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Survival-signaling pathway as a promising target for cancer chemotherapy

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Abstract The serine/threonine kinase AKT, also known as PKB or RAC-PK, is a key molecule for protecting cells from undergoing apoptosis. Several studies have suggested that the AKT-mediated survival-signaling pathway is an attractive target for cancer chemotherapy: (1) the AKT pathway is relatively inactive in resting cells; (2) amplification of the *AKT* gene occurs in some tumors; (3) loss of the tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) is common in tumors and its loss constitutively activates AKT; (4) AKT is activated at the cancer invasion front. To clarify which drugs exhibit their cytotoxicity by inhibiting the AKT pathway, we screened anticancer drugs that could downregulate phospho-AKT levels and AKT kinase activity. We found that UCN-01 (7-hydroxystaurosporine), heat-shock protein 90 (HSP90) inhibitors, and topotecan (10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin) possessed the ability to interfere with the AKT pathway. UCN-01 directly suppressed upstream AKT kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) ($IC_{50} < 33$ nM) both in vitro and in tumor xenografts. HSP90 inhibitors and topotecan suppressed AKT activity via indirectly downregulating PDK1 and phosphatidylinositide-3-OH kinase activities. Transfection of the constitutively active *AKT* complementary DNA into cells attenuated the cytotoxic effects of the drugs, indicating that inhibition of the AKT pathway plays an important role in exerting their cytotoxic effects. These results strongly suggest that the AKT-mediated survival-signaling pathway is a promising and attractive target for cancer chemotherapy.

Keywords Survival signal · AKT · Anticancer drugs · PDK1 · HSP90

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Introduction

The susceptibility of cells to undergo apoptosis appears to be dependent on the balance between proapoptotic and survival (antiapoptotic) signals. Diverse anticancer drugs induce apoptosis while they engage different intracellular targets and cause DNA damage. Therefore anticancer drugs may induce apoptosis not only by increasing proapoptotic signals but also by decreasing survival signals such as the AKT-mediated survival-signaling pathway.

Numerous reports have indicated that growth factors and cytokines stimulate cell survival [10, 15, 23]. After stimulation with growth factors and cytokines, phosphatidylinositide-3-OH kinase (PI3 K) is activated and phosphorylates phosphoinositides. The interaction of the phospholipid second messenger molecule generated, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5) P_3), with the pleckstrin homology domain of AKT recruits AKT to the plasma membrane, where it is phosphorylated at two key regulatory sites, Thr308 (by 3-phosphoinositide-dependent protein kinase (PDK)-1) and Ser473 (by an as-yet-unidentified Ser473 kinase, PDK2) residues. Phosphorylation at both residues is necessary for full activation of AKT and the subsequent regulation of biological responses, including apoptosis inhibition (by phosphorylation-dependent inactivation of proapoptotic BAD, a caspase family member caspase-9, Forkhead transcription

factors, and IκB kinase) [23] and cell-cycle progression (by phosphorylating and inactivating cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$) [9].

In this paper, we document the role of the AKT-mediated survival-signaling pathway in anticancer drug-induced apoptosis. We report that some anticancer drugs exhibit their cytotoxic effects by suppressing the AKT signaling pathway. Because transfection of the active form of *AKT* complementary DNA (cDNA) attenuates the cytotoxic effects of the drugs, inhibition of the survival-signaling pathway plays an important role in anticancer drug-induced apoptosis.

Materials and methods

Materials

UCN-01 (7-hydroxystaurosporine), radicicol, and oxime derivatives of radicicol (KF25706 and KF58333) were kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan). Geldanamycin was purchased from Sigma (St Louis, Mo.). Topotecan (10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin) was obtained from GlaxoSmithKline (King of Prussia, Pa.). Camptothecin was a generous gift from Yakult (Tokyo, Japan). Etoposide was kindly provided by Bristol-Myers Squibb (Tokyo, Japan).

Cell culture conditions

A549 and its camptothecin-resistant subline A549/CPT were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 μg/ml of kanamycin [14]. 293T, HT1080, and COS-7 cells were cultured in DMEM supplemented with 10% FBS [20].

Expression vector construction

Human wild-type (WT) *AKT1* cDNA, and dominant-negative *AKT1* cDNA and its deletion mutants were generated as described previously [9]. The human full-length *HSP90α* and *HSP90β* cDNAs in a pcDNA3.1/GS vector were purchased from Invitrogen (Carlsbad, Calif.) [20]. The Myc- and $(His)_6$-epitope-tagged N-terminal myristoylated active mouse *AKT1* cDNA in a pUSEamp vector was purchased from Upstate Biotechnology (Lake Placid, N.Y.). The Myc-tagged human WT *PDK1* cDNA in a pCMV3 vector was kindly provided by Drs. P. Hawkins and K. Anderson (Babraham Institute, Cambridge, UK). The HA-tagged PI3 K *p110α* subunit cDNA containing the membrane-targeting CAAX motif (active PI3 K) and its inactive mutant cDNA (kinase-dead PI3 K) in pcDNA3 vectors were kindly provided by Dr. M. Thelen (Istituto di Ricerca in Biomedicina, Bellinzona, Switzerland).

Western blot analysis

Western blot analysis was performed as described previously [8]. In brief, cells were electrophoresed and transblotted on to a nitrocellulose membrane. After blocking, the membranes were incubated with appropriate first antibodies. The membrane was then incubated with an appropriate peroxidase-conjugated second antibody and developed with the enhanced chemiluminescence mixture (Amersham, Little Chalfont, UK).

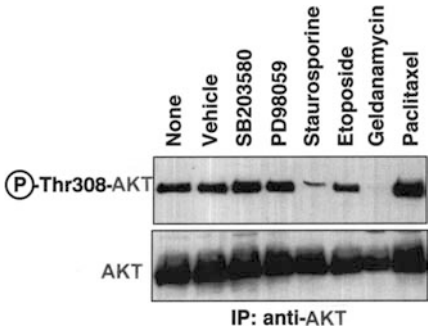


Fig. 1 Effects of chemotherapeutic drugs on phospho-AKT levels. 293T cells were transfected with wild-type *AKT* cDNA. After transfection for 24 h, cells were cultured in the medium alone (*None*) or medium containing DMSO (*Vehicle*), SB203580 10 μM, PD98059 30 μM, staurosporine 1 μM, etoposide 60 μg/ml, geldanamycin 10 μM, or paclitaxel 10 μM for 15 h. The immunoprecipitated AKT proteins were electrophoresed and immunoblotted with an anti-phospho-Thr308-AKT antibody (*upper*) or an anti-AKT antibody (*lower*) (*IP* immunoprecipitation)

Measurement of AKT, PDK1, and PI3 K kinase activities

AKT kinase activity was estimated by measuring incorporation of [γ-^{32}P]ATP into the peptide of glycogen synthase kinase-3. We measured PDK1 kinase activity using a PDK1 kinase assay kit (Upstate Biotechnology) according to the manufacturer's instructions. PI3 K activity was determined as previously described [14].

Results

Regulation of AKT-mediated survival-signaling pathway by heat-shock protein 90 (HSP90)

Screening of anticancer drugs with direct effects on the AKT pathway revealed that staurosporine and the HSP90 inhibitor geldanamycin possessed the ability to decrease the amount of phospho-AKT (Thr308) in 293T cells (Fig. 1).

We first examined the effect of HSP90 inhibitors (e.g. geldanamycin, radicicol, and radicicol analogs) on the AKT signaling pathway. HSP90 is an abundant and highly conserved protein involved in a diverse array of cellular processes. In contrast to other HSPs, HSP90 is not required for maturation or maintenance of most proteins in vivo. Most of the identified cellular targets are signaling proteins. HSP90 acts as a chaperone for unstable signal transducers and keeps them poised for activation until they are stabilized by conformational changes associated with signal transduction.

We have previously reported that AKT is a HSP90 client protein [20]. It forms a complex with HSP90 via its middle domain. The AKT-binding domain of HSP90 is different from the previously identified N-terminal ATP-binding and geldanamycin-binding domain and the C-terminal oligomerization domain. The results are supported by geldanamycin not inhibiting AKT binding to HSP90. When AKT–HSP90 binding is inhibited by overexpressing the binding domain-containing AKT

deletion mutant (1–309 AKT), AKT is inactivated, which results in increased sensitivity of the cells to apoptosis-inducing stimuli such as growth factor withdrawal and chemotherapeutic drugs [20]. Examining the role of HSP90–AKT binding has revealed that HSP90 protects AKT from dephosphorylation mediated by protein phosphatase 2A (PP2A) [20].

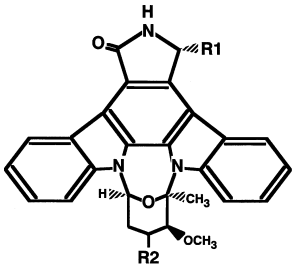
Endothelial nitric oxide synthase (eNOS) has been reported to bind to HSP90 through its middle domain [7]. Because AKT-dependent phosphorylation of eNOS at Ser1179 is promoted by HSP90 [7], HSP90 may function as a scaffold for AKT and its substrates. These facts indicate that the interaction of AKT with HSP90 might play an important role in AKT and its subsequent cellular functions. Thus we hypothesized that HSP90 inhibitors downregulate AKT signaling by inhibiting AKT–HSP90 binding. However, HSP90 inhibitors did not inhibit AKT–HSP90 binding. Moreover, HSP90 inhibitors did not directly affect AKT kinase activity in vitro. Therefore, we examined the effects of HSP90 inhibitors on upstream AKT kinases. We found that treatment of the cells with HSP90 inhibitors decreases the amount of PDK1 without directly inhibiting PDK1 kinase activity [8]. The kinase domain of PDK1 was found to be essential for complex formation with HSP90 in vivo and HSP90 inhibitors suppress PDK1–HSP90 complex formation.

Proteasome was involved in PDK1 degradation after inhibiting PDK1–HSP90 binding. Treatment with proteasome inhibitors increased the amount of detergent-insoluble PDK1 in HSP90 inhibitor-treated cells. Therefore HSP90 regulates the stability, solubility, and signaling of PDK1. These results indicate that HSP90 plays an important role in the AKT signaling pathway.

17-Allylaminogeldanamycin (17AAG) has been reported to suppress AKT kinase activity by inhibiting PI3 K activity in HER2-overexpressing breast cancer cells [4]. Moreover, 17AAG induces proteasome-dependent AKT degradation [4]. This adds to our previous reports of caspase-mediated cleavage of AKT [2, 19]. Thus HSP90 might be a promising target for developing new anticancer drugs that could block the antiapoptotic AKT signaling pathway.

Identification of upstream AKT kinase PDK1 as a molecular target for UCN-01

UCN-01 was originally isolated as a selective protein kinase C (PKC) inhibitor from the culture broth of *Streptomyces* species. Because UCN-01 exhibits potent antitumor activity in in vivo and in vitro tumor models [1], its clinical usefulness has been investigated in the USA. Although the PKC family was thought to be a major target of UCN-01 [1], it remains unclear whether PKC is the sole target of UCN-01 and how PKC inhibition is involved in its antitumor activity. As shown in Fig. 1, screening the AKT inhibitory drugs revealed that staurosporine possesses the ability to decrease levels of



Drug	R1	R2
Staurosporine	H	NH–
UCN-01	OH	NH–
CGP41251	H	

Drug	IC_{50} (nM)		
	PDK1	AKT	PKC
Staurosporine	220	830	4
UCN-01	< 33	491	< 3
CGP41251	1720	>1000	40

Fig. 2 Structure and IC_{50} values of staurosporine and its analogs (*PDK1* 3-phosphoinositide-dependent protein kinase-1, *PKC* protein kinase C)

phospho-AKT (Thr308). We thus examined the effects of the staurosporine analog UCN-01 on upstream AKT kinases and found that UCN-01 directly suppressed upstream AKT kinase PDK1 with an IC_{50} value of < 33 nM in in vitro and in vivo assays (Fig. 2) [21]. Moreover, we found that UCN-01 suppressed PDK1 kinase activity in vivo using xenografted NL-17 and PC-3 tumors. In contrast, UCN-01 had marginal effects on the activities of AKT and PI3 K. Overexpression of the active form of AKT diminished the cytotoxic effects of UCN-01, suggesting that UCN-01 may exert its cytotoxicity in part by inhibiting the PDK1-AKT survival pathway. A recent report also documented that staurosporine and its analog (CGP41251) suppress PDK1 kinase activity [10]. Because UCN-01 is the most powerful and selective PDK1 inhibitor (Fig. 2) and has already proved to have potent antitumor activity in vivo and in vitro [1], PDK1 may be a promising target for developing new anticancer drugs.

Topotecan exhibits its antitumor activity in part by interfering with the AKT-mediated survival-signaling pathway

Topotecan is a novel topoisomerase I inhibitor that has shown activity against numerous human tumor cell lines and xenografts. Topotecan has also shown clinical activity in small-cell and non-small-cell bronchogenic carcinoma, ovarian carcinoma, and myeloid leukemia, and has been approved in the USA for the treatment of relapsed ovarian cancer and small-cell lung cancer. When human lung cancer A549 cells were incubated with topotecan, cells underwent apoptosis with nuclear fragmentation [14]. Under these conditions, AKT is dephosphorylated in a dose-dependent manner. Camptothecin has the same ability to decrease phospho-AKT levels in A549 cells [14]. Measurement of AKT kinase activity showed that topotecan decreases cellular AKT kinase activity [14]. Interestingly, AKT dephosphoryla-

tion could not be seen in a topotecan-resistant A549/CPT cell line [14]. This suggests that AKT inactivation plays an important role in topotecan-mediated cytotoxic effects. This was supported by the results of a study showing that overexpression of the active form of AKT significantly suppressed topotecan-induced cell death while the kinase-dead form of *AKT* cDNA had no effect on sensitivity to topotecan [14].

To investigate the mechanism(s) of AKT dephosphorylation, we examined whether topotecan inactivates the upstream AKT kinases, PI3 K and PDK1. We found that topotecan suppresses PDK1 kinase activity and downregulates PI3 K activity in a dose-dependent manner [14]. These results indicate that topotecan exerts its cytotoxic effects in part by suppressing PI3 K and PDK1 kinase activity, which results in the turning off of the AKT-mediated survival-signaling pathway.

Reports have documented that topotecan inhibits angiogenic growth in the in vivo rat disc angiogenesis and mouse cornea angiogenesis models [6, 16]. Because the PI3K-AKT signaling pathway appears to play an important role in angiogenesis, we investigated the possibility that topotecan exerts its antiangiogenic effect by downregulating the PI3K-AKT signaling pathway. We found that topotecan downregulates phospho-AKT levels and in vitro migration of endothelial cells [15]. Because transfection of the constitutively active *AKT* cDNA attenuated the inhibitory effect of topotecan on endothelial migration, topotecan might exert its antiangiogenic activity by downregulating the PI3K-AKT signaling pathway [15]. These results suggest that the PI3K-AKT signaling pathway may be associated not only with cell survival but may also play a role in angiogenesis.

Discussion

After stimulation with growth factors and cytokines, AKT is recruited to the plasma membrane and is phosphorylated at Thr308 by PDK1 and at Ser473 by PDK2. The serine/threonine-specific phosphatase PP2A is known to inhibit AKT kinase activity. Thus the balance between PDK1/PDK2-mediated phosphorylation and PP2A-mediated dephosphorylation may determine AKT kinase activity in vivo. However, it is not clear whether AKT kinase activity is regulated by AKT-interacting protein(s). We initially attempted to identify the AKT-binding protein by AKT immunoprecipitation following immunoblot analysis. Fortunately, we found that AKT is associated with HSP90 in vivo.

HSP90 is an abundant and highly conserved protein involved in a diverse array of cellular processes. The AKT-binding domain of HSP90 is different from the N-terminal domain responsible for binding to ATP, geldanamycin, unfolded proteins, and small peptides, or the C-terminal oligomerization domain. These findings were supported by geldanamycin not inhibiting AKT binding to HSP90. The connecting middle domain of HSP90, where AKT could bind, was of variable length and missing in bacterial HSP90s. The middle domain of HSP90 might regulate cell growth and apoptosis by binding to AKT. This hypothesis was strengthened by the observation that *Escherichia coli* HSP90, which lacks the middle domain, does not suppress the decrease in viability of $HSP90^{-/-}$ yeast strains [17]. Because HSP90 is constitutively overexpressed at two to ten times higher levels in tumor cells, HSP90 could be associated with the upregulation of AKT kinase activity in tumor cells by preventing PP2A-mediated AKT dephosphorylation. Although we have not yet found the drugs that could inhibit AKT binding to HSP90, AKT–HSP90 binding would be an interesting target for developing new types of anticancer drugs (Fig. 3).

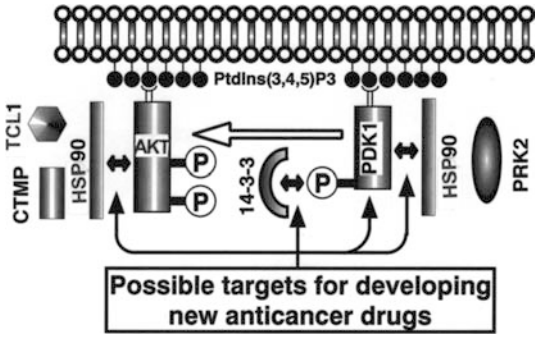


Fig. 3 Possible molecular targets for developing new AKT signaling inhibitors (*CTMP* carboxyl-terminal modulator protein, *HSP90* heat-shock protein 90, *PtdIns(3,4,5)P3* phosphatidylinositol 3,4,5-trisphosphate, *PDK1* 3-phosphoinositide-dependent protein kinase-1)

Reports indicate that suppressing the AKT pathway with the PI3 K inhibitor LY294002 or HSP90-interacting AKT deletion mutants synergistically enhances the cytotoxic effects of anticancer drugs [4, 20]. It has also been suggested recently that AKT is related to apoptosis resistance because it is relatively active in tumor cells (by amplification of the *AKT* gene or by the loss of *PTEN* gene) [5]. Thus suppressing the AKT pathway might improve the efficiency of anticancer drugs. However, it has not yet been clarified what types of drug exhibit their cytotoxicity by directly inhibiting the AKT pathway. Therefore we have started to examine the effects of anticancer drugs on the AKT pathway. During the screening, we have found that there is a loss of AKT after treatment of the cells with some anticancer drugs [19]. We have also found that some anticancer drugs have the ability to inhibit the AKT pathway by inactivating the upstream AKT kinases, PI3 K or PDK1 [8, 14, 21]. *N*-α-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) and the cyclooxygenase-2 inhibitor celecoxib have been reported to exert their cytotoxic effects by inhibiting AKT phosphorylation [3, 11], although it is not clear how TPCK and celecoxib downregulate AKT phosphorylation. TPCK and celecoxib have no effects on PI3 K. Thus TPCK and celecoxib might downregulate phospho-AKT by inhibiting PDK1. Therefore the upstream AKT kinase PDK1 would be an attractive

target for developing new anticancer drugs to inhibit the AKT pathway (Fig. 3).

In conclusion, we propose that therapies aimed at suppressing upstream AKT kinases, especially PDK1, would be the most successful approaches to make cancer cells vulnerable to apoptosis. Targeting AKT- or PDK1-interacting proteins (e.g. PRK2 [23], HSP90 [8, 20], 14-3-3 protein [22], protooncogene *TCL1* [12, 18], carboxyl-terminal modulator protein [13]) could also prove successful for developing new anticancer drugs (Fig. 3).

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