Article

Novel Lipid-Oligonucleotide Conjugates Containing Long-Chain Sulfonyl Phosphoramidate Groups: Synthesis and Biological Properties

Alina Derzhalova 1,2,†, Oleg Markov 2,†, Alesya Fokina 1,3,†, Yasuo Shiohama 4, Timofei Zatsepin 5,6,*, Masayuki Fujii 7,*, Marina Zenkova 2 and Dmitry Stetsenko 1,3,*

1 Department of Physics, Novosibirsk State University, 630090 Novosibirsk, Russia; alina.derzhalova@gmail.com (A.D.); alesya_fokina@mail.ru (A.F.)
2 Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, 630090 Novosibirsk, Russia; markov_oleg@list.ru (O.M.); marzen@niboch.nsc.ru (M.Z.)
3 Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, 63090 Novosibirsk, Russia
4 Tropical Biosphere Research Center, University of the Ryukyus, Okinawa 903-0213, Japan; h194005@comb.u-ryukyu.ac.jp
5 Skolkovo Institute of Science and Technology, Innovation Centre Skolkovo, 121205 Moscow, Russia; tsz@yandex.ru
6 Department of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia
7 Department of Biological & Environmental Chemistry, Kindai University, Iizuka, Fukuoka 820-8555, Japan; mfujii@fuk.kindai.ac.jp
* Correspondence: d.stetsenko@nsu.ru; Tel.: +7-383-363-4963
† These authors contributed equally to the manuscript.

Abstract: New lipid conjugates of DNA and RNA incorporating one to four [(4-dodecylphenyl)sulfonyl] phosphoramidate or (hexadecylsulfonyl)phosphoramidate groups at internucleotidic positions near the 3′ or 5′-end were synthesized and characterized. Low cytotoxicity of the conjugates and their ability to be taken up into cells without transfection agents were demonstrated. Lipid-conjugated siRNAs targeting repulsive guidance molecule a (RGMa) have shown a comparable gene silencing activity in PK-59 cells to unmodified control siRNA when delivered into the cells via Lipofectamine mediated transfection.

Keywords: therapeutic nucleic acid; drug delivery; nanoparticles; cytotoxicity; macrophages; cellular uptake; small interfering RNA; multiple sclerosis; repulsive guidance molecule a

1. Introduction

Nucleic acid derivatives such as antisense oligonucleotides or small interfering RNAs (siRNAs) are extensively studied as therapeutic agents that target biologically important RNAs; either mRNAs or noncoding RNAs such as micro-RNAs via either enzyme-mediated RNA cleavage [1–3] or steric blocking of RNA function [4,5]. While the former may be applicable to a wide range of diseases including some genetic disorders [6], cancer [7], viral [8] and bacterial [9] infections, where the target RNA can be eliminated, the latter is often employed where a fine tuning of the RNA activity is required such as in splice switching [10]. The progress in the development of oligonucleotide-based therapies in the past few years has resulted in the recent approval of several antisense and siRNA drugs by the FDA and EMA [11]. However, widespread application of therapeutic oligonucleotides still suffers from their relatively inefficient cellular uptake and often poor bioavailability [12,13]. The ways of promoting cellular delivery of oligonucleotides and their analogues include their structural modification [14,15], noncovalent binding [16] or covalent conjugation [17,18] to the molecules that could improve translocation through the outer membrane and direct intracellular trafficking of nucleic acids. Among these, lipids in a wider sense...
including biomolecules such as cholesterol, tocopherol (vitamin E) or fatty acids, and their synthetic analogues such as, e.g., DOTMA [19] have been shown to improve cellular uptake of oligonucleotides, most relevantly, in the form of lipid-oligonucleotide conjugates (LONs) [20–22].

In particular, lipid conjugates of antisense oligonucleotides and siRNAs have improved transfection ability and extended half-life in the bloodstream with beneficial impact on therapeutic activity in vivo [23–25]. We describe herein a series of novel DNA and RNA lipid conjugates, which have been chemically modified by one to four long-chain N-(sulfonyl)phosphoramidate groups, namely, [(4-dodecylphenyl)sulfonyl]-phosphoramidate group (δ) or (hexadecylsulfonyl)phosphoramidate group (η) at the internucleotidic position(s) near the 3′- or 5′-end of the oligonucleotide chain (Figure 1). The oligonucleotides were obtained by an automated phosphoramidite solid-phase synthesis protocol that employs the Staudinger reaction between the support-bound phosphate triester and the respective sulfonyl azide instead of usual aqueous iodine oxidation to introduce either of the lipophilic modifications following a previously developed procedure [26–28].

Figure 1. Structures and positions of hydrophobic groups in lipid-oligonucleotide conjugates used in this study: oligodeoxynucleotide (A); small interfering RNA (siRNA) (B).

The obtained lipid conjugates of oligodeoxynucleotides with one or two of the respective hydrophobic groups were nearly non-cytotoxic for either murine or human macrophages up to 20 μM concentration. Both types of lipid conjugates were able to enter the cells in a carrier free manner. Both features are beneficial for potential therapeutic application. The corresponding lipid conjugates of siRNAs with either two or four hydrophobic groups were designed to downregulate a validated target in multiple sclerosis, namely, repulsive guidance molecule a (RGMa) [29]. The conjugates with two groups were shown to possess silencing ability comparable to the unmodified siRNA when delivered by lipofectamine. This type of lipid conjugate may represent a useful addition to the inventory of therapeutic oligonucleotide derivatives.

2. Materials and Methods

2.1. Oligonucleotide Synthesis

Oligodeoxynucleotides and siRNAs containing [(4-dodecylphenyl)sulfonyl]phosphoramidate (δ) or (hexadecylsulfonyle)phosphoramidate (η) groups were assembled by automated solid-phase synthesis on a Mermaid MM-12 DNA/RNA synthesizer according to a modified β-cyanoethyl phosphoramidite protocol replacing aqueous iodine oxidation by a Staudinger reaction with either 4-dodecylbenzenesulfonyl azide or 1-hexadecanesulfonyl azide (0.5 M in acetonitrile for (δ) or in acetonitrile—THF (2:1 v/v) for (η), 120 min at
ambient temperature) in an appropriate cycle of chain elongation [27, 28]. Standard N-protected 5′-DMTr-3′-β-cyanoethyl-N,N′-phosphoramidites of deoxy, 2′-O-TBDMS-ribo and 2′-O-methylribonucleotides (Sigma-Aldrich Inc., St Louis, MO, USA) and 1000 Å CPG polymer supports (Glen Research Corp, Sterling, VA, USA) were used. After the completion of the synthesis, oligonucleotides were cleaved from support and deprotected by standard procedures followed by consecutive PAGE and RP-HPLC purification as described previously [30]. Molecular masses of the oligonucleotides were verified by ESI LC-MS (Table 1).

Table 1. Structures and molecular masses of oligonucleotides and their lipid conjugates (LONs).

| Designation | Oligonucleotide Sequence, 5′–3′ | Molecular Mass, Da² |
|-------------|-------------------------------|-------------------|
| ODN         | agtcgacttgctacc              | 5121.25           |
| ODN-Flu     | aagtcgacttgctacc-Flu         | 5120.98           |
| 1δ          | a6gtcgcacgctacc              | 5248.86           |
| 2δ          | a2g8tctcgacttgctacc          | 5746.18           |
| 18-F        | a6gtcgcagttgctacc-3′-Flu     | 5996.34           |
| 28-F        | a6g9tctcgacttgctacc-Flu      | 6303.85           |
| 1η          | a6gtcgcacgctacc              | 5407.83           |
| 2η          | a2g8tctcgacttgctacc          | 5696.18           |
| 1η-Flu      | a6gtcgcagttgctacc-3′-Flu     | 5976.34           |
| 2η-Flu      | a6g9tctcgacttgctacc-Flu      | 6263.85           |
| EGFP        | Sense CCAUCGCGAAGCACCCUGA     | 6698.11           |
| RGMa        | Antisense AGGUUGACCGUUGGAGGUG | 6812.10           |
| 2Δ-RGMa     | Sense CCAUCGCGAAGCACCCUGA     | 6563.99           |
| 4Δ-RGMa     | Antisense AGUUUGACCGUUGGAGGUG | 6806.20           |
| 2H-RGMa     | Sense CCAUCGCGAAGCACCCUGA     | 6563.99           |
| 4H-RGMa     | Antisense AGUUUGACCGUUGGAGGUG | 6806.20           |

1 Lowercase letters indicate deoxyribonucleotides, uppercase letters—ribonucleotides, underlined uppercase—2′-O-methylribonucleotides.
2 According to ESI LC-MS in the negative ions mode.
3 Control oligonucleotide with random sequence specially constructed not to affect gene expression in cells.
4 Flu—3′-terminal fluorescein label.
5 The symbols (δ), (η) mark the positions of dodecylphenyl or hexadecyl groups, respectively.
6 p—3′-terminal phosphate group.

4-Dodecylbenzenesulfonyl azide was obtained from a commercial supplier (Sigma-Aldrich Inc., St Louis, MO, USA). 1-Hexadecanesulfonoyl azide was prepared from the corresponding sulfonyl chloride and a slight excess of sodium azide in acetonitrile—THF (1:1 v/v) as a white solid as described in [31]. IR, 1H and 13C NMR spectra of the compound were consistent with the structure.

2.2. Dynamic Light Scattering (DLS) Characterization

Lipid-oligonucleotide conjugates (LONs) were analyzed at room temperature by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). Size was measured in a DTS0012 cuvette (Malvern Instruments Ltd., Malvern, UK). All the scattered photons were collected at a 173° scattering angle. Measurement conditions were: material protein (RI: 1.450; Absorption: 0.001), dispersant water (Viscosity: 0.8872 cP; RI: 1.330) temperature at 25 °C and equilibration time was 3 s. Each test was triplicated. Nanoparticle size was determined in deionized water at 5 μM oligonucleotide concentration.
cleotide concentration. The scattering intensity data was processed using the instrumental software to obtain the hydrodynamic diameter (Dh).

2.3. Cell Lines

RAW 264.7 cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich Inc., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GE Healthcare, Chicago, IL, USA) and 1% antibiotic/antimycotic mix (100 unit/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin) at 37 °C in a humidified atmosphere with 5% CO2 (standard conditions, SC) and passaged regularly to maintain exponential growth.

THP-1 cells were cultivated in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 1% antibiotic/antimycotic mix (100 unit/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin), 1% GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), 1% HEPES (Thermo Fisher Scientific, Waltham, MA, USA) and 1% glucose (Sigma-Aldrich Inc., St Louis, MO, USA) under SC and passaged regularly to maintain exponential growth.

To generate human macrophages (Mϕ) THP-1 cells were cultivated in complete RPMI medium in the presence of 200 nM PMA (Sigma-Aldrich Inc., St Louis, MO, USA) under SC for 48 h. Then, the culture medium was replaced with complete RPMI medium in the absence of PMA and cells were incubated for an additional 24 h under SC.

PK-59 human pancreatic cancer cells were cultivated in complete RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) under SC and passaged regularly to maintain exponential growth.

2.4. MTT Assay of Cell Viability

RAW264.7 cells were seeded in 96-well plates at a density of $5 \times 10^3$ cells/well in complete DMEM and incubated for 24 h under SC to adhere. THP-1 cells were seeded in 96-well plates at a density of $50 \times 10^3$ cells/well in complete DMEM medium supplemented with 200 nM PMA and incubated for 72 h under SC to generate human Mϕ as described above. Then, RAW264.7 and Mϕ cells were incubated in the corresponding complete culture media supplemented with 5, 10 and 20 µM of the respective lipid-oligonucleotide conjugate (LON) for an additional 24 h under SC. Cytotoxicity of LONs was assessed by MTT assay. Briefly, MTT solution (Sigma-Aldrich, Darmstadt, Germany) was added to the cells up to 0.5 mg/mL and the incubation was continued under SC for an additional 2 h. Then, the MTT-containing medium was aspirated and the formazan crystals formed in living cells were solubilized with 100 µL/well of DMSO. The absorbance of each well was read at the test and reference wavelengths ($\lambda$) of 570 and 620 nm, respectively, on a Multiscan RC plate reader (Thermo LabSystems, Helsinki, Finland). The percentage of living cells was calculated as follows:

$$\text{Living cells (\%) } = \frac{\text{OD}_{570\text{exp}} - \text{OD}_{620\text{exp}}}{\text{OD}_{570\text{cont}} - \text{OD}_{620\text{cont}}} \times 100, \quad (1)$$

where $\text{OD}_{570\text{exp}}$ and $\text{OD}_{570\text{cont}}$ correspond to optical density in experimental and control wells, respectively, at $\lambda$ 570 nm, and $\text{OD}_{620\text{exp}}$ and $\text{OD}_{620\text{cont}}$ correspond to optical density in experimental and control wells, respectively, at $\lambda$ 620 nm. All experimental points were run in triplicate for statistical analysis.

2.5. Flow Cytometry

Intracellular accumulation of the lipid-oligonucleotide conjugates labeled with fluorophores, namely $\delta$-Flu, $\varphi$-Flu, $\eta$-Flu and $\varphi$-Flu, was assessed in RAW264.7 and human Mϕ by flow cytometry. RAW264.7 cells were seeded into 24-well plates at a density of $80 \times 10^3$ cells/well in complete DMEM and cultivated overnight to adhere. THP-1 cells were seeded into 24-well plates at a density of $100 \times 10^3$ cells/well in complete RPMI medium supplemented with 200 nM PMA and incubated for 72 h under SC to generate human Mϕ as described above. Subsequently, RAW 264.7 and Mϕ were incubated
in the corresponding complete media supplemented with 5 μM of the respective lipid-oligonucleotide conjugate under SC for 24 h. After incubation, cells were detached from the plate with 2% trypsin (MP Biomedicals, Irvine, CA, USA), resuspended in a complete culture medium, centrifuged at 200 × g for 5 min, washed with PBS and fixed with 2% formaldehyde in a PBS buffer (10 min, room temperature). Cells were analyzed on a NovoCyte flow cytometer (ACEA Biosciences, Santa Clara, CA, USA) and the data were processed with a NovoExpress (ACEA Biosciences, Santa Clara, CA, USA). All experiments were run in triplicate for statistical analysis. The transfection efficiency (TE) was characterized by two values: percentage of fluorescence-positive cells in a population and mean fluorescent intensity (MFI) of cells in a sample.

2.6. Confocal Microscopy

RAW264.7 cells were seeded on coverslips (Marienfeld, Lauda-Königshofen, Germany) in 24-well plates at a density of 20 × 10^3 cells/well in complete DMEM medium and incubated for 24 h under SC to adhere. THP-1 cells were seeded on coverslips in 24-well plates at a density of 100 × 10^3 cells/well in complete RPMI medium supplemented with 200 nM PMA to obtain human Mφ as described above. Afterwards, RAW 264.7 cells and human Mφ were incubated in the corresponding complete media in the presence of 5 μM of the respective lipid-oligonucleotide conjugates under SC for 24 h. After incubation, coverslips with cells were washed twice with PBS, fixed with 3.7% formaldehyde in PBS buffer (15 min, 37 °C), and washed twice with PBS. The cells were stained with the Phalloidin-iFluor532 (Abcam, Cambridge, UK) or Phalloidin-TRITC (ECM Biosciences, Versailles, KY, USA) according to the manufacturer’s protocols. After staining, the cells were washed twice with PBS and placed on slides in a drop of ProLongTM Glass Antifade Mountant with NucBlueTM (Thermo Fisher Scientific, Waltham, MA, USA). Mounted samples were allowed to cure on a flat, dry surface for 18–24 h at room temperature in the dark. Intracellular localization of lipid-oligonucleotide conjugates was assessed by confocal fluorescent microscopy on an LSM710 (Zeiss, Oberkochen, Germany) using an αPlan-Apochromat 100x/1.46 Oil DIC M27 objective. Analysis of intracellular accumulation of LONs and Z-stacks was performed using ZEN software (Zeiss, Oberkochen, Germany). Confocal microscopic analysis was performed in three channels (blue, green, red). Fluorescence in the blue channel corresponded to DAPI (nuclei staining); the green channel corresponded to fluorescence of LONs labeled with fluorescein (Flu), and the red channel corresponded to Phalloidin-iFluor532 or Phalloidin-TRITC (cytoskeleton staining).

2.7. Gene Silencing

Experiments on siRNA-mediated silencing of repulsive guidance molecule a (RGMa) were carried out in the RGMa-expressing PK-59 human pancreatic cancer cell line. On the day of transfection, PK-59 cells were separately seeded in 24-well flat-bottomed plates at a density of 4 × 10^5 cells/well in 0.5 mL of growth medium without antibiotics. The cells were transfected with lipid-conjugated RGMa siRNAs carrying either two (passenger strand only) or four (both passenger and antisense strands) dodecylphenyl (Δ) or hexadecyl (H) groups at their 3'-end, or unmodified control RGMa siRNA (Table 1) at concentrations 1, 10, or 100 nM in a complex with Lipofectamine® 2000 (2 μL/well) according to the manufacturer’s instructions (Invitrogen Life Technologies Corporation, Frederick, MD, USA). The cells were then incubated at 37 °C in the atmosphere of 5% CO₂ in a humidified incubator. Cells were harvested and assayed 24 h after transfection.

The level of RGMa expression was quantified by real-time, two-step reverse transcriptase PCR (qRT-PCR) assay. Briefly, total RNA was extracted from the collected cells using ISOGNE (Nippon Gene, Ltd., Toyama, Japan), treated with DNase I® (ThermoFisher Scientific, Waltham, MA, USA) and retro-transcribed using Oligo (dT)₂₀ primer and ReverTra Ace® (Toyobo Co., Ltd., Japan). The resulting cDNA was quantified using the AriaMx Real-Time PCR System® (Agilent Technologies, Ltd., Santa Clara, CA, USA) using the following primers: 5’–TGGCCGCTCATCGACAATAA–3’, forward; and 5’–
GCTGTCCCCACCGTTCTTA–3′, reverse. Quantifications of RGMa mRNA expressions were normalized by the Comparative Ct Method (Ct) using 18S rRNA as an endogenous control to ensure accuracy; primers: 5′–GTAACCCGTTGAACCCCAT–3′, forward; and 5′–CCATCCAATCGGTAGTAGCG–3′, reverse [32]. All the data are the means of triplicate determinations. Each determination was calculated from triplicate measurements. Each error bar indicates a standard deviation of the triplicate determinations. Non-silencing siRNA targeting EGFP was used as a negative control, which was designed to have a minimum of 4 mismatches to all human, mouse and rat genes, and confirmed to have minimal targeting by genome-wide microarray analysis [33]. Significant differences in the results were determined by the two-tailed Student’s t-test using GraphPad Prism. A probability value of \( p < 0.05 \) was considered to be significant in the present study.

3. Results

3.1. Synthesis of Lipid-Oligonucleotide Conjugates (LONs) Incorporating Long-Chain (Sulfonyl)Phosphoramidate Groups

The synthesis of lipid conjugates of oligodeoxynucleotides having one or two ((4-dodecylphenyl)sulfonyl)phosphoramidate (δ) or (hexadecylsulfonyl)phosphoramidate (η) modifications of the phosphate linkages adjacent to the 5′-end (Figure 1A) was accomplished according to our previously reported protocols of automated solid-phase assembly via the phosphoramidite method employing the Staudinger reaction of the support-bound phosphite triester formed during the phosphoramidite coupling with 0.5 M solution of either 4-dodecylbenzenesulfonyl azide or 1-hexadecanesulfonyl azide in acetonitrile for 120 min at ambient temperature instead of the usual aqueous iodine oxidation [23,24]. Oligonucleotides containing 5′-dimethoxytrityl group (DMTr) were isolated by reverse-phased (RP) HPLC or, if necessary, purified to homogeneity by denaturing PAGE under the same conditions as normal phosphodiester oligodeoxynucleotides, followed by RP-HPLC to remove phosphodiester impurities. The molecular masses of the δ- and η-oligodeoxynucleotides obtained have been verified by ESI LC-MS (Table 1).

Sequences of siRNAs targeting RGMa mRNA were obtained from literature [25]. Lipid-siRNA conjugates containing 2–4 dodecylphenyl (δ) or hexadecyl (η) groups in the antisense and/or passenger chains were prepared according to the method above (Table 1). The 3′-overhangs of the siRNAs were modified by 2′-O-methyl diribonucleotides to increase enzymatic stability and avoid possible side reactions associated with the neighboring 2′-OH group when sulfonyl phosphoramidate groups were introduced into the overhang and at the 3′-terminal phosphate group (Figure 1B).

3.2. Spontaneous Nanoparticle Formation by LONs in Aqueous Media

As amphiphiles, lipid-oligonucleotide conjugates (LONs) can be expected to spontaneously form micelles in aqueous solutions. Indeed, dynamic light scattering (DLS) analysis revealed that both 2δ and 2η LONs formed nanoparticles in water characterized by mean diameters of 21.6 ± 8.8 and 79.7 ± 16.4 nm for 2η and 2δ, respectively (Figure 2). Interestingly, longer aliphatic chain (16-carbon vs. 12-carbon) at the internucleotidic position favored the formation of smaller nanoparticles, which may be responsible for a better cellular uptake of a more hydrophobic 2η LON (Figure 2).

3.3. LONs were Nontoxic to Murine or Human Macrophages at Therapeutic Concentrations

Introduction of lipophilic moieties into oligonucleotide chains can alter such properties of LONs as cytotoxicity. Cytotoxicity of the most hydrophobic LONs, namely, 2δ and 2η, was analyzed with respect to murine RAW264.7 and human THP-1-derived macrophages. Cells were incubated in complete media supplemented with different concentrations of one of LONs for 24 h followed by estimation of cell viability using colorimetric MTT assay. As depicted in Figure 3, both 2δ and 2η oligonucleotides were nontoxic to murine and human macrophages. \( IC_{50} \) obtained by extrapolation of dose-response curves ranged from 74 to 120 µM for 2δ and from 77 to 85 µM for 2η oligonucleotides, whereas the working
concentration of LONs in subsequent experiments was chosen to be 5 µM. It is worth mentioning that murine macrophages were more sensitive to LONs in comparison with human THP-1-derived macrophages (IC$_{50}$ were 74.2 ± 29.6 µM and 120.3 ± 31.6 µM for 2δ oligonucleotides, respectively; 77.0 ± 9.2 µM and 84.6 ± 17.2 µM for 2η oligonucleotides, respectively) (Figure 3).

Figure 2. Hydrodynamic diameters of nanoparticles formed spontaneously in water by 2δ (A) and 2η (B) lipid-oligonucleotide conjugates. Dynamic light scattering (DLS) data.

Figure 3. Dose-response curves of 2δ and 2η LONs for RAW264.7 (A,B) and human THP-1 induced macrophages (C,D). Cells were incubated with LONs in a complete medium for 24 h. The results are shown as a percentage of viable cells observed after cell incubation with LON relative to untreated cells (100%). All experimental points were run in triplicate for statistical analysis. Data are presented as MEAN ± SD. The dotted curve shows extrapolation to 50% of viable cells.

3.4. Conjugates with Longer Lipidic Chains Accumulated in Cells with Higher Efficiency

The ability of LONs to accumulate in eukaryotic cells in the absence of a transfection agent (gymnosis) was studied by flow cytometry using fluorescein-labeled LONs and murine macrophages RAW264.7 or THP-1-induced human macrophages. The efficiency...
of intracellular accumulation of LONs was assessed by two parameters: the number of fluorescein-positive cells in the population and average level of the conjugate content in the cells (mean fluorescence intensity, MFI).

Accumulation of LONs in RAW264.7 and THP-1-induced macrophages differed considerably. LONs accumulated in RAW264.7 cells with moderate efficiency: 20–50% of cells were transfected with a MFI of $6 \pm 11 \times 10^3$ RFU (Figure 4A,B). As expected, the cells incubated and control oligonucleotide ODN-F displayed the lowest efficiency: 10% of fluorescent cells with MFI of $4 \times 10^3$ RFU, which corresponded to the baseline level of oligonucleotide taken up by the macrophages and/or adhered to the cell surface. We observed that more hydrophobic LONs, that is, the ones containing either two hydrocarbon chains instead of only one, or those having a longer aliphatic group: hexadecyl instead of dodecylphenyl, were showing more efficient accumulation in RAW264.7 cells (Figure 4A,B: compare conjugates having two vs. one group, and η- vs. δ-modified LONs) than their less hydrophobic counterparts and unmodified control ODN-F.

A similar but much more prominent accumulation of lipid-conjugated oligodeoxynucleotides was observed in human THP-1-induced macrophages. LONs accumulated in these cells with higher efficiency both in terms of the number of transfected cells and mean fluorescence intensity (Figure 4C,D). Conjugates 1δ-, 1η-, and 1F containing one lipophilic group displayed similar transfection efficiency regardless of the type of lipid chains, either dodecylphenyl (δ) or hexadecyl (η): 43% cells were transfected with MFI of $8 \times 10^3$ RFU, which was fourfold higher as compared to the control ODN-F (Figure 4C,D). Increasing the number of lipophilic groups from one to two resulted in a significant enhancement of transfection efficiency. 2δ LON yielded 85% cells with fluorescence intensity $25 \times 10^3$ RFU whereas 2η conjugate was taken up with the highest efficiency, accumulating in nearly 100% cells with fluorescence intensity $150 \times 10^3$ RFU (Figure 4C,D), which is on par with the level achieved by liposome-mediated oligonucleotide delivery [34].

3.5. Lipid-Oligonucleotide Conjugates are Efficiently Taken up by Cells in the Absence of Transfection Agents and Localize Mostly in the Cytoplasm within Endosomes

Confocal microscopic analysis was carried out to verify and characterize intracellular localization of LONs. RAW 264.7 and human THP-1-induced macrophages were incubated with 5.0 μM fluorescein-labeled LONs in complete medium for 24 h. After incubation, cells
were stained with Phalloidin-iFluor532 or Phalloidin-TRITC (actin microfilaments) and NucBlue (nuclei) (Figures 5–7).

Figure 5. Intracellular localization of 2η LON in murine macrophages RAW264.7 (A) and THP-1-induced human macrophages (B). Analysis was performed by confocal fluorescent microscopy (αPlan-Apochromat 100x/1.46 Oil DIC M27 objective) 24 h post transfection. Data are presented as orthogonal projections. Blue signal is DAPI (nuclear staining), green signal is fluorescein-labeled 2η LON, red signal is Phalloidin-iFluor532 (A) or Phalloidin-TRITC (B). Scale bar 10 μm.

Figure 6. Intracellular accumulation of LONs in RAW264.7. Analysis of samples was performed 24 h after addition of fluorescein-labeled conjugates (5 μM) to the cells. Analysis was performed using αPlan-Apochromat 100x/1.46 Oil DIC M27 objective. Three-channel (BGR) pictures were obtained using staining by DAPI (nuclei staining) (B); fluorescein-labeled LONs (G); Phalloidin-iFluor532 (cytoskeleton staining) (R). Scale bars 20 μm. ODN-F—control unmodified oligonucleotide labeled with fluorescein.

Firstly, to prove that LONs do not expose on the cell membranes but do penetrate into the cells, confocal microscopic analysis was performed and Z-stack images were built. It was demonstrated that LON 2η, the most efficient one according to flow cytometry data, was indeed localized in the cytosol of RAW264.7 cells and THP-1-induced macrophages (Figure 5). In addition, LON 1η was shown to efficiently accumulate in KB-8-5 cells (see Supplementary Materials, Figure S4).
It was demonstrated that LON 2η, the most efficient one according to confocal microscopy (Figures 6 and 7) correlate well with the obtained flow cytometry data (Figure 4). Oligonucleotides containing two lipophilic groups accumulated in cells with much higher efficiency in comparison with LONs containing only one group, LON 2η with two hexadecyl groups being the best penetrator. LONs 1δ-F and 1η-F containing only one lipophilic modification accumulated in cells with efficiency not significantly higher than that of the control unmodified oligonucleotide ODN-F. However, these LONs penetrated into the cells whereas ODN-F mainly adhered to the cell membrane (Figure S5, Supplementary Materials).

In detail, LONs were evenly distributed in the cytosol, localizing mainly in endosomes; this was conjectured from the presence of numerous bright green fluorescent dots containing LONs in the cytosol of both cell lines tested (Figures 6 and 7).

3.6. Lipid-Conjugated RGMa siRNAs Demonstrated Gene-Silencing Ability Similar to Unmodified RGMa siRNA in PK-59 Human Pancreatic Carcinoma Cell Culture

The gene silencing ability of lipid-conjugated siRNAs in comparison with unconjugated siRNA was elucidated in human pancreatic carcinoma PK-59 cells expressing repulsive guidance molecule a (RGMa). RGMa is a glycoprotein, which is a potent inhibitor of axonal re-myelination and, thus, is regarded as a potential therapeutic target for multiple sclerosis (MS) treatment [35]. Inhibition of RGMa by human monoclonal antibodies promotes neuronal regeneration and repair [36]. RGMa siRNA sequence was obtained from literature [29]. Lipid-conjugated RGMa siRNAs were modified at the 3′-end by either two (passenger strand only) or four (both passenger and antisense strands) dodecylphenyl (Δ) or hexadecyl (H) groups. To compare silencing activity under similar conditions, transfection was carried out at concentrations of siRNA 1, 10 and 100 nM in the presence of Lipofectamine® 2000. The levels of RGMa expression as assessed by RT-PCR are shown in Figure 8.
Figure 8. Inhibition of RGMa expression in PK-59 human pancreatic carcinoma cell culture by lipid conjugates of RGMa siRNAs vs. unmodified siRNA (RGMa). The conjugates were modified near the 3’-end by two (passenger chain only) or four (both chains) lipophilic groups (Δ—dodecylphenyl, H—hexadecyl).

The gene silencing effect of siRNA conjugates 2Δ-RGMa and 2H-RGMa that carried two lipophilic groups at the 3’-end of the passenger strand was comparable to that of the unconjugated RGMa siRNA at a concentration of 100 nM. At the same time, the activity of the conjugates 4Δ-RGMa and 4H-RGMa containing four lipophilic groups at both 3’-ends of passenger and antisense strands was reduced as compared to the unmodified control siRNA.

4. Discussion

A previously developed methodology of Staudinger reaction-mediated in-line oligonucleotide modification [26–28] was employed herein to obtain lipophilic conjugates of oligodeoxynucleotides and siRNAs carrying one or more extended hydrocarbon chains, 4-dodecylphenyl or hexadecyl, attached to the internucleotidic phosphate groups via the (sulfonyl)phosphoramidate linkage, which is hydrolytically stable and carries negative charge under physiological conditions (Figure 1A,B). The scheme allows one to modify any internucleotidic or terminal phosphate group within a DNA or 2′-OMe RNA chain from the 5’– to 3’–end by a facile chemical reaction with a stable sulfonyl azide precursor during automated DNA/RNA assembly without recourse to a special phosphoramidite monomer, which is usually obtained by a multi-step synthesis [18,20,21]. The obtained lipid-oligonucleotide conjugates (LONs) can be conveniently isolated by the usual C18 reverse-phased HPLC, due to significant difference in retention times with unmodified oligonucleotides (see Supplementary Materials) for HPLC profiles and ESI-MS spectra. Our preliminary data have shown that such lipophilic groups are fully compatible with other phosphate group modifications such as phosphorothioate, mesyl phosphoramidate [27,37,38] and phosphoryl guanidine [39].

Notably, we have observed spontaneous nanoparticle formation in aqueous solution by LONs containing two adjacent hydrophobic chains, with nanoparticle size dependent on the type of the side-chain (Figure 2). Nanoparticle formation by oligonucleotides is thought to be essential for good cellular uptake [40], structured RNA studies [41] and design of stimuli-responsive drug delivery systems [42].

Low cytotoxicity is another beneficial characteristic of such LONs, which allows one to vary the concentration of an oligonucleotide over a wide range to optimize its therapeutic dosage for further in vitro and in vivo studies. We have confirmed that LONs
used in this study evinced low cytotoxicity. Calculated IC50 of studied LONs fell within the 75 to 120 µM range, which was significantly higher than concentrations of antisense oligonucleotides targeting therapeutically relevant RNAs commonly used in vitro and in vivo: nanomolar concentrations [43–46] or <5 µM [47] or therapeutic doses of siRNAs and antisense oligonucleotides approved by FDA and EMA for clinical practice: for siRNAs 21 nmol/kg for Patisiran [48] and 153 nmol/kg for Givosiran [49]; for antisense oligonucleotides <5 µmoles/kg for Golodirsen [50], Volanesorsen [51] and Inotersen [52]. It is worth noting that the increase in hydrophobicity of the conjugates have no influence on their cytotoxicity (Figure 3). This result prompts us to expect that the increase of the number of side-chains or introduction of more hydrophobic groups, for example, hydrocarbon chains longer than 16 carbon atoms into LONs will not increase significantly the overall cytotoxicity of conjugates.

We demonstrated that LONs accumulated in the cells with intracellular distribution analogous to that of oligonucleotides delivered by liposomal formulations (Figures 4 and 5) [53–55]. It appears that LONs penetrated through the outer membrane and localized in cytosol predominantly in endosomes 24 h after addition (Figures 6 and 7). It is known that endosomal escape of therapeutic oligonucleotides is one of the rate-limiting steps of their delivery and the key to successful intracellular performance of nucleic acid therapeutics [56]. Although longer term kinetics of LON accumulation (>24 h) was not studied, it can be assumed that such LONs will require longer times for their endosomal escape, similarly to cholesterol-conjugated siRNAs [57].

Cytoplasmic distribution of the developed LONs may be advantageous as far as the targets of therapeutic oligonucleotides (antisense oligonucleotides, siRNAs and anti-miRs) are localized in the cytosol such as mRNAs and many miRNAs. Application of the developed LONs to modulate splicing of pre-mRNA requires additional investigation including optimization of the number and length of the side-chains, selection of optimal delivery conditions etc. to achieve nuclear delivery of the conjugates. We demonstrated that dodecylphenyl and hexadecyl modifications did not significantly impede gene-silencing activity of lipophilic siRNAs delivered into cells by Lipofectamine (Figure 8). However, further studies on elucidating a wider spectrum of biological activities of siRNAs and antisense oligonucleotides carrying such (sulfonyl)phosphoramidate-based lipophilic groups are required.

One potential application for lipid-oligonucleotide conjugates as described herein could be the design of therapeutic oligonucleotides for the treatment of multiple sclerosis (MS), a severe neurodegenerative disease characterized by the destruction of the myelin sheaths of neurons [58]. Kitayama et al. [29] uncovered a link between the destruction of axonal myelin sheaths during MS and repellent guidance molecule a (RGMa): a glycoprotein, which is a powerful inhibitor of neuronal regeneration. Inhibition of RGMa by human monoclonal antibodies stimulate re-myelination and regeneration of damaged neurons in the laboratory in vivo model of MS [36]. Thus, reducing the level of RGMa by downregulation of the corresponding gene by antisense oligonucleotide or siRNA could be a promising strategy for therapeutic intervention in MS.

We studied RGMa inhibition in the RGMa-expressing PK-59 pancreatic cancer cell line using lipid-siRNA conjugates containing either two or four dodecylphenyl or hexadecyl chains at the 3′-end of one or both of the complementary RNA strands, respectively (Figure 1B; Table 1). It was evident that siRNA conjugates containing two adjacent lipid groups in the passenger strand were able to downregulate RGMa mRNA with a similar efficiency to that of the unmodified control RGMa siRNA at 100 nM concentration while those carrying four of either dodecylphenyl or hexadecyl groups were significantly inferior (Figure 8). We foresee that such therapeutic lipid-oligonucleotide conjugates (LONs) displaying low toxicity and improved cellular uptake may demonstrate high efficiency and minimal side effects in future experiments in vivo.
5. Conclusions

To conclude, we have obtained lipid conjugates of oligodeoxynucleotides and siRNAs carrying one, two or four hydrophobic ((4-dodecylphenyl)sulfonyl)phosphoramidate or (hexadecylsulfonyl)phosphoramidate groups at either 5′- or 3′-internucleotidic positions by a modified protocol of the phosphoramidite synthesis replacing usual aqueous iodine oxidation by the Staudinger reaction between a solid-supported phosphite triester and the corresponding long-chain sulfonyl azide.

The oligodeoxynucleotide conjugates were characterized by low cytotoxicity in either murine or human macrophages and improved cell uptake into the same in the absence of a transfection agent. Spontaneous formation of nanoparticles was observed in aqueous solutions of doubly-conjugated oligonucleotides having either of the two lipophilic modifications at the 5′-end, which may be responsible for the improved cellular uptake of the respective conjugates. Notably, the size of the nanoparticles decreased with the increase in the length of the hydrocarbon chain from dodecylphenyl to hexadecyl. Flow cytometry revealed the superior uptake of hexadecyl conjugates over the dodecyl ones into human macrophages. Intracellular localization of the conjugates according to confocal microscopy was predominantly cytosolic with the majority of the fluorescence within endosomes.

Finally, two adjacent lipophilic dodecylphenyl or hexadecyl groups placed within the 3′-terminal overhang and at the 3′-terminal phosphate of the passenger strand did not significantly impair the gene-silencing ability of siRNAs targeting repulsive guidance molecule (RGMa) mRNA in PK-59 cell culture, whereas the siRNAs carrying four of the same at the 3′-ends of both passenger and antisense strands were considerably less active. Thus, lipid-siRNA conjugates with optimized number and position of the lipid chains could be a prospective candidate for future experiments in an in vivo model of multiple sclerosis and, potentially, other diseases when RNA targeting is applicable.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2076-3417/11/3/1174/s1, Figures S1–S3: IR, 1H and 13C spectra of 1-hexadecanesulfonyl azide; reverse-phased HPLC profiles and ESI mass spectra of modified oligonucleotides, Figure S4: Intracellular accumulation of η-F conjugate in KB-8-5 cells, Figure S5: Intracellular accumulation of δ-F and η-F conjugates in RAW264.7 cells and THP-1-induced macrophages.

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