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Original article

Antioxidant xanthone derivatives induce cell cycle arrest and apoptosis and enhance cell death induced by cisplatin in NTUB1 cells associated with ROS

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ABSTRACT

In an effort to develop novel antioxidant as anticancer agents, a series of xanthones were prepared. In vitro screening, the synthetic xanthones revealed significant inhibitory effects on xanthine oxidase and ABTS radical-cation scavenging activity. The selective compounds **2** and **8** induced an accumulation of NTUB1 cells in the G_1 phase arrest and cellular apoptosis by the increase of ROS level. The combination of cisplatin and **2** significantly enhanced the cell death in NTUB1 cells. Compounds **2** and **8** did not show cytotoxic activity in selected concentrations against SV-HUC1 cells. The present results suggested that antioxidants **2** and **8** may be used as anticancer agent for enhancing the therapeutic efficacy of anticancer agents and to reduce their side effect.

1. Introduction

Xanthones are found in natural products and synthetic chemistry. Xanthone derivatives are characterized with diverse biological activities, including anti-inflammatory, anti-bacteria, anti-platelet, anti-hypertensive, vasorelaxing and cytotoxic effects, reported in the past decade [1–4]. In our previous papers, we have demonstrated that various synthetic xanthones revealed potential pharmacological activities [3–5].

Reactive oxygen species (ROS) are resulting in oxidation of various cell constituents as DNA, lipid, and proteins and consequently cause oxidative damage to cellular substance leading to cell death [6]. The oxidative damage of DNA induced by ROS lead to certain cancers, and ROS may also play a role in cell cycle progression. ROS is implicated in numerous pathological events including metabolic disorders, cellular aging, reperfusion damage of DNA, inflammation, atherosclerosis and carcinogenesis [7].

Xanthine oxidase (XO) is a key enzyme that catalyzes the oxidation of xanthine and hypoxanthine into uric acid and induces hyperuricemia and gout [8]. Antioxidant therapies have been recognized as a potential strategy for preventing acute CNS injury, cardiovascular diseases and asthma [8]. In the investigation on antioxidant agent, we found that synthetic xanthones significantly inhibited oxidative damage of DNA, XO activity, and ABTS radical-cation scavenging activity. For continual discovery of antioxidant agent as anticancer agent, we reported the synthesis, antioxidant activity, cytotoxicity against NTUB1 cells (human bladder cancer cell line) and SV-HUC1 cells (a SV-40 immortalized human uroepithelial cell line), and the structure–activity relationship of various synthetic xanthones in the present paper.

2. Chemistry

As shown in Schemes 1 and 2, compounds **1** and **7** were synthesized with appropriated benzoic acids, methoxybenzenes and related reagents. A mixture of **1** or **7** and 1,3-dibromopropane was reacted in appropriate solvents and then aminated with appropriate amines respectively, to give the final products, **2**–**6** or **8**–**14** (Schemes 1 and 2) [5].

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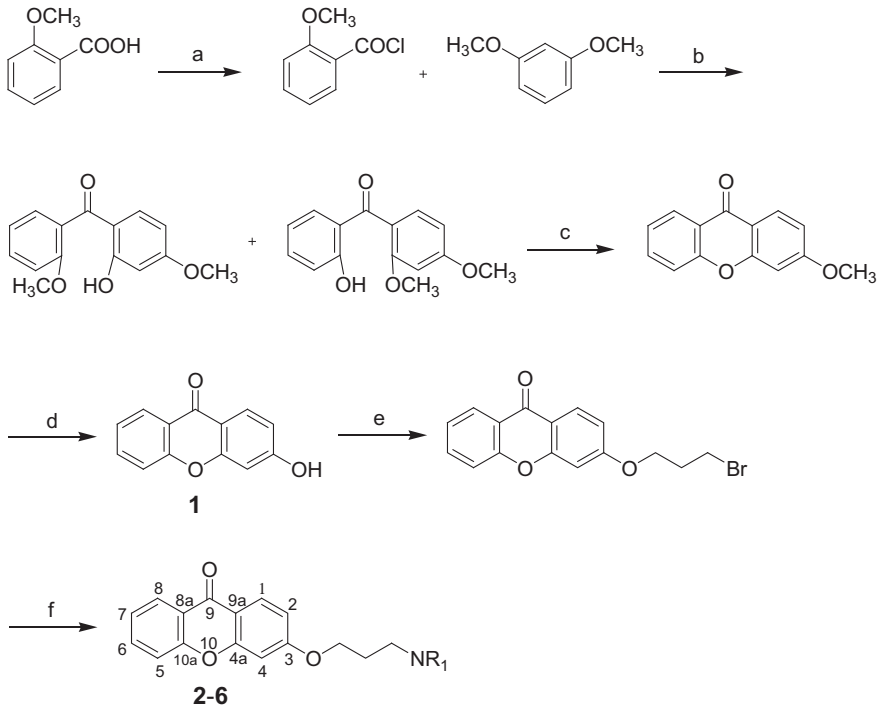
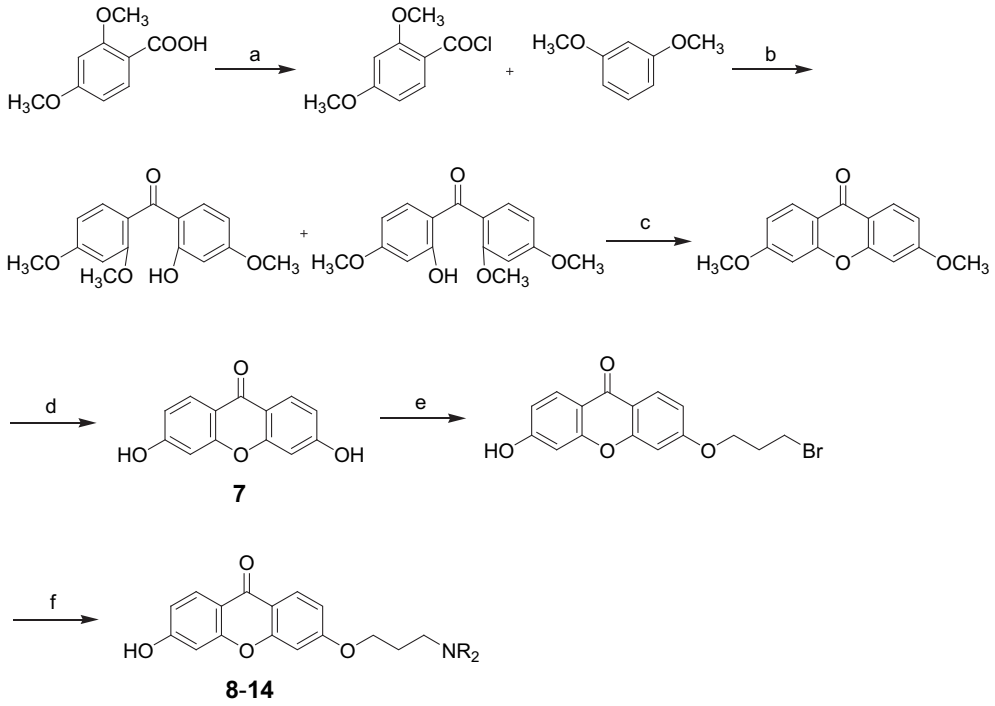


Scheme 1. Synthesis of aminated derivatives of 3-hydroxyxanthone. Reagents:(a) oxalyl chloride, anhydrous benzene, rt, 2 h; (b) $AlCl_3$, anhydrous ether, rt, 8 h; (c) pyridine, 10% tetramethylammonium hydroxide, reflux, 36 h; (d) phenol, HI, 160 °C, 8 h; (e) NaOH, H_2O, *n*-BuOH, 3-hydroxyxanthone, 1,3-dibromopropane, reflux, 5 h; (f) absolute EtOH, appropriate amine, stirred, 60–70 °C, 5 h. Substituents were described in Table 1.

3. Pharmacology

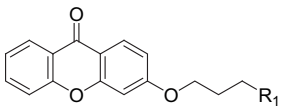
All synthesized compounds were tested for their antioxidant activity, including inhibition of oxidative DNA damage by agarose gel electrophoresis, inhibitory effect on xanthine oxidase, and ABTS radical-cation scavenging activity. The selected compounds **2** and **8** with potent antioxidant activity were further tested for cytotoxicity against NTUB1 and SV-HUC1 cells by MTT assay. The cytotoxic activity of **2** and **8** combined with cisplatin was also examined by MTT assay. For further evaluation of cytotoxic effect



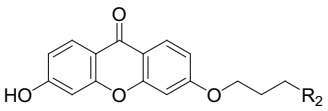
Scheme 2. Synthesis of aminated derivatives of 3,6-dihydroxyxanthone. Reagents:(a) oxalyl chloride, anhydrous benzene, rt, 2 h; (b) $AlCl_3$, anhydrous ether, rt, 8 h; (c) pyridine, 10% tetramethylammonium hydroxide, reflux, 36 h; (d) phenol, HI, 160 °C, 8 h; (e) NaOH, H_2O, *n*-BuOH, 3,6-dihydroxyxanthone, 1,3-dibromopropane, reflux, 5 h; (f) absolute EtOH, appropriate amine, stirred, 60–70 °C, 5 h. Substituents were described in Table 1.

and mechanisms of **2** and **8**, and the combination of **2** and **8** with cisplatin induced cell death in vitro, we first examined the effects of **2** and **8** on the intracellular ROS amount in NTUB1 cells and cell cycle progression determined by using fluorescence-activated cell sorting (FACS).

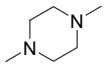
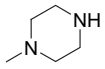
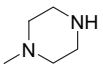
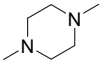
Table 1
Structure and basic data of xanthones.



2-6



8-14

Compounds	R_1	R_2	Formula	Yield %
2			$C_{21}H_{23}NO_3$	30
3			$C_{20}H_{21}NO_3$	40
4			$C_{21}H_{24}N_2O_3$	35
5			$C_{20}H_{22}N_2O_3$	38
6			$C_{20}H_{23}NO_3$	43
8			$C_{21}H_{23}NO_4$	33
9			$C_{20}H_{21}NO_4$	39
10			$C_{19}H_{19}NO_4$	35
11			$C_{22}H_{25}NO_4$	32
12			$C_{20}H_{23}NO_4$	40
13			$C_{20}H_{22}N_2O_4$	42
14			$C_{21}H_{24}N_2O_4$	39

4. Results and discussion

The inhibition of oxidative DNA damage by agarose gel electrophoresis, the inhibitory effect on xanthine oxidase, and ABTS radical-cation scavenging activity were used to evaluate the series of synthesized xanthones as antioxidants. As shown in Fig. 1, selected compounds, **2**–**6** and **8**–**11** showed protective effects of oxidative DNA damage caused by $O_2^{-}\cdot$ generated by xanthine (XA) and xanthine oxidase (XO). Compounds **2**–**6** and **8**–**11**, each showed protective effects on oxidative DNA damage caused by $O_2^{-}\cdot$ at 300 μM.

For determination of the antioxidant activity of the series of synthesized xanthones, ABTS radical-cation scavenging activity and XO inhibitory activity of **2**–**6** and **8**–**14** also were analyzed. As shown in Fig. 2, compound **2** with a piperidine ring substituted at C-3 of propoxy side chain and allopurinol (positive control) significantly inhibited the XO activity in a concentration-dependent manner with IC_{50} values of 37.8 ± 3.9 and 1.9 ± 0.7 μM respectively. Compounds **3**–**6** also showed concentration-dependent manner but possessed relatively weaker activities than that of **2** (Table 2). As shown in Fig. 3, compounds **8**–**14** and allopurinol all inhibit the XO activity in a concentration-dependent manner. Compound **14** with a methylpiperazine ring substituted at C-3 of propoxy side chain showed significant activity with an IC_{50} value of 44.4 ± 3.9 μM (Table 2). According to Figs. 2 and 3, a OH (**8**–**14**) or without a OH group substituted (**2**–**6**) at C-6 of xanthone moiety revealed no significant difference in the inhibitory effects on XO activity, but a piperidine or a piperazine ring substituted at C-3 of propoxy side chain of xanthone moiety enhanced the inhibitory effect on XO activity.

We also evaluated the ABTS radical-cation scavenging activities of **2**–**6** and **8**–**14**. As shown in Table 3, all synthesized xanthones showed ABTS radical-cation scavenging activities except for **3**. Among them **8** and **13** possessed stronger ABTS radical-cation scavenging activities with IC_{50} values of 69.3 ± 2.6 and 65.0 ± 4.1 μM respectively than that of the positive control, vitamin E (IC_{50} = 77.2 ± 3.5 μM). In turn, we found that compounds **5**, **8**, **13**, and **14** with a piperidine or piperazine ring substituted at C-3 of propoxy side chain showed significant ABTS radical-cation scavenging activities. As mentioned above, the synthesized xanthones with a piperidine or a piperazine ring substituted at C-3 of propoxy side chain enhanced the antioxidant activity.

The MTT microassay was used for the evaluation of cytotoxic properties of selective compounds, **2** and **8**, against NTUB1 cells. Fig. 4 showed the cell viability of **2** and **8** assessed by the MTT assay after treating with different concentrations of **2** and **8** for 72 h. The IC_{50} values of **2** and **8** were 31.2 ± 2.9 and 35.1 ± 4.0 μM respectively. The cell cycle progression of **2** and **8** was determined by using fluorescence-activated cell sorting (FACS) analysis in propidium iodide-stained NTUB1 cells. As shown in Figs. 5 and 6, NTUB1 cells were exposed to 40 and 60 μM of **2** and **8** respectively for 24 h. Obviously, G_1 arrest was induced, accompanied by an increased cell apoptosis. It also suggested that **2** and **8** revealed same cell cycle arrest in NTUB1 cells.

It was proven that ROS induced programmed cell death or necrosis, grand effect on gene expression and activated cell signaling cascades [9]. As shown in Fig. 7, NTUB1 cells were exposed to 20 and 40 μM of **2** and **8** respectively for 24 h, leading to significant enhancement of intracellular ROS levels. ROS cause adaptive cellular response to apoptosis or necrosis, depending on the level of ROS. It was well known that cellular ROS were essential to cell survival, but the effect of ROS on cells is complicated [10]. Experimentally, low concentrations of H_2O_2 caused a moderate enhancement in proliferation of many tumor cell lines, while higher levels resulted in slowed cell growth, cell cycle arrest,

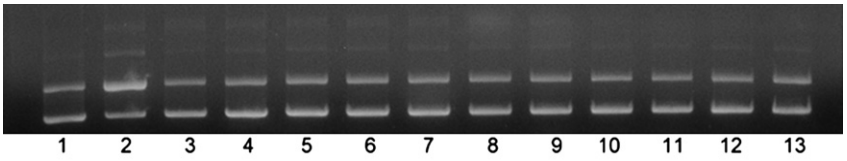


Fig. 1. Inhibition of DNA strand breaks induced by $O_2^{\bullet}$ (generated by XA/XO) in the presence of **2**–**6** and **8**–**11** studied by gel electrophoresis. Supercoiled plasmid pBR322 DNA (500 ng) in phosphate buffer (pH 7.4) solution was incubated for 20 min with XA/XO acting as the control. Lane 1, DNA (without XA/XO); lane 2, control; lane 3, control + SOD (300 μM); lane 4, control + quercetin (300 μM) serving as positive control; lane 5, control + **2** (300 μM); lane 6, control + **3** (300 μM); lane 7, control + **4** (300 μM); lane 8, control + **5** (300 μM); lane 9, control + **6** (300 μM); lane 10, control + **8** (300 μM); lane 11, control + **9** (300 μM); lane 12, control + **10** (300 μM); lane 13, control + **11** (300 μM).

apoptosis even necrosis [10]. Treatments of NTUB1 cells of **2** and **8** for 24 h exhibited G_1 arrest and increased the ROS levels about 250% compared with the control (Fig. 8). It indicated that **2** and **8** mediated through increased ROS in NTUB1 cells induced G_1 cell cycle arrest and apoptosis.

To enhance the therapeutic efficacy of anticancer agents and to reduce their side effect, antioxidants, such as pyrrolidine dithiocarbamate, epigallocatechin gallate, genistein, and vitamin E could enhance the cell death of various types of cancer cells [11].

Cisplatin has been successfully used as a chemotherapeutic agent against malignant solid tumors in the head and neck region. However, these have been barriers to the use of cisplatin in the clinical setting of head and neck cancer including nephrotoxicity and cisplatin resistance [12]. We hypothesized that antioxidants, **2** and **8** in combination with cisplatin may enhance tumor cell killing.

Thus we examined the effects of combination use of cisplatin with **2** and **8** respectively by MTT assay. As shown in Fig. 9, cisplatin in different concentrations (0.3, 1.0 and 3.0 μM) was cotreated with 15 and 30 μM **2** respectively. It clearly indicated that 30 μM **2** combined with cisplatin in all selected concentrations revealed synergistically significant cytotoxicities against NTUB1, while **8** did not show the same effects (Fig. 10). It indicated that the use of 30 μM **2** combined cisplatin of low level led to more significant cytotoxicity than that of cisplatin or **2** individually examined. The reduced dose of cisplatin may contribute to the decrease of side effects in clinic uses.

The MTT assay was also used for the evaluation of cytotoxicities of **2** and **8** against SV-HUC1 cells. Fig. 11 showed the cell viability of **2** and **8** assessed by the MTT assay after treating with different concentrations of **2** and **8** for 72 h, respectively. Compound **8** showed almost non-cytotoxic in all selected concentrations (0.3–50 μM) while **2** showed non-cytotoxic except for the concentration of 50 μM. It indicated that the clinic use of **2** and **8** may not be toxic for normal cells of urinary systems in selected levels except for **2** in higher levels (>30 μM). These results suggested that antioxidant **2** combined with low concentration of cisplatin may enhance the therapeutic efficacy of cisplatin and to reduce the side effect induced by cisplatin.

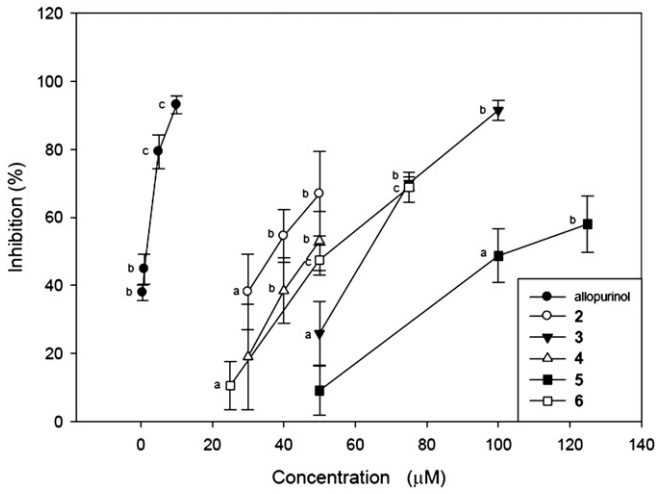


Fig. 2. Dose-dependent inhibition of XO by **2**–**6** and allopurinol. Data were presented as means ± s.e.m., $n = 3$–6. $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ compared to the control value, respectively.

5. Conclusion

In this study, we have synthesized a series of new compounds of xanthone derivatives and studied on their biological activities of antioxidation, xanthine oxidase inhibitory effect, ABTS radical scavenging, and cytotoxicities. This series of compounds revealed antioxidant effects, among them, compounds **2** and **8** showed potent antioxidant activities, and were selected for the determination of cytotoxicities against both NTUB1 and SV-HUC1 cells, cell cycle arrest, ROS level and combined use with cisplatin. The present results suggested that antioxidant **2** combined with low concentration of cisplatin may enhance the cell death of NTUB1 cells induced by cisplatin and reduced the side effect induced by cisplatin.

6. Experimental protocols

6.1. Chemistry, general information

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

Table 2
Xanthine oxidase inhibitory activities of **2**–**6** and **8**–**14**.[a]

Compounds	IC_{50} (μM)
2	37.8 ± 3.9
3	65.5 ± 2.6
4	47.3 ± 3.3
5	108.7 ± 6.3
6	56.5 ± 2.1
8	82.3 ± 2.8
9	68.8 ± 2.4
10	63.0 ± 1.4
11	72.9 ± 5.4
12	53.5 ± 2.8
13	91.9 ± 3.7
14	44.3 ± 3.9
Allopurinol	1.9 ± 0.7

[a] Data were presented as means ± s.e.m. ($n = 3$). Allopurinol was used as a positive control.

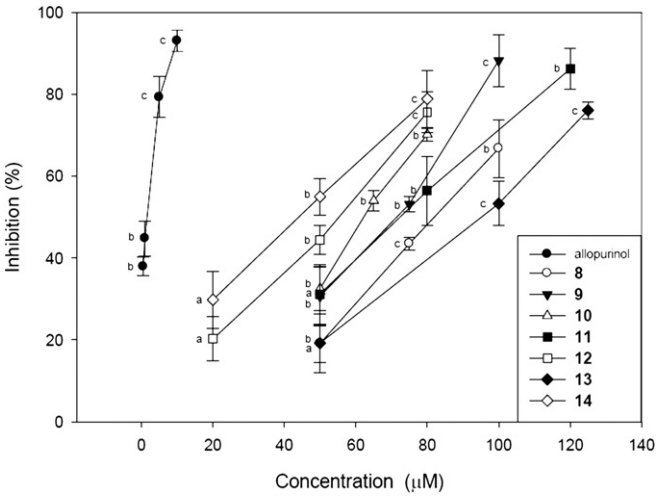


Fig. 3. Dose-dependent inhibition of XO by **8**–**14** and allopurinol. Data were presented as means ± s.e.m., $n = 3$–6. [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$ compared to the control value, respectively.

6.2. General procedure for the synthesis of xanthone derivatives

2-Methoxybenzoic acid (2.0 g, 13.14 mmol) in anhydrous benzene (60 mL) was treated with 5.0 mL of oxalyl chloride and thoroughly stirred at room temperature. After 2 h, the solvent and the excess reagent were removed under reduced pressure. The residue, 2-methoxybenzoyl chloride was dissolved in anhydrous ether (80 mL), 1,3-dimethoxybenzene (1.8 g, 13.03 mmol) and $AlCl_3$ (5.0 g) were added. After stirring for 8 h at room temperature, the mixture was hydrolyzed with ice-cold water (500 mL) containing concentrated HCl (45 mL) and extracted with $CHCl_3$. Solvent removal and purification with gel-column chromatography ($CHCl_3$) gave yellow oil, 2-hydroxy-4-methoxy-2′-methoxybenzophenone and 2,4-dimethoxy-2′-hydroxybenzophenone (2.20 g, 8.53 mmol, 65%). The yellow oil (2.20 g, 8.53 mmol) was treated with pyridine (100 mL), H_2O (50 mL) and aqueous 10% tetramethylammonium hydroxide (45 mL). The mixture was refluxed for 36 h, poured into ice, acidified with HCl, and extracted with ether. Purification with gel-chromatography ($CHCl_3$) and recrystallization ($CHCl_3$) gave colorless needle crystals, 3-methoxyxanthone (1.60 g, 7.08 mmol, 83%). A mixture of 3-methoxyxanthone (1.60 g, 7.08 mmol), phenol (42 mL) and HI (35 mL) was refluxed at 160 °C for 8 h, and the reaction mixture was poured into aqueous $NaHSO_3$ solution. The resulting yellow precipitate was collected, purified by silica gel-column chromatography ($CHCl_3$:CH_3OH) and recrystallized from CH_3OH to yield yellow needle crystals, 3-hydroxyxanthone (1.40 g, 6.0 mmol). 3-Hydroxyxanthone was reacted with dibromopropane (1.21 g, 6.0 mmol) to give **1**, 3-(3-bromopropoxy)xanthone. Compound **1** was then refluxed with ethanol and corresponding amines to yield **2**–**6**. 2,4-Dimethoxybenzoic acid was treated as the procedure mentioned above to yield 3-(3-bromopropoxy)-6-hydroxyxanthone (**7**) and 3,6-di(3-bromopropoxy)xanthone. Compound **7** was then refluxed with ethanol and corresponding amines to yield **8**–**14**.

Table 3
Free radical scavenging activities of **2**–**6** and **8**–**14** of ABTS.[a]

Compounds	IC_{50} (μM)
2	90.0 ± 4.9
3	Inactive
4	151.9 ± 1.6
5	75.1 ± 2.5
6	84.9 ± 2.1
8	69.3 ± 2.6
9	88.8 ± 1.8
10	107.9 ± 2.7
11	105.8 ± 1.6
12	80.5 ± 2.4
13	65.0 ± 4.1
14	76.5 ± 1.8
Vitamin E	77.2 ± 3.5

[a] Data were presented as means ± s.e.m. ($n = 3$). Vitamin E was used as a positive control.

6.2.1. 3-[3-(Piperidin-1-yl)-propoxy]xanthone (**2**)

Compound **1** (0.2 g, 0.60 mmol) in ethanol (30 mL) was added piperidine (0.51 g, 5.99 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol/*n*-hexane, 9:1) to afford **2** (0.061 g, 0.18 mmol, 30%) as yellow powder. IR (KBr) 1675, 1585 cm^{-1}; ^1H NMR (CD_3OD): δ 1.49 (2H, m, $-N(CH_2CH_2)_2CH_2$), 1.63 (4H, m, $-N(CH_2CH_2)_2-$), 2.04 (2H, m, $-OCH_2CH_2-$), 2.48 (4H, br.s, $-N(CH_2CH_2)_2-$), 2.54 (2H, t, $J = 6.0$ Hz, $-OCH_2CH_2CH_2-$), 4.14 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 6.96 (1H, dd, $J = 8.8$, 2.0 Hz, H-2), 6.99 (1H, d, $J = 2.0$ Hz, H-4), 7.41 (1H, td, $J = 8.0$, 1.2 Hz, H-7), 7.52 (1H, dd, $J = 8.8$, 0.8 Hz, H-5), 7.77 (1H, td, $J = 8.8$, 1.6 Hz, H-6), 8.11 (1H, d, $J = 8.8$ Hz, H-1), 8.20 (1H, dd, $J = 7.6$, 1.2 Hz, H-8). ^{13}C NMR (CD_3OD): δ 25.2 ($-N(CH_2CH_2)_2CH_2$), 26.5 ($-N(CH_2CH_2)_2-$), 27.2 ($-OCH_2CH_2-$), 55.5 ($-N(CH_2CH_2)_2-$), 56.9 ($-OCH_2CH_2CH_2-$), 68.3 ($-OCH_2-$), 101.8 (C-4), 115.1 (C-2), 116.3 (C-9a), 119.0 (C-5), 122.7 (C-8a), 125.3 (C-7), 127.2 (C-8), 128.8 (C-1), 136.1 (C-6), 157.7 (C-10a), 159.6 (C-4a), 166.4 (C-3), 178.0 (C=O). ESIMS m/z $[M + 1]^+$ 338. HRESIMS m/z $[M + 1]^+$ 338.1759 (calcd for $C_{21}H_{24}NO_3$, 338.1756).

6.2.2. 3-[3-(Pyrrolidin-1-yl)-propoxy]xanthone (**3**)

Compound **1** (0.2 g, 0.60 mmol) in ethanol (30 mL) was added pyrrolidine (0.43 g, 6.05 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol/*n*-hexane, 9:1) to afford **3** (0.078 g, 0.24 mmol, 40%) as pale-brown powder. IR (KBr) 1675, 1581 cm^{-1}; ^1H NMR (CD_3OD): δ 2.13 (4H, m, $-N(CH_2CH_2)_2$), 2.32 (2H, m, $-OCH_2CH_2-$), 3.44 (4H, br.s, $-N(CH_2CH_2)_2-$), 3.46 (2H, t, $J = 7.6$ Hz, $-OCH_2CH_2CH_2-$), 4.29 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 7.05 (1H, dd, $J = 9.2$, 2.4 Hz, H-2), 7.10 (1H, d, $J = 2.4$ Hz, H-4), 7.45 (1H, td, $J = 8.0$, 0.8 Hz, H-7), 7.57 (1H,

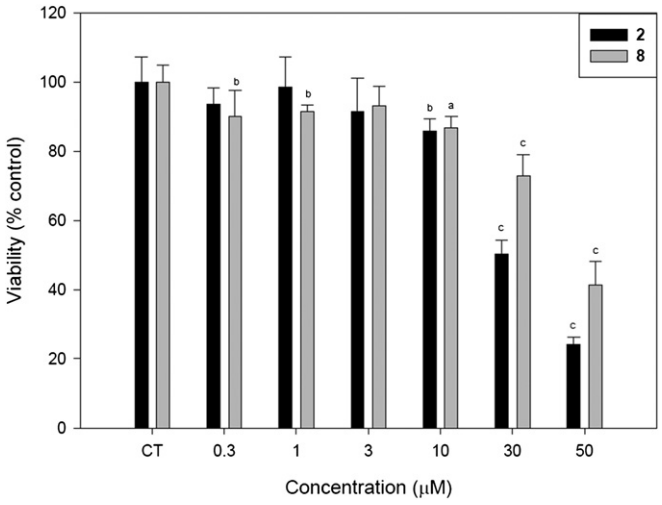


Fig. 4. Cytotoxicities of **2** and **8** against NTUB1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations for 72 h. The data were presented as means ± S.D. ($n = 3$). [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$ compared to the control value, respectively.

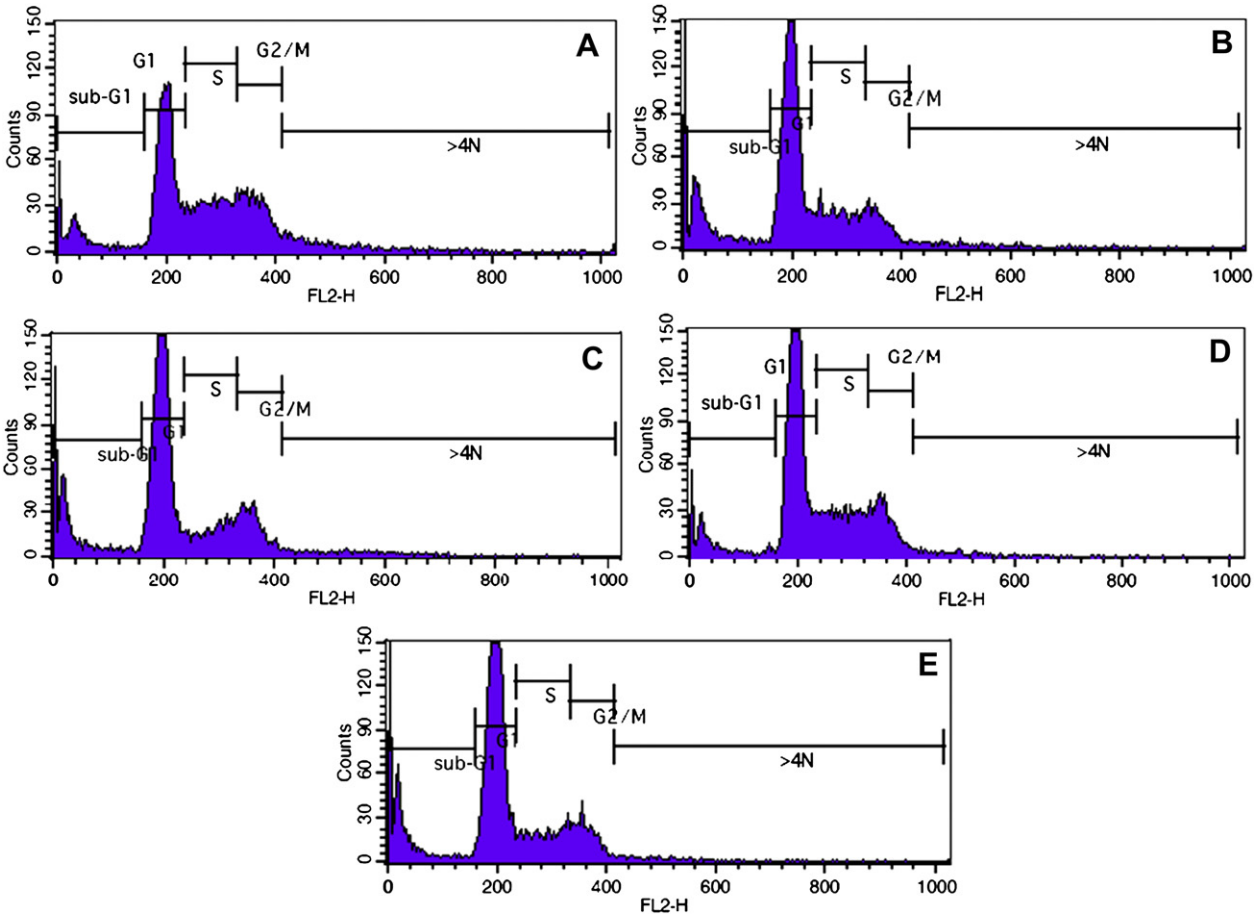


Fig. 5. Flow cytometry analysis of **2**- and **8**-treated NTUB1 cells. NTUB1 cells (8×10^5 cells/10 cm dish) were treated with absence of compounds (control, A), 40 μM **2** (B), 60 μM **2** (C), 40 μM **8** (D) and 60 μM **8** (E), respectively, for 24 h. At the time indicated, cells were stained with propidium iodide (PI), and DNA contents were analyzed by flow cytometry, apoptosis was measured by the accumulation of sub-G_1 DNA contents in cells. Results were representative of three independent experiments.

dd, $J = 8.4$, 0.8 Hz, H-5), 7.82 (1H, td, $J = 8.8$, 1.6 Hz, H-6), 8.18 (1H, d, $J = 8.8$ Hz, H-1), 8.24 (1H, dd, $J = 8.0$, 1.6 Hz, H-8). ^{13}C NMR (CD_3OD): δ 24.0 ($-N(CH_2CH_2)_2$), 26.9 ($-OCH_2CH_2-$), 53.6 ($-OCH_2CH_2CH_2-$), 55.4 ($-N(CH_2CH_2)_2$), 66.8 ($-OCH_2-$), 102.2 (C-4), 115.0 (C-2), 116.7 (C-9a), 119.1 (C-5), 122.7 (C-8a), 125.4 (C-7), 127.2 (C-8), 129.0 (C-1), 136.3 (C-6), 157.7 (C-10a), 159.6 (C-4a), 165.8 (C-3), 178.0 (C=O). ESIMS m/z $[M + 1]^+$ 324. HRESIMS m/z $[M + 1]^+$ 324.1599 (calcd for $C_{20}H_{22}NO_3$, 324.1600).

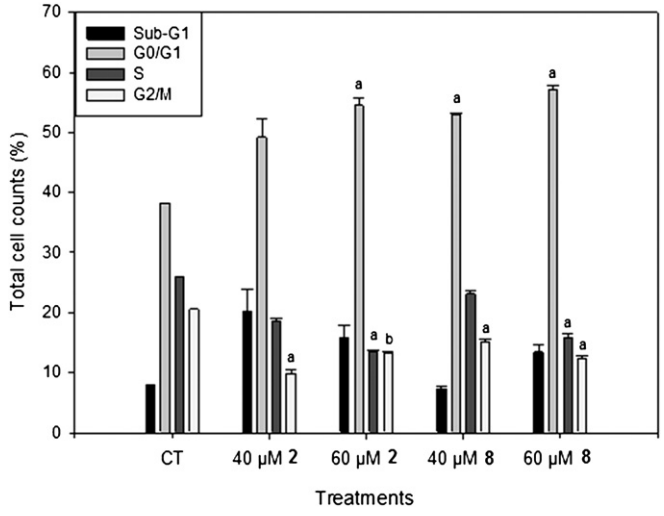


Fig. 6. Cell cycle distribution of NTUB1 cells treated with **2** and **8** for 24 h in different concentrations. Percentages of sub-G_1 cells and cells in G_0/G_1, S, and G_2/M phase were shown. Data refer to a representative experiment one of three. CT (control). $^a p < 0.05$, and $^b p < 0.01$ compared to the control value, respectively.

6.2.3. 3-[3-(4-Methylpiperazino)-propoxy]xanthone (**4**)

Compound **1** (0.2 g, 0.60 mmol) in ethanol (30 mL) was added 1-methylpiperazine (0.61 g, 6.09 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol/*n*-hexane, 9:1) to afford **4** (0.074 g, 0.21 mmol, 35%) as pale-yellow powder. IR (KBr) 1675, 1585 cm^{-1}; ^{1}H NMR (CD_3OD): δ 2.02 (2H, m, $-OCH_2CH_2-$), 2.29 (3H, s, $-NCH_3$), 2.57 (2H, t, $J = 7.6$ Hz, $-OCH_2CH_2CH_2-$), 2.57 (4H, br.s, $-N(CH_2CH_2)_2NCH_3$), 2.57 (4H, br.s, $-N(CH_2CH_2)_2NCH_3$), 4.13 (2H, t, $J = 6.4$ Hz, $-OCH_2-$), 6.90 (1H, d, $J = 9.6$ Hz, H-2), 6.93 (1H, s, H-4), 7.39 (1H, td, $J = 8.4$, 1.2 Hz, H-7), 7.48 (1H, dd, $J = 8.4$, 0.4 Hz, H-5), 7.75 (1H, td, $J = 8.8$, 1.6 Hz, H-6), 8.06 (1H, d, $J = 9.2$ Hz, H-1), 8.18 (1H, dd, $J = 7.6$, 1.2 Hz, H-8). ^{13}C NMR (CD_3OD): δ 27.2 ($-OCH_2CH_2-$), 45.9 ($-NCH_3$), 53.7 ($-N(CH_2CH_2)_2NCH_3$), 55.6 ($-N(CH_2CH_2)_2NCH_3$), 55.9 ($-OCH_2CH_2CH_2-$), 68.1 ($-OCH_2-$), 101.8 (C-4), 115.1 (C-2), 116.3 (C-9a), 119.0 (C-5), 122.6 (C-8a), 125.2 (C-7), 127.1 (C-8), 128.8 (C-1), 136.1 (C-6), 157.6 (C-10a), 159.5 (C-4a), 166.4 (C-3), 177.9 (C=O). ESIMS m/z $[M + 1]^+$ 353. HRESIMS m/z $[M + 1]^+$ 353.1867 (calcd for $C_{21}H_{25}N_2O_3$, 353.1865).

6.2.4. 3-[3-(Piperazino)-propoxy]xanthone (**5**)

Compound **1** (0.2 g, 0.60 mmol) in ethanol (30 mL) was added piperazine (0.52 g, 6.04 mmol) and then refluxed for 6 h. The mixture

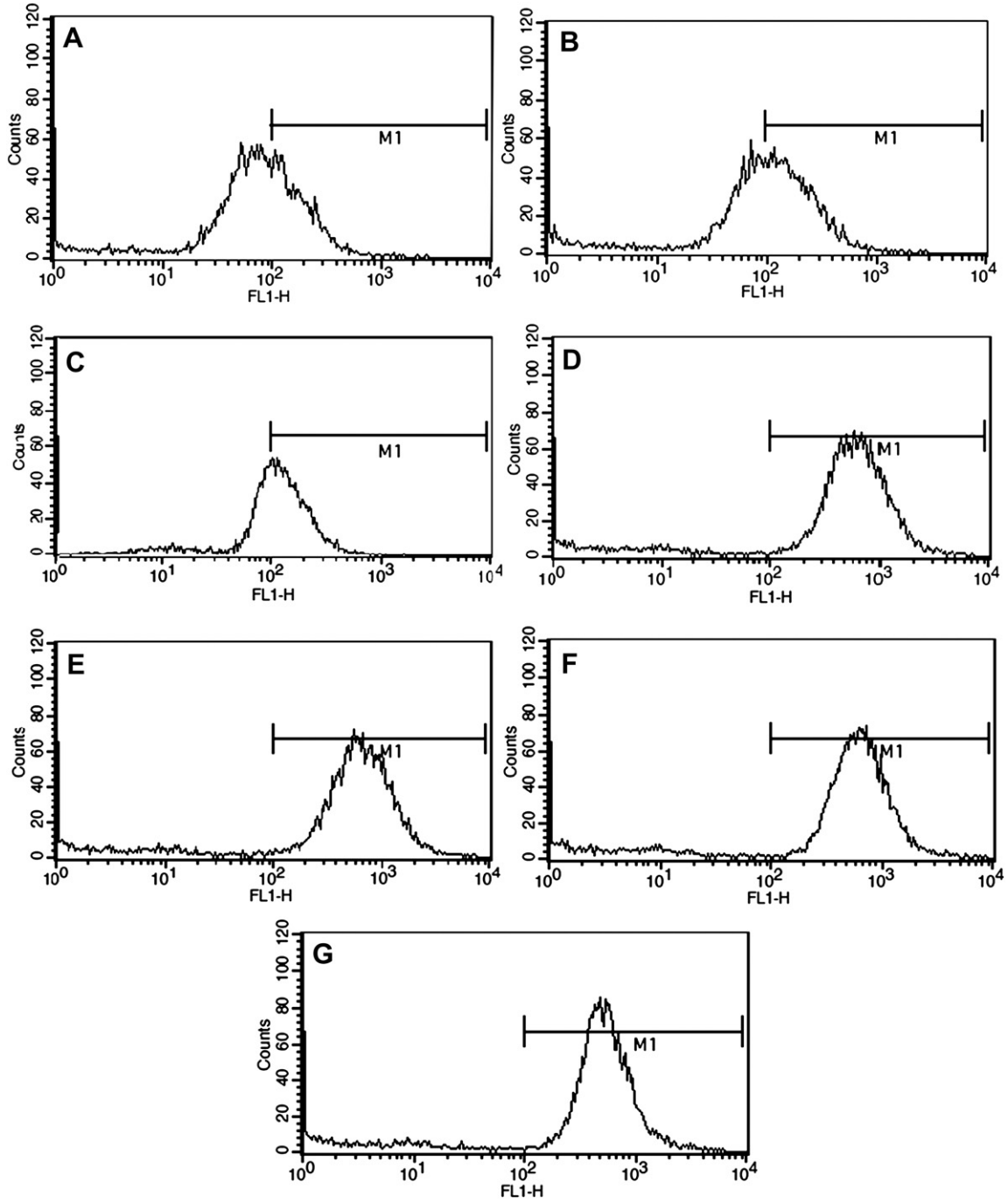


Fig. 7. The effect of **2** and **8** on the production of ROS in NTUB1 cells. (A) control, (B) 1 mM NAC, (C) 10 μM cisplatin, (D) 20 μM **2**, (E) 40 μM **2**, (F) 20 μM **8**, (G) 40 μM **8**. Cells were treated with 1 mM NAC, 10 μM cisplatin and different concentrations of **2** and **8** for 24 h, respectively, and the amount of ROS was assayed by H_2DCFDA staining. Results were repeated by three independent experiments.

was purified by column chromatography (silica gel and methanol/*n*-hexane, 9:1) to afford **5** (0.077 g, 0.23 mmol, 38%) as pale-yellow powder. IR (KBr) 1684, 1587 cm^{-1}; ^1H NMR (CD_3OD): δ 2.04 (2H, m, $-OCH_2CH_2-$), 2.51 (1H, br.s, −NH), 2.51 (4H, br.s, $-N(CH_2CH_2)_2NH$), 2.57 (2H, t, $J = 7.6$ Hz, $-OCH_2CH_2CH_2-$), 2.87 (4H, t, $J = 4.8$ Hz, $-N(CH_2CH_2)_2NH$), 4.16 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 6.96 (1H, dd, $J = 8.8$, 2.4 Hz, H-2), 6.99 (1H, d, $J = 2.0$ Hz, H-4), 7.41 (1H, td, $J = 8.0$, 0.8 Hz, H-7), 7.52 (1H, dd, $J = 8.4$, 0.8 Hz, H-5), 7.77 (1H, td, $J = 8.4$, 1.6 Hz, H-6), 8.11 (1H, d, $J = 9.2$ Hz, H-1), 8.20 (1H, dd, $J = 7.6$, 1.6 Hz, H-8). ^{13}C NMR (CD_3OD): δ 27.0 ($-OCH_2CH_2-$), 46.1 ($-N(CH_2CH_2)_2NH$), 54.9 ($-N(CH_2CH_2)_2NH$), 56.7 ($-OCH_2CH_2CH_2-$), 68.2 ($-OCH_2-$), 101.8 (C-4), 115.1 (C-2), 116.3 (C-9a), 119.0 (C-5), 122.7 (C-8a), 125.3 (C-7), 127.2 (C-8), 128.8 (C-1), 136.1 (C-6), 157.7 (C-10a), 159.6 (C-4a), 166.5 (C-3), 178.0 (C=O). ESIMS m/z $[M + 1]^+$ 339. HRESIMS m/z $[M + 1]^+$ 339.1708 (calcd for $C_{20}H_{23}N_2O_3$, 339.1709).

6.2.5. 3-[3-(Diethylamino)-propoxy]xanthone (**6**)

Compound **1** (0.2 g, 0.60 mmol) in ethanol (30 mL) was added diethylamine (0.44 g, 6.02 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol/*n*-hexane, 9:1) to afford **6** (0.084 g, 0.26 mmol, 43%) as brown powder. IR (KBr) 1653, 1559 cm^{-1}; ^1H NMR (CD_3OD): δ 1.07

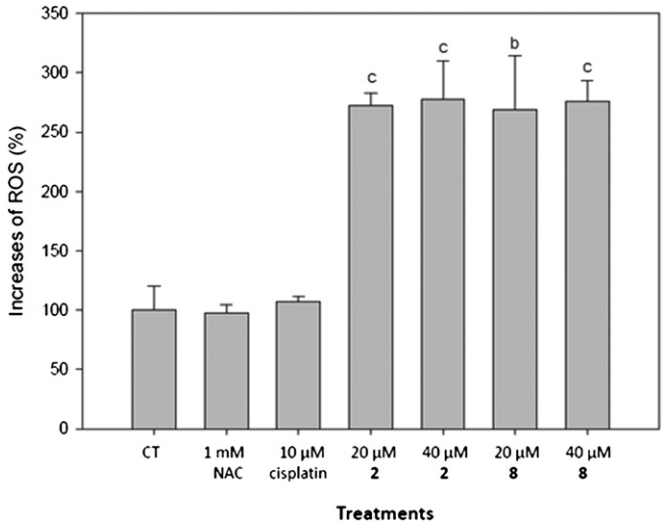


Fig. 8. The enhancement of **2** and **8** on the production of ROS in NTUB1 cells assayed by H_2DCFDA staining under the treatments of 1 mM NAC, 10 μM cisplatin and different concentrations of **2** and **8** for 24 h respectively. CT (control). Data were presented as means ± S.D. ($n = 3$). $^{b}p < 0.01$, and $^{c}p < 0.001$ compared to the control value, respectively.

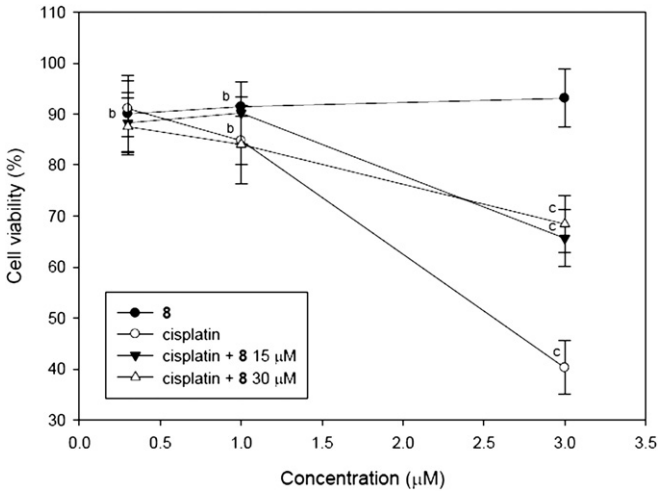


Fig. 10. Cytotoxicity of **8** combined with cisplatin against NTUB1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of **8** and cisplatin respectively, and 15, 30 μM **8** cotreated with cisplatin of different concentrations for 72 h. The data were presented as means ± S.D. ($n = 3$). $^{b}p < 0.01$, and $^{c}p < 0.001$ compared to the control value, respectively.

(6H, t, $J = 7.2$ Hz, $-N(CH_2CH_3)_2$), 1.98 (2H, m, $-OCH_2CH_2-$), 2.61 (4H, q, $J = 6.8$ Hz, $-N(CH_2CH_3)_2$), 2.67 (2H, t, $J = 6.0$ Hz, $-OCH_2CH_2CH_2-$), 4.11 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 6.91 (1H, dd, $J = 8.0, 1.6$ Hz, H-2), 6.92 (1H, d, $J = 1.6$ Hz, H-4), 7.38 (1H, td, $J = 8.0$, 1.2 Hz, H-7), 7.48 (1H, dd, $J = 8.4$, 0.8 Hz, H-5), 7.75 (1H, td, $J = 8.8$, 2.0 Hz, H-6), 8.07 (1H, d, $J = 8.0$ Hz, H-1), 8.17 (1H, dd, $J = 8.4$, 1.6 Hz, H-8). ^{13}C NMR (CD_3OD): δ 11.4 ($-N(CH_2CH_3)_2$), 26.9 ($-OCH_2CH_2-$), 47.8 ($-N(CH_2CH_3)_2$), 50.2 ($-OCH_2CH_2CH_2-$), 68.3 ($-OCH_2-$), 101.8 (C-4), 115.0 (C-2), 116.3 (C-9a), 119.0 (C-5), 122.6 (C-8a), 125.2 (C-7), 127.1 (C-8), 128.8 (C-1), 136.0 (C-6), 157.6 (C-10a), 159.6 (C-4a), 166.4 (C-3), 177.9 (C=O). ESIMS m/z $[M + 1]^+$ 326. HRESIMS m/z $[M + 1]^+$ 326.1753 (calcd for $C_{20}H_{24}NO_3$, 326.1756).

6.2.6. 3-Hydroxy-6-[3-(piperidin-1-yl)-propoxy]xanthone (**8**)

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added piperidine (0.49 g, 5.76 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **8** (0.067 g, 0.19 mmol, 33%) as yellow powder. IR (KBr) 3446, 1653, 1560 cm^{-1}; ^{1}H NMR (CD_3OD): δ 1.54 (2H, m, $-N(CH_2CH_2)_2CH_2$), 1.69 (4H, m, $-N(CH_2CH_2)_2CH_2$), 2.08 (2H, m, $-OCH_2CH_2-$), 2.64 (4H, br.s, $-N(CH_2CH_2)_2CH_2$), 2.69 (2H, t, $J = 7.6$ Hz, $-OCH_2CH_2CH_2-$), 4.13 (2H, t, $J = 6.0$ Hz, $-OCH_2-$), 6.71 (1H, d, $J = 2.4$ Hz, H-5), 6.80 (1H, dd, $J = 9.2$, 2.4 Hz, H-7), 6.90 (1H, d, $J = 2.4$ Hz, H-4), 6.92 (1H, dd, $J = 7.6$, 2.4 Hz, H-2), 8.01 (1H, d, $J = 8.8$ Hz, H-8), 8.07 (1H, d, $J = 8.4$ Hz, H-1). ^{13}C NMR (CD_3OD): δ 24.7 ($-N(CH_2CH_2)_2CH_2$), 26.1 ($-N(CH_2CH_2)_2CH_2$), 26.8 ($-OCH_2CH_2-$), 55.3 ($-N(CH_2CH_2)_2CH_2$), 56.7 ($-OCH_2CH_2CH_2-$), 67.9 ($-OCH_2-$), 101.8 (C-5), 103.6 (C-4), 114.2 (C-9a), 114.3 (C-7), 116.4 (C-8a), 116.5 (C-2), 128.6 (C-8), 128.8 (C-1), 159.5 (C-10a), 160.2 (C-4a), 165.6 (C-6), 168.8 (C-3), 177.4 (C=O). ESIMS m/z $[M + 1]^+$ 354. HRESIMS m/z $[M + 1]^+$ 354.1702 (calcd for $C_{21}H_{24}NO_4$, 354.1705).

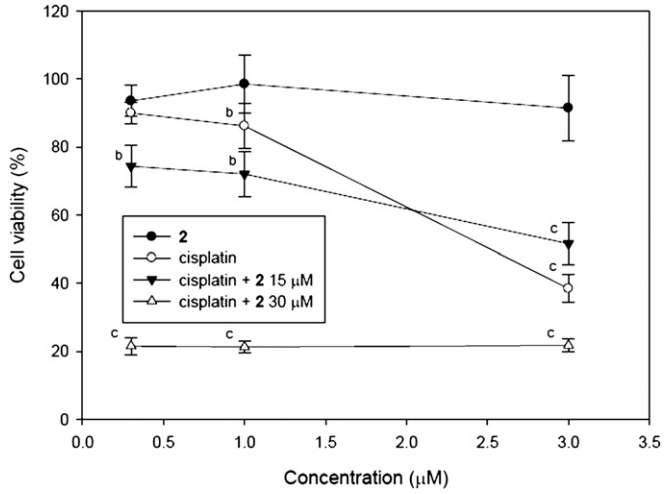


Fig. 9. Cytotoxicity of **2** combined with cisplatin against NTUB1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of **2** and cisplatin respectively, and 15, 30 μM **2** cotreated with cisplatin of different concentrations for 72 h. The data were presented as means ± S.D. ($n = 3$). $^{b}p < 0.01$, and $^{c}p < 0.001$ compared to the control value, respectively.

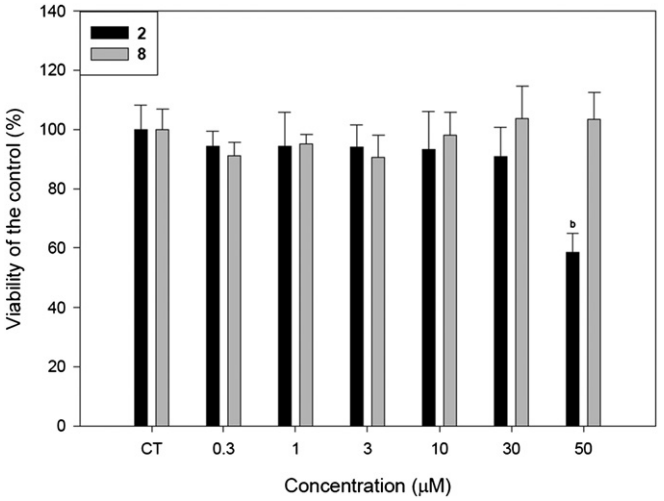


Fig. 11. Cytotoxicities of **2** and **8** against SV-HUC1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of **2** and **8** for 72 h. The data were presented as means ± S.D. ($n = 3$). $^{b}p < 0.01$ compared to the control value, respectively.

*6.2.7. 3-Hydroxy-6-[3-(pyrrolidin-1-yl)-propoxy]xanthone (**9**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added pyrrolidine (0.41 g, 5.76 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **9** (0.075 g, 0.22 mmol, 39%) as brown powder. IR (KBr) 3461, 1653, 1569 cm^{-1}; ^1H NMR (CD_3OD): δ 1.84 (4H, m, $-N(CH_2CH_2)_2$), 2.02 (2H, m, $-OCH_2CH_2-$), 2.63 (4H, m, $-N(CH_2CH_2)_2$), 2.71 (2H, t, J = 7.6 Hz, $-OCH_2CH_2CH_2-$), 4.06 (2H, t, J = 6.0 Hz, $-OCH_2-$), 6.54 (1H, d, J = 2.4 Hz, H-5), 6.69 (1H, dd, J = 8.8, 2.4 Hz, H-7), 6.82 (1H, d, J = 2.4 Hz, H-4), 6.84 (1H, dd, J = 8.0, 2.4 Hz, H-2), 7.92 (1H, d, J = 8.8 Hz, H-8), 8.01 (1H, d, J = 8.0 Hz, H-1). ^{13}C NMR (CD_3OD): δ 24.1 ($-N(CH_2CH_2)_2$), 29.0 ($-OCH_2CH_2-$), 54.0 ($-OCH_2CH_2CH_2-$), 55.0 ($-N(CH_2CH_2)_2$), 67.7 ($-OCH_2-$), 101.6 (C-5), 104.2 (C-4), 111.9 (C-9a), 113.8 (C-7), 116.3 (C-8a), 119.0 (C-2), 128.3 (C-8), 128.3 (C-1), 159.3 (C-10a), 160.8 (C-4a), 165.2 (C-6), 174.5 (C-3), 177.2 (C=O). ESIMS m/z $[M + 1]^+$ 340. HRESIMS m/z $[M + 1]^+$ 340.1551 (calcd for $C_{20}H_{22}NO_4$, 340.1549).

*6.2.8. 3-[3-(Cyclopropylamino)-propoxy]-6-hydroxyxanthone (**10**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added cyclopropylamine (0.33 g, 5.78 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **10** (0.065 g, 0.20 mmol, 35%) as brown powder. IR (KBr) 3420, 1684, 1569 cm^{-1}; ^1H NMR (CD_3OD): δ 0.40 (2H, m, $-NHCH(CH_\alpha H_\beta)_2$), 0.51 (2H, m, $-NHCH(CH_\alpha H_\beta)_2$), 2.04 (2H, m, $-OCH_2CH_2-$), 2.21 (1H, m, $-NHCH-$), 2.89 (2H, t, J = 7.2 Hz, $-OCH_2CH_2CH_2-$), 4.15 (2H, t, J = 6.4 Hz, $-OCH_2-$), 6.77 (1H, dd, J = 8.8, 2.4 Hz, H-2), 6.91 (1H, dd, J = 8.4, 2.4 Hz, H-7), 6.92 (1H, d, J = 2.0 Hz, H-5), 6.97 (1H, d, J = 2.4 Hz, H-4), 7.99 (1H, d, J = 8.8 Hz, H-1), 8.06 (1H, d, J = 8.4 Hz, H-8). ^{13}C NMR (CD_3OD): δ 5.9 ($-NHCH(CH_\alpha H_\beta)_2$), 29.8 ($-OCH_2CH_2-$), 31.3 ($-NHCH-$), 47.3 ($-OCH_2CH_2CH_2-$), 68.1 ($-OCH_2-$), 101.7 (C-4), 103.7 (C-5), 113.7 (C-9a), 114.3 (C-2), 116.3 (C-8a), 117.1 (C-7), 128.5 (C-1), 128.6 (C-8), 159.5 (C-4a), 160.3 (C-10a), 165.6 (C-3), 170.2 (C-6), 177.4 (C=O). ESIMS m/z $[M + 1]^+$ 326. HRESIMS m/z $[M + 1]^+$ 326.1395 (calcd for $C_{19}H_{20}NO_4$, 326.1392).

*6.2.9. 3-[3-(Cyclohexylamino)-propoxy]-6-hydroxyxanthone (**11**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added cyclohexylamine (0.57 g, 5.75 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **11** (0.067 g, 0.18 mmol, 32%) as brown powder. IR (KBr) 3420, 1684, 1571 cm^{-1}; ^1H NMR (CD_3OD): δ 1.17 (2H, m, $-NHCH(CH_2CH_2)_2CH_2$), 1.30 (4H, m, $-NHCH(CH_2CH_2)_2CH_2$), 1.67 (1H, m, $-NH-$), 1.81 (2H, m, $-OCH_2CH_2-$), 2.04 (4H, m, $-NHCH(CH_2CH_2)_2CH_2$), 2.72 (1H, m, $-NHCH-$), 2.96 (2H, t, J = 7.2 Hz, $-OCH_2CH_2CH_2-$), 4.05 (2H, t, J = 6.0 Hz, $-OCH_2-$), 6.51 (1H, d, J = 2.4 Hz, H-4), 6.67 (1H, dd, J = 8.8, 2.4 Hz, H-2), 6.76 (1H, d, J = 2.4 Hz, H-5), 6.78 (1H, dd, J = 8.8, 2.4 Hz, H-7), 7.91 (1H, d, J = 8.8 Hz, H-1), 7.97 (1H, d, J = 8.8 Hz, H-8). ^{13}C NMR (CD_3OD): δ 25.9 ($-NHCH(CH_2CH_2)_2CH_2$), 26.7 ($-NHCH(CH_2CH_2)_2CH_2$), 28.8 ($-OCH_2CH_2-$), 32.2 ($-NHCH(CH_2CH_2)_2CH_2$), 43.8 ($-NHCH-$), 58.2 ($-OCH_2CH_2CH_2-$), 67.4 ($-OCH_2-$), 101.6 (C-4), 104.2 (C-5), 111.9 (C-9a), 113.7 (C-2), 116.4 (C-8a), 119.2 (C-7), 128.3 (C-1), 128.3 (C-8), 159.2 (C-4a), 160.9 (C-10a), 164.9 (C-3), 174.7 (C-6), 177.1 (C=O). ESIMS m/z $[M + 1]^+$ 368. HRESIMS m/z $[M + 1]^+$ 368.1861 (calcd for $C_{22}H_{26}NO_4$, 368.1862).

*6.2.10. 3-[3-(Diethylamino)-propoxy]-6-hydroxyxanthone (**12**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added diethylamine (0.42 g, 5.74 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **12** (0.078 g, 0.23 mmol, 40%) as pale-brown powder. IR (KBr) 3446, 1675, 1569 cm^{-1}; ^1H NMR (CD_3OD): δ 1.13 (6H, t, J = 7.2 Hz, $-N(CH_2CH_3)_2$), 2.03 (2H, m, $-OCH_2CH_2-$), 2.73 (4H, q, J = 7.2 Hz, $-N(CH_2CH_3)_2$), 2.81 (2H, t, J = 6.0 Hz, $-OCH_2CH_2CH_2-$), 4.14 (2H, t, J = 6.0 Hz, $-OCH_2-$), 6.65 (1H, d, J = 2.4 Hz, H-4), 6.76 (1H, dd, J = 8.8, 2.0 Hz, H-2), 6.92 (1H, dd, J = 8.8, 2.4 Hz, H-7), 6.94 (1H, d, J = 2.4 Hz, H-5), 7.98 (1H, d, J = 8.8 Hz, H-1), 8.07 (1H, d, J = 8.8 Hz, H-8). ^{13}C NMR (CD_3OD): δ 9.8 ($-N(CH_2CH_3)_2$), 25.4 ($-OCH_2CH_2-$), 46.7 ($-N(CH_2CH_3)_2$), 49.0 ($-OCH_2CH_2CH_2-$), 66.7 ($-OCH_2-$), 100.6 (C-4), 102.6 (C-5), 112.0 (C-9a), 113.0 (C-2), 115.2 (C-8a), 116.5 (C-7), 127.3 (C-1), 127.4 (C-8), 158.3 (C-4a), 159.3 (C-10a), 164.3 (C-3), 170.2 (C-6), 176.2 (C=O). ESIMS m/z $[M + 1]^+$ 342. HRESIMS m/z $[M + 1]^+$ 342.1708 (calcd for $C_{20}H_{24}NO_4$, 342.1705).

*6.2.11. 3-Hydroxy-6-[3-(piperazino)-propoxy]xanthone (**13**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added piperazine (0.50 g, 5.81 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **13** (0.085 g, 0.24 mmol, 42%) as pale-yellow powder. IR (KBr) 3446, 1684, 1569 cm^{-1}; ^1H NMR (CD_3OD): δ 2.03 (2H, m, $-OCH_2CH_2-$), 2.50 (4H, br.s, $-N(CH_2CH_2)_2NH$), 2.56 (2H, t, J = 7.6 Hz, $-OCH_2CH_2CH_2-$), 2.87 (4H, t, J = 4.8 Hz, $-N(CH_2CH_2)_2NH$), 4.14 (2H, t, J = 6.0 Hz, $-OCH_2-$), 6.49 (1H, d, J = 2.4 Hz, H-5), 6.64 (1H, dd, J = 8.8, 2.0 Hz, H-7), 6.90 (1H, dd, J = 8.8, 2.4 Hz, H-2), 6.94 (1H, d, J = 2.4 Hz, H-4), 7.91 (1H, d, J = 8.8 Hz, H-8), 8.07 (1H, d, J = 8.8 Hz, H-1). ^{13}C NMR (CD_3OD): δ 27.0 ($-OCH_2CH_2-$), 46.0 ($-N(CH_2CH_2)_2NH$), 54.8 ($-N(CH_2CH_2)_2NH$), 56.7 ($-OCH_2CH_2CH_2-$), 67.9 ($-OCH_2-$), 101.7 (C-5), 104.4 (C-4), 110.9 (C-9a), 113.7 (C-7), 116.4 (C-8a), 120.2 (C-2), 128.1 (C-8), 128.3 (C-1), 159.4 (C-10a), 161.3 (C-4a), 165.2 (C-6), 177.2 (C-3), 177.2 (C=O). ESIMS m/z $[M + 1]^+$ 355. HRESIMS m/z $[M + 1]^+$ 355.1660 (calcd for $C_{20}H_{23}N_2O_4$, 355.1658).

*6.2.12. 3-Hydroxy-6-[3-(methylpiperazylamino)-propoxy]xanthone (**14**)*

Compound **7** (0.2 g, 0.57 mmol) in ethanol (30 mL) was added methylpiperazine (0.58 g, 5.79 mmol) and then refluxed for 6 h. The mixture was purified by column chromatography (silica gel and methanol) to afford **14** (0.082 g, 0.22 mmol, 39%) as yellow powder. IR (KBr) 3380, 1675, 1583 cm^{-1}; ^1H NMR (CD_3OD): δ 2.04 (2H, m, $-OCH_2CH_2-$), 2.32 (3H, s, $-NCH_3$), 2.55 (4H, br.s, $-N(CH_2CH_2)_2NCH_3$), 2.55 (4H, br.s, $-N(CH_2CH_2)_2NCH_3$), 2.60 (2H, t, J = 7.6 Hz, $-OCH_2CH_2CH_2-$), 4.17 (2H, t, J = 6.4 Hz, $-OCH_2-$), 6.79 (1H, d, J = 2.4 Hz, H-5), 6.86 (1H, dd, J = 8.8, 2.4 Hz, H-7), 6.96 (1H, dd, J = 8.8, 2.4 Hz, H-2), 6.99 (1H, d, J = 2.4 Hz, H-4), 8.06 (1H, d, J = 8.8 Hz, H-8), 8.10 (1H, d, J = 8.8 Hz, H-1). ^{13}C NMR (CD_3OD): δ 27.3 ($-OCH_2CH_2-$), 45.9 ($-NCH_3$), 53.6 ($-N(CH_2CH_2)_2NCH_3$), 55.6 ($-N(CH_2CH_2)_2NCH_3$), 55.9 ($-OCH_2CH_2CH_2-$), 68.0 ($-OCH_2-$), 101.8 (C-5), 103.3 (C-4), 114.6 (C-7), 115.1 (C-9a), 115.4 (C-2), 116.3 (C-8a), 128.7 (C-8), 128.9 (C-1), 159.6 (C-10a), 159.9 (C-4a), 165.9 (C-6), 166.6 (C-3), 177.5 (C=O). ESIMS m/z $[M + 1]^+$ 369. HRESIMS m/z 369.1815 (calcd for $C_{21}H_{25}N_2O_4$, 369.1814).

6.3. Assay of XO inhibitory activity

The XO activity with xanthine as the substrate was measured at 25 °C, according to the protocol of Kong and others [13] with modification. The assay mixture consisting of 50 μL of test solution, 60 μL of 70 mM phosphate buffer (pH 7.5), and 30 μL of enzyme solution (0.1 unit/mL in 70 mM phosphate buffer (pH 7.5)) was prepared immediately before use. After preincubation at 25 °C for 15 min, the reaction was initiated by addition of 60 μL of substrate solution (150 μM xanthine in the same buffer). The reaction was monitored for 5 min at 295 nm. The XO activity was expressed as micromoles of uric acid per minute.

6.4. ABTS radical scavenging assay

The scavenging activity of ABTS was measured according to the method described by Ng et al. [14] with some modifications. Briefly, ABTS was dissolved in deionized water to 7 mM in concentration, which was then mixed with 2.45 mM potassium persulfate.

The scavenging activity was determined by mixing with 180 μL of ABTS and 40 μL of selected compounds and positive control (vitamin E), followed by measuring absorbance at 630 nm.

6.5. Cell culture

NTUB1, a human bladder carcinoma cell line, was established from a high-grade bladder cancer by Yu et al. [15]. NTUB1 and stable transfected cells were maintained in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin-G, 100 μg/mL streptomycin and 2 mM L-glutamine. SV-HUC1 cells (a SV-40 immortalized human uroepithelial cell line) were obtained from ATCC and maintained in F12 medium supplemented with 10% fetal bovine serum, 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.

6.6. Cytotoxicity analysis by MTT assay

Cellular cytotoxicity of tested compounds was performed by using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., USA) (Hour et al. [16]). Briefly, the cells were plated at a density of 1×10^3 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 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 another 4 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 a microplate reader, MRX (DYNEXCO). The cell viability was expressed as the ratio to the un-treated control. The IC_{50} values of each group were calculated by the median-effect analysis and presented as mean ± standard deviation (SD).

6.7. Flow cytometry analysis

DNA content was determined following propidium iodide staining of cells as previously described by Huang et al. [17]. Briefly, 3×10^5 NTUB1 cells were plated and treated with indicated dose of compounds for 24 h, respectively. These cells were harvested by trypsinization, washed with 1 × PBS, and fixed in ice-cold methanol at −20 °C. After overnight incubation, the cells were washed with PBS and incubated with 50 μg/mL propidium iodide (Sigma Chemical Co., USA) and 50 μg/mL RNase A (Sigma Chemical Co., USA) 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).

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

Production of ROS was analyzed by flow cytometry as described previously (Pu at al., [18]). Briefly, cells were plated in 6-well plates. 10 μM dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) 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 by FACScan flow cytometer with a 525-nm band pass filter (Becton Dickinson).

6.9. Statistical analysis

Data were expressed as the means ± S. D. Statistical analysis was performed using student's *t*-test method for two group comparison. $P < 0.05$ was considered to be statistically significant.

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