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doi:10.1016/j.ymthe.2005.11.002

Design of Noninflammatory Synthetic siRNA Mediating Potent Gene Silencing *in Vivo*

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Available online 15 December 2005

Targeted silencing of disease-associated genes by synthetic short interfering RNA (siRNA) holds considerable promise as a novel therapeutic strategy. However, unmodified siRNA can be potent triggers of the innate immune response, particularly when associated with delivery vehicles that facilitate intracellular uptake. This represents a significant barrier to the therapeutic development of siRNA due to toxicity and off-target gene effects associated with this inflammatory response. Here we show that immune stimulation by synthetic siRNA can be completely abrogated by selective incorporation of 2′-*O*-methyl (2′OMe) uridine or guanosine nucleosides into one strand of the siRNA duplex. These noninflammatory siRNA, containing less than 20% modified nucleotides, can be readily generated without disrupting their gene-silencing activity. We show that, coupled with an effective systemic delivery vehicle, 2′OMe-modified siRNA targeting apolipoprotein B (apoB) can mediate potent silencing of its target mRNA, causing significant decreases in serum apoB and cholesterol. This is achieved at therapeutically viable siRNA doses without cytokine induction, toxicity, or off-target effects associated with the use of unmodified siRNA. This approach to siRNA design and delivery should prove widely applicable and represents an important step in advancing synthetic siRNA into a broad range of therapeutic areas.

Key Words: RNA interference, nucleic acid therapeutics, synthetic siRNA, chemical modification, inflammatory response, apolipoprotein B, interferons, liposomes

INTRODUCTION

Short interfering RNA (siRNA)[1] are double-stranded RNA duplexes that silence target gene expression through the process of RNA interference (RNAi) [1]. Gene silencing by this evolutionarily conserved mechanism results from the sequence-specific degradation of mRNA when bound within the RNA-induced silencing complex (RISC) by its complementary siRNA strand [2,3]. Naturally occurring siRNA are derived from the cleavage of long double-stranded RNA (dsRNA) by the endonuclease Dicer with subsequent unwinding and loading of single strands into the RISC. RNAi can also be mediated by the transfection of chemically synthesized siRNA [4] and there is currently great interest in developing synthetic siRNA that target disease-associated genes as therapeutic agents [1]. In this respect, several studies have now reported *in vivo* silencing of endogenous [5] and viral genes [6–9] using synthetic siRNA administered via a clinically viable route.

As part of the innate defense mechanism against invading pathogens, the mammalian immune system is activated by a number of exogenous RNA [10–12] and DNA species [13], resulting in the release of inflammatory cytokines and interferons (IFN) such as IFN-α. Initial studies on the off-target effects of siRNA focused on the induction of IFN responses from cell lines, most likely through activation of the dsRNA-dependent protein kinase (PKR) [14–16]. Our group [17] and others [18,19] have recently demonstrated that synthetic siRNA can also be potent activators of both the murine and the human innate immune system, particularly when associated with lipidic or polycation-based vehicles that facilitate intracellular delivery via endosomes. Although still poorly defined, immune recognition of siRNA is sequence dependent [17–19] and likely involves signaling through the endosomal Toll-like receptor-7 (TLR7) pathway [18]. The consequences

[1]*Abbreviations used:* apoB, apolipoprotein B, TLR, Toll-like receptors, sRNAsingle-stranded RNA, dsRNA, double-stranded RNA, siRNA, short interfering RNA, IFN-α, interferon-α, IL-6, interleukin-6, TNF-α, tumor necrosis factor-α, RNAi, RNA interference, β-gal, β-galactosidase, PBMC, peripheral blood mononuclear cells.

1525-0016/$30.00

of activating the innate immune response can be severe, particularly in more sensitive species, including humans [20,21], due to local and systemic inflammatory reactions that can be triggered by TLR ligands at very low doses. Toxicities associated with the administration of siRNA *in vivo* have been attributed to such a response [9,17].

Stabilization of synthetic siRNA against rapid nuclease degradation is often regarded as a prerequisite for *in vivo* and therapeutic applications. This is achieved using stabilization chemistries that were previously developed for ribozymes and antisense oligonucleotide drugs [22]. These include chemical modifications to the 2′-OH group in the ribose sugar backbone, such as 2′-*O*-methyl (2′OMe) and 2′-fluoro (2′F) substitutions, that are readily introduced as modified nucleotides during siRNA synthesis. A number of reports have shown that siRNA containing 2′OMe [23–25], 2′F [24–27], 2′-deoxy [26], or "locked nucleic acid" (LNA) [18,28] modifications can retain functional RNAi activity, indicating that these chemistries can be compatible with the RNAi machinery. However, these modifications to siRNA appear to be tolerated only in certain ill-defined positional or sequence-related contexts as, in many cases, their introduction has a negative impact on RNAi activity [18,23,25,26,28]. Therefore, the design of chemically modified siRNA has required a stochastic screening approach to identify duplexes that retain potent gene-silencing activity.

Poor uptake of exogenous nucleic acids by cells represents an additional barrier to the development of siRNA-based drugs. To develop a delivery vehicle that enhances intracellular uptake of nucleic acids and is suitable for systemic administration, we have encapsulated siRNA within liposomes termed stable nucleic acid lipid particles (SNALP). These systems are effective at mediating RNAi *in vitro* [9,17] and have been shown to inhibit viral replication at therapeutically viable siRNA doses in a murine model of hepatitis B [9]. These studies led to the discovery that chemical modification of the siRNA (greater than 90% modified nucleotides), originally designed to maximize serum stability and nuclease resistance, also abrogated the immunostimulatory properties of the unmodified duplex.

In this report, we demonstrate that only minimal 2′OMe modifications within one strand of the duplex are sufficient to abrogate fully the immunostimulatory activity of siRNA, irrespective of its sequence. By restricting the degree of chemical modification, noninflammatory siRNA that retain full RNAi activity can be readily generated. Using apolipoprotein B (apoB) as an endogenous gene target, we show that potent gene silencing can be achieved *in vivo* using these modified siRNA, without evidence of cytokine induction, immunotoxicity, or off-target effects associated with immune activation triggered by the unmodified siRNA.

RESULTS

2′-*O*-Methyl Modifications within ssRNA Abrogate Immune Stimulation

We have recently shown that synthetic siRNA can activate innate immune responses in a sequence-dependent manner [17] and that this deleterious side effect is abrogated in stabilized siRNA with extensive chemical modifications (>90% of nucleotides) throughout the ribose backbone [9]. To examine the extent and type of chemical modification required to inhibit immune cell activation by RNA, we selectively introduced 2′OMe nucleotides into the GU-rich immunostimulatory motif of a single-stranded RNA oligonucleotide (ssRNA) derived from a β-galactosidase control (β-gal) siRNA [17]. Oligonucleotide sequences used in these studies are provided in Table 1. 2′OMe modification of the 5 nucleotides comprising the immunostimulatory 5′-UGUGU-3′ motif (2′OMe GU) in the β-gal sense ssRNA completely abrogated IFN-α induction when we treated human peripheral blood mononuclear cell (PBMC) cultures with lipid encapsulated ssRNA (Fig. 1A). Inhibition of the interferon response was also achieved by selectively modifying either the two guanosine (2′OMe 2 × G) or the three uridine nucleotides (2′OMe 3 × U) within the motif. The

TABLE 1: RNA oligonucleotides

Name	Strand	Sequence 5′-3′
β-Gal	Native (S)	UUGAUGUGUUUAGUCGCUAUU
	2′OMe GU(S)	UUGAUGUGUUUAGUCGCUAUU
	2′OMe 3 × U(S)	UUGAUGUGUUUAGUCGCUAUU
	2′OMe 2 × G(S)	UUGAUGUGUUUAGUCGCUAUU
	2′OMe 2 × G 3′(S)	UUGAUGUGUUUAGUCGCUAUU
	Native (AS)	*UAGCGACUAAACACAUCAAUU
	2′OMe AC(AS)	*UAGCGACUAAACACAUCAAUU
apoB-1	Native (S)	GUCAUCACACUGAAUACCAAU
	2′OMe U(S)	GUCAUCACACUGAAUACCAAU
	2′OMe G(S)	GUCAUCACACUGAAUACCAAU
	2′OMe C(S)	GUCAUCACACUGAAUACCAAU
	2′OMe A(S)	GUCAUCACACUGAAUACCAAU
	Native (AS)	*AUUGGUAUUCAGUGUGAUGACAC
	2′OMe GU(AS)	*AUUGGUAUUCAGUGUGAUGACAC
	2′OMe U(AS)	*AUUGGUAUUCAGUGUGAUGACAC
	2′OMe G(AS)	*AUUGGUAUUCAGUGUGAUGACAC
apoB	Native (S)	GUGAUCAGACUCAAUACGAAU
mismatch	2′OMe U(S)	GUGAUCAGACUCAAUACGAAU
	Native (AS)	*AUUCGUAUUGAGUCUGAUCACAC
	2′OMe GU(AS)	*AUUCGUAUUGAGUCUGAUCACAC
vFLIP	Native (S)	GUGGUAUUGUUCCUCCUAAdTdT
	2′OMe GU(S)	GUGGUAUUGUUCCUCCUAAdTdT
	2′OMe U(S)	GUGGUAUUGUUCCUCCUAAdTdT
	Native (AS)	*UUAGGAGGAACAAUACCACdTdT
	2′OMe U(AS)	*UUAGGAGGAACAAUACCACdTdT
	2′OMe C(AS)	*UUAGGAGGAACAAUACCACdTdT

Unmodified (native) and 2′-*O*-methyl modified (2′OMe) RNA oligonucleotides corresponding to the sense (S) and antisense (AS) strands of β-gal control, apoB-1, apoB mismatch, and vFLIP siRNA are shown. 2′OMe-modified nucleotides are underlined, asterisks represent 5′ phosphates.

doi:10.1016/j.ymthe.2005.11.002

inhibitory effect of 2′-*O*-methylation, however, did not appear to require the direct modification of the nucleotides within the immunostimulatory GU-rich motif since selective modification of the two guanosine residues 3′ of the UGUGU motif, toward the end of the β-gal ssRNA (2′OMe 2 × G 3′), also resulted in complete abrogation of the interferon response in PBMC cultures (Fig. 1A). As described previously, the unmodified complementary antisense (AS) ssRNA sequence was inherently nonimmunostimulatory in these assays [17]. We achieved similar results using the transfection reagent polyethylenimine (PEI) to deliver the RNA to PBMC (Supplementary Fig. S1). We applied a similar approach to the modification of the constituent 21- and 23-base strands of an siRNA duplex targeting human and mouse apoB (apoB-1 siRNA) [5]. As predicted by its GU-rich nucleotide sequence [11,17], unmodified apoB-1(AS) ssRNA stimulated a strong IFN-α response in PBMC cultures, even at low concentrations (Fig. 1B). This response was fully inhibited by 2′OMe modification of either the 5 nucleotides comprising the 5′-GUGUG-3′ motif (2′OMe GU) or the 6 guanosine (2′OMe G) or 7 uridine (2′OMe U) residues in apoB-1(AS) ssRNA (Fig. 1B). The unmodified, complementary apoB-1 sense oligonucleotide (apoB-1(S)) encapsulated in lipid particles did not induce IFN-α in PBMC (Fig. 1B), although high doses of this oligonucleotide delivered as PEI polyplexes were found to activate a cytokine response (not shown). This weak response to PEI-complexed apoB-1(S) ssRNA was also inhibited by 2′OMe-uridine modification. These findings demonstrate that the selective incorporation of 2′OMe-modified nucleotides within ssRNA is sufficient to prevent stimulation of the interferon response from innate immune cells.

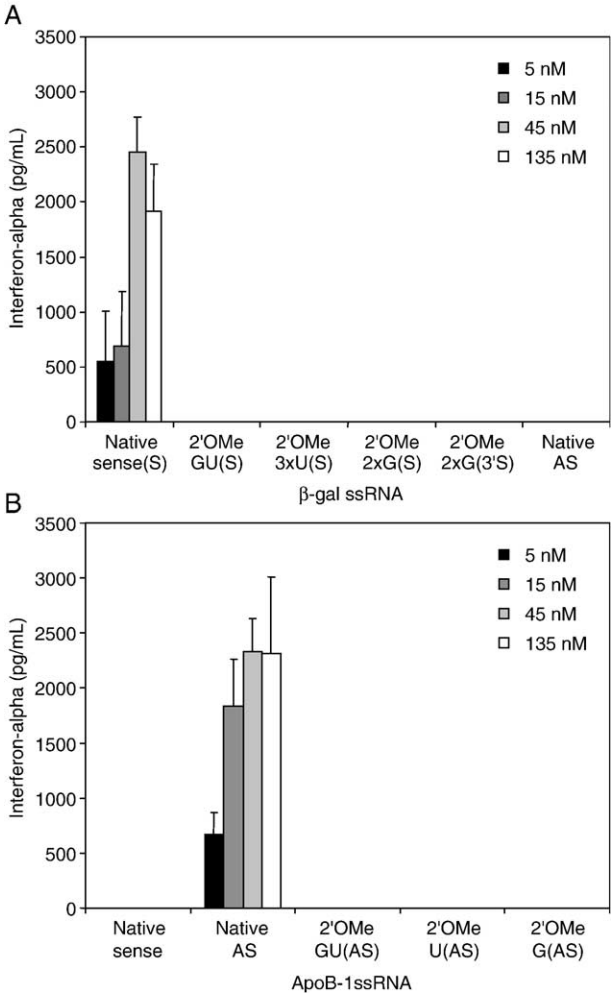

FIG. 1. 2′OMe modification abrogates ssRNA-mediated interferon induction in human PBMC. Liposome-encapsulated, unmodified (native) or 2′OMe U-, G-, or GU-modified ssRNA representing the sense (S) and antisense (AS) strands of (A) β-gal and (B) apoB-1 siRNA were cultured with PBMC at increasing concentrations (5–135 nM). Sequences are detailed in Table 1. IFN-α was assayed in culture supernatants at 24 h. Values are means + SD of triplicate cultures.

Selective Nucleotide Modifications within siRNA Abrogate Immune Stimulation

To examine whether selective 2′OMe modifications within siRNA duplexes also inhibited immune stimulation, we generated a series of β-gal and apoB-1 siRNA comprising 2′OMe-modified sense or AS strands annealed to their complementary unmodified oligonucleotides (see Table 1). As predicted by results for the constituent ssRNA (see Fig. 1A), double-stranded β-gal siRNA comprising the 2′OMe-modified UGUGU, 2 × G, or 3 × U sense strand annealed with the unmodified (nonimmunostimulatory) AS strand induced no detectable interferon response from human PBMC (Fig. 2A). Interestingly, selective 2′OMe modification of the complementary 5′-ACACA-3′ motif in the AS strand, juxtaposed to the unmodified 5′-UGUGU-3′ motif in the sense strand, also diminished the level of IFN-α induction despite the annealed duplex containing the unmodified (immunostimulatory) sense strand (Fig. 2A). We found similar results with modified apoB-1 duplex siRNA (Fig. 2B). Unmodified apoB-1 siRNA induced a strong IFN-α response in PBMC and this reaction was completely abrogated when 2′OMe GU-, U-, or G-modified AS strands were incorporated in the apoB-1 duplex. Strikingly, modified apoB-1 siRNA containing 2′OMe G- or U-modified sense strands annealed to the unmodified, immunostimulatory, AS strand were also rendered nonimmunostimulatory (Fig. 2B). Abrogation of cytokine induction by 2′OMe G or U modifications to the sense strands of modified apoB-1 siRNA appeared absolute as even high concentrations (675 nM, ~9 μg/ml) of modified siRNA failed to induce IFN-α or inflammatory cytokines such as TNF in PBMC cultures (Figs. 2B and 2C).

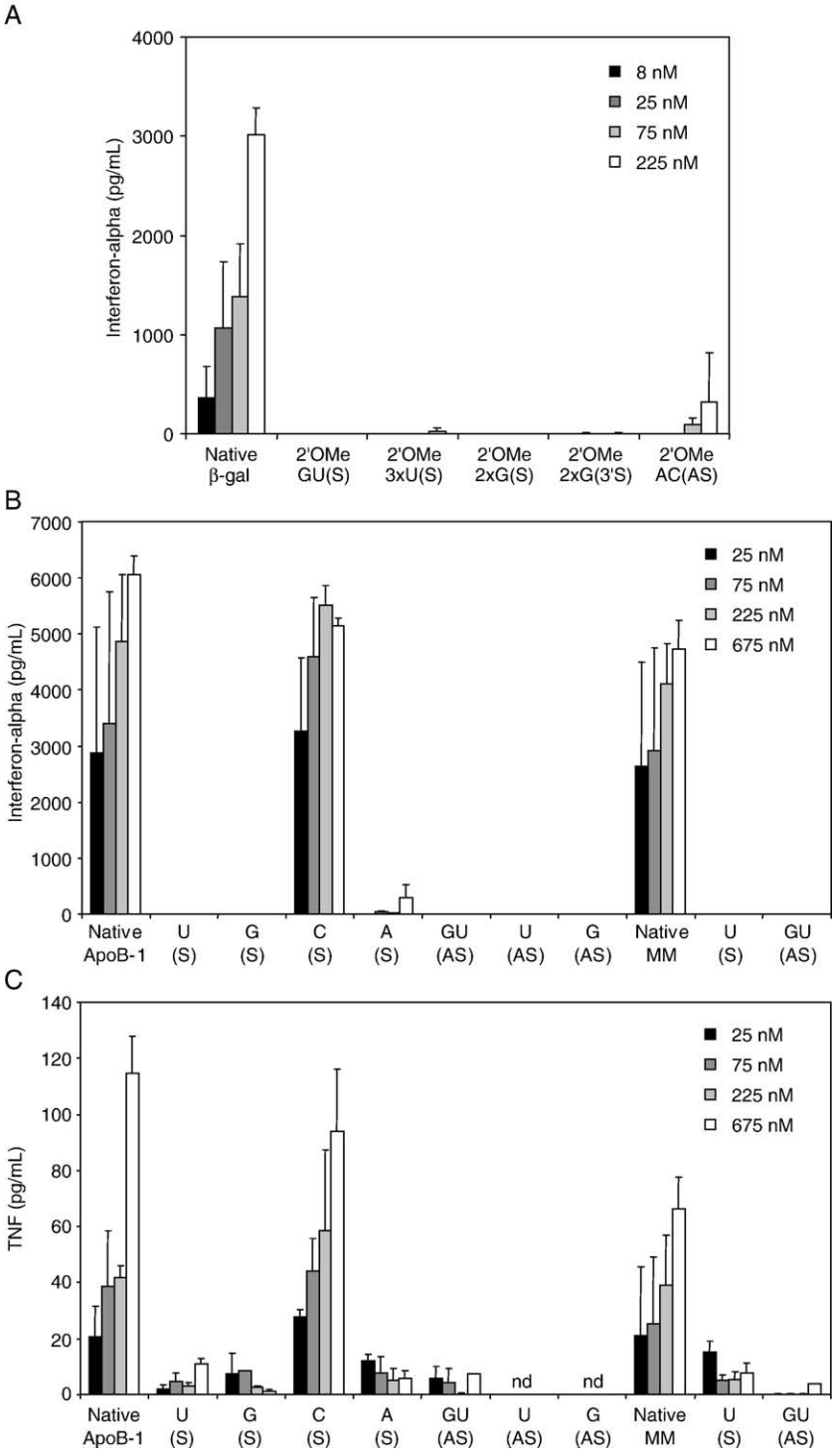

FIG. 2. Selective 2′OMe modifications to siRNA duplexes abrogate cytokine induction in human PBMC. (A, B) Interferon-α and (C) TNF induction from human PBMC cultured with increasing concentrations (8–675 nM) of encapsulated (A) β-gal or (B, C) apoB-1 or apoB mismatch siRNA. Cytokine responses to unmodified (native) siRNA were compared to duplexes containing 2′OMe-modified U, G, C, or A residues in either the sense (S) or the antisense (AS) strands as indicated (see Table 1 for siRNA sequences). Secreted cytokines were assayed after 24 h culture. Values are means + SD of triplicate cultures.

We did not observe the inhibitory effect of 2′-O-methylation on immune stimulation by siRNA with all patterns of modification, however, as apoB-1 siRNA containing 2′OMe-modified cytidine residues induced levels of cytokines similar to those induced by the native duplex (Fig. 2B). The incorporation of 2′OMe adenosine resulted in significant, but not absolute, inhibition of the cytokine response. These differences did not simply reflect the extent of chemical modification, as the 2′OMe G-, U-, C-, and A-modified apoB-1 contain 2, 5, 6, and 8 modified

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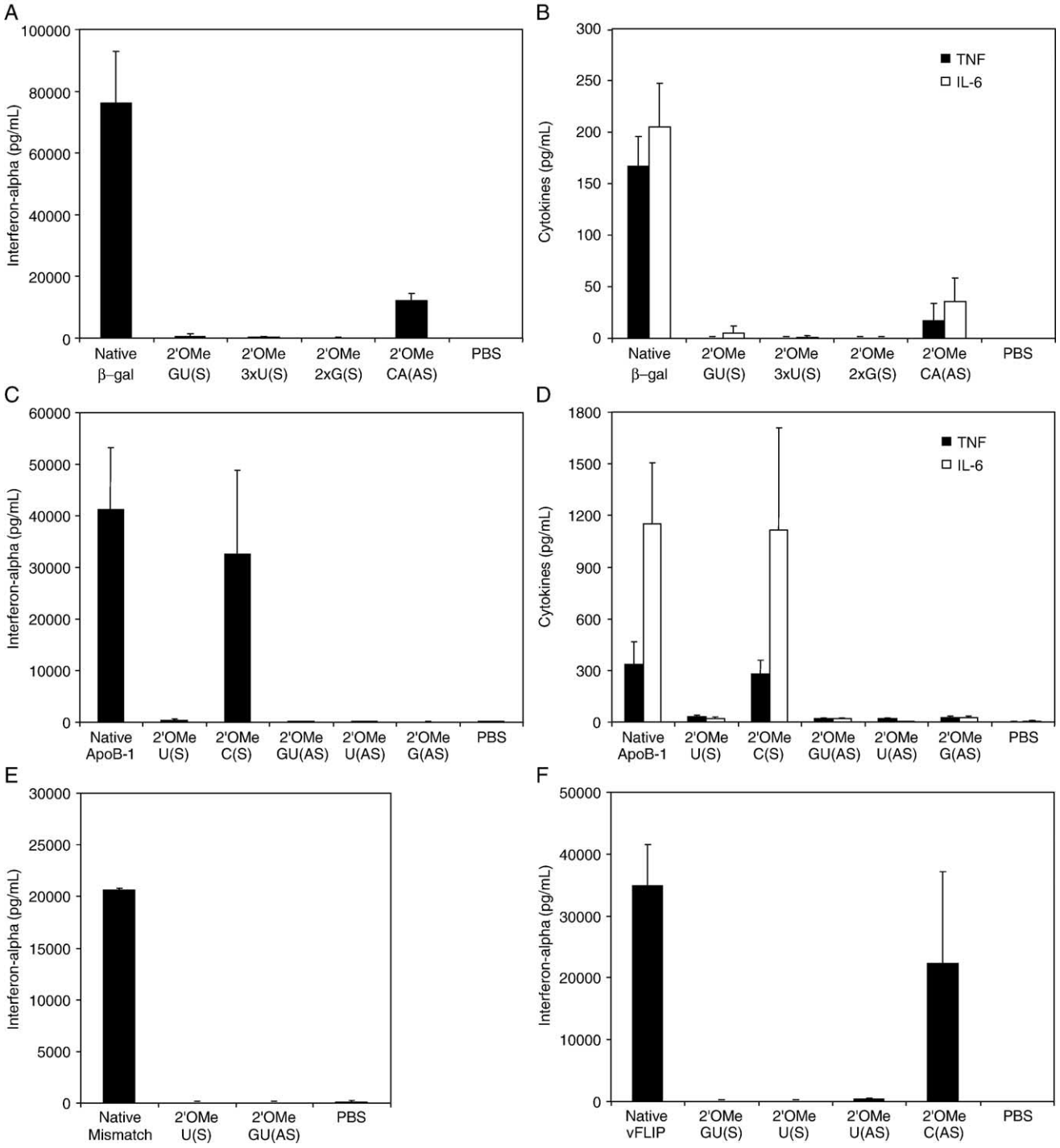

FIG. 3. Selective 2′OMe modifications to siRNA duplexes abrogates cytokine induction *in vivo*. (A, C, E, F) Serum interferon-α and (B, D) TNF and IL-6 levels 6 h after intravenous administration of encapsulated (A, B) β-gal, (C, D) apoB-1, (E) apoB mismatch, and (F) vFLIP siRNA. Responses to unmodified (native) siRNA were compared to duplexes containing 2′OMe-modified U, G, or C residues in either the sense (S) or the antisense (AS) strands as indicated (see Table 1 for siRNA sequences). All mice received 40 μg encapsulated siRNA. Values are means + SD from three or four animals. Lower levels of quantitation are 75 pg/ml for IFN-α, 30 pg/ml for TNF, and 60 pg/ml for IL-6.

nucleotides in the sense strand, respectively. This suggests that unmodified U and/or G residues may play a key role in immune recognition of the duplex siRNA.

To confirm that this approach to siRNA design would successfully inhibit inflammatory responses to siRNA *in vivo*, we assessed the immunostimulatory activity of the 2′OMe-modified β-gal and apoB-1 siRNA in mice. Intravenous administration of lipid-encapsulated β-gal (Figs. 3A and 3B) or apoB-1 (Figs. 3C and 3D) siRNA containing 2′OMe-modified guanosine or uridine residues in either sense or AS strands caused no detectable increase in serum IFN-α or inflammatory cytokines such as TNF. This was in marked contrast to the unmodified or cytosine-modified siRNA that induced substantial elevations in the level of these cytokines (Figs. 3C and 3D). We confirmed these striking effects of selective 2′OMe modification by applying a similar approach to modifying apoB mismatch [5] and vFLIP [29] siRNA sequences (see Table 1). For the apoB mismatch (Fig. 3E) and vFLIP siRNA duplexes (Fig. 3F), modifying either the GU-rich regions or only the uridine residues in either one of the RNA strands completely abrogated cytokine induction by the siRNA duplex. Inhibition of the cytokine response to modified apoB mismatch siRNA was also confirmed in human PBMC cultures (Figs. 2B and 2C). As with apoB-1, selective incorporation of 2′OMe cytosine residues into vFLIP siRNA did not substantially reduce the IFN-α response (Fig. 3F). Thus far, we have consistently observed, for each siRNA sequence tested, that the introduction of 2′OMe uridine or guanosine residues has generated a noninflammatory duplex. Five additional examples are provided in Supplementary Figs. S2–S4. Taken together, these findings support the conclusion that the underlying mechanism for immune recognition of short RNA duplexes appears to be largely conserved between mouse and humans [17,18]. Our results indicate that this mechanism can be profoundly disrupted in either species by the incorporation of as few as 2 2′OMe modified nucleotides within either strand of the RNA duplex.

Restricting Modifications to siRNA Sense Strand can Help Maintain RNAi Activity

We assessed the gene silencing activity of native and 2′OMe-modified apoB-1 siRNA *in vitro*. Unmodified apoB-1 encapsulated within liposomes caused potent, dose-dependent inhibition of apoB protein in HepG2 cell culture supernatants (Fig. 4). Estimated IC_{50} values (~1.5 nM) were in agreement with those established for this siRNA sequence using Oligofectamine transfection in a similar *in vitro* model [5]. Modified apoB-1 duplexes in which 2′OMe modifications were restricted to the nontargeting sense, or passenger, strand displayed apoB silencing activity broadly similar to that of the native siRNA (Fig. 4). In contrast, modifications to the targeting AS, or guide, strand severely impacted the RNAi activity of this duplex. Incorporation of 2′OMe uridine or guanosine residues in the AS strand abrogated apoB gene silencing, whereas the duplex containing the 5′-GUGUG-3′ modified AS strand displayed substantially reduced activity compared to the native or sense modified duplexes (estimated IC_{50} ~15 nM). Unmodified or modified apoB mismatch control siRNA yielded no significant inhibition of apoB protein expression (Fig. 4). A similar strategy of restricting 2′OMe modifications to the sense strands of β-gal 728 and luciferase siRNA also proved successful in generating noninflammatory siRNA that retained full RNAi activity (Supplementary Figs. S3 and S4). Although frequently reported to occur, it is not yet possible to predict how a particular pattern of chemical modifications may negatively impact RNAi activity [18,23,25,26,28]. Consistent with our findings on apoB-1, 2′OMe modification to the AS strand of an siRNA duplex, particularly at the 5′ end, has been shown to reduce its RNAi activity [25]. However, other groups have identified siRNA sequences

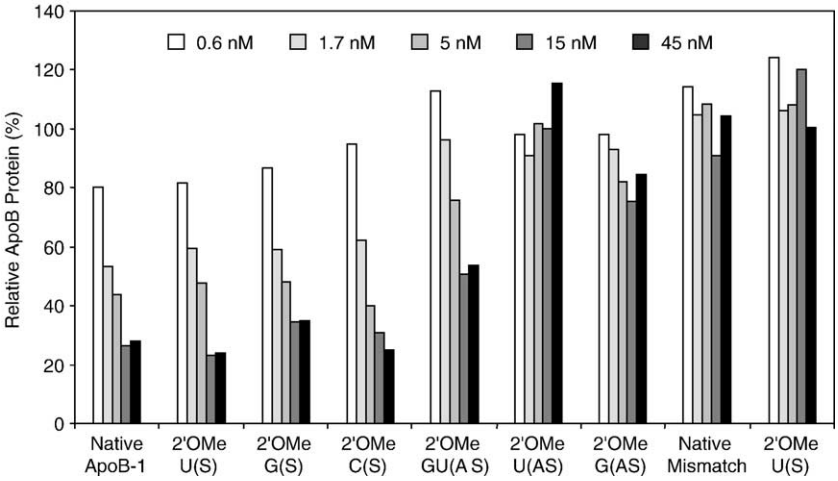

FIG. 4. *In vitro* silencing of apoB expression by 2′OMe-modified siRNA. HepG2 cells were treated with encapsulated apoB-1 or mismatch siRNA at the indicated concentrations (0.6–45 nM). Unmodified (native) apoB-1 siRNA was compared to apoB-1 duplexes containing 2′OMe-modified U, G, or C residues in the sense (S) strand or GU motif, U, or G residues in the antisense (AS) strand as indicated (see Table 1 for modified siRNA sequences). Unmodified and 2′OMe U(S) apoB mismatch siRNA served as control duplexes. ApoB protein in culture supernatants was measured by ELISA after 48 h. ApoB levels are expressed as % of PBS-treated control cultures. Each value is derived from means of duplicate cultures and is representative of three separate experiments.

doi:10.1016/j.ymthe.2005.11.002

that can tolerate extensive 2′OMe modifications to the AS strand [8,23]. Our results suggest that selective 2′OMe modifications, restricted to the sense strand of siRNA, offer a robust approach to overcoming the problem of immune activation by siRNA while reducing the chance of negatively impacting RNAi activity. It is envisaged that this approach can be applied to many, if not all, siRNA sequences with inherent capacity to stimulate the innate immune response. In our experience, this would encompass the majority of conventionally designed synthetic siRNA.

Potent RNAi Activity without Immune Stimulation *in vivo*

We assessed 2′OMe-modified apoB-1 siRNA for their ability to silence gene expression *in vivo*. We selected 2′OMe U(S)- and GU(AS)-modified apoB-1 as noninflammatory duplexes (see Figs. 2 and 3). This also afforded the opportunity to assess the impact of chemical modifications that reduced *in vitro* RNAi activity of the AS modified siRNA (see Fig. 4). We formulated native or 2′OMe-modified apoB-1 and mismatch siRNA in SNALP, shown previously to deliver siRNA to the liver [9]. For use in systemic applications, nucleic acid-based drugs require stabilization or protection from nuclease degradation. Encapsulation inside the lipid bilayer protected unmodified and otherwise labile siRNA from serum nuclease degradation for greater than 24 h at 37°C *in vitro*, implying that encapsulation offers adequate nuclease protection without the need for extensive chemical modification to the siRNA. By comparison, naked siRNA was fully degraded within 4 h under similar conditions (Fig. 5).

We administered encapsulated apoB siRNA intravenously to BALB/c mice at 5 mg/kg/day for 3 days. This regimen represents a 10-fold reduction in apoB-1 siRNA dose originally reported to be efficacious in experiments utilizing cholesterol-conjugated, chemically modified apoB-1 siRNA [5]. Animals receiving native, immunostimulatory apoB-1 or mismatch siRNA displayed overt symptoms of toxicity as evidenced by a loss of 10.5 and 9% of initial body weight, respectively, by day 3 (Fig. 6A) and mild deterioration in general body condition during the course of treatment (not shown). In contrast, treatment with the 2′OMe-modified siRNA was well tolerated with minimal (less than 1%) or no body weight loss (Fig. 6A). We confirmed abrogation of the innate cytokine response in these efficacy studies by in-life serum IFN-α analysis (Fig. 6B), and accordingly we attribute the toxicity associated with administration of the unmodified siRNA to the systemic cytokine response. Of note, cytokine levels and body weight loss induced by unmodified mismatch siRNA were lower than for the corresponding active apoB-1 duplex. The mismatch control in this case was generated by four G/C substitutions within the apoB-1 sequence [5], providing further evidence for the sequence-dependent effects on immune stimulation by RNA duplexes.

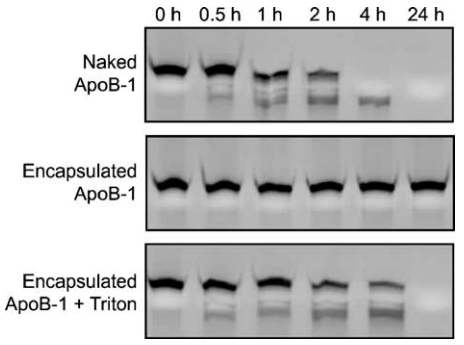

FIG. 5. Encapsulation of siRNA in lipid particles protects against serum nuclease degradation. Unmodified naked (top) or SNALP-encapsulated (middle) apoB-1 siRNA were incubated in 50% mouse serum at 37°C. Duplex integrity was assessed at indicated time points by nondenaturing PAGE analysis. Addition of Triton X to disrupt lipid particle integrity (bottom) restored siRNA nuclease sensitivity.

As a direct measure of RNAi-mediated knockdown, we determined apoB mRNA in the liver 2 days after final siRNA treatment (Fig. 6C). In both the native and the 2′OMe U(S)-modified apoB-1-treated groups, apoB mRNA levels were significantly reduced compared to PBS-treated animals (18 ± 2 and 18 ± 5% of PBS controls, respectively). By comparison, mice treated with 2′OMe GU(AS)-modified apoB-1 siRNA displayed less pronounced silencing of apoB mRNA (44 ± 4% of controls), which correlated with reduced *in vitro* RNAi activity of this modified siRNA (see Fig. 4). ApoB mRNA levels in the modified mismatch group were equivalent to those in PBS controls (Fig. 6C), while the native mismatch siRNA caused a modest reduction in apoB mRNA levels (79 ± 12% of PBS controls). The modest reduction in liver apoB mRNA observed with the native mismatch siRNA was apparent in three separate experiments (not shown) and correlated with interferon release and symptoms of toxicity associated with systemic administration and delivery of the unmodified siRNA.

Silencing of apoB mRNA in the liver resulted in proportional, sequence-specific reductions in serum apoB protein. Mice treated with native, 2′OMe U(S)- or GU(AS)-modified apoB-1 siRNA had serum apoB protein levels that were 26, 28, and 47% those of the PBS-treated animals, respectively (Fig. 6D). Functional silencing of apoB expression was reflected in significant reductions in serum cholesterol that also correlated with the relative potency of mRNA and protein knockdown. Mice treated with native or 2′OMe U(S)- or GU(AS)-modified apoB-1 siRNA displayed serum cholesterol levels that were 48, 51, and 69% of cholesterol levels in the PBS control group (Fig. 6E). Neither mismatch siRNA had any effect on serum cholesterol (Fig. 6E). In separate experiments, the noninflammatory 2′OMe G(S)-modified apoB-1 mediated similar reductions in apoB mRNA,

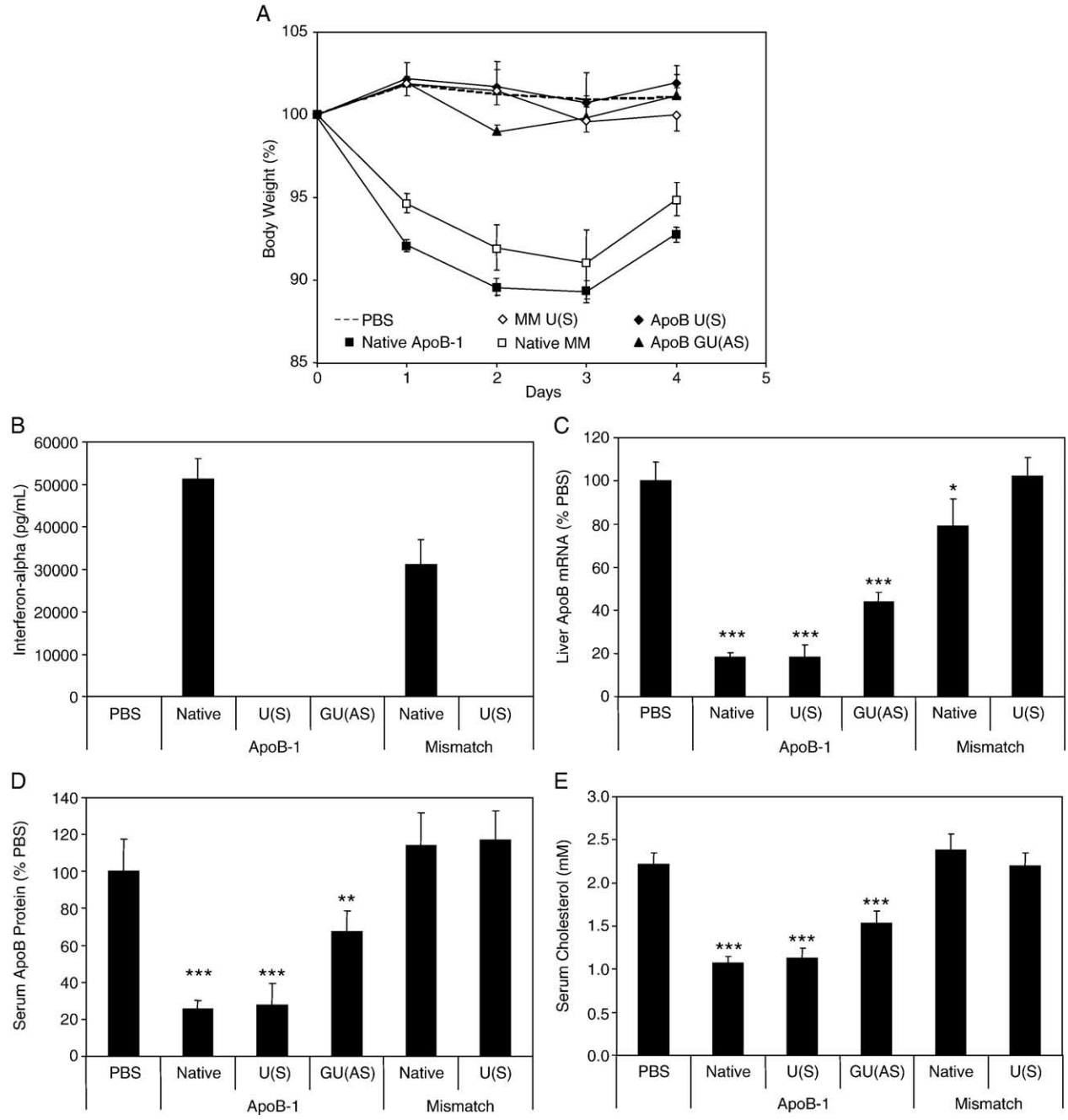

FIG. 6. Silencing of apoB expression *in vivo* without activation of the innate immune response. (A–E) *In vivo* effects following intravenous administration of encapsulated apoB-1 or mismatch siRNA in mice. Mice were treated on days 0, 1, and 2 with encapsulated unmodified (native) or 2′OMe U(S)- or GU(AS)-modified apoB-1 and native or 2′OMe U(S)-modified mismatch siRNA at 5 mg/kg per day. (A) Daily changes in body weight (% of day 0 weight) of apoB-1 (solid symbols) and mismatch (open symbols) siRNA-treated mice over the 4-day study period. (B) Serum IFN-α from test bleeds 6 h after initial treatment; lower level of quantitation 75 pg/ml. (C) apoB mRNA levels in liver, (D) apoB protein in serum, and (E) serum cholesterol (mM) 2 days after final siRNA treatment. C and D are expressed as % of apoB mRNA and apoB protein compared to PBS-treated animals. All values are means + SD of five animals. All data are representative of two separate experiments. Statistical significance versus PBS-treated animals: $*P < 0.05$, $**P < 0.005$, $***P < 0.00005$; unpaired two-tailed *t* test on group values prior to normalization to PBS.

doi:10.1016/j.ymthe.2005.11.002

protein, and serum cholesterol, in the absence of IFN induction (not shown).

Results from these studies demonstrate that lipid encapsulation of siRNA provides adequate serum stability for systemic applications and negates the need for extensive chemical modifications to the RNA. This, coupled with the effective delivery of the siRNA payload to the target organ, in this case the liver, facilitates the silencing of endogenous genes. This is exemplified in these studies by apoB, a protein that represents a potential therapeutic target for hypercholesterolemia. Importantly, the 2′OMe-modified siRNA, designed to be noninflammatory, displayed potency *in vivo* that is equivalent to that of the unmodified siRNA but without the immunotoxicity and other off-target effects associated with the systemic administration of unmodified siRNA. We believe that this approach will be generally applicable to a wide range of gene targets and shows great promise for use in a therapeutic setting.

DISCUSSION

Although naked siRNA can trigger RNA interference *in vivo* without apparent IFN induction [30], recent reports have shown that, when formulated with lipidic or polycation-based delivery systems, unmodified siRNA can be potent activators of the innate immune response [17–19]. TLR7, located within the endosomal compartment, has been implicated as the primary immune recognition pathway for siRNA [18] and this may, in part, explain why delivery vehicles that enter cells through the endosomal pathway can potentiate this response. Based on the finding that immune activation by siRNA is sequence dependent, we have shown previously that it is possible to design active siRNA with negligible immunostimulatory activity by selecting sequences that lack GU-rich motifs [17]. Although this strategy has proven successful, it significantly limits the number of novel siRNA sequences that can be designed against a given target. Furthermore, it currently requires some degree of screening due to the relatively ill-defined nature of putative RNA immunostimulatory motifs. In this report, we highlight a novel and robust approach to abrogating synthetic siRNA-mediated immune stimulation by selective incorporation of 2′OMe-modified nucleotides into the siRNA duplex. Remarkably, incorporation of as few as two 2′OMe guanosine or uridine residues in highly immunostimulatory siRNA molecules completely abrogated siRNA-mediated interferon and inflammatory cytokine induction in human PBMC and in mice *in vivo*. This degree of chemical modification represents ~5% of the native 2′-OH positions in the siRNA duplex. Since complete abrogation of the immune response required only one of the RNA strands to be selectively modified, 2′OMe modifications could be restricted to the sense strand of the duplex, thereby minimizing the potential for attenuating the potency of the siRNA. These findings have provided a simple rationale for the synthesis of nonimmunostimulatory siRNA based on native sequences with proven RNAi activity. We demonstrate that by combining selectively modified siRNA with an effective systemic delivery vehicle, potent silencing of an endogenous gene target can be achieved *in vivo* at therapeutically viable doses without the deleterious side effects associated with systemic activation of the innate immune response.

It is unclear at present how the introduction of 2′OMe nucleotides into siRNA prevents recognition of the duplex RNA by the immune system. Although there is evidence from gene-deficient mice that TLR7 is required for siRNA-mediated immune stimulation [18], the nature of the physical interaction between the receptor and the putative ligand is not known. This is also true for the defined TLR7/8 ligands that include guanosine analogues [31] and ssRNA containing GU or poly(U) motifs [11,12]. The mammalian immune system comprises a number of alternative signaling pathways that can also be activated by RNA species [10,32–35], and studies in cell lines suggest that siRNA may be a ligand for TLR3 [36] or dsRNA-dependent PKR [14,16] under certain conditions. Further work is therefore required to confirm the precise mechanism of siRNA-mediated immune activation before the molecular basis underlying the inhibitory effects of 2′OMe modification can be fully elucidated. Since the 2′-OH in the ribose backbone is a distinguishing feature of RNA, extensive chemical substitutions at this position may be anticipated to disrupt recognition of the modified nucleic acid by an RNA binding receptor pathway. However, our studies show that 2′OMe-modified siRNA are rendered nonimmunostimulatory despite retaining up to 95% of their native ribonucleotides, including those comprising defined immunostimulatory regions of the RNA. 2′OMe is considered to be a relatively bulky chemical group at the 2′ position that sits within the minor groove of an RNA duplex without significantly distorting its A-form helical structure [26,37]. This may be sufficient to disrupt interactions between the double-stranded RNA duplex and its putative immune receptor or accessory molecules. The *trans*-inhibitory effect of 2′-O-methylation, whereby 2′OMe-modified ssRNA annealed to unmodified immunostimulatory ssRNA generates a nonimmunostimulatory duplex, is consistent with such a hypothesis that implies recognition of the siRNA as a double-stranded molecule.

Recent work has demonstrated that human TLRs may be preferentially activated by pathogen-derived RNA based on their lack of modified nucleosides [32], which occur at much higher frequency in most mammalian RNA. The introduction of a variety of naturally occurring modified nucleosides into RNA was shown to inhibit TLR activation

and immune stimulation [32]. Our findings with 2′OMe-modified siRNA are consistent with this report, particularly with respect to the low degree of modification required to abrogate RNA-mediated immune stimulation and the differential effects of modifying uridine versus cytidine residues on the activation of primary immune cells. This suggests that 2′OMe-modified siRNA may avoid recognition by the innate immune system based on an evolutionary mechanism by which immune receptors distinguish pathogen-derived RNA from self-RNA.

A number of other stabilization chemistries are routinely used in synthetic siRNA design in an effort to confer nuclease resistance that may also influence immune recognition and RNAi. LNA that contain a 2′-O, 4′-C methylene bridge in the sugar ring have been shown to reduce partially the immunostimulatory activity of an siRNA [18]. We have found that siRNA containing inverted deoxy abasic end caps retain immunostimulatory activity [9]. No evidence of a *trans*-inhibitory effect was reported with LNA modified duplexes. These observations suggest that immune stimulation by siRNA is particularly sensitive to inhibition by 2′OMe modifications versus other, well-characterized, stabilization chemistries.

In this report, we demonstrate that both unmodified and 2′OMe-modified synthetic siRNA can mediate potent silencing of the endogenous gene target apoB when encapsulated in lipid particles and administered systemically. Intravenous administration of encapsulated unmodified or modified apoB-1 siRNA resulted in significant reductions in apoB mRNA levels in the liver and concomitant reductions in apoB protein in the blood. Importantly, given the interest in apoB as a therapeutic target for hypercholesterolemia, apoB silencing resulted in a significant reduction in serum cholesterol. Lipid encapsulation also confers excellent resistance to degradation by serum nucleases, enabling the *in vivo* use of minimally modified siRNA duplexes. Accordingly, we have been able to focus our attention on chemical modifications to siRNA that overcome the problem of immune activation. By preventing the induction of interferons and inflammatory cytokines we are able to limit the potential for nonspecific effects on gene expression while improving the tolerability of siRNA formulations. Specifically, we show that intravenous administration of encapsulated 2′OMe-modified siRNA is efficacious and well tolerated in mice. These findings, coupled with ongoing improvements in delivery technology, siRNA design, and our understanding of the mechanism of RNA interference, are important steps in the advancement of synthetic siRNA in a broad range of *in vivo* and therapeutic applications.

METHODS

siRNA. All siRNA used in these studies were chemically synthesized by Dharmacon (Lafayette, CO, USA) and received as desalted, deprotected oligonucleotides. Duplexes were annealed by standard procedures. Complementary strands at equimolar concentrations were heated to 90°C for 2 min and then cooled slowly at 37°C for 60 min. Formation of annealed duplexes was confirmed by nondenaturing PAGE analysis. All native and 2′OMe-modified sequences are listed in Table 1.

Lipid encapsulation of RNA. siRNA or ssRNA was encapsulated into liposomes by a process of spontaneous vesicle formation followed by stepwise ethanol dilution as described for pDNA by Jeffs *et al*. [38]. Liposomes were composed of the following lipids: synthetic cholesterol (Sigma, St. Louis, MO, USA), the phospholipid 1,2-distearoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL, USA), the PEG lipid PEG-cDMA (3-*N*-[(methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine), and the cationic lipid DLinDMA (1,2-dilinoleyloxy-3-(N,N-dimethyl)aminopropane) in the molar ratio 48:20:2:30. The lipids PEG-cDMA and DLinDMA [39] were synthesized in-house at Protiva Biotherapeutics. The resulting stabilized lipid particles were dialyzed in PBS and filter sterilized through a 0.2-μm filter prior to use. Particle sizes of each liposome preparation ranged from 100 to 130 nm and typically contained 90–95% of siRNA encapsulated within the liposome. Concentration and percentage encapsulation of formulated siRNA were determined using the membrane-impermeable fluorescent dye RiboGreen (Molecular Probes, Eugene, OR, USA) before and after the addition of detergent to disrupt the lipid bilayers [38].

Serum nuclease protection assay. Unmodified naked or lipid-encapsulated siRNA (0.25 mg/ml) were incubated in 50% mouse serum at 37°C. At the times indicated, aliquots were taken directly into gel loading buffer containing 0.1% SDS and frozen in liquid nitrogen. After the final sample was frozen, siRNA were run on a nondenaturing 20% polyacrylamide TBE gel and visualized by ethidium bromide staining. To confirm that nuclease protection of siRNA was conferred by lipid encapsulation, 0.1% Triton X-100 was added to disrupt lipid bilayer integrity immediately prior to incubation with serum.

Cell isolation and culture. Human PBMC were isolated from whole blood from healthy donors by a standard Ficoll–Hypaque density centrifugation technique. For immune stimulation assays, 3×10^5 freshly isolated PBMC were seeded in triplicate in 96-well plates and cultured in RPMI 1640 medium with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Liposome-encapsulated siRNA were added to cells at the indicated final nucleic acid concentration and culture supernatants were collected after 16–20 h and assayed for IFN-α, IL-6, and TNF-α by sandwich ELISA.

In vitro RNA interference assay. HepG2 cells were seeded into 24-well plates at 20,000 cells/well. To determine *in vitro* RNAi activity of 2′OMe-modified apoB siRNA, HepG2 cultures were treated, in duplicate, with encapsulated siRNA at five nucleic acid concentrations between 0.6 and 45 nM. Medium was changed 24 h after addition of siRNA and then the cultures were incubated for an additional 48 h. Human apoB protein levels were determined in culture supernatants by sandwich ELISA, as detailed in Soutschek *et al*. [5], using polyclonal goat anti-human apoB capture antibody (Chemicon International) and horseradish peroxidase-conjugated goat anti-human apoB-100 antibody (Academy Bio-medical) to detect bound apoB. ELISA plates were developed using TMB substrate and stopped with 2 N sulfuric acid and absorbance was read at 450 nm − 570 nm. A_{450} values were normalized against a standard curve generated from untreated HepG2 conditioned medium to define the linear range of the ELISA. Mean, residual apoB protein levels in siRNA-treated culture supernatants were calculated as a percentage of PBS-treated controls.

In vivo cytokine induction. Animal studies were completed in accordance with the Canadian Council on Animal Care guidelines following approval by the local Animal Care and Use Committee at Protiva Biotherapeutics. Six- to eight-week-old CD1 ICR mice (Harlan, Indianapolis, IN, USA) were subjected to a 3-week quarantine and acclimation

doi:10.1016/j.ymthe.2005.11.002

period prior to use. Encapsulated siRNA formulations were administered by standard intravenous injection in the lateral tail vein in 0.2 ml PBS. Blood was collected by cardiac puncture 6 h after administration and processed as plasma for cytokine analysis. In RNAi efficacy experiments, plasma was collected from 50-μl test bleeds 6 h after initial siRNA administration.

Cytokine ELISA. All cytokines were quantified using sandwich ELISA kits according to the manufacturer's instructions. These were mouse and human IFN-α (PBL Biomedical, Piscataway, NJ, USA), human IL-6 and TNF-α (eBioscience, San Diego, CA, USA), and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences, San Diego, CA, USA).

In vivo RNA interference. Groups of five Balb/C mice (Harlan) were treated once a day for 3 consecutive days with lipid-encapsulated siRNA (native, 2′OMe U(S), or 2′OMe GU(AS) apoB-1 and native or 2′OMe U(S) mismatch) at 5 mg/kg by standard intravenous injection via the lateral tail vein. Body weights and general observations were recorded throughout the duration of the studies. Forty-eight hours after the final siRNA treatment, mice were sacrificed. Blood was collected by cardiac puncture for serum analysis of apoB protein and cholesterol. Livers were weighed and collected into 6 ml RNALater (Sigma) for apoB mRNA analysis by QuantiGene assay (Genospectra, Fremont, CA, USA).

Serum cholesterol was measured using a commercial cholesterol detection kit according to the manufacturer's instructions (Thermo Electron Corp., Melbourne, Australia).

ApoB-100 was detected in serum from individual animals by sandwich ELISA using monoclonal mouse apoB-100 capture antibody LF3 (obtained from Dr. S. Young [40]). Bound apoB-100 was detected with polyclonal rabbit anti-mouse apoB (Biodesign International, Saco, ME, USA) and horseradish peroxidase-conjugated goat anti-rabbit Ig's (Jackson Immunoresearch; West Grove, PA, USA). Serum apoB levels were determined from A_{450} values using a standard curve generated with normal mouse serum to define the linear range of the ELISA and expressed as a percentage of the PBS-treated control group.

The QuantiGene assay (Genospectra) was used to quantify the reduction of mouse apoB mRNA in liver tissue after siRNA treatment. Small uniform tissue samples were taken from livers that had been collected 48 h after final injection and stored in RNALater (Sigma). Liver lysates were prepared according to the manufacturer's instructions and used directly for apoB and GAPDH mRNA quantification. The ratio of apoB to GAPDH mRNA was calculated for each liver sample and data were expressed as a group average relative to the PBS control group. Specific probe sets used for detection of mRNA were designed by Genospectra to target the following regions: for the apoB mRNA, positions 5183–5811 of Accession No. XM_137955; for GAPDH mRNA, positions 9–319 of Accession No. NM-008084.

ACKNOWLEDGMENTS

The authors acknowledge the expert technical assistance of Kevin McClintock, Matt Fedoruk, Vandana Sood, Jay Petkau, Lorne Palmer, Ed Yaworski, and Kieu Lam.

RECEIVED FOR PUBLICATION OCTOBER 7, 2005; REVISED NOVEMBER 2, 2005; ACCEPTED NOVEMBER 4, 2005.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.11.002.

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