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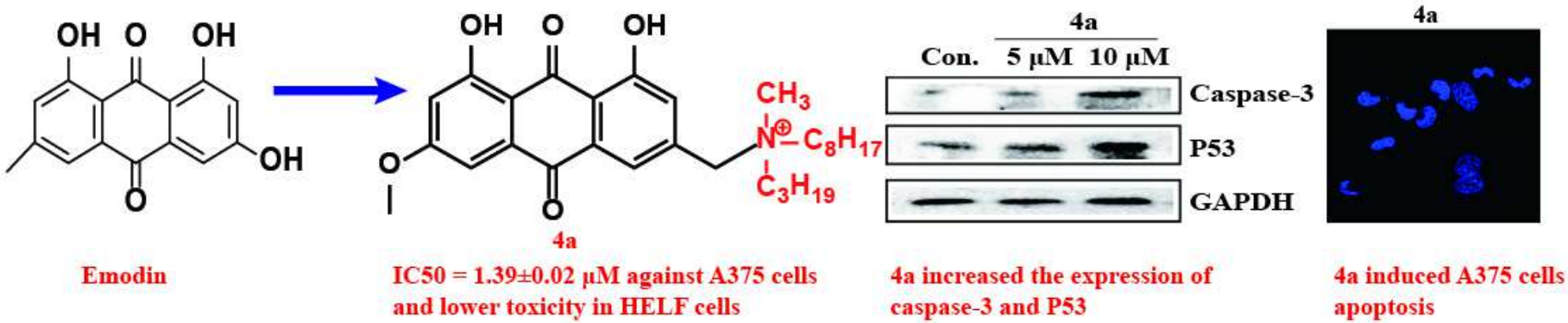
Graphical abstract

Fifteen new emodin derivatives were synthesized and investigated for their anticancer activities. Compound **4a** was shown to induce apoptosis in cancer cells *in vitro.*



Synthesis, SAR and pharmacological characterization of novel anthraquinone cation compounds as potential anticancer agents

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Abstract

Emodin, a natural anthraquinone derivative isolated from *Rheum palmatum L.*, has been demonstrated to exhibit good anti-cancer effect. In this study, a series of novel quaternary ammonium salts of emodin, anthraquinone and anthrone were synthesized and their anticancer activities were tested *in vitro*. The effects of emodin quaternary ammonium salts on cell viability, apoptosis, intracellular ROS, and mitochondrial membrane potential were investigated in A375, BGC-823, HepG2 and HELF cells. The results demonstrated that compound **4a** induced morphological changes and decreased cell viability. Apoptosis triggered by compound **4a** was visualized using DAPI staining and Annexin V-FITC/PI staining. Compound **4a**-induced apoptosis of A375 cells were showed to be associated with the dissipation of mitochondrial membrane potential ($\Delta\Psi m$) as a result of the up-regulation of P53 and Caspase-3. When cancer cells were treated with emodin derivative, their ability to generate reactive oxygen species (ROS) rose significantly and the mitochondrial membrane potential decreased. Additionally, confocal microscopy assay confirmed that compound **4a** was primarily located in the mitochondria of A375 cells. These results suggested that compound **4a** has the potential for use in cancer therapy.

Keyword: Quaternary ammonium salt; Reactive oxygen species; Anticancer activity; Quinoid structure; Synthesis.

1. Introduction

Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone) has been identified as a natural product from herbs used in traditional medicine for treatment of constipation jaundice, gastro-intestinal hemorrhage and ulcers in China for more than two thousand years. It has been shown to display a number of biological activities such as anti-tumor [1], antimicrobial [2], immunosuppressive [3], and enzyme inhibition [4]. Recently, pharmacological studies showed that emodin is capable of inhibiting cellular proliferation [5], and activation of caspase cascade and mitochondrial death pathways [6] in different cancer cells. .

In addition, chemical modifications of anthraquinone have been more commonly reported than those of emodin due to its accessibility. Shrestha *et al* [7] synthesized a series of novel analogs of cationic anthraquinones whose possible modes of interaction with cancer cells are intercalation of nucleic acids and disruption of cellular redox processes. Joksovic *et al* [8] synthesized a series of anthraquinone-chalcone hybrids which showed promising activity in inhibition of HeLa cells and low cytotoxicity against healthy MRC-5 cell lines. Lee *et al* [9] used ring fusion strategy to synthesize sulfur-substituted anthrax [1,2-c][1,2,5] thiadiazole-6,11-dione derivatives which not only induced apoptosis of cancer cells but also attenuated the ERK1/2 and p38 signaling pathways. Zhao *et al* [10] reported that an introduction of mustard group into hydrioxyanthraquinone moiety enhanced drug's anticancer activities based on rational . In addition, natural anthraquinones, such as Rubiadin and Soranjidiol [11], are reported to induce apoptosis in cancer cells.

These works indicate that quinoid structure may play an important role in anticancer activity. Figure **1** shows some structures of derivatives of anthraquinone mentioned above.

We previously reported that emodin quaternary ammonium salt derivatives showed significant anticancer activities against hepatoma cells both *in vitro* and *in vivo* [12,13]. We also reported that lipophilicity and the length of the carbon chains on the quaternary (4°) nitrogen atom are important factors and chemical moieties accounting for the anticancer activities of the emodin quaternary ammonium salt derivatives [14,15]. To fully address the structure-activity relationship (SAR) and further refine the molecular structure of the emodin derivatives for anticancer activities, a number of questions should be addressed first. (1) Can other alkyls, such as nonyl, bring better anticancer activity for emodin quaternary ammonium salts? (2) If lipophilic long carbon chain is substituted by polyoxyethylene chain to enhance the water solubility of emodin quaternary ammonium salt, how will the anticancer activity change? (3) what is the mechanism of action of these emodin quaternary ammonium salt derivatives? (4) Is quinoid structure necessary for the anticancer activities of emodin quaternary ammonium salts? In order to answer these questions, fifteen new quaternary ammonium salts of emodin, aloe emodin, anthraquinone and anthrone were synthesized and their anticancer activities were tested *in vitro*. In addition, the anti-cancer mechanism of compound **4a** was also evaluated against A375 cell line.

2. Materials and methods

2.1. General

The chemical agents and the apparatus to test the structures of compounds synthesized in this work are the same as those have been reported in our recent work [12,13,19].

2.2. Synthesis

2.2.1. The preparation of compound ***1*** (N-methylnonan-1-amine)

1-Bromononane (15 mL, 16.3 g, 79.1 mmol) in 15 mL ethanol was added into 50 mL aqueous methylamine (40%, excessive). The mixture was stirred at room temperature for 5 h. The mixture was washed by 80 mL distilled water for 3 times to remove unreacted methylamine and the water phase was extracted by $CHCl_3$ (30 mL×3). The organic phases were combined and dried by Na_2SO_4. After the solvent had been removed under reduced pressure, the residue (light yellow sticky liquid) was chromatographed on a silica gel column by using dichloromethane/ethanol (v/v 20:1) as eluents to give product ***1***.

Yield: 94.0%; Colorless sticky liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 3.69 (s, 1H, -NH), 2.61 (t, J = 6.8Hz, 2H, -NCH_2-), 2.45 (s, 3H, -NCH_3), 1.53 (m, 2H, -$NCH_2C\underline{H}_2$-), 1.26 (m, 12H, 6×-CH_2-), 0.87 (t, J = 6.8Hz, 3H, -CH_3); ESI-MS, m/z: 158.18$[M+H]^+$.

2.2.2. General procedure for preparation of compounds ***2a-2c***

Compound ***1*** (0.015 mol), brominated alkanes (0.015 mol) and NaOH (0.6 g, 0.015 mol) were dissolved in 30 mL ethanol. The mixture was stirred and refluxed for 5 h and then washed by 30 mL distilled water for 3 times. The water phase was

extracted by $CHCl_3$ (30 mL×3). The organic phases were combined and dried by Na_2SO_4. After the solvent had been removed under reduced pressure, the residue (light yellow sticky liquid) was chromatographed on a silica gel column by using dichloromethane as eluents to give product ***2***.

2.2.2.1. N-methyl-N-octylnonan-1-amine (***2a***) According to the general procedure, compound ***1*** was treated with 1-bromooctane and then purified on silica gel column to give compound ***2a***.

Yield: 94.1%; Colorless liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 2.33 (m, 4H, -C$\underline{H_2}$NC$\underline{H_2}$-), 2.23 (s, 3H, -NCH_3), 1.48 (m, 4H, 2×-$NCH_2C\underline{H_2}$-), 1.29 (m, 22H, 11×-CH_2-), 0.90 (m, 6H, 2×-CH_3); ESI-MS, m/z: 270.37$[M+H]^+$.

2.2.2.2. N-methyl-N-nonylnonan-1-amine (***2b***) According to the general procedure, compound ***1*** was treated with 1-bromononane and then purified on silica gel column to give compound ***2b*.**

Yield: 95.1%; Colorless liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 2.33 (t, *J* = 7.2Hz, 4H, -C$\underline{H_2}$NC$\underline{H_2}$-), 2.22 (s, 3H, -NCH_3), 1.47 (m, 4H, 2×-$NCH_2C\underline{H_2}$-), 1.27 (m, 24H, 12×-CH_2-), 0.88 (t, *J* = 7.2Hz, 6H, 2×-CH_3); ESI-MS, m/z: 284.34$[M+H]^+$.

2.2.2.3. N-methyl-N-nonyldecan-1-amine (***2c***) According to the general procedure, compound ***1*** was treated with 1-bromodecane and then purified on silica gel column to give compound ***2c*.**

Yield: 95.0%; Colorless liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 2.32 (t, *J* = 7.6Hz, 4H, -C$\underline{H_2}$NC$\underline{H_2}$-), 2.22 (s, 3H, -NCH_3), 1.47 (m, 4H, 2×-$NCH_2C\underline{H_2}$-), 1.28 (m, 26H, 13×-CH_2-), 0.89 (t, *J* = 6.8Hz, 6H, 2×-CH_3); ESI-MS, m/z: 298.39$[M+H]^+$.

2.2.3. The preparation of compound ***2d*** (N,N-dimethylnonan-1-amine)

4 mL aqueous dimethylamine (33%, m/m, excessive) reacted with 5 mmol 1-bromononane in 30 mL ethanol under stirring and reflux for 5 h, then the mixture was washed by distilled water (3×30 mL) to get rid of unreacted dimethylamine. The water phase was extracted by $CHCl_3$ (3×30 mL). The organic phases were combined and dried by Na_2SO_4. After the solvent had been removed under reduced pressure, the crude product (light yellow sticky liquid) was chromatographed on a silica gel column by using dichloromethane as eluents to give product ***2d***.

Yield: 95.0%; Colorless liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 3.61 (t, *J* = 8.8Hz, 4H, -C<u>H</u>$_2$NC<u>H</u>$_2$-), 3.51 (s, 6H, -N(CH_3)$_2$), 1.73 (m, 2H, -NC<u>H</u>$_2$C<u>H</u>$_2$-), 1.23 (m, 12H, 6×-CH_2-), 0.84 (t, *J* = 7.2Hz, 3H, -CH_3); ESI-MS, m/z: 172.24$[M+H]^+$.

2.2.4. General procedure for preparation of compound ***4a-4d***

Compound ***3*** (0.27 mmol) and equimolar tertiary amine (***2a-2d***) in 35 mL $CHCl_3$ were stirred and refluxed for 24 h. After removal of the solvent, the crude product was chromatographed on a silica gel column by gradient elution to give products ***4a-4d***, the eluent order were dichloromethane first and then dichloromethane/ethanol (v/v from 50:1 to 25:1).

2.2.4.1.

N-octyl-N-((4,5-dihydroxy-7-methoxy-9,10-anthraquinone-2-yl)methyl)-N-methylnonan-1-aminium bromide (***4a***). According to the general procedure, compound ***3*** was treated with N-methyl-N-octylnonan-1-amine and then purified on silica gel column to give compound ***4a***.

Yield: 63.7%; Purple red solid; m.p.135-138˚C; ^{1}H NMR (400MHz, $CDCl_3$) δ: 12.07 (s, 1H, OH), 12.04 (s, 1H, OH), 7.84 (s, 1H, Ar-H), 7.77 (s, 1H, Ar-H), 7.31 (d, *J = 2.4Hz*, 1H, Ar-H), 6.69 (d, *J* = 2.4*Hz*, 1H, Ar-H), 5.27 (s, 2H, $ArCH_2N^+$), 3.97 (s, 3H, OCH_3), 3.50 (t, *J* = 8.0 *Hz* ,4H, -$C\underline{H}_2N^+C\underline{H}_2$-), 3.35 (s, 3H, -$N^+CH_3$), 1.84 (m, 4H, 2×-$N^+CH_2C\underline{H}_2$-), 1.42-1.30 (m, 22H, 11×-CH_2,), 0.90 (t, *J* = 6.0*Hz*, 6H, 2×-CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 189.9, 180.1, 167.0, 165.5, 162.2, 136.2, 134.3, 133.4, 129.7, 122.9, 116.4, 109.7, 108.8, 106.8, 63.8, 61.0, 56.2, 48.2, 31.7, 29.3, 29.1, 26.3, 22.6, 14.0; Anal. Calcd for $C_{34}H_{50}BrNO_5 \cdot 0.6H_2O$: C 63.46, H 8.02, N 2.18; Found: C 63.48, H 7.88, N 1.93; ESI-MS, m/z: 552.51 $[M\text{-}Br]^+$.

2.2.4.2.

N-nonyl-N-((4,5-dihydroxy-7-methoxy-9,10-anthraquinone-2-yl)methyl)-N-methylnonan-1-aminium bromide (***4b***). According to the general procedure, compound ***3*** was treated with N-methyl-N-nonylnonan-1-amine and then purified on silica gel column to give compound ***4b***.

Yield: 67.5%; Purple red solid; m.p.110-112˚C; ^{1}H NMR (400MHz, $CDCl_3$) δ: 12.09 (s, 1H, OH), 12.07 (s, 1H, OH), 7.89 (s, 1H, Ar-H), 7.77 (s, 1H, Ar-H), 7.33 (d, *J* = 2.0*Hz*, 1H, Ar-H), 6.69 (d, *J* = 2.0*Hz*, 1H, Ar-H), 5.23 (s, 2H, $ArCH_2{}^+N$), 3.95 (s, 3H, OCH_3), 3.47 (m, 4H,-$C\underline{H}_2N^+C\underline{H}_2$-); 3.34 (s, 3H, -$N^+CH_3$), 1.88-1.74 (m, 4H, 2×-$N^+CH_2C\underline{H}_2$-), 1.43-1.26 (m, 24H, 12×-CH_2-), 0.88 (t, *J = 6.8Hz*, 6H, 2×-CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 189.9, 180.1, 167.1, 165.6, 162.1, 136.2, 134.3, 133.4, 129.6, 122.9, 116.4, 109.7, 108.8, 106.8, 63.7, 61.0, 56.2, 53.4, 48.2, 31.7, 29.4, 29.2, 29.1, 26.3, 22.7, 22.6, 14.0; Anal. Calcd for $C_{35}H_{52}BrNO_5 \cdot 1.3H_2O$: C 62.73, H 8.21, N

2.09; Found: C 62.78, H 8.00, N 1.87; ESI-MS, m/z: 566.50 $[M\text{-}Br]^+$.

2.2.4.3.

N-nonyl-N-((4,5-dihydroxy-7-methoxy-9,10-anthraquinone-2-yl)methyl)-N-methylde can-1-aminium bromide (***4c***). According to the general procedure, compound ***3*** was treated with N-methyl-N-nonyldecan-1-amine and then purified on silica gel column to give compound ***4c***.

Yield: 63.5%; Purple red solid; m.p.110-112˚C; ^{1}H NMR (400MHz, $CDCl_3$) δ: 12.12 (s, 1H, OH), 12.08 (s, 1H, OH), 7.88 (s, 1H, Ar-H), 7.78 (s, 1H, Ar-H), 7.35 (d, J=2.4*Hz*, 1H, Ar-H), 6.71 (d, J = 2.0*Hz*, 1H, Ar-H), 5.25 (s, 2H, $ArCH_2N^+$), 3.97 (s, 3H, OCH_3), 3.51(s, 4H,-C<u>H</u>$_2$N$^+$C<u>H</u>$_2$-), 3.35 (s, 3H, -N^+CH_3), 1.86-1.78 (m, 4H, 2×-N^+CH_2C<u>H</u>$_2$-), 1.42-1.29 (m, 26H, 13×-CH_2), 0.90 (t, J = 6.8*Hz*, 6H, 2×-CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 190.0, 180.3, 167.1, 165.6, 162.2, 136.0, 134.4, 133.5, 129.7, 122.8, 116.5, 109.8, 108.9, 106.8, 65.8, 61.0, 56.2, 48.4, 31.8, 31.7, 29.4, 29.3, 29.2, 29.1, 26.3, 22.7, 22.6, 14.0; Anal. Calcd for $C_{36}H_{54}BrNO_5\cdot 1.4H_2O$: C 63.04, H 8.35, N 2.04; Found: C 63.06, H 7.97, N 2.06; ESI-MS, m/z: 580.38 $[M\text{-}Br]^+$.

2.2.4.4.

N,N-dimethyl-N-((4,5-dihydroxy-7-methoxy-9,10-anthraquinone-2-yl)methyl)nonan-1-aminium bromide (***4d***). According to the general procedure, compound ***3*** was treated with N,N-dimethylnonan-1-amine and then purified on silica gel column to give compound ***4d***.

Yield: 65.5%; Bright yellow solid; m.p.160-162˚C; ^{1}H NMR (500MHz, $CDCl_3$) δ: 12.00 (s, 2H, OH), 7.79 (s, 1H, Ar-H), 7.76 (s, 1H, Ar-H), 7.21 (d, J = 2.5*Hz*, 1H,

Ar-H), 6.64 (d, *J* = 2.5*Hz*, 1H, Ar-H), 5.29 (s, 2H, $ArCH_2N^+$), 3.96 (s, 3H, OCH_3), 3.58 (t, *J* = 8.0*Hz*, 2H, $-N^+CH_2-$), 3.41 (s, 6H, $2\times-N^+CH_3$), 1.90-1.87 (m, 2H, $-N^+CH_2CH_2-$), 1.40-1.28 (m, 12H, $6\times-CH_2-$), 0.89 (t, *J* = 7.0*Hz*, 3H, $-CH_3$); ^{13}C NMR (400 MHz, $CDCl_3$) *δ*: 190.0, 180.3, 167.1, 165.6, 162.2, 136.4, 134.4, 133.5, 129.5, 123.1, 116.6, 109.7, 108.9, 106.8, 66.7, 64.6, 63.8, 56.2, 53.4, 51.4, 50.2, 31.8, 31.6, 29.3, 29.2, 29.1, 26.3, 23.0, 22.8, 22.6, 14.0; Anal. Calcd for $C_{27}H_{36}BrNO_5\cdot 0.4H_2O$: C 59.87, H 6.85, N 2.59; Found: C 59.85, H 7.15, N 2.67; ESI-MS, m/z: 454.37 $(M-Br)^+$.

2.2.5. The preparation of compound ***6a*** (N,N-dioctyl-N-((4,5,7-trimethoxy-9,10-anthraquinone-2-yl)methyl)octan-1-aminium bromide)

Compound ***3*** (100 mg, 0.260 mmol) and 0.1 mL trioctylamine (excessive) in 20 mL $CHCl_3$ were stirred and refluxed for 10 h. After removal of the solvent, the crude product was chromatographed on a silica gel column by using dichloromethane/ethanol (v/v from 20:1 to 25:1) as eluent to give ***6a*** (74 mg) as product.

Yield: 46.2%; Yellow solid; m.p.146-148˚C; ^{1}H NMR (400MHz, $CDCl_3$) *δ*: 8.43 (s, 1H, Ar-H), 7.60 (s, 1H, Ar-H), 7.29 (s, 1H, Ar-H), 6.73 (s, 1H, Ar-H), 5.23 (s, 2H, $ArCH_2N^+$), 4.16 (s, 3H, $-OCH_3$), 3.97 (s, 3H, $-OCH_3$), 3.95 (s, 3H, $-OCH_3$), 3.35 (t, *J* = 8.0 Hz, 6H, $3\times-N^+CH_2-$), 1.84 (m, 6H, $3\times-N^+CH_2CH_2-$), 1.32 (m, 30H, $15\times-CH_2-$), 0.87 (t, *J* = 6.8 Hz, 9H, $3\times-CH_3$); ^{13}C-NMR (400 MHz, $CDCl_3$) *δ*: 183.0, 180.7, 164.0, 161.8, 160.3, 135.8, 134.5, 133.3, 124.6, 124.2, 120.8, 118.0, 105.4, 102.1, 62.3, 59.6,

58.9, 58.2, 56.4, 55.9, 53.4, 31.6, 29.0, 26.5, 22.9, 22.6, 14.0; Anal. Calcd for $C_{42}H_{66}BrNO_5 \cdot 0.6H_2O$: C 66.75, H 8.96, N 1.85; Found: C 66.60, H 8.75, N 1.88; ESI-MS, m/z: 664.47$[M-Br]^+$.

2.2.6. The preparation of compound ***6b*** (2-(2-methoxyethoxy)-N-(2-(2-methoxyethoxy)ethyl)-N-((4,5,7-trimethoxy-9,10-anthraquinone-2-yl)methyl)ethanaminium bromide)

Compound ***5*** (150 mg, 0.384 mmol) and tris(3,6-dioxaheptyl)amine (124 mg, 0.384 mmol) in 20 mL methylglycol were stirred and refluxed for 24 h. After removal of the solvent, the crude product was chromatographed on a silica gel column by using dichloromethane/ethanol (v/v from 20:1 to 25:1) as eluent to give ***6b*** (86 mg) as product.

Yield: 31.4%; Yellow viscous liquid; ^{1}H NMR (400MHz, $CDCl_3$) δ: 8.22 (s, 1H, Ar-H), 8.04 (s, 1H, Ar-H), 7.34 (d, J = 2.8 Hz, 1H, Ar-H), 6.82 (d, J = 2.8 Hz, 1H, Ar-H), 5.23 (s, 2H, $ArCH_2N^+$), 4.17 (m, 6H, 3×$C\underline{H_2}OCH_3$), 4.14 (s, 3H, -OCH_3), 4.01 (s, 3H, -OCH_3), 3.98 (s, 3H, -OCH_3), 3.98-3.96 (m, 6H, 3×-N^+CH_2), 3.72-3.67 (m, 12H, 3×$C\underline{H_2}OC\underline{H_2}$), 3.39 (s, 9H, 3×$CH_2OC\underline{H_3}$**)**; ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 183.4, 181.1, 164.2, 161.8, 160.2, 136.1, 134.7, 133.3, 125.0, 124.8, 123.1, 118.2, 105.4, 102.3, 71.7, 71.5, 70.4, 70.3, 64.8, 58.9, 56.5, 55.9, 54.7; ESI-MS, m/z: 634.37$[M-Br]^+$; HRMS (ESI^+), calcd for $C_{33}H_{48}NO_{11}$ 634.3227 $[M-Br]^+$; Found, 634.3222.

2.2.7. The preparation of compound (1,3-bis((4-bromomethyl)benzyl)oxy)-8-hydroxy-6-methylanthraquinone (***7a***) and

(1,6-bis((4-bromomethyl)benzyl)oxy)-8-hydroxy-3-methylanthraquinone (***7b***)

Emodin 100mg(0.37 mmol) and K_2CO_3 102 mg(0.74 mmol) were dissolved in 100 mL acetone and the mixture was heat till reflux, then 1,4-bis(bromomethyl)benzene 195 mg (0.74 mmol) was added to react 2 h. When the mixture cooled to room temperature, dilute hydrochloric acid was added to make the solution acidic. After an addition of 200 mL water, a lot of precipitates were obtained as crude product by filtration. The crude was purified by column chromatography with dichloromethane as eluent to give the mixture of ***7a***+***7b*** (115 mg, the molar ration is about 2:1).

Yield: 49.1%; Orange solid; m.p.218-221˚C; ^{1}HNMR(500MH$_Z$,$CDCl_3$) δ: 13.24 (s, 0.5H, OH), 13.21 (s, 1H, OH), 7.81 (d, *J*=1.0Hz, 0.5H, Ar-H), 7.61 (m, 4H, $ArOC_6\underline{H}_4Br$), 7.58 (d, *J*=2.5Hz, 1.0H, Ar-H), 7.50 (t, *J*=2.5Hz, 2H, $ArOC_6\underline{H}_4Br$), 7.48 (d, *J*=2.5Hz, 2H, $ArOC_6\underline{H}_4Br$), 7.47 (m, 4H, $ArOC_6\underline{H}_4Br$), 7.44 (s, 1H, Ar-H), 7.41 (d, *J*=2.5Hz, 0.5H, Ar-H), 7.19 (s, 0.5H, Ar-H), 7.13 (d, *J*=0.5Hz, 1H, Ar-H), 6.90 (d, *J*=2.5Hz, 1H, Ar-H), 6.81 (d, *J*=2.5Hz, 0.5H, Ar-H), 5.30 (s, 2H, $ArOC\underline{H}_2C_6H_4CH_2Br$), 5.23 (s, 2H, $ArOC\underline{H}_2C_6H_4CH_2Br$), 5.21 (s, 1.0H, $ArOC\underline{H}_2C_6H_4CH_2Br$), 5.15 (s, 1.0H, $ArOC\underline{H}_2C_6H_4CH_2Br$), 4.55 (s, 2H, $ArOCH_2C_6H_4C\underline{H}_2Br$), 4.54 ($ArOCH_2C_6H_4\underline{CH}_2Br$), 2.50 (s, 1.5H, $ArCH_3$), 2.46 (s, 3H, $ArCH_3$); ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 187.2, 182.9, 165.2, 164.3, 164.1, 162.7, 161.6, 159.8, 147.0, 146.8, 138.2, 138.0, 137.7, 137.6, 136.5, 136.2, 135.9, 135.7, 135.5, 134.3, 132.3, 129.49, 129.48, 129.45, 128.1, 127.9, 127.0, 124.9, 121.5, 120.6, 120.0, 116.0, 114.8, 111.9, 108.3, 106.9, 106.8, 105.1, 70.8, 70.3, 70.0, 33.2, 33.1, 32.9, 32.8, 22.3, 22.0; ESI-MS, m/z: 637.26 $[M+H]^+$; HRMS

(ESI$^+$): calcd for $C_{31}H_{24}Br_2O_5$ $[M+H]^+$ = 637.0048; Found, 637.0062.

2.2.8. The preparation of N,N'-(((((8-hydro-6-methyl-9,10-anthraquinone-1,3-diyl)bis(oxy)bis(methylene))bis(4,1-phenylene))bis(methylene))bis(N-methyl-N-nonyldecan-1-aminium) bromide (***8a***) and N,N'-(((((8-hydro-3-methyl-9,10-anthraquinone-1,6-diyl)bis(oxy)bis(methylene))bis(4,1-phenylene))bis(methylene))bis(N-methyl-N-nonyldecan-1-aminium) bromide (***8b***)

The mixture of ***7a***+***7b*** 100 mg (0.16 mmol) was dissolved in 20 mL $CHCl_3$ and the mixture was heated to reflux, then equimolar tertiary amine (***2c***) was added to react for 8 h. After removal of the solvent, the residue was chromatographed on a silica gel column by gradient elution to give the mixture of ***8a+8b*** (the molar ratio is about 5:1), the eluent order were dichloromethane first and then dichloromethane/ethanol (v/v from 50:1 to 10:1)

Yield:26.1%; Orange solid; mp:128-130˚C;^{1}HNMR (400Hz, $CDCl_3$) δ: 13.21 (s, 1.0H, OH), 13.13 (s, 0.2H, OH), 7.74 (m, 2.0H, $OCH_2C_6\underline{H}_4CH_2N$), 7.65 (m, 2.4H, $OCH_2C_6\underline{H}_4CH_2N$), 7.61 (m, 2.4H, $OCH_2C_6\underline{H}_4CH_2N$), 7.52 (s, 1.2H, Ar-H), 7.35 (m, 0.6H, $OCH_2C_6\underline{H}_4CH_2N$), 7.33 (m, 2.0H, $OCH_2C_6\underline{H}_4CH_2N$), 7.19 (m, 0.4H, $OCH_2C_6\underline{H}_4CH_2N$), 7.09 (m, 1.2H, Ar-H), 7.02 (s, 1.0H, Ar-H), 6.61 (d, *J*=2.4Hz, 1.0H, Ar-H), 6.44 (d, *J*=2.4Hz, 0.2H, Ar-H), 5.71 (s, 0.4H, $ArOC\underline{H}_2C_6H_4CH_2N$), 5.37 (s, 0.4H, $ArOC\underline{H}_2C_6H_4CH_2N$), 5.29 (s, 2.0H, $ArOC\underline{H}_2C_6H_4CH_2N$), 5.26 (s, 2.0H, $ArOC\underline{H}_2C_6H_4CH_2N$), 5.21 (s, 0.4H, $ArOC\underline{H}_2C_6H_4CH_2N$), 5.11 (s, 0.4H, $ArOCH_2C_6H_4C\underline{H}_2N$), 5.08 (s, 2.0H, $ArOCH_2C_6H_4C\underline{H}_2N$), 4.94(s, 2.0H,

267 $ArOCH_2C_6H_4C\underline{H}_2N$), 3.49-3.34 (m, 9.6H, $2\times C\underline{H}_2NC\underline{H}_2$), 3.18 (s, 6.0H, $2\times NCH_3$),
268 3.14 (s, 1.2H, $2\times NCH_3$), 2.45 (s, 0.6H, $ArCH_3$), 2.39 (s, 3.0H, $ArCH_3$), 1.78 (s, 9.6H,
269 $2\times C\underline{H}_2CH_2NCH_2C\underline{H}_2$), 1.39-1.28 (m, 62.4H,
270 $2\times NCH_2CH_2(C\underline{H}_2)_6CH_3+2\times NCH_2CH_2(C\underline{H}_2)_7CH_3$), 0.89 (m, 14.4H, $4\times CH_3$); ^{13}C
271 NMR (400 MHz, $CDCl_3$) δ: 187.0, 182.4, 165.0, 163.4, 162.6, 161.5, 146.9, 138.4,
272 138.0, 137.3, 134.1, 133.7, 133.6, 132.2, 129.9, 129.8, 127.6, 127.2, 127.1, 126.9,
273 126.6, 126.3, 120.0, 115.1, 114.8, 104.9, 69.4, 64.4, 63.4, 60.8, 47.8, 35.9, 31.84,
274 31.80, 31.76, 29.5, 29.4, 29.31, 29.28, 29.23, 29.13, 29.10, 29.06, 26.5, 26.2, 25.8,
275 22.7, 22.6, 14.1; Anal. Calcd for $C_{71}H_{110}Br_2N_2O_5\cdot 0.5H_2O$: C 68.50, H 9.03, N 2.25;
276 Found: C 68.83, H 9.31, N 2.05; ESI-MS, m/z: 535.75$[M-2Br]^{2+}$.

277 2.2.9. The preparation of compound ***9*** (2-bromomethyl-9,10-anthraquinone)

278 2-methyl-9,10-anthraquinone 444 mg (2.0 mmol) and NBS 356 mg (2 mmol)
279 were dissolved in 60 mL CCl_4 and the mixture was stirred under reflux for 10
280 minutes, then 48 mg BPO (0.2 mmol) was added as initiator. The mixture was stirred
281 under reflux for another 24 h. After removal of the solvent under reduced pressure,
282 the faint yellow crude product was chromatographed on a silica gel column by using
283 dichloromethane/petroleum ether (v/v = 1:2) as eluent to give compound ***9*** (530 mg)
284 as product.

285 Yield: 88.3%; Faint yellow solid; m.p.196-198˚C; ^1H NMR(400 MHz, $CDCl_3$)
286 δ: 8.32-8.34 (M, 4H, Ar-H), 7.84-7.86 (m, 2H, Ar-H), 4.62 (s, 2H, $-CH_2Br$). ESI-MS,
287 m/z: 301.14 $[M+H]^+$.

288 2.2.10. General procedure for preparation of compounds ***10a*** and ***10b***

Compound ***9*** 110 mg (0.50 mmol) was dissolved in 20 mL chloroform and then 0.1 mL tertiary amine was added to the solution. The mixture was stirred under reflux for 7 h, and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column by gradient elution to give products ***10a*** and ***10b***, with dichloromethane first and then chloroform/ethanol (v/v from 40:1 to 10:1, based on the polarity of final products).

2.2.10.1. N-octyl-N-((9,10-anthraquinone-2-yl)methyl)-N-methyloctan-1-aminium bromide (***10a***). According to the general procedure, compound ***9*** was treated with N-methyl-N-octylnoctan-1-amine and then purified on silica gel column to give compound ***10a***.

Yield: 88.6%; Faint yellow solid; m.p.116-118˚C; ^{1}H NMR(400 MHz, $CDCl_3$) δ: 8.48 (d, *J* = 8.0Hz, 1H, Ar-H), 8.29-8.25 (m, 2H, Ar-H), 8.21-8.17 (m, 2H, Ar-H), 7.78 (t, *J* = 3.6Hz, 2H, Ar-H), 5.41 (s, 2H, $ArCH_2N^+$), 3.48 (t, *J* = 8.4Hz, 4H, $2\times{}^+NC\underline{H}_2C_7H_{15}$), 3.32 (s, 3H, $^+NCH_3$), 1.89-1.83 (m, 4H, $2\times{}^+NCH_2C\underline{H}_2C_6H_{13}$), 1.37-1.25 (m, 20H, $2\times-(C\underline{H}_2)_5CH_3$), 0.90 (t, *J* = 7.2Hz, 6H, $2\times-C\underline{H}_3$); ^{13}C NMR (400 MHz, $CDCl_3$) *δ*: 181.90, 180.88, 139.5, 134.50, 134.47, 134.3, 133.7, 133.4, 133.04, 132.97, 130.9, 128.2, 127.31, 127.29, 64.0, 60.0, 48.0, 31.6, 29.1, 26.3, 22.7, 22.6, 14.0; ESI-MS, *m/z*: 476, $(M-Br)^+$; HRMS (ESI^+): calcd for $C_{32}H_{46}NO_2$ $[M-Br]^+$ = 476.3523; Found, 476.3519.

2.2.10.2. N-decyl-N-((9,10-anthraquinone-2-yl) methyl)-N-methyldecan-1-aminium bromide (***10b***)**.**

According to the general procedure, compound ***9*** was treated with

N-methyl-N-decylndecan-1-amine and then purified on silica gel column to give compound ***10b***.

Yield: 90.1%; Faint yellow solid; m.p.119-121˚C; ^{1}H NMR(400 MHz, $CDCl_3$) δ: 8.58(d, *J* = 8.0Hz, 1H, Ar-H), 8.36 (d, *J* = 8.0Hz, 1H, Ar-H), 8.30-8.28 (m, 3H, Ar-H), 7.84 (t, *J* = 4.0Hz, 2H, Ar-H), 5.40 (s, 2H, $ArCH_2N^+$), 3.48 (t, *J* = 7.6Hz, 4H, $2\times{}^+NC\underline{H}_2C_7H_{15}$), 3.34 (s, 3H, $^+NCH_3$), 1.86-1.82 (m, 4H, $2\times{}^+NCH_2C\underline{H}_2C_8H_{17}$), 1.41-1.27 (m, 28H, $2\times-(C\underline{H}_2)_7CH_3$), 0.90 (t, *J* = 6.8Hz, 6H, $2\times-C\underline{H}_3$); ^{13}C NMR (400 MHz, $CDCl_3$) *δ*: 181.9, 180.9, 139.6, 134.51, 134.47, 134.3, 133.7, 133.4, 133.1, 133.0, 130.9, 128.3, 127.34, 127.31, 64.0, 60.9, 48.0, 31.8, 29.4, 29.2, 26.4, 22.7, 22.6, 13.9; ESI-MS, *m/z*: 532, $(M-Br)^+$; HRMS (ESI^+): calcd for $C_{36}H_{54}NO_2$ $[M-Br]^+$ = 532.4149; Found, 532.4144.

2.2.11. The preparation of compound ***12*** (3-((dimethylamino)methyl)-1,6,8-trimethoxyl-9,10-anthraquinone)

KOH 358 mg was dissolved in 5 mL water and added into the mixture of compound ***1*** 200 mg (1.27mmol) and TEBA 120 mg, then a solution of compound ***5*** (250 mg, 0.64 mmol) in 30 mL chloroform was added into the mixture. The mixture was refluxed under stirring for 8h and then extracted by $CHCl_3$ (3×15 mL). The organic phases were combined and dried by Na_2SO_4. After the solvent had been removed under reduced pressure, the crude product (light yellow solid) was chromatographed on a silica gel column by gradient elution to give product ***12***, the eluent order were dichloromethane first and then dichloromethane/acetone (v/v from 30:1 to 10:1).

Yield 36.5%; Yellow solid. m.p.52-54˚C; ^{1}H NMR (400MHz, $CDCl_3$) δ: 7.74 (s, 1H, Ar-H), 7.41 (s, 1H, Ar-H), 7.35 (d, *J* = 2.0*Hz*, 1H, Ar-H), 6.79 (d, *J* = 2.0*Hz*, 1H, Ar-H)，4.02 (s, 3H, -OCH_3), 3.98 (s, 3H, -OCH_3), 3.97 (s, 3H, -OCH_3) , 3.59 (s, 2H, ArCH_2N) , 2.25 (s, 3H, N-CH_3), 2.38 (t, *J* = 6.8Hz, 2H, -NCH_2), 1.53 (m, 2H, -$NCH_2C\underline{H}_2C_7H_{15}$), 1.27-1.44 (m, 12H, -$NCH_2CH_2C_6\underline{H}_{12}CH_3$), 0.89 (t, *J = 6.8Hz*, 3H, -CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) *δ*: 184.4, 181.9, 163.8, 161.7, 160.0, 136.6, 135.3, 134.4, 125.0, 122.8, 119.2, 118.6, 105.2, 102.0, 61.9, 57.6, 56.5, 55.9, 42.3, 32.2, 31.9, 29.6, 29.2, 27.4, 27.2, 26.4, 23.4, 22.7, 14.1; ESI-MS, m/z: 468.22 $[M+H]^+$; HRMS (ESI^+): calcd for $C_{28}H_{37}NO_5$ $[M+H]^+$ = 468.2750; Found, 468.2758.

2.2.12. The preparation of compound ***13*** (N-2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-N-methyl-N-((4,5,7-trimethoxy-9,10-anthra quinone-2-yl)methyl)nonan-1 aminium iodide

Compound ***11*** was prepared according to the document [20] and its structure was affirmed by HRMS (ESI^+) with the *m/z* being 260.9973 (calculated $(M+H)^+$ is 260.9982). A solution of compound ***12*** (150 mg, 0.32 mmol) and ***11*** (83.5 mg, 0.32 mmol) in 15 mL methylglycol was refluxed and stirred for 24 h. After the solvent had been removed under reduced pressure, the crude product (yellow solid) was chromatographed on a silica gel column by gradient elution to give product ***13***, the eluent order were dichloromethane first and then dichloromethane/ethanol (v/v from 25:1 to 4:1).

Yield 45.6%; Yellow solid, m. p. 138-140˚C; ^{1}H NMR(400MHz, $CDCl_3$) δ: 8.38 (s, 1H, Ar-H), 7.82 (s, 1H, Ar-H), 7.33 (d, *J* = 2.0 *Hz*, 1H, Ar-H), 6.82 (d, *J* = 2.0 *Hz*,

1H, Ar-H), 5.32 (s, 2H, NCH_2Ar), 4.17 (s, 3H, $-OCH_3$), 4.00 (s, 3H, $-OCH_3$) , 3.99 (s, 3H, $-OCH_3$) , 4.14 (m, 2H, $-C\underline{H}_2OH$), 3.93 (m, 2H, $-C\underline{H}_2CH_2OH$), 3.80 (m, 4H, OC_2H_4O), 3.73 (m, 2H, $NCH_2C\underline{H}_2O$), 3.70 (m, 2H, $NC\underline{H}_2CH_2O$), 3.66 (m, 2H, $NC\underline{H}_2C_8H_{17}$), 3.34 (s, 3H, $-NCH_3$), 1.40-1.29 (m, 14H, $NCH_2C_7\underline{H}_{14}CH_3$), 0.90 (t, J = 7.2*Hz*, 3H, $NC_8H_{16}C\underline{H}_3$); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 183.3, 180.7, 164.0, 161.9, 160.2, 135.9, 134.7, 132.9, 125.1, 124.7, 122.1, 117.9, 105.4, 102.3, 72.5, 70.5, 70.2, 65.2, 64.9, 62.5, 61.5, 61.1, 58.1, 56.4, 55.9, 31.8, 29.4, 29.2, 29.1, 26.3, 23.4, 22.6, 14.0; Anal. Calcd for $C_{34}H_{50}INO_8$: C 56.12, H 6.93, N 1.92; Found: C 56.04, H 7.29, N 1.53; ESI-MS, m/z: 600.16 $[M-I]^+$.

2.2.13. The preparation of compound ***15*** ***(***N-decyl-N-methyl-N-((4,5,7-trihydroxy-9-anthrone-2-yl)methyl)decan-1-aminium bromide)

A solution of compound ***14*** (323 mg , 0.5 mmol) in 15 mL acetic acid was heated to 100˚C, then a solution of $SnCl_2 \cdot 2H_2O$ (226mg, 1 mmol) in 10 mL c-HCl was added dropwise. The mixture was stirred and refluxed for another 3 h and then poured into 20 mL water. 15 mL $CHCl_3$ was used to extract the mixture twice. The organic phases were combined and then product ***15*** was deposited from $CHCl_3$.

Yield 25.4%; Dark-green solid, m. p. 126-128˚C. ^{1}H NMR(400MHz, DMSO) δ: 12.35 (s, 1H, Ar-OH), 12.23 (s, 1H, Ar-OH), 11.13 (s, 1H, Ar-OH), 7.11 (s, 1H, Ar-H), 7.08 (s, 1H, Ar-H), 6.52 (s, 1H, Ar-H), 6.31 (s, 1H, Ar-H), 4.53 (s, 2H, $-CH_2N$), 4.42 (s, 2H, $ArCH_2Ar$), 3.27-3.17 (m, 4H, $2\times NCH_2$), 2.97 (s, 3H, NCH_3), 1.76-1.68 (m, 4H, $2\times NCH_2C\underline{H}_2$), 1.30-1.26 (m, 20H, $2\times N^+CH_2CH_2(C\underline{H}_2)_5CH_3$), 0.86 (t, J = 6.8Hz, 6H,

377 2×-CH_3); ^{13}C NMR (400 MHz, CDCl3) δ: 191.3, 166.0, 165.3, 161.9, 145.3, 142.9,
378 135.5, 123.5, 119.3, 116.6, 108.8, 108.1, 101.6, 64.2, 61.3, 47.8, 31.6, 28.8, 26.2, 22.5,
379 22.0, 14.1; ESI-MS, m/z: 510.35 $(M-Br)^+$; HRMS (ESI+): calcd for $C_{32}H_{48}NO_4$
380 $[M-Br]^+$ = 510.3578; Found, 510.3557.

381 2.3. Cell lines and culture

382 A375, BGC-823, HepG2 and HELF cells obtained from our own laboratory were
383 cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin,
384 and 100 mg/mL strepto-mycin in a humidified atmosphere of 5.0% CO_2 at 37˚C.

385 2.3.1. MTT assay for cell viability

386 Cell viability was assessed by the MTT assay described as previously [21]. Briefly,
387 A375 cells in logarithmic growth phase were seeded in 96-well plates at a density of
388 1×10^4/well. After 24 h of incubation, cells were cultured in medium with different
389 concentrations of emodin derivatives for 24 h. After two times wash of cells by 200
390 mL PBS, 100 mL of 5 mg/mL MTT were added to each well, and then cells were for
391 incubated another 4 h. The MTT-formazon formed by metabolically viable cells was
392 dissolved in 150 mL of DMSO. After shaken for 10 min, the amount of reduced MTT
393 was measured by an ELISA reader at 492 nm. Results are the means of three
394 independent experiments performed in triplicate. The 50% inhibitory concentration
395 (IC 50) was determined as the anticancer drug concentration causing 50% reduction
396 in cell viability and calculated from the cytotoxicity curves.

397 2.4. Cell apoptosis measurement by DAPI nuclear staining and flow cytometry.

398 The method was similar to what we used previously [22]. A375 cells were

399 incubated with compound ***4a*** (1,2,4 μM) for 24 h, and then washed once with PBS,
400 fixed with cold methanol: acetic acid (3:1) for 10 min, and washed three times with
401 PBS. Then, these cells were treated with 1 μg/mL DAPI for at 37˚C 5 min. After
402 staining, the cells were examined and photographed at 364 nm by a fluorescence
403 microscope.
404 Apoptosis was detected using Annexin V-FITC/PI staining and was analyzed by flow
405 cytometry (FCM). HeLa cells were seeded in 6-well plates (10^5 cells per well) for 24
406 h and incubated with 1 μM compound ***4a***, Untreated cells were also used as control..
407 After 24 h incubation, the cells were collected, washed twice with PBS, carefully
408 trypsinized to avoid mechanical damage ofthe membrane and stained with 5 μM of
409 the solution containing Annexin V-FITCand PI for 15 min in the dark according to the
410 manufacture's instructions. Cells were than analyzed by using flow cytometry (BD
411 Bioscience, FACS AriaIII).
412 2.5. Western bloting analysis
413 A375 cells were treated for 24 h with various concentrations of compound ***4a*** (5
414 and 10 μM). Cell dishes were washed with cold phosphate-buffered saline and treated
415 with lysis buffer for several seconds at an ice-cold bath, and then cell lysates were
416 clarified by centrifugation at 12,000 g for 5 min under 4°C and the supernatants were
417 recovered. The concentrations of proteins were determined by the BCA method.
418 Samples with equivalent amounts of proteins were resolved by SDS-PAGE using a
419 10% gel. Proteins were transferred to a polyvinyldene difluoride (PVDF) membrane.
420 The membranes were washed for ten minutes with TBS and were blocked by blocking

421 solution. The blots were washed three times with TBST, and they were incubated with
422 specific primary antibodies diluted 1:1000 in TBST solution overnight at 4°C.
423 Subsequently, the blots were washed three times with TBST, and then the blots were
424 incubated with secondary antibody diluted 1:5000 in TBST solution for 2 h at 37°C.
425 The membranes were washed with TBST and TBS. Chemiluminescent signals were
426 generated using a Super Signal West Pico Chemiluminescent Substrate kit (Pierce),
427 and detected by using the ChemiDoc XRS system (Bio-Rad). The target proteins
428 expression was quantified by use of Image Lab analysis software
429 (Bio-Rad)

430 2.6. Measurement of intracellular ROS.

431 2,7-Dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, USA) was
432 frequently used to monitor intracellular ROS level. DCF-DA was cleaved
433 intracellularly by non-specific esterases and oxidized by ROS to the highly
434 fluorescent compound 2,7-dichlorofluorescein (DCF). After A375 cells was exposed
435 to various drugs for 24 h, washed once with PBS, treated cells were incubated with 20
436 μM DCF-DA in serum-free DMEM at 37˚C for 30 min. Then, Cells were washed
437 twice with ice-cold PBS and harvested, and the fluorescence intensity of the cells was
438 detected by flow cytometry. The average intensity of DCF stands for intracellular
439 ROS levels.

440 2.7. Measurement of mitochondrial membrane potential.

441 Mitochondrial membrane potential ($\Delta\Psi m$) was measured using
442 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (Invitrogen, USA) by flow cytometry as

443 described previously [23]. Briefly, the treated A375 cells were incubated with 50 nM
444 DiOC6 in serum-free DMEM at 37˚C for 30 min and then washed twice with ice-cold
445 PBS and harvested, and analyzed by flow cytometry.

446 2.8. Measurement of subcellular localization.

447 The fluorescent mitochondrial probe, Mito-Tracker Green, was used to measure
448 the subcellular localization of compound ***4a*** in A375 cells, as described previously
449 [24]. A375 cells (10^5 cells/mL) were plated in petri dish for 24 h, when reached 70%
450 confluence, they were exposed to compound ***4a*** (2 μM) at 37˚C for 24 h. Then, cells
451 were washed twice and incubated in the medium containing 100 nM of Mito-Tracker
452 Green (emitted at 488 nm), for 30 min. After washed twice with PBS, the intracellular
453 localization of compound ***4a*** (emitted at 568 nm) was detected by a laser confocal
454 microscope (Olympus FluoView FV1000, Japan).

455 2.9. Drug treatment of isolated mitochondria

456 A number of microliters of isolated mitochondria was treated with compound ***4a***
457 at indicated concentrations (0、1、10、50、100 μM) for 2 h at 37 ℃. Than the
458 mitochondria samples were washed once with MT buffer, and the pellets were
459 resuspended in 200 μL of DiOC6 and incubated for 15 min at 37 ℃. The optimal
460 DiOC6 staining concentration was indentified to be 500 nM. After washing twice with
461 MT buffer, the DiOC6(3)–stained mitochondria was resuspended in 50 μL of MT
462 buffer for HSFCM (a laboratory-built high sensitivity flow cytometer analysis).

463 2.9. Statistical analysis

464 All data were presented as mean±S.D of three determinations. Statistical analysis

was evaluated using Student's t-test and one-way analysis of variance. Multiple comparisons of the means were performed by the least significant difference (LSD) test. Differences were considered statistically significant at $P < 0.05$. Data variation analysis was made by using SPSS statistical software (version 16.0).

3. Results and discussion

3.1. Chemistry

Scheme 1 shows a synthetic route of four new tertiary amines **2a**-**2d** containing at least one *n*-nonyl chain. The tertiary amines reacted with emodin derivative **3** to give corresponding quaternary ammonium salts **4a**-**4d** (**scheme 2**). Compounds **6a** and **6b** were prepared from the reaction between emodin derivative **5** and corresponding tertiary amines. The synthetic method of compounds **3** and **5** had been reported in our earlier work [12,13].

Scheme 3 and **scheme 4** showed the synthetic routes of emodin bis-quaternary ammonium salts **8a+8b** and anthraquinone quaternary ammonium salts **10a**-**10b,** respectively.

In **scheme 5**, iodotriglycol **11** was obtained by a nucleophilic substitution by KI on chlorotriglycol. Compound **5** reacted with compound **1** to give an emodin tertiary amine **12** whose alkylation with iodotriglycol gave quaternary ammonium salt **13** as product. **13** is slightly water soluble. In **scheme 6**, **a**n emodin quaternary ammonium salt **14** was transformed into anthrone quaternary ammonium salt **15** by reduction with $SnCl_2$. Along with this reaction, demethylation of three methoxy groups of reactant took place, which was affirmed by the appearance of three peaks of hydroxyl in ^1H

NMR of compound **15**.

3.2. In vitro anticancer activity screening and structure-activity relationship

The *in vitro* cytotoxicity activity was evaluated for all of the synthesized compounds against human malignant melanoma cell line A375, human gastric cancer cell line BGC-823, human hepatoma cell line HepG2 and human embryonic lung fibroblast cell line HELF. All compounds were dissolved in DMSO, and then diluted with culture medium containing 0.1% DMSO for assay. Paclitaxel was used as a positive control. The results are listed in **Table 1**.

Compounds **4a**-**4d** all contain at least one long carbon chain with 9 carbon atoms. Compound **4d** showed the lowest anticancer activity due to its insufficient lipophilic property. Compounds **4a**-**4c** all contain two long carbon chains on the 4° N atom showed higher anticancer activities than that of paclitaxel, the positive control, on cancer cells. Moreover, Compound **4a**-**4c** demonstrated anticancer activities that are slightly higher than those of our previously reported emodin quaternary ammonium salts [12,13]. We observe here that quaternary ammonium salt with hydrocarbon chains containing 9 carbons exhibit highest anticancer activity compared to those containing 8 or 10 carbons. In addition, Emodin bis-quaternary ammonium salts **8a**+**8b** and two anthraquinone quaternary ammonium salts **10a**-**10b** also show high anticancer activities.

Compound **6a** and **6b** possess similar lengths of chains in 4° N atom, but they have big difference of water solubility. Compound **6b** is water soluble with a ClogP value of (2.40), much smaller than that of **6a** (9.77). As a result, compound **6a** shows

high anticancer activity and compound **6b** hardly possesses anticancer activity. Compound **13** also possesses similar length of chains in 4° N atom with those of compounds **4a**-**4c**. Unlike compounds **4a**-**4c** which show high anticancer activities and big ClogP values (from 6.85 to 7.90), compound **13** is slightly soluble in water with ClogP of 3.44 and exhibits low anticancer activity. Thus, we conclude that increasing aqueous solubility of the emodin 4° ammonium salt derivatives negatively impact the potency of these compounds against cancer cells.

At last, an emodin quaternary ammonium salt **14,** whose structure and anticancer activity have been reported previously by our group [13] was reduced into anthrone quaternary ammonium salt **15** by using $SnCl_2$ as reducing reagent. Compound **14** has anticancer activity against HepG2 cell line (10.28 μM/24h), but anthrone quaternary ammonium salt **15** exhibited no inhibitive activity against HepG2 cell line. Our early work [12] had proved that a transformation of methoxyl into hydroxyl could improve the anticancer activities of emodin derivatives, so the loss of anticancer activity of compound **15** is likely due to the lack of quinoid structure. Now we can draw a conclusion that quinoid structure plays an important role in the anticancer activities of emodin derivatives.

Since A375 cells exhibit well sensitivity to emodin quaternary ammonium salts, the growth-inhibitory mechanism of the most active compound **4a** *in vitro* on A375 cells was further investigated.

3.3. compound **4a** induced apoptosis in A375 cells

To assess whether compound **4a** induced morphological change, A375 cells

were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) after treatment with compound **4a** (1, 2, 4 μM) for 24 h, and detected by fluorescence microscopy. As shown in **Fig. 2A**, control cells showed even distribution of the stain and round homogeneous nuclei feature, while cells exposed to compound **4a** displayed typical changes in a dose-dependent manner including smaller and brighter stained nuclei with condensed chromatin forming apoptotic bodies.

To evaluate whether compound **4a** triggered cell apoptosis, AnnexinV-FITC/PI double staining kit was used to analyze apoptosis in HeLa cells after the cells were treated with 1 μM of compound **4a**. The results are shown in **Fig. 2B**. Different states of cell death were defined as follows: Lower-left quadrant stands for normal cells, lower-right quadrant represents early apoptosis cells, late apoptosis cells are localized in the upper-right quadrant, and the necrotic cells are localized in the upper-left quadrant. As shown in **Fig. 2B**, exposure to compound **4a** for 24 h increased the proportion of late apoptotic cells compared to negative control, with little increase in the number of early apoptotic cells, suggesting an increase in the number of necrotic cells. The results suggested that compound **4a** can effectively induce apoptosis/necrosis in HeLa cells.

3.4. Down –regulation the protein expression of cell apoptosis molecule by compound **4a** in A375

To confirm whether the anti-tumor effect of compound **4a** *in vitro* was related to the apoptosis activity, two proteins related to the apoptosis including caspase-3 and P53 were traced by western blot technique. As shown in **Fig. 2C**, the bands for these

553 indicator proteins in groups administrated with different concentrations of compound
554 **4a** (5 μM and 10 μM) increased in a dose-dependent manner. Using GAPDH as an
555 internal reference, the related protein expression levels were displayed under the **Fig.**
556 **2C**. Compared with the control group, A375 cells treated with compound **4a**
557 expressed considerably more caspase-3 and P53 ($^{*}P<0.05$). Caspase family of
558 cysteinyl-proteases plays a critical role in the initiation and execution of programmed
559 cell death. The P53 tumor suppressor gene is an apoptosis regulator, it can induce
560 tumor cells apoptosis. Thus, the over expression of caspase-3 and P53 in A375 cells as
561 we observed indicates that compound **4a** may possesse the ability to inhibit
562 proliferation and induce apoptosis of A375 cells via activating caspases and
563 upregulating P53.

564 3.5. Measurement of ROS generation

565 We next assessed the intracellular ROS production in the treated A375 cells using
566 DCF-DA fluorescence dye. As is shown in **Fig. 3A**, exposure of cells to 0.5 μM of
567 compound **4a** hardly elicited any elevation of cellular ROS level. However, 2.7 and
568 5.8-fold increase in the ROS level detected in cells treated with 1 and 2 μM of
569 compound **4a**, respectively, compared to those in untreated cells.

570 Our recent work [16] showed that there was not a direct correlation between
571 alkylatiing reactivitiesof emodin derivatives and their anticancer activities. Moreover,
572 many spectral experiments also confirmed that there was only a weak interaction
573 between emodin and DNA [17,18]. These results imply that DNA may not be the
574 main target of emodin derivatives. So in this paper, the abilities of emodin and its

quaternary ammonium salt derivatives to generate ROS are tested, with the result listed in **Fig. 3B.** Compound **c** whose structure is listed in **Fig 3B** is an emodin quaternary ammonium salt which has been reported in our early work [15]. Compound **c** is water-solubility but does not exhibit any anticancer activity. **Fig. 3B** also shows that compound c's ability to generate ROS is lower than that of emodin, which implies that the compound's hydrophilicity is unfavorable in intracellular generation of ROS. Since water-soluble compounds **6b** and **13** exhibit no or low anticancer activity, a conclusion can be drawn that there is a close relationship between the ability to generate ROS and anticancer activity. Both compound **4d** and **4a** are fat soluble and they contain one and two long carbon chains in N cation respectively. **Fig. 3B** shows that compound 4a has the highest capability to generate ROS (4a > 4d > emodin), which correlates to their respective anticancer activities *in vitro*. This experiment indicates that there is a positive correlation between anticancer activity and the ability to generate ROS. Emodin quaternary ammonium salts possess positive charge and quinoid structure. They are not only accumulated in the matrix of mitochondria due to relatively high mitochondrial membrane potential of cancer cells compared to normal cells, but are also capable of capturing electrons leaking from respiratory chain due to their quinoid structure. But before these functional moieties induce apoptosis of cancer cells intracellularly, penetration through various membranes of the cell is required. This rational requires the hydrophobic interactions between the compound and the fatty acyl domains of the lipid bilayer. So we conclude that positive charge, lipophilicity and quinoid structure

597 are three main reasons of emodin quaternary ammonium salts possessing high
598 anticancer activities. Compound **13** shows low lipophilicity and compound **15** has no
599 quinoid structure, so they exhibit low or no anticancer activity.

600 3.6. Mitochondrial membrane potential assay

601 Mitochondrial membrane depolarisation is a prelude of apoptosis, given that
602 compound **4a** induces cell apoptosis via the mitochondrial intrinsic pathway, and ROS
603 level is closely related with ΔΨm. As shown in **Fig. 4A**, compound **4a** significantly
604 (**P < 0.01) diminished the ΔΨm in a concentration-dependent manner compared to
605 control in A375 cells. The decrease in ΔΨm was evident starting at 1 μM with a 25%
606 change and reached a maximum of 38% at 4 μM compared to the untreated cells. The
607 38% decrease reflected the collapse of mitochondrial membrane potential. The result
608 indicated that compound **4a**-induced apoptosis could be related to contributions from
609 the mitochondrial pathway.

610 3.7. Intracellular localization assay

611 To observe whether compound **4a** can bind to the mitochondria, Mito-Tracker
612 Green, a mitochondria-specific dye was used for visualizing mitochondria, while
613 fluorescence of compound **4a** was imaged directly by a laser scanning confocal
614 microscopy. As shown in **Fig. 4B**, the punctate pattern of red fluorescence displayed
615 by the compound **4a** image corresponded to Mito-Tracker Green, indicating
616 compound **4a** was primarily localized to mitochondria but not in the nucleus.

617 3.8. compound **4a** induced apoptosis through mitochondria-mediated pathway

618 To identify whether compound **4a** can directly act on the mitochondria, mitochondria

isolated from HeLa cells was incubated directly with different concentrations of compound **4a** (0、1、10、50、100 μM) for 2 h , followed by staining with $DiOC_6$ (3) and then analyzed on the HSFCM (a laboratory-built high-sensitivity flow cytometry) to measure the ΔΨm change upon drug treatment. **Fig. 4C** shows the results obtained with compound **4a**, in the bivariate dot-plots of the green fluorescence versus the side scatter, by plotting the median and mean fluorescence intensity against the drug concentration, a dose-dependent ΔΨm decrease was observed for mitochondria treated with compound **4a**. **Fig. 4C** indicates that, when the isolated mitochondria was stimulated with 10 μM compound **4a** for 2 h, the median and mean fluorescence of the ΔΨm signal significantly decreased. These data suggest that compound **4a** could directly affect on mitochondria.

4. Conclusions

In conclusion, a series of emodin derivatives by the introduction of quaternary ammonium salts were designed and synthesized as potential anticancer reagents. Among these compounds, most of the quaternary ammonium salts (**4a, 4b, 4c, 6a, 8a+8b**) showed better anticancer activities compared with emodin. More interestingly, compound **4a** has high activity in A375 cells, and low toxicity to HELF cells. Molecular mechanistic studies demonstrated that **4a** may induce cell apoptosis by increasing generation of ROS, the loss of mitochondrial membrane potential (ΔΨm), and activation of caspase-3 and P53 in A375 cancer cells. The results obtained in this study can lead to better understanding of structure–activity relationships, enhanced chemical modifications and bioactivity of emodin, with the aim to improve its

641 anti-cancer activity.

642 ## Conflict of interest

643 The authors declare that there are no conflicts of interest.

644 ## Acknowledgments

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Figure Captions:

Table 1 In vitro cytotoxicity of emodin and its derivatives against various cancer cells and normal cell line

Scheme 1 The synthetic route of tertiary amine used in this paper

Scheme 2 The synthetic route of emodin mono-quaternary ammonium salt derivatives

Scheme 3 The synthetic route of emodin bis-quaternary ammonium salt derivatives

Scheme 4 The synthetic route of anthraquinone quaternary ammonium salt derivatives

Scheme 5 The synthetic route of water soluble emodin quaternary ammonium salt derivative.

Scheme 6 The synthetic route of anthrone quaternary ammonium salt derivative.

Fig. 1. The molecular structures of emodin and anthraquinone anticancer drugs.

Fig. 2 (A) Apoptosis of A375 cells detected by DAPI staining. Representative photographs of A375 cells after treatment with compound **4a** at different concentrations 0 μM (control,), 1μM, 2 μM and 4μM, respectively (magnification 400×). **(B)** Apoptosis of HeLa cells detected by flow cytometry after treatment with compound **4a** at concentrations 0 μM (control), 1μM,. **(C)** Western blot analysis for in vitro P53 and caspase-3 expressions of A375 cells after treatment with compound **4a** at different concentrations 0 μM (control), 5 μM, 10 μM.

Fig. 3. (A) Increase in intracellular ROS level in A375 cells. A375 cells were treated with different concentrations of compound **4a** at 0 μM， control, 1μM, 2 μM and 4μM respectively. ROS level in A375 cells was evaluated by measuring DCF fluorescent signals by flow cytometry and dose-dependent induction of mean fluorescence of DCF . Representative experiments, n=3, mean±SD. **p<0.01. **(B)** The abilities to generate ROS for emodin and its quaternary ammonium salt derivatives. A375 cells were treated with different compound (Control 0μM,; Emodin 45μM, c

747 50μM, **4d** 10μM, **4a** 2μM,) and the mean fluorescence of DCF. Representative experiments, n=3,

748 mean±SD. **p<0.01.

749 **Fig. 4**. **(A)** Effects of compound **4a** on mitochondrial membrane potential of A375 cells.

750 Treatment of A375 cells with compound **4a** at 0 μM (control), 1μM, 2 μM and 4μM respectively

751 resulted in a concentration-dependent decrease in mitochondrial ΔΨ m. Results are expressed as

752 the percent change of ΔΨ m of the treated cells compared to the untreated control. Statistical

753 difference from controls: **P < 0.01. **(B)** Isolated mitochondria of HeLa cells were treated

754 with compound **4a** at 1 μM, 10 μM, 50 μM and 100 μM detected by a laboratory-built high

755 sensitivity flow cytometer analysis. **(C)** Intracellular distribution of compound **4a**. A375 cells

756 were loaded with 2 μM **4a** and 100 nM MitoTracker Green. fluorescence images of mitochondria.

757 fluorescence of 4a. Overlay fluorescence. of A and Bright-field image of A375 cells were

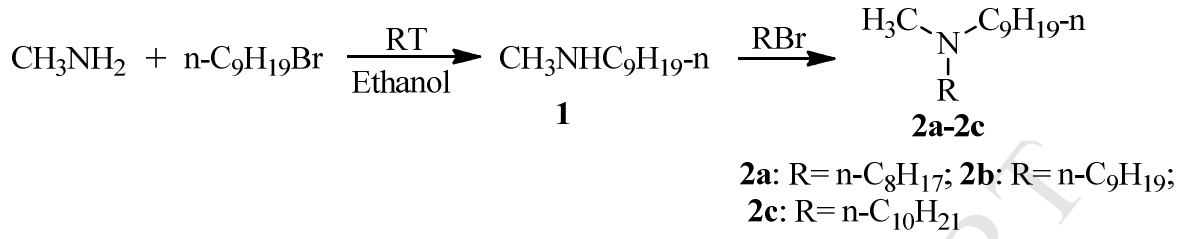
758 detected byconfocal microscopy. Scale bars represent for 20 μm.

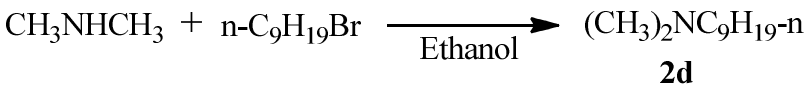
759

Table 1 ClogP values and *in vitro* activity of synthesized compounds against various cancer cells and normal cell.

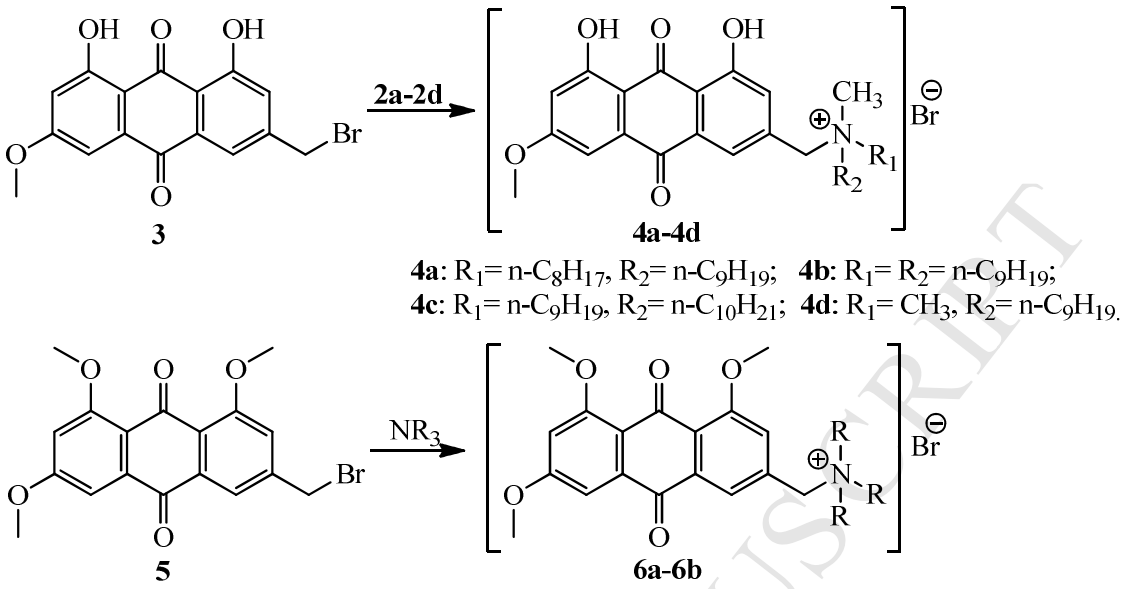
Compounds.	ClogP	IC_{50} (μM)			
		A375	BGC-823	HepG2	HELF
Emodin	3.62	44.91±2.32	>50	>50	46.27±4.11
4a	6.85	1.39±0.02	2.79±0.80	4.12±0.48	9.65±1.70
4b	7.38	2.63±0.76	3.93±0.79	7.39±1.26	9.83±1.85
4c	7.90	2.20±0.49	3.85±0.49	3.97±0.70	9.56±1.40
4d	3.14	9.87±0.96	9.76±0.49	10.42±1.11	21.55±1.68
6a	9.77	3.46±1.60	5.00±0.54	5.45±0.40	4.50±0.46
6b	2.40	>100	>100	>100	>100
8a+8b	16.16	1.55±0.05	-	1.46±0.04	1.71±0.06
10a	6.11	-	-	2.08±0.52	8.46±1.37
10b	8.22	-	-	1.57±0.76	5.84±0.62
13	3.44	28.94±0.64	32.67±0.37	37.66±0.36	40.35±.67
15	6.07	-	-	>100	>100
Paclitaxel	4.73	11.26±0.15	9.26±0.14	10.32±0.02	7.14±0.09

Scheme 1





Scheme 2



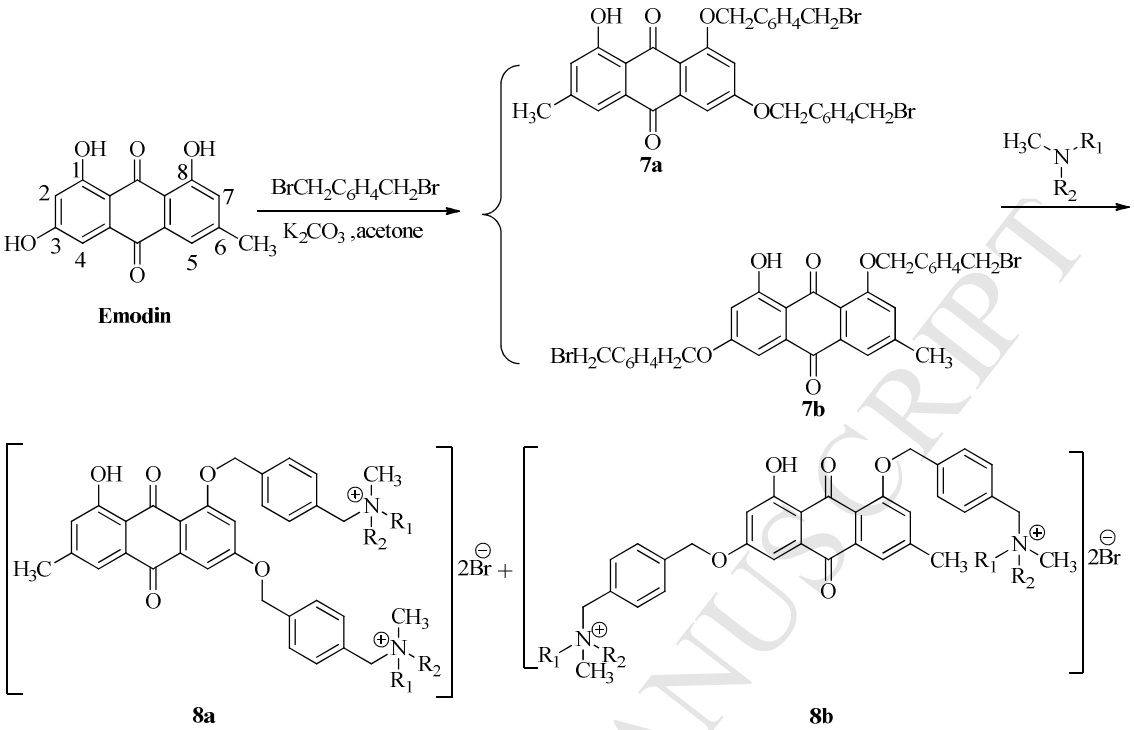
3 **4a-4d**

4a: R_1= n-C_8H_{17}, R_2= n-C_9H_{19}; **4b**: R_1= R_2= n-C_9H_{19};
4c: R_1= n-C_9H_{19}, R_2= n-$C_{10}H_{21}$; **4d**: R_1= CH_3, R_2= n-C_9H_{19}.

5 **6a-6b**

6a: R=n-C_8H_{17}; **6b**: R= -$CH_2CH_2OCH_2CH_2OCH_3$

10 **Scheme 3**

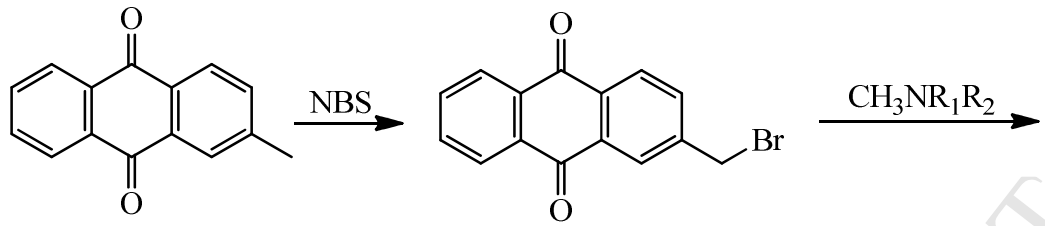


11 **8a** and **8b** R_1=n-C_9H_{19}, R_2=n-$C_{10}H_{21}$

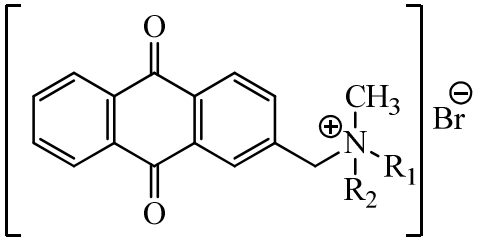
12

13

14 **Scheme 4**



2-methylanthraquinone **9**



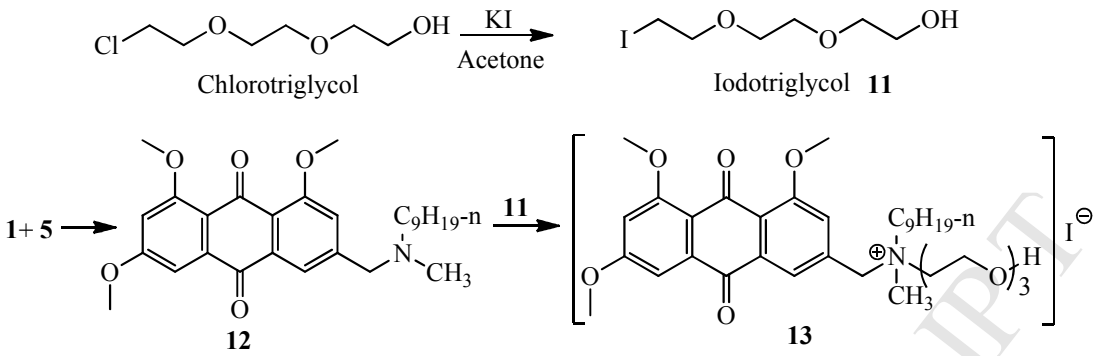
10a-10b

10a: R_1=R_2=n-C_8H_{17};
10b: R_1=R_2=n-$C_{10}H_{21}$;

15

16

17 **Scheme 5**



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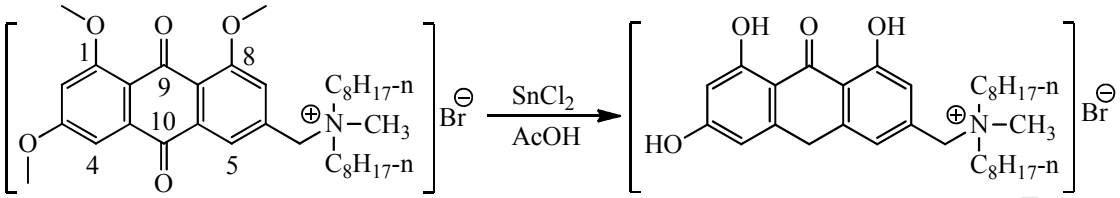
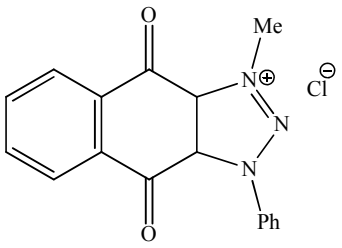
Scheme 6

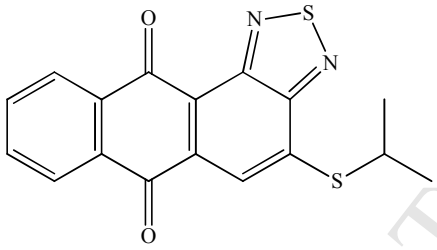


14 **15**

25 **Fig. 1**



Shrestha, Eur. J. Med. Chem., 77 (2014), 96



Lee, Eur. J. Med. Chem., 102 (2015), 661

26

Fig. 2

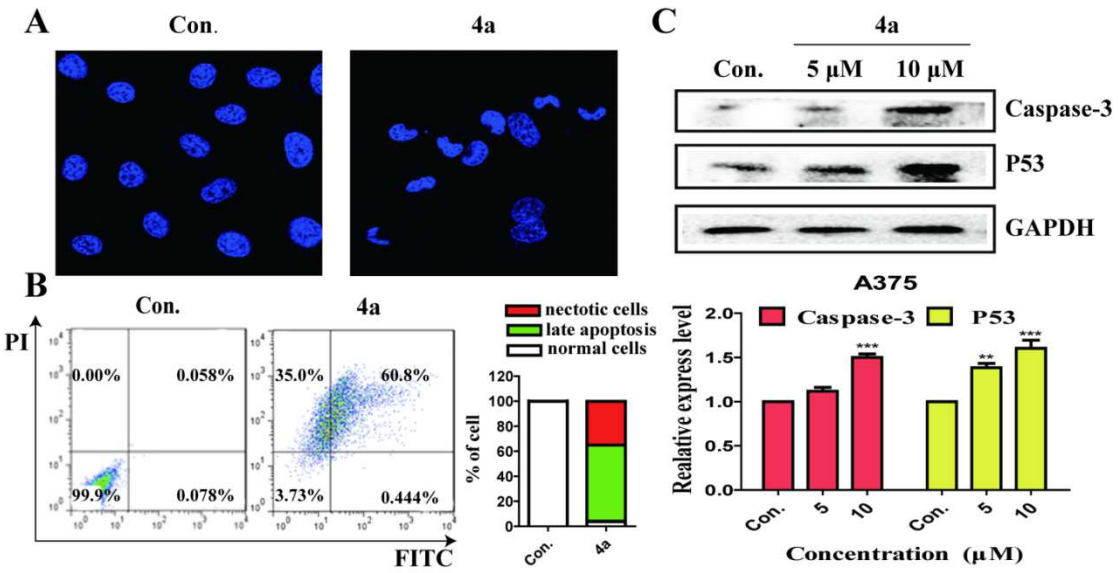


Fig. 3

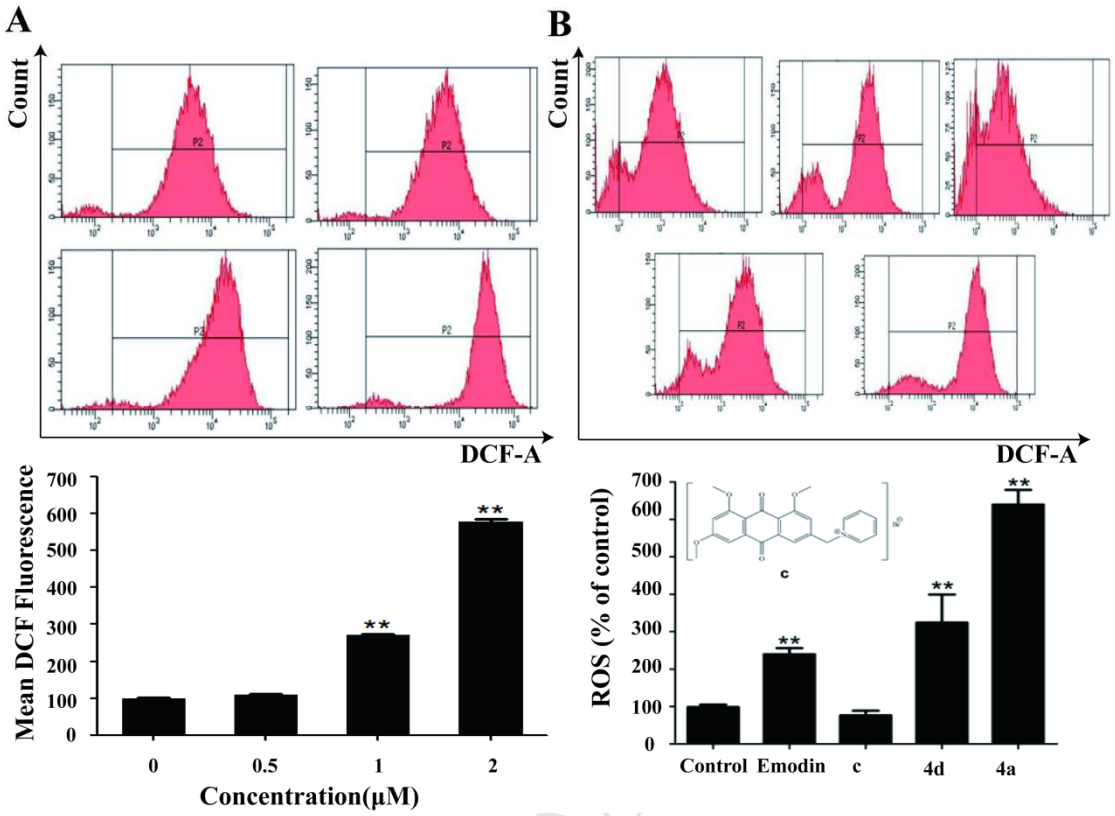
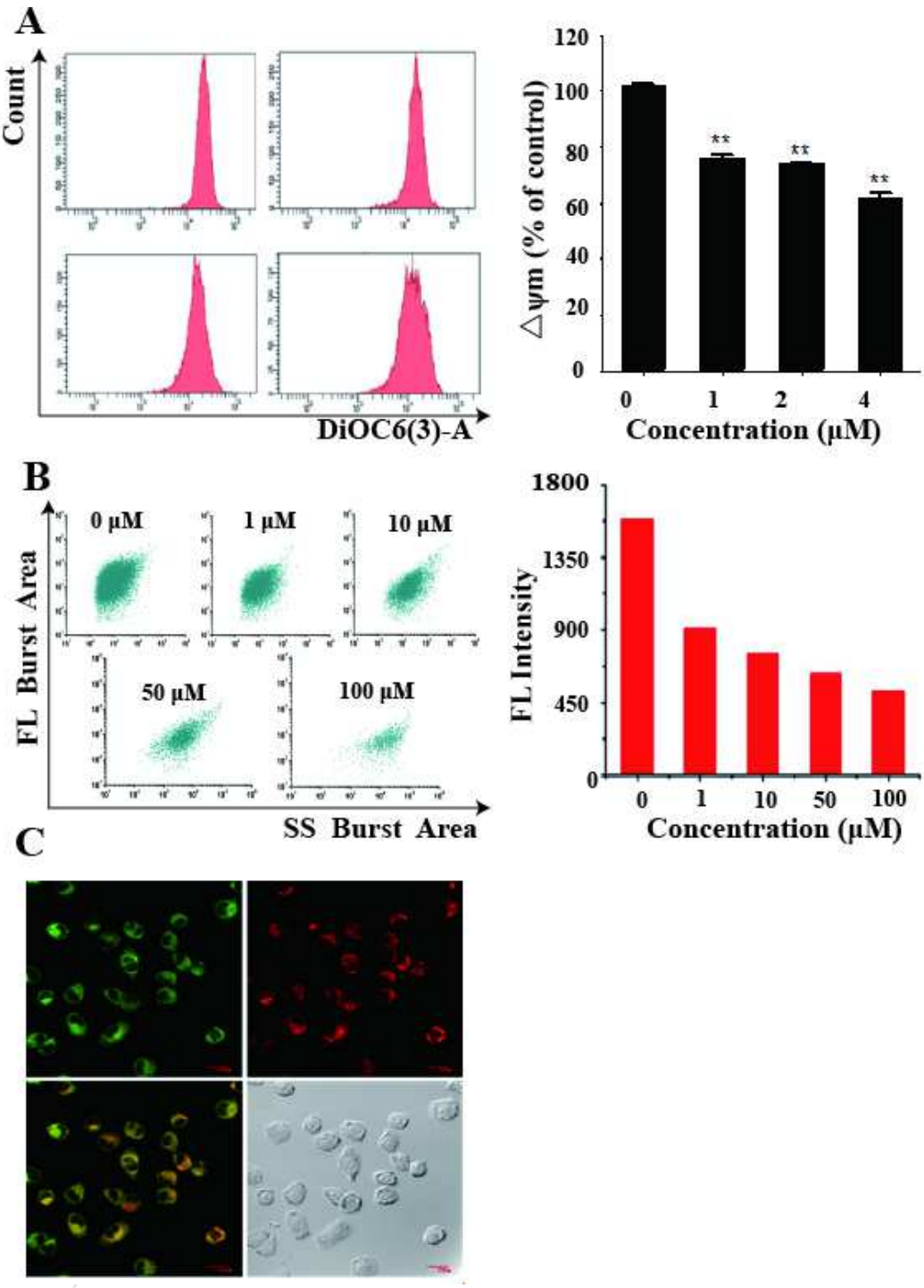


Fig. 4


A
Count
DiOC6(3)-A
△ψm (% of control)
120
100
80
60
40
20
0
**
**
**
0
1
2
4
Concentration (μM)
B
0 μM
1 μM
10 μM
50 μM
100 μM
FL Burst Area
SS Burst Area
FL Intensity
1800
1350
900
450
0
0
1
10
50
100
Concentration (μM)
C

Highlights

►Fifteen novel emodin derivatives were designed and synthesized.

► Compound **4a** showed significant antiproliferative activity *in vitro*.

►Compound **4a** induced apoptosis through caspase-3 and P53 activation.

►Compound **4a** directly effected on mitochondrial by generating ROS and decreasing ΔΨm.