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The convenient aqueous synthesis and biological evaluation of ortho-(3,4,5-trimethoxybenzoyl)-acetanilides as novel anti-cancer agents

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A series of new ortho-(3,4,5-trimethoxybenzoyl)-acetanilides were synthesised by the cross-coupling reaction catalyzed with Pd catalyst in aqueous, with polyethylene glycol as additive at very mild condition. The evaluation of these compounds as tubulin polymerization inhibitors indicated that most of these compounds were potential anti-cancer agents. Among them, compound **13**, 2-hydroxy-N-(5-methoxy-2-(3,4,5-trimethoxy benzoyl)phenyl) acetamide exhibited excellent antiproliferative activity against various human cancer cell lines (GI_{50}= 71nM for human HeLa cell line), and good activity for inhibiting tubulin polymerization (IC_{50} = 2.94 μM). The mechanism study indicated that compound 13 could arrest cell-cycle progression at the mitosis phase, block the formation of cdc2/cyclin B1 complex, down-regulated the p-Cdc 25C and the anti-apoptotic protein Bcl-2 and Bcl-XL, up-regulated the pro-apoptotic protein Bax and Bad.

Introduction

Microtubules are cytoskeletal filaments consisting of α, β-tubulin heterodimers and are essential in a wide range of cellular processes including cell shape maintenance, intracellular transport, cell signaling, separation of duplicated chromosomes during cell division, which are required for cell life cycle.[1-3] The crucial involvement of microtubules in mitosis makes them an attractive target for anticancer drugs.[4, 5]

There are many chemically diverse compounds targeting the tubulin-microtubule system.[6, 7] Among them, combretastatin A-4 (CA-4, 1), a natural *cis*-stilbene derivatives isolated from the bark of African willow tree Combretum caffrum in 1982,[8] is a typical example which strongly inhibits tubulin polymerization (with about 1.9 μM of the IC_{50}) by binding to the colchicine binding site, and then, the disruption of tubulin assembly results in mitotic arrest, and subsequent cancer cell death.[9] However, the *cis* double bond in CA-4 is prone to isomerize to the more thermodynamic stable, but less bioactive trans isomers (with the IC_{50} above 50μ M against tubulin polymerization).[10] For overcoming this disadvantage, numerous modifications to stabilize the conformation by replacement of the olefinic bridge with a ring or carbonyl have been developed in recent years.[11-13] Generally, several reaction steps were needed for the synthesis of these compounds, therefore, from both academically and practical standpoints, it would be very interested to develop a highly effective strategy for the preparation of the new effective antimitotic agents by simple, especially one-step procedure.

In view of direct synthesis being able to minimize waste/side product formation, transition-metal-catalyzed direct C-H bond functionalization/cross-coupling as a valuable tool for the modular and facile synthesis of structurally similar, yet diversified organic molecules has attracted increasing attention. A lot of papers have been published on transition metals (e.g., Pd, Ir, Rh, Ru, Cu, Fe, etc.) facilitating the cross-coupling reaction in recent years.[14] Recently, we and other groups reported the palladium-catalyzed cross-dehydrogenative coupling between anilides and aromatic aldehydes in toluene.[15-17] Subsequently, Novák described that the reaction could be performed mildly in aqueous with the addition of detergent, accompanying the reaction rate acceleration.[18, 19]

Although a lot of progress have been made in transition-metal-catalyzed carbon-carbon coupling reactions, there were very limited reports on the application of this methodology to the preparation of complex molecules and useful medical compounds. Herein, we disclose the synthesis of a series of new ortho-(3,4,5-trimethoxybenzoyl)-acetanilides catalyzed with Pd catalyst in aqueous with polyethyleneglycol as additive, especially the evaluation of them as tubulin polymerization inhibitors, and the study of their anticancer mechanism of these compounds.

The design strategy of these target compounds were showed in Figure 1. As 3,4,5-trimethoxy groups on the A-ring and the *cis*-orientation of the two aryl rings on olefinic bond were responsible for efficient binding to tubulin,[20, 21] we focused on the modification of B-ring and the bridging double bond, using the simple coupling reaction of commercially available 3,4,5-trimethoxybenzaldehyde and different anilides to obtaine these bioactive ortho-(3,4,5-trimethoxybenzoyl)-acetanilides.

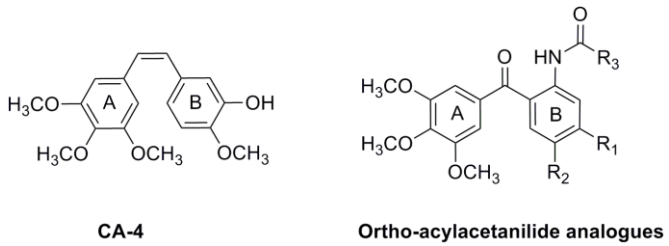


Figure. 1 structures of CA-4 and the Ortho-acylacetanilide analogues

Results and discussion

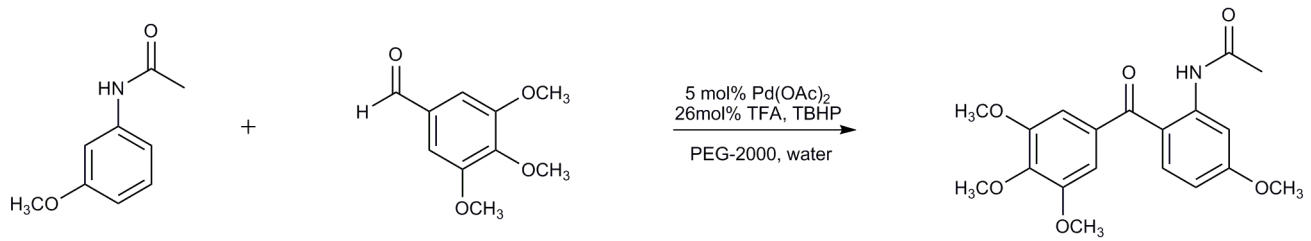
Chemistry

The study of one-step synthesis of the new ortho-acylacetanilides **1-12** is shown in Table 1 and Schemes 1. Inspired by Novák's method with the addition of detergent to accelerate the coupling reaction, and considering polyethylene glycol (PEG), which is soluble in both water and organic solvents, has been successfully used as reaction medium and additive in a variety of organic reactions,[22-27] we studied the reaction with 3-methoxyacetanilide and 3,4,5-trimethoxybenzaldehyde as the prototypical substrates, 5% $Pd(OAc)_2$ as the catalyst and polyethylene glycol as the additive. A screening of optimal conditions indicated that the use of PEG was rather significant (Table 1, entries 1-4: 5% mol of PEG-2000, 63% yield; 10% mol of PEG, 71% yield; 20% mol of PEG, 91% yield; 50% mol of PEG, trace product). The temperature effect was also important for the reaction; it could proceed smoothly at 40°C or 60°C, but only gave 38% yield at room temperature (Table 1, entries 3, 6-7). We also attempted to reduce the the amount of $Pd(OAc)_2$, however, low yields were obtained (Table 1, entry 8-9).

With this optimal reaction conditions, compounds **2-12** were prepared by the reaction of substituted anilides and 3,4,5-trimethoxybenzaldehyde (Schemes 1).

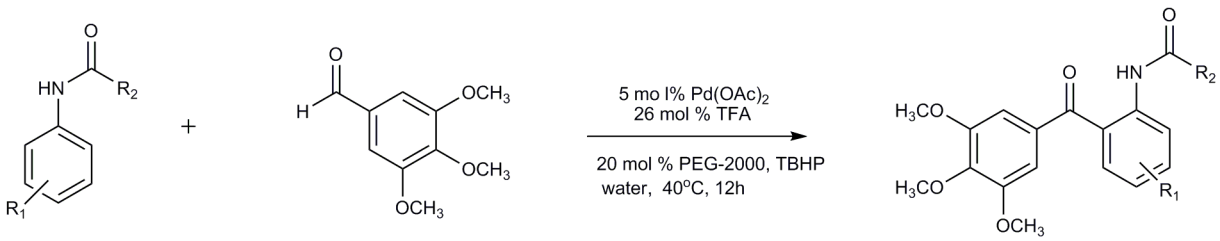
Compounds **13-17** were prepared with **12** as the intermediate as the route of Schemes 2. The reaction of **12** with CH_3OH in presence of CH_3ONa gave compound **13** in good yield.[28] Similarly, **12** reacted with methylamine, ethylamine and N,N-dimethylamine in the presence of potasium carbohydrate to provide compounds **14**, **15** and **16** in 86-90% yields, respectively.[29] Finally, compound **17** was obtained by the reaction of **16** with CH_3I in CH_2Cl_2.[30]

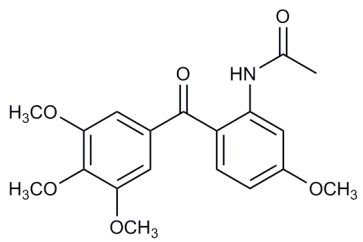
Table 1. Optimization of Reaction Conditions[a]



entry	$Pd(OAc)_2$ (equiv)	PEG-2000 (equiv)	H_2O (ml)	T （℃）	TFA (equiv)	TBHP (equiv)	Yield[b] (%)
1	5%	5%	1	40	26%	4.0	63
2	5%	10%	1	40	26%	4.0	71
3	5%	20%	1	40	26%	4.0	91
4	5%	50%	1	40	26%	4.0	trace
5[c]	5%	100%	0	40	26%	4.0	trace
6	5%	20%	1	rt	26%	4.0	38
7	5%	20%	1	60	26%	4.0	89
8	1.5%	20%	1	40	26%	4.0	trace
9	3%	20%	1	40	26%	4.0	47

[a]Reactions was carried out using amide (1.0 mmol), 3,4,5-trimethoxybenzaldehyde (1.0 mmol), $Pd(OAc)_2$, PEG-2000, tert-butylhydroperoxide TBHP (4.0 mmol), trifluoroacetic acid TFA (0.26 mmol, 26% mol), water (1.0 mL) under air for 12 h. [b]Isolated yields . [C]PEG-2000 was also as solvent.





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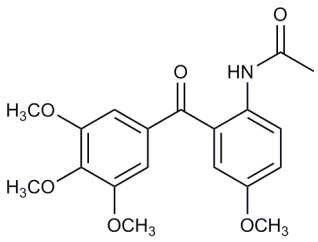

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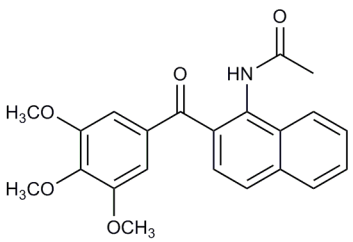

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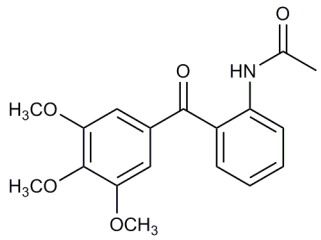

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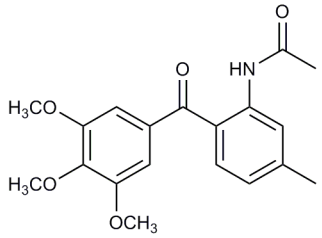

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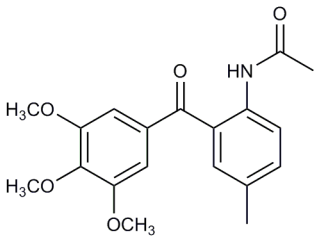

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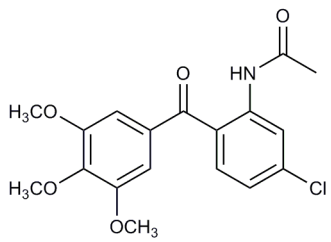

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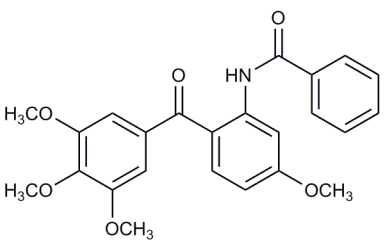

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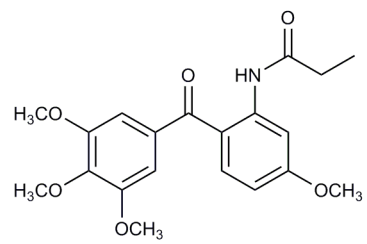

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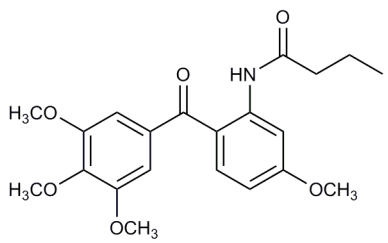

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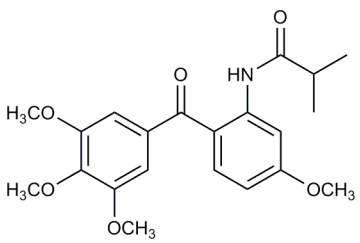

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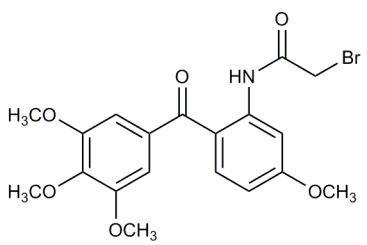
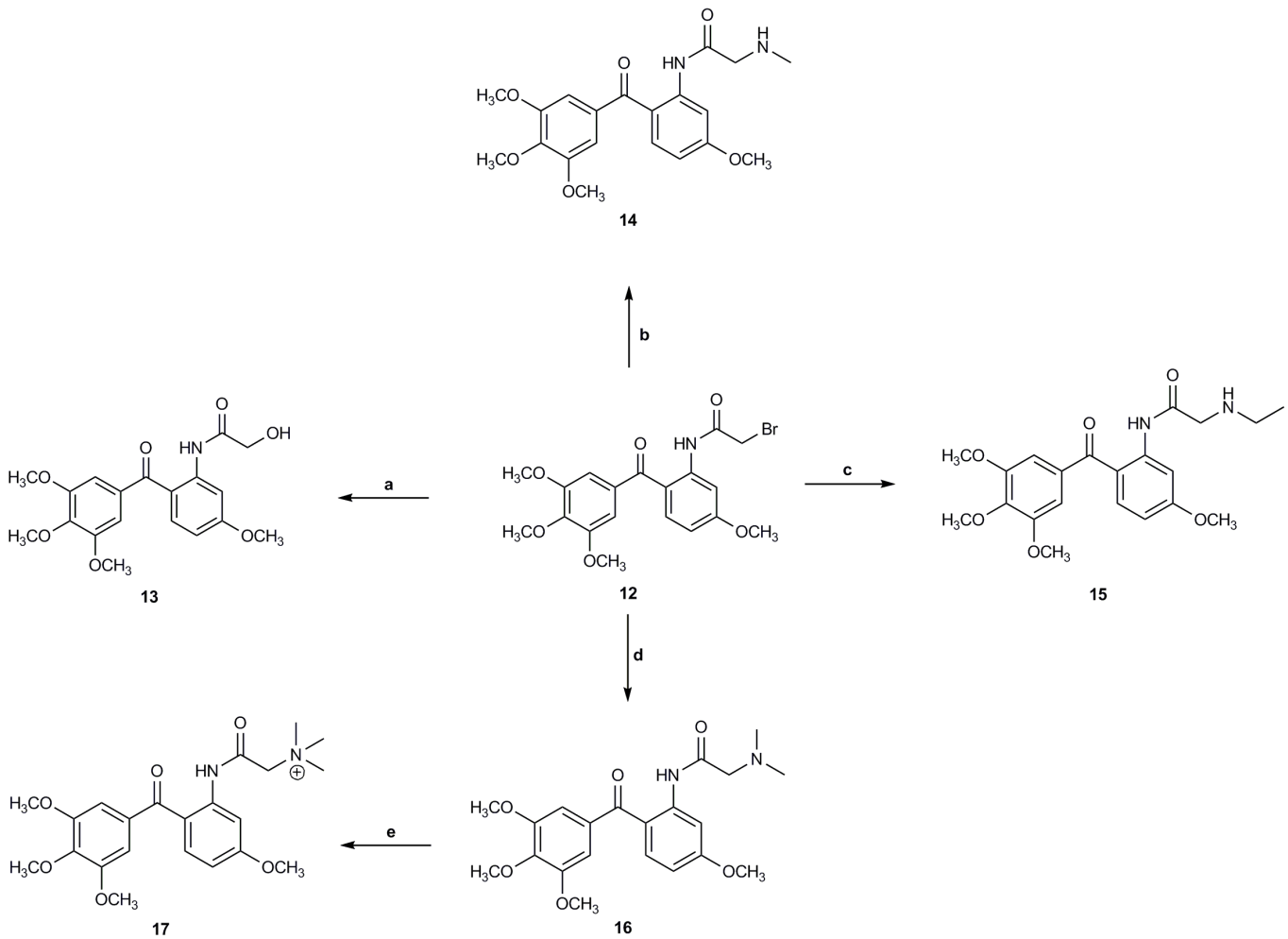


12, 76%

Scheme 1. Reaction conditions: Corresponding amide (1.0 mmol), 3,4,5-trimethoxybenzaldehyde (1.0 mmol), $Pd(OAc)_2$ (0.05 mmol, 5 mol %), PEG-2000 (0.2 mmol, 20 mol %), tert-butylhydroperoxide TBHP (4.0 mmol), trifluoroacetic acid TFA (0.26 mmol, 26 mol %), water (1.0 mL) at 40 ℃ under air for 12 h. Isolated yields (1-12) were reported (reaction times for each substrate were not optimized).



Scheme 2. conditions: (a) CH_3ONa, CH_3OH, reflux; (b)NH_2CH_3, K_2CO_3, 50℃; (c) $NH_2CH_2CH_3$, K_2CO_3 ,50℃; (d) $NH(CH_3)_2$,K_2CO_3 ,50℃; (e) CH_3I ,rt

In vitro cell growth inhibition

To evaluate the antiproliferative activities of all the synthesized compounds, human cancer cell lines HeLa (human epithelial cervical cancer line), A549 (non-small cell lung carcinoma), MCF-7 (human breast cancer cell line), and HCT-8 (human colon cancer cell line) were tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with CA-4 as the reference compound.

The results shown in Table 2 demonstrated that most of the synthesized compounds displayed good anti-proliferative activity with GI_{50} values in micromol to sub-micromol arrange. Among them, compounds **13** and **1** gave the best results (**13**, with 71 nM of GI_{50} value for HeLa cell line; **1**, GI_{50} = 113 nM for HeLa). A simple structure-activity relationship analysis showed that the anti-proliferative activity closely related to the substituted groups in B ring, and especially, the methoxy group in R_1 (C-3) position was necessary. Compounds **2-7**, with H, CH_3 and Cl in this position exhibited relatively poor activities with GI_{50} values from 0.90 to 36.8μM respectively for the four cell lines. On the other hand, compounds **9**, **10**, **11**, **13**, **14**, and **15**, which possess OCH_3 in R_1 position, showed moderate to very good activities (e.g., with 692 nM to 71 nM of GI_{50} values for HeLa, and 750 nM to 71 nM of GI_{50} values for A549). When moving the methoxy substitution from C-3 to the C-4 position of the B ring (compound **1** vs **2**), almost 10-fold decrease in antiproliferative activity was observed (from 113 nM to 1420 nM). Using the naphthalene ring to replace the benzene ring (compound **3** vs **4**), the antiproliferative activity (GI_{50} from 20.1μM to 4.88μM) was significantly increased.

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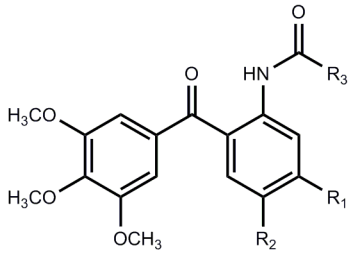
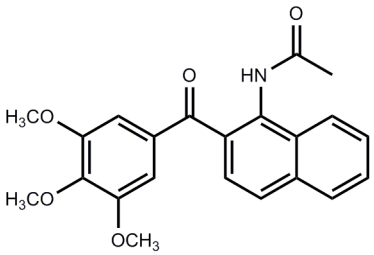
Table 2. Antiproliferative activity of compounds **1-11**,**13-17** and CA-4 against four human cancer cell lines[a].



1-2, 4-11, 13-17



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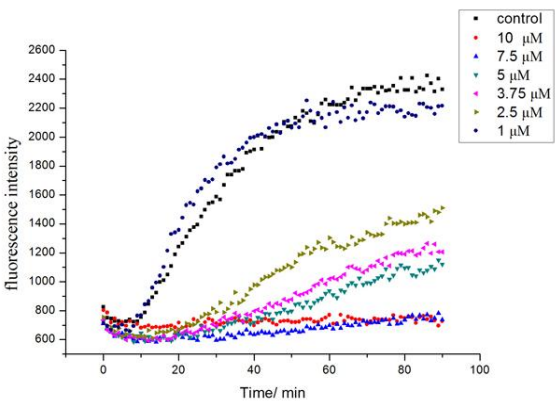
Compd.	R_1	R_2	R_3	GI_{50}(μM)			
				HeLa	A549	MCF-7	HCT-8
1	OCH_3	H	CH_3	0.113 ±0.11	0.18 ±0.01	0.88 ±0.02	0.34 ±0.06
2	H	OCH_3	CH_3	1.40 ±0.014	1.29 ±0.19	0.90 ±0.03	1.59 ±0.02
3	-	-	-	4.88 ±0.31	2.65 ±0.03	1.71 ±0.02	0.676 ±0.05
4	H	H	CH_3	20.1 ±2.15	10.4 ±0.16	14.2 ±1.05	16.8 ±1.10
5	CH_3	H	CH_3	23.5 ±1.16	13.4 ±0.62	16.1 ±3.17	20.5 ±0.56
6	H	CH_3	CH_3	2.15 ±0.15	2.00 ±0.35	3.56 ±0.22	2.59 ±0.69
7	Cl	H	CH_3	36.8 ±4.13	7.60 ±1.93	12.5 ±1.2	21.5 ±1.57
8	H	H	Ph	0.54 ±0.03	0.50 ±0.035	4.34 ±1.28	0.89 ±0.05
9	OCH_3	H	CH_2CH_3	0.32 ±0.01	0.36 ±0.064	2.34 ±0.03	1.68 ±0.86
10	OCH_3	H	n-propyl	0.23 ±0.02	0.50 ±0.03	2.60 ±0.4	0.84 ±0.06
11	OCH_3	H	isopropyl	0.38 ±0.05	0.50 ±0.102	0.91 ±0.01	1.23 ±0.75
13	OCH_3	H	CH_2OH	0.071 ±0.013	0.071 ±0.004	0.23 ±0.01	0.12 ±0.02
14	OCH_3	H	CH_2NHCH_3	0.692 ± 0.158	0.613 ±0.059	0.55 ±0.13	0.78±0.01
15	OCH_3	H	$CH_2NCH_2CH_3$	0.47 ±0.11	0.590 ±0.004	0.79 ±0.02	0.79 ±0.01
16	OCH_3	H	$CH_2N(CH_3)_2$	2.1 ±0.006	0.75 ±0.03	1.05 ±0.05	1.56 ±0.39
17	OCH_3	H	$CH_2N(CH_3)_3$	>10	21.4 ±3.39	31.5 ±2.59	35.6 ±0.45
CA-4	-	-	-	0.0028±0.06	0.018±0.05	0.009±0.01	0.025±0.02

[a] Cell lines were treated with different concentrations of the compounds for 48 h. Cell viability was measured by MTT assay as described in the Experimental Section. [b] GI_{50} values are indicated as the mean±SD (standard error) of three independent experiments.

Inhibition of tubulin polymerization

To investigate whether the antiproliferative activities of these compounds were related to their interaction with tubulin, Compounds **1**, **8**, **13**, and **15**, which showed relatively better activity, were choosed to study for their ability to block the assembly of tubulin (Table 3) by the method originally described by Bonne, D. et al with moderate modification,[31, 32] and CA-4 was examined as reference compound. We firstly use 10 μM of the tested compounds for the assays. At this concentration, compound **13** was found to inhibit tubulin polymerization the most (97.8%), while compounds **1**, **8**, **15** gave 59.3%, 32.9%, and 31.9% inhibitions, respectively. Then, the complete dose-response curves were obtained by assaying these compounds with inhibition rates and the results were summarized in Table 3. The representative raw data for the polymerization assay of compound **13** is shown in Figure 2. It can be seen that compounds **1** and **13** were effective tubulin polymerization inhibitors with IC_{50} values of 7.99 and 2.94 μM, respectively. Compounds, **8** and **15**, which had ralatively poor anti-proliferative activities, exihibited weaker ability to inhibit tubulin polymerization **(**with IC_{50} values more than 10 μM) than that of **1** and **13**, which suggest that the anti-proliferative activity of these compounds was correlated with their tubulin polymerization inhibition. Moreover, the chemical stability of the target compound **13** and CA-4 were evaluated by HPLC (see Supplementary Information). The results show that there is



no degradation of compound **13** up to 5 day at room temperature. While three impurities (should be the isomerize or decomposed products) of CA-4 in acetonitrile appeared in 24h, which is the same as those reported in literature[33]. These results indicated that replacement of the bridging double bond of CA-4 with carbonyl group could enhance the configurational stability.

Table 3. Effects of the selected compounds on tublin polymerization inhibition[a]

Compd	IC50 (μM)b
1	7.99 ±0.05
8	>10
13	2.94 ±0.02
15	>10
CA-4	1.2 ±0.2

[a]Inhibition of tubulin polymerization. Tublin was at 20 μM. [b] IC_{50} values are indicated as the mean±SD (standard error) of three independent experiments.

Figure 2. Effects of compound 13 on mircrotubule dynamics.

The morphological alterations of HeLa cells caused by 13

Base on the assay of antiproliferative activity and tubulin polymerization inhibition, we choose compound **13** for further biological study. Optical microscope was applied to identify the morphological alterations of HeLa cells after treated with **13**. As shown in Figure 3, when exposed to serial concentrations of **13** (0.01, 0.05, 0.1, and 0.5 μM) for 24 h and 48 h, the cells exhibited morphological alterations such as shrinkage, membrane blebbing and chromatin condensation that are normally associated with the occurrence of apoptotic cell death.

Analysis of cell cycle arrest

It is well-known that CA-4 blocks the cell cycle in the G_2/M phase due to microtubule depolymerization and cytoskeleton disruption. The cell growth inhibitory potency of compound **13** prompted us to evaluate its effects on the cell cycle (Figure.3). After HeLa cells were treated with DMSO (0.01%) or compound **13** at different concentrations (0.01, 0.05, 0.1, and 0.5 μM) for 24 h and 48 h, the analysis (Figure 4) of harvested cells by flow cytometry indicated that **13** resulted in significant cell-cycle arrest at G_2/M phase, showing a time and dose-dependent manner. The lowest concentration of **13** induced no significant distribution of HeLa cells through the cell cycle (22.0% of cells at G_2/M phase for 24 h, and 23.0% for 48 h). Whereas, when the concentration of **13** was increased to 0.05, 0.1, and 0.5 μM for 24 h, the percentage of cells at G_2/M phase correspondingly increased to 39.51%, 52.75%, and 56.06%, respectively (Figure. 4A), and the percentage of cells at G_2/M phase were 58.70%, 60.07%, and 63.86% (Figure. 4B) after treatment of compound **13** at the same concentration for 48 h. Futhermore, when the cells were exposed to the high concentration, the obvious typical apoptosis sub-peak was observed in DNA histogram. These results revealed that compound **13** could arrest cell-cycle progression at the mitotic phase, which is in agreement with the reported properties for most of the antimitotic agents

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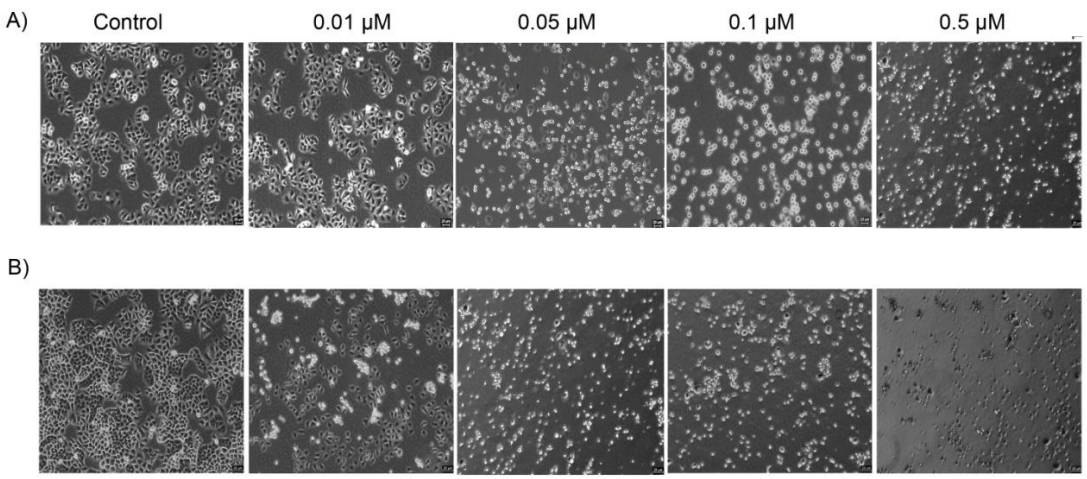

Figure 3. The micrographs of HeLa cells exposed to compound 13 (0.01, 0.05, 0.1, and 0.5 μM) or 0.01% DMSO (control) for 24 h (A) and 48 h (B), (magnification ×200).

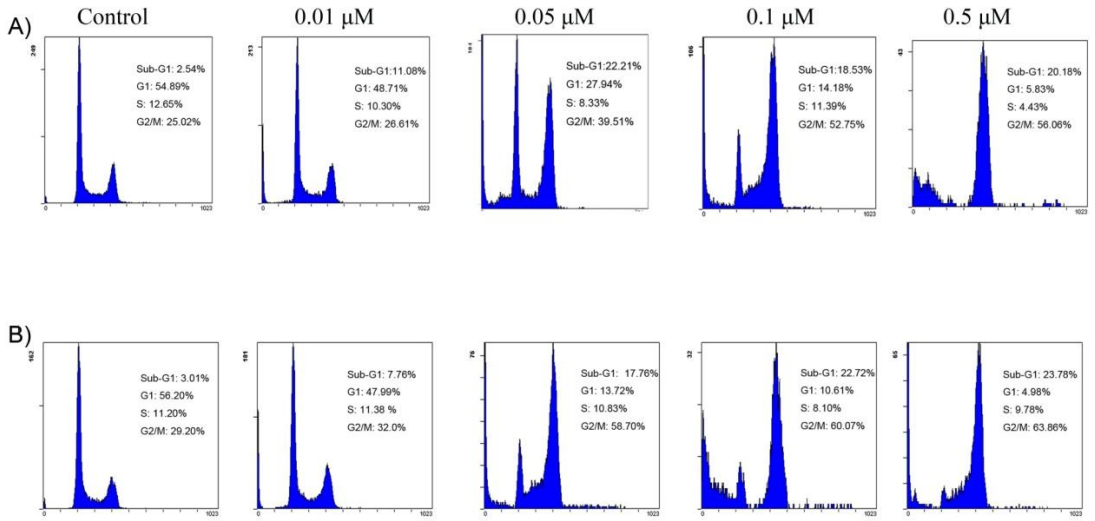

Figure 4. Effect of compound 13 on cell-cycle arrest of HeLa cells. Cells were treated with compound 13 (0.01, 0.05, 0.1, and 0.5 μM) or DMSO (0.01%) for 24 h (A)and 48 h (B). The DNA contents of each cell phase were analyzed by flow cytometry. The experiments were performed for three times, and the results of representative experiments are shown.

Effect of compound 13 on cell-cycle-related-protein expression

In view of the significant ability of compound **13** to induce a G_2/M arrest, we further studied the correlation between **13**-induced G_2/M arrest and alterations of proteins expression that regulate cell division. The formation of a cdc2/cyclin B1 complex is an important event for cell mitosis. Phosphorylation of cdc2 on Tyr15 and phosphorylation of cdc25c phosphatase on Ser216 negatively regulate the activation of the cdc2/cyclin B1 complex. Thus, dephosphorylation of these proteins is necessary to activate the cdc2/cyclin B1 complex, and is mediated by the phosphatase cdc25c, which dephosphorylates cdc2 and autodephosphorylates itself. Phosphorylation of cdc25c directly stimulates both its phosphatase and autophosphatase activities, a condition required to trigger cdc2/cyclin B1 induction of entry of cells into mitosis.[34, 35] Thus, the protein level of cyclin B1 and the status of both p-cdc2 and cdc25c were examined by Western blot analysis. As shown in Figure 5, treatment of HeLa cells with **13** at the lowest concentration (0.01μM) caused no significantly variations in cyclin B1, p-Cdc2, and Cdc25C. However, with the concentration increasing to 0.05, 0.1, and 0.5 μM, a dramatic decrease in the cyclin B1 expression, the phosphorylated form of Cdc2 (p-Cdc2) and Cdc25C, was observed. These results, which were perfectly consistent with the cell cycle arrest analysis, further illustrated the mechanism of the cell-cycle arrest effect of compound **13**.

Apoptosis assay

To evaluate the mode of cell death induced by **13**, a flow cytometry was performed using propidium iodide (PI), which only stains DNA and enters only dead cells, and the protein annexin-V, which selectively binds to the apoptotic cells that expose on their surface the phospholipid phosphatidylserine. Thus, this analysis is able to distinguish between live cells (annexin-V^-/PI^-), early apoptotic cells (annexin-V^+/PI^-), late apoptotic cells (annexin-V^+/PI^+), and necrotic cells (annexin-V^-/PI^+). As shown in Figure 6A, when HeLa cells were treated with **13** at different concentrations (0.01, 0.05, 0.1, and 0.5 μM) or DMSO (0.01%) for 24 h, comparing with the control cells, an accumulation of annexin-V positive cells was observed, which meaning the apoptotic cells were increased. And this tendency was also obvious after the cells treated with **13** for 48 h. These data showed **13** induce cell apoptosis in a concentration-and time-dependent manner.

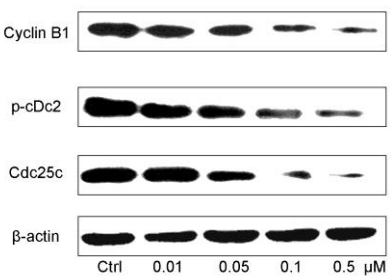

Figure 5. Effects of 13 on G_2/M regulatory proteins. HeLa cells were treated with 13 for 24 h at the concentrations of 0.01, 0.05, 0.1, and 0.5 μM. The cells were harvested and lysed for the detection of cyclin B1, p-Cdc2, Cdc25C expression by Western blot analysis, and β-actin was used as a control. The experiments were performed for three times, and the results of representative experiments are shown.

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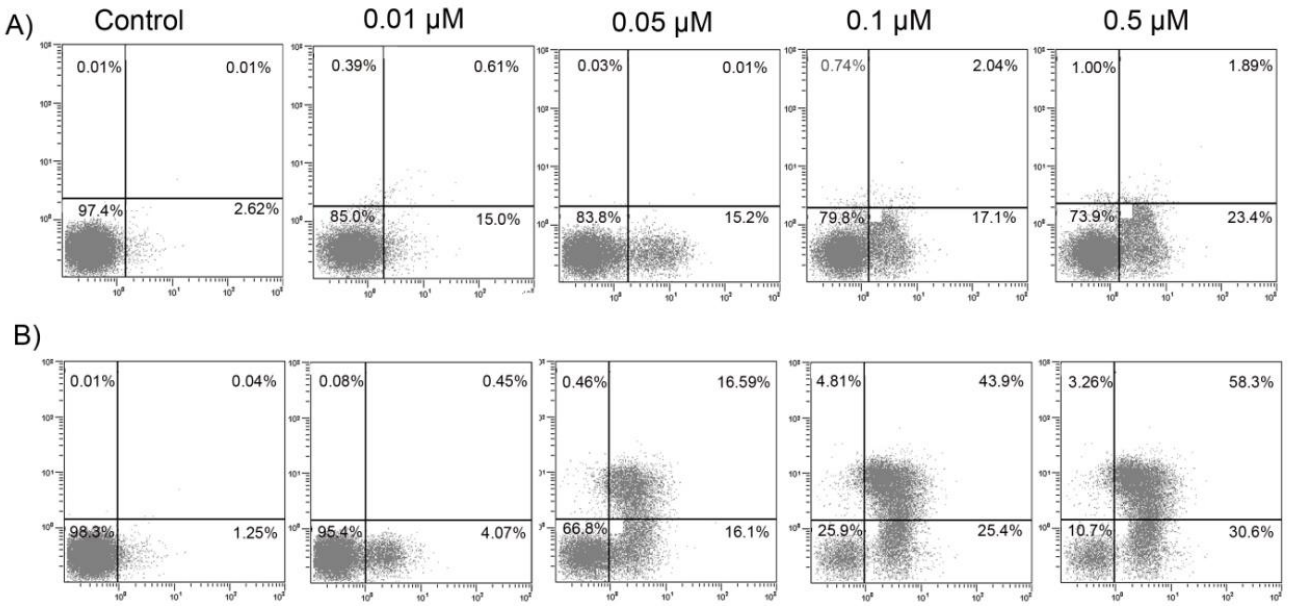

Figure 6. Effect of compound 13 on HeLa cell apoptosis. Cells were treated with compound 13 (0.01, 0.05, 0.1, and 0.5 μM) or DMSO (0.01%) for 24 h (A)and 48 h (B). The percentages of cells in each stage of cell apoptosis were quantitated by flow cytometry. (upper left quadrant) necrotic cells; (upper right quadrant) late apoptotic cells; (bottom left quadrant) live cells; and (bottom right quadrant) early apoptotic cells. The experiments were performed three times, and representative experiments are shown.

Effect of 13 on apoptosis-related-protein expression

It is well-known that tubulin polymerization inhibitors affect signaling pathways that involve the regulation of the Bcl-2 family of proteins.[36] Several pro-apoptotic family proteins (e.g., Bax, Bid, Bim, and Bad) promote the release of cytochrome C and further induce cell apoptosis, whereas anti-apoptotic members (Bcl-2, Bcl-XL) are capable of antagonizing the pro-apoptotic proteins and preventing the cells from apoptosis.[37-39] To evaluate the effects of **13** on apoptosis-related proteins, HeLa cells were treated with **13** at different concertrations (0.01, 0.05, 0.1, and 0.5 μM) for 24 h. As shown in Figure 7, the expression of the pro-apoptotic protein Bax and Bad was strongly up-regulated after the treatment, whereas the expression of the anti-apoptotic protein Bcl-2 and Bcl-XL was obviously down-regulated. These results illustrated that the anti-proliferative activity of **13** was due to the regulation of apoptosis-related protein and eventually led to cell death.

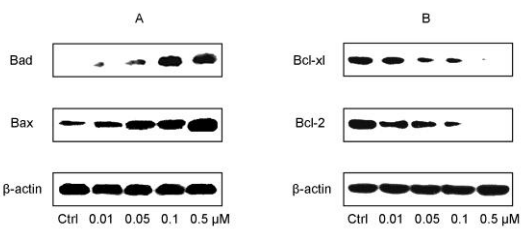

Figure 7. Effects of 13 on apoptosis-related proteins. HeLa cells were treated with 13 for 24 h at the concentrations of 0.01, 0.05, 0.1, and 0.5 μM. The cells were harvested and lysed for the detection of Bad, Bax, Bcl-xl, Bcl-2 expression by Western blot analysis, and β -actin was used as a control. The experiments were performed for three times, and the results of representative experiments are shown.

Conclusion

In conclusion, we have developed a new series of ortho-(3,4,5-trimethoxybenzoyl)-acetanilides by the simple cross-coupling reaction in aqueous solution with polyethylene glycol as additive at a very mild conditions. The evaluation of these compounds as anti-cancer agents exhibited excellent

antiproliferative activity against various human cancer cell lines and good activity for inhibiting tubulin polymerization. The study of the relation of the structure and activity indicated that the methoxyl group on C-3 position of B ring is crucial for the activity, and at the same time the acetyl group was helpful to improve their activity. It is worth noting that compound 13, which bears a hydroxyl in acetyl group, showed the best antiproliferative activity (GI_{50}= 71nM, for human HeLa cell line) and inhibited tubulin polymerization with IC_{50} value of 2.94 μM. The mechanism study indicated that compound 13 could arrest cell-cycle progression at the mitosis phase, block the formation of cdc2/cyclin B1 complex and down-regulated the p-Cdc 25C. The western blot analysis revealed the pro-apoptotic protein Bax and Bad was strongly up-regulated and the anti-apoptotic protein Bcl-2 and Bcl-XL was obviously down-regulated. The further study of compound 13 as anti-cancer agent in vitro and in vivo is in progress.

Experimental

Materials and methods

All reagents used in the synthesis were obtained commercially and used without further purification, unless otherwise specified. The ^{1}H NMR and ^{13}C NMR spectra were recorded using TMS as the internal standard on a Bruker BioSpin GmbH spectrometer at 400 and 101 MHz, respectively, and the coupling constants are reported in hertz. The reactions were monitored by thinlayer chromatography (TLC) on glass-packed precoated silica gel plates and visualized in an iodine chamber or with a UV lamp. Flash column chromatography was performed using silica gel (200−300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. The high-resolution mass spectra were obtained using a Shimadzu LCMS-ITTOF mass spectrometer. The term "dried" refers to the use of anhydrous sodium sulfate. The purity (≥95%) of the samples was determined by high-performance liquid chromatography (HPLC), conducted on a Shimadzu LC-20AT series system, a TC-C18 column (4.6 mm × 250 mm, 5 μm), eluted with a 40:60 acetonitrile/PBS mixture [25 mM NaH_2PO_4 (pH 3.0)], at a flow rate of 1 mL/min.

Preparation of substituted acetanilide substrates[40-42]

All the substituted anilides were prepared from their corresponding precursors with their corresponding acyl chlorides in CH_2Cl_2 according to the literature method without modifications.

General procedures for the coupling reactions[15, 18, 19]

Substituted anilide (1.0 mmol), 3,4,5-trimethoxybenzaldehyde (1.0 mmol), $Pd(OAc)_2$ (12 mg, 0.05 mmol) and PEG-2000 (400mg, 0.2 mmol) were loaded into a Schlenk tube, and then water (1.0 mL) was added. After the mixture was stirred for 2 minutes, TBHP (4.0 mmol) and trifluoroacetic acid (0.26 mmol) were added at the room temperature. The mixture was stirred at at 40 ℃ for 12 hours and then cooled to room temperature. Saturated solution of K_2CO_3 was added to the mixture and diluted with ethyl acetate. The organic layer was separated, washed with brine and concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel (200-300 mesh) to afford the desired product.

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide(1).

White solid; 91% yield; mp.156.7-157.9℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.30 (s, 1H), 8.35 (d, *J* = 2.4 Hz, 1H), 7.56 (d, *J* = 8.9 Hz, 1H), 6.89 (s, 2H), 6.59 (d, *J* = 8.9 Hz, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.87 (s, 6H), 2.24 (s, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 197.82, 169.41, 164.45, 152.83, 143.47, 141.55, 135.65, 134.40, 115.82, 108.81, 107.22, 105.12, 60.90, 56.28, 55.60, 25.37. HRMS found (ESI) $(M + H)^+$ 360.1440 $C_{19}H_{22}NO_6$, requires 360.1442. Purity: 98.3% (by HPLC).

N-(4-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide(2).

White solid; 71% yield; mp.144.4-145.6℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.98 (s, 1H), 8.39 (d, *J* = 9.1 Hz, 1H), 7.13 (dd, *J* = 9.1, 2.9 Hz, 1H), 7.07 (d, *J* = 2.9 Hz, 1H), 7.01 (s, 2H), 3.95 (s, 3H), 3.88 (s, 6H), 3.77 (s, 3H), 2.17 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.48, 168.72, 154.33, 152.86, 142.44, 132.93, 132.78, 125.99, 123.95, 119.02, 117.31, 107.79, 60.96, 56.32, 55.67, 24.81. HRMS found (ESI) $(M + H)^+$ 360.1442 $C_{19}H_{22}NO_6$, requires 360.1442. Purity: 98.1% (by HPLC).

N-(2-(3,4,5-trimethoxybenzoyl)naphthalen-1-yl)acetamide(3).

White solid; 53% yield; mp.209.7-211.1℃;^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 7.98 (d, *J* = 7.4 Hz, 1H), 7.86 (d, *J* = 6.0 Hz, 1H), 7.72 (d, *J* = 8.4 Hz, 1H), 7.59 (d, *J* = 3.5 Hz, 2H), 7.44 (d, *J* = 8.5 Hz, 1H), 7.13 (s, 2H), 3.95 (s, 3H), 3.85 (s, 6H), 2.23 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.14, 169.68, 152.86, 142.73, 134.78, 132.44, 132.11, 130.84, 129.00, 128.00, 127.63, 126.74, 125.55, 125.21, 124.51, 108.06, 60.91, 56.25, 23.64. HRMS found (ESI) $(M + H)^+$ 380.1495 $C_{22}H_{22}NO_5$, requires 380.1492. Purity: 99.3% (by HPLC).

N-(2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide(4).

White solid; 66% yield; mp.171.5-172.8℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.49 (s, 1H), 8.56 (d, *J* = 8.7 Hz, 1H), 7.57 (t, *J* = 6.5 Hz, 2H), 7.11 (t, *J* = 7.6 Hz, 1H), 6.97 (s, 2H), 3.95 (s, 3H), 3.87 (s, 6H), 2.22 (s, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 198.31, 169.03, 152.86, 142.28, 139.98, 133.91, 133.39, 132.82, 124.01, 122.08, 121.92, 107.75, 60.96, 56.31, 25.10. HRMS found (ESI) $(M + H)^+$ 330.1339 $C_{18}H_{20}NO_5$, requires 330.1336. Purity: 99.5% (by HPLC).

N-(5-methyl-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (5).

White solid; 73% yield; mp.148.5-150.2℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.71 (s, 1H), 8.44 (s, 1H), 7.49 (d, *J* = 7.9 Hz, 1H), 6.96 (s, 2H), 6.92 (d, *J* = 7.7 Hz, 1H), 3.95 (s, 3H), 3.79 (d, *J* = 73.4 Hz, 6H), 2.45 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.27, 169.08, 152.82, 145.42, 141.98, 140.32, 133.79, 133.22, 122.92, 122.06, 121.16, 107.57, 60.94, 56.28,

25.20, 22.07. HRMS found (ESI) (M + H)$^+$ 344.1494 $C_{19}H_{22}NO_5$, requires 344.1492. Purity: 98.7% (by HPLC).

N-(4-methyl-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (6).

White solid; 67% yield; mp.128.7-131.0℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 8.44 (d, *J* = 8.3 Hz, 1H), 7.40 (d, *J* = 10.6 Hz, 2H), 7.00 (s, 2H), 3.98 (s, 3H), 3.90 (s, 6H), 2.34 (s, 3H), 2.21 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.30, 168.93, 152.86, 142.25, 137.46, 134.53, 133.45, 132.94, 131.76, 124.22, 122.02, 107.76, 60.99, 56.34, 25.05, 20.71. HRMS found (ESI) (M + H)$^+$ 344.1490 $C_{19}H_{22}NO_5$, requires 344.1492. Purity: 96.1% (by HPLC).

N-(5-chloro-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide(7).

White solid; 48% yield; mp.158.6-160.1℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.66 (s, 1H), 8.71 (s, 1H), 7.54 (d, *J* = 8.4 Hz, 1H), 7.09 (d, *J* = 8.4 Hz, 1H), 6.94 (s, 2H), 3.96 (s, 3H), 3.89 (s, 6H), 2.24 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.68, 169.13, 152.97, 142.43, 141.27, 140.45, 133.99, 133.20, 122.22, 121.70, 121.62, 107.59, 61.01, 56.35, 25.19. HRMS found (ESI) (M + H)$^+$ 364.0946 $C_{18}H_{19}ClNO_5$, requires 364.0946. Purity: 99.8% (by HPLC).

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)benzamide(8).

White solid; 86% yield; mp.156.4-158.2℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.43 (s, 1H), 8.62 (d, *J* = 2.6 Hz, 1H), 8.17 – 8.03 (m, 2H), 7.64 (d, *J* = 8.9 Hz, 1H), 7.60 – 7.49 (m, 3H), 6.92 (s, 2H), 6.64 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.89 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.48, 166.19, 164.77, 152.89, 144.12, 141.42, 136.13, 134.63, 134.53, 132.13, 128.87, 127.44, 115.98, 109.38, 107.09, 104.91, 60.98, 56.32, 55.74. HRMS found (ESI) (M + H)$^+$ 422.1600 $C_{24}H_{24}NO_6$, requires 422.1598. Purity: 97.4% (by HPLC).

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)propionamide (9).

White solid; 81% yield; mp.141.7-143.1℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.37 (s, 1H), 8.41 (d, *J* = 2.6 Hz, 1H), 7.57 (d, *J* = 8.9 Hz, 1H), 6.89 (s, 2H), 6.59 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.92 (d, *J* = 7.0 Hz, 6H), 3.87 (s, 6H), 2.50 (q, *J* = 7.6 Hz, 2H), 1.29 (t, *J* = 7.6 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 197.93, 173.35, 164.52, 152.83, 143.69, 141.41, 135.74, 134.52, 115.72, 109.00, 107.12, 104.85, 60.94, 56.27, 55.62, 31.59, 9.45. HRMS found (ESI) (M + H)$^+$ 374.1599 $C_{20}H_{24}NO_6$ requires 374.1598. Purity: 99.9% (by HPLC).

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl) butyramide(10).

White solid; 76% yield; mp.90.7-92.1℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.37 (s, 1H), 8.41 (d, *J* = 2.6 Hz, 1H), 7.57 (d, *J* = 8.9 Hz, 1H), 6.89 (s, 2H), 6.59 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.92 (d, *J* = 7.3 Hz, 6H), 3.88 (s, 6H), 2.44 (t, *J* = 7.5 Hz, 2H), 1.88 – 1.74 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.95, 172.63, 164.55, 152.85, 143.70, 141.48, 135.76, 134.52, 115.70, 109.01, 107.18, 104.86, 60.95, 56.29, 55.64, 40.56, 18.89, 13.73. HRMS found (ESI) (M + H)$^+$ 388.1754 $C_{21}H_{26}NO_6$, requires 388.1755. Purity: 99.8% (by HPLC).

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)isobutyramide (11).

White solid; 69% yield; mp.117.1-118.8℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.48 (s, 1H), 8.44 (d, *J* = 2.6 Hz, 1H), 7.58 (d, *J* = 8.9 Hz, 1H), 6.86 (d, *J* = 19.9 Hz, 2H), 6.59 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 3H), 3.88 (s, 6H), 2.73 – 2.54 (m, 1H), 1.32 (s, 3H), 1.30 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.00, 176.72, 164.58, 152.84, 143.95, 141.38, 135.83, 134.58, 115.68, 109.14, 107.11, 104.68, 60.94, 56.28, 55.63, 37.46, 19.46. HRMS found (ESI) (M + H)$^+$ 388.1755 $C_2H_{26}NO_6$, requires 388.1755. Purity: 99.0% (by HPLC).

2-bromo-N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl) acetamide(12).

White solid ; 76% yield; mp.127.1-128.8℃ ; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.93 (s, 1H), 8.30 (d, *J* = 2.5 Hz, 1H), 7.61 (d, *J* = 8.9 Hz, 1H), 6.91 (s, 2H), 6.66 (dd, *J* = 8.9, 2.6 Hz, 1H), 4.03 (s, 2H), 3.93 (s, 3H), 3.92 (s, 3H), 3.88 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.56, 165.35, 164.30, 152.86, 142.45, 141.64, 135.73, 134.14, 116.64, 109.49, 107.30, 105.57, 60.97, 56.32, 55.70, 29.50. LCMS found (ESI) 437.0 (M + H)$^+$.

Synthesis of 2-hydroxy-N-(5-methoxy-2-(3,4,5-trimethoxy benzoyl)phenyl) acetamide(13).[18]

To a stirred solution of 2-bromo-N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl) acetamide (**12** ,60 mg) in CH_3OH (dry) at room temperature, CH_3ONa (30mg) was added and then refluxed for 6h. The mixture was evaporated under reduced pressure and the residue was purified by column chromatography (petroleum ether/ethyl acetate,5:1) to give the product as a white solid. 85% yield; mp.146.8-148.7℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.84 (s, 1H), 8.37 (s, 1H), 7.60 (s, 1H), 6.91 (s, 2H), 6.64 (d, *J* = 9.0 Hz, 1H), 5.36 (s, 1H), 4.31 (s, 2H), 4.19 – 3.68 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.54, 171.38, 164.33, 152.80, 142.29, 141.60, 135.62, 134.22, 116.56, 109.17, 107.31, 105.53, 62.97, 60.97, 56.29, 55.66. HRMS found (ESI) (M + H)$^+$ 376.1393 $C_{19}H_{22}NO_7$, requires 376.1391. Purity: 99.8% (by HPLC).

General procedure for the synthesis of 14-16.[29]

To a stirred solution of 2-bromo-N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl) acetamide (**12**,50 mg) in acetone, K_2CO_3 (30mg) and the corresponding amine were added. After stirred at 50℃ for 5h, the mixture was extracted with ethyl acetate (3×10ml), washed with brine (twice), dried over Na_2SO_4 and concentrated in vacue to give the crude product which was purified by column chromatography (petroleum ether/ethyl acetate,5:1) to give a white solid.

N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)-2-(methylamino)acetamide (14).

Yellow solid; 90% yield; mp.123.6-125.4℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.04 (s, 1H), 8.42 (d, *J* = 2.5 Hz, 1H), 7.55 (d, *J* = 8.8 Hz, 1H), 6.93 (s, 2H), 6.61 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.92 (d, *J* = 3.9 Hz, 6H), 3.87 (s, 6H), 3.43 (s, 2H), 2.55 (s, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 196.95, 171.76, 164.09, 152.77, 142.40, 141.48, 135.31, 134.48, 117.04, 108.91, 107.34, 105.45, 60.91, 56.27, 55.84, 55.59, 36.84. HRMS found (ESI) $(M + H)^+$ 389.1710 C_{20} $H_{25}N_2O_6$, requires 389.1707. Purity: 99.5% (by HPLC).

2-(ethylamino)-N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl) phenyl)acetamide(15).

Yellow solid; 86% yield; mp: 99.1-100.3℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.04 (s, 1H), 8.41 (d, *J* = 2.5 Hz, 1H), 7.53 (d, *J* = 8.8 Hz, 1H), 6.93 (s, 2H), 6.61 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.92 (d, *J* = 5.9 Hz, 6H), 3.87 (s, 6H), 3.46 (s, 2H), 2.75 (q, *J* = 7.1 Hz, 2H), 1.20 (t, *J* = 7.1 Hz, 3H). ^{13}CNMR (101 MHz, $CDCl_3$) δ 196.70, 172.20, 163.97, 152.76, 142.29, 141.45, 135.15, 134.49, 117.20, 108.86, 107.33, 105.44, 60.89, 56.26, 55.56, 53.54, 44.53, 15.19. HRMS found (ESI) $(M + H)^+$ 403.1866 $C_{21}H_{27}N_2O_6$, requires 403.1864. Purity: 98.8% (by HPLC).

2-(dimethylamino)-N-(5-methoxy-2-(3,4,5-trimethoxy benzoyl)phenyl) acetamide (16).

Yellow solid; 88%yield; mp.138.4-140.3℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.94 (s, 1H), 8.39 (d, *J* = 2.5 Hz, 1H), 7.53 (d, *J* = 8.8 Hz, 1H), 6.92 (s, 2H), 6.61 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.92 (d, *J* = 5.2 Hz, 7H), 3.87 (s, 6H), 3.14 (s, 2H), 2.42 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.80, 170.90, 164.00, 152.80, 142.28, 141.33, 135.30, 134.64, 117.21, 108.93, 107.17, 105.52, 64.34, 60.90, 56.28, 55.58, 46.07. HRMS found (ESI) $(M + H)^+$ 403.1866 $C_{21}H_{27}N_2O_6$, requires 403.1864. Purity: 97.6% (by HPLC).

Synthesis of 2-(5-methoxy-2-(3,4,5-trimethoxybenzoyl) phenylamino)-N,N,N-trimethyl-2-oxoethanaminium (17).[30]

To a stirred solution of 2-(dimethylamino)-N-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (50mg) in CH_2Cl_2, CH_3I (85mg) and triethylamine (35mg) were added and then the mixture was stirred at room temperature overnight. The solid was obtained from the reaction mixture was filtered and dried to give a white solid. 91% yield; mp.127.6-128.9℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.48 (s, 1H), 7.86 (d, *J* = 2.5 Hz, 1H), 7.48 (d, *J* = 8.8 Hz, 1H), 6.84 (s, 2H), 6.67 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.10 (s, 2H), 3.92 (s, 3H), 3.89 (s, 3H), 3.83 (s, 6H), 3.68 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.73, 163.76, 161.69, 152.74, 141.63, 140.00, 134.97, 133.55, 118.64, 110.11, 107.35, 65.20, 60.94, 56.38, 55.76, 55.10. HRMS found (ESI) (M^+) 417.2018 $C_{22}H_{29}N_2O_6$, requires 417.2020. Purity: 99.1% (by HPLC).

Cell culture and maintenance.

Four cancer cell lines, HeLa (human epithelial cervical cancer), A549 (non-small cell lung carcinoma), MCF-7 (human breast cancer cell line), and HCT-8 (human colon cancer cell line) were obtained from the laboratory animal center of Sun Yat-sen University, which were cultivated in the Dulbecco's modified Eagle medium (DMEM, GIBCO), containing 10% (v/v) heat inactivated fetal bovine serum (FBS, GIBCO), 100 IU/mL penicillin and 100 μg/mL streptomycin (GIBCO) in culture dish in a humidified atmosphere of 5% CO_2 at 37℃.

Antiproliferative Activity Assays in Vitro.

The antiproliferative activity of the target compounds were examined in four human cancer cell lines (HeLa, HCT-8, A549 and MCF-7) by MTT assay. When the cells grew in the logarithmic phase, 5×10^3 cells/well cells were harvested and plated into the 96-well plates for 24 h, and then the cells were exposed to different concentrations of the test compounds for 48 h in three replicates. Afterward, 20 μL of MTT (5mg/mL, Sigma) was added and incubated for another 4 h. Then, the suspension was discarded and 150 μL of DMSO was added to each well. After shooked the plateds to dissolve the dark blue crystals (formazan) for 10min, the absorbance at 570nm was measured using a multifunction mircoplate reader (Moleculardevices, Flex Station 3). All experiments were repeated at least three times. The data were calculated using Grap Pad Prism version 5.0. The IC_{50} values were fitted using a nonlinear regression model with a sigmodial dose response.

Flow cytometric analysis of cell-cycle .

For flow cytometric analysis of DNA content, HeLa cells were plated in 6-well plates (3×10^5 cells/well) and incubated in the presence or absence of compound **13** at various concentrations (0.01, 0.05, 0.1 and 0.5 μM) for 24 or 48 h. The cells were harvested with 0.25% trypsin and fixed with ice-cold 70% ethanol overnight. Ethanol was removed by centrifugation, and the cells were washed with cold 10% phosphate buffer solution (PBS), then, treated with RNAse A (100 μg/mL, Beyotime) at 37 ℃ for 30 min and DNA staining solution (PI, Beyotime) at 4 ℃ for 15 min. The DNA contents of 10,000 events were measured by flow cytometer (Beckman Coulter, Epics XL) at 488 nm. The data regarding the number of cells in different phases of the cell cycle were analyzed by EXPO32 ADC Analysis software.

Flow cytometric analysis of apoptotic cells.

HeLa cells were plated in 6-well plates (3×10^5 cells/well) and incubated in the presence or absence of compound **13** at various concentrations (0.01, 0.05, 0.1, and 0.5 μM) for 24 h or 48 h to induce cell apoptosis; 0.1% DMSO was used as a vehicle control. The percentages of apoptotic cells were estimated by staining with Annexin-V-FITC and PI (Beyotime Annexin-V-FITC Apoptosis Detection Kit) according to the manufacturer's instructions with moderate modification. After the cells were induced for 24 h or 48 h, both treated and untreated cells were harvested and incubated with 5 μL of Annexin-V-FITC in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ at Ph 7.4) at room temperature for 15 min. PI was then dropwise added to the medium at cold temperature, and the

mixture was incubated for 10min. Almost 10,000 events were collected for each sample and analyzed by flow cytometer (Beckman Coulter, Epics XL). The percentage of cells undergoing apoptosis was calculated using EXPO32 ADC Analysis software.

Tublin polymerization assay in vitro.

Tublin polymerization is followed by fluorescence enhancement due to the incorporation of a fluorescent reporter into microtubules as polymerization occurs. The tublin polymerization assay was monitored by an increase in fluorescence emission at 410 nm over a 60 minute period at 37 ℃ (excitation wavelength is 340 nm) using a modification of methods described by Bonne, D. et al. Purified brain tubulin polymerization kit was purchased from Cytoskeleton (BK110P, Denver, CO). The final buffer concentrations for tubulin polymerization contained 80.0 mM piperazine-N ,N′-bis(2-ethanesul-fonic acid)sequisodium salt (pH 6.9), 2.0 mM $MgCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)- N,N,N′,N′-tetraacetic acid (EGTA), 1 mM GTP, and 10.2% glycerol. Firstly, 5μL of test compounds were added in different concentrations, and then were warmed to 37 ℃ for 1 min. The reaction was initiated by the addition of 55μL of the tubulin reaction mix.

Western Blot Analysis.

HeLa cells (8.0×10^5 cells/dish) were incubated with or without at various concentrations (0.01, 0.05, 0.1, and 0.5 μM) for 24 h. After incubation, the cells were collected by centrifugation and washed twice with phosphate-buffered saline chilled to 0 ℃. The cells were then suspended in a lysis buffer at 0 ℃ for 30 min, and then centrifuged at 15,000 g for 30 min at 4 ℃. Total protein content was determined with the BCA protein kit (Pierce, Italy), and Bovine Serum Albumin (BSA) as a standard. The protein extracts were reconstituted in sample loading buffer (62 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled at 100 ℃ for 5 min. Equal amounts (50 μg) of denatured protein samples were loaded into each lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) on 8−12% polyacrylamide gradient, and then transferred onto a polyvinylidene difluoride (PVDF) membrane and then were blocked with 5% non-fat dried milk in PBS containing 1% Tween-20 for 2 h at room temperature followed by incubation with primary antibodies overnight and either horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies for 2 h. The blots were detected by ImageQuant LAS 4000 mini (Fujifilm) with the Super-Signal West Pico chemiluminescence substrate (Thermo Scientific, IL). Western blot analyses were performed using antibodies against cyclin B1 (Cell Signaling, Boston, U.S.A.), Cdc2 (Cell Signaling, Boston, U.S.A.). Cdc25C (Cell Signaling, Boston, U.S.A.), Bcl-2 (Cell Signaling, Boston, U.S.A.), Bax (Cell Signaling, Boston, U.S.A.), Bad (Santa Cruz, Texas, U.S.A.), Bcl-xl (Santa Cruz, Texas, U.S.A.). Densitometry analyses were performed using quantitative software (AlphaEase, Genetic Technology Inc. Miami, FL) with the control representing 1-fold as shown just below the data.

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