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Molecular mechanisms of converting K562/DNR cellular drug-resistance by bortezomib

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Abstract. – OBJECTIVES: The aim of this study was to observe the effects of bortezomib (PS341) on the expression of NF-κB (nuclear factor-kappa B), IκB (inhibitor kB) and P-gp (P-glycoprotein) of K562 cells induced by daunorubicin (K562/DNR).

MATERIALS AND METHODS: MTT method was used to determine the drug resistance of K562 cells and the cellular toxicity of bortezomib. Detect the expression of NF-κB, IκB and P-gp of K562/DNR 36 hours after receiving the treatment of 100 μg/ml DNR only or added with 0.4 μg/L, 4 μg/L and 40 μg/L bortezomib, and 12 hours and 24 hours after receiving the treatment of 100 μg/ml DNR only or added with 4 μg/L bortezomib by Western blot. Detect the apoptosis rate in each group by flow cytometry respectively and the activity of NF-κB was detected by ELISA method.

RESULTS: Compared with the control group, the expressions of NF-κB and P-gp in K562/DNR could be induced by DNR. When K562/DNR were cultured with bortezomib, the expressions of NF-κB and P-gp induced by DNR were significantly suppressed and this effcet increased with the increase of the concentration or the action time of bortezomib.

CONCLUSIONS: Proteasome inhibitor bortezomib could convert the cellular drug resistance to promote cell apoptosis, and this effect showed the characteristic of concentration-dependent and time-dependent pattern.

Key Words:
Proteasome inhibitor, NF-κB, IκB, P-gp, K562 cells.

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. NF-κB (nuclear factor-kappa B) is a transcription factor present in eukaryocytes universally and binds to cytoplasmic inhibitor κB (IκB) under normal condition. The phosphorylated IκB in response to stress reaction is recognized and degraded by proteasomes. Once NF-κB is released, then, enters the nucleus, it binds to specific κB sequences to induce or up-regulate multiple drug resistant gene (MDR1 genes) expression. The P-gp (P-glycoprotein) is an ATP-dependent drug pump coded by MDR1 genes which can reduce intracellular drug concentration by pumping the drug out of the cell, resulting in cells with drug-resistant properties[1-5].

Bortezomib (PS-341) is an antitumor drug of proteasome inhibitor family and mainly used to treat recurrent and refractory multiple myeloma in clinical practices[6-10], but its application in leukemia therapy is still in the period of theoretical and exploratory study[11-13]. In this study, the *in vitro* methods were used to examine the effects of various PS-341 action times on NF-κB, IκB and P-gp gene expression of drug-resistant leukemia cell strain K562/DNR. The molecular mechanisms and action properties for PS-341 to reverse drug-resistance were also explored in order to provide experimental evidence to overcome multiple drug resistance in leukemia therapy.

Materials and Methods

Cells

Human leukemia multiple drug resistant cell strain K562/DNR was kindly provided by Liu Yunpeng professor, the Head of Laboratory of Oncology Department, the first affiliated Hospital of China Medical University. The drug-resistant activity of this cell strain was induced and maintained by 0.5 mmol/L DNR.

Cell Culture

K562 drug sensitive strain (K562/S) and drug resistant strain K562/DNR were cultured in Roswell Park Memorial Institute (RPMI) 1640 media containing 12% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin

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at 37^0C, 5% CO_2 and saturated humidity. Experiments were started when cultured cells reached exponential growth phase after multi-passages. K562/DNR cells were cultured in media containing 0.5 mmol/L DNR to maintain their drug-resistance. Cells were cultured in drug-free media for 2 weeks before experiments.

Identification of Drug-Resistant Strains

The experiment was divided into two groups: K562/S group and K562/DNR group. Cell concentration was adjusted to 2×10^5/mL, 100 µl of cell suspension and various concentrations of DNR (0-400 µg/mL) were added to 96-well plates with 5 parallel wells for each concentration. A blank group and a drug-free control group were designed for each experimental group. 20 µL of MTT (5 g/L) was added to each well after 72 hours and the cells were continued to culture for 4 additional hours. The supernatant was discarded after centrifugation and 150 µL of dimethyl sulfoxide (DMSO) were added to terminate reaction. The samples were vortexed under light-proof condition for 15 minutes. Then, the absorbance (*A*) was detected at 570 nm using an automatic ELISA analyzer and used to calculate the growth inhibition rate.

$$\text{Growth inhibition rate} = \frac{\left(\begin{array}{c}\text{Control}\\ \text{group}\\ A\ \text{value}\end{array} - \begin{array}{c}\text{Experimental}\\ \text{group}\\ A\ \text{value}\end{array}\right)}{\left(\begin{array}{c}\text{Control}\\ \text{group}\\ A\ \text{value}\end{array} - \begin{array}{c}\text{Blank}\\ \text{group}\\ A\ \text{value}\end{array}\right)} \times 100\%$$

The median inhibitory concentration (IC_{50}) was calculated based on the growth inhibition rate obtained.

$$\text{Drug-resistance folds} = \frac{IC_{50}\ \text{(drug-resistant cells)}}{IC_{50}\ \text{(sensitive cells)}}$$

Determination of PS-341 Direct Cytotoxic Activity

MTT method was used to determine direct cytotoxic activity of PS-341 as described above. Different concentrations of PS-341 at 0.4 µg/L, 4 µg/L and 40 µg/L were added to each well. The absorbance of the blank control groups was designated as 100% survival rate. Relative survival rate (%) of each group = absorbance of the experimental group/the absorbance of the blank group×100%. The calculated PS-341 concentration at 90% survival rate was IC_{10}.

K562 drug-resistant cell strains were incubated with 100 µg/ml DNR alone or in combination with 0.4 µg/L, 4 µg/L and 40 µg/L PS-341 for 36h. Western blot was used to examine NF-κB, IκB and P-gp expression of each group. NF-κB p65 activity was assayed with ELISA method, and apoptosis was detected with flow cytometry, as follows.

Western Blot

1×10^7 cells were removed and 200 µl of cell disruption buffer were added in the tube containing cells. Cell total proteins were extracted and quantified with Larry method. 50 µg of protein samples was loaded onto 15% sodium dodecyl sulphate (SDS)-polyacrylamide gel for electrophoresis (100V, 1.5h). When bromophenol blue immersed into the bottom of the gel, the proteins were blotted onto the nitrocellulose membrane. The membrane was incubated with anti-NF-κB, IκB and P-gp antibodies and anti β-actin antibodies before adding alkaline phosphatase (AP) labeled corresponding secondary antibodies. The o-dianisidine, tetrazotized and β-naphthyl acid phosphate developers were used to develop colors for image analysis with a gel imaging analyzer.

ELISA

Nucleoprotein extraction and experimental procedure followed by the reagent kit instruction (Active motif).

Flow Cytometry

Cells were washed twice with cold PBS (phosphate buffered saline) and resuspended in 400 µ1×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. Propidium iodide (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.

Statistical Analysis

A gel imaging analyzer was used for gray intensity analysis. The expression for each parameter was presented as: absorbance of each parameter/β-actin absorbance ×100%. SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The *t*-test was used to compare means between the groups and χ^2-test were used to compare the apoptosis rates in each group.

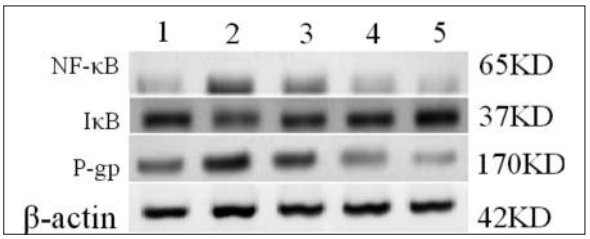

Figure 1. The express of NF-κB, IκB and P-gp levels as determined by Western blot after incubation with DNR alone or in combination with 0.4 μg/L, 4 μg/L and 40 μg/L PS-341, respectively, for 36h. (1: negative control group; 2: DNR group; 3: DNR + 0.4 μg/L PS-341 group; 4: DNR + 4 μg/L PS-341 group; 5: DNR + 40 μg/L PS-341 group).

Results

Drug-Resistance of K562/DNR Cells

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

Results of PS-341 Direct Cytotoxic Activity as Determined by MTT Method

The IC_{10} for PS-341 effect on cell strain K562/DNR was 4 μg/L. Since 90% cells survived when PS-341 concentration was below 4 μg/L, this concentration was considered to be non-cytotoxic.

Western Blot

After 36h incubation with DNR alone or in combination with 0.4 μg/L, 4 μg/L and 40 μg/L PS-341, respectively, the express of NF-κB, IκB and P-gp levels as determined by Western blot and the changes in NF-κB p65 activity shown in Figure 1, Figure 2 and Table I. Since 90% cells survived when PS-341 concentration was the IC_{10}, this concentration was considered to be non-cytotoxic. Therefore, the IC_{10} or less was selected as the concentration for PS-341 to reverse drug-resistance different times in this study. The direct cytotoxic activity of PS-341 was 4 μg/L,

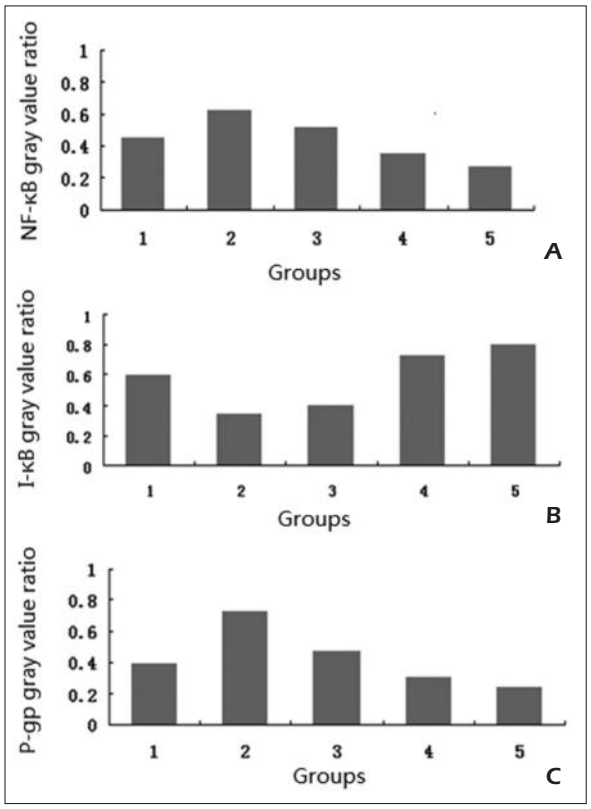

Figure 2. Western blot gray intensity analysis for the groups as described above. ***A,*** NF-κB gray intensity: compare with negative control group, DNR can up-regulate the expression of NF-κB, when PS-341 was added, the expression of NF-κB decreased, strength of PS-341 action increased with concentration in an apparent concentration -dependent manner. ***B***, IκB gray intensity: compare with the negative control group, DNR can down-regulate the expression of IκB, when PS-341 was added, the expression of IκB increased, strength of PS-341 action increased with concentration in an apparent concentration-dependent manner. ***C,*** P-gp gray intensity: compare with negative control group, DNR can up-regulate the expression of P-gp, when PS-341 was added, the expression of P-gp decreased, strength of PS-341 action increased with concentration in an apparent concentration-dependent manner;

Table I. Changes in NF-κB p65 activity after incubation with DNR alone or in combination with PS-341 in different concentration for 36h ($\bar{x} \pm s$, %).

PS-341 concentration	n	DNR group	DNR+PS-341group	t	p
0.4 μg/L	10	25.9 ± 2.46	20.3 ± 1.96	1.85	> 0.05
4 μg/L	10	26.9 ± 2.58	6.08 ± 2.53	3.98	< 0.01
40 μg/L	10	27.2 ± 2.35	4.58 ± 1.56	4.26	< 0.01

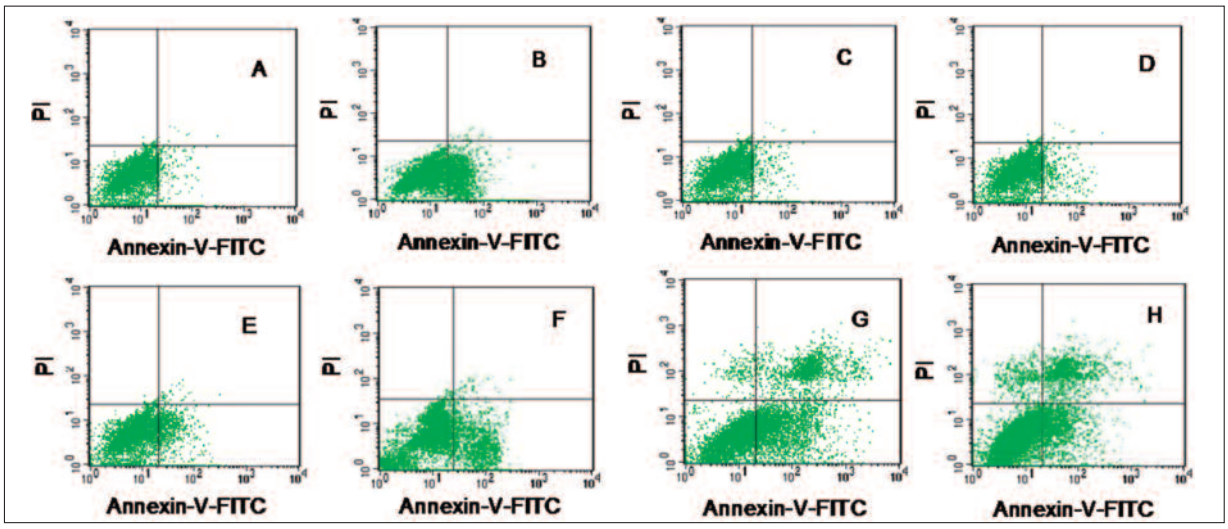

Figure 3. Apoptosis rates as determined with flow cytometry after incubation with DNR alone or in combination with PS-341 in different concentration for 36h. ***A,*** Negative control group. ***B,*** DNR group; ***C,*** 0.4 μg/L PS-341 group. ***D,*** 4 μg/L PS-341 group. ***E,*** 40 μg/L PS-341 group. ***F,*** DNR+0.4 μg/L PS-341 group; ***G,*** DNR+4 μg/L PS-341 group. ***H,*** DNR+40 μg/L PS-341 group; compare with negative control group, DNR group, 40 μg/L PS-341 group and DNR in combination with different concentration, apoptosis rate increased evident, but it's not as evident as it was in 0.4 μg/L PS-341 group and 4 μg/L PS-341 group; compare with DNR treated group, apoptosis rate induced by DNR increased when PS-341 added, and apoptosis rate increased with concentration of PS-341 in concentration-dependent manner; compare with 40 μg/L PS-341 group, apoptosis rate of DNR+40 μg/L PS-341 group increased.

analysed the data in Figure 1, compared to DNR group, the activity of NF-κB was inhibited distinctively in DNR + 4 μg/L PS-341 group, however, inhibiting effect weakened in DNR + 0.4 μg/L PS-341 group, so 4 μg/L was chosen to be the concentration to reverse cell drug-resistance for different times. After 12 h, 24 h and 36 h incubation with DNR alone or in combination with 4 μg/L PS-341, the express of NF-κB, IκB and P-gp levels as determined by Western blot and the changes in NF-κB p65 activity shown in Figure 4, Figure 5 and Table II.

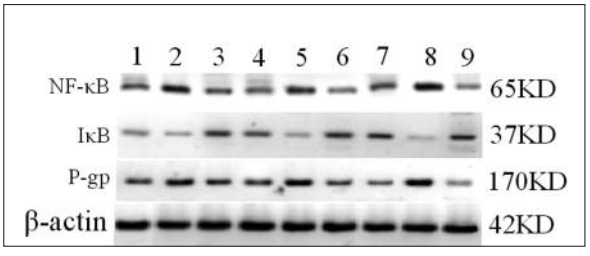

Figure 4. The express of NF-κB, IκB and P-gp levels as determined by Western blot after incubation with DNR alone or in combination with PS-341 for various times. (1: 12 h negative control group; 2: 12 h DNR group; 3: 12 h DNR+PS-341 group; 4: 24 h negative control group; 5: 24 h DNR group; 6: 24 h DNR+PS-341 group; 7: 36 h negative control group; 8: 36 h DNR group; 9: 36 h DNR+PS-341 group.

ELISA, Flow Cytometry

The flow cytometric results after incubation with DNR alone or in combination with PS-341 in different concentration for 36h were shown in Figure 3 and Table III.

The flow cytometric results after incubation with DNR alone or in combination with 4 μg/L PS-341 for various times were shown in Figure 6 and Table IV.

Table II. Apoptosis rates in each group after incubation with DNR alone or in combination with PS-341 in different concentration for 36h ($\bar{x} \pm S$).

Groups	n	Apoptosis rate (%)
Control group	5	4.25 ± 0.36
DNR group	5	22.47 ± 4.58*
0.4 μg/L PS-341 group	5	5.35 ± 1.78
4 μg/L PS-341 group	5	6.06 ± 2.58
40 g/L PS-341 group	5	23.26 ± 2.21*
DNR+0.4 μg/L PS-341 group	5	30.96 ± 5.21*,$
DNR+4 μg/L PS-341 group	5	43.58 ± 7.69*,#
DNR+40 μg/L PS-341 group	5	55.97 ± 9.25*,#,&

Note: *Compared to the control group; $p < 0.01$; $Compared to DNR group; $p < 0.05$; #Compared to DNR group, $p < 0.01$; &Compared to 40 μg/L PS-341 group, $p < 0.01$.

Table III. Changes in NF-κB p65 activity after incubation with DNR alone or in combination with PS-341 ($\bar{x} \pm s$, %).

Incubation time	n	DNR group	DNR+PS-341 group	t	p
12h	10	23.8 ± 2.27	15.3 ± 1.87	2.03	< 0.05
24h	10	25.4 ± 1.98	10.2 ± 1.69	3.02	< 0.01
36h	10	26.9 ± 2.58	6.08 ± 2.53	3.98	< 0.01

Discussion

Multi-drug resistance is the urgent problem to solve for leukemia therapy[14,15]. Hu et al[16] conducted *in vitro* culture of leukemic cells isolated from blood of 19 acute myelocytic leukemia patients with epirubicin, daunorubicin, darubicin and cytarabine for 16 hours. They found that MDR1 and P-gp expression were up-regulated in MDR1/P-gp positive cells, while MDR1 and P-gp low express in MDR1/P-gp negative cells. Similar phenomenon was observed in the *in vivo* study with daunorubicin/cytarabine standard chemotherapy regimen. They considered up-regulation of MDR1 expression as a normal reaction of leukemic cells in response to cytotoxic stress. Antitumor drugs were able to activate NF-κB while killing tumor cells. The activated NF-κB entered the nucleus and induced MDR1 gene expression. This could further facilitate leukemia drug-resistance. This experiment aimed to reveal whether proteasome inhibitor can inhibit proteasome activity, inhibit IκB degradation, prevent NF-κB release, inhibit NF-κB initiated genes transcription and reduce MDR1 expression, resulting in reduced P-gp production, converting leukemia cellular drug resistance by inhibiting NF- b pathway[17,18].

Table IV. Apoptosis rates in each group after incubation with DNR alone or in combination with PS-341 for various times ($\bar{x} \pm S$).

Groups	n	Apoptosis rate (%)
Control group	5	4.25 ± 0.36
DNR action 12h group	5	15.56 ± 4.12*
DNR+PS-341 12h group	5	35.23 ± 5.15#,$
DNR action 24h group	5	17.25 ± 2.89*
DNR+PS-341 24h group	5	40.26 ± 6.89#,$
DNR action 36h group	5	22.47 ± 4.58*
DNR+PS-341 36h group	5	43.58 ± 7.69#,$

Note: *Compared to the control group, $p < 0.05$; #Compared to the control group, $p < 0.01$; $Compared to corresponding DNR group, $p < 0.01$.

Certain reports involving K562 cell strains indicated that PS-341 is able to inhibit IκB degradation, prevent NF-κB release, inhibit NF-κB initiated genes transcription and reduce MDR1 expression by inhibiting proteasome activity, re-

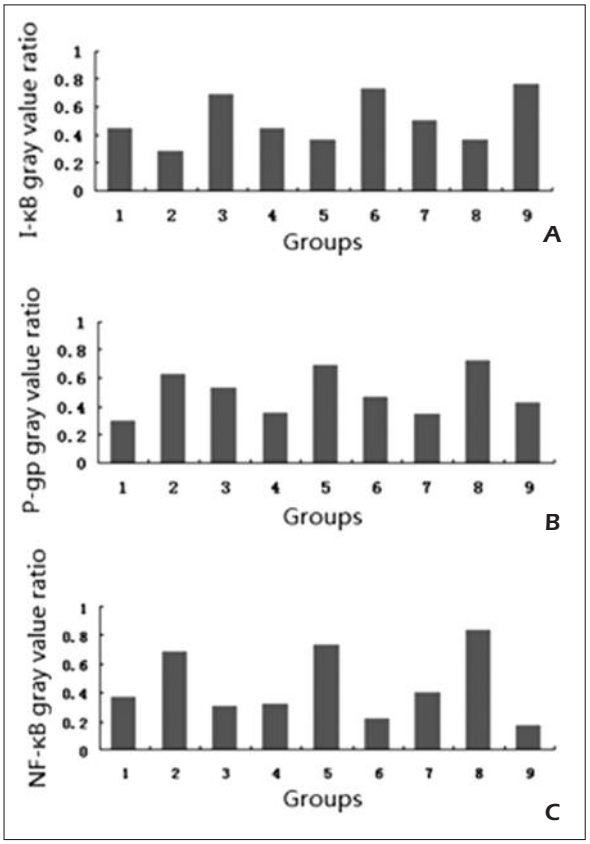

Figure 5. Western blot gray intensity analysis for the groups as described above. ***A,*** NF-κB gray intensity: compare with negative control group, DNR can up-regulate the expression of NF-κB, when PS-341 was added, the expression of NF-κB decreased, strength of PS-341 action increased in an apparent time-dependent manner; ***B,*** IκB gray intensity: compare with the negative control group, DNR can down-regulate the expression of IκB, when PS-341 was added, the expression of IκB increased, strength of PS-341 action increased in an apparent time-dependent manner; ***C,*** P-gp gray intensity: compare with negative control group, DNR can up-regulate the expression of P-gp, when PS-341 was added, the expression of P-gp decreased, strength of PS-341 action increased in an apparent time-dependent manner.

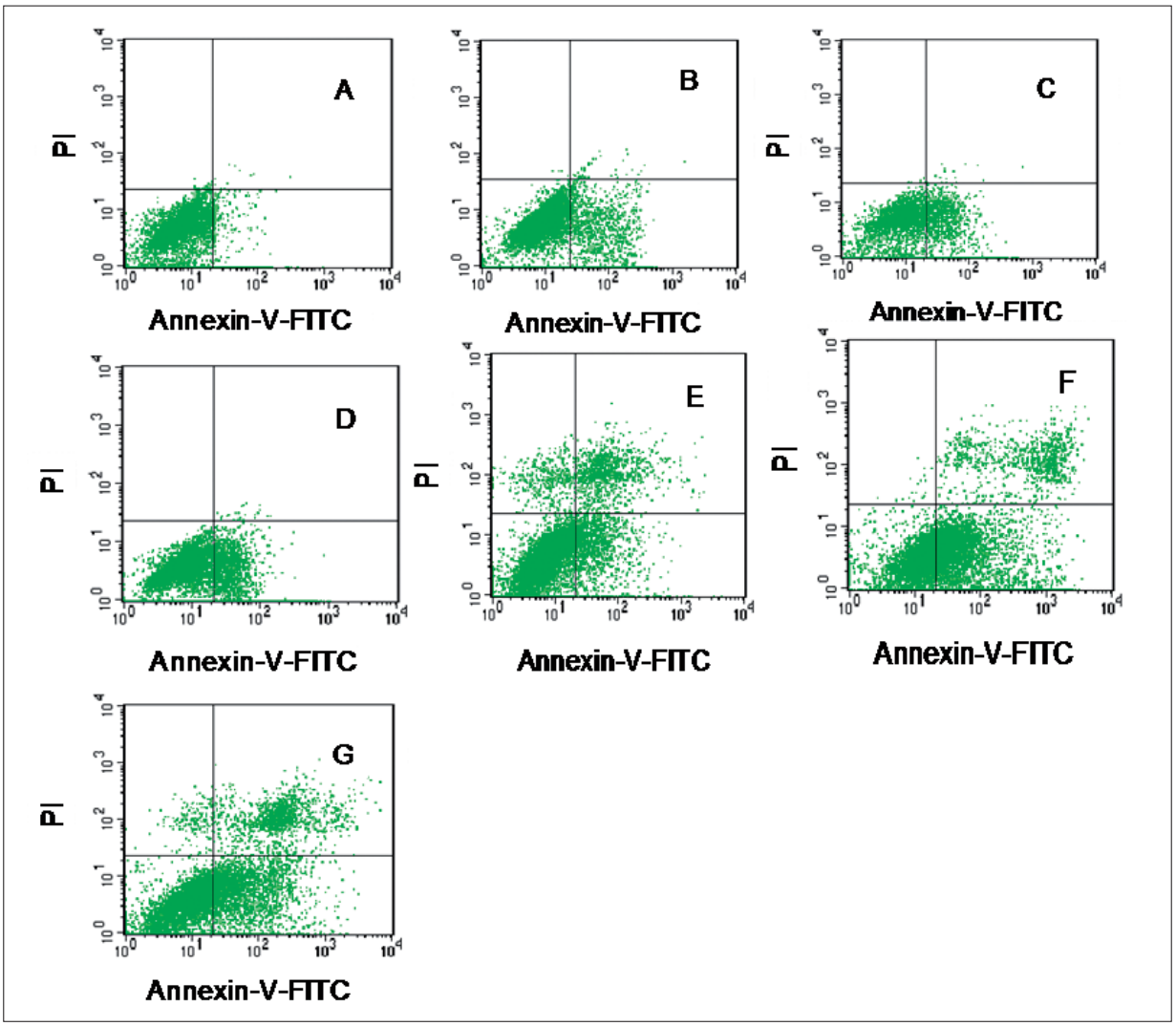

Figure 6. Apoptosis rates as determined with flow cytometry after incubation with DNR alone or in combination with PS-341 for various times. ***A,*** Negative control grou. ***B,*** DNR 1 2h group. ***C,*** DNR 24 h group. ***D,*** DNR 36 h group. ***E,*** DNR+4 μg/L PS-341 12 h group. ***F,*** DNR+4 μg/L PS-341 24 h group. ***G,*** DNR+4 μg/L PS-341 36 h group; compared to corresponding DNR group, apoptosis rate induced by DNR increased when PS-341 added, and apoptosis rate went higher as action time prolonged.

sulting in reduced P-gp production. No reports are found to examine whether PS-341 has similar effects on cell strain K562/DNR to reverse drug-resistance.

Zheng et al[19] reports PS-341 plus DNR had synergistic effect on antiproliferation (synergistic ratio > 1). In our study, multi-drug resistant cell strain K562/DNR was incubated with respective 100 μg/ml DNR alone or in combination with PS-341 as 0.4 μg/L, 4 μg/L and 40 μg/L for 36 h; K562/DNR was also incubated with respective 100 μg/ml DNR alone or in combination with PS-341 for 12 h, 24 h and 36 h to analyze the above parameters. The results showed that IκB increased, NF-κB and P-gp expression decreased significantly, and apoptosis rates increased gradually with high concentration prolonged PS-341 incubation time within certain time frame. It was suggested that PS-341 had made it possible for continuous binding between IκB and NF-κB by inhibiting IκB degradation. It could also decrease MDR1 expression and P-gp production by preventing entry of NF-κB into the nucleus and binding with MDR1 genes. As a result, more chemotherapeutic agents could enter the cells to increase apoptosis and reverse leukemic cell drug-resistance. The results also showed that strength of PS-341 action to reverse drug-resis-

tance increased gradually with time and concentration in an apparent time-dependent and concentration-dependent manner.

In this experiment, 100 μg/ml DNR was acted on K562/DNR, the expression of NF-κB and P-gp increased compared to control group. The experiment result accorded with Hu et al[16] as it was stated above, maybe there was same mechanism in it.

Certain studies showed that determination of NF-κB activity was more effective than quantitative analysis alone in demonstrating its anti-apoptotic effects. Because NF-κB needed to enter the nucleus to acquire transcription activity and activate 1 gene expression[20-22], both NF-κB expression level and NF-κB activity were determined in this study. The results showed that NF-κB activity tended to decrease and apoptosis tended to increase with prolonged PS-341 action, providing further evidence for PS-341 to reverse leukemic cell drug-resistance through NF-κB pathway.

This experiment showed that IC_{10} of cell strain K562/DNR acted on PS-341 was 4 μg/L. It means that when 0.4 μg/L PS-341 alone or 4 μg/L PS-341 alone adds, there is no apoptosis, but when combines with DNR, apoptosis rate increases, which can be regarded as it is able to reverse leukemic cell drug resistance in this way. It provides laboratory evidence for small dose proteasome inhibitor combining with traditional chemotherapeutics treat drug-resistant and refractory leukemia in clinical practices. In this study, it is also founded that apoptosis rate in 40 μg/L PS-341 alone group is higher than in control group, which explains that under this concentration, PS-341 could reserve drug-resistance on one hand, on the other hand, it has the effect to promote apoptosis. It is speculated that proteasome inhibitor plays a role in antitumour effect through activating the blocked pathway of drug-resistant cell[23].

Conclusions

As shown by the current findings, NF-κB was closely associated with leukemic multiple drug resistance. PS-341 was able to reverse leukemic cell drug resistance by targeting at NF-κB and enhance chemotherapeutic sensitivity. The finding is of significant values in preventing and overcoming leukemic cell multiple drug resistance and in exploring new therapeutic methods for leukemia treatment. Since PS-341 is expensive, how to choose the most appropriate concentration has become an urgent problem to solve in clinical practices. This experiment for clinical drug application provides certain evidence for laboratory, but further study is still needed *in vivo*.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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