Bortezomib-Induced Survival Signals and Genes in Human Proximal Tubular Cells

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ABSTRACT

Bortezomib has been introduced recently in the therapy of multiple myeloma (MM), a disease that is frequently associated with progressive renal failure. Because bortezomib-based therapy has been reported to lead to a rapid recovery of kidney function in patients with MM, we decided to study its direct effects in proximal tubular epithelial cells (PTCs) compared with glomerular mesangial cells (GMCs). After 24 h of stimulation, 50 nM bortezomib led to a 6.37-fold induction of apoptosis and markedly activated caspase-9 and -3 in GMCs but not in PTCs. In PTCs but not in GMCs, bortezomib led to a strong time-dependent degradation of IkB-α and to a long-lasting phosphorylation of both NF-κB p65 and extracellular signal-regulated kinase 1/2. Microarray analysis in bortezomib-treated PTCs revealed a time-dependent predominance of antiapoptotic genes compared with proapoptotic genes. Bortezomib (50 nM) induced heat shock protein (Hsp) 70 mRNA and protein levels in PTCs, whereas basal and bortezomib-stimulated Hsp70 protein expression was much weaker in GMCs. Moreover, bortezomib induced Bcl-2-associated athanogene (BAG) 3 mRNA and protein expression but inhibited BAG5 mRNA levels in PTCs. These data suggest that the reduced susceptibility of PTCs to bortezomib-induced cell apoptosis is because of cell type-specific effects of this compound on apoptosis/survival genes and pathways. The concept of bortezomib representing a blocker of both NF-κB activation and cell survival should be carefully examined in particular renal cell types.

The 26S proteasome, a multicatalytic protease complex expressed in the nucleus and cytoplasm of all eukaryotic cells, has traditionally been viewed as a recycler of damaged or misfolded proteins responsible for most nonlysosomal intracellular protein degradation. However, emerging data have revealed that besides degrading proteins tagged with ubiquitin, the proteasome plays a more varied and decisive role in cellular regulation than previously imagined (Ciechanover and Schwartz, 1998; Demartino and Gillette, 2007). Aside from its role as a protease, the proteasome also functions nonproteolytically in a variety of cellular processes including transcription, DNA repair, and chromatin remodeling (Demartino and Gillette, 2007). Furthermore, the normal degradation of proteins is crucial for maintenance of cellular homeostasis, and the proteasome plays a critical role in modulating intracellular levels of a variety of proteins such as tumor suppressors, oncogenes, and proteins that are involved in cell cycle regulation (Ciechanover and Schwartz, 1998). This is one reason why the 26S proteasome has received much attention as a potential therapeutic target and why various proteasome inhibitors have been actively studied for their antitumor effects (Adams, 2002).

Bortezomib (PS-341, Velcade), a boronic acid dipeptide, is a potent, selective, and reversible proteasome inhibitor that

ABBREVIATIONS: Bortezomib, PS-341, Velcade, C_{19}H_{25}BN_{4}O_{4}, [(R)-3-methyl-1-[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl)amino]propyl]-amino]butyl boronic acid; MM, multiple myeloma; PTC, proximal tubular epithelial cell; GMC, glomerular mesangial cell; HK, human kidney; DEG, differentially expressed gene; POR, polymerase chain reaction; NF, nuclear factor; ERK, extracellular signal-regulated kinase; Hsp, heat shock protein; BAG, Bcl-2-associated athanogene; HGNC, HUGO Gene Nomenclature Committee; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKB protein kinase B; PKG, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase kinase; ATF, activating transcription factor; PAGE, polyacrylamide gel electrophoresis; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal.
has been shown to induce cell death in many tumor models, including prostate, colon, and pancreatic cancer and multiple myeloma (MM) (Frankel et al., 2000; Cusack et al., 2001; Hideshima et al., 2001). Of these malignant diseases, the hematologic neoplasia MM is characterized by monoclonal proliferation of bone marrow plasma cells leading to a number of organ dysfunctions and symptoms such as bone pain or fracture, susceptibility to infection, anemia, and renal failure. Approximately 20 to 40% of newly diagnosed MM patients develop renal impairment, with up to 13% having renal failure requiring dialysis (Chanan-Khan et al., 2007; Kastritis et al., 2007). Several studies have shown that the severity of renal impairment significantly affects the prognosis of patients with MM (Chanan-Khan et al., 2007). With supportive measures and antymeloma treatment, renal failure is reversible in 25 to 58% of patients (Kastritis et al., 2007). New agents such as bortezomib, either as a monotherapy or in combination with dexamethasone, have been very efficient in newly diagnosed MM patients (Jagannath et al., 2005). Bortezomib can be safely used in relapsed patients with renal impairment, and two preliminary reports have been published recently showing that bortezomib combination therapy led to a reversal of acute multiple myeloma-induced renal failure (Mohrbacher and Levine, 2005; Ostermann et al., 2006). Additional studies revealed reversibility of renal failure in newly diagnosed MM patients treated with high-dose dexamethasone containing regimens and a more rapid improvement of renal function in the presence of bortezomib (Nozza et al., 2006; Kastritis et al., 2007). These findings are in line with a recent retrospective multicenter study performed with MM patients requiring dialysis for advanced renal failure, which suggested that bortezomib therapy is a well tolerated, effective option in the subgroup of MM patients with severe renal dysfunction (Chanan-Khan et al., 2007).

Albeit these clinical results are encouraging, there is still little information available regarding the direct cellular effects of bortezomib treatment in kidneys and the cell type-specific intracellular mechanisms induced by this proteasome inhibitor. These are important questions because, for reasons that remain to be fully elucidated, some cell types are less likely to protect these PTCs from apoptosis. Bortezomib affects a set of intracellular signaling events and pathways in epithelial PTCs compared with renal glomerular tubular cells (PTCs). Moreover, we investigated antiapoptotic mechanisms that are involved in MM (Batuman, 2007) and to gain insight into the molecular mechanisms affected by bortezomib along the involved in MM (Batuman, 2007) and to gain insight into the molecular mechanisms affected by bortezomib along the

### Materials and Methods

#### Chemicals and Reagents

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Bortezomib was kindly provided by Janssen-Cilag Pharma (Vienna, Austria). Unless otherwise indicated, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

#### Cell Culture

Human PTCs human kidney (HK-2, representing a human papillomavirus 16 E6/E7-immortalized PTC line from normal adult human kidney from passages 20 to 35 were cultured in keratinocyte-serum free medium containing 10% fetal bovine serum, 5 ng/ml recombinant epidermal growth factor, 0.05 mg/ml bovine pituitary extract, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sarközi et al., 2007). Rat GMCs from passages 25 to 40 were maintained in RPMI 1640 medium supplemented with 10% FCS, 5 ng/ml each of insulin and transferrin, 5 μg/ml selenite, 100 units/ml penicillin, and 100 μg/ml streptomycin (Schrämke et al., 1996). Wild-type LLC-PK1 cells, representing a proximal tubular epithelial cell line derived from the pig kidney, were used from passages 178 to 185. They were grown in Eagle’s minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1.2 mg/ml NaHCO3, and 5% fetal bovine serum, and split at a 1:10 ratio once a week (Schramek et al., 1993). All cells were cultured at 37°C in a humidified 5% CO2 atmosphere; after growth to subconfluent state, they were washed once, made quiescent by incubation in serum- and supplement-free medium for 48 h, and then used for experiments. Stimulated PTCs were cultured in a medium containing 10% fetal bovine serum in the absence of serum and any other growth supplements.

#### Flow Cytometric DNA Analysis

PTCs, LLC-PK1 cells, and GMCs were made quiescent by incubation in serum- and supplement-free medium for 24 h and then either left unstimulated or stimulated with 50 nM bortezomib in the absence of serum and any other growth supplements. Flow cytometry was performed using an Epics XL-MCL Flow Cytometry System (Beckman Coulter, Fullerton, CA). Data were collected from at least 10,000 events. Bortezomib did not increase autofluorescence of the cell populations tested (data not shown).

#### Western Blot Analysis

Whole-cell lysates were prepared using ice-cold radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% deoxycholic acid, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich), 0.1% SDS, 1% Triton X-100). Protein-matched samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride microporous membrane (Millipore Corporation, Billerica, MA). Immunoblotting was performed as described previously (Schramek et al., 1996; Sarközi et al., 2007). The following primary antibodies were applied: cleaved caspase-9 (Asp315), cleaved caspase-3 (Asp175), phosphonuclear factor (NF)-κB p65 (Ser536), phospho-p44/42 mitogen-activated protein kinase (all from Cell Signaling Technology Inc., Danvers, MA), NF-κB p65, IκB-α, AKT1/2/3, phospho-Akt1/2/3 (Ser473)-R, extracellular signal-regulated kinase (ERK) 2, Hsp70 (W27) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-actin (Sigma-Aldrich), and Bcl-2-associated anathogene (BAG) 3 (Alexis Laboratories).

#### Microarray Hybridization and Data Analysis

We used human exonic evidence-based oligonucleotide arrays from the Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/heeb.do). Total cellular RNA (vide infra) was reverse-transcribed using the CyScribe cDNA Post Labeling Kit (GE Healthcare, Chalfont St. Giles, UK). RNA from bortezomib-stimulated cells was labeled with Cy-3 (red) and RNA from unstimulated control experiments was labeled with Cy-5 (green). For each of the four different time points (4, 8, 16, and 32 h), a cobybridization of Cy-5- and
Cy-3-labeled reverse-transcribed RNA was performed in biological duplicates. Arrays were scanned with the Axon GenePix 4000B microarray scanner, and the images were analyzed with GenePix 6.0 software (Molecular Devices, Sunnyvale, CA). Raw data and array images were transferred to the Stanford Microarray Database for data storage and data preprocessing (http://genome-www5.stanford.edu/MicroArray/SMD). All experiments were performed according to the MIAME (Minimum Information About a Microarray Experiment) guidelines (Brazma et al., 2001). Mean log2 R/G values were used for analysis, and only spots with intensity values greater than 1.5 times the background values in either channel were considered for analysis. Of the 36,293 retrieved spots, 32,083 were mapped to HUGO Gene Nomenclature Committee (HGNC) Gene Symbols. The other 4210 spots represented expressed sequence tags and hypothetical proteins.

Functional gene categorization was based on gene ontology terms that were assigned to all annotated genes on the chip using the GO package from the Bioconductor R module. The set of genes associated with apoptosis was extracted, and gene expression values of those genes were further analyzed. Values of different clones assigned to a single gene cluster were averaged, and genes showing at least a 2-fold change in either direction in both replicate arrays at any time point were defined as differentially expressed.

To further evaluate the contribution of differentially expressed gene (DEGs) to anti- and proapoptotic processes, we determined the numbers of DEGs assigned to the respective gene ontology categories and compared those numbers with the background distribution of the apoptosis-associated reference gene set on the chip.

RNA Isolation and Real-Time PCR. Total cellular RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH). RNA quantity was estimated by spectrophotometric analysis. Reverse transcription was performed using 2 μg of total RNA, 1 μM random hexanucleotides as primer (Roche Diagnostics, Indianapolis, IN), and Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA) according to the manufacturer’s instructions.

Real-time PCR was performed using 2 μl of cDNA template from the reverse transcription reaction, 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and premade TaqMan Gene Expression Assays [human GAPDH (Hs99999905m1), human BAG3 (Hs00188713m1), and human BAG5 (Hs00191644m1); Applied Biosystems] according to the manufacturer’s instructions. Relative changes in gene expression (-fold change) were calculated using the 2-ΔΔCT method described by Livak and Schmittgen (2001).

Statistical Analyses. Two-sided Student’s t tests were used to assess the significance of differences of bortezomib administration on the percentage of apoptotic and necrotic cells in PTCs and GMCs compared with untreated cells. Percent values of apoptotic cells are expressed as mean ± S.E.M.

Results

PTCs Are Less Sensitive to Bortezomib-Induced Apoptosis Compared with GMCs. Bortezomib, which is a reversible inhibitor of the chymotrypsin-like activity of 26S proteasome, behaves as an efficient apoptotic agent in a number of tumor cells; therefore, it has been introduced in the therapy of MM (Hideshima et al., 2001). However, it has been noted earlier that one identical concentration of proteasome inhibitor has divergent effects on different cell types. Although proteasome inhibition induces apoptosis in rapidly proliferating tumor cells, the opposite response, protection from apoptosis, has been observed in differentiated and quiescent cells (Wojcik, 1999; Meiners et al., 2008). Because no information on direct effects of bortezomib in renal cells is available to date and because the final cellular outcome of bortezomib administration might largely depend on both drug concentration and the specific cell type affected by this treatment, we first studied bortezomib-induced apoptosis/necrosis in PTCs compared with GMCs, utilizing fluorescence-activated cell sorting analysis following annexin V/propiodium iodide staining. After 24 h of stimulation, 50 nM bortezomib led to a highly significant, 6.37-fold induction of apoptosis in GMCs but not in PTCs (Fig. 1A). GMC apoptosis increased from 2.13 ± 0.2% in unstimulated controls to 13.56 ± 2.22% in bortezomib-stimulated cells (n = 12; p < 0.0001). In contrast, PTC apoptosis showed only a slight 1.56-fold increase, from 2.98 ± 0.8% in unstimulated cells to 4.66 ± 1.43% in bortezomib-treated cells (n = 11; p = 0.316). In parallel, bortezomib led to a similar induction of necrosis after 24 h in both cell types (Fig. 1B). Bortezomib stimulated necrosis 2.69-fold in PTCs (from 4.31 ± 0.68% to 11.61 ± 1.16%; n = 11; p < 0.0001) and 2.74-fold in GMCs (from 6.19 ± 1.36% to 16.98 ± 1.75%; n = 12; p < 0.0001) (Fig. 1). Representative dot blots of annexin V/propiodium iodide staining of PTCs and GMCs are depicted in Fig. 1, C and D, respectively. GMC display increased fractions of early apoptotic annexin V-positive cells and necrotic propidium iodide-positive cells after treatment with bortezomib (Fig. 1D). In contrast, PTCs show only a slight increase in both the late apoptotic double-positive population and the necrotic propidium iodide-positive population (Fig. 1C). Compared with untreated PTCs, no difference in the early apoptotic annexin V-positive cell population is visible after bortezomib administration. These results from fluorescence-activated cell sorting analysis are corroborated by the finding that bortezomib led to a strong activation of caspase-9 and -3 in GMCs (Fig. 2). Bortezomib (50 nM) induced a time-dependent cleavage of caspase-9 (Fig. 2, top) and caspase-3 (Fig. 2, bottom) in GMCs. Bortezomib-stimulated cells exhibited a caspase-9 cleavage band visible in bortezomib-treated LLC-PK1 cells, which represents a proximal tubular cell line derived from pig (Fig. 4). Similar to PTCs, proximal tubular LLC-PK1 cells showed a low susceptibility to bortezomib-induced cell death. After 24 h of stimulation, LLC-PK1 cell apoptosis was increased from 1.56 ± 0.89% in unstimulated cells to 4.24 ± 0.94% in bortezomib-treated cells (n = 8; p = 0.015). Necrosis showed only a marginal induction, from 0.89% to 3.33 ± 0.58% in unstimulated controls to 4.48 ± 0.27% in bortezomib-stimulated cells (n = 8; p = 0.093). These results together suggest that the proximal tubular epithelial cell lines HK-2 and LLC-PK1 are less susceptible to bortezomib-induced apoptosis compared with mesenchymal GMCs.

Bortezomib Induces Long-Lasting NF-κB65 Phosphorylation on Ser536 in PTCs but Not in GMCs. NF-κB comprises a family of at least five proteins, which all contain a Rel homology domain mediating DNA binding, dimerization, and interaction with inhibitory factors known as IxB proteins (Perkins, 2007). The canonical NF-κB complex represents a heterodimer composed of p50 and p65 subunits and regulates many physiological processes, including cell death, apoptosis, adhesion, and proliferation (Perkins, 2007). Pro-
Grouped Bar Chart with Error Bars

- **A**: Apoptosis (%)
  - Control
  - Bortezomib (50 nM) 24 h
  - (n=11) (n=12)
  - Control vs. Bortezomib (50 nM) 24 h: 1.56 6.37 -fold vs. Control
  - NS

- **B**: Necrosis (%)
  - Control
  - Bortezomib (50 nM) 24 h
  - (n=11) (n=12)
  - Control vs. Bortezomib (50 nM) 24 h: 2.69 2.74 -fold vs. Control

- **C**: Annexin V - FITC and Propidium iodide
  - PTC control
  - PTC bortezomib
  - 96.8 0.6 vs. 93.6 0.9

- **D**: Annexin V - FITC and Propidium iodide
  - GMC control
  - GMC bortezomib
  - 71.5 6.5 vs. 71.5 6.5
teasome inhibitors have been reported to block IκB degradation, thereby sequestering NF-κB in the cytoplasm and down-regulating its transcriptional activity (Traenckner et al., 1994). In many cell types, apoptosis induced by administration of proteasome inhibitors is associated with both accumulation of IκB-α and inhibition of NF-κB activity (Dai et al., 2003; An et al., 2004). Because PTCs seem to be more resistant to bortezomib-induced cell apoptosis and because the effect of the drug on NF-κB activation probably depends on the nature and the cellular context of its induction, we next studied the effects of bortezomib on NF-κB in PTCs and LLC-PK1 cells compared with GMCs. Administration of 50 nM bortezomib led to a time-dependent reduction of IκB-α and inhibition of NF-κB activity (Fig. 3). In parallel, bortezomib (50 nM) time-dependently stimulated NF-κBp65 phosphorylation at Ser536 in PTCs and in GMCs (Fig. 3, middle). In PTCs, strong bortezomib-induced p65 RelA phosphorylation occurred as early as 8 h after administration and lasted for at least 36 h, whereas in GMCs, p65 RelA was only transiently phosphorylated after 8 h, which then again decreased to control levels after 16 h before p65 RelA phosphorylation was almost abolished after 24 and 36 h (Fig. 3). No alterations in NF-κBp65 protein expression associated with bortezomib treatment were detected in the two cell types investigated under these experimental conditions (Fig. 3, bottom). With respect to IκB-α degradation and NF-κBp65 phosphorylation at Ser536, similar results as described for PTCs were obtained in proximal tubular LLC-PK1 cells after 24 h of bortezomib administration (Fig. 4). Because reduction of IκB-α protein levels and phosphorylation of the p65 RelA subunit on Ser536 is associated with increased NF-κB transcriptional activity (Dolcet et al., 2006), these results suggest that in PTCs and LLC-PK1 cells, bortezomib represents an activator of NF-κB transcriptional activity.

Fig. 1. Bortezomib-induced effects on apoptosis and necrosis in PTCs compared with GMCs. A, effect of 50 nM bortezomib on apoptosis in PTCs and in GMCs after 24 h of stimulation compared with untreated controls. B, effect of 50 nM bortezomib on necrosis in PTCs and in GMCs after 24 h of stimulation compared with untreated controls. PTCs and GMCs were stained with a fluorescein isothiocyanate-conjugated annexin V and propidium iodide for 10 min and were then subjected to flow cytometric analysis, as outlined under Materials and Methods. C and D, representative dot blots of annexin V/propidium iodide staining of PTCs and GMCs, respectively.

Fig. 2. Bortezomib-induced time-dependent activation of caspase-9 and -3 in GMCs compared with PTCs. PTCs and GMCs were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 8, 12, 16, 24, and 36 h compared with untreated controls (8 and 36 h). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for cleaved caspase-9, cleaved caspase-3, and β-actin protein expression. The results from one representative Western blot of n = 5 separate experiments are depicted.

Fig. 3. Bortezomib-induced time-dependent degradation of IκB-α and phosphorylation of NF-κBp65 in PTCs compared with GMCs. PTCs and GMCs were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 8, 12, 16, 24, and 36 h compared with untreated controls (8 and 36 h). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for IκB-α protein expression, NF-κBp65 Ser536 phosphorylation, and NF-κBp65 protein expression. The results from one representative Western blot of n = 5 separate experiments are depicted.
BP65

PTCs, LLC-PK1 cells, and GMCs were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 24 h compared with untreated controls. Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for cleaved caspase-3 expression, β-actin protein expression, IkB-α protein expression, NF-κB phosphorylation, and NF-κBp65 protein expression. The results from one representative Western blot of n = 4 separate experiments are depicted. C, unstimulated control; B, bortezomib-stimulated.

Bortezomib Inhibits Akt/PKB Protein Expression in GMCs but Not in PTCs. The serine/threonine kinase Akt, also known as protein kinase B (PKB), represents the crucial link between phosphatidylinositol 3-kinase (PI3K) and the prevention of apoptosis (Manning and Cantley, 2007). Akt is able to enhance the survival of cells by blocking the function of proapoptotic proteins and processes via various mechanisms. Akt activation, for example, prevents the processing of procaspase-9 through its inhibitory effects on the function and expression of several Bcl-2 homology domain 3-only proteins, which exert their proapoptotic effects by binding to and inactivating prosurvival Bcl-2 family members (Manning and Cantley, 2007). Akt has also been found to directly phosphorylate Ser196 on human procaspase-9, and this phosphorylation correlates with a decrease in the protease activity of caspase-9 in vitro. In addition, it has been reported that under some conditions, the PI3K-Akt pathway can activate NF-κB survival signaling (Manning and Cantley, 2007). Thus, we next investigated the regulation of Akt/PKB in bortezomib-treated PTCs compared with GMCs. As depicted in Fig. 5, Akt showed a high Ser473 phosphorylation in PTCs both under basal and bortezomib-stimulated conditions. In contrast, we did not detect Akt/PKB phosphorylation in GMCs (Fig. 5, top). Akt/PKB protein levels were equally expressed in both cell types under unstimulated control conditions (Fig. 5, middle). Interestingly, administration of 50 nM bortezomib led to a time-dependent inhibition of Akt protein expression in GMCs, which started after 16 h and was maximal after 36 h (Fig. 5, middle). In contrast, no alteration of Akt protein expression was detected in bortezomib-treated PTCs. Equal loading during these experiments was assured by the fact that β-actin protein expression was equal and unaffected by bortezomib treatment during all conditions tested (Fig. 5, bottom).

Bortezomib Induces a Long-Lasting ERK1/2 Phosphorylation in PTCs but Not in GMCs. The mitogen-activated protein kinase family, especially the Raf-MEK1/2-ERK1/2 signaling module, represents another intracellular signaling system, which is important in the regulation of normal cell proliferation, cell survival, and cell differentiation (Torii et al., 2006). Moreover, ERK1/2 signaling acts cooperatively with PI3K/Akt signaling in regulating growth factor-stimulated cell cycle progression (Torii et al., 2006). Because ERK1/2 signaling also could be involved in the relative bortezomib resistance of PTCs compared with GMCs, we next investigated phosphorylation and expression of ERK1/2 in unstimulated and bortezomib-stimulated cells. After 24 h of PTC stimulation, bortezomib led to a concentration-dependent increase in ERK1/2 phosphorylation, which started at a concentration of 5 nM bortezomib and was maximal at 100 nM (data not shown). Bortezomib (50 nM) led to an induction of ERK1/2 phosphorylation in PTCs, which started after 8 h and lasted for at least 36 h (Fig. 6). In GMCs, bortezomib-stimulated, time-dependent ERK1/2 phosphorylation was equal and unaffected by bortezomib treatment during all conditions tested (Fig. 5, bottom).
loration started after 8 h, was highest after 12 h, but started to decrease after 16 h and already reached control levels after 36 h (Fig. 6). At the same time, bortezomib did not affect ERK2 protein expression in PTCs or in GMCs (Fig. 6, bottom). Thus, the time-dependent decrease of NF-κBphosphorylation, Akt protein expression, and ERK1/2 phosphorylation in bortezomib-treated GMCs is associated with the time-dependent activation of caspase-9 and -3. In bortezomib-stimulated PTCs, on the other hand, long-term activation of NF-κB and ERK1/2 and lack of Akt degradation could be involved in the relative resistance of these cells to apoptosis induction.

**Bortezomib-Mediated Differential Gene Expression in PTCs: Evidence for a Time-Dependent Predominance of Antiapoptotic Genes Compared with Proapoptotic Genes.** To gain further insight into the molecular mechanisms affected by bortezomib along the normal tu-tube, we next examined time-dependent global gene expression profiles in bortezomib-treated PTCs utilizing oligonucleotide microarray analysis. After growth to a subconfluent state, PTCs were washed once, made quiescent by incubation in serum- and supplement-free medium for 48 h, and were then stimulated with 50 nM bortezomib for 4, 8, 16, and 32 h and compared with unstimulated control cells. We identified 379 proapoptotic, 308 antiapoptotic, and 538 apoptosis-related cDNA clones on the array mapping to 210 (33%), 157 (25%), and 261 (42%) unique genes based on HGNC Gene Symbols, respectively (for a listing of GO terms and associated cDNA clones, see Table 1 at http://www.microarray.at; “Data” section; given are the clone identification, the National Center for Biotechnology Information gene identification, the HGNC gene symbol, the gene name, and the apoptosis-related gene ontology terms). Of the total 628 genes, 181 were differentially expressed at least at one time point after stimulation as depicted in Table 2 at http://www.microarray.at; “Data” section. Values are given as log2 R/G, where R represents the bortezomib-treated gene array intensity values, and G represents untreated intensity values. Genes are designated as proapoptotic (+), antiapoptotic (−), or apoptosis-related (○). The proportion of up-regulated genes involved in antiapoptotic processes (gray columns) compared with those representing proapoptotic potential (black columns) steadily increased over time, with a peak at 16 h after bortezomib treatment, whereas the number of down-regulated genes involved in pro- and antiapoptosis was balanced (Fig. 7). The distribution of DEGs up-regulated 16 h after treatment was significantly different from the reference set based on a χ² test (p value of 0.0496). Ten DEGs (45% of up-regulated genes) were involved in antiapoptotic processes, whereas only three (14%) had proapoptotic activity (Fig. 7). Up-regulated antiapoptotic genes included the BAG3, the hepatocyte growth factor, and the four heat shock proteins HSPA1B (heat shock 70-kDa protein 1B), HSPA5 (heat shock 70-kDa protein 5), HSP90B1 (heat shock protein 90-kDa β member 1), and HSPA9B (heat shock 70-kDa protein 9). A similar trend was observed at 32 h after treatment, with 45% of up-regulated genes being involved in antiapoptotic processes and 18% showing proapoptotic potential. The proportion of up-regulated pro- and antiapoptotic genes at the two earlier time points after 4 and 8 h of stimulation was more balanced and similar to the background distribution. The two antiapoptotic genes HSPA1B and BAG3 were up-regulated at all four time points (Table 2 at http://www.microarray.at; “Data” section). All of the antiapoptotic genes listed above were also up-regulated after 32 h of bortezomib treatment (Table 2 at http://www.microarray.at).

**Strong Basal and Bortezomib-Induced Hsp70 Protein Expression in PTCs but Not in GMCs.** Stress response or heat shock is a cellular adaptive response, which helps to maintain cellular homeostasis of living cells under stress. Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly conserved set of proteins, the Hsps or stress proteins (Arya et al., 2007). Because we found several Hsp genes to be up-regulated in bortezomib-stimulated PTCs compared with unstimulated controls and because HSPA1B gene showed the highest up-regulation in oligonucleotide microarray analysis (Table 1 at http://www.microarray.at; “Data” section), we next investigated basal and bortezomib-induced protein expression of Hsp70 in PTCs, LLC-PK1 cells, and GMCs using Western blot analysis. Interestingly, in quiescent PTCs and LLC-PK1 cells, a much higher basal Hsp70 protein expression was obtained compared with GMCs, where very low basal Hsp70 protein expression was detected under identical conditions (Figs. 8 and 10). In addition, 50 nM bortezomib led to a strong and time-dependent additional induction of Hsp70 proteins in PTCs, which started after 8 h and peaked after 36 h (Fig. 8). In contrast, bortezomib-induced Hsp70 protein expression was weaker in GMCs (Figs. 8 and 10) and peaked after 24 h (Fig. 8).

**Bortezomib Induces Up-Regulation of BAG3 mRNA and Down-Regulation of BAG5 mRNA in PTCs.** Proteins that share the BAG domain were originally identified by their ability to associate with the antiapoptotic protein Bcl-2.
Fig. 7. Distribution of differentially expressed pro-apoptotic (black), anti-apoptotic (gray), and apoptosis-related (white) genes 16 h after bortezomib treatment compared with the reference set. Gene expression \( \log_{2} R/G \) values are depicted in detail for up- and down-regulated anti- and proapoptotic genes. HGNC gene symbols are used for gene annotation.

Fig. 8. Time-dependent effects of bortezomib on Hsp70 protein expression in PTCs compared with GMCs. PTCs and GMCs were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 8, 12, 16, 24, and 36 h compared with untreated controls (8 and 36 h). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for Hsp70 and \( \beta \)-actin protein expression. The results from one representative Western blot of \( n = 3 \) separate experiments are depicted.
BAG family proteins were also found to interact with Hsp70 and are able to modulate, either positively or negatively, the functions of these chaperone proteins (Doong et al., 2002). BAG3, also known as CAIR-1 or Bis, has been reported to sustain cell survival and/or to down-modulate the apoptotic response in some cell types (Rosati et al., 2007). BAG5, on the other hand, represents the only BAG family member with four BAG repeats and has been reported to enhance dopaminergic neuronal degeneration through inhibition of the E3 ligase activity of parkin and the chaperone activity of Hsp70 (Kalia et al., 2004). Because we found evidence for up-regulation of BAG3 and down-regulation of BAG5 during microarray analysis of bortezomib-stimulated PTCs compared with untreated controls, we next studied mRNA expression of BAG3 and BAG5 in bortezomib-stimulated PTCs using real-time PCR analysis. As depicted in Fig. 9A, 50 nM bortezomib led to a strong 11.7- (n = 6) and 10.6-fold (n = 6) induction of BAG3 mRNA after 8 and 16 h, respectively. The p values of Student’s t tests comparing the ΔCt PCR values between treated and control samples were <0.001 at both time points. BAG5 mRNA expression, on the other hand, was significantly down-regulated after 8 (0.61-fold; n = 6) and 16 (0.54-fold; n = 6) h of bortezomib treatment (p values of 0.002 and <0.001 at the 8- and 16-h time points, respectively) (Fig. 9B). The strong stimulatory effect of bortezomib on BAG3 expression was verified at the protein level in PTCs and LLC-PK1 cells compared with a lower induction in GMCs (Fig. 10). Because these results corroborate the findings obtained in the oligonucleotide microarray assays, it is tempting to speculate that bortezomib administration is not only able to induce genes that are able to sustain cell survival and/or to down-modulate apoptosis (e.g., BAG3 and Hsp70) but also to inhibit genes that might be involved in the induction of cell injury and/or cell death (e.g., BAG5).

**Discussion**

This study presents two major findings with relevance for both basic and applied clinical research of current applications of the proteasome inhibitor bortezomib in MM patients with renal impairment. 1) Bortezomib modulates renal cellular function in a cell type-specific manner. 2) In human PTCs, bortezomib affects a set of intracellular signaling mechanisms and genes that are likely to protect this renal epithelial cell type from apoptosis rather than inducing PTC

![Fig. 9. Bortezomib-induced effects on BAG3 mRNA expression and on BAG5 mRNA expression in PTCs. PTCs were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 8 and 16 h compared with untreated controls (8 and 16 h). Thereafter, total RNA was isolated and used for real-time PCR analysis. A, cDNAs from six independent RNA isolations were applied for real-time PCR analysis of the BAG3 gene. Data are presented as -fold change above BAG3 mRNA control levels after normalizing to GAPDH expression. Each data point indicates the average of n = 6 independent experiments; error bars, S.E.M. B, cDNAs from six independent RNA isolations were applied for real-time PCR analysis of the BAG5 gene. Data are presented as -fold change compared with BAG5 mRNA control levels after normalizing to GAPDH expression. Each data point indicates the average of n = 6 independent experiments; error bars, S.E.M.](image1)

![Fig. 10. Bortezomib-induced effects on Hsp70 and BAG3 protein expression in PTCs, LLC-PK1 cells, and GMCs. Cells were serum- and supplement-starved for 48 h and were then exposed to 50 nM bortezomib for 24 h compared with untreated controls. Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for Hsp70, BAG3, and β-actin protein expression. The results from one representative Western blot of n = 2 separate experiments are depicted. C, unstimulated control; B, bortezomib-stimulated.](image2)
apoptosis. Furthermore, our results are consistent with the idea that proteasome inhibitors in general, specifically bortezomib, have the ability to act either as poisons, which induce apoptosis and cell death, or as remedies, which modulate cellular function (Wojcik, 1999; Meiners et al., 2008). Although rapidly proliferating cells with abnormal phenotypes are most sensitive to the proapoptotic action of proteasome inhibitors, differentiated cells are not or less sensitive, even when they form highly proliferating populations, such as bone marrow or epithelia. Different tumor cell lines also vary markedly in their sensitivity to proteasome inhibition, although the chymotrypsin-like activity of the 20S proteasome is inhibited to the same extent (Meiners et al., 2008). It is even possible to observe differential responsiveness to proteasome inhibition within the same cell type. A defined dose of proteasome inhibitors, for example, is capable of inducing apoptosis in proliferating but not in quiescent human leukemia HL60 cells (Drexler, 1997). Similar differential responsiveness to induction of apoptosis was observed in rat-1 fibroblasts, primary endothelial cells, and in tumor-derived lymphocytes and hepatocytes (in contrast to normal lymphocytes and hepatocytes) (Meiners et al., 2008). Although several mechanisms for proteasome inhibitor-mediated induction of apoptosis have been obtained and discussed, the molecular mechanisms of potential protective effects of proteasome inhibition in kidney cells are still largely unknown.

Bortezomib is currently used as a chemotherapeutic drug because of its ability to block NF-κB, a transcription factor constitutively activated in many types of human cancer. Recent studies revealed reversibility of renal failure in newly diagnosed MM patients treated with high-dose dexamethasone-containing regimens and a more rapid improvement of renal function in the presence of bortezomib (Nozza et al., 2006; Kastritis et al., 2007). Thus, we investigated direct cellular effects of bortezomib treatment and cell type-specific intracellular mechanisms induced by this proteasome inhibitor in PTCs, LLC-PK1 cells, and GMCs. We found evidence for distinct bortezomib-mediated protection pathways in PTCs and LLC-PK1 cells compared with GMCs. In the presence of bortezomib, PTCs showed long-term activation of NF-κB signaling and long-term phosphorylation of Akt and ERK1/2 protein kinases, associated with lack of caspase-9, caspase-3, and apoptosis induction. In contrast, bortezomib led to a strong time-dependent activation of caspase-9 and -3 in GMCs, which was followed by apoptosis induction after 24 h. Furthermore, GMCs did not present long-term phosphorylation of NF-κB, Akt, and ERK1/2 but showed reduced Akt protein expression at late time points after bortezomib administration, suggesting that the activation of either one of these survival pathways in PTCs could be involved in the resistance of these cells to bortezomib-induced apoptosis. The fact that almost identical results have been obtained in both PTCs (HK-2 cells) and LLC-PK1 cells strongly argues against a predominant involvement of E6 and E7 oncoproteins of HPV 16 in the observed processes in PTCs (Figs. 4 and 10).

Our data are in line with a recent study showing that proteasome inhibitors induce cell death but activate NF-κB in endometrial carcinoma cells and primary explant culture of endometrial adenocarcinoma (Dollet et al., 2006). In HepG2 cells, bortezomib also induced a decrease in IκB-α protein levels and concomitantly an increase in NF-κB DNA-binding activity (Calvaruso et al., 2006). In renal cell carci-
Bortezomib, Hsp72 interacted with and sequestered the pro-apoptotic factors p53, AIF, Bax, and Apaf-1 in HepG2 cells (Calvaruso et al., 2007). Although the nuclear levels of Hsp72, p53 and AIF steadily increased, the interaction of Hsp72 with these factors diminished during the 2nd day of treatment, which was associated with the activation of caspases (Calvaruso et al., 2007). All together, our data obtained in PTCs compared with GMs suggest that induction of Hsp70 mRNA and protein expression could be involved in the relative resistance of these human proximal tubular cells against bortezomib-induced apoptosis. This idea is further supported by the finding of a strong bortezomib-stimulated induction of BAG3 mRNA and protein expression in PTCs and LLC-PK1 cells (Figs. 9 and 10). Expression of BAG3 is elevated in some leukemia and solid tumors, and anti-sense-mediated reductions in BAG3 expression enhance the apoptotic response to chemotherapeutic drugs in some malignant cells (Rosati et al., 2007). Most recently, it has been reported that BAG3 was induced at the transcriptional level by proteasome inhibitors such as MG132 and lactacystin in cancer cell lines derived from different histology (Wang et al., 2008). Prohibition of BAG3 up-regulation using small interfering RNAs enhanced cytotoxicity induced by MG132, suggesting that induction of BAG3 might be an adaptive response to proteasome inhibition (Wang et al., 2008). The time-dependent massive bortezomib-stimulated expression of BAG3 mRNA levels in PTCs supports these results. BAG5 mRNA expression, on the other hand, is inhibited by bortezomib treatment in PTCs. This BAG family member has been reported to enhance dopaminergic neuronal degeneration through inhibition of the E3 ligase activity of parkin and the chaperone activity of Hsp70 (Kalia et al., 2004).

In summary, it is tempting to speculate that both bortezomib-stimulated transcription of cell protection genes and bortezomib-inhibited transcription of pro-apoptotic genes might support NF-κB, Akt/PKB, and/or ERK1/2 prosurvival signaling pathways to reduce or delay apoptotic cell death in PTCs. Identification of bortezomib-induced survival genes might yield novel therapeutic approaches for the stimulation of early proximal tubulointerstitial protection to avoid renal disease progression. Finally, the concept of bortezomib representing a blocker of both NF-κB activation and cell survival should be carefully examined in particular renal cell types.

References


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