

Akt/Protein Kinase B Signaling Inhibitor-2, a Selective Small Molecule Inhibitor of Akt Signaling with Antitumor Activity in Cancer Cells Overexpressing Akt

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Abstract

Accumulated studies have shown that activation of the Akt pathway plays a pivotal role in malignant transformation and chemoresistance by inducing cell survival, growth, migration, and angiogenesis. Therefore, Akt is believed to be a critical target for cancer intervention. Here, we report the discovery of a small molecule Akt pathway inhibitor, Akt/protein kinase B signaling inhibitor-2 (API-2), by screening the National Cancer Institute Diversity Set. API-2 suppressed the kinase activity and phosphorylation level of Akt. The inhibition of Akt kinase resulted in suppression of cell growth and induction of apoptosis in human cancer cells that harbor constitutively activated Akt due to overexpression of Akt or other genetic alterations such as PTEN mutation. API-2 is highly selective for Akt and does not inhibit the activation of phosphatidylinositol 3'-kinase, phosphoinositide-dependent kinase-1, protein kinase C, serum- and glucocorticoid-inducible kinase, protein kinase A, signal transducer and activators of transcription 3, extracellular signal-regulated kinase-1/2, or c-Jun NH₂-terminal kinase. Furthermore, API-2 potently inhibited tumor growth in nude mice of human cancer cells in which Akt is aberrantly expressed/activated but not of those cancer cells in which it is not. These findings provide strong evidence for pharmacologically targeting Akt for anticancer drug discovery.

Introduction

Akt, also named protein kinase B, represents a subfamily of the serine/threonine kinase. Three members, AKT1, AKT2, and AKT3, have been identified in this subfamily. Akt is activated by extracellular stimuli in a phosphatidylinositol 3'-kinase (PI3k)-dependent manner (1, 2). Full activation of Akt requires phosphorylation of threonine 308 in the activation loop and serine 473 in the COOH-terminal activation domain. Akt is negatively regulated by PTEN tumor suppressor. Mutations in *PTEN* have been identified in various tumors, which lead to activation of Akt pathway (1, 2). In addition, amplification, overexpression, and/or activation of Akt have been detected in a number of human malignancies (1, 2). Ectopic expression of Akt, especially constitutively active Akt, induces cell survival and malignant transformation, whereas inhibition of Akt activity stimulates apoptosis in a range of mammalian cells (1–4). Furthermore, activation of Akt has been shown to associate with tumor invasiveness and chemoresistance (5). These observations establish Akt as an attractive target for cancer therapy.

Here, we report the identification of a small molecule inhibitor of Akt pathway, Akt/protein kinase B signaling inhibitor-2 (API-2), by screening the National Cancer Institute (NCI) Diversity Set. API-2 potently inhibits Akt signaling in human tumor cells with aberrant

Akt, leading to inhibition of cell growth and induction of apoptosis. In a xenograft nude mice model, API-2 significantly inhibits tumor growth in Akt-overexpressing cells but not in the tumors with low levels of Akt.

Materials and Methods

Cell Lines and NCI Diversity Set. All cell lines used in this study were either purchased from American Type Culture Collection or described previously (4, 6). The NCI Structural Diversity Set is a library of 1,992 compounds selected from the approximately 140,000-compound NCI drug depository. In-depth data on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program Web site.⁵

Screening for Inhibition of Akt-Transformed Cell Growth. AKT2 transformed NIH3T3 cells or LXS vector-transfected NIH3T3 control cells (4) were plated into 96-well tissue culture plate. After treatment with 5 μ M NCI Diversity Set compound, cell growth was detected with CellTier 96 One Solution Cell Proliferation kit (Promega). Compounds that inhibit growth in AKT2-transformed but not LXS-transfected NIH3T3 cells were considered as candidates of Akt inhibitor and subjected to additional analysis.

In Vitro Protein Kinase, Cell Survival, and Apoptosis Assays. *In vitro* kinase was performed as described previously (7). Cell survival was assayed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega). Apoptosis was detected with annexin V, which was performed as described previously (7). Recombinant Akt and phosphoinositide-dependent kinase-1 (PDK1) were purchased from Upstate Biotechnology, Inc.

Antitumor Activity in the Nude Mouse Tumor Xenograft Model. Tumor cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks (2×10^6 cells/flank) of 8-week-old female nude mice as reported previously (8). When tumors reached about 100–150 mm³, animals were randomized and dosed i.p. with 0.2-ml vehicle of drug daily. Control animals received DMSO (20%) vehicle, whereas treated animals received injections of API-2 (1 mg/kg/day) in 20% DMSO.

Results

Identification of Small Molecule Inhibitor of Akt Signaling Pathway, API-2. Frequent alterations of Akt have been detected in human cancer, and disruption of Akt pathway induces apoptosis and inhibits tumor growth (9). Thus, Akt has been considered as an attractive molecular target for development of novel cancer therapeutics. To identify small molecule inhibitor(s) of Akt, we have evaluated a chemical library of 1,992-compounds from the NCI Diversity Set for agents capable of inhibition of growth in AKT2-transformed but not empty vector LXS-transfected NIH3T3 cells as described in "Materials and Methods." Repeated experiments showed that 32 compounds inhibited growth only in AKT2-transformed cells. The most potent of these compounds, API-2 (NCI identifier, NSC 154020), suppressed cell growth at a concentration of 50 nm. Fig. 1A shows the chemical structure of API-2, which is also known as triciribine (TCN;

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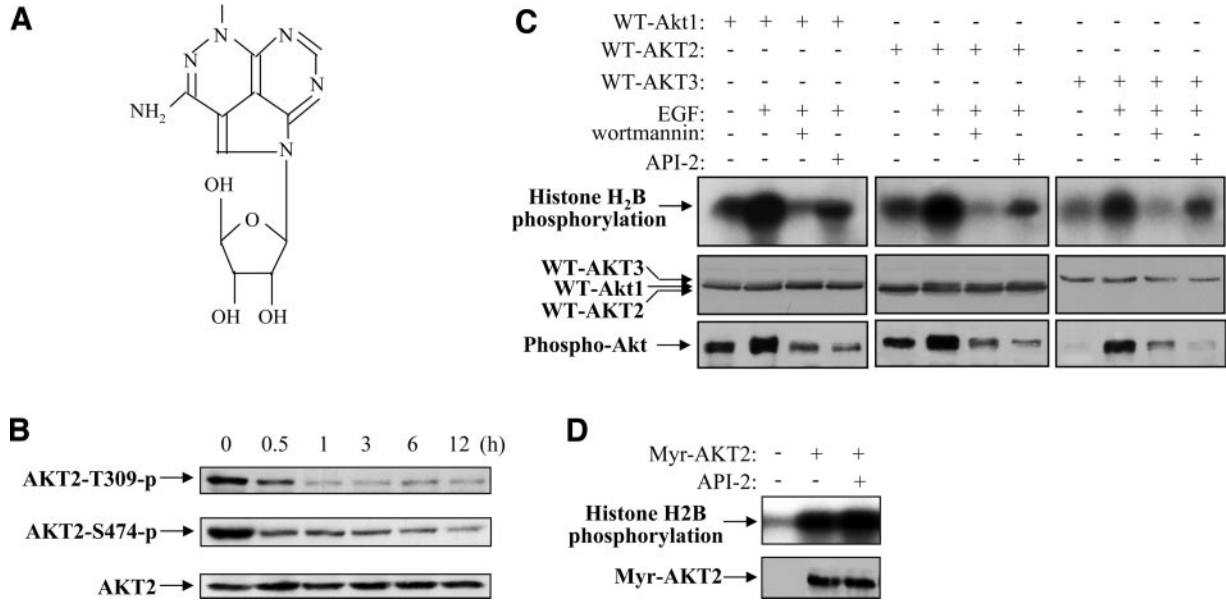
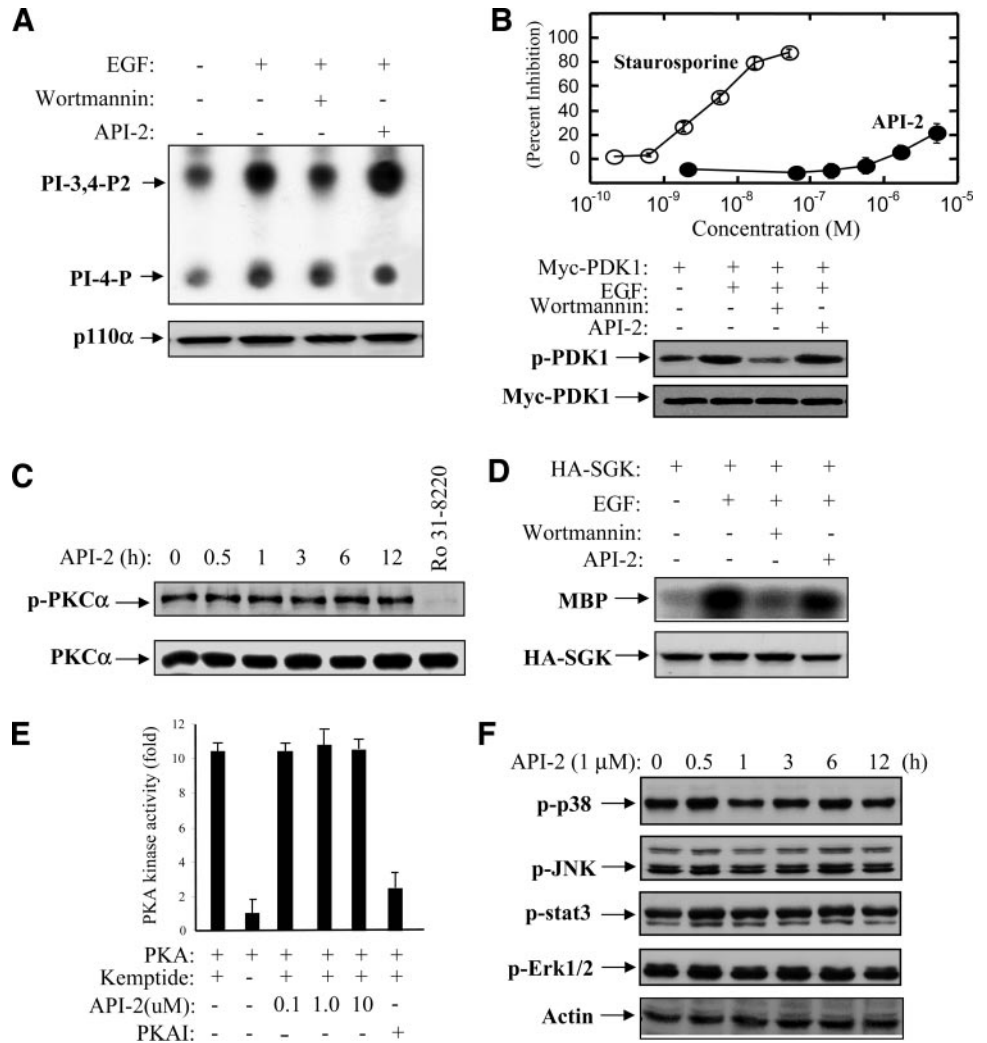


Fig. 1. Identification of API-2 (TCN) as a candidate of Akt inhibitor from the NCI Diversity Set. **A**, chemical structure of API-2 (TCN). **B**, API-2 inhibits phosphorylation levels of AKT2 in AKT2-transformed NIH3T3 cells. Wild-type (WT) AKT2-transformed NIH3T3 cells were treated with API-2 (1 μ M) for indicated times and subjected to immunoblotting analysis with anti-phospho-Akt-threonine 308 (AKT2-T309-p) and anti-phospho-Akt-serine 473 (AKT2-S474-p) antibodies (top and middle panels). Bottom panel shows expression of total AKT2. **C**, API-2 inhibits three isoforms of Akt. HEK293 cells were transfected with HA-Akt1, -AKT2, and -AKT3 and treated with API-2 (1 μ M) or wortmannin (15 μ M) before EGF stimulation; the cells were lysed and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (top) and immunoblotting analysis with anti-phospho-Akt-threonine 308 (bottom) antibody. Middle panel shows expression of transfected Akt1, AKT2, and AKT3. **D**, API-2 did not inhibit Akt *in vitro*. *In vitro* kinase assay of constitutively active AKT2 recombinant protein in a kinase buffer containing 1 μ M API-2 (Lane 3).

Fig. 2. API-2 does not inhibit PI3k, PDK1, and the closely related members of AGC kinase family. **A**, *in vitro* PI3k kinase assay. HEK293 cells were serum-starved and treated with API-2 (1 μ M) or wortmannin (15 μ M) for 30 min before EGF stimulation. Cells were lysed and immunoprecipitated with anti-p110 α antibody. The immunoprecipitates were subjected to *in vitro* kinase assay using phosphatidylinositol 4-phosphate as substrate. **B**, effect of API-2 on *in vitro* PDK1 activation (top panel). ●, inhibition by API-2. ○, inhibition by the positive control staurosporine, which is a potent PDK1 inhibitor (IC₅₀, 5 nM). Bottom panels are immunoblotting analyses of HEK293 cells that were transfected with Myc-PDK1 and treated with wortmannin or API-2 before EGF stimulation. The immunoblots were detected with indicated antibodies. **C**, immunoblotting analysis of phosphorylation levels of PKC α with anti-phospho-PKC α -T638 (top) and total PKC α (bottom) antibodies after treatment with API-2 or a nonselective PKC inhibitor Ro31-8220. **D**, *in vitro* SGK kinase assay. HEK293 cells were transfected with HA-SGK and treated with API-2 or wortmannin before EGF stimulation. *In vitro* kinase was performed with HA-SGK immunoprecipitates using MBP as substrate (top). Bottom panel shows the expression of transfected HA-SGK. **E**, PKA kinase assay. Immuno-purified PKA was incubated in ADB buffer (Upstate Biotechnology, Inc.) containing indicated inhibitors (API-2 or PKAI) and substrate Kempptide. The kinase activity was quantified. **F**, Western blot. OVCAR3 cells were treated with API-2 for indicated times. Cell lysates were immunoblotted with indicated anti-phospho-antibodies (panels 1-4) and anti-actin antibody (bottom).



Ref. 10). The fact that API-2 inhibited selectively AKT-2-transformed cells over untransformed parental cells prompted us to determine whether API-2 is an inhibitor of AKT2 kinase. To this end, AKT2 was immunoprecipitated with anti-AKT2 antibody from AKT-2-transformed NIH3T3 cells after treatment with API-2. AKT2 immunoprecipitates were immunoblotted with anti-phospho-Akt antibodies. As shown in Fig. 1B, API-2 significantly inhibited AKT2 phosphorylation at both threonine 309 and serine 474, which are required for full activation of AKT2 (1, 2). Because three isoforms of Akt share high homology and similar structure, we next evaluated the effect of API-2 on their kinase activities. HEK293 cells were transfected with hemagglutinin (HA)-Akt1, HA-AKT2, and HA-AKT3; serum-starved overnight; and treated with API-2 for 60 min before epidermal growth factor (EGF; 50 ng/ml) stimulation. Triple experiments showed that API-2 suppressed EGF-induced kinase activity and phosphorylation of Akt1, AKT2, and AKT3 (Fig. 1C). However, kinase activity of recombinant constitutively active AKT2 (Myr-AKT2) was not inhibited by API-2 in an *in vitro* kinase reaction (Fig. 1D), suggesting that API-2 does not directly inhibit Akt *in vitro* and that API-2 neither functions as ATP competitor nor as the substrate competitor that binds to active site of Akt.

API-2 Does Not Inhibit Known Upstream Activators of Akt. It has been well documented that Akt is activated by extracellular stimuli and intracellular signal molecules, such as active Ras and Src, through a PI3k-dependent manner. Therefore, API-2 inhibition of Akt could result from targeting upstream molecule(s) of Akt. Because PI3k and PDK1 are direct upstream regulators of Akt (1, 2), we next

examined whether API-2 inhibits PI3k and/or PDK1. HEK293 cells were serum-starved and then treated with API-2 or PI3k inhibitor wortmannin for 30 min before EGF stimulation. PI3k was immunoprecipitated with anti-p110 α antibody. The immunoprecipitates were subjected to *in vitro* PI3k kinase assay using phosphatidylinositol 4-phosphate as a substrate. As shown in Fig. 2A, the EGF-induced PI3k activity was inhibited by wortmannin but not by API-2. To evaluate the effect of API-2 on PDK1, we used an assay in which recombinant PDK1 promotes the threonine 309 phosphorylation of AKT2 peptides in the presence of lipid vesicles containing phosphatidylinositol (2). As shown in Fig. 2B, the assay was potently inhibited by the control PDK1 inhibitor staurosporine (IC₅₀, 5 nM). In contrast, API-2 displayed only 21% inhibition of the assay at the highest concentration tested (5.1 μ M). These data demonstrate that API-2 is not a potent inhibitor of PDK1. To further evaluate the effect of API-2 on PDK1 activation, we examined autophosphorylation level of PDK1 at serine 241, a residue that is phosphorylated by itself and is critical for its activity (1), after API-2 treatment of HEK293 cells. Triplicate experiments show that phosphorylation levels of PDK1 were not inhibited by API-2 (Fig. 2B). However, PI3k inhibitor wortmannin, as expected, inhibited EGF-stimulated PDK1 (Fig. 2B).

API-2 Is Highly Selective for the Akt over Protein Kinase C (PKC), Protein Kinase A (PKA), Serum- and Glucocorticoid-Inducible Kinase (SGK), Signal Transducer and Activators of Transcription, c-Jun NH₂-Terminal Kinase, p38, and Extracellular Signal-Regulated Kinase Signaling Pathways. Akt belongs to the AGC (PKA/PKC/PKC) kinase family, which also includes PKA,

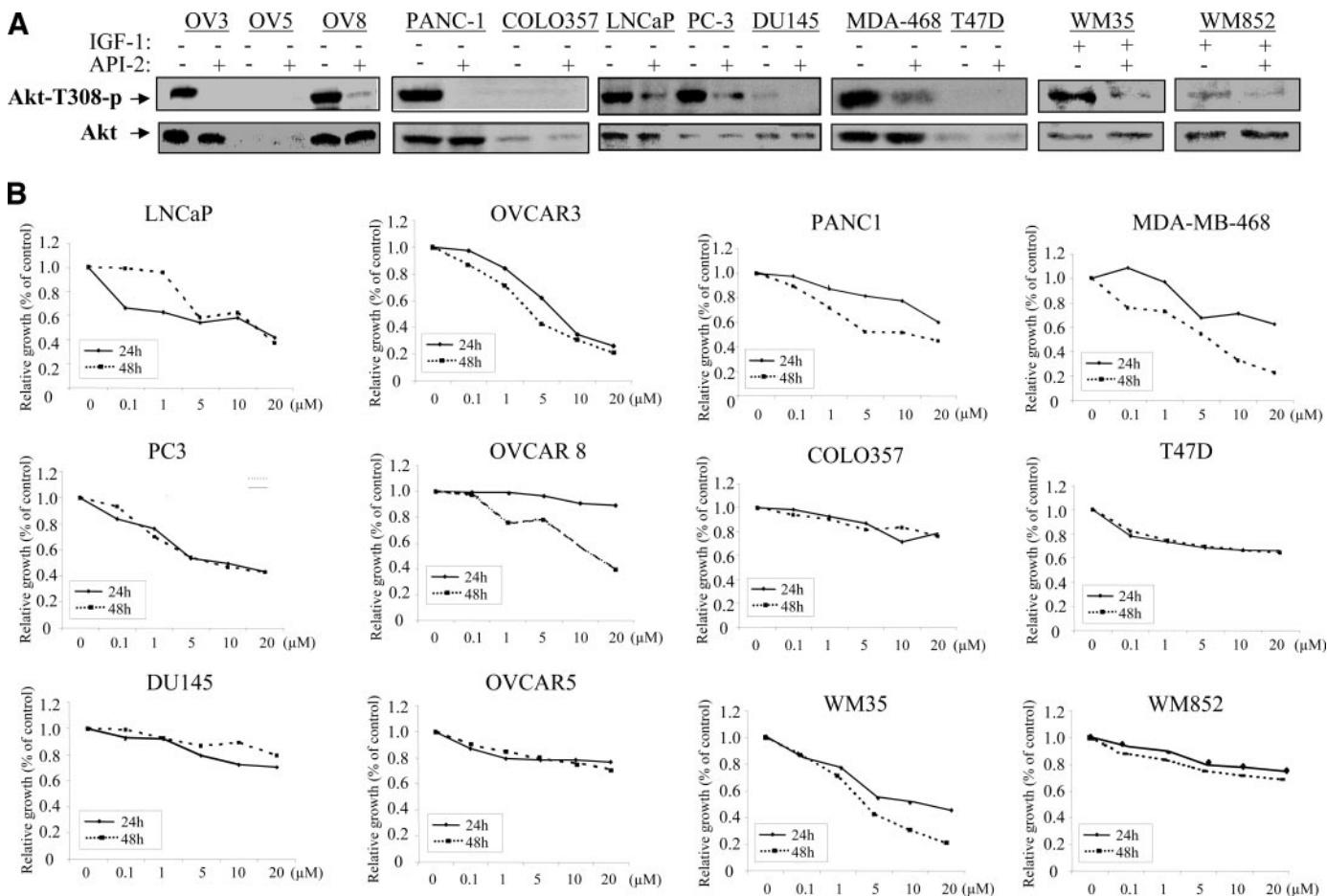


Fig. 3.

PKC, SGK, p90 ribosomal S6 kinase, p70^{S6K}, mitogen- and stress-activated protein kinase, and PKC-related kinase. Among AGC kinase family members, protein structures of PKA, PKC, and SGK are more close to Akt kinase than other members. Therefore, we next examined the effects of API-2 on the enzymatic activities of these three kinases. HEK293 cells were transfected with HA-tagged PKA, PKC α , or SGK. *In vitro* kinase assay and immunoblotting analysis showed that the kinase activities of PKA and PKC α were inhibited by PKAI and Ro 31-8220, a PKC inhibitor, respectively, whereas API-2 exhibited no effect on their activities (Fig. 2, C and E). Furthermore, EGF-induced SGK kinase activity was attenuated by wortmannin but not by API-2 (Fig. 2D). In addition, we determined whether API-2 has effect on other oncogenic survival pathways. Western blotting analyses with commercially available anti-phospho-antibodies revealed that phosphorylation levels of signal transducer and activators of transcription 3, c-Jun NH₂-terminal kinase, p38, and extracellular signal-regulated kinase-1/2 were not affected by API-2 treatment (Fig. 2F). These data indicate that API-2 specifically inhibits Akt signaling pathway.

API-2 Suppresses Cell Growth and Induces Apoptosis in Akt-Overexpressing/Activating Human Cancer Cell Lines. The ability of API-2 to selectively inhibit the Akt pathway suggests that it should inhibit proliferation and/or induces apoptosis preferentially in those tumor cells with aberrant expression/activation of Akt. Because activation of Akt in human malignancies commonly results from overexpression of Akt or *PTEN* mutations, API-2 was used to treat the cells that express constitutively active Akt, caused by overexpression of AKT2 (OVCAR3, OVCAR8, PANC1, and AKT2-transformed NIH3T3) or mutations of the *PTEN* gene (PC-3, LNCaP, and MDA-MB-468), and cells that do not (OVCAR5, DU-145, T47D, COLO357, and LXSNI-NIH3T3), as well as melanoma cells that are

activated by insulin-like growth factor-I to activate Akt or do not respond to growth stimulation by insulin-like growth factor-I (6). Immunoblotting analysis showed that phosphorylation levels of Akt were inhibited by API-2 only in the cells expressing elevated Akt or responding to insulin-like growth factor-I stimulation (Fig. 3A). Accordingly, API-2 inhibited cell growth to a much higher degree in Akt-overexpressing/activating cells compared with those with low levels of Akt. As shown in Fig. 3B, API-2 treatment inhibited cell proliferation by approximately 50–60% in Akt-overexpressing/activating cell lines, LNCaP, PC-3, OVCAR3, OVCAR8, PANC1, MDA-MB-468, and WM35, whereas only by about 10–20% in DU145, OVCAR5, COLO357, T47D, and WM852 cells, which exhibit low levels of Akt or do not respond to growth stimulation by insulin-like growth factor-I. Moreover, API-2 induces apoptosis by 8-fold (OVCAR3), 6-fold (OVCAR8), 6-fold (PANC1), and 3-fold (AKT2-NIH3T3). No significant difference of apoptosis was observed between API-2 and vehicle (DMSO) treatment in OVCAR5, COLO357, and LXSNI-NIH3T3 cells (Fig. 3C). Thus, API-2 inhibits cell growth and induces apoptosis preferentially in cells that express aberrant Akt.

API-2 Inhibits Downstream Targets of Akt. It has been shown that Akt exerts its cellular effects through phosphorylation of a number of proteins (1). More than 20 proteins have been identified as Akt substrates, including the members of Forkhead protein family (FKHR, AFX, and FKHL1), tuberlin/TSC2, p70^{S6K}, GSK-3 β , p21^{WAF1/Cip1}, p27^{kip1}, MDM2, Bad, ASK1, and IKK α , etc. We next examined whether API-2 inhibits downstream targets of Akt. Because anti-phospho-tuberlin, anti-phospho-Bad, anti-phospho-AFX, and anti-phospho-GSK-3 β antibodies are commercially available, we therefore determined the effect of API-2 on their phosphorylation induced by

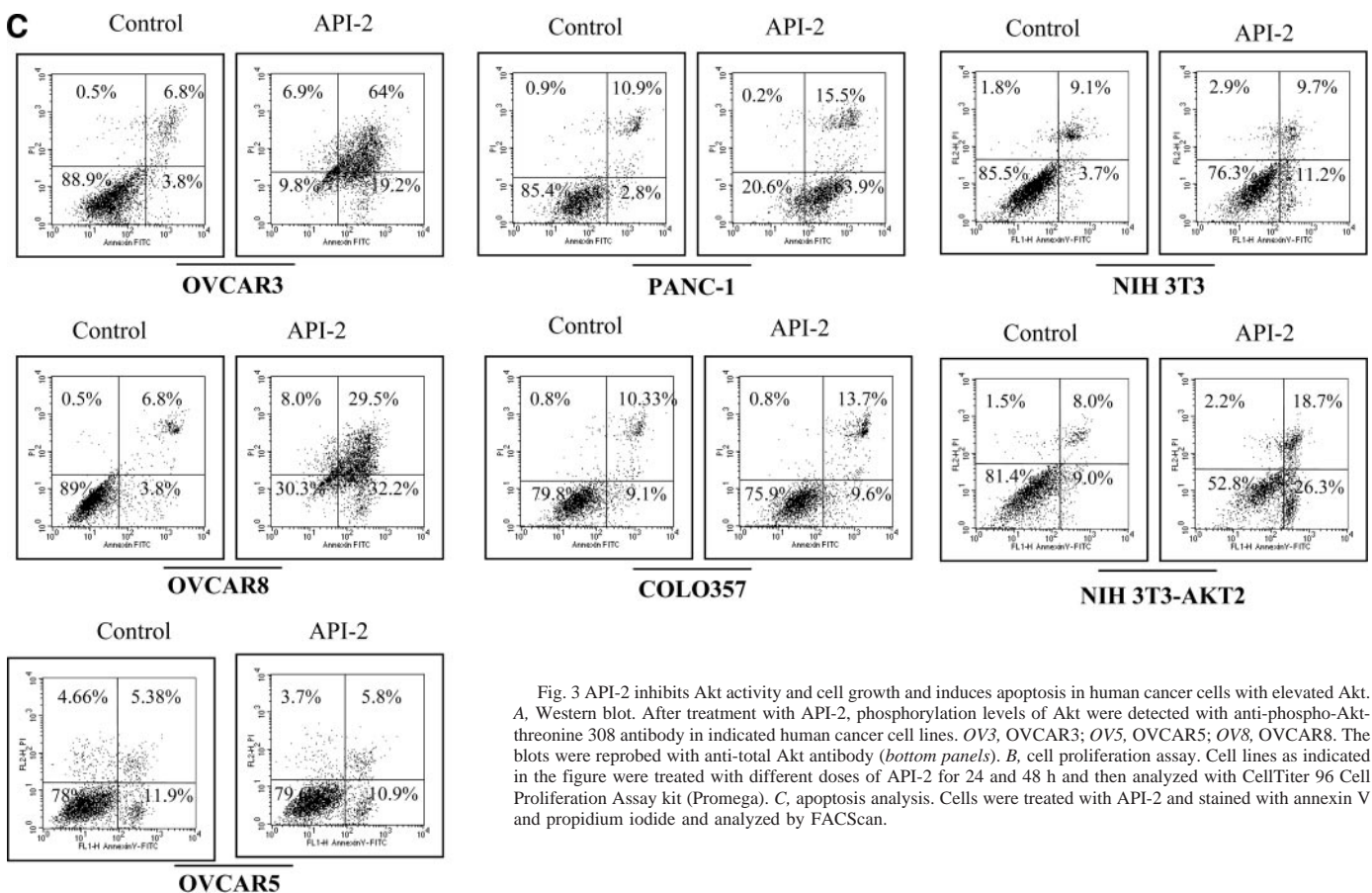


Fig. 3 API-2 inhibits Akt activity and cell growth and induces apoptosis in human cancer cells with elevated Akt. A, Western blot. After treatment with API-2, phosphorylation levels of Akt were detected with anti-phospho-Akt-threonine 308 antibody in indicated human cancer cell lines. OV3, OVCAR3; OV5, OVCAR5; OV8, OVCAR8. The blots were reprobbed with anti-total Akt antibody (bottom panels). B, cell proliferation assay. Cell lines as indicated in the figure were treated with different doses of API-2 for 24 and 48 h and then analyzed with CellTiter 96 Cell Proliferation Assay kit (Promega). C, apoptosis analysis. Cells were treated with API-2 and stained with annexin V and propidium iodide and analyzed by FACSscan.

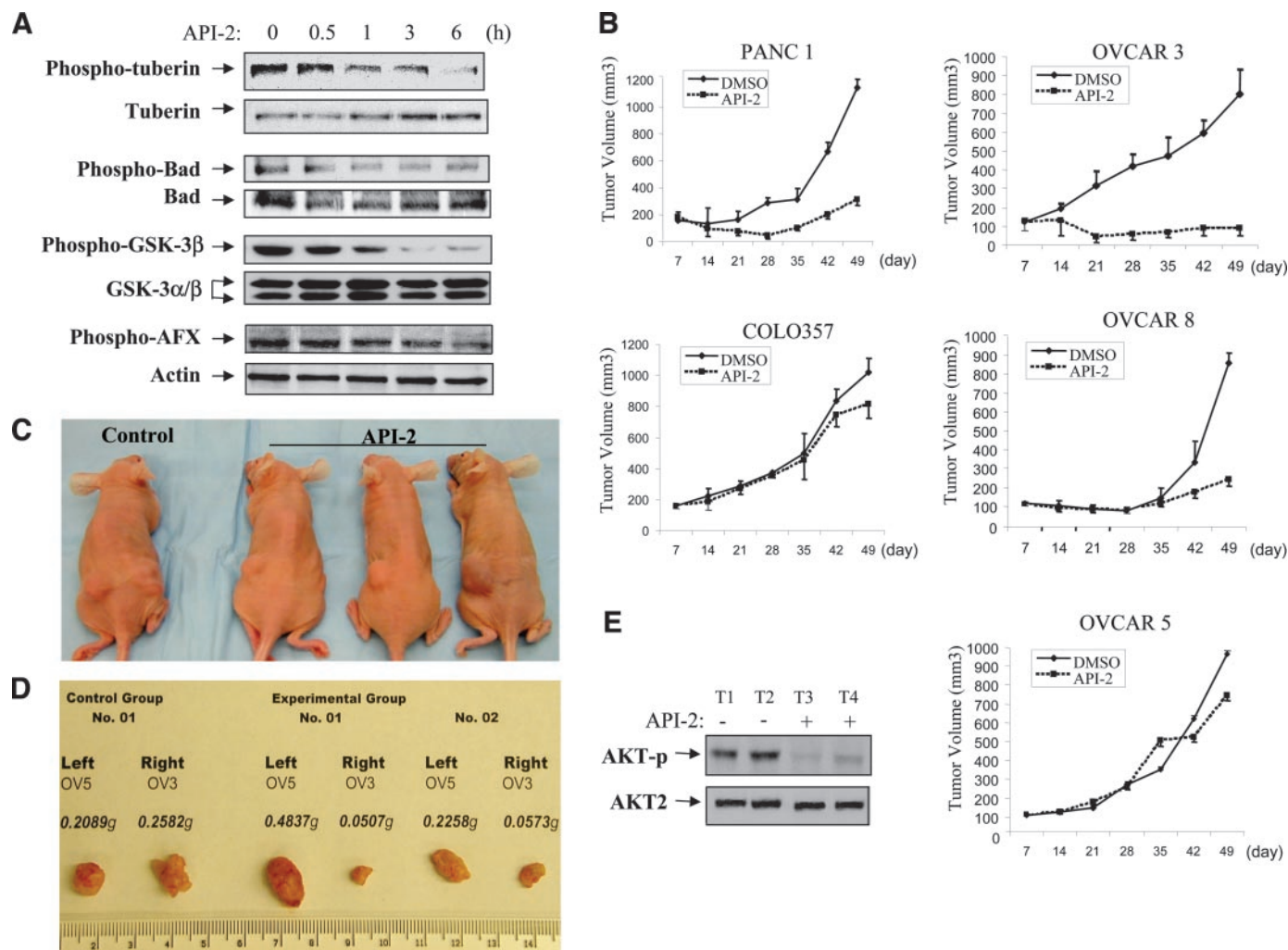


Fig. 4. API-2 inhibits downstream targets of Akt and exhibits antitumor activity in cancer cell lines with elevated Akt in mouse xenograft. **A**, API-2 inhibits Akt phosphorylation of tuberlin, Bad, AFX, and GSK-3 β . After treatment with API-2, OVCAR3 cells were lysed and immunoblotted with indicated antibodies. **B**, API-2 inhibits tumor growth. Tumor cells were s.c. injected into nude mice with low level of Akt cells on left side and elevated level of Akt cells on right side. When the tumors reached an average size of about 100–150 mm³, animals were treated with either vehicle or 1 mg/kg/day API-2 as described in “Materials and Methods.” Each measurement represents an average of 10 tumors. **C**, representation of the mice with OVCAR3 (right) and OVCAR5 (left) xenograft treated with API-2 or vehicle (control). **D**, examples of tumor size (bottom) and weight (top) at the end of experiment. **E**, immunoblotting analysis of tumor lysates with anti-phospho-Akt-serine 473 (top) and anti-AKT2 (bottom) antibodies in OVCAR3-derived tumors that were treated (T3 and T4) and untreated (T1 and T2) with API-2.

Akt. After API-2 (1 μ M) treatment, OVCAR3 cells were lysed and immunoblotted with the individual anti-phospho-antibody. Fig. 4A shows that API-2 considerably inhibited the phosphorylation levels of tuberlin leading to stabilization and up-regulation of tuberlin (11). The phosphorylation levels of Bad, GSK-3 β , and AFX were partially attenuated by API-2. These data suggest that API-2 induces cell death and cell growth arrest by inhibiting phosphorylation of its downstream targets. API-2 inhibition of Akt downstream targets at different degrees could be due to the fact that phosphorylation sites of these targets are also regulated by other kinase(s), for instance, Bad serine 136 is phosphorylated by PAK1 in addition to Akt (12).

API-2 Inhibits the Growth of Tumors in Nude Mice That Overexpress Akt. We have previously shown frequent overexpression/activation and/or amplification of AKT1 and AKT2 in human ovarian and pancreatic cancer (2). Inhibition of Akt pathway by inhibitors of PI3k, HSP70, Src, and farnesyltransferase resulted in cell growth arrest and induction of apoptosis (13, 14). A recent study showed that the tumor growth of xenografts with elevated Akt was also significantly inhibited by intratumoral injection of adenovirus of dominant-negative Akt (9). Because API-2 inhibits Akt signaling and induces apoptosis and cell growth arrest only in cancer cells with

elevated levels of Akt (Fig. 3), we reasoned that the growth of tumors with elevated levels of Akt should be more sensitive to API-2 than that of tumors with low levels of Akt in nude mice. To this end, we s.c. implanted Akt-overexpressing cells (OVCAR3, OVCAR8, and PANC-1) into the right flank and those cell lines that express low levels of Akt (OVCAR5 and COLO357) into the left flank of mice. When the tumors reached an average size of about 100–150 mm³, the animals were randomized and treated i.p. with either vehicle or API-2 (1 mg/kg/day). As illustrated in Fig. 4B, OVCAR5 and COLO357 tumors treated with vehicle grew to about 800–1,000 mm³ 49 days after tumor implantation. OVCAR3, OVCAR8, and PANC1 tumors treated with vehicle control grew to about 700–900 mm³ 49 days after tumor implantation. API-2 inhibited OVCAR3, OVCAR8, and PANC1 tumor growth by 90, 88, and 80%, respectively. In contrast, API-2 had little effect on the growth of OVCAR5 and COLO357 cells in nude mice (Fig. 4, B–D; data not shown). At a dose of 1 mg/kg/day, API-2 had no effect on blood glucose level, body weight, activity, and food intake of mice. In treated tumor samples, Akt activity was inhibited by API-2 without a change of total Akt content (Fig. 4E). Taken together, these results indicate that API-2 selectively inhibits the growth of tumors with elevated levels of Akt.

Discussion

In this study, we identified a small molecule inhibitor of Akt signaling, API-2, by screening the NCI diversity set. Treatment of human cancer cells with API-2 suppresses Akt signaling without obvious inhibitory effects on a number of other oncogenic kinases examined. In tumor xenograft mouse model, we further demonstrated that the antitumor effect of API-2 is selective for those tumors with aberrant Akt. Significantly, the high degree of efficacy was achieved at a low dose of API-2 and no compound related side effects were observed. In contrast, little efficacy was seen with tumors displaying low levels of Akt. API-2 is a synthetic small molecule compound identified previously and named TCN or tricyclic nucleoside (10). Previous studies have shown that API-2/TCN inhibits DNA synthesis and has antitumor and antiviral activity (15, 16). Our data indicate that API-2/TCN inhibition of Akt pathway plays a key role in its antitumor activity.

Phase I and II clinic trials of API-2/TCN have been conducted on advanced tumors (17, 18). API-2/TCN exhibited some side effects, which include hepatotoxicity, hypertriglyceridemia, thrombocytopenia, and hyperglycemia (17, 18). It is not clear whether the hyperglycemic effect of API-2/TCN relates to the inhibition of Akt activation. Recent knock-out mouse studies have shown that the mice deficient in *Akt2* are impaired in the ability of insulin to lower blood glucose because of defects in the action of insulin on skeletal muscle and liver. *Akt2*^{-/-} mice are born without apparent defects but develop peripheral insulin resistance and nonsuppressible hepatic glucose production, resulting in hyperglycemia accompanied by inadequate compensatory hyperinsulinemia (19). In contrast, *Akt1*-deficient mice did not display a diabetic phenotype (20). The mice are viable but display impairment in organismal growth. Such relatively subtle phenotypic change in *Akt1*^{-/-} mice suggests that *Akt2* and *Akt3* may substitute to some extent for *Akt1* (20). Although a high dose of API-2/TCN-induced hyperglycemia may be due to inhibition of AKT2 activation in human, the compound-exhibited potent stimulation of apoptosis and inhibition of tumor cell growth must result from inhibition of all three isoforms of Akt. The side effects of API-2/TCN have been shown to closely relate to the dose in the clinic trails (17, 18). Due to its severe side effects at high doses, API-2/TCN has been limited in the clinic. In this study, we demonstrated that low dose of API-2 effectively and selectively induces apoptosis and inhibits growth in tumor cells with elevated levels of Akt. In xenograft experiments, no visible side effects were observed in 50 mice treated with API-2 at concentration of 1 mg/kg/day, which significantly inhibited tumor growth in Akt-overexpressing cancer cells. These data indicate that API-2 at low doses could achieve antitumor growth without significant side effect in tumors with elevated Akt. Therefore, protocols for additional assessment of API-2/TCN in the clinic must incorporate careful patient selection based on Akt status in the tumor.

In summary, we have demonstrated that API-2/TCN is a potent and selective inhibitor of Akt signaling pathway in tumor cells. API-2 blocks Akt pathway, leading to the induction of apoptosis and cell growth arrest. Additional studies are required to elucidate the mech-

anism by which API-2/TCN blocks Akt activation. The ability of API-2 to inhibit growth of human tumor xenografts in nude mice provides validation for the development of drugs targeting Akt to treat cancers displaying elevated levels of Akt. Additional investigation is required to evaluate whether API-2/TCN is clinically useful in this setting.

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