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Hsp72 controls bortezomib-induced HepG2 cell death via interaction with pro-apoptotic factors

GIUSEPPE CALVARUSO, MICHELA GIULIANO, PATRIZIA PORTANOVA, ORNELLA PELLERITO, RENZA VENTO and GIOVANNI TESORIERE

Dipartimento di Scienze Biochimiche, Università degli Studi di Palermo, Via del Vespro 129, 90127 Palermo, Italy

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Abstract. The proteasome inhibitor bortezomib is an efficacious inducer of apoptosis in the hepatoma HepG2 cell line. This study shows that bortezomib increased in these cells the level of the survival factor Hsp72 in a time- and dose-dependent manner. In a first phase of treatment, Hsp72 rapidly increased so that at 24 h of incubation with 50 nM bortezomib its level was ~five-fold higher than the control. In this phase Hsp72 seemed to play a role in preventing HepG2 cell death, since it interacted with and sequestered the pro-apoptotic factors p53, AIF, Bax and Apaf-1. During a second day of treatment, although the nuclear levels of Hsp72, p53 and AIF increased, the interaction of Hsp72 with these factors diminished. In addition, bortezomib induced the activation of caspases, which stimulated Hsp72 degradation. In conclusion, in the second day of treatment with bortezomib the protective ability of Hsp72 decreased thus favouring the appearance of apoptosis.

Introduction

In all living organisms the ubiquitous class of Hsp70 includes protective factors which are involved in the response to a variety of stresses. In humans various isoforms of Hsp70 occur, one of which, the inducible form Hsp72, is involved in the repair of misfolded proteins and plays a key role against apoptosis induced by a variety of stimuli, such as heat shock, DNA damage, UV radiation, ceramide, reactive oxygen species, and proteasome inhibition. This action of Hsp72 seems to be dependent on its binding to some pro-apoptotic proteins (1-4).

Many authors have demonstrated that proteasome inhibitors are efficacious apoptotic agents in tumor cells (5-7). In particular, the boronic acid dipeptide bortezomib (formerly Velcade or PS-341), which is an effective inhibitor of the 26S proteasome, has been recently introduced in the therapy of multiple myeloma (8).

We have previously reported that bortezomib (20-100 nM) induces apoptosis in human hepatoma HepG2 cells and that at a concentration of 50 nM the effect occurs only in the second day of treatment (9). The lag-time required by bortezomib to trigger apoptosis could be dependent on some mechanisms activated by the cells to prevent the injury of the drug during the first phase of treatment. We have already demonstrated that one of these mechanisms is represented by the increase in the DNA-binding activity of NF-κB (10).

In the present study we showed that treatment of HepG2 cells with bortezomib induced a marked increase in the Hsp72 level which can contribute to the delay of apoptotic effects of the drug. This hypothesis is supported by the demonstration that in the first phase of bortezomib treatment, Hsp72 interacted with and sequestered some pro-apoptotic factors, such as p53, AIF, Bax and Apaf-1.

Materials and methods

Chemicals and reagents. Bortezomib (formerly Velcade or PS-341) was purchased from Millennium Pharmaceuticals (London, UK). Benzyloxy carbonyl-Val-Ala-Asp-fluoro-methylketone (z-VAD-fmk) was purchased from Promega (Italy), and all antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), except Hsp72 antibody (anti-Hsp70 rabbit pAb) which was supplied by Calbiochem (Germany). Stock solutions of bortezomib were prepared in DMSO and opportunely diluted in the culture medium. The final concentration of DMSO never exceeded 0.04%.

Cell cultures and preparation of protein extracts. Human hepatoma HepG2 cells, seeded on 100-mm tissue culture dishes ($1.0x10^6$ cells), were cultured for 24 h in RPMI-1640 medium. Then, bortezomib was added and the incubation was protracted for various times. At the end of the incubation, total cell extracts were obtained by dissolving the cells in lysis buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, pH 7.4) for 30 min at 4˚C in the presence of a mix of protease inhibitors. Cells were then sonicated three times for 10 sec and the lysates were centrifuged at 15,000 x g for 20 min at 4˚C.

Nuclear fractions were prepared by lysing the cells in Potter homogenizer in the presence of 20 mM HEPES,

Correspondence to: Professor Giovanni Tesoriere, Dipartimento di Scienze Biochimiche, Università degli Studi di Palermo, Via del Vespro 129, 90127 Palermo, Italy
E-mail: gtesor@unipa.it

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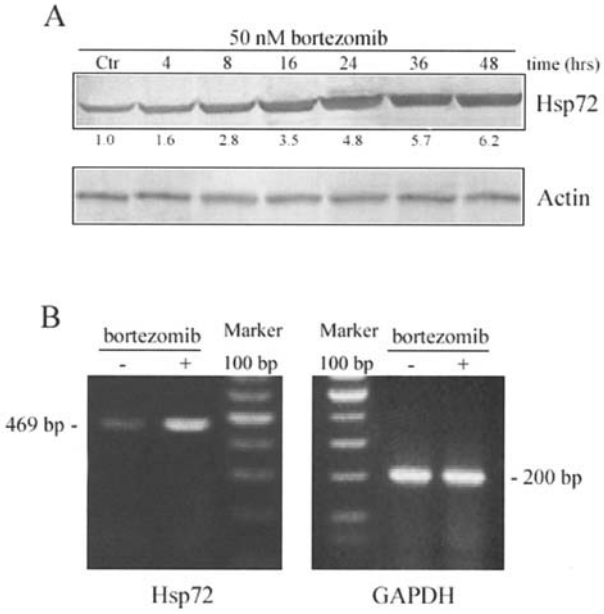

Figure 1. Effect of bortezomib on the Hsp72 level. (A) Western blot analysis to detect the expression level of Hsp72 was performed in HepG2 cells treated with 50 nM bortezomib for different times (4-48 h). Untreated cells were assumed as the control. The actin blot was included to show equal protein loading. (B) RT-PCR was performed in HepG2 cells untreated or treated with 50 nM bortezomib for 24 h using Hsp72-specific primers as reported in Materials and methods. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control of RT-PCR. Data are representative of four separate experiments.

containing 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and a mix of protease inhibitors, pH 7.5. Pellets were collected by centrifuging at 1,500 x g for 10 min and re-homogenized in the same buffer. After centrifugation at 800 x g for 10 min, nuclei were re-suspended in 50 mM Tris HCl, pH 7.4, containing 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS and were sonicated three times for 10 sec. The supernatants, obtained at 12,000 x g for 15 min, were used as nuclear extracts.

Western blotting and immunoprecipitation analysis. Western blot analysis was performed using specific antibodies as previously described (11). Actin immunodetection was performed to confirm that all lanes were loaded with similar amounts of protein. The relative intensities of protein bands were quantified by densitometric analysis using SMX Image software.

For immunoprecipitation analysis equal amounts of cells or nuclear extracts (500 μg of protein) were incubated for 3 h with 0.5 μl of specific antibody against Hsp72, or 2.0 μg of specific antibodies against p53, AIF, Apaf-1 and Bax. Then, to capture the immunocomplexes, 20 μl of Protein A/G plus agarose immunoprecipitation reagent (Santa Cruz Biotechnology Inc.) was added and the incubation was protracted overnight. The immunocomplexes were precipitated at 10,000 x g for 5 min, washed three times with lysis buffer and submitted to Western blotting.

RNA extraction and RT-PCR analysis. Total RNA was extracted from the cells (5-7x10^6 cells/sample) by the guanidine isothiocyanate method, as described by Chomczynski and Sacchi (12). RNA integrity was confirmed by agarose electrophoresis. Total RNA (1 μg) was reverse-transcribed using the GeneAmp kit for reverse transcriptase-polymerase chain reaction (RT-PCR) (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PCR conditions and the expected size of each RT-PCR amplicon were as described by Bhagat *et al* (13). The primers used were: for the Hsp72 gene, forward, 5'-GCTGACCAAGA TGAAGGAGATC-3' and reverse, 5'-GAGTCGATCTCCA GGCTGGC-3'; and for the GAPDH gene, forward, 5'-TGA CATCAAGAAGGTGGTGA-3' and reverse, 5'-TCCACC ACCCTGTTGCTGTA-3', respectively. Samples without reverse transcriptase, Taq polymerase or RNA were employed as internal controls for each RT-PCR assay. The amplified products were resolved by agarose gel electrophoresis (1% agarose-0.5 μg/ml ethidium bromide).

Results

Bortezomib increases the level of Hsp72 in HepG2 cells. Western blot analysis demonstrated that treatment of HepG2 cells with bortezomib induced a marked increase in the level of Hsp72. The effect was dependent on the dose of bortezomib, with the maximum increase observed at a concentration of 50 nM (not shown). As shown in Fig. 1A, the effect intensified progressively in the first 24 h of treatment. With 50 nM bortezomib the increase was already evident at 4 h of treatment while at 24 h Hsp72 content reached a level five-fold higher than the control. However, in the second day of treatment the level continued to increase, but more slowly.

In addition, RT-PCR showed that treatment with 50 nM bortezomib for 24 h also induced a marked increase in the mRNA level of Hsp72 (469 bp), which was equivalent to the extent of the effect observed for Hsp72 protein (Fig. 1B).

Hsp72 binds and sequesters some pro-apoptotic proteins in bortezomib-treated HepG2 cells. In a previous study (9), we demonstrated that in HepG2 cells bortezomib treatment increased the level of a number of apoptotic factors, such as p53, Bax, AIF, Apaf-1, Bid, Bim, c-Jun, caspase-3, caspase-8, cytochrome c and JNK. In order to demonstrate the interaction of Hsp72 with these proteins, immunoprecipitation analysis was performed using cell extracts from HepG2 cells grown for 24 h in the presence of 50 nM bortezomib. Immunocomplexes were obtained by means of a specific antibody against Hsp72 and resolved by Western blot analysis for the above-mentioned factors. As indicated in Fig. 2A, densitometric analysis demonstrated that bortezomib treatment increased the content of p53, Bax, AIF and Apaf-1 in the immunocomplexes (lane 4) by four- to seven-fold with respect to the control (lane 3), while much less consistent increases in the level of these proteins were observed in the total extracts (lanes 1 and 2).

To confirm these results, immunoprecipitation analysis was performed by using specific antibodies against p53, Bax, AIF and Apaf-1. The experiments, illustrated in Fig. 2B, demonstrated the presence of Hsp72 in the immunocomplexes and the increase in its level after bortezomib treatment.

In addition, Western blot analysis showed that Bid, Bim, c-Jun, caspase-3, caspase-8, cytochrome c and JNK, which

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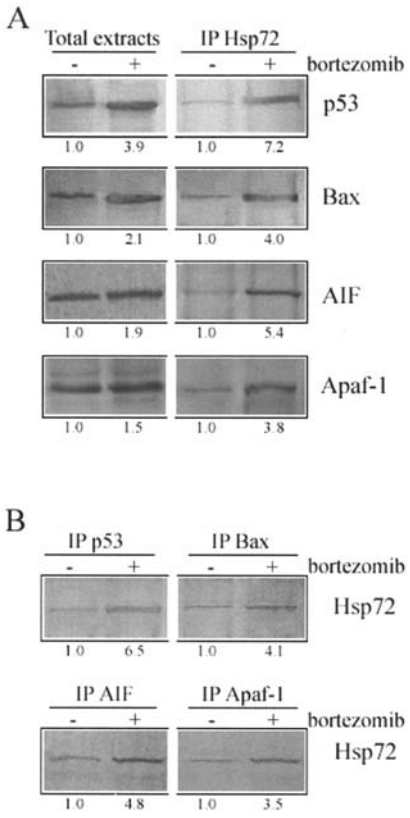

Figure 2. Interaction of Hsp72 with pro-apoptotic proteins in bortezomib-treated HepG2 cells. (A) HepG2 cells were treated with 50 nM bortezomib for 24 h. Then, Hsp72 was immunoprecipitated from the cell extracts using a specific antibody. The complexes were resolved by SDS-PAGE, immunoblotted and detected using specific antibodies against p53, Bax, AIF and Apaf-1 (lanes 3 and 4). Lanes 1 and 2 refer to the detection of the same pro-apoptotic factors in the total cell extracts. (B) p53, Bax, AIF and Apaf-1 were immunoprecipitated from cell extracts with specific antibodies. The immunocomplexes were resolved by SDS-PAGE and were probed with anti-Hsp72 antibody. Data are representative of three separate experiments.

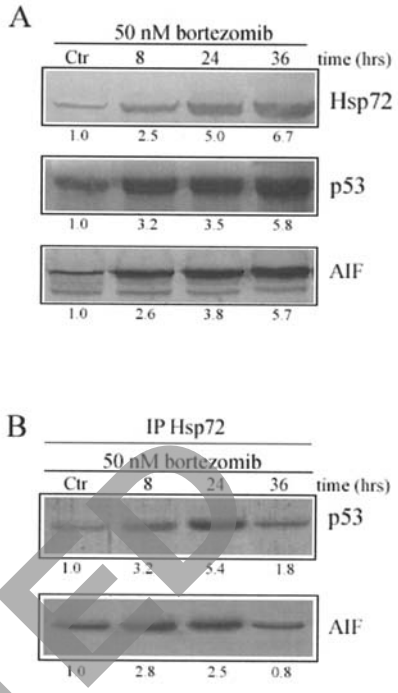

Figure 3. The time-dependent increase in the nuclear levels of p53 and AIF in bortezomib-treated HepG2 cells and the interaction of these factors with Hsp72. (A) Cells were treated with 50 nM bortezomib for different times (8-36 h). Then, the nuclear fraction was prepared as described in Materials and methods, and equal amounts of protein were analysed by Western blotting. (B) Nuclear extracts were immunoprecipitated with anti-Hsp72-specific antibody and analysed by Western blot analysis for p53 and AIF expression. The results are representative of three independent experiments.

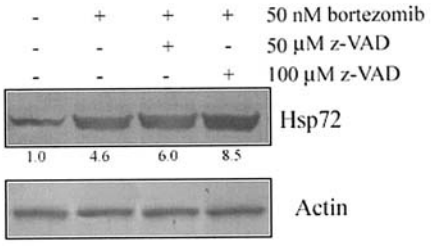

Figure 4. The effect of z-VAD on the Hsp72 level. HepG2 cells were incubated in the presence of 50 nM bortezomib for 16 h, then 50 or 100 μM z-VAD-fmk was added and the incubation was protracted for an additional 24 h. The total cell extracts were analysed by Western blotting for Hsp72. The actin blot was included to show equal protein loading for all the samples. The results are representative of four separate experiments.

are other factors involved in bortezomib-induced apoptosis in HepG2 cells, were absent in the Hsp72 immunocomplexes (not shown).

The interaction of Hsp72 with p53 and AIF decreases in the second phase of treatment. It is known that Hsp72, p53 and AIF are distributed differently in subcellular compartments and that their action in the apoptotic mechanism is related to translocation into the nucleus (14). Fig. 3A shows that bortezomib induced a time-dependent increase in the level of Hsp72, p53 and AIF in the nuclear fraction of HepG2 cells. The maximum effect for all these proteins was observed at 36 h of treatment. To note, the immunoassay performed in the Hsp72 immunocomplexes obtained from the nuclear fraction revealed that the content of p53 and AIF increased in the first phase of bortezomib treatment and then markedly decreased in the second day (Fig. 3B).

Caspase activities are involved in Hsp72 degradation. It has been reported that caspase activities are involved in Hsp72 degradation (15). Since bortezomib induced a marked activation of caspases (9), we analysed the effect of z-VAD, a general inhibitor of these activities, on the level of Hsp72. Fig. 4 shows that the addition of z-VAD to bortezomib-treated cells dose-dependently increased the level of Hsp72. This result indicates that in our experimental conditions caspases induced Hsp72 degradation.

Discussion

In a previous study we reported that hepatoma HepG2 cells are very sensitive to apoptosis induced by the proteasome inhibitor bortezomib. In our experimental conditions, upon treating the cells with bortezomib, apoptosis occurred in the second day of treatment. In the first 24 h apoptosis was most likely prevented by the increase in the level of the survival

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factors NF-κB (10), Hsp27 and the inducible isoform of Hsp70 (Hsp72) (9).

In particular, this research demonstrated that bortezomib induced in hepatoma HepG2 cells a progressive increase in the Hsp72 level which was already evident at 4 h of treatment and was dependent on new protein synthesis. This is in accordance with the observation that overexpression of Hsp72 confers a general resistance to various cellular stresses by inhibiting apoptosis (16).

The anti-apoptotic action of Hsp72 is often related to its ability to bind and sequester some pro-apoptotic factors (4). Thus, we employed the immunoprecipitation technique to analyse in HepG2 cells the possible interaction of Hsp72 with those factors whose level increased after bortezomib treatment and that were involved in apoptosis induced by the drug. We observed that consistent amounts of the pro-apoptotic factors p53, Bax, AIF and Apaf-1 interacted with Hsp72 in the first phase of treatment. Therefore, the increased level of Hsp72 occurring in the first 24 h prevented the action of the pro-apoptotic factors, thus contributing to counteract the apoptotic effects induced by bortezomib in HepG2 cells. Successively, coinciding with the apoptotic effect of bortezomib, the interaction of Hsp72 with the pro-apoptotic factors decreased. Particularly evident was the decrease in the nuclear interaction of Hsp72 with p53 and AIF observed in the second day of bortezomib treatment.

To explain this reduced interaction between Hsp72 and the pro-apoptotic factors, we hypothesize that this event is a consequence of post-translational modifications of Hsp72 occurring in the second phase of bortezomib treatment. Alternatively, HepG2 cells produce a factor which competes with p53 and AIF for the binding with Hsp72. Studies are in progress to verify these or other hypotheses.

Moreover, the observation that the addition of z-VAD, a general inhibitor of caspase activities, induced a further increase in the Hsp72 level indicated that in the second day of treatment, caspase activities were involved in the control of the Hsp72 content. This event could have contributed to limit the anti-apoptotic activity of Hsp72 in the second phase of the bortezomib treatment.

The present study suggests that a reduction in the anti-apoptotic action of Hsp72 could markedly improve the antitumoral efficacy of bortezomib.

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