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5-(2-Carboxyethenyl) isatin derivative induces G_2/M cell cycle arrest and apoptosis in human leukemia K562 cells

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ABSTRACT

Our previous study successfully identified that the novel isatin derivative (E)-methyl 3-(1-(4-methoxybenzyl)-2,3-dioxoindolin-5-yl) acrylate (HKL 2H) acts as an anticancer agent at an inhibitory concentration (IC_{50}) level of 3 nM. In this study, the molecular mechanism how HKL 2H induces cytotoxic activity in the human chronic myelogenous leukemia K562 cells was investigated. Flow cytometric analysis showed that the cells were arrested in the G_2/M phase and accumulated subsequently in the sub-G_1 phase in the presence of HKL 2H. HKL 2H treatment down-regulated the expressions of CDK1 and cyclin B but up-regulated the level of phosphorylated CDK1. Annexin-V staining and the classic DNA ladder studies showed that HKL 2H induced the apoptosis of K562 cells. Our study further showed that HKL 2H treatment caused the dissipation of mitochondrial membrane potential, activated caspase-3 and lowered the Bcl-2/Bax ratio in K562 cells, suggesting that the HKL 2H-causing programmed cell death of K562 cells was caused via the mitochondrial apoptotic pathway. Taken together, our data demonstrated that HKL 2H, a 5-(2-carboxyethenyl) isatin derivative, notably induces G_2/M cell cycle arrest and mitochondrial-mediated apoptosis in K562 cells, indicating that this compound could be a promising anticancer candidate for further investigation.

1. Introduction

Isatin derivatives possess a wide variety of biological activities like anticancer, antidepressant, anticonvulsant, antifungal, anti-HIV and anti-inflammatory activities [1–7]. Over the last few decades, many isatin-based anticancer agents were found to exhibit high cytotoxic activity. Among them, 5-bromo-3-o-nitrophenyl isatin hydrazone and a series of 5-bromo-(2-oxo-3-indolinyl) thiazolidine-2,4-diones substituted derivatives have been reported to exhibit anticancer activity against lymphocytic leukemia [8,9]; N-benzylation of 5,7-dibromoisatin can increase the cytotoxicity against U937 lymphoma cells [7]; and SU11248 (Sutent), a 5-fluoro-3-substituted isatin derivative, have even been approved by FDA in 2006 to be used clinically for the treatment of advanced renal carcinoma and gastrointestinal stromal tumors [10,11]. Even more, two groups [12–14] reported recently the synthesis of several N alkyl and C-5 sulfonamido isatin analogues and demonstrated that the cytotoxic activity of those compounds was achieved by inhibiting caspase-3/caspase-7 activity, thus opening the door for better understanding the underlying molecular mechanism how those anticancer agents induces cytotoxic activity of the cancer cells.

It was well known that some cytotoxic agents and/or DNA damaging agents induce cell cycle arresting of the related cancer cells and consequently inhibited the cell viability [15]. The cell cycle might be mediated by the activation of cyclin-dependent kinases (Cdks) which could be initiated with the formation of Cyclins–Cdks complexes [16]. Among these complexes, the cyclin B1/Cdk1 complex had been known to be the primary regulator of transition from the G_2 to M phase [17]. As for apoptosis, it is the result of a highly complicated cascade of cellular events including cell rounding and shrinkage, chromatin condensation, DNA fragmentation, shedding of smaller fragments from cells and detachment from the plate [18]. In particular, the mitochondria play a significant role in the

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occurrence of some cancer cell apoptosis when induced by chemotherapeutic agents [19–21]. It is well documented that the highly conserved cysteine-dependent aspartate-specific proteases caspase-3, caspase-8 and caspase-9 are and the key components of the apoptotic machinery and might have significant impact on the apoptotic progression [22].

We previously reported [23] the design, synthesis and *in vitro* cytotoxicity evaluation of 44 5-(2-carboxyethenyl) isatin derivatives as anticancer agents and identified that HKL 2H exhibits excellent cytotoxic activity against three different cancer cell lines including K562 (IC_{50} = 3 nM), HepG2 and HT-29. In the present study, the molecular mechanism of the cytotoxic activity of HKL 2H against the human chronic myelogenous leukemia K562 cells was investigated.

2. Materials and methods

2.1. Cell lines and culture conditions

K562 cell line was obtained from the Shanghai Institutes of Biological Sciences (Shanghai, China). Cells were grown at 37 °C in RPMI-1640 supplemented with 10% fetal bovine serum, 2.05 mM glutamine and 1% penicillin/streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2. The medium was replaced once every third days.

2.2. Cytotoxic activity assay

The cytotoxic activity was measured using the MTT assay as described previously [23]. Briefly, 100 μl of K562 cells were cultured in 96-well plates at a density of 5×10^4 cells/ml for 2 h. Different concentrations of HKL 2H (1–10,000 nM) were added to each well to culture for another 48 h. MTT assay was performed using thermo microplate reader. The DMSO-treated controls were calculated as a cell viability value of 100%. The inhibitory concentrations (IC_{50}) were obtained by nonlinear regression using GraphPad Prism 4.0. For each experiment, IC_{50} value was calculated from three independent assays.

2.3. Cell cycle analysis

For the DNA content analysis, 2×10^6 cells were cultured for 2 h and treated with HKL 2H (30 nM) for 3, 6, 12 and 24 h. respectively. Cells were collected and fixed with 1 ml of 70% ice-cold ethanol at −20 °C overnight. Cells then were washed with PBS, incubated with propidium iodide (PI, 1 mg/ml), RNase A (20 μg/ml) for 15 min in dark at room temperature and analyzed using a FACS Calibur system (version 2.0, BD) using the CELLQuest program (Becton Dickinson). Results were representatives of at least three independent experiments.

2.4. Flow cytometric analysis of apoptosis

Apoptotic cells were assayed by the Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. In brief, K562 cells were treated with DMSO or HKL 2H (30 nM) for 1 h. Cells were harvested, washed twice with ice-cold PBS and resuspended in 1 × Binding buffer at a concentration of 1×10^6 cells/ml. Cells were stained with 5 μl of Annexin-V-FITC and 5 μl of PI (50 μg/ml) for 15 min at room temperature in the dark and analyzed by flow cytometry.

2.5. DNA fragmentation assay

K562 cells were collected by centrifugation (2500 rpm, 5 min) after treatment with DMSO or HKL 2H (30 nM) for 6, 12, 24 and 48 h, respectively. Cell pellet was washed with 1 × PBS and re-suspended with 100 μl of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) for 1 min on ice. The solution was centrifuged (3000 rpm, 5 min) to obtain supernatant. 10 μl of 10% SDS solution was added in the pooled supernatant, treated with 25 μl 20 mg/ml RNase A and incubated at 56 °C for 2 h, and then incubated at 37 °C for another 2 h before the treatment with 10 μl of 25 mg/ml proteinase K. The supernatant was treated with 65 μl of 10 mM ammonium acetate and 500 μl of ice-cold ethanol, mixed thoroughly, seated in −80 °C for 1 h, and then centrifuged at 15,000 rpm for 20 min. The white pellet was washed with 200 μl of 80% ice-cold ethanol and air-dry for 10 min at room temperature. The pellet was dissolved with 50 μl of TE buffer and determined the DNA concentration. DNA pellets were electrophoresed for 100 min at 45 V on 2% agarose gel. The DNA fragments were visualized under ultraviolet light.

2.6. Flow cytometric analysis of mitochondrial membrane potential

To measure the effects of HKL 2H on mitochondrial membrane potential (ψm), K562 cells were treated with DMSO or HKL 2H (30 nM) for 18 and 36 h, respectively. After that, cells were stained with 100 nM tetramethylrhodamine methyl ester (TMRM; Invitrogen, USA) for 15 min at room temperature in the dark and analyzed by flow cytometry. TMRM is the most specific for measuring changes in the loss of mitochondrial membrane potential ($\Delta\psi m$).

2.7. Caspase-3 activity assay

Caspase-3 activity was evaluated by the Apo-ONE® Homogeneous Caspase-3/7 Assay Kit (Promega) according to the manufacturer's instructions. In brief, K562 cells were cultured in the 96-well with DMSO or HKL 2H (30 nM) for 24 and 48 h. Each well was added 100 μl of Apo-ONE® Homogeneous Caspase-3/7 buffer with 1 μl Caspase Substrate Z-DEVD-R110 (100×) for 18 h. Fluorochrome release after peptide cleavage was determined kinetically at room temperature (excitation: 485 nm and emission: 521 nm). Cell treated with staurosporine (1 μM) for 12 h were used as positive control.

2.8. Western blotting analysis

Anti-Bcl-2 and anti-Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other anti-antibodies were purchased from Cell Signaling Technology (CST, Boston, MA). Cells were lysed in a lysis buffer containing 10 mM Hepes-Na, 150 mM Na_2SO_4, 1 mM EDTA, 3% CHAPS, 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml each of aprotinin and leupeptin. For Western blot analysis of total cell lysates, samples were prepared by mixing an aliquot of cell lysates with an equal volume of 2× Laemmli's sample buffer and heating at 100 °C. Samples were separated by SDS–PAGE and electrotransfered to PVDF membranes (Millipore, Bedford, MA). The membranes were probed with a relevant antibody and incubated with Alexa Fluor® 680 Goat Anti-Mouse IgG (H+L) and Alexa Fluor® 680 Goat Anti-Rabbit IgG (H+L) followed by detection using Odyssey Western blotting detection system (Amersham Pharmacia Biotech).

3. Result

3.1. Morphological changes in HKL 2H-treated K562 cells

Treatment of the highly cytotoxic compound HKL 2H (IC_{50} = 3 - nM) (Fig. 1A) can cause K562 cells the morphology change of G_2/M phase arrest (such as elongation of the cells) and typical apoptotic morphology (such as cell shrinkage and/or blebbing) (Fig. 1B). As

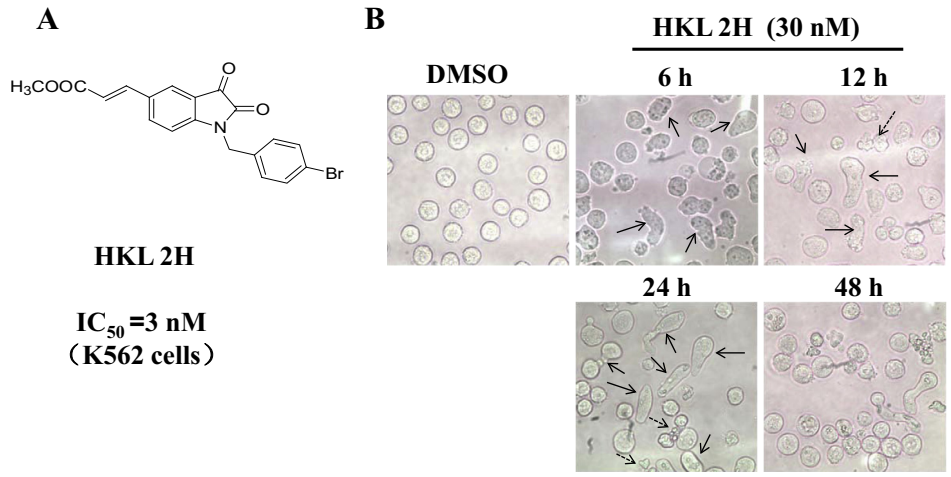

Fig. 1. Morphological changes in HKL 2H-treated K562 cells. (A) Structure and *in vitro* cell proliferation inhibitory activity of HKL 2H. (B) Morphological changes induced by HKL 2H (30 nM) treatment for 6, 12, 24 and 48 h were observed. Solid and dotted arrows showed the elongated cells and apoptotic cells, respectively.

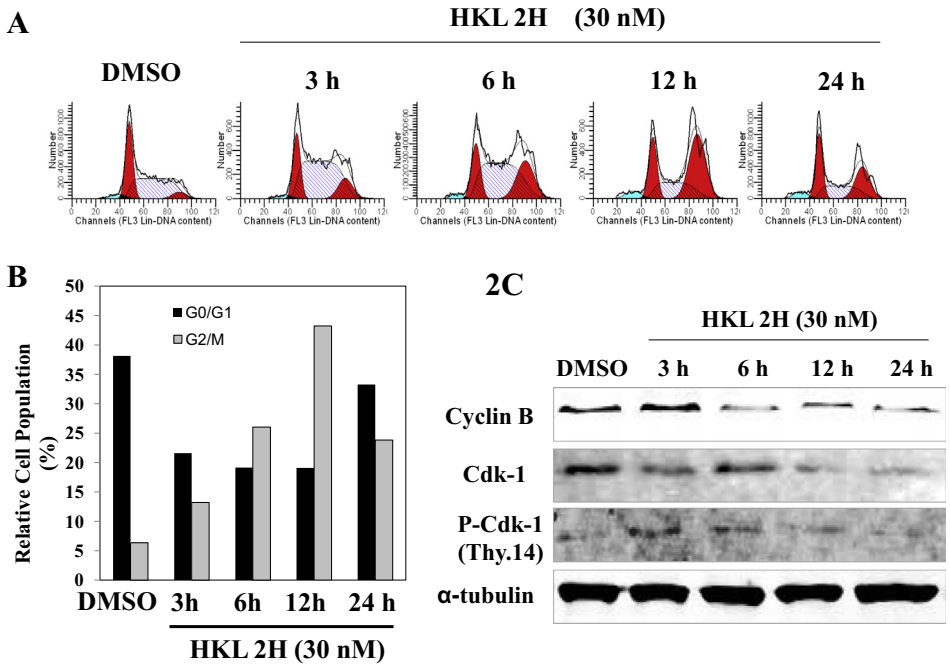

Fig. 2. HKL 2H induced the cell cycle arrest of K562 cells in G_2/M phase. (A) K562 cells were treated with 30 nM HKL 2H. At the time points indicated, cells were labeled with PI and their DNA content was determined using FACS analysis. The data was presented as mean of three independent experiments. (B) Representative histograms for cell cycle distribution in K562 cells. (C) The levels of the cell cycle regulatory proteins cyclin B, CDK1 and p-CDK1 (Thr14) were detected using Western blotting.

shown in Fig. 1B, the elongated cells (solid arrows) increased significantly as early as 6 h after HKL 2H treatment at a concentration of 30 nM as compared to DMSO-treated cells. The apoptotic cells (dotted arrows) were clearly observed in K562 cells after the treatment with HKL 2H for 24 h (Fig. 1B). These results indicated that HKL 2H might inhibit the cell proliferation of K562 cells through the cell cycle arrest step.

3.2. HKL 2H induced G_2/M cell cycle phase arrest in K562 cells

To determine whether the influence of HKL 2H on cell cycle distribution was related to the decrease in cell viability, flow cytometric analysis was performed. As shown in Fig. 2A, the exposure to HKL 2H caused a significant increased cell fraction in G_2/M phase and a decreased cell fraction in G_0/G_1 phase in a time-dependent manner. The percentage of cells in G_2/M phase increased by 6.8-fold after the cells were treated with 30 nM HKL 2H for 12 h (Fig. 2B). These results demonstrated that HKL 2H has cell proliferation inhibitory effect and can induce the cell cycle arrest of K562 cells in G_2/M phase.

We next checked the expression levels of two G_2/M-related proteins in HKL 2H-treated K562 cells (30 nM) by Western blotting assay. As shown in Fig. 2C, the expression level of cyclins B and cyclin-dependent kinase 1 (CDK1) decreased significantly in HKL 2H-treated K562 cells in a time-dependent manner. However, it was obvious that the phosphorylated CDK1 (p-CDK1) level was

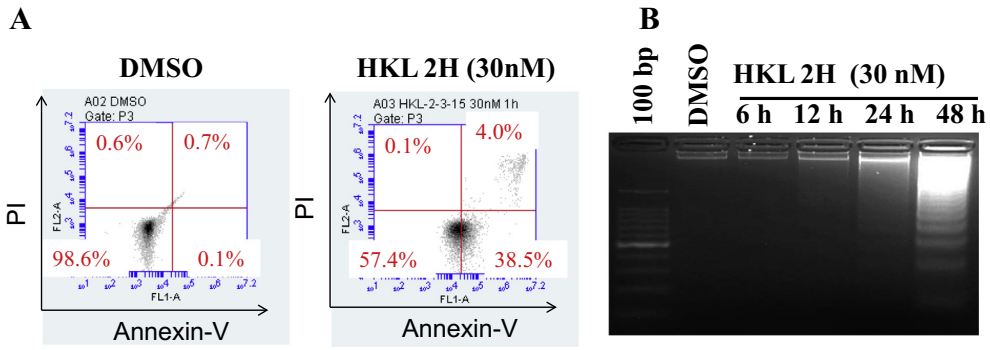

Fig. 3. HKL 2H induced apoptosis in K562 cells. (A) K562 cells were treated with 30 nM HKL 2H. At the time points indicated, cells were labeled with Annexin-V-FITC and PI and apoptosis was determined using FACS analysis. Data was shown as the mean of three independent experiments. The cell percentage in each phase of cell cycle was indicated. (B) DNA fragmentation after the exposure to HKL 2H. K562 cells were exposed to 30 nM HKL 2H for the indicated period. Genomic DNA was extracted as described in the experimental section, electrophoresed in a 2% agarose gel and visualized with ethidium bromide staining.

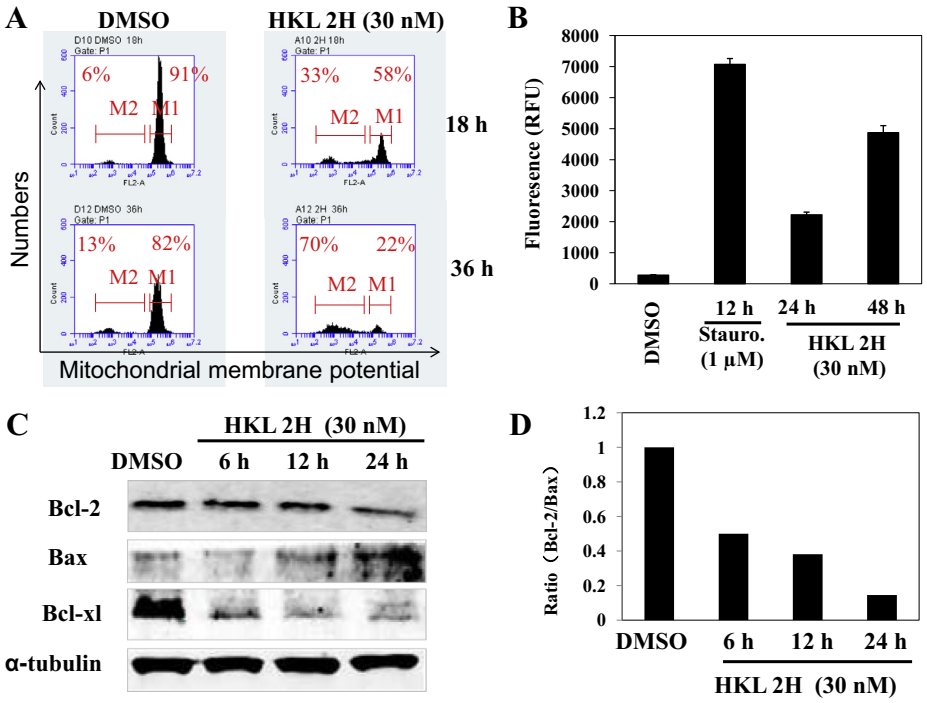

Fig. 4. Effects of HKL 2H treatment on mitochondrial membrane potential, the caspase-3 activity and the expression levels of Bcl-2 family proteins. (A) K562 cells were treated with 30 nM HKL 2H. At the time points indicated, cells were labeled with TMEM and the mitochondrial membrane potential was determined using FACS analysis. (B) Caspase-3 activation was quantified by means of a fluorescence-based method. (C) Expression of Bcl-2, Bax and Bcl-xL during HKL 2H-induced apoptosis. (D) Effect of HKL 2H on the ratio of Bcl 2/Bax in K562 cells.

upregulated instead. The result thus showed that HKL 2H can down-regulate cyclins B and CDK1 expressions but upregulate the expression level of p-CDK1 (Thr14) in K562 cells.

3.3. HKL 2H induced apoptosis in K562 cells

Flow cytometric analysis shown that apoptotic cells were observed in HKL 2H-treated K562 cells when double labelled with annexin-V-FITC and PI (Fig. 3A). The apoptotic rates (Annexin V+/PI−) in HKL 2H-treated K562 cells were increased to 38.5% of the total cells whereas only 0.1% cells were observed as apoptotic cells in the control. In HKL 2H-treated K562 cells, the DNA ladder pattern of internucleosomal fragmentation, another characteristic of apoptosis, was increased obviously in a time-dependent manner and maximized at 48 h after HKL 2H treatment (Fig. 3B). These results suggested that HKL 2H inhibited the proliferation of K562 cells by inducing apoptosis in a time-dependent manner.

3.4. HKL 2H induced apoptosis in K562 cells through the mitochondrial pathway

The mitochondria play a significant role in the occurrence of apoptosis of some cancer cells. To clarify whether the mitochondrial pathway is involved in the HKL 2H-induced apoptosis, K562 cells were treated with DMSO or HKL 2H (30 nM) for 18 and 36 h, respectively, and subjected to the membrane potential sensing dye TMRM. The mitochondrial membrane potential in HKL 2H-treated cells were decreased significantly from 58% (18 h) to 22% (36 h) whereas the loss of mitochondrial membrane potential in DMSO-treated cells only decreased from 91% (18 h) to 82% (36 h). This result showed that HKL 2H reduced the ratio of TMRM fluorescence of the cells and caused the dissipation of mitochondrial membrane potential (Fig. 4A), suggesting that HKL 2H-induced apoptosis was caused, at least in part, through the mitochondrial pathway.

The caspase cascade reaction is one of the most important events in the apoptosis process through the mitochondrial pathway [22]. To determine whether caspase-3 was involved in HKL 2H-induced apoptosis, K562 cells were treated with DMSO, 1 μM staurosporine (positive control) or 30 nM HKL 2H, respectively, and the caspase-3 activation was then determined. As shown in Fig. 4B, similar to the positive control, the caspase-3 activation was observed to increase significantly in HKL 2H-treated K562 cells. This result indicated that the caspase-3-dependent pathway was involved in the HKL 2H-induced apoptosis.

The loss of mitochondrial membrane potential is a pivotal event in the mitochondrial apoptosis pathway and regulated by Bcl-2 family, which comprises two functionally distinct groups: the apoptotic inhibitors including Bcl-2 and Bcl-xl and pro-apoptotic proteins such as Bax [24–26]. To investigate the role of Bcl-2 family members in HKL 2H-induced apoptosis in K562 cells, the effect of HKL 2H on the expressions of Bcl-2, Bax and Bcl-xL was investigated by Western blotting assay. Alpha-tubulin was used as an internal loading control. As shown in Fig. 4C, our data showed that the expression level of Bax protein increased significantly and the Bcl-xL protein level decreased dramatically in HKL 2H-treated K562 cells. Our data also showed that HKL 2H only caused a slight change in Bcl-2 expression, however the Bcl-2/Bax ratio was significantly downregulated in a time dependent manner (Fig. 4D) and peaked at 24 h when treated with HKL 2H (30 nM) (0.14-fold).

The dissipation of mitochondrial membrane potential, caspase-3 activation and the decrease of Bcl-2/Bax ratio in HKL 2H-treated K562 cells suggested the possible involvement of the mitochondrial apoptotic pathway in the programmed cell death in HKL 2H-treated K562 cells.

4. Discussion

In our previous study, HKL 2H has been found to be an excellent cytotoxic agent against human chronic myelogenous leukemia cells K562 (IC_{50} = 3 nM) [23]. In this study, the morphological changes, cell cycle arrest and apoptotic events of K562 cells when treated with HKL 2H were investigated.

Firstly, our study showed that HKL 2H caused typical G_2/M arrest morphological change. The elongated cells increased significantly compared to DMSO-treated cells (Fig. 1A), indicated that HKL 2H might inhibit the cell proliferation of K562 cells through the cell cycle arrest step.

The cell cycle is mediated by the activation of the cyclin-dependent kinases (Cdks). The cyclin/Cdk families have been found to play an important role in G_2 to M phase progression, particularly in the end of the G_2 phase. Both cyclin binding and phosphorylation by CDK are required for activation of the complex. In the present study, flow cytometric analysis showed that HKL 2H caused the cell cycle arrest of the K562 cells in G_2/M phase and G_2/M phase accumulation peaked at 24 h after HKL 2H treatment. Western blotting assay revealed a time-dependent decrease of cyclin B and CDK1 expression in HKL 2H-treated K562 cells. Our data showed that HKL 2H-induced sustained decrease in Cdk1 activity might be due to the phosphorylation of Cdk1 threonine 14, suggesting that the HKL 2H-induced modulations of G_2/M regulatory protein expression and activity contribute to HKL 2H-induced G_2/M arrest in tumor cells.

Our study also showed that HKL 2H caused typical apoptosis morphological change in K562 cells, gradually exhibiting cell rounding and shrinkage, vacuolization and even detachment from the bottom (Fig. 1B). This observation was further supported by finding nuclear fragmentation of K562 cells when treated with HKL 2H.

Mitochondria have been described to play a key role and perhaps even a central role in the apoptotic process. The caspase cascade reaction is one of the most important events in the apoptosis process through the mitochondrial pathway [22]. Among the caspase family, caspase-3 has been known to play a central role in apoptosis initiation [12]. The Bcl-2 family proteins, containing both pro-apoptotic and anti-apoptotic members, are known to regulate mitochondrial-mediated apoptosis. In the present study, we found that HKL 2H caused the dissipation of mitochondrial membrane potential and caspase-3 activation. Western blotting assay revealed a significant change of Bcl-2/Bax ratio in HKL 2H-treated K562 cells. Based on those observations, it was concluded that HKL 2H can induce mitochondrial dependent apoptosis in K562 cell.

In summary, the exposure of K562 cells to HKL 2H, a novel 5-(2-carboxyethenyl) isatin derivative results in cell growth inhibition which is concomitant with reversible G_2/M cell cycle arrest and apoptosis at doses as low as 3 nM. Our data reveal that HKL 2H possesses potential anti-cancer activity against human K562 cells by causing G_2/M arrest and enhancing mitochondrial-mediated apoptosis.

Acknowledgments

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