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Inhibition of hepatitis B virus replication by various RNAi constructs and their pharmacodynamic properties

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The strategy of RNA interference (RNAi)-based gene silencing has been suggested to have great potential in treating viral diseases. It provides new hope of being able to complement the limited therapeutic options currently available for chronic hepatitis B virus (HBV) infection. To advance such a strategy towards clinical use, the effects of various parameters on the anti-HBV efficiency of RNAi need to be well-defined. In this study, the efficacy and pharmacodynamic properties of different RNAi target sequences and constructs were examined. Several sequences were found to be effective in cell and animal models, achieving inhibition rates of approximately 80–90 %. Methyl-modified small interfering RNA (siRNA) molecules were found to be more stable inside cells than natural siRNA molecules and offered longer-lasting inhibitory effects. Both were effective at rather low doses (an equimolar ratio with HBV preS2–S protein expression vector). Plasmid DNA vectors were less dose-responsive, but their effectiveness *in vivo* lasted longer, for approximately 1 month. By analysing these different parameters and their possible mechanisms, some important issues in RNAi therapeutics that should assist the future development of clinical applications have been addressed.

INTRODUCTION

Hepatitis B is one of the most common liver diseases in the world. It is caused by hepatitis B virus (HBV) infection and may progress to cirrhosis (scarring) and hepatocellular carcinoma (Lok, 2002). A significant proportion of the population in South-East Asia and South Africa are chronic carriers of HBV, which poses a major threat to public health. The current treatment for chronic hepatitis B is to administer interferon, antiviral drugs such as lamivudine or a combination of these (Carreno *et al.*, 1992; Dienstag *et al.*, 2003; Lai *et al.*, 1997). However, these drugs have side effects, and the recurrence of viraemia after cessation of therapy and the development of escape mutants are major concerns (Tipples *et al.*, 1996). Therefore, new strategies for improved treatment of the disease are urgently needed.

It has been suggested that the preferred way of controlling the virus is to interfere with the viral gene replication and expression processes. Studies employing antisense and ribozyme technologies have demonstrated some promise, but the poor *in vivo* stability of these molecules, limited access to infected cells and secondary-structure interference of the DNA or RNA sequences all limited their clinical efficacy (Aoki *et al.*, 2003; Goodarzi *et al.*, 1990; von Weizsacker *et al.*, 1992).

Most recently, RNA interference (RNAi) technology has emerged not only as an extremely powerful tool for functional genomic studies, but also as a potentially useful method for developing specific gene-silencing therapeutics, especially for the treatment of viral diseases (Marathe *et al.*, 2000; Yang *et al.*, 2000). Specifically designed RNAi molecules can target viral mRNAs and initiate their degradation. Several pioneering studies have demonstrated great possibilities for treating serious viral diseases, including those caused by human immunodeficiency virus, HBV and hepatitis C virus (Adelman *et al.*, 2001; Gitlin *et al.*, 2002; Jacque *et al.*, 2002; Lee *et al.*, 2002; Wilson *et al.*, 2003). For HBV, RNAi molecules have been shown to have impressive inhibitory effects against viral gene transcription and expression (Giladi *et al.*, 2003; Hamasaki *et al.*, 2003; McCaffrey *et al.*, 2003; Ying *et al.*, 2003). The inhibitory effects were similar against both replication-competent and -incompetent HBV (Shlomai & Shaul, 2003). Therefore, this strategy could be used for treatment at various stages of disease progression. One recent study further demonstrated viral clearance from the liver of transgenic mice (Uprichard *et al.*, 2005), which provides high hopes for the use of RNAi molecules as HBV therapeutics.

However, to advance such a strategy towards clinical use, there are still many aspects of the molecule's pharmacological properties that need to be well-defined. Several construct

structures that have RNAi activity have been proposed, including double-stranded RNA (dsRNA) synthesized by chemical methods or T7 RNA polymerase, and small hairpin RNA transcripts from plasmid vectors containing the U6/H1 promoter (Brummelkamp *et al.*, 2002). Furthermore, some structural modifications have been made to the dsRNA constructs to improve molecular stability and efficacy (Chiu & Rana, 2003; Czauderna *et al.*, 2003; Morrissey *et al.*, 2005). All of these different structures may affect *in vivo* RNAi efficacy, as well as their pharmacokinetic/pharmacodynamic properties and the delivery requirements. These parameters are essential for the development of therapeutic applications based on RNAi mechanisms.

In this paper, we present a detailed comparison of the *in vitro* and *in vivo* activities and the pharmacodynamic behaviours of several different RNAi molecular constructs. Although they all had specific inhibitory effects *in vitro*, their stability *in vitro* and *in vivo* and their dose responsiveness and effective duration were distinctively different. These parameters all have to be taken into account when designing possible therapeutic regimes for chronic HBV treatment.

METHODS

Plasmid expressing HBV surface antigen. The HBV surface antigen expression plasmid (pHBs) encoding the preS2–S gene in the pcDNA3.1 vector was kindly provided by Dr Xiaoqiang He (Guangzhou Airforce Hospital, Guangzhou, China) (He *et al.*, 2003). The plasmid was amplified in the DH5α strain of *Escherichia coli* and purified by using a Qiagen Plasmid Giga kit.

RNAi constructs. Three RNAi sequences (s1, s2 and s3) starting with AA were designed based on the complete genome of HBV subtype *ayw* (GenBank accession no. U95551) and analysed by BLAST to ensure that they did not have significant nucleotide sequence identity to other genes. The three target sequences are listed in Fig. 1(a). Small interfering RNAs (siRNAs) with two different chemical structures (Ts and cTs) were synthesized by GenePharma. The Ts oligonucleotides with the native RNA structure were synthesized by using 2′-OH tert-butyldimethylsilyl (TBDMS) chemistry. The cTs oligonucleotides were synthesized by replacing rU and rC with 2′-*O*-methyl rU and 2′-*O*-methyl rC, respectively, whilst leaving rA and rG unchanged. All oligonucleotides were purified by HPLC and annealed to form duplexes before use. For construction of the siRNA expression plasmids, pSilencer 2.1-U6 neo vector (Ambion) was linearized with *Bam*HI and *Hin*dIII to facilitate directional cloning and purified to remove the digested insert. For each target gene, the designed complementary 64-mer oligonucleotide with a 5′ single-stranded overhang for ligation into the pSilencer vector was synthesized, annealed and cloned into the linearized vector.

Cell culture and transfection. The human hepatoma cell line HuH-7 and HEpG2.2.15 cells (Sells *et al.*, 1987) with integrated HBV *ayw* genome were obtained from the Shanghai Cancer Institute. Cells were maintained at 37 °C in an atmosphere of 5 % CO_2 in RPMI 1640 (Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS; Gibco-BRL). For transfection studies, HuH-7 cells (4×10^5 per well) were plated on six-well plates (Costar) for 24 h. The medium was removed and cells were washed twice with RPMI 1640 without FBS and treated with the transfection complex. The transfection complex for each well was prepared by adding 275 µl RPMI 1640 containing 5 µg pHBs and 28 pmol siRNA (or 6 µg siRNA expression plasmid) to 275 µl DOTAP liposome solution ($0{\cdot}5$ mmol l^{-1}). The resultant complex was incubated at room temperature for 10 min and then diluted to 2 ml with RPMI 1640.

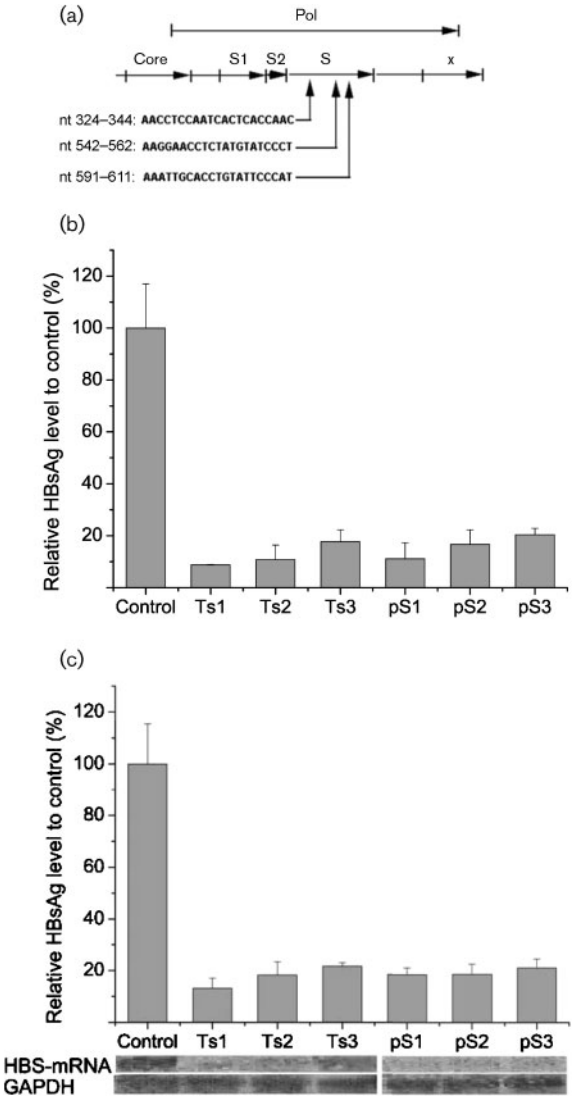


Fig. 1. Inhibition of HBsAg expression by different siRNA constructs *in vitro* and *in vivo*. (a) Schematics of the HBV open reading frame and the locations of the RNAi target sites. Arrows indicate the locations of the target sequences listed (sense strand). (b) Interference effects of siRNA or siRNA expression plasmids on HBsAg expression in HuH-7 cells. The amount of HBsAg in the culture medium was assayed 24 h after transfection. Data presented are means ± SD of three independent transfection experiments and are plotted as a percentage of the control level. (c) Interference effects of siRNA or siRNA expression plasmids against HBVsAg mRNA (HBS-mRNA) and HBsAg expression in mouse liver. Mice were injected hydrodynamically with 5 µg pHBs DNA together with 28 pmol siRNA (or 6 µg siRNA expression plasmid). The amount of HBsAg in the mouse liver homogenates was measured 24 h after co-transfection. Total RNA was extracted from the liver and Northern blot analysis was performed, using 20 µg total RNA in each lane.

This transfection medium (2 ml) was added to each well and incubated for 3 h. The transfection medium was then removed and cells were further incubated under the same conditions in complete medium (2 ml per well) with antibiotics (100 U penicillin ml^{-1} and 50 μg streptomycin ml^{-1}; Sigma). At specific time points, 50 μl culture medium was collected from each well and assayed for the amount of HBV surface antigen (HBsAg). Cells transfected with the same dose of irrelevant siRNA or siRNA expression plasmid were used as controls. For HEpG2.2.15 cell transfection, cells were cultured in 96-well plates and transfected by using Oligofectamine (Gibco-BRL) based on the manufacturer's protocol.

RNAi construct administration *in vivo*. For all *in vivo* experiments, we used 6–8-week-old female BALB/c mice. All animal experimental procedures were approved by the Shanghai Department of Experimental Animals Management. To test the inhibitory effect of RNAi constructs in liver, the RNAi constructs and/or pHBs were delivered to mice by hydrodynamic injection (Liu *et al.*, 1999; Zhang *et al.*, 1999).

To test the inhibitory effect of RNAi constructs in muscle, the RNAi constructs and pHBs in 40 μl 0·9 % saline were injected into mouse gastrocnemius muscle. Immediately after injection, two stainless-steel electrodes were inserted around the injection site and six electric pulses with 120 V cm^{-1} field strength and 60 ms pulse duration were applied.

HBV viral DNA quantification. HBV viral DNA copy numbers were determined by using an HBV fluorescent quantitative PCR kit (Daan Gene Co.). HBV DNA in 40 μl culture medium and cell lysates were extracted and PCRs were prepared according to the kit's protocol. PCRs were carried out in an ABI Prism 7000 using the following program: pre-denaturation at 93 °C for 2 min; 10 cycles of 93 °C for 45 s and 55 °C for 60 s; and 30 cycles of 93 °C for 30 s and 55 °C for 45 s. Negative-control and serially diluted standard samples were processed synchronously (for the standard curve, $r=0{\cdot}9991$). Results were expressed as copies (ml culture medium)$^{-1}$ (cell lysates).

RNA extraction and Northern blot analysis. Total RNA was extracted from pooled frozen liver samples by using a guanidinium thiocyanate/phenol-based protocol (Sangon). For Northern blot analysis, 20 μg total RNA was denatured, electrophoresed in 1·0 % agarose gel containing 37 % formaldehyde and transferred onto a nylon membrane filter (Hybond-N+; Amersham Biosciences). The probe was an 880 bp *Eco*RI fragment of HBsAg cDNA in the pHBs plasmid, labelled with horseradish peroxidase (HRP) by using a North2South Direct HRP Labelling and Detection kit (Pierce). Labelling was carried out strictly according to the kit's protocol, followed by stringent washing and autoradiography.

HBsAg and HBV surface antibody (anti-HBs) assays. Mouse blood samples were collected and sera were separated by low-velocity centrifugation. Levels of anti-HBs and HBsAg in the sera were determined by using the corresponding quantitative ELISA kits (Sino-American Biotechnology Co.). At the same time, for the detection of HBsAg concentration in tissues, mice were sacrificed at designated time points. The gastrocnemius muscle or liver was dissected and homogenized. The level of HBsAg in the tissue lysates was determined by using the quantitative ELISA kit (Sino-American Biotechnology Co.).

Statistical analysis. The results are presented as mean values ± SD and statistical analyses were carried out by using Student's *t*-test.

RESULTS

Design and screening of RNAi sequences against the HBsAg open reading frame

Three different 21 nt sequences in the HBsAg open reading frame (nt 324–344 for S1, nt 542–562 for S2, nt 591–611 for S3) along the HBV genome were selected as RNAi targets as shown in Fig. 1(a). siRNA oligonucleotides were synthesized and designated Ts1, Ts2 and Ts3. siRNA expression plasmids targeted towards these sites were constructed and named pS1, pS2 and pS3.

The three different sequences were screened for their inhibitory effect in the human hepatoma cell line HuH-7 co-transfected with pHBs (Fig. 1b). Cells treated with pHBs alone or with irrelevant siRNA or siRNA expression plasmid had substantial amounts of HBsAg (approximately 1·6 μg ml^{-1}) detectable in the culture medium. However, co-transfection of all three RNAi sequences in either short-stranded RNA duplex form (Ts) or in plasmid vectors (pS) resulted in a significant reduction in HBsAg expression (approx. 70–90 %) compared with the respective irrelevant RNAi controls.

For *in vivo* tests, pHBs and the various RNAi constructs were co-transfected into mouse livers by hydrodynamic injection. The results showed that HBsAg expression was greatly reduced in Ts and pS co-transfected animals (Fig. 1c). HBsAg mRNA levels were also checked by Northern blot analysis and the amount of intact mRNA was reduced significantly in Ts and pS co-transfected liver tissues, indicating that the inhibitory effect was indeed mediated through the degradation of HBsAg mRNA (Fig. 1c).

The actual inhibitory effects towards the three different target sequences were similar, so we selected the sequence S2 for subsequent studies to examine other parameters of the inhibitory effect.

Inhibitory effect of siRNA constructs in HEpG2.2.15 cell models

HEpG2.2.15 cells containing integrated HBV DNA were used as a cell model of HBV-infected hepatocytes. Cells were transfected, using Oligofectamine, with 80 nmol l^{-1} of the RNA duplex Ts2 and methyl-modified RNA duplex cTs2 and compared with irrelevant siRNA. At 36 h post-transfection, HBV DNA copy numbers and the amount of HBsAg in the culture medium were assayed. The results (Table 1) showed that Ts2 can interfere significantly with the transcription of HBV and reduce viral DNA copy numbers both in culture medium and in cell lysates (approx. 50–70 % reduction). Similar effects were found in the HBsAg level in culture medium. The efficiency of inhibition of the chemically modified siRNA (cTs2) was lower than that of the unmodified version (Fig. 2a), indicating that the chemical modification in the backbone of siRNA may affect its activity to a certain extent. However, the inhibitory effect of

Table 1. Inhibition of HBV DNA replication and expression in HEpG2.2.15 cells by siRNA

Sample	HBV DNA level ($\times 10^5$ copies ml^{-1})*		HBsAg in culture medium (%)*
	Culture medium	Cell lysates	
Control	4·40 ± 0·36	15·56 ± 1·38	100·00 ± 11·21
Ts2	2·03 ± 0·17†	3·86 ± 0·27†	36·01 ± 4·69†
cTs2	2·29 ± 0·31†	5·63 ± 0·73†	48·30 ± 6·81†

*Data represent means ± SD of three independent experiments performed in duplicate.
†Statistical differences between the experimental groups (Ts2 and cTs2) and the control were tested by using Student's *t*-test ($P < 0·05$).

cTs2 lasted longer than that of Ts2. The effect of Ts2 declined quickly and had disappeared completely by day 4, whilst the effect of cTs2 was maintained at approximately 50 % of the original level after 4 days (Fig. 2b). The longer effective duration suggested that cTs2 is probably more stable inside cells.

When the siRNA-expressing plasmid construct pS2 was used to transfect HEpG2.2.15 cells, the resultant inhibition was limited and highly variable (data not shown). This may be due to the low and variable transfection efficiencies of plasmids in HEpG2.2.15 cells.

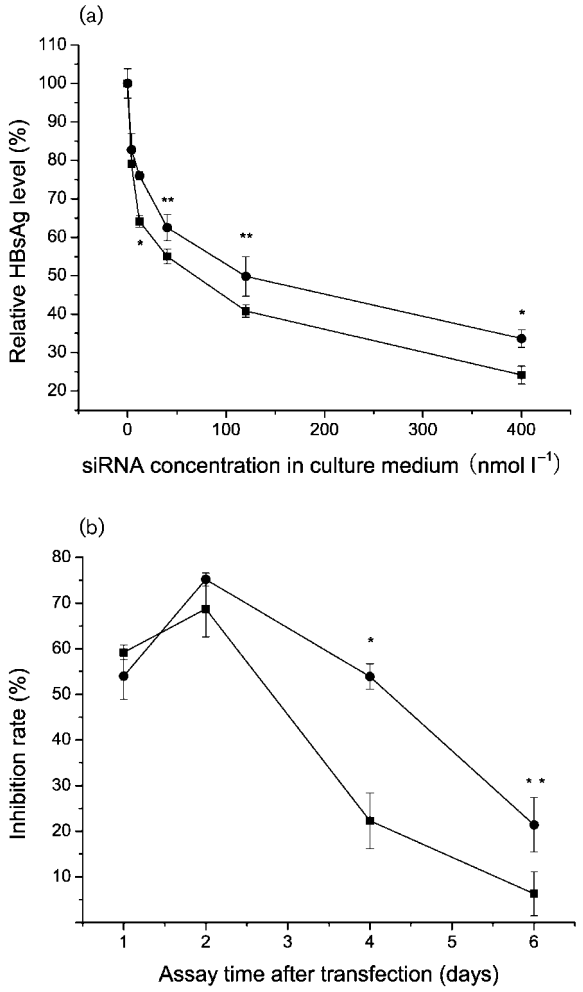


Fig. 2. Inhibition of HBV viral expression in HEpG2.2.15 cells by siRNA. (a) Dose-dependent inhibition of HBsAg expression by siRNA (Ts2, ■) and chemically modified siRNA (cTs2, ●). Cells were transfected with Ts2 or cTs2 at various concentrations and the amount of HBsAg secreted into the medium was assayed 36 h after transfection. Data represent means ± SD of three independent experiments performed in duplicate. (b) Kinetics of the inhibitory effects of Ts2 (■) and cTs2 (●). HEpG2.2.15 cells were transfected with 80 nmol Ts2 or cTs2 l^{-1} and the level of HBsAg secreted into the medium was measured 1, 2, 4 and 6 days after transfection. Data represent means ± SD of three independent experiments performed in duplicate. Statistical difference between the datasets for Ts2 and cTs2 was tested by using Student's *t*-test (*$P < 0·01$; **$P < 0·05$).

Dose–response profiles of various RNAi constructs *in vivo*

It is important to determine the dose–response properties of the various RNAi constructs before developing any *in vivo* application strategies. We explored the extent of inhibition after co-injection of pHBs with various amounts of Ts2, cTs2 and pS2 into mouse livers by using the hydrodynamic method. pHBs (5 μg) was co-injected with Ts2, cTs2 or pS2 at designated molar ratios. Irrelevant siRNA or siRNA expression plasmid was used as a control. HBsAg levels in the liver were examined 1 day after co-injection. The interference effects were clearly dose-dependent for all constructs: the higher the dose administered, the stronger the inhibitory effect exhibited (Fig. 3). Fifty per cent inhibition could be achieved by using the plasmid vector pS2 at 0·6–0·3 : 1 molar ratios and using Ts2 and cTs2 at a 1 : 1 molar ratio. The level of inhibition gradually reached a plateau at a 3 : 1 molar ratio for plasmid DNA vector and at a 10–100 : 1 molar ratio for siRNA.

Duration of the siRNA constructs' inhibitory effect *in vivo*

To examine the duration of the RNAi effect of various siRNA constructs delivered *in vivo*, we first administered

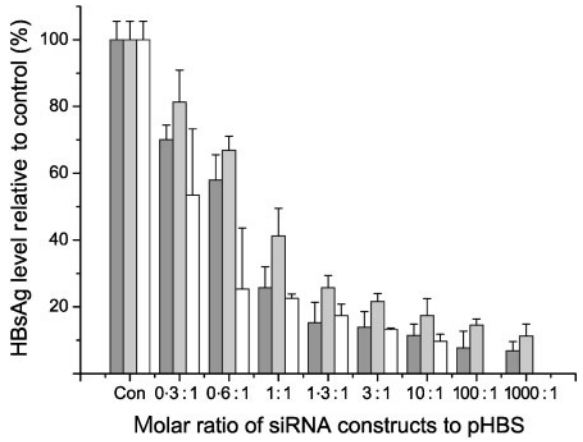
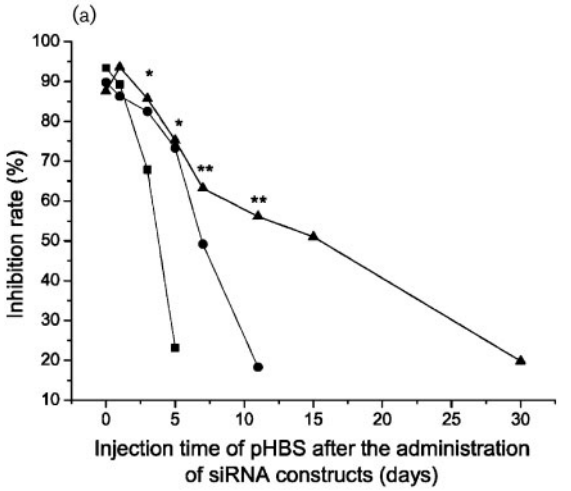
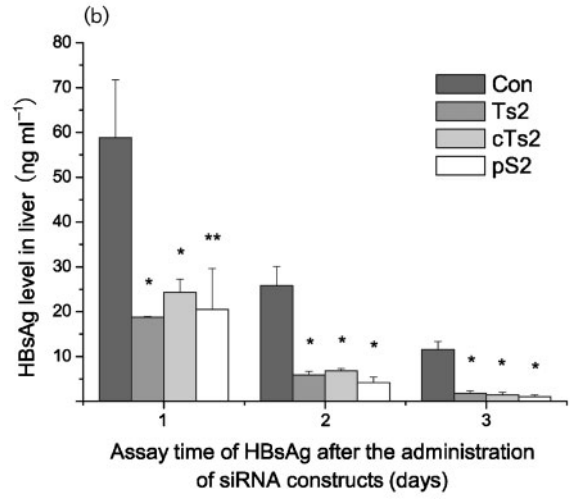


Fig. 3. Dose–response profiles of the inhibitory effect of various siRNA constructs *in vivo*. Mice were injected hydrodynamically with 5 μg pHBs together with Ts2 (dark-shaded bars), cTs2 (light-shaded bars) or pS2 (empty bars) at the indicated molar ratios. Irrelevant siRNA or siRNA expression plasmids were used as controls. The level of HBsAg was assayed 24 h after injection. Data represent means ± SD of three independent experiments performed in duplicate.

Ts2, cTs2 and pS2 by hydrodynamic injection and then injected pHBs at different time intervals. The level of HBsAg expression in liver was assayed 1 day after pHBs injection. As shown in Fig. 4(a), pre-administered Ts2 maintained its inhibitory activity for less than 5 days. The inhibitory effect reached a peak 1 day after injection, then decreased continuously until it disappeared almost completely after 5 days. cTs2, however, could maintain 70–80 % of its inhibitory activity for more than 5 days. Most interestingly, the effect of the siRNA expression plasmid was so long-lasting that, even 15 days after its administration, it could





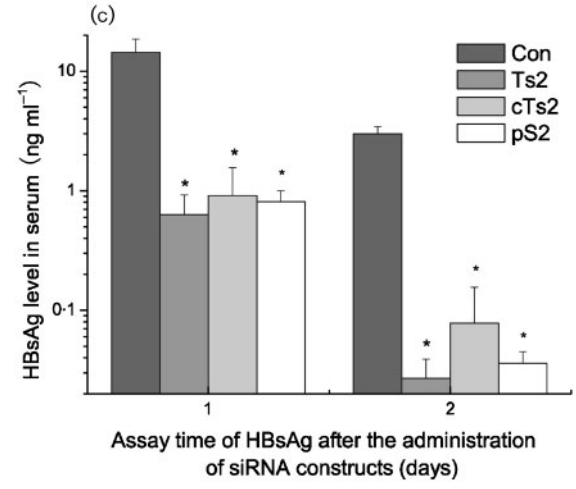


Fig. 4. Duration and efficacy of various siRNA constructs *in vivo*. (a) Inhibitory effect of pre-existing siRNA constructs against newly expressed HBsAg in mouse liver. Ts2 (0·28 nmol ■), cTs2 (0·28 nmol, ●) or pS2 (6 μg, ▲) was transfected into mouse livers at designated times prior to injection of 5 μg pHBs. The amount of HBsAg in the liver was measured 1 day after pHBs injection. *Statistical difference between the datasets of Ts2 and cTs2, $P<0{\cdot}05$; **statistical difference between the datasets of cTs2 and pS2, $P<0{\cdot}05$. (b) Inhibitory effect of siRNA constructs on pre-existing HBsAg expression in liver tissues. Mice were injected with 5 μg pHBs at day 0 by hydrodynamic transfection to establish animal models of pre-existing HBsAg expression. They were then injected with 0·28 nmol Ts2 or cTs2 or with 6 μg pS2 at day 1 and the level of HBsAg in the liver was measured over the following 3 days. Data are means ± SD of three independent injection experiments. Statistical differences between the experimental groups (Ts2, cTs2 and pS2) and the control (Con) were tested by using Student's *t*-test (*$P<0{\cdot}01$; **$P<0{\cdot}05$). (c) Inhibitory effect of siRNA constructs on pre-existing HBsAg expression in serum. Mice were treated as described above and the level of HBsAg in serum was measured over the following 2 days. Data are means ± SD of three independent injection experiments. Statistical differences between the experimental groups (Ts2, cTs2 and pS2) and the control were tested by using Student's *t*-test (*$P<0{\cdot}01$).

still interfere with freshly injected pHBs transcription and maintain 50 % inhibition of protein expression.

In order to examine the inhibitory effects of Ts2, cTs2 and pS2 against pre-existing HBsAg expression, we transfected mice hydrodynamically with 5 μg pHBs on day 0 and administered Ts2, cTs2 and pS2 after 24 h. The amount of HBsAg present in the liver and in serum was examined at different time points after RNAi construct administration. As shown in Fig. 4(b), the amount of protein detected decreased to less than 30–40 % of the original level and the inhibitory effects lasted for more than 3 days. Circulating HBsAg levels in the blood were also examined and similar inhibitory effects were observed (Fig. 4c). Interestingly, the reduction of HBsAg in serum was more significant than in liver samples.

Inhibition of HBsAg expression and anti-HBs induction in muscles *in vivo*

We also tested the inhibitory effects of siRNA constructs delivered intramuscularly by using electroporation. HBsAg expression in the muscles can reach up to 2·4 μg 1 day after injection of 10 μg pHBs by electroporation and can persist for several weeks. However, when the siRNA expression plasmids were delivered together, also by electroporation, expression levels of HBsAg were reduced significantly, to approximately 20 % of the original level. Again, the delivery of an irrelevant siRNA expression plasmid had no effect on the HBsAg expression level in the muscle (Fig. 5a).

The reduction of HBsAg expression in the muscles by siRNA expression plasmid also affected the induction of antibodies against the antigen. Fig. 5(b) shows the HBsAg-specific antibody titres detected in mouse serum 2 weeks after the delivery of pHBs (10 μg) with and without co-delivery of pS2 (20 μg). As expected, high serum-antibody titres resulted from the intramuscular electroporation of pHBs only. However, with co-delivery of pS2, the antibody response was almost undetectable.

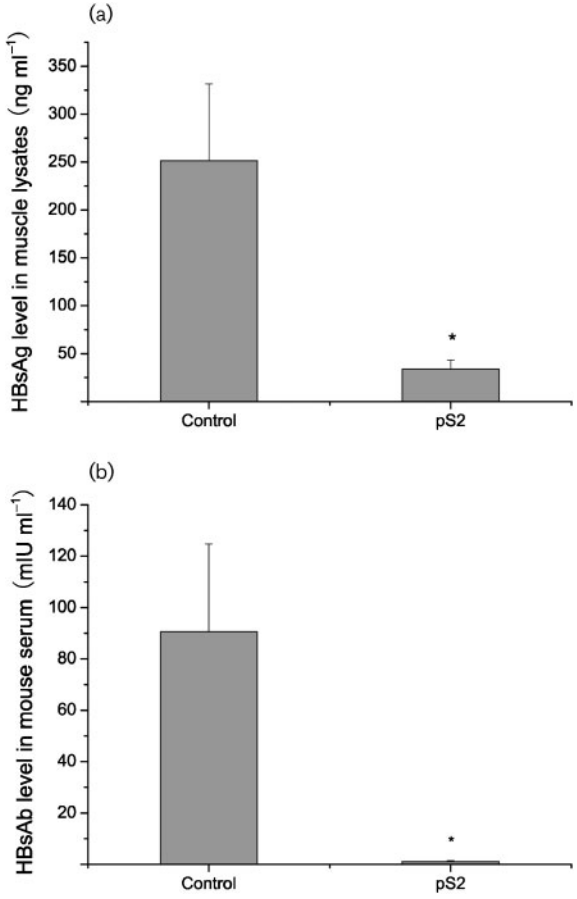


Fig. 5. Interference effects of siRNA expression plasmids on the production of HBsAg and induction of antibodies in mice. (a) pHBs (10 μg), together with 20 μg pS2 or an irrelevant siRNA expression plasmid, was injected into mouse muscle and transfected into cells by electroporation. The amount of HBsAg in the muscle was measured 24 h after injection. (b) pHBs (10 μg), together with 20 μg pS2 or an irrelevant siRNA expression plasmid, was transfected into mouse muscle by electroporation and the amount of anti-HBs in the serum was measured 2 weeks after injection. Data are means ± SD of two independent injection experiments with seven mice. Statistical differences between the experimental group and the control were tested by using Student's *t*-test (*$P < 0{\cdot}01$).

DISCUSSION

RNAi is a process of sequence-specific, post-transcriptional gene silencing found naturally in plants, yeasts and animal cells. Therapeutic applications that employ this gene-silencing mechanism are being suggested. Especially, the strategy is considered enormously useful for antiviral therapy with many advantages. In addition, it can work in the absence of active virus replication. RNAi activity is sequence-specific, hence minimizing many undesirable side effects such as those observed with conventional drug therapies. Another important advantage of the RNAi approach is that there are numerous potential targets for RNAi along the viral genome and RNAi cocktails targeted towards different genes may be even more efficacious. We aimed at the HBsAg-encoding region and screened three different sequences. All three were effective and could inhibit surface-protein synthesis to different extents in cell models. The reasons for the differences in RNAi efficacy are not well understood, but probably involve primary-sequence and secondary-structure effects of both the siRNA and targeted viral RNAs. In addition, the binding of viral and/or cellular proteins to the targeted RNA sequence may also prevent recognition by the RNA-induced silencing complex (Haasnoot *et al.*, 2003). Thus, it is still common practice to search empirically for ideal RNAi targets along the viral genome. Furthermore, in our studies, increased inhibitory efficacy was observed when the three RNAi sequences were used in combination at a relatively high dose (data not shown). We expect that rational design of siRNA cocktails will be desirable to ensure greater efficacy and to combat viral escape through mutation.

In order to find an optimal vector for the RNAi sequence, we tested three different molecular structures carrying the same RNAi sequence. The plasmid vector encoded a hairpin RNA sequence that could fold into a duplex RNAi structure after transcription. The siRNA duplexes were made synthetically by using traditional 2′-TBDMS chemistry and the modified siRNA oligonucleotides were synthesized by changing the native rU and rC to 2′-*O*-methyl rU and 2′-*O*-methyl rC, respectively, whilst the native rA and rG remained the same. In both *in vitro* and *in vivo* experimental models, we showed that the pharmacodynamics of these different structures were considerably different. The native siRNA duplex had the greatest inhibitory effect, but culminated quickly, whilst the methyl-modified siRNA duplexes were more stable inside cells and exerted their inhibitory effect over a longer period. This is consistent with the report of Soutschek *et al.* (2004) on chemically modified siRNAs. In their study, partial phosphorothioate backbone and 2′-*O*-methyl sugar modifications were reported to stabilize siRNAs *in vitro* and *in vivo*. Most interestingly, we found that plasmid vectors were very stable once delivered inside liver cells and maintained a longer inhibitory effect. Surprisingly, even 30 days after a single hydrodynamic injection, the RNAi-encoding plasmids were still available and able to inhibit HBsAg expression from freshly administered pHBs to a considerable degree.

These different constructs also behaved differently with respect to their delivery *in vivo*. Plasmid DNA, with a high molecular mass and highly charged characteristics, was hard to deliver into cells *in vitro*, as well as *in vivo*. The siRNA constructs, on the other hand, were much smaller and entered the target cells more easily. Their structure could be further modified to shield the surface charge and make them more hydrophobic for easier transport across the cell membrane. Most impressively, Soutschek *et al.* (2004) conjugated cholesterol to the 3′ end of the sense strand and reported improved delivery *in vivo*. However, the 2′-*O*-methyl modification of siRNAs tested in our experiment did not significantly improve the delivery by normal intravenous injection *in vivo* (data not shown). As a matter of fact, almost all earlier proof-of-concept studies in animals have had to use the hydrodynamic injection method, but the feasibility of using such an approach clinically is still not clear. Delivery is still the biggest and most pressing issue in the development of therapeutic applications of RNAi. In our study, in addition to applying a hydrodynamic method to test the RNAi efficacy in mouse liver, we used another highly efficient intramuscular gene-delivery method, electroporation (Aihara & Miyazaki, 1998; Rols *et al.*, 1998), to verify the inhibitory effect of our RNAi constructs on HBsAg expression. Significant inhibition was apparent at all levels, including mRNA transcription, HBsAg expression and anti-HBs production. Unfortunately, electroporation is also very difficult to apply to hepatic cells for gene delivery. However, these data again suggest that RNAi therapeutics have great potential once an efficient delivery method is available.

Using the hydrodynamic delivery method, we also looked into the dose–response and effective-duration characteristics of the different vectors. An equimolar ratio of siRNA to pHBs resulted in substantial inhibition of HBsAg expression. Increasing the dose further did not lead to significant improvement. However, as these siRNA constructs could only maintain their efficacy *in vivo* for less than 3–5 days, for therapeutic effects, they should probably be used frequently and repetitively. In contrast, for plasmid vectors, higher doses clearly resulted in better efficacy and, because these are stable inside liver cells and have a long duration of action, they could probably be used less frequently and at a higher dose.

In a mouse model with pre-existing HBsAg expression, we also demonstrated that RNAi administration could effectively clear some HBsAg from liver and serum. Interestingly, the reduction in HBsAg levels in serum was more significant than in liver samples. We suspect that this is because expressed HBsAg can accumulate in liver tissues, whilst secreted proteins are cleared quickly, and any reduction in supply would affect the serum concentration. We were also interested at looking into the possibility and effect of repeated RNAi administration in these models. However, because HBsAg expression resulting from pHBs hydrodynamic transfection was transient and usually disappeared within 5 days, it was difficult to observe the added effect of repeated RNAi administration *in vivo*.

To summarize, we have carried out detailed studies looking into several pharmacodynamic features of RNAi constructs for the treatment of HBV. We showed that RNAi sequences can be designed and screened to achieve effective inhibition in cell and animal models. Different constructs carrying the same RNAi sequences had different properties in terms of their activities *in vitro* and *in vivo*. Sequence, stability, method of delivery and several other issues are all important. These factors need to be taken into consideration when examining the clinical potential of these RNAi-based therapeutics.

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