Biomaterials 33 (2012) 5955–5965

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The interactions of amphiphilic antisense oligonucleotides with serum proteins and their effects on *in vitro* silencing activity

Arnaud E. Felber [a], Núria Bayó-Puxan [a,b], Glen F. Deleavey [b], Bastien Castagner [a], Masad J. Damha [b,**], Jean-Christophe Leroux [a,*]

[a] Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli Str. 10, 8093 Zurich, Switzerland
[b] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, QC H3A 0B8, Canada

ARTICLE INFO

Article history:
Received 3 April 2012
Accepted 10 May 2012
Available online 30 May 2012

Keywords:
Nucleic acid
Antisense oligonucleotide
Docosanoic acid
Albumin
Polymeric micelle
Fatty acid

ABSTRACT

Antisense oligonucleotides (AONs) are a class of compounds with high therapeutic potential. One of the challenges facing this platform is the development of effective techniques to achieve cellular delivery. AON conjugates, in which traditional AONs are attached to certain biomolecules, can exhibit improved intracellular bioavailability in the absence of delivery systems. In this study, the lipophilic moieties docosahexaenoic acid, cholesterol, and docosanoic acid (DSA) were conjugated to various phosphorothioated DNA and chemically-modified 2′-fluoro-arabinonucleic acid AONs *via* an amino-hexanol-linker added to the 5′-end of the molecule. The gene silencing potential of these compounds was evaluated *in vitro* in the absence or presence of a transfecting agent (polyion complex micelle). Incubation with sub-micromolar concentration of DSA-conjugates could, in the absence of serum proteins, downregulate more than 60% of the targeted mRNA under carrier-free and carrier-loaded delivery methods. Gene silencing activity of carrier-free DSA-conjugates was, however, decreased in a dose-dependent fashion by adding albumin in the transfection medium. Supplementing the medium with free fatty acid prevented the interaction of the DSA-conjugate with albumin, and restored its silencing activity. These findings suggest that strategies aiming at preventing the association of hydrophobized AONs to serum proteins at the site of action may improve their activity.

1. Introduction

Since the late 1970's starting with the pioneer work of Zamecnik and Stephenson, antisense oligonucleotides (AONs) have been investigated as a means to achieve targeted and specific knockdown of gene expression [1,2]. The success of AON therapeutics depends not only on the intracellular recognition event between the mRNA of the gene to be inhibited and the synthetic oligonucleotide drug, but also on many upstream pharmacokinetic processes. The anionic nature of AONs renders them almost impermeable to the negatively-charged cell membranes [3,4]. Additionally, unmodified AONs are rapidly degraded by nucleases [5]. In order to achieve the desired pharmacological effect, a state of the art AON drug should exhibit i) a favorable pharmacokinetic profile, ii) resistance to nucleases, iii) intracellular bioavailability, iv) high affinity for target mRNA, and v) potent activation of the cellular machinery (*i.e.*, ribonuclease H (RNase H)), to yield reliable and effective knockdown of the targeted gene. Attempts to imbue AONs with these desirable properties have produced an impressive number of backbone chemical modifications compatible with AON-mediated gene silencing (for reviews on these modifications, see Refs. [6,7]). The first generation of backbone chemical alterations utilized 2′-deoxyribonucleotide phosphorothioate (PS) modifications. It entailed AONs with increased stability in biological systems and significantly increased their biological half-life [8]. The second generation of AON constructs typically employed chemically-modified sugars, paired with a PS backbone, in order to further enhance stability towards nucleases, and in some cases potency. Common examples of chemically-modified sugars for these applications include 2′-fluoro, 2′-*O*-methyl and 2′-*O*-methoxyethyl analogs of RNA. The 2′-fluoro-arabinonucleic acid (2′F-ANA) modification is another example of a sugar chemical modification that can be readily applied to AON constructs [9,10]. 2′F-ANA improves the nuclease resistance of oligonucleotides [11], increases binding stability to target mRNA [12], and structurally mimics DNA when bound with a RNA target, preserving RNase H activation [10,12].

* Corresponding author. Tel.: +41 44 633 7310; fax: +41 44 633 1314.
** Corresponding author. Tel.: +1 514 398 7552; fax: +1 514 398 3797.
E-mail addresses: masad.damha@mcgill.ca (M.J. Damha), jleroux@ethz.ch (J.-C. Leroux).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2012.05.019

Today, most clinical trials with AONs and other nucleic acid drug candidates are performed in the absence of a delivery system [13]. Unfortunately, chemical modifications of the backbone structure do not generally solve permeability problems [14,15] and only modestly improve the pharmacokinetic and/or biodistribution profiles [16,17]. Strategies aimed at solving these problems can be divided into two categories: (i) the use of carrier-based systems and (ii) the chemical derivatization of the nucleic acid with functional molecules. The first approach consists of protecting and transporting the antisense drugs with a colloidal delivery system, such as viral capsids [18], lipoplexes (cationic lipids) [19], polyplexes (cationic polymers) [20], and different types of nanoparticles [21–24]. However, issues such as the toxicity of the formulating agents [25], and insufficient transfection efficacy *in vivo*, are hampering the clinical applicability of this approach to some extent [26]. On the other hand, strategies based on the chemical derivatization of nucleic acids involve the direct administration of an uncomplexed AON which is linked to a targeting ligand [27], cell penetrating peptide [28], or hydrophobic tail [29]. For example, it has been reported that covalent attachment of steroids and various other hydrophobic moieties to the 5′-end of AONs could extend their systemic circulation time and enhance cellular uptake [6,30,31]. These types of hydrophobized macromolecular structures are often referred to as amphiphilic AON conjugates. Even though promising results have been generated *in vitro* with such derivatives in the absence of transfecting agent, acceptable silencing levels are usually achieved with high nucleic acid doses (in the 2–10 μM range). Furthermore, the interactions between these amphiphilic conjugates and serum proteins, such as human serum albumin (HSA), have often not been well characterized [32].

In the present study, a library of amphiphilic AON derivatives was synthesized and characterized. The model AONs were all derived from the model Oblimersen (OB) sequence. OB is an 18-mer PS oligodeoxynucleotide complementary to the initial coding region of Bcl-2 mRNA, which was designed to inhibit the expression of the Bcl-2 oncoprotein [14]. The lipophilic moieties, docosahexaenoic acid (DHA), cholesterol (CHL), and docosanoic acid (DSA) were attached *via* an aliphatic amino-hexanol-linker to the 5′-end of PS-DNA AONs and PS-DNA analogs containing a fluorine substituent at the 2′-position of the arabinose sugar (*i.e.*, 2′F-ANA) (Scheme 1, Supplementary Fig. S1). CHL was selected as control due its extensively reported conjugation to nucleic acid [29,32,33]. DHA, an ω-3 polyunsaturated C_{22} natural fatty acid, was chosen due to the reported reduced side-effects and enhanced anti-tumor efficacy observed when conjugated to paclitaxel [34]. DSA was selected as polysaturated counterpart of DHA. The *in vitro* downregulation of Bcl-2 by the amphiphilic AONs was assessed using carrier-free and carrier-mediated transfection conditions. The selected nucleic acid carrier was pH-sensitive polyion complex micelle (PICM) targeting the transferrin receptor (*i.e.*, CD71). This nanosized system was previously shown to improve the intracellular bioavailability and efficacy of AONs and siRNAs [22,35]. The impact of serum proteins addition, and more specifically, HSA addition, on the transfection activity of the unformulated and formulated AONs was also examined, with the aim of identifying new strategies for improving the *in vivo* delivery of amphiphilic AONs.

2. Materials and methods

2.1. Materials

RPMI medium, Opti-MEM I medium, fetal bovine serum (FBS), trypsin, Lipofectamine™ 2000 (used according to the supplier's instructions), SYBR® Gold nucleic acid gel stain, and phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 150 mM NaCl, 3 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) were obtained from Invitrogen (Carlsbad, CA). Unmodified siRNA sequence (5′-GCA UGC GGC CUC UGU UUG AUU-3′, sense strand) was designed and synthesized by Dharmacon (Chicago, IL). Low-binding microcentrifugation tubes (DNA Lobind®) were purchased from Eppendorf-Vaudaux (Schönenbuch, Switzerland). DHA, DSA, cholesteryl chloroformate, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), *N*,*N*-Diisopropylethylamine (DIPEA), HSA, generation 5.0 poly(amido amine) (PAMAM), and decanoic acid were purchased from Sigma–Aldrich (Buchs, Switzerland). Acrylamide/bis-acrylamide solution (30%) was from Bio-Rad Laboratories (Hercules, CA). Boric acid was purchased from Hänseler AG (Herisau, Switzerland). Ethylenediaminetetraacetic acid (EDTA) was obtained from AppliChem (Darmstadt, Germany). Poly(ethylene glycol)-*b*-poly(propyl methacrylate-*co*-methacrylic acid) (PEG_{115}-*b*-P($PrMA_{22}$-*co*-MAA_{58})) (M_n 13,000; $M_w/M_n = 1.06$) and fragment antigen binding (Fab′)-modified PEG copolymer (Fab′-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62})) were synthesized as previously reported [35,36]. All other products, unless otherwise specified, were purchased from Fisher Scientific AG (Wohlen, Switzerland).

2.2. Synthesis of oligonucleotide

Standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of the oligonucleotides [37]. Syntheses were performed on an Applied Biosystems (Carlsbad, MA) 3400 DNA Synthesizer on a 1-μmol scale using Unylink CPG as the solid support (ChemGenes, Wilmington, MA). 2′F-ANA phosphoramidites were prepared as 0.15 M solutions in dry acetonitrile (ACN), and DNA phosphoramidites were prepared as 0.1 M in dry ACN. 5-ethylthiotetrazole (0.25 M in ACN, ChemGenes) was used to activate the phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid (TCA) in dichloromethane (DCM) for 110 s. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran (THF) and 16% *N*-methylimidazole in THF. Oxidation was done using 0.1 M I_2 in 1:2:10 pyridine:water:THF. Sulfurizations of PS backbone oligonucleotides were accomplished using a 0.1 M solution of xanthane hydride (TCI America, Portland, OR) in 1:1 (*v*/*v*) pyridine:ACN. The sulfurization step was allowed to proceed for 2.5 min, with new sulfurization reagent added to the column after 1.25 min. Phosphoramidite coupling times were 600 s for 2′F-ANA, with the exception of guanosine phosphoramidite, which was allowed to couple for 900 s. DNA coupling times were 110 s, and 270 s for guanosine. For amphiphilic AON conjugates preparation, monomethoxytrityl (MMT)-protected 6-amino-hexanol phosphoramidite (ChemGenes) was attached to oligonucleotide 5′-ends using a coupling cycle (without capping) with a coupling time of 600 s, followed by oxidation and detritylation (3% TCA in DCM, 240 s). Prior to the coupling with the corresponding aliphatic moiety (next section), oligomers were successively washed with DCM (1 × 5 min), 5% DIPEA in DCM (5 × 1 min), DCM (5 × 1 min) and ACN (5 × 1 min). Washes were removed by ultracentrifugation and final residual solvent was evaporated under reduced pressure.

2.3. Synthesis of amphiphilic AONs conjugates

The solid support-bound amino modified oligonucleotide (1.0 μmol) was placed in a low-binding microcentrifugation tube. *DHA coupling*: 150 μL of a DHA (0.25 M), PyBOP (0.25 M) and DIPEA (0.75 M) solution in anhydrous DMF was added to the AON and the reaction was stirred for 4 h at room temperature. *DSA coupling*: 150 μL of a DSA (0.25 M), PyBOP (0.25 M) and DIPEA (0.75 M) solution in anhydrous THF:DMF (9:1) was added to the AON, and the reaction was stirred for 4 h at 40 °C. *CHL coupling*: 150 μL of a cholesteryl chloroformate (0.25 M) and DIPEA (2.25 M) solution in anhydrous DCM:DMF (5:1) was added to the AON and the reaction was stirred for 4 h at room temperature. *bisDHA coupling*: Refer to Supplementary methods S1. For all conjugates, excess of reagents was removed by washing the solid support as follows: DMF (5 × 1 min), ACN (5 × 1 min). Washes were removed by ultracentrifugation, and final residual solvent was evaporated under reduced pressure. In all cases, lipophilic moiety coupling conversions were more than 75%, as determined by high-performance liquid chromatography (HPLC) (Supplementary Fig. S1).

2.3.1. Cleavage and purification

Deprotection and cleavage from the solid support was accomplished with 1 mL of 3:1 NH_4OH:EtOH for 48 h at room temperature [38]. Cleavage solution was removed under reduced pressure. The product was extracted with 2 × 250 μL H_2O and 2 × 250 μL EtOH. Solvent was removed, and the pellet was precipitated from 50 μL NaOAc (3 M, pH = 5.5) and 1 mL of cold *n*-BuOH for 3 h in a dry ice bath. The pellet was dissolved in 1 mL H_2O:ACN (1:1) and subjected to ultraviolet quantification. Purification of crude oligonucleotides was done either by preparative denaturing polyacrylamide gel electrophoresis (PAGE) using 24% acrylamide gels or by reverse phase HPLC. For the PAGE purification, gel bands were extracted overnight in diethylpyrocarbonate (DEPC)-treated autoclaved ultrapure deionized water, and lyophilized to dryness. Reverse phase HPLC purification was carried out on a Waters 1525 HPLC (Milford, MA) using a Varian Pursuit 5 (Agilent Technologies, Mississauga, ON, Canada) reverse phase C18 column (250 × 10 mm) with solvent A being 100 mM triethylammonium acetate in water supplemented with 5% ACN (pH 7.0), and solvent B being ACN. HPLC flow was set at 4 mL/min and a gradient was run for 30 min at 50 °C from 80:20 to 60:40 (solvent A:solvent B) for DHA conjugates, and from 70:30 to 20:80 (solvent A:solvent B) for DSA, CHL, and bisDHA conjugates. All purified oligonucleotides were desalted with Nap-25 Sephadex columns from GE