Original Article

Reversion of Multidrug-Resistance by Proteasome Inhibitor Bortezomib in K562/DNR Cell Line

Hui-han Wang[1], Ying-chun Li[1], Ai-jun Liao[1], Bei-bei Fu[1], Wei Yang[1], Zhuo-gang Liu[1*], Xiao-bin Wang[2]

[1]*Department of Hematology,* [2]*Department of Urology, Shengjing Hospital, China Medical University, Shenyang 110004, China*

DOI: 10.1007/s11670-011-0069-4

© Chinese Anti-Cancer Association and Springer-Verlag Berlin Heidelberg 2011

ABSTRACT

Objective: To observe the reversion of multi-drug resistance by proteasome inhibitor bortezomib in K562/DNR cell line and to analyze the possible mechanism of reversion of multidrug-resistance.

Methods: MTT method was used to determine the drug resistance of K562/DNR cells and the cellular toxicity of bortezomib. K562/DNR cells were cultured for 12 hours, 24 hours and 36 hours with 100 μg/ml DNR only or plus 4 μg/L bortezomib. The expressions of NF-κB, IκB and P-gp of K562/DNR were detected with Western blot method, the activity of NF-κB was tested by ELISA method and the apoptosis rate was observed in each group respectively.

Results: The IC_{50} of DNR 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. The IC_{10} of PS-341 on Cell strain K562/DNR was 4 μg/L. Therefore, 4 μg/L was selected as the concentration for PS-341 to reverse drug-resistance in this study. DNR induced down-regulation of IκB expression, up-regulation of NF-κB and P-gp expression. After treatment with PS-341, a proteasome inhibitor, the IκB degradation was inhibited, IκB expression increased, NF-κB and P-gp expression decreased in a time dependent manner. Compared to DNR group, the NF-κB p65 activity of DNR+PS-341 group was decreased. Compared to corresponding DNR group, DNR induced apoptosis rate increases after addition of PS-341 in a time dependent manner.

Conclusion: Proteasome inhibitor bortezomib can convert the leukemia cell drug resistance. The mechanism may be that bortezomib decreases the degradation of IκB and the expression of NF-κB and P-gp, therefore induces the apoptosis of multi-drug resistant cells.

Key words: Bortezomib; NF-κB; Multi-drug resistance; mdr1 gene; P-gp; K562 cells

INTRODUCTION

Multi-drug resistance is the obstacle for leukemia therapy, many researchers focus on how to reverse it.

Nuclear factor-kappa B (NF-κB) is a transcription factor present in eukaryocytes universally and binds to cytoplasmic 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-glycoprotein (P-gp) 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[1-4].

Bortezomib (PS-341) is an antitumor drug of proteasome inhibitor family and mainly used to treat recurrent and refractory multiple myeloma in clinical practices[5-7], but its application in leukemia therapy is still in the period of theoretical and exploratory study[8, 9].

In this study, we examined the effects of bortezomib on NF-κB, IκB and P-gp gene expressions of drug-resistant leukemia cell strain K562/DNR. The molecular mechanisms 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

Cell Lines and Reagents

Human leukemia multiple drug resistant cell strain K562/DNR was kindly provided by Pro. Liu Yunpeng, Laboratory of Oncology Department of the First Affiliated

Received 2010–08–23; **Accepted** 2011–01–16
This work was supported by Educational Commission of Liaoning Province, China (No. 20060985).
*Corresponding author.
E-mail: liuzg@sj-hospital.org

Hospital of China Medical University and maintained in media containing 0.5 mmol/L DNR.

Dimethylsulfoxide (DMSO) was the product of Amreson American Company. The rabbit anti-human NF-κB, IκB and P-gp antibodies were products of American SANTA CRUZ Company. Alkaline phosphatase labeled goat anti-rabbit and horse anti-mouse antibodies were products of Beijing Zhongshan Company. Mouse anti β-actin antibody was the product of American Sigma Company. Apoptosis kit was from Beijing Biosea Company. NF-κB ELISA kit was the product of American Active Motif. PS-341 was from Xian-Janssen Pharmaceutical, Ltd..

Cell Culture

Drug sensitive K562 strain (K562/S) and drug resistant strain K562/DNR were cultured in RPMI-1640 media supplemented with 12% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃, in a humidified atmosphere containing 5% CO_2. Experiments were started when cultured cells reached exponential growth phase after multi-passages. K562/DNR cells were maintained in media containing 0.5 mmol/L DNR and then cultured in DAN-free media for 2 weeks before experiments.

Cytotoxicity assessments by MTT Assay

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. MTT 20 μl (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 were added to terminate the reaction. The absorbance (A) was detected at 570 nm and the growth inhibition rate calculated as follows:

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

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

Drug-resistance folds = IC_{50} (drug-resistant cells)/IC_{50} (sensitive cells).

Determination of PS-341 Direct Cytotoxic Activity

MTT method was also used to determine the direct cytotoxic activity of PS-341 as described above. Bortezomib of different concentrations at 0.4 μg/L, 4 μg/L and 40 μg/L were added to each well. Relative survival rate (%) of each group = absorbance of the experimental group/the absorbance of the control group×100%. The calculated PS-341 concentration at 90% survival rate was IC_{10}. A concentration below IC_{10} was selected as the experimental concentration for PS-341 to reverse drug-resistance.

Examination of NF-KB, IKB and P-Gp Expressions by Western Blot Method

K562 drug-resistant cell strains were incubated with 100 μg/ml DNR alone or in combination with 4 μg/L PS-341 for 12 h, 24 h and 36 h. Western blot was used to examine NF-κB, IκB and P-gp expressions of each group. Cell disruption buffer 200 μl were added in the tube containing 1×10^7 cells. Cell total proteins were extracted and quantified with Larry method. 50 μg of protein samples was loaded onto 15% SDS-polyacrylamide gel for electrophoresis (100V, 1.5 h). When bromophenol blue reached to 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. 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%.

Detection of NF-κB Activity Using ELISA Method

NF-κB p65 activity was assayed with ELISA method using an NF-κB ELISA kit. Cell suspensions were centrifugated and the supernates were assayed immediately. The microtiter plate was pre-coated with an antibody specific to NF-κB p65. The supernates were added to the appropriate microtiter plate wells 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 incubated. Then a TMB substrate solution was added to each well. Only those wells that contain NF-κB p65, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric 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 the standard curve.

Detection of Apoptosis Using Flow Cytometry

Apoptosis rate of each groups were determined with flow cytometry followed the Apoptosis kit (Beijing Biosea Company) instruction. Cells were washed twice with cold PBSand resuspend in 400 μl 1× binding buffer at a concentration of 1×10^6 cells/ml. Add 5 μl of Annexin V–FITC and gently vortex the cells and incubate for 15 min at 4-8℃ in the dark. Add 10 μl of PI to tube for another 5 min at 4-8℃ in the dark. And then subject to flow cytometry.

Statistical Analysis

SPSS software was used for statistical analysis. The t-test was used to compare means between the groups incubation with DNR alone or in combination with PS-341 and χ^2-test were used to compare the apoptosis rates in each group after incubation with DNR alone or in combination with PS-341 for various times.

RESULTS

Cytotoxic Effect of Bortezomib on K562/DNR Cells

The IC_{50} of DNR K562/S and K562/DNR groups were

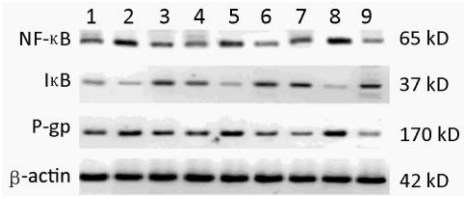

Figure 1. The expressions of NF-κB, IκB and P-gp determined by Western blot after incubation with DNR alone or in combination with bortezomib for various times, (1: 12 h negative control group; 2: 12 h DNR group; 3: 12 h DNR+bortezomib group; 4: 24 h negative control group; 5: 24 h DNR group; 6: 24 h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

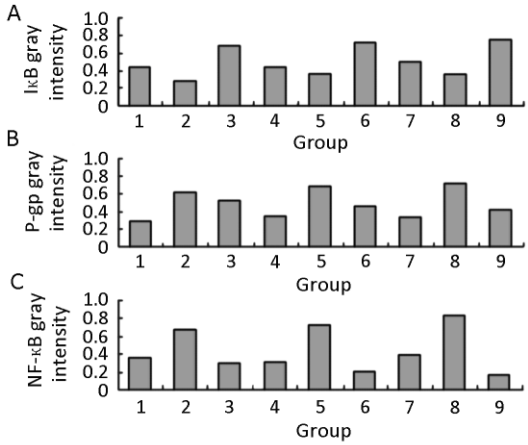

Figure 2. Western blot gray intensity analysis in K562/DNR cells with different treatment. A: IκB gray intensity; B: P-gp gray intensity; C: NF-κB gray intensity. Group 1: 12 h negative control; 2: 12 h DNR; 3: 12 h DNR+bortezomib; 4: 24 h negative control; 5: 24 h DNR; 6: 24 h DNR+ bortezomib; 7: 36 h negative control; 8: 36 h DNR; 9: 36 h DNR+ bortezomib.

1.16 μg/ml and 50.43 μg/ml, respectively. When 100 μg/ml DNR was added, K562/DNR cell survival rate was 80% and the cells demonstrated clear drug-resistant property. Therefore, 100 μg/ml was chosen as the DNR experimental concentration for each group.

The IC_{10} of PS-341 on Cell strain K562/DNR was 4 μg/L. Since 90% of the cells survived when PS-341 concentration was below 4 μg/L, this concentration was considered to be non-cytotoxic. Therefore, 4 μg/L was selected as the concentration for PS-341 to reverse drug-resistance in this study.

Changes of NF-κB, IκB and P-gp Expressions

After 12 h, 24 h and 36 h incubation with DNR alone or in combination with 4 μg/L PS-341, the expression of NF-κB, IκB and P-gp were determined by Western blot (shown in Figure 1 and Figure 2). Compared to the negative control group, DNR induced down-regulation of IκB expression, up-regulation of NF-κB expression and up- regulation of P-gp expression. After treatment with bortezomib, the IκB degradation was inhibited, NF-κB expression decreased and P-gp expression also decreased. The expressions changed in a time dependent manner.

NF-κB p65 Activity

Compared to DNR group, the NF-κB p65 activity of DNR and bortezomib combination group was decreased. The difference between the two groups had statistical significance. The activity of NF-κB p65 changes in a time dependent manner (Table 1).

Apoptosis of K562/DNR Cells

The flow cytometric results after incubation with DNR alone or in combination with 4 μg/L bortezomib for various times were shown in Figure 3 and Table 2. When compared to corresponding DNR group, DNR induced apoptosis rate increased after addition of bortezomib in a time dependent manner.

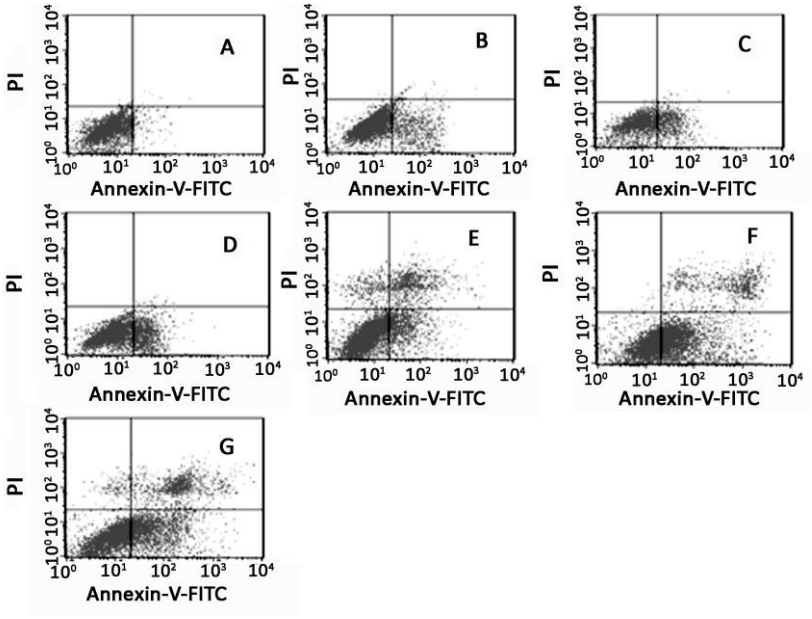

Figure 3. Apoptosis rates of K562/DNR cells determined with flow cytometry after incubation with DNR alone or in combination with bortezomib. A: Negative control group; B: DNR 12 h group; C: DNR 24 h group; D: DNR 36 h group; E: DNR+4 μg/L bortezomib 12 h group; F: DNR+4 μg/L bortezomib 24 h group; G: DNR+4 μg/L bortezomib 36 h group.

Table 1. Changes of NF-κB p65 activity after incubation with DNR alone or in combination with bortezomib in K562/DNR cells ($\bar{x}\pm s$, %)

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

Table 2. Apoptosis rates of K562/DNR cells treated with DNR alone or in combination with PS-341 for various times ($\bar{x}\pm s$, %)

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

*Compared to the control group, *P*<0.05; **Compared to the control group, *P*<0.01; #Compared to corresponding DNR group, *P*<0.01.

DISCUSSION

The proteasome inhibitor bortezomib (trade name Velcade®, previously known as PS-341), a boronic acid dipeptide, is a unique and specific inhibitor of the proteasome pathway, and mainly used proteasome pathway, and mainly used to treat recurrent and refractory multiple myeloma in clinical practices[10-13], but its application in leukemia therapy is still in the period of theoretical and exploratory study. It has functions in regulating cell cycles, degradation of intracellular active molecules and effecting intracellular signaling pathways by inhibiting the bioactivity of ubiquitin-proteasomes, and down-regulating multiple drug resistant gene (mdr1 genes) expression[14-18]. Therefore proteasome inhibition is a rational therapeutic approach both by itself and as a means to induce chemosensitization and overcome chemoresistance[19-21].

Multi-drug resistance is the obstacle for leukemia therapy, many researchers focus on how to reverse it. Although recent studies suggest that many signaling pathways are associated with the conventional cytotoxic drug resistance, the major cause is the over expression of P-gp. P-gp leads to increased drug efflux, reducing the concentration of chemotherapeutic drugs within leukemia cells, through which leukemia cells escape destruction by chemotherapy and cause drug resistance[22]. Many studies show that, P-gp over expression 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.

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, resulting 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. In this study, multi-drug resistant cell strain K562/DNR was incubated with 100 μg/ml DNR alone or in combination with bortezomib 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 prolonged PS-341 incubation time within certain time frame. It has been suggested that bortezomib can promote the continuous binding between IκB and NF-κB by inhibiting IκB degradation. It can 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 the strength of bortezomib action to reverse drug-resistance increased gradually with time in an apparent time-dependent manner.

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 needs to enter the nucleus to acquire transcription activity and to activate mdr1 gene expression[23-25], 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 treatment, providing further evidence for PS-341 to reverse leukemic cell drug-resistance through NF-κB pathway.

NF-κB and P-gp expression increased significantly when compared to the negative control group after addition of 100 μg/ml DNR to K562/DNR cell culture in this study. Hu et al.[26] 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 expressions were up-regulated in mdr1/P-gp positive cells, while mdr1 and P-gp began to 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 are able to activate NF-κB while killing tumor cells. The activated NF-κB enters the nucleus and induces mdr1 gene expression. This could further facilitate leukemia drug-resistance. The study findings are consistent with the above literature reports and suggest possible identical mechanisms of actions.

As shown by the current findings, NF-κB was closely associated with leukemic multiple drug resistance. Bortezomib 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.

Acknowledged

Liu Yunpeng Professor, the head of Laboratory of Oncology

Department, the first affiliated Hospital of China Medical University.

REFERENCES

1. Lövborg H, Oberg F, Rickardson L, et al. Inhibition of proteasome activity，nuclear factor-Kappa B translocation and cell survival by the antialcoholism drug disulfiram. Int J Cancer 2006; 118:1577-80.
2. Guzman ML, Neering SJ, Upchurch D, et al. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. Blood 2001; 98:2301-7.
3. Lei HY, Zhao XL. Clinical significance of NF-kappaB continual activity and expression of WT1 and MDR1 in acute nonlymphocytic leukemia. Zhongguo Shi Yan Xue Ye Xue Za Zhi (in Chinese) 2007; 15:253-7.
4. Fujita T, Washio K, Takabatake D, et al. Proteasome inhibitors can alter the signaling pathways and attenuate the P-glycoprotein-mediated multidrug resistance. Int J Cancer 2005; 117:670-82.
5. Ohashi K. Clinical implications of bortezomib in frontline treatment of newly-diagnosed multiple myeloma. Gan To Kagaku Ryoho 2008; 35:1029-32.
6. Wang H, Liu X, Xu B. Proteasome inhibitor induces apoptosis and influences the expression of Notch 1 and NF-kappaB in multiple myeloma RPMI8226 cells. Zhongguo Shi Yan Xue Ye Xue Za Zhi (in Chinese) 2008; 16:531-7.
7. Terpos E, Roussou M, Dimopoulos MA. Bortezomib in multiple myeloma. Expert Opin Drug Metab Toxicol 2008; 4:639-54.
8. Gil L, Styczynski J, Dytfeld D, et al. Activity of bortezomib in adult de novo and relapsed acute myeloid leukemia. Anticancer Res 2007; 27:4021-5.
9. McCloskey SM, McMullin MF, Walker B, et al. The therapeutic potential of the proteasome in leukemia. Hematol Oncol 2008; 26:73-81.
10. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003; 348:2609-17.
11. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008; 14:1649-57.
12. Rajkumar SV, Richardson PG, Hideshima T, et al. Proteasome inhibition as a novel therapeutic target in human cancer. J Clin Oncol 2005; 23:630-9.
13. Lightcap ES, McCormack TA, Pien CS, et al. Proteasome inhibition measurements: clinical application. Clin Chem 2000; 46:673-83.
14. Singh G, Alqawi O, Espiritu M. Metronomic PDT and cell death pathways. Methods Mol Biol 2010; 635:65-78.
15. Zineldeen DH, Uranishi H, Okamoto T. NF-kappaB signature on the aging wall. Curr Drug Metab 2010; 11:266-75.
16. Yamamura M, Hirai T, Yamaguchi Y. Proteasome inhibitor. Nippon Rinsho 2010; 68:1079-84.
17. Ludwig H, Khayat D, Giaccone G, et al. Proteasome inhibition and its clinical prospects in the treatment of hematologic and solid malignancies. Cancer 2005; 104:1794-807.
18. Yang HH, Ma MH, Vescio RA, et al. Overcoming drug resistance in multiple myeloma: the emergence of therapeutic approaches to induce apoptosis. J Clin Oncol 2003; 21:4239-47.
19. Ma MH, Yang HH, ParkerK, et al. The proteasome inhibitor PS-341 markedly enhances sensitivity of multiple myeloma tumor cells to chemotherapeutic agents. Clin Cancer Res 2003; 9:1136-44
20. Mitsiades N, Mitsiades CS, Richardson PG, et al. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. Blood 2003; 101:2377-80.
21. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001; 61:3071-6.
22. Li Y, Yuan H, Yang K, et al. The structure and functions of P-glycoprotein. Curr Med Chem 2010; 17:786-800.
23. Cilloni D, Martinelli G, Messa F, et al. Nuclear factor κB as a target for new drug development in myeloid malignancies. Haematologica 2007; 92:1224-9.
24. Yates LL, Górecki DC. The unclear factor-kappaB (NF-kappaB): from a versatile transcription factor to a ubiquitous therapeutic target. Acta Biochim Pol 2006; 53:651-62.
25. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clin Cancer Res 2007; 13:1076-82.
26. Hu XF, Slater A, Kantharidis P, et al. Altered multidrug resistance phenotype caused by anthracycline analogues and cytosine arabinoside in myeloid leukemia. Blood 1999; 93:4086-95.