GMZ-1 is a podophyllotoxin derivative that suppresses growth and induces apoptosis in adriamycin-resistant K562/A02 cells through modulation of MDR1 expression

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Abstract. The incidence of multidrug resistance (MDR) during cancer chemotherapy is a major challenge for treatment. With the aim of identifying drugs that are capable of targeting treatment-resistant cancer cells, the present study evaluated the efficacy of GMZ-1 in cancer chemotherapy using K562/A02, an MDR leukemia cell line. Cell viability and apoptosis were measured by MTT assay and flow cytometry/Giemsa staining, respectively. The expression levels of the MDR protein 1 (MDR1) gene transcript and protein in K562/A02 cells were determined by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. GMZ-1 suppressed the viability of various human cancer cell lines and induced apoptosis in the K562/A02 cell line in a time- and concentration-dependent manner. GMZ-1 toxicity may be associated with a decrease in MDR gene expression. These findings demonstrated that GMZ-1 may have efficacy as a potential antitumor drug to overcome leukemia cell resistance to apoptosis induced by chemotherapy.

Introduction

The majority of patients with cancer respond to initial chemotherapy (1); however, many patients subsequently relapse following this initial response. These patients are commonly characterized by the emergence of drug-resistant cells and consequent resistance to multiple anti-cancer agents, which may have various chemical structures and mechanisms of action (2). This phenomenon, defined as multidrug resistance (MDR), is a major cause of chemotherapy failure. There are several potential mechanisms of resistance; one involves elevated expression of membrane transporter proteins and, therefore, declined intracellular drug concentrations and cytotoxicity. Among these transporter proteins, MDR protein 1 (MDR1), encoded by the *MDR1* gene, has been associated with the resistance phenotype (3).

Various inhibitors of the drug efflux pump, including calcium channel blockers, anti-arrhythmics, antidepressants and antipsychotics, have been demonstrated to overcome drug resistance *in vitro* (4,5). However, a number of these were demonstrated to exhibit high toxicity in animal studies (6). Others that belong to the class of MDR modulators or chemosensitizers are less cytotoxic and are able to reverse MDR1-associated resistance (7).

Podophyllotoxin is an interesting lead in the development of anticancer antiviral agents. Toxicity issues and side effects cause its limited use. Etoposide (VP-16) and teniposide (VM-26), derivatives of podophyllotoxin, have been successfully used in combination chemotherapy. Cancers like small cell lung cancer, testicular cancer, acute leukaemia and malignant lymphoma responded to them well. However, these derivatives have not overcome limitations, such as narrow anticancer spectrum, low solvability and development of resistance. In addition, major side effects including gastroenteric reaction and leukopenia have restricted their usage. The present study designed and filtered a series of water soluble derivatives of podophyllotoxin. To the best of our knowledge, there has been no report on the role of podophyllotoxin or its analogues in MDR reversal, particularly in MDR leukemia K562/A02 cells. Therefore, a number of novel podophyllotoxin derivatives were synthesized and their cytotoxicity in K563/A02 cells was tested. The present study proposed that the novel derivative GMZ-1 may be an alternative to VP-16, a clinical anti-cancer agent (Fig. 1). In order to investigate this, the anti-proliferative capacity of GMZ-1 was assayed in a number of cancer cell lines; as GMZ-1 exhibited high

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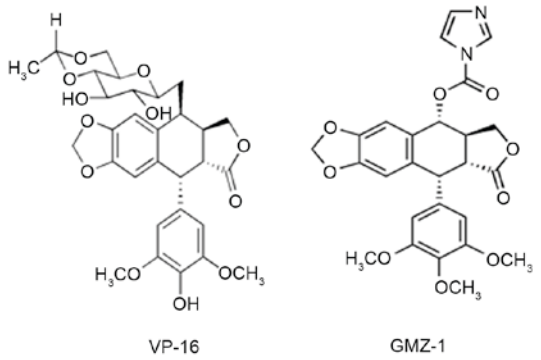

Figure 1. Chemical structures of VP-16 and GMZ-1.

toxicity towards K562, an MDR cell line, these cells were subsequently used to compare the effects of GMZ-1 and VP-16 *in vitro*. It was observed that GMZ-1 inhibited proliferation and induced apoptosis in K562/A02 cells in a time- and concentration-dependent manner. The present study additionally investigated the underlying mechanism of the anticancer activity of GMZ.

Materials and methods

Cell lines and culture. The K562 cell line (courtesy of Professor Hong Chen, Logistics University of Chinese People's Armed Police Forces, Tianjin, China) was a clone from human chronic myelogenous leukemia, previously established by alternate passages in nude mice and *in vitro* culture. HeLa, A549, MCF-7, HepG2, SKOV3, BGC-823, MGC-803 and the fibroblast cell line (3T3) were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). K562 was cultured in RPMI-1640 (catalog no. 31800-022) supplemented with 10% fetal bovine serum (catalog no. 10099141) (both from Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified 5% CO_2 atmosphere. The MDR leukemia cell line K562/A02 (courtesy of Professor Hong Chen also) was generated previously by incremental adriamycin (ADM) treatments refer to Yang's *et al* paper published in 1995 (1). K562/A02 was maintained in RPMI-1640 medium supplemented with 1 μg/ml ADM to maintain its MDR phenotype.

Cell viability measurement. In order to evaluate the anti-proliferative activity of GMZ-1 [molecular weight 508.15, white powder, insoluble in water, purity >98%, supplied by Professor Hong Chen (Tianjin Key Laboratory of Cardiovascular Remodeling and Target Organ Injury, Tianjin, China). GMZ-1 was synthesized with imidazole-2-carboxy to generate podophyllolox imidazole-2-carboxylate] in cancer cell lines, cell viability was measured by determining mitochondrial dehydrogenase activity using an MTT assay. Cells ($5x10^3$ cells/well) were plated in 96-well plates and cultured overnight. Triplicate wells were treated with concentrations of GMZ-1 (10, 1, 0.1 and 0.01 μmol/l) and VP-16 (10, 1, 0.1 and 0.01 μmol/l) (cat no. H32025583; prepared with normal saline; Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) or normal saline (control vehicle) for 48 h. To perform the MTT assay, 20 μl MTT solution (5 mg/ml in PBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well, followed by incubation for 4 h at 37˚C. A total of 150 μl/well dimethyl sulfoxide was added at room temperature for 10 min to dissolve the formazan precipitate. Absorbance was measured at a wavelength of 570 nm (Thermo Fisher Scientific, Inc.).

Flow cytometry analysis. K562/A02 cells ($5x10^3$ cells/well) were seeded in 6-well plates and cultured overnight. Triplicate wells were treated with 0.05, 0.10 or 0.20 μM GMZ-1 and 10 μM VP-16 or normal saline (vehicle control) for 12, 24 and 36 h. Cells were collected and fixed in 70% ethyl alcohol at 4˚C overnight, followed by washing in PBS and incubation with 10 μg/ml RNA se at 37˚C for 30 min. Cells were subsequently incubated with 10 μg/ml propidium iodide (PI) for 30 min in the dark on ice. The stained samples were analysed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Data was analysed using FlowJo version 7.6.3 software (FlowJo LLC, Ashland, OR, USA).

Giemsa staining. K562/A02 cells were treated with varying concentrations of GMZ-1 or normal saline (control vehicle) for 48 h, lifted from the plate and mounted on slides. Following rinsing with water, the slides were stained with Giemsa solution (BDH; Merck KGaA) for 5 min at room temperature. The slides were rinsed with water three times and the cells were observed under an inverted microscope (TMS; Nikon Corporation, Tokyo, Japan) at x400 magnification.

Examination of MDR1 gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from K562 or K562/A02 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and RT-qPCR was performed using a SuperScript One-Step RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) in a 50 μl reaction mix. The reaction mix contained 25 μl 2X RT-PCR buffer, 3 μl template RNA, 0.6 μl forward and reverse primers each (β-actin 540 bp, sense, 5'-GTGGGGCGCCCC AGGCACCA-3' and antisense, 5'-CTTCCTTAATGTCAC GCACGATTTC-3'; MDR-1, 150 bp, sense, 5'-GTGGGGCGC CCCAGGCACCA-3' and antisense, 5'-CTTCCTTAATGT CACGCACGATTTC-3'), 1 μl AMV/Taq mixture and 19.8 μl deionised water. The thermocycling reaction protocol was as follows: Reverse transcription for 35 min at 37˚C; pre-denaturation at 94˚C for 3 min; 30 cycles of qPCR (1 min denaturation at 94˚C, 30 sec annealing at 57˚C and 1 min extension at 72˚C); and 10 min final extension at 72˚C. PCR products were run on 1.5% agarose gels with 0.01% Gel Red (cat no. G5560; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), with β-actin (540 bp) as an internal standard. Band intensity was quantified using Gel-Pro Analyser 3.1 (Media Cybernetics, Inc., Rockville, MD, USA).

Examination of MDR1 protein expression by western blot analysis. Cells were lysed using rdioimmunoprecipitation acid lysis buffer (cat no. P0013B; Beyotime Institute of Biotechnology, Haimen, China) and the extracted protein was quantified with a bicinchoninic protein assay kit (cat no. P0010; Beyotime Institute of Biotechnology). A total of 30 μg/well cell extracts were separated by Bolt™ 12% Bis-Tris Plus 10-well gels (cat no. NW00120BOX; Thermo Fisher Scientific,

Table I. Cytotoxic activity of GMZ-1 on human cancer cells and fibroblasts.

	IC_{50} (μM)	
Cell line	GMZ-1	VP-16
HeLa	0.07±0.01	1.33±0.86
A549	0.18±0.07	1.06±0.73
MCF-7	0.14±0.05	2.36±0.53
HepG-2	0.093±0.012	2.03±0.55
SKOV3	0.12±0.04	3.43±0.87
BGC-823	0.083±0.009	2.06±0.59
MGC-803	0.089±0.011	3.61±0.85
3T3	0.34±0.07	17.36±2.29

Values are presented as the mean ± standard deviation (n=3). IC_{50}, half-maximal inhibitory concentration; VP-16, etoposide.

Table II. Reversion of drug resistance in K562/A02 cells.

	IC_{50} (μM)		
Drug	K562	K562/A02	Fold change
Adriamycin	0.26±0.10	28.62±4.27	110.08
VP-16	2.02±0.83	22.81±4.23	11.29
GMZ-1	0.08±0.02	0.12±0.03	1.52

Values are presented as the mean ± standard deviation (n=3). IC_{50}, half-maximal inhibitory concentration; VP-16, etoposide.

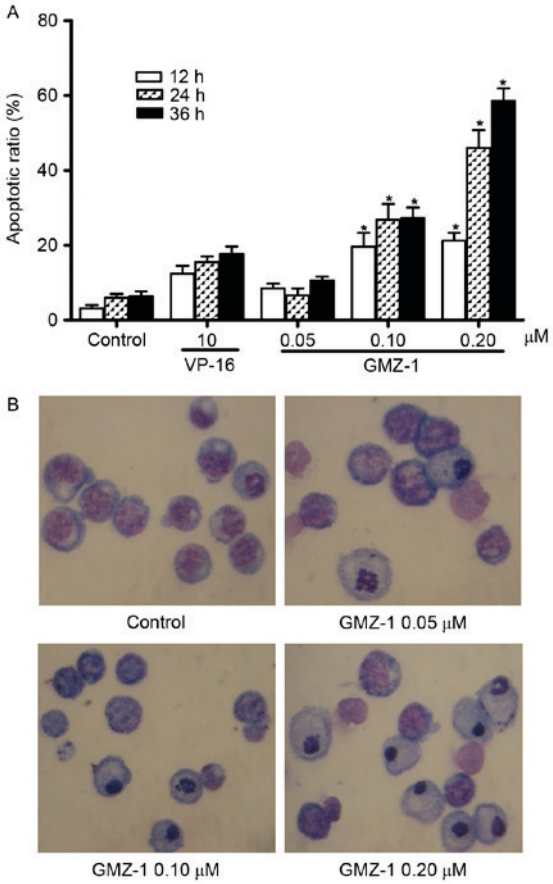

Figure 2. GMZ-1 induces apoptosis in K562/A02 cell line. (A) Flow cytometric analysis of K562/A02 cells treated with GMZ-1 or VP-16 for 12-36 h. Apoptosis was assessed by propidium iodide staining and flow cytometry. *P<0.05 vs. respective control. (B) Morphology of K562/A02 cells treated with increasing concentrations of GMZ-1. K562/A02 cells were treated with vehicle or different concentrations of GMZ-1 for 48 h and stained with Giemsa. Nuclear condensation, cytoplasmic shrinkage and the formation of apoptotic bodies were visible in GMZ-1 treated cells. Representative images of three independent experiments are presented. Magnification, x400.

Inc.), and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4˚C with MDR1/ABCB1 (E1Y7B) rabbit monoclonal antibody (cat no. 13342; Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:1,000 dilution or GAPDH (D16H11) XP® rabbit monoclonal antibody (cat no. 5174; Cell Signaling Technology, Inc.) at 1:1,000 dilution. Following washing, the membranes were incubated with a horse radish peroxidase-conjugated secondary antibody, anti-rabbit IgG, horse radish peroxidase-conjugated antibody (cat no. 7074; Cell Signaling Technology, Inc.) at 1:3,000 dilution at room temperature for 1 h and visualized using SuperSignal™ West Pico Plus Chemiluminescent substrate (cat no. 34580; Thermo Fisher Scientific, Inc.).

Statistics. All data are presented as the mean ± standard error. Differences between groups were analysed using a one-way analysis of variance followed by Student-Newman-Keuls and Least Significant Difference post hoc tests using SPSS version 20 software (IBM Corp., Armonk, NY, USA). P≤0.05 was considered to indicate a statistically significant difference.

Results

GMZ-1 reduces cancer cell viability. GMZ-1 demonstrated a marked effect on the viability of several cancer cell lines, and the half-maximal inhibitory concentration (IC_{50}) values following treatment for 48 h are presented in Table I. GMZ-1 displayed the highest efficacy in K562 and K562/A02 cells, with IC_{50} values of 0.08±0.02 and 0.12±0.03 μM 48 h following treatment, respectively (Table II). Therefore, the K562/A02 cell line was selected as a model to examine the impact of GMZ-1 on cell viability.

GMZ-1 induces apoptosis in K562/A02 cells. A number of anti-cancer drugs impact upon apoptosis-associated signaling pathways to induce apoptosis in cancer cells. In order to examine whether the reduced viability of K562/A02 cells was due to the induction of apoptosis, flow cytometry analysis of PI-stained cells was performed. K562/A02 cells were treated with 0.05, 0.10 or 0.20 μM GMZ-1 for 12, 24 or 36 h. The flow cytometry results indicated that GMZ-1 may induce apoptosis in K562/A02 cells in a time- and concentration-dependent manner (Fig. 2A). Quantification revealed a significant

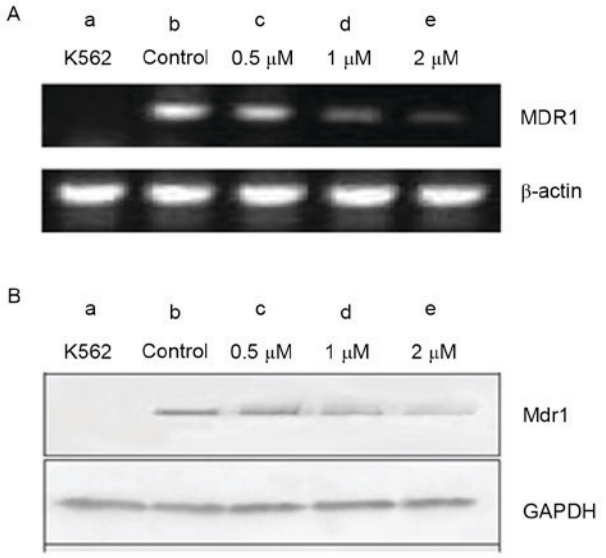

Figure 3. Quantification of MDR1 expression in GMZ-1-treated K562 and K562/A02 cells. (A) The level of *MDR1* mRNA in K562 and K562/A02 treated with various concentrations of GMZ-1 was determined using reverse transcription-quantitative polymerase chain reaction analysis. The expression of *MDR1* mRNA in (a) K562 cells and K562/A02 cells treated with (b) 0.1% DMSO, (c) 0.05 μM GMZ-1, (d) 0.1 μM GMZ-1 or (e) 0.2 μM GMZ-1 is presented. β-actin mRNA served as the loading control. (B) Expression of MDR1 protein in K562 and K562/A02 cells treated with various concentrations of GMZ-1, determined by western blotting. The expression of MDR1 protein in (a) K562 cells and K562/A02 cells treated with (b) 0.1% DMSO, (c) 0.05 μM GMZ-1, (d) 0.1 μM GMZ-1 or (e) 0.2 μM GMZ-1 is presented. GAPDH served as the loading control. MDR1, multidrug resistance protein 1; DMSO, dimethyl sulfoxide.

difference in the apoptotic rate between control cells and cells treated with GMZ-1 ($P<0.05$; Fig. 2A).

GMZ-1 treated K562/A02 cells were stained with Giemsa solution to observe whether GMZ-1 induced the characteristic morphology of apoptosis. The observed morphology in GMZ-1-treated K562/A02 cells included nuclear condensation, cytoplasmic shrinkage and the formation of apoptotic bodies (Fig. 2B), which were absent in the control cells. Consistent with the MTT assay, these results indicated that GMZ-1 may reduce the viability of K562/A02 cells via the induction of apoptosis.

Expression analysis of the MDR1 gene and MDR1 protein in K562 and K562/A02 cells. The *MDR1* gene, encoding MDR1, is associated with MDR. The present study examined MDR1 expression at the mRNA and protein levels in K562 and K562/A02 cells, using RT-qPCR and western blot analyses. Cells were treated with various concentrations of GMZ-1 (0.05, 0.1 and 0.2 μM) for 24 h. A marked increase in *MDR1* gene and MDR1 protein expression was observed in K562/A02 cells, compared with K562 cells (Fig. 3). Notably, *MDR1* and MDR1 expression decreased with an increasing concentration of GMZ-1, which suggested that K562 was an MDR1-negative cell line and K562/A02 was an MDR1-overexpressing cell line.

Discussion

Although novel chemotherapeutic drugs are being developed, chemotherapy remains a challenge in cancer treatment. This is partially due to the development of MDR. Among the numerous mechanisms underlying MDR, elevated expression of the *MDR1*-encoded MDR1 protein in cancer cells has been considered to be a frequent factor (8,9). MDR1 serves to remove the drug from the cells, thereby assisting in drug resistance. The weak potency and toxicity of developed MDR modulators have limited their clinical use. The few non-toxic compounds that downregulate the expression of MDR1 include curcumin (10), tryptanthrin (11), estrogen (12) and perospirone (13).

VP-16, an aryltetralinelignan, is a clinical antitumor drug used to treat testicular cancer and small cell lung cancer (14,15). VP-16 elicits a few adverse effects, including myelosuppression and the initiation of secondary leukaemia (14,16-18). In order to reduce damage to bone marrow cells, VP-16 has been combined with other compounds in animal studies, such as quercetin, dexrazoxane and wongonin (19-21).

The present study demonstrated that the novel podophyllotoxin derivative GMZ-1 exhibited increased efficacy compared with a traditional podophyllotoxin derivative (22). GMZ-1 has a similar IC_{50} value between MDR1-negative cell line K562 and overexpressing cell line K562/A02; however, it exhibits decreased cytotoxicity in human fibroblasts at therapeutic doses. Apoptosis is characterized by specific morphological changes including plasma membrane blebbing, chromatin condensation and fragmentation, and the emergence of apoptotic bodies. The results of the present study suggested that GMZ-1 may induce apoptosis in K562/A02 *in vitro* and significantly decrease MDR1 expression from 24 h. To the best of our knowledge, this is the first report demonstrating the suppressive effect of GMZ-1 on MDR1 expression in K562/A02 cells.

In conclusion, GMZ-1, as a novel derivative of podophyllotoxin, may have utility as an MDR modulator in adriamycin-resistant K562/A02 cells. It may serve as an alternative to the current treatment for treating patients with MDR1-overexpressing tumors. Further work is required to validate this drug, and to investigate whether GMZ-1 inhibits the functions of other ATP binding cassette transporters.

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