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Cuphiin D_1, the macrocyclic hydrolyzable tannin induced apoptosis in HL-60 cell line

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Abstract

Cuphiin D_1 (CD_1), a new macrocyclic hydrolyzable tannin isolated from *Cuphea hyssopifolia*, has been shown to exert antitumor activity both in vitro and in vivo. In this study, we explored the mechanism of the CD_1-induced antitumor effect on human promyelocytic leukemia (HL-60) cells. The results showed that CD_1 induced cytotoxicity in HL-60 cells and the IC_{50} was 16 μM after 36 h treatment. HL-60 cells treated with CD_1 for 36 h decreased the uptake of [^{3}H]-labeled thymidine, uridine and leucine in a dose dependent manner. Electron micrographs demonstrated that HL-60 cells treated with 16 μM CD_1 for 36 h exhibited chromatin condensation, indicating the apoptosis occurrence. Flow cytometric analysis demonstrated the presence of apoptotic cells with low DNA content, a decrease of cell population at G_2/M phase, and a concomitant increase of cell population at G_1 phase. CD_1 also caused DNA fragmentation and inhibited Bcl-2 expression in the HL-60 cells. These results suggest that the inhibition of Bcl-2 expression in HL-60 cell might account for the mechanism of CD_1-induced apoptosis.

Keywords: Cuphiin D_1; HL-60 cells; *Cuphea hyssopifolia*; Tannin; Apoptosis; Bcl-2

1. Introduction

Natural products have been used in traditional and folk medicine for therapeutic purposes. Several anticancer drugs derived from natural sources, including the well-known *Cantharanthus* alkaloids, colchicine, etoposide and taxol [1], have been used in cancer chemotherapy. Since cytotoxic and anti-proliferative drugs have had great success, and are likely to continue to play a major role in cancer treatment, we are interested in finding new active compounds from natural sources and studying their biological effects.

Cuphea hyssopifolia Humb. Bompl. et Kunth, a small shrub native to Central and South America, is used by Mexican Indians to treat stomach upset, syphilis and cancer [2]. Previously, we have demonstrated, both in vitro and in vivo, that cuphiin D_1 (CD_1) (Fig. 1), a new macrocyclic hydrolyzable tannin, originally isolated from *Cuphea hyssopifolia* exhibited an antitumor effect [3]. The effects of CD_1 on cell growth and differentiation vary, depending on the cell types and differentiation. CD_1 inhibited the growth of human carcinoma cell lines, including KB, HeLa, DU-145, Hep 3B and leukemia cell line HL-60, and prolonged the survival time of ascites S-180-bearing mice [3]. However, the underlying mechanism of the antitumor activity induced by CD_1 has not yet been clarified. In this paper, we attempted

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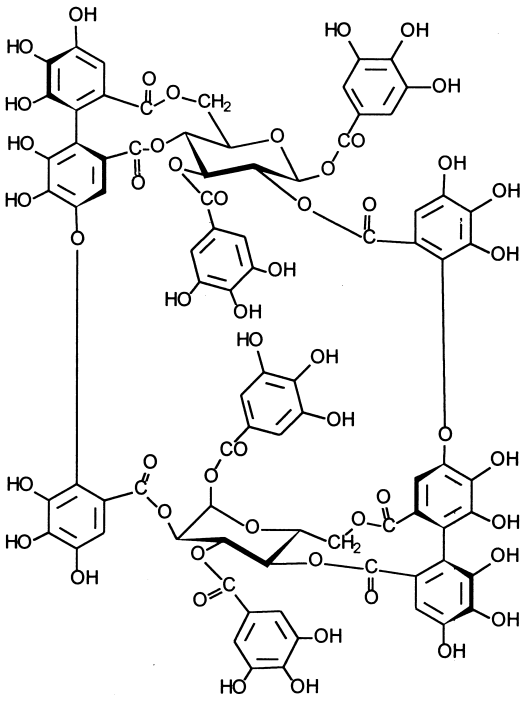

Fig. 1. Structure of cuphiin D_1 isolated from *Cuphea hyssopifolia*.

to determine whether CD_1 would induce HL-60 cell death in vitro.

Types of cell death include apoptosis and necrosis. Apoptosis is a programmed cell death that is fundamentally different from necrosis in terms of its morphological appearance, biochemical mechanism and mode of initiation [4]. Accordingly, we observed the morphology of the tumor cell in response to CD_1 treatment by electron microscopy, and then measured the DNA synthesis and fragmentation by biochemical analyses.

2. Materials and methods

2.1. Materials

Cuphiin D_1 (CD_1) was isolated from *Cuphea hyssopifolia* Humb. Bonpl. et Kunth, and was analytically pure as shown by high-performance liquid chromatography and nuclear magnetic resonance spectra [5]. Adriamycin was obtained from Sigma (St. Louis, MO). [^{3}H]Thymidine (NET-027), [^{3}H]uridine (NET-174) and [^{3}H]leucine (NET-460) were purchased from Du Pont-New England Nuclear (Boston, MA). All others reagents and chemicals were of the highest purity grade available.

2.2. Cell culture

Human promyelocytic leukemia (HL-60) cell line was obtained from American Type Cell Culture (ATCC) (Rockville, MD) and maintained in RPMI-1640 (GIBCO BRL Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 mg/l streptomycin, and 100 IU/ml penicillin (GIBCO BRL). The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

2.3. Cytotoxicity assays

The stock solution of CD_1 (2×10^4 μg/ml) was prepared by dissolving CD_1 in dimethyl sulfoxide (DMSO) and then storing it at 4°C until use. Serial dilutions of the stock solution were prepared in the culture medium in 96-well microtiter plates. CD_1 at the appropriate concentration was added to cell cultures (1×10^5 cells/well) for 36 h without renewal of the medium. The number of surviving cells was then counted by using the tetrazolium (MTT) assay [6]. Finally, the products were evaluated by measuring the optical density for each well at 600 nm, using an MRX microplate reader (Dynex technologies, Guernsey, Channel Islands, UK). The concentrations of drugs giving 50% growth inhibition (IC_{50}) were determined from three separate experiments. In the same part of experiment, HL-60 cells (2×10^5 cells/ml) were treated with different concentration of CD_1 (8, 16 or 32 μM) for 12, 24, 36, 48, 60 and 72 h separately. The viability of HL-60 cells was measured by the trypan blue exclusion.

2.4. Incorporation of thymidine, uridine and leucine

DNA, RNA and protein synthesis were measured by the cellular incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine, respectively. HL-60 cells (1×10^5 cells/well) were seeded into microtiter plates overnight and then incubated with medium containing a series of concentrations of CD_1 for 36 h. The cells were exposed to 2 μCi/ml radioactive precursors during the last 3 h of the incubation period.

Each incorporation of labeled precursors was stopped by adding 10% trichloroacetic acid and refrigerating the plates at 4°C. After 20 min, the plates were washed with ethanol and dried. The residues were dissolved in 1% sodium dodecyl sulfate in 0.3 N NaOH at 60°C for 30 min. The solutions were then counted in 2 ml Biofluor cocktail (Ecoscint H, National Diagnostics, Atlanta, GA) in a liquid scintillation counter (Beckman, LS-6500, Fullerton, CA). All experiments were performed in triplicates.

2.5. Electron microscopy

The HL-60 cell line was plated in 35-mm dishes and allowed to incubate overnight. Aliquots of CD_1 (16 μM) were added into the culture dish for 36 h. At the end of incubation, cell samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 2% buffered osmium tetroxide for 2 h and dehydrated in ethanol. Specimens for transmission electron microscopy (TEM) were embedded in epon. Thin sections were cut on an ultramicrotome (Reichert Ultracut E, Leica) and double stained with uranyl acetate and lead citrate. Electron micrographs were taken on a Hitachi H-600 electron microscope operating at 75 kV.

2.6. Flow cytometry analysis

After the appropriate treatment, HL-60 cells (2×10^6 cells/well) were harvested by centrifugation and washed with phosphate buffered saline (PBS). The cells were fixed with ice-cold 80% ethanol for 30 min, washed with PBS, and then treated with 0.25 ml of 0.5% Triton X-100 solution (containing 1 mg/ml RNase A) at 37°C for 30 min. Finally, 0.25 ml of 50 μg/ml propidium iodide was added to the sample for 30 min in the dark. Samples were run through a FACScan (Becton Dickinson, San Jose, CA). Results are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence.

2.7. Agarose gel electrophoresis

HL-60 cells (2×10^6 cells/well) exposed to CD_1 for 36 h were collected into tubes and then washed with PBS. The cells were incubated for 10 min in 200 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Sarkosyl) at room temperature, then centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant was incubated for 3 h at 56°C with 250 μg/ml proteinase K. Cell lysates were then treated with 2 mg/ml RNase A and incubated at 56°C for 1.5 h. DNA was extracted with one volume of chloroform/phenol/isoamyl alcohol (25:24:1), precipitated from the aqueous phase by centrifugation at $14\,000 \times g$ for 30 min at 0°C. An aliquot (10–20 μl) of this solution was transferred to a 2% agarose gel containing 0.5 μg/ml of ethidium bromide, and electrophoresis was carried out at 80 V for 2 h with TBE (×0.5) as running buffer. DNA in the gel was visualized under UV light.

2.8. Western blot analysis

HL-60 cells (2×10^6 cells/well) exposed to CD_1 for 36 h were collected into tubes and then washed with PBS. Protein samples were prepared and resolved by denaturing SDS-PAGE using standard methods. The proteins were transferred to nitrocellulose, and Western blot was performed by using antibody specific to human Bcl-2 or α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). A goat anti-rabbit or anti-mouse antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, SC-2007) and BCIP/NBT (BCIP/NBT, GIBCO BRL) were used to visualize protein bands.

3. Results

3.1. Cytotoxic effect of Cuphiin D_1

HL-60 cells were treated with a series of CD_1 concentrations for 36 h, and then cell growth or viability was evaluated by either an MTT assay or [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine incorporation. The results in Fig. 2 show that CD_1 decreased proliferation rates, and DNA, RNA, and protein synthesis in the HL-60 cell line, indicating a non-specific inhibitory action on cellular metabolism; and the IC_{50} of cytotoxicity effect was 16 μM. Moreover, cell viability was estimated with the trypan blue exclusion test. Fig. 3 shows that CD_1 inhibited HL-60 cell proliferation in a concentration- and time-dependent manner. At a low concentration (8 μM), CD_1 had a minor inhibition effect on HL-60 cell proliferation.

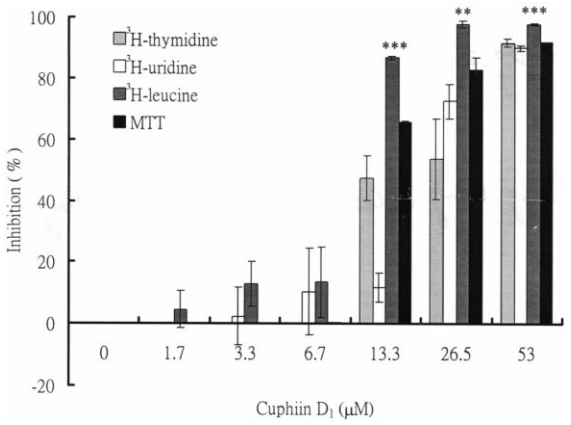

Fig. 2. Uptake of [^{3}H]thymidine, [^{3}H]uridine, [^{3}H]leucine compared with cytotoxicity by MTT on CD_1-treated HL-60 cells for 36 h. Difference in the inhibition index between the [^{3}H]leucine incorporation assay and MTT assay were significant for 13–53 μM. $***P < 0.005$; $**P < 0.01$; significantly different from control. $n = 3$.

When HL-60 cells were treated for more than 24 h, cytotoxicity was exhibited at 16 or 32 μM.

3.2. Effect of Cuphiin D_1 on morphology

As to morphology, intracellular damage caused by incubation with 16 μM CD_1 for 36 h was investigated by TEM. After 36 h of incubation with 16 μM CD_1, some cells still appeared normal, whereas others exhibited dramatic morphological alterations characteristic of apoptosis. The ultrastructure of CD_1-treated HL-60 cells showed CD_1 induced morphological changes characteristic of apoptosis, ie. disappearance of microvilli, cell shrinkage and chromatin condensation without disruption of organelles (Fig. 4B); and untreated HL-60 cells exhibited typical non-adherent fairly round morphology as shown in Fig. 4A. Numerous apoptotic bodies, which were membrane-enclosed vacuoles that had budded off the cytoplasmic extension, were also detected in CD_1-treated HL-60 cells by light microscopy observation. These apoptotic cells, as well as intact cells, excluding those with trypan blue dye, suggested that the cells were not necrosis.

3.3. Effect of Cuphiin D_1 on cell-cycle progression

HL-60 cells were treated with a range of CD_1 concentrations, and the cell-cycle distribution was examined after 24 or 36 h. When the dosage was 32 μM, the DNA content frequency histograms showed a Sub-G_1 peak and a progressive loss of the normal G_2/M phase (Fig. 5). Moreover, the data showed that CD_1 could induce DNA fragmentation (Sub-G_1) in HL-60 cells at 32 μM for 24 h (Fig. 5A). However, the low concentration (16 μM) had to be cultured for 36 h before HL-60 cells would show a Sub-G_1 peak (Fig. 5B).

3.4. Effect of Cuphiin D_1 on DNA fragmentation

DNA fragmentation is a characteristic feature of apoptosis [1]. Increased DNA fragmentation was apparent in HL-60 cells after treatment with 8–32 μM CD_1 for 36 h. A typical experimental result of agarose gel electrophoresis is shown in Fig. 6, where the effect of 16 and 32 μM CD_1 for 36 h treatment showed DNA fragment ladders. HL-60 cells apoptosis from CD_1 was also confirmed by flow cytometric analysis of DNA-stained cells (Fig. 5).

On the other hand, Bcl-2 protein levels were determined using Western blot analysis. The results showed that HL-60 cells exposed to 8 and 16 μM CD_1 for 36 h (Fig. 7) contained significantly lower levels of Bcl-2 protein compared to HL-60 cells treated with the same concentration of DMSO (0.3%). The above results indicated that decrease of Bcl-2 protein might be involved in mechanism of CD_1-induced apoptosis.

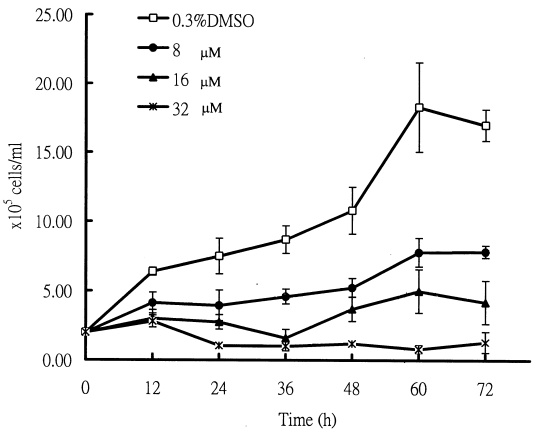

Fig. 3. Growth inhibition effects of HL-60 cells treated with various concentrations of CD_1 in a time-dependent manner by Trypan Blue dye exclusion test.

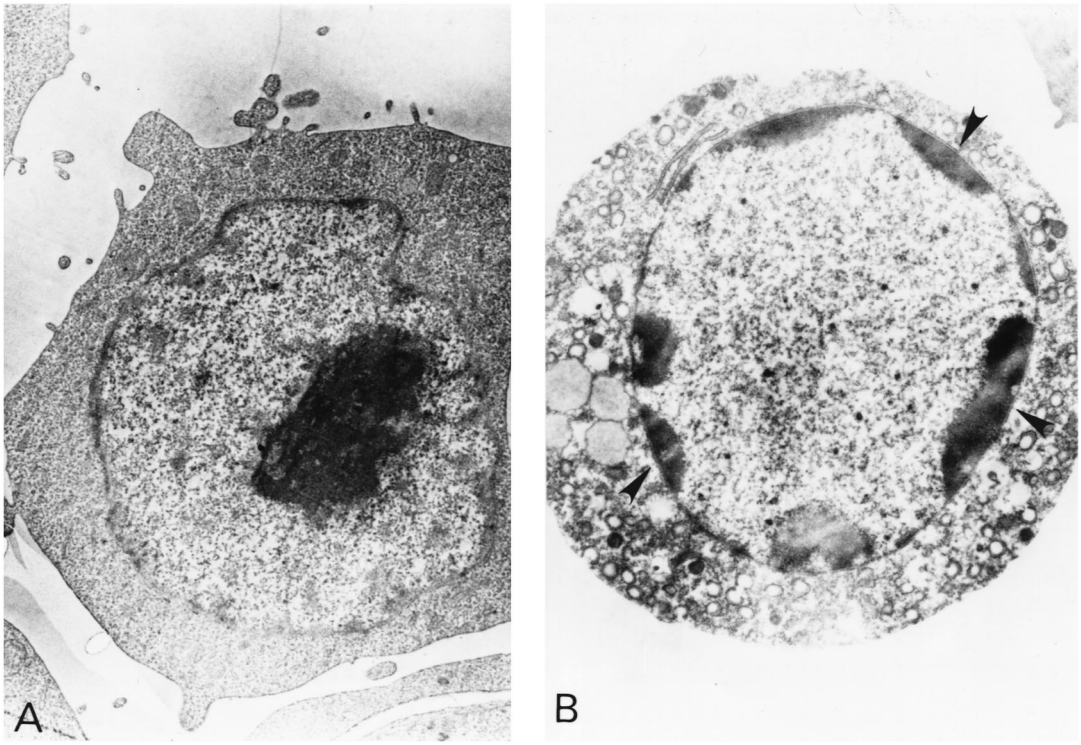

Fig. 4. Effect of CD_1 on the ultrastructure of HL-60 cells. (A) Control cells (5000×); (B) 16 μM of CD_1 for 36 h (6000×). Black arrows indicate chromatin condensation.

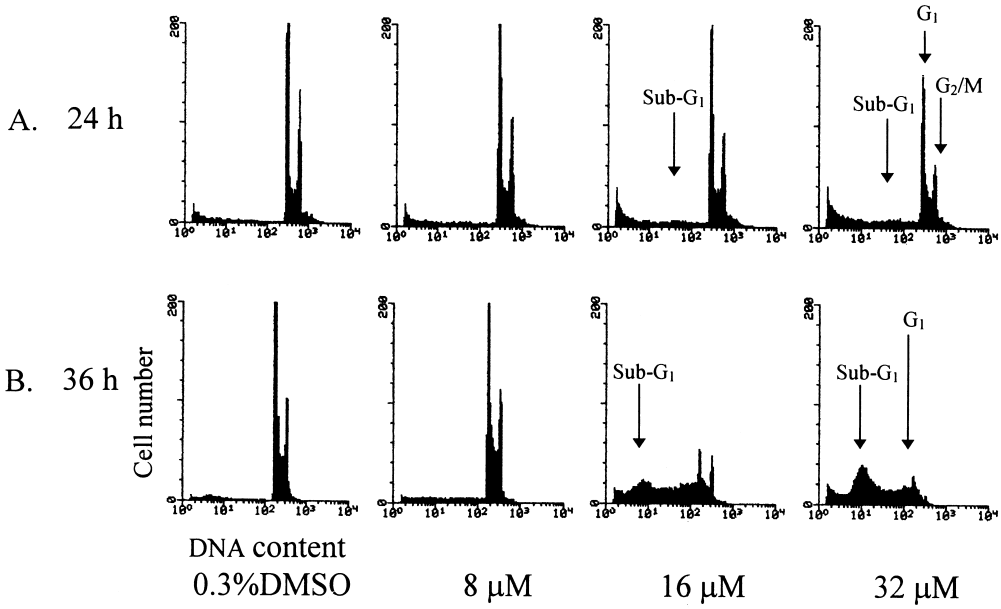

Fig. 5. DNA content frequency histograms of HL-60 cells after treatment with 8, 16 and 32 μM of CD_1 for (A) 24 h; and (B) 36 h.

Fig. 6. Effect of CD_1-induction of DNA fragmentation in HL-60 cells after treatment for 36 h. Lane 1, control (0.3% DMSO); lane 2, 32 μM; lane 3, 16 μM; lane 4, 8 μM.

4. Discussion

Many compounds have been shown to be capable of inhibiting proliferation of mammalian cells in culture. However only a small proportion of cytotoxic compounds demonstrate significant selectivity in vivo, even in the most chemosensitive animal tumor models. The new compound, CD_1, isolated from *Cuphea hyssopifolia,* has antitumor effects both in vitro and in vivo [3,5]. We had investigated the effects of CD_1 on proliferation, viability, and cell-cycle progression of representative CD_1 sensitive tumor cell lines [3].

The results for cellular uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine showed no cell-specific inhibitory action of CD_1 on HL-60 cells. The compound is most likely a general cytotoxic agent (Fig. 2). The results may be related to protein biosynthesis being strongly inhibited by CD_1. Other evidence revealed that the inhibition effects of [^{3}H]leucine incorporation were stronger than cytotoxicity for CD_1-treated HL-60 cells for 36 h (Fig. 2). According to the results of flow cytometry analysis and agarose gel electrophoresis, CD_1 indicated degenerative DNA phenomena (Figs. 5 and 6). Moreover, the morphology of CD_1-treated HL-60 cells showed many apoptotic cells, and chromatin condensation was observed by electron microscopy (Fig. 4). The data suggest that CD_1 can induce apoptosis in HL-60 cells. Some apoptosis-inducing agents have been shown to be selective for certain phases of the cell-cycle [7]. CD_1, however, appeared to induce apoptosis in all phases of the cell-cycle (Fig. 5). The finding is similar to that described for fostriecin and cisplatin which are known to be cell-cycle independent inducers of apoptosis in HL-60 cells [7].

Bcl-2 and its homologs are critical regulators of the cell death pathway. The first identified member of this gene family, Bcl-2, was discovered by virtue of its involvement in t(14; 18) chromosomal translocations commonly from B cell lymphomas [8]. Since then, overexpression of Bcl-2 has been reported in a wide variety of cancers [9]. Therefore, CD_1 exhibited to inhibit Bcl-2 expression (Fig. 7) is a good reason to develop it as an antitumor drug.

The structure of CD_1 was characterized as a gallate

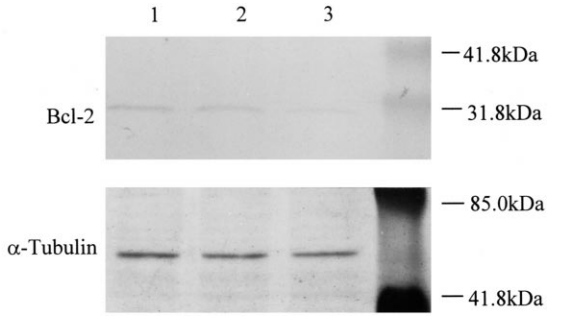

Fig. 7. Western blot analysis of Bcl-2 and α-tubulin proteins in CD_1-treated HL-60 cells for 36 h. α-Tubulin was as an internal control to identify the equal amount of proteins loading in each lane. Lane 1, control (0.3% DMSO); lane 2, 8 μ M; lane 3, 16 μM.

of woodfordin C or two gallates of oenothein B [3]. There is a possibility that CD_1 can elicit death signals through some specific receptor or enzyme. Some physiological and pharmacological actions of hydrolyzable tannins have been suggested via: (i), their complexation with metal ions (iron, manganese, vanadium, copper, aluminum, calcium, etc.); (ii), their antioxidant and radical scavenging activities; and (iii), their ability to complex with other molecules including macromolecules such as proteins and polysaccharides [10]. Recently, many biological and pharmacological activities of polyphenols have been reported. For example, woodfordin C, a macrocyclic hydrolyzable tannin is an inhibitor of topoisomerase II [11], and oenothein B was found to be a potent and specific inhibitor of poly-(ADP-ribose) glycohydrolase [12]. Conclusively, CD_1 is found to induce apoptosis due to inhibition of Bcl-2 expression in HL-60 cells by its distinctive structures, but the detailed mechanism is currently unclear. In the future, we will explore structure–activity analysis and the mechanism of antitumor effects.

Acknowledgements

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