

Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells

Christina Lorenz,^a Philipp Hadwiger,^{a,*} Matthias John,^a Hans-Peter Vornlocher^a and Carlo Unverzagt^b

^a*Alnylam Europe AG, Fritz-Hornschuch-Str. 9, D-95326 Kulmbach, Germany*

^b*Lehrstuhl für Bioorganische Chemie, Universität Bayreuth, D-95440 Bayreuth, Germany*

Received 27 May 2004; revised 7 July 2004; accepted 8 July 2004

Available online 29 July 2004

Abstract—Double-stranded short interfering RNAs (siRNAs) mediate post-transcriptional inhibition of gene expression in a variety of biological systems. However, human liver cells show poor uptake of these nucleic acids. In order to improve the delivery of siRNA into these cells without transfection agents, we have synthesized two series of lipophilic siRNAs conjugated with derivatives of cholesterol, lithocholic acid or lauric acid. The lipid moieties were covalently linked to the 5'-ends of the RNAs using phosphoramidite chemistry. The potency of these chemically modified siRNAs to inhibit reporter gene expression was further investigated in vitro with β -galactosidase expressing liver cells.

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The phenomenon of RNA interference was first discovered by Fire et al.¹ and describes post-transcriptional gene silencing mediated by long double-stranded RNAs. During RNA interference these RNAs are processed into small fragments of approximately 22 nucleotides by the enzyme complex Dicer.^{2,3} The so-termed short interfering RNAs (siRNA) get unwound and are then incorporated into the RNA-induced silencing complex (RISC), which uses the single strands to search for complementary mRNA sequences.⁴ Finally, the homologous mRNA is cleaved by RISC and further degraded by cellular nucleases, resulting in sequence specific inhibition of gene expression. As long double-stranded RNAs induce a nonspecific interferon response in mammalian cells, chemically synthesized siRNAs with 19–24 nucleotides in length provide a possibility to bypass Dicer processing and thus to induce RNA interference in higher biological systems, especially in human cells.^{5,6}

Potential siRNA therapeutics against liver cell-specific diseases such as hepatitis C, require improved cellular uptake of siRNA. The liver is involved in lipid and steroid metabolism. Thus, siRNA modified with lipophilic

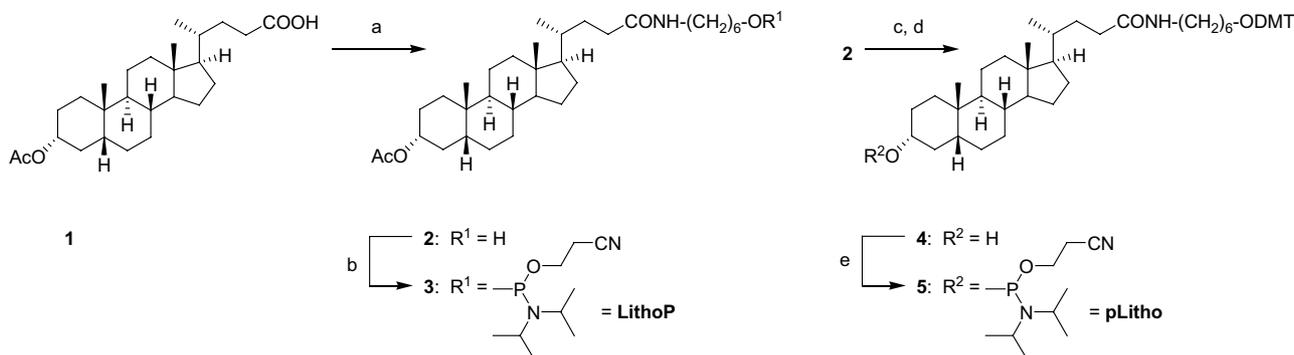
moieties may enhance siRNA uptake via a receptor-mediated mechanism or by an increased membrane permeability of the otherwise negatively charged RNA.

In this study we describe the synthesis of lipophilic siRNA conjugates. The cholesterol derivative (Chol **6**⁷) was chosen for modification, as Manoharan and co-workers have shown an increased uptake of cholesterol-modified antisense oligonucleotides in liver cells.⁸ Furthermore, derivatives of lithocholic acid (LithoP **3** and pLitho **5**) and 12-hydroxy lauric acid (Laurin **9** and C32 **11**) were synthesized. The lithocholic acid derivatives **3** (LithoP) and **5** (pLitho) used in this study were obtained as outlined in Scheme 1. A C6-linker was introduced by chemoselective coupling of 6-aminohexanol to acetylated lithocholic acid **1**⁹ with ethyl-dimethylaminopropyl-carbodiimide hydrochloride (EDCI) and *p*-dimethylaminopyridine (DMAP) in dichloromethane. Subsequently, the hydroxyl group of compound **2** was converted to the phosphoramidite **3** (LithoP) using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite.¹⁰

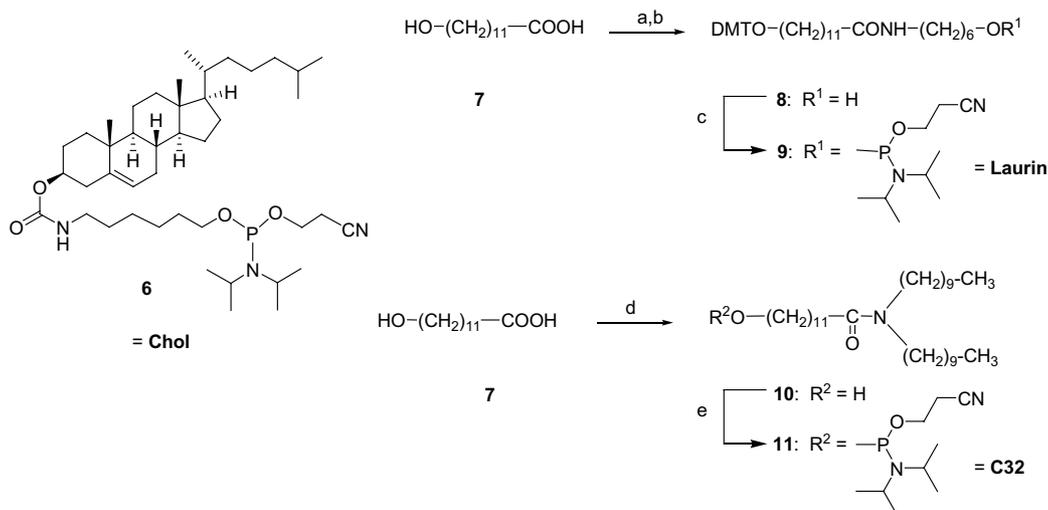
In order to obtain the derivative **5** (pLitho), the hydroxyl group of **2** was first protected by a dimethoxytrityl group (DMT). After deacetylation of the crude trityl-ether the resulting alcohol **4** was converted to the desired phosphoramidite **5**. The cholesterol derivative **6** (Chol, Scheme 2) was obtained according to the literature.⁷

Keywords: siRNA; RNAi; Cellular uptake; Solid phase synthesis.

* Corresponding author. Tel.: +49-9221-8276241; fax: +49-9221-8276299; e-mail: phadwiger@alnylam.de



Scheme 1. Synthesis of **3** (LithoP) and **5** (PLitho). Reagents and conditions: (a) 6-aminohexanol, DMAP, EDCl, CH₂Cl₂ (67%); (b) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂ (35%); (c) DMT-Cl, pyridine; (d) K₂CO₃, MeOH/CH₂Cl₂ (c + d: 83%); (e) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂ (76%).



Scheme 2. Phosphoramidite **6** (Chol); synthesis of Laurin **9** and C32 **11**. Reagents and conditions: (a) DMT-Cl, pyridine; (b) 6-aminohexanol, DMAP, EDCl, CH₂Cl₂; (a + b: 97%); (c) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂ (43%); (d) di-*n*-decylamine, DMAP, EDCl, CH₂Cl₂ (90%); (e) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂ (54%).

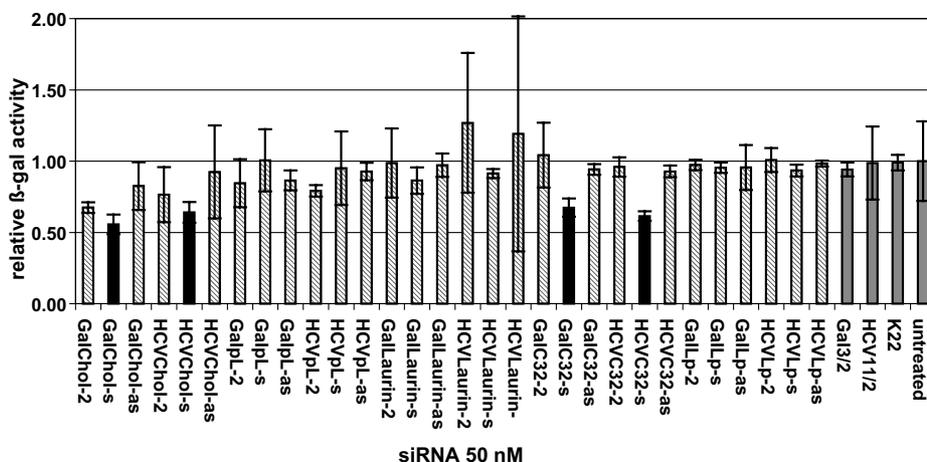
The synthesis of the two 12-hydroxy lauric acid derivatives **9** (Laurin) and **11** (C32), mimicking membrane lipids, was performed as indicated in **Scheme 2**. Chemo-selective elongation of the dimethoxytrityl ether of 12-hydroxy lauric acid **7** with 6-aminohexanol gave the amide **8**. Phosphitylation of the OH-function of **8** yielded compound **9** (Laurin). To obtain the second derivative, 12-hydroxy lauric acid **7** was reacted with di-*n*-decylamine, which was added in 2M excess resulting in the branched amide **10**. The final conversion of the hydroxyl function of **10** to a phosphoramidite group led to compound **11** (C32).

With the five lipid-containing phosphoramidites in hand, a series of 5'-modified siRNAs was synthesized on solid phase. The siRNA sequences were directed either against the 3'-untranslated region of hepatitis C-RNA (HCV 3'-UTR) or against *E. coli* β -galactosidase mRNA. The incorporation of the modifications 5 and 9 proceeded in efficiencies similar to commercially

siRNA:

	5'	cg GUG AAA UUA UCG AUG AGC GUG	3'	mRNA target
GalChol-s	5'	CHOL GUG AAA UUA UCG AUG AGC GUG	3'	sense
	3'	GC CAC UUU AAU AGC UAC UCG CAC	5'	antisense
GalChol-as	5'	GUG AAA UUA UCG AUG AGC GUG	3'	sense
	3'	GC CAC UUU AAU AGC UAC UCG CAC CHOL	5'	antisense
GalChol-2	5'	CHOL GUG AAA UUA UCG AUG AGC GUG	3'	sense
	3'	GC CAC UUU AAU AGC UAC UCG CAC CHOL	5'	antisense
HCVChol-s	5'	uc ACG GCU AGC UGU GAA AGG UCC	3'	mRNA target
	5'	CHOL ACG GCU AGC UGU GAA AGG UCC	3'	sense
	3'	AG UGC CGA UCG ACA CUU UCC AGG	5'	antisense
HCVChol-as	5'	ACG GCU AGC UGU GAA AGG UCC	3'	sense
	3'	AG UGC CGA UCG ACA CUU UCC AGG CHOL	5'	antisense
HCVChol-2	5'	CHOL ACG GCU AGC UGU GAA AGG UCC	3'	sense
	3'	AG UGC CGA UCG ACA CUU UCC AGG CHOL	5'	antisense

Scheme 3. Shown are the six exemplary siRNA conjugates obtained with a Chol **6** modification. The prefixes Gal and HCV indicate the target mRNA β -galactosidase or the HCV-3'-UTR, respectively. The extensions -s, -as and -2 refer to the modification attached to the sense (-s) or the antisense (-as) strand or both strands (-2). In a similar fashion the other four lipophilic compounds LithoP **3**, pLitho **5**, Laurin **9** and C32 **11** were used to obtain six conjugates each.



Scheme 4. The results of siRNA delivery experiments without transfection agents in β -Gal@Huh-7 cells with all 30 modified siRNAs are shown in this figure. The cells were divided into five wells and treated for 4 h with siRNAs at a concentration of 50 nM in serum free medium. Subsequently, the incubation medium was replaced by complete medium. After 48 h cells were lysed and β -galactosidase activity was measured in a standard chemoluminescence assay. The four most effective siRNAs for further studies are highlighted. Data are presented as mean values with the corresponding standard deviation of five assays. Gal3/2 and HCV11/2 are unmodified siRNAs possessing the same sequence as the modified siRNAs. K22 is an unmodified, nonspecific siRNA. Furthermore the β -gal activity value for untreated cells is shown.

available standard nucleotide phosphoramidites as judged by colorimetric analysis of the release of the dimethoxytrityl cation. The oligonucleotides were deprotected employing established procedures,¹⁴ purified by IEX HPLC and characterized by electrospray mass spectrometry (ESI-TOF-MS).

Each series of siRNAs contained one of the five possible modifications either at the 5'-end of the sense or the antisense strand or contained modifications at both strands. Altogether 15 modified siRNAs directed against the same sequence of HCV and 15 siRNAs targeting one region of the β -galactosidase mRNA were assembled (Scheme 3).

The complete panel of siRNAs was evaluated for the ability to inhibit gene expression in the human liver cell line β -Gal@Huh-7, most importantly, without the use of any transfection agent. These cells are derived from the human hepatoma cell line Huh-7^{11–13} and contain a stable expressed gene fusion consisting of a part of the HCV 3'-UTR and *lacZ*.¹⁵

Incubation of these cells with siRNAs against each of the two target sequences resulted in a decrease in β -galactosidase expression. It was found that siRNAs with a modified sense strand downregulated β -galactosidase expression to a higher extent than siRNAs with a modified antisense strand or two modified strands. Unmodified siRNAs did not reduce gene expression under identical conditions, although they were equally effective when transfected with lipofection (data not shown). Furthermore, two modifications were identified (Chol 6 and C32 11), which were superior in downregulation of β -galactosidase expression, independent of the siRNA sequence they were attached to (Scheme 4). Consequently, the four siRNAs, directed against the HCV 3'-UTR or the *lacZ* sequence and sense modified with

either Chol 6 or C32 11 (HCVChol-s, HCVC32-s, GalChol-s and GalC32-s) were selected for detailed studies, which will be reported in due course.

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