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# Lipid nanoparticle-encapsulated, chemically modified anti-adenoviral siRNAs inhibit hepatic adenovirus infection in immunosuppressed Syrian hamsters

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**RNA interference has demonstrated its potential as an antiviral therapy for treatment of human adenovirus (hAd) infections. The only existing viral vector-based system for delivery of anti-adenoviral artificial microRNAs available for *in vivo* use, however, has proven to be inefficient in therapeutic applications. In this study, we investigated the potential of stabilized small interfering RNA (siRNA) encapsulated in lipid nanoparticles (LNPs) for treatment of hepatic hAd serotype 5 (hAd5) infection in an hAd infection model using immunosuppressed Syrian hamsters. The siRNA sipTP$_{mod}$ directed against the adenoviral pre-terminal protein (pTP) and containing 2′-*O*-methyl modifications as well as phosphorothioate linkages effectively inhibited hAd5 infection *in vitro*. In light of this success, sipTP$_{mod}$ was encapsulated in LNPs containing the cationic lipid XL-10, which enables hepatocyte-specific siRNA transfer, and injected intravenously into hAd5-infected immunosuppressed Syrian hamsters. This resulted in a significant reduction of liver hAd5 titers, a trend toward reduced liver injury and inflammation, and reduction of viral titers in the blood and spleen compared with hAd5-infected animals that received a non-silencing siRNA. These effects were demonstrated in animals infected with low and moderate doses of hAd5. These data demonstrate that hepatic hAd5 infection can be successfully treated with anti-adenoviral sipTP$_{mod}$ encapsulated in LNPs.**

## INTRODUCTION

Human adenoviruses (hAds) belong to the family Adenoviridae within the genus Mastadenovirus and can be divided into seven species (A–G), made up of more than 100 different types.[1] Humans of any age can be infected with hAds, but young children and infants are most often affected.[2] Because of genetic heterogeneity resulting in different tissue tropisms, hAds cause various organ infections, mainly affecting the respiratory tract, the eyes, and the intestine,[3] but infections of the genitourinary tract, the heart, the brain, and the liver have also been observed.[2] In patients with an intact immune system, these infections are generally acute and self-limiting with mild symptoms, which is why they are usually treated symptomatically. In contrast to this course of infection, in immunocompromised patients, hAds can induce severe disease; for example, in patients with congenital immunodeficiency, after infection with human immunodeficiency virus, in children receiving chemotherapy for hematological cancer diseases, and in solid organ transplant recipients.[4–7] Allogeneic hematopoietic stem cell transplantation (HSCT) patients, usually young children, represent a group with a particularly high risk for life-threatening infection with hAds.[8,9] For example, Fisher et al.[10] found that, among 191 allogeneic HSCT recipients, 58 (30.4%) were infected with hAds. Fifteen of the patients died, and two-thirds of these deaths were related to progression of hAd disease.[10] Causes of death include multiorgan failure because of disseminated hAd infection or liver failure because of massive hAd replication and liver tissue damage.[11–14]

There is no US Food and Drug Administration (FDA)- or European Medicines Agency (EMA)-approved antiviral therapy for the treatment of hAd infections. As a result, the only therapy options are off-label use of drugs approved for other indications. Severe hAd infections are most commonly treated with the nucleotide phosphate cytosine analog cidofovir (CDV) and the orally bioavailable lipid-linked derivative of CDV, brincidofovir (BCV), whereas ribavirin and ganciclovir were used less frequently.[15] However, only a small majority of patients profits from the treatment.[16–18] Moreover, CDV is nephrotoxic,[19] and gastrointestinal toxicity upon oral application in a phase III clinical trial[20] was observed after treatment of patients with BCV. Another promising therapy for these infections is hAd-specific T cell therapy,[21] but because

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of its personalized nature, this therapy is costly and time consuming, limiting its application in patients.[22]

About a decade ago it was shown that hAd infections can be efficiently inhibited *in vitro* with anti-adenoviral small interfering RNAs (siRNAs), which induce post-transcriptional gene silencing via the conserved cellular mechanism of RNA interference (RNAi).[23,24] The nature of adenoviral infection and viral replication defined several adenoviral proteins as potential targets of therapeutic RNAi, and indeed it has been confirmed that hAd infection can be inhibited *in vitro* by silencing the adenoviral proteins DNA polymerase (Pol), pre-terminal protein (pTP), IVa2, E1A, and the hexon protein.[23–26] The most efficient inhibition of adenoviral replication by RNAi was achieved by targeting both pTP and Pol.[25] Both proteins play a central role in replication of the adenoviral genome, indicating that disturbing the adenoviral DNA replication machinery represents a potent approach to inhibit hAd infections. More recently, inhibition of hAd infection by RNAi was confirmed by our group in an *in vivo* model of hAd5-induced hepatitis using immunosuppressed Syrian hamsters.[27] In this approach, self-complementary adeno-associated virus (AAV) vectors of serotype 9 were used as carriers to deliver anti-adenoviral artificial microRNAs (amiRNAs) to the liver. However, strong inhibition of hepatic hAd infection was only observed when the vector was applied 2 weeks before the animals were infected with hAd5, whereas application of the AAV vectors concomitant with hAd5 infection resulted only in very low inhibition of hepatic hAd infection.

Lipid nanoparticles (LNPs) are non-viral lipid vesicles with a homogeneous lipid core.[28] They are one of the most commonly used systems for delivery of small-molecule drugs and nucleic acids.[28,29] In the last few years LNP-based delivery of nucleic acids has received a great deal of attention because it has been used as a delivery platform for the coronavirus disease 2019 (COVID-19) mRNA vaccines Spikevax[30] and Comirnaty,[31] and there is an FDA-approved LNP siRNA for treatment of hereditary transthyretin amyloidosis.[32] LNPs are composed of different components, such as ionizable lipids, cationic lipids, structural lipids (cholesterol and phospholipids), and poly(ethylene glycol)-anchored lipids to aid in covering and protecting the nucleic acids to be transported and to aid in passing through the cell and nuclear membranes.[28] The internalization and degradation of LNP, their recognition by the immune system, their duration in the blood circulation, and their biological distribution are affected by proteins surrounding the outer layer of LNPs as well as by the size and charge of the LNPs.[28,33] Because systemic application leads to efficient perfusion of the liver, LNPs have been used for delivery of siRNAs into hepatocytes. When in the circulation, some types of LNPs bind to serum proteins, including apolipoprotein E, which, in turn, binds to the low-density lipoprotein receptor. This receptor is highly expressed on hepatocytes and directs the LNP siRNA into these cells.[34,35] However, a fraction of the LNP siRNA is taken up by Kupffer cells, sinusoidal endothelial cells, and stellate cells, resulting in a significant amount of siRNA not accumulating in hepatocytes.[36] This can be largely avoided by changing the lipid composition in the LNP siRNAs; e.g., by using appropriate cationic aminolipids.[37]

In the present study, we investigated the therapeutic potential of LNP-mediated delivery of anti-adenoviral siRNA to the liver for treatment of hAd5 infection *in vivo*. Initial experiments revealed that two siRNAs, $sipTP_{mod}$ and $siPol\text{-}1_{mod}$, targeting the adenoviral *pTP* or *Pol* gene, which contained 2'-*O*-methyl residues, phosphorothioate linkages, and an optimized thermodynamic profile, efficiently inhibited hAd5 infection *in vitro*. For *in vivo* use, $sipTP_{mod}$ was encapsulated in LNPs containing the cationic aminolipid XL-10 (LNP-$sipTP_{mod}$) and applied concomitantly with hAd5 to immunosuppressed Syrian hamsters. This treatment resulted in distinct inhibition of hAd5 replication in the liver, reduction of hepatic inflammation, normalization of liver transaminase levels, as well as reduction of hAd titers in the blood serum compared with hAd5-infected animals that received a non-silencing control siRNA (LNP-$siContr_{mod}$).

## RESULTS

### Evaluation of anti-adenoviral siRNAs

Studies by other investigators and ourselves[25–27] have defined the adenoviral *pTP* and *Pol* genes as the best target genes for RNAi therapeutics for inhibition of hAd infection. We therefore designed two siRNAs (siPol-1 and siPol-2) targeting the adenoviral *Pol* gene and one siRNA targeting the adenoviral *pTP* gene (sipTP) (Figure 1A). The *Pol* and *pTP* gene target sequences were from adenovirus serotype 5 because it belongs to adenoviral subgroup C, the subgroup most frequently detected in patients with severe adenovirus infection.[38,39] To compare their silencing efficiencies, HeLa cells were infected with hAd5 at an MOI of 0.1, 1, or 2.5 and transfected with 30 nM sipTP, siPol-1, or siPol-2. Quantitative PCR to determine the number of viral genomes and plaque assays to determine the amount of infectious hAd5 showed that all three siRNAs had a strong effect of inhibiting adenoviral infection *in vitro* (Figures 1B and 1C). Relative to cells transfected with a non-silencing siContr, adenoviral replication was inhibited by siPol-1, siPol-2, and sipTP by approximately 98% (MOI 0.1) to 95% (MOI 2.5), as determined by qPCR, and by approximately 2 (MOI 0.1) to 1.5 (MOI 2.5) orders of magnitude, as determined by plaque assay. Thus, all siRNAs show high efficiency against hAd5.

To achieve high inhibitory efficiency *in vivo*, we next designed the siRNAs $siPol\text{-}1_{mod}$, $siPol\text{-}2_{mod}$, and $sipTP_{mod}$ (Figure 2A). Selected positions of these siRNAs were modified with 2′-*O*-methyl to suppress an immune response against the siRNA[40] and to confer stabilization against endonucleolytic degradation.[41] Furthermore, phosphorothioate linkages were introduced at the two ends of each strand to further increase protection against exonucleolytic degradation.[42] In addition, a single overhang structure at the 3′ end of the guide strand and optimization of the thermodynamic profile by introducing a DNA T at position 1 of the guide strand served to increase siRNA activity.[43] HeLa cells were then transfected with 30 nM siPol-1, siPol-2, and sipTP and their modified counterparts $siPol\text{-}1_{mod}$, $siPol\text{-}2_{mod}$, and $sipTP_{mod}$ as well as with siContr and its modified counterpart ($siContr_{mod}$) and infected with 0.1, 1, or 2.5 MOI of hAd5 to clarify whether the modifications in the siRNAs affect their ability to inhibit hAd5 replication. Quantitative PCR revealed similar

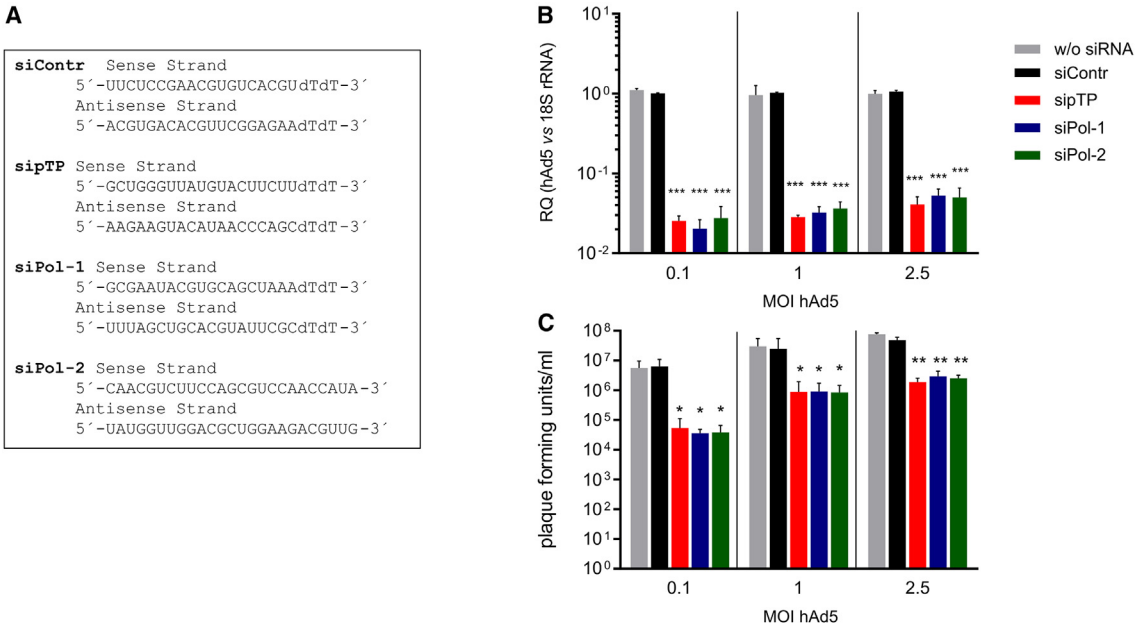


**Figure 1. Evaluation of the anti-adenoviral siRNAs sipTP, siPol-1, and siPol-2**

(A) Schematic of the tested anti-adenoviral siRNAs showing the sense and antisense strands of sipTP, siPol-1, siPol-2, and the non-silencing control siRNA (siContr). (B) Determination of inhibition of hAd5 replication with anti-adenoviral siRNAs by qPCR. HeLa cells were infected with hAd5 at an MOI of 0.1, 1, and 2.5 and, after 2 h, transfected with 30 nM sipTP, siPol-1, siPol-2, or siContr or with transfection reagent only (without [w/o] siRNA). After 48 h, cells were lysed. Supernatants were used for infection of HeLa cells that were lysed after 2 h, and their supernatants were used for quantification of infectious adenoviral genomes by qPCR. Fold change was calculated using the ΔΔCt method against siContr-treated cells with determination of genomic DNA of 18S rRNA for normalization. Significance against siContr-treated cells: ***$p < 0.001$. (C) Determination of inhibition of hAd5 replication with anti-adenoviral siRNAs by plaque assay. HeLa cells were treated as described in (B). Supernatants containing infectious hAd5 were directly quantified by plaque assay. Significance against siContr-treated cells: *$p < 0.05$ and **$p < 0.01$.

inhibition of hAd5 replication induced by sipTP and sipTP$_{mod}$ or siPol-1 and siPol-1$_{mod}$, respectively, indicating that modification of sipTP and siPol-1 had no effect on inhibition of hAd5 replication. In contrast, treatment of hAd5-infected cells with siPol-2$_{mod}$ resulted in up to 7-fold lower inhibition of hAd5 replication compared with use of siPol-2. The lower efficiency of siPol-2$_{mod}$ appears to be due to a shortening of siRNA from 25-mer to 19-mer rather than the modifications per se because the modification pattern of siPol-2$_{mod}$ was identical to that of sipTP$_{mod}$ and siPol-1$_{mod}$ (Figure 2B). Because of the lower efficiency, siPol-2$_{mod}$ was not investigated further.

Because severe adenoviral infections in humans can be caused by different hAd serotypes,[15] use of a specific siRNA that effectively recognizes a target sequence in multiple hAd subtypes would be beneficial for clinical application. As shown in Table 1, sipTP and siPol-1 perfectly matched their respective target sequences in the pTP and Pol mRNA of the adenoviral subgroup C serotypes 1, 2, 5, and 6. One mismatch within a target sequence is often tolerated by an siRNA;[44] therefore, we examined sipTP, which had only one mismatch relative to each to the pTP target sequences of hAd19 and hAd64 (subgroup D), hAd4 (subgroup E), and hAd41 (subgroup F), but not siPol-1, which has two or more mismatches to the Pol target sequence in these virus strains. HeLa cells were co-transfected with sipTP and a luciferase reporter plasmid containing the corresponding pTP target sequences of the four hAd or, as a control, with a luciferase reporter plasmid containing the pTP target sequence of hAd5. The pTP target sequences of hAd4, hAd19, hAd41, and hAd64 were efficiently recognized, as indicated by strong silencing of the luciferase reporter. Interestingly, a common mismatch in the sipTP target sequence of hAd4, hAd19, and hAd64 seems to even have increased the silencing activity of sipTP (Figure 2C). Finally, we verified the RNAi mechanism of sipTP$_{mod}$ by determining pTP mRNA expression in HeLa cells infected with 0.1 and 1 MOI of hAd5 and transfected with 30 nM of sipTP$_{mod}$ or siContr$_{mod}$. Compared with the control, treatment with sipTP$_{mod}$ resulted in 90.5% and 83% lower expression of pTP mRNA, respectively, as detected by qRT-PCR, demonstrating a strong silencing effect of sipTP$_{mod}$ (Figure 2D).

Based on these results showing strong and broad activity of sipTP, we decided to further investigate sipTP$_{mod}$ for its therapeutic efficacy against adenoviruses *in vivo*.

### Generation and characterization of LNP-sipTP$_{mod}$

The delivery of siRNAs *in vivo* is a key challenge for development of efficient siRNA therapies. LNPs, which consist of different lipid components and form particles smaller than 100 nm, play an important role in this. While the surface of these particles is surrounded by PEG lipids and is weakly positively charged, there is a largely

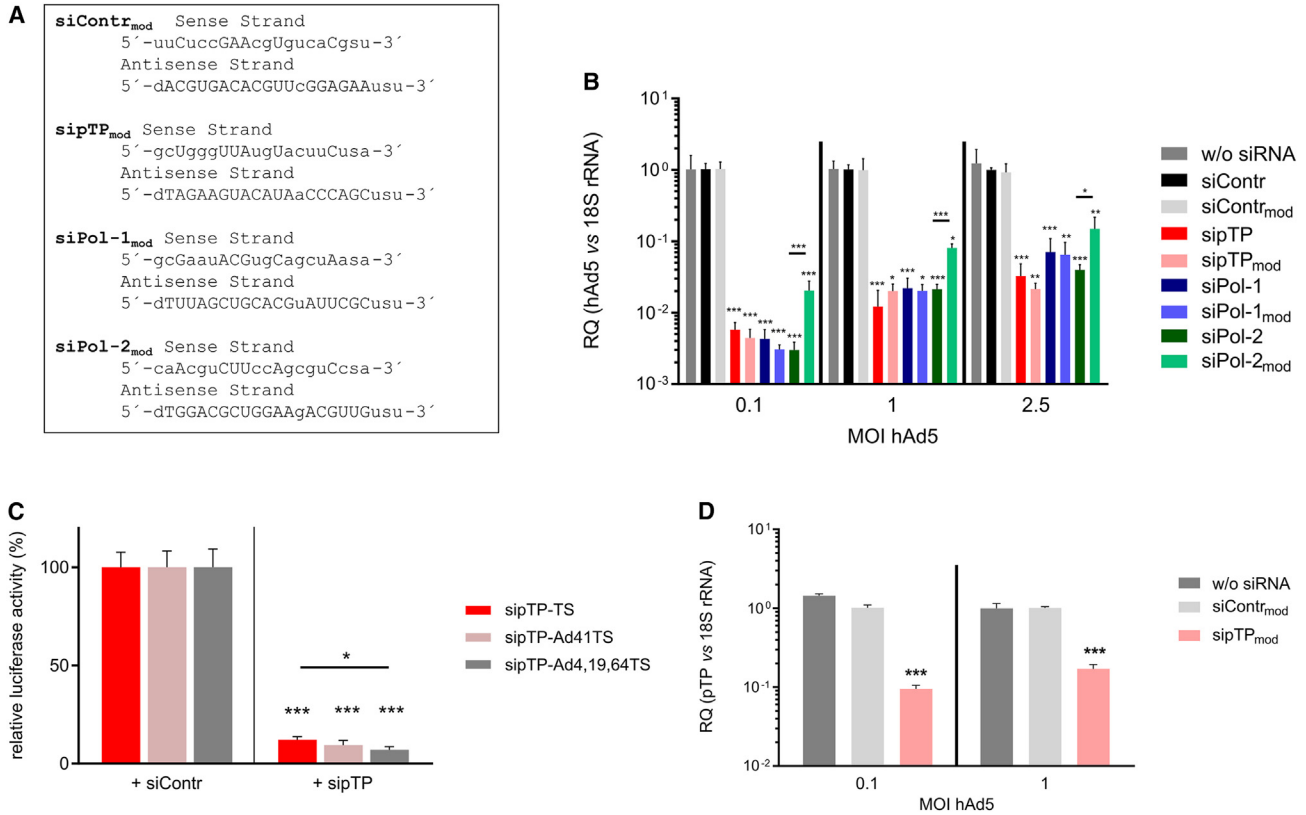


**Figure 2. Evaluation of the anti-adenoviral siRNAs sipTP$_{mod}$, siPol-1$_{mod}$, siPol-2$_{mod}$ and silencing of pTP of different adenoviral serotypes by sipTP**

(A) Schematic of the tested modified anti-adenoviral siRNAs sipTP$_{mod}$, siPol-1$_{mod}$, and siPol-2$_{mod}$ and siContr$_{mod}$. The sense and antisense strands of the sequences are shown. N, RNA residues; dN, DNA residues; n, 2′-*O*-methyl residues; s, phosphorothioate. (B) Inhibition of hAd5 replication by modified anti-adenoviral siRNAs. The siRNAs described in Figure 1B and their modified versions sipTP$_{mod}$, siPol-1$_{mod}$, siPol-2$_{mod}$, and siContr$_{mod}$ were used to infect HeLa cells and to quantify the inhibitory effect on hAd5 replication as described in Figure 1B. Significance of unmodified siRNAs versus siContr and modified siRNAs versus siContr$_{mod}$ or as indicated: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. (C) Silencing of pTP of different hAd serotypes by sipTP in a reporter gene assay. HeLa cells were co-transfected with sipTP and a hRLuc reporter plasmid containing the complete corresponding sipTP target site (sipTP-TS) from hAd5 or the target sites for the serotype hAd41 (sipTP-Ad41TS) or hAd4, hAd19, and hAd64 (sipTP-Ad4,19,64TS). The cells were harvested 48 h after transfection, and luciferase activity was determined. As a control, each sipTP-TS reporter plasmid was co-transfected with siContr. The siRNA silencing activity was calculated as a percentage of luciferase activity in samples treated with sipTP compared with samples treated with siContr. Significance against siContr-treated cells or as indicated: ***$p < 0.001$, *$p < 0.05$. (D) Determination of adenoviral pTP mRNA expression with anti-adenoviral siRNAs by qRT-PCR. HeLa cells were infected with hAd5 at an MOI of 0.1 and 1 and, after 2 h, transfected with 30 nM sipTP$_{mod}$, siContr$_{mod}$, or transfection reagent alone (w/o siRNA). After 48 h, total RNA was isolated, and the level of pTP mRNA was quantified. Fold change was calculated using the ΔΔCt method against siContr$_{mod}$-treated cells with determination of cellular 18S rRNA levels for normalization. Significance against siContr$_{mod}$-treated cells: ***$p < 0.001$.

hydrophobic core of inverted lipid micelles inside, which contains the siRNA.[45] It has been shown that LNPs containing the cationic aminolipid XL-10 (Figure S1) are able to specifically transport siRNAs into hepatocytes after intravenous (i.v.) administration.[37] Therefore, XL-10 was used in a cocktail with other lipids and sipTP$_{mod}$ and siContr$_{mod}$ to prepare the therapeutic LNP-sipTP$_{mod}$ and the control LNP-siContr$_{mod}$, respectively. The total lipid-to-siRNA ratio was 7:1 in the generated particles. Several parameters of the LNP siRNA preparations were determined before their use because they are crucial for their *in vivo* functionality. LNP-sipTP$_{mod}$ and LNP-siContr$_{mod}$ had a size of 83.2 and 88.4 nm, respectively, and the polydispersity index was 0.04 for each. The zeta potential was 0.7 mV for LNP-sipTP$_{mod}$ and 1.3 mV for LNP-siContr$_{mod}$. The drug encapsulation reached 90% and 89% for LNP-sipTP$_{mod}$ and LNP-siContr$_{mod}$, respectively. The drug concentration of both LNP siRNAs was 1.0 mg/mL. Both LNP siRNA preparations thus had very similar parameters in the range of typical LNP siRNA preparations.[37]

### The course of hAd5 infection in immunosuppressed Syrian hamsters in a low- and moderate-dose infection model

The Syrian hamster model has been developed as a standard model for investigation of hepatic hAd infections and analysis of therapeutic efficiency of anti-adenoviral drugs and biologicals *in vivo*.[27,46,47] This model was therefore chosen to determine the anti-adenoviral efficacy of LNP-sipTP$_{mod}$. In an initial experiment, we investigated the course and severity of hAd infection in Syrian hamsters as a function of viral dose because viral dose is a crucial factor affecting both disease parameters in the model.[27] For this purpose, Syrian hamsters were

**Table 1. Sequence comparison of sipTP, siPol-1, and siPol-2 with target sequences of corresponding pTP and Pol mRNAs in different hAd serotypes**

| Subgroup | Serotype(s) | GenBank | Adenoviral target sequence for sipTP (3′-5′) |
|---|---|---|---|
| A | 12 | X73487.1 | UUCUU*U*AUGU*C*UU*A*GGUGG |
| C | 1, 2, 5, 6 | AC_000017.1<br>AC_000007.1<br>AC_000008.1<br>LC068720.1 | UUCUUCAUGUAUUGGGUCG |
| D | 19, 64 | AB448774.1<br>JQ326208.1 | UUCUUCAUGUAUU*A*GGUCG |
| E | 4 | MN307143.1 | UUCUUCAUGUAUU*A*GGUCG |
| F | 41 | ON561778.1 | UUCUUCAUGUAUUGGGU*U*G |
| G | 52 | DQ923122.2 | UUCUUCAUGUA*C*U*A*GGUCG |
| | | | Adenoviral target sequence for siPol-1 (3′-5′) |
| A | 12 | X73487.1 | AA*C*U*C*AACGUG*U*AUAAGCG |
| C | 1, 2, 5, 6 | AC_000017.1<br>AC_000007.1<br>AC_000008.1<br>LC068720.1 | AAAUCGACGUGCAUAAGCG |
| D | 19, 64 | AB448774.1<br>JQ326208.1 | AA*C*UCGAC*C*UG*U*AUGAGCG |
| E | 4 | MN307143.1 | AA*U*U*C*AACGUGCAUGAGCG |
| F | 41 | ON561778.1 | AA*C*UCGACGUGCAUG*A*A*A*G |
| G | 52 | DQ923122.2 | AA*G*U*C*AACGUGCAUGAGCG |
| | | | Adenoviral target sequence for siPol-2 (3′-5′) |
| A | 12 | X73487.1 | *U*UACCA*U*CCUGCGAC*U*UU*G*UG*U*AA*U* |
| C | 1, 2, 5, 6 | AC_000017.1<br>AC_000007.1<br>AC_000008.1<br>LC068720.1 | AUACCAACCUGCGACCUUCUGCAAC |
| D | 19, 64 | AB448774.1<br>JQ326208.1 | *C*UACCA*C*CCGGCAACCUUCUA*C*AA*U* |
| E | 4 | MN307143.1 | *C*UACCA*C*CC*A*G*A*A*A*CCUU*U*U*A*CAA*U* |
| F | 41 | ON561778.1 | *U*UAACACCCGGAAAC*U*UUU*U*UGCAA*U* |
| G | 52 | DQ923122.2 | *C*UACCACCCCGCAACCUUCUGCAA*U* |

immunosuppressed with cyclophosphamide (CP) twice weekly. Animals were infected i.v. with $4 \times 10^{10}$ (low dose) or $4 \times 10^{11}$ (moderate dose) virus particles (vp) of hAd5 per kilogram. The animals were investigated for another 7 days (Figure 3A). No deaths were observed during the study period. After hAd5 infection, body weight remained unchanged in the low-dose group, whereas it decreased slightly in the animals that received the moderate hAd5 dose (Figure 3B). Replicating hAd5 was detected in the liver and serum at the low and moderate doses. Corresponding to the different doses of virus administered, the hAd5 titers in liver and serum were about 1 order of magnitude higher in the moderate-dose group than in the low-dose group (Figure 3C). These results demonstrate that systemic application of hAd5 at low and moderate doses induces productive hepatic hAd5 infection associated with delivery of virus into the bloodstream in immunosuppressed Syrian hamsters. Furthermore, the investigations show that the course of disease and the amounts of virus detected in the liver and serum correlate with the initial dose of virus injected.

### Concomitantly applied LNP-sipTP$_{mod}$ inhibits hAd5 replication in the liver of immunosuppressed Syrian hamsters in the low-dose infection model

First, we investigated the therapeutic efficacy of LNP-sipTP$_{mod}$ in the low-dose hAd5 infection model, assuming that low amounts of hAd5 in the liver may be more strongly inhibited by sipTP$_{mod}$ than high amounts. Syrian hamsters were divided into three groups and immunosuppressed a week prior to hAd5 infection. Two of the three groups were i.v. infected with $6 \times 10^{10}$ vp/kg hAd5. One of these groups was concurrently i.v. injected with 2 mg/kg LNP-sipTP$_{mod}$, whereas the other received 2 mg/kg of LNP-siContr$_{mod}$. All animals were sacrificed 7 days later (Figure 4A). During the investigational period of 7 days, both groups lost weight after infection, but weight loss was lower in the LNP-sipTP$_{mod}$ group (up to 7.6%) compared with the LNP-siContr$_{mod}$ group (up to 13.7%) (Figure 4B). Determination of hAd5 titers in the liver and serum revealed a significant reduction of virus burden in animals that were treated with LNP-sipTP$_{mod}$ compared

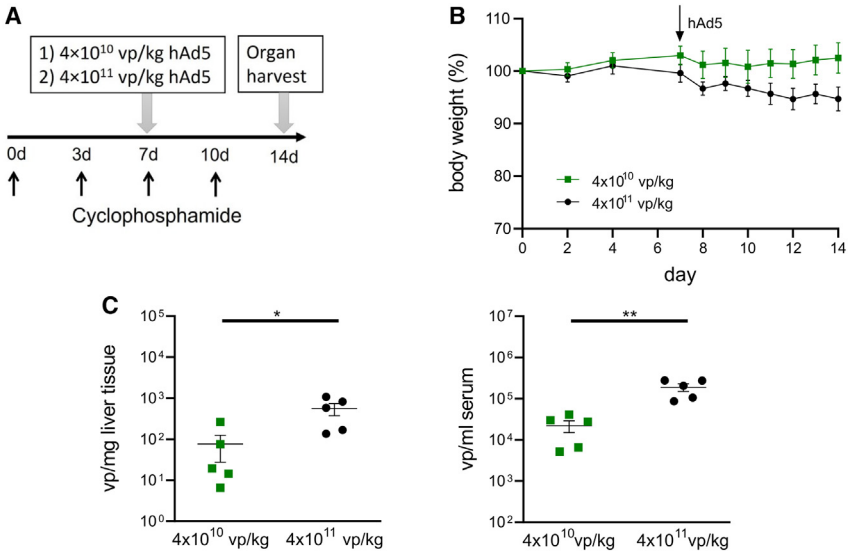


**Figure 3. Dose-dependent replication of hAd5 in immunosuppressed Syrian hamsters**

(A) Time course of CP and hAd5 injection. Syrian hamsters were divided into two groups (each with 5 animals) and immunosuppressed by repeated application of CP, as indicated. Both groups were infected with hAd5 on day 7 after the first CP injection via i.v. injection of the virus into the jugular vein. One group received hAd5 at a dose of $4 \times 10^{10}$ vp/kg and the other group hAd5 at a dose of $4 \times 10^{11}$ vp/kg. Animals were sacrificed and analyzed on day 14 after the first CP injection. (B) Development of body weight of immunosuppressed, hAd5-infected Syrian hamsters. Shown are the relative changes in body weight, beginning after the first CP injection. The time of adenoviral infection is marked by an arrow. (C) Measurement of liver tissue and serum titers of hAd5. Titers of infectious hAd5 in liver tissue and serum were determined by an *in vitro* hAd5 amplification qPCR protocol, as described under materials and methods. Significance: *$p < 0.05$, **$p < 0.01$.

with LNP-siContr$_{mod}$-treated animals (Figure 4C). Indeed, hAd5 titers were 3.4-fold lower in the liver and 9.4-fold lower in serum of LNP-sipTP$_{mod}$-treated animals than in LNP-siContr$_{mod}$-treated animals. The latter indicates that LNP-sipTP$_{mod}$ treatment reduced viremia. No changes were found in pTP-mRNA levels in the liver, as detected by qRT-PCR (Figure S2A). We also investigated the spleen and the heart for hAd5 infection, but viral titers were too low to obtain evaluable data (data not shown). Histological examination of liver sections showed reduced inflammation focus frequency with reduced focus size in LNP-sipTP$_{mod}$-treated animals compared with LNP-siContr$_{mod}$-injected animals (Figure 4D). Accordingly, the pathological score of liver tissue tended to be lower in LNP-sipTP$_{mod}$-treated animals than in LNP-siContr$_{mod}$-treated animals (Figure 4E). To characterize the inflammatory processes in the liver more comprehensively, we also measured a panel of inflammatory and immune mediators in the liver tissue by qRT-PCR. Samples were assessed for type II interferon response-related mediator interferon γ (IFN- γ) and the innate/adaptive mediators interleukin-6 (IL-6), IL-12, IL-1β, and tumor necrosis factor (TNF). All mediators were upregulated in hAd5-infected animals compared with uninfected immunosuppressed Syrian hamsters. More importantly, expression of IL-12 was significantly reduced, and expression of IFN-γ, IL-6, and IL-1β tended to be lower in LNP-sipTP$_{mod}$-treated compared with LNP-siContr$_{mod}$-treated animals (Figure 4F). To further investigate the influence of the siRNA therapy on liver damage, the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and the glutamate dehydrogenase (GLDH), representing biomarkers for liver injury, were measured in the serum of hAd5-infected animals treated with LNP-sipTP$_{mod}$ or LNP-siContr$_{mod}$ and in uninfected control animals. There were no differences in enzyme activity between animal groups (Figure 4G), suggesting that the liver damage induced by the low dose of hAd5 was too small to significantly increase enzyme activity.

### Concomitantly applied LNP-sipTP$_{mod}$ inhibits hAd5 replication and injury in the liver of immunosuppressed Syrian hamsters in the moderate-dose infection model

Encouraged by the data obtained in the low-dose hAd5 infection model and to analyze whether LNP-sipTP$_{mod}$ can also combat a more severe hepatic hAd5 infection, we next investigated the therapeutic efficacy of LNP-sipTP$_{mod}$ after infection of immunosuppressed Syrian hamsters with a 10-fold higher hAd5 dose of $6 \times 10^{11}$ vp/kg (Figure 5A). In this moderate-dose infection model, LNP-sipTP$_{mod}$-treated and LNP-siContr$_{mod}$-treated hAd5-infected animals lost body weight during the investigation period, but the latter lost more (Figure 5B). We also observed a significant reduction in viral titer in the liver and serum of LNP-sipTP$_{mod}$-treated compared with LNP-siContr$_{mod}$-treated hAd5-infected animals, whereas hepatic pTP-mRNA levels remained unchanged (Figure S2B). Compared with the low-dose model, the inhibitory effect of LNP-sipTP$_{mod}$ was even stronger, with a 19.6-fold reduction of virus titers in the liver and a 110-fold reduction of virus titers in the serum (Figure 5C). In addition, a 100-fold reduction of hAd5 titer was found in the spleen with LNP-sipTP$_{mod}$ treatment, whereas there was no difference in the heart. Reduction of hepatic hAd5 titers by siRNA treatment was confirmed by immunohistochemical staining of hAd5-positive cells in liver sections, showing that LNP-sipTP$_{mod}$-treated animals tended to have fewer hAd5-positive cells than siContr$_{mod}$-treated animals (Figure S3). Compared with LNP-siContr$_{mod}$-treated animals, there was slightly reduced liver pathology for animals treated with LNP-sipTP$_{mod}$, as indicated by a reduced pathological score (Figures 5D and 5E). As in the low-dose model, IFN-γ, IL-6, IL-12, IL-1β, and TNF were upregulated by hAd5 infection in the liver, whereas IL-12 levels were markedly reduced, and IL-6 and IL-1β tended to be expressed at lower levels in the liver tissue of LNP-sipTP$_{mod}$-treated animals compared with LNP-siContr$_{mod}$-treated animals (Figure 5F). In contrast to the low-dose model, the activities

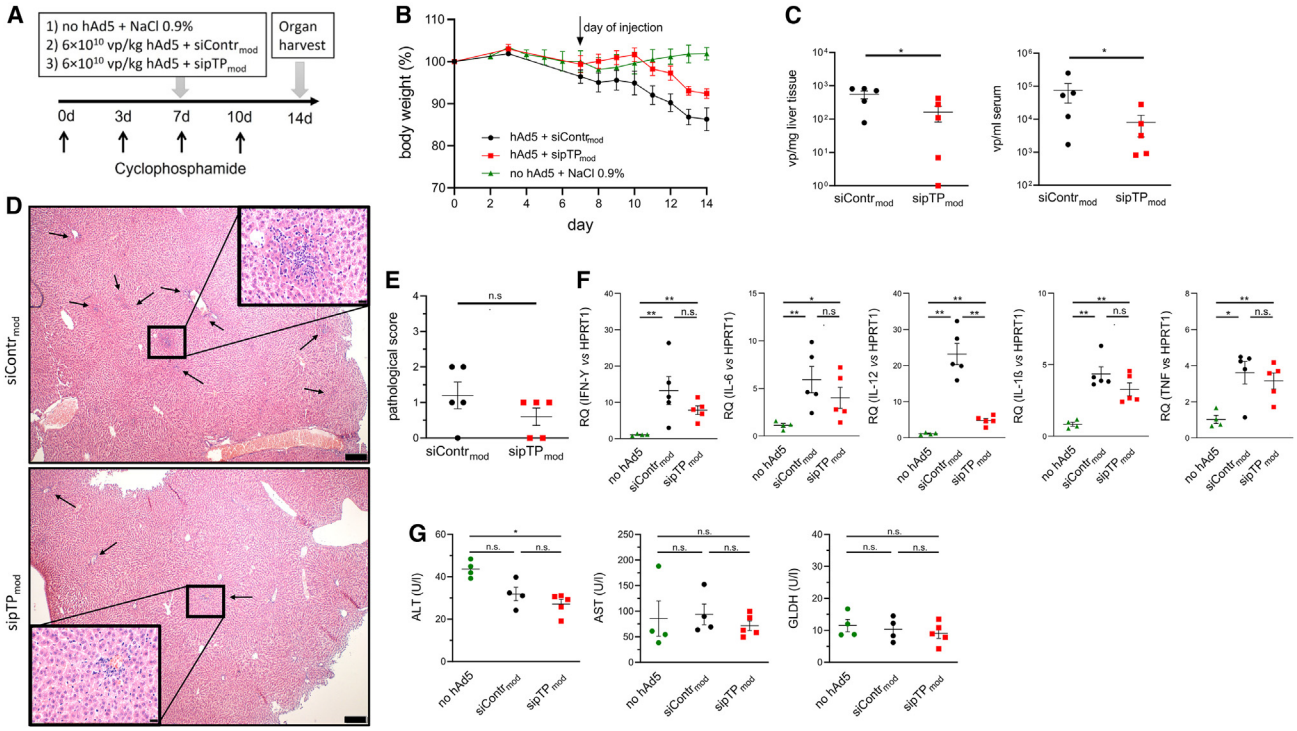


**Figure 4. Application of LNP-sipTP$_{mod}$ concomitant with hAd5 to immunosuppressed Syrian hamsters decreases hAd5 infection of the liver in a low-dose hAd5 infection model**

(A) Time course of CP application and hAd5 and LNP siRNA injection. Syrian hamsters were divided into three groups and immunosuppressed by repetitive CP application as indicated. One group received no hAd5 but 0.9% NaCl (n = 4), and two groups were infected with hAd5 at a dose of $6 \times 10^{10}$ vp/kg on day 7 after the first CP injection by injection of the virus into the left jugular vein. Simultaneously, animals in one of the hAd5-infected groups received 2 mg/kg LNP-siContr$_{mod}$ (siContr$_{mod}$) (n = 5), whereas the other group received 2 mg/kg LNP-sipTP$_{mod}$ (sipTP$_{mod}$) (n = 5). In each case, application was into the right jugular vein. All animals were sacrificed and analyzed on day 14 after the beginning of CP administration. (B) Development of body weight of immunosuppressed Syrian hamsters. Shown are relative changes of body weight beginning at the first CP injection. The time of injection is marked by an arrow. (C) The titers of hAd5 measured in liver tissue and serum. Titers of hAd5 in liver tissue and serum were determined by an *in vitro* hAd5 amplification qPCR protocol, as described under materials and methods. Significance: *$p < 0.05$. (D) Severity of liver tissue damage. Shown are H&E-stained, formalin-fixed liver slides and a representative of each hAd5-infected group. Arrows show foci of inflammation. Scale bars, 200 μm; scale bars of magnification, 20 μm. (E) Liver damage was assessed and given as a pathological score on a scale from 0 (no damage) to 5 (severe damage). n.s., not significant. (F) Expression of inflammatory and immune mediators in the liver. Samples were assessed for type II interferon response-related (IFN-γ) and innate/adaptive mediator (IL-6, IL-12, IL-1β, TNF, and IFN-γ) expression by qRT-PCR. Fold change was calculated via the ΔΔCt method against uninfected, immunosuppressed animals, with the housekeeping gene HPRT1 used to normalize the sample. Significance: *$p < 0.05$, **$p < 0.01$. (G) ALT, AST, and GLDH levels in serum of the three investigated groups. Significance: *$p < 0.05$. Note: serum of only 4 animals in the LNP-siContr$_{mod}$-treated group could be analyzed.

of ALT, AST, and GLDH tended to be higher in the liver of immunosuppressed hAd5-infected Syrian hamsters treated with LNP-siContr$_{mod}$ compared with uninfected animals, indicating that a hAd5 dose of $6 \times 10^{11}$ vp/kg was sufficient to induce severe liver injury (Figure 5G). Interestingly, in hAd5-infected hamsters treated with LNP-sipTP$_{mod}$ the AST levels remained at the level of uninfected hamsters.

In conclusion, these data demonstrate that LNP-sipTP$_{mod}$ can inhibit hepatic hAd5 infection *in vivo*.

## DISCUSSION

Severe hAd infections are generally treated symptomatically and in the context of specific antiviral therapy with antiviral drugs such as CDV, BCV, ribavirin, or ganciclovir,[15] but the therapeutic efficacy of these treatments remains limited, and the toxicity of the drugs poses a significant problem in clinical applications. Therefore, new therapies are needed. In a previous proof-of-concept study, we demonstrated that RNAi-induced gene silencing by anti-adenoviral amiRNAs delivered by AAV vectors is capable of inhibiting hepatic hAd5 infection *in vivo*.[27] Although this treatment was effective when used prophylactically, it had little effect when given at the time of hAd5 infection. Moreover, generation of AAV vectors is expensive and time consuming, and it has been shown that hepatic AAV vector-mediated gene transfer can induce fatal liver toxicity in patients.[48] Therefore, AAV vector-based RNAi therapeutics are rather unsuitable for clinical use in patients with severe hepatic hAd5 infection.

Here we demonstrate LNP-sipTP$_{mod}$ as a new powerful RNAi treatment to combat hepatic hAd5 infection. After i.v. administration of

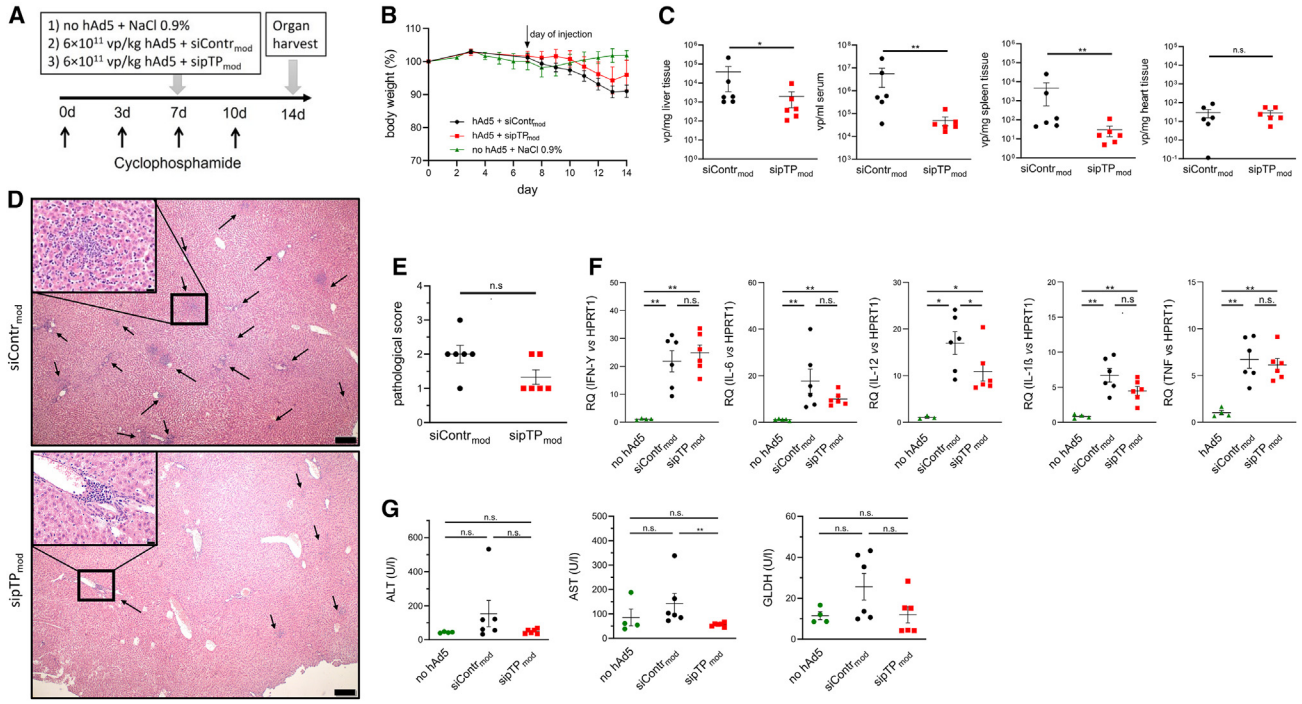


**Figure 5. Application of LNP-sipTP$_{mod}$ concomitant with hAd5 to immunosuppressed Syrian hamsters decreases hAd5 infection of the liver in a moderate-dose hAd5 infection model**

(A) Syrian hamsters were divided into three groups and treated and analyzed as shown in Figure 4A with the following exceptions: hAd5 was administered at a dose of $6\times10^{11}$ vp/kg, and each group of hAd5-infected animals comprised 6 animals. (B) Development of body weight of immunosuppressed Syrian hamsters. Shown are relative changes of body weight beginning at the first CP injection. The time of injection is marked by an arrow. (C) The titers of hAd5 measured in liver tissue, serum, spleen, and heart tissue are shown. Determination of hAd5 titers and testing of significance were performed as described in Figure 4C. Significance: *$p < 0.05$ and **$p < 0.01$. (D) The severity of liver tissue damage is shown in H&E-stained, formalin-fixed liver slides. A representative example of each hAd5-infected group is shown. The foci of inflammation are indicated by arrows. Scale bars, 200 μm; scale bars of magnification, 20 μm. (E) The liver damage shown here was assessed and given as a pathological score, as described in Figure 4E. (F) The expression of IL-6, IL-12, IL-1β, TNF, and IFN-γ in the liver was determined as described in Figure 4F. Significance: *$p < 0.05$, **$p < 0.01$. (G) The ALT, AST, and GLDH levels measured in serum. Significance: **$p < 0.01$. Note: the data shown for the uninfected group 1 (no hAd5) are the same as those shown in Figures 4B, 4F, and 4G.

a single dose of 2 mg/kg of LNP-sipTP$_{mod}$, hepatic hAd5 infection was markedly inhibited in the immunosuppressed Syrian hamster model, as evidenced by a decrease in hepatic hAd5 titers compared with animals treated with a siContr. Moreover, there was a trend toward reduced liver injury and inflammation.

Selection of appropriate viral target genes, efficient silencing of the target, and long-lasting efficacy are important challenges when selecting a suitable siRNA for the treatment of viral infections. It has been shown that genes of the adenoviral replication machinery, particularly *pTP* and *Pol*, are excellent targets for therapeutic RNAi.[25–27,49] Our data confirmed these results because the investigated siRNAs sipTP, siPol-1, and siPol-2 directed against the *pTP* and *Pol* genes strongly suppressed hAd5 infection *in vitro*. However, unmodified synthetic siRNAs, used in the initial *in vitro* investigation, are quickly degraded by nucleases.[50] This prevents long-lasting silencing of the target genes, which greatly limits use of this siRNAs for many clinical applications, including treatment of hAd5 infection. Chemical modifications, such as 2'-ribose modifications, 2'-*O*-methyl, 2'-fluoro and phosphorothioate linkages, have been widely used to prolong siRNA half-life.[29,51] While chemically modified siRNAs are more resistant to degradation by nucleases, the insertion of such modifications increases the risk that siRNA activity will be reduced.[51] Therefore, insertion of modifications into a siRNA must be well considered. Here we incorporated 2'-*O*-methyl residues and phosphorothioate linkages at selected positions within the siRNAs sipTP, siPol-1, and siPol-2. The pattern we created for positioning the chemical modifications in the siRNA used is a further development of the pattern used in patisiran,[52] the first siRNA-based drug approved by the FDA. It is based on numerous investigations and evaluations of the corresponding results carried out by the company Axolabs, which generated the siRNAs used in this study. These modifications did not affect the inhibitory effect of two of the siRNAs, sipTP$_{mod}$ and siPol-1$_{mod}$, on hAd5 infection.

Delivery of a siRNA into a target organ or cell *in vivo* or into patients is a major challenge in making this technology useful for clinical applications in humans. Considerable progress has been made in this area over the past 10 years. In this regard, LNPs used for encapsulation of siRNAs and N-acetylgalactosamine (GalNAc) ligand-modified

siRNAs were developed, which are currently the most widely used siRNA delivery platforms.[51] In this study, we used an LNP formulation containing the cationic amino-lipid XL-10, which has been shown to effectively transfer siRNAs into hepatocytes *in vivo*,[37,53] and successfully employed this LNP with the sipTP$_{mod}$ to inhibit hepatic hAd5 infection in immunosuppressed Syrian hamsters. Interestingly, we found a significantly greater reduction in hepatic hAd5 viral titers when the infected animals were treated with LNP-sipTP$_{mod}$ than after therapeutic application of anti-pTP amiRNAs expressing AAV9 vectors.[27] Indeed, using similar experimental protocols, AAV-amiRNA treatment reduced hepatic hAd5 titers by about 50%,[27] while LNP-sipTP treatment resulted in a 70.5% reduction in liver hAd5 titers (Figure 4C), indicating a better effect of LNP-sipTP. Reasons for this observation could be that LNP enter the liver more specifically and efficiently than AAV vectors but also that adenoviral pTP expression is suppressed more rapidly by siRNAs than by amiRNAs because siRNAs are released directly into the cytoplasm, whereas amiRNAs must be expressed from the vector genome and processed before they can knock down their target.

It should be noted that we were unable to detect sipTP$_{mod}$ in the liver at the end of the investigational period, 7 days after LNP-sipTP$_{mod}$ application (data not shown). This demonstrates that, despite the chemical modifications, sipTP$_{mod}$ was completely degraded in liver tissue by this time point. On the other hand, this observation makes it likely that maximal silencing of pTP, and thus the maximal effect of hAd5 inhibition *in vivo*, occurred within the first few days after LNP-sipTP$_{mod}$ administration. Re-application or multiple re-administrations of LNP-sipTP$_{mod}$ may therefore be advantageous to further increase inhibition of hepatic hAd5 infection and should be investigated in a follow-up study. This assumption is supported by another study showing a reduction of Hepatitis C Virus (HCV) titer in the serum of more than 4 orders of magnitude upon repeated LNP-mediated siRNA transfer.[54] Another approach to increase the efficiency of sipTP$_{mod}$ could be to increase its stability; for example, by introducing additional modifications. The guide strand would be particularly suitable for this because it has only a few modifications in the current configuration. However, it should be noted that the number and pattern of modifications are of great importance for the function of the siRNA,[55] which is why different patterns need to be evaluated.

Furthermore, we found that reduction of hepatic hAd5 infection by LNP-sipTP$_{mod}$ also resulted in lower virus titers in the circulation. This is important because adenoviral viremia can cause disseminated hAd5 infection with multi-organ disease, which increases the risk of fatal systemic disease for the patient.[56,57] Corresponding to the reduced hAd5 serum titers, we observed reduced viral infection of the spleen in LNP-sipTP$_{mod}$-treated hamsters. No effects were seen in the heart. This result in the heart contrasts with our previous study, in which we observed inhibition of cardiac hAd5 infection in immunosuppressed Syrian hamsters after prophylactic AAV9 vector-mediated administration of anti-pTP amiRNAs.[27] Although the reasons for these different results may be manifold, the fact that AAV9 vectors transduced the liver and the heart with high efficiency and therefore expressed the anti-pTP-amiRNAs also in cardiomyocytes, whereas the used LNPs preferentially target hepatocytes, may play a role. However, further investigations are necessary to better understand the mechanisms involved.

A surprising observation was that the inhibition of hepatic hAd5 infection by LNP-sipTP$_{mod}$ was lower in the model where animals were infected with a low dose of hAd5 than in animals infected with a moderate dose of hAd5. Kupffer cells, the tissue macrophages of the liver, may offer an explanation for this observation. As part of the innate cellular immune response, they form the first line of defense at the liver sinusoids against viruses that enter the liver via the portal blood circulation.[58] Thus, they bind and phagocytize hAd5. After i.v. administration, about 90% of the viruses are sequestered by Kupffer cells.[59] Compared with the model with moderate hAd5, it can be assumed that, in the model with low hAd5, a significantly higher percentage of the applied hAd5 is intercepted by the Kupffer cells. Fewer hepatocytes are thus infected, and the probability that hepatocytes are simultaneously infected with hAd5 and contain sipTP$_{mod}$ decreases. This could lead to the lower inhibition of hAd5 infection observed in this model. However, further studies are needed to confirm this proposed mechanism. The uptake of hAd5 by Kupffer cells could also be an explanation for the lack of pTP mRNA silencing in the liver by LNP-sipTP$_{mod}$. XL-10-containing LNP siRNAs are taken up by hepatocytes, whereas other liver cells, such as Kupffer cells, are not sensitive to this formulation (H-P.V., unpublished data). Accordingly, adenoviral pTP mRNA expression is not inhibited in Kupffer cells, which could mask pTP mRNA silencing in hepatocytes. The decrease in hepatic hAd5 titers after LNP-sipTP$_{mod}$ treatment does not contradict this conclusion because hAd5 infection in Kupffer cells is abortive,[59] and the hAd5 titers we measured in liver tissue are therefore exclusively from infected hepatocytes.

In addition to siRNAs encapsulated in LNPs, siRNAs can also be introduced into the liver in the form of GalNAc-conjugated siRNA. The trimeric GalNAc ligand in the conjugates binds to the asialoglycoprotein receptor, which is highly expressed on hepatocytes, resulting in efficient uptake and release of siRNA into hepatocytes.[55] The stability of naked GalNAc-conjugated siRNA *in vivo* is achieved by extensive chemical modifications of the nucleotides and by replacing phosphodiester bonds with phosphorothioate bonds.[60] GalNAc-siRNAs are simpler, smaller, and more defined than the LNP formulations[61] and can also be administered subcutaneously to the patient,[62] making them a potential alternative to the LNP-sipTP$_{mod}$ presented here. Because of these advantages, such systems may be developed for treatment of hAd infection in the future.

siRNAs as antiviral agents have been used against all types of viral genomes, double-stranded or single-stranded DNA/RNA,[63] and because of their large therapeutic potential against pathogenic viruses, some of them have already entered clinical trials for treatment of HCV (phase II),[64] respiratory syncytial virus (phase II),[65] hepatitis B virus (phase I),[66] and Ebola virus[67] infection. Our study provides the next step toward translation of anti-adenoviral RNAi for

treatment of hAd5 infection from the bench to the clinic. In conclusion, here we show that treatment of hAd5 infection with LNP-sipTP$_{mod}$ is suitable to inhibit hepatic hAd5 infection *in vivo*. The use of anti-adenoviral LNP-sipTP$_{mod}$ can therefore be considered a promising approach to combat severe hepatic hAd infection.

## MATERIALS AND METHODS

### Cell culture

HEK293 (human embryonic kidney) cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Biowest, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; c.c. pro, Oberdorla, Germany), L-glutamine (Sigma-Aldrich, Merck, Darmstadt, Germany), sodium pyruvate (Sigma-Aldrich), and 1% each of penicillin and streptomycin (AppliChem, Darmstadt, Germany). HeLa (human cervical carcinoma) cells were grown in minimum essential medium (MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with L-glutamine and supplemented with 5% FCS, 1% each of penicillin and streptomycin, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES; Sigma-Aldrich), and 0.1 mM Non Essential Amino Acids (NEAA; Thermo Fisher Scientific).

### Production of hAd5

The hAd5 stock batch was a kind gift from Stefan Weger (Institute of Virology, Campus Benjamin Franklin, Charité – Universitätsmedizin Berlin, Berlin, Germany). HAd5 was amplified on HEK293 cells, concentrated, and purified by CsCl gradient centrifugation, and desalted with PD-10 desalting columns (Cytiva Life Sciences, Freiburg im Breisgau, Germany). The viral titers were determined by photometric measurement of the optical density at 260 nm to count virus particles per milliliter and by standard plaque assay to count plaque-forming units (PFU) per milliliter on HEK293 cells, as described previously.[27]

### siRNAs

The online tool BLOCK-iT RNAi Designer from Thermo Fisher Scientific was used to select new siRNA against the adenoviral *pTP* and *pol* genes, resulting in design of sipTP and siPol-1, respectively. siPol-2,[25] which is also directed against the *pol* gene, and siContr, which does not match any sequence present in the viral or human genome,[49] has been described previously. The siRNAs were synthesized as siRNA duplexes with dTdT 3' overhangs (Eurofins Genomics Germany, Ebersberg, Germany). Chemically modified siRNAs sipTP$_{mod}$, siPol-1$_{mod}$, siPol-2$_{mod}$, and siContr$_{mod}$ were generated by Axolabs (Kulmbach, Germany). The sequences of unmodified and chemically modified siRNAs are listed in Figures 1A and 2A.

### Generation of LNPs with encapsulated siRNAs

For *in vivo* delivery, siContr$_{mod}$ and sipTP$_{mod}$ were encapsulated within LNPs, which contained the cationic aminolipid XL-10. The generation of the XL-10-containing LNPs with encapsulated siRNAs has been described previously.[37,53] Briefly, the T junction-based produced LNPs were a composition of a lipid mixture containing the aminolipid XL-10, 1,2-distearoyl-3-phosphatidylcholine (DSPC), α-[3'-(1,2-dimyristoyl-3-propanoxy)-carboxamide-propyl]-ω-methoxy-polyoxyethylene (PEG-c-DOMG), and cholesterol. The ratio of XL10:DSPC:cholesterol:PEG-c-DOMG was 50:10:38.5:1.5 mol %. The lipids were first mixed in ethanol, and the siRNA molecules were dissolved in an aqueous buffer. The total lipid-to-siRNA ratio was 7:1. Then both mixtures were mixed together, which led to self-assembly of the particles encapsulating the siRNAs. The particle size, the polydispersity index, and the zeta potential were determined by dynamic light scattering (DLS) method. The drug concentration was determined by measurement of the optical density 260 ($OD_{260}$) and drug encapsulation by oligo green assay. Generation of LNP siRNAs and all measurements on the LNP siRNAs were carried out by Axolabs (Kulmbach, Germany).

### Plasmids

Plasmids containing miR-TS were generated by insertion of annealed miR-TS primers into the 3' UTR of the *Renilla* luciferase (hRLuc) reporter cDNA psiCheck2 (Promega, Walldorf, Germany) via the *Xho*I and *Pme*I restriction sites. The primers were as follows: for sipTP-TS, 5'-TCGAGGCTGGGTTATGTACTTCTTCTTT-3' and 5'-AAAGAAGAAGTACATAACCCAGCC-3'; for sipTP-Ad41TS, 5'-TCGAGGTTGGGTTATGTACTTCTTCTTT-3' and 5'-AAAGAAGAAGTACATAACCCAACC-3'; and for sipTP-Ad4,19,64TS, 5'-TCGAGGCTGGATTATGTACTTCTTCTTT-3' and 5'-AAAGAAGAAGTACATAATCCAGCC-3'. The correctness of all plasmids was confirmed by sequencing.

### Luciferase reporter assays for detection of siRNA activity

HEK293 cells were seeded in 48-well plates. The next day, cells of one well were transfected with 50 ng Dual-Luciferase reporter plasmids containing the corresponding miR-TS and 30 nM of siRNAs using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). Firefly luciferase and hRLuc activity were analyzed after 48 h using the Dual-Luciferase reporter system (Promega) in a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany), as recommended by the manufacturer.

### Transfection of siRNAs and hAd5 infection

HeLa cells were seeded in 24-well plates. The next day, cells were infected with selected MOIs of hAd5 for 2 h in serum-free medium. The medium was replaced with complete medium, and siRNAs were transfected to a final concentration of 30 nM per well via Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). After 48 h, cells were lysed by three freeze-thaw cycles. Supernatants were used to directly quantify infectious hAd5 via plaque assay or to infect new HeLa cells seeded in 24-well plates for 2 h to determine the amount of infectious hAd5 particles by subsequent qPCR.

### Plaque assay

HEK293 cells were seeded in 12-well-plates and reached a confluent monolayer the next day. Cells were inoculated with log dilutions of virus-containing solution in serum-free medium for 1 h. The supernatant was discarded, and cells were overlaid with a 1:3 mixture of 5% low-melting agarose (Sigma-Aldrich) and complete medium.

After 10–14 days, plaques appeared, and the hAd5 titer was determined as PFUs per milliliter.

#### Quantitative PCR for detection of adenoviral DNA

After discarding the supernatant of hAd5-infected cells, the cells were lysed in PBS by three freeze-thaw cycles, transferred to a fresh tube, heat-inactivated at 95°C for 10 min, and centrifugated at 12,000 rpm for 10 min 1.5 μL of the supernatant was used directly in a qPCR for detection of hAd5 DNA using primers 5'-CACATCC AGGTGCCTCAGAA-3' and 5'-AGGTGGCGTAAAGGCAAATG-3' directed against the adenoviral *hexon* gene and the SsoFast EvaGreen Supermix (Bio-Rad, Feldkirchen, Germany). As a reference, the genomic DNA of 18S rRNA was analyzed using the primer pair 5'-CCCCTCGATGCTCTTAGCTG-3' and 5'-TCGTCTTCGAACC TCCGACT-3'. A qPCR reaction was carried out in duplicate in a C1000 Thermal Cycler and CFX96 real-time system (Bio-Rad). Relative hAd5 genome copy numbers were determined by the ΔΔCt calculation method.

#### Determination of infectious hAd5 titers in tissue

Three pieces of each animal organ (liver and spleen) and one piece of heart were separately homogenized in 0.4 mL DMEM using disposable plastic pestles followed by two freeze-thaw cycles. Serum from animals was obtained by centrifugation of whole blood and stored at −20°C. HAd5 titer was determined as described previously.[27] Briefly, HeLa cells were seeded in 24-well-plates and incubated the next day with 1:10 diluted virus solution for 2 h in serum-free medium. The medium was replaced with complete medium, and after 48 h, cells were washed with PBS. Virus was released from cells by three freeze-thaw cycles in PBS. For absolute quantification of viral titers, HeLa cells were infected in parallel with 500, 50, 5, and 0.5 vp hAd5 per cell (hAd5 standard). The number of viral genomes was determined by qPCR as described above.

### QUANTIFICATION OF hAd5 pTP mRNA AND IMMUNE MEDIATOR mRNA

Total RNA was isolated from liver tissue using TRIzol (Thermo Fisher Scientific) according to the manufacturer's recommendations. Two mg of the RNA was treated with 2–3 U DNaseI (New England Biolabs, Frankfurt am Main, Germany) for 1–2 h. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to reverse transcribe 400–800 ng DNaseI-treated RNA. hAd5 pTP mRNA was determined by qRT-PCR using the primer pair 5'-TCA ACTTCGCCGTGGACTTCT-3' and 5'-AGGTAGTTGAGGGTGGT GGC-3' with 18S rRNA expression for normalization of the *in vitro* data or with HPRT1[68] expression for normalization of the *in vivo* data. IL-6, IL-12, IL-1β, TNF, and IFN-γ expression was determined by qRT-PCR using the previously described primer pairs recognizing the respective sequences,[68] with determination of HPRT1 for normalization. SsoFast EvaGreen Supermix was used, and change of expression was calculated using the ΔΔCt method against $siContr_{mod}$-transfected HeLa cells for evaluation of the *in vitro* data or against NaCl 0.9%-treated and not hAd5-infected immunosuppressed animals.

#### Measurement of ALT, AST, and GLDH

Serum analysis for ALT, AST, and GLDH activity was done by Laboklin (Bad Kissingen, Germany).

#### Ethics statement for *in vivo* experiments

All *in vivo* procedures involving the use and care of animals were performed according to the European principles of laboratory animal care (Directive 2010/63/EU) and approved by the local ethics committee (Landesamt für Gesundheit und Soziales, Berlin, Germany).

#### Animal experiments

Male Syrian hamsters were obtained from Charles River Laboratories (Sulzfeld, Germany) and had a weight of about 110 g when starting immunosuppression with CP (Sigma-Aldrich). CP was administered intraperitoneally (i.p.) at an initial dose of 140 mg/kg and then twice weekly at a dose of 100 mg/kg. Seven days after the first CP injection, animals were anesthetized with isoflurane, the left jugular vein was prepared, and hAd5 was injected at a dose of $4 \times 10^{10}$ vp/kg or $4 \times 10^{11}$ vp/kg hAd5. In another experiment, $6 \times 10^{10}$ vp/kg of hAd5 (low-dose infection model) or $6 \times 10^{11}$ vp/kg of hAd5 (moderate-dose infection model) were i.v. injected 7 days after the first CP injection. In this experiment, the right jugular vein was prepared immediately after hAd5 infection, and 2 mg/kg of the LNP siRNA was injected. Immunosuppressed animals that were not hAd5 infected received NaCl 0.9% into the left jugular vein 7 days after the first CP injection. The surgical step was accompanied by analgesic treatment with subcutaneous injection of 0.5 mg/kg meloxicam (Mesolute, 5 mg/mL, CP-Pharma, Burgdorf, Germany). Animals were sacrificed for organ harvest 14 days after the first CP injection. The organs were dissected and rapidly frozen in liquid nitrogen or placed in 4% formalin.

#### Immunohistological examination

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut, and stained with hematoxylin and eosin (H&E) as described previously.[69] Semiquantitative histopathologic scoring was performed by a board-certified pathologist to quantify inflammatory lesions. A grade of 0 was given to a completely physiologic liver tissue. Grade 1 showed minimal (1–2 spots), grade 2 mild (2–5 spots), grade 3 moderate (5–10 spots), grade 4 severe (>10 spots), and grade 5 severe (<10 spots and/or large coalescing areas) of inflammation. Preparation of 4- to 5-μm sections of FFPE livers for immunohistochemical staining was performed as described previously.[27] Briefly, after blocking nonspecific antibody binding, sections were incubated overnight at 4°C with a monoclonal mouse anti-adenovirus antibody (ab3648, Abcam, Cambridge, UK) that recognizes the hAd5 E1A protein. Afterward, slides were incubated at room temperature for 30 min with a biotinylated secondary goat anti-mouse antibody (dilution 1:200 each; Vector Laboratories, Burlingame, CA, USA). An avidin-biotin-immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Eching, Germany) was used for immunolabeling, and diaminobenzidine tetrahydrochloride (DAB; Merck, Darmstadt, Germany) was used for viral protein visualization.

### Statistics

Results are expressed as means ± SEM. To test for statistical significance of *in vitro* data, an unpaired Student's t test was applied. Statistical significance of *in vivo* data was determined using the Mann-Whitney *U* test.

## DATA AVAILABILITY

Data will be supplied following reasonable requests.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.05.016.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

A.G. conducted *in vitro* and *in vivo* experiments with hAd5-infected immunosuppressed Syrian hamsters. B.D. and L.E. contributed to *in vivo* experiments with hAd5-infected immunosuppressed Syrian hamsters. R.K. and K.K. conducted histological and immunohistological investigations. J.K. contributed with resources. A.G. wrote the manuscript, which was reviewed, edited, and revised by K.K., R.K., H.-P.V., J.K., and H.F. H.F. conceived the project and was responsible for project administration and supervision.

## DECLARATION OF INTERESTS

A.G. and H.F. have a patent pending for anti-adenoviral modified siRNAs encapsulated within LNPs.

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