Proteasome inhibition attenuates hepatic injury in the bile duct-ligated mouse

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Anan, Akira, Edwina S. Baskin-Bey, Hajime Isomoto, Justin L. Mott, Steven F. Bronk, Jeffrey H. Albrecht, and Gregory J. Gores. Proteasome inhibition attenuates hepatic injury in the bile duct-ligated mouse. Am J Physiol Gastrointest Liver Physiol 291: G709–G716, 2006. First published June 22, 2006; doi:10.1152/ajpgi.00126.2006—Proteasome inhibition has recently been demonstrated to inhibit hepatic fibrogenesis in the bile duct-ligated (BDL) mouse by blocking stellate cell NF-κB activation. The effect of proteasome inhibition on liver injury, however, is unclear. Our aims were to assess the effect of the proteasome inhibitor bortezomib on liver injury in the BDL mouse. Liver injury was assessed in 7-day BDL mice treated with a single dose of bortezomib on day 4 after bile duct ligation. Despite NF-κB inhibition by bortezomib, liver injury and hepatocyte apoptosis were reduced in treated BDL mice. The antiapoptotic effect of bortezomib was likely mediated by an increase in hepatic cellular FLICE inhibitory protein (c-FLIP) levels, a potent antiapoptotic protein. Unexpectedly, numerous mitotic hepatocytes were observed in the bortezomib-treated BDL mice liver specimens. Consistent with this observation, PCNA immunoreactivity and cyclin A protein expression were also increased with bortezomib treatment. Bortezomib therapy was also associated with a decrease in numbers and activation of Kupffer cells/macrophages. In conclusion, these data suggest that the proteasome inhibitor bortezomib reduces hepatocyte injury in the BDL mouse by mechanisms associated with a reduction in hepatocyte apoptosis, a decrease in Kupffer cell/macrophage number and activation, and increased hepatocyte proliferation.

bortezomib; cellular FLICE inhibitory protein; cholestasis; hepatocyte proliferation; Kupffer cells.

CHOLESTASIS, defined as an impairment in bile formation, is a common occurrence in human liver diseases and results in progressive liver injury culminating in cirrhosis and liver failure. Unfortunately, the therapeutic options for treating this syndrome remain limited, in part, because the essential mechanisms mediating cholestatic liver injury remain incompletely understood. The cellular complexity of liver injury in cholestatic syndromes is highlighted by the role of the transcription factor NF-κB. Elevated concentrations of bile acids within the hepatocyte during cholestasis activate NF-κB (28). NF-κB induces cytoprotective gene expression in hepatocytes attenuating liver injury (21). The innate immune system also contributes to cholestatic liver injury with Kupffer cell activation and neutrophil infiltration (5, 17). Kupffer cell activation occurs by a NF-κB-dependent process (30, 36); in this context, NF-κB activation, by promoting expression of death ligands by Kupffer cells (i.e., Fas ligand and TNF-α), is cytotoxic to the liver (8, 30). Finally, stellate cell activation is responsible for the fibrosis that occurs in cholestasis (7). NF-κB activation is a pivotal feature of stellate cell activation (19), and inhibition of NF-κB activation in stellate cells induces their apoptosis, diminishing hepatic fibrosis (2, 10). Thus the global impact of inhibiting NF-κB pharmacologically on cholestatic liver injury remains unclear. On one hand, blocking NF-κB activation maybe expected to exacerbate liver injury by promoting hepatocyte damage. On the other hand, by inhibiting Kupffer cell and stellate cell activation, blocking NF-κB activation would be expected to reduce liver injury.

NF-κB is normally complexed to an endogenous inhibitor protein I-κB in the cytosol (21). NF-κB-activating stimuli result in phosphorylation of I-κB, leading to its dissociation from NF-κB and degradation by the proteasome (21). Freed from the inhibitor protein, NF-κB translocates to the nucleus where it functions as a potent transcription factor. Proteasome inhibition prevents I-κB degradation, thereby keeping NF-κB complexed to this inhibitory protein despite its phosphorylation (35). Thus proteasome inhibitors, which are already available in clinical practice, are potential drugs to modulate NF-κB activation in liver diseases. Indeed, we have recently demonstrated (2) that proteasome inhibitors will attenuate hepatic fibrogenesis in the bile duct-ligated (BDL) mouse by blocking NF-κB activation in stellate cells. However, the composite effect of inhibiting NF-κB on liver injury was not assessed in these studies, in particular, the effects on hepatocyte injury and Kupffer cell activation.

The overall objective of this study was to examine the effect of proteasome inhibition on liver injury in the BDL mouse. To address this objective, we formulated two questions, 1) What is the effect of proteasome inhibition on indices of hepatocytes injury, and 2) What is the effect of proteasome inhibition on Kupffer cell/macrophage activation. The results indicate that the proteasome inhibitor bortezomib reduces hepatocyte injury in the BDL mouse. Not only did this proteasome inhibitor reduce markers of Kupffer cell activation, but it also unexpectedly enhanced hepatocyte proliferation. Proteasome inhibition appears to be an attractive therapeutic strategy for cholestatic liver diseases.

MATERIALS AND METHODS

BDL mouse model of liver fibrosis. The use and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. C57/BL6 mice, 6–8 wk of age, were used for these studies. Common bile duct ligation was performed as described by us in detail previously (29). Bortezomib (Mayo Clinic Formulary, Rochester, MN) was administered at 0.1
mg/kg body wt by intraperitoneal injection on day 4 after BDL. Bortezomib was dissolved in saline as a vehicle, and control animals received saline alone. On day 7 after BDL, animals were killed, and serum and liver samples were removed for analysis.

**Serum assays.** Under deep anesthesia, blood samples were collected after 7 days of BDL. Serum bilirubin and alanine aminotransferase (ALT) determinations were performed using commercially available assay kits following the manufacturer’s instructions (Sigma Diagnostics kit; Sigma-Aldrich, St. Louis, MO). Serum TNF-α levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN).

**Histological analysis and TUNEL assay.** For histological review of hematoxylin and eosin (H&E)-stained liver sections, the liver was diced into 5 × 5-mm sections, fixed in 4% paraformaldehyde for 48 h, and then embedded in paraffin (Curtin Matheson Scientific, Houston, TX). Tissue sections (4 μm) were prepared with a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. H&E staining was performed according to standard techniques. Terminal deoxynucleotidyl transferase (TUNEL) assay was performed following the manufacturer’s instructions (in situ cell death detection kit; Boehringer Mannheim, Indianapolis, IN). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 30 random microscopic fields (×400).

**Immunohistochemistry and bile infarct area quantitation.** Immunolabeling was performed using formalin-fixed paraffin-embedded blocks (5-μm thick). Freshly cut sections were deparaffinized in xylene and rehydrated through sequentially graded ethanol steps. Antigen retrieval was performed by incubating the slides in 1 mmol/l EDTA (pH 8.0) using a household vegetable steamer for 60 min. For detection of CD68 and PCNA immunoreactivity, slides were incubated with proteinase K (20 μg/ml) for 10 min. Slides were next incubated in 3% hydrogen peroxide for 10 min at room temperature and then incubated in blocking buffer (5% normal goat serum in PBS-0.05% Tween) for 30 min, also at room temperature. The samples were next incubated in blocking buffer containing the desired dilution of antisera overnight at 4°C. Primary antibody recognizing CD68 was obtained from DAKO (MO-876; Carpinteria, CA), and the NF-κB (sc-372) and the PCNA antibodies (sc-7907) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dilutions for the primary antibodies were as follows: mouse MAb for CD68 (1:400), mouse MAb for NF-κB (1:200), and rabbit polyclonal antibody for PCNA (1:400). Negative control slides were incubated with nonimmune immunoglobulins under the same conditions. After washing three times with PBS-0.05% Tween, the slides were incubated with the peroxidase-conjugated secondary antibody (1:1,000) that was obtained from DAKO Envision system horseradish peroxidase (HRP) (diaminobenzidine, DAB) kit for 30 min at room temperature. Peroxidase activity was detected by incubating the samples with 3,3’-DAB as the chromogen. Slides subsequently were counterstained with hematoxylin. To accurately quantitate immunostained positive cells, slides were viewed by digital microscopy (Axioplan 2; Zeiss, Oberkochen, Germany). Digital pictures were captured through a video archival system using a digital TV camera system (Axiocam High Resolution color, Zeiss). With an automated software analysis program (KS400, Zeiss), the percent immunostained/field area of digital photomicrographs were quantitated (38). H&E-stained slides of liver tissue specimens from BDL mice with or without Bortezomib treatment were analyzed for bile infarcts. Bile-infarcted areas (the percent infarct/field area) were also quantitated using digital image analysis technology.

**Immunoblot analysis.** Cellular extracts of whole mouse liver were prepared and Western blot analysis was performed as described previously (1, 12). For Western blot analysis, 50 μg of protein extract per lane was added to an equal volume of 2× sample buffer and was size-fractionated on 12 or 15% SDS-PAGE, transferred to nitrocellulose, and detected using the ECL chemiluminescence kit (Amersham, Arlington Heights, IL). The following antibodies were used: monoclonal anti-mouse cyclin D1 (sc450) and polyclonal antibodies to cyclin A (sc596) and polyclonal rabbit anti-Mcl-1 from Santa Cruz Biotechnology; monoclonal antibodies to c-FLIP (Dave-2) from Alexis (San Diego, CA); and polyclonal mouse anti-Bcl-xL from ExalPHA Biologicals (Maynard, CA).

**NF-κB DNA binding activity.** The DNA binding activity of NF-κB in BDL mouse liver was quantified by an ELISA assay using the trans-AM NF-κB p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. Briefly, nuclear extracts from mice liver were prepared by using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL). The protein content in the two fractions was quantitated by the Bradford assay. Nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide (5’-AGTTAGGGAGCTTTCCCAGGC-3’) containing a consensus (5’-GGGACCTTCCC-3’) binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antisera specific for the activated form of p65. The ELISA assay was developed by employing an anti-IgG horseradish peroxidase conjugate and a developing solution provided by the kit. The optical density was determined at 450 nm. Background binding was subtracted from the value obtained for binding to the consensus DNA sequence.

**EMSA.** EMSA was performed using nuclear extracts prepared with the NE-PER kit (Pierce Biotechnology, Rockford, IL). Protein was quantitated (Bradford), and 5 μg nuclear extract was used per reaction. Binding reactions were incubated at room temperature for 30 min with 0.5 μl of double-stranded oligonucleotide with the NF-κB target sequence (26), which was labeled with the infrared fluorophore IRDye700 (Li-cor Biosciences, Lincoln, NE). Cold competitor was the same double-stranded sequence without label (Mayo DNA Synthesis Core Facility, Rochester, MN). Samples were separated on 5% polyacrylamide gels in 1× Tris-Borate EDTA buffer (130 mM Tris, pH 8.3, 4.5 mM boric acid, 2.5 mM EDTA). Imaging was performed in the gel cassette on the Odyssey infrared imaging system (Li-cor Biosciences). An EMSA performed in this manner yields a gel with an appearance dissimilar from an EMSA performed with radioactivity. The precast acrylamide gels have well markers attached to the glass, and these are apparent in the figure as vertical lines outlining the well. Also, as the oligonucleotide is labeled with a fluorescent probe, there is a moeity available for nonspecific binding to cellular proteins or large complexes.

**Kupffer cell isolation.** Kupffer cells were isolated from BDL and sham-operated mice. Kupffer cells were isolated by in situ digestion and cell separation on a discontinuous gradient of arabinogalactan (Larcoll, Sigma), followed by selective adherence on glass cover-slips (33) and Friedman and Roll (13). The viability of isolated Kupffer cells was >90% as assessed by trypan blue exclusion. Cell lysates from Kupffer cells were obtained after 24 h of plating. Kupffer cells were cultured in DMEM (GIBCO-Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 100 mmol/l insulin.

**RT-PCR.** Total RNA was obtained from whole liver or primary Kupffer cells using the TRIzol reagent (Invitrogen). For each RNA sample, a 10-μg aliquot was reverse transcribed into cDNA using oligo-dT random primers and Moloney Murine Leukemia virus reverse transcriptase. RT-PCR was performed using Taq polymerase (Invitrogen) as described by us previously (6). Primers used were as follows: A1, forward 5’-CCT GGA TGA CTA CAT CTA TTT TG-3’ (product size, 240 bp); c-FLIP, forward 5’-ACT GCA CAA CTC ACC GAG AA-3’, reverse 5’-CCA CTA TTC CAG GAC ACC AA-3’ (product size, 193 bp); TNF-α, forward 5’-AGT CCG GCC AGG TCT ACT TT-3’, reverse 5’-GTT GAC TGT CCC AGG ATC TT-3’ (product size, 232 bp); Fas-L, forward 5’-TTG GCC TAC CGG TGG TAT TT-3’, reverse 5’-CAC ACT CCT CGG CTC TTT TT-3’ (product size, 197 bp).
bp); inducible type nitric oxide synthase (iNOS), forward 5'-CAGCT-GGCGTGCAAAACCT-3', reverse; 5'-CATTTGGAATGGAACGGTTTCG-3'. 18S primers (Ambion, Austin, TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis. RT-PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular Probes). The results were expressed as a ratio of product copies per milliliter to copies per milliliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Data analysis. All data represent at least 3 independent experiments and are expressed as means ± SE unless otherwise indicated. Differences between groups were compared using two-way ANOVA and a post hoc Bonferroni test to correct for multiple comparisons.

RESULTS

Bortezomib blocks hepatocyte NF-κB activation in BDL mice. To examine the effects of bortezomib on hepatocytes in cholestasis, sham-operated and 7-day BDL mice were treated with or without bortezomib on day 4 after the initial surgical procedure. On day 7 after surgery, the animals were killed. Immunohistochemistry examination of liver specimens demonstrated NF-κB p65 subunit immunoreactivity predominantly in nuclei of hepatocytes following BDL. In contrast, immunoreactivity for NF-κB was excluded from the nucleus and localized to the cytoplasm in sham-operated animals, animals treated with bortezomib, and in BDL mice receiving bortezomib (Fig. 1A). In experiments complimentary to the p65 NF-κB immunohistochemistry, we also examined p65 NF-κB binding activity using a commercial ELISA-like assay. p65 NF-κB binding activity in nuclear extracts was significantly reduced in bortezomib-treated BDL mice compared with nuclear extracts from untreated BDL animals (P < 0.01) (Fig. 1B). We also assessed NF-κB activation in the BDL mouse liver by EMSA (Fig. 1C). The EMSA demonstrated that the nuclear protein extracts from the livers of BDL mice contained NF-κB capable of binding a fluorescent oligonucleotide, including the NF-κB consensus binding motif. Nuclear extracts from sham-operated mice or bortezomib-treated BDL mice did not bind the NF-κB consensus binding motif in this EMSA. To further assess the ability of this route, dose and timing of bortezomib administration to inhibit NF-κB activation, we

Fig. 1. Bortezomib (Bort.) blocks hepatocyte NF-κB activation in bile duct-ligated (BDL) mice. Immunostaining for NF-κB on liver tissue from sham and BDL mice was performed. A: immunoreactivity for NF-κB was observed in nuclei of hepatocytes in the untreated BDL mice (indicated by arrows). In contrast, immunoreactivity for NF-κB was observed in the cytoplasm in sham-operated animals, animals treated with bortezomib, and in BDL mice receiving bortezomib (Fig. 1A). B: as assessed by ELISA, p65 NF-κB binding activity using a commercial ELISA-like assay. p65 NF-κB binding activity in nuclear extracts was significantly reduced in bortezomib-treated BDL mice compared with nuclear extracts from untreated BDL animals (P < 0.01). C: NF-κB activation was assessed by EMSA. Nuclear extract (5 μg) was incubated with labeled NF-κB probe and resolved on a 5% polyacrylamide gel. Animals were sham-operated, BDL, or BDL and treated with bortezomib (BDL + bortezomib). Control experiments (not shown) confirmed binding specificity by the loss of binding in the presence of a 100-fold molar excess of unlabeled oligonucleotide. Total RNA was extracted from whole liver, and inducible-type nitric oxide synthase (iNOS) (D) and A1 (E) mRNA expression was quantitated by RT-PCR. The expression was normalized as a ratio using 18s mRNA as a housekeeping gene. A value of 1 for this ratio was arbitrarily assigned to the data obtained from sham-operated mice. iNOS and A1 expression was decreased in bortezomib-treated BDL mice compared with control BDL mice (P < 0.03 for iNOS; P < 0.05 for A1; n = 5 for each group).
measured the transcripts of two NF-κB target genes, iNOS and the antiapoptotic protein A1 (Fig. 1, D and E). Consistent with the immunohistochemistry, ELISA and EMSA analysis, iNOS and A1 mRNA expression were significantly reduced in bortezomib-treated mice compared with untreated BDL mice (\(P < 0.03\) for iNOS, \(P < 0.05\) for A1). Thus this route, dose, and timing of bortezomib administration were sufficient to at least partially block hepatic NF-κB activation.

Fig. 2. Liver injury is reduced in bortezomib-treated BDL mice. Seven days after the surgical procedure, blood samples and liver tissues were obtained from all mice. Serum alanine aminotransferase (ALT) values were examined as described in MATERIALS AND METHODS. A: ALT values were significantly lower in bortezomib-treated BDL mice than control BDL mice (\(\# P < 0.05\), \(n = 3\) for each experimental group). B: fixed liver specimens were analyzed by terminal dUTP nick-end labeling (TUNEL) assay to identify apoptotic hepatocyte. The number of TUNEL-positive hepatocytes were significantly lower in bortezomib-treated BDL mice than in control BDL mice (\(* P < 0.01\), \(n = 3\) for each group). C and D: fixed liver specimens from all mice were prepared for hematoxylin and eosin (H&E) staining. Bile infarcts were assessed by digital image analysis. Numerous bile infarcts were present in BDL mice (indicated by arrows). However, bortezomib treatment significantly reduced bile infarcts in the BDL mice (\(\# P < 0.05\), \(n = 3\) for each group). E: serum bilirubin levels were not significantly different between treated and untreated BDL mice.
Bortezomib attenuates liver injury following BDL. Liver injury was examined by measuring serum ALT concentrations. Serum ALT values were markedly increased in BDL mice as anticipated (Fig. 2A). However, serum ALT values were significantly reduced in bortezomib-treated BDL mice compared with untreated BDL mice (328 ± 129 vs. 898 ± 400 U/L, respectively, P < 0.05). Furthermore, bortezomib treatment following BDL reduced the number of TUNEL-positive hepatocyte compared with untreated BDL mice (5.6 ± 1.2 and 23.4 ± 4.7, respectively, P < 0.01) (Fig. 2B). Bile infarcts are confluent foci of hepatocyte feathery degeneration due to bile acid cytotoxicity and are a prominent feature of liver injury in the BDL mouse. Bile infarcts in the liver were assessed by conventional H&E staining and quantitated using digital image analysis. Histopathological examination of liver specimens demonstrated numerous bile infarcts in untreated BDL mice. However, bortezomib treatment significantly reduced bile infarcts in the BDL mice (Fig. 2, C and D). The reduction in liver injury by bortezomib could not be explained by a change in cholestasis, as serum bilirubin values were not reduced by bortezomib treatment (Fig. 2E). These data suggest that bortezomib attenuates hepatic injury in the BDL mice.

Recently, Chang et al. (9) have demonstrated that proteasome inhibition blocks liver injury by concanavalin A by enhancing cellular c-FLIP levels, a potent inhibitor of death receptor signaling. Indeed, c-FLIP was cytoprotective in these studies despite NF-kB inhibition. Based on these very recent observations, we also examined c-FLIP expression in BDL mice. Indeed, bortezomib markedly enhanced hepatic c-FLIP protein levels in this model (Fig. 3A). Consistent with the effect of a posttranslational effect of bortezomib as c-FLIP protein regulation, RT-PCR analysis demonstrated no change in liver c-FLIP mRNA in treated vs. untreated BDL mice (data not shown). Protein levels of Bcl-xL and Mcl-1 (Fig. 3, B and C) were also analyzed to assess the effects of bortezomib on other prominent antiapoptotic proteins of the liver, as murine hepatocytes do not express Bcl-2 (25). There was no change in hepatic Bcl-xL or Mcl-1 protein levels in bortezomib-treated vs. untreated BDL mice. These data support the concept that enhanced c-FLIP protein levels in bortezomib-treated animals may afford hepatic cytoprotective properties despite NF-kB inhibition.

Bortezomib induces hepatocyte proliferation in the BDL mice. Unexpectedly, examination of the liver histopathology specimens revealed numerous hepatocyte mitotic figures in bortezomib-treated BDL mice (Fig. 4A). Sham-operated and
bortezomib alone-treated animals had 0–1 mitotic figures/high power field (hpf), bortezomib-untreated BDL had 0–2 figures/hpf, and bortezomib-treated BDL animals had 7–9 figures/hpf. To further examine the possibility that bortezomib enhances hepatocyte proliferation following BDL, we assessed hepatocyte PCNA expression in liver specimens and cyclin A and D protein expression in hepatic protein extracts. PCNA-positive nuclei were assessed by immunohistochemistry and quantitated by using digital image analysis technology. PCNA-positive nuclei were increased in bortezomib-treated BDL mice compared with control BDL mice (P < 0.05) (Fig. 4B). Cyclin A, but not cyclin D, expression was increased in bortezomib-treated BDL mice compared with control BDL mice (Fig. 4C). Taken together, these findings suggest that bortezomib administration to the BDL mouse enhances hepatocytes proliferation.

Bortezomib reduces macrophage number and activation in the BDL mice. Kupffer cells, resident macrophages of the liver, and likely additional macrophage recruited to the liver, contribute to cholestatic liver injury (22, 33, 37). To investigate whether bortezomib reduces hepatic macrophage number in cholestatic liver injury, hepatic CD68 expression, a macrophage marker, was quantitated using digital image analysis technology. CD68 immunoreactivity was increased 14-fold in BDL mice compared with sham-operated mice (5.6 ± 2.33 and 0.4 ± 0.05, respectively), indicating either proliferation of resident Kupffer cells or recruitment of circulating macrophages to the liver. In bortezomib-treated BDL mice, in contrast, CD68 immunoreactivity was reduced by greater than 50% (5.6 ± 2.33 and 2.1 ± 0.63, respectively, P < 0.05) (Fig. 5, A and B). As Kupffer cells and likely recruited macrophages are a major source of proinflammatory cytokines and chemokines during liver inflammation (3), serum TNF-α levels were determined by an ELISA assay. Circulating serum TNF-α levels were found to be decreased in bortezomib-treated mice compared with BDL-untreated mice (30.59 ± 4.47 vs. 78.79 ± 21.14 pg/ml, respectively, P < 0.05) (Fig. 6A). To more directly assess hepatic macrophage activity, the liver cell populations were isolated from livers, and the mRNA was extracted (Fig. 6, B and C). Indeed, hepatic macrophage and TNF-α and Fas-L mRNA expression was also reduced in bortezomib-treated and -untreated animals (Fig. 6, B and C). Taken together, these data suggest that bortezomib therapy is associated with reduced hepatic macrophage activity in BDL mice.

DISCUSSION

The principal findings of this study relate to the potential therapeutic and/or deleterious effects of proteasome inhibition on cholestatic liver injury. We have previously demonstrated that proteasome inhibition attenuates fibrosis in this model by inducing stellate cell apoptosis. Therefore, this study focused on the effects of proteasome inhibition in hepatocytes and Kupffer cells/hepatic macrophages. The data indicate that administration of the proteasome inhibitor bortezomib to BDL mice 1) reduces hepatocyte apoptosis and liver injury, 2) enhances hepatocyte proliferation, and 3) reduces hepatic macrophage number and activation. These data suggest that proteasome inhibition may be a new and unexpected therapeutic strategy for the treatment of cholestatic syndromes.

NF-κB is activated in BDL mice (4), presumably by bile acids (32), and hepatoocyte-specific NF-κB inhibition exacerbates cholestatic liver injury in this model (28). Unexpectedly, proteasome inhibition, which prevents NF-κB activation, actually reduced cholestatic liver injury in the current studies. Indeed, treatment of BDL mice with bortezomib reduced hepatocyte apoptosis and all indices of hepatic damage examined (Figs. 1 and 2). Chang et al. (9) have recently demonstrated that proteasome inhibition also reduces liver injury by concanavalin A. In their studies, proteasome inhibition pre-

![Fig. 5. The number of Kupffer cells is reduced by bortezomib treatment in BDL mice. Seven days after the surgical procedure, liver tissues were obtained from mice and fixed in 4% paraformaldehyde. A: immunohistochemistry for CD68 was performed as described in MATERIALS AND METHODS. B: the percentage of CD68-positive area in the liver section was quantitated using digital image analysis. Immunoreactivity for CD68 was significantly reduced in bortezomib-treated BDL mice compared with control BDL mice (#P < 0.05, n = 3 for each group).](image)
Proteasome inhibition may be a pharmacological approach to overcoming the downstream inhibition of cyclin D. The accumulation of cyclin A in bortezomib-treated animals is also consistent with the known degradation of cyclins by the proteasome (14, 15). This potential role of cyclin A in stimulating hepatocyte proliferation in the presence of bile acids will require further study.

We have previously demonstrated a role for hepatic macrophages in hepatic injury following BDL in the mouse. Kupffer cells and likely recently recruited hepatic macrophages to the liver are “activated” in the BDL mouse and express death ligands such as TNF-α and Fas ligand (5). NF-κB is a potent activator of macrophages (31), and NF-κB activation is blocked by proteasome inhibitors (20). Therefore, the ability of bortezomib to block activation of hepatic macrophages is consistent with its pharmacological effects. Less well-understood is the reduction in hepatic macrophage numbers during bortezomib treatment of the BDL mouse. There are several potential explanations for this observation. First, bortezomib inhibition of hepatic damage may simply reduce their recruitment into the liver by inhibiting chemokine generation. Second, bortezomib, as a chemotherapeutic agent, may also simply inhibit proliferation of hepatic resident macrophages or Kupffer cells by blocking NF-κB activation. Finally, by blocking macrophage NF-κB activation, bortezomib may block production of the cytoprotective antiapoptotic molecule A1, and loss of A1 is sufficient to induce macrophage apoptosis (31). However, our TUNEL assay did not identify a large number of TUNEL-positive sinusoidal lining cells to support this possibility. Additional studies will be necessary to identify the mechanism(s) by which proteasome inhibition reduces hepatic macrophage numbers in obstructive cholestasis.

The mechanisms of liver injury in obstructive cholestasis are complex. Initially, there is an accumulation and retention of toxic bile acids in the liver due to inhibition of their transport into bile (16, 34). These toxic bile acids induce hepatocyes injury by triggering Fas-mediated injury (11). Hepatocyte injury then results in activation of the innate immune response with accumulation of activated macrophages and neutrophils in the liver (27). The injurious processes stimulate stellate cell activation and fibrosis of the liver. Proteasome inhibition with bortezomib appears to reduce all of these events by inhibiting hepatocytes apoptosis by a processes including but not limited to an associated increase in c-FLIP and reduction of activated hepatic macrophages and stellate cells. Whether these are all distinct effects of bortezomib or are interrelated is unclear from the current studies. Collectively, these observations suggest proteasome inhibitors warrant further investigation as hepatic protective agents in cholestasis.

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