B1, a novel topoisomerase II inhibitor, induces apoptosis and cell cycle G1 arrest in lung adenocarcinoma A549 cells

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In our previous studies, we demonstrated that 2,6-bis-(2-chloroacetamido) anthraquinone (B1) showed a highly significant cytotoxic effect. However, its influence in the cell cycle and apoptotic induction effects has not been investigated yet. Here we report the antiproliferative effect of B1, for which IC_{50} values were 0.57 μmol/l for lung cancer A549 cells, 0.63 μmol/l for colon cancer HT-29 cells, and 0.53 μmol/l for breast cancer MCF-7 cells. DNA topoisomerase II (Topo II), an essential enzyme in DNA synthesis and meiotic division, is highly expressed in cancer cells. Some currently used clinical anticancer drugs (doxorubicin and mitoxantrone) targeting Topo II are very effective antineoplastic agents. B1, sharing the basic structure of known Topo II inhibitors, demonstrated a significant inhibitory effect on Topo II bioactivity. In A549 cells, B1 increased apoptotic cell population with induction of Fas, Bax, and cleaved poly(ADP-ribose) polymerase and by reduction of Bcl-2 expression. Moreover, cell cycle analysis indicated that B1 induced G1 phase arrest through modulation of G1 cell cycle regulatory proteins, such as the downregulation of cyclin D1 and upregulation of Cip/p21, Kip1/p27, and p53. Thus, our study suggests that B1, with the ability to inhibit Topo II activity and cause cell cycle G1 arrest and apoptosis, has potential as a novel anticancer agent. *Anti-Cancer Drugs* **23:191–199** © **2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Lung cancer is the leading cause of cancer-related death in many developed countries. In Taiwan, it is the leading cause of death due to cancer in women and the second most frequent cancer-related death in men next to hepatocellular carcinoma [1]. The majority of patients present with advanced disease at diagnosis, and therefore have a very poor prognosis [2,3]. Despite a large amount of research, symptom relief and meaningful improvement in quality of life are still the primary goals of current chemotherapy. Therefore, the development of novel therapeutic agents is urgently needed. Anthraquinones occur widely in the plant kingdom and may possess various biological activities. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a natural anthraquinone, is purified from the roots and rhizomes of *Rheum palmatum* L and has been used as a traditional Chinese medicine for the treatment of constipation, infection, gallstones, hepatitis, and inflammation. Emodin has been reported to exhibit antiproliferative effects in lung, liver, colorectal, and prostate cancer cells, but its anticancer activity is not significant [4]. Commercial 1,4-disubstituted synthetic anthraquinones, such as mitoxantrone and adriamycin (doxorubicin), intercalate to the duplex DNA and damage DNA through inhibition of topoisomerase II (Topo II), resulting in apoptosis in cancer cells [5]. Using anthraquinone as a basic structure, we have developed and synthesized a series of difunctionalized tricyclic anthraquinones at the 1,4-position, 1,5-position, 1,8-position, and 2,6-position and tested their cytotoxic activities in our previous studies [6–11]. Among them, 2,6-bis-(2-chloroacetamido) anthraquinone (B1) has demonstrated outstanding cytotoxic effects in various cancer cell lines. However, the mechanism of this cytotoxic effect has not yet been clearly established.

The dysregulation of apoptosis may contribute not only to tumorigenesis but may also be the cause of the resistance of cancer cells to conventional chemotherapeutic drugs. There are two major apoptosis signal transduction pathways: the death receptor (external) and mitochondrial (internal) pathways. Fas (CD95/APO-1) and its death ligand (FasL) is the best characterized external apoptotic pathway. The interaction of Fas and FasL can activate initiator caspases (caspase-2, caspase-8, and caspase-9) leading to the proteolytic activation of downstream

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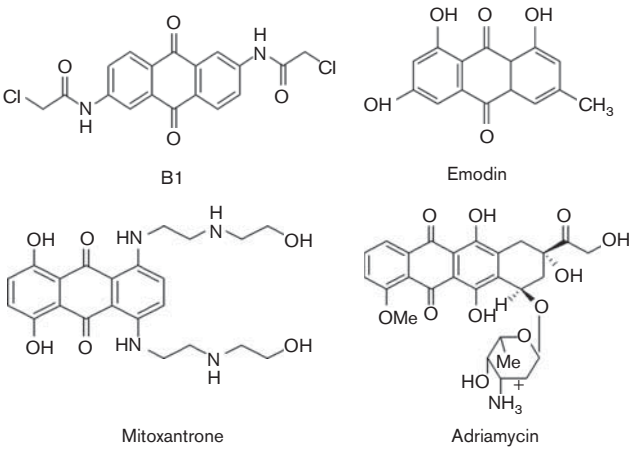
effector caspases (caspase-3, caspase-6, and caspase-7) that cleave specific substrates, such as poly(ADP-ribose) polymerase (PARP) [12,13]. The dysregulation of the cell cycle plays a critical role in malignant transformation and in the inefficacy of chemotherapy [14]. Because of this, cell cycle regulators could be a potential anticancer drug target. Compounds manipulating the cell cycle may be able to promote growth arrest, inhibit differentiation, and induce apoptosis.

Topo II plays a crucial role in the survival of all eukaryotic cells. It alters the topological state of DNA through the formation of a double-stranded cleavage, followed by DNA strand passage and relegation. Topo II is highly expressed during periods of rapid cell proliferation [15]. B1 shares the same tricyclic anthraquinone structure with clinically applied Topo II inhibitors such as mitoxantrone and adriamycin, and it is the leading agent in our small-molecule anticancer drug development plan. The aim of the present study was to reveal the cytotoxic molecular mechanism of B1 and its modulation of Topo II activity in human lung adenocarcinoma A549 cells.

Fig. 1



Chemical structures of 2,6-bis-(2-chloroacetamido) anthraquinone (B1), emodin (natural reference compound), mitoxantrone, and adriamycin (commercial reference compounds); all share the same basic tricyclic anthracene framework.

Materials and methods

Reagents

A series of 2,6-diamidoanthraquinone derivatives were synthesized by Dr Hsu-Shan Huang, School of Pharmacy, National Defense Medical Center. A stock solution of the 2,6-diamidoanthraquinone derivative (B1) was prepared in dimethylsulfoxide (DMSO), protected from light and stored at −20°C. Mitoxantrone, adriamycin, and emodin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and dissolved in DMSO. The chemical structures of B1, mitoxantrone, adriamycin, and emodin, all sharing the same basic tricyclic anthraquinone framework, are shown in Fig. 1. Before use, testing compound solutions were freshly prepared in a medium at the desired concentrations. Tetrazolium reagent (XTT) was purchased from Sigma Chemical Co. Anti-Kip1/p27, anti-Cip1/p21, anti-p53, anti-cyclin D1, anti-Fas, and anti-Bax were purchased from BD Technologies (Research Triangle Park, North Carolina, USA). Anti-Bcl-2, anti-caspase 3, and anticleaved PARP were purchased from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA), and anti-β-actin was purchased from Sigma Chemical Co.

Cell lines and cell culture

Human lung non-small-cell lung cancer (NSCLC) cell line A549, colon adenocarcinoma cell line HT-29, and breast adenocarcinoma cell line MCF-7 were obtained from Bioresource Collection and Research Center, Taiwan. All cell lines were cultured in minimum essential medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere comprising 95% air and 5% CO_2.

Cell cytotoxicity assay

Cell viability in response to various concentrations of B1 after 72 h of treatment was assessed by XTT assay. Briefly, 1×10^5 cells per well of A549, HT-29, and MCF-7 were plated into 24-well culture plates and grown for 24 h. The cells were then treated with different concentrations of testing compounds for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. DMSO alone was used as a control. After the desired treatment time, cells were collected by a brief trypsinization and measured by the colorimetric XTT method as described before [9,10]. Proliferation rates were obtained from B1-treated wells and compared with those not treated with B1 (100% control value). The data shown in the study are the mean of six independent experiments.

Assay of topoisomerase II activity

Topo II activity was measured using a Topo II drug screening kit (TopoGEN Inc., Columbus, Ohio, USA), which detects any covalent links between Topo II and DNA. The supercoiled DNA plasmid (pRYG) contained a 54 bp alternating repeat of purine/pyrimidine sequences, a high-affinity Topo II cleavage site [16]. The 20 μl final mixture included 1 μl pRYG DNA (supercoiled), 2 μl of 10× assay buffer, 2 U of Topo II, and 2 μl of different concentrations of B1 (1.0 and 10 μmol/l) or the Topo II inhibitor (50 μmol/l etoposide) as positive control. The reaction was incubated for 30 min at 37°C in cleavage buffer (30 mmol/l Tris-HCl, pH 7.6, 3.0 mmol/l ATP, 15 mmol/l mercaptoethanol, 8.0 mmol/l $MgCl_2$, 60 mmol/l NaCl) and terminated with 2 μl of 10% SDS. Proteinase K (2 μl) was then added to stop the reaction. After 15 min, 2 μl of sample-loading dye was added and then samples of DNA were extracted with a 26 μl mixture of chloroform

and isoamyl alcohol (1 : 24). Samples were subjected to electrophoresis on 2.0% agarose gel containing 0.5 μg/ml ethidium bromide at 45 V for 2 h.

Analysis of apoptosis by phase contrast microscopy and annexin V staining

Briefly, 5×10^4 of A549 cells were allowed to attach in a cell culture dish for 24 h, and the cells were treated with the indicated concentrations of B1 for an additional 48 h. Morphological changes were observed under a phase contrast microscope (Nikon Inc., Tokyo, Japan). Apoptosis rates were determined by the annexin V and propidium iodide (PI) double-staining method. In brief, 1.0×10^5 cells were treated with 0, 0.1, 0.5, and 5.0 μmol/l of B1 for 48 h; the cells were then harvested by trypsinization and washed with ice-cold PBS. After centrifugation and removal of the supernatants, cell pellets were resuspended in 100 μl 1 × annexin-binding buffer. After adding 5 μl of Alexa Fluor 488 annexin V (component A) and 1 μl of 100 μg/ml PI working solution, the cells were incubated at room temperature for 15 min before further addition of 400 μl of 1 × annexin-binding buffer. The stained cells were analyzed quantitatively using a Flow Cytometer (BD Biosciences, San Jose, California, USA).

Cell cycle analysis

A549 cells (1.0×10^5 cells/well) were treated with various concentrations of B1 (0, 0.1, 0.5, and 5.0 μmol/l) for 48 h. At the end of incubation, adherent cells were collected by centrifugation, washed with PBS twice, and fixed with 70% cold ethanol for 30 min at 4°C, and then resuspended in 1 ml of cell cycle assay buffer (0.05% ribonuclease A and 0.5% Triton) in a dark room for 1 h. After staining with PI, cell cycles were analyzed on a FACScan flow cytometer (Becton Dickinson Instruments, Franklin Lakes, New Jersy, USA) and calculated using the ModFit LT 2.0 program (San Diego, California, USA).

Western blot analysis

Cells were cultured without or with B1 for 48 h, and then both adherent and floating cells were harvested. After washing with ice-cold PBS and lysing with RIPA reagent (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min at 4°C, the cells were centrifuged at 13 500 rpm for 15 min. For western blotting, equal amounts of total protein were loaded onto SDS-PAGE, and separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, California, USA). To block nonspecific binding, the polyvinylidene fluoride membrane was soaked in PBS solution containing 5% nonfat milk at room temperature for 1 h. Membranes were probed for cell cycle and apoptosis regulatory protein levels using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibodies, and visualized by enhanced chemiluminescence.

Statistical analysis

The quantitative data were assessed by Student's *t*-test. Each value was the representative of at least three separate experiments with reproducible results. A *P*-value less than 0.05 was regarded as significant.

Results

B1 inhibited cancer cell growth

We used the colorimetric XTT assay to determine the antitumor effects of the testing compounds. In this study, human NSCLC cell line A549, human colon cancer cell line HT-29, and breast cancer cell line MCF-7 were used. To determine the antiproliferative effects, different concentrations (0.01, 0.1, 1.0, 10, and 100 μmol/l) of B1, emodin, mitoxantrone, and adriamycin were added and cocultured for 72 h. As shown in Fig. 2, 72 h of treatment of all the tested compounds showed potently antiproliferative effects in a dose-dependent manner. The effective doses of tested compounds that inhibited 50% cell growth (IC_{50}) of the A549 cell line are shown in Table 1. In addition, B1 treatment inhibited the growth of A549 cells in a dose-dependent and time-dependent manner (Fig. 3). B1 demonstrated an inhibition rate in A549 cells from 22 to 79% (Fig. 3a). Meanwhile, when the B1 concentration was held constant (1 μmol/l), the number of viable cells decreased steadily as the exposure time increased (Fig. 3b).

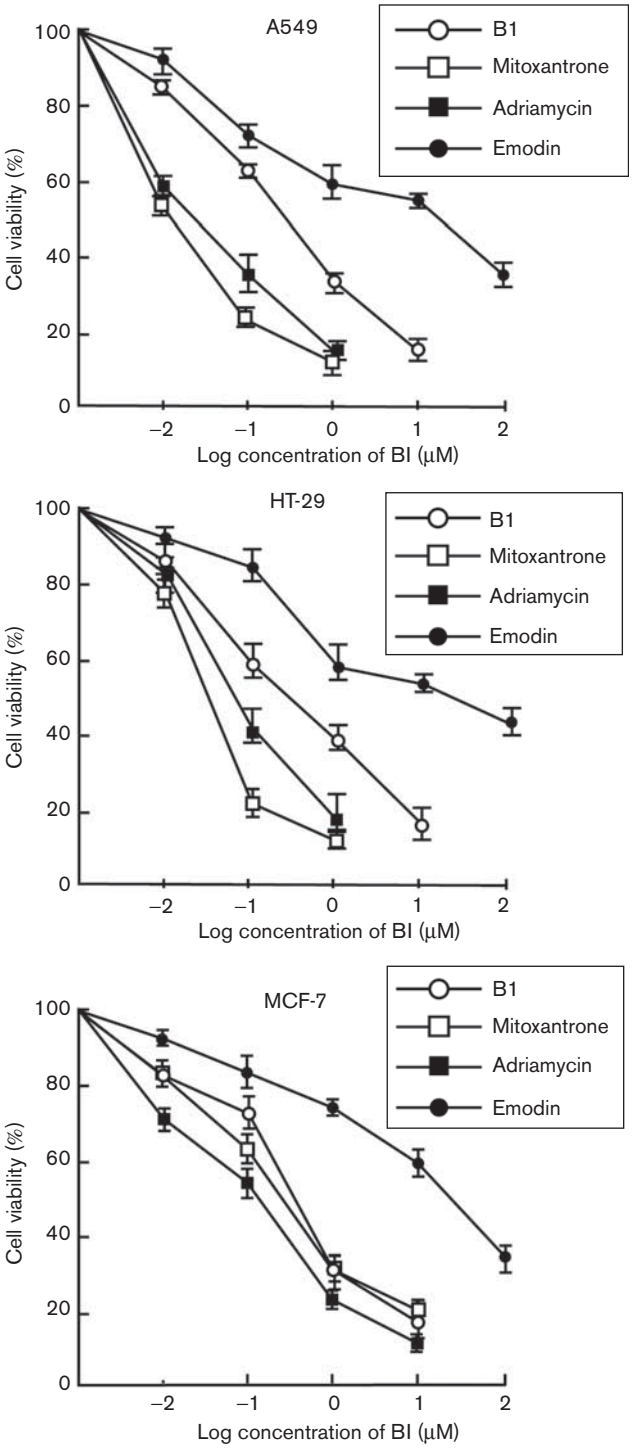
B1 inhibited the catalytic activity of topoisomerase II

The special plasmid (pRYG) contains alternating purine–pyrimidine DNA that is preferentially recognized by eukaryotic Topo II. The pRYG cleavage assay allows the detection of two kinds of Topo II inhibitors: those that promote formation of drug-induced Topo II–DNA cleavage complexes (Topo II poisons), and agents that interfere with the active site of the enzyme (catalytic inhibitors) [17]. Drug-induced Topo II inhibition was assessed using a commercial Topo II drug screening kit in which supercoiled pRYG plasmid DNA was incubated with increasing concentrations of B1 (1.0 and 10 μmol/l) or the commercial Topo II inhibitor, etoposide (50 μmol/l), plus purified human Topo II. The gel image presented in Fig. 4 shows a typical sample of this experiment. Our results showed that the inhibitory effect of 1.0 μmol/l B1 on the Topo II-dependent relaxation of the pRYG plasmid was more potent than 50 μmol/l etoposide.

B1 induced apoptosis in A549 cells

We used a phase contrast microscope to detect phenotypic changes of B1-treated A549 cells. After treatment with different concentrations of B1 for 48 h, the cells showed typical apoptotic morphological features such as membrane blebbing and cell shrinkage (Fig. 5a). The number of cells also diminished with increasing concentrations of B1. The morphological changes and cell cytotoxicity after B1 treatment could have been because of the induction of apoptosis. Next, we used an annexin

Fig. 2



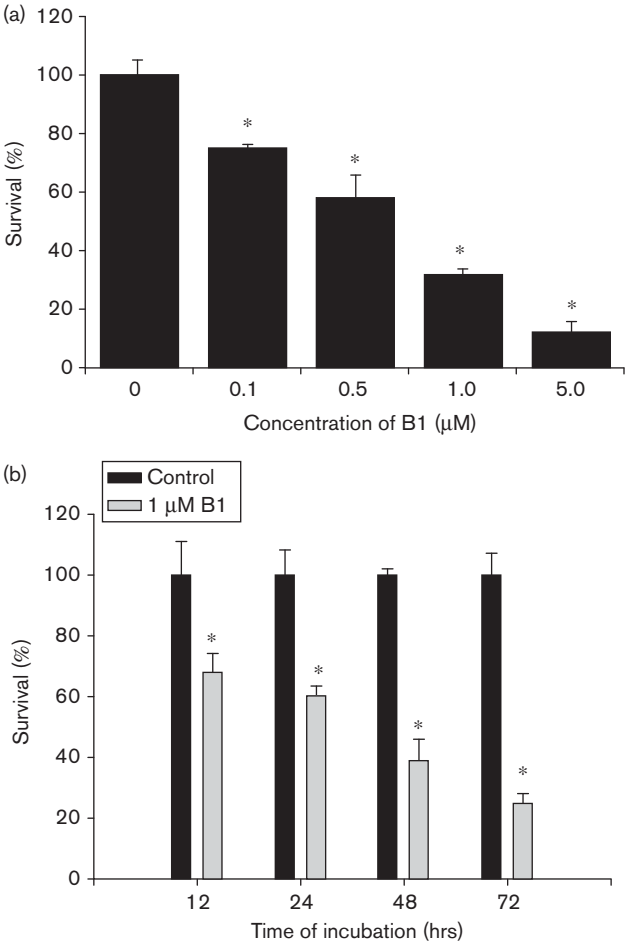
Dose–response effects of different anthraquinone analogs on cell growth of various human cancer cell lines. The human non-small-cell lung cancer cell line A549, colon cancer cell line HT-29, and breast cancer cell line MCF-7 were treated with various concentrations of testing compounds for 72 h, and cell viability was determined by the XTT assay. Cell viability values are expressed relative to those wells in which various compounds were not added (100% control value). Each point represents a mean value ± SD of at least six independent experiments. BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

Table 1 Cytotoxic activities of B1 in different human cancer cell lines

	Mean IC_{50} (μmol/l)[a]		
Drugs	A549	HT-29	MCF-7
B1	0.57	0.63	0.53
Emodin	35.56	53.22	43.16
Mitoxantrone	0.017	0.06	0.48
Adriamycin	0.027	0.08	0.42

Values represent an average of at least six independent experiments.
BI, 2,6-bis-(2-chloroacetamido) anthraquinone.
[a]IC_{50}, drug concentration inhibiting 50% of cellular growth following 72 h of drug exposure.

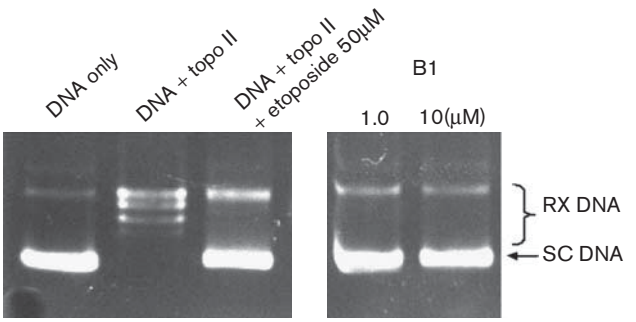
Fig. 3



The percentage of viable A549 cells after B1 treatment. A total of 1.0×10^5 cells per well were distributed in 24-well plates and treated the next day with 0.1, 0.5, 1.0, and 5.0 μmol/l of B1 for 72 h (a) or with 1 μmol/l of B1 for 12, 24, 48, and 72 h (b). The ability of B1 to inhibit cell proliferation was determined by XTT survival assay. The results are expressed as mean values ± SD of at least six independent experiments. *Significantly ($P < 0.05$) different from untreated controls. BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

V/PI staining assay, followed by flow cytometric analysis, to confirm the apoptotic effects of B1. After treatment with 0.1, 0.5, and 5.0 μmol/l of B1 for 48 h, the

Fig. 4



Topoisomerase II inhibition by 2,6-bis-(2-chloroacetamido) anthraquinone (B1) treatment. The inhibitory effect of B1 on DNA topoisomerase II (Topo II) catalytic activity was conducted with supercoiled plasmid pRYG, 2 U of Topo II in the absence or presence of B1 (1.0 and 10 μmol/l), and etoposide (50 μmol/l), respectively, and then analyzed on a 2% agarose gel. SC, supercoiled; RX, relaxed.

percentages of apoptotic (annexin V-positive/PI-negative) cells increased from 15.95 to 60.34% (Fig. 5b). Our data showed that the exposure to different concentrations of B1 increased the apoptotic cell percentage in a dose-dependent manner. This also suggested that apoptosis induction may be a major contributor to the cytotoxic effect of B1 in A549 cells.

B1 modulated apoptosis regulatory proteins

To further characterize the molecular mechanism of B1-induced apoptosis, we treated A549 cells with diverse concentrations of B1 and measured the accumulation of some key apoptosis regulatory proteins. A549 cells were treated with various concentrations of B1 for 48 h, and protein levels of Fas [18], Bcl-2, Bax [19], caspase-3, and cleaved PARP [20] were assessed by western blot analysis. As shown in Fig. 6, B1 treatment induced a concentration-dependent increase in Fas, Bax, and cleaved PARP, whereas the levels of Bcl-2 and caspase-3 were reduced.

B1 induced G1 cell cycle arrest in A549 cells

B1-induced cell growth inhibition *in vitro* could, in part, be because of the modulation of cell cycle progression. To test this, A549 cells treated with 0.1, 0.5, and 5.0 μmol/l of B1 for 48 h were stained with PI and then subjected to flow cytometry. We observed that B1 arrested A549 cells at the G1 phase in a dose-dependent manner (Fig. 7), suggesting that the inhibitory effect of B1 might be because of G_1 phase cell cycle arrest.

B1 modulated G1 cell cycle regulatory proteins

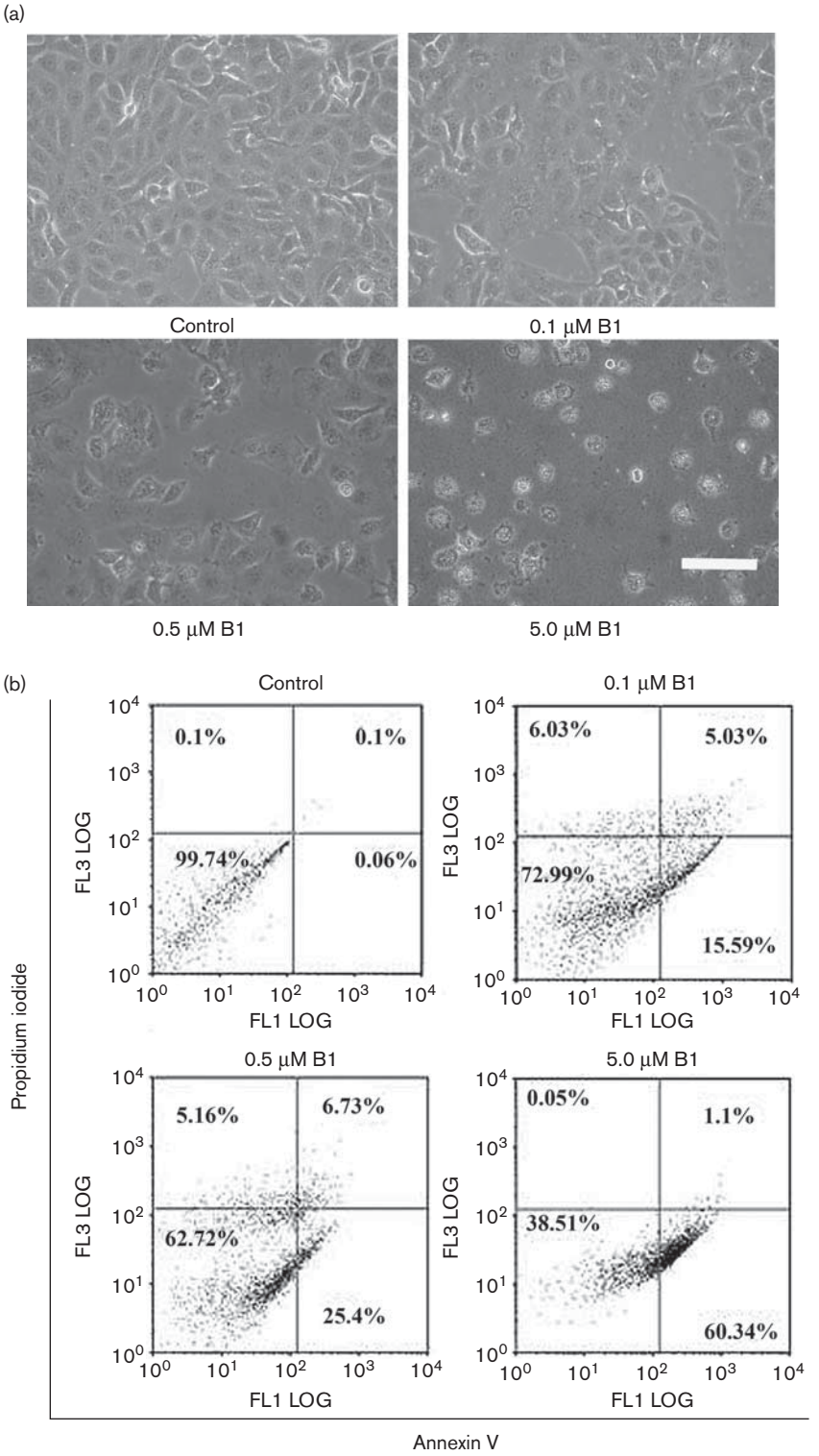
Western blot analysis revealed that B1 treatment resulted in a moderate-to-strong decrease in the expression of cyclin D1 and increases in the expressions of Cip1/p21 and p53 (Fig. 8), whereas Kip1/p27 was markedly activated at a low concentration (0.1 μmol/l) of B1. Overall, our results suggest that B1-induced G1 cell cycle arrest in A549 cells may be through an induction of p53, Cip1/p21, and Kip1/p27 and through a decrease in the expression of cyclin D1.

Discussion

We have previously reported the significant cytotoxic and human telomerase inhibitory effects of a series of newly synthesized diamidoanthraquinones in different human cancer cell lines [6,11]. Among the hundreds of compounds that we have synthesized, B1, a novel 2,6-diamidoanthraquinone, is the leading compound in our new anticancer drug development plan. In the present study, we focused on Topo II activity modulation, cell cycle regulation, and apoptosis induction abilities of B1 in A549 cells. There are two major topoisomerase forms: the type I (Topo I) enzyme, which makes transient single-strand DNA breaks, and the type II (Topo II) enzyme, which makes double-strand DNA breaks [21]. The function of Topo II is to solve topological problems arising during DNA replication, transcription, and re-combination, and it is highly expressed in lung neoplastic tissues, including A549 [22]. Because of its critical role in mitosis and high expression in cancer cells, Topo II has become an attractive target for molecular targeting therapies [22,23]. Topo II inhibitors are among the most effective anticancer drugs and show therapeutic potential in the treatment of NSCLC [24]. Our results revealed that B1 induced both time-dependent and concentration-dependent cytotoxicity in A549 cells (Fig. 3). A structural analog with commercial Topo II inhibitors (mitoxantrone and doxorubicin), B1 also showed remarkable Topo II inhibition in the DNA cleavage-relegation assay, and the inhibitory ability of 1.0 μmol/l B1 was stronger than that of 50 μmol/l etoposide (VP16) (Fig. 4).

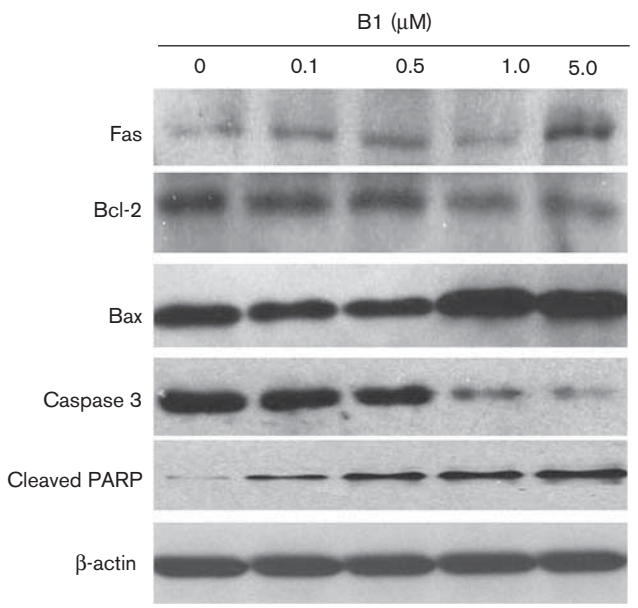
Apoptosis is a precisely regulated process that maintains physiological growth control and tissue homeostasis [25]. The understanding that most current chemotherapeutic agents act through the activation of the apoptosis signal pathway could be a major development in recent anti-cancer research. Our phase contrast microscopy observations showed characteristic evidence of apoptotic induction in B1-treated A549 cells (Fig. 5a). Two major pathways, the death receptor (extrinsic) and the mitochondria (intrinsic), have been described in mammals. During apoptotic induction, PARP is the major substrate of caspase-3, the central executor of the apoptotic process. Because of observations that neutralization of FasL consequently prevented doxorubicin-induced apoptosis in T-cell leukemia lines, the FasL/Fas pathway has been proposed as a critical factor in drug-induced apoptosis in multiple cancer cell types [26,27]. Our results are consistent with reports that Topo II inhibitors increase Fas and FasL expression, especially in cells bearing wild-type p53, such as A549 cells [28]. Bcl-2, the antiapoptotic protein of the Bcl-2 family, represses apoptosis. Bax, the death-promoting

Fig. 5



Effect of B1 on apoptotic induction in A549 cells. (a) Cells were treated with dimethylsulfoxide (as vehicle) or with different concentrations of B1 (0.1, 0.5, and 5.0 μmol/l) for 48 h. The morphological and apoptotic changes were observed using a phase contrast microscope (× 200). (b) After treatment with the indicated concentrations (0.1, 0.5, and 5.0 μmol/l) of B1 for 48 h, apoptosis was analyzed by flow cytometry for annexin V and propidium iodide (PI) dual stain, as described in Materials and methods. Cytograms of annexin V–FITC binding (abscissa) versus PI uptake (ordinate) stratified treated A549 cells into three district categories: (i) viable cells in the bottom left quadrant; (ii) early apoptotic cells in the bottom right quadrant (annexin V positive and PI negative); (iii) very late apoptotic cells with loss of cell membranous integrity in the top right quadrant (annexin V and PI positive). BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

Fig. 6



Effect of B1 on the expression of apoptosis regulatory proteins. A549 cells were exposed to dimethylsulfoxide or to 0.1, 0.5, 1.0, and 5.0 μmol/l of B1 for 48 h. At the end of treatment, total cell lysates were prepared and an equal amount of protein was separated by SDS-PAGE, followed by western blotting as described in Materials and methods. The membranes were probed with anti-Fas, Bcl-2, Bax, caspase 3, and cleaved poly(ADP-ribose) polymerase (PARP) antibodies or with a β-actin antibody serving as a loading control. BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

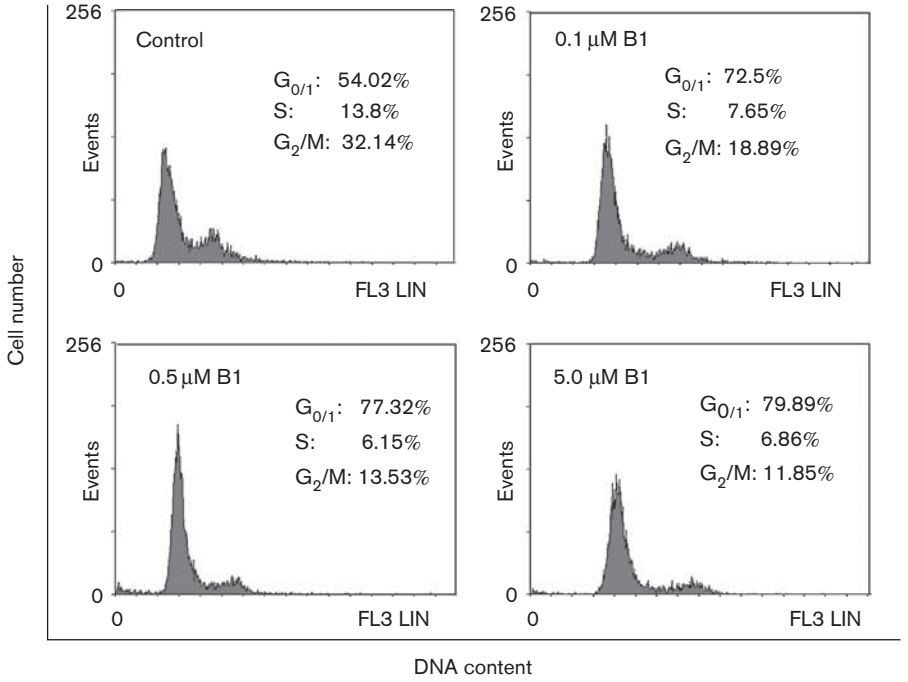
member of the Bcl-2 family, is considered as the requisite gateway to the mitochondrial apoptotic pathway [25]. Increasing the expression of the Bax gene through gene therapy has been shown to cause extensive apoptosis and suppress tumor growth of human lung adenocarcinoma cells, both *in vitro* and *in vivo* [29,30]. In addition, an elevated level of Bax expression has been shown to induce apoptosis in human pulmonary adenocarcinoma cells, which are resistant to the clinically available anticancer drug gefitinib (Iressa, a epidermal growth factor receptor tyrosine kinase inhibitor) [30]. Our results showed that B1 triggered apoptotic cell death of A549 cells in a dose-dependent manner (Fig. 5b), and this effect was associated with increased levels of Fas and Bax proteins and decreased levels of Bcl-2 content (Fig. 6). However, in contrast to one previous study [31], B1-treated cells increased the expression of apoptotic Bax signal. Further studies are necessary to determine whether the different side chain substitution or detection methods affected these results.

Control of the cell cycle is a tightly regulated process and involves a complex signal cascade. The dysregulation of the cell cycle is the hallmark of cancer, and it can be modulated by chemotherapeutic agents. The modulation of cell cycle regulatory proteins, including cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors, plays a crucial role in the inhibition of cancer cell proliferation by chemotherapeutic agents. The levels of D-type cyclins present as the integrator of a variety of extracellular mitogenic signals [32]. Overexpression of cyclin D1 has been reported in several types of human cancer including lung, esophagus, breast, bladder, and liver cancers [33]. The CDK inhibitory proteins fall into two families: INK4 and Cip/Kip [34]. Kip1/p27 is considered the main regulator of G1 phase CDK activity, and Cip1/p21 functions as a sensor of cytostatic signals and is mainly regulated by the p53 tumor suppressor protein [32,35]. In our study, we observed that B1 caused cell cycle G1 arrest and decreased protein expression of cyclin D1 in A549 cells, and these effects were associated with the induction of Cip1/p21, Kip1/p27, and p53 expression (Fig. 8). Emodin has the same basic structure as B1, and it has been shown to cause cytotoxicity in A549 cells through the reactive oxygen species-dependent ataxia-telangiectasia-mutated–p53–Bax pathway [36]. Although B1 also caused an upregulation of Bax and p53, further studies are needed to determine whether the ataxia-telangiectasia-mutated–p53–Bax pathway also plays a role in the anticancer effect of B1.

DNA topoisomerases are nuclear enzymes that allow cells to manipulate the topology of intracellular DNA. Topo II is considered to be a proliferative marker and also the enzyme necessary in meiotic division. All Topo II inhibitory agents in clinical use are Topo II poisons, which stabilize the Topo II–DNA cleavage complex. The stable Topo II–DNA complexes convert permanent double-stranded breaks and become targets for recombination and sister chromatid exchange, leading to the instability of chromosomes. When these permanent strand breaks are present in sufficient numbers, they may lead to cell death primarily by apoptosis [5,15,21].

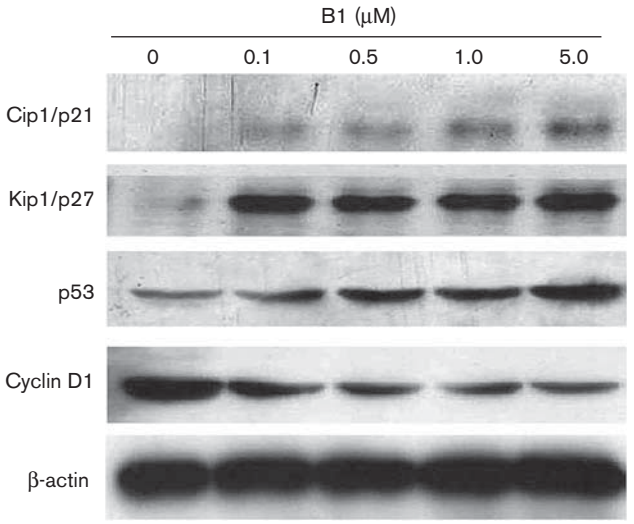
Anthracyclines were first considered to be inhibitors of Topo II in 1984, and now all clinically active anthracyclines are anthraquinones [21]. Most of the currently used Topo II-targeted drugs, such as mitoxantrone and doxorubicin, cause cell cycle arrest in G2 phase. Nevertheless, B1 induced cell cycle arrest in the G1 phase in this study (Fig. 7). The difference in mechanism between B1 and mitoxantrone or doxorubicin in cell cycle regulation is an interesting topic. A previous study showed that breast cancer cells treated with doxorubicin exhibited an increase in p53 activity, a decline in telomerase activity, and replicative senescence characterized by G0/G1 arrest [5]; this is consistent with our results. In addition, it has been reported that treatment with Topo II inhibitors in cells containing functional copies of p53 could result in G1 arrest, and this effect reflects a posttranslational modification that increases the half-life of p53 [37]. A549 cells, with wild-type p53, may share the same mechanism. We observed that B1-induced p53 activation contributed to the overexpression of Cip1/p21 and Kip1/p27, both being strong cyclin-dependent kinase inhibitors involved in G1 to S transition [5].

Fig. 7



Effect of B1 on cell cycle distribution. Cells were exposed to dimethylsulfoxide or to 0.1, 0.5, and 5.0 μmol/l of B1 for 48 h. At the end of the treatment, only attached cells were harvested and stained with propidium iodide followed by flow cytometric analysis as described in Materials and methods. BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

Fig. 8



Effect of B1 on the expression of cell cycle regulatory proteins. Cells were treated with dimethylsulfoxide or with 0.1, 0.5, 1.0, and 5.0 μmol/l of B1 for 48 h. The total cell lysates were prepared and an equal amount of protein was separated by SDS-PAGE. Membranes were probed for the expression levels of Cip1/p21, Kip1/p27, p53, and cyclin D1 by western blot analysis. BI, 2,6-bis-(2-chloroacetamido) anthraquinone.

Histone deacetylases (HDACs), modifying nucleosomal histones, play a key role in the regulation of gene transcription and may also be involved in cell cycle regulation, disruption of differentiation, and formation of human cancer. Consequently, there has been considerable effort to develop HDAC inhibitors (HDACi) as a new class of anticancer agents, suberoylanilide hydroxamic acid being the first approved HDACi for the treatment of cutaneous T-cell lymphoma [38]. HDACi has been shown to induce tumor cell cycle arrest in G1 phase and may interact directly with DNA Topo II [39,40]. In our previous studies, we found telomerase inhibition in a series of bis-substituted amidoanthraquinone derivatives [11,41–44]. In contrast, the inhibitory effect of B1 on telomerase and Topo II and the interaction between Topo II, telomerase, and HDAC will be conducted in our further studies.

In conclusion, we report, for the first time, that B1 (a newly synthesized 2,6-diamidoanthraquinone) has potent cell cytotoxic effects in human lung adenocarcinoma A549 cells. Our investigation shows that the molecular cytotoxic mechanism of B1 is through downregulation of Bcl-2, caspase 3, and cyclin D1 and through upregulation of Fas, Bax, Cip1/p21, Kip1/p27, and p53. In our preliminary study, B1 also demonstrated inhibitory effects

on HDAC and telomerase, suggesting the potential for the development of B1 as a new anticancer agent.

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Conflicts of interest

There are no conflicts of interest.

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