Akt Is Activated in Response to an Apoptotic Signal*

Damu Tang‡§, Hitoshi Okada‡, Jurgen Ruland¶, Lieqi Liu‡, Vuk Stambolic‡, Tak W. Mak§, and Alistair J. Ingram‡

From the 2Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 1Y2 and Amgen Institute, Toronto, Ontario M5G 2C1, Canada

Akt is a serine-threonine kinase known to exert antiapoptotic effects through several downstream targets. Akt is cleaved during mitochondrial-mediated apoptosis in a caspase-dependent manner. The reason for this is not clear, however, because Akt has not been demonstrated to be activated in response to mitochondrial apoptotic stimuli. Accordingly, we explored whether the well described mitochondrial apoptotic stimuli staurosporine (STS) and etoposide activate Akt and whether such activation impacts apoptosis. Both STS and etoposide activated Akt in NIH 3T3 cells, maximally at 8 and 2 h, respectively, preceding the onset of apoptosis and poly(ADP-ribose) polymerase cleavage. The overexpression of Akt delayed STS-induced apoptosis with an even more pronounced delay observed with overexpression of constitutively active Akt. Akt activation by proapoptotic stimuli lay upstream of mitochondria, because neither caspase inhibitors nor overexpression of Bcl-2 or Bcl-xL could prevent it. Activation depended on phosphatidylinositol 3-kinase activity, however. Conversely, inhibition of phosphatidylinositol 3-kinase with wortmannin sensitized cells to apoptosis initiated by STS. These data demonstrate that mitochondrial apoptotic stimuli also activate Akt and such activation modulates apoptosis in this setting.

Apoptosis is an important cellular response to irreparable damage from exposure to external stimuli or failure to receive survival signals (1). Common to the process is the activation of caspasas, aspartic acid-directed proteases (2). For apoptosis to effectively occur, survival pathways must be inhibited or fail to be activated. In this regard, Akt (also termed protein kinase B or PKB) has been found to be cleaved in a caspase-dependent manner during Jurkat cell apoptosis mediated by Fas ligation, UV irradiation, or etoposide (ETOP) (3). Akt is phosphorylated and activated by phosphatidylinositol 3-kinase (PI3K) in response to mitogens (especially Ras signaling) and adhesion (4, 5). It has been demonstrated that signaling through PI3K/Akt is necessary for transformation by activated mutant Ras (6, 7). Akt delivers an antiapoptotic survival signal by several mechanisms including the phosphorylation and inactivation of Bad (8) and caspase-9 (9) as well as stimulation of NF-xB activity through the activation of IKK (10–12) and inactivation of forkhead transcription factor pathways (13, 14). There is also evidence that the transforming potential of Akt depends on the protein kinase mTOR (15). Constitutive activation of the PI3K/Akt pathway has been observed in several human cancers including ovarian (16), breast (17, 18) and prostate cancers (19). Furthermore, the inhibition of Akt signaling can induce apoptosis in some human cancer cell lines (16, 20).

Importantly, it also seems that Akt signaling can affect the response to chemotherapeutic agents. Inhibition of Akt phosphorylation is seen with topotecan, and the re-expression of phosphorylated Akt renders human lung cancer cells less sensitive to topotecan treatment (21). Similarly, ovarian cancer cells overexpressing constitutively activated Akt are resistant to paclitaxel (22).

The observation that cleavage of Akt occurs during apoptosis (3) suggests that either a level of baseline Akt signaling is vital for cell survival or that Akt activation occurs during apoptosis and acts as a “brake” on the process. Consequently, we have studied the behavior of Akt during apoptosis and indeed find activation of Akt and show that this has functional importance.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—STS, ETOP, and the PI3K inhibitor wortmannin were from Sigma. STS and ETOP were used at concentrations of 0.1–1.0 and 10–100 μM, respectively. zVAD, a pan-caspase inhibitor, was from Enzyme Systems Products. Wortmannin and zVAD were dissolved in MeSO. Wortmannin and zVAD were used at final concentrations of 100 and 50 μM, respectively.

NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum (Life Technologies, Inc.), streptomycin (100 μg/ml), penicillin (100 units/ml), and 2 mM glutamine at 37 °C in 95% air/5% CO2.

Protein Isolation and Immunoblotting—For immunoblotting, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 10 mM NaF, 2.5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Roche Molecular Biochemicals). 50 μg of sample was then separated on a 10% SDS-polyacrylamide gel and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the indicated antibodies in 10 ml of antibody dilution buffer (1× Tris-buffered saline and 0.05% Tween 20 with 5% milk) with gentle rocking at room temperature for 1 h. Signals were detected using an ECL Western blotting kit (Amersham Pharmacia Biotech) and then exposed to x-ray film (X-OMAT, Eastman Kodak Co.). The primary antibodies and concentrations used were: antitotal Akt (1:500; New England Biolabs), anti-phospho-Akt (1:500; 4E2, New England Biolabs) and then exposed to x-ray film (X-OMAT, Eastman Kodak Co.). The primary antibodies and concentrations used were: antitotal Akt (1:500; New England Biolabs), anti-phospho-Akt (1:500; 4E2, New England Biolabs), anti-poly(ADP-ribose) polymerase (PARP) (1 μg/ml; Upstate Biotechnology), anti-GSK3α (1 μg/ml; Upstate Biotechnology), anti-phospho-GSK3α (1 μg/ml; Upstate Biotechnology), anti-HA (0.5 μg/ml; 12CA5, Roche Molecular Biochemicals), anti-Bcl-2 (1 μg/ml;

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‡ To whom correspondence should be addressed: Dept. of Medicine, 500-25 Charlton Ave. East, Hamilton Ontario L8N 1Y2. Tel.: 905-521-6151; Fax: 905-521-6153; E-mail: damu@mcmaster.ca.

§ The abbreviations used are: ETOP, etoposide; PI3K, phosphatidylinositol 3-kinase; NF-xB, nuclear factor xB; STS, staurosporine; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; TNF, tumor necrosis factor; GSK, glycogen synthase kinase; PDK, 3-phosphoinositide-dependent protein kinase.

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Santa Cruz Biotechnology, and anti-Bcl-x, (1 μg/ml; Transduction Laboratories).

Akt Activity Assay—Akt activity was measured in vitro using histone H2B as the substrate. After protein isolation from total cell lysate as described above, 200 μg of total protein was then incubated with anti-Akt antibody immobilized on protein G-Sepharose A beads (100; New England Biolabs) with gentle rocking overnight at 4 °C. The beads were washed three times with the lysate buffer and once with the kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM MgCl2) and resuspended in 50 μl of 1× kinase buffer supplemented with 2 μg of histone H2B and 1 μg/ml bovine serum albumin. Akt activity was then determined by the addition of 200 μl [γ-32P]ATP (1000 cpn/pmol) incubated for 30 min at 30 °C. The reaction was terminated with the addition of 3× SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% w/v SDS, 30% glycerol, 150 mM sodium orthovanadate, and 10 mM MgCl2) and resuspended in 50 μl of 1× kinase buffer supplemented with 2 μg of histone H2B and 1 μg/ml bovine serum albumin. Akt activity was then determined by the addition of 200 μl [γ-32P]ATP (1000 cpn/pmol) incubated for 30 min at 30 °C.

DNA fragmentation after exposure to STS or ETOP was assayed as published previously (23).

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Results

Mitochondrial Apoptotic Stimuli Lead to Akt Activation—Recently it was shown that the activation of Akt mediates survival of cells in various death settings. Forced expression of Akt protects neuronal cells from apoptosis induced by withdrawal of the survival factors insulin-like growth factor-1 or nerve growth factor (24, 25) and protects from interleukin-3 withdrawal-induced apoptosis in hematopoietic cells (26). The activation of Akt also protects cells from apoptosis induced by detachment from extracellular matrix and c-myc overexpression (7, 27). The common feature of these apoptotic processes, all inhibited by the forced expression of Akt, is that they are executed primarily through the mitochondrial apoptotic pathway.

NF-κB, which strongly protects against death receptor-induced apoptosis, is activated by TNFα ligation of death receptors in concert with the initiation of the death signal (12). The NF-κB activation observed in this setting is through the IκB/ Akt pathway (12). There is also evidence of NF-κB activation after ETOP treatment associated with an increase in Akt and a Bcl-2-like protein (28). Given this and the known ability of forcibly expressed Akt to inhibit mitochondrial apoptosis, we sought to determine whether well-established mitochondrial apoptotic stimuli would activate Akt. To address this, NIH 3T3 cells were treated initially with STS, a potent protein kinase inhibitor that induces apoptosis by directly stimulating cytochrome c release from mitochondria (29). STS exposure led to caspase activation and cell death as predicted (Fig. 1A, upper panel). PARP cleavage was demonstrable at 8 h, correlating well with the exponential increase in cell death observed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining beginning by 8 h. Interestingly, besides inducing apoptosis, however, STS also led to Akt activation detected by increasing levels of Ser-473 phosphorylation beginning by 2 h and maximally at 8–12 h (Fig. 1A). Three events are required for Akt activation: recruitment to the cell membrane through the N-terminal PH-domain, Thr-308 phosphorylation in the kinase domain, and Ser-473 phosphorylation in the C-terminal tail (30). Although phosphorylation on Ser-473 always correlates with in vivo Akt activation, to ensure that increases in phospho-Akt represented increases in Akt activity the phosphorylation of a histone H2B target protein in the presence of immunoprecipitated Akt was measured. Consistent with the phospho-Akt data, marked histone H2B phosphorylation was observed after 8 h of STS treatment (Fig. 1B). As expected, the positive control platelet-derived growth factor also led to marked histone H2B phosphorylation.

Because STS is a potent inhibitor of many protein kinases, it could activate Akt by the blockade of an Akt-directed protein kinase that delivers a negative phosphorylation signal. To address whether Akt activation in an apoptotic process induced by STS is unique, we examined ETOP-induced apoptosis, which is also known to be executed through the mitochondrial apoptotic pathway (31, 32). Akt phosphorylation was observed 2 h after the addition of ETOP to NIH 3T3 cells (Fig. 2, upper panel). The kinetics are different, however, in that maximal Akt phosphorylation induced by ETOP occurred at 2–4 h, which contrasts with the 8-h time point maximum seen with STS. Phosphorylation correlated with activity, as revealed by Akt-dependent phosphorylation on Ser-21 of glycogen synthase kinase 3-α (GSK3α), a known specific Akt target (33) (Fig. 2, lower panel). These data indicate that Akt activation, usually associated with the inhibition of cytochrome c release (34), occurs in the presence of apoptosis induced by agents that require cytochrome c release for their actions (32).

Akt Activation Protects against Mitochondrial Apoptotic Cell Death—To determine whether Akt activation is functional in mitochondrial apoptotic cell death, we wished to determine whether the activation of Akt affected cellular execution in these settings. HA-tagged bovine Akt, either in its native form (HA-Akt) or in a constitutively active form (HA-Akt-DD) generated by the substitution of Thr-308 and Ser-473 with an aspartic acid (D), was successfully overexpressed in NIH 3T3 cells infected with pBabe/HA-Akt, and pBabe/HA-Akt-DD in response to STS. PARP cleavage, and phosphorylation of both endogenous Akt and HA-Akt were examined in NIH 3T3 cells infected with pBabe, pBabe/H9262, and pBabe/H9262-Akt in response to STS stimulation. As expected, the treatment of vector retrovirus-infected (pBabe) cells with STS led to Akt phosphorylation, again maximal at 8–12 h, and PARP cleavage was again observed by 8 h (Fig. 3B). Overexpression of native HA-Akt led to the appearance of two phospho-Akt bands after STS treatment, with the slower migrating band being the HA-tagged overexpressed protein. PARP cleavage was clearly delayed by the overexpression of Akt, now occurring maximally between 8 and 12 h (Fig. 3B). The phospho-specific anti-Ser-473 antibody, 4E2, again detects the activation of endogenous Akt in NIH 3T3 cells infected with pBabe/H9262-Akt. The antibody also detects the DD form of Akt with Ser-473 substituted by a D residue even in untreated cells, indicating that the antibody recognizes the negative charge at residue 473 rather than the phosphorylated Ser-473 itself. However, 4E2 does have a much higher affinity for this region when Ser-473 is phosphorylated rather than replaced with a D substitution (data not shown). Overexpression of constitutively active Akt (HA-Akt-DD) was even more effective than overexpression of wild-type Akt at delaying PARP cleavage, with cleavage not observed until 24 h in this condition (Fig. 3B). These data indicate that the activa-
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FIG. 1. Activation of Akt by STS in NIH 3T3 cells. A, NIH 3T3 cells were incubated with 1.0 μM STS for the indicated times at 37 °C. Akt phosphorylation, apoptotic cell death, and PARP cleavage were measured as described under “Experimental Procedures.” All experiments were performed at least three times; representative blots are shown (upper panel), and the results showing Akt activity and apoptosis with error bars (n = 3 experiments) are shown graphically (lower panel). B, NIH 3T3 cells were mock-treated with Me2SO (DMSO), treated with 1 μM STS for 8 h, or treated with platelet-derived growth factor (PDGF) at 100 ng/ml for 30 min after serum starvation for 5 h. Cell lysate was prepared, and Akt was immunoprecipitated with an anti-Akt antibody as described under “Experimental Procedures.” The precipitated Akt was used to phosphorylate histone H2B in vitro and was detected by Western blot using an anti-Akt antibody.

activation of Akt in response to STS may have functional significance and suggests that Akt may act as a brake on apoptotic processes. This is consistent with the notion that survival functions are also initiated along with the activation of an apoptotic process. It should be noted that ultimately, by 24 h PARP cleavage is complete under all conditions, indicating that Akt activation functions to delay rather than diminish apoptosis.

We reasoned that if the activation of Akt functions as a brake to slow down apoptosis, inhibition of Akt activation might sensitize cells to apoptosis. NIH 3T3 cells were treated with or without the PI3K inhibitor wortmannin together with 0.1 μM STS or 10 μM ETOP. DNA fragmentation was observed at 12 h, with co-incubation with 100 nM wortmannin rendering it more prominent (Fig. 4A). Similarly, PARP cleavage was also observed at 12 h, again more prominently with wortmannin (Fig. 4B). Quantification of cell death with terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling confirmed that 19.4% of STS-treated cells were dead at 12 h, and this was increased to 35% with wortmannin co-incubation (Fig. 4, B and C). Similar data were observed with ETOP (data not shown). These results indicate that wortmannin-sensitive Akt activation in response to STS or ETOP indeed does have functional significance and may delay apoptosis.

STS-induced Akt Activation Takes Place Upstream of Mitochondria and Requires PI3K Activity—One can distinguish whether an apoptotic process is initiated through death receptors or mitochondria by determination of the relationship between cytochrome c release from mitochondria and caspase activation. Cytochrome c release from mitochondria depends on Bid cleavage by caspase-8 in the death receptor apoptotic pathway and is independent of caspase activity in the mitochondrial apoptotic pathway (29). Consequently, we sought to determine whether the inhibition of caspase activity would affect Akt activation in response to STS. zVAD, a broad spectrum caspase inhibitor, inhibited PARP cleavage but was without effect on Akt phosphorylation after 8 and 12 h of STS treatment of NIH
Subsequently we wished to determine whether STS-induced Akt activation depends on PI3-kinase activity. NIH 3T3 cells were treated with STS with or without 100 nM wortmannin. Wortmannin completely abolished STS-induced Akt activation (Fig. 5B), indicating the dependence on PI3-kinase activity for STS-induced Akt activation.

To demonstrate firmly that Akt activation in response to apoptotic stimuli occurs upstream of mitochondria, we wished to address whether inhibition of cytochrome \( c \) release would affect STS-induced Akt activation (Fig. 5B), indicating the dependence on PI3-kinase activity for STS-induced Akt activation.

To demonstrate firmly that Akt activation in response to apoptotic stimuli occurs upstream of mitochondria, we wished to address whether inhibition of cytochrome \( c \) release would affect STS-induced Akt activation. The expression of either Bcl-2 or Bcl-x\(_L\) is known to maintain the integrity of mitochondria and to prevent cytochrome \( c \) release in response to diverse apoptotic stimuli (35). NIH 3T3 cells infected with pBabe, HA-Akt, and HA-Akt-DD were treated with STS at 1 \( \mu \)M for the times indicated. Retrovirus pBabe-infected NIH 3T3 cells were also treated with platelet-derived growth factor (PDGF) as outlined in the Fig. 1 legend to provide a positive control. The cell lysate was prepared and Western blotted with the phospho-specific anti-Akt (4E2) antibody (first panel), an antitotal Akt antibody (second panel), an anti-PARP antibody (third panel), and an anti-actin antibody (fourth panel). All the experiments were repeated at least three times, and representative blots are shown.

DISCUSSION

Proliferation and death (apoptosis) are two sides of the same coin. Although their functions are opposed, they are tightly connected. Deregulation of the oncogenes \textit{myc}, \textit{E1A}, or \textit{E2F} drives cell cycle progression but may also promote apoptosis in certain conditions (36). The mitotic and proapoptotic properties of \textit{c-myc} require intact N-terminal transcriptional, DNA binding, and dimerization domains (37, 38). However, transcription-defective \textit{c-mycS} is also capable of promoting apoptosis as well as retaining the ability to promote cell proliferation (39). The functions of E1A in promoting proliferation and apoptosis are also linked and were both mapped to the N-terminal region that is involved in binding pocket proteins such as Rb (retinoblastoma protein), which is essential in the regulation of cell cycle progression (36, 40). Mutations in \textit{E2F} that prevent binding to Rb accelerate both cell cycle progression and promote apoptosis (41).

Conversely, apoptotic stimuli may activate counterbalancing survival functions. The binding of TNF\( \alpha \) to TNF receptor-1 activates caspase-8 and NF-\kappaB in a TNF receptor-associated death domain protein-dependent manner (42, 43). The activa-
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Akt is activated in response to an apoptotic signal, which can be mediated through the PI3K/Akt pathway. This pathway promotes cell survival through various mechanisms, including the inactivation of pro-apoptotic signals like Bad (8) and caspase-9 (9) and the stimulation of NF-κB activity (10–12). Akt-mediated inhibition has not been reported. This study examines the role of the Akt/protein kinase B pathway in cellular apoptosis induced by two diverse mitochondrial apoptotic stimuli: the potent protein kinase inhibitor (STS) and the DNA damage reagent (ETOP).

These agents were chosen because overexpression of Akt has been demonstrated to impair apoptosis induced by either STS (46) or ETOP (10). In response to STS and ETOP, Akt was activated. Although Akt activation occurs early after stimulation, 8 h for STS and 2 h for ETOP, late Akt cleavage in certain apoptotic settings (3). More importantly, we have demonstrated that this activation has a functional importance in that it delays and diminishes PARP cleavage and apoptosis (Figs. 3 and 4).

**Fig. 4.** Akt inhibition sensitizes to apoptosis. NIH 3T3 cells were incubated with 0.1 μM STS with or without 100 nM wortmannin (Wort) for 12 h at 37 °C. DNA fragmentation (A) and PARP cleavage and apoptosis (B) in response to STS were determined as described under “Experimental Procedures.” PARP cleavage and apoptosis with error bars (n = 3 experiments) are shown graphically in C. con, control.

**Fig. 5.** Pathways of STS-induced Akt activation. NIH 3T3 cells were pretreated with 50 μM zVAD (A) or wortmannin (Wort, B) for 30 min before the addition of STS (1 μM) for 8 h (B) or for the times indicated (A). Akt activation was determined by Western blot using the phospho-specific anti-Akt antibody. The PARP and actin levels in these cells were also examined by Western blot using specific antibodies (A). con, control.

**Fig. 6.** Overexpression of Bcl-2/Bcl-xL does not inhibit STS-induced Akt activation. NIH 3T3 cells were infected with retrovirus expressing either LacZ, human Bcl-2, or human Bcl-xL. The expression of Bcl-2 or Bcl-xL was determined by Western blot using specific antibodies (A). These cells were then either mock-treated or treated with 1 μM STS for 8 h. Akt activation was examined by Western blot using the phospho-specific anti-Akt antibody (B). The actin level in these cells is also shown (B).
tation of apoptosis seems to occur at a postmitochondrial level, given that Akt overexpression can prevent apoptosis initiated by the microinjection of cytochrome c (47, 48), although others have found that Akt prevents the release of cytochrome c from mitochondria (34). We find that Akt activation by STS occurs upstream of mitochondria, i.e. outside of apoptotic core machinery, in that it is not affected by either caspase inhibition (Fig. 5A) or Bcl-2/Bcl-xL overexpression (Fig. 6B). Consequently, we would not expect that all mitochondrial apoptotic signals activate Akt. This is consistent with our observation that no Akt activation is detected in NIH 3T3 cells treated with either UV or ionizing radiation (data not shown) and reflects observations of NF-κB activation in the death receptor apoptotic pathway. Except for the clear protective role of NF-κB activation in TNFα induced apoptosis, its role in other death receptor-induced apoptosis is not clear.

These data have implications for cellular transformation. A large and growing number of human tumors are associated with PI3K/Akt amplification or activation including cervical (18), ovarian (16), breast (17, 18), and prostate cancers (19), with PI3K/Akt overexpression or activation including cervical (49), ovarian (16), breast (17, 18), and prostate cancers (19), and the effect of preventing it.

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The data presented here add one more potential indication of the importance of Akt activity in tumor progression. Therapy-resistant cancers arising after radiotherapy or chemotherapy present major problems in cancer therapy. Chemotherapeutic agents like ETOP target the mitochondrial apoptotic pathway to execute tumor cells. The ability of ETOP to also activate Akt might lead to the emergence of cancers resistant to therapy. In this regard, overexpression of Akt in ovarian cancer cells led to paclitaxel resistance, which is associated with Bad inactivation and a failure of apoptosis (22), and the overexpression in lung cancer cells resulted in topotecan resistance (21). Conversely, the inhibition of PI3K/Akt signaling with wortmannin enhanced pancreatic carcinoma cell sensitivity to gemcitabine (50). If chemotherapy agents such as ETOP induce Akt activation, the activation of NF-κB might enable overcoming drug resistance.

In view of this, it is important to understand precisely how Akt is activated. Presently, this is not totally clear. Activation requires recruitment to the plasma membrane by ligation of the N-terminal PH-domain to the PI3-kinase substrates PI3,4,5-P3 and inositol 1,4,5-trisphosphate. This brings Akt in close proximity to 3-phosphoinositide-dependent protein kinase (PKD)-1, resulting in the phosphorylation of Thr-308 in the T loop domain. Phosphorylation of the Ser-473 in the C-terminal hydrophobic motif is also required for Akt activation, but the putative kinase PKD2 that phosphorylates Ser-473 of Akt has not been clearly identified (30). The debate whether PKD1 could also phosphorylate Ser-473 of Akt was settled in the negative by the demonstration of inhibition of phosphorylation of Thr-308 but not Ser-473 in PKD1−/− mouse embryonic stem cells in response to insulin-like growth factor-1 (51). Regardless of the identity of PKD2, our data indicate that STS at 1 μM does not inhibit its activity. Because STS inhibits numerous kinases with IC50 values within the nM or sub-nM range, this finding might offer valuable information for searching this important kinase.

The data presented here indicate that apoptosis-inducing agents including the chemotherapeutic drug etoposide also independently activate PI3K/Akt, and such activation delays apoptosis. Further work will be required to determine the precise mechanism of activation of PI3K/Akt by these agents and the effect of preventing it.
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