DC120, a Novel and Potent Inhibitor of AKT Kinase, Induces Tumor Cell Apoptosis and Suppresses Tumor Growth

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ABSTRACT

Protein kinase B/AKT kinase is the core component of the phosphatidylinositol 3-kinase/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, (S)-(N-1-amino-3-(2,4-dichlorophenyl)propan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl)thiazole-5-carboxamide (DC120), was identified to inhibit AKT activity in vitro with an EC50 of 153 nM by a fluorescence resonance energy transfer-based CyFRET assay. The antitumor effect of DC120 was tested on human CNE2 and MDA-MB-453 cell lines and the 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 increases in sub-G1, and annexin V-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, whereas CNE2 cells with knockdown of AKT1 expression by short hairpin RNA were more resistant to DC120. Of more importance, DC120 partially attenuated the phosphorylation levels of forkhead transcription factor (FKHR), FKHRL1, glycogen synthase kinase 3β, and mammalian target of rapamycin in a dose-dependent and time-dependent fashion and led to an increase in the nuclear accumulation of exogenous FKHR in cancer cells. In addition, DC120 at 20 mg/kg/day inhibited the CNE2 xenograft tumor growth with a treated group/control group ratio of 38.1%, accompanied by increasing terminal deoxynucleotidyl transferase-UTP nick-end labeling-positive cells in the tumor sample. In addition, DC120 induced a feedback loop to activate the mitogen-activated protein kinase pathway and treatment with mitogen-activated protein kinase kinase inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (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

Protein kinase B/AKT kinase, a serine/threonine kinase, is the core component of the phosphoinositide 3-kinase/AKT signaling pathway and therefore is 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; Manning and Cantley, 2007; Tokunaga et al., 2008; Deng et al., 2011; Janku et al., 2011).

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Abbreviations: MK-2206, 8-[4-(1-amino-3-(2,4-dichlorophenyl)propan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl)thiazole-5-carboxamidyl 3-piperidinylmethoxy]-1-(4-(5-(1-amino-3-(2,4-dichlorophenyl)propan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl)thiazole-5-carboxamidyl 3-(isopropylamino)propan-1-one; DC120, (S)-(N-1-amino-3-(2,4-dichlorophenyl)propan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl)thiazole-5-carboxamide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (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.
lished that hyperactivation of AKT kinase is a common event in many human cancers, and elevated AKT activity can also be detected in preneoplastic lesions (Balsara et al., 2004; Bellacosa et al., 2005; Caporal et al., 2008). Loss or mutation of tumor suppressor phosphatase and tensin homolog, amplification or mutation of phosphoinositide 3-kinase, 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. In addition, immunohistochemical analyses have shown that AKT activation is a poor prognostic factor in various cancers (LoPiccolo et al., 2007; Nakanishi and Ross, 2012; 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 chemotherapeutic agents or radiotherapy can reduce the apoptotic threshold and preferentially kill cancer cells (Crowell et al., 2007; Engel et al., 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 inhibitors targeting the ATP-binding site, pleckstrin homology domain, or protein substrate binding site of AKT. Several of them, such as 8-(4-(1-amino-2,3-dicyano-1,4-bis(methylthio)buta-2,3-diyldinyl) thiazole-5-carboxamide derivatives (Chang 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; Zhu et al., 2007; Wei et al., 2011). We screened hit compounds to a series of 2-(methylaminopyrimidinyl) propan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide (DC120) from these compounds by a fluorescence resonance energy transfer-based Z-LYTE assay. A fluorescence resonance energy transfer-based Z-LYTE kinase assay kit—Ser/Thr 6 peptide (Invitrogen, Carlsbad, CA) was used to evaluate the EC_{50} value of these 2-pyrimidyl-5-amidothiazole compounds for inhibition of AKT1 kinase. The reaction was performed on a 384-well plate with a 10-μM 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 an appropriate amount of AKT1 kinase with a serial 3-fold dilution of test compound. The final reaction concentration of ATP was 75 μM. After a 1-h incubation, a reaction was developed and terminated, and the fluorescence ratio was calculated according to the manufacturer’s protocol. Staurosporine was used as a positive control. A dose-response curve was fitted using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

**Materials and Methods**

**DC120 Preparation.** For all in vitro studies, compound DC120 (structure shown in Fig. 2A) was dissolved in DMSO at a concentration of 50 mM 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 50 mg/ml.

**Cell Culture and Reagents.** Human nasopharyngeal carcinoma CNE2 and human breast cancer MDA-MB-453 cells were cultivated in DMEM supplemented with 10% fetal bovine serum in a 5% CO₂ humidified atmosphere at 37°C. Glyceraldehyde-3-phosphate dehydrogenase, AKT, phospho-AKT (Ser473), phospho-AKT (Thr308), GSK3α/β, caspase-3, and horseradish peroxidase-conjugated second antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-FKHR, phospho-FKHR/L1, phospho-GSK3β, phospho-mTOR, cleaved caspase-3, mTOR, and chemiluminescence reagents were obtained from Cell Signaling Technology (Danvers, MA). Anti-Flag antibody, DAPI, MTT, and DMSO were purchased from Sigma (St. Louis, MO, USA).

**Z-LYTE Kinase Assay.** A fluorescence resonance energy transfer-based Z-LYTE kinase assay kit—Ser/Thr 6 peptide (Invitrogen, Carlsbad, CA) was used to evaluate the EC_{50} value of these 2-pyrimidyl-5-amidothiazole compounds for inhibition of AKT1 kinase. The reaction was performed on a 384-well plate with a 10-μM 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 an appropriate amount of AKT1 kinase with a serial 3-fold dilution of test compound. The final reaction concentration of ATP was 75 μM. After a 1-h incubation, a reaction was developed and terminated, and the fluorescence ratio was calculated according to the manufacturer’s protocol. Staurosporine was used as a positive control. A dose-response curve was fitted using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

**Annexin V-FLUOS Isothiocyanate Apoptosis Assay.** An annexin V-FLUOS Staining Kit (Roche, Basel, Switzerland) was used to evaluate DC120-induced apoptosis. Cells were cultured on a six-well plate and exposed to DC120. The cells were harvested and stained with annexin V-FLUOS solution and PI solution provided with the kit for 10 to 15 min. Apoptosis was immediately analyzed with a flow cytometer (Beckman Coulter, Fullerton, CA) at the wavelength of 488 nm.

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

**Cell Cycle Analysis.** For detecting DC120-induced apoptosis, cells were cultured in six-well plates and exposed to DC120. The cells were harvested and stained with annexin V-FLUOS solution and PI solution provided with the kit for 10 to 15 min. Apoptosis was immediately analyzed with a flow cytometer (Beckman Coulter, Fullerton, CA) at the wavelength of 488 nm.

**Plasmids and Transfection.** For inhibition of AKT activity, the short hairpin RNA (shRNA) sequences against AKT1 (5'-GC-
TACTTCCCTCCTAAGATG-3′ (Irie et al., 2005) were synthesized by Invitrogen Life Technologies (Shanghai, China) and cloned into the retroviral vector (pSUPER RNAi.puro). The CNE2 cells stably expressing AKTI 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 (Millipore) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol suggested by the manufacturer. After 24 h of transfection, the cells were used for further experiments.

**Immunofluorescence Staining.** For detecting the cellular localization of endogenous FKHR, cells were subcultured on a 24-well plate covered with 13 mm × 13 mm 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 Fluor 488 goat anti-mouse IgG antibody (diluted 1:400) for 1 h. For frozen sections of tumor tissue samples, the cells were incubated with anti-phospho-GSK3β antibody (1:200) at 4°C overnight and Alexa Fluor 488 goat anti-rabbit IgG antibody (1:400) for 1 h. After counterstaining with DAPI, the cells on coverslips or sections of tumor tissue were observed using a confocal microscope (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. Grading was as follows: −, for no immunofluorescence; +, for weak but definitely detectable immunofluorescence; +++, for weak but definitely detectable immunofluorescence; ++, for moderate immunofluorescence; and ++++, for intense immunofluorescence (Zhang et al., 2008; Deng et al., 2009).

**In Vivo Antitumor Activity.** BALB/c nude mice were obtained from Hunan Slac Jingda Laboratory Animal Co. Ltd (Changsha, China) 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 (Institute of Laboratory Animal Resources, 1996) and the United Kingdom Co-ordinating Committee on Cancer Research (1998). Tumor xenografts were established by 2 × 10^6 CNE2 cells injected subcutaneously into nude mice. Mice were randomly divided into three groups, and each group contained six mice. Treatments were initiated on day 7 after inoculation, by which time the tumor volume had reached ~50 mm^3; for each group, 8% solvent diluent (vehicle, negative control), 100 mg/kg CTX every 5 days (positive control), and DC120 (20 mg/kg/day) were administered intraperitoneally for 21 days. Tumor volumes and body weight of mice were observed. Tumor volumes were calculated by the formula: 0.5 × a × b^2 in millimeters, where a is the length and b is the width. When all control tumors developed to more than 2000 mg, the mice were sacrificed. After the tumor tissues were excised and weighed, the tissue samples were made into frozen sections with 4-mm-width immediately and fixed in ice-cold acetone. Then the frozen sections were stored at −80°C for future studies. Tumor growth inhibition (T/C percentage), which was used to evaluate the antitumor activity in vivo, 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.** An In Situ Cell Death Detection Kit (Roche) was used to evaluate DC120-induced apoptosis in vivo. Frozen tumor sections were incubated in blocking solution for 10 min and permeabilization solution for 2 min on ice. Then 50 μl of TUNEL reaction mixture was added to samples for 60 min at 37°C in a humidified atmosphere in the dark. After counterstaining with DAPI (1 μg/ml), frozen sections were observed using a confocal microscope. TUNEL-positive nuclei were stained green, and all other nuclei were stained blue (Markaryan et al., 2008).

**Statistical Analysis.** Student’s t test was used to evaluate the statistical significance of the result at the 95% confidence level, and P < 0.05 was considered to indicate statistical significance.
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 17/19-kD fragmentation that was also detected in CNE2 and MDA-MB-453 cells after DC120 treatment (Fig. 3D). These data indicated that DC120 indeed induced apoptosis in cancer cells, which was consistent with the results of the MTT growth inhibition assay.

**Growth Inhibition of DC120 on Cancer Cells Depending on AKT Activity.** To further confirm DC120 targeting of AKT kinase, retroviral vectors encoding shRNA sequences against AKT1 were stably transfected to CNE2 cells. The down-regulation of phospho-AKT and AKT1 was at least 75% (Fig. 4A, left), and the inhibitory rate in CNE2/AKT1 shRNA cells was obviously lower than that in control cells after treatment with DC120 \( (p < 0.01) \) (Fig. 4B, left). On the other hand, myr-AKT1 plasmids (constitutively activated

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**Fig. 1.** The effect of 2-(methylaminopyrimidinyl) thiazole-5-carboxamide compounds on the AKT kinase activity in a cell-free system. AKT1 kinase activity was determined using a Ser/Thr 6 peptide substrate, AKT1 kinase, ATP, and different concentrations of compounds in the presence of kinase buffer for 60 min as described under Materials and Methods with the Z'-LYTE assay. Staurosporine was used as a positive control. The dose-response curve was fitted using Prism 5.0.

DC130, \((S)-N-(1-amino-3-phenylpropan-2-yl)-2-(2-(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide; DC154, \((S)-N-(1-amino-3-(2,4-dichlorophenyl)propan-2-yl)-4-methyl-2\text{-}(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide; DC174, \((S)-N-(1-amino-3-(4-chlorophenyl)propan-2-yl)-2\text{-}(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide; DC175, \((S)-N-(1-amino-3-(1H-indol-3-yl)propan-2-yl)-2\text{-}(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide; DC181, \((S)-N-(1-amino-3-(4-hydroxyphenyl)propan-2-yl)-2\text{-}(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide; DC831, \((S)-N-(1-amino-3-p-tolylpropan-2-yl)-2\text{-}(methylamino)pyrimidin-4-yl) thiazole-5-carboxamide.

**Fig. 2.** DC120 inhibited cancer cell proliferation. A, chemical structure of 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 72 h. Each column represented the mean ± S.D. of triplicate determinations.
AKT1) were transfected into the MDA-MB-453 cells. In comparison with that in the control cells, the exogenous AKT and phospho-AKT expression significantly increased in MDA-MB-453/myr-AKT1 cells (Fig. 4A, right), and the inhibitory rate was also significantly increased after treatment with DC120 ($p < 0.01$) (Fig. 4B, right). To directly examine cell killing, the sub-G$_1$ fraction was tested using flow cytometric analysis. The results showed that DC120 decreased cell viability mainly because of the induction of apoptosis, as shown by the increase in sub-G$_1$ cells. In addition, we observed that the apoptotic cells increased more obviously in CNE2/vector cells compared with that in CNE2/AKT1 shRNA cells and in MDA-MB-453/myr-AKT1 cells compared with that in MDA-MB-453/vector cells, which was consistent with results for 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. Because 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 (Fig. 5, A and B). 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 the forkhead protein family (FKHR, FKHRL1, and AFX), GSK-3, endothelial nitric-oxide synthase, mTOR, p21, p27, MDM2, Bad, tuberin/TSC2, IKK, and others (Hers et al., 2011; Mannoury la Cour et al., 2011; Wu and Shih, 2011). Because inhibition of substrate phosphorylation can 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-3, and mTOR were all partially attenuated by DC120 dose de-
pendently 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 (Fig. 5, A and B). 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-MB-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 after treatment with DC120 (Fig. 5C). All these data indicated that DC120 could induce cell apoptosis by blocking the AKT downstream signaling pathway including AKT/GSK-3β, AKT/mTOR, and 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 8% solvent (negative control), DC120, or CTX (positive control) was administered intraperitoneally on day 7 after implantation. No obvious toxicity was observed in mice receiving 8% solvent treatment or 20 mg/kg/day DC120 treatment. Treatment with DC120 at 20 mg/kg/day could obviously suppress the tumor growth and the tumor growth inhibition (T/C percentage) was approximately 38.1% (Fig. 6A). Although the inhibitory rate of the positive control group (100 mg/kg CTX every 5 days) was 84.2%, obvious toxicity was observed, and three mice died during the treatment phase. To determine whether the growth inhibition of DC120 was caused by apoptosis in vivo, frozen tumor sections from CNE2-bearing nude mice were stained with TUNEL to identify the apoptotic cell population. As shown in Fig. 6B, treatment with 8% solvent did not appreciably induce apo-
ptosis, whereas DC120 at 20 mg/kg/day stimulated a substantially increased number of TUNEL-positive cells in CNE2 tumors, with an obvious increase in the ratio of apoptotic cells. Then we detected the 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/α/H11001 was ++ in the control group, whereas in the group treated with 20 mg/kg/day DC120, the intensity of fluorescence was +++ (Fig. 6C).

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

**Discussion**

Here we report 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 without affecting the total amount of JNK, P38, and ERK (Fig. 7A), possibly because DC120 induces a feedback loop to activate the MAPK pathway. The activation of feedback loops such as the MAPK pathway by DC120 prompted us to detect the apoptotic effect of the combination of the MEK inhibitor U0126 and DC120 on cancer cells. We noted that treatment with U0126 and DC120 inhibited phospho-ERK expression drastically (Fig. 7B). We also found that the two-drug combination resulted in more apoptotic cells than did treatment with either drug alone. Once combined with U0126, DC120-induced apoptosis increased from 13.9 to 27.5% in CNE2 cells and from 22.1 to 64.1% in MDA-MB-453 cells (p < 0.01) (Fig. 7C). These results indicated that U0126 effectively enhanced DC120-induced apoptosis in vitro.

<table>
<thead>
<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>
<td>—</td>
</tr>
<tr>
<td>DC120 (50 mg/kg/d)</td>
<td>1.61±0.33</td>
<td>38.1*</td>
</tr>
<tr>
<td>CTX (100 mg/kg/d)</td>
<td>0.41±0.07</td>
<td>84.2**</td>
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**Fig. 6.** Effects of DC120 on tumor growth of human CNE2 xenografts in nude mice. A, top, mice bearing established human CNE2 xenografts were given 8% solvent (vehicle group), 20 mg/kg/day DC120, or 100 mg/kg CTX every 7 days (positive group) intraperitoneally for 21 days. Points represent means of tumors (n = 6 for vehicle group and DC120 group; n = 3 for positive group), and bars represent S.D. 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 (arrows). Bottom, the relative numbers of apoptotic cells were determined by counting TUNEL-positive cells in three random fields (original magnification, ×40) for each sample. Columns represent means, and bars represent S.D. (*, p < 0.01 versus solvent). C, representative images of immunofluorescence staining of CNE2 xenograft frozen tumor sections for phospho-GSK3β protein. Original magnification, 40×.
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 (Figs. 5, A and B and 6C). We further observed that FKHR translocated into the nucleus upon the reversal of its phosphorylation induced by DC120 in cancer cells (Fig. 5C). Because the 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., (S)-1-(1H-indol-3-ylmethyl)-2-[5-(3-methyl-1H-indazol-5-yl)-pyridin-3-yl]oxy-ethylamine (A-443654), 4-[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-[(3S)-3-piperidinylmethoxy]-1H-imidazo[4,5-c]pyridin-4-yl]-2-methyl-3-butyn-2-ol (GSK690693), and GDC0068] tested and seems to be a sensitive marker of ATP-competitive AKT inhibition (Luo et al., 2005; Rhodes et al., 2008; Okuzumi et al., 2009). It might be caused by a feedback loop induced by DC120 or might be a direct consequence of DC120 binding to the ATP binding site of AKT (Okuzumi et al., 2009; Chandarlapaty et al., 2011; Chakrabarty et al., 2012). 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, 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 believed that combining of AKT inhibitors with other cancer therapeutic agents is a promising way to improve the tumor therapeutic window. It has been recently reported that combination treatment with MK-2206 (an allosteric AKT inhibitor) and selumetinib (AZD6244; a MEK inhibitor) is more effective than treatment with either drug alone in human non–small-cell lung cancer in vitro and in vivo (Meng et al., 2010). In addition, 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 noted that DC120 induced a feedback loop to activate the MAPK pathway and U0126 effectively enhanced DC120-induced apoptosis in vitro, which indicated that the combination of these two agents has a significant synergistic effect (Fig. 7). In addition, combination of DC120 with other anticancer agents, such as with everolimus (RAD001; a mTOR inhibitor) or radiotherapy is already being tested in our laboratory, and some exciting results have been achieved (data not shown). Exploring the efficacy of the combination of DC120 with other anticancer agents in further studies will be useful to...
confirm DC120 as a lead compound targeting AKT signal pathway or for further development.

In summary, our data provide evidence of a sustained antitumor effect and promising development for 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 cell 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 is clinically useful in this setting.

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

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