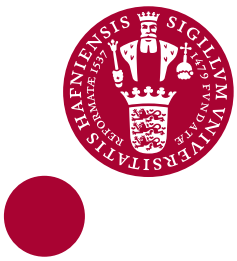

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Role of Cell-Penetrating Peptides in Intracellular Delivery of Peptide Nucleic Acids Targeting Hepadnaviral Replication

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Peptide nucleic acids (PNAs) are potentially attractive antisense agents against hepatitis B virus (HBV), although poor cellular uptake limits their therapeutic application. In the duck HBV (DHBV) model, we evaluated different cell-penetrating peptides (CPPs) for delivery to hepatocytes of a PNA-targeting hepadnaviral encapsidation signal (ε). This anti-ε PNA exhibited sequence-specific inhibition of DHBV RT in a cell-free system. Investigation of the best in vivo route of delivery of PNA conjugated to $(D\text{-}Arg)_8$ (P1) showed that intraperitoneal injection to ducklings was ineffective, whereas intravenously (i.v.) injected fluorescein-P1-PNA reached the hepatocytes. Treatment of virus carriers with i.v.-administered P1-PNA resulted in a decrease in viral DNA compared to untreated controls. Surprisingly, a similar inhibition of viral replication was observed in vivo as well as in vitro in primary hepatocyte cultures for a control 2 nt mismatched PNA conjugated to P1. By contrast, the same PNA coupled to $(D\text{-}Lys)_4$ (P2) inhibited DHBV replication in a sequence-specific manner. Interestingly, only P1, but not P2, displayed anti-DHBV activity in the absence of PNA cargo. Hence, we provide new evidence that CPP-PNA conjugates inhibit DHBV replication following low-dose administration. Importantly, our results demonstrate the key role of CPPs used as vehicles in antiviral specificity of CPP-PNA conjugates.

INTRODUCTION

Hepatitis B virus (HBV) remains the major cause of chronic hepatitis, still accounting for at least 600,000 deaths per year worldwide.[1] Currently, pegylated interferon-α and nucleos(t)ide analogs (NUCs) are the only drugs approved for the treatment of chronic HBV infection.[2,3] However, these NUCs targeting viral polymerase only have a virostatic effect because rebound of viral replication is common after therapy cessation.[4] The global prevalence of HBV infection together with the lack of effective treatment warrants the search for novel therapeutic options as a functional cure for chronic hepatitis B.

In this context, peptide nucleic acids (PNAs) represent promising candidates as inhibitors of HBV replication. PNAs are synthetic DNA mimics having a pseudopeptide backbone that are able to bind with high affinity to complementary DNA or RNA. Furthermore, their remarkable stability against enzymatic degradation in biological fluids makes them potentially attractive antiviral compounds.[5–9] PNAs are currently considered as "third generation" antisense and anti-gene agents. Importantly, PNAs can be designed to target other stages of HBV replication than viral polymerase, thus limiting the selection of drug-resistant mutants. However, limiting cellular uptake, owing to the fact that they are large hydrophilic molecules, has challenged the broad application of unmodified PNA as antiviral agents.

In the past decade, a series of natural and synthetic cell-penetrating peptides (CPPs) have been discovered and applied as membrane-permeable delivery vehicles for a wide range of cargo, including PNAs.[10–12] CPPs typically contain clusters of arginine or lysine residues sharing a small size, less than 30-aa residues, and cationic charge. Different studies revealed the potential of CPPs as promising nonviral gene carriers being used as "Trojan horses" to introduce therapeutically relevant cargo into cells. Indeed, oligoarginines are internalized by mammalian cells, and their ability to increase cellular uptake and antisense activity of CPP-PNA conjugates has been convincingly demonstrated in several studies.[13–15] The tetralysine tail was previously found to be important for the antisense activity of PNAs in cells and in vivo in a mouse model.[16,17] Additionally, the HIV-1-derived TAT peptide was recently shown to enhance intracellular distribution and antisense activity of a PNA oligomer targeting a direct repeat sequence of HBV following hydrodynamic injection of viral genomes into mice.[18] However, studies on the therapeutic efficacy of CPP-PNA

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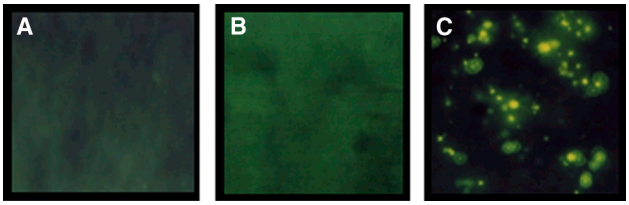

Figure 1. Uptake of CPP-PNA Conjugates following Different Routes of In Vivo Delivery

Representative fluorescence microscopy images of liver sections 48 hr after i.v or i.p. injection of fluorescein-PNA coupled to P1 $(D\text{-}Arg)_8$. (A–C) Fluorescent staining of livers from control uninjected duckling (hepatocyte autofluorescence) (A) or ducklings injected with fluorescein-P1-PNA via i.p. route (B) or via i.v. route (C).

conjugates have so far exclusively been conducted in mouse models, and the ability of these conjugates to inhibit the full replication cycle of hepadnaviruses in vitro and in vivo has not yet been investigated.

In the present study, we explored the ability of an antisense PNA targeting the duck HBV (DHBV) ε coupled to different CPPs to inhibit hepadnaviral replication. The DHBV model, a reference for evaluation of novel anti-HBV approaches, provides a unique opportunity to study the antiviral potential of CPP-PNA conjugates in vitro in primary duck hepatocyte culture (PDH) as well as in vivo in Pekin ducklings.[19] Having previously demonstrated the ability of this PNA-targeting hepadnaviral encapsidation signal ε to inhibit viral RT in a sequence-specific manner in a rabbit reticulocyte extract,[20] we conjugated this PNA to CPPs to increase hepatocyte penetration in a DHBV context because the major challenge of therapeutic PNA application is their inefficient cell uptake.[14]

We first defined the most effective in vivo route of delivery using a fluorescein-labeled CPP-PNA conjugate administrated to uninfected animals. On the basis of our previous results, we selected $(D\text{-}Arg)_8$ as CPP for PNA coupling, which exhibited a weaker inhibitory activity on DHBV egress compared to Decanoyl$(Arg)_8$.[21] Next, we compared the ability of different CPP-PNA conjugates to inhibit DHBV replication in vitro and in vivo. We report herein that intravenously (i.v.) injected $(D\text{-}Arg)_8$-PNA, in contrast to the intraperitoneal (i.p.) route of delivery, reached the liver and inhibited DHBV replication. Notably, our study also revealed that the choice of CPP as a vehicle for cellular delivery of its PNA cargo plays a crucial role in the specificity of hepadnaviral replication inhibition by CPP-PNA conjugates.

RESULTS

Optimization of CPP-PNA Conjugate Delivery Route In Vivo

To optimize the conditions for PNA in vivo administration to ducklings, the i.v. and i.p. PNA delivery route were investigated. The fluorescein-PNA coupled to P1 CPP was i.v. or i.p. injected into DHBV-free ducklings, and the hepatocyte-associated fluorescence was analyzed by fluorescence microscopy 48 hr later. As illustrated in Figure 1, little or no cell-associated fluorescence was detected after i.p. injection of fluorescein-P1-PNA (Figure 1B). By contrast, the fluorescein-PNA coupled to P1 CPP was efficiently delivered to the liver following i.v. injection (Figure 1C). This indicates that the injection via i.v. route of fluorescein isothiocyanate (FITC)-PNAs coupled to a cationic CPP led to a better liver delivery than the i.p. route. Fluorescence was predominantly detected in the hepatocytes and to some extent also in the kidney and spleen but not in the lungs.

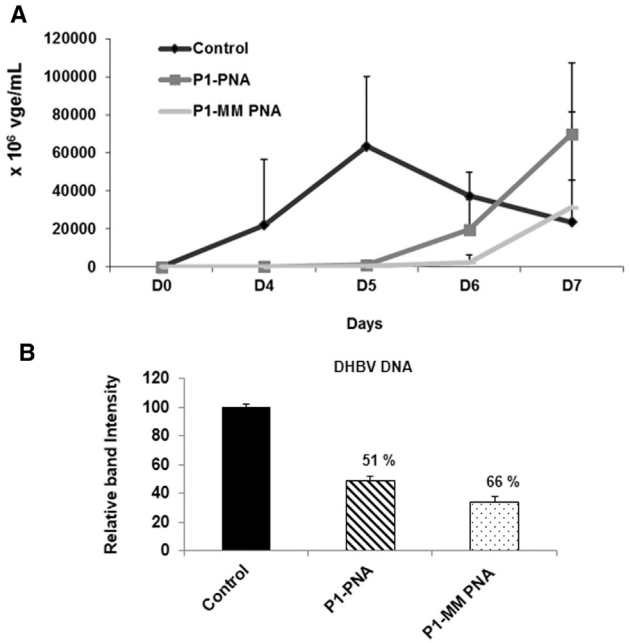

Figure 2. Effect of P1-PNA Conjugates on Viral Replication In Vivo

DHBV-infected ducklings received an i.v. injection of CPP-PNA conjugates daily during 6 consecutive days. (A) Serum DHBV DNA was monitored by dot-blot hybridization over a 6-day time course of treatment. The mean DHBV DNA titers in vge/mL for each group of ducks quantified by PhosphorImager scanning is represented. (B) Viral DNA was analyzed in liver samples at the end of a 6-day treatment with P1-PNA or P1-MM PNA. PhosphorImager quantifications of all DHBV DNA replicative forms from Southern blot analysis of intrahepatic DNA are represented and expressed as a percentage of inhibition, considering untreated controls as 100%. Percentages of inhibition are indicated.

Inhibition of DHBV Replication In Vivo by P1-PNA Conjugates and P1 Alone

DHBV-infected ducklings were randomly assigned into different treatment groups that received antisense PNA, as described in the therapeutic protocol. Viremia was followed daily in ducklings from all groups. The follow up of serum DHBV DNA titers in the untreated controls showed the typical evolution of viremia in the DHBV-infected ducklings, reaching a peak value at day 5 post-inoculation (p.i.), followed by a decrease starting from day 5 p.i. (Figure 2A). The administration of P1-PNA conjugates (1 μg/g/body weight [bw]/day) led to delayed and reduced viremia in treated ducklings, as measured by the decrease in the total virus release during the treatment, compared with the untreated controls. However, unexpectedly, the administration of a control mismatched PNA differing by only 2 nt and coupled to the same CPP (P1-MM-PNA conjugate) also

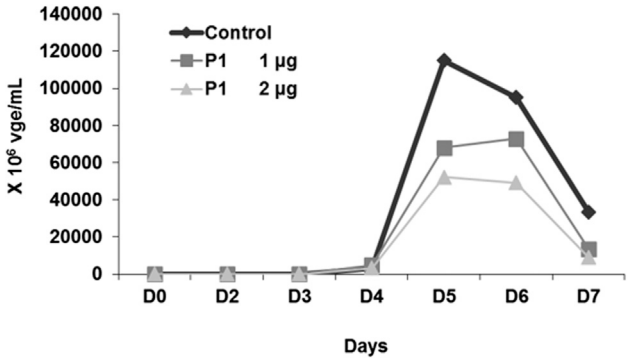

Figure 3. Effect of P1 Alone on Viremia

Serum DHBV DNA was analyzed by dot-blot hybridization during a 6-day time course of P1 $(D\text{-}Arg)_8$ administration at 1 or 2 µg/gbw/day to virus-infected ducklings. The mean DHBV DNA titers in vge/mL for each group of ducks are represented.

induced a decrease and delay in viremia in treated animals (Figure 2A).

Next, we analyzed the impact of treatment on intrahepatic viral replication. As illustrated in Figure 2B, the analysis of liver DNA showed that treatment with P1-PNA conjugates and its corresponding P1-MM PNA decreased viral DNA synthesis by 51% and 66%, respectively, compared to controls. Because the treatment with the 2 nt mismatched PNA sequence also led to a decrease in serum and liver DHBV DNA, we asked whether the observed inhibition of viral replication was due to an antiviral effect of P1 CPP alone. To this end, a similar experiment was performed as described above but using P1 alone. Briefly, DHBV-infected ducklings were i.v. treated with 1 or 2 µg/gbw/day of P1 for 6 days. Interestingly, the injection of 1 or 2 µg/gbw/day of P1 induced a decrease, in a dose-dependent manner, of viremia in treated animals compared with the untreated controls (Figure 3). No loss of weight was noticed within the different treatment groups compared with the untreated controls during the follow-up period (data not shown), indicating the absence of in vivo toxicity of P1-PNA conjugates or P1 CPP alone.

Altogether, these data implied that treatment of DHBV-infected ducklings by an anti-ε P1-PNA conjugate or its 2 nt MM PNA coupled to the same P1 CPP decreased DHBV replication in vivo. Because the P1 alone inhibited DHBV replication in vivo, this may explain the limited sequence specificity of P1-PNA conjugates.

In Vitro Effect of P1-PNA Conjugates or P1 CPP on DHBV Replication

To further investigate the inhibitory effect of P1-PNA conjugates or P1 alone, we have evaluated their antiviral efficacy in vitro in DHBV-infected primary hepatocyte cultures. The analysis of PDHs treated with P1-PNA conjugates or P1-MM PNA showed a similar decrease in the release of viral particles into cell culture supernatants, which was estimated to be 47% and 52% of inhibition, respectively, compared with untreated controls (Figures 4A and 4B). In addition, these results were confirmed by a decrease in all intracellular viral DNA replicative intermediates by about 59% and 60%, respectively, in P1-PNA conjugates or P1-MM PNA-treated cells (Figure 4C). Moreover, treatment of PDH by P1 alone also led to a decrease in DHBV DNA levels in both supernatants and cells by 37% and 44%, respectively, compared with the untreated controls (Figures 4A–4C). The observed antiviral effect was not related to any general cytotoxicity of the peptide or peptide-PNA conjugates because P1-PNA, P1-MM PNA conjugates, or P1 itself did not display significant toxicity in PDH cultures, as assessed by Neutral Red uptake test (Figure S1).

Thus, the in vitro study corroborated the inhibitory effect of P1-PNA and P1-MM PNA conjugates on DHBV replication observed in vivo. The cationic CPP $(D\text{-}Arg)_8$ alone, in the absence of its PNA cargo, exhibited anti-DHBV activity both in vitro in PDH and in vivo in ducklings, thereby suggesting that this antiviral effect may affect the antiviral specificity of CPP-PNA conjugates.

Antiviral Effect of CPP-PNA Conjugates Depends of CPPs Used as a Vehicle

To further explore the role of CPP used as a vehicle for PNA-mediated inhibition of DHBV replication in PDH, we analyzed the antiviral effect of the same anti-ε PNA, but coupled to another cationic CPP $(D\text{-}Lys)_4$ termed P2. In addition, the antiviral effect of this CPP alone was investigated. By using the same experimental approach as described above, the ability of this CPP and CPP-PNA conjugates to inhibit DHBV replication was analyzed in PDH cultures. As illustrated in Figure 5, treatment with P2-PNA conjugates led to the inhibition by about 48% of DHBV release in cell culture supernatants. In addition, Southern blot analysis showed a similar 48% decrease of intracellular DHBV DNA. Importantly, the inhibition was specific because a 2-base mismatch PNA conjugated to the same P2 CPP showed no marked inhibitory effect on released DHBV and on intracellular viral replication (Figures 5A–5C). Moreover, treatment with P2 alone resulted in neither the marked decrease of DHBV in cell culture supernatants nor the intracellular DHBV DNA content (Figure 5 and data not shown). These results, reproducibly observed in two independent experiments, are consistent with the conclusion that the antiviral effects of the CPP as such is responsible for the reduced sequence-specific activity of CPP-PNA conjugates. In contrast, in the absence of such inhibitory CPP action, the CPP-PNA conjugates are able to specifically inhibit DHBV replication.

Neither the $(D\text{-}Lys)_4$-PNA conjugate nor $(D\text{-}Lys)_4$ alone exhibited toxicity in PDH cultures, as assessed by Neutral Red uptake test (data not shown).

DISCUSSION

Understanding structure-activity relations is crucial for the development of CPP-PNA conjugates as antiviral agents. In this preclinical study, we explored the ability of CPP-PNA conjugates targeting the hepadnaviral encapsidation signal ε to inhibit viral replication in vitro and in vivo. Because the human HBV has an extremely

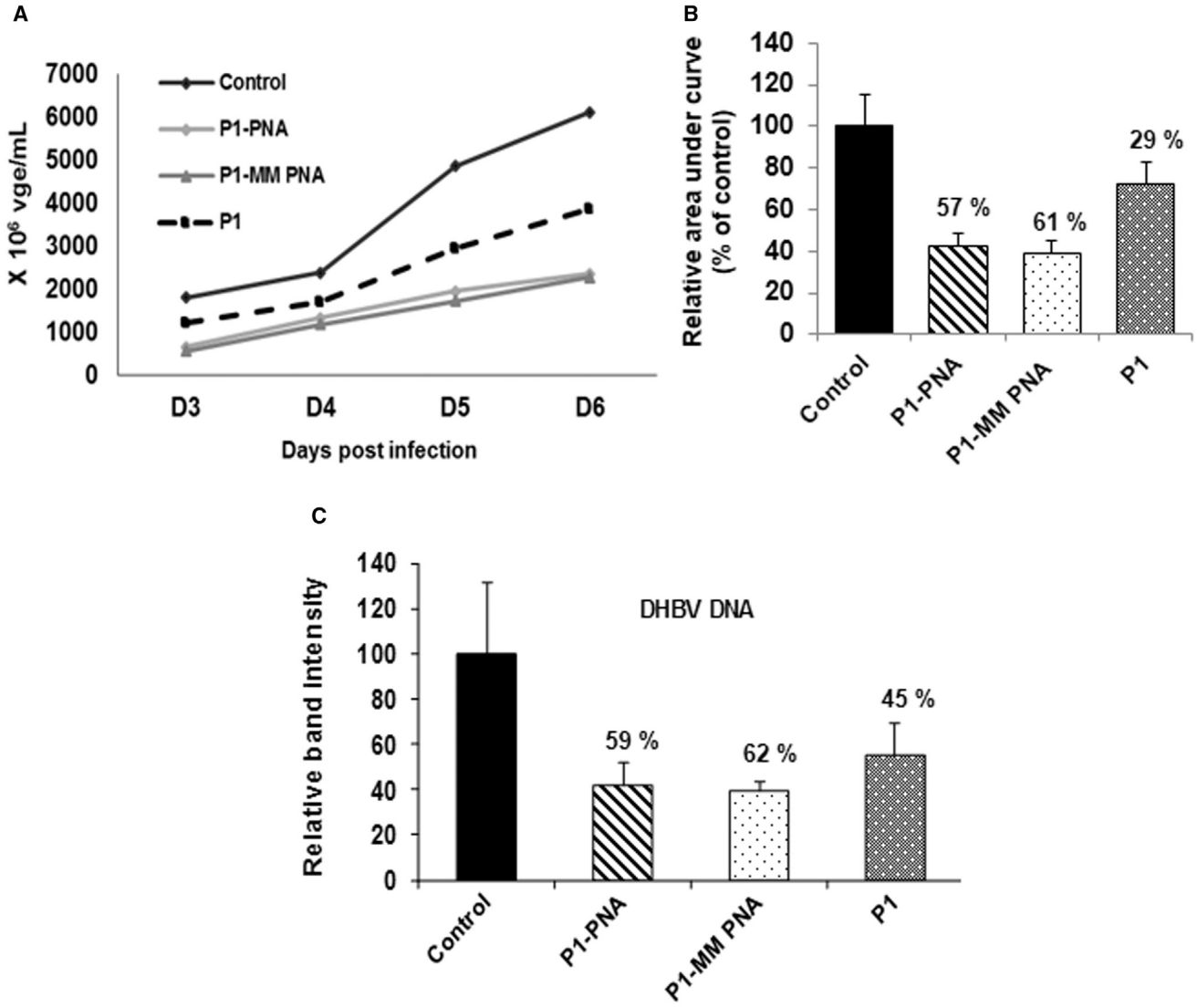

Figure 4. Analysis of Viral Replication In Vitro in PDH Cultures following Treatment with P1-PNA Conjugates or P1 Alone

Primary duck hepatocyte cultures were plated and infected with DHBV, followed by treatment with P1-PNA, P1-MM PNA, or P1 alone. (A) The viral release in supernatants of DHBV-infected PDHs was monitored between days 3 and 6 post-infection by dot-blot hybridization and quantified by PhosphorImager. (B) Relative areas under curve of viral release determined by the means of duplicate and compared to untreated cells (set as 100%) are represented. (C) PhosphorImager quantifications of DHBV DNA replicative forms from Southern blot analysis of intracellular DNA. The mean relative band intensity for each group was normalized against untreated control cells (set as 100%) and SDs are shown. Percentages of inhibition are indicated.

narrow host range, we used DHBV-infected PDH culture and Pekin ducks as model systems. The DHBV represents a reference model validated by us and others for the evaluation of novel anti-HBV approaches in preclinical studies.[19–27]

Using this in vivo model, our team has previously demonstrated that i.v. delivery of antisense phosphodiester oligodeoxynucleotides (ODNs) complexed to polyethylenimine can selectively block hepadnaviral replication in the liver.[23] Among various antisense approaches, PNAs are currently being considered as particularly promising third-generation antisense agents due to their unique structural features, high sequence selectivity, and very high biostability.[5,9] These findings provide a rationale for the development of an innovative, PNA-based approach for chronic hepatitis B treatment. We have initially shown that antisense PNA targeting the DHBV ε exhibits a potent, highly sequence-specific inhibitory effect on the DHBV RT in a cell-free transcription and translation system.[20]

Because one of the major limitations regarding therapeutic use of PNAs is poor transport across the cell membrane, we focused herein

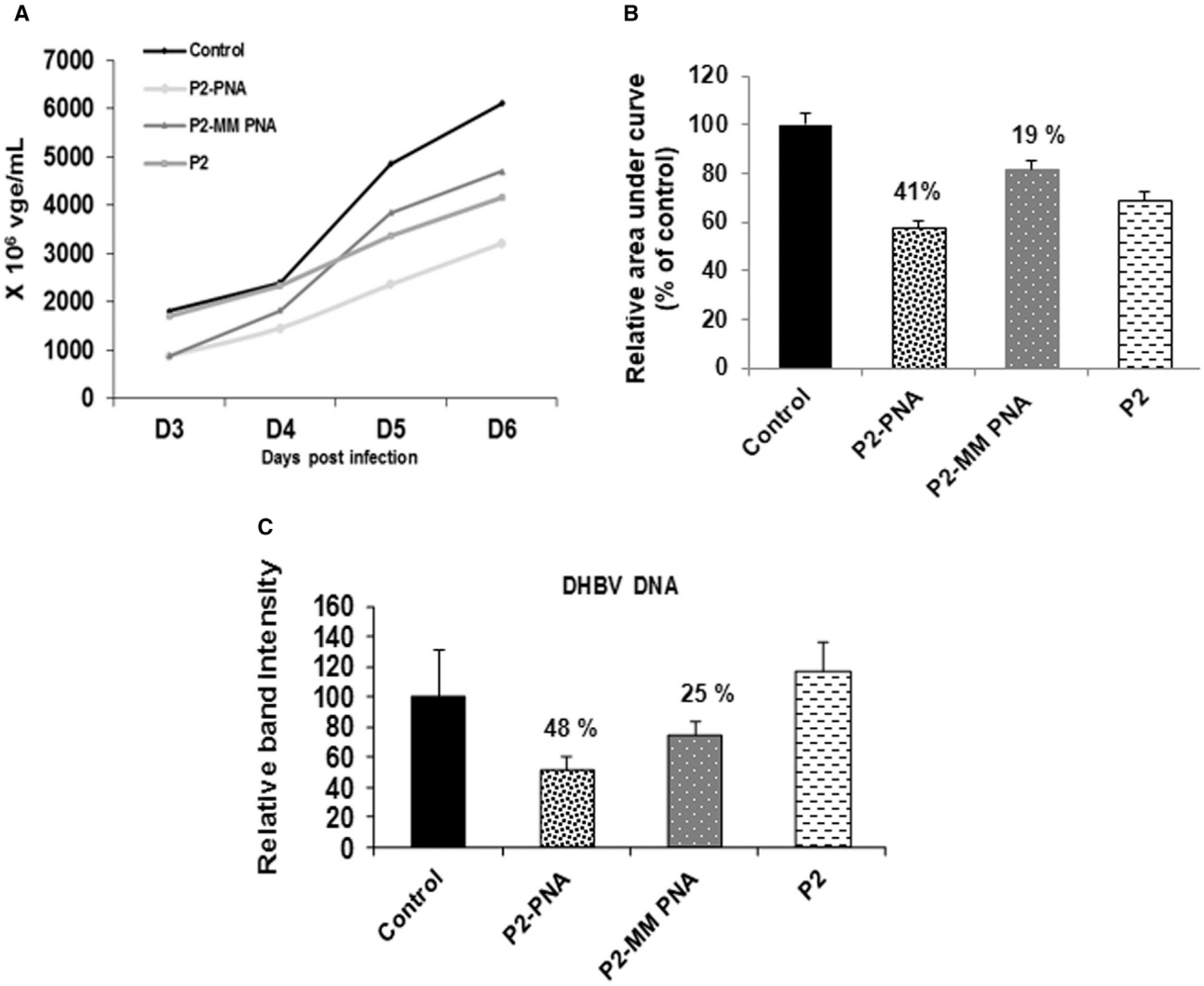

Figure 5. Effect of Treatment with P2-PNA Conjugates or P2 Alone on Viral Replication In Vitro in PDH Cultures

PDH cultures were plated, infected with DHBV, and treated with P2(D-Lys)$_4$-PNA, P2-MM PNA, or P2 alone. (A) Cell supernatants were collected and DHBV DNA was quantified between days 3 and 6 post-infection by dot-blot hybridization and quantified by PhosphorImager scanning. (B) Relative areas under the curve of viral release determined by the means of duplicate and compared to untreated cells (set as 100%) are shown. (C) Quantifications by PhosphorImager of DHBV DNA replicative forms from Southern blot analysis of intracellular DNA. The mean relative band intensity relative to untreated controls (set as 100%) and SDs are represented. Percentages of inhibition are represented.

on the intracellular delivery of anti-ε PNA coupled to CPPs such as oligoarginine or oligolylysine. Indeed, it has been reported that oligoarginine conjugation greatly enhances the cellular delivery and antisense activity of PNA.[15,28–30] In addition, PNAs bearing oligolysine tails exhibit enhanced cellular uptake, without compromising sequence selectivity.[16,17,31,32] However, in spite of numerous studies aiming at elucidating the mechanisms of CPP-PNA conjugate entry and uptake in cultured cells, data on in vivo activity of such conjugates are scarce and essentially limited to mouse models.[18,33,34]

In the present study, we initially examined the ability of PNA targeting the DHBV encapsidation signal ε to inhibit viral replication in vivo by comparing the uptake of fluorescein-labeled PNA coupled to P1 (D-Arg)$_8$ following i.p. or i.v. administration. Unlike the mouse model, in which an i.p. injection led to an effective PNA delivery,[34] we demonstrate here that this route of delivery was inefficient in the duck model. Indeed, hepatocyte-associated fluorescence was only observed following i.v. injection of fluorescein-P1-PNA into ducklings. Collectively, these results suggest that the pharmacokinetics of PNA may differ according to the mode of injection and animal model used.

Importantly, using a low CPP-PNA conjugate dose (1 μg/gbw/day), we found efficient liver uptake of such conjugates associated with

Table 1. Sequences of PNA and CPP-PNA Conjugates

Designation	CPP Alone or Conjugate	Sequence of Peptide or CPP-PNA Conjugates
P1	$(D\text{-}Arg)_8$	RRRRRRRRG
P2	$(D\text{-}Lys)_4$	KKKKG
Fluorescein-PNA	Flk-$(D\text{-}Arg)_8$-PNA	Flk-RRRRRRRRG-gcaatgtagacgtaa-K-NH_2
P1-PNA	$(D\text{-}Arg)_8$-PNA	RRRRRRRRG-gcaatgtagacgtaa-K-NH_2
P1-MM PNA	$(D\text{-}Arg)_8$-MM PNA	RRRRRRRRG-gcaaggtagacttaa-K-NH_2
P2-PNA	$(D\text{-}Lys)_4$-PNA	KKKKG-gcaatgtagacgtaa-K-NH_2
P2-MM PNA	$(D\text{-}Lys)_4$-MM PNA	KKKKG-gcaaggtagacttaa-K-NH_2

Sequences are shown in uppercase letters for peptides and in lowercase letters for PNA. PNAs are written from their N to C termini, and the N terminus corresponds to the 5′ end of a conventional oligonucleotide. The PNA sequence targets the epsilon of the DHBV sequence (position 2573–2587). The replaced nucleotides in the mismatched PNA are underlined. Flk, ε-fluoresceinated lysine.[45]

an antiviral effect following i.v. injection in ducklings. This is of particular interest because to date, the in vivo route of delivery and antisense efficacy of CPP-PNAs has exclusively been investigated in mice,[34] which weigh less than 30 g. For comparison, the neonatal Pekin ducklings used as a model in the present study grow extremely fast, weighing about 50 g at the beginning of treatment and reaching 150 g by its end (day 6). Thus, we provide novel evidence that i.v. injected CPP-PNA conjugates can be delivered into hepatocytes and exhibit a biological effect in larger animals than mice.

One of the main objectives of this study was to investigate whether the injection of CPP-PNA conjugates was able to inhibit hepadnaviral replication. The target region of antisense PNA was the bulge (asymmetric internal loop) and upper stem of the DHBV encapsidation signal ε located at the pregenomic viral RNA. Because ε is essential for hepadnavirus replication and RT of pregenomic RNA,[35,36] it is not surprising that the release of viral particles was markedly decreased. Moreover, all intrahepatic viral DNA forms, including the covalently closed circular DNA (cccDNA), were decreased following in vivo treatment with CPP-PNA conjugates. However, the 2-base mismatched PNA coupled to the same CPP (P1) and used as specificity control exhibited an almost similar antiviral effect. This was a rather unexpected finding because we have previously demonstrated that the same double-mismatched PNA, tested in the absence of CPP in a cell-free system for DHBV polymerase expression, had no effect on the RT reaction.[20] Thus, an inhibitory effect on viral replication of the CPP vehicle $(D\text{-}Arg)_8$ alone, affecting the specificity of CPP-PNA conjugates, was suspected. Indeed, it has been shown that some natural CPPs, which are rich in basic amino acids, exhibit different biologic activities, such as an antifungal or antimicrobial effect.[37] In addition, the antiviral activity of some CPPs has been reported toward enveloped viruses such as HSV and HIV[38–40] and, more recently by us, for a CatLip displaying an inhibitory effect on hepadnaviral secretion.[21,41] However, the contribution of the antiviral activity of CPPs to the antisense potential of CPP-PNA conjugates was not explored.

To address this issue, we compared the in vitro antiviral activity of anti-ε PNA conjugated to different CPPs using a PDH culture. Interestingly, our in vitro results were consistent with and reinforced the in vivo data because $(D\text{-}Arg)_8$ P1-PNA conjugates as well as 2 nt MM PNA conjugated to P1 and P1 alone inhibited DHBV replication in PDHs. By contrast, the same PNA targeting ε, but coupled to another CPP $(D\text{-}Lys)_4$ (P2), inhibited DHBV replication in a sequence-specific manner because neither P2-MM PNA conjugates nor P2 alone exhibited significant anti-DHBV activity. Collectively, our results suggest that the choice of a CPP used as a vehicle for intracellular delivery of PNAs may play an essential role in the ability of CPP-PNA conjugates to specifically inhibit hepadnaviral replication. Because some CPPs, such as $(D\text{-}Arg)_8$, exhibit an inhibitory effect on DHBV replication, this can have a major impact on antiviral activity and specificity of CPP-PNA conjugates. In the absence of significant antiviral activity of CPP, a sequence-specific inhibition of DHBV replication was observed for $(D\text{-}Lys)_4$ conjugated to the same PNA. A better understanding of the antiviral activities of different CPPs is essential for their therapeutic application as transport vehicles for PNA delivery, warranting further studies aiming at evaluating their mechanisms of action. In this regard, in vitro studies with additional controls to discriminate between peptide activity and PNA binding to a target viral nucleic acid sequence will be of particular importance.

Taken together, we provide here the first evidence that CPP-PNA conjugates injected i.v. can enter hepatocytes and inhibit hepadnaviral replication in vivo at a low dose, without observed toxicity, suggesting the potential usefulness of this approach for hepatitis B therapy. Importantly, our data strongly suggest that the difference in antiviral activity of some CPPs, in the absence of their PNA cargo, may play a key role in CPP-PNA conjugate specificity. Moreover, the results presented here demonstrate that conjugation of anti-ε PNA to $(D\text{-}Lys)_4$ led to the inhibition of hepadnaviral replication, without compromising sequence specificity. The anti-HBV potential of such and similar CPP-PNAs needs to be further explored in association with new-generation NUCs, novel inhibitors targeting viral morphogenesis and immune modulators in the context of innovative therapeutic strategies aiming at achieving a functional cure for chronic hepatitis B.

MATERIALS AND METHODS

Synthesis of PNA and CCP-PNA Conjugates

The PNAs were synthesized by Boc-solid phase chemistry as previously described,[15,42] and sequences are summarized in Table 1.

Virus

A pool of viremic sera from ducklings infected with the cloned and sequenced DHBV was used as an inoculum.[43] This inoculum was quantified into virus genome equivalents (vge) by quantitative dot-blot hybridization, as described previously.[22,23]

Animals

3-day-old DHBV-free Pekin ducklings originating from a commercial supplier were i.v. inoculated with a DHBV-positive serum

(4×10^9 vge per duck), as previously described.[22,24] Animal experimentation was performed in accordance with the guidelines of animal care and ethics of the National Veterinary School of Lyon (VetAgro Sup, Marcy L'Etoile, France).

PDH Cultures

Fetal hepatocytes were isolated from Pekin duck embryos as previously described.[43] DHBV infection was performed in serum-free William's medium, 24 hr after plating by incubation of hepatocytes with a pool of DHBV-positive duck serum (50 vge per cell). Hepatocytes were seeded on collagen-coated 12-well plates (Biocoat, Becton Dickinson, Heidelberg, Germany) at a density of 1×10^6 cells per well, and the growth medium was supplemented with 5% fetal calf serum and changed daily. Antisense PNAs (2 μM final) conjugated to CPPs or CPPs alone (2 μM final) were added to the culture medium daily starting from 2 hr post-infection, as previously described.[21] The PDHs were treated during 6 days and lysed on day 7, followed by viral replication analysis. Each experiment was repeated three to four times. The cellular toxicity was analyzed by daily examination of cells with a light microscope and cell toxicity test based on the neutral red dye uptake, as described.[44]

Optimization of CPP-PNA Conjugate Delivery In Vivo

For the in vivo study of PNA distribution, two different delivery routes were tested, i.e., the i.v. route via the occipital sinus and the i.p. route. 1 μg/gbw/day of fluorescein-labeled PNA coupled to $(D\text{-}Arg)_8$ CPP was injected via either the i.v. or i.p. route to two groups of four ducklings. 48 hr after fluorescein-PNA injection, all animals were sacrificed and frozen liver, spleen, lung, and kidney sections were analyzed by fluorescence microscopy (Leica, France, DMLB 100), as previously described.[23] The distribution of PNA administrated by both routes was compared.

Therapeutic Protocol

DHBV-injected ducklings were randomly assigned to 3 groups of four animals, which were either treated with CPP-PNA conjugates (1 μg/gbw/day) or untreated. Ducklings received a daily i.v. injection of CPP-PNA conjugates or CPP alone into the occipital sinus starting 2 hr after DHBV infection and daily during 6 consecutive days. Blood samples were collected daily during the viremia follow up. The toxicity was evaluated by daily follow up of animal weight. All animals were sacrificed at the end of therapy (day 6 p.i.), and liver necropsy samples were snap-frozen and stored at −80°C for molecular analysis.

Detection of Viral DNA

DHBV DNA was detected in duck serum or supernatant of PDH by dot-blot hybridization by using a full-length ^{32}P-labeled DHBV probe, as described previously.[24] Total liver DNA was obtained from 0.2 g of frozen tissue homogenized in liquid nitrogen as described.[24] DNA was extracted from frozen necropsy liver samples or PDH and 10 μg of the total DNA were subjected to electrophoresis on 1% agarose gel, followed by Southern blot analysis and hybridization with a ^{32}P-labeled genomic DHBV probe as previously described.[24,25] The EcoRI-linearized, cloned DHBV DNA was used as an additional control. Viral DNA was quantified by PhosphorImager scanning using ImageQuant software (Molecular Dynamics).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at https://doi.org/10.1016/j.omtn.2017.09.003.

AUTHOR CONTRIBUTIONS

B.N., N.R., and C.J. conducted the experiment. G.J.L., P.E.N., and L.C. designed the experiment and wrote the paper.

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