Cancer Chemother Pharmacol (2008) 61:549–558
DOI 10.1007/s00280-007-0500-1

ORIGINAL ARTICLE

Preclinical studies of the proteasome inhibitor bortezomib in malignant pleural mesothelioma

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Received: 7 February 2007 / Accepted: 11 April 2007 / Published online: 24 May 2007

Abstract Malignant pleural mesothelioma (MPM) is a highly lethal neoplasm that is resistant to chemotherapy. Bortezomib is an FDA-approved proteasome inhibitor that is currently under clinical investigation in multiple neoplasms but has not been studied extensively in MPM. In this report, we determine the biological and molecular response of cultured MPM cells to bortezomib alone and in combination with cisplatin or pemetrexed. We used four MPM cell lines (MS589, H28, H2052, JMN), a normal mesothelial cell line (HM3), and a lung cancer cell line (H23) in survival studies utilizing bortezomib, cisplatin, and pemetrexed alone and in combination by administering concurrently or by varying the order of administration. We determined the effect of bortezomib on the cell cycle, apoptosis, and on the expression of cell cycle proteins p21/WAF1 and p27/KIP1 and on apoptosis-related proteins IAP-1, IAP-2, survivin, and XIAP. Bortezomib was highly cytotoxic to MPM cells and induced both G_2/M and G_1/S cell cycle arrest. Apoptosis increased in a concentration- and time-dependent manner in 3 of 4 MPM cell lines. Bortezomib stabilized or increased protein levels of p21/WAF1 and IAP-1 and to a lesser degree p27/KIP1, IAP-2, XIAP, and survivin. In combination studies with cisplatin, bortezomib was generally synergistic at high concentrations and antagonistic at low concentrations. Bortezomib increased the cytotoxicity of cisplatin and pemetrexed in a concentration-dependent manner when administered prior to either. Bortezomib may improve outcome in MPM patients alone or in combination with standard chemotherapy but the order of administration is likely to be important. This study justifies further evaluation of bortezomib in MPM.

Keywords Mesothelioma · Bortezomib · Cisplatin · Pemetrexed · Inhibitor of apoptosis protein

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Introduction

Malignant pleural mesothelioma (MPM) is a highly lethal thoracic neoplasm with a universally dismal outlook. There are three distinct common histologic subtypes of MPM based on the microscopic appearance of the major malignant elements: epithelial, sarcomatoid, biphasic (mixed). The majority of MPMs are epithelial (50%). Without any treatment, the expected median survival of patients presenting with MPM is between 4 and 12 months [1, 2]. Aggressive cytoreductive therapy including surgery (i.e.,

extrapleural pneumonectomy, EPP) followed by combination of chemotherapy and radiation therapy (trimodality therapy) has been shown to prolong survival in selected patients with early MPM [3–6]. However, most patients do not undergo trimodality therapy due to insufficient cardiopulmonary reserve, advanced disease or lack of access to specialized centers.

MPM is exceedingly resistant to most chemotherapy regimens examined till date [7, 8] and radiation therapy is generally ineffective as a primary treatment as well [9]. Targeted therapy, such as anti-angiogenic drugs [10] and inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase [11], has proved equally ineffective at prolonging MPM patient survival despite substantial overexpression of the relevant molecular targets in MPM tumors. Pemetrexed (Alimta) and cisplatin combination chemotherapy was recently shown in a prospective randomized trial to be the best chemotherapy regimen for MPM [12]. However, this therapy increased only median survival from 9 to 12.4 months and the response rate was lower than 50% [12]. New therapies for MPM are desperately needed but the process of designing new drugs is expensive and time consuming. Emerging molecular targeted therapy (e.g., receptor tyrosine kinase c-Met [13] and others), or gene therapy-based treatment [14], may eventually prove superior in MPM but is not yet ready for clinical implementation.

As MPM is highly lethal, immediate improvement in patient outcome is likely to result by incorporating pre-existing drugs into current MPM treatment regimens. One potential class of drugs is the proteasome inhibitors. The proteasome is a large catalytic complex that is responsible for most non-lysosomal intracellular protein degradation. This structure is a promising target for cancer since it has been shown to impact the cell-cycle, apoptosis, proliferation, and other physiological processes by regulating the levels of central proteins such as CDC25A, CDC25B, CDC25C, MDM2, p21/WAF-1, p27/KIP1, and I-κB [15]. Bortezomib (VELCADE) is the first proteasome inhibitor to enter clinical trials. It was approved by the FDA in 2003 for the treatment of relapsed and refractory multiple myeloma, and is currently being evaluated alone and in combination with other chemotherapy in a number of solid tumors, such as lung cancer [15].

One family of proteins regulated by the proteasome is the inhibitor of apoptosis proteins (IAP). The IAP gene family includes eight members, five of which have been extensively studied in the context of cancer: IAP-1 (a.k.a. MIHC/cIAP2), IAP-2 (a.k.a. MIHB/cIAP1), XIAP (a.k.a. MIHA/hILP), livin (a.k.a. ML-IAP/KIAP), and survivin [16]. IAPs have been implicated in the promotion of cell survival [17, 18] as well as in the regulation of proliferation, cell-cycle, protein degradation, and gene transcription [19–22]. Many IAPs are highly expressed by multiple types of tumors [16] but, with the exception of survivin, also by normal adult tissues [23], suggesting that at least some of their physiological roles are cell- and tissue-type dependent.

We have previously discovered IAP-1 as a novel upregulated gene in MPM that is responsible in large part for the resistance of cultured MPM cells to cisplatin [24]. In a follow-up study, we have found that multiple IAPs are expressed by a large panel of MPM tumors and cell lines at the mRNA and protein levels. In addition, the expression levels of some of these genes are correlated with a number of clinical variables including patient survival [25]. Other investigators have also observed aberrant expression of IAPs in MPM [26–28]. In addition, we have shown that at least some IAPs are transcriptionally regulated by NF-κB in MPM [29]. Proteasome inhibitors, such as bortezomib, inhibit the degradation of I-κB which prevents the activation of NF-κB, a transcription factor that regulates survival proteins (including IAPs and others) used by the cancer cells to evade apoptosis. In fact, NF-κB is thought to be the primary target of bortezomib, particularly when combined with other chemotherapies that induce apoptosis [15].

These observations suggest that bortezomib may have efficacy in the treatment of MPM. Therefore, we performed survival and molecular studies of bortezomib alone and in combination with cisplatin and pemetrexed in cultured MPM cell lines ($n = 4$), a lung cancer control cell line, and in a normal mesothelial cell line. We also determined the effect of bortezomib (and other drugs) on protein levels of IAP family members since these proteins (and related pathways) are likely to be important in mediating multi-drug resistance in MPM.

Materials and methods

Cell culture and reagents

The biphasic MPM cell line MS589 was kindly provided by Jonathan A. Fletcher, Department of Pathology, Brigham and Women's Hospital (BWH). The bipahasic MPM cell line JMN [30, 31] and the normal primary human mesothelial cell line HM3 [32, 33] were kindly provided by James Rheinwald, Department of Dermatology, BWH. MPM cell lines H2052 (sarcomatoid), H28 (epithelial) and lung adenocarcinoma cell line H23 were purchased from the American Type Culture Collection (http://www.atcc.org). All tumor cell lines were grown in RPMI1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% calf serum and antibiotics. HM3 cells were expanded in culture by growing in M199/MCDB106 (1:1 (v/v) medium +15% newborn calf serum +0.4 μg/ml hydrocortisone +EGF 10 ng/ml). (M199 and MCDB106 medium

was obtained from Invitrogen Life Technologies and Cascade Biologics, Portland, OR, respectively.) This formulation is essential for the proliferation and expansion of HM3 cells [32, 33]. Prior to survival studies, HM3 cells were grown in medium lacking EGF for 24 h to halt proliferation in order to more closely model normal in vivo conditions. All cells were maintained in a humidified, 5% CO_2 incubator at 37°C. Cisplatin (*cis*-platinum(II)diammine dichloride) was purchased from Sigma Chemicals (St Louis, MO). Bortezomib was obtained from Millennium (Cambridge, MA). Pemetrexed was purchased from the Dana-Farber Cancer Institute Pharmacy (Boston, MA).

Survival studies

Cells were seeded in 24-well plates at a density of 20,000 cells/well (for cisplatin and bortezomib studies) or 3,000 cells/well (for pemetrexed studies) and were allowed to adhere overnight. For single agent studies, medium containing either bortezomib (0.9–1,000 nM), cisplatin (0.9–1,000 μM), or pemetrexed (0.9–1,000 μM) was applied for 48 h. Complete medium without any drug was used in control wells. For cisplatin and bortezomib single agent studies, fresh medium was added after 48 h for an additional 24 h after which the surviving cells in individual wells were fixed using 4% paraformaldehyde for 15 min at 4°C. Fixed cells were stained using 0.5% crystal violet (Sigma) in water for 15 min then washed in distilled water for 15 min and dried overnight. Crystal violet was eluted by incubation for 15 min at room temperature in Sorenson's solution (0.03 M sodium citrate, 0.02 M HCL, and 50% ethanol). The absorbance of the eluent was measured at 570 nm and was used as a measure of cell survival by combining absorbance data for individual wells ($n = 4$ per concentration) and stating the average relative to the average of control wells. For pemetrexed single agent studies, fresh medium was added after 48 h of drug exposure for an additional 24–72 h after which the cells were fixed and stained as described above. Colonies containing at least 50 cells were counted in individual wells ($n = 4$ per concentration), averaged, and expressed relative to the average of control. All experiments were repeated at least three times.

For drug combination studies utilizing bortezomib and cisplatin, survival analysis was performed exactly as above (for either agent individually) under three experimental conditions utilizing drug concentrations exactly three orders of magnitude apart (3.75–240 μM cisplatin/3.75–240 nM bortezomib) and a total drug exposure time of 48 h: (1) both drugs at the same time for 48 h, (2) cisplatin for 24 h followed by bortezomib for 24 h, and (3) bortezomib for 24 h followed by cisplatin for 24 h. The concentration of bortezomib was exactly 1,000 times lower than that of cisplatin based on the relative difference in the IC_{50} values for both drugs alone (i.e., nM compared to μM). All experiments were repeated at least three times.

To determine whether bortezomib would chemosensitize MPM cells to cisplatin or pemetrexed, cells were seeded as above and all wells were exposed to bortezomib (4 nM) for 24 h. Immediately following, standard cisplatin and pemetrexed survival analysis was performed as described above for either agent individually. All experiments were repeated at least 3 times.

Protein studies

Cells were seeded in T75 flasks and were allowed to adhere overnight. In one experiment, medium containing bortezomib at a range of concentrations (0.1–100 nM) was applied for 24 h. For the first of three drug combination studies, cells were exposed to either bortezomib (10 nM), cisplatin (30 μM), or the combination (10 nM bortezomib/30 μM cisplatin) for 24 h. For the second drug combination study, cells were exposed to bortezomib (10 nM) for 24 h followed by cisplatin (30 μM) for 24 h. The order of drug administration was reversed for the third study. In all cases, immediately after drug exposure protein was extracted using MPER buffer supplemented with HALT protease inhibitor cocktail (Pierce, Rockford, IL). Protein quantity was estimated using the Bio-Rad Protein Assay (Hercules, CA). For Western blot analysis, aliquots of cell lysates were combined with a 2X protein solubilization stock buffer (Invitrogen) supplemented with 5% 2-mercaptoethanol and denatured by boiling for 7 min. Samples containing 100 μg of protein per lane were separated using SDS-PAGE with pre-cast 10, 12, or 14% Tris/glycine polyacrylamide gels (Novex, San Diego, CA) and were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, IL) according to standard procedures. All subsequent incubations were carried out at room temperature unless stated otherwise. Nitrocellulose membranes were incubated with agitation overnight at 4°C in a blocking buffer consisting of 5% Quick Blocker (Chemicon, Hampshire, U.K.) in PBS/0.1% Tween-20. Blots were probed overnight at 4 °C with primary antibodies (diluted in PBS) specific for p21/WAF1 (1:1,000, Zymed Research antibodies from Invitrogen), p27/KIP1 (1:1,000, BD Biosciences, San Jose, CA), GAPDH (1:5,000, Ambion/Applied Biosystems, Foster City, CA), XIAP (1:1,000, Stressgen/Nventa, San Diego, CA), IAP-1/cIAP2 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), IAP-2/cIAP1 (1:1,000, Santa Cruz Biotechnology), survivin (1:2,000, R and D Systems, Minneapolis, MN), and caspase-3 (1:500, Chemicon International, Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) were diluted 1:5,000 in PBS and applied for 1 h. Membranes were washed after the primary and secondary

antibodies in three changes of 0.1% Tween 20 in PBS for 5 min each. Chemiluminescent detection was performed using ECL reagents (Amersham) and the size of the protein bands was estimated by comparison to Full Range Rainbow molecular marker standard (Amersham). All experiments were repeated at least three times.

Flow cytometry

Fluorescence-activated cell sorting (FACS) was used for cell cycle analysis and to quantify apoptotic cells. MPM cells (0.75 million/T25 flask) were exposed to bortezomib (1, 10, or 100 nM) for 24, 48, and 72 h and then fixed in cold 100% ethanol and stained with propidium iodide (for cell cycle analysis) or processed immediately (for apoptosis analysis). Quantification of apoptotic cells was accomplished using Annexin V/propidium iodide staining in kit format (Vybrant Apoptosis Assay Kit, Invitrogen) FACS was performed by the Dana-Farber/Harvard Cancer Center Core Facility using a Becton Dickinson FACScan analyzer. All experiments were repeated at least three times.

Statistical analysis

Survival curves were plotted using Graph Pad Prism v.4.0 (Graph Pad Software, San Diego, CA). Non-linear regression analysis was used to fit a standard sigmoidal dose response (variable slope) equation to log-transformed data (GraphPad Prism v.4.0) in order to obtain IC_{50} values (i.e., the theoretical concentration required to kill 50% of cells) as a relative measure of drug sensitivity. Isobolograms were constructed for the combination of cisplatin and bortezomib according to the method of Chou and Talalay [34] using CalcuSyn v.2.0. software (Biosoft, Cambridge, UK). Combination indices are automatically calculated and are used to determine whether the drug combination is synergistic (<1), additive (=1), or antagonistic (>1) at various concentrations.

Results

Cytotoxic effects of bortezomib in MPM

We performed survival studies using MPM cell lines (MS589, H28, H2052, JMN) and control cell lines representing normal mesothelial cells (HM3) and lung adenocarcinoma (H23) after exposure to bortezomib at a range of concentrations (Fig. 1a). Non-linear regression analysis of survival curves for all tumor cell lines produced an excellent fit ($R^2 = 0.70$ for H28, $R^2 = 0.94$–0.95 for others). Bortezomib was similarly toxic to all four MPM cell lines based on calculated IC_{50} values (cell line, IC_{50} value, 95% confidence interval (CI)): MS589, 2.7 nM, 2.0–3.6; H28, 3.8 nM, 2.0–7.8; H2052, 0.98, 0.68–1.4; JMN, 6.2 nM, 4.9–7.8. The H23 lung adenocarcinoma cell line was approximately 25 times more resistant to bortezomib compared to MPM cell lines ($IC_{50} = 82.6$ nM, 95% CI = 62.9–108.6). Bortezomib was minimally cytotoxic to normal mesothelial cells at all concentrations, particularly in the IC_{50} range of MPM cell lines (i.e., ~5–10 nM, a concentration range that was associated with >90% viability of HM3 cells).

Cell cycle analysis

Bortezomib induced a concentration-dependent G_2/M cell cycle arrest in H28 cells after 24 h of drug exposure that remained in effect, at least partially, for up to 72 h (Fig. 1b). Similar results were obtained for H2052 cells (data not shown). In contrast, there was a substantial accumulation of MS589 cells at G_1/S in both a concentration- and time-dependent manner in response to bortezomib (Fig. 1b). JMN cell cycle analysis did not produce easily interpretable data since this cell line displays substantial aneuploidy (data not shown). Protein levels of p21/WAF1 were stabilized to some degree in all MPM cell lines in response to escalating concentrations of bortezomib while those of p27/KIP1 displayed a similar trend in H2052 cells only (Fig. 1c). Protein levels of p27/KIP1 were stabilized only at low concentrations in MS589 cells. Interestingly, for H28 cells only those that were not exposed to bortezomib expressed detectable, albeit low, levels of p27/KIP1. There was no discernible difference in p27/KIP expression levels between control and bortezomib exposed JMN cells.

Analysis of apoptosis

Apoptotic cells were quantified in MPM cell lines after exposure to increasing concentrations of bortezomib for varying time periods (Fig. 2). Apoptotic cells clearly increased in a concentration- and time-dependent manner in H2052 cells (Fig. 2a) and in JMN cells (Fig. 2c). H28 cells displayed a concentration-dependent increase in apoptosis only at the 72 h time-point (Fig. 2b). MS589 cells did not display an increase in apoptosis in response to bortezomib at any concentration or time-point (Fig. 2d). Apoptosis, or lack thereof, was additionally confirmed in all MPM cell lines by monitoring the cleavage of caspase 3 (data not shown). In all cases, the relative extent of caspase 3 cleavage mirrored the increase in apoptotic cell number as quantified using Annexin V staining and FACS.

We determined the effect of proteasome inhibition on IAP protein levels in MPM cell lines exposed to increasing concentrations of bortezomib (Fig. 2e). IAP-1 protein levels increased in a concentration-dependent manner in 3 of 4

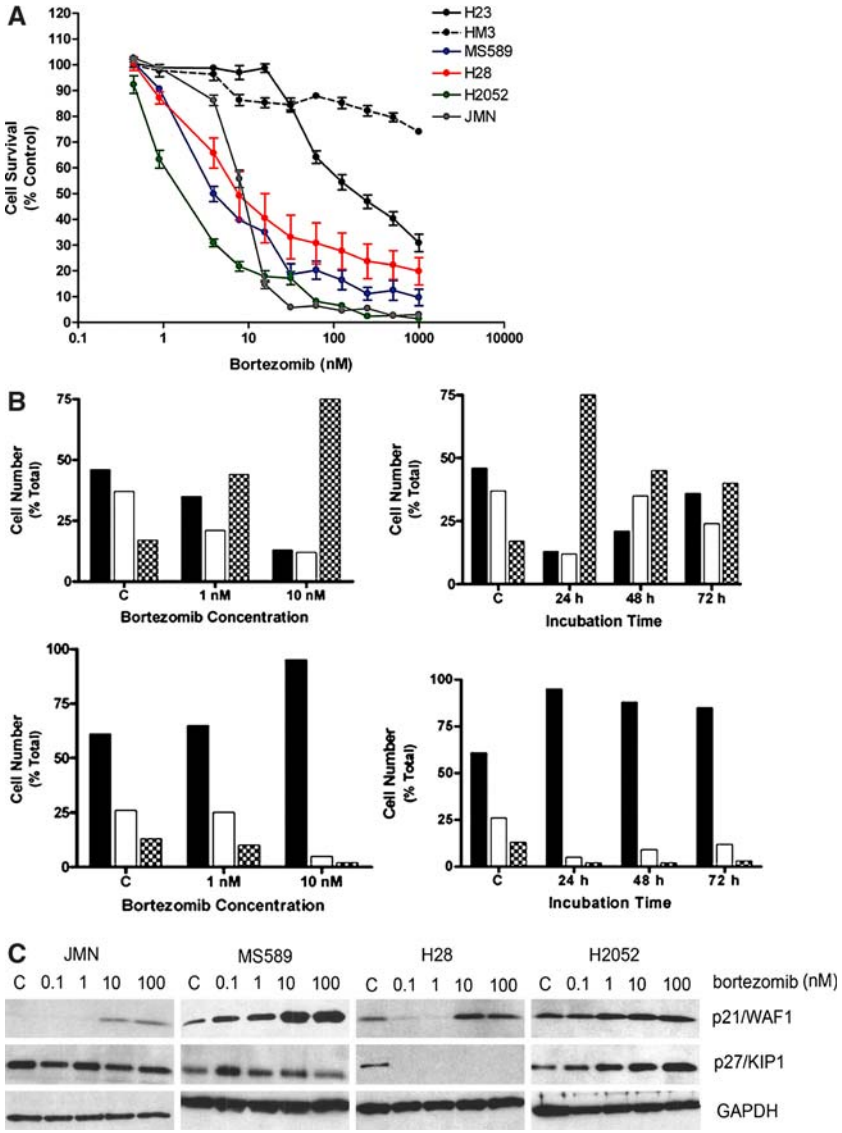

Fig. 1 Biological effects of bortezomib in cultured MPM and control cell lines. **a** Survival analysis of MPM cells (MS589, H28, H2052, JMN), lung adenocarcinoma cells (H23), and normal mesothelial cells (HM3) exposed to bortezomib for 48 h. *Error bar*, SEM. **b** Concentration-dependent (*left graphs*) and time-dependent (*right graphs*) cell cycle analysis of bortezomib exposed H28 MPM cells (*top graphs*) and MS589 MPM cells (*bottom graphs*) relative to unexposed controls ("C") *Solid bars*, G_1; *Clear bars*, S; *Hatched bars*, G_2. **c** Western blot analysis of cell cycle proteins p21/WAF1 and p27/KIP1 in MPM cells exposed to bortezomib for 24 h. Each experiment was repeated three times and a representative blot is shown. GAPDH was used as a loading control

MPM cell lines (MS589, H28, H2052). IAP-2 levels increased modestly in a single cell line (H28). XIAP and survivin protein levels in general were modestly elevated (or unchanged) relative to control at low bortezomib concentrations (0.1 and 10 nM) and decreased at high concentrations (10 and 100 nM).

Survival effects of bortezomib in combination with cisplatin

We performed survival studies to establish a baseline cytotoxicity profile for MPM cell lines exposed to cisplatin alone (Fig. 3a). Non-linear regression analysis of survival curves produced an excellent fit for all cell lines ($R^2 = 0.90$–0.98) and extrapolated IC_{50} values showed that two cell lines (MS589, H28) were relatively resistant to cisplatin ($IC_{50} = 20.0$ μM, 95% CI = 12.8–30.0 and $IC_{50} = 27.8$ μM, 95% CI = 16.0–48.5, respectively). The other two cell lines (H2052, JMN) were approximately 10- to 20-fold more sensitive to cisplatin ($IC_{50} = 0.41$ μM, 95% CI = 0.24–0.71 and $IC_{50} = 1.9$ μM, 95% CI = 1.2–3.0, respectively). Overall, the IC_{50} values for cisplatin in MPM cells were approximately 1,000-fold higher compared to those for bortezomib (i.e., nM compared to μM).

We performed survival analysis of MPM cells exposed concurrently to cisplatin and bortezomib over a range of concentrations (Fig. 3b and c). In each experiment, the concentration of bortezomib was exactly 1,000 times lower than that for cisplatin. We found that the cisplatin/bortezomib combination was antagonistic at low concentrations and synergistic at high concentrations for three MPM cell lines (MS589, H28, JMN) compared to either agent given alone; a representative experiment for MS589 cells is shown for the drug combination relative to cisplatin alone (Fig. 3b) and bortezomib alone (Fig. 3c). The drug combination in the remaining cell line (H2052) was generally

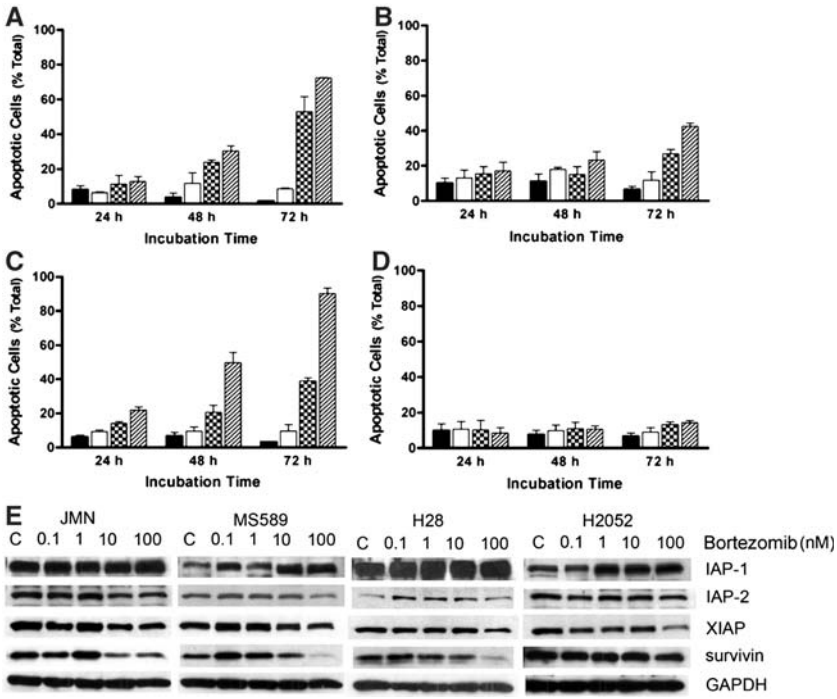

Fig. 2 Quantification of apoptotic MPM cells exposed to escalating concentrations of bortezomib. Total apoptotic cells, as determined by Annexin V staining, after 24 h, 48 h, and 72 h of bortezomib exposure in MPM cell lines H2052 (**a**), H28 (**b**), JMN (**c**), and MS589 (**d**). *Black bars*, unexposed control cells; *Clear bars*, 1 nM; *Hatched bars*, 10 nM; *Diagonal lines*, 100 nM. **e** Expression of IAP proteins in MPM cells exposed to escalating concentrations of bortezomib. Western blot analysis for IAP-1, IAP-2, XIAP, and survivin in MPM cell lines exposed for 24 h to 0.1 nM, 1 nM, 10 nM, or 100 nM bortezomib and for unexposed control cells ("C"). GAPDH was used as a loading control

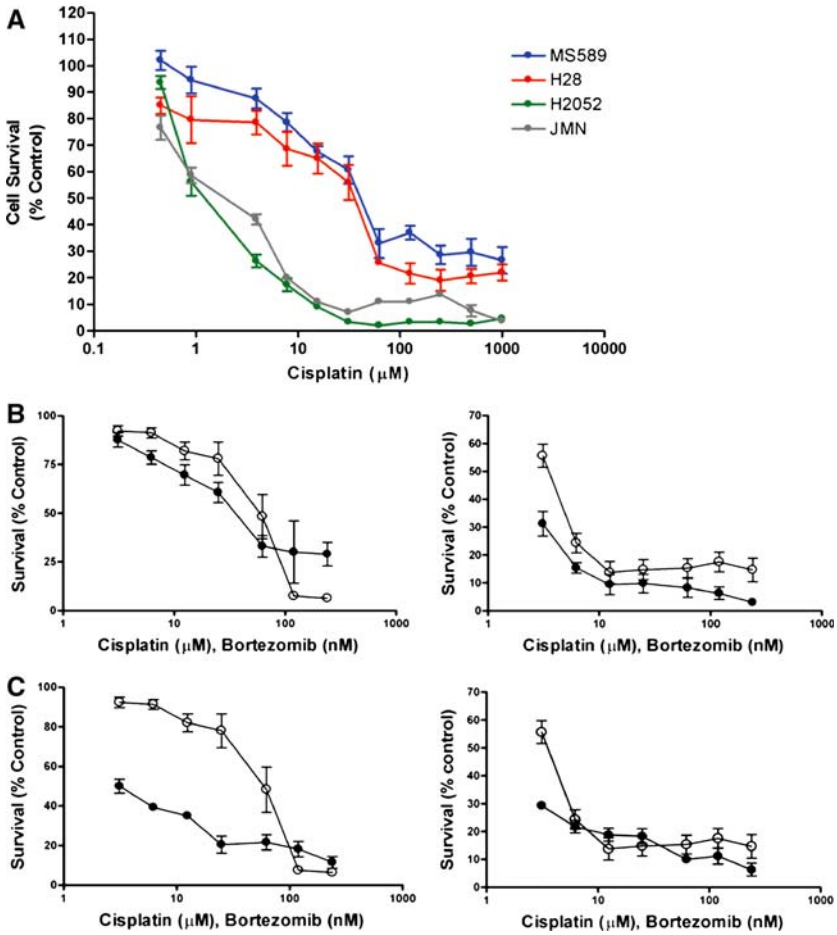

Fig. 3 Survival of cultured MPM cells exposed to cisplatin alone and in combination with bortezomib. **a** Survival analysis of MPM cells (MS589, H28, H2052, JMN) exposed to cisplatin for 48 h. *Error bar*, SEM. **b** Survival analysis of MS589 cells (*left graph*) and H2052 cells (*right graph*) exposed to cisplatin alone (*filled circles*) or cisplatin/bortezomib (*open circles*). *Error bars*, SEM. **c** Survival analysis of MS589 cells (*left graph*) and H2052 cells (*right graph*) exposed to bortezomib alone (*filled circles*) or cisplatin/bortezomib (*open circles*). *Error bars*, SEM

Table 1 Effects of combining bortezomib with cisplatin in MPM cell lines

MPM cell line	IC_{25} C.I.	IC_{50} C.I.	IC_{75} C.I.	IC_{90} C.I.
MS589	2.58	1.35	0.72	0.40
H28	2.77	1.63	0.95	0.56
JMN	3.56	1.52	0.71	0.36
H2052	1.46	1.59	1.74	1.91

Combination index (C.I.) values were calculated as described in the Methods for the IC_{25}, IC_{50}, IC_{75}, and IC_{90} concentrations of the combination of bortezomib and cisplatin. C.I. values >1 and <1 indicate drug antagonism and synergism, respectively

antagonistic at all concentrations (Fig. 3b and c). These results were additionally confirmed by constructing isobolograms and calculating combination indices (Table 1). In related studies we determined if varying the order of administration of cisplatin and bortezomib had any effect on MPM cell survival. In most cases, we found that cytotoxicity was similar or modestly lower when bortezomib preceded cisplatin, particularly at low concentrations (data not shown).

Expression of cell cycle proteins and apoptosis proteins in MPM cells exposed to cisplatin and/or bortezomib

We exposed MPM cells to either bortezomib, cisplatin, or to the combination for 48 h (i.e., "B", "CDDP", "B/CDDP", respectively, in Fig. 4). As expected, protein levels of p21/WAF1 and p27/KIP1 were generally stabilized in cells exposed to bortezomib alone compared to cells exposed to cisplatin alone. Interestingly, expression levels of both cell cycle proteins were attenuated in JMN and MS589 cells exposed to the drug combination compared to bortezomib alone, unlike in H28 and H2052 cells where they remained essentially unchanged. Protein levels of IAP genes were highly variable across all experiments and cell lines. In general, protein expression levels for most IAPs were unchanged, or more frequently substantially elevated, in MPM cell lines with some exceptions, e.g., survivin and XIAP expression in JMN and H28 cells and IAP-2 expression in H28 cells.

In related studies, the 48 h total exposure time was equally split by exposing cells to cisplatin for 24 h followed by bortezomib for 24 h or the reverse ("CDDP→B" and "B→CDDP", respectively, Fig. 4). In these experiments, p21/WAF1 and p27/KIP1 protein levels were essentially similar with some exceptions, e.g., p27/KIP1 protein was expressed at lower levels in JMN and H28 cells exposed to cisplatin followed by bortezomib compared to the reverse order of administration. Similarly, protein expression levels of IAP genes were essentially the same with three notable exceptions. When exposed to bortezomib followed by cisplatin, i) JMN cells expressed moderately more XIAP, ii) H28 cells expressed moderately less survivin, and iii) H2052 cells expressed substantially less survivin.

Chemosensitizing effects of bortezomib

We transiently exposed MPM cell lines to bortezomib and then performed survival analysis using cisplatin over a range of concentrations as in Fig. 3a. Non-linear regression analysis of all survival curves produced an excellent fit ($R^2 = 0.84–0.94$). We found that bortezomib was capable of sensitizing H28 cells (Fig. 5a) and MS589 cells (Fig. 5b) to cisplatin as seen in the lowering of the IC_{50} values to 5.8 μM (95% CI = 2.9–11.6) for H28 and to 7.7 μM (95% CI = 4.9–12.2) for MS589 (compared to 27.8 μM and

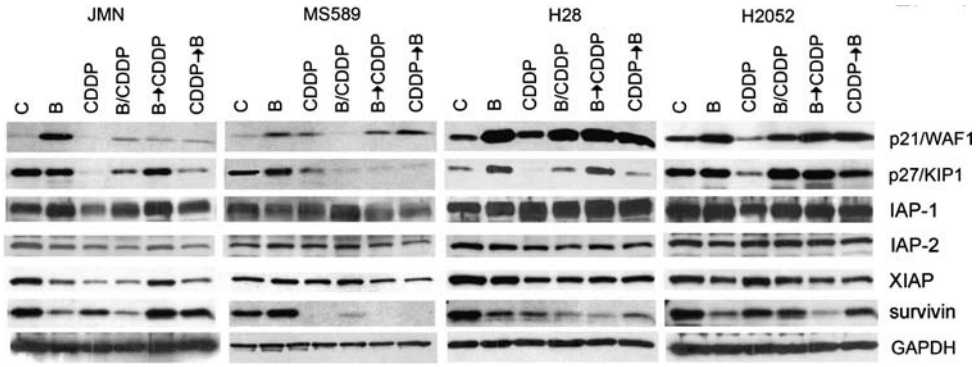

Fig. 4 Expression of cell cycle and apoptosis proteins in MPM cells exposed to cisplatin and bortezomib at concentrations approximating the IC_{50} value of each. Western blot analysis for p21/WAF1, p27/KIP1, IAP-1, IAP-2, XIAP, and survivin in MPM cell lines exposed for 48 h to either bortezomib ("B", 10 nM), cisplatin ("CDDP", 30 μM), or both drugs concurrently ("B/CDDP", 10 nM and 30 μM, respectively). (Note that p27/KIP1 is stabilized in H28 cells unlike in experiments shown in Fig. 1c. This likely relates to a longer drug incubation time, i.e., 24 h for Fig. 1c vs. 48 h for Fig. 5). In a separate experiment, MPM cells were sequentially exposed to bortezomib (10 nM) for 24 h followed by cisplatin (30 μM) for 24 h ("B→CDDP") or the cisplatin (30 μM) for 24 h followed by bortezomib (10 nM) for 24 h ("CDDP→B"). Results were compared to unexposed control cells ("C"). GAPDH was used as a loading control

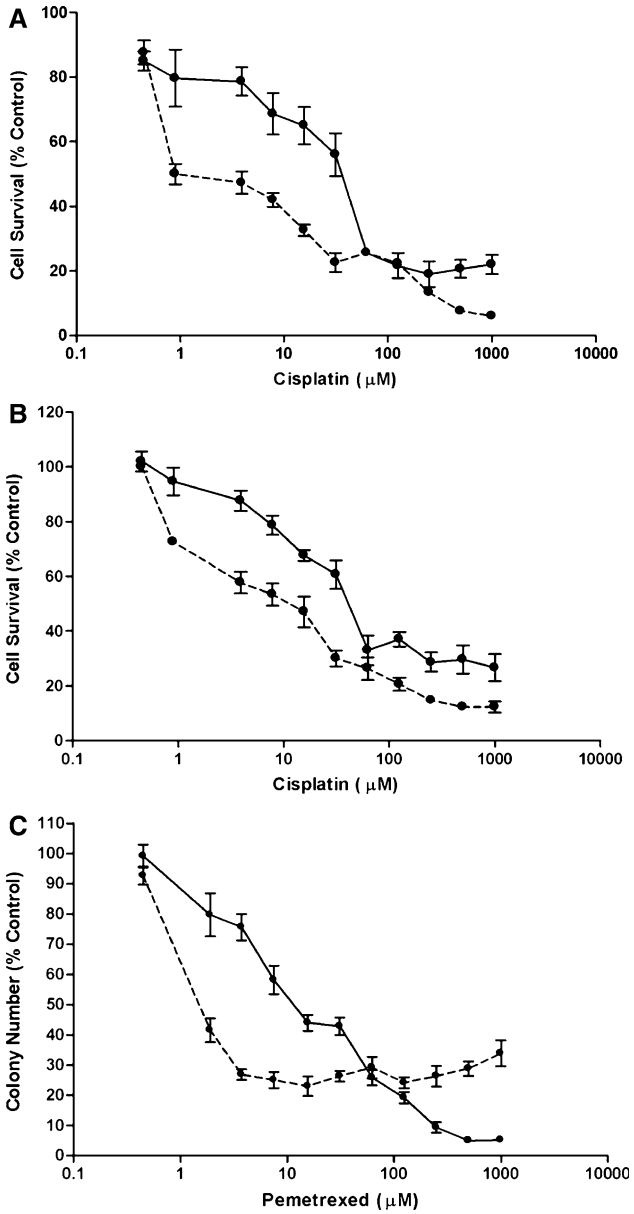

Fig. 5 Chemosensitizing effects of bortezomib. MPM cell lines H28 (**a**) and MS589 (**b**) were exposed to escalating concentrations of cisplatin alone for 48 h (*solid line*) or after 24 h exposure to bortezomib (4 nM, *dashed line*). In a separate experiment, the MPM cell line H28 (**c**) was exposed to escalating concentrations of pemetrexed alone for 48 h (*solid line*) or after 24 h exposure to bortezomib (4 nM, *dashed line*)

20.0 μM, respectively). Cisplatin survival curves for H2052 and JMN cell lines after transient exposure to bortezomib were essentially identical to those in Fig. 3a (data not shown). In a pilot study, we determined whether bortezomib would chemosensitize MPM cells to other drugs with activity in MPM such as pemetrexed. For these experiments we chose one MPM cell line that was associated with increased cisplatin toxicity after transient bortezomib exposure (MS589, Fig. 5b) and one that was not (JMN, data not shown). We found that bortezomib pre-treatment dramatically lowered the resistance of JMN cells to pemetrexed (Fig. 5c), but did not result in any change in MS589 cells (data not shown). Interestingly, this synergistic effect was lost at higher pemetrexed concentrations (>100 μM) after which point bortezomib pre-treatment appeared to be antagonistic compared to pemetrexed alone.

Discussion

In this report we have shown that bortezomib is cytotoxic to cultured MPM cells at concentrations within physiological relevance in MPM patients based on preclinical and clinical studies in other neoplasms (reviewed in [15]). Encouragingly, bortezomib was minimally toxic to normal mesothelial cells even at high concentrations. We have also produced evidence suggesting that the combination of bortezomib with other drugs active in MPM (i.e., cisplatin and pemetrexed) may increase the sensitivity of MPM tumors to chemotherapy and perhaps result in higher response rates and prolonged patient survival in a clinical setting compared to existing chemotherapy. These preliminary results support the additional study of bortezomib in MPM, particularly with respect to cancer associated pathways that are affected by the proteasome such as those that govern the cell cycle, proliferation, and apoptosis.

Of note, MPM cells were ~25-fold more sensitive to bortezomib (IC_{50} = 1–6 nM) compared to the H23 lung adenocarcinoma cell line (IC_{50} = 82.6 nM). Our IC_{50} value for H23 cells is consistent with that previously estimated (~94 nM) for a different lung adenocarcinoma cell line (A549) under essentially the same experimental conditions [35]. The increased sensitivity of MPM cell lines to bortezomib when compared to lung cancer cell lines is encouraging considering that bortezomib as a single agent does not improve on existing chemotherapy in lung cancer in clinical trials conducted thus far [36]. As for other solid malignancies in which bortezomib has limited activity as a single agent, bortezomib is likely to be more efficacious in lung cancer in combination with other chemotherapy [36], particularly when used as a chemosensitizing agent to drugs that promote apoptosis. This treatment paradigm is also consistent with our current findings in MPM.

Multi-center clinical trials of bortezomib and bortezomib/cisplatin in patients with MPM have been recently opened to accrual in Western Europe and are being planned in the U.S. However, preclinical data of bortezomib in MPM is limited. In fact, there are only two currently published studies that directly address the inhibition of the proteasome in mesothelial-derived tumors. In one study, Sun et al. [37] show that proteasome inhibition reduces cellular viability and increases apoptosis in MPM cells. These experiments are limited in that bortezomib was not used

[37]. In the second study, Borczuk and colleagues used molecular profiling to identify the ubiquitin-proteasome pathway as potentially important in biphasic malignant peritoneal mesotheliomas [38]. It is not known whether these results obtained from analyzing patients' peritoneal mesothelioma samples will be equally relevant in MPM patient tumors. However, in the same publication [38] the investigators demonstrated that bortezomib was equally cytotoxic in two MPM cell lines (H28 and 211-H).

The latter study [38] also noted a G1 cell cycle arrest (and p21/WAF1 induction) in 211-H (biphasic) MPM cells exposed to bortezomib, similar to our results in the biphasic MPM cell line MS589 (Fig. 1b). This is in marked contrast to the typical bortezomib-induced G_2/M cell cycle block observed in lung cancer [35, 36] and other tumor cell types [15]. It is not known whether a G_1/S cell cycle block is limited to MPM, or even biphasic MPM, or whether p21/WAF1 plays a causal role. The involvement of p21/WAF1 is highly plausible since MS589 cells exposed to bortezomib displayed the greatest degree of p21/WAF1 protein stabilization. However, we have not provided direct supporting evidence for this possibility in the current study. On the other hand, induction of p21/WAF1 and a G_2/M cell cycle block are generally considered to be reliable biomarkers of inhibition of the 26S proteasome indicating that stabilization of p21/WAF1 protein does not necessarily result in a G_1/S block in all tumor types.

Both of the previous studies [37, 38] showed that biphasic and epithelial MPM cells respond similarly to proteosomal inhibition, consistent with the current study (Fig. 1a). Sun et al. [37] further show that sarcomatoid MPM cells are more resistant to the proteasome inhibitor PSI compared to epithelial and biphasic MPM cells. This is in contrast to our results which show that (sarcomatoid) H2052 cells are actually the most sensitive to bortezomib cytotoxicity. If this finding is confirmed, it may indicate that bortezomib is more effective in sarcomatoid tumors than other proteasome inhibitors. Such a discovery would be particularly advantageous since sarcomatoid MPM tumors are the most aggressive and are associated with a worse patient outcome than either biphasic or epithelial MPM tumors.

Consistent with our results (Table 1), Borczuk et al. [38] found that bortezomib combined with other chemotherapy (oxaliplatin) was antagonistic at IC_{50} concentrations; combinational effects at higher concentrations were not determined in this study. These investigators also provide data indicating that bortezomib followed by oxaliplatin results in greater cytotoxicity in sequential studies and they suggest that this effect may be the most relevant in biphasic MPM [38]. Our results are generally consistent with these as we show that bortezomib can be used to chemosensitize MPM cells to cisplatin, although we observed this effect in both biphasic and epithelial MPM cells (Fig. 5).

The IAP family of proteins warrant additional study in the context of bortezomib for MPM [24, 25, 29] and other solid tumors [16, 19–22] including those in which bortezomib is currently under clinical investigation (e.g., carcinomas of lung, breast, colorectal, prostate). IAP protein levels in general are regulated by the proteasome and select IAPs can affect their own protein levels (and those of other IAPs) via a RING domain which exhibits E3 ubiquitin ligase activity and catalyzes the addition of ubiquitin moieties to proteins which in turn targets them for destruction by the proteasome [16]. IAPs are also positively regulated by NF-κB in MPM [29], a transcription factor whose inhibitor (I-κB) is degraded by the proteasome during the activation of NF-κB. Therefore, bortezomib could theoretically positively impact the stability of IAP proteins and/or negatively regulate the production of *de novo* IAP protein (via NF-κB). Our results (Fig. 2) provide strong evidence supporting the former possibility for IAP-1, the only IAP gene shown so far to be directly implicated in mediating MPM drug resistance [24]. (Off note, previous studies [38] report that bortezomib does not affect I-κB protein levels or NF-κB functionality in the single MPM cell line examined, 211-H.) Our findings suggest that IAP-1 facilitates at least some resistance to bortezomib in MPM cells. This conclusion is consistent with the fact that IAPs mediate survival of tumor cells to a functionally diverse group of chemotherapeutics [39]. Interestingly, XIAP and survivin protein levels decrease at high bortezomib concentrations which may imply alternative regulatory mechanisms and is consistent with previous studies [40–43]. While provocative, these preliminary observations will need to be expanded in additional studies to determine the expression patterns of IAPs under conditions of protracted bortezomib exposure (i.e., >24 h) in order to dissect the precise molecular mechanisms and physiological relevance of IAPs in MPM in the context of inhibition of the proteasome.

Acknowledgments This work was partly funded by grants to G.J.G., R.B., and B.Y.Y. from The International Mesothelioma Program (http://www.impmeso.org), to G.J.G. from the Mesothelioma Applied Research Foundation (MARF), to R.B. from the NCI (CA120528 and CA100315).

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