DC120, a novel and potent inhibitor of AKT kinase, induces tumor cells apoptosis and suppresses tumor growth

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ABBREVIATIONS:

PKB protein Kinase B;  
PI3K phosphoinositide 3-kinase;  
DMSO dimethyl sulfoxid;  
DAPI 4, 6-diamidine-2- phenylindole;  
PI propidium iodide;  
MAPKs mitogen-activated protein kinases;  
MTT 3-[4,5-dimethylthiazol-2 -thiazoly]-2,5-diphenyltetrazolium bromide  
CTX cyclophosphamide
Abstract

PKB/AKT kinase is the core component of the PI3K/AKT signaling pathway which is frequently hyperactivated in human cancers. We designed and synthesized a series of 2-pyrimidyl-5-amidothiazole compounds based on the ATP binding site of AKT, and the most potent compound DC120 was identified to inhibit AKT activity in vitro with EC50 of 153nM by a FRET-based Z'-Lyte assay. Then the anti-tumor effect of DC120 was tested on human CNE2 and MDA-MB-453 cell lines and CNE2 xenograft model. The results showed that DC120 could obviously inhibit the proliferation of CNE2 and MDA-MB-453 cells via induction of apoptosis, with the evidence of increasing sub-G1 and AnnexinV-positive cells, characteristic morphologic changes of apoptosis in the nucleus, and cleaved caspase-3. Further study showed that MDA-MB-453 cells transfected with constitutively activated AKT1 were more sensitive to DC120, while CNE2 cells with knock-down of AKT1 expression by shRNA were more resistant to DC120. More importantly, DC120 partially attenuated the phosphorylation levels of FKHR, FKHRL1, GSK-3β and mTOR in a dose-dependent and time-dependent fashion, and led to an increase in the nuclear accumulation of exogenous FKHR in cancer cells. And DC120 at 20 mg/kg/qd inhibited the CNE2 xenograft tumor growth with the T/C value of 38.1%, accompanied by increasing TUNEL-positive cells in tumor sample. Additionally, DC120 induced a feedback loop to activate MAPKs pathway, and treatment with MEK inhibitor U0126 and DC120 synergistically induced cancer cell apoptosis. These data provide validation for the development of DC120 to treat cancers displaying elevated levels of AKT.
Introduction

PKB/AKT kinase, a serine/threonine kinase, is the core component of the PI3K/ AKT signaling pathway and therefore involved in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis, autophagy, glucose metabolism, the repair of DNA double-strand breaks and tumorigenesis (Bellacosa et al., 2005; Deng et al., 2011; Janku et al., 2011; Manning and Cantley, 2007; Tokunaga et al., 2008). It is well established that hyperactivation of AKT kinase is a common event in many human cancers, and elevated AKT activity can also be detected in preneoplastic lesions (Bellacosa et al., 2005; Caporali et al., 2008; Balsara et al., 2004). Loss or mutation of tumor suppressor PTEN, amplification or mutation of PI3K, activation or mutation of growth factor receptors and oncogenes, and amplification of AKT itself are involved in activation of AKT in tumors (Tokunaga et al., 2008; Kalinsky et al., 2011; Whitehall et al., 2011). Activation of AKT promotes the development or progression of cancer as well as resistance to treatment with chemotherapy and/or radiation therapy. Also, immunohistochemical analyses have shown that AKT activation is a poor prognostic factor in various cancers (LoPiccolo et al., 2007; Nakanishi and Ross, 2011; Wei and Xu, 2011). Therefore, AKT is an attractive target for cancer therapy, and it has been proven that inhibition of AKT alone or in combination with conventional chemotherapeutics or radiotherapy can reduce the apoptotic threshold and preferentially kill cancer cells (Crowell et al., 2007; Engelman, 2009; Liu et al., 2012; Sun et al., 2011).

AKT kinase has been an attractive target for small molecular drug discovery. To
date, researchers have developed many AKT inhibitors, including targeting the ATP binding site, pleckstrin homology (PH) domain, or protein substrate binding site of AKT. Several of them, such as MK-2206, GDC0068, Perifosine, are currently in phase I to II trials alone or in combination to treat multiple forms of cancer (Hers et al., 2011; Richardson et al., 2011). In the present study, we analyzed the crystal structures of the published ATP-competitive AKT inhibitors bound to AKT kinases (Lin et al., 2006; McHardy et al., 2010; Seefeld et al., 2009; Zhu et al., 2007), optimized the screened hit compounds to a series of 2-(methylaminopyrimidinyl) thiazole-5-carboxamide derivatives (Chang et al., 2011). We screened out DC120 from these compounds by a FRET-based Z'-Lyte assay (EC50=153nM) and focused on the anti-tumor activity of this potent compound. Our data showed that DC120 exhibited inhibitory effect of proliferation in human nasopharyngeal carcinoma CNE2 cells and human breast cancer MDA-MB-453 cells via inducing cells apoptosis, and significantly reduced tumor growth of CNE2 xenografts. Also, we observed that inhibition of AKT kinase activity and blockade of AKT downstream signaling pathway in cancer cells were involved in antitumor activity of DC120. Moreover, we found that DC120 induced a feedback loop to activate MAPKs pathway, and the combination of MEK inhibitor U0126 and DC120 has a significant synergistic effect.
Materials and Regents

DC120 Preparation

For all in vitro studies, compound DC120 was dissolved in DMSO at a concentration of 50mM and stored at -20°C. For the tumor xenograft studies, DC120 was formulated in 8% solvent diluent (DMSO/ (Cremophor EL+ethanol), 1: 3) at a concentration of 50mg/ml. Its structure was shown in Figure 2A.

Cell Culture and Reagents

Human nasopharyngeal carcinoma CNE2 and human breast cancer MDA-MB-453 cells were cultivated in DMEM medium supplemented 10% fetal bovine serum in a 5% CO2 humidified atmosphere at 37°C. GAPDH, AKT, phospho-AKT(Ser473), phospho-AKT(Thr308), GSK3α/β, caspase-3 and horseradish peroxidase-conjugated second antibodies were purchased from Santa Cruz Biotechnology (California, CA, USA). Anti-phospho-FKHR, phospho-FKHRL1, phospho-GSK3β, phospho-mTOR, cleaved caspase-3, mTOR and chemiluminescence reagents were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Flag antibody, DAPI, MTT and DMSO were purchased from Sigma (St Louis, MO, USA).

Z’-LYTE™ Kinase Assay

A FRET-based Z’-LYTE kinase assay kit-Ser/Thr 6 peptide (Invitrogen Corporation, Carlsbad, CA, USA) was used to evaluate the EC50 value of these 2-pyrimidyl-5-amidothiazole compounds for inhibition of AKT1 kinase. The reaction was carried out in a 384-well plate with 10 μL reaction volume per well containing 2 μM Ser/Thr 6 peptide substrate in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM...
MgCl₂, 1 mM EGTA and appropriate amount of AKT1 kinase with a serial 3-fold dilution of test compound. The final reaction concentration of ATP was 75 μM. After 1 h incubation, reaction was developed and terminated, and the fluorescence ratio was calculated according to the manufacturer’s protocol. Staurosporine was used as a positive control. Dose response curve was fitted using Prism 5.0 from Graph-Pad Software.

**MTT assay**

Cells were seeded in 96-well-plate (Falcon, Lincon Park, NJ) at the 5000~8000 density per well. Then different concentrations of compounds were added to the medium and incubated for an indicated period. Cell viability was determined by MTT assay as described previously (Deng et al., 2009). The value of IC₅₀ was calculated by CalcuSyn software (Biosoft).

**Annexin V–FITC Apoptosis assay**

Annexin-V-FLUOS Staining Kit (Roche, Switzerland) was used to evaluate DC120-induced apoptosis. Cells were cultured in six-well plate and exposed to DC120. The cells were harvested and stained with Annexin-V-FLUOS Solution and PI Solution provided by the Kit for 10-15 min. Then apoptosis was analyzed by flow cytometry (Beckman Coulter, USA) at the wavelength of 488 nm immediately.

**DAPI nuclear staining assay**

For detecting DC120-induced apoptosis, cells were subcultured into a 24-well plate covered with 13mm×13mm slips and exposed to DC120. Then the cells were fixed with 10% absolute methanol permeabilized by 0.25% Triton X-100 and stained
with DAPI (1μg/mL) for 10 min. The morphologic changes of apoptosis-characteristic nuclei were examined by confocal microscopy (Olympus, Tokyo, Japan).

**Cell cycle analysis**

For detecting DC120-induced apoptosis, cells were cultured in six-well plate and exposed to DC120. The cells were harvested and fixed with 70% ethanol and incubated overnight at -4°C. Then the cells were washed and resuspended in 1 mL of staining solution (50 μg/mL PI, 50 μg/mL RNase) for 15 min. The PI fluorescence associated with DNA was measured by flow cytometer (Beckman Coulter). The percentages of nuclei in sub-G1 phase of the cell cycle were calculated by MultiCycle software.

**Western blot analysis**

The cells were harvested and lysed in 1× cell lysis buffer (Cell Signaling Technology) adding 1mM phenylmethanesulfonyl fluoride (PMSF) immediately before use. The protein concentration was estimated by Pierce BCA protein assay kit. Equal amounts of protein (20μg-40μg) were separated electrophoretically on 8%-15% SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore), and analyzed as previously described (Deng et al., 2009).

**Plasmids and transfection**

For inhibition of AKT activity, the hairpin RNA (shRNA) sequences against AKT1 (5’-GCTACTTCCTCCTCAAGATG -3’ (Irie et al., 2005) was synthesized by Invitrogen Life Technologies Corporation (Shanghai, China) and cloned into the
retroviral vector vector (pSUPER RNAi. puro). The CNE2 cells stably expressing AKT1 shRNA were established by infection with retrovirus-containing supernatants as described previously (Zhou et al., 2009). For activation of AKT activity, MDA-MB-453 cells were transiently transfected with activated myr-AKT1 (Upstate Biotechnology) using Lipofectamine 2000 (Invitrogen) according to the protocol suggested by the manufacture. After 24h of transfection, the cells were used for further experiments.

**Immunofluorescence staining**

For detecting the cellular localization of exogenous FKHR, cells were subcultured into a 24-well plate covered with 13mm×13mm slips and transiently transfected with pCDNA3-Flag-FKHR plasmids using Lipofectamine 2000 for 24 h. After DC120 treatment, cells were fixed and permeabilized. Then the cells were incubated with monoclonal anti-Flag antibody (diluted 1:500) for 2 h and Alexa Flour 488 goat anti-mouse IgG antibody (diluted 1:400) for 1h. For frozen sections of tumor tissue samples, the cells were incubated with anti-phospho-GSK3β antibody (1:200) at 4°C overnight and Alexa Flour 488 goat anti-rabbit IgG antibody (1:400) for 1h. After counterstaining with DAPI, the cells in coverslips or sections of tumor tissue were observed using confocal microscopy (Olympus). For frozen sections of tumor tissue samples, the intensity of immunofluorescence representing the expression of phospho-GSK3β protein was evaluated by repeated staining of the same specimens and by two observers. It was graded as (−) for no immunofluorescence, (±) for weak and indefinitely detectable immunofluorescence, (+) for weak but definitely
detectable immuno fluorescence, (++) for moderate immunofluorescence, and (++++) for intense immunofluorescence (Deng et al., 2009; Zhang et al., 2008).

In Vivo Antitumor Activity

BALB/c nude mice were obtained from Hunan Slac Jingda Laboratory Animal Co. Ltd and were 4 to 6 weeks old. All manipulations were performed under sterile conditions. The procedures involving mice and their care were in accordance with the National Institutes of Health Guide for the care and use of Laboratory Animals with the UKCCCR (UKCCCR, 1998). Tumor xenografts were established by 2×10⁶ CNE2 cells injected s.c. into nude mice. Mice were randomly divided into 3 groups and each group contained 6 mice. Treatments were initiated on day 7 after inoculation, by which time the tumor volume had reached ~50mm³. 8% solvent diluent (vehicle, negative control), 100mg/kg/q5d CTX (positive control), and DC120 (20 mg/kg/d) were administered intraperitoneally for 21d for each group. Tumor volumes and body weight of mice were observed. Tumor volumes were calculated by the formula: 0.5×a×b² in millimeters, where ‘a’ is the length and ‘b’ is the width. When all control tumors developed to more than 2,000 mg, nude mice were sacrificed. After the tumor tissues were excised and weighed, the tissue samples were made into frozen sections with 4mm-width immediately and fixed in cold acetone. Then the frozen sections were stored at -80°C for future studies. Tumor growth inhibition (T/C %), which was used to evaluate the tumor response to the drugs, was calculated using the ratio of the average tumor weight of the treated group (T) to the average tumor weight of the control group (C).
TUNEL staining assay

In Situ Cell Death Detection Kit (Roche, Switzerland) was used to evaluate DC120-induced apoptosis in vivo. Frozen tumor sections were incubated in blocking solution for 10 min and permeabilisation solution for 2 min on ice. Then 50 μl TUNEL reaction mixture was added on samples for 60 min at 37°C in a humidified atmosphere in the dark. After counter-staining with DAPI (1 μg/ml), frozen sections were observed using confocal microscopy (Olympus). TUNEL-positive nuclei were stained green, and all other nuclei were stained blue (Markaryan et al., 2008).

Statistic Analysis

The student’s t test was used to evaluate the statistical significance of the result at the 95% Confidence Level, and a P value less than 0.05 was considered to indicate statistical significance.
Results

DC120 inhibited AKT kinase activity in vitro

We used biochemistry method (Z'-LYTE™ Kinase Assay Kits- Ser/Thr 6 Peptide) to evaluate the effect of these 2-(methylaminopyrimidinyl) thiazole-5-carboxamide compounds on the AKT kinase activity in cell-free system. In this experiment, Z-LYTE™ Ser/Thr 6 Peptide was used as a substrate; Thus, the changes of Z-LYTE™ Ser/Thr 6 Peptide phosphorylation can directly reflect the AKT kinase activity. As expected, we screened out several compounds including compound DC120. The results showed DC120 could concentration-dependently suppress phosphorylation of Z-LYTE™ Ser/Thr 6 Peptide and the value of EC50 was about 153 nM, while EC50 value for Staurosporine (positive control) was about 122nM (Figure 1). We also used HTScan® Akt1 Kinase Assay Kit to confirm the AKT inhibitory effect of DC120. The results showed that DC120 could concentration-dependently suppress phosphorylation of eNOS (Ser1177) (Supplementary Table 1). To further test whether DC120 has off-target effects, we also employed KINOMEscan's in vitro competition binding assay to evaluate DC120 against a panel of distinct human protein kinases. The results showed that DC120 selectively inhibited AKT but not JNK, ERK, P38, EGFR, CDK2, etc. At the concentration of 100 nM DC120, the percentage of AKT kinase binding to the immobilized ligand was only 15% (Supplementary Table 2). Taken together, all these data indicated that DC120 could inhibit AKT kinase activity in vitro obviously.

DC120 suppressed proliferation and induced apoptosis in cancer cells
To investigated the cytotoxicity of DC120 in cancer cells, we did an MTT assay. DC120 displayed potent cytotoxicity in diversified cancer cell lines including nasopharyngeal carcinoma (CNE1, CNE2, HONE1), hepatocellular cancer (HepG2, SMMC7721, Bel-7402), Melanoma (SK-MEL-1, ME-4405), breast cancer (MDA-MB-453, MDA-MB-436, MDA-MB-435). The IC\textsubscript{50} values for all the tested tumor cell lines varied from 5 to 10\,\mu M (Supplementary Figure 1). In this study, we focused on the effect of DC120 on CNE2 and MDA-MB-453 cell lines which showed more aberrantly activated AKT signaling pathway in cells observed in our previous work. As shown in Figure 2, DC120 obviously suppressed cell viability in a dose-dependent manner. At 72 hr, the IC\textsubscript{50} values showed 7.09 \,\mu M in CNE2 cells and 6.48 \,\mu M in MDA-MB-453 cells (Figure 2B).

To confirm whether the growth inhibition of DC120 was caused by apoptosis in cells, the sub-G1 fraction and the percentage of Annexin V-positive cells were tested using flow cytometric analysis. After cancer cells treated with different concentrations of DC120, the sub-G1 fraction and the percentage of Annexin V-positive cells markedly increased. When the cells were exposed to 20 \,\mu M DC120 for 48 hr, the rates of sub-G1 fraction were from 0.3% up to 45.9% in CNE2 cells and from 0.9% up to 60.2% in MDA-MB-453 cells (Figure 3A); When the cells exposure to 20 \,\mu M DC120 for 44 hr, the percentage of Annexin V-positive cells were from 1.0% up to 47.2% in CNE2 cells and from 1.8% up to 61.1% in MDA-MB-453 cells (Figure 3B). Moreover, cell morphology stained with DAPI was observed to identify the apoptotic cell population. Figure 3C showed that treatment with 0.1% DMSO did...
not appreciably induce apoptosis in cells, but typical morphological changes associated with apoptosis-chromatin condensation, apoptotic body formation and DNA fragmentation were prevalently observed in DC120-treated CNE2 and MDA-MB-453 cells. Furthermore, procaspase-3 cleaved to yield a 17/19-kD fragmentation was also detected in CNE2 and MDA-MB-453 cells following DC120 treatment (Figure 3D). These data indicated that DC120 indeed induced apoptosis in cancer cells, which was consistent with the results of the MTT growth-inhibition assay.

The growth inhibition of DC120 on cancer cells depending on AKT activity

To further confirm DC120 targeting AKT kinase, retroviral vectors encoding short hairpin RNA (shRNA) sequences against AKT1 were stably transfected to CNE2 cells. The downregulation of phospho-AKT and AKT1 was at least 75% (Figure 4A left pannel), and the inhibitory rate in CNE2/ AKT1 shRNA cells was obviously lower than that in control cells following treatment with DC120 (p<0.01, Fig. 4B left pannel). On the other hand, myr-AKT1 plasmids (constitutively activated AKT1) were transfected into the MDA-MB-453 cells. In comparison with the control cells, the exogenous AKT and phospho-AKT expression significantly increased in MDA-MB-453 /myr-AKT1 cells (Fig 4A, right pannel), and the inhibitory rate was also significantly increased after treatment with DC120 (p<0.01, Fig. 4B right pannel).

To directly examine of the cell killing, the sub-G1 fraction was tested using flow cytometric analysis. The results showed that DC120 decreased cell viability mainly due to the induction of apoptosis, as shown by the increase of sub-G1 cells. Also we
observed that the apoptotic cells increased more obviously in CNE2/ vector cells when comparing with that in CNE2/AKT1 shRNA cells, and in MDA-MB-453/myr-AKT1 cells when comparing with that in MDA-MB-453/vector cells, which was consistent with the MTT assay. Thus, the data further confirmed that AKT kinase was indeed the target of DC120 treatment in cancer cells.

Effect of DC120 on phosphorylation of AKT and its downstream targets in cancer cells

As the AKT kinase activity is regulated by phosphorylation on two sites, threonine 308 in the activation loop of the catalytic domain and serine 473 in the COOH-terminal regulatory domain, we assessed the effect of DC120 on the phosphorylation status of AKT on Ser473 and Thr308 in cancer cells. The results showed that DC120 up-regulated phosphorylation of Ser473-AKT and Thr308-AKT in a dose- and time-dependent manner, without affecting the amount of AKT (Figure 5A, 5B). It has been stated that AKT exerts its cellular effects through phosphorylation of a number of substrate proteins. More than 20 proteins have been identified as AKT substrates, including the members of Forkhead protein family (FKHR, FKHRL1, and AFX), GSK-3β, eNOS, mTOR, p21, p27, MDM2, Bad, tuberin/TSC2, and IKKα, etc (Hers et al., 2011; Mannoury la Cour et al., 2011; Wu and Shih, 2011). As inhibition of substrate phosphorylation can really reflect inhibition of AKT activity, we examined whether DC120 could inhibit phosphorylation of downstream targets of AKT. As expected, the phosphorylation levels of FKHR, FKHRL1, GSK-3β or mTOR were all partially attenuated by DC120
dose-dependently and time-dependently without affecting the amount of total proteins in CNE2 cells and MDA-453 cells, although the Thr308 and Ser473 phosphorylation of AKT increased concomitantly (Figure 5A, 5B). More precisely, the reduction of phosphorylation of these proteins occurred within 1 h after exposure to 10 μM DC120 in CNE2 and MDA-MB-453 cells.

To further show the correlation between FKHR protein phosphorylation and its cellular localization, CNE2 cells and MDA-453 cells were transiently transfected with pCDNA3-Flag-FKHR plasmids, and treated with DC120 for the indicated times. Then we investigated the cellular localization of exogenous FKHR using immunofluorescence staining. We observed that exogenous FKHR protein mainly located in the cytoplasm without drug treatment, but translocated into the nucleus following treatment with DC120 (Figure 5C). All these data indicated that DC120 could induce cell apoptosis by blocking AKT downstream signaling pathway including AKT/GSK-3β, AKT/mTOR, AKT/FOXO in CNE2 cells and MDA-MB-453 cells.

**Antitumor Activity of DC120 in Vivo**

Because of the potent growth inhibition of DC120 in vitro, its antitumor properties were further examined in vivo. CNE2 xenografts were established and given i.p. administration of 8% solvent (negative control), DC120 or CTX (positive control) on day 7 after implantation. No obvious toxicity was observed in mice receiving 8% solvent treatment or 20mg/kg/d DC120 treatment. Treatment with DC120 at 20mg/kg/d could obviously suppress the tumor growth and the tumor growth inhibition (T/C %) was about 38.1% (Figure 6A). Although the inhibitory rate of the
positive control group (100mg/kg/q5d CTX) was 84.2%, the obvious toxicity was observed and three mice died during the phase of receiving the dosage treatment. To determine whether the growth inhibition of DC120 was caused by apoptosis in vivo, tumor frozen sections from CNE2-bearing nude mice were stained with TUNEL to identify the apoptotic cell population. As shown in Figure 6B, treatment with 8% solvent did not appreciably induce apoptosis, whereas DC120 at 20mg/kg/d stimulated a substantially increased number of TUNEL-positive cells in CNE2 tumors, the ratio of apoptotic cells increased up obviously. Then we detected effect of DC120 on the AKT signaling pathway in tumor samples. Immunofluorescence staining in tumor frozen sections showed that the intensity of fluorescence representing the protein level of phospho-GSK3β was (+++) in the control group, whereas in the group treated with 20 mg/kg/d of DC120, the intensity of fluorescence was (+) to (+++) (Figure 6C).

**U0126 enhanced the DC120-induced apoptosis**

In this study, we tested the effect of DC120 on the phosphorylation of MAPKs signaling pathway. The results revealed that phospho-JNK, phospho-P38, and phospho-ERK all increased obviously after DC120-treatment for 24hr in CNE2 and MDA-MB-453 cells, without affecting the total amount of JNK, P38, and ERK (Figure 7A). This might be DC120 induces a feedback loop to activate MAPKs pathway. The activation of feedback loops such as MAPKs pathway by DC120 prompted us to detect the apoptotic effect of the combination of MEK inhibitor U0126 and DC120 on cancer cells. We noticed that treatment with U0126 and DC120
inhibited phospho-ERK expression drastically (Figure 7B). We also found that the two-drug combination resulted in more apoptotic cells than either single-drug treatment alone. Once combined with U0126, DC120-induced apoptosis increased from 13.9% to 27.5% in the CNE2 cells and from 22.1% to 64.1% in the MDA-MB-453 cells ($p<0.01$, Figure 7C). These results indicated that U0126 effectively enhanced DC120-induced apoptosis in vitro.
Discussion

Here we reported that DC120, a new compound screened out from 2-pyrimidyl-5-amidothiazole ATP-competitive AKT inhibitors, possessed an antiproliferative effect on CNE2 and MDA-MB-453 cells and suppressed CNE2 xenografts tumor growth by inhibiting AKT kinase activity and blocking its signal pathway.

In this study, DC120 showed a potent antitumor activity. It not only showed its significantly inhibitory effect on CNE2 and MDA-MB-453 cells proliferation alone in vitro (Figure 2), but also potently suppressed CNE2 xenografts tumor growth without obvious toxicity at 20mg/kg/d in vivo (Figure 6A). Further study confirmed that DC120 decreased cell viability mainly due to induction of apoptosis, as demonstrated by increasing sub-G1 group cells, Annexin V-positive cells, characteristic morphological changes of apoptosis in the nucleus, cleaved caspase-3 as well as increasing TUNEL-positive cells in tumor samples (Figure 3, Figure 6B).

Also, the following data provided validation that DC120 could inhibit AKT kinase activity and block its signal pathway in vitro and in vivo. Firstly, we confirmed that DC120 suppressed AKT kinase activity obviously in cell-free system by biochemistry method (Figure 1B, Supplementary Table 1, Supplementary Table 2); Secondly, the cytotoxic effects of DC120 on cancer cells depending on AKT activity (Figure 4). In MDA-MB-453/myr-AKT1 cells, AKT signaling pathway is more hyperactivated, rendering the cells highly dependent on this pathway. While in CNE2/AKT1 shRNA cells, the situation is the opposite. Because DC120 could inhibit the AKT activity, the growth inhibition was much higher in MDA-MB-453/
myr-AKT1 cells but much lower in CNE2/AKT1 shRNA cells when compared with that in control cells. This result is concurrent with another selective AKT inhibitor-API-2, which much more potently inhibits cell growth in AKT-overexpressing/activating cells compared with those with low levels of AKT (Yang et al., 2004); Thirdly, the ability of AKT to phosphorylate its downstream targets including FKHR, FKHRL1, GSK-3β and mTOR was markedly decreased in the presence of the DC120 in vitro and in vivo (Figure 5A, 5B, Figure 6C). We further observed that FKHR translocated into the nucleus upon the reversal of its phosphorylation induced by DC120 in cancer cells (Figure 5C). Because reduction in phosphorylation of these downstream effectors occurred early, these effects could not result from induction of apoptosis but instead could conceivably cause apoptosis as a consequence. On the other hand, together with the decrease in phosphorylation of AKT downstream targets, we observed a concomitant increase in the Thr308 and Ser473 phosphorylation of AKT. This increase has been observed with other AKT inhibitors (i.e. A-443654, GSK690693, GDC0068) tested and seems a sensitive marker of ATP-competitive AKT inhibition (Luo et al., 2005; Okuzumi et al., 2009; Rhodes et al., 2008). This might be caused by a feedback loop induced by DC120 or a direct consequence of DC120 binding to the ATP binding site of AKT (Chakrabarty et al., 2012; Chandarlapaty et al., 2011; Okuzumi et al., 2009). However, as shown by the reduction in the phosphorylation of multiple AKT substrates, DC120 effectively inhibited AKT kinase activity in cells regardless of any feedback hyperphosphorylation of AKT. Taken together, all these data suggest that DC120 can
inhibit AKT kinase activity and phosphorylate its target effectors, block the AKT signaling pathway, and subsequently initiate apoptotic events.

It is largely admitted that collaborating AKT inhibitors with other cancer therapeutics is a promising way to improve tumor therapeutic window. It has been recently reported that combination treatment with MK-2206 (an Allosteric AKT Inhibitor) and AZD6244 (a MEK inhibitor) is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo (Meng et al., 2010). Additionally, MK-2206 showed synergistic responses in combination with molecular targeted agents such as erlotinib and lapatinib, or with cytotoxic agents such as doxorubicin, camptothecin, docetaxel and carboplatin in lung NCI-H460 or ovarian A2780 tumor cells in vitro and in vivo (Hirai et al., 2010). In the present study, we noticed that DC120 induced a feedback loop to activate MAPKs pathway and U0126 effectively enhanced DC120-induced apoptosis in vitro, which indicated that the combination of these two agents has a significant synergistic effect (Figure 7). Additionally, DC120 combination with other anticancer agents, such as with RAD001 (a mTOR inhibitor) or radiotherapy is already being conducted in our laboratory, and some exciting data has been achieved (data not shown). To explore the combination efficacy of DC120 with other anticancer agents in the further study, it will be useful for DC120 as a leading compound targeting AKT signal pathway or for further development.

In summary, our data provide evidence of a sustained antitumor effect and a promising development of DC120. DC120 is a potent inhibitor of AKT kinase in
tumor cells. Through inhibition of AKT kinase and blockade of its signal pathway, DC120 induces tumor cells apoptosis *in vitro* and inhibits tumor growth *in vivo*. These data provide validation for the development of DC120 to treat cancers displaying elevated levels of AKT. Further investigation is required to evaluate whether DC120 are clinically useful in this setting.
Acknowledgments

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Authorship Contributions

Participated in research design: Deng, Ding and Zhu

Conducted experiments: Deng, Yang, Chang, Tang, Qin, and Feng

Contributed new reagents or analytic tools: Chang and Ding

Performed data analysis: Deng, Yang, Tang and Zhu

Wrote or contributed to the writing of the manuscript: Deng, Chang, Ding and Zhu.
References


Footnotes

Rong Deng, Fen-Yang and Shao-hua Chang equally contributed to this article.

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Legends for figures

Figure 1 The effect of 2-(methylaminopyrimidinyl) thiazole-5-carboxamide compounds on the AKT kinase activity in cell-free system. AKT1 kinase activity was determined using Ser/Thr 6 peptide substrate, AKT1 kinase, ATP and different concentrations of compounds in the presence of kinase buffer for 60min as described in “Materials and Methods” by the Z’-LYTE assay. Staurosporine was used as a positive control. Dose response curve was fitted using Prism 5.0 from Graph-Pad Software.

Figure 2 Compound DC120 inhibited cancer cell proliferation. A, Chemical structure of compound DC120. B, Effect of DC120 on proliferation of CNE2 and MDA-MB-453 cells. Cells were cultured in a 96-well plate, exposed to different concentrations of DC120 and incubated for 72h. Each column represented the mean ± S.D. of triplicate determinations.

Figure 3 Compound DC120 induced cancer cell apoptosis. A, CNE2 and MDA-MB-453 cells were treated with different concentrations of DC120 for 48h. Then the PI fluorescence associated with DNA was measured by flow cytometer. B, AnnexinV/PI analysis of CNE2 and MDA-MB-453 cells treated with different concentrations of DC120 for 44 h. DC120 treatment increased the percentages of AnnexinV+/PI− (bottom right quadrant, D4) and AnnexinV+/PI− (top right quadrant, D2) cells. C, Characteristic apoptotic cells were presented in CNE2 cells treated with DC120 for 48 h and in MDA-MB-453 cells for 24 h. Magnification, ×100. D, cells
were treated with different concentrations of DC120 for 48 hr in the CNE2 cell line or 24 hr in MDA-MB-453 cell line. Western blot analysis was conducted and probed with cleaved caspase3 antibody or caspase3 antibody. Control: 0.1% DMSO.

Figure 4 The cytotoxic effects of Compound DC120 on cancer cells relying on AKT activity. A, The efficiency of AKT1 shRNA stably transfection in CNE2 cells and transient transfection of myr-AKT1 plasmid in MDA-MB-453 cells were analyzed by western blotting. B, Cell viability was assayed by MTT. CNE2/vector and CNE2/AKT1 shRNA cells were exposed to different concentrations of DC120 for 48 hr. And MDA-MB-453/vector and MDA-MB-453/myr-AKT1 cells were exposed to different concentrations of DC120 for 24 hr. Data are presented as mean ± SD of quadruplex determinations (**<0.01 vs *). C, The PI fluorescence associated with DNA was measured by flow cytometry. CNE2/vector cells and CNE2/AKT1 shRNA cells were treated with 20μM DC120 for 48 hr. MDA-MB-453/vector cells and MDA-MB-453/myr-AKT cells were treated with 20μM DC120 for 30 hr. The numbers represent the percentages of apoptotic sub-G1-phase cells. Data represent one of three independent experiments with similar results.

Figure 5 Effect of Compound DC120 on phosphorylation of AKT and its downstream targets in cancer cells. CNE2 cells or MDA-MB-453 cells were treated with different concentrations of DC120 for 24 h (A), or 10μM DC120 for different times (B). Total protein isolated was analyzed by immunoblotting with indicated antibodies. Results are representative of three different experiments. Control:
0.1% DMSO. C, CNE2 cells and MDA-MB-453 cells were transiently transfected with pCDNA3-Flag-FKHR plasmids, then treated with 10μM DC120 for the indicated times. The cellular localization of exogenous FKHR was examined using immunofluorescence. Magnification, ×100.

**Figure 6** Effects of Compound DC120 on tumor growth of human CNE2 xenografts in nude mice. A, Top, Mice bearing established human CNE2 xenografts were given i.p. administration of 8% solvent (vehicle group), 20mg/kg/d DC120, or 100mg/kg/ q7d CTX (positive group) for 21 d. Points, mean of tumors (n = 6 for vehicle group and DC120 group, n=3 for positive group); bars, SD. Bottom, tumor growth inhibition was calculated. *, p < 0.05; **, p < 0.01, significantly different from vehicle-treated animals (8% solvent) on day 21 of treatment. B, Top, Representative images of TUNEL staining of CNE2 xenograft frozen tumor sections after treatment. The apoptotic cells with DNA fragmentation were stained positively as green nuclei (arrowheads). Bottom, The relative apoptotic cells were determined by counting TUNEL positive cells in three random fields (at ×40magnification) for each sample. Columns, mean; bar, SD. (**<0.01 vs *). C, Representative images of immunofluorescence staining of CNE2 xenograft frozen tumor sections for phospho-GSK3β protein. Magnification, ×40.

**Figure 7** Treatment with U0126 and DC120 synergistically induced cancer cell apoptosis. A, The effect of DC120 on phosphorylation of JNK, ERK or P38 in cancer cells. CNE2 and MDA-MB-453 cells were treated with different concentrations of
DC120 for 24 h. **B**, The effect of the combination of DC120 and U0126 on ERK activation. Cells were treated with DC120, U0126, or combination at the indicated concentrations for 24 hr. **C**, DC120 and U0126 synergistically induced cancer cell apoptosis. Cells were treated with DC120, U0126, or combination at the indicated concentrations for 24 hr in CNE2 cells or 48hr in MDA-MB-453 cells. Then Annexin V–FITC apoptosis assay was performed. The numbers represent the percentages of AnnexinV-positive apoptotic cells. Data represent one of three independent experiments with similar results. Columns, mean; bar, SD. *, p<0.01, compared with the single agent treatments.
### Figure 6

#### A

**Inhibitory effect of DC120 on growth of human CNE2 xenografts in nude mice**

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<tr>
<th>Group</th>
<th>Weight of tumor (g)</th>
<th>Inhibitory rate (%)</th>
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<tbody>
<tr>
<td>Solvent (8%)</td>
<td>2.60 ± 0.37</td>
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<tr>
<td>DC120 (20mg/kg/qd)</td>
<td>1.61 ± 0.31</td>
<td>38.1*</td>
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<tr>
<td>CTX (100mg/kg/q5d)</td>
<td>0.41 ± 0.07</td>
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#### B

**Apoptotic cells (%)**

- Solvent (8%)
- DC120 (20mg/kg/d)

#### C

**p-GSK3β**

- Solvent (8%)
- DC120 (20mg/kg/d)**

**DAPI**

- Solvent (8%)
- DC120 (20mg/kg/d)**

**Overlap**

- Solvent (8%)
- DC120 (20mg/kg/d)**
Figure 7

A

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B

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<td>DC120 (10μM)</td>
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C

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Apooptotic Cell (%)