Induction of apoptosis and erythroid differentiation of human chronic myelogenous leukemia K562 cells by low concentrations of lidamycin

CHU ZHANG1*, LU-YING GUO2*, DAN MU2, JIAN-HUA GONG3 and JING CHEN2

1School of Pharmacy, China Pharmaceutical University, Nanjing, Jiangsu 210009; 2College of Life Sciences, North China University of Science and Technology, Tangshan, Hebei 063210; 3Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 210009, P.R. China

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Abstract. Apoptosis induction and differentiation of promyelocytic leukemic cells into mature cells are major strategies for the drug-based treatment of leukemia. Lidamycin (LDM) which is a member of the enediyne antibiotic family exhibits extreme cytototoxicity. In the present study, the induction of apoptosis and differentiation in human chronic myeloid leukemia K562 cells by low concentrations of lidamycin were investigated. K562 cells were treated with lidamycin at various concentrations for 48 h, and accumulated in the metaphase as determined in previous experiments. Cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay and the IC50 value of lidamycin was 0.1±3.2 nM. Induction of apoptosis was investigated morphologically by acridine orange/ethidium bromide (AO/EtBr) staining. Growth inhibition and apoptosis induction were observed in cells treated with low concentrations of lidamycin. In addition, western blot analysis revealed that treatment of the K562 cells with lidamycin at low concentrations upregulated the expression of caspase-8 and caspase-3. The induction of differentiation in human chronic myeloid leukemia K562 cells by lidamycin at low concentrations was also investigated. The nitroblue tetrazolium reduction ability of K562 cells was increased following treatment with lidamycin. Low concentrations of lidamycin triggered erythroid differentiation among K562 cells, indicated by morphological changes, increased hemoglobin content, and the expression of cell surface antigens such as CD71. Additionally the expression of GATA-binding factor 1 (GATA-1) protein in low concentration lidamycin-treated K562 cells was increased. The results of the present study suggest that a low-concentration lidamycin exerts effects on apoptosis and erythroid differentiation induction by increasing the expression of caspases and GATA-1 protein. Lidamycin may serve a positive role in relevant targeted chemotherapy and may represent a potential candidate for chronic myelogenous leukemia differentiation-inducing treatment.

Introduction

Chronic myelogenous leukemia (CML) is a lethal malignancy. The increased and unregulated growth of predominantly myeloid cells in the bone marrow is the main characteristic of CML. CML has a morbidity of 1-2 cases per 100,000 adults and is responsible for ~15% of all newly diagnosed cases of leukemia in adults (1). The majority of patients with CML fail to respond effectively to the current regimen of drug therapy due to occurrence of drug resistance. Bone marrow or allogenic stem cell transplantations are recent and effective treatments for CML, but they have a high risk of morbidity and mortality (2). CML places considerable burden on patients and the majority of chemotherapeutic drugs have long-term side effects (3,4). Therefore, it is important to continue research into novel therapeutic approaches for CML. With regard to the induction of apoptosis, it is generally believed that drugs cause the elimination of cancer cells. Apoptosis is a type of cell death used by multicellular organisms to prevent uncontrolled proliferation and to dispose of unwanted cells. Apoptosis is originally characterized by morphological changes, including membrane blebbing, DNA fragmentation, chromatin condensation, nuclear fragmentation, cell shrinkage and apoptotic body formation (5). Resistance to apoptosis is a reason for concern in developing effective chemotherapeutic agents in various types of cancer. In addition, differentiation-inducing therapy has been proven to be a promising strategy. To date, all-trans retinoic acid-based therapy of acute promyelocytic...
leukemia (APL) has been the best clinical application of differentiation therapy (6,7). However, the development of differentiation therapy for CML requires further investigation.

Lidamycin (LDM) is a macromolecular enediyne antibiotic (8). LDM has two parts: one is an enediyne chromophore (MW 843 Da) responsible for the extremely potent bioactivity of the antibiotic and the other is a non-covalently bound apoprotein (MW 10.5 kDa) (9,10). The two parts can be dissociated and reconstituted, and the biological activity of the rebuilt molecule is comparable to that of natural LDM (11,12). LDM can induce DNA damage (13). LDM is extremely cytotoxic and induces growth inhibition of transplantable tumors in mice (14-17). Furthermore, LDM exhibited a strong inhibitory effect on tumor metastasis and angiogenesis (18). At present, several clinical trials are in progress to assess the therapeutic efficacy of LDM in multiple cancer indications (19). To determine the function of LDM on CML, the present study investigated the effects of LDM on the K562 cell line as an experimental model for CML. The present study revealed that LDM was highly active in inhibiting cell growth and that a low concentration LDM could induce cell apoptosis by upregulating the expression of caspase-8 and caspase-3. Furthermore, a low concentration of LDM increases the cell hemoglobin contents and induces erythroid differentiation of K562 cells by upregulating the expression of GATA-1.

Materials and methods

Cell culture. The human K562 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (10 U/ml) and streptomycin (100 U/ml) at 37°C. The cells were accumulated in the metaphase in the following experiments.

Cell proliferation assay. A cell proliferation assay was performed using a Cell Counting Kit-8 (CCK-8; Beijing Zoman Biotechnology Co., Ltd., Beijing, China). In brief, the K562 cells were plated into 96-well plates at a density of 5,000 cells/well and incubated at 37°C overnight. Experimental cells were treated with various concentrations (10^{-12} to 10^{-6} M) of LDM for 48 h at 37°C. Next, 10 µl of CCK-8 reagent was added into each well. Cells were incubated for 2 h at 37°C and optical density was measured at 450 nm. The aforementioned experiments were performed in triplicate.

Morphological analysis of apoptotic cells. The K562 cells were seeded into 12-well plates at 5x10^4 cells/well and exposed to various concentrations of LDM for 48 h. Cell viability was assessed by CCK-8 assay. Results were derived from three independent experiments. CCK-8 assay showed the potent inhibitory effects of LDM on K562 cell proliferation. The dashed line shows the IC_{50} value which was calculated by GraphPad Prism 7 software. LDM, lidamycin; CCK-8, Cell Counting Kit-8.

Figure 1. Effect of LDM on the proliferation of K562 cells. Cells were exposed to various concentrations of LDM for 48 h. Cell viability was assessed by CCK-8 assay. Results were derived from three independent experiments. LDM, lidamycin; CCK-8, Cell Counting Kit-8.

In order to distinguish the apoptosis induction and look for the more effective drug dose, we used a lower concentration of LDM (0.001 nM) and a higher concentration of LDM (10 nM) in the following experiments.

Nitro blue tetrazolium (NBT) reduction experiment. The K562 cells were seeded into 12-well plates at 5x10^4 cells/well and exposed to LDM for 48 or 72 h. Next, cells were harvested and washed with PBS for 3 times. After 100 µl 0.2% NBT solution containing TPA (0.2 mg/ml) was added, cells were incubated in the dark at 37°C for 30 min and 1 ml cold PBS was added to terminate the reaction. The cells were then placed onto a glass slide and observed under a light microscope (magnification, x100). Three visual fields (each containing 200 cells) were randomly selected, and the percentage of blue punctate particle-positively stained cells was calculated using Mshot Image Analysis system (Micro-shot Technology Co., Ltd., Guangzhou, China).

Morphological analysis of K562 cells. The K562 cells were seeded into 12-well plates at 5x10^5 cells/well and exposed to LDM for 48 or 72 h. Morphological changes in the cells were evaluated by Wright-Giemsa staining using a fluorescence microscope (BH2 system; Olympus Corp.). Cells were washed with cold PBS and adjusted to a cell density of 5x10^5 cells/well. A total of 10 µl cells was placed onto a glass slide, mixed with Giemsa and observed under a microscope.
Detection of hemoglobin contents. The K562 cells were seeded onto 6-well plates at 2 ml/well and exposed to LDM for 48 or 72 h. All the cells were harvested and lysed, and the concentration of hemoglobin was determined using a spectrophotometer. The aforementioned experiments were performed in triplicate.

CD71 expression assay. The expression of CD71 on the surface of K562 cells was assessed using a flow cytometer. The K562 cells were seeded into 6-well plates at 2 ml/well and exposed to LDM for 48 or 72 h. Cells were then harvested and the cell count was adjusted to 1x10⁵ cells/ml. Following washing with PBS, cells were labeled with 20 μl PE-conjugated anti-CD71 (BD Biosciences, San Jose, CA, USA) for 15 min in the dark at room temperature. Cells were washed twice with PBS and resuspended in 500 μl PBS prior to measurement of CD71 expression. Fluorescence intensity was measured using FACSCanto II (BD Biosciences) in continuous mode (20).

Western blot analysis. The K562 cells were treated with LDM and harvested after 48 or 72 h. Harvested cells were washed with PBS and then lysed using RIPA buffer [25 mM Tris (pH 7.8), 2 mM EDTA, 20% glycerol, 0.1% Nonidet P-40 (NP-40), 1 mM dithiothreitol] and protease inhibitors. The protein concentrations of cell supernatants were determined with the BCA Protein Assay kit from Hyclone-Pierce. Proteins (30 μg/lane) were equally loaded to and separated by SDS-PAGE gel (15%). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Bedford, MA, USA). Blots were blocked for 60 min at room temperature with 5% non-fat milk powder and 0.1% Tween-20 in PBS and exposed overnight at 4°C using primary antibodies against caspase-8 (sc-56071; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), active caspase-8 (cat. no. 9748; Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-3 (sc-56052; Santa Cruz Biotechnology, Inc.), active caspase-3 (cat. no. 9661; Cell Signaling Technology, Inc.), GATA-1 (sc-266) and β-actin (sc-130065) (both from Santa Cruz Biotechnology, Inc.). All the primary antibodies were diluted at 1:1000; sc-2380 and sc-2379; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The bands were visualized using enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology, Inc.). Western blots were quantified via densitometry scanning using NIH Image software version 1.46 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Results are expressed as the mean ± standard deviation (SD) of three independent experiments. Treatment effects were analyzed using the unpaired Student’s t-test when comparing two variables, while inter-group and intra-group comparisons were conducted using one-way ANOVA test with post hoc contrasts by Student-Newman-Keuls test. A P-value of ≤0.05 was considered to indicate a statistically significant difference.

Results

Effects of LDM on the proliferation of K562 cells. Inhibition of cell proliferation was measured using CCK-8 assays. K562 cells were treated with different concentrations of LDM for 48 h. CCK-8 assays revealed that cell viability was reduced following treatment with LDM and the IC₅₀ value was 0.1±3.2 nM which was calculated by GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA (Fig. 1). Since LDM has extreme cytotoxicity to many cancer cells as well as normal cells, in order to reduce its toxicity, we explored the effects of low dose LDM. In our experiment, we chose 0.01 or 0.001 nM as the low concentration.

Induction of apoptosis by LDM in K562 cells. AO/EB staining was used to detect the apoptosis of K562 cells. AO can insert the DNA of whole cells and show green fluorescence, while EB only penetrates the impaired cell membrane and shows orange red fluorescence. Fluorescence increases in an apoptotic cell. Under a fluorescence microscope, the nuclei of the control cells were large and round without condensation or fragmentation. By contrast, following exposure to LDM for 48 h, the majority of cells presented with typical morphological changes of apoptosis, including chromatin condensation or shrunk nuclei (Fig. 2). In the group treated with higher concentrations of LDM, condensed nuclei were observed. The live cells appeared green in color with undamaged nuclei, while the early apoptotic cells exhibited condensed nuclei and were green in color with bright green dots in their nuclei, and the late apoptotic cells were orange in color. In addition, the number of cells also decreased in the 0.1 and 1 nM LDM groups, respectively.

In addition, flow cytometry (FACS) analysis was carried out to determine the percentage of apoptosis. Unfortunately,
the results were unsatisfactory and this will be repeated in future studies.

**Effects of LDM on the activation of apoptosis-related proteins in K562 cells.** Apoptosis is induced by the activation of a group of cysteine proteases called ‘caspases’, which cleave proteins during cell death. According to the function of caspases, they are grouped into two different subfamilies. One subfamily includes the initiation (caspase-8 and -9) and execution (caspase-3, -6 and -7) of the apoptotic program (initiator and executioner caspases). Caspase-8 and caspase-9 are caspases upstream of the apoptosis signal transduction process, while caspase-3 is downstream, all of which are executioner molecules of cell apoptosis. The activation of initiator caspase-8 will, in turn, activate caspase-3. Caspase-3 is an executioner caspase in the last and irreversible phase of the apoptotic caspase-dependent pathway. The expression of caspase-8 and caspase-3 in K562 cells treated with 0.01, 0.1 and 1 nM LDM was investigated by western blot analysis. As shown in Fig. 3, treatment with LDM for 48 h led to upregulation and activation of caspase-8 and caspase-3 in K562 cells compared to controls.

**Effect of LDM at low concentrations on the differentiation induction of K562 cells.** To assess whether a low concentration of LDM could induce differentiation of K562 cells, the NBT reduction experiment was performed. Differentiated K562 cells can reduce NBT to dark blue diformazan particles, which can easily be observed under a light microscope. K562 cells were treated with 0.001, 0.1 and 10 nM LDM for 48 or 72 h. The NBT-positive percentages of the control group were 7.6±1.5 and 10.3±1.7% at 48 and 72 h, respectively, while the NBT-positive percentages of K562 cells were 45.7±4.2, 52.5±2.7 and 28.4±5.6% in the 0.001, 0.1 and 10 nM groups, respectively, at 48 h; and 59.4±3.1, 73.1±1.9 and 32.8±2.8% in the 0.001, 0.1 and 10 nM groups, respectively, at 72 h. To note, most cells died in the 10 nM group, thus the NBT reduction rate was decreased (Fig. 4).

**Effect of LDM at low concentrations on the induction of erythroid differentiation of K562 cells**

*Morphological changes of K562 cells.* Wright-Giemsa staining revealed that a low concentration of LDM induced distinct morphological changes in K562 cells. In control groups, K562...
cells showed blue purple staining. While some cells showed pale pink in the 0.001 and 0.1 nM LDM groups. This reflected some changes in cellular components. In addition, the cell volume also changed considerably. Most cells died in the 10 nM LDM group. The cytoplasm of K562 cells following treatment with 0.1 and 0.001 nM LDM for 48 or 72 h became more ample than that of the control group cells. These typical morphological changes showed a differentiation tendency for K562 cells (Fig. 5).

**LDM increases the hemoglobin contents of K562 cells.** The results of the hemoglobin content assay demonstrated that the level of hemoglobin in K562 cells was increased following treatment with LDM for 48 or 72 h. In the 0.001 and 0.1 nM LDM groups, the hemoglobin contents of K562 cells were markedly increased, compared with those in the control group. Additionally, most cells died in the 10 nM group, thus there was a decrease in the hemoglobin content (Table I).

**LDM increases the expression level of CD71 in K562 cells.** The CD71 antigen is a classical erythroid differentiation marker expressed on the cell surface. Flow cytometric analysis demonstrated that LDM increased the expression level of CD71 in K562 cells. Compared with the control cells, the expression of CD71 was 10.1, 16.7 and 5.4% in the 0.001, 0.1 and 10 nM groups, respectively, at 48 h; and 26.3, 71.4 and 9.3% in the 0.001, 0.1 and 10 nM groups, respectively, at 72 h. The expression of CD71 in the 0.1 nM LDM-treated K562 cells showed blue purple staining. While some cells showed pale pink in the 0.001 and 0.1 nM LDM groups. This reflected some changes in cellular components. In addition, the cell volume also changed considerably. Most cells died in the 10 nM LDM group. The cytoplasm of K562 cells following treatment with 0.1 and 0.001 nM LDM for 48 or 72 h became more ample than that of the control group cells. These typical morphological changes showed a differentiation tendency for K562 cells (Fig. 5).

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**Table I. Influence of LDM on the hemoglobin content of K562 cells.**

<table>
<thead>
<tr>
<th>Concentration of LDM (nmol/l)</th>
<th>n</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>0.273±0.024</td>
<td>0.341±0.052</td>
</tr>
<tr>
<td>0.001</td>
<td>3</td>
<td>0.703±0.204*</td>
<td>0.827±0.174*</td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
<td>0.833±0.314*</td>
<td>1.157±0.162*</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.562±0.116*</td>
<td>0.614±0.332*</td>
</tr>
</tbody>
</table>

*P<0.05, *P<0.01 vs. control (0 nmol/l) group. LDM, lidamycin.
cells was significantly higher than that in the other group cells (Fig. 6). These results clearly demonstrated that low concentration LDM could induce the differentiation of K562 cells into erythroid lineage.

**Activation of GATA-1 is associated with the erythroid-inducing differentiation mechanism in K562 cells by LDM at a low concentration.** GATA-1 is a member of the GATA transcription factor family and is a key mediator of the development of specific types of blood cells from their precursor cells. Based on the erythroid-inducing differentiation effects of LDM on K562 cells and the significant role of GATA-1 in erythropoiesis, GATA-1 protein expression was detected by western blot analysis. The results demonstrated that following treatment with LDM for 48 and 72 h, the GATA-1 protein expression in the K562 cells was increased compared with that in the control cells, while it was decreased in the 10 nM group as most of the cells died (Fig. 7).

**Discussion**

Chronic myelogenous leukemia (CML) is a lethal hematological disorder originating from a small number of leukemia
of K562 cells. The most important factor in the mechanism of that LDM at low concentrations could induce the apoptosis morphologically by AO/EB staining. The results revealed present study, the induction of apoptosis was investigated to be an efficient strategy for the treatment of cancer. In the of cancer, induction of apoptosis in cancer cells is known 0.1±3.2 nM. Since the inhibition of apoptosis is a hallmark of cancer, induction of apoptosis in cancer cells is known to be an efficient strategy for the treatment of cancer. In the present study, the induction of apoptosis was investigated morphologically by AO/EB staining. The results revealed that LDM at low concentrations could induce the apoptosis of K562 cells. The most important factor in the mechanism of apoptosis is the activation of caspases. Caspases are proteases that participate in the control of cell growth and apoptosis (22). Caspase-8 and caspase-9 are caspases upstream of the apoptosis signal transduction process, while caspase-3 is downstream, and all of these are effector molecules of cell apoptosis (23). The present study analyzed the protein expression level of caspase-8 and caspase-3 by western blot analysis. The results demonstrated that a low concentration of LDM could increase the expression of caspase-8 and caspase-3 in K562 cells. Furthermore, the fact that LDM at a low concentration increased NBT reduction capacity indicated that it could induce the differentiation of K562 cells. Subsequently, morphological changes, including decreased nuclear to cytoplasm ratio following LDM treatment, were observed under a light microscope. The hemoglobin concentration in LDM-treated K562 cells was further evidenced by a colorimetry assay. Flow cytometric analysis revealed that LDM could increase the expression level of CD71 in K562 cells. These phenotypic changes are frequently taken as indications for cellular erythroid differentiation.

Malignant hematopoiesis is characterized by a lack of differentiation capacity. Cell differentiation is a process of gene selective expression. The presence of hemoglobin in a cell indicates that the cell gene is selectively expressed, indicating that the cell has differentiated. The effect of cell differentiation is often associated with the dysfunction of the transcription factors in the signal transduction pathway. GATA-binding factor 1 (GATA-1), NF-E2, T-cell acute lymphocytic leukemia protein 1 (TAL1) and Kruppel like factor 1 (KLF1) are major erythroid-specific transcription activators that bind to the β-globin locus and regulate transcription of the globin gene (24). GATA-1 is a major regulator of the expression of other erythroid-specific activators in human erythroid K562 cells. During red blood cell maturation, GATA-1 activates nearly all erythroid-specific genes, while silencing genes associated with the immature proliferative red blood cell precursor cells (25,26). Hemoglobin is a specific protein. The presence of hemoglobin indicates that cells have differentiated into red blood cells. In order to investigate the mechanism of erythroid differentiation of LDM on K562 cells, the present study detected the hemoglobin contents and the GATA-1 protein expression in LDM-treated K562 cells. The results revealed that LDM at a low concentration could increase the hemoglobin contents and upregulate the GATA-1 protein expression in K562 cells.

In conclusion, a major finding of the present study was that low concentrations of lidamycin could upregulate and activate the caspase-8 and caspase-3 signaling pathway, which in turn may have contributed toward the induction of apoptosis and cancer cell growth inhibition. On the other hand, LDM at low concentrations upregulated the GATA-1 protein expression of K562 cells, which in turn may have contributed toward the induction of K562 cell erythroid differentiation. These results indicate that lidamycin may serve a positive role in challenging the differentiation-inducing therapy of chronic myelogenous leukemia.

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Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions
JC and JHG conceived and designed the study. CZ and LYG performed the experiments and prepared the manuscript. DM performed the image and data analysis. JC and JHG revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the work are appropriately invested and resolved.

Ethics approval and consent to participate
are appropriately investigated and resolved. ensuring that the accuracy or integrity of any part of the work and that the authors declare that they have no competing interests

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