Pseudosubstrate Peptides Inhibit Akt and Induce Cell Growth Inhibition

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Received April 1, 2003; Revised Manuscript Received November 21, 2003

ABSTRACT: We have designed peptide inhibitors that potently inhibit Akt both *in vitro* and inside cells. These peptide inhibitors are selective for Akt versus other closely related kinases. The peptides inhibit the *in vitro* phosphorylation of a biotinylated Bad peptide by Akt with potency up to 100 nM. We have shown that the binding between Akt1 and these peptide inhibitors requires MgATP. Mutating the two putative Akt phosphorylation sites to Ala (nonsubstrate) in these peptides increases the inhibitory potency while mutating the sites to aspartic acid (phosphorylation mimetic) reduces the potency. When delivered into cells, these peptide inhibitors can inhibit cellular Akt activity and cell growth. Thus, these Akt-specific peptide inhibitors provide prototypes for peptide mimetic drugs as well as very useful tools to dissect cellular functions of Akt.

Akt1 is a Ser/Thr[1] protein kinase that was first discovered as the human homologue of the transforming gene in Akt-8 oncogenic virus, which was isolated from a spontaneous thymoma in AKR mouse (*1*, *2*). Since the discovery of human Akt1 (also called PKB), two additional mammalian Akt isoforms, Akt2 and Akt3, have been identified (*3*−*6*). Knockout experiments with Akt1 and Akt2 revealed distinct phenotypes (*7*−*10*), possibly due to differential tissue expression or different functions of the Akt isoforms.

Akt is activated when phosphorylated at two sites: (1) Thr 308 in the activation loop motif that is common to many kinases and (2) at Ser 473, a residue in a hydrophobic motif that is common to many ACG family kinases (*11*). Akt activation is mediated through the PI3′K pathway in response to a number of mitogenic stimuli (*12*) as well as apoptotic signals such as Fas activation by Fas ligand (*13*), hypoxia (*14*), and oxidative stress (*15*). Once activated, PI3′K phosphorylates PtdIns(4)P or PtdIns(4,5)P_2 (PIP2) to form PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 (PIP3). The tumor suppressor PTEN, on the other hand, is a lipid phosphatase that reverses the function of PI3′K, decreases the amount of PIP3, and thus prevents Akt activation (*16*, *17*). When present, PIP3 recruits PH domain containing proteins, such as Akt and PDK1, to the plasma membrane. Once colocalized, PDK1 partially activates Akt by phosphorylation on Thr 308. In a second activity, PDK2 completes the activation via phosphorylation at Ser 473 (*16*, *17*). In addition to its localization function, the binding of PIP3 to the PH domain of Akt induces a conformational change that reveals the activating phosphorylation sites (Thr 308 and Ser 473) to PDK1 and PDK2. This conformational change may also cause an increase in the intrinsic catalytic activity of Akt (*18*). Multiple candidates for the PDK2 activity have been identified. These PDK2 candidates include ILK1 (*19*), MAPK AP kinase 2 (*20*), and raft-associated PDK2 (*21*). Akt has also been shown to autophosphorylate itself at Ser 473 (*22*). The impact on Akt2 activation by the phosphorylations on Thr 309 (corresponding to Thr 308 in Akt1) and Ser 474 (corresponding to Ser 473 in Akt1) is described in the recent publications of the Akt2 crystal structure. The unphosphorylated, inactive Akt2 displays a disordered structure at the catalytic site. The phosphorylations at Thr 309 and Ser 474 lock Akt2 in an ordered and active conformation (*23*, *24*). Recently, HSP90 (*25*), CTMP (*26*) and TRB3 (*27*, *28*) have also been reported to regulate Akt activity through various mechanisms.

Akt modulates cellular functions by phosphorylating its target proteins. Akt phosphorylates and inactivates key components in the apoptotic cascade such as Bad (*29*−*31*), Caspase 9 (*32*), and ASK1 (*33*). Akt also downregulates the expression of proapoptotic proteins such as the Fas ligand through phosphorylation and inactivation of the forkhead transcription factors FOXO3 (FKHRL1) and FOXO4 (AFX) (*34*−*36*). Another important downstream target of Akt is GSK3. Inactivation of GSK3 upon phosphorylation by Akt leads to protection from apoptosis (*18*). Furthermore, Akt has been shown to regulate the transcription factor NFκB. NFκB is inhibited by IκB. Akt induces the phosphorylation and degradation of IκB (*37*−*40*). The reduction of IκB increases NFκB activity, thereby upregulating the expression of antiapoptotic proteins such as cIAP1 and cIAP2. In addition, Akt directly modulates mitochondria function by promoting the interaction between hexokinase and the voltage-dependent anion channel, which prevents cytochrome *c* release (*41*). Recently, Akt was also reported to phospho-

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[1] Abbreviations: Ser, serine; Thr, threonine; Ala, alanine; PI3′K, phosphatidylinositol 3′-kinase; PtdIns(4)P, phosphatidylinositol 4-phosphate; PIP2 or PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PIP3 or PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; IKKα, IκB kinase α; PKB, protein kinase B; NMP, *N*-methylpyrrolidone; ESI-MS, electrospray ionization mass spectrography; MALDI-MS, matrix-assisted laser desorption ionization mass spectrography.

10.1021/bi034515p CCC: $27.50
Published on Web 01/15/2004

rylate and stabilize PED/PEA-15, an antiapoptotic protein (*42*).

In addition to inhibiting apoptosis, Akt enhances the malignant phenotype by inducing cell growth. The forkhead transcription factor, FOXO4, induces cell cycle arrest at G1 through upregulation of $p27^{Kip1}$. By inactivating FOXO4, Akt reduces the $p27^{Kip1}$ level and allows cells to proceed into the S phase (*43*). Studies with Akt1 knockout murine embryonic stem cells revealed additional Akt functions in promoting G2/M progression (*44*). Recently, Akt was shown to activate p70S6 kinase through phosphorylation of TSC2, which destabilizes the mTOR inhibitory complex of TSC1−TSC2 (*45*−*48*). Furthermore, the activation of the mTOR−p70S6 kinase pathway was shown to be critical for Akt-induced transformation (*49*). Akt also increases cellular metabolism through phosphorylation and inactivation of GSK3 in addition to other pathways (*50*).

Tumor cells are under apoptotic pressure due to deregulation of many cell-growth-related functions. Elevation of activated Akt is one strategy that tumor cells use to survive. Indeed, Akt isoforms are overexpressed in a variety of human tumors including lung, breast, ovarian, gastric, and pancreatic carcinomas (*5*, *51*−*58*). Increased Akt expression also correlates with disease progression (*3*, *52*). To further support this notion, *PTEN* mutations, which result in increased Akt activity, have been described in a wide variety of malignancies, including breast cancer, prostate cancer, melanoma, glioblastoma multiforme, and endometrial cancer (*59*−*70*). PI3′K−Akt activity also promotes angiogenesis (*71*). In addition, Akt overexpression could result in drug resistance in cancer cells. PI3′K inhibitors and overexpression of PTEN have been shown to inactivate Akt and sensitize tumor cells to chemotherapy or radiotherapy (*72*−*74*). Furthermore, expression of constitutively active Akt can transform cells and promote tumorigenesis (*75*, *76*). Thus, it is not surprising that blocking Akt expression can reduce tumor growth (*77*). All of these data support the idea that Akt inhibitors might be useful in the treatment of cancer.

Developing selective small molecule kinase inhibitors, which typically bind to the ATP site, is challenging due to the similarity of this site in many kinases. In contrast to ATP site inhibitors, pseudosubstrate peptide inhibitors have been proven to be selective and potent for many kinases (*78*, *79*). This selectivity derives from the much larger peptide−kinase contact region that has evolved to discriminate between various protein substrates. In this study, we have developed a series of Akt peptide inhibitors based on the optimal peptide substrate consensus sequence (*80*). We have shown that these peptides bind with Akt only in the presence of MgATP. They selectively inhibit Akt activity *in vitro*. When delivered into mammalian cells, they also inhibit cellular Akt and induce growth inhibition in cells.

EXPERIMENTAL PROCEDURES

Materials. PKA was purchased from Panvera (Madison, WI); CDC2, PKCδ, and PKCγ were purchased from Calbiochem (San Diego, CA); SGK, p70S6K, p90S6K, Src, and MAPK AP kinase 2 were purchased from Upstate Biotechnology (Charlottesville, VA). Phosphotidylserine and dioleyl-*sn*-glycerol were purchased from Avanti Polar Lipids (Alabaster, AL). Strepavidin FLASH plates were purchased from Perkin-Elmer Life Sciences (Boston, MA). PDK1 was expressed and purified in a baculoviral system (unpublished results). λ-Protein phosphatase was purchased from New England BioLabs (Beverly, MA).

Substrates. Kinase substrates were custom synthesized by Genemed Synthesis, Inc. (South San Francisco, CA) except for the Src substrate, which was purchased from Promega (Madison, WI; catalog no. V6480). The substrates had the general structure of biotin-Ahx-peptide. The Akt assay used EELSPFRGRSRSAPPNLWAAQR; the PKA assay used kemptide (LRRASLG); the CDC2 assay used PKTPKKAKKL; the SGK assay used RPRAATF; the PKCδ and PKCγ assays used neurogranin (ERMRPRKRQGSVRRRV); the p70S6K assay used RRRLSDLRA; and the p90S6K assay used KKKNRTLSVA.

Akt1 Purification. His-Akt1 contains an N-terminal 6×His tag, a thrombin cleavage site, and the wild-type Akt1 sequence of 1−480 amino acids. It was coexpressed for 48 h with PDK1 using a bacculovirus/Sf9 expression system. His-Akt1 expression was optimized for both expression level and specific activity by adjusting the multiplicity of infection of each baculovirus. Insect cells were lysed in buffer A [20 mM Tris, 10% (w/v) glycerol, and 1 mM DTT, pH 7.4] supplemented with 150 mM KCl and complete protease inhibitor cocktail lacking EDTA (Roche, Mannheim, Germany). His-Akt1 was purified to homogeneity using a two-step procedure. His-Akt1 was bound to a peptide affinity column based on peptide inhibitor 2 coupled to Affi-Gel-10 (Bio-Rad Laboratories, Hercules, CA; manuscript in preparation) in buffer A supplemented with 1 mM ATP and 2 mM $MgCl_2$. Akt was eluted by buffer A supplemented with 1 mM EDTA and 200 mM arginine. Akt was further enriched using Sepharose fast-flow (Amersham Biosciences, Piscataway, NJ) anion-exchange chromatography by developing a shallow gradient of 10−500 mM KCl in buffer A.

Dephosphorylation by λ-Protein Phosphatase. His-Akt1 (50 μM) was incubated at room temperature with 0.5 unit of λ-protein phosphatase in 25 mM Tris, pH 7.7, 10% (w/v) glycerol, 400 mM KCl, 0.2 mM manganese chloride, and 0.1% β-mercaptoethanol in a total volume of 550 μL for various times as indicated. After the λ-protein phosphatase treatment, Akt1 protein from each reaction was purified to remove the phosphatase at each time point using a Ni affinity column (Qiagen) eluted with 20 mM Tris, pH 7.4, 10% (w/v) glycerol, 400 mM KCl, 0.1% β-mercaptoethanol, and 200 mM imidazole.

In Vitro Phosphorylation by PDK1 and MAPK AP Kinase 2. Akt1 (6 μM) was incubated at room temperature for 2 h with 1 μM PDK1 and 30 nM MAPK AP kinase 2 in kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 0.01% Triton X-100) containing 0.5 mM ATP in a final volume of 30 μL. Twenty nanomolar phosphorylated Akt1 was characterized in the Akt kinase assay as described.

Peptide Synthesis. Peptides were assembled on a 430A-automated synthesizer (Applied Biosystems, Foster City, CA) using standard Fastmoc deprotection/coupling cycles with preloaded Wang resin (0.1−0.25 mmol). Cartridges containing N^{α}-Fmoc amino acids (1 mmol) with side-chain protection (Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Asp and Glu: *tert*-butyl ester; Asn, Cys, Gln, and His, trityl; Lys and Trp, *tert*-butyloxycarbonyl; Ser, Thr, and Tyr, *tert*-butyl ether) were activated with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-

tetramethyluronium hexafluorophosphate (1 mmol), 1-hydroxybenzotriazole (1 mmol), and diisopropylethylamine (2 mmol) in *N*-methylpyrrolidone (NMP). The activated amino acid was coupled for 30 min following removal of the N-terminal Fmoc group with 20% piperidine in NMP. N-Terminal acetylation was accomplished with acetic acid (1 mmol) coupled for the Fmoc amino acids. The peptides were cleaved and deprotected by shaking with reagent K (trifluoroacetic acid:water:thioanisole:phenol:ethanedithiol: triisopropylsilane, 80:5:5:5:2.5:2.5) (*81*) for 3 h at ambient temperature. The crude peptides were recovered by precipitation with ether following evaporation under reduced pressure. The peptides were purified on a preparative HPLC running Unipoint analysis software (Gilson, Inc., Middleton, WI) on a 25 × 200 mm radial compression column containing Delta-Pak C_{18} packing (Waters, Inc., Taunton, MA) with a flow rate of 20 mL/min. The peptides were eluted with a linear gradient of 0.1% trifluoroacetic acid/water and acetonitrile. Fractions containing the peptide were pooled and lyophilized. The purity of the final products were confirmed by reverse-phase analytical HPLC on a Hewlett-Packard 1050 series system with diode array and fluorescence detection (Agilent Technologies, Palo Alto, CA) eluted with a linear gradient of 0.1% trifluoroacetic acid/water and acetonitrile on a 4.6 × 250 mm YMC ODS-AQ, 5 μm, 120 Å column (Waters Inc.). The identity of the products was confirmed by electrospray ionization mass spectrography (ESI-MS) on a Finnigan SSQ7000 (Finnigan Corp., San Jose, CA) or by matrix-assisted laser desorption ionization mass spectrography (MALDI-MS) on a Voyager DE-PRO (Applied Biosystems).

Molecular Modeling. A model of human Akt was developed using the Homology module in the Insight suite of software (Accelrys Inc.). The crystal structure of PKA bound with ATP and peptide inhibitor was used as the template. In brief, the sequences of PKA and Akt were aligned. In the structurally conserved regions (helices and β-strands), the coordinates of the identical amino acids were copied from the crystal structure to the homology model. The coordinates of the backbone of the other matching residues were transferred to the model. The conformations of the side chains of these residues were chosen to follow the path of the corresponding residue from the PKA structure. The coordinates of the remaining residues (the insertions and deletions) were chosen from a database of crystallographically observed peptide conformations. The model was then briefly minimized. The structure of the peptides in the model was developed from the structure of the peptide inhibitor in the X-ray structure of PKA.

K_d Measurement. Fluorescence polarization measurements were performed at 25 °C in either a polarizing spectrofluorometer (Photon Technologies International, London, Ontario) using 0.35 mL in quartz cuvettes or an analyst polarizing spectrofluorometer (LJL Biosystems, Sunnyvale, CA) using 0.15 mL per well in 96-well plates. His-Akt1 was diluted to the indicated concentrations in buffer containing 10 nM Oregon green-labeled Akt inhibitory peptides and incubated at 25 °C for 30 min prior to measurements. The buffer contained 20 mM Tris, 100 mM KCl, 10% (w/v) glycerol, 1 mM DTT, 0.4 mM ATP, and 1 mM $MgCl_2$, pH 7.4. Data were analyzed by Origin software (Microcal Software, Inc., Northhampton, MA).

Akt Kinase Assay. The kinase assay uses His-Akt1 and a biotinylated mouse Bad peptide as substrate. The kinase assay was carried out at room temperature for 30 min in 50 μL of reaction buffer [20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 5 μM ATP (K_m = 40 μM), 5 μM peptide (K_m = 15 μM), 1 mM DTT, 60 ng of Akt1, and 0.5 μCi of [γ-^{33}P]ATP] in the presence of different concentrations of Akt peptide inhibitors. Each reaction was stopped by adding 50 μL of termination buffer (0.1 M EDTA, pH 8.0, and 4 M NaCl). The biotinylated Bad peptides were immobilized on streptavidin-coated FLASH plates. After being washed with PBS−Tween 20 (0.05%), the ^{33}P-phosphopeptide captured on the FLASH plates was measured with a TopCount Packard Instruments γ counter (Packard Instruments, Boston, MA).

Kinase Selectivity Assays. p70S6K and p90S6K were assayed using the condition of Akt kinase assay except that 2 nM p70S6K or 100 pM p90S6K was used. Other enzymes were tested in 25 mM HEPES buffer, pH 7.4, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM DTT, and 0.0075% (w/v) Triton X-100. PKCγ and PKCδ required 90 μg/mL phosphatidylserine and 18 μg/mL dioleyl-*sn*-glycerol. The PKCγ assay was run in the presence of 1 mM $CaCl_2$, and Src was tested in the presence of 1 mM $MnCl_2$. PKA, CDC2, SGK, and Src assay buffers contained 0.05% (w/v) gelatin. Enzymes, peptide substrates, ATP, and [γ-^{33}P]ATP were in the following concentrations: (1) PKA assay, 0.44 nM enzyme, 3.5 μM peptide substrate, 7.5 μM ATP, and 7.5 μCi/mL [γ-^{33}P]ATP; (2) CDC2 assay, 0.022 nM enzyme, 2.5 μM peptide substrate, 5 μM ATP, and 20 μCi/mL [γ-^{33}P]ATP; (3) SGK assay, 0.83 nM enzyme, 1 μM peptide substrate, 10 μM ATP, and 10 μCi/mL [γ-^{33}P]ATP; (4) Src assay, 3.2 nM enzyme, 6 μM peptide substrate, 5 μM ATP, and 5 μCi/mL [γ-^{33}P]ATP; (5) PKCγ assay, 2.1 nM enzyme, 5 μM peptide substrate, 10 μM ATP, and 10 μCi/mL [γ-^{33}P]ATP; (6) PKCδ assay, 2 nM enzyme, 5 μM peptide substrate, 10 μM ATP, and 10 μCi/mL [γ-^{33}P]ATP. Reactions in 50 μL were carried out at room temperature for 30 min and then stopped by the addition of 50 μL of stopping buffer (4 M NaCl and 0.1 M EDTA, pH 8.0). Reactions were transferred to FLASH plates, incubated at room temperature for 10 min, then washed three times with phosphate-buffered saline containing 0.05% Tween 20, and counted on a TopCount Packard Instruments γ counter (Packard Instruments, Boston, MA). IC_{50}s were calculated using the sigmoidal curve fit with the Assay Explorer software (MDL, Los Angeles, CA).

Delivery of Peptide Inhibitors into Cells. (A) BioPORTER: The peptide inhibitors were delivered into cells using BioPORTER reagent (Gene Therapy Systems, San Diego, CA) according to the vendor's instructions. Specifically, the cells were washed once with serum-free media. The peptide solutions were used to hydrate the dried BioPORTER reagent. Five minutes after coincubation at room temperature, the peptide−BioPORTER complex was vortexed gently, mixed with serum-free media, and added to the cells. Four hours after incubation, the medium was changed to the complete medium for further culture. (B) Poly-D-Arg as Membrane Transporter Peptide: HeLa cells were incubated with six FAM-Aha-poly-D-Arg-Aha-peptides (Fl-dr-peptide) in the complete medium.

GSK3 Western Blot. Cells were harvested and lysed with brief sonication in insect cell lysis buffer (BD Pharmingen,

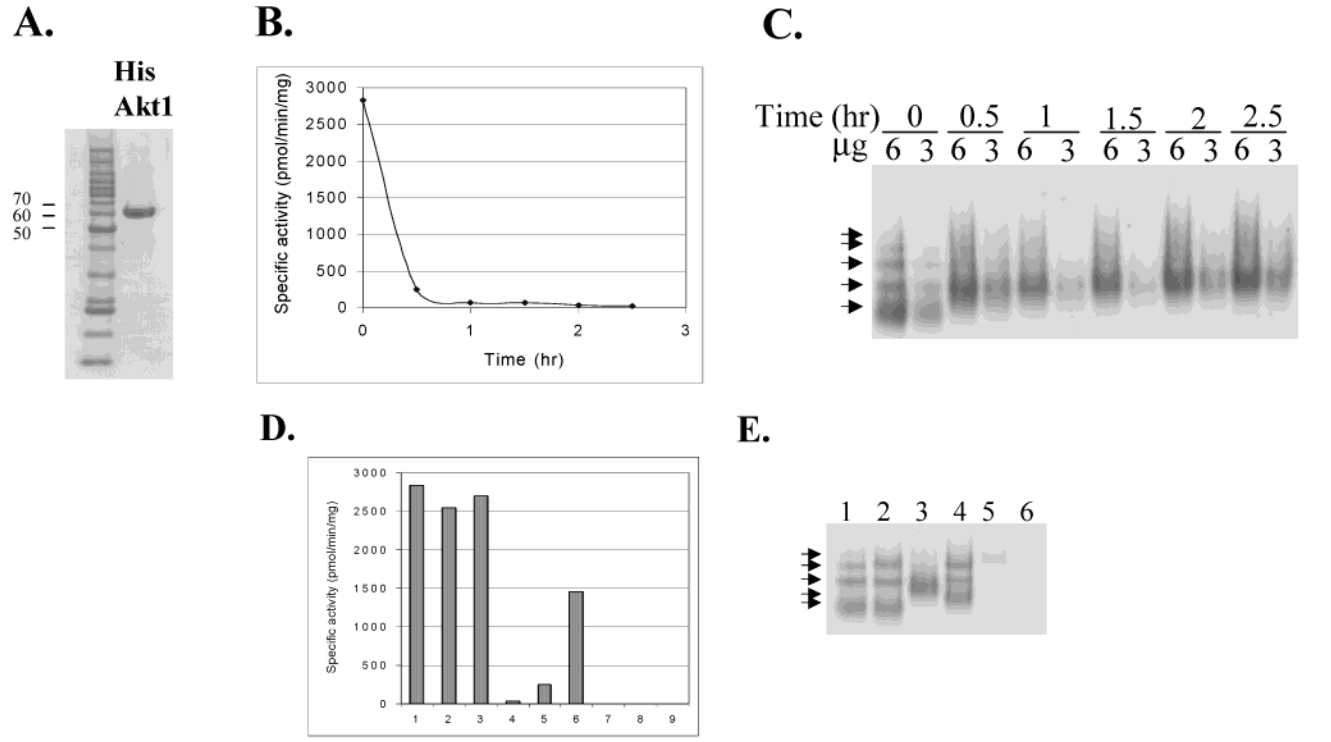


FIGURE 1: Characterization of His-Akt1. (A) Characterization of purified His-Akt1 on SDS−PAGE. (B) Dephosphorylation of His-Akt1 by λ-protein phosphatase. Akt1 (50 μM) was incubated with 0.5 unit of λ-protein phosphatase at room temperature for various times as indicated. After the λ-protein phosphatase treatment, Akt1 protein from each reaction was purified to remove the phosphatase at each time point using a Ni affinity column (Qiagen), and 20 nM purified Akt1 protein was used in the Akt kinase assay as described in the Experimental Procedures. (C) Characterization of His-Akt1 after λ-protein phosphatase treatment on native PAGE. (D) Rephosphorylation of His-Akt1 by PDK1 and MAPK AP kinase 2. Akt1 (6 μM) was treated with λ-protein phosphatase for 2 h (Akt1-2h) and purified with a Ni affinity column. The purified His-Akt1 or Akt1-2h, was *in vitro* phosphorylated by PDK1 and MAPK AP kinase 2. Then 20 nM phosphorylated Akt1 was characterized in the Akt kinase assay as described. Other proper controls at the step of *in vitro* phosphorylation were included as indicated: 1, purified His-Akt1; 2, purified His-Akt1 + ATP; 3, purified His-Akt1 + ATP + PDK1 + MAPK AP kinase 2; 4, His-Akt1-2h; 5, His-Akt1-2h + ATP; 6, His-Akt1-2h + ATP + PDK1 + MAPK AP kinase 2; 7, PDK1 + ATP; 8, MAPK AP kinase 2 + ATP; 9, PDK1 + MAPK AP kinase 2 + ATP. (E) Characterization of His-Akt1 after various treatments on native PAGE. Each His-Akt1 (3 μg) was loaded on native PAGE. Lanes: 1, purified His-Akt1; 2, purified His-Akt1 + ATP + PDK1 + MAPK AP kinase 2; 3, His-Akt1 treated with λ-protein phosphatase for 2 h; 4, His-Akt1 treated with λ-protein phosphatase for 2 h, purified, and then rephosphorylated with ATP, PDK1, and MAPK AP kinase 2; 5, PDK1 alone; 6, MAPK AP kinase 2 alone.

San Diego, CA; 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM $NaPP_i$) with the addition of 1× protease inhibitor cocktail (BD Pharmingen, San Diego, CA; 16 μg/mL benzamidine hydrochloride, 10 μg/mL phenanthroline, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 1 mM PMSF) and 1 μM microcystin LR (Sigma, St. Louis, MO). For western blotting, 40 μg of total protein was loaded per lane on an SDS−PAGE and electrophoresed under reducing conditions prior to transfer to PVDF membrane (Invitrogen, Carlsbad, CA). The western blot was performed with phospho-GSK3α/β (Ser21/9) antibody (Cell Signaling, Beverly, CA) (1:1000) and GSK-3β antibody (Santa Cruz, Santa Cruz, CA) (1:2000).

AlamarBlue Assay. The cells on 96-well plates were gently washed with 200 μL of PBS. AlamarBlue reagent (Biosource International, Carmarillo, CA; catalog no. DAL1100) was diluted 1:10 in normal growth media. The diluted AlamarBlue reagent (100 μL) was added to each well on the 96-well plates and incubated until the reaction was complete as per the manufacturer's instructions. Analysis was performed using an *fmax* fluorescence microplate reader (Molecular Devices, Sunnyvalle, CA), set at the excitation wavelength of 544 nm and emission wavelength of 595 nm. Data were analyzed using SOFTmax PRO software provided by the manufacturer.

RESULTS

Characterization of His-Akt1. His-Akt1 expression was optimized for the highest specific activity by adjusting the multiplicity of infection of the Akt virus and the PDK1 virus. His-Akt1 was purified to homogeneity and displayed a single band on denaturing SDS−PAGE (Figure 1A). *In vitro* phosphorylation and dephosphorylation experiments were carried out to characterize the Akt activity. The specific activity of His-Akt1 was reduced ~70-fold after the treatment with λ-protein phosphatase to remove the activating phosphorylations at Thr 308 and Ser 473 (Figure 1B). LC-MS analysis of tryptic fragments confirmed the presence of phosphates at Thr 308/Ser 473 and the loss of both phosphates after 30 min of λ-protein phosphatase treatment (data not shown). When the protein was analyzed by native PAGE to reveal the posttranslational modifications, multiple bands were seen, with a predominant band migrating the fastest (at the bottom, Figure 1C). Gel scanning densitometry indicated that this bottom band comprised ~70% of the total protein. Upon λ-protein phosphatase treatment, the bottom band was converted to more slowly migrating species. The kinetics of this transition correlated well with the loss of

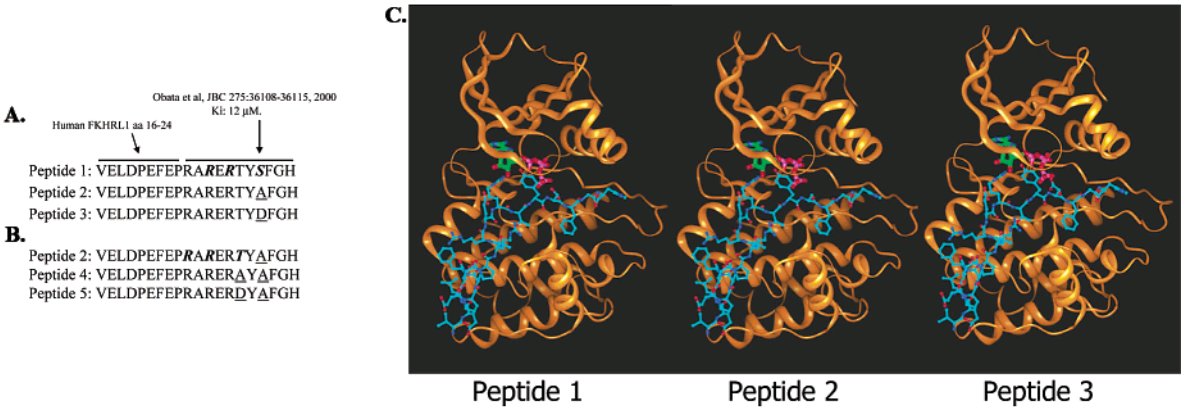


FIGURE 2: Sequences of the peptide inhibitors and molecular models of the binding between peptide inhibitors and Akt1. (A, B) Sequences of the peptide inhibitors. (C) Molecular models of the binding between peptide inhibitors and Akt1.

specific activity (Figure 1B,C). This suggests that the bottom band represents the most active species that was phosphorylated at both Thr 308 and Ser 473. *In vitro* phosphorylation of purified His-Akt1 using PDK1 and MAPK AP kinase 2, in an attempt to further activate Akt, yielded neither more active His-Akt1 nor any changes in the migration pattern on the native PAGE. This suggests that purified His-Akt1 was already fully activated and highly, if not completely, phosphorylated at Thr 308 and Ser 473 (Figure 1D,E). In vitro rephosphorylation of the dephosphorylated His-Akt1 by PDK1 and MAPK AP kinase 2 resulted in the appearance of an additional band between the original bottom two bands in native PAGE and restored the specific activity to ~60% of the fully active protein (Figure 1D,E). The newly appearing band may represent the protein with phosphate at Thr 308 and Ser 473, whereas the original bottom band may have additional phosphates at other sites. Indeed, the acquisition of the phosphorylation at Thr 308 and Ser 473 was confirmed by LC-MS analysis of tryptic fragments (data not shown). All of these data indicate that His-Akt1, as initially purified, was fully active.

Pseudosubstrate Peptides Inhibit Akt. Obata and colleagues reported an optimal peptide substrate sequence, ARKRERTYSFGHHA (AKTide-2T), that binds to the substrate binding site of mouse Akt1 and inhibits it with a K_i of 12 μM (*80*). To achieve a more potent peptide inhibitor of Akt1, we made a hybrid peptide (peptide 1) between amino acids 16−24 of human FOXO3 (AF03285) and the peptide AKTide-2T, with the putative phosphorylation site Ser at position 17 (Figure 2A). To compare the relative affinity of this hybrid peptide substrate with its nonphosphorylatable variant, the putative phosphorylation site Ser 17 in peptide 1 was changed to either alanine in peptide 2 or aspartate in peptide 3 (Figure 2A). Molecular models of these peptides bound to human Akt1 are shown in Figure 2C. The peptides can be modeled into the peptide-binding groove of Akt1 in such a way that the side chains of the peptide amino acids make favorable, complementary interactions with the Akt1 protein. As expected, either Ser (peptide 1) or Ala (peptide 2) is small enough to fit into the substrate-binding cleft. However, the model of the Ser → Asp mutation in peptide 3 suggests that the large side chain of aspartate would fit poorly into the binding cleft. Therefore, the Asp peptide would be expected to bind with less affinity when compared to the other two peptides.

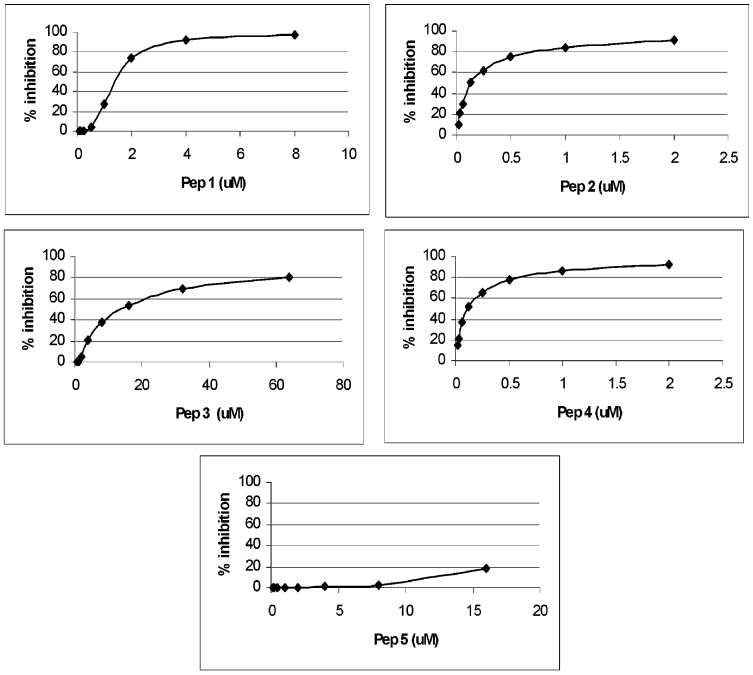
These peptides were tested for their ability to inhibit Akt1 kinase activity in an *in vitro* kinase assay. The inhibition curves by the three peptides are shown in Figure 3A. Peptide 1 inhibits Akt with a K_i of 1.11 μM, a 10-fold improvement over AKTide-2T, indicating higher affinity due to additional interactions between the peptide and Akt1. Peptide 2 with the Ser → Ala mutation at the phosphorylation site is the most potent inhibitor with a K_i of 0.11 μM. This is probably because Akt1 binds the peptide but can neither phosphorylate nor release the peptide when it has the Ser → Ala mutation. Peptide 3 with the Ser → Asp mutation is the least potent, possibly because the Asp carboxylate would occupy the same space as the γ-phosphate of ATP (Figures 2 and 3). These peptides also inhibit Akt2 or Akt3 (data not shown).

These peptide sequences have a second putative Akt phosphorylation site at Thr 15. We mutated the Thr to Ala (peptide 4) or Asp (peptide 5) from peptide 2 (Figure 2B). The K_i was also measured as before. Results analogous to the Ser 17 mutations were obtained where peptide 4 was as potent as peptide 2 and peptide 5 was the least potent inhibitor (Figure 3). This suggests that either Thr 15 or Ser 17 can bind with Akt1 at the catalytic site.

The above result shows that increasing the length of the peptide by nine amino acids increases the binding affinity between the peptide and Akt1 by 10-fold. We examined the minimal peptide length required for the maximal affinity. A series of peptides were synthesized; each was three amino acids shorter than the predecessor. No significant change was observed when the peptide is shortened to 17 amino acids. However, any peptide less than 17 amino acids exhibited dramatically less affinity than the original 20 amino acid peptide. Therefore, a peptide must be at least 17 amino acids in length to inhibit Akt with maximal affinity (Figure 4).

Binding between Peptide Inhibitors and Akt Requires MgATP. We examined the requirements for the interaction between the peptides and Akt. Fluorescence polarization is used as an indicator of binding between the Oregon green-labeled peptide and the Akt1 protein (see the Experimental Procedures). No binding was observed between peptide 2 and Akt1 in the absence of MgATP. In the presence of MgATP, peptide 2 bound to Akt1 with a K_d of 260 nM (Figure 5A). This suggests that Akt binds with MgATP before it binds with its protein substrate. This is consistent with kinetic data from other kinases suggesting that the

A.



B.

	Peptides	Ki (Akt1)
1:	VELDPEFEPRARER T Y S F GH	1.11 uM
2:	VELDPEFEPRARER T Y A F GH	0.11 uM
3:	VELDPEFEPRARER T Y D F GH	10.6 uM
4:	VELDPEFEPRARER A Y A F GH	0.095 uM
5:	VELDPEFEPRARER D Y A F GH	>16 uM

FIGURE 3: Pseudosubstrate peptides inhibit Akt1 *in vitro*. The Akt kinase assay was carried out as described in the Experimental Procedures. Different concentrations of the inhibitors were included in the kinase reaction. The percentage of inhibition from each reaction was plotted against inhibitor concentrations, and IC_{50}s were derived from an exponential fit of the curve. K_i was calculated according to the Michaelis−Menten equation. (A) Titration curves. (B) K_i values.

Peptides	Ki
Ac- ERTYAFGH	>100 μM
Ac- RARERTYAFGH	15.86 μM
FEPRARERTYAFGH	1.97 μM
Ac- FEPRARERTYAFGH	1.44 μM
Ac- DPEFEPRARERTYAFGH	0.26 μM
VELDPEFEPRARERTYAFGH	0.18 μM
Ac- VELDPEFEPRARERTYAFGH	0.098 μM

FIGURE 4: Minimal-length requirement for the peptide inhibitors. Different lengths of the peptide inhibitor 2 were synthesized as described. An acetyl group was added to the amino- terminal end of some of these peptides to increase their stability for cellular assays. These peptides were tested in the Akt1 assay as described in Figure 3.

reaction is ordered and requires the first addition of ATP prior to the protein substrate (*82*).

We measured the binding constants between these peptides and Akt using Akt titration experiments. Figure 5 shows that the dissociation constants of these peptides agree very well with their potency in inhibiting Akt activity *in vitro* (Figures 3 and 5B,C).

Selectivity of the Peptide Inhibitors. Each kinase has its own signature in the substrate-binding groove. Since these peptide inhibitors were designed to bind to the substrate-binding site, we expected them to be selective inhibitors of Akt. Kinases can be classified on the basis of the similarity of their catalytic sites. We tested the peptides against kinases that represent different classes. We selected two groups of kinases for the selectivity testing. Like Akt, SGK, p70S6K, p90S6K, PKA, PKCδ, and PKCγ are all from the AGC kinase family. Cdc2 and Src represent other families of kinases that regulate cell growth. Indeed, we did not observe any inhibition at a concentration of 30 μM on p70S6K, p90S6K, PKA, PKCδ, PKCγ, Cdc2, or Src. Interestingly, we observed inhibition on SGK by peptide 1. This is not surprising since SGK and Akt share many common downstream targets (*34*, *83*, *84*). However, both peptide 2 and peptide 4 showed significant selectivity for Akt when compared to SGK, suggesting the possibility of designing selective pseudosubstrate peptide inhibitors even among the kinases that share some common substrates (Table 1).

Pseudosubstrate Peptides Inhibit Akt Functions in Cells and Inhibit Cell Growth. We delivered the peptide inhibitors into HeLa cells using the BioPORTER reagent and examined the effect on cellular Akt activity. Using a fluorescent tag-labeled peptide, we estimated the delivery efficiency to be over 90% (data not shown). Since GSK3 is one of the major downstream phosphorylation targets of Akt, we used GSK3 phosphorylation as an indication of cellular Akt activity. Two hours after the delivery process, total cell lysates were prepared, and GSK3 phosphorylation was assessed with phospho-GSK3 antibody. Both peptide 2 and peptide 3 inhibited GSK3 phosphorylation in HeLa cells in a dose-dependent manner. Consistent with the *in vitro* kinase inhibition potency, peptide 2 was more potent than peptide 3 in inhibiting GSK3 phosphorylation. In the same experiment, total GSK3 protein levels were not changed by treatment with the peptide inhibitors (Figure 6A).

Blocking Akt expression by antisense oligonucleotide induces cell growth inhibition and apoptosis in mammalian

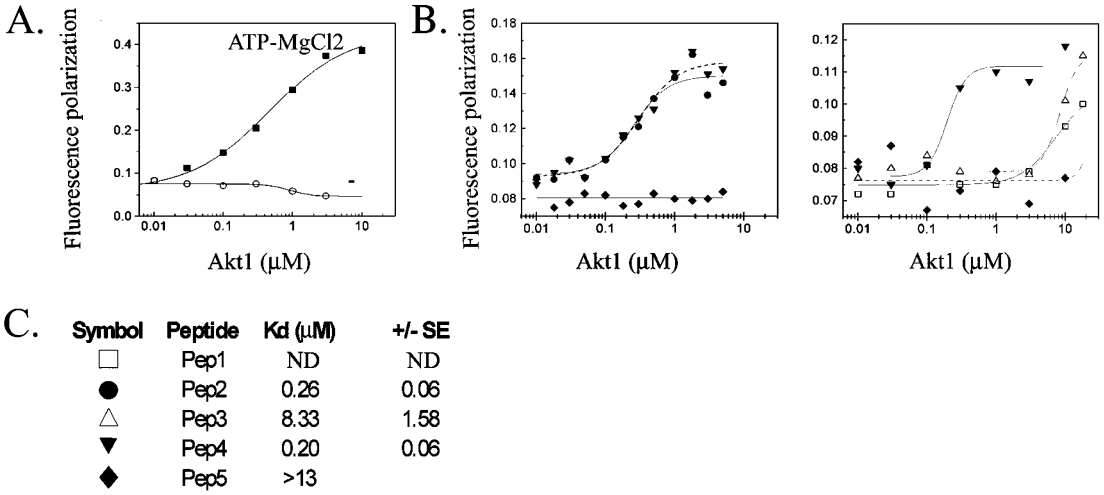


FIGURE 5: Binding between peptide inhibitors and His-Akt1. Fluorescence polarization was used as an indication of binding between the 10 nM Oregon green-labeled peptides and Akt1. The fluorescence polarization value was plotted vs different concentrations of His-Akt1, and the K_d was derived as described in the Experimental Procedures. (A) The binding between peptide 2 and His-Akt1 requires ATP−$MgCl_2$. Key: filled square, binding in the presence of 1 mM ATP−$MgCl_2$; open circle, binding in the absence of ATP−$MgCl_2$. (B) K_d measurements in the presence of 1 mM ATP−$MgCl_2$. Symbols for each peptide are shown in (C). The data are representative of several experiments using His-Akt1. (C) K_d for each peptide.

Table 1: Selectivity of Akt Peptide Inhibitors[a]

	K_i (μM)								
	Akt1	p70S6K	p90S6K	PKA	Cdc2	Src	SGK	PKCδ	PKCγ
peptide 1	1.11	>30	>30	>34	>33	>33	1	>42	>30
peptide 2	0.11	>30	>30	>34	>33	>33	>36	>42	>30
peptide 4	0.095	>30	>30	>34	>33	>33	12.5	>42	>30

[a] Various kinase assays were carried out as described in the Experimental Procedures. Different concentrations of the inhibitors were included in the kinase reactions. The cpm from each reaction was plotted against inhibitor concentration, and IC_{50} was derived from exponential fit of the curve. K_i was calculated according to the Michaelis−Menten equation.

cells (*85*). We examined the phenotype of cells after the delivery of the peptide inhibitors into HeLa cells. We did not observe cell growth inhibition by peptide 2 or peptide 3 (data not shown). Studies with Akt small molecule inhibitors have demonstrated that a minimum of 16 h of Akt inhibition is required for the maximal effect on cell growth inhibition (Y. Luo, unpublished results). The peptide inhibitors may not be stable long enough to induce growth inhibition. Indeed, when we examined the GSK3 phosphorylation at 4 h post delivery, no reduction was observed by peptide 2. This suggests that peptide 2 is not stable after 4 h in cells (Figure 6A).

To achieve a continuous delivery of the peptide inhibitors into cells, we used poly(D-arginine) as a membrane translocation peptide (MTP) to facilitate the cell takeup of the peptide inhibitors. Fusion peptides containing a fluorescent tag (Fl), poly-D-arginine (dr), and each peptide inhibitor sequence were synthesized. Fluorescence microscopic examination indicated that more than 90% of HeLa cells (Figure 6B) or MiaPaCa cells (data not shown) took up the fusion peptides. A dose-dependent inhibition on the GSK3 phosphorylation was also observed with Fl-dr-peptide 2, while the negative control Fl-dr-peptide 5 had no effect (Figure 6C). In addition, significant growth inhibition was observed with Fl-dr-peptide 2 in both HeLa and MiaPaCa cells, while no growth inhibition was induced by Fl-dr-peptide 5 (Figure 7). This is consistent with the fact that continuous incubation of cells with Fl-dr-peptide 2 would allow a partial but persistent Akt inhibition, which results in partial growth inhibition.

DISCUSSION

We have shown that, by increasing the length of the suboptimal, 11 amino acid substrate to 17−20 amino acids with the appropriate sequence, we can increase the affinity of the peptide for Akt dramatically. This may be explained by the increased amino acid contacts between the longer peptides and Akt in the molecular model. When delivered continuously into cells, peptide 2 can inhibit Akt within cells. The Akt peptide inhibitors, peptides 1, 2, and 4, are very specific. As expected for pseudosubstrate peptide inhibitors, these peptide inhibitors do not inhibit the kinases in the AGC family (p70S6K, p90S6K, PKA, and PKC isoforms) to which Akt belongs. Taken together with the fact that Fl-dr-peptide 2 inhibits Akt function in cells, we conclude that the cell growth inhibition we observed was indeed due to the specific Akt inhibition by Fl-dr-peptide 2.

Most small molecule kinase inhibitors are competitive with ATP. They bind to the ATP-binding pocket that is common to all kinases. While significant differences may exist between ATP-binding sites of different kinases, allowing for compounds to be selective, kinases that belong to the same family often have similar ATP-binding sites (e.g., there are no differences between the ATP site residues of Akt and p70S6K). Furthermore, ATP-competitive kinase inhibitors often cross-react with distantly related kinases, and these

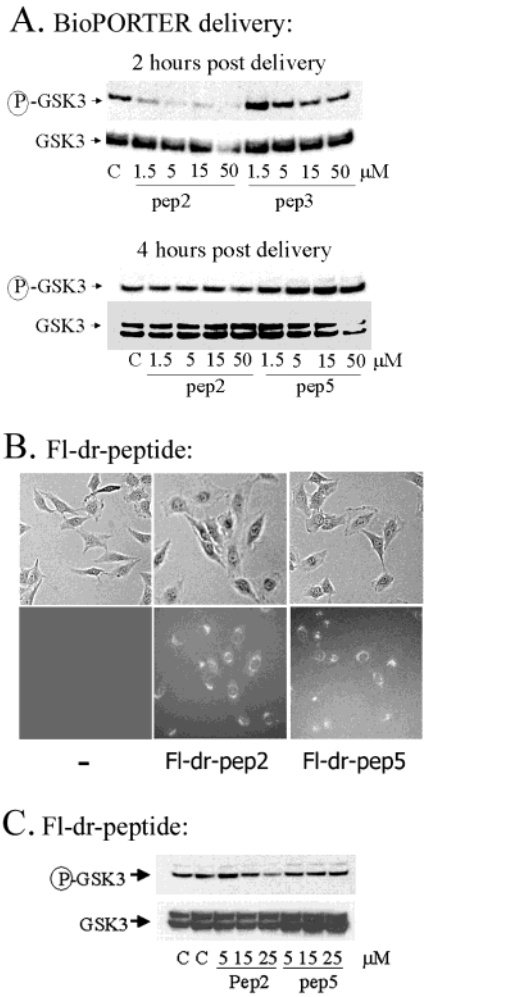


FIGURE 6: Akt peptide inhibitors inhibit Akt inside cells. (A) Peptide inhibitors as indicated in each lane were delivered into HeLa cells via BioPORTER agents. Two hours or 4 h post delivery, cell lysates were prepared and analyzed by immunoblotting using the indicated antibodies. (B) HeLa cells were incubated with 25 μM Fl-dr-peptide 2 or Fl-dr-peptide 5 for 5 h. The cells were examined using a fluorescent microscope. The top panel shows the bright fields, and the bottom panel shows the fluorescent fields. (C) HeLa cells were incubated with Fl-dr-peptides as indicated. Three hours after the incubation, cell lysates were prepared and analyzed by immunoblotting using the indicated antibodies. In both (A) and (C), the upper panels show GSK3 phosphorylation. The lower panels show total GSK3 protein levels.

interactions cannot be predicted by primary sequence (*86*). Thus, it is difficult to identify a specific small molecule inhibitor that only inhibits the target kinase. Indeed, commonly used kinase inhibitors, such as H89 and LY294002, inhibit several other kinases in addition to their target kinases (*87*). A peptide inhibitor, on the other hand, binds to a protein substrate-binding pocket that differs from kinase to kinase. The unique binding interaction provides a high degree of specificity to the target kinase. This was demonstrated with these Akt peptide inhibitors.

Much of the interrogation of Akt function relies on experiments that overexpress constitutively active Akt or, alternatively, knock out Akt function using genetic manipulation or dominant negative Akts. While valuable, these studies are often inadequate to accurately predict the result of pharmacological inhibition of Akt. The main reason for this inadequacy is the inability to inhibit the three cellular

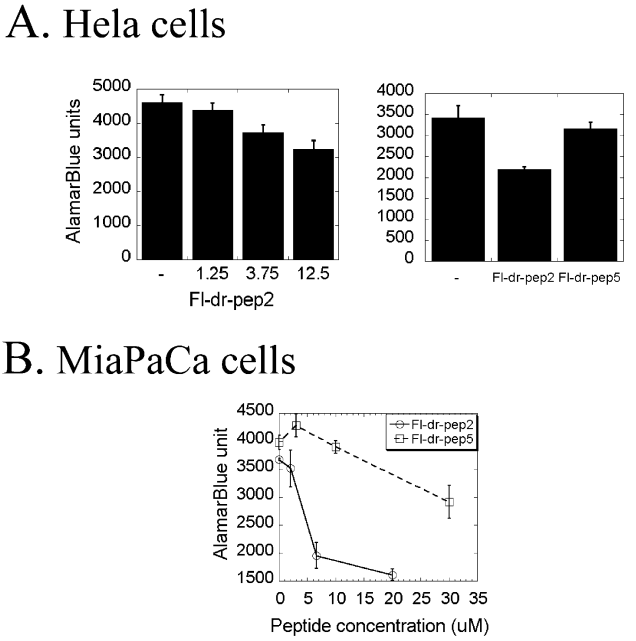


FIGURE 7: Akt peptide inhibitor 2 inhibits cell growth. Cells were plated on 96-well plates with 7500 cells/well for MiaPaCa-2 cells or 5000 cells/well for HeLa cells 20 h before the peptide incubation. The cells were incubated with either Fl-dr-peptide 2 or Fl-dr-peptide 5 at the indicated concentration for 48 h. The AlamarBlue assay was carried according to the vendor's instruction.

Akts simultaneously. Use of small molecule inhibitors to examine the phenotype of Akt inhibition suffers, as do many kinase inhibition studies, from a lack of very selective inhibitors. With the newly developed peptide−protein delivery technology, peptide inhibitors become powerful tools to dissect the function of the target kinase within cells. This is particularly useful in the case of Akt, where partially redundant multiple isoforms are present in nature and share the same substrates. These peptide inhibitors will be used to further refine our understanding of the phenotype resulting from the pharmacological manipulation of Akt.

ACKNOWLEDGMENT

We thank Leo Barrett for synthesizing peptides and Tilman Oltersdorf and Ron deJong (IDUN Pharmaceutical Inc.) for baculoviruses that encode Akt1 and PDK1.

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BI034515P