Ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate exhibits anti-proliferative activity and induces apoptosis in promyelocytic leukemia HL-60 cells

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Abstract. Furoquinolone and its derivatives exhibit anti-microbial, anti-allergic, anti-inflammatory and anticancer properties. The present study investigated the anti-tumor activity of synthesized intermediates of furoquinolone in human promyelocytic leukemia HL-60 cells. The biological effects of the active compound ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (compound 131) were examined in HL-60 cells. The following properties were analyzed: Cell survival, cell cycle profile, caspase-3 activity, Bax and Bcl-2 expression, the amount of intracellular Ca^{2+}, the number of reactive oxygen species (ROS) and the mitochondrial membrane potential. Compound 131 (50% cytotoxic concentration, 23.5 μM) significantly reduced the proliferation of HL-60 cells and was revealed to induce apoptosis in HL-60 cells in a concentration-dependent manner. Moreover, this was associated with the activation of caspase-3, upregulation of Bax, an increase in intracellular Ca^{2+} and ROS production, and a decrease in mitochondrial membrane potential and Bcl-2 expression levels. Compound 131, a novel 4,5-dihydrofuran-3-carboxylate, induced apoptosis in HL-60 cells via the increase of intracellular Ca^{2+} and ROS to alter the mitochondrial membrane potential and the protein level of Bax and Bcl-2, as well as activating caspase-3. The results of the current study indicate that compound 131 may represent a promising compound for the development of anti-leukemia therapeutics.

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Introduction

Leukemia is a malignancy affecting leukocytes and can be subdivided into myeloid and lymphocytic leukemia. The mortality rate of chronic myeloid leukemia has reduced since imatinib methylate was approved as the treatment. However, total acute myeloid leukemia-associated mortalities have continued to rise over the past 20 years (1). Numerous furoquinoline alkaloids have been identified and studied, including dictamnine, confusameline, skimmianine and kokusaginine, all of which belong to the Rutaceae family (2). The majority of furoquinoline alkaloids influence multiple biological processes, such as antifungal activity (3), antiplatelet aggregation (4) and Ca^{2+} influx suppression (5). Acrophylline and acrophyllidine have structures that are similar to furoquinolone, and can be isolated from *Acronychia*, *Dictamnus*, *Ptelea*, *Glycosmis* and *Ruta* plants (6), which exhibit anti-microbial and anticancer activities (7,8). Acrophyllidine and its synthetic derivatives also exhibit significant anti-allergic activity via suppressing mast cell degranulation (9). Moreover, ethyl 2-(3-hydroxyanilino)-4-oxo-4,5-dihydrofuran-3-carboxylate, an intermediate in furoquinolone synthesis, exhibits anti-inflammatory activity (10) and ethyl 2-[N-p-chlorobenzyl-(2'-methyl)] anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01007) has also been revealed to induce apoptosis in mouse leukemia (WEHI-3) and human cervical cancer (CaSki) cell lines (11,12). Notably, JOT01006 activates BCL2 antagonist/killer 1, poly(ADP-ribose) polymerase 1 and caspase-3, resulting in apoptosis and inhibiting the migration of human cervical cancer HeLa cells (13). However, anticancer activity of the intermediates in furoquinolone synthesis is rarely reported in treating acute myeloid leukemia.

The present study aimed to characterize the anti-proliferative and apoptotic activity of ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (compound 131) in acute promyelocytic leukemia HL-60 cells. The current results indicate that compound 131 induces apoptosis in HL-60 cells, and this was associated with increased intracellular Ca^{2+}, increased reactive oxygen species (ROS), activation of caspase-3 and a decrease in mitochondrial membrane potential. Hence, compound 131 may represent a novel target for treating acute promyelocytic leukemia.

Materials and methods

Cells. Human promyelocytic leukemia HL-60 and plasma cell leukemia ARH-77 cells (Bioresource Collection and Research Centre) were cultured in RPMI-1640 medium plus 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C and 5% CO_2. Vero cells (a monkey kidney epithelial cell line) (Bioresource Collection and Research Centre) were cultivated in Eagle's Minimum Essential Medium (Thermo Fisher Scientific, Inc.) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C, 5% CO_2.

Reagents. Ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (compound 131) was manufactured and refined using high-performance liquid chromatography as described in a previous study (10). Briefly, a mixture of diethyl malonate (32.0 g, 0.2 mol) in 50 ml tetrahydrofuran (THF) with chloroacetyl chloride (11.3 g, 0.1 mol) in 100 ml THF was incubated at 10-12˚C for 1 h followed by 40-45˚C for 1 h; ethyl 2-ethoxy-4-oxo-4,5-dihydrofuran-3-carboxylate was produced post cooling. Finally, the ethoxy group in the compound was substituted with aniline after stirring at room temperature for 1 h and heating on a water bath at 80˚C for 3 h to yield ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (compound 131). After the product of the reaction had been confirmed by performing via thin layer chromatography on silica gel-protected aluminum sheets (Type 60 F254; Merck KGaA) in which the spots were detected using a UV-lamp, the reaction was further mixed with 100 cc of ice water to form a precipitate; white crystals of compound 131 (18.29 g; yield, 74%; melting point, 115-116˚C) were generated after the precipitate was recrystallized from 90% ethanol at room temperature ˚C for 1-2 days. The structure of compound 131 (Fig. 1) was confirmed via mass spectrometry (*m/z*) as follows: 3267.87 (-NH-), 1695.26 (C4=O), 1672.59 (C3-CO-OEt); UV λmax nm (MeOH) (log ε): 297 (4.523); 1H-NMR (200 MHz, $CDCl_3$) δ: 1.24 (3H, *t*, *J*=7 Hz, H-2''), 4.20 (2H, *q*, *J*=7 Hz, H-1''), 4.67 (2H, *s*, H-5), 7.25 (5H, *m*, H-2', H-3', H-4', H-5', H-6'), 10.264 (1H, *s*,-NH-); 13C-NMR (200 MHz, DMSO-d6) δ: 14.67 (C-2''), 59.37 (C-1''), 75.30 (C-5), 86.99 (C-3), 123.48 (C-2', C-6'), 126.35 (C-4'), 129.27 (C-3', C-5'), 135.24 (C-1'), 164.18 (C-2), 177.34 (C-3''), 188.84 (C-4).

MTT assay. HL-60, ARH-77 or Vero cells (1x10^4/well) were cultured in 96-well plates in the presence or absence of compound 131 (0, 5, 25 and 50 μM) at 37˚C for 2 days followed by the addition of 20 μl 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) per well for another 4-h incubation at 37˚C. After removing the cultured media, the reduced form of MTT in the cells was solubilized using 150 μl dimethyl sulfoxide for 15 min. The survival rate of treated cells was measured as the ratio of the optical density $(OD)_{(570\text{-}630\ nm)}$ of treated cells to that of mock cells, similar to the calculation of the 50% cytotoxic concentration (CC_{50}).

Cell cycle analysis and caspase-3 fluorimetric assay. A total of 2x10^5 HL-60 cells were treated with compound 131 (0, 5, 25 or 50 μM) for 48 h at 37˚C. Subsequently, cells were collected after centrifugation at 100 x g (Kubota Corporation) for 5 min at room temperature and cell cycle profile analysis was performed using propidium iodide (PI) staining and caspase-3 activity analysis using BD ApoAlert Caspase Fluorescent assay kit (cat. no. 51-6632AK and 51-6632BK; BD Biosciences) as described in our previous report (14,15). PI-stained cells were analyzed at a wavelength of 488 nm by flow cytometry (BD Biosciences). The supernatants of treated cell lysates were added to the wells of the caspase-3 assay with BD ApoAlert Caspase Fluorescent assay kit for a 2-h incubation at 37˚C according to the manufacturer's protocol. Subsequently, caspase-3 activity was examined using a fluorescent substrate and a fluorescent plate reader (BioTek China) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Western blotting assay. The cells treated with compound 131 were harvested and washed with ice-cold PBS by centrifuging at 100 x g (Kubota Corporation) for 5 min at 4˚C, and then mixed with the RIPA lysis buffer (cat. no. R0278, Sigma-Aldrich; Merck KGaA) in microfuge tubes for 30 min at 4˚C. The lysate of treated cells was collected after centrifugation at 12000 x g for 20 min at 4˚C, and the protein concentration was determined using coomassie protein assay reagent (cat. no. 27813, Sigma-Aldrich; Merck KgaA) at an absorbance of 280 nm. A total of 20 μg of lysate per lane from the cells treated with compound 131 was dissolved in 2X SDS-PAGE sample buffer (Sigma-Aldrich; Merck KGaA), boiled at 95˚C for 5 min, loaded in 12% SDS-PAGE gels and then analyzed using a vertical electrophoresis system (Bio-Rad Laboratories, Inc.). The separated proteins in the gels were transferred to nitrocellulose papers, and were blocked with 5% skim milk at room temperature overnight. The immune reaction on the blots was performed using a 1:2,000 dilution of anti-caspase-3 (cat. no. 9662), anti-Bax (cat. no. 2772), anti-Bcl-2 (cat. no. 3498) and anti-β-actin (cat. no. 4970) antibodies in at room temperature overnight, and 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (cat. no. 7076) (Cell Signaling Technology, Inc.) at room temperature for 2 h. The immune-reactive bands were developed using enhanced chemiluminescence solution (Amersham Pharmacia Biotech Ltd.), as previously described (14,15).

Cytoplasmic-free Ca^{2+}, intracellular ROS and mitochondrial membrane potential ($\Delta\Psi_m$) assays using flow cytometry. To detect intracellular calcium levels, treated cells were collected and incubated with 10 μM Fluo-3/AM for 30 min at 37˚C in the dark and analyzed at 526 nm by flow cytometry (16). In the intracellular ROS assay, cells stained with

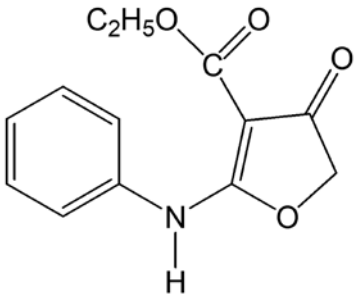

Figure 1. Chemical structure of compound 131 as ascertained by nuclear magnetic resonance spectroscopy. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate.

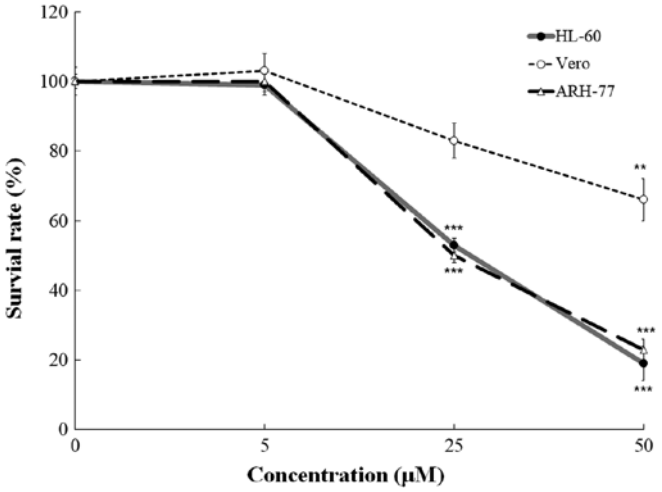

Figure 2. Survival rates of HL-60 cells and Vero cells in response to compound 131. HL-60, Vero and ARH-77 cells were treated with compound 131 at the indicated concentrations for 48 h. The cells were analyzed using an MTT assay; absorbance values at 570 nm were recorded. Survival data exhibited represent percentages of absorbance values of treated cells with compound 131 normalized to PBS-treated cells (Control) . **P<0.01; ***P<0.001 vs. PBS-treated cells. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate.

2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were examined using flow cytometry as described in our previous study (17). To measure the $\Delta\Psi_m$, compound 131-treated cells were analyzed with $DiOC_6$ staining at 37˚C for 30 min and examined using flow cytometry (17).

Statistical analysis. Data from three independent experiments of mock cells and compound 131-treated cells were analyzed by one-way ANOVA followed by Scheffe's post hoc test using SPSS 12.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with compound 131 results in cell growth inhibition and apoptosis in HL-60 cells. Initially, 23 synthesized intermediates of furoquinolone at 50 μM were screened for anti-proliferative activity against HL-60 and Vero cells; only compound 131 (ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate) exhibited a significant inhibitory effect on the proliferation of HL-60 (but not Vero cells) (Fig. S1). Subsequently, the cells were treated with compound 131 at 0, 5, 25 and 50 μM for 48 h at 37˚C; the cell viability was tested using the MTT assay to determine the CC_{50} values of compound 131 on the proliferation of HL-60, ARH-77 and Vero cells. The survival rates of HL-60 and ARH-77 cells treated with compound 131 were significantly lower compared with treated Vero cells (Fig. 2). The CC_{50} values of compound 131 were 23.5 μM for HL-60, 24.2 μM for ARH-77 and 87.0 μM for Vero cells. These results demonstrated that compound 131 exhibits significant anti-proliferative effects against HL60 and ARH-77 cells.

Activation of caspase-3 in HL-60 cells following treatment with compound 131. To determine whether compound 131 initiated apoptosis, the cell cycle and caspase-3 activity of treated cells were further analyzed using flow cytometry, western blotting and caspase-3 enzymatic activity assays (Figs. 3 and 4). Cell cycle analysis indicated that compound 131 increased the percentage of cells in the sub-G_1 phase (apoptotic fraction) in a concentration-dependent manner in HL-60 cells (Fig. 3A). The percentage of apoptotic cells (sub-G_1 fraction) was 0.2, 1.6, 75.4 and 80.0% in cells treated with compound 131 at 0, 5, 25 and 50 μM, respectively (Fig. 3B). Moreover, western blot analysis of cell lysates indicated that compound 131 treatment increased the protein levels of pro- and active forms of caspase-3, and also upregulated Bax and downregulated Bcl-2 in a concentration-dependent manner (Fig. 4A and B). Fluorescence assays of caspase-3 enzymatic activity revealed that caspase-3 enzymatic activity in compound 131-treated cells was higher compared with mock cells by 6-, 43- and 140-fold for 5, 25 and 50 μM, respectively (Fig. 4C). The current results indicate that compound 131 significantly promotes caspase-3 and Bax activation, but suppresses Bcl-2 expression, in apoptotic cells.

Treatment with compound 131 results in increases of intracellular calcium and ROS in HL-60 cells. Intracellular Ca^{2+} accumulation and ROS generation serve a critical role in apoptosis (17-20). The effects of compound 131 on intracellular Ca^{2+} and ROS levels in HL-60 cells were examined (Figs. 5 and 6). Following treatment with compound 131 (0, 5, 25 or 50 μM) at 37˚C for 48 h, cells were harvested, stained with Fluo-3/AM or DCFH-DA and analyzed using flow cytometry. Compound 131 treatment resulted in the increase of intracellular Ca^{2+} release from the ER in HL-60 cells. A 39.2% increase resulted from treatment with 5 μM, a 93.8% increase from treatment with 25 μM and an 80.4% increase following treatment with 50 μM compared with PBS-treated cells, respectively (Fig. 5). In addition, compound 131 significantly increased the production of intracellular ROS compared with mock cells (Fig. 6). The results indicate that compound 131 treatment significantly stimulates an increase in intracellular Ca^{2+} and ROS levels in HL-60 cells.

Treatment with compound 131 results in a decrease of mitochondrial membrane potential in HL-60 cells. The disruption of mitochondrial membrane potential (MMP) has been reported to correlate with ROS generation (21-23). Treated

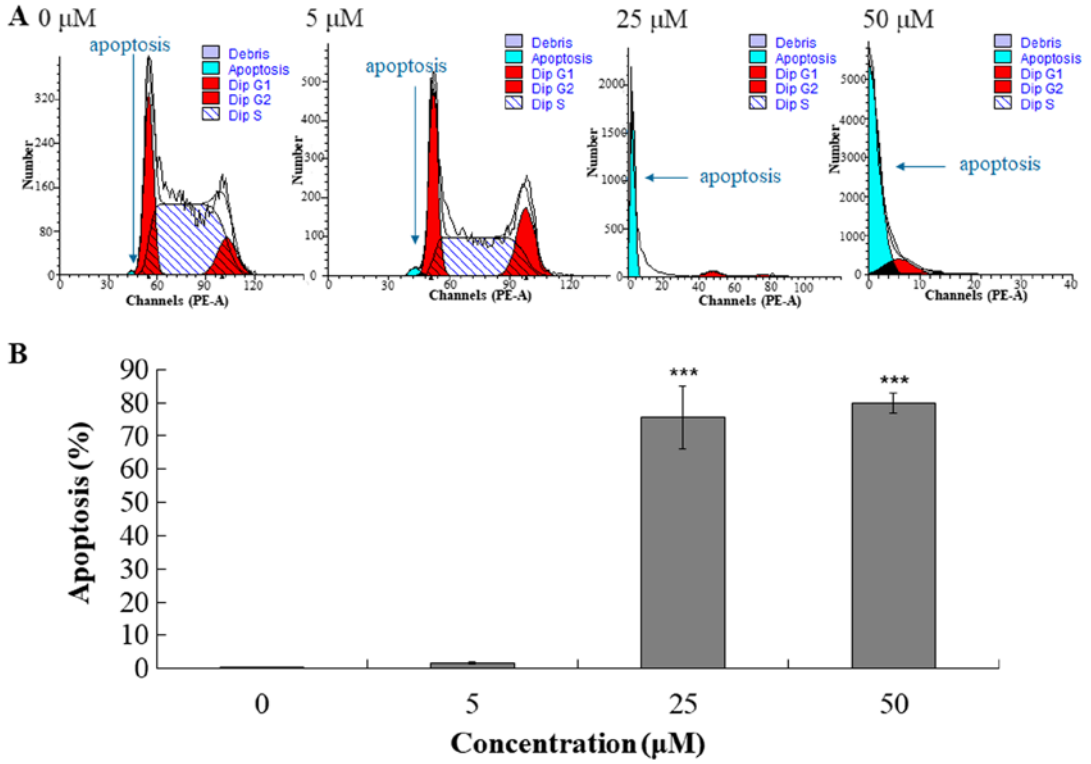

Figure 3. Cell cycle profiles and apoptotic rates of HL-60 cells treated with compound 131. Cells were treated with compound 131 at 37˚C for 48 h. (A) Cells stained with propidium iodide were analyzed using flow cytometry. (B) Proportions of apoptotic HL-60 cells after compound 131 treatment are presented as a bar graph. ***P<0.001 vs. PBS-treated cells. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate.

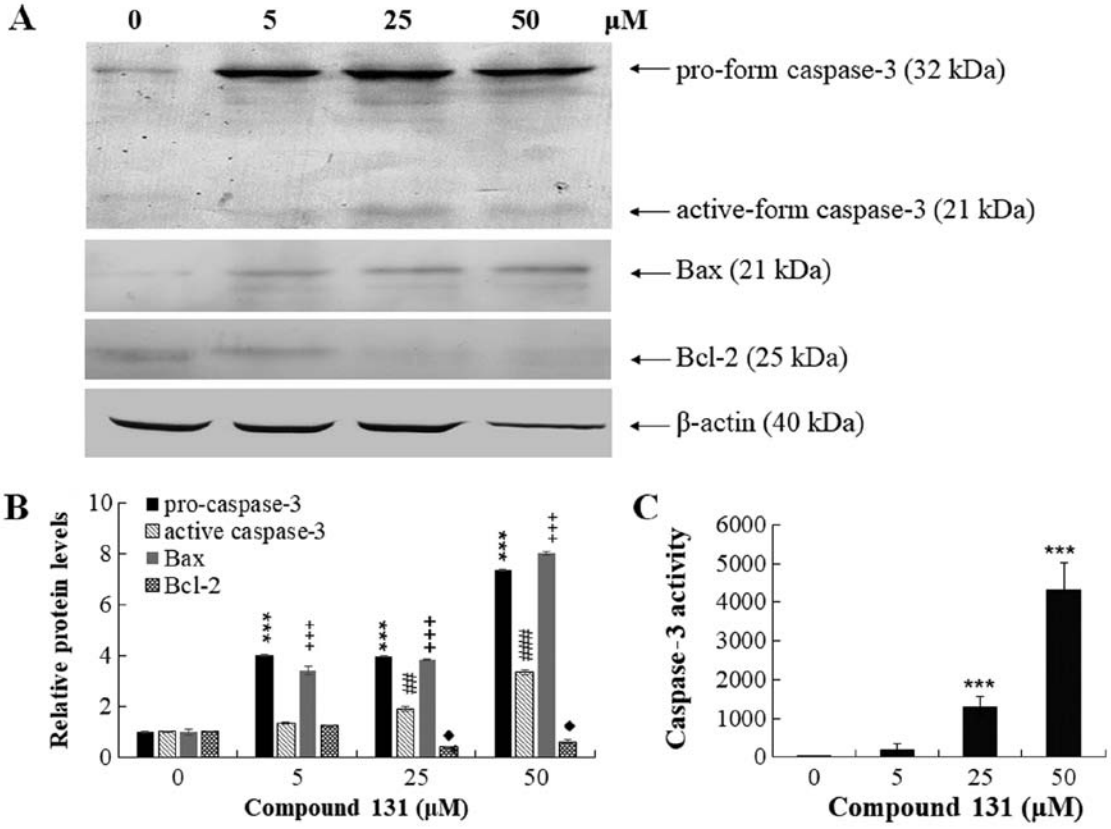

Figure 4. Addition of compound 131 results in caspase-3 activation and Bax upregulation in HL-60 cells. Treated cells were harvested after a 2-day incubation with compound 131 at 37˚C. (A) Lysates of treated HL-60 cells were analyzed using western blotting with anti-caspase-3, anti-Bax and anti-Bcl-2 antibodies. (B) Relative level of each immuno-reactive band was quantified and normalized to the protein level of β-actin. (C) Lysates reacted with fluorescent substrates at 37˚C for 2 h; then, the fluorescence intensity of each well was analyzed using a fluorescent plate reader (BioTek China) (excitation at 380 nm and emission at 460 nm). $^{◊}$P<0.05 ***P<0.001; $^{##}$P<0.01; $^{###}$P<0.001; $^{+++}$P<0.001 vs. PBS-treated cells. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate.

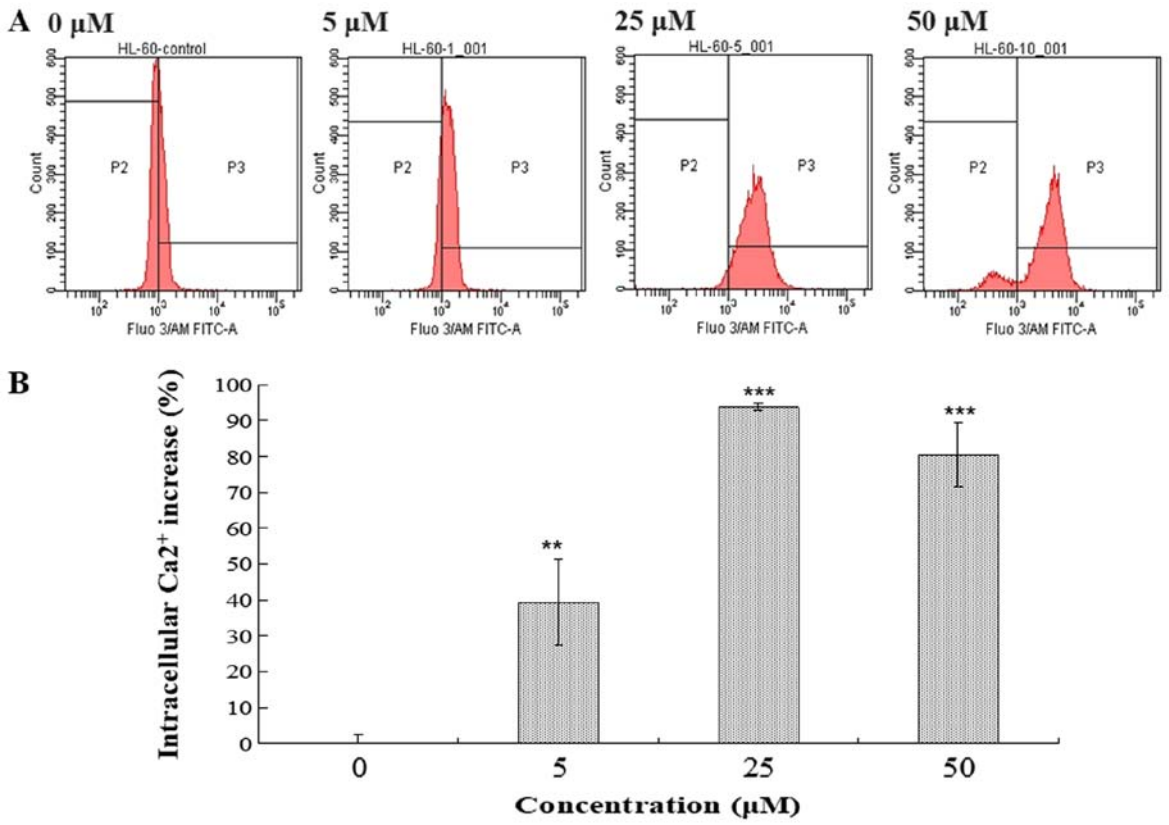

Figure 5. Intracellular calcium production of HL-60 cells is influenced by compound 131. Treated cells were stained using Fluo-3/AM. (A) Intracellular calcium levels in HL-60 cells were analyzed by flow cytometry (absorbance at 526 nm). (B) Relative increases in the intracellular calcium levels were quantified and are presented as a bar graph. **P<0.01; ***P<0.001 vs. PBS-treated cells. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate.

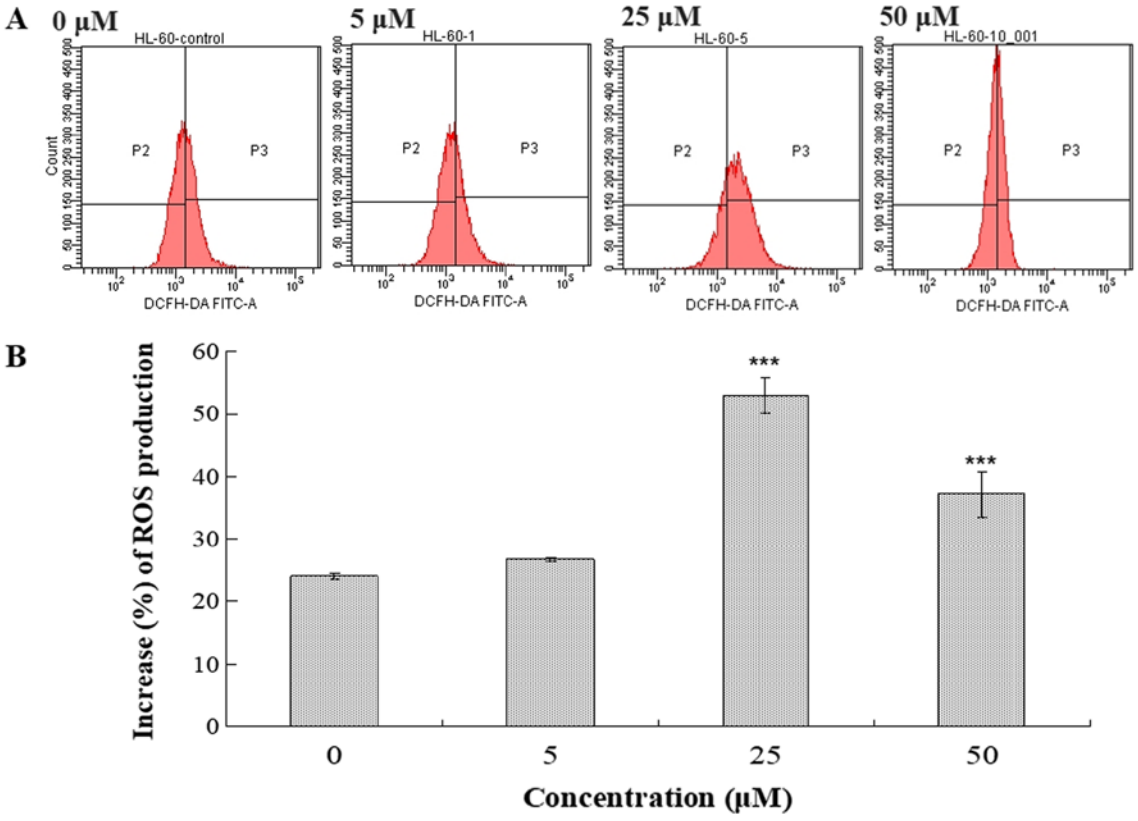

Figure 6. Intracellular ROS production of HL-60 cells is influenced by compound 131. (A) Cells were stained with DCFH-DA and examined by flow cytometry. (B) Increment ratio of intracellular ROS in treated cells is shown by bar graph. ***P<0.001 vs. PBS-treated cells. ROS, reactive oxygen species; compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate.

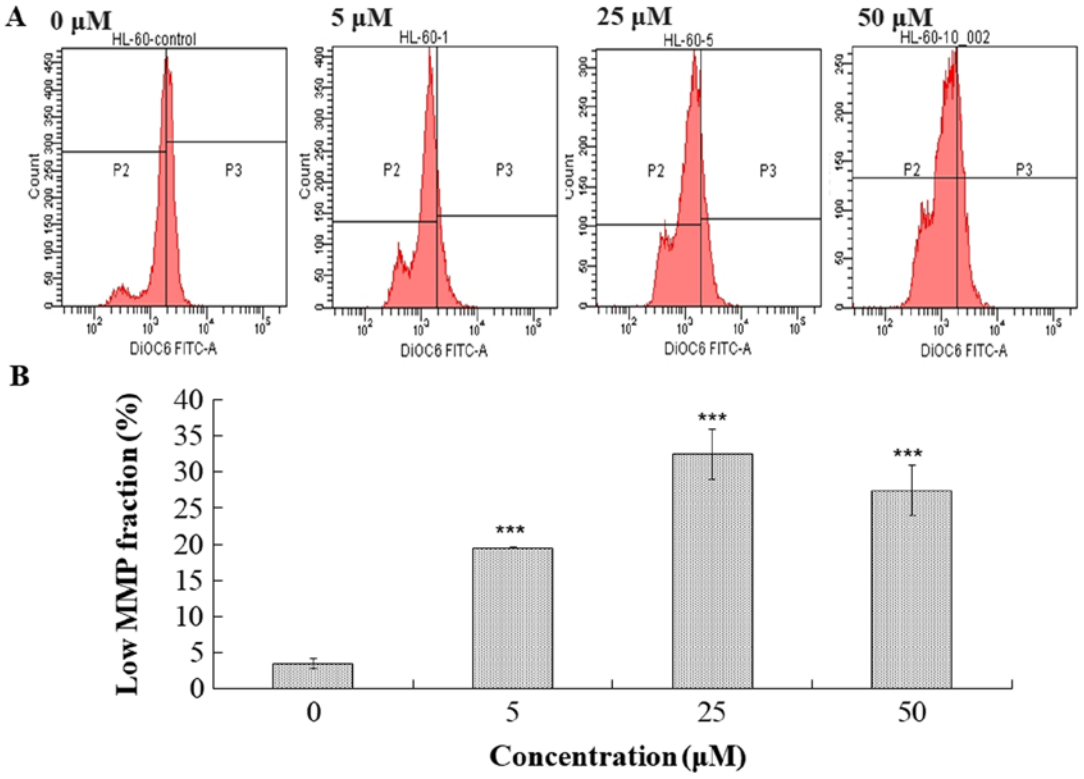

Figure 7. MMP detection in compound 131-treated HL-60 cells. (A) Treated HL-60 cells were stained with $DiOC_6$ and analyzed by flow cytometry. (B) Percentages of the MMP decrease in treated HL-60 cells are illustrated by bar graph. ***P<0.001 vs. PBS-treated cells. Compound 131, ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate; MMP, mitochondrial membrane potential.

cells were tested to determine the MMP levels by staining with $DiOC_6$ and analysis using flow cytometry (Fig. 7). Cells treated with compound 131 exhibited a significant decrease in $DiOC_6$ intensity by 19.6% for 5 μM, 32.5% for 25 μM and 27.45% for 50 μM. Thus, it was revealed that treatment with compound 131 also resulted in a decrease in MMP in HL-60 cells.

Discussion

To the best of our knowledge, this is the first report to demonstrate that ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (compound 131), an intermediate of furoquinoline synthesis, exerts anti-leukemic effects and induces apoptosis via the upregulation of caspase-3 and Bax in HL-60 cells. These effects are also associated with an increase in intracellular calcium, an increase in ROS levels and a decrease in MMP expression. The mechanism of compound 131-induced apoptosis was similar to previous reports of apoptosis induced by 4,5-dihydrofuran-3-carboxylate derivatives (11,12). In addition, a structure-function association study demonstrated that ethyl -2-(3-methoxyanilino)-4-oxo-4,5-dihyfrofuran-3-carboxylate and ethyl-2-(3-oxyanilino)-4-oxo-4,5-dihyfrofuran-3-carboxylate exhibited lower anti-proliferative properties than compound 131 (ethyl 2-anilino-4-oxo-4,5-dihydrofuran-3-carboxylate) (data not shown). The aforementioned result indicates that the aniline group in compound 131 serves a key role in its anti-proliferative activity against HL60 cells. Ethyl 2-[N-p-chlorobenzyl-(2'-methyl)] anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01007) triggered the mitochondria-dependent pathway, which was significantly correlated with cytoplasmic Ca^{2+} levels in human cervical CaSki cancer cell apoptosis (11). Moreover, ethyl 2-[N-m-chlorobenzyl-(2'-methyl)] anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01006) induced the increase in ROS production that resulted in the caspase-dependent apoptosis of human cervical cancer cells (13). In addition to anticancer activity, the intermediates of furoquinoline synthesis and furoquinoline derivatives have been revealed to exhibit diverse pharmacological effects, such as 5-HT2 receptor antagonist activity (24), vasorelaxation via the suppression of calcium influx (25), anti-allergic effects (9) and the blocking of outward K^+ current and Na^+ channels (5). A previous study revealed that the intermediates of furoquinoline synthesis exhibited moderate inhibitory effects on the growth of human ovarian cancer A2780 cells (26).

In summary, the present results indicated that compound 131 inhibits the proliferation and induces apoptosis in HL-60 cells; moreover, it was associated with the production of intracellular Ca^{2+} and ROS, and reduced the mitochondrial membrane potential. Compound 131, a novel 4,5-dihydrofuran-3-carboxylate, represents a promising compound that may inform the development of new anti-leukemia agents. The current results also revealed the biological properties of compound 131 and indicated the mechanisms underlying its anti-leukemia effects.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

ACH, CSL, JCL, HCL, WHL and CWL designed and conducted the experiments. ACH, CSL, and JCL performed data analysis. ACH and CWL wrote the first draft of the manuscript. All authors reviewed and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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