Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors

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We developed a high-throughput HTRF (homogeneous time-resolved fluorescence) assay for Akt kinase activity and screened approx. 270000 compounds for their ability to inhibit the three isoforms of Akt. Two Akt inhibitors were identified that exhibited isoenzyme specificity. The first compound (Akt-I-1) inhibited only Akt1 (IC_{50} 4.6 μM) while the second compound (Akt-I-1,2) inhibited both Akt1 and Akt2 with IC_{50} values of 2.7 and 21 μM respectively. Neither compound inhibited Akt3 nor mutants lacking the PH (pleckstrin homology) domain at concentrations up to 250 μM. These compounds were reversible inhibitors, and exhibited a linear mixed-type inhibition against ATP and peptide substrate. In addition to inhibiting kinase activity of individual Akt isoforms, both inhibitors blocked the phosphorylation and activation of the corresponding Akt isoforms by PDK1 (phosphoinositide-dependent kinase 1). A model is proposed in which these inhibitors bind to a site formed only in the presence of the PH domain. Binding of the inhibitor is postulated to promote the formation of an inactive conformation. In support of this model, antibodies to the Akt PH domain or hinge region blocked the inhibition of Akt by Akt-I-1 and Akt-I-1,2. These inhibitors were found to be cell-active and to block phosphorylation of Akt at Thr^{308} and Ser^{473}, reduce the levels of active Akt in cells, block the phosphorylation of known Akt substrates and promote TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand)-induced apoptosis in LNCap prostate cancer cells.

Key words: Akt, allosteric inhibitor, inhibitor, kinase, pleckstrin homology domain, protein kinase B (PKB).

INTRODUCTION

Akt/PKB (protein kinase B) is a serine/threonine kinase which has a key role in the regulation of survival and proliferation [1–8]. There are three isoforms of human Akt (Akt1, Akt2 and Akt3) and they all have an N-terminal PH (pleckstrin homology) domain and a kinase domain separated by a 39-amino-acid hinge region. The PH domains have approx. 60 % identity and the kinase domains are > 85 % identical [9]. The hinge region is the least conserved at approx. 28 % identity. The Akt active-site residues, described in a recent report on the crystal structure of Akt2 containing an ATP analogue and a peptide substrate [10], are the same in all three isoenzymes. Based on the high degree of homology between the AGC protein kinase family members, the identification of specific active-site inhibitors has been predicted to be difficult. The identification of Akt isoenzyme-specific inhibitors seemed to be an even greater challenge.

Activation of the Akt survival kinase cascade [7] can begin with the stimulation of a cell-surface receptor, followed by the recruitment of PI3K (phosphoinositide 3-kinase) to the plasma membrane. Once localized to the plasma membrane, PI3K phosphorylates PtdIns(4,5)P_2 to generate the second messenger PtdIns(3,4,5)P_3. The dual-specificity phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) modulates the activation state of the pathway by removing the 3′-hydroxyl phosphate from PtdIns(3,4,5)P_3. Akt and its upstream activating kinase PDK1 (phosphoinositide-dependent kinase 1) are localized to the plasma membrane through protein–protein interactions and the binding of their PH domains to PtdIns-(3,4,5)P_3. Following co-localization, PDK1 activates Akt1 by phosphorylating Thr^{308} on the activation loop. Phosphorylation of Akt1 at Ser^{473} by a putative PDK2 also contributes to activation, by stabilizing the active conformation of Akt [11].

In the absence of a PtdIns(3,4,5)P_3-containing lipid bilayer, Akt exists in a 'closed' conformation and cannot be phosphorylated by PDK1. The PH domain is thought to occlude the activation loop, including Thr^{308}, and block access of PDK1. If the PH domain is deleted, Akt can be phosphorylated by PDK1 in the absence of PtdIns(3,4,5)P_3-containing membranes [12,13]. A crystal structure of full-length Akt has yet to be reported, but an Akt2 kinase domain structure has been determined [11]. This protein adopts an inactive conformation characterized by a 20° rotation of the N and C lobes of the kinase domain relative to each other. Another characteristic of this inactive conformation is that Phe^{294} of the DFG (Asp-Phe-Gly) motif points into the ATP-binding pocket. This misalignment of the lobes and redirection of Phe^{294} were predicted to block binding of peptide substrate and ATP.

Abbreviations used: cdk2, cyclin-dependent kinase 2; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; FGFR, fibroblast growth factor receptor; FLT, Fms-related tyrosine kinase; GSK3, glycogen synthase kinase 3; HTRF, homogeneous time-resolved fluorescence; MAPK, mitogen-activated protein kinase; MAPKK1, MAPK kinase 1; MEK, MAPK/ERK kinase; PDK, phosphoinositide-dependent kinase; PEG, poly(ethylene glycol); PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SGK, serum- and glucocorticoid-inducible kinase; SH, Src homology; TCL1, T-cell leukaemia/lymphoma 1; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand.

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Akt activation is likely to play a critical role in tumorigenesis, as indicated by the many different ways and the frequency with which this can occur. Activation can occur when production of growth factors is elevated or when growth factor receptors are overexpressed or mutationally activated. Activation also occurs when functional PTEN levels are reduced through mutation or deletion or when PI3K p110α, Akt1 or Akt2 are overexpressed or activated. PTEN mutations leading to activation of Akt are common in human cancers [14–16], and are most frequently observed in mammary carcinomas, melanomas, and endometrial, prostate and thyroid tumours. PTEN germline mutations are also associated with a number of cancer syndromes, such as Cowden's disease [17,18], and Bannayan–Riley–Ruvalcaba [19], Proteus [20] and Lhermitte–Duclos syndromes [21].

Because of the strong evidence that activation of the Akt cell-survival pathway is important in the generation and maintenance of the tumour phenotype, we hypothesized that inhibitors of Akt might induce apoptosis alone or in combination with standard cancer chemotherapeutics. To test this hypothesis, we screened our compound collection for Akt inhibitors. Two isoenzyme-specific Akt inhibitors were identified and were shown to require an intact kinase and PH domain for inhibition.

EXPERIMENTAL

Enzyme cloning

The human PDK1 gene was amplified by PCR from human liver cDNA (Clontech) using the 5′ primer, 5′-GCTGGGTACCATGGAATACATGCCGATGGAAGCCAGGACCACCAGCCAG-3′, and the 3′ primer, 5′-GTGAGAATTCTCACTGCACAGCGGCGTC-3′. The 5′ primer contained a *Kpn*I site and a middle T tag [22] and the 3′ primer contained an *Eco*RI site and stop codon. The resultant PCR product was cloned into pBlueBac4.5.

The human Akt1 gene was amplified by PCR from a human spleen cDNA (Clontech) using the 5′ primer, 5′-CGCGAATTCAGATCTACCATGAGCGACGTGGCTATTGTG-3′, and the 3′ primer, 5′-CGCTCTAGAGGATCCTCAGGCCGTGCTGCTGGC-3′. The 5′ primer included an *Eco*RI and a *Bgl*II site. The 3′ primer included an *Xba*I and a *Bam*HI site for cloning purposes. The resultant PCR product was subcloned into pGEM3Z (Promega) as an *Eco*RI/*Xba*I fragment. For expression and purification purposes, a middle T tag was added to the 5′ end of the full-length Akt1 gene using the PCR primer, 5′-GTACGATGCTGAACGATATCTTCG-3′. The resulting PCR product encompassed a 5′ *Kpn*I site and a 3′ *Bam*HI site which were used to subclone the fragment in-frame with a biotin tag containing insect cell expression vector, pS2neo (A.T.C.C., Manassas, VA, U.S.A.).

ΔPH-Akt1 was constructed by deleting amino acids 4–129 from Akt1 by PCR deletion mutagenesis using the full-length Akt1 gene in the pS2neo vector as a template. The PCR was carried out in two steps using overlapping internal primers 5′-GAATACATGCCGATGGAAAGCGACGGGGCTGAAGAGATGGAGGTG-3′ and 5′-CCCCTCCATCTCTTCAGCCCCGTCGCTTTCCATCGGCATGTATTC-3′ which encompassed the deletion, and 5′ and 3′ flanking primers which encompassed the *Kpn*I site and middle T tag on the 5′ end. The final PCR product was digested with *Kpn*I and *Sma*I and was ligated into the appropriately prepared pS2neo vector, effectively replacing the 5′ end of the clone with the deleted version.

The human Akt2 gene was amplified by PCR from human thymus cDNA (Clontech) using the N-terminal oligonucleotide primer (5′-AAGCTTAGATCTACCATGAATGAGGTGTCTGTC-3′) and the C-terminal oligonucleotide primer (5′-GAATTCGGATCCTCACTCGCGGATGCTGGC-3′). These primers included a 5′ *Hin*dIII/*Bgl*II site and a 3′ *Eco*RI/*Bam*HI site for cloning purposes. The resultant PCR product was subcloned into the *Hin*dIII/*Eco*RI sites of pGem3Z (Promega). For purification purposes, a middle T tag was added to the 5′ end of the full-length Akt2 using the PCR primer, 5′-GGTACCATGGAATACATGCCGATGGAAAATGAGGTGTCTGTCATCAAAG-3′. The resultant PCR product was subcloned into the PS2neo vector as described above.

ΔPH-Akt2 (deletion of amino acids 4–131) was constructed by PCR deletion mutagenesis using the full-length Akt2 gene in the pGEM3Z vector as template. PCR was performed using the 5′ oligonucleotide primer, 5′-GGTACCATGGAATACATGCCGATGGAAAATGAGACGACTGAGGAGATGGAAGTGGC-3′, that encompassed the deletion and included a *Kpn*I site and the middle T tag. The 3′ PCR primer, 3′-GCCAGCATCCGCGAGTGAGGATCCGAATTC-5′, included a 3′ *Bam*HI and a *Eco*RI site. The resultant PCR product was digested with *Kpn*I and *Sma*I and the 5′ 350 bp fragment was isolated. PS2neo/Akt2 was then digested with *Kpn*I and *Sma*I to generate the vector plus the 3′ end of the Akt2 gene. The PCR fragment was ligated into this, creating PS2neo/mtAkt2ΔPH.

The human Akt3 gene was amplified by PCR of adult brain cDNA (Clontech) using the N-terminal oligonucleotide primer, 5′-GAATTCAGATCTACCATGAGCGATGTTACCATTGTG-3′ and the C-terminal oligonucleotide primer, 5′-TCTAGATCTTATTCTCGTCCACTTGCAGAG-3′. These primers included a 5′ *Eco*RI/*Bgl*II site and a 3′ *Xba*I/*Bgl*II site for cloning purposes. The resultant PCR product was cloned into the *Eco*RI and *Xba*I sites of pGEM4Z (Promega). For purification purposes, a middle T tag was added to the 5′ end of the full-length Akt3 clone using the PCR primer, 5′-GGTACCATGGAATACATGCCGATGGAAAGCGATGTTACCATTGTGAAG-3′. The resultant PCR product encompassed a 5′ *Kpn*I site which allowed in-frame cloning with the insect cell expression vector, pS2neo.

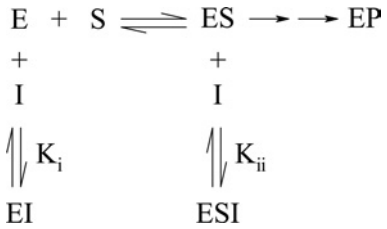
Akt proteins were expressed with a biotinylation tag for use as PDK1 substrates in an HTRF (homogeneous time-resolved fluorescence) assay (see below). The following oligonucleotides were used to insert the biotinylation tag into the *Kpn*I site of pS2neo (A.T.C.C.): 5′-GTACGATGCTGAACGATATCTTCGAAGCCCAGAAGATCGAGTGGCACG-3′ and 5′-GTACCGTGCCACTCGATCTTCTGGGCTTCGAAGATATCGTTCAGCATC-3′. The Akt genes were cloned into the remaining *Kpn*I site of the resulting vector. Both strands of all DNA constructs described were sequenced.

Enzyme expression and purification

The DNA containing the cloned Akt1, Akt2, Akt3 and ΔPH-Akt1 genes in the pS2neo expression vector was purified and used to transfect *Drosophila* S2 cells (A.T.C.C.) by the calcium phosphate method. Pools of antibiotic (G418, 500 μg/ml)-resistant cells were selected. Cell cultures were expanded to a 1.0 litre volume (approx. 7.0×10^6 per ml), and biotin and $CuSO_4$ were added to a final concentration of 50 μM and 500 μM respectively. Cells were grown for 72 h at 27 °C and were harvested by centrifugation at 500 ***g*** for 10 min. ΔPH-Akt2 and PDK1 were cloned into pBlueBac (Invitrogen) and expressed in Sf9 cells, according to the manufacturer's instructions. The cell paste was frozen at − 70 °C until needed.

Cell paste from 1 litre of S2 or Sf9 cells was lysed by sonication in 50 ml of buffer A {50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], 10 μg/ml benzamidine, 5 μg/ml each of leupeptin, aprotinin and pepstatin, 10 % (v/v) glycerol and 1 mM DTT (dithiothreitol)}. The soluble fraction was purified

on a Protein-G–Sepharose fast-flow (Amersham Biosciences) column loaded with 9 mg/ml anti-(middle T) monoclonal antibody and eluted with 75 μM EYMPME (Glu-Tyr-Met-Pro-Met-Glu) peptide in buffer A containing 25 % (v/v) glycerol [22]. Akt-containing fractions were pooled and the protein purity was estimated to be approx. 95 % by SDS/PAGE. The protein was biotinylated quantitatively as judged by binding to streptavidin–agarose. The purified protein was quantified using a standard Bradford protocol [22a] and then flash-frozen in liquid nitrogen and stored at − 70 °C.



Scheme 1

Akt activation

Lipid vesicles were prepared from PtdIns(3,4,5)P_3, dipalmitoyl (Matreya; 1775-1) and a synthetic phospholipid blend of DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids; 790595). A 1.0 mg/ml solution of PtdIns(3,4,5)P_3 and a 16.7 mg/ml suspension of DOPC/DOPS in 10 mM Hepes (pH 7.4) were prepared immediately before use. The DOPS/DOPC suspension (800 μl) and the PtdIns(3,4,5)P_3 solution (964 μl) were mixed and then diluted in 18.2 ml of 10 mM Hepes, pH 7.4, and mixed thoroughly. This mixture [400 μM DOPS, 400 μM DOPC and 40 μM PtdIns(3,4,5)P_3] was subjected to five freeze–thaw cycles using liquid nitrogen and a 50 °C water bath, and aliquots were stored at − 20 °C. Lipid vesicles prepared and stored this way maintained reproducible activation properties.

The Akt isoforms and deletion mutants were activated by PDK1 *in vitro* under the following reaction conditions: 1.0 μM Akt, 40 nM PDK1, 1× lipid vesicles (described above), 50 mM Tris/HCl, pH 7.4, 1.0 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 μM PKA (protein kinase A) Inhibitor Peptide (UBI), 1.0 μM microcystin LR, 0.1 mM ATP, 10 mM $MgCl_2$ and 0.325 mg/ml BSA. The final volume was 2.4 ml, and incubation was allowed to proceed at room temperature (22 °C) for 3.0 h, when it was stopped by the addition of 0.1 ml of 0.5 M EDTA. These activation conditions resulted in complete phosphorylation of Thr[308] and some phosphorylation of Ser[473]. Aliquots of the activated Akt protein constructs were frozen in liquid nitrogen and were stored at −70 °C.

Kinase assays

Kinase activity was measured in a homogeneous assay in a 96-well format. Detection was performed by HTRF using an EuK-labelled anti-phospho(S21)–GSK3 (glycogen synthase kinase 3) α antibody (New England Biosciences) and streptavidin-linked XL665 fluorophore which bound to the biotin moiety on the substrate peptide (biotin–GGRARTSSFAEPG) [23]. Final reaction conditions were 50 mM Hepes, pH 7.5, 0.1 % (v/v) PEG [poly-(ethylene glycol)], 0.1 mM EDTA, 0.1 mM EGTA, 0.1 % (w/v) BSA, 2 mM β-glycerol phosphate, 0.5 μM substrate peptide, 150 μM ATP, 10 mM $MgCl_2$, 50 mM KCl, 5 % (v/v) glycerol, 1 mM DTT, 2.5 % (v/v) DMSO, 10 μg/ml benzamidine, 5 μg/ml each of pepstatin, leupeptin and aprotinin, 5 μM test compound and 45–200 pM activated enzyme in a 40 μl volume. The reaction was started with the addition of enzyme.

We also employed a standard [γ-^{33}P]ATP kinase assay which was used for the mechanism of inhibition studies. Buffer conditions were the same for the two assays. Enzyme concentrations varied from 5 to 50 nM, depending on the isoenzyme, and ATP concentrations were 150 μM for IC_{50} determinations and 300 μM for the peptide competition experiments. The GSK3 substrate peptide was used at 10 μM for the IC_{50} determinations and 30 μM for the ATP competition experiments. Reactions were stopped by acidification, radiolabelled product was collected on Whatman P81 96-well filter plates (Polyfiltronics; 7700-3312), washed nine times with 200 μl of 0.75 % H_3PO_4 and twice with water, and the plates were dried. A volume of 30 μl of high-capacity scintillation fluid (Packard Microscint 20) was added, and the phosphorylated substrate was quantified on a Packard TopCount. Alternatively, radiolabelled product was detected using Streptavidin FlashPlate® PLUS (NEN Life Sciences; SMP103). In this case, the EDTA-stopped reactions were transferred to the FlashPlate and placed on a plate shaker for 10 min. Contents of the wells were then removed, and each well was rinsed twice with TBS (Tris-buffered saline). An additional three washes were conducted over the course of 15 min, and then the plates were quantified in a Packard TopCount.

The mechanism of inhibition of Akt inhibitors was determined at a fixed concentration of ATP (300 μM) or peptide (30 μM), while the concentration of the second substrate was varied. The entire raw data set was simultaneously fitted with the following equation for linear mixed-type inhibition using SigmaPlot software:

$$v = \frac{V_{max} \cdot [S]}{K_s \cdot \left(1 + \frac{[I]}{K_i}\right) + [S] \cdot \left(1 + \frac{[I]}{K_{ii}}\right)} \quad (1)$$

where I is the inhibitor, S is the varied substrate, K_i and K_{ii} are the binding constants for the interaction of I with the enzyme (E) and with the ES complex respectively. K_i and K_{ii} are defined with respect to the varied substrate S according to Scheme 1. For a substrate competitive inhibitor, we expect that $K_{ii} \gg K_i$, whereas the reverse would be true for an uncompetitive inhibitor.

IC_{50} values for PDK1 phosphorylation of Akt isoenzymes were measured in an HTRF format similar to that described above for Akt. The antibody used to detect product was an anti-(phospho-Akt Thr[308]) (Cell Signaling Technology; catalogue no. 9275, lot 2) labelled with Lance Eu-W1024 (Wallac; #AD0096) at 12:1 molar ratio in sodium carbonate buffer, pH 9.5, for 16 h at 4 °C. The mixture was desalted into Tris/HCl, pH 7.8, and 150 mM NaCl containing 0.05 % sodium azide. The HTRF kinase reactions contained 200 nM biotinylated Akt1, Akt2 or ΔPH-Akt1, 0.5 nM PDK1 and 10 μM PtdIns(3,4,5)P_3, 100 μM DOPC and 100 μM DOPS in vesicle form as described above. The assay buffer used and the subsequent HTRF detection conditions were the same as those described for PDK1 activation of Akt. Reactions were incubated for 12 min at room temperature.

The anti-(phospho-Akt Thr[308]) antibody recognized phosphorylated Akt3 very poorly in Western blots and in the HTRF format. This required us to develop a 'coupled' assay for measuring PDK1 phosphorylation of Akt3. PDK1 was assayed as described above using Akt3 as a substrate (190 nM). This part

of the assay was incubated at room temperature for 40 min. The reaction was stopped by adjusting the EDTA concentration to 30 mM, and then the entire reaction mixture was diluted 1:1000 in assay buffer without EDTA. The diluted reaction containing 2 nM Akt3 was then assayed for Akt activity in the standard HTRF assay using the GSK3-derived peptide substrate as described above.

Antibody effects on Akt inhibitor activities

Akt1 and Akt2 IC_{50} values for inhibitors were determined using the HTRF assay in the presence of anti-Akt antibodies or control antibodies. Two anti-Akt antibodies were tested. Akt2Hinge is a rabbit polyclonal antibody produced to a 38-amino-acid peptide corresponding to the hinge region between the PH domain and the kinase domain of Akt2. Akt1PH (Upstate Biotechnology; catalogue no. 06-608, lot 18 766) is a sheep polyclonal antibody made against amino acids 1–149 of Akt1. Antibodies against unrelated proteins (GSK3 and Rab6) were used as controls. The anti-GSK3 antibody (Upstate Biotechnology; catalogue no. 06-391, lot 21 204) is a sheep polyclonal antibody against a peptide corresponding to amino acids 471–483 of GSK3ι and Rab6 (Santa Cruz Biotechnology; catalogue no. Sc-310, lot I297) is a rabbit polyclonal against the C-terminal domain of human Rab6. Akt1 or Akt2 was incubated with antibody (50–100 μg/ml) on ice for 3 h, and then diluted into kinase assay buffer. Final antibody concentrations ranged from 200 to 400 ng/ml and the Akt concentration was 200 nM.

Cell-based assays for inhibition of Akt phosphorylation

LNCaP (prostate cancer) and C33A (cervical carcinoma) cells were plated in 10-cm-diameter dishes, and when they reached 70–80 % confluence, the medium [RPMI 1640/10 % (v/v) FBS (foetal bovine serum) for LNCaP; MEMα (minimal essential medium α)/10 % FBS for C33A; both from Gibco] was exchanged, and the test compound was added. Incubation of both cell lines with the inhibitors for 1 h was sufficient to achieve maximum inhibition of Akt phosphorylation. Incubation with inhibitor for longer periods was required to see maximum inhibition of downstream events or caspase induction. Following inhibitor treatment, the medium was removed and the cells were washed gently with PBS. The washed cells were scraped from the plates, pelleted and washed again with PBS. The cell pellet was resuspended in lysis buffer [20 mM Tris/HCl, pH 8, 140 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 1 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM NaF, 0.5 mM Na_3VO_4, 1 μM microcystin LR, and 1× P8340 protease inhibitor cocktail (Sigma)], placed on ice for 15 min and gently vortex-mixed. Insoluble material was sedimented (100 000 ***g***, 4 °C, 20 min), and the supernatant was stored at − 70 °C until use.

The lysate supernatant was immunoprecipitated as follows. For anti-Akt1 immunoprecipitates, lysates were mixed with Santa Cruz Biotechnology Sc-7126 (D-17) in NETN buffer [100 mM NaCl, 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5 % (v/v) Nonidet P40] and Protein-A/G–agarose (Santa Cruz Biotechnology, Sc-2003) was added, for anti-Akt-2, lysates were mixed in NETN buffer with anti-Akt-2–agarose (Upstate Biotechnology; 16-174), and for anti-Akt-3, lysates were mixed in NETN buffer with anti-Akt-3–agarose (Upstate Biotechnology; 16-175). The immunoprecipitates were incubated overnight at 4 °C and washed, and the proteins were separated by SDS/PAGE.

Western blots were performed using the following antibodies: anti-Akt (Cell Signaling Technology), anti-(Phospho-Akt Ser^{473}) (Cell Signaling Technology), anti-(Phospho-Akt Thr^{308}) (Cell Signaling Technology), anti-Bad (Santa Cruz Biotechnology), anti-$p27^{Kip1}$ (Upstate Biotechnology) and anti-$p21^{Cip1/Waf1}$ (BD Pharmingen). After incubating with the appropriate primary antibody diluted in PBS + 0.5 % (w/v) non-fat dried milk at 4 °C overnight, blots were washed, incubated with horseradish-peroxidase-tagged secondary antibody in PBS + 0.5 % (w/v) non-fat dried milk for 1 h at room temperature. Proteins were detected with ECL® (enhanced chemiluminescence) reagent (Amersham Biosciences; RPN2134).

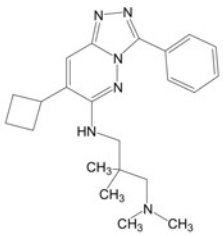
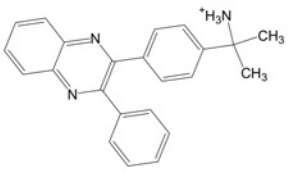
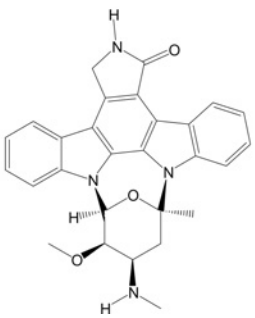
Immunoprecipitated Akt kinase assays

The activity of the three Akt isoenzymes obtained from drug-treated C33A cells was determined in a 96-well-plate-based HTRF assay in which an individual Akt isoenzyme was captured with an antibody. A Reacti-bind Protein-A-coated clear strip plate (Pierce; catalogue no. 15 132 CH51416) was prepared by coating each well with isoenzyme-specific antibodies. The isoenzyme-specific antibodies were produced using antigens composed of fragments of Akt (Akt1, amino acids 113–151; Akt2, amino acids 102–156 and Akt3, amino acids 101–152) fused to glutathione S-transferase, expressed in *Escherichia coli* and purified on glutathione resin. The antibodies were raised in rabbits and were affinity-purified. Antibodies were allowed to bind for 2 h at 4 °C with gentle shaking and then the plates were washed twice with lysis buffer (Tris/HCl, pH 7.4, 150 mM NaCl, and 0.1 % Tween 20). Lysates from C33A cells treated with inhibitor were prepared as follows: each well of a 96-well plate (Corning Costar; #3598) was seeded with 6.0×10^4 C33A cells and the following day, dilutions of inhibitors in medium were added. After incubation with the inhibitors for 5 h, 100 μl of lysis buffer containing 1× phosphatase inhibitor cocktail I (Sigma; catalogue no. P2850) and II (Sigma; catalogue no. P5726), and protease inhibitor cocktail (Sigma; catalogue no. P8340) were added to each well, the plate was frozen at − 70 °C and then thawed on ice. These lysates were transferred to the antibody-coated plates, and the plates were incubated overnight at 4 °C with shaking. The plate was then washed twice with lysis buffer and twice with assay buffer [50 mM Hepes, pH 7.5, 1 % (v/v) PEG-6000, 1.0 mM EDTA, 1.0 mM EGTA, 20 mM β-glycerophosphate, 50 mM KCl, 150 μM ATP, 10 mM $MgCl_2$, 5 % (v/v) glycerol, 1 mM DTT, 0.75× protease inhibitor cocktail and 0.1 % BSA], followed by incubation in assay buffer and GSK3 peptide. HTRF detection of product was as described above.

Caspase 3 assays

LNCaP cells (1×10^4 cells per well) were seeded in a 96-well plate and maintained for 72 h before testing. Inhibitor stocks were prepared in DMSO, and final DMSO concentrations were 0.1 %. The final inhibitor concentrations were 25 μM for both Akt-I-1 and Akt-I-1,2, and 15 μM for LY294002. TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand)/Apo2L (Research Diagnostics) was used at 0.5 μg/ml. Cells were pre-incubated with drugs for 1.5 h before the addition of TRAIL, and incubations continued for another 4 h. Plates were centrifuged at 500 ***g*** for 10 min, the media were removed and 50 μl of lysis buffer (ApoAlert Caspase-3 fluorescent assay kit; Clontech) added to each well. Plates were incubated at 4 °C for 20 min and then frozen at − 70 °C for 1 h. Plates were thawed at room temperature, the caspase 3 substrate added (50 μM final concentration) and then incubated for 6–18 h at 37 °C. Product formation was quantified by measuring the absorbance at 400 and 505 nm.

Table 1 Inhibition of Akt isoenzymes and PH domain deletion constructs

Identifier	Structure	IC_{50} (μM)					
		Akt1	ΔPH-Akt1	Akt2	ΔPH-Akt2	Akt3	SGK
Akt-I-1		4.6	>250	>250	>250	>250	>250
Akt-I-1,2		2.7	>250	21.0	> 250	>250	>250
Staurosporine		0.12	0.029	0.10	0.018	0.057	0.57

RESULTS

Identification of PH-domain-dependent and isoenzyme-specific Akt inhibitors

We developed an Akt kinase assay in which a phosphorylated biotin-peptide product was detected by HTRF using an EuK-coupled antibody specific for the phosphopeptide and streptavidin-linked XL665 fluorophore [23]. Approx. 270000 compounds were screened for their ability to inhibit Akt1, Akt2 or Akt3. Most of the inhibitors identified were members of structural classes known for ATP-competitive inhibitors, such as staurosporine, and tri-substituted imidazoles and pyrroles [24]. Much to our surprise, several compounds were discovered to have very unique and unpredicted properties, and two compounds were characterized further (Table 1). Akt-I-1 only inhibited Akt1, and Akt-I-1,2 inhibited both Akt1 and Akt2. Even high concentrations (250 μM) of Akt-I-1 and Akt-I-1,2 did not inhibit Akt3. In addition, they did not inhibit forms of Akt1 and Akt2 that lacked the PH domain (ΔPH-Akt1 and ΔPH-Akt2). Because the kinase domains of the three Akt isoforms are highly homologous (~85%) and the active-site residues are identical, these data suggest that Akt-I-1 and Akt-I-1,2 inhibit by direct interactions with the PH domain and/or binding to a site that is only formed in the presence of the PH domain.

We also screened a panel of 121 kinase inhibitors obtained from Calbiochem (list available upon request from S. F. Barnett) for inhibition of Akt1. The following compounds exhibited greater than 50% inhibition at 50 μM: bisindolylmaleimide III (66%), K252a (93%), K252b (97%), PD 169316 (65%), Ro-31-8425 (58%), Ro-31-8220 (89%), SB 202190 (60%), staurosporine (97%) and H-89 (85%). IC_{50} values were determined for staurosporine and Ro-31-8220 (Tables 1 and 2).

Table 2 Effect of PH-domain- and hinge-specific antibodies on inhibitor IC_{50} values

Rab6 and GSK3 are control antibodies that do not bind to Akt. Hinge and Akt PH domain refer to antibodies which bind the Akt2 hinge and PH domains respectively. n/a, not applicable.

	Akt1 IC_{50} (μM)			Akt2 IC_{50} (μM)				
Antibody	None	GSK3*	Akt1 PH domain	None	Rab6	GSK3	Hinge	Akt1 PH domain
Akt-I-1	2.0	1.7	>50	n/a	n/a	n/a	n/a	n/a
Akt-I-1,2	3.4	2.8	>50	11	13	9	>50	>50
Staurosporine	0.08	0.09	0.07	0.07	0.07	0.07	0.04	0.07
Ro-31-8220	5.2	7.0	3.1	3.7	4.0	4.6	2.7	5.3

Akt-I-1 and Akt-I-1,2 are specific inhibitors of Akt

Akt-I-1 and Akt-I-1,2 were assayed for their ability to inhibit a number of tyrosine and serine/threonine kinases. The kinases tested and their GenBank® accession numbers were as follows: PDK1 (AF017995), IGFR (insulin-like growth factor receptor) kinase (X04434), KDR (kinase insert domain protein receptor; vascular endothelial growth factor receptor 2)

Table 3 Kinetic parameters for Akt-I-1 and Akt-I-1,2 competition were determined using a linear mixed-type inhibition model

Kinase	Inhibitor	Substrate	$K_i \pm$ S.E.M. (μM)	$K_{ii} \pm$ S.E.M. (μM)	Mechanism
Akt1	Akt-I-1	ATP	2.9 ± 1.2	8.2 ± 3.9	Mixed-type
	Akt-I-1	Peptide	3.7 ± 0.72	4.8 ± 2.2	Mixed-type
	Akt-I-1,2	ATP	0.99 ± 0.16	5.9 ± 2.0	Mixed-type
	Akt-I-1,2	Peptide	7.6 ± 5.5	2.0 ± 0.71	Mixed-type
	Staurosporine	ATP	0.055 ± 0.001	$(2.2 \pm 0.2) \times 10^9$	Competitive
	Staurosporine	Peptide	0.112 ± 0.008	0.085 ± 0.046	Mixed-type
ΔPH-Akt1	Staurosporine	ATP	0.0074 ± 0.0015	$> 6.8 \times 10^8$	Competitive
	Staurosporine	Peptide	0.044 ± 0.019	0.054 ± 0.017	Mixed-type
Akt2	Akt-I-1,2	ATP	14 ± 0.58	46 ± 6.4	Mixed-type
	Akt-I-1,2	Peptide	26 ± 7.1	17 ± 2.4	Mixed-type
	Staurosporine	ATP	0.050 ± 0.011	2.0 ± 0.79	Competitive
	Staurosporine	Peptide	0.072 ± 0.016	0.068 ± 0.017	Mixed-type
ΔPH-Akt2	Staurosporine	ATP	0.0076 ± 0.0007	0.846 ± 0.072	Competitive
	Staurosporine	Peptide	0.033 ± 0.0084	0.024 ± 0.007	Mixed-type

(AF035121), FLT (Fms-related tyrosine kinase) -1 (X51602), FLT-4 (X69878), FGFR (fibroblast growth factor receptor) 1 (M34641), FGFR2 (M55614), Src (K03218), TIE-2 (tyrosine kinase with Ig and epidermal growth factor homology domains 2) (L06139), PDGFR (platelet-derived growth factor receptor) (J03278), p38 (L35263), PKC (protein kinase C) θ (L07032), Lck (M36881), ZAP70 (T-cell receptor zeta-chain-associated tyrosine kinase involved in T-cell development and activation) (L05148), SGK (serum- and glucocorticoid-inducible kinase) (Y10032), PKA (M34181) and PKC (X52479). IC_{50} values determined for these kinases were all greater than 40 μM. Of special note was the lack of activity against SGK and PKA. SGK has the most closely related kinase domain to Akt and shares approx. 53 % amino acid identity. Most (12/14) of the active site residues in Akt1, SGK and PKA are identical, and the two that differ are functionally similar and provide backbone interactions [11,25]. These results demonstrated that Akt-I-1 and Akt-I-1,2 were not only Akt-isoenzyme-specific and PH-domain-dependent, but were also specific for Akt with respect to other closely related kinases.

Competition with ATP and peptide

The data shown so far suggested that it was possible that these inhibitors bound outside the active site and were not competitive with ATP or peptide substrate. We first investigated the ability of these inhibitors to compete with ATP for binding. Staurosporine was included in this analysis as an example of an ATP-competitive inhibitor. The kinase activity of Akt1, Akt2, ΔPH-Akt1 and ΔPH-Akt2 was assayed at various concentrations of ATP in the presence of increasing concentrations of Akt-I-1, Akt-I-1,2 or staurosporine. A similar analysis was performed in which peptide substrate concentrations were varied as a function of inhibitor concentration. The data sets from each experiment were simultaneously fitted to a linear mixed type inhibition model (eqn 1 and Scheme 1) and the K_i, K_{ii} (Table 3) and K_m values (Table 4) were determined.

Staurosporine exhibited the expected competitive mechanism with respect to ATP for all four forms of Akt, as indicated by K_{ii} values much greater than the K_i values. K_i and K_{ii} values for staurosporine competition with peptide were similar, suggesting non-competitive binding. The inhibition pattern for Akt-I-1 and Akt-I-1,2 was significantly different from that observed with staurospoine. Akt-I-1 exhibited a pattern consistent with a mixed-type mechanism of inhibition for Akt1 with respect to both ATP and peptide, with K_i differing from K_{ii} by less than 3-fold. A similar result was obtained with Akt-I-1,2 on both Akt1 and Akt2. Overall, this analysis indicated that Akt-I-1 and Akt-I-1,2 were inhibiting Akt by a mechanism different from that of staurosporine and that they were neither ATP- nor peptide-competitive inhibitors.

Table 4 Akt K_m values for ATP and peptide substrate

Kinase	K_m ATP $\pm$ S.E.M. (μM)	K_m peptide $\pm$ S.E.M. (μM)
Akt 1	132 ± 17	3.2 ± 0.51
ΔPH-Akt1	26 ± 1.5	7.4 ± 0.1
Akt 2	254 ± 38	3.4 ± 0.47
ΔPH-Akt2	36 ± 1.0	16 ± 7.1

The K_m values for the four different forms of Akt are shown in Table 4. The ATP K_m values for the PH-domain-deleted forms of Akt1 and Akt2 were significantly less than those observed for the full-length proteins (5- and 7-fold respectively). The peptide K_ms for ΔPH-Akt1 and ΔPH-Akt2 were greater than those of the full-length proteins (2.3- and 4.7-fold respectively). These results suggest that the PH domain is interacting with the kinase domain in a way that affects ATP and peptide binding.

Effect of PH-domain-specific antibodies on Akt inhibition by Akt-I-1 and Akt-I-1,2

To explore the PH domain requirement for compound inhibition, we tested the ability of PH domain/hinge- or hinge-directed antibodies to block the activity of Akt inhibitors. Unrelated antibodies of the same type, species and concentration were included as controls. A mouse polyclonal PH domain/hinge (amino acids 1–145)-specific antibody, as well as a mouse polyclonal antibody against the hinge region of Akt2, were evaluated for their ability to change the IC_{50} values of Akt-I-1 and Akt-I-1,2. The PH-domain-directed antibody completely blocked inhibition of Akt1 by both compounds, and the Akt2 hinge-specific antibody blocked the inhibition of Akt2 by Akt-I-1,2 (Table 2), but had no effect on staurosporine or Ro-31-8220 IC_{50} values. These antibodies did not affect k_{cat} under conditions of limiting substrate (results not shown), indicating that the antibodies were not interfering with the binding of ATP or the substrate peptide. The control antibodies had no effect on the ability of the compounds to block the activity of Akt. These results, along with the inability of Akt-I-1 and Akt-I-1,2 to inhibit ΔPH-Akt1 and ΔPH-Akt2, highlighted the importance of the PH domain and hinge region for inhibitor function.

Akt-I-1 and Akt-I-1,2 block PDK1 phosphorylation of Akt *in vitro*

Akt is activated by PDK1 when the two kinases are brought into proximity at the plasma membrane following growth factor stimulation and an increase in PtdIns(3,4,5)P_3. Binding of Akt to PtdIns(3,4,5)P_3 is thought to result in a conformational change that moves the PH domain relative to the kinase domain and results in increased accessibility of Thr^{308} in the activation loop. PDK1 was assayed for its ability to phosphorylate Thr^{308} (or equivalent) in the activation loop of Akt1, Akt2, Akt3 and ΔPH-Akt1 in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 and either Akt-I-1 or Akt-I-1,2. Akt-I-1 blocked PDK1 phosphorylation of Akt1, but not that of the other three forms of Akt, while Akt-I-1,2 blocked PDK1 phosphorylation of Akt1 and Akt2, but not Akt3 or ΔPH-Akt1 (Table 5). IC_{50} values for inhibition of PDK1 phosphorylation of Akt were similar to those determined for inhibition of Akt phosphorylation of peptide substrate. Both

Table 5 Inhibitor effects on PDK1 phosphorylation of Akt activation loop

	PDK1 substrates (IC_{50}s, μM)			
Identifier	Akt1	ΔPH-Akt1	Akt2	Akt3
Akt-I-1	1.7	> 250	> 250	> 250
AKT-I-1,2	4.1	> 250	17	> 250

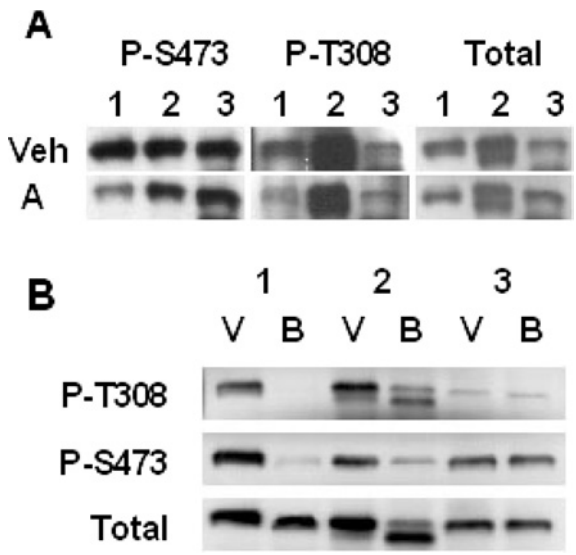


Figure 1 Inhibition of Akt phosphorylation in C33A cells

C33A cells were treated with Akt-I-1 (**A**) or Akt-I-1,2 (**B**) as described in the Experimental section, and the individual Akt isoforms were immunoprecipitated. The phosphorylation status of each isoform was determined by Western blot analysis using antibodies specific for phospho-Thr^{308} (P-T308) and phospho-Ser^{473} (P-S473). The phospho-Ser^{473} blots were stripped and probed for total Akt levels. Veh/V, DMSO vehicle; A, Akt-I-1; B, Akt-I-1,2. The numbers 1, 2 and 3 refer to the Akt isoenzymes.

of the inhibitors were tested for their ability to inhibit PDK1 phosphorylation of a peptide derived from the Akt activation loop. No inhibition was detected at concentrations up to 40 μM, further ruling out direct inhibition of PDK1 by these compounds (results not shown). These results demonstrated that the inhibitors bound to Akt in a way which blocked the access of PDK1 to Thr^{308} (or equivalent), and that Akt activation was not required for inhibitor binding.

Akt-I-1 and Akt-I-1,2 block phosphorylation of Akt Thr^{308} and Ser^{473} in cells and reduced active Akt levels

We next evaluated the ability of our inhibitors to block the phosphorylation of Akt isoenzymes in cell-based experiments. C33A cells (human cervical carcinoma) were treated with inhibitors, the individual Akt isoenzymes were immunoprecipitated, and changes in their phosphorylation state were evaluated by Western blot analysis (Figure 1). Treatment of the cells for 2 h with 20 μM Akt-I-1 reduced the amount of phospho-Ser^{473} and phospho-Thr^{308} present on Akt1, and did not significantly reduce the level of phosphorylation of the other two isoenzymes. Treatment of the cells with Akt-I-1,2 (50 μM, 2 h) reduced the amount of phosphorylation on both Akt1 and Akt2. Akt3 phosphorylation did not change in response to treatment with these Akt inhibitors. These results directly parallel those observed

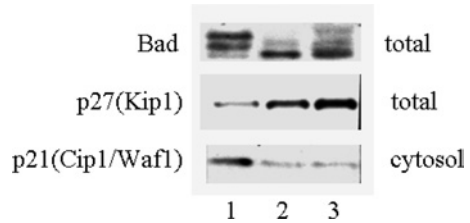


Figure 2 Effects of inhibiting Akt1 and Akt2 on downstream signalling events

LnCaP cells were treated with vehicle (lane 1), 20 μM Akt-I-1,2 (lane 2) or 10 μM LY294002 (lane 3) for 18 h. Total cell lysates were prepared and Western blots were performed to determine the levels and migration patterns of Bad and p27^{Kip1}. Cytosolic extracts were prepared, and the relative amounts of p21$^{Cip1/Waf1}$ present following inhibitor treatment was determined by Western blot analysis.

in vitro, and demonstrate that these inhibitors enter cells, bind to a subset of the Akt isoenzymes and prevent phosphorylation by PDK1.

The reduction in the levels of phosphorylation on Thr^{308} and Ser^{473} observed when cells were treated with the inhibitors was expected to result in reduced Akt activity. This was confirmed by determining the levels of Akt activity in C33A cells utilizing an isoenzyme-specific immunoprecipitation kinase assay. Treatment of cells with 25 μM Akt-I-1 for 5 h reduced the levels of Akt1 activity by 70%, while the levels of Akt2 and Akt3 activity were unaffected. Treatment with Akt-I-1,2 (25 μM, 5 h) reduced the levels of Akt1 activity by approx. 90%, and the Akt2 activity levels dropped by 70%. Akt3 activity levels were not reduced following treatment with Akt-I-1,2. These results also demonstrate that the isoenzyme specificity observed *in vitro* was maintained in cells.

Inhibition of Akt1 and Akt2 affects downstream signalling events

Akt-I-1,2 is not very potent, but it did provide the first opportunity to test an Akt-specific small-molecule inhibitor for effects on downstream signalling. LNCaP cells have been reported to express only Akt1 and Akt2 [26], and we have confirmed this (results not shown). Use of this cell line avoided the potential complications of interpretation resulting from the fact that we are not inhibiting Akt3. The effects of inhibiting Akt1 and Akt2 with Akt-I-1,2 on the downstream effectors Bad, p27^{Kip1} and p21$^{Cip1/WAF1}$ were analysed (Figure 2). Treatment of LNCaP cells with 20 μM Akt-I-1,2 for 5 h increased the amount of the faster-migrating species of Bad, p27^{Kip1} protein levels increased and cytoplasmic p21$^{Cip1/WAF1}$ protein levels decreased consistent with previous reports. None of these effects were observed following treatment with Akt-I-1 (results not shown), suggesting that inhibition of Akt1 alone is not sufficient to affect the regulation of downstream substrates.

Caspase activation following combination treatment with TRAIL and Akt inhibitor

It has been demonstrated previously that LNCaP cells are resistant to TRAIL-induced apoptosis, but that treatment with a PI3K inhibitor can sensitize these cells to TRAIL-induced apoptosis [27,28]. Again, Akt-I-1,2 is not very potent, but its specificity provides the opportunity to determine if Akt is a major contributor to the resistance to TRAIL or whether PI3K and other PDK1 substrates are major contributors. Treatment of the cells with Akt-I-1,2 in combination with TRAIL resulted in a similar level of caspase 3 activation as with that following treatment with a

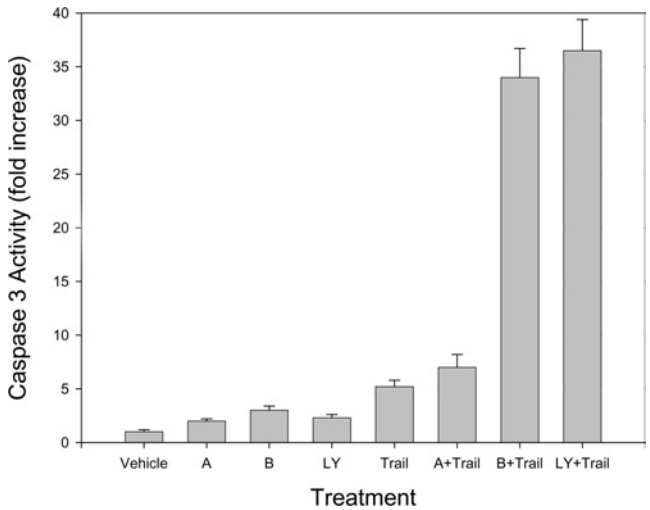


Figure 3 Caspase 3 activity in LNCaP cells treated with Akt inhibitors or LY294002 in the presence or absence of TRAIL

LNCaP cells were treated with either vehicle, Akt-I-1 (A, 25 μM), Akt-I-1,2 (B, 25 μM) or LY294002 (LY, 15 μM) in the presence or absence of TRAIL. Each caspase 3 assay was performed in triplicate and results are means $\pm$ S.D.

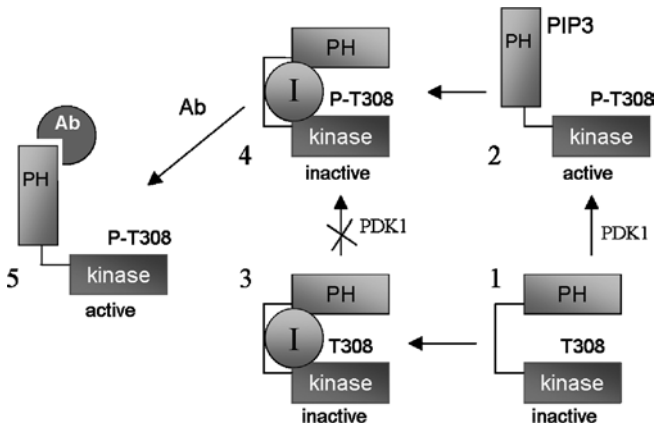


Figure 4 Model for Akt inhibition by PH-domain-dependent inhibitors

Inhibitors bind Akt outside of the active site and interact with the PH domain and/or hinge region. Inactive Akt (1) can be phosphorylated by PDK1 and activated (2) or bind inhibitor (3). Binding of inhibitor at (3) prevents phosphorylation of Thr^{308} by PDK1. Inhibitor can bind to activated Akt (4) and block phosphorylation of peptide substrates. Antibodies bound to the PH domain or hinge region (5) prevent inhibitor binding.

combination of LY294002 and TRAIL (Figure 3). These results indicate that reducing the levels of active of Akt1 and Akt2 is sufficient to sensitize these cells to TRAIL-induced apoptosis to a level similar to that observed with the LY294002.

DISCUSSION

Kinases are important therapeutic targets that are involved in many disease states, and significant resources have been expended identifying therapeutically useful kinase inhibitors [29,30]. Kinase activities are tightly regulated in cells, and the modes of regulation are diverse and overlapping. Phosphorylation is common, and can regulate the accessibility of the active site to ATP and protein substrates, and regulate protein–protein interactions. These phosphorylations and protein–protein interactions can regulate the specific activity of kinases, as well as their movement in and out of organelles, and movement from the cytoplasm to the plasma membrane. The intracellular localization will affect which additional regulatory events the kinase is exposed to. These modes of regulation, while highly specific, may be difficult to replicate with small-molecule inhibitors.

There are, however, modes of kinase inhibition that can be duplicated with small-molecule inhibitors. These modes are pseudosubstrate inhibition, inhibition by nucleotide competitive protein domains and mechanisms involving protein domains which lock the kinase into an inactive conformation [31]. Twitchin is an example of a kinase inhibited by an extended pseudosubstrate sequence which binds in the active-site cleft [32]. A similar mechanism of inhibition has been observed for PKA [33] and MLCK (myosin light-chain kinase) [34]. A nucleotide competitive mechanism of inhibition has been observed in the p27^{Kip1}–cdk2 (cyclin-dependent kinase 2)–cyclin A complex [35]. In this case, a tyrosine located in the C-terminus of p27^{Kip1} protrudes into the adenine-binding pocket of cdk2 and blocks ATP binding. Most synthetic inhibitors that have been developed to date mimic these mechanisms. They bind in or very near the active sites, and are competitive with ATP and/or peptide substrate binding [24,29,30,36]. The best example of a kinase that can be locked into an inactive conformation by another protein domain is c-Src and closely related family members. These kinases have SH (Src homology) 2 and SH3 domains that mediate binding to phosphotyrosine and proline-rich sequences respectively. In the inhibited state, the SH2 domain interacts with a C-terminal peptide containing a phosphorylated tyrosine. This interaction positions the SH3 domain so that it can interact with the linker region between the kinase domain and the SH2 domain. These intramolecular interactions stabilize a conformation in which the kinase active site is disrupted and unable to support catalysis [37,38]. It is possible that the Akt inhibitors we have identified stabilize a conceptually similar inactive conformation involving the PH domain and the approx. 39-amino-acid linker region connecting the PH and kinase domains.

A model describing the inhibition of Akt by our compounds is presented in Figure 4. There are three key observations which define this model. First, the PH domain was required for the activity of these inhibitors. Akt protein constructs lacking the PH domain were not inhibited by our inhibitors at concentrations of up to 250 μM. There are two possible models to explain the PH/hinge domain requirement. One is that some or all of the contacts required for inhibitor binding are on the PH domain. Alternatively, the PH domain may induce a specific conformation in the kinase domain to which the inhibitors bind. The second key observation came from kinetic analyses demonstrating that these inhibitors are non-competitive with respect to ATP and peptide substrate binding. The third observation was that there appeared to be a conformational change involved in inhibitor binding. Polyclonal antibodies against either the PH domain/hinge or the hinge region blocked inhibition. This could be due to direct competition between the antibodies and the inhibitors for binding. An alternative possibility is that antibody binding prevents Akt from adopting the conformation to which the inhibitors bind. Another indication of a conformation change came from the observation that the inhibitors could bind to unactivated Akt and block the phosphorylation of Akt Thr^{308} by PDK1. Inhibitors may prevent access of PDK1 to the activation loop of Akt by inducing local changes near the active site, global changes affecting the interaction between the Akt domains or blocking PH domain–lipid interaction.

The discovery that the inhibitors can block the phosphorylation of Thr^{308} by PDK1 was surprising, but the model presented in Figure 4 can accommodate this observation. What is more difficult

to incorporate into the model is the observation that these inhibitors block the phosphorylation of Ser^{474} in cell-based assays. Recently, the determination of an Akt2 kinase domain crystal structure and Akt activation studies utilizing Ser^{474}-containing peptides have clarified the regulatory role of phosphorylated Ser^{474} [10,11]. The hydrophobic amino acids surrounding the phosphorylated Ser^{474} bind in a hydrophobic cleft on the N-terminal lobe of the kinase domain formed in part by the αC-helix. This binding results in a restructuring of the active site to an active state. Phosphorylation of Ser^{474} in cells may require certain protein–protein interactions that are blocked when inhibitors are bound to Akt, or binding of the inhibitors may result in a conformation in which the phosphorylated residues are more exposed to phosphatases. Additional studies will be required to determine how these inhibitors reduce Ser^{474} phosphorylation.

Allosteric kinase inhibitors have been reported previously: PD 098059 binds to MAPKK1 [MAPK (mitogen-activated protein kinase) kinase 1] {MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]} 1 and blocks activation and phosphorylation by c-Raf and MEK kinase [39], and is not competitive with ATP or ERK [40]. BIRB 796 is a potent allosteric inhibitor of p38 MAPK which competes indirectly with ATP [41]. Binding of both of these inhibitors have been reported to involve conformational changes. PD 098509 does not bind to MAPKK1 in the activated conformation, but does bind to certain mutants of MAPKK1 which mimic the activated state [42]. BIRB 796 binds to a conformation of p38 MAPK in which the conserved DFG active site motif is in the 'out' position [41]. Although there are a number of similarities between the mechanism of inhibition by these inhibitors and our allosteric Akt inhibitors, the binding sites for the inhibitors are likely to be distinct. The Akt inhibitors bind to both the active and inactive forms of the enzyme, do not inhibit Akt3 and have an absolute requirement for the PH domain.

The importance of kinase specificity in the development of therapeutics has received a great deal of attention. Inhibition of off-target kinases at therapeutically relevant concentrations may result in toxicity and an unacceptably narrow therapeutic index. However, there may be cases where inhibiting multiple members of a kinase class has advantages, particularly in the case of cancer therapeutics. STI571 (imatinib mesylate; Gleevec) was developed as an inhibitor of BCR (breakpoint cluster region)–Abl [Abelson murine leukaemia viral (v-abl) oncogene homologue 1] and shown to be efficacious in the treatment of CML (chronic myelogenous leukaemia) [43]. STI571 also inhibits KIT (v-kit Hardy–Zuckerman 4 felin sarcoma viral oncogene homologue, tyrosine kinase), a receptor tyrosine kinase activated in GISTs (gastrointestinal stromal tumours), and was shown to be efficacious in this tumour type [44]. Tyrosine kinase inhibitors with activity against EGFR (epidermal growth factor receptor) and ErbB2 may be more efficacious than inhibitors against individual receptors [45,46]. In contrast, ATP-competitive Akt inhibitors are most likely to inhibit other members of the ACG kinase family. Inhibition of the most closely related kinases, such as PKA, PKC and SGK may lead to broad non-specific cytotoxicity. The allosteric inhibitors described in the present paper appear to be very specific. They do not inhibit closely related kinases nor do they inhibit all of the Akt isoenzymes indiscriminately. The isoenzyme specificity seen with our inhibitors *in vitro* was also observed in cells. This suggests that the unique binding site of these inhibitors is present in cells and that the lipid and protein interactions of Akt in cells do not interfere with the allosteric and isoenzyme-specific mode of inhibition seen *in vitro*. Taken together, our data demonstrate that it should be possible to develop highly specific Akt inhibitors for therapeutic use that have minimal off-target activities.

Activation of the Akt cell-survival pathway has been shown in a number of cell lines to block TRAIL-induced apoptosis. LNCap cells is one example [27,28]. This suggests that activation of the Akt pathway may result in resistance to therapies designed to activate the TRAIL receptor. We have shown that inhibition of Akt1 and Akt2 in LNCaP cells sensitizes these cells to TRAIL-induced apoptosis. It is possible that Akt inhibitors could be used in the clinic in combination with TRAIL therapies to enhance tumour cell killing.

The functions and expression patterns of the Akt isoforms are not identical, and the relative contributions of individual Akt isoenzymes to tumorigenesis are the subject of intense research. One functional difference between the isoforms is their interactions with other proteins. For example, Akt1 and Akt2 bind to all three members of the TCL1 (T-cell leukaemia/lymphoma 1) family of proto-oncogenes [TCL1, MTCP1 (mature T-cell proliferation 1) and TCL1b], while Akt3 only interacts with TCL1 [47]. The functional differences between Akt isoforms have also been studied in knockout mice. Akt1-knockout mice are healthy, but have impaired foetal and post-natal growth [48], while Akt2-knockout mice have normal growth characteristics, but have a mild insulin-resistance phenotype [49]. Finally, the expression patterns of the isoforms have been shown to vary in tumours. All three Akt isoforms are expressed in most tissues, but the relative ratios vary considerably [7].

Akt2 is the most commonly observed isoform overexpressed in tumours [50,51], while Akt1 [52] and Akt3 [26] overexpression appears to be less common. It remains to be determined which Akt isoenzyme inhibition profile will yield the optimum impact on cancer cell survival and minimize toxicity. However, it is clear that the ability to inhibit a subset of the Akt isoenzymes will provide an opportunity for widening the therapeutic window of survival signalling inhibitors.

REFERENCES

1 Mitsiades, C. S., Mitsiades, N., Koutsilieris, M., Nicholson, K. M., Anderson, N. G., Neri, L. M., Borgatti, P., Capitani, S., Martelli, A. M., Brazil, D. P. and Hemmings, B. A. (2004) The Akt pathway: molecular targets for anti-cancer drug development. Curr. Cancer Drug Targets **4**, 235–256

2 Vivanco, I. and Sawyers, C. L. (2002) The phosphatidylinositol 3-kinase-Akt pathway in human cancer. Nat. Rev. **2**, 489–501

3 Brazil, D. P. and Hemmings, B. A. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem. Sci. **26**, 657–664

4 Simpson, L. and Parsons, R. (2001) PTEN: Life as a tumor suppressor. Exp. Cell Res. **264**, 29–41

5 Vanhaesebroeck, B. and Alessi, D. R. (2000) The P13K–PDK1 connection: more than just a road to PKB. Biochem J. **346**, 561–576

6 Besson, A., Robbins, S. M. and Yong, V. W. (1999) PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. Eur. J. Biochem. **263**, 605–611

7 Chan, T. O., Rittenhouse, S. E. and Tsichlis, P. N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. Annu. Rev. Biochem. **68**, 965–1014

8 Alessi, D. R. and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. **8**, 55–62

9 Masure, S., Haefner, B., Wesselink, J. J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R. and Richardson, A. (1999) Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. Eur. J. Biochem. **265**, 353–360

10 Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A. and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. Nat. Struct. Biol. **9**, 940–944

11 Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A. and Barford, D. (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol. Cell **9**, 1227–1240

12 Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr. Biol. **7**, 261–269

13 Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science **277**, 567–570
14 Sulis, M. L. and Parsons, R. (2003) PTEN: from pathology to biology. Trends Cell Biol. **13**, 478–483
15 Bonneau, D. and Longy, M. (2000) Mutations of the human PTEN gene. Human Mutat. **16**, 109–122
16 Yeh, J. J. and Sellers, W. R. (2000) PTEN – Regulator of phosphoinositide 3-kinase signal transduction. In Tumor Suppressor Genes in Human Cancer (Fisher, D. E., ed.), pp. 231–251, Humana Press Inc, Totowa
17 Liaw, D., Marsh, D. J., Li, J., Dahia, P. L. M., Wang, S. I., Zheng, Z. M., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M. et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. **16**, 64–67
18 Marsh, D. J., Coulon, V., Lunetta, K. L., Rocca-Serra, P., Dahia, P. L. M., Zheng, Z. M., Liaw, D., Caron, S., Duboue, B., Lin, A. Y. et al. (1998) Mutation spectrum and genotype–phenotype analyses in Cowden disease and Bannayan–Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. Human Mol. Genet. **7**, 507–515
19 Arch, E. M., Goodman, B. K., Van Wesep, R. A., Liaw, D., Clarke, K., Parsons, R., McKusick, V. A. and Geraghty, M. T. (1997) Deletion of PTEN in a patient with Bannayan–Riley–Ruvalcaba syndrome suggests allelism with Cowden disease. Am. J. Med. Genet. **71**, 489–493
20 Zhou, X. P., Hampel, H., Thiele, H., Gorlin, R. J., Hennekam, R. C. M., Parisi, M., Winter, R. M. and Eng, C. (2001) Association of germline mutation in the PTEN tumour suppressor gene and Proteus and Proteus-like syndromes. Lancet **358**, 210–211
21 Kwon, C. H., Zhu, X. Y., Zhang, J. Y., Knoop, L. L., Tharp, R., Smeyne, R. J., Eberhart, C. G., Burger, P. C. and Baker, S. J. (2001) Pten regulates neuronal soma size: a mouse model of Lhermitte–Duclos disease. Nat. Genet. **29**, 404–411
22 Grussenmeyer, T., Scheidtmann, K. H., Hutchinson, M. A., Eckhart, W. and Walter, G. (1985) Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. U.S.A. **82**, 7952–7954
22a Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
23 Kolb, A. J., Kaplita, P. V., Hayes, D. J., Park, Y. W., Pernell, C., Major, J. S. and Mathis, G. (1998) Tyrosine kinase assays adapted to homogeneous time-resolved fluorescence. Drug Discov. Today **3**, 333–342
24 Garcia-Echeverria, C., Traxler, P. and Evans, D. B. (2000) ATP site-directed competitive and irreversible inhibitors of protein kinases. Med. Res. Rev. **20**, 28–57
25 Madhusudan, Trafny, E. A., Xuong, N. H., Adams, J. A., Teneyck, L. F., Taylor, S. S. and Sowadski, J. M. (1994) cAMP-dependent protein kinase: crystallographic insights into substrate recognition and phosphotransfer. Protein Sci. **3**, 176–187
26 Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J. and Roth, R. A. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J. Biol. Chem. **274**, 21528–21532
27 Nesterov, A., Lu, X., Johnson, M., Miller, G. J., Ivashchenko, Y. and Kraft, A. S. (2001) Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. J. Biol. Chem. **276**, 10767–10774
28 Rokhlin, O. W., Guseva, N. V., Tagiyev, A. F., Glover, R. A. and Cohen, M. B. (2002) Caspase-8 activation is necessary but not sufficient for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in the prostatic carcinoma cell line LNCaP. Prostate **52**, 1–11
29 Dumas, J. (2001) Protein kinase inhibitors: emerging pharmacophores 1997–2000. Expert Opin. Ther. Pat. **11**, 405–429
30 Bridges, A. J. (2001) Chemical inhibitors of protein kinases. Chem. Rev. **101**, 2541–2571
31 Taylor, S. S. and Radzio-Andzelm, E. (1997) Protein kinase inhibition: natural and synthetic variations on a theme. Curr. Opin. Chem. Biol. **1**, 219–226
32 Kobe, B., Heierhorst, J., Feil, S. C., Parker, M. W., Benian, G. M., Weiss, K. R. and Kemp, B. E. (1996) Giant protein kinases: domain interactions and structural basis of autoregulation. EMBO J. **15**, 6810–6821
33 Taylor, S. S., Buechler, J. A. and Yonemoto, W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. **59**, 971–1005
34 Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyck, L. F., Taylor, S. S. and Kemp, B. E. (1992) Structural basis of the intrasteric regulation of myosin light chain kinases. Science **258**, 130–135
35 Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J. and Pavletich, N. P. (1996) Crystal structure of the $p27^{Kip1}$ cyclin-dependent-kinase inhibitor bound to the cyclin A–Cdk2 complex. Nature (London) **382**, 325–331
36 Scapin, G. (2002) Structural biology in drug design: selective protein kinase inhibitors. Drug Discov. Today **7**, 601–611
37 Xu, W. Q., Harrison, S. C. and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src. Nature (London) **385**, 595–602
38 Sicheri, F., Moarefi, I. and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck. Nature (London) **385**, 602–609
39 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. **270**, 27489–27494
40 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., Saltiel, A. R., Alessi, D. R., Cuenda, A. and Cohen, P. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689
41 Pargellis, C., Tong, L., Churchill, L., Cirillo, P. F., Gilmore, T., Graham, A. G., Grob, P. M., Hickey, E. R., Moss, N., Pav, S. and Regan, J. (2002) Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. Nat. Struct. Biol. **9**, 268–272
42 Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. **273**, 18623–18632
43 Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., Sawyers, C. L. and Fry, D. W. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia: Mechanism of action of erbB tyrosine kinase inhibitors. N. Engl. J. Med. **344**, 1031–1037
44 Demetri, G. D. (2002) Identification and treatment of chemoresistant inoperable or metastatic GIST: experience with the selective tyrosine kinase inhibitor imatinib mesylate (ST1571). Eur. J. Cancer **38** (Suppl. 5), S52–S59
45 Arteaga, C. L. (2003) ErbB-targeted therapeutic approaches in human cancer. Exp. Cell Res. **284**, 122–130
46 Arteaga, C. L. (2003) Molecular therapeutics: is one promiscuous drug against multiple targets better than combinations of molecule-specific drugs? Clin. Cancer Res. **9**, 1231–1232
47 Laine, J., Kunstle, G., Obata, T. and Noguchi, M. (2002) Differential regulation of Akt kinase isoforms by the members of TCL1 oncogene family. J. Biol. Chem. **277**, 3743–3751
48 Cho, H., Thorvaldsen, J. L., Chu, Q. W., Feng, F. and Birnbaum, M. J. (2001) Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J. Biol. Chem. **276**, 38349–38352
49 Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q. W., Crenshaw, E. B., Kaestner, K. H., Bartolomei, M. S., Shulman, G. I. and Birnbaum, M. J. (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). Science **292**, 1728–1731
50 Page, C., Lin, H. J., Jin, Y., Castle, V. P., Nunez, G., Huang, M. and Lin, J. Y. (2000) Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. Anticancer Res. **20**, 407–416
51 Bellacosa, A., DeFeo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M. H., Dubeau, L., Scambia, G., Masciullo, V. et al. (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int. J. Cancer **64**, 280–285
52 Staal, S. P. (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc. Natl. Acad. Sci. U.S.A. **84**, 5034–5037

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