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Experimental Study on the Mechanism of Reversal of Leukemia Multidrug Resistance by Proteasome Inhibitor Bortezomib

Ying-chun LI
Hui-han WANG
Hong-yu PAN
Ai-jun LIAO
Wei YANG
Zhuo-gang LIU
Xiao-bin WANG

Department of Hematology, Shengjing Hospital, China Medical University, Shenyang 110004，Liaoning Provence, China.

Correspondence to: Zhuo-gang LIU
E-mail: liuzg@sj-hospital.org

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Received June 9, 2010; accepted June 22, 2010.

E-mail: 2008cocr@gmail.com
Tel (Fax): 86-22-2352 2919

OBJECTIVE In this study, we applied multidrug resistant leukemia cell line expressing mdr1-mRNA to observe changes in mdr1-mRNA, the P-gp, cell cycle and apoptosis before and after bortezomib was used, in order to explore the mechanism of reversal of leukemia multidrug resistance by the proteasome inhibitor bortezomib.

METHODS Flow cytometry (FCM) was used to detect the intracellular drug concentration, expression of P-gp, cell apoptosis and cell cycle status of K562/DNR cells before and after treatment with different concentrations of bortezomib. Fluorescence quantitative PCR was applied to detect the mdr1-mRNA expression in K562/DNR and K562/S cells.

RESULTS Bortezomib could increase the intracellular DNR content in K562/DNR cells, but showed no effect in K562/S cells. 5-100 nmol/L bortezomib could significantly reduce the P-gp/mdr1-mRNA expression in K562/DNR cells *in vitro*, and showed a dose-dependent effect. There was a statistically significant difference ($P < 0.05$) between different concentration groups and the control group. P-gp/mdr1-mRNA expression was negatively correlated with cell apoptosis ($r = -0.912$ and $P < 0.01$). After treatment with different concentrations of bortezomib for 24 h, K562/DNR cells in G2 + M phases were significantly increased, while cells in G0 + G1 phases and S phase were significantly decreased, accompanied by an increased apoptotic rate.

CONCLUSION Bortezomib can induce G0 + G1 phase to G2 + M phase, and thereby enhance the chemosensitivity of leukemia, and may also reverse the multidrug resistance in leukemia mediated by P-gp overexpression encoded by mdr1 gene. This confirms that bortezomib can reverse leukemia multidrug resistance at the levels of nucleic acid and protein molecules.

KEY WORDS: proteasome inhibitor, bortezomib, multi-drug resistance, K562 cells.

Introduction

Leukemia multi-drug resistance (MDR) is a major problem in chemotherapy and recurrence. To overcome MDR is an important way to improve the efficacy of leukemia therapy. MDR is defined as the drug resistance in tumor cells to not only the contacted drug, but also other drugs with different structures and affections. It is a complex

process mediated by a variety of factors. Currently, it is believed that the 3 main mechanisms involved in MDR are: *i)* membrane glycoprotein-mediated drug efflux pump; *ii)* apoptosis-regulating gene-mediation; *iii)* MDR enzyme-mediation. Among them, P-glycoprotein (P-gp) overexpression encoded by the mdr1 gene causes increased efflux and decreased efficacy of the anticancer drug remains to be a main reason for the formation of drug resistance[1]. The proteasome inhibitor bortezomib (trade name Velcade®, previously known as PS-341) can bind to the 26S subunit of the proteasome complex in a highly selective and reversible fashion[2], regulate the progress of cell cycle and cell apoptosis through various pathways, down-regulate mdr1 gene expression, thereby improving treatment outcome and reversing drug resistance[3-13]. However, current studies on bortezomib mostly use chemotherapy-sensitive tumor cell lines, while application of multidrug resistant tumor cell line is rare. In this study, we used a multidrug resistant leukemia cell line expressing mdr1-mRNA to observe the effects of bortezomib on the levels of mdr1-mRNA and its encoded P-gp, as well as dynamic changes in cell cycle and apoptosis, in order to further clarify the molecular mechanism of bortezomib to reverse multidrug resistance of leukemia.

Materials and Methods

Cell line and cell culture

K562/S was chemotherapy-sensitive acute human leukemia cell line and K562/DNR was a leukemia cell line expressing a multidrug resistance gene mdr1, derived from K562/S by stimulation with 0.5 mmol/L daunorubicin (DNR). Both cell lines were generous gifts from the Oncology Laboratory of the First Affiliated Hospital of China Medical University. K562/S and K562/DNR cells were cultured in RPMI-1640 with 100 U/ml penicillin, 100 μg/mL streptomycin and 12% FBS, 37°C, saturated humidity, supplied with 5% CO_2/95% air. One μg/mL DNR was added to K562/DNR cells and the cells were used for experiments after at least 1 wk of being cultured in the absence of DNR.

Reagents

RPMI-1640 was purchased from Hyclone Company (United States), fetal bovine serum was purchased from Sigma Company (United States), Bortezomib was purchased from Xi'an Janssen, DNR was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd., mdr1-mRNA detection kit was purchased from Shanghai DaAn Biotechnology Co., Ltd., anti-p-glycoprotein PE was purchased from BD company (United States), Annexin V-FITC apoptosis kit was purchased from Jingmei Biological Engineering Co., Ltd., flow cytometry cell cycle analysis kit was purchased from Shanghai ZhuoKang Biotechnology Co., Ltd.

Identification of drug-resistance in K562/DNR cells using MTT method

K562/S and K562/DNR cells (2×10^8/L) were seeded in 96-well plate (100 μL/well). After 12 h of culture, DNR was added and cells were continuously cultured for 68 h. Then 20 μL MTT was added into each well, incubated for 4 h, centrifuged at 2000 r/min for 10 min, discarded supernatant, added 150 μL DMSO into each well and measured absorbance OD values at 540 nm wavelength with a microplate reader. The inhibitory rate, half inhibitory concentration (IC_{50}) and MDR fold were calculated using the following formulas.

Cell inhibitory rate (%) = (1 - OD value of the experimental group/ control OD value) × 100%

Fold of MDR = IC_{50} of MDR cells/ IC_{50} of drug-sensitive cells

Determination of intracellular drug concentration by flow cytometry

Bortezomib (final concentration of 10 nmol/L) was added to the experimental group and DNR was added to both the treatment and the control groups at final concentration of 5 μmol/L, incubated at 37°C for 90 min, centrifuged, discarded supernatant, washed 2 times with pre-cooled PBS, added fresh medium and measured the intracellular DNR fluorescence intensity on flow cytometry.

Detection the expression of mdr1-mRNA by Quantitative fluorescence PCR

About 5×10^8 K562/DNR and K562/S cells after treatment by 0 nmol/L, 5 nmol/L, 10 nmol/L, 50 nmol/L and 100 nmol/L bortezomib for 24 h were collected and total RNA was extracted. Reverse transcription (RT) was performed according to the instructions of the kit. Upstream primer for MDR gene was 5'-AAA AGT GAA AAA GAT AAG AAG GAA AAG AAA-3', and downstream primer was 5'-CAC CAT ATA CAA CTT GTC AAG CCA A-3'. Fluorescent probe sequence was 5'-FAM-TTG AAT AGC GAA ACA TTG AAA ATA CAC TGA CAG TTG-TAMRA-3'.

Detection of intracellular P-gp by flow cytometry

About 2×10^5 K562/DNR cells after treatment by 0 nmol/L, 5 nmol/L, 10 nmol/L, 50 nmol/L and 100 nmol/L bortezomib for 24 h were collected to make cell suspension. Twenty μL anti-p-glycoprotein PE was added, incubated at 25°C for 30 min in the dark, washed 3 times with PBS, and detected the relative fluorescence intensity (MFI) and positive rate through flow cytometry.

Detection of cell apoptosis by flow cytometry

K562/DNR cells after treatment by 0, 5 , 10 , 50 and 100 nmol/L bortezomib for 24 h were collected. Apoptosis rate of each groups were determined with flow cytometry followed by apoptosis kit instruction.

Cell cycle analysis by flow cytometry

K562/DNR cells after treatment by 0, 5 , 10 , 50 and 100 nmol/L bortezomib for 24 h were collected, washed by PBS, fixed in 70% ethanol, stained by propidium iodide (PI) and tested the distribution of cells in G1, S and G2 + M phases by flow cytometry.

Statistical analysis

Experimental data were presented as mean ± standard deviation ($\overline{x} \pm s$) and SPSS 13.0 statistical software was applied for linear correlation, analysis of variance and comparisons between groups.

Results

Drug resistance of K562/DNR cells

After treatment with different concentrations of DNR for 68 h, the K562/DNR cells exhibited significantly higher resistance to DNR than the parental K562/S cells. The IC_{50} to DNR of these 2 were respectively (29.74 ± 2.61) μg/mL and (1.08 ± 0.09) g/mL, yielding a drug resistance fold of 27.5 times.

Effect of bortezomib on intracellular accumulation of DNR

Compared with the K562/S cells (Fig.1a), the peak of K562/DNR cells (Fig.1c) shifted to the left, and the average fluorescence intensity of cells decreased, with a wide and skewed distribution curve. This indicated that the average concentration of intracellular DNR in K562/DNR cells reduced with more drug resistance. Compared to corresponding untreated K562/DNR cells (Fig.1c), the peak of after bortezomib treatment (Fig.1d) shifted to the right, with increased average fluorescence intensity in the cells and good normal distribution, suggesting that bortezomib increased DNR accumulation in the drug resistant leukemia cells, i.e., drug resistance in a portion of the cell was corrected (Fig.1). It indicated that bortezomib executes its function through increasing the intracellular concentration of chemotherapy drugs in the K562/DNR cells, while having no significant effect on the K562/S cells.

Expression of mdr1-mRNA before and after bortezomib treatment

The results of fluorescence quantitative PCR were shown in Table 1. After treatment by different concentrations of bortezomib for 24 h, the mdr1-mRNA expression in K562/DNR cells had a downward trend, showing dose-dependence. ANOVA analysis detected a significant difference between different concentration groups and the control group ($P < 0.05$). K562/S cells also expressed mdr1-mRNA at a very low level, bortezomib showed no significantly inhibitory effect since there

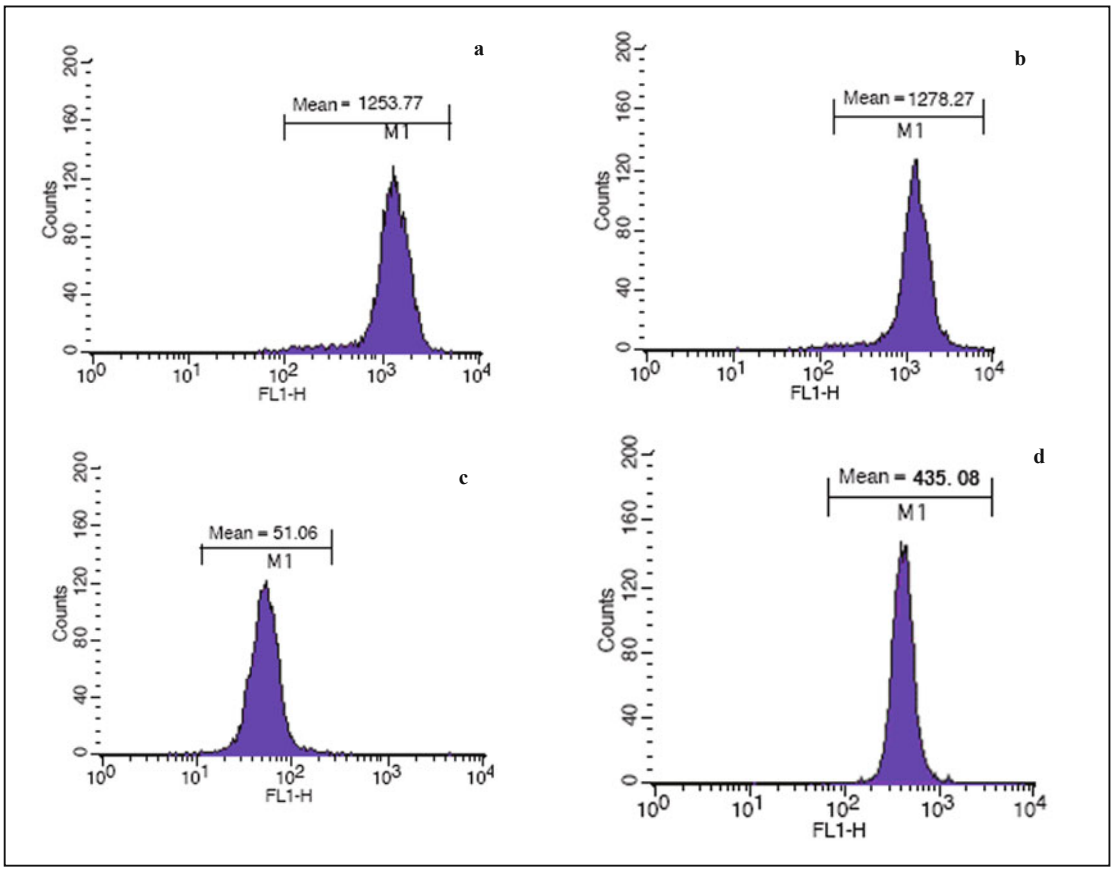

Fig.1. Effects of bortezomib on intracellular DNR concentration in K562/S and K562/DNR cells using FCMS. a, DNR content in K562/S cells; b, DNR content in K562/S cells after bortezomib treatment; c, DNR content in K562/DNR cells; d, DNR content in K562/DNR cells after bortezomib treatment.

were no statistically significant differences between different concentration groups and the control group ($P > 0.05$).

Table 1. Level of mdr1-mRNA expression before and after different concentrations of bortezomib treatment ($n = 3$ and $\bar{x} \pm s$).

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 ± 107.25*
10	3798.48 ± 51.67	48927.11 ± 52.84*
50	3769.89 ± 57.48	16874.84 ± 98.17*
100	3765.94 ± 54.37	12637.51 ± 53.83*

*, $P < 0.05$ *vs.* Control; mdr1-mRNA gene copy number in unit of: gene copy/mL.

P-gp/mdr1mRNA expression and correlation with apoptosis in K562/DNR cells before and after bortezomib treatment

Detection of P-gp by flow cytometry was shown in Table 2 and Fig.2. After treatment by different concentrations of bortezomib for 24 h, the P-gp positive expression rate and relative fluorescence intensity (MFI) of the K562/DNR cells both had a trend to decrease, showing dose-dependence. ANOVA analysis detected significant differences between the different concentration groups and the control group (bortezomib 0 nmol/L) ($P < 0.05$). Compared with untreated K562/DNR (bortezomib 0 nmol/L) cells, the peak in cells treated by 5-100 nmol/L final concentration of bortezomib gradually shifted to the left and the average fluorescence intensity of the cell gradually decreased, with a more widened and skewed distribution curve. This suggests that bortezomib could down-regulate the P-gp expression in K562/DNR cells.

Detection of Anexin-V by flow cytometry is shown in Table 2 and Fig.3. After treatment by different concentrations of bortezomib for 24 h, K562/DNR cells underwent apoptosis, showing dose-dependence. In addition, cell apoptotic rate was higher in cells with lower expression level of P-gp/mdr1mRNA. Linear correlation analysis detected a negative correlation between P-gp and cell apoptotic rate ($r = -0.912$ and $P < 0.01$).

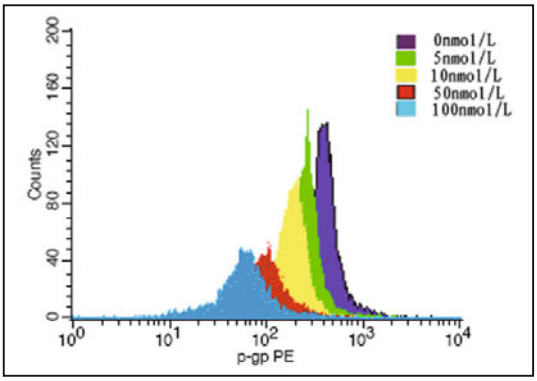

Fig.2. P-gp expression on cell surface of K562/DNR after treated by different concentrations of bortezomib for 24 h.

Effects of bortezomib on K562/DNR cell apoptosis and cell cycle distribution

Results of flow cytometry to detect cell cycle are shown in Table 3 and Fig.4. After treatment by different concentrations of bortezomib for 24 h, K562/DNR cells G2 + M phase increased while these in G0 + G1 and S phases decreased with increased drug concentration, accompanied by increased apoptotic rate of the cells. This suggests that bortezomib can induce cell cycle transi-

Table 2. P-gp expression status and apoptosis of K562/DNR cells before and after treatment by different concentrations of bortezomib ($n = 3$ and $\bar{x} \pm s$).

Bortezomib concentration (nmol/L)	mdr1mRNA	P-gp expression on cell surface of K562/DNR		Cell apoptotic rate (%)
		Positive rate (%)	MFI	
0 (control)	240709.20 ± 240.27	86.55 ± 1.52	393.37 ± 7.417	0.3
5	129830.90 ± 107.25*	81.14 ± 0.79	253.19 ± 11.06*	9.79
10	48927.11 ± 52.84*	68.26 ± 3.43*	209.53 ± 13.49*	10.95
50	16874.84 ± 98.17*	60.03 ± 5.80*	95.05 ± 7.89*	60.74
100	12637.51 ± 53.83*	54.53 ± 4.81*	58.53 ± 6.86*	73.25

*, $P < 0.05$ *vs.* Control

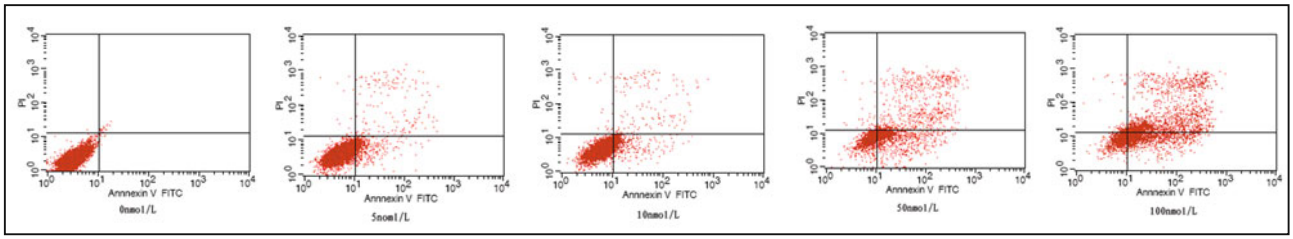

Fig.3. Detection of apoptosis in K562/DNR cells by FCMS after treatment by different concentrations of bortezomib for 24 h.

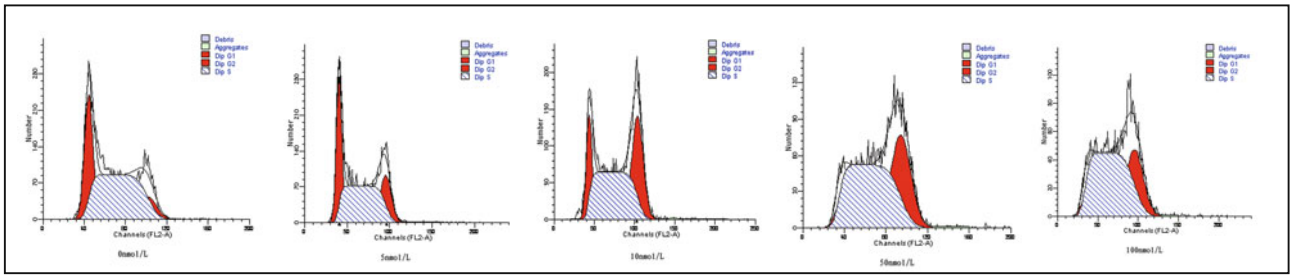

Fig.4. Detection of cell cycle phase distribution change in K562/DNR cells by FCMS after treatment by different concentrations of bortezomib for 24 h.

tion from G0 + G1 phase to G2 + M phase, entering cell proliferate cycle, and thus improve the chemotherapy sensitivity of leukemia cells.

Table 3. Changes in cell cycle phase distribution in K562/DNR after treatment by different concentrations of bortezomib for 24 h (%).

Bortezomib concentration (nmol/L)	G0/G1 phase	S phase	G2/M phase	Cell apoptotic rate (%)
0 (control)	30.13	59.03	10.84	0.30
5	29.93	50.78	19.29	9.79
10	16.63	50.76	32.61	10.95
50	4.18	59.85	35.97	60.74
100	4.45	65.57	29.98	73.25

Discussion

Although recent studies suggest that many signaling pathways are associated with the conventional cytotoxic drug resistance, the major cause is the overexpression of P-gp. Overexpression of mdr1 gene and its product P-gp lead to increased drug efflux, reducing the concentration of chemotherapeutic drugs within leukemia cells, through which leukemia cells escape destruction by chemotherapy and cause tolerance[14]. Many studies show that, P-gp overexpression is correlated with poor prognosis, high recurrence rate, drug resistance and short survival, therefore reversing the MDR induced by P-gp is greatly significant in tumor therapy.

The latest research shows[15-17] that NF-κB could regulate P-gp-mediated drug resistance. A 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.[16] found the mdr1mRNA expression in human breast cancer cell line MCF27 could be regulated by NF-κB/p65 and c-Fos transcription factor protein complex. Proteasome inhibitor bortezomib proteasome could significantly inhibit NF-κB activation by preventing degradation of I-κB (a negative regulator of NF-κB). Though it may cause decreased expression of MDR1 gene, reduced P-gp and thereby reversing MDR in leukemia cells. Our results confirmed that after treated with different concentrations of bortezomib for 24 h, the expression of P-gp/MDR1-mRNA in K562/DNR cells decreased and had dose-dependence. It suggests that bortezomib can modulate leukemia MDR at nucleic acid and protein levels. This is consistent with results by Fekete et al.[18] Cells that have acquired MDR have strong survival ability and are able to live in a toxic environment[19]. This study suggests that bortezomib can increase the accumulation of DNR in drug resistant leukemia cells, it checks up with the decreasing of P-gp/MDR1-mRNA.

Apoptosis is a strictly organized programmed cell death[20,21]. Apoptosis dysfunction leads to population expansion of the redundant cell. However, because that chemotherapy and radiotherapy-induced tumor cell death is mostly through activation of tumor cell apoptosis[22], inhibition of apoptosis is likely to cause resistance of cancer cells. As a response to chemotherapy and radiotherapy, tumor cells increase the production of survival proteins that could inhibit cell apoptosis and protect cells from death[23]. Adams et al.[24] found that inhibition of proteasome activity could cause accumulation of p53, p27, pro-apoptotic factor Bax in the cell to activate the mitochondrial apoptosis pathway. Some literatures also reported that proteasome inhibitor bortezomib could induce tumor cell arresting in G2/M phase, increase p27 expression and increase the permeability of mitochondrial outer membrane through inhibiting NF-*k*B and stabilizing p53, p21, p27, Bax, caveolin-1 and I*k*B-α, thereby inducing cell death through joint action of caspase-dependent and independent pathways[25-27]. Bortezomib used in this study could induce apoptosis of K562/DNR. P-gp MFI and the apoptosis rate were negatively correlated, indicating that bortezomib reverses MDR through the interaction of a variety of pathways involved in MDR.

Tumor cells at different proliferation stages could exist in 1 tumor cell population. Chemotherapy sensitivity of tumors is correlated with proliferation rate, cell cycle time and doubling time. Generally, tumors with a high rate of proliferation, cell cycle time and short doubling time are expected to have better treatment efficacy, or may even be cured by chemotherapy. Even in tumors sensitive to chemotherapy, existence of some

non-sensitive non-proliferating cells often becomes the source of recurrence later, since these cells can enter proliferation cycle again after the sensitive proliferating cells are killed. In this study we showed that bortezomib significantly increased distributions in G2 + M phase and S phase while significantly reducing distribution in G0 + G1 phase. Furthermore, proliferation rate was increased, cell cycle and doubling time were shortened, and ratio of non-sensitive non-proliferating cells was reduced, thereby increasing the sensitivity to chemotherapy in multidrug resistant leukemia cells and reversing the MDR. In addition, these data further suggest that bortezomib can also reverse the NF-κB-mediated anti-apoptosis mechanism in MDR.

In summary, proteasome inhibitor bortezomib can induce multidrug resistant leukemia cells entering the G2 + M phase from G0 + G1 phase; and thereby enhance the chemosensitivity of leukemia. In addition, it may also reverse the MDR in leukemia medicated by mdr1 gene and P-gp overexpression or anti-tumor cell apoptosis mechanism; this confirms that bortezomib can regulate leukemia MDR at nucleic acid and protein levels. Further studies are needed to prove the above hypothesis. Elimination of multidrug resistance leukemia cells are the main challenge to treat AML since recurrence in many leukemia patients is due to the presence of drug-resistant residual leukemia cells. The potential of bortezomib as an ideal drug to eliminate these multidrug resistance leukemia cells needs to be further explored.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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