

Enhanced Delivery of Cisplatin to Intraperitoneal Ovarian Carcinomas Mediated by the Effects of Bortezomib on the Human Copper Transporter 1

Danielle D. Jandial, Salman Farshchi-Heydari, Christopher A. Larson, Gregory I. Elliott, Wolfgang J. Wrasidlo, and Stephen B. Howell

Abstract Purpose: The copper transporter 1 (CTR1) is a major influx transporter for platinum drugs. However, the accumulation of cisplatin in human ovarian carcinoma cells is limited by the fact that cisplatin triggers the down-regulation and proteasomal degradation of CTR1, thereby limiting its own uptake. We sought to determine whether proteasome inhibition using bortezomib would prevent human CTR1 (hCTR1) degradation and increase platinum accumulation in ovarian cancer cells.

Experimental Design: The effects of bortezomib on human hCTR1 expression and cisplatin accumulation were measured by Western blot, flow cytometric, and confocal digital imaging analyses. Platinum accumulation was measured by inductively coupled plasma mass spectrometry and bortezomib concentrations by liquid chromatography/mass spectrometry.

Results: Bortezomib blocked the cisplatin-induced down-regulation of hCTR1 in a concentration-dependent manner and increased cisplatin uptake 1.6- to 2.4-fold. Median effect analysis showed a combination index of 0.37 at 50% cell kill, indicating a high level of synergy. The effect of bortezomib was muted in cells lacking both alleles of *CTR1*, showing that bortezomib was working primarily through its effect on blocking hCTR1 degradation. I.p. administration of bortezomib produced a peritoneal/plasma area under the curve ratio of 252 in a murine model. I.p. administration of bortezomib before i.p. cisplatin increased platinum accumulation in peritoneal tumors by 33% ($P = 0.006$).

Conclusions: Proteasomal inhibition prevented cisplatin-induced down-regulation of hCTR1 in ovarian cancer cells and enhanced drug uptake and cell killing in a synergistic manner. Bortezomib shows a large pharmacologic advantage when administered i.p. There is a strong rationale for the combined i.p. administration of bortezomib and cisplatin.

The pharmacologic advantage of administering cisplatin by the i.p. route for the treatment of ovarian cancer was shown many years ago (1). Multiple randomized trials have shown that i.p. therapy can increase progression-free and overall survival in ovarian cancer (2–4), and this approach is now considered standard practice for the treatment of patients with stage III ovarian cancer following optimal debulking surgery (5). The incremental benefit, however, is small, and novel pharmacologic strategies to improve the efficacy of i.p. therapy are needed.

The goal of infusing drugs directly into the peritoneal cavity is to safely expose tumor confined to this compartment to higher concentrations of the drug for longer periods of time than can be achieved with systemic administration. The magnitude of the advantage of i.p. drug administration is a function of the rate of clearance of the drug from the peritoneal cavity relative to its clearance from the systemic circulation (6). The ideal drug has a very long peritoneal residence time but is rapidly cleared from the blood once it leaks into the systemic circulation. In the case of cisplatin, the advantage, expressed as the area under the curve (AUC) ratio, is in the range of 12-fold (1). The rapid plasma clearance of bortezomib makes it a potential candidate for i.p. chemotherapy; once in the systemic circulation, >90% of bortezomib is cleared within 15 minutes (7, 8).

Recent studies from this and other laboratories have shown that the copper transporter 1 (CTR1) is a major influx transporter for the platinum-containing drugs. Deletion of the *yCTR1* gene in *Saccharomyces cerevisiae* markedly reduces the accumulation of all three clinically available platinum-containing chemotherapeutic agents and attenuates their cytotoxicity (9, 10). Previous studies have shown that the cellular accumulation of cisplatin is impaired in mouse embryonic fibroblasts in which both *mCTR1* alleles have been disrupted

Authors' Affiliation: Department of Medicine and the Rebecca and John Moores Cancer Center, University of California, San Diego, La Jolla, California
Received 8/8/08; revised 9/30/08; accepted 10/1/08.

Grant support: CA95298 and T32 CA121938 from the NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Presented in part at the 2008 meeting of the American Association for Cancer Research.

Requests for reprints: Stephen B. Howell, Moores UCSD Cancer Center, 3855 Health Sciences Drive, CA 92093-0819. Phone: 858-822-1110; Fax: 858-822-1111; E-mail: showell@ucsd.edu.

© 2009 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-08-2081

Translational Relevance

There is an urgent need to improve the efficacy and reduce the toxicity of i.p. chemotherapy for patients with ovarian cancer. Multiple randomized clinical trials have shown improved survival in treatment arms with i.p. cisplatin. Despite these advances, the incremental benefit of i.p. chemotherapy is small, and novel pharmacologic strategies are needed to maximize the efficacy of this therapy. This study introduces the concept that proteasomal blockade with bortezomib can increase cisplatin uptake and antitumor efficacy. Further, this study shows a highly favorable difference in exposure for the peritoneal cavity versus the plasma when bortezomib is administered by the i.p. route. Taken together, our findings suggest that i.p. bortezomib can modulate platinum delivery into ovarian cancer tumors, thereby increasing antitumor activity. This study provides a novel basis on which to develop a clinical strategy that addresses the challenge of improving the efficacy of i.p. therapy for ovarian cancer patients.

(11). Similarly, forced overexpression of human CTR1 (hCTR1) in human ovarian carcinoma cells enhances cisplatin uptake (12), and increased hCTR1 expression in human small cell lung cancer cells has been reported to enhance the uptake of cisplatin, carboplatin, and oxaliplatin (13). However, the accumulation of cisplatin in human ovarian carcinoma cells seems to be limited by the fact that cisplatin binding triggers the rapid down-regulation of CTR1 and thereby limits its own uptake (14). Studies from this laboratory of the mechanism have shown that the loss of hCTR1 is due to proteasomal degradation and that it can be prevented by pretreating the cells with the proteasomal inhibitors MG-132 or lactacystin (14). However, whether this results in greater platinum drug influx has not been previously investigated.

Bortezomib is a dipeptidyl boronic acid that potently inhibits the 20S proteasome. It is currently approved for the treatment of multiple myeloma and mantle cell lymphoma but is of interest with respect to ovarian cancer for several reasons. First, like other proteasome inhibitors, bortezomib may be able to prevent the cisplatin-induced down-regulation of hCTR1 and thus increase uptake into tumor cells. Second, bortezomib is predicted to have a favorable AUC ratio when administered by the i.p. route. Third, there is evidence that proteasomal inhibitors can sensitize malignant cells to a variety of standard cancer therapeutics (15). Finally, ovarian epithelial malignancies express higher amounts of the proteasomal subunits compared with benign ovarian tumors *in vivo*, an effect which is also seen *in vitro* when ovarian cancer cells are compared with immortalized ovarian surface epithelium. Further, there are differentially high levels of ubiquitin proteasome stress in ovarian cancer cells compared with their nonmalignant counterparts, making them particularly susceptible to proteasome inhibition-induced apoptosis (16). Proteasomal inhibition in ovarian cancer cells has been shown to increase cisplatin DNA adduct formation, block nucleotide excision repair of these adducts, prevent cisplatin-induced transcriptional up-regulation of the excision nuclease ERCC-1, and potentiate cisplatin-mediated apoptosis (17, 18).

The aim of the current study was to determine whether hCTR1 expression could be exploited to enhance the efficacy of i.p. administered cisplatin. We report here that bortezomib indeed blocks the cisplatin-induced down-regulation of its major influx transporter, hCTR1, and that this is associated with an increase in cisplatin uptake and enhanced cell killing of human ovarian cancer cells. In addition, the interaction between these two drugs is highly synergistic, and i.p. administration of bortezomib produces an AUC ratio of 252 in a murine model.

Materials and Methods

Drugs and reagents. Cisplatin (Platinol) was a gift from Bristol-Myers Squibb. The clinical formulation containing 3.33 mmol/L cisplatin was kept in the dark at room temperature. Bortezomib (Velcade; Millenium Pharmaceuticals) was obtained from the Moores University of California in San Diego Cancer Center pharmacy at a concentration of 1 mg/mL. FITC-conjugated goat antirabbit antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. Hoechst 33342 dye for nuclear staining and Alexa 488-conjugated goat antirabbit antibody were purchased from Invitrogen Molecular Probes. Horseradish peroxidase-conjugated donkey antirabbit antibody was purchased from Amersham Pharmacia. Protein concentration was measured using Bradford's reagent from Bio-Rad, Inc. All other chemicals and reagents were obtained from Thermo Fisher.

Cell lines and assays. The 2008 human ovarian cancer cell line (19, 20) was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO₂. Wild-type mouse embryo fibroblasts or fibroblasts in which both alleles of *CTR1* had been knocked out were grown in DMEM high-glucose supplemented with 20% heat-inactivated fetal bovine serum, 1× nonessential amino acids, 55 μmol/L 2-mercaptoethanol, 50 mg/L uridine, and 110 mg/mL pyruvate. All media and supplements were obtained from Thermo Fisher. For *in vivo* studies, a subline of the 2008 cells was engineered to express green fluorescent protein (21) and was inoculated i.p. into *nu/nu* mice. Sensitivity to cisplatin was determined by seeding 5,000 cells per well into 96-well plates. After 24 h, increasing concentrations of the drug were added to the media. After the appropriate drug exposure, the media were replaced with drug-free media and the cells were allowed to grow for 5 d. Surviving cells were then fixed using 50% trichloroacetic acid for 1 h at 4°C and stained using 0.4% sulforhodamine B dye. After rinsing away unbound dye with 1% acetic acid, bound dye was solubilized using 10 mmol/L Tris-HCl and read at 515 nm on a Versamax absorbance microplate reader (Molecular Devices). The optical density data were processed as previously reported (22). The interaction between cisplatin and bortezomib was investigated in four independent experiments using median effect analyses with the assistance of Calcsyn software (Biosoft, Inc.).

Measurement of hCTR1 expression levels. Expression levels of hCTR1 were quantified by Western blot, flow cytometric, and confocal image analysis as previously reported (14, 23). The anti-hCTR1 antibodies utilized are described in detail elsewhere (24). A polyclonal rabbit anti-hCTR1 antibody that reacts with the carboxy terminus (Novus Biologicals) was utilized for immunohistochemistry and flow cytometry, and a polyclonal rabbit anti-hCTR1 antibody that reacts with the amino terminus (BioCarta, Inc.) was used for Western blotting. Western blots were done using crude membrane preparations. For flow cytometric analysis the cells were fixed using 2% paraformaldehyde, washed with 5% bovine serum albumin in PBS, and permeabilized with 0.3% Triton X-100 (Fisher Scientific). Cells were blocked for 1 h at room temperature with 5% bovine serum albumin/PBS and then stained with anti-hCTR1 antibody diluted to a final concentration of 1:1,000 overnight at 4°C and then with a 1:1,000 dilution of Alexa

488-conjugated IgG for 45 min at room temperature. The cells were run on a Becton Dickinson FACSCalibur flow cytometer, and results were analyzed using CellQUEST software and normalized to cells stained only with secondary antibody. For deconvolution digital immunofluorescent confocal microscopy, cells were seeded on coverslips and treated with 2 $\mu\text{mol/L}$ cisplatin for 5 min with or without a prior 4-h pretreatment with 50 nmol/L bortezomib. The cells were then washed, fixed in 3.7% paraformaldehyde at room temperature for 30 min, permeabilized with 0.3% Triton X-100, and stained with anti-hCTR1 antibody diluted 1:500 in 5% bovine serum albumin/PBS for 4 h followed by FITC-conjugated antirabbit antibody diluted 1:1,000 and Hoescht 33342 dye diluted 1:20,000 for 1 h.

Measurement of whole cell and tumor platinum content. After exposure of cells to cisplatin *in vitro* or *in vivo*, cells were digested in 70% nitric acid overnight, diluted to a final 5% nitric acid concentration with water containing 1 ppb indium as an internal standard and 0.1% Triton X-100. Platinum content was then measured by inductively coupled mass plasma spectroscopy (Element 2, Perkin Elmer Life Sciences) and normalized to protein concentrations as determined from corresponding cell lysates using the Bradford assay as previously reported (23).

In vivo pharmacology and pharmacokinetics. All animal studies were approved by the University of California Institutional Animal Care and Use Committee and done in accordance with NIH guidelines. To assess the effect of bortezomib on cisplatin accumulation in 2008 cells *in vivo*, female *nu/nu* mice (Charles River Laboratories) were inoculated i.p. with 5×10^6 2008/green fluorescent protein cells. When there was evidence of i.p. tumor growth by optical imaging with the IVIS 200 (Caliper Life Sciences), typically 4 wk postinoculation, the animals were divided randomly into two groups of 8 to 10 mice each and treated with a single i.p. injection of 1 mg/kg bortezomib (the published maximum tolerated dose in mice) followed 4 h later by a single i.p. injection of 10 mg/kg cisplatin. Both injections were diluted with sterile 0.9% NaCl to a final volume of 500 μL to allow for adequate peritoneal distribution. Animals were sacrificed 2 h after cisplatin injection and all tumors were removed for further analysis. The pharmacokinetics of i.p. administered bortezomib was determined in non-tumor-bearing BALB/c mice (Charles River Laboratories). Plasma and i.p. fluid samples were obtained from three mice per time point at 1, 2, 4, 8, 16, 32, 64, and 95 min after bolus i.p. injection of bortezomib 1 mg/kg in 1 mL total volume. Following centrifugation for 10 min at $16,100 \times g$ to remove formed elements, supernates were extracted by vortexing at high speed for 1 min with equal volumes of 0.1% formic acid in acetonitrile. Samples were then centrifuged for 10 min at $16,100 \times g$, and the supernatant transferred to vials for measurement of bortezomib concentration using a liquid chromatography and tandem mass spectrometry assay developed in this institution. Pharmacokinetic parameters were calculated using standard formulas for clearance and volume of distribution. Terminal half-life was estimated using the best fit of the monoexponential peritoneal fluid decay curve. AUC and AUC ratios for the peritoneal cavity relative to plasma were calculated using the trapezoidal rule. All parameters were calculated using Microsoft Excel (Microsoft Corp.).

Bortezomib assay. The organic phase of each sample was run on an Agilent 1100 Series LC/MSD Trap coupled to a Supelco Discovery HS C18 column. Each run was 30 min at a flow rate of 0.2 mL/min with a 10-min column equilibrium period. The mobile phase was analyzed by linear gradient elution starting with 95% water (0.1% trifluoroacetic acid) for 25 min, ending in an isocratic elution with 95% acetonitrile for 5 min. The high performance liquid chromatograph eluent was introduced via electrospray ionization using a TurboIonSpray interface set at 350°C. Ionization was assisted with a nebulizer and ionspray dry gas set at 25 psi and 10 L/min, respectively. Octopole RF amplitude and skimmer voltages were set at 200 V and 40 V, respectively. Data were acquired in full-scan ion mode. An m/z of 367.0 for native bortezomib, which corresponds to an observed $[M + H - H_2O]^+$ mass, was reported as previously described (25). No internal standard was used. Calibration

curves consisting of a range of bortezomib concentrations from 250 nmol/L to 125 $\mu\text{mol/L}$ were constructed before each run from standards isolated from stock concentrations of bortezomib prepared in serum. The linear range of the assay was from 1 to 100 $\mu\text{mol/L}$. The lower limit of quantitation was 1 $\mu\text{mol/L}$. An Agilent Technologies CE Chemstation Rev. B.0.1.03 was used to control the instrument and collect data. Agilent Technologies Quantanalysis was used for data analysis.

Statistical methods. Tests for statistical significance were done with the NCSS Statistical Software package. Group means were compared using Student's *t*-test. Confidence intervals (95%) were calculated where appropriate.

Results

Effect of bortezomib on cisplatin-induced down-regulation of hCTR1. hCTR1 has previously been shown to be rapidly internalized and degraded in the presence of even low concentrations of cisplatin in ovarian carcinoma cells (14, 26). This degradation is mediated by the proteasome, an effect that can be inhibited with the proteasome inhibitors lactacystin, proteasome inhibitor 1, and MG-132 (11). In that bortezomib is currently the only clinically available proteasome inhibitor, we sought to determine its ability to block the cisplatin-induced down-regulation of hCTR1. Human ovarian carcinoma 2008 cells were exposed to 2 $\mu\text{mol/L}$ cisplatin for 5 minutes, with or without a 4-hour pretreatment with increasing concentrations of bortezomib, and hCTR1 expression was quantified by Western blot. As shown in Fig. 1A, hCTR1 down-regulation triggered by clinically relevant concentrations of cisplatin was blocked by bortezomib in a concentration-dependent fashion; a concentration of 10 nmol/L bortezomib was sufficient to substantially reduce cisplatin-induced down-regulation. Flow cytometric analysis (Fig. 1B) revealed that whereas hCTR1 expression was decreased to 49% of control upon exposure to 2 $\mu\text{mol/L}$ cisplatin for 5 minutes, this decrease was limited to just 11% when cells had previously been exposed to 50 nmol/L bortezomib. Figure 1C presents confocal microscopic images that confirm that staining for hCTR1 disappeared upon exposure to cisplatin and that this was prevented by pretreatment with bortezomib. Thus, as documented by three different analytic techniques, bortezomib was able to prevent the cisplatin-induced degradation of hCTR1 in a manner similar to that previously shown for other proteasome inhibitors not available for use in patients.

Effect of bortezomib on cisplatin accumulation in vitro. To determine whether the persistence of hCTR1 at normal levels permitted increased cellular accumulation of cisplatin, 2008 cells were exposed for 5 minutes to increasing concentrations of cisplatin with or without a prior 4-hour exposure to 50 nmol/L bortezomib, and the total cellular platinum accumulation was measured by inductively coupled plasma mass spectroscopy. The range of cisplatin concentrations tested was selected to reflect those attainable in patients following i.p. instillation of this drug. As shown in Table 1, prior exposure to bortezomib increased the cellular accumulation of platinum accumulation at all concentrations tested; the 1.6-fold (95% confidence interval, 1.3- to 2.6-fold) and 2.4-fold (95% confidence interval, 1.8- to 3.1-fold) increases observed with 30 and 100 $\mu\text{mol/L}$ cisplatin, respectively, were statistically significant.

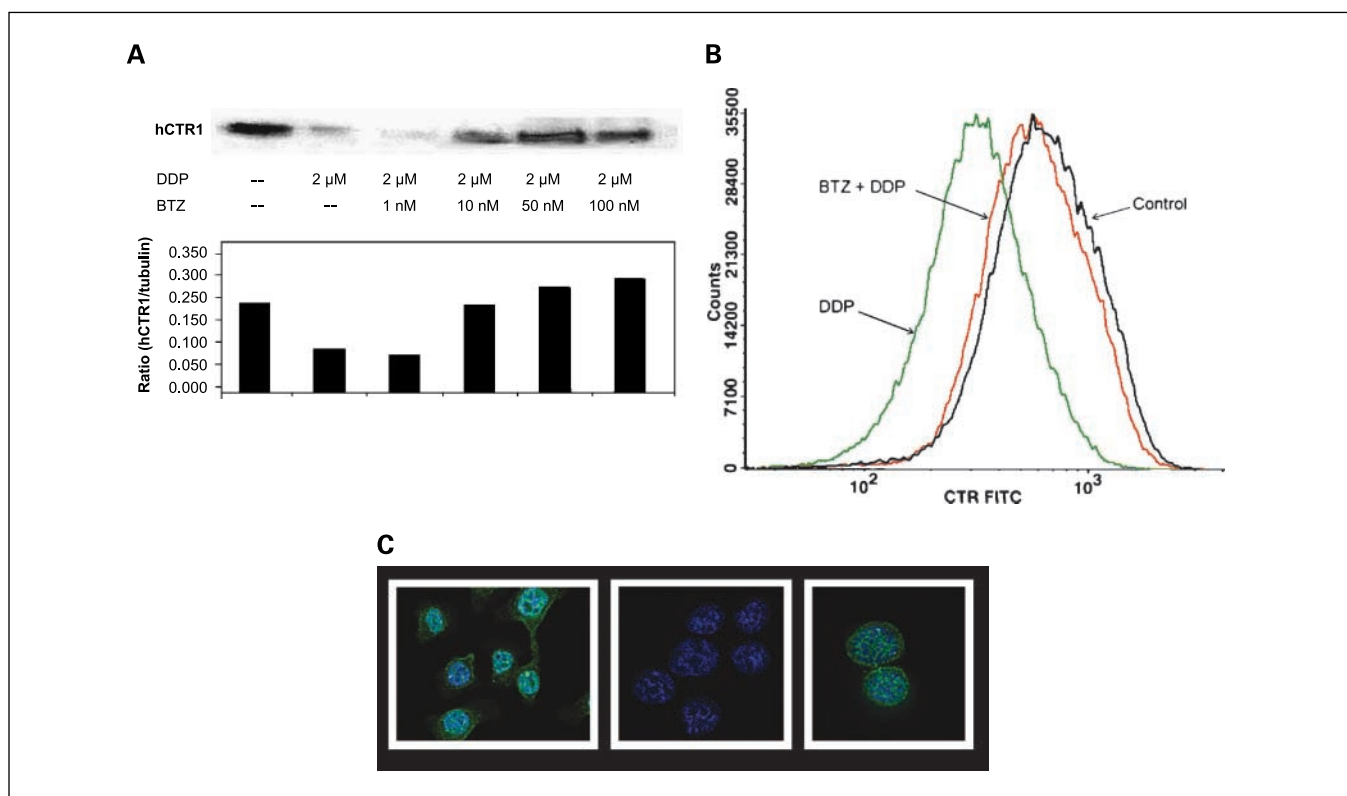


Fig. 1. Effect of bortezomib pretreatment on the level of hCTR1 in the presence of cisplatin. *A*, Western blot analysis of hCTR1 expression. 2008 ovarian cancer cells exposed to 2 μ mol/L cisplatin for 5 min preceded by a 4-h exposure to the indicated bortezomib concentrations. Graph shows ratio between hCTR1 and β -actin expression. *B*, flow cytometric and *(C)* confocal digital microscopic analyses of hCTR1 levels in 2008 ovarian cancer cells exposed to 50 nmol/L bortezomib for 4 h followed by 2 μ mol/L cisplatin for 5 min (*right panel*) or 2 μ mol/L cisplatin alone (*center panel*) as compared with untreated cells (*left panel*). Green, hCTR1; blue, nucleus stained with 4', 6'-diamidino-2-phenylindole. Results are representative of three independent experiments; image intensity was normalized to cells stained only with secondary antibody.

Thus, the ability of bortezomib to prevent cisplatin-induced degradation of hCTR1 was accompanied by a substantial increase of cisplatin uptake into tumor cells.

Inhibition of the proteasome with bortezomib has the potential of changing the expression of many proteins in the cell, which could potentially modulate cisplatin influx or efflux. To determine the extent to which the effect of bortezomib on cisplatin uptake was dependent on CTR1, experiments were conducted using wild type *CTR1*^{+/+} mouse embryo fibroblasts and similar cells isolated from an embryo in which both alleles of *CTR1* had been knocked out. As shown in Table 2, a 4-hour exposure to 50 nmol/L bortezomib followed by a 30-minute exposure to cisplatin increased whole cell platinum content in the *CTR1*^{+/+} cells by 2.7-fold (95% confidence interval, 1.3- to 4.0-fold) over cells treated with cisplatin alone. However, no difference in cellular platinum content was detected in the *CTR1* knockout cells. Thus, the effect of bortezomib on cisplatin uptake can be largely attributed to its prevention of CTR1 degradation.

Effect of bortezomib on cisplatin accumulation in vivo. To assess whether i.p. administered bortezomib could increase tumor accumulation of cisplatin *in vivo*, human ovarian cancer 2008 cells expressing green fluorescent protein were inoculated i.p. and allowed to form small tumor nodules on the peritoneal surface over a period of 4 to 6 weeks. Tumors were harvested from mice which had received either 10 mg/kg cisplatin i.p. alone (*n* = 107 tumors from 9 mice) or 10 mg/kg

cisplatin preceded 4 hours earlier by an i.p. injection of 1 mg/kg bortezomib (*n* = 134 tumors from 11 mice). Tumors were harvested 2 hours after cisplatin injection, and platinum content was measured by inductively coupled plasma mass spectroscopy. Values are expressed as nanogram platinum per milligram tumor wet weight. Figure 2A shows a scattergram of all platinum content values. It is apparent that, irrespective of prior bortezomib treatment, larger tumor nodules tended to accumulate lower amounts of platinum per milligram wet weight. However, for any given tumor size, the scattergram suggests that prior injection of bortezomib increased the extent of cisplatin accumulation; this effect was observed in both small and large tumor nodules. Figure 2B presents the

Table 1. Effect of bortezomib on platinum uptake into 2008 cells

Cisplatin concentration, μ mol/L	Fold increase in platinum	95% CI
10	2.1	0.8, 3.3
30	1.6	1.3, 2.6*
100	2.4	1.8, 3.1*

Abbreviation: 95% CI, 95% confidence interval.
**P* < 0.05.

Table 2. Effect of bortezomib on platinum uptake into MEF CTR+/+ versus MEF CTR-/- cells

Cell line	Fold increase in platinum	95% CI
MEF CTR +/+	2.7	1.3, 4.0*
MEF CTR -/-	0.7	0.4, 1.0

Abbreviation: MEF, mouse embryonic fibroblast.

* $P < 0.05$.

data in the form of a histogram showing the mean \pm SE platinum content for all tumor nodules. The mean level of platinum in the mice treated with bortezomib was 33% ($P = 0.006$) higher than in mice receiving only cisplatin. Thus, the ability of bortezomib to enhance cisplatin accumulation in ovarian cancer cells *in vitro* was also observed *in vivo* under conditions where both drugs were administered by the i.p. route.

Synergy between bortezomib and cisplatin. If bortezomib really prevents cisplatin from inducing the degradation of its own influx transporter, and this results in increased cisplatin uptake, one would expect these two drugs to interact synergistically with each other with respect to cytotoxicity. Previous studies have reported synergism between bortezomib and a number of chemotherapeutic agents other than cisplatin although the mechanistic basis for this synergism was not identified (27, 28). The interaction of bortezomib and cisplatin was examined with respect to cytotoxicity to 2008 cells using the technique of median effect analysis previously described by Chou and Talalay (29). There was a marked degree of synergy between these two compounds with a combination index of 0.37 at the IC_{50} concentration. Synergy was present at all levels of cell kill (data not shown).

Pharmacokinetics of i.p. bortezomib. Bortezomib was able to enhance the cellular accumulation and cytotoxicity of cisplatin when used at concentrations found in patient plasma following i.v. injection. However, we hypothesized that, because of the short plasma half-life of bortezomib, i.p. administration of bortezomib would yield much higher levels of bortezomib

exposure for tumor cells growing on peritoneal surfaces relative to those in plasma. The pharmacokinetic profile of i.p. administered bortezomib was investigated in non-tumor-bearing BALB/c mice. As shown in Fig. 3, following i.p. administration peritoneal bortezomib concentrations decreased in a monoexponential manner. Cytotoxic concentrations of bortezomib ($>1.6 \mu\text{mol/L}$) were detectable in the peritoneal cavity even at 95 minutes. In contrast, bortezomib was detectable in the plasma only sporadically between 9 and 14 minutes. The ratio of the exposure for the peritoneal cavity relative to that of plasma (AUC ratio) was 252. The pharmacokinetic parameters, estimated using a noncompartment model, are presented in Table 3.

Discussion

The results of this study indicate that blockade of the proteasome in human ovarian cancer cells impairs the ability of cisplatin to trigger the degradation of its own influx transporter. This, in turn, results in greater cellular accumulation of cisplatin and a synergistic interaction between cisplatin and bortezomib with respect to cell killing. The results also show that there is a large pharmacologic advantage to be gained by administering bortezomib by the i.p. route, and that this strategy resulted in greater cisplatin accumulation in ovarian cancer tumor nodules *in vivo* than was attained with i.p. cisplatin alone. These insights into the pharmacology of the interaction between cisplatin and bortezomib provide the basis for the development of a novel strategy to improve the efficacy of i.p. chemotherapy and overcome acquired cisplatin resistance.

Upon binding of cisplatin to hCTR1, the transporter is internalized and degraded within minutes by a process that involves macropinocytosis and proteasomal degradation (14). This suggests that cisplatin induces a conformational change that renders the protein recognizable to the ubiquitin-proteasome degradation machinery. The triggering mechanism is not currently known, but similar findings have been described in *S. cerevisiae* where exposure to copper activates degradation of yCTR1, an effect that is thought to be mediated by the E3 ubiquitin ligase homologue Rsp5 (30). Our results

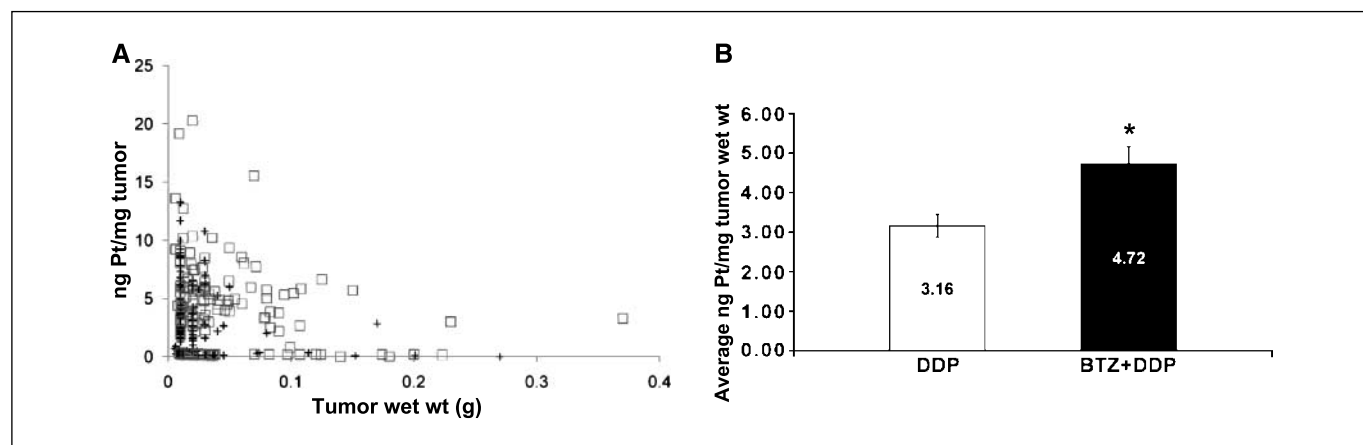


Fig. 2. Effect of bortezomib on tumor accumulation of cisplatin *in vivo*. *A*, scattergram of nanogram platinum per milligram tumor wet weight as a function of tumor weight. +, no prior bortezomib; □, 1 mg/kg bortezomib i.p. 4 h prior to injection of cisplatin by the i.p. route. *B*, mean nanogram platinum per milligram tumor wet weight. Vertical bars, SE.

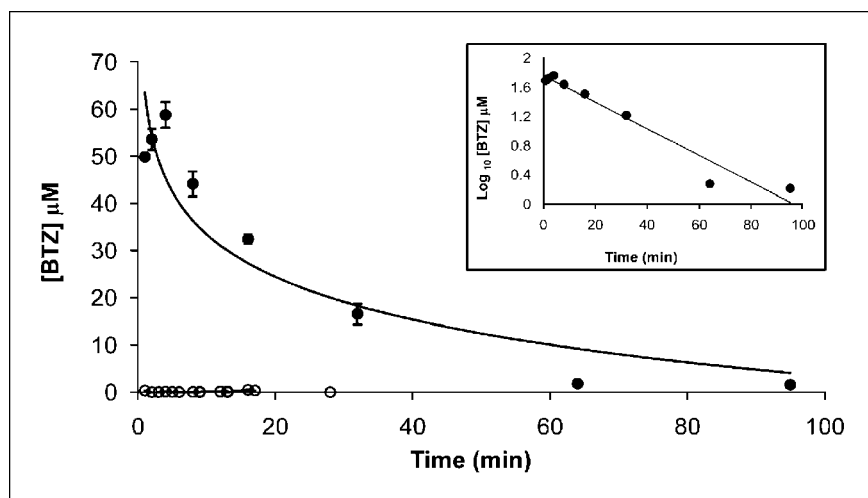


Fig. 3. Peritoneal and plasma concentrations of bortezomib as a function of time following i.p. injection. Closed circles, peritoneal concentration; open circles, plasma concentration. Inset, a log-linear plot of peritoneal bortezomib concentration as a function of time. Each time point represents data from at least 3 mice. Vertical bars, SE.

are consistent with the concept that interaction of cisplatin with hCTR1 triggers its ubiquitination followed by proteasomal degradation in human ovarian cancer cells as well; a proteasome inhibitor would be expected to increase the fraction of hCTR1 that is ubiquitinated. The ability of bortezomib to maintain hCTR1 expression and increase cellular cisplatin accumulation suggests that the both the putative ubiquitinated and nonubiquitinated forms of hCTR1 remain functional with respect to their ability to transport cisplatin. Although hCTR1 protein expression levels were restored to near control levels in the presence of bortezomib, the increases in cellular platinum uptake was relatively modest (2.4-fold in 2008 cells, 2.7-fold in CTR^{-/-} cells). This may be accounted for by the fact that only a small fraction of hCTR1 is located on the plasma membrane at any one time; it may be that changes in the level of plasma membrane hCTR1, the component most likely responsible for initial cisplatin influx, do not track exactly with changes in whole cell CTR1. Nevertheless, despite the fact that bortezomib can reasonably be expected to alter the levels of a variety of proteins that might modulate cisplatin uptake and cytotoxicity, the current results show that its effect on cisplatin uptake was largely dependent on the presence of CTR1 because enhanced platinum content was not seen in cells that do not express CTR1.

A previous study by Mimnaugh et al. (17) revealed that inhibition of the proteasome with ALLnL increased not only

whole cell cisplatin uptake but also DNA adduct formation by ~50% in highly cisplatin-resistant A2780 human ovarian carcinoma cells. Mimnaugh's observation can be interpreted as an effect resulting from inhibition of hCTR1 degradation because cisplatin-induced down-regulation of hCTR1 similar to that observed in 2008 cells has now been reported in a variety of other cell lines including ovarian carcinoma A2780 (26) and murine embryonic fibroblasts.¹ At present, it is not clear how cisplatin gains access to the nucleus and DNA; however, this result suggests that blockade of proteasome function does not impair trafficking of cisplatin from the cell membrane to critical cytotoxic targets.

The proteasome mediates the degradation of a wide array of cellular proteins, including the controlled degradation of regulatory proteins important for cellular homeostasis and damaged or misfolded proteins targeted for degradation by the ubiquitin-proteasome pathway. The effects of proteasome inhibition are thus similarly broad in scope, and a number of key signaling pathways and processes including cell cycle regulation, transcription, and apoptosis are modulated. Thus, despite the apparent dependence of bortezomib-enhanced platinum delivery on the expression of hCTR1, the marked synergism that occurs with bortezomib and cisplatin is likely mediated by a variety of other mechanisms as well. Modulation of nuclear factor- κ B signaling is among the best characterized effects of the drug, and constitutively expressed nuclear factor- κ B has been shown in a number of cancer cell lines, including ovarian cancer (reviewed in ref. 31). Bortezomib inhibits chemotherapy-induced nuclear factor- κ B activation through its effect on the inhibitor I- κ B, thus leading to enhanced chemosensitivity and increased apoptosis (7, 31). Bortezomib has also been noted to affect the level of expression of the proapoptotic protein Noxa and to produce estrogen receptor stress-related apoptosis (32). Frankel et al. (33) reported that bortezomib was more toxic to ovarian cancer cells grown as multicellular spheroids than cells grown as a monolayer, suggesting that bortezomib can circumvent the multicellular resistance pathways. It has also been shown to increase the

Table 3. Pharmacokinetic parameters for i.p. bortezomib

AUC ratio	252
Peritoneal C _{max} (mean)	63.4 μmol/L
Plasma C _{max} (mean)	2.4 μmol/L
t _{1/2} with i.p. delivery	34.9 min
t _{1/2} with i.v. delivery*	15 min
Peritoneal drug clearance	2.2 mL/min

Abbreviations: C_{max}, maximum concentration; t_{1/2}, terminal half-life.

* Data from refs. 7, 8.

¹ Larson CA, Blair BG, Safaei R, Howell SB. The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs. *Mol Pharmacol* 2008 Nov 7. [Epub ahead of print].

depth of penetration of radiolabeled gemcitabine and 5-fluorouracil *in vitro* using multicellular spheroids, and decrease tumor interstitial fluid pressure allowing for greater drug penetration *in vivo* (34). Thus, the ability of bortezomib to increase cisplatin accumulation in ovarian tumors in this xenograft model may be the result of multiple interdependent effects resulting from proteasomal inhibition.

Although the combination of bortezomib and cisplatin has not been extensively studied, two phase 1 trials combining i.v. bortezomib (on days 1, 4, 8, and 11) and i.v. carboplatin (on day 1) in platinum-sensitive and platinum-resistant ovarian cancer patients have been reported (35, 36). Few responses were noted in either study, but there is evidence in other tumor types to suggest that the clinical effects of bortezomib in combination with other agents can be highly sequence-dependent (37–39). Similarly, our results suggest that if bortezomib is to augment the antitumor effect of cisplatin or carboplatin by hCTR1 modulation, it will likely need to be given on a schedule that will allow for maximal proteasomal inhibition prior to administration of the cisplatin or carboplatin. Although hCTR1 levels in tumors were not assessed in either study, given that carboplatin and bortezomib were coadministered on day 1, the pharmacokinetics and pharmacodynamics of these two drugs would likely not allow for optimal enhancement of platinum tumor delivery by the hCTR1 mechanism. It will be important in future clinical trials to investigate dosing schedules in which bortezomib is given prior to cisplatin such that the effect of proteasomal inhibition on hCTR1 and subsequent platinum accumulation is maximized.

The relative advantage of administering bortezomib by the i.p. route was 252-fold in this murine model, and the duration of exposure to detectable concentrations of the drug was at least 6-fold greater in the peritoneal cavity than in plasma. These results suggest that the i.p. route is highly favorable as an approach to increasing tumor exposure and reducing systemic toxicity. The much greater exposure attained for the tumor on the surface of the peritoneum provides a basis for selective modulation of cisplatin uptake when both agents are administered by the i.p. route. Additional opportunity for selectivity is derived from the observation that many types of transformed

cells are differentially susceptible to the cytotoxic effects of proteasomal inhibition (40–43). In many cases actively proliferating cells were found to be more susceptible to proteasome inhibitor-mediated apoptosis than quiescent cells (44, 45), a factor that should favor killing of proliferating ovarian cancer cells relative to the quiescent mesothelium on which they grow but may not favor the killing of quiescent tumor stem cells. Dysregulation of the ubiquitin-proteasome pathway in malignant cells has also been postulated to play a role in this selectivity. With regard to the potential ability of bortezomib to increase cisplatin uptake into other normal tissues, relatively few other tissues have been shown to express hCTR1, as detected by immunohistochemical staining, its expression being limited largely to the pancreatic islet cells, colonic epithelium, and gastric mucosa (24). The effects of bortezomib and cisplatin treatment in these cell populations have not been tested, but the fact that plasma concentrations of bortezomib are very low at all points following i.p. administration suggests a limited likelihood of excessive toxicity to normal tissues.

In conclusion, this study shows a novel use of bortezomib to modulate the molecular pharmacology of cisplatin and thereby increase tumor accumulation and killing. Further, these effects can be maximized for i.p. tumors while at the same time limiting the potential for systemic toxicity by delivering both cisplatin and bortezomib via the i.p. route. Future studies are needed to investigate whether this combined regimen and subsequent enhanced platinum tumor accumulation results in a survival advantage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Dennis Thiele for generously providing the pair of *CTR1^{+/+}* and *CTR1^{-/-}* mouse embryo fibroblasts used in this study, Dennis Young at the Moores Cancer Center Core facilities for his assistance with flow cytometry, and Kersi Pestonjamp at the Microscopy Core for his assistance with confocal imaging and analysis.

References

- Howell SB, Pfeifle CL, Wung WE, et al. Intraperitoneal cisplatin with systemic thiosulfate protection. *Ann Intern Med* 1982;97:845–51.
- Armstrong DK, Bundy B, Wenzel L, et al. A. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med* 2006;354:34–43.
- Elit L, Oliver TK, Covens A, et al. A. M. Intraperitoneal chemotherapy in the first-line treatment of women with stage III epithelial ovarian cancer: a systematic review with metaanalyses. *Cancer* 2007; 109:692–702.
- Markman M, Bundy BN, Alberts DS, et al. Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup study of the Gynecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. *J Clin Oncol* 2001;19: 1001–7.
- Cannistra SA. Intraperitoneal chemotherapy comes of age. *N Engl J Med* 2006;354:77–9.
- Howell SB. Section XV Principles of chemotherapy, 4. Regional chemotherapy. In: Holland JF, Frei EJ, Bast RC, Jr., Kufe D, Morton R, Weischelbaum RR, editors. *Cancer medicine*. 3rd ed. Philadelphia: Lea & Febiger; 1993. p. 640–52.
- Adams J, Kauffman M. Development of the proteasome inhibitor Velcade (bortezomib). *Cancer Invest* 2004;22:304–11.
- Ogawa Y, Tobinai K, Ogura M, et al. Phase I and II pharmacokinetic and pharmacodynamic study of the proteasome inhibitor bortezomib in Japanese patients with relapsed or refractory multiple myeloma. *Cancer Sci* 2008;99:140–4.
- Ishida S, Lee J, Thiele DJ, et al. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci U S A* 2002;99:14298–302.
- Lin X, Okuda T, Holzer A, et al. The copper transporter CTR1 regulates cisplatin uptake in *Saccharomyces cerevisiae*. *Mol Pharmacol* 2002;62:1154–9.
- Holzer AK, Manorek GH, Howell SB. Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin. *Mol Pharmacol* 2006;70:1390–4.
- Holzer AK, Samimi G, Naerdemann, et al. Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells. *Proc Am Assoc Cancer Res* 2005;46:347.
- Song IS, Savaraj N, Siddik ZH, et al. Roles of copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and resistant cells. *Mol Cancer Ther* 2004;3: 1543–9.
- Holzer AK, Howell SB. The internalization and degradation of human copper transporter 1 following cisplatin exposure. *Cancer Res* 2006;66:10944–52.
- Voorhees PM, Orłowski RZ. The proteasome and proteasome inhibitors in cancer therapy. *Annu Rev Pharmacol Toxicol* 2006;46:189–213.
- Bazzaro M, Lee MK, Zoso A, et al. Ubiquitin-proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. *Cancer Res* 2006;66:3754–63.

17. Mimnaugh EG, Yunmbam MK, Li Q, et al. Prevention of cisplatin-DNA adduct repair and potentiation of cisplatin-induced apoptosis in ovarian carcinoma cells by proteasome inhibitors. *Biochem Pharmacol* 2000;60:1343–54.
18. Yunmbam MK, Li QQ, Mimnaugh EG, et al. Effect of the proteasome inhibitor ALLnL on cisplatin sensitivity in human ovarian tumor cells. *Int J Oncol* 2001;19:741–8.
19. Disaia PJ, Sinkovics JG, Rutledge FN, et al. Cell-mediated immunity to human malignant cells. *Am J Obstet Gynecol* 1972;114:979–89.
20. Hamilton TC, Winker MA, Louie KG, et al. Augmentation of adriamycin, melphalen, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* 1985;34:2583–6.
21. Katano K, Safaei R, Samimi G, et al. Expression of the copper efflux transporter ATP7B in human ovarian carcinoma cells alters cisplatin and carboplatin sensitivity and cellular pharmacology. *Proc Am Assoc Cancer Res* 2003;44:806.
22. Monks A, Scudiero D, Skehan P, et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991;83:757–65.
23. Holzer AK, Samimi G, Katano K, et al. The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. *Mol Pharmacol* 2004;66:817–23.
24. Holzer AK, Varki NM, Le QT, et al. Expression of the human copper influx transporter 1 in normal and malignant human tissues. *J Histochem Cytochem* 2006;54:1041–9.
25. Pekol T, Daniels JS, Labutti J, et al. Human metabolism of the proteasome inhibitor bortezomib: identification of circulating metabolites. *Drug Metab Dispos* 2005;33:771–7.
26. Holzer AK, Katano K, Klomp LW, et al. Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. *Clin Cancer Res* 2004;10:6744–9.
27. Ma C, Mandrekar SJ, Alberts SR, et al. A phase I and pharmacologic study of sequences of the proteasome inhibitor, bortezomib (PS-341, Velcade), in combination with paclitaxel and carboplatin in patients with advanced malignancies. *Cancer Chemother Pharmacol* 2007;59:207–15.
28. Voorhees PM, Dees EC, O'Neil B, et al. The proteasome as a target for cancer therapy. *Clin Cancer Res* 2003;9:6316–25.
29. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006;58:621–81.
30. Liu J, Sitaram A, Burd CG. Regulation of copper-dependent endocytosis and vacuolar degradation of the yeast copper transporter, *ctr1p*, by the *rsp5* ubiquitin ligase. *Traffic* 2007;8:1375–84.
31. Cusack JC. Rationale for the treatment of solid tumors with the proteasome inhibitor bortezomib. *Cancer Treat Rev* 2003;29 Suppl 1:21–31.
32. Fribley AM, Evenchik B, Zeng Q, et al. Proteasome inhibitor PS-341 induces apoptosis in cisplatin-resistant squamous cell carcinoma cells by induction of Noxa. *J Biol Chem* 2006;281:31440–7.
33. Frankel A, Man S, Elliott P, et al. Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. *Clin Cancer Res* 2000;6:3719–28.
34. Grantab R, Tannock I. Pretreatment with bortezomib might overcome cell adhesion mediated drug resistance by improving drug penetration in solid tumors. Los Angeles (CA): American Association for Cancer Research Annual Meeting; 2007.
35. Aghajanian C, Dizon DS, Sabbatini P, et al. Phase I trial of bortezomib and carboplatin in recurrent ovarian or primary peritoneal cancer. *J Clin Oncol* 2005;23:5943–9.
36. Ramirez PT, Landen CN, Jr., Coleman RL, et al. Phase I trial of the proteasome inhibitor bortezomib in combination with carboplatin in patients with platinum- and taxane-resistant ovarian cancer. *Gynecol Oncol* 2008;108:68–71. Epub 2007 Oct 1.
37. Ma C, Mandrekar SJ, Alberts SR, et al. A phase I and pharmacologic study of sequences of the proteasome inhibitor, bortezomib (PS-341, Velcade), in combination with paclitaxel and carboplatin in patients with advanced malignancies. *Cancer Chemother Pharmacol* 2007;59:207–15.
38. Mack PC, Davies AM, Lara PN, et al. Integration of the proteasome inhibitor PS-341 (Velcade) into the therapeutic approach to lung cancer. *Lung Cancer* 2003;41:S89–96.
39. Mortenson MM, Schlieman MG, Virudachalam S, et al. Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapy in the A549 non-small-cell lung cancer cell line. *Cancer Chemother Pharmacol* 2004;54:343–53.
40. Masdehors P, Omura S, Merle-Beral H, et al. Increased sensitivity of CLL-derived lymphocytes to apoptotic death activation by the proteasome-specific inhibitor lactacystin. *Br J Haematol* 1999;105:752–7.
41. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 2003;348:2609–17.
42. Orłowski RZ, Eswara JR, Lafond-Walker A, et al. Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. *Cancer Res* 1998;58:4342–8.
43. Soligo D, Servida F, Delia D, et al. The apoptogenic response of human myeloid leukaemia cell lines and of normal and malignant haematopoietic progenitor cells to the proteasome inhibitor PSI. *Br J Haematol* 2001;113:126–35.
44. Drexler HC. Activation of the cell death program by inhibition of proteasome function. *Proc Natl Acad Sci U S A* 1997;94:855–60.
45. Lopes UG, Erhardt P, Yao R, et al. p53-dependent induction of apoptosis by proteasome inhibitors. *J Biol Chem* 1997;272:12893–6.