

Biological and physicochemical characterization of siRNAs modified with 2',2'-difluoro-2'-deoxycytidine (gemcitabine)^{†‡}

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The use of synthetic short interfering RNAs (siRNAs) is currently a method of choice to manipulate gene expression in mammalian cells. Efforts aimed at improving siRNA biological activity, including increased silencing properties, higher substrate specificity and cellular stability, lower cytotoxicity, and improved target delivery, have been made through the introduction of various chemical modifications into the siRNA strands. In these studies, we present the synthesis of oligoribonucleotides with the single replacement of a cytidine unit for 2',2'-difluoro-2'-deoxycytidine (gemcitabine, dFdC) and the use of them in a series of siRNAs for gene silencing experiments. The dFdC modifications are located in six different positions of the antisense strand, which are crucial for siRNA silencing activity. The results indicate a position-dependent tolerance for the dFdC modification. Gemcitabine units present in the “seed region”, at positions 1 or 8, resulted in only a ~15% silencing activity in the corresponding duplexes. The dFdC unit at position 10 virtually switched off the silencing activity (below 10%), while the dFdC unit at the positions 2, 4 or 5 produced duplexes of silencing potential comparable to that of the non-modified duplex (70% silencing). The dFdC modification had little impact on the structure of the siRNA duplexes, as determined by circular dichroism analysis, while melting experiments showed their lower thermal stability.

1. Introduction

RNA interference (RNAi) is a powerful biological process for the sequence-specific silencing of gene expression in diverse eukaryotic cells. RNAi has tremendous potential for functional genomics and the development of novel gene-specific therapies.^{1–3} Specific gene silencing may be induced by synthetic short interfering RNAs, which are 21–23-nucleotide (nt) long duplexes consisting of sense (non-guide, passenger) and antisense (guide) complementary strands, with two unpaired nucleotides (2-nt overhangs) at each 3'-end (Fig. 1). siRNA duplexes are designed to mimic the RNase III processing intermediates of naturally-expressed dsRNAs, such as miRNAs, to effectively enter the RNAi pathway.^{4–7} Naturally processed siRNAs or miRNAs are characterized by the presence of 5'-phosphates and 3'-terminal overhangs, which are important for their biological activity.^{8,9} Synthetic siRNA duplexes with 5'-hydroxyl ends are rapidly phosphorylated inside cells by the cellular kinase Clp1.¹⁰ An siRNA duplex

molecule, after introduction into the cell, is complexed by a nucleoprotein effector complex RISC (RNA-induced silencing complex). Then, one of the two strands works as the guide strand and directs the complex to a complementary sequence of the target mRNA, while the other one is endonucleolytically degraded during the assembly process.^{11,12} The core of the effector complex contains one of the Argonaute (Ago) proteins, which are members of the PAZ/PIWI family.^{13,14} Depending on the nature of the Ago protein and on the degree of complementarity between the siRNA and the target sequence, the association of human RISC with mRNA results in different silencing modes, as endonucleolytic cleavage, translational repression, deadenylation or sequestration to the P-body compartments.¹⁵ Among the four Ago proteins coded in the human genome (hAgo1–4), only hAgo2 is endonucleolytically active.^{14,16,17} Ago proteins are composed of four functional domains: amino-terminal N, PAZ, and C-terminal MID and PIWI. The catalytic site of the slicer is situated at the PIWI domain, the MID domain recognizes and anchors the 5'-phosphate of the guide strand, whereas the 2-nt overhang at the 3'-end is recognized by the PAZ domain.^{18–21} Ago-mediated target mRNA cleavage requires Watson–Crick base pairing between the guide strand of siRNA and the target strand, spanning both the seed region (positions from 2nd to 8th nucleotide) and the cleavage site (phosphate group opposite to the internucleotide linkage located between 10th and 11th nucleotide, counted from the 5'-end of the guide strand).⁴ Recently, Wang *et al.* reported structural studies that reveal the molecular dynamics

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[†] This paper is dedicated to Professor Wojciech J. Stec on the occasion of his 70th Birthday. This article is part of a themed issue on Biophosphates.

[‡] Electronic supplementary information (ESI) available: Silencing activity of siRNAs (1 nM) in HeLa cells; cytotoxicity of siRNA duplexes in HeLa cells; nucleolytic stability of siRNA duplexes in 10% FBS. See DOI: 10.1039/b9nj00746f

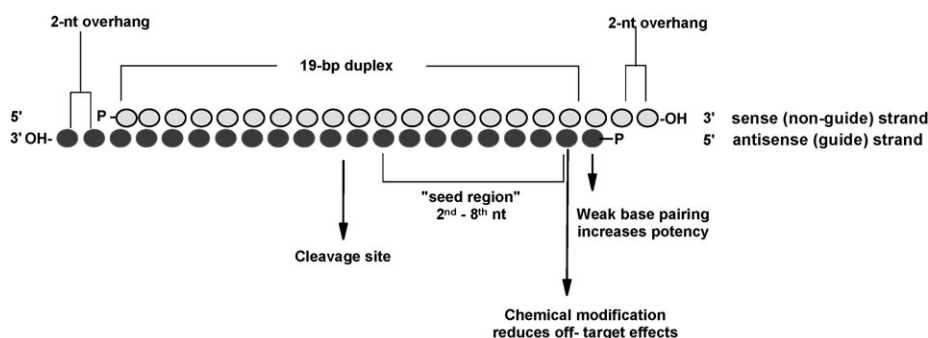


Fig. 1 Short interfering RNA (siRNA) structure with indicated sites crucial for silencing activity, which represent potential positions for chemical modification.

of *Thermus thermophilus* Ago protein as it binds to and slices its RNA target.^{19,22,23}

Along with efforts aimed at explaining the mechanism of the RNA gene silencing process,^{18–23} tremendous emphasis has been placed on the improvement of the most important features of siRNAs: (i) silencing potency, (ii) cellular uptake, (iii) nucleolytic stability, (iv) target specificity and (v) elimination of immune and non-specific “off-target” effects. A possible approach to reach this goal is the introduction of chemical modification into the duplex.^{24–26} A variety of chemical modifications have been proposed and have led to a significant growth in understanding of the mechanism of RNAi. Widely used in siRNAs are modifications of the ribose moiety; especially extensively modified is the 2'-OH group.^{27–32} Chiu and Rana, in one of the earliest studies on chemically modified siRNA, have shown that, while preservation of the A-form duplex structure is important for its silencing activity, the 2'-OH group is not required.²⁸ In general, 2'-modifications are more or less tolerated, depending on the number and position in siRNA strands. Functional siRNAs can contain fluorine at the 2'-position, *e.g.*, 2'-F-RNA is one of the best known siRNA modifications, and several replacements are tolerated throughout the sense and antisense strands.^{28,29,33} Such modifications strongly favour the A-form helical structure of the duplex and significantly increase the stability of siRNAs in serum.³⁴ Another one, a 2'-F-ANA modification, with the opposite stereochemistry of the fluorine (in the *ara* position), developed as a DNA-mimic, is still well tolerated in siRNA duplexes, including the fully modified sense strand and in partial modifications of the antisense strand.^{35,36}

In the present study, we describe the synthesis of six siRNA duplexes with a single modification in the antisense strand, where a cytidine unit is replaced with 2',2'-difluoro-2'-deoxycytidine (dFdC, gemcitabine, IUPAC name: 4-amino-1-(2-deoxy-2,2-difluoro- β -D-erythro-pentofuranosyl)-pyrimidin-2(1H)-one). Gemcitabine, an analog of deoxycytidine (dC), is an anticancer drug that is widely used in the treatment of prevalent human cancers.^{37,38} dFdC is also a potent sensitizer to radiation, and this activity requires metabolic transformation to the corresponding triphosphate (dFdCTP) and incorporation of dFdCp into DNA.³⁹ The modification was introduced into several positions important for siRNA activity. The gene silencing activity, thermodynamic properties, nucleolytic stability and cytotoxicity of the

modified siRNAs were compared to those of the parent, non-modified (NM) duplex. We did not expect any significant structural changes of the RNA duplexes carrying the dFdC modification, as the structural features of gemcitabine fit well to the A-type RNA conformation.⁴⁰ Rather, we were interested in to what extent a single gemcitabine unit present in the “seed region” or in the cleavage site modulates the activity and thermodynamic stability of the corresponding siRNA duplexes.

2. Results and discussion

2.1 Selection of the model siRNA sequence and sites for dFdC modification

In our previous studies, we designed several siRNAs for target sequences of human/mouse/rat (h/m/r) beta-secretase (BACE1) mRNA and validated their silencing activity in cellular models.⁴¹ All of those siRNAs had a conventional duplex structure: a 19-bp stem and 2-nt overhangs (TT) at the 3'-ends. For the present studies, we chose the duplex h/m **Gem** of moderate activity for the target sequences of human (629–659) and mouse (602–632) BACE1 mRNA. Importantly, in the antisense strand, the molecule **Gem** siRNA has several cytidine units at positions considered important for silencing activity. For the modification, we chose positions 1, 2, 4, 5, 8 and 10 (counting from the 5'-end), and replaced the respective cytidine units with a dFdC unit (Fig. 2). The 3'-O-phosphoramidite monomer of the protected 2',2'-difluoro-2'-deoxycytidine was prepared according to Scheme 1. All RNA oligonucleotides (with and without gemcitabine substitution) were synthesized in house using solid phase phosphoramidite chemistry.^{42,43} After the routine synthesis, the oligonucleotides were deprotected, purified and assembled into duplexes with the non-modified sense RNA strand (details are given in the Experimental section).

2.2 Silencing activity of dFdC-modified siRNAs

To investigate the silencing activity of siRNA duplexes, we applied a dual fluorescence reporter system.⁴⁴ This model system is based on a measurement of the relative fluorescence intensity of the enhanced green fluorescent protein (EGFP) and the coral-derived (*Discosoma spp.*) red fluorescent protein (RFP), expressed from plasmids delivered exogenously into

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUACCACC-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUACCACC-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUA₂CCACC-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUAC₂ACC-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUACCA₂CC-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUACCA₂C-5'

5'- AGCUUUGUGGAGAUGGUGGT-3'
3'-TTUCGAAACACCUCUACCACC-5'

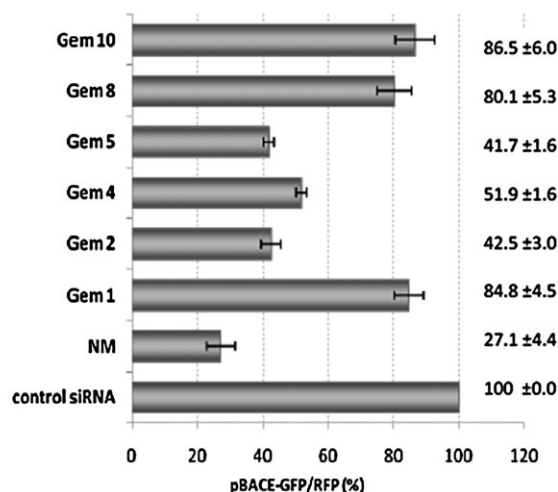
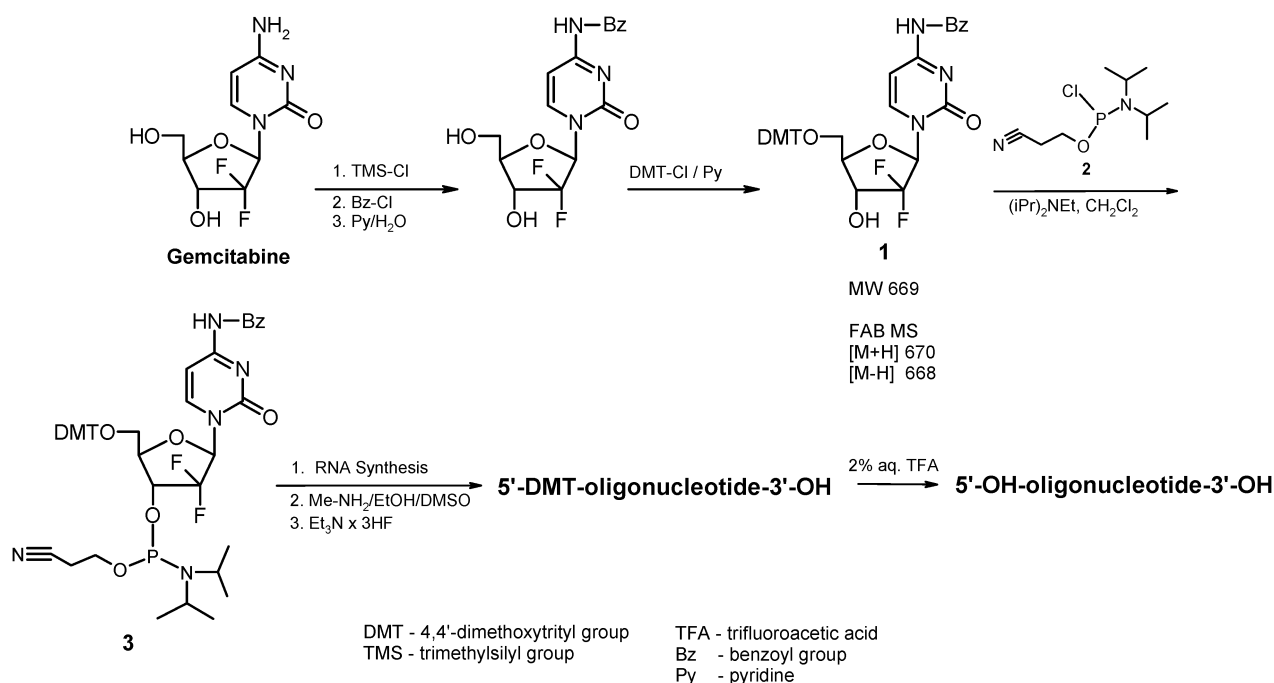


Fig. 2 The sequences of siRNAs used in this study (with indicated dFdC-modified sites) and their silencing activity in HeLa cells (5 nM siRNAs), presented as percentages of GFP expression of the control cells transfected with non-silencing control siRNA.



Scheme 1 A schematic representation of 2',2'-difluoro-2'-deoxycytidine phosphoramidite synthesis, RNA oligonucleotide preparation and deprotection. More detailed information is included into the Experimental section.

HeLa cells. Two reporter plasmids were used: commercially available pDsRed2-N1 (BD Biosciences) and pBACE1-GFP plasmid coding a fusion protein BACE1-GFP, kindly provided by Dr Weihong Song (The University of British Columbia, Vancouver, Canada).⁴⁵ All transfection experiments were performed with Lipofectamine 2000 (Invitrogen) on a 96-well plate in a 48 h assay. After transfection of the plasmids and of the screened siRNA (Fig. 2) (used in 1 or 5 nM concentrations) into HeLa cells, the cells were incubated for 48 h, lysed and the relative fluorescence of GFP to RFP determined. The results of the screening experiments are shown in Fig. 2. While the parent, non-modified duplex (NM) silences the expression of the target gene by *ca.* 70%,

all the dFdC-modified duplexes are less active. Duplexes **Gem2**, **Gem4** and **Gem5**, modified at positions 2, 4 and 5, respectively, are remarkably active (50–60% silencing). In contrast, dFdC modifications at positions 1, 8 and 10 are detrimental to the activity of siRNA duplexes (**Gem1**, **Gem8** and **Gem10**, respectively), of which the silencing effect is below 20%.

2.3 Cytotoxicity of dFdC-modified siRNAs

Chemically modified RNA duplexes may induce numerous cytotoxic effects.^{27,31} In the case of the screened **Gem1–Gem10** siRNAs, the cytotoxic effects might originate from the nature

of the introduced modified unit, as gemcitabine is the very effective anticancer drug.^{37–40} Therefore, the influence of the dFdC modification on cell viability was evaluated by screening the toxicity of siRNAs **Gem1–Gem10** for HeLa cells. The siRNA duplexes were used at five chosen concentrations (1, 5, 10, 20 and 50 nM) with an appropriate amount of transfection reagent Lipofectamine 2000. Cell viability was assessed by the MTT assay. Untreated HeLa cells were used as a positive control. Virtually no toxic effect for any sample was observed (ESI, Fig. S2†), and the cytotoxicities of non-modified siRNA and the dFdC-modified duplexes were similar. The biggest, but still minor, effect on cell viability (~10–15% reduction) was observed for siRNAs at concentrations above 20 nM.

2.4 Nucleolytic stability of modified siRNAs in 10% FBS

The increased resistance of siRNAs to endo- and exonucleolytic cleavage is a primary factor that decides on the basic features of RNAi, such as the efficiency of cellular uptake, the duration of gene expression inhibition and the dosing schedules required to achieve therapeutic effects. We examined the stability of siRNA duplexes with dFdC modification in 10% active fetal bovine serum (FBS). All the duplexes (**NM** and **Gem1–Gem10**) (Fig. 2) were radioactively labelled (³²P) at the 5'-end of the sense strand and treated with 10% FBS in RPMI medium (the same medium composition as for HeLa cell culturing) at 37 °C. Aliquots were collected after incubation for 0, 1, 5, 15, 30, 60, 120, 240, 360 min and 24 h, and examined for the presence of the intact duplex by native 20% polyacrylamide gel electrophoresis (PAGE). The stability of modified siRNAs (**Gem1–Gem10**) in 10% FBS was comparable to the stability of the reference **NM** duplex (half-life longer than 6 h). The respective data are shown in the ESI (Fig. S3).† Besides, the stability of the single-stranded dFdC-containing oligonucleotides was also screened. All of them were unstable in these conditions and were hydrolyzed during the first minute, indicating that a single modification within the chain does not prevent ssRNA from undergoing endonucleolytic cleavage.

2.5 Analysis of the structure of the dFdC-modified duplexes by circular dichroism

Circular dichroism (CD) spectra were collected for all the dFdC-modified siRNA duplexes and compared with the CD spectrum of the non-modified duplex. The majority of CD spectra indicated a typical A-type structure of the double-stranded RNA (a maximum of the positive Cotton effect at 268 nm and a crossover point at 240 nm). The obtained result was expected, based on the earlier report of Konerding *et al.*,⁴⁰ who determined the structural effect of the dFdC substitution on model DNA of the Okazaki fragment. Respective NMR studies revealed that the dFdC sugar conformation differed from unmodified deoxycytidine and adopted the C3'-endo sugar ring puckering (RNA-like A-type sugar ring conformation). While the majority of duplexes adopted the typical A-type RNA structure, in the CD spectrum recorded for duplex **Gem4**, with the dFdC substitution at position 4, a significantly lower intensity of the maximum of

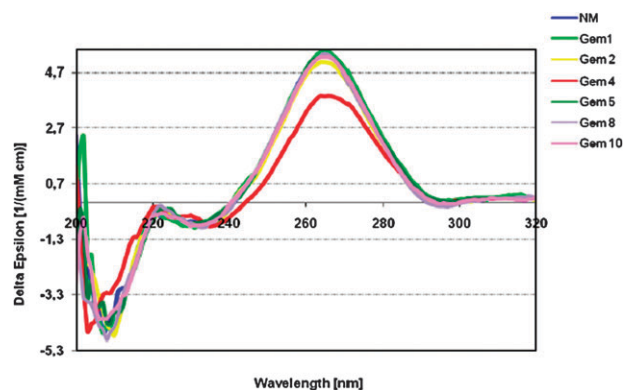


Fig. 3 CD spectra of the dFdC-modified siRNA duplexes. Reagents and conditions: 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA buffer (pH 7.4), temperature 25 °C, duplex concentration 2 μ M.

the positive Cotton effect at 268 nm and a shift of the crossover point from 240 to 245 nm were observed, suggesting that the structure of this duplex is distorted from the A-RNA type (Fig. 3).

2.6 Hybridization studies

UV-monitored thermal dissociation profiles were determined for all the dFdC-modified siRNAs and for the non-modified duplex (Fig. 4). Melting temperatures (T_m) were calculated as the maximum of the first derivative $\Delta A/\Delta T$ for the thermal dissociation profiles. The biggest difference in the thermal stability ($\Delta T_m = 6$ °C) and, thus the most severe decrease in stability of the modified duplex in comparison to the non-modified congener, was observed for the **Gem10** duplex, with the dFdC modification in the central position of the antisense strand. A similar decrease of T_m (~5 °C) was found for **Gem1**, **Gem2** and **Gem8**, with the dFdC modification at the 1st, 2nd and 8th position, counting from the 5'-end of the antisense strand. Based on the two-state van't Hoff model, the melting temperatures ($T_{m, cal}$) and standard thermodynamic parameters (ΔH° , ΔS° and ΔG°) were calculated (Table 1).⁴⁶ From a thermodynamic point of view, the binding affinity constant, K_a , is defined in terms of the free energy of

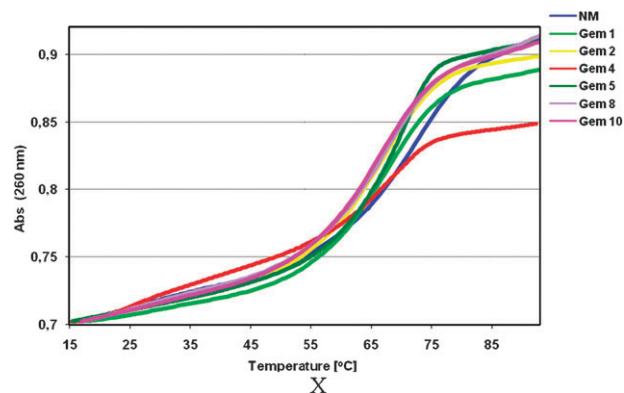


Fig. 4 UV-monitored thermal dissociation profiles for non-modified duplex **NM** and siRNA duplexes with dFdC modifications. Oligonucleotide concentration 2 μ M, 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA buffer (pH 7.4), dissociation at a temperature gradient of 1 °C min⁻¹.

Table 1 Melting temperatures and calculated thermodynamic parameters for the thermal dissociation of screened siRNA duplexes

Name	$T_{m \text{ exp}}/^{\circ}\text{C}$	$T_{m \text{ cal}}/^{\circ}\text{C}$	$\Delta H^{\circ}/\text{kcal mol}^{-1}$	$\Delta S^{\circ}/\text{cal K}^{-1} \text{ mol}^{-1}$	$\Delta G^{\circ}/\text{kcal mol}^{-1}$
NM	72.6	70.55	−66.59	−164.92	−15.44
Gem 1	67.4	66.09	−68.18	−172.15	−14.79
Gem 2	67.5	66.32	−70.12	−177.73	−15.00
Gem 4	68.2	nd	nd	nd	nd
Gem 5	70.1	68.22	−83.09	−214.56	−16.54
Gem 8	67.6	65.42	−73.18	−187.37	−15.08
Gem 10	66.8	64.84	−68.86	−174.91	−14.62

binding (ΔG°), $K_a = \exp(-\Delta G^{\circ}/RT)$, where R is the gas constant and T is the absolute temperature. The free energy of binding is, in turn, defined by the enthalpy (ΔH°) and entropy (ΔS°) changes and $\Delta G = \Delta H^{\circ} - T\Delta S^{\circ}$. Therefore $K_a = \exp[-(\Delta H^{\circ} - T\Delta S^{\circ})/RT] = \exp[-\Delta H^{\circ}/RT] - \exp[\Delta S^{\circ}/R]$. The binding affinity can be enhanced by driving ΔH° more negative, by driving ΔS° more positive or by a combination of both effects. The binding enthalpy primarily reflects the strength of the interactions of the siRNA strands in the duplex (hydrogen bonds, base stacking, *etc.*) relative to those existing with solvent molecules for the single strands. On the other hand, the entropy change mainly reflects two contributions: changes in solvation entropy and changes in conformational entropy.⁴⁷

Considering the melting profile recorded for **Gem4**, we observed some pre-melting and a low hyperchromic effect; so in this case, we could not calculate standard thermodynamic parameters using the two-state van't Hoff model. Comparing the binding enthalpy of the non-modified ($\Delta H^{\circ} = -66.59 \text{ kcal mol}^{-1}$) and the dFdc-modified siRNAs **Gem1**, **Gem2**, **Gem5**, **Gem8** and **Gem10**, one can postulate stronger interactions of the siRNA strands in the modified duplexes, with the strongest interactions in the **Gem5** (C5 \rightarrow dFdc) and **Gem8** (C8 \rightarrow dFdc) duplexes. By CD spectroscopy, we did not observe any conformational changes for **Gem1**, **Gem2**, **Gem5**, **Gem8** and **Gem10**, in comparison to the NM duplex. Therefore, in our opinion, the more positive entropy changes for the NM duplex ($\Delta S^{\circ} = -164.92 \text{ cal K}^{-1} \text{ mol}^{-1}$) in comparison to dFdc-modified siRNAs (ΔS° in the range from $-172.15 \text{ cal K}^{-1} \text{ mol}^{-1}$ for **Gem1** to $-214.56 \text{ cal K}^{-1} \text{ mol}^{-1}$ for **Gem5**) reflect changes in the solvation entropy. Upon strand binding, desolvation occurs, water is released and a gain in solvent entropy is observed.

3. Conclusions

In this paper, we have described the application of 2',2'-difluoro-2'-deoxycytidine (dFdc, gemcitabine) modification in the single substitution of a cytidine residue in the antisense strand of the siRNA duplex. Encouraged by already published results concerning fluorine-modified siRNAs and the unique characteristics of gemcitabine,⁴⁸ we decided to examine the tolerance of such modifications at six sites of the antisense strand crucial for siRNA silencing activity: in the “seed region” (positions 2–8) and at the cleavage site. The “seed region” is especially important for mRNA target recognition, and complementarity to this sequence is often sufficient to obtain the desired silencing. Detailed analysis has shown a position-dependent tolerance for antisense strand modification.

Gemcitabine was moderately or poorly tolerated in some positions of the “seed region” of the guide strand. Modifications at positions 1 and 8 had a strong impact on efficacy, while silencing activity was only *ca.* 15% (for comparison, 70% GFP silencing by the non-modified duplex). A similar situation was observed for siRNA with gemcitabine at position 10, where activity declined to below 10%. Gemcitabine substitution at the positions 2, 4 and 5 caused only moderate changes in silencing efficiency in comparison to the non-modified duplex. CD spectra for most of the screened duplexes indicated a typical A-shaped RNA structure, but the melting temperature parameters of the modified duplexes were decreased by 5–6 $^{\circ}\text{C}$.

From already published data, we know that gemcitabine cytotoxicity is the consequence of an alteration in DNA polymerase-mediated DNA synthesis following dFdc mis-incorporation. dFdc adopts an RNA-like ribose conformation (C3'-*endo*).⁴⁰ In addition, the geminal difluoro group of dFdc substantially alters the electrostatic surface of the duplex at the site of substitution. Such substantial changes may be potentially important for protein binding. Furthermore, the steric clashes may also contribute to a change in the siRNA silencing activity, as difluorine substitution alters the size of the modified moiety relative to the non-modified moiety.⁴⁰ We suppose that changes in the silencing activity of the studied siRNAs originate from intrinsic gemcitabine properties that are the consequence of a disturbance in the RNA–protein interaction rather than in RNA–RNA recognition.

4. Experimental

4.1 Preparation of the phosphoramidite derivative of the dFdc monomer and the synthesis of gemcitabine-modified RNA oligonucleotides (Scheme 1)

5'-Dimethoxytrityl- N_4 -benzoyl-2',2'-difluoro-2'-deoxycytidine (**1**) (1 equiv., 0.4 mmol, 270 mg) was dissolved in anhydrous acetonitrile (6 ml). A 0.5 M solution of ethylthiotetrazole (1.6 equiv., 0.65 mM, 1.3 ml) in anhydrous acetonitrile and 2-cyanoethyl-bis-(N,N' -diisopropyl)phosphoramidite (**2**) (1.43 equiv., 0.57 mmol, 0.2 ml) were added, and the reaction mixture stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC and ^{31}P NMR. When 5'-dimethoxytrityl- N_4 -benzoyl-2',2'-difluoro-2'-deoxycytidine (**1**) was consumed (~ 2 h), the mixture was applied to a silica gel column, and the product (**3**) eluted with 20% hexane in CH_2Cl_2 , followed by MeOH in CHCl_3 (0–5% gradient). The desired product was identified by ^{31}P NMR (CDCl_3): δ 154.1,

152.1, and FAB MS calculated for: $C_{46}H_{50}O_8N_5P_1F_2$ 869.33, found 870 $[M + H]^+$, 868 $[M - H]^-$. The synthesis of all RNA oligonucleotides, unmodified and containing dFdC units, was performed according to the phosphoramidite approach^{42,43} on a Gene World DNA synthesizer (Department of Bioorganic Chemistry, CMMS, PAS). The synthesis was carried out on a 200 nmol scale using appropriately protected phosphoramidite derivatives of thymidine, cytidine, uridine, guanosine, adenosine and 2',2'-difluoro-2'-deoxycytidine (**3**), LCA-CPG as a solid support and 5-benzylmercaptopotetrazole in anhydrous acetonitrile (0.25 M) as an activator. The synthesis had a prolonged coupling time (up to 600 s) for the modified unit. The coupling efficiency was determined by the DMT-cation assay.

4.2 Deprotection and purification of oligonucleotides; assembly of siRNAs

Oligonucleotides were cleaved from the solid support as 5'-DMT-derivatives, and then deprotected and purified according to a described procedure.⁴⁹ Support-bound oligonucleotides were treated with 33% ethanolic methylamine and DMSO (Sigma-Aldrich) 1:1 (v:v) mixture for 15 min at 65 °C, then with triethylamine-trihydrofluoride (Sigma-Aldrich) for 15 min at 65 °C. The reaction mixture was frozen for 30 min at -20 °C, quenched with cold 1.5 M ammonium bicarbonate and poured into a conditioned SepPak cartridge (Waters). Shorter oligomers were eluted with 14% CH_3CN in 50 mM NaOAc and 50 mM NaCl. The remaining oligomers were treated with 2% aqueous TFA for 15 min at room temperature and washed with water, 1 M NaCl, and water. The product was eluted from the cartridge with 30% CH_3CN . The desired oligonucleotides were obtained in 90–95% yield. The structure and purity of the oligomers were confirmed by MALDI-TOF mass spectroscopy and 20% polyacrylamide/7M urea gel electrophoresis. siRNA duplexes were assembled in phosphate saline buffer (PBS, without Ca^{2+} and Mg^{2+}) by mixing equimolar amounts of complementary oligonucleotides, heating at 95 °C for 2 min and slow cooling down to room temperature (2 h). The formation of the resulting duplexes was confirmed by 4% agarose electrophoresis.

4.3 Cell cultures and transfections

HeLa cells were cultured in RPMI 1640 medium (Gibco, Paisley) supplemented with 10% FBS (Gibco, BRL, Paisley) and with antibiotics (penicillin 100 units ml^{-1} , streptomycin 100 mg ml^{-1} , Polfa) at 37 °C and 5% CO_2 . 24 h prior to the experiment, cells were plated in 96-well black well plates with a transparent bottom (Perkin-Elmer) at a density of 15 000 cells per well. Before transfection, the cell medium was replaced with the one free of antibiotics. The cells were cotransfected with DNA plasmids: pDsRed2-N1 (BD Biosciences), 15 ng $well^{-1}$, pBACE-EGFP-C1,⁴⁵ 70 ng $well^{-1}$ and siRNA (1 or 5 nM) dissolved in Opti-MEM1 medium (Gibco, Paisley), and complexed with the transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were incubated in the transfection mixture for 5 h, and then the mixture was replaced with fresh culturing medium containing antibiotics. After a 48 h incubation at 37 °C in an

atmosphere of 5% CO_2 , the cells were washed with PBS and lysed with NP-40 buffer (150 mM NaCl, 1% IGEPAL, 50 mM Tris-HCl (pH 7.0), 1 mM PMSF) overnight at 37 °C. Cell lysates were used for fluorescence determination.

4.4 Dual fluorescence assay

Fluorescence values of enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) fluorophores were measured in cell-culturing plates using a Synergy HT (BIO-TEK) reader. Data quantification was done with KC4 software. Excitation and emission wavelengths for GFP and RFP were as follows: $\lambda_{ex} = 485/20$ nm, $\lambda_{em} = 528/20$ nm for GFP and $\lambda_{ex} = 530/25$ nm, $\lambda_{em} = 590/30$ nm for RFP. The siRNA activity was calculated as a ratio of GFP to RFP fluorescence values. The level of GFP fluorescence of control cells (transfected with pDsRed2-N1 and pBACE-GFP plasmids, and control non-silencing siRNA) was taken as a reference (100% GFP expression). Each siRNA activity value given on the plots is an average of the mean value from three independent experiments.

4.5 Cytotoxicity determination

The cytotoxicity of siRNA duplexes for HeLa cells was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (Sigma-Aldrich) (activity of the mitochondrial respiratory chain).⁵⁰ Cells were plated and transfected as described above (siRNAs at 1, 5, 10, 20 and 50 nM concentrations). As a background control, the cells were treated with Lipofectamine 2000 only. After 48 h of incubation at 37 °C and 5% CO_2 , 25 μl of MTT solution (5 mg ml^{-1} in PBS) was added to each well and incubated for additional 2 h at 37 °C. Finally, 95 μl of lysis buffer (20% SDS, 50% aqueous dimethylformamide, pH 4.5) was added to each well and incubated overnight at 37 °C. The absorbance of a given sample was measured at 570 nm, with the reference wavelength 630 nm (plate reader Synergy HT, BIO-TEK). The percentage of living cells (PLC) was calculated from the equation: $PLC = A_{Spl} \times 100 / (A_{control\ cells})$, where A_{Spl} is the absorbance of a given sample of cells treated with siRNA and $A_{control\ cells}$ is the absorbance of the cells untreated with siRNAs. Each data point represents the mean of three independent measurements.

4.6 Melting profiles and thermodynamic parameters calculations

All UV absorption measurements were carried out in a 1 cm path length cell with a UV/Vis 916 spectrophotometer equipped with a Peltier Thermocell (GBC, Australia). Complementary oligonucleotides were mixed in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA buffer (pH 7.4) at final concentration of siRNA of 2 μM . The duplexes were heated at 96 °C and strand association was achieved by cooling down to 5 °C with a temperature gradient of 0.4 °C min^{-1} . Melting profiles were recorded under heating from 5 to 96 °C with a temperature gradient of 1 °C min^{-1} . The melting temperatures were calculated by the first-order derivative method. Thermodynamic parameters were obtained by numerical fitting of the recorded curves (MeltWin software, version 3.5).

4.7 CD spectra recording

CD spectra in the range 200 to 350 nm were recorded on a CD dichrograph (Jobin-Yvon) at 25 °C in the same buffer as in the melting experiments at a duplex concentration of 2 µM, using a 5 mm path length cell, 2 nm bandwidth and a 1–2 s integration time. Each spectrum was smoothed with a 25-point algorithm (included in the manufacturer's software, version 2.2).

4.8 The stability of siRNAs in 10% FBS

The unmodified ssRNA sense strand was 5'-labelled with [γ -³²P] ATP by T4 polynucleotide kinase (Amersham) (incubation of 3 nmol of RNA with 10 units of enzyme for 1 h at 37 °C). Duplexes were formed by annealing equal molar ratios of sense and antisense strands to form seven types of siRNA (Fig. 2). Duplex formation was confirmed by 20% PAGE under native conditions. Then duplexes (3 pmols per reaction) were diluted in RPMI medium (Gibco) supplemented with 10% FBS (Gibco) and incubated at 37 °C. Aliquots of 10 µl were collected after 0, 1, 5, 15, 30, 60, 120, 240, 360 min and 24 h, diluted in 1× loading buffer (Fermentas), frozen in liquid N₂ and kept at –20 °C until analysis time. Control sample "0" was prepared with ice cold FBS and RPMI medium, collected and immediately frozen. Samples were separated on 20% PAGE under non-denaturing conditions. Substrate and degraded products were quantified using a G-Box (SynGene, Cambridge, UK) instrument and GeneTools 4.0 software.

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