RESEARCH ARTICLE

Inhibition Effects of Lamellarin D on Human Leukemia K562 Cell Proliferation and Underlying Mechanisms

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Abstract

Lamellarin D (LamD) is a marine alkaloid with a pronounced cytotoxicity against a large panel of cancer cells, affecting cell growth and inducing apoptosis. However, the molecular mechanisms of action of this compound are poorly understood. In this study, the anticancer efficacy of LamD was investigated in human leukemia K562 cells. The results showed suppressed cell proliferation and induction of G0/G1-phase arrest,while expression of CDK1, and activity of smad3 and smad5 were reduced, but that of p27, p53 and STGC3 was increased. LamD induced cell apoptosis through activation of caspases-8/-3, inhibition of survivin and Bcl-2, suggesting that this compound may also act through a caspase-independent pathway. Moreover, LamD inhibited the secretion of TGF-β, IL-1β, IL-6, IL-8 and other inflammatory cytokines and the transcriptional activity of transcription factor NF-ϰB in human leukemia K562 cells.Taken together, our results suggest that LamD-mediated inhibition of leukemia cell proliferation may be related to the induction of apoptosis and the regulation of cell cycle, tumor-related gene expression and cytokine expression, which may provide a new way of thinking for the treatment leukemia.

Keywords: Lamellarin D - proliferation - apoptosis - K562 cells - leukemia

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Introduction

Leukemia is a type of clonal hematopoietic stem cell malignancy and one of the cancers with high incidence in China (Wang et al., 2013; Davis et al., 2014). Due to uncontrolled proliferation, differentiation disturbance, blocked apoptosis and other mechanisms of action, the clonal leukemia cells not only massively accumulate in the bone marrow and other hematopoietic tissues but also infiltrate to other tissues and organs, while the normal hematopoiesis is suppressed (Manola, 2013; Itzykson et al., 2013). Clinically, varying degrees of anemia, hemorrhage, infection, fever, enlargement of the liver, spleen and lymph nodes, and ostealgia can be observed. Despite the significant progresses made in the diagnosis and treatment, its overall prognosis is still poor.

The ocean is a vast treasure house of resources and a source of natural medicines with huge potential. Extracts from some of the marine animals have anti-tumor activity or cytotoxicity (Lai et al., 2013; Prabhu et al., 2012; Tohme et al., 2011). Lamellarins are pyrrolidine alkaloids extracted from prosobranch mollusk lamellaria sp. and the most active among the identified series of lamellarin compounds (Plisson et al., 2012). It is a new Topoisomerase 1 inhibitor following Camptothecin with strong cytotoxicity. It is believed that Lamellarins can not only regulate cell growth, survival, differentiation, proliferation, migration and other cell processes but also participate in a variety of signal transduction pathways (Chittchang et al., 2009; Gallego et al., 2008). This study examined the effect of Lamellarin D on leukemia K562 cell proliferation and the mechanisms of action in order to lay the experimental foundation for further clinical applications.

Materials and Methods

Cell culture and drug treatment

Human leukemia K562 cells were cultured in RPMI 1640 containing 10% FBS in an incubator under 37°C and 5% CO_2 conditions.

Cell proliferation assay

5×10^5/mL cells were added 100μl to each well of the 96-well plate. Culture the cells in an incubator under 37°C and 5% CO_2 conditions for 24h. Then, add 1, 2, 10, 20 and 100μM of harmine separately to continue the culture for 24h. Change to fresh media and add 20μl MTS to incubate for 2h. Determine the absorbance at 490nm with a microplate reader.

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Determination of apoptosis change with flow cytometry

3×10^5 cells were cultured in a 6-well plate for 24h and then incubate with 1μM and 2μM of Lamellarin D for 24h. After digestion and centrifugation, add Annexin V/PI and incubate at room temperature for 15min. Then, wash with PBS and determine with flow cytometry.

Determination of cell cycle change with flow cytometry

3×10^5 cells were cultured in a 6-well plate for 24h and then incubate with 1μM and 2μM of Lamellarin D for 24h. After digestion and centrifugation, wash twice with PBS. Fix with 1mL of pre-cooled 70% ethanol at 4°C overnight; discard the ethanol and wash once with PBS. Add 400μl PI staining buffer containing RnaseA, and incubate at room temperature for 15min. Wash with PBS and determine with flow cytometry.

Western Blot assay

3×10^5 cells were cultured in 6-well plate for 24h and then incubate with 1μM and 2μM of Lamellarin D for 24h. Extract cell total proteins with RIPA lysis method and determine protein concentration with BCA method. Apply 100μg protein sample onto 10% SDS-PAGE for electrophoresis and transfer the proteins onto nitrocellulose membrane (150mA, 1h). Block in 5% skim milk for 1h and add the primary antibodies (Survivin, 1:400; bcl-2, 1:400; CDK1 1:400; p53, 1:400; p27, 1:400; *p*-smad3, 1:200; *p*-smad5, 1:200; caspase-3, 1:200; caspase-8, 1:200; β-actin, 1:4000) to incubate at 4°C overnight. After washing for 3 times with TBST, add the secondary antibody and incubate for 1h prior to ECL fluorescence imaging.

Fluorescent quantitative real-time PCR assay

3×10^5 cells were cultured in a 6-well plate for 24h and then incubate with 1μM and 2μM of Lamellarin D for 24h. Extract total RNA from the cells with Trizol method and synthesize the first strand cDNA with reverse transcription technique. Carry out fluorescent quantitative PCR of GAPDH for the target gene in all samples and the reference gene to obtain the relative expression differences, respectively. Carry out 40 cycles of PCR at 94°C 75s, 56°C 40s and 72°C 50s. See Table 1 for the respective detection genes and primer sequences.

Table 1. Primer Sequences for Real-Time PCR Study

Gene	Sequences (5'-3')
Survivin	Forward: GCATGGGTGCCCCGACGTTG-
	Reverse:GCTCCGGCCAGAGGCCTCAA
Bcl-2	Forward: GGCTGGGATGCCTTTGTG
	Reverse: GCCAGGAGAAATCAAACAGAGG
CDK1	Forward: GGATGTGCTTATGCAGGATTCC
	Reverse: ATGTACTGACCAGGAGGGATAG
p53	Forward: GAGGTTGGCTCTGACTGTACC
	Reverse: TCCGTCCCAGTAGATTACCAC
p27	Forward: TGCAACCGACGATTCTTCTACTCAA
	Reverse: CAAGCAGTGATGTATCTGATAAAC
CDKN1B	Forward:TGGAGAAGCACTGCAGAGAC
	Reverse: GCGTGTCCTCAGAGTTAGCC
STGC3	Forward:CCTCTCCTCTCCCGTCAGTC
	Reverse: CATTGTGTGGCTTCCGTATCT
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC
	Reverse: GAAGATGGTGATGGGATTTC

Determination of cytokine levels with liquid chip method

3×10^5 cells were cultured in a 6-well plate for 24h and then incubate with 1μM and 2μM of Lamellarin D for 24h. Change to fresh serum-free media and continue the culture for 24h to collect the supernatant. Determine cytokine TGF-β, IL-1β, IL-6 and IL-8 levels in all treatment groups with liquid chip technique.

Reporter gene assay

3×10^5 cells were cultured in a 6-well plate for 24h and then transfect NF-κb luciferase plasmid for 6h using Lipo2000 method; incubate with 1μM and 2μM of Lamellarin D for 24h and determine cellular NF-κb transcriptional activity in accordance with the kit instructions.

Statistical analysis

SPSS16.0 statistical software was use to data analysis and represent the data as mean ± standard deviation ($\bar{x}\pm s$). Carry out one-way ANOVA and determine the statistical significance using $P<0.05$.

Results

Effects of Lamellarin D on tumor cell proliferation, apoptosis and cell cycles

MTS assay results showed that the proliferation of Lamellarin D-treated K562 cells significantly decreased (Figure 1). After the K562 cells have been treated with 1μM and 2μM Lamellarin D for 24h, the tumor inhibition rate was 4.3% and 9.6%, respectively, which was non-toxic concentration (inhibition rate<10%). Accordingly, the above 2 concentrations were used in this study as the treatment groups; while 0μM was used as the control group in order to avoid the interference of Lamellarin D-mediated tumor inhibition with the results.

To study the role of Lamellarin D on apoptosis, we selected 1μM and 2μM of Lamellarin D for the study. After treatment with 1μM and 2μM of Lamellarin D for 24h, the apoptosis rates were significantly higher than control group ($p<0.05$). As showed in Figure 2,the cell cycle was arrested in G0/G1 phase; the percentage of G0/G1 phase for 1μM and 2μM groups were significantly higher than control group ($P<0.05$).

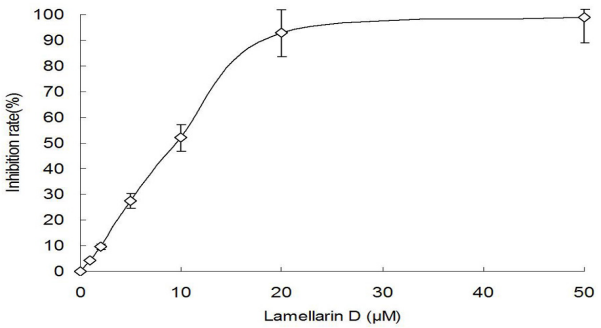

Figure 1. The Effects of Lamellarin D on Proliferation of K562 Cells. K562 cells were treated with Lamellarin D at different concentrations (0, 1, 2,10,20 and 100 μM) for 72 h. Proliferation of tumor cells was measured using the MTS method,and the inhibition rate (%) was calculated. Values were presented as the means±SD, n=5

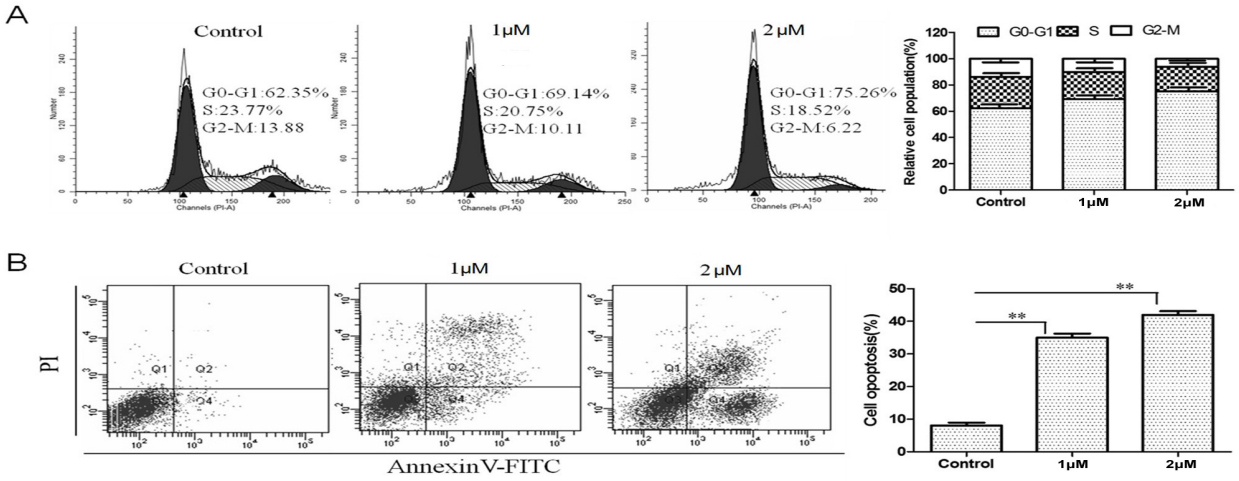

Figure 2. The Effects of Lamellarin D on Cell Cycle and Opoptosis of K562 Cells. A:Cells were treated with 1μM and 2μM Lamellarin D for 24h.PI staining was used to analyze the cell cycle distribution;B:1μM and 5μM Lamellarin D exposure in K562 cells as assessed by Annexin V-fluorescein isothiocyanate and propidium iodide (PI) double staining and fluorescence-activated cell sorter analysis. The statistical significance was considered as *p<0.05 and **P < 0.01 where compared with control. Values were presented as the means±SD, n=3; #compared to the control group, p>0.05, *compared to the control group, p<0.05

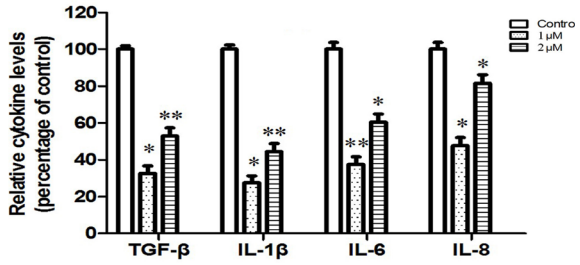

Figure 3. The Effects of Lamellarin D on Cytokines Expression of K562 cells. Cells were treated with 1μM and 2μM Lamellarin D for 24h,then cytokine TGF-β, IL-1β, IL-6 and IL-8 levels were detected by liquid chip method. The statistical significance was considered as *p<0.05 and **p<0.01 where compared with control

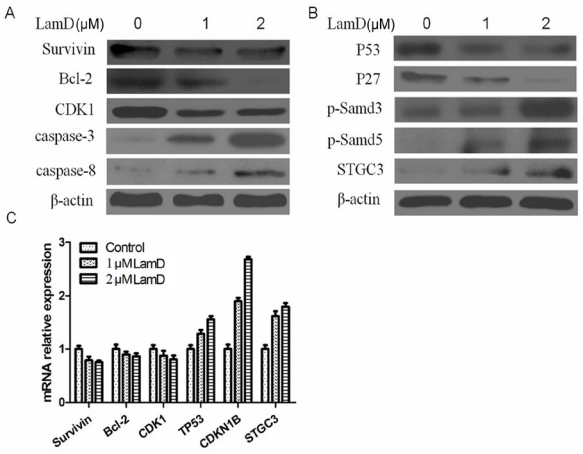

Figure 4. The effect of Lamellarin D on Expression of tumor Related Gene in K562 Cell Lines. A,B:Immunoblottings for the proteins associated with proliferation.Immunoblot analyses were performed on the lysates of K562 cells that had been incubated with 1μM and 2μM Lamellarin D for 48h. Equal protein loading was verified by β-actin immunoblotting. Results were confirmed by repeated experiments. β-actin was as the internal control. C: K562 cells treated with 1μM and 2μM Lamellarin D for 48h, total cellular RNA were collected and subjected to real time-PCR analysis. The statistical significance was considered as *p<0.05 and **p<0.01 where compared with control

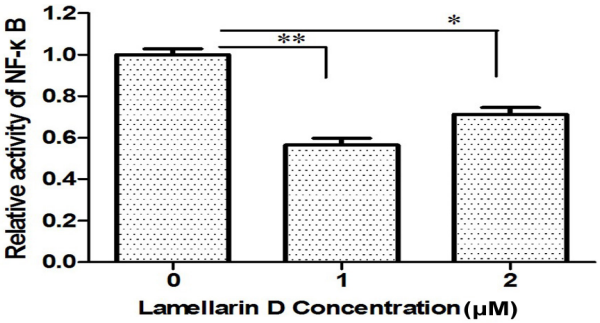

Figure 5. The effect of Lamellarin D on NF-κB Transcriptional Activity in K562 Cell Lines. The Effect of Lamellarin D on NF-κB transcriptional activity in K562 Cells by reporter gene assay. Activity of control cells was regarded as 100%.Cells were treated with Lamellarin D at 1μM and 2μM for 24h.The statistical significance was considered as **P < 0.01 where compared with control

Effects of Lamellarin D on cytokine expression in tumor cells

Liquid chip results showed the changes of cytokine expression in 1μM and 2μM groups, respectively when compared with the control group as follows: TGF-β expression decreased by 67.2% and 47.1% (P<0.05); IL-1β expression decreased by 72.5% and 55.4% (P<0.05); IL-6 expression decreased by 62.5% and 39.4% (P<0.05); IL-8 expression decreased by 52.3% and 18.5% (P<0.05).

Effects of Lamellarin D on the expression of tumor cell proliferation-related genes Real-time PCR results showed the changes of mRNA expression in 1μM and 2μM groups, respectively when compared with the control group as show in Figure 4. Western blotting results showed that the changes of protein expression in 1μM and 2μM groups, respectively when compared with the control group as follows: the protein expression of Survivin, bcl-2, CDK1, p-smad3 and p-smad5 decreased significantly; the protein expression of STGC3, caspase-3/8, p27 and p53 increased significantly (Figure 4).

Effects of Lamellarin D on NF-κB activation in K562 cells

NF-κB is a transcriptional regulator that plays a

central role in responses to inflammatory signaling. Phosphorylation of NF-κB p65 is an important step for its transcriptional activity. Thus, we examined whether Lamellarin D could suppress transcriptional activity. Cells were pretreated without or with Lamellarin D (1μM and 2μM) and for 24 hours, and then detected by Reporter gene assay. As shown in Figure 5, NF-κb transcriptional activity in 1μM and 2μM groups decreased by 43.5% and 28.7%, respectively when compared with the control group ($P<0.05$).

Discussion

Tumorigenesis and tumor development are a multi-step, multi-stage and multi-factor complex process. Elucidation of the mechanisms of drug-mediated inhibition on tumor cell growth and proliferation has important significance for the treatment of tumors and the improvement of prognosis and there was a decreased mRNA and protein expression of tumor related gene such as CDK1, Survivin, Bcl-2 and so on. (Vinken et al., 2013; Banerjee et al., 2012; Zhu et al., 2014). Previous studies have shown that Lamellarin D is an alkaloid capable of inhibiting a variety of human tumors and has important significance in the treatment of tumors (Banerjee et al., 2012). By observing the effects of Lamellarin D on human leukemia K562 cells, this study found that Lamellarin D significantly inhibited tumor cell proliferation, induced apoptosis, and arrested cell cycle in G0/G1 phase.

The results of this study showed that Lamellarin D not only significantly up-regulated the expression of capase-3/8, p53 and p27 but also down-regulated the expression of Survivin, bcl-2 and CDK1, suggesting that the effects of Lamellarin D including inhibition of tumor proliferation, induction of apoptosis and arresting of cell cycle may be correlated with its ability to regulate the cell cycle-related genes. TGF-β/*p*-smad3/*p*-smad5 signal transduction pathway is able to regulate not only cell survival, differentiation, proliferation, metabolism and other basic processes but also tumor-related gene expression to further change the cell cycle process and effectively inhibit cell growth. Lamellarin D-mediated tumor suppression may be related to the inhibition of TGF-β/*p*-smad3/*p*-smad5 signal transduction activity.

Meanwhile, our experiments also found that Lamellarin D inhibited the secretion of TGF-β, IL-1β, IL-6, IL-8 and other inflammatory cytokines and the transcriptional activity of transcription factor NF-κB in human leukemia K562 cells. Inflammatory cells play important roles in tumor metastasis; IL-6, IL-1β, IL-8 and other inflammatory cytokines stimulate tumor cell proliferation (Chanmee et al., 2014; Peppicelli et al., 2014; Voronov E et al., 2014; El-Kadre et al., 2013). TGF-β can induce the expression of multiple transcription factors and up-regulate tumor-related gene expression, subsequently promoting tumorigenesis (Taylor et al., 2013; Li et al., 2014). IL-6 is an important inflammatory cytokine linking inflammation with cancer (El-Kadre et al., 2013). IL-6 has tumor promoting effect in breast cancer cells through NFκB-IL-6-STAT3 cascade (Kojima et al., 2013). IL-1β regulates tumorigenesis by activating Zeb1 (Li et al., 2013; Sakthivel et al., 2013). Among a large number of signaling pathways linking inflammation with tumors, NF-κB is a core molecule involved in inflammation-induced metastasis (Vilela et al., 2014; Deng et al., 2012). There is evidence demonstrating that the activation of NF-κB is related to the induction of many transcription factors (e.g. Slug, Snail, Twist and ZEB1/ZEB2) involved in tumorigensis.

In summary, this study found that Lamellarin D-mediated inhibition of leukemia cell proliferation may be related to the induction of apoptosis and the regulation of cell cycle, tumor-related gene expression and cytokine expression, which may provide a new way of thinking for the treatment leukemia.

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