Biochemical and Biophysical Research Communications 439 (2013) 179–186

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Proteasome inhibitor MG132 enhances TRAIL-induced apoptosis and inhibits invasion of human osteosarcoma OS732 cells

Xiucheng Li [a], Tao Huang [a,*], Guangjian Jiang [b], Weihua Gong [b], Hao Qian [a], Chunping Zou [a]

[a] Department of Orthopedics, The First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning, PR China
[b] Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

ARTICLE INFO

Article history:
Received 16 August 2013
Available online 29 August 2013

Keywords:
Osteosarcoma
MG132
TRAIL
Apoptosis
Invasion

ABSTRACT

MG132 as a proteasome inhibitor could induce apoptosis in various cancer cells. This study aimed to discuss the effect of proteasome inhibitor MG132 on the TRAIL-induced apoptosis of human osteosarcoma OS732 cells. MG132 and TRAIL were applied on OS732 cells respectively or jointly. Cell survival rates, changes of cellular shape, cell apoptosis and cell invasion were analyzed, respectively, by 3-(4,5)-dimethylthiahiazo(-z-y1)-2,5-di-phenytetrazoliumromide (MTT) assay, inverted phase contrast microscope, flow cytometry, and transwell invasion chamber methods. The protein levels of DR5, caspase-3, caspase-8, $p27^{kip1}$ and MMP-9 were measured by Western blot analysis. The results indicated that combination of MG132 and TRAIL had the effect of up-regulating expression of DR5, caspase-3, caspase-8 and $p27^{kip1}$, down-regulating expression of MMP-9 and inducing apoptosis as well as suppressing the ability of invasion of OS732 cells. The survival rate of combined application of 10 μM MG132 and 100 ng/ml TRAIL on OS732 cells was significantly lower than that of the individual application ($p < 0.01$). Changes of cellular shape and apoptotic rates also indicated the apoptosis-inducing effect of combined application was much stronger than that of individual application. Cell cycle analysis showed combination of MG132 and TRAIL mostly caused OS732 cells arrested at G2–M-phase. The invasion ability of OS732 cells was restrained significantly in the combined group compared with the individual group and control group.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Osteosarcoma is the most common malignant tumor of bone, usually occurring in children and adolescents and early pulmonary metastasis is liable to occur with bad prognosis [1]. Five-year disease-free survival has increased up to 70% with combination of limb salvage and neoadjuvant chemotherapy [2–4]. Unfortunately, the resistance to chemotherapy and metastatic spread has become the most difficult obstacle of the therapy. It was reported the intrinsic resistance to apoptosis is one important mechanism by which osteosarcoma cells can escape therapeutic control [5–7]. Therefore, novel, safe and more effective anti-cancer treatments are needed for patients with chemotherapy-resistant osteosarcoma.

Most human tumor cells are sensitive to the apoptotic induced by TRAIL. However, many osteosarcomas are resistant to TRAIL [8]. Induction of apoptosis requires additional treatment with other chemotherapeutic agents that damage normal cells and tissues. Thus, safe and more effective adjuvant treatments for osteosarcoma are needed. The ubiquitin–proteasome pathway, responsible for mediating most intracellular proteolysis, plays a crucial role in the regulation of many normal cellular processes, including the cell cycle, differentiation and apoptosis [9]. Apoptosis in cancer cells is closely connected with the activity of ubiquitin–proteasome pathway. MG132 acts as a blocker in ubiquitin–proteasome pathway. The ubiquitin–proteasome pathway is the major pathway of intracellular protein degradation, more than 80% of intracellular proteins, including a variety of proteins that regulate the cell cycle [10]. Recent reports have demonstrated that a variety of tumor cells can be sensitized to TRAIL-induced apoptosis by combining TRAIL with proteasome inhibitors such as bortezomib [11,12]. In our research, we investigated susceptibility to TRAIL in human osteosarcoma OS732 cells, and examined the efficacy of combination therapy with TRAIL and the proteasome inhibitor MG132 and also examined changes of TRAIL receptors DR5,caspase-3, 8, $p27^{Kip1}$ and MMP-9 after treatment to clarify the related molecular mechanisms.

2. Materials and methods

2.1. Reagents

OS732 was purchased from Orthopaedics Graduate School in Beijng Jishuitan Hospital (Beijing, China). RPMI-1640 medium were

* Corresponding author. Fax: +86 024 83282997.
E-mail addresses: huangtao@mail.cmu.edu.cn, cmuhuangtao@126.com (T. Huang).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.08.066

bought from Gibco (Gaithersburg, MD, USA). Trypsin and MTT were purchased from Sigma (St. Louis, MO, USA). z-VAD-fmk, z-IETD-fmk and MG132 were purchased from Calbiochem (Gibbstown, NJ, USA); Rabbit polyclonal to DR5, rabbit monoclonal antibody to caspase-3 and rabbit polyclonal to caspase-8 were bought from Abcam (Cambridge, UK). Rabbit antibody to MMP-9 was bought from Proteintech Group (Chicago, IL, USA), and $p27^{Kip1}$ was bought from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture and research methods

OS732 was cultured in RPMI-1640 medium with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. We selected the concentration of 1, 10, 100 ng/ml for the single TRAIL group; the concentration of 0.1, 1, 10 μM for the MG132 group, TRAIL (100 ng/ml) and MG132 (10 μM) for the combined group, meanwhile setting PBS blank control group. All trials were repeated three times.

2.3. Measurement of the survival rates of tumor cells with MTT method

5×10^5/ml of cells were seeded in the 96-well plate with 200 μl each well, and added to the culture medium containing agents of different concentrations or control PBS with 100 μl each well, each concentration for parallel 4 wells after adherence. After culturing for 24 h, 48 h, 72 h, newly made-up 20 μl MTT was added, and continued to incubate at 37 °C for 4 h, then the supernatant was discarded and dissolved in 150 μl DMSO. The absorptance was measured at 540 nm wavelength after mixed. Survival rate of tumor cells (%) = experimental group A value/control group A value × 100%.

2.4. Observation of the morphology of apoptotic cells

The morphology, number and adherence of tumor cells were directly observed with inverted phase contrast microscope.

2.5. Determination of cell apoptosis

After culturing for 24 h, apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit. Cells were detached by trypsinization and washed three times in PBS, centrifuged at 1000*g* for 5 min and resuspended in 195 μl Annexin V-FITC binding buffer. 5 μl Annexin V-FITC was added and mixed. Then, the OS732 cells were stained in the dark for 10 min at room temperature. After that, cells were centrifuged at 1000*g* for 5 min and resuspended in 190 μl of Annexin V-FITC binding buffer. Last, 10 μl propidium iodide staining solution was added and mixed. The OS732 cells were kept on ice in the dark and immediately subjected to flow cytometry analysis. The data were analyzed using the Cell Quest software. The experiment was repeated three times.

2.6. Cell cycle analysis

Cell cycle distribution was determined by DNA content analysis after PI staining. After treating for 24 h, OS732 cells (10^5 per condition) were harvested and fixed with cold 70% ethanol at −20 °C overnight. Cells were incubated with PI at room temperature for 1 h. Flow cytometric determination of DNA content was analyzed by a FACScan (BD FACSCalibur) flow cytometer. For each sample, 10,000 events were stored. The fractions of the cells in G_0–G_1, S, and G_2–M phases were analyzed using CELL Quest software. The experiment was repeated three times.

2.7. Determination of the invasion ability

The invasive ability of OS732 cells was calculated by the number of cells passed through a polycarbonate membrane (8-μm pore size). The chamber was washed with serum-free medium, and then 20 μl matrigel was added to evenly cover the surface of the polycarbonate membrane. 200 μl preprocessed DMEM medium with 10% FBS containing 2×10^5 cells was seeded in the upper chamber of the Transwell invasion system while 600 μl DMEM medium with 10% FBS was added into the lower chamber. Then placing the transwell invasion system into a cell culture incubator for 24 h, taking out the upper chamber, and removing the cells on the upper surface of the membrane with a sterile cotton swab, those cells that invaded to the lower surface of the membrane were stained with 1% crystal violet. The results are presented as the mean ± SD, with three repeated experiments for each group.

2.8. Western blot analysis

The methods for Western blot have been described previously [13]. After electrophoresis, protein blots were transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBST and incubated with primary antibody in TBST containing 5% BSA overnight at 4 °C. After washing three times with TBST, the membrane was incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST. The detected protein signals were visualized by an ECL system.

2.9. Statistic method

Statistical analysis was performed using Windows SPSS 17.0 software. Data were given as mean ± SD and three independent experiments were analyzed. Data were compared using standard ANOVA methodology for repeated measurement, followed by Student–Newman–Keuls test. Differences were considered statistically significant at the 5% level ($p < 0.05$).

3. Results

3.1. Changes of survival rates of tumor cells

When the concentration of TRAIL was used respectively at 1, 10, 100 ng/ml, we did not observe a very significant dose-inhibition effect between different groups ($p > 0.05$) (Fig. 1A). However, when the concentration of MG132 was used respectively at 0.1, 1, 10 μM, the cell growth was obviously suppressed between different groups ($p < 0.05$) (Fig. 1B). With the combination of 10 μM MG132 and 100 ng/ml TRAIL, the survival rate was significantly lower ($p < 0.01$), compared with individual group, showing that the combined use of TRAIL and MG132 have stronger inhibition effect than the single agent (Fig. 1C).

3.2. Morphological changes of apoptosis of OS732 cells

Under the inverted phase contrast microscope, the normal OS732 cells were attached to the dish, the cells looked like fusiform and angular (Fig. 2A). With 10 μM MG132 or 100 ng/ml TRAIL, only part of cells became small and round (Fig. 2B and C), however, with the combined application of 10 μM MG132 and 100 ng/ml TRAIL, chromatin and cytoplasm were condensed, and many cells became non-adherent and suspended in the culture medium (Fig. 2D). Cell death was blocked in the combination of TRAIL and MG132 after using of a pan-caspase inhibitor z-VAD-fmk (150 μM) or a

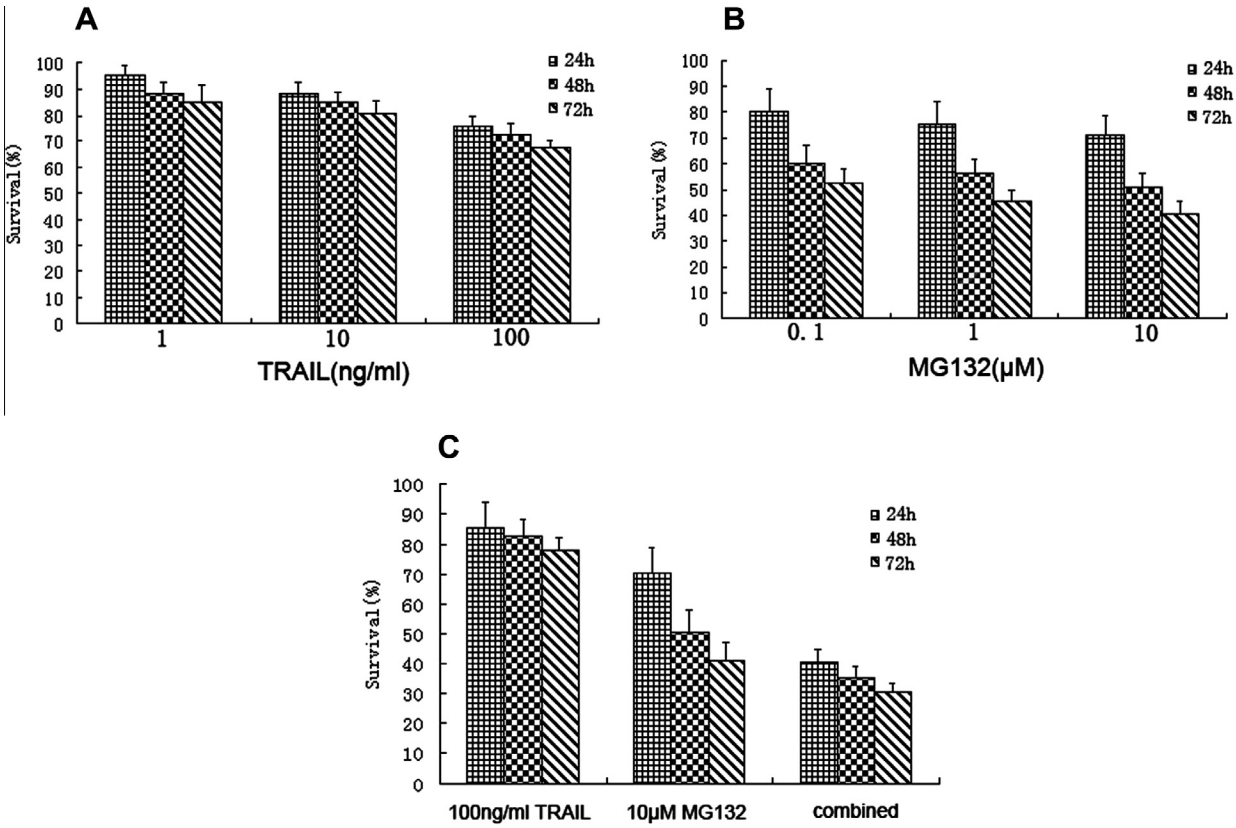

Fig. 1. The inhibition effect on OS732 after 24 h, 48 h, 72 h measured by MTT. (A) The survival rate of OS732 with different concentration of TRAIL. (B) The survival rate of OS732 with different concentration of MG132. (C) The survival rate of OS732 with combination of 100 ng/ml TRAIL and 10 μM MG132.

caspase-8 inhibitor z-IETD-fmk (150 μM) (Fig. 2E), indicating that this cell death requires activation of caspases.

3.3. Effect of TRAIL and MG132 used individually or jointly on apoptosis of OS732 cells

Annexin V and PI staining results showed that the number of apoptotic cells in the combination of 100 ng/ml TRAIL and 10 μM MG132 group was more than that in the blank control group and the individual group ($p < 0.01$). These data indicated that the combination of TRAIL and MG132 could induce more apoptosis on OS732 cells (Fig. 3A and B).

3.4. Cell cycle of OS732 cells treated with TRAIL and MG132 individually or jointly

Combined treatment of TRAIL and MG132 resulted in an increase of cell numbers at G_2–M phase and a decrease of the cell numbers at G1 phase. Combined group resulted in 44.8% of cells that arrested at G_2–M phase, only 22.3% of cells at G_2–M phase in the control group ($p < 0.05$) (Fig. 3C).

3.5. Effect of TRAIL and MG132 used individually or jointly on the invasive ability of OS732 cells

The transwell invasion chamber experiments showed that the number of OS732 cells passing through the polycarbonate membrane in the combination of 100 ng/ml TRAIL and 10 μM MG132 group was significantly less than that in the control group and the individual group ($p < 0.01$) (Fig. 3D and E). These data indicated that the combination of TRAIL and MG132 could diminish the invasion of OS732 cells than used individually.

3.6. The expression of DR5, caspase-3, caspase-8, $p27^{Kip1}$ and MMP-9 of OS732 cells

After exposure to 100 ng/ml TRAIL, 10 μM MG132 or combination of 100 ng/ml TRAIL and 10 μM MG132 for 72 h, expression of DR5, caspase-3, caspase-8, $p27^{Kip1}$ and MMP-9 in OS732 cells was tested. We observed the increasing levels of DR5, caspase-3, caspase-8 and $p27^{Kip1}$, in both 100 ng/ml TRAIL group and 10 μM MG132 group. The levels of DR5, caspase-3, caspase-8 and $p27^{Kip1}$ in the combined group were much higher than the individual group and control group ($p < 0.05$) (Fig. 4A–D), however, the expression of MMP-9 in the combined group was much lower than the individual group and control group ($p < 0.05$) (Fig. 4C and D).

4. Discussion

Tumor necrosis factor related apoptosis-inducing ligand is a member of the TNF super family of cytokines [14]. TRAIL induces apoptosis through interactions with its death domain containing receptors death receptor 4 (DR4) and death receptor 5 (DR5) [15,16]. Once activated, TRAIL receptors recruit FADD as a major cellular adaptor protein. FADD engages the initiator protease caspase-8. FADD triggers the auto-activation of caspase-8 and subsequently leads to the activation of downstream caspases such as caspases-3, -7, and -9 resulting in the cleavage of cellular substrates and which leads to the ultimate cell death. TRAIL is a potentially important anticancer agent, since it selectively kills malignant cells while leaving normal cells unaffected [17]. Similar to other TNF family members such as TNF-a and Fas-L, TRAIL induces apoptosis by interactions with its death domain containing receptors death receptor 4 (DR4) and death receptor 5 (DR5) [18,19]. However, unlike most cancers, many osteosarcomas are

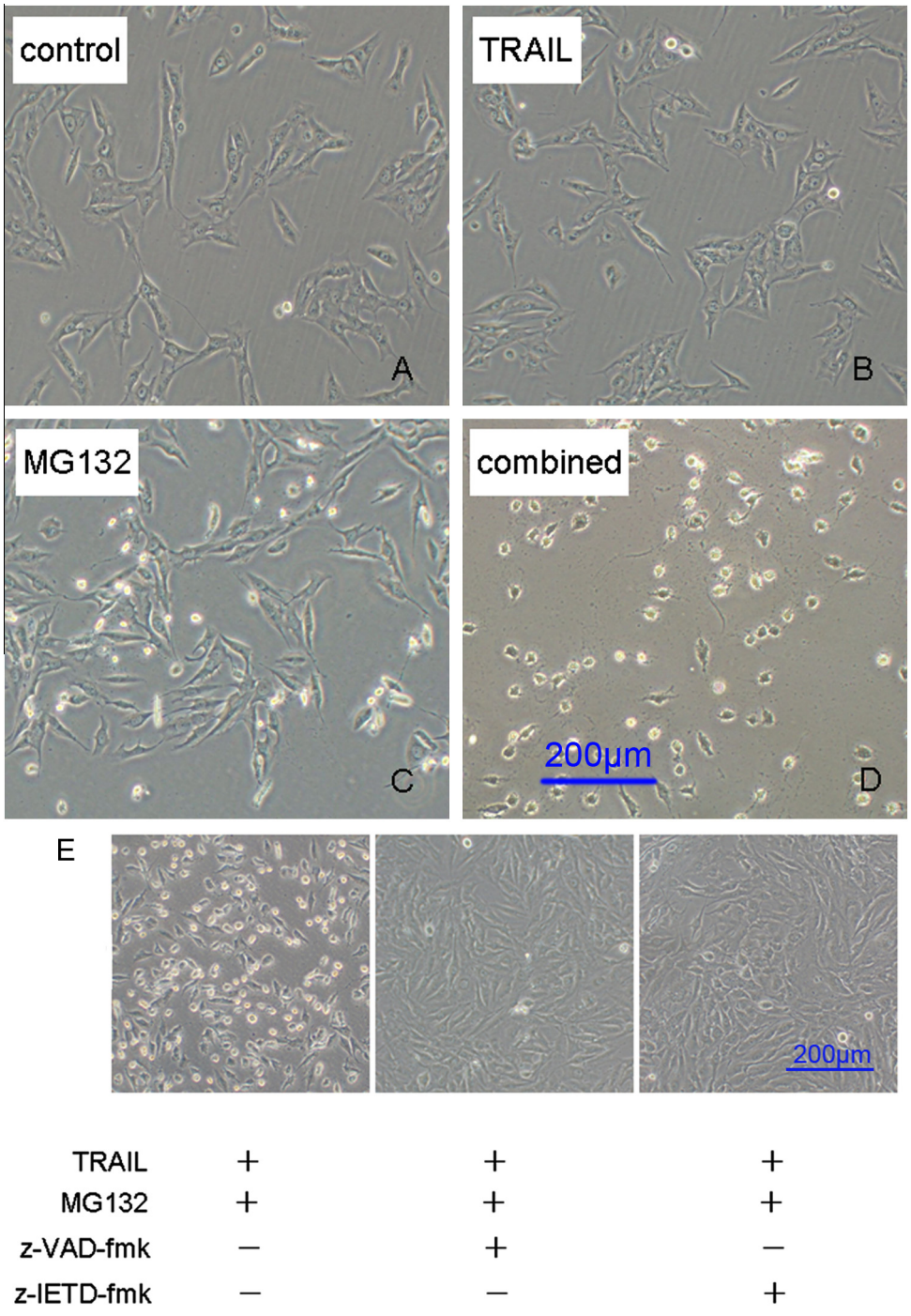

Fig. 2. Morphological changes of OS732 cells after 48 h with different drugs. (A) Morphological appearance of OS732 under inverted phase contrast microscope 100×. (B) Morphological changes of OS732 treated with 100 ng/ml TRAIL under inverted phase contrast microscope 100×. (C) Morphological changes of OS732 treated with 10 μM MG132 under inverted phase contrast microscope 100×. (D) Morphological changes of OS732 treated with combination of 100 ng/ml TRAIL and 10 μM MG132 under inverted phase contrast microscope 100×. (E) OS732 cells were co-treated with TRAIL (100 ng/ml) and MG132 (10 μM) in the presence or absence of z-VAD-fmk (150 μM) or z-IETD-fmk (150 μM) and photographed under the microscope 100×.

resistant to TRAIL. When using TRAIL individually, we found the apoptosis rate is very low which was consistent with the previous research and further confirmed the tolerance of OS732 cells to TRAIL (Fig. 1A).

Ubiquitin-mediated protein degradation is an important part of numerous cellular processes, involving cell-cycle regulation, signal transduction, gene transcription and apoptosis. As novel anti-cancer agents, proteasome inhibitors are currently under intensive investigation [20,21]. Several proteasome inhibitors exert anti-tumor activity in vivo and induce apoptosis in tumor cells in vitro [22,23]. There are two pathways in apoptosis: the cell surface death receptor pathway and the mitochondria-initiated pathway. In the cell surface receptor pathway, activation of caspases following their recruitment to the death-inducing signaling complex is the critical event that transmits the death signal. Apoptosis is an intrinsic cell death program that is involved in the regulation of various physiological and pathological processes [24,25]. Several reports have shown a close correlation between apoptosis and the inhibition of the ubiquitin–proteasome pathway [26–28]. MG132 is a natural triterpene proteasome inhibitor extracted from

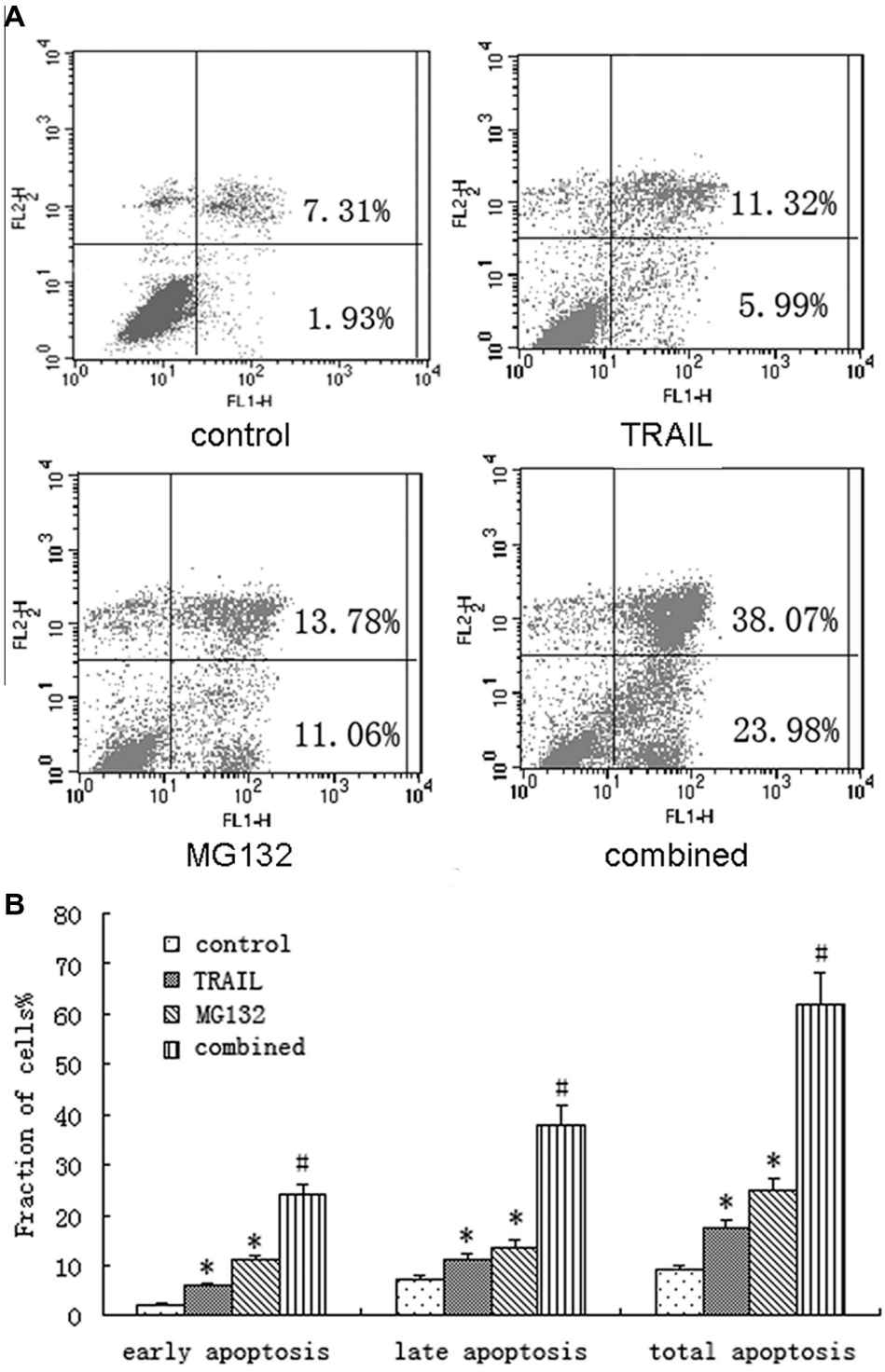

Fig. 3. Apoptosis in OS732 cells treated with different drugs after 24 h. (A) The four groups (blank control group, 100 ng/ml TRAIL group, 10 μM MG132 group, 100 ng/ml TRAIL and 10 μM MG132 group) were incubated for 24 h, cells were stained with FITC-conjugated Annexin V and PI, followed by flow cytometric analysis. Early apoptotic populations are found in the lower-right quadrants, while necrotic or late apoptotic cells are localized in the upper-right quadrant. (B) The percentage of early apoptosis, late apoptosis, and total cell death in blank control group, TRAIL group, MG132 group as well as the combination group of TRAIL and MG132 group for 24 h. The mean ± SD of the results were obtained from three independent experiments ($^{*}p < 0.01$ versus the control group, $^{\#}p < 0.01$ versus TRAIL and MG132 group). (C) TRAIL and MG132 induces G_2–M-phase arrest in OS732 cells. OS732 cells were exposed to TRAIL and MG132 individually or jointly. After exposure, cells (1×10^5) were stained with propidium iodide as described above. Each bar represented cell cycle distributions. The mean ± SD of the results were obtained from three independent experiments ($^{*}p < 0.05$ versus the control group). (D) Invasion in OS732 cells treated with different drugs after 24 h. The crystal violet staining of the OS732 cells that passed through the polycarbonate membrane (blank control group, 100 ng/ml TRAIL group, 10 μM MG132 group, 100 ng/ml TRAIL and 10 μM MG132 group). (E) The number of cells passed through transwell invasion chamber. All experiments were repeated three times. ($^{*}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus TRAIL and MG132 group).

a Chinese medicinal plant. It is a peptide aldehyde that inhibits 20S proteasome activity by covalently binding to the active site of the beta subunits and effectively blocks the proteolytic activity of the 26S proteasome complex [29,30]. MG132 inhibits the growth of tumor cells by inducing the cell cycle arrest as well as triggering apoptosis [31]. Our research indicated that MG132 increased

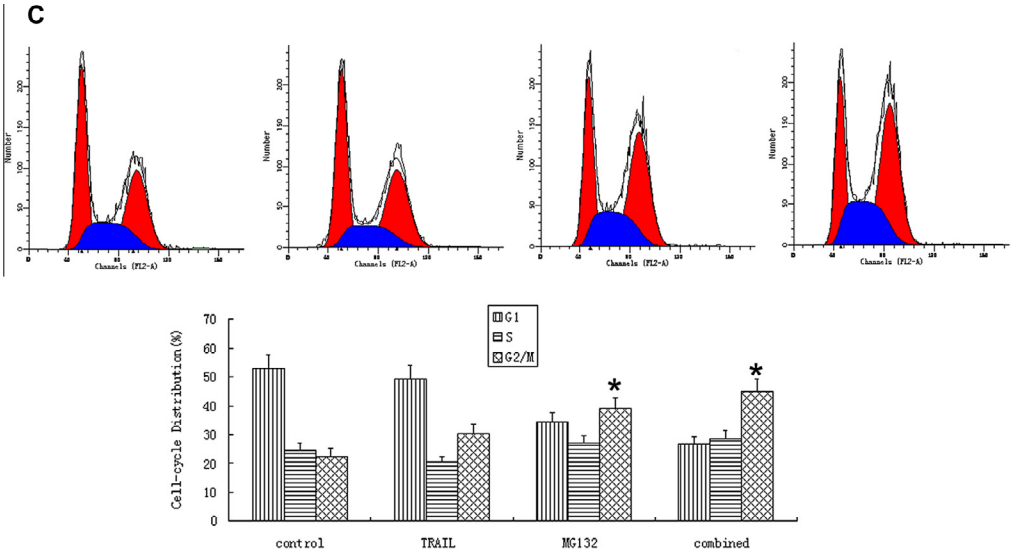

Fig. 3 (*continued*)

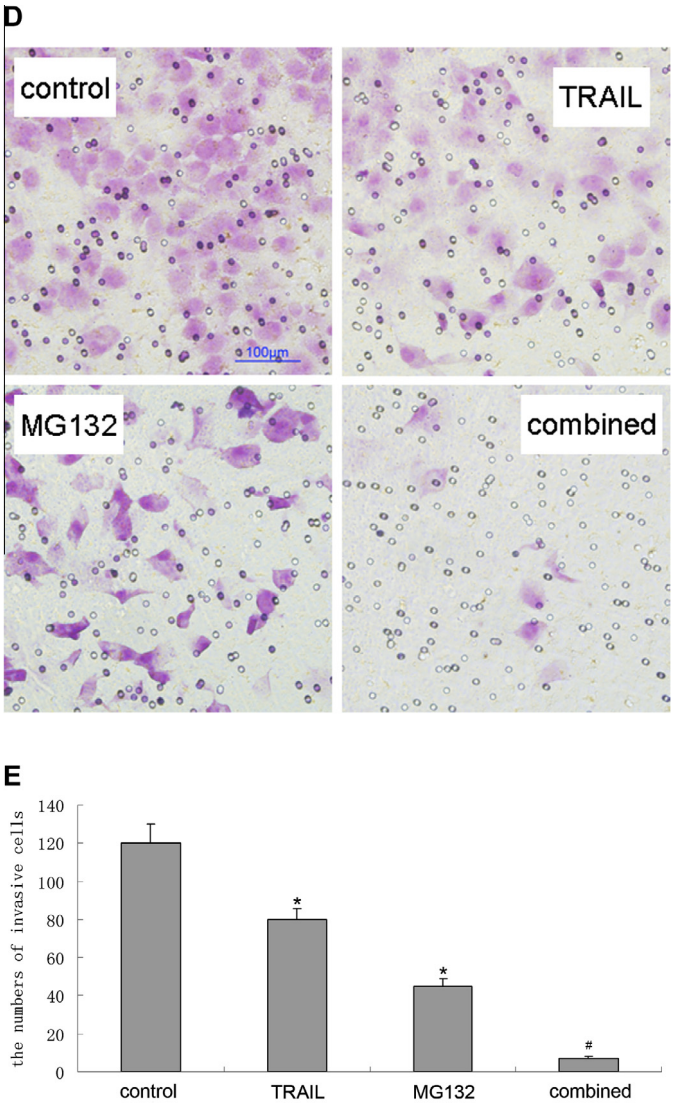

Fig. 3 (*continued*)

apoptosis rate in dose- and time-dependent manner, with the concentration increased from 0.1 μM to 10 μM, the survival rate decreased from 80.28% to 70.62% after 24 h (Fig. 1B). Therefore, MG132 could promote the OS732 cells apoptosis, but the effect was not very strong which was also reflected from the results of FCM. $P27^{Kip1}$ is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, which is degraded by the ubiquitin–proteasome system. By the accumulation of $p27^{Kip1}$, proteasome inhibitor induced apoptosis is accompanied [32]. We found that application of TRAIL and MG132 individually or jointly could increase the expression of $p27^{Kip1}$ in OS732 cells, which is consistent with previous studies that over-expression of $p27^{Kip1}$ leads to apoptosis in various cancer cell lines (Fig. 4C and D) [33,34]. Accumulation of $p27^{Kip1}$ may play an important role in inducing apoptosis.

Some researches had revealed that combination of TRAIL and chemotherapy drugs could improve sensitivity to TRAIL in osteosarcoma cells, not including the combination of TRAIL and proteasome inhibitors applied in the OS732 cells [35,36]. So we proposed whether there will be a combined effect using MG132 and TRAIL jointly in the OS732 cells owe to increasing the sensitivity to TRAIL and apoptosis rate. MTT results showed that the combination of TRAIL and MG132 may have stronger inhibition effect than single agent (Fig. 1C). The effects of the joint group in inducing apoptosis were stronger than single application group (Figs. 2A–D and 3A and B). However, Cell apoptosis was blocked in the combination of TRAIL and MG132 after using of a pan-caspase inhibitor z-VAD-fmk or a caspase-8 inhibitor z-IETD-fmk (Fig. 2E), indicating that this cell apoptosis requires activation of caspases. Our findings provide useful information regarding the combined treatment of TRAIL and proteasome inhibitor MG132 that they have a synergistic apoptotic effect contrast with applied individually. In addition, osteosarcoma is characterized by its strong invasion and early hematogenous metastasis, which is the major reason of both treatment failure and death. Therefore, it is important to detect the molecular mechanism for invasion and metastasis of osteosarcoma. Our data indicated that the combination of MG132 and TRAIL had a greater effect in diminishment of invasion of OS732 cells than MG132 and TRAIL used individually (Fig. 3D and E) and we also found that the expression level of MMP-9 was significantly decreased in combined group than in individual group and control group (Fig. 4C and D). To identify the mechanism of the

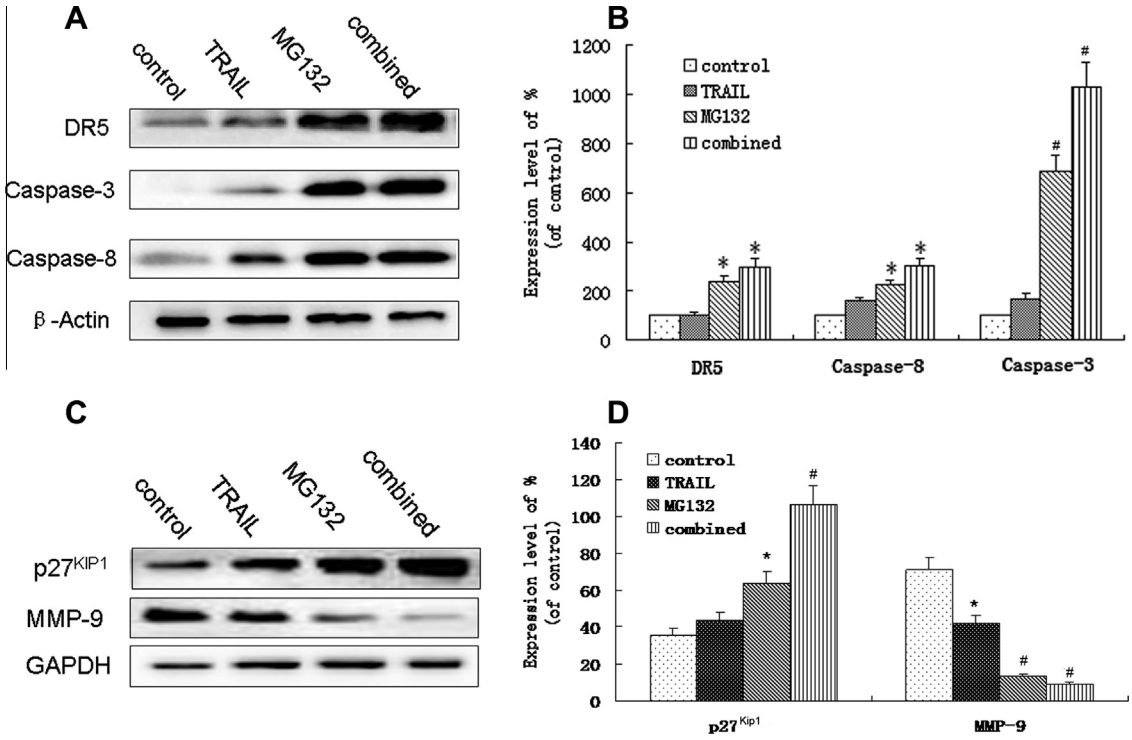

Fig. 4. Western blot analysis the expression of DR5, caspase-3,8, $p27^{Kip1}$, MMP-9. (A) The levels of DR5, caspase-3,8 were analyzed by Western blot analysis. There was an increase of DR5, caspase-3,8 obviously in the combination group of 100 ng/ml TRAIL and 10 μM MG132 group. (B) The mean ± SD of the results were obtained from three independent experiments. ($^{*}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus TRAIL and MG132 group). (C) The levels of $p27^{Kip1}$ and MMP-9 were analyzed by Western blot analysis. There was an obviously up-regulation of $p27^{Kip1}$ and down-regulation of MMP-9 in the combination group of 100 ng/ml TRAIL and 10 μM MG132 group. (D) The mean ± SD of the results were obtained from three independent experiments ($^{*}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus TRAIL and MG132 group).

combination treatment of MG132 and TRAIL restores sensitivity to TRAIL-induced apoptosis, we investigated changes of TRAIL receptors DR5 and caspase-3, 8 after treatment with TRAIL and MG132 individually or jointly. Our results found out that the expression of DR5, caspase-3,8 are increased (Fig. 4A and B), suggesting that the synergistic effect is mediated via the activation of caspase pathway. We deduced MG132 could increase the expression of DR5, the number of TRAIL receptor increasing, thus increasing the sensitivity to TRAIL. Our findings may throw light on the treatment of osteosarcoma. However, drug trial in vitro may be different from that in vivo given the impact of human pharmacokinetics. Usually, drug tests are more complex in vivo. For this reason, our findings may need further researches to clarify the molecular mechanisms underlying the present important results, but this research provided us with a promising treatment which will bring hope to the clinical treatment of the osteosarcoma patients.

Acknowledgment

This paper was supported by the natural science foundation of science and technology department of Liao Ning province (2013021065).

References

[1] D.D. Rasalkar, W.C. Chu, V. Lee, B.K. Paunipagar, F.W. Cheng, C.K. Li, Pulmonary metastases in children with osteosarcoma: characteristics and impact on patient survival, Pediatr. Radiol. 41 (2011) 227–236.

[2] W. Choeyprasert, R. Natesirinilkul, P. Charoenkwan, S. Sittipreechacharn, Carboplatin and doxorubicin in treatment of pediatric osteosarcoma: a 9-year single institute experience in the northern region of Thailand, Asian Pac. J. Cancer Prev. 14 (2013) 1101–1106.

[3] G. Bacci, A. Balladelli, E. Palmerini, M. Alberghini, P. Pollastri, S. Galletti, M. Mercuri, P. Picci, Neoadjuvant chemotherapy for osteosarcoma of the extremities in preadolescent patients: the Rizzoli Institute experience, J. Pediatr. Hematol. Oncol. 30 (2008) 908–912.

[4] G. Bacci, S. Ferrari, M. Mercuri, A. Longhi, N. Fabbri, S. Galletti, C. Forni, A. Balladelli, M. Serra, P. Picci, Neoadjuvant chemotherapy for osteosarcoma of the extremities in patients aged 41–60 years: outcome in 34 cases treated with adriamycin, cisplatinum and ifosfamide between 1984 and 1999, Acta Orthop. 78 (2007) 377–384.

[5] R. Yang, R. Sowers, B. Mazza, J.H. Healey, A. Huvos, H. Grier, M. Bernstein, G.P. Beardsley, M.D. Krailo, M. Devidas, J.R. Bertino, P.A. Meyers, R. Gorlick, Sequence alterations in the reduced folate carrier are observed in osteosarcoma tumor samples, Clin. Cancer Res. 9 (2003) 837–844.

[6] P.D. Cole, A.K. Smith, B.A. Kamen, Osteosarcoma cells, resistant to methotrexate due to nucleoside and nucleobase salvage, are sensitive to nucleoside analogs, Cancer Chemother. Pharmacol. 50 (2002) 111–116.

[7] W. Guo, J.H. Healey, P.A. Meyers, M. Ladanyi, A.G. Huvos, J.R. Bertino, R. Gorlick, Mechanisms of methotrexate resistance in osteosarcoma, Clin. Cancer Res. 5 (1999) 621–627.

[8] J. Sun, Z.M. Fu, C.Q. Fang, J.H. Li, Induction of apoptosis in osteogenic sarcoma cells by combination of tumor necrosis factor-related apoptosis inducing ligand and chemotherapeutic agents, Chin. Med. J. (Engl.) 120 (2007) 400–404.

[9] K. Chen, H.H. Cheng, R.J. Zhou, Molecular mechanisms and functions of autophagy and the ubiquitin–proteasome pathway, Yi Chuan 34 (2012) 5–18.

[10] S. Meiners, M. Laule, W. Rother, C. Guenther, I. Prauka, P. Muschick, G. Baumann, P.M. Kloetzel, K. Stangl, Ubiquitin–proteasome pathway as a new target for the prevention of restenosis, Circulation 105 (2002) 483–489.

[11] R. Koschny, H. Holland, J. Sykora, H. Erdal, W. Krupp, M. Bauer, U. Bockmuehl, P. Ahnert, J. Meixensberger, W. Stremmel, H. Walczak, T.M. Ganten, Bortezomib sensitizes primary human esthesioneuroblastoma cells to TRAIL-induced apoptosis, J. Neurooncol. 97 (2010) 171–185.

[12] R. Popat, T. Plesner, F. Davies, G. Cook, M. Cook, P. Elliott, E. Jacobson, T. Gumbleton, H. Oakervee, J. Cavenagh, A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma, Br. J. Haematol. 160 (2013) 714–717.

[13] D. Wessel, U.I. Flugge, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, Anal. Biochem. 138 (1984) 141–143.

[14] M.H. Moon, J.K. Jeong, J.S. Seo, J.W. Seol, Y.J. Lee, M. Xue, C.J. Jackson, S.Y. Park, Bisphosphonate enhances TRAIL sensitivity to human osteosarcoma cells via death receptor 5 upregulation, Exp. Mol. Med. 43 (2011) 138–145.

[15] J. Ursini-Siegel, W. Zhang, A. Altmeyer, E.N. Hatada, R.K. Do, H. Yagita, S. Chen-Kiang, TRAIL/Apo-2 ligand induces primary plasma cell apoptosis, J. Immunol. 169 (2002) 5505–5513.
[16] C.S. Mitsiades, S.P. Treon, N. Mitsiades, Y. Shima, P. Richardson, R. Schlossman, T. Hideshima, K.C. Anderson, TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications, Blood 98 (2001) 795–804.
[17] R.M. Locklin, E. Federici, B. Espina, P.A. Hulley, R.G. Russell, C.M. Edwards, Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis, Mol. Cancer Ther. 6 (2007) 3219–3228.
[18] E. Szliszka, D. Jaworska, M. Ksek, Z.P. Czuba, W. Krol, Targeting death receptor TRAIL-R2 by chalcones for trail-induced apoptosis in cancer cells, Int. J. Mol. Sci. 13 (2012) 15343–15359.
[19] S. Fossati, J. Ghiso, A. Rostagno, TRAIL death receptors DR4 and DR5 mediate cerebral microvascular endothelial cell apoptosis induced by oligomeric Alzheimer's Abeta, Cell Death Dis. 3 (2012) e321.
[20] J. Jiang, Regulation of Hh/Gli signaling by dual ubiquitin pathways, Cell Cycle 5 (2006) 2457–2463.
[21] H.K. Kinyamu, J. Chen, T.K. Archer, Linking the ubiquitin–proteasome pathway to chromatin remodeling/modification by nuclear receptors, J. Mol. Endocrinol. 34 (2005) 281–297.
[22] V. Milacic, S. Banerjee, K.R. Landis-Piwowar, F.H. Sarkar, A.P. Majumdar, Q.P. Dou, Curcumin inhibits the proteasome activity in human colon cancer cells in vitro and in vivo, Cancer Res. 68 (2008) 7283–7292.
[23] J.B. Almond, G.M. Cohen, The proteasome: a novel target for cancer chemotherapy, Leukemia 16 (2002) 433–443.
[24] A. Mutlu, A.V. Gyulkhandanyan, J. Freedman, V. Leytin, Activation of caspases-9, -3 and -8 in human platelets triggered by BH3-only mimetic ABT-737 and calcium ionophore A23187: caspase-8 is activated via bypass of the death receptors, Br. J. Haematol. 159 (2012) 565–571.
[25] I. Budihardjo, H. Oliver, M. Lutter, X. Luo, X. Wang, Biochemical pathways of caspase activation during apoptosis, Annu. Rev. Cell Dev. Biol. 15 (1999) 269–290.
[26] N. Rastogi, D.P. Mishra, Therapeutic targeting of cancer cell cycle using proteasome inhibitors, Cell Div. 7 (2012) 26.
[27] K.R. Landis-Piwowar, Proteasome inhibitors in cancer therapy: a novel approach to a ubiquitous problem, Clin. Lab. Sci. 25 (2012) 38–44.
[28] M. Demasi, F.R. Laurindo, Physiological and pathological role of the ubiquitin–proteasome system in the vascular smooth muscle cell, Cardiovasc. Res. 95 (2012) 183–193.
[29] V.K. Wong, S.S. Cheung, T. Li, Z.H. Jiang, J.R. Wang, H. Dong, X.Q. Yi, H. Zhou, L. Liu, Asian ginseng extract inhibits in vitro and in vivo growth of mouse lewis lung carcinoma via modulation of ERK-p53 and NF-kappaB signaling, J. Cell. Biochem. 111 (2010) 899–910.
[30] L. Ji, Y. Chen, T. Liu, Z. Wang, Involvement of Bcl-xL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes, Toxicol. Appl. Pharmacol. 231 (2008) 393–400.
[31] H. Yang, D. Chen, Q.C. Cui, X. Yuan, Q.P. Dou, Celastrol, a triterpene extracted from the Chinese "Thunder of God Vine", is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice, Cancer Res. 66 (2006) 4758–4765.
[32] B. An, R.H. Goldfarb, R. Siman, Q.P. Dou, Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts, Cell Death Differ. 5 (1998) 1062–1075.
[33] X. Wang, M. Gorospe, Y. Huang, N.J. Holbrook, $P27^{Kip1}$ overexpression causes apoptotic death of mammalian cells, Oncogene 15 (1997) 2991–2997.
[34] Y. Katayose, M. Kim, A.N. Rakkar, Z. Li, K.H. Cowan, P. Seth, Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27, Cancer Res. 57 (1997) 5441–5445.
[35] J. Gerspach, K. Pfizenmaier, H. Wajant, Therapeutic targeting of CD95 and the TRAIL death receptors, Recent Pat. Anticancer Drug Discov. 6 (2011) 294–310.
[36] C. Zanchi, V. Zuco, C. Lanzi, R. Supino, F. Zunino, Modulation of survival signaling pathways and persistence of the genotoxic stress as a basis for the synergistic interaction between the atypical retinoid ST1926 and the epidermal growth factor receptor inhibitor ZD1839, Cancer Res. 65 (2005) 2364–2372.