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Characterization of Proapoptotic Compounds from the Bark of *Garcinia oblongifolia*

Chao Feng,[†] Sheng-Xiong Huang,[‡] Xue-Mei Gao,[§] Hong-Xi Xu,*[,⊥,∥] and Kathy Qian Luo*[,▽]

[†]Department of Chemical and Biomolecular Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

[‡]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, People's Republic of China

[§]Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, Yunnan University of Nationalities, Kunming 650031, Yunnan, People's Republic of China

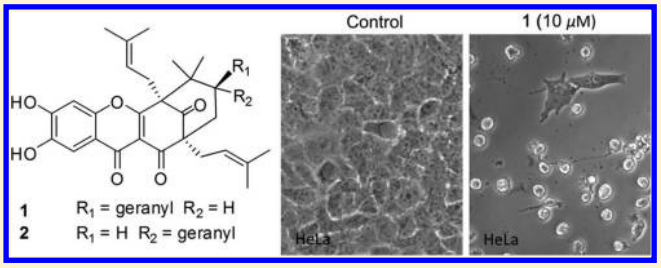
[⊥]School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, People's Republic of China

[∥]Engineering Research Center of Shanghai Colleges for TCM New Drug Discovery, Shanghai 201203, People's Republic of China

[▽]Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457

Supporting Information

ABSTRACT: Twenty compounds from *Garcinia oblongifolia* were screened for proapoptotic activity using FRET-based HeLa-C3 sensor cells. Among them, oblongifolins F and G (**1** and **2**), 1,3,5-trihydroxy-13,13-dimethyl-2*H*-pyran[7,6-*b*]-xanthone (**3**), nigrolineaxanthone T (**4**), and garcicowin B (**5**) showed significant proapoptotic activity at a concentration of 10 μM. Bioassessments were then performed to evaluate the potential of these compounds for therapeutic application. All five compounds showed significant cytotoxicity and caspase-3-activating ability in cervical cancer HeLa cells, with compounds **1** and **2** having the highest potencies. All five compounds specifically induced caspase-dependent apoptosis, which could be prevented by the pan-caspase inhibitor zVAD-fmk. In particular, **3** induced apoptosis through mitotic arrest. Compounds **1**−**5** displayed similar IC_{50} values (3.9−16.5 μM) against the three cancer cell lines HeLa, MDA-MB-435, and HepG2. In addition, compounds **1**, **2**, and **4** exhibited similar and potent IC_{50} values (2.4−5.1 μM) against several breast and colon cancer cell lines, including those overexpressing either HER2 or P-glycoprotein. HER2 and P-glycoprotein are known factors that confer resistance to anticancer drugs in cancer cells. This is the first study on the cytotoxicity, caspase-3-activing ability, and specificity of proapoptotic compounds isolated from *G. oblongifolia* in HeLa cells. The potential application of these compounds against HER2- or P-glycoprotein-overexpressing cancer cells was investigated.



Many cancer chemotherapy drugs, such as camptothecin, etoposide, paclitaxel, and vinblastine, are derived from plants, and they kill cancer cells by promoting apoptosis.[1−4] Apoptosis, or programmed cell death, is a controlled cell suicide death process that allows the human body to destroy damaged or unwanted cells in an orderly manner.[5] During apoptosis, apoptotic cells degrade their cellular components and fragment into small apoptotic bodies, which are engulfed by the surrounding cells.[6] Because apoptosis does not cause an inflammatory response in the surrounding tissues, only compounds that induce apoptosis but not necrosis have the therapeutic potential for development into useful anticancer drugs.

The challenge of identifying proapoptotic compounds from plants is that there are a large number of compounds in each organism; however, the majority of compounds do not have an apoptotic effect. Therefore, a more efficient, cell-based, high-throughput drug screening platform is necessary to guide the isolation process toward a single bioactive compound.

To find potential proapoptotic compounds from plant extracts, a sensor cell line named HeLa-C3 was developed that can express stably a fusion protein consisting of three parts, a cyan fluorescent protein (CFP), a 16-amino-acid linker containing the cleavage site of caspase-3 (DEVD), and a yellow fluorescent protein (YFP).[7] These sensor cells emit green light under normal growth conditions because of fluorescence resonance energy transfer (FRET), and these sensor cells emit blue light when caspase-3 is activated during apoptosis via cleavage of the FRET sensor linker into two parts. This color change allows for rapid identification of proapoptotic compounds using a fluorescent plate reader in a high-

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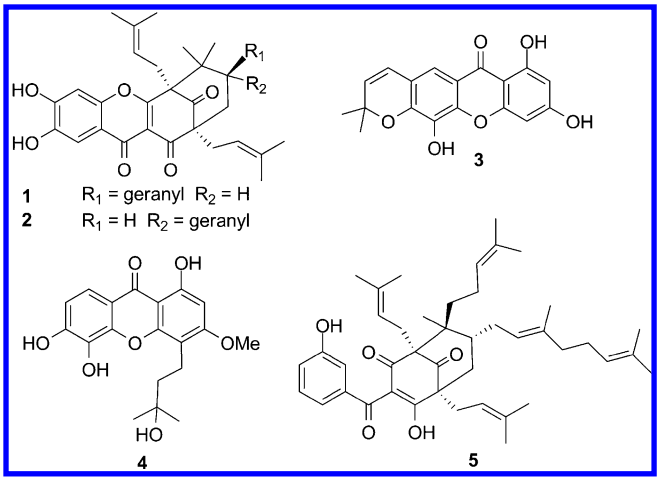
throughput manner.[8] The YFP/CFP emission ratio has been used to indicate the caspase-activating ability of test samples. In normal cells, the ratio is usually greater than 5, whereas in apoptotic cells, the ratio is less than 3.[8]

In previous studies, a number of plant samples were screened using the FRET sensor HeLa-C3 cells-based apoptotic assay. One plant tested was *Garcinia oblongifolia* Champ. ex Benth. (Clusiaceae). The bioassay-guided fractionation of *G. oblongifolia* led to the isolation and identification of six new compounds, including oblongifolins E−G, together with 12 known compounds from the bark of this plant.[9] Another two known compounds, garcicowin B[10] and oblongifolin U,[11] were also isolated.

In the present study, these 20 compounds from the bark of *G. oblongifolia* were screened using HeLa-C3 sensor cells to identify the substances with the highest proapoptotic effect. Furthermore, a series of bioassessments was performed to evaluate the therapeutic potential of the active compounds.



RESULTS AND DISCUSSION

Cytotoxicity Study of *G. oblongifolia* Compounds. Twenty compounds isolated from the bark of *G. oblongifolia* were screened for proapoptotic ability at a concentration of 10 μM using the FRET-based HeLa-C3 sensor cells. Of these compounds, oblongifolins F and G (**1** and **2**), 1,3,5-trihydroxy-13,13-dimethyl-2*H*-pyran[7,6-*b*]xanthone (**3**), nigrolineaxanthone T (**4**), and garcicowin B (**5**) decreased the YFP/CFP emission ratio to 3 within 72 h (Figure 1). By comparison, to achieve a similar reduction of the YFP/CFP emission ratio, the minimal concentration necessary for the clinical anticancer drug etoposide was 50 μM (data not shown). Because compounds **1**−**5** showed potential therapeutic activity with the FRET-based apoptotic assay, they were selected for further bioactivity analysis.

The five candidate compounds (**1**−**5**) were tested for cytotoxicity against cervical cancer HeLa cells using the MTT assay. Cell viabilities at 0 h were standardized as 100. After 72 h treatment, the cell viability of the control group increased to over 300; however, the cell viabilities of the groups treated with the five compounds at 10 μM concentration were significantly decreased to approximately 50 or lower ($p < 0.001$), which was more than six times less than the cell viability of the control group (Figure 2). Of the five candidate compounds, **1** and **2** displayed the highest potencies. The viability of cells treated with **1** or **2** was more than 30 times lower than the control cells, a cytotoxic effect similar to paclitaxel (Figure 2). These data show that the five candidate compounds effectively kill cancer cells.

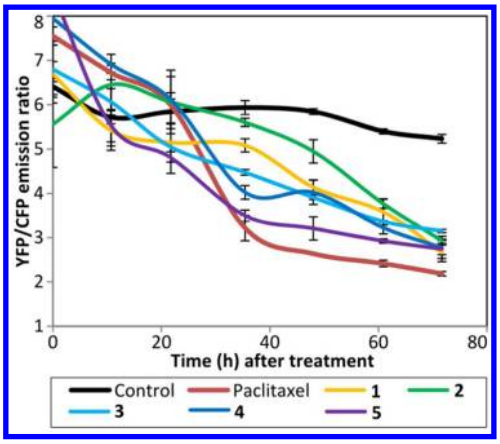


Figure 1. Screening data for proapoptotic compounds from *Garcinia oblongifolia* using HeLa-C3 sensor cells. The active compounds were defined as those that effectively induced apoptosis by decreasing the YFP/CFP emission ratio to 3 or lower within 72 h. The culture medium containing 0.1% DMSO was used as the vehicle control, whereas paclitaxel (500 nM) was used as the positive control. Twenty compounds isolated from *G. oblongifolia* were tested for their proapoptotic activity at a concentration of 10 μM. Compounds **1**−**5** were the most potent agents. The graphs of the other 15 compounds investigated with no significant proapoptotic activity are not included in this figure for clarity.

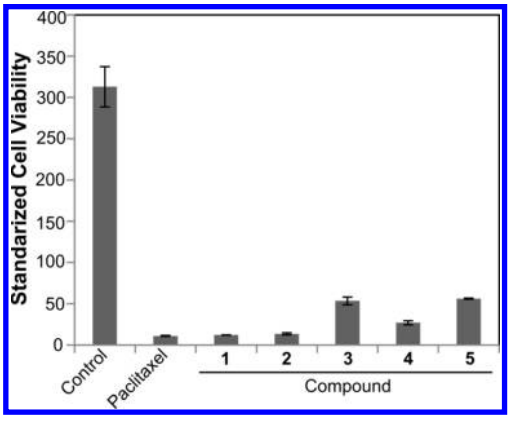


Figure 2. Cytotoxicity assay data for compounds **1**−**5**. These five compounds were tested for cytotoxicity in cervical cancer HeLa cells using the MTT assay. The active compounds decreased cell viability after treatment. Compared to the vehicle control (medium plus 0.1% DMSO), all five compounds showed significant cytotoxicity ($p < 0.001$) against HeLa cells at 10 μM after 72 h treatment. Paclitaxel (500 nM) was used as the positive control. For each sample, the cell viability at 0 h was normalized to "100".

Induction of Caspase-Dependent Apoptosis. The caspase-3-activating ability of the candidate compounds was investigated using a caspase-3 activity assay. In this assay, the fluorescence levels from the cleaved substrate of caspase-3 correlate with the caspase-3 proteolytic activity in the cells. The results indicated that the activity of caspase-3 increased significantly in HeLa cells treated with 10 μM compounds **1**−**5** for 24 h (Figure 3). The highest caspase-3 activity was detected in the cells treated with **1** or **2**, which is consistent with the cytotoxicity results measured with the MTT assay. These data suggest that compounds **1**−**5** effectively induced apoptosis in cancer cells.

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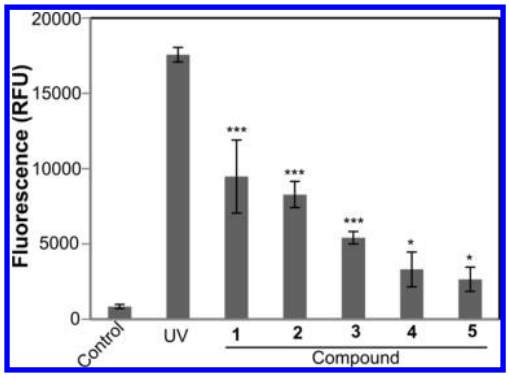

Figure 3. Caspase-3 activity assay data for compounds **1**–**5**. The caspase-3 proteolytic activity was tested in HeLa cells after 24 h treatment with each of the five compounds (10 μM). The effective compounds activated caspase-3, resulting in high fluorescence readings. Compared to the vehicle control (medium plus 0.1% DMSO), all five compounds significantly activated caspase-3 within 24 h (*p < 0.05, ***p < 0.001). Cells treated with UV for 3 min and collected after 6 h were used as the positive control.

The proapoptotic effects of the candidate compounds were validated by examining the cellular and nuclear morphology. The specificity of the tested compounds for inducing caspase-dependent apoptosis was confirmed using the pan-caspase inhibitor zVAD-fmk. As shown in Figure 4, after 72 h, the control cells grew into a confluent monolayer with good cell attachment and a normal DNA staining pattern. By contrast, the compound-treated cells showed a much lower cell density with many small cells and cells that were detached from the plate (examples are indicated by arrows in Figure 4). The minority of cells that remained on the plate displayed stressed cell morphology, and their chromosomal DNA became condensed or fragmented, which appeared as shining spots after being stained with the fluorescent DNA dye Hoechst 33342. The observed cell shrinkage, DNA condensation, and fragmentation are typical phenomena of apoptosis. However, when the cells were cotreated with the pan caspase inhibitor zVAD-fmk, the changes in the cell and nuclear morphology were greatly reduced, except that the cell density remained lower than the control cells and the cells displayed stressed morphology. These findings indicate that compounds **1**–**5** specifically induced caspase-dependent apoptosis, but not necrosis, in HeLa cells.

Compound 3 Arrests Cells in Mitosis and Then Induces Apoptosis. It was observed that the size of the nucleus, as visualized by Hoechst-stained chromosomal DNA, was larger in the cells treated with compound **3** than in the cells treated with compounds **1**, **2**, **4**, and **5** (Figure 4). The same change was also observed in the cells cotreated with **3** and z-VAD-fmk (Figure 4). This phenomenon may result from mitotic arrest, which blocks cells in the G2/M phase of the cell cycle, induced by **3**. Certain anticancer drugs, such as paclitaxel

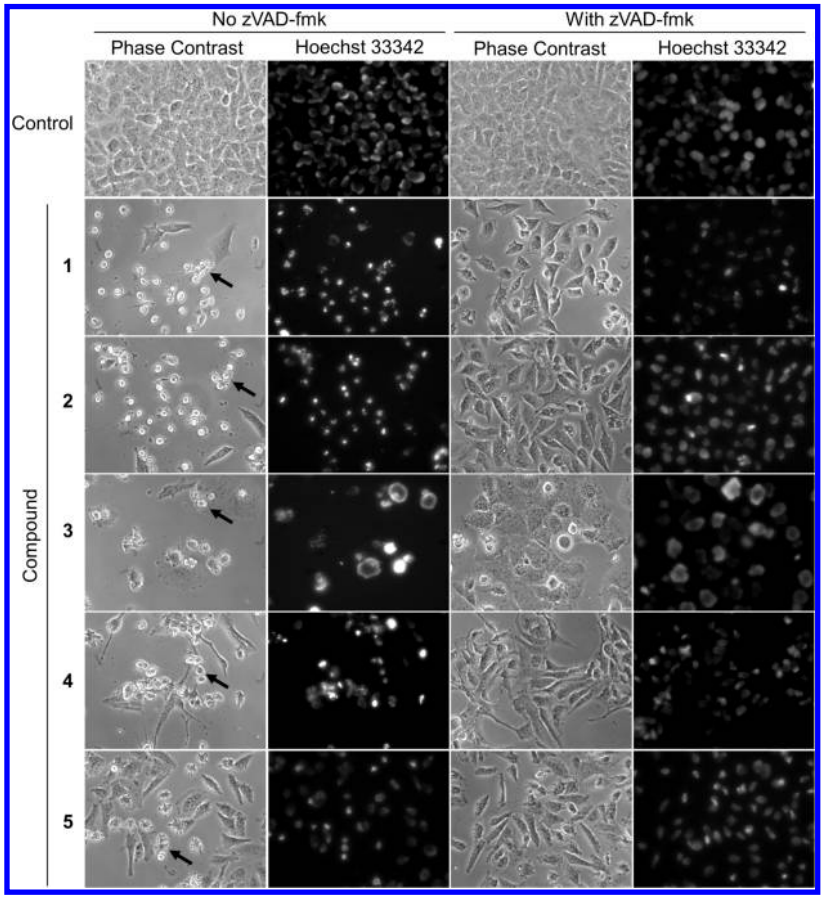

Figure 4. The pan-caspase inhibitor zVAD-fmk prevented apoptosis induced by compounds **1**–**5**. HeLa cells were treated with compound (10 μM) only or compound plus zVAD-fmk (50 μM) for 72 h. The cells treated with medium (containing 0.1% DMSO) only or medium plus zVAD-fmk were used as the controls. The cells with shrunken morphology and condensed or fragmented DNA as visualized by Hoechst 33342 were considered apoptotic cells, and the examples are indicated by black arrows.

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and the vinca alkaloids, cause mitotic arrest. These drugs interfere with tubulin dynamics during mitosis, resulting in mitotic arrest and subsequent apoptotic cell death.[12−14] It is possible that **3** acts in a similar manner.

To test this hypothesis, the initial cell morphology changes after treatment with **3** were examined. From Figure 5, it is

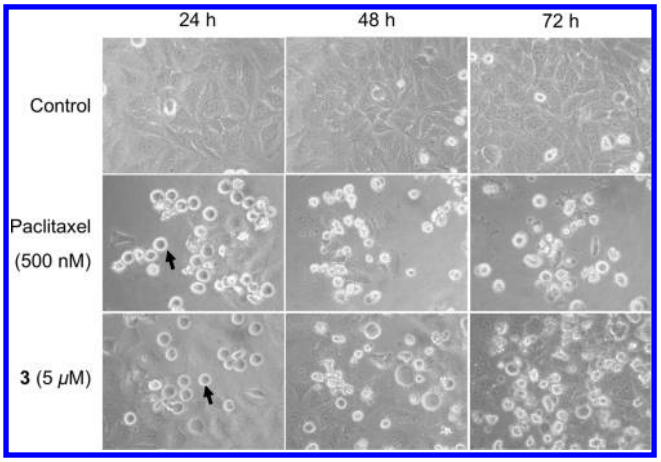

Figure 5. HeLa cells treated with compound **3** show a rounded morphology prior to apoptosis. HeLa cells were treated with **3** (5 μM) and imaged at 24, 48 and 72 h. Medium (containing 0.1% DMSO) only was used as the vehicle control. Paclitaxel (500 nM) was used as the positive control. Examples of the rounded cells after treatment with paclitaxel or **3** are indicated by the black arrows.

evident that **3**-treated HeLa cells showed a rounded morphology before apoptosis, a phenomenon also observed in paclitaxel-treated cells. This indicated that **3** may induce mitotic arrest in a manner similar to paclitaxel in HeLa cells.

Second, flow cytometry was used to analyze the cell cycle of cells treated with **3** or **3** plus the pan-caspase inhibitor zVAD-fmk. As shown in Figure 6, the majority of the control cells were in the G0/G1 phase, whereas a small portion of the control cells were in the G2/M phase (29.2%), and a few control cells were either in the sub-G1 phase or undergoing apoptosis (0.9%). However, after treatment with **3** for 24 h, more cells (51.6%) were in the G2/M phase, and the portion of apoptotic cells also increased from 0.9% to 12.7%. The percentage of apoptotic cells also increased to 51.8% and 53.0% after cells were treated with **3** for 48 and 72 h, respectively. Furthermore, when the cells were cotreated with **3** and the pan-caspase inhibitor zVAD-fmk for 24, 48, and 72 h, the majorities of cells (85.2%, 79.1%, and 72.4%) were in the G2/M phase of the cell cycle and were not undergoing apoptosis. This showed that compound **3** induced apoptosis through mitotic arrest in HeLa cells.

Comparison of Growth Inhibitory Effects of Compounds 1−5. The concentration required for inhibiting 50% of cell growth (IC_{50}) of compounds **1**−**5** was measured in three representative cancer cell lines, HeLa (cervical cancer), MDA-MB-435 (melanoma), and HepG2 (liver cancer). As shown in Table 1, the IC_{50} values of these compounds against these three types of cancer cells ranged from 3.9 to 16.5 μM. Compounds **1** and **2** exhibited the highest potency against HeLa cells with IC_{50} values of 3.9−4.2 μM. Compound **3** had the lowest IC_{50} value of 3.9 μM in MDA-MB-435 cells. Compound **4** was equally effective against HeLa (IC_{50} 4.7 μM) and HepG2 cells (IC_{50} 4.8 μM), and compound **5** was more potent against

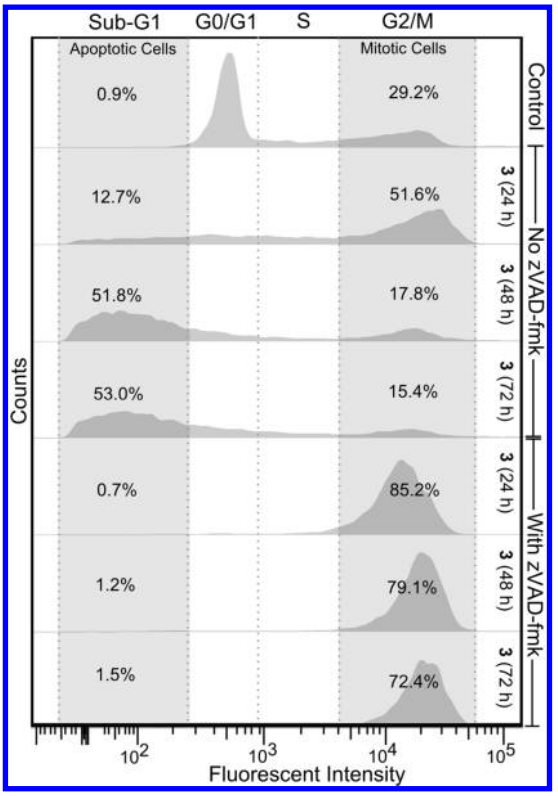

Figure 6. Compound **3** induces apoptosis through mitotic arrest. HeLa cells were treated with **3** (20 μM) only or **3** plus the pan-caspase inhibitor zVAD-fmk (20 μM). Cells treated with medium (containing 0.1% DMSO) only were used as control. The cells were collected, stained with propidium iodide (PI), and analyzed for DNA content by flow cytometry. The cells were grouped into different phases (sub-G1, G0/G1, S, and G2/M) of the cell cycle by arbitrary cutoffs of the fluorescence intensity (dotted lines). The percentages of apoptotic (sub-G1) and mitotic (G2/M) cells of each sample were counted by arbitrary gates and are shown in the shaded areas, respectively.

Table 1. IC_{50} Values of Compounds 1−5 against Three Representative Cancer Cell Lines

	IC_{50} (μM)[a]		
compound	HeLa	MDA-MB-435	HepG2
1	4.2 ± 0.6	16.5 ± 0.7	7.7 ± 0.2
2	3.9 ± 0.5	16.5 ± 0.8	7.7 ± 0.2
3	4.9 ± 0.6	3.9 ± 1.8	10.1 ± 9.8
4	4.7 ± 2.6	9.0 ± 0.5	4.8 ± 0.5
5	10.8 ± 0.6	12.6 ± 6.5	6.2 ± 0.2

[a]Mean ± SD (n = 3).

HepG2 cells (IC_{50} 6.2 μM). The IC_{50} data suggest that compounds **1**−**5** have both a broad spectrum and selectivity against certain types of cancer cells.

Compounds 1, 2, and 4 May Treat Drug-Resistant Cancer Cells. Human epidermal growth factor receptor 2 (HER2) and P-glycoprotein are factors that confer resistance to chemotherapeutic agents in cancer cells.[15,16] Therefore, clinically used anticancer drugs with the potential to treat HER2- and P-glycoprotein-overexpressing carcinomas have high therapeutic value. To explore the potential of the candidate compounds against HER2- and P-glycoprotein-overexpressing cancer cells, their IC_{50} values were measured in four different cancer cell lines, MCF7, MCF7-HER2, HCT

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Table 2. IC_{50} Values of Compounds 1−5 against Four Cell Lines, Including HER2- and P-Glycoprotein-Overexpressing Cells

	IC_{50} (μM)[a]			IC_{50} (μM)		
compound	MCF7	MCF7-HER2[b]	*t*-test[d]	HCT 116	HCT-15[c]	*t*-test[e]
1	3.0 ± 0.7	5.1 ± 3.6	–[f]	2.9 ± 1.2	4.9 ± 1.2	–
2	2.6 ± 0.5	2.9 ± 1.6	–	2.9 ± 1.5	4.0 ± 0.7	–
3	11.7 ± 4.2	34.4 ± 16.9	–	4.7 ± 3.4	5.1 ± 2.0	–
4	3.0 ± 0.4	3.8 ± 2.0	–	3.7 ± 0.8	2.4 ± 0.4	–
5	10.8 ± 0.8	19.1 ± 7.3	–	6.0 ± 1.5	9.6 ± 0.1	$p < 0.05$

[a]Mean ± SD (n = 3). [b]MCF7-HER2, a HER2-overexpressing breast cancer cell line, was compared to MCF7. [c]HCT-15, a P-glycoprotein-overexpressing colon cancer cell line, was compared to HCT 116. [d]*t*-test between the IC_{50} values for the MCF7 and MCF7-HER2 cells for the indicated compound. [e]*t*-test between the IC_{50} values for the HCT 116 and HCT-15 cells for the indicated compound. [f]"–" indicates that $p > 0.05$.

116, and HCT-15. MCF7 is a breast cancer cell line with no expression of HER2, whereas MCF7-HER2 is derived from MCF7 with medium to high exogenous expression of HER2.[17,18] HCT 116 and HCT-15 are colon cancer cell lines. HCT 116 expresses low levels of multidrug-resistant *mdr*-1/P-glycoprotein, whereas HCT-15 is one of two cell lines with the highest level of expression of *mdr*-1/P-glycoprotein of the 60 cell lines utilized by the U.S. National Cancer Institute's Anticancer Drug Screen.[19] Previous studies from our laboratory showed that MCF7-HER2 cells are resistant to etoposide, HCT-15 cells are resistant to paclitaxel, and MCF7-HER2 and HCT-15 cells are both resistant to vinblastine.[20] By comparison, the IC_{50} values of compounds **1**−**5** in the four cell lines were all in the micromolar range (Table 2), similar to etoposide.[20] The MCF7-HER2 cells also showed no significant resistance to any of the five compounds, whereas the HCT-15 cells showed resistance to **5** only. In particular, compounds **1**, **2**, and **4** displayed similar and potent IC_{50} values (2.4−5.1 μM) against all four cell lines (Table 2), indicating their potential use for treating HER2- and P-glycoprotein-overexpressing cancers.

EXPERIMENTAL SECTION

General Experimental Procedures. The procedures for fractionating and isolating the proapoptotic compounds from the bark of *Garcinia oblongifolia* are described in the Supporting Information. The purity of the compounds used in this study was confirmed as at least 95% by HPLC and NMR spectroscopy. For biological tests, the optical density (OD) and fluorescent readings were obtained using a PerkinElmer Victor 3 plate reader. The phase contrast and fluorescence images were taken with a Nikon Eclipse TE200-U inverted fluorescence microscope equipped with a SPOT 12.0 Monochrome w/o IR-18 camera. The results are shown as either representative examples or mean values (with standard deviation) from three independent experiments. A two-tailed Student's *t*-test was used to compare the different groups. Two groups with $p < 0.05$ were considered significantly different.

Cell Culture. The HeLa, HepG2, MCF7, and HCT 116 cell lines were obtained from the Biology Department of the Hong Kong University of Science and Technology. The HeLa-C3 stable cell line was generated from HeLa cells.[7,8] The MDA-MB-435 and MCF7-HER2 cell lines were kindly provided by Dr. Xiao-Feng Le from the Department of Experimental Therapeutics, University of Texas MD Anderson Cancer Center, Houston, TX, USA. The HCT-15 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) (ATCC CCL-225). The HeLa cells were cultured in minimum essential medium (MEM). The HeLa-C3 cells were grown in MEM with 500 μg/mL Geneticin. The HepG2 and MCF7 cells were cultured in MEM with 1 mM sodium pyruvate. The MDA-MB-435 cells were grown in DMEM. The MCF7-HER2 cells were cultured in DMEM with 500 μg/mL Geneticin. The HCT-15 cells were grown in RPMI 1640, and HCT 116 cells were cultured in McCoy's 5A modified medium. All culture media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured in a 5% CO_2 humidity incubator at 37 °C. The culture media, FBS, antibiotics, and Geneticin were obtained from Invitrogen (Carlsbad, CA, USA).

Screening for Proapoptotic Compounds. The screening study was performed as previously described.[9] Briefly, each dried sample was dissolved in DMSO as a stock solution. The stock concentration was at least 1000 times higher than the working concentration. Before testing a sample for proapoptotic activity, 7500 HeLa-C3 cells in 100 μL of culture medium were seeded into a well in a 96-well plate. After 12−16 h of incubation, the plate was measured with a plate reader to obtain the emission fluorescence intensity of the YFP and CFP. The excitation wavelength was 440 ± 10 nm, and the emission wavelength was 486 ± 8 nm for CFP and 535 ± 8 nm for YFP. Another well of the plate with culture medium only (100 μL) was treated and measured in the same manner to obtain the background readings. After subtracting the background readings, the net fluorescence intensity readings were used as the initial time point 0 h. Next, the old medium was removed, and freshly prepared culture medium (100 μL) containing the test sample was added. Culture medium containing 0.1% DMSO was used as the vehicle control, whereas paclitaxel (500 nM) was used as the positive control. Next, the plate was read repeatedly at the time points indicated. The data acquisition period was up to 72 h, and the YFP/CFP emission ratio was then calculated. If the YFP/CFP emission ratio decreased to below 3, this suggested that a significant level of apoptotic cell death had occurred.[8]

MTT Assay. Cells were seeded into a 96-well plate and treated under various sampling conditions as described above. For the viability assay, MTT powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS at a concentration of 5 mg/mL, and 10 μL of MTT solution was added to each well. After 2 h incubation at 37 °C, 100 μL of 10% SDS solution with 0.01 M HCl was added to each well to dissolve the purple crystals produced by the viable cells. After 24 h of incubation at 37 °C, the OD readings at 595 nm were measured with a plate reader.

Caspase-3 Activity Assay. Cells were treated under different conditions, collected, and lysed in lysis buffer [20 mM Tris-HCl, pH 7.5, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 130 mM NaCl, 10 mM sodium pyrophosphate, 1% Triton X-100, and the following protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA): 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL PMSF, and 2 mM EDTA] for 15 min at 4 °C. The cell lysate was centrifuged at 13 000 rpm for 15 min at 4 °C. The supernatant was collected, and the protein concentration was determined using the Bradford method with a Bio-Rad protein assay kit. The protein concentration of the supernatant was then adjusted to 5 μg/μL with lysis buffer, and the caspase-3 activity assay was performed with a 96-well plate. For each assay, 10 μL of supernatant containing 50 μg of soluble protein (10 μL of lysis buffer without protein was tested in parallel to measure the background reading) was combined with 89 μL of assay buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) and 1 μL of the fluorometric caspase-3 substrate (Ac-DEVD-AMC, 1 μg/μL, BD Biosciences, San Diego, CA, USA). The mixture was incubated at 37 °C in the dark for 1 h. The fluorescence intensity was measured using a plate reader with an excitation wavelength of 355 nm and an emission wavelength of 460

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nm. The caspase-3 proteolytic activity was determined as the net reading of fluorescence per microgram (μg) of protein.

Fluorescence Microscopy. Cells were treated with a test compound only or a test compound plus pan-caspase inhibitor zVAD-fmk (Promega #G7232, Madison, WI, USA) for 72 h. The cells were then stained with the fluorescent DNA dye Hoechst 33342 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Next, the cells were washed three times with PBS and imaged for both phase contrast and fluorescence to visualize the DNA.

Cell Cycle Analysis. The cells were treated under various conditions, trypsinized, collected, and rinsed with ice-cold PBS. The cells were then fixed in 70% ethanol (−20 °C, overnight), washed with ice-cold PBS, and treated with 200 μL of RNase (1 mg/mL, 37 °C, 30−60 min). Next, 800 μL of propidium iodide (PI) staining buffer (PBS containing 0.1 mg/mL PI and 1% Triton X-100) was added to stain the cells in the dark (4 °C, 30 min). The stained cells were analyzed using a flow cytometer (BD FACSVantage SE).

IC_{50} Determinations. The IC_{50} values were determined using the method described in the literature with some modifications.[18,20,21] To test the IC_{50} value of a compound against a cell line, the growth inhibition effect of this compound at different concentrations was first examined. The compound was dissolved in DMSO as a stock solution, and its concentration was at least 1000-fold higher than the concentration of the working solution. The working solution was further diluted into a series of 3-fold dilutions with at least seven concentrations tested for each compound. To test the growth inhibition effect at a certain concentration, 3000 cells in 100 μL of culture medium were seeded into the well of a 96-well plate. Another well containing culture medium only (100 μL) was treated in parallel to obtain a background reading. After an overnight culture, the old medium was replaced with 100 μL of fresh medium containing the test compound at a particular concentration. The plate was further incubated, and cell viability was later measured using the MTT assay. From the MTT assay, the OD values of the control group (medium containing 0.1% DMSO) at 0 h (C_0) and 72 h (C_{72}) together with the compound-treated group at 72 h (T_{72}) were obtained. The inhibition rates at different compound concentrations were calculated as $1 - [(T_{72} - C_0)/(C_{72} - C_0)]$ and plotted against logarithmic concentrations for a sigmoidal fit. The IC_{50} is defined as the concentration of a compound inhibiting 50% of cell growth. At the IC_{50}, $1 - [(T_{72} - C_0)/(C_{72} - C_0)] = 50\%$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The fractionation and isolation procedures for the proapoptotic compounds from the bark of *G. oblongifolia* are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(H.-X. Xu) Tel: +86-21-51323089. Fax: +86-21-51323089. E-mail: xuhongxi88@gmail.com.
*(K. Q. Luo) Tel: +65-67904257. Fax: +65-67911761. E-mail: kluo@ntu.edu.sg.

Notes

The authors declare no competing financial interest.

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