ZGDHu-1 and fludarabine have a synergistic effect on apoptosis of chronic lymphocytic leukemia cells

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Abstract. Previously, it was demonstrated that the novel proteasome inhibitor N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1) possesses activity against chronic lymphocytic leukemia (CLL). In the present study, we attempted to assess whether this drug has a synergistic effect with fludarabine on the apoptosis of CLL cells. Annexin V/PI staining, mitochondrial membrane potential (ΔΨm) and reactive oxygen species (ROS) levels were examined by flow cytometry in short-term cell culture of blood cells from untreated newly diagnosed patients ex vivo. Expression of active caspase-3 and the Bcl-2/Bax ratio for determination of apoptosis were also investigated by flow cytometry and western blot analysis. Our results revealed that the ZGDHu-1 may induce the apoptosis of CLL cells through the mitochondrial pathway and its pro-apoptotic effect is CLL-specific, not affecting normal lymphocytes. Most importantly, a combination of ZGDHu-1 and a non-cytotoxic dose of fludarabine had a synergistic apoptotic effect. To some extent, caspase-3 activation may be involved in the mechanism of the ZGDHu-1 synergistic cytotoxic effect with fludarabine, as well as the cleavage of PARP, consequently leading to apoptosis. Notably, the rate of apoptosis caused by ZGDHu-1 alone or in combination with fludarabine was independent of prognostic markers of CLL disease such as ZAP-70 and CD38 expression or clinical Rai classification stage. In conclusion, ZGDHu-1 exhibited a significant synergistic effect with fludarabine to induce the apoptosis of CLL cells, which implies a possible clinical application.

Introduction

Chronic lymphocytic leukemia (CLL), the most common type of adult leukemia in Western countries, characterized by the accumulation of malignant CD19⁺CD5⁺ cells, is a highly heterogeneous disease with variable prognoses and clinical course (1-3). Some patients never require treatment and are indolent, while other patients have aggressive disease and require intensive treatment soon after diagnosis (4,5). Traditionally, symptomatic CLL patients can be effectively treated with fludarabine, glucocorticoids, alkylating agents or monoclonal antibodies. However, despite these therapeutic regimens, CLL disease is still incurable. Thus, the discovery of novel, less toxic and more effective drugs for CLL patients is in urgent need. Moreover, relapsed or refractory CLL patients have limited therapeutic treatment options.

To discover new drugs, the use of plant-derived substances or immunomodulatory drugs was reported to have a therapeutic effect in CLL treatment. Additionally, new candidates for CLL therapies include histone deacetylase inhibitors, Bcl-2 inhibitors and proteasome inhibitors have also been developed (6,7). These latter agents may induce CLL cell apoptosis partly through pro-apoptotic and anti-apoptotic family members (6,7). Among these drugs, fludarabine, an inhibitor of STAT1 activity and DNA synthesis inhibitor, is the most effective drug for the treatment of CLL disease, especially for routine treatment failure patients. However, toxic side effects in the clinic are extremely evident (8). The toxic effects of fludarabine include immunosuppression marked by a decrease in the CD4⁺/CD8⁺ ratio, and the development of myelosuppression, opportunistic infections or gastrointestinal toxicities which include vomiting, nausea and hepatic lesions have also been reported (8).

ZGDHu-1 [N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide] (Fig. 1) is a tetrazine compound (9), which has been reported by our group to exhibit antitumor activity (10). It has been identified as a potential proteasome inhibitor (11). It was demonstrated that ZGDHu-1 induces the apoptosis of B lymphocytes from CLL patients (12). In the present study, we investigated ZGDHu-1 used alone or combined with fludarabine in regards to the ex vivo apoptotic effects on CLL cells and normal lymphocytes derived from peripheral blood. We examined the apoptosis of CLL cells, loss of mitochondrial membrane potential (ΔΨm), phosphatidylserine (PS) translocation across the plasma membrane (13,14) and accumulation of reactive oxygen species (ROS) (15). At the same time, the percentage
of active caspase-3-expressing cells, intracellular Bcl-2 and Bax expression were also investigated. Subsequently, we also analyzed the correlation between these apoptotic effects with clinical indices, such as lactate dehydrogenase (LDH), ZAP-70 or CD38 expression, lymphocyte count, β2-microglobulin level and Rai classification status.

Materials and methods

Patients. Twenty-five untreated, newly diagnosed CLL patients were enrolled. CLL diagnosis was carried out according to clinical examination, morphological and immunological criteria. After informed consent, peripheral blood cells were obtained from the CLL patients. The present study was approved by the Zhejiang Provincial People's Hospital Ethics Committee. Patient characteristics are summarized in Table I.

Reagents and instruments. ZGDHu-1 compound (Fig. 1) with a purity of >95% was kindly provided by Dr Wei-Xiao Hu (Pharmaceutical College of Zhejiang University of Technology, China) as previously reported (12). ZGDHu-1 was dissolved in dimethylsulfoxide (DMSO) as a stock solution (1 mg/ml) and stored at -20°C. Antibodies against Bcl-2 (SC-7382), Bax (SC-2774), caspase-3 (SC-9746), β-actin (SC-4967) for western blot analysis were purchased from Cell Signaling Technology (Beverly, MA, USA). Fludarabine, DMSO, 3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dihydrodorhodamine-123 (DHR), broad spectrum caspase inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) and Ficoll-Hypaque liquid were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fludarabine, DMSO, 3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dihydrodorhodamine-123 (DHR), broad spectrum caspase inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) and Ficoll-Hypaque liquid were purchased from Sigma-Aldrich (St. Louis, MO, USA) as previously reported (12). The apoptosis kit of propidium iodide (PI), Annexin V and the IntraPrep™ permeabilization kit were all from ImmunoTech Company (Marseille, France). JC-1 (5,5',6,6'-tetrachloro-1',1',3,3'-tetraethyl benzimidazlyl carbocyanine iodide) was purchased from BioTeam Inc. Company as previously reported (12). CLL cells were stained with the following mAbs: anti-CD19-PerCP CY 5.5 (ID3), anti-CD5-APC (53.7.3) (both from BD Pharmingen, San Diego, CA, USA), anti-ZAP-70 Alexa Fluor® 488 (SBZAP) and anti-CD38-FITC (T16); both from Immunotech), anti-active caspase-3-PE (C92-605; BD Pharmingen), anti-Bax-FITC (SC20067; Santa Cruz Biotechnology, CA, USA); and anti-Bcl-2-PE (Bcl-2/100; BD Pharmingen). Cells were analyzed with Navios FACS (Beckman Coulter, Miami, FL, USA).

Cell viability assay. Cell viability was analyzed with an MTT assay as previously described (16). Cells (at a density of 5x10³/ml) were incubated with ZGDHu-1 (50, 100, 150, 200 and 250 ng/ml) alone or in combination with fludarabine (1 µg/ml) on 96-well plates for 72 h. The control group was incubated only with drug-free medium with 0.05% DMSO solution (v/v). Then, MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C, the medium was aspirated and then 150 µl DMSO solution was added to each well. The plate was then measured by the M680 microplate reader (Bio-Rad, Hercules, CA, USA) at a reference 630 nm wavelength and a test 570 nm wavelength. All experiments were performed in triplicate and repeated at least three times. The cell viability was expressed as a percentage of the DMSO-treated control samples as previously reported (12).

Lymphocyte purification and culture. EDTA-K2 anticoagulant blood samples were obtained from the CLL patients and healthy controls during a routine diagnosis at the Zhejiang Provincial People's Hospital. B lymphocytes were isolated immediately by using Ficoll gradient centrifugation. After a 1-h incubation at 37°C, in a 5% CO₂ condition, adhesive mononuclear cells were removed. The non-adherent lymphocytes were washed with Hank's solution (Biochrom, Berlin, Germany). Then the T lymphocytes were removed using anti-CD3 Dynabeads® (Dynal, Merseyside, UK). The B lymphocytes were further purified by flow cytometric sorting based on CD19 antibody staining (ImmuneTech, Coulter, USA) as previously reported (12). B lymphocytes were then counted in a Neubauer Counting Chamber with trypan blue to exclude dead cells. The cells were then resuspended in RPMI-1640 medium with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin G and 0.1 mg/ml streptomycin (Sigma-Aldrich) in 75 cm² flasks at a density of 1-4x10⁶/ml and cultured at 37°C in 5% CO₂ as previously reported (12). The cells were further divided into four groups. The first group served as the control without any treatment. The second group was treated with 100 ng/ml ZGDHu-1 for 0-5 days. The third group was treated with fludarabine (1 µg/ml) for 0-5 days. The fourth group was treated with both ZGDHu-1 (100 ng/ml) and fludarabine (1 µg/ml) for 0-5 days. At the end of each time-point, the cells were harvested for FACS analysis or lysed for western blot analyses.

FACS. CLL cells were stained with the following mAbs: anti-CD19-PerCP CY 5.5 (ID3), anti-CD5-APC (53.7.3), anti-ZAP-70 Alexa Fluor® 488, anti-CD38-FITC (T16), anti-active caspase-3-PE (C92-605; BD Pharmingen), anti-Bax-FITC (SC20067) and anti-Bcl-2-PE (Bcl-2/100; BD Pharmingen). The stained cells were analyzed with Navios FACS. Then 10,000 cells for each sample were counted and then CD19 and CD5 antibody staining was carried out and the CD19⁺ CD5⁺ double-positive cell population was gated for the following analysis. For the apoptosis detection, the percentage of Annexin V-positive (+) and PI-negative (-) cells was detected using the Annexin V kit. The mitochondrial potential (ΔΨm) was measured with JC-1 dye. The intracellular accumulation of ROS was assessed with the fluorescent dye DHR. The percentage of active caspase-3-positive cells in the control cells was calculated. The Bcl-2 and Bax expression was examined for each sample. Then the Bcl-2/Bax ratio for the CD19⁺CD5⁺ cells was calculated. The CD19⁺CD5⁺ populations were regarded as non-leukemic cells. To determine the frequency of prognostic factors, the percentage of CD38 and ZAP-70 was detected for each sample. Patients were defined as ZAP-70-positive when the ZAP-70 expression was >20% in leukemic cells. Patients were defined as CD38-positive, when the CD38 expression was at least 20% in leukemic cells.

Western blot analysis. The treated CLL and control cells were collected and lysed in 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
phenylmethylsulfonyl fluoride, 0.5% Triton-X and protease inhibitor cocktail (Pierce, Rockford, IL, USA) on ice for 30 min. Then, the same amount of proteins was loaded and separated by SDS-PAGE gel, transferred to nitrocellulose membranes at 100 V for 2 h and then blocked with PBS solution with 5% non-fat milk for 1 h. After PBS washing, the membrane was incubated with the primary monoclonal antibodies against human Bcl-2, Bax and caspase-3 (1:1,000 dilution) for 1 h, incubated with the primary monoclonal antibodies against non-fat milk for 1 h. After PBS washing, the membrane was blocked with PBS solution with 5% non-fat milk for 1 h. After PBS washing, the membrane was incubated with the primary monoclonal antibodies against human Bcl-2, Bax and caspase-3 (1:1,000 dilution) for 1 h, respectively. The β-actin expression was used as the control. Then, the rabbit anti-mouse IgG antibodies (1:1,000 dilution) were used as the secondary antibody. The ECL kit (Pierce) and the GDS-8000 imaging system (UVP, Upland, CA, USA) were used for the visualization of the immunoreactive bands.

**Evaluation of the combination index.** A combination index (CI) calculated based on the Chou-Talalay method was used to evaluate the synergism between ZGDHu-1 and fludarabine (2,17-19). The following formula was used: CI = (sum of single agent treatment/specific apoptosis of combined treatment). The percentage of specific apoptosis was examined by using the following formula: Specific apoptosis = (drug induced apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis) x 100%. CI <1, CI =1 and C >1 were regarded as synergistic, additive or infra-additive, respectively (20,21). The percentage of active caspase-3 cells was estimated for these populations. In the present study, 7 of the 25 patients for whom the non-leukemic cells were higher than 10% in the peripheral blood were analyzed.

**Statistical analysis.** Data from individual experiments are presented as mean ± SD. The statistical analysis was carried out using SPSS10.0 software. The Wilcoxon test was used for two independent variables; Mann-Whitney U and Spearman's tests were used for two independent group and two variable correlations, respectively. P<0.05 was considered to indicate a statistically significant result.

**Results**

**Effects of ZGDHu-1 and fludarabine on the viability of CLL cells.** To investigate the synergistic effect of fludarabine and ZGDHu-1, we used the classical MTT assay to evaluate the cytotoxic effects of the two drugs. Firstly, we screened the minimal fludarabine cytotoxic concentration. After a series of preliminary studies with the aim to reduce the in vitro cytotoxicity above controls at a mean level of <10% after a 3-day treatment, a 1 µg/ml concentration of fludarabine was regarded as being non-cytotoxic. Consistent with previous studies (22), fludarabine did not show significant cytotoxicity at the concentration of 1 µg/ml compared to the controls (5.24±1.33%). But when concentrations of 2 and 2.5 µg/ml (30.5±8.05%; 41.7±7.25%, respectively) were used, its cytotoxicity was significantly increased (Fig. 2). Furthermore, ZGDHu-1 treatment also caused an increase in the cytotoxicity to CLL cells in a dose-dependent manner; 5.8±1.34% at 50 ng/ml compared to the controls (5.24±1.33%). But when concentrations of 2 and 2.5 µg/ml (30.5±8.05%; 41.7±7.25%, respectively) were used, its cytotoxicity was significantly increased (Fig. 2). In the following synergistic effect studies, the treatment duration was set at 3 days at a concentration of 100 ng/ml for ZGDHu-1 and 1 µg/ml for fludarabine.

**Combination of ZGDHu-1 and fludarabine induces the apoptosis of CLL cells.** To better support the MTT assay in regards to cell death triggered by ZGDHu-1 alone and in combination with fludarabine, CLL cells were evaluated for apoptosis by FACS analysis following Annexin-V and PI staining. As shown in Fig. 3, the apoptotic cell population was significantly increased after the CLL cells were treated with ZGDHu-1 and/or fludarabine on day 3 compared to the control (no treatment). Notably, the combination of ZGDHu-1 and fludarabine significantly increased the apoptotic population when compared to this population in the cells treated with ZGDHu-1 alone (20.5±4.56% for ZGDHu-1; 39.5±7.45% for fludarabine + ZGDHu-1). Thus, in the following synergistic effect studies, the treatment duration was set at 3 days at a concentration of 100 ng/ml for ZGDHu-1 and 1 µg/ml for fludarabine.

**Table I. Characteristics of the CLL patients.**

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tr>
<td>Male</td>
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<tr>
<td>I</td>
<td>6</td>
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<tr>
<td>II</td>
<td>8</td>
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<tr>
<td>III</td>
<td>6</td>
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<tr>
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<tr>
<td>Range</td>
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<tr>
<td>CD38*, n (%)</td>
<td>6 (24)</td>
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<tr>
<td>ZAP-70*, n (%)</td>
<td>8 (32)</td>
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</table>

**Figure 1. Molecular structure of ZGDHu-1.**

**Combination of ZGDHu-1 and fludarabine induces the apoptosis of CLL cells.** To better support the MTT assay in regards to cell death triggered by ZGDHu-1 alone and in combination with fludarabine, CLL cells were evaluated for apoptosis by FACS analysis following Annexin-V and PI staining. As shown in Fig. 3, the apoptotic cell population was significantly increased after the CLL cells were treated with ZGDHu-1 and/or fludarabine on day 3 compared to the control (no treatment). Notably, the combination of ZGDHu-1 and fludarabine significantly increased the apoptotic population when compared to this population in the cells treated with ZGDHu-1 and fludarabine alone. To further investigate whether ZGDHu-1 and fludarabine induced CLL cell apoptosis through the caspase-dependent pathways, we treated CLL cells with ZGDHu-1 and/or fludarabine in the presence of the broad spectrum caspase inhibitor, Z-VAD-fmk. We found that the apoptotic cell population was significantly reduced after pretreatment with Z-VAD-fmk (Fig. 3A and B). These data indicate that ZGDHu-1 induced cell apoptosis through the caspase-dependent pathway.
Effect of the combination of ZGDHu-1 and fludarabine on the mitochondrial pathway through the change in ROS. As we know, ROS play an important role in the mitochondrial pathway during cell apoptosis (15). The ROS level in CLL cells was examined following treatment with ZGDHu-1 alone or in combination with fludarabine. In the present study, the ROS level was detected by DHR staining and FACS method. As shown in Fig. 4A and B, following ZGDHu-1 treatment alone and in combination with fludarabine on day 3 and 5, the ROS levels were significantly increased (all P<0.05). However, treatment with fludarabine for 3 or 5 days did not significantly increase the ROS level in the CLL cells. Additionally, the...
ROS scavenger glutathione (GSH) was also investigated as to whether it could suppress the apoptosis of CLL cells mediated by ZGDHu-1 or the combination of ZGDHu-1 and fludarabine. Pretreatment with GSH (100 µM) for 2 h significantly blocked ZGDHu-1-induced ROS generation (Fig. 4A and C, P<0.05). In contrast, GSH did not inhibit the pro-apoptotic effects of ZGDHu-1 on the CLL cells (Fig. 4D and E). Overall, these results suggest that ZGDHu-1 may induce the apoptosis of CLL cells by altering the ROS level.

**Effect of the combination of ZGDHu-1 and fludarabine on the mitochondrial pathway through ΔΨm.** To further investigate whether ZGDHu-1 induces CLL cell apoptosis through the mitochondrial pathway, we analyzed the ΔΨm after ZGDHu-1 and/or fludarabine treatment by FACS analysis. After the CLL cells were treated with ZGDHu-1 and/or fludarabine on day 3 and 5, then the CLL cells stained with JC-1 (10 µmol/l) were detected for the ΔΨm level. As shown in Fig. 5B, the percentage of cells with low ΔΨm in the ZGDHu-1 + fludara-
bine-treated culture was significantly higher than that of the ZGDHu-1 group and the fludarabine group, indicating that ZGDHu-1 synergistically acts with fludarabine inducing CLL cell apoptosis through the mitochondrial pathway.

Effect of the combination of ZGDHu-1 and fludarabine on the mitochondrial pathway through the Bcl-2 family. As known, Bcl-2 family proteins play an important role in the control of membrane permeability of mitochondria and caspase activation (23). The expression levels of anti-apoptotic factor Bcl-2 and pro-apoptotic Bax factor in CLL cells on day 3 and 5 were detected by FACS (Fig. 6A) and western blot analysis (Fig. 6B). Following exposure to ZGDHu-1 alone and in combination with fludarabine, the Bcl-2 expression was significantly decreased while the Bax expression was not changed. However, when CLL cells were exposed to fludarabine alone, the expression of Bcl-2 and Bax was not changed (Fig. 6C and D). Overall, these data suggest that ZGDHu-1 induces the apoptosis of CLL cells through the intrinsic mitochondrial pathway, which was different from fludarabine.

Combination of ZGDHu-1 and fludarabine synergistically increases caspase-3 activity in CLL cells. Caspase activation, which is a key event in apoptosis and the downstream signaling pathway of the mitochondrial pathway, also plays an important role in fludarabine-induced cytotoxicity (24). Among the caspase family members, caspase-3 serves as an effector caspase, causing cleavage of a variety of proteins including polyADP-ribose polymerase (PARP), a well-known caspase substrate. Our previous study indicated that caspase-3 is involved in the apoptotic effect induced by ZGDHu-1 in CLL cells (12). In the present study, the minimal caspase-3 activation was observed with fludarabine (1 µg/ml) treatment (Fig. 7). When CLL cells were incubated with a combination of ZGDHu-1 and fludarabine, caspase-3 activation exhibited a 2-fold increase on day 3 compared to the CLL cells treated either with fludarabine or ZGDHu-1 alone (P<0.01, Fig. 7A). Furthermore, cleavage of PARP also exhibited a 2-fold increase on day 3 compared to that in the CLL cells treated either with fludarabine or ZGDHu-1 alone (Fig. 7B). When CLL cells were exposed to ZGDHu-1 or ZGDHu-1 + fludarabine in the presence of the broad spectrum caspase inhibitor Z-VAD-fmk, Z-VAD-fmk significantly blocked ZGDHu-1-induced caspase-3 activation as well as the cleavage of PARP (Fig. 7B). Moreover, Z-VAD-fmk pre-treatment also partially attenuated the ZGDHu-1-induced apoptotic effects on the CLL cells by PS externalization (Fig. 3A and B), but did not affect the ΔΨm or the Bcl-2/Bax ratio (data not shown). Subsequently, to evaluate the combined effect of ZGDHu-1 and fludarabine more precisely, the combination index (CI) was calculated where a CI <1 indicates a synergistic effect. Twenty-two patients in the ZGDHu-1 + fludarabine group of the 25 analyzed patients had a synergistic effect (Table II). In addition, CI =1 was noted in one patient and CI >1 in two patients, representing an additive or infra-additive effect, respectively. Overall, our data suggest that there is a synergistic effect between these two drugs.

ZGDHu-1-induced apoptosis rate of CLL cells is independent of ZAP-70 and CD38 expression. Traditionally, high CD38 expression, high ZAP-70 expression, immunoglobulin heavy chain genes (IgVH) gene and cytogenetic abnormalities (especially deletions of 11q and 17p) are all associated with the poor prognosis of CLL patients and can define patients who are in an aggressive status (4,5,25-27). To assess whether
the rate of ZGDHu-1-induced apoptosis is associated with the prognosis of CLL, the apoptotic parameters in ZAP-70high vs. ZAP-70low groups and CD38high vs. CD38low groups were compared. However, a significant difference in the

Table II. Combination indices (CIs) of 25 CLL patients treated with ZGDHu-1 and fludarabine.

<table>
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<th>Patient no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>CI</td>
<td>0.8</td>
<td>0.9</td>
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<td>0.5</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
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<td>0.9</td>
<td>1.0</td>
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<tr>
<th>Patient no.</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
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<tbody>
<tr>
<td>CI</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
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<td>0.7</td>
<td>0.6</td>
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<td>0.6</td>
<td>0.9</td>
<td>0.8</td>
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The CI calculated for the percentage of active caspase-3 expression in CD19+CD5+ cells on day 3 in ex vivo cultures with ZGDHu-1, fludarabine and the combination of ZGDHu-1 and fludarabine, separately. CI value = sum of the specific apoptosis of the single drug treatment/specific apoptosis of the combined treatment. The specific apoptosis percentage = (drug induced apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis) x 100%. CI <1, CI =1 and CI >1 are defined as synergistic, additive and infra-additive, respectively.
percentage of caspase-3-positive CLL cells cultured with ZGDHu-1 between the ZAP-70 \textsuperscript{high} and ZAP-70 \textsuperscript{low} groups was not found (Fig. 8B), also between the CD38 \textsuperscript{high} and CD38 \textsuperscript{low} patients (Fig. 8C). Moreover, the rate of apoptosis caused by ZGDHu-1 as a single agent or in combination with fludarabine in early (I-II) stage compared to advanced (III-IV) stages disease was assessed (Fig. 8E). In the present study, there was no significant difference between these groups. That is to say, the rate of ZGDHu-1-induced apoptosis was independent of these prognostic markers, such as lymphocytosis, LDH or the β2-microglobulin level (Fig. 8A and D).

Discussion

CLL is the most common leukemia in Western countries, characterized by the accumulation of malignant B lymphocytes following the failure to undergo apoptosis. Despite huge progress in treatment, it is still an incurable disease. Currently, purine analogs are widely used for the treatment of CLL and have achieved a higher remission rate (28,29). Fludarabine has been shown to have multiple functions such as interference with DNA synthesis and repair, apoptosis induction and cell cycle regulation in leukemia cells (30). However, the toxic effect of fludarabine such as severe opportunistic infections, myelosuppression and gastrointestinal toxicities including vomiting, nausea and hepatic lesions have been widely reported (6). Thus, reducing the fludarabine toxicity by lowering its dose and exploring new drug are desperately needed. In the present study, we highlight that the use of fludarabine in combination with ZGDHu-1 may reduce the fludarabine dose due to the synergistic effect of the two drugs. The combination of ZGDHu-1 and fludarabine may be useful for the maintenance therapy of CLL patients, as it can sensitize CLL cells to low-dose fludarabine without increasing the risk of long-term side effects on the immune system or other opportunistic infections.

ZGDHu-1, a potential proteasome inhibitor (14), showed significant cytotoxicity on malignant B lymphocytes isolated from CLL patients in a dose-dependent manner, but not on normal B lymphocytes of healthy controls. Moreover, ZGDHu-1 may induce the apoptosis of CLL cells by increasing the mitochondrial membrane permeability, production of ROS, activation of caspase-3 and a decrease in the Bcl-2/Bax ratio (15). Moreover, to the best of our knowledge, this is the first study to demonstrate that ZGDHu-1 may increase the percentage of apoptotic cells in combination with fludarabine and this synergistic effect of ZGDHu-1 with fludarabine was assessed based on the CI. Previous data demonstrated that ZGDHu-1 may induce the apoptosis of CLL cells through the mitochondrial pathway. Moreover, in the present study, the mitochondrial pathway also played an important role in the combination of ZGDHu-1 with fludarabine. As we know, Bcl-2 is regarded as a classical...
marker for the intrinsic apoptosis pathway and the major anti-apoptotic protein of the Bcl-2 family. Overexpression of Bcl-2 may inhibit the apoptosis partly by suppressing ROS generation or by inhibiting the mitochondrial permeability transition (13-15). Overall, compared to fludarabine, ZGDHu-1 had no side effects. Moreover, it had a significant synergistic effect with fludarabine to induce the apoptosis of CLL cells partly through the mitochondrial pathway (31).

As we know, caspase-3 can be cleaved into the 17- and 12-kDa subunits during cleavage and activation (32). In the present study, in CLL cells, ZGDHu-1 induced the complete cleavage of caspase-3 and this effect was inhibited by the caspase inhibitor Z-VAD-fmk. In addition, caspase-3 was activated partly through the intrinsic pathway. Our results revealed that caspase-3 activation may participate in the synergistic apoptotic combined effect of ZGDHu-1 and fludarabine.

Additionally, several prognostic markers, such as lymphocyte count, LDH elevation, β2-microglobulin, IgVH, ZAP-70 and CD38, are widely used for CLL patients (4,25). In the present study, we assessed whether the apoptosis rate caused
by ZGDHu-1 is different between CLL patients with favorable and unfavorable prognosis. In the present study, the rate of ZGDHu-1-induced apoptosis of CLL cells was independent of ZAP-70 or CD38 expression and the clinical Rai classification status. Furthermore, it did not correlate with lymphocytosis, LDH and β2-microglobulin. Overall, our data imply that ZGDHu-1 may be equally effective in CLL patients with both a favorable and poor prognosis.

In conclusion, the present study indicates that ZGDHu-1 may be used as a single agent or in combination with fludarabine for the treatment of CLL patients. One of the mechanisms of ZGDHu-1 synergism with fludarabine appears to be associated with the triggering of caspase-3 activation that makes CLL cells more susceptible to apoptosis. Thus, ZGDHu-1 combined with fludarabine is a promising treatment strategy for CLL patients.

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