Inhibition of Akt kinase activity by a peptide spanning the βA strand of the protooncogene TCL1

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Running Title: The “Akt-in” peptide inhibits Akt kinase activity
**Summary**

Akt plays a central role in the regulation of cellular anti-apoptosis underlying various human neoplastic diseases. We have previously demonstrated that TCL1 (a protooncogene underlying human T cell prolymphocytic leukemia) interacts with Akt and functions as an Akt kinase co-activator. With the aim to develop an Akt kinase inhibitor, we hypothesized that a peptide, which spans the Akt binding site, binds to Akt and modulates Akt kinase activity and its downstream biological responses. Indeed, we demonstrated that a peptide, named “Akt-in” (Akt inhibitor, NH$_2$-AVTDHPDRLWAWEKF-COOH, encompassing the βA strand of human TCL1) interacted with Akt and specifically inhibited its kinase activity. Nuclear magnetic studies suggested that interaction of Akt-in with the pleckstrin homology domain (PH) of Akt caused conformational changes on the variable loop 1 of Akt, the locus mediating phosphoinositide binding. Consistently, interaction of Akt-in with the Akt PH domain prevented phosphoinositide binding, hence, inhibited membrane translocation and activation of Akt. Moreover, Akt-in inhibited not only cellular proliferation and anti-apoptosis *in vitro*, but also *in vivo* tumor growth without any adverse effect.

The roles of Akt, which possesses a PH domain, in intracellular signaling were well-established. Hence, Akt inhibitors create an attractive target for anticancer therapy. However, no effective inhibitors specific for Akt have been developed. Akt-in, which inhibits association of PtdIns with Akt, is the first molecule to demonstrate specific Akt kinase inhibition potency. The observation will facilitate the design of specific
inhibitors for Akt, a core intra-cellular survival factor underlying various human neoplastic diseases.
Introduction

Akt (also known as protein kinase B, PKB), a central component of the PI3K signaling pathways, has emerged as a pivotal regulator of many cellular processes (1-4). Three highly homologous Akt isoforms (Akt1, Akt2 and Akt3) exist in mammals. Akt is composed of three functionally distinct regions: an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal hydrophobic region. The PH domain is a small 100-120-residue module found in many proteins involved in cell signaling or cytoskeletal rearrangement. The PH domain of Akt is similar to other proteins, and it consists of seven β strands forming two orthogonal antiparallel β sheets that are closed with the C-terminal α-helix (5-9).

In response to growth factors and other extra-cellular stimuli, Akt is activated by the lipid products [PtdIns (3,4,5)P$_3$ and its immediate breakdown product PtdIns (3,4)P$_2$] of phosphoinositide 3'-kinase (PI3K), which phosphorylates the 3-OH position of the inositol core of inositol phospholipids (PtdIns) (10-12). Recent structural studies have located the binding pocket of PtdIns (1,3,4,5)P$_4$[the polar head group of PtdIns (3,4,5)P$_3$] between variable loops 1 (VL1, the loop between the β1 and β2 strands) of the Akt PH domain (5,7,8,13). An association with PtdIns (3,4,5)P$_3$ induces a conformational change of Akt, which allows phosphoinositide-dependent protein kinase 1 (PDK1) to access and phosphorylate threonine 308 (Thr308) (14,15) in the so-called activation loop. It is also regulated by phosphorylation events within the conserved C-terminal hydrophobic motif (10,15). Both serine 473 (Ser473) phosphorylation and membrane anchoring are required for Thr 308 phosphorylation (16) and complete activation of Akt (10,15). Over 20 molecules have been identified as potential physiological substrates of Akt,
including GSK3α (Glycogen Synthesis Kinase3α), GSK3β, FKHR (Fork Head Transcription Factor), BAD, and eNOS (endothelial nitric oxide synthase) (1,17).

Activation of Akt promotes cell survival (18); thus it could be the underlying mechanism for numerous human neoplastic diseases including lung, ovarian, and prostate cancers (1,19). Activation of Akt is also induced in the mutation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene. PTEN antagonizes PI3Kinase function by the reduction in the levels of both PtdIns (3,4,5)P$_3$ and PtdIns (3,4)P$_2$. Mutation of PTEN are implicated in several tumor types including glioblastoma, endometrial tumors, and Cowden’s syndrome(20,21).

We have demonstrated that the protooncogene TCL1 is an Akt kinase co-activator (22-25). TCL1 contains two distinct functional motifs responsible for Akt association and homodimerization. Both Akt association and homodimerization of TCL1 are required for the complete function of TCL1 to enhance Akt kinase activity. TCL1 binds to Akt and activates Akt via a transphosphorylation reaction (26,27). TCL1 oncogene was first implicated in human T-cell prolymphocytic leukemia (T-PLL), a chronic adulthood leukemia (28). Under physiological conditions, TCL1 expression is limited to early developmental stages such as the immune system (24,28,29).

Since the PI3 kinase-Akt pathway is involved in various human neoplastic diseases, Akt represents an attractive target for drug development (19,30). A small peptide was proven to effectively modulate activity of kinases effectively (31-33). One class of Akt inhibitors under development is based on the cross reactivity between known kinase inhibitors (e.g. PKA or
PI3 kinase) (34-36); however, these drugs are not specific for Akt. With the aim to develop a putative Akt kinase inhibitor, we hypothesized that a peptide, which is spanning the Akt binding site, binds to Akt and modulates Akt kinase activity along with its downstream biological responses.

Based on the binding domain of TCL1 with Akt, we identified characterized a peptide, encompassing the βA strand of TCL1, interacted with Akt, inhibits Akt kinase activity. Akt-in prevented PtdIns binding to Akt, and consequently it inhibited membrane translocation of Akt and its downstream biological responses. Given the pivotal role of Akt kinase as a core intracellular survival factor implicated in the molecular mechanisms of human neoplastic diseases, the results could help to design Akt kinase specific inhibitors for therapeutic approaches.
**Experimental Procedures**

**Peptide Design**

**Akt-in peptides**

Akt-in: NH$_2$-AVTDHPDRLWAEKF–COOH (amino acid positions 10-24 of human TCL1)

TAT-Flag Akt-in: NH$_2$-YGRKKRRQRRR-DYKDDDDK–AVTDHPDRLWAEKF–COOH

**Control peptides**

βC: NH$_2$-EKQHAWLPLTIE-COOH (amino acid positions 29-40 of human TCL1)

TAT-βC: NH$_2$-YGRKKRRQRRR-EKQHAWLPLTIE-COOH

TAT-Flag: NH$_2$-YGRKKRRQRRR-DYKDDDDK-COOH

For functional assays, the Akt-in peptide was fused with TAT (YGRKKRRQRRR) (37). The peptides were either purchased from Sigma Genosys Japan and ABI SynthAssist (USA) or synthesized using N-alpha-FMOC-protected amino acids and standard 1-benzotriazolylxy-trisdemethylaminophosphoniumhexafluorophosphate (BOP/HOBt) N-hydroxybenzotriazole-coupling methods as reported previously (38).

**Co-immunoprecipitation assay**

Co-immunoprecipitation assays were performed as described previously (22). Briefly, Akt1, Akt2, or Akt3 in pCMV6 were transfected into 293 cells (ATCC). Then the cells were harvested, lysed, and pre-cleaned with Protein G/A agarose mixture (50% v/v, ProG/A, Pharmacia). Flag-Akt-in or control peptides (βC) at 400 µM were added to the cell lysates, incubated at 4 ºC for 3 hours, incubated with ProG/A
preconjugated with anti Flag M2 antibody (Sigma). The resultant immune precipitants were washed and run on SDS PAGE and immunoblotted with anti-HA antibody (3F10, Boehringer Mannheim).

**GST-pull down assay**

293T cells (ATCC) were transfected with 10 µg of Flag-tagged wild Type Akt3, PH domain, or C-terminal Akt3 (27). The cell lysates were immunoprecipitated with anti-Flag antibody (Flag M2, Sigma) bound to ProG/A (27). Fifty ng of GST fusion proteins were incubated with 20µl of immobilized Akt3, PH domain, or C-terminal Akt. The samples were run on SDS-gel, and immunoblotted with anti-GST antibody (Pharmacia). The results were consistent in at least three independent experiments. GST-fusion Akt-in was generated by subcloning with the corresponding nucleotide into pGEX4T-2 vectors (Pharmacia). All nucleotide sequences were verified before the experiments.

**GST-competition assay**

Recombinant GST-Akt-in fusion protein was generated by pGEX 4T-2 Vector (Pharmacia) using oligonucleotide pairs (5’- aattgcagtcaccgaccgccgacgcctggtggcctgggagaagtctagg-3’). 0.1µg of Akt (activated, Upstate Biotechnology) were incubated with TAT-Flag, TAT-Akt-in, or TAT-βC at the concentration of 0, 50, 100, or 250 µM in Hepes Binding Buffer (20mM Hepes pH 7.0, 150mM NaCl, 0.5µg/µlBSA). Then, 0.1 µg of GST-Akt-in was added and incubated for additional 20 min at 4°C. Twenty µl of immobilized Akt beads (Cell Signaling) were added to the sample, and washed five times with Hepes Binding Buffer in the presence of 0.1% NP40 and resolved onto SDS gel, and immunoblotted by anti-Akt.
(Cell Signaling) or anti-GST (Pharmacia Amersham Biosystem Inc.) antibodies using ECL (Amersham).

**Kinetics of Akt-in**

The kinetics of Akt-in with the human Akt2-PH domain (amino acid 1-125 of human Akt2) was performed using the Applied Biosystems 8500 Affinity Chip Analyzer. Briefly, His-fusion protein of human Akt2-PH domain was generated using pQE30 (Qiagen) by PCR. 1.25 pg of GST fusion proteins (Akt-in or wild type TCL1) were spotted onto the Protein A/G Affinity Chips preconjugated with anti-GST antibody (Sigma). Fifty µM of His-Akt2-PH domain was applied and the dissociation constant was calculated using data analysis software (Applied Biosystems). The values (mean±SD) were calculated from the 80 measurements.

**In vitro Akt kinase assay**

**In vitro** Akt kinase assays were performed using the Akt kinase assay kit (Cell Signaling) (22). Briefly, the immobilized Akt was incubated with 0, 200, or 400 µM of indicated peptides for 2 hours, then IVK reaction was performed for 4 min. at 30 °C. The samples were heat denatured and separated on SDS-PAGE, and immunoblotted with anti-phosphoGSK or anti-Akt (Cell Signaling) using ECL (Amersham).

**PKA kinase assay**

**In vitro** PKA kinase assays were performed using Peptag (Promega) as described previously (22). Indicated concentrations of peptides (Akt-in or TAT-Flag control peptide) were incubated with 25 ng PKA with 100 ng BSA for 1 h., in the presence or absence of 2 µM PKA inhibitor.
(Calbiochem #116805), followed by the kinase reaction for 20 min. at 26 °C and separated on 0.8% TBE agarose gel.

**In vitro PDK1 kinase assay**

Purified baculovirus-derived recombinant His-PDK-1-WT (1.0 µg/reaction, (22)) was incubated with or without indicated concentration of peptides (Akt-in, or TAT-Flag) in 20 µl of reaction mixture containing 20 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 10mM MgCl₂, 0.5mM EGTA, 1mM DTT, 100µM cold ATP, and 3µ Ci of [γ-³²P] ATP for 10min at 30 °C. The reactions were analyzed by autoradiography after Coomassie staining (shown in the bottom panel).

**Phosphorylation of Akt, BAD, FKHR, or p44/42 MAP kinase in 293 cells**

293 cells (ATCC) were cultured in 60 mm dish, and transfected (or non transfected in Fig. 3B) with 5 µg of m-BAD (Fig. 3C, pEBG-mBad, Cell Signaling) using calcium phosphate transfection as previously described (22). 24 hours after transfection, the cells were serum starved (0.2% FBS) and treated with either control (TAT-Flag) or Akt-in (TAT-Akt-in) at 50 µM for additional 12 h. The cells were stimulated with or without 20 ng/ml of PDGF for 8 min. (in Fig. 3C) or 5 min. (in Fig. 3B), lysed with Brij lysis buffer in the presence of phosphatase inhibitors (22), and the resultant samples were resolved on 4-20% SDS gel (Daiich kagaku). Then they were immunoblotted with indicated antibodies purchased from Cell Signaling (Akt, #9272, phospho-Ser473 Akt #9271, phospho-Ser308 Akt #9275, Ser-256 FKHR #9461, FKHR #9462, P-Ser-136 BAD, Cell Signaling #9295, BAD #9292, anti-p44/42 MAP kinase #9102, anti-phospho-p44/42 MAP kinase #9106, anti-38 MAP kinase #9212, phospho-p38 MAP kinase antibody # 9216), and detected by ECL (Amersham).
NMR (Nuclear Magnetic Resonance) experiment

NMR spectra were recorded on 0.25 - 0.3 ml [Shigemi tubes pre-coated with a silicon solution (Sigma)] samples of 0.05 mM $^{15}$N-labeled Akt2-PH dissolved in the conditioning buffer [10 mM Tris/H$_2$O (pH 7.4), 300 mM NaCl, 0.1 mM benzamidine, 0.1 mM EDTA, with 5-10% $^2$H$_2$O for the lock], in the presence or absence of 20 mM Akt-in. NMR experiments were carried out at 10°C on a Bruker AVANCE 600 spectrometer equipped with 5 mm z-shielded gradient $^1$H-$^13$C-$^{15}$N triple resonance cryogenic probe. $^1$H chemical shifts were directly referenced to the resonance of DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt), while $^{15}$N chemical shifts were indirectly referenced with the absolute frequency ratios ($^{15}$N/$^1$H) = 0.101329118. In all experiments, the $^1$H carrier was centered on the water resonance and a WATERGATE sequence was incorporated to suppress the solvent resonance. All NMR spectra were acquired in the phase-sensitive mode with Digital Quadrature Detection in the F2 dimension and hypercomplex States-TPPI method in F1 dimension, and processed using Gifa (version 4.22) software. [$^1$H,$^{15}$N]-HSQC spectra were recorded using a time domain data size of 64 $t_1$ * 1 K $t_2$ complex points and 32 transients per complex $t_1$ increment.

PtdIns (3, 4, 5) P$_3$ lipid-protein pull-down assay

A lipid-protein pull-down assay was performed using PIP Beads [PtdIns (3,4,5) P$_3$, Echelon Bioscience Incorporated]. Indicated peptides (Akt-in or βC control) were incubated with 50ng of Akt kinase (unactivated, Upstate Biotechnology, #14-279) with 400ng/ml BSA for 2 hours with gentle agitation at 4°C. TAT-Flag control was added to adjust
the final peptide concentration to be equal throughout the samples. Then, 25μl PIP Beads were added to each sample and incubated for an additional 16 h. The reactions were then washed four times with washing buffer (10mM Hepes, pH 7.4, 0.25% NP-40, 140 mM NaCl), resolved on SDS gel, and immunoblotted by ECL (Pharmacia).

**Membrane translocation experiment**

293 cells (ATCC) were grown on a poly-L-lysine coated cover glass and were transfected with 1 μg HA-Akt1 or Akt-PH-GFP or Btk (Bruton tyrosine kinase)-PH-GFP in a mammalian expression vector (39) using FuGENE6 (Roche Diagnostics). Six hours after the transfection, 50 μM Akt-*in* or TAT-Flag control was added and incubated for 16 hours. The cells were then serum starved (0.5%), and then, incubated for an additional 24 h. The cells were treated with or without 50 nM wortmannin for 20 min., stimulated with 50ng/ml PDGF-AB (Sigma, 3226) for 10 min., fixed with 4% paraformaldehyde, stained with 10ng/ml FITC-conjugated anti-HA antibody (12CA5, MBL) or phospho-Ser 473 antibody (587-F11, Cell Signaling), and examined using a confocal microscope (Nikon).

**Proliferation assay**

Cell growth was assessed by a colorimetric method using WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (347-07621, Dojin) according to the manufacturer’s instructions. Briefly, 2000 T4 cells were seeded into each well of 96-well plate. The peptides (TAT-βC or TAT-Akt-*in*) were added at indicated concentrations. Three days later, WST-8
regent was added, incubated for 4 h. more at 37°C, and the absorbance was determined using a microplate reader (BioRad).

Cell Death Assay and Mitochondrial Permeability Transition Assay

T4 cells (human T cell leukemia cells) were treated with indicated concentration of peptide (TAT-Flag control or TAT-Akt-in) for 24 h. The cells were transfected with either myr-Akt (Upstate Biotechnology #17-253) or a control. Cell death was assessed by staining with 2µg/ml of Propidium Iodide (PI). Mitochondrial Permeability Transition was verified by staining with Rhodamine 123 (Molecular Probe) at 5µM for 15 min. at 37°C (26) and analyzed using FACS (Cell Quest).

In vivo Tumor Growth

Fibrosarcoma cells (QRsP-11 cells, 2 X 10⁵ cells per mouse) were subcutaneously transplanted into syngeneic C57BL/6 mice (eight mice in each group) (40). Two micromoles indicated peptides (TAT-Akt-in, TAT-Flag, or PBS) per mouse were injected directly into the tumor on days 5, 7, 10, 12, 14, 17, and 19. In vivo cell growth was calculated based on the tumor diameter. On day 9 after transplantation, the tumors were resected, fixed in formalin, embedded in paraffin, staining with Hematoxylin-Eosin (H&E), TUNEL (Tdt-mediated dUTP nick end labeling, #MK500, Takara), or phospho Akt (Ser473) monoclonal antibody (587F11, Cell Signaling).
Results

Peptide Design of Akt-in and the Structure of TCL1

TCL1 forms a closed symmetrical β-barrel structure, consisting of eight antiparallel β-strands (41) (**Fig. 1A**). In our previous studies, we showed that the surface composed of βA and βE strands of TCL1 mediated the interaction with Akt (22,26) (42) (**Fig. 1A**, top surface). Both dimerization and Akt interaction are essential for the full function of TCL1 to activate Akt (26). We hypothesized that a peptide, which spans the Akt binding sequences, can modulate Akt kinase activity and its downstream signals. We designed a peptide (named Akt-in, Akt inhibitor, position 10-24 of human TCL1, NH$_2$-AVTDHPDRLWAWEKF-COOH), which encompasses the βA strand of TCL1 for further study (**Fig. 1B**). For functional assays, the Akt-in peptide (amino acid positions 10-24 of TCL1, **Fig. 1B**) was fused with TAT (YGRKKRRQRRR) and/or Flag epitope (DYKDDDDK). The TAT fusion protein which contains an NH$_2$-terminal 11-amino acid protein transduction domain (PTD) from the human immunodeficiency virus, can be efficiently imported to the cytosol (37,43). By immunohistochemical staining, either TAT-controls or TAT-Akt-in peptides were efficiently imported to cytosol (data not shown).

**Akt-in specifically interacts with three Akt isoforms through Pleckstrin Homology Domain.**

Since TCL1 interacts with all three Akt isoforms (22,27), we first investigated whether Akt-in can interact with all three isoforms of Akt. In pull-down assays, Akt-in did interact with them (**Fig. 2**, panels A-C, Akt1, Akt2, and Akt3, respectively).

Wild type TCL1 interacts with Akt through an N-terminal pleckstrin homology (PH) domain (22,23). **Akt-in** encompasses the βA strand of TCL1,
the locus mediating TCL1-Akt interaction. Therefore, it is predicted that Akt-in interacts with Akt via a PH domain.

In pull-down experiments, Akt-in bound the full-length wild type Akt (Fig. 2D, lane 2) and the PH domain of Akt (Fig. 2E, lane 2), but not the C-terminal Akt (Fig. 2F, lane 2). Furthermore, control peptides (GST protein, or βC peptide) did not interact with either the full-length Akt or the PH domain confirming the specificity of Akt-in (Fig. 2D-F, lanes 1 and 4, βC peptide and GST protein, respectively). In GST-competition assays, specific binding of Akt-in with Akt was further demonstrated by the dose-dependent inhibition by Akt-in (Fig. 2G, lanes 5-8), but not by control peptides (Fig. 2G, lanes 1-4 or lanes 9-12, TAT-Flag or TAT-βC, respectively).

The kinetics study of Akt-in interaction with the Akt-PH domain was performed by Spot Matrix SPR technology. The dissociation constant (Kd) of Akt-in with the Akt-PH domain was 18 ± 4.8 μM (Akt-in, mean ± SD) and the Kd of wild type TCL1 with the Akt-PH domain was 5.4 ± 1.4 μM.

**Akt-in specifically inhibits Akt kinase activity.**

Akt is regulated by multiple site phosphorylation. Akt kinase assays performed in vitro showed that the addition of Akt-in compromised Akt kinase activity as assessed by the phosphorylation levels of GSK3α in a dose-dependent manner (Fig. 3A, top panels).

To determine the specific inhibition of Akt-in on Akt activation in intact cells, 293 cells were treated with TAT-Akt-in and stimulated with PDGF. Akt-in treatment inhibited phosphorylation of PDGF-induced Akt activation at Thr 308 (top row, lanes 2-5) or Ser 473 (second row panel,
lanes 2-5). However, *Akt-in* did not inhibit the levels of phosphorylation of P38 MAP kinase (fourth row, lanes 2-5). Control peptide (50 μM TAT-Flag) showed no inhibition in this experiment (lane 6 of each panel).

TAT-*Akt-in* treatment of the 293 cells inhibited the phosphorylation of Akt at Ser 473 (**Fig. 3C**, compare the top row lanes 6 with lane 4, TAT-Flag control peptide vs. TAT-*Akt-in* after PDGF stimulation). In the same experiment, TAT-*Akt-in* treatment inhibited the phosphorylation levels at serine 136 of BAD (the fifth row) or serine at 256 of FKHR (the seventh row) after PDGF stimulation. To determine the specificity of *Akt-in* on Akt phosphorylation, we have examined the phosphorylation levels of p44/42 MAP kinase (the third row). *Akt-in* treatment did not inhibit p44/42 MAP kinase suggesting the specificity of *Akt-in* on Akt phosphorylation. Consistently, TAT-*Akt-in* treatment of QRsP-11 fibrosarcoma cells (40) inhibited PDGF-induced Akt phosphorylation on both Ser473 and Thr308 detected by immunoblotting (data not shown).

Further demonstrating the specificity of *Akt-in*, phosphorylation of PDK1 was examined. PDK1 phosphorylates Thr308 of Akt, which is a crucial step for activating Akt (14,15,44). Using recombinant PDK1, *Akt-in* did not inhibit PDK1 kinase activity as measured by the phosphorylation of PDK1 in a dose-dependent manner (**Fig. 3D**). *Akt-in* did not inhibit phosphorylation of PDK1 substrates (data not shown), consistent with the previous studies that demonstrated wild type TCL1 does not interact with PDK1 (22).

The catalytic domain of Akt is structurally similar to PKA (cyclic AMP dependent kinase) (7). To further determine the specificity of *Akt-in*, an *in vitro* PKA kinase assay was carried out. The inhibitory effect of *Akt-in*
was specific to Akt, since Akt-in did not inhibit PKA activity (Fig. 3E). In order to further support that Akt-in specifically inhibited Akt kinase activity, we examined the effect of Akt-in on PKC kinase activity using an in vitro kinase assay. Akt-in did not inhibit phosphorylation of PKC (Protein Kinase C) kinase activity in PKC kinase assay in vitro (data not shown).

**Akt-in interacts with the Akt PH domain, and therefore, induces conformational changes on the locus for the phosphoinositide binding.**

In order to further determine the precise binding site of Akt-in with the PH domain of Akt, a nuclear magnetic resonance (NMR) mapping study was performed. In the presence of Akt-in, [β5 (L62, M63, K64, T65, and 66E), β6 (V74 and I75) strands, and α-helix (E98, M100, R101, I103, and M105)] showed significant resonance shift, suggesting Akt-in interacts with these regions. In addition, amino acid residues R15, G16, Y18, and I19 [located on the variable loop 1 (VL1, the loop formed between the β1 and β2 strand)], [T21 and R23] (β2 strand), L52 (β4), and [S56 and A58] (β5 strand) showed above the threshold level of resonance shift only in the presence of Akt-in, but not wild type TCL1 (Fig. 4A-D, and data not shown). The amino acid residues (K14, E17, T21, R23, R25) on (and around) the VL1 (variable loop 1, located between β1 and β2) of PH domain were responsible for PtdIns binding (5,8,9)(Fig. 4E). The results together suggested that Akt-in interacted with PH domain through the β5, β6 strands, and caused deviations on the amino acid residues on VL1, the locus involved in the PtdIns binding.
Akt-in inhibits phosphoinositide (3,4,5) P₃ binding to Akt, and consequently, it inhibits membrane translocation and activation of Akt.

The product of PI3kinase activation, PtdIns (3,4,5) P₃ or PtdIns(3,4)P₂, triggers the activation by interacting with the Akt PH domain (1,8). PtdIns is abundant in the plasma membrane, and translocalization of Akt to the plasma membrane is one of the critical steps for the initiation of Akt activation (45,46). NMR mapping study suggested that Akt-in could induce the conformational change at the locus responsible for PtdIns binding. The results prompted us to investigate whether Akt-in can affect the interaction of PtdIns (3, 4, 5) P₃ with Akt.

In lipid-protein pull-down assays using PtdIns beads [PI (3, 4, 5)P₃], the presence of Akt-in prevented the association of PtdIns (3, 4, 5)P₃ with Akt in a dose-dependent manner (Fig. 5A, compare lanes 1-3 with 4-6, Akt-in vs. TAT-Flag control, respectively).

Membrane translocation of Akt is mediated through the interaction of PtdIns (3,4, 5) P3 with Akt, which triggers the activation of Akt (45,46). Therefore, we next examined whether TAT-Akt-in could inhibit the membrane translocation of Akt in the intact cells (Fig. 5B). As predicted by the result of lipid-protein pull-down assays, TAT-Akt-in treatment of the 293 cells (g-i) prevented PDGF-induced membrane translocation of Akt from the cytosol (g) and inhibited phosphorylation of Akt (Ser473) (h and i, P-Ser473 Akt and the overlay view of Akt (HA) plus P-Ser473 Akt, respectively]. In contrast, control peptide (TAT-Flag) treatment (m-r) did not inhibit PDGF-induced membranous translocation of Akt (m, green), phosphorylation of Akt at Ser-473 (n, red), and the overlay view of Akt (HA) plus P-Ser473 Akt (o, yellow). Wortmannin treatment of the cells completely inhibited the Akt activation and membrane translocation in this
In order to examine the specificity of the inhibitory effect on membrane translocation by Akt-in, we compared the PDGF induced membrane translocation of Akt (Akt-PH-GFP) or Btk (Bruton tyrosine kinase, Btk-PH-GFP,(39)), both of which contains a PH domain and are also known to be translocalized upon PDGF stimulation.

Akt-in treatment of the cells potently inhibited translocation of the GFP-fused Akt (Akt-PH-GFP) from the cytosol to plasma membrane after PDGF stimulation (Fig. 5C, compare b and d, control peptide or Akt-in treated cells after the PDGF stimulation, respectively). However, Akt-in treatment of the cells did not inhibit translocation of Btk-PH-GFP from the cytosol to plasma membrane after PDGF stimulation. The results indicated Akt-in specifically inhibited the membrane translocation of Akt from the cytosol upon PDGF stimulation.

**Akt-in prevents the biological consequences of Akt activation.**

We next examined whether Akt-in could inhibit the biological effects of Akt activation (17,47,48). Treatment of T4 cells by TAT-Akt-in not only prevented cellular proliferation (Fig. 6A), but also inhibited cell survival in a dose-dependent manner (Fig. 6B). The inhibitory effect was Akt dependent, since introduction of myr-Akt (a constitutive active form of Akt) reversed inhibition of the proliferation assay as well as the cell death assay (Fig. 6B and data not shown). Similar enhancement of apoptosis was observed with DNA content analysis and annexin V staining (data not shown). Moreover, TAT-Akt-in could inhibit stabilization of mitochondrial outer membrane depolarization using Rhodamine 123 staining (Fig. 6C).
**Akt-in inhibits tumor growth in vivo.**  

Finally, we investigated whether Akt-in inhibits tumor growth in vivo. QRsP-11 fibrosarcoma cells (40) (49) were transplanted subcutaneously into syngeneic C57BL/6 mice and TAT-Akt-in (or TAT-Flag) was directly injected into the tumor (2 micromole per mouse, three times per week starting from day 5). TAT-Akt-in treatment suppressed tumor growth when compared to the control groups (Fig. 7A). Importantly, no obvious side effects were observed by the weight gain (Fig. 7A, inlet). On day 19, the mean tumor volume from the TAT-Akt-in treated mice was significantly smaller than the tumor from the control peptide treated mice (240±223 mm$^3$ vs. 1428±319 mm$^3$, p <0.01, TAT-Akt-in treated vs. control peptide treated mice, respectively). Microscopically, the TAT-Akt-in treated tumors resulted in an increased number of degenerated cells with apoptotic cells, which correlated with lower phosphorylation of P473 staining when compared to the control peptide treated tumor [Fig. 7B, TAT-Akt-in or TAT-Flag control peptide treated tumors, the second row (H&E), the third row (TUNEL), and the fourth row (Phospho Ser 473 staining)]. Moreover, TAT-Akt-in treated mice significantly prolonged their mean survival time (48.3±9.4 days vs. 37.3±5.9 days, p<0.05, Akt-in vs. control peptide treated mice, respectively).


Discussion

We have previously demonstrated that the protooncogene TCL1 is an Akt kinase coactivator (22) (23). In yeast two hybrid assays and co-immunoprecipitation assays, we showed that TCL1 interacted with Akt through the PH domain, and activated Akt (22,26-28). TCL1 forms a closed symmetrical β-barrel structure, consisting of eight antiparallel β-strands (41)(Fig. 1A). By mutational studies, we demonstrated that both Akt interaction and dimerization of TCL1 are required for complete function of TCL1 to enhance Akt kinase activity (26,42). We hypothesized that a peptide spanning the Akt binding sequences of TCL1, which lacks the ability to form an oligomeric complex of Akt/TCL1 complex (22), but retains the ability for interaction with Akt, can down-modulate the Akt activation. We demonstrated that a peptide (named Akt-in, Akt inhibitor, encompassing the βA strand of TCL1) specifically interacted with the Akt-PH domain and inhibited Akt kinase activity.

As demonstrated by GST pull-down experiments and NMR studies, Akt-in interacted with Akt-PH domain. NMR mapping techniques further defined the molecular interaction of Akt-in with the Akt-PH domain at the amino acid level. The results indicated that similar to wild type TCL1, Akt-in, encompassing βA strand of wild type TCL1, interacted with Akt-PH domain via the β5, β6 strands, and the α-helix of Akt-PH domain(25). Moreover, interaction of Akt-in with Akt-PH domain caused above the threshold levels of the resonance shifts of the amino acid residues on VL1, the β5 strand, β6 strand, and α-helix of Akt-PH domain. The significant resonance shifts were detected only in the presence of Akt-in, suggesting the specific interaction between Akt-in and Akt-PH domain, consistent with the observation that Akt-in specifically interacted with Akt-PH domain in co-immunoprecipitation
This conformational change is not induced by wild type TCL1, since wild type TCL1 interacted with the Akt-PH domain via both \(\beta A\) and \(\beta E\) strands of TCL1, which can help to stabilize the interaction. The deviations on the VL1 by Akt-\(in\) could also be induced by the indirect conformational change due to the very hydrophobic C-terminal end of Akt-\(in\), which is protected from water by the \(\beta\)-barrel structure in wild type (native form) TCL1; hence it remains unexposed to the solvent. In lipid protein pull-down experiment using PtdIns beads, the interaction of Akt-\(in\) with the Akt PH domain, dramatically decreased the affinity of Akt for PtdIns. Since binding of phosphatidylinositol 3,4,5-triphosphate to the PH domain of Akt induces a conformational change, a prerequisite for triggering the Akt activation (8), dissociation of PtdIns with the Akt PH domain by Akt-\(in\) could explain the inhibitory effects on Akt activation observed in vitro. In intact cells, disruption of PtdIns with the Akt PH domain resulted in the failure of translocalization of Akt to the plasma membrane and activation of Akt. The Akt kinase properties depend on its ability to bind PtdIns (45,46,50). Ser473 phosphorylation as well as membrane anchoring are required to achieve full Thr 308 phosphorylation (10,15). Thus, Akt-\(in\) inhibited PtdIns binding to the Akt PH domain, and consequently it failed to activate Akt.

The N-terminal PH domain of Akt, which is common to over 100 signaling molecules, provides a lipid binding module to direct Akt to PI3K-generated phosphoinositide PtdIns (3, 4, 5)\(P_3\) or PtdIns (3,4) \(P_2\) (1,2,51). PH domains are located in the N-terminal portion of Akt, and among the three Akt isoforms, over 90% are conserved at the amino acid level. Recently, the molecular interaction of PtdIns with the Akt PH domain has been clarified. Milburn et al. reported by crystallography that interaction of Akt with PtdIns resulted in a conformational change of VL1 (variable loop 1 between the \(\beta1\)
and the β2 strand of PH domain), which is involved in the PtdIns binding (8).

By structural and functional studies, Thomas et al. described that D4 phosphate interacted with K14, D3 phosphate with K14, R23, and R25, and D1 phosphate with Y18, I19, and R23. It is noteworthy that the main interactions were mediated through binding to the D3-and D4-phosphates, whereas the D5-phosphate had no significant interactions (9). In conjunction with our findings demonstrated that PtdIns (1,3,4,5)P$_4$ [the polar head group of PtdIns (3,4, 5)P$_3$] interacted with PH domain of Akt through the variable loops VL1 (5,8,9,13,25) (Fig. 4E).

Akt is one of the effector kinases that is activated by the product of PI3kinase activation, PtdIns (3,4,5)P$_3$ and its immediate breakdown product PtdIns (3,4)P$_2$ (2,10-12). Association of PtdIns with the Akt PH domain induces conformational change, which is a prerequisite to the phosphorylation of Thr 308 by PDK1 (10,52). PDK1 phosphorylates Akt at Thr 308, which is required to initiate Akt activation (44,53). The findings that Akt-in did not inhibit kinase activity of PDK1 suggested that Akt-in could directly inhibit Akt kinase activity. The observation is consistent with one that Akt-in could not bind to PDK1 in GST pull-down experiment (data not shown); therefore, in vitro PDK1 kinase assays did not demonstrate inhibition.

The full ranges of target molecules of Akt-in that harbor PH domain remain to be elucidated. In co-immunoprecipitation assays, wild type TCL1 interacted solely with Akt, but not with PDK1 or PKA(22). Consistently, Akt-in did not interact with PDK1 in GST pull-down experiments (data not shown). NMR mapping studies indicated that Akt-in interacted with Akt-PH domain via β4 and β5 strands. It is notable that the amino acid sequences of the PH domain at (and around) the β4 and β5 strands (the locus mediating
the Akt-in interaction) appear to be divergent among the PH domains (13,25,51). In addition, the study of the PH domain revealed that in contrast to the VL1 loop of the Akt-PH domain, which contains a short β1-2 typed loop, the VL1 of PDK1 has a long β1-2 typed loop (13). Although we could not completely exclude the possibility that Akt-in suppressed other kinases, Akt-in did not inhibit PKA, PKC, PDK1, p42/44 MAPK, or p38 MAP. These observations further suggested that Akt-in interacts solely with Akt, and specifically inhibits Akt kinase activity.

In vitro, Akt-in compromised Akt-dependent cellular proliferation, stabilization of mitochondrial permeability transition, and anti-apoptosis. In addition to T4 cells (human T cell leukemia cells), other cell lines such as P3HR-1 (EBV transformed human B cell line, HTB), 293T (human kidney CRL-11268), or WiDr cells (human colon adenocarcinoma cells, CCL-218) inhibited cellular proliferation following treatment with Akt-in. However, the inhibitory effect seemed stronger on TCL1 negative cell lines than on TCL1 positive cell lines since T4 cells, 293T, or WiDr cells are negative on TCL1b or MTCP1, other isoforms of TCL1 family proteins determined by Western blotting. Due to its considerably lower Kd (5.4 μM vs. 18.0 μM, wild type TCL1, vs. Akt-in, respectively), Akt-in may not efficiently remove or replace native TCL1 molecules from Akt, which explains the decrease in kinase inhibition potency in TCL1 positive cell lines. Moreover, it is possible that once wild type TCL1 binds and enhances Akt kinase activity, a limited window appears for exogenous Akt-in to silence or inactivate the elevated level of Akt kinase activity.

Three isoforms of the TCL1 protooncogene exist and they are 30% homologous at the amino acid level (28). Based on alignment of the peptide
sequences of Akt-in (10-24 of human TCL1), the sequences (P15-L18W19) appear to be conserved among the TCL1 family proteins. It is logical to speculate that the corresponding amino acid sequences in the other members of the TCL1 family [8-22 (GVPPGRLWIQRP) in TCL1B and 5-19 (GAPPDHLWVHQEG) in MTCP1] may also inhibit Akt kinase activity. The roles of phosphoinositide binding to the PH domain in intracellular signaling are well established (1,2,51,54). In this regard, mutations of PTEN (20), which activates Akt by accumulating PtdIns, can be a promising target for potential therapy with Akt-in. Given the roles of the PH domain in cellular signaling (51,55), further modification of the Akt-in design may help to develop specific inhibitors for the signaling molecules that harbor PH domain.

Akt activation promotes cell survival, which affects tumor progression and resistance to chemotherapy and radiation in cancers (2,56,57). Hence, Akt inhibitor is believed to be an attractive target for anticancer therapy (19,30). Several attempts have been undertaken to develop Akt inhibitors (34-36,58,59). Previous evidences suggested that Akt kinase activity could be down modulated by protein-protein interactions (Hsp90 or CTMP) (60-62). Moreover, a small peptide was proved to effectively modulated kinase activity of AGC family kinases (32,33). However, no effective inhibitors specific for Akt have been developed.

We showed that Akt-in did not inhibit kinase activities of p44/42 MAPK, PDK, PKA, P38 MAP kinase, or PKC. Moreover, Akt-in did inhibit the membrane translocation of Akt, but not Btk. Both contain a PH domain and are also known to be translocalized upon PDGF stimulation. We cannot completely exclude the possibility that Akt-in may inhibit other PH bearing kinases. However, observation together supported the notion that
Akt-in binds specifically with Akt, and therefore, inhibits Akt kinase activity.

In order to develop a more powerful form of inhibitor, we created a dimer of Akt-in (2XAkt-in, repetitive sequences of 10-23 of TCL1, NH₂-VTDHPDRLWAWEK-GGG-VTDHPDRLWAWEK –COOH). 2XAkt-in efficiently inhibited Akt kinase activity in a dose-dependent manner in Akt kinase assay performed in vitro. Dissociation constant of 2XAkt-in was almost two times higher than the Kd of monomeric Akt-in (18±4.8 vs. 10±2.7 µM, Akt-in vs. 2XAkt-in, mean±SD, respectively) using Applied Biosystems 8500 Affinity Chip Analyzer. The results may facilitate to develop more efficient Akt inhibitors for therapeutic approaches.

Akt-in, which inhibits association of PtdIns with Akt, is the first molecule to demonstrate specific Akt kinase inhibition potency. Akt-in inhibited anti-apoptosis and tumor cell growth in vivo without any toxic effects. Given the pivotal role of Akt kinase as a core intracellular survival factor in the molecular mechanisms of human neoplastic diseases, the results should facilitate the design Akt specific inhibitors for human cancer therapy.
Fig. 1. Structure-Based Alignment of TCL1 and Akt-in

A. Akt-in that encompasses the βA strand, which forms the interface for Akt interaction, is shown with TCL1. The structural study demonstrated that TCL1 forms a β-barrel structure consisting of eight anti-parallel β strands (41). Previously, we demonstrated that the surface, which consists of both βA and βE strands, formed an interface mediating the Akt interaction (26). We hypothesized that a peptide spanning the Akt binding site binds to Akt and modulates Akt kinase activity along with its downstream biological responses. In the current study, we generated a peptide, named “Akt-in” (Akt inhibitor, NH$_2$-AVTDHPDRLWAKEF-COOH, encompassing the βA strand of human TCL1) to test our hypothesis.

B. Amino acid sequence of Akt-in (Akt inhibitor) is shown with the alignment of the sequence of TCL1 (41). Akt-in consists of 15 amino acids (position 10-24 of TCL1, NH$_2$-AVTDHPDRLWAKEF-COOH, shaded gray), which spans the crucial binding site for Akt interaction [Aspartic Acid (D) 16, bold and underlined](22,26).
**Fig. 2.** *Akt-in* specifically interacts with the Akt Pleckstrin Homology Domain.

**A-C.** *Akt-in* interacted with all three Akt isoforms in co-immunoprecipitation experiments.

Since wild type TCL1 interacted with three isoforms of Akt, we examined whether *Akt-in* can interact with the three isoforms of Akt (Akt1, Akt2, or Akt3). Flag-*Akt-in* or control peptides (Flag-βC) were added to the cell lysates from the Akt transfected cells (Akt1, Akt2, or Akt3). The resultant samples were immunoprecipitated with anti Flag M2-agarose gel (A2220, Sigma), and immunoblotted by Akt antibody (Cell Signaling). *Akt-in*, but not control peptides (TAT-Flag), could interact with Akt1 ([A], lane 2), Akt2 ([B], lane 2), and Akt3 ([C], lane 3).

**D-F.** In pull down assays, *Akt-in* interacted with the Akt PH domain.

GST-*Akt-in* was incubated with either Flag-full-length Akt, Flag-PH domain of Akt, or Flag-C-terminal Akt, then immunoprecipitated with Flag M2-agarose gel, immunoblotted using anti-GST antibody. Analogous to full length TCL1 (lane 3 in panels **E** and **F**), *Akt-in*, bound the full-length Akt, the Akt PH domain, but not the Akt C-terminal (lane 2 in panels **E** and **F**). Control peptides (βC peptide or GST) showed no background (lanes 1 or 4 in each panel).

**G.** *Akt-in*, but not the control peptide (TAT-βC or TAT-Flag), specifically bound Akt.

To support that *Akt-in* specifically interacted with the Akt PH domain, GST-competition assays were performed. *Akt-in*, but not the control peptides (TAT-βC or TAT-Flag), specifically bound to Akt. The
addition of Akt-in (0, 50, 100, 250 µM, lanes 5-8, respectively), but not the control peptides (TAT-Flag, lanes 1-4) or (TAT-βC, lanes 9-12), inhibited the association of Akt with Akt-in in a dose-dependent manner. The results (A-G) were consistent in three independent experiments.
Fig. 3. *Akt-in* inhibits Akt kinase activity.

**A.** To examine whether the interaction of *Akt-in* with Akt modulate activation process of Akt, in vitro kinase assays were conducted. Akt kinase assays *in vitro* using recombinant Akt and *Akt-in* inhibited the phosphorylation of GSK3α (top panel 0, 100, 200, or 400 µM of peptide) in a dose dependent manner. Control peptide (TAT-Flag) showed no inhibition (lanes 4-6).

**B.** 293 cells were treated with TAT- *Akt-in* for 12 hours and stimulated with 20 ng/ml PDGF and phosphorylation of p38 MAP kinase was examined by immunoblotting (nonphosphorylated Akt, phospho-Ser473 Akt, phospho-Thr308 Akt, nonphosphorylated p38 MAP kinase, or phosphorylated p-38MAP kinase). *Akt-in* treatment inhibited phosphorylation of Akt at Thr 308 (top row, lanes 2-5) or Ser 473 (second row panel, lanes 2-5). However, *Akt-in* did not inhibit phosphorylation of P38 MAP kinase (fourth row, lanes 2-5). Control peptide (50 µM TAT-Flag) showed no inhibition in this experiment (lane 6 of each panel).

**C.** TAT-*Akt-in* treatment of 293 cells inhibited the phosphorylation of Ser 473 Akt (compare the top row lanes 6 with lane 4, TAT-Flag control peptide vs. TAT-*Akt-in* after PDGF stimulation), Ser 136 BAD (compare row 5 lanes 6 with lane 4, TAT-Flag control peptide vs. TAT-*Akt-in* after PDGF stimulation), or Ser 256 FKHR (compare row 7 lanes 6 with lane 4, control peptide vs. *Akt-in* after PDGF stimulation) after 10 minutes of PDGF stimulation. However, TAT-*Akt-in* did not inhibit phosphorylation of p44/42 MAPK indicating the specificity of *Akt-in* on Akt phosphorylation (compare row 3 lanes 6 with lane 4, TAT-Flag control peptide vs. TAT-*Akt-in* after PDGF stimulation). The amount of the non-phosphorylated
forms was verified to determine that an equal amount of proteins was loaded onto the gel {Akt (row 2), p44/42 MAPK (row 4), BAD (row 6), or FKHR (row 8)}.

**D.** PDK1 kinase assay *in vitro* was performed using Akt (unactivated form, upstate biotechnology) as a substrate (22). Increasing amounts of Akt-*in* did not inhibit PDK1 phosphorylation (lanes 2-4, vs. lanes 5-7, TAT-Flag control vs. Akt-*in*, respectively). Since PDK1 is an upstream kinase that activates Akt at Thr308. The results suggested that Akt-*in* directly inhibited Akt kinase activity, but not through the inhibition of PDK1 activation.

**E.** To further examine the specificity of Akt-*in* to Akt, PKA kinase assays were performed using Peptag (Promega). Akt-*in* did not inhibit PKA kinase activity in *in vitro* PKA kinase assays as measured by phosphorylation levels of Kemptide (lanes 3-6 vs. lanes 9-12, Akt-*in* vs. TAT-Flag control, respectively). PKA inhibitor (Calbiochem #116805) inhibited the kinase reaction (lanes 2 and 8). The results (A-E) were consistent in at least two independent experiments.
**Fig. 4.** Akt-in interacted with the Akt-PH domain, and consequently, induced conformational changes.

**A-D.** Interactions of Akt-in with the PH domain of human Akt2 (Akt2-PH) are shown. From top to bottom: different views (180° rotation around the vertical axis) of the NMR 3D structure of Akt2 are presented as ribbons (right panels) or Van der Waals surface diagrams (left panels). PtdIns (1,3,4,5)P$_4$ (shown in blue) as well as Akt-in (shown in green) are depicted as ball-and-stick representations. The position of PtdIns (1,3,4,5)P$_4$ was deduced from NMR (5) and X-ray (9) studies. The conformation of Akt-in is presented from the complex of Akt2-PH with wild type TCL1(25). Colored residues indicate the footprint in the presence of Akt-in as determined by NMR mapping: red residue represented significant deviation in the chemical shift of the amide group, while orange residues represented a modest shift in the study. Unaffected residues are shown in gray.

**E.** Sequence alignment of human Akt-PH domain of human and mouse Akt1, Akt2, and Akt3 are shown. The amino acid residues responsible for binding to the phosphates of PtdIns are shaded blue with arrows (5,8,9,13). The amino acids responsible for PtdIns bindings are located at (and around) VL1 (the variable loop between β1 and β2 strands of PH domain). The footprints of Akt-in that are deduced from the NMR mapping study are shown in red arrow with shades. The amino acid residues, which showed above the threshold levels of the resonance shift specific to Akt-in are indicated by green arrow with shades.
Fig. 5. Akt-in prevents association of PtdIns (3,4,5)P₃ with Akt, and inhibits membrane translocation and activation of Akt.

A. NMR mapping studies prompted us to investigate whether Akt-in can affect the interaction of PtdIns (3, 4, 5) P₃ with Akt. In lipid-protein pull down assays, increasing amounts of Akt-in (0, 200, or 400 µM) prevented association of Akt with PtdIns (3,4, 5) P₃ (lanes 1-3) in a dose-dependent manner (compare lanes 1-3 with 4-6, Akt-in vs. TAT-Flag control, respectively).

B. HA-tagged Akt transfected 293 cells were treated with TAT-Akt-in (g-l) or control TAT-Flag peptide (m-r) and examined by double immunostaining using anti-HA (Akt, top panels), anti-phospho-Ser473 (middle panels), or overlay view of anti-HA plus anti-phospho-Ser473 antibodies (lower panels). TAT-Akt-in treatment (g-l) not only prevented membrane translocation of Akt (g) from the cytosol, but also inhibited phosphorylation of Akt Ser-473 after PDGF stimulation (h and i, P-Ser473 and the overlay view of Akt plus P-Ser473, respectively). Control peptide (TAT-Flag) treatment (m-r) did not inhibit PDGF-induced membranous translocation of Akt (m, green), phosphorylation of Akt at Ser-473 (n, red), and the overlay view of Akt (HA) plus P-Ser473 (o, yellow). Wortmannin treatment completely inhibited membrane translocation from the cytosol and activation of Akt upon PDGF stimulation (a-c).

C. TAT-Akt-in treatment of the cells potently inhibited translocation of the GFP-fused Akt (Akt-PH-GFP) from the cytosol to plasma membrane after the PDGF stimulation (compare b and d, TAT-Flag control peptide or TAT-Akt-in treated cells after PDGF stimulation, respectively). TAT-Akt-in
did not inhibit translocation of Btk (Btk-PH-GFP,(39)) from the cytosol to plasma membrane after PDGF stimulation. Control peptide (TAT-Flag) did not inhibit translocation of Akt-PH-GFP or Btk-PH-GFP from cytosol to membrane after PDGF stimulation (b and f, Akt-PH-GFP or Btk-PH-GFP transfected cells). The results (A-C) were consistent in at least two independent experiments.
Fig. 6. *Akt-in* inhibits the biological effects of Akt activation *in vitro*.

A. MTT assay to examine cellular proliferation was performed using human T cell leukemia (T4) cells *in vitro*. TAT-Akt-*in*, but not the control peptide (TAT-Flag), could inhibit cell growth of human T cell leukemia (T4) cells at 3, 6.25, 12.5, 25, or 50 µM concentrations in a dose-dependent manner.

B. TAT-Akt-*in*, but not control peptide (TAT-Flag), inhibited cell survival in a dose-dependent manner. The inhibitory effect could be reversed by introducing an active form of Akt (*myr-Akt*, △) suggesting that *Akt-in* inhibited Akt. Cell viability could be reversed by adding *myr-Akt*, which indicates *Akt-in* inhibited Akt activation to induce cell death.

C. Using Rhodamine 123 (Rh 123), a lipophilic cationic fluorochrome, we analyzed the effects of *Akt-in* on mitochondrial transmembrane potential (MTP). TAT-Akt-*in* could enhance mitochondrial depolarization after treatment with dexamethasone. Mean Fluorescence Intensity (MFI) staining with Rhodamine 123 dye was 29.3, 33.3, or 42.7 (no induction, TAT-Flag control, or TAT-Akt-*in*, respectively). Approximately 25% of cell death was induced with dexamethasone alone. The results (A-C) were consistent in at least two independent experiments.
Fig. 7. Akt-in suppresses tumor growth in vivo.

A. To verify the in vivo effect of Akt-in for the potential treatment for the neoplastic diseases, QRsP-11 fibrosarcoma cells were transplanted subcutaneously into C57BL/6 mice. The peptides (TAT-Akt-in, TAT-Flag control peptide) were directly injected into the tumor on the days shown by the arrowheads. TAT-Akt-in inhibited tumor growth, while the control peptide (TAT-Flag) or PBS showed no inhibition. Eight mice in each arm were examined. No adverse effects were observed based on body weight measurement (shown in inlet).

B. Macroscopic appearances of QRsP-11 fibrosarcoma on day 9 after the transplantation are shown (first row, gross appearance of TAT-Akt-in and the TAT-Flag control peptide treated tumors). High power views of the tumor from the TAT-Akt-in treated mouse shows degenerated cells with apoptotic cells (indicated by arrows) are often present (H&E, second row) compared to the tumor cells treated with the control peptide. The presence of apoptotic cells was further determined by the TUNEL method (third row). TAT-Akt-in treatment efficiently inhibited phosphorylation of Akt at Ser473 compared to the control peptide treated tumor (fourth row). PBS treatment did not affect tumor growth (data not shown).
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References

1. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) Annu Rev Biochem 68, 965-1014
2. Cantley, L. C. (2002) Science 296, 1655-1657
3. Brazil, D. P., and Hemmings, B. A. (2001) Trends Biochem Sci 26, 657-664
4. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Biochem J 335, 1-13
5. Auguin, D., Barthe, P., Auge-Senegas, M. T., Stern, M. H., Noguchi, M., and Roumestand, C. (2004) J Biomol NMR 28, 137-155
6. Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A., and Barford, D. (2002) Mol Cell 9, 1227-1240
7. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Nat Struct Biol 9, 940-944
8. Milburn, C. C., Deak, M., Kelly, S. M., Price, N. C., Alessi, D. R., and Van Aalten, D. M. (2003) Biochem J 375, 531-538
9. Thomas, C. C., Deak, M., Alessi, D. R., and van Aalten, D. M. (2002) Curr Biol 12, 1256-1262
10. Vanhaesebroeck, B., and Alessi, D. R. (2000) Biochem J 346 Pt 3, 561-576.
11. Frech, M., Andjelkovic, M., Ingly, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1997) J Biol Chem 272, 8474-8481
12. James, S. R., Downes, C. P., Gigl, R., Grove, S. J., Holmes, A. B., and Alessi, D. R. (1996) Biochem J 315 (Pt 3), 709-713
13. Ferguson, K. M., Kavran, J. M., Sankaran, V. G., Fournier, E., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (2000) Mol Cell 6, 373-384
14. Lawlor, M. A., Mora, A., Ashby, P. R., Williams, M. R., Murray-Tait, V., Malone, L., Prescott, A. R., Lucocq, J. M., and Alessi, D. R. (2002) Embo J 21, 3728-3738
15. Collins, B. J., Deak, M., Arthur, J. S., Armit, L. J., and Alessi, D. R. (2003) Embo J 22, 4202-4211
16. Scheid, M. P., and Woodgett, J. R. (2003) FEBS Lett 546, 108-112
17. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev 13, 2905-2927
18. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Toke, K., Kadowaki, T., and Hay, N. (2001) Genes Dev 15, 2203-2208.
19. Luo, J., Manning, B. D., and Cantley, L. C. (2003) Cancer Cell 4, 257-262
20. Cantley, L. C., and Neel, B. G. (1999) Proc Natl Acad Sci U S A 96, 4240-4245.
21. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998) Cell 95, 29-39
22. Laine, J., Kunstle, G., Obata, T., Sha, M., and Noguchi, M. (2000) Mol Cell 6, 395-407
23. Pekarsky, Y., Koval, A., Hallas, C., Bichi, R., Tresini, M., Malstrom, S., Russo, G., Tsichlis, P., and Croce, C. M. (2000) Proc Natl Acad Sci U S A 97, 3028-3033
24. Gold, M. R. (2003) Trends Immunol 24, 104-108
25. Auguin, D., Barthe, P., Royer, C., Stern, M. H., Noguchi, M., Arold, S. T., and Roumestand, C. (2004) J Biol Chem 279, 35890-35902
26. Kunstle, G., Laine, J., Pierron, G., Kagami, S., Nakajima, H., Hoh, F., Roumenstand, C., Stern, M.-H., and Noguchi, M. (2002) Mol. Cell. Biol. 22, 1513-1525
Laine, J., Kunstle, G., Obata, T., and Noguchi, M. (2002) J. Biol. Chem. 277, 3743-3751.

Pekarsky, Y., Hallas, C., and Croce, C. M. (2001) Oncogene 20, 5638-5643.

Narducci, M. G., Fiorenza, M. T., Kang, S. M., Bevilacqua, A., Di Giacomo, M., Remotti, D., Picchio, M. C., Fidanza, V., Cooper, M. D., Croce, C. M., Mangia, F., and Russo, G. (2002) Proc Natl Acad Sci U S A 99, 11712-11717.

Lock, R. B. (2003) Int J Biochem Cell Biol 35, 1614-1618.

Barr, R. K., Boehm, I., Attwood, P. V., Watt, P. M., and Bogoyevitch, M. A. (2004) J Biol Chem 279, 36327-36338.

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) Curr Biol 9, 393-404.

Datta, K., Sundberg, C., Karumanchi, S. A., and Mukhopadhyay, D. (2001) Cancer Res 61, 1768-1775.

Narducci, M. G., Fiorenza, M. T., Kang, S. M., Bevilacqua, A., Di Giacomo, M., Remotti, D., Picchio, M. C., Fidanza, V., Cooper, M. D., Croce, C. M., Mangia, F., and Russo, G. (2002) Proc Natl Acad Sci U S A 99, 11712-11717.

Lock, R. B. (2003) Int J Biochem Cell Biol 35, 1614-1618.

Barr, R. K., Boehm, I., Attwood, P. V., Watt, P. M., and Bogoyevitch, M. A. (2004) J Biol Chem 279, 36327-36338.
50. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) J Biol Chem 272, 31515-31524.
51. Lemmon, M. A., Ferguson, K. M., and Abrams, C. S. (2002) FEBS Lett 513, 71-76
52. Kobayashi, T., and Cohen, P. (1999) Biochem J 339 (Pt 2), 319-328
53. Toker, A., and Newton, A. C. (2000) Cell 103, 185-188
54. Yu, J. W., Mendrola, J. M., Audhya, A., Singh, S., Keleti, D., DeWald, D. B., Murray, D., Emr, S. D., and Lemmon, M. A. (2004) Mol Cell 13, 677-688
55. Lemmon, M. A., and Ferguson, K. M. (2000) Biochem J 350 Pt 1, 1-18
56. Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R., and Muller, W. J. (2001) Mol Cell Biol 21, 2203-2212
57. Razzini, G., Berrie, C. P., Vignati, S., Broggini, M., Mascetta, G., Brancaccio, A., and Falasca, M. (2000) Faseb J 14, 1179-1187
58. Reuveni, H., Geiger, T., Geiger, B., and Levitzki, A. (2000) J Cell Biol 151, 1179-1192
59. Katome, T., Obata, T., Matsushima, R., Masuyama, N., Cantley, L. C., Gotoh, Y., Kishi, K., Shiota, H., and Ebina, Y. (2003) J Biol Chem 278, 28312-28323
60. Brazil, D. P., Park, J., and Hemmings, B. A. (2002) Cell 111, 293-303
61. Maira, S. M., Galetic, I., Brazil, D. P., Kaech, S., Ingley, E., Thelen, M., and Hemmings, B. A. (2001) Science 294, 374-380
62. Sato, S., Fujita, N., and Tsuruo, T. (2000) Proc Natl Acad Sci U S A 97, 10832-10837.
A

![Graph showing mean tumor volume over days after tumor cell injection.](image)

- Control (n=8)
- PBS (n=8)
- Akt-in (n=8)

B

**Macroscopic View**

Control (Day 9) vs. Akt-in (Day 9)

- H & E
- Tunel Staining
- P-Ser473 Akt
Inhibition of Akt kinase activity by a peptide spanning the \( \beta \)A strand of the protooncogene TCL1

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