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Antimultidrug-resistant effect and mechanism of a novel CA-4 analogue MZ3 on leukemia cells

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A major issue in the treatment of leukemia is resistance to chemotherapeutic drugs. The most common mechanism encountered in the laboratory is the increased efflux of hydrophobic cytotoxic drugs that is mediated by a family of energy-dependent transporters. Besides, resistance to apoptosis can also cause failure in the treatment of leukemia. Recently, we have introduced 4-(4-bromophenyl)-2,3-dihydro-*N*,3-bis(3,4,5-trimethoxyphenyl)-2-oxoidmidazole-1-carboxamide (MZ3) as a novel synthesized combretastatin A-4 analogue which is a potent and specific compound against leukemia cells both *in vitro* and *in vivo*. Aim of this study was to evaluate the effect of MZ3 on multidrug-resistant (MDR) cancer cells of leukemia, and explore the antimultidrug-resistant mechanisms. Here, we observed that the MDR leukemia cell models investigated, overexpressing MDR1 (P-gp), were hypersensitive against MZ3. Parental K562, HL60 cells and MDR1-overexpressing K562R, HL60R cells were employed in this study. MZ3 hypersensitivity was confirmed to be based on great apoptosis induction and cell cycle arrest at unaltered intracellular drug accumulation. Cell proliferation assay demonstrated that, compared with HL60 and K562 cells, HL60R and K562R cells exhibited 1.3-fold and 2.4-fold resistance to MZ3, showing 26.9-fold and 92.2-fold resistance to daunorubicin (DNR) respectively. Moreover, real-time RT-PCR result showed that MZ3 impacted the transcription of MDR1 gene and western blotting results indicated that MZ3 can activate apoptosis on MDR cells by downregulating the anti-apoptotic protein XIAP levels and inducing the decrease in the phosphorylation state of ERK. Summarizing, our data demonstrate that MZ3 can inhibit the MDR function of leukemia cells, and it exerts the effect through altering the transcription of MDR1 genes and downregulating the anti-apopotic protein levels. MZ3 may be a potential candidate for further research and development in anti-MDR territory.

1. Introduction

Resistance to chemotherapeutic drugs is a major issue in the treatment of acute leukemia (Steinbach et al. 2007). Some patients fail to respond to chemotherapy and others relapse with resistant disease. Frequently, tumor cells exhibit resistance not only against a single class of drugs but against diverse chemotherapeutics, even with unrelated modes of action. This phenomenon, known as multidrug resistance (MDR), is based on three major mechanisms in cells (Szakacs et al. 2006): first, decreased uptake of water-soluble drugs such as folate antagonists, nucleoside analogues and cisplatin, which require transporters to enter cells; second, various changes in cells that affect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis and altered metabolism of drugs; and third, increased energy-dependent efflux of hydrophobic drugs that can easily enter the cells by diffusion through the plasma membrane.

Of these mechanisms, the one that is most commonly encountered in the laboratory is the increased efflux of a broad class of hydrophobic cytotoxic drugs that is mediated by one of a family of energy-dependent transporters, known as ATP-binding cassette (ABC) transporters. The best-characterized ABC transporter is P-gp, which is encoded by the multidrug resistance gene 1 (MDR1 or ABCB1). *In vitro* it confers resistance to many drugs, which are used in the treatment of acute leukemia such as doxorubicin (DOX), daunorubicin (DNR), vincristine, vinblastine, methotrexate, mitoxantrone and others (Szakacs et al. 2006).

It is now recognized that the inability of the cells to undergo apoptosis contributes in several ways to the genesis and progression of cancer may also represent a critical cause of tumor drug resistance. P-gp did not seem to play a major role as a specific inhibitor of apoptosis. In fact, the P-gp inhibitor verapamil reversed only partially the resistance to DOX-induced apoptosis of the MDR cells Notarbatolo et al. 2002). So the other important mechanism is that resistance to apoptosis causes failure in the treatment of leukemia (Hao et al. 2003). It is well known that the antiapoptotic proteins may contribute a lot to resistance against chemotherapeutic drugs.

Combretastatin A-4 (CA-4), a naturally occurring stilbene derived from the South African tree *Combretum caffrum*, shows potent cytotoxicity against a broad spectrum of human cancer cell lines, including multidrug-resistant cancer lines (Gwaltney et al. 2001). CA-4P (disodium combretastatin-A-4-3-*O*-phosphate), a water-soluble prodrug, is a novel antitumor vascular targeting agent and is the first combretastatin analogue to enter clinic trials (Dowlati et al. 2002). Numerous studies on the structure-activity relationships of CA-4 have demonstrated that the cis-orientation between the diaryl groups is essential for its strong cytotoxicity. The newly designed and synthesized cis-restricted analog, 4-(4-bromophenyl)-2,3-dihydro-*N*,3-bis(3,4,5-trimethoxyphenyl)-2-oxoidmi-dazole-1-carboxamide (MZ3) exhibited strong and specific cytotoxic activity against a wide variety of human leukemia cell lines and their MDR-positive cells both *in vitro* and *in vivo* (Fang et al. 2007). MZ3 exposure potently arrested cancer cells in the G2/M phase of the cell cycle and induced apoptosis via the mitochondrial pathway.

Here, we demonstrate that the potent antimultidrug-resistant activity of MZ3 is associated with P-gp drug pumper, and can also down-regulate antiapoptotic proteins to promote apoptosis.

2. Investigations and results

2.1. Cytotoxicity of MZ3 on drug-sensitive and -resistant leukemia cells

To evaluate the antimultidrug-resistant effect on leukemia cells, cytotoxic activity of MZ3 were tested using a panel of chemosensitive tumor cell lines and their chemoresistant sublines. Representing cytotoxicity curves are shown in Fig. 1. The IC_{50} values showed that HL60R and K562R cells were resistant to DNR (26.9-fold and 92.2-fold) compared with their parental cells. In contrast, MZ3 is almost equally potent toward the parental HL60 and K562 cells ($IC_{50} = 2.65$ and $3.38\ \mu M$) and corresponding MDR-positive cells ($IC_{50} = 3.46$ and $8.00\ \mu M$) (Table). Summarizing, MZ3 showed strong cytotoxic effect on both drug-sensitive and its MDR-positive leukemia cells, which suggested that MZ3 has promising anti-multidrugresistant activity.

Table: Cytotoxicity of MZ3 and DNR on drug resistant human leukemia cell lines

Cell line	$IC_{50}(\mu M)$	
	MZ3	DNR
HL60	2.65 ± 0.19	0.09 ± 0.01
HL60R	3.46 ± 0.48	2.42 ± 0.24
Drug resistant-fold	1.31	26.89
K562	3.38 ± 0.12	0.02 ± 0.01
K562R	8.00 ± 0.29	1.90 ± 0.14
Drug resistant-fold	2.37	92.23

Cells were exposed with various concentrations of MZ3 and DNR for 72 h

2.2. MZ3 cause G2/M-phase arrest and finally induce apoptosis

Recently, we have shown that MZ3 induce apoptosis in HL60 cells (Fang et al. 2007). In order to determine whether MZ3 has a similar effect on drug-resistant cells, we analyzed the effect of MZ3 on cell cycle distribution and apoptosis by flow cytometry analysis. As shown in Fig. 2, MZ3 treatment induced a strong G2/M phase arrest in both drug-sensitive and its MDR-positive cells. In HL60 and HL60R cells, G2/M phase distribution was 41.5% and 41.5%, after treatment with MZ3 (16 μM) for 24 h respectively. In K562 and K562R cells, G2/M phase distribution was 36.9% and 49.2% after treatment with MZ3 (16 μM) for 24 h respectively. Accordingly, Fig. 3 shows that the MZ3-induced apoptosis of both drug-sensitive and corresponding MDR-positive cells. About 92.9% HL-60 cells, 64.4% HL-60R cells, 68.9% K562 cells and 89.9% K562R cells were detected to be undergoing apoptosis following the treatment of MZ3 at 16 μM for 48 h.

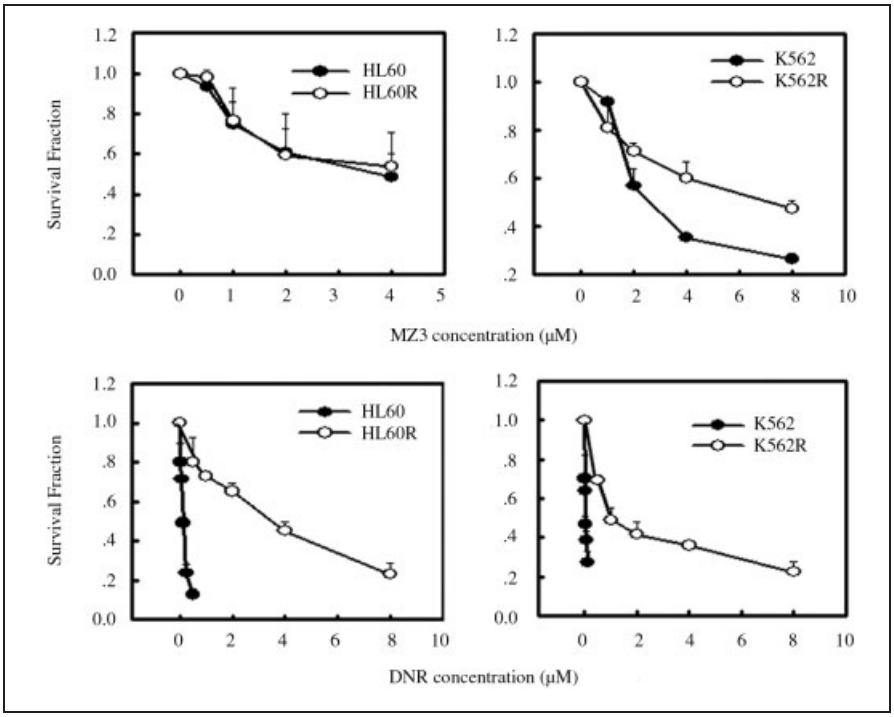

Fig. 1:
Survival fraction of MZ3 on human leukemia cell lines. Cells were treated with the indicated concentrations of MZ3 and DNR for 72 h

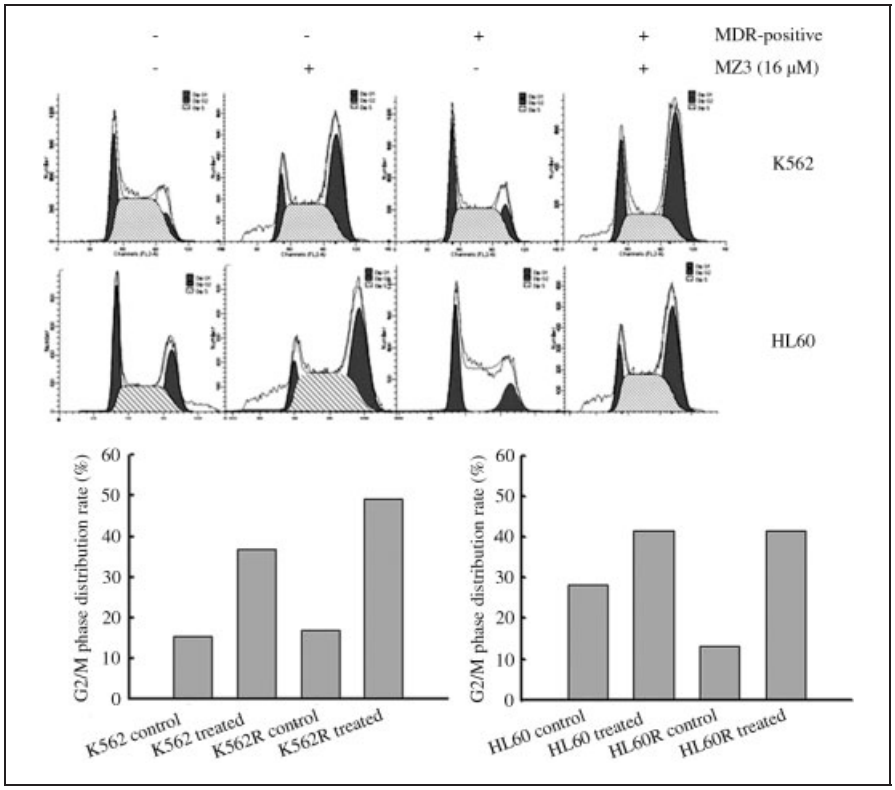

Fig. 2:
MZ3 causes G2/M-phase arrest in HL60, HL60R, K562 and K562R cells. HL60, HL60R, K562 and K562R cells were treated with 16.0 μM MZ3 for 24 h and analyzed propidium iodide-stained DNA content by flow cytometry

2.3. MZ3 can induce MDR1 down-regulated in drug-resistant cells

To characterize the resistant cell lines, we used Real-Time PCR to identify mRNAs that were differentially expressed between the parental and daughter cell lines. Figure 4A shows that MDR1 (ABCB1), but not MRP1, was primarily up-regulated among the two resistant cell lines. We found a 10^4-fold increase on HL60R cells and a 10^5-fold increase on K562R cells in the MDR1 mRNA level compared with their parental counterparts, which suggested that MDR1 up-regulation may play a major role in the acquired DNR resistance. In order to determine whether MZ3 has an effect on the mRNA level of MDR1, we analyzed the effect on mRNA level of MDR1 on resistance cells, and found that the MDR1/GAPDH mRNA level was

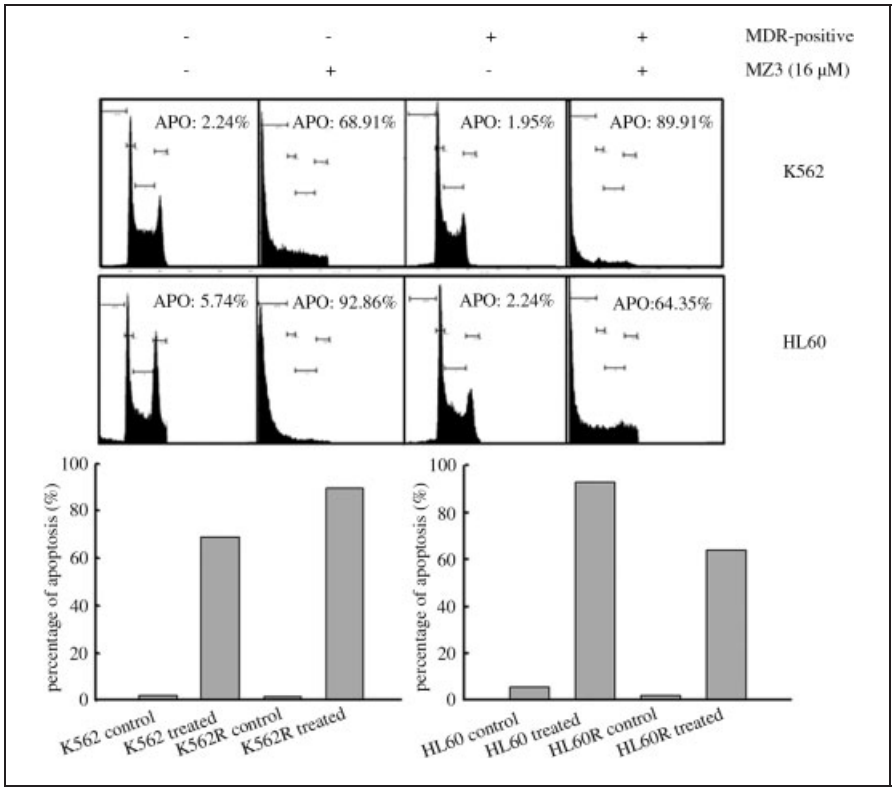

Fig. 3:
MZ3 induces Apoptosis in HL60, HL60R, K562 and K562R cells. HL60, HL60R, K562 and K562R cells were treated with 16.0 μM MZ3 for 48 h and analyzed for propidium iodide-stained DNA content by flow cytometry

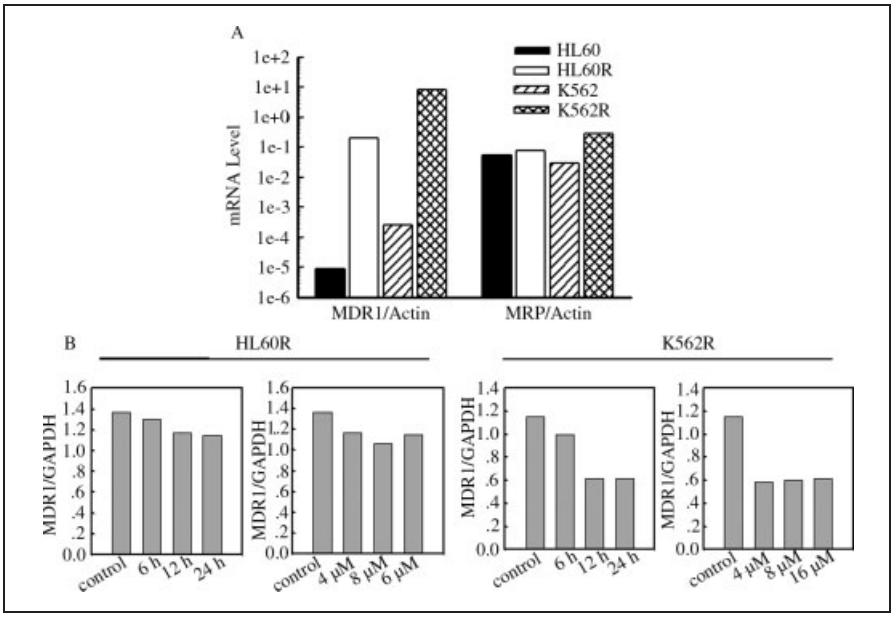

Fig. 4:
MZ3 reduce MDR1 transcription of MDR cells. A, The mRNA of HL60/HL60R cells and K562/K562R cells were analyzed by Real-Time RT-PCR. Amplification products obtained using actin-specific, MDR1-specific and MRP-specific oligonucleotide primers, respectively. B, The time and dosage effect of MZ3 on the reduction in MDR1 transcription in HL60R and K562R cells. Cells were treated with indicated dosage of MZ3 for the indicated time and analyzed by Real-Time RT-PCR. Amplification products obtained using GAPDH-specific and MDR1-specific oligonucleotide primers, respectively

reduced after treated with MZ3 (Fig. 4B). MDR1/GAPDH mRNA level was reduced by 34.2% after treatment with MZ3 8 μM for 12 h in HL60 cells, and 46.6% after treatment with MZ3 8 μM for 12 h in K562 cells.

2.4. Impact of P-gp drug-transporter overexpression on intracellular accumulation of MZ3

To examine whether the hypersensitivity of MDR1-overexpressing cells is based on increased drug uptake, MZ3 levels in HL60, K562, HL60R and K562R cells were determined. As shown in Fig. 5, the amount of MZ3 was not obviously decreased in both drug-sensitive and corresponding MDR-positive cells.

2.5. Effect of MZ3 on the antiapoptotic proteins

Previously we have demonstrated that MZ3-induced apoptosis was mediated by mitochondrial permeability changes that lead to capase-dependent cytotoxicity in HL60 cells (Fang et al. 2007). To demonstrate whether the antiapoptotic proteins among those play an important role in MZ3's antimultidrug-resistant mechanism in MDR-positive cells, the expression of BAX, p-AKT, XIAP, p-ERK was measured in both drug sensitive cell lines HL60, K562 and their MDR-positive cell lines HL60R, K562R treated with MZ3 16.0 μM for 24 h. As shown in Fig. 6, MZ3 (16.0 μM, 24 h) decreased p-ERK and XIAP protein levels in both four cell lines, increased the expression of BAX in drug sensitive cells but not so obvious in MDR-positive cells, and failed to regulate the activity of p-AKT in both drug sensitive and corresponding MDR-positive cells.

3. Discussion

Up to now, the escape of cancer cells from chemotherapy through activation of MDR mechanisms is a major reason for systemic cancer treatment failure. Among those, the effectiveness of many clinically useful drugs is limited by the fact that they are substrates for the efflux pumps P-gp/MDR1. Recently, we have reported that MZ3, which is a novel synthesized combretastatin A-4 analogue exerts a

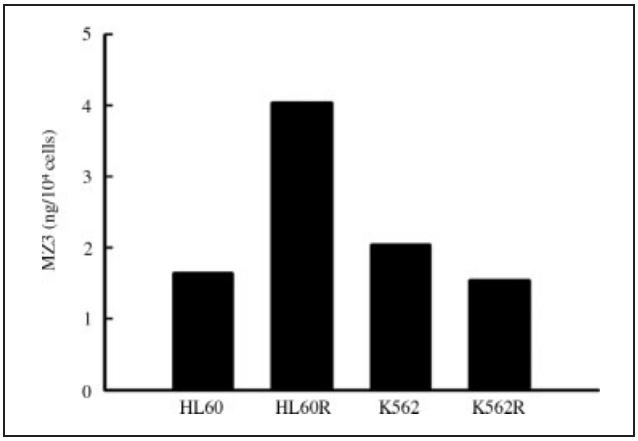

Fig. 5: Impact of drug-transporter overexpression on intracellular accumulation of MZ3. MZ3 levels of HL60/HL60R cells and K562/K562R cells were measured by LC-MS/MS after 2 h incubation on the concentration of 8.0 μM

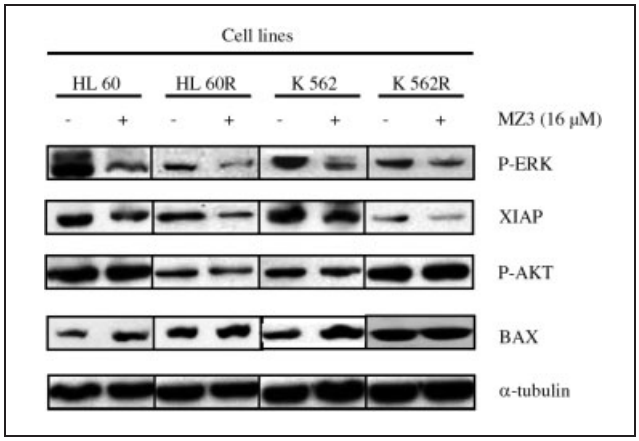

Fig. 6: MZ3's effect on protein expression of p-ERK, XIAP, p-AKT and BAX. Cells were harvested after treated by 16.0 μM MZ3 for 24 h, and Equal amounts (40 μg/lane) of cellular protein were fractionated on Tris-Glycine pre-cast gels and electroblotted onto Immobilon – P Transfer Membrane, followed by immunoblotting with anti-p-ERK, XIAP, p-AKT, and BAX, α-tubulin(B) antibodies

specific activity against human leukemia cell lines (Fang et al. 2007). In this study, MZ3 exhibited high antitumor activity in multidrug resistance cancer cells, caused a strong G2/M phase arrest and finally induced apoptosis similarly in drug sensitive cells and corresponding MDR-positive cells. This finding suggests that MZ3 may have promising antimultidrug-resistant activity in leukemia cells. The feature is distinct from those of DNR because MDR-positive leukemia cells are much more resistant to these chemotherapeutic agents.

So far, numerous studies have confirmed that the expression of P-gp is an adverse prognostic factor for complete remission and survival in adult leukemia (Schaich et al. 2005). P-gp, a member of the ABCB subfamily, stands out among ABC transporters by conferring the strongest resistance to the widest variety of compounds. Three-dimensional modeling of human MDR1/P-gp indicates that these glycoproteins function as efficient, ATP-dependent gate-keepers, which scan the plasma membrane and its inner leaflet to flip lipophilic substrates to the outer membrane leaflet (Mahaderan and List et al. 2004). Several agents with hyperactivity against MDR cells, as for example DNR (Litman et al. 1997), are MDR1 transport substrates. In case of MZ3, it showed that MDR1/GAPDH mRNA level was reduced after treated in MDR-positive leukemia cells. Meanwhile, intracellular accumulation of MZ3 is not obviously decreased in MDR-positive cells in contrast to drug sensitive cells after treatment. This suggests that MZ3 may not act as MDR1 transport substrate.

Recent investigations have demonstrated that the coordinated over-expression of the different IAPs may play a role in tumor cell resistance to drug induced apoptosis (Notarbartolo et al. 2004). The IAPs are a recently identified group of antiapoptotic proteins, including XIAP. They have been shown to protect cells from a range of apoptotic triggers including Fas ligation, Bax, activated caspases, cytochrome c, TNFa, some chemotherapeutic agents, viral infection, and radiation (McEleny et al. 2002). XIAP expression has been demonstrated to be associated with a shorter duration of remission and overall survival in acute myeloid leukemia (Tamm et al. 2000). Our results exhibited that MZ3 obviously downregulated XIAP and upregulated BAX in drug-sensitive leukemia cells. In contrast, there was an obvious protein expression decrease of XIAP but not BAX in MDR-positive leukemia cells after treatment with MZ3, indicating that the regulation of XIAP expression plays an important role in MZ3-induced apoptosis in MDR-positive leukemia cells.

Phosphorylation of ERK is normally associated with a pro-liferative response. However, certain chemotherapeutic drugs such as paclitaxel can induce the phosphorylation of ERK at the same residues, which is associated with the prevention of apoptosis (McCubrey et al. 2007). Our data showed that MZ3 obviously downregulated the phosphorylation level of ERK but not AKT in both drug-sensitive and MDR-positive leukemia cells suggested that the phosphorylation of ERK but not AKT may be involved in the MZ3-mediated antimultidrug-resistance process.

In summary, the present study demonstrated that MZ3 inhibits the MDR function of leukemia cells, and exerts the effect by altering the transcription of MDR1 genes. MZ3 can induce apoptosis in MDR-positive leukemia cells by downregulating the XIAP and p-ERK proteins levels. MZ3 may be a potential candidate for further research and development in anti-MDR territory.

4. Experimental

4.1. Chemicals

MZ3 was synthesized according to Cheng et al. (2005), dissolved in DMSO (8.0 mM stock solution) and stored at −20 °C, and further diluted with the appropriate assay medium immediately before use.

4.2. Cell culture

The human leukemia cell lines (K562 and HL60) were obtained from ATCC, and two drug resistant cell lines (K562R and HL60R) were endowed by the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences). All cell lines were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) with 10% heat-inactivated newborn calf serum (NCS, Gibco) at 37 °C in 5% CO_2.

4.3. Cytotoxicity assay

Cells were cultured in 96-well plates and the cytotoxic activity of MZ3 and daunorubicin on K562, K562R, HL60, HL60R cells was detected using the MTT assay. After treatment in 96-well plates, MTT solution (2.5 mg/ml in RPIM-1640, Sigma, St. Louis, MO) was added (20.0 μl/well), and the plates were incubated for an additional 4 h at 37 °C. The purple formazan crystals were dissolved in 100.0 μl DMSO. After 5 min, the plates were read on a Multiskan Spectrum (Thermo Electron Corporation, Marietta, Ohio) at 570 nm. Assays were performed in triplicate on three independent experiments. The concentration of drug inhibiting for 50% of cells (IC_{50}) was calculated using the software of Dose-Effect Analysis with Microcomputers.

4.4. Flow cytometry

Four human leukemia cell lines (2×10^6) were collected by centrifugation, washed with PBS, and fixed with 70% ethanol respectively. The fixed cells were harvested by centrifugation at 800 rpm for 10 min and resuspended in 100.0 μl of PBS containing 50.0 mg/ml RNase (Amersco, Solon, Ohio), then incubated at 37 °C for 1 h. After incubation, the cells were stained with 200.0 mg/ml propidium iodide (PI, Sigma, St. Louis, MO) at 4 °C for 30 min. The fluorescence of cells (2×10^4) was measured with FACSCalibur (Becton Dickinson, Lincoln Park, NJ) (Yang and Reynolds et al. 2005).

4.5. Real-Time PCR

Total RNA was extracted from sample cells with TRIzol. The RNA was precipitated by isopropyl alcohol and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified RNA using oligo(dT) priming (Thermoscript RT kit; Invitrogen), followed by SYBR-Green real-time PCR. The primers are as follows: MDR1, 5′-ctcatcgtttgtctacagttcgt-3′,5′-gctttctgtcttgggcttgt-3′; MRP, 5′-catgaaggccatcggactct-3′,5′-caggtccacgtgcagaca-3′; GAPDH, 5′-gagtcaacggatttggtcgt-3,5′-ttgattttggagggatctcg-3′. The threshold cycle for PCR products was defined as the cycle at which the SYBR-Green fluorescent signal was 20 standard deviations above background. Relative quantification of gene transcription was performed using the comparative CT method (or DDCT, method available in the user bulletin of ABI Prism 7700 sequence detection system) with GAPDH and β-actin as the control. Melting dissociation was performed to evaluate the purity of the PCR product.

4.6. Drug accumulation assay

Four human leukemia cell lines were cultured at 37 °C in a 5% CO_2 atmosphere. After treated with MZ3 for 2 h, cells were harvested and quickly washed three times with ice-cold PBS, and then cell pellets were resuspended in 0.5 ml DDW with three freeze-thaw cycles at −20 °C until there was no integrity cell under microscope. Supernatant liquid containing MZ3 was analyzed by the LC-MS/MS method after centrifuge for 15 min (15000 r/min).

4.7. Western analysis

Proteins of four human leukemia cell lines were extracted in radioimmunoprecipitation assay buffer (50.0 mM NaCl, 50.0 mM Tris-HCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS) and 40.0–80.0 μg of total protein was loaded per lane. Proteins were fractionated on 10–15% Tris-Glycine pre-cast gels, transferred to nitrocellulose membrane (Pierce Biotechnology, Rockford, IL), and probed with primary antibodies and then HRP-labeled secondary antibodies. Proteins were visualized using ECL (Pierce Biotechnology).

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