Basic Medical Research

Effect of matrix metalloproteinase-9 inhibitors in hepatitis B virus replication

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ABSTRACT

BACKGROUND Hepatitis B virus (HBV) remains difficult to eradicate due to the persistence of covalently closed circular DNA (cccDNA). Matrix metalloproteinase-9 (MMP-9) enhances HBV replication, but the effects of its inhibition remain unexplored. This study aimed to investigate the effects of MMP-9 inhibitors on HBV replication markers.

METHODS Primary hepatocyte cultures were obtained from the livers of 6 *Tupaia javanica*. Cultures were infected with HBV from human sera and divided into control and intervention groups. The intervention group received MMP-9 inhibitors at 1, 3, and 7 nM. The control group received phosphate-buffered saline. Levels of hepatitis B surface antigen (HBsAg), HBV DNA, cccDNA, MMP-9, interferon alpha and beta receptor subunit 1 (IFNAR1), and interferon beta (IFN-β) were measured in both groups before and 72 hours post-intervention.

RESULTS MMP-9 inhibitor administration at 1, 3, and 7 nM consistently reduced HBsAg, HBV DNA, cccDNA, and MMP-9 levels, though not statistically significant. Median HBV DNA levels at 1, 3, and 7 nM were 7.05, 5.29, and 5.98 $\times 10^3$ copies/ml, respectively. Mean cccDNA levels at 1, 3, and 7 nM were 14.15, 11.04, and 13.94 $\times 10^3$ copies/ml, respectively. The 3 nM dose increased IFNAR1 levels, while the 7 nM dose increased IFN-β, but neither change was significant. Among the tested doses, 3 nM showed the most favorable effects despite the lack of significance.

CONCLUSIONS MMP-9 inhibitor suppressed HBsAg, HBV DNA, cccDNA, and MMP-9 while increasing IFNAR1 and IFN-β *in vitro*.

KEYWORDS hepatitis B, IFNAR1, interferon beta, matrix metalloproteinase-9

Chronic hepatitis B (CHB) is a global health concern that can cause liver cirrhosis and hepatocellular carcinoma. In 2022, the World Health Organization estimated 254 million people had hepatitis B, with approximately 1.2 million new infections and 1.1 million deaths annually, mostly owing to complications such as acute liver failure, cirrhosis, and cancer. Despite the availability of antiviral therapy, only 13% of CHB cases are diagnosed and <3% receive treatment by the end of 2022.[1] Within the hepatitis B virus (HBV) replication cycle, covalently closed circular DNA (cccDNA) forms in the hepatocyte nucleus,[2] acting as a stable minichromosome and replication center for HBV and as a precursor to viral protein formation. Its persistence and stability make the complete elimination of HBV infection challenging.[3,4]

Current therapies for hepatitis B, such as nucleos(t)ide analogs, effectively suppress viral replication but

have minimal impact on cccDNA.[5] Interferon (IFN), a signaling protein produced in response to bacterial, viral, or parasitic infections,[6,7] is an alternative therapy for direct antiviral action and indirect stimulation of the immune response against HBV. To our knowledge, IFN is the only approved HBV therapy known to degrade cccDNA.[8]

HBV infection induces the production of host factors, such as matrix metalloproteinase-9 (MMP-9),[9] which can enhance HBV replication by impairing the IFN pathway.[10] IFN plays a crucial role in HBV eradication; however, MMP-9 production may impair this process by attenuating the IFN response. Despite the established role of MMP-9 in HBV pathogenesis, only a few studies have evaluated the potential therapeutic effects of MMP-9 inhibition. Therefore, this study aimed to explore the potential of a novel therapeutic strategy of MMP-9 inhibitors by investigating its effect on HBV replication.

Tupaia species have been recognized as suitable animal models of HBV infection because of their genetic and physiological similarities with primates.[11] Recent studies have demonstrated that primary hepatocyte cultures from *Tupaia javanica* support HBV replication, including the detection of hepatitis B surface antigen (HBsAg), HBV DNA, and cccDNA following infection with human HBV.[12–14] These findings suggest that *T. javanica* is a promising model for HBV research.

METHODS

This *in vitro* experimental study used primary Tupaia javanica hepatocyte (PTH) cultures. Six *T. javanica* were maintained in a quarantine facility and underwent a 1-week acclimatization period before the experiment. Preliminary mapping indicated that 30 million hepatocytes were required. Based on our preliminary experiments, each *T. javanica* yielded approximately 5–7.5 × 10^6 hepatocytes, which justified the use of six animals in this study. Wild adult male *T. javanica* (40–60 g) were included, whereas those with prior HBV infection or positive for HBsAg were excluded. Laboratory analyses measured HBsAg, HBV DNA, cccDNA, MMP-9, interferon alpha and beta receptor subunit 1 (IFNAR1), and interferon beta (IFN-β). The study was conducted at the Primate Research Center, IPB University (PRC-IPB), Indonesia and was approved by the Animal Care and Use Committee of PRC-IPB under the approval protocol number IPB PRC-22-B002.

Isolation and primary culture of *Tupaia* hepatocytes

Prior to hepatocyte isolation, *Tupaia* were anesthetized with ketamine (5 mg/100 g body weight [BW]) and xylazine (1 mg/100 g BW). Blood was collected by exsanguination, and livers were obtained via laparotomy. The livers were pre-perfused with 10 ml pre-perfusion medium (500 ml phosphate-buffered saline [PBS], 2.5 ml 1M ethylene glycol tetraacetic acid (5 mM), and 2% penicillin-streptomycin). Pre-perfusion was performed 5 times until the liver appeared pale. Perfusion was then performed using 10 ml of perfusion medium (50 ml Dulbecco's Modified Eagle Medium, 500 µl 100 × $CaCl_2$, and 1% collagenase). The liver was sliced, minced, incubated at 37°C for 15 min, and centrifuged at 400 × *g* for 15 min. The supernatant was discarded and the cells were resuspended in 10 ml of pre-perfusion medium, followed by cell counting. Each flask received 10^6 cells. Hepatocyte culture media (Hepatocyte Basal Medium [HBM] CC-3199, and Hepatocyte Culture Medium CC-4182 [Lonza Bioscience, USA], with 20% fetal bovine serum) were added to the wells, followed by incubation at 37 °C and 5% CO_2.

HBV infection of the PTH

HBV was obtained from the serum of patients with CHB (HBV DNA level >10^6 IU/ml) and resuspended in HBM. On Day 20, when hepatocyte culture reached 80% confluence, HBV was added at 10^6 IU/ml and incubated for 20 hours at 37°C and 5% CO_2. The cells were then washed with PBS and fresh culture medium was added. A second 48-hour incubation was required to ensure that the released virus in the supernatant was from an infected hepatocyte.[13,14] After 48 hours, the MMP-9 inhibitor (ab142180; Abcam, UK) was administered at 1, 3, or 7 nM. These concentrations were selected based on our preliminary study, in which a wider dose range (1, 3, 5, and 7 nM, as well as 1, 3, 5, and 7 µM), with only 1, 3, and 7 nM demonstrating a consistent antiviral effect.

Isolated hepatocytes were divided into intervention and control groups. Supernatant samples were analyzed for HBV DNA, quantitative HBsAg (qHBsAg), MMP-9, IFNAR1, and IFN-β levels before and 72 hours after MMP-9 inhibitor administration. cccDNA levels were measured in hepatocytes at the same time points.

Detection method

Quantitative HBsAg levels were measured using a chemiluminescent enzyme immunoassay

method with an automated immunoassay system (HISCL-5000; Sysmex Co., Ltd., Indonesia). HBV DNA and cccDNA concentrations were measured by real-time polymerase chain reaction (PCR) using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). The concentrations of MMP-9 and IFNAR1 were measured using an enzyme-linked immunosorbent assay (ELISA) with an ELISA reader (Reed Biotech Ltd., China). IFN-β concentration was also measured by ELISA using an ELISA reader (Wuhan Feiyue Biotechnology Co., Ltd., China).

Statistical analysis

Data were analyzed using SPSS software version 25.0 (IBM Corp., USA). Normality of the data was evaluated using the Shapiro–Wilk test. For normally distributed data, comparisons between two groups were performed using independent *t*-tests. For non-normally distributed data, the Mann–Whitney *U* test was used. Statistical significance was set at $p<0.05$.

RESULTS

A preliminary study determined that 1, 3, and 7 nM were the optimal MMP-9 inhibitor doses, with 72 hours post-administration as the ideal assessment time. The effects of these inhibitors were evaluated in PTH cultures before and after HBV infection and MMP-9 inhibitor administration.

The HBV-infected PTH cultures showed detectable levels of qHBsAg, HBV DNA, and cccDNA. MMP-9 concentrations were higher in HBV-infected PTH cultures than in uninfected cultures, although the difference was not statistically significant (median 68.26 *versus* 67.31, $p = 1.000$). HBV-infected PTH cultures showed lower IFNAR1 concentrations than the uninfected cultures, but the differences were not significant (mean 576.32 *versus* 720.59, $p = 0.284$). IFN-β concentrations were higher in HBV-infected PTH cultures than in uninfected cultures, but this increase was not statistically significant (mean 7.69 *versus* 5.35, $p = 0.140$) (Table 1).

Treatment with the MMP-9 inhibitor at all doses resulted in lower qHBsAg, HBV DNA, and cccDNA levels than those treated with a placebo. qHBsAg levels were lower in cultures treated with MMP-9 inhibitors than in those treated with the placebo. However, these reductions lacked statistically significant (median 0.07 *versus* 0.08, $p = 0.548$ for 1 nM; median 0.07 *versus* 0.08, $p = 0.222$ for 3 nM; median 0.06 *versus* 0.08, $p = 0.095$ for 7 nM) (Figure 1a). The greatest reduction in HBV DNA levels was observed at 3 nM following MMP-9 inhibitor treatment with no significant difference observed (median 7.05 *versus* 7.68, $p = 0.548$ for 1 nM; median 5.29 *versus* 7.68, $p = 0.056$ for 3 nM; median 5.98 *versus* 7.68, $p = 0.056$, 7 nM) (Figure 1b). Similarly, cccDNA levels were reduced the most at 3 nM, but the differences were not statistically significant (mean 14.15 *versus* 14.92, $p = 0.839$ for 1 nM; mean 11.04 *versus* 14.92, $p = 0.125$ for 3 nM; mean 13.94 *versus* 14.92, $p = 0.778$ for 7 nM) (Figure 1c). Treatment with the MMP-9 inhibitor reduced MMP-9 concentrations across all doses compared to those receiving the placebo, with the lowest reduction observed at 7 nM. However, no statistically significant differences were observed (median 69.06 *versus* 69.53, $p = 1.000$ for 1 nM; median 68.9 *versus* 69.53, $p = 0.690$ for 3 nM; median 66.99 *versus* 69.53, $p = 0.548$ for 7 nM) (Figure 1d). Among the

Table 1. Replication and inflammation marker before and after HBV infection to PTH

Parameters	HBV infection		*p*
	Pre-infection	48 hours post-infection	
qHBsAg (IU/ml), median (IQR)	-	0.84 (0.82–0.88)	-
HBV DNA (×10³ copies/ml), median (IQR)	-	51.45 (50.55–61.95)	-
cccDNA (×10³ copies/ml), mean (SD)	-	19.21 (4.91)	-
MMP-9 (pg/ml), median (IQR)	67.31 (66.75–97.31)	68.26 (66.92–68.74)	1.000*
IFNAR1 (pg/ml), mean (SD)	720.59 (69.89)	576.32 (172.81)	0.284†
IFN-β (pg/ml), mean (SD)	5.35 (0.28)	7.69 (1.73)	0.140†

cccDNA=covalently closed circular DNA; HBV=hepatitis B virus; IFNAR1= interferon alpha and beta receptor subunit 1; IFN-β=interferon beta; MMP-9=matrix metalloproteinase-9; IQR=interquartile range; PTH=primary Tupaia javanica hepatocyte; qHBsAg=quantitive hepatitis B surface antigen; SD=standard deviation

*Mann–Whitney test; †independent *t*-test

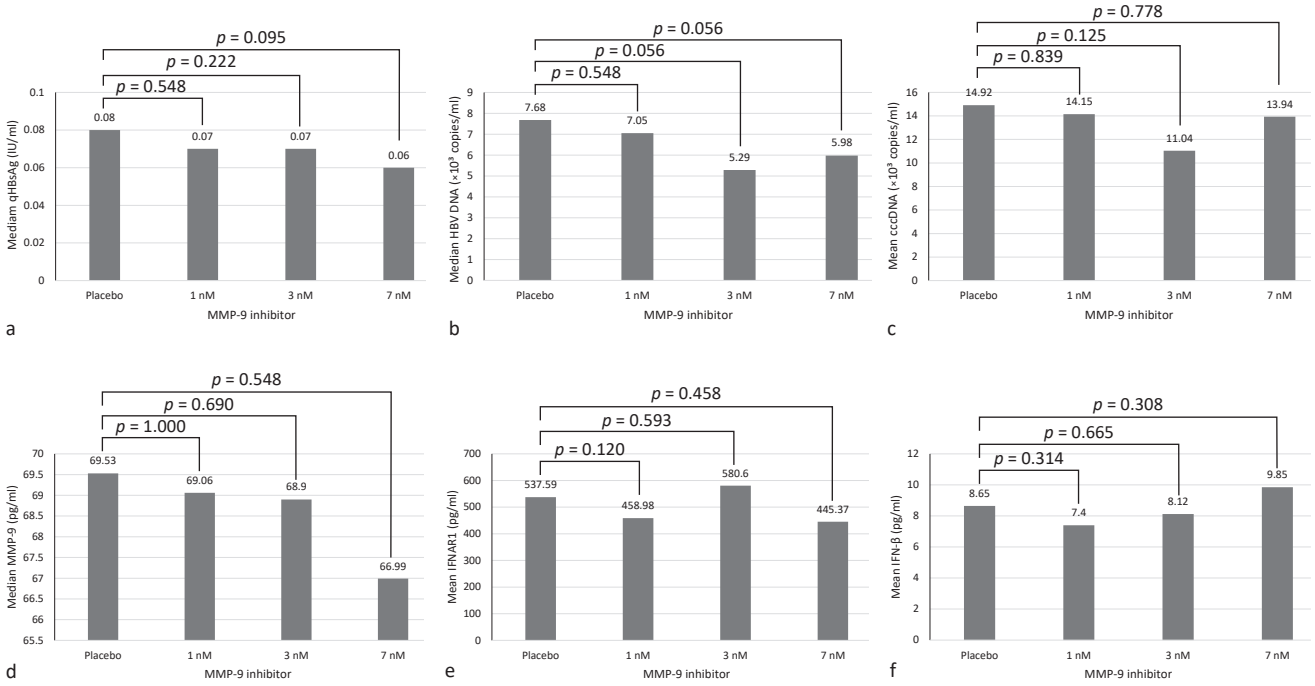

Figure 1. qHBsAg (a), HBV DNA (b), cccDNA (c), MMP-9 (d), IFNAR1 (e), and IFN-β (f) before and after administration of MMP-9 inhibitor. cccDNA=covalently closed circular DNA; HBV=hepatitis B virus; IFN-β=interferon beta; IFNAR1= interferon alpha and beta receptor subunit 1; MMP-9=matrix metalloproteinase-9; qHBsAg=quantitive hepatitis B surface antigen

infected cultures treated with the MMP-9 inhibitor, 1 nM and 7 nM had lower IFNAR1 levels than the placebo (mean 458.98 *versus* 537.59, p = 0.120, 1 nM; mean 445.37 *versus* 537.59, p = 0.458 for 7 nM). In contrast, the 3 nM dose increased IFNAR1 levels (mean 580.60 *versus* 537.59, p = 0.593). However, these differences did not reach statistically significant (Figure 1e). After MMP-9 inhibitor treatment, 1 nM and 3 nM doses lowered IFN-β levels compared to placebo (mean 7.40 *versus* 8.65, p = 0.314 for 1 nM; mean 8.12 *versus* 8.65, p = 0.665 for 3 nM), while the 7 nM dose increased IFN-β levels (mean 9.85 *versus* 8.65, p = 0.308 for 7 nM). However, these differences were not statistically significant (Figure 1f).

DISCUSSION

Detection of qHBsAg, HBV DNA, and cccDNA in HBV-infected PTH cultures suggests that *T. javanica* hepatocytes are susceptible to human HBV infection and support HBV replication. These findings aligned with those of a study by Kalista et al,[14] who demonstrated similar results by detecting HBsAg, HBV DNA, and cccDNA in PTH cultures infected with human HBV *in vitro*. MMP-9 concentrations were higher in HBV-infected PTH cultures than in uninfected cultures, indicating that MMP-9 levels increased during HBV infection. This result supports previous studies suggesting that HBV triggers the increased production of various host factors, including MMP-9, which is produced mainly by leukocytes. Under normal physiological conditions, MMP-9 levels are typically low. However, HBV-induced inflammation can significantly upregulate its expression. A study by Li et al[9] reported that MMP-9 levels were significantly higher in patients with CHB than in healthy individuals, which was linked to inflammation severity. These findings demonstrate that HBV-induced inflammatory processes may drive MMP-9 upregulation.[9] Similarly, Chen et al[10] found that HBV activates MMP-9 expression in peripheral blood mononuclear cells and in macrophages differentiated from Tohoku Hospital Pediatrics-1 cells. Our findings align with these findings, as we also observed increased MMP-9 concentrations in human HBV-infected PTH cultures.

Lower IFNAR1 concentrations in HBV-infected PTH cultures suggest that HBV infection reduces IFNAR1 levels. This aligns with studies indicating that MMP-9, which is upregulated during HBV infection, disrupts IFN signaling by phosphorylating, ubiquitinating, and altering its subcellular distribution and subsequent

degradation. Consequently, IFNAR1 cannot bind to IFN, thereby impairing the IFN-mediated antiviral response.[10]

In contrast, IFN-β concentrations were higher in PTH cultures, contradicting the theory that HBV infection lowers IFN-β owing to MMP-9-mediated Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway inhibition.[10] This discrepancy is likely owing to the acute-phase timing of IFN-β assessment, as IFN levels typically rise early in infection.[15] Treatment with the MMP-9 inhibitor reduced qHBsAg, HBV DNA, and cccDNA levels compared with the placebo, indicating that the MMP-9 inhibitor may reduce HBV replication.

Previous studies have shown that MMP-9 facilitates HBV replication by degrading IFNAR1 and disrupting IFN signaling through the repression of the JAK-STAT pathway.[10] In this study, MMP-9 concentrations were consistently lower in HBV-infected *T. javanica* hepatocyte cultures treated with MMP-9 inhibitors (1, 3, and 7 nM) than in those treated with placebo, suggesting that MMP-9 inhibitors effectively reduce MMP-9 levels. IFNAR1 concentrations in inhibitor-treated cultures varied; 1 and 7 nM doses lowered IFNAR1 levels, whereas 3 nM increased them compared to the placebo. Similarly, IFN-β concentrations were lower at 1 and 3 nM but higher at 7 nM compared to placebo. Because MMP-9 degrades IFNAR1, its inhibition is expected to preserve IFNAR1, restore JAK-STAT pathway activation, and enhance IFN-stimulated gene activation, leading to increased production of antiviral proteins against HBV. Findings from HBV-infected PTH cultures suggest that while a 7 nM dose does not improve efficacy, a 3 nM dose demonstrates more favorable effects than both the 1 nM and 7 nM doses. These findings suggest that the 3 nM dose of MMP-9 inhibitor may offer a more favorable balance of efficacy in suppressing HBV replication than lower or higher doses. Although additional *in vivo* and clinical studies are needed, this dose may serve as a starting point for future therapeutic developments targeting MMP-9 in HBV infections.

A limitation of this study was the use of *T. javanica* hepatocyte cultures infected *in vitro* rather than *in vivo*. Future studies should involve *in vivo Tupaia* models to establish chronic infections before isolating the hepatocytes for further analysis. In conclusion, MMP-9 inhibitor administration reduced HBsAg, HBV DNA, cccDNA, and MMP-9 levels, while increasing IFNAR1 and IFN-β levels *in vitro*. Further studies are needed to determine the optimal dose to effectively suppress HBV replication.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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