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Disarmed anthrax toxin delivers antisense oligonucleotides and siRNA with high efficiency and low toxicity

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ABSTRACT

Inefficient cytosolic delivery and vector toxicity contribute to the limited use of antisense oligonucleotides (ASOs) and siRNA as therapeutics. As anthrax toxin (Atx) accesses the cytosol, the purpose of this study was to evaluate the potential of disarmed Atx to deliver either ASOs or siRNA. We hypothesized that this delivery strategy would facilitate improved transfection efficiency while eliminating the toxicity seen for many vectors due to membrane destabilization. Atx complex formation with ASOs or siRNA was achieved *via* the in-frame fusion of either *Saccharomyces cerevisiae* GAL4 or *Homo sapien sapien* PKR (respectively) to a truncation of Atx lethal factor (LFn), which were used with Atx protective antigen (PA). Western immunoblotting confirmed the production of: LFN-GAL4, LFn-PKR and PA which were detected at ~45.9 kDa, ~37 kDa, and ~83 kDa respectively and small angle neutron scattering confirmed the ability of PA to form an annular structure with a radius of gyration of 7.0 ± 1.0 nm when placed in serum. In order to form a complex with LFn-GAL4, ASOs were engineered to contain a double-stranded region, and a cell free *in vitro* translation assay demonstrated that no loss of antisense activity above 30 pmol ASO was evident. The *in vitro* toxicity of both PA:LFn-GAL4:ASO and PA:LFn-PKR:siRNA complexes was low (IC_{50} > 100 μg/mL in HeLa and Vero cells) and subcellular fractionation in conjunction with microscopy confirmed the detection of LFn-GAL4 or LFn-PKR in the cytosol. Syntaxin5 (Synt5) was used as a model target gene to determine pharmacological activity. The PA:LFn-GAL4:ASO complexes had transfection efficiency approximately equivalent to Nucleofection® over a variety of ASO concentrations (24 h post-transfection) and during a 72 h time course. In HeLa cells, at 200 pmol ASO (with PA:LFN-GAL4), 5.4 ± 2.0% Synt5 expression was evident relative to an untreated control after 24 h. Using 200 pmol ASOs, Nucleofection® reduced Synt5 expression to 8.1 ± 2.1% after 24 h. PA:LFn-GAL4:ASO transfection of non- or terminally-differentiated THP-1 cells and Vero cells resulted in 35.2 ± 19.1%, 36.4 ± 1.8% and 22.9 ± 6.9% (respectively) Synt5 expression after treatment with 200 pmol of ASO and demonstrated versatility. Nucleofection® with Stealth RNAi™ siRNA reduced HeLa Synt5 levels to 4.6 ± 6.1% whereas treatment with the PA:LFn-PKR:siRNA resulted in 8.5 ± 3.4% Synt5 expression after 24 h (HeLa cells). These studies report for the first time an ASO and RNAi delivery system based upon protein toxin architecture that is devoid of polycations. This system may utilize regulated membrane back-fusion for the cytosolic delivery of ASOs and siRNA, which would account for the lack of toxicity observed. High delivery efficiency suggests further *in vivo* evaluation is warranted.

Abbreviations: ASOs, antisense oligonucleotides; Atx, anthrax toxin; BME, beta-mercaptoethanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; E7, early 7; EEA1, early endosomal antigen 1; LFn, Atx lethal factor domain I; GFP, Green fluorescent protein; PKR, *Homo sapien sapien* protein kinase-R; HPV, human papilloma virus; ILVs, intraluminal vesicles; LBPA, lysobisphosphatidic acid; LAMP2, lysosome associated membrane protein 2; LF, lethal factor; MVBs, multivesicular bodies; PEI, poly(ethyleneimine); PNS, post-nuclear supernatant; PA, protective antigen; GAL4, *Saccharomyces cerevisiae* galactose metabolism DNA binding protein; SANS, small-angle neutron scattering; (si)RNA, small interfering; Synt5, Syntaxin5; t-GFP, turbo-GFP.

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1. Introduction

Inefficient cytosolic delivery, suboptimal pharmacokinetics and pharmacodynamics and vector toxicity contribute to the limited application of antisense oligonucleotides (ASOs) and small interfering (si)RNAs as routinely used clinical tools [1,2]. A variety of non-viral delivery systems have been explored in relation to the intracellular delivery of siRNA and ASOs. However, to date, despite three antisense drugs being licensed by the FDA [3,4,5], no ASO intracellular delivery systems have entered into routine clinical use [1,2].

Anthrax toxin (Atx) has evolved to mediate the cytosolic delivery of macromolecules [6] and the attenuation of its intrinsic toxicity using recombinant technology is facile *i.e.* using recombinant PCR to remove Lethal Factor (LF) domains II–IV to give rise to the truncated protein LFn [7]. The translocation of LFn into the cytosol requires *Bacillus anthracis* protective antigen (PA) and has been described [6–10]. This operation requires the association of PA with one of its three known receptors (β1-integrin [11], tumor endothelial marker 8 (TEM8) [12] or capillary morphogenesis gene-2 (CMG-2) [13], which are almost ubiquitously expressed in mammals. PA has been reported to form homoheptamers [6] or homooctamers prior to, or during cell receptor association [14], which are then trafficked onto the limiting membrane of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) [8,9,10]. A pH-driven PA conformational change results in PA membrane insertion (pre-pore to pore transition) [15] and the translocation of LFn over the ILV limiting membrane, into the lumen of the ILV. A subsequent back-fusion event between the ILV and the limiting membrane of the MBV releases LF(n) into the cytosol [8,9,10]. Given that all of the events culminating in back-fusion and LFn cytosolic release are highly regulated, it may be possible to exploit these trafficking events for not only the transport of peptides into the cytosol [16,17,18] but also the movement of nucleic acids, without the aid of any polycationic condensing agents [19,20,21].

Consequently, the aim of this study was to evaluate the potential of Atx components to deliver ASOs and siRNA to the cytosol without generating the toxicity resulting from non-specific membrane destabilization common to many forms of non-viral delivery technology. A cartoon representing the proposed delivery strategy, and the membrane back-fusion event acting as an "airlock" into the cytosol, is shown (Fig. 1) and is based upon the trafficking of LF [8,9,10]. To this end, ASOs or siRNA was joined to LFn *via* the in-frame fusion of a DNA or RNA binding protein requiring *Saccharomyces cerevisiae* galactose metabolism DNA binding protein (GAL4) (for ASOs) [21] or *Homo sapien sapien* protein kinase-R (PKR) [22] (for RNA) to be fused in frame to LFn and used in conjunction with PA.

For the first time, we report the ability of disarmed Atx to deliver ASOs and siRNA to a variety of primate cell lines (HeLa, THP-1 and Vero) without the aid of any polycationic or lipidic helpers. The PA, LFn-GAL4, LFn-PKR proteins and their complexes with ASO and Stealth RNAi™ siRNA were characterized in relation to structure and general toxicity in Hela and Vero cells *in vitro*. Fluorescence microscopy and subcellular fractionation were used to verify cytosolic entry of LFn-GAL4 and LFn-PKR or their Texas Red®-labeled analogues and the trafficking of PA to lysobisphosphatidic acid (LBPA) positive endocytic vesicles over time. Finally the ability of the disarmed Atx to deliver ASOs and siRNA to a variety of primate cell lines (HeLa, THP-1 and Vero) was studied by measuring the knockdown of Syntaxin5 (Synt5) expression as a model to quantitate pharmacological activity in relation to Nucleofection® (and lipofection) as a benchmark available commercially to anyone wishing to gauge the efficiency of their system in relation to the data reported herein.

2. Materials and methods

2.1. Oligonucleotides

ASOs are described (Table 1) and were supplied by Invitrogen (Paisley, UK). The antisense sequence against Synt5 has previously been published [23]. Synt5 specific Stealth RNAi™ siRNA modified RNA (4392420; Life Technologies, Paisley, UK) was purchased commercially.

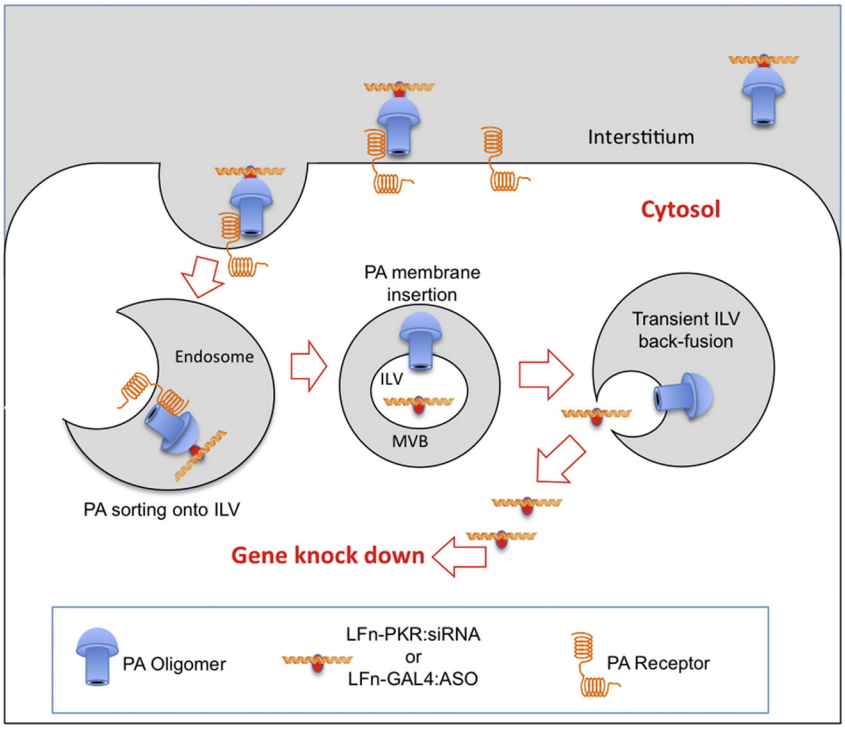

Fig. 1. Exploiting membrane back-fusion for the delivery of ASOs and siRNA. This cartoon is adapted from data describing the cytosolic translocation of LF(n) [6–10]. For the purposes of this study it is used to illustrate the possible route taken to the cytosol by the Atx derived delivery systems described herein *i.e.* PA:LFn-GAL4:ASO or PA:LFn-PKR:siRNA.