Bioorganic & Medicinal Chemistry 17 (2009) 7265–7274

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Ursolic acid derivatives induce cell cycle arrest and apoptosis in NTUB1 cells associated with reactive oxygen species

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ARTICLE INFO

Article history:
Received 6 May 2009
Revised 21 August 2009
Accepted 22 August 2009
Available online 27 August 2009

Keywords:
Synthesis
Ursolic acid derivatives
Cytotoxicity
NTUB1
ROS
Cell cycle
Anticancer
Seco-compound
SAR

ABSTRACT

Twenty-three ursolic acid (**1**) derivatives **2**–**24** including nine new **1** derivatives **5**, **7**–**11**, **20**–**22** were synthesized and evaluated for cytotoxicities against NTUB1 cells (human bladder cancer cell line). Compounds **5** and **17** with an isopropyl ester moiety at C-17-COOH and a succinyl moiety at C-3-OH showed potent inhibitory effect on growth of NTUB1 cells. Compounds **23** and **24** with *seco*-structures prepared from **1** also showed the increase of the cytotoxicity against NTUB1 cells. Exposure of NTUB1 to **5** (40 μM) and **23** (20 and 50 μM) for 24 h significantly increased the production of reactive oxygen species (ROS) while exposure of NTUB1 to **5** (20 and 40 μM) and **23** (20 and 50 μM) for 48 h also significantly increased the production of ROS while exposure of cells to **17** did not increase the amount of ROS. Flow cytometric analysis exhibited that treatment of NTUB1 with **5** or **17** or **23** led to the cell cycle arrest accompanied by an increase in apoptotic cell death after 24 or 48 h. These data suggest that the presentation of G1 phase arrest and apoptosis in **5**- and **23**-treated NTUB1 for 24 h mediated through increased amount of ROS in cells exposed with **5** and **23**, respectively, while the presence of G2/M arrest before accumulation of cells in sub-G1 phase in **5**-treated cells for 48 h also due to increased amount of ROS in cells exposed with **5**. The inhibition of tubulin polymerization and cell cycle arrest at G2/M following by apoptosis presented in the cell cycle of **23** also mediates through the increase amount of ROS induced by treating NTUB1 with **23** for 48 h.

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1. Introduction

Triterpenoids abundantly exist in plant kingdom. The triterpenoids, ursolic acid (**1**) and **1** derivatives, have been reported to have antitumor,[1,2] antiinflammatory,[3] antiviral,[4] and antioxidant activities.[5,6]

Reactive oxygen species (ROS) are resulting in oxidation of various cell constituents as DNA, lipid, and proteins and consequently these oxidations may cause damage to the cellular substance leading to cell death as the ultimate consequence. ROS have been implicated in a number of disease including various forms of non-hormone dependent cancers, atherosclerosis, ischemic reperfusion injury, neurodegenerative diseases, chronic inflammatory disease, such as rheumatoid and psoriatic arthritis, and some factors underlying the aging process itself. ROS might also play a role as signaling molecules and as such they may have a role in cell cycle progression.[7]

Several anticancer agents, such as arsenic trioxide, doxorubicin, bleomycin, cisplatin, 5-Fu, and paclitaxel have been shown to induce ROS generation in cancer cells. Various mechanisms have been described, including respiratory chain disruption, redox cycling, or p53-mediated mitochondrial oxidase activation.[8]

Recently, several structures and cytotoxic relationships have been reported.[9] However, a series of structures and cytotoxic relationships of **1** derivatives did not appear in literature. Based on the above reason, we synthesized a series of **1** derivatives, evaluated their cytotoxicities against human NTUB1 cells, and discussed their structures and cytotoxic relationships and mechanisms of action.

2. Results and discussion

2.1. Chemistry

Leaves of loquat were extracted with NaOH solution. The NaOH solution was separated by partition with organic solvent. The

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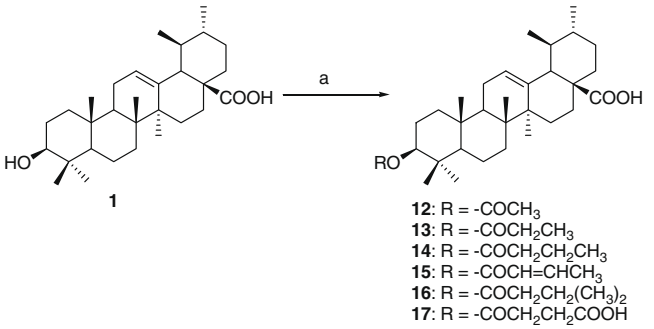
doi:10.1016/j.bmc.2009.08.046

aqueous layer was neutralized with HCl solution to yield a precipitate. The precipitate was separated and purified by silica gel chromatography to yield **1**. Compound **1** in acetone reacted with K_2CO_3 and various alkyl halides to afford different esters (**2**–**11** and **19**) (Scheme 1). The hydroxyl group at C-3 in the ring A was treated with various anhydrides to form different esters (**12**–**17**) (Scheme 2). Compound **1** was oxidized to the 3-keto compound **18** with CrO_3 in DMF and the treatment of 3-oxo-derivative **18** with *m*-chloroperbenzoic acid (*m*-CPBA) afford lactone **20**. Compound **18** in anhydrous MeOH/benzene was esterified with trimethylsilyldiazomethane ($TMSCHN_2$) at room temperature to yield **19** (Scheme 3). Lactone **20** was cleavaged in the appropriate amount of MeOH and H_2SO_4 as the catalyst to yield *seco*-compound **21** and cleavaged with KOH in MeOH to yield *seco*-compound **22** (Scheme 3). *seco*-Compound **22** in MeOH reacted with H_2SO_4 to yield *seco*-compound **23**. Compound **23** in anhydrous MeOH/benzene was esterified with trimethylsilyldiazomethane ($TMSCHN_2$) at room temperature to yield **24** (Scheme 3). The known **1** derivatives, **2**–**4**, **6**, **12**–**19**, **23**, and **24**, were identified by spectroscopic data and compared with those data reported in literatures while the new **1** derivatives were also characterized by various spectroscopic methods and compared with those data reported in literatures.



Scheme 2. Reagents and conditions: (a) anhydrous acetic acid or various carboxylic acid, DCC, DMAP, pyridine.

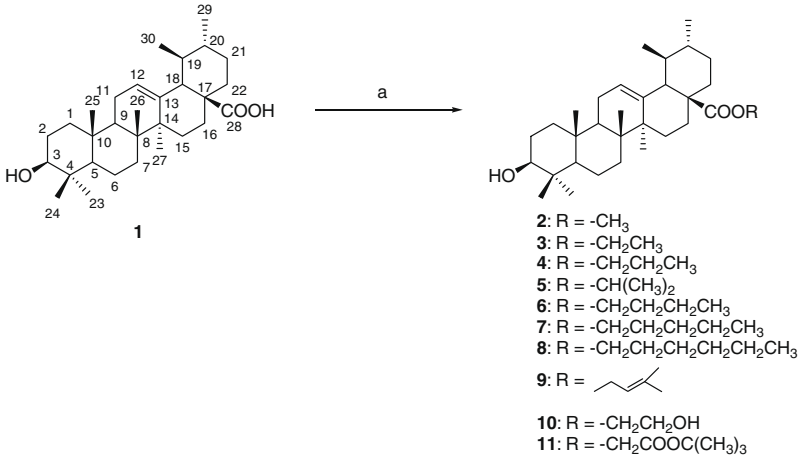
2.2. Biological results and discussion

Cytotoxicities of **1** and its derivatives against NTUB1 cells were studied, and cisplatin was used as the positive control. As shown in Tables 1 and 2, compound **1** and most of its derivative showed significant cytotoxic activities against NTUB1 cells. The esterification of C-17-COOH of **1** and **18** with methyl halide such as **2** and **19** decreased the cytotoxic activity against NTUB1 cells, but the esterification of **21** such as **24** enhanced the cytotoxicity against NTUB1 cells. The C-17-COOH of **1** esterified with the increased alkyl chain of halides such as **3**–**8**, **10** or prenyl halide such as **9** indicated that all these compounds revealed stronger cytotoxicities than that of **2** against NTUB1 cells. The hydroxylation of C-17 ethyl ester of **3** such as **10** attenuated the cytotoxicity against NTUB1 cells. The acetylation of C-3-OH of **1** enhanced the cytotoxic activity while the C-3-OH of **1** modified to succinic ester, such as **17**, potently enhanced the cytotoxicity activity against NTUB1 cells. Increasing carbon chain of ester moieties attenuated the cytotoxic effect while increasing the unsaturation of the same carbon chain of ester such as **15** enhanced cytotoxic effect. The oxidation of C-3-OH (**1** and **2**) to keto group (**18** and **19**) increased cytotoxicities while lactone derivative **20** obtained from **18** and 3,4-*seco*-compounds **21** and **22** obtained from **20** weakened cytotoxic activities. The 3,4-*seco*-compound obtained from **22**, such as **23**, indicated the potently cytotoxic activity and its dimethyl ester further enhanced the inhibitory effect on the cell growth. It clearly indicated that the 3,4-*seco*-compound with dimethyl ester moieties substituted as C-3 and C-17 obtained from cleavage of ring A displayed stronger and concentration-dependent effect against NTUB1 cell growth.

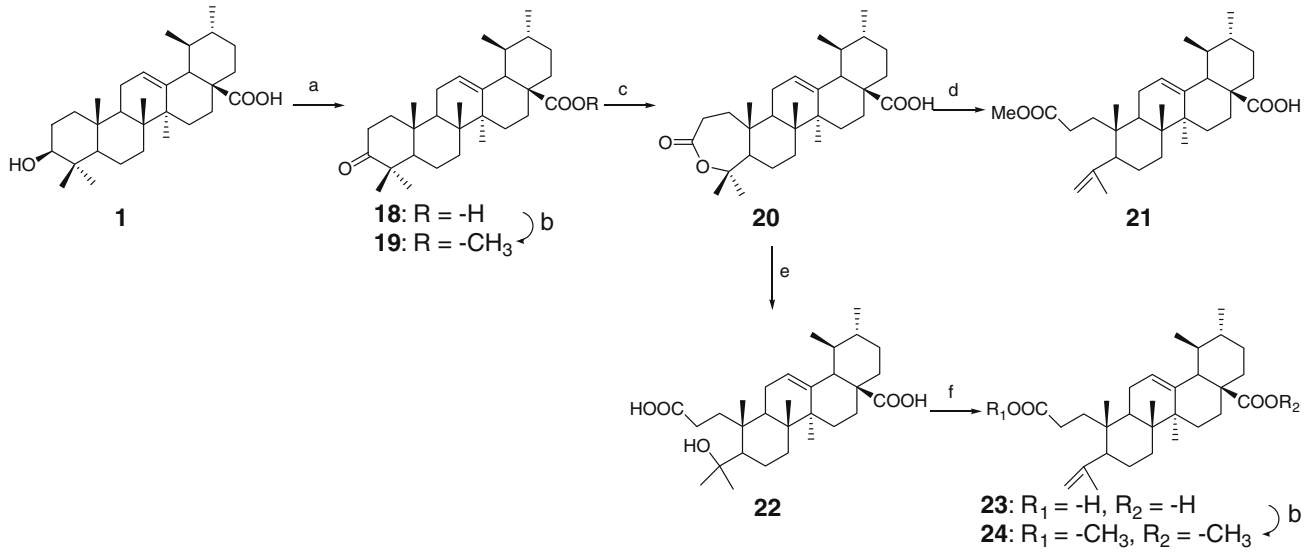
For further evaluated the cytotoxic effect of **1** derivatives and mechanisms of induced cancer cell death in vitro, cytotoxicities of selective compounds **5**, **17**, and **23** against PC3 and A549 were studied and compared with those of cytotoxicities against NTUB1 cells (Fig. 1). As shown in Fig. 1, **5**, **17**, and **23** did not exhibited stronger cytotoxic effects against PC3 and A549 than those of cytotoxicities against NTUB1 cells while 50 μM **5**, **17**, and **23** indicated same cytotoxic effect against these three human cancer lines.

ROS induce programed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signaling cascades.[10] We first examined the effect of **5** and **23** on intracellular ROS levels in NTUB1 cells. Exposure of cells to 10 μM cisplatin, 40 μM **5**, and 20 and 50 μM **23** for 24 h caused a significant increase in intracellular ROS while this effect was inhibited by *N*-acetyl-cysteine [NAC, a thiol antioxidant agent (Fig. 2)]. Exposure of cells to 10 μM cisplatin, 20 and 40 μM **5**, and 20 and 50 μM **23** for 48 h also caused a significant increase in intracellular ROS while this effect did not inhibit by NAC (Fig. 3).

ROS cause a wide range of adaptive cellular responses ranging from transient growth arrest to permanent growth arrest, to apoptosis or to necrosis, dependent on the level of ROS. These responses to allow organism to remove damage caused by ROS or allow organisms to remove damage cells.[7]

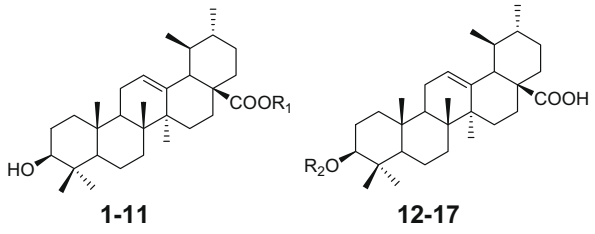


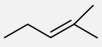
Scheme 1. Reagents and conditions: (a) alkyl halide, K_2CO_3, $(CH_3)_2CO$.



Scheme 3. Reagents and conditions: (a) CrO_3, H_2SO_4, DMF; (b) $TMSCHN_2$, MeOH, and benzene; (c) *m*-CPBA, $CHCl_3$; (d) MeOH, H_2SO_4; (e) KOH, MeOH; (f) H_2SO_4, MeOH.

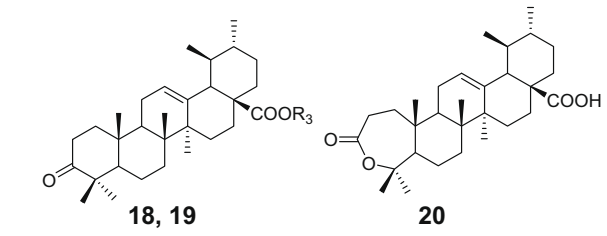
Table 1
Cytotoxicities of **1**–**17** against NTUB1 cells[a] (IC_{50} values in μM)

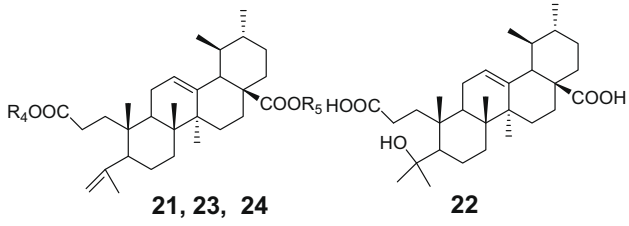


Compound	R_1	R_2	
Cisplatin			3.27 ± 0.10
1	H		29.44 ± 1.90
2	CH_3		37.13 ± 0
3	CH_2CH_3		13.45 ± 0
4	$CH_2CH_2CH_3$		18.28 ± 0
5	$CH(CH_3)_2$		7.97 ± 0.48
6	$CH_2CH_2CH_2CH_3$		15.64 ± 1.31
7	$CH_2CH_2CH_2CH_2CH_3$		27.79 ± 1.63
8	$CH_2CH_2CH_2CH_2CH_2CH_3$		26.16 ± 2.51
9			10.93 ± 2.01
10	CH_2CH_2OH		19.53 ± 0
11	$CH_2COOC(CH_3)_3$		>30 μM (64.85%)
12		$COCH_3$	14.27 ± 2.14
13		$COCH_2CH_3$	>30 μM (72.53%)
14		$COCH_2CH_2CH_3$	>30 μM (76.47%)
15		$COCH{=}CHCH_3$	11.94 ± 0
16		$COCH_2CH_2(CH_3)_2$	30.98 ± 4.14
17		$COCH_2CH_2COOH$	8.65 ± 2.89

[a] Data are presented as mean ± SD (n = 5). Compounds **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. When 50% inhibition could not reached at the highest concentration, then % of viability is given in parentheses.

Table 2
Cytotoxicities of **18**–**24** against NTUB1 cells[a] (IC_{50} values in μM)





Compound	R_3	R_4	R_5	
18	H			21.44 ± 15.50
19	CH_3			29.57 ± 4.65
20				>30 μM (63.91%)
21		CH_3	H	>30 μM (62.77%)
22				>30 μM (56.04%)
23		H	H	25.49 ± 1.46
24		CH_3	CH_3	15.63 ± 1.82

[a] Data are presented as mean ± SD (n = 5). Compounds **18**–**24** 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. When 50% inhibition could not reached at the highest concentration, then % of viability is given in parentheses.

Whereas cell proliferation and differentiation are specifically in the G1 phase and the G_1–S transition in the cell cycle, the oncogenic progress exerts its greatest effect by targeting particular regulators of G1 phase progression.[11] The effect of positive control cisplatin, **5**, **17**, and **23** on cell cycle progression was determined by using fluorescence-activated cell sorting (FACS) analysis in propidium iodide-stained NTUB1 cells. As shown in Figure 4, treatment with 10 and 20 μM cisplatin for 24 h led to a dose-dependent accumulation of cells in the G1 phase with a concomitant increase in the population of sub-G1 phase. Treatment with 20 and 40 μM **5** and 20 and 50 μM **23** for 24 h induced G1 phase arrest in a dose-dependent manner, accompanied by an increase in the apoptotic cell death while treatment with 20–40 μM **17** for 24 h induced G2/M arrest before accumulation of cells in sub-G1 phase. As shown in Figure 5, treatment with 10 μM cisplatin for 48 h re-

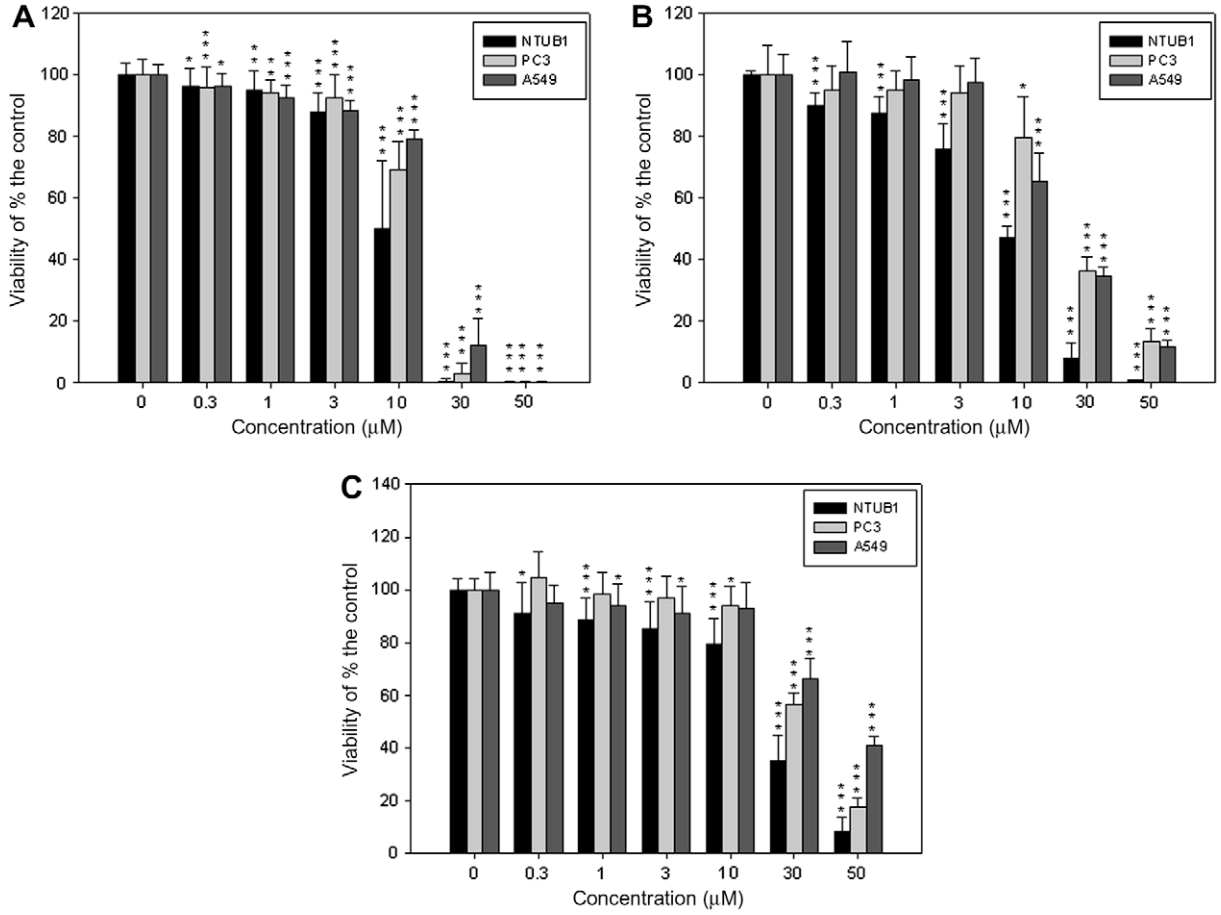


Figure 1. Cytotoxicities of **5** (A), **17** (B), and **23** (C) against NTUB1, PC3, and A549 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of compounds for 72 h. The data shown represent mean ± SD ($n = 3$) for one experiment performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control value, respectively.

sulted in S phase arrest while treatment with 20–40 μM **5** and 20–50 μM **23** for 48 h arrest G2/M phase before accumulation of cells in sub-G1 phase.

It has been known that cellular ROS are essential to cell survival, but the effect of ROS on cell is complex. Experimentally, a low concentration of H_2O_2 causes a moderate increase in proliferation of many tumor cell lines, whereas a higher level results in slowed growth, cell cycle arrest, and apoptosis or even necrosis.[12] Treatment of cells with **5** for 24 and 48 h exhibited induced G1 phase and G2/M arrest, respectively, and increased the amount of ROS in cells. It indicated that the cell cycle arrest and apoptosis induced by **5** were correlated with ROS. Future investigation should address the detailed mechanism of action inducing the cell cycle arrests at G1 or G2/M phase and apoptosis. Treatment of cells with 20 and 40 μM **17** for 24 h induces G2/M arrest before accumulation of cells in sub-G1 phase. Further investigation should also address the detailed mechanism of action of cell cycle arrest at G2/M phase and apoptosis. The induction of increased amount of ROS and G1 phase arrest by treatment of cells with 20 and 50 μM **23** for 24 h. It also showed that the induction of G1 phase arrest by treatment of cells with **23** for 24 h is due to the increased amount of ROS induced by **23** in cells. Treatment of cells with 20 μM **23** for 48 h resulted in G2/M phase arrest.

All inhibition of tubulin polymerization has been implicated in G2/M phase cell cycle arrest in various cancer cell lines.[13] To identify these findings, we investigated whether treatment of cells with 20 μM **23** for 48 h affects in vivo microtubular architecture (Fig. 6). Microtubule assembly was analyzed by indirect immunofluorescence staining using an anti-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Untreated NTUB1 cells (control) showed diffuse staining throughout the cytoplasm and dense perinuclear staining. Treatment with taxol resulted in a distinctive microtubule bundle that was likely due to stabilization of the rigid microtubule network. Treatment with Compound **23**, however, resulted supported that cells treated with **23** for 48 h prevents microtubule assembly in vivo by inhibiting tubulin polymerization.

3. Conclusion

Twenty-three ursolic acid (**1**) derivatives were synthesized and a several of synthetic compounds indicated significant cytotoxic effects against NTUB1 cells. Compounds **5** and **23** revealed a partial mechanism by which **5** and **23** mediated through generation of ROS in NTUB1 cells induce inhibition of tubulin polymerization, G2/M and G1 cell cycle arrest, and apoptosis. Our study may not elucidate the detailed mechanism of synthetic ursolic acid derivatives induced inhibition of tumor cell growth, but may encourage the development of novel, efficient, and less toxic anticancer agents targeting microtubules.

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 spec-

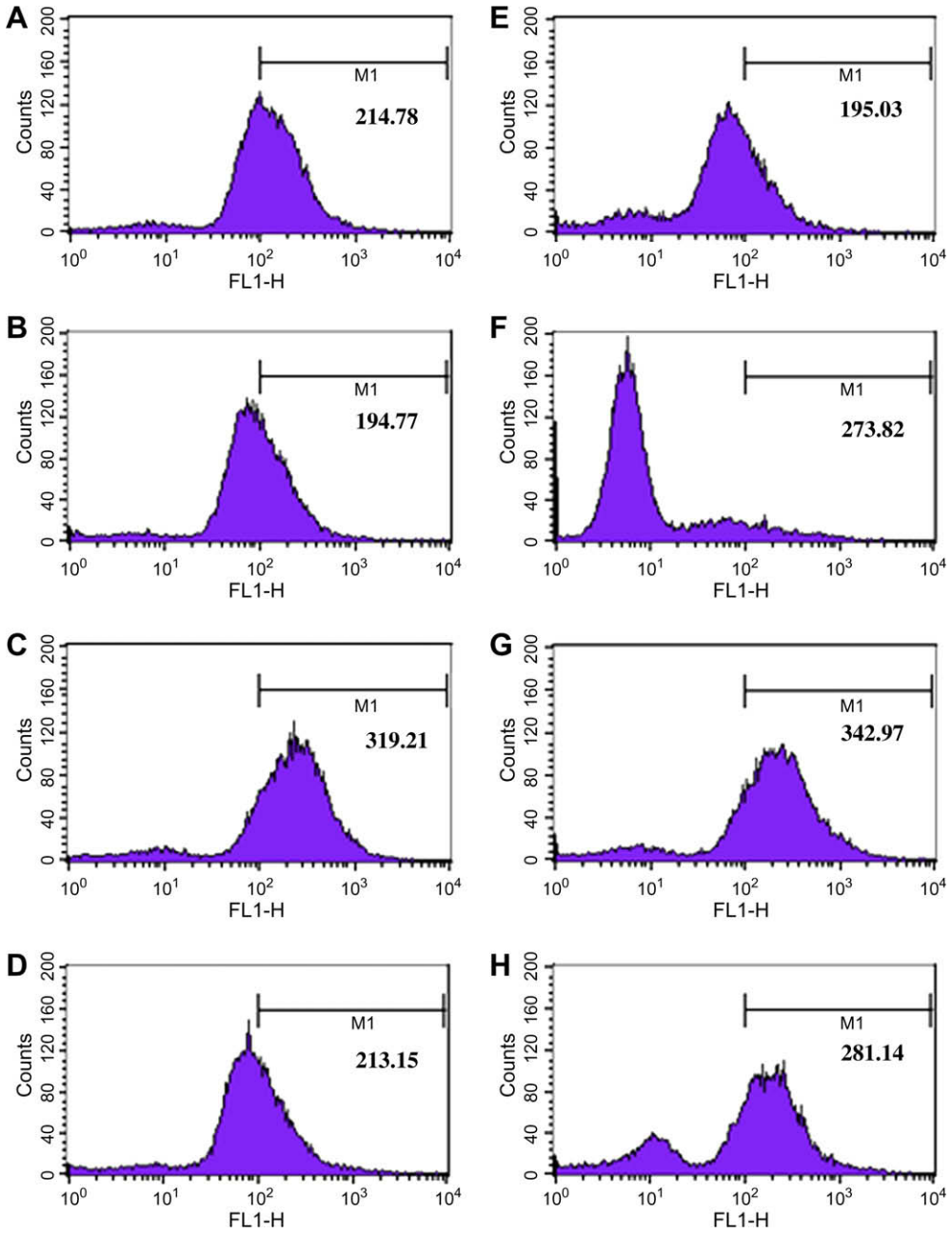


Figure 2. The effect of **5** and **23** on the production of ROS in NTUB1 cells. (A) Control; (B) 1 mM NAC; (C) 10 μM cisplatin; (D) 10 μM **5**; (E) 20 μM **5**; (F) 40 μM **5**; (G) 20 μM **23**; (H) 50 μM **23**. Cells were treated 1 mM NAC, 10 μM cisplatin, and different concentrations of **5** and **23** for 24 h, respectively, and the amount of ROS was assayed by DCFH-DA staining. Results were repeated by three independent experiments.

trometer, and mass 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.

4.2. Extraction of ursolic acid (1)

The method for extracting ursolic acid from leaves of loquat (*Eriobotrya japonica*) has been reported. We used modified method to isolate large amount of ursolic acid (**1**). Leaves of loquat (10 kg) were extracted by methanol. The MeOH extract was shaken under ultrasonic wave for 90 min at 50 °C. The extract was added 1% NaOH solution to form salt and filtered. The solution was neutralized with *c*-HCl to afford light yellow solid. The solid was purified by silica gel chromatography and recrystallized for several times to give ursolic acid (**1**) (21.9 g).

4.3. General procedure for esterification at C-17 carboxylic acid of 1

To a solution of **1** (30 mg, 0.07 mmol) in acetone was added K_2CO_3 (20 mg, 0.14 mmol) and different alkyl halide (0.14 mmol). The reaction mixture was stirred at the room temperature overnight. The mixture was concentrated to dryness under reduced pressure, diluted with water (30 mL), and extracted with DCM (30 mL × 3). The organic phase was dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The crude product was purified by chromatography using EtOAc/*n*-hexane to afford purified compounds **2**–**11**.

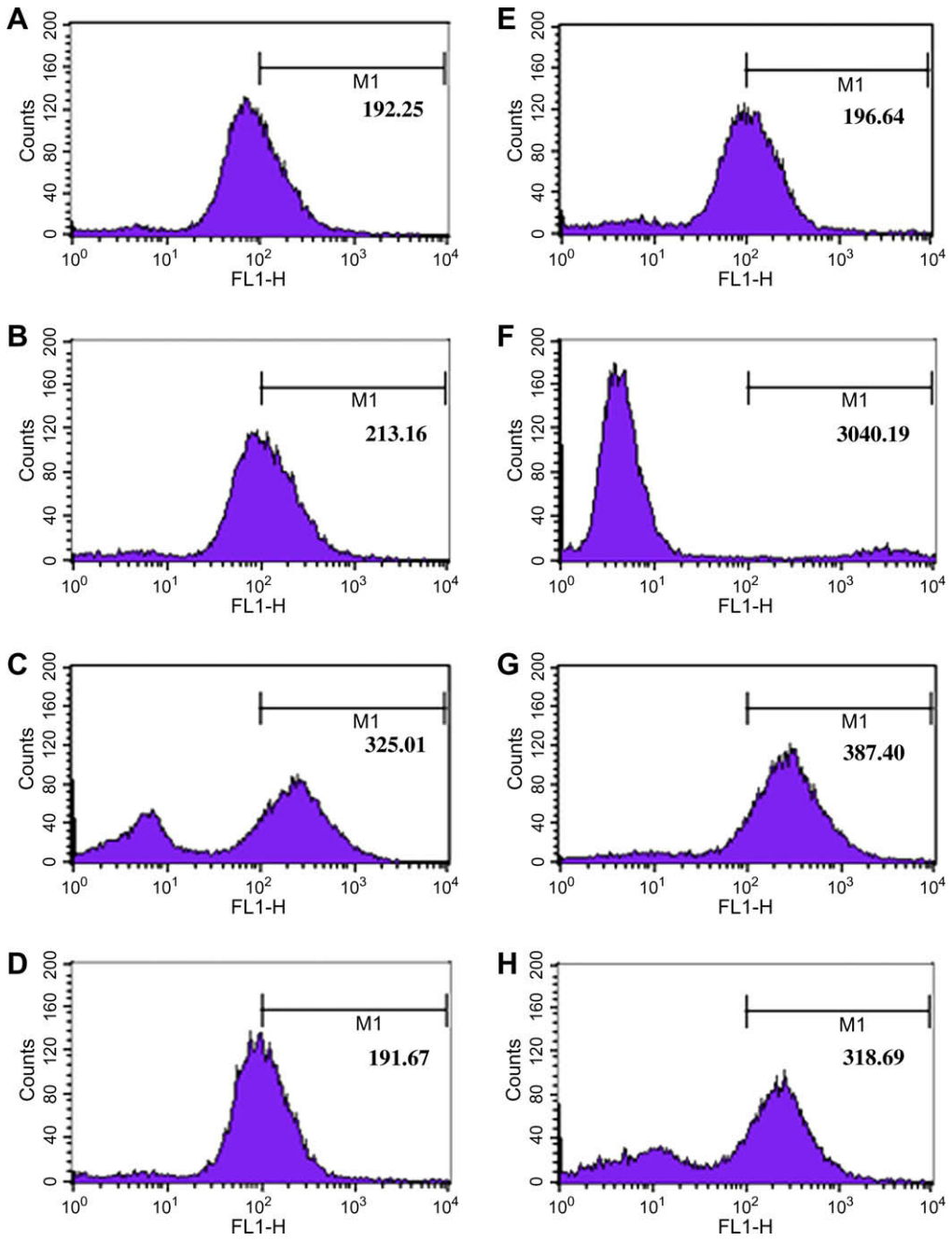


Figure 3. The effect of **5** and **23** on the production of ROS in NTUB1 cells. (A) Control; (B) 1 mM NAC; (C) 10 μM cisplatin; (D) 10 μM **5**; (E) 20 μM **5**; (F) 40 μM **5**; (G) 20 μM **23**; (H) 50 μM **23**. Cells were treated 1 mM NAC, 10 μM cisplatin, and different concentrations of **5** and **23** for 48 h, respectively, and the amount of ROS was assayed by DCFH-DA staining. Results were repeated by three independent experiments.

4.3.1. Isopropyl 3β-hydroxyurs-12-en-28-oate (5)

Compound **5** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using isopropyl iodide as alkyl halide moiety. Compound **5** was obtained as a white solid (49.2 mg, 0.10 mmol, 90%), $[\alpha]_D^{25}$ = +50. IR (KBr): 3447, 1715 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.77 (3H, m, H-24), 0.82 (3H, s, H-25), 0.85 (3H, d, J = 6.4 Hz, H-30), 0.91 (3H, s, H-26), 0.93 (3H, d, J = 6.4 Hz, H-29), 0.98 (3H, s, H-27), 1.07 (3H, s, H-23), 1.16 (3H, d, J = 6.0 Hz, –CH(CH_3)$_2$), 1.19 (3H, d, J = 6.0 Hz, –CH(CH_3)$_2$), 2.21 (1H, d, J = 11.2 Hz, H-18), 3.21 (1H dd, J = 10.8, 5.2 Hz, H-3α), 4.91 (1H, m, –CH(CH_3)$_2$), 5.24 (1H, t, J = 4.0 Hz, H-12).^{13}C NMR ($CDCl_3$): δ 15.5 (C-24), 15.6 (C-25), 17.0 (C-11), 17.3 (C-26), 18.3 (C-6), 21.2 (C-29), 21.7 (–CH(CH_3)$_2$), 21.8 (–CH(CH_3)$_2$), 23.3 (C-27), 23.4 (C-30), 24.1 (C-16), 27.2 (C-2), 28.0 (C-15), 28.1 (C-23), 30.7 (C-21), 33.2 (C-7), 36.6 (C-22), 36.9 (C-10), 38.6 (C-4), 38.7 (C-1), 38.9 (C-20), 39.1 (C-19), 39.6 (C-8), 42.1 (C-14), 47.6 (C-9), 47.7 (C-17), 52.8 (C-18), 55.2 (C-5), 66.9 (–COOCH(CH_3)$_2$), 79.0 (C-3), 125.5 (C-12), 138.1 (C-13), 176.9 (C-28). EIMS (70 eV) m/z (% rel. int.): 498 $[M]^+$ (3). HREIMS: calcd for $C_{33}H_{54}O_3$: 498.4073, found: 498.4071.

4.3.2. Pentyl 3β-hydroxyurs-12-en-28-oate (7)

Compound **7** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using pentyl iodide as alkyl halide moiety. Compound **7** was obtained as a white solid (48.5 mg, 0.09 mmol,

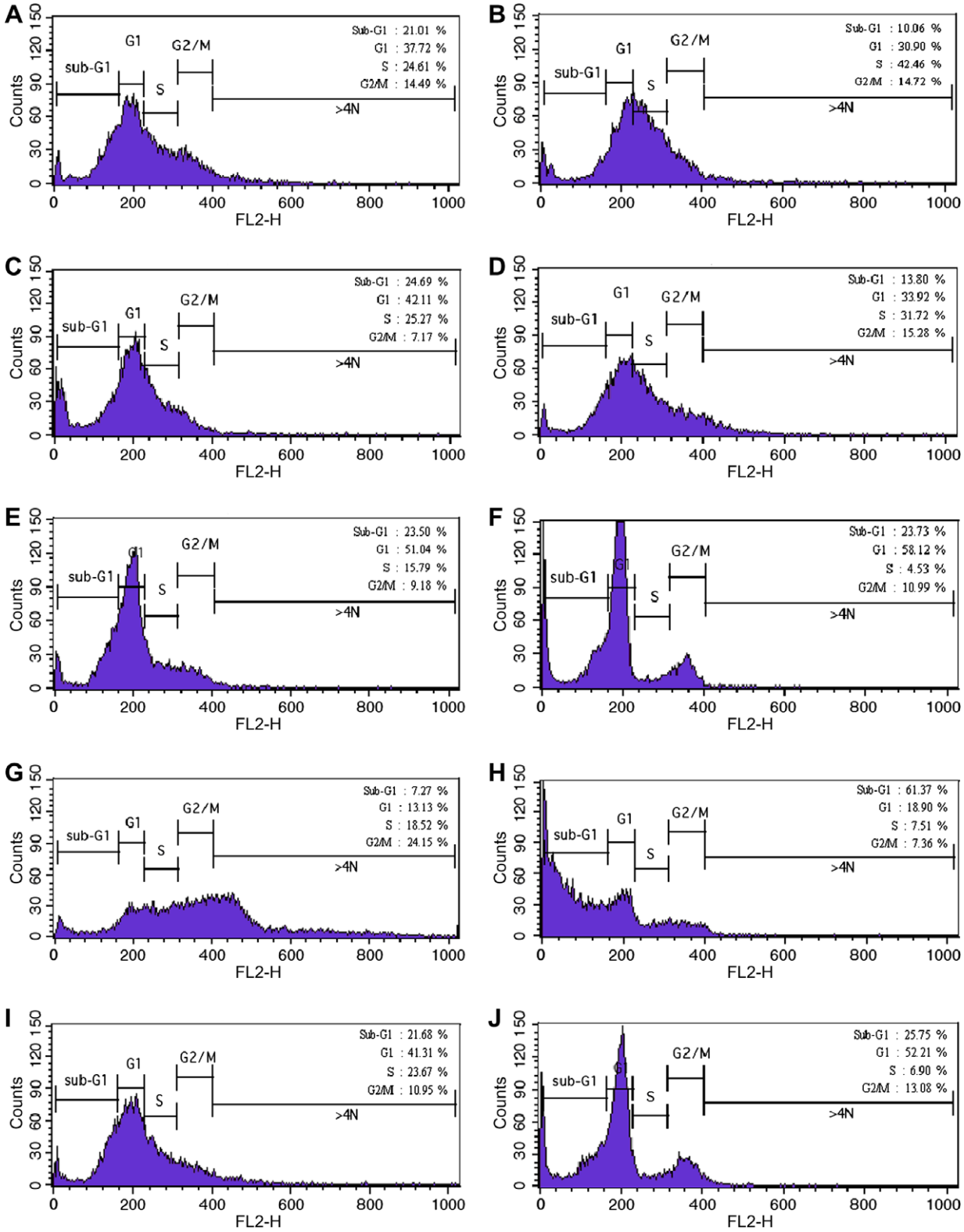


Figure 4. Flow cytometry analysis of cisplatin, **5**-, **17**-, and **23**-treated NTUB1 cells. NTUB1 cells (8×10^5 cells/10 cm dish) were treated with absence of cisplatin or compounds (control, A), 10 μM cisplatin (B), 20 μM cisplatin (C), 10 μM **5** (D), 20 μM **5** (E), 40 μM **5** (F), 20 μM **17** (G), 40 μM **17** (H), 20 μM **23** (I), or 50 μM **23** (J) 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-G1 DNA contents in cells. The control cells were treated with medium. Results were representative of three independent experiments.

84%), $[\alpha]_D^{25}$ = +38. IR (KBr): 3447, 1718 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.75 (3H, s, H-25), 0.77 (3H, s, H-24), 0.85 (3H, d, J = 6.4 Hz, H-30), 0.91 (3H, s, H-26), 0.91 (3H, t, J = 7.2 Hz, $-CH_2CH_3$), 0.93 (3H, d, J = 6.4 Hz, H-29), 0.98 (3H, s, H-27), 1.07 (3H, s, H-23), 2.22 (1H, d, J = 10.8 Hz, H-18), 3.21 (1H, dd, J = 10.8, 4.8 Hz, H-3α), 3.97 (2H, m, $-COOCH_2-$), 5.23 (1H, t, J = 3.6 Hz, H-12). ^{13}C NMR

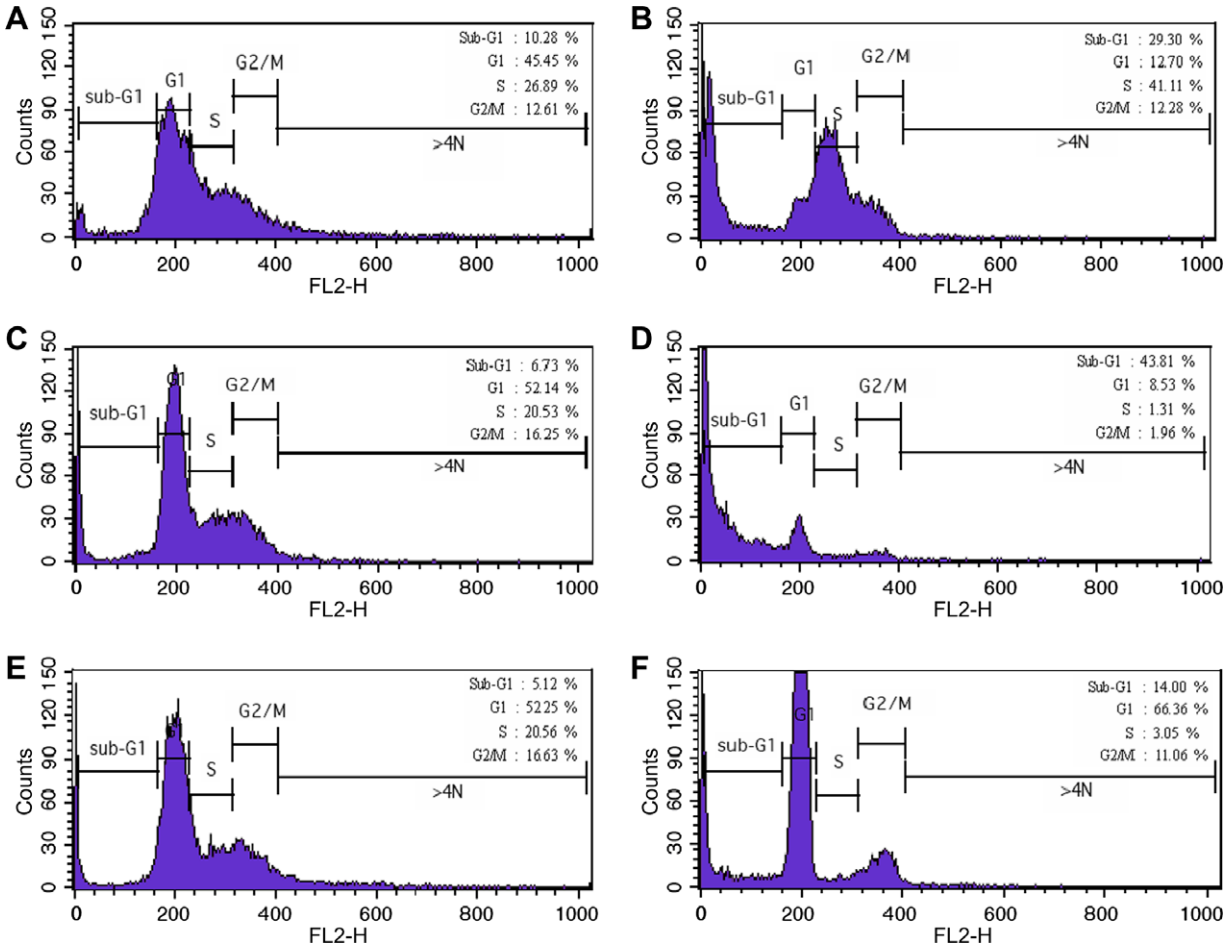


Figure 5. Flow cytometry analysis of cisplatin, **5**-, **17**-, and **23**-treated NTUB1 cells. NTUB1 cells (8×10^5 cells/10 cm dish) were treated with absence of cisplatin or compounds (control, A), 10 μM cisplatin (B), 20 μM **5** (C), 40 μM **5** (D), 20 μM **23** (E), or 50 μM **23** (F) for 48 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-G1 DNA contents in cells. The control cells were treated with medium. Results were representative of three independent experiments.

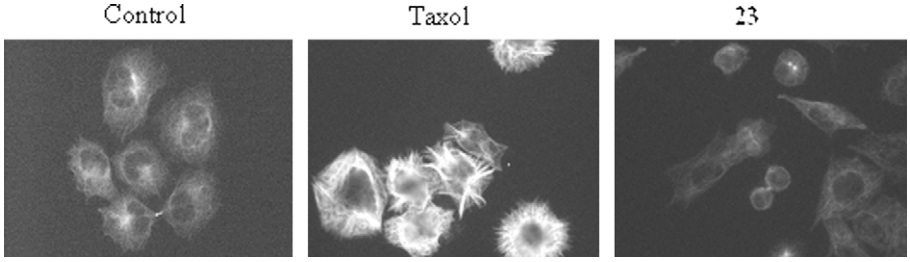


Figure 6. Effect of taxol and **23** on microtubule assembly. NTUB1 cells were treated with 10 nM taxol and 50 μM **23** for 24 h, and stained with anti-α-tubulin antibody and Rodamine-conjugated secondary antibody. The cellular microtubules were observed by Axioskop 2 plus fluorescence microscope.

($CDCl_3$): δ 14.0 ($-CH_2CH_3$), 15.4 (C-24), 15.6 (C-25), 17.0 (C-11), 17.1 (C-26), 18.3 (C-6), 21.2 (C-29), 22.3 ($-CH_2CH_3$), 23.3 (C-27), 23.5 (C-30), 24.2 (C-16), 27.2 (C-2), 28.0 (C-15), 28.1 ($-CH_2CH_2CH_3$), 28.2 (C-23), 28.3 ($-OCH_2CH_2-$), 30.7 (C-21), 33.0 (C-7), 36.7 (C-22), 36.9 (C-10), 38.6 (C-1), 38.7 (C-4), 38.9 (C-20), 39.1 (C-19), 39.5 (C-8), 42.0 (C-14), 47.5 (C-9), 48.0 (C-17), 52.8 (C-18), 55.2 (C-5), 64.3 ($-COOCH_2-$), 79.0 (C-3), 125.5 (C-12), 138.2 (C-13), 177.6 (C-28). EIMS (70 eV) *m/z* (% rel. int.): 526 $[M]^+$ (3). HREIMS: calcd for $C_{35}H_{58}O_3$: 526.4385, found: 526.3910.

4.3.3. Hexyl 3β-hydroxyurs-12-en-28-oate (8)

Compound **8** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using hexyl iodide as alkyl halide moiety. Compound **8** was obtained as a white solid (26.5 mg, 0.05 mmol, 45%), $[\alpha]_D^{25}$ = +34. IR (KBr): 3446, 1718 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.75 (3H, s, H-25), 0.77 (3H, s, H-24), 0.86 (3H, t, *J* = 7.6 Hz, $-CH_2CH_3$), 0.88 (3H, d, *J* = 6.4 Hz, H-30), 0.91 (3H, s, H-26), 0.93 (3H, d, *J* = 6.4 Hz, H-29), 0.98 (3H, s, H-27), 1.07 (3H, s, H-23), 2.22 (1H, d, *J* = 11.2 Hz, H-18), 3.21 (1H, dd, *J* = 9.6, 4.8 Hz, H-3α), 3.97 (2H, m, $-COOCH_2-$), 5.23 (1H, t, *J* = 3.6 Hz, H-12). ^{13}C NMR ($CDCl_3$): δ 14.0 ($-CH_2CH_3$), 15.4 (C-24), 15.6 (C-25), 17.0 (C-11), 17.1 (C-26), 18.3 (C-6), 21.2 (C-29), 22.6 ($-CH_2CH_3$), 23.3 (C-27), 23.5 (C-30), 24.2 (C-16), 25.7 ($OCH_2CH_2CH_2-$), 27.2 (C-2), 28.0 (C-15), 28.1 (C-23), 28.5 ($-CH_2CH_2CH_3$), 30.7 (C-21), 31.4 ($-OCH_2CH_2-$), 33.0 (C-7), 36.7 (C-22), 36.9 (C-10), 38.6 (C-1), 38.7 (C-4), 38.9 (C-20),

39.1 (C-19), 39.5 (C-8), 42.0 (C-14), 47.5 (C-9), 48.0 (C-17), 52.8 (C-18), 55.2 (C-5), 64.3 (–$COOCH_2$–), 79.0 (C-3), 125.5 (C-12), 138.2 (C-13), 177.6 (C-28). EIMS (70 eV) *m/z* (% rel. int.): 540 $[M]^+$ (5). HREIMS: calcd for $C_{36}H_{60}O_3$: 540.4542, found: 540.4538.

4.3.4. 3′-Methyl-2′-butenyl 3β-hydroxyurs-12-en-28-oate (9)

Compound **9** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using 1-bromo-3-methyl-2-butene as alkyl halide moiety. Compound **9** was obtained as a white solid (17.5 mg, 0.03 mmol, 30%), $[\alpha]_D^{25}$ = +36. IR (KBr): 3447, 1720 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.75 (3H, s, H-25), 0.77 (3H, s, H-24), 0.85 (3H, d, *J* = 6.4 Hz, H-30), 0.92 (3H, s, H-26), 0.94 (3H, d, *J* = 6.4 Hz, H-29), 0.99 (3H, s, H-27), 1.07 (3H, s, H-23), 1.68 (3H, s, –CH=C(CH_3)$_2$), 1.74 (3H, s, –CH=C(CH_3)$_2$), 2.23 (1H, d, *J* = 11.2 Hz, H-18), 3.23 (1H, dd, *J* = 10.8, 4.8 Hz, H-3α), 4.48 (1H, dd, *J* = 10.4, 7.2 Hz, –COOC*H*H–), 4.51 (1H, dd, *J* = 10.4, 7.6 Hz, –COOCH*H*–), 5.24 (1H, t, *J* = 4.0 Hz, H-12), 5.30 (1H, t, *J* = 7.6 Hz, –C*H*=C(CH_3)$_2$). ^{13}C NMR ($CDCl_3$): δ 15.4 (C-24), 15.6 (C-25), 17.0 (C-11), 17.1 (C-26), 18.0 (–CH=C(*C*H_3)$_2$), 18.3 (C-6), 21.2 (C-29), 23.3 (C-27), 23.5 (C-30), 24.2 (C-16), 25.8 (–CH=C(CH_3)$_2$), 27.2 (C-2), 28.0 (C-15), 28.1 (C-23), 30.7 (C-21), 33.1 (C-7), 36.6 (C-22), 37.0 (C-10), 38.6 (C-1), 38.7 (C-4), 38.8 (C-20), 39.1 (C-19), 39.5 (C-8), 42.1 (C-14), 47.6 (C-9), 48.0 (C-17), 52.9 (C-18), 55.2 (C-5), 61.0 (–$COOCH_2$–), 79.0 (C-3), 119.1 (–*C*H=(CH_3)$_2$), 125.5 (C-12), 138.1 (–CH=*C*(CH_3)$_2$), 138.2 (C-13), 177.5 (C-28). ESIMS (70 eV) *m/z* (% rel. int.): 547. HRESIMS: calcd for $C_{35}H_{56}O_3Na$: 547.4127, found: 547.4123.

4.3.5. 2′-Hydroxyethyl 3β-hydroxyurs-12-en-28-oate (10)

Compound **10** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using 2-chloroethanol as alkyl halide moiety. Compound **10** was obtained as a white solid (28.5 mg, 0.06 mmol, 52%), $[\alpha]_D^{25}$ = +35. IR (KBr): 3433, 1718 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.76 (3H, s, H-25), 0.77 (3H, s, H-24), 0.86 (3H, d, *J* = 6.4 Hz, H-30), 0.91 (3H, s, H-26), 0.94 (3H, d, *J* = 6.4 Hz, H-29), 1.01 (3H, s, H-27), 1.09 (3H, s, H-23), 2.24 (1H, d, *J* = 10.8 Hz, H-18), 3.21 (1H, dd, *J* = 11.2, 4.8 Hz, H-3α), 3.79 (2H, m, –$COOCH_2$–), 4.08 (1H, ddd, *J* = 11.6, 5.6, 3.2 Hz, –C*H*HOH), 4.20 (1H, ddd, *J* = 11.6, 6.0, 3.6 Hz, –CH*H*OH), 5.26 (1H, t, *J* = 3.6 Hz, H-12). ^{13}C NMR ($CDCl_3$): δ 15.4 (C-24), 15.6 (C-25), 17.0 (C-11), 17.1 (C-26), 18.3 (C-6), 21.1 (C-29), 23.3 (C-27), 23.5 (C-30), 24.2 (C-16), 27.2 (C-2), 27.9 (C-15), 28.1 (C-23), 30.6 (C-21), 33.0 (C-7), 36.7 (C-22), 36.9 (C-10), 38.6 (C-1), 38.7 (C-4), 38.8 (C-20), 39.1 (C-19), 39.5 (C-8), 42.2 (C-14), 47.5 (C-9), 48.3 (C-17), 53.0 (C-18), 55.2 (C-5), 61.4 (–CH_2OH), 66.0 (–$COOCH_2$–), 79.0 (C-3), 125.3 (C-12), 138.9 (C-13), 177.9 (C-28). EIMS (70 eV) *m/z* (% rel. int.): 500 $[M]^+$ (3). HREIMS: calcd for $C_{32}H_{52}O_4$: 500.3865, found: 500.3863.

4.3.6. *tert*-Butoxycarbonylmethyl 3β-hydroxyurs-12-en-28-oate (11)

Compound **11** was prepared from **1** (50 mg, 0.11 mmol) following the general procedure described for esterification at C-17 carboxylic acid using chloroacetic acid *tert*-butyl ester as alkyl halide moiety. Compound **11** was obtained as a white solid (38.9 mg, 0.07 mmol, 62%), $[\alpha]_D^{25}$ = +20. IR (KBr): 3447, 1733 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.72 (3H, s, H-25), 0.78 (3H, s, H-24), 0.86 (3H, d, *J* = 6.4 Hz, H-30), 0.91 (3H, s, H-26), 0.94 (3H, d, *J* = 6.4 Hz, H-29), 0.98 (3H, s, H-27), 1.08 (3H, s, H-23), 1.45 (9H, s, –C(CH_3)$_3$), 2.25 (1H, d, *J* = 11.2 Hz, H-18), 3.21 (1H, dd, *J* = 9.6, 3.6 Hz, H-3α), 4.42 (2H, m, –$COOCH_2$–), 5.25 (1H, t, *J* = 3.6 Hz, H-12). ^{13}C NMR ($CDCl_3$): δ 15.4 (C-24), 15.6 (C-25), 16.9 (C-26), 17.0 (C-11), 18.3 (C-6), 21.2 (C-29), 23.3 (C-27), 23.5 (C-30), 24.2 (C-16), 27.2 (C-2), 28.0 (–OC(*C*H_3)$_3$), 28.0 (–OC(*C*H_3)$_3$), 28.1 (C-23), 29.7(–OC(*C*H_3)$_3$), 30.6 (C-21), 33.0 (C-7), 36.5 (C-22), 36.9 (C-10), 38.6 (C-1), 38.7 (C-4), 38.8 (C-20), 39.1 (C-19), 39.5 (C-8), 42.0 (C-14), 47.6 (C-9), 48.0 (C-17), 52.7 (C-18), 55.2 (C-5), 60.9 (–$COOCH_2$–), 79.0 (C-3), 125.7 (C-12), 138.0 (C-13), 167.1 (–CH_2CO–), 176.7 (C-28). EIMS (70 eV) *m/z* (% rel. int.): 570 $[M]^+$ (6). HREIMS: calcd for $C_{36}H_{58}O_5$: 570.4283, found: 570.4291.

4.4. General procedure for esterification at C-3 hydroxy group of 1

Compound 1 (50 mg, 0.11 mmol) was dissolved in different anhydride (1 mL) and pyridine (1 mL), and the solution was stirred at room temperature for 6 h. The reaction mixture was added water (10 mL) and partitioned with DCM (15 mL × 3). The organic solution was dried over Na_2SO_4 and concentrated in vacuo to give crude product. The crude product was purified by chromatography on a column of silica gel eluted with EtOAc/*n*-hexane to obtain compounds **12**–**17**.

4.5. 4-Hydroxy-3,4-*seco*-ursan-12-en-28-oic acid 3,4 lactone (20)

A mixture of **18** (150 mg, 0.3 mmol) and 70–75% 3-chloroperoxybenzoic acid (200 mg, 0.8–0.9 mmol) in $CHCl_3$ (10 mL) was stirred at room temperature for 72 h. More chloroform (20 mL) was added and the organic layer was washed with aq KI (5%), aq Na_2SO_3, water, aq $NaHCO_3$ solutions. The solution was extracted with $CHCl_3$ (30 mL × 3), dried over Na_2SO_4, and concentrated in vacuo to give the crude product. The crude product was purified by chromatography on a column of silica gel eluted with EtOAc/DCM (1:6) to obtain **20** (91 mg, 0.19 mmol, 59%) as a white powder, $[\alpha]_D^{25}$ = +49. IR (KBr): 3448, 1756, 1714 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.80 (3H, s, H-25), 0.85 (3H, d, *J* = 6.4 Hz, H-30), 0.95 (3H, d, *J* = 6.0 Hz, H-29), 1.01 (3H, s, H-26), 1.07 (3H, s, H-27), 1.26 (3H, s, H-24), 1.28 (3H, s, H-23), 2.20 (1H, d, *J* = 11.2 Hz, H-18), 5.25 (1H, t, *J* = 3.6 Hz, H-12). ^{13}C NMR ($CDCl_3$): δ 13.6 (C-25), 16.8 (C-26), 17.0 (C-11), 19.7 (C-24), 20.8 (C-27), 21.1 (C-30), 23.3 (C-29), 23.9 (C-6), 24.0 (C-16), 27.9 (C-15), 29.6 (C-7), 30.5 (C-21), 31.8 (C-2), 36.6 (C-22), 37.0 (C-9), 38.8 (C-20), 39.0 (C-19), 42.1 (C-14), 45.3 (C-8), 47.9 (C-17), 52.5 (C-5), 55.2 (C-18), 74.9 (C-4), 125.3 (C-12), 138.1 (C-13), 175.8 (C-3), 182.8 (C-28). EIMS (70 eV) *m/z* (% rel. int.): 470 $[M]^+$ (2.3). HREIMS: calcd for $C_{30}H_{46}O_4$: 470.3395, found: 470.2825.

4.6. Methyl 3,4-*seco*-ursan-4(23),12-dien-28-oic 3-oat (21)

A mixture of **18** (100 mg, 0.2 mmol) and 70–75% 3-chloroperoxybenzoic acid (100 mg, 0.4–0.5 mmol) in $CHCl_3$ (10 mL) was stirred at room temperature for 72 h. The mixture was concentrated to dryness under reduced pressure, added MeOH (10 mL) and *c*-H_2SO_4 (three drops), and stirred at room temperature for 24 h. The mixture was concentrated to dryness under reduced pressure again, washed with aq $NaHCO_3$ solution, extracted with $CHCl_3$ (20 mL × 3), dried over Na_2SO_4, and concentrated in vacuo to give the crude product. The crude product was purified by chromatography on a column of silica gel eluted with EtOAc/*n*-hexane (1:3) to obtain **21** (22.0 mg, 0.05 mmol, 21%) as a white powder, $[\alpha]_D^{25}$ = +8. IR (KBr): 3461, 1735, 1693 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82 (3H, s, H-27), 0.86 (3H, d, *J* = 6.4 Hz, H-30), 0.92 (3H, s, H-25), 0.94 (3H, d, *J* = 6.4 Hz, H-29), 1.09 (3H, s, H-26), 1.72 (3H, s, H-24), 2.25 (1H, d, *J* = 11.2 Hz, H-18), 3.65 (3H, s, –$COOCH_3$), 4.64 (1H, s, –C=C*H*H), 4.86 (1H, s, –C=CH*H*), 5.26 (1H, t, *J* = 3.2 Hz, H-12). ^{13}C NMR ($CDCl_3$): δ 17.0 (C-11), 17.2 (C-26), 19.5 (C-25), 21.1 (C-29), 23.4 (C-27), 23.4 (C-24), 23.5 (C-30), 24.0 (C-16), 24.3 (C-6), 28.0 (C-15), 28.5 (C-2), 30.6 (C-21), 31.6 (C-1), 33.9 (C-7), 36.7 (C-22), 37.7 (C-9), 38.8 (C-20), 39.1 (C-19), 39.2 (C-8), 39.2 (C-10), 42.4 (C-14), 48.0 (C-17), 50.3 (C-5), 51.6 (–$COOCH_3$), 52.6 (C-18),

113.6 (C-23), 125.7 (C-12), 137.9 (C-13), 147.3 (C-4), 174.6 (C-28), 183.4 (C-3). EIMS (70 eV) *m/z* (% rel. int.): 484 $[M]^+$ (23). HREIMS: calcd for $C_{31}H_{48}O_4$: 484.3552, found: 484.3549.

4.7. 3,4-*seco*-Ursan-4-hydroxy-12-en-3,28-dioic acid (22)

Compound 20 (30 mg, 0.06 mmol) in 5% methanolic KOH was kept at room temperature for 48 h. The organic layer was removed under pressure. The mixture was added water (10 mL) and extracted with EtOAc (10 mL × 3) to give the crude product. The crude product was purified by column chromatography eluted with acetone:DCM (1:3) and MeOH to obtain **22** (15 mg, 0.03 mmol, 48%) as a white powder, $[\alpha]_D^{25}$ = +35. IR (KBr): 3447, 1690 cm^{-1}. ^1H NMR (CD_3OD): δ 0.89 (3H, d, J = 6.4, H-30), 0.91 (3H, d, J = 6.4 Hz, H-29), 0.97 (3H, s, H-25), 1.10 (3H, s, H-26), 1.14 (3H, s, H-27), 1.25 (3H, s, H-24), 1.27 (3H, s, H-23), 2.22 (1H, d, J = 11.6, H-18), 2.30 (1H, m, H_α-2), 2.49 (1H, m, H_β-2), 5.27 (1H, t, J = 3.6 Hz, H-12). ^{13}C NMR (CD_3OD): δ 17.7 (C-26), 17.8 (C-11), 18.1 (C-25), 20.6 (C-29), 21.6 (C-6), 23.4 (C-27), 23.8 (C-30), 24.1 (C-16), 28.3 (C-24), 29.2 (C-15), 30.4 (C-2), 31.8 (C-21), 32.7 (C-7), 33.7 (C-23), 35.7 (C-22), 38.1 (C-20), 40.1 (C-19), 40.6 (C-10), 42.1 (C-14), 43.8 (C-8), 49.0 (C-17), 52.9 (C-18), 53.0 (C-5), 54.4 (C-9), 76.2 (C-4), 127.2 (C-12), 139.4 (C-13), 181.7 (C-28 and C-3). ESIMS (70 eV) *m/z*: 511 $[M+Na]^+$. HRESIMS: calcd for $C_{30}H_{48}O_5Na$: 511.3399, found: 511.3402.

4.8. Cell culture and MTT assay for cell viability/proliferation

NTUB1, a human bladder cancer cell line, was established from a high-grade bladder cancer,[14] PC3, and A549 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (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 the tested compound with cisplatin, a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay was performed.[15] Briefly, the cells were plated at a density of 1800 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 the test compound with or without various concentrations of cisplatin (Pharmacia & Upjohn, Milan, Italy) 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}s of each group were calculated by the median-effect analysis and presented as mean ± standard deviation (SD).

4.9. Flow cytometry analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described.[16] Briefly, 8×10^5 cells were plated and treated with various concentrations of cisplatin and various concentrations of **5**, **17**, and **23** for 24 or 48 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 propidium iodide (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).

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

Production of ROS was analyzed by flow cytometry as described previously.[17] Briefly, cells were plated and treated as indicated conditions. Ten-micromolar dichlorofluorescein diacetate (DCFH-DA; 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).

4.11. Immunofluorescence microscopy

NTUB1 cells plated on coverslips were treated with no compound as control, 10 nM taxol, 50 μM compound **23** 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 anti-α-tubulin monoclonal antibody (Sigma, Co) in PBS and incubated for 3 h at room temperature. Then cells were washed with PBS and reincubated with Rodamine-conjugated secondary antibody (Sigma, Co) in dark room for 1 h at room temperature. After being washed with PBS, coverslips were mounted with 80% glycerol in PBS and examined with Axioskop 2 plus fluorescence microscope.[18]

4.12. Statistical analysis

Data were expressed as means ± 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.

Acknowledgment

This work was supported by a research grant from the National Science Council of the Republic of China (NSC95-2320-B-037-037).

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