

Design, Synthesis and Anti-tubulin Activity of Novel Dinitro Diphenyl Ether Derivatives as Potent Anticancer Agent

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Abstract

In this study, a series of Dinitrodiphenyl Ether derivatives (1b-5b) were synthesized and their biological activities were also evaluated as potential tubulin inhibitors. Among them, compound 3b ($IC_{50}=1.26 \pm 0.84 \mu M$) exhibited the most potent anti-proliferative activity of MCF-7 cancer cell line *in vitro*, which was comparable to that of colchicine ($IC_{50}=0.93 \pm 0.46 \mu M$) as reference. And it also showed the most potent growth inhibitory activity against tubulin ($IC_{50}=5.10 \pm 0.81 \mu M$), which was also compared with colchicine ($IC_{50}=3.42 \pm 0.63 \mu M$). Molecular docking simulation results demonstrated that 3b could bind to the colchicine binding site of tubulin and effectively inhibit tubulin polymerization. Moreover, the subsequent cell cycle arrest and apoptosis assay further confirmed that 3b could cause MCF-7 cell cycle arrest in the middle and later stages of mitosis and thus leading to cell apoptosis. To sum up, we recommend 3b as the best candidate of potential anti-cancer agent among these new compounds. Based on the preliminary results, compound 3b with potent inhibitory activity in tumor growth may be a potential anti-cancer agent.

Keywords: Anti-cancer; Dinitrodiphenyl ether derivatives; Anti-tubulin

Introduction

Microtubules are major cytoskeletal components of eukaryotic cells that are involved in multiple cellular processes [1]. It always combined with other protein to assemble spindle, centriole, flagellum, nerviduct etc. Microtubules are critical elements in a variety of fundamental cell functions, including sustained shape, transportation of vesicles and protein complexes, and regulation of motility and cell division [2]. Small molecules inhibitors can cause mitotic arrest and induce apoptosis via interfere with the dynamics of tubulin polymerization and depolymerization. Hence, microtubules have become an attractive pharmacologic target for anticancer drug discovery [3].

The three characterized binding sites of tubulin are the taxane, vinca alkaloid and colchicine sites [4]. The well-characterized clinically used microtubule inhibitors are the taxanes and the Vinca alkaloids. Taxanes, such as paclitaxel and docetaxel, are newer antimetabolic agents that stabilize microtubules and induce a net polymerization. The established class of Vinca alkaloids, such as vincristine, vinblastine, and vinorelbine, bind to the tubulin dimer, block the formation of new microtubules, and lead to the depolymerization of existing microtubules [5]. However, the colchicine binding site inhibitors that are successfully used in clinical tumor treatment has not been reported.

The first colchicine site inhibitor, colchicine, was extracted from the poisonous meadow saffron *Colchicum autumnale* L, but its high toxicity has limited its therapeutic applications [6]. Combretastatin A-4 (CA-4), a natural product isolated by Pettit and co-workers in 1982 from the South African bush willow tree *Combretum caffrum*, strongly inhibits the polymerization of tubulin by binding to the colchicine site [7]. However, CA-4 exerts potent cytotoxicity against a variety of human cancer cells including multidrug resistant (MDR) cancer cell lines. Despite its very potent cytotoxic and antitubulin activities *in vitro*, CA-4 as an antimetabolic agent showed poor antitumor effects *in vivo* models due to its poor water solubility [8]. Given that, the researchers have to looking for other CA-4 analogues which can address its limitations [9,10].

It is widely reported that Schiff's base is a promising skeleton that has shown the good antitubulin activity. It displays a variety of biological activities, such as anti-cancer, anti-inflammatory, anti-tuberculosis, anti-fungal, activities, and as herbicide [11,12]. Their broad biological properties are largely due to the exocyclic-RC=N-bond. The introduction of substituents to the terminal bonds of Schiff's base remains an area of pharmacological interest to activate Schiff's base. For instance, Zhao et al. synthesized a series of 2,6-dinitro-4-(trifluoromethyl) phenoxyacylaloxime derivatives which share the similar part with it [13]. And among them, compound 2h showed the most potent anti-proliferative activity against cancer cell lines *in vitro* and significant tubulin polymerization inhibitory activity. The author considered that the incorporation of 1,3-dinitro-5-(trifluoromethyl) benzene could contribute to the binding of target compounds with tubulin. Because trifluoromethyl possess lipophilicity to penetrate the cell membrane. And the isosteric substitution of hydrogen by the strong electron-withdrawing effect of CF₃ groups may increase the lipophilicity and thus enhance the rate of cell penetration, which is a very important feature in drug delivery *in vivo*, referring to prokaryotic as well as eukaryotic cells.

Meanwhile, dinitrobenzene, a widely used chemical fragment, always been considered to drug design. Zhao et al. synthesized a series of flavone and isoflavone derivatives which contains dinitrobenzene moiety and evaluated them as kinesin spindle protein inhibitors [14].

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George et al. reported that the Dinitrodiphenyl Ether sulfonamides shows antikinoplastid antimetabolic activity and can increase metabolic stability against parasites [15]. Though structure-activity relationship analysis, Fennell et al. synthesized a series of antimalarial agent which contain Dinitrodiphenyl Ether moieties and determined the target as tubulin [16].

Our current work continues efforts to design and synthesize a series of Dinitrodiphenyl Ether compounds as novel heterocyclic CA-4 analogs and to evaluate their biological activities for further drug development. These compounds strongly inhibited the growth of cancer cell lines and tubulin polymerization by binding to the colchicine site of tubulin and caused cells to arrest in the G2/M phase of the cell cycle.

Experiment

Materials and measurements

All chemicals and reagents used in current study were analytical grade. Thin layer chromatography (TLC) was performed on the glass-backed silica gel sheets (Silica Gel 60 GF254) and visualized in UV light (254 nm). Separation of the compounds by column chromatography was carried out with silica gel 60 (200-300 mesh ASTM, E. Merck). The quantity of silica gel used was 50-100 times the weight charged on the column. Melting points were determined on an XT4 MP apparatus (Taike Corp., Beijing, China). All the Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Bruker PX500 or DPX300 model Spectrometer in DMSO- d_6 at 25°C with TMS and chemical shifts were reported in parts per million (δ). The solvent signals allotted as internal stands. Elemental analyses were performed on a CHNeO-Rapid instrument. All the compounds gave satisfactory chemical analyses ($\pm 0.4\%$).

General procedure for the synthesis of compounds 1b-5b

N-(4-(2,6-dinitro-4-(trifluoromethyl)phenoxy)-3-methoxybenzylidene)-2-methylaniline (1b): Yellow powder, yield 78.2%, mp: 89-90°C. 1H NMR (300 MHz, $CDCl_3$): δ 8.78(s, 2H, Ar-H), 8.20(s, 1H, C-H), 7.15-7.05 (m, 6H, Ar-H), 6.90(d, $J=12.1$ Hz, 1H, Ar-H), 4.09 (s, 3H, Ar-O- CH_3), 2.15 (s, 3H, Ar- CH_3). ESI-TOF, calcd for $C_{22}H_{16}F_3N_3O_6$ ($[M+Na]^+$) 498.2320, found 498.1562. Anal. Calcd for $C_{22}H_{16}F_3N_3O_6$: C, 55.58; H, 3.39; N, 8.84; Found: C, 55.69; H, 3.48; N, 8.92.

N-(4-(2,6-dinitro-4-(trifluoromethyl)phenoxy)-3-methoxybenzylidene)-2-fluoroaniline (2b): Yellow powder, yield 68.8%, mp: 84-86°C. 1H NMR (300 MHz, $CDCl_3$): δ 8.82(s, 2H, Ar-H), 8.25 (s, 1H, C-H), 7.10-7.02 (m, 6H, Ar-H), 6.98(d, $J=11.7$ Hz, 1H, Ar-H), 4.25 (s, 3H, Ar-O- CH_3), 2.19 (s, 3H, Ar- CH_3). ESI-TOF, calcd for $C_{21}H_{13}F_4N_3O_6$ ($[M+Na]^+$) 502.3420, found 502.2584. Anal. Calcd for $C_{21}H_{13}F_4N_3O_6$: C, 52.62; H, 2.73; N, 8.77; Found: C, 52.89; H, 2.68; N, 8.72.

N-(4-(2,6-dinitro-4-(trifluoromethyl)phenoxy)-3-methoxybenzylidene)-2,4-difluoroaniline (3b): Yellow powder, yield 88.5%, mp: 86-87°C. 1H NMR (300 MHz, $CDCl_3$): δ 8.90 (s, 2H, Ar-H), 8.29(s, 1H, C-H), 7.18-7.09 (m, 6H, Ar-H), 6.98(d, $J=12.5$ Hz, 1H, Ar-H), 3.99 (s, 3H, Ar-O- CH_3), 2.15 (s, 3H, Ar- CH_3). ESI-TOF, calcd for $C_{21}H_{12}F_5N_3O_6$ ($[M+Na]^+$) 520.3325, found 520.2893. Anal. Calcd for $C_{21}H_{12}F_5N_3O_6$: C, 50.72; H, 2.43; N, 8.45; Found: C, 50.77; H, 2.50; N, 8.62.

3-bromo-N-(4-(2,6-dinitro-4-(trifluoromethyl)phenoxy)-3-methoxybenzylidene)-5-(trifluoromethyl)aniline (4b): Yellow powder, yield 80%, mp: 90-92°C. 1H NMR (300 MHz, $CDCl_3$): δ 8.85 (s, 2H, Ar-H), 8.23(s, 1H, C-H), 7.16-7.05 (m, 6H, Ar-H), 6.86 (d, $J=11.9$

Hz, 1H, Ar-H), 3.90 (s, 3H, Ar-O- CH_3), 2.28 (s, 3H, Ar- CH_3). ESI-TOF, calcd for $C_{22}H_{12}BrF_6N_3O_6$ ($[M+Na]^+$) 631.2410, found 631.2522. Anal. Calcd for $C_{22}H_{12}BrF_6N_3O_6$: C, 43.44; H, 1.99; N, 6.91; Found: C, 43.50; H, 2.10; N, 7.12.

N'-(4-(2,6-dinitro-4-(trifluoromethyl)phenoxy)-3-methoxybenzylidene)benzohydrazide (5b): Yellow powder, yield 65%, mp: 83-85°C. 1H NMR (300 MHz, $CDCl_3$): δ 8.86(s, 2H, Ar-H), 8.24(s, 1H, C-H), 7.14-7.00 (m, 6H, Ar-H), 6.84(d, $J=11.2$ Hz, 1H, Ar-H), 3.95 (s, 3H, Ar-O- CH_3), 2.20 (s, 3H, Ar- CH_3). ESI-TOF, calcd for $C_{22}H_{15}F_3N_4O_7$ ($[M+Na]^+$) 527.3712, found 527.3805. Anal. Calcd for $C_{22}H_{15}F_3N_4O_7$: C, 52.39; H, 3.00; N, 11.11; Found: C, 52.50; H, 3.10; N, 11.23.

Anti-proliferation activity

The anti-proliferation activity of the prepared compounds against three cancer cell lines, MCF-7, A549 and MDA-MB-231 and one normal cell line, L02 were evaluated as described elsewhere with some modifications. Target tumor cell line was grown to log phase in DMEM medium supplemented with 10% fetal bovine serum. After diluting to 2×10^4 cells mL^{-1} with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The subsequent incubation was permitted at 37°C, 5% CO_2 atmosphere for 24 hours before the cytotoxicity assessments. Tested samples at pre-set concentrations were added to 6 wells with colchicine as positive reference because it can bind to the active site of tubulin nicely. After 48 hours exposure period, 40 μL of PBS containing 2.5 mg mL^{-1} of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well. Four hours later, 100 μL extraction solutions (10% SDS-5% isobutyl alcohol-0.01 M HCl) was added. After an overnight incubation at 37°C, the optical density was measured at a wavelength of 570 nm on a microplate reader ELx 800 (BioTek, Highland Park, Winooski, VT, USA). In all experiments three replicate wells were used for each drug concentration. Each assay was carried out at least three times. The results are summarized in Table 1.

Effects on tubulin polymerization assay

To evaluate the effect of the compounds on tubulin assembly *in vitro*, varying concentrations were pre-incubated with 10 μM tubulin in glutamate buffer at 30°C and then cooled to 0°C. After addition of GTP, the mixtures were transferred to 0°C cuvettes in a recording spectrophotometer and warmed-up to 30°C and the assembly of tubulin was observed turbid metrically. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after 20 minutes incubation.

Cell cycle assay

MCF-7 cells were plated in 6-well plates (5.0×10^3 cells/well) and incubated at 37°C for 24 hours. Exponentially growing cells were then incubated with the compound 3b at 1 μM , 3 μM and 10 μM . Untreated cells (control) or cells treated with the compounds solvent (DMSO) were included. DMSO was used at the highest concentration used in the experiments. After then, cells were centrifuged and fixed in 70% ethanol at 4°C for at least 12 hours and subsequently resuspended in PBS containing 0.1 mg mL^{-1} RNase A and 5 μg mL^{-1} propidium iodide (PI). Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry using FACScan cytofluorometer (PT. Madagasi Brosa Inc. Jl. Batang Hari NO.73, Propinsi Sumatera Utara, Indonesia) plotting at least 20,000 events per sample. The percentage of cells in the G1, S and G2/M phases of the cell cycle and the percentage of cells in the sub-G1 peak were determined using the Flowjo 7.6.1

software after cell debris exclusion.

Apoptosis

For Annexin V/PI assays, MCF-7 cells were stained with Annexin V-FITC and PI and then monitored for apoptosis by flow cytometry. Briefly, 0.5×10^6 cells were washed with PBS and stained with 5 μ L of Annexin V-FITC and 2.5 μ L of PI (5 μ g mL⁻¹) in 1x binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl₂) for 30 minutes at room temperature in the dark. Apoptotic cells were quantified using a FACScan cytofluorometer (PT. Madagasi Brosa Inc. Jl. Batang Hari No. 73, Propinsi Sumatera Utara, Indonesia.). Statistical analysis was done using Flowjo 7.6.1 software. Both early apoptotic (AnnexinV-positive, PI-negative) and late apoptotic (double positive of Annexin V and PI) cells were detected.

Results and Discussion

Chemistry

The synthesis of Dinitrodiphenyl Ether derivatives followed the general reaction pathway outlined in Scheme 1. 0.1 mol vanillina and 0.1 mol phenylamine derivatives were dissolved in 50 mL ethyl alcohol and refluxing for a few hours until vanillina disappeared. Then, cooling the solution to room temperature to afford the pure products (1a-5a). After then, 0.01 mmol 4-trifluoromethyl-3,5-dinitrochlorobenzene and 0.01 mmol 1a-5a were dissolved in 20 mL DMF and stirred at room temperature for several hours. Then, pouring it into ice and stirring until the solution was dissolved in ice-water completely. After filtering, raw products were obtained and recrystallized to offered pure drugs (1b-5b) and the chemical structures were shown in Figure 1. All the compounds were reported for the first time. All of the synthetic compounds gave satisfactory analytical and spectroscopic data. ¹H NMR and ESI-MS spectra were consistent with the assigned structures.

Bioassay

Anti-proliferative activity: All synthesized Dinitrodiphenyl Ether derivatives 1b-5b were evaluated for their ability of anti-proliferative activities against three cancer cell lines, MCF-7, A549 and MDA-MB-231 and one normal cell lines, L02. The results are summarized in

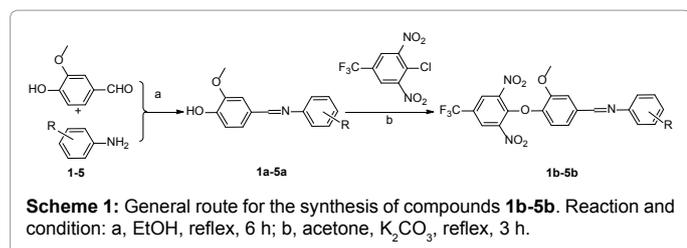
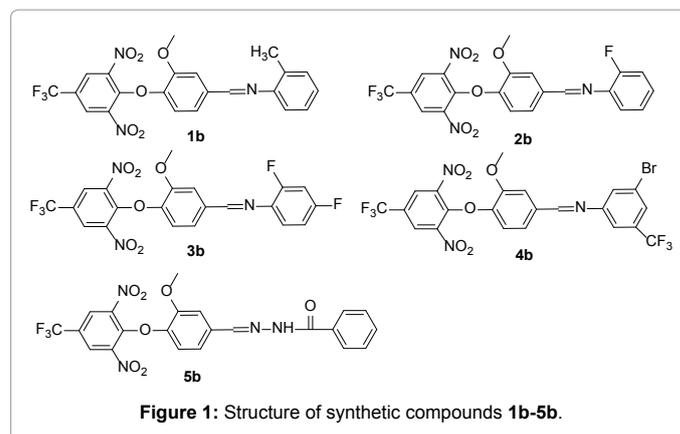


Table 1. In general, all of them showed remarkable inhibition effects on cancer cell proliferation, especially for compound **3b** (IC₅₀=1.26 ± 0.84 μ M) which showed a comparable effect to colchicine (IC₅₀=0.93 ± 0.46 μ M) against MCF-7 cells through inhibiting tubulin polymerization. Additionally, they showed low cytotoxicity towards non-cancer cells when compared with colchicine.

Effects of compound 3b on tubulin polymerization: Then we performed the tubulin assembly assay to examine whether the compounds interact with tubulin and inhibit tubulin polymerization *in vitro*. As is shown in Table 1, **3b** showed the most potent anti-tubulin activity and their 50% tubulin polymerization inhibition are 5.10 ± 0.81 μ M, which are similar to that of colchicine (IC₅₀=3.42 ± 0.63 μ M). In the binding model, compound **3b** is nicely bound to the colchicine binding site of tubulin via one hydrogen bond with ALA 250 and one π bond with CYS 241 (Figure 2A), and the binding energy was -52.8116 kcal. For Figure 2B, the binding poses of colchicine to tubulin, two hydrogen bonds with VAL 181 and CYS241, and one π bond with CYS241 were formed to stabilize the binding between colchicine and tubulin. The binding energy between them was -55.3687 kcal. In view of the binding energy and binding poses, the interaction between compound **3b** is similar to that of colchicine. The molecular docking results argue that compound **3b** may be a potential anti-tubulin inhibitor via binding to the colchicine binding site of tubulin and then inhibit tubulin polymerization.

Effects of compound 3b on MCF-7 cell cycle distribution: Considering that tubulin could be the target of **3b**, we detected the effect of **3b** on cell cycle distribution. As shown in Figure 3, MCF-7 cells were treated with **3b** at different concentrations for 24 h could arrest cell cycle in G2/M phase in a time-dependent manner. When the drug concentration was increased to 10 μ M, 23.34% cells were arrest in G2/M phase.



Compound	IC ₅₀ ± SD ^a (μ M)				Tubulin ^b
	MCF-7	A549	MDA-MB-231	L02	
1b	3.13 ± 1.25	4.36 ± 1.22	3.86 ± 0.98	78.6 ± 1.87	11.86 ± 0.87
2b	4.45 ± 0.68	4.98 ± 0.55	5.51 ± 0.96	86.3 ± 3.35	6.84 ± 0.96
3b	1.26 ± 0.84	2.17 ± 0.46	3.28 ± 0.53	98.6 ± 3.54	5.10 ± 0.81
4b	5.47 ± 1.27	1.43 ± 1.09	5.54 ± 1.14	76.5 ± 1.56	8.46 ± 1.27
5b	2.53 ± 0.41	3.63 ± 0.71	3.56 ± 0.27	88.9 ± 2.36	10.47 ± 1.75
Colchicine	0.93 ± 0.46	1.25 ± 0.67	2.11 ± 0.97	3.15 ± 0.56	3.42 ± 0.63

^aInhibition of the growth of tumor cell lines; ^bInhibition of tubulin polymerization

Table 1: Inhibition (IC₅₀) of MCF-7, A549, MDA-MB-231 cells proliferation and tubulin polymerization by compounds **1b-5b**.

Effects of compound 3b on MCF-7 cell apoptosis: We next treated MCF-7 cells with varying concentrations of compound 3b and analysed cells for changes in apoptotic markers by flow cytometer *in vitro*. As shown in Figure 4, after treated with increasing concentrations of 3b, the percentage of killed cells has a significant increase. When the concentration increased to 10 μ M, 5.13% and 33.2% cells presented as early and later apoptosis, respectively.

Conclusion

In this paper, a series of novel Dinitrodiphenyl Ether derivatives compounds (1b-5b) were synthesized and evaluated their anti-cancer activities. These compounds exhibited potent cell growth inhibitory activities and anti-proliferative activities against human MCF-7, A549 and MDA-MB-231 cell lines. Among them, compound 3b showed the most potent inhibition activity with IC_{50} values of 1.26 ± 0.84 μ M against human MCF-7 cell line, which was comparable to that of colchicine ($IC_{50} = 0.93 \pm 0.46$ μ M). *In vitro* tubulin polymerization assay and molecular docking simulation results demonstrated that 3b could bind to the colchicine binding site of tubulin and effectively inhibit tubulin polymerization. Moreover, the subsequent cell cycle arrest

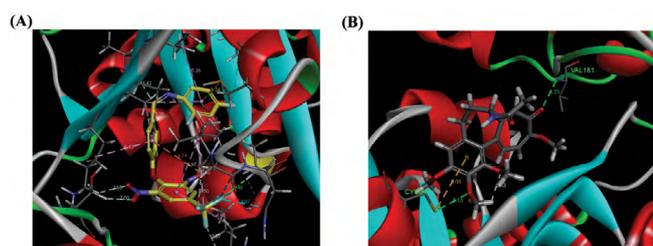


Figure 2: The binding mode between the active conformation of compound 3b, colchicine and the target tubulin (PDB code: 1sa0) provided by the CDOCKER protocol (Discovery Studio 3.5, Accelrys, Co. Ltd).

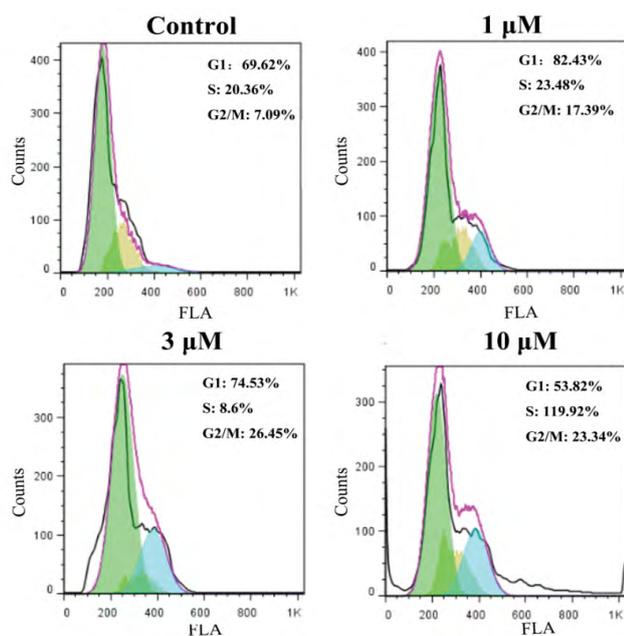


Figure 3: Effect of 3b on the cell cycle distribution of MCF-7 cells (Green, G1 phase; yellow, S phase; blue, G2/M phase).

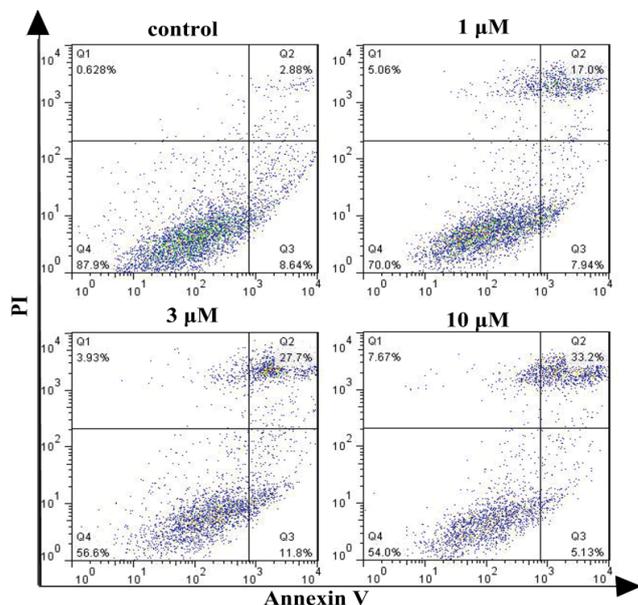


Figure 4: Annexin V/PI dual-immuno-fluorescence staining of MCF-7 cells treated with 3b for 24 h.

and apoptosis assay further confirmed that 3b could cause MCF-7 cell cycle arrest in the middle and later stages of mitosis and thus leading to cell apoptosis. To sum up, we recommend 3b as the best candidate of potential anti-cancer agent among these new compounds.

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