Blockade of Hsp27 Overcomes Bortezomib/Proteasome Inhibitor PS-341 Resistance in Lymphoma Cells

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Abstract

Bortezomib (PS-341), a selective inhibitor of proteasome, induces apoptosis in various tumor cells, but its mechanism of action is unclear. Treatment with PS-341 induces apoptosis in SUDHL6 (DHL6), but not SUDHL4 (DHL4), lymphoma cells. Microarray analysis shows high RNA levels of heat shock protein-27 (Hsp27) in DHL4 versus DHL6 cells, which correlates with Hsp27 protein expression. Blocking Hsp27 using an antisense strategy restores the apoptotic response to PS-341 in DHL4 cells; conversely, ectopic expression of wild-type Hsp27 renders PS-341-sensitive DHL6 cells resistant to PS-341. These findings provide the first evidence that Hsp27 confers PS-341 resistance.

Introduction

The ubiquitin-proteasome signaling pathway regulates cell growth, differentiation, and apoptosis; target proteins are ubiquitinated by activating (E1)/conjugating enzymes (E2s), ligases (E3s), and then degraded by the 26S proteasomal multicatalytic protein complex (1–3). Importantly, inhibition of proteasome function has led to the development of novel antitumor agents (1, 2, 4), e.g., Bortezomib/PS-341, a reversible proteasome inhibitor, shows remarkable anticancer activity in various cancer cell types (2, 3), including MM cells that are resistant to conventional therapies (5). Our recent preclinical and clinical studies in MM have led to the FDA approval of Bortezomib/PS-341 for the treatment of MM refractory to conventional therapies (6). Although resistance to another proteasome inhibitor MG-132 has been reported in Burkitt’s lymphoma cells (7), the mechanism of PS-341 resistance in any cell type is undefined. Overexpression of Bcl-2 family of proteins confers drug resistance (8), and our recent study showed that PS-341 overcomes the Bcl-2-mediated protection against conventional therapies in MM (9). Hsp-27 functions in a manner similar to Bcl-2 (10, 11) and also confers resistance to various anticancer drugs (11, 12); however, the role of Hsp27 in providing protection against PS-341-induced cell death is undefined. In the present study, we show that Hsp27 confers PS-341 resistance in DHL4 lymphoma cells, and blocking Hsp27 using an antisense to Hsp27 (AS-Hsp27) restores sensitivity to PS-341 in PS-341-resistant DHL4 cells. These findings provide the first evidence of potential mechanisms of PS-341 resistance and suggest a therapeutic advantage of using an AS-Hsp27 to overcome PS-341 resistance.

Materials and Methods

Cell Culture and Reagents. Human SUDHL4 (DHL4) and SUDHL6 (DHL6) lymphoma cells were kindly provided by Dr. Margaret Shipp (Dana-Farber Cancer Institute, Boston, MA). Cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were treated with various concentrations of PS-341 for different time intervals (Millennium Pharmaceuticals, Boston, MA).

Cell Viability Assays. Cell viability was assessed by MTT (Chemicon International, Inc., Temecula, CA) assay according to manufacturer’s instructions (Roche Molecular Biochemicals), and as described previously (13).

Quantification of Apoptosis. Dual fluorescence staining with DNA-binding fluorochrome HO and PI was used to quantitate the percentage of apoptotic (PI+ HO+) cells using flow cytometry (The Vantage, Becton Dickinson), as described previously (13). Apoptosis was also assessed by Annexin V staining.

Western Blotting. Protein lysates were prepared and subjected to Western blot analysis, as described previously (14). Briefly, equal amounts of proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were then transferred to nitrocellulose filters, blocked by incubation in 5% dry milk in PBST (0.05% Tween 20 in PBS), and probed with anti-Hsp-70, anti-Hsp70, anti-PARP, or antitubulin (Santa Cruz Biotechnology, Santa Cruz, CA) Abs (Sigma, St. Louis, MO). Blots were then developed by enhanced chemiluminescence (Amer sham, Arlington Heights, IL).

Oligonucleotide Array Analysis. The analysis was performed as described previously (15). Affymetrix U133A arrays were hybridized with biotinylated in vitro transcription products (10 μg/Chip), as per manufacturer’s instructions. Fluidic station 400 (Affymetrix, Santa Clara, CA) was used for washing and staining the arrays. The DNA Chips were then analyzed using a Gene Array Scanner (Affymetrix). CEL files are obtained using Affymetrix Microarray Suite 5.0 software. The DNA-Chip Analyzer (Dchip; Ref. 16) was used to normalize all CEL files to a baseline array with overall median intensity, and the model-based expression (Perfect match only) was used to compute the expression values. Analysis identified signals varying by ±2-fold (lower bound) with a 90% confidence interval.

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5 Internet address: www.dchip.org, version 1.3.
PS-341 for 24, 48, and 72 h, followed by analysis for cell viability, apoptosis, and protein expression as described above.

**Proteasome Inhibition Assay.** Total cell lysates from DHL4 and DHL6 lymphoma cells were analyzed for 20S proteasome activity, as described previously (18).

**Results and Discussion**

**Effect of PS-341 on the DHL4 and DHL6 Viability.** We first determined whether treatment of DHL4 and DHL6 lymphoma cells affects the viability of these cells. Cells were treated with various doses (1–10 nM) of PS-341 for 24 h, harvested, and analyzed for cell viability by an MTT assay. As seen in Fig. 1A, PS-341 significantly decreases the cell viability in DHL6 cells (IC50 = 5 nM); in contrast, PS-341 did not alter the viability of DHL4 cells (P = 0.05, as determined by one-sided Wilcoxon rank-sum test).

To determine whether PS-341 triggers apoptosis, DHL4 and DHL6 cells were treated with 10 nM PS-341 for 24 h, harvested, and analyzed for apoptosis by two methods: (a) dual staining with PI and HO; and (b) PARP cleavage, a hallmark of apoptosis (19). As seen in Fig. 1B, treatment of cells with PS-341 triggers a significant increase in percentage of apoptotic (PI+ HO-) cells in DHL6 but not DHL4 cells [median percentage of apoptotic cells: 74.3 ± 4.3% in DHL6 cells (P < 0.003) and 7.2 ± 0.5% in DHL4 cells (P < 0.004)]. These results were confirmed using PARP cleavage assays. As seen in Fig. 1C, PS-341 triggers proteolytic cleavage of PARP into M, 85,000 fragment in DHL6, but not DHL4, cells. Taken together, our results suggest that PS-341 induces apoptosis in DHL6 cells, whereas DHL4 cells are resistant to PS-341.

To exclude the possibility that this event is specific to PS-341, we performed similar experiments using another proteasome inhibitor MG-132. DHL4 and DHL6 cells were treated with MG-132 (0.2–1 μM) for 24 h, harvested, and analyzed for cell viability. As seen in Fig. 1D, MG-132 decreased the viability of DHL6 cells; however, no such effects of MG-132 were observed in DHL4 cells. Together, these findings demonstrate that DHL4 cells are resistant, and DHL6 cells are sensitive to proteasome inhibitors.
Hsp27 Is Differentially Expressed in DHL4 versus DHL6 Cells. To determine the molecular mechanisms mediating PS-341-resistance in DHL4 cells, we performed oligonucleotide arrays using both PS-341-sensitive DHL6 and PS-341-resistant DHL4 cells. Total cellular RNA was prepared and subjected to oligonucleotide microarray profiling, followed by data analysis using DNA Chip Analyzer (Dchip; Ref. 16). A total of 238 genes were up-regulated (≥2-fold), and 231 genes were down-regulated in DHL4 compared with DHL6 cells. Given that previous studies have shown the role of Hsp27 in conferring drug resistance, we examined whether Hsp27 similarly affects PS-341 sensitivity in lymphoma cells. As shown in Fig. 1E, Hsp27 transcripts were highly expressed in DHL4 versus DHL6 cells (3–4-fold higher; \( P < 0.006, n = 3 \)).

We next determined whether differences in the Hsp27 mRNA levels correlate with alterations in Hsp27 protein levels. Lysates from DHL4 and DHL6 cells were subjected to immunoblotting with anti-Hsp27 Abs. As seen in Fig. 1F (top panel), PS-341-resistant DHL4 cells express significantly higher Hsp27 protein levels compared with PS-341-sensitive DHL6 cells. Reprobing the blots with antitubulin Ab showed equal protein loading in each lane (Fig. 1F, bottom panel). Alterations in Hsp27 protein levels therefore correlate with changes in mRNA levels. These findings are consistent with another study suggesting that Hsp27 expression may be associated with sensitivity to proteasome inhibitors (20).

Functional Significance of Hsp27 Expression. We next directly examined whether inhibition of Hsp27 affects responsiveness to PS-341. DHL4 cells were transfected with antisense to Hsp27 (AS-Hsp27) and analyzed for Hsp27 protein expression. As seen in Fig. 2A (top panel), the exogenous expression of AS-Hsp27 markedly reduces the expression of endogenous Hsp27 without altering cellular Hsp70 protein levels (bottom panel). These data confirmed the functional specificity of AS-Hsp27. To determine whether inhibition of Hsp27 in DHL4 cells restores sensitivity to PS-341, AS-Hsp27-transfected DHL4 cells were treated with PS-341 (10 nm) for 24, 48, and 72 h, followed by assessment of cell viability using an MTT assay. As seen in Fig. 2B, treatment of AS-Hsp27-transfected cells with PS-341 significantly decreased cell viability; median viability was 93.4 ± 4.3% at 24 h for empty vector-transfected cells versus 53.7 ± 3.1% in AS-Hsp27-transfected cells (\( P = 0.05 \), as determined by one-sided Wilcoxon rank-sum test). To further confirm specificity of AS-Hsp27, cells were also treated with scrambled ODNs and similarly analyzed for PS-341-sensitivity. No significant difference in cell viability was observed in cells treated with PS-341 and scrambled ODNs (data not shown).

To confirm whether PS-341 induces apoptosis in AS-Hsp27-transfected DHL4 cells, cells were analyzed by both Annexin V staining and PARP cleavage activity. As seen in Fig. 2C, PS-341 triggers apoptosis in AS-Hsp27-transfected but not in either nontransfected or control vector-transfected DHL4 cells. Importantly, transfection of AS-Hsp27 alone does not induce apoptosis in DHL4 cells. As seen in Fig. 2D, examination of PARP cleavage demonstrated similar results: PS-341 induces proteolytic cleavage of PARP in AS-Hsp27-, but not in control vector-, transfected DHL4 cells. Taken together, these findings demonstrate that inhibition of Hsp27 by AS-Hsp27 restores PS-341 responsiveness in otherwise PS-341-resistant lymphoma cells.

Overexpression of Hsp27 (WT) Confers Resistance to PS-341 in PS-341-sensitive DHL6 Cells. Given that Hsp27 protein levels are significantly lower in PS-341-sensitive DHL6 cells vis-à-vis PS-341-resistant DHL4 cells, we next asked whether exogenous expression of Hsp27 wild-type (WT) would confer PS-341-resistance in PS-341-sensitive DHL6 cells. As seen in Fig. 3A (top panel), transfections of Hsp27 (WT), but not control vector, led to a marked increase in the Hsp27 protein levels. Expression of Hsp27 (WT) does not alter the endogenous tubulin protein levels (Fig. 3A, bottom panel), confirming the functional specificity of Hsp27. Importantly, as seen in Fig. 3B, Hsp27 (WT)-transfected cells survive significantly longer than control vector-transfected DHL6 cells after treatment with PS-341 (\( P = 0.05 \), as determined by Wilcoxon rank-sum test). Together, these findings indicate that high Hsp27 expression is associated with resistance to PS-341 in MM cells; conversely, low expression of Hsp27 correlates with PS-341 sensitivity.

Proteasome Inhibition in Tumor Cells. It is known that PS-341 mediates its effects by inhibiting cellular proteasomes (1, 2); however, whether proteasome inhibition is universally required for PS-341-
triggered apoptosis is unclear. On the basis of our present findings that PS-341 triggers differential biological responses in DHL4 versus DHL6 cells, we next determined whether PS-341 induces similar alterations in proteasome activity using a substrate specific for measuring chymotrypsin-like activity in these cells. As seen in Fig. 3C, treatment with PS-341 (10 nM) for 24 h led to 82 and 88% inhibition of proteasome activity in DHL4 and DHL6 cells, respectively. Similar results were observed when cells were treated with higher concentrations of PS-341 (20 nM) for a shorter time period (6 h; data not shown). Together, these data confirm that: (a) the proteasome inhibition pathway is not defective in PS-341-resistant DHL4 cells; (b) proteasome inhibition is not correlated with apoptosis; and (c) the mechanism whereby Hsp27 confers PS-341 resistance is independent of proteasome activity. Whether PS-341 triggered proteasome inhibition alone is sufficient to induce cytotoxicity in other cell types remains to be defined.

The mechanisms mediating the cytoprotective function of Hsp27 are unclear. We and others have shown that Hsp27 regulates mitochondrial apoptotic signaling, inhibits the release of cyto-c/Smac and subsequent activation of caspase cascade (10, 12). Hsp27 functions in a manner similar to another mitochondria-resident protein Bcl-2; both prevent the release of cyto-c and block apoptosis. Preliminary examination of the Bcl-2 and Hsp27 protein levels in PS-341-resistant DHL4 versus PS-341-sensitive DHL6 cells shows differential expression patterns of Hsp27 but not of Bcl-2 (data not shown). Moreover, previous study showed that PS-341 overcomes Bcl-2-mediated cytoprotective effects (5). Together, these results indicate that Hsp27, but not Bcl-2, is likely to confer PS-341 resistance in lymphoma cells. Another study also showed that Hsp27 blocks cyto-c release by maintaining the integrity of actin network; Hsp27 prevents the translocation of proapoptotic factors from the actin cytoskeleton to mitochondria, where they can trigger cyto-c release. In the present study, blocking Hsp27 restores sensitivity to PS-341 in PS-341-resistant DHL4 lymphoma cells; however, the underlying mechanism mediating this event remains to be defined. It is likely that Hsp27 interacts with either Bcl-2/Bax or cytoskeleton proteins to block PS-341-initiated apoptotic signaling in these cells.

In summary, our results demonstrate the following: (a) DHL4, but not DHL6, lymphoma cells are resistant to PS-341; (b) Hsp27 is highly expressed in DHL4 versus DHL6 cells; (c) Hsp27 confers PS-341 resistance in DHL4 lymphoma cells; (d) blocking Hsp27 by AS-Hsp27 restores sensitivity to PS-341 in PS-341-resistant DHL4 cells; and (e) conversely, overexpression of Hsp27 (WT) increases resistance to PS-341 in PS-341-sensitive DHL6 lymphoma cells. Our results therefore suggest that AS-Hsp27 may be used to enhance or restore sensitivity of tumor cells to PS-341.

References
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