ZGDHu-1 promotes apoptosis of chronic lymphocytic leukemia cells

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Abstract. Chronic lymphocytic leukemia (CLL) is a low-grade lymphoid malignancy incurable with conventional modalities of chemotherapy. We aimed to examine the proapoptotic effects of a novel proteasome inhibitor, N,N'-di-(m-methylphenyi)-3,6- dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1), on CLL cells. B lymphocytes were isolated from CLL patients and normal healthy controls, and treated with various concentrations of ZGDHu-1 for different days. CLL cell viability was detected by MTT assay. The apoptosis, mitochondrial membrane potential ($\Delta\Psi m$) and reactive oxidative species (ROS) were examined by flow cytometry. The expression of caspase-3 and Bcl-2/Bax ratio was detected by Western blotting. ZGDHu-1 significantly reduced the viability of CLL cells and induced apoptosis in comparison to the control cells (both $P<0.05$). Normal peripheral B cells were resistant to the apoptosis-inducing effects of ZGDHu-1. Apoptosis induced by ZGDHu-1 was accompanied with generation of ROS, loss of $\Delta\Psi m$, downregulation of Bcl-2 and increase of caspase-3 cleavage. Results of this study indicate that ZGDHu-1 is a promising specific treatment for CLL in the clinic.

Introduction

Chronic lymphocytic leukemia (CLL), is a low-grade B-cell malignancy predominantly affecting adults over 50 years old (1), and the majority are men. It is identified by the gradual accumulation of a monoclonal population of $CD5^+CD19^+$ B lymphocytes, which arise primarily from a failure of apoptosis (2,3). Clinically, the CLL is very heterogeneous that some patients may live for many years, while others may rapidly die of progressive and chemotherapy-resistant diseases.

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At present, there is no effective cure for CLL, and thus novel therapies have been urgently demanded for these patients with poor prognosis (4). New targeted therapies in CLL and other hematological malignancies include Bcl-2 inhibitors, histone deacetylase inhibitors and proteasome inhibitors (5,6). These latter agents induced cell apoptosis partly through modifying the balance between pro-apoptotic and anti-apoptotic family members. Apoptosis of cells is mainly characterized by morphological changes such as loss of mitochondrial membrane potential ($\Delta\Psi m$), and phosphatidylserine (PS) translocation across the plasma membrane (7,8) and accumulation of reactive oxygen species (ROS) in cells (9). The drug bortezomib is currently the only proteasomal inhibitor used clinically to successfully treat multiple myeloma alone or in combination of other drugs. Smolewski *et al* (10) found that bortezomib triggered caspase-dependent apoptosis and resulted in reduction of the expression of Bcl-2, Mcl-1 and XIAP. However, treatment of CLL patients with bortezomib generated no objective responses due to the dietary flavonoid quercetin present in the plasma, which blocks the effects of the drug to kill the CLL cells (11,12). Patients with CLL genermally had poor prognosis due to lack of effective treatments.

ZGDHu-1, [N,N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide] (Fig. 1) is a tetrazine compound, synthesized by Wei-Xiao Hu (Pharmaceutical College of Zhejiang University of Technology, China) who obtained the Patent of China (13), possess anti-tumor activity (14) and has been identified as a potential proteasome inhibitor (15). There is currently no report on effects of ZGDHu-1 on the treatment of CLL.

In this study, by using this novel proteasome inhibitor ZGDHu-1, we investigated the effects of ZGDHu-1 on lymphocytes isolated from CLL patients. We found ZGDHu-1 specifically reduced the viability and enhanced apoptosis in CLL cells without affecting the normal peripheral B cells, which may ascribe to the up-regulation of ROS and mitochondria membrane permeability in the CLL cells.

Materials and methods

Patients. Twenty CLL patients (12 males and 8 females) aged 58-85 years (with median age of 63 years) were enrolled in this study. CLL was diagnosed and confirmed according to definition of the World Health Organization (WHO) classification.

Only those patients showing no previous treatments within the last 6 months were included in the present study. All 20 patients were sampled prior to the commencement of any treatment and at least 2 weeks after the transfusion. Age-matched controls were obtained from 10 healthy donors. This study was approved by the Zhejiang Provincial People's Hospital research ethics committee and all the patients and healthy controls signed an informed consent form.

Main reagents and instruments. The ZGDHu-1 compound (Fig. 1) with purity >95% was kindly provided by Dr Wei-Xiao Hu (Pharmaceutical College of Zhejiang University of Technology, China) and was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (1 mg/ml) and stored at -20˚C. Antibodies against Bcl-2, Bax, caspase-3, β-actin in western blotting were manufactured by Cell Signaling Biotechnology (Beverly, MA, USA), while phycoerythrin (PE)-conjugated anti-human CD19 monoclonal antibody and the PE mouse immunoglobulin G1 k (IgG1 k) isotopes control were from American Beckman-Coulter Inc. DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dihydrorhodamine-123 (DHR), broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk) and Ficoll-Hypaque were all purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). The apoptosis assay kit of annexin V and propidium iodide (PI) and the IntraPrept™ permeabilization kit were from the Bender MedSystems Inc. and the Immunotech Company (France), respectively. The JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetrethyl benzimidalyl carbocyanine iodide) was from BioTeam Inc. Flow cytometry was performed on a Beckman EPICS-XL FACS (Miami, FL, USA).

Lymphocyte purification and culture. EDTA-K_2 anticoagulant blood samples were obtained from the CLL patient and healthy controls during a routine diagnosis at the Leicester Royal Infirmary. B lymphocytes were isolated immediately using Ficoll gradient centrifugation according to the manufacturer's instructions. After 1 h of incubation at 37˚C in 5% CO_2, adhesive mononuclear cells were removed. Those non-adherent lymphocytes were thoroughly washed with the Hank's solution (Biochrom, Berlin, Germany). T lymphocytes were removed using anti-CD3 dynabeads (Dynal, Merseyside, UK). The purification of B lymphocytes was assessed by flow cytometry with anti-CD19 antibodies (Immunotech, Coulter, USA). This cell preparation contained about 95% CD19 (B lymphocyte antigen) positive cells.

The purified cells were counted in Neubauer plate by trypan blue exclusion of dead cells, re-suspended in the RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, 100 U/ml penicillin G and 0.1 mg/ml streptomycin (Sigma, USA) in 75 cm^3 flasks at a density of 1 to $4x10^6$/ml. Cells were then incubated at 37˚C in an atmosphere of 5% CO_2 in the media, or that added either ZGDHu-1 or z-VAD.fmk. At the indicated days of culture, cells were analyzed for apoptosis, or lysed for western blotting.

Cell viability assay. Effects of ZGDHu-1 on viability of primary cells were assayed by color reaction with MTT assay as described previously (16). Cells (at density of $5x10^5$/ml) were incubated with ZGDHu-1 at different concentrations (50, 100, 150, 200, 250 ng/ml) in 96-well plates for 72 h, respectively. The control group received drug-free medium with 0.05% DMSO (v/v). After the treatment, MTT (5 mg/ml) was added to each well and incubated for 4 h at 37˚C, and then the medium was removed, followed by addition of 150 μl DMSO to each well. The optical density was measured by a microplate reader M680 (Bio-Rad, Hercules, CA, USA) at a reference wavelength of 630 nm and a test wavelength of 570 nm. All experiments were performed in triplicates and repeated at least three times. The cell viability was expressed as a percentage of the DMSO-treated control samples.

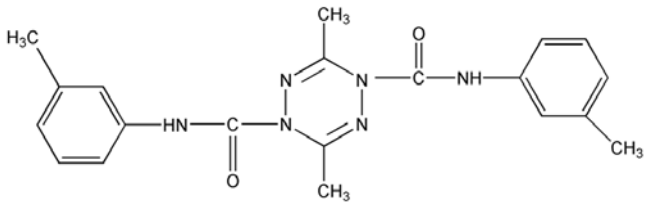

Figure 1. The molecular structure of ZGDHu-1.

Apoptosis assays. The apoptosis was determined by assays of annexin V in the presence of PI for PS externalization (17) and Hoechst 33258 (18). The annexin V assay followed a protocol provided with the annexin V kit. CLL cells were stained with 10 μl annexin V and 10 μl PI for 15 min at the room temperature in the dark, and the fluorescence signals of annexin V and PI were measured by flow cytometry on the FL1 and FL3 channels with gating for $CD19^+$ lymphocytes based on CD19 and side scattering, respectively. Only annexin V-positive (+) and PI-negative (-) cells were defined as apoptotic.

For Hoechst 33258 staining, CLL cells were plated into eight-chamber slides at $9x10^4$ cells per well until fully differentiated. Then they were washed extensively with serum-free RPMI 1640 after the drug exposure at the indicated times, and then washed once with PBS, fixed by 4% paraformaldehyde for 10 min, finally stained with 10 μg/ml of Hoechst 33258 (Applygen Tech Inc, Beijing, China) for 10 min. The morphologic change of apoptosis of CLL cells was evaluated under a fluorescence microscopy (Olympus BX61) with 350-370 nm excitation and 465 nm emission.

Measurement of ΔΨm. To measure ΔΨm, CLL cells were stained with lipophilic cationic JC-1 for 30 min at 37˚C in the dark. The JC-1 dye selectively accumulates in the mitochondria of healthy cells as aggregates, which emit the red fluorescent signal at 590 nm wavelength in response to 488 nm light excitation. Upon the onset of apoptosis, the mitochondrial potential was disrupted (19), and thus the JC-1 dye can no longer accumulate in the mitochondria but remains in the cytoplasm in a monomeric form, which emits green fluorescence at 525 nm in response to 488 nm light excitation. In the experiments, cells were washed in PBS and then resuspended in a total volume of 400 μl, and differential distribution of the

red and green forms of JC-1 dye can be easily analyzed by flow cytometry with gating for $CD19^+$ lymphocytes based on CD19 and side scattering.

ROS assay. The formation of mitochondrial ROS was tested by measuring oxygen consumption in the mitochondrial isolates with the fluorescent dye DHR. DHR is nonfluorescent and is oxidized to the fluorescent rhodamine-123 by various reactive oxygen species. DHR was dissolved in DMSO before it had been purged with nitrogen for 30 min. For staining, the density of suspended CLL cells was adjusted to $0.5x10^6$ cells/ml, and DHR was added to the assay medium in the absence of BSA or aprotinin, with gently stirring. The assay medium containing the DHR was then kept in the dark for 30 min at 37˚C. The intracellular accumulation of ROS was assessed at FL1 with flow cytometry with gating for $CD19^+$ lymphocytes based on CD19 and side scattering.

Bcl-2 and Bax assay. CLL cells were collected and washed in PBS. Briefly, a pellet of 10^6 CLL cells was fixed in 100 μl IntraPrepa™ permeabilization kit I solution for 15 min at the room temperature, then washed by adding 1 ml of PBS containing 1% BSA. For labeling, cells were permeabilized with 100 μl IntraPrep™ permeabilization kit II solution for 5 min and incubated with an anti-human Bcl-2-PE and Bax-PE antibody for 30 min at the room temperature. Unbound antibody was removed by washing twice in PBS/BSA. A total of about 5,000 cells were analyzed for organ fluorescence through a 575 nm-wavelength filter by flow cytometry.

Western blot analysis. The treated CLL cells were collected and lysed in a buffer contained 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 mM phenylmethyisulfonylfluoride, 0.5% Triton-X as well as protease inhibitor cocktail (Pierce, Rockford, IL, USA) on ice for 20 min. The protein concentrations of cell lysates were measured with the Lowry method (20). The proteins were equally loaded on and separated by SDS-PAGE gel, and then transferred to nitrocellulose membranes with moisture transfer technique at 100 V for 2 h. The membranes were blocked by incubation with 5% defatted milk PBS solution for 1 h. After washing, the membranes were incubated in a solution of monoclonal antibodies against human Bcl-2, Bax and caspase-3 (in dilution 1:1000) for 1 h, respectively. The β-actin was used as internal references. The rabbit anti-mouse IgG antibodies (1:1000) were used as the secondary antibody. Immunoreactive bands were visualized by the ECL kit (Pierce) and the gray densities were measured with GDS-8000 imaging system (UVP, USA). The western blot analysis shown in figures are representative results obtained in at least three separate experiments.

Statistical analysis. All values are expressed as mean ± SD. The data were analyzed using Statistical Program for Social Sciences (SPSS) software (version 13.0, SPSS Inc., Chicago, IL, USA). Analysis of variance with a post-hoc Dunn test was performed for multiple comparisons. The difference was considered to be significant at P-value <0.05.

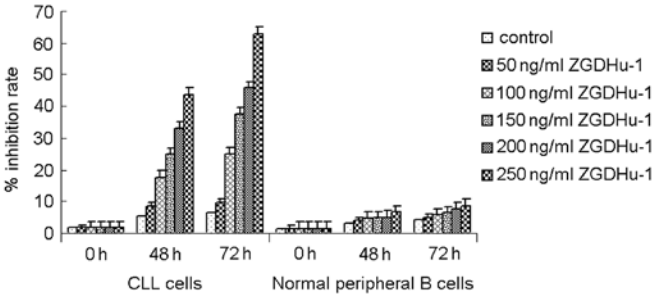

Figure 2. ZGDHu-1 reduces viability of CLL cells. Freshly isolated and purified CLL cells, and normal peripheral B cells were treated with different concentration of ZGDHu-1 for 48 or 72 h. Cell viabilities were determined by MTT assay. For each case, experiments were carried out in triplicate. Results are expressed as mean ± SD, n=3, *P<0.05 vs. respective control.

Results

The effects of ZGDHu-1 on the viability of CLL cells. To evaluate the effect of ZGDHu-1 on the viability of primary CLL cells, the cells (randomly from 10 patients, n=10) were treated by ZGDHu-1 at different concentrations and their viability were examined by MTT assay. The treatment of ZGDHu-1 dose-dependently reduced the viability of CLL cells from 94.2±4.9% at 50 ng/ml to 52.3±15.4% at 250 ng/ml for 48 h treatment (Fig. 2). Moreover, the viability for 72 h treatment at 250 ng/ml concentration was only 40.2±17.4% of CLL cells with ZGDHu-1 treatment. However, this inhibitive effect of ZGDHu-1 was not detected in normal peripheral B cells which were treated by ZGDHu-1 at the same conditions, suggesting that cytotoxic effects of ZGDHu-1 on CLL cells are specific.

MTT assay also showed that the treatment of CLL cells with ZGDHu-1 at 200-250 ng/ml for 72 h reduced the viability of CLL cells by about 50%, with an IC_{50} of 236.6 ng/ml. Thus, in the following studies, the treatment duration was set at 72 h (3 day).

ZGDHu-1 induces apoptosis of CLL cells. Next, we investigated whether the growth inhibition by ZGDHu-1 was caused by apoptosis. The CLL cell morphology was examined. The Hoechst 33258 staining experiments showed that CLL cells treated with ZGDHu-1 at 100 ng/ml and 200 ng/ml presented morphology changes of typical apoptosis characteristics (Fig. 3A). The pyknosis (nucleus condensing) and karyorrhexis (nucleus fragmenting) were observed in ZGDHu-1 treated cells.

The apoptosis was further quantified by the externalization of PS, assessed by annexin V-PI double staining at indicated time. The percentage of annexin V+/PI- cells (CD19-gated) increased to 16.3 and 30.6% in CLL cells at 3 days of 100 ng/ml and 200 ng/ml ZGDHu-1 treatment, respectively, being significantly higher than that of untreated control (all P<0.01). While for normal B lymphocytes, ZGDHu-1 treatment did not induce early apoptosis (Fig. 3B and C). These data suggested that ZGDHu-1 appeared to have significant pro-apoptotic activity specifically against CLL cells.

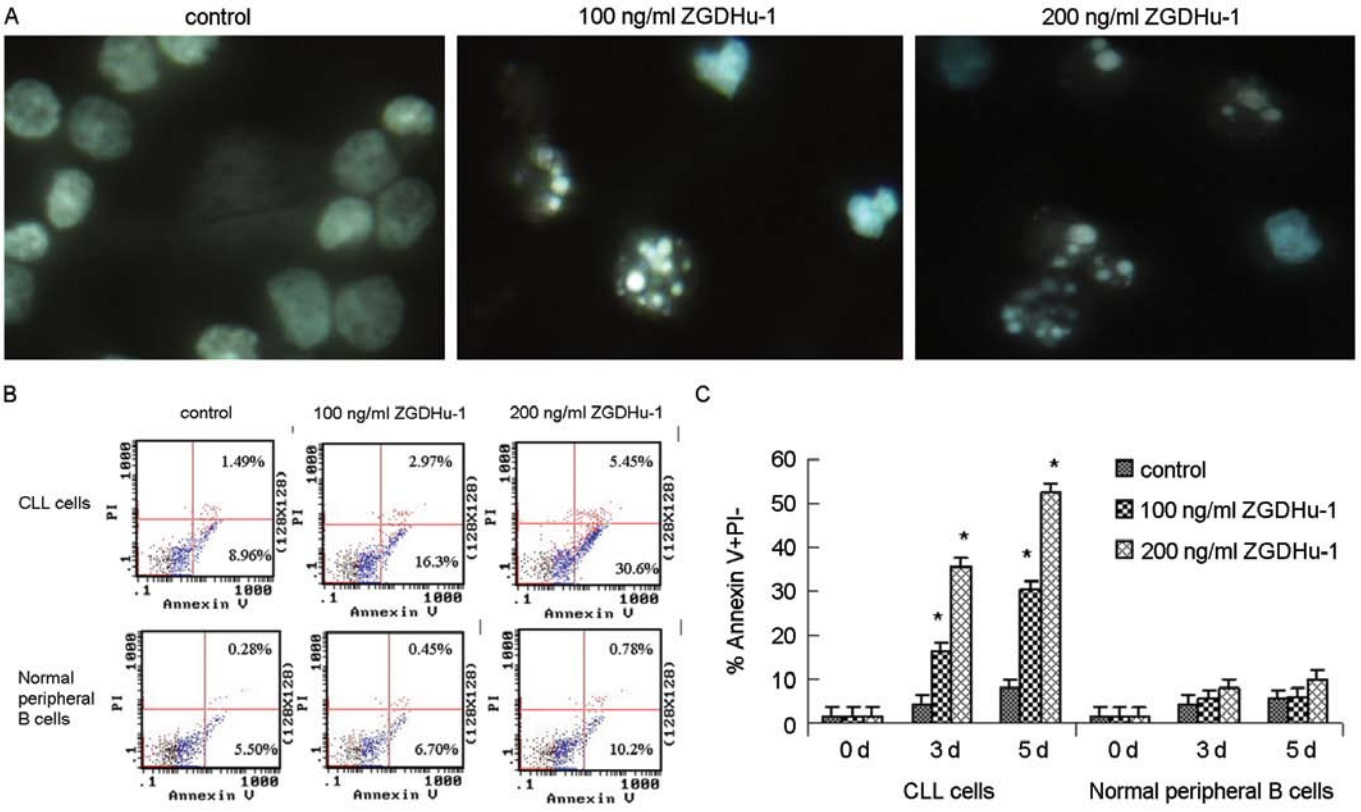

Figure 3. ZGDHu-1 induces apoptosis in CLL cells. Freshly isolated and purified CLL cells were cultured in the drug-free medium with 0.05% (v/v) DMSO (control) and incubated with 100 ng/ml ZGDHu-1, 200 ng/ml ZGDHu-1 for different times. (A) Apoptotic cell morphology was measured by the Hoechst 332583 staining after 3-days of treatment. Many of the treated cells displayed characteristic apoptotic nuclear morphology. (B) Early apoptosis was also measured by the annexin V assay with flow cytometry after a 3-day incubation. (C) The summarized apoptotic data. Results are expressed as means ± SD, n=10, *P<0.01 vs. respective control.

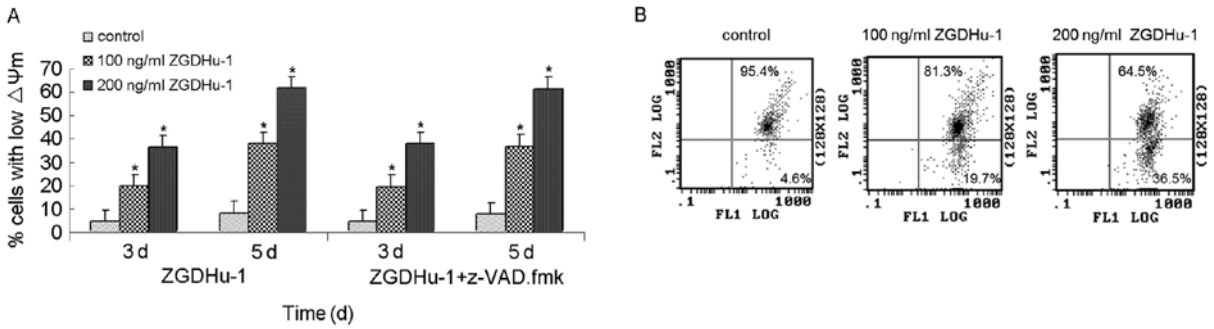

Figure 4. The effect of ZGDHu-1 on ΔΨm of CLL cells. (A) Freshly isolated purified CLL cells were cultured in drug-free medium with 0.05% (v/v) DMSO (control) and incubated with 100 ng/ml ZGDHu-1, 200 ng/ml ZGDHu-1 for 3 or 5 days. ΔΨm was measured by JC-1 staining with flow cytometry. The presented data reveal that exposure to ZGDHu-1 caused an early decreased in ΔΨm in a dose-dependence manner. Results are expressed as means ± SD, n=10 *P<0.05 vs. respective control. (B) Representative data of 10 independent experiments are shown following exposure to ZGDHu-1 alone for 3 days. Control indicates drug-free medium with 0.05% DMSO (v/v), 100 ng/ml ZGDHu-1, 200 ng/ml ZGDHu-1.

Effect of ZGDHu-1 on mitochandrial pathway. To search for the indication of mechanisms involved in apoptosis, we examined the effects of ZGDHu-1 on ΔΨm and on the expression of Bcl-2 family members. The treatment with ZGDHu-1 (100 ng/ml or 200 ng/ml for 3 or 5 days) had higher percentage of low ΔΨm in CLL cells in comparison to the controls (Fig. 4, P<0.05). The adding of caspase inhibitor z-VAD.fmk did not inhibit this effect of ZGDHu-1.

The membrane permeability of mitochondria is directly controlled by Bcl-2 family proteins, which function as the central regulators of caspase activation (21). To examine whether ZGDHu-1 may change the mitochondrial membrane properties through affecting these Bcl-2 family members, we measured the expression levels of anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax of CLL cells after 3 or 5 days of incubation with ZGDHu-1. Both flow cytometry and western blot experiments indicated that, following the exposure to ZGDHu-1, Bcl-2 expression was decreased while that of Bax was not changed (Fig. 5A and B). In the flow cytometry assay, the treatment with 200 ng/ml ZGDHu-1 for 3 days

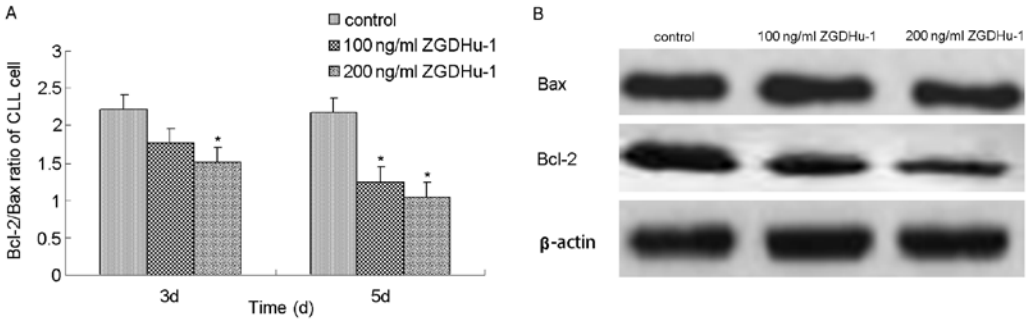

Figure 5. The effect of ZGDHu-1 on Bcl-2 and Bax of CLL cells. Freshly isolated purified CLL cells were cultured in the culture medium and incubated with 100 ng/ml ZGDHu-1, 200 ng/ml ZGDHu-1 for 3 or 5 days. (A) Bcl-2 and Bax of CLL cells were measured by flow cytometry, respectively. The presented data reveal that ZGDHu-1 induced negative modulation of Bcl-2/Bax ratio. Results are expressed as the means ± SD, n=10, *P<0.05 vs. respective control. (B) The protein levels of Bcl-2 and Bax in CLL cells were measured by Western blot after 3-days of treatment.

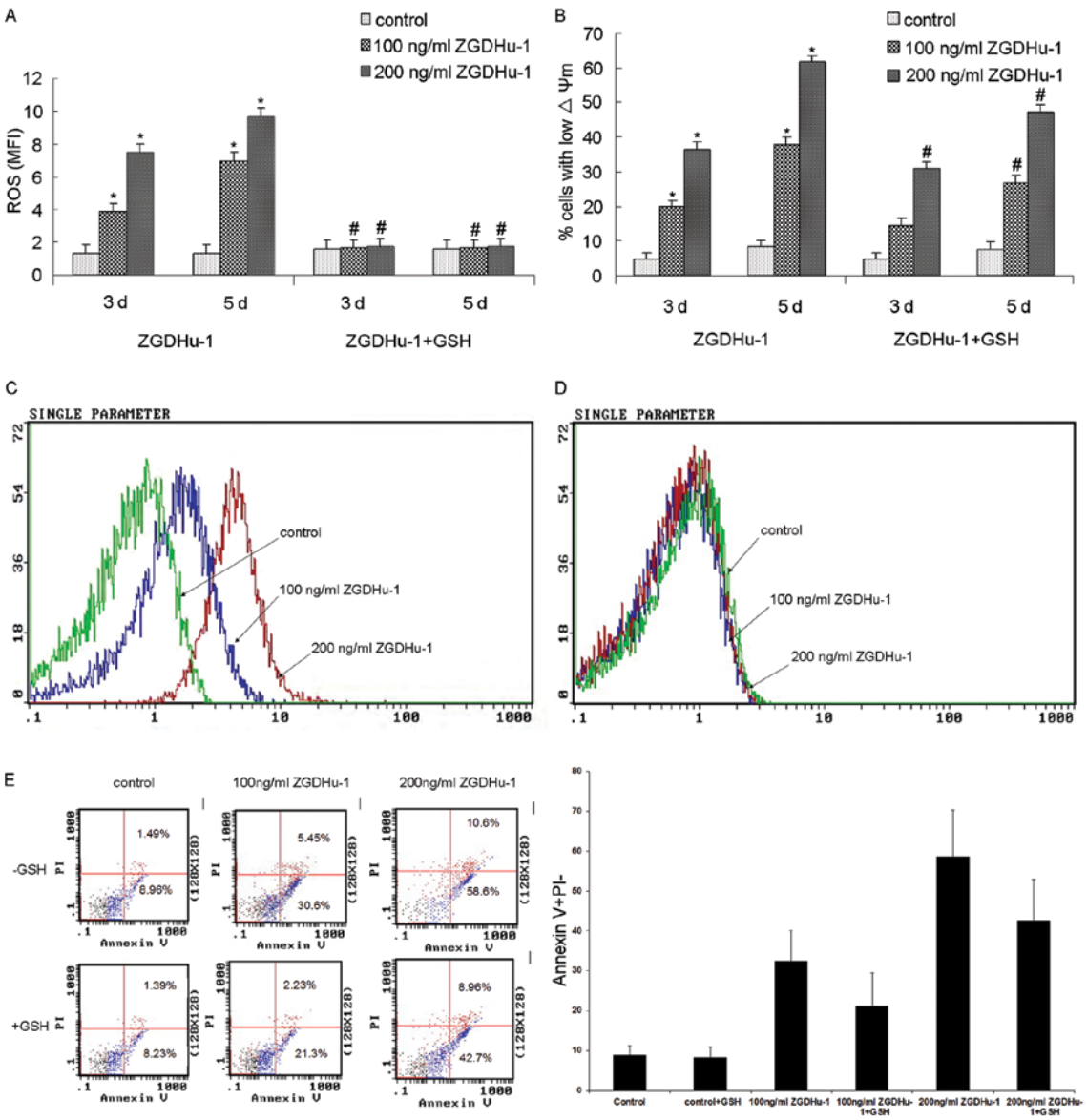

Figure 6. The effect of ZGDHu-1 on ROS of CLL cells. (A) Freshly isolated purified CLL cells were cultured in drug-free medium with 0.05% (v/v) DMSO (control) and incubated with ZGDHu-1 either alone or in the presence of 100 μM GSH for 3 and 5 days. ROS was measured by DHR staining with flow cytometry. The mean fluorescence was enhanced by ZGDHu-1 in comparison to control alone. (B) The effect of GSH on ZGDHu-1-induced a decrease in ΔΨm. Freshly isolated purified CLL cells were treated as indicated above. ΔΨm was measured by JC-1 staining with flow cytometry. The presented data reveal that GSH partly inhibited ZGDHu-1-induced a decrease in ΔΨm. Representative data of 10 independent experiments are shown following exposure to ZGDHU-1 (100 or 200 ng/ml) alone for 3 days in the absence of (C) or in the presence of 100 μM GSH (D). Control indicates culture medium alone. (E) The effect of GSH on the ZGDHu-1-induced apoptosis of CLL cells analyzed by the annexin V assay with flow cytometry after a 3-day incubation. Data are presented as the means ± SD, n=10. *P<0.05 between treated and control; #P<0.05 between ZGDHu-1 and ZGDHu-1+GSH.

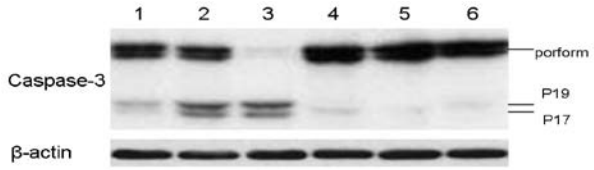

Figure 7. The effect of ZGDHu-1 on caspase-3 of CLL cells. CLL cells were pre-treated by z-VAD.fmk for 1 h at 37˚C then treated with ZGDHu-1 either alone or in the presence of z-VAD.fmk for 3 days. At the end of treatment, cells were collected and both the nuclear and cytosolic extracts were prepared. Caspase-3 was measured by Western blotting. Band 1, control; 2, 100 ng/ml ZGDHu-1; 3, 200 ng/ml ZGDHu-1; 4, 100 μM z-VAD.fmk; 5, 100 ng/ml ZGDHu-1+ 200 μM z-VAD.fmk; 6, 200 ng/ml ZGDHu-1+200 μM z-VAD.fmk.

decreased the percentage of Bcl-2-positivity of CLL cells (CD19-gated) from 50.27±12.53% to 30.3±9.68%, and that of untreated control was 25.4±7.43%. These data suggested that ZGDHu-1 induced cell apoptosis through an intrinsic mitochondrial pathway.

ZGDHu-1-induced change of ROS in CLL cells. ROS also plays a role during cell apoptosis. We further examined whether the level of ROS in CLL cells was affected by ZGDHu-1 with DHR staining in the flow cytometry assay. As shown by Fig. 6, treatment with ZGDHu-1 at 100 ng/ml and 200 ng/ml for 3 or 5 days ZGDHu-1 significantly induced ROS generation (all P<0.05). We also tested whether ROS scavenger glutathione (GSH) could suppress the ZGDHu-1-induced apoptosis of CLL cells. Pretreatment with GSH (at 100 μM) for 2 h could significantly block ZGDHu-1-induced ROS generation (Fig. 6A, all P<0.05), and partly inhibited ZGDHu-1-induced increasing percentage of low ΔΨm CLL cells (Fig. 6B-D, all P<0.05). However, GSH did not significantly inhibit the pro-apoptotic effects of ZGDHu-1 on CLL cells (Fig. 6E). These results suggested that ZGDHu-1 induced ROS generation might underlie its effect on promoting CLL cell apoptosis.

Role of caspase-3 in ZGDHu-1-induced apoptosis. We further examined whether the caspase-3 is involved in the apoptosis process induced by of ZGDHu-1 in CLL cells. CLL cells were exposed to ZGDHu-1 either alone or in the presence of the broad spectrum caspase inhibitor z-VAD.fmk. ZGDHu-1 treatment could induce the cleavage of caspase-3 in CLL cells (Fig. 7, lanes 2 and 3). However, pre-treatment with z-VAD.fmk significantly blocked ZGDHu-1-induced caspase-3 cleavage (Fig. 7).

Moreover, pre-treatment with z-VAD.fmk also partially attenuated the ZGDHu-1-induced apoptosis of CLL cells, as assessed by the PS externalization (Fig. 8A and B). However, neither the ΔΨm (Fig. 8C) nor the Bcl-2/Bax ratio (Fig. 8D) was decreased by z-VAD.fmk, indicating that Bcl-2/Bax acti-

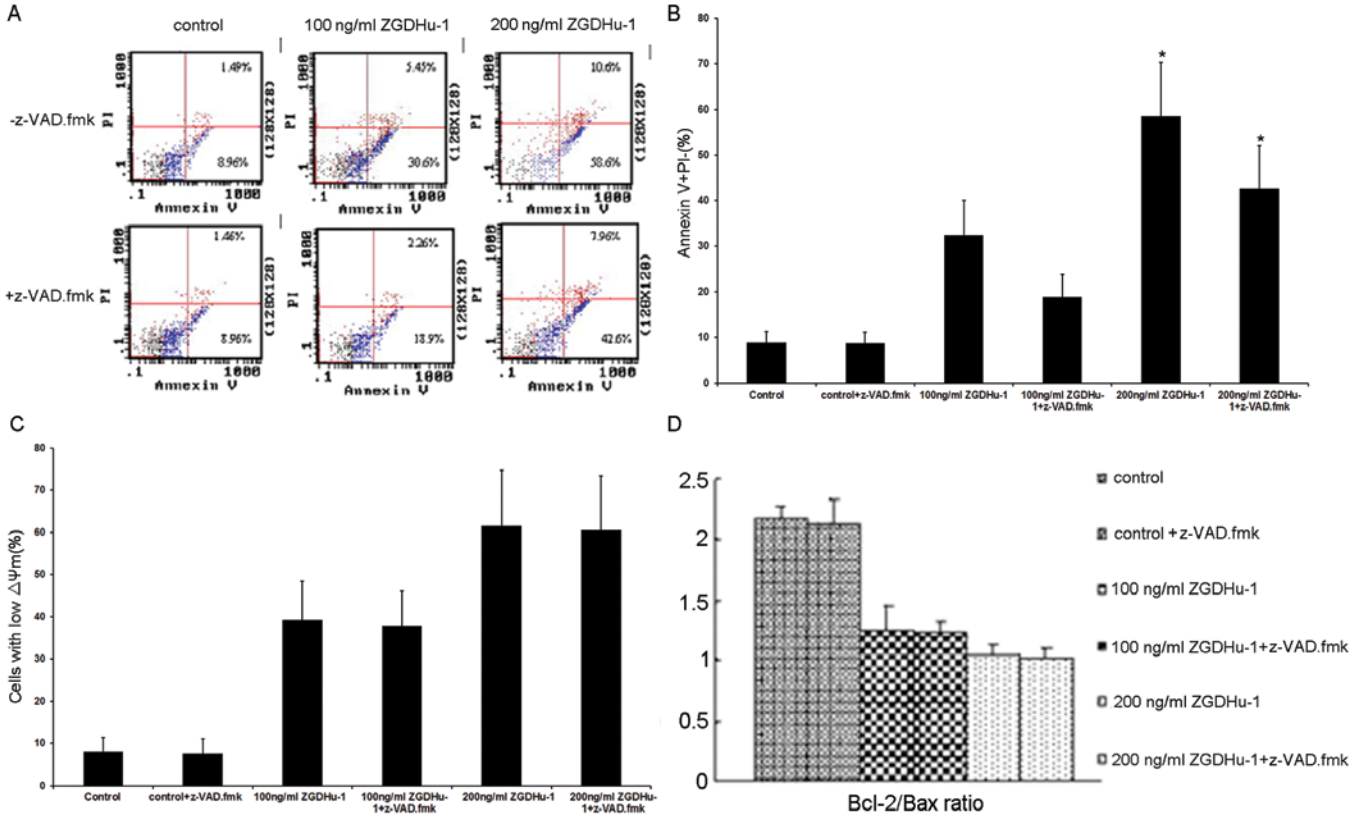

Figure 8. Effects of caspase inhibitor z-VAD.fmk on ZGDHu-1-induced apoptosis in CLL cells. CLL cells were pre-incubated with 200 μM z-VAD.fmk for 1 h at 37˚C and then treated with 100 or 200 ng/ml ZGDHu-1 for 3 days. (A,B) Apoptotic cell death was measured by the annexin V assay with flow cytometry. The pro-apoptotic effect of ZGDHu-1 on CLL cells was attenuated by z-VAD.fmk. (C) Changes of ΔΨm was accessed by JC-1 staining with flow cytometry. (D) Bcl-2 and Bax levels were measured with flow cytometry, respectively. Data are expressed as the means ± SD, n=10. *P<0.05 between ZGDHu-1 and ZGDHu-1+GSH.

vation occurred upstream of the activation of caspases. Thus, it appeared that the effects of z-VAD.fmk on the ZGDHu-1-induced cell apoptosis and caspase-3 cleavage correlated to some extent.

Discussion

CLL is the most common leukemia in the world and is characterized by the accumulation of quiescent malignant B lymphocytes failure to undergo apoptosis. There are few effective treatments for CLL at present. In this study, we firstly reported that ZGDHu-1, a potential proteasome inhibitor and an anti-tumor agent (14), showed cytotoxic effects in a dose-dependent manner with an IC_{50} of 236.6 ng/ml on primary B lymphocytes isolated from CLL patients but not from normal healthy donors. Moreover, ZGDHu-1 induced apoptosis in CLL cells by increasing mitochondrial membrane permeability, producing ROS, cleavage and activation of caspase-3, and decreasing Bcl-2/Bax ratio.

In this study, ZGDHu-1 specifically induced apoptosis in primary CLL cells. Based on a decrease in $\Delta\Psi m$, the apoptosis in CLL cells induced by ZGDHu-1 was closely related to mitochondrial pathway. Bcl-2, as the major anti-apoptotic protein of Bcl-2 family, was significantly reduced by the treatment of ZGDHu-1, further implying that intrinsic apoptosis pathway is involved. Overexpression of Bcl-2 potently inhibits apoptosis in response to many cytotoxic insults, among others by suppressing the generation of ROS, inhibiting the mitochondrial permeability transition and cytochrome C release (22-27). In this study, cytochrome C release was not detected. However, we already proved that permeability of $\Delta\Psi m$ was increased with the treatment of ZGDHu-1. Bcl-2 proteins modulate the activity of caspases, the effector proteases which comprise the final common pathway of programmed cell death. Caspase-3, a 32 kDa zymogen, is cleaved into 17 kDa and 12 kDa subunits during cleavage and activation (28). At concentration of 200 ng/ml, ZGDHu-1 induced complete cleavage of caspase-3 in CLL cells, which was inhibited by the pretreatment of caspase inhibitor z-VAD.fmk, which also attenuated apoptosis induced by ZGDHu-1. Caspase-3 was involved in ZGDHu-1-induced apoptosis through intrinsic pathway. This is in accordance with previous reports (29-31) that the proteasome inhibitor triggered a caspase-dependent apoptosis. It is very interesting that above pro-apoptotic effect of ZGDHu-1 on CLL cells did not occur in normal B lymphocytes. We do not know the reason for this specific mechanism. Normal B lymphocytes might lack a specific target molecule of ZGDHu-1 which is required to activate the intracellular apoptotic pathway.

We have previously found Bcl-2 to be high constitutively expressed in CLL patients (32). In this study, after exposure to ZGDHu-1, Bcl-2 was significantly reduced, but Bax was unaffected. Recent studies stressed these genes was associated with PI3K/NF-κB pathway on the regulation of CLL cell survival (33). It is implied that down-regulation of NF-κB-dependent genes might be involved in the pro-apoptotic mechanism of ZGDHu-1. However, further study are needed to clarify this hypothesis.

The intracellular redox status, depending on GSH levels and ROS generation, is important in stabilizing mitochondria functions. Our results suggest that ZGDHu-1 appears to affect the intracellular redox status and regulate the mitochondria through elevating the level ROS. Whether this cellular signaling pathway underlies the process of ZGDHu-1 induced CLL cell apoptosis remains to be further elucidated.

Collectively, the results demonstrate that ZGDHu-1 significantly reduced the viability of CLL cells and ZGDHu-1 could effectively trigger caspase-dependent apoptosis by elevating the level ROS and the loss of $\Delta\Psi m$. Based on these findings, especially its non-toxicity to normal B-lymphocytic cells, we propose that the compound ZGDHu-1 may potentially function as a novel anti-CLL agent.

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