UNIVERSITY OF CALIFORNIA SAN DIEGO

Delivery of RNAi Therapeutics: Building Multifunctional RNAi Triggers for Extra-Hepatic Targeting and Endosomal Escape

A dissertation submitted in partial satisfaction of the

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Biomedical Sciences

by

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2019

The Dissertation of Aaron David Springer is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

Delivery of RNAi Therapeutics: Building Multifunctional RNAi Triggers for Extra-Hepatic Targeting and Endosomal Escape

by

Aaron David Springer

Doctor of Philosophy in Biomedical Sciences

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Professor Steven F. Dowdy, Chair

The discovery of RNAi and the subsequent demonstration that synthetic short interfering RNA (siRNA) could silence all mRNA expression in a sequence dependent manner offered tremendous potential as a therapeutic to treat all genetic disease. However, siRNA is both too large (>14,000 Da) and too charged (40+ phosphates) to passively diffuse across the cell membrane, requiring a targeting domain to deliver the siRNA therapeutic. Conjugation of siRNA

to *tris*-N-acetylgalactosamine (GalNAc) targeting liver asialoglycoprotein receptor (ASGPR) revolutionized RNAi therapeutics and the problem of hepatic delivery can now be considered solved. However, siRNA therapeutics have not seen the same success in extra-hepatic tissues as the biology of these tissues has proven more difficult for siRNA targeting and delivery.

To address the issue of siRNA delivery, our laboratory developed short interfering ribonucleic neutrals (siRNN) whose charged phosphodiester backbone has been neutralized by bioreversible phosphotriester groups. The first generation siRNN utilized a *t*-butyl-s-acyl-2-thioethyl (tBu-SATE) phosphotriester group that allowed enhanced *in vivo* GalNAc-siRNA delivery. To adapt this technology for extra-hepatic delivery, we modified the *t*Bu-SATE phosphotriester to allow site-specific conjugation through copper catalyzed Click chemistry. Conjugation of mannose to siRNNs effectively delivered siRNN to CD206+ macrophages and elicited a robust RNAi response in a model of tumor-associated macrophages (TAMs). Unfortunately, additional ligand/receptor pairs like mannose/CD206 and GalNAc/ASGPR that are tissue specific, highly expressed, and rapidly internalize are extremely limited. An alternative to small ligands is the use of antibodies. Traditional antibody targeted therapies often rely on conjugations that result in a poorly defined, heterogeneous mixtures of antibody-drug conjugates. To conjugate siRNNs to antibodies in a quantitative manner, we developed a site-specific enzymatic conjugation strategy. Unfortunately, the resulting antibody RNA conjugates (ARC) failed to deliver the siRNN into the cytoplasm, likely due to endosomal entrapment. To avoid entrapment, endosomal escape domains (EED) were incorporated into the ARC through a hydrazone conjugation phosphotriester to form an ARC-EED. Taken together, this work describes the development of novel, well defined, site-specific, multifunctional, and multivalent siRNN conjugates capable of extra-hepatic targeting and endosomal escape.

CHAPTER 1

THE CURRENT STATE OF RNAi THERAPEUTICS

THE CURRENT STATE OF RNAi THEAPEUTICS

ABSTRACT

The discovery of RNAi afforded a new and powerful approach to post-translational gene regulation. The subsequent discovery that exogenous small interfering RNAs (siRNAs) could reproduce this effect opened up a new class of therapeutics with exquisite target selectivity, potency, and the potential to treat genetic and pandemic disease. Despite the promise of RNAi therapeutics, siRNA has a long list of unfavorable drug-like properties and, critically, has no ability to enter cells. Early attempts to mask the siRNA and deliver to cells *in* vivo utilized complex formulations of cationic lipids and polymers for nanoparticle delivery. While this approach saw limited preclinical success, nanoparticles are plagued with poor biodistribution, tissue penetration, and systemic toxicities. Conjugation of siRNAs to *tris*-N-acetylgalactosamine (GalNAc) revolutionized the siRNA therapeutic field by providing a small, monomeric, soluble siRNA delivery method. GalNAc-siRNA therapeutics are in several late stage clinical trials with promising initial results. Despite this success, extra-hepatic targeting and endosomal escape remain major hurdles for the field to overcome if the potential of RNAi therapeutics is to be realized. While advances have been made in these fields, construction of a multifunctional siRNA molecule capable of extra-hepatic targeting and endosomal escape poses a challenge in itself. To this end, my thesis project has sought build upon our small interfering ribonucleic neutral (siRNN) platform to develop conjugation strategies that provide rapid, modular and well-characterized multifunctional siRNA therapeutics.

INTRODUCTION

RNA interference (RNAi) was discovered in 1998 when it was found that injection of double stranded RNA (dsRNA) could silence endogenous genes in *C. elegans* (Fire et al., 1998a). This discovery revolutionized the scientific community's understanding of gene regulation by revealing a litany of cellular pathways where small, non-coding dsRNAs alter gene expression. RNAi spans both plants and animal kingdoms (Shabalina and Koonin, 2008) and as much as 5% of the human genome is devoted to encoding the ~2,000 micro RNAs (miRNA) that regulate ~30% of the expressed human genome (Jinek and Doudna, 2009; Lee et al., 2006). These RNAi pathways are responsible for modulating a diverse array of cellular processes and their dysregulation is involved in cardiovascular disease, neurological disorders, and cancer (Lu et al., 2008). In addition to these endogenous processes, RNAi plays an important role in cellular response to exogenous assaults from viral nucleic acids (Wilson and Doudna, 2013).

The primary unit in RNAi is the RNA induced silencing complex (RISC), and is minimally composed of a single stranded RNA (ssRNA)(20-30 nt) and an Argonaut (Ago) family protein. The RISC complex silences mRNA expression through target degradation or transcriptional repression in a sequence specific manner determined by the Ago-bound ssRNA (Wilson and Doudna, 2013). The origins of the ssRNA within the RISC complex can be divided primarily into endogenous/genomic origins (miRNA) and exogenous/synthetic (siRNA) (Carthew and Sontheimer, 2009). Both of these sources converge in their mechanism of action following successful loading into the RISC complex, but their disparate origins serve to highlight important cellular machinery that impact and influence current therapeutic applications of RNAi.

Production of functional miRNA begins with transcription of primary-miRNA (pri-miRNA) (de Rie et al., 2017; Kim and Kim, 2007). Pri-miRNA consist of at least 1,000 nt and are composed of single or clustered hairpin loops characterized by 3' and 5' single stranded overhangs and a ~10 nt distal loop (Saini, Griffiths-Jones, and Enright, 2007). The base of each hairpin loop is recognized by the DiGeorge syndrome critical region gene 8 (DGCR8) protein

that recruits and positions an RNase III family enzyme, Drosha, for endonucleolytic cleavage (Y.-K. Kim and Kim 2007; Jinju Han et al. 2006). The resulting 65-70 nt pre-miRNA is exported from the nucleus to the cytoplasm by the Exportin 5/RanGTP complex (Lund and Dahlberg, 2006; Denli et al., 2004).

Following export from the nucleus into the cytoplasm, pre-miRNA is recognized by transactivation response element (TAR) RNA-binding protein (TRBP) (Chendrimada et al., 2005). Efficient binding of the pre-miRNA by TRBP requires association with three dsRNA binding domains (DRBD) and mutations in TRBP resulting in loss of a single DRBD significantly reduce pre-miRNA binding (Fareh et al., 2016). TRBP is tightly bound to Dicer (Chendrimada et al., 2005; MacRae et al., 2008), a multi-domain enzyme containing a PAZ domain (present in PIWI, Argonaut, and Zwille proteins) and multiple tandem RNase III domains (Ha and Kim, 2014; Jinek and Doudna, 2009; Wilson et al., 2015; Chakravarthy et al., 2010; Y. Kim et al., 2014). Co-recognition by Dicer and TRBP allows for efficient processing of pre-miRNA that represents only ~0.01% of cellular RNA (Peltier and Latham, 2008). The PAZ domain of Dicer recognizes the 2 nt 3'-overhang of pre-miRNA and the spacing between PAZ binding and the RNase III domain in Dicer acts as a molecular ruler, cleaving the loop from the hairpin structure and determining the 21-23 nt length of the final, mature miRNA (Yan et al., 2003; MacRae et al., 2006; MacRae, Zhou, and Doudna, 2007; Tian et al., 2014; Bernstein et al., 2001; Zhang et al. 2004). Dicer and TRBP load mature miRNA into an Ago protein to form the RISC-loading complex (RLC) (MacRae et al., 2008; Yoda et al., 2010).

Coincident with miRNA loading is selection of a Guide (Antisense) strand from the miRNA duplex to be loaded into Ago while the other strand, termed the Passenger (Sense) strand is discarded. For miRNAs, strand selection is determined in part by thermodynamic stability at the 5'-end or the presence of a 5'-U in the first position (Khvorova, Reynolds, and Jayasena, 2003).

The mature RISC complex binds to mRNA targets with specificity determined by the

Guide strand sequence (Ha and Kim, 2014). Guide strand nucleotides 2-8 make up the Seed sequence that is responsible for initiating binding to the target mRNA prior to full strand interaction (Birmingham et al., 2006; Anderson et al., 2008; Schirle and MacRae, 2012). Argonaut proteins provide the Guide strand structural context for initial binding of the Seed region to the target mRNA by pre-forming these nucleotides in an A-form helical shape (Schirle and MacRae, 2012; Schirle, Sheu-Gruttadauria, and MacRae, 2014), thereby dramatically lowering the activation energy needed for initial base pairing compared to a free antisense oligonucleotides (Salomon et al., 2015). The majority of miRNAs bind their target sequence within the 3' UTR though less frequent miRNA binding sites have been found in the 5'-UTR and promoter sites of mRNAs (Huntzinger and Izaurralde, 2011; Ipsaro and Joshua-Tor, 2015; Xu et al., 2014). The Seed region of the Guide strand and the target mRNA often contain base pairing mismatches that do not interfere with RNAi activity, allowing a single miRNA Guide strand to regulate gene expression for many different mRNAs (Martin et al., 2014). Guide strands that contain mismatches lead to translational inhibition of target mRNA expression through recruitment of additional cellular machinery that leads to mRNA sequestration within cytoplasmic processing (P)-bodies (Eulalio, Huntzinger, and Izaurralde, 2008). In contrast, perfect complementarity can lead to mRNA strand scission when loaded into Ago2 (Ha and Kim, 2014; Jo et al., 2015; Jonas and Izaurralde, 2015). Of the four human Ago family members (Ago1,2,3,4), Ago2 is the only Argonaute family protein that contains catalytic activity capable of mRNA strand scission (Rivas et al., 2005; Song et al., 2004; Liu et al., 2004; Meister et al., 2004).

In contrast to miRNA, exogenous dsRNA from viral or synthetic origins is processed entirely within the cytoplasm by Dicer into 21-23 nt dsRNA (**Figure 1.1**) (Bernstein et al., 2001). These dsRNA are loaded into Ago family proteins and silence pathogenic viral replication. The action of this 21-23 nt dsRNA can be replicated synthetically to target any mRNA target as

demonstrated in Tuschl's lab (Elbashir et al., 2001; Fire et al., 1998b). These short interfering RNA (siRNA) are characterized by 2 nt 3'-overhangs, 5'-phosphate groups, and are composed of a Passenger and Guide strand, similar to miRNA. siRNA is recognized by the same cellular machinery as miRNA and are efficiently loaded by TRBP into all Ago family proteins (Ohrt et al., 2012). The exact cellular machinery involved in siRNA processing is determined by the structure of the synthetic siRNA. Standard 21/21 dsRNA with 2 nt 3'-overhangs can bypass Dicer processing entirely and be loaded directly into Ago2 (Murchison et al., 2005). Longer 25-27 nt dsRNA triggers characterized by a single blunt end and an opposing 2 nt 3'-overhang are recognized by the PAZ domain in Dicer and are thus termed Dicer substrates (Sakurai et al., 2011). In contrast to miRNA, siRNA are designed to target a specific gene of interest and therefore contain complete complementarity to the target mRNA, allowing for catalytic activity when loaded into Ago2. siRNA-induced RNAi has become standard in the study of gene function and has great potential therapeutic applications.

Evaluating the Therapeutic Potential of RNAi

Traditional small molecule therapeutics have played a major role in the treatment of a variety of diseases, but fall far short of being able to drug the entire proteome. Additionally, small molecule therapeutics have no ability to adapt to changes in target binding sites resulting from mutation or target pathway adaptation, such as in cancer, that can render a small molecule therapeutic ineffective. Development of new small molecule therapeutics is a time consuming and costly process that is not possible for every patient's individual mechanism of resistance. The nature of small molecule therapeutics also makes them susceptible to rapid kidney filtration and degradation in the liver, requiring frequent dosing to achieve prolonged efficacy. With these issues in mind, siRNA therapeutics offer a promising alternative that has the potential to overcome these limitations and improve patient care for a variety of previously incurable or difficult diseases.

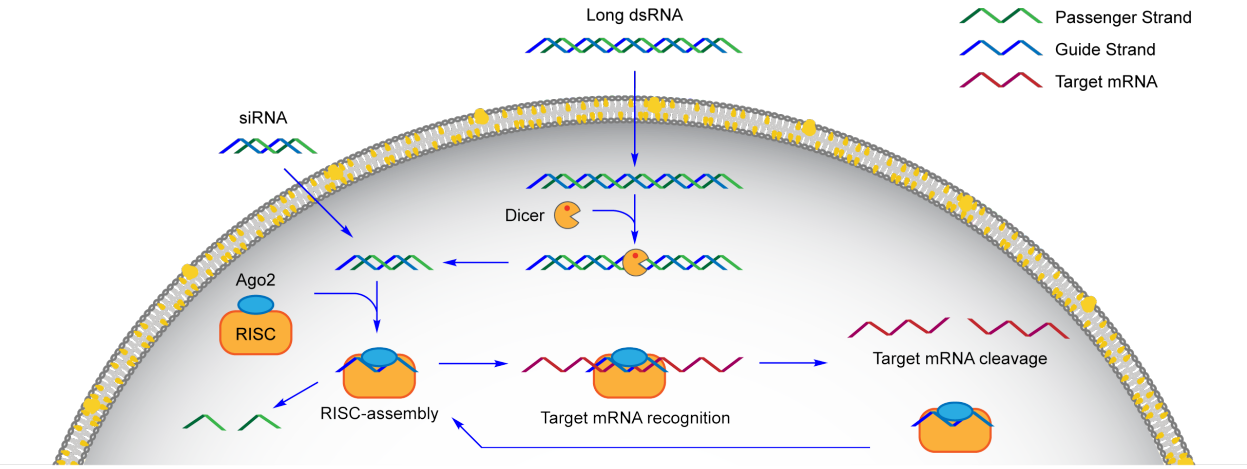


Figure 1.1. The Mechanism of RNA Interference (RNAi).
Viral long double-stranded RNA is cleaved in the cytoplasm into siRNA by the enzyme Dicer. Alternatively, synthetic siRNA can also be introduced into the cell for entry into the RNAi pathway in a Dicer-independent manner. Cytoplasmic siRNA is incorporated into RISC, resulting in the cleavage of the Passenger strand by Argonaute 2 (Ago2). The cleaved Passenger strand is then ejected and degraded, thereby activating the RISC-siRNA complex. The activated complex seeks out, binds to, and degrades target mRNA, leading to silencing of the corresponding gene. The activated RISC-siRNA complex is then recycled for the continued degradation of identical mRNA targets.

siRNAs have many promising therapeutic attributes, including an EC_{50} in the picomolar (10^{-12}) range and exquisite target selectivity for all mRNAs (Bumcrot et al., 2006). In contrast, traditional small molecule therapies have been unable to target transcription factors and many oncogenes, whereas siRNAs have the potential to target previously intractable cMyc and Kras mutants with relatively simple sequence screens (**Figure 1.2**). As a result, siRNA has the potential to treat a wide variety of human diseases from cancer to pandemic viral outbreaks to Parkinson's Disease (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017). Moreover, siRNAs have the potential to pharmaco-evolve their targeting sequence to keep pace with mutations in diseases driven by genetic change, such as cancer and influenza, a feat that no other clinical modality can perform (Dowdy, 2017). Due to the catalytic mechanism of action of siRNAs, a single dose is capable of prolonged pharmacodynamic effects, with a single subcutaneous dose showing efficacy past 6/9 months for liver diseases (Alnylam, 2017c; Fitzgerald et al., 2017a). siRNAs can also be synthesized in a scalable manner, allowing for rapid production of siRNAs targeting any mRNA (Beaucage and Iyer, 1992).

Despite the promise of siRNA as a potential therapeutic, it has several significant attributes that limit its therapeutic utility. The 40 negative charges of the siRNA phosphodiester backbone and the 14,000 Dalton (Da) size prevent siRNA molecules from crossing the cellular membrane (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017). These attributes also make siRNAs pharmacokinetically unfavorable, as naked siRNA is removed from the bloodstream by the kidneys within minutes of injection into mice and humans (Merkel et al., 2009). Additionally, native double stranded siRNAs are recognized as invading nucleic acids by multiple cellular defense mechanisms, including extracellular Toll-Like Receptors (TLR-3, -7, -8) and intracellular sensors retinoic acid inducible gene (RIG-I) and melanoma differentiation associated protein 5 (MDA-5) (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017; Gantier and Williams, 2007; Iversen et al., 2013; Juliano et al., 2014).

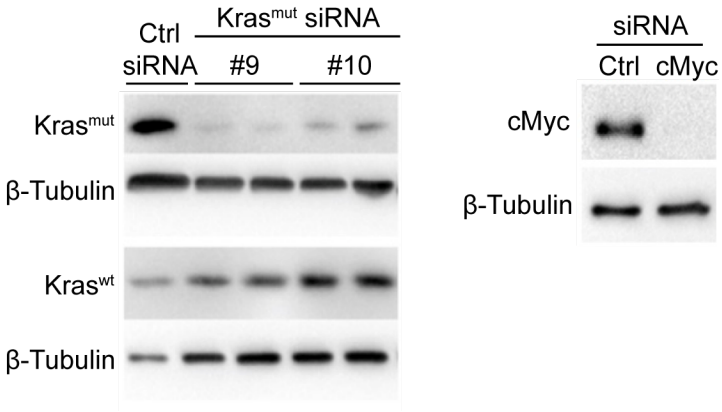


Figure 1.2. Targeting "Undruggable" Oncogenes by RNAi.
Selective Kras^{mut} RNAi response with siRNA perfectly matching Kras^{mut} and bearing mismatches to Kras^{wt} at positions 9 and 10 on the guide strand in MDA-MB-231 breast cancer cells at 48 h after lipofection. cMyc RNAi response in MDA-MB-231-cMyc-HA breast cancer cells at 48 h after lipofection.

These difficulties necessitate the use of delivery agents to both assist siRNAs to cross the lipid bilayer and to remain in circulation for longer periods of time. Consequently, the major obstacle prohibiting effective RNAi therapeutics has been delivery. Unsurprisingly there has been significant attention and investment of time and resources to address the delivery problem by harnessing and developing a wide array of technologies (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017).

Early Work on RNAi Therapeutics

The majority of early solutions to RNAi therapeutic delivery focused on lipid nanoparticles (LNP) and synthetic nanoparticles (NPs) that were predicated on several decades worth of nanoparticle delivery approaches developed for large DNA gene therapy vectors (Whitehead, Langer, and Anderson, 2009; Juliano, 2016; Zimmermann et al., 2006). These nanoparticle delivery systems are extremely diverse in their compositions and properties, but generally employ a strategy where cationic lipid or polymer molecules interact and condense with the anionic siRNA backbone to compact siRNAs into large particles surrounded by delivery agent molecules.

LNPs and NPs serve to mask the siRNA charge, protect it from degradation by RNases and facilitate endosomal escape into the cytoplasm. Nanoparticles also allowed for the use of minimally modified siRNA backbones that primarily contained native 2'-hydroxyl groups. LNPs and NPs have a number of advantages, including increased circulation time, and avoidance of innate immune activation. These characteristics have enabled nanoparticle-based siRNA delivery methods to function with limited success in a variety of animal models (Schroeder et al., 2010). Tissue specificity of LNPs and NPs is determined primarily by their size as nanoparticles tend to accumulate in the liver due to the large fenestrations within the hepatic vasculature that allow for escape and accumulation in the hepatic tissue (Sarin, 2010; Akinc et al., 2009;

Schroeder et al., 2010). Attempts to direct the cellular delivery of LNPs and NPs beyond these tissues utilize additional motifs such as antibodies, cell targeting ligands, and a variety of lipids.

Despite their potential benefits, nanoparticle siRNA delivery systems are fraught with significant problems that limit their use for delivery of siRNA in humans. The size and mass of siRNA nanoparticles, in the range of hundreds of nanometers in size and on the order of 100,000,000 Daltons (100 megaDa) in size are in far excess (~5,000x) larger than the siRNA molecules they are attempting to deliver (Meade and Dowdy, 2009). The massive size of the particles limits biodistribution and also gravely reduces their diffusion coefficient through the interstitial spaces of tissues, limiting tissue exposure (Whitehead, Langer, and Anderson, 2009). Toxicity poses another significant problem for nanoparticle delivery systems, as cationic polymer and lipid based systems are known to be toxic (Shim and Kwon, 2010). This toxicity arises from the unnatural lipid and polymer composition as well as the excessive cationic charge.

Despite decades of work, to date only a single LNP siRNA formulation has received FDA approval. Onpattro (Patisiran) was developed by Alnylam targeting liver hepatocytes to treat peripheral nerve disease (polyneuropathy) caused by hereditary transthyretin-mediated amyloidosis (hATTR). Despite the success of this first in class RNAi therapeutic, Alnylam is not pursuing any subsequent LNP formulations and their pipeline is focused on well-defined, monomeric siRNA delivery. Indeed, taking the disadvantages of LNPs into consideration, it is no surprise that monomeric siRNA delivery is the preferred mode of delivery going forward.

Improving the Chemical Design of siRNAs

Despite RNAi's promising therapeutic features, the limitations caused by its size, charge, nuclease instability, and innate immune stimulation contribute to a poor drug profile that must be addressed before the therapeutic potential of siRNAs can be realized. Traditional small molecule CMC optimization strategies fail to fully address these limitations as siRNAs have

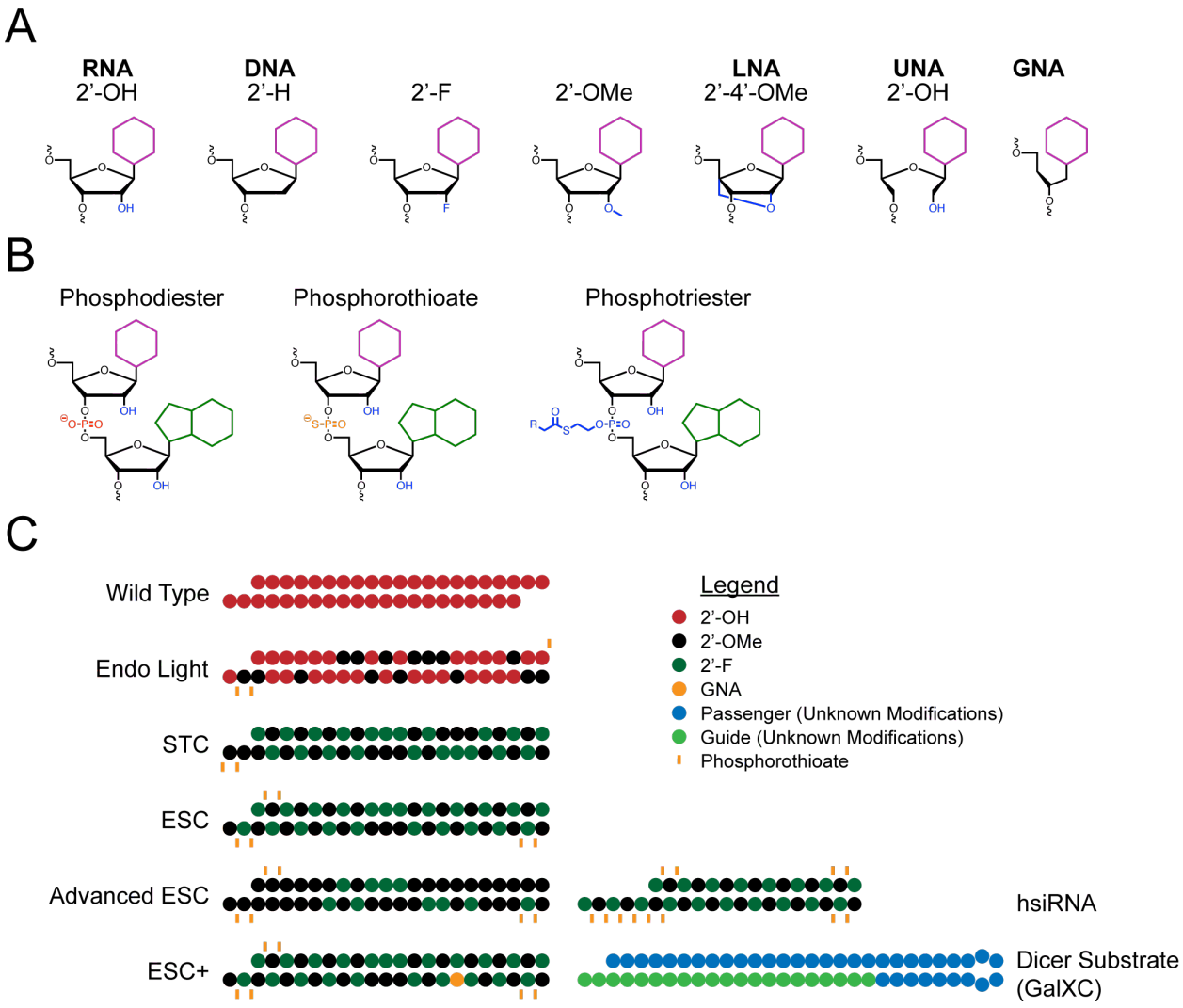
several biological requirements for activity. First, TRBP contains three double-stranded RNA binding domains (DRBDs) that bind in a sequence-independent manner to the minor groove of A-form, double stranded RNA through 2'-OH and charged phosphodiester backbone contacts without direct contact to any of the nucleobases (Ryter and Schultz, 1998). Second, Ago2 binds the 5'-terminal phosphate of the Guide strand through strong mid-domain binding with multiple interactions along the phosphate backbone and 2'-OH in the central groove, and PAZ-domain binding to the terminal 3'-OH (Schirle and MacRae 2012; Schirle, Sheu-Gruttadauria, and MacRae, 2014; Rettig and Behlke, 2012). Third, structural limitations of Ago2 require ~19 nucleotides for proper binding to both the mid and PAZ domain, with shorter oligonucleotides resulting in significantly reduced RNAi activity (Hagopian et al., 2017). As a result, chemical modifications to siRNAs must maintain or closely mimic the properties of a double stranded, A-form RNA with a charged phosphodiester backbone and a Guide strand of at least 19 nucleotides to maintain efficient RNAi activity.

Fortunately, the siRNA backbone is amenable to some types of modifications to improve its stability from attack by RNases and reduce activation of the innate immune system. First, incorporation of 2'-Fluoro (2'-F) and 2'-Hydroxymethyl (2'OMe) modifications greatly reduces the ability of RNases to degrade the siRNA (**Figure 1.3**). 2'-F and 2'-OMe modifications closely mimic the biophysical properties of the 2'-OH group and are highly tolerated by TRBP and Ago2 (Dowdy, 2017). In fact, with the exception of one kidney siRNA (QUARTZ), most, if not all, siRNAs in clinical trials today utilize fully 2'-modified siRNAs (Fitzgerald et al., 2017b; Rettig and Behlke, 2012). However, care must be taken to prevent placement of a 2'-OMe modification on the passenger strand opposite the cleavage position, as the OMe group prevents passenger strand cleavage and loading into Ago2 (Matranga et al., 2005). 2'-modifications also dramatically reduce the ability of TLR-3/7/8 to recognize the siRNA as a foreign nucleic acid invader (Gantier and Williams, 2007; Fitzgerald et al., 2017b; Robbins, Judge, and MacLachlan, 2009; Rettig and Behlke, 2012). Second, similar to their role in antisense oligonucleotides

(ASOs), placement of single or multiple phosphorothioates on the extreme 5'- and 3'-ends of each strand greatly improves stability, potency, and durability of RNAi response *in vivo* (**Figure 1.3**). Terminal phosphorothioates are also well tolerated by TRBP as these locations lie outside of its minor groove binding area (Ryter and Schultz, 1998). Third, although siRNAs differ between various groups, four general RNAi triggers have emerged: 1) the classic 21/21 nucleotide Passenger/Guide template with 19 base pairs and two 2 nt 3'-overhangs, 2) 21/23 nucleotides with a blunt end on the 5'-Guide strand end and a 2 nt 3'-overhang on the opposite end, 3) a short 15/20 duplex with a 5 nt unpaired tail on the 3'-end of the Guide strand that represents the minimum substrate for TRBP loading, and 4) dicer substrates that are longer with 26/28 nucleotides with a short single stranded nucleotide loop near the equivalent of the 5' end of the Guide strand (**Figure 1.3**) (Khvorova and Watts, 2017).

Perhaps the most significant contribution to siRNA potency has been 2'modification of the entire siRNA with 2'-F and 2'-OMe that has primarily been driven by the chemists at Alnylam Pharmaceuticals. Early iterations of fully 2'modified siRNA utilized an alternating 2'-F and 2'-OMe pattern (standard template chemistry, STC) to replace all 2'-OH groups (**Figure 1.3**) (Huang, 2017). This was followed up with enhanced stability chemistry (ESC)(D'Souza and Devarajan, 2015a) that improved upon STC by adding on two terminal phosphorothioates at each 5' end of the Passenger strand (Kallanthottathil et al., 2013; Maier et al., 2016). Direct comparisons between STC and ESC chemistries using several siRNA sequences and mRNA targets showed a 5-10 fold higher potency for ESC siRNAs vs. STC siRNAs *in vivo* (Alnylam, 2014). This work was followed by yet more refinement to generate Advanced ESC by reducing the total 2'-F content to a mere 9-10 positions out of 44 (Schlegel et al., 2017a). Importantly, 2'-F modifications must remain at key positions, including position 2 on the guide strand and a short patch on the passenger strand at the cleavage site.

Figure 1.3. Therapeutic siRNA Modifications.
A) Modifications to the ribose 2'-position: native RNA 2'-Hydroxyl (OH); native DNA 2'-Deoxy (H); 2'-Fluoro (F); 2'-Hydroxymethyl (OMe); 2',4'- Bicyclic containing O-Methylene bridge or locked nucleic acid (LNA); deletion of the ribose C2-C3 bond or unlocked nucleic acid (UNA). Deletion of ribose or (S)-glycol nucleic acid (GNA). **B)** Phosphate backbone modifications: native RNA, anionic charged phosphodiester (achiral phosphorus); charged phosphorothioate (chiral phosphorus); neutral phosphotriester (chiral phosphorus, becomes achiral after intracellular conversion to charged phosphodiester).**C)**. siRNA structure modifications (Passenger depicted 5' to 3' over Guide strand 3' to 5' from left to right): native 21/21 fully 2'-OH siRNA (Wild Type); partially 2'-OMe modified 21/23 with phosphorothioates on 3' end of Passenger and Guide (Endo Light); fully 2'-modified 21/23 with alternating 2'-F and 2'-OMe modifications, 2'-F group near cleavage position of Passenger strand, phosphorothioate pairs on 3'-end of Guide strand only, Standard Template Chemistry (STC); fully 2'-modified 21/23 identical to STC with additional phosphorothioate insertions at the 5'-end of each strand, Enhanced Stability Chemistry (ESC); fully 2'-modified 21/23 similar to ESC with 2'-F content reduced to minimum require positions, (Advanced ESC); fully 2'-modified 21/23 identical to ESC with addition of GNA at position 7 on the Guide strand to reduce off target knockdown (ESC+), fully 2'-modified 15/20 with alternating 2'-F and 2'-OMe pattern and terminal pairs of phosphorotioates on each end of the Passenger strand and 2 phosphorotioates on the 5'-end and 6 phosphorothioates on the3'-end of the Guide strand (hydrophobic siRNA, hsiRNA); 37/21 Dicer substrate with unknown 2'-mod pattern, Passenger strand forms 17 nucleotide hairpin loop (GalXC).


A
RNA
2'-OH
DNA
2'-H
2'-F
2'-OMe
LNA
2'-4'-OMe
UNA
2'-OH
GNA
B
Phosphodiester
Phosphorothioate
Phosphotriester
C
Wild Type
Endo Light
STC
ESC
Advanced ESC
ESC+
Legend
2'-OH
2'-OMe
2'-F
GNA
Passenger (Unknown Modifications)
Guide (Unknown Modifications)
Phosphorothioate
hsiRNA
Dicer Substrate
(GalXC)

Together, Advanced ESC modifications reduced the EC_{50} by an additional ~8 fold and perhaps even more importantly, resulted in a much longer duration of RNAi response, on the order of 6 to 9 month durations (Alnylam, 2017c). Other modifications have also been investigated to reduce siRNA off target effects, including incorporation of DNA, LNA, 2'-F-5'-methyl modifications, 5'-vinylphosphonate (5'-VP) (Prakash et al., 2016; Parmar et al., 2016), AU pairing at the 5' end of the guide strand, and glycol nucleic acid (GNA) modifications within the seed region (ESC+) (**Figure 1.3**) (Schlegel et al., 2017b). One modification in particular, 5'-VP, has also been shown to increase potency of modified siRNA 3-10 fold *in vivo* by increasing the 5'-VP-siRNA affinity for Ago2 (Elkayam et al., 2017; Parmar et al., 2016, 2016; Prakash et al., 2016). Together, these modifications have greatly increased the serum and RNase stability of siRNA while reducing its innate immune activity and off target effects. However, kidney clearance and tissue targeting remain obstacles to RNAi therapeutics that must be overcome for non-nanoparticle RNAi therapeutics to become viable.

CURRENT STATE OF RNAi THERAPEUTICS

The current state of the art and proto-typical siRNA therapeutic is a fully 2'-modified siRNA conjugated to *tris*-N-acetylgalactosamine (GalNAc) that avidly binds to the highly expressed hepatic asialoglycoprotein receptor (ASGPR) (Morell et al., 1971; Nair et al., 2014). GalNAc-siRNA conjugates have been thoroughly investigated in preclinical rodent and NHP models, and are currently being tested in multiple clinical trials sponsored by three biotech companies. None of this would have been be possible if it were not for the convergence of 50+ years of prior work on ASGPR and 50+ years of nucleic acid chemistry. GalNAc-siRNA conjugates serve as a simple solution to the delivery problem for liver hepatocytes and have shown the RNAi (and ASO) field the path forward for targeting other tissue types.

Identification of ASGPR as a Target for RNAi Therapeutics

ASGPR, also known as hepatic binding protein (HBP) or the Ashwell Morell receptor (AMR), was the first animal lectin to be detected (Grewal, 2010; Stockert, Morell, and Scheinberg, 1974). ASGPR was first "accidently" discovered as early as 1965 by Gilbert Ashwell and Anatol Morell in the course of studying a circulating glycoprotein, ceruloplasmin, in rabbits (Ashwell, 2008). An attempt to determine ceruloplasmin's circulating half-life by removing terminal sialic acids and radiolabelling the resulting terminal galactose led instead to the observation that asialo-ceruloplasmin rapidly disappeared from serum and was fully recoverable in the liver within 5-10 min (Morell et al., 1966, 1968). By 1968, Ashwell and Morell had determined that galactose was the necessary terminal sugar residue for binding to the yet unnamed ASGPR. Removal or oxidation of the terminal galactose by β-galactosidase or galactose oxidase, respectively, inhibited clearance of the labeled asialoceruloplasmin. Localization was observed specifically in hepatocytes with total exclusion from Kupfer cells, suggesting that clearance of asialoceruloplasmin is unique from heat killed controls and indicating a specific mechanism (Morell et al., 1971; Morell et al., 1968). By the early 1970's, ASGPR activity had been isolated to membrane fractions of rat and rabbit liver and was determined to be pH sensitive with an absolute requirement for Ca^{2+} to maintain binding activity (Pricer and Ashwell, 1971; Morell and Scheinberg, 1972). Blood type specific agglutination confirmed ASGPR as a lectin and demonstrated for the first time that the affinity for N-acetylgalactosamine (GalNAc) was higher than galactose (Sarkar et al., 1979; Novogrodsky and Ashwell, 1977).

In 1970, partial replacement of sialic acid residues on asialoglycoproteins indicated that at least two galactose residues were required for rapid clearance of asialoglycoproteins (Hickman et al., 1970; Van Den Hamer et al., 1970). Additionally, preferential clearance among various co-injected asialoglycoproteins and peptides suggested that both the number of galactose residues and their arrangement play a role in ligand binding (Morell et al., 1971; Van

Lenten and Ashwell, 1972). These early studies went on to show that binding depended on the type of sugar (GalNAc > Gal), and number of sugars with 4 = 3 > 2 > 1 (Kawaguchi et al., 1980; Steer and Ashwell, 1980; Lee et al., 1983; Baenziger and Fiete, 1980). Recent studies have shown that geometrical spacing between the sugars is also important (D'Souza and Devarajan, 2015b). X-ray crystal structures of the extracellular domain of ASGPR revealed a shallow carbohydrate binding pocket, explaining the requirement for multivalency (Meier et al., 2000; Mammen, Choi, and Whitesides, 1998).

GalNAc binding to ASGPR occurs at the sinusoidal surface of the hepatocyte (Pricer and Ashwell, 1971; D'Souza and Devarajan, 2015a). Hepatocytes contain ~500,000 receptors per cell (Stockert, Morell, and Scheinberg, 1974; Steer and Ashwell, 1980; Schwartz et al., 1981; Schwartz, Rup, and Lodish, 1980), though only 5-10% of the receptor population is present at the cell surface at any one time (Steer and Ashwell, 1980; Pricer and Ashwell, 1976). GalNAc binding initiates on diffuse monomeric ASGPR receptors, followed by rapid local aggregation of ligand bound receptors leading to larger scale aggregation in clathrin coated pits, and proceeding to endocytosis (Steer and Ashwell, 1980; Kolb-Bachofen, 1981; Weigel, 1980; Schwartz, Fridovich, and Lodish, 1982; Stockert et al., 1980). Early studies noted that the ASGPR half-life was much longer than the bound asialoglycoproteins (Tanabe, Pricer, and Ashwell, 1979). Subsequent studies showed that endosomal acidification during maturation led to dissociation of the GalNAc ligand from ASGPR followed by GalNAc degradation in the lysosome (Gregoriadis et al., 1970) and recycling of ASGPR to the cell surface, allowing for rapid and continued binding of additional serum asialoglycoproteins (**Figure 1.4**) (Bridges et al., 1982; Geuze et al., 1983; Wall, Wilson, and Hubbard, 1980).

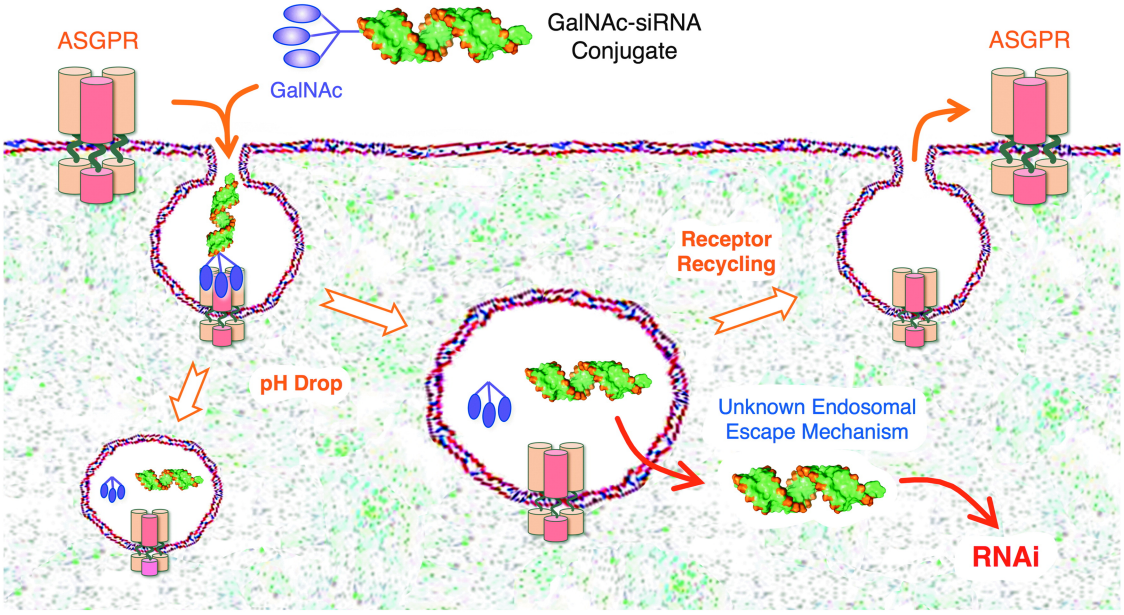


Figure 1.4. GalNAc-siRNA Delivery to Hepatocytes.
Delivery of GalNAc-siRNA conjugates into hepatocytes. Approximately 10^6 ASGPRs reside on the surface of liver hepatocytes. Upon binding sialyl-GalNAc molecules, ASGPRs are rapidly internalized into hepatocytes by endocytosis. Due to a pH drop, GalNAc-siRNA conjugates are released from ASGPR into the lumen of the endosome, and ASGPR recycles back to the hepatocyte surface. GalNAc and the linkers are rapidly degraded off of the siRNA conjugate and by a currently unknown mechanism, a small fraction of free siRNA, likely <1%, escapes across the endosomal lipid bilayer membrane into the cytoplasm of the hepatocyte. Once in the cytoplasm, siRNAs are rapidly loaded by transactivation responsive RNA-binding protein into Ago to induce robust and sustained RNAi responses. GalNAc, *tris-N*-acetylgalactosamine; ASGPR, asialoglycoprotein receptor; siRNA, short-interfering RNA. The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers. [Taken from Springer and Dowdy, *Nucleic Acid Therapeutics* 2018]

Early Work on GalNAc-Nucleic Acid Conjugates Targeting the Liver

The clinical possibilities of ASGPR were realized as early as 1971 when delivery of non-glycoproteins to the liver was accomplished via conjugation to asialofetuin (Rogers and Kornfeld, 1971). Liver targeting of protein-lactose conjugates in 1978 demonstrated that decoration of proteins with galactose ligands residues was sufficient for delivery (Wilson, 1978). The first targeted-delivery of a biologically active molecule to hepatocytes *in vivo* was demonstrated in 1979 when injection of asialofetuin-linked trifluorothymidine reduced hepatic *Ectromelia* viral DNA replication 3-fold in rats (Fiume et al., 1979). Delivery of additional antivirals (Fiume et al., 1980), LDL (Attie, Pittman, and Steinberg, 1980), and diphtheria toxin (Simpson, Cawley, and Herschman, 1982) demonstrated that ASGPR targeting is capable of delivering diverse cargo and eliciting an array of biological responses *in vivo*. In the following decades, asialoglycoproteins, galactose, and galactose derivatives including GalNac were widely investigated to deliver biologically active glycopeptides (Baenziger and Fiete, 1980), glycolipids (Rensen et al., 2004), small molecules (Seymour et al., 2002; Rohlff et al., 1999), nucleoside analogues (Fiume et al., 1979, 1980; Fiume, Busi, and Mattioli, 1983; Rohlff et al., 1999), plasmid DNA (Wu and Wu, 1987, 1988b, 1988a; Plank et al., 1992; Merwin et al., 1994), and ASOs to liver hepatocytes (Wu and Wu, 1992; Hangeland et al., 1995; Biessen et al., 1999; Prakash et al., 2014; Yu et al., 2016).

Throughout the 1980s and 1990s, plasmid DNA was delivered using poly-lysine nanoparticles. Early work in the late 1980s showed that delivery utilizing asialo-orosomucoid-poly-lysine conjugates targeted plasmid DNA to hepatocytes *in vitro* (Wu and Wu, 1987, 1988a). The first *in vivo* delivery utilizing this asialo-orosomucoid-poly-lysine approach showed rapid and selective expression of plasmid encoded protein in the hepatocytes 24 hours post intravenous injection (Wu and Wu, 1988b; Wu, Wilson, and Wu, 1989). By the early 1990s, structurally defined DNA-binding conjugates were created using multivalent galactose and GalNAc. This reduced the complexity and size of the previous asialo-orosomucoid conjugate nanoparticles

and effectively delivered DNA to hepatocytes *in vivo* (Plank et al., 1992; Merwin et al., 1994). Despite these advances, plasmid transfection efficiency was very low, with over 50% of the DNA degraded in the lysosome (Orrantia and Chang, 1990). Co-injection with defective viral particles increased plasmid DNA delivery 500-fold (Plank et al., 1992), highlighting the importance of endosomal escape in ASGPR-mediated delivery, a rate-limiting issue that persists today.

Early work characterizing ASGPR targeting showed that GalNAc valency and positioning played an important role in ASGPR targeting. In 1995, a neoglycopeptide, YEE(ah-GalNAc)$_3$, was used as a targeting ligand to deliver a short, neutral methylphosphonate 8-mer tester oligonucleotide (Hangeland et al., 1995). Linker length and sugar arrangement continued to be optimized until in 1999, Biessen's lab refined a tris-Galactoside structure that was used to deliver lipids and antisense oligonucleotides (ASOs) (Biessen et al., 1999; Sliedregt et al., 1999). Tris-GalNAc structure activity relationship, looking at linker length and configurations, was extensively interrogated using ASO and siRNA conjugates in the early 2000's (Prakash et al., 2016; Migawa et al., 2016). It was later shown that sequential conjugation of GalNAc sugars on nucleosidic linkages had similar potency to tri-antennary GalNAc conjugates, allowing for more flexible GalNAc delivery platforms (Rajeev et al., 2015; Matsuda et al., 2015; Sebestyén et al., 2015). The GalNAc approach was subsequently shown to enhance hepatocyte delivery of ASOs by ~10-fold vs. free ASOs in preclinical models, resulting in a dramatic dose reduction (Prakash et al. 2014).

Combining the Pieces of the Puzzle to Develop GalNAc-siRNA Conjugates

While chemical modifications have greatly stabilized siRNA against RNases and innate immune responses, these modifications are still insufficient to deliver these large (14 kDa), charged (40 phosphates) macromolecules across the lipid bilayer and into cytoplasm. Building on 50 years of ASGPR and 30+ years of galactose delivery studies (Morell et al., 1971; Sehgal

et al., 2015), Monoharan's group at Alnylam put together the pieces of the puzzle and conjugated *tris*-GalNAc to siRNA to achieve significant RNAi activity in liver hepatocytes *in vivo* (Dowdy, 2017; Khvorova and Watts, 2017; Juliano, 2016). Unlike complicated LNP formulations, GalNAc-siRNA conjugates are a simpler, smaller and compositionally defined approach for hepatic delivery. A complete GalNAc-siRNA can be synthesized on a solid-state oligonucleotide synthesizer, and chemically defined by mass spectrometry (Rajeev et al., 2015).

The high number and rapid turnover of ASGPR receptors is thought to contribute to successful GalNAc delivery of siRNAs. GalNAc-siRNA conjugates bind ASGPR and are rapidly internalized into clathrin-coated endosomes (**Figure 1.4**). As the endosomal pH drops, the GalNAc-siRNA is released from ASGPR. ASGPR is recycled back to the cell surface, while the GalNAc-siRNA remains in the lumen of the endosome. GalNAc is cleaved from the siRNA by endosomal glycosidases by 1 hr and the linker arms are degraded by 4 hr (Prakash et al., 2014). The vast majority of free siRNA remains trapped in the endosome, while a very small amount (<<1%) is able to traverse the endosomal membrane through an unknown mechanism to enter the cytoplasm and induce an RNAi response (**Figure 1.4**). In addition to the mystery of how the siRNA actually escapes the endosome to enter the cytoplasm, it is unknown how increasing the 2'-OMe content increases the duration of the RNAi response compared to higher 2'-F content siRNA. These mechanistic details aside, together, GalNAc-siRNA and GalNAc-ASO delivery studies performed by many groups have shown the overall exquisite superiority of this delivery approach.

Success and Failures of siRNA in Clinical Trials

Three biotech companies are currently performing clinical trials using GalNAc-siRNA conjugates: Alnylam Pharmaceuticals, Arrowhead Pharmaceuticals, and Dicerna Pharmaceuticals. While there were several significant setbacks early on, overall data from the next generation of RNAi triggers suggest a promising future for the field of RNAi therapeutics.

Alnylam currently has six GalNAc-siRNA conjugates in clinical trials, including three in ongoing phase III trials for a variety of liver hepatocyte diseases. Alnylam performed the first GalNAc-siRNA clinical trials with Revusiran (ALN-TTRsc), an earlier STC chemical modification pattern targeting the transthyretin (TTR) gene to treat TTR-mediated amyloidosis (ATTR) (Hawkins et al., 2015a; Butler et al., 2016; Zimmerman et al., 2014; Zimmermann et al., 2017a). Phase I clinical trials administered subcutaneous single ascending dose (SAD) and multiple ascending dose (MAD) in a 2:1 drug to placebo randomized patient population. 9 out of 19 MAD patients showed a dose independent elevation of liver aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT)(Zimmermann et al., 2017b). Levels of AST and ALT returned to normal with continued dosing and no anti-drug antibodies were observed in the MAD treatment group.

A phase II and open label extension (OLE) reported ~90% reduction of TTR serum levels after multiple dosing with a sustained knockdown of TTR beyond 90 days in hereditary ATTR (hATTR) patients with cardiomyopathy (Hawkins et al., 2015b; Butler et al., 2016). Following a 12 month treatment, five of nine patients had met the primary endpoint goal of a stable 6 min walk distance (6-MWD). However, phase II OLE data revealed that 20% of Revusiran treated patients experienced peripheral neuropathy, prompting an ad hoc investigation of Alnylam's ongoing phase III ENDEAVOUR study by a Data Monitoring Committee (DMC) (Alnylam, 2017b). The DMC found an imbalance in mortality of 16 deaths in the Revusiran arm compared to 2 deaths in the placebo arm (2:1 drug to placebo patient distribution), leading Alnylam to discontinue all ongoing Revusiran dosing on October 5, 2016. Ultimately, the DMC found no conclusive evidence for drug related neuropathy, but could not exclude the possibility of a drug related effect on mortality in the ENDEAVOUR study (Alnylam, 2017b).

Despite the discontinuation of Revusiran, the ionizable LNP based sister product, Patisiran, targeting TTR, completed a phase III APOLLO study (Alnylam, 2018e) and in August,

2018, Patisiran was given FDA and EMA approval, under the name Onpattro, as the first approved siRNA therapeutic (Commissioner, 2018; Alnylam, 2018h). Despite the difference in formulation and route of administration, Patisiran's approval demonstrates long term safety and efficacy of liver targeted TTR knockdown using siRNA. Patisiran patients (n=225) represented a diverse population from 19 countries and presented with a total of 39 different TTR mutations. Patisiran was administered intravenously at 0.3 mg/kg every 3 weeks for 18 months. The primary endpoint in the study was the modified Neuropathy Impairment Score +7 (mNIS+7), an assessment of motor strength, reflexes, sensation, nerve conduction and postural blood pressure. Patisiran treated patients saw a 6-point improvement in mNIS+7 score while the placebo control saw a 28-point decline over an 18 month period. Patisiran treated patients also saw an increase in quality of life above their own baseline while AEs and SAEs were not significantly different between Patisiran and Placebo groups (Alnylam, 2018d). Alnylam has announced plans to seek label expansion for Patisiran in ATTR for amyloidosis patients with cardiomyopathy in early 2019 (Alnylam, 2018g) as well as additional New Drug Application (NDA) submissions in Japan (Alnylam, 2018d).

Following the failure of Revusiran, Alnylam has reported the incidence of AE and SAE across their platform as of 2016 and found low incidence of AE (15.2%) consisting of mild transient injection site reactions (ISR), as well as a low incidence (2.2%) of SAE consisting of mild, asymptomatic, reversible liver function test (LFT) increases >3-fold above upper limit of normal (ULN)(Alnylam, 2017b). Alnylam continues to see this liver enzyme SAE across several of its RNAi drugs. Further advances in stability chemistry that led to the Advanced ESC and ESC+ platforms are expected to reduce these SAEs.

Revusiran utilized the less stable STC backbone (**Figure 1.3**), requiring high and frequent dosing, resulting in extensive patient exposure to the drug of 28 g per year (Alnylam, 2017b). However, all subsequent GalNAc-siRNA conjugates in clinical trials use the much more stable and potent ESC platform. The follow on for TTR therapy, Vutrisiran (previously ALN-

TTRsc02), is in in early clinical development and has completed a phase I clinical trial. A randomized ascending fixed dose (5-300 mg) in 80 healthy volunteers reported a maximum mean TTR knockdown of 97% maintained over 320 days. No SAEs or study discontinuations due to AE were reported, though AEs remain high in both arms (77% for Vutrisiran and 50% for placebo), likely due to the nature of the TTR disease (Taubel et al., 2018). Due to the dramatically increased stability and duration of RNAi responses by ESC siRNAs, Vutrisiran knockdown of TTR supports a low dose of 25 mg/quarter to achieve knockdown comparable to Patisiran. The projected dose for Vutrisiran represents an annualized dose of 100 mg compared to 28 g for Revusiran. Alnylam has announced plans to begin recruitment of 160 participants for a phase III HELIOS-A clinical trial in December 2018 with Vutrisiran dosing beginning in late 2019 and primary completion dates estimated for early 2021 (Alnylam, 2018g).

Givosiran (previously ALN-AS1) (Chan et al., 2015) is a GalNAc-siRNA conjugate that targets the ALAS1 gene to treat Acute Hepatic Porphyria (AHP) (Sardh et al., 2018). Givorsiran has completed a phase I clinical trial and is currently under both a phase I/II OLE and a phase III ENVISION trial. Phase I clinical trial data revealed no SAEs attributed to Givosiran with subcutaneous doses as high as 5 mg/kg monthly. Monthly dosing with 2.5 mg/kg saw a lowering of target biomarkers aminolevulonic acid (ALA) and porphobilinogen (PBG) and an 83% reduction in annualized attack rate (AAR) and 88% reduction in hemin use relative to placebo. Increasing the monthly dose to 5 mg/kg did not show increased reduction of ALA and PBG levels.

Preliminary results from an ongoing phase I/II OLE trial following the 2.5 mg/kg monthly injection reveal enhanced clinical activity in patients treated with Givosiran up to 25 months (mean = 13.6 months) with sustained lowering of ALA and PBG of 87% and 83%, respectively, following 12 months of treatment (Alnylam, 2018f). Patients who had continued Givosiran treatment after the phase I trial (n=12) saw a reduction in AAR of 93% and a reduction in annualized hemin use of 94% relative to pre-treatment results. Similarly, patients who crossed

over from the placebo group of the phase I study (n=4) saw mean AAR and hemin use reductions of 95% and 98%, respectively. Seven of sixteen patients have achieved an AAR of zero with a mean of 11.3 months of treatment compared to a pretreatment AAR of 15.2. SAEs have been reported in four patients with a single case of anaphylactic response attributed to Givosiran dosing.

A phase III ENVISION clinical trial is currently ongoing and Alnylam plans to announce topline results in early 2019 (Alnylam, 2018g). Givosiran has been granted Breakthrough Therapy designation by the U.S. Food and Drug Administration (FDA), PRIME designation by the European Medicines Agency (EMA), and orphan disease designations in both the U.S. and EU (Sardh, et al. 2018). Alnylam has announced that they expect to file and an NDA with the FDA and a Marketing Authorization Application (MAA) with the EMA in mid-2019 (Alnylam, 2018g, 2018c).

Inclisiran (previously ALN-PCSsc) (Gaudet, 2016; Strat et al., 2016) is a first-in-class PCSK9 synthesis inhibitor for treating hypercholesterolemia. Inclisiran completed a phase II ORION-1 study, the largest randomized, placebo controlled study for an investigational RNAi therapeutic to date (Alnylam, 2017b). Patients were dosed with 300 mg Inclisiran subcutaneously at day 1 and day 90, resulting in a time averaged reduction in LDL-C of 51% over the following 6 month period (day 90-270) (Ray et al., 2017a, 2017b). No drug related differences in liver function test (LFT) were observed between the Inclisiran and placebo arms (Alnylam, 2017b). Following these positive results, Alnylam is moving forward with a phase III trial using a 300 mg maintenance dose every 6 months following initial dosing (Ray et al., 2017a, 2017b). In contrast, hypercholesterolemia patients currently taking statins are required to dose every day vs. Inclisiran's anticipated twice yearly dosing. Alnylam, in partnership with The Medicines Company, plans to announce topline results from the ORION 9, 10, and 11 studies in mid to late 2019 and file an NDA in the U.S. around the end of 2019 pending positive results in the ORION studies (Alnylam, 2018g).

Fitusiran (previously ALN-AT3sc) targets anti-thrombin 3 (AT3) for the treatment of Hemophilia A and B (Pasi et al., 2017). Completion of a phase I study showed no drug related SAEs and a reduction in anti-thrombin (AT) of 70-89% following monthly dosing of 0.225-1.8 mg/kg, resulting in peak thrombin levels within the lower range of healthy participants (Pasi et al., 2017). A phase II OLE study dosing patients for ≤20 months and a median of 11 months showed a reduction in AT of 80% with only 2 drug related SAEs observed in patients with pre-existing conditions (Pasi et al., 2017).

In all patients who received Fitusiran, thrombin levels remained at the lower end of normal for the duration of observation. Annualized bleeding rate (ABR) was zero in the Fitusiran + inhibitor (factor VII or bypassing agents) group compared to an ABR of 20 in the Fitusiran only group and an ABR of 38 in the inhibitor only group (Pasi et al., 2017). Impressively, 48% of Fitusiran + inhibitor patients remained bleed free for the duration of observation and 67% of patients experienced zero spontaneous bleeds. In a separate, smaller study, patients receiving Fitusiran required reduced dosing of FVII or bypassing agents for perioperative management following dental work (Negrier, et al. 2018). The ATLAS phase III study is currently enrolling to further study Fitusiran's safety and efficacy. Funding and development of Fitusiran was transferred wholly to Sanofi in January 2018 (Alnylam, 2018b) with continued support for the ATLAS phase III clinical trial from Alnylam (Alnylam, 2018g).

Lumasiran (previously ALN-GO1) targets glycolate oxidase (GO) for treatment of Primary Hyperoxaluria Type 1 (PH1)(Liebow et al., 2017; Carney, 2016). In a phase I/II trial (n=20), following monthly doses of 1 or 3 mg/kg or quarterly 3 mg/kg doses, patients saw a mean maximal reduction in urinary oxalate of 64%, supporting a once quarterly dosing regimen and the potential to normalize urinary oxalate levels and halt PH1 disease progression (Alnylam, 2018i). Alnylam has received an Accelerated Development path from the FDA for Lumisiran and has announced plans to complete enrollment for a phase III ILLUMINATE-A study and

initiate two additional phase III studies (ILLUMINATE-B and –C) to look at pediatric applications in mid 2019 (Alnylam, 2018a).

Alnylam's clinical pipeline also has two early clinical trial GalNAc-siRNA conjugates. Cemdisiran (previously ALN-CC5), targets complement component C5 (CC5) for the treatment of complement-mediated disease (Alnylam, 2017a). Cemdisiran is currenlty undergong a phase II clinical trial for patients with Atypical Hemolytic-Uremic Syndrome (aHUS) and a phase I/II trial for patients with paroxysmal nocturnal hemoglobinuria (PNH) (Alnylam, 2017a). ALN-HBV02 (Also VIR-2218) targeting all Hepetitis B Virus (HBV) RNA transcripts is being produced in partnership with Vir Biotechnology and is currently recruiting for a phase I/II (Alnylam, 2018j).

Another biotech, Arrowhead Pharmaceuticals, uses two types of GalNAc conjugates. The first generation was a dynamic polyconjugate (DPC) that utilized an endosomolytic peptide (butyl and amino vinyl ether, PBAVE, or melittin) masked with GalNAc through a pH sensitive carboxy dimethyl maleic anhydride (CDM) linkage (Rozema et al., 2007; Wong et al., 2012). The siRNA is conjugated to cholesterol and is co-injected with the DPC as combination therapy. The cholesterol-siRNA forms a large aggregate (low-density lipoprotein) in blood that is transported to and taken up by the liver, whereas the GalNAc-DPC is taken up specifically by ASGPR in the liver. When both are present in the same endosome, the GalNAc-DPC facilitates endosomal escape of the cholesterol-siRNA conjugates (Rozema et al., 2007; Wong et al., 2012).

Arrowhead's lead DPC compound into clinical trials was ARC-520 along with a related ARC-521 that contained two siRNAs targeting different regions of the X gene in Hepatitis-B infection (HBV) (Wooddell et al., 2013; Yuen et al., 2015) ARC-520/521 have undergone multiple phase I and phase II trials, alone and in combination with the antiviral entecavir (Yuen et al., 2018). However, in late 2016, the FDA halted five ongoing clinical trials involving ARC-520 due to a nonhuman-primate (NHP) death in one of Arrowhead's preclinical studies, likely due to toxicity from the DPC (Buchanan, 2016). Despite the termination of the clinical trials,

results showed a rapid, six log suppression (>99.9%) of HBV DNA in all Hepatitis B e-antigen positive (HBeAg) treatment naïve patients.

Treatment of HBeAg-negative, naive patients reduced HBV DNA below the limit of detection. Single dose treatment of ARC-520 also inhibited covalently closed circular DNA (cccDNA)-derived mRNA expression and reduced viral protein production by 99%. Additionally, some patients developed and expressed antibodies against hepatitis B surface antigen (HBsAg) (Yuen et al., 2015). Despite the regulatory shutdown of the ARC-520/521 clinical trials, Arrowhead showed that HBV is susceptible to RNAi therapeutics and gleaned a large volume of clinical data on how to best treat HBV with siRNA therapeutics.

Arrowhead's next generation delivery platform, called Targeted RNAi Molecule (TRiM), removes the problematic active endosomal escape agent (PBAVE, melittin) in favor of direct conjugation of GalNAc targeting domains (Wooddell et al., 2018). While the exact nature of the TRiM siRNAs have not been made public, Arrowhead reports that numerous tailored design chemistries have been incorporated to generate highly robust RNAi triggers. Building off of their prior RNAi clinical experience, Arrowhead has partnered with Jansen to move ARO-HBV into clinical trials. ARO-HBV targets both the X gene and the S gene of HBV. Importantly, the X gene targeting sequence is present on all integrated forms of HBV, whereas the ARC-520 siRNA gene target was not.

Using multiple sequence targets present on both cccDNA and integrated HBV, ARO-HBV reduces the opportunity for HBV to develop resistance to the RNAi therapeutics and allows the drug to tackle both forms of the virus (Wooddell et al., 2018). ARO-HBV is currently recruiting and undergoing a phase I/II clinical trial. Arrowhead also has begun a phase I clinical trial involving ARO-AAT to treat alpha-1 antitrypsin (AAT) related liver disease (Wooddell et al., 2018) and is also currently partnered with Amgen to begin recruitment for a phase I trial for AMG 890, a treatment for cardiovascular disease patients with elevated lipoprotein(a). In addition, Arrowhead has several preclinical GalNAc TRiM programs.

Dicerna Pharmaceuticals has developed a proprietary GalNAc delivery platform that differs from both Alnylam and Arrowhead. Dicerna's "GalXC" technology utilizes an altered siRNA structure and tetra-antennary GalNAc, rather than the more widely used tri-antennary pattern (Dicerna, 2019). Monomeric GalNAcs are covalently linked to four nucleotides on the single stranded loop of their dicer substrate siRNAs (**Figure 1.3**) (Dicerna, 2019). The Guide strand is annealed to this altered passenger strand forming a mature GalNAc-dicer substrate siRNA conjugate containing a nick at the 5' end of the guide strand separating it from the looped passenger strand sequence. This approach provides simple "on column" oligonucleotide manufacturing with proper orientation of the four GalNAc ligands. Using this GalXC platform, Dicerna has developed several preclinical candidates with one, DCR-PHXC, advancing to phase I clinical trials for the treatment of Primary Hyperoxaluria (PH). Dicerna also has multiple GalNAc-siRNA conjugates in preclinical development including a planned phase I trial in 2019 for DCR HBVS, targeting HBV.

TARGETING BEYOND THE LIVER

GalNAc targeting has revolutionized the RNAi therapeutics field to such an extent that hepatic delivery can be considered solved. However, extra-hepatic targeting remains a formidable obstacle to treatment of genetic disease by RNAi Therapeutics. Targeting of extra-hepatic tissues has taken on four strategies: 1) local delivery, 2) delivery with lipid nanoparticles, 3) targeting extracellular receptors with natural and synthetic ligands/peptides, and 4) targeting extracellular components using antibodies and other large binding proteins.

Local delivery is an attractive approach for RNAi therapeutics as transdermal, intravitreal, and intranasal administration offer high bioavailability, reduced adverse effects, and simple formulations. The first clinical trials for an siRNA therapeutic was carried out by Opko Health (Bevasiranib) in 2004 for the treatment of age-related macular degeneration (AMD) and diabetic macular edema (DME). Bevasiranib was an unmodified 21/21 siRNA and was

administered via intravitreal injection. Bevasiranib completed phase I and II clinical trials but failed its phase III trial for AMD due to poor performance in reducing vision loss (Garba and Mousa, 2010; Dejneka et al., 2008). Further analysis of Bevasiranib's mechanism of action revealed that RNAi was unlikely to be the mechanism of action and instead, Bevasiranib acts through innate immune activation of TLR3 receptors (Kleinman et al., 2008). Additional clinical trials utilizing intravitreal delivery were carried out by Allergan (AGN-745) (Burnett and Rossi, 2012; Kaiser et al., 2010), Pfizer (PF-655) (Burnett, Rossi, and Tiemann, 2011), and Sylentis (Bamosiran & SYL1001) (Veronica Ruz et al., 2014; Benitez-Del-Castillo et al., 2016; Sylentis, 2016; Martínez et al., 2014) with similar results. Quark Pharmaceuticals has an ongoing Phase III trial for the treatment of non-arteritic anterior ischemic optic neuropathy (NAION).

Local administration to the Lungs via nebulizer or inhaler was explored by Zebecor Pharma and Alnylam for the treatment of asthma and respiratory synsytial virus in lung transplant patients with bronchial obliterans syndrome, respectively (Alvarez et al., 2009; Alnylam, 2018; Zamora et al., 2011; Fujita et al. 2013). Despite promising clinical trial results, both companies have discontinued development (Gottlieb et al., 2016; Liao et al., 2017). Transdermal delivery has been explored as a local delivery method for a variety of skin diseases with clinical stage trials conducted by Transderm Pharma (TD101) (Leachman et al., 2010; Deng et al., 2016), RXi pharmaceuticals (RXI-109) (RXi Pharmaceuticals Corp., 2017; Chakraborty et al., 2017), OLiX pharma (OLX101) (OliX 2017), and Siranomics,Inc (STP705) (Kaczmarek, Kowalski, and Anderson, 2017). Despite positive clinical results, only RXi pharmaceuticals (RXI-109) and Siranomics (STP705) are continuing development of their treatments for hypertrophic scars in phase II trials. While local delivery of siRNA therapeutics has seen limited success, many of these therapeutics suffer from innate immune activation and low stability that plagued early siRNA stability chemistries and local delivery does not represent a viable approach on its own going forward.

To avoid the problems of low stability and innate immune responses, early efforts looked to lipid nanoparticles to protect the siRNA cargo from nuclease degradation (Schroeder et al., 2010) and innate immune response activation. These nanoparticle have utilized diverse formulations including lipids, polymers and inorganic material (Zhou et al., 2013), and often involve addition of 4-5 components at exacting ratios, each with their own toxicity profiles (Schroeder et al., 2010). The size of the nanoparticles limits kidney filtration, increasing circulation time compared to naked siRNA. The size of the nanoparticles also leads to accumulation in the liver, driven primarily by uptake through the hepatic reticuloendothelial system (Akinc et al., 2009; Schroeder et al., 2010).

In order to truly target all tissues, specific extracellular ligand/target interactions need to be utilized. Effective targeting of a tissue via receptor or extracellular target must meet three criteria in order to be a viable method of targeting siRNA therapeutics. First, a receptor or extracellular target must be expressed on the cell type of interest in high enough numbers to deliver a sufficient payload of siRNA to each cell. Low abundance receptors provide comparatively few opportunities for delivery and make the challenge of RNAi therapeutics more difficult. Second, the receptor or target of interest must internalize through some mechanism at as high a rate as possible. A receptor with low internalization results in rapid receptor saturation and subsequent clearance of unbound circulating therapeutic, requiring more frequent dosing or alternate dosing mechanisms. Conversely, high internalization rates can make up for relatively low numbers of target receptor and provide more opportunity for cytoplasmic delivery. Third, the receptor or target of interest must be selective for or highly overexpressed on the target tissue compared to off target tissues. Targeting an abundant and fast internalizing receptor that is present on all cell types results in delivery of the siRNA drug to all tissues. While off target effects in undesired tissues can be mitigated to some degree through target selection and siRNA sequence decisions, non-specific delivery effectively sequesters most of the therapeutic

in non-productive tissues. This can lead to prohibitively high doses and may lead to significant toxicity.

Several attempts in academia and Industry have been made to unlock additional tissues of interest for siRNA therapeutics in hopes of "solving" delivery and treatment of that tissue in a manner similar to what GalNAc has accomplished in the liver. IONIS Pharmaceuticals targets the glucagon like protein-1 (GLP-1) receptor for pancreatic β-islet delivery of ASOs (Monia, Brett et al., 2017), a technology that could be utilized for siRNA delivery with little modification. The GLP-1 receptor is a class 2 G-protein coupled receptor that is expressed on β-islet cells in the pancreas. Glucagon like peptide-1 (GLP-1) is released into circulation following nutrient stimulation in the intestines. Binding of GLP-1 by its receptor (GLPR) activates the receptor and leads to endocytosis and sorting into lysosomes (Kuna et al., 2013). IONIS has filed a patent on GLP-1 peptide-ASO conjugates as well as other small molecule, peptide, and antibody conjugates targeting the GLP-1 receptor (Monia, Brett et al., 2017). Despite limited numbers of receptors, GLP-1 mediated delivery has shown successful β-islet cell targeting with little silencing in off target tissues (Ämmälä et al., 2018). Success with ASOs may not translate into successful delivery of siRNA as ASOs are capable of endosomal escape through a mechanism termed gymnosis that siRNA is incapable of replicating, potentially limiting the utility of GLP-1 ligands on their own.

Targeting tumors in a specific and efficient manner offers the promise of truly precision RNAi medicine. Folate Receptor α (Frα) is overexpressed on epithelial cancers including breast (Zhang et al., 2014), lung (Cagle et al., 2013), ovary (Kalli et al., 2008), kidney (Parker et al., 2005), and colon cancers (Jun Yang, Vlashi, and Low, 2012) as well as some hematological malignancies (Lynn et al., 2015). Levels of FRα in normal tissues are significantly lower (Srinivasarao, Galliford, and Low, 2015; Parker et al., 2005) with the exception of apical membranes of proximal renal tubules in the kidneys (Fisher et al., 2008). Folate is bound in its oxidized form by FRα with high affinity (Kd = 10^{-9} M), leading to rapid FRα endocytosis. Folate

dyes and small molecule conjugates are rapidly taken up in tumors but are also rapidly cleared by the kidneys, posing a potential problem for RNAi therapeutics (Vlashi et al., 2013; Yang et al., 2007, 2006). Despite the rapid kidney clearance, dye labeled miRNA- and siRNA-folate conjugates have demonstrated successful targeting and labeling of tumors *in vivo* (Thomas et al., 2009; Zhang et al., 2008). Tumor accumulation and gene knockdown have been demonstrated *in vitro* and in subcutaneous tumor models (Orellana et al., 2017) and several patents have been filed on its use for transmembrane transport (Low, Horn, and Heinstein, 1992; Low and Horn, 1990). Alnylam has filed a patent on folate delivery as well as a synthetic strategy for site-specific conjugation through a defined carboxylic acid. Work by Alnylam has shown an *in vivo* EC_{50} of 0.1-1 nM though differences in tumor growth remain modest and kidney filtration remains high (Manoharan et al., 2008)(Low, Horn, and Heinstein, 1992; Low and Horn, 1990).

Targeting of small molecule chemotherapeutics and imaging agents using folate has been the subject of several clinical trials and folate remains an attractive targeting domain for siRNA therapeutics though obstacles including rapid kidney filtration and competitive binding by free serum folate remain. In addition to these problems, folate-siRNA conjugates must escape the endosome. Comparisons of ASGPR and Frα suggest that potential folate-siRNA internalization is >100 fold less, indicating that whatever mechanism GalNAc-siRNA escapes the endosome through may not be sufficient for folate-siRNA delivery.

Other tumor selective or overexpressed extracellular receptors have been described for selective targeting of therapeutics and imaging agents, among them are integrins αvβ3, αvβ5, and α5β1 (Desgrosellier and Cheresh, 2010; Cox, Brennan, and Moran, 2010; Rathinam and Alahari, 2010). The activity of these integrins is mediated through binding of extracellular matrix (ECM) proteins via a tripeptide Arg-Gly-Asp (RGD) motiff, prompting efforts to use this motiff for tumor targeting (Pierschbacher and Ruoslahti, 1984; Meyer et al., 2006). Various strategies have been implemented to increase the binding and selectivity of the RGD peptide as well as to

increase their pharmakokinetics, metabolic stability, and biodisctribution. These strategies have employed cyclization, introduciton of flanking amino acids, stereochemical modualtion of the each amino acid, and N-methylation (Aumailley et al., 1991; Chatterjee, Rechenmacher, and Kessler, 2013; Chatterjee et al., 2008; Dechantsreiter et al., 1999). Merk developed a potent agonist (Cilengitide, cyclic (RGDf-*n*MeVal) of the αvβ3 integrin utilizing these strategies for the treatment of glioblastoma multiforme, but failed to show efficacy in a phase III trial (Mas-Moruno, Rechenmacher, and Kessler, 2010). Despite the failure in the clinic to elicit a therapeutic response, Cilengitide demonstrated potent αvβ3 binding and RGD peptides and their derivatives continue to be explored for targeted delivery of siRNA in nanoparticle formulations (Huang et al., 2015; Y. Sakurai, Hada, and Harashima, 2016) as well as direct conjugates (Alam et al., 2011; Cen et al., 2018; He et al., 2017; Liu et al., 2016). While RGD peptides and their drivatives may be able to effectvly target siRNA to tumors, they ignore the problem of endosomal escape that must be adressed in addition to targeting in order to achieve sucessful RNAi activity.

Targeting extracellular receptors with small ligands poses a strategic problem in that binding affinity is determined primarily by the receptor of interest. Binding affinity can be increased through ligand structure modification but the maximum binding affinity of the receptor cannot be surpassed. An alternative approach looks to reverse the binding protein-ligand relationship and utilize antibodies to target extracellular antigens of interest. Antibodies allow for binding affinity to be adjusted through binding protein optimization rather than ligand optimization, allowing any extracellular protein to be targeted, regardless of its own binding capabilities. Additionally, targeting siRNA therapeutics with antibodies allows the field to take advantage of decades of target/antibody validation for a variety of diseases, offering immediate candidates for RNAi therapeutics in the clinic. However, despite the promise of antibody-siRNA therapeutics, significant challenges arise in the construction of well-defined and stable antibody-siRNA conjugates.

Genentech first described a defined conjugation scheme with their THIOMAB platform and demonstrated efficacy in a variety of tumor cells as well as in subcutaneous models (Cuellar et al., 2015). However, prediction of success *in vitro* based on receptor numbers or internalization route was inconsistent and *in vivo* translations of *in vitro* success yielded poor tumor reduction at best. This work demonstrated that successful targeting of an internalizing extracellular receptor is not sufficient for cytoplasmic delivery. Subsequently, Takeda Pharmaceuticals demonstrated delivery of a Transferrin (CD71) receptor targeting antibody fragment (Fab)-siRNA conjugate (Sugo et al., 2016). Robust knockdown of target myostatin mRNA (50-70% knockdown) and concomitant increases in muscle mass were observed in mice following intravenous, intraparitoneal, or subcutaneous injections.

While both Genentech and Takeda have seen limited success in development and implementation of antibody-siRNA conjugates and their derivatives, it is unclear how these siRNA therapeutics are escaping from the endosome to affect their targets in the cytoplasm. Receptor number and internalization biology are not predictive of success using these modalities and it is clear that whatever intrinsic biological property allows endosomal escape is rare and cell line/tissue type specific. In order to go beyond these biological barriers, mechanisms for endosomal escape must be implemented or siRNA therapeutics will forever be relegated to hepatic delivery and rare special cases.

ESCAPING THE ENDOSOME

Despite the success of GalNAc, the mechanism of endosomal escape for GalNAc-siRNA conjugates is poorly understood and explanations remain speculative. It remains to be seen whether this phenomena can be reproduced outside the liver or whether GalNAc-siRNA endosomal escape is a hepatocyte-restricted phenomena. In the absence of this understanding, effective extra-hepatic delivery may require endosomal escape moieties. Escape from the endosome represents the rate-limiting step for delivery of nucleic acids (**Figure**

1.5) (Varkouhi et al., 2011; Wiethoff and Middaugh, 2003; Cho, Kim, and Park, 2003; Vaidyanathan, Orr, and Banaszak Holl, 2016). Indeed, even within the field of ASGPR delivery, it was discovered early on that delivery to the hepatocytes was largely improved following the addition of endosomal lytic viral particles (Varkouhi et al., 2011; Wiethoff and Middaugh, 2003; Cho, Kim, and Park, 2003; Vaidyanathan, Orr, and Banaszak Holl, 2016). Considering the comparatively low numbers and slow turnover of extra-hepatic receptors, endosomal escape functionality may be an absolute requirement to overcome the biological barriers of the endosome. Much effort has been made to describe mechanisms of endosomal escape and to engineer solutions to overcome the endosomal compartment. These efforts can be broadly categorized as cationic polymers and domains that function through a proposed "proton-sponge" mechanism, peptides and proteins that disrupt or permeabilize the endosome, and hydrophobic interaction mechanisms.

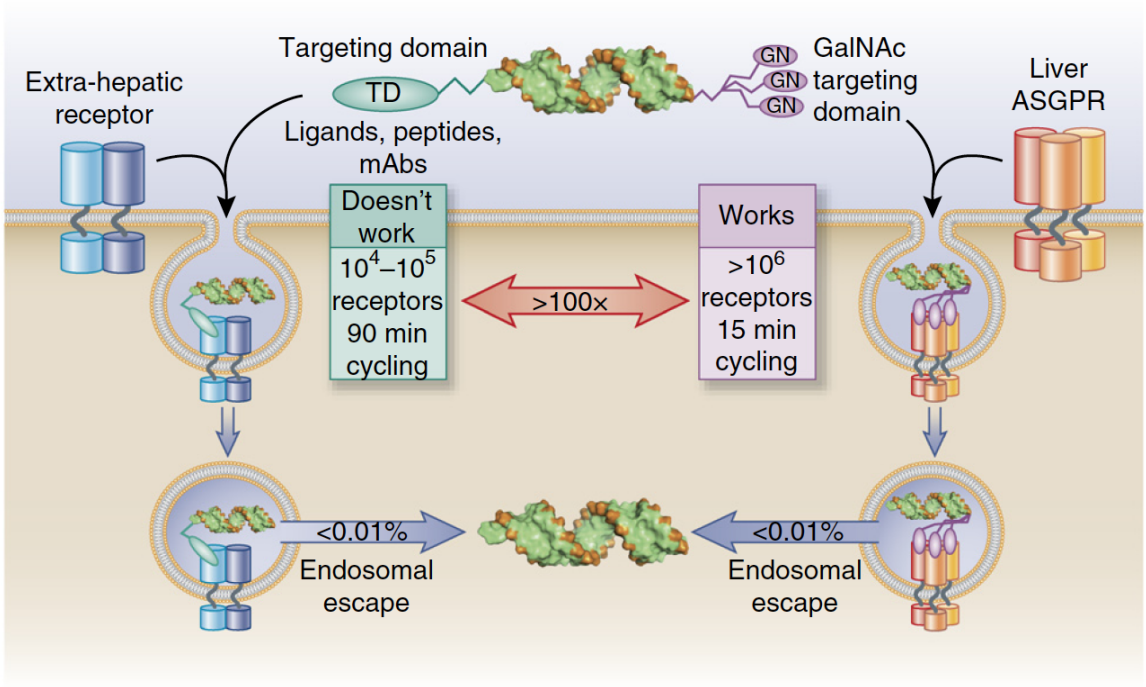


Figure 1.5. Endosomal Escape of *tris*-GalNAc-siRNA.
Tris-GalNAc binding to liver ASGPR (~10^6/hepatocyte) induces endocytosis (~15 min) where a small fraction of the siRNA or ASO cargo escapes into the cytoplasm to induce selective RNA drug responses. In contrast, targeting non-hepatic cell surface receptors (10^4–10^5) that have a much slower rate of endocytosis (~90 min) has proven extremely difficult. Assuming there is no endosomal escape advantage in ASGPR endosomes, ASGPR brings in ~100-fold more siRNAs/ASOs into hepatocytes than is mathematically possible in any other ligand–receptor pair. Consequently, development of next-generation RNA-based therapeutics needs to incorporate new chemistries, materials and/or mechanisms of enhancing endosomal escape ~100-fold. [Taken from Dowdy, *Nature Biotechnology* 2017]

Endocytosis can proceed through a variety of mechanisms including clatherin-dependant and -independent endocytosis, calveolae-mediated endocytosis, and macropinocytosis (Bus et al., 2017; Rejman, Bragonzi, and Conese, 2005; Rehman, Zuhorn, and Hoekstra, 2013; Gabrielson and Pack, 2009; Gonçalves et al., 2004). Internalization via clatherin-dependant and clatherin-independent endocytosis leads to rapid acidification of the early endosome by vacuolar-type ATPase (V-ATPase, "proton pump") to a pH of 6-6.5. Early endosomes are then directed to sorting endosomes where the content can either be recycled back to the extracellular matrix (exocytosis) or to other intracellular pathways via the trans-golgi network (Shukla et al., 2016). Early endosomes can also mature to late endosomes with an internal pH of 5-5.5 before fusion with lysosomes, where the lysosomal milieu drops the pH to 4-4.5 to allow for lysosomal hydrolases to degrade the endosomal contents. In addition to endosomal acidification during maturation, the inner leaflet of the endosome changes composition from the initial extracellular membrane composition of sphingolipids, sterols (cholesterol), and glyocerophospholipids to a more anionic composition of increasing bis(monocyclglycero) phosphate (BMP) (van Meer, Voelker, and Feigenson, 2008). Each of these pathways offer opportunities for increasing cytoplasmic siRNA availability through endosomal escape as well as inhibition of non-productive pathways or degradation (Gonçalves et al., 2004; Shukla et al., 2016; Wattiaux et al., 2000).

Cationic Polymers and the Proton Sponge

The earliest mammalian gene delivery using cationic polymers was demonstrated in 1962 by Szyblaska and Szyblaska using spermine to deliver DNA (Szybalski and Szybalski, 1962). Additional polymers were investigated in the following decades and in 1995, poly(ethylene imine) (PEI) became the gold standard in gene delivery agents (Boussif et al., 1995). Despite the simplicity and efficiency of PEI, toxicity was a major issue and the following decades saw a boon in new cationic polymers.

Cationic polymers are an attractive delivery agent because of their simplicity and the reliability of electrostatic interactions between DNA and the charged amino groups within the polymers. Electrostatic condensation leads to the formation of nano-scale particles, masking the charge of the DNA (Kabanov et al., 1991; Bloomfield, 1996). These nanoparticles are taken up typically via endocytosis, though the exact mechanism depends on the size of the particle as well as a polymer and cell type (Rejman et al., 2004; von Gersdorff et al., 2006; Midoux et al., 2008). Generally, in the absence of ligand mediated receptor targeting, cationic particles associate nonspecifically with anionic surface glycoproteins prior to internalization (Payne et al., 2007; Yameen et al., 2014). For sensitive cargos such as DNA, escape from the endosomal pathways needs to occur early to avoid the degradation process.

Early attempts to explain the mechanism of endosomal escape for these cationic polyplexes looked toward the pH buffering capabilities of PEI other cationic polymers within the early endosome (Boussif et al., 1995; Behr, 1997). Following the development of PEI in 1995, its creators postulated that as the endosome acidified, the secondary and tertiary amines of the cationic polymer would absorb protons, leading to further pumping of protons into the endosomes followed by chloride ions and water, causing endosomal swelling and membrane disruption (Boussif et al., 1995). Proponents of this theory, termed the “proton sponge”, cite as evidence the reduced acidification and increased endosomal chloride concentration following PEI transfection (Sonawane, Szoka, and Verkman, 2003) as well as the reduced transfection efficiencies in the presence of V-ATPase inhibitors (Kichler et al., 2001). Additionally, reducing the buffering capacity of PEI by substitution of tertiary amines with quaternary amines in PEI drastically reduces transfection efficiency (Akinc et al., 2005).

Despite the evidence provided by its proponents, the proton sponge effect is unlikely to play a major role in cationic polyplex endosomal escape. Attempts to engineer superior cationic polymers guided by the proton sponge hypothesis failed to achieve increased functionality and often resulted in greatly reduced activity instead. Increasing the buffering capacity of these

cationic polymers within the range of endosomal pH led to the production of poly(2-methyl-acrylic acid 2-[(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester (pDAMA). However, rather than increasing endosomal escape, pDAMA had poor transfection efficiencies, strongly suggesting that the proton sponge effect is not sufficient for endosomal escape (Funhoff et al., 2004). Further, comparison of different poly(methacrylate)s using varied ratios of primary, secondary, tertiary, and quaternary amines showed that formulations with high primary amine content and poor buffering capacity at endosomal pH led to higher transfection rates, calling the validity of the proton sponge effect into question (Sprouse and Reineke, 2014; Li et al., 2013; Smith et al., 2011; Trützschler et al., 2018; Zhu et al., 2010).

Membrane rupture due to endosomal swelling as described in the proton sponge effect is unlikely to occur as experimental and computational modeling showed that typical PEI concentrations were insufficient to burst endosomes (Won, Sharma, and Konieczny, 2009; Benjaminsen et al., 2013). However, local membrane disruptions via PEI aggregate-membrane interaction have been observed by electron microscopy and suggest an alternative mechanism for endosomal escape mediated by interactions between polyplex aggregates and the inner leaflet of the endosome (Zhu et al., 2010; Bieber et al., 2002; Mishra, Webster, and Davis, 2004; Jonker et al., 2017; Rehman, Hoekstra, and Zuhorn, 2013; Vaidyanathan, Orr, and Banaszak Holl, 2016; Rattan et al., 2013). Experimental and computational studies report that PEI interaction with anionic lipids allows for penetration into hydrophobic core and anionic lipid translocation, facilitating transport of charged nucleic acids across the membrane (Zhang et al., 2014; Kwolek et al., 2016). Regardless of the mechanism, PEI and other cationic polymers and lipid formulations have high associated toxicities that prevent their use going forward into clinical trials. Cationic polymers intercalate into the endosomal membrane (Vaidyanathan, Orr, and Banaszak Holl, 2016) and are unable to be cleared by the cell, instead accumulating in the lipid membranes of mitochondria and the endoplasmic reticulum (Grandinetti, Ingle, and Reineke, 2011; Grandinetti, Smith, and Reineke, 2012).

Defined Endosomal Escape Domains

More direct attempts at endosomal escape have sought to harness naturally occurring membrane disrupting and penetrating agents from a variety of sources spanning viral, bacterial, plant and animal/human origins. The first demonstration of a protein translocating into the cytoplasm was in 1988 when two independent groups reported that full length HIV-1 *trans*-activator of transcription (TAT) could be efficiently internalized by a cell to modulate transcription of the HIV-1 promoter (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Shortly after this discovery, in 1991, the homeodomain of Antennapedia, a homeoprotein in *D. melanogaster*, was demonstrated to also have cell penetrating activity (Joliot et al., 1991). Discovery of these two translocating proteins prompted analysis of their similarities and efforts to identify the minimal amino acid sequence required for their cell penetrating activity. It was subsequently discovered that the 9 basic amino acids of TAT and the 16 amino acids from the third helix of the Antennapedia homeodmain (later termed Penetratin) were sufficient for transduction (Ezhevsky et al., 1997; Vivès, Brodin, and Lebleu, 1997; Derossi et al., 1994, 1996). Discovery of these two peptides prompted the discovery and design of more than 100 different chimeric and synthetic peptides, including derivatives of TAT and Penetratin capable of membrane translocation (Derossi et al., 1994; Pooga et al., 2001; Lindgren and Langel, 2011; Marín et al., 2011; Koren and Torchilin, 2012). These peptides are termed cell penetrating peptides (CPPs) or protein transduction domains (PTDs). These PTDs are typically 5-30 amino acids in length and enter cells without specific receptor interactions (Raucher and Ryu, 2015). The most successful of these PTDs contain 4-9 basic arginine residues clustered together (van den Berg and Dowdy, 2011), whereas as lysine residues were ineffective.

The *in vivo* delivery potential of PTDs was first demonstrated in the late 1990s when the Dowdy lab generated TAT-PTD recombinant fusion protein with β-galactosidase (βgal) and reported successful TAT-βgal delivery to most tissues within the mouse, including the brain

(Schwarze et al., 1999). Importantly, a control β-gal lacking the TAT-PTD was excluded from all tissues, demonstrating TAT mediated *in vivo* delivery for the first time. Subsequently, PTDs have been shown to deliver a variety of cargos including peptides/proteins, antisense oligonucleotides, small drugs, charge neutral peptide nucleic acids (PNAs) and morpholinos (PMOs) (Schwarze et al., 1999; Lewin et al., 2000; Gait, 2003; Wadia and Dowdy, 2005; van den Berg and Dowdy, 2011; Bechara and Sagan, 2013). These cargoes have been complexed with PTDs through covalent linkage, using disulfides and thioester bonds, as well as non-covalently through formation of large complexes.

The endosomal properties of these early PTDs were improved through carful design to produce next generation peptides. A second generation Penetratin, R6-Penetratin, adds arginine residues to the N-terminus of the original peptide and has shown increased endosomal disruption (Abes et al., 2007). A Penetratin analog, EB1, forms amphipathic α-helices at low pH and was more effective than Penetratin alone when delivering siRNA nanoparticles (Lundberg et al., 2007). Other similar PTDs include the singling peptide of bovine prion protein (bPrPp) that forms β-structures when interacting with anionic membranes and is able to transport large nanoparticles out of the endosome. Amphipathic Sweet Arrow Peptide (SAP) is an engineered proline rich, gamma-zein-related sequence that has low cytotoxicity and good translocation properties (Fernández-Carneado et al., 2004; del Pozo-Rodríguez et al., 2009).

Work in the Dowdy lab Improved TAT delivery by screening amino acid additions to the TAT peptide. Addition of hydrophobic tryptophan and phenylalanine increased cytoplasmic delivery of a cargo peptide (Lönn and Dowdy, 2015). While discrete hydrophobic domains may aid in siRNA endosomal escape, hydrophobic modification of the siRNA duplex itself has been explored by several groups, often taking inspiration from ASOs. ASOs are capable of endosomal escape *in vivo* through a process termed gymnosis (Stein et al., 2010). While the exact mechanism of gymnosis is not well understood, it is thought that the unstructured ssRNA and the increased hydrophobicity from the fully phosphorothioate backbone and 2'-modifications

aides in endosomal membrane translocation. The Khvorova lab utilizes a structurally modified siRNA duplex that is composed of 20/15, Guide/Passenger strands, with a 5 nt unpaired, fully phosphorothioated, 3'-Guide strand and a lipid or cholesterol domain conjugated to the 3'-end of the truncated passenger strand (**Figure 1.3**). This modified dsRNA contains 5 additional phosphorothioate modifications and 7 fewer nucleotides than the typical 21/21 siRNA, increasing the overall hydrophobicity, decreasing its size, and increasing flexibility of the molecule along the 3'-end of the Guide strand (Osborn and Khvorova, 2018). These modifications increase the overall ASO-like properties of the molecule and form a hydrophobic siRNA (hsiRNA) (**Figure 1.3**) that is still recognized by the RNAi machinery to induce an RNAi response (Alterman et al., 2015). *In vitro* and *in vivo* studies have demonstrated enhanced delivery of these hsiRNA and biodistribution studies have shown systemic delivery to the kidneys, liver, and heart (Osborn and Khvorova, 2018). Perhaps the best delivery of these hsiRNAs has been shown in the CNS for the treatment of Huntington's (Alterman et al., 2015). Following intraventricular injection, brain knockdown can be observed in a manner similar to the successful ASO treatments of CNS diseases. However, these CNS treatments are plagued by injection site toxicity and poor diffusion throughout the brain. It is also likely that despite reduction of overall charge to only 20 phosphodiesters, hydrophobic modifications and endosomal escape domains form nanoparticles in solution. Indeed, these methods are plagued by many of the problems of nanoparticles including limited biodistribution. While a promising approach that has seen gradual improvements in toxicity and biodistribution, there remains a lot of work to be done before effective hydrophobic endosomal escape domains can be utilized beyond the liver, kidneys, and CNS.

While many of these PTDs and hydrophobic domains operate under unknown mechanisms, known mechanisms of viral, bacterial, plant and animal endosomal disruption have allowed for the discovery and design of efficient endosomal escape domains. Endosomal escape via membrane fusion is a mechanism employed by many enveloped viruses including

Influenza, West Nile, and Herpes Simplex viruses. These fusion events are mediated by membrane integral peptides that form a hydrophobic random coil at neutral pH and rapidly undergo conformational change to drive membrane insertion and fusion following endosomal acidification (Marsh and Helenius, 1989; Horth et al., 1991)..

Membrane fusion following Influenza internalization through the action of Hemagglutinin (HA) is well characterized and serves as a model for membrane fusion in general (IWilson, Skehel, and Wiley, 1981; Bullough et al., 1994; Chen et al., 1998; Bizebard et al., 1995). Hemagglutinin is a trimeric integral membrane protein consisting of two disulfide-linked subunits, HA1 and HA2. The last 20-25 amino acids of HA2 are termed the "fusion peptide" and are highly conserved (Nobusawa et al., 1991). This fusion peptide consists almost entirely of hydrophobic residues and only contains three acidic amino acids. At neutral pH, the mature form the HA protein sequesters the hydrophobic residues of the fusion peptide, preventing premature membrane insertion (Chen et al., 1998). Following endocytosis and endosomal acidification, conformational changes present the fusion protein, allowing for membrane insertion in the endosomal as well as viral membranes. Insertion of the fusion peptide alone is sufficient to cause small (<2.6 nm in diameter) stable pores that can persist for minutes to hours, though these pores alone are insufficient for viral escape (Shangguan, Alford, and Bentz, 1996). Endosomal acidification also triggers a dramatic conformational change causing the entire HA protein to hinge onto itself, mechanically drawing the viral and endosomal membranes together, resulting in fusion (Tatulian et al., 1995; Gray and Tamm, 1997; Bentz, 2000; Chernomordik et al., 1999). In the absence of the HA1 subunit, this conformational change does not occur and membrane fusion is drastically reduced (Gray and Tamm, 1997).

The endosomal escape properties of the HA2 fusion peptide have been harnessed for nucleic acid polyplex delivery alone (Wagner et al., 1992; Subramanian et al., 2002; Lear and Degrado, 1987) and in conjunction with poly(L-lysine) (PLL) for enhanced escape (Wagner et al., 1992). Variants of the HA2 peptide have been formulated to have greater activity at

endosomal pH and include cationic peptides KALA and GALA that have shown efficacy with different drug and nucleic acid formulations *in vitro* (Wyman et al., 1997; Lee, Jeong, and Park, 2001; Han and Yeom, 2000; Min et al., 2006; Parente, Nir, and Szoka, 1990; Futaki et al., 2005; Sasaki et al., 2008; Kakudo et al., 2004; Simões et al., 1999; Parente, Nir, and Szoka, 1988).

Other endosomal escape agents derived from fusogenic viral proteins include influenza fusogenic peptide diINF-7 (Oliveira et al., 2007; Mastrobattista et al., 2002), gp41 (Kwon, Bergen, and Pun, 2008), L2 peptide (Kämper et al., 2006), and major envelope protein (E) (Kimura and Ohyama, 1988) that have been utilized to aid escape for siRNA nanoparticles, fusion proteins, PEI polyplexes, and gene delivery. An analog to herpes simplex virus glycoprotein H (gpH) is a known fusogenic peptide has been reported to increase trans-gene expression 30-fold *in vitro* (Tu and Kim, 2008).

Bacteria have also been used as a source of inspiration as several bacteria escape the endosome using exotoxins as part of their pathogenicity (London, 1992). *Listeria monocytogenes* produces listeriolysin O (LLO) that interacts with cholesterol at low pH to form pores in cholesterol containing membranes (Mandal and Lee, 2002; Tweten, 2005). LLO is known as a pore forming hemolysin (Glomski et al., 2002) but has very poor cytosolic stability. Utilizing this cytosolic instability and pH dependency, LLO has been modified to have very little cytotoxic activity (Decatur and Portnoy, 2000) and has been used as an endosomal escape agent with LNP and cationic polymers (Lorenzi and Lee, 2005; Kullberg, Owens, and Mann, 2010; Walton, Wu, and Wu, 1999; Saito, Amidon, and Lee, 2003). *Pseudomonas aerugneosa* produces a single chain endotoxin with three major domains called exotoxin A (ETA). Domain II induces endosomal escape for the entire bacterium (Teter and Holmes, 2002; Rasper and Merrill, 1994; Prior, FitzGerald, and Pastan, 1992) and domain II alone has been utilized in fusion proteins to deliver immunotoxins and other fusion proteins (Prior, FitzGerald, and Pastan, 1992; Jia et al., 2003; Bruell et al., 2003). Shiga toxin and cholera toxin operate in a unique mechanism involving retrograde transport to the endoplasmic reticulum followed by

translocation to cytosol (Sandvig et al. 2004). Diphtheria toxin has membrane translocation activity in addition to pore forming activity and has shown the ability to enhance PEI polyplex escape (London, 1992; Kakimoto et al., 2009).

Beyond viruses and bacteria, endosomal escape agents have been derived from plants and animal origin. Melittin is a cationic peptide and the major component of bee venom. In solution, melittin it forms an amphipathic α-helix to destabilize membranes via pore formation. Melittin has been used as a endosomolytic agent in several formulations to increase endosomal escape (Legendre and Szoka, 1993; Bettinger et al., 2001). Of note, this mechanism is not pH dependent, allowing melittin high activity, but also resulting in high toxicity due to cellular membrane disruption that results in cell death (Ogris et al., 2001; Dempsey, 1990). Several attempts to mask the immunogenicity and activity of melittin using pH labile domains have demonstrated reduce toxicity while retaining endosmolytic activity (Boeckle et al., 2006; Bettinger et al., 2001). Additional insect venom sources have been used for endosomal escape including an attenuated membrane lytic spider venom peptide, M-lycotoxin (L17E), that has demonstrated the ability to deliver large macromolecules including antibodies into the cytoplasm (Akishiba et al., 2017). Plants produce a variety of ribosomal-inactivating protein (RIP) members. Examples of RIPs include Ricin, Saporin and Gleonin, whose mechanisms are largely unknown though they have been used to facilitate release of large and small molecules (Sun et al., 2004; Day et al., 2002; Vago et al., 2005; Hartley and Lord, 2004; Stirpe, 2013).

Despite the considerable work and advances made in the field of endosomal escape, translation to the clinic has remained limited largely due to continued problems with immunogenicity and low stability. To date, only a single endosomal escape agent, melittin, has been utilized in a clinical trial to enhance delivery of and siRNA therapeutic. Arrowhead therapeutics utilized melittin-GalNac to release cholesterol-siRNA nanoparticles from the endosome in their DPC technology. However, the immunogenicity and cytotoxicity, even in a pH sensitive protected form, was too high and the technology was pulled form clinical trials

following safety concerns (Buchanan, 2016). The company has since moved away from melittin and all other endosomal escape agents in its clinical trials, highlighting the need for safe and effective agents.

In addition to problems with immunogenicity, stability, and toxicity, many of these viral and bacterial agents have been removed from their functional context, limiting their utility. Perhaps most glaring of all is the HA2 fusion peptide. While insertion of HA2 fusion peptide into the membrane of the endosome does cause small pores to form, in the absence of the full HA1 and HA2 domains, the mechanical force required for the HA membrane fusion mechanism is absent. The small (<2.6 nm) pores formed by fusion peptide insertion are sufficient for water and small dyes to escape the endosome but is far below the size required for larger macromolecules like siRNA (~6 nm) to pass. It stands to reason that under these considerations, the HA2 peptide operates in a greatly reduced manner compared to its original functionality and all derivatives of this peptide may suffer the same deficiencies.

In order to fully realize the full potential of siRNA therapeutics, highly efficient endosomal escape agents need to be developed that have low immunogenicity and toxicity. These agents need to also be easy to use and produce, and function in the context of the highly charged siRNA. Unfortunately, the current state of the art falls far short of these requirements as illustrated by the dearth of endosomal escape considerations within the clinic today.

SMALL INTERFERING RIBONUCLEIC NEUTRALS (siRNNS)

Although PTDs have been utilized to deliver a wide array of macromolecular cargos *in vitro*, *in vivo,* and in >25 clinical trials, few have reported successful delivery of siRNA using PTDs (Wadia and Dowdy, 2005; Lönn and Dowdy, 2015). Delivery via PTD association has largely been accomplished through non-covalent complexes of PTDs and siRNA into nanoparticles, an approach that suffers from the same problems associated with traditional nanoparticle delivery systems (Nakase, Tanaka, and Futaki, 2013). A more elegant and

desirable approach is direct, covalent, conjugation of a PTD and siRNA to form a small, monomeric, soluble PTD-siRNA molecule capable of self-delivery. However, successful construction of a PTD-siRNA comes with its own share of problems as the dense cationic charge that is critical to PTD delivery and the 40x negative charges of the siRNA molecule effectively neutralize each other and abolish PTD activity (**Figure 1.6**) (Glover, Lipps, and Jans, 2005; Gonçalves, Kitas, and Seelig, 2005; Jiang et al., 2004). Further, electrostatic interactions between siRNA and cationic PTDs lead to aggregation and formation of nanoparticles or precipitates (Moschos et al., 2007). To overcome the obstacles to PTD-siRNA construction, work was carried out by the Dowdy lab to neutralize the phosphate backbone to allow for construction of small, monomeric, soluble PTD-siRNA.

Initial efforts by the Dowdy lab utilized a TAT-PTD fusion protein with a dsRNA Binding Domain (DRBD) termed TAT-DRBD (**Figure 1.6**) (Eguchi et al., 2009). This approach demonstrated *in vivo* delivery of an siRNA and effective treatment of glioblastoma in a mouse model (Michiue et al., 2009). However, despite this success, aggregation at concentrations needed for systemic dosing occurred, reducing its therapeutic utility. Despite these problems, this method demonstrated that masking of the negative charges on the siRNA allowed for effective PTD mediated delivery *in vivo*. With this in mind, the Dowdy lab sought to develop a novel synthetic approach to masking the negative charge on the siRNA backbone.

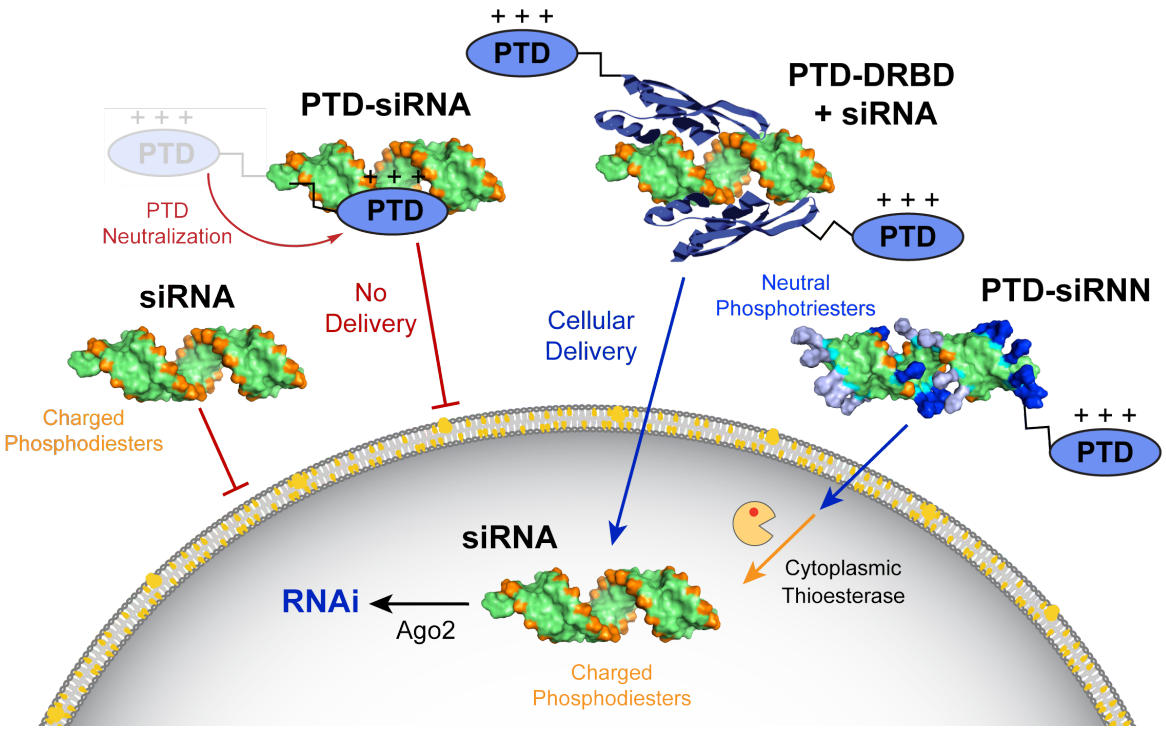


Figure 1.6. Overcoming the siRNA negative charge for PTD-mediated siRNA delivery. siRNA

The size (14,000 Da) and negative charge of the phosphodiester backbone prevent wild type siRNA from crossing the cellular membrane unassisted. **PTD-siRNA:** Conjugation of a cationic peptide transduction domain (PTD) to an anionic siRNA results in neutralization of the PTD and no cellular delivery. **PTD-DRBD + siRNA:** PTD-DRBD fusion proteins are able to deliver siRNA across the cellular membrane. The double-stranded RNA binding domains (DRBDs) of the fusion protein mask the negative charge of the siRNA phosphodiester backbone and allow cellular delivery siRNA by the fused PTD. **PTD-siRNN:** Short interfering ribonucleic neutrals (siRNNs) contain bioreversible phosphotriester groups that neutralize the negative charge of the phosphodiester backbone. This charge neutralization enables conjugated PTDs to deliver the monomeric, soluble siRNA prodrug across the cellular membrane. Upon cytosolic entry, ubiquitous cytoplasmic-restricted thioesterases convert the neutral phosphotriester groups into charged phosphodiester groups, resulting in a charged siRNA capable of Ago2 loading and RNAi response induction.

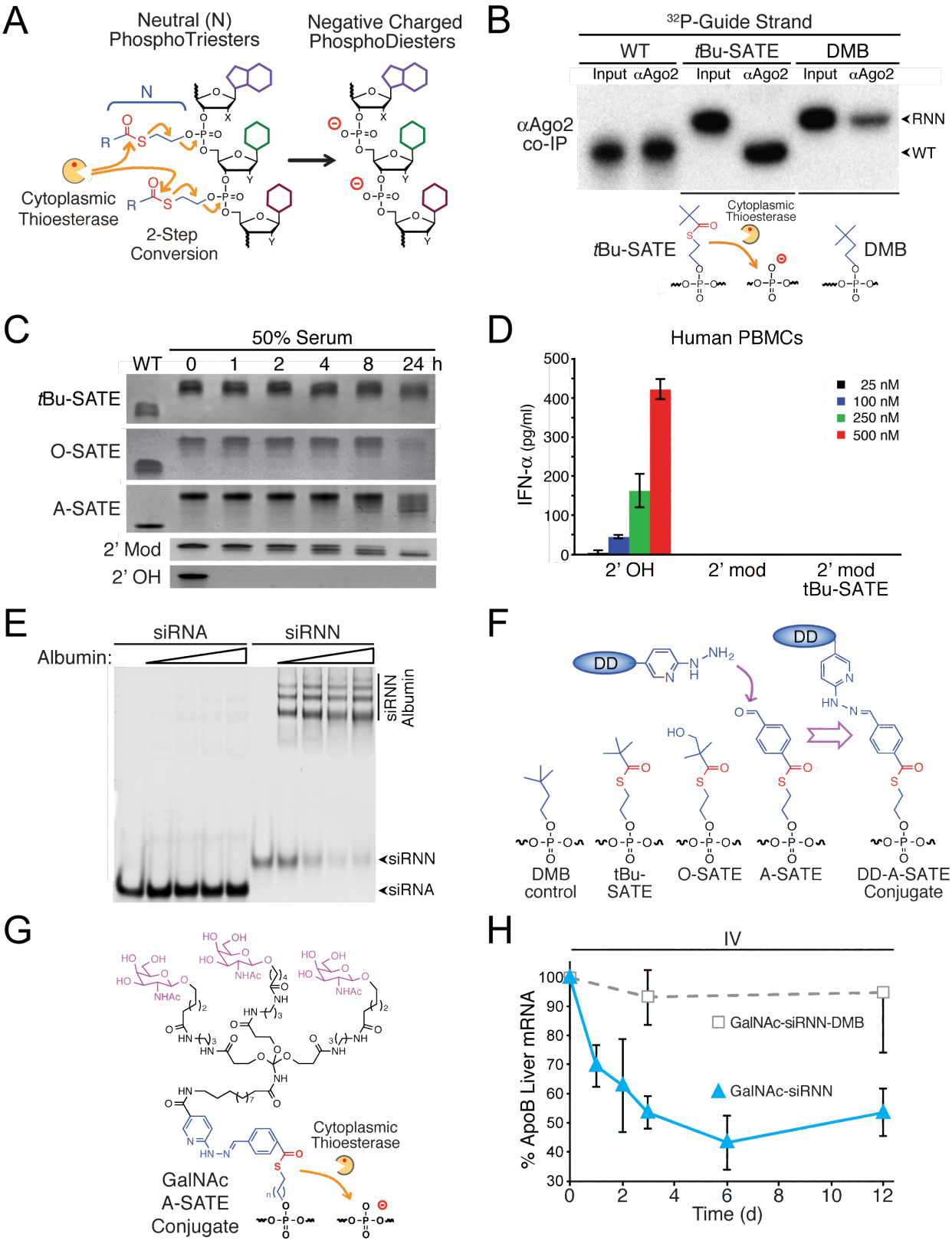
To determine the degree of backbone neutralization required for successful PTD delivery, the Dowdy lab performed a test with irreversible methyl phosphotriester modifications (Meade, 2010). siRNAs containing varying numbers of neutral methyl phosphotriesters were synthesized with a terminal cyanine dye (Cy3) and conjugated to TAT-PTDs via a hydrazone linkage. Cells were treated with these TAT-siRNA-Cy3 conjugates followed by trypsonization to remove surface-bound material and analyzed by flow cytometry to determine siRNA-Cy3-uptake. Analysis revealed that only siRNA-PTDs with a theoretical overall positive charge (~70% methyl phosphotriester neutralization) were taken up into the cells, predominantly into the endosomes. While these results demonstrated the ability of a synthetic approach to mask the phosphate backbone for PTD delivery, methyl triesters are irreversible and thus unable to mediate an RNAi response as RNAi requires a negatively charged backbone (Behlke, 2008; Schirle and MacRae, 2012; Vuković et al., 2014).

Methyl phosphotriesters were chosen for their synthetic simplicity and compatibility with solid-state synthesis; however, neutral phosphotriester modifications capable of RNAi induction must be bioreversible. Bioreversible phosphotriesters must be stable in the extracellular environments but rapidly convert into the bioactive charged phosphodiester siRNA upon entry into the cytoplasm. To accomplish this goal, the Dowdy lab chose to use a *t*-butyl-S-acyl-2-thioethyl (*t*Bu-SATE) phosphotriester group that was originally developed for a mononucleotide inhibitor of HIV (Puech et al., 1993; Lefebvre et al., 1995; Gröschel et al., 2002). The thioester bond in the *t*Bu-SATE is stable to extracellular esterases, but is rapidly cleaved by cytoplasmically restricted thioesterases (**Figure 1.7A**) (Zeidman, Jackson, and Magee, 2009). Cleavage of the thioester bond in the *t*Bu-SATE initiates a rapid, two-step, conversion of the neutral *t*Bu-SATE-phosphotriester into a charged phosphodiester bond. While this approach demonstrated a theoretical possibility of neutral bioreversible siRNA therapeutics, considerable synthetic problems remained, requiring a multitude of orthogonal solutions before these bioreversible *t*Bu-SATEs could be incorporated into an oligonucleotide. The Dowdy lab

developed solutions to each problem and successfully synthesized *t*Bu-SATE-siRNA bioreversible prodrugs, termed short interfering riobonucleic neutrals (siRNNs).

Importantly, *t*Bu-SATE siRNAs are bioreversible and are capable being loaded into Ago2 by TRBP to induce a robust RNAi response following transfection into cells (**Figure 1.7B**) (Meade et al., 2014). Additionally, *t*Bu-SATEs are stable in serum for >24 hr, do not stimulate the innate immune response, and have a long half life in serum due to increased serum albumin binding capabilities (**Figure 1.7C-E**). The *t*Bu-SATE technology is also amenable to modification distal to the thioester bond, allowing for modulation of solubility and addition of conjugation handles for site selective conjugation of PTDs and other cargos (**Figure 1.7F**). This conjugation capability was utilized to conjugate *tris*-GalNAc for GalNAc-siRNN delivery *in vivo*. Systemic delivery via tail vein injection showed enhanced and prolonged knockdown of liver target mRNA (**Figure 1.7H**) (Meade et al., 2014). Taken together, these results demonstrated effective masking of the problematic siRNA phosphodiester backbone in a bioreversible manner, opening up the possibility of conjugating cationic PTDs as well as any other targeting or endosomal escape domains. This capability provides a platform for the construction of diverse and multifunctional RNAi therapeutics in a way that has not been seen before.

Figure 1.7. Short Interfereing Ribonucleic Neutrals (siRNN).
A) Phosphotriester cleavage by cytoplasmic thioesterase initiates a two-step conversion that resolves as a charged phosphodiester linkage. **B)** Anti-Ago2 co-immunoprecipitation from cells transfected with ^{32}P-labeled guide strand containing control wild-type charged phosphodiester (WT), six *t*Bu-SATE or six control irreversible DMB phosphotriester oligonucleotides, duplexed to wild-type passenger strands and analyzed by denaturing gel electrophoresis. Note conversion of ^{32}P-labeled *t*Bu-SATE phosphotriester guide strand into wild-type phosphodiester guide strand. Input, ^{32}P-labeled single guide strand. **C**) Serum stability analysis of single-stranded oligonucleotides containing 9× *t*Bu-SATE, O-SATE or A-SATE phosphotriester groups vs. wild-type phosphodiester 2'-OH or 2'-F/O-Me (2'-Mod) RNA incubated in 50% human serum at 37 °C for indicated times. **D)** Analysis of IFN-α induction in human PBMCs at 24 h post-treatment with highly stimulatory β-gal 2'-OH siRNA (2'-OH), 2'-modified siRNA (2'-mod) and O-SATE (14×) phosphotriester siRNN (2'-mod *t*BU-SATE). **E)** Charged siRNA and neutral siRNNs were assayed for albumin binding by incubation with 0, 0.5, 1.0, 1.5 or 2.0 mg/ml serum albumin then separated by gel electrophoresis mobility shift and ethidium bromide staining. Note that due to charge neutralization, siRNNs do not stain as efficiently as charged siRNAs. **F)** Structures of the main phosphotriester groups used in this study. Hydrazine containing delivery domain (DD) peptides are conjugated to siRNNs via chemically reactive aldehyde A-SATE phosphotriester group. Cleavage of DD-A-SATE by thioesterase removes both phosphotriester group and conjugated peptide. **G)** Structure of GalNAc targeting domain conjugated to A-SATE phosphotriester group. **H)** Single intravenous dose kinetic comparison of GalNAc-siRNN ApoB vs. irreversible control GalNAc-siRNN-DMB ApoB by qRT-PCR (25 mg/kg; n = 3, each time point). Values normalized to β2-microglobulin from water treated control group (n = 5). Error bar indicates s.d. [Adapted from Meade *et. al*. *Nature Biotechnology* 2014]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Neutral (N)
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Human PBMCs
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tBu-SATE
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SATE
O-SATE
A-SATE
DD-A-SATE
Conjugate
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Thioesterase
GalNAc
A-SATE
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IV
% ApoB Liver mRNA
100
90
80
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GalNAc-siRNN-DMB
GalNAc-siRNN
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2
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6
8
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CONCLUSIONS

The advances made to the understanding of RNAi over the past two decades have elevated the field from a single miRNA effect in *C. elegans* to the first FDA approved RNAi therapeutic, with several additional candidates on the very near horizon. Indeed, the dream of safe and effective RNAi therapeutics is flourishing with the advent of GalNAc targeting to the liver, so much so that targeting genetic disease within the hepatocytes can be considered solved. However, despite the rapid pace of progress toward eradicating so many hepatic diseases, progress toward treating tissues outside the liver remains limited. Targeting domains to extra-hepatic receptors often fail to make the transition from academic study to the rigors of clinical validation. Unfortunately, the kinetics of GalNAc targeting with 10^6 receptors per cell recycling every 15 min are not replicated outside the liver, representing a serious biological limitation when working with even well validated targeting domains (**Figure 1.6**). Finally, in the event of successful targeting and internalization, escape from the endosome remains an unsolved problem. The success of GalNAc in the absence of an endosomal escape domain is an unexplained phenomenon, one that may not be present in all biological systems. These limitations present a field starved of extra-hepatic targeting with no viable option to overcome the biological limitations of the target receptors themselves. In order to continue the RNAi revolution, considerable efforts will need to be made to address the issues of extra-hepatic targeting and endosomal escape.

Current efforts to address the problem of extra-hepatic delivery have seen some success in the case of pancreatic β-islet targeting with GLP-1-ASOs, but this technology has not been utilized in the clinic for siRNA therapeutics so its clinical utility in RNAi remains to be seen. Efforts to target tumors with folate, RGD peptides, and other peptides remain inconclusive and the question of how these targeting domain-siRNA conjugates will escape the endosome remains unanswered. Endosomal escape domains continue to advance though stability, toxicity, and immunogenicity problems remain. Even with potent and effective targeting and

endosomal escape domains, their application to siRNA therapeutics pose considerable problems in their construction. To this end, I developed strategies for site specific, well defined, multifunctional, and multivalent construction of RNAi triggers for targeting of tumor associated macrophages (**Chapter 3**) and construction of antibody RNA conjugates (ARCs) to target tumors (**Chapter 4**).

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CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Phosphoramidite Synthesis

All phosphoramidites with phosphotriester groups were synthesized using the general synthetic protocol in (**Figure 2.1**). Specific methods for synthesizing each of the phosphoramidites used in this work are described in Meade *et al.* (Meade et al., 2014). An example protocol for the synthesis of 5'-O-(4,4'-Dimethoxytrityl)-2'-F-uridine 3'-O-[(S-pivaloyl-2-thioethyl) N,N-diisopropylphosphoramidite] is as follows: N,N-Diisopropylethylamine (DIEA) (Sigma Aldrich) (0.907 mL, 5.47 mmol) was added dropwise over 10 min to a magnetically stirred cooled solution (-78 C) of 5'-O-(4,4'- Dimethoxytrityl)-2'-F-uridine (2 g, 3.65 mmol, RI CHEMICAL). A solution of bis- (N,N-diisopropylamino)-chlorophosphine (1.23 g, 95% purity, 4.38 mmol, Sigma Aldrich) in dry CH_2CL_2 (5 mL) was then added dropwise over 10 min and the reaction mixture was allowed to warm to room temperature while stirring was maintained (1 hr). S-(2-hydroxyethyl) thiopivaloate (0.71 g, 4.3 mmol) was added portion wise followed by ethyl thiotetrazole (8.76 mL, 0.25 M solution in acetonitrile (ACN), 2.19 mmol) and the reaction was stirred for 2-12 hr. Then the reaction mixture was washed with brine (2 x 20 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* and the residue was subjected to flash silica gel column purification on a combi-flash instrument (Teledyne Isco) using hexane-ethyl acetate (0.5% triethylammonium acetate) as the solvent (0-70%). The fractions containing the products were pooled together and evaporated to dryness. The foamy residue was re-dissolved in benzene, frozen and lyophilized, affording a final product as a colorless powder (~2.2 g), 80% yield as diastreomeric mixture.

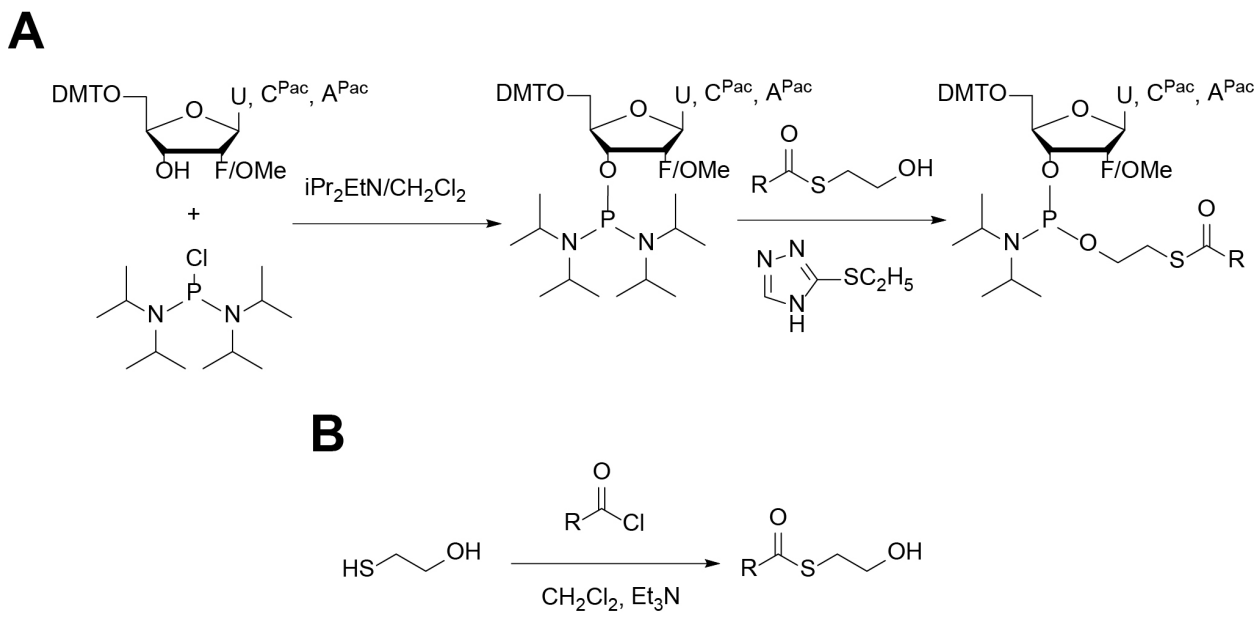
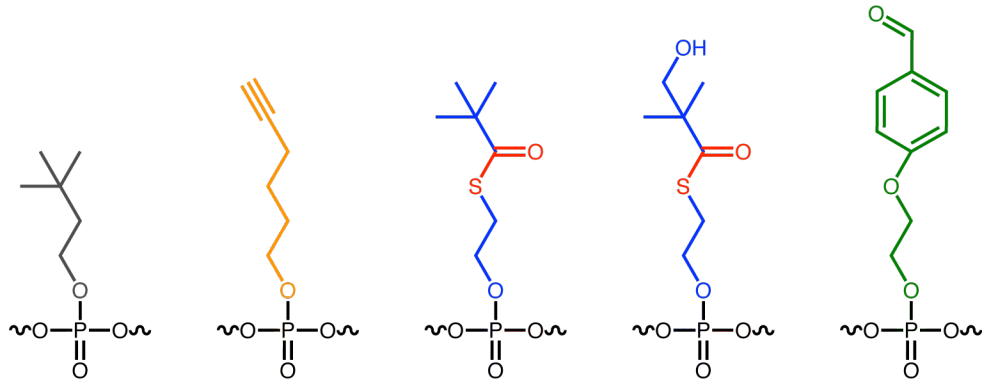


Figure 2.1. General Scheme for RNN Phosphoramidite Synthesis.
A) General synthetic routes for U, C, and A phosphoramidites containing SATE groups. **B)** General synthetic scheme of the SATE alcohol used in SATE phosphoramidite synthesis

Figure 2.2. Phosphotriester Structures and Oligonucleotide Sequences. A) Structures of phosphotriester groups discussed in this dissertation. All structures are shown in the deprotectedform utilized for biological studies. At the time of oligonucleotide synthesis, O-SATE hydroxyl group is protected by a tert-butyldimethylsilyl (TBDMS) group and the benzaldehyde present on the Ax is protected by an acetal. DMB, Kx, and AX are *irreversible* phosphotriester groups that do not contrain a thioester bond. Nomenclature: DMB = 2,2-DiMethylButyl; KX = irreversible alkyne; SATE = s-Acycl-ThioEthyl; AX = *irreversible* Aldehyde. **B)** siRNA sequences (5' to 3' orientation) and phosphotriester patterns discussed in this dissertation. All Purines (A and G) are 2'-hydroxymethyl modified on the ribose sugar and all Pyrimidines (U and C) are 2'-Fluoro modified on the ribose sugar. P#() and G#() nomenclature represents the Passenger (P) or Guide (G) strand and the number (#) of phosphotriester insertions. Nomenclature in parentheses represents specific phosphotriester modifications (D = DMB, S = SPTE, Kx, Ax), the specific number of each modification (KX2 = two Kx insertions), and the presence of phosphorothioate pairs (t) at the 5' and 3' ends. Sequence coloration represents placement of a phosphotriester at a specific position 3' of the indicated base. Coloration matches phosphotriester type to oligonucleotide name. Phosphorothioate positions are indicated by underlined (A) nucleotides, with the phosphorothioate modification present on the phosphate 3' to the underlined base. Oligonucleotide sequences with only Kx and/or Ax modifications without SPTE phosphotriesters are termed "siRNA" while oligonucleotides with SPTE modifications with or without Ax and/or Kx phosphotriesters are termed "siRNN" when discussed in this dissertation.

A



B

PLK1 Passenger Strand Oligonucleotides

P2(tKX1AX1t)-	**AGAAGAUGCUUCAGACAGAUT**
P3(tKX2AX1t)-	**AGAAGAUGCUUCAGACAGAUT**
P2(tKX2t)-	**AGAAGAUGCUUCAGACAGAUT**
P6(tKX1S4AX1t)-	**AGAAGAUGCUUCAGACAGAUT**
P6(tKX2S3AX1t)-	**AGAAGAUGCUUCAGACAGAUT**
P6(tKX2S4t)-	**AGAAGAUGCUUCAGACAGAUT**

PLK1 Guide Strand Oligonucleotides

G(tt)-	**UCUGUCUGAAGCAUCUUCUUT**
G6(tS5D1t)-	**UCUGUCUGAAGCAUCUUCUUT**

GAPDH Passenger Strand Oligonucleotides

P2(tKX1AX1t)-	**UCUACAUGUUCCAGUAUGAUT**
P3(tKX2AX1t)-	**UCUACAUGUUCCAGUAUGAUT**
P2(tKX2t)-	**UCUACAUGUUCCAGUAUGAUT**
P6(tKX1S4AX1t)-	**UCUACAUGUUCCAGUAUGAUT**
P6(tKX2S3AX1t)-	**UCUACAUGUUCCAGUAUGAUT**
P6(tKX2S4t)-	**UCUACAUGUUCCAGUAUGAUT**

GAPDH Guide Strand Oligonucleotides

G(tt)-	**UCAUACUGGAACAUGUAGAUT**
G6(tS5D1t)-	**UCAUACUGGAACAUGUAGAUT**

Luciferase Passenger Strand Oligonucleotides

P2(tKX1AX1t)-	**CCUACAUUCUGGAGACAUAUT**
P3(tKX2AX1t)-	**CCUACAUUCUGGAGACAUAUT**
P2(tKX2t)-	**CCUACAUUCUGGAGACAUAUT**
P6(tKX1S4AX1t)-	**CCUACAUUCUGGAGACAUAUT**
P6(tKX2S3AX1t)-	**CCUACAUUCUGGAGACAUAUT**
P6(tKX2S4t)-	**CCUACAUUCUGGAGACAUAUT**

Luciferase Guide Strand Oligonucleotides

G(tt)-	**UAUGUCUCCAGAAUGUAGGUT**
G6(tS5D1t)-	**UAUGUCUCCAGAAUGUAGGUT**

Oligonucleotide Synthesis

All oligonucleotides synthesis was carried out on a BioAutomation Mermade-6 oligonucleotide synthesizer (BioAutomation). Oligonucleotide synthesis reagents include: Activator = 0.25 M 5-Benzylthio-1H-tetrazole (BTT) in ACN (Glen Research, 30-3170); Cap A = 5% Phenoxyacetic anhydride (w/v), 90% THF (v/v), 10% pyridine (Glen Research, 40-4210); Cap Mix B = 16% 1-Methylimidazole (v/v), 84% THF (Glen Research, 40-4220); Deblock = 3% Trichloroacetic acid (w/v) in DCM (VWR, EM-BI0830-0950); Oxidizing Reagent = 0.02 M Iodine in 70% THF (v/v), 20% pyridine, 10% water (VWR, EM-BI0420-4000); Sulfurizing Reagent = 0.05 M 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (Sulfurizing Reagent II) in 40% pyridine (v/v), 60% ACN (Glen Research, 40-4137-52); Anhydrous ACN (VWR, AX0151-1). Commercially available amidites used are: dT-CE (Glen Research, 10-1030), 2'-OMe-5'-O-DMTr-PAC-A-CE (Glen Research, 10-3601), 2'-Fluoro-5'-O-DMTr-PAC-C-CE (Carbosynth, PD-158882), 2'-OMe-5'-O-DMTr-iPr-PAC-G-CE (Glen Research, 10-3621), and 2'-Fluoro-5'-O-DMTr-U-CE (Carbosynth, PD09874). Phosphoramidites were coupled at concentrations and time following manufacturer recommendations. For modifications, 5'-DBCO-TEG phosphoramidite (Glen Research, 10-1941-90), 5'-IRDye 800 phosphoramidite (LI-COR Biosciences, 4000-33), 5'-Thiol modifier phosphoramidite (Glen Research, 10-1936), 5'-Aldehyde modifier phosphoramidite (Glen Research, 10-1933), and Cy3 phosphoramidite (Glen Research, 10-5913) were coupled following manufacturer's recommendation. All Phosphotriester phosphoramidites were coupled at 100 mM with two coupling cycles of 6 min each. CPG supports used were dT-Q-CPG 500 (Glen Research, 21-2230) and Universal Q SynBase 500/110 (Link Technologies Ltd., 2300-C001) at 1 µmol scale. Manual detritylation was accomplished by flowing 1 ml of deblock solution through the CPG column into 3 ml of 100 mM p-toluenesulfonic acid in anhydrous ACN followed by 2 mL anhydrous ACN wash. Absorbance readings at 498 nm were measured to quantify full-length oligonucleotide yield by DMT concentration and ensure full-length coupling.

Primary Oligonucleotide Deprotection

For all wild type (2'-OH) oligonucleotide deprotection, CPG was incubated in 1 mL of AMA (Ammonium Hydroxide/40% Aqueous Methylamine (1:1)) (Sigma-Aldrich, 295531) for 1 hr at 65° C. For all SATE-containing oligonucleotides, CPG was incubated in 1 mL of 10% diisopropylamine (v/v) (Sigma-Aldrich, 386464) 90% methanol for a 4 hr at room temperature (RT). For all oligonucleotides with only irreversible phosphotriesters (AX, KX, and DMB phosphotriesters), CPG was incubated in 3:1 ammonium hydroxide:ethanol for 2 hr at 65° C. After deprotection, oligonucleotide solutions were placed in a centrifugal evaporator for drying.

For 2'O-TBDMS deprotection, oligonucleotides were dissolved in 100 µl of anhydrous DMSO. To each oligonucleotide solution, 125 µL of 98% triethylamine trihydrofluoride (Sigma Aldrich, 344648) was added and reactions were left at room temperature for 4 hr. After 4 hr, oligonucleotides were precipitated by the addition of 35 µl of 3 M sodium acetate and 1 mL of 1-Butanol. Oligonucleotides were then incubated at -80° C for 2 hr. After incubation, the oligonucleotides were centrifuged at 16,000 g for 5 min, supernatant was aspirated, oligonucleotide pellets were dissolved in 1 mL of water and desalted with NAP-10 columns (GE Healthcare, 83-468).

For TBDMSO-SATE deprotection, oligonucleotides were dissolved in 219 µl of anhydrous DMSO. To each oligonucleotide solution, 31 µL of 98% triethylamine trihydrofluoride was added and reactions were incubated at RT for 1 hr per TBDMSO-SATE on the oligonucleotide. After deprotection, oligonucleotides were precipitated by the addition of 35 µL of 3 M sodium acetate and 1 mL of 1-butanol. The oligonucleotides were then incubated at -80° C for 2 hr. After incubation, the oligonucleotides were centrifuged at 16,000 g for 5 min, followed by aspiration of the supernatant. The oligonucleotides pellets were dissolved in 250 µL of 50% ACN for purification by RP-HPLC.

Oligonucleotide Purification

All oligonucleotides were purified by RP-HPLC on an Agilent 1200 Series Analytical HPLC with an Agilent SB-C18 column (9.4 x 150 mm) (Agilent, 883975-202). Linear gradients were run from 50 mM triethylammonium acetate (TEAA) pH 7.0 in water to 90% ACN/ 10% water at a flow rate of 2 ml/min. Length and steepness of gradient varied with number and type of SATE groups present on oligonucleotides. For DMT-On purifications, DMT-oligonucleotide HPLC peaks were collected, analyzed for the presence of full-length SATE oligonucleotides by MALDI-TOF mass spectrometry, and selected fractions were pooled and frozen on dry ice and lyophilized twice to remove TEAA.

Mass Spectrometry

Oligonucleotides were analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). 10 pmol of RNA/RNN was spotted with 1 µL of matrix from a 20 mg/mL solution of 2',4',6'-Trihydroxyacetophenone (Sigma-Aldrich, 91928), 20 mM ammonium citrate dibasic (Sigma-Aldrich, 09831) in 50% ACN/ 50% water. Spectra were collected in negative mode with accelerating voltage =20,000 V, grid = 90%, guide wire = 0.15%, 40 nsec delay time. >200 shots were collected for each sample.

Secondary Oligonucleotide Deprotection and Desalting

For all oligonucleotides aside form those containing an acetal-AldSATE phosphotriester, detritylation and removal of acetal protection from any present acetal-AX phosphotriesters was accomplished by treatment with 200 uL 80% acetic acid and heating at 65° C for 1 hr. Following deprotection, oligonucleotides were frozen and lyophilized until dry.

For oligonucleotides containing an acetal-AldSATE phosphotriester, detritylation and removal of acetal protection was accomplished by treatment with 200 µl 80% formic acid and

incubation at room temperature for 4 hr. Following deprotection, oligonucleotides were frozen and lyophilized until dry.

Following lyophilization, deprotected oligonucleotides were dissolved in 20% ACN and desalted with NAP-10 columns (GE Healthcare Life Sciences 17-0854-02). Desalted oligonucleotides were dried in a centrifugal evaporator. Once dry, completed oligonucleotides were dissolved in 50% ACN, quantified, and stored at -20° C.

Gel Electrophoresis

Single stranded RNA (ssRNA) oligonucleotides were analyzed by denaturing gel electrophoresis using 15% acrylamide/7 M Urea denaturing gels and stained with methylene blue for visualization. dsRNA oligonucleotides were hybridized by heating to 65° C for 2 min and allowed to cool to room temperature. dsRNA analysis was performed by non-denaturing gel electrophoresis using 15% acrylamide non-denaturing gels and ethidium bromide staining for visualization.

Cell Cycle Analysis

Cell cycle position following Plk1 mRNA knockdown was determined by flow cytometry of cells stained with propidium iodide (PI) using a BD LSR-II flow cytometer. For PI staining, >50,000 cells of interest were washed with PBS and removed from a tissue culture plate using trypsin-EDTA 0.05% (Life Tech, 25300054). Cells were pelleted and resuspended in 70% EtOH and incubated over night at 4^{o} C. After 12 hr, cells were pelleted and resuspended in 0.1% propidium iodide, 0.02% sodium azide in PBS. Cells were incubated at 37^{o} C for 30 min before FACS analysis.

Peptide Synthesis

All protected amino acids and coupling reagents were purchased from Nova Biochem or Bachem, BOC 6-hydrazino-nicotinic acid (Solulink, s-3003-500), and Fmoc-N-amido-dPEG6-acid (Quanta Biodesign, 10063). Endosomal Escape Peptides: EED1-HyNic, BEED-HyNic, HA2-HyNic, Endoporter-HyNic, Acid Melitin-HyNic (nGM3). Lysine peptides: KAYA-PEG6-Azide, KAYA-PEG6-HyNic, KAYA-Flourescein, K-PEG6-Azide, K-PEG18-Azide. Peptide synthesis was performed at 25 μM scale using Fmoc solid phase peptide synthesis on Symphony Quartet peptide synthesizer (Ranin) and rink-amide MBHA resin as solid support. All HyNic peptides were cleaved and deprotected using standard conditions (92.5% TFA, 2.5% acetone, 2.5% water, 2.5% TIS) for 2 hr. Crude peptides were precipitated with cold diethylether and purified by RP-HPLC on an Agilent 1200 Series Preparative HPLC Prep-C18 with a Prep-C18 30 × 250 mm column (Agilent, 410910-302). Peptide purity was confirmed by mass spectrometry using α-CHCA matrix (Sigma-Aldrich, 70990) and an Applied Biosystems Voyager-DE PRO MADLI-TOF mass spectrometer.

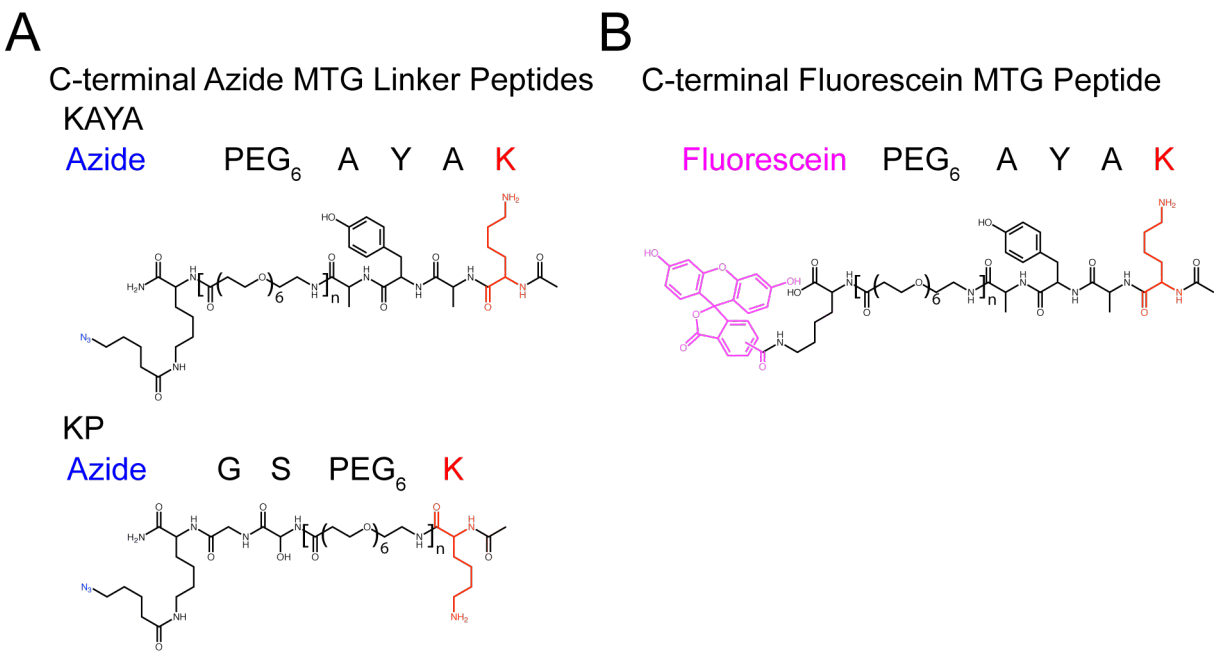


Figure 2.3. Structure of MTG Linker Peptides.
A) Structures of C-terminal Azide microbial transglutaminase (MTG) lysine donor peptides. PEG_6 spacing amino acids were inserted as n=1 or n=3. **B)** Structures of C-terminal 5(6)-Carboxyfluorescein labeled (MTG) lysine donor peptides. PEG_6 spacing amino acids were inserted as n=1.

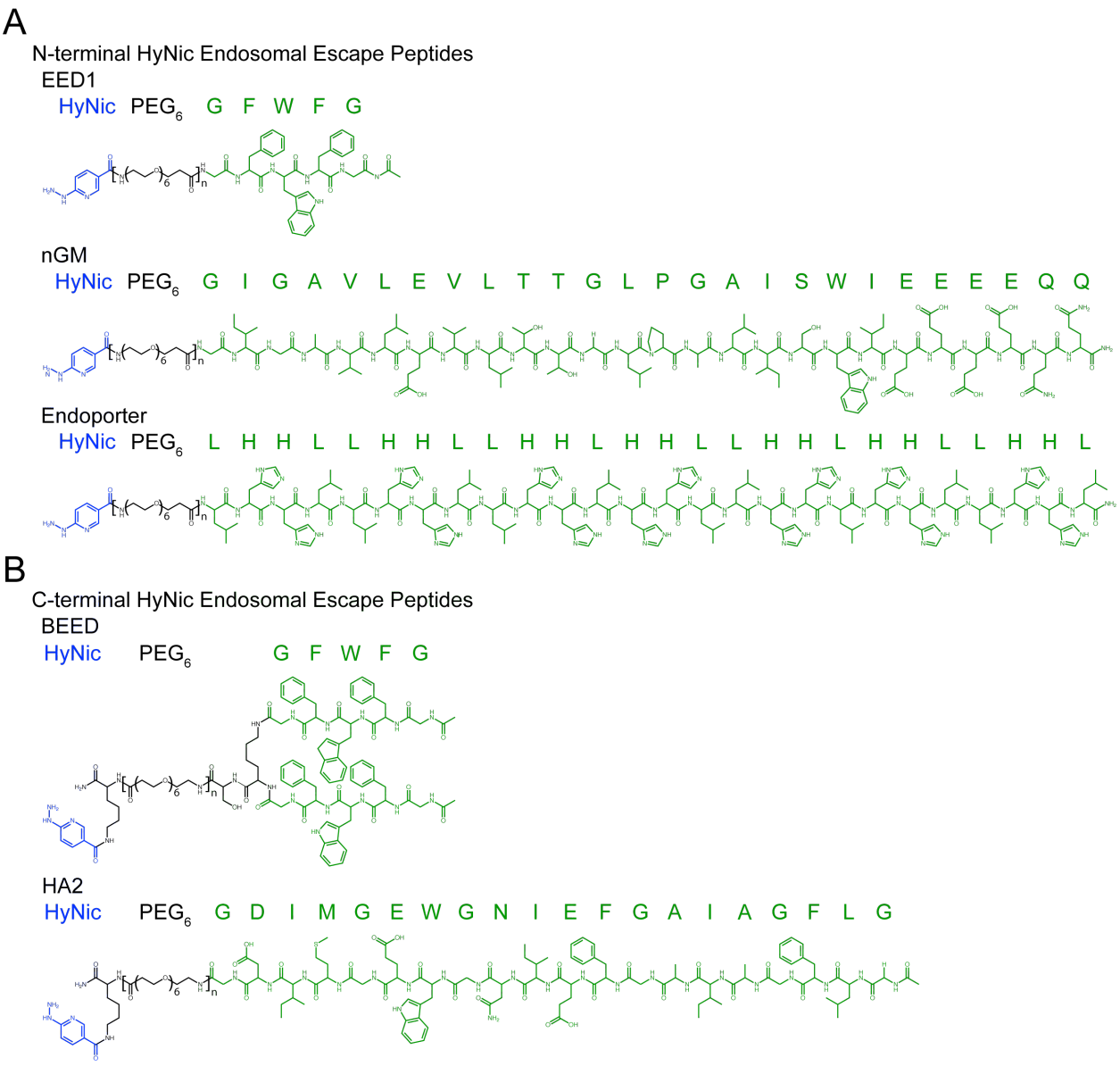


Figure 2.4. Structure of Endosomal Escape Peptides.
A) Structures of C-terminal HyNic Endosomal Escape peptides. PEG_6 spacing amino acids were inserted as n=1 or n=3. **B)** Structures of N-terminal Endosomal Escape peptides. PEG_6 spacing amino acids were inserted as n=1 or n=3.

siRNA Conjugation

For all hydrazinonicotinic acid (HyNic) conjugations, Peptide-HyNic was reacted with oligonucleotides bearing either AX or AldSATE phosphotriester insertions at a ratio of 10:1 (peptide:aldehyde conjugation site). Reactions were carried out above 0.5 mM oligonucleotide concentration in a 1% Analine (Tokyo Chemical Industry, A0463) solution in 50% ACN/50% Water at RT for 1 hr. Conjugates were purified by FPLC using a Superdex-75 10/300GL column (GE Healthcare) with a flow rate of 0.7 mL/min 50 mM TEAA (pH 7.0) in water. Fractions were checked by MALDI-TOF, pooled, frozen on dry ice, and lyophilized to yield the final product. Lyophilates were resuspended in 50% ACN/50% water and duplexed with complimentary single stranded RNN (ssRNN) oligonucleotides. Peptide-oligonucleotide conjugates were analyzed by PAGE and methylene blue staining.

For all Targeting Domain (TD)-azide conjugations, single stranded oligonucleotides bearing KX modification, and TD-azide were lyophilized together at a 10:1 ratio (azide:alkyne). One hour prior to use, a solution of 12 mM CuSO4 and 60 mM THPTA (Glen Research, 50-1004) was made and allowed to complex at RT for 1 hour. The $CuSO_4$/THPTA solution was diluted to a final concentration of 3 mM $CuSO_4$,15 mM THPTA in a solution of 50 nM (+)-Sodium L-Ascorbate (Sigma Aldrich, A7631), 10% *t*-butanol (JT Baker, 9065) in degassed water. This final reaction mixture was used to resuspend the TD-azide/oligonucleotide-alkyne pellet to a final concentration of 500 uM oligonucleotide-alkyne, 5 mM TD-azide. The reaction was carried out at 65 C for 45 min followed by purification by FPLC using a Superdex-75 10/300GL column (GE Healthcare) with a flow rate of 0.7 mL/min 50 mM TEAA (pH 7.0) in water. Fractions were checked by MALDI-TOF, pooled, frozen on dry ice, and lyophilized to yield the final product. Lyophilates were resuspended in water and duplexed with complimentary ssRNN oligonucleotides. TD-oligonucleotide conjugates were analyzed by PAGE and methylene blue staining.

qRT-PCR

For macrophage RNA isolation, a 24 well plate containing ~250,000 cells per well was washed 1x with PBS and lysed using a Illustra RNAspin isolation kit (GE Healthcare, 25050071) following manufacturer's recommendation. 1 µg of RNA was used to synthesize cDNA with iScript cDNA Synthesis Kit (Bio-Rad, 170-8891) and qRT-PCR was performed on 2.5 ng of cDNA with SYBR Green PCR Master Mix (Applied Biosystems, 4309155) on Applied Biosystems 7300 Real-Time PCR System. mGAPDH primers: forward, 5′-CTCCGGGTGATGCTTTTCCT; reverse, 5′- ACATGTAAACCATGTAGTTGAGGT. B2M primers: forward, 5′-ACCGTCTACTGGGATCGAGA; reverse, 5′-TGCTATTTCTTTCTGCGTGCAT.

Isolation of Bone Marrow Derived Macrophages (BMDM)

Bone Marrow Derived Macrophages (BMDM) were isolated from C57B/6 mice (Envigo) between 10-12 weeks of age. Mice were sacrificed by CO_2 followed by cervical dislocation. Femurs and tibias were removed from each mouse and sterilized with 70% ethanol prior to being placed into cold PBS. In a sterile flow hood, bone marrow was extracted by removing the ends of each bone, followed by flushing of the interior of each bone with cold, serum free, RPMI-1640 (Life Technologies, 27016021) using a 25 G needle and 10 mL syringe. The resulting mixture of bone marrow and cold RPMI was collected and the process was repeated a second time from the other end of each bone. Bone marrow samples were centrifuged at 400 x g for 5 min at 4° C. Supernatant was discarded and the bone marrow pellet was resuspended in 1 mL Red Blood Cell Lysis Buffer (eBiosciences, 00-4333-57) and allowed to incubate at RT for 5 min. 25 mL cold serum free RPMI was added to the lysis reaction and the resulting cell suspension was filtered through a 40 µm cell strainer into a new sterile tube. The bone marrow filtrate was then centrifuged at 400 x g for 5 min at 4° C before the supernatant was discarded and the resulting pellet resuspended in 15-20 mL Culture Medium (RPMI + 10% FBS + 1x

Penicillin-Streptomycin (Invitrogen, 15140122) +40 ng/mL M-CSF (PeproTech, 315-02)). Cells were then plated on a 15 cm plate (Fisher Scientific, 08-757-148) at a density of 2.5 x 10^7 cells/plate in a final volume of 20 mL culture medium. After 4 days, additional M-CSF was added to a final concentration of 20 ng/mL. After 2 additional days (6 days since harvest), cells were scraped off the plate, counted, and plated at either 125,000 cells/well in a 48 well or 250,000 cells/well in a 24 well tissue culture plate in culture medium containing 20 ng/mL M-CSF and 20 ng/mL IL-4 (Pepro Tech, 214-14). After two additional days (8 days since harvest), BMDM cells were stained for CD206 and F4/80 expression to confirm M2 macrophage polarization and were subsequently treated. All animals were maintained, treated, and euthanized in accordance with the UCSD Institutional Animal Care and Use Committee.

CD206 and F4/80 Staining of BMDM

BMDM cells had their media aspirated and were washed with 1x PBS followed by removal from their plate using Accutase (Innovative Cell Technologies Inc., AT104) at 37° C for 45-60 min followed by gentle pipetting to dissociate the BMDM cells fully. Culture plates were rinsed with additional Culture medium (RPMI + 10% FBS + 1x P/S) and the cell suspension was centrifuged at 600 x g for 3 min at 4° C. The resulting supernatant was aspirated and cells were resuspended with cold Staining Buffer (HBSS (-Ca -Mg) + 5 mM EDTA + 20 mM HEPES + 2% FBS). Cells were centrifuged again at 600 x g for 3 min at 4° C followed by aspiration of the supernatant and resuspension in 500 µL cold Staining Buffer containing Fc Block (Purified anti-mouse CD16/32 Antibody, BioLegend, 101301) at a final concentration of 1 µg/mL. Fc Block was incubated with the BMDM cells for 10 min on a rotisserie at 4C. Cells were divided into 100,000 cell aliquots before staining with Propidium Iodide (10 µg/mL), APC-anti-mouse CD206 antibody (5 µg/mL) (Biolegend, 141707), and BV 421 anti-mouse F4/80 antibody (2 µg/mL) (Biolegend, 123131). Staining was carried out at 4C in the dark on a rotisserie for 20 min. Cells were washed with 1 mL cold Staining Buffer and centrifuged at 600 x g for 3 min at 4° C before

discarding the supernatant. Cells were washed twice with 1 mL Cold Staining buffer and centrifuged each time at 600 x g for 3 min at 4C before being resuspended in cold staining buffer before being filtered through a 100 µm filter prior to FACS analysis.

Treatment of BMDM

For treatment of BMDM with transfected Man9-siRNA, 25 nM Man9-siRNA targeting GAPDH mRNA was complexed with lipofectamine RNAiMAX transfection reagent (Invitrogen, 13778030) in 100 µL Opti-MEM transfection media (ThermoFisher Scientific, 31985070) at RT for 30 min. The lipofection suspension was added dropwise to BMDM in 400 uL Optimem in a 24 well plate. Treatments were left on the cells for 72 hr before GAPDH mRNA knockdown was assayed by qRT-PCR.

For mannose-TRC knockdown, BMDM cells were washed 2x with PBS followed by addition of 500 µL Optimem media. To each well, Mannose-TRC or control treatment was added following dilution in 10 µL of Opti-MEM media. Treatments were left on the cells for 72 hr before GAPDH mRNA knockdown was assayed by qRT-PCR. For statistical analyses, data are expressed as mean ± standard deviation, as indicated, and compared by Student's t-test. Statistical significance was assigned at $P < 0.05$.

Mannose Binding Studies

48 hours post IL-4 polarization, BMDM cells were removed from a 24 well plate using Accutase and 125,000 cells were treated with PBS control or 1, 3, 10, 33, 100, or 333 nM Cy3-labeled mannose ligand in 50 µL of Staining Buffer (see cell staining methods) at 4° C in the dark on a rotisserie for 90 min. Mannose ligands include Mannose-3-azide and Mannose-9-azide conjugated to a Cy3-DBCO (Click Chemistry Tools, A140), and Mannose-3-siRNA-Cy3, Mannose-3-siRNN-Cy3, Mannose9-siRNA-Cy3, and Mannose9-siRNA-Cy3 labeled with a Cy3 phosphoramidite on the Guide strand of the duplexed oligonucleotide. Cells were washed 2x

with Staining buffer and run on a LSR II for FACS analysis. For binding competition studies, BMDM cells were preincubated with PBS control, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 mg/mL mannan (from saccromyces cerevisiae, Sigma, m7504) for 30 min prior to 333 nM Mannose-Cy3 ligand treatment.

Mannose-TRC Internalization Studies

48 hr post IL-4 polarization, 125,000 BMDM cells were treated in a 48 well plate with 1, 5, 10, or 100 nM Mannose-3-Cy3, Mannose-9-Cy3, Mannose-3-siRNA-Cy3, Mannose-3-siRNN-Cy3, Mannose-9-siRNA-Cy3, Mannose-9-siRNN-Cy3, or control PBS control treatment for 2-3 hr at 37° C to allow for cell binding and internalization. BMDM cells were then removed from the plate using trypsin and proteinase K (Fischer, BP1700100) to remove all membrane protein bound ligand. Cells were washed with cold Staining Buffer and centrifuged at 600 x g for 3 min at 4° C prior to suspension in Staining buffer and FACS analysis. For binding competition studies, BMDM cells were preincubated with PBS control, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 mg/mL mannan for 30 min prior to 10 nM Mannose-Cy3 ligand treatment.

Mannose-TRC Toxicity Assay

BMDM cells were treated with 1nM, 10nM, 100nM, 1μM, and 10 μM nM Man9-siRNA or Man9-siRNN in RPMI + 10% FBS + 20 ng/mL M-CSF in a 48 well plate (125,000 cells/well). Cells were trpysinized at 24 and 48 hr and incubated at RT with (0.05 mg/mL) PI for 30 min before FACS analysis. Dead BMDM cell control was established by heating BMDM cells to 65^{o} C for 5 min prior to staining with PI. Cell Viability was determined by FACS forward and side scatter analysis.

Activated Peritoneal Macrophage Model

For *in vivo* mannose-TRC knockdown studies, randomly chosen 10-12-week-old female C57B/6 mice (Envigo) were injected intraperitoneally with 3.85% Brewers thioglycollate (80 µL/g body weight) (VWR/GE Healthcare, 90000-294). After 3 days, Mannose-9-TRC (5 mg/kg), GalNAc-9-TRC (5 mg/kg), and siRNA (5 mg/kg) targeting mGAPDH or Luciferase was administered intraperitoneally in 250 µL saline. Treatments were repeated on day 4 (total dose 10 mg/kg, each). 5 days after thioglycollate injection, mice were sacrificed and activated peritoneal macrophages were collected by peritoneal lavage with 2 x 5 mL cold Harvest Buffer (HBSS w/o Ca and Mg + 5 mM EDTA + 20 mM HEPES). For FACS sorting, activated peritoneal macrophages were stained as described for BMDM staining and sorted on a BD FACS Aria II by CD206+/F4/80+. Cells were enriched for the top 50% CD206+ population. GAPDH knockdown was assayed by qRT-PCR. For statistical analyses, data are expressed as mean ± standard deviation, as indicated, and compared by Student's t-test. Statistical significance was assigned at $P < 0.05$. All animals were maintained, treated, and euthanized in accordance with the UCSD Institutional Animal Care and Use Committee.

Figure 2.5. Antibody Expression Plasmid Maps.
Representative plasmid maps for heavy chain (Upper) and light chain (Lower) production. Heavy chain variable regions (HCv) were cloned into both human heavy chain IgG1 and IgG4 heavy chain constant (HCc) regions. Light chain variable regions (LCv) were cloned into both kappa (κ) and lambda (λ) human light chain constant (hLCc) regions. CMV promoters drive expression of heavy and light chains with BGH polyA tails.

Antibody Production

Amino acid sequences for the variable regions of antibodies targeting CD33, PSMA, EGFR, HER2 and Transferrin Receptor (CD71) were collected and codon optimized for the ExpiCHO expression system. Variable regions were cloned into a pcDNA3 backbone into both κ or λ light chains and IgG1 or IgG4 heavy chain subtypes. Microbial transglutaminase recognition sequences (LLQGA) were cloned onto the C-terminus of LCs and HCs to provide a site-specific conjugation site. Antibody expression was driven from a Cytomegalovirus (CMV) promoter and BGH polyA signals were used on the 3'-end of the heavy and light chain transcripts. Heavy chain signal peptide: MEFGLSWVFLVALFRGVQC. Light chain signal peptide: MDMRVPAQLLGLLLLWLSGARC. Cloning was carried out using the InFusion HD Cloning System (Takara Bio USA, 639645). Antibodies were produced using the ExpiCHO Expression system (ThermoFischer) following manufacturer protocols. Heavy chain (HC) and light chain (LC) plasmids were transfected into the ExpiCHO cells in a 2:1 HC:LC ratio.

Microbial Transglutaminase (MTG) Antibody Conjugations

For all antibody conjugations, MTG reactions were carried out at a ratio of 50:1 Lysine peptide: LLQGA site (1 mg/mL antibody, 666 μM peptide). Reactions were carried out in 162.5 mM NaCl, 12.5 mM (+)-Sodium L-Ascorbate, 25 mM Tris-HCl, pH 8 at 37C for 1 hr. Conjugates were purified by FPLC using an Enrich-SEC 650 10/300 column (Biorad, 780-1650) using PBS at a flow rate of 1.8 mL/min. Fractions were checked by reducing SDS-PAGE electrophoresis and pooled before concentration and solvent exchange to 1x PBS using regenerated cellulose 30K amicon ultra spin filtration cartridges (Millipore, UFC503024). Concentration was checked by BCA assay and peptide conjugated mAbs were stored at -20^{o} C for further use.

Antibody Binding Assay

For antibody binding studies, Flourescein-KAYA peptide was conjugated to an αCD33 antibody using MTG as described. 50,000 THP-1 or Jurkat cells were incubated with increasing concentrations of αCD33- Flourescein antibody for 20 min at 4C in RPMI before being washed with 0.1% sodium azide in PBS and analyzed for Flourescein signal.

ARC DBCO Conjugations

For all DBCO-Oligonucleotide conjugations, oligonucleotides bearing 5'-terminal DBCO-TEG modifications (Glen Research, 10-1941-90) were duplexed, frozen, and lyophilized before being resuspended in PBS solution containing antibody-peptide-azide conjugate (>1mg/mL) + 40 mM arginine-HCl. Reactions were carried out at a 5:1 ratio (DBCO:azide) at 37 C for 1 hour. For oligonucleotides modified with additional groups/peptides, HyNic conjugations and purification was carried out prior to oligonucleotide duplex and DBCO conjugation. Oligonucleotide-DBCO-Peptide-Antibody (ARC) conjugates were purified by FPLC using an Enrich-SEC 650 10/300 column using 10% isopropanol/90% water at a flow rate of 1.8 mL/min. Fractions were checked by reducing SDS-PAGE electrophoresis and pooled before concentration and solvent exchange to 1x PBS using 30K amicon ultra spin filtration. Concentration was checked by BSA Bradford assay and ARCs were stored at -20C for further use.

ARC Cellular Transduction

A431-dGFP cells were plated in a 48 well plate at 50,000 cells/well in 150 µL complete DMEM (10% FBS, Pen/Strep). After 24 hr, media was aspirated and replaced with purified αEGFR-ARC-siRNN-3xnGM, αEGFR-antibody, or siRNN-3xnGM targeting GFP diluted in complete DMEM + 50 µg/mL Gentamicin (Gold Biotechnology, G-400-1). At day 1-3, cells were assayed for GFP knockdown by FACS analysis.

ARC Preclinical Animal Models

For *in vivo* antibody distribution studies, randomly chosen 28-week-old female NRG mice (Jackson Laboratory) were injected subcutaneously in each flank with 2 x 10^6 A431-Luc cells in a volume of 100 µL HBBS buffer. After 20 days, mice with tumor volume of 300 mm^3 were injected intravenously with 23 µg purified αCD33 or αEGFR antibody-IR800 conjugates suspended in 100 µL PBS. 24 hr post injection, mice were euthanized and their organs harvested and imaged for IR800 fluorescence (PerkinElmer, IVIS Spectrum). All animals were maintained, treated, and euthanized in accordance with the UCSD Institutional Animal Care and Use Committee.

For *in vivo* ARC distribution studies, randomly chosen 9-10-week-old female Nu/nu mice (Jackson Laboratory) were injected subcutaneously in each flank with 2.5 x 10^6 THP-1-dGFP-Luc cells in a volume of 100 µL 1:1 Matrigel:PBS. After 23-40 days, d-Luciferin (150 mg/kg) was administrated intraperitoneally and luciferase expression was monitored by live-animal imaging (PerkinElmer, IVIS Spectrum). After luciferin injection animals were anesthetized with isoflurane, placed in the IVIS imaging chamber, and kept under isoflurane anesthesia during imaging. Bioluminescent images were acquired for 10-20 min after luciferin injection. Following imaging, mice were returned to their cages for recovery. Mice with tumor volumes of 250 mm^3 were injected intravenously with 54 µg of purified αCD33 ARC-siRNA-IR800, or ARC-siRNN-IR800 in 100 µL PBS, or PBS only (Mock). Mice with tumor volumes of 250 mm^3 were injected intraperitoneally with 100 µg of purified αCD33 antibody-IR800 conjugate in 1.5 mL PBS or, PBS only (Mock). IR800 signal and luciferase expression were monitored daily, as described, by IVIS Spectrum imaging. Blood samples were collected by puncturing the superficial temporal vein with a 4 mm lancet. Blood was stabilized against coagulation by addition of sodium citrate to a final concentration of 0.01 M before centrifugation at 3,000 x g for 20 min at 4^o C. Plasma was removed from the pelleted blood samples and stored at -80 C prior to analysis by SDS

PAGE. 7 days post treatment, mice were euthanized and their organs harvested and imaged for IR800 fluorescence.

For *in vivo* ARC knockdown studies, randomly chosen 9-week-old female Nu/nu mice (Jackson Laboratory) were injected subcutaneously in each flank with 2.5 x 10^6 THP-1-dGFP-Luc cells in a volume of 100 µL 1:1 Matrigel:PBS. After 24-28 days, d-Luciferin (150 mg/kg) was administrated intraperitoneally and luciferase expression was monitored as described by live-animal imaging. Mice with tumor volumes of 250 mm^3 were injected intraperitoneally with 0.21 mg/kg ARC-siRNA-3xnGM or 0.12 mg/kg ARC-siRNA-3xnGM conjugate in 1 mL PBS or, PBS only (Mock). siRNA and siRNN sequences were targeting Plk1 mRNA. Luciferase expression was monitored daily, as described, by IVIS Spectrum imaging. Tumor volume was monitored daily by caliper measurement (tumor volume = (Width^2 x Length)/2).

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CHAPTER 3

TARGETING M2 MACROPHAGES WITH MANNOSE-siRNA CONJUGATES

Targeting M2 Macrophages with Mannose-siRNA Conjugates

ABSTRACT

Tumor associated macrophages (TAMs) promote an immunosuppressive tumor environment, increased angiogenesis and metastasis, limit the efficacy of various forms of anti-cancer therapies, and correlate strongly with reduced patient survival in a variety of solid tumors. Given their abundance within the tumor and their role in tumor progression and patient survival, TAMs represent an attractive target in the treatment of cancer. Short interfering RNA (siRNA) therapeutics offer tremendous potential to target the immunosuppressive and tumor promoting pathways in TAMs. However, the chemical properties of siRNA limit its bioavailability and necessitate a targeting domain to deliver the siRNA therapeutic and to escape the endosome. Targeting of TAMs with multivalent mannose has been demonstrated clinically with Tilmanocept, a multivalent mannose ligand that binds and labels M2 macrophages via CD206 binding. CD206 is highly overexpressed on the surface of TAMs compared to other tissues and is internalized continuously, providing a route for siRNA delivery. Addition of mannose targeting and endosomal escape domains requires multifunctional, site-specific conjugation schemes for the construction of well-defined, monomeric siRNA therapeutics. Previous work in our lab provides a flexible and robust platform for modifying the phosphate backbone of the siRNA to create short interfering ribonucleic neutrals (siRNN). These modifications can be designed to include conjugation sites that can be placed anywhere along the siRNN molecule. Here I describe site-specific, multifunctional, and multivalent conjugation strategies for the construction of targeted siRNA conjugates (TRC) capable of self-delivery and induction of an RNAi response in a model of TAMs.

INTRODUCTION

Tumor associated macrophages (TAMs) consist primarily of alternately polarized macrophages (M2) (Mills et al., 2000), and are a major cellular component of both mouse and human tumors (Pollard, 2004; Lewis and Pollard, 2006; Qian and Pollard, 2010). Macrophages are differentiated into the M2 phenotype following IL-4 and IL-13 exposure, leading to up regulation of anti-inflammatory IL-10, down regulation of pro-inflammatory cytokines, and up regulation of pathways that suppress adaptive immune responses (Biswas and Mantovani, 2010; Sica and Mantovani, 2012). M2 macrophages also have poor antigen presenting capabilities and effectively suppress T-cell activation (Mills et al. 2000). Immune suppression is mediated in part by expression of programmed cell death ligand 1 (PD-L1), PD-L2, CD80, and CD86 that bind to immune checkpoint receptors programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), inhibiting the activity of CD8+ T cells (Noy and Pollard, 2014; Mantovani et al., 2017).

In humans, several meta analyses have revealed that high levels of TAMs correlate with reduced patient survival in a variety of solid tumors (Bingle, Brown, and Lewis, 2002; Zhang et al., 2012; Guo et al., 2016; Mei et al., 2016; Yin et al., 2017; Zhao et al., 2017). TAMs promote tumor progression through secretion of pro-angiogenic factors VEGF-A and TIE2 (Lewis, Harney, and Pollard, 2016; Williams, Yeh, and Soloff, 2016; Zhou et al., 2015; Hambardzumyan, Gutmann, and Kettenmann, 2016; Noy and Pollard, 2014; Matsubara et al., 2013). TAMs induce transient openings in tumor neovessels, promoting distant metastases from solid tumors (Kitamura, Qian, and Pollard, 2015; Lewis, Harney, and Pollard, 2016; Pollard, 2004; Robinson et al., 2009). TAMs also limit the efficacy of various forms of anti-cancer therapies (De Palma and Lewis, 2011; De Palma and Lewis, 2013) and surprisingly increase in number following chemotherapy, thereby contributing to relapse (Williams, Yeh, and Soloff, 2016; Kurahara et al., 2012; Hughes et al., 2015).

Given their abundance within the tumor and their role in tumor progression and patient survival, TAMs represent an attractive target for therapeutic modulation in the treatment of cancer. RNAi therapeutics offer the potential to modulate gene expression within the M2 TAMs. siRNA has an EC_{50} in the picomolar (10^{-12} M) range with exquisite target selectivity for all mRNA (Bumcrot et al., 2006). Due to the catalytic properties and mechanism of action of siRNAs, a single dose is capable of prolonged pharmacodynamic effects in non-dividing cells such as TAMs. These unique characteristics give siRNA the potential to target genes and pathways within TAMs that are currently difficult to treat or are undruggable with traditional small molecule therapeutics or antibodies. siRNAs can also be synthesized in a scalable manner, allowing for rapid production of siRNAs targeting any mRNA (Beaucage and Iyer, 1992).

Directly inhibiting factors responsible for TAM mediated immune suppression and tumor progression, including Arginase, IL10, and TGFβ, EGF, and VEGF represents an attractive strategy for cancer therapy using RNAi therapeutics (Mills and Ley, 2014; Kaneda, et al., 2016a; Kaneda et al., 2016b). Integrin α4β1 and its activator PI3Kγ inhibit antitumor immunity and stimulate tumor growth (Kaneda et al., 2016; Kaneda et al., 2016). Pharmacologic and genetic blockade of α4β1 and PI3Kγ in mouse models of pancreatic cancer resulted in dendritic cell maturation, inhibition of immunosuppressive myeloid cell polarization and restored antitumor T cell-mediated immunity, providing a model for potential RNAi therapeutic applications (Foubert et al., 2017).

In addition to RNAi monotherapy, siRNA may also improve existing therapies, including immune checkpoint inhibitors. Checkpoint inhibitors that reboot CD8+ T cells in tumors have seen dramatic success in some tumor types in the clinics with several checkpoint inhibitors gaining FDA approval (Farkona, Diamandis, and Blasutig, 2016; Khalil et al., 2016). Intravital microscopy of tumors following αPD1 antibody administration shows that while T cells are initially targeted, these therapeutic antibodies are transferred to TAMs within 24 hr. Fc-γ receptor (FcγR) blockade prolongs the binding of αPD1 antibodies to tumor infiltrating T cells

(Arlauckas et al., 2017). RNAi knockdown of FcγR in TAMs could effectively enhance αPD1 antibody treatments in responding patients.

Despite the potential of RNAi therapeutics to treat cancer by targeting immunosuppressive pathways in TAMs, siRNA is prevented from crossing the lipid bilayer due to both its size (~14 kDa) and 40x negative charges (Juliano, 2016; Dowdy, 2017; Khvorova and Watts, 2017). These attributes also make siRNAs pharmacokinetically highly unfavorable, as naked siRNA is removed from the bloodstream by the kidneys within minutes of injection into mice and humans (Merkel et al., 2009). Additionally, native (2'-OH) double stranded siRNAs are recognized as invading nucleic acids by multiple cellular defense mechanisms, including extracellular Toll-Like Receptors (TLR-3, -7, -8) and intracellular sensors, retinoic acid inducible gene (RIG-I) and melanoma differentiation associated protein 5 (MDA-5) (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017; Gantier and Williams, 2007; Iversen et al., 2013; Juliano et al., 2014). These difficulties necessitate the use of delivery agents to both assist siRNAs to cross the lipid bilayer and to remain in circulation for longer periods of time. Thus, the major obstacle prohibiting effective RNAi therapeutics has been delivery into the cytoplasm of cells. Consequently, there has been significant attention and investment of time and resources to address the delivery problem by harnessing and developing a wide array of technologies (Juliano, 2016; Dowdy, 2017; Khvorova and Watts, 2017).

To address these problems our lab developed small interfering ribonucleic neutrals (siRNNs) containing neutralizing phosphotriester groups. This technology was based on an HIV mononucleoside prodrug inhibitor containing a bioreversible *t*-butyl-S-acyl-2-thioethyl (*t*Bu-SATE) neutralizing the phosphate (Lefebvre et al., 1995; Gröschel et al., 2002). These neutralizing phosphotriesters are converted into charged phosphodiesters by intracellular restricted thioesterases to yield a wild type siRNA that can be loaded into RISC to induce and RNAi response. This technology also increases serum stability >24 hr, prevents innate immune stimulation, and increases *in vivo* circulation time (Meade et al., 2014). The *t*Bu-SATE

technology is also amenable to modification, allowing for modulation of solubility and addition of conjugation handles for site selective addition of PTDs and other cargos.

Despite the pharmacologic improvements made to siRNA through the addition of charge neutralizing phosphotriester groups, the siRNN molecule is unable to self deliver into the cytoplasm of the cell, a problem that the entire siRNA therapeutics field faces. Conjugation of *tris*-N-acetalgalactosamine (GalNAc) targeting hepatocyte asialoglycoprotein receptor (ASGPR) has shown effective long term knockdown of target mRNA in a dose dependent manner and is currently the state of the art in the field of RNAi therapeutics (**see Chapter 1**) (Meade et al., 2014). The success of GalNAc targeting and delivery of siRNNs demonstrated the potential for this technology to affect robust RNAi activity. However, despite the success of GalNAc targeted siRNNs in our lab and GalNAc-siRNA conjugates in the clinic, targeting extra-hepatic tissues remains a major obstacle to the field of RNAi therapeutics.

In order to effectively target siRNA therapeutics to all tissues, targeting domains and ligands must meet three criteria:

1) A receptor or extracellular target must be highly expressed on the cell type of interest to deliver a sufficient payload of siRNA. Low abundance receptors provide comparatively few opportunities for delivery and make the challenge of RNAi therapeutics more difficult.

2) The receptor or target of interest must internalize at as high a rate as possible. A receptor with low internalization results in rapid receptor saturation and subsequent clearance of unbound circulating RNAi therapeutic, requiring more frequent dosing or modified dosing methodology. Conversely, high internalization rates can make up for relatively low numbers of target receptor and provide more opportunity for cytoplasmic delivery.

3) The receptor or target of interest must be selective for or highly overexpressed on the target tissue compared to off-target tissues. Targeting a receptor that is present

on all cell types results in non-specific systemic delivery of the siRNA, effectively sequestering much of the therapeutic in non-productive tissues.

M2 macrophages express high levels of CD206 (mannose receptor, MR) on their cell surface (Luo et al., 2006; Mantovani et al., 2002). CD206 contains eight C-type lectin domains (CTLDs) that bind glyco-conjugates terminated in mannose, fucose, or GlcNAc in a Ca^{2+} dependent manner (Taylor et al., 1992; Taylor et al., 2005). Multiple CTLDs are needed for high affinity binding as single CTLDs retain weak sugar binding in isolation (Taylor et al., 1992; Taylor and Drickamer, 1993). Multivalent mannose ligands show increasing affinity for CD206 and can achieve binding in the range of Kd = 10^{-11} nM (~11.2 average mannose valency) (Azad et al., 2015). CD206 is a highly effective endocytic receptor, recycling constantly between the plasma membrane and the early endosomal compartment, with only 10-30% of the total cellular CD206 population present on the cell surface at a time (Azad et al., 2015; Gazi and Martinez-Pomares, 2009).

Several methods for labeling M2 macrophages using CD206 targeting have been utilized and are in development, including an αCD206 nanobody (Movahedi et al., 2012; Blykers et al., 2015), and several small peptides (Jaynes et al., 2015;Scodeller et al. 2017). Targeting of CD206 with multivalent mannose is a strategy that has received FDA approval in the form of Tilmanocept, a ^{99m}Tc-labelled multivalent mannose imaging agent for lymph node mapping (Azad et al., 2015). Analysis of Tilmanocept activity *in vivo* showed binding and labeling of sentinel lymph nodes within 10 min following submucosal or prostate injection, demonstrating the ability for rapid CD206 targeting in humans (Salem et al., 2006; Méndez et al., 2003). Given the proven clinical success of Tilmanocept, targeting CD206+ M2 TAMs with multivalent mannose ligands is a promising delivery strategy for siRNA therapeutics.

While mannose targeting of CD206 provides a strategy for cellular targeting and internalization, escape from the endosome remains an obstacle as the siRNA molecule cannot

cross the endosomal membrane. The current state of the art GalNAc-siRNA therapeutics escape the endosome in through an unknown mechanism that may not be replicated in all cell types. In order to enhance endosomal escape, addition of endosomal escape domains (EEDs) may be required. To this end, we have sought to incorporate multiple bioconjugation chemistries into the siRNN technology to produce multifunctional siRNN molecules capable of potent CD206 targeting and endosomal escape.

RESULTS & DISCUSSION

Site Specific Conjugation of Mannose Targeting Domains

To facilitate site-specific conjugation of a mannose targeting domain (TD), a phosphotriester with a terminal alkyne (Kx) was designed for copper catalyzed Click bioconjugation. Kx-modified phosphoramidites were synthesized by the same synthetic route used to produce *tBu*-SATE phosphoramidites (**Figure 2.1**), but with an irreversible 1-hexyne group in place of the *t*Bu-SATE modification (**Figure 3.1A)**. Kx phosphotriesters were efficiently incorporated into the siRNN under standard solid state oligonucleotide synthesis conditions and tolerated ultra-mild (10% DIA, 90% MeOH, anhydrous, RT, 4 hr) and standard (NH4OH, H2O, 65 C, 2 hr) primary deprotection conditions. Final yields of oligonucleotides with a single and multiple Kx phosphotriester insertions were similar to those of 2'-mod oligonucleotides.

As previously noted, phosphotriesters are susceptible to nucleophilic attack by the ribose 2'-OH resulting in strand cleavage. Therefore, for stable insertion of *t*Bu-SATE as well as Kx phosphotriester groups, modification of the 2'-position is a requirement. For this reason, all siRNA and siRNN oligonucleotides synthesized and discussed here are fully modified with 2'-F pyrimidines and 2'-OMe purines (**Figure 1.3A**). Fully 2'-modified siRNA is well tolerated by the RNAi machinery and has become an industry standard for all therapeutic siRNAs (**See Chapter 1**). Additionally, all siRNA sequences discussed here contain two stabilizing phosphorothioate backbone modifications on each of the 5'- and 3'-ends of the Passenger and Guide strands.

Induction of RNAi by siRNNs containing *t*Bu-SATE phosphotriesters relies on the bioreversible properties of the S-acyl-2-thioethyl linkage for conversion to wild type phosphodiesters. However, use of irreversible phosphotriesters is tolerated at the 5'- and 3'-terminal ends of the Passenger strand and the 3'-end of the Guide strand and maintains RNAi activity (Meade et al., 2014a). Addition of TDs, including GalNac, by conjugation through a 5'-terminal irreversible phosphotriester maintains RNAi activity *in vivo*. Similarly, insertion of irreversible Kx phosphotriester groups on the terminal end did not interfere with RNAi activity following lipofection into cells (**Figure 3.1F)**.

Conjugation through the Kx phosphotriester group was carried out at a ratio of 10:1 TD to Kx phosphotriester. Conjugation of multivalent *tris*-mannose (Man3) and 9-mer mannose (Man9) proceeded to >90% completion within 45 min at 65 C or 1 hr at RT (**Figure 3.1 B-D**). Conjugation of mannose and non-targeting control GalNAc (GN3 and GN9) to oligonucleotides with two Kx phosphotriesters resulted in >90% of the final product bearing two TDs (**Figure 3.1E)**. Insertion of two Kx phosphotriesters clustered on the 5'-end or single insertions on the 5'- and 3'-ends of the Passenger strand resulted in similar conjugation efficiencies, suggesting that the first conjugation of a TD does not interfere with the second conjugation site. Ratios of TD to Kx conjugation site varied slightly between TD batches, requiring 15:1 TD to Kx phosphotriester ratios in some cases.

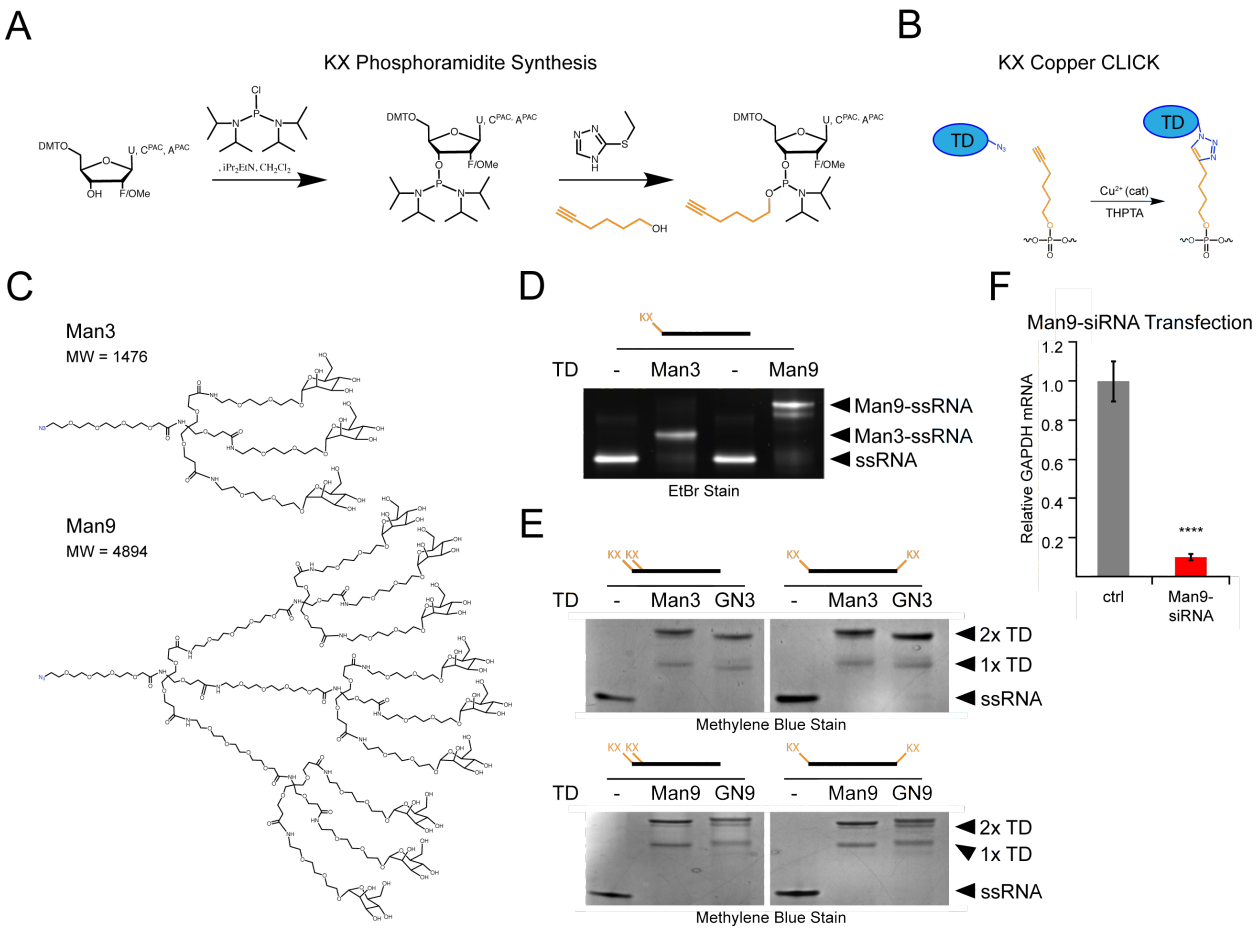


Figure 3.1. Copper Click Conjugations and Mannose Targeting Domains.
A) Synthetic routes for Kx U, C, and A phosphoramidites. **B)** The copper catalyzed CLICK reaction between azide modified targeting domain (TD) and an alkyne functionalized siRNN results in the formation of a stable triazole linkage. The reaction occurs readily in aqueous buffers with Cu^{2+}, (+) sodium-L-ascorbate, and tris-hydroxypropyltriazolylmethylamine (THPTA). **C)** Structures of trimeric mannose (Man3) and 9-mer mannose (Man9) TDs. **D)** Conjugation of Man3 and Man9 TDs to a single stranded RNA (ssRNA) oligonucleotide containing one Kx phosphotriester. 0.2 nmol of each sample analyzed by urea denaturing PAGE visualized by ethidium bromide staining (EtBr). **E)** Conjugation of Man3 and Man9 TDs and non-targeting control trimeric GalNAc (GN3) and 9-mer GalNAc (GN9) to ssRNA oligonucleotide containing two clustered Kx (Left) or two opposing Kx (Right) phosphotriesters. Urea PAGE analysis visualized by methylene blue stain. **F)** qRT-PCR analysis of BMDM cells treated with Man9-siRNA lipofection targeting GAPDH at 72 hr (25 nM; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (ctrl) group. Error bar indicates s.d. ****$P < 0.0001$; two-tailed Student's *t*-test.

Conjugation of mannose and GalNAc domains through a Kx phosphotriester resulted in a well defined targeted siRNA conjugate (TRC). However, purification of the TRC is necessary to remove excess unconjugated mannose TD that may compete for CD206 binding and reduce TRC delivery. HPLC purification of multivalent mannose-siRNN conjugates resulted in reduction of free Man9 from Man9-siRNA or Man9-siRNN as determined by MALDI-TOF analysis, though residual Man9 could not be entirely removed. Due to the difference in size of the free TD (1,476-4,894 Da) (**Figure 3.1C**) and the mannose TRC (~8,500-12,000 Da), size exclusion chromatography (SEC) was explored for purification. SEC spin column purification resulted in final yields >80% but poor removal of excess TD by MALDI-TOF analysis. The poor purification by spin column SEC may have been due to the small resin volume as purification was improved with FPLC SEC purification. SEC FPLC purification resulted in 70-90% yield and improved purification away from excess TD, though residual TD remains even following repeat SEC purification.

With this, we have demonstrated efficient synthesis and incorporation of Kx phosphotriester groups at several positions along the siRNN Passenger strand without alteration of RNAi capabilities. Conjugation proceeded efficiently at low TD:Kx ratios and allowed for multiple Kx conjugations along the same Passenger strand. Purification by SEC FPLC allowed for removal of the copper Click reaction mixture and a majority of free TD. Residual free TD did not appear to inhibit the activity of mannose TRC and likely represents a small percentage of the starting material that is visible due to the sensitivity of MALDI-TOF mass spectrometry.

Binding and Internalization of Mannose Targeted siRNA Conjugates (TRC)

Rapid screening of large numbers of mannose TRCs requires a robust model that expresses high levels of CD206 and replicates the cellular biology of M2 macrophages for mannose TD binding and internalization. In order to facilitate *in vitro* screening we utilized a

bone marrow derived macrophage (BMDM) model that provides a homogenous macrophage population and recapitulates the M2 phenotype (Zhang, Goncalves, and Mosser, 2008). *In vivo*, IL-4 polarizes macrophages into a $CD206^{+}/F4/80^{+}$ M2 phenotype. Analysis of BMDM cells extracted from C57B/6 mice (12 weeks old) revealed >90% $F4/80^{+}$ polarization after 4 days of M-CSF exposure. Addition of IL-4 *in vitro* resulted in 10-fold induction of CD206 within 48 hr (**Figure 3.2A**). These results indicate that we have a M2 macrophage population that expresses high levels of our target CD206 receptor.

To test binding of multivalent mannose by BMDM cells *in vitro*, Man3 and Man9 TDs were conjugated to a cyanine dye (Cy3). Both Man3-Cy3 and Man9-Cy3 conjugates labeled BMDMs in a dose dependent manner (**Figure 3.2B**). Binding occurred in the majority of the BMDM population though high doses of TD-Cy3 revealed a subpopulation that binds CD206 10-15 fold lower than the majority of BMDM cells, suggesting a subpopulation of cells that bind mannose ligands at a significantly reduced capacity. Cy3 dyes are hydrophobic and can lead to nonspecific membrane association, independent of receptor-ligand binding. To ensure that the observed dose dependent increase in Cy3 signal was mediated by CD206, binding was competed with mannan, a mannose linear polymer and known CD206 ligand. Man3-Cy3 and Man9-Cy3 labeling of BMDM cells was reduced by mannan in a dose dependent fashion (**Figure 3.2C**). A small amount of residual Cy3 labeling remained at the highest doses of mannan indicating that there was some contribution from nonspecific Cy3 incorporation. Regardless, these results indicate that the observed increase in Cy3 signal was primarily mediated by CD206 specific binding of Man3 and Man9.

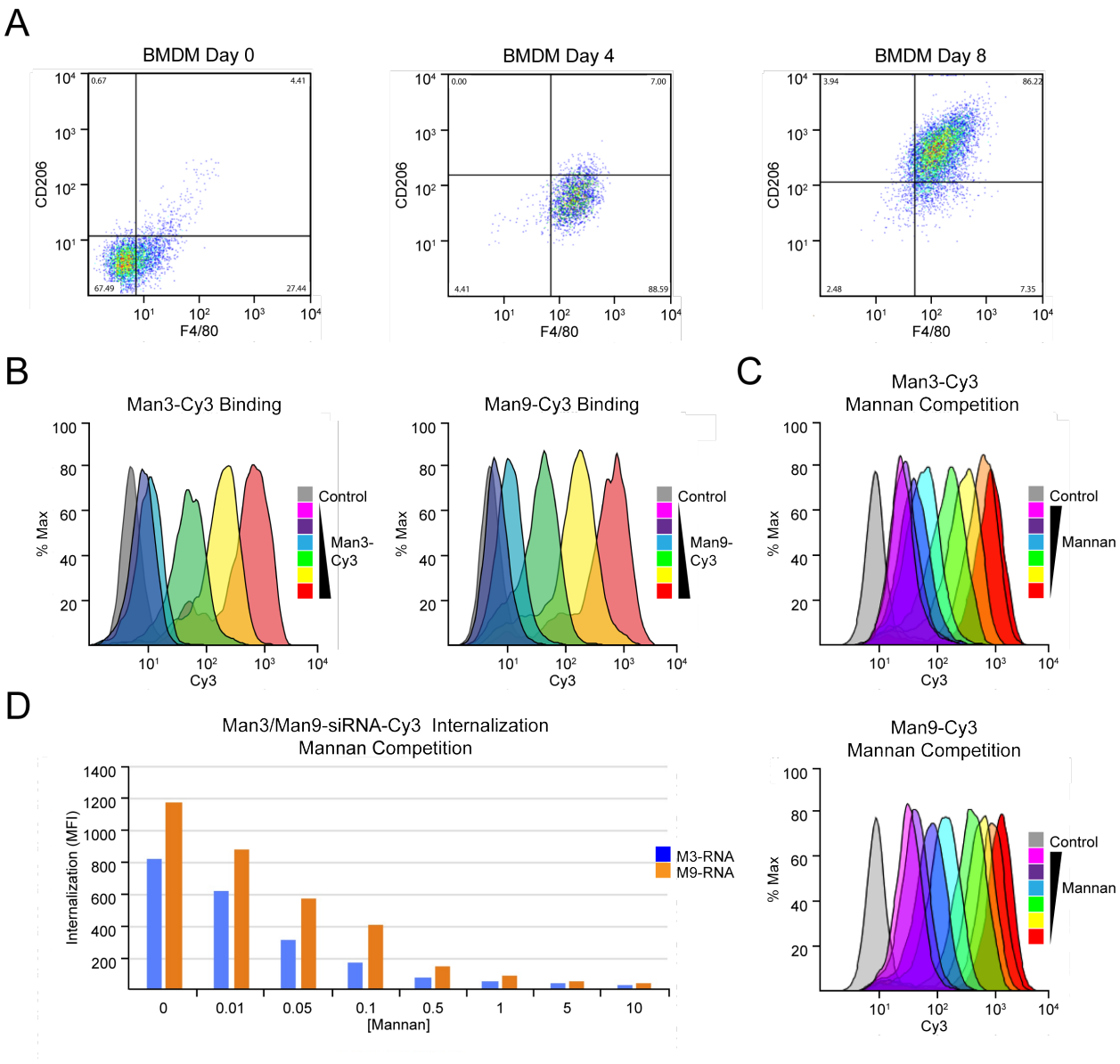


Figure 3.2. ***In Vitro*** **Mannose Binding and Internalization.**
A) FACS analysis of CD206 and F4/80 induction in bone marrow derived macrophages (BMDM) from C57B/6 mice. BMDM were in 40 ng/mL M-CSF for 4 days before M-CSF was reduced to 20 ng/mL. 20 ng/mL IL-4 was added to the media after 6 days to polarize BMDM into M2 phenotype. **B)** Binding assay of Man3-Cy3 (Left) and Man9-Cy3 (Right) treated BMDM cells. 0 nM (Grey), 1 nM (Pink), 3 nM (Blue), 10 nM (Teal), 33 nM (Green), 100 nM (Yellow), and 333 nM (Red). **C)** Competitive binding assay of Man3-Cy3 (Top) and Man9-Cy3 (Bottom) treated BMDM cells. BMDM were pretreated with indicated concentration of mannan followed by Man3-Cy3 or Man9-Cy3 treatment (333 nM). **D)** Competitive internalization assay of Man3-siRNA-Cy3 and Man9-siRNA-Cy3 treated BMDM cells. BMDM were pretreated with mannan followed by Man3-Cy3 or Man9-Cy3 treatment (10 nM).

While multivalent mannose bound to BMDM cells, productive delivery of RNAi therapeutics requires internalization of the target receptor and the bound TRC. To determine whether mannose-TRCs are internalized, BMDMs were treated with Man3-siRNA-Cy3 or Man9-siRNA-Cy3 for 4 hr before trypsin cleavage of the extracellular receptor population. Both Man3-siRNA-Cy3 and Man9-siRNA-Cy3 were effectively internalized at 10 nM (**Figure 3.2D**). Internalization was competed away by pre-treatment with excess mannan in a dose dependent fashion. Mannose-TRC internalization was robust with only a 25% reduction in internalized Cy3 signal following 0.01 mg/mL mannan competition. While precise quantification of mannan molarity is difficult without characterization or manufacturer specifications, a comparison of mannose species in solution reveals 0.01 mg/mL mannan represents a >60 fold excess of mannose for Man9-TRC and >180 fold excess mannose for Man3 TRC. This result may indicate that the dendrimeric orientation of our mannose TDs has a higher affinity for CD206 than linear mannose polymers or that the siRNA and siRNN may play an additional role in binding and internalization of the mannose TRCs. In either case, the residual mannose TDs in our purified TRCs did not appear to hinder binding or internalization in a significant way.

Targeting Bone Marrow Derived Macrophages (BMDMs)

To determine whether mannose-TRCs are able to self-deliver *in vitro*, BMDMs were treated with charged Man3-siRNA and neutral Man3-siRNN targeting murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and assayed for mRNA knockdown by qPCR. Treatment of BMDM with Man3-TRCs targeting GAPDH resulted in >50% knockdown of GAPDH mRNA while off target sequence control Man3-TRCs did not affect GAPDH mRNA levels, indicating that mannose-TRCs are capable of gene silencing in a sequence specific manner (**Figure 3.3A**). Comparison of Man3-siRNA and Man-9-siRNA with a non-targeting *tris*-GalNAc (GN3) or 9-mer GalNAc (GN9) siRNA resulted in robust mRNA silencing in mannose-TRCs and no significant change in GAPDH mRNA levels following GalNAc non targeting controls (**Figure**

3.3B). These results indicate that TRC induction of target mRNA silencing was mediated by mannose-TD delivery and not a non-specific effect such as phagocytosis.

Binding and internalization of mannose ligands by CD206 depends heavily on valency as previously demonstrated (**Figure 3.2**). To determine the effects of mannose valency on siRNA and siRNN delivery, dose curves were carried out comparing Man3- and Man9-TRCs. Dose dependent knockdown of target GAPDH mRNA occurred for both Man3- and Man9-TRCs (**Figure 3.3C**). Comparisons between charged Man3-siRNA and Man9-siRNA conjugates revealed greater knockdown in Man9-siRNA, with an EC_{50} = 20 nM and a maximum knockdown of ~80% at 50 nM. Similar results were seen in neutral Man3-siRNN and Man9-siRNN conjugates (**Figure 3.3D**). In both siRNA and siRNN delivery, Man9-TRC performed better than Man3-TRC, consistent with CD206 binding and internalization results (**Figure 3.2**). Comparisons between siRNA and siRNN knockdown revealed slightly greater knockdown for siRNA conjugates as compared to siRNN. This difference may have been a result of increased membrane association imparted by hydrophobic *t*Bu-SATE phosphotriester groups in the siRNN. Membrane association may sequester TD-siRNN away from CD206 at low concentrations, resulting in reduced delivery and knockdown.

To determine the cellular toxicity of the mannose-TRCs conjugates, BMDMs were incubated with increasing doses of Man9-siRNA or Man9-siRNN and assayed for membrane integrity and cell viability (**Figure 3.4A**). Membrane integrity was assayed by propidium iodide (PI) incorporation. Across all tested doses, Man9-siRNA had no detectable dose dependent loss of membrane integrity compared to PBS treated control at 24 or 48 hr. However, Man9-siRNN treatment revealed minor loss of membrane integrity in a large percentage of the BMDM population and an increase in the dead cell population at 10 μM. Cell viability revealed similar results, with no observable impact on cell viability after Man9-siRNA treatment, while Man9-siRNN treatment resulted in a 40% reduction in cell viability at 10 μM (**Figure 3.4B**). The increase in toxicity between siRNA and siRNN may have been a result of the increased

hydrophobicity and membrane association/disruption from the *t*Bu-SATE groups. It is unlikely that the observed toxicity was due to *t*Bu-SATE thioesterase enzymatic products as the thiirane ring and pivalic acid products have maximum tolerate doses >6,500 and >30,000 fold higher, respectively, than the highest therapeutic doses of siRNN conjugates (12 total t-Bu-SATE groups) (Wiley Reference Works, 2000.; Meade et al., 2014). Given these results and the previously described EC_{50} (**Figure 3.3**), our mannose TRCs demonstrated a therapeutic index >500.

Given that mannose valency affects binding, internalization, and knockdown in BMDMs, we sought to further investigate increased mannose valency as well as altered orientations of the mannose-TDs along the siRNA/siRNN backbone. siRNN passenger strands with two 5'-clustered Kx or two opposing 5'- and 3'- terminal Kx phosphotriesters were synthesized and conjugated to mannose and GalNAc TDs (**Figure 3.1E**). Treatment of BMDMs with Man3, clustered 2xMan3 (Man6a), and opposing 2xMan3 (Man6b) TRCs revealed greatly enhanced knockdown of target GAPDH mRNA, with both Man6a and Man6b orientations resulting in >80% knockdown of GAPDH mRNA at 10 nM (**Figure 3.5A**). Similar treatments with Man9, clustered 2xMan9 (Man18a), or opposing 2xMan9 (Man18b) TRCs resulted in only modest improvements in knockdown of GAPDH mRNA with Man18a-siRNA and Man18b-siRNA compared to Man9-siRNA (**Figure 3.5B**). These results indicate that increasing mannose valency beyond Man9 does not improve dosing or increase the magnitude of target mRNA knockdown. Comparisons between all mannose valencies and orientations revealed Man6a/b and Man9 as the most promising candidates for mannose-TD delivery of RNAi therapeutics *in vitro*. These results are consistent with binding data for multivalent mannose radiolabelling Tilmanocept ligand (Azad et al., 2015). Improvements in binding affinity plateau at ~11 mannose residues (average) while reduction to ~7 average mannose residues only reduces binding by 10%.

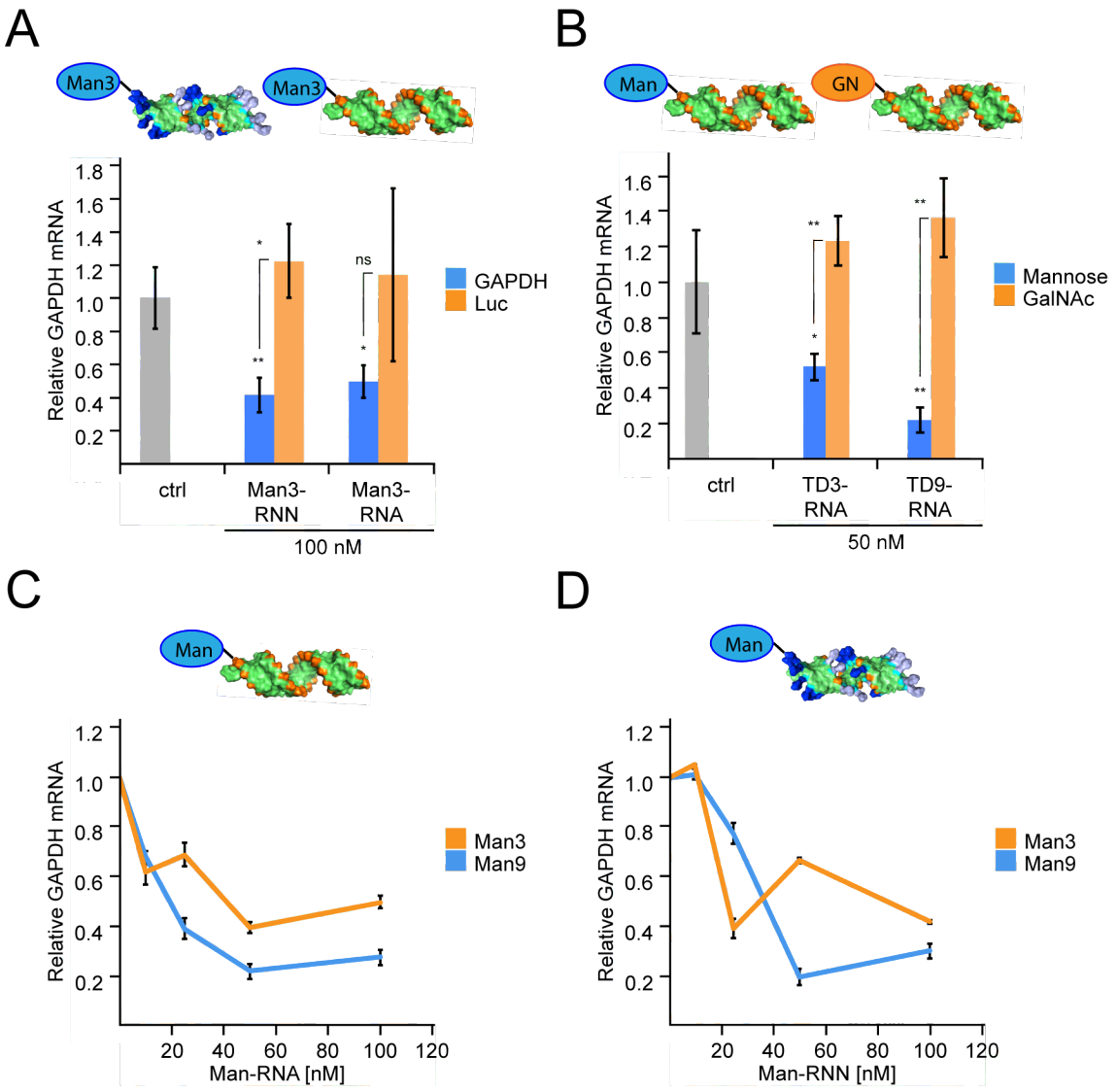


Figure 3.3. *In Vitro* Delivery of Mannose TRC.
A) *In vitro* administration into BMDM cells of charged Man3-siRNA and neutral Man3-siRNN targeting GAPDH and Luciferase (Luc) at 72 hr analyzed by qRT-PCR (100 nM; *n* = 2, each group). Values normalized to β2-microglobulin internal control and PBS treated control (ctrl) group. Error bar indicates s.d. *P<0.05, ***P* < 0.01; two-tailed Student's *t*-test. **B)** *In vitro* administration into BMDM cells of trimeric and 9-mer targeting domains (TD- mannose, GalNAc (GN)). TD3-siRNA and TD9-siRNA targeting GAPDH at 72 hr analysed by qRT-PCR (50 nM; *n* = 2 GN9-siRNA, *n* = 3 each group). Values normalized to β2-microglobulin internal control and PBS treated control (ctrl) group (*n* = 5). Error bar indicates s.d. *P<0.05, ***P* < 0.01; two-tailed Student's *t*-test. **C)** Dose curve comparison of charged Man3-siRNA vs. Man9-siRNA targeting GAPDH at 72 hr analyzed by qRT-PCR (10-100 nM; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group (*n* = 3). Error bar indicates s.d. **D)** Dose curve comparison of neutral Man3-siRNN vs. Man9-siRNN targeting GAPDH at 72 hr analyzed by qRT-PCR (10-100 nM; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group (*n* = 3). Error bar indicates s.d.

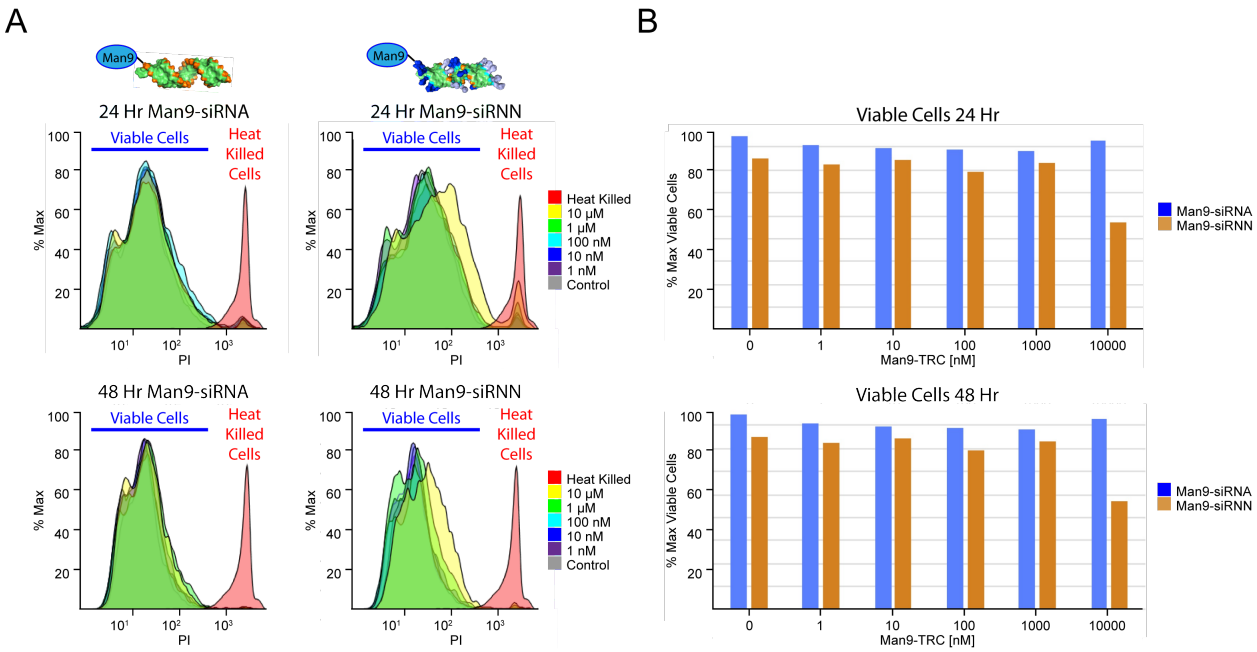


Figure 3.4. *In Vitro* Mannose Targeted RNA Conjugate Toxicity.
A) FACS analysis of membrane integrity in BMDM cells treated with charged Man9-siRNA or neutral Man9-siRNN at 24 and 48 hr. Membrane integrity assayed by FACS for propidium iodide (PI) uptake compared to live untreated (Control) and dead cell control (heat killed cells). **B)** *In vitro* analysis of BMDM cell viability following treatment with charged Man9-siRNA or neutral Man9-siRNN (Man9-TRC, collectively) at 24 and 48 hr. Viability determined by forward and side scatter FACS analysis.

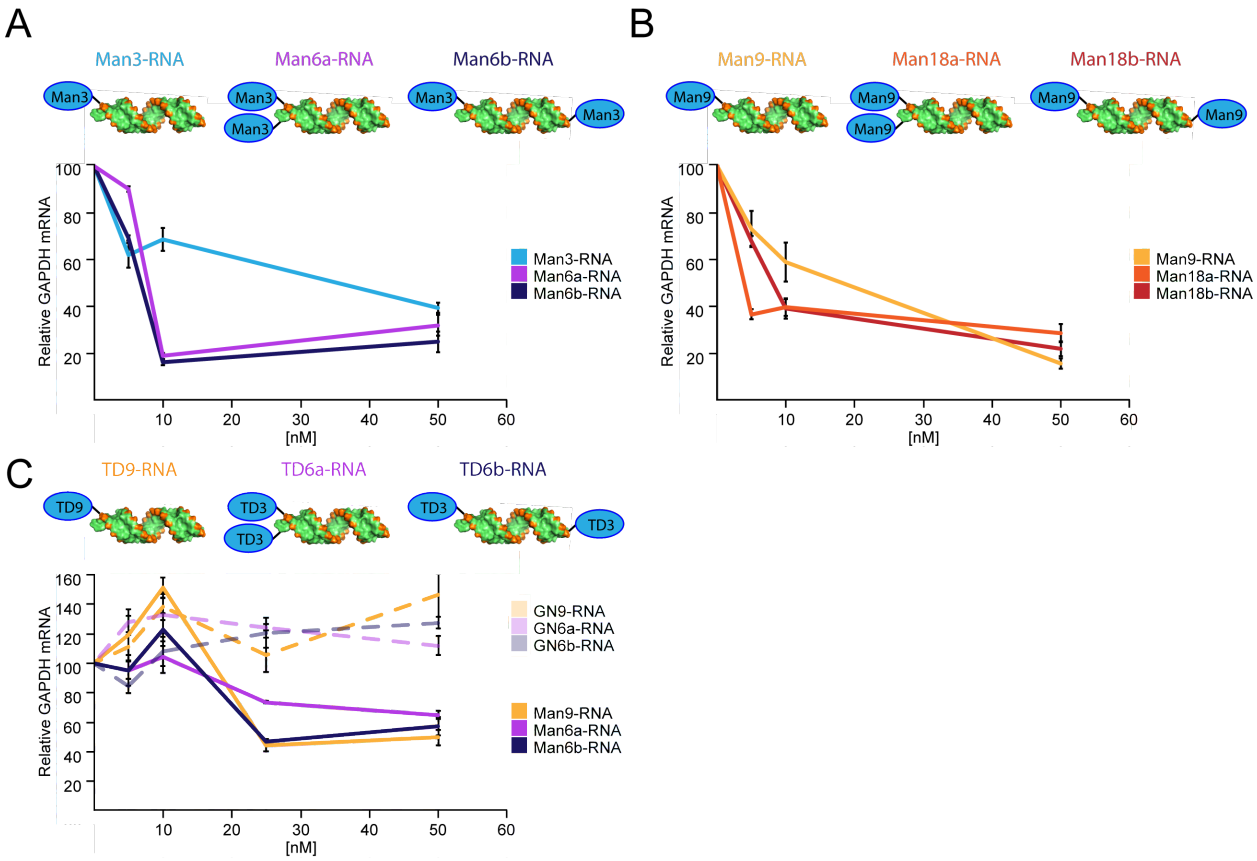


Figure 3.5. ***In Vitro*** **Multivalent Mannose Delivery.**
A) *In vitro* dose curve comparison of charged Man3-siRNA vs. clustered Man6a-siRNA vs. opposing Man6b-siRNA targeting GAPDH at 72 hr analyzed by qRT-PCR (5-50 nM; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group (*n* = 3). Error bar indicates s.d. **B)** *In vitro* dose curve comparison of charged Man9-siRNA vs. clustered Man18a-siRNA vs. opposing Man18b-siRNA targeting GAPDH at 72 hr by qRT-PCR (5-50 nM; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group (*n* = 3). Error bar indicates s.d. **C)** *In vitro* dose curve comparison of charged Man9-siRNA vs. clustered Man6a-siRNA vs. opposing Man6b-siRNA targeting GAPDH at 72 hr by qRT-PCR (5-50 nM; *n* = 3, each group). Knock down of GAPDH mRNA compared to non-targeting GalNAc controls (GN9-siRNA, GN6a-siRNA, GN6b-siRNA). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group (*n* = 3). Error bar indicates s.d.

To ensure that mannose delivery remained specific to CD206 targeting, Man9- and Man6a/b-siRNA conjugates were compared to their GalNAc non-targeting controls (GN9- and GN6a/b-siRNA). Consistent with previous results, knockdown of GAPDH mRNA remained specific for mannose targeting, with no knockdown observed in the GalNAc controls (**Figure 3.5C**).

Interestingly, the maximal knockdown was only 60%, compared to >90% for transfection (**Figure 3.1F**). Review of mannose binding data (**Figure 3.2B**) revealed a subpopulation that bound TD-Cy3 10-15 fold lower than the majority of the BMDM population. The reduction in maximal knockdown may have been due to this $CD206^{low}$ population within the BMDM cells that was poorly targeted by mannose-TRCs, but is efficiently transfected independent of CD206 expression. This $CD206^{Low}$ subpopulation artificially reduced maximal knockdown and represents an artifact of bulk RT-qPCR analysis, masking a potentially deeper knockdown in $CD206^{High}$ populations and highlighting minor differences between BMDM cell preparations.

Endosomal Escape Domains

For RNAi therapeutics, escape from the endosome represents the rate-limiting step in siRNA delivery. While multivalent mannose ligands successfully deliver their siRNA cargo into the cell to elicit an RNAi response, it is not known how these macromolecules are escaping the endosome into the cytoplasm. siRNAs have no bioavailability on their own and addition of neutralizing phosphotriesters to form siRNNs is insufficient to allow passage through the lipid bilayer due to the size of the siRNA/siRNN molecule (14-20 kDa) (See Chapter 1). With this in mind, whatever mechanism the mannose-TRC escapes the endosome by is likely very inefficient and affects only a small fraction of the overall mannose-TRC population. The addition of endosomal escape domains (EEDs) to the TD-siRNA molecule may increase the fraction of the siRNA population that escapes the endosome and reduce the therapeutic dose needed for induction of RNAi. To this end, we utilized an endosomal escape domain consisting of

hydrophobic GFWFG that was previously screened in the lab (EED1) and a branched version termed BEED (**Figure 3.6B**) (Lönn and Dowdy, 2015).

To facilitate conjugation of EEDs, we utilized a phosphotriester with a terminal benzaldehyde conjugation handle (Ax). Conjugation through an Ax phosphotriester was carried out with hydrazide modified peptides that react with the terminal benzaldehyde to form a stable bis-aryl hydrazone linkage (HyNic) linkage (**Figure 3.6A**). Despite the irreversible nature of the Ax phosphotriester group, previous work has shown that insertion at the 3'- and 5'- ends of the Passenger strand is well tolerated and does not alter induction of RNAi responses *in vitro* or *in vivo* (Meade et al., 2014b). HyNic conjugation of EED1 and BEED1 proceeded to >90% completion after 1 hr at RT (**Figure 3.6C-D**).

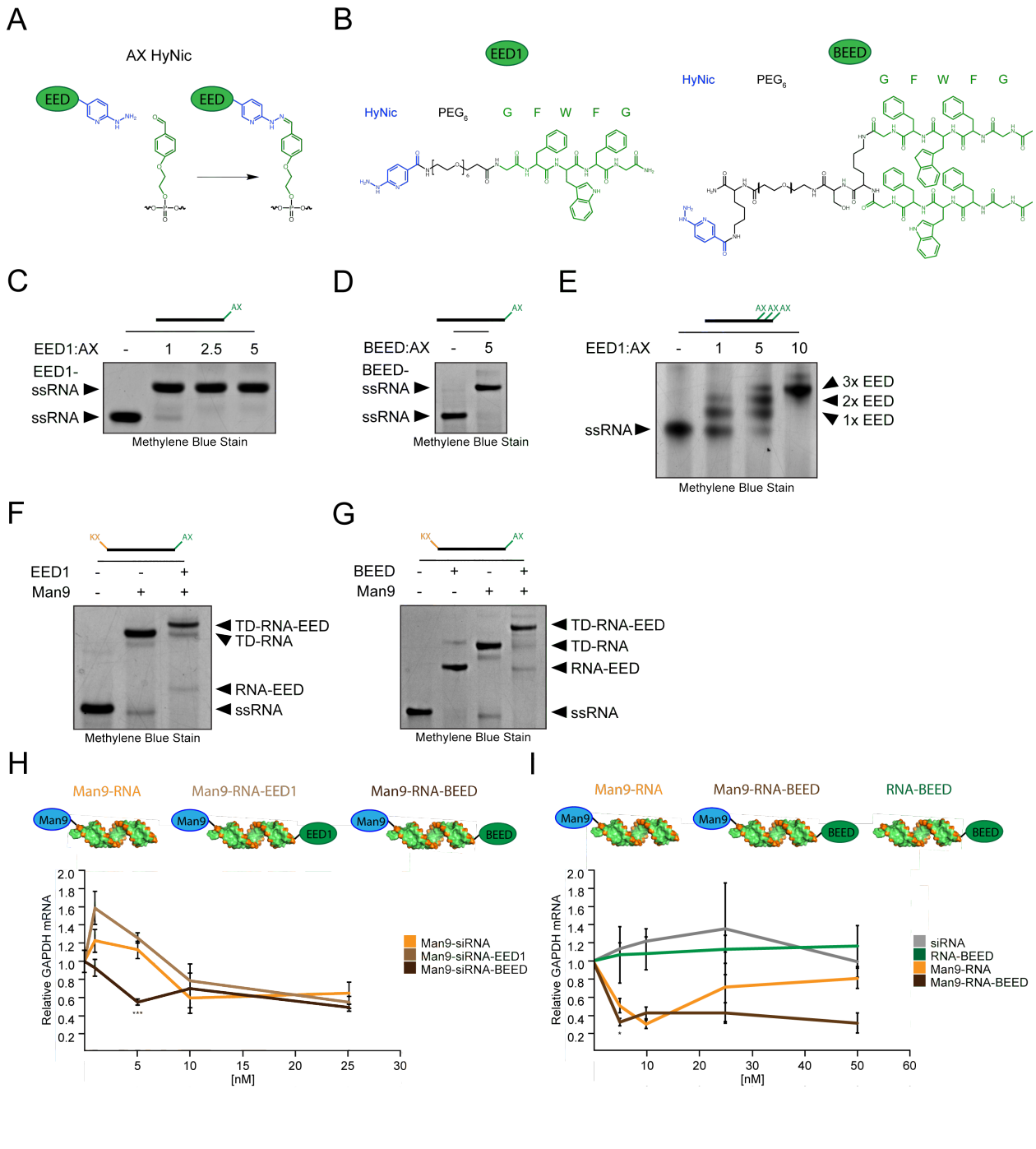
Many EEDs require high concentrations or coordination between several peptides to efficiently mediate endosomal escape (See Chapter 1). To enhance the endosomal escape properties of EED1 we sought to increase EED valency on the TRC through peptide branching and multivalent conjugation on the siRNN itself. Synthesis of a siRNN with three clustered Ax conjugation handles on the 3'-end of the Passenger strand allowed for multivalent conjugation of EEDs (**Figure 3.6E**). Modulation of valency through phosphotriester conjugation allows for modification to the number and placement of EEDs. Coupled with monomeric EED1 and branched BEED, valency can be modulated from 1-6 EED1 peptides per Passenger strand. However, due to the hydrophobic nature of EED1, multivalent conjugation did not yield a soluble product for TRCs with greater than 2 EED1 peptides. Despite the large anionic charge of the siRNA and siRNN, the clustering of so much hydrophobicity led to large-scale aggregates and precipitation, limiting the EED1 valency of soluble TRC-EED molecules to two.

Construction of multivalent and multifunctional RNAi therapeutics required multiple orthogonal, site-specific conjugations. The Kx and Ax phosphotriester modifications allow for Click and HyNic conjugation chemistries, respectively. These conjugation chemistries were unreactive and stable under the conjugation conditions of the opposing phosphotriester. This

allowed for sequential conjugation of a TD through a Kx Click reaction followed by a HyNic conjugation through an Ax phosphotriester to yield a multifunctional Passenger strand with both targeting and endosomal escape domains in defined and selective positions (**Figure 3.6F-G)**. Sequential conjugation could be carried out with Click or HyNIC in any order but required purification after each conjugation. Purification of the final TRC-EED was carried out by SEC FPLC and resulted in >99% excess EED removal from the final product. SEC separation of the final TRC-EED product revealed a monomeric conjugate, free of aggregates or particles.

To determine if hydrophobic EED1 was capable of improving the activity of Man9-siRNA, BMDM cells were treated with Man9-siRNA, Man9-siRNA-EED1 or Man9-siRNA-BEED. Man9-siRNA and Man9-siRNA-EED1 showed no significant difference in knockdown of GAPDH mRNA and reached a maximum knockdown of 40% by 10 nM. In contrast, Man9-siRNA-BEED resulted in significantly improved knockdown, shifting the dose curve to the left with a maximum response of 40% knockdown at 5 nM (**Figure 3.6H**). To ensure that the effect seen by the Man9-siRNA-BEED was not due to any non-specific effects of the BEED peptide, knockdown was compared between siRNA, siRNA-BEED, Man9-siRNA, and Man9-siRNA-BEED (**Figure 3.6I**). Treatment with siRNA and siRNA-BEED did not result in knockdown of GAPDH mRNA indicating that conjugation of endosomal escape BEED is not sufficient alone for delivery into cell. Both Man9-siRNA and Man9-siRNA-BEED resulted in robust knockdown of 50-70% of GAPDH mRNA at 5 nM. While the improvement in knockdown at 5 nM that BEED provided was reduced, Man9-siRNA-BEED treatment revealed significantly improved knockdown at 5 nM (*P<0.05)*, consistent with previous results. Together, these results demonstrated that addition of hydrophobic EEDs improve mannose-TRC delivery, potentially through enhanced endosomal escape. Valency appeared to play a role in hydrophobic EED activity, consistent with other EEDs (See chapter 1). However, this effect was modest and construction of soluble TRC-EEDs presents a limitation to EED valency without further modification of the siRNN.

Figure 3.6. Testing Endosomal Escape Domains *In Vitro*.
A) Schematic of the hydrazinonicotinic acid (HyNic) conjugation reaction between hydrazide-modified endosomal escape domain (EED) and a benzaldehyde functionalized phosphotriester (Ax) resulting in the formation of a stable bis-aryl hydrazone linkage. The reaction occurs readily in aqueous and organic solutions containing 1% aniline. **B)** Structure of EED1 and Branched EED1 (BEED) endosomal escape domains. **C)** Conjugation of hydrazide functionalized EED1 domain to single stranded RNA (ssRNA) oligonucleotide containing one Ax phosphotriester. Increasing EED1 peptide relative to AX phosphotriester yielded >90% conjugation. **D)** Conjugation of hydrazide functionalized BEED domain to ssRNA oligonucleotide containing one Ax phosphotriester. Reaction proceeded to >90% efficiency. **E)** Conjugation of hydrazide functionalized EED1 domain to ssRNA oligonucleotide containing three clustered Ax phosphotriesters. Increasing EED1 peptide relative to AX phosphotriester yields >90% conjugation at all three Ax phosphotriesters. **F)** Sequential multi-domain conjugation of an azide functionalized Man9 targeting domain (TD) followed by a hydrazide functionalized EED1 to ssRNA oligonucleotide with one Kx and one Ax phosphotriester on each end of the Passenger strand. Both HyNic and Click reactions proceeded to >90% efficiency yielding >80% final product with TD and EED domains. **G)** Sequential multi-domain conjugation of an azide functionalized Man9 TD followed by a hydrazide functionalized BEED to a RNA oligonucleotide with one Kx and one Ax phosphotriester on each end of the Passenger strand. Both HyNic and Click reactions proceeded to >90% efficiency yielding >80% final product with TD and EED domains. **H)** *In vitro* dose curve comparison of charged Man9-siRNA vs. Man9-siRNA-EED1 vs. Man9-siRNA-BEED targeting GAPDH at 72 hr analyzed by qRT-PCR (1-25 nM; $n = 3$, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group ($n = 3$). Error bar indicates s.d. Significance indicated between Man9-siRNA and Man9-siRNA-BEED. ***$P<0.001$; two tailed Student's t-test. **I)** *In vitro* dose curve comparison of charged siRNA vs. Man9-siRNA vs. siRNA-BEED vs. Man9-siRNA-BEED targeting GAPDH at 72 hr by qRT-PCR (1-25 nM; $n = 3$, each group). Values normalized to β2-microglobulin internal control and PBS treated control (0 nM) group ($n = 3$). Error bar indicates s.d. Significance indicated between Man9-siRNA and Man9-siRNA-BEED. *$P<0.05$; two tailed Student's t-test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Targeting M2 Macrophages *In Vivo*

Resident macrophages are present in all tissues of the body, but are difficult to isolate in large numbers for study or treatment of a pure population. Peritoneal macrophages represent a large population of resident macrophages that are easily harvested and purified (Edwards et al., 2006). Peritoneal macrophage cell numbers increase following IP injection of sterile Brewer's thioglycollate, leading to monocyte invasion, and polarization to M2 macrophages (Zhang, Goncalves, and Mosser, 2008; Gundra et al., 2014). IP thioglycollate can produce >10^7 cells with macrophages accounting for approximately 70% of the population. Thioglycollate induced peritoneal macrophages express high levels of CD206 (Gundra et al., 2014) and represent an attractive model for screening mannose-TRCs *in vivo*.

Injection of thioglycollate into the peritoneal space of C57B/6 mice (12 weeks old) resulted in >1-2x10^7 cells per mouse at day 3-5 with ~70% of all cells $CD206^+$/$F4/80^+$ indicating an M2 phenotype (**Figure 3.7A**). These results were similar to the reported yields and macrophage percentages for IP administration of Brewers thioglycollate (Zhang, Goncalves, and Mosser, 2008). IP administration of two sequential doses of 5 mg/kg (10 mg/kg total) Man9-siRNA into C57B/6 mice on day 3 and 4 post thioglycollate injection resulted in 35% knockdown of GAPDH in the bulk cell population on day 5 post thiolglycollate injection (**Figure 3.7B**). Similar IP administration of 2 x 5 mg/kg Man9-siRNN showed no significant knockdown of GAPDH in the bulk cell population. CD206 receptor expression determines cellular uptake of mannose-TRCs and knockdown is predicted to be higher in $CD206^{High}$ macrophages. However, enrichment of $CD206^{high}$/$F4/80^{high}$ macrophages (top 50%) resulted in no significant improvement of GAPDH knockdown as compared to bulk cell samples (**Figure 3.7B**). This result indicates that the mechanism of uptake of mannose-TRCs in thioglycollate activated peritoneal macrophages may not be CD206 dependent. Additionally, whatever mechanism is responsible for mannose-TRC uptake is less active towards the more hydrophobic Man9-

siRNN. This result may have been due to nonspecific hydrophobic interactions with epithelial cells in the peritoneal space, sequestering mannose siRNN TRCs from the macrophage population.

To further test for specificity of delivery, Man9-siRNA was compared to non-targeting siRNA and GN9-siRNA controls. No significant difference was found between Man9-siRNA and non-targeting controls, indicating that the mechanism of delivery was not specific for CD206 binding (**Figure 3.7C**). Importantly, the off target Man9-siRNA luciferase control did not result in any GAPDH knockdown, indicating that despite nonspecific uptake, the observed response was siRNA sequence specific due to induction of RNAi. The lack of specificity of mannose-TRC delivery may be restricted to this thioglycollate activated peritoneal macrophage model. Activation by thioglycollate results in a macrophage population with increased phagocytic capacity and macrophages that are harvested in this model exhibit an atypical appearance due to the increased phagocytosis of the thyoglycollate material (Zhang, Goncalves, and Mosser, 2008). This increase phagocytic capacity was likely responsible for the nonspecific uptake and RNAi activity of the non-targeted siRNA and GN9-siRNA controls.

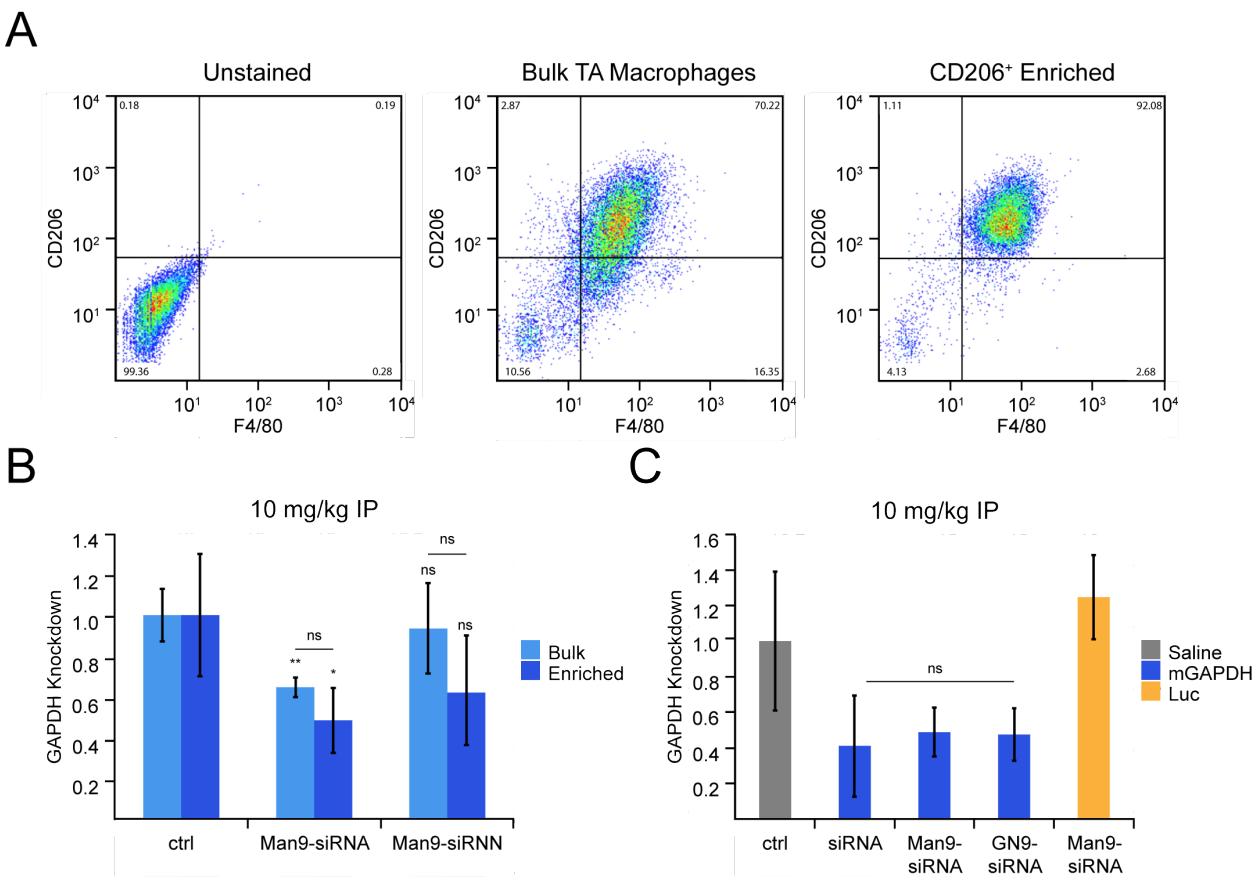

Figure 3.7. *In Vivo* Treatment of Activated Peritoneal Macrophages.
A) FACS analysis of CD206 and F4/80 profiles for thioglycollate activated (TA) macrophages from C57B/6 mice 5 days post IP administration of thioglycollate. FACS sorting of bulk cells by CD206 and F4/80 expression (highest 50%, CD206$^+$ Enriched) enriched for a CD206$^+$ and F4/80$^+$ population. **B)** *In vivo* IP administration of charged Man9-siRNA vs. neutral Man9-siRNN targeting GAPDH into C57B/6. 2x 5 mg/kg injections were administered on day 3 and 4 post thioglycollate injection for a total of 10 mg/kg per mouse. GAPDH mRNA knockdown compared between bulk peritoneal macrophages (Bulk) and CD206+/F4/80+ sorted cells (Enriched) populations. GAPDH mRNA analyzed at day 5 post thioglycollate injection by qRT-PCR (2 x 5 mg/kg; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (ctrl) group. Error bar indicates s.d. *P<0.05, **P < 0.01; two-tailed Student's *t*-test. **C)** *In vivo* IP administration of targeting Man9-siRNA vs. non-targeting GN9-siRNA targeting GAPDH and Luciferase off target control (Luc) into C57B/6. 2x 5 mg/kg injections were administered on day 3 and 4 post thioglycollate injection for a total of 10 mg/kg per mouse. GAPDH mRNA analyzed at day 5 post thioglycollate injection by qRT-PCR (2x 5 mg/kg; *n* = 3, each group). Values normalized to β2-microglobulin internal control and PBS treated control (ctrl) group. Error bar indicates s.d. two-tailed Student's *t*-test.

CONCLUSIONS

Tumor associated macrophages (TAMs) consist primarily of alternately polarized macrophages (M2) and are a major cellular component of both mouse and human tumors. TAMs promote an immunosuppressive tumor environment, increased angiogenesis and metastasis, limit the efficacy of various forms of anti-cancer therapies, and correlate strongly with reduced patient survival in a variety of solid tumors. Given their abundance within the tumor and their role in tumor progression and patient survival, TAMs represent an attractive target for therapeutic modulation in the treatment of cancer. siRNA offers the potential to treat cancer through targeting of TAMs and potent knockdown of mRNA involved in a variety of immunosuppressive pathways. However, the biophysical properties of siRNA limit its bioavailability and necessitate the use of TDs to achieve tissue specificity and cellular delivery.

Targeting of TAMs with multivalent mannose has been demonstrated clinically with the radiolabeled Tilmanocept imaging agent, a multivalent mannose ligand that binds and labels M2 macrophages via CD206 binding. CD206 is highly overexpressed on the surface of TAMs compared to other tissues and internalizes continuously, providing a route for siRNA delivery. However, following internalization, endosomal escape remains an unsolved problem and may require addition of endosomal escape domains. Addition of mannose targeting and endosomal escape domains requires multifunctional, site-specific conjugation schemes for the construction of well-defined, monomeric siRNA therapeutics. Previous work in our lab provides a flexible and robust platform for modification the phosphate backbone of the siRNA to create siRNN molecules. These modifications can be designed to include conjugation sites that can be placed anywhere along the siRNN molecule. Here I have described site-specific, multifunctional, and multivalent conjugation strategies for the construction of TRCs. I have also demonstrated robust binding and internalization of mannose TRCs as well as CD206 mediated siRNA delivery and RNAi activity in a model of M2 TAMs.

Here I described novel Kx phosphotriester synthesis and efficient and site-specific incorporation into an siRNN. Kx phosphotriesters mediated efficient copper catalyzed Click conjugation to multivalent mannose targeting and GalNAc non-targeting domains. Utilizing a well-established *in vitro* bone marrow derived macrophage (BMDM) model, I demonstrated that mannose TRCs bind to and are internalized by M2 macrophages in a CD206 dependent manner. Treatment of BMDMs *in vitro* resulted in robust knockdown of $CD206^+$ cells. Increasing the valency of the mannose TDs improved delivery and knockdown of target mRNA. Importantly, target mRNA knockdown was specific for mannose targeting and siRNA sequence, indicating a true RNAi response rather than off target effects.

We next utilized HyNic conjugation chemistry to attach endosomal escape domains (EEDs) onto the mannose-TRC. Incorporation of these hydrophobic peptides was limited to two per siRNN in order to preserve solubility. Multivalent EEDs provided enhanced delivery of mannose-TRCs over monovalent EED, suggesting that valency improves their activity. Incorporation of branched EEDs provided enhanced mannose-TRC delivery, moving the dose curve to the left. However, the observed effect was slight and improvements in siRNN chemistry that allow for greater solubility and incorporation of more EEDs may enhance this effect. Additionally, advances in potency and solubility of the EEDs themselves are likely to improve TRC activity.

Mannose-TRCs failed to provide evidence of *in vivo* efficacy due in part to the limitations of the activated peritoneal macrophage model and TRCs may prove effective in more robust murine TAM models. While *in vivo* delivery of mannose-TRCs was unsuccessful, this study provides a strategy for the construction of well-defined, site specific, and multifunctional targeted siRNA conjugates. Advances in endosomal escape, siRNN solubility, and alternate tissue TDs can be readily incorporated into this platform, allowing for rapid and efficient adaptation for treatment of a variety of diseases.

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CHAPTER 4

BUILDING MULTIFUNCTIONAL ANTIBODY-RNA CONJUGATES (ARCs) FOR EXTRA-HEPATIC TARGETING AND ENDOSOMAL ESCAPE

Building Multifunctional Antibody-RNA Conjugates (ARCs) for Extra-Hepatic Targeting and Endosomal Escape

ABSTRACT

Short interfering RNA (siRNA) therapeutics offer tremendous potential to treat all genetic disease. However, the chemical properties of siRNAs limit their bioavailability and necessitate a targeting domain to deliver the siRNA therapeutic as well as a mechanism to escape the endosome. siRNA conjugates of *tris*-N-acetalgalactosamine (GalNAc) have demonstrated robust and prolonged RNAi activity in clinical trials. Targeting of siRNA therapeutics to the hepatocytes has revolutionized the siRNA therapeutic field, but similar therapeutics in extra-hepatic tissues have not seen the same success due to lack of extra-hepatic targeting domains and inefficient endosomal escape. Antibodies offer an attractive tool for targeting a variety of extra-hepatic tissues and advances made in the field of antibody-drug conjugates (ADCs) offer insights into the problems and solutions that may affect the construction of antibody-RNA conjugates (ARCs). However, following delivery of siRNAs by ARCs, siRNA has no ability to escape the endosome and so requires additional endosomal escape functionality to be an effective therapeutic. Well-defined, site-specific conjugation of an antibody and endosomal escape domains to an siRNA requires multifunctional conjugation strategies and has not been demonstrated prior to this work. Previous work in our lab has provided a flexible and robust platform for modification the phosphate backbone of the siRNA to create short interfering ribonucleic neutral (siRNN) molecules. These modifications can be placed anywhere along the siRNN molecule and designed to include conjugation sites. Here I describe a novel site-specific, multifunctional, and multivalent conjugation strategy for the construction of ARCs.

INTRODUCTION

The discovery of RNA interference (RNAi) in 1998 (Fire et al., 1998) and the subsequent demonstration in 2001 that exogenous short interfering RNA (siRNA) could affect potent post-transcriptional gene regulation provided the potential for a new approach to treat human disease (Elbashir et al., 2001). siRNA-induced RNAi responses offer an EC_{50} in the picomolar (10^{-12} M) range with exquisite target selectivity for all mRNA (Bumcrot et al., 2006). These unique characteristics provide the potential to treat a wide variety of human diseases from cancer to pandemic viral outbreaks to Parkinson's Disease (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017).

Despite the potential of RNAi therapeutics, siRNA is prevented form crossing the lipid bilayer due to both its size (~14 kDa) and 40x negative charges (Juliano, 2016; Dowdy, 2017; Khvorova and Watts, 2017). These attributes also make siRNAs pharmacokinetically highly unfavorable, as naked siRNA is removed from the bloodstream by the kidneys within minutes of injection into mice and humans (Merkel et al., 2009). Additionally, native (2'-OH) double stranded siRNAs are recognized as invading nucleic acids by multiple cellular defense mechanisms, including extracellular Toll-Like Receptors (TLR-3, -7, -8) and intracellular sensors, retinoic acid inducible gene (RIG-I) and melanoma differentiation associated protein 5 (MDA-5) (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017; Gantier and Williams, 2007; Iversen et al., 2013; Juliano et al., 2014). These difficulties necessitate the use of delivery agents to both assist siRNAs to cross the lipid bilayer and to remain in circulation for longer periods of time. Thus, the major obstacle prohibiting effective RNAi therapeutics has been delivery into the cytoplasm of cells. Consequently, there has been significant attention and investment of time and resources to address the delivery problem by harnessing and developing a wide array of technologies (Juliano, 2016; Dowdy, 2017; Khvorova and Watts, 2017).

To address these problems our lab developed small interfering ribonucleic neutrals (siRNNs) containing neutralizing phosphotriester groups based on a mononucleotide HIV

prodrug inhibitor containing a bioreversible *t*-butyl-S-acyl-2-thioethyl (*t*Bu-SATE) group (Puech et al., 1993; Lefebvre et al., 1995; Gröschel et al., 2002). These neutralizing phosphotriesters are converted into charged phosphodiesters by intracellular restricted thioesterases to yield a wild type siRNA that can be loaded into RISC to induce an RNAi response. This technology increases serum stability >24 hr, avoids innate immune stimulation, and increases *in vivo* circulation time (Meade et al., 2014). The *t*Bu-SATE technology is also amenable to modification, allowing for modulation of solubility and addition of conjugation handles for site selective conjugation of PTDs and other cargos. Conjugation of a siRNN to *tris*-N-acetalgalactosamine (GalNAc) targeting hepatocyte asialoglycoprotein receptor (ASGPR) showed effective long-term knockdown of target mRNA in a dose dependent manner (Meade et al., 2014).

GalNAc-siRNA conjugates have been thoroughly investigated in preclinical rodent and NHP models, and clinical trial data reveals robust and prolonged knockdown of target mRNA with concomitant disease treatment. GalNAc targeting has revolutionized the RNAi therapeutics field to such an extent that hepatic delivery can be considered solved. However, targeting and delivery of siRNA therapeutics outside of the liver has not seen the same success and remains a major hurdle to treatment of extra-hepatic diseases.

In order to effectively target siRNA therapeutics to all tissues, targeting domains and ligands must meet three criteria:

4) A receptor or extracellular target must be highly expressed on the cell type of interest to deliver a sufficient payload of siRNA. Low abundance receptors provide comparatively few opportunities for delivery and make the challenge of RNAi therapeutics more difficult.

5) The receptor or target of interest must internalize at as high a rate as possible. A receptor with low internalization results in rapid receptor saturation and subsequent clearance of unbound circulating RNAi therapeutic, requiring more frequent dosing or

modified dosing methodology. Conversely, high internalization rates can make up for relatively low numbers of target receptor and provide greater opportunity for cytoplasmic delivery.

6) The receptor or target of interest must be selective for or highly overexpressed on the target tissue compared to off-target tissues. Targeting a ubiquitous receptor results in non-specific systemic delivery of the siRNA, sequestering much of the therapeutic in non-productive tissues.

Efforts to target nucleic acids to extra-hepatic tissues have tested a variety of receptor/ligand pairs including glucagon-like peptide-1 (GLP-1) targeting pancreatic β-islet cells, and folate and Arg-Gly-Asp (RGD) peptides for tumor targeting (Ämmälä et al., 2018; Alam et al., 2011; Cen et al., 2018; Manoharan, Rajeev and Jayaraman, 2008). However, receptor/ligand pairs that meet the criteria for effective therapeutic targeting are limited and their clinical efficacies have yet to be demonstrated for siRNA therapeutic applications. An attractive alternative to these endogenous receptor/ligand pairings is the use of monoclonal antibodies (mAbs) that have several decades worth of clinical validation for a wide array of therapeutic targets. Given their ability to bind antigens with superb specificity and their widespread use as therapeutic agents, mAbs have been potent targeting agents for Antibody-Drug Conjugates (ADCs) and Radionuclide Antibody Conjugates (RACs).

Antibodies have been utilized for selective delivery of chemotherapeutics in the form of ADCs for nearly 60 years, with the first clinical trials starting in the 1980s (Perez et al., 2014) and the first FDA approved ADC, gemtuzumab ozogamicin (Mylotarg), in 2000 (Sievers et al., 2001), followed by three more ADC approvals in 2011 (brentuximab vedotin, Adcetris) (Senter and Sievers, 2012), 2013 (ado-trastuzumab emtansine, Kadcyla) (Lambert and Chari, 2014) and 2017 (inotuzumab ozogamicin, Besponda) (Research, 2017). Since 2013, >30 ADCs have entered clinical trials for oncologic applications (Beck et al., 2017; Mullard, 2013). ADCs face

many of the challenges that siRNA therapeutics currently face and the solutions provided for each problem have potential applications for siRNA therapeutics as Antibody RNA Conjugates (ARCs).

Antibody targeted therapeutics allow for superb binding to a broad array of cellular targets as ADCs effectively deliver their cargo to cells with extracellular antigen numbers in the range of 5,000 to >3 million per cell (Bross et al., 2001; Sievers, 2003; Phillips et al., 2008; Junttila et al., 2011; Beck et al., 2017). To overcome the limitations imposed by low abundance surface antigen, the cytotoxic payload on ADCs must have potency in the picomolar (10^{-12} M) range (Chari et al., 2014; Lambert, 2016; Beck et al., 2017). The high potency of these chemotherapeutics offers superb target activity at low delivered doses, a property that is shared with siRNA.

ADC therapeutics rely heavily on effective conjugation of the chemotherapeutic agent without appreciably altering the mAb binding properties or pharmacokinetics. Early conjugation efforts relied on alkylation of reduced inter-chain disulfides and acylation of lysine residues. However, these methods resulted in a poorly defined drug to antibody ratio (DAR) and can result in aberrant inter-chain disulfide formations, compromising mAb binding properties (Panowski et al., 2014). Improvements in site-specific conjugation have allowed for greater definition of DAR. Most second and third generation ADCs in clinical trials use site-specific cysteine or lysine linkages with a DAR of 3.5-4.0 (Lambert and Berkenblit, 2018; Beck et al., 2017). Genetically engineered cysteine residues avoid reduction of native disulfide bonds and allow greater control of the final DAR (Sievers and Senter, 2013). Various forms of this technology are currently being used by Genentech (Junutula et al., 2008), Seattle Genetics (Jeffrey et al., 2013), Novartis (Voynov et al., 2010), MedImmune (Beck et al., 2013), Kirin, and Pfizer. Several companies including PolyTherics (now Abzena), ThioLogics, Igenica Biotherapeutics, and Sorrento Therapeutics, utilize native cysteine re-bridging that retains mAb

binding characteristics and ADC activity without the need for engineered conjugation sites (Beck et al., 2017; Bryant et al., 2015; Maruani et al., 2015; Behrens et al., 2015).

Despite the improvements in DAR definition, most ADCs with lysine or cysteine linkages, including all current FDA approved ADCs, have measurable drug loss during prolonged circulation. Shedding of chemotherapeutic cargo through a retro-Michael reaction results in systemic exposure, reduced anti-tumor activity, and increasing toxicity. This mechanism is described for both cysteine linked ADCs and lysine linked SMCC thioether linked ADCs (Lyon et al., 2014; Dere et al., 2013). Hydrolysis of the succinimide ring prevents the retro-Michael reaction from occurring, limiting chemotherapeutic shedding. Various methods have been developed to achieve succinimide ring hydrolysis, including incubation of the final ARC at pH 9.2 (Tumey et al., 2014), use of self-hydrolizing maleimides (Lyon et al., 2014; Fontaine et al., 2015), use of N-aryl maleimide agents (Christie et al., 2015), and use of maleimide alternatives, such as sodium 4-((4-(cyanoethynyl) benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (CBTF) (Kolodych et al., 2015), and 2-(maleimidomethyl)-1,3-dioxanes (Dovgan et al., 2016). It has also been found that inclusion of maleimide conjugation sites within a cationic surface charge region of the mAb leads to hydrolysis of the succinimide ring and reduced cargo shedding from ADCs (Shen et al., 2012).

Other methods for site selective conjugation that reduce chemotherapeutic shedding involve incorporation of unnatural amino acids selenocysteine (Xiuling Li et al., 2015), paraacetylphenylalanine (pAMF) (Zimmerman et al., 2014), as well as azide containing amino acids (VanBrunt et al., 2015). These methods utilize a variety of biocompatible conjugation strategies but require protein engineering and supplementing unnatural tRNA for their incorporation (Kline et al., 2015; Tian et al., 2014). Additional methods include glycan remodeling, glyco-conjugation (Ekholm et al., 2016; Okeley et al., 2013; Zuberbühler et al., 2012; Zhou et al., 2014; Xiuru Li, Fang, and Boons, 2014; Qasba, 2015; van Geel et al., 2015), and N-terminal serine conjugation (Thompson et al., 2015).

An alternative to chemical conjugations is the use of enzymes, such as formylglycine-generating enzyme (FGE) (Albers et al., 2014; Drake et al., 2014), sortase A (SrtA)(Beerli et al., 2015), and microbial transglutaminase (MTG). MTG belongs to a family of enzymes that catalyze isopeptide reactions between the ε-amine group on lysine and the terminal acyl group of glutamine. It has been shown that MTG recognizes and preferentially catalyzes reactions between glutamine and lysine residues within specific amino acid sequence motifs. Peptide library and viral expression vector screens coupled with structural analysis of native MTG substrates have revealed amino acid patterns for optimal MTG recognition that have guided the design of site-specific MTG conjugation handles (Caporale et al., 2015; van Buggenum et al., 2016; Siegmund et al., 2015). In all cases it has been found that enzymatic selectivity is higher for the glutamine residue compared to lysine residue. Implementation of MTG recognition sequences allows for rapid and selective conjugation at desired sites with minimal off-target conjugation, even on large proteins containing free native lysine and glutamine residues (Caporale et al., 2015; van Buggenum et al., 2016; Siegmund et al., 2015). Pfizer currently has an ADC candidate (PF-06664178) in clinical trials utilizing MTG conjugation (Strop et al., 2016; Strop et al., 2013). MTG has also been used for conjugation of DNA to protein complexes (Strop, 2014) and is a promising potential route for ARC production.

Linker selection is critical in mAb-targeted therapies and will be critical for the design of ARCs as well. Linkers must be stable enough to take advantage of the mAb's long circulating half-life, while efficiently releasing their cargo following cellular internalization by endocytosis (Chari et al., 2014; Lambert, 2016). Non-cleavable linker chemistries such as thioether bonds require lysosomal degradation of the targeting mAb and leave residual amino acid tags on the chemotherapeutic cargo (Erickson et al., 2006; Doronina et al., 2006). Enzyme-cleavable linkers are serum stable and utilize lysosome-restricted enzymes that recognize specific amino acid or carbohydrate motifs, allowing for more rapid release of ADC cargo without the need for full degradation of the targeting mAb. Citrulline-valine linkers are specifically cleaved by

cathepsin B and β-glucuronide linkers are recognized by β-glucuronidase within the lysosome (Sanderson et al., 2005; Doronina et al., 2006; Erickson et al., 2006; Jeffrey et al., 2010).

While irreversible and enzyme-cleavable linkers limit cargo shedding in circulation, the required lysosomal degradation may not be suitable for siRNA therapeutic candidates, potentially limiting their use in ARCs (Heydrick et al., 1991; Dominska and Dykxhoorn, 2010). An attractive alternative is a chemically labile linker that takes advantage of the acidifying and reductive environment of the endosome (Yang et al., 2006). Both acid-labile hydrazone and disulfide-based linkers are commonly used, but are comparatively less stable, with serum half-lives of approximately 48-72 hr and 24 hr, respectively (Boghaert et al., 2008; Senter, 2009). Early ADCs with cleavable linkers had measurable drug loss in circulation and toxicity profiles similar to standard chemotherapeutic regimens (Beck et al., 2017; Senter, 2009). Shedding of cytotoxic cargos due to unstable linker chemistries led to the voluntary withdrawal of the first FDA approved ADC, Mylotarg, by Pfizer, though Mylotarg was recently reapproved in 2017 by the FDA at a lower dose. Following this temporary withdrawal, the stability of cleavable linkers has been improved and their incorporation into ARCs remains a viable approach.

Taken together, the advances made in site-specific conjugation and linker chemistries offer the potential to build well-defined ARCs in a way that has previously not been possible. Early efforts to produce effective ARCs relied on electrostatic interaction between positively charged peptides, such as protamine, and the negative backbone of the siRNA molecule, resulting in heterogeneous aggregation that negatively affects drug clearance, maximum tolerated doses, and efficacy (Mehta et al., 2015; Bäumer et al., 2016; Hamblett et al., 2004; Song et al., 2005; Yao et al., 2012). Genentech first utilized ADC chemistry technologies to develop an siRNA conjugation scheme with their engineered cysteine THIOMAB platform and demonstrated efficacy in a variety of *in vitro* tumor models, but showed very poor delivery in subcutaneous tumor models (Cuellar et al., 2015). Prediction of success *in vitro* based on receptor number or internalization route was inconsistent and *in vivo* translations of *in vitro*

success yielded modest tumor reduction at best. While mAb mediated siRNA delivery was accomplished in select cell lines, this work demonstrated that successful targeting of an internalizing extracellular receptor is not sufficient for cytoplasmic delivery. However, this technology represented the first site-specific conjugation of siRNA to a mAb, in stark contrast to the poorly defined protamine predecessors. Subsequently, Takeda Pharmaceuticals demonstrated delivery of a transferrin (CD71) receptor targeting Fab-siRNA conjugate (Sugo et al., 2016). The siRNA cargo is conjugated through a free cysteine generated after enzymatic cleavage of the parental mAb. Robust knockdown of target myostatin mRNA (50-70%) and concomitant increases in muscle mass were observed in mice following intravenous, intraperitoneal, or subcutaneous injections. This work provided an example of targeted delivery *in vivo* for a variety of systemic administration routes and demonstrated the potential for extra-hepatic delivery of siRNA therapeutics.

While both Genentech and Takeda observed varying levels of success with mAb and Fab targeted siRNAs, it is unclear how these therapeutics are escaping from the endosome to affect their targets in the cytoplasm. Unlike ADC drug toxins that are capable of diffusing out of the endosome, siRNAs are unable to cross the membrane of the endosome to escape into the cytoplasm. The inability to cross lipid membranes allows siRNA to avoid inherent toxicity, but also sequesters the siRNA therapeutic in the endosome, preventing RNAi. Escape from the endosome represents the rate-limiting step for delivery of nucleic acids (Varkouhi et al., 2011; Wiethoff and Middaugh, 2003; Cho, Kim, and Park, 2003; Vaidyanathan, Orr, and Banaszak Holl, 2016; Dowdy, 2017). In order to efficiently escape the endosome, mechanisms for endosomal escape must be implemented into ARCs. To this end, we have sought to engineer clinically validated mAbs with site selective conjugation handles for quantitative addition of siRNA. We provide a conjugation strategy for a flexible, modular construction of ARCs that allows for a variety of linker chemistries and the inclusion of site-specific endosomal escape domain conjugations.

RESULTS & DISCUSSION

Production of Therapeutic Monoclonal Antibodies

Design, development, and production of monoclonal antibodies (mAb) with high binding affinities and good therapeutic properties can take years of work and considerable expenditures. To avoid lengthy screening processes and allow for rapid development of ARCs targeting multiple extracellular antigens, the amino acid sequences for FDA approved mAbs were obtained from the patent literature. Additionally, in order to allow for flexible design and addition of site-specific MTG conjugation handles, we opted to produce our own mAbs. To conserve mAb glycosylation, ensure proper protein folding, and produce sufficient mAb yields for *in vivo* work, we utilized the ExpiCHO expression system (Zhong et al., 2018). Amino acid sequences for the variable regions of mAbs targeting CD33, PSMA, EGFR, HER2 and Transferrin Receptor (CD71) were collected and codon optimized for the ExpiCHO expression system. Variable regions were cloned into both κ or λ light chains (LC) and IgG1 or IgG4 heavy chain (HC) subtypes. MTG recognition sequences were cloned onto the C-terminus of LCs and HCs to provide a site-specific conjugation site.

Expression of full-length mAbs from plasmid DNA can require optimization for each mAb to be expressed. Due in part to the difference in final protein size, HC fragments express at a lower level than LC fragments when driven from their own promoters. Disequilibrium in HC and LC production can lead to incomplete mAbs and poor overall yields. To ensure production of complete and properly folded mAbs, an excess of HC plasmid over LC plasmid was utilized at a ratio of 2:1 (HC:LC) though in some cases higher ratios up to 5:1 were required for proper mAb expression.

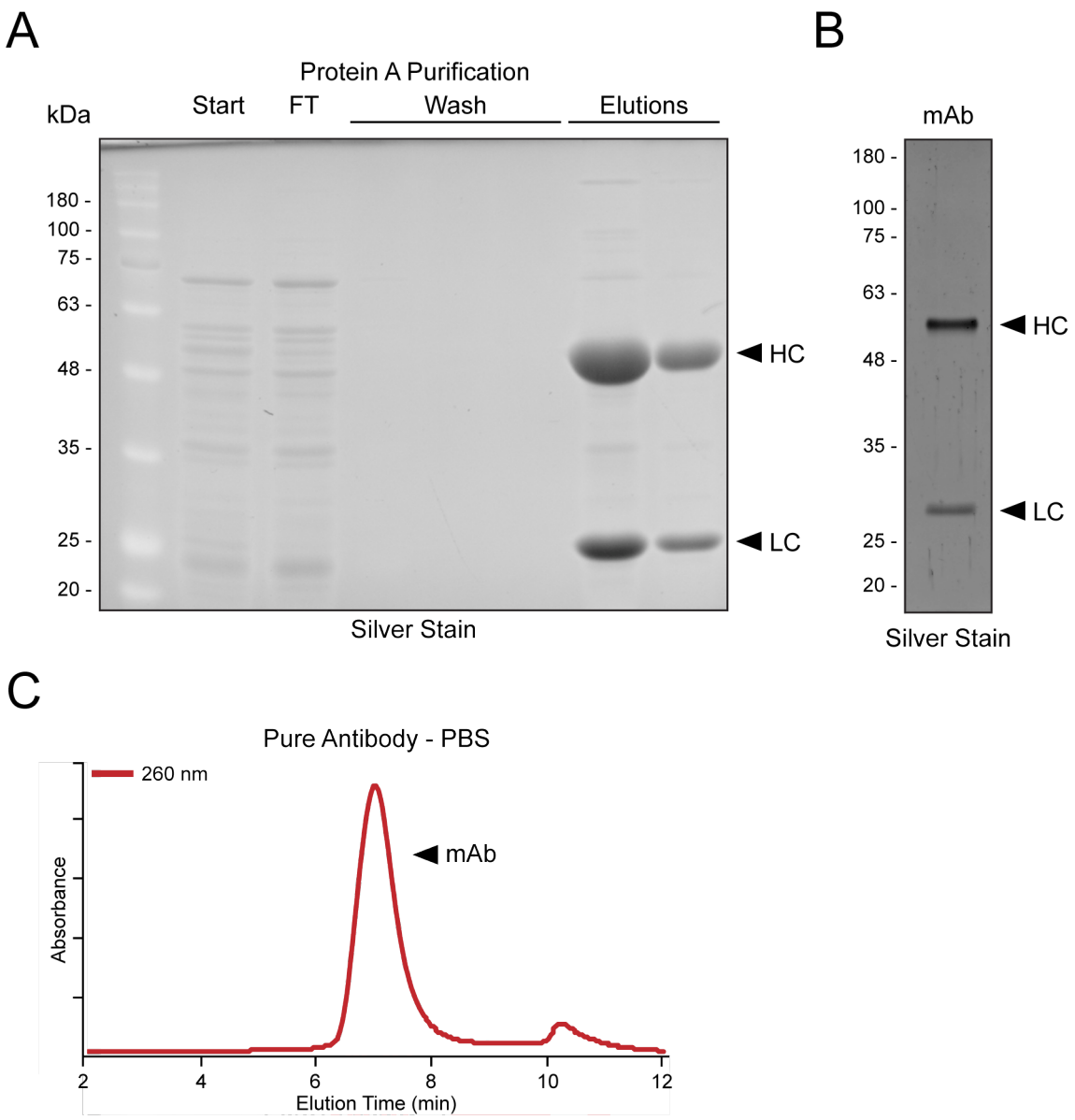


Figure 4.1. Antibody Production and Purification.
A) Protein A purification. SDS PAGE analysis of Protein A fractions revealed starting lysate (Start) contains IgG heavy chain (HC) bands (50 kDa) and light chain (LC) bands (25 kDa). Flow through (FT) revealed depletion of antibody bands. Wash fractions did not contain antibody bands. Elution fractions contained HC and LC bands. **B)** SDS PAGE analysis of pooled elution fractions showed a final product free of contaminating proteins. **C)** FPLC size exclusion chromatogram of purified αCD33 antibody showed a pure sample with no aggregates or contaminants (absorption at 260 nm).

Protein expression systems require purification of the desired protein from the final cellular and extracellular milieu. Protein-A binds avidly to the Fc region of mAbs and is denatured at low pH, allowing for subsequent release of the mAb. Utilizing a Protein A resin, mAbs were efficiently captured from the ExpiCHO supernatant and immobilized on the resin. Washes of the resin removed unwanted proteins with a low level loss of mAb (**Figure 4.1A**). Elution from the Protein A resin under low pH (pH 3.0) conditions resulted in a highly pure mAb with little contaminating protein (**Figure 4.1B**). Proper mAb folding following neutralization of the Protein A elution buffer was retained with few aggregates (<0.01%) observed in the final mAb product (**Figure 4.1C**). Yields varied between batches and specific mAbs, but ranged from 1 mg to ~35 mg per 35 mL ExpiCHO culture.

Conjugation of Linker Peptides by Microbial Transglutaminase (MTG)

A critical issue facing mAb conjugations is site specificity, with traditional lysine or cysteine conjugations resulting in a range of DAR from 0-8. Each of these species has a different solubility and pharmacokinetic profile. Conjugation within the variable regions may reduce or destroy binding capabilities. To produce mAb conjugates with a defined DAR and site-specific conjugation, conjugation tags or handles can be engineered into the mAb that are recognized by microbial transglutaminases (MTG). Several MTG recognition sequences have been published based on sequence recognition as well as structural mimics of natural MTG substrates (Strop et al., 2013, 2016; Caporale et al., 2015) However, we found that many of these sequences yielded significant mAb conjugation to itself and to other mAbs resulting in significant aggregation. In screening several amino acid sequences based on the literature, we found that the MTG recognition sequence LLQGA resulted in the least self-conjugation and aggregation while maintaining MTG activity.

The activity of enzymes depends in part on their concentration, with high concentrations leading to increased off-target activity, while low concentrations can require prohibitively long reaction times. To determine the proper concentration of MTG, mAbs were reacted in vast excess of linker peptide to MTG conjugation handle (200:1) while the amount of MTG in solution was varied. To measure on and off-target linker incorporation with high sensitivity, a Flourescein labeled peptide was utilized, allowing for detection of non-specific linker conjugation in <1% of the mAb sample. The lowest levels of MTG suggested by the literature (0.126 mU/mL) (Caporale et al., 2015) revealed <5% conjugation of the Flourescein linker to the HC. Increasing the concentration of MTG 10x (1.26 mU/mL) improved conjugation to 65% and increasing the MTG concentration to 3.15 mU/mL allowed for 80% HC conjugation (**Figure 4.2A-B**). Increasing the concentration of MTG beyond 3.15 mU/mL saw diminishing improvements in HC conjugation while off-target LC linker-peptide incorporation, as observed by LC- Flourescein signal on an SDS-PAGE gel, continued to increase to >20%. Additionally, higher concentrations of MTG increased self-conjugation, aggregation, and precipitation of the reaction mixture. Self-conjugation was observed as a faster migrating species on SDS PAGE that prevented incorporation of the linker peptide and represents a non-productive species in subsequent conjugations. Aggregation was observed as a complex, slow migrating banding pattern >100 kDa representing crosslinking of the HCs and LCs to one another, preventing dissociation on SDS PAGE (**Figure 4.2A**).

MTG recognition sequences theoretically provide a conjugation site with faster kinetics than off-target glutamines, but the kinetics need to be balanced with the fact that MTG retains slow promiscuous activity for native glutamine and lysine residues on the mAb. To determine the optimal conjugation time to attain complete site-specific conjugation, while limiting off-target conjugation, a time course was carried out on the MTG reaction. It was found that in the presence excess linker to MTG conjugation site (200:1), the MTG reaction proceeds to completion within 1 hr, while off-target conjugation remains minimal. Additional time beyond 1

hr did not increase site specific conjugation and only increased off-target conjugation species (**Figure 34.2C**)

Linker sequences for MTG conjugation have also been published for the lysine donor peptide in the MTG reaction (Strop et al., 2013, 2016; Caporale et al., 2015). Comparisons between the published recognition sequences and simple Lysine-PEG (KP) linker peptides synthesized in our lab resulted in similar conjugation efficiencies and kinetics and so KP peptides were chosen for ARC construction. The ratio of linker peptide to mAb-MTG conjugation handle plays a major role in MTG off-target conjugation activity. In the absence of excess linker peptide, MTG randomly conjugates lysine and glutamine residues across the mAb surface resulting in significant self-conjugation and aggregation. SDS PAGE analysis of MTG reactivity in the absence of linker peptide revealed almost complete depletion of the monomeric HC band (**Figure 4.2A**).

To determine the appropriate ratio of linker peptide to mAb, MTG reactions were carried out in increasing concentrations of linker peptide relative to mAb-MTG conjugation site (**Figure 4.2D**). A ratio of 100:1 linker peptide to MTG conjugation site provided >90% HC conjugation with only minor self-conjugation and aggregation. It was also noted that the incidence of self-conjugation and aggregation species varied between batches of the same mAb, a property that became a screening point for later batches (**Figure 4.2E**).

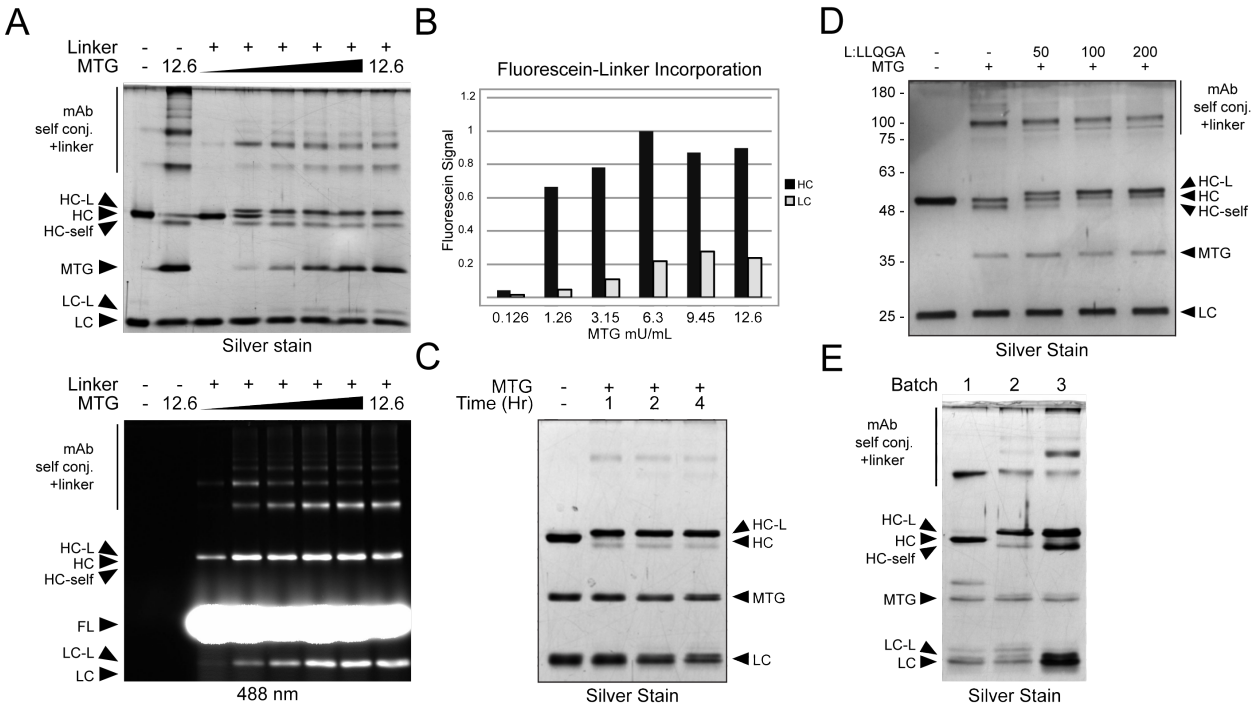


Figure 4.2. MTG Conjugation Refinement.
A) MTG concentration curve with a Flourescein-linker peptide (FL) at 200:1 linker to MTG conjugation site (2 per mAb) ratio carried out at RT for 2.5 hr. 0.5 µg of each sample was run on a 10% SDS PAGE and analyzed by silver stain and fluorescence at 488 nm. MTG concentrations: 0.126, 1.26, 3.15, 6.3, 9.45, and 12.6 mU/mL MTG. **B)** Quantification of FL signal incorporation into LC and HC bands. **C)** MTG reaction time course with 200:1 linker to MTG conjugation site (2 per mAb) ratio at RT with 3.15 mU/mL MTG. 0.5 µg of each sample was run on a 10% SDS PAGE and silver stained. **D)** Linker-antibody ratio (L:LLQGA) test with 3.15 mU/mL MTG enzyme carried out at RT for 1 hr. 0.5 µg of each sample was run on a 10% SDS PAGE and silver stained. **E**) Batch testing of IgG1 αCD33 antibodies with 200:1 linker to MTG conjugation site (2 per mAb) ratio with 3.15 mU/mL MTG for 1 hr at RT. 0.5 µg of each sample was run on a 10% SDS PAGE and silver stained.

Purification of Antibody-Linker Conjugates

Purification of antibody-linker (mAb-L) conjugates away from excess linker peptide and MTG is critical for efficient downstream conjugations and long-term storage of mAb-L intermediates as long-term exposure to MTG will result in mAb self-conjugation and aggregation. Purification yields >90% are desirable in order to produce mAb-L intermediates in high enough quantity for large-scale conjugations and *in vivo* testing. MTG activity led to slow accumulation of off-target conjugations between and within mAbs, leading to aggregation and precipitation in reactions beyond 4 hr (**Figure 4.2A,C**). Protein A purification was tested for mAb-L purification from MTG. Protein A followed by sample concentration and solvent exchange into PBS by 30 kDa spin filtration resulted in mAb-L yields between 70-90%. Unfortunately, Protein A bead purification did not remove all MTG from the final product (**Figure 4.3A**), leading to continuous off-target conjugation and precipitation. Alternatively, purification by 30 kDa pore size spin filtration and 40 kDa Zeba Size Exclusion Chromatography (SEC) spin column filtration removed the need for solvent exchange or sample concentration steps and resulted in yields between 70-80%. However, these methods failed to remove MTG from the final product (**Figure 4.3B**). Increasing the pore size of the spin filtration purification to 100 kDa to allow more MTG to be removed resulted in yields <20%.

An alternative purification strategy of SEC filtration using FPLC allowed for visualization of each reaction component by spectrographic analysis and collection of desired fractions (**Figure 4.3C**). The mAb-L peak eluted at 6.5-7.5 min and separated cleanly from the MTG peak at 8-8.5 min. SDS PAGE analysis of the collected SEC fractions revealed pure mAb-L without major contamination from unconjugated mAb or MTG (**Figure 34.3D**). Concentration and solvent exchange into PBS using a 30 kDa pore size spin filters resulted in >90% yield. Long-term storage did not result in aggregation or precipitation, indicating that MTG had been fully removed.

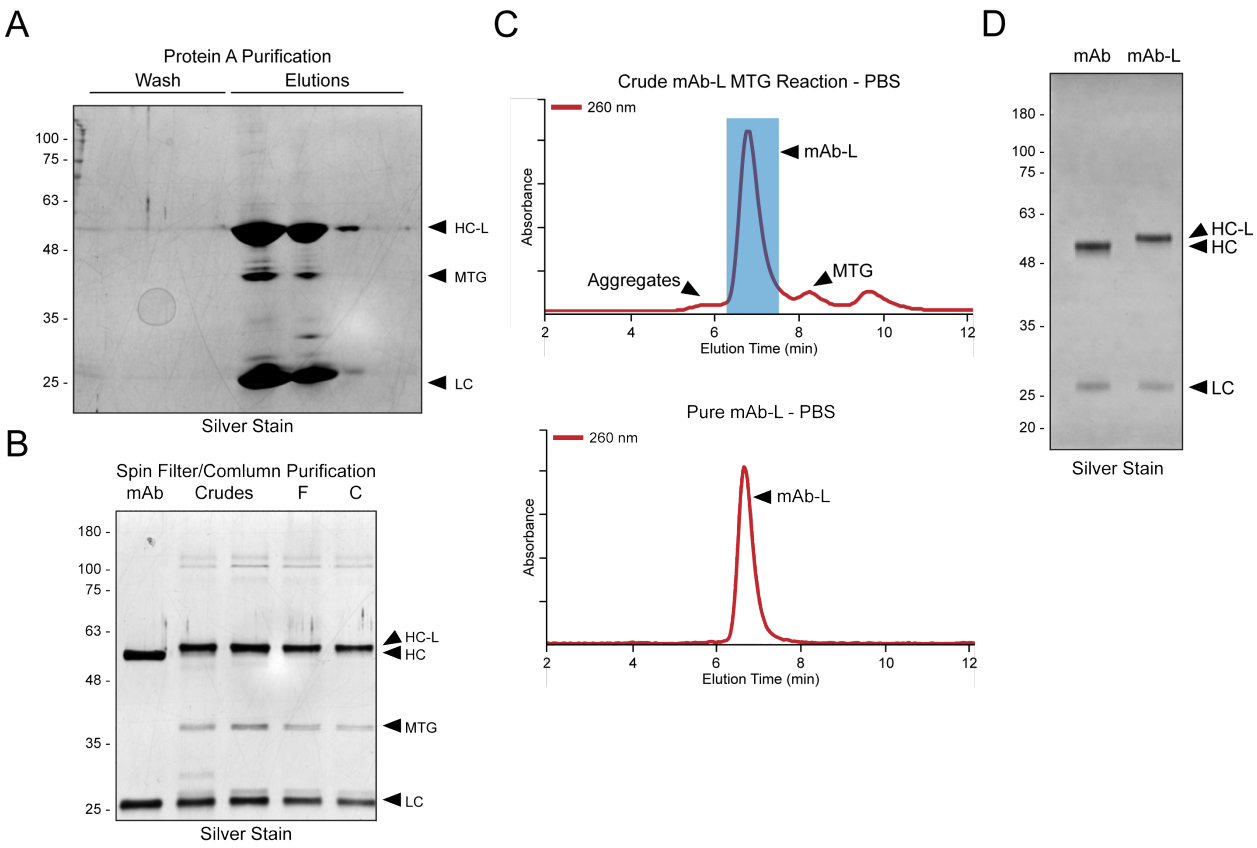


Figure 4.3. Purification and Analysis of Antibody-Linker Conjugate.
A) Protein A purification of an antibody-linker (mAb-L) washes and elutions. A set volume of each fraction was run in each lane of a 10% acrylamide SDS PAGE gel followed by silver stain analysis. Heavy chain-linker (HC-L) and light chain (LC) bands were the major products. Microbial transglutaminase (MTG) was not fully removed. **B)** Crude mAb-L conjugates were purified by 30k amicon ultra spin filtration (F) and 40k Zeba size exclusion spin columns (C). Equal portions of input and purified product were run in each well to compare yields. Gels were analyzed by silver stain. **C)** (Upper panel) Size Exclusion Chromatography (SEC) purification of crude mAb-L MTG reaction. Collected fractions are highlighted in blue. Aggregates eluted at 5.5 min. Antibody-linker conjugate (mAb-L) eluted at 6.5-7.5 min. MTG eluted at 8-8.5 min. (Lower panel) SEC analysis of purified mAb-L revealed a single peak without MTG contamination. **D)** Pooled fractions from SEC purification. 0.5 µg starting antibody (mAb) and an equal portion of purified antibody linker (mAb-L) revealed >90% yield and >90% conjugation of linker to heavy chain (HC-L).

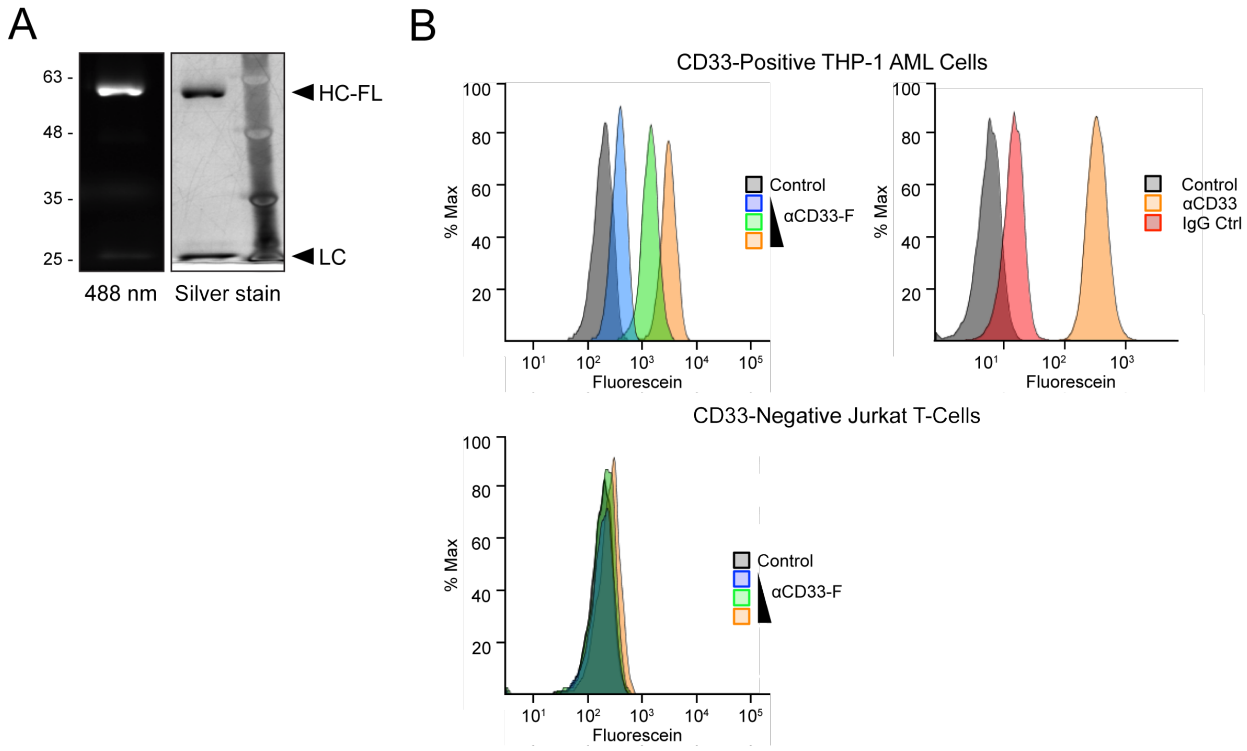


Figure 4.4. Antibody-Linker Binding.
A) 15% SDS PAGE gel analysis of a αCD33-Flourescein MTG conjugation following FPLC SEC purification. Flourescein linker (FL) imaging by 488 nm (Left panel) and silver stain analysis (Right panel). **B)** FACS analysis of αCD33-Flourescein (αCD33-F) binding to CD33+ THP-1 cells in a dose dependent manner. Off-target control IgG-Flourescein binding to THP-1 cells compared to αCD33-F mAb. CD33 negative Jurkat cells showed no binding of the αCD33-F mAb.

Binding of Antibody-Linker Conjugates

Conjugation of peptides to mAbs may disrupt their binding capabilities by direct interference with antigen binding or indirectly through alteration of mAb structure. Site specific conjugation aims to limit these potential problems by directing conjugation to regions of the mAb that do not interfere with its binding. To test the hypothesis that the site and method of conjugation we have utilized has not altered mAb binding or specificity, a linker peptide labeled with a Flourescein fluorophore was conjugated to a αCD33 mAb. Purification and SDS PAGE analysis revealed a pure mAb-Flourescein conjugate without free Flourescein peptide and with a single Flourescein fluorophore conjugated to each HC of the mAb (**Figure 4.4A**). Treatment of CD33+ THP-1 cells with a αCD33- Flourescein mAb resulted in a dose-dependent increase in Flourescein labeling (**Figure 4.4B**). Treatment with a Flourescein-labeled IgG isotype control did not result in a marked increase in Flourescein signal. αCD33- Flourescein treatment of CD33 negative Jurkat cells also saw no significant binding. Taken together, these results indicate that conjugation of a peptide linker at the C-terminus of the HC does not interfere with mAb binding and that the observed binding was specific for CD33.

Conjugation of Endosomal Escape Domains (EEDs)

Escape from the endosome is the rate-limiting step for delivery of RNAi therapeutics and represents a major challenge to the field. A variety of endosomal escape domains (EED) have been developed utilizing a myriad of proposed escape mechanisms including pore formation, membrane disruption, and hydrophobic interactions. Endosomal escape domains have been utilized in delivery of fusion proteins and peptides as well as in nanoparticle delivery. To construct monomeric siRNA therapeutics, a site-specific and efficient mechanism of conjugation is needed to incorporate endosomal escape domains onto the siRNA. To facilitate conjugation of EEDs, we utilized a phosphotriester with a terminal benzaldehyde conjugation handle (Ax).

Conjugation through an Ax phosphotriester was carried out with hydrazide modified peptides that react with the terminal benzaldehyde to form a stable bis-aryl hydrazone linkage (HyNic) linkage (**Figure 4.5A)**.

HyNic conjugation allowed for addition of diverse EEDs ranging in size from ~800 Da to over 5 kDa (**Figure 2.4**). Conjugation proceeded to completion at room temperature in aqueous and organic solutions containing 1% aniline within 1 hr and was compatible with a variety of EEDs (**Figure 4.5A-C**). Each of these peptides conjugated >90% at EED to Ax conjugation site ratios $\geq$2.5:1 to form soluble, monomeric siRNN-EED conjugates (**Figure 4.5B,C**). Many of these EEDs require high concentrations or coordination between several peptides to efficiently mediate endosomal escape. To test the hypothesis that multivalency enhances the activity of EEDs, we increased EED valency on the siRNN itself to facilitate greater pore formation and membrane disruption through coordination of multiple EEDs. Synthesis of an siRNN with three clustered Ax phosphotriesters on the 3'-end of the Passenger strand allowed for multivalent conjugation of EEDs (**Figure 4.5D**). Modulation of valency through phosphotriester conjugation allows for easy modification of the number and placement of EEDs without having to synthesize additional multivalent branched EED peptides. The modular nature of oligonucleotide synthesis and Ax phosphotriester incorporation provides the opportunity for rapid screening of structure activity relationships through multivalency and varying EED placement along the siRNN molecule. Due to the hydrophobic nature of many of these EEDs, multivalent conjugation did not yield a soluble product for bivalent Endoporter or HA2 peptides, or tetravalent EED1, EED2, and nGM peptides. Despite the large anionic charge of the siRNA and siRNN, the clustering of so much hydrophobicity led to large-scale aggregation and precipitation. Purification by SEC FPLC allowed for effective purification of the final siRNN-EED molecule and capture of free EED peptides for recycling (**Figure 4.5E**).

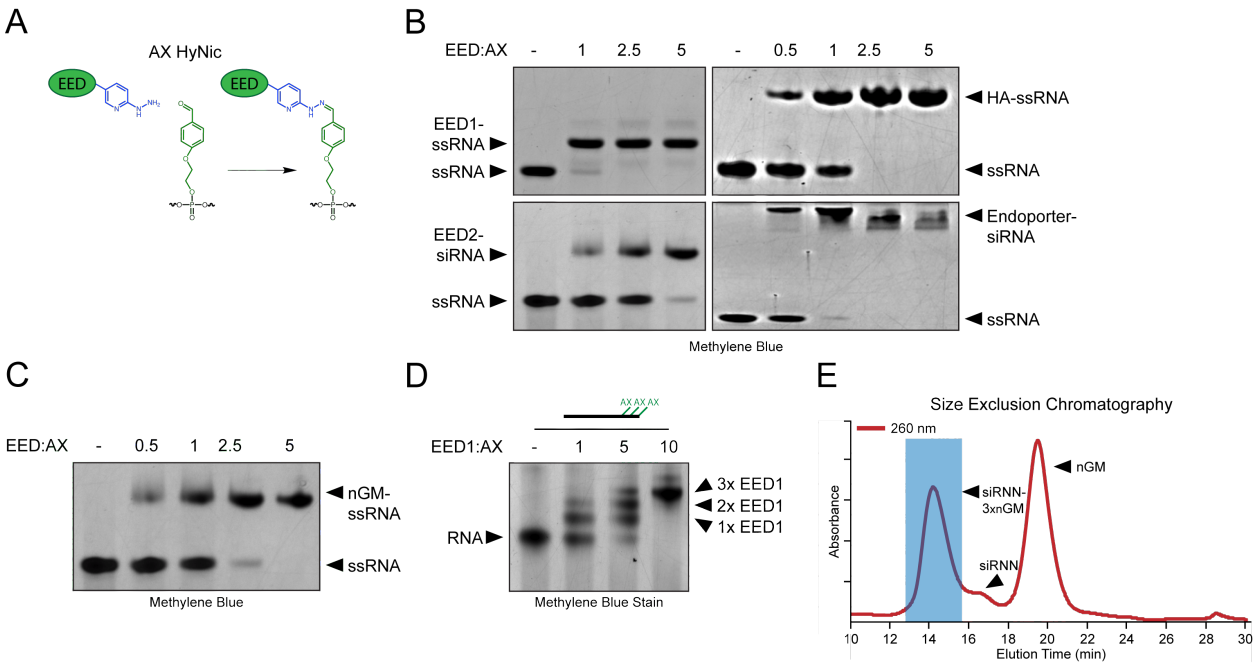


Figure 4.5. HyNic Conjugation of Endosomal Escape Domains.
A) Schematic of hydrazinonicotinic acid (HyNic) conjugation of an endosomal escape domain (EED) to an Ax phosphotriester. **B)** HyNic conjugation of EEDs at increasing EED to Ax conjugation site ratios (EED:AX) drove conjugation to >90% completion at low ratios. EED peptides included EED1, EED2, HA2, and Endoporter. 0.2 nmol of each sample is analyzed by 15% acrylamide/7 M urea denaturing PAGE analysis and visualized with methylene blue. **C)** HyNic conjugation of a pH dependent melitin derivative (nGM) at increasing EED to Ax conjugation site ratios (EED:AX) drove conjugation to >90% completion at low ratios. 0.2 nmol of each sample is analyzed by 15% acrylamide/ 7 M urea denaturing PAGE analysis and visualized with methylene blue. **D)** Conjugation of EED1 to an oligonucleotide with three Ax conjugation sites. Increasing concentration of EED1 drove reaction to >90% completion with a final product of 3x EED1 on a single oligonucleotide. 0.2 nmol of each sample is analyzed by 15% acrylamide/ 7 M urea PAGE analysis and visualized with methylene blue. **E)** Size exclusion chromatography (SEC) profile for a purification of a siRNN-3xnGM. Blue shading denotes fractions collected and pooled to from the final product, separate from unconjugated siRNN and excess nGM peptide.

Unfortunately, unconjugated siRNN eluted close to siRNN-EED conjugates. Therefore, mixed fractions were discarded, resulting in yields between 70-80%.

Conjugation and Assembly of Antibody-RNA Conjugates (ARC)

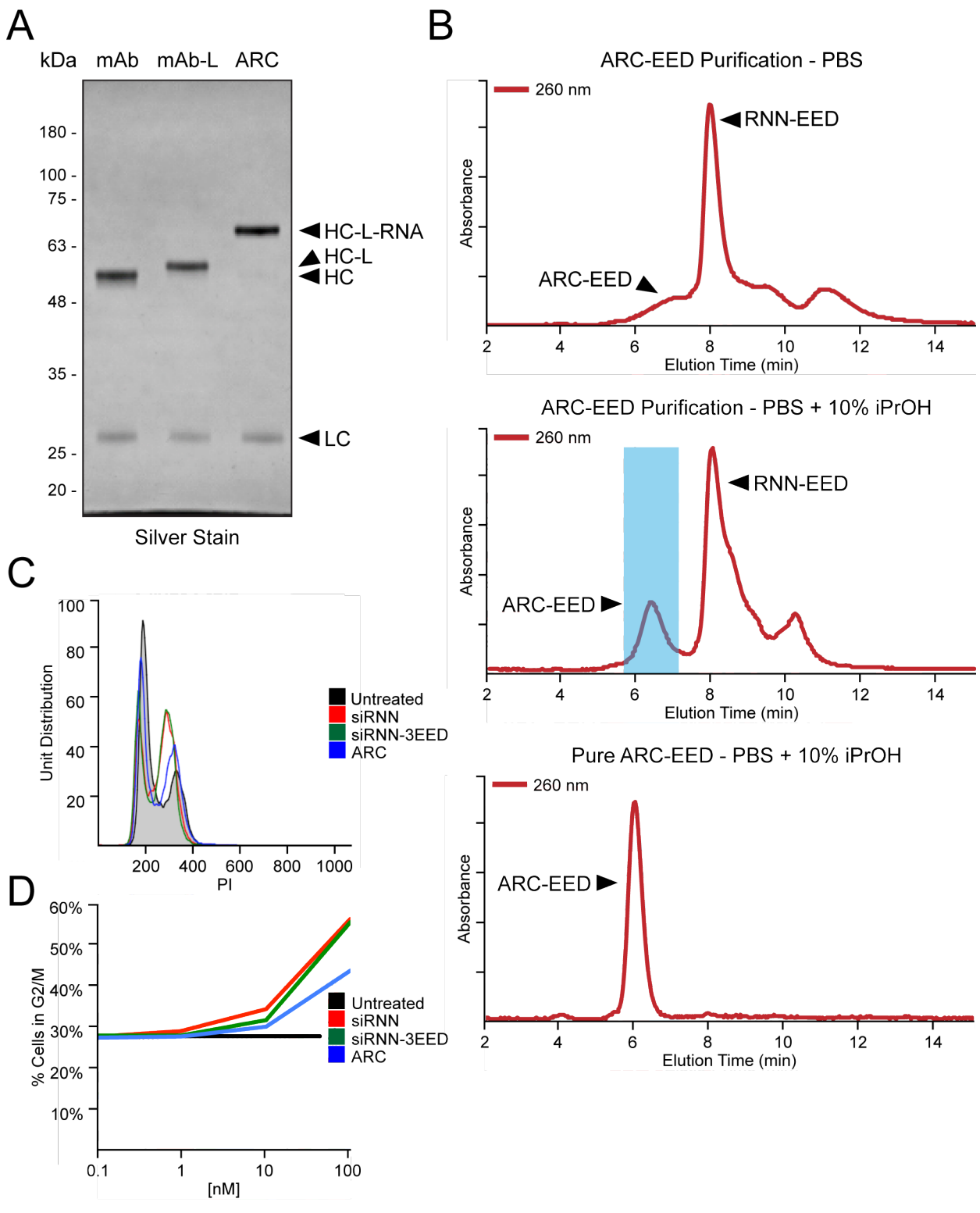
Conjugation of the mAb-L and siRNN-EED pieces to build a full mAb-linker-siRNN-EED (ARC-EED) molecule requires a conjugation chemistry that uses mild conditions to preserve mAb structure and binding. The conjugation chemistry must also be compatible with solid-state oligonucleotide synthesis reagents for efficient incorporation into the oligonucleotide and must be unreactive under HyNic conjugation conditions to allow for sequential site-selective conjugation of EEDs and mAb-L. Additionally, this conjugation step requires near equimolar ratios of each reactant, as high ratios of excess siRNN-EED may be prohibitively expensive to produce. Finally, the reaction must proceed at concentrations that allow both the mAb-L and siRNN-EED to remain soluble. To address these needs, we utilized the copper free CLICK dibenzylcyclooctyl (DBCO) conjugation chemistry. Incorporation of a commercially available 5'-terminal DBCO modification into the Passenger strand resulted in high yields of multifunctional siRNN capable of both HyNic and CLICK conjugation. The DBCO terminal group did not interfere with or degrade during HyNic conjugation and efficiently conjugated to the mAb-L species to form a fully formed ARC-EED (**Figure 4.6A**). Conjugation at a ratio of 2.5:1 siRNN-EED to mAb-L conjugation site (2 per mAb-L) resulted in yields of >90% conjugation within 1 hr at 37 C. The reaction proceeded slower at RT and did not result in complete conjugation. Conjugation at temperatures higher than 37 C resulted in mAb denaturation and aggregation.

Purification of the final ARC-EED by SEC FPLC in PBS running buffer resulted in poor separation of ARC-EED from excess siRNN-EED (**Figure 4.6B**). The poor separation may have been due to transient hydrophobic interactions between the EED groups and hydrophobic regions of the final ARC-EED. However, addition of 10% isopropanol (iPrOH) to the running buffer allowed for complete separation of the ARC-EED product from aggregates and excess

siRNN-EED (**Figure 4.6B**). The addition of iPrOH may increase separation by allowing greater solvent interaction with the hydrophobic EEDs and/or disrupting hydrophobic interactions between the ARC-EED and the siRNN-EED. Analysis of the pooled fractions by SEC FPLC showed a single peak corresponding to monomeric ARC-EED without aggregates or degradation species (**Figure 4.6B**). Final yield was 30-60% of initial mAb-L. Most yield loss was likely due to membrane adhesion during sample concentration by spin filtration. Spin filtration utilized regenerated cellulose membranes and exploration of additional membrane options did not result in increased yields. Larger batches had consistently higher yields as membrane adhesion represented a large percentage of overall material at reactions below 1 mg, while larger batches were able to saturate the membrane with a smaller percentage of overall material.

ARCs lacking endosomal escape domains were purified by the same method, resulted in similar yields, and remained soluble following concentration to >1 mg/mL. However, the addition of EEDs caused ARC-EEDs to crash out of solution within 1-4 hr following concentration, representing a major obstacle to the production of ARCs. Conjugation of hydrophobic chemotherapeutics poses a similar challenge in the ADC field, with highly hydrophobic cargos leading to precipitation and aggregation. To address the problem of ADC solubility, excipients such as sugars and amino acids are often added. Addition of 40 mM arginine to the DBCO reaction and the final purified ARC-EED resulted in improved solubility of ARC-EEDs with low numbers of hydrophobic EEDs. However, addition of hydrophobic SPTE phosphotriesters to the siRNN molecule or increased numbers of EEDs resulted in precipitation following concentration, even in the presence of arginine. Addition of sucrose and histidine was also investigated, but resulted in increased precipitation. In the absence of concentration by spin filtration, the final product remained soluble but in a relatively large volume (800 µL – 1.5 mL).

Figure 4.6. Antibody RNA Conjugates.
A) Sequential conjugation of Linker (L) and siRNA with a 5'-Passenger strand DBCO modification onto an αEGFR antibody (mAb). DBCO conjugations were carried out at a 2.5:1 siRNN-EED to MTG conjugation tag (2 per mAb-L) ratio at 37 C for 1 hr. 0.5 µg of each purified sample was loaded into each lane of a 10% SDS PAGE gel and silver stained for analysis. Sequential conjugation showed >90% conjugation of antibody-linker (mAb-L) and antibody RNA conjugate (ARC). **B)** Bulk purification of ARC conjugation reaction by SEC. Purification with PBS resulted in poor separation (Upper panel). Addition of 10% isopropanol (iPrOH) resulted in increased peak separation (Middle panel). Collected fractions are highlighted in blue and reveal a pure peak without contaminating aggregates or siRNN-EED (Lower panel). **C)** Transfection of siRNN, siRNN-3xEED, and antibody RNA conjugate (ARC) targeting PLK1 mRNA in A431 cells. Knockdown of PLK1 resulted in mitotic arrest, revealing a G2/M peak increase by propidium iodide (PI) staining and FACS analysis. siRNN, siRNN-3xEED, and ARC transfections all resulted in an increase in the G2/M peak at ~350 units and a reduction in the G1/G0 peak at ~175 units. **D)** Quantification of the Area under the curve by percentage if G2/M arrested cells.


A
kDa
mAb
mAb-L
ARC
180 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
HC-L-RNA
HC-L
HC
LC
Silver Stain
B
ARC-EED Purification - PBS
260 nm
RNN-EED
ARC-EED
Absorbance
Elution Time (min)
ARC-EED Purification - PBS + 10% iPrOH
260 nm
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Pure ARC-EED - PBS + 10% iPrOH
260 nm
ARC-EED
Absorbance
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C
Unit Distribution
PI
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siRNN
siRNN-3EED
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D
% Cells in G2/M
[nM]
Untreated
siRNN
siRNN-3EED
ARC

The concentration of the final product could be improved without spin filtration by increasing the size of the batch to >1-3 mg, but production of such large batches proved costly and difficult. Despite these issues, a purified ARC and ARC-EED was produced and could be administered to mice via intraperitoneal injection.

In Vitro Testing of ARCs

Conjugation of large peptides and macromolecules to an siRNA can alter its ability to induce an RNAi response if the added macromolecules interfere with loading or processing by the RNAi machinery. In constructing the ARC-EED, we added a >150 kDa mAb to the 5'-end of the Passenger strand and several EEDs to the 3'-end of the same strand. The conjugations utilized here were irreversible and so may interfere with RNAi activity. To test whether addition of a mAb and EEDs to the siRNN altered its ability to induce and RNAi response, siRNN, siRNN-EED, and ARC-EED were transfected into A431 cells and assayed for knockdown of target PLK1 activity. Knockdown of PLK1 leads to G2/mitotic (M) arrest and can be assayed by propidium iodide (PI) staining and FACS analysis for an increased 4n DNA peak. Control transfection of siRNN and siRNN-EED led to an equal increase in the percentage of cells in the G2/M peak as compared to untreated cells, suggesting that 3'-addition of EEDs to the Passenger strand does not affect induction of RNAi (**Figure 4.6C,D**). Transfection of the ARC-EED resulted in a slight increase in the G2/M peak, but to a lesser extent than the siRNN or siRNN-EED transfections. The knockdown of PLK1 by ARC-EED suggests that irreversible 5'-conjugation of a mAb to the siRNN does not interfere with RNAi induction, while the magnitude of the response suggests that the transfection was not as efficient as the siRNN and siRNN-EED. This reduction in efficiency was likely due to the mAb interfering with proper nanoparticle formation, rather than interference with the RNAi response.

In vitro treatment of antigen positive cells was carried out with αCD33, αEGFR, and αHer2 ARC and ARC-EED. Cell Lines were targeted with PLK1 and GFP siRNA sequences.

Unfortunately, for all three CD33, EGFR and Her2 targeting ARC and ARC-EED, no RNAi response was observed for any mRNA target. A slight reduction in growth and an increased G2/M population on FACS was observed for αEGFR ARC-EED, though this effect was also seen in cells treated with αEGFR mAb alone. This effect was likely due to the source of our mAb as the EGFR mAb was developed to have a therapeutic effect on its own, without a cargo. If the ARC-EEDs have an effect, it is slight and may be difficult to distinguish from that of the targeting mAb itself.

Biodistribution of ARCs

Endogenous and therapeutic mAbs have long half-lives *in vivo*, owing in part to recognition and binding by Fc receptors (FcR) and their molecular weight above the renal threshold (Sanz et al., 2004; Roopenian and Akilesh, 2007). In contrast, siRNA and to a lesser extent, siRNNs, have a relatively short half-life. While mAbs have exquisite selectivity for their target antigens, oligonucleotides are rapidly cleared by the kidneys and taken up non-specifically by scavenger receptors in the liver. To investigate the changes to biodistribution that conjugation has on our mAbs, we labeled αEGFR and αCD33 mAbs with an IR800 infrared dye (mAb-IR800). Purified mAb-IR800 wes injected into NRG mice with subcutaneous EGFR+ A431-Luc tumors. αEGFR mAb-IR800 localized strongly to the tumors as well as the liver and spleen. Unfortunately, off-target αCD33 mAb-IR800 showed a similar biodistribution, though tumor fluorescence was slightly lower than the αEGFR mAb-IR800 (**Figure 4.7A-D**). This non-specific biodistribution may be due to the poor vascularity of the subcutaneous tumor trapping mAbs in a non-specific manner. The spleen contains FcR+ immune cells that may bind the Fc region of our mAbs in an IgG specific, but antigen non-specific manner, explaining the observed IR800 fluorescence. Some fluorescence was observed in the kidneys and may be a result of free IR800 (**Figure 4.7A**). It is unlikely that intact mAb-IR800 would be filtered by the kidneys due to its size being in excess of the ~60 kDa size cutoff for kidney filtration. In contrast, a

much smaller EGFR targeting centyrin (14 kDa) RNA conjugate (total mass = <35 kDa) is below this threshold and exhibits strong kidney accumulation and low tumor targeting (**Figure 4.7A,B,D**).

The binding, biodistribution, and kinetics of mAbs may be altered following the conjugation of our siRNA or siRNN cargo. To test this, Nu/Nu mice with CD33+ THP-1-Luc tumors were injected with αCD33 mAb-IR800, αCD33 ARC-siRNA-IR800, and αCD33 ARC-siRNN-IR800 and observed daily for 7 days. IVIS imaging revealed strong IR800 signal in the liver and tumors of mAb-IR800 injected mice within 24 hr that persisted for 5 days (**Figure 4.8A**). Dissection of the organs revealed strong liver and tumor IR800 signal 7 days post injection (**Figure 4.8C**). In contrast, injection of IR800 labeled αCD33 ARC-siRNA and αCD33 ARC-siRNN conjugates revealed more widespread biodistribution at day 1, followed by strong liver IR800 signal that persisted through the 7 day observation period (**Figure 4.8B**). No IR800 signal was observed in the tumors of αCD33 ARC-siRNA or αCD33 ARC-siRNN treated mice (**Figure 4.8C**). Plasma samples taken from these mice 30 min post injection revealed a strong IR800 signal that disappeared entirely by 24 hr, suggesting that the ARC-siRNA and ARC-siRNN were rapidly cleared from circulation and accumulated in the liver, sequestered from their tumor targets (**Figure 4.8D**).

Imaging of IR800 dyes is useful for tracking biodistribution in live animals; however, IR800 labeling itself can alter biodistribution as hydrophobic dye conjugates are taken up efficiently by the liver. While mAb-I800 retained its tumor targeting capabilities, the addition of IR800 to an ARC may enhance liver accumulation in addition to the oligonucleotide, preventing any tumor targeting that an unlabeled ARC may possess. Despite these caveats these results demonstrate that the addition of nucleic acids to the mAb changes the distribution and half-life of the mAb to be more similar to that of the oligonucleotide cargo, potentially limiting the therapeutic potential of the ARC without further modification.

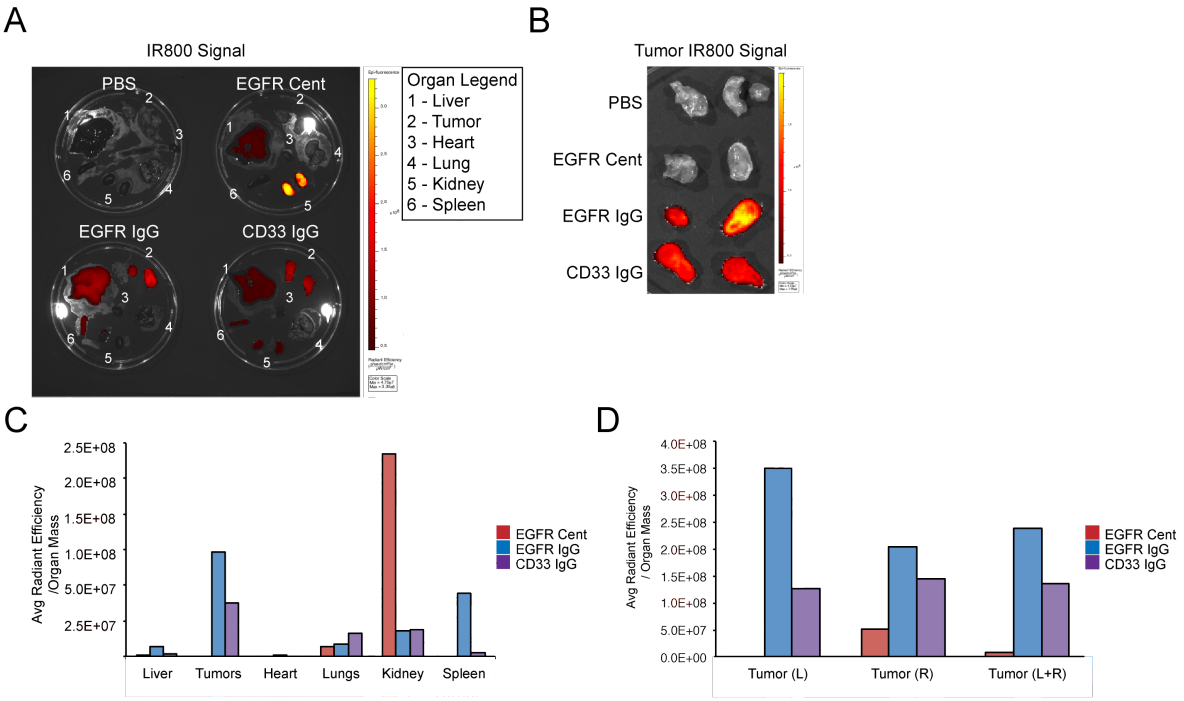

Figure 4.7. *In Vivo* Biodistribution of IR-800 Antibodies.
A) NRG mice with subcutaneous A431-Luc tumors (300 mm^3) were injected IV with αEGFR centyrin-IR800 (EGFR Cent) (10 µg), αEGFR-IP800 (EGFR IgG) (23 µg) or αCD33-IR800 (CD33 IgG) (23 µg). Equal moles of IR800-tagged molecules were injected into each mouse. I800 imaging was carried out on an IVIS. Mice were dissected and imaged at 24 hr for IR800 signal. **B)** IR800 imaging of each dissected tumor. **C)** Quantification of organ IR800 signal normalized to tissue mass. **D)** Quantification of tumor IR800 signal normalized to tumor mass.

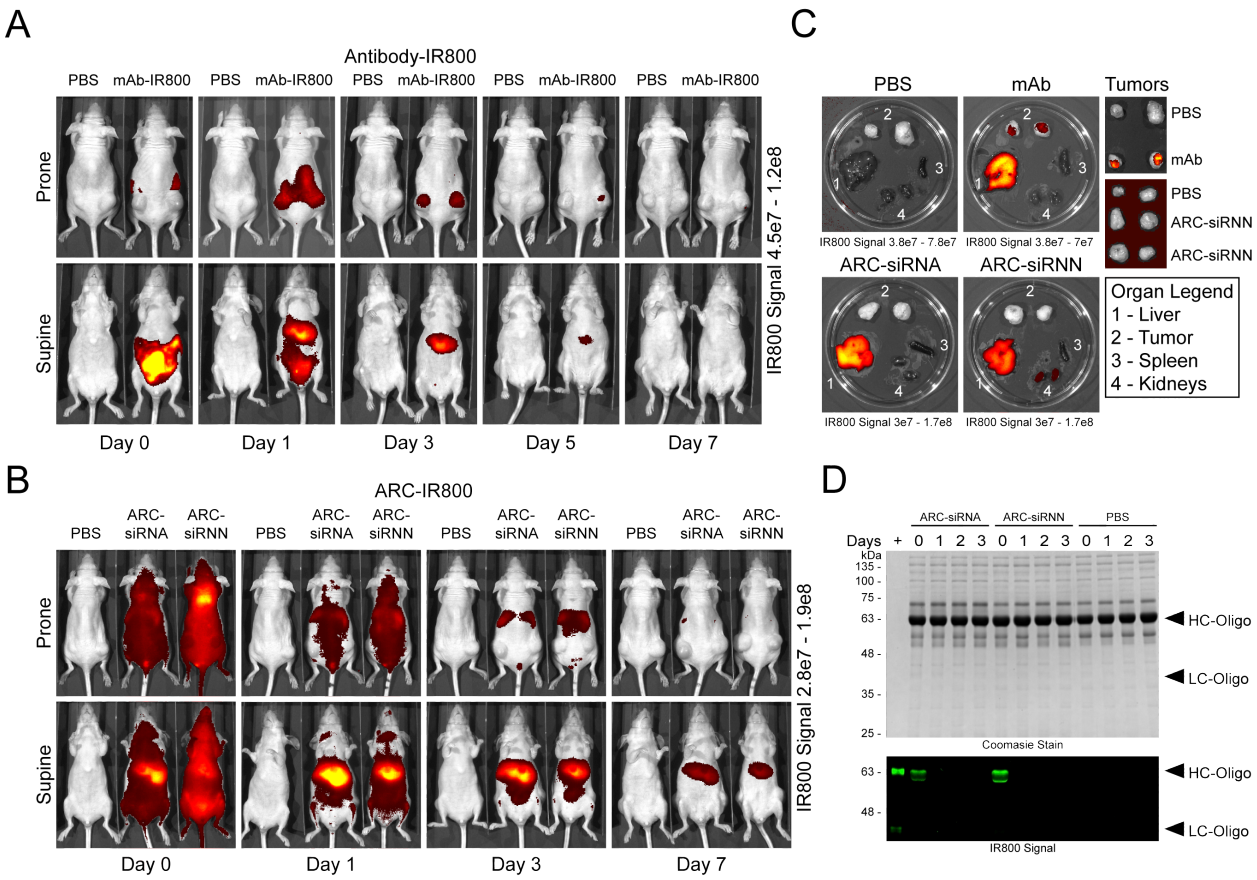

Figure 4.8. ARC Biodistribution.
A) Nu/Nu mice with subcutaneous THP-1-Luc tumors (250 mm^3) were injected with αCD33-IR800 (mAb-IR800) or PBS. 100 µg of mAb-IR800 was injected IP in a volume of 1-1.5 mL. Mice were imaged at 1 min post injection and every day for 7 days before mice were sacrificed, dissected and imaged at 7 days for IR-800 signal. **B)** Nu/Nu mice with subcutaneous THP-1-Luc tumors (250 mm^3) were injected IV with αCD33-siRNA (ARC-siRNA) or αCD33-siRNN (ARC-siRNN). 54 µg of IR800-tagged molecules were injected in each mouse. Mice were imaged every day for 7 days before mice were sacrificed, dissected and imaged for IR800 signal. **C)** Imaging of bulk organ IR800 dye uptake 7 days following treatment. Organs are taken from mice in Panel A and B. Tumor imaging of IR800 signal range is 3.6e7 – 4.8e7 (upper panel) and 1.3e7 - 1.7e8 (lower panel). **D)** Plasma samples from mice post injection of IR800 labeled ARC-siRNA and ARC-siRNN. Submandibular blood samples were taken 30 min after injection and each day following injection for 3 days. Blood samples were collected in sodium citrate anticoagulant for centrifugation and plasma preparation. Samples were stored at -80 C prior to analysis on SDS PAGE for IR800 signal. IR800 signal was absent in samples after 30 min post injection.

In Vivo Knockdown of ARCs

Therapeutic ADCs target extracellular receptors that are highly specific to the cancer of interest, but are often expressed in low numbers on the cell surface and so utilize cargos with EC_{50} in the picomolar range that are able to effectively kill their target cells at low delivered doses. Similarly, RNAi therapeutics have an EC_{50} in the picomolar range allowing them to affect their target cells even with very low levels of delivery. Whole animal imaging of IR800 can give a false negative for IR800 accumulation as illustrated by comparative images of a whole mouse and its organs after 7 days (**Figure 4.8A,C**). The whole mouse imaging showed no signal after 7 days while organ imaging revealed IR800 signal throughout the liver and tumors. Given the potency of RNAi and the limitations of IR800 imaging, knockdown of target mRNA may be possible with quantities of ARC-EED that are not detectable by IVIS imaging of IR800 at early time points.

To test if ARC-EED is capable of delivering sufficient siRNA/siRNN into the cytoplasm of a target cell, Nu/Nu mice bearing CD33+ THP1-Luc subcutaneous tumors were injected with αCD33 ARC-siRNA-3xnGM or αCD33 ARC-siRNN-3xnGM targeting PLK1 mRNA (**Figure 4.9A).** IVIS imaging was carried out daily to analyze luciferase expression and tumor volume was determined by caliper measurement. Luciferase signal and tumor volume increased over time in all mice (**Figure 4.9B-C**). PBS control treated mice showed significant variability in luciferase signal and tumor growth. No significant reduction in luciferase signal or tumor growth was observed in either αCD33 ARC-EED treated mouse. These results suggest that in its current form, ARC-EEDs may be unable to deliver RNAi therapeutics to the cytoplasm of their target cells.

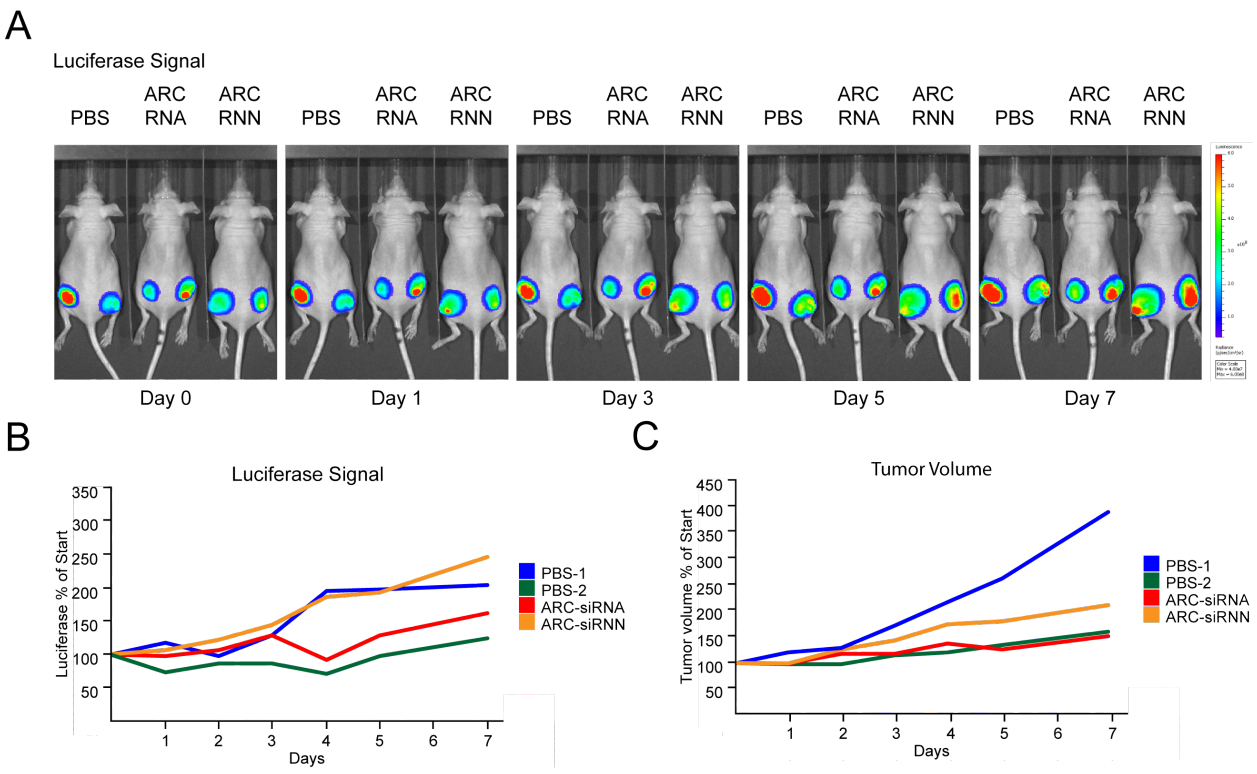

Figure 4.9. ***In Vivo*** **Knockdown of PLK1 in Subcutaneous Tumors.**
A) NU/Nu mice with subcutaneous THP1-Luc tumors (250 mm^3) were injected IP with αCD33-siRNA-3xGM3 (ARC-siRNA) (0.21 mg/kg), αCD33-siRNN-3xGM3 (ARC-siRNN)(0.12 mg/kg), or PBS control in a volume of 1-1.5 mL. Both ARC siRNA cargos target PLK1-5. Mice were imaged daily for luciferase signal and tumor volume for 7 days. **B)** Quantification of tumor luciferase expression by IVIS imaging. **C)** Tumor volume over time measured by caliper.

CONCLUSIONS

siRNA therapeutics have tremendous potential to treat a wide variety of genetic diseases. The chemical properties of siRNAs require a targeting domain to deliver the siRNA therapeutic to the tissue of interest and an EED to escape the endosome. siRNA conjugated to GalNAc effectively targets the hepatocytes and elicits robust and long term RNAi responses in clinical trials. However, extra-hepatic delivery of siRNA therapeutics has not seen the same success and targeting domains for extra-hepatic tissues are greatly needed. mAbs offer an attractive tool for targeting a variety of extra-hepatic tissues and advances made in the fields of ADCs offer insights into the problems and solutions that face the construction of ARCs. However, unlike membrane permeable chemotherapeutics, siRNAs have no ability to escape the endosome and require an additional endosomal escape mechanism. Well-defined, site-specific conjugation of a mAb and endosomal escape domains to a siRNA requires multifunctional conjugation strategies and has not been demonstrated prior to this work. Previous work in our lab provides a flexible and robust platform for modification of the siRNA phosphodiester backbone to create siRNN molecules. These modifications can be designed to include conjugation sites that can be placed anywhere along the siRNN molecule. Here I have described a novel site-specific, multifunctional, and multivalent conjugation strategy for the construction of ARCs.

Construction of the full mAb-siRNN-EED molecule required several well defined site-specific conjugation and purification strategies. mAb conjugation to the siRNN was mediated by an intermediate peptide linker that was enzymatically conjugated to an engineered conjugation handle present on the C-terminus of the HC by MTG. The final mAb-L contained two conjugation sites and retained binding activity and specificity following conjugation (**Figure 4.4A**). A variety of EEDs were efficiently conjugated to the Passenger strand as monovalent or multivalent siRNN-EEDs. Final conjugation of the mAb-L and siRNN-EED was accomplished utilizing copper-free DBCO CLICK chemistry. Despite the addition of bulky EEDs and

conjugation to a >150 kDa mAb, siRNN-EED and ARC-EED retained RNAi activity *in vitro* following transfection (**Figure 4.6C**).

Final purification of the ARC-EED presented a challenge, as initial SEC separation of ARC-EED from siRNN-EED was low and final ARC-EED rapidly precipitated following purification. Addition of 10% isopropanol allowed for increased SEC peak separation and addition of arginine increased solubility of the final product. However, precipitation problems remained for ARC-EEDs containing highly hydrophobic siRNN-EED groups, limiting the types and numbers of EEDs available and necessitating intraperitoneal injections *in vivo*.

In vivo biodistribution studies showed proper localization of mAb-IR800 conjugates to subcutaneous tumors, though tumor targeting appeared to be non-specific, perhaps due to the limitations of subcutaneous tumor models. However, biodistribution of ARC-siRNA and ARC-siRNN revealed rapid clearance from circulation (presumably by the liver) and no tumor localization, suggesting that the biodistribution and pharmacokinetics of ARCs are dominated by the siRNA/siRNN cargo, rather than the mAb (**Figure 4.8A-D**). This may be addressable by use of hydrophilic OSATE phosphotriester groups (Meade et al., 2014). Additionally, ARC-EEDs showed no anti-tumor activity in subcutaneous tumor models, suggesting that the current generation of ARCs is not capable of delivery and RNAi *in vivo*.

Despite the failures of the first generation of ARCs, this work developed a robust and flexible approach to construction of site-specific, multivalent, and multifunctional siRNN conjugation that builds upon the siRNN platform. Advances in endosomal escape domains and linker chemistries can be immediately applied to this platform, allowing for rapid development of the next generation of ARCs. The flexibility of the siRNN phosphotriester platform allows for modification of the siRNN surface for modulation of pharmacokinetics and biodistribution. Advances made in the phosphotriester technology may provide increased circulation times, reduced non-specific liver accumulation, and biodistribution more similar to their mAb targeting domain alone.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

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ABSTRACT

The discovery of RNAi and the subsequent discovery that exogenous small interfering RNAs (siRNAs) could affect powerful post transcriptional gene regulation opened up a new class of therapeutics with exquisite target selectivity, potency, and the potential to treat genetic and pandemic viral disease. Despite the promise of RNAi therapeutics, siRNA has a long list of unfavorable drug-like properties and, critically, has no ability to enter cells on its own. Conjugation of siRNAs to *tris*-N-acetylgalactosamine (GalNAc) revolutionized the siRNA therapeutic field by providing a small, monomeric, soluble siRNA delivery method. Despite this success, extra-hepatic targeting and endosomal escape remain major hurdles for the field to overcome if the potential of RNAi therapeutics is to be realized. To address these challenges, I have built upon our laboratory's previous work on small interfering ribonucleic neutrals (siRNN) to develop conjugation strategies that provide rapid, modular, and well-characterized multifunctional siRNA therapeutics. I utilized this platform to create mannose targeted RNA conjugates (TRCs) capable of delivering siRNA into CD206+ models of tumor-associated macrophages (TAMs). Mannose-TRCs elicited robust silencing of their target mRNA *in vitro*, but failed to produce a specific RNAi response *in vivo* due to limitations with the *in vivo* model. Small ligands like GalNAc and mannose that are capable of targeting specific and highly expressed receptors are rare and antibodies offer greater opportunity for extra-hepatic targeting. I developed a site specific and quantitative strategy for conjugation of siRNN to therapeutic antibodies to form antibody RNA conjugates (ARCs). ARCs maintained antibody binding and RNAi activity following transfection, but failed to deliver siRNA or silence target genes on their own. Addition of endosomal escape domains (EED) required development of additional conjugation chemistries and resulted in multifunctional TRCs and ARCs. Mannose-TRC-EEDs saw a slight increase in RNAi activity with the addition of EEDs, while ARCs saw no benefit *in*

vitro or *in vivo*. Addition of EEDs also presented problems with solubility in both the ARC and TRC platforms. Taken together, this work provides a framework for the development of next-generation ARCs and TRCs and highlights problems that need to be addressed in future work.

INTRODUCTION

The discovery of RNA interference (RNAi) in 1998 (Fire et al., 1998) and the subsequent demonstration in 2001 that exogenous short interfering RNA (siRNA) could affect potent post transcriptional gene regulation provided the potential for a new and highly selective way to treat human disease (Elbashir et al., 2001). siRNA has many promising attributes as a therapeutic, including an EC_{50} in the picomolar (10^{-12}) range and exquisite target selectivity for all mRNA (Bumcrot et al., 2006). While traditional small molecule therapies struggle to target transcription factors and many oncogenes, siRNA has the potential to target all mRNA, including cMyc and Kras mutants (**Figure 1.2**). As a result, siRNA has the potential to treat a wide variety of human disease, from cancer to pandemic viral outbreaks to Parkinson's Disease (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017). Moreover, siRNA has the potential to pharmaco-evolve the targeting sequence to keep pace with mutations in diseases driven by genetic change, such as cancer and influenza, a feat that no other clinical modality can perform (Dowdy, 2017). Due to the catalytic effect of siRNA, a single dose is capable of prolonged pharmacodynamic effects, with a single subcutaneous GalNAc-siRNA dose showing efficacy past 6/9 months in liver hepatocytes (Fitzgerald et al., 2017; Alnylam, 2017). siRNAs can also be synthesized in a scalable and sequence-independent manner, allowing for rapid production of siRNAs targeting any mRNA target (Beaucage and Iyer, 1992).

However, despite the promise of siRNA as a potential therapeutic, it has several attributes that dramatically limit its therapeutic utility. The 40 negative charges of the siRNA phosphodiester backbone and the 14,000 Dalton (Da) size prevent siRNA molecules from crossing the cellular or endosomal membranes (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017). These attributes also make siRNAs pharmacokinetically (PK) unfavorable, as naked siRNA is removed from the bloodstream by the kidneys within minutes of injection into a mouse (Merkel et al., 2009). Additionally, native (2'-OH) double stranded siRNAs are recognized as invading nucleic acids by multiple cellular defense mechanisms, including

extracellular Toll-Like Receptors (TLR-3, -7, -8) and intracellular sensors, retinoic acid inducible gene (RIG-I) and melanoma differentiation associated protein 5 (MDA-5) (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017; Gantier and Williams, 2007; Iversen et al., 2013; Juliano et al., 2014). These difficulties necessitate the use of delivery agents to both assist siRNAs crossing cell membranes and to remain in circulation for longer periods of time. Consequently, the major obstacle prohibiting effective RNAi therapeutics has been delivery. Unsurprisingly there has been significant attention and investment of time and resources to solve the delivery problem by harnessing and developing a wide array of technologies (Dowdy, 2017; Juliano, 2016; Khvorova and Watts, 2017).

To address these problems, our lab developed small interfering ribonucleic neutrals (siRNNs) containing neutralizing phosphotriester groups based on a mononucleotide HIV prodrug inhibitor containing a bioreversible *t*-butyl-S-acyl-2-thioethyl (*t*Bu-SATE) group (Puech et al., 1993; Lefebvre et al., 1995; Gröschel et al., 2002). These neutralizing phosphotriesters are converted into charged phosphodiesters by intracellular restricted thioesterases to yield a wild type siRNA that can be loaded into RISC to induce an RNAi response. This technology increases serum stability >24 hr, avoids innate immune stimulation, and increases *in vivo* circulation time (Meade et al., 2014). While neutralization of the charged phosphodiester backbone with phosphotriesters significantly improved the drug-like properties of siRNA therapeutics, siRNNs still require a delivery domain for tissue targeting and delivery into the cytoplasm. Fortunately, *t*Bu-SATE technology is amenable to modification, allowing for addition of conjugation handles for site selective conjugation of targeting domains (TD) and endosomal escape domains (EED). Conjugation of a siRNN to GalNAc targeting hepatocyte asialoglycoprotein receptor (ASGPR) showed effective long term knockdown of target mRNA in a dose dependent manner (Meade et al., 2014).

Beyond our lab, GalNAc targeting has revolutionized the RNAi therapeutic field with several phase III clinical trials underway. The ability of GalNAc to effectively deliver siRNA into

hepatocytes has been so successful that hepatic delivery of siRNA therapeutics can be considered solved. Despite this success, advances in extra-hepatic delivery of siRNA have been limited, necessitating development of extra-hepatic targeting domains. Efforts to target nucleic acids to extra-hepatic tissues have tested a variety of ligand/receptor pairs including glucagon-like peptide-1 (GLP-1) targeting pancreatic β-islet cells, and folate and Arg-Gly-Asp (RGD) peptides for tumor targeting (Ämmälä et al., 2018; Alam et al., 2011; Cen et al., 2018; Manoharan, Rajeev and Jayaraman, 2008). However, ligand/receptor pairs that are highly expressed and rapidly internalize are limited and the clinical efficacy of current extra-hepatic targeting domains have yet to be demonstrated for siRNA therapeutic applications.

An attractive alternative to these endogenous receptor/ligand pairings is the use of monoclonal antibodies (mAbs) that have several decades worth of clinical validation for a wide array of therapeutic targets. Given their ability to bind antigens with superb specificity and their widespread use as therapeutic agents, mAbs have been potent targeting agents for a variety of therapeutics including Antibody-Drug Conjugates (ADCs) and Radionuclide Antibody Conjugates (RACs). ADCs have had 60+ years of development with four FDA approvals and >65 ongoing clinical trials (Beck et al., 2017; Mullard, 2013). ADCs have faced many of the challenges that siRNA therapeutics currently face and the solutions provided for each problem have potential applications for siRNA therapeutics as Antibody RNA Conjugates (ARCs).

Genentech first utilized ADC chemistry technologies to develop an siRNA conjugation scheme with their engineered cysteine THIOMAB platform and demonstrated efficacy in a variety of *in vitro* tumor models but showed very poor delivery in subcutaneous tumor models (Cuellar et al., 2015). Prediction of success *in vitro* based on receptor number or internalization route was inconsistent and *in vivo* translations of *in vitro* success yielded modest tumor reduction at best. While mAb mediated siRNA delivery was accomplished in select cell lines, this work demonstrated that successful targeting of an internalizing extracellular receptor is not

sufficient for cytoplasmic delivery. However, this technology represented the first site-specific conjugation of siRNA to a mAb.

While the Genentech ARCs demonstrated successful targeting and internalization, their limited activity was likely due to endosomal entrapment, prohibiting cytoplasmic availability and subsequent RNAi induction. Escape from the endosome represents the rate-limiting step for delivery of siRNA therapeutics and a major obstacle towards successful implementation of ARCs and other targeted siRNA therapeutics. To effectively deliver siRNA therapeutics, endosomal escape mechanisms will have to be employed. A variety of endosomal escape domains have been developed in our lab and others that function through a variety of membrane disruption, pore formation, and unknown mechanisms to enhance endosomal escape of nanoparticles, fusion proteins, peptides, and other macromolecular cargo. In order to take advantage of endosomal escape domains, multifunctional conjugation schemes are needed to conjugate both targeting domains and endosomal escape moieties to a single siRNA.

CONCLUSIONS

Mannose Targeted RNA Conjugates

In order to construct RNAi therapeutics capable of both tissue targeting and endosomal escape, I developed a phosphotriester modification (Kx) capable of copper catalyzed CLICK conjugation chemistry. The Kx phosphoramidite was easily synthesized and incorporated into solid-state oligonucleotide synthesis to produce siRNN molecules with single and multiple insertions along the siRNN backbone. Conjugation of mannose targeting domains (TD) to the Kx phosphotriester yielded >90% conjugation and was easily purified by size exclusion chromatography (SEC) FPLC. Despite the irreversible nature of the Kx phosphotriester and the added size of the mannose TD, RNAi activity was maintained. Addition of Kx phosphotriesters on the 5'- and 3'- ends of the passenger strand localizes the resulting TD away from the major groove where TRBP makes contact (Ryter and Schultz, 1998). Additionally, the PEG linkers

within the mannose TD provide distance between the siRNA backbone and the bulk of the TD. These properties allow for unhindered TRBP binding and loading of the siRNA into Ago2. Following Ago2 loading, the passenger strand is cleaved and removed, leaving an unmodified Guide strand for RNAi. These results were consistent with the maintained RNAi activity of siRNNs containing irreversible Ax phosphotriesters for GalNAc TD conjugates (Meade et al., 2014).

Cyannine (Cy3) dye labeled mannose-TD labeled and was internalized into bone marrow derived macrophages (BMDM) alone and following conjugation with siRNA/siRNN to form targeted RNA conjugates (TRC). Importantly, Cy3 signal was competed away with addition of mannan, a known CD206 ligand, indicating that CD206 was responsible for the observed TRC binding and internalization (**Chapter 3**). Further, mannose-TRCs were capable of eliciting an RNAi response in BMDM. Increasing mannose valency improved RNAi knockdown with 3 < 6 = 9 = 18 *in vitro*. Importantly, TRCs with off target luciferase siRNA sequences or non-targeting control GalNAc TDs did not elicit an RNAi response, indicating CD206 mediated delivery and siRNA sequence dependent RNAi response. Interestingly, M2 macrophages express a macrophage galactose lectin (MGL) that binds to GalNAc (Kawasaki et al., 1986; Suzuki et al., 1996). Despite expression of MGL, GalNAc-TRCs did not result in knockdown of target mRNA, highlighting the importance of selecting a receptor with productive internalization, in addition to cell type specificity and ligand binding.

Surprisingly, mannose-TRCs delivered their siRNA cargo into the cytoplasm despite having no endosomal escape domain (EED). If we assume that a treatment of 10 nM in 500 µL with 2.5 x 10^5 cells where every molecule of mannose-TRC is delivered evenly to all cells over the 72 hr treatment period, then 1.2 x 10^7 molecules would have been delivered into each cell. RNAi requires only 1,000-5,000 molecules of siRNA per cell to elicit a robust knockdown of target mRNA (Wittrup et al., 2015). This represents <0.01-0.05% of the total TRC population, indicating that the mechanism of endosomal escape for mannose TRC is exceedingly inefficient.

Addition of endosomal escape domains EED1 and BEED resulted in soluble mannose TRC-EEDs. Only the BEED peptide enhanced RNAi knockdown suggesting that valency plays an important role in endosomal escape. However, mannose TRCs with higher EED valency were insoluble, requiring advances in EED technology before the full potential of endosomal escape domains is fully realized. A concern with this approach was the formation of nanoparticles due to the hydrophobicity of the BEED; however, SEC analysis showed monomeric mannose-TRC-BEED conjugates without major aggregation or nanoparticle peaks.

The failure of mannose-TRC to elicit a mannose specific response in the activated peritoneal macrophage model was likely a result of increased macrophage phagocytosis, rather than the inability of the mannose-TRC to deliver. Prior characterization of this model has shown increased macrophage phagocytic capacity (Zhang, Goncalves, and Mosser, 2008). This increase in phagocytosis likely led to nonspecific uptake of the mannose-TRC as well as our non-targeting siRNA and GalNAc-TRC controls. Importantly, despite the absence of CD206 specific uptake, GAPDH knockdown was still dependent on GAPDH siRNA sequence, indicating that RNAi was the cause of the observed GAPDH knockdown. Despite the potential for siRNA delivery into highly phagocytic macrophages, this model is highly artificial and the large localized population of macrophages seen in this model is not recapitulated *in vivo*. Alternate models of M2 TAMs may prove more reliable for mannose TRC screening, but care will have to be taken to avoid non-specific tumor targeting mechanisms due to rapid growth and poor vascularity in subcutaneous tumor models (Kamb, 2005; Mak, Evaniew, and Ghert, 2014).

Antibody-RNA Conjugates

To expand the potential of RNAi therapeutics beyond the liver and the limited available receptor ligand pairs, we also investigated the use of therapeutic monoclonal antibodies (mAb) for targeting of siRNA therapeutics as antibody-RNA conjugates (ARCs). We leveraged the decades of therapeutic mAb development by utilizing clinically validated mAbs as a starting

point for ARCs. We were able to express a variety of mAbs targeting CD33, Her2, EGFR, PSMA, and others using the ExpiCho expression system. Yield and quality differences between batches and specific mAbs presented a limitation on some mAbs but yield and quality could be optimized in the event of a validated candidate and were adequate for our purposes. Further, this system allowed for rapid screening and engineering of amino acid recognition sequences for microbial transglutaminase (MTG) conjugation.

Conjugation of linker peptides via MTG resulted in >90% conjugation in a site-specific manner. Purification by size exclusion chromatography (SEC) FPLC resulted in a pure antibody-linker conjugate (mAb-L) that was free of excess linker peptide and MTG enzyme. Removal of excess MTG enzyme proved critical for the production of monomeric mAb-L as trace amounts of MTG were sufficient to aggregate and precipitate the mAb-L product through continued nonspecific MTG activity. Importantly, conjugation of peptides was site-selective for the C-terminal heavy chain tag that was engineered and MTG conjugation did no disrupt mAb binding or specificity.

Conjugation of EEDs to the siRNN was demonstrated for single and multiple Ax HyNic conjugation sites and resulted in >90% conjugation of a variety of EEDs. This conjugation chemistry has proven to be highly versatile and compatible with a wide range of peptide, carbohydrate, and protein cargos. EED hydrophobicity limited solubility of several multivalent siRNN-EEDs and represents a limiting factor to the production of highly multivalent EED conjugates. This limitation will likely require advances in solubility within the endosomal escape domains themselves as well as in the ARC overall.

Conjugation of siRNN-EED to mAb-L was carried out using DBCO Click chemistry and resulted in >90% conjugation to produce an ARC with a drug to antibody ratio (DAR) of 2 with endosomal escape domains covalently linked to the siRNN. Purification of the final ARC and ARC-EED required addition of 10% isopropanol (iPrOH) to separate ARC-EED from unconjugated siRNN-EED. The inclusion of iPrOH allowed for greater disruption of hydrophobic

interactions between hydrophobic regions of the ARC-EED and the siRNN-EED, allowing for separation by SEC. Following purification, ARC-EED precipitated out of solution, but solubility was restored following addition of excipients to the DBCO reaction and final purification mixture. Despite the effects of excipients on solubility, concentration of the final ARC-EED construct led to poor yields (20-60%) and continued precipitation in ARCs containing multiple EEDs and high numbers of hydrophobic *t*Bu-SATE phosphotriesters. Yield loss was less in larger batches, as irreversible ARC-EED binding to the spin filtration membranes was the primary cause of yield loss. Solubility of the final ARC and ARC-EED proved to be a difficult problem to solve and limited the doses that could be attained *in vitro* and *in vivo*. In order for ARCs to become a viable therapeutic, solubility will need to be addressed, likely through modifications to every part of the ARC construction rather than any one piece alone.

Despite the addition of a full mAb and several EEDs, transfection of siRNN-3xEED and ARC-EED resulted in RNAi activity, indicating that TRBP and Ago2 tolerated the increased bulkiness of the siRNA prior to Passenger strand cleavage. However, treatment *in vitro* and *in vivo* with ARC or ARC-EED alone failed to elicit an RNAi response, likely due to entrapment within the endosome as well as poor biodistribution. Addition of monovalent and multivalent EEDs to the ARC did not improve endosomal escape and may have been due to insufficient endosomal disruption or insufficient dosing due to low solubility. Additionally, irreversible conjugation to the endosomal escape domains may also limit escape as covalent linkage to the EED may localize the siRNN to the membrane and the EED pore, but restrict exit into the cytoplasm.

In vivo biodistribution studies showed that MTG conjugation of an IR800 dye to a mAb did not alter tumor targeting. However, conjugation of siRNA, siRNN, or siRNN-EED to the mAb altered the biodistribution significantly to resemble that of untargeted siRNN, with rapid liver accumulation and no tumor targeting. These results match current information given at several Oligonucleotide Therapeutic Society (OTS) conferences where ARCs with a DAR = 2 are taken

up rapidly by scavenger receptors in the liver while ARCs with a DAR = 1 are able to target tumors and tissues with less liver accumulation. To address these points, ARCs with a DAR = 1 reduce the delivered siRNA cargo by half, increasing the required doses. While an increase in required dosing may be preferred for unmodified siRNA due to complete loss of delivery with DAR = 2 ARCs, the siRNN platform provides greater opportunity to modify the biodistribution properties of the oligonucleotide and so DAR 2 ARC species should not be entirely abandoned.

In conclusion, my thesis work has shown the first multifunctional, multivalent, site-specific conjugation strategy for functionalization of siRNA and siRNNs with both targeting and endosomal escape domains. This was accomplished by building upon the siRNN platform to achieve a flexible and modular toolset capable of efficient and selective conjugation of a wide array of macromolecules. Additionally, this work represents the first ARC with defined conjugation of siRNA with endosomal escape domains. This work provides a platform to build the next generation of TRCs and ARCs and allows advances in mAb, linker, endosomal escape, and siRNN surface modifications to be implemented immediately without the need for new process development.

FUTURE DIRECTIONS

The mAb-linker-siRNN-EED platform is highly modular, with the ability to easily swap out each piece without the need for modification of the remaining components. This allows for rapid implementation of advances in the fields of antibody targeting, linker chemistry, siRNN stability and potency, and EED potency and solubility. Indeed, this capability may prove necessary as construction of a therapeutic ARC-EED will likely require advances to be made for each component to enhance potency, solubility, biodistribution, and tissue specificity.

Extra-Hepatic Targeting

Targeting RNAi therapeutics to extra-hepatic tissues will require identification of tissue specific antigens and receptors capable of targeting by ligands or mAbs. While mAbs have demonstrated great efficacy in delivering chemotherapeutics as ADCs, the properties of chemotherapeutics and siRNA therapeutics may require different antibody screening criteria. Antibody screening specific for siRNA delivery may allow for selection of more productive internalization pathways that allow for greater endosomal escape.

In ADCs, hydrophobic chemotherapeutics are capable of diffusion through endosomal membranes, allowing activity without need for endosomal escape domains. Additionally, chemotherapeutics are capable of diffusing out from the cell and into neighboring cells resulting in the bystander effect. The bystander effect can lead to off target toxicity, but also allows for greater tumor penetration. This characteristic is beneficial to ADCs as clinical dosimetry of radiolabelled mAbs reveals that <0.01% of the injected dose/g of tumor binds in the tumor. Further, the majority of delivered ARC binds within the perivascular tumor region without penetrating into the bulk of the tumor (Lambert and Berkenblit, 2018; Esteban et al., 1987). While mAbs offer superb target specificity and longevity in circulation, their size limits their tissue penetration.

Tumor penetration can be improved by using smaller mAb derivatives that retain the binding specificity of the parental mAb. Fragment antigen-binding (Fab) is a class of mAb derivative composed of one constant and one variable domain of each of the heavy and light chains of a parental antibody. Several Fab formats have been utilized in clinical investigations (Juweid et al., 2000; Becker et al., 1995; Gulec et al., 1995) and have demonstrated increased tumor penetration despite reduced serum half lives (Behr and Goldenberg, 1996). Takeda demonstrated successful *in vivo* delivery of siRNA using a Fab derived from an αTransferrin Receptor (CD71) mAb, illustrating that smaller mAb derivatives are a viable alternative to full mAb targeting of siRNA (Sugo et al., 2016). Similar mAb derivatives have been developed for

clinical targeting applications including single chain variable fragments (svFv) consisting of variable regions of the heavy and light chains linked together with an engineered peptide linker (Bird et al., 1988; Huston et al., 1993). Compared to intact mAbs, scFvs exhibit greater penetration and more even tumor distribution, making them an attractive targeting agent (Colcher et al., 1990; Yokota et al., 1993). scFvs also demonstrate greater tumor to normal tissue ratios compared to full mAbs, but are rapidly cleared from circulation due to their small size, leading to severe nephrotoxicity from conjugated toxic cargo (Behr and Goldenberg, 1996). Clustering of scFvs has demonstrated improved avidity and efficacy for tumor targeting with reduced kidney clearance (Colcher et al., 1990; Yokota et al., 1993; Plückthun and Pack, 1997; Adams et al., 1993; Wu et al., 1996; Adams et al., 1998; Beresford et al., 1999; Pavlinkova et al., 1999; Goel et al., 2000). Multimerization of Fabs and scFvs to improve binding affinity and tumor penetration, and reduce kidney filtration, has taken a variety of simple and exotic forms (Cuesta et al., 2010), each with their own potential for siRNA therapeutics.

Multimerization of Fab and scFv domains for ARC purposes could be accomplished through protein engineering of multivalent proteins as well as through conjugation of single scFV and Fab domains to multiple phosphotriester groups on a single siRNN. While HyNic and DBCO conjugations are compatible with antibody and antibody derived targeting domains, copper Click chemistry can lead to protein misfolding and aggregation, limiting the utility of the Kx phosphotriester in these applications. While the EED peptides can be adapted for Kx conjugation, leaving the Ax phosphotriesters available for HyNic conjugation to Fab-Linker and scFv-Linker conjugates, an alternative approach is to utilize more stable engineered binding proteins that can withstand the harsher conditions of copper Click chemistry.

Single chain nanobodies from the *Camelidae* family are small (~14 kDa) binding proteins that are amenable to selection by phage display and exhibit increased thermal and chemical stability compared to mAbs (Muyldermans, 2013). Specifically, nanobodies have demonstrated structural stability and retained binding capabilities following copper catalyzed Click conjugation

conditions (Ta et al., 2015, 2016). Targeting of siRNA and siRNN conjugates need not be limited to mAbs alone, and the plethora of antibody derivatives and engineered binding proteins offers the opportunity to fine tune binding, tissue penetration, serum half life, toxicity, and solubility beyond what I have demonstrated in my dissertation work.

Next-Generation Linker Chemistries

The ARCs discussed here have utilized an irreversible PEG linker that was chosen for simplicity, stability, and ease of production during initial ARC development. However, the irreversible nature of this linker does not allow for rapid siRNN release from the bulky mAb, sequestering the siRNN cargo throughout much of the endocytic pathway. The entrapped siRNN cargo is only released from the mAb following lysosomal degradation of the mAb or peptide linker. While siRNA and siRNNs with phosphorothioates and fully 2'-modified structures are stable in the lysosomal environment for >24 hr (Nair et al., 2017), the harsh lysosomal environment may damage or degrade the EEDs prior to siRNN endosomal escape. Also, the kinetics of siRNA/siRNN release via nonspecific mAb/linker degradation may be too slow or inefficient to allow potent RNAi.

An alternative to non-cleavable linkers is to utilize linkers that degrade under conditions specific to the endosome or lysosome (**Figure 5.1**). Comparisons between cleavable and non-cleavable linkers demonstrated enhanced anti-tumor activity in ADCs with cleavable linker chemistries (Kovtun et al., 2006). While the biophysical properties of chemotherapeutics and siRNA/siRNNs are quite different, cleavable linkers represent an opportunity for more rapid release of siRNN-EED cargo from the mAb at earlier stages in the endocytic pathway, potentially increasing endosomal escape and RNAi activity.

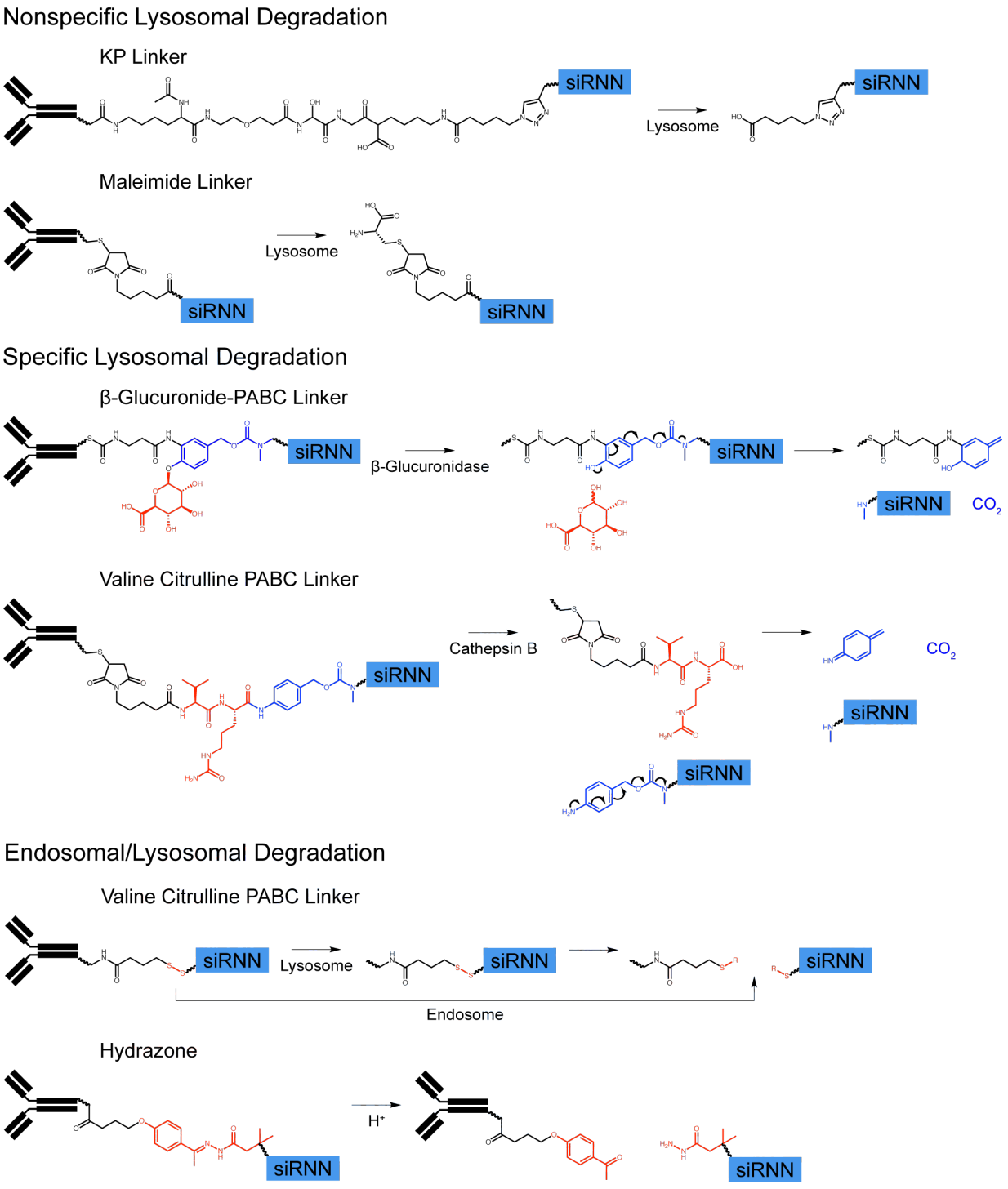

Figure 5.1. ADC Linkers for ARC Adaptation.
Structures and mechanisms of siRNN release for potential ADC linker chemistry adaptations. Linkers are representative and do not preclude modification for solubility and stability purposes. Nonspecifc lysosomal degradation relies on degradation of peptide bonds in the targeting antibody or peptide linker. Specific lysosomal degradation relies on enzymatic recognition sequences within the linker. Endosomal/lysosomal degradation relies on chemical instability within the endosomal/lysosomal compartments.

One promising approach is to use pH sensitive chemistries such as hydrazone linkers that have been utilized in ADCs for decades. However, the lower stability of early hydrazone linkers led to measurable shedding of ADC cargo into the bloodstream (Senter, 2009). This instability was a major contributing factor in the observed toxicity of the first FDA approved ADC, gemtuzumab ozogamicin (Mylotarg) (van Der Velden et al., 2001). This toxicity initially prompted Pfizer to voluntarily pull Mylotarg from the market in 2010, though Mylotarg has since been reapproved by the FDA in 2017 following an adjustment to a lower dose, suggesting that a hydrazone linker may be safe for ARCs. Additionally, inotuzumab ozogamicin (Besponsa) was approved in 2017 with an identical acid-labile 4-(4-acetylphenoxy) butanoic acid hydrazone linker (Research 2017) and shows good stability in human plasma and serum (DiJoseph et al., 2004, 2006; Takeshita et al., 2009). These results indicated that despite the observed linker degradation in serum, the levels are low enough to avoid fatal toxicity and achieve sufficient payload to the target cells of interest.

Another chemically labile approach is to utilize a reducible disulfide linker that takes advantage of the reducing environment of the endosomal and lysosomal compartments (Yang et al., 2006). Reducible disulfide linkers have been utilized in a variety of ADCs in clinical trials and demonstrate efficient delivery and release of chemotherapeutic cargo (Erickson et al., 2010; Lu et al., 2016). While shedding of cargo can be detected for both hydrazone and disulfide linkers, toxicity from cargo shedding is not a concern with ARCs, as untargeted siRNN has no bioavailability or immunogenicity and is effectively inert without the targeting antibody. siRNN shedding is only a concern for target competition with mAbs that do not have siRNN cargo. However, ADCs face this same problem and retain efficacy, suggesting that binding competition with free antibody this is not a substantial issue.

An alternative to chemically labile linkers is to take advantage of the abundant hydrolytic enzymes of the lysosome that have the ability to recognize specific amino acid sequences and

patterns of carbohydrates. The lysosomally restricted nature of these enzymes allows for highly stable linkers that undergo cleavage only within the lysosome. Dipeptide linkers such as valine-citruline (Val-Cit) and phenylalanine-lysine (Phe-Lys) show high stability in circulation and rapid release of cargo after recognition by lysosomal proteases such as cathepsin B (Dubowchik and Firestone, 1998; Dubowchik et al., 2002). This type of dipeptide linker is utilized in the FDA approved ADC brentuximab vedotin (Senter and Sievers, 2012). Another enzyme labile linker takes advantage of the high lysosomal levels and general overexpression of β-glucuronidase in some tumors (Jeffrey et al., 2007; Albin et al., 1993; de Graaf et al., 2002). Linkers containing a β-glucuronide group are serum stable, are rapidly degraded in the lysosome, and have been utilized in several ADCs to link a variety of chemotherapeutic cargos (Jeffrey et al., 2010). One advantage of β-glucuronide linkers is that they are much more hydrophilic than the previously discussed linkers, increasing the solubility of future ARCs, and making this strategy particularly attractive (Kim and Kim, 2015).

Improving siRNN and ARC Biodistribution

The siRNN platform was originally developed for TAT mediated delivery *in vitro* and was further optimized for GalNAc mediated hepatocyte delivery *in vivo*. The relatively small size (<20 kDa) leads to rapid kidney filtration of intravenously administered charged GalNAc-siRNA, while the hydrophobic *t*Bu-SATE phosphotriester decorated neutral GalNAc-siRNN is able to maintain a longer half life through serum albumin binding (Meade et al., 2014). The approach of increasing siRNA circulation time through hydrophobic modifications has been expanded elsewhere through increasing phosphorothioate content and conjugation of hydrophobic cholesterol and phosphatidylcholine-docosahexanoic acid (DHA) (Osborn and Khvorova, 2018). These modifications improve serum half-life through cholesterol association to low density lipoprotein (LDL), and DHA association with high-density lipoprotein (HDL) and albumin. Comparisons between cholesterol and DHA linked siRNA therapeutics reveal differences in half

life as well as biodistribution, suggesting that modifications to hydrophobicity and structure can have distinct impacts on the *in vivo* properties of siRNA therapeutics.

While increasing hydrophobicity and optimizing hydrophobic structure has shown promising results in our lab and others, these technologies have been primarily applied to small siRNA conjugates that are susceptible to rapid clearance by the kidneys. ARCs are much larger and are not prone to renal filtration as a result of their increased size. With this in mind, the serum albumin binding of the *t*Bu-SATE phosphotriester (Meade et al., 2014) and other hydrophobic modifications may be redundant with the pharmacokinetic properties of the conjugated antibody. Further, the hydrophobicity imparted by these modifications is a liability as maintaining solubility of ARCs with these modifications is difficult. IR800 imaging of ARC-siRNA and ARC-siRNN revealed that both accumulate primarily in the liver (**Figure 4.8**), suggesting that increased solubility of the wild type phosphodiester backbone does not alter liver accumulation. However, IR800 has strong liver biodistribution properties on its own and may have overshadowed subtler biodistribution differences between siRNA and siRNN *in vivo* that could alter unlabeled ARC biodistribution.

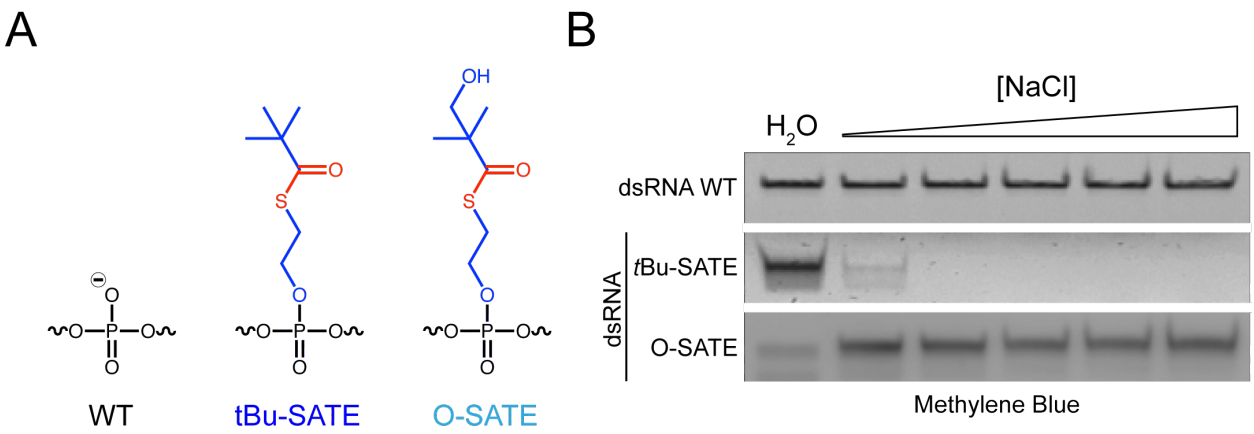

Figure 5.2. *t*Bu-SATE and O-SATE Structures and Solubility.
A. Structure of *t*Bu-SATE and O-SATE phosphotriesters. **B.** Comparative solubility analysis of wild-type phosphodiester siRNA and siRNNs containing 18x *t*Bu-SATE or O-SATE phosphotriesters. Wild-type siRNAs and O-SATE siRNNs remains soluble at high salt concentrations, but *t*Bu-SATE siRNNs suffer from hydrophobic collapse. [NaCl] = 100, 200, 300, 400, 500 mM.

The phosphotriester platform allows for incremental modulation of surface hydrophobicity of the final siRNN between charged phosphodiester and hydrophobic *t*Bu-SATE. Indeed, neutral hydrophilic phosphotriesters have already been developed through addition of a primary alcohol to the *t*Bu-SATE, yielding a hydrophilic O-SATE (**Figure 5.2**). O-SATEs have been shown to increase siRNN solubility at physiologic salt concentrations, maintain *in vivo* activity of GalNAc-siRNNs, and represent a viable approach to increasing siRNN solubility while retaining the charge masking properties of phosphotriesters (Meade et al., 2014). In addition to the O-SATE phosphotriester, >100 phosphotriester groups have been synthesized by our lab with varying functionalities, hydrophobicities, structures, and stabilities, offering a platform to screen for phosphotriesters that improve the biodistribution of ARCs. While the mechanisms behind the current ARC liver accumulation are not well understood, the phosphotriester technology provides a platform for improving the pharmacokinetics of ARCs.

Improving siRNN Potency and Stability

While targeting and biodistribution are critical to the future success of TRCs and ARCs, the importance of siRNA potency cannot be overlooked. Due to the modular nature of the siRNN ARC and TRC platforms, advancements made in the field of siRNA therapeutics can be rapidly integrated for immediate improvements in the ARC platform. Despite utilizing the same *tris*-GalNAc targeting ligand across the past decade, the field of RNAi therapeutics has seen vast improvements potency and reduction of toxicity. These improvements have come primarily from increased stability of the siRNA molecule and reduction in off target effects. Inclusion of phosphorothioates on the ends and full modification of the 2'-OH position of the ribose sugar with 2'-Fluoro (2'-F) and 2'OMethyl (2'-OMe) groups greatly improved stability and reduced immunogenicity to allow *in vivo* delivery (Nair et al., 2014). Since this milestone, improvements in phosphorothioate and 2' modification patterns towards reduced 2'-F content has seen dramatic reduction in dosing requirements (**Figure 1.3**) (Schlegel et al., 2017). The current

siRNN and ARC platform utilizes 2'-F pyrimidines and 2'-OMe purines and updating the 2'-modification pattern to include advances made in the field should reduce dosing requirements similarly for ARCs.

Further improvements to siRNA potency have been made through structural analysis of Ago2. A 5'-phosphate group is required for proper Ago2 recognition of the Guide strand (Schirle et al., 2016). Synthetic addition of a 5'-monophosphate to a GalNAc-siRNA conjugates is ineffective as 5'-phoshpates are rapidly cleaved by lysosomal acid phosphatases (Parmar et al., 2016). As a result, the 5'-phosphate must be added by intracellular kinases prior to proper Ago2 binding. Clp1 kinase is the primary kinase for siRNA 5'-phosphorylation (Weitzer and Martinez, 2007). The extensive modification of therapeutic siRNA that is necessary for *in vivo* delivery impairs Clp1 activity, causing 5'-phosphorylation to be a rate limiting step in siRNA mediated RNAi (Kenski et al., 2010). A 5'-phosphate analogue, 5'-(E)-vinylphosphonate (5'-VP), has been developed as a stable mimetic of the 5'-phosphate group and structural analysis shows that 5'-VP is well accommodated by the 5'-nucleotide binding pocket in Ago2 (Parmar et al., 2018; Elkayam et al., 2017). Addition of 5'-VP to hydrophobically modified siRNA platforms resulted in enhanced siRNA accumulation, RNAi activity, and duration of effect in liver and kidney. Additionally, following the addition of 5'-VP, RNAi activity was seen in the heart, where siRNAs without the 5'-VP modification had no activity. Taken together, inclusion of 5'-VP into our siRNNs, ARCs, and TRCs may improve RNAi activity and allow potent gene knockdown in currently intractable tissues.

Improving Endosomal Escape

GalNAc-siRNA conjugates are able to elicit a robust *in vivo* response within hepatocytes in the absence of endosomal escape domains. The mechanism of GalNAc-siRNA endosomal escape is not understood and has not been replicated clinically in other targets of siRNA therapeutics. Mannose-TRC is another system where an RNAi effect is seen without

endosomal escape, though the mechanism for this is also not understood. Despite, the preliminary *in vitro* success of mannose-TRCs, the unfortunate reality is that no other ligand-receptor pair in the human body is capable of matching the combination of high receptor expression (10^6 per cell) and rapid turnover (15 min) of hepatic ASGPR. In contrast to the biology of ASGPR, most receptors are expressed in the range of $<10^4$-10^5 and recycle every ~90 min (Wiley, 1988; Berkers, van Bergen en Henegouwen, and Boonstra, 1991; Schoeberl et al., 2002). Comparing these systems suggests that in order to overcome the low and slow receptor biology of the majority of receptors, endosomal escape will have to be greatly enhanced. Unfortunately, most endosomal escape domains result in modest improvements of 5-10 fold delivery, while the biology of the ASGPR suggests that for most receptors, improvements of >100-fold endosomal escape enhancement will be necessary for extra-hepatic RNAi to be viable.

Increasing the valency of endosomal escape domains may improve their activity sufficiently for extra-hepatic targeting, as suggested within this work (**Chapter 3).** However, multivalent hydrophobic endosomal escape domains pose serious problems for solubility at higher valency, limiting their utility. I primarily utilized hydrophobic endosomal escape domains due to the tendency for cationic endosomal escape domains to interact with the anionic phosphodiester backbone. While the siRNN technology has reduced this tendency somewhat, the number of *t*Bu-SATE phosphotriesters required for full neutralization and prevention of aggregation with cationic peptides leads poor solubility at physiologic salt concentrations (**Figure 5.2**). Hydrophilic O-SATE phosphotriesters offer one possible solution to this problem by increasing siRNA solubility while maintaining phosphate masking.

Another strategy is to mask the cationic charges on the EED to allow for conjugation of minimally neutralized siRNN. This strategy would also prevent premature membrane disruption and associated toxicity of cationic EEDs. To effectively mask the cationic charge of EEDs, a bioreversible protecting group must be used. These protecting groups must be stable in serum

to avoid premature membrane activity and siRNN aggregation, but rapidly convert to wild type cationic amino acids. To address these needs, our lab is currently exploring alternative protecting group strategies.

Treating Cancer

Due to the high mutational rate of cancer, many chemotherapeutics, small molecule inhibitors, and extracellular antibody therapies fail to fully eradicate cancer from patients, resulting in recurrent disease. Unfortunately, these current therapies are limited to the druggable genome that represents a very restricted <5% of all genes, providing very few tools to deal with the diverse biology of cancer. These current approaches are also unable to pharmaco-evolve their activity to adapt to mutations in advanced and recurrent disease. Given the long development times for new drugs, truly personalized medicine is impossible with current therapeutic modalities. Taken together, the cancer therapeutic landscape is in need of a new approach.

In stark contrast to the current therapeutic approaches, RNAi offers the ability to target all mRNA including previously “undruggable” targets. In addition, unlike any previous modality, siRNA is able to adapt to changing mutations by simply changing its target sequence to match the new genetic landscape. Additionally, siRNA can target cancer specific mutations, limiting off target effects in healthy tissue. Furthermore, siRNA therapies offer the ability to simultaneously target multiple oncogenes, cancer specific mutations, and pathways necessary for tumor survival to achieve synthetic lethality in the tumor (Michiue et al., 2009; Kacsinta and Dowdy, 2016).

Despite all the promise of RNAi therapeutics, the fundamental problems of targeting and delivery into the cytoplasm remain potent obstacles. Improvements to targeting, linker chemistry, siRNA stability and potency, and endosomal escape will all have to be utilized together to overcome the numerous obstacles that stand in the way of RNAi therapeutics. My

dissertation work described here outlines many of the problems that RNAi therapeutics face and provides a framework where incremental improvements to each piece of the ARC and TRC platforms can be rapidly implemented to achieve the ultimate goal of treating cancer and other genetic diseases with RNAi therapeutics.

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