The potential of toxin-based drug delivery systems for enhanced nucleic acid therapeutic delivery

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Abstract.

Introduction: The potential of gene replacement therapy has been underscored by the market authorisation of alipogene tiparvovec (Glybera) and GSK2696273 (Strimvelis) in the EU and recombinant adenovirus-p53 (Gendicine) in China. Common to these systems is the use of attenuated viruses for "drug" delivery. Whilst viral delivery systems are being developed for siRNA, their application to antisense delivery remains problematic. Non-viral delivery remains experimental, with some notable successes. However, stability and the "PEG dilemma", balancing toxicity and limited (often liver-tropic) PK-PD, with the membrane destabilising activity, necessary for nucleocytosolic access and transfection remain a problem.

Areas Covered: Here we review the use of attenuated protein toxins as a delivery vehicle for nucleic acids, their relationship to the PEG-dilemma, and their biological properties with specific reference to their intracellular trafficking.

Expert opinion: The possibility of using attenuated toxins as antisense and siRNA delivery systems has been demonstrated *in vitro*. Systems based upon attenuated anthrax toxin have been shown to have high activity (equivalent to nucleofection) and low toxicity whilst not requiring cationic "helpers" or condensing agents, divorcing these systems from the problems associated with the PEG dilemma. It remains to be seen whether these systems can operate safely, efficiently and reproducibly, *in vivo* or in the clinic.

Key words.

Antisense, Drug Delivery, Endocytosis, Gene Therapy, siRNA, Toxin.

Abbreviations.

(AAV) Adeno-Associated Virus, (ASO) Antisense oligonucleotide, (Atx) Anthrax toxin, (BoNT) *Clostridium botulinum* toxin, (CPPs) Cell penetrating peptides, (C) Catalytic domain, (CMG2) Capillary morphogenesis protein 2 (CT) Cholera toxin, (DEAE) Diethyl aminoethyl, (DT) Diphtheria toxin, (EF) Oedema factor, (EF-2) Elongation factor-2, (EGFR) Epidermal growth factor receptor, (EMA) European Medicines Agency, (FDA) U.S. Food and Drug Administration, (Gel) Gelonin, (ILV) Intraluminal vesicle, (IPEC) Interpolyelectrolyte complexes, (ITGB1) Integrin beta-1, (LF) Lethal Factor, (LPLD) Lipoprotein lipase deficiency, (LPL) Lipoprotein lipase, (MVBs) Multivesicular bodies, (NSF) N-ethylmaleimide-sensitive factor, (PA) Protective antigen, (PAP) Pokeweed antiviral protein, (PEA) Pseudomonas exotoxin A, (PEG) Polyethylene glycol, (PEI) Poly(ethyleneimine), (PK-PD) Pharmacokinetics and pharmacodynamics, (PLL) Poly(L-lysine), (PNA) Protein nucleic acid, (PT) Pertussis toxin, (R) Receptor-binding domain, (RIPs) Ribosome-inactivating proteins, (RNAi) RNA interference, (RT) Ricin holotoxin, (RTAC) RT a chain, (RTBC) RT b chain, (SNARE) Soluble NSF attachment protein receptors, (ST) Shiga toxin, (SLNs) Solid core lipid nanoparticles, (T) Trans-membrane domain, (TEM8) Tumour endothelial marker 8, (TeNT) *Clostridium tetani* toxin, (VAMP) Vesicle-associated membrane protein.

Article highlights.

This paper covers:

1) The deferential between the challenges associated with gene and siRNA / antisense delivery.

2) Why this differential is important within the context of existing rate limits to non-viral therapy *i.e.* the PEG dilemma.

3) The use of protein toxins as part of non-viral DNA delivery systems.

4) How toxins navigate the endomembrane system.

5) Recent successes using toxins to deliver siRNA and antisense oligonucleotides.

1.0 Introduction.

Nucleic acid drugs may be thought of loosely as gene replacement "drugs" or entities that can directly modulate the expression of existing genes [1]. The former is referred to as gene replacement therapy or "gene therapy" whilst there are many ways to achieve the modulation of gene expression via the application of conventional or, nucleic acids "drugs" [1]. The term "antisense" within the context of a gene-modulating tool first appeared in PubMed in 1972, [2] some 26 years before the appearance of the term RNA interference (RNAi) [3].

Antisense therapeutics: Antisense gene modulation requires the binding of single stranded antisense oligonucleotide (ASO) or analogues thereof [4], to mRNA, which has the effect of blocking mRNA expression and also (in some instances), inducing the destruction of the mRNA via RNase H [5]. Target specificity (hybridisation), is driven by the sequence of the ASO which is typically a single stranded molecule, of 19-21 base pairs in length. The antisense sequence is in the reverse complementary orientation to the target sequence, hence the designation "antisense" relative to the "sense" configuration of the mRNA sequence [2]. This is distinct from an "antigene" or "gene silencing" strategy, which seeks to prevent the synthesis of mRNA via the hybridisation of an oligonucleotide within the major groove of the target gene [6]. This gene silencing operation is limited by: (1) a requirement for either a homopurine or homopyrimidine gene sequence to foster the Hoogsteen (or reverse Hoogsteen) bonding necessary to generate a stable DNA triplex and (2) the thermodynamic stability of the triplex, which often requires additional stabilisation to remain intact [6]. Consequently, gene silencing *per se* will not be further considered herein, though a wealth of literature exists covering this subject [6&7].

RNAi therapeutics: The phenomena of RNAi requires (evolutionarily) well-conserved, specific machinery to mediate gene down-regulation, and may have evolved as a way to interrupt viral replication [3]. RNA interference (RNAi) requires the cytoplasmic delivery of double stranded RNA (or a suitable RNA analogue), which interacts with the RNase III enzyme Dicer [8]. Dicer cleaves double-stranded RNA into siRNA. Cleaved siRNA is typically 22-25 base pairs long and contains a characteristic 2 base 3' overhang, making this molecule suitable for loading into the RNA-induced silencing complex (RISC) [9]. Once loaded into RISC, one of the RNA strands is removed and degraded, leaving a "guide strand" that will, by virtue of reverse complementarity, hybridise specifically with "target" message RNA [9]. Guide

strand selection is driven by the base pairing stability, *i.e.* the GC content, of the 5' terminus of the RNA duplex, suggesting a role for Dicer during the selection of the guide strand [9]. The liberation of the RISC associated non-guide strand from the RISC::RNA complex (and RNA duplex) allows the hybridisation of the RNA guide strand :: RISC complex with the target mRNA, and facilitating the activities of the *Piwi* domain of the Argonaute 2 protein "slicer", mediating mRNA cleavage and expressional silencing [9].

1.1 Clinical relevance of gene replacement or modulating technologies.

The first licensed gene therapy was an adenovirus-based medicine that delivered a functional copy of the P53 gene via recombinant adenovirus-p53 ((Gendicine); Shenzhen SiBiono Gene Technologies) to treat head and neck squamous cell carcinoma and was licensed for use in China in 2003 [10]. The successful licensing of GSK2696273 (GSK) [11] and alipogene tiparvovec (UniQure) [12] within the European Union underscore the prospect of gene therapy becoming a reality. GSK2696273 was licensed to treat severe combined immunodeficiency due to adenosine deaminase deficiency in April 2016 [11] and is a gammaretrovirus containing the human adenosine deaminase gene. Alipogene tiparvovec utilises a non-replicating Adeno-Associated Virus (AAV) 1 vector to deliver a functional copy of the lipoprotein lipase (LPL) gene to treat lipoprotein lipase deficiency (LPLD) [12]. The U.S. Food and Drug Administration (FDA) approval of three antisense drugs; Fomivirsen (Vitravene, previously ISIS 2922) [13], Alicaforsen (previously ISIS 2302) [14], & Mipomersen (Kynamro, previously ISIS 301012) [15], the voluntary withdrawal from the market of Fomivirsen in 2002 [16], and the failure of the European Medicines Agency (EMA) to approve Mipomersen in 2013 [17], points to much unlocked potential associated with these technologies. Although there has not been a licenced medicine based upon siRNA technology, there are several potential medicines undergoing phase I, II and III evaluation, the most notable of which are listed (Table 1). To date there are 138 clinical studies linked to the word "antisense" (on https://clinicaltrials.gov), and 41 trials using the term "siRNA". The progress of various RNAi-based drugs through clinical trial has also recently been reviewed [18].

Many of the problems associated with the use of both siRNA and antisense technologies may be solved through the use of medicinal chemistry; stabilising molecules against nuclease degradation and reducing immunogenicity. The need for advanced drug delivery technology [19], enhancing pharmacokinetics and pharmacodynamics (PK-PD), cell targeting and intracellular (cytosolic) translocation is also becoming apparent for not only siRNA but also antisense agents [20]. However, even if these problems are solved in such a way that the bioaccumulation of these entities is circumvented, cytosolic access circumventing the polyethylene glycol (PEG) dilemma remains an issue [20,21,22]. The pleiotropic nature of protein targets must also be considered. Undesirable, indirect (as opposed to direct) effects of gene knockdown may be problematic, though may be less of a concern if the intended target is exogenous (*i.e.* viral in origin). Before these problems are evident, a larger one remains to be solved, and that is the safe and reproducible delivery of a nucleic acid drug to the correct intracellular compartment in the appropriate tissue type.

1.2 Is advanced drug delivery technology needed?

There are several rate limits that have prevented ASO and siRNA technologies from becoming a first line medicine, and at the centre of this problem lies bioavailability [20]. Bioavailability is a problem on account of the susceptibility of both DNA and RNA to nuclease attack in biological fluids, including those within the endolysosome and the cytosol [4,19]. Further, the PK-PD associated with these molecules and their inability to cross biological membranes such as phospholipid bilayers contribute significantly to this problem. Medicinal chemistry has gone a long way to addressing the stability of these potential "drugs" in biological fluids and the intracellular environment, yet the problems of cell or even tissue targeting as well as access to the cytosol remain [4, 20, 21, 22].

In the instance of siRNA and ASOs, backbone modification may negate the need for nuclease stabilising agents, potentially impacting upon the "PEG dilemma", described later in this article [22,23] (section 1.4). Where exogenous genes are being targeted *i.e.* those of viruses, target specificity should be exquisite, driven by the sequence of the drug. However, to realise the full potential of these therapeutic modalities, the ASO / siRNA needs to be in the correct compartment within the correct cell [20, 22]. Given that after endocytic uptake, from a topological point of view, the ASO is still on the wrong side of a biological membrane *i.e.* within the lumen of an endocytic vesicle, (and is heading towards the catabolic endolysosome), something needs to be done to improve ASO cytosolic access [24].

If the toxicity and intracellular dynamics of ricin toxin are considered, especially ricin toxin a chain (RTAC) relative to ricin holotoxin (RT) [25, 26], the benefits, *vis-a-vis* enhanced navigation of the endomembrane system, cytosolic delivery and target assimilation become obvious. This is reflected in the enhanced IC_{50} of RT relative to RTAC. RT shows a 1000+ times decrease in IC_{50} relative to RTAC in Vero [25], a 126 times decrease in IC_{50} in THP-1 cells [25] and an approximately 5-fold decrease in B16F10 cells [26]. These numbers are lent further poignancy when the efficiency of the RT b chain (RTBC)'s ability to deliver RTAC to the ER (and eventually the cytosol) is considered [25]. In the instance of RT, only 5% of the internalised ricin has been measured within the Golgi (*en route* to the cytosol), with the balance following the default route to the endolysosome and catabolic destruction or being subject to exocytosis [27]. Regardless of the efficiency of this cytosolic delivery system, and considering the enzymatic potency of RTAC, the benefits of cytosolic delivery are here underscored. If the toxicity of type II ribosome-inactivating proteins (RIPs) *i.e.* RIPS with lectinic activity are considered, relative to type I RIP's such as gelonin [26], again the enhancements in toxicity are profound.

The need for the cytosolic delivery of other "large molecules" was further underscored when the intracellular trafficking of both synthetic- (non-cationic), and bio-polymers was examined. In the instance of fluorescently labelled -HPMA, -dextrin, -dextran or -bovine serum albumin [28], fluorescence can be readily detected within (endocytic) membrane delineated compartments, specifically those identified by the late endocytic marker LAMP1 and LAMP2 [28]. This is in agreement with the literature defining the subcellular localisation (over time) of radiolabeled polymers, colloidal gold and IgG [29, 30] as defined by subcellular fractionation and microscopy. The distribution of these macromolecules is in contrast to fluorescently labelled, attenuated anthrax toxin [23], which can be readily detected within the cytosol both microscopically and using subcellular fractionation, controlling for non-specific membrane interactions at the cell surface giving false positive readings during the microscopy [23].

In relation to efficiency, it is interesting to note that *in vitro,* often as much as 20% gene knock-down has been reported at higher concentrations of ASOs and RNAi analogues without any sort of cytosolic delivery system [23]. However, it also needs to be noted that the efficiency of cytosolic access is highly cell type dependent [31], that these cells are in exponential growth phase and, in all probability, are not representative of the *in vivo* milieu [4]. Without the aid of a delivery system, ASOs may be dependent upon "leaky" vesicle fusion to escape the endocytic system [32]. Within healthy cells, leaky vesicle fusion appears to be an event that is of low probability, on account of the need to: (1) compartmentalise and contain the content of the endolysosome, as well as (2) efficiently sort cargo within the cell *i.e.* during lysosome biogenesis [33]. It may also account for the relative inefficiency and high doses of ASOs / siRNA analogues required clinically.

1.3 Advanced drug delivery strategies for nucleic acid therapeutics.

The potential of not only gene or protein replacement therapy, but also being able to control the expression levels of exogenous as well as endogenous genes is vast. However in order to realise the potential of these phenomena, the safe, efficient and reproducible introduction of functional nucleic acids into the nucleocytosolic compartments of human cells needs to be realised *in vivo* [24]. Given the enormous humanitarian and commercial potential of realising the above ambition, the scientific community has been trying to develop both viral and non-viral nucleic acid delivery technology for roughly 30 years [21, 22]. These efforts have met with varying degrees of success [22]. Given that the market for advanced drug delivery systems was estimated to be worth $227.3 billion USD by 2020 [34] and that this figure is aside from the value of the medicine being delivered, there is plenty of impetus to move this technology forward. The market for RNAi based medicines was, in 2011, forecast to reach $4.04bn by 2017 prior to a single siRNA based drug being FDA approved [35].

Since the 1980s there has been an explosion in the literature base, describing the generation and characterisation of non-viral drug delivery systems, evaluating first the interaction of DNA with (model histones *i.e.* poly(L-lysine) (PLL)) [36] and then the transfection potential of numerous polycations. The interactions of a large molecule such as DNA with molecules as simple as water are complex and have been described in some detail [37]. However, the ability to formulate DNA into transfection competent interpolyelectrolyte complexes (IPEC) [37,38] (or polyplexes) [39] is even more complex, though possible to describe and reproduce. These endeavours have evolved from the use of protamine sulphate [40], diethyl aminoethyl (DEAE)-dextran [41], to PLL [42], poly(ethyleneimine) (PEI) [43] and eventually to cell penetrating peptides (CPPs) [44], amphiphilic polymers [45] and dendrimers [46].

Similarly, much interest has been shown in the use of cationic and "helper" lipids to deliver DNA, forming "lipoplexes" [47]. The chemistry of these formulations is also complex with fatty acid tail length, degree of saturation and other modifications [48], as well as the method of formulation (such as nitrogen to phosphate ratio) impacting upon transfection efficiency [47]. As previously described the non-viral advanced drug delivery field has moved as far as phase III clinical trials (Table 1) and one notable success is GalNAk [49]. GalNAk utilises the high density of a liver parenchymal cell-specific receptors (*i.e.* the asialoglycoprotein receptors) expressed upon hepatocytes [50]. These receptors are expressed at densities of approximately $5x10^5$ per cell though this number varies in a liver location dependent way [50]. The asialoglycoprotein receptor, which usually scavenges spent proteinatious material from the systemic circulation for delivery and catabolism within the endolysosome

[51], interacts with the three galactose groups conjugated to the siRNA duplex [49]. This makes GalNAk constructs very good at delivering siRNA to hepatocytes, though there is not an obvious way for the siRNA conjugated to this construct to exit the endomembrane system once subject to endocytic capture. As there is a measurable benefit to using this technology *in vivo* [49], it is obvious that more siRNA is reaching its target. It is possible that the GalNAk is increasing the intravascular *i.e.* intraendosomal concentration of siRNA which is then better able to exploit "leaky" vesicle fusion [32]. The prospect of being able to target non-hepatocyte cells is limited. However at this time, and in the absence of evidence and empirical study, this commentary is at this point in time conjecture.

There have been many rate limits identified when investigating the use of polyplex and lipoplex-based delivery systems, which have issues with stability, PK-PD and toxicity [52]. These rate limits are a product of the charge associated with these molecules and in the instance of IPEC "polyplexes" have been termed the PEG-dilemma [22] (see section 1.4).

Stepping out from the PEG-dilemma and in addition to GalNAk, there are solid core lipid nanoparticles (SLNs) also in phase I-III clinical trials (Table 1) for the delivery of siRNA, as well as liposomal technologies based upon Smarticles [53]. SLNs are lipid particles typically 100 nm in diameter stabilised via the addition of several types of agent such as cholesterol or surfactants [54]. It is this stabilisation that's proposed to give these entities an advantage over polymeric or cationic systems [54].

1.4 The poly(ethylene glycol) (PEG) dilemma.

The use of charged material to deliver nucleic acids to the nucleocytosolic compartment is now relatively well understood and has reached a dichotomy attempting to balance toxicity with transfection activity [22]. Both toxicity and transfection activity are products of the intrinsic positive charge and the density of positive charges within a macromolecule. In brief this is due to the need for the molecule to breach or rupture membranes delineating specific intracellular compartments in order to mediate transfection. This is caused by positively charged groups within the delivery system, which interact with negatively charged membrane components [22]. This binding causes membrane destabilisation not only within the endolysosomal compartment (allowing the release of proapoptotic enzymes [45]) but also at the plasma membrane [55&56]. Given the more recent discoveries regarding the biology of the endolysosomal compartments, the "proton sponge hypothesis seems an unlikely explanation of membrane destabilisation [57]. Polycation mediated plasma membrane damage also causes toxicity and cell death in a concentration dependent way [26,55,56&58] and in this instance there is little scope for a proton sponge-like effect to disrupt membrane.

As it has been widely reported that an excess of positive charge is necessary for transfection activity, there is a balancing act to be performed between membrane disruption resulting in toxicity and membrane disruption resulting in transfection. This conundrum was explored further by creating A-B block co-polymers containing positively charged blocks interspersed with neutrally charged blocks (consisting of poly(ethylene glycol) (PEG)). The results of these explorations have been very well reviewed recently [22] and in brief, the greater the proportion of PEG, the less toxicity was observed by the lowered the transfection activity. Conversely, when the proportion of PEG was decreased, the toxicity increased, as did the transfection activity [22]. Beyond this there is also the issue of PK-PD, which is also influenced by change density, with positively charged material being sequestered to the liver very rapidly, or in the instance of charged particulates such as IPEC, accumulating in the

lungs or spleen [56,57&59]. This makes accessing other tissue difficult limiting the range of conditions that can be treated using this technology.

2.0 Evolved solutions to cytosolic access, circumventing the PEG dilemma.

Many different types of toxin have evolved to exploit membrane-recycling events to evade destruction in the endolysosome and to access the nucleocytosolic compartment where the target for the toxin resides. It is often possible to physically (using recombinant technology), separate the toxin "warhead" from its "rocket motor" or proteinatious intracellular delivery system [23,60-63]. For several years it has been postulated that it may be possible to use the rocket motor protein architecture necessary to access the nucleocytosolic compartment for drug delivery, once the toxin's warhead has been removed [23,60-63]. Below, some of the well-characterised protein toxins documented to access the cytosol are discussed in relation to their potential to deliver nucleic acids.

Protein toxins have evolved to exploit several different mechanisms to access a variety of topologically discrete compartments. Some of the better-known pathways are shown (Figure 1), which documents the intracellular trafficking of RT [25], anthrax toxin (Atx) [64], shiga toxin (ST) [65], cholera toxin (CT) [66] and diphtheria toxin (DT) [67]. These toxins are further detailed with regard to their receptor affinity (Table 2).

2.1 Accessing the cytosol via the endoplasmic reticulum (ER).

RT is a heterodimer, initially synthesised as a single, inactive, 576 amino acid chain by the castor bean (*Ricinus communis*). The single prepro-RT amino acid chain is subject to proteolytic processing and glycosylation in the castor bean ER and Golgi [68]. Mature (lethal) RT consists of a 267 residue RTAC and a 262 residue RTBC, held together by a disulphide bond between Cys^{294} (on RTAC) and Cys^{318} (on RTBC) [69]. RTAC attenuation is facile and has been undertaken to produce a potential anti-RT vaccine [70].

RT cellular internalisation is driven by an association between RTBC and numerous receptors containing terminal *N*-acetylgalactosamine or beta-1,4-linked galactose (Table 2) [71]. This association can result in the binding of 10^6-10^8 RT molecules per cell [71], a number significantly higher *vis-a-vis* the distribution of the asialoglycoprotein receptor [50]. Given the abundance of RT receptors, it is not surprising that RT is internalised via several coat proteins including clathrin, caveolin and actin resulting in RT translocation to the Golgi via both recycling (Rab11 positive) and sorting endosomes (Rab5, EEA1 positive). This retrograde transport step results in the translocation of approximately 5-10% of the internalised RT to the Golgi apparatus [27]. A second retrograde transport event results in the translocation of RT from the Golgi apparatus to the ER, where RTAC is reductively "cleaved" from RTBC, prior to its retro-translocation, possibly utilising the *Sec*61p translocon, into the cytosol [72]. RTAC hydrolyses the *N*-glycosidic bond attaching the adenine residue at position 4324 to the 28S rRNA within the sarcin-ricin loop, inhibiting protein synthesis [73].

Similar to RT, Pseudomonas exotoxin A (PEA) [74], CT [66], ST [65] and pertussis toxin (PT) [75] as well as several type I RIPs, that is RIPs with no cell binding domain *i.e.* pokeweed antiviral protein (PAP) [76] or Gelonin (Gel) [26], also have cytosolic

targets. CT, PEA, ST and PT have all been reported to translocate via the Golgi and ER *en route* to the cytosol, utilising a variety of retrograde transport strategies. It is less clear how the type I RIPs achieve cytosolic translocation, though there are reports of the C-terminus of PAP being necessary for cytosolic translocation and toxicity [76], when studied using a yeast expression system [76]. Here, the propensity of various PAP truncations and point mutation were examined in relation to PAP's ability to navigate ER exit and depurinate ribosomes [76]. What is not clear is how a type I RIP exits the endocytic system that by default would deliver the protein to the endolysosome and destruction. It is possible that leaky vesicle fusion [31] is also responsible for the cytosolic translocation of the type I RIPs. If this is the case then, as mentioned earlier, the reduced toxicity of the type I RIPs (or RTAC without RTBC) [25] relative to the type II RIPs, may shed light on the benefits of utilising a cytosolic delivery system for membrane impervious therapeutics with cytosolic targets such as ASO or RNAi agents. CT, PEA, ST and PT receptors are summarised (Table 2).

2.2 Accessing the cytosol from the endosome.

Diphtheria toxin (DT) is a virulence factor produced by *Corynebacterium diphtheriae* and is synthesised as a single polypeptide precursor of 535 amino acids [77]. The precursor molecule is cleaved into an A and a B subunit, linked by two internal disulfide bonds (Cys^{186} interacting with Cys^{201} and Cys^{461} with Cys^{471}). The DT B subunit consists of a trans-membrane (T) domain and a receptor-binding (R) domain. The DT A subunit contains the catalytic (C) domain responsible for inducing cell death [77]. The association of the R domain with the heparin-binding epidermal growth factor precursor (HB-EGF) receptor [77] facilitates the endocytic capture of DT (Table 2). The nucleation of multiple T domains in response to the acidification of the endosome [77], drives their insertion into the vesicle limiting membrane, and forms a pore through which the C-domain of the DT A chain may translocate (to the cytoplasm) inhibiting protein synthesis by deactivating elongation factor (EF)-2 [77].

DT has been modified for use as a cytotoxic therapeutic by mutating its R domain, enhancing its specificity for the epidermal growth factor receptor (EGFR) [78] or, in the instance of Denileukin diftitox (Ontak®) (Eisai Medical Research Inc.) [79], an interleukin 2 receptor binding DT mutant, used in the management and treatment of cutaneous T-cell lymphoma (licensed by the FDA in 1999) [79]. Resimmune (A-dmDT390-bisFv(UCHT1)) (Angimmune, LLC) is an experimental anti-T cell immunotoxin containing elements of DT, which has binding specificity for CD3 [80].

Clostridium tetani toxin (TeNT) and *C. botulinum* toxin (BoNT) are responsible for tetanus and botulism (respectively) [81]. BoNT has seven distinct serotypes (A, B, C_1, C_2, D, E, F, G), which have protease activity targeting the pre-synaptic neuroreceptor soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex responsible for neutortransmitter (acetylcholine) release [81,82]. This SNARE complex consists of SNARE domains contributed by SNAP25 (hydrolysed by serotypes E, C, A), Synaptobrevin or vesicle-associated membrane protein (VAMP) (hydrolysed by serotypes G,B,D,F) and Syntaxin 1 (hydrolysed by serotype C) proteins [81]. Cleavage of these target molecules prevents neurotransmitter release. The toxin is synthesised as a 150 KDa molecule, which like RT is subject to proteolytic cleavage to produce a 100 KDa heavy chain and a 50 KDa light chain, responsible for proteolytic activity. After receptor binding, a membrane translocation event from the endosome to the cytosol occurs which, similar to DT, requires the multimerisation and membrane insertion of the heavy chain, followed by the unfolding and translocation of the light chain [81]. *C. botulinum* toxin serotypes A and B are used clinically, with botulinum toxin A being marketed as

Botox (Allergan), Dysport (Ipsen), Xeomin (Merz Pharma) whilst serotype B is marketed as Myobloc (Solstice Neuroscience) [83].

2.3 Accessing the cytosol via multivesicular bodies (MVBs).

Atx is a virulence factor of *Bacillus anthracis*, and may be considered an AB toxin, containing three protein chains [84]. The first of these, the protective antigen (PA) chain, may be thought of as a B chain, facilitating both cellular uptake and the cytosolic delivery of the A chains (oedema factor (EF) and Lethal Factor (LF)) [84]. In the wild PA83 is subject to serum or cell surface proteolytic cleavage by a furin-like protease at an RKKR motif (residues 164-167) to release PA63, which forms both homo-octamers and homo-heptamers [85, 86]. The low incidence octameric form displays: (i) enhanced translocase activity (ii) longer serum stability and (iii) decreased aggregation and inactivation relative to the heptamer [86]. Further, mutants have been documented that exclusively force PA octameric assembly, making this phenomenon accessible and highly reproducible (*i.e.* PA83 $K^{245}G$, $R^{252}N$ and $D^{512}K$ or PA83 $K^{245}N$, $R^{252}S$ and $D^{512}K$) [87]. It is not known if the octameric mutants adopt (1) an identical conformation to those previously described [85, 87] and (2) display enhanced serum stability relative to the more physiological octamers, though their translocase activity has been documented both *in vitro* and *in vivo* [85, 87]. The $PA63_{(7/8)}$ complex interacts with cellular receptors including; capillary morphogenesis protein 2 (CMG2) [88], integrin beta-1 (ITGB1) [89] and tumour endothelial marker 8 (TEM8) [90] at the cell surface (Table 2). TEM8 and CMG2 are almost ubiquitously expressed throughout the body, and drive the capture, tissue distribution and internalisation of PA [91]. After endocytic internalisation, the oligomeric, receptor bound, PA pre-pore undergoes a pH driven conformational transition, resulting in the membrane insertion of the 2β2-2β3 loops from each PA molecule. In the instance of heptameric PA assemblies, these loops combine to form a 14-stranded trans-membrane β-barrel, [92] across which, LF and EF may translocate. LF::PA oligomer association is via the flexible PA Phe-clamp loop [93] located on the interior of the pre-pore and pore, distal to the cell membrane. Within a multivesicular body (MVB) PA is sorted onto an intraluminal vesicle (ILV) (figure 1) [64] allowing the translocation of material from the lumen of the MVB into the lumen of an ILV. This translocation event is driven by the pH gradient across the endosomal membranes and requires the unfolding on LF [94]. As the lumen of the ILV is topologically equivalent to the cytosol, an internal vesicle recycling “back-fusion” event has been documented to be responsible for the cytosolic release of LF and EF [64]. Once back-fusion has occurred, the contents of the MVB are digested within the endolysosome (figure 1). Uptake of PA has been found to be almost ubiquitous [95 & 96] with PA demonstrating a plasma t½ of 53 min following injection of 100μg *i.v.* into BALB/CJ mice [95], further the attenuation of both EF and LF is facile, removing domains II-IV leaving LFn and EFn respectively [97].

2.4 Attenuated toxins as nucleic acid delivery systems.

A variety of toxins have been explored as agents that may enhance the delivery of nucleic acids, and a number of different strategies have been adopted. In most cases a polycation has been conjugated to either an attenuated version of the toxin or to the toxin B chain. Occasionally a polycation like PLL is used to condense plasmid DNA that has already associated to the toxin system. DT [98 & 99], AT [100], PEA,

[101&102], and CT [103 & 104], have all been used in this way. All of these systems were designed to deliver therapeutic genes, in the form of plasmids, rather than synthetic oligonucleotides and as a consequence required a way to prevent the degradation of labile plasmids in biological fluids. However this would also have several other effects including the formation of IPECs which may be up to 120nm in diameter [38], and too large to pass through toxin pores, like PA [93] or the *Sec*61p translocon [105]. In addition, the surface charge of the IPEC may also impact upon pore translocation [106] as well as the thermodynamic stability of the complex, as several different A chains need to unfold in order to pass through their relevant pore to perform membrane translocation [94] (*i.e.* the PA pore or the *Sec*61p translocon). The heteromeric PA protein assembly is shown (Figure 2). In addition to the above, it is also known that an excess of positive charge may also radically alter the PK-PD of a molecule [59], increase its toxicity and impart a membrane destabilsing effect required for transfection [22] (see section 1.4). Consequently, it is difficult to discern from the above experiments (*i.e*. [98-104]) how much transfection was due to the polycation and how much was due to the toxin and further, how much was due to a synergistic effect resulting from the effects of both toxin pore formation and membrane interaction, and polycation membrane interaction. A similar problem exists with the experiments utilising TeNT heavy chain chemically conjugated to PLL to mediate gene delivery [107]. A separate study expressing fragment of TeNT within muscle cells avoided the use of polycations but concluded that the inclusion of the TeNT fragment encoded by a plasmid injected into muscle mass did not have any influence on the expression of the transfected gene [108].

If the deliverable (in this instance a protein nucleic acid (PNA)) was derivative with lysine then it was shown that PA protein could be used as a delivery system [109], though what is not clear from this study is how the PNA would associate with the PA. Further, as it is known that PA may stress membranes [110], it is also possible that the 8 lysine residues attached to the PNA were serving to further stress the membrane (to the point of rupture) rather than facilitating the entry of the PNA into the PA pore.

Two studies stand out from this group in that they have not utilised polycations to augment toxin-mediated delivery of nucleic acids. The first used ST-like Vero toxin, fused to a DNA binding domain without polycations and reported the delivery plasmid DNA to the nucleus, monitored via the tracking of fluorescent DNA labelled with Oregon Green [111]. Another study, from the author's laboratory, negated the use of polycations (with anthrax toxin) by using either antisense or siRNA as the deliverable [23]. Here the problem of nuclease resistance was overcome via chemical modification of the oligonucleotide and it was shown that despite the minimum diameter of the PA pore being 6Å [93] and the size of the LFn::ASO construct ASO having a radius of gyration of about 25Å, [23] delivery was achieved *in vitro* at high efficiency (equivalent to nucleofection) with no measurable toxicity [23]. The PA pore and the conjugation strategy binding the LFn to either the siRNA or ASO are shown (Figure 2). These data may indicate that the PA pore is more dynamic than has been previously imagined.

3.0 Conclusion.

The PEG dilemma has been and remains a considerable rate limit when considering the non-viral delivery of nucleic acids. When non-viral gene replacement therapy is considered using toxin fragments to guide a replacement gene either to a subset of cells or to guide the DNA through the endomembrane system, polycations have been

used to protect DNA from nuclease attach. This means that many of the limits associated with the PEG dilemma remain. As siRNA and antisense oligonucleotides can be chemically modified to enhance their resistance to nuclease attack, toxin fragments have been successfully deployed to enhance transfection efficiency without impacting upon toxicity. If this can be shown to be applicable to the *in vivo* delivery of these therapeutic agents we may be one step closer to moving antisense and siRNA medicines into becoming mainstream therapeutic modalities.

4.0 Expert opinion.

What potential does this research hold and what are the goals? The potential value, both humanitarian and commercial, associated with being able to safely and efficiently modulate gene expression is vast. This value is not limited to the clinical arena but is also apparent within the agricultural and veterinary fields, impacting upon sectors as diverse as drug development and food security. Of the may ways to modulate gene expression antisense is one of the simplest, requiring the delivery of an often-polar macromolecule to the cytosolic compartment (containing target mRNA) is antisense therapy. Given that only three antisense drugs have been licenced by the FDA since the first in 1998, it is evident that there are problems with this rationale.

Key weaknesses with antisense therapeutics? Given that cells have evolved to exclude exogenous RNA and DNA (*i.e.* viroids, viruses etc.), it is not surprising that "naked" antisense drugs are not very effective as their intrinsic intracellular trafficking, assuming they can assimilate their target cells, is suboptimal. The cellular uptake of "naked" nucleic acids results in their endolysosomal accumulation and their subsequent enzymatic destruction. Consequently some sort of drug delivery system is required to 1) favourably modulate the PK-PD of antisense molecules (towards the intended target cell type) and 2) manipulate their subcellular targeting diverting them from the endolysosomal compartment to the cytosol and their target (mRNA). In the instance of antisense chemical modification can and has been used to stabilise these molecules in biological fluid, eliminating the need for enzymatic stabilisation from the delivery system. Whilst drug specificity is built into both antisense and siRNA sequence, target cell specificity is still desirable as it reduces the amount of drug needed to elicit the desirable therapeutic effect whilst reducing the possibility of unwanted side effects.

What is needed to achieve the above goals? Many research groups have dedicated a lot of time to developing delivery systems and strategies for antisense, siRNA and gene replacement therapies. The use of polycations as nucleic acid delivery vehicles has been known for many years.

What are the challenges? It has become apparent that it is difficult navigating; toxicity, intracellular targeting, stability, complement activation and PK-PD when using either cationic polymers or lipidic formulations for DNA delivery. The highly entropic nature of polymer-membrane interactions also makes defining specific mechanisms of activity responsible for the delivery of nucleic acids to the nucleocytosolic compartment difficult.

Future research? The ability of many different protein toxins to access the cytosol has also been extensively studied and many of the specific molecular mechanisms responsible for their intracellular trafficking are well understood. As polycations mediated protection from nucleases for ASOs and siRNA can be countered with innovative changes to oligonucleotide chemistry, and the possibility of nucleic acid

pore transit has been demonstrated with a similar efficiency to nucleofection, the possibility of the cytosolic delivery of siRNA and antisense agents using attenuated protein toxins is now a reality. This is important as toxin delivery circumvents many of the problems associated with the PEG dilemma. What remains to be seen is if these systems can function safely and with high efficiency *in vivo* and eventually in the clinic. Whilst there will undoubtedly be further problems to overcome, such as cell targeting and protein immunogenicity, solutions may already exist, such as protein PEGylation, or as the Leppla group recently demonstrated, with drugs that can control the immune response to toxins [112], and further recombination strategies to facilitate cell type specific targeting.

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*[93] Jiang, J., Pentelute B. L., Collier R. J., *et al.,* (2015), Atomic structure of Anthrax protective antigen pore elucidates toxin translocation. Nature **521**: 545–549, doi:10.1038/nature14247.

[94] Krantz B. A., Trivedi A.D., Cunningham K., *et al.,* (2004) Acid-induced Unfolding of the Amino-terminal Domains of the Lethal and Edema Factors of Anthrax Toxin, J. Mol. Biol. **344**, 3: 739-56

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*[96] Dadachova E. Rivera J., Revskaya E., *et al.* (2009), *In vitro* evaluation, biodistribution and scintigraphic imaging in mice of radiolabeled anthrax toxins. Nucl. Med. Biol. **37**: 755–761, doi: 10.1016/j.nucmedbio.2008.07.001

[97] Aurora N. & Leppla S.H. (1993), Residues 1-254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. J. Biol. Chem. **268: 3334–3341.

*[98] Uherek C., Fominaya J., Wels W. (1998), Modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery, J Biol Chem., **273**: 8835-41.

This paper used a variant of the T protein fused to GAL4 (DNA binding) for the delivery of a plasmid that was condensed using poly(lysine). Transgene expression (luciferase) was enhanced by the addition of chloroquin and a dependence upon a specific receptor used by their toxin construct was observed.

[99] Fisher K.J., & Wilson J.M. (1997), The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer, Biochem J., **321: 49–58

This study used the trans-membrane domain of DT which was chemically coupled to poly(lysine) and used to condense a plasmid. This paper didn't really explore the system's trafficking beyond the use of chloroquin which didn't seem to dramatically enhance transfection.

[100] Gaur R., Gupta P.K., Goyal A., *et al.* (2002), Delivery of nucleic acid into mammalian cells by anthrax toxin. Biochem. Biophys. Res. Commun., **297: 1121–7

This paper used Atx LFn fused to GAL 4 to bind DNA which was condensed with poly(lysine). Transfection was evident through both luciferase and GFP expression though beyond the omission of PA (which decreased transfection activity), little attention was given to the intracellular trafficking of this system or the influence of PLL upon transfection.

*[101] Fominaya J., & Wels W. (1996), Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. J Biol. Chem., **271**: 10560-8

This paper examines the ability of PEA to augment plasmid gene delivery. Again both GAL4 (to bind the plasmid DNA to the PEA fragment) and poly(lysine) (to condense the plasmid) were used. It is interesting that the PEA "trans-membrane domain" domain was shown to augment endosomal escape when it is now known that PEA achieves cytosolic access via the Golgi and ER. Again receptor specificity was demonstrated, which, given the non-specific interaction between PLL and membranes seems strange.

*[102] Fominaya J., Uherek C., Wels W. (1998), A chimeric fusion protein containing transforming growth factor-alpha mediates gene transfer via binding to the EGF receptor. Gene Therapy, **5**: 521–30

This paper reports a similar strategy to the one above documenting their modular construct increasing the transfection activity 150-fold relative to a PLL plasmid complex alone. Of interest was the cell type dependent activity of the "membrane destabilising domain" in relation to the use of chloroquine.

*[103] Barrett L.B., Logan A., Berry M., *et al.,* (1999), Targeted transfection of neuronal cells using a poly(D-lysine)-cholera-toxin b chain conjugate. Biochem Soc Trans., **27**: 851–7

Here CTBC was chemically conjugated to poly(D-lysine) and the results of transfection with and without serum were documented. There is no attempt made to analyse intracellular trafficking.

[104] Barrett L.B., Berry M., Ying W.B., *et al.* (2004), CTb targeted non-viral cDNA delivery enhances transgene expression in neurons, J. Gene Med. **6**: 429–38

Here a 36-fold increase in transfection level relative to poly(lysine) and plasmid alone is reported in a differentiated neuron cell line using a CTBC fused to poly(D-lysine).

[105] Voorhees R. M., Fernández I.S., Scheres S.H., *et al.,* (2014), Structure of the Mammalian Ribomsome-Sec61 Complex to 3.4 A Resolution, Cell, ***157***: 1632-43, doi: 10.1016/j.cell.2014.05.024

[106] Zornetta I., Brandi L., Janowiak B., *et al.* (2010), Imaging the cell entry of the anthrax oedema and lethal toxins with fluorescent protein chimeras, Cell Microbiol. **12**: 1435-45, doi: 10.1111/j.1462-5822.2010.01480.x

[107] Knight A., Carvajal J., Schneider H., *et al.,* (1999) Non-viral neuronal gene delivery mediated by the HC fragment of tetanus toxin, Eur. J. Biochem., **259**: 762-9

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[109] Wright D.G., Zhang Y., Murphy J.R. (2008) Effective delivery of antisense peptide nucleic acid oligomers into cells by anthrax protective antigen, Biochem. Biophys. Res. Commun., **376,** 1: 200-5, doi: 10.1016/j.bbrc.2008.08.124

[110] Nablo, B. J., Panchal R.G., Bavari S., *et al.,* (2013) Anthrax toxin-induced rupture of artificial lipid bilayer membranes, J. Chem. Phys., **139**: 065101

***[111] Facchini L. M. and Lingwood C. A. (2001) A Verotoxin 1 B Subunit-Lambda CRO Chimeric Protein Specifically Binds Both DNA and Globotriaosylceramide (Gb3) to Effect Nuclear Targeting of Exogenous DNA in Gb3 Positive Cells, Experimental Cell Research 269: 117–129, doi:10.1006/excr.2001.5297

Reported are the results of the generation of a Vero toxin-Cro (DNA binding domain) fusion used for the nuclear import of reporter DNA bound to the Cro domain. This was intended to circumvent the cytosolic degradation of plasmid DNA en route to the nucleus which was identified by the authors as a rate limit. A dependence on Gb3 (the receptor) was demonstrated and the fluorescent DNA imaged microscopically within the cell. Brefeldin A (blocking Golgi transport) inhibited the nuclear transport of the fluorescent labelled DNA. The data is presented in the form of micrographs, which makes quantitation of the effectiveness of this strategy hard. Regardless this is a very interesting paper as no condensing agents were used. It is also interesting that the focus of this paper was the delivery of plasmids and that no transfection (utilising a plasmid) was shown.

***[112] Liu S., Liu J., Ma Q., (2016), Solid tumor therapy by selectively targeting stromal endothelial cells, Proc Natl Acad Sci USA, **113**, 28: E4079-87, doi: 10.1073/pnas.1600982113

This paper is of interest as it describes an experimental method of countering protein toxin (anthrax toxin) immunogenicity in vivo.

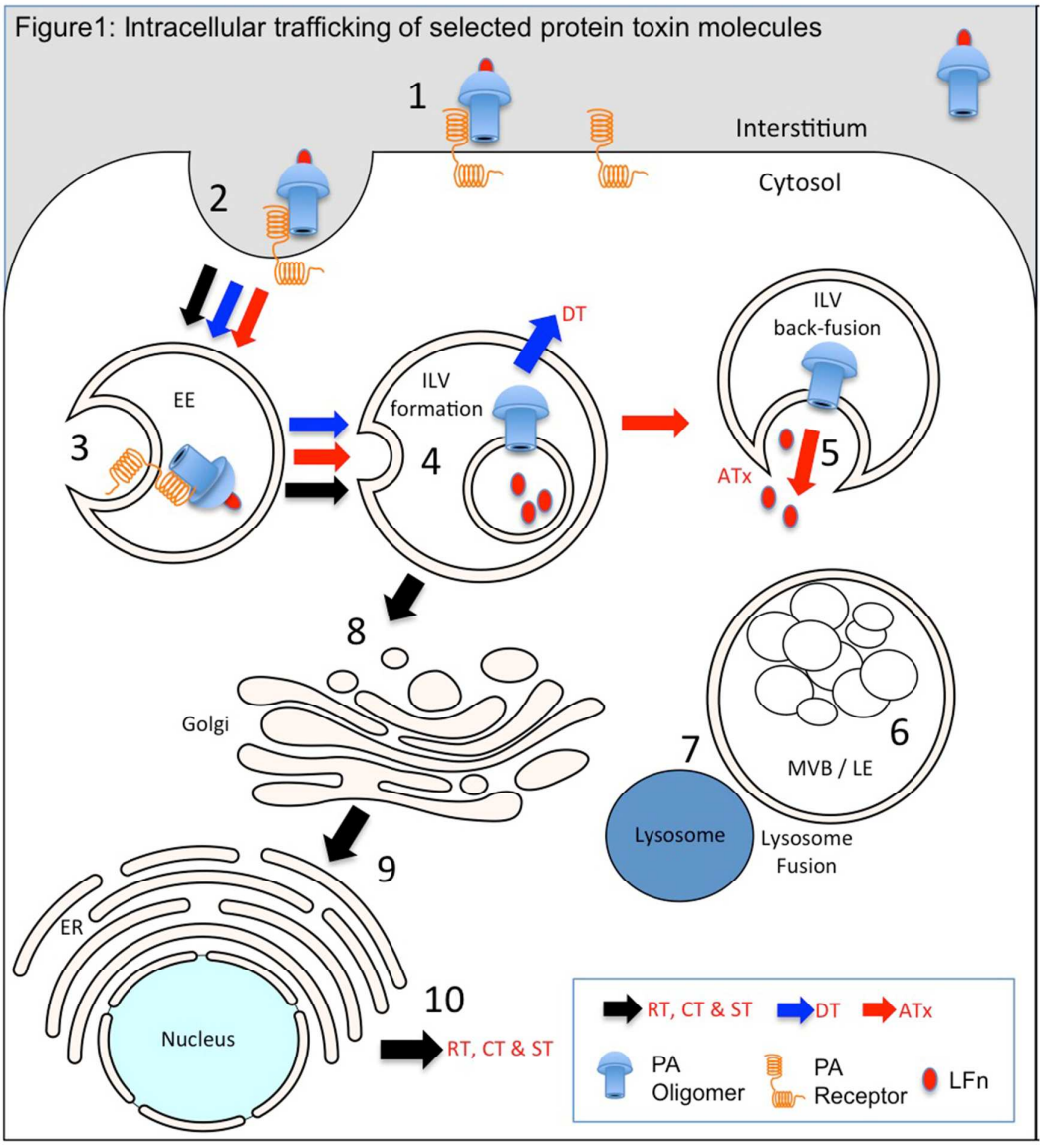

Figure 1: (1) Receptor association mediates cell uptake. (2) Internalisation may be driven via a number of different coat proteins and is toxin dependent. (3) Receptor sorting within early endosomes (EE). (4) Cargo sorting and Anthrax PA prepore to pore transition is driven by the drop in luminal pH. This triggers a shift in PA conformation and pushes the pore into the lipid bilayer of the ILV. The drop in luminal pH is also thought to drive the translocation of cargo into the ILV lumen. This is also the point of membrane insertion of the DT B subunit which allows the "C" domain of the A chain into the cytosol. (5) ILV recycling or "back fusion" releases Anthrax LFn into the cytosol. (6) ILVs concentrate in multivesicular bodies (late endosomes) prior to lysosomal fusion are digested after (7) endolysosome formation. (8) RT can retro-translocate from either the recycling endosome or the sorting endosome to the

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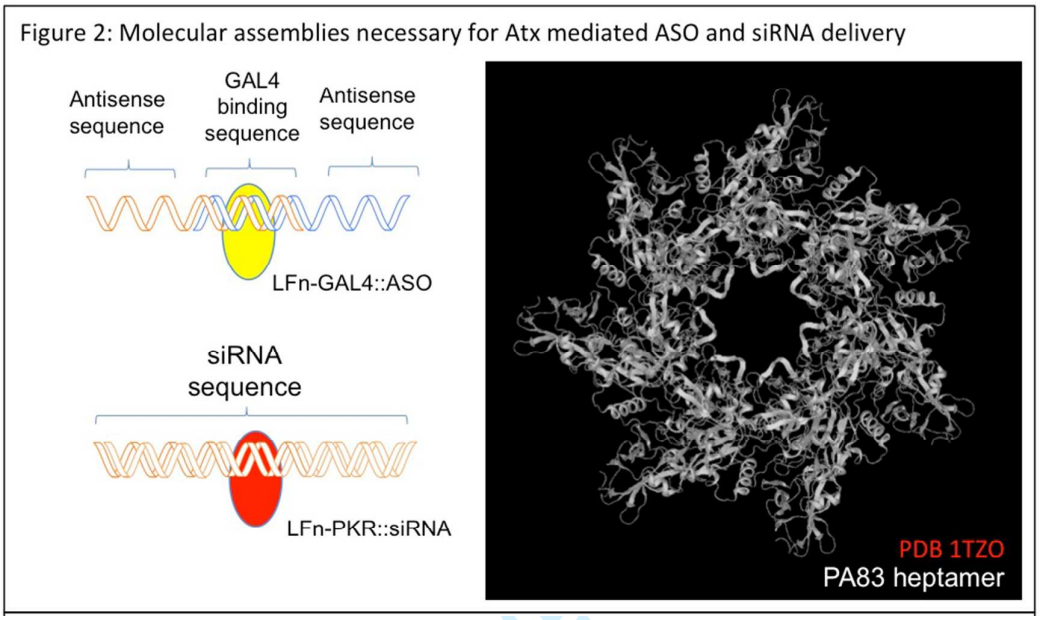

Figure 2. Shown are the components of the attenuated Atx delivery system used for siRNA and ASO delivery. Here Anthrax Lethal Factor has been truncated to leave (approximately) domain 1 (n-terminal amino acids 1-255) which was fused to either the *Saccharomyces cerevisiae* transcription factor GAL4 (to bind a specific sequence of double stranded DNA) or *Homo sapien sapien* protein kinase R (PKR) for binding double stranded RNA. ASOs were assembled in such a way to produce both a double stranded GAL4 binding sequence as well as a single stranded antisense sequence. These were assembled and introduced to recombinant PA83, which has been documented to form both homo-heptamers and homo-octamers. These two elements were then allowed for a supramolecular assembly, which was introduced to cells in culture. Adapted from [23, 85-87].

Trial	Disease	Drug	Delivery System	Progress
NCT01591356	advanced, recurrent cancer	siRNA-EphA2-DOPC	DOPC	Phase 1 (recruiting)
NCT00938574	Advanced Solid Tumours	Atu027 targeting protein kinase N3	Liposome	Phase 1 (complete)
NCT01437007	Primary or Secondary Liver Cancer	TKM-080301	Liposome	Phase 1 (complete)
NCT00882180	Advanced Solid Tumours With Liver Involvement	ALN-VSP02 targeting VEGF and KSP	Lipid Nanoparticle	Phase 1 (complete)
NCT02314052	Hepatocellular Carcinoma (HCC)	DCR-MYC siRNA against MYC	Lipid Nanoparticle	Phase 1b/2
NCT02227459	Moderate to Extensive Hepatic Fibrosis	L02-s0201 siRNA Against HSP47	Vitamin A-Coupled Lipid Nanoparticle	Phase 1b/2 (active)
NCT02595983	Transthyretin (TTR) cardiac amyloidosis	Revusiran (ALN-TTRsc)	GalNAc-conjugated RNAi	Phase 2 (recruiting)
NCT02597127	Atherosclerotic cardiovascular disease	ALN-PCSSC	LDL Cholesterol	Phase 2 (recruiting)
NCT02319005	Transthyretin (TTR) mediated Familial Amyloidotic Cardiomyopathy	Revusiran (ALN-TTRsc)	GalNAc-conjugated RNAi	Phase 3 (recruiting)
NCT01960348	TTR-mediated Amyloidosis	Patisiran (ALN-TTR02)	Lipid Nanoparticle	Phase 3 (active)

Table 1: Select, Recent, Completed, Active or Recruiting Clinical Trails Using siRNA

Table 1: Select examples of recent and on going clinical trials using siRNA as a therapeutic. Adapted from [18 and www.clinicaltrials.gov. (using “siRNA” as a search term)]

Toxin	Structure	Receptors	Target
Ricin toxin	AB	Surface proteins containing terminal *N*-acetylgalactosamine or beta-1,4-linked galactose	Ribosome (28s rRNA)
Anthrax toxin	AB	Tumour Endothelial Marker (TEM) 8, capillary morphogenesis gene (CMG)2, Integrin β1	LF-MAP Kinase kinase EF- Adenylate cyclase
Shiga toxin	AB_5	Globotriaosylceramide 3 (G_{b3})	Ribosome (28s rRNA)
Cholera toxin	AB_5	Galactosyl-*N*-acetylgalactosaminyl-(sialyl)-galactosylgluco- sylceramide 1 (G_{M1})	Heterotrimeric G protein
Pseudomonas Exotoxin A	AB_5	Low-density lipoprotein receptor-related protein 1 (LRP 1), LRP1B	Elongation Factor (EF) -2
Diphtheria toxin	AB	Heparin-binding epidermal growth factor precursor (HB-EGF) receptor	Elongation Factor (EF) -2
Tetanus toxin	AB	Dissialogangliosides (GD2 and GD1b)	VAMP

Table 2: Selected Protein Toxin Characteristics

Table 2: Select protein toxins and their cellular receptors. Adapted from [61, 69, 74].

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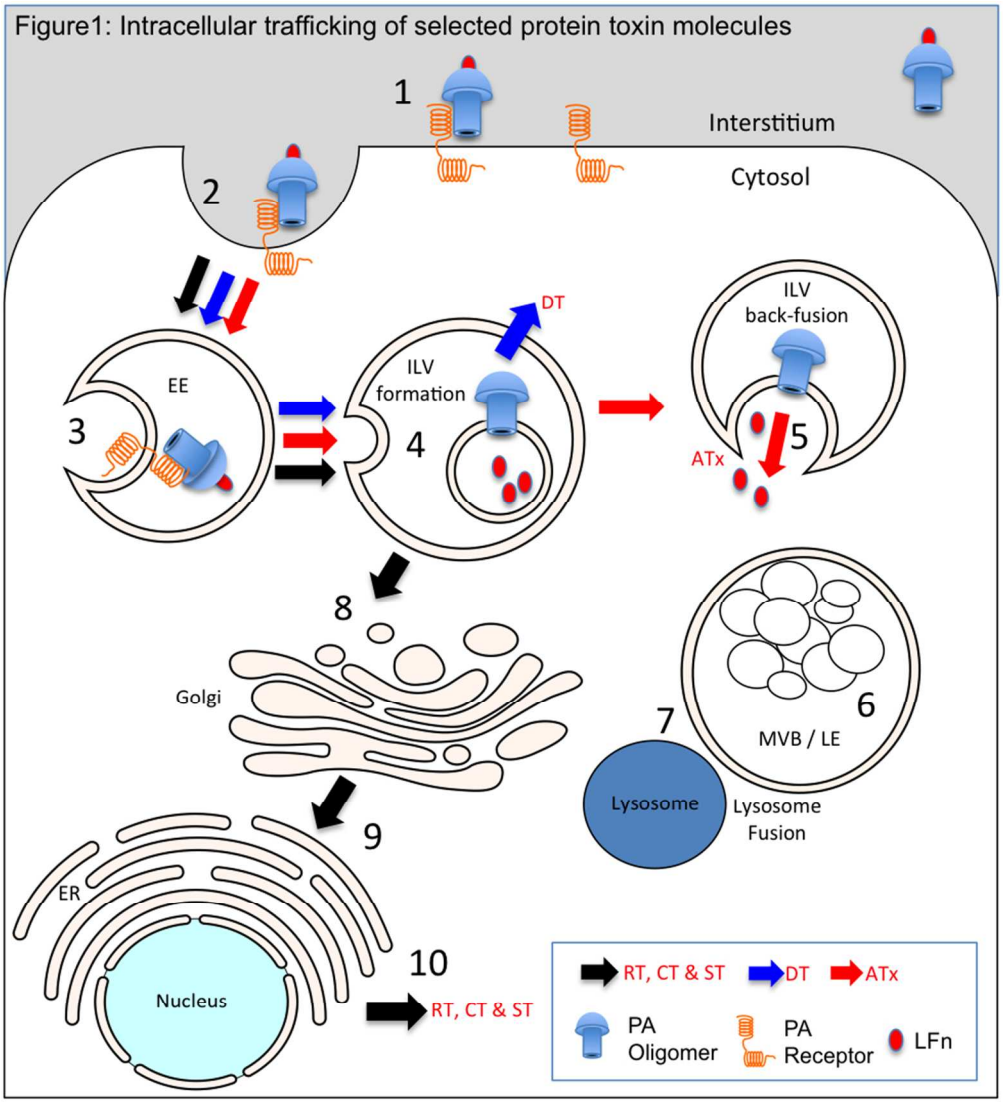

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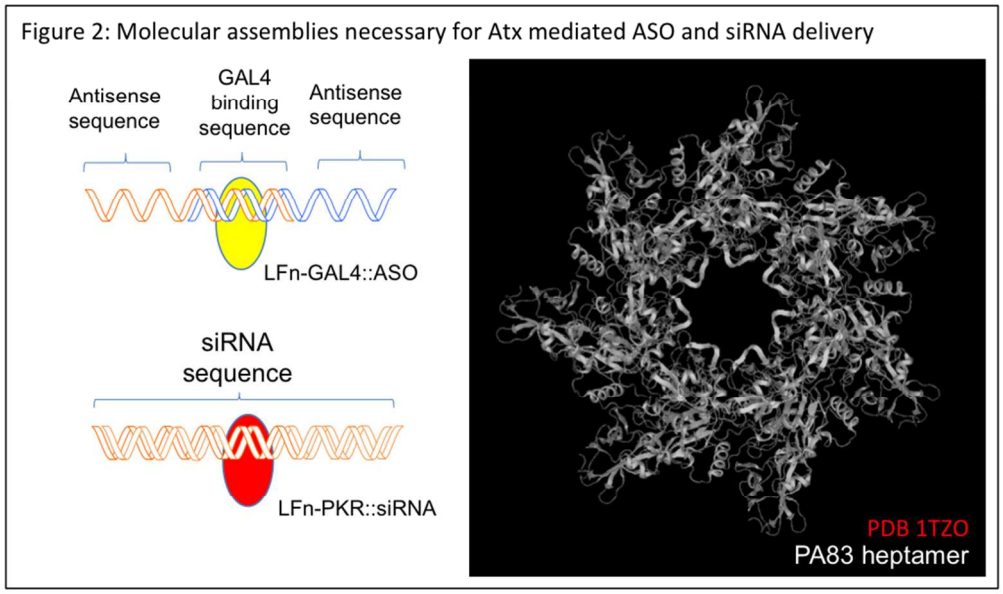

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