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# Evaluation of 4-phenylamino-substituted naphthalene-1,2-diones as tubulin polymerization inhibitors

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ABSTRACT

A series of 4-phenylamino-substituted naphthalene-1,2-dione derivatives were prepared and evaluated as effective antiproliferative agents. MTT assays showed that the compounds with a methyl group on the nitrogen linker exhibited potent antiproliferative activities against human cancer cells. The mechanistic study revealed that these compounds could induce mitochondrial depolarization, which resulted in intracellular ROS production, and they also acted as tubulin polymerization inhibitors. Moreover, the typical compound could arrest A549 cells in the G2/M phase, resulting in cellular apoptosis and induced mitotic arrest in A549 cells through disrupting microtubule dynamics.

Malignant tumors, commonly known as cancer, are a major disease that is the second most serious threat to human life and health after cardiovascular disease, and cancer has become the world's leading "life killer." It is estimated that by 2020, the number of deaths worldwide may double due to cancer, and approximately 84 million people will die from cancer in the next 10 years.[1,2] With the rapid increase in cancers worldwide, the existing drugs are far from meeting the needs. Therefore, in the past several decades, many anticancer agents aimed at various targets have been developed.[3–6] Among them, tubulin polymerization or depolymerization inhibitors have attracted much attention, as tubulin-microtubule systems play a critical role for a wide range of cellular processes and represent a prominent cancer drug target. The use of taxane and vinca alkaloids to treat a variety of human cancers in the past decades are such an example.[7] At the same time, new tubulin inhibitors are also being developed. For example, MPC-6827, a quinazoline amino-substituted derivative, could effectively inhibit the polymerization of tubulin in vitro and disrupt the formation of microtubules in a variety of cancer cell lines, and has reached phase II for the treatment of recurrent glioblastoma.[8–11] Since cancer is prone to drug resistance, from the view point of both the academic and applied science realms, the discovery and development of highly effective and low toxicity candidate drug molecules for the treatment of cancer is still important.

Reactive oxygen species (ROS) are closely related to tumorigenesis and treatment. In cancer cells, growth and proliferation are encouraged under the condition of a modest rise in the intracellular ROS. On the other hand, apoptosis is also induced at higher levels of ROS. Small molecules that modulate antioxidant levels and/or enhance intracellular ROS could disturb the cellular oxidative environment and induce cell death. Thus, the utilization of ROS-inducing small molecules to target cancer has been considered as a potential strategy. The enhancement of intracellular ROS could be achieved by endogenous antioxidant inhibition, thus modulating the functions of proteins responsible for maintaining redox homeostasis in the presence of small molecule ROS.[12] Quinones are one of the major sources of ROS in cells, and many quinone derivatives, such as mitomycin C,[13] geldanamycin,[14] mitoxantrone,[15] and eoxynyboquinone,[16] have been used as anticancer agents. Recently, Potter et al. reported that β-lapachones are potent, reversible, and selective inhibitors of human liver carboxylesterase (CEs) with $K_i$ values in the nanomolar range, which are ubiquitous enzymes that are responsible for the metabolism of xenobiotics including drugs such as the anticancer drugs irinotecan and oseltamivir.[17] Inspired by the structure and anticancer activity of compound MPC-6827, we proposed that 4-phenylamino-substituted naphthalene-1,2-diones should possess the antiproliferative activity against cancer cells. To further promote the application of these compounds in the treatment of cancer, and to investigate the structure-activity relationships, herein, we reported the evaluation of a series 4-phenylamino-substituted naphthalene-1,2-diones as antiproliferative agents as well as their mechanism of action.

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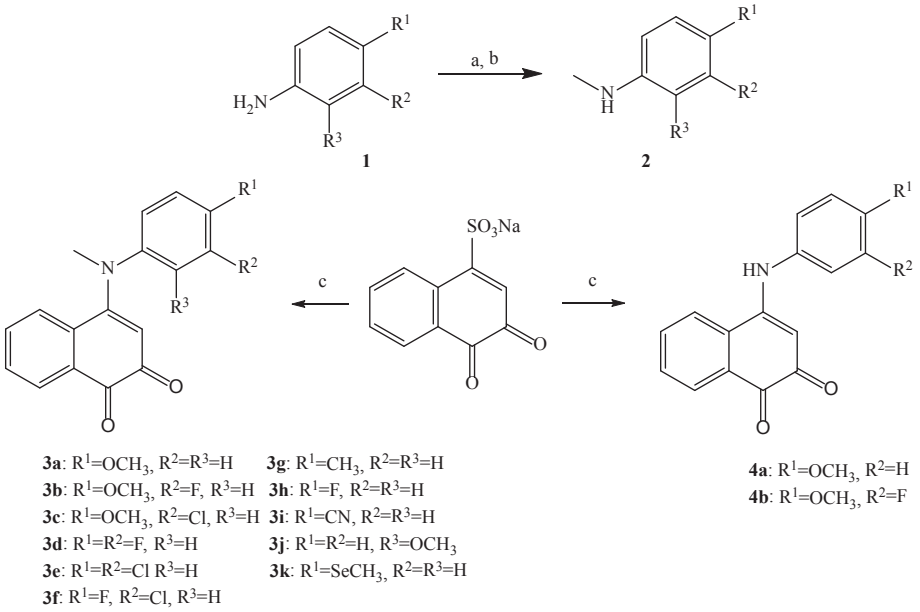



**Scheme 1.** The synthesis of 4-phenylamino-substituted naphthalene-1,2-diones. Reagents and conditions Reagents and conditions: (a) Na, MeOH, $(CH_2O)_n$. (b) $NaBH_4$, 60 °C. (c) **1** or **2**, $H_2O$, rt.

Compounds **3a**, **3g**, **3h** and **4a** were previously synthesized and evaluated as selective inhibitors of human liver CEs.[17] To study their activity of anti-tubulin aggregation, we prepared **3a–k** and **4a–b** (Scheme 1) according the method described in the same literatures.[17,18] Briefly, aniline derivatives reacted with paraformaldehyde in the presence of sodium, methanol and sodium borohydride to afford N-methyl aniline derivatives. The commercial sodium 3,4-dioxo-3,4-dihydronaphthalene-1-sulfonic acid reacted with the N-methyl aniline derivatives gained above or other aniline derivatives to afford target compounds **3** or **4** in water under mild conditions.

To evaluate the antiproliferative activities of the 4-(phenylamino) naphthalene-1,2-dione derivatives, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed toward five human cancer cell lines (A549, nonsmall cell lung carcinoma; HeLa, human epithelial cervical cancer cell line; HepG2, human hepatoma carcinoma cells; RKO and HCT-116, human colon cancer cell lines), and the results are listed in Table 1. Among the tested compounds, **3a** (4-((4-methoxyphenyl)(methyl)amino)naphthalene-1,2-dione) and **3b** exhibited potent antiproliferative activities with $IC_{50}$ values ranging from 0.091 to 0.471 μM. The substitutes on the ortho position of the methoxy group, especially larger groups, seem unfavorable for the activity; **3c**, with Cl at the $R_2$ position, gave relatively weak activity (0.684–1.441 μM). Compounds **3d**, **3e** and **3f** containing halogen groups exhibited weak activities, which suggested that the methoxy at the 4-position of the phenylamino moiety is necessary. Compound **3g**, with methyl group at the 4-position of the phenylamino moiety exhibited good activity with 1.091–2.352 μM of the $IC_{50}$ values. However, cyano group at the 4-position provided very poor activity (**3i**: > 10 μM of the $IC_{50}$ values for five cancer cell lines). The position of methoxy at the phenylamino moiety is also important to the activity; **3j**, with methoxy group at the 2-position, exhibited 5.924–6.853 μM of the $IC_{50}$ values, which was much weaker than its isomer **3a**. We previously reported the synthesis and evaluation of selenium-containing 4-anilinoquinazoline derivatives as novel antimitotic agents. Among them, the optimal compound, N,2-dimethyl-N-(4-(methylselanyl)phenyl)quinazolin-4-amine, exhibited $IC_{50}$ values of 2–9 nM against six human cancer cell lines including A549 and Hct116.[19] However, compound **3g**, with $SeCH_3$ at the 4-position of the phenylamino moiety, provided moderate activities, with the $IC_{50}$ values ranging from 1.005 to 3.515 μM. To examine the effect of methyl group on the linker nitrogen,

**Table 1**
Antiproliferative activities (μM) of **3a–3k** and **4a–4c** against five human cancer cell lines.[a,b]

| | A549 | HELA | HEPG2 | RKO | HCT116 |
|---|---|---|---|---|---|
| **3a** | 0.097 ± 0.004 | 0.091 ± 0.001 | 0.096 ± 0.001 | 0.116 ± 0.010 | 0.471 ± 0.025 |
| **3b** | 0.093 ± 0.019 | 0.092 ± 0.014 | 0.101 ± 0.008 | 0.332 ± 0.022 | 0.106 ± 0.012 |
| **3c** | 0.684 ± 0.015 | 0.864 ± 0.044 | 0.785 ± 0.015 | 0.784 ± 0.059 | 1.441 ± 0.011 |
| **3d** | 2.154 ± 0.036 | 4.081 ± 0.324 | 6.546 ± 0.063 | 4.641 ± 0.158 | 6.229 ± 0.327 |
| **3e** | 4.933 ± 0.351 | 4.059 ± 0.036 | 5.091 ± 0. 358 | 2.208 ± 0.321 | 3.851 ± 0.253 |
| **3f** | 1.036 ± 0.063 | 1.985 ± 0.095 | 2.468 ± 0.117 | 1.447 ± 0.021 | 8.4245 ± 0.215 |
| **3g** | 1.091 ± 0.011 | 1.230 ± 0.012 | 1.295 ± 0.012 | 1.371 ± 0.015 | 2.352 ± 0.012 |
| **3h** | 1.63 ± 0.021 | 1.59 ± 0.027 | 1.89 ± 0.025 | 1.713 ± 0.031 | 1. 915 ± 0.023 |
| **3i** | > 10 | > 10 | > 10 | > 10 | > 10 |
| **3j** | 6.463 ± 0.035 | 6.910 ± 0.032 | 6.751 ± 0.037 | 5.924 ± 0.032 | 6.853 ± 0.042 |
| **3k** | 1.003 ± 0.081 | 1.661 ± 0.142 | 3.515 ± 0.092 | 2.572 ± 0.157 | 2.242 ± 0.106 |
| **4a** | 0.159 ± 0.071 | 0.416 ± 0.056 | 0.776 ± 0.041 | 2.490 ± 0.114 | 2.496 ± 0.098 |
| **4b** | 6.632 ± 0.072 | 4.905 ± 0.0188 | 3.967 ± 0.039 | 8.407 ± 0.603 | 8.591 ± 0.548 |
| Colchicine | 0.023 ± 0.003 | 0.051 ± 0.006 | 0.046 ± 0.005 | 0.043 ± 0.006 | 0.039 ± 0.005 |
| **MPC6827** | 0.005 ± 0.001 | 0.007 ± 0.002 | 0.006 ± 0.002 | 0.005 ± 0.001 | 0.009 ± 0.002 |

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

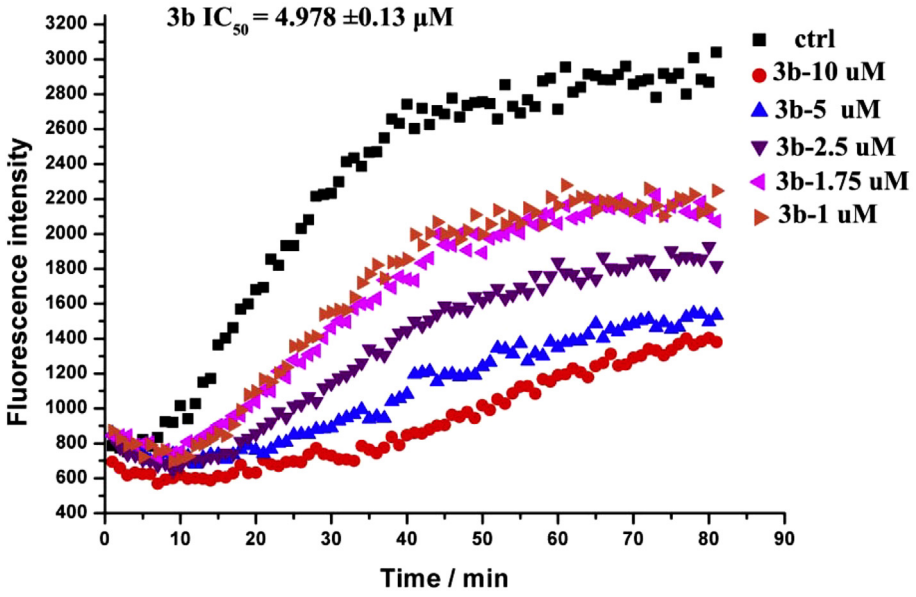



**Fig. 1.** Effects of compounds **3b** on microtubule dynamics. Purified tubulin protein at 10 μM in a reaction buffer was incubated at 37 °C in the absence (control) or presence of **3b** at the indicated concentrations (ranging from 1 to 10 μM). The experiments were performed for three times.

compounds **4a** and **4b**, without methyl on the nitrogen, were prepared and evaluated. It can be seen that the two compounds provided slightly weaker antiproliferative activities than their homologues **3a** and **3b**, which shows the same trend as the literature.[20]

Considering the structural similarity of 4-phenylamino-substituted naphthalene-1,2-diones with the tubulin polymerization inhibitor MPC-6827, we proposed that these compounds should also possess the ability to inhibit tubulin polymerization. As compounds **3a** and **3b** showed better anti-proliferative activity than the others in the initial cytotoxicity screening, we chose **3b** as the typical for further study, which may have better stability due to the presence of the fluorine atom on the 3-position of the aniline moiety. We incubated purified, unpolymerized,

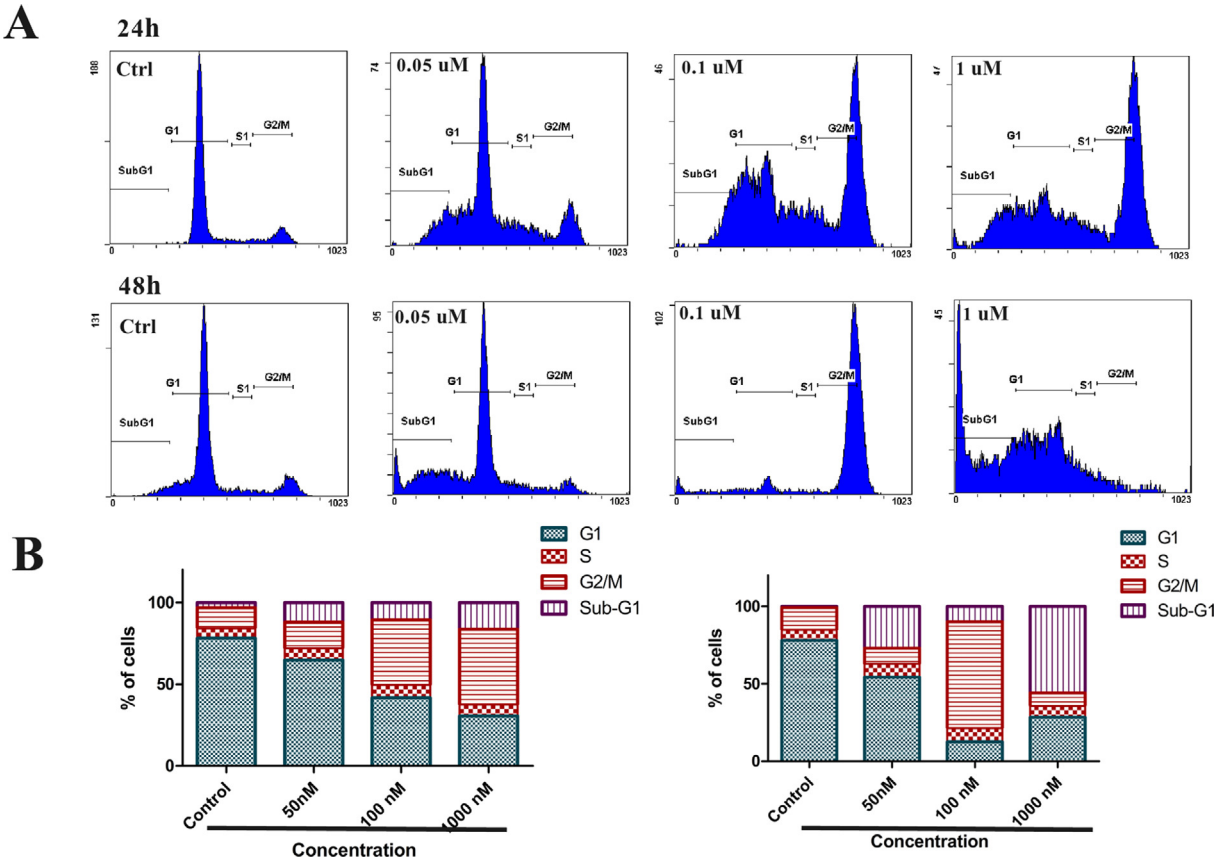



**Fig. 2.** Effect of **3b** on cell cycle arrest. (A) The A549 cells were treated with compound **3b** at 0.05, 0.10, or 1.0 μM for 24 h or 48 h. (B) Quantitative analysis of the percentage of cells in each cell cycle phase was carried out by EXPO32 ADC analysis software. The experiments were performed three times, and the results of representative experiments are shown.

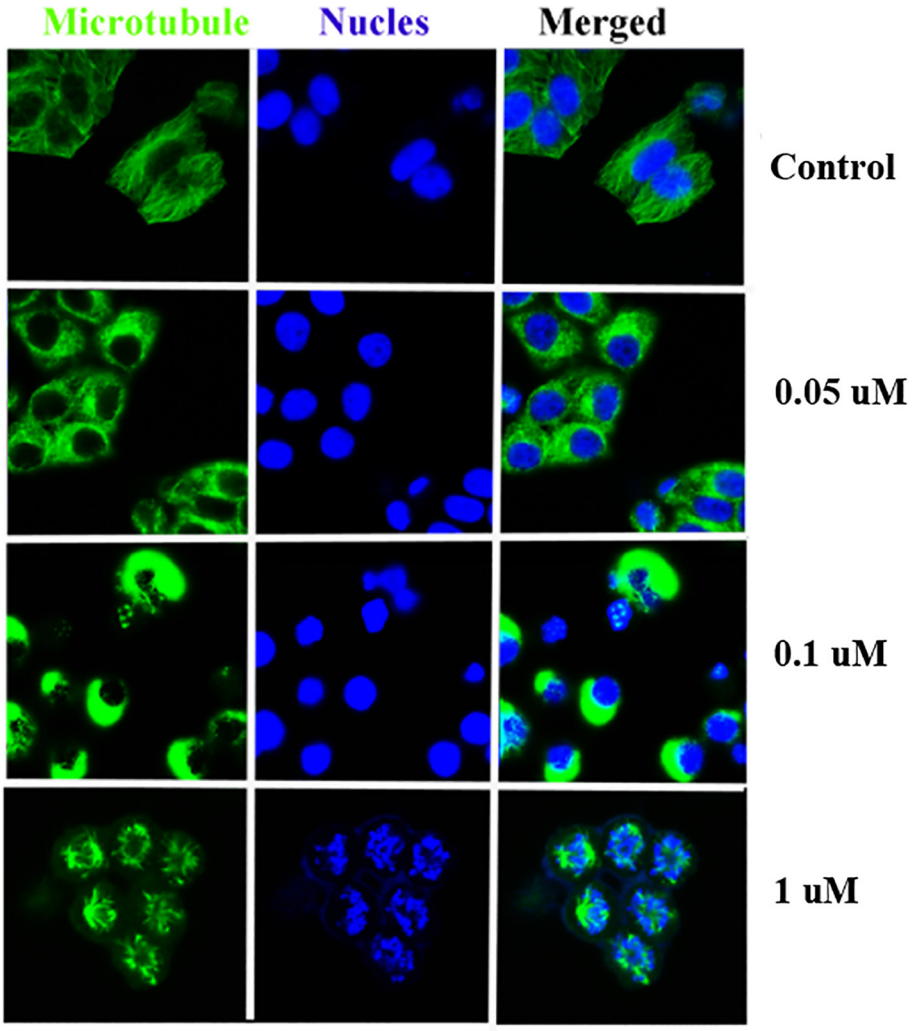



**Fig. 3. 3b** disrupted the organization of the cellular microtubule network at the indicated concentrations. A549 cells were plated in confocal dishes and incubated with **3b** at 0.05, 0.10, and 1.0 μM for 24 h, followed by a direct microscopy assay. The detection of the fixed and stained cells was performed with a LSM 570 laser confocal microscope (Carl Zeiss, Germany). The experiments were performed three times, and the results of the representative experiments are shown.

and microtubule-associated protein-rich tubulin with various concentrations of **3b** and measured the polymerization according to the literature method reported by Bonne et al.[21,22] It can be seen from the results in Fig. 1 that the fluorescence intensity at 410 nm of the control increased with the extension of time, while in the presence of **3b** at concentrations of 1, 1.75, 2.5, 5 and 10 μM, respectively, the fluorescence intensity increases significantly more slowly than that of the control, which indicated that tubulin polymerization was indeed inhibited. The inhibitory concentration that reduces polymerized tubulin by 50% is 4.978 ± 0.13 μM, which indicated that 4-phenylamino-substituted naphthalene-1,2-diones are effective tubulin polymerization inhibitors.

It is known that tubulin-destabilizing agents block the cell cycle in the G2/M phase due to microtubule depolymerization and cytoskeleton disruption.[23] To investigate whether the cytotoxicity induced by the 4-(phenylamino)naphthalene-1,2-dione derivatives was due to cell cycle arrest, we performed flow cytometry analysis using human A549 non-small cell lung carcinoma cells to evaluate the arrest effect on the G2/M transition. After the cells were treated with compound **3b** at 0, 0.05, 0.1, or 1 μM for 24 h, the cells were harvested, and the cell cycle phases were analyzed. As the results show in Fig. 2, **3b** significantly arrested the cell cycle at the G2/M phase in a dose-dependent manner with a concomitant decrease in G1 phase cells. After treatment for 24 h at a concentration of 0.1 μM, 39.88% of the G2/M phase was arrested, and the data rose to 46.18% when the concentration was increased to 1 μM. When the incubation time was extended to 48 h, the G2/M cell cycle arrest effect was more obvious at 0.1 μM (68.79%). However, when exposed at a concentration of 1 μM, the sub-G1 peak (a characteristic hypodiploid peak) was the main peak (55.94%), which implied that most cells underwent DNA fragmentation.

The tubulin-microtubule system is very important in the maintenance of cellular morphology. To determine whether compound **3b** could affect microtubule dynamics in living cells, we performed a laser confocal microscopy assay that involved the disruption of microtubule dynamics with A549 cells. The results in Fig. 3 clearly show the heavy disruption of the microtubule system in A549 cells after treatment with different concentrations of compound **3b** compared with the control. The microtubule network became disorganized with the treatment of compound **3b** and was completely disrupted as the concentration of **3b** increased. When the concentration was increased to 1.0 μM, the microtubule spindles had obviously shrunken around the center of the cells; the cytoplasm and the chromosomes appeared more irregular than in the cells treated with the 0.05 and 0.1 μM concentrations. In comparison, the control cells predominantly exhibited at interphase of the cell cycle, which was characterized by uncondensed chromosomes and the regularly assembled, slim, and fibrous microtubules wrapped

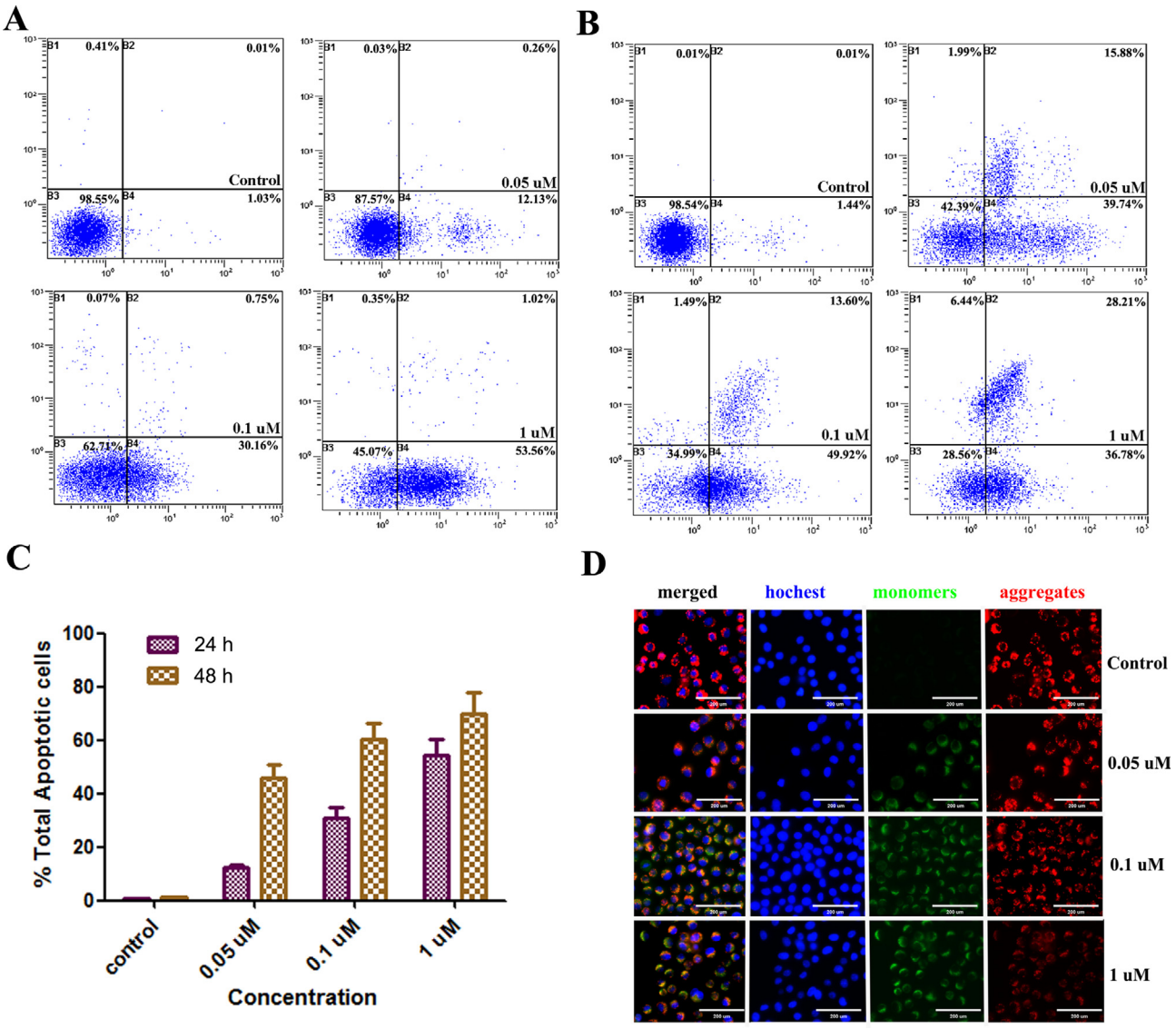



**Fig. 4.** Compound **3b** induced apoptosis and decreased the mitochondrial membrane potential in a dose- and time-dependent manner. Dot plot representation of Annexin-V-FITC-fluorescence (x-axis) vs PI-fluorescence (y-axis) of the apoptotic A549 cells (Annexin-V positive) cells, treated with compound **3b** (0.05, 0.1, and 1.0 μM) for 24 h (A) or 48 h (B). A549 cells were treated with **3b** at the indicated concentration (0.05, 0.1, and 1.0 μM) or DMSO (0.01%) for 24 h, followed by incubation with the fluorescence probe JC-1 for 30 min. Then, the cells were analyzed by fluorescence microscope (D). The experiments were performed at least three times, and the results of the representative experiments.

around the cell nucleus.

Apoptosis play an important role in tumor biology. To evaluate the ability of compound **3b** to induce the apoptosis of cancer cells, we carried out flow cytometry assay using propidium iodide (PI) and fluorescent immunolabeling of the protein annexin-V (V-FITC) with A549 cells and the result was shown in Fig. 4. At the concentrations of 0.05, 0.1 and 1.0 μM of **3b** for 24 h, the early and late apoptosis cells were found to be 12.39%, 30.91%, 54.58% comparing with 1.04% of the control (DMSO, 0.01%), respectively (Fig. 4A). The total apoptosis cells increased to 55.62%, 63.52%, 64.99% when the incubation time was extended to 48 h (Fig. 4B), respectively.

Mitochondrial dysfunction plays a vital role in the progression of apoptosis,[24] To investigate the possible involvement of mitochondrial dysfunction in **3b**-induced apoptosis of the cells, the quantitative mitochondrial transmembrane potential (MMP) assay was performed with JC-1 staining. The results in Fig. 4d demonstrated that when the A549 cells were exposed to the concentrations at 0.05, 0.10, and 1.0 μM for 24 h, a rapid collapse of MMP was detected in contrast to the control (DMSO, 0.01%), which accumulate fluorescent dye from its red-aggregated to green-monomeric forms.

To evaluate the ability of **3b** to generate ROS in cancer cells, an assay with an oxidation-sensitive fluorescent probe (DCFH-DA) was performed, and the results are shown in Fig. 5. The green fluorescence reflects the level of intracellular ROS, which was hardly observed in the control group. After compound **3b** was added to the A549 cell line for 12 h, the intensity of the green fluorescence increased significantly in contrast to the control group. Furthermore, there is also a dose-dependent relationship between **3b** and the ROS. It was obvious that the intensity of the green fluorescence became progressively brighter when the concentration of compound **3b** was increased.

To evaluate whether the new 4-phenylamino-substituted naphthalene-1,2-diones could act as carboxylesterase inhibitors, compound **3b**, which exhibited potent antiproliferative activities for human cancer cell lines, was chosen for the carboxylesterase inhibition assay according the method reported in literature[25] using **3a** and **3h** as the reference compounds. The results in Table 2 indicated that all the three compounds were reasonable inhibitors of human liver carboxylesterase with the Ki values range from 15.6 to 92.3 nM.

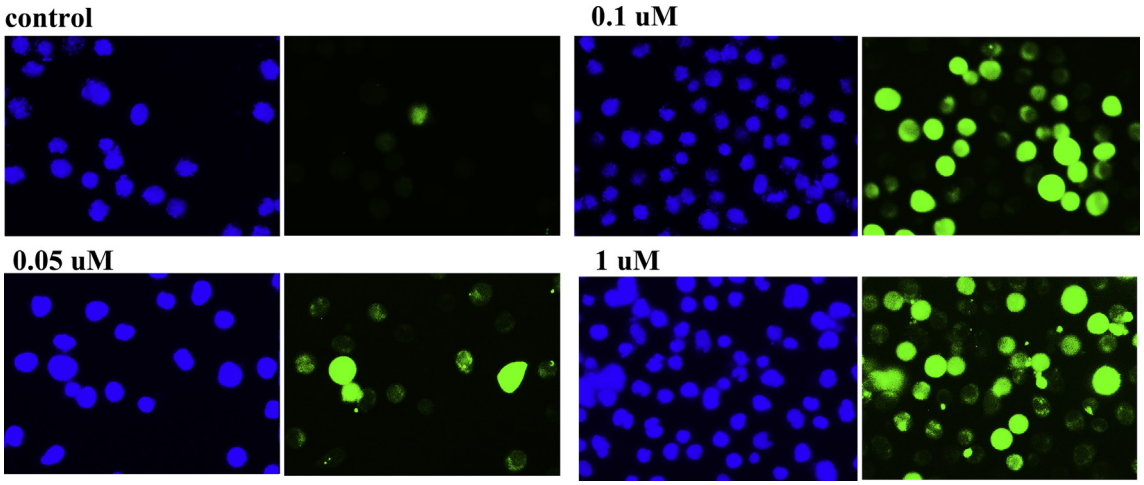
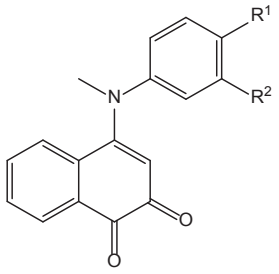



**Fig. 5.** The A549 cells were treated with **3b** at different concentrations (0.1, 0.5, and 1 μM) or DMSO (0.01%) for 12 h using an oxidation-sensitive fluorescent probe (DCFH-DA). The green fluorescence intensity, which corresponded to the intracellular ROS level, was measured using a microplate reader with the excitation and emission wavelengths set at 488 and 525 nm, respectively.

**Table 2**
Esterase Inhibition data for Compound **3a**, **3b** and **3h**.




| Comp. | $R^1$ | $R^2$ | hCE1 $K_i$ (nM ± SE; *o*-NPA) |
|---|---|---|---|
| **3a** | $OCH_3$ | H | 87.5 ± 5.1 (81.6 ± 4.7)[a] |
| **3b** | $OCH_3$ | F | 92.3 ± 4.3 |
| **3h** | $CH_3$ | H | 15.6 ± 0.7 (11.1 ± 0.2)[a] |

[a] The literature value (brackets) in Ref. 17.

**Table 3**
Anti-proliferative activities of compound **3a** and **3b** towards human normal cell lines.[a]

| | **3a** | **3b** |
|---|---|---|
| $IC_{50}$ (μM) against LO2 cell lines | 4.096 ± 0.009 | 3.566 ± 0.013 |
| $IC_{50}$(μM) against A549cell lines | 0.097 ± 0.004 | 0.093 ± 0.019 |
| Selectivity ratio | 42.23 | 38.34 |

[a] Data are presented as the mean ± SE from the dose-response cures of at least three independent experiments; selectivity ratio = ($IC_{50}$ LO2 cell)/($IC_{50}$ A549).

As low toxicity to normal cells is one of the important indicators for evaluating anti-tumor lead compounds, we further evaluated the activities of **3a** and **3b** against LO2, the human normal cells. The results in Table 3 indicated that **3a** and **3b** provided very low cytotoxicity against LO2 cells (**3a**: 4.096 μM; **3b**: 3,566 μM). The selectivity factor values (RF: 42.23 and 38.34) suggested **3a** and **3b** possess low toxicity towards human normal cell lines.

In a conclusion, developing effective antitumor drugs is an important task for pharmacologists. Inspired by the report that β-lapachones are reversible selective inhibitors of human liver carboxylesterase, which is responsible for the metabolism of some drugs such as the anticancer drugs irinotecan and oseltamivir, we synthesized and evaluated a series of 4-phenylamino-substituted naphthalene-1,2-dione derivatives as effective antiproliferative agents. The MTT assay results indicated that some of the compounds, especially **3a** and **3b**, exhibited potent antiproliferative activities against five human cancer cells with $IC_{50}$ values below the micromolar level. We have studied the antiproliferative mechanism of these compounds, such as inducing mitochondrial depolarization, resulting in intracellular ROS production, and acting as tubulin polymerization inhibitors. All of these results indicated that compound **3a** or **3b** has the potential to act as a lead compound for further evaluation, especially as a combination drug with irinotecan and oseltamivir that are easily metabolized by liver carboxylesterase.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.07.047.

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