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Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay

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Abstract—Gambogic acid (**2**), a natural product isolated from the resin of *Garcinia hurburyi* tree, was discovered to be a potent apoptosis inducer using our cell- and caspase-based high-throughput screening assays. Gambogic acid was found to have an EC_{50} of 0.78 μM in the caspase activation assay in T47D breast cancer cells. The apoptosis-inducing activity of gambogic acid was further characterized by a nuclear fragmentation assay and flow cytometry analysis in human breast tumor cells T47D. Gambogic acid was found to induce apoptosis independent of cell cycle, which is different from paclitaxel that arrests cells in the G2/M phase. To understand the structure–activity relationship (SAR) of gambogic acid, derivatives of **2** with modifications to different function groups were prepared. SAR studies of gambogic acid, as measured by the caspase activation assay, showed that the 9,10 carbon–carbon double bond of the α,β-unsaturated ketone is important for biological activity, while the 6-hydroxy and 30-carboxy group can tolerate a variety of modifications. The importance of the 9,10 carbon–carbon double bond was confirmed by the traditional growth inhibition assay. The high potency of **2** as an inducer of apoptosis, its novel mechanism of action, easy isolation and abundant supply, as well as the fact that it is amenable to chemical modification, makes gambogic acid an attractive molecule for the development of anticancer agents.

1. Introduction

Programmed cell death, or apoptosis, plays important roles in normal cell development and tissue homeostasis.[1,2] Apoptosis can be activated by two major pathways: the extrinsic pathway, in which specific cell death receptors located on the cell surface membrane are activated by specific ligands; and the intrinsic pathway where mitochondria are primarily involved.[3] Caspases play a crucial role in the execution of apoptosis with caspase 3 as the key enzyme in both the extrinsic and intrinsic pathways.[4–6] It has been found that many cancer cells, while containing caspases, lack part of the molecular machinery that activate the caspase cascade, which results in a loss of the capacity to undergo apoptosis.[7]

Many current clinically used anticancer drugs are known to kill tumor cells through the induction of apoptosis. Therefore, the discovery and development of apoptosis inducers could lead to new anticancer agents.[8] For example, it has been reported recently that retinoids can induce apoptosis and inhibit cell proliferation,[9,10] and several retinoids are currently being evaluated as preventive or therapeutic agents in clinical studies. In addition, compounds that kill tumor cells by inducing apoptosis may overcome tumor resistance to conventional anti-cancer agents. For example, *trans*-[$PtCl_2$(*n*,*n*-dimethylamine)(isopropylamine) has been found to have high cytotoxic activity for cisplatin-resistant cell lines and to induce apoptosis in these cells.[11] Several natural products also have been reported recently to induce apoptosis in cancer cells, including chloptosin from the culture broth of *Streptomyces*,[12] apoptolidin from *Nocardiopsis* sp,[13] and a streptonigrin derivative from a *Micromonospora* strain.[14]

In our effort to discover and develop novel apoptosis inducers as potential anti-cancer drugs, we have created a cell-based high-throughput screening (HTS) assay for the identification of apoptosis inducers using a novel fluorescent caspase 3 substrate.[15] The screening technology was designed for the discovery of compounds that activate caspase 3 through any apoptotic mechanism. Recently, we reported the discovery and SAR studies of substituted *N*-phenyl nicotinamides, exemplified

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by 6-methyl-*N*-(4-ethoxy-2-nitro-phenyl)-pyridine-3-carboxamide (**1**), as potent inducers of apoptosis using our HTS assay.[16] Applying the same assay, we have identified gambogic acid (**2**) (Chart 1) as a novel apoptosis inducer from a commercial library.

Gambogic acid is a natural product isolated from the gamboge resin of *Garcinia hanburyi* tree in Southeast Asia. The resin is used as a traditional medicine as well as a color material for painting. The structure of gambogic acid was elucidated mainly by detailed NMR spectrum analysis[17] and was confirmed recently by X-ray crystallographic analysis[18] (Fig. 1). It contains a unique 4-oxatricyclo[4.3.1.0]decan-2-one ring system which is found in the natural products isolated from plants in the genus *Garcinia*.[19] The first total synthesis of a compound containing this system, 1-*O*-methylforbesione (Chart 1), has been reported recently.[20] Gambogic acid and several related compounds have been reported previously to have cytotoxic activities.[21] In addition, gaudichaudione A, a structurally related cytotoxic xanthone (Chart 1), has been reported recently to induce apoptosis in Jurkat cells through mitochondrial destabilization and caspase-3 activation.[22] Herein, we report the discovery and characterization of gambogic acid as a potent apoptosis inducer in several cancer cell lines, as well as the SAR studies of gambogic acid as an inducer of apoptosis.

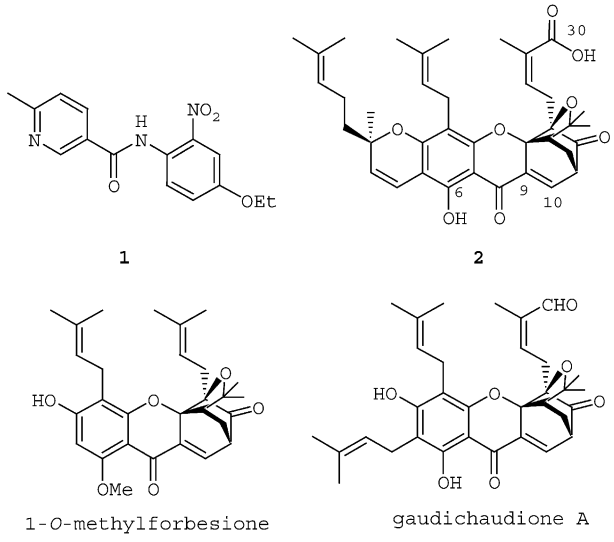


Chart 1.

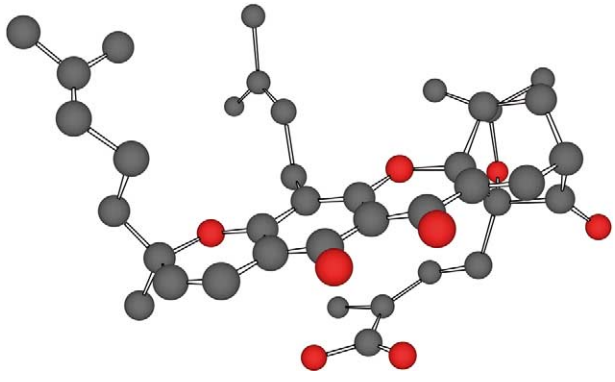


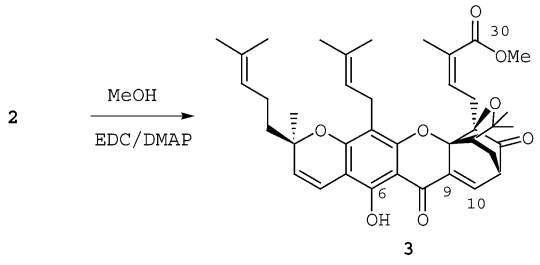
Figure 1. X-ray crystallographic structure of gambogic acid in ORTEP representation excluded the hydrogen.[18]

2. Results and discussion

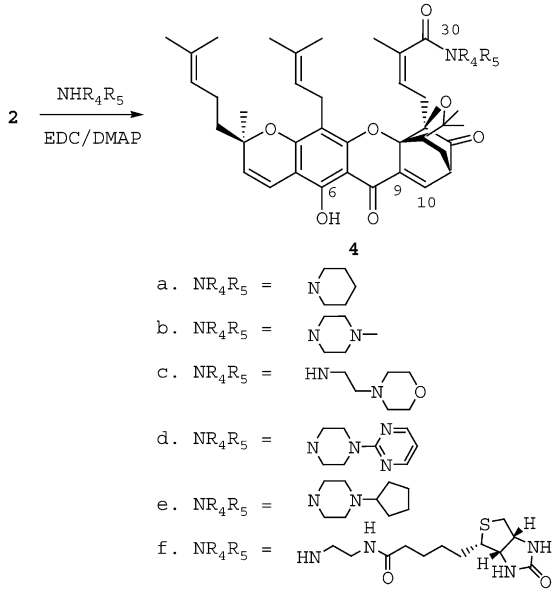
2.1. Chemistry

Gambogic acid (**2**) was isolated in overall yield of approximately 5% from the easily and widely available gamboge resin. It was purified by converting the crude extract from the gamboge resin into pyridine salt, followed by recrystallization.[17] The structure of gambogic acid was confirmed by X-ray crystallographic analysis and is shown in Figure 1.[18] There are many functional groups in the structure of **2** which potentially could be modified. To understand the basic SAR of gambogic acid as an inducer of apoptosis, we elected to modify the 30-carboxy, 6-hydroxy, and 9,10-double bond in the α,β-unsaturated ketone.

The modification of 30-carboxy is depicted in Schemes 1 and 2. Reaction of **2** with methanol in the presence of DMAP and EDC produced the methyl ester of gambogic acid (**3**) in 95% yield (Scheme 1). Coupling of **2** with various amines in the presence of DMAP and EDC, produced the corresponding amides of gambogic acid (**4**) (Scheme 2).



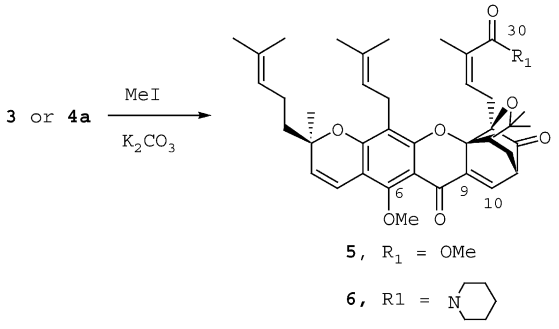
Scheme 1.



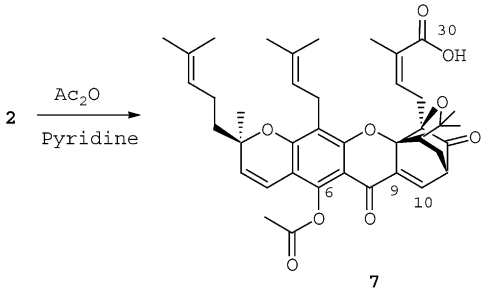
Scheme 2.

The 6-hydroxy group was modified as shown in Schemes 3 and 4. Reaction of methyl gambogate (**3**) with methyl iodide in the presence of K_2CO_3, produced the 6-*O*-methyl derivative of methyl gambogate (**5**) (Scheme 3). Similarly, reaction of compound **4a** with methyl iodide produced the 6-*O*-methyl derivative **6**. Reaction of **2** with acetic anhydride in pyridine produced 6-*O*-acetyl derivative of gambogic acid (**7**) (Scheme 4).

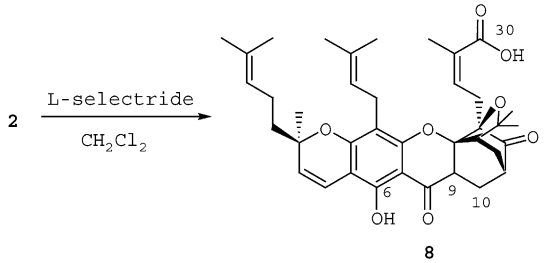
The 9,10 carbon–carbon double bond of the α,β-unsaturated ketone in gambogic acid was modified as shown in Schemes 5 and 6. Using L-selectride,[23] the 9,10-double bond in **2** was selectively reduced to produce 9,10-dihydro-gambogic acid (**8**) (Scheme 5). The proton in position 10 of **2** has a chemical shift of 7.43 ppm, which is distinct from all the other vinyl protons, and reduction of the 9,10-double bond resulted in the selective disappearance of this signal. Reaction of **2** with cyclohexyl cuprate, which was prepared from cyclohexyl magnesium chloride and CuI,[24] resulted in the addition of a cyclohexyl group to the 10-position to produced compound **9**.



Scheme 3.



Scheme 4.



Scheme 5.

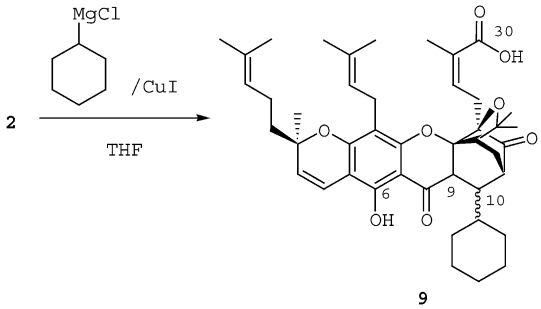
2.2. HTS assay

Gambogic acid was identified as an inducer of apoptosis from a commercial compound library using our cell- and caspase 3-based HTS assays as described previously.[16] Briefly, 10 μM of test compounds were incubated with T47D cells in a 96-well microtiter plate for 24 h to induce apoptosis; then *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110 fluorogenic substrate[25] was added and the cells were incubated for 3 h. The fluorescence was measured using a fluorescent plate reader, employing excitation at 485 nm and emission at 525 nm, and the amount of caspase activation was determined. Compounds that induce apoptosis and activate the caspases will produce a fluorescent signal that is higher than the background in untreated cells (signal/background ratio). Compounds found to give a ratio of >3 are considered active and retested in triplicate for confirmation. Compounds confirmed to be active are then tested at several concentrations to provide a dose response and the caspase activation activity (EC_{50}, concentration of compound at which the ratio of caspase activity is 50% of maximum) calculated. Gambogic acid was found to induce apoptosis and activate caspases in T47D cells with a ratio of approximately 14 versus untreated cells. This level of activation is one of the highest observed in our assay, indicating that gambogic acid is highly efficacious in activating caspases in cells. In comparison, *N*-phenyl nicotinamides, another series of apoptosis inducer identified using the same assay,[16] has a ratio of around 6. Gambogic acid was found to have an EC_{50} value of 0.78 μM in T47D cells.

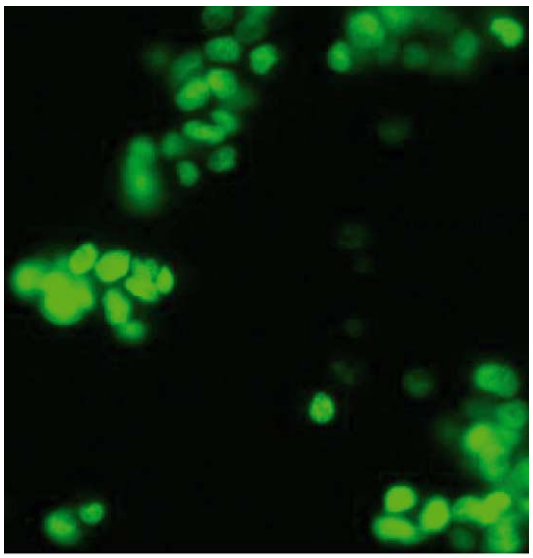
2.3. Characterization of gambogic acid

The ability of gambogic acid to induce apoptosis was further characterized in two cell-based apoptosis assays. Nuclear fragmentation is one of the hallmarks of caspase-mediated apoptosis.[26] T47D cells treated with 10 μM of gambogic acid for 24 h followed by staining with Syto16, a fluorescent DNA dye, were found to have condensed and fragmented nuclei (Fig. 2b). In contrast, cells treated with solvent (DMSO) showed normal nuclei (Fig. 2a). These results showed that gambogic acid induced nuclear fragmentation, a key marker of cellular apoptosis.

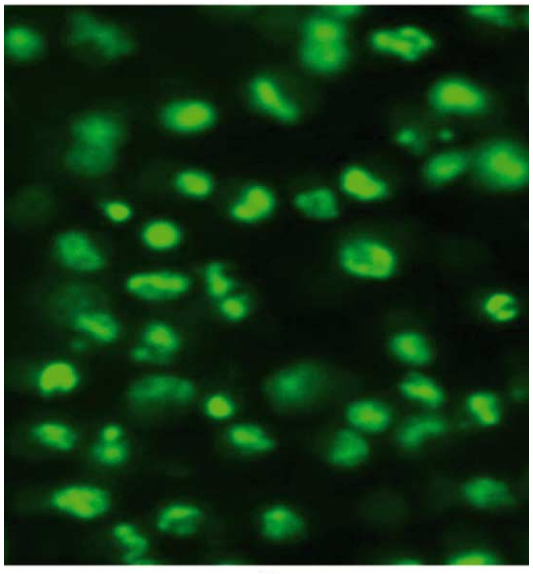
The apoptosis-inducing activities of gambogic acid were also characterized by flow cytometry. T47D cells were treated with 10 μM of gambogic acid for 24 h, then



Scheme 6.



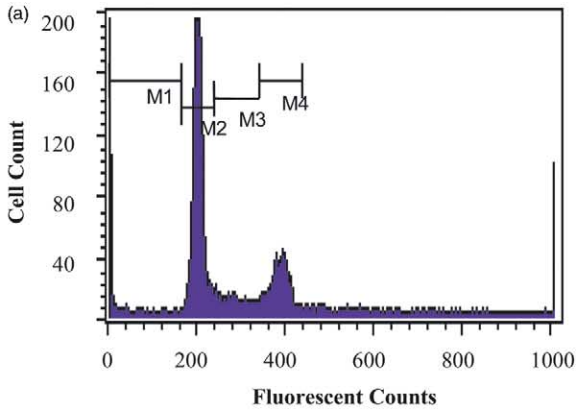
(a)

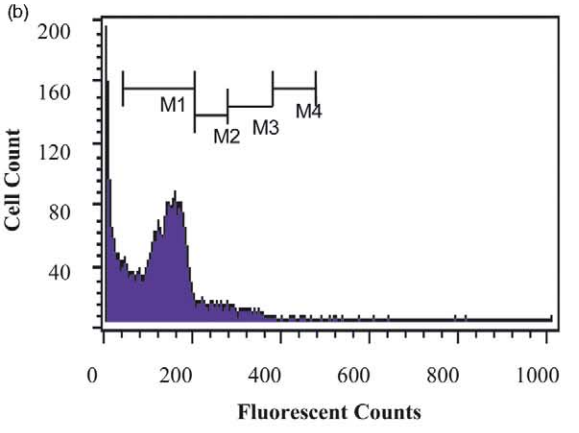


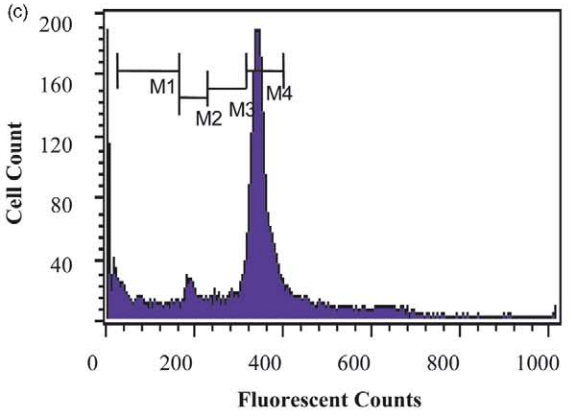
(b)

Figure 2. (a) and (b) depict fluorescent micrographs of T47D cells treated with gambogic acid and stained with a fluorescent DNA probe, Syto16. (a) depicts control cells. (b) depicts cells treated with 10 μM of gambogic acid for 24 h, showing shrunken and fragmented nuclei.

stained with propidium iodide, and analyzed by flow cytometry. Figure 3A shows that control cells are mostly in the G_1 phase. Cells treated with gambogic acid for 24 h, however, showed that most of the cells have sub-diploid DNA contents and are apoptotic (Fig. 3B). In comparison, cell treated with 100 nM of paclitaxel, a well known microtubule stabilizing molecule, for 24 h showed that few cells are in the G1 phase, most of the cells are in the G2/M phase, as well as some cells have sub-diploid DNA contents which are apoptotic (Fig. 3C). These results suggest that, in contrast to paclitaxel, apoptosis induced by gambogic acid is not preceded by arrest in any specific phase of the cell cycle.



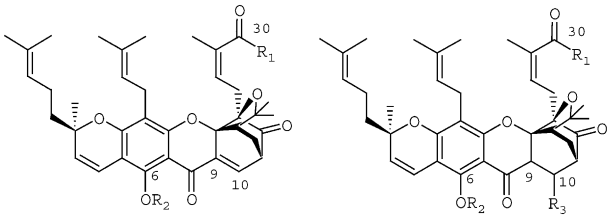


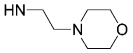
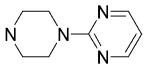
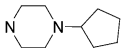
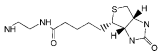


	M1	M2	M3	M4
3A	2.5	45.3	6.3	15.5
3B	85.6	11.3	3.2	0.3
3C	9.9	5.7	5.2	48.5

Figure 3. (a)–(c) depict drug-induced apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (a) Control cells showing most of the cells in G1 (M2). (b) Cells treated with 10 μM of gambogic acid for 24 h showing most of the cells have sub-diploid DNA content (M1). (c) Cells treated with 100 nM of paclitaxel for 24 h showing a reduction in the G1 (M2), an increase in the G2/M (M4) and cells with sub-diploid DNA content (M1).

Table 1. SAR of gambogic acid and derivatives in caspase activation assay



Compd	R_1	R_2	R_3	EC50 (μM)[a]		
				T47D	ZR751	DLD-1
2	OH	H	NA	0.78±0.02	1.64±0.04	0.89±0.01
3	OMe	H	NA	0.44±0.01	1.29±0.07	0.64±0.02
4a		H	NA	0.21±0.01	0.78±0.01	ND
4b		H	NA	1.39±0.15	3.85±0.56	2.70±0.01
4c		H	NA	0.68±0.05	1.76±0.15	1.39±0.03
4d		H	NA	0.24±0.01	0.78±0.05	0.51±0.01
4e		H	NA	0.38±0.01	1.24±0.04	0.67±0.02
4f		H	NA	0.56±0.03	1.14±0.04	1.86±0.05
5	OMe	Me	NA	0.80±0.42	2.31±1.00	1.37±0.03
6		Me	NA	0.42±0.09	1.01±0.14	0.43±0.02
7	OH	Ac	NA	0.51±0.05	1.39±0.03	1.00±0.01
8	OH	H	H	>10	>10	>10
9	OH	H		>10	>10	>10

NA, not applied; ND, not determined.
[a] Data are the mean of three or more experiments and are reported as mean±standard error of the mean (SEM).

2.4. Structure–activity relationship

The derivatives of gambogic acid were tested by the HTS caspase activation assay using three tumor cell lines, T47D, ZR751 and DLD-1. Table 1 summarizes the EC_{50} data of these compounds.

Gambogic acid was found to activate caspases and induce apoptosis with an EC_{50} value of 0.78, 1.64 and 0.89 μM in T47D, ZR751 and DLD-1 cell lines, respectively. The carboxy group was found to tolerate many modifications. The acidic carboxy group can be converted into a neutral group such as ester **3** and amide **4a**, or a basic group such as amide **4b**, without much effect on apoptosis inducing activity. The basic groups in amide **4b**–**4e** were designed to improve the solubility of the compounds in acidic media. It is interesting to note that relatively large modifications of the carboxy group, such as in compounds **4c**–**4f**, are well tolerated, indicating that the area around the carboxy may not be involved in the binding of gambogic acid to its targets. Based on the crystal structure of gambogic acid (Fig. 1), the xanthone ring structure is planar and there are two different faces, the top and bottom. The two prenyl chains and the polycyclic ring on top consist of the hydrophobic face, while the carboxylic acid and the carbonyl in the polycyclic ring on the bottom consist of the hydrophilic face.[18] The tolerance of the carboxy group toward many different modifications suggests that the hydrophilic face of gambogic acid might not be important for binding to its biological targets. Applying this SAR information, the carboxyl group was used to

prepare compounds which are useful for the identification of the molecular target(s) of gambogic acid, such as the biotin derivative **4f**, which was found to have similar activity as that of **2**.

Derivatives with modification of the 6-hydroxy group by either methylation or acylation produced compounds **5**, **6**, and **7**. These compounds were found to have similar activity as the corresponding 6-hydroxy compound. For example, compound **5** had similar activity as compound **3** in the three cell lines tested. Compound **7** had similar activity as that of gambogic acid **2.** Therefore, the 6-hydroxy is not important for the apoptosis inducing activity of gambogic acid. Interestingly, the 6-hydroxy group was found to form strong intra-molecular hydrogen bond with the 8-carbonyl group as indicated by the far downfield chemical shift (12.66 ppm for gambogic acid) of the hydrogen at the 6-hydroxy group.

Derivatives of gambogic acid without the 9,10 carbon–carbon double bond in the α,β-unsaturated ketone, such as compounds **8** and **9**, were found to be inactive in the caspase activation assay in all three cancer cell lines tested at concentrations up to 10 μM. These compounds are more than 10-fold less active than gambogic acid in T47D cells, indicating that the 9,10-double bond in the α,β-unsaturated ketone is critical for activity. It is possible that gambogic acid-induced apoptosis is a two-step process. First, gambogic acid binds to its molecular target(s); second, a nucleophile presented in the target attacks the carbon–carbon double bond in the α,β-unsaturated ketone. This Michael addition results in the covalent attachment of gambogic acid to its target, which then activates the apoptosis signal and leads to the activation of caspase cascade and cell death.

The caspase activation activities of gambogic acid and its derivatives in the breast cancer line ZR751 and colon cancer cell line DLD-1 were found to parallel those of T47D. In general DLD-1 and ZR751 were slightly less sensitive toward the gambogic acid and derivatives than T47D in this assay (roughly of 2–4-fold less sensitive than T47D cells). Interestingly, this is different from the *N*-phenyl nicotinamides, which induce apoptosis through interacting with tubulin, similar to that of vinblastine and paclitaxel. ZR751 was found to be more sensitive toward the *N*-phenyl nicotinamides than T47D in the caspase activation assay.[16]

Gambogic acid **2** and derivative **8** were also tested by the traditional inhibition of cell proliferation (GI_{50}) assay to confirm that the active compound can inhibit the growth of tumor cells. Table 2 shows that gambogic acid is a potent inhibitor of cell proliferation. Gambogic acid (**2**) has GI_{50} values of 0.04 and 0.03 μM in the T47D and DLD-1 cancer cells, respectively. Compound **8**, without the 9,10-double bond in the α,β-unsaturated ketone, was found to be inactive up to 10 μM. Therefore, compound **8** is at least 250-fold less active than gambogic acid in growth inhibition assay, confirming that the 9,10-double bond in the α,β-unsaturated ketone is important for the apoptosis-inducing activity of gambogic acid.

Table 2. Inhibition of cell proliferation by gambogic acid **2** and derivative **8**

Compd	GI_{50} (μM)[a]	
	T47D	DLD-1
2	0.04±0.01	0.03±0.01
8	>10	>10

[a] Data are the mean of three experiments and are reported as mean ±standard error of the mean (SEM).

3. Conclusion

In conclusion, gambogic acid, isolated from the resin of *G. hurburyi* tree, was identified as a potent apoptosis inducer through our cell-based high throughput screening assays. The ability of gambogic acid to induce apoptosis was further characterized in nuclear fragmentation and flow cytometry assays in T47D cells. The flow cytometry results indicate that gambogic acid does not induce apoptosis through cell cycle arrest. These data suggest that, similar to that of gaudichaudione A,[22] a structurally related compound, gambogic acid has a different mechanism of action than many of the current natural product anticancer drugs, including the taxanes and vinca alkaloids, which induce apoptosis through interaction with tubulin and G_2/M cell-cycle arrest.[27,28] Through SAR studies of gambogic acid, it has been found that the 30-carboxy and 6-hydroxy are not important for its apoptosis-inducing activity, while the 9,10 carbon–carbon double bond of the α,β-unsaturated ketone is critical for its apoptosis-inducing activity and cytotoxicity.

Since gambogic acid can be isolated in good yield from a tree resin, it is readily available from a renewable natural resource. This should avoid the supply problem encountered frequently in the development of natural products, such as paclitaxel. Gambogic acid has a different mechanism of action from many other natural anticancer agents and provides the possibility of combination treatment with other anticancer drugs. The understanding of the SAR of **2** led to the design and synthesis of reagents such as biotin-labeled derivative **4f** for the identification of the molecular target(s) of gambogic acid, as well as novel derivatives that have been found to have potent in vivo anti-tumor activity.[29] Due to the interesting apoptotic properties of **2** and derivatives, we are following up with further animal efficacy studies, as well as target identification and validation.

4. Experimental

4.1. General methods and materials

The ^{1}H NMR spectra were recorded at Varian 300 MHz. Chemical shifts are reported in ppm (δ) and *J* coupling constants are reported in Hz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA, USA). Reagent grade solvents were

used without further purification unless otherwise specified. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh) using reagent grade solvents. Syto16 and *N*-(2-aminoethyl)biotinamide were obtained from Molecular Probes (Eugene, OR, USA). *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110 was prepared as described in US patent 6,335,429.[25] Human breast cancer cells T47D and ZR751, and human colon cancer cells DLD-1, were obtained from American Type Culture Collection (Manassas, VA, USA).

4.2. Isolation and purification of gambogic acid (2)

Dry powder of gamboge resin (140 g), from the *G. hurburyi* tree was extracted with methanol (600 mL) at room temperature for one day. The mixture was filtered and the extraction was repeated two more times with methanol (600 mL). The combined filtrate was concentrated under reduced pressure to yield crude extract (122 g) as a yellow powder. The crude extract (120 g) was dissolved in pyridine (120 mL), then warm water (30 mL) was added to the stirred solution. After cooling to room temperature, some precipitate was observed. Hexane (120 mL) was added to the mixture and the mixture was filtered. The solid was collected and washed with hexane and dried. The yellow salt was purified by repeated recrystallization (six times) from ethanol to yield the pyridine salt of gambogic acid as yellow prisms (7.5 g, 6.25%), mp 141–143 °C. ^{1}H NMR ($CDCl_3$): 8.61 (m, 2H), 7.71 (t, J = 7.5 Hz, 1H), 7.55 (d, J = 6.6 Hz, 1H), 7.31 (m, 2H), 6.59 (d, J = 10.2 Hz, 1H), 6.09 (t, J = 7.2 Hz, 1H), 5.37 (d, J = 10.2 Hz, 1H), 5.04 (brs, 2H), 3.49 (m, 1H), 3.38–3.20 (m, 2H), 3.06–2.85 (m, 2H), 2.52 (d, J = 9.6 Hz, 1H), 2.31 (m, 1H), 2.01 (m, 2H), 1.76–1.30 (m, 29H).

The gambogic acid pyridine salt (6.3 g) was dissolved in ether (100 mL) and shaken with aqueous HCl (1N, 50 mL) for 1 h. The ether solution was then washed with water (2×50 mL), dried and evaporated to yield gambogic acid (5.47 g, 98%). ^{1}H NMR ($CDCl_3$): 12.66 (s, 1H), 7.43 (d, J = 6.9 Hz, 1H), 6.48 (d, J = 10.2 Hz, 1H), 5.97 (t, J = 7.5 Hz, 1H), 5.26 (d, J = 10.2 Hz, 1H), 4.91 (m, 2H), 3.37 (m, 1H), 3.24–2.98 (m, 2H), 2.81 (d, J = 6.6 Hz, 1H), 2.41 (d, J = 9.0 Hz, 1H), 2.20 (m, 1H), 1.88 (m, 1H), 1.63 (s, 3H), 1.60 (s, 3H), 1.58 (s, 3H), 1.53 (s, 3H), 1.51 (s, 3H), 1.43 (s, 3H), 1.26 (s, 3H), 1.18 (s, 3H). Anal. calcd for $C_{38}H_{44}O_8$: C, 72.59; H, 7.05. Found: C, 72.41; H, 7.24.

4.2.1. Methyl gambogate (3). A mixture of gambogic acid (200 mg, 0.32 mmol), DMAP (78 mg, 0.64 mmol), EDC (123 mg, 0.64 mmol) and methanol (102 mg, 3.2 mmol) in THF (5 mL) was stirred at room temperature for 3 h. The solution was poured into water (10 mL), then extracted with ethyl acetate (3×10 mL). The combined organic layer was dried and concentrated to yield the crude product, which was purified by chromatography (SiO_2, EtOAc–hexane 1:5) to yield compound **3** (196 mg, 95%). ^{1}H NMR ($CDCl_3$): 12.85 (s, 1H), 7.54 (d, J = 6.9 Hz, 1H), 6.67 (d, J = 10.5 Hz, 1H), 5.94 (t, J = 6 Hz, 1H), 5.43 (d, J = 10.2 Hz, 1H), 5.05 (m, 2H), 3.49 (m, 1H), 3.43 (s, 3H), 3.35–3.10 (s, 2H), 3.00 (t, J = 7.2 Hz, 1H), 2.52 (d, J = 10.2 Hz, 1H), 2.32 (q, J = 4.8 Hz, 1H), 2.02 (m, 1H), 1.74 (s, 3H), 1.69 (s, 3H), 1.67 (s, 3H), 1.65 (s, 3H), 1.63 (s, 3H), 1.55 (s, 3H), 1.44 (s, 3H), 1.29 (s, 3H). Anal. calcd for $C_{39}H_{46}O_8 \cdot 0.5H_2O$: C, 71.86; H, 7.21. Found: C, 71.56; H, 7.46.

4.2.2. 1-Gambogylpiperidine (4a). A mixture of gambogic acid (200 mg, 0.32 mmol), DMAP (39 mg, 0.32 mmol), EDC (123 mg, 0.64 mmol) and piperidine (54.2 mg, 0.64 mmol) in THF (3 mL) was stirred at room temperature for 6 h. The solution was poured into water (10 mL), then was extracted with ethyl acetate (3×10 mL). The combined organic layer was dried and concentrated to yield the crude product, which was purified by chromatography (SiO_2, EtOAc–CH_2Cl_2 1:8) to yield compound **4a** (187 mg, 84%). ^{1}H NMR ($CDCl_3$): 12.87 (s, 1H), 7.53 (d, J = 6.9 Hz, 1H), 6.68 (d, J = 10.2 Hz, 1H), 5. 43 (d, J = 10.5 Hz, 1H), 5.40 (t, J = 6.0 Hz, 1H), 5.05 (m, 2H), 3.54–3.33 (m, 2H), 3.28 (d, J = 6.9 Hz, 1H), 3.11 (t, J = 8.4 Hz, 1H), 2.50 (d, J = 9.6 Hz, 1H), 2.46–2.17 (m, 3H), 2.00 (m, 1H), 1.75–1.72 (m, 6H), 1.68 (s, 3H), 1.65 (bs, 6H), 1.56 (s, 3H), 1.43 (s, 3H), 1.25 (s, 3H). Anal. calcd for $C_{42}H_{47}NO_7 \cdot H_2O$: C, 72.34; H, 7.77; N, 1.96. Found: C, 71.97; H, 7.30; N, 1.88.

Compounds **4b**–**4f** were prepared by a method similar to that described for the preparation of compound **4a**.

4.2.3. 1-Gambogyl-4-methylpiperazine (4b). Compound **4b** was prepared from gambogic acid and *N*-methyl piperazine (64%). ^{1}H NMR ($CDCl_3$): 12.85 (s, 1H), 7.52 (d, J = 6.6 Hz, 1H), 6.66 (d, J = 9.9 Hz, 1H), 5.42 (t, J = 10.5 Hz, 1H), 5.05 (m, 2H), 3.62 (m, 1H), 3.40 (m, 2H), 3.28–3.17 (m, 4H), 2.50–1.98 (m, 7H), 2.23 (s, 3H), 1.72 (bs, 6H), 1.66 (s, 3H), 1.63 (bs, 6H), 1.53 (bs, 3H), 1.41 (s, 3H), 1.23 (s, 3H). Anal. calcd for $C_{43}H_{54}N_2O_7 \cdot 0.5H_2O$: C, 71.74; H, 7.70; N, 3.89. Found: C, 71.39; H, 7.45; N, 3.51.

4.2.4. *N*-(2-Morpholin-4-yl-ethyl)gambogamide (4c). Compound **4c** was prepared from gambogic acid and 2-(4-morpholinyl)ethylamine (56%). ^{1}H NMR ($CDCl_3$): 12.86 (s, 1H), 7.54 (d, J = 6.6 Hz, 1H), 6.68 (d, J = 10.2 Hz, 1H), 6.56 (t, J = 5.1 Hz, 1H), 5.46 (d, J = 10.2 Hz, 1H), 5.28 (d, J = 7.5 Hz, 1H), 5.05 (brs, 1H), 3.68 (t, J = 4.2 Hz, 4H), 3.47 (m, 1H), 3.71–3.17 (m, 4H), 2.68 (t, J = 6.6 Hz, 2H), 2.54 (d, J = 9.6 Hz, 1H), 2.48–2.44 (m, 6H), 2.36–2.30 (m, 1H), 2.01–2.00 (m, 3H), 1.74 (s, 6H), 1.67 (s, 3H), 1.65 (s, 6H), 1.61 (s, 3H), 1.44 (s, 3H), 1.28 (s, 3H). Anal. calcd for $C_{44}H_{56}N_2O_8 \cdot H_2O$: C, 69.63; H, 7.64; N, 3.69. Found: C, 69.89; H, 7.79; N, 3.77.

4.2.5. 1-Gambogyl-4-(2-pyrimidyl)piperazine (4d). Compound **4d** was prepared from gambogic acid and 4-(2-pyrimidyl)piperazine (50%). ^{1}H NMR ($CDCl_3$): 12.85 (s, 1H), 8.31 (d, J = 4.8 Hz, 2H), 7.52 (d, J = 6.9 Hz, 1H), 6.66 (d, J = 10.2 Hz, 1H), 6.52 (t, J = 4.8 Hz, 1H), 5.44 (m, 1H), 5.42 (d, J = 10.2 Hz, 1H), 5.07 (m, 1H), 3.90–3.20 (m, 8H), 1.77 (s, 3H), 1.72 (s, 3H), 1.68 (s, 3H), 1.65 (bs, 6H), 1.56 (s, 3H), 1.42 (s, 3H), 1.25 (s, 3H). Anal. calcd for $C_{46}H_{54}N_4O_7 \cdot 1.5H_2O$: C, 68.88; H, 7.10; N, 6.98. Found: C, 68.89; H, 6.82: H, 6.80.

4.2.6. 1-Gambogyl-4-cyclopentylpiperazine (4e). Compound **4e** was prepared from gambogic acid and 4-cyclopentylpiperazine (40%). ^{1}H NMR ($CDCl_3$): 12.87 (s, 1H), 7.54 (d, J = 6.6 Hz, 1H), 6.68 (d, J = 10.5 Hz, 1H), 5.48 (m, 1H), 5.45 (d, J = 6.3 Hz, 1H), 5.07 (m, 1H), 3.65–3.18 (m, 7H), 1.74 (bs, 6H), 1.69 (s, 3H), 1.65 (bs, 6H), 1.59 (s, 3H), 1.43 (s, 3H), 1.26 (s, 3H). Anal. calcd for $C_{47}H_{60}N_2O_7$: C, 73.79; H, 7.91; N, 3.66. Found: C, 73.42; H, 7.72; N, 3.82.

4.2.7. *N*-(2-Gambogylaminoethyl)biotinamide (4f). Compound **4f** was prepared from gambogic acid and *N*-(2-aminoethyl)biotinamide (23%). ^{1}H NMR ($CDCl_3$): 12.92 (s, 1H), 7.58 (d, J = 6.9 Hz, 1H), 7.05–6.90 (m, 2H), 6.68 (d, J = 10.5 Hz, 1H), 6.15 (bs, 1H), 5.46 (d, J = 10.5 Hz, 1H), 5.27 (m, 2H), 5.03 (m, 2H), 4.49 (m, 1H), 4.32 (m, 1H), 3.58–2.00 (m, 14H), 1.77 (bs, 3H), 1.73 (bs, 3H), 1.69 (bs, 6H), 1.65 (bs, 6H), 1.45 (bs, 3H), 1.29 (bs, 3H). Anal. calcd for $C_{50}H_{64}N_4O_9S{\cdot}2.5H_2O$: C, 63.74; H, 7.38; N, 5.96. Found: C, 64.05; H, 7.49; N, 5.56.

4.2.8. Methyl 6-*O*-methylgambogate (5). A mixture of methyl gambogate **3** (70 mg, 0.11 mmol), anhydrous K_2CO_3 (0.5 g), methyl iodide (1 mL) in acetone (5 mL) was stirred at room temperature for 70 h. After evaporation to near dryness, water (30 mL) was added into the mixture and it was extracted with ethyl acetate (3×10 mL). The combined organic layer was dried and concentrated to yield the crude product, which was purified by chromatography (SiO_2, EtOAc–hexane 1:4) to yield compound **5** (69 mg, 96%). ^{1}H NMR ($CDCl_3$): 7.41 (d, J = 6.9 Hz, 1H), 6.64 (d, J = 9.9 Hz, 1H), 5.93 (m, 1H), 5.52 (d, J = 10.2 Hz, 1H), 5.05 (m, 2H), 3.79 (s, 3H), 3.40 (s, 3H), 3.45–3.18 (m, 3H), 2.95 (d, J = 9.6 Hz, 1H), 2.26 (m, 1H), 2.02 (m, 1H), 1.73 (s, 3H), 1.67 (s, 3H), 1.66 (d, 3H), 1.52 (s, 3H), 1.42 (s, 3H), 1.27 (s, 3H). Anal. calcd for $C_{40}H_{48}O_8$: C, 73.15; H, 7.37. Found: C, 73.23; H, 7.70.

4.2.9. 1-(6-*O*-Methylgambogyl)piperidine (6). Compound **6** was prepared from **4a** and methyl iodide by a method similar to that described for the preparation of compound **5** (83%). ^{1}H NMR ($CDCl_3$): 7.40 (d, J = 6.9 Hz, 1H), 6.64 (d, J = 10.2 Hz, 1H), 5.53 (d, J = 10.2 Hz, 1H), 5.34 (t, J = 6.3 Hz, 1H), 5.12–5.00 (m, 2H), 3.80 (s, 3H), 3.52 (m, 1H), 3.38–3.31 (m, 4H), 3.11 (t, J = 5.4 Hz, 2H), 2.50–1.98 (m, 6H), 1.73 (s, 3H), 1.70 (bs, 3H), 1.65 (s, 3H), 1.63 (bs, 6H), 1.53 (s, 3H), 1.42 (s, 3H), 1.22 (s, 3H). Anal. calcd for $C_{44}H_{55}NO_7{\cdot}0.5H_2O$: C, 73.50; H, 7.78; N, 1.94. Found: C, 73.25; H, 7.61; N, 1.82.

4.2.10. 6-*O*-Acetylgambogic acid (7). A mixture of gambogic acid (154 mg, 0.24 mmol) and Ac_2O (0.3 mL, 3.2 mmol) in pyridine (3 mL) was stirred at room temperature for 4 days. The mixture was diluted (EtOAc–hexane 1:1) (80 mL), washed with water, 2 N HCl and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography (EtOAc–hexane 2:1) to yield compound **7** as a yellow solid (47 mg, 29%). ^{1}H NMR ($CDCl_3$): 7.44 (d, J = 6.9 Hz, 1H), 6.66 (t, J = 6.6 Hz, 1H), 6.40 (d, J = 10.2 Hz, 1H), 5.60 (d, J = 10.5 Hz, 1H), 5.13 (t, J = 6.9 Hz, 1H), 5.04 (t, J = 6.9 Hz, 1H), 3.46 (m, 1H), 3.34 (d, J = 6.9 Hz, 1H), 2.67–2.50 (m, 3H), 2.39 (s, 3H), 2.33–2.27 (m, 1H), 2.08–1.98 (m, 2H), 1.73 (s, 3H), 1.71 (s, 3H), 1.65 (s, 6H), 1.54 (s, 3H), 1.40 (s, 3H), 1.6 (s, 3H), 1.29 (s, 3H). Anal. calcd for $C_{40}H_{46}O_9{\cdot}0.5H_2O$: C, 70.67; H, 6.91. Found: C, 70.29; H, 6.62.

4.2.11. 9,10-Dihydrogambogic acid (8). To a solution of gambogic acid (324 mg, 0.5 mmol) in methylene chloride (30 mL) was added L-selectride solution (10 mL, 10 mmol) dropwise at −78 °C. It was stirred at −78 °C for 1 h. The reaction mixture was quenched with ice water and acidified with 30 mL of 1 N HCl. The mixture was allowed to warm to room temperature and it was diluted with ethyl acetate (50 mL). The resulting mixture was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography (EtOAc–hexane 1:3) to yield compound **8** (78.6 mg, 25%). ^{1}H NMR ($CDCl_3$): 11.96 (s, 1H), 6.67 (d, J = 10.2 Hz, 1H), 6.54 (t, J = 6.6 Hz, 1H), 5.46 (d, J = 10.2 Hz, 1H), 5.13–5.05 (m, 2H), 3.33–3.16 (m, 3H), 2.85 (m, 1H), 2.60 (d, J = 8.7, 1H), 2.43 (s, 1H), 2.08 (m, 1H), 1.97 (s, 3H), 1.74 (s, 3H), 1.67 (s, 6H), 1.64 (s, 3H), 1.57 (s, 3H), 1.37 (s, 3H), 1.36 (d, J = 6.9, 3H), 1.31–1.22 (m, 5H), 1.14 (s, 3H). Anal. calcd for $C_{38}H_{46}O_8$: C, 72.68; H, 7.31. Found: C, 72.36; H, 7.35.

4.2.12. 10-Cyclohexyl-9,10-dihydrogambogic acid (9). To a solution of gambogic acid (80 mg, 0.13 mmol) in THF (5 mL) was added a solution of cyclohexylcuprate (1.2 mmol) in THF prepared from cyclohexylmagnesium chloride and CuI at 0 °C. The mixture was stirred for 2 h and the cooling bath was allowed to slowly warm to room temperature. The reaction mixture was quenched with 2 N HCl and diluted (EtOAc–hexane 1:1) (80 mL). The resulting mixture was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography (EtOAc–hexane 1:3) to yield compound **9** as an oily solid (9 mg, 10%). ^{1}H NMR ($CDCl_3$): 6.61 (d, J = 10.2 Hz, 1H), 6.14 (t, J = 6.0 Hz, 1H), 5.39 (d, J = 10.2 Hz, 1H), 5.20 (t, J = 6.6 Hz, 1H), 5.06 (t, J = 7.2 Hz, 1H), 3.64 (m, 1H), 3.35–3.10 (m, 3H), 2.82 (brs, 2H), 2.67–2.61 (m, 2H), 1.76 (s, 3H), 1.72 (s, 3H), 1.68 (s, 3H), 1.66 (s, 3H), 1.56 (s, 3H), 1.44 (s, 3H), 1.94–1.25 (m, 15H). Anal. calcd for $C_{44}H_{56}O_8.H_2O$: C, 72.30; H, 7.93. Found: C, 72.50; H, 7.91.

4.3. Caspase activation assay (EC_{50})

The potency of gambogic acid and its derivatives as inducers of apoptosis was measured by our caspase-based cell assay as previously described.[16] Briefly, human breast cancer cell lines T47D, ZR75-1 and human colon cancer cell line DLD-1 were treated with various concentrations of gambogic acid and its derivatives and incubated at 37 °C for 24 h. The samples were then treated with the fluorogenic substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110, and incubated for 3 h. The fluorescent signal was measured using a fluorescent plate reader (Model Spectrafour Plus Tecan). The EC_{50} (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The Caspase Activation activity (EC_{50}) in the three cancer cell lines, T47D, ZR751 and DLD-1, are summarized in Table 1.

4.4. Cell growth inhibition assays (GI_{50})

The potency of gambogic acid and its derivatives as inhibitors of cell proliferation was measured as previously described.[16] Briefly, T47D and DLD-1 cells were treated with various concentrations of gambogic acid and derivatives. The samples were incubated at 37 °C for 48 h, then treated with CellTiter-Glo™ reagent (Promega). The samples were mixed by agitation and incubated at room temperature for 10–15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument). GI_{50} values were calculated from dose–response curves using XLFit3 (IDBS) software. The GI_{50} for compounds **2**and **8** are summarized in Table 2.

4.5. Nuclear fragmentation assay

A nuclear fragmentation assay was performed as previously described.[16] Briefly, T47D cells were treated with 10 μM of gambogic acid for 24 h, followed by staining of the nucleus with Syto16, a fluorescent DNA dye. The cells were then observed under fluorescent microscope. The nuclei of T47D cells treated with solvent (DMSO) showed normal nuclei (Fig. 2a). In contrast, T47D cells treated with 10 μM of gambogic acid have shrunken and fragmented nuclei (Fig. 2b).

4.6. Measurement of apoptosis by flow cytometry

Cell cycle analysis was performed as previously described.[16] Briefly, T47D cells were treated with 10 μM of gambogic acid, or 100 nM of paclitaxel, and incubated for 24 h at 37 °C. Control cells were treated with the solvent (DMSO). After the 24 h incubation, cells were treated with propidium iodide and RNAse A, and analyzed on a flow cytometer. All flow cytometry analyses were performed on FACScalibur (Becton Dickinson) using Cell Quest analysis software. On the *x*-axis is plotted the fluorescence intensity and on the *y*-axis is plotted the number of cells with that fluorescence intensity. The T47D control cell population profile is shown in Figure 3a with most of the cells in the G_1 (M2) phase of the cell cycle. After treatment with 10 μM of gambogic acid for 24 h, most of the cells are in the M_1 region (Fig. 3b), indicating that most of the cells have sub-diploid DNA. The sub-diploid amount of DNA (M1) is indicative of apoptotic cells that have undergone DNA degradation or fragmentation. In comparison, T47D cells treated with paclitaxel shows that most of the cells are in the G2/M (M4) phase, with an increase in the sub-diploid DNA content (Fig. 3c), indicating that treatment with paclitaxel for 24 h mainly causes G2/M arrest of T47D cells.

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