A Small Molecule Inhibits Akt through Direct Binding to Akt and Preventing Akt Membrane Translocation *

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Donghwa Kim 1, Mei Sun 1, Lili He 1, Qing-Hua Zhou 1, Jun Chen 5, Xia-Meng Sun 4, Gerold Bepler 6, Said M. Sebti 7, and Jin Q. Cheng 1,2

From the Departments of 1Molecular Oncology, 2Thoracic Oncology, and 3Drug Discovery, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33612 and the 4Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin 300052, China

The Akt pathway is frequently hyperactivated in human cancer and functions as a cardinal nodal point for transducing extracellular and intracellular oncogenic signals and, thus, presents an exciting target for molecular therapeutics. Here we report the identification of API-1, a selective inhibitor, as a novel Akt/thr308 and ser473) levels of constitutively active Akt, mTORC2. Notably, the kinase activity and phosphorylation (e.g. phosphatidylinositol-dependent kinase-1, and members of the Akt family. In contrast, API-1 had no effects on mTORC2. Furthermore, API-1 directly inhibited the activity of the kinase activation loop of Akt and blocks Akt membrane translocation. Furthermore, API-1 treatment of cancer cells results in inhibition of mTORC2 and activates Akt through an Akt- and phosphatidylinositol 3,4,5-trisphosphate, produced by conversion of phosphatidylinositol 3,4-diphosphate by conversion of phosphatidylinositol 3-kinase, mTORC2. Notably, Akt phosphorylation at Thr(P)308 and Ser(P)473 domains of Akt, which Akt is elevated but not of those cancer cells in which it is inhibited by API-1. API-1 directly inhibits the activity of Akt and blocks Akt membrane translocation and activation the Akt pathway (13).

Akt phosphorylates and/or interacts with a number of molecules to exert its cellular functions, which include roles in cell proliferation, survival, migration, and differentiation (14). Several lines of evidence demonstrate that Akt is a critical player in tumor development. Hyperactivation of the Akt pathway has been detected in up to 50% of all human tumors (15, 16) and is closely associated with chemoresistance (17). Therefore, Akt has been an attracting target for anti-cancer drug discovery (17). A recent study identified a recurring somatic mutation within the PH domain of AKT1 in human breast, colorectal, and ovarian cancers that results in a glutamic acid to lysine substitution protein and apyrase phosphatase that reduces intracellular levels of phosphatidylinositol 3,4,5-trisphosphate by converting it to phosphatidylinositol 4,5-diphosphate, thereby inhibiting Akt membrane translocation and activation the Akt pathway (13).

Akt was first described as the cellular homologue of the product of the v-akt oncogene (1), and it has three members, Akt1/ PKBα, Akt2/PKBβ, and Akt3/PKBγ (2–5). Activation of Akt depends on the integrity of the pleckstrin homology (PH) domain, which mediates its membrane translocation, and on the phosphorylation of Thr308 in the activation loop and Ser473 in the regulatory domain. The kinase activity of Akt depends on the integrity of the pleckstrin homology (PH) domain, which mediates its membrane translocation, and on the phosphorylation of Thr308 in the activation loop and Ser473 in the regulatory domain. Full activation of Akt requires phosphorylation of Thr308 and Ser473 (6). Phosphoinositides phosphatidylinositol 3,4-diphosphate (7, 8) and phosphatidylinositol 3,4,5-trisphosphate (6) are produced by conversion of phosphatidylinositol 4,5-diphosphate by conversion of phosphatidylinositol 4-kinase (9). Recent studies indicate that the rictor-mTOR complex 2 (mTORC2) integrates Akt activation (10, 11), which is frequently activated in human cancers (12). Activation of Akt results in phosphorylation of the Akt substrate substrate-catalyzed Akt phosphorylation in mTORC2. This phosphorylation is important for Akt membrane translocation and activation the Akt pathway (13).

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This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, part of the p-Akt immunoblot from Fig. 4D was reused as part of the p-Akt-T308 immunoblot from the same figure. The authors state that this image reuse does not affect the overall conclusions of the study.
substitution at amino acid 17 (E17K) in the lipid binding pocket (18). Lys-17 alters the electrostatic interactions of the pocket and forms new hydrogen bonds with a phosphoinositide ligand. This mutation activates AKT1 through aberrant pathological localization to the plasma membrane, transforms cells, and induces leukemia in mice (18). Furthermore, the E17K substitution reduces the sensitivity to an allosteric Akt kinase inhibitor (18).

In the present report we identified a small molecule Akt inhibitor, API-1, by screening the compound libraries obtained from NCI/Developmental Therapeutics Program Open Chemical Repository, National Institutes of Health (NCI/DTP) using a cell-based assay. API-1 binds to the Akt PH domain and inhibits Akt membrane translocation, which leads to inhibition of Akt-regulated cell growth and cell survival. In a xenograft nude mouse model, API-1 inhibits growth of tumors with hyperactivated Akt but not in those with low levels of phospho-Akt.

EXPERIMENTAL PROCEDURES

Cell Lines, Compounds, and Plasmids—All cell lines used in this study were either purchased from the ATCC or described previously (19–21). All 2300 compounds were from the NCI/DTP Open Chemical Repository (nci.nih.gov). HA-tagged Akt1, Akt2, and Akt3 expression plasmids have been described previously (21). Wild-type human AKT1 construct was created by reverse transcription-PCR using MCF10A RNA as the template. The PCR products were cloned to BamH1-EcoRI sites of pCMV-Myc-Tag2 vector (Stratagene). The AKT1 primers used for PCR were: forward, 5′-H11032-ATGAGC-GACGTGGCTATTGTGAAGG-3′/H11032, and reverse, 5′-H11032-CTCGC-CCCCCGTTGGCGTACTCC-3′/H11032. AKT1-E17K plasmid was obtained by converting G to A at nucleotide 49 of wild-type AKT1 using the QuikChange site-directed mutagenesis kit (Stratagene). GFP-Akt and GFP-PH domain expression plasmids were created by ligation of Akt and Akt-PH cDNAs into pEGFP-C1 vector (Clontech).

Screening for Inhibition of Akt-transformed Cell Growth—AKT2-transformed NIH3T3 cells or LXSN vector-transfected NIH3T3 control cells (19) were plated into a 96-well tissue culture plate. After treatment with 5 μM concentrations of each compound, cell growth was detected with a CellTier 96 One Solution Cell Proliferation kit (Promega). Compounds that inhibit growth in AKT2-transformed but not LXSN-transfected NIH3T3 control cells were considered as candidates of Akt inhibitors and subjected to further analysis.

In Vitro Protein Kinase and Apoptosis Assays—In vitro kinase assay was performed as described (20, 21). The AKT1 primers used for PCR were: forward, 5′-ATGAGC-GACGTGGCTATTGTGAAGG-3′, and reverse, 5′-CTCGC-CCCCCGTTGGCGTACTCC-3′. AKT1-E17K plasmid was obtained by converting G to A at nucleotide 49 of wild-type AKT1 using the QuikChange site-directed mutagenesis kit (Stratagene). GFP-Akt and GFP-PH domain expression plasmids were created by ligation of Akt and Akt-PH cDNAs into pEGFP-C1 vector (Clontech).

API-1 inhibits Akt phosphorylation in vitro and in vivo. FIGURE 1. Identification of API-1 as an Akt inhibitor. A, shown is the chemical structure of API-1. HA- mutant (HA-Akt1, -AKT2, and -AKT3) and wild-type (HA-WT-Akt1) Akt-transfected HEK293 cells were treated with API-1 or EGF before lysis and immunoprecipitation with anti-HA antibody. The immunoprecipitates were subjected to an in vitro kinase assay. B, shown is a Western blot showing expression of Akt kinases in OVCAR3 cells, which express hyperactivated Akt. Patient serum was used as a positive control. C, shown is a Western blot showing expression of Akt kinases in NIH3T3 cells transfected with HA-WT-Akt1, -AKT2, and -AKT3 and treated with API-1 or EGF before lysis and immunoprecipitation with anti-HA antibody. The immunoprecipitates were subjected to an in vitro kinase assay. D, shown is an in vitro kinase assay of recombinant constitutively active Akt protein in a kinase buffer containing the indicated concentrations of API-1. Compound E, an ATP-mimic multiple kinase inhibitor, was used as the positive control. The API-1 kinase activity was inhibited by 60% at 12.8 μM. The experiment was repeated three times.
API-1 and Akt Protein Binding Assay—The assay for API-1 binding to Akt was performed essentially as previously described for other kinase inhibitors that contain an amino group (22–24). API-1 was immobilized on Sepharose beads (GE Healthcare) through covalent linkage using its amino group (Fig. 1A). Briefly, NHS-activated Sepharose (1 ml) was equilibrated in DMSO and then incubated with 1 mM API-1 and 100 mM triethylamine (the ratio of volumes for coupling solution/Sepharose beads is 0.5:1). The coupling reaction was allowed to proceed on an end-over-end shaker for 16 h. Free NHS groups were blocked with 0.8 M aminoethanol and then alternated washing with two buffers (0.1 M Tris-HCl, pH 8.0, and 0.1 M acetate, 0.5 M NaCl, pH 4.0) (22, 23). The coupled affinity Sepharose beads were incubated with 400 ng of recombinant Akt1 (Upstate Biotechnology) or GST fusion proteins (e.g. GST-PH, GST-KD (kinase domain), or GST-CT (C terminus) of Akt) overnight at 4 °C in buffer containing 50 mM Tris-HCl, pH 7.5, 50, 100, or 150 mM NaCl, 0.2% Nonidet P-40, 5% glycerol, 1.5 mM MgCl₂, 25 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml aprotinin. Subsequently, the beads were washed with the same buffer for 4 times and eluted by heat-denaturing with the sample buffer. Binding protein was separated by 10% SDS-PAGE and immunoblotted with anti-Akt1 and -GST antibodies. NHS-activated Sepharose beads coupling with unrelated compound (BMS-354825) was used as a negative control and compound E (a pan-kinase inhibitor) as a positive control. Both compounds contain an amino group.

Anti-tumor Activity in the Nude Mouse Tumor Xenograft Model—Tumor cells were harvested, resuspended in phosphate-buffered saline, and injected subcutaneously into the right and left flanks (2 × 10⁶ cells/flank) of 8-week-old female
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nude mice as reported previously (21). When tumors reached about 100 mm³, animals were randomized and dosed intraperitoneal with vehicle or drug daily. Control animals received DMSO (20%), and treated animals were injected intraperitoneal with API-1 (10 mg/kg/day) in 20% DMSO.

**RESULTS AND DISCUSSIONS**

**Identification of a Small Molecule Akt/PKB Inhibitor-1 (API-1)**—The fact that aberrant activation of the Akt pathway occurs in almost 50% all the human malignancy (15, 16) and inhibition of Akt induces cell growth arrest and apoptosis prompted industry and academia to develop Akt inhibitors as anti-cancer drugs (25, 26). Although several Akt inhibitors have been reported, many lack anti-tumor activity in vivo. A lipid-based non-selective Akt inhibitor, perifosine, has been evaluated in phase I and II studies (27, 28). However, in neither study was modulation of Akt assessed. A recent phase II study of perifosine in pancreatic cancer was terminated as a result of unacceptable adverse events during the first stage (29). Therefore, there is an unmet need to develop potent and selective Akt inhibitors that are void of inhibiting other kinase activities with minimal adverse effect. To identify small a molecule inhibitor(s) of Akt, we have evaluated 2300 compounds from the NCI/DTP Open Chemical Repository for agents capable of inhibition of AKT2-transformed but not empty vector LXSN-transfected NIH3T3 cells as described under “Experimental Procedures.” Triple experiments showed that 32 compounds inhibited growth only in AKT2-transformed cells. We previously characterized one of them, named API-2/triciribine, which is a pan-
Akt inhibitor, with anti-tumor activity in vitro and in vivo and currently in phase I clinical trial (21). In the present study, we characterized a second Akt inhibitor, API-1. API-1 specifically inhibits the kinase activity and phosphorylation (Thr(P)308 and Ser(P)473) levels of Akt in living cells. Fig. 1A shows the chemical structure of API-1 (Cancer Chemotherapy National Service Center (NSC) 177223; pyrido[2,3-d]pyrimidines), which is structurally related to the antibiotic sangivamycin (30). Although the sangivamycin has been shown to have anti-tumor activity (31–33), NSC 177223/API-1 has not been tested in cancer cells including NCI 60 cell lines (nih.gov). Because API-1 inhibited AKT2-transformed cells over untransformed parental cells, we first examined whether API-1 is an inhibitor of AKT2 kinase and whether it also inhibits the other two members of Akt family. HEK293 cells, which are commonly used to robustly express the protein of interest, were transfected with HA-tagged wild-type Akt1, AKT2, and AKT3. After serum starvation overnight, cells were treated with API-1 for 60 min before EGF stimulation and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to in vitro kinase assay. Fig. 1B shows that API-1 inhibited EGF-induced kinase activity of Akt1, AKT2, and AKT3.

We next examined if API-1 decreases phospho-Akt levels in living cells. OVCAR3 cells, which express elevated levels of phospho-Akt, were treated with different doses of API-1 for 3 h. Immunoblotting analysis with anti-phospho-Akt-S473 antibody showed that API-1 efficiently reduced the phosphorylation levels of Akt with an IC50 of ~0.8 μM. However, total Akt levels were not changed (Fig. 1C). Furthermore, we examined if API-1 directly inhibits Akt kinase activity in vitro. Recombinant constitutively active Akt protein was incubated with Akt/SGK substrate peptide (Upstate Biotechnology) in a kinase buffer containing different amounts of API-1 and compound E, a pan-kinase ATP-competitive inhibitor as positive control. Triple experiments showed that API-1 did not reduce in vitro Akt kinase activity at concentrations that inhibit pAkt in cell culture, whereas a high dose (e.g. 50 μM) of API-1 decreased Akt activity about 20% (Fig. 1D), suggesting that API-1 functions neither as ATP nor substrate competitor.

API-1 Directly Binds to Akt Protein and Inhibits Akt Membrane Translocation—To explore the mechanism by which API-1 inhibits Akt, we performed a protein kinase-compound binding assay because API-1 contains an amino group that has been shown to bind to NHS-activated Sepharose (22–24). After immobilization, compound-bound Sepharose beads were incubated with recombinant Akt protein. After washing and elution, the products were immunoblotted with the indicated antibodies. Fig. 2A shows that API-1 and compound E (positive control), but not BMS-354825 (negative control), pulled down API-1 inhibited tumor growth in vitro and in vivo. Recombinant PKA was incubated in ADB buffer (Upstate Biotechnology) containing the indicated inhibitors (API-1 or PKA1) and substrate Kemptide. The kinase activity was quantified. B and C, API-1 does not inhibit PKC or SGK. Recombinant PKC or SGK protein was incubated with API-1 or compound E, kinase assay was started by adding SGK substrate peptide and [γ-32P]ATP. The kinase activity was quantified (B). HEK293 cells were transfected with HA-SGK and treated with the indicated inhibitors for 3 h. Cells were lysed and immunoblotted with the indicated antibodies (C). D, shown is the effect of API-1 on phosphorylation of ERK, p38, JNK, and Stat3. OVCAR3 cells were treated with API-1 for 3 h and immunoblotted with the indicated antibodies.
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A

HA-myr-Akt1  -Akt1-E40K  -myr-AKT2

API-1 (μM):
0  2  10  0  2  10  0  2  10

p-H2B
p-Akt-T308
Akt

B

Myc-WT-hAkt1  Myc-hAkt1-E17K

EGF:  +  +  +  +  -  -  -  -  -

API-1 (μM):
0  2  5  10  0  2  5  10

p-H2B
p-Akt-T308
p-Akt-S473
myc-hAkt1

C

API-1 (10 μM):
0  0.75  1.5  3  5  9

p-Akt-S473
p-Akt-T308
Akt
p-GSK3β
GSK3β
p-S6
S6

D

OVCAR3

API-1:  -  +
p-Akt-T308
Akt
p-BAD-S136
BAD
p-FOP

E

Myc-wt-AKT1

EGF:  +  +  +

API-1 (μM):
0  2

H2B

Myc-AKT1

F

API-1 (μM):
0  2  10

H2B

AKT1-E17K

G

Myc-WT-hAkt1  Myc-hAkt1-E17K

EGF:  -  +  +  +  -  -  -  -  -

API-1 (μM):
0  2  5  10  0  2  5  10

p-AKT1-S473
Myc-AKT1
API-1 inhibits tumor growth in vitro and in vivo

Akt. To define the domain(s) of Akt that interacts with API-1, we generated GST-PH, -KD, and -CT fusion proteins and incubated them with API-1. Immunoblotting analysis of the eluted products revealed that the PH domain of Akt bound to API-1 in a buffer containing 50, 100, or 150 mM NaCl (Fig. 2B).

Because Akt activation is initiated by PH domain binding to phosphatidylinositol 3,4,5-trisphosphate in the cell membrane, we reasoned that API-1 inhibits Akt through blockage of its membrane translocation. To this end, HeLa cells, which are commonly used for immunofluorescence staining, were transfected with Myc-Akt and then treated with or without API-1 for 30 min before stimulation with IGF1. Immunofluorescence staining revealed that Akt membrane translocation induced by IGF1 was abrogated by API-1 (Fig. 2C). To further demonstrate API-1 inhibition of Akt through targeting the PH domain, we transfected HeLa cells with the GFP-Akt and GFP-PH domain of Akt. Before stimulation with IGF1, cells were treated with and without API-1 for 1 h and examined under a fluorescence microscope. Fig. 2D shows that IGF1-induced GFP-Akt and GFP-PH membrane translocation was largely attenuated by API-1. Moreover, endogenous AKT2 membrane translocation induced by EGF was also inhibited by API-1 (supplemental Fig. S1). Collectively, these findings indicate API-1 inhibition of Akt through binding to the Akt-PH domain and blocking Akt membrane translocation.

API-1 does not inhibit upstream activators of Akt

Activated by extracellular stimuli and intracellular molecules through a PI3K-dependent manner, Akt will activate PDK1 leading to induction of Akt. Therefore, API-1 inhibition of Akt upstream regulators of Akt was examined next. For this end, we examined if API-1 inhibited the upstream regulators of Akt. Similar results were obtained. As shown in Fig. 3A, cells were serum-starved overnight, treated with wortmannin, and then uniformly transfected with Myc-AKT1 and Myc-AKT1-E17K was immunoprecipitated with Myc antibody. The immunoprecipitates were subjected to PDK1 kinase assay using phosphatidylinositol 4-phosphate as substrate. As shown in Fig. 3A, the EGF-induced PDK1 activity was inhibited by wortmannin but not by API-1.

To evaluate the effect of API-1 on PDK1, we performed an in vitro PDK1 kinase assay using SGK as a readout (Upstate Biotechnology). Unlike PDK1 inhibitor UCN-01 (34), API-1 had no effect on in vitro PDK1 kinase activity (Fig. 3B). To further evaluate the effect of API-1 on PDK1 activity in living cells, we examined the phosphorylation level of PDK1-Ser473, a residue that is autophosphorylated and is critical for its activation (35). After API-1 treatment of OVCAR3 cells, immunoblotting analysis shows that phosphorylation levels of PDK1 were inhibited by UCN-01 but not API-1 (Fig. 3C).

In addition to PDK1, Rictor-mTOR (mTORC2) complex activates Akt by phosphorylation of Ser473, whereas Raptor-mTOR (mTORC1) negatively regulates Akt by phosphorylation of IRS1 (10, 37). Because API-1 did not inhibit PDK1, we next examined whether mTORC1 and mTORC2 complexes are affected by API-1. Co-immunoprecipitation and immunoblotting experiments show that API-1 had no effects on the interaction of Rictor-mTOR and Raptor-mTOR, whereas it inhibits phospho-mTOR-S2841 (Fig. 3D), which is resulted from inhibition of Akt-TSC2-Rheb-mTOR cascade (11). These finding further suggest that API-1 directly inhibits Akt.

API-1 is selective for the Akt over the AGC Kinase Members PPA, PKC, and SGK and Other Signaling Molecules ERK, JNK, p38, and Stat3—In addition to Akt, the AGC (PKA/PKG/ PKC) kinase family also includes PKA, PKC, SGK, p90 ribosomal S6 kinase, p70 S6K, mitogen-activated protein kinase, and PKC-related kinases. The protein structures of PKA, PKC, and SGK are similar to those of Akt than other members. Therefore, we examined the effects of API-1 on the enzymes (Fig. 4, A and B). API-1 inhibits rapidly phosphorylation of increasing members of the AGC kinase family, including SGK with a concentration of 10 μM. Figure 4 C shows that the kinase activity of SGK was inhibited by PKAI and competitively, but not by API-1. In addition, we carried out an in vitro SGK kinase assay in HEK293 cells, which were transfected with HA-tagged SGK, which showed that EGF-induced SGK kinase activity was attenuated by wortmannin but not API-1 (Fig. 4C). To evaluate the effect of API-1 on the PKC and PKA activation in living cells, OVCAR3 cells were treated with the indicated doses of API-1 or a specific inhibitor of PKC and PKA, and immunoblotting analysis revealed that phosphorylation levels of PKC and PKA were not inhibited by API-1 (Fig. 4D).

To determine whether API-1 has effects on other oncogenic survival pathways, OVCAR3 cells were treated with API-1 (10 μM) for different times and immunoblotted with commercially available anti-phospho-antibodies. We did not observe detectable changes of phosphorylation levels of Stat3, JNK, p38, and ERK1/2 after API-1 treatment (Fig. 4E). These data suggest that API-1 is a specific Akt inhibitor.

FIGURE 5. API-1 inhibits constitutively active Akt and its downstream targets. A, API-1 inhibited constitutively active Akt. HEK293 cells were transfected with HA-myr-Akt1, HA-Akt1-E40K, and HA-myr-AKT2. After treatment with API-1 for 1 h, cells were lysed and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to in vitro kinase assay (top) and immunoblotting with the indicated antibodies (middle and bottom panels). Note that the second lane from the left (lane 2) shows no significant change of pAkt as compared with lane 1 (middle panel), which is due to higher expression of myr-Akt1 in the transfection (bottom panel). B, kinase activity and phospho-Thr473 and -Ser473 of Akt1-E17K were inhibited by API-1. Myc-tagged human WT AKT1 and AKT1-E17K were introduced into HEK293 cells. After serum starvation overnight, the cells were treated with API-1 at indicated concentration for 1 h. After EGF stimulation of WT-AKT1-transfected cells for 30 min, the cells were subjected to in vitro kinase and immunoblotting analysis as described in panel A. C and D, API-1 inhibited downstream targets of Akt. OVCAR3 and H661 cells were treated with API-1 (10 μM) and immunoblotted with the indicated antibodies. GSK3β, glycogen synthase kinase 3. E–G, API-1 is more potent than API-2. HEK293 cells were transfected with wild-type Myc-AKT1 (E) and constitutively active myc-AKT1-E17K (F). After 36 h incubation, cells were serum-starved overnight. WT-AKT1-transfected cells were treated with API-1 (left) or API-2 (right) for 30 min and subsequently stimulated with 15 ng/ml EGF for 15 min. Immunoblotting was carried out with anti-myc antibody, and the immunoprecipitates were subjected to in vitro kinase assay (top). Akt kinase activity was quantified (middle). The bottom panels show expression of transfected plasmids. The experiments were repeated three times. Shown is a Western blot analysis of pAkt (G) in HEK293 cells that were transfected with Myc-AKT1 and Myc-AKT1-E17K and treated with or without API-1 or API-2 at the indicated doses for 1 h.
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API-1 Inhibits Constitutively Active Akt, Including Naturally Occurring Mutant AKT1-E17K and Its Downstream Targets—Because API-1 abrogates kinase activity and phosphorylation of Akt in living cells and binds to the PH domain of Akt, we assumed API-1 may inhibit AktE140K and AktE17K but not Myr-Akt as Glu<sup>40</sup> and Glu<sup>17</sup> locate in the PH domain, whereas myristoylation (myr) could directly bind to membrane. To this end we transfected HEK293 cells with HA-tagged myr-Akt1, myr-AKT2, and Akt1E40K and myc-tagged Akt1E17K. After serum starvation overnight, cells were treated with or without API-1. Akt were immunoprecipitated with anti-HA or antimyc antibody. The immunoprecipitates were subjected to in vitro kinase assay and immunoblotting analysis with anti-phospho-Akt-Thr<sup>308</sup> antibody. Unexpectedly, API-1 inhibited all four forms of constitutively active Akt (Fig. 5, A and B), suggesting that API-1 also interferes with myristoylation signal by binding to Akt. Nevertheless, API-1 inhibition of AKT1-E17K is clinically significant because the E17K mutation, which was detected in human tumors, leads to constitutively activation of AKT1 through aberrant pathological localization to the plasma membrane (18). An allosteric Akt kinase inhibitor AKT1/2 inhibitor VIII (22) could not efficiently inhibit AKT1-E17K (18).

Akt exerts its cellular function through phosphorylation of a number of proteins (6, 14). Thus, we next examined whether API-1 inhibits downstream targets of Akt. Because Akt Phosphatase and Tensin Homolog (PTEN) gene, mTOR/p70<sup>S6K</sup>, Bad, and Forkhead Box O3a (FOXO3a) are major Akt targets, we treated OVCAR3 and COLO357 cells that do not contain hyperactivated Akt (Fig. 5, C and D). These results indicate that API-1 inhibits cell growth and induces apoptosis preferentially in the cells that express aberrant Akt.

API-1 Inhibits Tumor Growth in Vitro and in Vivo—We and others have previously shown that overexpression and overexpression of Akt are frequent events in human cancer (2, 15, 16) and that anti-Pten Akt significantly inhibits tumor cell growth (42). Therefore, inhibition of Akt pathway by inhibitors of Akt, PI3K, HSP70, Src, and farnesyltransferase resulted in cell growth arrest and induction of apoptosis (20, 39, 40). Because API-1 inhibits Akt signaling and induces apoptosis and cell growth arrest in cancer cells with elevated levels of Akt (Fig. 6), we reasoned that the growth of tumors with elevated levels of Akt should be more sensitive to API-1 than that of tumors with low levels of Akt in nude mice. To this end, we subcutaneously implanted tumors with hyperactivated Akt (OVCAR3 and PANC-1) into the left flank and those tumors that express low levels of activated Akt (OVCAR5 and COLO357) into the right flank of mice. When the tumors reached an average size of about 100 mm<sup>3</sup>, the animals were randomized and treated intraperitoneal with either vehicle or API-1 (10 mg/kg/day). As illustrated in Fig. 7, A–C, OVCAR3 and PANC1 tumors treated with vehicle control continued to grow. API-1 inhibited OVCAR3 and PANCl tumor growth by 70 and 50%, respectively (Fig. 7, B and C). In contrast, API-1 had little effect on the growth of OVCAR5 and COLO357 cells in nude mice (Fig. 7, A–C). At
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A

Control

Left: PANC1
Right: COLO357

API-1

Left: OV3
Right: OV5

B

OVCAR3

Tumor Volume (mm³)

0 100 200 300 400 500 600

Treatment time (weeks)

1 2 3 4 5 6

DMSO

API-1

OVCAR5

Tumor Volume (mm³)

0 100 200 300 400 500 600

Treatment time (weeks)

1 2 3 4 5 6

DMSO

API-1

COLO357

Tumor Volume (mm³)

0 50 100 150 200 250 300

Treatment time (weeks)

1 2 3 4 5 6

DMSO

API-1

C

PANC1

COLO357

OVCAR3

OVCAR5

Control

API-1

Control

API-1

Control

API-1

Control

API-1

D

Tumor weight (g)

PANC1

COLO357

OV3

OV5

p-Akt T308

1.0 1.0 0.2 0.3

Control

API-1

Akt
a dose of 10 mg/kg/day, API-1 had no effects on blood glucose level, body weight, activity, and food intake of mice (data not shown). In treated tumor samples, phosphorylation levels of Akt were reduced by API-1 about 70% without a change of total Akt content (Fig. 7D). Taken together, these results indicate that API-1 selectively inhibits the growth of tumors with hyperactivated Akt.

In the last several years, through combinatorial chemistry, high throughput and virtual screening, and traditional medicinal chemistry, several inhibitors of the Akt pathway have been identified (25, 26). Lipid–based inhibitors of Akt were the first to be developed, including perifosine (43), PX-316 (44), and phosphatidylinositol ether lipid analogues (45), which were designed to interact with the PH domain of Akt. In addition, several Akt antagonists have been identified using high throughput screening of chemical libraries and rational design. These inhibitors include 9-methoxy-2-methylellipticinium acetate (46), the indazole-pyridine A-443654 (47), isoform-specific allosteric kinase inhibitors (48), and Akt/PKB signaling inhibitor-2 (API-2), also called triciribine/TCN (21). TCN is a tricyclic nucleoside that has previously been evaluated clinically and raises the possibility that lower doses that inhibit Akt may result in a clinical response with less toxicity in those patients whose tumors have hyperactivated Akt is undergoing phase I trials in leukemia. Unlike API-1, a selective inhibitor of Akt and has not only activated wild-type Akt, but also constitutively active Akt mutants including myr-Akt1, myr-Akt2, and E40K-Akt1 as well as the recurring mutant AKT1-E17K.

In summary, we have identified an Akt inhibitor, API-1, that inhibits Akt by binding to the PH domain of Akt and blocking Akt membrane translocation. As a result, API-1 selectively induces apoptosis and inhibits cell growth. The ability of API-1 to inhibit growth of human tumor xenografts in nude mice provides validation for the development of drugs targeting Akt to treat cancers displaying hyperactivated Akt. Further investigation is needed to evaluate whether API-1 is clinically useful in this setting. In addition, API-1 could be further chemically modified and optimized to develop it into a more effectively therapeutic agent.
Fig. S1

α-AKT2/OVCAR3

Starvation

API-1/EGF

WITHDRAWN

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