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Original article

Synthesis and biological activity evaluation of emodin quaternary ammonium salt derivatives as potential anticancer agents

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ABSTRACT

Twenty-six emodin derivatives (17 novel) which attach quaternary ammonium salt were synthesized and evaluated for their anticancer activities *in vitro* and *in vivo*. Compounds **11g** + **12g** and **11h** + **12h** had more significant antiproliferative ability against three cancer cell lines and low cytotoxicity to HELF. **11g** + **12g** and **11h** + **12h** induced AGS cell apoptosis and arrested cell cycle at the G_0/G_1 phase in a dose-dependent manner. Furthermore, the activities of the caspase-3, -9 enzymes were increased in the treated cells. *In vivo* studies revealed that compounds **11g** + **12g** and **11h** + **12h** showed significant anti-tumor activity compared with controlled group.

1. Introduction

Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone) has been used as a traditional medicinal herb in the treatment of constipation jaundice, gastro-intestinal hemorrhage and ulcers in China for about two thousand years [1]. Recently pharmacological studies have been reported that emodin is capable of inhibiting cellular proliferation [2], inducing cell differentiation [3] and apoptosis [4], and activating of caspase cascade pathway [4,5] and mitochondrial death pathway [6,7] in different cancer cells.

However, emodin itself is not sufficient to effect anti-tumor activity and the chemical modification is necessary. Teich [8] and Gu [9,10] introduced a cationic side chain in 4- and 6-positions of emodin respectively to enhance the interaction between drug molecules and their targets (DNA), and the cations both came from protonating various amino groups in the side chain. Since quaternary ammonium salt can keep positive charge at any pH range, perhaps there is a stronger electrostatic interaction by quaternary ammoniums than by protonated tertiary or secondary amines. Our recent work [11] shows that the emodin derivatives can perform good anticancer activities by introducing a long quaternary ammonium salt side chain on methyl of 6-position of emodin. This result indicates that long quaternary ammonium salt side chain is a good pharmacophore for anticancer activities of emodin.

In this paper, a long quaternary ammonium salt side chain is introduced into 1- or and 8-positions or 6-position of emodin to test the effects of pharmacophore positions on anticancer activities. Since the hydroxyl in 1- or 8-position of emodin can stabilize the radical anion by forming hydrogen bond, the protecting groups of hydroxyls of a series of emodin derivatives are removed to enhance their redox activities in this work.

2. Results and discussion

2.1. Chemistry

Emodin **1** was methylated with 1 equivalent methyl sulfate to give physcion **2**, **2** was then alkylated with 1,2-dibromoethane in acetone in the presence of K_2CO_3 to give a mixture of **3** and **4**. Unfortunately, the dialkylated product of **2** was difficult to obtain even when excessive 1,2-dibromoethane was used. It is very likely that the alkylation of one hydroxyl can block the alkylation of the other hydroxyl. **3** and **4** are position isomers which cannot be separated by column chromatography. The corresponding quaternary ammonium salts **5** and **6** are obtained by treatment of the mixture of **3** and **4** with a series of tertiary amines. **5** and **6** are also position isomers which can not be separated by column chromatography (Scheme 1). Quantum chemistry calculation shows that **3** is more stable than **4**. In addition, methoxyl is a stronger electron-donor group than methyl, so the electronic density of hydroxyl in 1-position is larger than that in 8-position.

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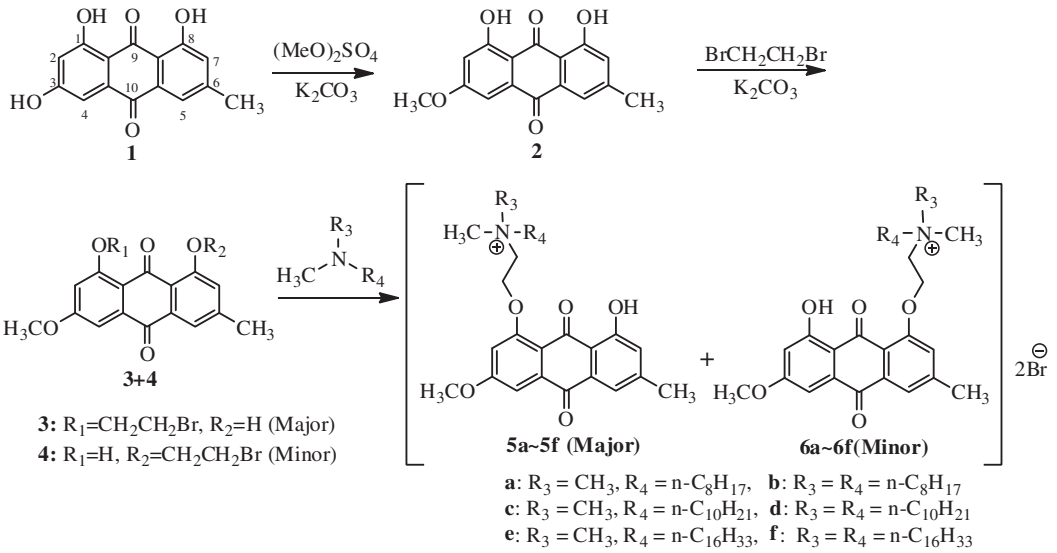


Scheme 1. The synthetic route of emodin derivatives **5** and **6**.

That is to say, **3** is not only a thermodynamic main product but also a kinetic main product. ^{1}H NMR spectrum shows that the proportion of **3** to **4** is about 1.9:1, while the proportion of **5** to **6** sometimes decreases to 1.4:1 (such as **5a**:**6a**). It indicates that **3** and **4** may interconvert by exchanging their 2-bromoethyl, the course of which is proposed in Scheme 2.

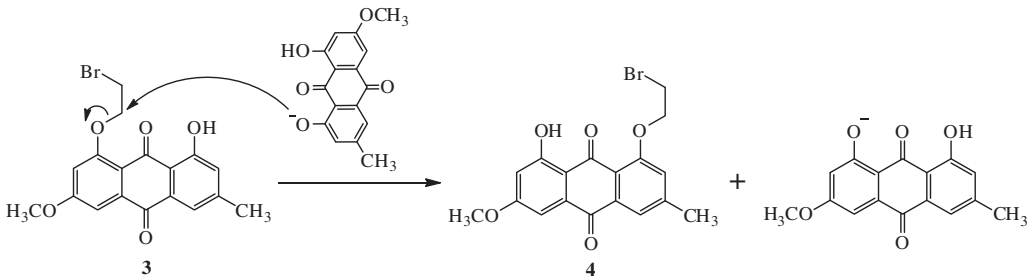
The synthesis of **8** was carried out according to Ref. [12]. If the acid hydrolysis of **8** with HBr aqueous solution (47%) is carried out at reflux temperature for a few hours, the methyl protecting groups at site 1 and site 8 will both be removed [12]. In this paper, at room temperature, only one methyl could be removed even though HBr aqueous solution was much excessive by acid hydrolysis, so a mixture of **9** and **10** was obtained. **9** and **10** were also position isomers which could not be separated by column chromatography. The mixture of **9** and **10** reacted with a series of tertiary amines to give a quaternary ammonium salt mixture of **11** and **12** (Scheme 3). **11** and **12** were also position isomers which could not be separated by column chromatography. **8** reacted with a series of tertiary amines to also give a series of quaternary ammonium salts **13** as products (Scheme 3). Thereinto, the synthesis of **13a**–**e**, **13h** and **13j** was reported in our recent work [11], but all the cytotoxic activities of the above seven compounds to HepG2, BGC, AGS and HELF have never been tested before; the synthesis of compound **13g** and **13i** and their cytotoxic activities to AGS, BGC and HELF was reported in another of our recent works [13]. To our knowledge, the synthesis of compound **13f** has never been reported before.

2.2. Cytotoxic activity

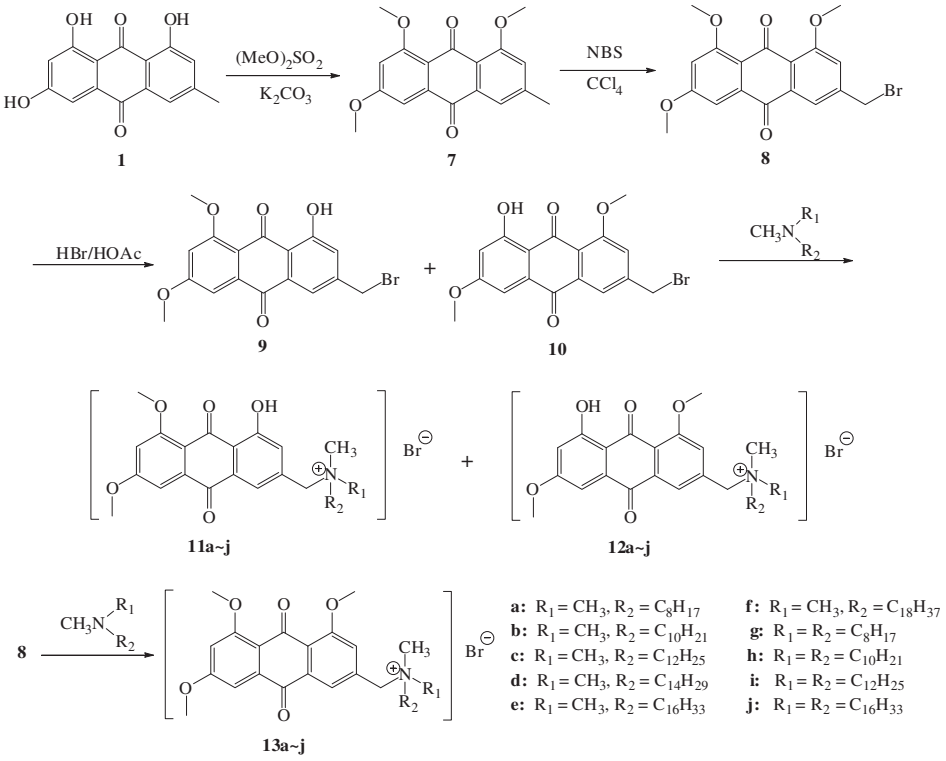
The *in vitro* cytotoxic activities were evaluated for all synthesized compounds against HepG2, BGC-823, AGS and HELF cell lines. Emodin, paclitaxel, compounds **11a** + **12a**–**11j** + **12j** and **13a**–**j** were dissolved in DMSO or diluted with culture medium containing 0.1% DMSO, respectively. The controlled cells were treated with culture medium containing 0.1% DMSO. Paclitaxel was used for positive control. If 50% inhibition could not be reached at the highest concentration, then >70 μM was given. The results are listed in Table 1. Since **5a**–**f** and **6a**–**f** show no cytotoxic activities to all cancer cell lines, Table 1 does not include these compounds.

The anticancer activities of **11**, **12** and **13** are all much higher than that of emodin, which indicates that the quaternary ammoniums group can improve the anticancer activity of emodin. But **5** and **6** shows no activities to all cancer cell lines (so it was not shown in the date), which implies that the positions of pharmacophores are very important. The length of quaternary ammoniums chains is also an essential factor. **11g** + **12g** and **11h** + **12h** show the highest activities of **11a** + **12a**–**11j** + **12j**, **13g** and **13h** also show the highest activities of **13a**–**j**, which indicates that the quaternary ammoniums groups that possess two octyls or decyls are the most effective pharmacophores of all quaternary ammoniums groups used in the work.

Human cancer cells, HepG2, BGC-823, AGS and HELF were used in the study. To quantitate the effects of emodin derivatives on cell growth, cell viability was first assayed by the reduction of MTT at



Scheme 2. The course of interconversion between **3** and **4**.



Scheme 3. The synthetic route of emodin derivatives **11**, **12** and **13**.

24 h after the addition of control medium or various concentrations of emodin derivatives. Cancer cells exhibited a clear concentration-dependent growth inhibition with emodin derivatives, though with varying sensitivity (Table 1). The IC_{50} values against cancer cell lines (HepG2, BGC-823 and AGS) of emodin were 14-times higher than that of compounds **11g** + **12g** and **11h** + **12h**. However, the cytotoxicity increased about 1.5-times by treating HELF cells with compound **11h** + **12h**. The result indicated that compounds **11g** + **12g** and **11h** + **12h** showed significant activities against cancer cells (HepG2, BGC-823 and AGS) and lower cytotoxicity against HELF, especially with **11h** + **12h** (Fig. 1).

Table 1
The *in vitro* activity of synthesized compounds against various cancer cell lines.

Compound	IC_{50} (μM)[a]			
	HepG2	BGC	AGS	HELF
Emodin	> 70[b]	> 70	> 70	> 70
11a + **12a**	37.71 ± 6.4	17.16 ± 5.4*	38.43 ± 5.5	>70
11b + **12b**	22.53 ± 3.4	28.93 ± 4.3	10.22 ± 4.2*	13.10 ± 4.8
11c + **12c**	10.18 ± 3.2*	6.03 ± 2.4**	14.86 ± 5.7	19.61 ± 3.2
11d + **12d**	5.61 ± 4.6**	8.36 ± 3.7**	14.36 ± 4.5	20.10 ± 2.2
11e + **12e**	9.91 ± 5.1**	21.18 ± 2.4	11.63 ± 2.4	10.74 ± 3.6
11f + **12f**	22.77 ± 7.2	12.09 ± 3.3*	21.26 ± 2.5	65.64 ± 3.3
11g + **12g**	2.11 ± 1.9**	5.43 ± 2.7**	4.97 ± 5.6**	4.81 ± 2.2**
11h + **12h**	3.41 ± 3.5**	3.87 ± 1.5**	2.91 ± 1.9**	6.14 ± 1.3**
11i + **12i**	11.78 ± 6.9	10.13 ± 4.6	12.43 ± 1.8	10.13 ± 4.4*
11j + **12j**	>70	>70	>70	>70
13a	>70	50.43 ± 3.6	>70	>70
13b	54.84 ± 3.5	47.72 ± 2.9	36.90 ± 1.7*	>70
13c	22.39 ± 5.3	26.95 ± 3.7*	14.54 ± 4.4	48.23 ± 3.1*
13d	12.78 ± 2.5*	12.95 ± 2.4	12.47 ± 2.6*	26.84 ± 1.2
13e	16.13 ± 4.0*	20.37 ± 5.7	12.17 ± 3.9*	23.07 ± 4.0
13f	19.24 ± 2.4	14.34 ± 3.9*	8.15 ± 3.5**	19.21 ± 3.4*
13g	10.28 ± 2.3*	10.71 ± 3.1*	6.72 ± 5.0**	11.83 ± 2.8
13h	14.12 ± 2.0	7.75 ± 2.4**	10.18 ± 4.4	5.77 ± 3.3**
13i	26.24 ± 2.1	4.13 ± 3.5**	16.88 ± 1.9*	35.85 ± 4.0
13j	>70	>70	33.45 ± 5.5	>70
Paclitaxol	10.32 ± 0.02**	9.26 ± 0.14**	15.40 ± 0.03**	7.14 ± 0.09**

Data are presented as mean SD ($n = 6$). *$P < 0.05$, **$P < 0.01$. Compared with control group.

[a] The concentration of the drug that inhibited cells growth by 50%.

[b] When 50% inhibition could not reached at the highest concentration, then >70 μM was given.

2.3. Analysis of structure–activity relationship of different emodin derivatives

According to the above MTT result, the mixtures of **11** and **12** showed higher activities than **13**. In order to study the structure–activity relationship of emodin derivatives, the structures of two emodin derivatives are optimized and the results are listed in Fig. 2. One (A) has two methoxyls both in 1- and 8-positions, the other (B) has one hydroxyl in 1-position and one methoxyl in 8-position. In Fig. 2(b), there is no orbital repulsion among three bonds (C1–O1, C2–O2 and C3–O3), so the three cycles of emodin derivatives are coplanar. But in Fig. 2(a), O1 atom is repulsed by lone-pairs of both O2 and O3 atoms, so O1 atom deflects from the plane that contains C2–O2 and C3–O3 obviously, which makes the three cycles not coplanar. Fig. 2 indicates that the anthracene rings of **11** and **12** are planar, but that of **13** is nonplanar. Since planar structure is favorable to drug molecules to intercalate into the base pairs of DNA, **11** and **12** showed higher anticancer activities. In addition, anthraquinones is easy to produce hydroxyl radical to break the chains of DNA through redox reactions, so the following semi-reactions are calculated and the relative energies of reactants and products are listed in the brackets.

The equations (1) and (2) give reaction heat 0.0446 a.u. (117.1 kJ/mol) and 0.0572 a.u. (150.2 kJ/mol) respectively, which indicates that the hydroxyl in 1- or 8-position facilitates the anthraquinone to obtain an electron, the reason of which is that the hydroxyl can stabilize the oxygen anions by forming an H-bond. That is to say, if

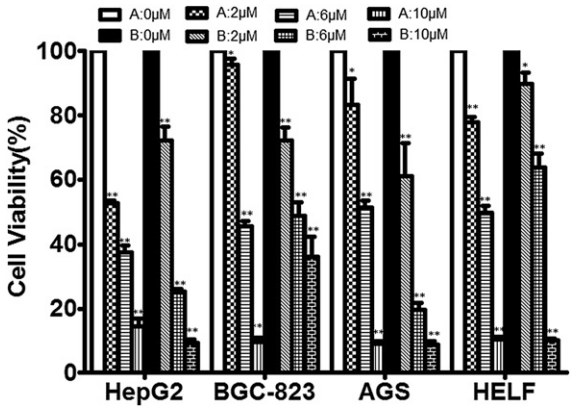


Fig. 1. Inhibition of cell viability by compounds **11g** + **12g** (A) and **11h** + **12h** (B) on HepG2, BGC-823, AGS and HELF cells. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with control.

the emodin derivatives possess one hydroxyl in its 1- or 8-position, redox reaction will take place easily. It is well known that the redox reactions of emodin derivatives can produce ROS or hydroxyl radical to break the chains of DNA, which will improve the anticancer activities of emodin derivatives, so **11** and **12** have higher activities than **13**.

2.4. Investigation of apoptosis

AGS and HepG2 cells were both highly sensitive to **11g** + **12g** and **11h** + **12h** (Table 1), in this paper, AGS cell line was selected for all further experiments.

2.4.1. Fluorescence staining detection of cell apoptosis

Apoptosis cells had many morphologic changes, such as chromosomal condensation and internucleosomal fragmentation. The morphologic changes in the cells after treatment with compound **11g** + **12g** and **11h** + **12h** were assessed by fluorescence microscopy using acridine orange/ethidium bromide (AO/EB) staining. Treatment of AGS cells with compounds **11g** + **12g** and **11h** + **12h** for 24 h resulted in significant chromosomal condensation, internucleosomal fragmentation and morphological changes, indicating that the cytotoxic action of compounds **11g** + **12g** and **11h** + **12h** were due to their abilities to induce apoptosis (Fig. 3).

2.4.2. Measurement of caspase-3 and caspase-9 activity

Caspases are a family of cysteine proteases that play a central role during the executional phase of apoptosis. To explore whether emodin derivatives induced apoptosis by the activation of caspases, appropriate substrates were used to detect specific caspase activities. The results shown in Fig. 4 indicated that caspase-9 and -3 were activated in AGS cells after 24 h treatment with compounds **11g** + **12g** and **11h** + **12h**.

2.5. Investigation of cell cycle distribution

To further evaluate the effects of emodin on cell cycle progression, we performed flow cytometry analysis and the results were summarized in Figs. 5 and 6. Compared with control, the addition of emodin derivatives increased G_0/G_1 phase cells ($p < 0.05$) and decreased G_2/M phase cells ($p < 0.05$) in a dose-dependent manner. These results demonstrated that compounds **11g** + **12g** and **11h** + **12h** could inhibit the G_1–S phase transition and arrest cells at G_0/G_1 phase.

2.6. Anti-tumorigenic activities of compounds *11g* + *12g* and *11h* + *12h* against H22 xenograft tumor growth

We examined the effects of compounds **11g** + **12g** and **11h** + **12h** on the growth of H22 hepatic carcinomas tumors. H22 hepatic carcinomas were established in Kunming mice within 6–8 d and subjected to treatment with vehicle (0.9% sodium chloride), cyclophosphamide (CTX, 1.5 mg/mL), compounds **11g** + **12g** (5, 10, 20 mg/kg) and **11h** + **12h** (5, 10, 20 mg/kg) once per day for 8 days. The high dose of compound **11g** + **12g** (20 mg/kg) and the medium dose of compound **11h** + **12h** (10 mg/kg) showed significant anti-tumor activities (48.6 ± 3.4% and 49.9 ± 2.5%) compared with control ($P < 0.05$; Fig. 7). Meanwhile, the growth of tumor was also inhibited by the low dose of compound **11g** + **12g** (5 mg/kg, Fig. 7).

3. Conclusion

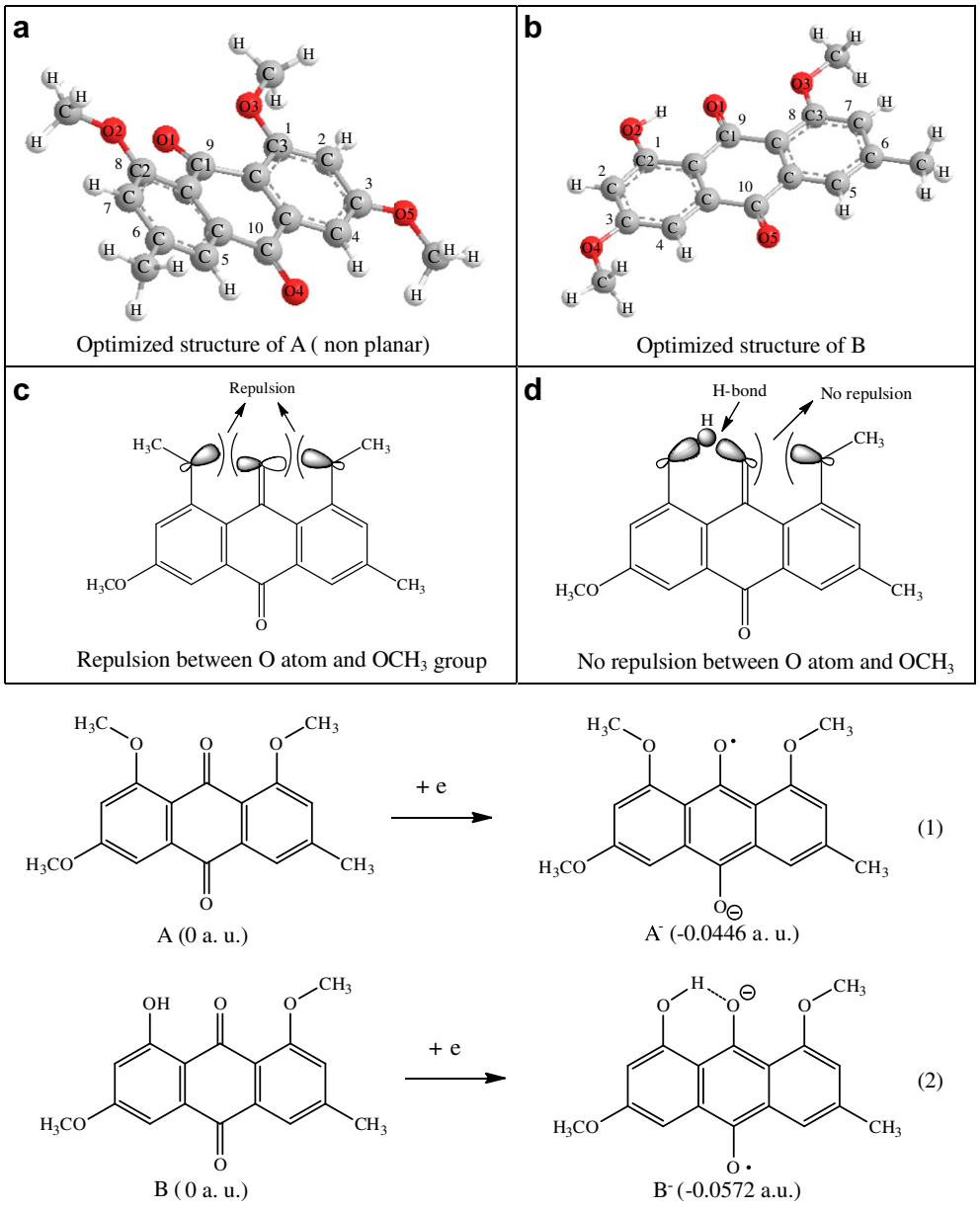
More and more studies have shown that emodin has significant anti-tumor effects and exhibits cytotoxic activities against various kinds of cancer cell lines *in vitro*, which could be explained through the activation of caspases [4], inducting of p53 and p21 expression [14], surviving inhibition [15]. Another emodin-induced apoptotic mechanism involves down-regulation of the cellular inhibitor of apoptosis genes and the inhibition of NF-κB activities [16].

The above study results demonstrate that (1) emodin can be modified to more potent bioactive derivatives. The anticancer activities of **11a** + **12a**–**11j** + **12j** are a little higher than those of **13a**–**j**, which indicates that replacing the methoxyl of 1- or 8-position by hydroxyl increases the anticancer activities. (2) Compounds **5a**–**f** and **6a**–**f** show no cytotoxic activity to all cancer cell lines (data are not showed). These phenomena indicate that introduction of quaternary ammoniums to 1- and 8-positions has no effects on the anticancer activity of emodin. (3) But the number and length of long chains are very important. Compounds **11a** + **12a**–**11j** + **12j** can be divided into two classes, with one containing only one long carbon chain (**11a** + **12a**–**11f** + **12f**, C8–C18) in quarter N atom, with the other containing two long carbon chains in quarter N atom (**11g** + **12g**–**11j** + **12j**). To sum up, the compounds containing two long carbon chains exhibited higher activities than those containing only one long carbon chain. (4) The two long carbon chains emodin derivatives that contain length of carbon chains between eight carbon atoms (compound **11g** + **12g**, **13g**) and ten carbon atoms (compound **11h** + **12h**, **13h**) had more effective anticancer activities than others (compound **11i** + **12i**, **13i**, etc.). (5) Compound **11j** + **12j** shows very low cytotoxicity against all cancer cell lines, which is likely because large bulks of two cetyls hold back the interaction between drug molecule and target molecule. (6) Preliminary mechanistic studies demonstrated that compounds **11g** + **12g** and **11h** + **12h** might inhibit cell growth by inducing apoptosis, arresting cell cycle progression at the G_0/G_1 phase in AGS cells and increasing the activities of caspase-3 and caspase-9. (7) The *in vivo* tests demonstrated that certain emodin derivatives could provide anti-tumor activities. These findings can serve as the basis for further researches on the chemical modifications, structure–activity relationships and bioactivities of emodin.

4. Experiment

4.1. General

Emodin was purchased from China Xi'an Sino-Herb Bio-technnology Co. Ltd., in 62% purity. After 2 repeats of wash (by 5% $NaHCO_3$) and extraction (2% Na_2CO_3), the purity of the emodin can reach about 90%. Silica gel (100–200 mesh) used in column



The equation (1) and (2)

Fig. 2. The structures and orbital interaction of derivatives of emodin.

chromatography was provided by China Tsingtao Marine Chemistry Co. Ltd. Compound **7** and **8** were prepared according to a previous reference [12]. All solvents were distilled before their usage, and other reagents were obtained from commercial suppliers in analytically chemically pure forms.

Melting points were measured with WRS-1B micromelting apparatus and were uncorrected. ^{1}H NMR spectra were recorded by BRUKER AV (400M) and BRUKER AV (500M) spectrometer with TMS as an internal standard in $CDCl_3$. Electrospray ionization (ESI) mass spectra were measured on an Agilent 1100 IC/MSD TrapXCT and were reported as *m*/*z*. The chemical shift values were on δ scale and the coupling constants (*J*) in Hz. Elemental analyses were conducted by the Service Center of Elemental Analysis of Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences. The IR spectra were recorded by Perkin–Elmer 2000 FT-IR spectrometer.

All geometry optimizations of reactants and products were performed by B3LYP method with Gaussian 98 program and 6-311G (d) basis set for all atoms. All calculated details are available from authors.

4.2. Synthesis

4.2.1. The preparation of physcion 2 (1, 8-dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone)

Emodin 2.499 g (9.25 mmol) and K_2CO_3 1.341 g (9.70 mmol) were solved in 100 mL acetone. The solution was heated to 60 °C and then a solution of 1 mL dimethyl sulfate in 10 mL acetone was added slowly drop by drop. The mixture was stirred at the reflux temperature for 12 h. Then the reaction was stopped and 50 mL water was added to decompose unreacted dimethyl sulfate. The yellow precipitate was separated from water by filtration and the

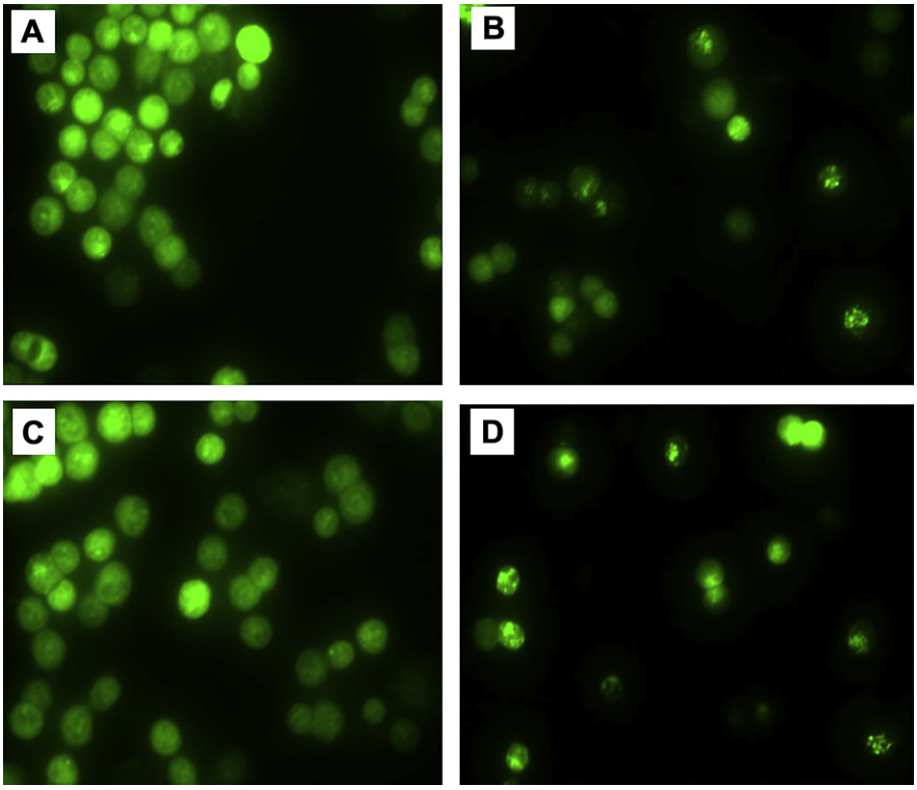


Fig. 3. Fluorescence images of acridine orange and ethidium bromide-stained AGS cells. Apoptotic cells were observed based on morphological changes in AGS cells after treatment with compounds **11g** + **12g** (A) and **11h** + **12h** (B) at different concentrations 0 μM (**11g** + **12g**, control, A), 3 μM (**11g** + **12g**, B), 0 μM (**11h** + **12h**, C), 3 μM (**11h** + **12h**, D) for 24 h.

residue was purified by column chromatography with CH_2Cl_2/petroleum ether (v/v = 1:1) elution. A yellow solid was obtained.

Yield: 81.3%; yellow solid; mp: 208–210 °C (documental value: 205–207 °C [2]); IR (KBr) ν: 3065, 2987, 2840, 1675, 1633, 1567, 1480, 1325, 1166, 980, 875 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 12.31 (s, 1.0H, OH), 12.11 (s, 1.0H, OH), 7.63 (d, J = 1.2 Hz, 1.0H, Ar–H), 7.37 (d, J = 2.8 Hz, 1.0H, Ar–H), 7.08 (d, J = 0.8 Hz, 1.0H, Ar–H), 6.69 (d, J = 2.4 Hz, 1.0H, Ar–H), 3.94 (s, 3.0H, OCH_3), 2.45 (s, 3.0H, Ar–CH_3); ESI-MS m/z 285.1 $(M + H)^+$.

4.2.2. The preparation of 3 [1-(2-bromoethoxy)-8-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone] and 4 [8-(2-bromoethoxy)-1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone]

Physcion **2** 500 mg (1.76 mmol) and K_2CO_3 1.0 g (7.25 mmol) were solved in 50 mL acetone, and the solution was stirred at reflux temperature. The 1,2-dibromoethane 0.3 mL (3.5 mmol) was dripped into the mixture and the mixture was stirred at reflux temperature for 12 h, then the reaction was stopped and the reaction mixture was poured into 50 mL distilled water. The yellow precipitate was separated from water by filtration and the residue was purified by column chromatography with CH_2Cl_2 elution. A yellow solid containing **3** and **4** was obtained. The peak areas of ^1H NMR show that the proportion of **3** to **4** is about 1.9:1.

Yield: 65.2%; yellow solid; mp: 176–178 °C; IR (KBr) ν: 3436, 3107, 2937, 1672, 1633, 1595, 1320, 1196, 898, 608 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.29 (s, 1.0H, OH), 13.08 (s, 1.9H, OH), 7.82 (s, 1.0H, Ar–H), 7.58 (d, J = 1.2 Hz, 1.9H, Ar–H), 7.51 (d, J = 2.8 Hz, 1.9H, Ar–H), 7.31 (d, J = 2.4 Hz, 1.0H, Ar–H), 7.14 (s, 1.0H, Ar–H), 7.09 (s, 1.9H, Ar–H), 6.78 (d, J = 2.8 Hz, 1.9H, Ar–H), 6.71 (J = 2.4 Hz, 1.0H, Ar–H), 4.47 (t, J = 6.4 Hz, 2.0H, ArO–$\underline{CH_2}CH_2Br$), 4.45 (t, J = 6.8 Hz, 3.8H, ArO–$\underline{CH_2}CH_2Br$), 3.99 (s, 5.7H, $\overline{OCH_3}$), 3.92 (s, 3.0H, OCH_3), 3.79 (t, J = 6.8 Hz, 5.8H, ArO–$CH_2\underline{CH_2}Br$), 2.50 (s, 3.0H, Ar–CH_3), 2.44 (s, 5.7H, Ar–CH_3); ESI-MS m/z 391.0 $(M + H)^+$.

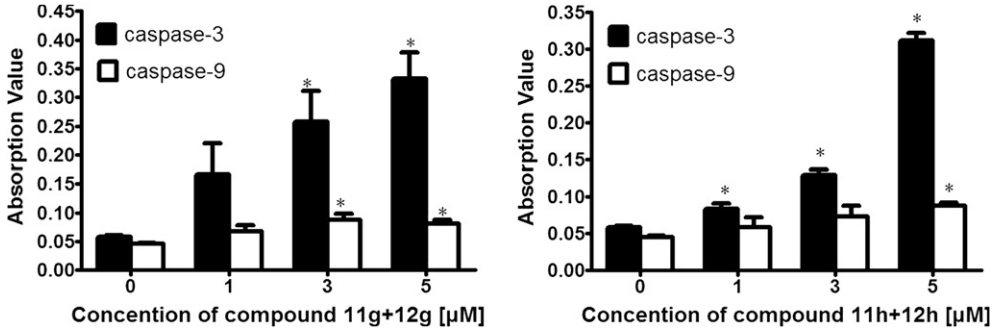


Fig. 4. Measurement of caspase-9 and caspase-3 activity. Dose-dependent induction of caspase-9 and caspase-3 in human AGS cell lines. Data are mean ± SD (n = 6). *P < 0.05 compared with control.

*4.2.3. General procedure for the preparation of **5** and **6***

The mixture of **3** and **4** 98 mg (0.25 mmol) was added into 15 mL methylglycol, and was stirred at 125 °C. Till **3** and **4** were dissolved, 76–150 mg tertiary (about 0.40–0.50 mmol) was added into the solution. The reaction mixture was stirred at reflux temperature till the detected spot of the mixture of **3** and **4** became weak (about 16–24 h). And then the mixture was concentrated under reduced pressure and the residue was chromatographed on a silica gel column by gradient elution to produce **5a**–**j** and **6a**–**j**. The eluent order was dichloromethane first and then chloroform/ethanol (v/v from 50:1 to 10:1).

*4.2.3.1. N-(2-((8-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N,N-dimethyloctan-1-aminium bromide (**5a**) and N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl) oxy) ethyl)-N,N-dimethyloctan-1-aminium bromide (**6a**).* According to the general procedure, **3** and **4** were treated with *N*,*N*-dimethyloctan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give compounds **5a** and **6a**, the proportion of which is about 1.4:1.

Yield: 55.7%; yellow solid; mp: 202–204 °C; IR (KBr) ν: 3426, 3000, 2930, 2852, 1670, 1627, 1593, 1320, 1162, 996, 599 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.14 (s, 1.0H, OH), 12.95 (s, 1.4H, OH), 7.79 (s, 1.0H, Ar–H), 7.54 (s, 1.4H, Ar–H), 7.45 (s, 1.4H, Ar–H), 7.33 (s, 1.0H, Ar–H), 7.28 (s, 1.0H, Ar–H), 7.05 (s, 1.4H, Ar–H), 6.94 (s, 1.4H, Ar–H), 6.66 (s, 1.0H, Ar–H), 4.75 (s, 4.8H, ArO–$\underline{CH_2}CH_2N$), 4.45 (s, 4.8H, ArO–$CH_2\underline{CH_2}N$), 4.00 (s, 4.2H, OCH_3), 3.92 (s, 3.0H, OCH_3), 3.83 (s, 4.8H, N($\underline{CH_2}$)–C_7H_{15}), 3.57 (s, 14.4H, $N(CH_3)_2$), 2.51 (s, 4.2H, Ar–CH_3), 2.43 (s, 3.0H, Ar–CH_3), 1.26–1.88 (m, 28.8H, $(\underline{CH_2})_6CH_3$), 0.87–0.88 (m, 7.2H, $(CH_2)_6$–$C\underline{H_3}$); Anal. Calcd for $C_{28}H_{38}BrNO_5$: C 61.31, H 6.98, N 2.55; found: C 61.65, H 7.33, N 2.12; ESI-MS *m*/*z* 469.0 $(M - Br)^+$.

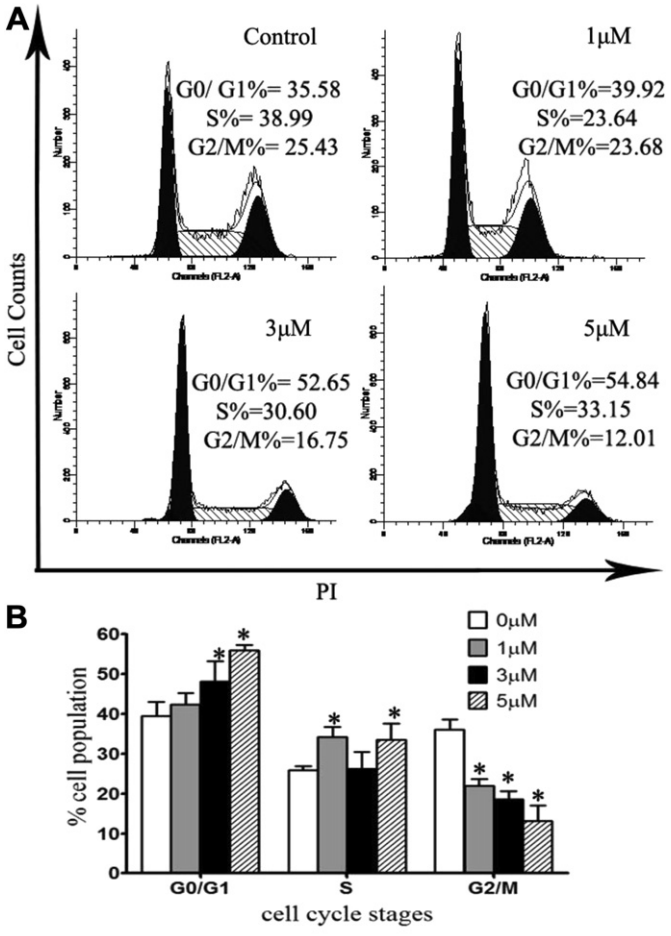


Fig. 5. Investigation of cell cycle distribution. (A) Demonstration of apoptosis by flow cytometric analysis. Untreated AGS cells (control). Cells were treated with increasing concentrations of compound **11g** + **12g** (1 μM, 3 μM and 5 μM) for 24 h. (B) Dose-dependent induction of apoptosis by compound **11g** + **12g**. $^*P < 0.05$, compared with control.

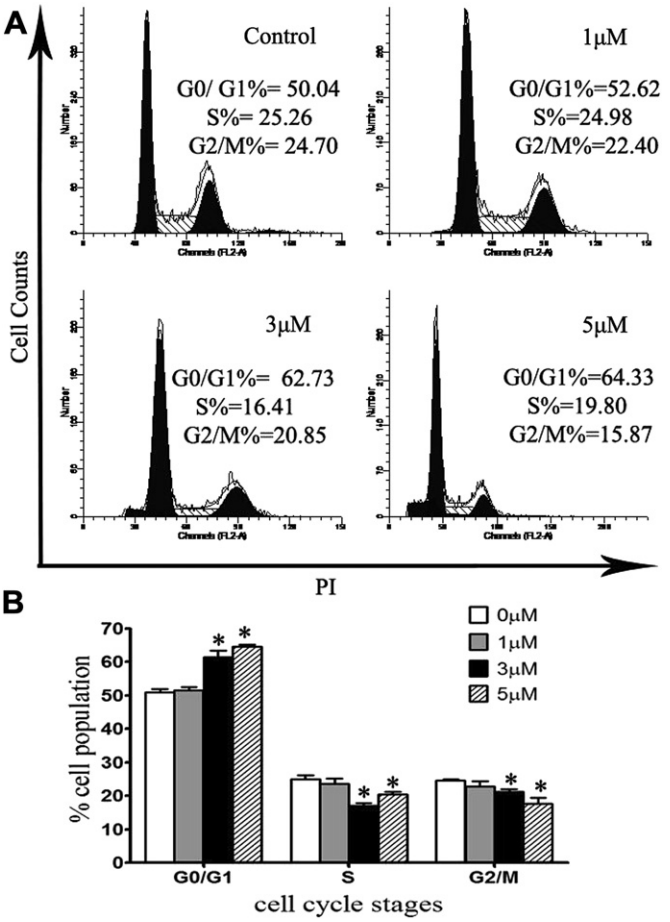


Fig. 6. Investigation of cell cycle distribution. (A) Demonstration of apoptosis by flow cytometric analysis. Untreated AGS cells (control). Cells were treated with increasing concentrations of compound **11h** + **12h** (1 μM, 3 μM and 5 μM) for 24 h. (B) Dose-dependent induction of apoptosis by compound **11h** + **12h**. $^*P < 0.05$, compared with control.

*4.2.3.2. N-(2-((8-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N,N-dimethyldecan-1-aminium bromide (**5b**) and N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl)oxy) ethyl)-N,N-dimethyldecan-1-aminium bromide (**6b**).* According to the general procedure, **3** and **4** were treated with *N*,*N*-dimethyldecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give **5b** and **6b**, the proportion of which is about 1.5:1.

Yield: 54.5%; yellow solid; mp: 210–212 °C; IR (KBr) ν: 3414, 3026, 2928, 2854, 1670, 1633, 1599, 1335, 1260, 995, 600 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.13 (s, 1.0H, OH), 12.95 (s, 1.5H, OH), 7.81 (s, 1.0H, Ar–H), 7.55 (s, 1.5H, Ar–H), 7.47 (s, 1.5H, Ar–H), 7.32 (s, 1.0H, Ar–H), 7.29 (s, 1.0H, Ar–H), 7.06 (s, 1.5H, Ar–H), 6.94 (s, 1.5H, Ar–H), 6.67 (s, 1.0H, Ar–H), 4.75 (s, 5.0H, ArO–$\underline{CH_2}CH_2N$), 4.46 (s, 5.0H, ArO–$CH_2\underline{CH_2}N$), 4.00 (s, 4.5H, OCH_3), 3.92 (s, 3.0H, OCH_3),

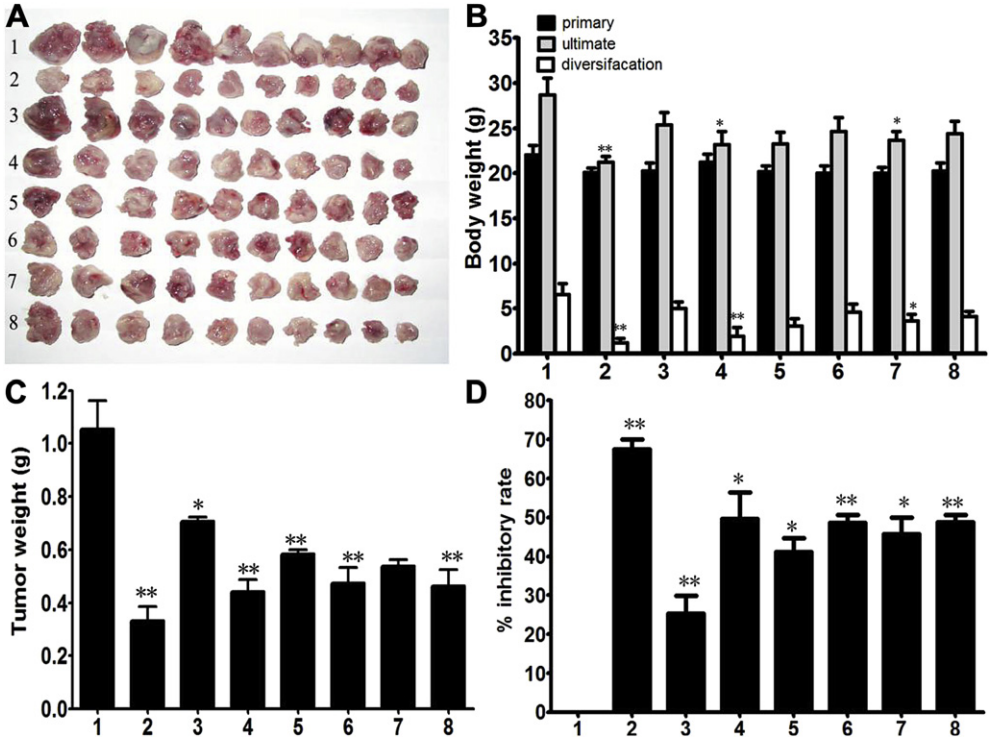


Fig. 7. Effect of anti-tumor *in vivo*. H22 tumor-bearing mice were administered i.p. 7 days with normal saline (1) CTX at 30 mg/kg (2) Compound **11h** + **12h** at 5, 10 and 20 mg/kg (3–5). Compound **11g** + **12g** at 5, 10 and 20 mg/kg (6–8). The figure shows the tumor mode (A) and body weight (B) of the control and therapeutic groups, (C) shows the tumor weight and (D) shows the inhibitory mice after administration. Data represent means ± S.D. from three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the normal saline (1).

3.83 (s, 5.0H, N($C\underline{H}_2$)–C_9H_{19}), 3.56 (s, 15.0H, $N(CH_3)_2$), 2.51 (s, 4.5H, Ar–CH_3), 2.43 (s, 3H, Ar–CH_3), 1.26–1.46 (m, 40.0H, $(C\underline{H}_2)_8CH_3$), 0.87 (t, J = 6.8 Hz, 7.5H, $(CH_2)_8$–$C\underline{H}_3$); Anal. Calcd for $C_{30}H_{42}BrNO_5 \cdot 4.5H_2O$: C 54.79, H 7.82, N 2.13; found: C 54.48, H 7.74, N 1.90; ESI-MS m/z 497.0 $(M - Br)^+$.

*4.2.3.3. N-(2-((8-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N,N-dimethylhexadecan-1-aminium bromide (**5c**) and N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl)oxy) ethyl)-N,N-dimethylhexadecan-1-aminium bromide (**6c**).* According to the general procedure, **3** and **4** were treated with *N*,*N*-dimethylhexadecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give **5c** and **6c**, the proportion of which is about 1.5:1.

Yield: 54.5%; yellow solid; mp: 225–227 °C; IR (KBr) ν: 3400, 3020, 2923, 2853, 1675, 1635, 1597, 1335, 1216, 993, 596 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.11 (s, 1.0H, OH), 12.93 (s, 1.5H, OH), 7.83 (s, 1.0H, Ar–H), 7.57 (s, 1.5H, Ar–H), 7.50 (s, 1.5H, Ar–H), 7.31 (s, 1.0H, Ar–H), 7.30 (s, 1.0H, Ar–H), 7.07 (s, 1.5H, Ar–H), 6.94 (s, 1.5H, Ar–H), 6.67 (d, J = 2.4 Hz, 1.0H, Ar–H), 4.75 (m, 5.0H, ArO–$C\underline{H}_2CH_2N$), 4.48 (m, 5.0H, ArO–$CH_2C\underline{H}_2N$), 4.00 (s, 4.5H, OCH_3), 3.93 (s, 3.0H, OCH_3), 3.83 (t, J = 8.4 Hz, 5.0H, N($C\underline{H}_2$)–$C_{15}H_{31}$), 3.56 (s, 15.0H, $N(CH_3)_2$), 2.52 (s, 4.5H, Ar–CH_3), 2.44 (s, 3.0H, Ar–CH_3), 1.24–1.46 (m, 70.0H, $(C\underline{H}_2)_{14}CH_3$), 0.88 (t, J = 7.2 Hz, 7.5H, $(CH_2)_{14}$–$C\underline{H}_3$); Anal. Calcd for $C_{36}H_{54}BrNO_5 \cdot 3H_2O$: C 60.49, H 8.46, N 1.96; found: C 60.58, H 8.11, N 1.71; ESI-MS m/z 581.0 $(M - Br)^+$.

*4.2.3.4. N-(2-((8-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N-methy-N-octyloctan-1-aminium bromide (**5d**) and N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl)oxy)ethyl)-N-methyl-N-octyloctan-1-aminium bromide (**6d**).* According to the general procedure, **3** and **4** were treated with *N*-decyl-*N*-methyldecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give compounds **5d** and **6d**, the proportion of which is about 1.8:1.

Yield: 54.5%; yellow solid; mp: 188–190 °C; IR (KBr) ν: 3436, 3080, 2980, 2847, 1667, 1633, 1611, 1301, 1195, 931, 609 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.14 (s, 1.0H, OH), 12.97 (s, 1.8H, OH), 7.81 (m, 1.0H, Ar–H), 7.56 (d, J = 5.6 Hz, 1.8H, Ar–H), 7.47 (m, 1.8H, Ar–H), 7.36 (s, 1.0H, Ar–H), 7.30 (m, 1.0H, Ar–H), 7.06 (s, 1.8H, Ar–H), 6.99 (d, J = 2.4 Hz, 1.8H, Ar–H), 6.68 (m, 1.0H, Ar–H), 4.77 (m, 5.6H, ArO–$C\underline{H}_2CH_2N$), 4.40 (m, 5.6H, ArO–$CH_2C\underline{H}_2N$), 4.01 (m, 5.6H, OCH_3), 3.93 (s, 3.0H, OCH_3), 3.73 (t, J = 5.6 Hz, 11.2H, 2× N($C\underline{H}_2$)–C_7H_{15}), 3.50 (s, 8.4H, N–CH_3), 2.51 (s, 5.4H, Ar–CH_3), 2.44 (s, 3.0H, Ar–CH_3), 1.26–1.80 (m, 67.2H, 2× $(C\underline{H}_2)_6CH_3$), 0.88 (t, J = 7.2 Hz, 16.8H, 2× $(CH_2)_6$–$C\underline{H}_3$); Anal. Calcd for $C_{35}H_{52}BrNO_5 \cdot 0.5H_2O$: C 64.11, H 8.15, N 2.14; found: C 64.08, H 7.99, N 1.99; ESI-MS m/z 566.9 $(M - Br)^+$.

*4.2.3.5. N-Decyl-N-(2-((8-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N-methyldecan-1-aminium bromide (**5e**) and N-decyl-N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl)oxy)ethyl)-N-methydecan-1-aminium bromide (**6e**).* According to the general procedure, **3** and **4** were treated with *N*-methyl-*N*-octyloctan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give **5e** and **6e**, the proportion of which is about 1.7:1.

Yield: 54.5%; yellow solid; mp: 176–177 °C; IR (KBr) ν: 3410, 3127, 2926, 2854, 1677, 1629, 1596, 1334, 1218, 933, 580 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 10.53 (s, 2.7H, OH), 7.77 (s, 1.0H, Ar–H),

7.57 (d, J = 1.2 Hz, 1.7H, Ar–H), 7.47 (d, J = 2.4 Hz, 1.7H, Ar–H), 7.30 (d, J = 2.8 Hz, 1.0H, Ar–H), 7.21 (s, 1.0H, Ar–H), 7.06 (s, 1.7H, Ar–H), 6.88 (d, J = 2.8 Hz, 1.7H, Ar–H), 6.68 (d, J = 2.8 Hz, 1.0H, Ar–H), 4.56 (m, 5.4H, ArO–$C\underline{H}_2CH_2N$), 4.48 (m, 5.4H, ArO–$CH_2C\underline{H}_2N$), 3.98 (s, 5.1H, OCH_3), 3.92 (s, 3.0H, OCH_3), 3.06 (m, 5.4H, N($C\underline{H}_2$)–C_9H_{19}), 2.93 (m, 5.4H, N($C\underline{H}_2$)–C_9H_{19}), 2.82 (d, J = 4.8 Hz, 8.1H, N–CH_3), 2.49 (s, 3.0H, Ar–CH_3), 2.43 (s, 5.1H, Ar–CH_3), 1.22–1.28 (m, 86.4H, 2× ($C\underline{H}_2$)$_8CH_3$), 0.87 (t, J = 7.2 Hz, 16.2H, 2× $(CH_2)_8$–$C\underline{H}_3$); Anal. Calcd for $C_{39}H_{60}BrNO_5 \cdot 0.5H_2O$: C 65.81, H 8.64, N 1.97; found: C 65.82, H 8.70, N 1.87; ESI-MS m/z 704.0 $(M + H)^+$.

*4.2.3.6. N-Hexadecyl-N-(2-((8-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-1-yl)oxy)ethyl)-N-methylhexadecan-1-aminium bromide (**5f**) and N-hexadecyl-N-(2-((1-hydroxy-3-methoxy-6-methyl-9,10-anthraquinone-8-yl)oxy)ethyl)-N-methylhexadecan-1-aminium bromide (**6f**).* According to the general procedure, **3** and **4** were treated with *N*-hexadecyl-*N*-hexadecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 10:1) as eluent to give compounds **5f** and **6f**, the proportion of which is about 1.7:1.

Yield: 54.5%; yellow solid; mp: 153–155 °C; IR (KBr) ν: 3430, 2920, 2850, 1682, 1633, 1599, 1333, 1219, 994, 595 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.15 (s, 1.0H, OH), 12.97 (s, 1.7H, OH), 7.84 (s, 1.0H, Ar–H), 7.59 (s, 1.7H, Ar–H), 7.52 (d, J = 2.4 Hz, 1.7H, Ar–H), 7.35 (s, 1.0H, Ar–H), 7.33 (m, 1.0H, Ar–H), 7.07 (m, 1.7H, Ar–H), 7.00 (m, 1.7H, Ar–H), 6.68 (m, 1.0H, Ar–H), 4.77 (m, 5.4H, ArO–$C\underline{H}_2CH_2N$), 4.41 (m, 5.4H, ArO–$CH_2C\underline{H}_2N$), 4.02 (s, 5.1H, OCH_3), 3.93 (s, 3.0H, OCH_3), 3.73 (m, 10.8H, 2× N($C\underline{H}_2$)–$C_{15}H_{31}$), 3.49 (s, 8.1H, N–CH_3), 2.53 (s, 3.0H, Ar–CH_3), 2.44 (s, 5.1H, Ar–CH_3), 1.22–1.80 (m, 151.2H, 2× ($C\underline{H}_2$)$_{14}CH_3$), 0.88 (t, J = 6.8 Hz, 16.2H, 2× $(CH_2)_{14}$–$C\underline{H}_3$); Anal. Calcd for $C_{51}H_{84}BrNO_5 \cdot H_2O$: C 68.89, H 9.75, N 1.58; found: C 69.09, H 9.63, N 1.21; ESI-MS m/z 791.1 $(M - Br)^+$.

4.2.4. The preparation of 9 (6-(bromomethyl)-8-hydroxy-1,3-dimethoxyanthracene-9,10-dione) and 10 (6-(bromomethyl)-1-hydroxy-3,8-dimethoxyanthracene-9,10-dione)

8 110.5 mg (0.28 mmol) was solved in the solution of 10 mL chloroform and 20 mL glacial acetic acid. 2 mL HBr aqueous solution (47%) was then dripped slowly into the mixture under N_2 atmosphere. After being stirred for another 36 h at room temperature, the yellow precipitate was extracted by 50 mL chloroform. The chloroform solution was washed by water (50 mL × 2) and was concentrated under reduced pressure. Then the residue was chromatographed on a silica gel column by CH_2Cl_2 to give **9** and **10**, the proportion of which is about 1:1.

Yield: 75.1%; orange solid; mp: 197–199 °C (documental value: 199–201 °C [12]); IR (KBr) ν: 3435, 3050, 2943, 2846, 1676, 1633, 1596, 1327, 1214, 922, 596 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.17 (s, 1.0H, OH), 13.12 (s, 1.0H, OH), 7.95 (d, J = 1.6 Hz, 1.0H, Ar–H), 7.77 (d, J = 1.6 Hz, 1.0H, Ar–H), 7.48 (d, J = 2.4 Hz, 1.0H, Ar–H), 7.37 (d, J = 1.6 Hz, 1.0H, Ar–H), 7.33 (d, J = 2.4 Hz, 1.0H, Ar–H), 7.29 (d, J = 1.6 Hz, 1.0H, Ar–H), 6.81 (d, J = 2.8 Hz, 1.0H, Ar–H), 6.72 (d, J = 2.4 Hz, 1.0H, Ar–H), 4.54 (s, 2.0H, Ar–CH_2–Br), 4.46 (s, 2.0H, Ar–CH_2–Br), 4.09 (s, 3.0H, OCH_3), 4.04 (s, 3.0H, OCH_3), 4.00 (s, 3.0H, OCH_3), 3.92 (s, 3.0H, OCH_3); ESI-MS m/z 377.1 $(M + H)^+$.

*4.2.5. General procedure for the preparation of **11** and **12***

The mixture of **9** and **10** 100 mg (0.27 mmol) and 0.27 mmol tertiary amine were added into 25 mL chloroform, till **9** and **10** were dissolved, 76–150 mg tertiary (about 0.40–0.5 mmol) was added into the solution, and stirred at refluxed temperature for 10–12 h. Then the mixture was concentrated under reduced pressure and the residue was chromatographed on a silica gel column by gradient elution to give products **11a–j** and **12a–j**. Proportions of **11** to **12** are all about 1:1. The eluent order was dichloromethane first and then chloroform/ethanol (v/v from 50:1 to 5:1).

*4.2.5.1. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyloctan-1-aminium bromide (**11a**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyloctan-1-aminium bromide (**12a**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethyloctan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 30:1), chloroform/ethanol (v/v 5:1) as eluent to give compounds **11a** and **12a**.

Yield: 95.3%; orange solid; mp: 196–197 °C; IR (KBr) ν: 3429, 3017, 2925, 2854, 1671, 1632, 1595, 1334, 1265, 966, 608 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 12.95 (s, 1.0H, OH), 12.94 (s, 1.0H, OH), 8.42 (s, 1.0H, Ar–H), 7.78 (s, 1.0H, Ar–H), 7.72 (s, 1.0H, Ar–H), 7.66 (s, 1.0H, Ar–H), 7.22 (s, 1.0H, Ar–H), 7.13 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.70 (d, J = 1.6 Hz, 1.0H, Ar–H), 6.64 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.44 (s, 2.0H, Ar–CH_2–N), 5.31 (s, 2.0H, Ar–CH_2–N), 4.18 (s, 3.0H, OCH_3), 4.00 (s, 6.0H, OCH_3), 3.93 (s, 3.0H, OCH_3), 3.62 (m, 4.0H, 2× N–CH_2–C_7H_{15}), 3.43 (s, 6.0H, N–$(CH_3)_2$), 3.38 (s, 6.0H, N–$(CH_3)_2$), 1.27 (s, 24H, 2× ($C\underline{H}_2$)$_6$–CH_3), 0.89 (t, J = 6.4 Hz, 6H, 2× $(CH_2)_6$–$C\underline{H}_3$); Anal. Calcd for $C_{27}H_{36}BrNO_5 \cdot 1.5H_2O$: C 57.75, H 7.00, N 2.49; found: C 57.80, H 6.58, N 2.32; ESI-MS m/z 454.8 $(M - Br)^+$.

*4.2.5.2. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyldecan-1-aminium bromide (**11b**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyldecan-1-aminium bromide (**12b**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethyldecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 30:1), chloroform/ethanol (v/v 5:1) as eluent to give compounds **11b** and **12b**.

Yield: 78.4%; orange solid; mp: 189–191 °C; IR (KBr) ν: 3400, 3016, 2924, 2854, 1676, 1632, 1595, 1334, 1266, 924, 608 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.04 (s, 1.0H, OH), 13.00 (s, 1.0H, OH), 8.59 (s, 1.0H, Ar–H), 7.79 (s, 1.0H, Ar–H), 7.72 (s, 1.0H, Ar–H), 7.66 (s, 1.0H, Ar–H), 7.38 (s, 1.0H, Ar–H), 7.26 (s, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.77 (d, J = 1.6 Hz, 1.0H, Ar–H), 6.69 (d, J = 2.4 Hz, 1.0H, Ar–H), 5.37 (s, 2.0H, Ar–CH_2–N), 5.20 (s, 2.0H, Ar–CH_2–N), 4.21 (s, 3.0H, OCH_3), 4.02 (s, 3.0H, OCH_3), 4.00 (s, 3.0H, OCH_3), 3.93 (s, 3.0H, OCH_3), 3.58 (s, 4.0H, 2× N–CH_2–C_9H_{19}), 3.39 (s, 6.0H, N–$(CH_3)_2$), 3.35 (s, 6.0H, N–$(CH_3)_2$), 1.26 (m, 32.0H, 2× ($C\underline{H}_2$)$_8$–CH_3), 0.88 (t, J = 6.4 Hz, 6.0H, 2× $(CH_2)_8$–$C\underline{H}_3$); Anal. Calcd for $C_{29}H_{40}BrNO_5 \cdot 2.5H_2O$: C 57.33, H 7.47, N 2.31; found: C 57.14, H 6.99, N 2.01; ESI-MS m/z 483.0 $(M - Br)^+$.

*4.2.5.3. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyldodecan-1-aminium bromide (**11c**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyldodecan-1-aminium bromide (**12c**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethyldodecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 20:1) as eluent to give compounds **11c** and **12c**.

Yield: 79.4%; orange solid; mp: 185–187 °C; IR (KBr) ν: 3402, 3015, 2923, 2852, 1672, 1632, 1595, 1334, 1266, 925, 609 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.08 (s, 1H, OH), 13.05 (s, 1.0H, OH), 8.61 (s, 1.0H, Ar–H), 7.82 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.69 (s, 1.0H, Ar–H), 7.41 (d, J = 2.0 Hz, 1.0H, Ar–H), 7.28 (d, J = 2.4 Hz, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.80 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.72 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.38 (s, 2.0H, Ar–CH_2–N), 5.20 (s, 2.0H, Ar–CH_2–N), 4.24 (s, 3.0H, OCH_3), 4.05 (s, 3.0H, OCH_3), 4.03 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.60 (m, 4.0H, 2× N–CH_2–$C_{11}H_{23}$), 3.41 (s, 6.0H, N–$(CH_3)_2$), 3.37 (s, 6.0H, N–$(CH_3)_2$), 1.27 (m, 40.0H, 2×

($C\underline{H}_2$)$_{10}$–CH_3), 0.90 (t, J = 6.0 Hz, 6.0H, 2× (CH_2)$_{11}$–$C\underline{H}_3$); Anal. Calcd for $C_{31}H_{44}BrNO_5 \cdot 1.5H_2O$: C 60.29, H 7.67, N 2.27; found: C 60.12, H 7.19, N 2.05; ESI-MS m/z 512.1 $(M - Br)^+$.

*4.2.5.4. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyltetradecan-1-aminium bromide (**11d**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyltetradecan-1-aminium bromide (**12d**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethyltetradecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 20:1) as eluent to give compounds **11d** and **12d**.

Yield: 66.8%; orange solid; mp: 191–193 °C; IR (KBr) ν: 3386, 3017, 2921, 2851, 1670, 1632, 1596, 1335, 1267, 924, 609 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.08 (s, 1.0H, OH), 13.05 (s, 1.0H, OH), 8.60 (s, 1.0H, Ar–H), 7.82 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.69 (s, 1.0H, Ar–H), 7.40 (d, J = 2.0 Hz, 1.0H, Ar–H), 7.27 (d, J = 2.4 Hz, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.79 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.71 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.38 (s, 2.0H, Ar–CH_2–N), 5.22 (s, 2.0H, Ar–CH_2–N), 4.23 (s, 3.0H, OCH_3), 4.05 (s, 3.0H, OCH_3), 4.03 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.60 (m, 4.0H, 2× N–CH_2–$C_{13}H_{27}$), 3.41 (s, 6.0H, N–(CH_3)$_2$), 3.37 (s, 6.0H, N–(CH_3)$_2$), 1.27 (m, 48.0H, 2× ($C\underline{H}_2$)$_{12}$–CH_3), 0.90 (t, J = 5.6 Hz, 6.0H, 2× (CH_2)$_{13}$–$C\underline{H}_3$); Anal. Calcd for $C_{33}H_{48}BrNO_5 \cdot 1.5H_2O$: C 61.39, H 7.96, N 2.17; found: C 61.36, H 7.50, N 2.02; ESI-MS m/z 539.2 $(M - Br)^+$.

*4.2.5.5. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethylhexadecan-1-aminium bromide (**11e**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethylhexadecan-1-aminium bromide (**12e**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethylhexadecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 20:1) as eluent to give compounds **11e** and **12e**.

Yield: 97.8%; orange solid; mp: 195–196 °C; IR (KBr) ν: 3430, 3018, 2919, 2850, 1670, 1632, 1596, 1335, 1267, 924, 609 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.01 (s, 1.0H, OH), 13.00 (s, 1.0H, OH), 8.52 (s, 1.0H, Ar–H), 7.80 (s, 1.0H, Ar–H), 7.75 (s, 1.0H, Ar–H), 7.68 (s, 1.0H, Ar–H), 7.32 (d, J = 2.0 Hz, 1.0H, Ar–H), 7.21 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.75 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.68 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.43 (s, 2.0H, Ar–CH_2–N), 5.28 (s, 2.0H, Ar–CH_2–N), 4.21 (s, 3.0H, OCH_3), 4.03 (s, 3.0H, OCH_3), 4.02 (s, 3.0H, OCH_3), 3.94 (s, 3.0H, OCH_3), 3.60 (m, 4.0H, 2× N–CH_2–$C_{15}H_{31}$), 3.412 (s, 6.0H, N–(CH_3)$_2$), 3.38 (s, 6.0H, N–(CH_3)$_2$), 1.26 (m, 56.0H, 2× ($C\underline{H}_2$)$_{14}$–CH_3), 0.90 (t, J = 5.6 Hz, 6.0H, 2× (CH_2)$_{15}$–$C\underline{H}_3$); Anal. Calcd for $C_{35}H_{52}BrNO_5 \cdot 0.5H_2O$: C 64.11, H 8.15, N 2.14; found: C 64.04, H 7.89, N 1.89; ESI-MS m/z 567.1 $(M - Br)^+$.

*4.2.5.6. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyloctadecan-1-aminium bromide (**11f**) and N-(1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)-N,N-dimethyloctadecan-1-aminium bromide (**12f**).* According to the general procedure, **9** and **10** were treated with *N*,*N*-dimethyloctadecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 25:1) as eluent to give compounds **11f** and **12f**.

Yield: 87.8%; orange solid; mp: 189–191 °C; IR (KBr) ν: 3428, 3013, 2919, 2850, 1670, 1632, 1596, 1334, 1267, 925, 609 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.09 (s, 1.0H, OH), 13.06 (s, 1.0H, OH), 8.64 (s, 1.0H, Ar–H), 7.82 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.69 (s, 1.0H, Ar–H), 7.42 (d, J = 2.0 Hz, 1.0H, Ar–H), 7.29 (s, 1.0H, Ar–H), 6.81 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.72 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.37 (s, 2.0H, Ar–CH_2–N), 5.19 (s, 2.0H, Ar–CH_2–N), 4.24 (s, 3.0H, OCH_3), 4.06 (s, 3.0H, OCH_3), 4.03 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.60 (m, 4.0H, 2× N–CH_2–$C_{17}H_{35}$), 3.41 (s, 6.0H, N–(CH_3)$_2$), 3.37 (s, 6.0H, N–(CH_3)$_2$), 1.27 (m, 64.0H, 2× ($C\underline{H}_2$)$_{16}$-CH_3), 0.90 (t, J = 5.6 Hz, 6.0H, 2× (CH_2)$_{17}$–$C\underline{H}_3$); Anal. Calcd for $C_{37}H_{56}BrNO_5 \cdot 1.0H_2O$: C 64.15, H 8.44, N 2.02; found: C 64.56, H 7.96, N 1.87; ESI-MS m/z 595.0 $(M - Br)^+$.

*4.2.5.7. N-((8-Hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyl-N-octyloctan-1-aminium bromide (**11g**) and N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyl-N-octyloctan-1-aminium bromide (**12g**).* According to the general procedure, **9** and **10** were treated with *N*-methyl-*N*-octyloctan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 25:1) as eluent to give compounds **11g** and **12g**.

Yield: 66.4%; orange powder; mp: 91–93 °C; IR (KBr) ν: 3401, 3009, 2925, 2855, 1674, 1633, 1596, 1335, 1265, 923, 607 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.04 (s, 2.0H, OH), 8.65 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.75 (s, 1.0H, Ar–H), 7.71 (s, 1.0H, Ar–H), 7.40 (d, J = 1.6 Hz, 1.0H, Ar–H), 7.27 (d, J = 2.0 Hz, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.79 (d, J = 1.6 Hz, 1.0H, Ar–H), 6.71 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.33 (s, 2.0H, Ar–CH_2–N), 5.20 (s, 2.0H, Ar–CH_2–N), 4.29 (s, 3.0H, OCH_3), 4.05 (s, 3.0H, OCH_3), 4.02 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.50 (m, 8.0H, 2× N–($C\underline{H}_2$–C_7H_{15})$_2$), 3.35 (s, 3.0H, N–CH_3), 3.31 (s, 3.0H, N–CH_3), 1.43–1.30 (m, 48.0H, 4× ($C\underline{H}_2$)$_6$–CH_3), 0.92–0.89 (m, 12.0H, 4× (CH_2)$_7$–$C\underline{H}_3$); Anal. Calcd for $C_{34}H_{50}BrNO_5 \cdot 1.0H_2O$: C 62.76, H 8.06, N 2.15; found: C 62.27, H 7.57, N 1.92; ESI-MS m/z 553.2 $(M - Br)^+$.

*4.2.5.8. N-Decyl-N-((8-hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyldecan-1-aminium bromide (**11h**) and N-decyl-N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyldecan-1-aminium bromide (**12h**).* According to the general procedure, **9** and **10** were treated with *N*-decyl-*N*-methyldecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 25:1) as eluent to give compounds **11h** and **12h**.

Yield: 79.6%; orange powder; mp: 81–83 °C; IR (KBr) ν: 3413, 3006, 2925, 2854, 1675, 1633, 1596, 1329, 1265, 924, 608 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.02 (s, 2.0H, OH), 8.63 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.74 (s, 1.0H, Ar–H), 7.71 (s, 1.0H, Ar–H), 7.38 (d, J = 2.0 Hz, 1.0H, Ar–H), 7.26 (d, J = 2.0 Hz, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.77 (d, J = 2.0 Hz, 1.0H, Ar–H), 6.69 (d, J = 2.0 Hz, 1.0H, Ar–H), 5.35 (s, 2.0H, Ar–CH_2–N), 5.22 (s, 2.0H, Ar–CH_2–N), 4.21 (s, 3.0H, OCH_3), 4.04 (s, 3.0H, OCH_3), 4.02 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.49 (m, 8.0H, 2× N–($C\underline{H}_2$–C_9H_{19})$_2$), 3.35 (s, 3.0H, N–CH_3), 3.30 (s, 3.0H, N–CH_3), 1.41–1.23 (m, 64.0H, 4× ($C\underline{H}_2$)$_8$–CH_3), 0.90 (t, J = 5.6 Hz, 12.0H, 4× (CH_2)$_9$–$C\underline{H}_3$); Anal. Calcd for $C_{38}H_{58}BrNO_5 \cdot 0.7H_2O$: C 65.07, H 8.54, N 2.00; found: C 64.97, H 8.16, N 1.76; ESI-MS m/z 609.2 $(M - Br)^+$.

*4.2.5.9. N-Dodecyl-N-((8-hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyldodecan-1-aminium bromide (**11i**) and N-dodecyl-N-((1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methyldodecan-1-aminium bromide (**12i**).* According to the general procedure, **9** and **10** were treated with *N*-dodecyl-*N*-methyldodecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 30:1) as eluent to give compounds **11i** and **12i**.

Yield: 72.3%; orange powder; mp: 81–83 °C; IR (KBr) ν: 3434, 3009, 2924, 2853, 1675, 1633, 1596, 1323, 1265, 922, 607 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 13.04 (s, 2.0H, OH), 8.64 (s, 1.0H, Ar–H), 7.77 (s, 1.0H, Ar–H), 7.75 (s, 1.0H, Ar–H), 7.72 (s, 1.0H, Ar–H), 7.39 (s, 1.0H, Ar–H), 7.29 (s, 1.0H, Ar–H, overlapped with $CHCl_3$), 6.78 (s, 1.0H, Ar–H), 6.70 (s, 1.0H, Ar–H), 5.34 (s, 2.0H, Ar–CH_2–N), 5.21 (s, 2.0H, Ar–CH_2–N), 4.22 (s, 3.0H, OCH_3), 4.04 (s, 3.0H, OCH_3), 4.02 (s, 3.0H, OCH_3), 3.95 (s, 3.0H, OCH_3), 3.50 (m, 8.0H, 2×

N–(C$\underline{H}_2$–$C_{11}H_{23}$)$_2$), 3.36 (s, 3.0H, N–CH_3), 3.31 (s, 3.0H, N–CH_3), 1.27 (m, 80.0H, 4× (C$\underline{H}_2$)$_{10}$–CH_3), 0.90 (t, J = 5.6 Hz, 12.0H, 4× $(CH_2)_{11}$–C$\underline{H}_3$); Anal. Calcd for $C_{42}H_{66}BrNO_5 \cdot H_2O$: C 66.12, H 8.98, N 1.84; found: C 66.02, H 8.73, N 1.63; ESI-MS m/z 665.1 $(M - Br)^+$.

*4.2.5.10. N-Hexadecyl-N-((8-hydroxy-1,3-dimethoxy-9,10-anthraquinone-6-yl)methyl)-N-methylhexadecan-1-aminium bromide (**11j**) and N-hexadecyl-N-(1-hydroxy-3,8-dimethoxy-9,10-anthraquinone-6-yl)-N-methylhexadecan-1-aminium bromide (**12j**).* According to the general procedure, **9** and **10** were treated with *N*-hexadecyl-*N*-methylhexadecan-1-amine, and then purified on silica gel column using dichloromethane, chloroform/ethanol (v/v 50:1), chloroform/ethanol (v/v 30:1) as eluent to give compounds **11j** and **12j**.

Yield: 61.7%; orange powder; mp: 76–78 °C; IR (KBr) ν: 3391, 2923, 2853, 1675, 1633, 1596, 1327, 1265, 924, 606 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 12.99 (s, 2.0H, OH), 8.58 (s, 1.0H, Ar–H), 7.76 (m, 3.0H, Ar–H), 7.33 (d, J = 2.4 Hz, 1.0H, Ar–H), 7.24 (d, J = 1.6 Hz, 1.0H, Ar–H), 6.74 (s, 1.0H, Ar–H), 6.67 (d, J = 1.6 Hz, 1.0H, Ar–H), 5.37 (s, 2.0H, Ar–CH_2–N), 5.26 (s, 2.0H, Ar–CH_2–N), 4.22 (s, 3.0H, OCH_3), 4.03 (s, 3.0H, OCH_3), 4.01 (s, 3.0H, OCH_3), 3.94 (s, 3.0H, OCH_3), 3.50–3.30 (m, 14.0H, 2× N–(C$\underline{H}_2$–$C_{11}H_{23}$)$_2$ and 2× N–CH_3), 1.42–1.27 (m, 112.0H, 4× (C$\underline{H}_2$)$_{14}$–CH_3), 0.90 (t, J = 5.6 Hz, 12.0H, 4× $(CH_2)_{15}$–C$\underline{H}_3$); Anal. Calcd for $C_{50}H_{82}BrNO_5 \cdot 0.8H_2O$: C 68.91, H 9.67, N 1.61; found: C 68.67, H 9.23, N 1.34; ESI-MS m/z 777.2 $(M - Br)^+$.

*4.2.6. The preparation of **13f** (N-((1,3,8-trimethoxy-9,10-anthraquinone-6-yl)methyl)-N,N-dimethyloctadecan-1-aminium bromide)*

The compound **8** (100 mg, 0.26 mmol) and *N*,*N*-dimethyloctadecan-1-amine (80.6 mg, 0.27 mmol) were added into 25 mL chloroform, and then the mixture was stirred at refluxed temperature for 24 h. Then the mixture was concentrated under reduced pressure and the residue was chromatographed on a silica gel column by gradient elution to give product **13f**. The eluent order was dichloromethane first and then chloroform/ethanol (v/v from 30:1 to 5:1).

Yield: 90.2%; orange powder; mp: 180–181 °C; IR (KBr) ν: 2923, 2852, 1662, 1598, 1466, 1337, 1245, 1020, 752 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ: 8.43 (s, 1.0H, Ar–H), 7.72 (s, 1.0H, Ar–H), 7.30 (d, J = 2.5 Hz, 1.0H, Ar–H), 6.79 (s, 1.0H, Ar–H), 5.28 (s, 2.0H, Ar–CH_2–N), 4.17 (s, 3.0H, OCH_3), 3.990 (s, 3.0H, OCH_3), 3.987 (s, 3.0H, OCH_3), 3.58 (s, 2.0H, N–CH_2–$C_{17}H_{35}$), 3.36 (s, 6.0H, N–$(CH_3)_2$), 1.41–1.27 (m, 32.0H, (C$\underline{H}_2$)$_{16}$–CH_3), 0.90 (t, J = 7.0 Hz, 3.0H, $(CH_2)_{17}$–C$\underline{H}_3$); Anal. Calcd for $C_{38}H_{58}BrNO_5 \cdot 1.8H_2O$: C 63.28, H 8.61, N1.94; found: C 63.23, H 8.25, N 1.93; ESI-MS m/z 609.0 $(M - Br)^+$.

4.3. Cell lines and culture

HepG2, BGC-823, AGS and HELF were all obtained from our own laboratory. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in a RPMI 1640 cell culture medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (10 mg/mL).

4.4. Cell viability assay

Tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Sigma] assays were used to evaluate the inhibitory effect on cell viability induced by emodin derivatives. In brief, cells (1×10^4) were seeded on 96-well plates with RPMI medium containing 10% fetal bovine serum in a final volume of 0.12 mL, incubated overnight, and then treated with drugs for 24 h. After the drug treatment, MTT solution was added to each well and incubated for 3–5 h before the medium was removed. DMSO was then added and plates were shaken for 10 min at room temperature. Cell viability was determined by measuring absorbance at 490 nm on an ELISA reader. Each test was repeated at least three times. The concentration of the compound, which gives 50% growth inhibition value, corresponds to the IC_{50}.

4.5. Apoptosis tests

4.5.1. Assessment of nuclear morphology

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine orange/ethidium bromide (AO/EB) staining. AGS cells grown in 6-well plates at seeding densities of 2×10^5 cells were treated with compounds **11g** + **12g** (3 μM) and **11h** + **12h** (3 μM) for 24 h. After washing twice with phosphate buffered saline (PBS), 25 μL cells were incubated with 1 μL of a mixture (1:1) of AO/EB solutions at room temperature in the dark. Stained cells were visualized with a Nikon fluorescence microscope measuring at 515 nm.

4.5.2. 4.5.2Caspase-3 and caspase-9 enzyme assay

The activities of caspase-3 and caspase-9 proteins were determined following the test kit protocol. In brief, after being treated with compounds **11g** + **12g** and **11h** + **12h** for 24 h, cells (10^5/mL) were harvested, washed once with PBS and centrifuged at 500 *g* for 5min, and then 25 μL of lysis buffer were added to the cells on ice for 10 min. Cell lysates were clarified by centrifugation at 4 °C, 12,000 *g* for 10 min, and 10 μL of supernatant were incubated with 80 μL of react buffer, 10 μL of caspase-3 substrate Ac-DEVD-pNA or caspase-9 substrate Ac-LEHD-pNA at 37 °C for 60–120 min in dark. Activities of caspase-3 and caspase-9 were described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

4.6. Assay for cell cycle distribution

AGS cells were cultured in 96-well plates at a concentration of 5×10^5/well. After 24 h, cells were treated with compounds **11g** + **12g** and **11h** + **12h**, in parallel with cells grown in the absence of emodin derivatives to determine effects on cell cycle distribution. Cells were collected and fixed with 70% ice ethanol. Cell pellets were suspended in 8 μL of 10 mg/mL RNase (Sigma Chemical). After incubated in the dark at 37 °C for 30 min, 10 μL of propidium iodide (Sigma Chemical) were added and incubated in the dark at room temperature for 30 min. Data acquisition and analysis were performed on a flow cytometry (BD FACS Calibur).

4.7. Evaluation of therapeutic effect in vivo

Briefly, the method was similar to what we used previously [17]. Kunming mice (16–19 g body weight, 6–8 weeks old) were housed in a temperature controlled room and given free access to water and standard laboratory chow during the study period. After inoculation with murine hepatoma H22 cells for 24 h, tumor-bearing mice were randomly divided into negative control (normal saline), positive control (CTX, 1.5 mg/mL), compound **11g** + **12g** treatment (5, 10, 20 mg/kg), and compound **11h** + **12h** treatment (5, 10, 20 mg/kg). Ten mice are used for experiments for each group. Tumor inhibition was determinated by injection to mice at a dose of 0.2 mL/10 g body weight for eight successive days. The mice were weighed prior to sacrifice. All the animals were killed at the end of the 8th day and the tumor were weighted at the time of sacrifice. The tumor inhibitory rates were then calculated.

4.8. Statistical analysis

The data were presented as means ± standard deviations of three determinations. Statistical analysis was performed by using Student's *t*-test and one-way analysis of variance. Multiple comparisons of the means were done by the least significance

difference (LSD) test. A probability value of $P < 0.05$ was considered significant. All computations were made by employing SPSS statistical software (version 13.0).

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