BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 689–697

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

SZ-685C, a marine anthraquinone, is a potent inducer of apoptosis with anticancer activity by suppression of the Akt/FOXO pathway

Gui'e Xie[1–3]*, Xun Zhu[1–3]*, Qing Li[1,2,4], Minghui Gu[1,2], Zhenjian He[1–3], Jueheng Wu[1–3], Jun Li[1,2,5], Yongcheng Lin[1,2,4], Mengfeng Li[1–3], Zhigang She[1,2,4] and Jie Yuan[1,2,5]

[1]*Key Laboratory of Tropical Disease Control (Sun Yat-sen University), Ministry of Education, Guangzhou, China,* [2]*Guangdong Province Key Laboratory of Functional Molecules in Oceanic Microorganism (Sun Yat-sen University), Bureau of Education, Guangzhou, China,* [3]*Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China,* [4]*School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou, China, and* [5]*Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China*

Background and purpose: The aims of this study were to investigate the anti-cancer activity of SZ-685C, an anthracycline analogue isolated from marine-derived mangrove endophytic fungi, and to explore the molecular mechanisms underlying such activity.
Experimental approach: The effect of SZ-685C on the viability of cancer cell lines was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. SZ-685C-induced apoptosis was assessed by Annexin V-fluorescein isothiocyanate/propidium iodide staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay and analysis of caspase activation. The effect of SZ-685C on the Akt/FOXO pathway was studied using Western blotting analysis, and the *in vivo* anti-tumour efficacy was examined in an MDA-MB-435 breast cancer xenograft model.
Key results: SZ-685C suppressed the proliferation of six cancer cell lines derived from human breast cancer, prostate cancer, glioma and hepatoma (IC_{50} values ranged from 3.0 to 9.6 μM) and the growth of breast cancer xenografts in mice. SZ-685C had a direct apoptosis-inducing effect through both the extrinsic and intrinsic apoptotic pathways, as shown by activation of caspase-8 and 9 as well as effector caspase-3 and poly (ADP-ribose) polymerase. Phosphorylation of Akt and its downstream effectors, forkhead box protein O1 and forkhead box protein O3a, was down-regulated in SZ-685C-treated cancer cells. Furthermore, the pro-apoptotic protein Bim was up-regulated by SZ-685C treatment consistent with FOXO dephosphorylation.
Conclusions and implications: SZ-685C could induce apoptosis through the Akt/FOXO pathway, which consequently leads to the observed anti-tumour effect both *in vitro* and *in vivo.* Our data suggest that SZ-685C may be a potentially promising Akt inhibitor and anti-cancer drug candidate.
British Journal of Pharmacology (2010) **159,** 689–697; doi:10.1111/j.1476-5381.2009.00577.x; published online 28 January 2010

Keywords: SZ-685C; breast cancer; apoptosis; Akt

Abbreviations: Ac-DEVD-AFC, 7-amino-4-trifluoromethylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; ER, oestrogen receptor; FITC, fluorescein isothiocyanate; FOXO1, forkhead box protein O1; FOXO3a, forkhead box protein O3a; IETD-*p*NA, L-isoleucyl-L-glutamyl-L-Threonyl-L-aspartic-p-nitroanilide acid amide; LEHD-*p*NA, L-leucine-L-glutamyl-L-histidyl-L-aspartic-p-nitroaniline acid amide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

Correspondence: Jie Yuan, Zhongshan School of Medicine; Zhigang She, School of Chemistry, Sun Yat-sen University 74 Zhongshan Road II, Guangzhou, Guangdong 510080, China. E-mail: yuanjie@mail.sysu.edu.cn; cesshzhg@mail.sysu.edu.cn
*These authors contributed equally to this work.
Received 7 June 2009; revised 22 September 2009; accepted 25 September 2009

Introduction

Over the decades, much effort has been directed to using natural products as a source of novel anticancer drugs in the fight against the challenge that many cancers remain incurable by currently available therapeutic approaches. In recent

years, marine microorganisms are increasingly attracting attention of the pharmaceutical community as they produce a wide variety of metabolites that are structurally unique and pharmacologically active (Schwartsmann *et al.*, 2003; D'Incalci *et al.*, 2004; O'Hanlon, 2006; Banerjee *et al.*, 2008). The chemical and biological diversities of marine microorganisms are considerable, and thus they may represent a promising resource for identifying new anticancer drugs (Simmons *et al.*, 2005). Moreover, the fact that microorganism-derived metabolites can be obtained from manufacturing culture systems makes drug discovery based on marine microbiology a pharmaceutically promising approach (Amador *et al.*, 2003).

As cancer development largely results from uncontrolled growth of malignant cells, in which cell proliferation surpasses cell death, deregulation of apoptosis, which occurs frequently in a vast majority of cancer types, has become a non-negligible target for anticancer strategies. Pro-apoptotic compounds, derived either from synthetic chemistry or natural sources, are under active investigation for their therapeutic effects, and for their mode of actions as well, against various cancers. Apoptosis, a form of programmed cell death, is a suicidal process executed via a cascade of caspase activation in an ordered and regulated manner (Hengartner, 2000; Fulda and Debatin, 2006). Activation of the caspase cascade can be triggered by two known pathways, namely, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is initiated by ligation of transmembrane death receptors with their ligands to activate membrane-proximal caspases such as caspase-8 and 10, which in turn cleave and activate effector caspases such as caspase-3 and 7. The intrinsic pathway is triggered by release of mitochondrial proteins, such as cytochrome c, from disrupted mitochondria. Once released, cytochrome c forms a complex with Apaf-1 and pro-caspase-9, which in return induces activation of caspase-9 and subsequently activates effector caspases. Apoptotic cells are usually characterized by several distinctive morphological and biochemical changes, including cell shrinkage, chromatin condensation, presence of phosphatidylserine on cell membrane surface, DNA fragmentation, protein cleavage at specific locations and increased mitochondrial membrane permeability (Fesik, 2005; Ghobrial *et al.*, 2005; Fulda and Debatin, 2006).

On the other hand, cell death and survival are under modulation of a network of transmembrane and/or intracellular signals, in which the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a prominent mediator of the survival, proliferation and other malignant phenotypes in a wide variety of cancers, and suppression of the PI3K/Akt pathway usually halts cell proliferation and results in cell death. Briefly, Akt is phosphorylated in response to PI3K activation, and the phosphorylated Akt promotes cell survival, proliferation and possibly other malignant properties such as motility and invasiveness through phosphorylating downstream targets, including anti-apoptotic protein Bad and transcriptional factors forkhead box protein (FOXO) and NF-κB (Brunet *et al.*, 1999; Romashkova and Makarov, 1999; Vivanco and Sawyers, 2002). Significantly increased Akt phosphorylation has been found in a wide variety of cancers (Yamamoto *et al.*, 2004; Lu *et al.*, 2006). The link between activation of the PI3K/Akt pathway and cancer development and progression makes the signalling pathway a promising target for cancer chemotherapy (LoPiccolo *et al.*, 2007). Development of cytotoxic drugs that target the PI3K/Akt pathway has become an increasingly attractive strategy for cancer therapy.

SZ-685C is a natural, biologically active substance isolated from the secondary metabolites of the mangrove endophytic fungus No. 1403 collected from the South China Sea (She *et al.*, 2008). Interestingly, SZ-685C exhibits structural similarity to epiadriamycin, an anti-cancer cytotoxic drug used widely in the clinic. Nonetheless, the biological functions of SZ-685C remain largely unknown. In this study, we investigated the anti-cancer activity of SZ-685C and analysed its underlying molecular mechanisms. Our data demonstrated that the compound selectively caused apoptosis in malignant cells and that such a pro-apoptotic effect might involve inhibition of the PI3K/Akt pathway in target cells.

Methods

Preparation of SZ-685C

SZ-685C was prepared and purified from mangrove endophytic fungus No. 1403 as previously reported, and its structure was identified by interpretation of spectral data (MS, ^{1}H NMR, ^{13}C NMR, 2D NMR) and X-ray single crystal diffractive technique (Figure 1) (She *et al.*, 2008). The compound was dissolved in 0.5% dimethylsulphoxide (DMSO) at a concentration of 1 mM as stock solution and diluted according to experimental requirements when used.

Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-435, prostate adenocarcinoma cell line PC-3, glioma cell line LN-444 and hepatoma cell lines Hep-3B and Huh-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 μg·mL^{-1} streptomycin and 100 units·mL^{-1} penicillin (Invitrogen). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability assay

Cells were seeded in 96-well flat-bottom plates at a density of 1×10^4 cells per well and cultured in a humidified incubator for 24 h, followed by exposure to various concentrations of SZ-685C for 48 h. Subsequently, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

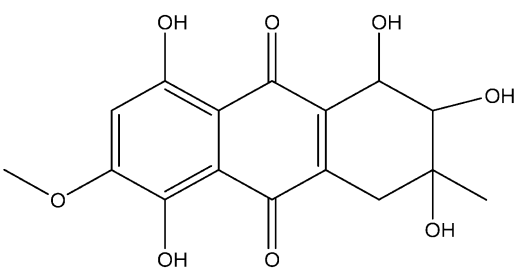

Figure 1 Chemical structure of SZ-685C.

(MTT) reagent (Genview, Houston, TX, USA) dissolved in phosphate-buffered saline (PBS) (pH 7.4) at a concentration of 5 mg·mL^{-1} was added to each well, and the cells were incubated for additional 4 h. The MTT-formazan crystals formed were dissolved in 150 μL DMSO (Sangon Biotech, Shanghai, China), and the absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader. Cell growth inhibition was determined using the following formula according to a previously published method: growth inhibition (%) = (1-OD of treated cells/OD of control cells) × 100% (Moon *et al*., 2000). The half maximal inhibitory concentration (IC_{50}) was calculated by Bliss's software (Bliss, 1935), and the data were analysed by SPSS.

Annexin V-FITC (fluorescein isothiocyanate)/propidium iodide (PI) staining assay

Cells (3×10^5) were collected, washed and stained after treatment with SZ-685C at final concentrations of 50%, 100% and 200% of IC_{50} for 8 h, according to the manufacturer's protocol (Keygen Biotech, Nanjng, China). Briefly, cells were washed twice with PBS and incubated in 500 μL binding buffer containing Annexin V-FITC and PI in the dark for 10 min at room temperature. The stained samples were then analysed on a FACSort flow cytometer as instructed by the manufacturer (Becton Dickinson, San Jose, CA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay

The TUNEL assay for *in situ* detection of apoptosis was performed by using the DeadEnd™ Fluorometric TUNEL System assay kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Cells were plated in 24-well flat-bottom plates at a density of 1×10^5 cells per well, treated with SZ-685C at different concentrations of 50%, 100% and 200% IC_{50} for 24 h. Following SZ-685C treatment, cells were fixed in 4% paraformaldehyde at 4°C for 25 min. Fixed cells were then permeabilized in 0.1% Triton X-100 and labelled with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. After rinsing with PBS, nuclei were counterstained with PI (1 μg·mL^{-1}) for 15 min. The localized green fluorescence of apoptotic cells was detected by fluorescence microscopy with an inverted microscope (Zeiss Axiovert100M, Carl Zeiss, Germany). Ten randomly chosen microscopic fields were captured. Experiments were performed in triplicate.

Western blotting analysis

After treatment with SZ-685C at different concentrations for 48 h, cells in each dish, including dead cells floating in medium, were harvested and lysed in 1× sampling buffer. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay kit (Pierce Biotech, Rockford, IL, USA). An aliquot of the denatured supernatant containing 30 μg of protein was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes. Membranes were blocked with blocking buffer (Tris-buffered saline, i.e. TBS, containing 5% non-fat milk) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with the following specific primary antibodies: mouse anti-human caspase-8, mouse anti-human caspase-9 (BD Biosciences, San Jose, CA, USA); rabbit anti-human caspase-3 (Abcam, Cambridge, MA, USA); rabbit anti-human poly (ADP-ribose) polymerase (PARP), rabbit anti-human phospho-Akt (ser473), rabbit anti-human Akt, rabbit anti-human phospho-FOXO3a (ser253), rabbit anti-human FOXO3a, rabbit anti-human phospho-FOXO1 (ser256), rabbit anti-human Bim (Cell Signaling Technology, Beverly, MA, USA); and mouse anti-human GAPDH (ProteinTech, Chicago, IL, USA). Further incubation with appropriate horseradish peroxidase-conjugated secondary antibodies, depending on the primary antibody used, was performed for 1 h at room temperature. Detection of staining signals was performed by using the enhanced chemiluminescence kit (Pierce Biotech). Experiments were performed in triplicate.

Caspase activity assay

Activity of caspase-8 and 9 was measured by using a caspase colorimetric assay kit (Keygen Biotech). After treatment of SZ-685C at different concentrations for 48 h, cells were harvested, washed with PBS and then resuspended in chilled lysis buffer. After incubation on ice for 40 min, cells were centrifuged for 1 min at 10 621× *g*. The supernatant was collected and protein concentration was determined by a Bradford protein assay kit (Keygen Biotech). Subsequently, 150 μg of each protein sample was diluted with 50 μL lysis buffer and added to 50 μL of 2× reaction buffer containing 10 mM dithiothreitol in a 96-well plate. Five microlitres of a colorigenic substrate, IETD-*p*NA (L-isoleucyl-L-glutamyl-L-Threonyl-L-aspartic-p-nitroanilide acid amide) or LEHD-*p*NA (L-leucine-L-glutamyl-L-histidyl-L-aspartic-p-nitroaniline acid amide), was added to each well, and the plate was incubated at 37°C in the dark for 4 h. ODs were determined at 405 nm using a microplate reader (Biotek, Winooski, VT, USA).

Caspase-3 activity was determined by using a fluorometric assay kit as described by the manufacturer (Roche, Nutley, NJ, USA). Cleavage of the fluorogenic substrate, Ac-DEVD-AFC (7-amino-4-trifluoromethylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) by caspase-3 was measured at an excitation wavelength of 380 nm and an emission wavelength of 490 nm. The fold increase in caspase activity was determined by comparing results with those obtained with non-induced control.

Xenografted tumour model and anti-tumour effect of SZ-685C in vivo

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Female BALB/c-nu mice (18–20 g) were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine, and were housed in barrier facilities on a 12 h light/dark cycle. On day zero, human breast cancer MDA-MB-435 cells (5×10^6 cells in 0.1 mL per mouse) were inoculated subcutaneously in the right mammary gland. On day 5, the formed tumours were measured, and the mice were randomly divided into a

treatment group and a control group. The treatment group received i.p. doses of 50 mg·kg^{-1} body weight of SZ-685C in 200 μL 0.5% DMSO solution every 3 days, while animals in the vehicle-control group received i.p. 200 μL 0.5% DMSO solution per mouse. Tumours were measured every 3 days in double-blinded manner, and the tumour sizes were assessed by measuring perpendicular diameters with a digital caliper. The tumour volumes (mm^3) were calculated using the following formula: volume = width × width × length × π/6. Data were presented as the means ± SD of six mice in each group. At the endpoint of the experiment, all the animals were killed, and the tumours were dissected, weighed and subjected to pathological examination.

Statistical analysis

The data given in the text are expressed as means ± SD. For the MTT assay, statistical analyses used Student's *t*-test. Statistical significances of the differences in tumour volumes between treatment and control groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons.

The drug and molecular target nomenclature used in this paper conform with the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2008)

Results

Growth inhibition of human cancer cells by SZ-685C

The effect of SZ-685C on the growth of human breast cancer cell lines, MCF-7 and MDA-MB-435, was investigated using the MTT assay. After treatment with SZ-685C for 48 h, cultured MCF-7 and MDA-MB-435 cells exhibited markedly inhibited growth, compared with control cells treated with vehicle, in a dose-dependent manner (Figure 2). Calculated

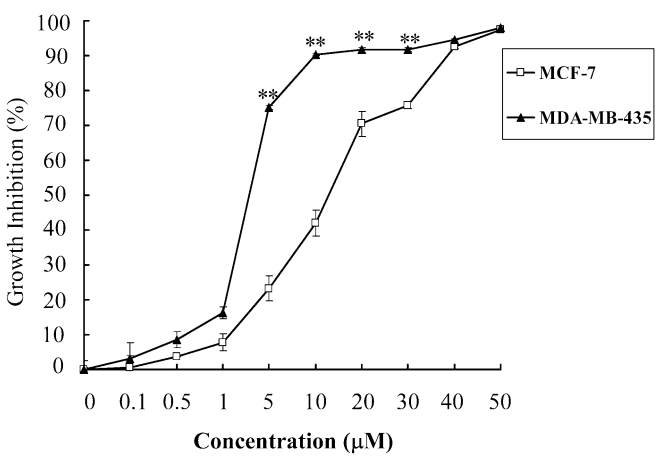

Figure 2 Effect of SZ-685C on growth of breast cancer cell lines MCF-7 and MDA-MB-435. Cells were seeded in 96-well plates and incubated with different concentrations of SZ-685C as noted for 48 h at 37°C. Cell viabilities were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data points are presented as means ± SD of triplicate experiments. The Student's *t*-test was performed to compare the growth of MCF-7 and MDA-MB-435 cells; $^{**}P < 0.01$; significant difference between two cell lines.

IC_{50} values, that is, concentrations of SZ-685C required for decreasing the growth rate of the cells by 50%, were 7.5 μM for MCF-7 and 3.0 μM for MDA-MB-435 cells respectively.

We further evaluated the effect of SZ-685C on the viability of cell lines of three other types of human cancer, including PC-3 (prostate adenocarcinoma), LN-444 (glioma), Hep-3B and Huh-7 (hepatoma), and the results revealed significant inhibitory effects of SZ-685C on all four tested cell lines, with IC_{50} values <10 μM after 48 h of treatment (Table 1).

SZ-685C induced apoptosis in breast cancer cells

To determine whether the loss of cell viability induced by SZ-685C was associated with apoptosis, we performed Annexin V-FITC/PI binding assay, which evaluates phosphatidylserine turnover from the inner to the outer lipid layer of the plasma membrane, an event typically associated with apoptosis. As shown in Figure 3, after 8 h treatment with SZ-685C at concentrations ranging from 50% to 200% IC_{50}, the numbers of apoptotic MCF-7 and MDA-MB-435 cells, as revealed by Annexin V binding, increased in a dose-dependent manner, indicating a pro-apoptotic activity of SZ-685C.

When TUNEL assays were performed to assess DNA fragmentation as a late event in the process of apoptosis in MCF-7 and MDA-MB-435 breast cancer cells after treatment with SZ-685C, dose-dependent TUNEL staining of fragmented DNA as a result of apoptosis was demonstrated (Figure 4). The results of two apoptosis assays, that is, the Annexin V-binding and TUNEL assays, strongly suggested that the death of breast cancer cells treated with SZ-685C was a consequence of cell apoptosis selectively caused by the compound as an apoptosis inducer, rather than a result of non-specific cytotoxicity.

Activation of both the extrinsic and intrinsic apoptotic pathways by SZ-685C

To further understand the apoptotic pathway(s) involved in SZ-685C-induced cell death, key caspases were examined using Western blotting and enzymatic (colorimetric or flurometric) activity assays. As shown in Figure 5A, SZ-685C treatment dose-dependently led to activating cleavage of caspase-8, displaying a dramatic increase of cleaved bands accompanied by a reduction in the level of the pro-caspase-8. Consistent with the results of the Western blotting analysis, the enzymatic activity of caspase-8 showed a dose-dependent increase with SZ-685C treatment (Figure 5B). Concurrently, cleavage of caspase-9 precursor and increased caspase-9 activity were also detected (Figure 5). These results indicated

Table 1 IC_{50} values of SZ-685C on various cancer cell lines

Cell line	*IC_{50} (μM)*
MCF-7	7.5
MDA-MB-435	3.0
PC-3	4.1
LN-444	7.8
Hep-3B	3.2
Huh-7	9.6

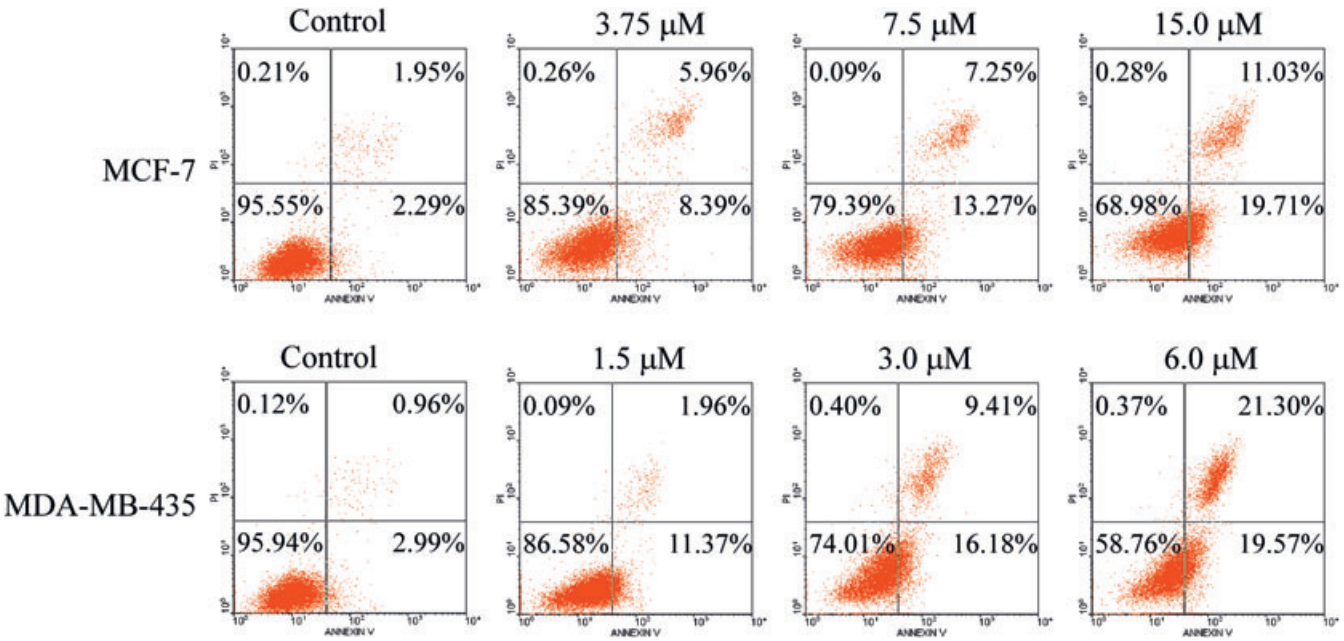

Figure 3 Annexin V-FITC/PI staining of MCF-7 and MDA-MB-435 cells treated with SZ-685C. Cells were exposed to different concentrations (50%, 100% and 200% of IC_{50}) of SZ-685C for 8 h. Cells were collected and subjected to Annexin V-FITC/PI staining and analysed by flow cytometry. FITC, fluorescein isothiocyanate; PI, propidium iodide.

that both caspase-8- and caspase-9-dependent apoptotic pathways were activated by SZ-685C.

Caspase-3, one of the major effector caspases, was also examined in MDA-MB-435 cells before and after SZ-685C treatment. Cleaved, activated caspase-3 was detected after 48 h treatment with 6.0 μM SZ-685C (Figure 5). It has been widely reported that MCF-7 cells do not express detectable levels of caspase-3 due to a deletion in the *casp-3* gene (Vegran *et al*., 2006). The apoptotic phenomenon induced by SZ-685C and the cleavage of PARP in MCF-7 cells may be mediated by other effector caspases such as caspase-6 and 7. Cleavage of PARP from 116 to 85 kDa was clearly demonstrated after SZ-685C treatment both in MDA-MB-435 and MCF-7 cells (Figure 5A). Taken together, these data suggest that apoptosis induced by SZ-685C in breast cancer cells may involve both intrinsic and extrinsic pathways.

Inhibition of Akt phosphorylation and its downstream substrates by SZ-685C

In order to further understand the signalling cascade that mediates the pro-apoptotic effect of SZ-685C on breast cancer cells, changes in the phosphorylation status of Akt were investigated. As shown in Figure 6, for both tested breast cancer cell lines, in which Akt was constitutively activated, SZ-685C treatment resulted in a dose-dependent decrease of phospho-Ser473-Akt without affecting total Akt expression.

We next examined the effect of SZ-685C on FOXO1 and FOXO3a phosphorylation, as Akt has been shown to negatively regulate the activity of forkhead transcriptional factors (Brunet *et al*., 1999). As shown in Figure 6, phosphorylation of FOXO1 and FOXO3a was dose-dependently inhibited by SZ-685C treatment. As phosphorylation suppresses the transcriptional activity of FOXO, we further examined the expression of Bim, a pro-apoptotic BH3-only protein and a target gene of FOXO3a to determine whether inhibition of FOXO was associated with increase of Bim expression. As shown in Figure 6, in parallel with reduced FOXO phosphorylation, Bim was up-regulated in response to SZ-685C treatment.

Anti-tumour effect of SZ-685C in vivo

To evaluate the anti-tumour activity of SZ-685C *in vivo*, nude mice subcutaneously inoculated with MDA-MB-435 cells were used. When the tumour volumes were assessed on day 3 after inoculation, all group of animals were found to have developed s.c. tumours, with a mean volume (±SD) of approximately 30 mm^3. Following i.p. injection with SZ-685C, a marked inhibition of the growth of the xenografted tumours was observed (Figure 7). The differences in tumour volumes between the vehicle and the 50 $mg \cdot kg^{-1}$ SZ-685C treatment groups were statistically significant from day 20 of treatment ($P < 0.05$) to the end of the experiment. After 35 days of drug administration, the average tumour weight in the control group was 1.69 ± 0.18 g, whereas the average tumour weight in SZ-685C-treated group was 0.65 ± 0.08 g, exhibiting a 61% inhibition of tumour growth. No signs of adverse effects, such as discomfort, behavioural changes or weight loss, were observed in SZ-685C-treated animals.

Discussion

Anthracyclines are a class of anti-cancer chemotherapeutic drugs derived from *Streptomyces* and are among the most effective anti-cancer treatments ever developed against various types of human cancer. Aggressive use of

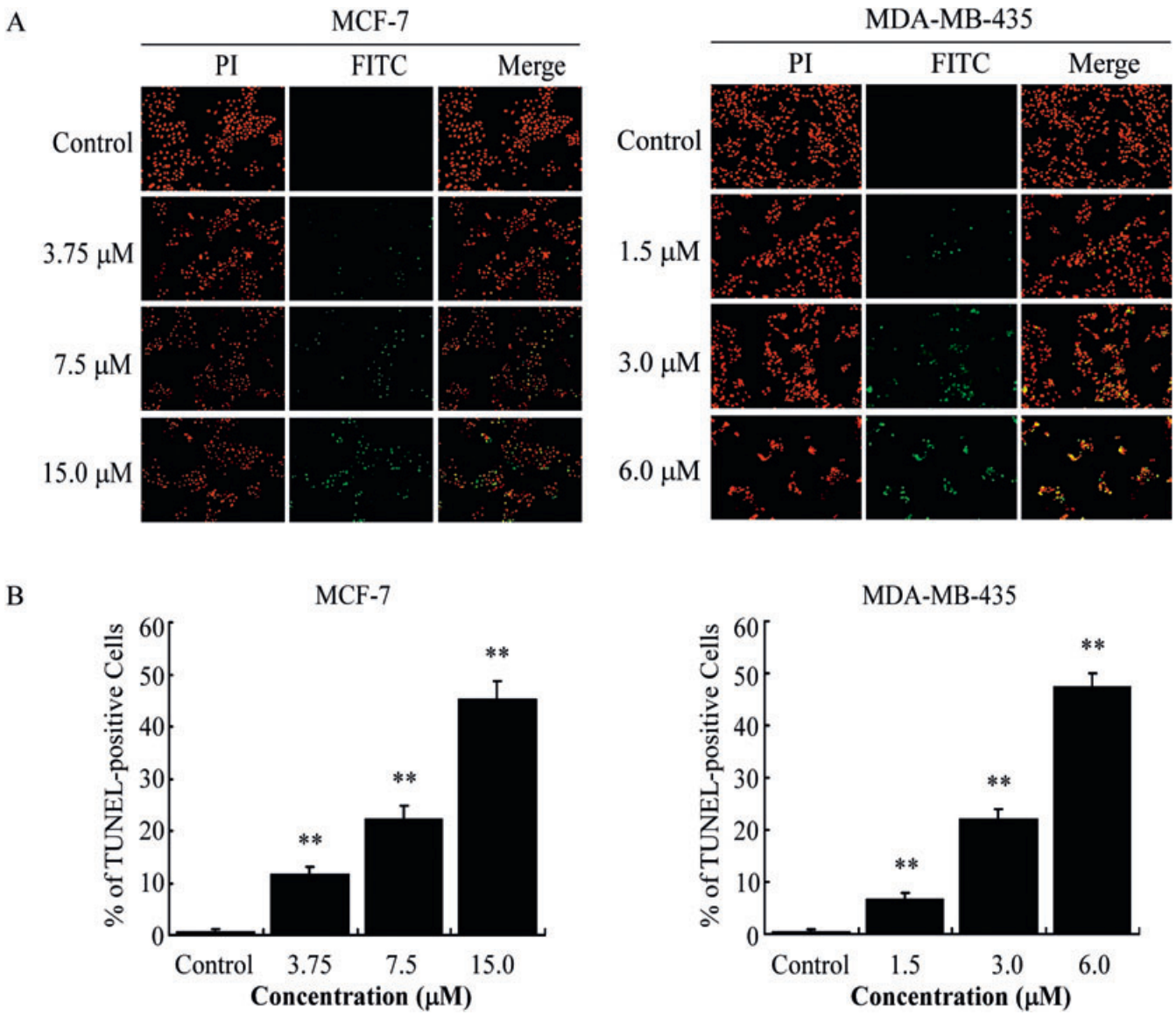

Figure 4 TUNEL assay of MCF-7 and MDA-MB-435 breast cancer cells treated with SZ-685C. (A) Cells were treated with different concentrations of SZ-685C as noted for 24 h and labelled with fluorescein-12-dUTP (red) and PI counterstaining (green). (B) Apoptotic index as determined by counting and calculating the percentages of TUNEL-positive cells in 10 fields. Data for the quantitative assessment of apoptosis are expressed as means ± SD. $^{**}P < 0.01$, compared with control. FITC, fluorescein isothiocyanate; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

anthracyclines in the clinic is frequently prevented by their severe adverse side effects, including cardiotoxicity (Shadle *et al.*, 2000; Schimmel *et al.*, 2004; Gianni *et al.*, 2008) and myelosuppression (Hofland *et al.*, 2005), as well as the tendency to induce multidrug resistance (Chien and Moasser, 2008; Perez, 2009). Therefore, identification of new anthracenedione derivatives with improved pharmacological and toxicological profiles is an ongoing effort in the community. Based on our previous preliminary investigation on a large number of metabolites derived from marine microbes for their effects on cancer cell growth and comparative efficacies on malignant versus normal epithelial cells, SZ-685C emerged from 69 compounds, all of which exhibited significant inhibition on malignant cells to various degrees, as one of those that displayed mildest cytotoxic effects on normal cells (data not shown). In this study, we have demonstrated that SZ-685C selectively kills cancer cells via activating both caspase-8- and caspase-9-based apoptotic mechanisms by suppressing, at least in part, the phosphorylation of Akt, and thus might represent a novel candidate for future development of effective, safe anti-cancer drugs.

As our data show, SZ-685C displayed potent cytocidal effect on six tested cancer cell lines including MCF-7 and MDA-MB-435 breast cancer cells, with IC_{50} < 10 μM. Interestingly, of two breast cancer cell lines tested, MCF-7 is relatively well differentiated and oestrogen-dependent, whereas MDA-MB-435 is an invasive and oestrogen-independent line. Thus, it is noteworthy that the oestrogen receptor (ER)-negative, highly invasive MDA-MB-435 cells were highly sensitive to SZ-685C treatment (IC_{50} = 3.0 μM). As oestrogen-independent breast

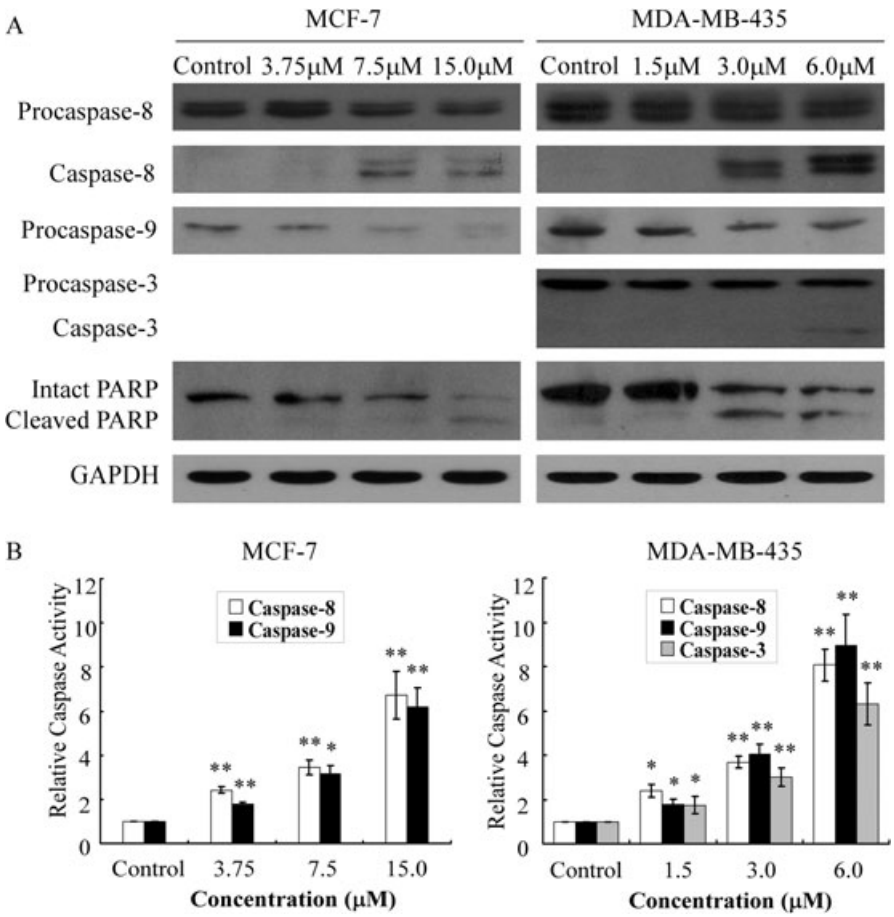

Figure 5 Activation of caspases and poly (ADP-ribose) polymerase (PARP) in MCF-7 and MDA-MB-435 breast cancer cells by SZ-685C. (A) Western blotting analysis of caspases and PARP in MCF-7 and MDA-MB-435 after SZ-685C treatment at different concentrations for 48 h using antibodies against caspase-8, 9, 3 and PARP. GAPDH was used as an internal control. (B) Enzymatic activities of caspases in SZ-685C-treated MCF-7 and MDA-MB-435 cells, as assessed with colorimetric or fluorometric assay. The fold increases in the activities of caspase-8, 9 and 3 were determined by comparison with those of the vehicle-treated control cells. Results are presented as means ± SD. *$P < 0.05$, **$P < 0.01$; significant differences compared with the control.

cancer is more resistant to anti-oestrogen therapy and associated with poor prognosis (Chang *et al.*, 2003), the equally, if not more, effective inhibition against MDA-MB-435 cells by SZ-685C as compared with that against the ER-positive, less invasive and more differentiated MCF-7 cells (IC_{50} = 7.5 μM) warrants further exploration of the possibility to treat ER-negative breast cancer.

The Akt pathway plays key roles in sustaining survival against the death programme in cancer cells, and it has been widely shown that hyperactivation of Akt is a common event in many human cancer types and that activation of the PI3K/Akt pathway has been reported to contribute to chemotherapeutic resistance in human cancers (West *et al.*, 2002; Lee *et al.*, 2005; Lu *et al.*, 2006; LoPiccolo *et al.*, 2007). This pathway therefore represents an important target for anticancer therapies. Identification of an effective and yet safe therapeutic inhibitor of PI3K or Akt, however, remains a major challenge. Our study shows that SZ-685C inactivates Akt, which, in turn, reduces phosphorylation of FOXO1 and FOXO3a, consequently leading to increased expression of its downstream target genes, such as Bim (Weidinger *et al.*, 2008). Up-regulation of Bim, which can bind to Bcl-2 and Bcl-XL via its BH3 domain, thereby sequestering Bcl-2 from the pro-apoptotic Bax and Bad, and initiating the apoptotic cascade (Gilley *et al.*, 2003; Sunters *et al.*, 2003), has been seen in SZ-685C-treated breast cancer cells. Thus, although it requires more in-depth studies to clarify whether the compound is a direct or indirect Akt inhibitor, the pharmacological and toxicological profiles of SZ-685C as partly revealed in this study, as well as its *in vivo* effectiveness in inhibiting xenografted tumour growth, provide reasonable optimism that the compound could become an attractive anti-Akt and anti-cancer drug candidate.

Acknowledgements

This work was supported by High-Tech Research (863) projects (Ministry of Science and Technology, China) (2007AA09Z448, 2007AA09Z431), a grant from the National Natural Science Foundation of China (40806059), a grant from the Natural Science Foundation of Guangdong Province, China (8151008901000014), a Key Project of Science & Technology Planning of Guangdong Province (2007A03260001), a

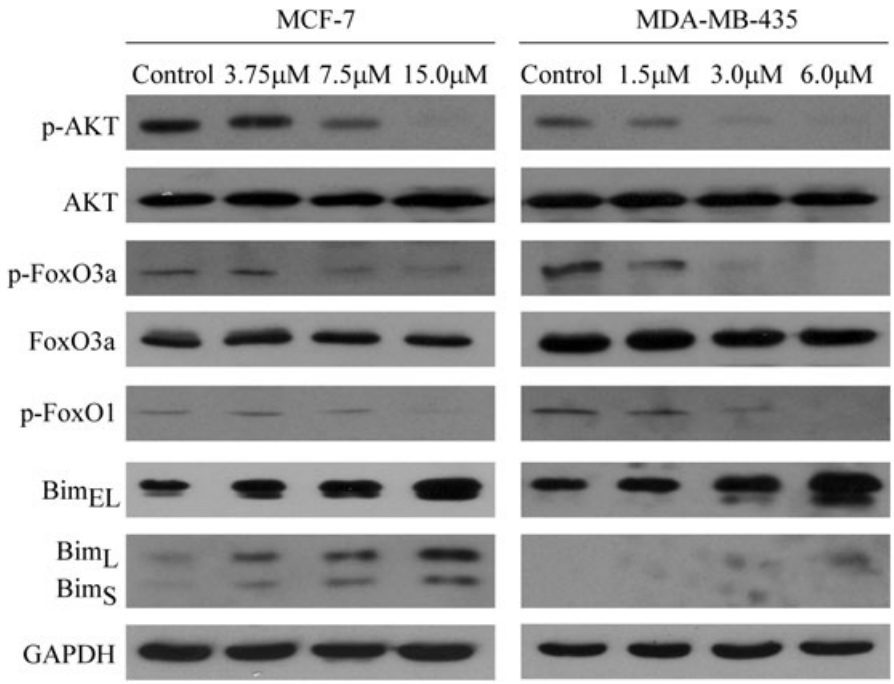

Figure 6 Effect of SZ-685C on the Akt signalling pathway in MCF-7 and MDA-MB-435 cells. Cells were treated with different concentrations for 48 h as noted. Western blotting analysis was performed using antibodies against phospho-Akt (Ser473), total Akt, phospho-FOXO3a (Ser 253), FOXO3a, phospho-FOXO1 (Ser256) and Bim, with GAPDH used as a loading control. FOXO1, forkhead box protein O1; FOXO3a, forkhead box protein O3a.

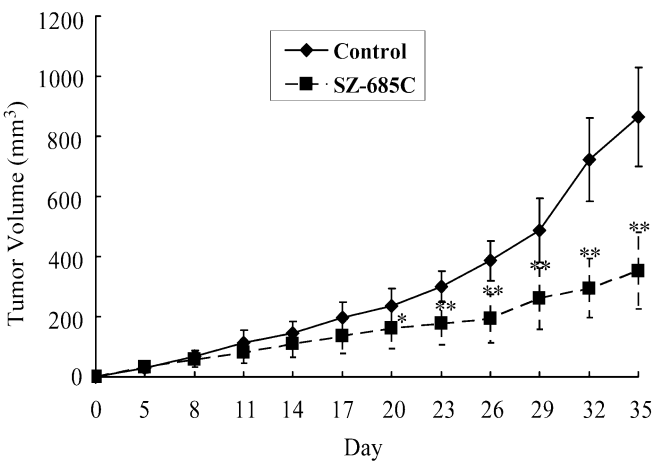

Figure 7 *In vivo* anti-tumour effect of SZ-685C on xenografted MDA-MB-435 tumour in nude mice. Viable MDA-MB-435 cells (5×10^6 cells per mouse) were inoculated subcutaneously in the right mammary gland of female nude mice. After solid tumour formation, the mice received an i.p. injection of SZ-685C (50 mg·kg^{-1}) or vehicle (0.5% dimethylsulphoxide) every 3 days for 1 month. Results are presented as means ± SD. *P < 0.05 and **P < 0.01; significant differences compared with the control; one-way ANOVA, followed by Dunnett's multiple comparisons test.

Key Science and Technique Research Project of Guangzhou Municipality (2006Z3-E4081), a Key Science and Technique Research Project of Zhuhai Municipality (PC20071076), a Basic Research Project of Ministry of Science and Technology, China (2007FY210600), and University-industry cooperation projects of Guangdong Province and Ministry of Education (2008B090500171).

Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Amador ML, Jimeno J, Paz-Ares L, Cortes-Funes H, Hidalgo M (2003). Progress in the development and acquisition of anticancer agents from marine sources. *Ann Oncol* **14**: 1607–1615.

Banerjee S, Wang Z, Mohammad M, Sarkar FH, Mohammad RM (2008). Efficacy of selected natural products as therapeutic agents against cancer. *J Nat Prod* **71**: 492–496.

Bliss C (1935). The calculation of the dose-mortality curve. *Ann Appl Biol* **22**: 134–167.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS *et al*. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**: 857–868.

Chang J, Clark GM, Allred DC, Mohsin S, Chamness G, Elledge RM (2003). Survival of patients with metastatic breast carcinoma: importance of prognostic markers of the primary tumor. *Cancer* **97**: 545–553.

Chien AJ, Moasser MM (2008). Cellular mechanisms of resistance to anthracyclines and taxanes in cancer: intrinsic and acquired. *Semin Oncol* **35** (Suppl. 2): 1–14.

D'Incalci M, Simone M, Tavecchio M, Damia G, Garbi A, Erba E (2004). New drugs from the sea. *J Chemother* **16** (Suppl.): 86–89.

Fesik SW (2005). Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer* **5**: 876–885.

Fulda S, Debatin KM (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* **25**: 4798–4811.

Ghobrial IM, Witzig TE, Adjei AA (2005). Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin* **55**: 178–194.

Gianni L, Herman EH, Lipshultz SE, Minotti G, Sarvazyan N, Sawyer DB (2008). Anthracycline cardiotoxicity: from bench to bedside. *J Clin Oncol* **26**: 3777–3784.

Gilley J, Coffer PJ, Ham J (2003). FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* **162**: 613–622.

Hengartner MO (2000). The biochemistry of apoptosis. *Nature* **407**: 770–776.

Hofland KF, Thougaard AV, Sehested M, Jensen PB (2005). Dexrazoxane protects against myelosuppression from the DNA cleavage-enhancing drugs etoposide and daunorubicin but not doxorubicin. *Clin Cancer Res* **11**: 3915–3924.

Lee S, Choi EJ, Jin C, Kim DH (2005). Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. *Gynecol Oncol* **97**: 26–34.

LoPiccolo J, Granville CA, Gills JJ, Dennis PA (2007). Targeting Akt in cancer therapy. *Anticancer Drugs* **8**: 861–874.

Lu S, Ren C, Liu Y, Epner DE (2006). PI3K-Akt signaling is involved in the regulation of p21 (WAF/CIP) expression and androgen-independent growth in prostate cancer cells. *Int J Oncol* **28**: 245–251.

Moon IJ, Choi K, Choi YK, Kim JE, Lee Y, Schreiber AD *et al*. (2000). Potent growth inhibition of leukemic cells by novel ribbon-type antisense oligonucleotides to c-myb1. *J Biol Chem* **275**: 4647–4653.

O'Hanlon LH (2006). Scientists are searching the seas for cancer drugs. *J Natl Cancer Inst* **98**: 662–663.

Perez EA (2009). Impact, mechanisms, and novel chemotherapy strategies for overcoming resistance to anthracyclines and taxanes in metastatic breast cancer. *Breast Cancer Res Treat* **114**: 195–201.

Romashkova JA, Makarov SS (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signaling. *Nature* **401**: 86–90.

Schimmel KJ, Richel DJ, van den Brink RB, Guchelaar HJ (2004). Cardiotoxicity of cytotoxic drugs. *Cancer Treat Rev* **30**: 181–191.

Schwartsmann G, Da Rocha AB, Mattei J, Lopes R (2003). Marine-derived anticancer agents in clinical trials. *Expert Opin Investig Drugs* **12**: 1367–1383.

Shadle SE, Bammel BP, Cusack BJ, Knighton RA, Olson SJ, Mushlin PS *et al*. (2000). Daunorubicin cardiotoxicity: evidence for the importance of the quinone moiety in a free-radical-independent mechanism. *Biochem Pharmacol* **60**: 1435–1444.

She ZG, Chen SP, Lin YC, Yuan J, Pang JY, Li MF *et al*. (2008). SZ-685C preparation method and antitumor application. Application No: 00810028628.3, Aplication Date: 2008.6.6.

Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH (2005). Marine natural products as anticancer drugs. *Mol Cancer Ther* **4**: 333–342.

Sunters A, Fernzyxonefiveninexyzndez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA *et al*. (2003). FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* **278**: 49795–49805.

Vegran F, Boidot R, Oudin C, Riedinger JM, Bonnetain F, Lizard-Nacol S (2006). Overexpression of caspase-3 splice variant in locally advanced breast carcinoma is associated with poor response to neoadjuvant chemotherapy. *Clin Cancer Res* **12**: 5794–5800.

Vivanco I, Sawyers CL (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**: 489–501.

Weidinger C, Krause K, Klagge A, Karger S, Fuhrer D (2008). Forkhead box-O transcription factor: critical conductors of cancerzyxonethreetwoxyz fate. *Endocr Relat Cancer* **15**: 917–929.

West KA, Castillo SS, Dennis PA (2002). Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat* **5**: 234–248.

Yamamoto S, Tomita Y, Hoshida Y, Morooka T, Nagano H, Dono K *et al*. (2004). Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. *Clin Cancer Res* **10**: 2846–2850.