

Tumor cell sensitization to apoptotic stimuli by selective inhibition of specific Akt/PKB family members

Deborah DeFeo-Jones,¹ Stanley F. Barnett,¹
Sheng Fu,¹ Paula J. Hancock,¹
Kathleen M. Haskell,¹ Karen R. Leander,¹
Elizabeth McAvoy,¹ Ronald G. Robinson,¹
Mark E. Duggan,² Craig W. Lindsley,²
Zhijian Zhao,² Hans E. Huber,¹
and Raymond E. Jones¹

¹Department of Cancer Research and ²Technology Enabled Synthesis Group, Department of Medicinal Chemistry, Merck Research Laboratories, West Point, Pennsylvania

Abstract

Recent studies indicate that dysregulation of the Akt/PKB family of serine/threonine kinases is a prominent feature of many human cancers. The Akt/PKB family is composed of three members termed Akt1/PKB α , Akt2/PKB β , and Akt3/PKB γ . It is currently not known to what extent there is functional overlap between these family members. We have recently identified small molecule inhibitors of Akt. These compounds have pleckstrin homology domain-dependent, isozyme-specific activity. In this report, we present data showing the relative contribution that inhibition of the different isozymes has on the apoptotic response of tumor cells to a variety of chemotherapies. In multiple cell backgrounds, maximal induction of caspase-3 activity is achieved when both Akt1 and Akt2 are inhibited. This induction is not reversed by overexpression of functionally active Akt3. The level of caspase-3 activation achieved under these conditions is equivalent to that observed with the phosphatidylinositol-3-kinase inhibitor LY294002. We also show that in different tumor cell backgrounds inhibition of mammalian target of rapamycin, a downstream substrate of Akt, is less effective in inducing caspase-3 activity than inhibition of Akt1 and Akt2. This shows that the survival phenotype conferred by Akt can be mediated by signaling pathways independent of mammalian target of rapamycin in some tumor cell backgrounds. Finally, we show that inhibition of both Akt1 and Akt2 selectively sensitizes tumor cells, but not normal cells, to apoptotic stimuli. [Mol Cancer Ther 2005;4(2):271–9]

Introduction

The most compelling evidence implicating the phosphatidylinositol-3-kinase (PI-3-K)/Akt pathway in the development of human cancer comes from studies of the *PTEN* tumor suppressor gene (1). *PTEN* is one of the most frequently mutated tumor suppressors in human cancer (2). *PTEN* functions primarily as a lipid phosphatase that reduces the levels of phosphatidylinositol 3,4,5-trisphosphate. Phosphatidylinositol 3,4,5-trisphosphate functions as a ligand that binds to the pleckstrin homology (PH) domain of several proteins in the PI-3-K signal transduction pathway, including Akt/PKB (hereafter identified as Akt), promoting protein-membrane association. Membrane association of Akt family members results in their activation by phosphorylation on threonine 308 and serine 473 (Akt1 numbering). Phosphorylation of threonine 308 is mediated by the phosphoinositide-dependent kinase 1 whereas phosphorylation of serine 473 is mediated by an unknown kinase. Phosphorylation of these residues results in increased Akt kinase activity toward a number of substrates that promote cellular growth and survival and block proapoptotic signals. In the absence of *PTEN* function, cells exhibit elevated Akt activity and are less sensitive to apoptotic stimuli (3, 4). Additional evidence that implicates Akt activity in dysregulated cellular growth comes from studies that show that Akt1 is one of the major downstream effectors of *PTEN* in embryonic stem cells. Deletion of Akt1 was shown to partially reverse the aggressive growth of *PTEN* (–/–) embryonic stem cells *in vivo*, suggesting that Akt1 plays an essential role in *PTEN*-controlled tumorigenesis (5). Inappropriate Akt activation can also occur through PI-3-K up-regulation or enhanced growth factor receptor signaling. In human ovarian cancer, overexpressed and/or activated Akt2 was shown to be a common occurrence (6). Additional studies on human cancers (prostate, glioma, breast, and colon) support the conclusion that activated Akt is causally involved in tumorigenesis (7–14).

Recent mouse gene knockout studies indicate that *Akt1*, *Akt2*, and *Akt3* are functionally distinct. Mice that are *Akt1* (–/–) are reduced in size and display increased spontaneous apoptosis in the testes and thymus whereas mice that are *Akt2* (–/–) are of normal size but are impaired in the ability to maintain glucose homeostasis (15–17). *Akt3* (–/–) mice have no gross observable phenotype but display a uniformly reduced brain size (18). Additional studies have indicated that the gene expression of the three isoforms of Akt is differentially regulated. Akt1 and Akt2 seem to be ubiquitously expressed whereas the expression of Akt3 seems to be more restricted (19, 20). Other functional differences occur in human T cells where the three Akt isoforms are differentially regulated by members of the *TCL1* oncogene family (21).

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Requests for reprints: Raymond E. Jones, Department of Cancer Research, Merck Research Laboratories, Building 26-462, West Point, PA 19486. Phone: 215-652-7637; Fax: 215-993-3398. E-mail: raymond_jones@merck.com

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The relative contribution of the individual Akt isoforms to PI-3-K/PTEN-mediated tumorigenesis is currently unknown. Inhibition of Akt activation can be achieved by inhibiting PI-3-K with inhibitors such as LY294002 or wortmannin. However, PI-3-K inhibition indiscriminately affects not just all three Akt isozymes but also other PH domain-containing signaling molecules that are dependent on phosphatidylinositol 3,4,5-trisphosphate lipids. Recently, we identified compounds that reversibly inhibit both the activity as well as the activation of Akt. These allosteric inhibitors exhibit isozyme specificity and dependence on the PH domain (22). Compounds in one structural series preferentially inhibit the activity of Akt1 or Akt2 whereas a second structural series inhibits the activity of both Akt1 and Akt2. Here, we report that inhibition of both Akt1 and Akt2 results in a greater sensitization of tumor cells to apoptotic stimuli than inhibition of either isoform alone. This sensitization is not reversed by overexpression of a functionally active Akt3.

Materials and Methods

Measurement of Akt Kinase Activity

Purified recombinant human Akt1, Δ PH-Akt1, Akt2, and Akt3 proteins were monitored for kinase activity in the presence or absence of Akt inhibitors. Activation and kinase activity of cellular Akt was analyzed by homogeneous assay in a 96-well format. Detection was done by homogeneous time-resolved fluorescence using a europium chelate (Perkin-Elmer, Boston, MA) [Eu(K)]-labeled phospho(S21)-GSK3 α antibody (Cell Signaling Technologies, Beverly, MA) and streptavidin-linked XL665 fluorophore which binds to the biotin moiety on the substrate peptide (biotin-GGRARTSSFAEPG). Final reaction conditions were 50 mmol/L HEPES (pH 7.5), 0.1% polyethylene glycol, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% bovine serum albumin, 2 mmol/L β -glycerol phosphate, 0.5 μ mol/L substrate peptide, 150 μ mol/L ATP, 10 mmol/L MgCl₂, 50 mmol/L KCl, 5% glycerol, 1 mmol/L DTT, 2.5% DMSO, 10 μ g/mL benzamidine, 5 μ g/mL pepstatin, 5 μ g/mL leupeptin, 5 μ g/mL aprotinin, test compound as indicated, and 45 to 200 pmol/L activated enzyme in a 40 μ L volume. The reaction was started with the addition of enzyme.

Cell Culture and Immunoprecipitation

LNCaP and MDA-MB468 cell lines were maintained in RPMI 1640 supplemented with 10% FCS. Cells were exposed to Akti-1, Akti-2, Akti-1/2, or LY294002 for 2 to 4 hours. Normal human prostate epithelial, human umbilical vascular endothelial cell, and human mammary epithelial cells were maintained in media recommended by the manufacturer (Cambrex BioScience, Baltimore, MD). For Akt1 immunoprecipitation, 100 μ g of cell extract protein prepared in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris/HCl (pH 7.4), 1 mmol/L EDTA, 1% NP40, 0.1% SDS, 1 mmol/L N-ethylmaleimide, 10 mmol/L β -glycerol phosphate, 10 mmol/L NaF, 0.5 mmol/L NaVO₄, 1 μ mol/L microcystin, and protease inhibitor cocktail; Calbiochem, San Diego, CA] were incubated with 1 μ g of anti-Akt1 mouse monoclonal

antibody (Cell Signaling Technology) and NETN [100 mmol/L NaCl, 20 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, 0.5% NP40] in a total volume of 50 μ L. Akt2 and Akt3 immunoprecipitations were done using 200 μ g of cell lysate mixed with 3 μ g of anti-Akt2 antibody (F-7, Santa Cruz Biotechnology, Santa Cruz, CA) or 3 μ g of anti-Akt3 antibody (M-14, Santa Cruz Biotechnology). To each cell lysate/antibody mixture an equal volume of Protein A/G PLUS Agarose (Santa Cruz Biotechnology) was added. The immunoprecipitates were placed on a rotary shaker at 4°C overnight. Immunoprecipitated pellets were washed thrice in NETN and electrophoresed in 8% polyacrylamide SDS denaturing precast Novex gels (Invitrogen, Carlsbad, CA). Protein transfer to nitrocellulose filters was according to a protocol described by the manufacturer. Anti-total-Akt, anti-phospho-Akt serine 473, and anti-phospho-Akt threonine 308 antibodies were purchased from Cell Signaling Technology. Secondary horseradish peroxidase-tagged antibody and enhanced chemiluminescence (Amersham, Piscataway, NJ) detection reagents were from Amersham. Protein band intensities were determined using a Kodak Image Station 440 CF instrument (Perkin-Elmer). MCF7 cells, maintained in α -MEM media, were used for insulin-like growth factor I stimulation of the Akt pathway. Cells were placed in serum-free media for 15 hours before the addition of 25 μ mol/L Akti-1/2a or 20 μ mol/L LY294002. The treated cells were incubated for an additional 2 hours before the addition of 1 nmol/L final concentration of heregulin β 1 (Lab Vision Corp; NeoMarkers, Inc., Fremont, CA). Incubation was continued for an additional 30 minutes before the addition of lysis buffer. Antibodies (extracellular signal-regulated kinase, BD-PharMingen, San Diego, CA; phospho-extracellular signal-regulated kinase, Promega, San Luis Obispo, CA) were used according to the recommendation of the manufacturer.

Caspase-3 Assay

In a 96-well plate, 1×10^4 cells per well were seeded and maintained for 72 hours before testing. Drug stocks were prepared in DMSO. The final concentration of DMSO in the assay was 0.1%. LY294002 and camptothecin were purchased from Sigma (St. Louis, MO). Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/Apo2L was purchased from Research Diagnostics, Flanders, NJ. Cells were incubated in the presence of compounds for 18 hours. In experiments utilizing the death receptor pathway, cells were preincubated with compounds for 1.5 hours before the addition of TRAIL/Apo2L. Incubations were continued for an additional 4 hours. Plates were centrifuged, the media was removed, and 50 μ L of cell lysis buffer (Clontech ApoAlert caspase-3 fluorescent assay kit) were added to each well. Plates were kept at 4°C for 20 minutes and then at -70°C for 1 hour. Plates were thawed to room temperature and caspase-3 substrate (final concentration, 50 μ mol/L) added in 2 \times reaction buffer [200 mmol/L HEPES (pH 7.6), 1 mmol/L EDTA, 10 mmol/L DTT]. Plates were further incubated for 6 to 18 hours at 37°C. Caspase activity was measured using a Spectra Max Gemini fluorescence plate reader (Molecular Devices Corporation, Sunnyvale, CA).

Results

Identification of Isoform-Specific Akt Inhibitors

A high-throughput kinase screen utilizing purified activated Akt1 and a peptide substrate derived from the Akt substrate glycogen synthase kinase 3- α led to the identification of a series of allosteric, isoform-specific Akt inhibitors (22). A representative example of the series is shown in Table 1 (Akti-1/2a). Further characterization of Akti-1/2a in enzyme kinetic assays showed that it is not competitive with ATP, that its inhibitory activity required the presence of the PH domain, and that it was 8-fold selective for Akt1 over Akt2. Furthermore, whereas Akti-1/2a blocked Akt phosphorylation by phosphoinositide-dependent kinase 1, phosphoinositide-dependent kinase 1 itself was not inhibited (22). In addition, Akti-1/2a did not seem to significantly affect any other upstream activator of Akt owing to Akt3 activity remaining unchanged in cells treated with this compound (see below). A medicinal chemistry effort to explore the structure-activity relationship around the Akti-1/2a scaffold led to the identification of more potent compounds with varying specificity for the Akt isozymes. Compounds were classified as inhibitors of Akt1 (Akti-1), inhibitors of Akt2 (Akti-2), and inhibitors of both Akt1 and Akt2 (Akti-1/2). The specificity of the

compounds for the Akt isozymes observed in enzyme assays is maintained in cell-based assays of Akt activation that used the C33a cervical carcinoma cell line which contains all three isoforms of Akt as well as the prostate LNCaP/Akt3 cell line where we introduced the Akt3 gene (Table 1 and Fig. 1). The binding of the Akt inhibitors, although PH domain-dependent, is not directly to the PH domain. Our studies with tritium-labeled Aktis show that they do not bind to isolated PH domain taken from Akt but bind only to the intact Akt protein. In addition to phosphoinositide-dependent kinase 1 (a PH domain-containing protein) mentioned above, these compounds were tested against and showed no inhibitory activity toward protein kinase A, protein kinase C, and serum and glucocorticoid regulated kinase ($IC_{50} > 50 \mu\text{mol/L}$; data not shown). These results imply that the Akt binding site is formed from an interaction between multiple domains within the Akt protein.

Inhibition of the Activation of Akt in LNCaP Tumor Cells

Initial studies to analyze the effect of inhibiting the individual isoforms of Akt on tumor cell survival were done using LNCaP prostate tumor cells. LNCaP cells are PTEN-negative and express Akt1 and Akt2 but no Akt3

Table 1. IC_{50} of recombinant Akt activity / EC_{50} of endogenous Akt activation

Compound	Enzyme assay				C33a cell assay			
	Akt1	Akt2	Akt3	$\Delta\text{PH-Akt1}$	Akt1	Akt2	Akt3	
Akti-1/2a		1,600	12,500	>50,000	>50,000	10,000	20,000	>50,000
Akti-1		760	23,340	>50,000	>50,000	1,140	>10,000	>10,000
Akti-2		19,120	330	21,870	>50,000	>10,000	4,150	>10,000
Akti-1/2		60	210	2,120	>50,000	310	2,090	>10,000

NOTE: Purified recombinant human Akt1, Akt2, Akt3, and $\Delta\text{PH-Akt1}$ proteins were monitored for kinase activity in the presence or absence of the Aktis. Kinase activity was determined by homogenous time-resolved fluorescence using a europium chelate-labeled anti-phospho-(serine 21)-glycogen synthase kinase α peptide antibody and streptavidin-linked XL665 fluorophore that binds to the biotin moiety on the peptide substrate (biotin-GGRARTSSFAEPG). See ref. 22 for further details. The cervical carcinoma cell line C33a contains all three isoforms of Akt. C33a cells plated in a 96-well format were treated with Aktis for 5 hours. The media was aspirated, replaced with an equal volume of lysis buffer, and the cells lysed by freeze-thaw. A volume of lysate was identified for each Akt isoform that provided equivalent kinase activity in the absence of inhibitors. Data represent the average of at least three experiments. In all cases the SE did not exceed 22% of the reported value. Values are in nanomoles per liter.

(ref. 23 and our data). To evaluate the functional contribution of Akt3 in the LNCaP cell background, we established a clone of LNCaP cells (LNCaP/Akt3) that overexpresses this isoform. Analysis of all three isoforms of Akt in this cell line showed them to be phosphorylated and kinase active (Figs. 1 and 2).

To confirm the isoform specificity of our inhibitors, LNCaP/Akt3 cells were treated for 5 hours with the individual compounds, cell extracts were prepared, and immunoprecipitations were done using isoform-specific Akt antibodies. The immunoprecipitates were then analyzed by immunoblotting using antibodies specific for Akt phospho-T-308 or phospho-S-473. Phospho-S-473 blots were subsequently stripped and reprobed with non-phosphospecific Akt antibodies to ensure that equivalent amounts of protein were analyzed in each gel lane. As shown in Fig. 2, all three isoforms of Akt are phosphorylated on T-308 and S-473 (Akt1 numbering) in the LNCaP/Akt3 cells (vehicle, *V lanes*). Treatment of these cells with our allosteric Akti-1 inhibitor blocked phosphorylation of Akt1 T-308 and S-473 but showed little or no effect on the phosphorylation of these residues in Akt2 or Akt3 (*lanes Akti-1*). Similarly, Akti-2 inhibited phosphorylation of Akt2 T-308 and S-473 but had no observable effect on the phosphorylation of these residues in Akt1 or Akt3 (*lanes Akti-2*). Finally, Akti-1/2 caused a marked inhibition in the phosphorylation of these residues in both Akt1 and Akt2 but having little or no effect on Akt3 (*lanes Akti-1/2*). In contrast, the PI-3-K inhibitor LY294002 eliminated or dramatically reduced phosphorylation of T-308 and S-473 residues on all three isoforms of the Akt family (data not shown). These results are consistent with the data obtained utilizing the cervical carcinoma cell line C33a which endogenously expresses all three isoforms of Akt (Table 1). In addition, other cell lines (ovarian A2780, breast MD-MBA468, and kidney Hek293) that express all three isoforms of Akt were similarly analyzed. In all cases

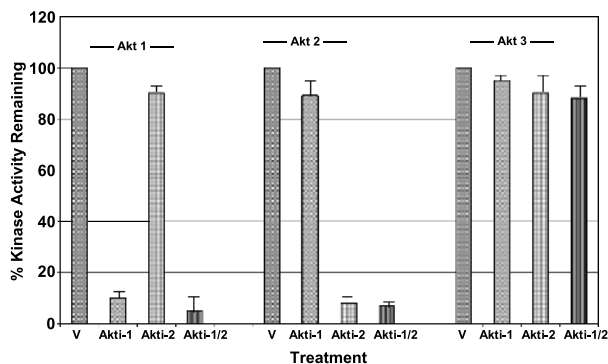


Figure 1. Inhibition of Akt1, Akt2, and Akt3 kinase activities in LNCaP/Akt3 cells. LNCaP/Akt3 cells were treated with vehicle [V, 0.1% DMSO], Akti-1 (12 $\mu\text{mol/L}$), Akti-2 (12 $\mu\text{mol/L}$), or Akti-1/2 (5 $\mu\text{mol/L}$) for 5 h. Cell lysates were incubated in 96-well plates coated with Akt isoform-specific antibodies. Kinase activity of individual Akt isozymes (indicated across the top) was analyzed as described in Materials and Methods. Akt kinase activity is expressed as a percentage of the activity in vehicle-treated cells. Each assay was done in triplicate. Data are representative of many independent assays.

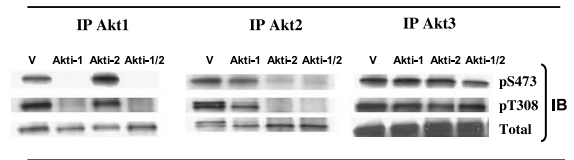


Figure 2. Akt inhibitors block phosphorylation of Akt in LNCaP/Akt3 cells. Immunoblot analysis was done to determine the phosphorylation state of the amino acid residues that are critical for kinase activity in the three Akt isoforms. LNCaP/Akt3 cells were incubated with vehicle [V, 0.1% DMSO], Akti-1 (12 $\mu\text{mol/L}$), Akti-2 (12 $\mu\text{mol/L}$), or Akti-1/2 (5 $\mu\text{mol/L}$). Akt isoform-specific immunoprecipitates (*IP*, as indicated) from cell lysates were analyzed by immunoblotting using phosphospecific antibodies for threonine 308 and serine 473 (Akt1 numbering). *Total*, total Akt detected with a non-phosphospecific Akt antibody. The data for Akt1, Akt2, and Akt3 are from different gels.

the results were similar to those shown for the LNCaP/Akt3 cells in Fig. 1 and 2 (data not shown). The assay used in Fig. 1 is amenable to automation and was used to determine EC_{50} values for inhibition of Akt phosphorylation/activation in cells.

Aktis Are Specific Inhibitors of Akt in Cells

To further show that these compounds are direct inhibitors of Akt activation by phosphoinositide-dependent kinase 1 and not acting through inhibition of an upstream effector, additional experiments were done. The breast carcinoma line MCF7 has elevated levels of the HER2 receptor, is PTEN-positive, and expresses both Akt1 and Akt2 proteins. Treatment of these cells with heregulin results in activation of both the PI-3-K and Ras-Raf-Mek signaling pathways (Fig. 3). Pretreatment of these cells with the dual Akti-1/2a inhibitor followed by stimulation with heregulin resulted in a significant reduction in phosphorylation of Akt1 and Akt2 but having no observable effect on the phosphorylation status of extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2 (Fig. 3). As expected, the phosphorylation of Akt induced by heregulin was also dramatically inhibited by the PI-3-K inhibitor LY294002.

Induction of Caspase-3 Activation by Aktis

It has been previously shown that LNCaP cells are resistant to TRAIL-induced apoptosis but that treatment with a PI-3-K inhibitor can sensitize these cells to TRAIL (24, 25). We therefore analyzed what effect the isoform-specific inhibitors would have on sensitization of LNCaP cells to TRAIL-induced apoptosis. As a surrogate for apoptosis induction, we analyzed the enzymatic activity of the effector caspase-3 in compound-treated and untreated cells. LNCaP cells were treated with either vehicle or TRAIL, with or without the different isoform-specific Aktis. Prior experiments done in our lab utilizing the PI-3-K inhibitor LY294002 in combination with TRAIL established that the highest level of caspase-3 activation occurred within 3 to 4 hours of TRAIL addition (data not shown). In order to compensate for possible shifts in time to maximal caspase-3 activation with our inhibitors, we stopped the assay at different time points after the addition of TRAIL. At the end of each time period the fold increase in

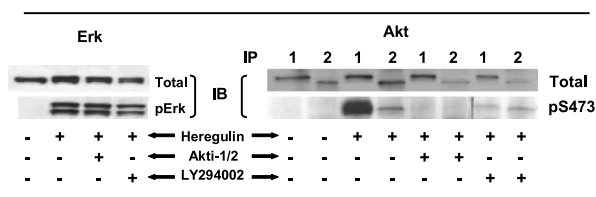


Figure 3. Akt inhibitors are specific for the PI-3-K signal transduction pathway in human breast tumor-derived MCF7 cells. MCF7 cells were treated with Akti-1/2a (20 $\mu\text{mol/L}$) or LY294002 (20 $\mu\text{mol/L}$) for 10 min before the addition of heregulin (10 ng/mL). Immunoprecipitations of cell lysates using extracellular signal-regulated kinase-specific antibodies or isoform-specific Akt antibodies were analyzed by Western blot using corresponding phosphospecific antibodies.

caspase-3 activity over untreated cells was determined. Figure 4 shows that treatment with TRAIL alone resulted in a 5-fold or less increase in caspase-3 activity over this time period. When LNCaP cells were treated with the Akti-1/2 dual inhibitor alone, a statistically nonsignificant increase in caspase-3 activity over untreated cells occurred (bar E). However, when Akti-1/2 was combined with TRAIL a dramatic increase of over 40-fold in caspase-3 activity occurred compared with the caspase activity seen with either TRAIL or compound alone. This high level of synergy in caspase-3 induction with Akti-1/2 and TRAIL treatment requires the inhibition of both Akt1 and Akt2. Treatment of LNCaP cells with Akti-1 or Akti-2 inhibitors, at concentrations that gave equivalent inhibition of the individual isozymes (as shown in Fig. 2), resulted in less than half the induction in caspase-3 activity. However, cotreatment of these cells with a combination of Akti-1 and Akti-2 in the presence of TRAIL generated an increase in caspase-3 activity similar to what was achieved with Akti-1/2.

Activated Akt3 Is Not Able to Compensate for Inhibition of Akt1 and Akt2

Owing to LNCaP cells lacking Akt3, we used the LNCaP/Akt3 line to determine whether Akt3 could rescue the cells from TRAIL-induced apoptosis in the presence of our Akti-1/2 inhibitor. As shown in Fig. 5A, overexpression of Akt3 had a statistically nonsignificant impact on the induction of caspase-3 activity when compared with the parental LNCaP cells lacking Akt3. These data show that activated Akt3, despite being grossly overexpressed (see Fig. 5A, inset), does not significantly prevent caspase-3 activation in cells where Akt1 and Akt2 have been inhibited. We next carried out experiments designed to show that the activation of caspase-3 activity observed in combination with TRAIL would extend to combinations with a clinically relevant chemotherapeutic, the topoisomerase I inhibitor camptothecin (Fig. 5B). The level of induction of caspase-3 activity in both LNCaP and LNCaP/Akt 3 cell lines treated with camptothecin alone is less than 3-fold. However, combination of Akti-1/2 or LY294002 with camptothecin resulted in a greater than 10-fold increase in caspase-3 activation. This level of induction results in a 50% to 60% loss in viable cell numbers as

measured by a standard cytotoxicity assay (Vialight plus, Cambrex; data not shown). The similar synergistic effect observed in both the LNCaP parental and LNCaP/Akt3 cells shows once again that the presence of activated, exogenously overexpressed Akt3 does not provide protection of these cells from caspase-3 induction when Akt1 and Akt2 are inhibited. The inability of Akt3 to provide protection from apoptosis is also suggested by the fact that Akt 1/2 inhibitors, in combination with chemotherapeutics, are potent inducers of apoptosis in multiple cell types with endogenous Akt3 (see below).

Akt1/2 Inhibition Broadly Sensitizes Tumor Cells to a Variety of Therapies

We next evaluated a number of different cancer therapeutics in a variety of tumor cell backgrounds to examine how broadly inhibition of the Akt isoforms would impact tumor cell apoptosis. The results are presented in Table 2. All drug concentrations as well as radiation levels chosen resulted in little or no induction of caspase-3 activity when used as single agents. In all cases, addition of Akti-1 or Akti-2 in combination with different treatments was less effective at increasing caspase-3 activation than combinations with Akti-1/2. We found this to be the case for tumor cell lines derived from breast, ovarian, prostate, and colon carcinomas. Combinations with Akti-1/2 were found to be broadly active, although the relative enhancement of caspase-3 activation varied from additive to synergistic, depending on which tumor line and/or therapy was used. Additionally, our Akt1/2 inhibitor was as effective at inducing caspase-3 activity as LY294002 in cells endogenously expressing Akt3. As LY294002 is a potent inhibitor of all three isoforms, this further supports our finding that Akt3 is not protective when Akt1 and Akt2 are inhibited. Similar data were obtained when standard growth assays

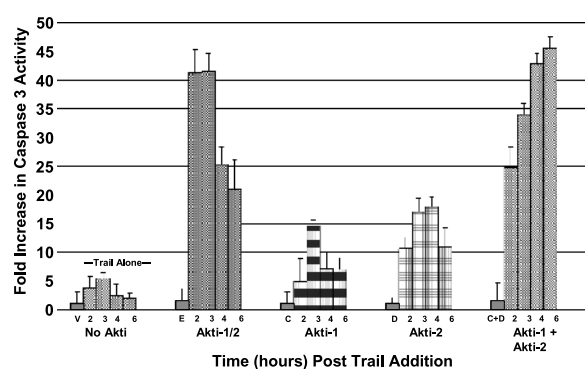


Figure 4. Maximal caspase-3 induction is achieved by inhibition of both Akt1 and Akt2 in combination with TRAIL. Inhibition of Akt1 and Akt2 in the presence of TRAIL generates a greater caspase-3 response than inhibition of either Akt isoform alone. LNCaP cells were treated with Akt inhibitors and TRAIL as described in Materials and Methods. Cells were incubated with Akti-1/2 (5 $\mu\text{mol/L}$), Akti-1 (12 $\mu\text{mol/L}$), Akti-2 (12 $\mu\text{mol/L}$), or a combination of Akti-1 and Akti-2 (each at 12 $\mu\text{mol/L}$) for 1.5 h followed by addition of 0.5 $\mu\text{g/mL}$ TRAIL. Caspase-3 activity was determined at 2, 3, 4, or 6 h after TRAIL addition. V, E, C, D, and C + D, average caspase-3 activity over 6 h in cells treated with vehicle, Akti-1/2, Akti-1, Akti-2, or Akti-1 plus Akti-2, respectively, in the absence of TRAIL.

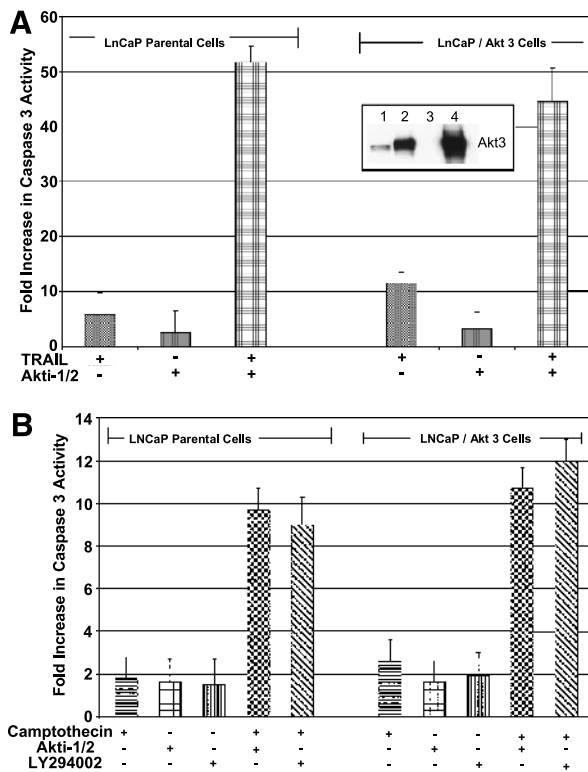


Figure 5. Overexpressed Akt3 does not rescue cells from caspase-3 activation induced by combinations of Akt1/2 inhibitors and either TRAIL or camptothecin. **A**, parental LNCaP and LNCaP/Akt3 cells were treated with either TRAIL (0.5 $\mu\text{g}/\text{mL}$) or Akti-1/2 (5 $\mu\text{mol}/\text{L}$) or a combination of TRAIL and Akti-1/2. *Inset*, immunoblot with isoform-specific Akt3 antibody using the following amounts of total cell lysate: *lane 1*, 50 μg of MDA-468 (a representative cell line that contains physiologic levels of Akt3); *lane 2*, 10 μg of LNCaP/Akt3; *lane 3*, 50 μg of parental LNCaP; and *lane 4*, 50 μg of LNCaP/Akt3. **B**, parental LNCaP and LNCaP/Akt3 cells were treated with either Akti-1/2 (5 $\mu\text{mol}/\text{L}$) or the PI-3-K inhibitor LY294002 (20 $\mu\text{mol}/\text{L}$) in the presence or absence of topoisomerase inhibitor camptothecin (0.1 $\mu\text{mol}/\text{L}$).

(Vialight Plus or Alamar) were used as an end point (data not shown). To further validate that the enhanced caspase-3 activation described above was mediated by Akt inhibition and was not a consequence of some off-target activity of the compounds, we generated an LNCaP cell line, LNCaP/ $\Delta\text{PHmyrAkt}$, that expresses a constitutively activated form of Akt1 which lacks its PH domain. Akt isozymes lacking their PH-domains are not inhibited by our Akt inhibitors (Table 1 and data not shown). The level of expression of the

total $\Delta\text{PHmyrAkt1}$ protein in this cell line is slightly less than the expression level of endogenous Akt (Fig. 6, *inset*). Treatment of these cells with Akti-1, Akti-1/2, or LY294002 in the presence or absence of TRAIL failed to give rise to any significant increase in caspase-3 activation as compared with the LNCaP parental cell line (Fig. 6). This result supports the conclusion that the sensitization to caspase-3 induction by combination treatment is mediated by inhibition of both Akt1 and Akt2 and not by an off-target activity since reintroduction of a constitutively activated, noninhibited form of Akt1 that strongly protects against caspase-3 activation.

Inhibition of Akt Preferentially Sensitizes Tumor Cells

To determine if inhibition of Akt would also result in enhanced activation of caspase-3 in normal cells in the presence of a standard chemotherapeutic, we investigated what effect Akti-1/2 alone or in combination with camptothecin would have on human primary umbilical vein endothelial, normal human prostate epithelial, and human mammary epithelial cells (Fig. 7). Treatment of cells with Akti-1/2 or camptothecin alone resulted in little or no increase in caspase-3 activity in the human primary cells as well as in the human prostate and colon tumor cell lines LNCaP and HT29, respectively. More importantly, the combination of Akti-1/2 and camptothecin had little or no effect on normal cells but caused a greater than 10-fold increase in caspase-3 activity in the tumor cell lines demonstrating Akti-selectivity for tumor cells.

Comparative Activity of Akti-1/2 and Rapamycin

It has been recently reported that inhibition of mammalian target of rapamycin (mTOR) is sufficient to reverse chemoresistance in a mouse model of lymphoma (26) and to reverse prostatic intraepithelial neoplasia formation in a prostate model (27), both driven by activated Akt. To address the issue of whether the induction of caspase-3 activity described above is mediated by the downstream Akt substrate mTOR, we did the following caspase activation experiment. Four different tumor cell lines were evaluated for caspase-3 activation after treatment with Akti-1/2 or the mTOR inhibitor rapamycin in the presence or absence of camptothecin (Fig. 8). In all cases combination treatment with Akti-1/2 was superior to combination treatment with rapamycin in inducing caspase-3 activity. Only in MCF7 cells did rapamycin show more than an additive effect. This result provides evidence that pathways other than mTOR contribute to the prosurvival/antiapoptotic effects of Akt activation.

Table 2. Maximal induction of caspase-3 activity is achieved by inhibition of both Akt1 and Akt2

Cell line	Cotreatment	Fold caspase induction over cotreatment alone				
		Akti-1	Akti-2	Akti-1 + Akti-2	Akti-1/2	LY294002
A2780	Doxorubicin	3.7	2.8	10	6.9	5
MDA-MB468	Camptothecin	1.5	1.1	2.2	2.4	2
BT474	None	1.8	1.2	3.5	3	nd

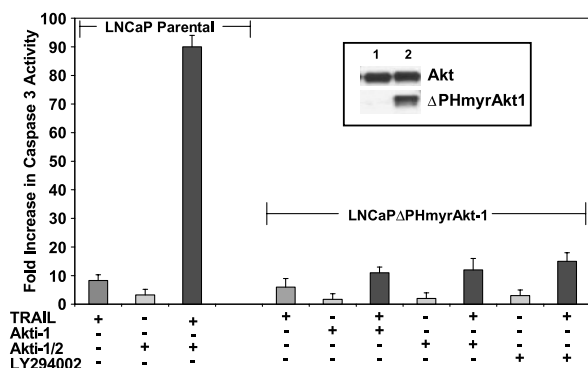


Figure 6. Apoptosis induction by Akti treatment is not due to off-target activity. Constitutively activated Akt inhibits Akti-mediated caspase-3 activation. Parental LNCaP cells and LNCaP/ Δ PHmyrAkt1 cells were treated with Akti-1, Akti-1/2, or LY294002 in the absence or presence of TRAIL. TRAIL (0.5 μ g/mL), Akti-1 (12 μ mol/L), Akti-1/2 (5 μ mol/L), and LY294002 (20 μ mol/L). *Inset*, Western blot analysis of total Akt proteins present in parental LNCaP (*lane 1*) and LNCaP/ Δ PHmyrAkt1 (*lane 2*) cells. Ten micrograms of total cell protein were analyzed.

Discussion

The PI-3-K signaling pathway is deregulated in most human cancers and provides critical survival signals during tumor initiation and progression (1). Overexpression of growth factors, activation of growth factor receptors, mutations in *Ras* and *PI-3-K*, inactivation of *PTEN*, and, less frequently, overexpression of Akt contribute to pathway activation. Akt in turn has multiple downstream effectors, including mTOR, p27^{KIP1}, mdm2, and Bcl-2 family members, which promote cell growth, cell cycle progression, and suppression of apoptosis.

Many PI-3-K pathway components have been shown in animal models to function as oncoproteins or tumor suppressors that, when activated or deleted, are sufficient to promote tumorigenesis. However, animal models provide much less guidance as to which of

these proteins provide the best opportunity for interfering with tumor progression in the clinic. Inhibitors of upstream and downstream pathway components, such as growth factor receptors and mTOR, respectively, have entered the clinic, with decidedly mixed results. Whereas ErbB antagonists, including the monoclonal antibody Herceptin and the small molecule inhibitor Iressa, show efficacy in patients with overexpression of ErbB2 and mutations in *EGFR*, respectively, the majority of patients with advanced cancers do not respond to these agents. Interestingly, the analyses of downstream pathways, including Akt and MapK, in biopsies from treated patients from multiple studies revealed that inhibition of Akt is a prerequisite for response. More specifically, patients in which phospho-Akt levels did not change in response to treatment with epidermal growth factor receptor or ErbB2 antagonists, whether small molecule or antibody, did not show objective responses. Whereas the mechanism of this resistance in the clinic has not been identified yet, it is certainly plausible that mutations downstream of ErbB receptors, such as the deletion of *PTEN*, would render an ErbB antagonist ineffective (28, 29). Owing to *PTEN* mutation so common in human cancers (>50%), it would seem that targeting of a component downstream of *PTEN*, such as *Akt*, would hold more promise in the clinic.

Inactivation of *PTEN* in human tumor cell lines results in increased sensitivity to inhibition of mTOR, one of the many effectors of Akt. Similarly, some animal tumor models suggest that mTOR may be a critical contributor to tumorigenesis. In prostate neoplasia and lymphoma models driven by activated Akt, the inhibition of mTOR by rapamycin or one of its derivatives is sufficient to reverse the activity of Akt (26, 27). Rapamycin-based inhibitors are currently in various stages of clinical development but again have shown very limited efficacy, with promising results to date seen only in renal cell carcinoma.

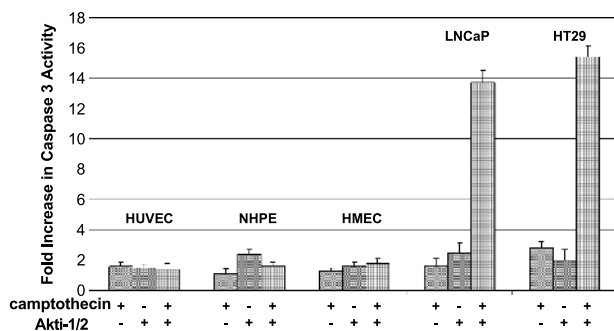


Figure 7. Akt inhibitors preferentially induce caspase-3 activity in tumor cells. Caspase-3 activity was determined in normal human primary cells and tumor cell lines treated individually with Akti-1/2 (5 μ mol/L), camptothecin (0.1 μ mol/L), or with a combination of Akti-1/2 (5 μ mol/L) and camptothecin (0.1 μ mol/L). Normal human umbilical vein endothelial cells (*HUVEC*), normal human prostate epithelial cells (*NHPE*), normal human mammary epithelial cells (*HMEC*), human prostate tumor (*LNCaP*), and human colon tumor (*HT29*) cell lines were analyzed. Representative example taken from three separate experiments.

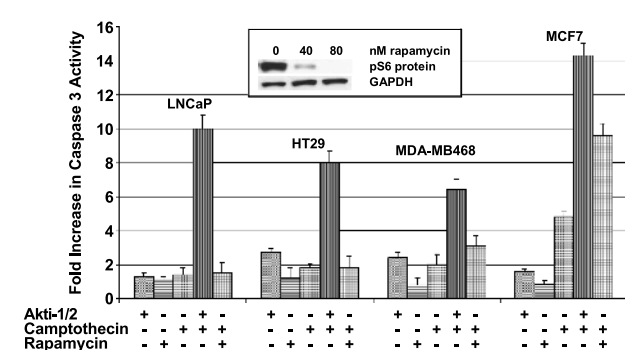


Figure 8. Inhibition of Akt1 and Akt2 is superior to the inhibition of mTOR in inducing caspase-3 activity in different tumor cell lines. Human prostate (*LNCaP*), colon (*HT29*), and breast (*MDA-MB468* and *MCF7*) tumor cell lines were treated with Akti-1/2 (5 μ mol/L) or rapamycin (0.08 μ mol/L) either alone or in combination with camptothecin (0.1 μ mol/L). *Inset*, extracts from LNCaP cells treated with different concentrations of the mTOR inhibitor rapamycin were analyzed for the presence of phospho-S6 protein by immunoblot.

Table 3. Inhibition of Akt1 and Akt2 synergizes with multiple therapeutics in inducing caspase-3 activity

Cell line	Cotreatment	Fold caspase induction by Akt1/2 over cotreatment alone
BT474	Herceptin	2.5
BT474	γ -IR (8 Gy)	22
MDA-MBA468	Camptothecin	2.4
HT29	γ -IR (20 Gy)	9
HT29	Camptothecin	7.7
A2780	Purvalanol	5.2
A2780	Doxorubicin	6.9

NOTE: Maximal induction of caspase-3 in combination drug treatments requires inhibition of Akt1 and Akt2. The ovarian A2780 and MDA-MB468 cell lines contain all three isoforms of Akt. The breast BT474 and colon HT29 cell lines contain only Akt1 and Akt2. The concentration of cotreatment was selected to give ~2-fold increase in caspase-3 activity over untreated cells: doxorubicin, 2 μ mol/L; camptothecin, 0.1 μ mol/L; herceptin, 2 μ mol/L; purvalanol, 2 μ mol/L; Akti-1, 12 μ mol/L; Akti-1/2, 5 μ mol/L. LY294002 (20 μ mol/L), an inhibitor of PI-3-K, blocks the activation of all Akt isoforms.

Based on these data and the fact that several other Akt effectors besides mTOR are known oncogenes, we expect that inhibition of the pathway at the level of Akt will result in superior efficacy compared with the survival targeting agents currently in clinical development. The major questions regarding the targeting of Akt are the tolerability of Akt inhibition, the utility of Akt inhibitors in mono-versus combination therapy, and the role of the individual isozymes. Animal models provide very limited guidance, as the critical double and triple *Akt* gene knockout animals are still under development and none of the currently available *Akt* knockout animals have been crossed with tumor models to evaluate the effect of Akt inhibition on tumorigenesis. The analysis of single *Akt* knockout animals suggests that inhibition of Akt may result in increased levels of apoptosis in some tissues, growth inhibition (Akt1), and, probably most critical, interference with glucose homeostasis (Akt2).

To evaluate the utility of Akt inhibitors as antineoplastic agents and the role of the individual isozymes, we have developed a series of Akt inhibitors with selectivity for subsets of isozymes. In this report, we show how these inhibitors cooperate with chemotherapeutics and biologics in cell culture. We find that simultaneous inhibition of Akt1 and Akt2 is superior to inhibition of a single isozyme, as measured by caspase-3 induction. Akt3, on the other hand, does not seem to be able to compensate for loss of Akt 1/2 survival signaling whether present at physiologic levels (MDA-MB468 and A2780 cells) or highly overexpressed (LNCaP/Akt3 cells). Inhibition of Akt1 and Akt2 is as effective as inhibition of PI-3-K by LY294002 in these caspase induction models. Whereas the physiologic function of Akt3 is not clear at present, the ability to avoid inhibition of Akt3 may provide for a greater therapeutic window for Akt inhibitors and avoid potential toxicity to the central nervous system where Akt3 is the dominant isozyme.

When used as single agents, Akt1/2 dual inhibitors show limited proapoptotic activity in cell culture, although some cell lines, including LNCaP and BT474, are highly sensitive to Akt inhibition alone (data not shown). For the majority of cell lines maximal caspase induction is seen only when combining Akt1/2 dual inhibitors with chemotherapeutics, such as the topoisomerase inhibitor camptothecin, or biologics, such as the death receptor ligand, TRAIL. Similar cooperation between Akt inhibitors and standard cancer treatments was seen with a wide variety of agents as well as γ -radiation (Table 3 and data not shown). As predicted for inhibitors of the PI-3-K pathway, Akt inhibitors are broadly active chemosensitizers in culture. Such compounds have thus the potential to reverse at least one cause of multidrug resistance in the clinic. Similarly, it has been recently shown (26) that blocking mTOR sensitizes tumor cells to killing by conventional chemotherapy agents. Our comparison of Akt inhibitors with rapamycin showed that Akt inhibitors gave consistently better caspase induction despite some dependence on the cell background.

In summary, we have shown that dual inhibitors of Akt1/2 cooperate with standard and experimental cancer treatments. Whereas caspase induction in cell culture does not predict clinical efficacy, Akt inhibitors do hold the promise of being broadly effective agents, particularly in combination therapy. The question of whether, and how much, Akt inhibition will be tolerated in the clinic remains to be answered. Preliminary *in vivo* experiments in rodents indicate that simultaneous inhibition of Akt1 and Akt2 is well tolerated. Future experiments will have to address the question of which combinations of Akt1/2 dual inhibitors and chemotherapeutics provide the best therapeutic window *in vivo*.

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