Plumbagin reduces chronic lymphocytic leukemia cell survival by downregulation of Bcl-2 but upregulation of the Bax protein level

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Abstract. Chronic lymphocytic leukemia (CLL) is the most common leukemia in Western countries, and mainly originates from an accumulation of abnormal B cells caused by the dysregulation of cell proliferation and apoptosis rates. The aberration of apoptosis-related genes in CLL cells results in defective apoptosis of CLL cells in response to traditional therapeutic medicine. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a natural compound from *Plumbago zeylinica*, has been shown to exhibit pro-apoptotic activities in tumor cells. In the present study, we report that plumbagin effectively inhibited CLL cell viability with a lower dose compared to fludarabine, and inhibited cell proliferation in a dose-dependent manner. In addition, plumbagin promoted accumulation of MEC-1 cells in the S phase, and blocked cell cycle transition of HG3 cells from G0/G1 to S phase. Molecularly, plumbagin markedly induced CLL cell apoptosis through reduction of Bcl-2, but through an increase in the Bax protein level. These results suggest that plumbagin may be considered as a potential anticancer agent for CLL therapy.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults, particularly in Western countries (1). However, the incidence of this disease is gradually increasing in China (2). CLL is characterized by an accumulation of abnormal B cells, resulting from the dysregulation of proliferation and apoptosis rates (3,4). Despite the advance in pathobiologic research and the development of effective treatment regimens, CLL is still largely an incurable disease (5).

It has been reported that p53 is inactivated in 10-15% of CLL patients decreasing cell apoptosis and accelerating disease development (6). Additionally, the anti-apoptotic protein Bcl-2 family is overexpressed, while pro-apoptotic proteins such as Bax and Bcl-xL are underexpressed in CLL cells (1,7). Bcl-2 upregulation increases the Bcl-2/Bax ratio, which further inhibits the caspase-dependent apoptosis of CLL cells (8-10). Therefore, the abnormal expression of apoptosis-related genes has restricted the application of chemotherapeutic or immunotherapeutic medicine and weakened the therapeutic outcome in CLL patients.

Recently, the introduction and subsequent approval of targeted kinase inhibitors (idelalisib and ibrutinib) has altered the standard of care for CLL patients and have acquired outstanding efficacy (11,12). In addition, other novel small-molecule inhibitors, such as venetoclax (ABT-199), an inhibitor of Bcl-2 currently in clinical trials, have the potential to improve therapy for CLL patients (12). Therefore, it is essential to develop novel agents to target the abnormal activation of genes for CLL treatment.

Plumbagin, a natural compound from *Plumbago zeylinica*, has been shown to function as an anti-bacterial, anti-atherosclerotic as well as an anticancer agent (13,14). It also exhibits pro-apoptotic activities in different tumor cells and animal models both *in vitro* and *in vivo* (15). In the present study, we explored the possible anticancer activity of plumbagin in CLL cells by analyzing its effects on cell viability, cell cycle regulation, proliferation and apoptosis, as well as the expression of apoptosis-related signaling molecules. Our data showed that plumbagin reduced CLL cell survival by downregulating Bcl-2, but upregulating the Bax level, suggesting that plumbagin may be considered as a promising agent for the treatment of CLL.

Materials and methods

Patients and samples. CLL samples were obtained from the Affiliated Hospital of Xuzhou Medical College according to the diagnostic criteria for CLL between September 2013 and October 2015, while 6 healthy volunteers served as a normal

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control group. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from 35 CLL patients.

All procedures performed in the study involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Cell lines and reagents. CLL cell lines, HG3 and MEC-1, a kind gift from Anders Rosén at Linköping University, were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37˚C in a 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). The Bcl-2 and Bax antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and anti-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG and rabbit anti-mouse IgG were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Xiongben, Japan). The Cell-Light EdU Apollo® 567 In Vitro Imaging kit was from Ruibo Biotechnology (Guangzhou, China). Annexin V and 7-AAD double-labeled apoptosis detection kit and propidium iodide (PI) were obtained from eBiosience (San Diego, CA, USA). Plumbagin was purchased from Sigma-Aldrich.

Quantitative real-time PCR. Extracted RNA from the CLL samples was reverse-transcripted to cDNA which was used for quantification of mRNA expression of Bcl-2 and Bax by real-time PCR with GADPH as internal control. Amplification was performed in triplicate on LightCycler® R480 II (Roche Life Science) in a total volume of 20 μl. The primers for Bcl-2, Bax and GADPH were as follows: Bcl-2 (F), 5'-ACGACTTCTC CCGCCGCTA-3' and (R), 5'-ACCCCACCGAACTCAAAGA AG-3'; Bax (F), 5'-AGAGGATGATTGCCGCCGT-3' and (R), 5'-CAACCACCCTGGTCTTGGATC-3'; GAPDH (F), 5'-TGA AGGTCGGAGTCAACGGATT-3 and (R), 5'-CCTGGAAGA TGGTGATGGGATT-3'. qPCR reaction was performed according to the SYBR-Green qPCR SuperMix manuals. The relative mRNA expression of target genes was calculated by the comparative Ct method which was performed using the following formula: Relative expression = $2^{-\Delta\Delta Ct}$.

CCK-8 analysis of cell viability. Three thousand cells in 100 μl of medium were seeded into 96-well plates with three replicates. The cells were incubutated with 0.625, 1.25, 2.5, 5, 10 and 20 μM of plumbagin or fludarabine for 48 h, or incubated with 10 μM of plumbagin or fludarabine for 0, 20, 40, 60 and 80 h, and then CCK-8 reagent (5 μl) was added into each well to incubate for an additional 4 h. The cells were exposed to measure the absorbance at 450 nm by a microplate reader (Wellscan MK-3; Labsystems, Dargon, Finland).

5-Ethynyl-2'-deoxyuridine (EdU)-incorporation for detecting cell proliferation analysis. EdU-incorporation is a method for labeling DNA *in vivo* during DNA replication. The HG3 and MEC-1 cells were pretreated with 0, 2.5, 5 and 10 μM of plumbagin for 48 h, and then incubated with EdU for an additional 3 h. After fixation, permeabilization and staining according to the kit manual, the cells were observed and the number of EdU-positive cells was calculated under a microscope.

Cell cycle analysis. The HG3 and MEC-1 cells following the same treatments with plumbagin were collected and fixed in 70% ethanol on ice for 10 min, rinsed with phosphate-buffered saline (PBS) and incubated with 100 mg/ml RNase A (0.25 mg/ml) for 15 min. After washing with PBS for two times, the cells were further incubated with 50 μg/ml PI at room temperature for 10 min, and then the cells were subjected to cell cycle analysis.

Analysis of cell apoptosis. The HG3 and MEC-1 cells following the same treatments with plumbagin were collected and then resuspended in 1× binding buffer at 1-5x10^6/ml, and incubated with Annexin V-APC and 7-AAD for 10 min at room temperature for analysis of apoptosis by flow cytometry. Early apoptotic cells were labeled with Annexin V and late apoptotic cells were double labeled with Annexin V and 7-AAD.

Immunoblotting. The HG3 and MEC-1 cells following the same treatments with plumbagin were collected and proteins were extracted for western blotting. Equal amount of protein lysates were subjected to 10-12% SDS-PAGE, and then transferred to a 0.45-μm pore size polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was probed with primary antibodies at 4˚C overnight and secondary antibodies at room temperature for 1 h. Bound antibodies were detected using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) and visualized by ImageQuant LAS 4000 (GE Healthcare, Fairfield, CT, USA).

Statistical analysis. The results are representative of experiments repeated at least three times and quantitative data are expressed as means ± SEM. Student's t-test and ANOVA test were used to analyze the difference between groups. $P<0.05$ was considered statistically significant, and $P<0.01$ as very significant. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Increased Bcl-2/Bax ratio in CLL patients. The mRNA expression of Bcl-2 and Bax was determined in 35 CLL patients and 6 healthy donors. Bcl-2 exhibited an increasing trend in Rai II-IV patients compared with the control group, particularly in Rai III/IV patients ($P<0.05$; Fig. 1A). By contrast, Bax showed a decreasing trend in the CLL patients, but without statistical significance (Fig. 1B). However, the expression ratio of Bcl-2/Bax was found to be significantly increased (3.78-fold) in the Rai III/IV patients compared with this ratio in the control group ($P<0.05$; Fig. 1C).

Plumbagin decreases cell viability and inhibits cell proliferation. To investigate the potential growth inhibition of plumbagin in CLL cells, the HG3 and MEC-1 cell lines were cultured with 0.625, 1.25, 2.5, 5, 10 and 20 μM of plumbagin

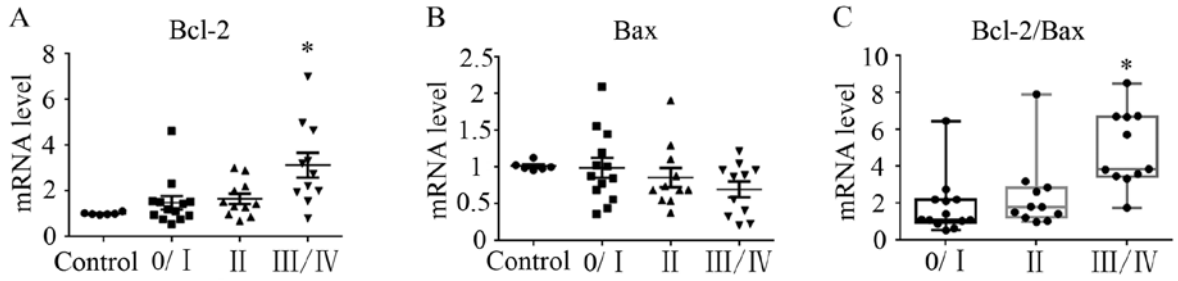
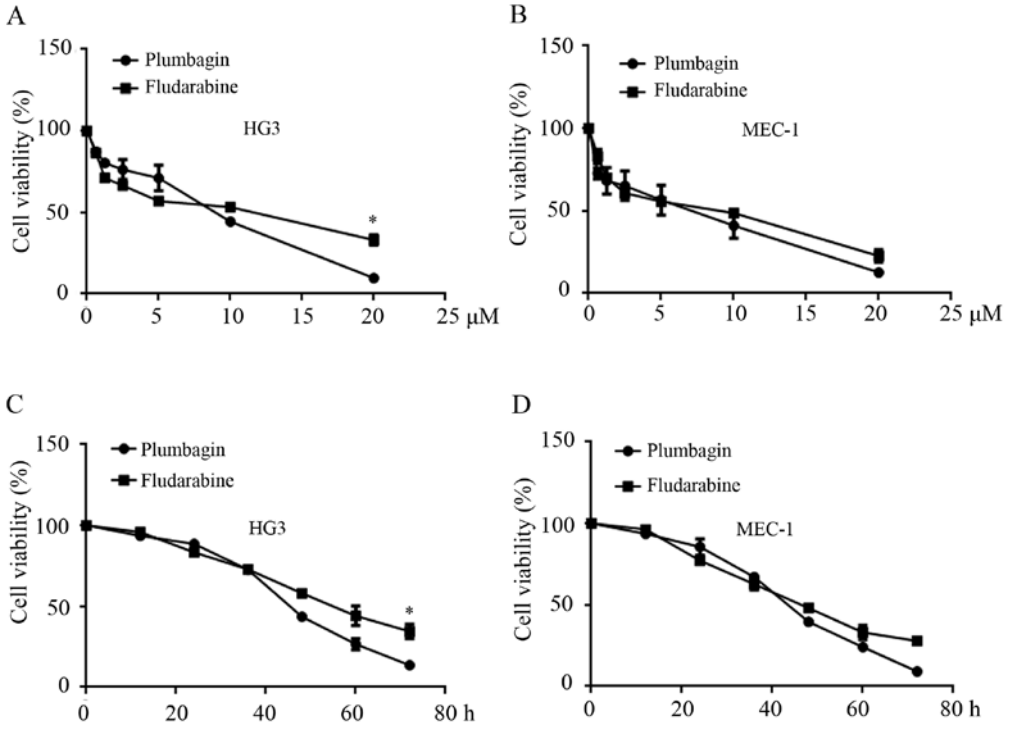


Figure 1. mRNA expression of Bcl-2 and Bax in CLL patients. RNA was isolated from the PBMCs of healthy donors or CLL patients for measuring mRNA expression of (A) Bcl-2 and (B) Bax by quantitative real-time PCR. *P<0.05 (P=0.035). (C) The ratio of Bcl-2/Bax was calculated by comparing the $2^{-\Delta\Delta Ct}$ value in CLL patients. *P<0.05 (P=0.025), compared with the control group.



E

	IC_{50} (μM)	
Cell line	Plumbagin	Fludarabine
HG3	8.5 ± 0.5	11.5 ± 0.7
MEC-1	7.5 ± 0.8	10.2 ± 0.5

Figure 2. Measurement of CLL cell viability. (A) The HG3 and (B) MEC-1 cells were grown into a 96-well plate at $1x10^3$ cells/well and treated with the indicated concentrations of plumbagin or fludarabine for 48 h, and then incubated with CCK-8 reagent for 4 h. Relative absorbance was measured by a microplate reader. (C) The same HG3 and (D) MEC-1 cells were treated with 10 μM of plumbagin or fludarabine for up to 80 h, and the cell viability was measured one time every 20 h. *P<0.05 (P=0.024 and P=0.031 respectively in HG3 and MEC-1 treated groups. (E) The IC_{50} values were calculated using three independent experiments. *P<0.05 (P=0.042 in HG3 cells), compared with fludarabine.

for 48 h, and the cell viability was determined by CCK-8 assay. As shown in Fig. 2A and E, HG3 cells treated with plumbagin and fludarabine presented decreased cell viability in a dose-dependent manner, while the cells treated with plumbagin showed a lower IC_{50} value (8.5±0.5 μM) compared with fludarabine (11.5±0.7 μM) (P<0.05). Similarly, under the same conditions, MEC-1 cells were more susceptible to plumbagin (IC_{50}=7.5±0.8 μM) compared to fludarabine (IC_{50}=10.2±0.5 μM) (Fig. 2B and E). Next, HG3 and MEC-1 cells were incubated with 10 μM of plumbagin or fludarabine for 0, 20, 40, 60 and 80 h. Almost all CLL cells showed growth retardation at 72 h when treated with plumbagin, while 34.5±0.7% (P<0.05) and 28±0.3% (P<0.05) of HG3 and MEC-1 cells presented strong cell viability after the same treatment with fludarabine (Fig. 2C and D).

To assess the effect of plumbagin on cell proliferation, we performed EdU incorporation assay with HG3 and MEC-1 cells in the presence or absence of plumbagin. As expected,

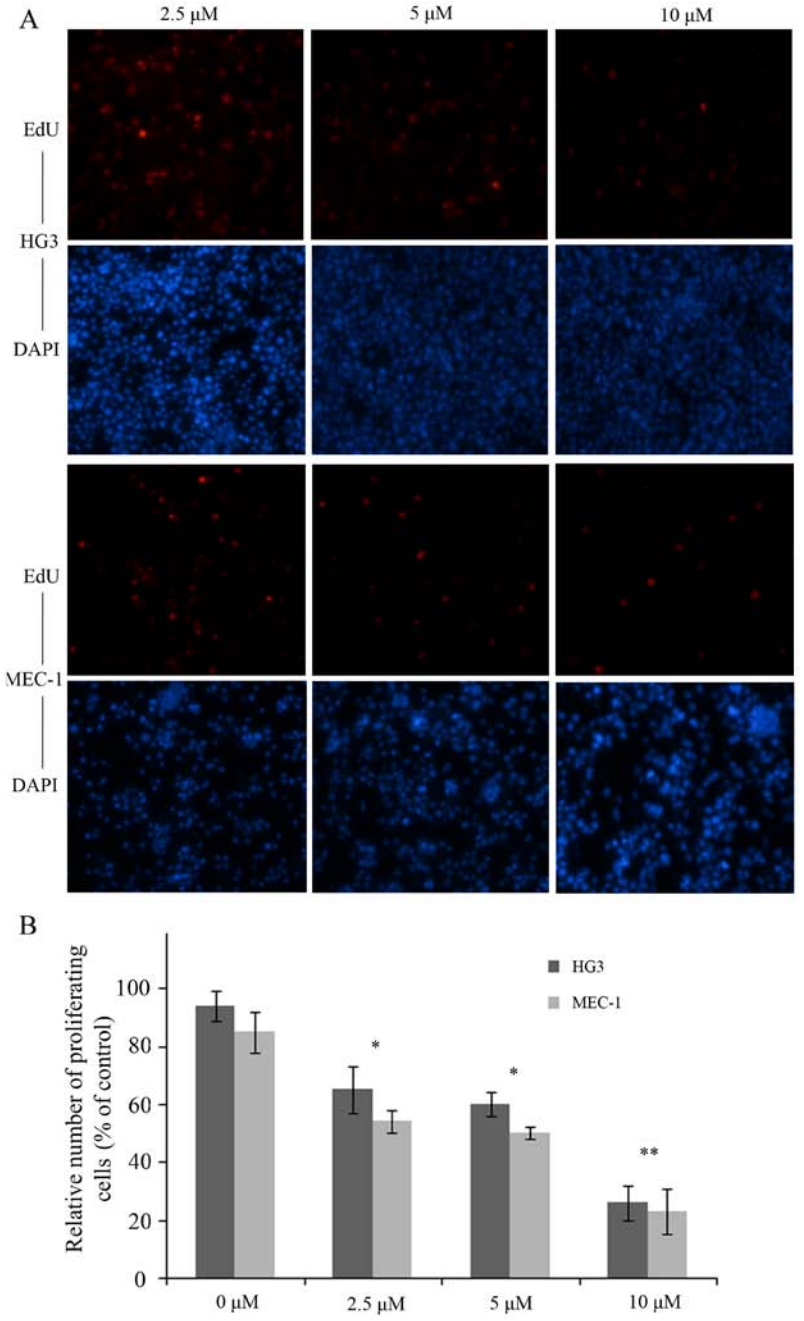


Figure 3. Analysis of CLL cell proliferation. The HG3 and MEC-1 cells were treated with the indicated concentrations of plumbagin for 48 h, and then incubated with EdU for an additional 3 h. After fixation, permeabilization and staining, the cells were observed (A) and the numbers of EdU-positive cells were calculated (B). *P<0.05 [P=0.042 (HGE cells) or P=0.025 (MEC-1 cells) at 2.5 μM, and P=0.038 or P=0.02 at 5 μM plumbagin] and **P<0.01 [P=0.0012 (HGE cells) or P=0.0001 (MEC-1 cells) at 10 μM plumbagin], compared with the control.

the numbers of EdU-positive cells distributed as 68, 62 and 30% in HG3 cells vs. 58, 54 and 28% in MEC-1 cells at 2.5, 5 and 10 μM of plumbagin, were significantly decreased in a dose-dependent manner (P<0.05, P<0.05, P<0.01; Fig. 3).

Plumbagin induces cell cycle arrest in the G0/G1 or S phase. To investigate the role of plumbagin in cell cycle progression, HG3 and MEC-1 cells treated with plumbagin were collected and stained with PI for cell cycle analysis via flow cytometry. The distribution of the cell cycle in the HG3 cells exhibited no obverse changes (38-41% in G1, 19-27% in S and 35-40% in G2/M phases) when treated with 0, 2.5 and 5 μM of plumbagin. However, ~60% of the HG3 cells were arrested in G0/G1 phase following treatment with 10 μM of plumbagin (P<0.01; Fig. 4A and B). Notably, MEC-1 cells were blocked at the S phase following the same treatment, and showed an increase from 33 to 53% compared with the control when treated with 10 μM of plumbagin (P<0.01; Fig. 4A and C).

Plumbagin promotes cell apoptosis. To assess whether plumbagin induced these growth-suppressive effects on CLL cells by apoptosis, we collected HG3 and MEC-1 cells treated with plumbagin for double-staining using Annexin V-APC and 7-AAD. As expected, plumbagin significantly induced cell

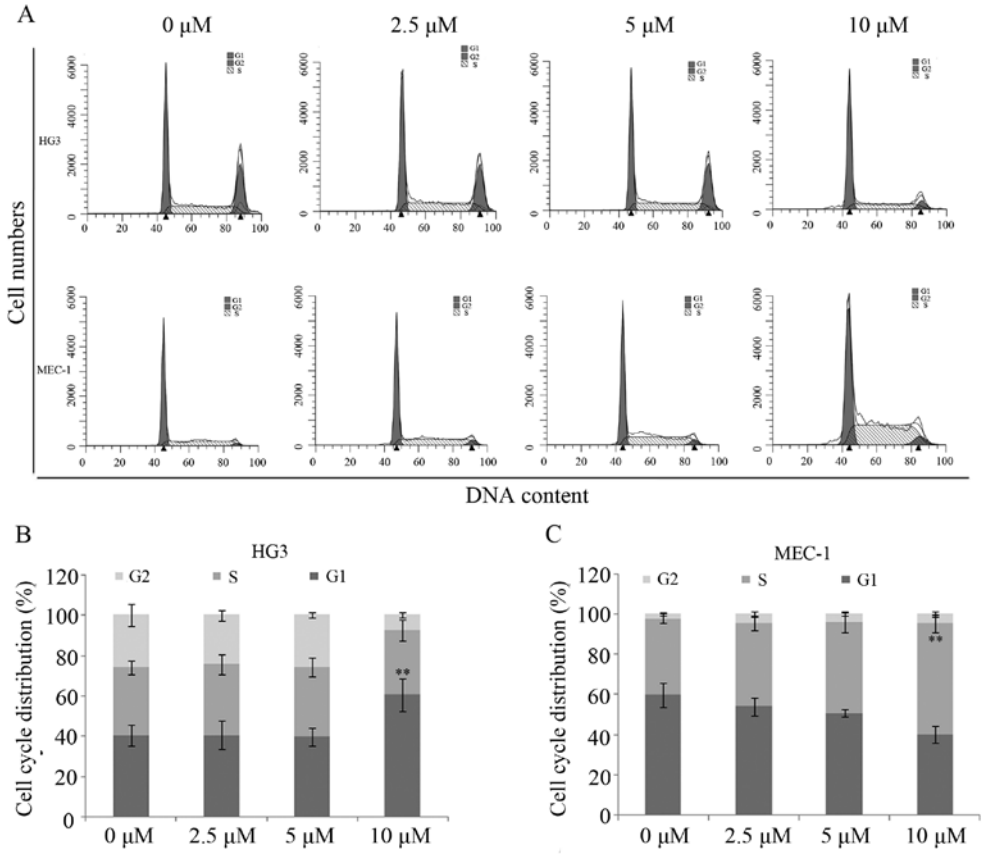


Figure 4. Cell cycle arrest induced by plumbagin. (A) The HG3 and MEC-1 cells were treated with the indicated concentrations of plumbagin. After 48 h, the cells were collected and stained with PI for cell cycle analysis by flow cytometry. (B and C) Three independent experiments were performed for statistical analysis of the distribution of the cell cycle. **P<0.01 [P=0.007 (HGE cells) or P=0.009 (MEC-1 cells) at 10 μM plumbagin], compared with the control.

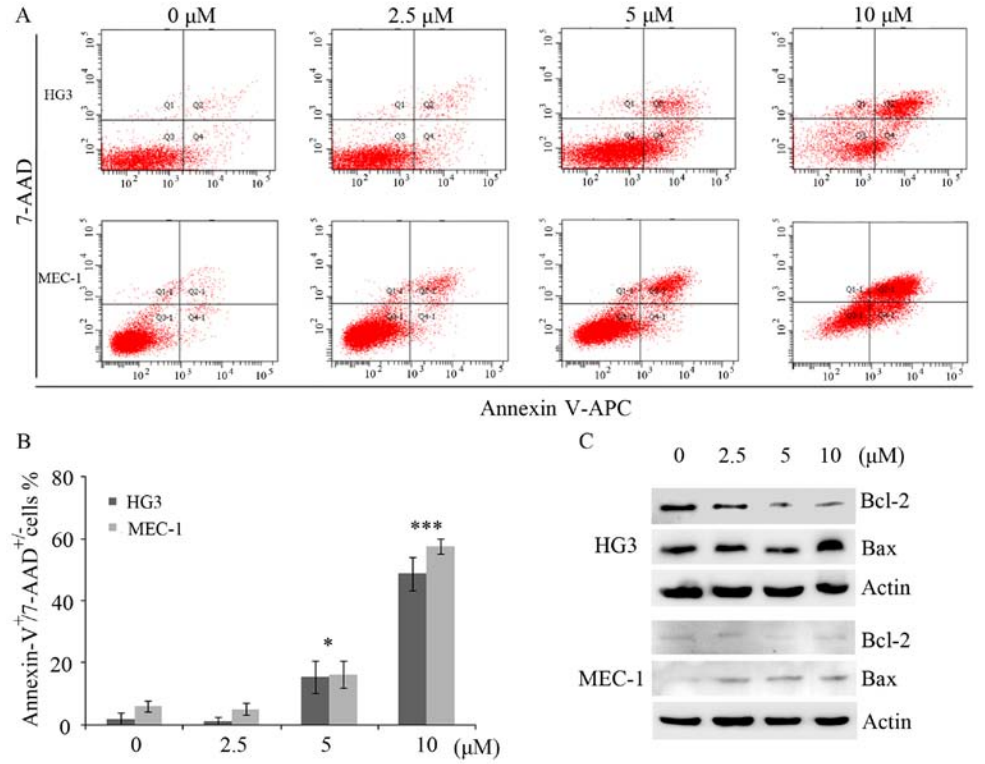


Figure 5. Cell apoptosis induced by plumbagin. (A) The HG3 and MEC-1 cells were treated with the indicated concentrations of plumbagin. After 48 h, the cells were double-stained with Annexin V-APC and 7-AAD, and then subjected to flow cytometry. (B) The apoptotic efficiency was calculated by three independent experiments. (C) Cell lysates were analyzed to assess the expression of Bcl-2 and Bax. *P<0.05 [P=0.043 (HGE cells) or P=0.048 (MEC-1 cells) at 5 μM plumbagin] and ***P<0.001 [P=0.0002 (HEG cells) or P=0.0004 (MEC-1 cells) at 10 μM plumbagin], compared with the control.

apoptosis in a dose-dependent manner, both in the HG3 and MEC-1 cell lines. In addition, statistical analysis showed that the number of apoptotic cells treated with 5 or 10 μM of plumbagin was 18±0.7 or 50±0.5% in the HG3 cells, and 19±0.5 or 58±0.2% in the MEC-1 cells ($P<0.05$; $P<0.001$; Fig. 5A and B). Furthermore, western blotting showed that plumbagin treatment notably decreased the expression of Bcl-2 and increased Bax in the HG3 cells. Similarly, the expression of Bcl-2 was slightly decreased, while the Bax level was strongly elevated even following treatment with a dose of 2.5 μM of plumbagin in the MEC-1 cells (Fig. 5C).

Discussion

Chronic lymphocytic leukemia (CLL) is the most common human leukemia, representing 30% of all cases (16). CLL cells show higher expression of Bcl-2 (17), higher activity of the PI3K/Akt pathway (18) as well as constitutive activation of NF-κB than normal lymphocytes (19). Approximately 10-15% of CLL patients reveal structural aberrations or point mutations in locus 17p13, containing TP53 (6). These activated proteins or abnormal genes result in the defective apoptosis of CLL cells in response to traditional therapeutic medicine. Thus, a personalized therapeutic approach based on genetic and molecular status could be preferable to the comprehensive treatment of CLL. Accordingly, several agents targeting B cell receptor signaling pathway kinases have entered clinical trials such as idelalisib and ibrutinib, separately targeting phosphoinositide 3-kinase (PI3K) or Bruton's tyrosine kinase (BTK). Moreover, oblimersen sodium (G3139) specific for the Bcl-2 mRNA sequence and obatoclax (GX15-070) or ABT-199 antagonistic for Bcl-2 protein have been applied in clinical phase I/II study. Other targeting agents have also been assessed for use in CLL therapy such as histone deacetylase (HDAC) inhibitors, cyclin-dependent kinase inhibitors and proteasome inhibitors (11,20).

Plumbagin, a potential anticancer agent, has been reported to regulate cell proliferation and apoptosis in the development of cancer (21,22). As reported, plumbagin induced colon cancer cell death via a Bak-dependent pathway (23), and promoted tongue squamous cell carcinoma cell arrest at the G2/M phase and apoptosis via p38 MAPK- and PI3K/Akt/mTOR-mediated pathways (24). Plumbagin also inhibited prostate xenograft development via targeting PKCε and Stat3 (25). In the present study, we found that plumbagin inhibited CLL cell viability with a lower dose compared to fludarabine, and inhibited cell proliferation in a dose-dependent manner. As reported, plumbagin showed anticancer activity in human osteosarcoma (MG-63) cells via the inhibition of S phase checkpoints (20). Consistently, the present study showed that plumbagin caused a marked accumulation of MEC-1 cells in the S phase. Notably, most of the HG3 cells, another CLL cell line, were blocked at the G0/G1 phase following the same plumbagin treatment. These results suggest that plumbagin regulated various cell cycle-related proteins, and finally induced cell cycle arrest.

It has been reported that the overexpression of anti-apoptotic proteins such as Bcl-2, Bag-1 and Mcl-1 results in CLL cell accumulation or apoptotic resistance (26,27). In agreement with previous results, the majority of the Chinese CLL patients in the present study showed a high Bcl-2 mRNA, but a slightly low Bax level as determined by qPCR. Further statistical analysis also showed an increasing trend for the Bcl-2/Bax ratio in the CLL patients, particularly for those in Rai III/IV phase, suggesting that the Bcl-2/Bax ratio is associated with the development of CLL. Fortunately, after long-time incubation with plumbagin, both CLL cell lines presented an increased apoptotic trend in a dose-dependent manner by downregulation of Bcl-2 and upregulation of Bax. These results suggest that plumbagin induced CLL cell apoptosis probably via a decrease in the ratio of Bcl-2/Bax. Nevertheless, the targeted proteins or regulatory mechanism of plumbagin in regards to Bcl-2 or Bax is unclear, deserving further study.

In summary, our results showed that plumbagin effectively decreased CLL cell viability, inhibited cell proliferation and blocked cell cycle progression using a lower dose compared to fludarabine. In addition, we found that plumbagin markedly induced CLL cell apoptosis by reducing Bcl-2, but by increasing the Bax level. These results suggest that plumbagin decreases the ratio of Bcl-2/Bax, thereby killing CLL cells.

Acknowledgements

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