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Cytotoxicity and Apoptogenic Activity of A Novel Synthetic Iron Chelator 1-(*N*-Acetyl-6-Aminohexyl)-3-Hydroxy-2-Methylpyridin-4-One (CM1) In Human Leukemic Cells

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Abstract

An interruption of the iron metabolism with chelators can lead to a significant inhibition of cancer cell growth. 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one or CM1, is a novel synthetic bidentate iron chelator which was successfully synthesized by our group. We have studied the characteristics and iron-chelating activity of this compound. Nevertheless, the anti-cancer activity of the chelator is largely unknown. In this study, we demonstrated the cytotoxicity and apoptogenic activity of CM1 against human leukemic cell lines-HL-60 and U937. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for the cytotoxicity study. The results showed that CM1 inhibited the cell growth and metabolic activity of the leukemic cells. Flow cytometric analysis clearly demonstrated the dose and time-response of CM1-induced apoptosis in these two cells. CM1 arrested the cell populations in the sub G_1 phase after 24 hours of exposure. The cancer cells induced by the compound significantly decreased mitochondria membrane potential ($\Delta\psi_m$), and increased the activation of caspase-2,-3,-8 and caspase-9 activities. Possibly, CM1 would interact with nonheme iron-containing enzymes, such as ribonucleotide reductase and depleting intracellular iron essential for fast dividing cancer cells, leading to cell apoptosis. The CM1 may act as a reducing agent and help to maintain the CM1-Fe^{2+} complex which can generate radicals.

Keywords: Iron; Hydroxypyridinone; Leukemic cells; Cytotoxicity; Apoptogenic activity

Introduction

Iron is essential to the cell viability of normal and cancer cells [1]. It is important in DNA synthesis because it modulates ribonucleotide reductase activity [2,3]. Iron is also crucial for normal mitochondrial electron transport and oxidative phosphorylation [4]. Many data from several sources suggest that iron depletion may be a useful target in the treatment of cancer, particularly those of a hematopoietic origin [5-7]. In cell cycle studies, iron chelator-treated cells are arrested in different phases of the cell cycle depending upon the cell type and the concentration and time of the exposure to chelators [8-10]. In addition, many reports have demonstrated that iron chelators induce apoptosis in several types of proliferating cells [11-13]. Therefore, it has been proposed that iron chelators are promising anti-proliferative agents in the treatment of human cancers. We have synthesized and characterized the chemical structure of a new bidentate iron chelator, 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1), which is a 3-hydroxypyridin-4-one (HPO) derivative [14]. Previously, we reported that CM1 was able to chelate plasma non-transferrin bound iron (NTBI) in iron-loaded mice effectively, and was non-toxic to normal peripheral blood mononuclear cells (PBMC) and hepatocytes [15]. Nevertheless, the biological activity of CM1 is largely unknown. In this study, we investigated the cytotoxicity and apoptogenic activity of the CM1.

Materials and Methods

Cell cultures

HL-60 cells (human promyelocytic leukemic cell line) and U937 cells (human leukemic monocyte lymphoma cell line) were maintained in RPMI 1640 medium (Gibco™, Life Technologies, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco™, Life Technologies, USA), and were incubated at 37°C in the humidified atmosphere of incubator containing 5% CO_2.

Iron chelator treatment

The CM1 was prepared in the stock solution as 60 mM in phosphate buffer saline (PBS), and filtered through 0.2 μm membrane (cellulose type). Cells were treated with tested compounds in selected concentrations (0-600 μM) at 37°C for the indicated time.

Cytotoxicity and cell growth inhibition study

Cytotoxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT (Invitrogen™, Life Technologies, USA) assay [16]. The product was solubilized with dimetylsulfoxide (DMSO) into a colored solution, and the absorbance was measured at 540 nm with a microplate reader (Synergy™ H4, BioTek, Singapore). Cell-growth inhibition studies were done by trypan blue exclusion assay [17].

Determination of cell cycle distribution and cell apoptosis induction

Cell cycle distribution studies were performed with propidium

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iodide (PI) staining (Invitrogen™, Life Technologies, USA) staining [18,19]. Apoptotic cells were detected with fluorescent probes, ApopNexin™ FITC Apoptosis Detection kit (Millipore, Canada) [20], and both assays were analyzed using a flow cytometer (FACSCanto II, B.D Bioscience, USA).

Assessment of mitochondria membrane potential

Mitochondrial membrane potentials ($\Delta\psi_m$) were measured by staining the cells with $DiOC_6$(3) (3,3'-dihexyloxacarbocyanine iodide) [21]. Briefly, 5×10^5 treated cells were incubated for 20 minutes at 37°C in 500 µl of 100 nM $DiOC_6$(3), and this step was immediately followed by analysis on a flow cytometer, with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Caspase activity assay

Treated cells (5×10^6 cells) were lysed in 50 µl of chilled cell lysis buffer and kept on ice for 10 minutes. Cell lysates were centrifuged for 1 minute at 10,000×g to collect the cytosolic extract. Assay protein concentration was measured by Bradford's method [22]. The cytosol extract was diluted to a concentration of 50-200 µg protein per 50 µl cell lysis buffer (1-4 mg/ml). The reaction buffer was added to each sample, as well as the substrates that were contained in the caspase colorimetric sampler kit (Norvex®, Life Technologies, USA), and they were then incubated at 37°C for 2 hours, before absorbance was measured at 405 nm using a microplate reader.

Statistical analysis

The results were expressed as mean ± SEM. Statistical significance was determined using a one-way analysis of variance (ANOVA), in which $p<0.05$ was considered significant.

Results

Cytotoxicity study of CM1 in human leukemic HL-60 and U937 cell lines

We found that the higher concentrations of CM1 significantly decreased viability of HL-60 and U937 cells (Figure 1). The IC_{50} of CM1 in HL-60 and U937 cells after a 24-hour period was 300 and 330 µM, respectively. In addition, we determined cell numbers using trypan blue exclusion assay, and the results showed that the numbers of treated cells significantly decreased in a dose and time-dependent manner (Figure 2).

Effect of CM1 on cell cycle distribution and apoptosis induction

Apparently, the accumulation of both cells in the sub G_1 phase was increased by the iron chelator treatment (Figure 3). Basically, the accumulation of cells in the sub G_1 phase indicates DNA fragmentation, which is a common marker of apoptosis. In order to investigate the type of cell death induced by CM1, phosphatidylserine (PS) externalization were then investigated for their involvement in CM1-induced human leukemic cells apoptosis. After 24 or 48 hours of treatment, Anexin V/FITC and PI staining were performed and analyzed using a flow cytometer. Results in Figure 4 showed that CM1 markedly induced cell apoptosis in HL-60 cells for up to 65 ± 3 % of the apoptotic cells, after they were treated with CM1 (600 µM) for 24 hours. Treatment with 300 µM CM1 achieved partial induction of cell apoptosis over 24-hour period, but increased the apoptotic cells significantly after 48 hours of treatment. Additionally, the treated U937 cells showed a slight increase in the percentages of the apoptotic cells, and were found respond in a dose and time-dependent manner. Therefore, the experimental results in the study suggested that CM1 treatment could arrest cell cycle and induce apoptosis in human leukemic HL-60 and U937 cells. $SubG_1$ peak indicates DNA fragmentation and late apoptosis event. Anexin V/FITC staining indicates apoptotic cell population expressing phosphatidylserine (PS) exposure. We expected that CM1 treatment would cause cell death by apoptosis, or/and necrosis, giving high $subG_1$ peak that was not related to the Anexin V positive cells (apoptotic cells).

Dose-response of CM1 on mitochondria membrane potentials ($\Delta\psi_m$) alteration

Mitochondrial membrane potential (ψΔm) indicates mitochondrial function and energy production. Decrease in Δm can give rise to release of cytochrome c, which is one of the apoptosis markers. Early event in apoptosis appears to be the reduction of $\Delta\psi_m$. To further understand this, the treated cells were investigated for $\Delta\psi_m$ alteration

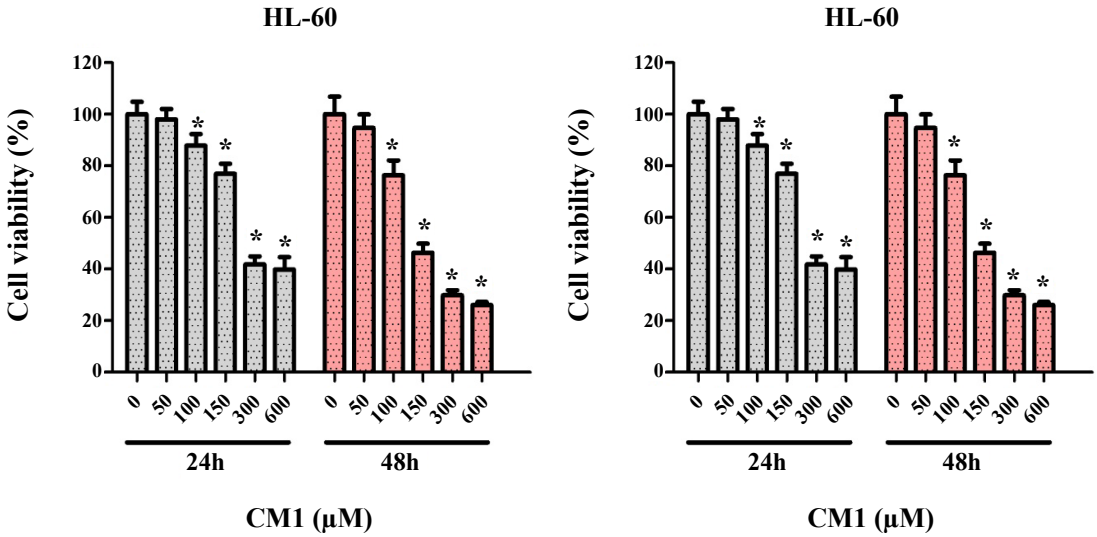


Figure 1: Cytotoxicity test of CM1 treatment on HL-60 and U937 cells for 24 and 48 hours. Data were expressed as mean ± SEM. *$p<0.05$ when compared to non-treatment.

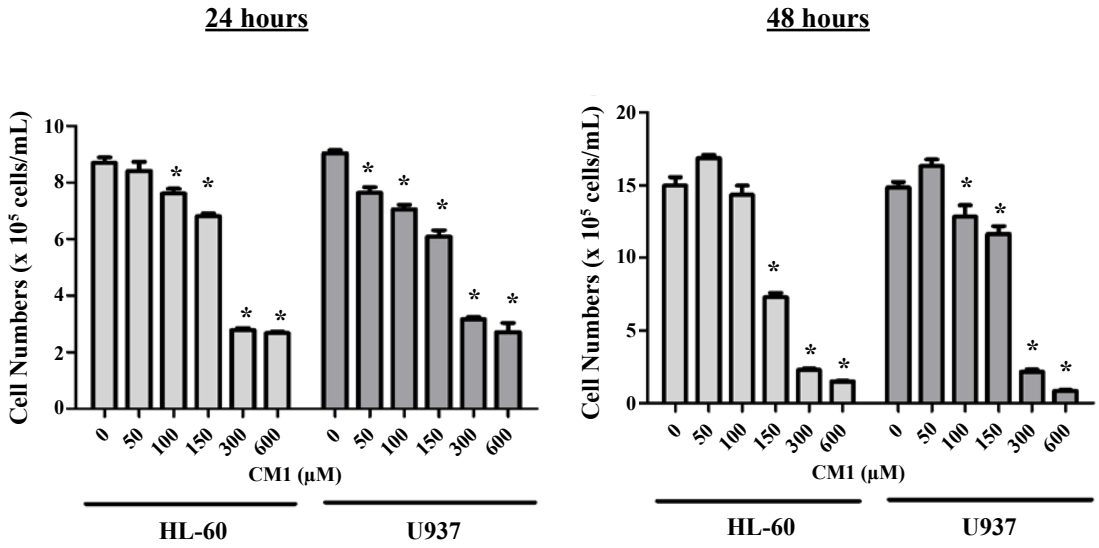


Figure 2: Numbers of HL-60 and U937 cells after being treated with CM1 (0-600 μM) for 24 and 48 hours. Data were expressed as mean ± SEM. *p<0.05 when compared to non-treatment. Regarding to U937 cells treated with CM1 at 150 μM for 48 hours, the cell viability was decreased around 40% (p<0.05), while numbers of the cells were decreased around 22% (Δ1.6×10^5 cells/ml). Possibly, U937 cells would be more resistant to 150 μM CM1 treatment even incubation for 48 hours. Most of the cells treated with higher CM1 concentrations (300 and 600 μM) died at 24 hours, and the remaining cells were not able to divide as usual.

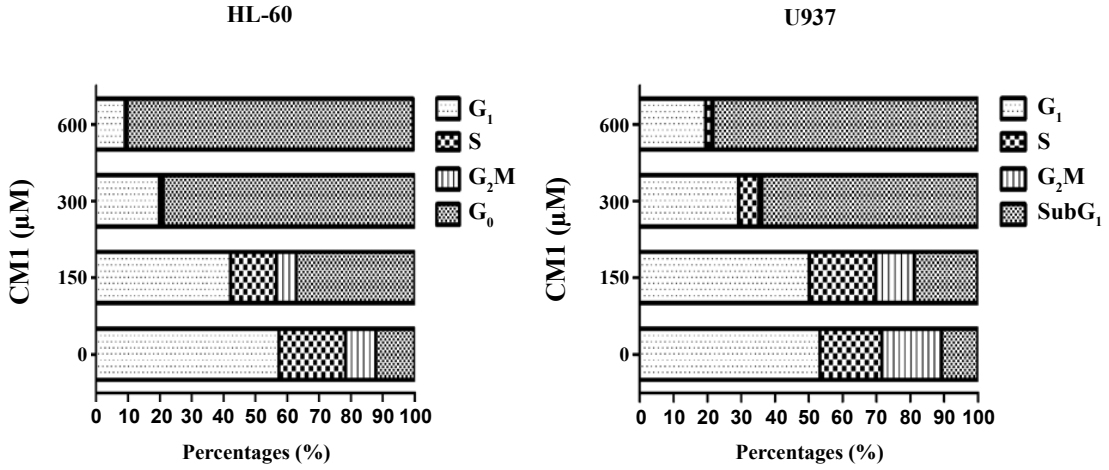


Figure 3: Percentage of cells in each phase of HL-60 and U937 cells treated with CM1 (0-600 μM) for 24 hours. Cells were stained with PI and analyzed with a flow cytometer. *p<0.05 when compared to non-treatment.

with $DiOC_6(3)$ staining. Loss of $DiOC_6(3)$ uptake indicated a decrease of $\Delta\psi_m$ value that was observed in response to apoptotic proceeding. Flow cytometric analysis showed that the percentage of decreasing $DiOC_6(3)$-accumulated cells was elevated in both leukemic after 24 hours of CM1 exposure (Figure 5). ψΔm values of the HL-60 and U937 cells were decreased in concentration-dependent manner. Significant decrease in ψΔm of the HL-60 and U937 cells was maximal (29.10 ± 0.06 and 29.23 ± 0.03 %, respectively), when the cells were treated with 600 μM CM1. It is possible that the cells may not be induced apoptotic death *via* mitochondria-independent (extrinsic) pathway, resulting in less efficiency of ψΔm. This experiment could suggest that CM1 significantly decrease $\Delta\psi_m$, which is an important marker for apoptotic processes.

Activation of caspase activity in human leukemic cells

Cell apoptosis can be marked by phosphatidylserine (PS) exposure, increase of caspase enzyme activity, DNA fragmentation, apoptotic protein expression and mitochondrial membrane damage. Following 24 hours of treatment of the cells with the different concentrations of CM1, activities of caspase-2,-3-8 and caspase-9 were found to be elevated in 300 μM and 600 μM compared to those of non-treated cells (Figure 6). Treatment with 300 μM CM1 tended to increase activity of the caspase enzymes (1.3, 1.3, 1.17 and 1.2 fold for caspase-2, 3, 8 and 9, respectively) of HL-60 cells when compared to non-treatment. Similarly, the treatment tended to increase activity of the caspase enzymes (1.9, 2.3 and 1.14 fold for caspase-2, 3 and 8, respectively) of U937 cells. Therefore, 300 μM CM1 could induce apoptosis in these two cells. Under the same treatment, dramatically decreased viability of the cells may come from both cell apoptosis and cell necrosis.

Discussion and Conclusions

Iron plays an important role at the active site of ribonucleotide

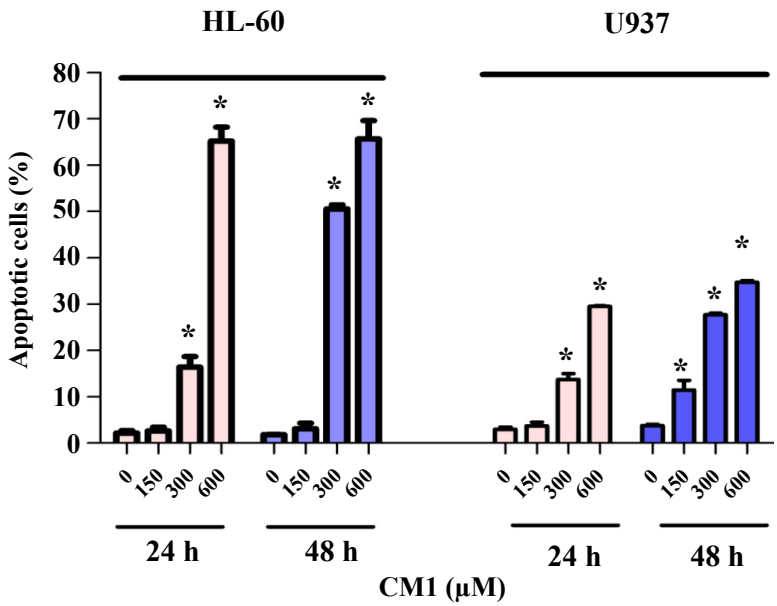


Figure 4: Percentage of apoptotic cells in HL-60 and U937 cells after being treated with CM1 (0-600 μM) for 24- and 48-hour periods. Cells were stained with Anexin V/FITC and PI and analyzed using flow cytometry. Data were expressed as mean ± SEM. **p* <0.05 when compared to non-treatment.

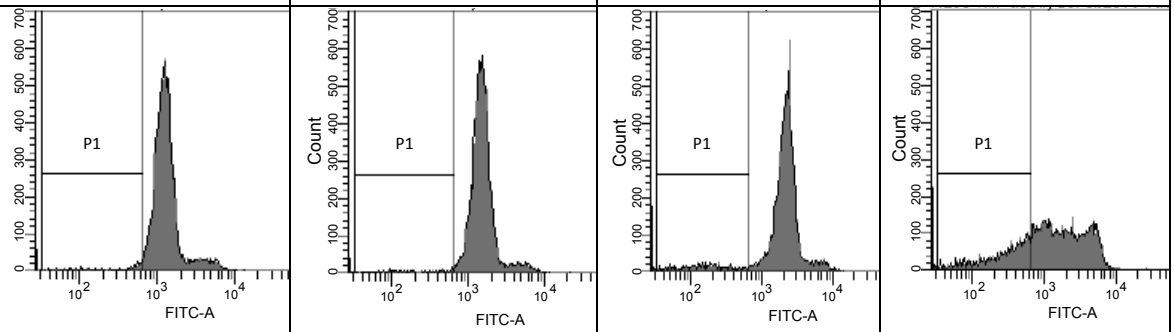
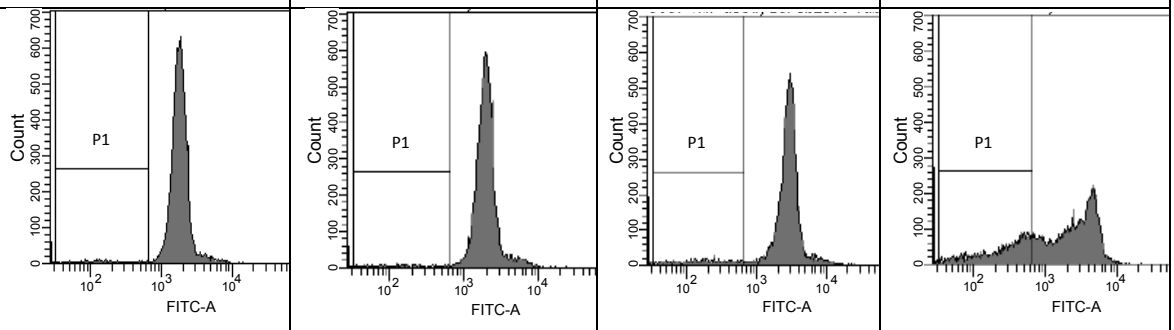
Cells	Measure	CM1 concentrations (M)			
		0 μM	150 μM	300 μM	600 μM
HL-60	Histogram				
	$\Delta\psi_m$ (%)	0	3.23±0.03	9.93±0.09*	29.10±0.06*
U937	Histogram				
	$\Delta\psi_m$ (%)	0	2.93±0.03	9.63±0.09*	29.23±0.03*

Figure 5: Flow cytometric histogram and percent decrease of $\Delta\psi_m$ (mean ± SEM) in HL-60 and U937 cells treated with CM1 (0-600 μM) for 24 hours. * *p*<0.05 when compared to non-treatment.

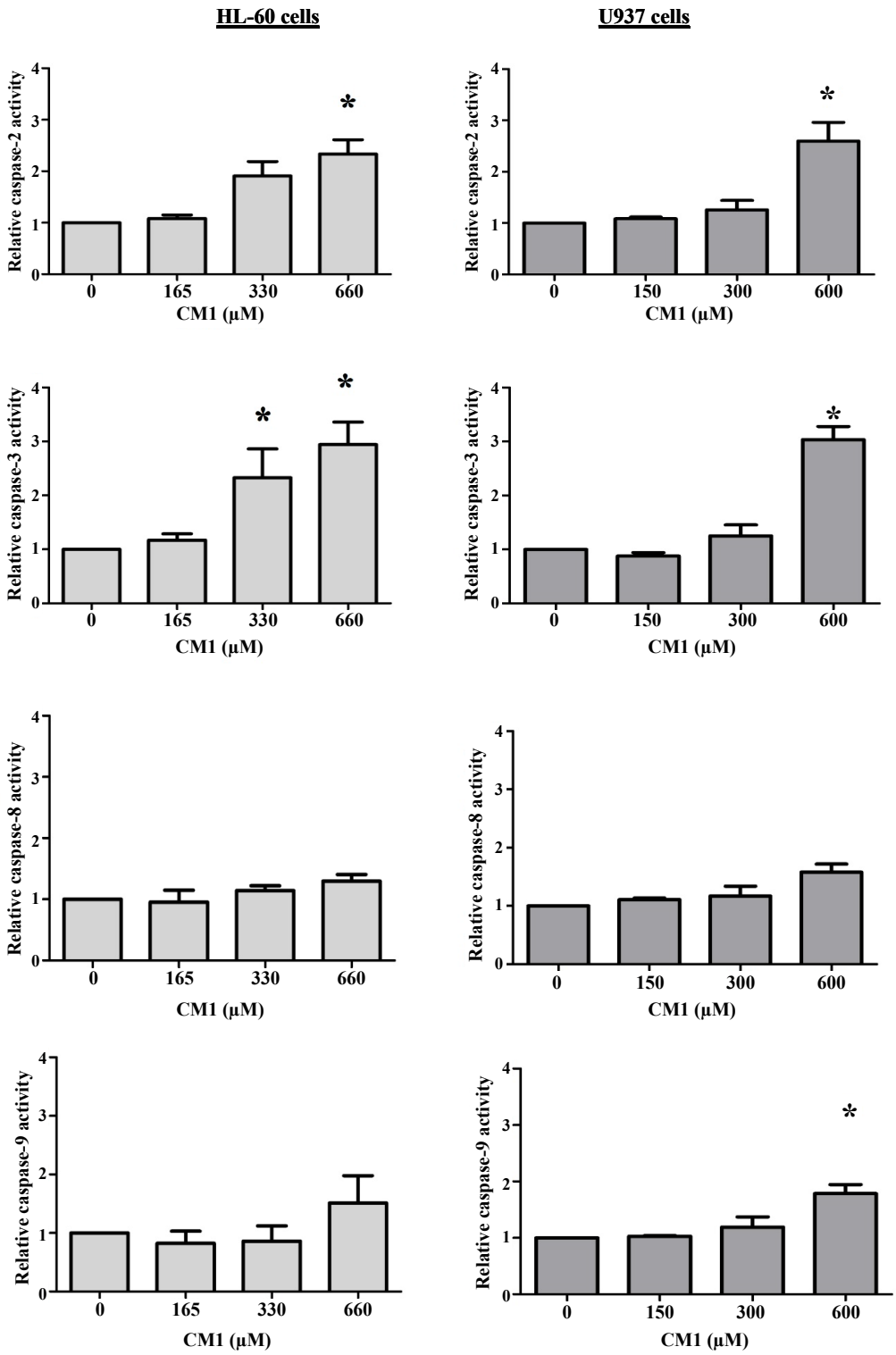


Figure 6: Relative activity of caspase-2, 3, 8 and 9 enzymes of HL-60 and U937 cells treated with CM1 (0-600 μM) for 24 hours. Data were expressed as mean ± SEM. *p<0.05 when compared to non-treatment.

reductase in DNA replication [23-26], and can affect expression of the molecules involved in cell cycle control [27-31]. Cellular iron depletion is a potent way to inhibit cell proliferation [32-34]. Iron chelators showed inhibitory effect on the proliferation of hematopoietic malignant cells, resulting in induction of cancer cells apoptosis [35-37]. For instance, tachpyridine, desferrioxamine (DFO) and dipyridyl can activate a caspase cascade pathway. Induction of apoptosis by tachpyridine is characterized by an early activation of caspase-9, followed by the sequential activation of caspase-3, caspase-8, and the mitochondrial pathway makes an important contribution to iron chelator-mediated

cell death [38]. Our preliminary results demonstrated that CM1 was effective in removal of plasma NTBI in iron-loaded mice, and found to be less toxic to normal peripheral blood mononuclear cells (PBMC) and cultured hepatocytes. In examining cytotoxicity and apoptogenic activity of the CM1, we evaluated its effect on the proliferation of human leukemic HL-60 and U937 cells using MTT and trypan blue exclusion assays. Promising iron chelators such as DFO, deferiprone (DFP) and deferasirox (DFX) exhibit anti-proliferative effect on HL-60 and U937 cells [40-43]. We found that CM1 seemed to be less effective in inhibiting the division of HL-60 and U937 cells than CM1 at equivalent doses. Nonetheless, CM1 is more lipophilic (K_{part}=0.53) and less toxic (LD_{50}>5.0 g/kg) than DFP (K_{part}=0.17; LD_{50}=1.2 g/kg), the compound would be used more efficiently *in vivo,* as results of more cell-penetrating capacity and safety. The anti-leukemic mechanism is supposed to be iron deprivation-induced apoptosis [43,44], and activation of caspase activity during apoptosis [38,45]. Other iron chelators also show such anti-leukemic activity [46-48]. Here, we have showed that CM1 strongly inhibited cell viability and decreased numbers of leukemic cells that responded in dose- and time-dependent manners. Flow cytometric analysis elucidated that CM1 significantly increased the accumulation of cell populations in the sub G_1 phase. Probably, CM1 would be an inducer of apoptosis at high concentrations over 24 hours of exposure in HL-60, while apoptosis of U937 cells was partially induced by CM1 as well. In consistent, CM1 significantly decreased mitochondria membrane potential with high concentrations (>300 μM) in both leukemic cells. It might suggest that CM1-induced apoptosis mediated the mitochondrial pathway. Most importantly, CM1 strongly enhanced activities of all the studied caspase enzymes of HL-60 cells with a high concentration. Apparently, caspase-2 and caspase-3 were activated in U937 cells with the medium and high concentrations of CM1.

It can be concluded that CM1 could have anti-proliferative and apoptogenic activities in human leukemic cells. Nonetheless, other biological activities of the CM1 should be further investigated.

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