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Leukocyte-specific protein 1 inhibits Bortezomib induced apoptosis in multiple myeloma cells

FANG Yinghui[1], REN Zijia[1], MA JIajia[1], GUO Yugang[1], ZHAO Ying[1], XU Lu[1], FANG Fang[1], WANG Yongqing[2], XIAO Weihua[1]

(1. *Hefei National Laboratory for Physical Sciences at Microscale, and School of Life Sciences, University of Science and Technology of China, Hefei 230027, China*;
2. *Department of Hemotology, the First Affiliated Hospital, Anhui Medical University, Hefei 230022, China*)

Abstract: To investigate the roles of anti-apoptosis by leukocyte-specific protein 1 (LSP1) in Multiple Myeloma cells (MM), RT-PCR and immunoblotting were used to assess the gene expression in MM cell lines, IM9 and KAS6. Plasmids containing either sh-RNA targeting LSP1 or full-length cDNA coding for human LSP1 were constructed and transfected into IM9 and KAS6 cells, respectively. Cell apoptosis rate induced by Bortezomib was measured by PI/Annexin V staining and FACS assay. The results shows that LSP1 is highly expressed in IM9 cells but undetectable in KAS6 cells and that is closely correlated with their abilities of anti Bortezomib-induced apoptosis. Knockdown LSP1 in IM9 cell leads to significant reduction of anti Bortezomib-induced apoptosis compared with its parent control cells. By contrast, overexpression of LSP1 in KAS6 cells remarkably increases its anti-Bortezomib ability compared with control KAS6 cells. RT-PCR shows that p53 is suppressed and Bcl-xL is up-regulated by LSP1 in MM cells. In conclusion, LSP1 inhibites Bortezomib-induced apoptosis in multiple myelomas by suppressing multiple pro-apoptosis genes.

Key words: LSP1; Bortezomib; drug resistant; apoptosis

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LSP1 抑制万珂诱导的多发性骨髓瘤细胞凋亡

方颖慧[1],任子甲[1],马佳佳[1],郭雨刚[1],赵 莹[1],
徐 璐[1],方 芳[1],王永庆[2],肖卫华[1]

(1.合肥微尺度物质科学国家实验室,中国科学技术大学生命科学学院,安徽合肥 230027;
2.安徽省医科大学血液病科,安徽合肥 230022)

摘要:淋巴细胞特异性蛋白-1(LSP1)在部分多发性骨髓瘤中表达升高,但其在肿瘤中的作用仍知之甚少.研

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Biography: FANG Yinghui, female, born in 1984, master. Research field: cell biology. E-mail: fang580@mail.ustc.edu.cn

Corresponding author: XIAO Weihua, Prof. E-mail: xiaow@ustc.edu.cn

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究了 LSP1 在多发性骨髓瘤中抗新型抗肿瘤药物万珂(Bortezomib)诱导细胞凋亡的作用及机制.筛选 LSP1 高表达和低表达的多发性骨髓瘤细胞 IM9 和 KAS6 作为实验模型.应用 RNA 干扰基因沉默 IM9 细胞中的 LSP1,或在 KAS6 细胞中转染 LSP1 表达质粒,用 Bortezomib 等化疗药物处理细胞后,PI/Annexin V 染色并用流式细胞仪检测和分析细胞凋亡率.同时 RT-PCR 方法检测和分析被 LSP1 所影响的重要细胞凋亡相关基因的变化.结果发现,LSP1 在多发性骨髓瘤细胞 IM9 和 KAS6 中差异性表达高和低,与 Bortezomib 诱导的细胞凋亡效率密切相关.利用 RNA 干扰敲低 IM9 细胞中 LSP1,可显著增强 IM9 对 Bortezomib 的敏感性, 同时在 KAS6 中转染 LSP1 表达质粒,可降低 Bortezomib 诱导的细胞凋亡.对部分重要凋亡基因的 RT-PCR 检测发现,LSP1 可诱导 BCL-xl 基因表达,同时抑制 p53 表达.因此,发现 LSP1 可通过调节凋亡基因的表达促进肿瘤的抗药性.

关键词:LSP1;Bortezomib;药物耐受性;凋亡

0 Introduction

Leukocyte Specific Protein 1 (LSP1) is specifically expressed in leukocyte cells and endothelial cells. LSP1 has been involved in cell migration and cytoskeletal re-organization through interacting with F-actin. High expression of LSP1 leads to the formation of hair-like structures enriched in F-actin bundles and inhibits motility; low expression of LSP1 positively regulates cell migration[1]. Increased endothelial permeability is required for the transmigration of leukocytes through the endothelium and LSP1 may function as a regulator of endothelial contraction for increased permeability[2-3]. Furthermore, expression of LSP1 is up-regulated during granulocytic and monocytic differentiation of myeloid cells in vitro[4]. LSP1 negatively regulates Mac-1 mediated adhesion and superoxide production in neutrophils[5-7], providing additional evidence for LSP1 being a critical regulator in inflammatory response. Recently, LSP1 was identified as a genetic marker for breast and lung cancers[8-9], which implicates the potential roles of LSP1 in tumorigenesis. Indeed, constitutive expression of LSP1 was also found in multiple myeloma[4]. However, its roles are still unknown.

Multiple myeloma (MM) is a progressive neoplastic disease. It is characterized as bone marrow plasma cells with a integrity monoclone of immunoglobulin (IgG, IgA, IgD or IgE) or Bence Joens protein (free of kappa or gamma light chain) excessive proliferation. Lack of effective treatment for MM patients with recurrent disease is the main cause of deaths. Bortezomib, a new class of antitumor drug as 26S proteasome inhibitor, has been shown having good safety and efficacy for treatment of multiple myeloma, other hematologic malignancies and aggressive solid tumor[10-12]. As a 26S proteasome inhibitor, Bortezomib selectively kills tumor cells by inhibition of the NF-kB pathway, promotion of ER stress-induced apoptosis, induction of p53-dependent apoptosis and disruption of the regulation of cell cycle-regulating proteins[13]. Although Bortezomib has a good performance in treating multiple myeloma, still at least 25%～30% of clinical cases showed with resistance.

In the present study, we report that the expression of LSP1 is closely correlated with anti-Bortezomib-induced apoptosis in MM cells. Genetic alternation of LSP1 by knowdown or overexpression can lead to an increase or reduce of Bortezomib induced apoptosis in the MM cells, respectively. Moreover, LSP1 suppresses the expression of p53 and induces Bcl-xL expression. These results implicate a novel function of LSP1, which is to inhibit Bortezomib-induced cell death in multiple myeloma cells through inducing BCL-xL but suppressing p53 expression.

1 Materials and methods

1.1 Reagents

Bortezomib was produced by Ben Venue

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Laboratories Inc and distributed by Xian' Janssen Pharmaceutical Ltd. Apoptosis assay kit AnnexinV/PI was purchased from Shengwujinmei Inc.

1.2 Cell culture and transfection

Multiple myeloma cell line KAS6 was obtained from ATCC (Manassas, Virginia, USA), maintained in complement RPM1640 supplimented with 1 μg/L human recombinant IL-6 (hrIL-6) (R&D System, Boston, USA) and 10% new-born bovine serum. IM9 was also purchased from ATCC and maintained in the same medium as for KAS6 except without hrIL-6. For drug treatment, the cells were cultured with 6 well plates for 24 h before 10 μmol/L Bortezomib was added and continuously cultured for the indicated time period. Transfection of IM9 and KAS6 cells was performed using Transfast (Promega, USA) according to manufacturer's instruction and the stably transfected cell lines were obtained by culturing transfected cells in the presence of 800 mg/L of G418 (Invitrogen, CA) for 3～4 weeks.

1.3 Plasmids

The plasmids, pU6-shScramble and pU6-shLSP1, containing either a scramble or a siRNA sequence specific targeting LSP1 coding region, were generated by inserting the corresponding sequence into pU6 + 27 vector on Sal1/XbaI enzyme sites. The LSP1- shRNA sequence is: 5′-TCG ACT ACA CCC AGG CCA TCG AGA TTC AAG AGA TCT CGA TGG CCT GGG TGT ATT TTT T -3′, which was composed of 19 bases of specifically targeted sequence (underlined bases), 9 bases of interval stem loop (italics), 19 reverse repeat sequence (underlined bases) and termination signal TTTTT. RT-PCR was used to obtain human LSP-1 full-length cDNA with the primer set of LSP1_Fw: 5′- CGG GAT CC ATG G CGG AGG CTT CGA GTG AC- 3′ and LSP1-Re: 5′-GC A CCG GTT CAG AAG CGA GAT GGG ACG CC TAG-3′, where underlining indicates enzyme sites, frame tagging indicates either LSP1 start coding ATG or stop coding TAG. The cDNA was cloned into pcDNA3.1 vector and the resulting plasmid was designed as pc-LSP1. All the plasmids were confirmed by DNA sequencing.

1.4 RT-PCR

Total RNA was extracted from the cells using the TRIZOL agent according to manufacturer's instruction (Invitrogin, USA). cDNA was synthesized from 2 μg of total RNA in a buffer supplied with the reverse transcriptase (RT) (Invitrogin) containing 900 μmol/L dNTP (Sanggong), 500 ng random primers (Sanggong) and 4 μL 0.1 mol/L DTT in a final volume of 20 μL. PCR was performed using 2 μL of cDNA with the EasyTaq polymerase (Transgenbiotech). PCR product was run on a 1.5% agarose gel with a 100 bp marker (Invitrogen) and stained with ethidium bromide. The primers used for the detection of gene expression are as follows: LSP1: Sense: 5′-AGG AGC ACC AGA AAT GTC AGC-3′, Anti-sense: 5′-GAG CGG TTT AGG GAC TCG G-3′; p53: Sense: 5′-GCT GCT CAG ATA GCG ATG GTC -3′, Anti-sense: 5′-CAT GTA GTT GTA GTG GAT GGT GGT AC-3′; Bcl-xl: Sense: 5′-CGG GCA TTC AGT GAC CTG AC-3′, Anti-sense: 5′-TCA GGA ACC AGC GGT TGA AG-3′; 18S rRNA: Sense: 5′-TGA GAA ACG GCT ACC ACA TCC-3′, Anti-sense: 5′-GCA CCA GAC TTG CCC TCC-3′.

1.5 Immunoblot analyses

The cells were harvested, washed with PBS, and lysed using lysis buffer [50×10^{-3} mol/L Tris-HCl (pH 7.4), 150×10^{-3} mol/L NaCl, 1% NP40, 5×10^{-3} mol/L EDTA, 5×10^{-3} mol/L NaF, 2×10^{-3} mol/L Na_3VO_4, 1×10^{-3} mol/L PMSF, 5 mg/L leupeptine, and 5 mg/L aprotinin]. For the detection of LSP1, GAPDH, and p53, cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with indicated specific antibodies, including anti-LSP1 (cell signaling, rabbit IgG), and anti-

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GAPDH (santa cruz, mouse IgG), and anti-p53 (santa cruz, mouse IgG). Horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence detection (Pierce Chemical Co., Rockford, IL) were used to detect the specific immuno-reactive proteins.

1.6 Apoptsis assay

The cells were were seeded into 6-well plates at a density of 6×10^5 cells/well. In order to observe a putative potentiation of apoptosis with Bortezomib, cells were pretreated with or without (control) 10 nmol/L Bortezomib for 24 h. At the end of the treatment, cells were rapidly centrifuged and apoptosis was assessed using AnnexinV-FITC Apoptosis Detection Kit Ⅱ "AnnexinV-PI" (Jingmei shengwu) as described by the manufacturer. Samples were analysed on FACSCalibur (American Becton Dickinson). The data represented three independent experiments and triplicate samples in each experiment.

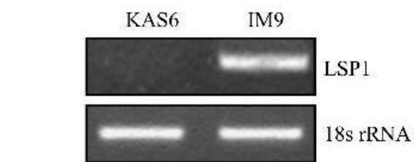
2 Results and discussion

2.1 The expression level of LSP1 is correlated with the apoptosis rate induced by the Bortezomib in multiple myeloma cells

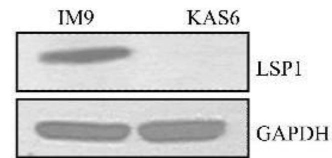
Human MM cell lines, IM9 and KAS6, were screened for their expression level of LSP1 by RT-PCR (Fig. 1(a)) and Western Blot (Fig. 1(b)). The LSP1 is highly expressed in IM9, but not in KAS6 (Fig. 1(a) and (b)). To investigate the effect of Bortezomib-induced apoptosis on these cell lines, both IM9 and KAS6 were cultured for 24 h either with or without 10 μmol/L Bortezomib before being harvested for apoptosis assay (Fig. 1 (c)). The results indicated that KAS6 expressing a low level of LSP1 shows a strong induction of apoptosis compared with IM9 cell that expresses a high level of LSP1 (Fig. 1 (c)), indicating a potential relationship between LSP1 expression level and chemotherapy resistance in MM cells.

2.2 LSP1-knockdown potentiates the sensitivity to Bortezomib in multiple myeloma cells

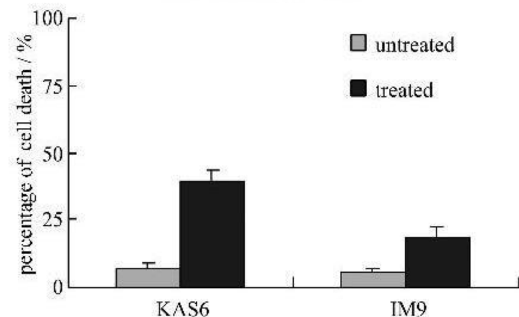
To further investigate the relationship between expression level of LSP1 and its drug-resistance abilities in multiple myeloma cells, the stable cell lines, IM9/sh-LSP1 and IM9/sh-Scramble that transfected with either pU6-siLSP1 or pU6-Scramble constructed, respectively, were established. The expression levels of LSP1 in IM9/sh-Scramble and IM9/sh-LSP1 cells were confirmed in both mRNA and protein levels by RT-PCR (Fig. 2(a)) and Western Blot (Fig. 2(b)) respectively. After culturing the cells for 24 h either with or without 10 μmol/L Bortezomib treatment, the cells were harvested and subjected for apoptosis assay. As shown in Fig. 2(c), IM9/sh-LSP1 cell demonstrates a significantly increased apoptosis rate induced by Bortezomib when compared with IM9/sh-Scramble cell, while the untreated cells show no differences between IM9/sh-Scramble and IM9/sh-LSP1.



(a) LSP1 mRNA is differentially expressed in KAS6 and IM9 cells



(b) LSP1 protein is differentially expressed in KAS6 and IM9 cells



(c) Bortezomib induces appoptosis in KAS6 and IM9 cells

Fig. 1 High expression of LSP1 increased Bortezomib resistance in multiple myeloma

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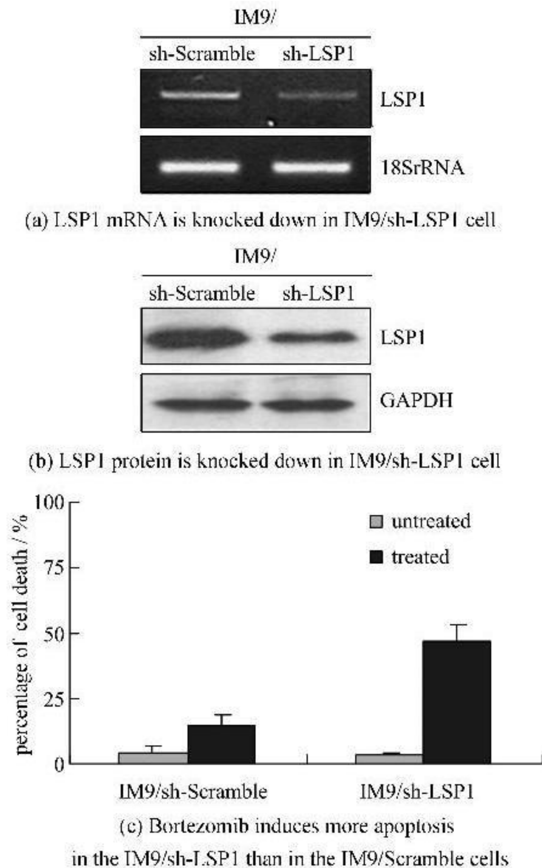
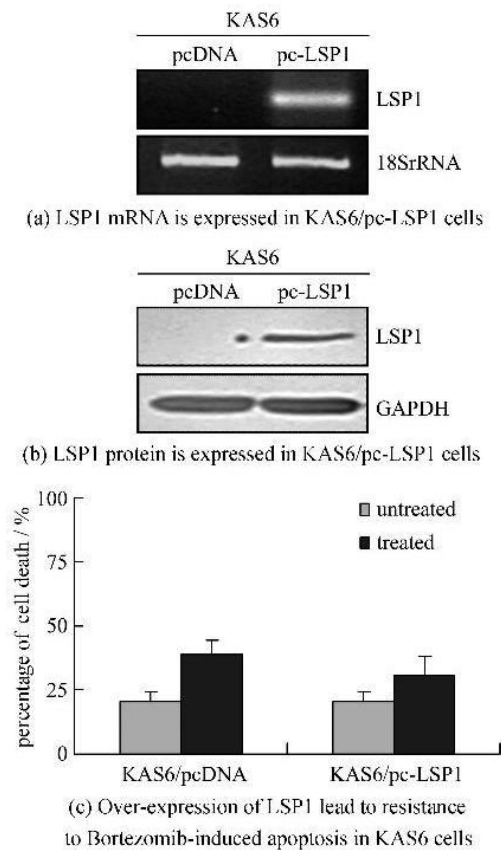


(a) LSP1 mRNA is knocked down in IM9/sh-LSP1 cell

(b) LSP1 protein is knocked down in IM9/sh-LSP1 cell

(c) Bortezomib induces more apoptosis in the IM9/sh-LSP1 than in the IM9/Scramble cells

Fig. 2 LSP1 knockdown potentiates sensitivity to Bortezomib in IM9



(a) LSP1 mRNA is expressed in KAS6/pc-LSP1 cells

(b) LSP1 protein is expressed in KAS6/pc-LSP1 cells

(c) Over-expression of LSP1 lead to resistance to Bortezomib-induced apoptosis in KAS6 cells

Fig. 3 Over-expression of LSP1 enhances the inhibitory activity of Bortezomib-induced apoptosis in KAS6 cells

2.3 LSP1 inhibited Bortezomib-induced apoptosis in multiple myeloma cells

To further confirm the Bortezomib resistance role of LSP1 in multiple myeloma cells, either control vector pc-DNA3.1 or LSP1 expression was constructed. pc-DNA and pc-DNA-LSP1, were transiently transfected into KAS6 cells indicated as KAS6/pcDNA and KAS6/pc-LSP1, respectively. Western blot and RT-PCR were applied to monitor the expression of LSP1 in these cells (Fig. 3(a) and (b)). The cells were either treated or untreated with 10 μmol/L Bortezomib for 24 h before being harvested for apoptosis assay (Fig. 3(c)). The results showed that over-expression of LSP1 in KAS-6/pc-LSP1 cells leads to a significantly enhanced resistance to Bortezomib-induced apoptosis compared with KAS6/pcDNA cells, but no differences between these cells without Bortezomib treatment (Fig. 3(c)).

Taking together, the data above strongly suggest that the expression level of LSP1 is closely correlated with Bortezomib resistance in MM cells, indicating a critical role of LSP1 in anti-Bortezomib-induced apoptosis in MM cells.

2.4 LSP1 suppresses p53 expression and induces Bcl-xl expression in MM cells

To investigate the mechanisms of LSP1 in anti-Bortezomib-induced apoptosis in MM cells, the expression levels of p53 and Bcl-xL were detected by RT-PCR (Fig. 4(a)). As shown in Fig. 4 (a), the expression levels of p53 transcription were significantly increased in IM9 cells with LSP1-knockdown when compared with the control line IM9/sh-Scramble. Conversely, p53 transcription was decreased in KAS6/pc-LSP1 when compared with the control KAS6/pcDNA cells. In contrast with the changes of p53, Bcl-xL

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that has been defined as an important anti-apoptosis gene was reduced in LSP1-knockdown IM9 cells, but elevated in LSP1-overexpression KAS6 cells when compared with their corresponding control cells. In addition, the protein level of p53 was also assessed by Western blot with IM9/sh-Scramble and IM9/sh-LSP1 cells either treated or untreated with 10 μmol/L Bortezomib for 24 h (Fig. 4(b)), while consistent results were obtained in the KAS6/pc-LSP1 and KAS6/pcDNA cells (data not shown). These results demonstrated that knockdown of LSP1 induced the elevation of p53 proteins, and more pronuced accumulation of p53 by Bortezomib was observed when treating the cells with Bortezomib (Fig. 4(b)). On the other hand, consistent results on the p53 expression were obtained in the KAS6/pc-LSP1 and KAS6/pcDNA cells (data not shown). The above results indicate that LSP1 may act as a mediator in response to Bortezomib-induced cell apoptosis through suppressing p53 and inducing Bcl-xL.

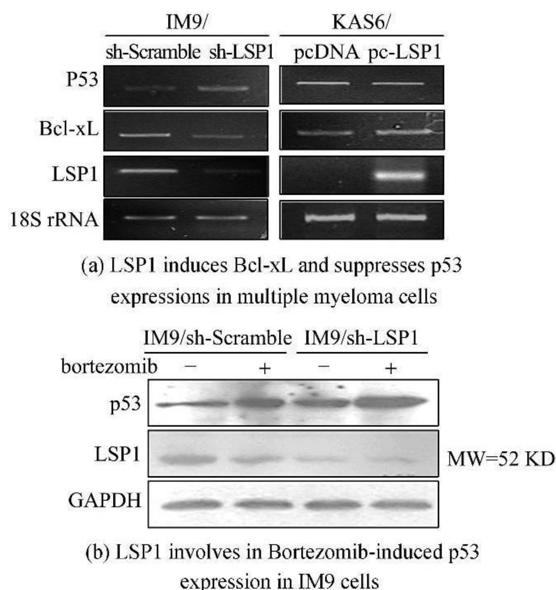

Fig. 4 LSP1 suppresses p53 but induces Bcl-xL expression in IM9 cells

3 Conclusion

Bortezomib is a specific inhibitor of the chymotryptic activity of proteasome. It has shown threapeutic efficacy in patients with multiple myeloma and malignant lymphoma[14-16]. It has been illustrated to inhibit NF-κB, which acts as a transcription factor for antiapoptotic proteins such as c-IAP2 and survivin, and is also known to stabilize p53 and cell cycle proteins, such as p21 and p27[17-19].

The present study demonstrated for first time that LSP1 inhibits Bortezomib induced apoptosis in multiple myeloma cells through suppressing p53 and inducing Bcl-xL expressions, which are important regulators of apoptosis, suggesting that LSP1 may play an important role in Bortezomib-resistence in multiple myeloma cells. However, further study is needed for this putative mechanism.

References

[1] Jongstra-Bilen J, Jongstra J. Leukocyte-specific protein 1 (LSP1): A regulator of leukocyte emigration in inflammation[J]. Immunologic Research, 2006, 35: 65-73.

[2] Liu L, Cara D C, Kaur J, et al. LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration[J]. The Journal of Experimental Medicine, 2005, 201: 409-418.

[3] Luscinskas F W, Ma S, Nusrat A, et al. The role of endothelial cell lateral junctions during leukocyte trafficking[J]. Immunological Reviews, 2002, 186: 57-67.

[4] Li Y, Cuerrero A, Howard T H. The actin-binding protein, lymphocyte-specific protein 1, is expressed in human leukocytes and human myeloid and lymphoid cell lines[J]. The Journal of Immunology, 1995, 155: 3 563-3 569.

[5] Hannigan M, Zhan L, Ai Y, et al. Leukocyte-specific gene 1 protein (LSP1) is involved in chemokine KC-activated cytoskeletal reorganization in murine neutrophils in vitro[J]. Journal of Leukocyte Biology, 2001, 69: 497-504.

[6] Coxon A, Rieu P, Barkalow F J, et al. A novel role for the β_2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation [J]. Immunity, 1996, 5: 653-666.

[7] Serrander L, Larsson J, Lundqvist H, et al. Particles binding β_2-integrins mediate intracellular production of oxidative metabolites in human neutrophils

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independently of phagocytosis [J]. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1999, 1452: 133-144.

[8] Easton D F, Pooley K A, Dunning A M, et al. Genome-wide association study identifies novel breast cancer susceptibility loci[J]. Nature, 2007, 447: 1 087-1 093.

[9] Hu Zhibin, Chen Jiaping, Tian Tian, et al. Genetic variants of miRNA sequences and non small cell lung cancer survival [J]. The Journal of Clinical Investigation, 2008, 118(7): 2 600-2 608.

[10] Field-Smith A, Morgan G J, Davies F E. Bortezomib (Velcade?) in the treatment of multiple myeloma[J]. Therapeutics and Clinical Risk Management, 2006, 2 (3): 271-279.

[11] Hayslip J, Chaudhary U, Green M, et al. Bortezomib in combination with celecoxib in patients with advanced solid tumors: A phase I trial[J]. BMC Cancer, 2007, 7: 221, doi: 10.1186/1471-2407-7-221.

[12] Utecht K N, Kolesar J. Bortezomib: A novel chemotherapeutic agent for hematologic malignancies [J]. American Journal of Health-System Pharmacy, 2008, 65(13): 1 221-1 231.

[13] Nencioni A, Grunebach F, Patrone F, et al. Proteasome inhibitors: Antitumor effects and beyond [J]. Leukemia, 2007, 21: 30-36.

[14] Richardson PG, Sonneveld P, Schuster M W, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma [J]. The New England Journal of Medicine, 2005, 352(24): 2 487-2 498.

[15] O'Connor O A, Wright J, Moskowitz C, et al. Phase Ⅱ clinical experience with the novel proteasome inhibitor Bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma [J]. Journal of Clinical Oncology, 2005, 23 (4): 676-684.

[16] Goy A, Younes A, McLaughlin P, et al. Phase Ⅱ study of proteasome inhibitor Bortezomib in relapsed or refractory B-cell non-Hodgkin's lymphoma [J]. Journal of Clinical Oncology, 2005, 23 (4): 667-675.

[17] Pham L V, Tamayo A T, Yoshimura L C, et al. Inhibition of constitutive NF-nB activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis[J]. The Journal of Immunology, 2003, 171: 88-95.

[18] Ling Y H, Liebes L, Jiang J D, et al. Mechanisms of proteasome inhibitor PS-341-induced G (2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines[J]. Clinical cancer research, 2003, 9: 1 145-1 154.

[19] 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[J]. Cancer Research, 2001, 61: 3 071-3 076.

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