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siRNAs Modified with Boron Cluster and Their Physicochemical and Biological Characterization

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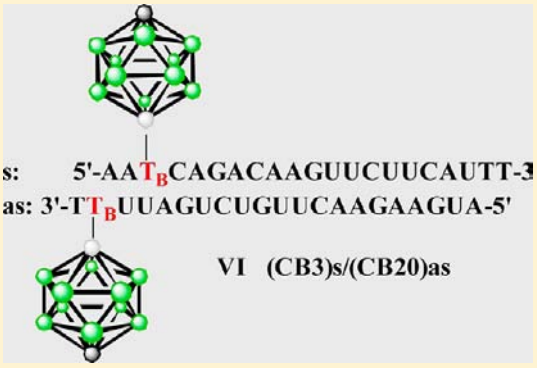
Supporting Information

ABSTRACT: RNA interference (RNAi) technology provides a powerful, yet selective, molecular tool to reduce the expression of genes in eukaryotic cells. Despite the success associated with the effective use of siRNA duplexes for gene silencing, there is a need to improve their properties. These properties, related mainly to migration through the cell membranes, stability of siRNA *in vivo*, and specificity of their silencing activity, can be improved by chemical modifications of siRNA backbone. In this study, we examined the physicochemical and biological properties of siRNA duplexes targeted against BACE1 gene modified at various positions with a lipophilic boron cluster ($C_2B_{10}H_{11}$, CB). The lipophilicity and resistance to enzymatic degradation of the modified oligomers was higher than the unmodified counterparts. As measured in a dual fluorescence assay (BACE1-GFP/RFP), the carboranyl siRNAs (CB-siRNAs) were as active as the parent nonmodified duplexes and their toxicity toward HeLa cells was also similar. The helical structure of CB-siRNAs remained unchanged upon boron cluster introduction, as determined by CD and UV melting experiments.



INTRODUCTION

The phenomenon of RNA interference (RNAi) is a specific silencing of gene expression in eukaryotic cells by evocation of enzymatic degradation of a corresponding mRNA (mRNA). Short interfering RNAs (siRNAs), double-stranded RNA oligomers of 21−23 nucleotides in length, are involved in this process. Exogenously delivered siRNAs are localized in the cytoplasm where they bind to the RISC complex. Upon cleavage and removal of the sense strand of siRNA, the remaining antisense strand of the duplex operates as a guide to bring the RISC complex into the complementary sequence of the target mRNA. Once the target mRNA is associated to the RISC, it is cleaved by the Ago ribonuclease, so the translation is inhibited.[1,2] Currently, many efforts are underway to find efficient methods of delivering siRNA molecules into the target cells under *in vivo* conditions. Conventional methods for siRNA delivery, such as encapsulation of siRNA with lipid nanoparticles,[3] cationic complexes,[4] inorganic nanoparticles,[5] RNA nanoparticles, and dendrimers,[6] often activate immunogenic response or induce cytotoxicity. Recently, an interesting approach for the preparation of a microsponge cargo for siRNA was proposed by Lee et al.[7] They generated polymeric RNA, that could be folded into dense particles easily delivered into the target cells and cleaved there into the siRNA subunits. Another approach to improve pharmacokinetic properties of siRNA relies on chemical modifications of the RNA chain.[8−10] This approach not only improves the silencing activity of siRNA duplexes, but also reduces the "off-target" effects originating from the nonspecific target gene silencing.[11] We have already reported appreciable influence of base-modified rare nucleosides on the silencing activity of siRNAs directed toward genes coding BACE1[8,12,13] and PS1 proteins.[14]

In this report we present the synthesis as well as physicochemical and biological characteristics of novel siRNA duplexes containing a boron cluster as the modifying entity.[15,16] For this purpose, thymidine phosphoramidite containing carboranyl pendant ($C_2B_{10}H_{11}$ (CB)) at position N3 (Scheme 1) was prepared and used for synthesis of several RNA oligonucleotides corresponding to the sense and antisense strands of siRNA (Table 1). We used mRNA of BACE1 protein as a target. The BACE1 protein contributes to the formation of beta-amyloid peptides (Aβ-peptides) identified in amyloid plaques of patients with Alzheimer's disease (AD).[17,18] According to the amyloid cascade hypothesis,[19] the inhibition of the Aβ-peptide production may offer an effective approach for AD treatment and prevention.

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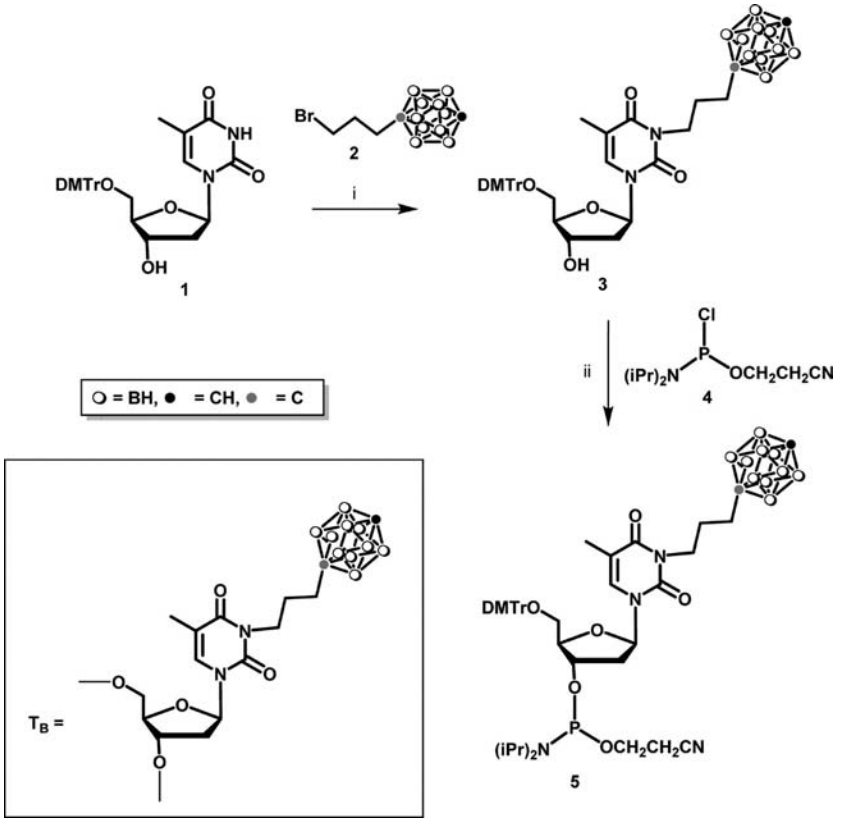
Scheme 1. Synthesis of the Modified Monomer 5 Bearing Boron Cluster, 5′-*O*-Dimethoxytrityl-3-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]thymidine 3′-*O*-(*N*,*N*-diisopropyl-*β*-cyanoethyl)phosphoramidite[a]



[a] i. **2**, K_2CO_3/DMF; ii. **4**, diisopropylethylamine/CH_2Cl_2.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from Aldrich Chemical Co. and were used without further purification unless otherwise stated. Thymidine was purchased from Pharma-Waldhof GmbH (Düsseldorf, Germany). Flash chromatography was performed on silica gel 60 (230−400 mesh, Aldrich Chemical Co.). R_f values refer to analytical TLC performed using precoated silica gel 60 F254 plates purchased from Sigma-Aldrich (Steinheim, Germany) and developed in the solvent system indicated. Compounds were visualized with UV light (254 nm) or a 0.5% acidic solution of $PdCl_2$ in HCl/methanol for boron-containing derivatives. The yields are not optimized.

Methods. *Nuclear Magnetic Resonance (NMR).* ^{1}H NMR and ^{31}P NMR spectra were recorded on a BrukerAvance DPX 250 MHz spectrometer equipped with the BB inverse probehead; the spectra for ^{1}H and ^{31}P nuclei were recorded at 250.13 and 101.26 MHz, respectively. Tetramethylsilane was used as a standard for ^{1}H NMR and 75% aqueous solution of H_3PO_4 was used as standard for ^{31}P NMR. The following abbreviations were used to describe the multiplicities: s = singlet, d = doublet, dd = double doublet, t = triplet, dt = double triplet, q = quartet, quin = quintet, bs = broad singlet, m = multiplet. Coupling constants are reported in Hertz (Hz).

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra of compounds **3** and **5** were recorded on a Finnigan MAT (Bremen, Germany). The m/z was measured in a positive and negative modes. The calculated m/z corresponds to calculated values based on the average mass of the elements consisting natural isotopes. The MALDI-TOF MS spectra of screened oligonucleotides **(CB3)s**, **(CB20)as**, **(CB20)s**, **(F1)s**, **(F21)as**, and **(F1)(CB20)s** were recorded on a Voyager Elite mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). Spectra were obtained in the linear, negative ion mode at the acceleration voltage of 20 kV. The energy of the laser beam was set slightly above threshold level.

Ultraviolet Spectroscopy Measurements (UV). UV measurements were performed on a GBC Cintra10 UV−vis spectrometer (Dandenong, Australia). Samples for UV experiments, ca. 0.5 ODU_{A260} of each compound were dissolved in 96% C_2H_5OH or methylene chloride. The measurement was performed at ambient temperature.

Infrared Spectroscopy (FT-IR) Measurements. Infrared absorption spectra were recorded using Smart iTR diamond attenuated total reflectance (ATR) attachment on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific) equipped with a ETC EverGlo* source for the IR range, Ge-on-KBr beam splitter, and DLaTGS detector. Samples to be analyzed were placed on a diamond ATR element in the solid form. For data acquisition and processing was used Omnic 8.1 software program

*Synthesis of 5′-O-Dimethoxytrityl-3-N-[(1,12-dicarba-closo-dodecaboran-1-yl)propan-3-yl]thymidine (**3**).* 1-(1,12-Dicarba-*closo*-dodecaboran-1-yl)-3-bromopropane (**2**) (100 mg, 0.38 mmol) was added to a solution of 5′-*O*-dimethoxytritylthymidine (**1**) (200 mg, 0,38 mmol) and potassium carbonate (52 mg, 0.38 mmol) in anhydrous DMF (5 mL). Then the reaction mixture was stirred at room temperature under argon. The reaction progress was monitored by TLC using

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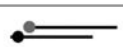
Table 1. Sequences and MALDI-TOF MS Data of Oligoribonucleotides Used for the Preparation of siRNAs I-XIII[a]

No	Symbol	Schemes	Sequence of oligoribonucleotides	Calculated Molecular mass	MALDI-TOF m/z
I	s/as		s: 5'-AAUCAGACAAGUUCUUCAUTT-3' as: 3'-TTUUAGUCUGUUCAAGAAGUA-5'	6597 6654	6597 6657
II	(CB20)s/as		s: 5'-AAUCAGACAAGUUCUUCAUT$_B$T-3' as: 3'-TTUUAGUCUGUUCAAGAAGUA-5'	6782 6654	6777 6657
III	(CB3)s/as		s: 5'-AAT$_B$CAGACAAGUUCUUCAUTT-3' as: 3'-TTUUAGUCUGUUCAAGAAGUA-5'	6780 6654	6775 6657
IV	s/(CB20)as		s: 5'-AAUCAGACAAGUUCUUCAUTT-3' as: 3'-TT$_B$UUAGUCUGUUCAAGAAGUA-5'	6597 6839	6597 6833
V	(CB20)s/(CB20)as		s: 5'-AAUCAGACAAGUUCUUCAUT$_B$T-3' as: 3'-TT$_B$UUAGUCUGUUCAAGAAGUA-5'	6782 6839	6777 6833
VI	(CB3)s/(CB20)as		s: 5'-AAT$_B$CAGACAAGUUCUUCAUTT-3' as: 3'-TT$_B$UUAGUCUGUUCAAGAAGUA-5'	6780 6839	6775 6833
VII	(F1)s/as		s: 5'-FAAUCAGACAAGUUCUUCAUTT-3' as: 3'-TTUUAGUCUGUUCAAGAAGUA-5'	7136 6654	7141 6657
VII I	s/(F21)as		s: 5'-AAUCAGACAAGUUCUUCAUTT-3' as: 3'-FTTUUAGUCUGUUCAAGAAGUA-5'	6597 7193	6597 7190
IX	(CB20)s/(F21)as		s: 5'-AAUCAGACAAGUUCUUCAUT$_B$T-3' as: 3'-FTTUUAGUCUGUUCAAGAAGUA-5'	7321 6654	7321 6657
X	(CB3)s/(F21)as		s: 5'-AAT$_B$CAGACAAGUUCUUCAUTT- 3'as: 3'-FTTUUAGUCUGUUCAAGAAGUA-5'	6780 7193	6775 7190
XI	(F1)s/(CB20)as		s: 5'-FAAUCAGACAAGUUCUUCAUTT-3' as: 3'-TT$_B$UUAGUCUGUUCAAGAAGUA-5'	7136 6839	7141 6833
XII	(F1)(CB20)s/as		s: 5'-FAAUCAGACAAGUUCUUCAUT$_B$T-3' as: 3'-TTUUAGUCUGUUCAAGAAGUA-5'	7321 6654	7321 6657
XII I	(F1)(CB20)s/(CB20)as		s: 5'-FAAUCAGACAAGUUCUUCAUT$_B$T-3' as: 3'-TT$_B$UUAGUCUGUUCAAGAAGUA-5'	7321 6839	7321 6833

[a]The position of the modified nucleoside is marked as T_B and indicated at scheme as a black dot. The fluorescein residue is marked as a **F** and indicated at scheme as a grey dot. Non-modified siRNA duplex s/as is listed as no **I**.

CH_2Cl_2:CH_3OH (90:10, v/v) as an eluent. After completion of the reaction (usually 72 h), the solvent was evaporated to dryness under vacuum yielding the crude product **3** which was purified by silica gel column chromatography (230−400 mesh) using a linear gradient of methanol in methylene chloride as an eluent. Yield: 80%, TLC (CH_2Cl_2/CH_3OH, 90:10 v/v): R_f = 0.36; UV (C_2H_5OH): λ_{sh} = 235 nm, λ_{min} = 254 nm, λ_{max} = 270 nm; ATR-IR (cm^{-1}): ν = 2950 ($CH_3^{asym.}$), 2919 ($CH_2^{asym.}$), 2869 ($CH_2^{sym.}$), 2849 ($CH_3^{sym.}$), 2603 (BH), 1696, 1668, 1607 ($CH^{arom.}$), 1631 (CO), 1460 (CH_3, CH_2), 1250 (CO); ^{1}H NMR (250.131 MHz, $CDCl_3$) [ppm]: δ = 1.50 (m, 2H, $\underline{CH_2}$-carb), 1.93 (d,3H, CH_3, J = 1.20 Hz), 2.25−2.50 (m, 2H, H-2′), 2.90, 2.97 (2d, 6H, CH_3O), 3.30−3.55 (m, 2H, H-5′), 3.70−3.85 (m, 5H, H-4′, H-3′, $NCH_2\underline{CH_2}CH_2$, CH carboranyl), 4.60 (m, 2H, $N\underline{CH_2}CH_2CH_2$), 6.19 (t, 1H, H-1′, J = 6,70 MHz), 6.80−7.21 (m, 8H, H^{arom}, DMTr), 7.29−7.32 (m, 5H, H^{arom}, Ph); MS (FAB, Gly, +Ve): m/z (%) = 728.5 (100%) (calcd for $C_{36}H_{48}B_{10}N_2O_7$, 728.5).

*Synthesis of 5′-O-Dimethoxytrityl-3-N-[(1,12-dicarba-closo-dodecaboran-1-yl)propan-3-yl]thymidine 3′-O-(N,N-diisopropyl-2-cyanoethyl)phosphoramidite (**5**).* Compound **3** (76.27 mg, 0.10 mmol) was dissolved in anhydrous methylene chloride freshly distilled over CaH_2 (5.06 mL). To the resultant solution, *N,N*-diisopropylethylamine (67.60 mg, 0.52 mmol, 90.50 μL) was added, followed by addition of the phosphitylating agent [2-*O*-cyanoethyl)(*N,N*-diisopropylamino) chlorophosphine (**4**)] (74.14 mg, 0.31 mmol, 69.87 μL). The reaction mixture was stirred at ambient temperature under argon and its progress was monitored by TLC using CH_2Cl_2:MeOH (2:98, v/v) as an eluent. After reaction completion (usually 2.5 h), the reaction was quenched with anhydrous methanol (1.3 mL). The resultant solution was diluted with methylene chloride (10 mL) and washed with 5% sodium bicarbonate (3 × 5 mL). The organic layer was dried over magnesium sulfate, then the drying agent was filtered off and washed with methylene chloride containing 1% triethylamine (3 × 3 mL). The filtrate and washings were combined and evaporated to dryness under vacuum. The crude product **5** (ca. 100 mg) was immediately purified by silica gel column chromatography (15 g, 230−400 mesh) and eluted with a gradient of methanol in methylene chloride (0−4%, ca. 60 mL). Fractions containing **5** were collected and concentrated under vacuum. The resulting oil was dissolved in methylene chloride (0.5 mL), cooled to −20 °C, and added dropwise to cooled to −20 °C petroleum ether (25 mL). The obtained precipitate was separated by centrifugation and dried under high vacuum; yield 51.42 mg (53%); TLC (CH_2Cl_2/MeOH, 98:2 v/v)): R_f = 0.48; ATR-IR (cm^{-1}): ν = 2960 ($CH_3^{asym.}$), 2923 ($CH_2^{asym.}$), 2869 ($CH_2^{sym.}$), 2849 ($CH_3^{sym.}$), 2601 (BH), 1701, 1666, 1607 ($CH^{arom.}$), 1642 (CO), 1461 (CH_3, CH_2), 1250 (CO); δ = ^{31}P NMR: 148.6, 148.6 (0.8:1.0); MS (FAB, NBA, +Ve): m/z (%) = 929.8 $[M+2]^+$ (calcd. for $C_{45}H_{64}B_{10}N_4O_8P$, 927.55).

Synthesis and Purification of RNA Oligonucleotides. The oligoribonucleotides isosequential and complementary to mRNA of BACE1 (Table 1) were synthesized according to the routine phosphoramidite approach[20] using LCA CPG glass support and commercially available nucleoside phosphoramidites (ChemGenes). 3′-Phosphoramidite of 5′-*O*-dimethoxytrityl-3-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]-thymidine (**5**) was obtained according to the described above procedure. Synthesis of the unmodified and modified oligoribonucleotides in 0.1 μmole scale was performed on a Gene World DNA synthesizer (K&A, Germany) under the conditions recommended by the manufacturer. Oligonucleo-

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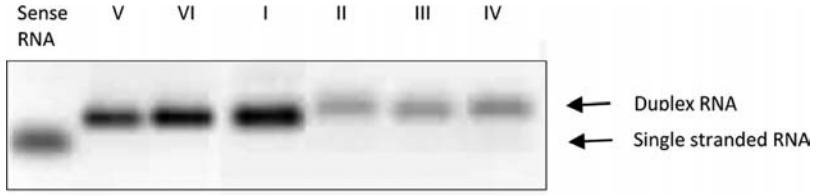

Figure 1. Electrophoretic analysis of siRNA duplexes **I-VI** in 4% agarose gel. Visualization of the siRNA duplexes was achieved with ethidium bromide. Single stranded RNA exhibits faster electrophoretic mobility than double stranded siRNAs.

Table 2. Melting Temperature and Thermodynamic Parameters of Modified siRNA Duplexes (II-VI) and Nonmodified Control (I)[a]

no	symbol	Tm_D (°C)	Tm calculated (°C)	$-\Delta H$ [kcal/mol]	$-\Delta S$ [cal/K·mol]	$-\Delta G°_{37\,°C}$ [kcal/mol]	$\Delta\Delta G°$ [kcal/mol]
I	**s/as**	64.2 (±0.6)	62.7 (±0.1)	116.4 (±10.6)	318.4 (±31.6)	17.7 (±0.8)	0.00
II	**(CB20)s/as**	60.3 (±0.4)	59.4 (±0.1)	112.6 (±11.2)	310.2 (±33.9)	16.3 (±0.7)	1.32
III	**(CB3)s/as**	64.0 (±0.2)	62.7 (±0.3)	120.1 (±6.3)	329.4 (±18.4)	18.0 (±0.5)	−0.32
IV	**s/(CB20)as**	63.9 (±0.0)	62.7 (±0.3)	118.4 (±6.8)	324.6 (±20.0)	17.8 (±0.6)	−0.09
V	**(CB20)s/ (CB20)as**	61.6 (±0.2)	60.2 (±0.7)	98.6 (±23.1)	267.5 (±68.6)	15.6 (±1.8)	2.06
VI	**(CB3)s/ (CB20)as**	64.1 (±0.2)	63.2 (±0.3)	123.2 (±3.7)	338.0 (±11.1)	18.4 (±0.3)	−0.73

[a]The sequences of the siRNAs are according to Table 1. Symbols: $\mathbf{Tm_D}$, melting temperature of dissociation, calculated using the first derivative of the melting curve function; $\mathbf{Tm_{calc}}$, melting temperature obtained using the function to the experimental curve (MeltWin); $-\Delta H$,$-\Delta S$, $\Delta G°_{37°C}$, respectively, enthalpy, entropy, and Gibbs free energy, calculated using MeltWin program; $\Delta\Delta G°$, differences in values of $\Delta G°_{37°C}$ between the samples studied and the control. The values given are averages of at least three independent measurements ± standard deviation.

tides were cleaved from the solid support as 5′-DMT-derivatives, deprotected and RP-HPLC purified according to the described procedure.[13] The molecular mass of the oligomers was confirmed by MALDI-TOF mass spectrometry (MALDI-TOF MS, Table 1) and their purity was assessed by 20% polyacrylamide/7 M urea gel electrophoresis (PAGE) (data not shown).

Assembly of siRNA. siRNA duplexes **I-XIII** (Table 1) were assembled in phosphate saline buffer (PBS, without Ca^{2+} and Mg^{2+}) by mixing equimolar amounts of complementary oligonucleotides, heating the mixture at 95 °C for 2 min, and slow cooling down to room temperature (ca. 2 h). Formation of the resulting duplexes was confirmed by 4% agarose gel electrophoresis (Figure 1).

Melting Profiles and Thermodynamic Calculations. All absorbance measurements were carried out in a 1 cm path length cell with a UV/VIS 916 spectrophotometer equipped with a Peltier Thermocell (GBC, Australia). The siRNA duplexes **I-VI** (the reference and those containing exclusively the carboranyl-modification) were dissolved in 10 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA buffer (pH 7.4) at final concentration of 1 μM. The UV measurements were performed in the range of 15−96 °C with a temperature gradient of 1 °C/min. The melting temperatures were calculated by the first order derivative method. Measurements were performed three times using fresh solutions of oligonucleotides each time. Thermodynamic parameters: melting temperature (Tm_{calc}), enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG) were determined using *MeltWin* software, version 3.5 (Table 2, Figure 2).

Circular Dichroism Measurements. CD spectra were recorded on a CD6 dichrograph (Jobin-Yvon) at 25 °C in the same buffer as in melting experiments at the duplex concentration of 1 μM using a 5 mm path length cell, 2 nm bandwidth, and 1−2 s integration time. Each spectrum was smoothed with a 25 point algorithm (included in the manufacturer's software, version 2.2) after averaging of at least three scans (Figure 3).

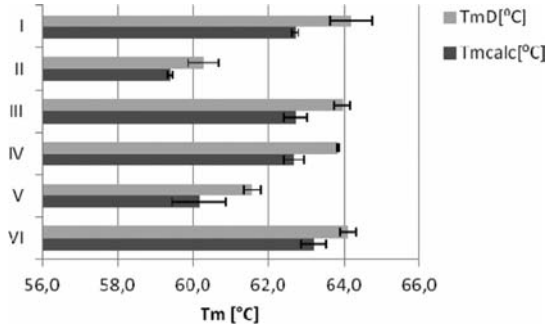

Figure 2. Graphical representation of the Tm values (°C) of siRNA duplexes **I-VI**. Light gray bars present the values of Tm_D, melting temperature of dissociation, calculated using the first derivative; dark gray bars show values of melting temperature Tm_{calc} obtained by the fitting to the experimental melting curve (MeltWin).

Cell Culture and Transfection Conditions. HeLa (human cervical carcinoma) cells were cultured in RPMI (GIBCO, BRL, Paisley) supplemented with 10% FBS (GIBCO, BRL, Paisley) and antibiotics (penicillin 100 units/mL, streptomycin 100 mg/mL, Polfa) at 37 °C and 5% CO_2. Twenty-four hours before the experiment, the cells were placed in a 96-well plate (plates with black walls and transparent bottom, Perkin-Elmer) at the density of 15 × 10^3 cells per well. Directly before the transfection, the cell medium containing antibiotics was replaced with the new one, free of antibiotics. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) at a ratio 1.5:1 (1.5 μL of Lipofectamine 2000 per 1 μg of nucleic acid) according to the manufacturer's protocol. For a dual fluorescence assay (DFA), HeLa cells were co-transfected with DNA plasmids: reporter plasmid pDsRed-N1 (BD Biosciences) (15 ng/well) and pBACE-GFP (100 ng/well)[12,17,21] and siRNAs (1 nM) dissolved in OPTI-MEM medium (GIBCO, BRL, Paisley). After 5 h of incubation, transfection mixture was replaced with fresh medium with antibiotics. After next 48 h incubation at 37 °C in 5% atmosphere of CO_2, the cells were washed three times with the

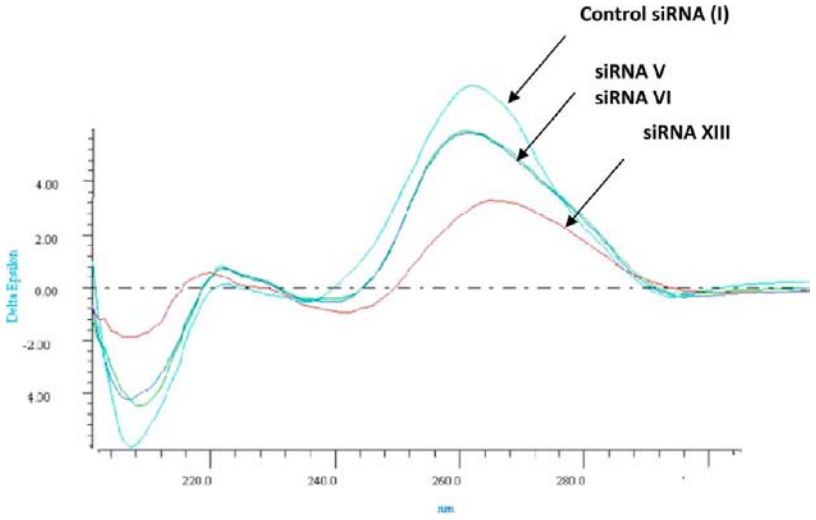

Figure 3. Circular dichroism (CD) spectra of nonmodified siRNA (**I**) and siRNA duplexes **V**, **VI**, and **XIII** containing boron clusters in both the sense and antisense strands. Duplexes were prepared in 10 mM Tris-HCl, 100 mM NaCl, and 10 mM $MgCl_2$ buffer (pH 7.4), at concentration of 1 μM. The measurements were performed at 25 °C. Sequences of the corresponding duplexes are given in Table 1.

PBS buffer (without Ca^{2+} and Mg^{2+}) and lysed with the NP-40 buffer (150 mM NaCl, 1% IGEPAL, 50 mM Tris-HCl, pH 7.0, 1 mM PMSF) overnight at 37 °C. The prepared cell lysates were used for fluorescence determination.

Dual Fluorescence Assay. Fluorescence values of EGFP and RFP (red fluorescent protein) were determined using a Synergy HT reader (BIO-TEK). Quantification of data was done with the KC4 software. Excitation and emission wavelengths for each protein were as follows: EGFP λ_{Ex} = 485/20 nm and λ_{Em} = 528/20 nm, RFP λ_{Ex} = 530/25 nm, and λ_{Em} = 590/30 nm. The silencing activity of siRNA duplexes was calculated as the ratio of EGFP to RFP fluorescence values according to the following equation: Activity of siRNA (%) = 100% − (sample EGFP-X/RFP: control EGFP-X/RFP) × 100% were EGFP-X means the fluorescence value of EGFP-X fusion protein, X = BACE1. An average value of fluorescence was the mean of eight repeats calculated after eliminating extreme values. Each siRNA activity value given on the plots is the average of the mean values from three independent experiments. The level of fluorescence (EGFP/RFP) in control cells (transfected with pDsRed-N1 and pEGFP-BACE plasmid and control (nonsilencing siRNA duplex) was taken as a reference (100%)[12] (Figure 4).

Cytotoxicity. The toxicity of siRNA duplexes **I**-**VI** in HeLa cells was measured using the MTT assay (Figure 5). The cells were plated at density 12000 cells per well (siRNAs in 1, 2, and 5 nM concentrations) in 96-well plates. The cells treated with Lipofectamine 2000 only were taken as a control (100% viability). After transfection, the cells were incubated for 36 h at 37 °C in 5% CO_2, then the MTT solution in PBS (5 mg/mL) was added to each well. After that, cells were incubated for 2 h at 37 °C in 5% CO_2. Finally, 95 μL of the lysis buffer (NP-40, 20% SDS, 50% aqueous dimethylformamide, pH 4.5) was added to each well and incubated overnight at 37 °C. The plate absorbance was measured at two wavelengths: 570 nm and reference 630 nm (the plate reader Synergy HT, BIO-TEK).

Determination of logP values. The log*P* values of three representative oligoribonucleotides, nonmodified antisense strand of siRNA (as), as well as two carboranyl-containing strands (**CB3**)**s** and (**CB20**)**as** (Table 1), were determined by analysis of their HPLC mobility, according to the procedure described previously by Teijeiro et al.[22] The chromatographic

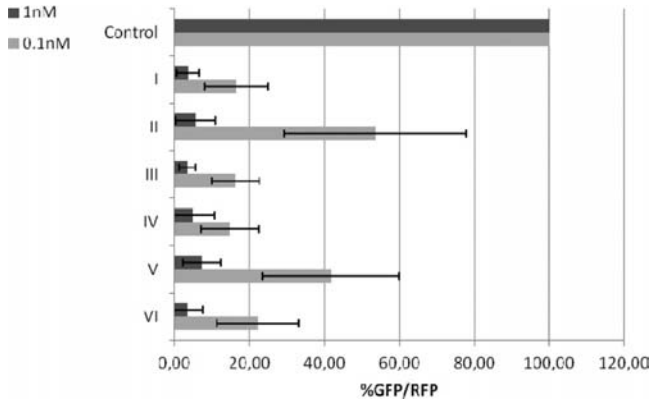

Figure 4. Silencing activity of modified siRNA duplexes measured in a dual fluorescence assay, at two concentrations of 1 nM (dark gray bars) and 0.1 nM (light gray bars). HeLa cells were transfected with pEGFP-BACE1, pDsRED-N1 plasmids and screened siRNAs (control nonsilencing siRNA **I** and duplexes **II**-**VI**). The level of relative EGFP/RFP fluorescence of the cells transfected with control nonsilencing siRNA was used as 100%. The results are mean values from three independent experiments.

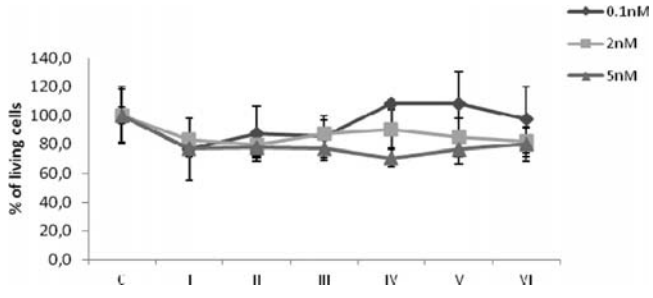

Figure 5. HeLa cells viability 36 h after transfection. The concentrations of siRNAs used in transfection experiments are given in the chart below. The means ± standard deviation are given.

analyses were performed on a reverse phase RP-18 (5 μm) LiChrosphere 100 Å (250 mm/4 mm) column, using an HPLC instrument with UV detection at λ = 254 nm. Compounds were dissolved in water to the final concentration 0.2 μM. Retention

times of unmodified oligonucleotides were measured at four different methanol–water mixtures (25%, 24%, 23%, and 22% of methanol) at flow rate 1 mL/min. The retention time of the modified oligonucleotides was measured from 55% to 45% of methanol in methanol–water mixtures at flow rate 1 mL/min. For each compound, a plot of retention time against composition of the mobile phase was generated. The intercept of the plot (k'_w) corresponds to the retention time of the compound in 100% water. All the measurements were carried out at ambient temperature. Finally the log*P* was calculated using the following equation: $\log P_{o/w} = 1.882 \log k'_w - 1.346$, where $k'_w = (t_R - t_0)/t_0$; t_0 – retention time of methanol (1.78 min); t_R – retention time of the solute; k'_w – extrapolated "*k*" value at 100% water[22] (Table 3).

Table 3. Log*P* Values for Three Representative RNA Oligonucleotides: Nonmodified Antisense RNA Strand (as), and Two RNA Oligonucleotides Modified with Borate Clusters, Namely (CB3)s and (CB20)as[a]

symbol	methanol [%]	Rt [min]	*k'*	log *k'*	log K'_w	log *P*
as	25.00	5.4	2.03	0.71	20.01	4.29
	24.00	7.6	3.27	1.18		
	23.00	14.3	7.03	1.95		
	22.00	36.9	19.73	2.98		
(CB3)s	53.00	5.6	2.15	0.76	10.06	2.99
	51.00	6.8	2.82	1.04		
	50.00	7.7	3.33	1.20		
	49.00	9.1	4.14	1.42		
	48.00	10.9	5.12	1.63		
(CB20)as	51.00	6.9	2.88	1.06	10.68	3.11
	50.00	8.0	3.49	1.25		
	49.00	9.3	4.22	1.44		
	48.00	10.8	5.07	1.62		
	47.00	12.7	6.13	1.81		

[a]Third column shows the percentage of methanol, in which the RNA oligonucleotide was eluted from the RP-HPLC column using isocratic conditions. K'_w – extrapolated "*k*" value at 100% water. The last column shows the values of log*P*, calculated according the methodology described in the Materials and Methods sections.

Delivery of Modified siRNA into Cells, Microscopic Observations. HeLa cells were transfected with a fluorescent CB-siRNA duplex **(F1)(CB20)s/(CB20)as (XIII)** bearing the boron clusters at the 3′-ends of both the sense and antisense strands, carrying the fluorescent tag at the 5′-end of the sense strand (Table 1). The delivery of the CB-siRNA **XIII** was observed either in the presence of Lipofectamine 2000 or without the carrier. The 1 μL of Lipofectamine 2000 was used for 1.5 μg of nucleic acids. Transfection mixture of siRNA was prepared in reduced OPTI-MEM medium (Gibco) in appropriate concentrations of 300, 500, and 1000 nM. SiRNA with Lipofectamine 2000 was initially incubated for 20 min at room temperature and then for 4, 6, 12, and 24 h at 37 °C in the atmosphere of 5% CO_2. After incubation, the delivery of the modified siRNA duplexes was tested using a fluorescent microscope and the respective images were collected (Figure 6).

Stability of CB-siRNAs against 3′-Exonuclease PDE I Analyzed by MALDI-TOF MS. Samples of the carboranyl-bearing RNA oligonucleotides s, **(CB20)s** and **(CB3)s** (0.1 ODU_{A260} in 10 μL of water) were mixed with PDE I (1 μL, 0.1 mU) and incubated at 37 °C. After 10, 20, 40, and 240 min, 1

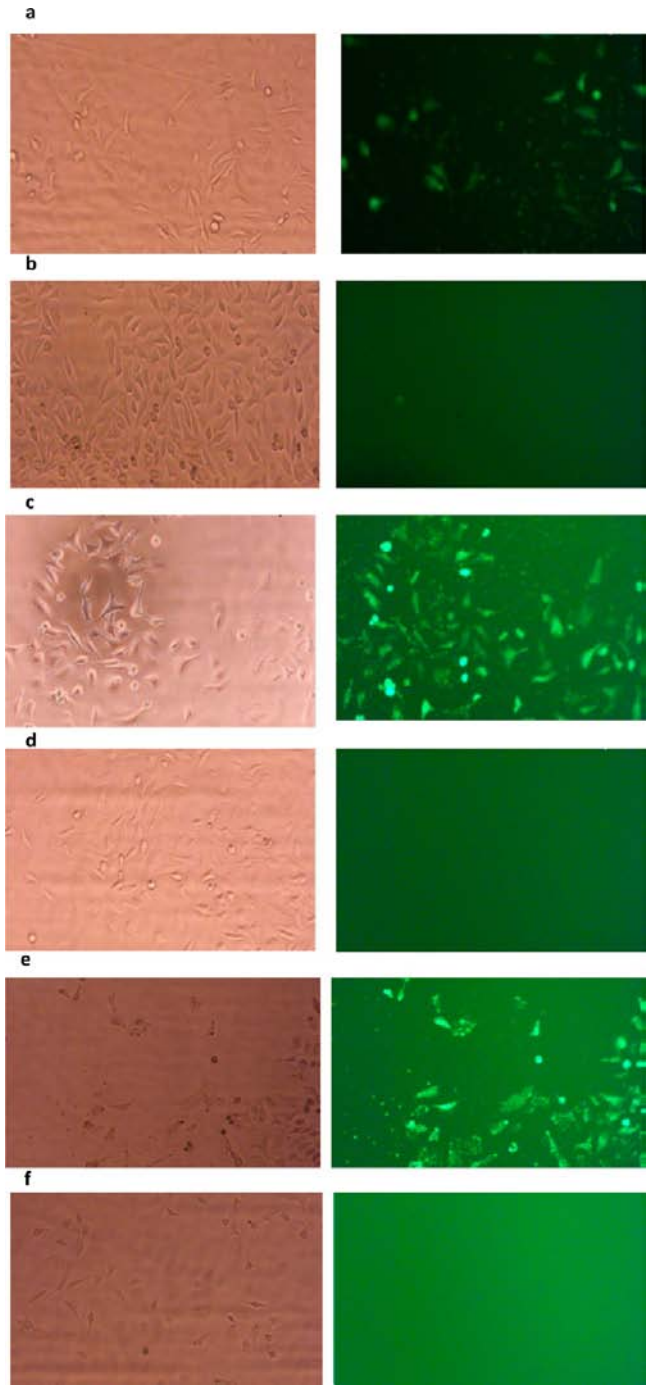

Figure 6. siRNA cellular delivery evaluated by the fluorescence microscopy. Images of HeLa cells transfected with siRNA **XIII** at a concentration of 300 nM (pictures a and b), 500 nM (pictures c and d), and 1000 nM (pictures e and f). Images of HeLa cells transfected with Lipofectamine 2000 pictures a, c, e and without the carrier b, d, f. Images show results of incubation of HeLa cells with modified siRNA **XIII** during 6 h of incubation at 37 °C in 5% CO_2; results after 4 and 12 h of incubation are not shown. Phase contrast on the left, right red filter 20× zoom lens.

μL aliquots were withdrawn, mixed with 1 μL of the matrix [2,4,6-trihydroxyacetophenone (10 mg mL^{-1} in water–acetonitrile 1:1)–diammonium citrate (50 mg mL^{-1} in water), 8:1 v/v] and applied directly to the sample plate. After 10 min of drying/crystallization the samples were

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analyzed by MALDI-TOF mass spectrometry using Voyager Elite instrument (PerSeptiveBiosystems, USA).

RESULTS AND DISCUSSION

Synthesis of Modified Monomer, 5′-*O*-Dimethoxytrityl-3-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)propan-3-yl]thymidine 3′-*O*-(*N,N*-diisopropyl-2-cyanoethyl)-phosphoramidite (5). The modified monomer[23] used for the incorporation of the boron cluster into the siRNA oligomers was synthesized in two steps (Scheme 1). First, the modification of 5′-*O*-dimethoxytritylthymidine (**1**) at position 3 was performed. For that purpose, **1**, obtained according to the literature procedure,[24] was treated with 1-(1,12-dicarba-*closo*-dodecaboran-1-yl)-3-bromopropane (**2**) in the presence of K_2CO_3 in DMF solution providing intermediate 5′-*O*-dimethoxytrityl-3-*N*-[(1,12-dicarba-*closo*-dodecaboran-1-yl)-propan-3-yl]thymidine (**3**). The boron cluster donor, 1-(1,12-dicarba-*closo*-dodecaboran-1-yl)-3-bromopropane (**2**), was obtained in the reaction of 1-(1,12-dicarba-*closo*-dodecaboran-1-yl)-3-hydroxypropane[25] with carbon tetrabromide and triphenylphosphine in methylene chloride as described earlier.[26] The alkylation of **1** with **3** at N3 was performed analogously to the method described previously with the exception that instead of carboranylalkyltosylates, bromide **2** was used.[27] Using of bromide **2** extends the range of alkylating agents available for modification of pyrimidine nucleosides at N3 with boron clusters. 5′-*O*-Dimethoxytrityl-3-*N*-[(1,12-dicarba-*closo*-dodecacarboran-1-yl)propan-3-yl]thymidine 3′-*O*-(*N,N*-diisopropyl-2-cyanoethyl)phosphoramidite (**5**) was synthesized according to the standard literature procedure[28] in the reaction of **3** with (2-*O*-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphine (**4**) with yield >50%.

Synthesis of anti-BACE1 siRNAs Modified with Boron Clusters. A set of RNA strands was synthesized to prepare thirteen siRNAs (**I-XIII**), directed toward mRNA of human BACE1 protein (Table1). The modified thymidine unit bearing the boron cluster at N3 was introduced at the position 3 or 20 of the siRNA sense strand, counting from the 5′-end [oligomers **(CB3)s** and **(CB20)s**]. The antisense strand was modified at position 20 [the oligomer **(CB20)as**]. These three modified RNA strands allowed us to obtain siRNA duplexes listed in rows **II-VI** in Table 1. Besides, the RNA strand **(F1)(CB20)s** bearing both the boron cluster and the fluorescent tag was prepared. Another two fluorescently labeled RNA strands **(F1)s** and **(F21)as** were prepared as controls. In this way we prepared the fluorescently labeled siRNA duplexes **VII-XIII**. All the measurements were referred to the nonmodified duplex **I** (s/as). The molecular mass of all tested RNA oligonucleotides was confirmed by MALDI-TOF mass spectrometry (Table 1, SI Figures S13−S18). The purity of RNA strands was assessed electrophoretically in 20% polyacrylamide/7 M urea denaturing gel (data not shown). All siRNAs used in the experiments have the typical structure of 19 base pair (bp) fully complementary duplex with 2-nucleotide (nt) overhangs at each 3′-end, typically with two thymidine units (TT). This approach is intended for the proper interaction of RNA duplex with proteins of RNAi machinery.[29] The formation of siRNA duplexes was confirmed by electrophoretic analysis in 4% agarose gel (Figure 1).

Thermodynamic Properties of siRNA Duplexes Modified with Boron Cluster. The UV-melting experiments were designed to determine the influence of the carboranyl clusters on the thermodynamic stability of the siRNA duplexes **I-VI**. Absorbance measurements were performed in triplicate in the temperature gradient of 1 °C from 15 to 96 °C. The obtained melting profiles allowed us to use the two-state model[30] and to calculate the thermodynamic parameters, such as enthalpy, entropy, and Gibbs' free energy of the siRNA duplex transition to single RNA strands, with the use of the *MeltWin* program. The respective melting temperatures and thermodynamic data are shown in Table 2 and Figure 2. In general, no significant differences ($\Delta\Delta G^\circ$ = 2.79 kcal/mol) are observed in the thermodynamic stability of the studied siRNA duplexes. Interestingly, the less stable are the siRNA **II** and **V** duplexes containing the boron cluster at their 5′-ends, i.e., composed of the **(CB20)s** strand. The stability of the remaining duplexes is similar to the stability of the reference siRNA. This result is rather surprising, at least in the case of duplexes **III** and **VI**, as in these complexes the thymidine residues are not involved in the molecular recognition with complementary units due to the absence of the hydrogen at their N3 donor centers. Probably in this case the boron cluster function offers interstrand interactions with other acceptor or donor centers of nucleobases, which may compensate for the lack of the T_B-A Watson−Crick base pairing. We hypothesize that the observed effect of boron cluster on the duplex stability can be attributed to the charge distribution within the cage resulting in "hydride-like" character of the hydrogens in some B−H groups of the cluster. This enables these hydrogen atoms to form unconventional hydrogen bonds, namely, dihydrogen bonds, also called proton−hydride bonds.[16] In addition, since the hydride-like hydrogens cannot form classical hydrogen bonds, boron clusters such as 1,12-dicarba-*closo*-dodecaborane are highly hydrophobic, which in turn may alter the hydration shell participating in the observed properties of the boron cluster containing single- and double-stranded oligonucleotides. In the duplexes **II** and **V** the boron cluster unit is positioned in the dangling 3′-ends. In this case the general effects of the steric distortion may play a dominant role, although it seems that the observed effects are sequence dependent. This hypothesis needs further experiments and molecular modeling to confirm such boron cluster−nucleobases interactions.

Analysis of the siRNA Structure by Circular Dichroism. Rana and co-workers have reported that RNAi in the RISC complex exclusively requires the A-form helix structure of the target mRNA and its guiding antisense strand.[31,32] We aimed to compare the structure of the nonmodified parent duplex **I** and the boron cluster-containing duplexes **II-VI**. The circular dichroism (CD) spectra of the duplexes **I-VI** are shown in Figure 3. The CD curves of siRNA duplexes modified with carboranyl pendants exhibit deviations from the reference A-type RNA helix curve (control siRNA **I**). The intensities of the Cotton effects at 260 nm (positive band) and at 207 nm (negative band) are smaller for carboranyl-modified duplexes **V**, **VI**, and **XIII** than those for the control siRNA. The most visible differences are noticed for the CD spectrum of duplex **XIII** containing two boron clusters and the fluorescein residue within the siRNA backbone. These results suggest that the presence of structurally constrained substituents has significant influence on the siRNA duplex structure deformation. This phenomenon could be due to some changes of angle between neighboring bases. This is especially important for the RNA duplexes, where the second and third nearest neighbors contribute together more to intensity of the CD bands than the nearest neighbors in DNA.[33]

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Silencing Activity of siRNAs Measured in a Dual Fluorescence Assay. The thymidine unit bearing the boron cluster was introduced at the ends of the sense and antisense strand to test the tolerance of RNAi for this modification.[34] The silencing activity of siRNA duplexes **I-VI** was determined in a dual fluorescence reporter system,[35] already developed for our target BACE1 gene.[12] This assay is based on comparison of the levels of fluorescence intensity of enhanced green fluorescent protein (EGFP) fused with BACE1 and the red fluorescent protein (RFP) expressed from exogenously delivered plasmids cotransfected with siRNA. In our case, the HeLa cells were transfected with pEGFP-BACE1[12,21] and pDsRED-N1, as well as with siRNAs **I-VI** listed in Table 1. The silencing activity of siRNA duplexes in HeLa cells was tested at two concentrations—1 nM and 0.1 nM. The results of the fluorescence measurements are shown in Figure 4. It is shown that all the duplexes are as active as the parent duplex, when used at higher concentration (1 nM). However, at lower siRNA concentrations some differences are visible. A small loss of activity is observed for the duplexes **II** and **V** with the carboranyl groups at their 5′-ends (i.e., on the 3′-end of the sense strand). This effect can be attributed to the steric hindrance of the boron cluster-modified duplex ends altering the interactions with the components of the RISC complex. This may result in lower loading of such duplexes into RISC. Our findings are in agreement with previously reported data that such pendant groups as puromycin or fluorescein located at the 3′-overhangs of each strand or both strands to limited extent only alter the silencing activity of the modified duplexes.[12,35,36]

Cytotoxicity of CB-siRNA Duplexes. These experiments aimed to check whether carboranyl-bearing siRNAs exhibit toxic properties toward mammalian cells. The toxicity of the siRNA duplexes **I-VI** in HeLa cells was measured using the MTT assay. The obtained results (shown at Figure 5) indicate that viability of HeLa cells treated with screened modified siRNA duplexes is similar, independent of the siRNA used and regardless of their concentration (0.1−5.0 nM).

Log*P* values. The log*P* values were determined for the unmodified RNA oligonucleotide coding the antisense strand of siRNA **I** and for two modified RNA oligonucleotides, namely, **(CB3)s** and **(CB20)**, modified with boron cluster at positions 3 of the sense strand and 20 of the antisense strand, respectively. These RNA strands are components of the duplexes **II-VI**. The RNA oligonucleotides were subjected to RP-HPLC analysis and eluted from a C-18 column with methanol−water mixtures (Figures S19−S21 in Supporting Information). As shown in Table 3 nonmodified RNA was eluted with the 22−25% methanol−water mixture, while the modified oligonucleotides were eluted with more "organic" methanol−water mixtures containing 47−53% of methanol. The results indicate that compounds modified with boron clusters are more lipophilic and exhibit higher affinity to the column support than the unmodified RNA. The remaining data, namely, the k', logk', logk'_w, and log*P* were also calculated; however, their comparison between nonmodified RNA and CB-RNAs, **(CB3)s** and **(CB20)as**, is not adequate due to the different eluting power of the eluting solvent mixtures. The pronounced effect of highly lipophilic boron cluster modification[16] on lipophilic properties of boron cluster-bearing oligonucleotides[15] has been already reported[37−40] and is consistent with present observations.

Cellular Uptake of CB-siRNA Evaluated by Fluorescence Microscopy. An ability of modified siRNA duplexes to cross the cellular membrane was studied in the model system of HeLa cells. The cells were transfected with fluorescent CB-siRNA duplex **(F1)(CB20)s/(CB20)as** bearing the boron clusters at the 3′-ends of both the sense and antisense strands and with a fluorescent tag at the 5′-end of the sense strand (Table 1, duplex **XIII**). The delivery of the CB-siRNA was tested either in the presence of Lipofectamine 2000 or without the carrier. The HeLa cells were incubated with siRNA **XIII** for 4, 16, 12, and 24 h. Figure 6 shows the fluorescent images of the transfected cells. None of them show the presence of fluorescence in the cells treated exclusively with carboranyl-siRNA. In contrary, the expected fluorescent siRNAs are visible in the cells treated with fluorescent siRNA delivered in the presence of Lipofectamine 2000 carrier. These results indicate that the boron cluster pendants most probably do not interact with the negatively charged cell membrane and do not facilitate the duplex siRNA transfer into the cell.

Stability of CB-siRNAs against 3′-Exonuclease PDE I analyzed by MALDI-TOF MS. Nucleolytic stability of the carboranyl-bearing RNA strands **(CB20)s** and **(CB3)s** was evaluated in the presence of the snake venom phosphodiesterase (svPDE) exhibiting 3′-exonucleolytic activity. For comparison nucleolytic stability of the nonmodified sense strands of the reference duplex **I** was also analyzed. The oligonucleotides were incubated with the 3′-exonuclease for a given time and the reaction mixtures were analyzed by the MALDI-TOF mass spectrometry. The products were identified by assigning to the molecular peaks (m/z) present in the spectrum. The digestion of the nonmodified strands of the reference duplex was more effective (T1/2−20 min) than of the carboranyl-bearing RNA strands (T1/2 ca. 190 min for **(CB20)s** and T1/2 ca. 60 min for **(CB3)s**) (data shown in Supporting Information, Figures S22, S23). While for the strand bearing carboranyl residue at its 3′-end the cleavage was stalled at the modification site, resulting in formation of the negative ion of (CB20)s-pT of m/z 6473, we could observe the residual m/z peak of 5986 corresponding to the product of the cleavage of the RNA chain upstream of the carboranyl-residue (removal of pTCBpT-3′ fragment). This shorter product is formed due to the "hopping" properties of the 3′-exonuclease through modified fragments of the nucleic acid chain.[41,42] Interestingly, the cleavage of the **(CB3)s** RNA strand was also exhibited to some extent (T1/2 ca. 60 min), despite the location of the boron cluster at the 5′-end of the RNA chain, which is opposite the cleavage site of svPDE. These results suggest that the presence of the carboranyl-modified units in the siRNA backbone may increase the duplex stability in the presence of nucleases independent of their location, which might be beneficial for their potential *in vivo* applications. Our assumption is based on the published data which show that increase of the stability of one siRNA strand by introduction of the 2′-fluoro-uridine and 2′-fluoro-cytidine (2′-FU and 2′-FC) units is beneficial for the entire siRNA complex with respect to its nucleolytic stability in the HeLa cell extract.[31]

CONCLUSIONS

Successful incorporation of 3-*N*-[(1,12-dicarba-*closo*-dodecacarboran-1-yl)propan-3-yl]thymidine, a novel boron cluster modification, into the RNA chain opened the possibility to obtain siRNA duplexes bearing carboranyl pendants in their 3′- and 5′-end strategic positions. These siRNAs are as thermodynamically stable as the parent nonmodified duplex, with the highest ΔTm of 4 °C. Their helical structure differs from the parent siRNA duplex. However, the silencing activity

is basically not changed as compared to the reference unmodified duplex. The only observed difference in thermodynamic stability and silencing activity is attributed to siRNA duplexes possessing boron cluster at their 5′-end (in the 3′-end of the sense strand). Probably, the introduction of a large boron cluster group at this position reduces affinity of the siRNA duplex to the RISC complex. Modified duplexes have cytotoxicity as low as that of the reference siRNA duplex. Although carboranyl-modified RNAs exhibit substantially higher lipophilicity compared to unmodified RNA strands it seems that this feature does not facilitate siRNA uptake through the cell membrane in this case. The main advantage of the boron cluster modification relies on the protection of the siRNA duplex against nucleolytic degradation, nonaltered silencing potential, and low cytotoxicity of CB-siRNAs, which may be beneficial in biological application.

■ ASSOCIATED CONTENT

Supporting Information

ATR-IR, ^{1}H NMR, FAB-MS spectra of compound **3**; ATR-IR, ^{31}P NMR, FAB-MS spectra of compound **5**; RP-HPLC traces and MALDI-TOF MS spectra of oligonucleotides **(CB3)s**, **(CB20)as**, **(CB20)s**, **(F1)s**, **(F21)as**, **(F1)(CB20)s**; RP-HPLC profiles of the RNA oligonucleotides **(CB3)s** and **(CB20)as** eluted from a C-18 column with methanol−water mixtures for the log*P* determination; MALDI-TOF MS analysis of oligonucleotides **s**, **(CB3)s**, and **(CB20)s** RNA digested in the presence of the 3′-exonuclease PDE I. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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