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Anthraquinone derivatives induce G2/M cell cycle arrest and apoptosis in NTUB1 cells

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ABSTRACT

Thirteen anthraquinone derivatives **5**–**17** including two 3-(3-alkylaminopropoxy)-9,10-anthraquinone (NHA) derivatives **5** and **6**, and 11 1-hydroxy-3-(3-alkylaminopropoxy)-9,10-anthraquinone (MHA) derivatives **7**–**17** were synthesized, evaluated for cytotoxicities against two cancer cell lines, and assayed the generation of reactive oxygen species (ROS) in NTUB1 cells (a human bladder carcinoma cell line). Compound **9** bearing a pyrrolidinyl group induced the stronger cytotoxic effect than those of other synthesized NHA and MHA derivatives. Exposure of NTUB1 cells to, **9**, **13**, and **17** for 24 h significantly increased the production of ROS, respectively. Flow cytometric analysis exhibited that the exposure of NTUB1 cells to the selective **9** led to the G2/M phase arrest accompanied by an increase of apoptotic cell death after the incubation for 24 h. Compound **9** induced up-regulation of cyclinB1 and p21 expressions. Biological results suggested that the induction of G2/M arrest, apoptosis, and cell death by **9** may associate with increased expression of p21 and cyclin B1, elevation of Bax and p53 levels, and generation of ROS in the cell. In conclusion, these series of compounds may be used as anticancer agents.

1. Introduction

Anthraquinone derivatives have many biological activities including antimicrobial,[1] antileukemia,[2,3] and antitumor activities.[4] Previously we have reported the synthesis and cytotoxic effects of a series of 1-hydroxy-3-(ω-alkylaminopropoxy)-9,10-anthraquinone,[5] 1,3-dihydroxy-9,10-anthraquinone (DHA), 1-hydroxy-3-(3-alkylaminopropoxy)-9,10-anthraquinone (MHA), and 3-(3-alkylaminopropoxy)-9,10-anthraquinone (NHA) derivatives.[6] Doxorubicin and daunorubicin, well-known analogues of anthraquinones, are used as anticancer drugs. It is valuable to continuously synthesize other series of anthraquinone derivatives and evaluate their anticancer activities.

Reactive oxygen species (ROS) are involved in multistage carcinogenesis. ROS may cause oxidative DNA damage and loss of DNA repair capacity.[7,8] It was reported that a radical therapeutic approach targeted cancer cells by ROS-mediated mechanism.[9] A moderate increase of ROS may promote cell proliferation. The amount of ROS in cancer cells is higher than that in normal cells. When the increase of ROS in cancer cells reaches the toxic threshold, it may overwhelm the antioxidant capacity of the cell and trigger cell death. Many ROS-modulating agents such as arsenic trioxide and elesclomol are used in cancer treatment because of inducing the ROS accumulation in cancer cells.

In recent years, emodin, 1,3,8-trihydroxy-6-methyl-anthraquinone, inducing cytotoxicity through a ROS-dependent and p53 mechanism was reported.[10] As part of our efforts to continually develop potential anticancer agents, we further synthesized a new series of NHA and MHA derivatives and reported their structures and cytotoxic relationships against two cancer cell lines, induction of the ROS accumulation, and mechanism of action in the present paper.

2. Results and discussion

2.1. Chemistry

As shown in Scheme 1 and Table 1, a new series of MHA and NHA derivatives were synthesized by the method described

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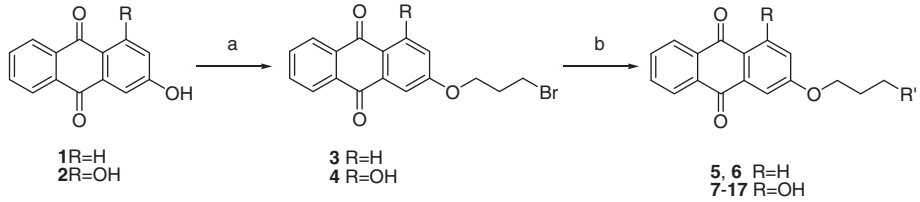
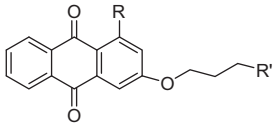
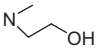
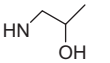
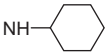
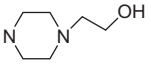
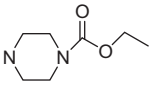


Scheme 1. Reagents: (a) 1,3-dibromopropane, KOH, MeOH (b) various amines.

Table 1
Structure, yield, and cytotoxicity (ED_{50} values in μM)[a] of NHA and MHA derivatives



Compound	R	R′	Yield (%)	NTUB1	PC3
5	H		15	—	—
6	H		30	24.45 ± 0.88	22.93 ± 2.43
7	OH		35	>50 (23.20 ± 6.08)	>50 (19.20 ± 9.08)
8	OH		41	18.87 ± 1.02	13.95 ± 1.43
9	OH		33	9.72 ± 1.21	7.64 ± 0.68
10	OH		25	12.30 ± 3.05	8.89 ± 0.09
11	OH		28	12.00 ± 1.19	9.07 ± 0.00
12	OH		42	32.62 ± 0.00	28.48 ± 0.11
13	OH		40	20.40 ± 1.71	17.73 ± 0.68
14	OH		44	8.37 ± 0.30	9.12 ± 0.32
15	OH		30	>30 (15.54 ± 6.73)	>50 (44.36 ± 3.84)
16	OH		28	>50 (18.75 ± 9.97)	>50 (18.54 ± 5.03)
17	OH		40	50.90 ± 2.23	>30 (12.44 ± 7.57)
Cisplatin				3.27 ± 0.10	4.56 ± 0.76

[a] Cytotoxity was assessed by the MTT assay after treating with different concentrations of compounds for 72 h. Data were presented as mean ± SD (n = 5).Compound **1**–**17** or cisplatin dissolved in DMSO, were diluted with culture medium containing 0.1% DMSO, respectively. The control cells were treated with culture medium containing 0.1% DMSO. Cisplatin was used as a positive control. —, not determined. When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses.

elsewhere.[5] Briefly, potassium salt of **1** or **3** was allowed to react with 1,3-dibromoalkane in appropriate solvent and then aminated with appropriate amines to give various NHA and MHA derivatives.

2.2. Biological results and discussion

Cytotoxic activities of synthesized NHA (**5** and **6**) and MHA (**7**–**17**) derivatives were studied against NTUB1 (a human bladder carcinoma cell line) and PC3 (a human prostate cancer cell line) cancer cell lines. Results are listed in Table 1, most of these anthraquinone derivatives **5**–**17** showed cytotoxic activities against the cancer cell line used in Table 1. Among these synthesized NHA and MHA derivatives, **9** showed the strongest cytotoxic activity against PC3 cells with an ED_{50} value of 7.64 ± 0.68 μM. Compound **14** indicated the most potent cytotoxic activity against NTUB1 cells with an ED_{50} value of 8.37 ± 0.30 μM.

Cytotoxic activities of MHA derivatives **7**–**17** bearing a hydroxy group at C-1 were stronger than those of NHA derivatives **6** except for **7**, **12**, and **15**–**17**. Most compounds with a hydroxy group at C-1 enhanced cytotoxic effects against all cancer cell lines used in this study. Compound **8** showed stronger cytotoxic effect against cancer cell lines used in Table 1 than those of **7**. It clearly indicated that an increase of carbon number of alkyl amine group such as **8** enhanced the cytotoxicity against those cancer cell lines used in Table 1. Compound **9** bearing a pyrrolidinyl or cyclohexylamino side chain, such as **9** and **14**, revealed potent cytotoxic activity than those of compounds bearing other heterocyclic ring. The hydroxylation at C-2 of the *N*-alkylamino side chain of **8** such as **11** enhanced the

cytotoxicity against NTUB1 and PC3 cancer cell lines. Compounds with a piperazinyl moiety such as **15–17** did not display significant cytotoxic activities against cancer cell lines used in Table 1.

For further evaluation of cytotoxicities on NTUB1, PC3, and SV-HUC1 (an immortalized normal human uroepithelial cell line) cells, cells were treated with different concentrations of selective **9** (3 and 10 μM), **13** (10 and 30 μM), and **17** (30 and 50 μM). The results showed that **9** revealed less cytotoxicity against SV-HUC1 cells than those of selective compounds against NTUB1 and PC3 cells (Fig. 1A–C).

We further examined the effect of **9**, **13**, and **17** on intracellular ROS generation in NTUB1 cells. It reported that exposure of NTUB1 cells to cisplatin, used as positive control, increased ROS accumulation. *N*-acetyl cysteine (NAC), a thiol antioxidant, was used as the negative control. As shown in Figure 2A–C, the exposure of NTUB1 cells to 20 μM of cisplatin and different concentrations of **9**, **13**, and **17** for 24 h caused an increase of the ROS accumulation in a dose-dependent manner. Treatment of NTUB1 cells with 1 mM of NAC attenuated the oxidation of H_2DCFDA (2,7-dichlorodihydrofluorescein) in NTUB1 cells. It suggested that these compounds are able to generate intracellular ROS and subsequently induced cell death.

Effects of cisplatin and **9** on the cell cycle progression were determined by using fluorescence-activated cell sorting (FACS) analysis in propidium iodide (PI)-stained NTUB1 cells. As shown in Figure 3A and B, NTUB1 cells treated with 20 μM of cisplatin for 24 h led to an accumulation of cells in G1 and S phases with the concomitant increased the population of sub-G1 phase. NTUB1 cells was exposed to 10 and 20 μM **9**, for 24 h induced G1 and G2/M phase arrest with the concomitant decrease and increase of the population of sub-G1 phase, respectively.

As shown in Figure 4A, exposure of NTUB1 cells to 1 mM of NAC, analyzed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method, exhibited slightly inhibitory cell growth while exposure of NTUB1 with **9** combined with NAC significantly increased the cell growth of NTUB1 cells. Immuno-fluorescence staining with H_2DCFDA showed that NAC attenuated the fluorescent intensity through reducing the ROS accumulation in NTUB1 cells (Fig. 4B). Doxorubicin could induce the ROS accumulation in cancer cells[11] used as the positive control. It also supported that the cytotoxicity induced by **9** associated with the ROS accumulation in NTUB1 cells.

Results of FACS analysis showed that exposure of NTUB1 cells to 20 μM **9** induced the G2/M phase arrest. The cell morphology of NTUB1 cells was further examined by Immuno-fluorescence staining with 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 5). Doxorubicin was used as a contrast because it was reported inducing G2/M phase arrest and apoptosis.[12] After treatment of 20 μM **9** for 24 h, the tetraploid DNA increased in NTUB1 cells. Most of NTUB1 cells did not exhibit the progression to the mitosis (Fig. 5). It suggested that 20 μM **9** may influence the expression of protein in G2 phase of NTUB1 cells.

Progression of the cell cycle is controlled by the actions of various types of cyclins and cyclin-dependent protein kinase (Cdks). As progression of the cell cycle moves into G2 phase, Cdk1 and B-type cyclin (B1 abd B2) complexes play important roles in the G2/M transition and M phase progression.[13] p21 is present during G1 phase while its levels decrease during S phase and increase during G2 phase.[14] Western blot analysis (Fig. 6) indicated that NTUB1 cells were treated with 20 μM **9** for 24 h induced up-regulation of cyclin B1 and p21 expressions in NTUB1 cells while NTUB1 cells

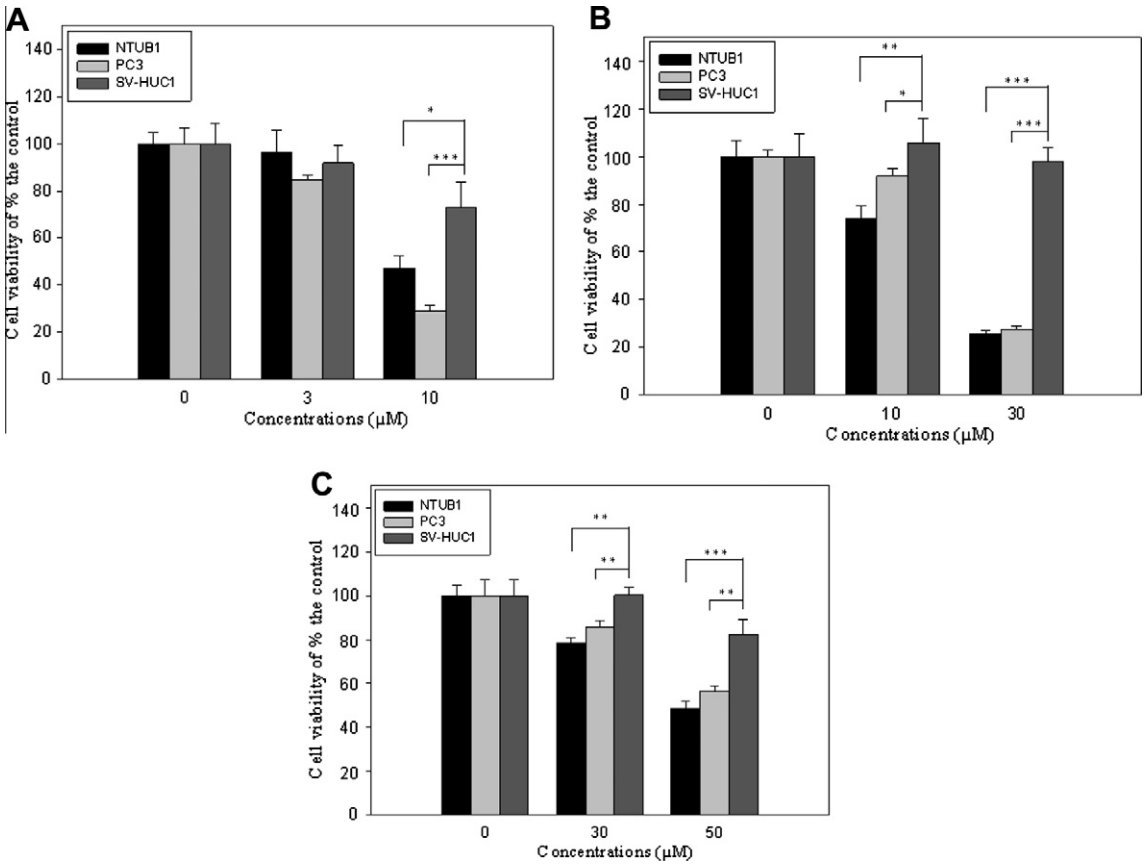


Figure 1. Cytotoxicities of **9** (A), **13** (B), and **17** (C) against NTUB1, PC3, and SV-HUC1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of compounds for 72 h. The data shown represent mean ± SD (n = 3) for one experiment performed in triplicate. $^{*}P$ <0.05, $^{**}P$ <0.01, and $^{***}P$ <0.001 compared to the control value, respectively.

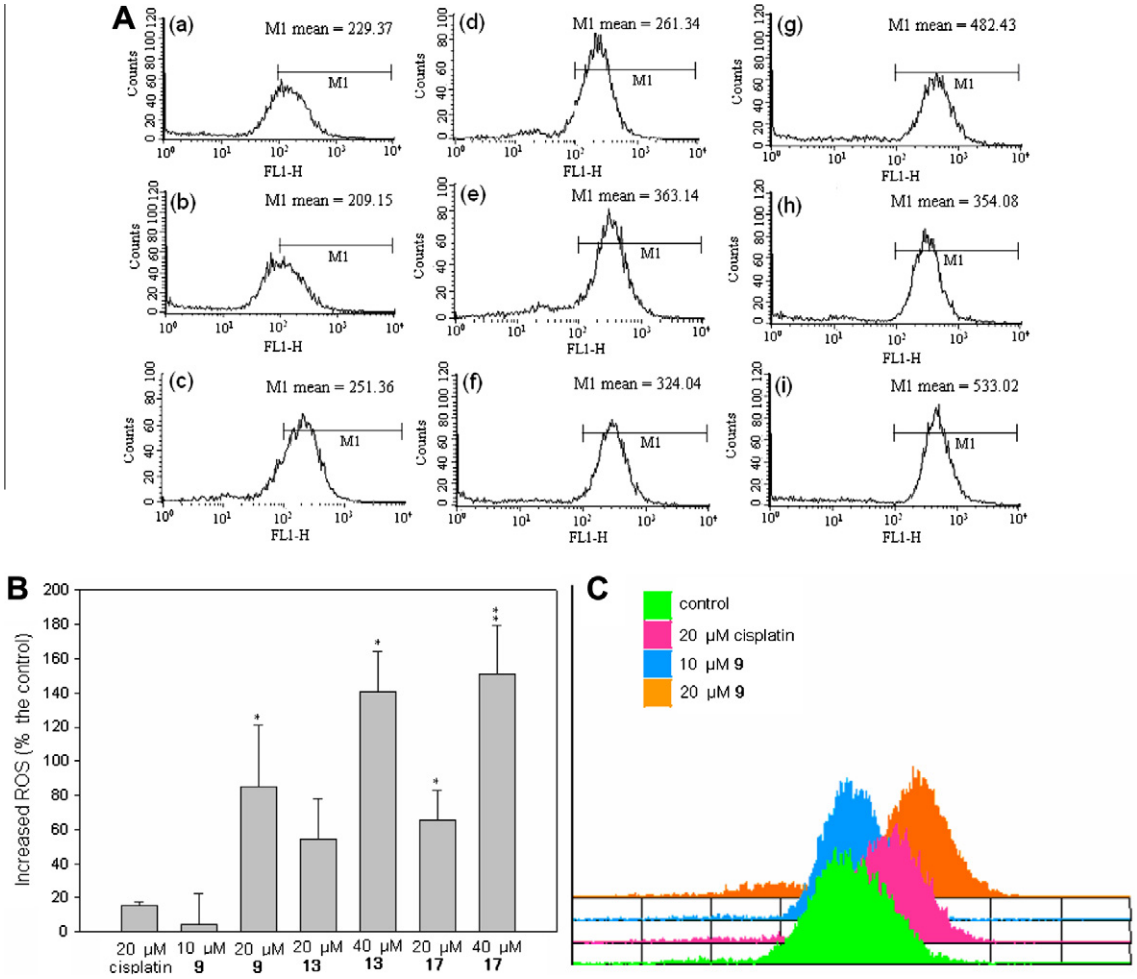


Figure 2. The effect of **9**, **13**, and **17** on the production of ROS in NTUB1 cells. (A) (a) control; (b) 1 mM NAC (*N*-acetyl cysteine, the negative control); (c) 20 μM cisplatin (the positive control); (d) 10 μM **9**; (e) 20 μM **9**; (f) 20 μM **13**; (g) 40 μM **13**; (h) 20 μM **17**; (i) 40 μM **17**. Cells were treated 1 mM NAC, 20 μM cisplatin, and different concentrations of **9**, **13**, and **17** for 24 h, respectively, and the amount of ROS was assayed by H_2DCFDA staining. (B) Results were repeated by three independent experiments and calculated the control as 0% increased ROS. $^{*}P$ value <0.05 and $^{**}P$ <0.01 compared to amount of increased ROS of the control, respectively. (C) Overlapped data of control, 20 μM cisplatin, and 10 μM and 20 μM **9**.

were treated with 10 μM **9** for 24 h induced down-regulation of cyclinB1 and up-regulation of p21 expressions in NTUB1 cells.

Treatment of androgen-sensitive (LNCaP) cells with kuguacin J resulted in significant G1-phase arrest of cell cycle progression, along with reduction of cyclinD1, cyclinE, cdk2, and CDK4, and increase of p21 and p27 at the protein level.[15] The above result suggested that the cell cycle arrest of NTUB1 cells at G1 phase induced by 10 μM **9** may correlate with up-regulation of p21 expression and independent with the decrease of expression of cyclinB1.

Cell cycle progression is well regulated by the timing of the expression of cell-specific cyclins. It was reported that treatment with deoxypodophyllotoxin (DPPT) in Hela cells induced G2/M phase arrest.[16] In the Western blot analysis, treatment with DPPT in Hela cells resulted in a rapid increase in cyclin B1 expression was observed within 3 h of DPPT treatment that remained elevated for up to 24 h. The expression of cyclin B1 returned to near control level by 48 h.[16] Based on the above results, it suggested that the cell cycle arrest of NTUB1 cells at G2/M phase induced by 20 μM **9** may correlate with up-regulation of cyclin B1 and p21 expressions in NTUB1 cells. It needs to examine the regulation of cyclin B1 by the timing of the expression of cyclinB1.

The tumor suppressor p53 may activate mitochondrial-mediated apoptosis by inducing the expression of proapoptotic genes, including Bax, PUMA, and Noxa.[17] Western blot analysis (Fig. 6), demonstrated that the expression of p53 and Bax decreased and increased after 24 h of 10 and 20 μM **9** exposure to NTUB1 cells. In contrast the GAPDH level, an internal control, did not change. FACS analysis demonstrated that a decreased and a marked accumulation of sub-G1 peaks induced by 10 and 20 μM **9** in NTUB1 cells. It suggested that the apoptosis induced by 20 μM **9** in NTUB1 cells may correlate with elevation of Bax and p53 levels detected after 24 h of 20 μM **9** treatment but the cell death or inhibition of cell growth induced by 10 μM **9** in NTUB1 cells may correlate G1 phase arrest and may be independent with down-regulation expression of P53 and Bax. It needs to further examine the detailed molecular mechanism of cell cycle arrest at G1 phase induced by 10 μM **9**.

The level p53 is controlled by Mdm2 protein which degrades p53 soon after its synthsis.[18] When the cells are suffering certain types of genotaxic stress, ATM or Chk2 can phosphorylate p53 at multiple sites, thus preventing Mdm2-mediated degradation.[18] It also needs to study the levels of phosphor-ATM, phosphor-Chk2, and phpspho-p53 after 20 μM **9** exposure in NTUB1 cells.

3. Conclusion

It was reported that some NHA derivatives did not exert stronger cytotoxicities than those of MHA derivatives.[19] We further synthesized a series of MHA and NHA derivatives and studied their

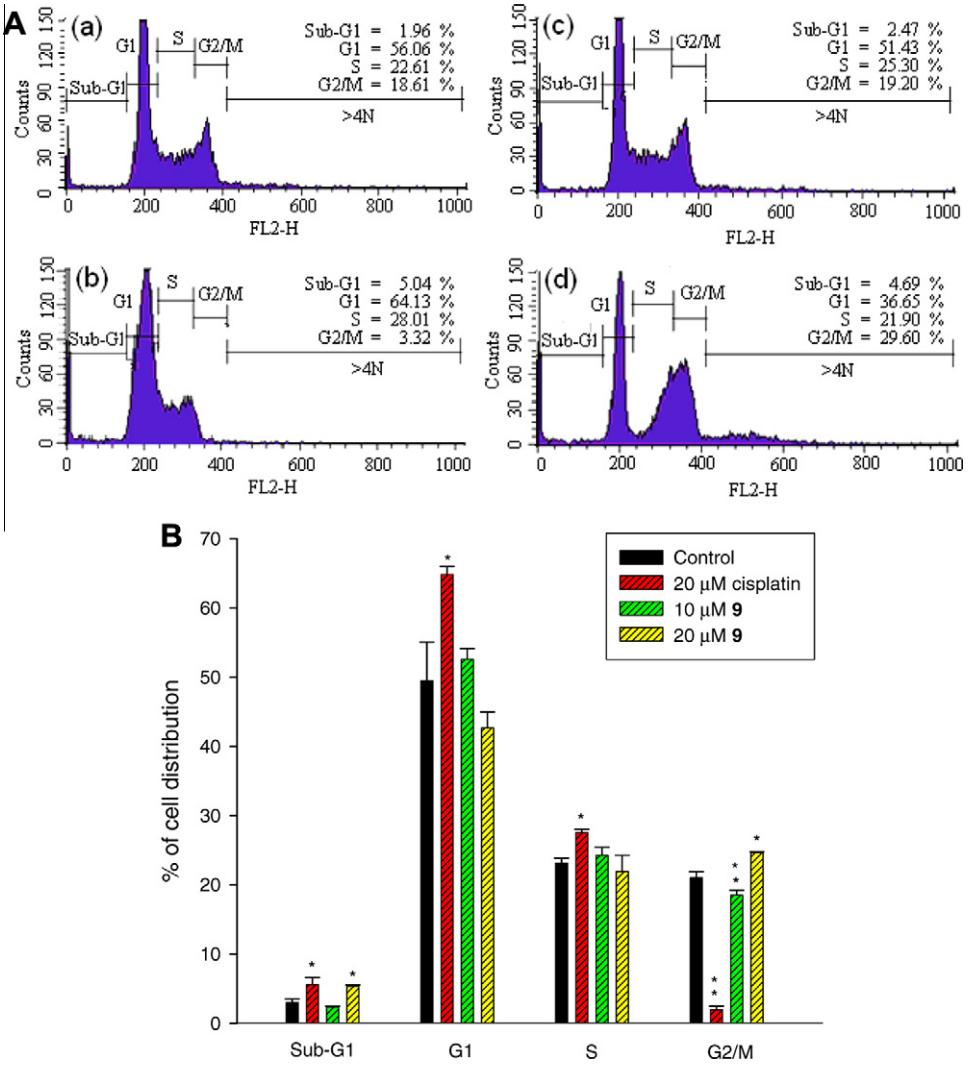


Figure 3. Flow cytometry analysis of cisplatin and **9**-treated NTUB1 cells. (A) NTUB1 cells (3×10^5 cells/ 6 cm dish) was treated with no compound (control) (a), cisplatin 20 μM (b), 10 μM **9** (c), and 20 μM **9** (d) for 24 h. At the time indicated, cells were stained with propidium iodide (PI), DNA contents were analyzed by flow cytometry, and apoptosis was measured by the accumulation of sub-G1 DNA contents in cells. The control cells were treated with medium. Results were representative of three independent experiments. (B) The bars showed the distribution of **9**-treated NTUB1 cells in the phases of the cell cycle. Values shown were means ± SD ($n = 4$) of the percentage of cells in individual phases of the cell cycle from four independent experiments. $^{*}P$ <0.05 and $^{**}P$ <0.01 compared to the control value, respectively.

cytotoxicities against two kinds of cancer cell lines. Among these synthesized MHA and NHA derivatives, compound **9** revealed the most potent cytotoxicity against cancer cell lines used in **Table 1**. Exposure of 10 μM of **9**, 10 and 30 μM of **13**, and 30 and 50 μM of **17** to SV-HUC1 cells, respectively, showed less cytotoxic effects than those of NTUB1 and PC3 cells treated with corresponding concentrations of **9**, **13**, and **17**. In the ROS assay, exposure of NTUB1 cells to 20 μM of **9** induced the increase of generation of ROS than those of 20 μM of **13** and **17**. NAC, an antioxidant, antagonized the cytotoxicity of **9** in NTUB1 cells. It showed that **9** exhibited the cytotoxicity in NTUB1 cells associated with the ROS accumulation. Compound **9** induced G2/M arrest and apoptosis may correlate with the increased expression p21 and cyclin B1 in 20 μM **9**-treated NTUB1 cells, and up-regulation of Bax and p53 expressions in 20 μM **9**-treated NTUB1 cells. The induction of cell death through G2/M phase arrest and apoptosis by 20 μM **9** may correlate with accumulation of ROS and up-regulation of p21, cyclin B1, p53, and Bax expression. Our study may not elucidate the detailed mechanism of this series of anthraquinone derivatives induced inhibition of tumor cell growth, but it may encourage the development of additional series of anthraquinone derivatives as anticancer agent.

4. Experimental section

4.1. General experimental procedures

IR spectra were determined with a Perkin–Elmer system 2000 FTIR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR were recorded on a Varian UNITY-400 spectrometer, and masses were obtained on a JMX-HX 100 mass spectrometer. Elemental analyses were within ±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.

4.2. 3-[3-(Dimethylamino)propoxy]-9,10-anthraquinone (5)

Compound **3** (60 mg, 0.17 mmol) in EtOH (40 mL) was added dimethylamine (130 mg, 1.7 mmol) and then refluxed for 1 h. The product was purified by column chromatography (silica gel and MeOH) to give **5** (7 mg, 0.03 mmol, 15%). IR (KBr) 3384, 1676, 1654, 1590 cm^{-1}. ^{1}H NMR (CD_3OD): δ 2.29 (2H, t, $-OCH_2CH_2-$), 2.98 (6H, s, Me), 3.41 (2H, t, $J = 8.0$ Hz, $-CH_2N(CH_3)_2$), 4.34 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 7.41 (1H, dd, $J = 8.8$, 2.8 Hz, H-2), 7.77 (1H, d, $J = 2.8$ Hz, H-4), 7.87 (2H, m, H-6, and H-7), 8.26 (3H, m, H-1, H-5,

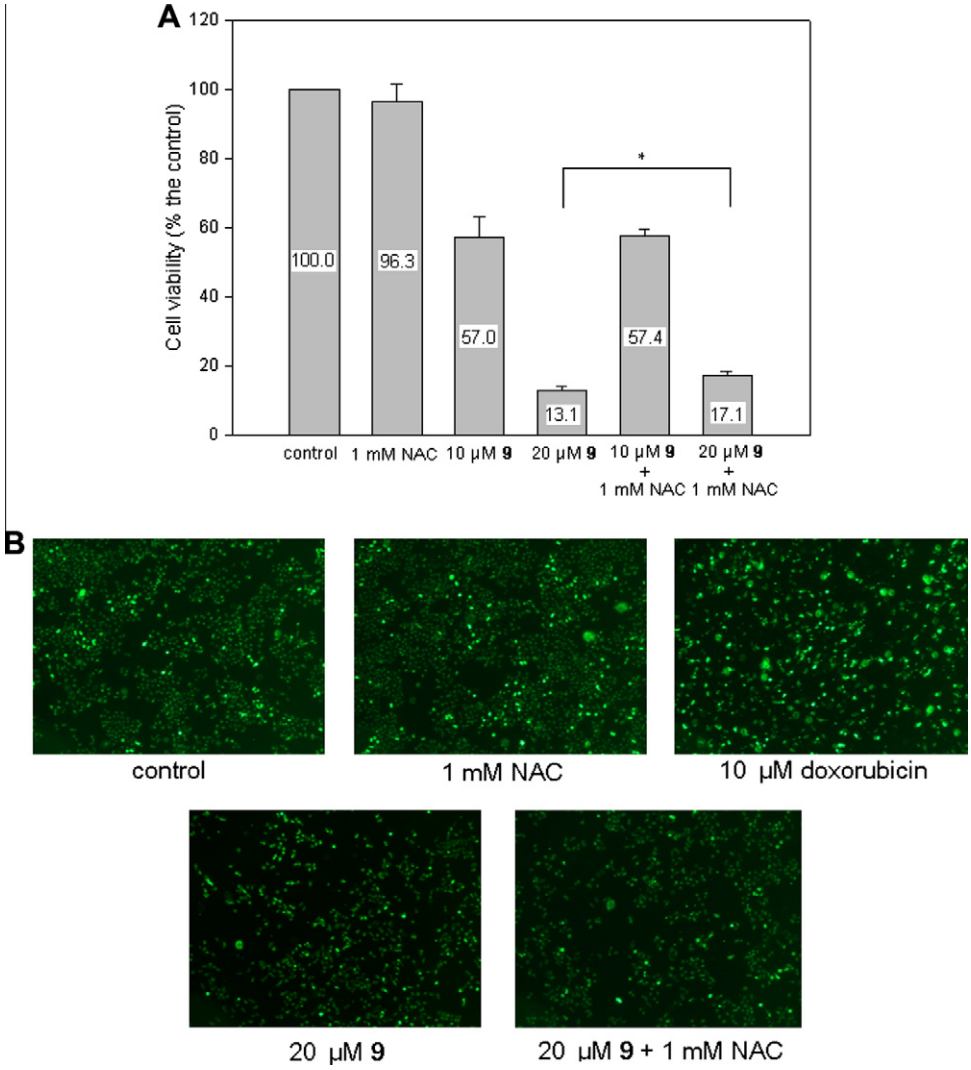


Figure 4. (A) Cytotoxicity of **9** and **9** combined with NAC. Cell viability was assessed by the MTT assay after treating with no compound (control), NAC, **9**, and the combination of NAC and **9** for 72 h. The data shown represent mean ± SD ($n = 3$) for one experiment performed in triplicate. *P value <0.05 compared to the control value. (B) Immunofluorescence staining with H_2DCFDA validated NAC antagonizing the ROS accumulation in **9**-treated NTUB1 cells. NTUB1 cells were incubated with no compound, NAC, doxorubicin, **9**, and the combination of NAC and **9** for 24 h, stained with H_2DCFDA and microscoped.

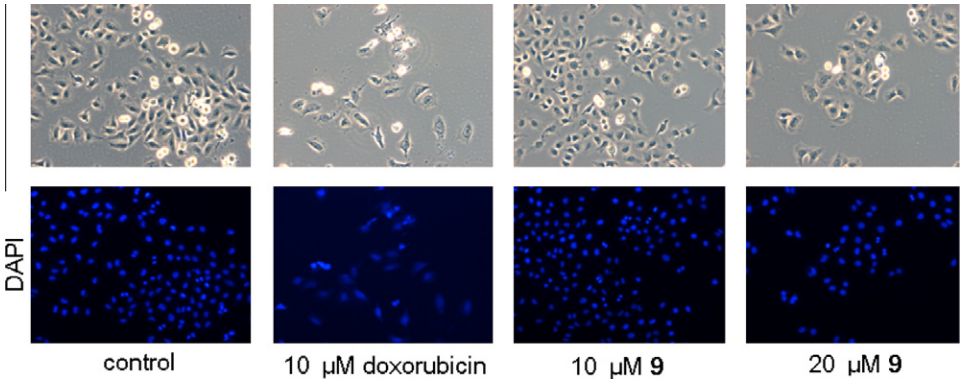


Figure 5. Immuno-fluorescent staining of NTUB1 cells with DAPI after the treatment with no compound (control), 10 μM doxorubicin, and 10 μM and 20 μM **9** for 24 h.

and H-8). ^{13}C NMR (CD_3OD): δ 25.6 (–OCH_2CH_2–), 43.7 (2 × Me), 56.5 (–$CH_2N(CH_3)_2$), 66.8 (–OCH_2–), 112.1 (C-4), 122.1 (C-2), 128.0 (C-5 and C-8), 128.6 (C-9a), 130.8 (C-1), 134.1 (C-8a), 134.9 (C-10a), 135.1 (C-6), 135.6 (C-7), 135.9 (C-4a), 164.8 (C-3), 183.3 (C-9), 184.2 (C-10). EIMS (70 eV) m/z (%rel. int.): 309(12) $[M]^+$. HREIMS m/z $[M]^+$ 309.1367 (calcd for $C_{19}H_{19}NO_3$, 309.1364).

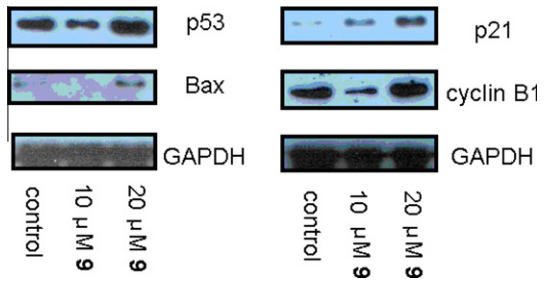


Figure 6. Regulations of p53, Bax, p21, and cyclinB1 in **9**-treated NTUB1 cells were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

4.3. 3-[3-(Ethylamino)propoxy]-9,10-anthraquinone·HCl (6)

Compound **3** (60 mg, 0.17 mmol) in EtOH (40 mL) was added ethylamine (130 mg, 1.7 mmol). The mixture was treated and purified as **5** to yield **6** (15 mg, 0.05 mmol, 30%). IR (KBr) 3447, 1677, 1655, 1590 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.21 (3H, t, J = 7.3 Hz, Me), 2.10 (2H, m, $-OCH_2CH_2-$), 3.01 (2H, q, J = 7.2 Hz, $-CH_2CH_3$), 3.12 (2H, t, J = 7.6 Hz, $-CH_2NH-$), 4.31 (2H, t, J = 6.0 Hz, $-OCH_2-$), 7.45 (1H, dd, J = 8.8, 2.8 Hz, H-2), 7.62 (1H, d, J = 2.8 Hz, H-4), 7.92 (2H, m, H-6 and H-7), 8.19 (3H, m, H-1, H-5, and H-8), 8.45 (2H, br s, NH_2^+). ^{13}C NMR (DMSO-d_6): δ 11.0 (Me), 25.4 ($-OCH_2CH_2-$), 42.1 ($-CH_2CH_3$), 43.5 ($-CH_2NH-$), 65.6 ($-OCH_2-$), 110.7 (C-4), 121.1 (C-2), 126.5 (C-5), 126.7 (C-8), 126.7 (C-9a), 129.6 (C-1), 133.0 (C-8a), 133.1 (C-10a), 134.3 (C-6), 134.7 (C-7), 135.0 (C-4a), 163.0 (C-3), 181.3 (C-9), 182.4 (C-10). EIMS (70 eV) m/z (%rel. int.): 309 (11) $[M]^+$. HREIMS m/z $[M+1]^+$ 310.1441 (calcd for $C_{19}H_{20}NO_3$, 310.1443).

4.4. 1-Hydroxy-3-[3-(ethylamino)propoxy]-9,10-anthraquinone ·HCl (7)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added ethylamine (90 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **7** (25 mg, 0.08 mmol, 35%). IR (KBr) 3447, 1675, 1635, 1591 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.22 (3H, t, J = 6.8 Hz, Me), 2.14 (2H, m, $-OCH_2CH_2-$), 2.97 (2H, m, $-CH_2CH_3$), 3.07 (2H, m, $-CH_2NH-$), 4.28 (2H, t, J = 6.4 Hz, $-OCH_2-$), 6.90 (1H, d, J = 2.4 Hz, H-2), 7.22 (1H, d, J = 2.4 Hz, H-4), 7.94 (2H, m, H-6 and H-7), 8.20 (2H, m, H-5 and H-8), 8.87 (2H, br s, NH_2^+), 12.76 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 11.1 (Me), 25.3 ($-OCH_2CH_2-$), 42.0 ($-CH_2CH_3$), 43.4 ($-CH_2NH-$), 66.0 ($-OCH_2-$), 106.8 (C-2), 107.7 (C-4), 110.3 (C-9a), 126.5 (C-8), 127.0 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-6), 134.8 (C-7), 134.9 (C-4a), 164.6 (C-1), 165.0 (C-3), 181.7 (C-10), 186.3 (C-9). EIMS (70 eV) m/z (% rel. int.): 325 (6) $[M]^+$. HREIMS m/z $[M+1]^+$ 326.1391 (calcd for $C_{19}H_{20}NO_4$, 326.1392).

4.5. 1-Hydroxy-3-[3-(propylamino)propoxy]-9,10-anthraquinone·HCl (8)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added propylamine (130 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **8** (31 mg, 0.09 mmol, 41%). IR (KBr) 3447, 1675, 1634, 1590 cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.92 (3H, t, J = 7.2 Hz, Me), 1.67 (2H, m, $-CH_2CH_3$), 2.18 (2H, m, $-OCH_2CH_2-$), 2.86 (2H, m, $-NHCH_2-$), 3.06 (2H, m, $-CH_2NH-$), 4.27 (2H, t, J = 6.4 Hz, $-OCH_2-$), 6.86 (1H, d, J = 2.4 Hz, H-2), 7.17 (1H, d, J = 2.4 Hz, H-4), 7.92 (2H, m, H-6 and H-7), 8.17 (2H, m, H-5 and H-8), 9.11 (2H, br s, NH_2^+), 12.67 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 11.5 (Me), 19.4 ($-CH_2CH_3$), 25.6 ($-OCH_2CH_2-$), 44.3 ($-NHCH_2-$), 48.9 ($-CH_2NH-$), 66.5 ($-OCH_2-$), 107.2 (C-2), 108.1 (C-4), 110.7 (C-9a), 126.9 (C-8), 127.4 (C-5), 133.3 (C-8a and C-10a), 135.1 (C-6), 135.2 (C-7), 135.3 (C-4a), 165.0 (C-1), 165.4 (C-3), 182.1 (C-10), 186.7 (C-9). EIMS (70 eV) m/z (%rel. int.): 339 (7) $[M]^+$. HREIMS m/z $[M+1]^+$ 340.1550 (calcd for $C_{20}H_{22}NO_4$, 340.1549).

4.6. 1-Hydroxy-3-[3-(pyrrolidin-1-yl)propoxy]-9,10-anthraquinone·HCl (9)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added pyrrolidine (150 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **9** (25 mg, 0.07 mmol, 33%) IR (KBr) 3421, 1670, 1637, 1589 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.15 (2H, m, $-OCH_2CH_2-$), 2.29 (2H, m, $-CH_2CH_2-$), 2.55 (2H, m, $-CH_2CH_2-$), 2.86 (2H, m, $-NCH_2-$), 3.33 (2H, m, $-NCH_2-$), 3.90 (2H, m, $-CH_2N-$), 4.23 (2H, t, J = 6.4 Hz, $-OCH_2-$), 6.69 (1H, d, J = 2.4 Hz, H-2), 7.32 (1H, d, J = 2.4, H-4), 7.81 (2H, m, H-6 and H-7), 8.29 (2H, m, H-5 and H-8), 12.10 (1H, br s, $\overset{\oplus}{N}H$), 12.86 (1H, s, OH-1). ^{13}C NMR ($CDCl_3$): δ 23.5 ($-CH_2CH_2-$), 25.5 ($-OCH_2CH_2-$), 53.1 ($-CH_2NH-$), 54.0 ($-N(CH_2CH_2)_2$), 65.5 ($-OCH_2-$), 107.0 (C-2), 107.6 (C-4), 110.6 (C-9a), 126.9 (C-8), 127.4 (C-5), 133.4 (C-8a and C-10a), 134.3 (C-6), 134.4 (C-7), 135.1 (C-4a), 164.6 (C-1), 165.4 (C-3), 182.3 (C-10), 186.9 (C-9). EIMS (70 eV) m/z (%rel. int.): 351 (8) $[M]^+$. HREIMS m/z $[M]^+$ 351.1467 (calcd for $C_{21}H_{21}NO_4$, 351.1470).

4.7. 1-Hydroxy-3-{3-[(2-hydroxyethyl)methylamino]propoxy}-9,10-anthraquinone·HCl (10)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added *N*-methylethanolamine (170 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **10** (20 mg, 0.06 mmol, 25%). IR (KBr) 3421, 1670, 1637, 1589 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.22 (2H, m, $-OCH_2CH_2-$), 2.82 (3H, s, $-NCH_3$), 3.33 (4H, br s, $-CH_2NCH_2-$), 3.77 (2H, m, $-NCH_2CH_2OH$), 4.25 (2H, t, J = 6.4 Hz, $-OCH_2-$), 5.37 (1H, t, J = 5.0 Hz, $-CH_2OH$), 6.90 (1H, d, J = 2.4 Hz, H-2), 7.21 (1H, d, J = 2.4 Hz, H-4), 7.92 (2H, m, H-6 and H-7), 8.19 (2H, m, H-5 and H-8), 10.06 (1H, s, $\geqslant\overset{\oplus}{N}H$), 12.76 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 23.1 ($-OCH_2CH_2-$), 52.6 ($>NCH_2-$), 55.2 ($>NCH_2-$), 56.9 ($>NCH_3$), 66.1 ($-OCH_2-$), 106.7 (C-2), 107.7 (C-4), 110.6 (C-9a), 126.5 (C-8), 126.9 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-6), 134.8 (C7), 134.9 (C-4a), 164.6 (C-1), 164.9 (C-3), 181.6 (C-10), 186.3 (C-9). EIMS (70 eV) m/z (%rel. int.): 355 (2) $[M]^+$. HREIMS m/z $[M+1]^+$ 356.1500 (calcd for $C_{20}H_{22}NO_5$, 356.1498).

4.8. 1-Hydroxy-3-[3-(2-hydroxypropylamino)propoxy]-9,10-anthraquinone·HCl (11)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added 1-amino-2-propanol (170 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **11** (21 mg, 0.06 mmol, 28%). IR (KBr) 3420, 1670, 1630, 1590 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.13 (3H, d, J = 6 Hz, Me), 2.17 (2H, m, $-OCH_2CH_2-$), 2.80 (1H, m, $-NHCHH-$), 3.00 (1H, m, $-NHCHH-$), 3.10 (2H, m, $-CH_2NH-$), 3.99 (1H, m,$-CH_2CH(OH)-$), 4.27 (2H, t, J = 6.4 Hz, $-OCH_2-$), 5.40 (1H, br s, $>CHOH$), 6.89 (1H, d, J = 2.4 Hz, H-2), 7.20 (1H, d, J = 2.4 Hz, H-4), 7.94 (2H, m, H-6 and H-7), 8.17 (2H, m, H-5 and H-8), 8.71 (H, br s, $>\overset{\oplus}{N}HH$), 8.94 (H, br s, $>\overset{\oplus}{N}HH$), 12.76 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 21.1 (Me), 25.0 ($-OCH_2CH_2-$), 44.3 ($-NHCH_2-$), 53.5 ($-CH_2NH-$), 62.2 ($>CHOH$), 66.1 ($-OCH_2-$), 106.8 (C-2), 107.7 (C-4), 110.3 (C-9a), 126.5 (C-8), 126.9 (C-5), 132.9 (C-8a), 132.9 (C-10a), 134.7 (C-6), 134.8 (C-7), 134.9 (C-4a), 164.6 (C-1), 165.0 (C-3), 181.6 (C-10), 186.3 (C-9). EIMS (70 eV) m/z (% rel. int.): 355

(1) $[M]^+$. HREIMS *m/z* $[M+1]^+$ 356.1500 (calcd for $C_{20}H_{22}NO_5$, 356.1498).

4.9. 1-Hydroxy-3-[3-(piperidin-1-yl)propoxy]-9,10-anthraquinone·HCl (12)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added piperidine (190 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **12** (34 mg, 0.09 mmol, 42%). IR (KBr) 3447, 1670, 1654, 1589 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.40 (H, m, $-NCH_2CH_2CHH-$), 1.77 (5H, m, $-NCH_2CH_2CHH-$), 2.22 (2H, m, $-OCH_2CH_2-$), 2.90 (2H, m, $-NCH_2-$), 3.22 (2H, m, $-NCH_2-$), 3.48 (2H, m, $-CH_2N<$), 4.27 (2H, t, *J* = 6.4 Hz, $-OCH_2-$), 6.91 (1H, d, *J* = 2.4 Hz, H-2), 7.23 (1H, d, *J* = 2.4 Hz, H-4), 7.95 (2H, m, H-6 and H-7), 8.23 (2H, m, H-5 and H-8), 9.80 (H, br s, $\overset{\oplus}{N}H$), 12.77 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 21.8 ($-OCH_2CH_2-$), 22.9 ($-N(CH_2CH_2)_2-$), 23.8 ($-NCH_2CH_2CH_2-$), 52.5 ($-N(CH_2CH_2)_2-$), 53.5 ($-CH_2N<$), 66.6 ($-OCH_2-$), 107.2 (C-2), 108.2 (C-4), 110.8 (C-9a), 127.0 (C-8), 127.4 (C-5), 133.4 (C-8a and C-10a), 135.2 (C-6), 135.3 (C-7), 135.3 (C-4a), 165.1 (C-1), 165.4 (C-3), 182.1 (C-10), 186.8 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 365 (5) $[M]^+$. HREIMS *m/z* $[M+1]^+$ 366.1706 (calcd for $C_{22}H_{24}NO_4$, 366.1705).

4.10. 1-Hydroxy-3-[3-(morpholin-4-yl)propoxy]-9,10-anthraquinone·HCl (13)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added morpholine (190 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **13** (32 mg, 0.09 mmol, 40%). IR (KBr) 3420, 1670, 1637, 1588 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.24 (2H, m, $-OCH_2CH_2-$), 3.09 (2H, m, $-NCH_2-$), 3.30 (2H, br s, $-NCH_2-$), 3.47 (2H, m, $-CH_2N-$), 3.80 (2H, m, $-CH_2CH_2O-$), 3.98 (2H, m, $-CH_2CH_2O-$), 4.28 (2H, t, *J* = 6.0 Hz, $-OCH_2-$), 6.91 (1H, d, *J* = 2.4 Hz, H-2), 7.22 (1H, d, *J* = 2.4 Hz, H-4), 7.94 (2H, m, H-6 and H-7), 8.20 (2H, m, H-5 and H-8), 10.95 (1H, br s, $\overset{\oplus}{N}H$), 12.77 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 22.8 ($-OCH_2CH_2-$), 51.1 ($N(CH_2CH_2)_2O$), 53.2 ($-CH_2NH-$), 63.2 ($N(CH_2CH_2)_2O$), 66.1 ($-OCH_2-$), 106.7 (C-2), 107.7 (C-4), 110.3 (C-9a), 126.5 (C-8), 127.0 (C-5), 132.9 (C-8a and C-10a), 134.7 (C-6), 134.8 (C-7), 134.9 (C-4a), 164.6 (C-1), 164.9 (C-3), 181.7 (C-10), 186.3 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 367 (3) $[M]^+$. HREIMS *m/z* $[M+1]^+$ 368.1499 (calcd for $C_{21}H_{22}NO_5$, 368.1498).

4.11. 1-Hydroxy-3-[3-(cyclohexylamino)propoxy]-9,10-anthraquinone·HCl (14)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added cyclohexylamine (220 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **14** (37 mg, 0.10 mmol, 44%). IR (KBr) 3446, 1670, 1637, 1577 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.11 (1H, m, $-CH_2CH_2CHH-$), 1.26 (4H, m, 2 × $-CH_2CH_2-$), 1.60 (1H, m, $-CH_2CH_2CHH-$), 1.78 (2H, m, $-CH_2CH_2-$), 2.08 (2H, m, $-CH_2CH_2-$), 2.12 (2H, m, $-OCH_2CH_2-$), 3.04 (1H, m, $-NHCH-$), 3.12 (2H, m, $-CH_2NH-$), 4.29 (2H, t, *J* = 6.0 Hz, $-OCH_2-$), 6.92 (1H, d, *J* = 2.4 Hz, H-2), 7.25 (1H, d, *J* = 2.4 Hz, H-4), 7.95 (2H, m, H-6 and H-7), 8.22 (2H, m, H-5 and H-8), 8.49 (2H, br s, $>\overset{\oplus}{N}HH$), 12.80 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 23.8 ($-CH_2-$), 24.7 ($-OCH_2CH_2-$), 25.4 ($-CH_2-$), 28.6 (2 × $-CH_2CH_2-$), 40.9 ($-NHCH-$), 56.0 ($-CH_2NH-$), 65.9 ($-OCH_2-$), 106.8 (C-2), 107.6 (C-4), 110.4 (C-9a), 126.5 (C-5), 127.0 (C-8), 132.9 (C-8a and C-10a), 134.8 (C-4a and C-6), 134.9 (C-7), 164.6 (C-1), 164.9 (C-3), 181.7 (C-10), 186.4 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 379 (10) $[M]^+$. HREIMS *m/z* $[M+1]^+$ 380.1860 (calcd for $C_{23}H_{26}NO_4$, 380.1862).

4.12. 1-Hydroxy-3-[3-(4-methylpiperazin-1-yl)propoxy]-9,10-anthraquinone (15)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added methylpiperazine (220 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **15** (25 mg, 0.07 mmol, 30%). IR (KBr) 3420, 1670, 1636, 1589 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.60 (2H, m, $-OCH_2CH_2-$), 2.20 (3H, br s, $-CH_3$), 2.74 (4H, br s, 2 × $-NCH_2CH_2-$), 2.94 (4H, br s, 2 × $-NCH_2CH_2-$), 3.40 (2H, m, $-CH_2N-$), 4.18 (2H, t, *J* = 6.0 Hz, $-OCH_2-$), 6.17 (1H, d, *J* = 2.4 Hz, H-2), 7.37 (1H, d, *J* = 2.4 Hz, H-4), 7.80 (2H, m, H-6 and H-7), 8.27 (2H, m, H-5 and H-8), 12.86 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 43.6 (Me), 49.7 (2 × $-NCH_2CH_2-$), 53.6 (2 × $-NCH_2CH_2-$ and $-CH_2N-$), 66.5 ($-OCH_2-$), 107.3 (C-2), 107.7 (C-4), 126.8 (C-5), 127.4 (C-8), 133.4 (C-10a), 133.5 (C-8a), 134.2 (C-6), 134.4 (C-7), 135.1 (C-4a), 165.3 (C-1 and C-3), 182.5 (C-10), 186.8 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 380 (3) $[M]^+$. HREIMS *m/z* $[M]^+$ 380.1739 (calcd for $C_{22}H_{24}N_2O_4$, 380.1736).

4.13. 1-Hydroxy-3-{3-[4-(2-hydroxyethyl)piperazin-1-yl]propoxy}-9,10-anthraquinone (16)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added ethanolpiperazine (290 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **16** (25 mg, 0.06 mmol, 28%). IR (KBr) 3446, 1670, 1637, 1577 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.10 (2H, br s, $-OCH_2CH_2-$), 3.20 (2H, br s, $-CH_2NH-$), 3.48 (10H, br s, 10 × $-NCH_2CH_2-$), 3.78 (2H, br s, $-CH_2OH-$), 4.28 (2H, t, *J* = 6.0 Hz, $-OCH_2-$), 6.93 (1H, d, *J* = 2.4 Hz, H-2), 7.24 (1H, d, *J* = 2.4 Hz, H-4), 7.95 (2H, m, H-6 and H-7), 8.24 (2H, m, H-5 and H-8), 12.78 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 23.1 ($-OCH_2CH_2-$), 48.2 ($-NCH_2CH_2-$), 48.7 ($-NCH_2CH_2-$), 52.9 ($-CH_2N-$), 55.2 (2 × $-NCH_2CH_2-$), 58.0 ($-CH_2OH-$), 66.0 ($-OCH_2-$), 106.8 (C-2), 107.8 (C-4), 110.3 (C-9a), 126.4 (C-5), 127.0 (C-8), 132.9 (C-8a), 132.9 (C-10a), 134.7 (C-4a), 134.8 (C-6), 134.9 (C-7), 164.6 (C-1), 164.9 (C-3), 182.7 (C-10), 186.3 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 410 (5) $[M]^+$. HREIMS *m/z* $[M+1]^+$ 411.1918 (calcd for $C_{23}H_{27}N_2O_5$, 411.1920).

4.14. 4-[3-(4-Hydroxy-9,10-dioxo-9,10-dihydro-anthracen-2-yloxy)-propyl]-piperazine-1-carboxylic acid ethyl ester·2HCl (17)

Compound **4** (80 mg, 0.22 mmol) in EtOH (40 mL) was added 1-piperzine-carboxylic acid ethyl ester (350 mg, 2.2 mmol). The mixture was treated and purified as **5** to yield **17** (39 mg, 0.09 mmol, 40%). IR (KBr) 3446, 1670, 1637, 1588 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.18 (3H, t, *J* = 6.0 Hz, Me), 2.23 (2H, m, $-OCH_2CH_2-$), 3.07 (4H, m, 2 × $-NCH_2CH_2-$), 3.26 (2H, m, $-CH_2N-$), 3.57 (4H, m, 2 × $-NCH_2CH_2-$), 4.07 (4H, m, $-OCH_2CH_3$ and $>NCH_2-$), 4.28 (2H, t, *J* = 6.0 Hz, $-OCH_2-$), 6.91 (1H, d, *J* = 2.4 Hz, H-2), 7.22 (1H, d, *J* = 2.4 Hz, H-4), 7.94 (2H, m, H-6 and H-7), 8.18 (2H, m, H-5 and H-8), 9.11 (1H, br s, $>\overset{\oplus}{N}H$), 10.83 (1H, br s, $>\overset{\oplus}{N}H$), 12.77 (1H, s, OH-1). ^{13}C NMR (DMSO-d_6): δ 14.5 (Me), 23.0 ($-OCH_2CH_2-$), 42.3 (2 × $-NCH_2CH_2-$), 50.5 (2 × $-NCH_2CH_2-$), 52.9 ($-CH_2N-$), 61.4 ($-OCH_2CH_3$), 66.0 ($-OCH_2-$), 106.7 (C-2), 107.8 (C-4), 110.4 (C-9a), 126.5 (C-5), 127.0 (C-8), 132.9 (C-8a), 132.9 (C-10a), 134.7 (C-4a), 134.8 (C-6), 134.9 (C-7a), 154.3 (C=O), 164.5 (C-1), 164.9 (C-3), 181.7 (C-10), 186.3 (C-9). EIMS (70 eV) *m/z* (% rel. int.): 439 (22) $[M+1]^+$. HREIMS *m/z* $[M+1]^+$ 439.1872 (calcd for $C_{24}H_{27}N_2O_6$, 439.1869).

4.15. Assays of NTUB1, PC3, and SV-HUC1 cell lines

NTUB1, an immortalized human urothelial carcinoma cell line, was established from a high-grade bladder cancer.[20] PC3 (a human prostate cancer cell line), and SV-HUC1 (a SV-40 immortalized human uroepithelial cell line) were obtained from ATCC. The cells were maintained in RPMI 1640 (for NTUB1 and PC3 cells) or F12 medium (for SV-HUC1) supplemented with 10% FBS, 100 unit/mL

penicillin-G, 100 μg/mL streptomycin and 2 mM L-glutamine. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

For evaluating the cytotoxic effect of tested compounds and cisplatin, a modified MTT assay was performed.[21–23] Briefly, the cells were plated at a density of 1000 (for NTUB1 and PC3 cells) and 5000 (for SV-HUC1 cells) cells/well in 96-well plates and incubated at 37 °C overnight before drug exposure. Cells were then cultured in the presence of graded concentrations of tested compounds at 37 °C for 72 h. At the end of the culture period, 50 μL of MTT (2 mg/mL in PBS) was added to each well and allowed to react for 3 h. Following centrifugation of plates at 1000 × g for 10 min, media were removed and 150 μL DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540 nm using MRX (DYNEXCO) microplate reader. The cell viability was expressed as a percentage to the viable cells of control culture condition. The IC_{50} values of each group were calculated by the median-effect analysis and presented as mean ± standard deviation (SD).

4.16. Quantitative analysis of intracellular reactive oxygen species (ROS)

Production of ROS was analyzed by flow cytometry as described previously.[22] Briefly, cells were plated and treated as indicated conditions. 10 μM H_2DCFDA (Molecular Probes, Eugene, OR) was added to the treated cells 30 min prior harvest. The cells were collected by trypsinization and washed with PBS. The green fluorescence of intracellular DCF (2′,7′-dichlorofluorescein) was then analyzed immediately by FACScan flow cytometer with a 525-nm band pass filter (Becton Dickinson).

4.17. Cell cycle analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described.[22–24] Briefly, 3×10^5 cells were plated and treated with 20 μM cisplatin (Pharmacia & Upjohn, Milan, Italy), various concentrations of **9** for 24 h, respectively. These cells were harvested by trypsinization, washed with 1 × PBS, and fixed in ice-cold MeOH at −20 °C. After overnight incubation, the cells were washed with PBS and incubated with 50 μg/mL PI (Sigma, Co) and 50 μg/mL RNase A (Sigma, Co) in PBS at room temperature for 30 min. The fractions of cells in each phase of cell cycle were analyzed using FACScan flow cytometer and Cell Quest software (Becton Dickinson).

4.18. Immunofluorescence microscopy

NTUB1 cells (3×10^5 cells) plated on the 6-cm dish were treated no compound as control and indicated compounds for 24 h. After treatment, cells were fixed with 2% formaldehyde/PBS for 20 min, washed with PBS, and cold methanol (−20 °C) for 3 min. After washing with PBS, cells were added H_2DCFDA in PBS and incubated for 30 min at 37 °C. Then cells were washed with PBS and stained with DAPI (Sigma, Co) in dark room for 1 h at room temperature. After being washed with PBS, cells were examined with Axioskop 2 plus fluorescence microscope.

4.19. Western blot analysis

Cells were harvested by trypsinization and resuspended with suitable amount of PBS adjusted with the cell numbers. The cells were mixed with equal volume of 2 × sample buffer and boiled for 10 min twice to denature the proteins. Cell extracts were separated by SDS–PAGE. The proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20). The membranes were incubated with specific antibodies at 4 °C overnight. The membranes were washed with TBST buffer and incubated with secondary antibody at room temperature for another 1 h. Signals were detected by chemiluminescence ECL reagent after TBST wash and visualized on Fuji SuperRX film.

4.20. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the student's *t*-test method for two group comparison, with $p < 0.05$ was considered to be statistically significant.

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