Inhibition of Cellular Proteasome Activities Mediates HBX-Independent Hepatitis B Virus Replication *In Vivo*[▽]

Zhensheng Zhang,¹ Eun Sun,¹ Jing-hsiung James Ou,² and T. Jake Liang^{1*}

Liver Diseases Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892,¹ and Department of Microbiology and Immunology, University of Southern California, Los Angeles, California 14853²

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The X protein (HBX) of the hepatitis B virus (HBV) is essential for HBV productive infection *in vivo*. Our previous study (Z. Hu, Z. Zhang, E. Doo, O. Coux, A. L. Goldberg, and T. J. Liang, *J. Virol.* 73:7231–7240, 1999) shows that interaction of HBX with the proteasome complex may underlie the pleiotropic functions of HBX. Previously, we demonstrated that HBX affects hepadnaviral replication through a proteasome-dependent pathway in cell culture models. In the present study, we studied the effect of the proteasome inhibitor MLN-273 in two HBV mouse models. We demonstrated that administration of MLN-273 to transgenic mice containing the replication-competent HBV genome with the defective HBX gene substantially enhanced HBV replication, while the compound had a minor effect on wild-type HBV transgenic mice. Similar results were obtained by using C57BL/6 mice infected with recombinant adenoviruses expressing the replicating HBV genome. Our data suggest that HBV replication is subjected to regulation by cellular proteasome and HBX functions through the inhibition of proteasome activities to enhance HBV replication *in vivo*.

Human hepatitis B virus (HBV) infection is a global health problem, and over 350 million people are chronically infected with HBV worldwide (9). HBV is a member of the *Hepadnaviridae* family, which includes the hepatitis viruses of the woodchuck, ground squirrel, tree squirrel, Pekin duck, and heron. HBV has a fourth open reading frame, termed the hepatitis B virus X (HBX) gene. The HBX gene is well conserved among the mammalian hepadnaviruses and codes for a 16.5-kDa protein. The protein can activate the transcription of a variety of viral and cellular genes (1, 6). Since HBX does not bind to DNA directly, its activity is thought to be mediated via protein-protein interactions. HBX has been reported to modulate a wide variety of host functions, and a variety of the interacting partners of HBX have been identified (3, 27). Signaling through calcium has been implicated in mediating the function of HBX in viral replication (4). Recent data suggested that nuclear hormone receptors, especially of the retinoid X receptors (RXRs) and peroxisome proliferator-activated receptors (PPARs), may interact with HBX to mediate the transcription and the replication of HBV (5, 8).

The ubiquitin-proteasome pathway is involved in diverse cellular functions and specifically has been shown to be involved in the life cycle of many viruses. Various viral gene products have been shown to interact with the ubiquitin-proteasome pathway to modulate the cellular environment for the advantage of the viruses (2, 11, 21, 24). We have previously demonstrated that the proteasome complex is a cellular target of HBX, and HBX inhibits the peptidase activities of the proteasome (15, 31). The inhibitory effect of HBX on proteasome was also shown by Stohwasser et al. (26). In the woodchuck

model, we demonstrated that the X-defective mutants of woodchuck hepatitis virus (WHV) are not completely replication defective and behave like attenuated viruses (32). Using recombinant adenoviruses or baculoviruses expressing replicating HBV or WHV genomes with or without a functional X gene, we determined the effects of proteasome inhibitors on the functions of the X protein in hepadnaviral replication and demonstrated that proteasome inhibitors restored the replication defect of X-negative HBV and WHV (30). On the other hand, Garcia et al. recently showed that proteasome inhibition blocks HBV release in cell culture, presumably by depletion of free cellular ubiquitin (10).

In the present study, we tested the effect of a proteasome inhibitor, MLN-273, on HBV replication in the HBV transgenic mice as well as in C57BL/6 mice infected with recombinant HBV adenovirus (ad-HBV). MLN-273 (Millennium Pharmaceuticals, Inc., Cambridge, MA) is a novel, small-molecule dipeptidyl boronic acid proteasome inhibitor with properties similar to those of bortezomib, which has been developed as a chemotherapeutic agent in multiple myeloma and other malignancies (16). MLN-273 has the advantage of a longer half-life than bortezomib and is hence a better therapeutic agent for *in vivo* studies. Recent studies reported that MLN-273 potently and markedly inhibits the proteasomal protease activities of *Mycobacterium tuberculosis* and thus sensitizes *M. tuberculosis* to therapeutic and immune interventions (7). This drug can also completely arrest *Plasmodium* parasite growth, making it a potential therapeutic agent against malarial infections.

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* Corresponding author. Mailing address: Liver Diseases Branch, NIDDK, National Institutes of Health, 10 Center Drive, Room 9B16, Bethesda, MD 20892-1800. Phone: (301) 496-1721. Fax: (301) 402-0491. E-mail: JLiag@nih.gov.

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MATERIALS AND METHODS

Reagents. The proteasome-specific inhibitor MLN-273 was provided by Millennium Pharmaceutical, Inc. (Cambridge, MA). Mice were injected intravenously (i.v.) with a standard 0.9% saline solution of the formulated MLN-273,

FIG. 1. Experimental protocols of HBV mouse models. (A) HBV transgenic mice. Eight-week-old HBV transgenic mice expressing either the wild-type (HBVWT) or X-negative (HBVX⁻) HBV genome were injected twice i.v. with MLN-273 at a dose of 0.8 mg/kg or with PBS as the control. (B) HBV-adenoviral infection. Eight-week-old C57BL/6 mice were inoculated with ad-HBV or ad-HBVX⁻ i.v. at a dose of 1×10^8 PFU/ml, followed by two i.p. injections of proteasome inhibitor MLN-273 at a dose of 0.8 mg/kg or PBS as the control. Serum and liver samples were harvested at various time points postinjection.

freshly prepared before each injection. The peptide Suc-Leu-Leu-Val-Tyr-AMC (LLVY), a substrate for proteasome activity assay, was obtained from Bachem (Torrance, CA).

Animals. Two mouse models were used in this study. The HBV transgenic mice were developed as described previously (wild-type HBV [HBVWT], Tg05 and Tg08 lines; HBX-defective HBV genome [HBVX⁻], Tg31) (28), and the C57BL/6 mice were obtained from Charles River Laboratories (Boston, MA). The mice were bred and maintained at the National Institutes of Health (NIH) animal facility. For injections in HBV transgenic mice, MLN-273 was injected twice a week i.v. at different doses ranging from 0.5 to 1.2 mg/kg of body weight (Fig. 1A). C57BL/6 mice were inoculated with the ad-HBV virus, followed by intraperitoneal (i.p.) injection of MLN-273 twice. The serum and liver samples were harvested according to the schedule illustrated in Fig. 1B.

Proteasome activity assay. The proteasome complexes from the livers of treated mice were extracted and purified, and the proteasome activities were tested using Suc-Leu-Leu-Val-Tyr-AMC (LLVY) as a substrate (14). In brief, 3 μ g of the purified proteasomes was incubated with 0.1 mM LLVY in a 50- μ l total volume of reaction buffer (20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP) at 37°C for 20 min. The reactions were stopped by adding 1 ml of 1% SDS. The resulting fluorescence was measured with a spectrofluorimeter (Packard Instruments, Downers Grove, IL).

Viral replication assay. To test the effect of MLN-273 on HBV replication and transcription, the livers from mice that were treated with MLN-273 or phosphate-buffered saline (PBS) were harvested at different time points for viral RNA and DNA analyses. RNA was prepared by the guanidium isothiocyanate-phenol method (TRIzol RNA isolation kit [Invitrogen, Carlsbad, CA]), analyzed by 1% formaldehyde agarose gel electrophoresis, and hybridized with a ³²P-labeled HBV-specific probe. Viral replicative intermediates associated with intracellular core particles were isolated by ultracentrifugation of cell lysate through a 30% sucrose cushion and analyzed by Southern blot hybridization as described previously (12).

Western blot detection of HBV core protein. Twenty-five milligrams of liver was homogenized in extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 1% aprotinin) and centrifuged at $13,000 \times g$ for 15 min at 4°C to remove cellular debris. After measurement of the protein concentration, Western blotting with anti-core antibody was performed as previously described (14).

Production of the ad-HBV recombinant virus. A recombinant adenovirus expressing the HBV genome was generated using the AdEasy system and was provided by U. Protzer (13, 25). A 1.3x genomic length wild-type or an X-defective mutant genome of HBV DNA was cloned into an adenovirus vector to generate the recombinant HBV adenovirus. The recombinant viruses were produced in 293T cells, and the viruses were purified using the Fast-Trap adenovirus purification and concentration kits (Millipore Corp., Billerica, MA).

Serum HBV DNA quantification and HBV antigen assays. The sera from MLN-273- or PBS-treated mice were harvested at certain time points and analyzed for HBV DNA quantification using the COBAS TaqMan HBV test (Roche Molecular Diagnostics), hepatitis B surface antigen (HBsAg) assay (Auszyme [Abbott Laboratories, Abbott Park, IL]), and hepatitis B e antigen (HBeAg) assay (ETI-EBK Plus [DiaSorin Inc., Stillwater, MN]).

Statistical analysis. Student's *t* test was used to analyze differences between two groups. A *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

MLN-273 had been extensively tested in mice and showed a good safety profile, except for weight loss at high doses. To choose an appropriate dose for injection, we tested the toxicities of MLN-273 at different doses in HBV transgenic mice. Using 8-week-old HBV transgenic mice, MLN-273 was tested at doses of 0.5, 0.8, 1.0, and 1.2 mg/kg of body weight. The treated mice were weighed daily for 2 weeks. At the highest dose of 1.2 mg/kg, 1 of 5 mice died and the rest lost over 20% of their body weight. At the dose of 1.0 mg/kg, the mice lost about 10% of their body weight. At the doses of 0.5 and 0.8 mg/kg, the mice lost 5 to 10% of their body weight. To test for the toxicity of MLN-273 in these mice at these doses, blood counts and chemistry were examined. There were no significant differences in mice with or without treatment, indicating that MLN-273 is not toxic at the doses used, which is consistent with the animal toxicology data of this drug from the manufacturer. Therefore, MLN-273 at a dose of 0.8 mg/kg was selected for further experiments.

By using a recombinant adenovirus expressing HBV (ad-HBV) and a recombinant baculovirus expressing WHV (bv-WHV), we have previously demonstrated that proteasome inhibitors substantially enhance viral replication of the HBVX⁻ virus but not that of the wild-type HBV, suggesting an important role of proteasome pathways in HBX-dependent viral replication (30). To study this effect *in vivo*, the proteasome inhibitor MLN-273 was then tested in HBV transgenic mice expressing wild-type or X-negative HBV.

Eight-week-old HBV transgenic mice were injected with 0.8 mg/kg of MLN-273 i.v. on days 0 and 3. Serum samples harvested at weeks 0, 1, and 4 postinjection were analyzed for HBV DNA, HBeAg, and HBsAg levels. In the transgenic mice expressing wild-type HBV (HBVWT), the serum HBV DNA and HBeAg levels were slightly increased after MLN-273 injection, and the serum HBsAg level did not change much with treatment (Fig. 2A). Consistent with previous reports, transgenic mice with the HBX-defective HBV genome (HBVX⁻) showed much lower HBV DNA levels than the HBVWT mice. In HBVX⁻ mice, both HBV DNA and HBeAg levels increased significantly (>100-fold for HBV DNA, ~10-fold for HBeAg) at week 1 postinjection. As expected, this effect disappeared at week 4. In contrast, serum antigen HBsAg levels did not show any change in the course of the experiment (Fig. 2B), consistent with our previous *in vitro* studies (30) showing that HBsAg levels do not correlate with HBV DNA levels. The

FIG. 2. Effect of MLN-273 on serum HBV markers in transgenic mice. HBV transgenic mice at the age of 8 weeks were treated with MLN-273 at a dose of 0.8 mg/kg or with PBS, and serum and liver samples were harvested at different time points as described. In HBVWT mice (Tg05 and Tg08) (A) and HBVX⁻ mice (Tg31) (B), serum HBV DNA (top panels), serum HBeAg (middle panels), and serum HBsAg (bottom panels) levels are shown. Results are from three independent experiments. **, $P < 0.001$; OD, optical density.

increase in serum HBeAg level likely reflected the increase in serum HBV DNA titer, because viral transcription was not affected by the proteasome inhibitor treatment (see Fig. 3). Studies on other transgenic mouse models have also shown that HBeAg, which is a component of the virion, can be degraded *in vivo* to protein species with HBeAg reactivities (20). Our previous study concerning the effect of proteasome inhibitors on HBV replication *in vitro* showed that HBeAg production was minimally affected by proteasome inhibitor treatment, whereas viral replication was markedly affected (30). This observation is consistent with the fact that serum HBeAg has been used as a marker of viremia *in vivo* prior to the development of sensitive HBV quantification assays (17).

To further test the effect of MLN-273 on HBV replication, transcription, and protein expression, livers were harvested from the transgenic mice at week 1 after injection. Cytoplasmic

viral core particles were isolated, and core-associated viral DNA was extracted for the analysis of replicative intermediates. MLN-273 had little effect on viral replication in the liver of the HBVWT mice. Similar to serum HBV DNA levels, viral replication and core protein levels in the HBVX⁻ livers were much lower than those in HBVWT livers. MLN-273 treatment significantly increased and restored the HBV replication and core protein levels to almost the same levels as those of HBVWT mice (Fig. 3A and 3C). However, MLN-273 treatment had little effect on the viral transcripts, including the 3.5-, 2.4-, and 2.1-kb mRNAs (Fig. 3B). This observation supports the notion that MLN-273 acts on viral replication without affecting viral transcription in an HBX-dependent manner.

To confirm that the proteasome activities were indeed inhibited by MLN-273 in the HBV transgenic mice, these mice were treated with MLN-273 at doses of 0.4 and 0.8 mg/kg. At

FIG. 3. Effect of MLN-273 on hepatic HBV viral replication, transcription, and protein expression in transgenic mice. Livers from the HBV transgenic mice were harvested for DNA, RNA, and protein analyses. (A) Effect on HBV replication. HBV core-associated DNA was extracted for the detection of replicative intermediates by Southern blot analysis with an HBV-specific probe. Cloned HBV DNA was used as a marker. RC, relaxed circular DNA; SS, single-stranded DNA. The intracellular HBV DNA levels were quantitatively measured using real-time qPCR assay. (B) Effect on HBV transcription. Total RNA was isolated for the detection of viral transcripts by Northern blotting. 28S and 18S RNAs were used as loading controls. (C) Effect on HBV core protein. Liver proteins were extracted for the detection of HBV core by Western blotting (top panel). β -Actin was used as a loading control (bottom panel). Results are from three independent experiments.

24 h postinjection of MLN-273, the livers were harvested and proteasomes were purified for proteasome activity determination. Without MLN-273 treatment, the proteasome activity of the HBVWT mice was somewhat lower than that of the

FIG. 4. Effect of MLN-273 on hepatic proteasome activities of HBV transgenic mice. HBV transgenic mice at the age of 8 weeks were injected i.v. with MLN-273 at doses of 0.4 and 0.8 mg/kg. At 24 h postinjection of MLN-273, the livers were harvested and the proteasomes were purified for proteasome activity determination. Results are from three independent experiments. *, $P < 0.01$; **, $P < 0.001$, compared to the control.

HBVX⁻ mutant mice (0.584 ± 0.15 versus 0.748 ± 0.21 pmol/s; $P < 0.01$) (Fig. 4). This minor effect could possibly be due to inhibition of the proteasome activities by the X protein from the wild-type HBV genome (14). In both HBVWT and HBVX⁻ mice, MLN-273 treatment reduced the proteasome activities significantly, but the magnitude of reduction appeared to be greater in the HBVX⁻ mice (Fig. 4). These data indicate that MLN-273 was indeed active in inhibiting the proteasome activities *in vivo*.

The HBV transgenic mouse experiments demonstrated that proteasome inhibitor MLN-273 enhanced viral replication markedly in the HBVX⁻ mice but only slightly in the HBVWT transgenic mice. To further confirm this finding, we applied another HBV mouse model, in which a recombinant adenovirus containing a replication-competent HBV genome can be inoculated into C57BL/6 mice and initiate HBV replication in the mouse liver (25). Eight-week-old C57BL/6 mice were inoculated with 1×10^8 PFU/ml of ad-HBV i.v., followed by two injections of MLN-273 i.p. at a dose of 0.8 mg/kg on days 0 and 1. Serum samples were harvested on days 2, 4, and 6 and analyzed for HBV DNA, HBeAg, and HBsAg levels. In mice injected with ad-HBV, there was no significant difference with or without MLN-273 treatment (Fig. 5). In mice infected with ad-HBVX⁻, the levels of serum HBV DNA and HBeAg were much lower (around 35%) than those in mice infected with ad-HBV, but HBsAg levels were comparable. However, MLN-273 treatment significantly enhanced both serum HBV DNA and HBeAg levels in ad-HBV-infected mice by 10- to 20-fold as early as 1 day postinjection but had little effect on HBsAg levels (Fig. 5). To ensure that measurement of serum HBV DNA represents authentic replicating HBV and not injected recombinant ad-HBV, we showed that by adenovirus-specific PCR, adenoviral DNA (detection limit of 10^3 copies/ml) could no longer be detected in serum 2 days after injection. Therefore, the detected HBV DNA indeed represents authentic replicating HBV DNA.

FIG. 5. Effects of MLN-273 on HBV replication in C57BL/6 mice infected with HBV adenoviruses. C57BL/6 mice at the age of 8 weeks were infected with ad-HBVWT (A) and ad-HBVX⁻ (B) and treated with proteasome inhibitor MLN-273 at a dose of 0.8 mg/kg or with PBS as the control. Serum and liver samples were harvested at different time points and assayed for serum HBV DNA (top panel), serum HBeAg (middle panel), and serum HBsAg (bottom panel) levels. Results are from three independent experiments. *, $P < 0.01$.

Our study demonstrates that cellular proteasome plays an important role in HBV replication *in vivo* and that this effect is mediated by the function of HBX. This result is consistent with our previous findings demonstrating the structural and functional interaction of HBX with the proteasome complex in cell culture models. In these *in vivo* experiments, we showed that the effect of proteasome inhibition on HBV replication resides in a posttranscriptional step. It is possible that the assembled core particles containing the replicative intermediates are targets of cellular proteasome degradation. HBX, functioning like a proteasome inhibitor (14), blocks this crucial step during productive HBV infection *in vivo*. Further studies are necessary to confirm this possibility and to characterize the mechanism of these functional interactions. Proteasome has also been linked to interferon-mediated suppression of HBV replication *in vitro* and *in vivo* (22), suggesting that proteasome may play an important role in the innate antiviral response in addition to its role in antigen presentation and activation of adaptive immunity. Cellular proteasome has already been shown to play a direct antiviral role in

other viral infections (18, 19, 23, 29). Therefore, targeting the interaction between HBX and proteasome may provide a novel therapeutic strategy for anti-HBV development.

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ADDENDUM IN PROOF

After the submission of our manuscript, Bandi et al. published a similar paper (P. Bandi, M. L. Garcia, C. J. Booth, F. V. Chisari, and M. D. Robek, *Antimicrob. Agents Chemother.* **54**:749–756, 2010) concerning the effect of proteasome inhibitor on an HBV transgenic mouse model. Bandi et al.

showed inhibition of wild-type HBV replication by bortezomib, and in contrast we found minor increased HBV replication by MLN-273. We are not sure of the reason for the difference and suspect it may be due to the different proteasome inhibitors and HBV transgenic mouse model used for each study.

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