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The proteasome inhibitor bortezomib reverses P-glycoprotein-mediated leukemia multi-drug resistance through the NF-κB pathway

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Multi-drug resistance (MDR) is one of the obstacles for leukemia therapy, the major cause is an overexpression of P-glycoprotein (P-gp) leading to increased drug efflux. We investigated the reversion of multi-drug resistance and the possible mechanism by which the proteasome inhibitor bortezomib affects the expression of the multi-drug resistance gene mdr1 in the K562/DNR cell line. The drug resistance of the cells and the cellular toxicity of bortezomib were confirmed by MTT. Intracellular drug concentrations and cell apoptosis were detected by flow cytometry. The expression of mdr1 mRNA was examined by fluorescence quantitative PCR. The expression levels of nuclear factor-kappa B (NF-κB), inhibitor of NF-κB (IκB) and P-gp were detected by western blotting, and NF-κB activity was detected by ELISA. DNR-induced apoptosis increased in a dose-dependent manner after adding bortezomib. Bortezomib decreased IκB degradation, decreased NF-κB and NF-κB p65 activity, reduced P-gp/mdr1 mRNA expression, and increased the intracellular DNR concentration in K562/DNR cells *in vitro*. The bortezomib reversed leukemic multi-drug resistance in a dose-dependent manner as the result of decreasing IκB degradation, thus preventing the translocation of NF-κB into the nucleus and leading the down-regulation of mdr1 and a reduction in P-gp expression. Therefore, the intracellular drug concentration increased, and then apoptosis was induced.

1. Introduction

Multi-drug resistance (MDR) results to recurrent and refractory leukemia and many researchers focus on how to reverse MDR. MDR is a complex process mediated by a variety of factors. P-Glycoprotein (P-gp) is an ATP-dependent drug pump encoded by the multi-drug resistance gene (mdr1 gene). P-gp can reduce intracellular drug concentrations by pumping the drug out of the cell, resulting in cells with drug resistance properties (Fujita et al. 2005; Lei and Zhao 2007; Leonard et al. 2002). Nuclear factor-kappa B (NF-κB) is a transcription factor universally present in eukaryocytes that binds to the cytoplasmic inhibitor of NF-κB (IκB) under normal conditions. Phosphorylated IκB is degraded by the proteasome. Once NF-κB is released, it enters the nucleus and then binds to specific κB sequences to induce or up-regulate mdr1 gene expression.

Bortezomib (trade name Velcade®, previously known as PS-341) is an antitumor drug belonging to the proteasome inhibitor family and is primarily used to treat recurrent and refractory multiple myeloma in clinical practice (Ohashi 2008; Terpos et al. 2008; Wang et al. 2008). It can bind to the 26S subunit of the proteasome complex in a highly selective and reversible fashion (Richardson et al. 2006) and can regulate the cell cycle and cell apoptosis through various pathways, thereby improving treatment outcomes and reversing drug resistance (Bold et al. 2001; Ludwig et al. 2005; Mitsiades et al. 2003; Singh et al. 2010; Yamamura et al. 2010; Yang et al. 2003; Zineldeen et al. 2010). Current studies on bortezomib typically focus on multiple myeloma and lymphoma; the use of bortezomib to treat leukemia is still in the theoretical and exploratory study phase (Gil et al. 2007; Liao et al. 2008; McCloskey et al. 2008). The research on bortezomib typically uses chemotherapy-sensitive tumor cell lines, and the use of multidrug-resistant tumor cell lines is rare. In this study, we used a multidrug-resistant leukemia cell line expressing the mdr1 gene to observe the effects of bortezomib on the levels of mdr1 mRNA and the protein encoded by this mRNA, P-gp, as well as the dynamic changes in cell apoptosis, to further clarify the molecular mechanisms by which bortezomib reverses the multidrug resistance of leukemia and to provide experimental evidence on how to overcome multiple drug resistance using bortezomib for the treatment of leukemia.

2. Investigations and results

2.1. Drug resistance of K562/DNR cells

The IC_{50} values for DNR for the K562/S and K562/DNR groups were 1.16 μg/mL and 50.43 μg/mL, respectively. The drug-resistance fold was 43.47; thus, the K562/DNR cell line was drug resistant. When 100 μg/ml DNR was added, the K562/DNR cell survival rate was 80%, and cells demonstrated clear drug resistance properties. Therefore, 100 μg/ml was chosen as the experimental DNR concentration for each group.

2.2. Direct cytotoxic activity of bortezomib as determined by the MTT method

The IC_{10} for bortezomib effect on K562/DNR was 10 nmol/L. Since 90% of cells survived when the bortezomib concentra-

Table: Level of mdr1 mRNA expression before and after treatment with different doses of bortezomib (n = 3 and $\bar{x} \pm s$,*: compared to the control group $P < 0.05$)

Bortezomib concentration (nmol/L)	K562/S	K562/DNR
0 (control)	3773.65 ± 57.65	240709.20 ± 240.27
5	3783.45 ± 55.24	$129830.90 \pm 107.25^{*}$
10	3798.48 ± 51.67	$48927.11 \pm 52.84^{*}$
50	3769.89 ± 57.48	$16874.84 \pm 98.17^{*}$
100	3765.94 ± 54.37	$12637.51 \pm 53.83^{*}$

(mdr1 mRNA gene copy number in units of gene copies/ml.)

tion was below 10 nmol/L, this concentration was considered to be non-cytotoxic. Therefore, 10 nmol/L was selected as the bortezomib concentration to reverse drug resistance in this study.

2.3. Apoptosis of the K562/DNR cell line

The flow cytometric results after incubation with DNR alone or in combination with bortezomib at various concentrations are shown in Fig. 1. Relative to the DNR group, the DNR-induced apoptosis rate increased after the addition of bortezomib in a dose-dependent manner ($P < 0.01$).

2.4. Effect of bortezomib on the intracellular accumulation of DNR

Compared with the K562/S cells (Fig. 2a), the peak of K562/DNR cells (Fig. 2c) shifted to the left, and the average fluorescence intensity of the cells decreased, with a wide and skewed

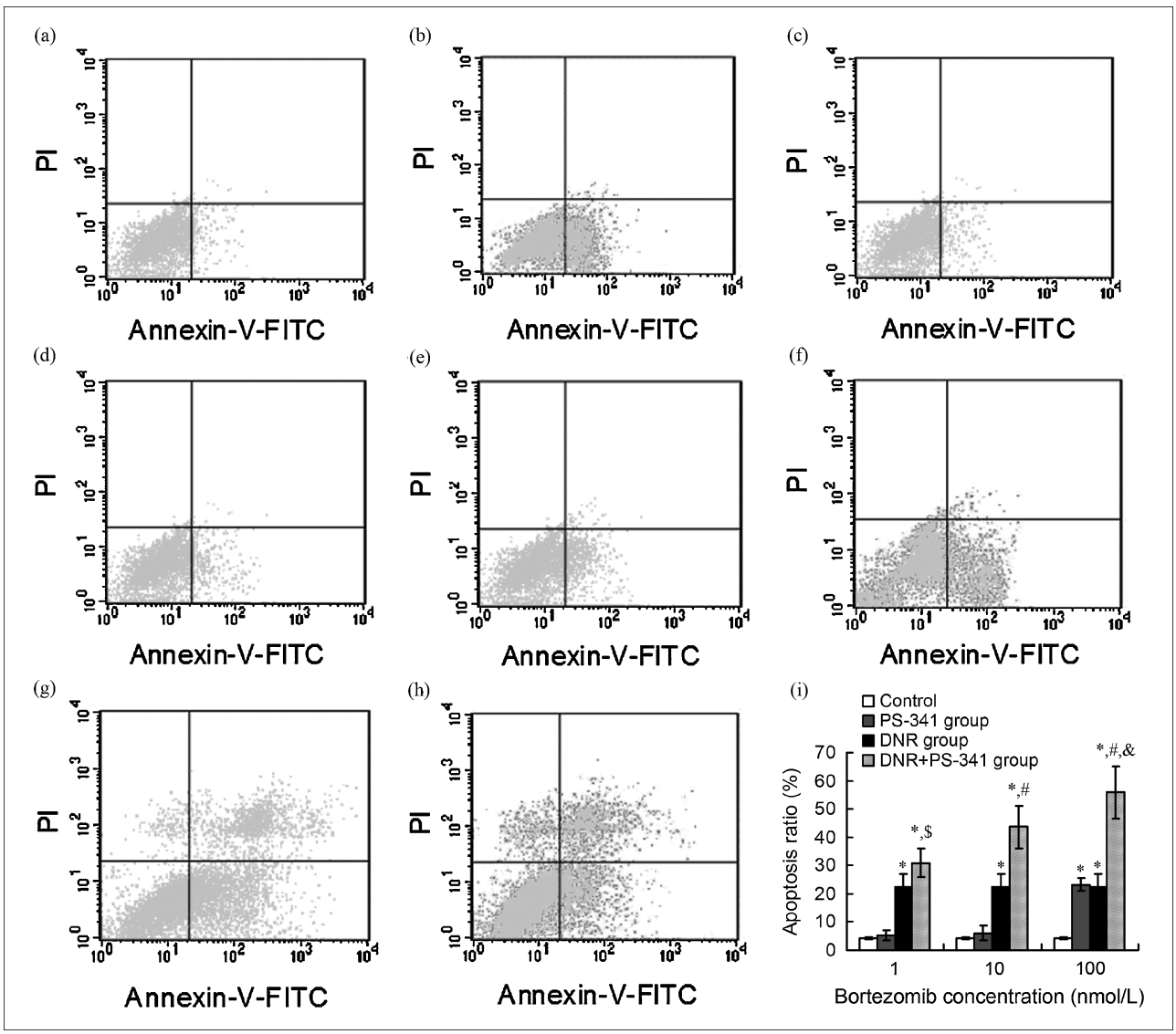

Fig. 1: Apoptosis rates determined by flow cytometry after incubation with DNR alone or in combination with bortezomib at various concentrations in K562/DNR cells for 36 h (n = 5). (a) Negative control; (b) DNR group; (c) 1 nmol/L bortezomib group; (d) 10 nmol/L bortezomib group; (e) 100 nmol/L bortezomib group; (f) DNR + 1 nmol/L bortezomib group; (g) DNR + 10 nmol/L bortezomib group; (h) DNR + 100 nmol/L bortezomib group; and (i) bortezomib at different concentrations: 1 nmol/L, 10 nmol/L, 100 nmol/L. Relative to the negative control group, the apoptosis rates were increased in the DNR group, the 100 nmol/L bortezomib group and the DNR + bortezomib at different concentration groups ($P < 0.01$,*), whereas the apoptosis rates were not significantly different in bortezomib groups with the doses less than 10 nmol/L. Relative to the DNR-treated group, the apoptosis rates increased after the addition of bortezomib in a dose-dependent manner (comparison of groups b and f, $P < 0.05$,$; comparison of groups b and g, $P < 0.01$,#; comparison of groups b and h, $P < 0.01$,#; comparison of groups e and h, $P < 0.01$,&)

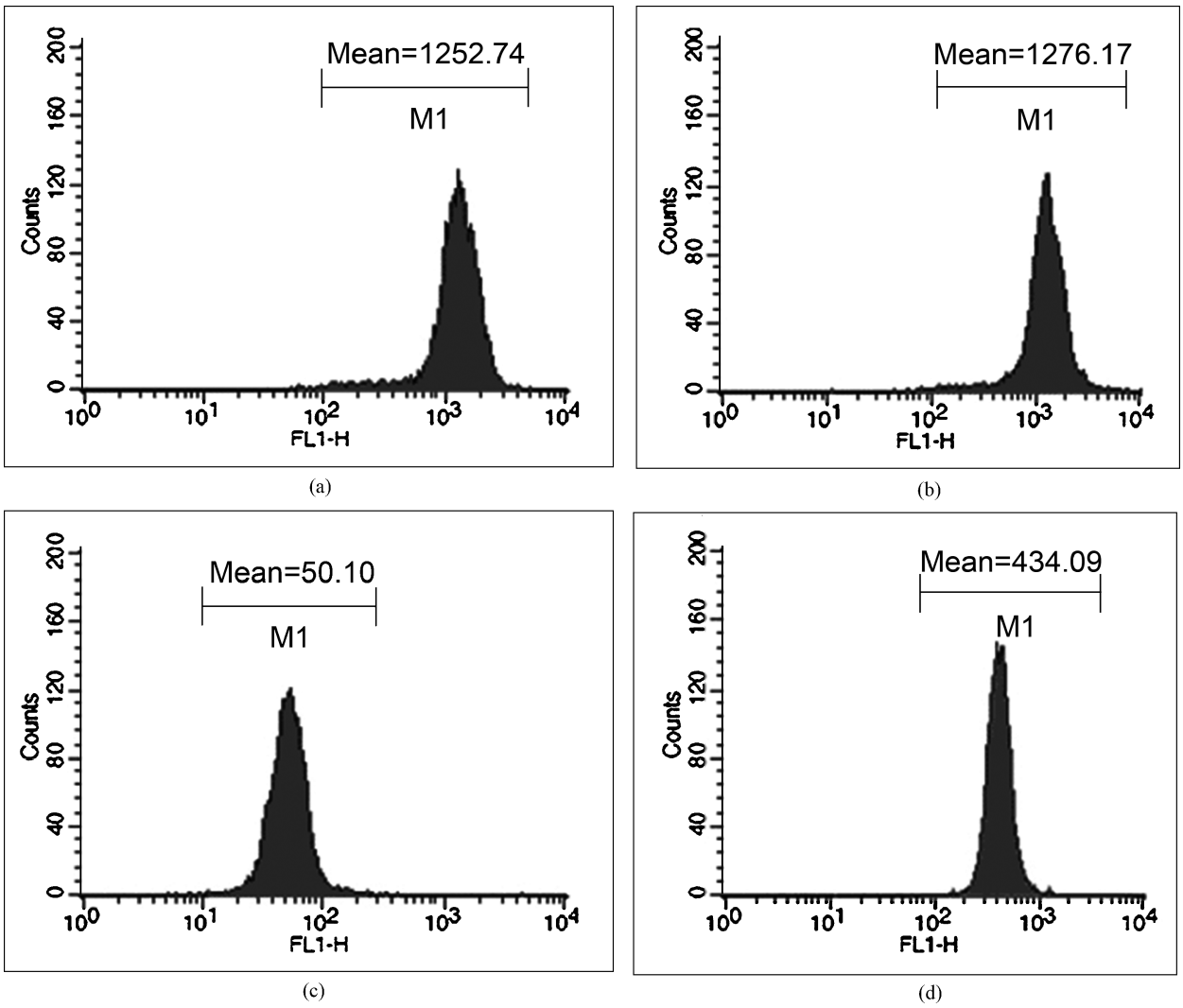

Fig. 2: Effects of bortezomib on the intracellular DNR concentration in K562/S and K562/DNR cells using FCMS. (a) DNR content in K562/S cells; (b) DNR content in K562/S cells after bortezomib treatment; (c) DNR content in K562/DNR cells; and (d) DNR content in K562/DNR cells after bortezomib treatment. The results indicate that bortezomib increased the intracellular concentration of the chemotherapy drug in the K562/DNR cells, whereas it had no significant effect on the K562/S cells

distribution curve. This result indicates that the average concentration of intracellular DNR in K562/DNR cells decreased with increased drug resistance. Relative to the peak for the corresponding untreated K562/DNR cells (Fig. 2c), the peak of after bortezomib treatment (Fig. 2d) shifted to the right, with increased average fluorescence intensity in the cells and a normal distribution, suggesting that bortezomib increased DNR accumulation in the drug-resistant leukemia cells and that the drug resistance was reversed (see Fig. 2). These results indicate that bortezomib executes its function through increasing the intracellular concentration of chemotherapy drugs in K562/DNR cells, whereas it had no significant effect on the K562/S cells.

2.5. Expression of mdr1 mRNA before and after bortezomib treatment

The results of fluorescence quantitative PCR are shown in the Table. After treatment with different concentrations of bortezomib for 24 h, the mdr1 mRNA expression in K562/DNR cells decreased in a dose-dependent manner. ANOVA analysis detected a significant difference between different the bortezomib-treated groups and the control group ($P < 0.05$). K562/S cells also expressed mdr1 mRNA at a very low level, and bortezomib had no significant inhibitory effect, as there were no statistically significant differences between the different bortezomib-treated groups and the control group ($P > 0.05$).

2.6. Expression levels of NF-κB, IκB and P-gp

After incubation with DNR alone or in combination with bortezomib at various concentrations, the expression levels of NF-κB, IκB and P-gp were determined by western blotting (Fig. 3). Compared to the negative control group, DNR was able to induce the down-regulation of IκB expression, the up-regulation of

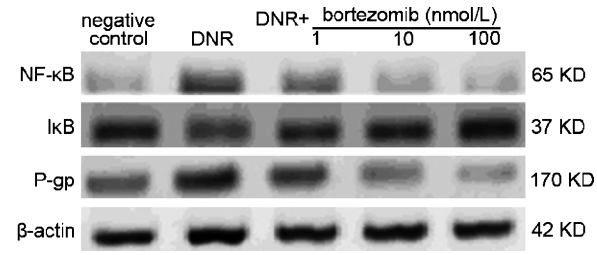

Fig. 3: The expression levels of NF-κB, IκB and P-gp as determined by western blotting after incubation with DNR alone or in combination with bortezomib at various concentrations (1: negative control group; 2: DNR group; 3: DNR + 1 nmol/L bortezomib group; 4: DNR + 10 nmol/L bortezomib group; and 5: DNR + 100 nmol/L bortezomib group). From the western blot gray intensity analysis, relative to the negative control, DNR was able to induce the down-regulation of IκB expression, the up-regulation of NF-κB expression, and the up-regulation of P-gp expression. After the addition of bortezomib, IκB degradation was inhibited, IκB expression increased, NF-κB expression decreased and P-gp expression decreased. The expression levels changed in a dose-dependent manner

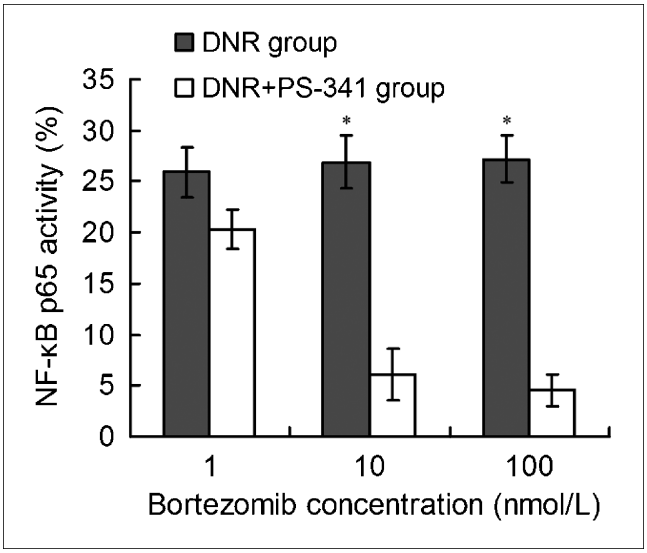

Fig. 4: Changes in NF-κB p65 activity after incubation with DNR alone or in combination with bortezomib at different concentrations (n = 10): 1 nmol/L, 10 nmol/L 100 nmol/L. The NF-κB p65 activity of DNR in combination with bortezomib decreased in a dose-dependent manner (*$P < 0.01$)

NF-κB expression, and the up-regulation of P-gp expression. After the addition of bortezomib, IκB degradation was inhibited, IκB expression increased, NF-κB expression decreased and P-gp expression decreased. The expression levels changed in a dose-dependent manner.

2.7. NF-κB p65 activity

Compared to the DNR group, the NF-κB p65 activity in cells treated with DNR in combination with bortezomib was decreased (Fig. 4). The difference between the two groups was statistically significant. The activity of NF-κB p65 changed in a dose-dependent manner.

3. Discussion

Bortezomib, a boronic acid dipeptide, is a unique and specific inhibitor of the proteasome pathway. Preclinical studies show that the cytotoxic and growth inhibitory effects of bortezomib are correlated with proteasome inhibition and do not overlap with the effect of other chemotherapeutic agents (Adams et al. 1999). Proteasome inhibition is a rational therapeutic approach both by itself and as a means to induce chemosensitization and overcome chemoresistance. As noted earlier, many cytotoxic agents activate the anti-apoptotic NF-κB pathway, and blockade of this induction by proteasome inhibition enhances the antitumor efficacy of these drugs (Ma et al. 2003; Mitsiades et al. 2003). In addition, several strategies by which tumor cells survive the effects of chemotherapy can be similarly abrogated. Based on this rationale, bortezomib has been combined with a variety of chemotherapeutic drugs, including carboplatin (Aghajanian et al. 2005), docetaxel (Messermith et al. 2006), irinotecan (Ryan et al. 2006), melphalan (Berenson et al. 2006), pegylated liposomal doxorubicin (Orlowski et al. 2005), and thalidomide (Barlogie et al. 2004). Bortezomib has also been incorporated into more complex regimens, such as paclitaxel and carboplatin (Ma et al. 2007) and gemcitabine and cisplatin (Voortman et al. 2007). From these studies, it seems possible to conclude that bortezomib has generally been successfully when combined with other agents without significantly increasing toxicity and without the need for large dose adjustments. In several cases, these combinations have shown evidence of enhanced activity and may reverse drug resistance. In preclinical studies, tumor resistance to conventional chemotherapy agents was demonstrated to be overcome by the addition of bortezomib, highlighting the importance of developing combination treatment for multiple myeloma (MM) and other malignancies (Cusack 2003; Hideshima et al. 2001). Current studies on bortezomib focus on multiple myeloma and lymphoma, and determining whether bortezomib can overcome the drug resistance that occurs during in leukemia therapy is still in the exploratory study period. We designed experiments to investigate the reversion of multi-drug resistance and the possible mechanism by which the proteasome inhibitor bortezomib functions in the treatment of leukemia.

Although recent studies suggest that many signaling pathways are associated with conventional cytotoxic drug resistance, the major cause of such drug resistance is the overexpression of P-gp. Overexpression of the mdr1 gene and its product P-gp lead to increased drug efflux, reducing the concentration of chemotherapeutic drugs within leukemia cells, as a result of which, leukemia cells escape destruction (Li et al. 2010). Therefore, reversing the MDR induced by P-gp is a significant challenge in leukemia therapy. The latest research shows (Bentires-Alj et al. 2003; Lee et al. 2007) that NF-κB can regulate P-gp-mediated drug resistance. An NF-κB binding site was identified in the first exon of the mdr1 promoter region, suggesting that mdr1 may be a downstream gene of NF-κB. Ogretmen et al. found the mdr1 mRNA expression in the human breast cancer cell line MCF27 can be regulated by NF-κB/p65. Certain studies involving the K562 cell lines have indicated that bortezomib is able to inhibit IκB degradation, prevent NF-κB release, inhibit NF-κB-initiated gene transcription and reduce mdr1 expression by inhibiting proteasome activity, thus resulting in reduced P-gp production. No reports detailing the ability of bortezomib to reverse drug resistance in K562/DNR cells were found.

In this study, the multi-drug resistant cell line K562/DNR was incubated with DNR alone or in combination with bortezomib at different concentrations to analyze the above parameters. The results showed that bortezomib decreased the degradation of IκB, prevented NF-κB from entering the nucleus, and decreased the activity of NF-κB p65. These effects resulted in the down-regulation of mdr1 gene expression and reduced the expression of the ATP-dependent drug pump P-gp encoded by the mdr1 gene. Therefore, the intracellular DNR concentration increased, inducing the apoptosis of multi-drug resistant cells. These results are significant in preventing and overcoming leukemic cell multiple drug resistance and in exploring new therapeutic methods for leukemia treatment.

4. Experimental

4.1. Cell lines and cell culture

The K562/S cell line is a chemotherapy-sensitive human leukemia cell line, and the K562/DNR cell line is a leukemia cell line expressing the mdr1 multidrug resistance gene that was derived from K562/S by stimulation with 0.5 mmol/L daunorubicin (DNR). Both cell lines were kindly provided by Professor Liu Yunpeng, head of the Laboratory of Oncology Department, the First Affiliated Hospital of China Medical University. K562/S and K562/DNR cells were cultured in RPMI-1640 containing 12% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 and saturated humidity. K562/DNR cells were cultured in media containing 0.5 mmol/L DNR to maintain their drug resistance.

4.2. Reagents

Bortezomib was purchased from Xi'an Janssen Co., Ltd. The mdr1 mRNA detection kit was purchased from Shanghai DaAn Biotechnology Co., Ltd. The Annexin V-FITC apoptosis kit was purchased from Jingmei Biological Engineering Co., Ltd. The primary fetal rabbit anti-human NF-κB, IκB and P-gp antibodies were products of Santa Cruz Biotechnology,

alkaline phosphatase-labeled goat anti-rabbit and horse anti-mouse secondary antibodies were products of Beijing Zhongshan Company, and the mouse anti-β-actin antibody was the products of Sigma. The NF-κB ELISA kit was obtained from Active Motif.

4.3. Identification of drug resistance in K562/DNR cells using the MTT method

Experiments were done with a K562/S group and a K562/DNR group. The cell density was adjusted to 2×10^5/mL, and 100 μl of cell suspension and various concentrations of DNR (0–400 μg/mL) were added to 96-well plates with five parallel wells for each DNR concentration. A blank group and a drug-free control group were designed for each experimental group. MTT (20 μL of 5 g/L) was added to each well after 72 h, and the cells were cultured for 4 additional hours. The supernatant was discarded after centrifugation, and 150 μL of DMSO was added to terminate the reaction. The samples were vortexed under light-proof conditions for 15 min. The absorbance (*A*) was detected at 570 nm using an automatic ELISA analyzer and was used to calculate the growth inhibition rate.

$$\text{Growth inhibition rate} = \frac{(\text{control group } A \textit{ value} - \text{experimental group } A \textit{ value})}{(\text{control group } A \textit{ value}\text{-blank group } A \textit{ value})} \times 100\%.$$

The median inhibitory concentration (IC_{50}) was calculated based on the obtained growth inhibition rate.
Drug-resistance folds = IC_{50} (K562/DNR)/IC_{50} (K562/S).

4.4. Determination of the direct cytotoxic activity of bortezomib using the MTT method

The MTT method was used to determine the direct cytotoxic activity of bortezomib as described above. Different concentrations of bortezomib were added to each well containing K562/DNR cells. The absorbance of the blank control groups was designated as 100% survival. The relative survival rate (%) of each group = absorbance of the experimental group/the absorbance of the blank group × 100%. The calculated bortezomib concentration resulting in a 90% survival rate was the IC_{10}. A concentration below the IC_{10} was selected as the experimental concentration for bortezomib to reverse drug resistance.

4.5. Detection of cell apoptosis by flow cytometry

K562/DNR cells were incubated with 100 μg/ml DNR alone or in combination with 1 nmol/L, 10 nmol/L, or 100 nmol/L bortezomib for 36 h. The apoptosis rate of each group was determined by flow cytometry. Cells were washed twice with cold PBS and resuspended in 400 μl 1 × binding buffer at a density of 1×10^6 cells/ml. Then, 5 μl of Annexin V–FITC were added, and the mixture was gently vortexed and incubated for 15 min at 4–8 °C in the dark. PI (10 μl) was added to each tube, and the samples were then incubated for another 5 min at 4–8 °C in the dark, after which the cells were analyzed by flow cytometry.

4.6. Detection of intracellular drug concentrations by flow cytometry

Bortezomib was added to the experimental group, and DNR was added to both the experimental and the control groups. The cells were then incubated at 37 °C for 90 min and centrifuged. The supernatant was discarded, and the cells were washed twice with pre-cooled PBS. Fresh medium was added, and the intracellular DNR fluorescence intensity was measured by flow cytometry.

4.7. Detection of the expression of mdr1 mRNA by quantitative fluorescence PCR

Approximately 5×10^8 K562/DNR and K562/S cells after treatment with 0 nmol/L, 5 nmol/L, 10 nmol/L, 50 nmol/L or 100 nmol/L bortezomib for 24 h were collected, and total RNA was extracted. Reverse transcription (RT) was performed according to instructions of the kit. The forward primer for the mdr1 gene was 5′-AAAAGTGAAAAAGATAAGAAGGAAAAGAAA-3′, and the reverse primer was 5′-CACCATATACAACTTGTCAAGCCAA-3′. The fluorescent probe sequence was 5′-FAM-TTGAATAGCGAAA CATTGAAAATACACTGACAGTTG-TAMRA-3′.

4.8. Examination of NF-κB, IκB and P-gp expression levels using western blotting

K562/DNR cells were incubated with 100 μg/ml DNR alone or in combination with 1 nmol/L, 10 nmol/L, or 100 nmol/L bortezomib for 36 h. Western blotting was used to examine the NF-κB, IκB and P-gp expression levels of each group. The expression levels for each protein are presented as the absorbance of the protein/β-actin absorbance × 100%.

4.9. Detection of NF-κB activity by ELISA

NF-κB p65 activity was assayed by ELISA using an NF-κB ELISA kit. Nucleoprotein extraction and the experimental procedure were completed as instructed by the reagent kit. Cell suspensions were centrifuged, and the supernatants were assayed immediately. The microtiter plate was pre-coated with an antibody specific to NF-κB p65. The supernatants were added to the appropriate microtiter plate wells along with a biotin-conjugated polyclonal antibody preparation specific for NF-κB p65. Next, avidin-conjugated to horseradish peroxidase (HRP) was added to each microplate well, and the plates were incubated. Then, a TMB substrate solution was added to each well. Only those wells that contained NF-κB p65, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a color change. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at 450 nm. The concentration of NF-κB p65 in the samples was then determined by comparing the OD values of the samples to a standard curve.

4.10. Statistical analysis

Experimental data are presented as the mean ± standard deviation ($\bar{x} \pm s$), and SPSS 13.0 statistical software was used for the statistical analysis.

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