Bortezomib Inhibits Hepatitis B Virus Replication in Transgenic Mice

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Pharmacological modulation of cellular proteins as a means to block virus replication has been proposed as an alternative antiviral strategy that may be less susceptible than others to the development of viral drug resistance. Recent evidence indicates that the ubiquitin-proteasome pathway interacts with different aspects of the hepatitis B virus (HBV) life cycle in cell culture models of virus replication. We therefore examined the effect of proteasome inhibition on HBV replication in vivo using HBV transgenic mice. The proteasome inhibitor bortezomib (Velcade) inhibits proteasome activity in vivo and is used therapeutically for the clinical treatment of multiple myeloma. We found that a single intravenous dose of 1 mg of bortezomib/kg of body weight reduced virus replication for as long as 6 days. The inhibition of HBV by bortezomib was dose dependent and occurred at a step in replication subsequent to viral RNA and protein expression. The reduction in HBV replication did not result from nonspecific hepatocellular toxicity and was not mediated indirectly through the induction of an intrahepatic interferon response. Thus, pharmacological manipulation of the ubiquitin-proteasome pathway may represent an alternative therapeutic approach for the treatment of chronic HBV infection.

Despite the availability of an effective vaccine, more than 350 million people worldwide are chronically infected with hepatitis B virus (HBV), and many of them develop serious liver diseases, such as cirrhosis and hepatocellular carcinoma. Several nucleoside or nucleotide analogs are currently approved for the treatment of chronic HBV infection; these interact with the viral polymerase (Pol) and act as competitive substrate inhibitors to block reverse transcription of the pregenomic viral RNA to DNA (reviewed in reference 5). However, Pol inhibitors, such as lamivudine, can be rendered ineffective due to resistance mutations arising within Pol that are subsequently selected for during antiviral treatment (2). One approach to potentially circumvent antiviral resistance is combination therapy with agents that target viral proteins and the cellular proteasome pathway may represent an alternative therapeutic approach for the treatment of chronic HBV infection.

Like other viruses, HBV interacts with the ubiquitin-proteasome pathway in multiple ways during its replication cycle. The HBx protein binds to a number of proteasome subunits, including XAPC7, PSMA7, and PSMC1 (14, 15, 35, 47), and this interaction has functional consequences for proteasome function and HBV replication. HBx decreases the chymotryptic and trypsin-like activities of the proteasome, resulting in reduced rates of degradation of ubiquitinated proteins (14). By binding to the α4/αMC6 proteasome subunit, HBx inhibits the activation of the proteasome by the interferon (IFN)-induced regulatory subunit PA28 (35). The interaction of HBx with the proteasome may be functionally important for virus replication, since the replication defect of an HBx-deficient HBV mutant is suppressed by proteasome inhibition in cell culture (46). We have also recently found that the IFN-regulated proteasome catalytic subunits can influence the specificity of the HBV-specific CD8 T-cell response (30) and that depletion of free cellular ubiquitin through proteasome inhibition blocks HBV release in cell culture (7).

To study the role of proteasome activity in HBV replication using a more physiological model, we treated 1.3-genomelength HBV transgenic mice with a proteasome inhibitor that...
has been well characterized previously with respect to its activity in vivo (1, 16, 20). Bortezomib (Velcade) is a potent proteasome inhibitor that is currently used as a therapy for patients with multiple myeloma (29). Surprisingly, we found that a low dose of bortezomib rapidly reduced HBV replication in the mice. Therefore, inhibition of proteasome activity may represent an alternative or complementary therapeutic approach for chronic HBV infection.

**MATERIALS AND METHODS**

**Animals.** HBV transgenic mice (strain 1.3.32) have been described previously (8, 9). These animals encode a 1.3-overlength copy of the HBV genome (serotype ayw) and reproduce the virus replication cycle from gene expression through virion release. Groups of mice in all experiments were matched for age (8 to 12 weeks), sex, and HBcAg levels as determined by an enzyme-linked immunosorbent assay (ELISA) (International Immunodiagnostics, Foster City, CA; Abbott Laboratories, Abbott Park, IL). All animals were housed in specific-pathogen-free rooms under strict barrier conditions. Mice were injected intravenously (i.v.) with the indicated dose of bortezomib diluted in 200 μL of 0.9% NaCl containing 10 mg/mL mannitol. Control animals received the same volume of diluent without any drug. All procedures were performed in accordance with the Animal Care and Use guidelines of The Scripps Research Institute and Yale University.

**Intracellular HBV RNA analysis.** Total-RNA preparation, tissue was homogenized in GTC solution (4.2 M guanine phosphate, 25 mM sodium citrate [pH 7.3], 0.5% sarcosyl) containing 100 mM β-mercaptoethanol (4). The pH of the lysate was adjusted to 4.0 with 0.1 volume 2 M sodium acetate (pH 4.0); the lysates were extracted twice with a 2:1 mixture of phenol (pH 4.0)-chloroform and chloroform. The DNA was then precipitated with an equal volume of isopropanol and washed in 80% ethanol. For Southern hybridization, 20 μg of DNA was digested with HindIII, separated by agarose gel (1.4%) electrophoresis, and transferred to a nylon membrane utilizing standard protocols. Membranes were hybridized with a 32P-labeled DNA probe consisting of the entire HBV (strain ayw) genome.

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**Real-time reverse-transcription PCR (RT-PCR).** One microgram of total RNA prepared from snap-frozen mouse liver tissue was reverse transcribed with random hexamers using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Real-time PCR was performed using Applied Biosystems 7500 real-time PCR system as follows. Reaction mixtures contained 100 ng reverse-transcribed RNA, 12.5 μL SYBR green reaction mix (Applied Biosystems), and 200 nM sense and antisense primers in a final volume of 25 μL. The primers used for PCR were as follows: GAPDH, 5'-TCT GGA GAG CTC TGC CGT GAT G'-3' (sense) and 5'-CCA GTG AGC TTC CCG TTC AG-3' (antisense); ISG15, 5'-CAA TGG CCT GGG ACC TAA A'-3' (sense) and 5'-CTT CTT CAG TCG TCA CAG CTG CAT-3' (antisense); Usp18, 5'-CTC CGT CTT GTG TAG ACT CT-3' (sense) and 5'-GGGA TCT GCC GGC GAG AGG ACT A'-3' (antisense); TGF-β, 5'-AGC ACA AGC GTC A-3' (sense) and 5'-GAA AAT GTT GGC TAA TGC TCC-3' (antisense). After an initial incubation at 95°C for 5 min, PCR amplification was performed by cycling 50 times for 1 min at 95°C, followed by 1 min at 60°C. Relative gene expression was quantified by the ΔΔCt method using the 7500 System Sequence Detection software (Applied Biosystems).

**RNease protection assay.** The RNease protection assay for intrahepatic cytokine expression was performed exactly as described previously (8, 11).

**HBcAg.** Liver tissue was homogenized in 2× SDS sample buffer, and expression of liver HBV core antigen (HBcAg) was determined by Western blotting using an HBcAg-specific antibody (Dako, Carpinteria, CA). Protein loading was controlled by immunoblotting the same membrane with an antibody specific for β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Serum HBV DNA analysis.** Mouse serum (5 μl) was diluted 1:100 in TNE buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA) and applied to a nitrocellulose membrane using a Hybrid-Dot manifold (BedaL Research Labs, Inc.). The membrane was denatured in 0.2 M NaOH–1.5 M NaCl for 10 min and then neutralized in 0.2 M Tris–HCl–1.5 M NaCl for 5 min. Nuclease acids were cross-linked on the membrane by UV irradiation and were hybridized with a radiolabeled HBV DNA probe as described above. Virion-associated DNA was visualized with a phosphorimager (FujiFilm, Tokyo, Japan) and quantified with ImageGauge software, version 4.22 (FujiFilm).

**Histology.** Liver tissue was fixed in 10% zinc-buffered formalin, processed by routine methods, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections of liver were evaluated for expression of HBV protein using a NeoMarkers HBcAg-specific monoclonal antibody (1:300; LabVision, Fremont, CA) and the chromogen diaminobenzidine tetrahydrochloride by routine immunohistochemistry methods (Research Histology, Department of Pathology, Yale University School of Medicine).

**Proteasome activity assay.** Proteasomes were purified from homogenized mouse liver tissue, and activity was measured using the fluorogenic protease substrate N-succinyl-Leu-Leu-Val-Tyr-amino-4-methylcoumarin as previously described (30).

**Statistical analysis.** Unless otherwise noted in the figure legends, experiments were performed using groups of four mice. This sample size is sufficient to detect an 80% reduction in HBV DNA levels using Student’s t test with a significance level (alpha) of 0.05 and a power (1 – β) of 0.80, assuming standard deviations equivalent to 50% of the mean data levels (22). A two-sample Student t test using the two-tailed distribution was used to determine significant differences in serum HBV DNA and antigen levels. P values of <0.05 were considered statistically significant.

**RESULTS**

**Bortezomib inhibits HBV replication in transgenic mice.** A single dose of 1 mg/kg bortezomib inhibits proteasome activity in mice by 80 to 90% in multiple organs, including the liver (1, 16, 20). This dose of bortezomib was shown to inhibit proteasome activity maximally 6 h after injection, with activity returning to baseline levels approximately 40 h later (20). Because the activity of the drug may differ in different mouse strains, we first confirmed that this dose of bortezomib also inhibited proteasome activity in the livers of HBV transgenic mice (Fig. 1A). We then injected HBV 1.3.32 transgenic mice i.v. with a single dose of saline or 1 mg/kg bortezomib, and we monitored HBV replication by Southern blot analysis of liver HBV DNA replication intermediates 1 day after administration of the drug. Prior to injection, the groups of mice were matched for serum HBcAg levels such that no significant difference in expression was noted between the two groups (Fig. 1B). In contrast to serum HBcAg expression prior to injection, we found significant reductions in levels of HBV DNA replication intermediates in the liver 1 day following bortezomib administration (Fig. 1C).

**Time course and dose response of HBV inhibition.** We next monitored HBV replication at 1, 2, 3, and 6 days after administration of bortezomib. Levels of HBV DNA replication intermediates in the liver were reduced within 24 h after injection of the drug and remained suppressed for at least the next 2 to 3 days before returning to near-baseline levels by day 6 (Fig. 2A). However, there was no significant change in the level
of HBV 3.5- and 2.1-kb mRNA expression compared to that of GAPDH or the steady-state level of total HBcAg protein in the liver (Fig. 2A). Under these conditions, we also observed only transient mild liver damage as measured by sALT levels, which peaked at 180 U/liter at day 2 postinjection and returned to normal levels by day 6 (Fig. 2A). Therefore, bortezomib inhibits HBV replication by a mechanism that does not reduce HBV gene expression or result in the destruction of a large number of hepatocytes.

We then determined the dose of bortezomib required to inhibit HBV replication in the transgenic mice. As we found in the preceding time course experiment, levels of HBV DNA replication intermediates in the liver were reduced 24 h after a single i.v. injection of 1 mg/kg bortezomib (Fig. 2B). However, virus replication was no longer blocked when a 5-fold-lower dose (0.2 mg/kg) was administered. We also observed no re-
duction in the level of HBV replication when a 5-fold-higher dose was used (5 mg/kg) but instead found a slight increase in the level of HBV DNA replication intermediates that was neither statistically significant \((P = 0.16)\) nor reproducible in a second experiment (data not shown). Again, expression of HBV 3.5- and 2.1-kb mRNAs and the steady-state level of total HBcAg were not significantly changed in the liver at all doses of the drug tested (Fig. 2B). Evidence of mild hepatocellular damage, as measured by sALT activity, was present only at the highest (5-mg/kg) dose of the drug (Fig. 2B). The animals that received 5 mg/kg bortezomib also showed visible signs of stress or illness at this dose, including ruffled fur, labored breathing, and hunched posture (data not shown). These animals were therefore euthanized, and longer treatments at the higher dose were not tested.

**Bortezomib treatment reduces levels of serum HBV DNA but not those of secreted antigens.** To determine if bortezomib administration altered the release of secreted viral antigens, serum HBcAg and HBsAg levels were measured by ELISA. In general, the drug only modestly changed the amounts of these antigens in mice that were assayed 1, 2, 3, and 6 days after injection with 1 mg/kg bortezomib (Fig. 3A) or 24 h after a single dose of 0.2, 1, or 5 mg/kg bortezomib (Fig. 3C). HBcAg levels in animals given 5 mg/kg bortezomib were reduced approximately 50%, and this reduction was statistically significant \((P = 0.04)\). There was also a consistent trend of increased HBsAg levels in mice 24 h after treatment with 1 mg/kg bortezomib, but this difference failed to achieve significance in two independent experiments \((P = 0.23, P = 0.07)\). Thus, consistent with the fact that bortezomib did not change HBV 3.5- and 2.1-kb RNA or HBcAg protein levels in the liver, with some minor exceptions, the production and release of the secreted viral antigens were also generally not significantly affected by the drug.

In contrast to the results for secreted viral antigens, mice given a single dose of 1 mg/kg bortezomib had serum HBV DNA levels that were maximally reduced to 8% of untreated levels at 3 days posttreatment \((P = 0.03)\) (Fig. 3B). There was also a slight increase in HBV DNA levels in the sera of mice treated with 5 mg/kg bortezomib for 24 h (Fig. 3D) that was consistent with the increased levels of liver HBV replication intermediates (Fig. 2B), but this difference also failed to achieve statistical significance \((P = 0.22)\). Therefore, the reduction in serum HBV DNA levels in mice treated with bortezomib correlated with the decrease in levels of HBV DNA replication intermediates in the liver.

**Histological analysis of bortezomib-treated mice.** We also determined if bortezomib treatment induced inflammation or other histological abnormalities in the livers of HBV transgenic mice. Zinc-formalin-fixed liver tissues isolated from representative mice from the time course (Fig. 2A) and dose-response (Fig. 2B) experiments were sectioned and stained with hematoxylin-eosin. At all time points and at all doses of the drug examined, there was no evidence of significant hepatitis or other pathology in the liver (Fig. 4), consistent with the relatively modest ALT elevation in the bortezomib-treated mice. Furthermore, consistent with the similar HBcAg levels measured by Western blotting (Fig. 2), there were no significant differences in the number or distribution of HBcAg-positive hepatocytes between mice injected with saline and mice injected with bortezomib (Fig. 5). Thus, the reduced levels of HBV replication cannot be explained by a large loss of HBV-producing hepatocytes.
Inhibition of HBV replication by bortezomib does not occur through an intrahepatic inflammatory cytokine response. Because IFN-α/β and IFN-γ also inhibit HBV replication without reducing viral mRNA expression or hepatocyte viability in the transgenic mice (8), we next determined if bortezomib administration induced intrahepatic IFN production by examining the expression of IFN-regulated genes in the liver. Quantitative RT-PCR was performed using total RNA isolated from the liver to monitor the expression of three representative IFN-inducible genes: ISG15, Usp18, and TGTP. The expression of ISG15 and Usp18 is preferentially induced by IFN-α/β, while IFN-γ preferentially induces TGTP (38). In mice from the time course experiment (Fig. 2A), the expression of these genes was not induced by bortezomib; instead, their expression levels were transiently decreased 24 h after drug injection (Fig. 6A). In addition, we also examined the intrahepatic expression of other inflammatory cytokines (including tumor necrosis factor alpha [TNF-α], TNF-β, interleukin-1α [IL-1α], IL-1β, and IFN-γ) by an RNase protection assay, and we found that the expression of these genes was not increased by bortezomib administration (Fig. 6B). As observed in the preceding time course analysis, the expression of IFN-stimulated genes also was not induced in the livers of mice from the dose-response experiment but rather was decreased (Fig. 6C). Thus, the bortezomib-mediated inhibition of HBV replication does not appear...
to occur through indirect intrahepatic induction of inflammatory cytokines. This conclusion is consistent with the absence of liver inflammation seen in the histological analysis (Fig. 4).

**DISCUSSION**

We found that bortezomib administration effectively reduced the levels of HBV DNA replication intermediates in the livers of HBV transgenic mice and the levels of HBV DNA in the sera. Furthermore, this block occurred at a step downstream of HBV mRNA and protein expression. The inhibition of HBV replication by bortezomib in the transgenic mice was surprising, since we have previously found that although proteasome inhibition blocks HBV release in cell culture, it does not affect the levels of intracellular DNA replication intermediates (7, 31). Although the reason for the difference between cell culture and mice is unclear, it is possible that the proteasome-sensitive aspect of the HBV replication cycle is more accurately reflected in the liver in vivo. For example, the inhibitory effect may be due to a gene(s) that is expressed in a strictly differentiation specific manner, and therefore this effect may be regulated differently in the liver and in cell culture. Alternatively, the antiviral mechanism may depend on a mediator produced by an extrahepatic tissue, and it would therefore be apparent only in vivo. However, we cannot rule out the possibility that the antiviral effect on HBV is restricted to mice, or is limited to the specific transgenic mouse line (1.3.32) used for these experiments. Additional studies of different HBV transgenic mouse lines, chimpanzees, and/or humans would be needed to determine whether this antiviral activity extends to chronic infections in other organisms.

In addition to measuring HBV DNA levels in the liver and serum, we also assessed the levels of secreted viral antigens (HBeAg, HBsAg) in the serum after bortezomib treatment. Consistent with the fact that we did not observe a change in the expression of the 3.5- and 2.1-kb HBV mRNAs in the liver, we also found little to no change in the levels of serum HBe and HBs antigens at the 1-mg/kg dose. The slight increase in HBsAg levels is consistent with previous results obtained in cell culture (7) and may reflect stabilization of the protein by proteasome inhibition.

We observed that the inhibition of HBV replication was sensitive to the dose of bortezomib administered to the animal. The dose at which we observed the inhibition of HBV (1 mg/kg) has been shown previously to inhibit proteasome activity in multiple mouse tissues (including the liver) by approximately 85% (1, 16, 20). Not surprisingly, a 5-fold-lower dose of bortezomib did not affect baseline levels of HBV replication. Somewhat unexpected was the observation that a 5-fold-higher dose also did not reduce virus replication. There are a number of possible explanations for this result, including systemic and/or local effects of the drug at the higher dose that mask the effects of proteasome inhibition on HBV replication. Alternatively, different levels of proteasome inhibition may differentially affect positive or negative factors for virus replication. A mechanism such as this has precedence in other drugs. For example, celecoxib loses anti-inflammatory activity in rats at high doses, because at high concentrations it induces, rather than inhibits, NF-κB activation (25).

Although the precise mechanism of HBV inhibition is not known at this time, there are a number of possibilities. First, it is possible that the ubiquitin-proteasome pathway influences some aspect of pregenomic RNA (pgRNA) encapsidation that is disrupted by proteasome inhibition. Interestingly, HBV RNA encapsidation and Pol priming require the activity of cellular chaperones (12, 13), and the activity of these proteins is intimately linked with the ubiquitin-proteasome pathway (44). Second, hepadnaviral Pol proteins have a short half-life...
and we have found that HBV Pol expression is enhanced by proteasome inhibition in cell culture (unpublished data). Therefore, proteasome inhibition may perturb the level of functional Pol available to mediate HBV DNA synthesis. Third, it is possible that proteasome inhibition interferes with HBx function, although this may be an unlikely explanation, since 1.32 transgenic mice express low levels of HBx mRNA and contain no detectable levels of HBx protein (9). Finally, it should also be noted that we cannot rule out the possibility that bortezomib inhibits HBV replication through a proteasome-independent mechanism.

Interestingly, IFN-α/β and IFN-γ also potently block HBV replication in transgenic mouse hepatocytes at a similar step in the virus life cycle (36, 37). Thus, one potential explanation for our finding is that bortezomib indirectly inhibits HBV replication by inducing an inflammatory cytokine response in the liver. However, we were unable to detect induction of IFN-α/β- or IFN-γ-activated genes, increased inflammatory cytokine mRNA expression, or inflammation in the livers of bortezomib-treated mice. Therefore, while it is difficult to rule out entirely, inhibition of HBV in vivo by bortezomib does not appear to be due to the induction of an IFN response in the liver.

Several studies have demonstrated that inhibitors of cellular proteins exploited by viral pathogens represent a viable strategy for antiviral therapy. For example, inhibitors of Abl-family tyrosine kinases and the ErbB-1 kinase block poxvirus replication in vivo (28, 39). Similarly, downregulation of the Raf/MEK/extracellular signal-regulated kinase (ERK) signaling cascade with a MEK inhibitor results in impaired propagation of influenza virus (19, 27). Furthermore, α-glucosidase inhibitors exhibit antiviral activity against several enveloped viruses, including HBV and dengue virus (3, 23). We have shown that proteasome inhibition suppresses HBV replication in transgenic mice. Overall, these results support recent evidence of the interaction between the HBV replication cycle and the ubiquitin-proteasome pathway, and they imply that viral or cellular proteins required for replication may be regulated by the proteasome. Our results further suggest the possibility that inhibitors of the proteasome or other proteasome-dependent cellular pathways required for HBV replication may be therapeutically useful in the treatment of chronic infection.


