The Effects of Curcumin-based Compounds on Proliferation and Cell Death in Cervical Cancer Cells

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Abstract. *Aim: To systematically investigate the effects of a class of curcumin-based compounds on cancer cell viability, proliferation, and apoptosis. Materials and Methods: Cytotoxicity and anti-proliferative potency were estimated by the trypan blue exclusion assay and WST-1 cell proliferation assay, respectively. Cell death pathways were discriminated according to plasma membrane integrity and lipid asymmetry cell profiles using a F2N12S and CYTOX AADvanced double staining flow cytometry-based assay. Results: Nine compounds (**2-10**) exhibit 13- to 58-fold better cytotoxic and anti-proliferative potencies than curcumin towards HeLa cells. In this cervical cancer cell model, dienone and 1-methylpiperidone serve as the favorable central linkers; 5-methylisoxazol-3-yl and 3-methylisoxazol-5-yl act as the optimal terminal aromatic moiety. Finally, the effects of compounds **6** and **10** on HeLa cells' plasma membrane integrity and lipid asymmetry suggest that the early cytotoxic effect of these compounds is due to a stimulation of apoptosis.*

Curcumin (**1**, Figure 1), extracted from *Curcuma longa* (turmeric) of the *Zingiberaceae* family, represents a typical example of dietary natural products displaying a wide range of interesting medicinal properties. The low cancer incidence in Asia has been, at least partly, demonstrated to be associated with the Asian diet (including turmeric). The anticancer potential of curcumin was first reported by Kuttan and co-workers in 1985 (1). Curcumin has already entered several human clinical studies for potential prevention and treatment of cancer (2, 3) but its clinical development has been slown-down by its poor pharmacokinetic profile (4, 5). Numerous curcumin-based compounds have been explored as potential anticancer agents (6-8). Recently, we reported a class of potential curcumin-based anticancer agents, structurally characteristic of a metabolically-stable monoketone central linker and two terminal basic nitrogen-containing heteroaromatic rings (9). They exhibited better cytotoxicity than curcumin against two androgen-refractory PC-3 and DU-145 human prostate cancer cell lines. Intriguingly, the four most promising curcumin analogs were demonstrated to be more potent than curcumin also against an aggressive cervical cell line (HeLa) and showed no discernible cytotoxicity towards normal mammary epithelial cells. These results spurred us to systematically investigate i) their cytotoxicity and anti-proliferative activity towards the HeLa cell line; and ii) their inductive ability towards cell apoptosis.

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Key Words: Curcumin-based compounds, cytotoxicity, anti-proliferative activity, apoptosis, cancer.

Materials and Methods

Synthesis of curcumin and its analogs. Curcumin was synthesized by Claisen-Schmidt condensation of aromatic aldehyde with acetylacetone according to the procedure illustrated in the literature (10). All curcumin-based compounds were synthesized following the identical procedures, as we described elsewhere (9). The structures of the synthesized compounds were characterized by interpreting their ^{1}H-nuclear magnetic resonance spectroscopy (NMR) data, which are consistent with those reported in the literature (9). The structures were also confirmed by comparison with the authenticated sample by thin layer chromatography using different solvent systems (dichloromethane-methanol=95:5; hexane-ethyl acetate=2:1).

Cell culture. HeLa line was initially purchased from the American Type Culture Collection (ATCC™, Manassas, VA, USA). The HeLa cervical cancer cell line was routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultures were maintained in a high humidity environment supplemented with 5% carbon dioxide at a temperature of 37˚C.

Trypan blue dye exclusion assay. HeLa cells were plated in 24-well plates at a density of 20,000 each well in 10% FBS RPMI-1640 medium. The cells were then treated with curcumin, or synthesized analogs separately at different doses for 3 days, while equal treatment volumes of DMSO were used as vehicle control. Cells were stained using a 0.4% trypan blue solution and counted with a

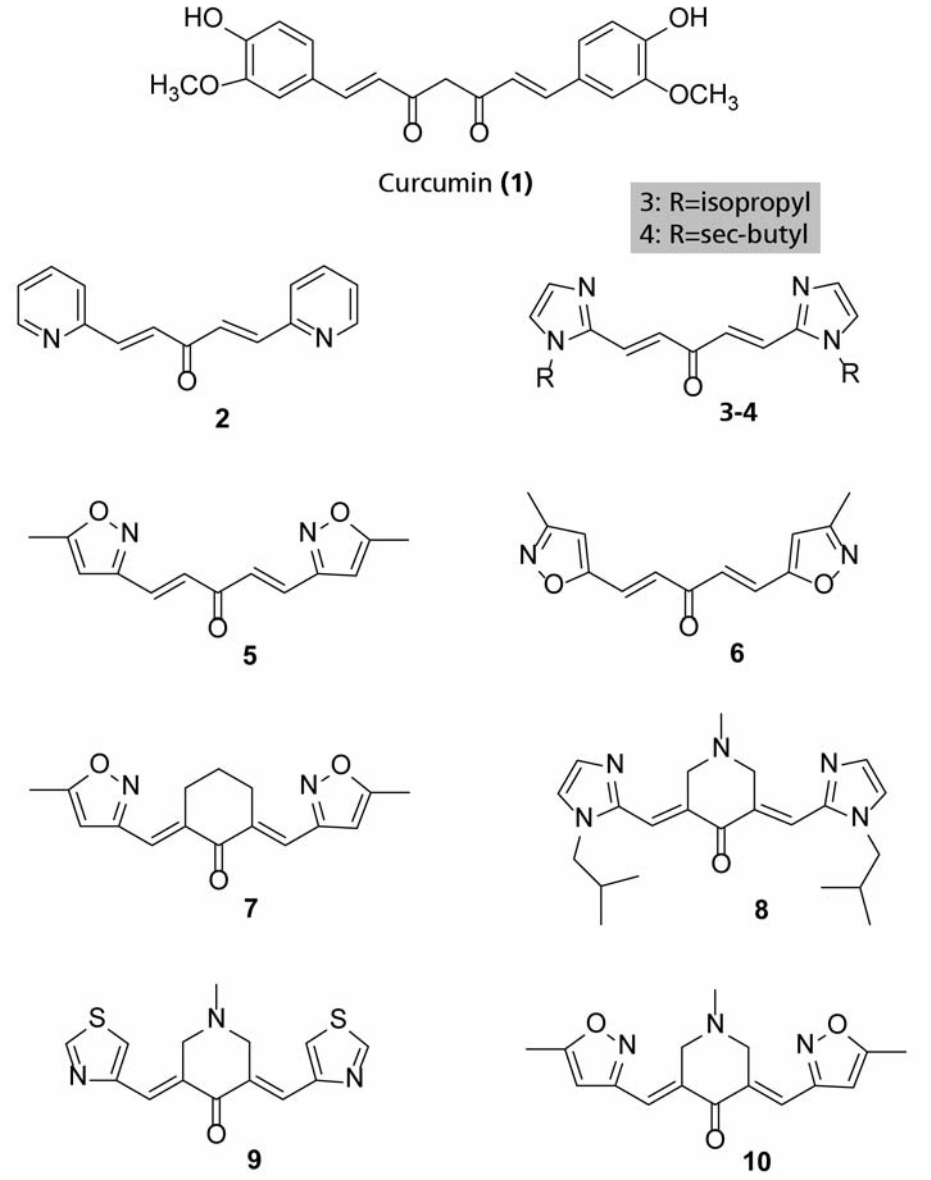

Figure 1. *Structures of curcumin and its analogs.*

cell viability analyzer (Beckman Coulter, Miami, FL, USA). The ratio of drug-treated viable cell numbers to vehicle-treated viable cell numbers was defined as percentage viability and variation between replicate experiments was not greater than 5%. For this assay, the half-maximal inhibitory concentration (IC_{50}) value was considered the concentration of each compound that triggers the loss of plasma membrane integrity of 50% of the cell population under the experimental conditions, and was the average of triplicate determinations. For calculating the IC_{50} values, a linear inhibition curve was obtained for each compound using at least five dosages. Significance of the difference in values was determined using the Student's *t*-test and considering $p<0.05$ as the threshold of significance.

WST-1 cell proliferation assay. HeLa cells were plated in 96-well plates at a density of 3,200 each well in 200 μl of culture medium. The cells were then treated with curcumin, or synthesized analogs separately at different doses from 0.1 μM to 1 μM for 3 days, while equal treatment volumes of DMSO were used as vehicle control. The cells were cultured in a CO_2 incubator at 37˚C for three days. Ten microliters of the premixed WST-1 cell proliferation reagent (Clontech Laboratories, Inc., Mountain View, CA, USA) was added to each well. After mixing gently for one minute on an orbital shaker, the cells were incubated for additional 3 h at 37˚C. The absorbance of each well was measured using a microplate-reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) in absorbance mode at a wavelength of 430 nm.

Table I. *Cytotoxicity and antiproliferative activity towards HeLa aggressive cervical cancer cells.*

Compounds	Cytotoxicity		Antiproliferative activity	
	IC_{50}, μMa	IC_{50} (curcumin)/IC_{50} (compound)	IC_{50} (μM)	IC_{50} (curcumin)/IC_{50} (compound)
Curcumin	10.46±1.97	-	13.33±1.52	-
2	0.97±0.22	15	0.69±0.08	19
3	0.42±0.16	25	0.57±0.02	23
4	0.65±0.17	16	0.75±0.02	18
5	0.30±0.02	35	0.37±0.11	36
6	0.23±0.04	46	0.25±0.03	53
7	0.57±0.18	18	0.55±0.02	24
8	0.43±0.05	24	0.68±0.03	20
9	0.82±0.07	13	0.78±0.08	17
10	0.24±0.07	43	0.23±0.03	58

IC_{50}: Drug concentration effective in inhibiting 50% of the cell viability measured by the trypan blue exclusion assay or by the WST-1 cell proliferation assay after 3 days' exposure, and is expressed as mean±SEM by at least three independent experiments.

4-N,N-Diethylamino-6-(N,N,N-dodecyl-methylamino-sulfopropyl)-methyl-3-hydroxyflavone (F2N12S) and CYTOX AADvanced double staining assay. HeLa cells were plated in 24-well plates at a density of 200,000 each well in 400 μl of culture medium. After 3 h of cell attachment, the cells were then treated with each test compound at different concentration for 15 hours, while equal treatment volumes of dimethyl sulfoxide (DMSO) were used as vehicle control. The cells were cultured in CO_2 incubator at 37˚C for 15 h. Both detached and floating cells were collected in a centrifuge tube by centrifugation at rcf value 450 ×*g* for 3 to 5 min. The collected cells were re-suspended with 500 μl hank's balanced salt solution (HBSS) to remove proteins which may affect flow signal and centrifuged again. After discarding the supernatant, the collected cells were re-suspended with 300 μl HBSS and stained with 0.3 μl of F2N12S (Molecular Probes, Inc., Eugene, OR 97420 USA) for 3-5 min followed by 0.3 μl Sytox AAdvanced (Molecular Probes, Inc., Eugene, OR 97420 USA) for an additional 5 min. The fluorescence intensity of the two probes was further measured in individual HeLa cells using an Attune flow cytometer (Life Technologies, Grand Island, NY, USA) 0.5 to 1 h after staining.

Results

The cytotoxicity of the panel of compounds towards HeLa cells was first evaluated using a trypan blue dye exclusion assay. Equal treatment volumes of DMSO were used as vehicle controls and curcumin was used as positive control. Cell numbers were counted with a cell viability analyzer (Beckman Coulter). The effect of DMSO on cytotoxicity in HeLa cells has been explored in our laboratory and the optimum volume of DMSO was found to be 0.05% (Figure 2). According to the percentage inhibitory rate in our preliminary assay for 32 compounds at 10 μM and 1 μM final concentrations, nine compounds (**2-10**, Figure 1) were selected for an accurate determination of their IC_{50} values. IC_{50} values for compounds **6** and **10** with 1, 2 and 3 days drug exposure were obtained and the optimal drug exposure time was identified as being 3 days (Figures 3 and 4). Consequently, in our subsequent experiments, the cells were treated with each of test compound at different doses for 3 days, while equal treatment volumes of DMSO (0.05%) were used as vehicle control. As shown in Table I, the acquired IC_{50} values for these nine promising compounds fall into a range between 0.21 μM and 0.97 μM, while the IC_{50} value for curcumin is 10.5 μM. These data imply that this panel of compounds showed excellent cytotoxicity towards HeLa cells with an optimum IC_{50} value of 0.21 μM, *i.e.* 50-times more potent than curcumin. It should be noted that we also confirmed a higher cytotoxicity of compounds **2**, **4**, **6**, and **8** *vs.* curcumin towards HeLa cells, as we previously reported (9).

The *in vitro* anti-proliferative activity of these nine compounds (**2-10**) against HeLa cells was evaluated using a WST-1 cell proliferation assay. Again, three days was determined as the most favorable exposure time based on the IC_{50} values for compounds **6** and **10** obtained from 1 to 3 days of drug treatment. As illustrated in Table I, the IC_{50} values for these selected compounds range from 0.2 μM to 0.78 μM, while that for curcumin was found to be 13.33 μM. As shown in Table I both trypan blue exclusion assay and the WST-1 cell proliferation assay led to the consensus that these nine compounds have 13- to 58-fold higher *in vitro* cytotoxic and anti-proliferative potency than curcumin towards the aggressive HeLa cervical cancer cell line.

F2N12S and CYTOX AADvanced double staining flow cytometry-based assay was chosen to discriminate HeLa cells dying from apoptosis from those dying from necrosis in response to increasing concentrations of compounds **6** and **10**. HeLa cells were incubated with the test compound for 15 h. Staurosporine was used as a specific apoptotic inducer and positive apoptotic control in all these experiments (not shown). As shown in Figures 5 and 6, compounds **6** and **10** induced significant levels

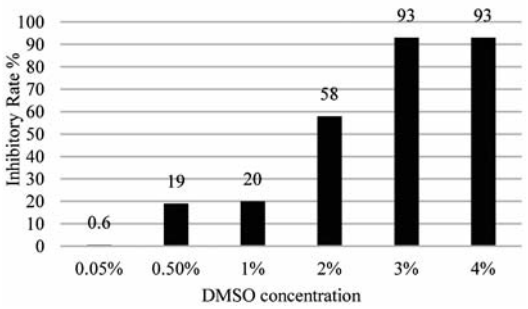

Figure 2. *Cell growth inhibition by dimethyl sulfoxide (DMSO) in HeLa cells.*

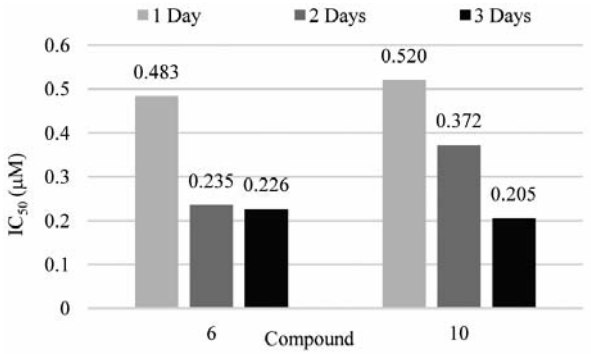

Figure 3. *Time-dependent cytotoxicity of compounds **6** and **10** on HeLa cells.*

of apoptotic cell death in the HeLa cancer cell line in a dose-responsive manner. For example, a 15-h exposure of HeLa cells to 30 μM of compound **6** led to 42% early apoptotic cells and 36% late apoptotic/necrotic cells; 20 μM of compound **10** also induced significant apoptosis, with 54% early apoptotic cells and 23% late apoptotic/necrotic cell. Both apoptotic and necrotic cell populations increased in response to increasing concentration of both compound **6** and **10** (0-100 μM final concentration range). However, the increase rate of the apoptotic rate was maximized between 0 and 20 μM of both compounds **6** and **10**, while the increase rate of the necrotic rate started to reach its maximum after cell incubation with 30 μM of the same compounds.

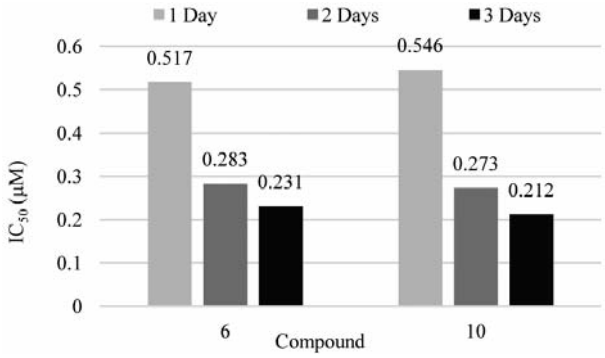

Figure 4. *Time-dependent anti-proliferative activity of compounds **6** and **10** on HeLa cells.*

Discussion

The structure–activity relationship reveals that the terminal heteroaromatic rings and the central monoketone linker have profound impacts on the cytotoxicity and anti-proliferative activity of curcumin-derived compounds. Both linear dienone and 1-methylpiperidone are favorable central linkers for the activity against HeLa cells. *N*-Containing heteroaromatics, including pyridine-2-yl, 1-sec-butyl-1*H*-imidazol-2-yl, 5-methylisoxazol-3-yl, 3-methylisoxazol-5-yl, and thiazol-2-yl, have significant contribution to the enhanced potency. In particular, 5-methylisoxazol-3-yl and 3-methylisoxazol-5-yl are the most favorable terminal aromatic moiety for the potent cytotoxicity in HeLa cells.

The linear five-carbon dienone (1,4-dien-3-one) and 1-*sec*-butyl or 1-isobutyl-1*H*-imidazol-2-yl clearly serve as the optimal central linker and nitrogen-containing heteroaromatic rings, respectively, for the potency of compounds **6** and **10**. Finally, the results from our flow cytometry-based assay strongly suggest that the primary cytotoxic effect of compounds **6** and **10** occurs through apoptosis: apoptotic markers were predominant in dead cell after treatment with a low dosage of these compounds, while necrotic markers were predominant after treatment with high dosages.

Conclusion

In conclusion, nine curcumin-based compounds (**2-10**) were identified as potential anti-cervical cancer agents, exhibiting 13- to 58-fold better *in vitro* potency than curcumin in both trypan blue exclusion assay and the WST-1 cell proliferation against aggressive cervical cancer cells. Both linear dienone and 1-methylpiperidone are pinpointed as the favorable central linkers for this activity. 5-Methylisoxazol-3-yl and 3-methylisoxazol-5-yl act as the most favorable terminal aromatic moiety for the potent cytotoxicity against HeLa cells. F2N12S and CYTOX AADvanced double staining assay suggest that the two most promising compounds can induce apoptotic cell death.

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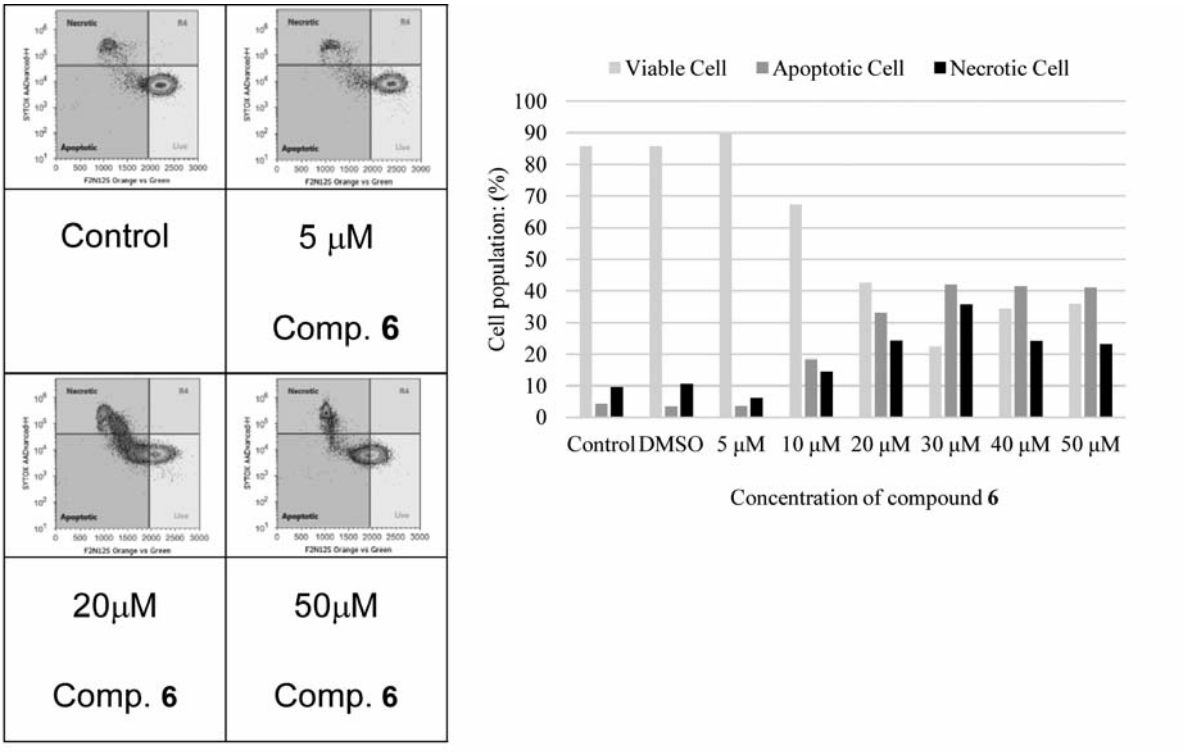

Figure 5. *Evolution of viable, apoptotic and necrotic HeLa cells populations in response to increasing dosages of compound* ***6***.

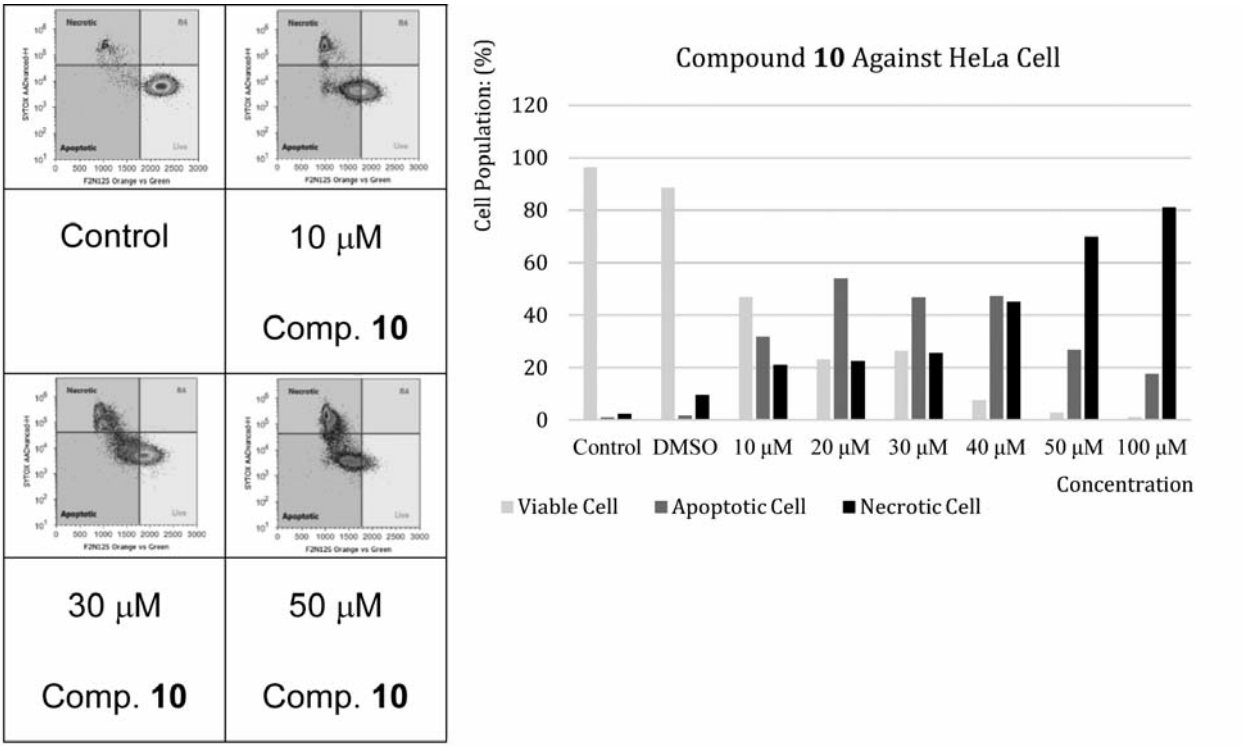

Figure 6. *Evolution of viable, apoptotic and necrotic HeLa cells populations in response to increasing dosages of compound* ***10***.

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