International Journal of Pharmaceutics 532 (2017) 21–28

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Research Paper

Amphiphilic lipopeptide significantly enhances uptake of charge-neutral splice switching morpholino oligonucleotide in spinal muscular atrophy patient-derived fibroblasts

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ARTICLE INFO

Keywords:
Peptide amphiphile
Cell-penetrating peptide
Splice switching oligonucleotides
Cubosomes
SAXS
Cryo-TEM

ABSTRACT

Splice-switching antisense oligonucleotides (SSOs) are emerging therapeutics with two SSOs recently approved by the FDA for Duchenne muscular dystrophy and spinal muscular atrophy. SSOs are administered without any delivery vector and require large doses to achieve the therapeutic benefit, primarily due to their poor cellular uptake. Although cell-penetrating peptides (CPP) have shown great potential in delivering SSOs into cells, their capacity as delivery vector is limited. Here we have studied the effect of lipid conjugation on the cell permeability of a known CPP (ApoE). Myristic acid was coupled at the N-terminus of ApoE to a C-terminal cysteine residue. The myristoylated ApoE (Myr-ApoE) was conjugated to a maleimide functionalised phosphorodiamidate morpholino oligonucleotide (PMO). The Myr-ApoE-PMO conjugate showed no cytoxicity and had significantly higher efficiency in cell permeability with 30% higher splice-switching activity compared to ApoE-PMO. The self-assembly properties of this amphiphilic lipopeptide-PMO conjugate was assessed. Transmission electron microscopy showed formation of nanoparticles with amphiphile behaviour and spherical structure. The self-assembly of Myr-ApoE-PMO into nanoparticles enabled it to better bind to cell membranes and to be more efficiently taken up by fibroblast cells. These results showed that modification of physico-chemical properties of peptides to produce peptide amphiphiles enhances cellular uptake and can be used as an efficient delivery vector for therapeutic SSOs.

1. Introduction

Antisense technology has shown great promise over the past decade as a viable gene-targeted therapy for various diseases. There are many different subclasses of antisense oligonucleotides (ASOs) that are broadly divided based on their mechanism of action. Amongst the ASOs, single-stranded splice-switching oligonucleotides (SSOs) are emerging as an excellent platform for development of RNA-based therapeutics. SSOs act via a steric blocking mechanism and target pre-mRNA in the cell nucleus and alter its splicing in favour of producing a therapeutically relevant protein (Du et al., 2007). The recent FDA approval of Eteplirsen (Aartsma-Rus and Krieg, 2017) (an SSO based on PMO) and Spinraza (2′-*O*-methoxyethyl-phosphorothioate) in late 2016 (Biogen, 2016) for the treatment of Duchenne muscular dystrophy and spinal muscular atrophy (SMA), respectively, are testament to the great therapeutic potential of SSOs.

Several SSOs with different chemical structure have been developed, which include 2′-O-methyl-phosphorothioate (2′-OMe/PS), 2′-*O*-methoxyethyl-phosphorothioate (2′-MOE/PS), phosphorodiamidate morpholino oligonucleotide (PMO) and peptide nucleic acids (PNA) (Jarver et al., 2014). PMO is the most commonly used SSO due to its stability, target-specificity and lack of toxicity (Winkler, 2013). Despite the great therapeutic potential of SSOs, their effective delivery across the cell membrane to their intracellular target remains the most significant barrier to their successful clinical translation and application.

A number of delivery systems for SSOs have been developed amongst which peptides, in particular cationic peptides, have shown the most promise in delivery of SSOs such as PMO across biological membranes (Amantana et al., 2007). Cationic peptide delivery vehicles, often referred to as cell-penetrating peptides (CPPs), are a group of non-viral carriers that have gained increasing attention since their discovery in 1994 (Derossi et al., 1994). CPPs have the ability to rapidly translocate into cells with low toxicity and, due to their small size, specificity

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http://dx.doi.org/10.1016/j.ijpharm.2017.08.116
Received 14 June 2017; Received in revised form 20 August 2017; Accepted 27 August 2017
Available online 31 August 2017

and ability to deliver large bio-cargo, have shown great promise as delivery vector for ASO *in vitro* and *in vivo* (Amantana et al., 2007; Madani et al., 2011). Most CPPs are poly-cationic and taken up in the cells by energy-dependent endocytosis through interaction with either negatively charged glycans or scavenger receptors which are present on the cell surface (Ezzat et al., 2015; Juks et al., 1848).

Other cationic delivery systems such as cationic lipids have also been developed for delivery of negatively charged ASOs. Cationic lipids rely on formation of complex through electrostatic interactions between the positively charged carrier and negatively charged ASO (Tajik et al., 2017). These cationic delivery vectors are not suitable for use in cellular delivery of charge-neutral SSOs such as PMO. Therefore, the development of peptide vector for PMO delivery requires covalent conjugation (Farrelly-Rosch et al., 2017; Shabanpoor and Gait, 2013). Many studies have reported that covalent conjugation of SSOs to cationic CPP improves cell uptake both *in vitro* and *in vivo* (Farrelly-Rosch et al., 2017; Shabanpoor and Gait, 2013; Shabanpoor et al., 2017; Betts et al., 2012; Shabanpoor et al., 2015; Lehto et al., 2014) but these require large doses which increases the risk of toxicity due to the destabilizing effect that the highly positive charged CPPs have on the biological membrane (Amantana et al., 2007).

Therefore, in order to realise the true therapeutic potential of SSOs, there is a need to develop a safe and an efficient peptide-based delivery system with improved pharmacokinetic properties. One such approach is to develop peptide amphiphiles (PAs) by addition of a lipid moiety to the peptide instead of increasing the positive charge of the peptide. PA are being explored as biomaterials for drug delivery due to their increased tendency to insert into membranes compared to the peptide alone (Epand, 1997) and high capacity for interfacial adsorption and spontaneous formation of nano-assemblies (Zhao et al., 2010). The improved amphiphilicity and compatibility of PAs with cell membranes enable the delivery of cargoes into cells *via* endocytosis. In addition, the self-assembly of PAs facilitates the peptide presentation at high density on the surface of nanostructures (Hamley, 2015).

One of the first PAs was proposed by Kunitake (1992) and was composed of a linker, a spacer and hydrophobic tail. PAs combine the structural features of amphiphilic compounds with the bioactive function of peptides. The long alkyl chain in the PA structure provides self-assembly to minimize unfavourable interactions with the surroundings and association with other hydrophobic substrates. Cellular delivery of negatively charged ASOs, such as small interfering RNA, has been dramatically improved by attaching a lipid moiety to the CPP (Nakase et al., 2012; Lehto et al., 2010; Tönges et al., 2006). This activity enhancement may be a result of increased complexation capacity in combination with increased endosomal escape (Lehto et al., 2010). A study by Järver et al. (2015) showed that established lipopeptides can form nanoparticles with PMO and dramatically improve its cellular uptake. Lehto et al. (2010) showed that the N-terminally stearylated $(RxR)_4$ peptide complexed with 2-OMe modified ASO, significantly promotes splice correction. PA-nucleic acid conjugates that self-assembled into fiber-shaped nanostructures were shown to bind oligonucleotides with higher affinity and specificity than the corresponding oligonucleotide (ON) duplexes (Guler et al., 2005).

In this study, we investigated whether the covalent attachment of a hydrophobic lipidic moiety (myristic acid) to a CPP conjugated to PMO assists this hybrid biomolecule to form a complex by self-assembly and increase cellular uptake and bioactivity of the PMO. We have used a PMO that targets survival motor neuron 2 gene (*SMN2*) which is a backup gene in SMA patients who have deletion or mutation in *SMN1* gene. *SMN2* is a nearly identical copy of *SMN1* but with a point mutation in intron-7. This results in exclusion of exon-7 in 90% of *SMN2* RNA transcripts during RNA splicing. The PMO can bind to and convert *SMN2* RNA transcripts without exon-7 (Δ7*SMN2*) to full-length *SMN2*. This increases the level of full-length functional SMN protein which has a significant therapeutic benefit for SMA patients. We used ApoE as a CPP, which is a 10 amino acid long fragment of Apolipoprotein E, LRKLRKRLLR. We have demonstrated the efficiency of ApoE to deliver PMO into different cell types in our previous studies (Farrelly-Rosch et al., 2017; Shabanpoor et al., 2017). We chemically synthesised ApoE and myristoylated-ApoE (PA) and conjugated them to PMO. Following assessment of cytotoxicty and bioactivity, we carried out the biophysical characterisation of this PA-PMO conjugate to investigate the influence of the lipid moiety on the structure and self-assembly properties of the peptide-PMO. The self-assembly behaviour of PA-PMO was evaluated by light and X-ray scattering, surface tension measurements and electron microscopy.

Finally, in order to further improve the efficacy of the PA-PMO we used a nanoparticle-based system using glycerol monooleate (GMO). GMO readily forms cubosomes, lipidic inverse bicontinous cubic liquid crystalline phase (Q_{II}) particles, which offer an effective way to encapsulate peptides and proteins (Meikle et al., 2017; Conn and Drummond, 2013) or oligonucleotide therapeutic agents (Tajik et al., 2017; Mulet et al., 2013) in a lipid based environment. The unique structural architectures and structural stability of cubosomes, their dual polar/apolar nature, tunable structural parameters and the ability of sustained release of cargo make them promising candidates for delivery of bioactive cargos. A number of reviews highlight the promising properties and application of lipidic cubic phases for drug delivery (Shah et al., 2001; Clogston and Caffrey, 2005; Rizwan et al., 2010; Zhen et al., 2012). Recently, Meikle et al. (2017) successfully loaded antimicrobial peptides into cubosomes and also demonstrated the release of amino acids from these lipidic cubic phases. Herein, we formulated PA-PMO into the lyotropic liquid crystalline lipid GMO and explored the influence of the liquid crystalline mesophases on the activity of PA delivery of PMO into cells. These data bring insight into the structure activity relationship of PA-PMO and PA-PMO-GMO carriers and may aid in improving PA delivery vehicles of charge neutral splice-switching PMO.

2. Material and methods

2.1. Materials

9-Fluroenylmethoxycarbonyl (Fmoc) protected L-α-amino acids and 2-(6-Chlor-1H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium-hexafluorophosphat (HCTU) were obtained from GL Biochem (Shanghai, China). *N,N*-dimethylformamide (DMF), methanol, diethyl ether, dichloromethane and piperidine were obtained from Merck (Kilsyth, Australia). Trifluoroacetic acid (TFA) was obtained from Auspep (Melbourne, Australia). Fmoc-Rink Amide SpheriTide resin (100–200 mesh) was purchased from Applied Biosystems (Melbourne, Australia); 3,6-dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), diisopropylethylamine (DIEA), 1,2,4,5 benzenetetracarboxylic dianhydride were obtained from Sigma-Aldrich (Sydney, Australia). Acetonitrile was purchased from Merck. NH_4HCO_3 and $(NH_4)_2CO_3$ were from BDH Laboratory Supplies (Poole, UK). Myristic acid, Oxymapure, Pluronic F108 (poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide), PEO-PPO-PEO) were purchased from Sigma Aldrich, and monoolein (GMO) was purchased from Nu-Check Prep (Elysian, USA).

2.2. Peptide and lipopeptide (PA) synthesis

ApoE peptide and the PA, (Myr-ApoE), with C-terminal cysteine were synthesized by Fmoc solid-phase peptide synthesis (SPPS) (Merrifield, 1963; Atherton, 1989). The peptides were prepared on a Rink Amide SpheriTide resin (100–200 mesh, loading = 0.6-0.8 mmol/g), and were synthesized on a 0.1 mmol scale. HTCU (4 eq) in the presence of DIEA (8 eq) was used to activate Fmoc protected amino acids. Fatty-acid modified peptide was prepared by coupling myristic anhydride at the N-terminus of the resin-bound ApoE. Peptides were cleaved from the resin with TFA:DODT:H_2O:TIPS (94:2.5:2.5:1, v/v) for 2 h at room temperature, precipitated in ice cold diethyl ether and