

CHINESE MEDICAL SCIENCES JOURNAL

ORIGINAL ARTICLE

ZM-66, a New Podophyllotoxin Derivative Inhibits Proliferation and Induces Apoptosis in K562/ADM Cells[△]

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Key words: ZM-66; podophyllotoxin; multidrug resistance; P-glycoprotein; apoptosis

Objective To investigate the anti-tumor effect of ZM-66 on multidrug-resistant leukemic cell line K562/ADM.

Methods The K562/ADM cells were treated with varying concentrations (0, 1, 2, 4×10^{-3} mmol/L) of ZM-66 or etoposide for 24 hours. The proliferation was detected by Sulforhodamine B Sodium Salt (SRB) assay and apoptosis was detected by flow cytometry analysis and fluorescent staining. In addition, the expression levels of p53 and bax genes in K562/ADM cells were detected by RT-PCR analysis. The level of P-glycoprotein (P-gp), P53 and Bax protein in K562/ADM cells were detected by Western blot assay.

Results SRB assay demonstrated that etoposide had little inhibitory effect on K562/ADM cells, whereas ZM-66 (1, 2, 4×10^{-3} mmol/L) had significantly inhibitory effect on K562/ADM cells (all $P < 0.01$). The acridine orange/propidium iodide dual staining showed that there were typical condensation of chromatin and nuclear fragmentation nuclei with red color in ZM-66 treated cells. Flow cytometric analysis showed that there was a significantly increase of apoptotic cells in K562/ADM cells after treated with ZM-66. RT-PCR showed that the p53 and bax mRNA expression levels in K562/ADM cells treated with ZM-66 at 1, 2, 4×10^{-3} mmol/L were higher than those in the cell without treatment. Western blot showed that the P53 and Bax protein expression levels in K562/ADM cells treated with ZM-66 at 2, 4×10^{-3} mmol/L were higher than those in the cell without treatment. But the P-gp protein expression level in K562/ADM cells treated with ZM-66 at 2, 4×10^{-3} mmol/L was gradually lower than those in the cell without treatment.

Conclusion ZM-66 is able to induce cell death by apoptosis *in vitro*, as a result of the reverse of the

apoptosis resistance in drug-resistant K562/ADM cells by modulating expression of key factors associated with apoptosis induction.

Chin Med Sci J 2014; 29(3):174-179

MULTIPLE drug resistance (MDR) is often characterized by a decrease in the intracellular accumulation of anticancer agents and over-expression of P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1) which belong to the superfamily of ATP-binding cassette transporters. These transporters are involved in ATP-dependent efflux of the cytotoxic agents from the cells.¹ P-gp located in the human chromosome 7q21-23 is a 170 kDa glycoprotein encoded by MDR1 gene and acts as an energy dependent drug-efflux pump in plasma membrane of cells. Here, it can pump chemotherapeutics out of leukemic cells and decrease intracellular drug concentrations which result in resistance.²⁻⁴ P-gp overexpression appears to be closely correlated with poor prognosis for cancers. Therefore, a successful inhibition of the P-gp transporter function or its expression may increase the intracellular accumulation of anticancer drugs and therefore overcome MDR. It has been verified that a variety of compounds can inhibit drug efflux from the cells.⁵ But most have little or no therapeutic potential because of the high toxicity *in vivo* at the doses required to reverse MDR. Extensive structure modifications have been carried out on podophyllotoxin to reduce its high toxicity. Two less toxic semisynthetic podophyllotoxin analogues, etoposide and teniposide have been used for the treatment of a broad spectrum of tumors. However, their intrinsic shortcomings, such as MDR and toxicity, mean that they are not suitable for clinical treatment of cancer. ZM-66, a new podophyllotoxin derivative synthesized by Chen Hong laboratory, has the advantages of effectiveness and stability. In this study, we investigated the anti-proliferative and apoptosis-inducing effects of ZM-66 to resist human leukemic K562/ADM cells, and discussed the possible mechanisms.

MATERIALS AND METHODS

Cell lines and reagents

Adriamycin-selected and P-gp positive multidrug resistant chronic myelogenous leukemia K562/ADM cell line and K562 cells were obtained from Institute of Hematology of Chinese Academy of Medical Sciences. They were cultured in the RPMI-1640 medium, with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, in humidified air at 37°C with 5% CO₂. To maintain drug resistance of K562/ADM cells, adriamycin was supplemented at regular intervals, but was omitted 2 weeks before any

experiment.

ZM-66 (Fig. 1) was dissolved in dimethyl sulfoxide. The control cells were only treated with the same amount of vehicle. The final dimethyl sulfoxide concentration never exceeded 0.1% (v/v), in either control or treated samples.

Cell viability assay

K562 and K562/ADM cells were plated in triplicate in 96-well plates with 5000 cells/well. After overnight incubation, triplicate wells were treated with varying concentrations (0, 1, 2, 4×10⁻³ mmol/L) of ZM-66 and etoposide (Jiangsu Hengrui Medicine Co., Ltd., China) respectively for 24 hours. The effects on cell growth were examined by the Sulforhodamine B Sodium Salt (SRB) cytotoxicity assay. In brief, cells were fixed with 80% trichloroacetic acid solution for 1 hour, and 0.4% SRB (Sigma Chemical Co., St. Louis, MO, USA) was added to each well. After 10 minutes incubation, the plates were washed, and dye was dissolved in 10 mmol/L buffer (pH=10.5) and the absorbance was measured by an Enspire 2300 Multilabel Reader (Perkin Elmer, MA, USA) at a wavelength of 570 nm.

Fluorescent staining of nuclei for K562/ADM cells

K562/ADM cells from exponentially growing cultures were seeded within 24-well culture plates. The cells were treated with ZM-66 (4×10⁻³ mmol/L) or without ZM-66 for 24 hours. After treatment, cells were washed with PBS and fixed in MeOH-HAc (3:1, v/v) for 10 minutes at 4°C. Cells were then stained with Hoechst 33342 (5 µg/ml in PBS) for 10 minutes at room temperature, and examined in a fluorescent microscope (Olympus XSZ-D2, Japan) with an excitation wavelength of 365 nm.⁶

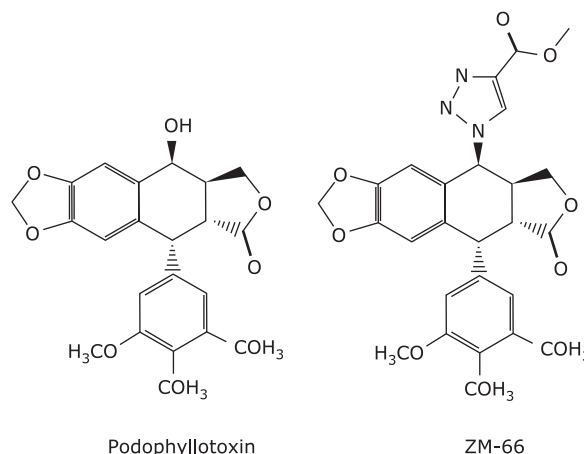


Figure 1. Chemical structure of podophyllotoxin and ZM-66.

Fluorescent staining for K562/ADM cells

Cells from exponentially growing cultures were seeded within 24-well culture plates. The cells were treated with ZM-66 (4×10^{-3} mmol/L) or without ZM-66 (control) for 24 hours. After treatment, cells were washed with PBS, and stained with acridine orange (AO, 10 μ g/ml in PBS, Sigma Chemical Co.) and propidium iodide (PI, 10 μ g/ml in PBS, Sigma Chemical Co.) for 10 minutes at 37°C. Cells were then washed with PBS, and examined in a fluorescent microscope (Olympus XSZ-D2) with an excitation wavelength of 490 nm.⁷

Flow cytometry assay for K562/ADM cells

K562/ADM cells (1×10^6) were incubated at 37°C with different concentrations (0, 2, 4×10^{-3} mmol/L) of ZM-66 for 12 or 24 hours. Cells were harvested and resuspended in PBS, incubated with 10 μ g/ml RNase A (Sigma Chemical Co.), and stained with 25 μ g/ml PI. The number of apoptotic cells was measured by a Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Cells with DNA content less than G_1 in the cell cycle distribution were counted as hypodiploid cells.

Analysis of expression levels of p53 and bax mRNA by RT-PCR

Eppendorf tubes and tips required for RT-PCR were soaked in 0.1% diethylenetriamine solution (Sigma Chemical Co.) for 24 hours, dried and sterilized for use. Procedure of RNA extraction was provided by manufacturer of TRIzol reagent (Shanghai Shengong Bioengineering Co., Ltd., China). Cells were collected and pipetted in TRIzol reagent for full lysis. Following 5 minutes of incubation at 15°C–30°C, chloroform (0.2 ml/ml TRIzol) was added which mixed completely with the lysate after vibration for 15 seconds. The lysate was then incubated at 15°C–30°C for 2–3 minutes, and subjected to centrifugation at 10 000 $\times g$ for 15 minutes at 2°C–8°C. The water phase supernatant was transferred to another clean tube, incubated with isopropyl alcohol for 10 minutes at 15°C–30°C, and was centrifuged at 10 000 $\times g$ for 10 minutes at 2°C–8°C. The RNA pellet was washed with 75% ethanol by centrifugation at 7500 $\times g$ for 5 minutes at 2°C–8°C. After drying at room temperature, the RNA pellet was dissolved in RNase-free water and incubated at 55°C for 10 minutes.

A total of 500 ng of RNA was transcribed into cDNA which was amplified using the following primers: sense p53 (5'-TCTGGGACAGCCAAGTCTGT-3') and antisense p53 (5'-GGAGTCTTCCAGTGTGATGA-3'); sense bax (5'-CGTCC-ACCAAGAAGCTGAGCG-3') and antisense bax (5'-AGCACTCCGCCACAAAGATG-3'). RT-PCR was performed in a 50 μ l

reaction system (2 $\times$ RT-PCR buffer 25 μ l, template RNA 3 μ l, primer 0.6 μ l each, AMV/Taq mixture 1 μ l, and sterilized deionised water 19.8 μ l) according to the following steps: reverse transcription at 37°C for 35 minutes, pre-denaturation at 94°C for 3 minutes, 30 cycles of PCR (denaturation at 94°C for 1 minute, anneal at 57°C for 30 seconds, and extension at 72°C for 1 minute), and extension at 72°C for 10 minutes. The amplification product was then loaded on 1.5% agarose gel containing 0.3 mg/L ethidium bromide to perform electrophoresis, with β -actin (540 bp) as an internal standard. The band intensity was determined from scanned images by using Gel-Pro Analyser 3.1 Automated Digitising System software (Media Cybernetics, USA). RNA amounts were normalized against the β -actin mRNA level.

Analysis of expression levels of P53, Bax and P-gp protein by Western blot

The total protein extracted from K562/ADM cell were equally loaded onto 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, USA). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBST) and incubated overnight with anti-P53, anti-Bax, and anti-P-gp (Beijing Biosynthesis Biotechnology Co. Ltd., China). The membranes were then washed with PBST and incubated for 1 hour with a secondary antibody and chemiluminescent horseradish peroxidase substrate (Thermo, USA).

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All experiments were repeated at least three times, and quantitative data were presented as mean $\pm$ SD. Statistical significance between the two groups was determined by homogeneity test of variances and Student's *t*-test. $P < 0.01$ was considered statistically significant.

RESULTS

Effect of ZM-66 on the proliferation of K562 and K562/ADM cells

SRB assay demonstrated that the cell viability was decreased along with the increase of ZM-66 and etoposide treated dose in K562 cells, but no significantly difference between two groups was observed ($P = 0.318$). However, compared with etoposide which had little inhibitory effect on K562/ADM cells at each concentration, ZM-66 had significantly inhibitory effect on K562/ADM cells ($P < 0.01$, Fig. 2), and no cross resistance to ZM-66 was observed.

Effect of ZM-66 on the apoptosis of K562/ADM cells

In the control group, the nuclei of K562/ADM cells were slightly stained, but no apoptotic nuclei were observed. In contrast, ZM-66 treated cells showed typical apoptosis morphology characterized by chromatin condensation and nuclear fragmentation (Fig. 3A-B). The result of AO/PI staining showed that, compared with the control group, there were typical condensation of chromatin and nuclear fragmentation nuclei with red color in ZM-66 treated cells (Fig. 3C-D).

As shown in Fig. 4, compared with cells untreated with ZM-66, there was just a slight increase of hypodiploid cells in K562/ADM cells after treated with ZM-66 for 12 hours (19.2%), but the proportion of hypodiploid cells significantly increased at 24 hours (69.3%). The treatment resulted in a hypodiploid peak (A_0 region) which probably due to the presence of apoptotic cells.

Effect of ZM-66 on the expression of p53, bax, and P-gp mRNA and protein in K562/ADM cells

The expression bands of p53 and bax mRNA and protein in K562/ADM cells treated with ZM-66 at 0, 1, 2, 4×10^{-3} mmol/L

for 24 hours were visible and became more thicker along with the increased concentration (Fig. 5A-B). But the P-gp protein expression level in K562/ADM cells treated with ZM-66 at 0, 1, 2, 4×10^{-3} mmol/L for 24 hours were gradually lower along with the increased concentration (Fig. 5C).

DISCUSSION

Human K562/ADM, one of multidrug-resistant leukemic cell line, is unable to respond to adriamycin, to undergo apoptosis and recover their sensitivity to adriamycin, daunorubicin, and etoposide.⁸ The use of cytotoxic agents is often accompanied by development of MDR tumor phenotype. A major determinant of MDR is the overexpression of drug efflux pumps, namely, the P-gp and MRP1. The overexpression of P-gp was shown to be resistant against various clinically relevant compounds, including anthracyclines and vinca alkaloids. Typical P-gp modulators, such as calcium channel blockers, anti-arrhythmics, antidepressants, antipsychotics, and others reverse MDR by competitively binding to P-gp. However, most of these compounds have had disappointing results in clinical treatment because of their dose-limiting

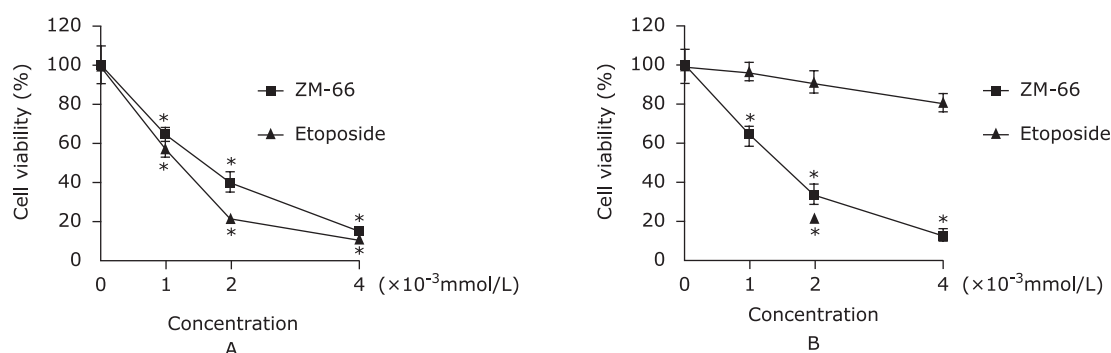


Figure 2. Effects of ZM-66 and etoposide on cell viability in K562 (A) and K562/ADM (B) cells.

Cells were exposed to the drugs for 24 hours and cell viability was determined by Sulforhodamine B Sodium Salt assay. Results were derived from three independent experiments.

* $P < 0.01$ compared with initial concentration of drug.

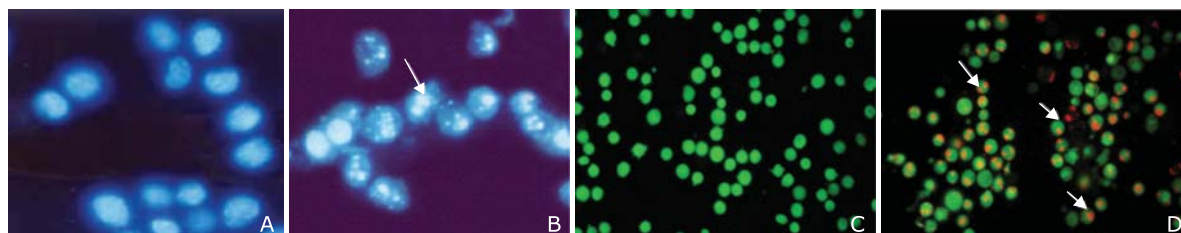


Figure 3. Morphological observation of K562/ADM cells under a fluorescent microscope.

A. Untreated cells were stained with Hoechst 33342 method.

B. ZM-66 treated cells were stained with Hoechst 33342 method. Chromatin condensation and nuclear fragmentation could be observed (arrow).

C. Untreated cells were stained with acridine orange/propidium iodide (AO/PI) method.

D. ZM-66 treated cells were stained with AO/PI method. The apoptotic cells exhibited red condensed chromatin or fragmented chromatin (arrows).

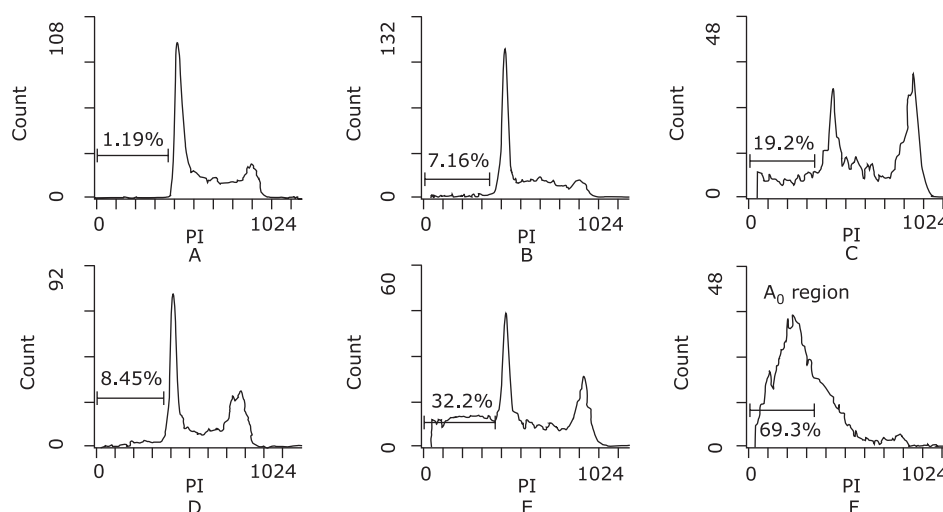


Figure 4. Flow cytometric analysis results of K562/ADM cells.

A, B, C. K562/ADM cells incubated with 0 (A), 2×10^{-3} (B), 4×10^{-3} mmol/L (C) ZM-66 for 12 hours;

C, D, E. K562/ADM cells incubated with 0 (A), 2×10^{-3} (B), 4×10^{-3} mmol/L (C) ZM-66 for 24 hours.

The horizontal axis: total number of cells; the vertical axis: number of apoptotic cells.

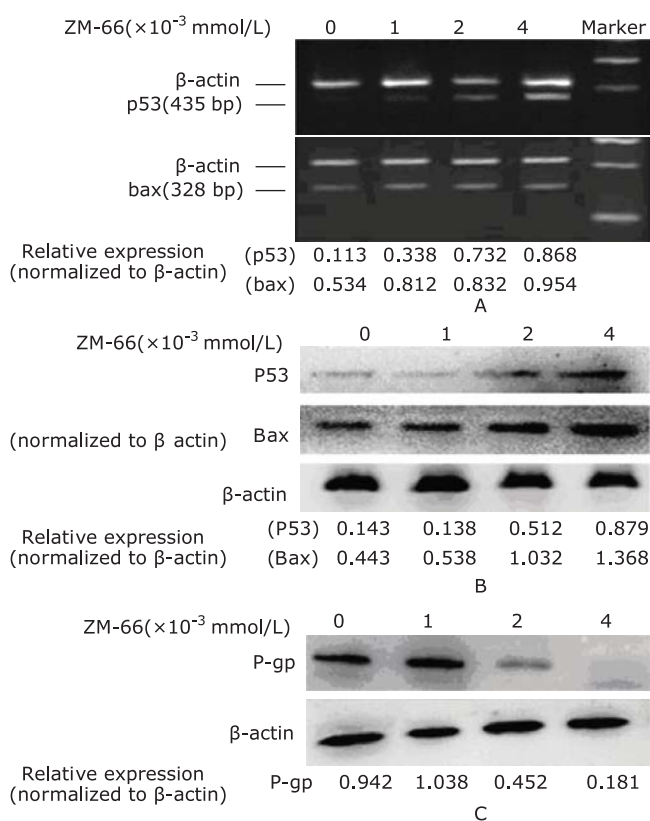


Figure 5. Effects of ZM-66 on p53, bax mRNA and P53, Bax, P-glycoprotein (P-gp) protein expressions in K562/ADM cells.

A. RT-PCR analysis of p53 and bax mRNA expressions in K562/ADM cells incubated with ZM-66 for 24 hours;

B. Western blot analysis of P53 and Bax protein expressions in K562/ADM cells incubated with ZM-66 for 24 hours;

C. Western blot analysis of P-gp protein expression in K562/ADM cells incubated with ZM-66 for 24 hours.

toxicity.⁹⁻¹¹ Therefore, we designed and synthesized a series of novel derivatives of the podophyllotoxin that aim directly at P-gp. In our study, the novel derivative, ZM-66, showed significant anti-tumor activity against K562 cells and K562/ADM cells. Furthermore, we found the K562/ADM cell line rendered resistant towards etoposide but not towards ZM-66.

K562/ADM cells treated with ZM-66 for 24 hours were found to have hyper-condensed nuclei which were brightly stained by Hoechst 33342. As is known, apoptosis is a process of cell suicide, characterized by specific morphological changes such as condensation of chromatin, blebbing of the plasma membrane, the appearance of apoptosis bodies, and biochemical properties involving fragmentation of chromatin. These results suggested that ZM-66 could induce K562/ADM cells apoptosis *in vitro*.

It has been widely accepted that apoptosis is an active gene-directed cellular suicide mechanism and many human genes contribute to its regulation, such as p53 gene, c-myc, bcl-2 gene family.¹² Among these genes, p53 plays a pivotal role in cellular responses to DNA damage, stimulating the expression of P21, which impedes cell cycle progression at G1/S check point. Subsequently, the cell fate can be decided, depending on the extent of DNA damage. A slight damage can be repaired by DNA repair mechanism. However, an extensive damage is unable to be repaired, and will ultimately initiate signal cascade leading to apoptosis.¹³ P53 is known to induce apoptosis by activating the expression of downstream target genes such as bax.¹⁴ In our study, we observed that ZM-66 could up-regulate p53 gene and bax gene. Meanwhile, the expression of P53 and Bax protein were up-regulated in

K562/ADM cells, the results showed that ZM-66 could induce P53-dependent apoptosis.

To further characterize the effects of ZM-66 treatment on multidrug-resistant leukemia cells, we examined the level of expression of P-gp by Western blot analysis. We found that P-gp expression was decreased after the treatment. The finding revealed that ZM-66 could increase the accumulation of itself in the MDR cells by inhibiting expression of P-gp. We speculated that the P-gp might be an appropriate target for the compound to produce a marked anti-MDR effect. Some observations from the present study suggest that etoposide was demonstrated to be a substrate of human P-gp, and one of the important mechanisms of its resistance is an overexpression of P-gp in cancer cells.¹⁵⁻¹⁷ A key report shows that P-gp significantly affect the pharmacokinetics of etoposide and/or etoposide glucuronide.¹⁸ Therefore, the result which P-gp expression was decreased revealed that the sensibility of etoposide to K562/ADM cells may be increased after ZM-66 treatment.

In conclusion, the present study demonstrated that ZM-66 directly interacts with the transcription of anti-apoptotic genes and reverses the expression of P-gp in K562/ADM cells, which in turn may contribute to the induction of apoptosis and tumor cell growth inhibition. This result indicated that ZM-66 can play a positive role against P-gp-overexpressing cells. However, further studies are needed to explore more detailed anti-tumor activity and mechanisms of ZM-66.

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