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Synthesis and biological evaluation of 2′,5′-dimethoxychalcone derivatives as microtubule-targeted anticancer agents

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ABSTRACT

A series of novel 2′,5′-dimethoxylchalcone derivatives including 18 new compounds were synthesized and evaluated for cytotoxicities against two human cancer cell lines, NTUB1 (human bladder cancer cell line) and PC3 (human prostate cancer cell line). All these derivatives except for **21** exhibited significant cytotoxic effect against NTUB1 and PC3 cell lines. Compounds **13** and **17** with 4-carbamoyl moiety showed potent inhibitory effect on growth of NTUB1 and PC3 cells. Flow cytometric analysis demonstrated that treatment of NTUB1 cells with 1 μM **13** and **17** induced G1 phase arrest accompanied by an increase in apoptotic cell death of NTUB1 cells after 24 h. Treatment of PC3 cells with 1 μM and 3 μM **13**, and 1 μM and 3 μM **17** induced S and G1, and G1 and G2/M phase arrests, respectively, accompanied by an increase in apoptotic cell death. These data suggested that **13** and **17** with different 4-carbamoyl moiety displayed same cell cycle arrest in NTUB1 cells while different doses of **13** and **17** revealed different cell cycle arrest in PC3 cells. Cell morphological study of **17** indicated that more cells rounding up or dead associated with tubulin polymerization. Compound **17** showed an increased α-tubulin level in polymerized microtubule fraction in a dose-dependent manner while 500 nM paclitaxel also showed similar effect in NTUB1 cells by Western blot analysis. The result suggested that **17** may be used as microtubule-targeted agents.

1. Introduction

Microtubules display an important role in a variety of cellular process including mitosis and cell division. Various anti-mitotic agents interfering with the natural dynamics of tubulin, the major protein component of microtubules, polymerization, and depolymerization inhibit cancer cell growth.[1]

Anti-mitotic agents such as paclitaxel stabilize microtubules by preventing the depolymerization of tubulin. The vinca alkaloids and colchicine inhibit the polymerization of tubulin. Disruption of tubulin dynamics leads to cell cycle arrest in the G2/M phase and induction of apoptosis.[1] Anti-mitotic compounds have been used clinically in the treatment of different cancers, a major problem of anti-mitotic agent, such as taxanes and vinca alkaloids in clinical application is the development of drug resistance.[1] Therefore, it needs to find and develop effective tubulin inhibitors for treating multidrug-resistant (MDR) tumors.

Chalcones have been characterized with multiple biological activities including anti-inflammatory, anti-malaria, anti-protozoal, anti-bacterial, nitric oxide inhibitory, tyrosinase inhibitory, cytotoxic, anti-leishmanial, and anti-oxidant activities in the past decade.[2–8] In our previous study, various 2′,5′-dialkoxylchalcones were synthesized and demonstrated their structures and cytotoxic relationships against A549, Hep 3B, HT-29, and MCF-7 cells.[8,9] Several reports have delineated the SAR of colchicine–tubulin binding. These studies have shown that the important structural features of colchicine molecule (Fig. 1) for binding to tubulin are the methoxy groups of the A ring and the carbonyl of the C ring.[10] In addition, a (*E*)-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one was reported to be an effective antimitotic agent at concentration of 4 nM in an in vitro HeLa cell test system.[10] Based on the above reason, we further synthesized a series of 2′,5′-dialkoxylchalcones with a carbonyl or carbamoyl group substituted at C-4 of the B ring, evaluated their cytotoxicities against human NTUB1 (human bladder cancer cell line) and PC3 (human prostate cancer cell line) cells, and discussed the structures and cytotoxicity and mechanism of action.

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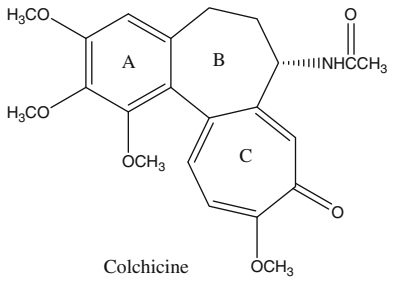


Figure 1. Structure of colchicine.

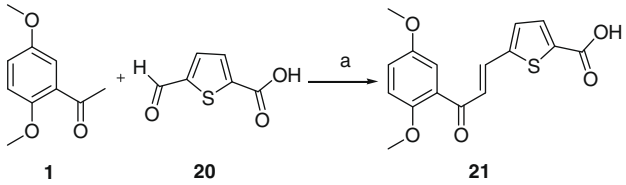
2. Chemistry

As shown in Schemes 1 and 2, chalcones **3** and **21** were prepared by using Claisen–Schmidt condensation and hydrolysis with methanolic 8% KOH solution. The compound **3** in acetone was added potassium carbonate and various alkyl halides in appropriate conditions to esterify C-4-COOH of **3** and afford various esters 4–9. Treatment of **3** in CH_2Cl_2 with various amines in the presence of *N*-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) afford various amides **10–19**. These procedures gave products with good yields (Table 1).

3. Results and discussion

Cisplatin shows more cytotoxicity against human transitional carcinoma but about 30–50% of advanced bladder cancer did not respond to cisplatin in chemotherapy.[11] Based on resistances of chemotherapy with cisplatin, it would like to find other new agents for cancer treatment. Cytotoxicities of **3** derivatives against NTUB1 and PC3 cell lines were studied and cisplatin was used as the positive control. As shown in Table 2, compound **3** and most of its derivatives showed significantly cytotoxic activities against NTUB1 cells while **21** had no significant cytotoxicity. The esterification of C-4-COOH of **3** enhanced the cytotoxicity against NTUB1 and PC3 cells except for **6**, **8**, and **9**. It indicated that increase of alkyl chain, unsaturated or branched chain, and aromatic functional group in the ester moiety attenuate the cytotoxicity against the same cancer cell lines. The amidation of C-4-COOH of **3** enhanced the cytotoxic effect against the two human cancer cell lines used in Table 2 while **12**, **13**, and **17** significantly enhanced the cytotoxicity against NTUB1 and PC3 cells. It clearly revealed that compounds possessed of 4-propyl, 4-(2-methylethyl), and 4-tetrahydropyrrolyl carbamoyl groups significantly enhanced the cytotoxicity against cancer cell lines used in Table 2.

As shown in Figure 2, compound **17** was selected to assay two additional cell lines, A549 and SV-HUC1. Compound **17** showed more potent effect against NTUB1 and PC3 cell lines than that of A549 cell line. It indicated that **17** displayed the specific cytotoxicity against cancer cells in the urinary system. In addition, compound **17** exhibited less cytotoxicity against human normal uroepithelial SV-HUC1 cells than those of NTUB1 and PC3 cell lines

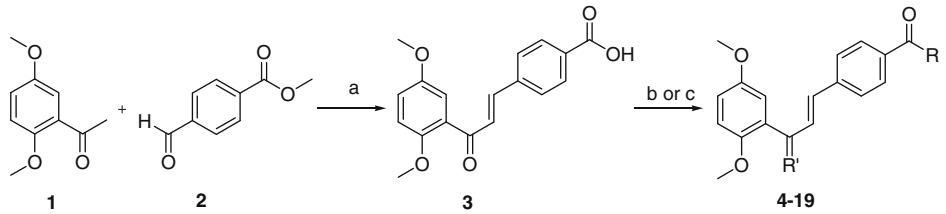


Scheme 2. Reagents: (a) 8% KOH in H_2O, MeOH

except for 0.3 μM **17** against NTUB1 and PC3 cells. It suggested that **17** was a good candidate for developing as cancer chemotherapeutic agents for treating cancer related to urinary system.

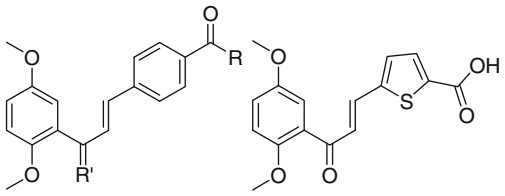
The effect of positive control, cisplatin, **13**, and **17** on cell cycle progression was determined by using fluorescence-activated cell sorting (FACS) analysis in propidium iodide-stained NTUB1 and PC3 cells. As shown in Figures 3 and 4, NTUB1 cells treated with 20 μM cisplatin for 24 h led to an accumulation of cells in G1 and S phases with the concomitant increase of the population of sub-G1 phase. NTUB1 cells were exposed to 1 μM **13** and **17**, respectively, for 24 h induced G1 phase arrest compared to the respective control value, accompanied by an increase in apoptotic cell death, respectively. PC3 cells treated with 20 μM cisplatin for 24 h led to an accumulation of cells in S phase with the concomitant increase of apoptotic cell death. Treatment of PC3 cells with 1 μM **13** and **17**, respectively, induced S and G1 phase arrests, respectively, compared to respective control value, accompanied by an increase of apoptotic cells while exposure of PC3 cells to 3 μM **13** and **17**, respectively, induced G1 and G2/M phase arrests, respectively, compared to the respective control value, accompanied by an increase of the apoptotic cells death (Figs. 5 and 6). It suggested that the increase of cell number of G2/M phase, accompanied by an increase of apoptotic cell death. It also suggested that **13** and **17** at lower dose revealed same cell cycle arrest in NTUB1 cells while their at higher dose indicated different cell cycle arrest in PC3 cells. In addition, cell morphological and immuno-fluorescence studies were also performed for demonstrating the vital role in the cell differentiation.

As shown in Figure 7, NUTB1 cells treated with different concentrations of **17** for 24 h showed more cell death and rounding up after treatment. Microtubules play an important role in the cell differentiation. If the function of microtubules is blocked by chemicals or compounds, such as paclitaxel, cell differentiation will arrest and be not progressed into the normal pattern of the cell cycle control. Paclitaxel was reported as a microtubule-targeted tubulin-polymerizing agent (MTPA) blocks the function of microtubules.[12] To determine whether **17**, like paclitaxel, induced the formation of stable microtubules bundles in cultured cells, the immuno-fluorescent localization of tubulin was assessed.[13] As shown in Figure 8, compound **17** induced microtubule bundle formation in NTUB1 cells and mimicked the effect of paclitaxel used as the positive control.[14] NTUB1 cells treated with 1 μM **17** showed more mitotic arrest cell and influence on the microtubule formation than those of 0.3 μM **17** while paclitaxel showed more potent stabilization of the tubulin assembly and inhibition on the



Scheme 1. Reagents: (a) 8% KOH in H_2O, MeOH; (b) various alkyl halides, K_2CO_3, acetone; (c) various amines, anhydrous HOBt, EDCI.

Table 1
Structures and yields of all synthesized compounds



3-19 **21**

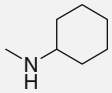
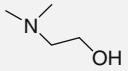
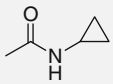
Compound	R	R′	Mp (°C)	Yield (%)	Molecular formula
3	–OH	O	214–215	63.0	$C_{18}H_{16}O_5$
4	$-OCH_3$	O	118–119	92.8	$C_{19}H_{18}O_5$
5	$-OCH_2CH_3$	O	118–119	45.9	$C_{20}H_{20}O_5 \cdot 1/2H_2O$
6	$-OCH_2CH_2CH_3$	O	Oil	40.8	$C_{21}H_{22}O_5$
7	$-OCH(CH_3)_2$	O	Oil	85.6	$C_{21}H_{22}O_5 \cdot 1/4H_2O$
8	$-OCH_2C_6H_5$	O	Oil	87.6	$C_{25}H_{22}O_5$
9	$-OCH_2CH{=}CH(CH_3)_2$	O	Oil	85.5	$C_{23}H_{24}O_5$
10	$-NHCH_3$	O	217–218	50.6	$C_{19}H_{19}NO_4$
11	$-NHCH_2CH_3$	O	213–214	47.8	$C_{20}H_{21}NO_4$
12	$-NHCH_2CH_2CH_3$	O	Oil	30.0	$C_{21}H_{23}NO_4 \cdot 1/2H_2O$
13	$-NHCH(CH_3)_2$	O	227–228	60.7	$C_{20}H_{23}NO_4$
14	$-N(CH_3)_2$	O	Oil	48.8	$C_{20}H_{21}NO_4$
15		O	280–281	66.7	$C_{24}H_{27}NO_4$
16	$-N(CH_2CH_3)_2$	O	Oil	98.2	$C_{22}H_{25}NO_4 \cdot 1/4H_2O$
17		O	Oil	68.4	$C_{22}H_{23}NO_4 \cdot 1/4H_2O$
18		O	Oil	79.1	$C_{21}H_{23}NO_5$
19			282–283	76.2	$C_{24}H_{26}N_2O_3 \cdot 1/4H_2O$
21			237–238	59.1	$C_{16}H_{14}O_5S$

Table 2
Cytotoxicities of chalcone derivatives against NTUB1 and PC3 cancer cell lines[a]

Compound	NTUB1 IC_{50} (μM) ± SD	PC3 IC_{50} (μM) ± SD
Cisplatin	3.27 ± 0.10	4.56 ± 0.76
3	5.70 ± 0.68	7.24 ± 0.58
4	3.51 ± 0.45	4.85 ± 0.11
5	4.49 ± 0.98	5.70 ± 0.43
6	6.01 ± 0.30	26.93 ± 0.00
7	4.42 ± 0.50	6.74 ± 1.34
8	13.26 ± 2.86	17.95 ± 0.50
9	13.41 ± 0.78	>50 μM
10	2.20 ± 0.37	2.13 ± 0.21
11	3.76 ± 0.40	4.18 ± 0.23
12	2.26 ± 0.31	1.50 ± 0.22
13	1.97 ± 0.39	1.58 ± 0.08
14	3.89 ± 0.21	4.58 ± 1.05
15	2.25 ± 0.57	6.30 ± 0.59
16	2.69 ± 0.47	3.79 ± 0.56
17	1.26 ± 0.25	0.53 ± 0.00
18	4.42 ± 0.15	5.75 ± 1.27
19	3.57 ± 0.47	4.26 ± 0.81
21	>50 μM	n.d

[a] Positive control: cisplatin; n.d, not determined. $n = 3–5$.

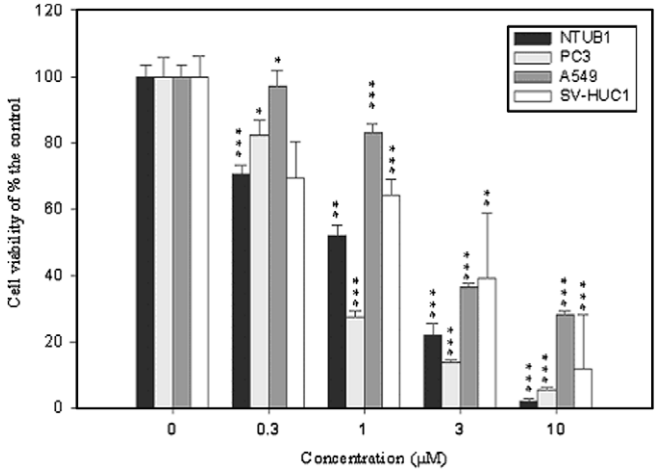


Figure 2. Cytotoxicities of **17** against NTUB1, PC3, A549, and SV-HUC1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of **17** for 72 h. The data shown represent mean ± SD ($n = 3$) for one experiment performed in triplicate. $^{*}P$ <0.05, $^{**}P$ <0.01, and $^{***}P$ <0.001 compared to the respective control values, respectively.

cell division. Following **17** binding to α-tubulin, it inhibited microtubule (MT) dynamic instability, cell cycle G2/M phase transition, mitotic arrest, and NTUB1 cell death through apoptosis (Fig. 4).

In the Western blot analysis of α-tubulin (Fig. 9), NTUB1 cells were treated with 500 nM paclitaxel and more high different concentrations of **17** for 6 h (short time treatment) and collected the supernatant and pellet for assays. Paclitaxel, used as the positive control, certainly stabilized the tubulin assembly. α-Tubulin in the supernatant showed a decreased level in a dose-dependent manner after treatment with various concentrations of **17** while in the pellet increased in a dose-dependent manner except for that

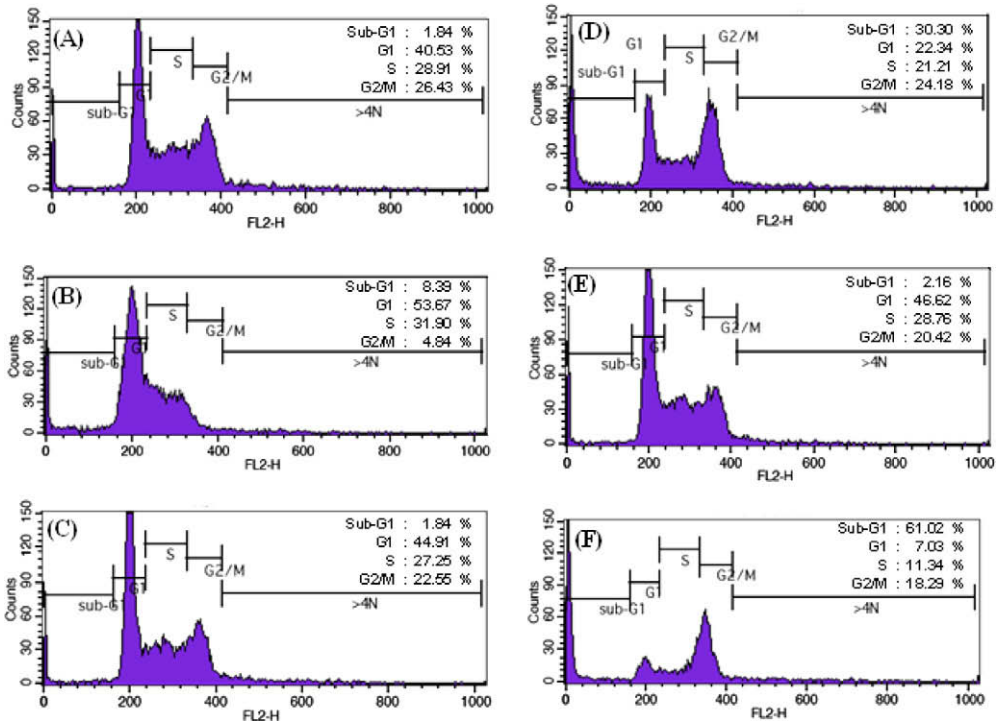


Figure 3. Flow cytometry analysis of the cell cycle with the treatment of **13** and **17**. NTUB1 cells (7×10^5 cells/10 cm dish) was treated with no compound (A), cisplatin 20 μM (B), 1 μM **13** (C), 3 μM **13** (D), 1 μM **17** (E), and 3 μM **17** (F) for 24 h. At the time indicated, cells were 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.

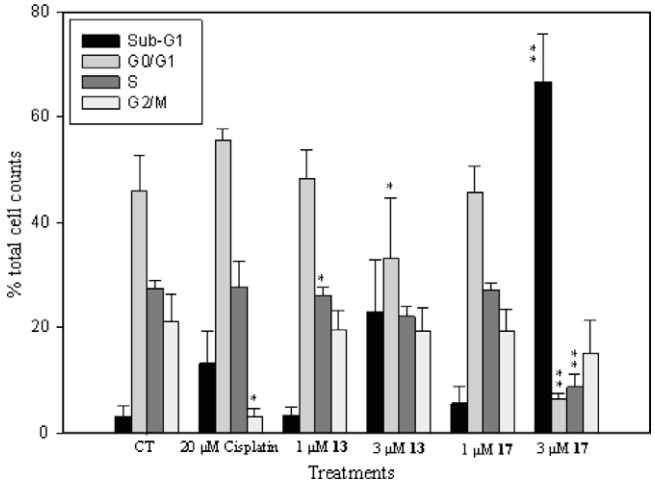


Figure 4. Cell cycle distribution of NTUB1 cells treated for 24 h with **13** and **17** in different concentrations. Cisplatin was used as reference drug. Percentages of sub-G1 cells and cells in G0/G1, S, and G2/M phase are shown. Data refer to a representative experiment one of three. CT (control), $^{*}P$ <0.05, $^{**}P$ <0.01 compared to the respective control values.

of 10 μM. It suggested that **17** could influence tubulin assembly in the molecular action similar to paclitaxel.

4. Conclusion

A series of 2′,5′-dimethoxylchalcones including 18 new compounds were synthesized and examined their biological activities. In the MTT assays, all derivatives except for **21** showed significantly cytotoxic effect against NTUB1 and PC3 cancer cell lines. Among all these derivatives, compound **17** with a carbamoyl group substituted at C-4 of B ring was found to have strongest cytotoxicity against NTUB1 and PC3 cancer cell lines and showed less cytotoxicity against SV-HUC1 and A549 cells. It indicated **17** specifically inhibited the growth of cancer cells in urinary system and less toxicity for normal cells. It also indicated that the carbamoyl group substituted at C-4 of the B ring of chalcone moiety may also important structural feature for binding to tubulin. It suggested that **17** may be used as MTPAs.

5. Experimental section

5.1. 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 mass were obtained on a JMX-HX 100 mass spectrometer. Elemental analyses measured with Vario EL III were within ±0.4% of the theoretical values for all elements listed. Chromatography was performed using a flash-column technique on Silica Gel 60 supplied by E. Merck.

5.2. 4-Carboxyl-2′,5′-dimethoxychalcone (3)

2,5-Dimethoxyacetophenone (**1**) (450.0 mg, 2.5 mmol) and methyl 4-formylbenzoate (**2**) (410.4 mg, 2.5 mmol) were dissolved in MeOH (50 mL), and added 8% KOH in H_2O (50 mL). The reaction mixture was stirred at room temperature for 24 h and neutralized with 10% HCl solution (100 mL) to form yellow precipitate. The yellow precipitate was filtered and washed with appropriate amount of water. The crude product was purified by chromatography using EtOAc/*n*-hexane (2:1), and crystallized by EtOAc/*n*-hexane (1:4) to afford **3** (491.6 mg, 63.0%) as a yellow solid, IR (KBr): 1681, 1597 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.82 (3H, s, OCH_3), 3.89 (3H, s,

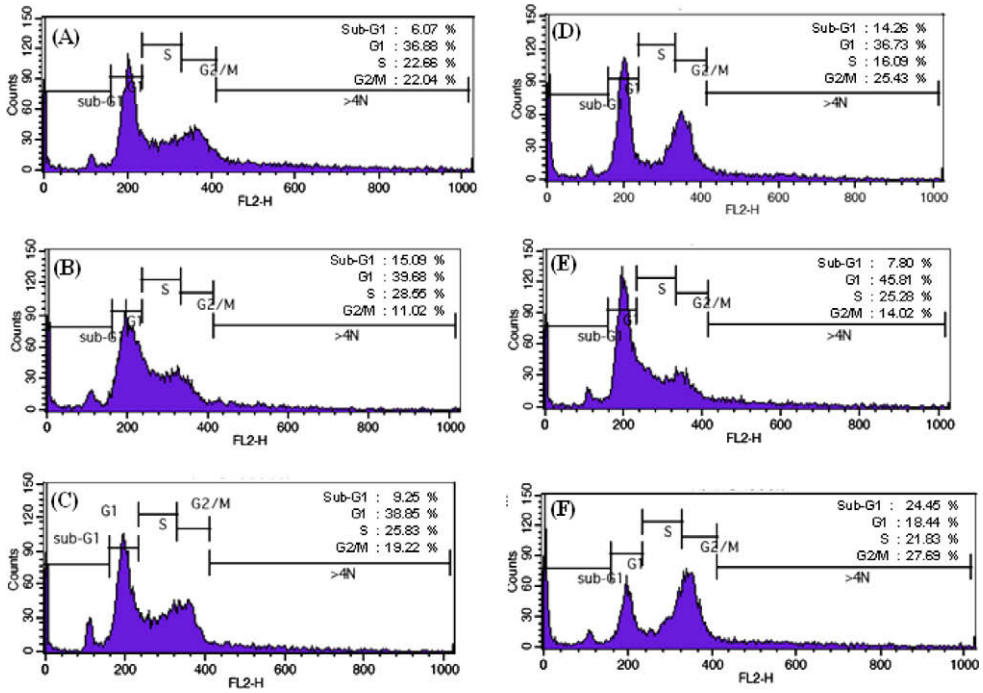


Figure 5. Flow cytometry analysis of the cell cycle with the treatment of **13** and **17**. PC3 cells (7×10^5 cells/10 cm dish) was treated with no compound (A), cisplatin 20 μM (B), 1 μM **13** (C), 3 μM **13** (D), 1 μM **17** (E), and 3 μM **17** (F) for 24 h. At the time indicated, cells were stained 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.

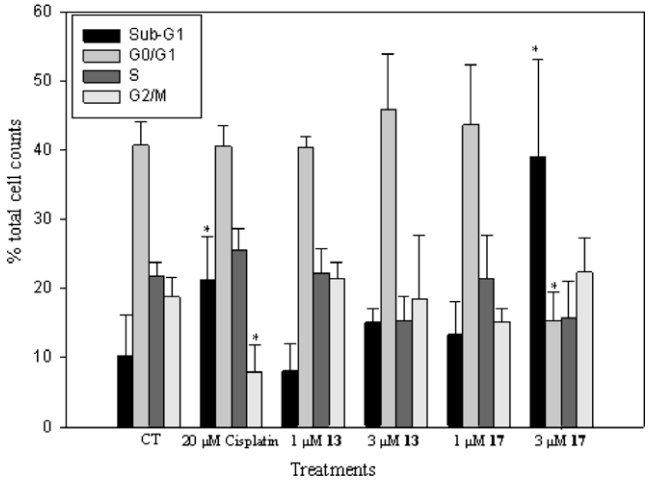


Figure 6. Cell cycle distribution of PC3 cells treated for 24 h with **13** and **17** in different concentrations. Cisplatin was used as reference drug. Percentages of sub-G1 cells and cells in G0/G1, S, and G2/M phase are shown. Data refer to a representative experiment one of three. CT (control), $^{*}P$ <0.05, $^{**}P$ <0.01 compared to the respective control values.

OCH_3), 6.96 (1H, d, J = 8.8 Hz, H-3′), 7.06 (1H, dd, J = 9.0, 3.2 Hz, H-4′), 7.23 (1H, d, J = 3.2 Hz, H-6′), 7.55 (1H, d, J = 16.0 Hz, H-α), 7.68 (1H, d, J = 15.6 Hz, H-β), 7.69 (2H, dd, J = 8.8, 1.6 Hz, H-2 and 6), 8.13 (2H, dd, J = 8.8, 1.6 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 55.9 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.8 (C-1′ and 4′), 119.8 (C-α), 128.3 (C-2 and 6), 129.3 (C-3 and 5), 130.2 (C-4), 140.4 (C-1), 141.0 (C-β), 152.8 (C-5′), 153.7 (C-2′), 171.0 (–COOH), 191.8 (C=O). ESIMS: m/z 313 $[M+H]^+$. Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.10; H, 5.18.

5.3. Procedure A for synthesis of C-4 ester compounds 4–9

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and alkyl halide (0.38 mmol). The reaction mixture was stirred at the room temperature for preparing **4**–**6** or refluxed for preparing **7**–**9** overnight. The mixture was concentrated to dryness under reduced pressure, neutralized with 10% HCl solution, and extracted with CH_2Cl_2. 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 with EtOAc/*n*-hexane, and crystallized with acetone/*n*-hexane (1:4) to afford purified products.

5.3.1. 4-Methoxycarbonyl-2′,5′-dimethoxychalcone (4)

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and methyl iodide (0.38 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to afford **4** (58.2 mg, 92.8%) as a yellow solid, IR (KBr): 1721, 1598 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.81 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.92 (3H, s, $COOCH_3$), 6.95 (1H, d, J = 8.8 Hz, H-3′), 7.05 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.21 (1H, d, J = 3.2 Hz, H-6′), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.64 (2H, d, J = 8.0 Hz, H-2 and 6), 7.65 (1H, d, J = 16.4 Hz, H-β), 8.05 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 52.2 ($COOCH_3$), 55.8 (OCH_3), 56.4 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.6 (C-1′ and 4′), 119.6 (C-α), 128.1 (C-2 and 6), 128.9 (C-3 and 5), 130.0 (C-4), 139.5 (C-1), 141.2 (C-β), 152.8 (C-5′), 153.6 (C-2′), 166.5 ($COOCH_3$), 191.8 (C=O). ESIMS: m/z 327 $[M+H]^+$. Anal. Calcd for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56. Found: C, 69.95; H, 5.62.

5.3.2. 4-Ethoxycarbonyl-2′,5′-dimethoxychalcone (5)

To a solution of **3** (100 mg, 0.32 mmol) in acetone was added K_2CO_3 (88.5 mg, 0.64 mmol) and ethyl iodide (0.64 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to

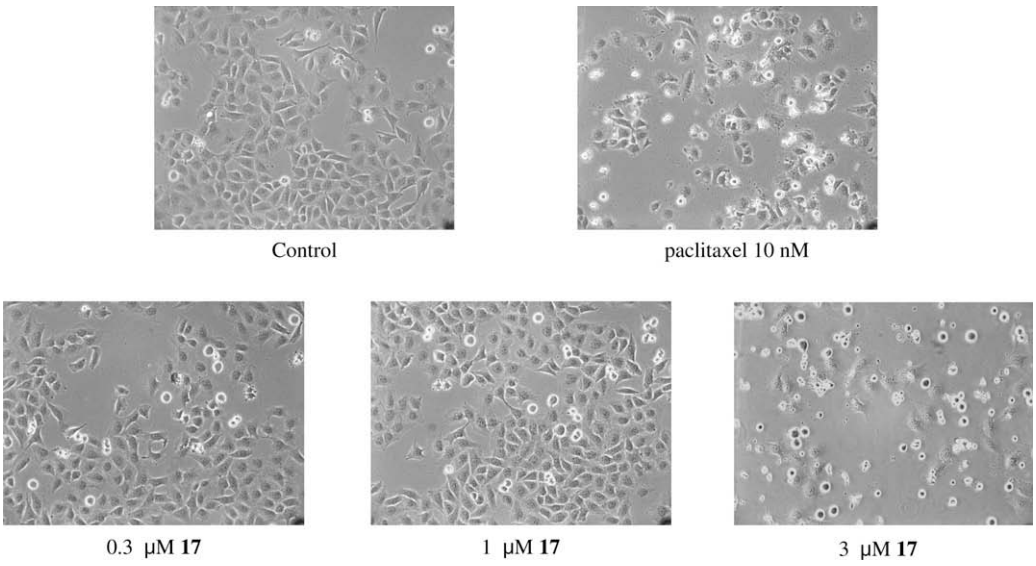


Figure 7. Cell morphologies of NTUB1 cells after treatment without compound (control), 10 nM paclitaxel, and different concentrations of **17** for 24 h. NTUB1 cells (7×10^5 cells/10 cm dish) were treated with 0.3, 1, and 3 μM of **17** for 24 h while treatment without compound was used as the control. As the concentration increased, most NTUB1 cells rounded up and died.

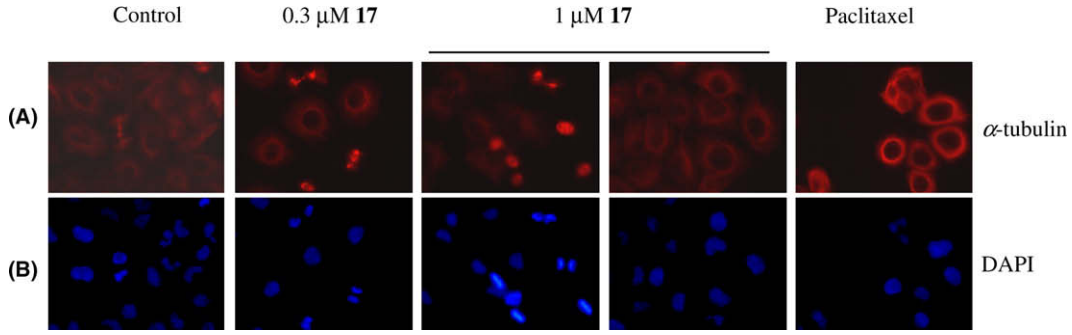


Figure 8. (A) Immuno-fluorescent microscopy of α-tubulin after treatment with only medium, 10 nM of paclitaxel, and different concentrations of compound **17**. NTUB1 cells were coated on cover slide with 100% serum in six-well plate (10^5 cells/well) before incubating overnight and treated with compounds for 24 h. Cells were fixed by 2% formaldehyde/PBS, washed with PBS, fixed with cold methanol, washed with PBS, added α-tubulin monoclonal antibody, washed with PBS, added several conjugated mouse antihuman antibody, washed with PBS, mounted on the slide with 80% glycerol/PBS, and microscoped. (B) Immuno-fluorescent microscopy of DNA after treatment with only medium, 10 nM of paclitaxel, and different concentrations of **17**. The protocol was performed as mentioned above while the antibody was replaced by DAPI stain.

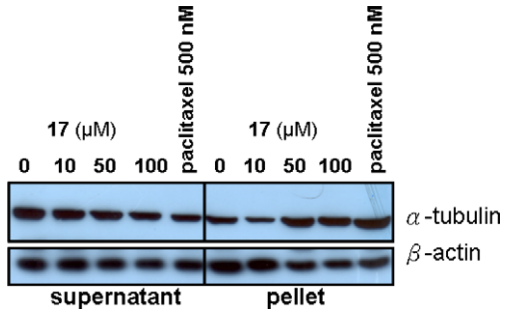


Figure 9. Western blotting of α-tubulin in NTUB1 cells. NTUB1 cells were treated with 500 nM paclitaxel or different concentrations of **17** for 6 h, collected, suspended with PBS, and assayed for α-tubulin assembly in the supernatant and pellet by western blotting. In this assay, β-actin was used as the internal control.

afford **5** (50.0 mg, 45.9%) as a yellow solid, IR (KBr): 1715, 1599 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.34 (3H, t, J = 7.2 Hz, CH_3), 3.81 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 4.38 (2H, q, J = 7.2 Hz, $COOCH_2$–), 6.95 (1H, d, J = 8.8 Hz, H-3′), 7.05 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.21 (1H, d, J = 3.2 Hz, H-6′), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.64 (2H, d, J = 8.4 Hz, H-2 and 6), 7.65 (1H, d, J = 16.4 Hz, H-β), 8.06 (2H, dd, J = 8.4, 1.6 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 14.3 (CH_3), 55.8 (OCH_3), 56.4 (OCH_3), 61.1 ($COOCH_2$–), 113.4 (C-6′), 114.4 (C-3′), 119.6 (C-1′ and 4′), 119.6 (C-α), 128.1 (C-2 and 6), 128.8 (C-3 and 5), 130.0 (C-4), 139.4 (C-1), 141.3 (C-β), 152.8 (C-5′), 153.6 (C-2′), 166.0 ($COOCH_2$–), 191.9 (C=O). ESIMS: m/z 341 $[M+H]^+$. Anal. Calcd for $C_{20}H_{20}O_5 \cdot 1/2H_2O$: C, 68.75; H, 6.06. Found: C, 68.71; H, 6.49.

5.3.3. 4-Propoxycarbonyl-2′,5′-dimethoxychalcone (6)

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and propyl iodide (0.38 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to afford **6** (27.8 mg, 40.8%) as a yellow oil, IR (KBr): 1715, 1602 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.04 (3H, t, J = 7.2 Hz, CH_3), 1.80 (2H, m, CH_2CH_3), 3.81 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 4.29 (2H, t, J = 6.4 Hz, $COOCH_2$–), 6.95 (1H, d, J = 9.2 Hz, H-3′), 7.05 (1H, dd, J = 9.2, 3.2 Hz, H-4′), 7.22 (1H, d, J = 3.2 Hz, H-6′), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.65 (2H, d, J = 8.4 Hz, H-2 and 6), 7.66 (1H, d, J = 16.4 Hz, H-β), 8.06 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 10.5 (CH_3), 22.1 (CH_2CH_3), 55.8 (OCH_3), 56.4 (OCH_3), 66.7 ($COOCH_2$–), 113.4 (C-6′), 114.4 (C-3′), 119.7 (C-1′ and 4′), 119.7 (C-α), 128.1 (C-2 and 6), 128.8 (C-3 and 5), 130.0 (C-4), 139.4 (C-1), 141.3 (C-β), 152.8 (C-5′), 153.7 (C-2′), 166.1

(*C*OOCH$_2$–), 191.9 (C=O). ESIMS: *m/z* 355 [M+H]$^+$. Anal. Calcd for $C_{21}H_{22}O_5$: C, 71.17; H, 6.26. Found: C, 71.15; H, 6.39.

5.3.4. 4-Isopropoxycarbonyl-2′,5′-dimethoxychalcone (7)

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and isopropyl iodide (0.38 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to afford **7** (58.2 mg, 85.6%) as a yellow oil, IR (KBr): 1711, 1603 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.37 (6H, d, J = 6.0 Hz, $CH_3 \times 2$), 3.81 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 5.26 (1H, m, COOC*H*$(CH_3)_2$), 6.95 (1H, d, J = 9.2 Hz, H-3′), 7.05 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.21 (1H, d, J = 3.2 Hz, H-6′), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.64 (2H, d, J = 8.8 Hz, H-2 and 6), 7.66 (1H, d, J = 16.0 Hz, H-β), 8.05 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 21.9 ($CH_3 \times 2$), 55.9 (OCH_3), 56.5 (OCH_3), 68.6 (COO*C*H$(CH_3)_2$), 113.4 (C-6′), 114.4 (C-3′), 119.7 (C-1′ and 4′), 119.7 (C-α), 128.1 (C-2 and 6), 128.8 (C-3 and 5), 130.0 (C-4), 139.3 (C-1), 141.4 (C-β), 152.8 (C-5′), 153.7 (C-2′), 166.1 (*C*OOCH$(CH_3)_2$), 191.9 (C=O). ESIMS: *m/z* 355 [M+H]$^+$. Anal. Calcd for $C_{21}H_{22}O_5 \cdot 1/4H_2O$: C, 70.28; H, 6.32. Found: C, 70.64; H, 6.26.

5.3.5. 4-Benzyloxycarbonyl-2′,5′-dimethoxychalcone (8)

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and benzyl bromide (0.38 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to afford **8** (67.7 mg, 87.6%) as a yellow oil, IR (KBr): 1717, 1601 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.81 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 5.38 (2H, s, $COOCH_2$–), 6.95 (1H, d, J = 8.8 Hz, H-3′), 7.05 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.22 (1H, d, J = 3.2 Hz, H-6′), 7.35 (5H, m, –C_6H_5), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.64 (2H, d, J = 8.4 Hz, H-2 and 6), 7.66 (1H, d, J = 16.0 Hz, H-β), 8.09 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 55.9 (OCH_3), 56.5 (OCH_3), 66.9 (COO*C*H$_2$–), 113.4 (C-6′), 114.4 (C-3′), 119.7 (C-1′ and 4′), 119.7 (C-α), 128.2–128.3 (tertiary aromatic carbon × 5), 128.6 (C-2 and 6), 129.0 (C-3 and 5), 130.2 (C-4), 135.9 (quaternary aromatic carbon × 1), 139.6 (C-1), 141.2 (C-β), 152.8 (C-5′), 153.7 (C-2′), 165.8 (*C*OOCH$_2$–), 191.8 (C=O). ESIMS: *m/z* 403 [M+H]$^+$. Anal. Calcd for $C_{25}H_{22}O_5$: C, 74.61; H, 5.51. Found: C, 74.63; H, 5.63.

5.3.6. 4-(3-Methyl-but-2-enyloxycarbonyl)-2′,5′-dimethoxychalcone (9)

To a solution of **3** (60 mg, 0.19 mmol) in acetone was added K_2CO_3 (52.5 mg, 0.38 mmol) and 1-bromo-3-methyl-2-butene (0.38 mmol). The mixture was treated as procedure A for synthesis of C-4 ester to afford **9** (62.5 mg, 85.5%) as a yellow oil, IR (KBr): 1714, 1600 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.78 (3H, s, CH_3), 1.79 (3H, s, CH_3), 3.81 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 4.83 (2H, d, J = 7.2 Hz, $COOCH_2$–), 5.47 (1H, m, C*H*=C$(CH_3)_2$), 6.95 (1H, d, J = 8.8 Hz, H-3′), 7.05 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.21 (1H, d, J = 3.2 Hz, H-6′), 7.51 (1H, d, J = 15.6 Hz, H-α), 7.63 (2H, d, J = 8.0 Hz, H-2 and 6), 7.65 (1H, d, J = 16.0 Hz, H-β), 8.06 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 18.1 (CH_3), 25.8 (CH_3), 55.9 (OCH_3), 56.5 (OCH_3), 62.1 (COO*C*H$_2$–), 113.4 (C-6′), 114.4 (C-3′), 118.5 (*C*H=C$(CH_3)_2$), 119.7 (C-1′ and 4′), 119.7 (C-α), 128.1 (C-2 and 6), 128.8 (C-3 and 5), 130.1 (C-4), 137.1 (CH=*C*$(CH_3)_2$), 139.4 (C-1), 141.4 (C-β), 152.8 (C-5′), 153.7 (C-2′), 166.1 (*C*OOCH$_2$–), 191.9 (C=O). ESIMS: *m/z* 381 [M+H]$^+$. Anal. Calcd for $C_{23}H_{24}O_5$: C, 72.61; H, 6.36. Found: C, 72.60; H, 6.48.

5.4. Procedure B for synthesis of C-4 amide compounds 10–19

A mixture of **3** (156 mg, 0.5 mmol), HOBt (135.1 mg, 1.0 mmol), EDCI (191.7 mg, 1.0 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added methylamine (1.0 mmol), and stirred at the room temperature for 2 h. The reaction mixture was concentrated in vacuo to give the crude product. The crude product was purified by chromatography with EtOAc/*n*-hexane, and crystallized with EtOAc to afford purified products.

5.4.1. 4-Methylcarbamoyl-2′,5′-dimethoxychalcone (10)

A mixture of **3** (156 mg, 0.5 mmol), HOBt (135.1 mg, 1.0 mmol), EDC (191.7 mg, 1.0 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added methylamine (1.0 mmol) and treated as procedure B to afford **10** (82.3 mg, 50.6%) as a yellow solid, IR (KBr): 3326, 1652, 1546 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.10 (3H, d, J = 4.8 Hz, $NHCH_3$), 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.39 (1H, br s, CONH–), 6.94 (1H, d, J = 9.2 Hz, H-3′), 7.04 (1H, dd, J = 9.2, 3.2 Hz, H-4′), 7.19 (1H, d, J = 3.2 Hz, H-6′), 7.47 (1H, d, J = 16.0 Hz, H-α), 7.61 (2H, d, J = 8.4 Hz, H-2 and 6), 7.62 (1H, d, J = 16.0 Hz, H-β), 7.78 (2H, d, J = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 26.9 ($NHCH_3$), 55.8 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.5 (C-1′ and 4′), 119.5 (C-α), 127.4 (C-2 and 6), 128.4 (C-3 and 5), 135.7 (C-4), 138.0 (C-1), 147.5 (C-β), 152.7 (C-5′), 153.6 (C-2′), 167.5 (CONH–), 192.0 (C=O). ESIMS: *m/z* 326 [M+H]$^+$. Anal. Calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 69.23; H, 5.95; N, 4.21.

5.4.2. 4-Ethylcarbamoyl-2′,5′-dimethoxychalcone (11)

A mixture of **3** (156 mg, 0.5 mmol), HOBt (135.1 mg, 1.0 mmol), EDC (191.7 mg, 1.0 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added ethylamine (1.0 mmol) and treated as procedure B to afford **11** (81.0 mg, 47.8%) as a yellow solid, IR (KBr): 3326, 1641, 1544 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, t, J = 7.2 Hz, CH_3), 3.50 (2H, m, NHC*H*$_2$–), 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.26 (1H, br s, CONH–), 6.94 (1H, d, J = 8.8 Hz, H-3′), 7.04 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.20 (1H, d, J = 3.2 Hz, H-6′), 7.47 (1H, d, J = 16.0 Hz, H-α), 7.62 (2H, d, J = 8.4 Hz, H-2 and 6), 7.63 (1H, d, J = 16.0 Hz, H-β), 7.78 (2H, d, J = 8.0 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 14.8 (CH_3), 35.0 ($NHCH_2$–), 55.8 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.5 (C-1′ and 4′), 119.5 (C-α), 127.4 (C-2 and 6), 128.4 (C-3 and 5), 135.9 (C-4), 138.0 (C-1), 141.5 (C-β), 152.7 (C-5′), 153.6 (C-2′), 166.7 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 339 (100). Anal. Calcd for $C_{20}H_{21}NO_4$: C, 70.48; H, 6.24; N, 4.13. Found: C, 69.87; H, 6.28; N, 4.07.

5.4.3. 4-Propylcarbamoyl-2′,5′-dimethoxychalcone (12)

A mixture of **3** (156 mg, 0.5 mmol), HOBt (135.1 mg, 1.0 mmol), EDC (191.7 mg, 1.0 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added propylamine (1.0 mmol) and treated as procedure B to afford **12** (52.9 mg, 30.0%) as a yellow oil, IR (KBr): 3329, 1641, 1544 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.99 (3H, t, J = 7.6 Hz, CH_3), 1.65 (2H, m, C*H*$_2$CH$_3$), 3.43 (2H, m, NHC*H*$_2$–), 3.81 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 6.22 (1H, br s, CONH–), 6.95 (1H, d, J = 8.8 Hz, H-3′), 7.02 (1H, dd, J = 8.8, 3.2 Hz, H-4′), 7.20 (1H, d, J = 3.2 Hz, H-6′), 7.48 (1H, d, J = 16.0 Hz, H-α), 7.63 (2H, d, J = 8.8 Hz, H-2 and 6), 7.64 (1H, d, J = 16.0 Hz, H-β), 7.78 (2H, d, J = 8.8 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3), 22.9 (CH_2CH_3), 41.8 (NCH_2–), 55.9 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.6 (C-1′ and 4′), 119.6 (C-α), 127.4 (C-2 and 6), 128.4 (C-3 and C-5), 136.0 (C-4), 138.0 (C-1), 141.5 (C-β), 152.7 (C-5′), 153.7 (C-2′), 166.8 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 353 (100). Anal. Calcd for $C_{21}H_{23}NO_4 \cdot 1/2H_2O$: C, 69.58; H, 6.68; N, 3.87. Found: C, 70.60; H, 6.68; N, 3.85.

5.4.4. 4-(2-Methylethyl)carbamoyl-2′,5′-dimethoxychalcone (13)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added isopropylamine (1.0 mmol) and treated as procedure B to afford **13** (68.7 mg, 60.7%) as a yellow solid, IR (KBr): 3320, 1638, 1538 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.26 (6H, d, J = 6.4 Hz, –CH(C*H*$_3$)$_2$),

4.29 (1H, m, NHC*H*(CH_3)$_2$), 6.02 (1H, d, *J* = 7.6 Hz, CONH–), 6.94 (1H, d, *J* = 8.8 Hz, H-3′), 7.04 (1H, dd, *J* = 9.0, 3.2 Hz, H-4′), 7.20 (1H, d, *J* = 3.2 Hz, H-6′), 7.47 (1H, d, *J* = 16.0 Hz, H-*α*), 7.62 (2H, d, *J* = 8.0 Hz, H-2 and 6), 7.63 (1H, d, *J* = 16.8 Hz, H-*β*), 7.77 (2H, d, *J* = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): *δ* 22.8 (CH_3), 29.7 (CH_3), 42.0 (NH*C*H(CH_3)$_2$), 55.8 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.5 (C-1′ and 4′), 119.5 (C-*α*), 127.4 (C-2 and 6), 128.4 (C-3 and 5), 136.1 (C-4), 138.0 (C-1), 141.5 (C-*β*), 152.7 (C-5′), 153.6 (C-2′), 165.9 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 353 (100). Anal. Calcd for $C_{20}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96. Found: C, 71.57; H, 6.68; N, 3.86.

5.4.5. 4-Dimethylcarbamoyl-2′,5′-dimethoxychalcone (14)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added dimethylamine (1.0 mmol) and treated as procedure B to afford **14** (53.0 mg, 48.8%) as a yellow oil, IR (KBr): 3485, 1633 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 3.09 (6H, m, –N(CH_3)$_2$), 3.81 (3H, s, –OCH_3), 3.86 (3H, s, –OCH_3), 6.94 (1H, d, *J* = 9.2 Hz, H-3′), 7.03 (1H, dd, *J* = 9.2, 3.2 Hz, H-4′), 7.19 (1H, d, *J* = 3.2 Hz, H-6′), 7.44 (2H, d, *J* = 8.4 Hz, H-2 and 6), 7.45 (1H, d, *J* = 15.6 Hz, H-*α*), 7.51 (2H, d, *J* = 8.0 Hz, H-3 and 5), 7.63 (1H, d, *J* = 16.0 Hz, H-*β*). ^{13}C NMR ($CDCl_3$): *δ* 35.3 and 39.5 (N(CH_3)$_2$), 55.8 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.5 (C-1′ and 4′), 119.5 (C-*α*), 127.6 (C-2 and 6), 128.3 (C-3 and 5), 136.3 (C-4), 137.7 (C-1), 141.8 (C-*β*), 152.7 (C-5′), 153.6 (C-2′), 170.9 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 339 (100). Anal. Calcd for $C_{20}H_{21}NO_4$: C, 70.48; H, 6.24; N, 4.13. Found: C, 70.10; H, 6.45; N, 4.16.

5.4.6. 4-Cyclohexylcarbamoyl-2′,5′-dimethoxychalcone (15)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added cyclohexylamine (1.0 mmol) and treated as procedure B to afford **15** (84.0 mg, 66.7%) as a yellow solid, IR (KBr): 3312, 1637, 1540 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 1.24 (2H, m, –CH_2–), 1.44 (2H, m, –CH_2–), 1.66 (2H, m, –CH_2–), 1.76 (2H, m, –CH_2–), 2.04 (2H, m, –CH_2–), 3.81 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.98 (1H, m, —NHC*H*<), 6.00 (1H, d, *J* = 7.6 Hz, CONH–), 6.95 (1H, d, *J* = 8.8 Hz, H-3′), 7.05 (1H, dd, *J* = 8.8, 3.2 Hz, H-4′), 7.21 (1H, d, *J* = 3.2 Hz, H-6′), 7.48 (1H, d, *J* = 16.0 Hz, H-*α*), 7.63 (2H, d, *J* = 7.6 Hz, H-2 and 6), 7.64 (1H, d, *J* = 16.4 Hz, H-*β*), 7.77 (2H, d, *J* = 8.4 Hz, H-3 and 5). ^{13}C NMR ($CDCl_3$): *δ* 24.9 (–CH_2– × 2), 25.5 (–CH_2– × 1), 33.2 (–CH_2– × 2), 48.8 (—NHCH<), 55.9 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.4 (C-3′), 119.6 (C-1′ and 4′), 119.6 (C-*α*), 127.4 (C-2 and 6), 128.4 (C-3 and 5), 136.2 (C-4), 138.0 (C-1), 141.5 (C-*β*), 152.7 (C-5′), 153.7 (C-2′), 165.8 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 393 (100). Anal. Calcd for $C_{24}H_{27}NO_4$: C, 73.26; H, 6.92; N, 3.56. Found: C, 73.31; H, 7.03; N, 3.55.

5.4.7. 4-Diethylcarbamoyl-2′,5′-dimethoxychalcone (16)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added diethylamine (1.0 mmol) and treated as procedure B to afford **16** (115.5 mg, 98.2%) as a yellow oil, IR (KBr): 3568, 1627 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 1.12 (3H, m, CH_3), 1.24 (3H, m, CH_3), 3.25 (2H, m, NCH_2–), 3.54 (2H, m, NCH_2–), 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.94 (1H, d, *J* = 8.8 Hz, H-3′), 7.03 (1H, dd, *J* = 9.2, 3.2 Hz, H-4′), 7.19 (1H, d, *J* = 2.8 Hz, H-6′), 7.39 (2H, d, *J* = 8.4 Hz, H-2 and 6), 7.44 (1H, d, *J* = 15.6 Hz, H-*α*), 7.60 (2H, d, *J* = 8.0 Hz, H-3 and H-5), 7.63 (1H, d, *J* = 15.6 Hz, H-*β*). ^{13}C NMR ($CDCl_3$): *δ* 12.9 (CH_3), 14.2 (CH_3), 39.3 (NCH_2–), 43.2 (NCH_2–), 55.8 (OCH_3), 56.4 (OCH_3), 113.3 (C-6′), 114.4 (C-3′), 119.4 (C-1′ and 4′), 119.4 (C-*α*), 126.9 (C-2 and 6), 128.4 (C-3 and 5), 135.9 (C-4), 138.7 (C-1), 141.9 (C-*β*), 152.6 (C-5′), 153.6 (C-2′), 170.5 (–CONH–), 192.1 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 367 (100). Anal. Calcd for $C_{22}H_{25}NO_4 \cdot 1/4H_2O$: C, 71.04; H, 6.91; N, 3.77. Found: C, 71.23; H, 6.93; N, 3.65.

5.4.8. 4-Tetrahydropyrrolylcarbamoyl-2′,5′-dimethoxychalcone (17)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added pyrrolidine (1.0 mmol) and treated as procedure B to afford **17** (80.0 mg, 68.4%) as a yellow oil, IR (KBr): 3466, 1619 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 1.89 (4H, m, –CH_2CH_2–), 3.42 (2H, m, NCH_2–), 3.60 (2H, m, NCH_2–), 3.79 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.94 (1H, d, *J* = 8.8 Hz, H-3′), 7.02 (1H, dd, *J* = 8.0, 3.2 Hz, H-4′), 7.18 (1H, d, *J* = 3.2 Hz, H-6′), 7.44 (1H, d, *J* = 16.0 Hz, H-*α*), 7.52 (2H, d, *J* = 8.0 Hz, H-2 and 6), 7.59 (2H, d, *J* = 8.4 Hz, H-3 and 5), 7.62 (1H, d, *J* = 15.6 Hz, H-*β*). ^{13}C NMR ($CDCl_3$): *δ* 24.3 (–CH_2CH_2–), 26.3 (–CH_2CH_2–), 46.2 (NCH_2–), 49.5 (NCH_2–), 55.8 (OCH_3), 56.4 (OCH_3), 113.3 (C-6′), 114.3 (C-3′), 119.4 (C-1′ and 4′), 119.4 (C-*α*), 127.6 (C-2 and 6), 128.1 (C-3 and 5), 136.5 (C-4), 138.5 (C-1), 141.8 (C-*β*), 152.6 (C-5′), 153.5 (C-2′), 168.9 (CONH–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 365 (97), 295 (100). Anal. Calcd for $C_{22}H_{23}NO_4 \cdot 1/4H_2O$: C, 71.43; H, 6.40; N, 3.79. Found: C, 71.67; H, 6.71; N, 3.63.

5.4.9. 4-(2-Hydroxyethyl)methylcarbamoyl-2′,5′-dimethoxychalcone (18)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added *N*-methylethanolamine (1.0 mmol) and treated as procedure B to afford **18** (93.6 mg, 79.1%) as a yellow oil, IR (KBr): 3403, 1610 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 2.45 (OH), 3.05 (3H, s, NCH_3), 3.10 (1H, m, NC*H*H–), 3.42 (1H, m, NCH*H*–), 3.71 (2H, m, C*H*$_2$OH), 3.80 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.93 (1H, d, *J* = 9.2 Hz, H-3′), 7.03 (1H, dd, *J* = 8.8, 3.2 Hz, H-4′), 7.19 (1H, d, *J* = 3.2 Hz, H-6′), 7.45 (1H, d, *J* = 16.0 Hz, H-*α*), 7.46 (2H, d, *J* = 8.0 Hz, H-2 and 6), 7.61 (2H, d, *J* = 9.2 Hz, H-3 and 5), 7.62 (1H, d, *J* = 15.6 Hz, H-*β*). ^{13}C NMR ($CDCl_3$): *δ* 38.6 (NCH_3), 51.0 (NCH_2–), 55.8 (OCH_3), 56.4 (OCH_3), 60.6 (–CH_2OH), 113.3 (C-6′), 114.4 (C-3′), 119.5 (C-1′ and 4′), 119.5 (C-*α*), 127.8 (C-2 and 6), 128.3 (C-3 and 5), 136.7 (C-4), 137.0 (C-1), 141.6 (C-*β*), 152.7 (C-5′), 153.6 (C-2′), 172.5 (–CON–), 192.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 369 (31), 295 (100). Anal. Calcd for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.79. Found: C, 66.78; H, 6.52; N, 3.45.

5.4.10. *N*-Cyclopropyl-4-[3-cyclopropylimino-3-(2′,5′-dimethoxy-phenyl)-propenyl]-benzamide (19)

A mixture of **3** (100 mg, 0.32 mmol), HOBt (86.5 mg, 0.64 mmol), EDC (122.7 mg, 0.64 mmol) was dissolved with CH_2Cl_2, and stirred for 10 min. The mixture was added cyclopropylamine (1.0 mmol) and treated as procedure B to afford **19** (95.3 mg, 76.2%) as a yellow solid, IR (KBr): 3286, 1640, 1536 cm^{-1}. ^{1}H NMR ($CDCl_3$): *δ* 0.60 (2H, m, –C*H*$_2$CH$_2$–), 0.84 (4H, m, –C*H*$_2$C*H*$_2$–), 0.96 (2H, m, –CH_2C*H*$_2$–), 2.67 (1H, m, NHC*H*–), 2.87 (1H, m, =NHC*H*–), 6.28 (1H, br s, CONH–), 6.42 (1H, d, *J* = 16.0 Hz, H-*α*), 6.70 (1H, d, *J* = 2.4 Hz, H-6′), 6.95 (2H, m, H-3′ and 4′), 7.12 (1H, d, *J* = 16.4 Hz, H-*β*), 7.42 (2H, d, *J* = 8.4 Hz, H-2 and 6), 7.66 (2H, d, *J* = 8.4 Hz, H-3 and H-5).^{13}C NMR ($CDCl_3$): *δ* 6.8 (–(–CH_2– × 2), 9.9 (–CH_2–), 10.0 (–CH_2–), 23.1 (–NHCH–), 36.2 (=NCH–), 55.8 (OCH_3), 56.4 (OCH_3), 112.6 (C-6′), 114.5 (C-3′), 115.0 (C-1′ and 4′), 125.7 (C-*α*), 127.1 (C-2), 133.5 (C-5 and C-6), 133.7 (C-3), 135.6 (C-1), 139.6 (C-*β*), 150.5 (C-5′), 153.6 (C-2′), 164.6 (C=N–), 168.3 (CONH–). EIMS (70 eV) *m/z* (% rel. int.):

390 (31). Anal. Calcd for $C_{24}H_{26}N_2O_3 \cdot 1/4H_2O$: C, 72.98; H, 6.76; N, 7.09. Found: C, 73.01; H, 6.69; N, 7.10.

5.5. 3-(3-Thiophene)carboxyl-1-(2,5-dimethoxyphenyl)prop-2-en-1-one (21)

2,5-Dimethoxyacetophenone (450.0 mg, 2.5 mmol) and 5-formyl-2-thiophenecarboxylic acid (**20**) (390.4 mg, 2.5 mmol) were dissolved in MeOH (50 mL), and added 8% KOH in H_2O (50 mL). The reaction mixture was stirred at room temperature for 72 h and neutralized with 10% HCl solution (100 mL) to form yellow precipitate. The yellow precipitate was filtered and washed with appropriate amount of water. The crude product was purified by chromatography using EtOAc/*n*-hexane (2:1), and crystallized with EtOAc to afford **21** (469.6 mg, 59.1%) as a yellow solid. ^{1}H NMR (CD_3OD): δ 3.80 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 7.12 (1H, m, H-3′), 7.14 (1H, m, H-4′), 7.15 (1H, m, H-6′), 7.40 (1H, d, J = 15.6 Hz, H-α), 7.42 (1H, d, J = 3.2 Hz, H-5), 7.70 (1H, d, J = 15.6 Hz, H-β), 7.71 (1H, d, J = 3.2 Hz, H-4). ^{13}C NMR (CD_3OD): δ 56.2 (OCH_3), 56.9 (OCH_3), 114.8 (C-6′), 115.5 (C-3′), 120.7 (C-4′), 129.1 (C-α), 132.8 (C-5), 135.1 (C-4), 135.8 (C-β), 147.5 (C-1), 154.3 (C-5′), 155.2 (C-2′), 193.2 (C=O). ESIMS: m/z 319 $[M+H]^+$. Anal. Calcd for $C_{16}H_{14}O_5S$: C, 60.37; H, 4.43; S, 10.07. Found: C, 60.09; H, 4.42; S, 10.27.

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

NTUB1, an immortalized human urothelial carcinoma cell line, was established from a high-grade bladder cancer.[15] PC3 (a human prostate cancer cell line), A549 (a human lung adenocarcinoma epithelial 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, PC3, and A549 cells) or F12 medium (for SV-HUC1) 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 tested compounds and cisplatin, a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay was performed.[16,17] 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 tested compounds at 37 °C for 72 h. At the end of the culture period, 50 μL of MTT (2 μg 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} value of each group were calculated by the median-effect analysis and presented as mean ± standard deviation (SD).

5.7. Cell cycle analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described.[17,18] Briefly, 6×10^5 cells were plated and treated with 20 μM cisplatin (Pharmacia & Upjohn, Milan, Italy) and various concentrations of **13** and **17** 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 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).

5.8. Immuno-fluorescence staining

NTUB1 cells were seeded onto serum-coated coverslides. The cells were treated as indicated conditions and processed as described previously.[17] Briefly, The cells were fixed with 2% formaldehyde/PBS for 20 min followed by cold methanol permeabilization for 3 min. The cells were washed with 1× PBS five minutes for two times. The cells were then incubated with α-tubulin (Oncogene, CP06) primary antibody overnight. The cells were washed with 1× PBS five minutes for three times. Goat-anti-rabbit-rhodamine secondary antibody and 1 μg/mL 4′-6-diamidino-2-phenylindole (DAPI) were incubated for 1 h at room temperature. The slides were washed with 1× PBS, mounted with mounting solution (80% glycerol/PBS) and photographed by microscopy.

5.9. Western blot analysis for α-tubulin

NTUB1 cells were seeded at a density of 1×10^6/100-mm^2 dishes and treated with indicated concentrations of test agents for 6 h. The cells were then washed with cold PBS and lysed by adding lysis buffer (20 mM Tris–HCl (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, 20 g/mL aprotinin, 20 g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 0.5% Nonidet P-40). The cell lysates were centrifuged at 15,000*g* for 10 min at 4 °C. Soluble and assembled tubulin could be separated in supernatant and pellet fraction, respectively.[19] The supernatants and pellets were dissolved in an SDS–PAGE sampling loading buffer and heated at 95 °C for 10 min; the resulting material was subjected to electrophoresis on 10% SDS–polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% skim milk/TBST overnight at 4 °C. The relative amounts of tubulins were detected by anti-tubulin monoclonal antibody (Oncogene) and horseradish peroxidase-conjugated secondary antibody (Jackson). β-Actin (AC-15, Novus Biologicals) was used for control. Detection of immunoreactive signal was accomplished with Western blot chemiluminescent reagent (Millipore).

5.10. Statistical analysis

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

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