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ASX RELEASE

ddRNAi Drug for Hepatitis C Safe and Deliverable Reported in New Scientific Paper.

Sydney June 28, 2012. Benitec Biopharma today announced that important peer-reviewed preclinical safety and delivery results from the hepatitis C program using Benitec Biopharma's ddRNAi gene silencing technology licensed to Tacere Therapeutics have been published in the prestigious journal *Molecular Therapy.* This follows the previously announced publication of the first paper from this program in December last year that demonstrated the capability of the lead compound to efficiently inhibit the hepatitis C virus in model systems.

The authors of the current study included researchers from Tacere, Pfizer and the University of Pennsylvania. They reported that a single intravenous injection of TT-034, Tacere's second generation anti-hepatitis C virus (HCV) ddRNAi-based drug, produced safe and sustained levels of shRNA in non-human primates (NHPs), the gold standard animal model for human toxicology testing. Furthermore, TT-034 successfully delivers its payload to nearly all of the hepatocytes within the liver. Importantly, none of the 18 animals dosed with the second generation vector demonstrated any signs of toxicity. The authors concluded that on this basis a clinical trial of the drug for treatment of HCV infection is warranted. Specifically, the authors state on the basis of these results: "...clinically feasible doses of TT-034 administered to patients should be sufficient to confer therapeutic benefit."

The full text of the paper is attached and is available on-line at www.nature.com/mt/journal/vaop/ncurrent/pdf/mt2012119a.pdf

"Clearly, TT-034 is well positioned to move into the clinic. We believe that the unique therapeutic modality has the potential to be transformative in the way patients infected with HCV are treated," said Dr David Suhy, Tacere's Research Director and lead author on the paper.

Benitec Biopharma's CEO, Dr Peter French, said, "These results indicate that TT-034 is likely to be a safe and effective single shot therapeutic for hepatitis C, a debilitating viral disease that affects 2% of the world's population. The next step of the program is to enter a clinical trial, and we are in active discussion with Tacere's management to determine the optimal path to the clinic for this key program."

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About Benitec Biopharma

Benitec Biopharma Ltd is developing novel treatments for chronic and life-threatening conditions based on targeted gene silencing activity using a transformational technology: DNA-directed RNA interference (ddRNAi) - sometimes called expressed RNAi. The technology's potential to address unmet medical needs and to cure disease results from its demonstrated ability to permanently silence genes which cause the condition. Importantly, this technology's target gene and related gene pathways will rarely have presented as a therapeutic avenue for research for the traditional small molecule agents, currently accounting for the majority of today's pharmaceutical products.

Founded in 1997 and trading publicly since 2001, Benitec Biopharma is listed on the Australian Securities Exchange (ASX) under the symbol "BLT". Benitec Biopharma aims to deliver a range of novel ddRNAi-based therapeutics to the clinic in partnership with the pharmaceutical industry. Besides a focused R&D strategy in infectious diseases, cancer and chronic cancer-associated pain, Benitec Biopharma is pursuing programs with licensees that have advanced to pre-clinical and/or clinical trials.

Benitec Biopharma videos can be viewed at www.youtube.com/user/BenitecNews

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original article

Safe, Long-term Hepatic Expression of Anti-HCV shRNA in a Nonhuman Primate Model

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The hepatitis C virus (HCV) chronically infects 2% of the world population and effective treatment is limited by long duration and significant side-effects. Here, we describe a novel drug, intended as a "single-shot" therapy, which expresses three short hairpin RNAs (shRNAs) that simultaneously target multiple conserved regions of the HCV genome as confirmed *in vitro* by knockdown of an HCV replicon system. Using a recombinant adeno-associated virus (AAV) serotype 8 vector for delivery, comprehensive transduction of hepatocytes was achieved *in vivo* in a nonhuman primate (NHP) model following a single intravenous injection. However, dose ranging studies performed in 13 NHP resulted in high-expression levels of shRNA from wild-type (wt) Pol III promoters and dose-dependent hepatocellular toxicity, the first demonstration of shRNA-related toxicity in primates, establishing that the hepatotoxicity arises from highly conserved features of the RNA interference (RNAi) pathway. In the second generation drug, each promoter was re-engineered to reduce shRNA transcription to levels that circumvent toxicity but still inhibit replicon activity. *In vivo* testing of this modified construct in 18 NHPs showed conservation of hepatocyte transduction but complete elimination of hepatotoxicity, even with sustained shRNA expression for 50 days. These data support progression to a clinical study for treatment of HCV infection.

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INTRODUCTION

Currently ~170 million people worldwide are chronically infected with the hepatitis C virus (HCV). HCV infects the human liver and the disease slowly progresses in chronically infected patients from an asymptomatic condition to end-stage liver cirrhosis in about one in four patients, at which time liver transplantation is required.[1] The current prolonged treatment with interferon and ribavirin cures the disease in ~50% of patients and in combination with the new generation of protease inhibitors may result in an increased cure rate of up to 75% in highly controlled clinical trials.[2,3] However, the required treatment duration remains prolonged (6–12 months) and often results in significant side-effects. Because the HCV genome is comprised of a single RNA molecule and the intracellular portion of its infectious cycle occurs strictly within the cytoplasm, HCV is an ideal candidate for therapeutics based on RNA interference (RNAi). There have been numerous reports on the use of siRNAs and/or short hairpin RNAs (shRNAs) to inhibit HCV RNA replication in replicon model systems.[4–7] Indeed, up to 80-fold decreases in HCV RNA levels have been observed, as well as the clearance of replicating HCV RNA in >98% of cells. Yet, because HCV is replicated with a self-encoded RNA-dependent RNA-Polymerase which lacks proof-reading activity, it is prone to mutant generation which can result in a loss of RNAi activity through the accumulation of nucleotide point mutations within the siRNA target sequence.[8–10] To counteract the generation of escape mutants for HCV and other viruses, several groups have documented success using viral vectors capable of expressing multiple shRNA species against the viral genome targets.[11–13]

The initial optimism surrounding the development of shRNA therapeutics against human diseases has been tempered by reports suggesting that high levels of hairpin expression can cause toxicity *in vivo*. Grimm *et al.* were among the first to demonstrate dose-dependent shRNA-induced hepatocellular toxicity and death in murine models.[14] This was followed by a number of other *in vivo* studies involving degeneration of murine striatal tissues[15,16] or central nervous system neurons.[17] It was hypothesized that liver damage was caused by high levels of expression of certain "toxic" shRNA sequences, resulting in a competition for cellular enzymes or pathways required to process endogenous miRNA species. To address this problem, different strategies have been employed by multiple groups to abrogate these toxic effects, including modelling the shRNA into the context of naturally occurring miRNA backbones[16] or Alu-repeats,[18] changing the type of viral vector used for delivery,[17] or by using endogenous promoters that possess weak transcriptional activity.[19–21]

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Direct modification of the regulatory elements within the endogenous promoters to attenuate shRNA transcription and accumulation has not been previously described. All vertebrate small nuclear RNA type promoters like U6 are organized into a proximal region, containing the proximal sequence element (PSE), a distal region containing the enhancer, and a TATA box located between the PSE and the transcription initiation nucleotide; all three regions contribute significantly to binding of the transcription complex. Despite a high degree of sequence similarity between the conserved elements of other Pol III promoters, exchanging the promoter-specific PSE from U6-7 with analogous sequences in the U6-8 promoter significantly reduced transcriptional activity as compared to the native promoter, demonstrating that these hybrid promoters have the potential to have reduced transcriptional activity.[22]

In this article, we describe an optimized therapeutic against HCV that expresses three shRNAs from a single vector. The simultaneous targeting of three highly conserved regions of the HCV viral genome substantially reduces the likelihood of escape mutants. A key to the success of this approach is an efficient delivery system that can transduce the majority of hepatocytes, thus eliminating all replicating virus in the HCV-infected cells and simultaneously providing prophylaxis to uninfected cells. Wild-type (wt) adeno-associated virus (AAV) is a small, nonpathogenic, replication-incompetent virus that has been used in a wide variety of clinical studies. We used an AAV serotype 8 capsid which has been shown to transduce hepatocytes with a high level of efficiency in mouse models[23,24] and in Rhesus macaques[25] and has also been used successfully in a recent clinical study for hemophilia B.[26] Durability of transgene expression following AAV transduction of liver is robust, often lasting years following a single injection.[27] Furthermore, we used self-complementary vectors, wherein the transgene expression cassette is packaged as a double-stranded AAV genome, thus eliminating the rate-limiting second strand synthesis for AAV transduction and enhancing transgene expression.[28,29] These features minimize the dose of vector required to achieve robust transduction and expression within liver tissues *in vivo*.

Our first generation molecule, termed TT-033 (aka PF-0488818), possessed potent activity against HCV in a replicon model and demonstrated almost complete penetration of hepatic tissues following intravenous injection in nonhuman primates (NHPs). Yet liver toxicity was observed in a dose-dependent manner, which we hypothesized was due to the strong transcriptional activity of each of the Pol III promoters and led to an overabundance of shRNA. A simple reduction in the dose of vector administered led to incomplete transduction of hepatocytes, potentially leaving a reservoir of HCV positive cells for continued viral replication and infection of untransduced cells.

To test the hypothesis that too much shRNA caused toxicity, transcriptional activity was significantly reduced from each Pol III promoter in our second generation molecule, TT-034 (aka PF-05095808), by exchanging a less active U6-7 PSE regulatory element into each promoter. Despite measurably reduced levels of shRNA, the construct was nonetheless still effective at maintaining efficient inhibition of the HCV replicon model. *In vivo* testing demonstrated that the vector was capable transducing NHP hepatocytes at levels comparable to the TT-033, but was now devoid of hepatotoxicity. Thus, re-engineering of the expression cassette has resulted in an agent that maintains efficacy and that is safe over a range of doses in a NHP model. These data predict that the product will be safe and efficacious in clinical testing.

RESULTS

Individual shRNA sequences were designed to target highly conserved HCV sequences from the (+) RNA genome across multiple genotypes (**Figure 1a**). Each shRNA was subcloned into an expression cassette comprised of three independent transcriptional cassettes under the control of three unique members of the U6 promoter family.[22] In order to verify RNAi activity of each shRNA, cells were cotransfected with the expression plasmid along with a reporter plasmid in which one of the targeted HCV sequences was fused downstream of the firefly luciferase open-reading frame. Although the shRNA were designed to target sequences within the (+) RNA of HCV, the virus also uses a (−) strand RNA genome

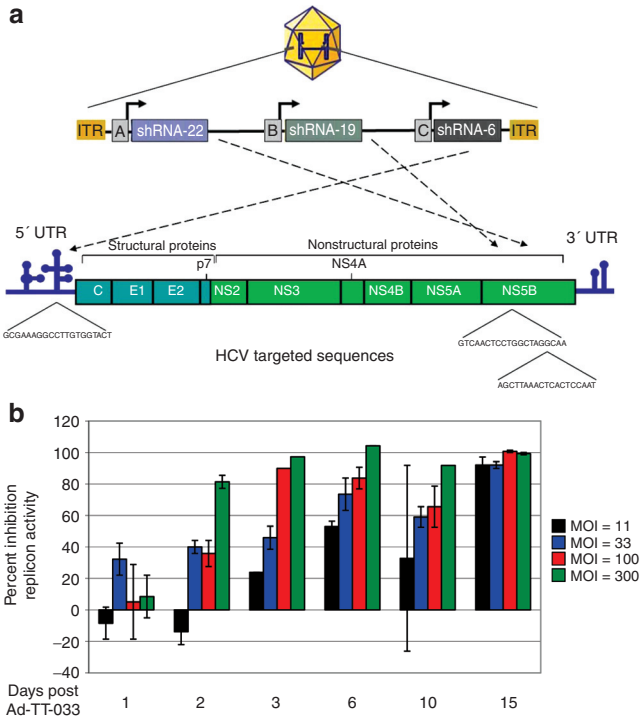


Figure 1 ***In vitro*** **anti-hepatitis C virus (HCV) inhibitory activity of short hairpin RNAs (shRNAs) against a HCV replicon model system.** (**a**) Schematic illustration of the TT-033 vector and expression cassette demonstrating the presence of three independent transcription cassettes, the order of the shRNA as well as the corresponding targeted regions and sequences within the HCV genome. A = U6-9 promoter; B = U6-1 promoter; C = U6-8 promoter. (**b**) Inhibition of renilla luciferase enzymatic activity in a Huh7-based HCV replicon model in which the reporter gene has been fused into the RNA genome. Replicon-containing cells were transduced with the indicated multiplicity of infection (MOI) of Ad-TT-033 and renilla activity monitored for up to 15 days following transduction. Each data point represents an average of $n = 8$ wells of independently transduced cells. Percent inhibition was calculated as the amount of renilla activity as compared to untransduced wells of cells. Figure 1a is reprinted with permission. Copyright© American Society for Microbiology, Antimicrobial Agents and Chemotherapy (2012) 56:1364-1375, doi: 10.1128/AAC.05357-11.

intermediate during the replication cycle; in theory both strands of the RNAi duplex could be active. Testing revealed that while shRNA-6 cleaved both the (–) and (+) RNA of the HCV genome, shRNA-22 and shRNA-19 only efficiently cleaved the (+) strand (**Supplementary Figure S1**).

One of the principal limitations of testing the activity of shRNA expression plasmids against the HCV replicon system is that Huh-7 cells, the only known cell line that supports robust replicon activity, is recalcitrant to typical transfection methods. In addition, although AAV8 is highly tropic for liver tissues *in vivo*, it is very inefficient at transducing tissue culture cells. Thus, to determine if the shRNA inhibited replicon activity, the expression cassette for TT-033 was subcloned into a transfer vector for production of recombinant adenovirus, known for broad tropism and efficient transduction of cells *in vitro*. Huh-7 cells harboring the HCV replicon were infected with different multiplicities of infection (MOIs) of Ad-TT-033 and the levels of replicon inhibition measured at various time points after vector administration. The data demonstrated that HCV replicon activity can be inhibited in a time- and dose-dependent manner (**Figure 1b**); a MOI of 11 was sufficient to inhibit replicon activity by ~100% at day 15. Cells transduced with an adenovirus stock expressing GFP demonstrated no appreciable changes in replicon activity at MOIs up to 100 (data not shown) indicating that the effect was specific to expression of the shRNA and not a result of the adenovirus delivery vehicle.

Transduction and toxicity assessment of TT-033 in Cynomolgus monkeys

A 50-day non-GLP dose range finding study with TT-033, a vector comprised of a self-complementary expression cassette (**Figure 1**) packaged in the serotype 8 AAV capsid, was conducted by administering a single intravenous injection into 13 Cynomolgus monkeys at doses ranging from 1.25×10^{11} to 1.25×10^{13} vector genomes per kilogram body weight (vg/kg). Before dosing, serum samples from the animals were screened for the prevalence of neutralizing antibodies against AAV8 to prevent confounding effects that may potentially arise from an unintended immune clearance of the vector leading to a lack of efficient transduction. Animals with levels of pre-existing serum antibody titers against AAV8 that were >1:5 were excluded from the study. During the course of the experiment, blood was collected weekly for clinical chemistry analysis in order to assess liver toxicity. Dose-dependent increases in alanine aminotransferase (ALT) (**Figure 2a,b**) were observed in NHPs administered 3.75×10^{11} vg/kg or greater of TT-033. Other dose-dependent changes consistent with hepatic injury included elevations in aspartate aminotransferase and alkaline phosphatase as well as decreases in albumin and total cholesterol (**Supplementary Figure S2a–d**). The parameters changed from baseline levels starting on or after day 21 post injection, peaked by day 36, and were trending toward resolution by day 50. One of the animals that received the highest dose of 1.25×10^{13} vg/kg was sacrificed at day 42 due to morbidity, and on gross and histological examination was found to have liver injury characterized by marked bridging hepatic necrosis. These serum chemistry data, which indicate hepatic damage, are consistent with results obtained in a 121-day acute toxicity study

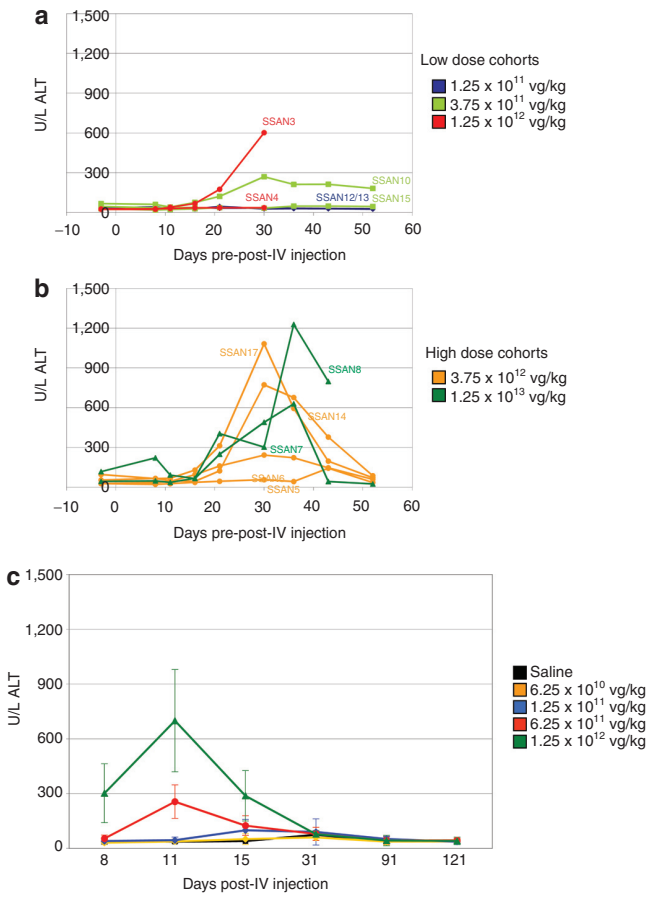


Figure 2 Alanine aminotransferase (ALT) activity in Cynomolgus monkeys and mice dosed with TT-033. Serum was collected at various time points following a single dose of TT-033 administered intravenously to Cynomolgus monkeys into either the cephalic or saphenous vein at (**a**) low dose levels of 1.25×10^{11} vg/kg (blue diamonds, $n = 2$), 3.75×10^{11} vg/kg (light green squares, $n = 3$) or 1.25×10^{12} vg/kg (red circles, $n = 2$) or (**b**) at high-dose levels of 3.75×10^{12} vg/kg (orange circles, $n = 4$) to 1.25×10^{13} vg/kg (green triangles, $n = 2$). ALT measurements from individual animals are shown for each data point. For data in (**a**) and (**b**), individual data points are plotted for each monkey and are demarcated using study specific animal numbers (SSAN). For the murine study (**c**), TT-033 was injected intravenously into the tail vein at dose levels of 6.25×10^{10} vg/kg (orange diamonds), 1.25×10^{11} vg/kg (blue diamonds), 6.25×10^{11} vg/kg (red circles) and 1.25×10^{12} vg/kg (green triangles). Mice dosed with saline (black squares) served as the negative control. Murine serum was collected at terminal necropsy at each time point. The plotted data average and standard deviation at each point are based from $N = 5$ mice per dosing cohort.

performed in female mice by administering a single intravenous injection of TT-033 ranging from 6.25×10^{10} to 1.25×10^{12} vg/kg (**Figure 2c** and **Supplementary Figure S3a–d**). Consistent with previous AAV studies that demonstrate more rapid onset of transgene expression in rodents than NHPs,[30] the progression of hepatocellular toxicity was faster in the murine model; the change in analogous parameters occurred as early as day 8, peaked at day 11, and resolved back to normal levels by day 31. A cohort of mice dosed with empty AAV8 capsids at protein levels consistent with the TT-033 dose of 1.25×10^{12} vg/kg showed no significant changes in ALT levels from baseline values at day

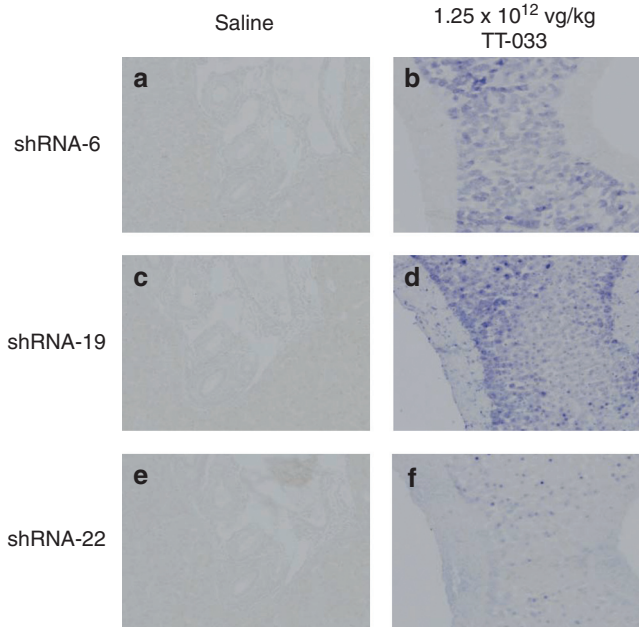


Figure 3 Distribution and homogeneity of short hairpin RNA (shRNA) expression in serial liver sections from Cynomolgus monkeys dosed with TT-033. Representative *in situ* hybridization (ISH) analysis of shRNA expression levels in hepatocytes using locked nucleic acid (LNA)-based probes against the hairpin sequences against (**a** and **b**) shRNA-6, (**c** and **d**) shRNA-19 and (**e** and **f**) shRNA-22. Tissues for (**a**,**c**, and **e**,) were taken from SSAN1, a monkey administered saline as a negative control while (**b**,**d**, and **f**) came from serial liver sections of SSAN3, a monkey dosed with 1.25×10^{12} vg/kg of TT-033.

8 indicating that the capsid was not responsible for the hepatic toxicity (data not shown).

The NHP model showed a strong correlation between dose and the severity of liver lesions detected by histopathology (**Supplementary Figure S4**). Test article-related hepatic lesions consisted of irregular liver surfaces with prominent lobular markings on gross examination and apoptosis of hepatocytes, bile duct hyperplasia, deposition of brown pigment, Kupffer cell hyperplasia, mitotic figures in liver cells, bridging necrosis, and hepatocellular regeneration on the microscopic examination. In addition, the murine model demonstrated dose-independent findings in the adrenal glands with cortical degeneration and vacuolation as early as day 8, which persisted through day 30 but cleared by day 91. Similarly, a transient mineralization of blood vessels of the myocardium was noted in high-dose cohorts of mice sacrificed at day 11, but was reduced in frequency and severity at later timepoints and absent altogether by day 121. There were no clinical findings or mortality associated with these microscopic adrenal and cardiac findings. Significantly, neither adrenal vacuolation nor myocardium mineralization occurred at any dose in the NHP model.

In situ hybridization analysis (ISH) was performed using locked nucleic acid (LNA) probes that were able to qualitatively detect the presence of expressed shRNA within liver cells. ISH analysis of serial sections of day 30 hepatic tissue from SSAN3, dosed with 1.25×10^{12} vg/kg TT-033, reveals cytoplasmic staining and a uniform pattern of distribution of each of the shRNAs across nearly 100% of the hepatocytes whereas no staining is noted in the surrounding vascular wall and perivascular connective tissue within the hepatic parenchyma (**Figure 3b,d,** and **f**). No shRNA staining was noted in hepatic tissues from a saline treated animal (**Figure 3a,c,** and **e**). Quantitative measurements of vector transduction and shRNA expression was assessed by quantitative PCR (QPCR) in liver biopsies collected at day 15 from the Cynomolgus monkeys and in livers harvested at the conclusion of the experiment. Weaker probe binding appears to be the cause of lightly stained tissues in qualitative shRNA-22 ISH analysis (**Figure 3**) as QPCR measurements demonstrate equivalent expression of all three shRNA in SSAN3 (**Figure 4c**). Within individual animals, all three lobes of the liver sampled show equivalent levels of vector DNA as assessed by QPCR analysis, indicative of homogeneous distribution across the liver (**Figure 4a**).

Because recombinant AAV vectors are nonintegrating and maintained as episomes, previous studies demonstrate that hepatocyte damage and regeneration often results in the loss of vector from AAV-transduced cells.[14] Indeed, although QPCR analysis of TT-033 DNA showed dose-dependent transduction of the liver in the day 15 biopsies, day 50 TT-033 levels were markedly decreased in animals that received high doses of the test article and experienced hepatocyte damage as indicated by the presence of elevated levels of liver enzymes (**Figures 2a** and **Figure 4a**). At low doses where hepatocellular toxicity was not observed, durable levels of transduction were sustained to day 50, the longest time point measured in these experiments. Within individual animals, QPCR analyses of shRNA demonstrate equivalent levels of all three hairpins being produced in hepatic tissues (**Figure 4b,c**). With the exception of the high-dose animals, the levels of shRNA measured within the biopsies are directly reflective of the level of TT-033 DNA. Despite possessing exceptionally high numbers of TT-033 DNA copies/cell, the animals in the highest dose cohorts appeared to reach a threshold of shRNA expression. As vector is lost via hepatocyte damage and death, the ability to sustain high levels of transgene expression is also abrogated as evidenced by a concomitant decrease in shRNA levels in day 50 tissues. For example, in animals dosed with 3.75×10^{12} vg/kg (**Figure 4a**), day 50 TT-033 DNA and shRNA levels were maintained at comparable levels to day 15 biopsy samples in SSAN 5 and SSAN6. These same animals showed negligible changes in ALT levels over that same time frame (**Figure 2a**). Conversely, copies of TT-033 DNA and shRNA measured at day 50 dropped from the levels detected in day 15 biopsies in SSAN14 and SSAN17 and were concomitant with significant elevations of ALT in those animals. Thus, despite efficient and near complete transduction of hepatocytes, the loss of vector and increased hepatic enzyme levels in serum indicate that shRNA-mediated hepatic toxicity resulted from high doses of TT-033. Yet, simply reducing the amount of vector administered (**Figure 4a**) resulted in incomplete hepatocyte transduction potentially leaving a subset of HCV-infected cells untreated and at risk for infecting other nontransduced normal hepatocytes.

Engineering promoters to eliminate toxicity

Given the dose-dependent toxicity findings, we hypothesized that lowering the transcriptional levels of shRNA expression per promoter could reduce toxicity while preserving the ability to use doses sufficient to ensure complete transduction of hepatocytes.

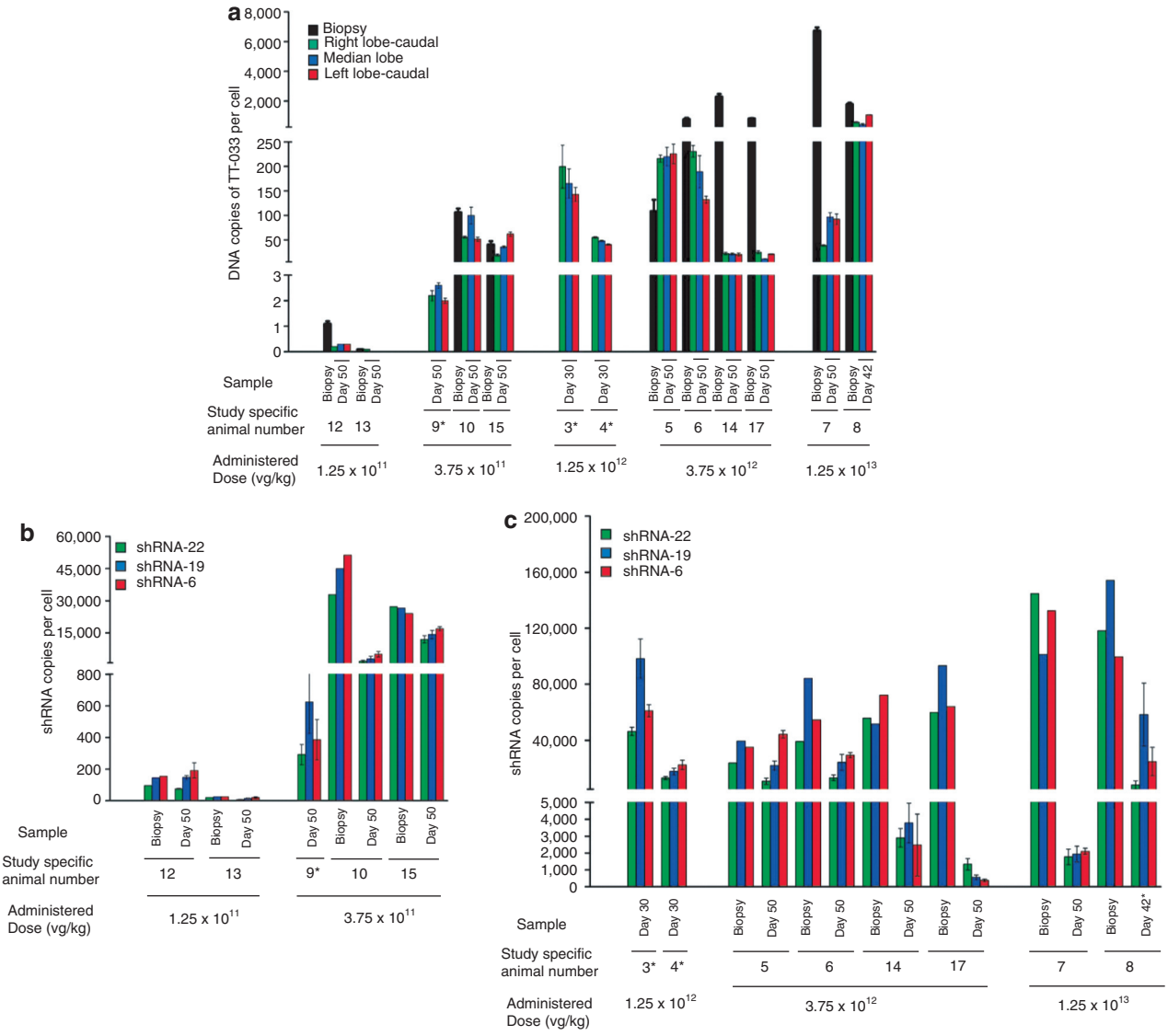
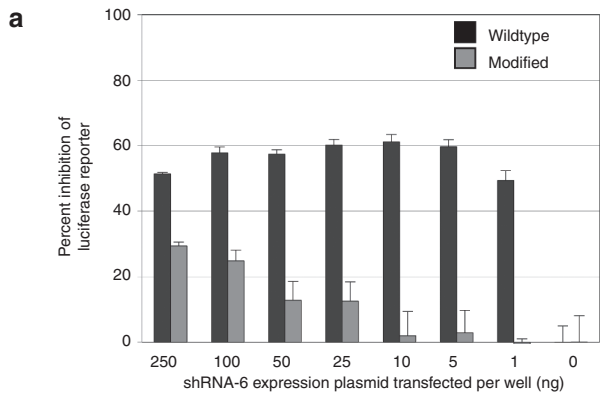
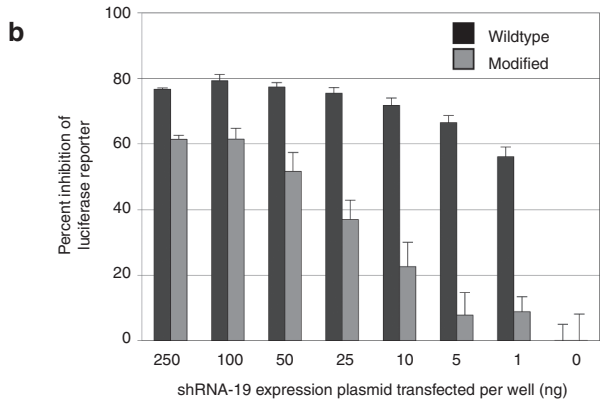
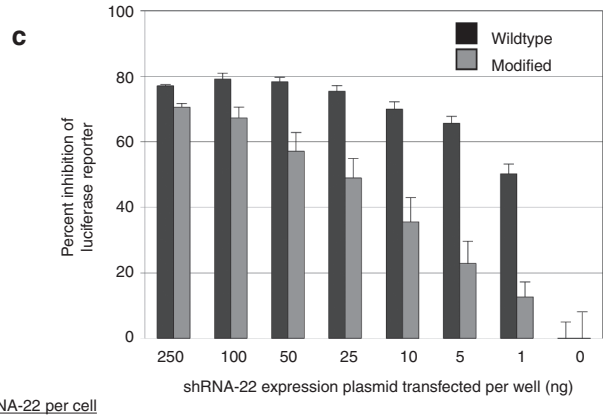


Figure 4 Transduction and short hairpin RNA (shRNA) expression in hepatic cells from Cynomolgus monkeys dosed with TT-033. Hepatic tissues were analyzed for either DNA or shRNA levels following intravenous administration of a single dose of TT-033 into either the cephalic or saphenous vein at dose levels ranging from 1.25×10^{11} vg/kg to 1.25×10^{13} vg/kg. Samples of liver were collected from biopsies performed 15 days following vector dosing, or from tissues of the right, medial, or left lobe taken at necropsy of the specified day. Biopsies were not collected from monkeys with asterisks next to the animal identifier number. (**a**) Genomic DNA was purified from each sample and DNA copies of TT-033, a measure of transduction, was assessed by QPCR. Each data point represents an average analysis from four independent aliquots of each genomic DNA sample. Levels of DNA from biopsy material are shown in black while material sampled from the right, medial or left lobe are represented by the green, blue and red columns, respectively. (**b** and **c**) RNA was collected and purified individually from each of the samples and shRNA-22 (green), shRNA-19 (blue), or shRNA-6 (red) expression levels examined using QPCR procedures. For biopsy samples, the data represents analysis in triplicate from RNA extracted from the individual sample. For tissues collected at necropsy, each data point and associated standard deviation represents the combined average from the independent analysis of one RNA sample taken from each of the three lobes.

As activity of the U6-8 promoter was significantly reduced by replacing the wt PSE region with the corresponding PSE from U6-7,[22] similar changes were engineered into the promoters of PF-0488818. The individual shRNA and all other components of the drug remained unchanged; this new molecule was labelled TT-034. RNAi activity from the hybrid promoters was tested by cotransfecting tissue culture cells with luciferase expression reporter constructs and plasmids encoding for individual shRNA transcribed by either hybrid or wt U6 promoters. Because even low levels of shRNA can lead to high levels of reporter inhibition, transfection conditions included a range of the shRNA expression plasmid. The percentage of luciferase reporter inhibition mediated by shRNA-6 expression from the wt U6-8 promoter remained relatively unchanged despite titrating down the input plasmid from 250 to 1 ng per transfected well (**Figure 5a**) Likewise, use of wt Pol III promoters to drive shRNA-19 (**Figure 5b**) and shRNA-22 (**Figure 5c**) expression resulted in sustained inhibition until transfected quantities are reduced to 10 ng per well or below. Conversely, even at 250 ng per well, reduced inhibition levels were measured in transfection mixtures using the plasmids containing the hybrid







shRNA-22 per cell								
Wildtype promoter	45,585	43,710	n/d	n/d	3,398	n/d	2,423	n/d
Modified promoter	5,197	2,174	n/d	n/d	432	n/d	48	n/d

n/d=not determined

Figure 5 Changes in short hairpin RNA (shRNA) expression and inhibitory activity from constructs with modified Pol III promoters. The activity of individual shRNA from expression plasmids coding for (**a**) shRNA-6, (**b**) shRNA-19, or (**c**) shRNA-22 that are driven from either wt Pol III promoters (black) or modified promoters (gray) were tested against their cognate luciferase reporter constructs. A Renilla expression plasmid was also included in each transfection mixture to normalize for differences in transfection efficiency. A nonspecific plasmid DNA was used to normalize the amount of nucleic acids used for each transfected condition. The percent inhibition was calculated by comparison of luciferase activity in cells transfected with an identical reporter and a U6-1 plasmid lacking an expressed shRNA sequence. Each analysis represents data collected from four replicate transfections ± SD.

Pol III promoters. More importantly, the ability to inhibit the reporter plasmid dropped far more quickly as the amount of the shRNA expression plasmid is decreased. For instance, ~70% inhibition of the shRNA-22 luciferase reporter could be achieved by transfection of 100 ng of the plasmid containing the hybrid Pol III promoters, whereas only 5 ng of the corresponding plasmid with wt promoters was required to achieve the same level of inhibition: a 20-fold difference.

To prove the hypothesis that lowered RNAi activity results from decreased shRNA production with the hybrid promoters, shRNA-22 levels were measured in RNA harvested from parallel wells using QPCR. Cells transfected with 100 ng of wt expression plasmid express an average of 43,710 copies/cell (**Figure 5c**). The same amount of transfected hybrid Pol III promoter plasmid yielded 16-fold less shRNA-22. Cells transfected with either 10 ng or 1 ng of the wt plasmid expressed 3,398 and 2,423 copies/cell, respectively, comparable to the level of shRNA-22 measured in cells transfected with 100 ng of the expression construct with the hybrid promoters.

Inhibition of a HCV replicon model with TT-034

As with the first generation compound, an adenovirus variant of TT-034 was tested for the ability of the shRNA to inhibit HCV replicon activity. Huh-7 cells harboring the HCV replicon were infected with different MOIs of Ad-TT-034 and the levels of replicon inhibition measured at various time points after vector administration. The data demonstrate that despite reduced shRNA expression, HCV replicon activity can be efficiently inhibited by TT-034 (**Figure 6a**). In order to quantify the amount of each shRNA required for inhibition of replicon activity, parallel wells of cells treated at identical conditions were harvested for RNA, and shRNA levels were measured by QPCR. Shown in **Figure 6b**, levels of each shRNA accumulate through day 3 and then drop off, likely because adenovirus is nonintegrating and is diluted out as cells divide. Though, we are taking static measurements of shRNA to measure the dynamic process of replicon inhibition, the results from these experiments, particularly cells treated with an MOI as little as 30, suggest that between 20 and 220 copies of each shRNA per cell may be sufficient to abrogate replicon activity (**Figure 6b**).

Transduction and toxicity assessment of TT-034 in Cynomolgus monkeys

A follow-up 50-day, non-GLP Cynomolgus monkey study was then performed on 18 NHPs to assess transduction and toxicity after a single intravenous administration of the modified compound TT-034. A parallel cohort of six NHPs was dosed with the first generation compound, TT-033, in order to assess any differences in transduction efficiency and shRNA expression through day 15. Following administration of TT-034, a mild elevation in serum ALT and aspartate aminotransferase was noted to occur at day 4 in high-dose animals (**Figure 7a** and **Supplementary Figure S5a**). This transient increase resolved to baseline levels by day 11. Because the previous onset of liver enzyme increases in response to TT-033 did not occur until after day 20 (**Figure 2a,b**), it is likely that the transient day 4 response in this animal is unrelated to the expression of the shRNA. More importantly, and unlike the

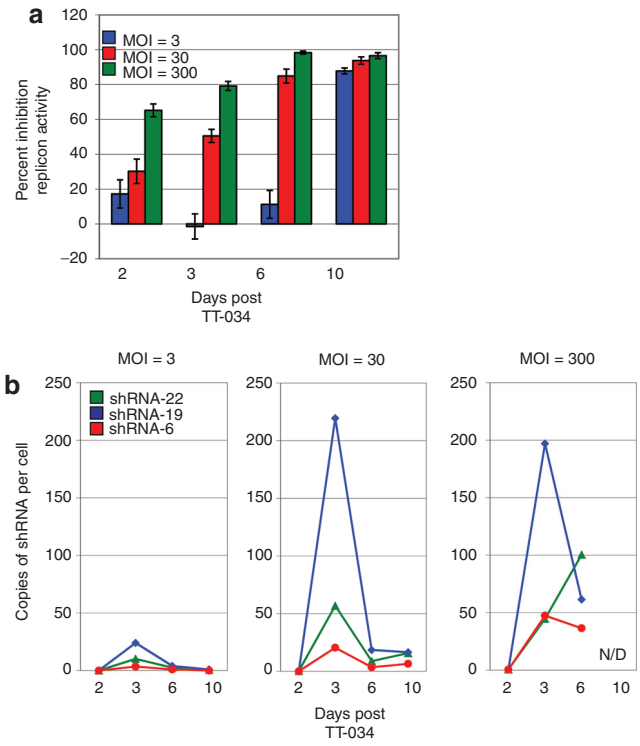


Figure 6 Anti-hepatitis C virus (HCV) inhibitory activity of short hairpin RNAs (shRNAs) against a HCV replicon model system. (**a**) Inhibition of renilla luciferase enzymatic activity in a Huh7-based HCV replicon model in which the reporter gene has been fused into the RNA genome. Replicon-containing cells were transduced with the indicated multiplicity of infection (MOI) of Ad-TT-034 and renilla activity monitored for up to 10 days following transduction. Each data point represents an average of $N = 8$ wells of independently transduced cells. Percent inhibition was calculated as the amount of renilla activity as compared to untransduced wells of cells. (**b**) RNA from parallel treated wells was collected and purified and served as template material for quantification. Levels of shRNA-22 (green), shRNA-19 (blue), or shRNA-6 (red) expression were quantified using QPCR procedures. Each shRNA analysis point represents data collected from four aliquots from the same RT reaction. N/D = not determined as RNA samples were not collected at this time point.

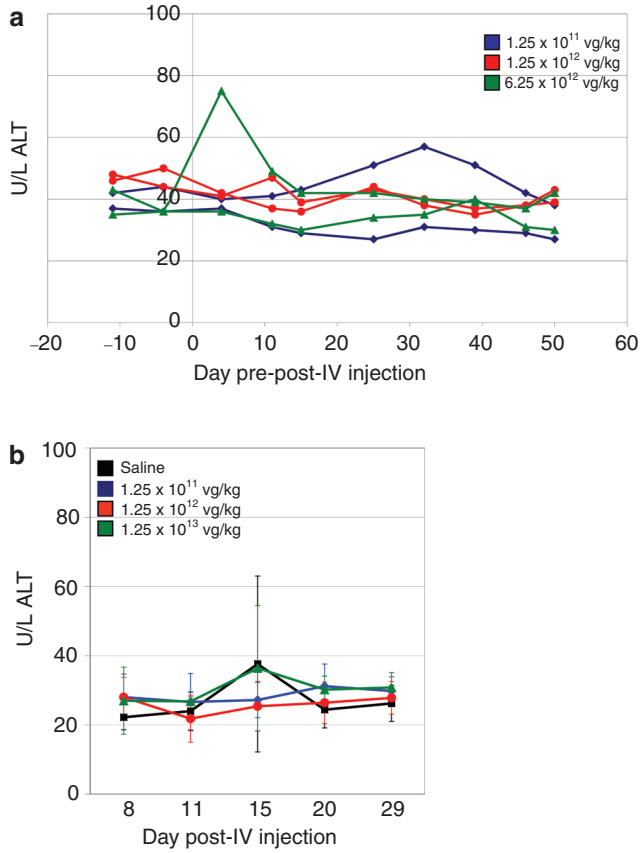
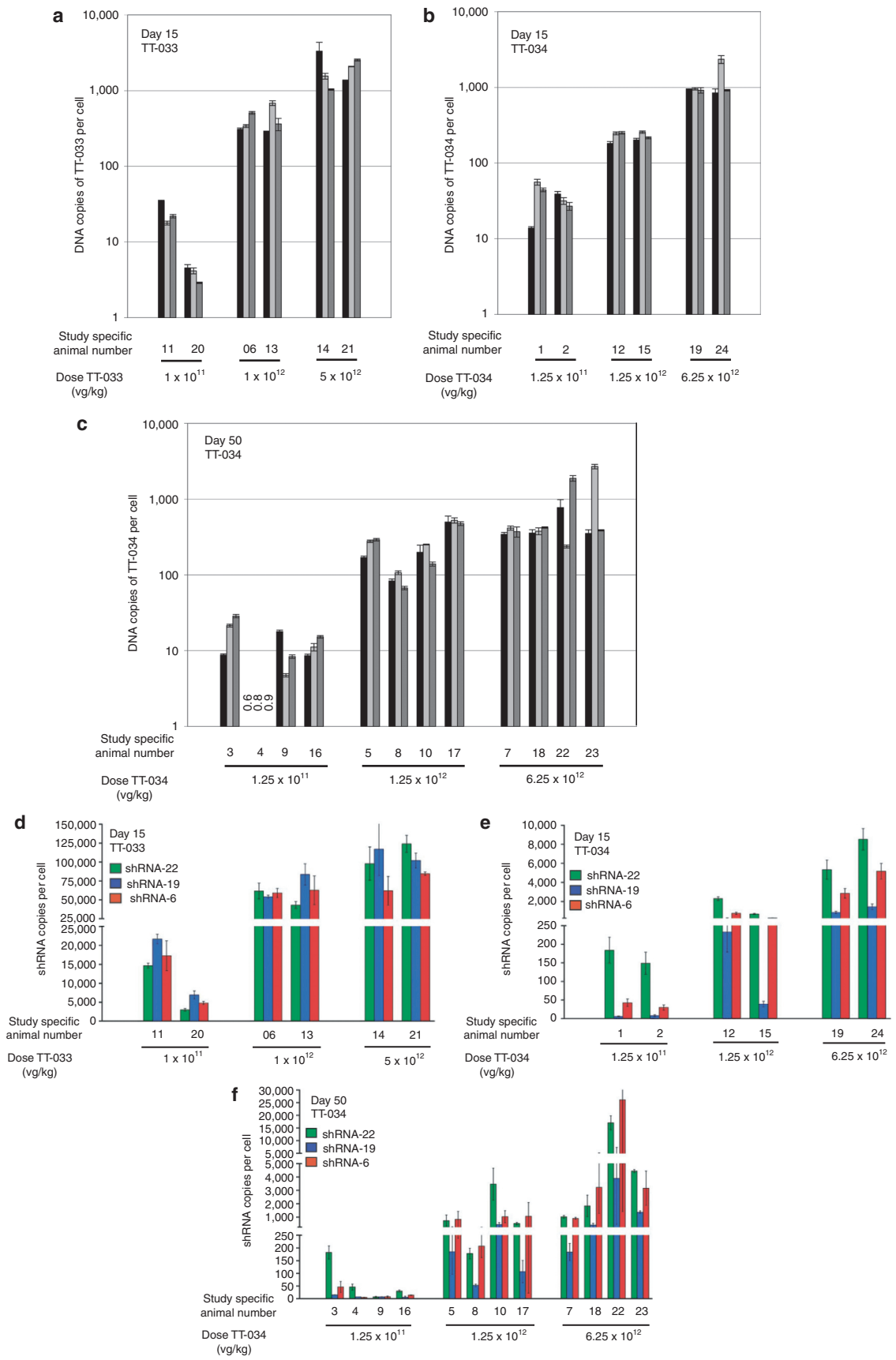


Figure 7 Alanine aminotransferase activity in Cynomolgus monkeys and mice dosed with TT-034. (**a**) Serum was collected at various time points following a single intravenous dose of TT-034 administered to Cynomolgus monkeys in either the cephalic or saphenous vein at dose levels of 1.25×10^{11} vg/kg (blue diamonds), 1.25×10^{12} vg/kg (red circles), or 6.25×10^{12} vg/kg (green triangles). Data from individual serum samples was assessed in quadruplicate. At each dose, the raw values from two males were averaged and two females were averaged and then plotted respectively. The modest rise in ALT levels in the high dose cohort at day 4 occurs within the male cohort. (**b**) For the murine study, TT-034 was injected intravenously through the tail vein at dose levels of 1.25×10^{11} vg/kg (blue diamonds), 1.25×10^{12} vg/kg (red circles), or 1.25×10^{13} vg/kg (green triangles) and serum collected at terminal necropsy at each time point. Serum from saline treated animals (black squares) served as a negative control. The plotted data average and standard deviation are based from $n = 5$ mice per dosing cohort.

first generation drug TT-033 which demonstrated dose-dependent shRNA related liver injury at doses of 3.75×10^{11} vg/kg or above, all remaining animals in the TT-034 cohorts showed no changes in markers of hepatic injury throughout the course of the experiment, despite being dosed with TT-034 at concentrations as high as 6.25×10^{12} vg/kg (**Figure 7a** and **Supplementary Figure S5b–d**). There were also no microscopic changes found by histopathology analysis in the liver that were related to the administration of TT-034. The lack of toxicity as a result of TT-034 administration was confirmed by a parallel 30-day acute toxicity study performed in a murine model. Despite significant changes in liver function tests in animals dosed with TT-033 at 6.25×10^{11} vg/kg or higher (**Figure 2c** and **Supplementary Figure S3a–d**), mice dosed with the second generation compound at levels up to 1.25×10^{13} vg/kg, 50-fold higher, showed no changes in any liver function test parameters as compared to saline control (**Figure 7b** and **Supplementary Figure 6a,b**).

Because the composition of the viral capsid, the primary determinant of transduction, remains unchanged between the two molecules, it was predicted that equivalent doses would result in a similar number of DNA copies of the respective vectors per cell. Indeed, although the doses of test article were slightly different, the day 15 TT-033 (**Figure 8a**) and day 15 TT-034 (**Figure 8b**) liver transduction levels were within two-fold of one another. However, QPCR analysis of shRNA levels demonstrated a marked reduction in levels of shRNA expressed from TT-034-transduced cells as compared to their TT-033 counterparts, a finding anticipated based on the manipulation of the U6 promoters (**Figure 8d-f**). For instance, day 15 animals dosed with 1.25×10^{12} vg/kg of TT-033 produced, on average, 52,358 copies of shRNA-22 per cell, 68,724 of shRNA-19 per cell, and 60,809 copies of shRNA-6 per cell; while a corresponding dose of 1×10^{12} vg/kg TT-034 produced 1,223, 196, and 782 copies/cell, respectively. Because shRNA-mediated toxicity has been eliminated, the levels of TT-034 DNA are largely



maintained through day 50 with a slight decrease across the same dosing cohorts from day 15 (**Figure 8b** versus **Figure 8c**). In addition, QPCR analysis demonstrates similar shRNA levels between comparably dosed animals at day 15 and 50 (**Figure 8e** versus **Figure 8f**), reaffirming that no hepatocellular toxicity has occurred and demonstrates that steady state levels of shRNA are likely attained at the earlier timepoint.

DISCUSSION

Even with the recent introduction of oral HCV protease inhibitors,[2,3] and associated sustained virologic responses of up to 75% in combination with interferon/ribavirin, HCV-infected patients still face a prolonged course of treatment with considerable side-effects.[31] The development of an effective shRNA approach could provide a therapeutic option that requires only a single injection, is devoid of the debilitating side-effects associated with current treatment regimens and has the potential to help provide benefit to patients with viral pools refractory to current treatment options. Alternatively, a shRNA approach could be used in combination therapies to markedly reduce the number of HCV-infected cells in the liver and allow for more efficient use of the current clinical drugs.

Treatment of infectious diseases by RNAi-based therapeutics provides an especially difficult challenge considering that the mechanism of action is dependent upon sequence specificity and that the replication machinery employed by many viruses generates a large pool of heterogeneous genomes. Fatefully positioned mutations within target sequences could quickly abrogate the inhibitory activity of a single RNAi inhibitor and give rise to a nonresponding virion subpopulation. Each of the shRNA in TT-034 was carefully chosen to inhibit a different, well-conserved area of the HCV genome and, much like the combination of small molecule drugs used in the treatment of HIV with highly active antiretroviral therapy, simultaneous expression of the shRNA thus provides a pool of therapeutic sequences that target multiple regions of the HCV genome simultaneously, thereby creating a "cocktail in one drug." A more detailed report describing the activity of TT-034 against the most abundant individual point mutations found in the clinic and HCV replicon variants other than genotype 1 has recently been published.[32]

Given that HCV infects an ever-changing subset of hepatocytes within a human liver, infection is focal and likely transient in the liver[33] and variable between patients,[34] a key requirement for an antiviral RNAi therapeutic to be curative would be the ability to transduce a high proportion of the hepatocytes. Failure to do so might permit untransduced, HCV-infected cells to continue shedding virus and potentially leaves uninfected cells at risk and vulnerable to nascent infection. Thus, a delivery method must be used that efficiently transduces nearly all hepatocytes, hence our choice of AAV8. Biodistribution analysis of over 30 different tissues from the cynomolgus monkeys dosed with TT-033 demonstrates that nearly 90% of AAV8 vector administered localizes to the hepatic tissues (data not shown, additional manuscript in preparation). Furthermore, transduction must be accomplished from a single administration of the therapeutic, as repeat dosing is untenable because of the humoral immune response which is generated by the memory B cells in response to the large infusion of viral capsids. **Figures 3** and **4a** and demonstrate that transduction of TT-033 is near homogeneous across hepatic tissues from a single administration of the drug.

Our work demonstrates that when sufficient doses of vector were used to ensure broad transduction of hepatocytes, wt Pol III promoters drive transcription of the shRNA at sufficiently high levels to cause hepatocellular toxicity in both murine and NHP models. To our knowledge, this is the first description of shRNA-mediated toxicity shown to occur in NHPs and argues this toxicity may well be conserved in humans. Previous studies have suggested that shRNA-mediated saturation of Exportin-5, an essential component of the endogenous RNAi cellular machinery responsible for translocation of miRNA from the nucleus to the cytoplasm, can lead to a global dysregulation of endogenous miRNA processing and perhaps account for liver toxicity.[14] While the experiments presented here do not definitively identify the specific mechanism of shRNA-mediated toxicity, a parallel TT-033 study (data not shown) in a murine model demonstrated that a large proportion of mature miRNA levels are not perturbed by TT-033 induced hepatocellular toxicity thus suggests that the shRNA had not induced toxicity by saturation of Exportin-5. Regardless, a strategy was required to keep the level of AAV8 vector administered at sufficiently high levels to maximize hepatocyte coverage while concomitantly reducing the cellular toxicity from the expressed shRNA. Several different approaches have been described that alleviate toxicity due to overexpression of shRNA. For example, shRNA directed against the mRNA sequences for the mutant huntingtin protein have been modeled into miRNA backbones, resulting in reduced striatal toxicity, but no compromise of the RNAi activity of the sequence. This corrective effect was attributed to a lowering of overall levels of mature shRNA species in which the hairpins associated with the precursor have been cleaved.[15,16] In the current study, engineering minor changes into the regulatory promoter elements of all three promoters to reduce transcriptional levels obviated the need to redesign shRNA sequences or use a delivery vehicle other than AAV8, a highly hepatotropic serotype. The resulting vector, TT-034, expresses the same three shRNA sequences at significantly reduced levels compared to TT-033, thus allowing the use of high levels of vector to transduce a large

Figure 8 Transduction and short hairpin RNA (shRNA) expression in hepatic cells from Cynomolgus monkeys dosed with TT-033 or TT-034. Cohorts of Cynomolgus monkeys were injected with either (**a**) TT-033 at doses of 1×10^{11}, 1×10^{12}, or 5×10^{12} vg/kg on day 0 or (**b**,**c**) TT-034 at doses of 1.25×10^{11}, 1.25×10^{12}, or 6.25×10^{12} vg/kg on day 0 and then sacrificed on either (**a**, **b**) day 15 or (**c**) day 50. Hepatic tissues from the left lobe (black columns), medial lobe (light gray), or right lobe (dark gray) were harvested at necropsy. Genomic DNA was collected and purified from the livers of each of the animals and was measured for transduction using QPCR procedures. Each analysis represents data collected from three aliquots of the genomic DNA samples. Sample from animal number 4 (**c**) resulted in 0.6, 0.8, and 0.9 DNA copies per hepatocyte from the left, medial, and right lobes, respectively. (**d**,**e**,**f**) RNA was collected and purified individually and shRNA-22 (green), shRNA-19 (blue), or shRNA-6 (red) expression levels examined using QPCR procedures. Each bar and associated SD represents the combined average from the independent analysis of one RNA sample taken from each of the three lobes.

percentage of hepatocytes, without associated shRNA toxicity. The reduction of shRNA levels from the modified U6 promoters was demonstrated on an activity level from a luciferase reporter system (**Figure 5**) as well as physically shown by QPCR analysis of liver tissues from NHPs (**Figure 8d–f**).

The results of *in vitro* replicon experiments demonstrated potent anti-HCV activity of each of the shRNA expression constructs. The ability to approximate the levels of shRNA required to inhibit HCV replicon activity, as well as quantify the level of shRNAs produced in NHPs, suggest that clinically feasible doses of TT-034 administered to patients should be sufficient to confer therapeutic benefit. Yet, the numbers of shRNA species required for inhibition of HCV on a per cell basis may actually be even lower than suggested by the transient replicon experiments (**Figure 6**); the adenovirus shRNA expression vector delivered to replicon containing cells is nonintegrating and thus was diluted amongst daughter cells as a result of cell division while in cell culture. The ability to use a shRNA approach with a vector which confers long-term expression *in vivo* results in durable expression; indeed, data from our unpublished IND enabling studies demonstrates persistent shRNA levels out to 180 days, the duration of the experiments in the murine and Cynomolgus models (data not shown). Furthermore, it is important to note that while the replicons produce hundreds or thousands of copies of the HCV genome per cell[35,36] chronically infected human hepatocytes harbor only 5–7 copies/cell on average.[33,37] Finally, the process of RNAi is catalytic; one active species loaded into the RNA-induced silencing complex can result in multiple rounds of degradation.

Testing the ability to knock down HCV infection *in vivo* in a relevant NHP model system remains an ongoing challenge, as the only species other than man that supports reliable viral replication is the chimpanzee. Due to the inability to perform extensive toxicology and histopathology on chimpanzees, the replicon continues to be the industry accepted model for anti-HCV activity. Murine models in which chimeric livers comprised in part of human hepatocytes can support replication of HCV *in vivo* but are exceptional fragile, limiting the ability to produce large numbers of viable animals and results in substantial variability.[38] In summary, we have shown that complete transduction of hepatocytes with TT-034 in a NHP, at levels that yield 100% inhibition in the replicon models. These doses of the modified vector, which can be delivered by a single injection, were not associated with toxicity in either mice or NHPs. These data justify the progression of this molecule to the clinic for the treatment of chronically infected HCV patients.

MATERIALS AND METHODS

Selection of targetable sequences from (+) strand of the HCV genome. HCV genotypes differ by as much as 31–34% in their nucleotide sequences, whereas subtypes (species within a given genotype) may differ by 20–23% based on full-length genomic sequence comparisons. Thus, regions of the viral genome with a high degree of conservation must be identified and carefully chosen to ensure that the clinical candidate will cast the broadest therapeutic net possible. To select shRNA sequences, a Jotun Hein bioinformatics alignment using MegAlign software (DNAStar) of all published independent full-length or near-full-length HCV sequences in the Lawrence Livermore database was performed (http://hcv.lanl.gov/content/index). In October 2003, when the analysis was initially performed, there were ~100 such sequences populated in that specific database. Although the distribution of sequences was skewed toward genotype 1 viruses, sequences representing genotypes 1 through 6 were analyzed. In the most optimal case, there would be a minimum of 21 nucleotides (nt) which would maintain absolute identity across all species analyzed for alignment. In the absence of having conservation of absolute identity, the stringency may be reduced to 80% of all sequences tested. The G/C base content must fall within a 35–65% range. The selected sequences must have a general lack of cross specificity to endogenous human targets (non-HCV) when queried against sequence databases.

Cynomolgus studies. Male and female Cynomolgus monkeys (*Macaca fascicularis*) were obtained and housed at SNBL USA (Everett, WA). SNBL USA is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), has an Animal Welfare Assurance issued by the Office of Laboratory Animal Welfare (OLAW), is registered with the United States Department of Agriculture (USDA), and has an Institutional Animal Care and Use Committee (IACUC) responsible for SNBL USA's compliance with applicable laws and regulations concerning the humane care and use of laboratory animals. Before the studies, the animals were given rigorous physical examinations and evaluated for clinical pathology parameters. Test article was administered intravenously after being diluted to the appropriate dose concentration with a solution consisting of 180 mmol/l sodium chloride, 10 mmol/l sodium phosphate, and 0.001% Poloxamer, pH 7.3. Intravenous bolus injection using an integrated straight needle was followed by a 2-ml flush with diluent. Serum chemistry analysis was performed on an Olympus AU640 analyzer. During necropsy, liver specimens were harvested, snapped frozen in liquid nitrogen and shipped to Tacere for analysis of DNA and shRNA levels. For histopathology analysis, a variety of tissues were collected, trimmed, placed in cassettes and fixed for 24 ± 2 hours in 4% paraformaldehyde at 2–8 °C prior to further processing. One set of perfused tissues was processed into paraffin blocks. The second set of tissues was immersed in 0.5 mol/l sucrose diluted in TBS for 48 hours at 2–8 °C, then mounted in OCT and frozen. OCT-mounted tissues were stored at −60 °C until observation by the pathologist for histopathology.

Mouse studies. Murine studies for animals dosed with TT-033 were conducted at Covance Laboratories (Harrogate, UK). The study was conducted in accordance with the requirements of the Animals (Scientific Procedures) Act 1986 (UK). Mice of the Crl: CD-1 (ICR) strain were obtained from Charles River UK Limited (Margate, UK). Murine studies for TT-034 were performed on male CD-1 mice at Pfizer (Andover, MA) and the study was conducted in accordance with the *In Vivo* Guidelines of Pfizer Global Research & Development (St Louis, MO). Test article was administered intravenously after being diluted to the appropriate dose concentration with a solution consisting of 180 mmol/l sodium chloride, 10 mmol/l sodium phosphate, and 0.001% Poloxamer, pH 7.3. For both studies, up to 200 µl was the maximal volume given by lateral caudal tail vein injection. Serum chemistry analysis was performed on samples collected at each necropsy point. During necropsy, liver specimens were harvested, snapped frozen in liquid nitrogen for analysis of DNA and shRNA levels.

Neutralizing antibody screening. Cynomolgus monkeys were prescreened for the presence of neutralizing antibodies, high titers of which have the potential to limit transduction. Animals in the TT-033 dose range finding study were screened using an AAV8 reporter virus (Children's Hospital of Philadelphia) that expressed the β-lactamase gene as previously described.[39] For experiments performed with TT-034, serum samples were screened with a modified protocol that used an AAV8 reporter vector that delivered the sequences encoding for luciferase (Virovek, Hayward, CA).

TT-033 plasmid construction. The pSiren Shuttle plasmid (Takara Biosciences-Clontech, Mountain View, CA), which contains the U6-1 wt promoter, was used for expression of single short hairpin molecules. Target

sequences of 19 nt for shRNA-19 were synthesized as duplex primer pairs with ends that are compatible with the 5′ *BamH*I and 3′*EcoR*I restriction sites available in the pSsh expression vector. When the first nucleotide of the guide strand to be produced was a pyrimidine, a purine was added, as the transcription complex that uses the U6 promoter prefers to initiate on this nucleotide. Two U6 related promoters, U6-8 and U6-9, were identified whose promoter activity was equal to U6-1 in an *in vitro* transcription assay.[22] Each promoter was synthesized in a PCR and individually cloned into a derivative of the U6-1 expression vector resulting in pU6-8 and pU6-9 expression vectors. A plasmid containing all three Pol III promoters, in the order U6-9, U6-1, U6-8, was constructed with two stretches of 400 base pairs of the hypoxanthine-guanine phosphoribosyltransferase gene (*HPRT*) intron 1 to provide spacer sequences between the three promoters. In order to generate stuffer sequences, an 800 base pair fragment, corresponding to a portion of the intron 1 sequence of the HPRT, was isolated using PCR amplification and anchored PCR used to split the amplified fragment into two equivalently sized stuffers. Similar regions from HPRT have been used for stuffer fragments on other recombinant viral vectors.[40] Each promoter has unique enzyme sites for rapid insertion of shRNAs. Three specific shRNA-coding DNA duplexes were inserted behind each promoter. Long-DNA oligonucleotide primers were annealed and cloned sequentially into the vector. Primer sequences for each of the three hairpins consisted of:

shRNA-6 top: 5′-GATCCCGCGAAAGGCCTTGTGGTACTGAAGC TTGAGTACCACAAGGCCTTTCGCTTTTTTCTAGAGAATTCTG-3′

shRNA-6 bottom 5′-CAGAATTCTCTAGAAAAAAGCGAAAGG CCTTGTGGTACTCAAGCTTCAGTACCACAAGGCCTTTCGCGGG ATC-3′

shRNA-19 Top: 5′-CGAGGATCCGTCAACTCCTGGCTAGGCAAT TTGTGTAGTTGCCTAGCCAGGAGTTGACTTTTTTCTAGAGAA-3′

shRNA bottom 5′- TTCTCTAGAAAAAAGTCAACTCCTGGCTA GGCAACTACACAAATTGCCTAGCCAGGAGTTGACGGATCC TCG-3′

shRNA-22 Top 5′- ACACTAGTATTGGAGTGAGTTTAAGC TGAAGCTTGAGCTTAAACTCACTCCAATTTTTTTAGATCTGT 3′

shRNA-22 bottom 5′- ACAGATCTAAAAAAATTGGAGTGAGTTT AAGCTCAAGCTTCAGCTTAAACTCACTCCAATACTAGTGT 3′.

The entire expression cassette was designed to have flanking sequences for insertion into inverted terminal repeat (ITR)-containing vectors for AAV production. For generation of self-complementary vectors used in the construction of this specific product, the wt AAV2 right ITR was coupled with a nonresolvable left ITR from AAV4, which was commercially synthesized within the context of a pUC vector. The intervening sequences between the two ITRs was bounded by *Not*I restriction enzyme sites for easy removal and replacement with TT-033 expression cassette.

Modification of plasmid to create TT-034. Pairs of long oligonucleotides were synthesized that contained the PSE sequences from the U6-7 promoter. The termini of the hybridized PSE-7 sequences to be inserted into the U6-1 promoter were designed to have overhangs complementary with *Nde*I and *BamH*I cleaved sequences. Likewise, *BstE*II and *BamH*I compatible overhangs were designed for PSE-7 hybridized primer pairs that were to be inserted into the U6-8 promoter. Following ligation, the modified plasmids were sequenced to verify that the PSE-7 sequence had been properly inserted. Because there were no convenient internal restriction sites to generate a PSE-7 exchange into the U6-9 promoter, the entire promoter (including TATA box) was generated using oligonucleotide primer stitching via PCR. All clones were sequence verified and it was discovered that sequences downstream from the PSE-7 sequences within the U6-9 promoter did not correspond to the endogenous U6-9 promoter. Instead, a design error had led to these sequences being replaced with the endogenous U6-8 sequences. Encompassing nearly 50 nt, and including the TATA box of the promoter, this hybrid promoter nonetheless remains active with the desired properties of reduced transcription levels and thus the sequence was not changed in future iterations.

Luciferase reporters: The vector pGL3-CTRL (Promega, Madison, WI.) was used for the construction of the firefly luciferase fusion reporter plasmids. This vector has an XbaI site and an FseI site between 2 and 22 nt downstream, respectively of the translational stop codon of the luciferase coding sequence, convenient for ligating duplex primer pairs encoding for HCV target sequences on the same strand that transcribes the luciferase reporter.

Adenovirus production. Replication-deficient adenoviral vectors (E1/E3 deleted) were produced at Vector Biolabs (Philadelphia, PA). Briefly, the expression cassette for either TT-033 or TT-034 was subcloned into a shuttle vector and then homologous recombination was used to generate the recombinant viral vector. Primary stocks were amplified in HEK293 cell monolayers; cells were harvested and lysed and the virus purified using two sequential cesium chloride gradients. The final product was desalted, titered both spectrophotometrically for viral particles and plaque formation assay for PFU/IFU, and tested for sterility.

AAV production. The AAV helper free system (Stratagene, San Diego, CA) was used for construction of AAV-derived vectors and production of the AAV virus. The plasmid pAAV-RC from this system encodes the AAV2 capsid protein and was modified for production of AAV8 pseudotyped vectors. The complete sequence of AAV8 was obtained from the NCBI database (accession number AF513852). The capsid portion of the sequence was synthesized (Blue Heron Technologies, Bothell, WA) and directionally cloned into the pAAV-RC vector. Self-complementary AAV8 vectors were generated by modifying pAAV-MC from the Stratagene system by exchanging the wt AAV2 ITR at the 5′ end of the plasmid with a mutant ITR from AAV4. The wt AAV2 ITR from the 3′ end of the vector plasmid remained unchanged. Recombinant AAV was generated by transient transfection of HEK293 cells, and purified by combined column chromatography and cesium chloride gradient centrifugation as has been previously described,[41] however with the modification that Poros 50HQ resin (Invitrogen, Carlsbad, CA) was used for the chromatography step. The purified vector was essentially free of empty capsids. Vector preps were titered by QPCR and titers were expressed in terms of vector genomes/ml (vg/ml). The QPCR primers used for titering were: Forward: 5′-AGCTCCACCCTCACTGGTTTTT-3′ Reverse: 5′- CAAGCATGATCAGAACGGTTGA -3′ and Probe: 5′-FAM-TTGTTGAACCGGCCAAGCTGCTG-TAMRA-3′ (Applied Biosystems, Foster City, CA). Serial dilutions of a linearized DNA plasmid encoding for the expression cassette of TT-034 was used as a standard for absolute quantification.

Determination of AAV copy numbers (transduction of hepatic tissues). Genomic DNA was isolated using the QIAamp kit (Qiagen, Valencia, CA) and the resultant quantification of nucleic acids was performed using the PicoGreen dsDNA quantitation assay (Invitrogen). AAV vector genome copy number was determined from 0.5 -50 ng of genomic DNA using quantitative real-time PCR with a forward and reverse primer that sit on the boundaries of the HPRT stuffer region and U6-8 promoter region, respectively and a probe that spanned the junction of these two elements. The QPCR primers used for biodistribution studies were: Forward: 5′-TAAGGGCAGGGAATTGATCTAGAA-3′ Reverse: 5′-GGGCACCCTGGGTAAAGG-3′ Probe: 5′-FAM-AAGCTAGTGGT ACCGGTCCTACGCGG-TAMRA-3′ (Applied Biosystems). Because the sequences within the amplicon did not change between the two expression cassettes, the same primer/probe set was used to quantify copies of TT-033 and TT-034. Primers/probes were validated to ensure no cross reactivity to genomic DNA sequences from mouse and non human primate species. Serial dilutions of a linearized DNA plasmid encoding for the expression cassette was used as a standard for absolute quantification.

Determination of shRNA copy numbers. RNA was purified from tissues using a modified procedure with Trizol (Invitrogen). The protocol was followed as outlined by the manufacturer with the exception that the concentration of magnesium is adjusted to 111 mmol/l by the addition of $MgCl_2$ prior to the final precipitation step. Custom QPCR assays were developed for each of the hairpins used in this study. Based upon established design rules[42,43] the miRNA loop assay was altered using the Applied Biosystems Custom Taqman Small RNA assays with specificity tailored to shRNA-22. Forty five nanogram of total RNA from each liver sample was utilized for the reverse transcription reaction which was performed with idenitcal conditions to the miRNA detection assay and a portion of the products used for the subsequent QPCR assay. Mature RNAi species for shRNA-6 and shRNA-19 were measured using a custom variation of the Mircury assay developed by Exiqon (Woburn, MA). A standard curve was generated for each species (*i.e.*, 19 nt, 20 nt, etc.) utilizing a dilution series of purified RNA templates specific for the corresponding assay. A portion of the reverse transcribed products were used in the subsequent QPCR assay.

Cell lines and transfections. HEK293 cells were seeded in 12-well tissue culture plates at 8×10^4 cells/well on the day before transfection. Transfections with shRNA expression plasmids and luciferase reporter plasmids were performed using Turbofect transfection reagent (Fermentas, Glen Burnie, MD) according to the manufacturer's instructions. Each condition was tested with $N = 4$ independent transfections unless otherwise specified. For experiments using firefly luciferase fusion reporter plasmids, 250 ng/well of the reporter plasmid and 150 ng/well of shRNA expression plasmid were added into the mixture along with 25 ng/well of a renilla expression plasmid pRL-SV40 (Promega); the latter used to normalize for differences in transfection efficiency. After a 48-hour incubation period, cells were harvested and assayed for dual luciferase activity (Promega). For replicon experiments, Huh-7.5 cells (licensed from Apath LLC, St Louis, MO) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mmol/l sodium pyruvate, 1× nonessential amino acids, and 1× penicillin/streptomycin. The Huh-7-derived cell line supports replication of subgenomic RNA from the con1 strain of HCV genotype 1b, which is engineered to encode the firefly luciferase reporter gene and neomycin phosphotransferase gene (licensed from RebLikon, Schriesheim, Germany). Replicon 1b cells were maintained in Huh-7.5 medium supplemented with G418 at 500 µg/ml. Luciferase activity was measured using the Britelite assay system (Perkin Elmer, Waltham, MA) for Firefly luciferase and the Renilla luciferase assay system (Promega) for Renilla activity.

In situ *hybridization.* Exiqon's miRCURY LNA microRNA ISH Optimization Kit (FFPE), v1.3 was used for *in situ* analysis of all three hairpins delivered by TT-033. Paraffin embedded Cynomolgus liver sections were cut at 6 µm and mounted on clean, positively charged slides, allowed to air dry for 2 hours and placed at 4 °C overnight, then baked at 60 °C for 45 minutes the night before the experiment. Slides were deparafinized with three 5-minute immersions in Xylene, followed by subsequent ethanol (ETOH) baths: three times with 100% ETOH, two times with 95%, two times with 70% and then rinsed once with PBS. The tissue was digested with Proteinase K (300 µl/slide) at a final concentration of 15 µg/ml in Proteinase K buffer [5 mmol/l Tris–HCl (pH 7.4), 1 mmol/l EDTA, 1 mmol/l NaCl] for 10 minutes at 37 °C in a slide hybridizer. The Proteinase K reaction was stopped with two holds of PBS-T and slides were dehydrated in new alcohol baths: two times with 70% ETOH, two times with 95%, and three times with 100% ETOH. Slides were air-dried on clean paper towel in the hood for ~15 minutes. DIG-labeled LNA probes were denatured at 90 °C for 4 minutes, then snap chilled on an ice bath and added to 1× Hybridization buffer. The following ISH probes, double DIG-labeled and enhanced with LNA, were used: shRNA-22: 5′-DIG-agcttaaactcactccaat-DIG-3′; shRNA-19: 5′-DIG-gtcaactcctggctaggcaa-DIG-3′; shRNA-6: 5′-DIG-gcgaaaggccttgtggtact-DIG-3′. Slides were incubated with 25–50 µl of LNA double-DIG labeled probe at a final concentration of 40–60 nmol/l for 1 hour at 57 °C. The slides were then washed in 5-minute increments with one wash of in 5× SSC, twice in 1× SSC, and twice with 0.2× SSC at the hybridization temperature, and then once with 0.2× SSC at room temperature, and then washed once with PBS-T. Tissue sections were then incubated in blocking buffer, DIG Wash and Block Buffer Set (Roche, Indianapolis, IN) for 15 minutes. Sheep anti-DIG-AP was added at 1:500, diluted in blocking buffer and 0.2% sheep serum, and incubated for 60 minutes. Slides were then washed three times with PBS-T and then incubated with NBT/BCIP substrate (Roche) in AP buffer (100 mmol/l Tris–HCl, 100 mmol/l NaCl, 5 mmol/l $MgCl_2$, 0.05% Tween-20, pH 9.5) containing 0.2 mmol/l Levamisole, for 2 hours in a humidifying chamber at 30 °C. The alkaline phosphatase reaction was stopped using KTBT buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 10 mmol/l KCl), 2 × 5 minutes. Following two 5-minute washes with water and dehydration protocol with two immersions in 70% ETOH, two with 95% and three times with 100%, coverslips were added to the slides with Eukitt mounting medium (Electron Microscopy Sciences, Hatfield, PA).

SUPPLEMENTARY MATERIAL

Figure S1. *In vitro* anti-HCV inhibitory activity of shRNAs against a surrogate model system.

Figure S2. AST, ALP, albumin, and cholesterol activity in Cynomolgus monkeys dosed with TT-033.

Figure S3. AST, ALP, albumin, and cholesterol activity in mice dosed with TT-033.

Figure S4. Histopathology of liver tissues from Cynomolgus monkeys treated with TT-033.

Figure S5. AST, ALP, albumin, and cholesterol activity in Cynomolgus monkeys dosed with TT-034.

Figure S6. AST and ALP in mice dosed with TT-034.

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