Annoglabayin, a Novel Dimeric Kaurane Diterpenoid, and Apoptosis in Hep G2 Cells of Annomontacin from the Fruits of *Annona glabra*

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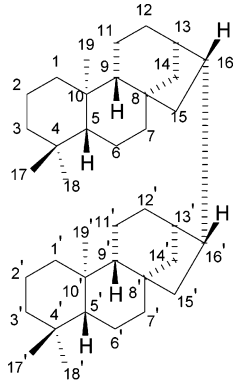
Annoglabayin (**1**), a novel *Annona* dimeric kaurane diterpenoid, has been isolated from *Annona glabra*, and its structure was determined on the basis of spectroscopic analysis. Annoglabayin (**1**) contains a unique carbon bridge between two nor-*ent*-kaurane monomeric units. The dose−response of **2** in Hep G2 cells indicated that **2** increased DNA damage. In addition, our results showed that **2** induced a noticeable decrease in mitochondrial transmembrane potential during treatment. These results indicate that **2** produces apoptotic events in Hep G2 cells, through inducing changes in mitochondria.

Annona glabra L. (Annonaceae), commonly known as "pond apple", is a tropical tree distributed mainly in the Americas and in Southeast Asia and cultured in the southern part of Taiwan. It has been used as an insecticide and a parasiticide.[1,2] In the course of screening for biologically and chemically novel agents from Formosan Annonaceous plants, we found that *Annona glabra* produced a novel dimeric kaurane diterpenoid named annoglabayin (**1**). Previous studies have investigated the chemical constituents of the Formosan Annonaceous plants and have identified kaurane diterpenoids, annoglabasin A−F, a novel dioxoaporphine alkaloid, annobraine, and 38 known compounds from the fresh fruits and stems of *A. glabra*.[3−5] In this paper, we report the isolation and structural elucidation of annoglabayin (**1**) and the apoptotic inducing capability of annomontacin (**2**) on a human hepatoma cell line, Hep G2. The apoptotic process in Hep G2 cells induced by **2** was caused by a significant decrease in mitochondrial transmembrane potential, an early and irreversible stage in apoptosis.[6]



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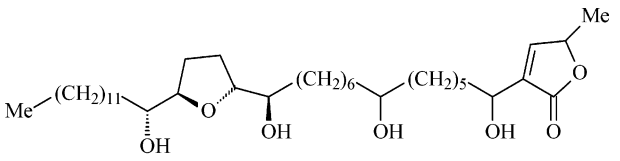

Annomontacin (**2**)

Annoglabayin (**1**), obtained as white powder, was assigned the molecular formula $C_{38}H_{62}$ by HREIMS at *m/z* $[M]^+$ 518.4848 (calcd for $C_{38}H_{62}$, 518.4852) and HRFABMS at *m/z* $[M + K]^+$ 557.4481 (calcd for $C_{38}H_{62}K$, 557.4489). The ^1H NMR spectrum of **1** showed three tertiary methyl groups at δ 0.79 (3H, s), 0.84 (3H, s), and 0.99 (3H, s) and two methine protons at δ 2.52 and 2.64, indicating that **1** was probably an *ent*-kaurane diterpene (16β-hydro-*ent*-kauran-17-oic acid) possessing a carboxylic acid moiety at C-17.[1] The ^{13}C NMR spectrum and a DEPT experiment indicated that **1** had a total of 19 carbons, with the skeleton consisting of 19 carbons, consistent with a nor-*ent*-kaurane diterpenoid. The carbons of the nor-*ent*-kaurane diterpene were assigned from ^{13}C NMR and DEPT experiments, as three methyls at δ 17.4 (C-19), 21.6 (C-18), and 33.6 (C-17), nine methylenes, four methines at δ 41.4 (C-13), 45.3 (C-16), 56.0 (C-5), and 56.2 (C-9), and three quaternary carbons at δ 33.2 (C-4), 39.3 (C-10), and 45.1 (C-8). The ^1H and ^{13}C NMR spectra of **1** were similar to those of 16β-hydro-*ent*-kauran-17-oic acid except for the carbonyl function.[1]

The structure **1** was also supported by 2D NMR experiments. COSY correlations were observed between H-16 and both H-13 and H-15. The HETCOR experiment showed that the carbon signals at δ 41.4 for C-13 and 45.3 for C-16 were correlated to the proton signals at δ 2.53 for H-13 and 2.64 for H-16, respectively. Thus, these two portions of the dimeric nor-*ent*-kaurane were proved to be carbon linked at the C-16 position of the A-portion and at the C-16′ position of the B-portion. The dimer structure was further

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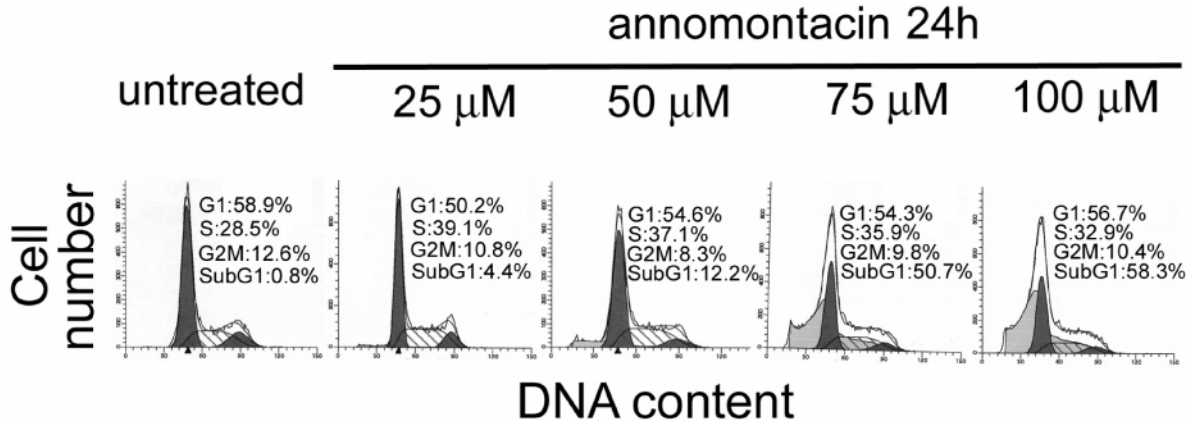
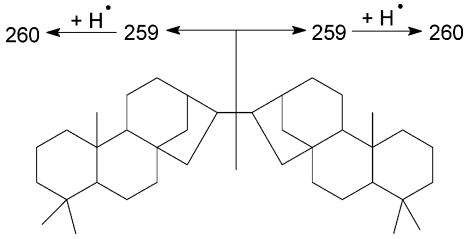


Figure 1. Effect of compound **2** on cell cycle of the Hep G2 cell line. Hep G2 cells were treated with the indicated concentrations of **2** for 24 h. After treatment, cells were collected, fixed with methanol, stained with propidium iodide, and analyzed by flow cytometry. Data on each sample represent the percentage of cells in the G1, S, G2M, and SubG1 phases of the cell cycle, respectively. Tests were performed at least three times, and a representative experiment is presented.

Scheme 1. MS Fragment Ions of **1** (m/z)



confirmed by a FABMS fragment at 260 [M − $C_{19}H_{31}$ + H]$^+$ (Scheme 1). Thus, the structure of **1** was determined to be (16→16′)-bis-16β-hydro-*ent*-kaurane and has been named annoglabayin.

In previous reports on plants of the Annonaceae, dimeric diterpenoids have been characterized as comprising one *ent*-kaurane and one labdane unit and were isolated only from *Xylopia* species.[7−9] Compound **1** is the first dimer with a new skeleton comprising two nor-*ent*-kaurane units isolated from a plant in the Annonaceae. This type of dimeric *ent*-kaurane has been isolated previously from the traditional Chinese medicine "Bei-Mu", prepared from the bulbs of *Fritillaria thunbergii* (Liliaceae) and related species.[10,11] Annomosin A (16β-hydroxy-19-al-*ent*-kauran-17-yl 16β-hydro-19-al-*ent*-kauran-17-oate) was the first dimer comprising two *ent*-kaurane monomeric units isolated from a plant in the Annonaceae.[12]

To evaluate the anticancer effects of annomontacin (**2**), we first examined whether the induction of apoptotic-relevant DNA damage occurred following the addition of **2** in the human hepatoma cell line, Hep G2. The DNA content of annomontacin (**2**) treated Hep G2 cells was determined by treatment with propidium iodide and measuring stained DNA by flow cytometry. The DNA histograms and the percentages of cells in each phase of the cell cycle are shown in Figure 1. Compared with untreated Hep G2 cells, treatment with 25, 50, 75, and 100 μM of **2** for 24 h resulted in a dose−responsive increase in the subG1 population, extending from 0.8 to 4.4, 12.2, 50.7, and 58.3%, respectively, and indicative of apoptosis.

We further examined whether **2** affected the morphology of Hep G2 cells. During 3−24 h treatment with 100 μM **2**, the percentage of cells with a normal morphology in the untreated groups was 68.4−78.9%. This was determined by a flow cytometric method that measures cellular shape changes using forward scatter (FSC) and side scatter (SSC) parameters (Figure 2). Fewer than 20% of untreated cells

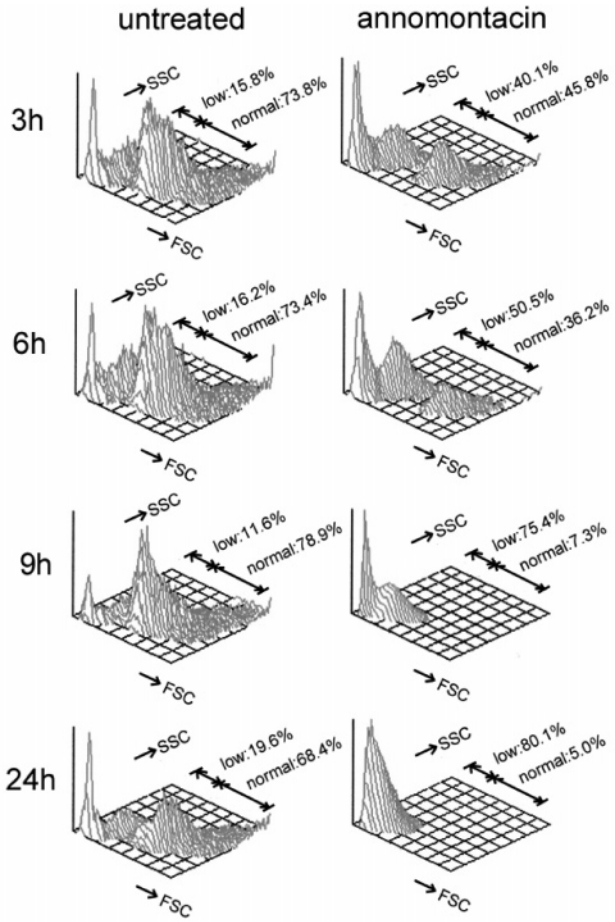


Figure 2. Effect of compound **2** on cellular shape change of Hep G2 cells. Hep G2 cells were treated with or without 100 μM compound **2** for 3, 6, 9, and 24 h. Cells were trypsinized and analyzed by flow cytometry. Cell size and granularity were monitored by flow cytometry, using forward scatter (FSC) and side scatter (SSC), respectively. Representative flow cytometric 3D-histograms of Hep G2 cells are shown, where the x-axis shows FSC and the y-axis is SSC. The percentages of normal and low cellular shape change were calculated from the FSC and SSC of each sample by gating out the population of non-shape-changed cellular debris. Tests were performed at least three times, and a representative experiment is presented.

exhibited cellular shape changes during the treatment period. In the presence of 100 μM **2**, the percentage of cells with a low cellular shape change was elevated initially (40.1%, 3 h) and increased with time. The highest percent-

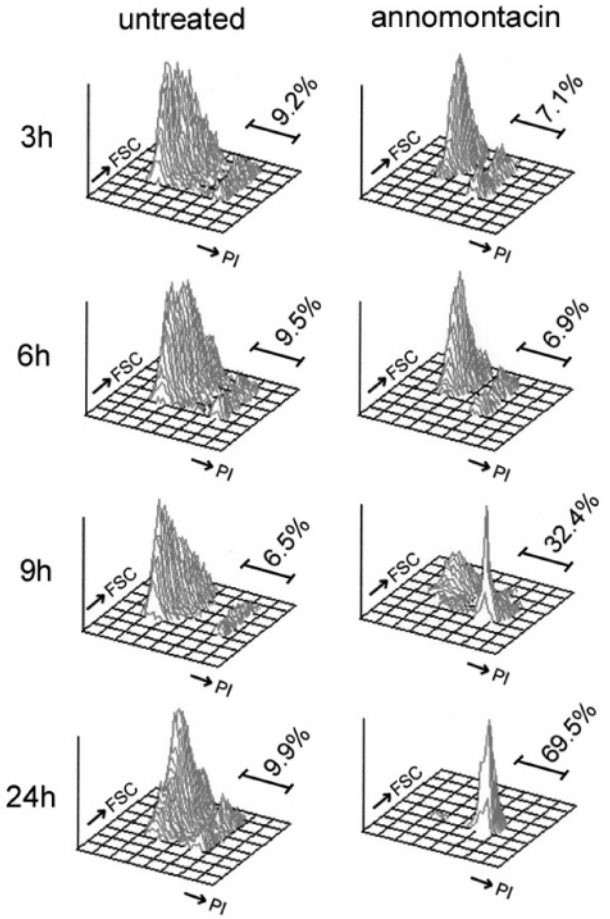


Figure 3. Effect of compound **2** on cellular membrane integrity of the Hep G2 cell line. Hep G2 cells were treated with or without 100 μM compound **2** for 3, 6, 9, and 24 h. Cells were trypsinized, stained with 5 μg/mL propidium iodide (PI), and analyzed by flow cytometry. Representative flow cytometric 3D-histograms of Hep G2 cells are shown, where the *x*-axis shows PI and the *y*-axis is forward light scatter (FSC). The percentage of cells that expressed membrane damage was calculated on each sample by gating out the population of non-shape-changed cellular debris. Tests were performed at least three times, and a representative experiment is presented.

age (80.1%) of low cellular shape change occurred in annomontacin-treated Hep G2 cells at 24 h.

The above results indicate that treatment with compound **2** may increase the rate of cell death. We further investigated the integrity of the cellular membrane during treatment with **2**. Ten percent of cells in the untreated groups exhibited cellular membrane damage as a result of trypsinization and experimental manipulation (Figure 3), 32.4% of Hep G2 cells exhibited cellular membrane damage at 9 h of treatment with 100 μM compound **2**, and 69.5% showed membrane damage at 24 h.

A decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) has been reported as an early event in apoptosis; this can be detected by a decline in rhodamine 123 fluorescence.[13] To measure changes in the $\Delta\Psi_m$, Hep G2 cells treated with 100 μM **2** and untreated Hep G2 cells were stained with rhodamine 123, a fluorescent dye reflecting $\Delta\Psi_m$,[13] at different times after annoglabayin treatment. The mean fluorescence intensity of rhodamine 123 in untreated groups during the experimental period ranged from 528−550 (Figure 4). For the annomontacin-treated cells, the rhodamine 123 fluorescence decreased to 367 at 3 h and to 75 at 24 h, post-treatment.

Our results suggest that compound **2** interacts with mitochondria, leading to several functional alterations in Hep G2 cells, some or all of which may be key factors in the induction of apoptosis. The mechanisms responsible for this action remain to be determined.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeOH using a JASCO V-530 spectrophotometer. Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra (all in $CDCl_3$) were recorded with Varian NMR spectrometers, using TMS as an internal standard. LRFABMS and LREIMS spectra were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. Flow cytometry analysis was done using a Becton-Dickinson FACS-Calibur flow cytometer. Labeling dyes such as propidium iodide (PI) and rhodamine 123 were used to investigate the events involved in apoptosis.

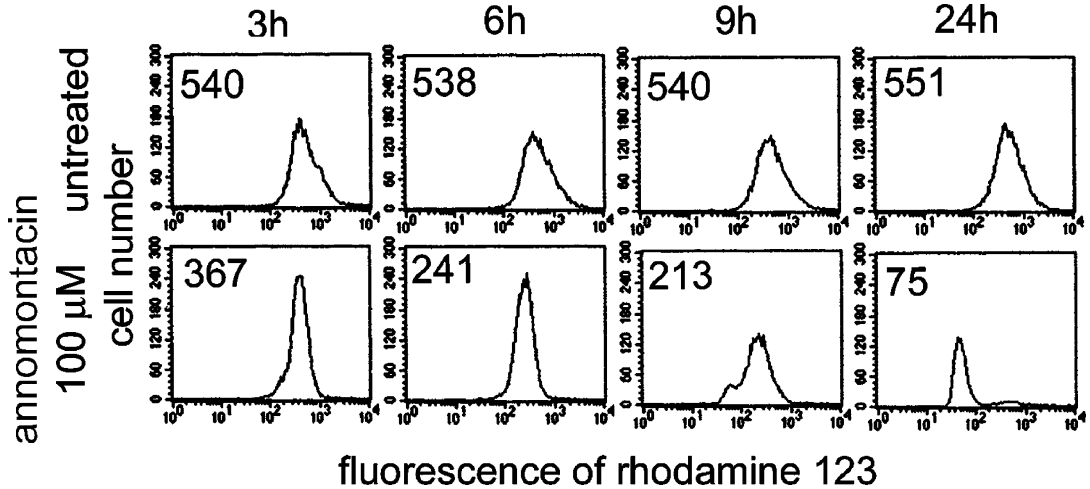


Figure 4. Effect of compound **2** on mitochondrial transmembrane potential ($\Delta\Psi_m$) of Hep G2 cells. Cells were treated with or without 100 μM of compound **2** for 3, 6, 9, and 24 h. Cells were trypsinized, stained with rhodamine 123, and analyzed by flow cytometry. Data represent the mean fluorescence intensity of rhodamine 123 in Hep G2 cells. These experiments were performed at least three times, and a representative experiment is presented.

Table 1. ^{13}C NMR (400 MHz), ^{1}H NMR (100 MHz), COSY, NOESY, and HMBC Data of Annoglabayin (**1**) in $CDCl_3$

C#	δ_C	δ_H	mult., *J* (Hz)	COSY	NOESY	HMBC
1(1′)	40.4, t	0.74	td, 13.2, 4.4	H-2(2′)		C-3(3′), C-19(19′)
		1.78	m			
2(2′)	18.3, t	1.50	m	H-1(1′)		
				H-3(3′)		
3(3′)	38.1, t	1.05	m	H-2(2′)	H-5(5′)	
		1.90	br d, 11.6		17(17′)-Me	
4(4′)	33.2, s					
5(5′)	56.0, d	1.08	m	H-6(6′)	H-9(9′)	C-1(1′), C-4(4′), C-10(10′), C-17(17′), C-18(18′), C-19(19′)
					H-3a(3a′)	
6(6′)	20.7, t	1.33	m	H-5(5′)		
		1.51	m	H-7(7′)		
7(7′)	42.0, t	1.23	m	H-6(6′)		C-8(8′)
8(8′)	45.1, s					
9(9′)	56.2, d	0.78	m		H-5(5′)	C-8(8′), C-12(12′)
10(10′)	39.3, s					
11(11′)	18.6, t	1.45	m	H-12(12′)		C-12(12′)
12(12′)	31.3, t	1.52	m	H-11(11′)	H-16(16′) 19(19′)-Me	
12(12′)				H-13(13′)		
13(13′)	41.4, d	2.53	br s	H-12(12′) H-14(14′) H-16(16′)		
14(14′)	40.9, t	1.51	m	H-13(13′)		C-8(8′), C-9(9′), C-12(12′)
15(15′)	44.7, t	1.69	m	H-16(16′)		C-8(8′), C-9(9′)
16(16′)	45.3, d	2.64	t, 8.8	H-13(13′) H-15(15′)	H-12(12′)	C-12(12′), C-13(13′), C-15(15′)
17(17′)	33.6, q	0.99	s		H-3b(3b′)	C-3(3′), C-4(4′), C-5(5′), C-18(18′)
18(18′)	21.6, q	0.84	s		19(19′)-Me	C-3(3′), C-4(4′), C-5(5′), C-17(17′)
19(19′)	17.4, q	0.79	s		H-12(12′) 18(18′)-Me	C-1(1′), C-5(5′), C-9(9′), C-10(10′)

Plant Material. The fruits of *A. glabra* were collected from Fooyin University, Kaohsiung Hsien, Taiwan, in September 2003. A voucher specimen was characterized by Dr. Horng-Liang Lay of the Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan, and deposited in the Fooyin University, Kaohsiung Hsien, Taiwan.

Extraction and Isolation. Steps for extraction and chromatographic fractionation of the fresh fruits were identical to those reported previously.[14] The fresh fruits of *A. glabra* (2.0 kg) were extracted with MeOH at room temperature repeatedly. The combined MeOH extracts (ca. 400 g) were evaporated and partitioned to yield $CHCl_3$ (ca. 250 g) and aqueous extracts. The $CHCl_3$ layer was further separated into six fractions by column chromatography on silica gel with gradients of *n*-hexane−EtOAc (*n*-hexane−EtOAc, 4:1, to pure EtOAc) and EtOAc−MeOH (pure EtOAc to EtOAc−MeOH, 10:1). Fraction 3 (76.3 g) eluted with *n*-hexane−EtOAc (1:8) was repeatedly subjected to silica gel CC and yielded annomontacin (**2**) (234.0 mg). Other fresh fruit (2.0 kg) was extracted with MeOH. The extracts were concentrated in vacuo and partitioned between $CHCl_3$ and H_2O. The organic layer was separated by silica gel column chromatography using gradient elution of $CHCl_3$−MeOH. Further purification by preparative TLC on silica gel with hexane−EtOAc (2:1) afforded annoglabayin (**1**) as a white powder (43.0 mg). Compound **2** has been characterized by comparison of its spectroscopic data with literature values.[15−17]

Annoglabayin ((16→16′)-bis-16*β*-hydro-*ent*-kaurane) (1): white powder; mp 125−127 °C; $[\alpha]^{25}_D$ −5.2° (*c* 0.75, $CHCl_3$); IR (neat) ν_{max} 1450, 1365, 1115 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m/z* 557 $[M + K]^+$ (16), 260 $[M - C_{19}H_{31} + H]^+$ (65); EIMS (30 eV) *m/z* 518 $[M]^+$ (1), 259 $[M - C_{19}H_{31}]^+$ (73), 243 (4), 192 (21), 123 (100), 109 (64), 107 (28), 91 (35); HRFABMS *m/z* 557.4481 $[M + K]^+$ (calcd for $C_{38}H_{62}K$, 557.4489); HREIMS *m/z* 518.4848 $[M]^+$ (calcd for $C_{38}H_{62}$, 518.4852).

Cell Culture and Drug Treatments. Hep G2 cells were seeded into the 60 mm tissue culture dishes (5×10^5 cells/dish) 48 h before the experiment. The basal medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 250 μg/mL amphotericin B. The stock solution of compound **2** (200 mM) was dissolved in DMSO, and experimental concentrations were prepared in the basal medium. The final concentration of DMSO in the medium was 0.1%. Cells were exposed to medium containing various concentrations of **2** for different periods.

Propidium Iodide Staining of Cellular DNA. Adherent and floating Hep G2 cells were pooled, washed with PBS, fixed in PBS−MeOH (1:2, v/v) solution, and maintained at 4 °C for at least 18 h. After an additional wash with PBS, the cell pellets were stained with the fluorescent probe solution containing PBS, 50 μg of propidium iodide/mL, and 50 μg of DNase-free RNaseA/mL for 30 min at room temperature in the dark. Cells were then analyzed using a FACS-Calibur cytometer (Becton-Dickinson, San Jose, CA) with excitation at 488 nm and gating out of doublets and clumps using pulse processing and collection of fluorescence emission above 580 nm. The percentage of cells undergoing DNA damage was defined by the percentage of cells in the subdiploid region of the DNA distribution histograms.

Shape Change Assay. Hep G2 cells were treated with or without compound **2** (100 μM) for 3, 6, 9, and 24 h. After treatment, the cells were harvested by trypsinization and analyzed using a Becton-Dickinson FACS-Calibur flow cytometer. The shape change of the cells was calculated from the normal and low forward light scatter and side scatter of each sample, using CellQuest software, and expressed as a percentage.

Analysis of the Cellular Membrane Integrity. To evaluate the integrity of the cellular membrane after treatment with compound **2**, trypsinized cells were stained for 10 min with 5 μg/mL propidium iodide. Stained cells were excited by exposure to an argon laser at 488 nm, collection of fluorescence emission was above 580 nm, and at least 10 000 cells were counted with a FACS-Calibur flow cytometer, using CellQuest software. Using this method, damage to the integrity of the cell membrane indicating cell death was determined by propidium iodide staining. In addition, propidium iodide staining measures cells that have become necrotic. By using propidium iodide staining, cells were classified as either intact or having membrane damage.

Measurement of Mitochondrial Transmembrane Potential by Flow Cytometry. Rhodamine 123 is a fluorescent dye that is incorporated into mitochondria in a transmembrane potential-dependent manner.[13] The culture medium was replaced with new medium containing 5 μM rhodamine 123 for 30 min in the dark when the cells became 80% confluent. After incubation with rhodamine 123, the cells were harvested by trypsinization, and the mitochondrial transmembrane poten-

tial, determined by the fluorescence level of rhodamine 123, was analyzed using a Becton-Dickinson FACS-Calibur flow cytometer.

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