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Antiviral effect of peptoids on hepatitis B virus infection in cell culture

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ABSTRACT

Although antimicrobial peptides have been shown to inactivate viruses through disruption of their viral envelopes, clinical use of such peptides has been hampered by a number of factors, especially their enzymatically unstable structures. To overcome the shortcomings of antimicrobial peptides, peptoids (sequence-specific *N*-substituted glycine oligomers) mimicking antimicrobial peptides have been developed. We aimed to demonstrate the antiviral effects of antimicrobial peptoids against hepatitis B virus (HBV) in cell culture. The anti-HBV activity of antimicrobial peptoids was screened and evaluated in an infection system involving the HBV reporter virus and HepG2.2.15-derived HBV. By screening with the HBV reporter virus infection system, three (TM1, TM4, and TM19) of 12 peptoids were identified as reducing the infectivity of HBV, though they did not alter the production levels of HBs antigen in cell culture. These peptoids were not cytotoxic at the evaluated concentrations. Among these peptoids, TM19 was confirmed to reduce HBV infection most potently in a HepG2.2.15-derived HBV infection system that closely demonstrates authentic HBV infection. In cell culture, the most effective administration of TM19 was virus treatment at the infection step, but the reduction in HBV infectivity by pre-treatment or post-treatment of cells with TM19 was minimal. The disrupting effect of TM19 targeting infectious viral particles was clarified in iodixanol density gradient analysis. In conclusion, the peptoid TM19 was identified as a potent inhibitor of HBV. This peptoid prevents HBV infection by disrupting viral particles and is a candidate for a new class of anti-HBV reagents.

1. Introduction

Hepatitis B virus (HBV) is the principal causative agent of chronic hepatitis. Despite the availability of effective vaccines in many countries, HBV infection has spread worldwide, causing chronic infection in approximately 300 million patients (Cui et al., 2023). In chronic hepatitis B patients, liver inflammation leads to cirrhosis, and accumulation of viral genome integration into host chromosomes leads to development of hepatocellular carcinoma (HCC). The primary infection routes of HBV are exposure to HBV-contaminated blood or body fluids, sexual contact with HBV-infected individuals, and passage from mother to infant at birth. Currently, two kinds of anti-HBV agents are clinically available: interferon (IFN)-α and nucleoside analogs (NAs). IFN-α inhibits HBV at multiple steps of the virus life cycle, with direct antiviral or immunomodulatory effects. Nevertheless, it is associated with a limited response rate and undesirable side effects. NAs inhibit reverse transcription and reduce synthesis of HBV DNA from pregenomic RNA (pgRNA), suppressing progeny virus production. Although NAs control hepatitis and prevent disease progression, HBV covalently closed circular DNA (cccDNA) in HBV-infected hepatocytes cannot be eradicated and production of HBV-associated proteins from integrated HBV genome cannot be blocked by NAs (Nassal, 2015). Thus, the current therapeutic goal for chronic hepatitis B patients is suppression of production of hepatitis B surface antigen (HBsAg), which is considered to reduce the risk of HCC development (Fanning et al., 2019; Terrault et al., 2018; Yim and Lok, 2006). To this end, long-term treatment with NAs is

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the only practically available treatment, though it is closely related to a risk of emergence of drug-resistant mutations (Ghany and Doo, 2009; Liang et al., 2015). Therefore, new therapeutic strategies for chronic hepatitis B are needed.

Antimicrobial peptides are universal host defense components of the innate immune system that recognize and eliminate pathogens and are promising lead materials for new antiviral therapies (Zasloff, 2002). In mammals, cathelicidins and defensins are two major classes of antimicrobial peptides. LL-37 is the only known cathelicidin in humans and has been reported to have a broad range of antimicrobial properties (Ahmed et al., 2019; Zanetti, 2004). It forms an amphipathic structure of hydrophilic and hydrophobic residues and disrupts bacterial membranes or viral envelopes (Alagarasu et al., 2017; Fernandez et al., 2023; Henzler Wildman et al., 2003; Matsumura et al., 2016; Tripathi et al., 2013; Wang et al., 2008). However, clinical use of LL-37 is limited primarily to topical applications, and systemic administration is difficult because of its high protease sensitivity. In addition, the high immunogenicity of these peptides renders them susceptible to antibody induction. To overcome these shortcomings of antimicrobial peptides, nonnatural peptidomimetics called 'peptoids' have been developed. Peptoids are sequence-specific *N*-substituted glycine oligomers characterized by resistance to proteases and reduced immunogenicity (Chongsiriwatana et al., 2008; Diamond et al., 2021). These peptoids have been shown to be active against bacteria, including ESKAPE pathogens and persister cells, fungi, parasites, and viruses (HSV-1 and SARS-CoV) (Chongsiriwatana et al., 2011; Diamond et al., 2021; Kumar et al., 2023; Lin et al., 2022; Nielsen et al., 2022). In this study, to explore a new class of anti-HBV reagents, we screened a peptoid library and identified candidates with anti-HBV properties. We also investigated the antiviral mechanisms of the identified peptoids by using an HBV production and infection system *in vitro*.

2. Materials and methods

2.1. Cell culture

HepG2.2.15 cells with an integrated HBV genome that continuously produce infectious HBV have been described previously (Murayama et al., 2021; Yamada et al., 2020). HepG2-NTCPsec+ (HepG2-NTCP) cells that were transduced sodium taurocholate-cotransporting polypeptide (NTCP) have also been described previously (Konig et al., 2019; Murayama et al., 2021; Washizaki et al., 2022). To assess cell viability, the water-soluble tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8) was used as described previously (Matsumura et al., 2016; Yamada et al., 2020).

2.2. Synthesis and purification of peptoids

Peptoids were synthesized using the submonomer method and as previously described for TM peptoids (Diamond et al., 2021; Zuckermann et al., 1992).

2.3. HBV reporter virus

The HBV reporter virus HBV/NL (genotype C, accession number: AB246345), which encodes NanoLuc luciferase (NL) in its genome, was used (Nishitsuji et al., 2015). To produce HBV/NL, two plasmids, HBV-NL (encoding the 1.2-fold HBV genome replacing the HBe/HBc region with NL) and HBV-dEdelS (encoding the 1.2-fold HBV genome that cannot be encapsidated because of a modified encapsidation signal and that cannot express all HBsAg species because of modification of the start codons), were prepared (Murayama et al., 2021; Washizaki et al., 2022). These plasmids were transfected into HepG2-NTCP cells with Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, MA), and HBV/NL generated in the culture medium was harvested one week later. The collected medium was passed through a 0.45-μm filter to remove cell debris and concentrated with Amicon Ultra15 centrifugal filter units (100 kDa, Merck Millipore, Tullagreen, Ireland). HBV/NL in the concentrated medium was purified by an iodixanol density gradient (Honda et al., 2021; Murayama et al., 2021). The fraction of peak infectivity in the gradient was used as inoculum. The viral titer was measured by real-time PCR with a primer and probe set designed to target the NL region after treatment with DNase (RQ1 RNase-Free DNase, Promega, Madison, WI) (Murayama et al., 2021). One week after infection of HepG2-NTCP cells with the HBV reporter virus, infectivity was estimated by measuring the luciferase activity of the infected cells using a Nano-Glo Luciferase Assay System (Promega).

2.4. Cell culture-generated HBV

HepG2.2.15 cells were cultured without G418, and the culture medium was collected and passed through a 0.45-μm filter to remove cell debris. HepG2.2.15-derived HBV was concentrated and purified by an iodixanol density gradient (Honda et al., 2021; Murayama et al., 2021). The HBV DNA titer was measured by real-time PCR with a primer-probe set targeting the HBs region by using the extracted DNA from the culture medium (Honda et al., 2021). HBV was inoculated into HepG2-NTCP cells at 100 genome equivalents (GEq) per cell. The inoculum was washed out after 16 h of incubation, and the infected cells were cultured for 12 days.

2.5. Immunostaining of HBV-infected cells

To detect HBV infection, HBV-infected cells were fixed with 4 % paraformaldehyde and permeabilized. HBV core protein-positive cells were visualized by staining with rabbit polyclonal anti-HBc antibody (AUSTRAL Biologicals, San Ramon, CA) and Alexa Fluor 555-conjugated anti-rabbit IgG (Thermo Fisher Scientific). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.6. HBV infection study in human primary hepatocytes

PXB-cells (PhoenixBio, Hiroshima, Japan) isolated from primary human hepatocytes-transplanted urokinase-type plasminogen activator/severe combined immunodeficient mice (PXB-mice, PhoenixBio) were grown in the culture medium provided by the same manufacturer and used for the evaluation of HBV infection in primary human hepatocytes (Murayama et al., 2021). The HBV genotype C derived from the PXB-mice serum collected after HBV infection was also obtained from the same manufacturer (PhoenixBio) and used as an inoculum to primary human hepatocytes at 2 GEq/cell (Ishida et al., 2015). To assess the infection of HBV, HBsAg and Hepatitis B e antigen (HBeAg) were measured by chemiluminescent enzyme immunoassay using commercial assay kits (Lumipulse G1200, Fujirebio, Tokyo, Japan). The HBV DNA titer in the culture medium was also measured by real-time PCR with a primer and probe set designed to target the HBs region after treatment with DNase (RQ1 RNase-Free DNase, Promega) (Honda et al., 2021).

2.7. Iodixanol density gradient analysis

HBV/NL was concentrated with Amicon Ultra15 centrifugal filter units and subjected to iodixanol density gradient (10–40 %) centrifugation at 38,000 rpm for 16 h at 4 °C in an SW 41 Ti rotor. Fractions were collected from the top of the gradient, and the density was measured for each fraction. HBsAg in each fraction was measured by Lumipulse G1200. Hepatitis B core-related antigen (HBcrAg), consisting of 3 proteins (HBcAg consists of HBV particles, HBeAg, and a 22-kDa precore protein) encoded by the precore/core region, was also measured by chemiluminescent enzyme immunoassay (Lumipulse G1200). The NL DNA was measured by real-time PCR with a primer and probe set

designed to target the NL region after treatment with DNase (RQ1 RNase-Free DNase) (Murayama et al., 2021).

2.8. Statistical analysis

Statistical analysis was performed by one-way ANOVA and multiple comparison tests using GraphPad PRISM 9 software (GraphPad Software, La Jolla, CA). *P* values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Screening for peptoids with anti-HBV effects

The HBV reporter virus HBV/NL, which contains NanoLuc luciferase as a reporter in its genome, was used to screen 12 peptoids (TM1, TM2, TM4, TM5, TM8, TM9, TM10, TM12, TM14, TM15, TM18, and TM19; Fig. 1) for anti-HBV effects. As a negative control, the C-terminal peptide fragment [15–34] of crotalicidin (Ctn), which is known to have no antiviral activity, was used. The human cathelicidin LL-37, a representative antimicrobial peptide, was also used as a peptide comparator.

To evaluate the effects of peptoids on production of HBV and infection of the viruses produced, HepG2-NTCP cells transfected with plasmids for HBV/NL production were treated with the peptoids or control peptides (Ctn and LL-37). These agents were administered at a concentration of 10 μM beginning at 24 h after transfection. After 7 days of culture of the transfected cells, the HBsAg level in the culture medium was measured. The HBsAg level in the culture medium when treated with peptoids or LL-37 was comparable to that of Ctn-treated cells, except for TM1-treated cells (Fig. 2A). To assess the infectivity of the generated HBV reporter viruses, the culture medium harvested after 7 days of culture with peptoids or peptides was used to infect naïve HepG2-NTCP cells. After 7 days of culture, the activity of NanoLuc luciferase in the infected cells was evaluated. The infectivity of HBV/NL produced in the cells treated with the peptoids TM1, TM4, and TM19 was reduced to less than 50 % of that in the Ctn-treated. Treatment with the antiviral peptide LL-37 reduced infectivity to 58.8 % of that in the Ctn-treated (Fig. 2B). The viability of the peptoid-treated cells was over 90 %, while that of the LL-37-treated cells was 77.6 ± 6.9 % of that of

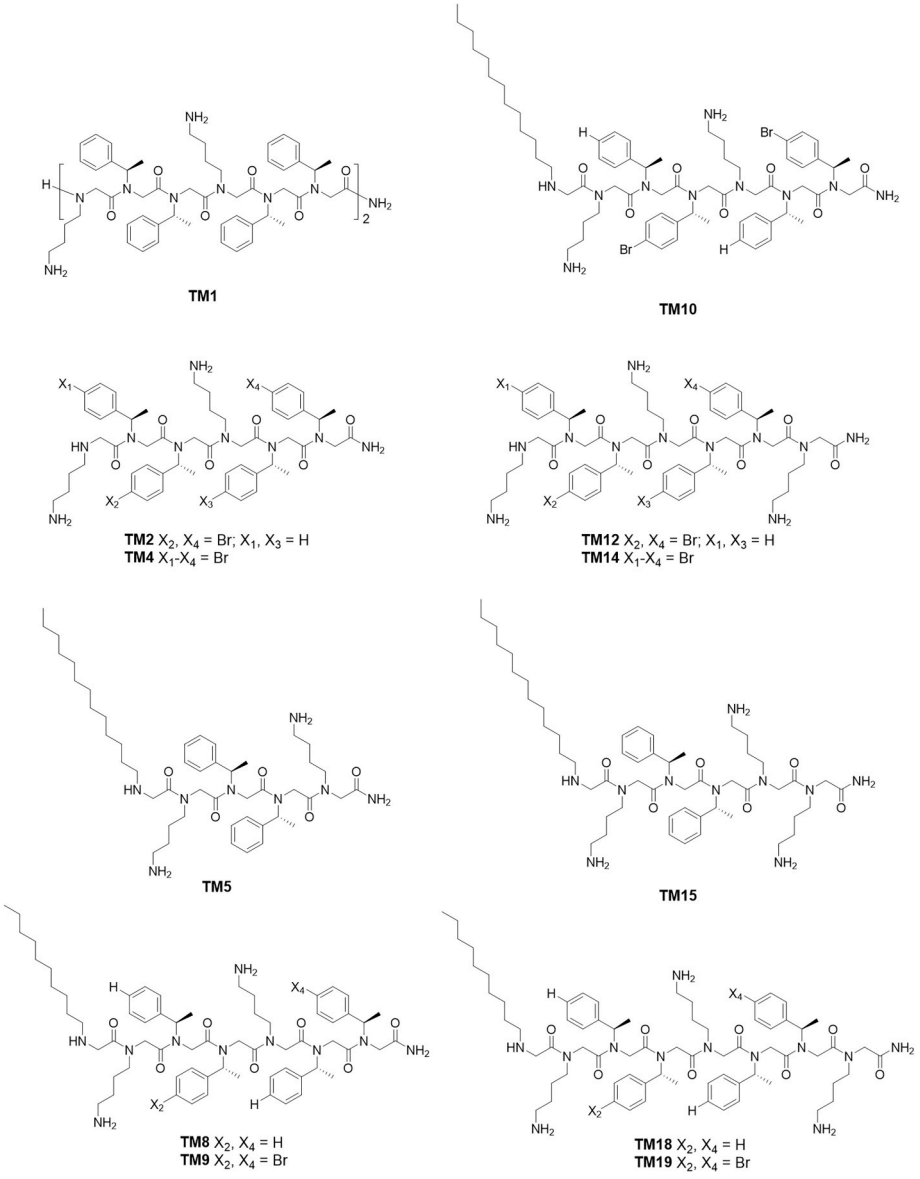

Fig. 1. Chemical structures of the peptoids.
The chemical structures of the peptoids used in this study are shown.

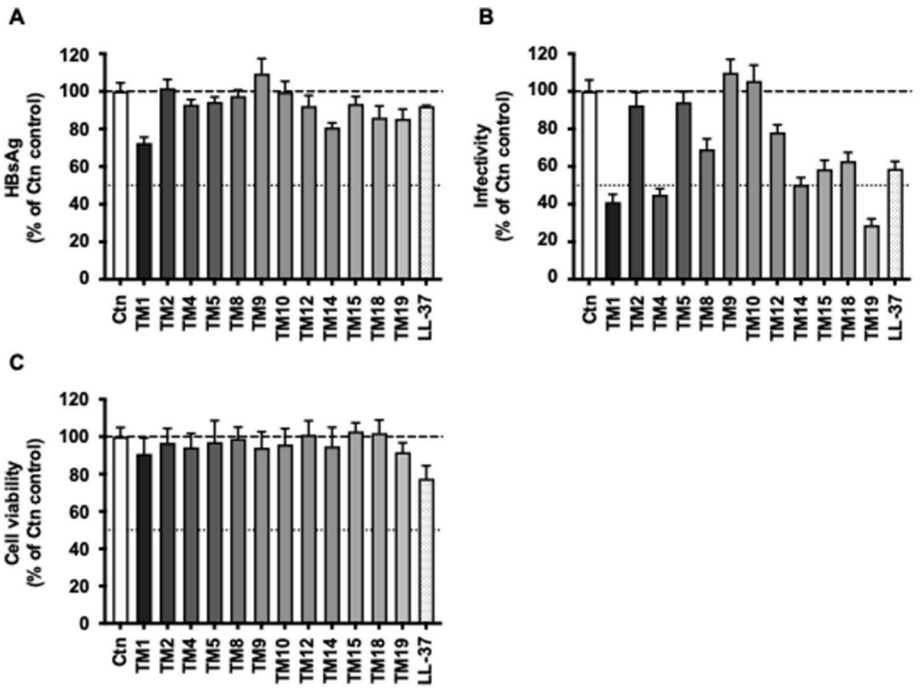

Fig. 2. Screening for the anti-HBV effects of peptoids on HBV production.
(A) The anti-HBV effects of peptoids on HBV production were evaluated. Plasmids for HBV/NL were transfected into HepG2-NTCP cells, and the transfected cells were cultured with the indicated peptoids and control peptides at a concentration of 10 μM. After 7 days of culture, HBsAg levels in the culture medium were measured. The percentages of HBsAg levels in peptoid-treated or LL-37-treated cells compared to Ctn-treated cells are indicated. (B) To assess the infectivity of the generated HBV/NL, the culture medium at 7 days after transfection was used to infect naïve HepG2-NTCP cells. The infected HBV/NL was washed out at 16 h after infection, and infectivity was estimated by measuring the NanoLuc luciferase activity in the infected cells at 7 days after infection. The percentages of luciferase activity of peptoid-treated or LL-37-treated cells compared to Ctn-treated cells are indicated. (C) The cytotoxicity of the peptides and peptoids was evaluated. HepG2-NTCP cells were treated with the indicated peptoids and control peptides for 7 days at a concentration of 10 μM, and cell viability was assessed by the WST-8 assay. The percentages of absorbance (450 nm) of peptoid-treated or LL-37-treated cells compared to Ctn-treated cells are indicated.

the Ctn-treated cells (Fig. 2C).

3.2. Anti-HBV effects of peptoids on cell culture-generated HBV

We selected 3 peptoids, TM1, TM4, and TM19, that reduced HBV/NL infection to less than 50 % of that with control peptide treatment and confirmed the anti-HBV effects of these peptoids in the infection system of HepG2.2.15-derived HBV. In this assay, HepG2-NTCP cells were pre-treated with peptoid or control peptides for 24 h prior to infection. The HepG2.2.15-derived HBV purified by the iodixanol density gradient was treated with peptoid or control peptides for 1 h at 37 °C and then used to infect HepG2-NTCP cells. After 16 h of infection, the inoculum was washed out, and the infected cells were treated with peptoid or control peptides during culture for 12 days. The HBsAg level in the culture medium was monitored to assess HBV production from the infected cells. The HBsAg level in the LL-37-treated cells was substantially reduced compared to that in the Ctn-treated cells (Fig. 3A). Treatment with the peptoids TM1, TM4, or TM19 also reduced HBsAg levels. Among the 3 peptoids, TM19 most potently reduced the HBsAg level, followed by TM4. Immunostaining of the infected cells with an anti-HBc antibody indicated a decrease in the number of HBc-positive cells when treatment with the peptoids, similar to the reduction in the HBsAg level (Fig. 3B). Cytotoxicity was observed in LL-37-treated cells, similar to the data in Fig. 2C. This cytotoxicity might affect the interpretation of the data on the anti-HBV effects of LL-37.

To clarify the point at which the peptoids affect the HBV lifecycle, the treatment period was divided into 3 phases: pre-treatment of cells, virus treatment prior to and during infection, and post-treatment of infected cells (Fig. 3C). The peptoid or control peptides was administered in one of the following 3 phases: the pre-treatment step, in which the peptoid or control peptides was administered to HepG2-NTCP cells for 24 h before infection; the virus treatment step, in which HepG2.2.15-derived HBV was treated with peptoid or control peptides for 1 h at 37 °C and then used to infect HepG2-NTCP cells for 16 h; and the post-treatment step, in which the peptoid or control peptides was administered during 11 days of culture of HBV-infected cells after washing. Substantial reductions in HBsAg levels were detected in the HepG2.2.15-derived HBV-infected cells after virus treatment with TM19 or LL-37 (Fig. 3D). However, the effects of pre-treatment or post-treatment with TM19 and LL-37 were minimal.

3.3. Dose dependency of the anti-HBV effect of the peptoid TM19

To assess dose dependency, HepG2.2.15-derived HBV was treated with serially diluted TM19 for 1 h and then used to infect HepG2-NTCP cells for 16 h (the virus treatment step in Fig. 3A). Twelve days after infection, a dose-dependent reduction in HBsAg level was observed after treatment with TM19 at concentrations of 1–30 μM (Fig. 4A). Cell viability was over 80 % of that of Ctn-treated cells at the evaluated concentrations of TM19 (Fig. 4B).

3.4. Effect of TM19 on HBsAg production from HepG2.2.15 cells

To confirm the minimal effects of the peptoid on HBsAg production, HepG2.2.15 cells were treated with TM19 or control peptides (Ctn and LL-37) at a concentration of 10 μM. After 7 days of culture of HepG2.2.15 cells with TM19 or control peptides, the HBsAg levels in the culture medium were measured. The HBsAg levels in the culture medium of TM19-and LL-37-treated cells were comparable to those of Ctn-treated cells (Supplemental Fig. S1). The viability of TM19-treated cells was over 90 %, although that of LL-37-treated cells was 88.6 %.

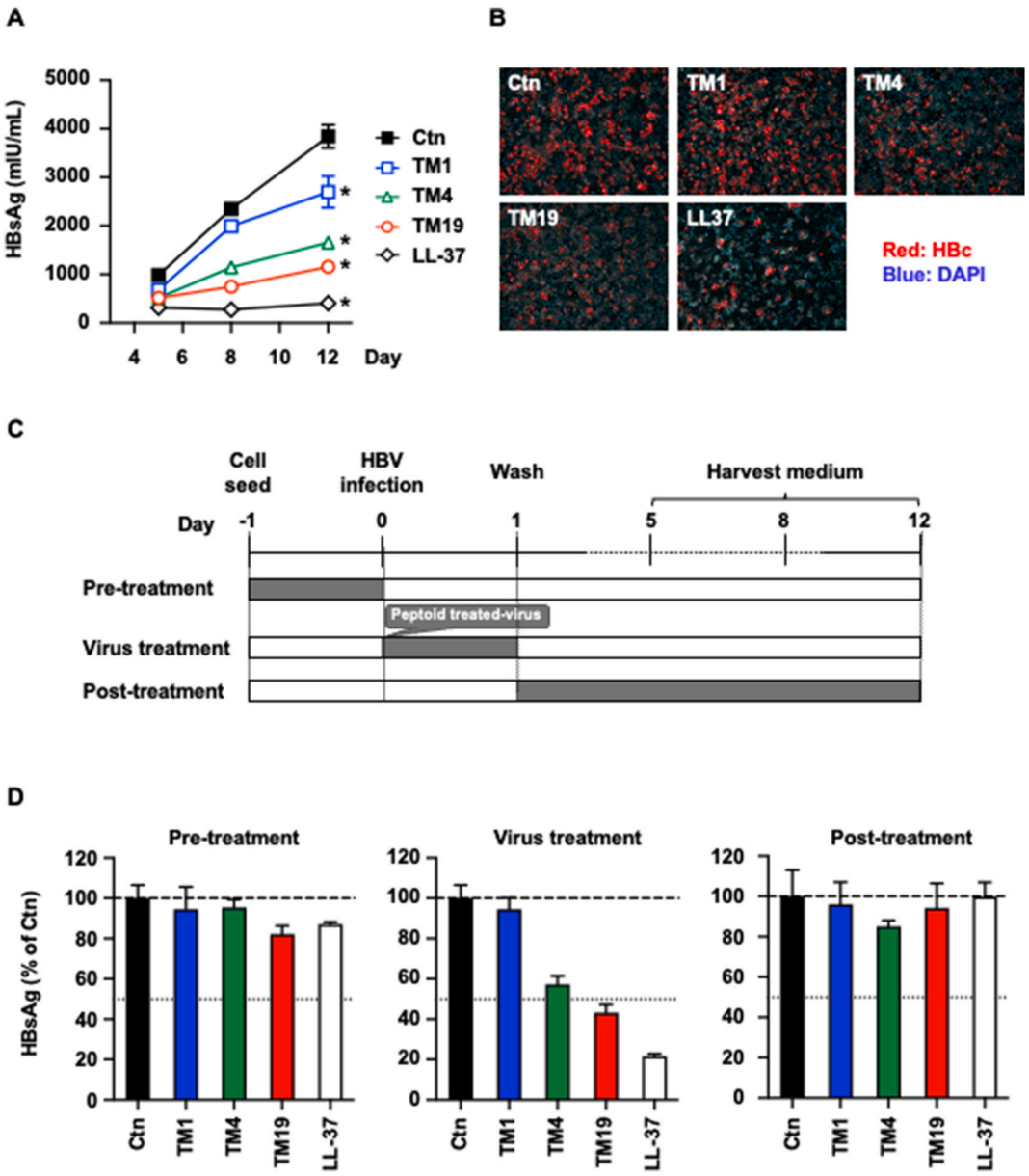

Fig. 3. Anti-HBV effects of peptoids on cell culture-generated HBV

(A) The anti-HBV effects of peptoids on cell culture-generated HBV were evaluated. HepG2-NTCP cells were incubated with the indicated peptoid or control peptide (10 μM) for 24 h prior to infection. HepG2.2.15-derived HBV was treated with peptoid or control peptide (10 μM) at 37 °C for 1 h before infection and then used to infect HepG2-NTCP cells at 100 GEq/cell (virus treatment) for 16 h with peptoid or control peptide (10 μM). After 16 h of infection, the infected cells were cultured with peptoid or control peptide (10 μM) for 11 days. HBsAg levels in the culture medium were measured after treatment with the indicated peptoid or control peptide on Days 5, 8, and 12 post-infection. *P < 0.0001 compared to the Ctn control. (B) HBc-positive cells were detected by immunostaining with the anti-HBc antibody on Day 12 post-infection. (C) The treatment period was divided into 3 phases: pre-treatment of cells (pre-treatment), virus treatment prior to and during infection (virus treatment), and post-treatment of infected cells (post-treatment). The peptoid or control peptide was administered in one of these 3 phases: pre-treatment, virus treatment, and post-treatment. (D) After administration of the peptoid or control peptide in one of three phases, the HBsAg level in the culture medium was measured on Day 12 post-infection. The percentages of HBsAg levels in peptoid-treated cells compared to Ctn-treated cells are indicated.

3.5. Anti-HBV effects of TM19 on HBV infection to human primary hepatocytes

To confirm the anti-HBV effects of TM19, HBV was treated with TM19 or control peptides (Ctn and LL-37) for 1 h at 37 °C before infection and then used to infect human primary hepatocytes with TM19 or control peptides for 16 h (the virus treatment step in Fig. 3A). After 11 days of culture of human primary hepatocytes, substantial reductions in HBsAg levels were detected in HBV-infected cells after treatment with TM19 or LL-37 (Fig. 5A). The levels of HBeAg and HBV DNA were also measured, and significant reductions of these parameters were also detected after treatment with TM19 or LL-37 (Fig. 5B and C).

3.6. Mechanism of the anti-HBV effect of the peptoid TM19

The antimicrobial peptide LL-37 is known to exhibit antiviral effects against several viruses by disrupting their envelopes. Thus, we analyzed the effect of TM19 on the viral particles of HBV by using iodixanol density gradient analysis. The HBV/NL generated in cell culture was treated with Ctn or TM19 and applied to the iodixanol density gradient. Fractions were collected after centrifugation, and HBsAg, HBcrAg, NL DNA, and the infectivity of HBV/NL in each fraction were evaluated. In

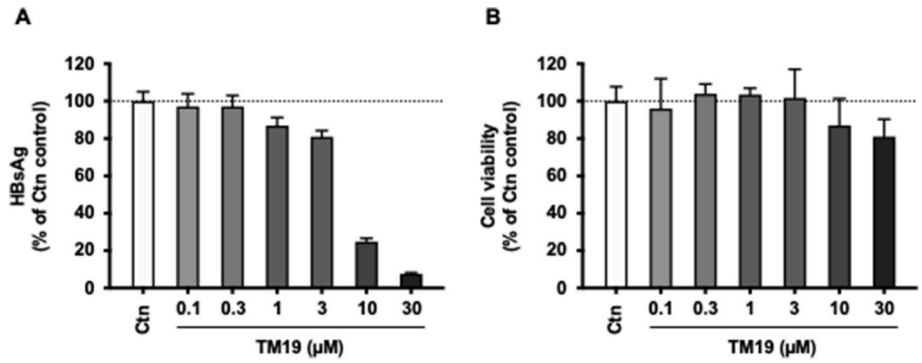

Fig. 4. Dose-dependent anti-HBV effects of peptoid TM19.
(A) To assess the dose dependency, HepG2.2.15-derived HBV was treated with serially diluted peptoid TM19 (30–0.1 μM) for 1 h prior to infection and then used to infect HepG2-NTCP cells at 100 GEq/cell. The infected HBV/NL was washed out at 16 h after infection, and infectivity was estimated by measuring the HBsAg level in the culture medium on Day 12 post-infection. The percentages of HBsAg levels compared to those in Ctn-treated cells are indicated. (B) Cell viability was assessed by the WST-8 assay on Day 12 post-infection. The percentages of absorbance (450 nm) compared to those in the Ctn-treated cells are indicated.

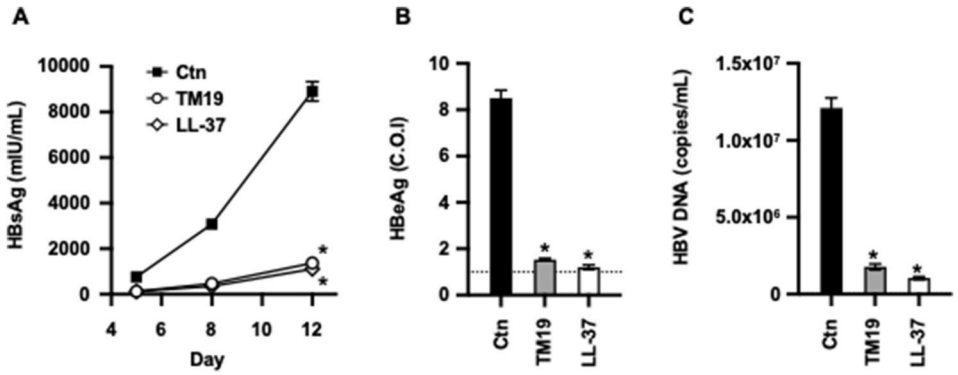

Fig. 5. Anti-HBV effects of TM19 on HBV infection to PXB cells.
(A) The anti-HBV effect of peptoid TM19 on HBV infection in human primary hepatocytes was evaluated. HBV was treated with TM19 or control peptides (10 μM) for 1 h at 37 °C before infection and then infected to PXB cells at 2 GEq/cell with peptoid or control peptides for 16 h, and infected cells were cultured for 11 days without peptoid or control peptides. HBsAg levels in the culture medium were measured on days 5, 8, and 12 after infection. *P < 0.0001 compared to the Ctn control. (B) HBeAg in the culture medium was measured on day 12 after infection after 20-fold dilution. *P < 0.0001 compared to the Ctn control. (C) HBV DNA in the culture medium was measured on day 12 after infection. *P < 0.0001 compared to the Ctn control.

the density gradient profile of Ctn-treated HBV/NL, HBsAg peaked in Fraction 14. HBcrAg had 3 peaks: fractions 8, 14, and 16. NL DNA had 2 peaks: fractions 14 and 16. The peak of infectivity was for Fraction 14. From these data, the infectious particles were mainly in Fraction 14, whereas naked viral particles (unenveloped capsids) were in Fraction 16 (Fig. 6A). After treatment with TM19, the peaks of HBcrAg, NL DNA, and infectivity in Fraction 14 disappeared, though the peak of HBsAg in Fraction 14 was not affected (Fig. 6B). These data indicate that the infectious particles of HBV were disrupted by treatment with TM19.

4. Discussion

In recent years, peptoids, peptide mimics characterized as oligomers of *N*-substituted glycines, have been developed as new drug discovery platforms that can overcome the problems of antimicrobial peptides as therapeutics. Unlike conventional peptides, peptoids have side chains on their backbone amide nitrogens rather than on their backbone α-carbons, rendering them less sensitive to proteases and thus less susceptible to degradation. In this study, we identified the peptoid TM19 as a potent inhibitor of HBV.

By screening using an HBV reporter virus production and infection system, several peptoids, including TM19, were found to reduce infectivity without exerting cytotoxicity. Notably, they did not alter the production levels of HBsAg when used to treat HBV reporter virus-producing cells. HBsAg is the main component of the HBV envelope. However, HBsAg also forms structures smaller than infectious viral particles (Dane particles), such as sphere or filament forms, and these forms are more abundant than Dane particles. This observation suggests that these peptoids only affect the Dane particle but not the number of small structures. To confirm the anti-HBV effects of these peptoids, we selected three peptoids, TM1, TM4, and TM19, and evaluated their anti-HBV effects in a HepG2.2.15-derived HBV infection system that closely mimics authentic HBV infection. Continuous administration of these peptoids before, during, and after infection with HepG2.2.15-derived HBV inhibited infection. Among the evaluated peptoids, TM19 exhibited the most potent anti-HBV effects, with levels of HBsAg reduction in culture supernatants and HBV-infected cell counts comparable to those of LL-37.

The antimicrobial peptide LL-37 exhibits antiviral effects by disrupting the lipid bilayer of the viral envelope (Alagarasu et al., 2017; Henzler Wildman et al., 2003; Matsumura et al., 2016; Tripathi et al., 2013). As with LL-37, we expected the peptoids to target the viral envelope and disrupt infectious viral particle production (Diamond et al., 2021; Tate et al., 2023). Thus, to determine the point at which these peptoids affect the HBV lifecycle, we administered them at different phases in HepG2.2.15-derived HBV infection: pre-treatment, virus treatment, and post-treatment. Among these 3 phases of treatment, treatment of viruses prior to and during infection with these peptoids reduced HBV infection, whereas treatment of cells before and after infection with these peptoids did not reduce HBV infection. These data

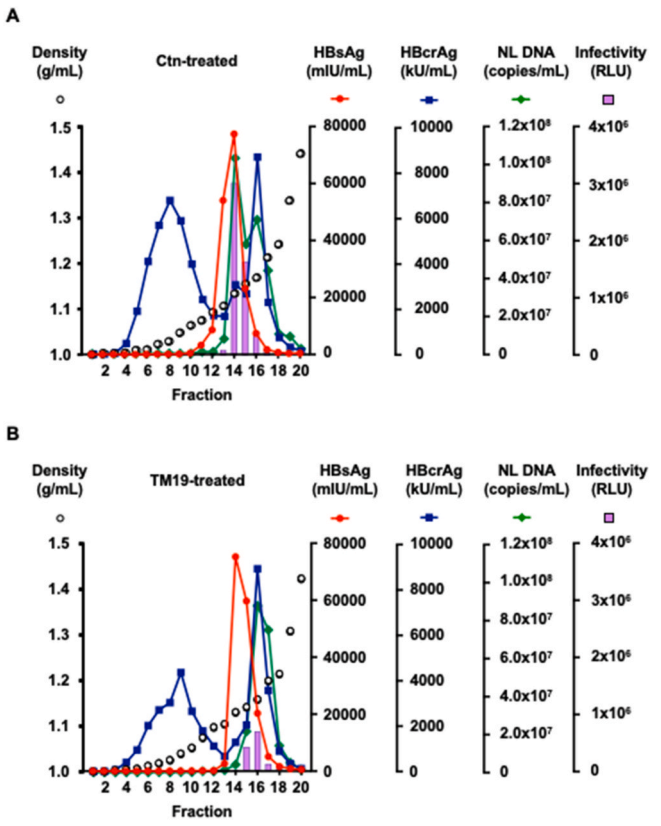

Fig. 6. Iodixanol density gradient analysis of TM19-treated HBV/NL.
HBV/NL was treated with control peptide Ctn (A) or peptoid TM19 (B) at 37 °C for 1 h at a concentration of 10 μM. The treated HBV/NL was analyzed by iodixanol density gradient. Twenty fractions were taken after centrifugation, and levels of HBsAg, HBcrAg, and NL DNA and the infectivity of HBV/NL in each fraction were determined.

suggest that peptoids with anti-HBV effects seem to inhibit the infection step of HBV or, similar to LL-37, disrupt the viral particles themselves. In this evaluation, the most potent anti-HBV effect was found for TM19. Furthermore, the anti-HBV effects of TM19 were dose dependent, and substantial reductions in HBsAg production were detected at concentrations of 10 and 30 μM. In comparison with peptides, peptoids have been proven to have efficient cellular membrane permeability, protease resistance, and low immunogenicity (Astle et al., 2008; Seo et al., 2012). Therefore, peptoids are promising candidates for anti-HBV agents.

The anti-HBV effect of treatment with peptoids coupled with the unaffected HBsAg production level for HBV/NL-producing cells after transfection led us to consider the direct impact on viral particles. Iodixanol density gradient analysis revealed that TM19 treatment eliminated the peaks of infectivity accompanied by peaks of HBcrAg and NL DNA but did not affect the peak of HBsAg. These data suggest that TM19 only disrupts infectious HBV particles (Dane particles) but does not affect the small structures of HBsAg, such as its sphere or filament forms, which are much more abundant in comparison with Dane particles. The difference in structure and lipid distribution between Dane particles and small HBsAg structures may be responsible for the specific disruption of the HBV infectious particles (Gilbert et al., 2005; Satoh et al., 2000). Taken together, the evidence indicates that the peptoid TM19 exhibits anti-HBV effects by eliminating infectious viral particles via disruption of viral envelopes, as reported for other viruses (Diamond et al., 2021; Tate et al., 2023). If the disruption of the envelope of HBV by peptoids could be observed directly by electron microscopy, similar to other viruses, this hypothesis will be proven (Brice et al., 2018; Diamond et al., 2021). Further study will be needed in the future.

In this study, peptoids were revealed to act as inhibitors of HBV infection. The clinical importance of entry inhibitors in chronic hepatitis B patients is considered to be limited. The well-investigated entry inhibitor myrcludex-B results in limited improvement in clinical conditions and HBV DNA and HBsAg levels (Bogomolov et al., 2016). On the other hand, the clinical significance of HBV entry inhibitors is firmly established in preventing mother-to-infant transmission or recurrence of hepatitis B after liver transplantation. In such situations, hepatitis B immunoglobulin (HBIG) has been approved and is used to prevent HBV infection (Doi and Kanto, 2021; Ishigami et al., 2015; Yi et al., 2016; Zoulim, 2003). However, it is expensive because it is fabricated from biomaterials. Therefore, the use of chemically synthesized peptoids as alternatives to HBIG may be considerably advantageous. In this study, TM19 exhibited slight cytotoxicity at the highest dose (30 μM), although it was not significant. Even if it is not so severe, it cannot be denied that such cytotoxicity may lead to potential side effects if it is used clinically. Thus, further screening or modification of TM19 may be necessary to identify more promising candidates with reduced cytotoxicity and/or improved inhibitory effect to increase the therapeutic window.

5. Conclusion

The anti-HBV peptoid TM19 was identified as a potent entry inhibitor of HBV via a viral-targeting strategy. This peptoid prevents HBV infection by disrupting viral particles without cytotoxic effects. Such peptoids are candidates for a new class of anti-HBV agents.

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CRediT authorship contribution statement

Asako Murayama: Formal analysis, Investigation, Methodology, Writing – original draft. **Hitomi Igarashi:** Data curation, Investigation. **Norie Yamada:** Investigation. **Hussein Hassan Aly:** Investigation. **Natalia Molchanova:** Resources. **Jennifer S. Lin:** Resources, Writing – review & editing. **Hironori Nishitsuji:** Resources, Methodology. **Kunitada Shimotohno:** Resources, Methodology. **Masamichi Muramatsu:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Annelise E. Barron:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Takanobu Kato:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

A.E.B. is a shareholder and member of the Board of Directors of

Maxwell Biosciences; N.M. is a consultant for Maxwell Biosciences.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2024.105821.

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