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Original article

Synthesis and cytotoxic effect of 1,3-dihydroxy-9,10-anthraquinone derivatives

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Abstract – 1,3-Dihydroxy-9,10-anthraquinone (**4**) was reacted with epichlorohydrin or 1,ω-dibromo-alkane to yield 1-hydroxy-3-(2,3-epoxypropoxy)-9,10-anthraquinone (**5**) and 1-hydroxy-3-(3-chloro-2-hydroxypropoxy)-9,10-anthraquinone (**6**) or 1-hydroxy-3-(ω-bromoalkoxy)-9,10-anthraquinone. Ring-opening of the epoxide (**5**) or 1-hydroxy-3-(ω-bromoalkoxy)-9,10-anthraquinones with appropriate amines, afforded various 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-anthraquinones. The synthetic compounds were tested in vitro inhibition of human T-24, Hep 3B, Hep G2, SiHa, HT-3, PLC/PRF/5 and 212 cells. Almost all compounds showed significant inhibitory activity against several different cancer cell lines. Structure–activity analysis indicated epoxidation of the hydroxyanthraquinone increased cytotoxicity against tumour cells, but ring-opening of the epoxide group with amine did not enhance the cytotoxic activity. The phosphatidylserine (PS) externalization and DNA fragmentation in SiHa cells were significantly observed after 48 h incubation with selected compound **19**. The results show that **19** cause cell death by apoptosis. © 2000 Éditions scientifiques et médicales Elsevier SAS

anthraquinone / antitumour / apoptosis

1. Introduction

Apoptosis is considered to be the major process responsible for cell death in various physiological events. It acts as a regulating mechanism of tissue growth, where it balances cell proliferation [1]. Recently, apoptosis has become a focus of interest in oncology because a disregulation of the apoptotic process can prompt malignancy of tumours [2, 3]. As part of our efforts to continually develop potent antitumour agents which may lead to tumour cell apoptosis, we have designed and synthesized a new series of 1,3-dihydroxy-9,10-anthraquinone (DHA) derivatives. In the present paper, we report the synthesis and cytotoxic effects against several different cancer cell lines in vitro and cytotoxicity through apoptosis, of various DHA derivatives and discuss their structure–activity relationships.

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2. Chemistry

A series of new DHA derivatives were synthesized by general synthetic routes, as shown in *figure 1*. In general, the best method available for the synthesis of oxygenated anthraquinones is the condensation of phthalic anhydride (**1**) with 1,3-dimethoxybenzene (**2**) to form a mixture of 1,3-dimethoxybenzoylbenzoic acid (**3a**) and 1-hydroxy-3-methoxybenzoylbenzoic acid (**3b**). A mixture of **3a** and **3b** were dehydrated with a molten mixture of anhydrous aluminum chloride and sodium chloride to give DHA (**4**) [4]. 1-Hydroxy-3-(2,3-epoxypropoxy)-9,10-anthraquinone (**5**), 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-anthraquinones, and 1-hydroxy-3-(ω-alkylamino-alkoxy)-9,10-anthraquinones were synthesized by a method described elsewhere [5]. Briefly, DHA (**4**), was allowed to react with 1 eq. of sodium hydroxide in 2-propanol and excess of epichlorohydrin to yield the epoxide **5** as the major product and 1-hydroxy-3-(3-chloro-2-hydroxypropoxy)-9,10-anthraquinone (**6**) as

the side product. Ring opening of **5** with appropriate amines in refluxing appropriate solvent afforded various 1-hydroxy-3-(3-alkylamino-2-hydroxy-propoxy)-9,10-anthraquines. Potassium salt of **4** was allowed to react with **1**, ω-dibromoalkane in appropriate solvent and then animated with appropriate amines to give various 1-hydroxy-3-(ω-alkylaminoalkoxy)-9,10-anthraquines (*figure 1*).

3. Biological results and discussion

Cytotoxic activities of a series of DHA derivatives were studied against a number of cancer cell types. The results are listed in *table I and table II*. All the synthetic DHA derivatives, shown in *table II*, indicated significant inhibitory activities against all cancer cell types, except that **15** indicated insignificant inhibitory activities against Hep3B, PLC/PRF/5 and 212 cells in vitro. As shown in *table II*, an increase in the length of the *N*-substituted alkyl chain did not significantly enhance the cytotoxicity against all the cell lines in *table II*, except that **13** significantly enhanced the cytotoxicity against SiHa cells due to an increase of the *N*-substituted alkyl chain from three to four carbons. *N*-substitution with a more bulky group of three or four carbons significantly enhanced the cytotoxicity, except for the cytotoxicity against T-24 cells. It clearly indicated that lipophilicity did not affect the cytotoxicity of compounds with different *N*-substituted carbon side chains, while it did affect the cytotoxicity of compounds with same *N*-substituted carbon side chains.

DHA (**4**) did not show cytotoxic effects against T-24, Hep 3B, Hep G2, SiHa, HT-3, PLC/PRF/5 or 212 cells in vitro. As shown in *table II*, epoxidation of DHA markedly increased the in vitro cytotoxic effects against T-24, SiHa, HT-3, PLC/PRF/5 and 212 cells, while ring-opening of the epoxide with dimethylamine and *tert*-butylamine enhanced the cytotoxic effects against SiHa cells. As shown in *table II*1-hydroxy-3-(ω-alkylaminoalkoxy)-9,10-anthraquinones did not show stronger cytotoxic effects against the cancer cell lines used in *table II* than those of 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-anthraquinones except for the cytotoxic effect against SiHa cells. It suggested that the alcoholic group of 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-anthraquinones is required for the cytotoxic effects against T-24, Hep 3B, Hep G2, PLC/PRF/5 and 212 cells in vitro.

In order to study the cytotoxic mechanism of the DHA derivatives on tumour cells, changes of phosphatidylserine (PS) externalization and DNA fragmentation in SiHa cells after the selected **19** treatment were analysed. Growing evidence indicates that an early event in apoptosis is the migration of the PS from the inner to the outer leaflet of plasma membrane [6–8]. In the study, **19** (4 μg/mL) were incubated with SiHa cells for 48 h. The percentage of apoptotic cells was quantified by flow cytometry. As shown in *figure 2*, the percentage of apoptotic cells for **19**, obtained from the flow cytometric histograms was 66.6%, indicating that SiHa cytoplasma did indeed exhibit PS externalization in response to **19**. In addition, DNA fragmentation is generally used to characterize cell death by apoptosis [9–11]. Thus, apoptosis of the SiHa cells after **19** treatment was also studied by DNA fragmentation. DNA fragmentation in SiHa cells was significantly observed after 48 h incubation with **19** (4 μg/mL) (*figure 3*).

4. Conclusions

These results indicate that **19** causes cell death by apoptosis and the other compound of this series of DHA derivatives may also induce cell death by apoptosis. Further experiments are needed to elucidate its mechanism of action. Thus, this series of DHA derivatives with novel cell apoptosis might be developed as anti-cancer agents.

5. Experimental protocols

Melting points (uncorrected) were determined with a Yanaco micro-melting point apparatus. IR spectra were determined with a Hitachi model 260-30 IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra [δ (ppm), J (Hz)] were determined with a Varian Unity-400 spectrometer. Mass spectra were determined with a Jeol JMS-D-100 mass spectrometer. Elemental analyses were within $\pm 0.4\%$ of the theoretical values, unless otherwise noted. Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck.

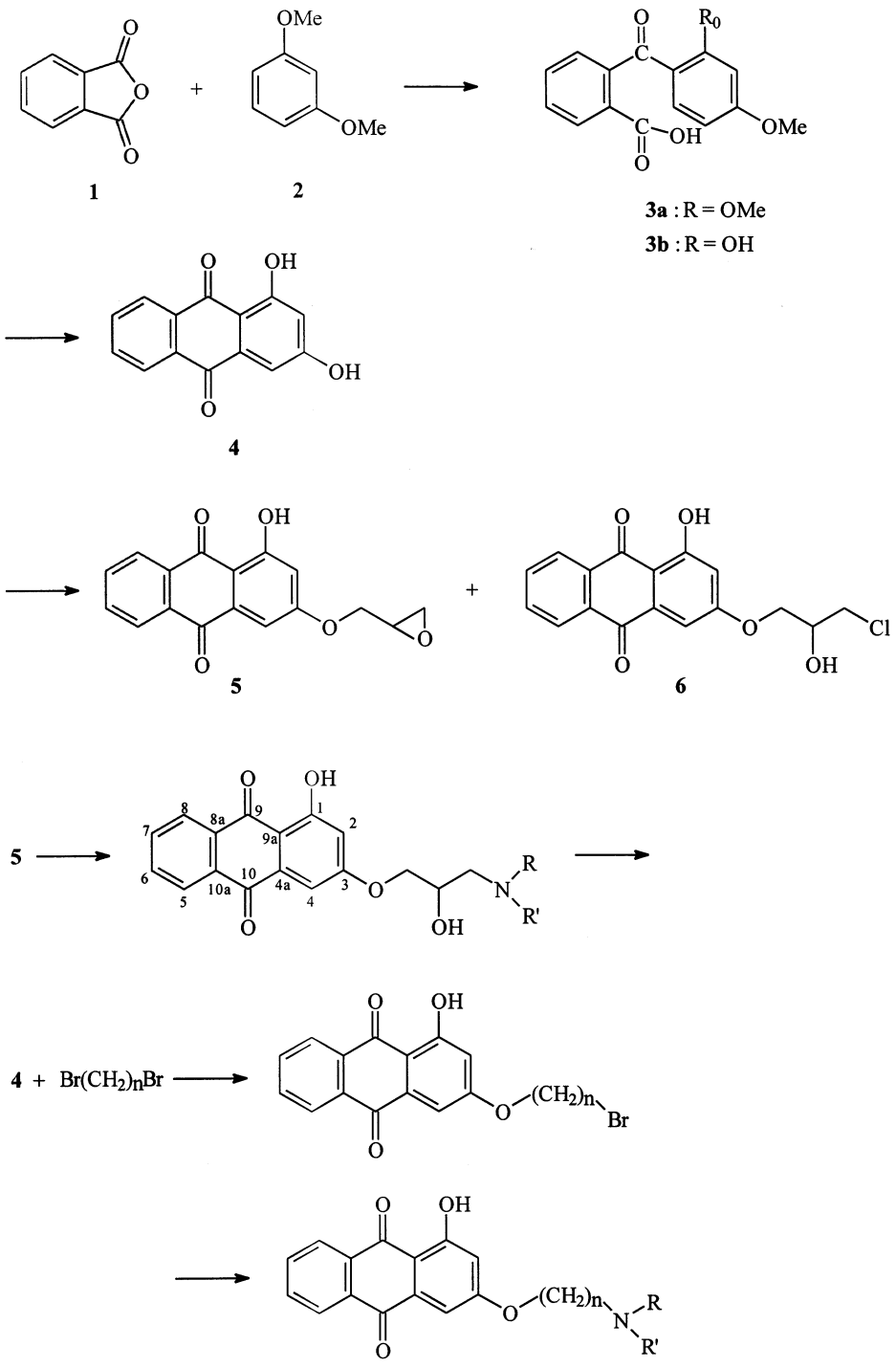
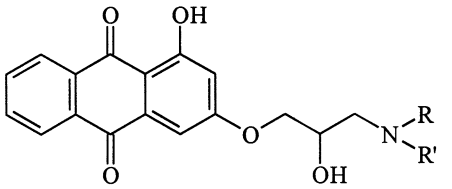
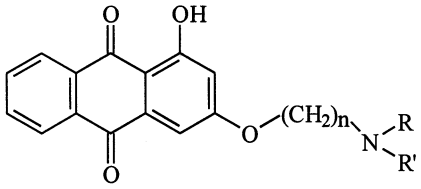


Figure 1. Synthesis of DHA derivatives.

Table I. DHA derivatives



7-15



18-20

Compd	*R*	*R′*	*n*	mp °C	Rcrystn solvent	% Yield	Formula	Anal.
5				181–183	$CHCl_3$	57	$C_{17}H_{12}O_5$	C, H
6				148–149	$CHCl_3$	24	$C_{17}H_{13}ClO_5$	C, H
7	CH_3	CH_3		109–111	EtOAc	65	$C_{19}H_{19}NO_5 \cdot 1/3H_2O$	C, H, N
8	C_2H_5	C_2H_5		amorph		71	$C_{21}H_{23}NO_5$	C, H, N
9	H	$CH(CH_3)_2$		amorph		70	$C_{20}H_{21}NO_5 \cdot HCl \cdot 1/2H_2O$	C, H, N
10	H	$(CH_2)_2CH_3$		200–203	MeOH	44	$C_{20}H_{21}NO_5 \cdot HCl$	C, H, N
11	H			amorph		73	$C_{20}H_{19}NO_5 \cdot HCl$	C, H, N
12	H	$CH(CH_3)CH_2CH_3$		amorph		83	$C_{21}H_{23}NO_5 \cdot HCl \cdot 1/3H_2O$	C, H, N
13	H	$C(CH_3)_3$		amorph		64	$C_{21}H_{23}NO_5 \cdot HCl \cdot 1/2H_2O$	C, H, N
14	H	$(CH_2)_3CH_3$		amorph		78	$C_{21}H_{23}NO_5 \cdot HCl$	C, H, N
15	H			amorph		61	$C_{23}H_{25}NO_5 \cdot HCl \cdot 1/3H_2O$	C, H, N
18	C_2H_5	C_2H_5	2	amorph		63	$C_{20}H_{21}NO_4 \cdot HCl \cdot 1/3H_2O$	C, H, N
19	CH_3	CH_3	3	amorph		78	$C_{19}H_{19}NO_4$	C, H, N
20	C_2H_5	C_2H_5	3	amorph		91	$C_{21}H_{23}NO_4 \cdot HCl \cdot H_2O$	C, H, N

*5.1. 1,3-Dihydroxy-9,10-anthraquinone (DHA) (**4**)*

To a stirred solution of phthalic anhydride (**1**) (6.0 g, 40.5 mmol) in anhydrous CH_2Cl_2 (500 mL) was added anhydrous $AlCl_3$ (15 g, 112.5 mmol) in small portions and then dropwise 1,3-dimethoxybenzene (**2**) (6.7 g, 48.5 mmol) at r.t. for 18 h. The reaction mixture was poured into ice water (500 mL) containing concentrated HCl (500 mL) and extracted with $CHCl_3$. Removal of the solvent gave a colourless powder (mixture of **3a** and **3b**) (9.66 g).

To a stirred molten mixture of anhydrous $AlCl_3$ (80 g, 600 mmol) and NaCl (50 g, 855.4 mmol) was added mixture of **3a** and **3b** (9.66 g) in small portions at 140–150 °C. The reaction temperature was raised to 160–165 °C and maintained for 6 h with continuous stirring. After cooling, the melt mass was poured into a mixture of ice water (500 mL) and concentrated HCl (500 mL) and allowed to stand overnight. The resulting precipitate was extracted with EtOAc [12]. The EtOAC extracts was purified by column chromatography (silica gel and C_6H_6-EtOAc (2:1) to give **4**, orange needles (Me_2CO), 5.6 g (23.33 mmol, 57.6%), mp 265–269 °C. The IR, MS and NMR were identified with an authentic sample of DHA [13].

*5.2. 1-Hydroxy-3-(2,3-epoxypropoxy)-9,10-anthraquinone (**5**) and 1-hydroxy-3-(3-chloro-2-hydroxypropoxy)-9,10-anthraquinone (**6**)*

To a solution of 0.56 g (10 mmol) sodium hydroxide in water (1 mL) was added 2-propanol (100 mL) and **4** (2.4 g, 10 mmol). To the above mixture was then added an excess of epichlorohydrin (18.5 g, 199.9 mmol) and the mixture was refluxed for 18 h with stirring. The product was purified by column chromatography (silica gel–$CHCl_3$) and crystallized from $CHCl_3$ to give two yellow needles **5** (1.70 g, 5.74 mmol) and **6** (0.79 g, 2.38 mmol). Compound **5**: IR (KBr) 1680, 1640 cm^{-1}; ^{1}H-NMR($CDCl_3$) δ2.80 (dd, $J = 2.8$, 4.8 Hz, 1H, C*H*H in the epoxide ring), 2.95 (t, $J = 4.8$ Hz, 1H, CH*H*), 3.39–3.43 (m, 1H, CH in the epoxide ring), 4.05 (dd, $J = 6.0$, 11.2 Hz, 1H, OC*H*H), 4.40 (dd, $J = 2.8$, 11.2 Hz, 1H, OCH*H*), 6.73 (d, $J = 2.8$ Hz, 1H, H-2), 7.38 (d, $J = 2.8$

Table II. Cytotoxicity of various DHA derivatives (ED_{50} values in μg/mL).

Compound	Cell line[a]						
	T-24	Hep 3B	Hep G2	SiHa	HT-3	PLC/PRF/5	212
5	0.51	n.d.	n.d.	0.80	0.53	0.51	0.16
6	1.66	n.d.	n.d.	4.18	1.98	2.08	0.63
7	1.20	1.95	0.72	0.67	n.d.	1.71	1.14
8	1.44	2.31	0.83	0.85	n.d.	2.01	1.32
9	1.55	n.d.	n.d.	1.18	1.47	1.67	0.38
10	1.75	1.97	n.d.	1.00	n.d.	2.02	0.90
11	1.40	1.76	n.d.	1.13	n.d.	1.58	0.88
12	1.96	1.86	n.d.	1.17	n.d.	1.85	1.04
13	2.36	1.88	0.72	0.65	n.d.	1.51	0.70
14	1.96	2.59	n.d.	1.47	n.d.	2.91	1.58
15	3.11	7.95	n.d.	1.99	n.d.	4.77	5.76
18	1.54	2.05	n.d.	1.20	n.d.	2.12	1.28
19	1.31	2.31	0.83	0.36	n.d.	1.71	1.21
20	2.33	2.57	n.d.	1.57	n.d.	2.60	1.31
Cisplatin						5.3	1.3
Actinomycin D	1.5×10^{-3}			8×10^{-4}	5.6×10^{-4}	1.4×10^{-3}	

[a] For significant activity of the DHA derivatives, an $ED_{50} < 4.0$ μg/mL is required; n.d., not determined.

Hz, 1H, H-4), 7.77–7.80 (m, 2H, H-6 and H-7), 8.25–8.29 (m, 2H, H-5 and H-8), 12.84 (s, 1H, OH-1); ^{13}CNMR ($CDCl_3$) δ44.5 (CH_2 in the epoxide ring), 49.6 (CH in the epoxide ring), 69.3 (OCH_2), 107.5 (C-2), 107.8 (C-4), 111.2 (C-9a), 126.8 (C-8), 127.4 (C-5), 133.5 (C-8a and C-10a), 134.2 (C-7), 134.3 (C-6), 135.1 (C-4a), 165.0 (C-1), 165.3 (C-3), 182.3 (C-10), 186.8 (C-9); MS *m/z* 296 (M^+, 75). Compound **6**: IR (KBr) 3505, 1676, 1641 cm^{-1}; ^{1}H-NMR (acetone-d_6) δ3.76–3.86 (m, 2H, CH_2Cl), 4.24–4.37 (m, 3H, C*H*OH and OCH_2), 4.84 (d, $J = 5$ Hz, 1H, CHO*H*), 6.85 (d, $J = 2.6$ Hz, 1H, H-2), 7.31 (d, $J = 2.6$ Hz, 1H, H-4), 7.87–7.97 (m, 2H, H-6 and H-7), 8.21–8.31 (m, 2H, H-5 and H-8), 12.85 (s, 1H, OH-1); ^{13}C NMR (acetone-d_6) δ47.3 (CH_2Cl), 71.0 (CHOH), 71.7 (OCH_2), 108.2 (C-2), 109.4 (C-4), 112.2 (C-9a), 128.1 (C-8), 128.6 (C-5), 134.9 (C-8a), 135.0 (C-10a), 136.1 (C-6 and C-7), 136.7 (C-4a), 167.1 (C-1), 167.2 (C-3), 183.2 (C-10), 188.5 (C-9); MS *m/z* 332 (M^+, 10).

5.3. General procedure for obtaining **7–15** [5]

To **5** was added various appropriate amines and ethanol. The mixture was refluxed for 2 h with stirring. The product was purified by column chromatography and crystallized from appropriate solvent or dissolved in 10% HCl–EtOAc solution, yielding various 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-anthraquinones or their hydrochloride salts. Treatment of **5** (0.55 g, 1.85 mmol) by this procedure yielding various 1-hydroxy-3-(3-alkylamino-2-hydroxypropoxy)-9,10-

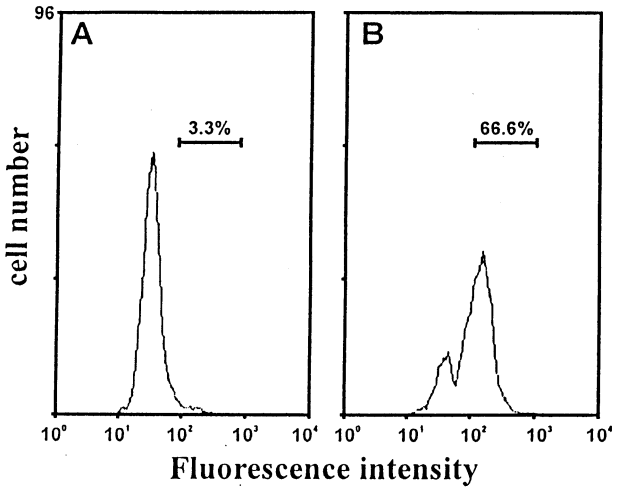


Figure 2. Apoptosis of the SiHa cells after **19** treatment was analysed by flow cytometric analysis. The quantitative analysis of apoptotic populations of SiHa cell by MC-540 staining. Cells (2×10^5) were seeded into six-well trays. After treatment with medium (A) or **19** (4 μg/mL) (B), cell pellets were resuspended in HBS buffer and stained with dye MC-540. Diluted cell suspension was assayed by FACScan. The percentage of apoptotic cells is shown in the panels.

Figure 3. Apoptosis of the SiHa cells after **19** treatment was analysed by DNA fragmentation. Cells (1.5×10^6) cultured in culture medium were treated with **19** for 48 h. C, control; lane 1, cells with **19** (2 μg/mL) treatment, lane 2, cells with **19** (4 μg/mL) treatment; M, 100 bp marker.

anthraquinones: **7** (0.41 g, 1.20 mmol), **8** (0.52 g, 1.41 mmol), **9** (0.49 g, 1.25 mmol), **10** (0.34 g, 0.87 mmol), **11** (0.53 g, 1.50 mmol), **12** (0.71 g, 1.74 mmol), **13** (0.27 g, 0.66 mmol), **14** (0.31 g, 0.76 mmol) and **15** (0.22 g, 0.51 mmol) (*table I*).

*5.4. 1-Hydroxy-3-[3-(dimethylamino)-2-hydroxypropoxy]-9,10-anthraquinone·1/3 H_2O (**7**)*

Yellow powder: IR (KBr) 3106, 1686, 1626 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ2.36 [s, 6H, $N(CH_3)_2$], 2.39–2.45 (m, 1H, C*H*HN), 2.52–2.65 (m, 1H, CH*H*N), 4.07–4.16 (m, 3H, OCH_2 and C*H*OH), 6.73 (d, $J = 2.5$ Hz, 1H, H-2), 7.39 (d, $J = 2.5$ Hz, 1H, H-4), 7.71–7.82 (m, 2H, H-6 and H-7), 8.21–8.31 (m, 2H, H-5 and H-8), 12.83 (bs, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ45.5 (CH_3), 61.5 (CH_2N), 65.7 (CHOH), 70.9 (OCH_2), 107.3 (C-2), 108.0 (C-4), 111.0 (C-9a), 126.8 (C-8), 127.4 (C-5), 133.5 (C-8a and C-10a), 134.1 (C-7), 134.3 (C-6), 135.0 (C-4a), 165.4 (C-1 and C-3), 182.4 (C-10), 186.8 (C-9); MS m/z 341 (M^+, 5).

*5.5. 1-Hydroxy-3-[3-(diethylamino)-2-hydroxypropoxy]-9,10-anthraquinone (**8**)*

Yellow powder: IR (KBr) 3116, 1681, 1621 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.06 [t, $J = 7.1$ Hz, 6H, $2 \times CH_3$), 2.49–2.78 (m, 4H, $N(CH_2)_2$], 4.01–4.11 (m, 3H, OCH_2 and C*H*OH), 6.72 (d, $J = 2.6$ Hz, 1H, H-2), 7.38 (d, $J = 2.6$ Hz, 1H, H-4), 7.71–7.82 (m, 2H, H-6 and H-7), 8.20–8.32 (m, 2H, H-5 and H-8), 12.81 (bs, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ11.9 (CH_3), 47.4 (CH_2CH_3), 55.7 (CH_2N), 67.2 (CHOH), 71.9 (OCH_2), 106.7 (C-2), 107.8 (C-4), 110.2 (C-9a), 126.5 (C-8), 127.0 (C-5), 133.0 (C-8a and C-10a), 134.7 (C-6 and C-7), 134.8 (C-4a), 164.7 (C-1), 165.6 (C-3), 181.7 (C-10), 186.3 (C-9); MS m/z 369 (M^+, 1).

*5.6. 1-Hydroxy-3-[3-(isopropylamino)-2-hydroxypropoxy]-9,10-anthraquinone hydrochloride·1/2 H_2O (**9**)*

Orange powder: IR (KBr) 3475, 1681, 1626 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ1.27 [d, $J = 5.6$ Hz, 3H, $CH(CH_3)_2$], 1.28 [d, $J = 5.6$ Hz, 3H, $CH(CH_3)_2$], 3.00 (dd, $J = 8.8$, 12.4 Hz, H, C*H*H$\overset{\oplus}{N}$H$_2$), 3.15 (dd, $J = 2.8$, 12.4 Hz, 1H, CH*H*$\overset{\oplus}{N}$H$_2$), 3.34 [m, 1H, NHC*H*$(CH_3)_2$], 4.16–4.23 (m, 2H, OCH_2), 4.24–4.28 (m, 1H, C*H*OH), 5.99 (s, 1H, CHO*H*), 6.91 (d, $J = 2.4$ Hz, 1H, H-2), 7.23 (d, $J = 2.4$ Hz, 1H, H-4), 7.91–7.95 (m, 2H, H-6 and H-7), 8.15–8.22 (m, 2H, H-5 and H-8); ^{13}C-NMR (DMSO-d_6) δ18.3 and 18.7 ($CH_3 \times 2$), 46.4 (CH_2NH), 49.9 [*C*H$(CH_3)_2$], 65.0 (CHOH), 70.7 (OCH_2), 106.8 (C-2), 107.8 (C-4), 110.4 (C-9a), 126.5 (C-8), 127.0 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-7), 134.8 (C-6), 134.9 (C-4a), 164.6 (C-1), 165.0 (C-3), 181.6 (C-10), 186.3 (C-9); MS m/z 355 (M^+, 2).

*5.7. 1-Hydroxy-3-[3-(n-propylamino)-2-hydroxypropoxy]-9,10-anthraquinone·hydrochloride (**10**)*

Red prisms: IR (KBr) 3465, 1696, 1621 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ0.92 (t, $J = 7.6$ Hz, 3H, CH_3), 1.63–1.72 (m, 2H, CH_2CH_3), 2.87–2.94 (m, 2H, $NH_2CH_2CH_2$), 2.98–3.05 (m, 1H, C*H*HNH_2), 3.14–3.19 (m, 1H, CH*H*$\overset{\oplus}{N}$H$_2$), 4.15–4.22 (m, 2H, OCH_2), 4.23–4.28 (m, 1H, C*H*OH), 5.98 (s, 1H, CHO*H*), 6.91 (d, $J = 2.4$ Hz, 1H, H-2), 7.24 (d, $J = 2.4$ Hz, 1H, H-4), 7.90–7.97 (m, 2H, H-6 and H-7), 8.16–8.24 (m, 2H, H-5

and H-8), 8.72 (bs, 1H, $\overset{\oplus}{N}HH$), 8.87 (bs, 1N, $\overset{\oplus}{N}HH$), 12.75 (s, 1H, OH-1); ^{13}C-NMR (DMSO-d_6) δ11.5 (CH_3), 20.7 (CH_2CH_3), 51.0 (NH_2CH_2), 51.1 ($CHOHCH_2\overset{\oplus}{N}H_2$), 66.7 (CHOH), 72.0 ($OCH_2$), 108.3 (C-2), 109.0 (C-4), 128.1 (C-8), 128.5 (C-5), 135.0 (C-8a and C-10a), 135.9 (C-7), 136.0 (C-6), 136.9 (C-4a), 166.8 (C-1), 181.6 (C-10), 186.3 (C-9); MS m/z 355 (M^+, 2).

5.8. 1-Hydroxy-3-[3-(cyclopropylamino)-2-hydroxypropoxy]-9,10-anthraquinone·hydrochloride (**11**)

Yellow powder: IR (KBr) 3465, 1691, 1631 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.75–0.93 (m, 4H, CH_2 in the cyclopropyl ring), 2.76 (bs, 1H, CH in the cyclopropyl ring), 3.09–3.14 (m, 1H, C*H*HNH), 3.24–3.7 (m, 1H, CH*H*NH), 4.16–4.25 (m, 3H, OCH_2 and C*H*OH), 5.98 (s, 1H, CHO*H*), 6.94 (d, $J = 2.4$ Hz, 1H, H-2), 7.27 (d, $J = 2.4$ Hz, 1H, H-4), 7.91–7.98 (m, 2H, H-6 and H-7), 8.18–8.25 (m, 2H, H-5 and H-8), 8.91 (bs, 2H, $N\overset{\oplus}{H}_2$), 12.78 (s, 1H, OH-1); ^{13}C-NMR (DMSO-d_6) δ3.1 and 3.2 (CH_2 in the cyclopropyl ring), 30.0 (CH in the cyclopropyl ring), 50.0 (CH_2NH_2), 64.5 (CHOH), 70.7 (OCH_2), 106.8 (C-2), 107.8 (C-4), 110.4 (C-9a), 126.5 (C-8), 127.0 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-4a), 134.8 (C-7), 134.9 (C-6), 164.6 (C-1), 165.0 (C-3), 181.6 (C-10), 186.3 (C-9); MS m/z 353 (M^+, 3).

5.9. 1-Hydroxy-3-[3-(1-methylpropylamino)-2-hydroxypropoxy]-9,10-anthraquinone hydrochloride·1/3 H_2O (**12**)

Yellow powder: IR (KBr) 3435, 1705, 1626 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.91 (t, $J = 7.6$ Hz, 3H, CH_2CH_3), 1.27 (d, $J = 6$ Hz, 3H, $CHCH_3$), 1.47–1.57 (m, 1H, C*H*HCH$_3$), 1.83–1.89 (m, 1H, CH*H*CH$_3$), 2.99–3.06 (m, 1H, C*H*CH$_3$), 3.12–3.21 (m, 2H, $CH_2\overset{\oplus}{N}H_2$), 4.12–4.20 (m, 2H, OCH_2), 4.28–4.36 (m, 1H, C*H*OH), 6.01 (s, 1H, CHO*H*), 6.78 (d, $J = 2.8$ Hz, 1H, H-2), 7.08 (d, $J = 2.8$ Hz, 1H, H-4), 7.84–7.91 (m, 2H, H-6 and H-7), 8.05–8.11 (m, 2H, H-5 and H-8), 8.74 (bs, 1H, $\overset{\oplus}{N}HH$) or 8.81 (bs, 1H, $\overset{\oplus}{N}HH$), 9.09 (bs, 1H, $\overset{\oplus}{N}HH$) or 9.21 (bs, 1H, $\overset{\oplus}{N}HH$), 12.64 (s, 1H, OH-1); ^{13}C-NMR (DMSO-d_6) δ9.7 (CH_2CH_3), 14.9 ($CHCH_3$), 25.0 (CH_2CH_3), 46.5 (CH_2NH), 55.0 (CH), 64.8 (CHOH), 70.6 (OCH_2), 106.7 (C-2), 107.7 (C-4), 110.2 (C-9a), 126.3 (C-8), 126.8 (C-5), 132.7 (C-8a and C-10a), 134.4 (C-4a), 134.6 (C-7), 134.7 (C-6), 164.5 (C-1), 164.9 (C-3), 181.3 (C-10), 186.1 (C-9); MS m/z 369 (M^+, 2).

5.10. 1-Hydroxy-3-[3-(tert-butylamino)-2-hydroxypropoxy]-9,10-anthraquinone hydrochloride·1/2 H_2O (**13**)

Yellow powder: IR (KBr) 3335, 1681, 1636 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ1.33 [s, 9H, $C(CH_3)_3$], 2.93–3.00 (m, 1H, C*H*H$\overset{\oplus}{N}H_2$), 3.14–3.19 (m, 1H, CH*H*$\overset{\oplus}{N}H_2$), 4.16–4.20 (m, 1H, C*H*OH), 4.23–4.26 (m, 1H, OCH_2), 6.00 (bs, 1H, CHO*H*), 6.90 (d, $J = 2.4$ Hz, 1H, H-2), 7.23 (d, $J = 2.4$ Hz, 1H, H-4), 7.89–7.96 (m, 2H, H-6 and H-7), 8.14–8.21 (m, 2H, H-5 and H-8), 8.55 (bs, 1H, $CH_2N\overset{\oplus}{N}HH$), 8.94 (bs, 1H, $CH_2\overset{\oplus}{N}HH$), 12.74 (s, 1H, OH-1); ^{13}C-NMR (DMSO-d_6) δ25.0 ($CH_3 \times 3$), 43.8 (CH_2NH_2), 56.5 [$C(CH_3)_3$], 65.2 (CHOH), 70.6 (OCH_2), 106.8 (C-2), 107.7 (C-4), 110.4 (C-9a), 126.5 (C-8), 126.9 (C-5), 132.9 (C-8a and C-10a), 134.6 (C-4a), 134.7 (C-7), 134.8 (C-6), 164.6 (C-1), 165.0 (C-3), 181.6 (C-10), 186.3 (C-9); MS m/z 369 (M^+, 1).

5.11. 1-Hydroxy-3-[3-(n-butylamino)-2-hydroxypropoxy]-9,10-anthraquinone hydrochloride (**14**)

Yellow powder: IR (KBr) 3445, 1691, 1636 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.90 (t, $J = 7.3$ Hz, 3H, CH_3), 1.24–1.43 (m, 2H, CH_2CH_3), 1.56–1.68 (m, 2H, $\overset{\oplus}{N}H_2CH_2CH_2$), 2.87–3.00 (m, 2H, $\overset{\oplus}{N}H_2CH_2CH_2$), 3.08–3.33 (m, 2H, $CH_2\overset{\oplus}{N}H_2$), 4.17–4.20 (m, 2H, OCH_2), 4.25–4.31 (m, 1H, C*H*OH), 5.97 (bs, 1H, CHO*H*), 6.91 (d, $J = 2.6$ Hz, 1H, H-2), 7.23 (d, $J = 2.6$ Hz, 1H, H-4), 7.91–7.99 (m, 2H, H-6 and H-7), 8.14–8.25 (m, 2H, H-5 and H-8), 8.75 (bs, 1H, $\overset{\oplus}{N}HH$), 8.91 (bs, 1H, $\overset{\oplus}{N}HH$), 12.75 (s, 1H, OH-1); ^{13}C-NMR (DMSO-d_6) δ13.5 (CH_3), 19.3 (CH_2CH_3), 27.3 ($NHCH_2CH_2$), 47.0 (CH_2NH_2), 49.1 ($NHCH_2CH_2$), 64.6 (CHOH), 70.7 (OCH_2), 106.8 (C-2), 107.8 (C-4), 110.4 (C-9a), 126.5 (C-8), 126.9 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-4a and C-7), 134.8 (C-6), 164.6 (C-1), 165.0 (C-3), 181.6 (C-10), 186.3 (C-9); MS m/z 369 (M^+, 3).

5.12. 1-Hydroxy-3-[3-(cyclohexylamino)-2-hydroxypropoxy]-9,10-anthraquinone hydrochloride·1/3 H_2O (**15**)

Yellow powder: IR (KBr) 3475, 1686, 1616 cm^{-1}; ^{1}H-NMR (CD_3OD) δ1.29–1.47 (m, 6H, $CH_2 \times 3$ in the cyclohexane ring), 1.87–2.19 [m, 4H, $CH(CH_2)_2$ in the cyclohexane ring], 3.13–3.24 (m, 3H, $CH_2\overset{\oplus}{N}H_2$ and CH in the cyclohexane ring), 4.19–4.21 (m, 2H, OCH_2), 4.24–4.30 (m, 1H, C*H*OH), 6.86 (d, $J = 2.6$ Hz, 1H, H-2), 7.40 (d, $J = 2.6$ Hz, 1H, H-4), 7.88–7.90 (m, 2H,

H-6 and H-7), 8.24–8.34 (m, 2H, H-5 and H-8); ^{13}C-NMR (CD_3OD) δ25.5, 25.6 and 26.1 ($CH_2 \times 3$ in the cyclohexane ring), 30.3 and 30.5 [$CH(CH_2)_2$ in the cyclohexane ring], 48.0 (CH_2NH_2), 58.9 (NH_2CH), 66.7 (CHOH), 71.7 (OCH_2), 108.1 (C-2), 108.8 (C-4), 127.8 (C-8), 128.2 (C-5), 134.8 (C-8a and C-10a), 135.6 (C-7), 135.7 (C-6), 136.6 (C-4a), 166.5 (C-1 and C-3), 183.5 (C-10), 188.2 (C-9); MS m/z 395 (M^+, 5).

5.13. 1-Hydroxy-3-[2-(bromo)-ethoxy]-9,10-anthraquinone (16)

To a solution of KOH (0.31 g, 5.5 mmol) in MeOH (100 mL) was added **4** (1.22 g, 5.1 mmol) and 1,2-dibromoethane (47.27 g, 251.6 mmol) and the mixture was stirred for 36 h under reflux. The reaction mixture was evaporated and the organic material was purified by column chromatography (silica gel; C_6H_6–EtOAc, 5:1). The purified product was crystallized from $CHCl_3$ to give **16**, yellow powder (0.63 g, 1.81 mmol, 35.3%).

Compound **16**: ^{1}H-NMR ($CDCl_3$) δ 3.68 (t, J = 6.1Hz, 2H, CH_2Br), 4.43 (t, $J = 6.1$ Hz, 2H, OCH_2), 7.26 (d, $J = 2.5$ Hz, 1H, H-2), 7.38 (d, $J = 2.5$ Hz, 1H, H-4), 7.74–7.84 (m, 2H, H-6 and H-7), 8.25–8.32 (m, 2H, H-5 and H-8), 12.84 (s, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ28.1 (CH_2Br), 68.3 (OCH_2), 107.4 (C-2), 107.6 (C-4), 111.3 (C-9a), 126.8 (C-8), 127.4 (C-5), 133.4 (C-8a and C-10a), 134.2 (C-6), 134.3 (C-7), 135.2 (C-4a), 164.6 (C-1), 165.3 (C-3), 183.3 (C-10), 186.9 (C-9).

5.14. 1-Hydroxy-3-[2-(bromo)-propoxy]-9,10-anthraquinone (17)

To a solution of KOH (0.18 g, 3.2 mmol) in MeOH (50 mL) was added **4** (0.48 g, 2.0 mmol) and 1,3-dibromopropane (20.32 g, 101.1 mmol), and the mixture was treated as in the procedure of **16** to give **17**, yellow powder (0.29 g, 0.80 mmol, 40.0%).

Compound **17**: ^{1}H-NMR ($CDCl_3$) δ 2.38 (m, 2H, CH_2), 3.62 (t, $J = 6.4$ Hz, 2H, CH_2Br), 4.26 (t, $J = 6.4$ Hz, 2H, OCH_2), 6.72 (d, $J = 2.8$ Hz, 1H, H-2), 7.38 (d, $J = 2.8$ Hz, 1H, H-4), 7.76–7.82 (m, 2H, H-6 and H-7), 8.26–8.31 (m, 2H, H-5 and H-8), 12.87 (s, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ29.3 (CH_2Br), 31.9 (CH_2), 66.2 (OCH_2), 107.2 (C-2), 108.0 (C-4), 111.0 (C-9a), 126.8 (C-8), 127.4 (C-5), 133.5 (C-8a and C-10a), 134.2 (C-6), 134.3 (C-7), 135.1 (C-4a), 165.3 (C-1), 165.4 (C-3), 182.4 (C-10), 186.8 (C-9).

5.15. General procedure for obtaining 18–20 [14]

To **16** or **17** was added appropriate amines and ethanol. The mixture was refluxed for 2 h with stirring. The product was treated as in the procedure for **7–15** to give **18** (0.26 g, 0.64 mmol), **19** (0.25 g, 0.32 mmol) and **20** (0.12 g, 0.30 mmol).

5.16. 1-Hydroxy-3-[2-(diethylamino)-ethoxy]-9,10-anthraquinone hydrochloride·1/3 H_2O (18)

Yellow powder: IR (KBr) 3355, 1681, 1644 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.49 (t, $J = 7.6$ Hz, 6H, $CH_3 \times 2$), 3.22–3.36 (m, 4H, $CH_2CH_3 \times 2$), 3.53 (dd, $J = 4.0$, 9.2 Hz, 2H, CH_2N), 4.72 (t, $J = 4.4$ Hz, 2H, OCH_2), 6.73 (d, $J = 2.4$ Hz, 1H, H-2), 7.29 (d, $J = 2.4$ Hz, 1H, H-4), 7.75–7.81 (m, 2H, H-6 and H-7), 8.22–8.28 (m, 2H, H-5 and H-8), 12.73 (bs, 1H, $\overset{\oplus}{N}H$), 12.79 (s, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ8.56 ($CH_3 \times 2$), 46.4 ($CH_2CH_3 \times 2$), 50.4 (CH_2NH), 63.5 (OCH_2), 107.3 (C-2), 107.4 (C-4), 111.5 (C-9a), 126.9 (C-8), 127.4 (C-5), 133.3 (C-8a and C-10a), 134.3 (C-7), 134.4 (C-6), 135.2 (C-4a), 163.5 (C-1), 165.3 (C-3), 182.0 (C-10), 186.8 (C-9); MS m/z 339 (M^+, 12).

5.17. 1-Hydroxy-3-[3-(dimethylamino)-propoxy]-9,10-anthraquinone (19)

Brown powder: IR (KBr) 1686, 1621 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.96–2.03 (m, 2H, CH_2), 2.26 (s, 6H, $CH_3 \times 2$), 2.47 (t, $J = 6.4$ Hz, 2H, CH_2N), 4.14 (t, $J = 6.4$ Hz, 2H, OCH_2), 6.67 (d, $J = 2.4$ Hz, 1H, H-2), 7.33 (d, $J = 2.4$ Hz, 1H, H-4), 7.73–7.81 (m, 2H, H-6 and H-7), 8.22–8.28 (m, 2H, H-5 and H-8), 12.83 (bs, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ27.1 (CH_2), 45.4 (CH_3), 56.0 (CH_2NH), 67.1 (OCH_2), 107.2 (C-2), 108.1 (C-4), 110.6 (C-9a), 126.7 (C-8), 127.3 (C-5), 133.4 (C-8a), 133.5 (C-10a), 134.0 (C-7), 134.2 (C-6), 134.9 (C-4a), 165.4 (C-1), 165.7 (C-3), 182.4 (C-10), 186.6 (C-9); MS m/z 325 (M^+, 16).

5.18. 1-Hydroxy-3-[3-(diethylamino)-propoxy]-9,10-anthraquinone hydrochloride·H_2O (20)

Yellow powder: IR (KBr) 3365, 1681, 1626 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.46 (t, $J = 7.2$ Hz, 6H, $CH_3 \times 2$), 2.44–2.51 (m, 2H, CH_2), 3.13–3.26 [m, 6H, $CH_2N(CH_2)_2$], 4.23 (t, $J = 6.6$ Hz, 2H, OCH_2), 6.66 (d, $J = 2.4$ Hz, 1H, H-2), 7.29 (d, $J = 2.4$ Hz, 1H, H-4),

7.77–7.79 (m, 2H, H-6 and H-7), 8.23–8.28 (m, 2H, H-5 and H-8), 12.38 (bs, 1H, NH), 12.82 (s, 1H, OH-1); ^{13}C-NMR ($CDCl_3$) δ8.51 ($CH_3 \times 2$), 23.8 (CH_2), 46.8 (CH_2CH_3), 49.2 (CH_2NH), 65.8 (OCH_2), 107.0 (C-2), 107.7 (C-4), 111.2 (C-9a), 126.8 (C-8), 127.4 (C-5), 134.4 (C-8a and C-10a), 134.2 (C-7), 134.3 (C-6), 135.1 (C-4a), 164.7 (C-1), 165.4 (C-3), 182.2 (C-10), 186.8 (C-9); MS m/z 353 (M^+, 35).

5.19. Tumour cell growth inhibition assays

The cytotoxicity was determined by colourimetric MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-1H-tetrazolium bromide) assay as described previously [15]. Briefly, cells (5×10^3 per well) were plated in the 96-well plates and incubated in medium for 6 h. Fifty microlitres of serial tested drug dilutions were added. The cells were incubated for 5 or 6 days at 37 °C and then pulsed with 10 μL of MTT (5 mg/mL; Sigma, St. Louis, MO) and incubated for an additional 4 h at 37 °C. Reduced MTT was measured spectrophotometrically by a Dynatech MR5000 microplate reader (Dynatech Laboratories, VA) at 550 nm after lysis of cells with 100 μL of 10% SDS in 0.01 M HCl. Control wells contained medium plus cells (total absorbance) or medium alone (background absorbance) cell death was calculated as the percentage of MTT inhibition:

$$\%\ \text{inhibition} = \frac{1 - \text{mean experimental absorbance}}{\text{mean control absorbance}} \times 100$$

PLC/PRF/5 cells were established from a human hepatoma and known to produce HBs Ag continuously in culture fluids [16]. Hep 3B cells, human hepatitis B surface antigen (HBs Ag) contains an integrated hepatitis B virus genome [17]. Human hepatoma, PLC/PRF/5, Hep 3B and Hep G2, T-24 cells, human cervical carcinoma, HT-3 and SiHa cells and 212 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) [18, 19], containing 10% foetal bovine serum (FBS; Gibco BRL), 2 mM 1-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The 212 cells (an inducible Ha-*ras* oncogene transformed the NIH/3T3 cell line) were maintained in minimum essential alpha medium (MEM; Gibco BRL), containing 10% calf serum (Gibco BRL) and antibiotics [20]. For the microassay, the growth medium was supplemented with 10 mM HEPES buffer pH 7.3 and incubated at 37 °C in a CO_2 incubator.

5.20. Flow cytometry

For cell apoptosis analysis, cells (2×10^6) were seeded into six-well trays. The cells were treated with a tested drug for 48 h and harvested. The cell pellets were resuspended in Hepes-buffer saline (HBS, 160 mM NaCl, 2.7 mM KCl, 6 mM glucose, 10 mM Hepes, 0.1% BSA, pH 7.3) and stained with dye Merocyanine 540 (MC540; Acros, Belgium) in the dark for 10 min. MC540, similar to Annexin V, can bind phosphatidylserine of extroverted inner membrane component during cell apoptosis and is used as the indicator of cell apoptosis [21, 22]. The cell suspension was diluted with 0.5 mL HBS buffer and 5×10^3 cells were assayed by the software CELLFIT of the fluorescence-activated cell sorter (FACScan; Becton-Dickinson immunocytometry system, CA) [23].

5.21. DNA fragmentation assay

After tested drug (8 μg/mL) treatment for 48 h, cells in 150-mm plates were harvested and washed with PBS. After the addition of 100 μL lysis buffer [1% of NP-40 (Sigma) in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5] and mixing, the cell lysates were centrifuged at 14 000 rpm for 5 min and the supernatants were collected. The supernatants were incubated with 50 μL of RNase A (20 mg/mL) and 20 μL of SDS (10%) at 56 °C for 2 h. Then, 35 μL of proteinase K (20 mg/mL) was added and incubated at 37 °C overnight. DNA fragments were precipitated after the addition of 150 μL of 10 M NH_4OAc and 1.2 mL of 100% ethanol at −20°C overnight. After centrifuging and drying, the DNA pellets were resuspended in 15 μL Tris-EDTA buffer and electrophoresed on a 1% agarose gel in TBE buffer at 30 V for 8 h, DNA ladder was observed after staining with ethidium bromide solution and exposing to the UV light [15].

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