Apoptotic Effects of Polyprenylated Benzoylphloroglucinol Derivatives from the Twigs of *Garcinia multiflora*

Xin Liu,[†] Ting Yu,[‡] Xue-Mei Gao,[†,⊥] Yan Zhou,[†] Chun-Feng Qiao,[†] Yong Peng,[§] Shi-Lin Chen,[§] Kathy Qian Luo,*[,‡] and Hong-Xi Xu*[,†]

Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Shatin, N. T., Hong Kong, People's Republic of China, Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457, Institute of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100083, People's Republic of China, and School of Chemistry and Biotechnology, Yunnan Nationalities University, Kunming 650031, People's Republic of China

Received March 9, 2010

With bioassay-guided fractionation, five new polyprenylated benzoylphloroglucinol derivatives, garcimultiflorone D (**1**), 18-hydroxygarcimultiflorone D (**2**), garcimultiflorone E (**3**), garcimultiflorone F (**4**), and isogarcimultiflorone F (**5**), and five known compounds, guttiferone E (**6**), guttiferone F (**7**), aristophenone A (**8**), isoxanthochymol (**9**), and morelloflavone (**10**), were isolated from the acetone extract of the twigs of *Garcinia multiflora*. The compounds were evaluated for their apoptotic effects against HeLa-C3 cells, which have been genetically engineered to produce a fluorescent biosensor capable of detecting caspase-3 activation. Compounds **1** and **3**−**9** activate caspase-3 in HeLa-C3 cells within 72 h after treatment at a concentration of 100 μM or lower. In particular, compounds **6**, **8**, and **9** showed strong apoptosis-inducing effects at a concentration of 25 μM.

Since Wyllie et al. discovered the significance of apoptosis to cell death over 30 years ago, tremendous efforts for unraveling the relationship between apoptosis and killer diseases such as cancer and evaluating potential apoptosis-inducing anticancer drugs have become a major focus among cancer research groups worldwide.[1] Apoptosis is an induced cell suicidal process that happens in multicellular organisms and allows organisms to eliminate unwanted or damaged cells.[2] Basic cancer research has made remarkable progress in the realization of cancer biology and cancer genetics and demonstrated that most anticancer agents could induce apoptosis.[3] Activation of caspase-3 is pivotal for apoptosis, and assaying caspase-3 activity has been widely used as a tool for detecting programmed cell death.[4,5]

In our previous studies, a series of bioactive xanthones and benzophenone derivatives were isolated from the *Garcinia* genus and screened by apoptotic assays.[6−9] Through continuous efforts in searching for anticancer agents from natural sources, the acetone extract of the twigs of *Garcinia multiflora* Champ. (Guttiferae) was found to possess good apoptotic effects against HeLa-C3 cells. *G. multiflora*, a small evergreen tree distributed in South China and Hong Kong, contains xanthones,[10] benzophenone derivatives,[11,12] and biflavonoids[13,14] as the main components and exhibits a variety of bioactivities, such as anti-inflammatory,[11] anti-HIV,[14] antioxidant,[10,15] and antituberculosis activities.[13] Bioactivity-directed fractionation of this plant led to the isolation and identification of five new benzophenone derivatives, garcimultiflorone D (**1**), 18-hydroxygarcimultiflorone D (**2**), garcimultiflorone E (**3**), garcimultiflorone F (**4**), and isogarcimultiflorone F (**5**), and five known compounds, guttiferone E (**6**), guttiferone F (**7**), aristophenone A (**8**), isoxanthochymol (**9**), and morelloflavone (**10**). Their apoptotic effects against HeLa-C3 cells were evaluated.

Results and Discussion

The combined crude acetone extract (215 g) was subjected to a D101 (granularity: 0.25−0.84 mm) resin column, and the column was eluted with 60% and 95% EtOH, successively. The 95% EtOH fraction (46 g) was found to have significant apoptotic activity. Therefore, this fraction was selected for further purification. Chromatographic purification on a silica gel column and semi-preparative HPLC yielded five new (**1**−**5**) and five known compounds (**6**−**10**) (Figure 1).

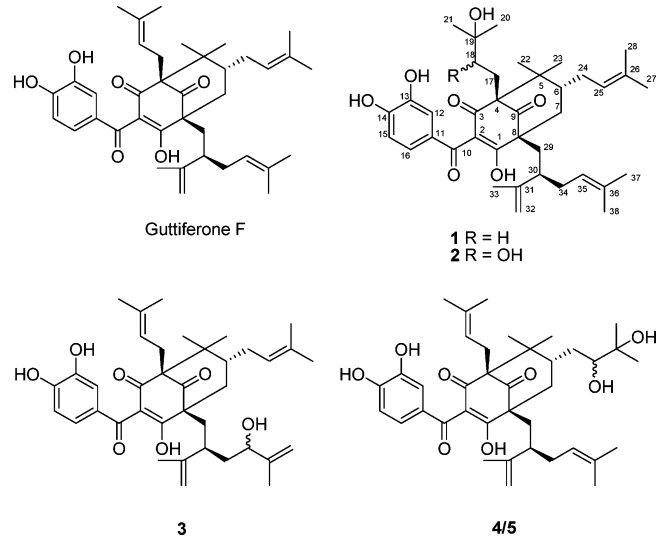

Figure 1. Compounds isolated from *Garcinia multiflora*.

Garcimultiflorone D (**1**) was obtained as a yellow, amorphous powder and showed a deprotonated molecular ion peak at *m*/*z* 619.3635 [M − H]$^-$ in the HR-ESITOFMS, corresponding to the formula $C_{38}H_{51}O_7$ (calcd 619.3635). The IR spectrum showed absorption bands at 1716, 1658, and 1600 cm^{-1} for three carbonyl groups, which were confirmed by signals at δ 194.0, 195.5, and 210.9 in the ^{13}C NMR spectrum. Its ^{1}H, ^{13}C, and DEPT NMR spectra, together with a HSQC spectrum, revealed the presence of nine methyl, seven methylene, seven methine, and 15 quaternary carbons. The ^{1}H NMR of **1** revealed the presence of one terminal double bond [δ 4.47 (1H, s) and 4.48 (1H, s)] and one 1,3,4-trisubstituted benzene ring [δ 6.70 (1H, d, J = 8.4 Hz), 6.99 (1H, dd, J = 2.0 and 8.4 Hz), and 7.18 (1H, d, J = 2.0 Hz)]. The ^{13}C

* To whom correspondence should be addressed. Tel: +852-34062873. Fax: +852-35527333. E-mail: kluo@ntu.edu.sg; xuhongxi@hkjcicm.org.
[†] Hong Kong Jockey Club Institute of Chinese Medicine.
[‡] Nanyang Technological University.
[§] Chinese Academy of Medical Science and Peking Union Medical College.
[⊥] Yunnan Nationalities University.

10.1021/np100156w
Published on Web 08/03/2010

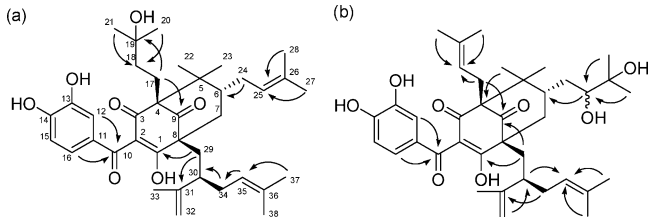

Figure 2. Key HMBC correlations of **1** (a) and **4** and **5** (b).

NMR spectrum of **1** showed the presence of a bicyclo[3.3.1]nonane-2,4,9-trione moiety[15,16] and was similar to that of guttiferone F (**7**),[18] the difference being the chemical shifts of C-18, C-19, and C-20. This suggested that the core bicyclic system of **1** was the same as that in **7** and that these differences could only be due to the modification of the side chain attached at C-4. The ^{1}H NMR data of **1** were also similar to those of **7**,[18] except for H-18 [δ 2.70 (1H, m) and 2.55 (1H, m)] of **1** and H-18 [δ 5.03 (1H, m)] of **7**, which indicated the existence of a 3-hydroxy-3-methylbutyl group at C-4 of **1**. This postulation was supported by HMBC correlations as shown in Figure 2. The relative configuration of C-6 was deduced from the ^{13}C NMR chemical shift of Me-22 (δ 27.4), which was due to the γ-gauche interaction shielding of the axial methyl by the C-6 substituent when this group is equatorial.[16] The absolute configuration of **1**, which had a negative optical rotation ($[\alpha]_D$ −53.6), was tentatively postulated with reference to the previously known analogues and the hypothesis that a positive or negative $[\alpha]_D$ value is attributed only to the bicyclo[3.3.1]nonane system orientation.[11,19] All these assignments were further confirmed by HSQC, HMBC, and NOESY NMR analyses. Thus, the structure of garcimultiflorone D (**1**) was assigned as shown in Figure 1.

18-Hydroxygarcimultiflorone D (**2**) was isolated as a yellow gum. The HR-ESIMS of **2** afforded a $[M + H]^+$ (calcd of $C_{38}H_{53}O_8$, 637.3740) ion at *m/z* 637.3751, implying a molecular formula of $C_{38}H_{52}O_7$. The IR spectrum showed the presence of OH (3551 cm^{-1}) and carbonyl (1733, 1716, and 1683 cm^{-1}) groups. Comparison of the ^{13}C NMR data of **2** (Table 1) with those of **1** suggested that their structures were closely related, except for the difference between C-18 (δ 73.8) of **2** and C-18 (δ 27.1) of **1**. This evidence indicated the presence of OH-18, which was supported by HMBC correlations between H-20 (δ 1.11) and C-18 (δ 73.8). Compound **2** was thus elucidated as 18-hydroxygarcimultiflorone D. Its structure was deduced from the ^{1}H−^{1}H COSY and ROESY experiments (Supporting Information) and ^{13}C NMR assignments and was confirmed by DEPT, HSQC, and HMBC analyses (Table 1 and Supporting Information).

Garcimultiflorone E (**3**) was isolated as a yellow gum. The HR-ESIMS of **3** afforded a $[M + H]^+$ ion at *m/z* 619.3635 (calcd of $C_{38}H_{51}O_7$, 619.3635). Its molecular formula was deduced to be $C_{38}H_{52}O_8$, which was supported by the ^{1}H, ^{13}C, and DEPT NMR data. The IR spectrum exhibited bands corresponding to hydroxy (3587 cm^{-1}) and both conjugated (1683 cm^{-1}) and nonconjugated (1733 cm^{-1}) carbonyl groups. The ^{1}H NMR data of **3** were similar to those of guttiferone F (**7**), except for the difference between H-35 [δ 3.86 (1H, m)] and H-37 [δ 4.79 (2H, br s)] of **3** and H-35 [δ 5.03 (1H, m)] and H-37 [δ 1.65 (3H, s)] of **7**, which indicated the existence of a 2-hydroxy-3-methylbutenyl group at C-30 of **3**. This finding was also supported by the ^{13}C NMR data of C-35 (δ 75.2), C-36 (δ 147.6), and C-37 (δ 113.1) and HMBC correlations between H-37/C-36, H-38/C-36, H-35/C-37, and H-34/C-29. The structure of garcimultiflorone E was further confirmed by the ^{1}H−^{1}H COSY, ROESY, DEPT, HSQC, and HMBC experiments (Supporting Information).

Garcimultiflorone F (**4**) and isogarcimultiflorone F (**5**) were obtained initially as a mixture by reversed-phase C_{18} silica gel column and semipreparative HPLC (MeOH−H_2O, 75:25, with 0.1% formic acid in MeOH, 3 mL/min) and then separated by an additional semipreparative HPLC (MeOH−MeCN−H_2O, 50:25:25, with 0.1% formic acid in MeOH, 3 mL/min). Compounds **4** and **5** gave the same molecular formula of $C_{38}H_{52}O_8$, which was deduced from their HR-ESIMS. Analyses of their ^{1}H and ^{13}C NMR data indicated that **4** and **5** have similar structures and might be a pair of epimers with a polyprenylated benzoylphloroglucinol backbone. The NMR data indicated that both **4** and **5** have one prenyl group, one 2,3-dihydroxy-3-methylbutyl group, one lavanduyl group, conjugated and nonconjugated carbonyl groups, and two quaternary carbons (Table 1). The presence of a 1,3,4-trisubstituted benzene ring in **4** and **5** was evident from the proton resonances at δ 6.68 (1H, d, J = 8.4 Hz), 6.98 (1H, dd, J = 1.9 and 8.4 Hz), and 7.22 (1H, d, J = 1.9 Hz). The HMBC experiments of these two compounds supported the existence of the prenyl, 2,3-dihydroxy-3-methylbutyl, and lavanduyl groups affixed to C-4, C-6, and C-8 of the bicyclo[3.3.1]nonane-2,4,9-trione moiety, respectively (Figure 2). Although the only structural difference between **4** and **5** was found to be the opposite configuration at C-25, the ^{13}C NMR chemical shift difference between these two compounds lies in the bicyclo[3.3.1]nonane-2,4,9-trione moiety and the C-24 side chain, which were caused by the different configuration of C-25 in **4** and **5** and the different hydrogen bonds of OH-25 and carbonyl groups at C-3 and C-9. However, the exact configurations of OH-25 in **4** and **5** have not been identified. Thus, compounds **4** and **5** were elucidated as garcimultiflorone F and isogarcimultiflorone F, respectively (Figure 1).

The structures of the known compounds were elucidated as guttiferone E (**6**),[8] guttiferone F (**7**),[18] aristophenone A (**8**),[20] isoxanthochymol (**9**),[12] and morelloflavone (**10**),[21] respectively, by comparing their spectroscopic data with reported data.

The biological activity of compounds **1**−**10** was evaluated for their apoptosis-inducing effects using genetically engineered HeLa-C3 cells that possess a fluorescent biosensor capable of detecting caspase-3 activation. This fluorescence resonance energy transfer (FRET)-based biosensor is highly sensitive to the activation of caspase-3 in intact living cells. These cells emit green fluorescent light under normal growth conditions and shift to blue fluorescent light emission under caspase-3 activation during apoptosis when the sensor proteins inside the cells are cleaved. This biosensor was constructed by fusing a CFP (cyan fluorescent protein) and a YFP (yellow fluorescent protein) with a specialized linker containing the caspase-3 cleavage sequence, DEVD.[4] On the basis of our previous results, the emission ratio of YFP/CFP is usually between 6 and 8 in normal cells, and this ratio will decrease to a value of 3 or below when a great majority of the cells undergo a caspase-3-dependent apoptotic cell death.[22] Therefore, any compounds that can reduce the YFP/CFP emission ratio to 3 or below were considered to be capable of activating apoptosis.

As shown in Table 2, five new compounds and five known compounds were tested at a concentration of 25, 50, and 100 μM. Among these compounds, morelloflavone (**10**) did not show apoptosis-inducing effects and did not induce HeLa-C3 cell death at a concentration of 100 μM. 18-Hydroxygarcimultiflorone D (**2**) was found to induce HeLa-C3 cell death before caspase-3 activation (the cell morphologies of HeLa-C3 treated with compound **2** are not shown) and did not reduce the YFP/CFP emission ratio to 3 or below within 72 h treatment at the indicated concentrations, suggesting that this compound may induce caspase-independent apoptosis in HeLa-C3 cells. Isoxanthochymol (**9**) was found to induce HeLa-C3 cell death at 50 and 100 μM without reducing the YFP/CFP emission ratio of treated HeLa-C3 cells to a value below 3 within 72 h of treatment, but did show apoptosis-inducing effects at 25 μM, which meant that this compound may induce HeLa-C3 cell death without activating caspase-3 at higher concentrations. Other compounds were found to reduce the YFP/CFP emission ratio below 3 within 72 h of treatment at 100 μM and lower concentrations, especially compounds **6** and **8**, which were shown to be strong apoptosis inducers with the YFP/CFP emission ratio below 3 within

Table 1. ^{13}C and ^{1}H NMR Data (100 and 400 MHz, methanol-d_4) for Compounds **1**−**5**

	garcimultiflorone D (**1**)		18-hydroxygarcimultiflorone D (**2**)		garcimultiflorone E (**3**)		garcimultiflorone F (**4**)		isogarcimultiflorone F (**5**)	
position	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)
1	196.0		195.8		195.2		194.3		197.6	
2	117.8		118.0		118.1		118.8		118.0	
3	194.0		193.9		193.5		196.6		196.8	
4	69.7		67.3		70.0		69.8		65.5	
5	50.2		50.0		50.1		50.9		50.3	
6	48.0	1.49, m	48.1	1.46, m	48.0	1.46, m	44.9	1.74, m	43.0	1.78, m
7	43.9	2.25, br d (13.8)	43.9	2.25, m	43.5	2.26, br d (13.9)	48.4	2.26, dd (6.2, 13.8)	42.6	2.30, br d (14.0)
8	59.8		59.0		59.0		58.9		58.0	
9	210.9		211.0		210.9		210.8		210.7	
10	195.5		195.8		195.8		196.5		196.1	
11	129.6		129.8		129.8		130.3		129.5	
12	117.4	7.18, d (2.0)	117.5	7.18, d (2.1)	117.3	7.20, d (2.0)	117.1	7.22, d (1.9)	117.3	7.16, d (2.1)
13	146.1		152.3		146.2		146.2		146.2	
14	152.4		146.0		152.3		152.3		152.5	
15	115.1	6.70, d (8.4)	115.3	6.71, d (8.3)	115.0	6.70, d (8.3)	115.0	6.68, d (8.4)	115.0	6.67, d (8.3)
16	125.1	6.99, dd (2.0, 8.4)	125.2	7.08, dd (2.1, 8.3)	125.2	7.01, dd (2.0, 8.3)	125.3	6.98, dd (1.9, 8.4)	125.3	6.95, dd (2.1, 8.3)
17	29.5	1.32, m	27.1	2.70, dd (9.1, 13.1)	27.1	2.67, dd (9.0, 13.2)	26.6	2.66, m	27.0	2.70, dd (9.1, 13.3)
18	27.1	2.70, m	73.8	3.11, d (1.4)	121.6	5.06, m	121.5	5.03, m	121.3	5.05, m
19	71.3		77.0		135.6		135.6		135.7	
20	29.2	1.15, s	25.4	1.11, s	26.4	1.70, s	26.4	1.72, s	24.6	1.70, s
21	29.1	1.13, s	25.4	1.11, s	18.3	1.69, s	18.3	1.68, s	18.3	1.65, s
22	27.4	0.99, s	27.4	0.99, s	27.3	0.98, s	27.1	1.00, s	27.3	1.03, s
23	23.2	1.15, s	23.3	1.15, s	23.1	1.13, s	23.0	1.12, s	23.1	1.14, s
24	30.3	2.12, m	30.4	1.28, m	30.3	2.07, m	35.6	1.85, m	31.9	1.50, m
25	125.6	4.08, m	125.8	4.87, m	125.7	4.88, m	82.3	3.03, dd (1.1, 10.6)	77.0	3.17, dd (1.6, 5.7)
26	133.6		133.5		133.5		73.8		73.8	
27	25.9	1.65, s	25.9	1.64, s	25.9	1.66, s	25.4	1.02, s	26.4	1.77, s
28	18.2	1.49, s	18.2	1.49, s	18.2	1.51, s	24.8	1.08, s	26.3	1.07, s
29	37.9	2.01, m	38.2	2.03, m	37.5	2.07, m	36.9	2.00, m	37.5	1.98, m
30	45.5	2.55, m	41.4	3.02, m	41.6	2.44, m	45.2	2.61, m	45.2	2.64, m
31	149.3		148.7		148.6		149.5		149.5	
32	113.4	4.47, s	114.2	4.55, br s	113.7	4.46, br s	113.0	4.45, br s	113.0	4.47, br s
33	17.8	1.58, s	18.1	1.61, s	17.9	1.63, s	18.2	1.56, s	18.3	1.58, s
34	42.8	1.15, m	36.2	1.71, m	39.5	1.62, m	33.6	2.01, m	33.4	2.02, m
35	121.4	5.05, m	121.6	5.06, m	75.2	3.86, t (7.1)	124.2	5.00, m	124.2	5.03, m
36	135.7		135.5		147.6		132.6		132.7	
37	26.4	1.74, s	26.3	1.71, s	113.1	4.79, br s	25.9	1.64, s	25.8	1.65, s
38	18.3	1.69, s	18.3	1.67, s	16.7	1.71, s	18.2	1.58, s	18.2	1.58, s

Table 2. Apoptosis-Inducing Effects of Test Compounds at 72 h[a]

	apoptotic effect at		
compound	100 μM	50 μM	25 μM
1	+	+	−
2*	−	−	−
3	+	−	−
4	+	+	−
5	+	−	−
6	+	+	+
7	+	+	−
8	+	+	+
9*	−	−	+
10	−	−	−

[a] "+" means the YFP/CFP emission ratio of compound-treated HeLa-C3 cells was below or equal to 3 at 72 h. "−" means the YFP/CFP emission ratio of compound-treated HeLa-C3 cells was above 3 at 72 h. The compounds that are labeled with "*" may induce cell death without activating caspase-3 at certain concentrations.

72 h at 25 μM. Compounds **1**, **4**, and **7** reduced the YFP/CFP emission ratio to a value below 3 within 72 h at 50 μM, while compound **3** did this at 100 μM. Compound **5** reduced the ratio to a value below 3 at 100 μM and to 3.3 at 50 μM.

To confirm the results of the in vivo apoptotic assay, we conducted a cell morphology analysis. HeLa-C3 cells were treated with compounds **6**, **8**, and **9** at 25 μM, **1**, **4**, **5**, and **7** at 50 μM, and **2** at 100 μM, for a period of three days. An anticancer drug, paclitaxel, at 500 nM was used as a positive control to treat HeLa-C3 cells. At three time points after the drug treatment (24, 48, and 72 h), the cell morphology was observed and recorded. The cells that received no drug treatment were used as negative control and had normal attached cells, while those treated with the apoptotic inducer paclitaxel shrank and detached from the culture plate. Similar cell shrinkage and detachment phenomena were also observed in HeLa-C3 cells treated with compounds **6**, **8**, and **9** at 25 μM, **1**, **4**, **5**, and **7** at 50 μM, and **3** at 100 μM (Figure 3). Since both caspase-3 activation and cell shrinkage are exclusive events only occurring in apoptotic cells, we conclude that these compounds are capable of inducing apoptotic cell death in HeLa-C3 cells.

We then determined the cytotoxicity of the five new compounds by measuring their IC_{50} values on HeLa cells. As shown in Table 3, compounds **1**, **3**, **4**, and **5** had strong HeLa cell growth inhibition effects, with IC_{50} values below 20 μM.

In summary, the results of the bioactivity assay showed that, except for compounds **2** and **10**, the compounds had mild or strong apoptotic effects against HeLa-C3 cells. Furthermore, the cytotoxicity test of the five new compounds showed that compound **1**, **3**, **4**, and **5**, which had apoptosis-inducing effects against HeLa-C3 cells, also had strong HeLa cell growth inhibition effects.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. Ultraviolet absorption spectra were recorded using a Perkin-Elmer Lambda L14 spectrometer, while infrared spectra were obtained from a Perkin-Elmer 577 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AV-400 spectrometer with TMS as internal standard. HR-MS were obtained using a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer, with an electrospray ion source (Waters, Milford, MA) connected to a lock-mass apparatus performing a real-time calibration correction. Column chromatography was performed with silica gel 60 (200−300 mesh, Merck), Sephadex LH-20 (Pharmacia), and reversed-

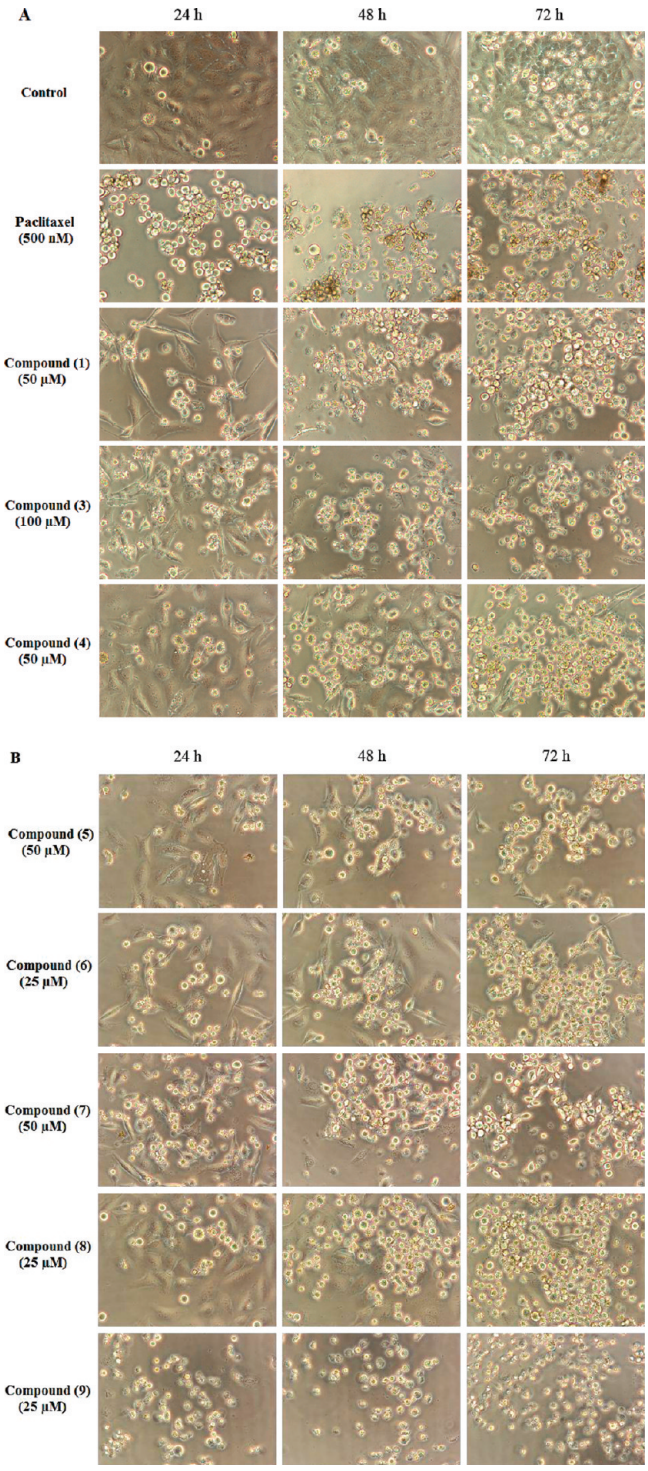

Figure 3. Cell morphological changes during the course of treatment. (A) HeLa-C3 cells were treated with compounds **1** and **4** at 50 μM and **3** at 100 μM, with an anticancer drug, pacilitaxel, at 500 nM (serving as a positive control), or without any drug (control) for 24, 48, and 72 h. (B) HeLa-C3 cells were treated with compounds **5** and **7** at 50 μM and **6**, **8**, and **9** at 25 μM for 24, 48, and 72 h.

phase C_{18} silica gel (250 mesh, Merck). Precoated TLC sheets of silica gel 60 GF^{254} were used. An Agilent 1200 Series machine equipped with Alltima C_8 columns or Zorbax SB-C_8 columns (4.6 × 250 mm 5 μm) was used for HPLC analysis, and semipreparative Alltima C_8 columns or Zorbax SB-C_8 columns (9.4 × 250 mm 5 μm) were used in sample preparation. Spots were visualized by heating the silica gel plate and spraying with 10% H_2SO_4 in EtOH.

Table 3. IC_{50} Values of Compounds **1**−**5**

compound	IC_{50} (μM)
1	17.5 ± 0.7
2	23.0 ± 2.6
3	14.3 ± 0.2
4	14.9 ± 2.6
5	12.4 ± 2.4

Plant Material. The twigs of *G. multiflora* were collected on Diaoluo Mountain, Hainan Province, People's Republic of China, in May 2006. The sample was authenticated by one of the authors (C.F.Q.), and a voucher specimen (CMED-047301) has been deposited at Hong Kong Jockey Club Institute of Chinese Medicine.

Extraction and Isolation. Air-dried and powdered twigs of *G. multiflora* (3.2 kg) were extracted with acetone (20 L, three times a day for 4 days) at room temperature. After evaporating the solvents under vacuum at 45 °C, a residue (215 g) was obtained. The crude acetone extract was subjected to a D101 (granularity: 0.25−0.84 mm) resin column and then washed with 60% and 95% EtOH, successively. The 95% EtOH fraction (46 g) was subjected to column chromatography over silica gel (200−300 mesh) and eluted with *n*-hexane−EtOAc (from 1:30 to 0:1) to give fractions A−I and **10** (4.3 g). Fractions D, E, and G were shown to possess the most potent apoptotic activities. Fraction D was subjected to reversed-phase C_{18} silica gel column chromatography and eluted in a step gradient manner with MeOH−H_2O (from 55:45 to 95:5) to give subfractions D1−D4. Fraction D2 was purified by semipreparative HPLC (MeOH−H_2O, 75:25, with 0.1% formic acid in MeOH, 3 mL/min) to give **2** (2 mg) and a mixture of **4** and **5** (12 mg). Further semipreparative HPLC was performed (MeOH−MeCN−H_2O, 50:25:25, with 0.1% formic acid in MeOH, 3 mL/min) to yield pure **4** (3 mg) and **5** (4 mg). Fraction D3 was subjected to passage over a Sephadex LH-20 column and eluted with MeOH, to yield compound **6** (113 mg) and another fraction, D5. This fraction was further separated by semipreparative HPLC (MeOH−H_2O, 75:25, with 0.1% formic acid in MeOH, 3 mL/min) to yield compound **3** (7 mg). Compound **9** (10 mg) was obtained from fraction D4 by recrystallization in MeOH. Fraction E was subjected to silica gel column chromatography and eluted with *n*-hexane−acetone (from 10:1 to 3:1) to give **7** (303 mg) and **8** (1.7 g). Fraction G was subjected to reversed-phase C_{18} silica gel column chromatography and eluted in a step gradient manner with MeOH− H_2O (from 55:45 to 95:5) to produce subfractions G1−G5. Fractions G4 and G5 were combined and subjected to passage over a Sephadex LH-20 column eluted with MeOH to yield four fractions, G6−G9. The last fraction was purified by semipreparative HPLC (MeOH−H_2O, 85:15, with 0.1% formic acid in MeOH, 3 mL/min) to yield compound **1** (22 mg).

Garcimultiflorone D (1): yellow gum; $[\alpha]_D^{20}$ −53.6 (*c* 0.48, MeOH); UV (MeOH) λ_{max} (log ε) 209 (2.30) nm; IR (KBr) ν_{max} 3627, 2931, 1716, 1685, 1600, 1446, 1375, 1118 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 1; HR-ESIMS (negative ion) *m*/*z* 619.3635 (calcd for $C_{38}H_{51}O_7$ [M − H]$^-$, 619.3635).

18-Hydroxygarcimultiflorone D (2): yellow gum; $[\alpha]_D^{20}$ −33.3 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 329 (1.51) nm; IR (KBr) ν_{max} 3551, 2341, 1733, 1716, 1683, 1473, 1456 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 1; HR-ESIMS (positive ion) *m*/*z* 637.3751 (calcd for $C_{38}H_{53}O_8$ [M + H]$^+$, 637.3740).

Garcimultiflorone E (3): yellow gum; $[\alpha]_D^{20}$ −43.6 (*c* 0.41, MeOH); UV (MeOH) λ_{max} (log ε) 203 (0.65) nm; IR (KBr) ν_{max} 3587, 3566, 2358, 1733, 1683, 1473, 1288 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 1; HR-ESIMS (positive ion) *m*/*z* 587.3733 (calcd for $C_{38}H_{51}O_5$ [M + H]$^+$, 587.3736).

Garcimultiflorone F (4): yellow gum; $[\alpha]_D^{20}$ −68.7 (*c* 0.49, MeOH); UV (MeOH) λ_{max} (log ε) 203 (0.61) nm; IR (KBr) ν_{max} 3587, 2341, 1716, 1697, 1683, 1473, 1373 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 1; HR-ESIMS (positive ion) *m*/*z* 637.3749 (calcd for $C_{38}H_{52}O_8$ [M + H]$^+$, 637.3740).

Isogarcimultiflorone F (5): yellow gum; $[\alpha]_D^{20}$ −46.0 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 204 (0.63) nm; IR (KBr) ν_{max} 3587, 2341, 1716, 1697, 1635, 1473, 1373 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 1; HR-ESIMS (positive ion) *m*/*z* 637.3733 (calcd for $C_{38}H_{52}O_8$ [M + H]$^+$, 637.3740).

Bioassays. All the test samples were dissolved in DMSO to make stock solutions. The concentration of each stock was at least 1000 times higher than the working concentration. HeLa-C3 cells, which can detect apoptotic cell death involving caspase-3 activation, were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, in a 5% CO_2 humidity incubator at 37 °C. To test compounds with apoptotic activity, 7500 HeLa-C3 cells were seeded in each well of a 96-well plate. After 12−16 h, the old medium was removed, and 100 μL of fresh medium containing the testing compound at a designated concentration was added. The plate was read by a Perkin-Elmer Victor reader with excitation wavelength at 440 ± 10 nm and emission wavelength at 486 ± 8 nm for CFP and 535 ± 8 nm for YFP at the indicated time point. The data-acquisition duration was up to 72 h. The YFP/CFP emission ratio was calculated. In addition, the cell morphological images were captured at 24, 48, and 72 h after drug treatment with a Leica DMIL HC equipped with a Leica DFC300FX camera. If the YFP/CFP emission ratio was decreased to a value of 3 or below and cell shrinkage was observed, then significant apoptotic cell death was considered to have occurred. The screening of all samples was conducted in triplicate, and all experiments were repeated three times.

The IC_{50} values of the five new compounds were measured with the MTT assay. MTT powder was dissolved in PBS at a concentration of 5 mg/mL. For the MTT assay, 10 μL of MTT solution was added into each well of a 96-well plate. After 2 h of incubation at 37 °C, 100 μL of 10% SDS solution with 0.01 M HCl was added to dissolve the purple crystals. After 24 h of incubation, the optical density (OD) readings at 595 nm were measured using a plate reader. About 2500 HeLa cells suspended in 100 μL of MEM medium were seeded respectively in a 96-well plate. After 24 h of incubation, the medium was changed by adding fresh medium containing various concentrations of each compound into the 96-well plate. The concentrations applied ranged from 100 to 1.6 μM, which was achieved by doing 2-fold dilutions six times. The OD values of the control group at 0 and 72 h together with the compound-treated groups at 72 h from the MTT assay were measured using a plate reader.

Acknowledgment. This research is funded by the Hong Kong Jockey Club Charities Trust and Nanyang Technological University, School of Chemical and Biomedical Engineering, start-up funds.

Supporting Information Available: ^{1}H, ^{13}C, and 2D NMR spectra of compounds **1**−**5** are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Wyllie, A. H.; Kerr, J. F.; Currie, A. R. *Int. Rev. Cytol.* **1980**, *68*, 251–306.
(2) Kerr, J. F.; Wyllie, A. H.; Currie, A. R. *Br. J. Cancer* **1972**, *26*, 239–257.
(3) Lowe, S. W.; Lin, A. W. *Carcinogenesis* **2000**, *21*, 485–495.
(4) Luo, K. Q.; Yu, V. C.; Pu, Y.; Chang, D. C. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 1054–1060.
(5) Samali, A.; Zhivotovsky, B.; Jones, D.; Nagata, S.; Orrenius, S. *Cell Death Differ.* **1999**, *6*, 495–496.
(6) Huang, S. X.; Feng, C.; Zhou, Y.; Xu, G.; Han, Q. B.; Qiao, C. F.; Chang, D. C.; Luo, K. Q.; Xu, H. X. *J. Nat. Prod.* **2009**, *72*, 130–135.
(7) Han, Q. B.; Zhou, Y.; Feng, C.; Xu, G.; Huang, S. X.; Li, S. L.; Qiao, C. F.; Song, J. Z.; Chang, D. C.; Luo, K. Q. *J. Chromatogr. B* **2009**, *877*, 401–407.
(8) Han, Q. B.; Yang, N. Y.; Tian, H. L.; Qiao, C. F.; Song, J. Z.; Chang, D. C.; Chen, S. L.; Luo, K. Q.; Xu, H. X. *Phytochemistry* **2008**, *69*, 2187–2192.
(9) Xu, G.; Feng, C.; Zhou, Y.; Han, Q. B.; Qiao, C. F.; Huang, S. X.; Chang, D. C.; Zhao, Q. S.; Luo, K. Q.; Xu, H. X. *J. Agric. Food Chem.* **2008**, *56*, 11144–11150.
(10) Chiang, Y. M.; Kuo, Y. H.; Oota, S.; Fukuyama, Y. *J. Nat. Prod.* **2003**, *66*, 1070–1073.
(11) Chen, J.-J.; Ting, C.-W.; Hwang, T.-L.; Chen, I.-S. *J. Nat. Prod.* **2009**, *72*, 253–258.
(12) Chien, S. C.; Chyu, C. F.; Chang, I. S.; Chiu, H. L.; Kuo, Y. H. *Tetrahedron Lett.* **2008**, *49*, 5276–5278.
(13) Lin, Y. M.; Flavin, M. T.; Cassidy, C. S.; Mar, A.; Chen, F. C. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2101–2104.
(14) Lin, Y.-M.; Anderson, H.; Flavin, M. T.; Pai, Y.-H. S.; Greenwood, E. M.; Pengsuparp, T.; Pezzuto, J. M.; Schinazi, R. F.; Hughes, S. H.; Chen, F.-C. *J. Nat. Prod.* **1997**, *60*, 884–888.
(15) Wu, J. H.; Tung, Y. T.; Chyu, C. F.; Chien, S. C.; Wang, S. Y.; Chang, S. T.; Kuo, Y. H. *J. Wood Sci.* **2008**, *54*, 383–389.
(16) Hamed, W.; Brajeul, S.; Mahuteau-Betzer, F.; Thoison, O.; Mons, S.; Delpech, B.; Van Hung, N.; Sevenet, T.; Marazano, C. *J. Nat. Prod.* **2006**, *69*, 774–777.
(17) Gustafson, K. R.; Blunt, J. W.; Munro, M. H. G.; Fuller, R. W.; McKee, T. C.; Cardellina, J. H.; McMahon, J. B.; Cragg, G. M.; Boyd, M. R. *Tetrahedron* **1992**, *48*, 10093–10102.
(18) Fuller, R. W.; Blunt, J. W.; Boswell, J. L.; Cardellina Ii, J. H.; Boyd, M. R. *J. Nat. Prod.* **1999**, *62*, 130–132.
(19) Marti, G.; Eparvier, V.; Moretti, C.; Susplugas, S.; Prado, S.; Grellier, P.; Retailleau, P.; Guéritte, F.; Litaudon, M. *Phytochemistry* **2009**, *70*, 80–90.
(20) Cuesta-Rubio, O.; Padron, A.; Castro, H. V.; Pizza, C.; Rastrelli, L. *J. Nat. Prod.* **2001**, *64*, 973–975.
(21) Jabit, M. L.; Wahyuni, F. S.; Khalid, R.; Israf, D. A.; Shaari, K.; Lajis, N. H.; Stanslas, J. *Pharm. Biol.* **2009**, *47*, 1019–1026.
(22) Tian, H.; Ip, L.; Luo, H.; Chang, D. C.; Luo, K. Q. *Br. J. Pharmacol.* **2007**, *150*, 321–334.

NP100156W