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7-Diethylamino-3(2′-benzoxazolyl)-coumarin is a novel microtubule inhibitor with antimitotic activity in multidrug resistant cancer cells

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ABSTRACT

Microtubules are a proven target for anticancer drug development because they are critical for mitotic spindle formation and the separation of chromosomes at mitosis. We here report a novel synthetic microtubule inhibitor 7-diethylamino-3(2′-benzoxazolyl)-coumarin (DBC). DBC causes destabilization of microtubules, leading to a cell cycle arrest at G_2/M stage. In addition, human cancer cells are more sensitive to DBC (IC_{50} 44.8–475.2 nM) than human normal fibroblast (IC_{50} 7.9 μM), and DBC induces apoptotic cell death of cancer cells. Furthermore, our data show that DBC is a poor substrate of drug efflux pumps and effective against multidrug resistant (MDR) cancer cells. Taken together, these results describe a novel pharmacological property of DBC as a microtubule inhibitor, which may make it an attractive new agent for treatment of MDR cancer.

1. Introduction

Microtubules, composed of mainly α-/β-tubulin heterodimers, are critical elements in a variety of fundamental cell functions, including sustained shape, transportation of vesicles and protein complexes, and regulation of motility and cell division [1,2]. The polymerization, stabilization, and dynamic properties of microtubules are influenced by interactions with microtubule-associated proteins [3]. Microtubules play crucial roles in the regulation of the mitotic apparatus through their dynamic changes during mitotic spindle formation and separation of chromosomes. Thus, microtubules are considered to be a proven target for anticancer drug development [1,4]. Microtubule inhibitors interfere with the microtubule dynamics which results in the inhibition of chromosome segregation in mitosis and consequently the inhibition of cell division [5,6]. At the present time, there are two types of microtubule inhibitors which are clinically used for cancer therapy: taxanes (paclitaxel and docetaxel) and *Vinca* alkaloids (vincristine, vinblastine, and vinorelbine). Taxanes promote a net polymerization, leading to stabilize microtubules and *Vinca* alkaloids bind to the tubulin dimer and to microtubule ends, block the formation of new microtubules, and lead to the depolymerization of existing microtubules [1,4].

Although the taxanes and *Vinca* alkaloids are effective for the treatment of various malignant tumors, their efficacies are limited by multidrug resistance (MDR) [7]. The MDR phenotype is often mediated by overexpression of drug efflux pumps, including *P*-glycoprotein (*P*-gp) and the multidrug resistance proteins (MRPs), in the plasma membranes of cancer cells [7,8]. These efflux pumps are able to reduce the intracellular concentrations of taxanes and *Vinca* alkaloids to a nontoxic level. Therefore, there are enormous efforts to identify and develop a novel microtubule inhibitor able to overcome MDR. As a result, epothilones have been evaluated as novel microtubule inhibitors which exert potent antiproliferative activity in human cancer cells with MDR [9,10]. Among them, ixabepilone (aza-epothilone B, Ixempra™) has recently been approved by the Food and Drug Administration for treatment of taxane-refractory metastatic breast cancer, or in combination with capecitabine for patients with advanced breast cancer refractory to anthracyclines and taxanes [11,12].

Recent report shows the antiproliferative potential of 7-diethylamino-3(2′-benzoxazolyl)-coumarin (DBC; Fig. 1A) [13]; however, the exact molecular mechanism has not been studied. Here we reexamined the antiproliferative activity of DBC and investigated its possible molecular mechanism. In addition, we evaluated whether MDR can be overcome by DBC treatment. Our data herein demonstrate a novel pharmacological property of DBC

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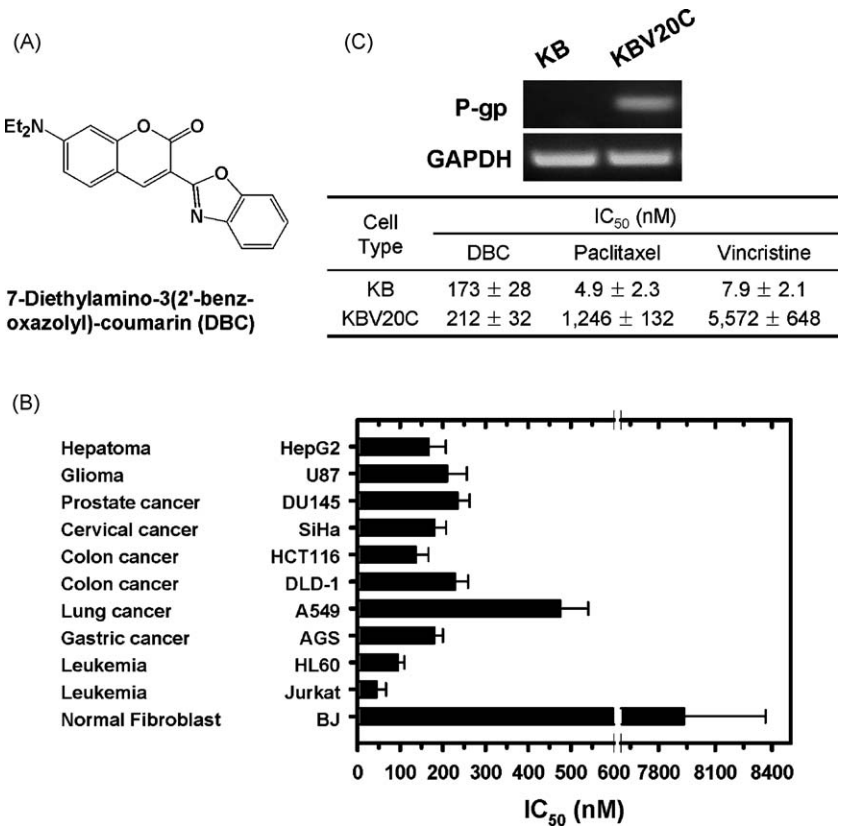

Fig. 1. Antiproliferative effect of DBC on various human cancer cell lines. (A) Chemical structure of 7-diethylamino-3(2′-benzoxazolyl)-coumarin (DBC). (B) Each cell was incubated with various concentrations of DBC for 48 h. Cell viability was determined by MTS assay as described in Section 2. Data were expressed as the mean ± SD from three independent experiments. (C) mRNA level of *P*-gp was analyzed in KB and KBV20C cells. And, KB and KBV20C cells were cultured in the presence of DBC, paclitaxel, or vincristine for 48 h, and cell viability was determined.

as a microtubule inhibitor: DBC treatment leads to a dramatic inhibition of tubulin polymerization and subsequent induction of G_2/M phase arrest in both MDR-negative and -positive cancer cells.

2. Materials and methods

2.1. Cell culture

Human cancer cell lines (KB, HepG2, U87, DU145, SiHa, HCT116, DLD-1, A549, AGS, HL60, and Jurkat) and human normal fibroblast BJ cells were grown in DMEM or RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). *P*-gp expressing KBV20C cells were maintained in the medium containing additional 20 nM vincristine (Sigma Chemical, St. Louis, MO) as described previously [14].

2.2. Reagents and cytotoxicity test

7-Diethylamino-3(2′-benzoxazolyl)-coumarin (DBC) was prepared as described previously [13]. Paclitaxel, vincristine and nocodazole were obtained from Sigma. All chemicals were dissolved in dimethyl sulfoxide (Sigma) and stored at −70 °C until use. Cytotoxicity was determined by the MTS assay (Promega, Madison, WI) according to the manufacturer's instruction.

2.3. Cell cycle analysis

KBV20C cells were treated with the indicated concentrations of paclitaxel or DBC for 24 h. Cells were harvested, washed twice with ice-cold PBS and fixed in 70% ethanol at −20 °C overnight. Cells were washed with PBS, incubated with 100 μg/ml RNase A at 37 °C for 30 min, stained with 25 μg/ml propidium iodide (PI) solution and analyzed by flow cytometry.

2.4. In vitro microtubule assembly assay

DBC was assayed at 1, 3, or 10 μM for effects on tubulin polymerization with Tubulin polymerization assay kit (#CDS01-B, Cytoskeleton Inc., Denver, CO) according to the manufacturer's recommended procedure. Incubation was carried out at 37 °C in a microplate reader (Victor 3, PerkinElmer) with absorbance readings at 355 nm every 30 s for 30 min.

2.5. Immunocytochemistry

KBV20C cells plated on coverslips were treated with the indicated concentrations of tested agents for 24 h. After fixing with methanol, the cells were blocked with 5% normal goat serum in PBS for 1 h, and then further incubated with anti-α-tubulin antibody (Cell signaling, Beverly, MA) for 2 h at room temperature. After PBS washing, the cells were incubated with Alexa-594 labeled secondary antibody (Molecular Probes, Eugene, OR) in the dark room for 1 h. The slides were sealed using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) for nuclei counterstaining. Cellular microtubules were observed with a confocal microscope (D-ECLIPSE C1, Nikon, Japan).

2.6. Apoptosis assays

After treatment of cells with DBC or paclitaxel for the indicated time points, the cells were harvested, and the extent of apoptosis

was determined by flow cytometric analysis using Annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA), according to the manufacturer's description. Poly(ADP-ribose) polymerase (PARP) cleavage was analyzed by immunoblot analysis.

2.7. Immunoblot analysis

Cells were lysed by incubation in 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 for 20 min at 4 °C and centrifuged at 15,000 × *g* for 15 min at 4 °C. Cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies to cyclin B1, phospho-cdc2, cdc2, Aurora A, phospho-histone H3 (Ser^{10}), α-tubulin (Cell signaling, Beverly, MA), phospho-Aurora A (Thr^{288}) (Novus Biologicals, Littleton, CO), MPM-2, and PARP (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed with TBS-0.1% tween 20, incubated for 1 h with a secondary antibody, and visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL).

2.8. RT-PCR

Total RNA was extracted using the easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology, Sungnam, Korea), and the integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) reaction using the ImProm-II Reverse Transcription System (Promega) and Taq polymerase (Solgent, Daejeon, Korea). The primer sets for *P*-gp were 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′; and the primer sets for GAPDH were 5′-CTCATGACCACAGTCCATGCCATC-3′ and 5′-CTGCTTCACCACCTTCTTGATGTC-3′.

3. Results

3.1. DBC inhibits proliferation of MDR cancer cells

A series of 7-diethylaminocoumarin compounds including DBC were recently synthesized and their antiproliferative potentials against human umbilical vein endothelial cells and several cancer cells were evaluated [13]. Among them, DBC showed the most potent effect against cancer cells [13]; however, the exact molecular mechanism has not been studied. To clarify a molecular mechanism by which DBC inhibits the proliferation of cancer cells, we first reexamined the effect of DBC on the proliferation of various human cancer cell lines. As shown in Fig. 1B, cell growth was inhibited to various degrees in the presence of DBC, having IC_{50} between 44.8 and 475.2 nM. DBC showed a marked antiproliferative effect against Jurkat and HL60, human leukemia cancer cells ($IC_{50} < 100$ nM), a moderate effect against HepG2, hepatocellular carcinoma cell, U87, glioma cell, DU145, prostate cancer cell, SiHA, cervical cancer cell, HCT116 and DLD-1, colon cancer cells, and AGS, gastric cancer cell (100 nM $< IC_{50} <$ 300 nM), and a weak activity against human lung cancer cell A549 with IC_{50} 475.2 nM. However, the growth of all cancer cell lines tested was more sensitive to DBC than the normal cell line, BJ (a human normal fibroblast), for which the IC_{50} value of DBC was 7.9 μM. The results indicate that DBC has a broad spectrum of antiproliferative activity toward various cancer cell lines. Next, we examined the effect of DBC on the proliferation of MDR cancer cells. The activity of DBC against MDR cells expressing *P*-gp drug efflux pump was assessed in KBV20C cells derived from human KB epidermoid carcinoma cells. As shown in Fig. 1C, high expression of *P*-gp was observed in KBV20C cells, but not in its parental KB cells. Concomitantly, KBV20C cells were more resistant to the well known microtubule inhibitors, paclitaxel and vincristine, which are good substrate for *P*-gp, than KB cells. In addition, the intracellular accumulation of rhodamine 123, a well known fluorescent *P*-gp substrate, was decreased by less than 10% in KBV20C cells, compared to KB cells (Supplemantary data Fig. S1), indicating that the overexpressing *P*-gp in KBV20C cells is functional. However, DBC showed similar antiproliferative effect against both KB and KBV20C cells, with IC_{50} 173 and 212 nM, respectively. In parallel, there are no significant differences in DBC accumulation between KB and KBV20C cells (Supplementary data Fig. S1). These results strongly indicate that DBC is a poor substrate of *P*-gp drug efflux pump and retains substantial activity against *P*-gp overexpressing MDR cancer cell lines.

3.2. DBC induces mitotic arrest by activation of cdc2/cyclin B1 in MDR cancer cells

To further analyze the antiproliferative effect of DBC, its effect on the cell cycle progression was next investigated. KBV20C cells were treated with various concentrations of DBC or paclitaxel for 24 h and then cell cycle analysis was performed. As shown in Figs. 2A and B, DBC treatment led to a dramatic increase in the cell population at G_2/M phase in a dose-dependent manner, and its potency was higher than that of paclitaxel. In addition, DBC was able to induce G_2/M arrest in KB cells with similar potency (data not shown). Next, we investigated the possible link between DBC-induced G_2/M phase arrest and alteration in G_2/M regulatory protein expression. It has been well demonstrated that activation of Cdc2 kinase is necessary for G_2/M transition of cell cycle, which requires accumulation of cyclin B protein and dephosphorylation of Cdc2 [15–17]. We first examined the level of cyclin B1 and phosphorylation level of cdc2 in DBC-treated KBV20C cells. As shown in Fig. 2C, DBC treatment caused an increase in cyclin B1 expression and a decrease in phosphorylated cdc-2 level in a dose dependent manner. We further examined the status of phosphorylated polypeptides found only in mitotic cells using MPM-2 antibody. After treatment with DBC, significant elevation in the levels of MPM-2 phosphoepitopes was observed in KBV20C cells (Fig. 2C). These results further support that DBC treatment can induce the cell cycle arrest at mitotic stage, resulting from activation of cdc2/cyclin B1 in MDR cancer cells.

3.3. Aurora kinases are activated by DBC treatment

Recent reports have demonstrated that Aurora kinases are essential for cell cycle regulation and high-fidelity mitosis [18,19]. Aurora-A kinase is necessary for mitotic spindle assembly and balanced chromosome segregation between daughter cells, and Aurora-B kinase is a "chromosomal passenger" protein that plays an important role in regulating mitosis [18,20]. Because specific inhibition of Aurora kinases can lead to errors in chromosome alignment and segregation, resulting in mitotic arrest, Aurora kinases are appreciated as promising targets for development of antitumor drugs [19,21]. To examine whether DBC-induced G_2/M arrest was due to the inhibition of Aurora kinases, we determined phosphorylation level of Aurora-A (Thr^{288}), which is an autophosphorylation site and essential for its kinase activity [22]. DBC treatment gradually increased phosphorylation level of Thr^{288} as well as expression of Aurora-A kinase (Fig. 2D). Next, we

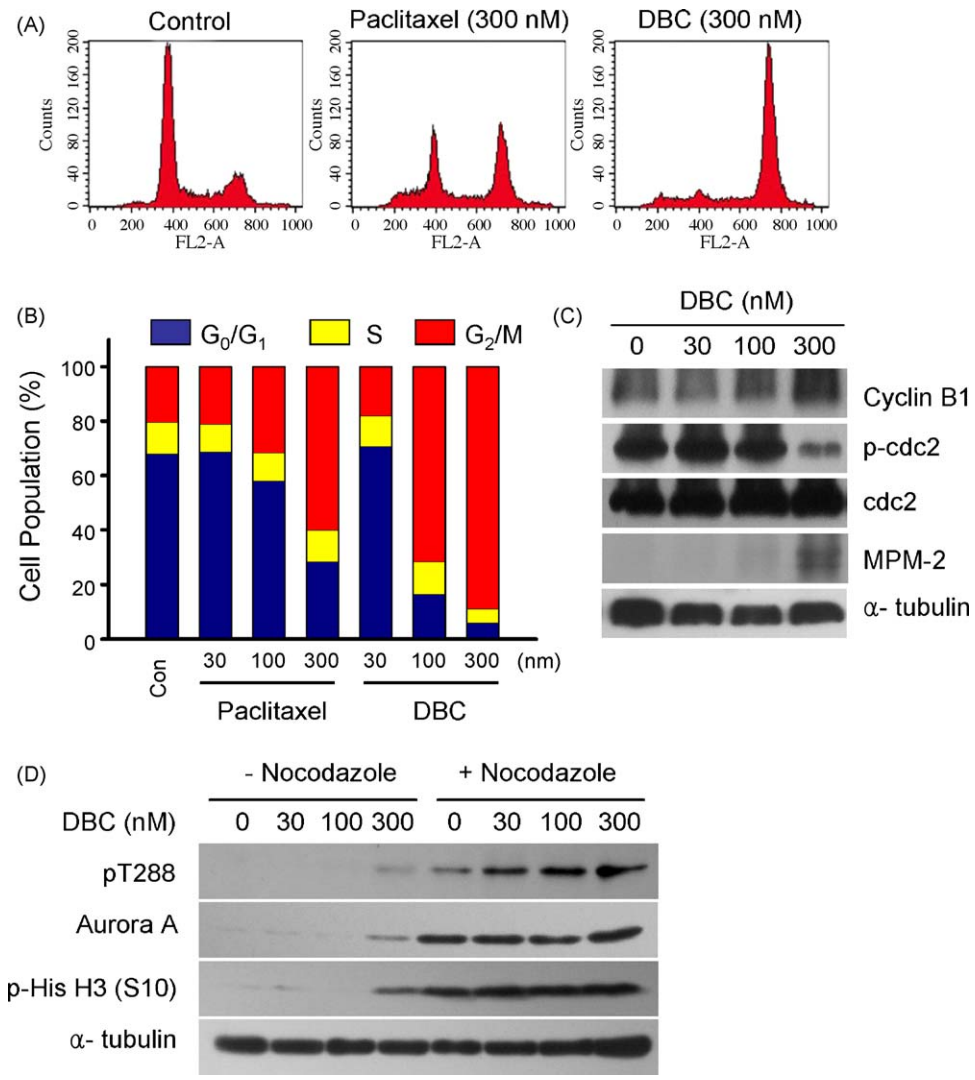

Fig. 2. G_2/M arrest of MDR cancer cells by DBC treatment. (A) and (B) KBV20C cells were treated with the indicated concentrations of paclitaxel or DBC for 24 h. After staining with propidium iodide, cell cycle stage was analyzed using flow cytometry. The cell population (%) at each cell cycle phase was represented. (C) After treatment of KBV20C cells with DBC for 24 h, expression and phosphorylation level of G_2/M regulators were examined with immunoblot analysis. (D) KBV20C cells were treated with the indicated concentrations of DBC for 24 h in the presence or absence of nocodazole (200 nM). And then phosphorylation level of Aurora A (Thr^{288}) and histone H3 (Ser^{10}), and expression level of Aurora A were analyzed.

determined the activation of Aurora-B kinase activity by measuring phosphorylation level of histone H3 (Ser^{10}) which is a well known substrate for Aurora-B kinase [23]. As shown in Fig. 2D, phosphorylation levels of histone H3 (Ser^{10}) were also increased by DBC treatment. These results indicate that DBC-induced G_2/M arrest is not mediated by inhibition of Aurora kinases, but on the contrary Aurora kinases are able to be activated by DBC, resulting from mitotic arrest. To further confirm this notion, we examined the effect of DBC in the presence of nocodazole, a well known mitotic arrester. Nocodazole treatment, which is enough for G_2/M arrest of KBV20C cells (Supplementary data Fig. S2), led to a dramatic increase in phosphorylation level of both Aurora-A (Thr^{288}) and histone H3 (Ser^{10}), as well as expression level of Aurora A, which was not inhibited by DBC treatment, but slightly increased (Fig. 2D).

3.4. DBC induces depolymerization of microtubules in MDR cancer cells

To investigate the molecular mechanism by which DBC induces mitotic arrest, we tested the effect of DBC on tubulin polymerization. As shown in Fig. 3A, DBC treatment abrogated tubulin polymerization in a dose-dependent manner. Similarly, treatment with vincristine, a well known microtubule destabilizer, led to a decrease in tubulin polymerization. However, treatment with a microtubule stabilizer paclitaxel led to a dramatic increase in tubulin polymerization. This result indicates that DBC may directly bind to tubulin, leading to inhibition of microtubule formation. We further examined the effect of DBC on cellular microtubule networks by using immunofluorescence techniques. As shown in Fig. 3B, the microtubule network exhibits normal arrangement and organization in KBV20C cells in the absence of drug treatment. Treatment with paclitaxel (0.1 μM) or vincristine (0.1 μM) did not make significant changes in microtubule arrangement and organization (Fig. 3B). However, DBC (0.1 μM) treatment led to dramatic changes in microtubule configuration; microtubule network in cytosol was disrupted and chromosome was also abnormally aggregated (Fig. 3B). Similar pattern was observed when the cells were treated with high concentration of vincristine (1 μM) (Fig. 3B). However, treatment with 1 μM paclitaxel dramatically promoted microtubule polymerization with an increase in the density of cellular microtubules (Fig. 3B). Altogether, these findings from *in vitro* tubulin

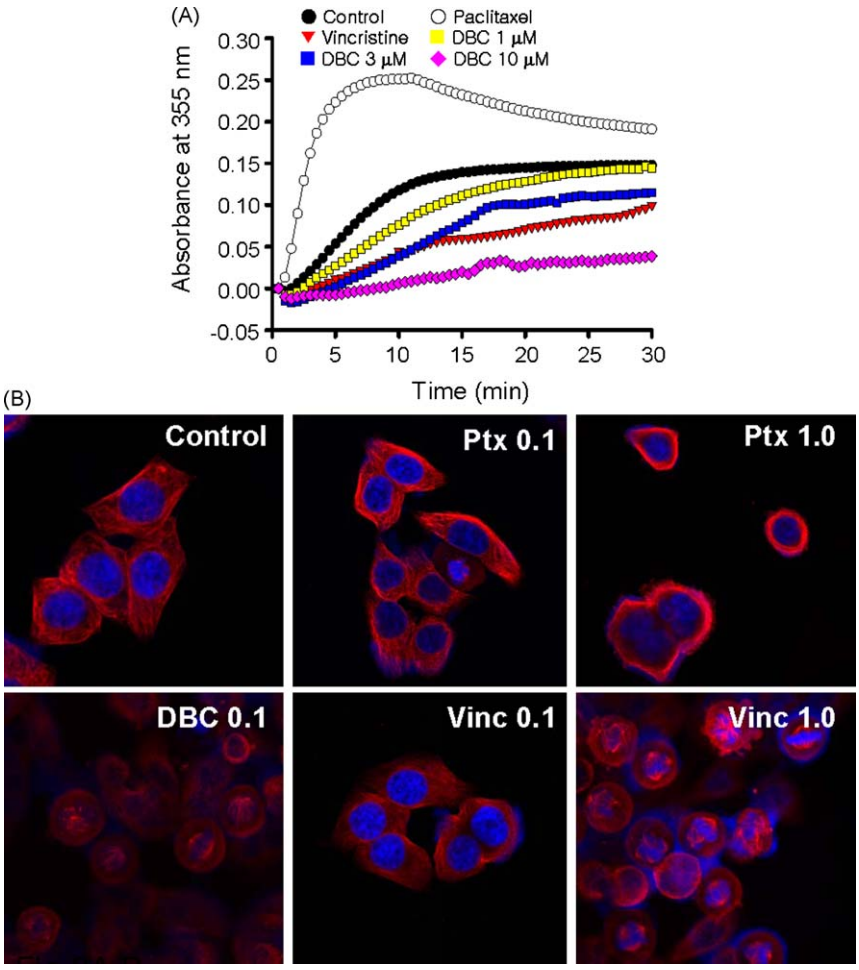

Fig. 3. DBC induces depolymerization of microtubules in MDR cancer cells. (A) Purified tubulin protein was incubated at 37 °C in the absence (control) or presence of drugs [paclitaxel (5 μM), vincristine (5 μM) or DBC (1, 3, 10 μM)]. Microtubule polymerization was analyzed by measuring absorbance at 355 nm every 30 s for 30 min. (B) After incubation of KBV20C cells with the indicated concentrations of each drug, the cells were fixed and reacted with α-tubulin antibody. And then the cells were incubated with Alexa-594 labeled secondary antibody. Cellular microtubules were observed with a confocal microscope.

polymerization and immunofluorescence strongly suggest that DBC inhibits microtubule formation and destabilizes microtubules through direct binding with tubulin components.

3.5. DBC induces apoptotic cell death in MDR cancer cells

We next examined the apoptotic potential of DBC on MDR cancer cells. Treatment of KBV20C cells with paclitaxel increased the number of Annexin V^+/PI^- cells (early apoptosis) up to 38% at 24 h, and gradually increased the number of Annexin V^+/PI^+ cells (late apoptosis) (Fig. 4A and B). However, DBC treatment led to a 78% increase in the number of Annexin V^+/PI^- cells at 24 h and the rapid appearance of cells in late apoptosis (Fig. 4A and B), indicating that DBC induces apoptotic cell death and its potency is higher than that of paclitaxel in MDR cancer cells. To further confirm the apoptotic potential of DBC, PARP cleavage, another maker of apoptotic cell death, was analyzed in the presence of increasing concentrations of DBC. As shown in Fig. 4C, the cleaved PARP fragment appeared in a dose-dependent fashion. Taken together, these results indicate the apoptotic potential of DBC in MDR cancer cells.

4. Discussion

Microtubules are an attractive target for chemotherapeutic agents. We here describe an anti-mitotic potential of the novel synthetic coumarin-based compound DBC. DBC has a broad-spectrum ability to inhibit growth of various human cancer cell lines as well as drug resistant cancer cells.

In this study, we show that microtubules are molecular target of DBC. DBC inhibits tubilin polymerization *in vitro* and destabilizes microtubules through direct binding with microtubule components, resulting in G_2/M arrest of cancer cells (Figs. 2 and 3). It has been well demonstrated that the complex of cdc2 kinase with cyclin B regulates the onset of mitotic phase and activation of cdc2 kinase at the G_2-M transition requires accumulation of cyclin B and dephosphorylation of cdc2 [15,16]. Here we demonstrate that in addition to directly disrupting microtubules, DBC treatment leads to an abnormal accumulation of cyclin B1 and dephosphorylation of cdc2 kinase (Fig. 2C). These changes are accompanied by the appearance of a mitosis-specific marker MPM-2 epitope (Fig. 2C), indicating that DBC induces cell cycle arrest at the mitotic phase. However, our data show some discrepancy between G_2/M arrest (Fig. 2B) and MPM-2 Western blot (Fig. 2C). Although DBC (100 nM) induces a massive blockade of cells in the G_2/M phase of the cell cycle (Fig. 2B), there are no detection of MPM-2 at the same concentration (Fig. 2C). Based on this result, we suggest that low concentration of DBC may block the cell cycle at the G_2 phase and high concentration of DBC may induce mitotic arrest. Recent reports have demonstrated that Aurora kinases are essential for cell cycle regulation and high-fidelity mitosis [18,19] and their specific inhibition can lead to errors in chromosome alignment and

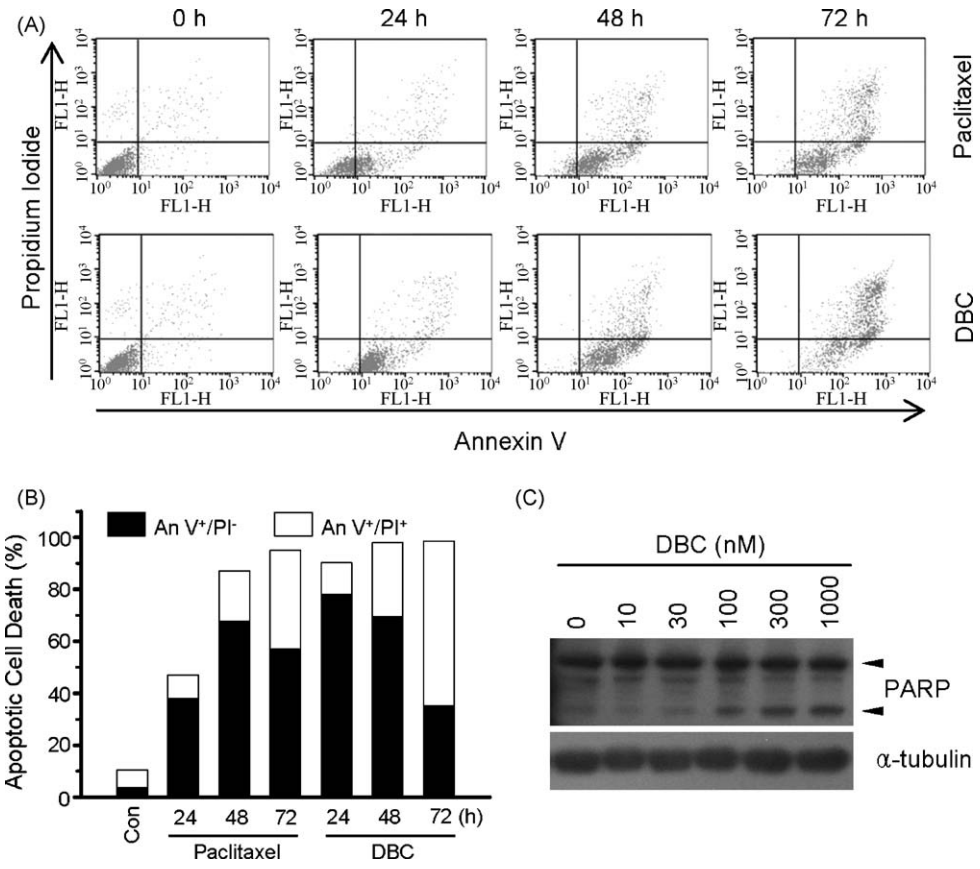

Fig. 4. DBC induces apoptotic cell death in MDR cancer cells. (A) and (B) KBV20C cells were treated with 300 nM paclitaxel or 300 nM DBC for the indicated time period. The cells were harvested, subsequently incubated with fluorescence-labeled Annexin V and PI, and subjected to flow cytometry analysis as described in Section 2. Apoptotic cell death was evaluated as the percentage of Annexin V^+/PI^- cells (early apoptosis) and Annexin V^+/PI^+ cells (late apoptosis). (C) After treatment of KBV20C cells with DBC, PARP cleavage in cell lysates was examined by immunoblot analysis.

segregation to induce mitotic arrest [19,21]. However, DBC-induced G_2/M arrest seems not to be mediated by inhibition of Aurora kinases, but on the contrary DBC treatment leads to an accumulation of active Aurora kinase and histone H3 (Ser^{10}), resulting from mitotic arrest (Fig. 2D).

Chemotherapy is the most effective treatment for patients who suffer from metastatic cancers. However its effectiveness is seriously limited by MDR which is mainly due to the overexpression of drug efflux pumps including *P*-gp. *P*-gp actively transport anticancer drugs from the inside to the outside of cancer cells and prevent the intracellular accumulation of anticancer drugs inside cancer cells necessary for cytotoxic activity. Therefore, for more effective chemotherapy, it is of great interest to develop a novel anticancer drug which is not substrate of *P*-gp. In the present study, DBC has similar antiproliferative potency against both MDR negative and MDR positive cells (Fig. 1C). In addition, our data show that DBC exerts apoptotic potential in MDR cancer cells (Fig. 4). These findings suggest that DBC is a poor substrate of *P*-gp drug efflux pump and MDR, at least induced by *P*-gp overexpression, may be overcome by DBC treatment. However, whether DBC overcomes drug resistance induced by tubulin mutations and/or different isotype expressions still needs to be investigated.

In summary, our data provide strong evidences that the novel coumarin-based compound, 7-diethylamino-3(2′-benzoxazolyl)-coumarin, exerts a broad spectrum antiproliferative effect by inhibiting microtubule functions and triggering apoptosis. In addition, these effects of DBC are also observed in *P*-gp over-expressing MDR cancer cells, indicating that drug resistance caused at least by *P*-gp overexpression might be overcome by DBC. Altogether, these pharmacological properties of DBC will prompt us to further develop as a novel microtubule inhibitor for treatment of multidrug resistant cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pepi.2008.04.016.

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