoptosis was directly correlated with the inhibition of AKT observed at 20 $\mu\text{mol/L}$ by Western blot for both initial hits, compounds **2** and **21** (data not shown; Supplementary Fig. S2). The four analogues of compound **2** were tested for their ability to inhibit cellular AKT activity (Fig. 2C) and to induce apoptosis (Table 2). All compounds (**2** and **24–27**) induced apoptosis and inhibited AKT phosphorylation. Finally, to directly determine the affinities of the lead compounds for the target PH domain, *in vitro* binding assays were performed using SPR spectroscopy. Figure 2A shows representative sensorgrams obtained for the direct binding of compounds **24** and **27** and K_D was calculated (Table 2). Compounds **25** and **26** did not seem to bind directly to the PH domain of AKT. These results correlate with a very weak inhibition of cellular AKT and weak induction of apoptosis. On the contrary, compound **27** exhibited all the characteristics of an AKT inhibitor with an IC_{50} of 6.3 ± 0.9 $\mu\text{mol/L}$ in Panc-1 cells, a strong induction of apoptosis at 20 $\mu\text{mol/L}$, and some cellular cytotoxicity. These data correlate with a low K_D for the compound to the PH domain as measured by SPR spectroscopy. Interestingly, again, the measurement of the K_i seems to be the most reliable and predictive assay for compound cellular efficacy. Finally, for selectivity purposes, we also tested the binding of **27** to the PH domain of PDK1 and obtained a K_D of 90.1 $\mu\text{mol/L}$ and a K_i of 5.5 $\mu\text{mol/L}$ (data not shown). These values correlated well with the GOLD score obtained for the compound to the PH domain of PDK, which was found to be 53.5 compared with 60.7 for the PH domain of AKT. These data suggest that compound **27** may represent a selective compound for AKT with some activity on PDK1 at higher concentrations.

Mechanism of action of compound 27

Our data strongly suggest that **27** is an active inhibitor of AKT function. Table 2 shows its biochemical properties, and Fig. 3A and B summarizes the effects of **27** in BxPC-3 cells on AKT function (IC_{50} , 8.6 ± 0.8 $\mu\text{mol/L}$) and on its downstream targets. The compound was able to reduce the phosphorylation of AKT mainly on its Ser⁴⁷³ residue and less strongly on Thr³⁰⁸ residue without affecting total AKT protein expression, whereas GSK3 β and p70S6K were inhibited in a dose-dependent manner by compound **27**. The phosphorylation of PDK1 on Ser²⁴¹ (34) was slightly affected by compound **27** at higher concentrations. Again, these data agreed with the SPR results and confirmed the possible selectivity of **27** for AKT at low concentrations. To define the mechanism of action of **27**, the fluorescent analogue **28** was used (scheme 3 and synthesis details are located in Supplementary Materials; ref. 35). The addition of the fluorescent NBD moiety did not alter the binding of **28** to the protein (Fig. 1D), and **28** inhibited AKT phosphorylation in a fashion similar to **27** in BxPC-3 cells (Fig. 3C). Finally, using confocal microscopy, compound **28** was found to be mainly located in the cytosol and/or lipid vesicles, potentially trapping AKT in the cytosol (Fig. 3C).

In vivo activities of the lead compound 27

Preliminary studies showed no toxicity of single doses up to 250 mg/kg, which was the maximum dose for compound **27** that could be conveniently administered i.p. Antitumor activity was measured against BxPC-3 pancreatic cancer xenografts in *scid* mice with compound **27** administered at a dose of 125 mg/kg i.p., twice a day for 5 days (Fig. 4A).

Compound **27** showed significant antitumor activity with cessation of tumor growth and even regression during the course of treatment. Tumor growth resumed at its original rate when the drug was removed (Fig. 4A). A single i.p. dose of **27** of 125 mg/kg caused significant inhibition of tumor AKT measured as phospho-Ser⁴⁷³-AKT. This dose produced up to 70% inhibition at 6 hours, and 50% inhibition at 12 hours, with a return to untreated levels by 24 hours (Fig. 4B). These results correlated well with the plasma concentrations of **27** after the single dose (Fig. 4C). Indeed, between 1 and 6 hours, a peak corresponding to compound **27** was detected in the plasma.

Discussion

Because of its roles in cellular apoptosis and survival pathways, AKT has emerged as an attractive therapeutic target (2,3). There have been many attempts to inhibit AKT via its kinase domain (13). We and others have recently developed an alternative approach targeting the PH domain of the protein (25,36,37). The PH domain is essential for the binding of cytosolic AKT to PtdIns(3,4,5)P₃, formed by the activity of PtdIns 3-K, in the plasma membrane, thus allowing the phosphorylation and activation of AKT. In this study, we have identified a novel small-molecule compound that inhibits AKT at low micromolar concentrations in pancreatic cell lines and exhibits good antitumor activity in a mouse xenograft model.

The virtual screen gave an initial set of four active compounds and directed two-dimensional searching yielded an additional 2 active compounds from a set of 22 commercially available analogues. Four of the 12 active molecules included a novel *N*-thiadiazole, aryl sulfonamide moiety, whereas 4 of the 5 compounds in the set that contained this moiety were active in inhibiting AKT in cells. To increase the delivery and potency of these hits, four new derivatives were synthesized based on the ADMET predictions. The addition of an acetamide group at a position in the molecule where modeling predicted there would be no direct interaction with the PH domain produced a small improvement in activity measured by inhibition of phospho-Ser⁴⁷³-AKT in cells over that of the parent compound. However, the addition of a dodecanoylamide resulted in a considerably more potent compound (**24**) with an IC₅₀ of 10 μmol/L in Panc-1 cells. Due to its long aliphatic chain, it was proposed that the compound may embed into the cellular membrane. Subsequent cleavage of the amide bond would then allow for the release of the compound into the cytoplasm. To test this hypothesis, compound **27** was synthesized, harboring a noncleavable dodecyl tail. This compound exhibited the most effective reduction of cell phospho-Ser⁴⁷³-AKT, with an IC₅₀ of 6.8 μmol/L. These data strongly suggest that **24** is not a prodrug for **2** and that the aliphatic chain on **27** helps the compound to enter the cells despite the poor predicted ADMET properties (high log *P* and low Caco-2 permeability).

To probe the mechanism of action of **27**, a fluorescence derivative, compound **28**, containing a fluorescent NBD substituent was studied in pancreatic cancer cells. The NBD fluorophore has previously been used for real-time reporting of cation transport in biological systems (38) and for understanding the mechanism of action of viridins, such as wortmannin (39). Compound **28** bound to the PH domain of AKT and was an inhibitor of cell phospho-Ser⁴⁷³-AKT, albeit more weakly than compound **27**. Confocal microscopic studies revealed that although some of the compounds localized within the cellular membrane, a large amount was also present in the cytoplasm. The most likely explanation for the activity of **28**, and by analogy **27**, is that the compounds localize in the cytoplasm where they bind to the PH domain of AKT preventing translocation of AKT to the cellular membrane. As expected, treatment with compound **27** also resulted in reduced levels of cellular phospho-Ser⁹ GSK3β and phospho-Thr³⁸⁹-p70S6K, two direct downstream targets of phospho-Ser⁴⁷³-AKT. Finally, compound **27** did not affect the autophosphorylation of PDK1 strongly and did not

bind well to the PH domain of PDK1 as determined using SPR ($K_D = 90.5 \mu\text{mol/L}$). These data may reflect some selectivity for the PH domain of AKT compared with the PH domain of PDK.

These same downstream targets were also inhibited *in vivo* by compound **27** in BxPC-3 tumor xenografts after a single dose of 125 mg/kg of **27**. Maximum inhibition was observed 4 to 6 hours after administration of compound **27** corresponding to its peak plasma concentration. There was also antitumor activity with complete cessation of tumor growth and even some regression during administration of the compound.

In conclusion, we have shown that *in silico* modeling can be a useful tool in the identification of novel small-molecule compounds targeting specific motifs and domains, such as the PH domains. The modeling allowed the identification of a novel chemical scaffold that bound to the expressed PH domain of AKT and *in vitro* in pancreatic cancer cell lines. The compounds inhibited AKT and the activation of its downstream targets. Derivatization of one of the compounds led to the identification of a novel generation of active small molecules that may represent a good starting point and chemical probe for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Modeling of the interactions between compound **2** and **21** with the PH domain of AKT. *A*, two-dimensional interaction map for compound **2** and AKT PH domain-binding site. Docking of the ligand Ins(1,3,4,5)P₄ into the binding pocket with FlexX, GOLD, and Glide could reproduce the crystal structure very well, with RMSD of 0.69 Å, 0.71 Å, and 0.69 Å, respectively. *B*, docking results of Ins(1,3,4,5)P₄, compound **2**, and compound **21** bound to the PH domain of AKT. *Dashed line*, hydrogen-bonding interactions between the small molecules and the protein. *Blue*, positively charged protein surface; *red*, negatively charged surface; *green*, Ins(1,3,4,5)P₄; *purple*, compound **2**; *yellow*, compound **21**. *C*, docking results of **2**, **24**, **27**, and **28**. *Dashed line*, the hydrogen-bonding interactions between the small molecules and the protein-binding site. *Blue*, positively charged electrostatic molecular surface of the protein; *red*, negatively charged surface; *magenta*, compound **2**; *yellow*, compound **24**; *green*, compound **27**. *D*, docking of **28** compared with its parent compound, **27**. Compound **27** (*purple*) and its fluorescent-tagged analogue, compound **28** (*green*), are docked in the PH domain of AKT.

Figure 2.

Biochemical properties of compound **2** and its derivatives. **A**, direct binding characteristics of **24** and **27**. SPR spectroscopy was used to determine the direct binding affinity of the compounds. The PH domain of AKT was flown through and the compounds were injected. A representative sensorgram is shown for compounds **24** (left) and **27** (right). Increasing concentrations (in μmol/L) were tested (low at the bottom, increasing to the top). K_D s were calculated using a 1:1 Langmuir model. **B**, graphs obtained during competition binding assays with PtdIns(3,4,5)phosphate-containing liposomes. Increasing concentrations of the compounds reduced the binding of the PH domain of AKT to the bound liposome surface. The K_i was defined as the concentration of compound tested that inhibited 50% of the binding. **C**, inhibition of phospho-Ser⁴⁷³-AKT by compounds **24**, **25**, **26**, and **27** in Panc-1 cells as measured by Western blotting analysis. V/C-, cells treated with vehicle control, nonstimulated; V/C+, cells treated with vehicle control, stimulated with insulin-like growth factor-I (IGF-I; 100 ng/mL for 20 min). Cells were incubated with 20 or 50 μmol/L of compounds **24**, **25**, **26**, or **27** and stimulated (+) before lysis.

Figure 3. Mechanism of action of compound **27**. *A*, representative Western blot for **27** activity in BxPC-3 pancreatic cells. Cells were treated with the compound at the indicated concentrations for 4 h. Cells were then stimulated and total cell lysates were loaded on 7.5% SDS-PAGE. *B*, quantification of phospho-Ser⁴⁷³-AKT/β-actin in BxPC-3 cells. Compound **27** exhibits an IC₅₀ of 8.6 ± 0.8 μmol/L. Columns, mean of at least three separate experiments in triplicate; bars, SD. ***, $P < 0.01$; **, $P < 0.05$. *C*, detection of **28** in BxPC-3 cells using confocal microscopy (*top*) and Western blotting for phospho-Ser⁴⁷³-AKT, AKT, and β-actin for the determination of **28** activity in BxPC-3 cells (*bottom*). V/C-, cells treated with vehicle control, nonstimulated; V/C+, cells treated with vehicle control, stimulated with IGF-I (100 ng/mL for 20 min).

Figure 4.

Antitumor activity and inhibition of tumor AKT by compound **27**. *A*, *scid* mice with 150 mm³ s.c. BxPC-3 pancreatic cancer xenografts were treated with vehicle (▲) or with compound **27** (□; 125 mg/kg in 2.5% ethanol, 20% Trappsol in water) by i.p. injection twice a day for 5 d (arrows). Points, mean of eight mice per group; bars, SE. **, $P < 0.05$, compared with vehicle control. *B*, *scid* mice with 300 mm³ s.c. BxPC-3 pancreatic cancer xenografts administered **27** (125 mg/kg in 2.5% ethanol, 20% Trappsol in water) as a single dose and tumor phospho-Ser⁴⁷³-AKT, total AKT, phospho-Ser²¹-GSK3β, phospho-Ser⁹-GSK3β, and phospho-Thr³⁸⁹-p70S6K measured by Western blotting at various times. *C*, plasma concentration for **27** was measured after the same time after a single i.p. injection of **27** at 125 mg/kg.

Predicted *in silico* properties and ADME properties of analogues of compound 2

N#Nc1ccc(cc1)S(=O)(=O)c2ccccc2
 2. $R=NH_2$
 24. $R=NHCO(CH_2)_3CH_3$
 25. $R=NHCOCH_3$
 26. $R=NHCO(CH_2)_3CH_3OCH_3$
 27. $R=(CH_2)_3CH_3$

[#]Caco-2 permeability is calculated for pH of 7.4 and rpm of 500.

Table 2

Biochemical and biological activities of compound 2 and its derivatives

Compound number	K_D^* and K_i^* ($\mu\text{mol/L}$)	pAKT inhibition [†] (IC_{50} , $\mu\text{mol/L}$)	Apoptosis [‡] at 20 $\mu\text{mol/L}$ (%)	Cell survival [§] (IC_{50} , $\mu\text{mol/L}$)
2	$K_D = 0.45 \pm 0.1$ $K_i > 50.0$	20/25	$24.3 \pm 3.2/25.7 \pm 2.6$	NI/NI
24	$K_D = 19.6 \pm 4.9$ $K_i = 21.8 \pm 1.8$	10/15	$28.7 \pm 0.3/20.0 \pm 1.5$	127/90
25	$K_D = \text{NB}$ $K_i > 50$	$>50/>50$	$6.8 \pm 0.9/10.3 \pm 2.1$	NI/NI
26	$K_D = \text{NB}$ $K_i > 50$	$>50/>50$	$11.4 \pm 0.5/18.7 \pm 3.1$	NI/NI
27	$K_D = 40.8 \pm 2.5$ $K_i = 2.4 \pm 0.6$	$6.3 \pm 0.9/10$	$40.0 \pm 2.9/31.3 \pm 1.6$	65/30

NOTE: All biological tests were made in Panc-1 (numbers on the left) and MiaPaCa-2 (number on the right) pancreatic cell lines.

Abbreviations: NI, not inhibitory; NB, not binding.

* K_D and K_i ($\mu\text{mol/L}$) were determined using purified AKT PH domain and SPR spectroscopy (Biacore 2000). The K_i for PdIns(3,4,5)trisphosphate was 0.26 $\mu\text{mol/L}$.

† Inhibition of AKT was measured by Western blots using specific antibodies against phospho-Ser⁴⁷³-AKT.

‡ Percentage of apoptosis was obtained by a morphologic assay at 20 $\mu\text{mol/L}$.

§ Cell survival was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.