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2′-*O*-Lysylaminohexyl Oligonucleotides: Modifications for Antisense and siRNA

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A novel type of oligonucleotide has been developed, characterized by the attachment of a lysyl moiety to a 2′-O-aminohexyl linker. A protected lysine building block was tethered to 2′-O-aminohexyluridine, and the product was converted into the corresponding phosphoramidite. Up to six modified nucleosides were incorporated in dodecamer DNA and RNA oligonucleotides using standard phosphoramidite chemistry. Each of the building blocks contributes one positive charge to the oligonucleotide instead of the negative charge of a wild-type nucleotide. Thermal denaturation profiles indicated a stabilizing effect of 2′-O-lysylaminohexyl chains that was more pronounced in RNA duplexes. Incubation of the oligonucleotides with 5′-exonuclease revealed an exceptionally high stability against enzymatic degradation. Incorporation of up to three modifications into functional antisense and siRNA oligonucleotides targeted at ICAM-1 showed that the gene-silencing activity was higher with an increasing number of lysylaminohexyl nucleotides. Compared with wild-type antisense or siRNA, compounds with three modifications led to equal or higher ICAM-1 downregulation.

Introduction

Sequence-specific gene silencing can be afforded by either antisense oligonucleotides or short interfering RNA (siRNA). Although widely used in scientific experiments, their successful therapeutic application has been prevented mainly by insufficient pharmacokinetic characteristics. The traditional major field of oligonucleotide application has been the antisense approach.[1,2] Several agents have been tested in clinical trials, but so far only one product for local administration, fomivirsen, has reached the market. During the last decade, many of the unfulfilled hopes for a rational specific therapy at the genetic level have been shifted to the newly available siRNA technology. Despite having different molecular mechanisms of action, the emerging problems and necessary precautions to be taken are very similar for both antisense- and siRNA-based therapeutics.[3]

A myriad of work has been reported on chemical modifications of antisense oligonucleotides aimed at their application as drugs. Phosphorothioate modified oligonucleotides have improved stability against enzymatic degradation,[4] rendering them the most important antisense molecules of the first generation of antisense-type drug-development candidates. The positive effects of 2′ substitutions at the sugar moiety[5–8] led to the most significant modification within the second generation of antisense drug candidates. Oligonucleotides bearing an alkyl substituent at position 2′ of the sugar moiety proved to be stable against DNA- or RNA-cleaving enzymes, owing to hindrance of nuclease attack at the phosphate groups.

Zwitterionic oligonucleotides constitute a further promising type of nucleic acid modification not yet used in clinical drug development.[9,10] It has been shown by our group and others that a decrease in the electrostatic repulsion between the polyanionic nucleic acid backbones can be achieved by the introduction of cationic groups. Because amino groups are protonated at physiological pH, they are extremely well suited for this purpose. One initial approach consisted of the conjugation of aminohexyl chains to C5 of uridine.[10] The hybridization affinity for the DNA complementary strand was increased and was shown to be largely independent of the ionic strength of the buffer solution used for the determination of melting temperatures. Our own approach was a follow-up to our work on O2′-modified oligonucleotides[5,11] and was based on the finding that a chain length of six ethylene units in a 2′-*O*-aminoalkyl group provides an optimum chance to achieve interstrand charge neutralization in addition to intrastrand interaction. A systematic study of the biophysical properties of 2′-*O*-aminohexyl-modified oligonucleotides showed a slight decrease in affinity for the complementary strand relative to unmodified control,[12] but the destabilizing effect on duplexes was significantly lower than that observed with 2′-*O*-heptyl modifications. These results prove that the decrease of net charge has favorable effects on duplex stability and can compensate for the decrease in stability due to the steric bulk of the substituent. In addition, this type of modification exhibited increased nuclease stability, with three modified nucleotides at the 5′ end of the

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oligonucleotide chain providing full protection over the course of 30 min. 2′-*O*-Aminoalkyl-modified adenosine and uridine building blocks have been used for the preparation of zwitterionic oligonucleotides by other groups as well.[13,14] Structure-based studies showed that the cationic modification prevents the binding of a metal ion that is required for the enzyme to efficiently catalyze the phosphoryl transfer reaction.[15]

Apart from their application as moieties introducing zwitterionic character into nucleotides, 2′-*O*-aminoalkyl groups have been widely used as anchors for ligand attachment.[15–17] A series of changes in molecular characteristics has been described based on the ligand approach at this and other attachment sites, which enhance interstrand binding or improve physical parameters like hydrophobicity, and pharmacokinetic parameters such as biodistribution and cellular uptake. It has been firmly established that an increase in cellular uptake of oligonucleotides can be achieved by conjugation or complexation of the oligonucleotide to poly-L-lysine.[18–21] A tail of one, two, or four lysine moieties at the 5′ end of a PNA oligonucleotide improved cellular uptake,[22] with the extent of uptake dependent on the number of conjugated lysine monomers. Similarly, we have shown that target downregulation of a phosphorothioate oligonucleotide increases when lysine[23] tails with growing length are tethered to the 3′ end.

There is abundant information that charge neutralization by the introduction of basic groups has an overall positive effect on oligonucleotide properties. With respect to the zwitterionic approach, each 2′-*O*-aminoalkyl-modified nucleotide, in which the ammonium neutralizes the phosphate, will formally leave the overall charge of the oligonucleotide unchanged. Consequently, a modification at O2′ containing two amino groups will formally contribute one positive charge per building block, in contrast to the negative charge of the native nucleotide. Provided that base-pairing and duplex-forming properties are not overly impaired, this oligonucleotide charge reversal will ultimately result in a system of positively charged oligonucleotides.

In the work reported herein, based on our experience with the zwitterionic 2′-*O*-aminohexyl modifications, we acylated this amino group with lysine and obtained 2′-*O*-lysylaminohexyl-modified amidites, which were used to synthesize a series of oligonucleotides. Each such building block has the ability to formally neutralize the negative charge of two phosphate groups. The distance between the two amino groups of lysine in this type of modification corresponds to the intrastrand distance of two phosphate groups. The position in the minor groove also allows interstrand stabilization with the negative phosphate groups of a counterstrand. Thus, the selective use of 2′-*O*-lysylaminohexyl amidites, which allow distribution of the cationic charge at any position over the whole strand, constitutes a precise tool to modify the ionic properties of nucleic acids.

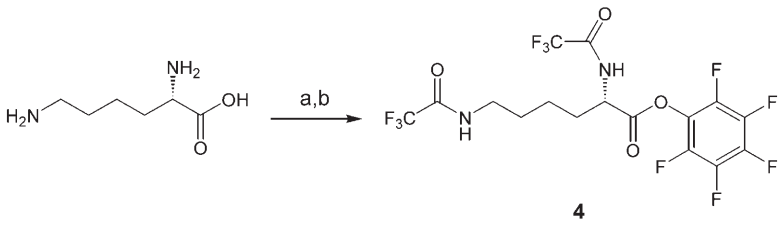
Results and Discussion

Synthesis

The synthesis of the lysyl-modified nucleoside **5** proceeded in a straightforward manner from the previously described 2′-*O*-aminohexyl nucleoside **1**.[12] The phthaloyl protecting group was removed by hydrazinolysis in boiling methanol to give free amine **2** (see Scheme 2 below). Quenching with carbonate buffer (pH 9) prevented loss of the pyrimidine nucleobase. The active ester method was used to attach the lysyl moiety. Before attachment, both lysine amino groups were protected as trifluoroacetates. This group has been shown to be well suited for oligonucleotide synthesis and may be removed under conditions used for the cleavage of exocyclic base protecting groups.[24,25] A number of methods have been reported for the trifluoroacetylation of lysine. Reaction in trifluoroacetic anhydride leads to extensive racemization,[26] whereas reaction with trifluoroacetic anhydride in dry trifluoroacetic acid under cooling selectively acylates the α-amino group.[27] Trifluoroacetic acid ethyl ester and thioethyl ester at pH 12 both react selectively at the terminal amino group.[28] To protect both amino functionalities at the same time, trifluoroacetic acid ethyl ester can be used at 50 °C. Alternatively, trifluoroacetic acid methyl ester and tetramethylguanidine yield *N*,*N*-trifluoroacetyl-L-lysine (**3**).[29] Compound **3** was prepared in 50% yield using the latter method. Pentafluorophenyl ester **4** was synthesized from **3** by using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and pentafluorophenol in dry ethyl acetate. Crystallization yielded 71% active ester **4** (Scheme 1). Addition of ester **4** to a solution of amine **2** and stirring for 18 h resulted in the formation of lysine nucleoside **5** in excellent yield (Scheme 2). Compound **5** was treated with cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to give phosphoramidite **6**.[30] After extensive drying, this nucleoside building block could be stored at −20 °C and remained stable for months.

In total, the preparation of the corresponding lysylaminohexyl phosphoramidite **6** was achieved in excellent yield despite the steric hindrance at position O3′ by the bulky O2′ substituent. This steric hindrance also had to be overcome in solid-phase oligonucleotide synthesis by prolonging coupling times to 15 min, a modification of the synthesis protocol that ultimately resulted in coupling yields of over 90%.

The use of cationic building blocks allows clear strategic planning of the positioning of cationic groups along the nucleotide strand. For an initial systematic study, the oligonucleo-



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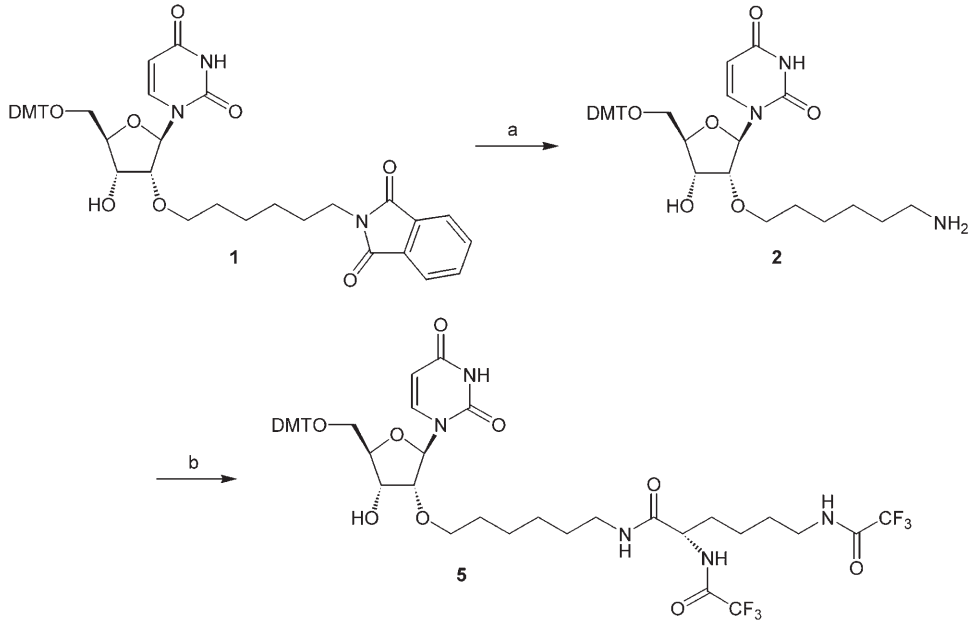
Scheme 1. Reagents and conditions: a) $F_3CCOOCH_3$, tetramethylguanidine, 0 °C, 18 h; b) pentafluorophenol, DCC, EtOAc, 0 °C, 1 h.



Scheme 2. Reagents and conditions: a) H_2NNH_2, MeOH, Δ, 4 h; b) **4**, EtOAc, room temperature, 18 h.

tides given in Tables 1 and 2 were synthesized. Up to five modified uridine nucleotides (compound **6**) were attached to the 5′ end of thymidine or uridine dodecamers (oligonucleotides **7**–**20**, Tables 1 and 2). Additionally, oligonucleotides with three and four lysylaminohexyl nucleotides distributed uniformly over thymidine dodecamers (**13** and **14**) were prepared. In contrast to mixed sequences, homopolymeric duplexes dT_n–dA_n and rU_n–rA_n give characteristic CD curves even at short length and are well suited to determine the influence a given modification has on the duplex melting temperature (T_m). The oligonucleotides obtained were purified and characterized by a standard RP HPLC method. Expected data were obtained for all oligonucleotides prepared, with retention times increasing as the number of lysylaminohexyl chains increased. Additionally, MALDI-ToF mass spectrometry confirmed successful incorporation of lysylaminohexyl building block **6**, and asserted that no cleavage of the amide bond occurred. Furthermore, no side products arising from transamination during capping or from Michael addition of the acrylonitrile generated by deprotection of the cyanoethyl groups[13] were detected.

Table 1. Duplex transition temperatures (T_m) of oligonucleotides **14**–**20** and unmodified rU_{12} with complementary RNA.

Compd	Sequence[a]	T_m [°C][b]
rU_{12}	UUUUUUUUUUUU	22.5
15	*U*UUUUUUUUUU	23.6
16	*U*U*UUUUUUUUU	24.5
17	*U*U*U*UUUUUUUU	27.5
18	*U*U*U*U*UUUUUUU	26.9
19	*U*U*U*U*U*UUUUUU	26.5
20	*U*U*U*U*U*U*UUUUU	26.6

[a] *U** = 2′-*O*-lysylaminohexyluridine. [b] Measured in 0.15 M NaCl/0.01 M Tris-HCl (pH 7.0).

Table 2. Experimental and calculated duplex transition temperatures (T_m) of oligonucleotides **7**–**14** and unmodified dT_{12} with complementary DNA.

Compd	Sequence[a]	$T_{m(exptl)}$ [°C][b]	$T_{m(calcd)}$ [°C][c]
dT_{12}	TTTTTTTTTTTT	34.7	31.0
7	*U*TTTTTTTTTT	33.6	25.1
8	*U*U*TTTTTTTTT	32.4	22.7
9	*U*U*U*TTTTTTTT	30.4	20.6
10	*U*U*U*U*TTTTTTT	28.3	18.6
11	*U*U*U*U*U*TTTTTT	26.2	16.8
12	*U*U*U*U*U*U*TTTTT	N.D.	15.2
13	TT*U*TTT*U*TTT*T	17.6	19.6
14	T*U*TT*U*TT*U*TT*T	9.5	17.7

[a] *U** = 2′-*O*-lysylaminohexyluridine. [b] Measured in 0.15 M NaCl/0.01 M Tris-HCl (pH 7.0). [c] Calculated temperatures for dT–rU hybrids were obtained with the nearest-neighbor model.

CD spectroscopy

To study the secondary structure of modified oligonucleotides, circular dichroism (CD) spectra were recorded. The CD curves of single-stranded RNA oligonucleotides **15**–**20** were not altered by the addition of 2′-*O*-lysylaminohexyl chains (Figure 1). The weak base-stacking strength of pyrimidine homopolymers leads to relatively low band intensities, which in turn are not influenced by the lysylaminohexyl chains. In contrast to that, band intensities of single-stranded DNA oligonucleotides **7**–**14** progressively decrease with increasing number of 2′-*O*-lysyla-

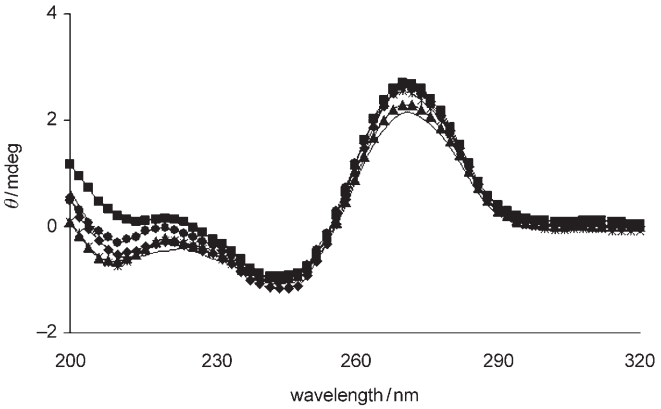


Figure 1. CD spectra of single-stranded **15**–**20.** **15**: ——, **16**: ◆, **17**: ■, **18**: ▲, **19**: ●, **20**: ∗.

minohexyl chains (Figure 2). This influence on the secondary structure of unpaired DNA oligonucleotides is apparently due to the change of the base from thymine to uracil, rather than by the basic substituents at O2′.

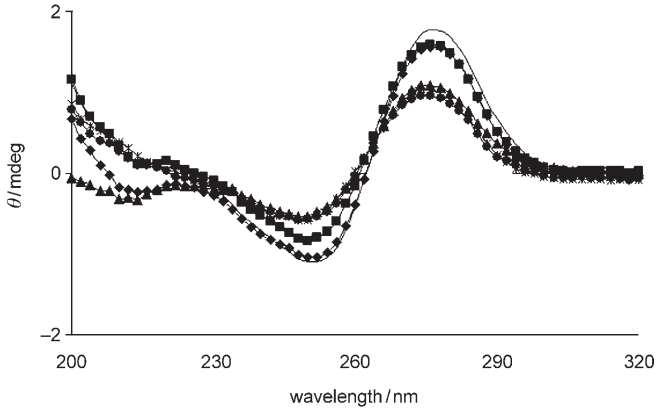


Figure 2. CD spectra of single-stranded **7**–**11**. **7**: ——, **8**: ◆, **9**: ■, **10**: ▲, **11**: ●, **12**: *.

To study the effects on the secondary structure of duplex oligonucleotides, CD spectra of an equimolar mixture of **7**–**20** and their DNA or RNA complement were recorded. Under the chosen strand and salt concentrations, dA or rA and dT or rU oligonucleotides only form duplex complexes.[31] For the formation of triplexes, a higher salt concentration and a surplus of one of the single strands is needed. Again, the rU_{12} derivatives **15**–**20** complexed to their complementary rA_{12} strand all exhibit CD curves with only minimal differences between them (Figure 3). The CD spectra are characteristic of an RNA–RNA duplex, exhibiting a strong positive band at 270 nm and a less intense negative band centered at 248 nm. The strong band in the far-UV region (205 nm) is also often displayed in A-form oligonucleotide CD curves.

The gradual changes in duplex secondary structure of DNA oligonucleotides (Figure 4) are more apparent than for single-stranded **7**–**14**. The typical double peak of dA_{12}–dT_{12} between

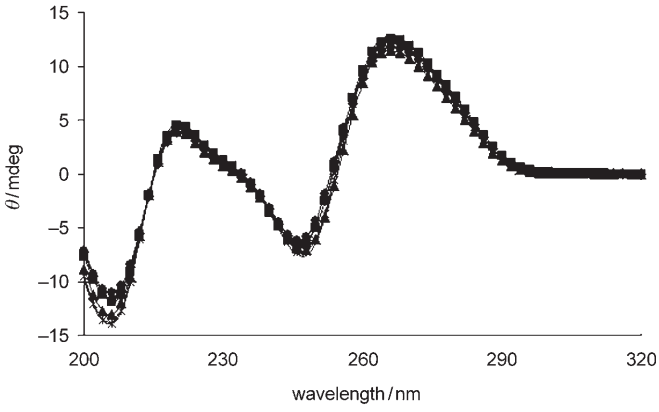


Figure 3. CD spectra of **15**–**20** with complementary RNA. **15**: ——, **16**: ◆, **17**: ■, **18**: ▲, **19**: ●, **20**: *.

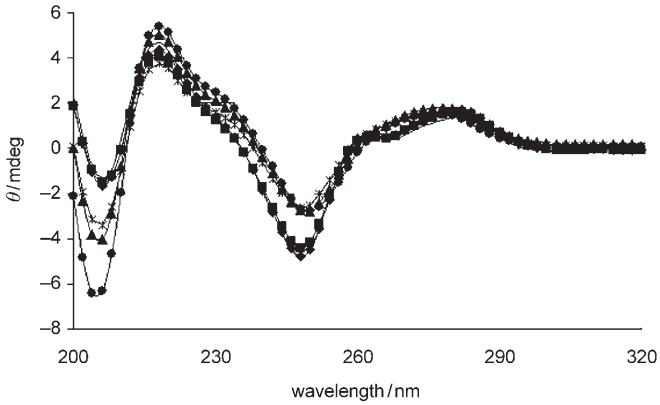


Figure 4. CD spectra of **7**–**11** with complementary DNA. **7**: ——, **8**: ◆, **9**: ■, **10**: ▲, **11**: ●, **12**: *.

300 and 260 nm, still prominently featured in the CD spectrum of **7**, gradually disappears if more than three 2′-*O*-lysylaminohexyluridine nucleotides are present. In this regard, the placement of the modified nucleotides either adjacent to each other or distributed over the strand is irrelevant. Substitution of thymidine for uridine also abolishes this double peak, but in a mixed oligonucleotide consisting of 50% dU and 50% dT, both peaks are still clearly present.[31] Likewise, a gradual substitution of thymidine by 2′-*O*-aminohexyluridine has a less pronounced effect on the CD curve.[12] Furthermore, the negative band at 248 nm steadily decreases from 4.83 mdeg for **7** over 4.40 mdeg for **9** to 2.48 mdeg for **11**. Again, the changes between **7**, **8**, **9**, **13**, and **14** are only minor, but for **10** and **11**, the band intensity is decreased by nearly 50% relative to control dA_{12}–dT_{12}. The negative band between 210 and 200 nm, a typical feature of A-form nucleic acids, shows an explicit jump in intensity between oligonucleotides **10** and **11**. Summarizing these changes, the CD spectrum of dA_{12}–dT_{12}, a classical B-DNA-type curve, shifts towards a spectrum that is a mixture of A- and B-form DNA.

The changes caused by the incorporation of 2′-*O*-lysylaminohexyluridine nucleotides in deoxythymidine homododecamers are not only due to the group at O2′, but may also originate from the nucleobase change. Alterations caused by the presence (thymine) or absence (uracil) of the 5-methyl group have been examined thoroughly and are well described in the literature.[31,32] In terms of T_m, this difference results in a mean decrease of about 0.5 °C per modification.[31] Some changes in the CD curve occur, predominantly in the region between 260 and 290 nm. In addition, the 2′-hydroxy function considerably changes the ribose conformation and consequently, the oligonucleotide characteristics. 2′-Deoxyribonucleic acids generally adopt a C2′-*endo* (southern) conformation while ribonucleic acids prefer the C3′-*endo* (northern) sugar pucker. These differences are reflected in different helical properties and often in changes in duplex stability. DNA usually shows B-form characteristics, while RNA favors the A-form. 2′-*O*-Alkyl-modified oligonucleotides have been shown to adopt the C3′-*endo* conformation and consequently the A-form.[13,33] NMR data of phosphoramidite building block **5** strongly suggest a C3′-*endo* con-

formation. The H1′–H2′ coupling constant of only 3.5 Hz together with the shifts of the ribose signals is an indication of this sugar conformation.

Melting behavior

CD and UV melting curves were recorded to quantify duplex stability of all modifications prepared. For all oligonucleotides tested except for the fully neutralized DNA–RNA hybrid **12**, sigmoid curves resulted, indicating that only the transition from double-stranded to single-stranded oligonucleotides occurred. For the RNA derivatives **15**–**20**, a slight but gradual increase in T_m from 22.5 to 27 °C was found (Figure 5 and Table 1). With

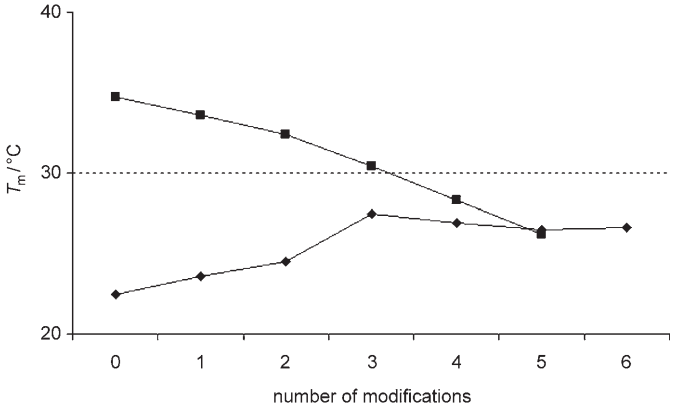


Figure 5. Influence of 2′-*O*-lysylaminohexyl chains on the melting temperature of DNA (dT_{12}–dA_{12}, ■) and RNA (rU_{12}–rA_{12}, ◆) oligonucleotides.

the incorporation of three cationic nucleotides (in **17**), a plateau was reached, and more modified building blocks had no further influence on the denaturation temperature. Two opposing effects influence duplex stability: the steric bulk of the substituent at O2′ lowers the T_m, whereas the cationic amines decrease the electrostatic repulsion between the two strands, leading to an increase in the denaturation temperature. 2′-*O*-Alkyl nucleotides have been shown to destabilize duplexes with increasing length. For 2′-*O*-hexadecyl-modified oligonucleotides with similarly bulky substituents, T_m values were over 4 °C lower per modification than for the unmodified control.[33]

For **7**–**11**, a considerable decrease in T_m was observed with increasing number of lysylaminohexyl-modified nucleotides (Figure 5 and Table 2). Despite the greater steric bulk of the lysylaminohexyl side chains, the extent of T_m decrease is very similar to that of the aminohexyl series.[12] The duplex destabilization per modification amounts to 1.7 °C for an oligonucleotide with five lysylaminohexyl-modified nucleotides compared with 1.5 °C for the aminohexyl series. In both cases, duplexes are much more stable than those containing oligonucleotides with 2′-*O*-alkyl substitutions.[33] For a proper comparison, expected T_m values of rU–dT mixed sequences with the complementary dA_{12} were calculated using nearest-neighbor parameters deducted from the literature.[34] These calculations take the destabilizing effects of uridine substitution and adverse sugar conformation into account. In contrast to what is expected for RNA–DNA hybrids, the first two modified nucleotides only had a minor effect on duplex stability, whereas the incorporation of additional 2′-*O*-lysylaminohexyl nucleotides led to a decrease of ~2 °C. This shows that the lysylaminohexyl chains actually stabilize the duplex, but the RNA-like character of the nucleotide used for attachment significantly lowers the affinity for the counter-strand.

For **13** and **14**, in which the modified nucleotides are distributed uniformly along the oligonucleotides, the T_m values were significantly lower than for **9** and **10**, which respectively have the same number of consecutive modifications at the 5′ terminus. They are also significantly lower than the calculated values. Clearly, isolated RNA-like nucleotides and a scattered bulk of lysylaminohexyl chains in the minor groove have a more pronounced impact on duplex stability than accumulated substituents.

Enzymatic degradation

The biological stability of the modified oligonucleotides was investigated using 5′-exonuclease isolated from calf spleen. No degradation of test oligonucleotides **7** and **9** was detected after incubation for 30 min with 200 μU 5′-exonuclease, indicating that as little as one terminal modification is sufficient for effective protection against enzymatic degradation. Unmodified T_{12} is cleaved to a high extent (90%) under these conditions, and a derivative of **7** with 2′-*O*-aminohexyl chains in place of the 2′-*O*-lysylaminohexyl groups is still considerably unstable, with 15% degradation.[12] The extent of protection is superior to that observed with the zwitterionic aminohexyl modifications, for which three 5′-terminal modifications are necessary to achieve a similar protection against exonuclease activity.[12]

In vitro target downregulation

For an initial assessment of the influence of 2′-*O*-lysylaminohexyl nucleotides on in vitro efficacy, we prepared a series of DNA and RNA oligonucleotides targeted at the intracellular adhesion molecule ICAM-1 (Table 3). The sequences of both the antisense and the siRNA molecules were taken from the literature. The respective phosphorothioate antisense oligonucleotide has been used in several clinical trials and is also known under the name alicaforsen. It is currently being developed as an enema formulation for the treatment of Crohn's disease and ulcerative colitis.[35] The wild-type siRNA sequence used in this study has been shown to silence the expression of ICAM-1 in primary cultures of human venous endothelial cells.[36–38]

We incorporated one, two, and three modified uridine nucleotides into antisense and siRNA agents at the positions indicated in Table 3. The human cell line ECV304, which has been shown to express ICAM-1,[39,40] was selected for gene knockdown studies. All oligonucleotides were complexed to the transfection agent lipofectamine 2000, likely abolishing any possible differences in cell membrane permeation of wild-type and modified oligonucleotides. Although improved cellular uptake may result from incorporation of the cationic nucleo-

Table 3. Sequences of antisense and siRNA oligonucleotides targeted at ICAM-1 and used for in vitro testing.

Compd		Antisense (phosphorothioate backbone)[a]
21		GCCCAAGCTGGCATCCGTCA
22		GCCCAAGC*U**GGCATCCGTCA
23		GCCCAAGC*U**GGCATCCG*U**CA
24		GCCCAAGC*U**GGCA*U**CCG*U**CA
Compd	siRNA[a]	
25	antisense:	5′-UAGAGGUACGUGCUGAGGCdTdT-3′
	sense:	3′-dTdTAUCUCCAUGCACGACUCCG-5′
26	antisense:	5′-UAGAGGUACG*U**GCUGAGGCdTdT-3′
	sense:	3′-dTdTAUCUCCA*U**GCACGACUCCG-5′
27	antisense:	5′-UAGAGG*U**ACGUGC*U**GAGGCdTdT-3′
	sense:	3′-dTdTAU*C**U**CCAUGCACGAC*U**CCG-5′
28	antisense:	5′-*U**AGAGG*U**ACGUGC*U**GAGGCdTdT-3′
	sense:	3′-dTdTA*U**CUCCA*U**GCACGAC*U**CCG-5′

[a] *U** = 2′-*O*-lysylaminohexyl nucleotide.

tides, the number of lysine chains used in this study is supposedly not sufficient to effectively ensure membrane passage without the help of a transfection agent. The extent of target downregulation was assessed with western blot 24 h after transfection. ICAM-1 protein levels were quantified by densitometry and normalized to the corresponding actin bands. Scrambled control oligonucleotides had no significant effect.

For both antisense and siRNA, the in vitro effect was dependent on the number of cationic nucleotides. For antisense agents **21**–**24**, considerable differences in ICAM-1 levels were detected (Figure 6). Surprisingly, **22**, with one thymidine nucleotide substituted for the modified uridine building block, had only minimal effect on ICAM-1 expression. Modifications at O2′ impede RNAse H mediated degradation when located at internal sites as in oligonucleotide **22**, which may explain the low antisense effect. However, with more heavily modified nucleotides, the gene-silencing effect became more pronounced, and the oligonucleotide with three lysine chains even surpasses the effect of phosphorothioate **21** ($p < 0.05$ for 200 nM). Because the melting temperature mirroring the mRNA target affinity is lowered by the incorporation of 2′-*O*-lysylaminohexyluridines, it can be speculated that the increasing effect is due to some enhancement of pharmacokinetic properties such as increased cellular uptake, higher re-release from the endosome, or higher stability against enzymatic degradation. While it seems unlikely that cellular uptake is increased when used in conjunction with a transfection agent, the higher nuclease stability certainly plays a role. In addition, a higher rate of endosomal escape due to weaker interaction with the cationic liposomes might be suspected.

For siRNA sequences **25**–**28**, the variations of the target protein levels were smaller. Again, when only one lysylaminohexyl nucleotide was present in each of the two strands (duplex **26**), the observed gene silencing effect was significantly lower relative to wild-type RNA (duplex **25**) at a concentration of 50 nM (Figure 7). No significant difference was observed between **27** and **28**, which respectively have two and three modified nucleotides in each strand. In siRNA, the effect of modified nucleotides depends on their position within the strand. The siRNA effector molecule RISC incorporates the double-stranded RNA and elects one of the two strands as guiding or antisense

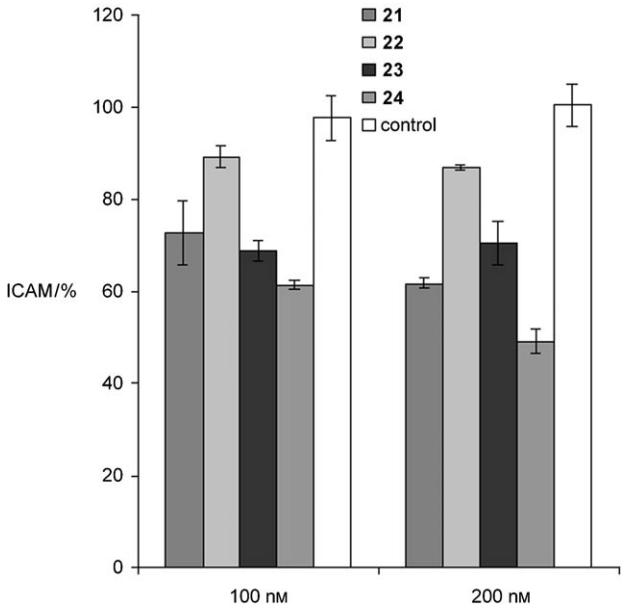


Figure 6. Downregulation of ICAM-1 by antisense oligonucleotides with up to three 2′-*O*-lysylaminohexyl modifications (**22**–**24**) compared with unmodified **21**. ICAM-1 protein levels were determined 24 h after transfection of the human endothelial cell line ECV304. ICAM-1 downregulation is reported relative to mock transfected cells (100%); control is a scrambled non-hybridizing phosphorothioate oligonucleotide. Reported values are the means of at least triplicate experiments; error bars represent the standard deviation.

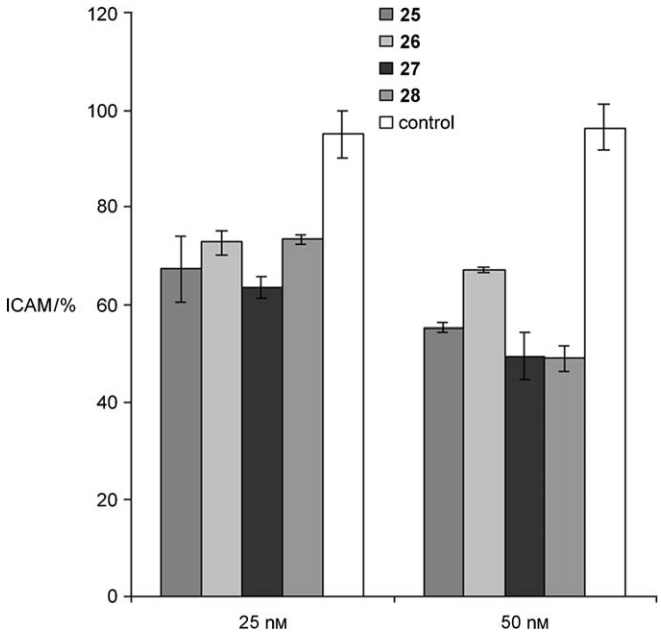


Figure 7. Downregulation of ICAM-1 by siRNA oligonucleotides with up to three 2′-*O*-lysylaminohexyl modifications (**26**–**28**) compared with unmodified **25**. ICAM-1 protein levels were determined 24 h after transfection of the human endothelial cell line ECV304. ICAM-1 downregulation is reported relative to mock transfected cells (100%); control is a scrambled non-hybridizing double-stranded RNA oligonucleotide. Reported values are means of at least triplicate experiments; error bars represent the standard deviation.

strand based on the thermodynamic profile. It has been shown that the relative duplex stability of the terminal two to five nucleotides is crucial for the selection of the guiding strand.[41] In that respect, oligonucleotide **28** is predicted to be poorly suited for gene silencing, because the two nucleotides at the 5′ end of the antisense strand are substituted for the modified uridines, which have a slightly higher affinity to the counter-strand. Nevertheless, the effect of **28** is not significantly different from that of **27**, indicating that the adverse effect due to lower RISC strand bias is countered by a positive effect, possibly by higher resistance against degradation or higher cytosolic availability.

These findings highlight the fact that not only the extent of charge neutralization is important, but also the localization in the particular sequence. A broader analysis including diverse sequences and targets, and a careful optimization of the number and positioning of the 2′-*O*-lysylaminohexyl modifications is necessary for a detailed assessment of the value of these modifications for nucleic acid based drug development. For an in-depth investigation of a possible enhancement in cellular uptake or endosomal escape, the development of other modified nucleotide building blocks is required to be able to introduce a greater number of lysylaminohexyl chains into a functional oligonucleotide sequence.

Conclusion

Oligonucleotides containing 2′-*O*-lysylaminohexyl nucleotides have the potential to neutralize not only one, but two phosphate moieties per modification and consequently allow for a precise modulation of the overall charge of single-stranded antisense or double-stranded siRNA oligonucleotides, as well as the exact charge allocation in the strand. 2′-*O*-Lysylaminohexyl nucleotides increase duplex stability in RNA duplexes, whereas in DNA duplexes, an increasing number of modified nucleotides consecutively lowers the duplex stability, apparently caused by the RNA characteristic of the O2′-modified building block. This indicates that the cationic 2′-*O*-lysylaminohexyl nucleotides are especially suited for use in siRNA, but the incorporation of several modified nucleotides at the 5′ end was also well tolerated in DNA oligonucleotides, effectively preventing nuclease attack. In vitro data prove that the incorporation of several 2′-*O*-lysylaminohexyl nucleotides in an antisense or siRNA oligonucleotide is possible without the loss of, or even achieving higher, gene-silencing activity. These findings indicate that the cationic nucleotide modifications presented herein might be a useful approach for the development of nucleic acid based drugs.

Experimental Section

Reagents for organic syntheses were purchased from Merck or Aldrich in standard quality and were used without purification. Reagents for oligonucleotide syntheses were purchased from Carl Roth GmbH (Karlsruhe, Germany). Melting points were measured in a Büchi melting point apparatus and are uncorrected. Anhydrous solvents were obtained as follows: THF was held at reflux on sodium and then distilled; pyridine, CH_2Cl_2, and triethylamine (TEA) were distilled from CaH. For DNA synthesis, CH_3CN was heated over CaH and distilled. Purified H_2O was obtained from a Milli-Q apparatus. NMR spectra were recorded on a Bruker AC-200 MHz. Shifts are reported relative to the solvent peak ($CHCl_3$ in $CDCl_3$: $\delta=$ 7.26 ppm (^{1}H) and 77.00 ppm (^{13}C), DMSO in [D$_6$]DMSO: $\delta=$ 2.50 ppm (^{1}H) and 39.50 ppm (^{13}C)), coupling constants are in Hz. Thin-layer chromatography (TLC) was performed on silica gel 60-F_{254} precoated aluminum plates from Merck. Column chromatography was performed with Merck silica gel 60. Elemental analyses were done at the Institut für Organische Chemie der Johann Wolfgang Goethe-Universität (Frankfurt am Main, Germany) with a Heraeus CHN Rapid instrument.

2′-*O*-(6-Aminohexyl)-5′-*O*-(dimethoxytrityl)uridine (2): 5′-Dimethoxytrityl-2′-(6-phthaloylaminohexyl)uridine (**1**, 4.0 g, 5.16 mmol)[12] was suspended in dry MeOH (20 mL), and hydrazine hydrate (1.0 mL, 80%) was added. The mixture was heated at reflux for 4 h. After cooling, $NaHCO_3/K_2CO_3$ buffer (20 mL, pH 9.0) was added, and MeOH was removed in vacuo. The aqueous solution was extracted with CH_2Cl_2. The organic phase was washed with buffer, dried with $NaSO_4$, and the solvent was removed. Vacuum drying afforded free amine **2** (3.10 g, 92%) as a yellowish foam that was used without purification. ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 7.72 (d, $J=$8.0 Hz, 1H, H-6), 7.40–6.80 (m, 13H, DMT), 5.80 (d, $J=$ 2.90 Hz, 1H, H-1′), 5.28 (d, $J=$8.0 Hz, 1H, H-5), 4.14 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.88 (m, 1H, H-2′), 3.73 (s, 6H, OCH_3), 3.56 (m, 2H, OCH_2), 3.35–3.15 (m, 2H, H-5′), 1.55–1.40 (m, 2H, CH_2N), 1.40–1.15 ppm (m, 8H, CH_2); ^{13}C NMR ([D$_6$]DMSO): $\delta=$163.34 (C4), 158.02 (DMT), 150.43 (C2), 144.46 (DMT), 140.00 (C6), 135.24 (DMT), 134.98 (DMT), 129.66 (DMT), 127.81 (DMT), 127.59 (DMT), 126.74 (DMT), 113.15 (DMT), 101.44 (C5), 86.88 (C1′), 85.83 (DMT), 82.58 (C4′), 80.78 (C2′), 69.74 (OCH_2), 68.30 (C3′), 62.60 (C5′), 54.95 (OCH_3), 41.02 (CH_2), 32.69, 28.90, 26.03, 25.17 and 20.27 ppm (5× CH_2); ESIMS: 646.6 [*M*+1] $C_{36}H_{43}N_3O_8$: calcd C 66.96, H 6.71, N 6.51; found C 66.81, H 6.73, N 6.35; DMT = dimethoxytrityl.

***N*$^{\alpha}$,*N*$^{\varepsilon}$-Bis(trifluoroacetyl)-L-lysine (3)**: Under Ar atmosphere, L-lysine hydrochloride (20.0 g, 0.11 mol) was mixed with trifluoroacetic acid methyl ester (70.4 g, 0.55 mol) and cooled to 0 °C. *N*,*N*,*N*′,*N*′-Tetramethylguanidine (38.0 g, 0.33 mol) was added, and the mixture was stirred for 18 h. The resulting emulsion was dried in vacuo and the residue was dissolved in an ice–H_2O mixture. HCl was added, and the solution was extracted with EtOAc. Collected organic phases were dried with $MgSO_4$, and the solvent was removed in vacuo. Crystallization was done from a mixture of $CHCl_3$, EtOH, Et_2O, and petrol ether and gave product **3** (18.55 g, 50%) as colorless crystals; fp = 111–112 °C; $[\alpha]=-6.85$ ($c=$2, EtOH); ^{1}H NMR ([D$_6$]acetone): $\delta=$8.80–8.30 (m, 2H, NH), 4.57–4.49 (m, 1H, H-2), 3.43–3.36 (m, 2H, H-6), 2.05–1.80 (m, 2H, H-3), 1.75–1.45 ppm (m, 4H, H-3, H-4); ^{13}C NMR ([D$_6$]acetone): $\delta=$172.05 (C1), 157.77 (q, $J=$ 36.2 Hz, $COCF_3$), 157.59 (q, $J=$36.2 Hz, $COCF_3$), 117.15 (q, $J=$ 285.5 Hz, CF_3), 116.97 (q, $J=$285.5 Hz, CF_3), 53.37 (C2), 39.87 (C6), 31.09 (C3), 28.96 (C5), 23.68 ppm (C4); ESIMS: 339.1 [*M*+1].

***N*$^{\alpha}$,*N*$^{\varepsilon}$-Bis(trifluoroacetyl)-L-lysinepentafluorophenylester (4)**: A mixture of *N*,*N*′-dicyclohexylcarbodiimide (3.72 g, 18 mmol) and pentafluorophenol (3.65 g, 19.8 mmol) in dry EtOAc (60 mL) was stirred for 10 min at 0 °C. A cooled solution of bis(trifluoroacetyl)-L-lysine **3** (6.1 g, 18 mmol) in EtOAc was added, and the resulting mixture was stirred for 1 h at 0 °C. Precipitated dicyclohexylurea was filtered off and washed with EtOAc. The filtrate was dried in vacuo, and the residue was dissolved in a small amount of EtOAc and crystallized in the refrigerator. Filtration afforded ester **4** (6.41 g, 71%) as colorless crystals. ^{1}H NMR ([D$_6$]DMSO): $\delta=$11.02

(m, 1H, NH), 9.04 (m, 1H, NH), 6.52 (m, 1H, H-2), 2.83 (m, 2H, H-6), 2.27 (m, 2H, H-3), 1.30–0.95 ppm (m, 4H, H-4, H-5); ESIMS: 520.2 [M+NH_3], 503.0 [M−1].

2′-*O*-{6-[Bis(trifluoroacetyl)-L-lysyl]aminohexyl}-5′-*O*-(dimethoxytrityl)uridine (5): 2′-*O*-Aminohexyl-modified nucleoside **2** (1.40 g, 2.16 mmol) was dissolved in dry EtOAc (25 mL), and lysine ester **4** (1.10 g, 2.16 mmol) was added. The mixture was stirred at room temperature for 18 h. Solvent was removed in vacuo, and the residue was purified by flash chromatography using CH_2Cl_2/EtOH 20:1 as mobile phase. Evaporation of the respective fractions gave product **5** (1.80 g, 86%) as colorless foam. ^{1}H NMR ([D_6]DMSO): δ = 11.40 (bd, J = 1.8 Hz, 1H, NH), 9.49 (d, J = 7.9 Hz, 1H, NH), 9.40 (bt, J = 5.3 Hz, 1H, NH), 7.74 (d, J = 8.1 Hz, 1H, H-6), 7.40–6.80 (m, 9H, DMT), 6.90 (d, J = 8.8 Hz, 4H, DMT), 5.80 (d, J = 3.5 Hz, 1H, H-1′), 5.28 (dd, J = 2.0, 8.0 Hz, 1H, H-5), 5.14 (d, J = 6.6 Hz, 1H, OCH_3), 4.18 (m, 2H, H-2′, H-2_{Lys}), 3.97 (m, 1H, H-4′), 3.90 (m, 1H, H-3′), 3.74 (s, 6H, OCH_3), 3.56 (m, 2H, OCH_2), 3.37–3.00 (m, 6H, H-5′, H-6_{Lys}, CH_2N), 1.68 (m, 2H, H-3_{Lys}), 1.60–1.20 (m, 12H, H-4_{Lys}, H-5_{Lys}, 4× CH_2); ^{13}C NMR ([D_6]DMSO): δ = 169.74 (CO), 162.94 (C4), 158.13 (DMT), 156.27 (q, J = 36.5 Hz, $COCF_3$), 156.10 (q, J = 35.6 Hz, $COCF_3$), 150.25 (C2), 144.61 (DMT), 140.17 (C6), 135.33 and 135.07 (DMT), 129.74, 127.85, 127.68 and 126.76 (DMT), 115.93 (q, J = 286.2 Hz, CF_3), 115.80 (q, J = 286.1 Hz, CF_3), 113.22 (DMT), 101.43 (C5), 87.15 (C1′), 85.87 (DMT), 82.60 (C4′), 80.88 (C2′), 69.83 (OCH_2), 68.47 (C3′), 62.61 (C5′), 55.00 (OCH_3), 53.28 (*C*HCO), 38.91 and 38.49 (CH_2N), 30.67, 29.04, 28.86, 27.68, 26.09, 25.05, 22.68 ppm (CH_2); ESIMS: 966.2 [M+1] $C_{46}H_{53}F_6N_5O_{11}$·0.14 CH_2Cl_2: calcd C 56.68, H 5.49, N 7.16; found C 56.72, H 5.60, N 6.96.

2′-*O*-{6-[Bis(trifluoroacetyl)-L-lysyl]aminohexyl}-3′-*O*-[(2-cyanoethoxy)-(diisopropylamino)phosphanyl]-5′-*O*-(dimethoxytrityl)uridine (6): Dried uridine derivative **5** (1.0 g, 1.03 mmol) was dissolved under moisture-free conditions in a mixture of ethylenediisopropylamine (530 mg, 4.12 mmol) and dry CH_2Cl_2 (10 mL). Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite was added dropwise, and the mixture was stirred for 3 h at room temperature. The solvent was removed in vacuo, and the residue was purified by MPLC using a linear gradient of petrol ether/EtOAc (1:3→0:1) with 2% TEA. Evaporation of the respective fractions gave diastereomeric phosphoramidite **6** (1.19 g, 98%) as a colorless foam. The proportion of diastereomers as judged by ^{1}H NMR was 35:65. ^{1}H NMR ($CDCl_3$): δ = 9.54 (bs, NH), 8.01 (d, J = 9.8 Hz, 0.35H, H-6), 7.92 (d, J = 8.1 Hz, 0.65H, H-6), 7.59 (m, 1H, NH), 7.42–7.20 (m, 9H, DMT), 7.14 (m, 1H, NH), 6.82 (m, 4H, DMT), 6.00 (d, J = 3.4 Hz, 0.65H, H-1′), 5.95 (d, J = 2.5 Hz, 0.35H, H-1′), 5.24 (m, 1H, H-5), 4.65–3.20 (m, 20H, H-2′, H-3′, H-4′, H-5′, OCH_3, OCH_2, $CH(CH_3)_2$, H-2_{Lys}, H-6_{Lys}), 2.74, 2.63 and 2.44 (3 m, 4H, OCH_2CH_2CN), 1.80–1.00 ppm (m, 36H, H-3_{Lys}, H-4_{Lys}, H-5_{Lys}, CH_2, CH_3); ^{13}C NMR ($CDCl_3$): δ = 169.94 (CO), 163.34 (C4), 158.79 (DMT), 157.25 (q, J = 36.4 Hz, $COCF_3$), 150.49 (C2), 144.32 and 144.18 (DMT), 140.12 and 140.06 (C6), 135.99 and 135.08 (DMT), 130.23, 128.22, 127.99 and 127.24 (DMT), 117.79 and 116.85 (CN), 115.98 (q, J = 279.0 Hz, CF_3), 113.28 (DMT), 102.44 (C5), 87.80 and 87.57 (C1′), 87.23 and 87.08 (CPh_3), 82.83 (C2′), 82.48 (C4′), 70.81 (OCH_2), 69.07 (d, J = 14.9 Hz, C-3′), 61.78 and 61.68 (C5′), 58.21 and 58.11 (OCH_2), 55.24 (OCH_3), 53.00 (CHCO), 45.40 and 45.28 (CH_2OP), 43.32 and 43.21 ($CH(CH_3)_2$), 39.59 and 39.28 (CH_2N), 32.02, 29.09, 28.92, 28.06, 26.11, 25.34, 24.70, 24.57 and 24.44 (CH_2), 22.99 and 22.94 (CH_3), 22.86 (CH_2), 20.22 ppm (CH_2CN); ^{31}P NMR ($CDCl_3$): δ = 151.00 and 150.67 ppm; ESIMS: 1165.5 [M−1] $C_{55}H_{70}F_6N_7O_{12}P$: calcd C 56.65, H 6.05, N 8.41; found C 56.84, H 6.53, N 7.96.

Synthesis of oligonucleotides

Oligonucleotides were prepared on an ABI 392B DNA synthesizer at the 1.0-μM scale according to the recommended standard synthesis protocol. Tetrazol (0.45 M) was used as activator, and iodine (0.1 M) in py/THF/H_2O, as oxidizing reagent. Capping was done before oxidation. 2′-*O*-Aminohexyl-modified nucleotides were dissolved in anhydrous CH_3CN to give 0.1 M solutions. Coupling of 2′-modified phosphoramidites was achieved with an extended coupling time of 15 min, which resulted in an average yield of 92%, determined by trityl cation assay. Oligonucleotides were prepared in DMT-off mode and cleaved from solid support using concentrated ammonia (1 h, room temperature). Deprotection was afforded by heating the resulting solution at 55 °C for 18 h. Ammonia was removed in vacuo, and the residue was redissolved in 0.5 mL H_2O.

HPLC analysis and purification

Analytical HPLC of a 20-μL sample (0.2 mg mL^{-1}) was performed on an ET 250/8/4 Nucleosil 100-5 C_{18} column (Macherey–Nagel) at a flow rate of 1 mL min^{-1} and a linear gradient of 10→40% B over 30 min (A: 0.1 M triethylammonium acetate in H_2O, B: 0.1 M triethylammonium acetate in 80% CH_3CN). Preparative HPLC was done on an Aquapore Octyl Prep 20 cartridge 250×10 mm (Applied Biosystems) at a flow rate of 4 mL min^{-1} and the same gradient as mentioned above.

CD spectroscopy

Concentrations of purified and desalted oligonucleotides were determined by UV/Vis at λ = 260 nm. Molar extinction coefficients were calculated by addition of nucleotides (ε = 184 800 cm^{-1} M^{-1} for homoadenosine dodecamers, ε = 105 600 cm^{-1} M^{-1} for uridine/thymidine dodecamers). CD spectra were recorded on a Jasco J-710 spectropolarimeter. Oligonucleotides were diluted to a concentration of 9 μM in a solution of 0.15 M NaCl and 0.01 M Tris-HCl (pH 7.0) in a total volume of 200 μL. Complementary strands were hybridized for 5 min at 80 °C, then cooled to room temperature to ensure duplex formation. Measurements were done in a quartz cuvette with a path length of 1 mm. The wavelength range was set to 320–200 nm with a scanning speed of 50 nm min^{-1}. For determination of T_m values, the duplex solution was heated from 0 to 80 °C with a slope of 50 °C h^{-1}. Reported denaturation temperatures are the mean values of triplicate experiments with SD < 1.0 °C.

Nuclease stability test

PDE incubation buffer was added to a solution of uridine/thymidine dodecamer (2.71 nmol, 0.33 OD) in an Eppendorf cap 25 μL. The solution was diluted with Milli-Q purified H_2O to a total volume of 240 μL. After 10 min incubation at 37 °C, 10 μL (200 μU) phosphodiesterase solution (calf spleen, Boehringer Mannheim, 4 U mL^{-1}, diluted 1:200) was added. After mixing briefly, the resulting solution was incubated at 37 °C for 30 min. PDE stop buffer (50 μL) was added, and the mixture was heated at 90 °C for 10 min. Quantitation of degradation was done using HPLC as described above.

In vitro ICAM-1 downregulation

The human cell line ECV304 was obtained from the European Collection of Cell Cultures (Wiltshire, UK). Cells were cultured in a 1:1 mixture of C6 medium and astrocyte conditioned medium (ACM).[42] C6 medium consists of a 1:1 mixture of IMDM (Gibco, Invitrogen, Carlsbad, CA, USA) and Ham's F-12 (Gibco), supplemented with 7.5% NBS (Gibco), 7 mM L-glutamine (Gibco), 5 $\mu g\,mL^{-1}$ transferrin (Sigma), 5 $\mu g\,mL^{-1}$ (0.5 $U\,mL^{-1}$) heparin (MP Biomedicals, OH, USA), 1% (100 $U\,mL^{-1}$, 100 $\mu g\,mL^{-1}$) penicillin/streptomycin (Gibco), and 0.1% (0.25 $\mu g\,mL^{-1}$) amphotericin B (Sigma). For the production of ACM, C6 cells derived from rat glioma, obtained from the German Cancer Research Center Heidelberg (DKFZ, Heidelberg, Germany), were grown in gelatin-coated tissue flasks (Greiner bio-one GmbH, Kremsmünster, Austria; gelatin from Sigma) in C6 medium. The supernatant of C6 cultures was collected every other day.

ECV304 were seeded on gelatin-coated six-well plates (Falcon, Bedford, MA, USA) 24 h before transfection. Oligonucleotides were complexed to lipofectamine 2000 according to the manufacturer's instructions. Phosphorothioate backbone antisense agents were used at final concentrations of 100 and 200 nM, and siRNA at concentrations of 25 and 50 nM. Scrambled control oligonucleotides were GACGCATCGCGCCTACATCG (antisense) and GGUCAGACGAGUGAGUUCGdTdT (siRNA, antisense strand) with CGAACUCACUCGUCUGACCdTdT (siRNA, sense strand). Oligonucleotide lipoplexes were added to the cells, and after 4 h, medium containing NBS was added to give a 5% NBS concentration. Cells were lysed in 8 M urea buffer 24 h after transfection. ICAM-1 protein levels were quantified by western blotting with actin as internal standard and correlated to mock transfected cells. Antibodies were from Santa Cruz (ICAM-1, HRP-conjugated secondary antibodies) and Sigma (actin); blots were visualized by chemiluminescence using ECL Plus and Hyperfilm (both GE Healthcare, UK). Exposed films were scanned with a G-710 gel scanner (Bio-Rad, CA, USA) and quantified with Quantity One software (Bio-Rad). All experiments were conducted at least in triplicate. Significance levels were calculated using analysis of variance (one-way ANOVA).

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Keywords: antisense agents · drug delivery · medicinal chemistry · oligonucleotides · siRNA agents

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