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Medicinal Chemistry of siRNA Delivery

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1. Overview of siRNA: New Opportunities and New Challenges

In 1998, Mello and Fire discovered RNA interference (RNAi[a]), an endogenous cellular pathway for regulation of gene transcription (Figure 1).[1] Double stranded RNA (dsRNA) is first cleaved by the cytosolic nuclease Dicer to generate short interfering RNA (siRNA). These siRNAs are then loaded into the RNA-induced silencing complex (RISC). Within RISC, the passenger strand is degraded while the guide strand is loaded into Argonaut 2 (Ago2), the active nuclease component of RISC. The guide strand binds the complementary sequence of the target mRNA and catalyzes the cleavage of this mRNA, thus silencing the translation of the target mRNA into protein. Tuschl demonstrated in 2001 that synthetic siRNA could also activate RNAi.[2] Subsequently, the application of siRNA for interrogating fundamental cellular pathways has become a standard method.

In 2006, only 8 years after their discovery, Mello and Fire were awarded the Nobel Prize in Physiology or Medicine for their discovery of RNAi. By this time the application of RNAi for investigation of cellular pathways was commonplace. The RNAi mechanism and the ability to control gene transcription with synthetic siRNA had transformed the way target identification and validation were conducted. It was the identification of nonviral delivery vectors such as lipofectamine that enabled this broad based application of the technology.[3] The oligonucleotide siRNA is a large (~13 kDa), hydrophilic, charged molecule and as such requires transfection vehicles to penetrate the cell membrane and to gain access to cytosolic RISC. While transfection vehicles such as lipofectamine are generally adequate for in vitro cell-based work, the identification of well-defined and well tolerated delivery vehicles would be required for in vivo delivery of siRNA. Decades of work in delivery of other oligonucleotides such as DNA and antisense oligonucleotides provided a framework for the discovery of novel siRNA delivery vehicles.[4] Leveraging 20 years of history in this difficult area has allowed numerous examples of successfully delivered siRNA in rodent models. These delivery systems, typically liposomes, now provide a basis for wide scale in vivo target validation work.[5] However, safety requirements for a well tolerated therapeutic are much higher than that required for rodent proof of concept studies and will necessitate a significant commitment to the task of identifying optimal siRNA delivery vehicles.

As this Perspective is focused upon the delivery of siRNA, the methods used to identify gene sequences, screen oligonucleotide sugar stabilization chemistries, and methods to understand RISC/Ago2 enzyme binding kinetics and catalytic activity are out of scope here. Delivery strategies for siRNA generally fall into two categories: local delivery to the target tissue and systemic delivery. Both of these modalities have significant hurdles to safe and effective delivery. This Perspective will focus on the requirements for systemic delivery of siRNA. In general, a delivery vehicle will have to control the appropriate biodistribution of the siRNA and enable the safe transport of the oligonucleotide to the cytosol of the target cells. We will highlight some of the published strategies to achieve this. In common to most of them is the requirement for rational medicinal chemistry. While the physical properties of

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[a] Abbreviations: siRNA, short interfering ribonucleic acid; RNAi, ribonucleic acid interference; dsRNA, double stranded ribonucleic acid; RISC, RNA-induced silencing complex; Ago2, Argonaut 2; mRNA, messenger ribonucleic acid; kDa, kilodalton; SAR, structure−activity relationship; ADMET, absorption, distribution, metabolism, excretion, toxicology; DNA, deoxyribonucleic acid; RES, reticuloendothelial system; LNP, lipid nanoparticle; PEG, polyethylene glycol; pDNA, plasmid DNA; SNALP, stable nucleic acid lipid particle; ApoB, apolipoprotein B; KSP, kinesin spindle protein; VEGF, vascular endothelial growth factor; iv, intravenous; PEI, poly(ethylenimine); DLinDMA, 1,2-dilinoleyloxy-3-dimethylaminopropane; DSPC, distearoyl phosphatidylcholine; N/P, nitrogen/phosphorus; nm, nanometer; DSDMA, 1,2-distearyloxy-*N*,*N*-dimethyl-3-aminopropane; DODMA, 1,2-dioleyloxy-*N*,*N*-dimethyl-3-aminopropane; DLenDMA, 1,2-dilinolenyloxy-*N*,*N*-dimethylaminopropane; MIT, Massachusetts Institute of Technology; NHP, non-human primate; TTR, transthyretin; DPhyPE, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DODAG, *N*′,*N*′-dioctadecyl-*N*-4,8-diaza-10-aminodecanoylglycine amide; DOTAP, (*N*-[1-(2,3-Dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium; LDP, lipid−polycation−DNA; ROS, reactive oxygen species; UNC, University of North Carolina; HDL, high density lipoprotein; ApoE, apolipoprotein E; CDP, cyclodextrin containing polycation; Tf, transferrin; AD, adamantane; PAsp-DET, poly(aspartamide)diaminoethane; DPT, diaminopropane; PAMAM, poly(amido)amine; cSCK, cationic shell-cross-linked knedel-like; MFCs, multifunctional carriers; PDI, polydispersity index; ASGR, asialoglycoprotein receptor; PVE, polyvinylether; NAG, *N*-acetylgalactosamine; CDM, carboxydimethylmaleic anhydride; DPC, dynamic polyconjugate; PLL, poly-L-lysine; DMMAn, dimethylmaleic anhydride; PS, passenger strand; GS, guide strand; SEC, size exclusion chromatography; TFF, tangential flow filtration; GPC, gel permeation chromatography; MALLS, multiangle (laser) light scattering; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; PAGE, polyacrylamide gel electrophoresis; DLS, dynamic light scattering; cryo-EM, cryogenic electron microscopy; SAXS, small-angle X-ray scattering; DSC, differential scanning calorimetry; ITC, isothermal calorimetry; EPR, enhanced permeability and retention; PK, pharmacokinetics; PD, pharmacodynamics; TLR, Toll-like receptor; ALT, alanine transaminase; AST, aspartate transaminase; RSV, respiratory syncytial virus; HCC, hepatocellular carcinoma; ASCO, American Society of Clinical Oncology; DLT, dose-limiting toxicity; LDL, low density lipoprotein; PKN3, protein kinase N3; RRM2, M2 subunit of ribonucleotide reductase; PET, positron emission tomography.

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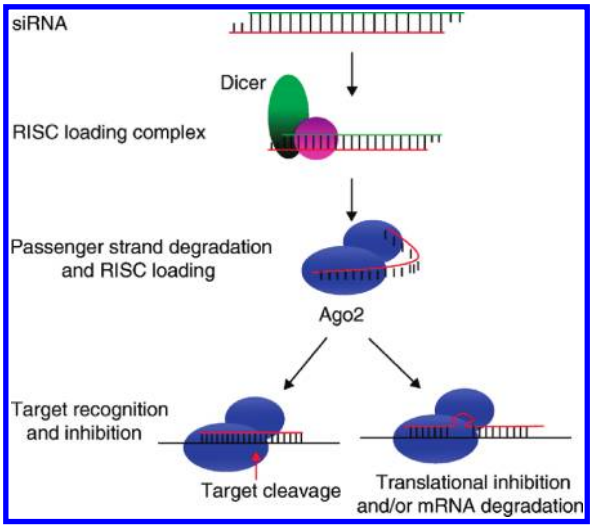

Figure 1. RNAi mechanism. Adapted and reproduced with permission from *Expert Opinion on Biological Therapy* **2009**, *9*, 609.[5a] Copyright 2009 Informa Healthcare.

the various delivery vehicles are vastly different from traditional small molecule drugs, we note that the fundamental process of optimization is quite similar. This Perspective will cover two general classes of systemic nonviral siRNA delivery vehicles: (a) nonconjugated macromolecular assemblies and (b) covalently bound siRNA conjugates. We will focus on the application of medicinal chemistry principles, such as structure−activity relationships (SAR), and highlight issues of absorption, distribution, metabolism, excretion, and toxicology (ADMET). In addition, we will discuss the current and future aspects of how siRNA delivery research is conducted. Finally, we will speculate on the future role of synthetic and medicinal chemistry in the realization of the potential of RNAi.

2. siRNA Delivery

a. Noncovalent Delivery Vehicles. The most advanced type of siRNA delivery vehicles consist of macromolecular assemblies, primarily containing cationic lipids that facilitate transfection. These systems are typically carryovers from decades of work on plasmid DNA gene transfection and single strand antisense oligonucleotides.[4] As such, the literature on cationic lipid design for gene transfection and antisense is far richer than that described for siRNA delivery. There are significant differences between siRNA and DNA delivery. For instance, siRNA is much smaller than DNA and does not require entry into the nuclear compartment. There are also significant differences between siRNA and antisense. For instance, siRNA is double-stranded and recognition is mediated through the RISC complex. With that in mind, this Perspective will focus only on those lipids that have been used to deliver siRNA. Additionally, while the composition and formulation of macromolecular assemblies are critical to designing optimal delivery vehicles, for the purposes of this Perspective we have chosen to focus on the primary chemical SAR of components wherever possible. This is complicated by the multivariate nature of these macromolecular assemblies. Properties such as size, shape, charge, etc. are known to have significant effects on biological properties such as biodistribution, recognition by reticuloendothelial system (RES), opsonization, toxicity, and efficacy. While we will examine several systems of particular

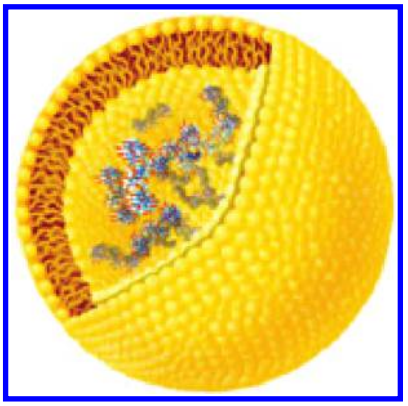

Figure 2. Three dimensional rendering of a lipid nanoparticle (LNP). Reproduced with permission from Macmillan Publishers Ltd.: *Nature* **2007**, *450*, 1117 (http://www.nature.com/nature/index.html).[4a] Copyright 2007 Nature Publishing Group, a Division of Macmillan Publishers Ltd.

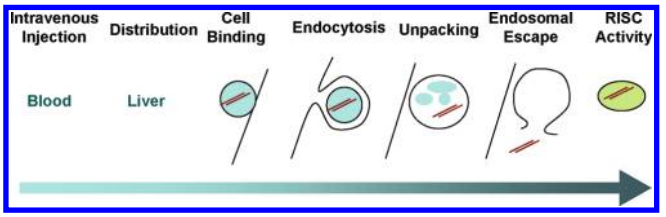

Figure 3. Barriers to LNP mediated transfection. Cationic lipids are proposed to be responsible for both cell binding and endosomal escape.

interest, lipid based delivery systems for siRNA delivery have been recently reviewed in detail elsewhere.[6]

Where nonviral gene transfection typically relies on cationic lipoplex formulations, the smaller size of siRNA has the potential to allow for more control of lipid nanoparticle (LNP) formation. Control over size, lamellarity, surface charge, etc. is now possible by variation of formulation conditions and by optimization of noncationic lipid component identity and ratios. Typically LNPs consist of a cationic lipid, a PEGylated lipid, cholesterol, neutral helper lipids (or fusogenic lipids), and the siRNA (Figure 2). An important component for transfection is the cationic lipid, as this lipid is proposed to be responsible for both cell association and endosomal escape. The polyethylene glycol (PEG) lipid is required to stabilize the particle. Additionally, the PEG lipid can be optimized to improve circulation half-life and systemic exposure. The cholesterol is typically required for lipid bilayer stability, but recent literature reports may implicate a more direct involvement in siRNA transfection mechanisms.[7] Neutral helper lipids are thought to play a role in particle structure, uniformity, stability, and possibly endosomal escape.

The mechanism of lipid nanoparticle mediated transfection relies on several critical steps (Figure 3). While distribution can be largely controlled by variation of formulation parameters, cell membrane association is thought to require a partial positive surface charge, as the cell membranes are negatively charged surfaces.[8] This is typically accomplished by inclusion of cationic lipids into the composition. From a design perspective, the challenge here is to have enough charge to adequately drive cell association without carrying too much positive charge, as this is thought to decrease particle stability and possibly to trigger immune system

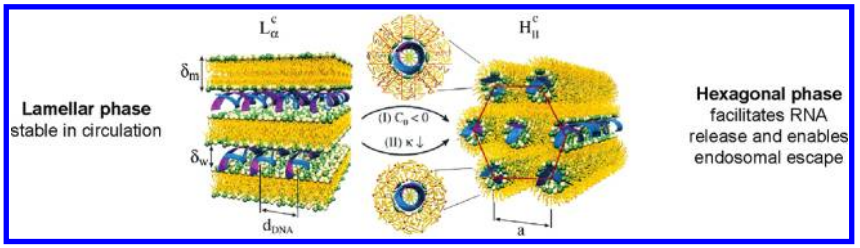

Figure 4. Lamellar to hexagonal phase transition is proposed as necessary to drive endosomal membrane fusion.[9] Adapted and reproduced with permission from AAAS, from Koltover, I.; Salditt, T.; Rädler, J. O.; Safinya, C. R. An inverted hexagonal phase of cationic liposome−DNA complexes related to DNA release and delivery. *Science* **1998**, *281*, 78−81 (http://www.sciencemag.org/cgi/content/abstract/281/5373/78).[12] Copyright 1998 AAAS.

activation and toxicity.[9] Once particles are associated with cell membranes, they are efficiently endocytosed into early endosomes.

The second key step for LNP mediated transfection is endosomal escape. Much of the mechanistic work regarding endosomal escape has been conducted with pDNA cationic liposomes. Szoka proposed a mechanism that relied on the formation of charge-neutral ion pairs between the cationic lipid vector and the anionic endosomal membranes.[10] Cullis later implicated a second step beyond the formation of charge-neutral ion pairs: the conversion of lamellar phases to nonlamellar phases and subsequent enhancement of endosomal release.[11] Safinya has described structural elements that guide lamellar to nonlamellar phase transitions, in particular the conversion to inverted hexagonal phases.[12] The fundamental mechanisms for pDNA release should hold for siRNA containing cationic lipid nanoparticles with the cationic lipid remaining a key driver for this process.[6] While the mechanistic details of endosomal escape remain somewhat poorly understood, rational design of cationic lipids has been conducted by evoking some of the above design principles: tuning pK_a for enhancement of charge neutral ion pairing and maximizing lamellar to nonlamellar phase transitions (Figure 4).[9,12,15]

From both a clinical development and preclinical validation perspective, the stable nucleic acid lipid particle (SNALP) technology has been significantly advanced by Tekmira (previously Protiva). This LNP delivery vehicle is currently employed by Tekmira (ApoB siRNA) and Alynlam (KSP/VEGF siRNAs) in ongoing Phase I clinical trials. Additionally, the technology was recently licensed by Roche, and they anticipate initiation of Phase I clinical development later this year. In 2006, Zimmerman et al. published their research detailing knockdown of ApoB protein in non-human primates upon iv injection of ApoB siRNA SNALP.[13]

The SNALP system evolved from earlier plasmid DNA/PEI delivery vehicles reported by Protiva.[14] Modifications to the earlier system included using a novel cationic lipid DLinDMA along with a novel synthetic PEG lipid PEG-C-DMA. The final ratio of lipids employed by the SNALP described by Zimmerman and co-workers is 48:40:10:2 (cholesterol/DLinDMA/DSPC/PEG-C-DMA). This optimized SNALP was prepared with an N/P ratio (amine/phosphate) equal to 6. Nanoparticles prepared under these conditions have been reported to be < 100 nm in size with a narrow polydispersity.

MacLachlan et al. reported the identification of DLinDMA as an optimal cationic lipid for siRNA delivery (Figure 5).[9] It was demonstrated by fluorescence microscopy that SNALPs containing the more basic cationic lipid DSDMA resulted in greater internalization of Cy3 labeled

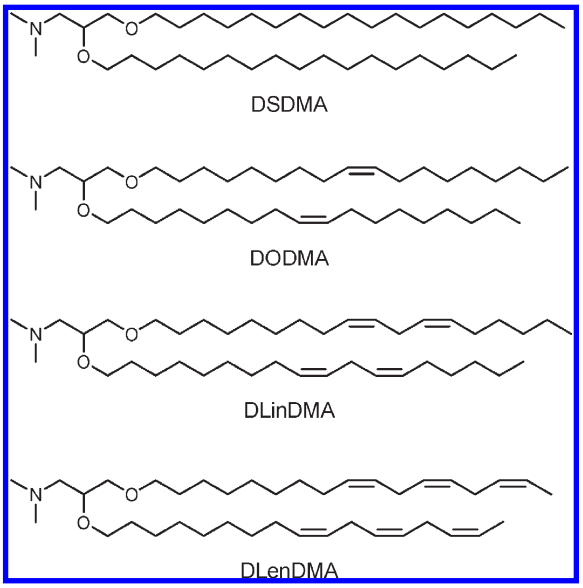

Figure 5. Structures of Tekmira cationic lipids.

siRNA. Optimization of pK_a is thought to be critical, as increasing pK_a can lead to particles that are charged at physiological pH which can lead to decreased systemic circulation half-lives and increased levels of toxicity. The headgroup needs to be protonated in the endosome to drive fusion with the endosomal membranes. Theoretically, head groups that have a pK_a of 6−7 would have the optimal combination of properties. DLinDMA falls into this category with a measured surface pK_a of 6.7 as reported by the Tekmira team.

As mentioned above, the headgroup of the cationic lipid must first be protonated at endosomal pH to initiate endosomal escape. MacLachlan et al. have proposed that the cationic lipid driven mechanism of endosomal escape is facilitated by conversion of the lamellar phase of the lipid nanoparticle to a hexagonal phase, which is much more prone to fusion with the endosomal membrane.[9,12] This hexagonal phase transition is governed by the degree of saturation of the lipid chains, with linoleyl being optimal.[9] The unsaturated chains adopt a more inverted conelike shape which is known to favor hexagonal phases by induction of a negative surface curvature.[12] By combination of the optimal headgroup pK_a and optimal phase transition biophysical properties, DLinDMA was identified as a promising cationic lipid for siRNA transfection and was the cationic lipid that was chosen by Zimmerman et al. for systemic delivery of ApoB.

Tekmira and Alnylam recently described the preparation of latently biodegradable cationic lipids derived from cyclic ketals, typified by DLin-K-DMA (Figure 6).[15] While in vivo

data describing the terminal half-life and thus biodegradable nature of this lipid was not described, the authors report improvements of in vivo efficacy employing this cationic lipid. As with the prior DLinDMA work, this manuscript outlines a rational medicinal chemistry approach to lipid optimization centering on pK_a and lipid biophysics tuning. Additional SAR of DLin-K-DMA revealed that the one carbon homologue DLin-KC2-DMA (Figure 6) resulted in further improvements in efficacy. While this lipid did demonstrate modest improvements in lamellar to hexagonal phase transition temperature and a subtle increase in amine pK_a, the authors could not rule out that the improved efficacy might be a result of in vivo considerations such as opsonization to plasma proteins.

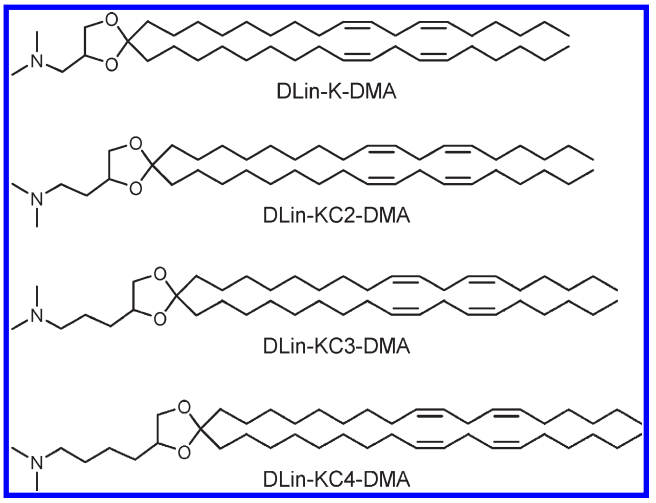

Figure 6. Structures of DLin-K-DMA and homologues.

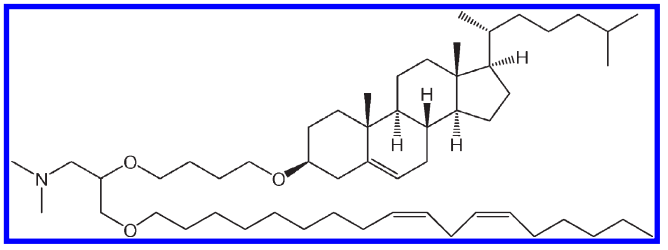

Figure 7. Structure of CLinDMA.

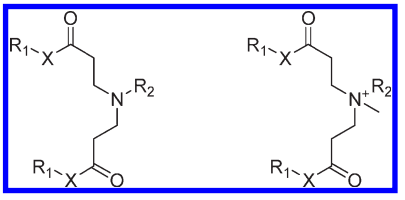

Figure 8. General structure of lipidoid library where R_1 = alkyl chain, X = O, N, and R_2 is variable, including additional amine sites.

The work of Tekmira (recently expanded to AlCana) is an example of cationic lipid medicinal chemistry in that they reduced, mechanistically, siRNA transfection into measurable attributes of the cationic lipid and explored the effect of structural changes on these attributes, the very essence of rational SAR-driven medicinal chemistry. The vast majority of published work on cationic lipid design tends to skip over issues of mechanism and focus solely on efficiency of siRNA transfection. Given the wealth of additional variables that are consistently changed (i.e., lipid components and their effect on particle properties), this leaves interpretation of the data ambiguous at best. Nonetheless, a number of unique cationic lipids with promising in vivo activity have been disclosed in the literature, a few of which are described below.

The Sirna chemistry team (currently Merck) identified novel cationic lipids that are a unique extension of the earlier lipids.[16] The most significant difference between the CLinDMA series of Sirna and DLinDMA is the presence of a cholesterol-linked lipid chain that is incorporated directly into the cationic lipid (Figure 7). Details concerning the discovery and optimization of this series of lipids will be forthcoming.

Juxtaposed against the Tekmira strategy is that employed by Langer and Anderson at MIT in conjunction with Alnylam.[17] This group has taken a combinatorial approach to cationic lipid identification by preparation of libraries of what they termed "lipidoids" (Figure 8). These are non-glycerol-based cationic lipids that vary in alkyl chain length and number of cationic groups. This empirical approach to cationic lipid identification led this team to identify efficacious siRNA delivery systems. The lipid that displayed the highest activity was $98N_{12}$-5(1) shown in Figure 9. LNPs containing $98N_{12}$-5(1) were further optimized into LNP01 which demonstrated robust efficacy in mouse, rat, and NHP across several gene targets.[18]

A very recent report from the MIT/Alnylam collaborators details a second generation library of lipid-like materials (Figure 10).[19] This novel library relied on epoxide ring-opening reactions with amines to generate a series of novel cationic lipids. One of these lipids, C12-200 (Figure 11), demonstrated increased efficacy in mice (with Factor VII siRNA, $ED_{50} \approx 0.01$ mpk). This lipid was subsequently formulated with TTR siRNA, and this formulation was dosed in cynomolgus monkeys. Liver biopsies were taken, and greater than 50% mRNA knockdown was observed at 0.03 mpk.

In 2006, Atugen (now Silence Therapeutics) described the identification of a novel cationic lipid AtuFect01 (Figure 12).[20] Liposomes prepared from this cationic lipid, the neutral helper lipid DPhyPE and the PEG−lipid DSPE−PEG (50:49:1, no cholesterol inclusion in these liposomes) displayed good cell uptake and endosomal release at 10 nM as determined by microscopy studies. The group at Atugen did not disclose

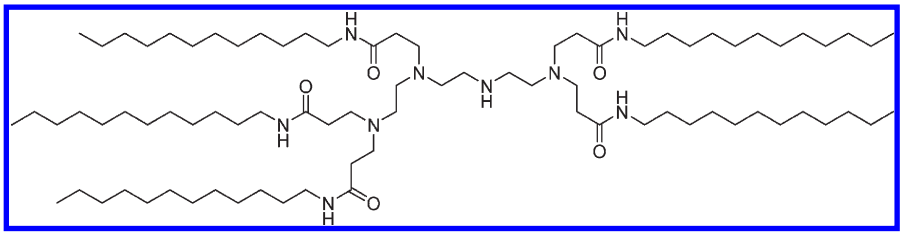

Figure 9. $98N_{12}$-5(1).

how they arrived at AtuFect01 other than to mention that the multiamine headgroup facilitated strong association with the negatively charged siRNA.

A recent report by Andrew Miller describes his group's efforts at optimization of traditional pDNA delivery cationic lipids based on CDAN.[21] The cationic lipid DODAG combines the multivalent headgroup that was originally developed for pDNA delivery with a glycine amide based lipid to generate DODAG (Figure 13). Importantly, the multivalent headgroup has been optimized to balance the pK_a of the amines, much in the way that the Tekmira group did with DLinDMA. DODAG based LNPs demonstrated reasonable in vivo activity in mice.

An alternative siRNA delivery system has been developed by Huang et al. at the University of North Carolina.[22,23] This system employs features of both cationic polyplexes and lipid nanoparticles. siRNA is mixed with protamine and calf thymus DNA, and the resulting polyplex was combined with preformed liposomes consisting of DOTAP (or DSGLA) and cholesterol. The resulting lipid−polycation−DNA (LDP) nanoparticles were then treated with PEG lipids, some ratio of which contained the targeting ligand anisamide (σ receptor ligand). The group's early work was conducted with DOTAP as the cationic lipid, but the group has recently reported improved efficacy with cationic lipid DSGLA (Figure 14).[24] The authors speculate that the improved anticancer efficacy with DSGLA is a result of off-target ERK1/2 activity and possibly induction of reactive oxygen species (ROS). Given the complexity of the formulation and the inclusion of multiple components that are capable of driving endosomal escape (protamine and cationic lipid), it is hard to delineate the role that the cationic lipid is playing in transfection. Optimization of the lipid via traditional medicinal chemistry may prove difficult until a more detailed mechanistic understanding of this system is developed. It is interesting that both lipids are quaternized salts, as these are thought to be more toxic than tertiary amine based lipids. To the best of our knowledge, the team at UNC has not looked at tertiary amine based cationic lipids in the LDP system.

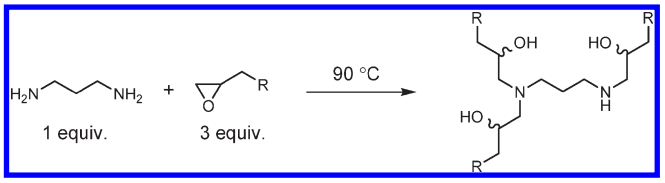

Figure 10. Novel lipid-like library.

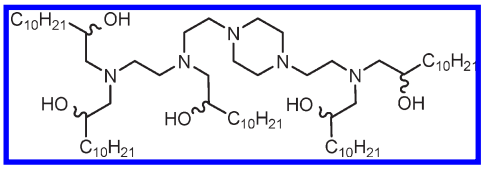

Figure 11. Structure of C12−200.

While the majority of literature around optimization of LNP components relates to cationic lipids, there are examples of optimization of other components. Of particular interest is the role of the PEG-lipid. This lipid is thought to play a key role in formation and stabilization of the nanoparticle along with controlling elements of biodistribution and efficacy. Several groups have reported efforts to optimize the performance of LNPs by conducting SAR studies on the PEG-lipid. Heyes and MacLachlan published on the optimization of the SNALP PEG-lipid.[25] Glycerol based acyl lipids have been used extensively in the past, but the presence of hydrolytically labile ester bonds can potentially be problematic for the preparation of lipid nanoparticles in aqueous environments. By replacing the ester bonds with hydrolytically stable ether bonds, the MacLachlin group

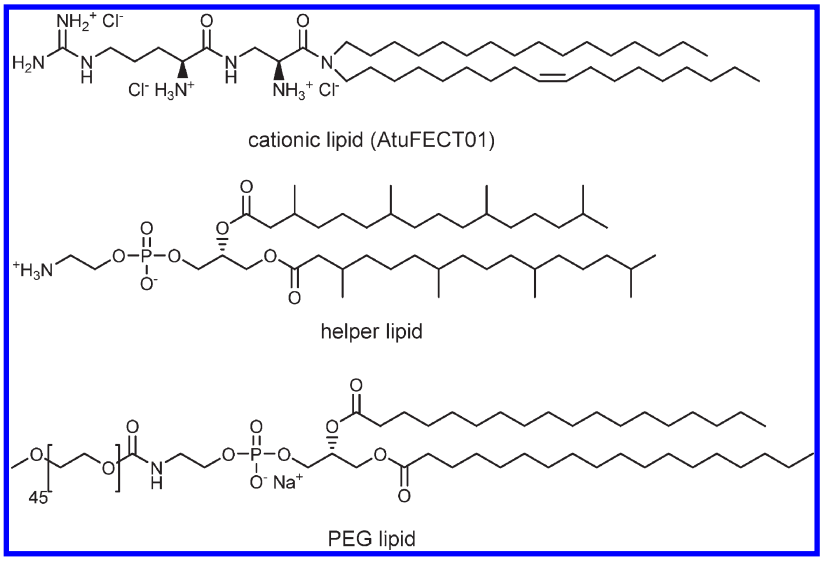

Figure 12. Atugen lipids.

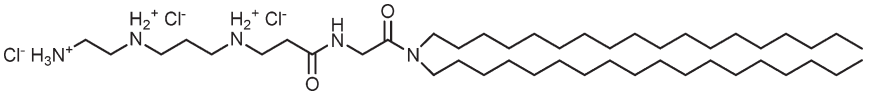

Figure 13. Structure of DODAG.

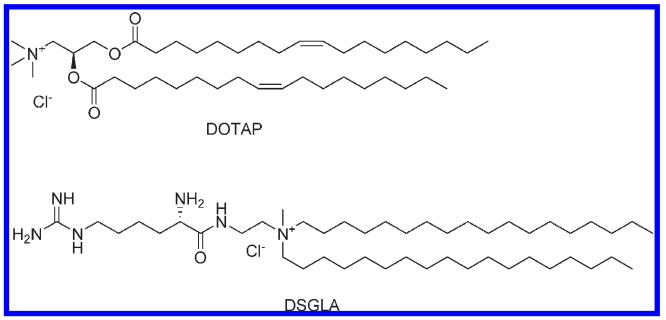

Figure 14. LDP cationic lipids.

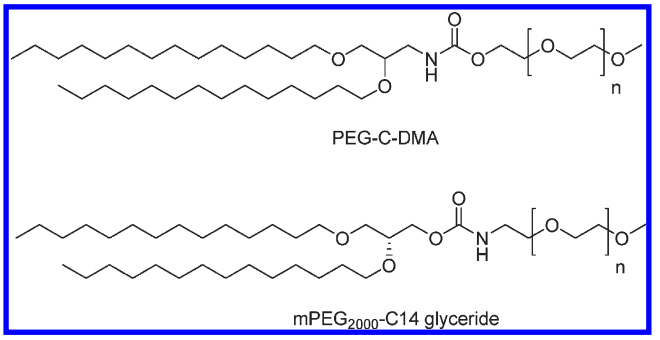

Figure 15. Optimized PEG lipids.

developed a series of PEG-lipids that were more stable to chemical hydrolysis. Alkyl chain length was also found to be important for optimal biodistribution/efficacy. The rate at which the PEG-lipid dissociates from the particle is thought to influence the circulation half-life. Additionally, some level of PEG lipid diffusion would likely be required for cell surface association and endocytosis. Tuning the rate of diffusion of PEG-lipids can therefore have a profound impact on in vivo performance of a given LNP. In the case of the SNALP formulations, the Tekmira team found that C14 alkyl chain ethers were optimal. The carbamate-based PEG-C-DMA (Figure 15) has been chosen as the PEG-lipid of choice for their more advanced in vivo work. The optimization of the lipidoid LNPs toward LNP01, discussed above, centered around PEG-lipid SAR. The optimal PEG-lipid resulted in an $mPEG_{2000}$-C14 glyceride that is very similar to PEG-C-DMA.

A highly provocative manuscript was published in 2009 by Chen that proposed that LNP-mediated transfection does not occur via an endosomal pathway but by an unknown mechanism that accounts for $< 5\%$ of siRNA that is internalized inside cells.[7] The role of cholesterol is intimately associated with this pathway, as depletion of cholesterol from cell membranes resulted in abolishment of siRNA activity. While this is an interesting possibility, it does not fully account for the observations by MacLachlin and others with regard to the impact of lamellar to hexagonal phase transition on transfection efficiency. However, if true, this could open new avenues for direct cytosolic delivery of siRNA via an unidentified pathway. In principle, such a pathway may be subject to systematic optimization. Interestingly, there have been reports of direct cytosolic delivery of lipophilic molecules via the SR-BI receptor.[26] Given the role of this receptor in HDL cholesterol transport, it is enticing to imagine that it may be related to the cholesterol depletion studies reported by Chen.

The future of cationic lipid design may very well rest with resolution of the mechanistic pathways that these lipids utilize for transfection. While DNA gene transfection agents have been reported for decades, the fairly recent interest in siRNA delivery has cast a new spotlight on the identification of new and improved cationic lipids. Clearly there is much interest in this area as demonstrated by the increasing number of patent applications. Unfortunately, given the proprietary nature of this research, there is much yet to be disclosed in scientific manuscript form. Some fundamental questions that we may begin to ask could be as follows: How do we increase cell uptake without increasing the charge of the particle? How can we better increase the efficiency of endosomal escape? Can design elements of the cationic lipid itself alter biodistribution, and can we harness that for improved delivery to a target tissue? For instance, data recently presented by Alnylam scientists implicate the recruitment of endogenous ApoE by LNPs in targeting hepatocytes.[27] Findings such as these will undoubtedly play into our collective ability to address the questions posed above.

While lipid nanoparticles represent the most advanced macromolecular siRNA delivery vehicles, a number of groups have published on nonconjugated siRNA polyplexes as viable in vivo delivery options. The most advanced of these is the cyclodextrin containing polycation (CDP) system from the laboratories of Mark Davis at California Institute of Technology (Figure 16).[28,29] The cationic nature of the polymer allows for association with negatively charged siRNA. The exposed cyclodextrin groups can then be capped with functionalized adamantanes. These adamantanes contain PEG groups, some of which contain a targeting ligand (transferrin). This technology was licensed to Calando (subsidiary of Arrowhead), which advanced a product candidate into Phase I studies, the preliminary results of which were recently published.[30] That trial is currently on hold, and Arrowhead is currently looking to partner this with another company. Significant SAR on the CDP revealed that the nature and density of the cationic portion of the polymer impact both efficacy and toxicity. While the details of this SAR will not be covered here, Davis et al. have published an excellent review of this work.[31]

The extension of DNA polyplexes with polyethylenimine (PEI)[32] to the delivery of siRNA has been conducted by a number of polymer groups. Trends in this area are (1) a movement toward biodegradable endosomolytic polymers to help attenuate toxicity and (2) covalent linkage of the siRNA to the polymer. The next section will explicitly discuss these covalent polyconjugates, but it is worth noting a few of the more interesting noncovalent siRNA polyplexes. Kataoka has developed a poly(aspartamide)diaminoethane (PAspDET) based system for delivery of plasmid DNA.[33] While the authors previously described a poly(aspartamide)diaminopropane (DPT) system for siRNA delivery,[34] the DET polymer design for plasmid DNA delivery is worth mentioning here. Specifically, the diaminoethane groups are thought to exist in different protonation states depending on the pH of the environment they reside in (Figure 17). Importantly at physiological pH, the monoprotonated gauche form of the diamine is favored. This monoprotonated form was shown to be nonlytic and therefore relatively nontoxic. Upon pH reduction to ~ 5, the diamine adopts an anti-diprotonated form that is endosomolytic and facilitates transfection. The control polymer that contains the diaminopropane group was shown to be less efficacious and more toxic than the DET derivative, presumably because of its propensity to be doubly protonated at physiologic pH, resulting in increased

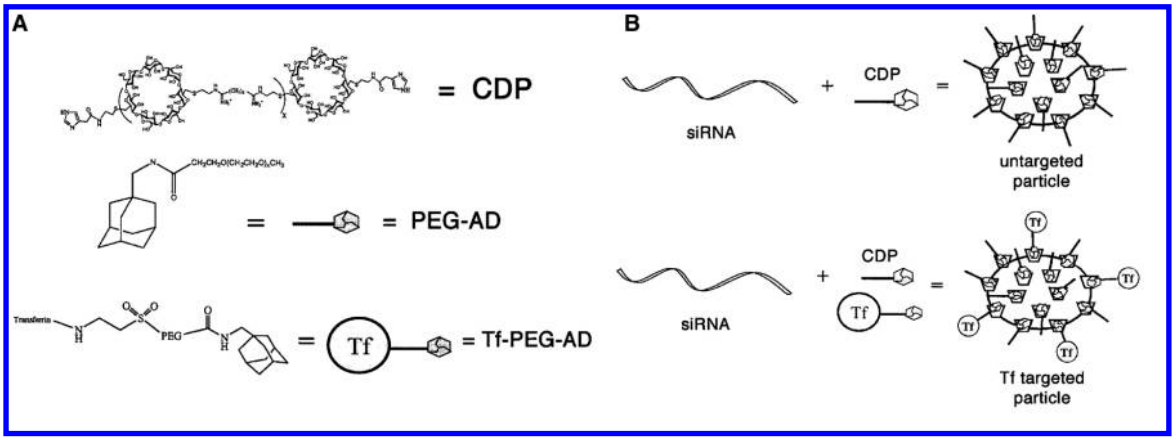

Figure 16. Cyclodextrin containing polycation (CDP) siRNA delivery system. Reproduced with permission from *Cancer Research* (Hu-Lieskovan, S.; Heidel, J. D.; Bartlett, D. W.; Davis, M. E.; Triche, T. J. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* **2005**, *65*, 8984−8992 (Figure 1)).[28] Copyright 2005 American Association for Cancer Research.

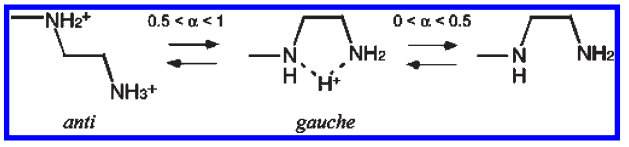

Figure 17. Two-step protonation of the 1,2-diaminoethane moiety in the side chain of PAsp(DET). Reproduced with permission from *Journal of the American Chemical Society*.[33] Copyright 2008 American Chemical Society.

cation-derived toxicity and a reduced proton sponge effect in mediating endosomal escape. The proton sponge effect relates to a buffering effect in the range of endosomal pH 7.4−5.1, where the increasing protonation of amines upon decrease of the pH in the endosome induces an influx of counterions (chloride) and water. A buildup of osmotic pressure is proposed to result in endosomal membrane rupture.[35]

Additional polyplexes worthy of mention are the polyethylene glycol-block-polylysine system also developed by Katoaka,[36] the poly(amido)amine PAMAM dendrimers recently developed by Peng, Minko, and Uhrich/Roth,[37] endosomolytic diblock copolymers developed by Stayton,[38] polycation/endosomolytic peptide copolymers prepared by Wagner,[39] cSCK nanoparticles published by Wooley,[40] acid-degradable polyhydroxylated polymers used to encapsulate siRNA disclosed by Frechet,[41] the application of amphiphilic scorpion-like polymeric micelles to siRNA delivery developed by Uhrich,[42] and multifunctional carriers (MFCs) originally reported on by Lu and now the basis of the startup company Surfagen.[43] All of these polyplexes employ various strategies to increase rates of endosomal release while maintaining cell viability.

b. Conjugated siRNA Delivery Vehicles. The previous section discussed the delivery of oligonucleotides via the formulation of several chemical components. Those individual components each provided distinct functions in the biodistribution and cellular transfection of siRNA. Chemists have engineered methods for incorporating those functional components into covalently bound or conjugated complexes. These chemical conjugates can be based on polymeric scaffolds, peptides, or the oligonucleotide itself. It is important to note that the use of polymers as chemical scaffolds will incorporate some degree of polydispersity, expressed as a polydispersity index (PDI). These can be mixtures of polymers with a range of molecular weight, monomer incorporation, tacticity, and conjugation regiochemistry.

Some of the early designs in gene delivery incorporated the delivery elements of a polymer framework, conjugation of nucleic acids through linkers, engineering of cleavable moieties, and the utility of targeting agents to facilitate cellular uptake.[44] Descriptions of polymer frameworks include poly(acrylates), poly(esters), and poly(ethers), all of which could contain reactive groups such as amines and carboxylic acids. The design of cleavable moieties could leverage change in pH, enzymatic action, and oxidation. An approach to cellular targeting has also been described via natural receptor-mediated endocytosis, with the well-known tissue selective asialoglycoprotein receptor (ASGR) present on hepatocytes. Others have noted the requirement of an endosomolytic peptide for the intracellular delivery of oligonucleotides.[45] Most recently an application of these design elements was used for the delivery of siRNA, and published by the biotechnology company Mirus,[46] subsequently purchased by Roche in July 2008.

Rozema et al. utilized a polyvinylether (PVE) amino polymer as the scaffold to which the oligonucleotide, a cellular targeting ligand, and polyethylene glycol (PEG) are covalently bound (Figure 18).[47] These components are engineered to be cleavable from the PVE scaffold within different cellular environments. The oligonucleotide is bound to the PVE through a linker that contains a disulfide bond, proposed to be cleaved within the reducing environment of the cytosol. The targeting ligand used is *N*-acetylgalactosamine (NAG), a monosaccharide with high affinity to the ASGR located on hepatocytes. An acid-labile linker composed of carboxydimethylmaleic anhydride (CDM) serves as the covalent attachment of the NAG ligand to the PVE backbone. This CDM linker strategy also serves to conjugate PEG molecules on the PVE as well for in vivo systemic stability. The CDM design is engineered into these polymer conjugates to provide masking of the lytic amino polymer throughout iv injection to cellular internalization. Assuming that the pathway for transfection is through receptor-mediated endocytosis, once the conjugate is subjected to the lowered pH of the endosome, the CDM-NAG and CDM-PEG covalent bonds are cleaved, resulting in a demasked PVE amino polymer that may trigger lysis of the endosome.

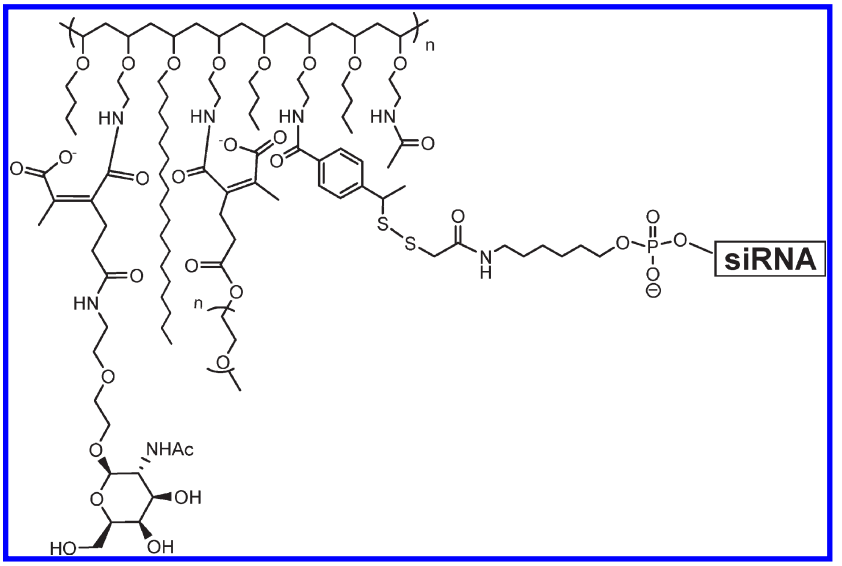

Figure 18. Representative structure of the polydispersed siRNA polymer conjugate reported by Mirus Corp.

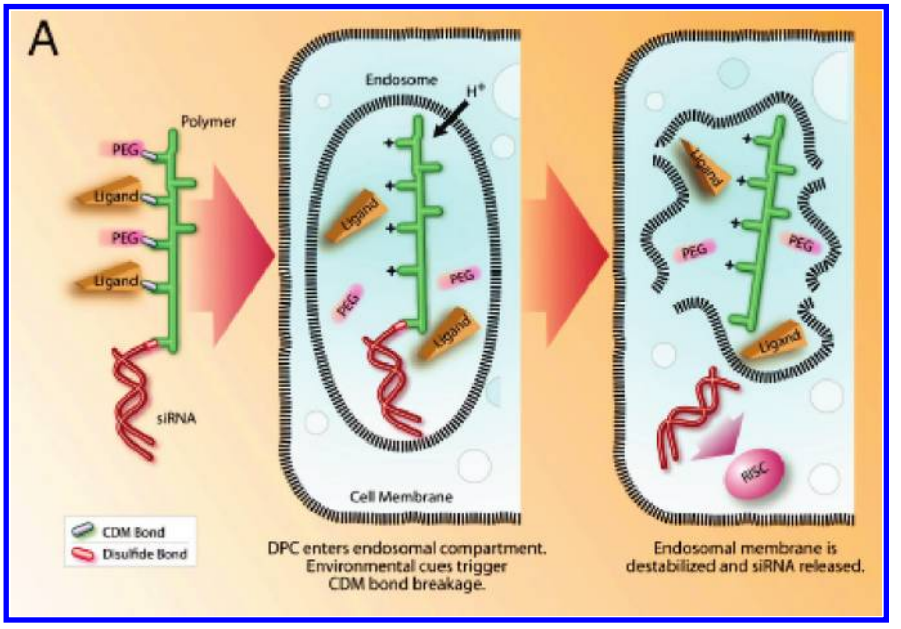

Figure 19. Components of the siRNA polyconjugate and the proposed mechanism of siRNA delivery. Schematic showing the siRNA dynamic polyconjugate (DPC), its cellular uptake, disassembly in the low pH environment of the endosome, and release of the siRNA into the cytoplasm of the target cell. Reproduced with permission from *Proceedings of the National Academy of Sciences of the United States of America* (Rozema, D. B.; Lewis, D. L.; Wakefield, D. H.; Wong, S. C.; Klein, J. J.; Roesch, P. L.; Bertin, S. L.; Reppen, T. W.; Chu, Q.; Blokhin, A. V.; Hagstrom, J. E.; Wolff, J. A. Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12982−12987)[46] Copyright 2007 National Academy of Sciences, U.S.A.

This event allows delivery of the disulfide conjugated PVE-bound siRNA to the cytoplasm, where it is proposed to be cleaved by glutathione. The free siRNA can then be loaded into the RISC complex, and gene silencing may ensue (Figure 19). Notably the authors reported in vivo knockdown activity in mice for two different genes, as well as phenotypic changes.

Other variations to this theme of polymer conjugates have been published, such as the work from the Wagner group. This team had previously published on the design and development of polymer polyplexes, which are derived from noncovalent complexes utilizing ionic interactions between the negatively charged siRNA and cationic polymers such as poly-L-lysine (PLL) and polyethylenimine (PEI).[39] Recently the group disclosed the extension of this work into covalent conjugates.[48] Here they used PLL as the polycation for siRNA binding and packing, and the lytic peptide melittin to induce endosomal escape (Figure 20). The melittin peptide was masked with multiple pH-responsive dimethylmaleic anhydride moieties. Both the oligonucleotide and the melittin peptide were individually bound through covalent bioreducible disulfide bonds, and the whole complex was stabilized with PEG units through amide bonds to the PLL amines. The presumed mechanism of action once again relates to the pH 5 environment of the endosome, triggering demasking of the dimethylmaleic anhydride moieties to expose the lytic melittin peptide. Upon rupture of the endosome and delivery of the conjugate to the cytoplasm, disulfide bond reduction allows the release of the duplex siRNA. The

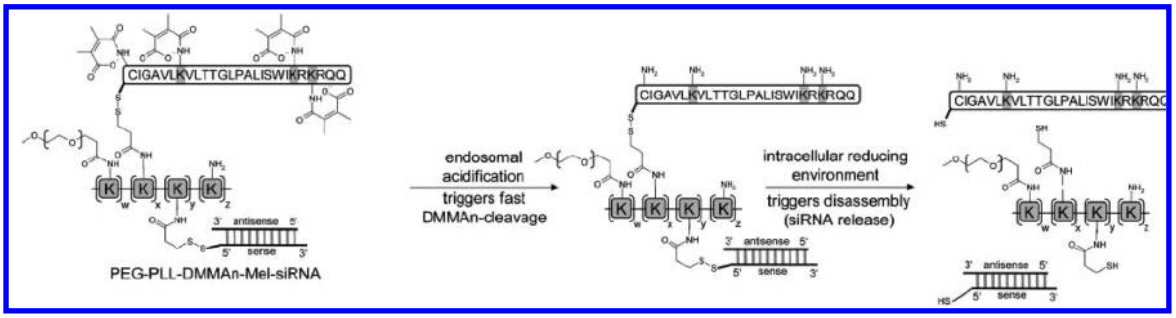

Figure 20. pH-triggered cleavage of DMMAn masking groups and release of siRNA upon disulfide cleavage in reducing environment. Reproduced with permission from *Molecular Pharmaceutics*.[48] Copyright 2009 American Chemical Society.

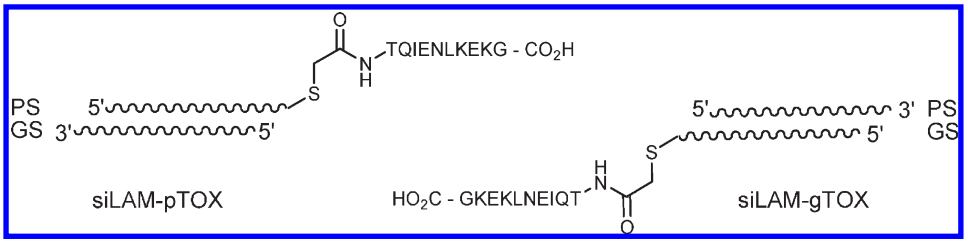

Figure 21. Structure of covalent linkage between siLAM (lamin A/C-directed siRNA)[51] and the bacterial peptide TQIENLKEKG; passenger strand (PS), guide strand (GS).

authors disclose that this particular delivery system was highly toxic in mice, and an assessment of in vivo activity did not appear feasible.

Others have published on the use of peptide−oligonucleotide conjugates for the delivery of siRNA to HeLa cells as well.[49] Focused on the hypothesis that limited efficacy stems from intracellular trapping of payload siRNA, a recent report by Sczakiel and Gait et al. described the in vitro application of a signaling peptide conjugated to siRNA via a stable thioether linkage.[50] As an alternative approach to endocytosis, they pursued the phosphorothioate-mediated caveosomal uptake pathway by incorporating the signal peptide TQIENLKEKG for transmembrane transport of bacterial protein toxin (Figure 21). They showed increased efflux of the siRNA−peptide conjugate from perinuclear organelles, with subsequent increased cytoplasmic delivery and activity in cells.

The Alnylam team has shown early on that conjugates of oligonucleotides with cholesterol, as well as other lipophilic moieties, behave as transfection agents both in vitro and systemically in vivo (Figure 22).[52] This group has since published in the recent patent literature an extension of this cholesterol−siRNA conjugate theme.[53] Here they demonstrate the synthesis of siRNA conjugates with cationic lipids (DLin-KC2-DMA, vide supra) as presumably the endosomolytic component, covalently bound to the oligonucleotide (Figure 23). In addition to hybridizing cholesterol conjugates of siRNA, the description of conjugating targeting ligands onto the oligonucleotide is also included. Similar to the Mirus work, targeting ligands such as higher order NAG sugars are conjugated onto oligonucleotides, as well as folate targeting ligands. The endosomolytic components and targeting ligands are conjugated onto oligonucleotides via typical labile covalent linker strategies, such as disulfides, ketals, and acyl hydrazones.

A very good review of conjugated siRNA delivery systems was recently published for those that wish to read further the details of this approach.[54]

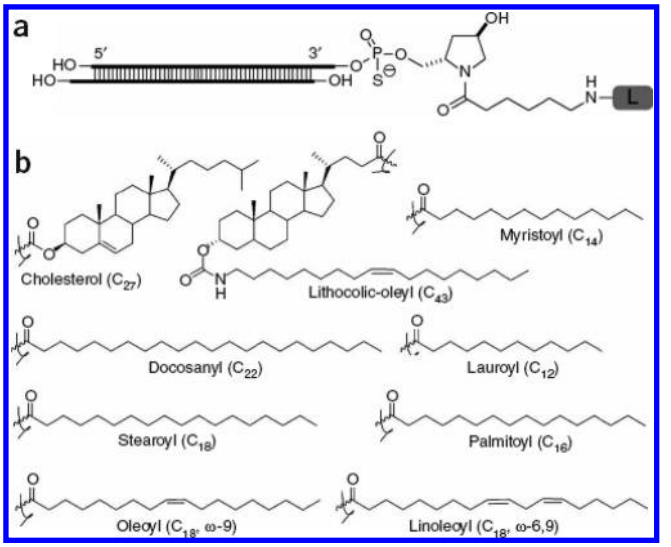

Figure 22. (a) Structure of lipid conjugated siRNAs with (b) desired lipophiles (L). Reproduced with permission from Macmillan Publishers Ltd.: *Nat. Biotechnol.* **2007**, *25*, 1149 (http://www.nature.com/nbt/index.html).[52] Copyright 2007 Nature Publishing Group, a Division of Macmillan Publishers Ltd.

3. Practice of siRNA Therapeutics Discovery

Since its discovery, the utility and application of siRNA in the pharmaceutical industry have evolved considerably. Early progress was made from an in vitro method of target identification to target validation in mice that was both restricted to disorders of the liver and greatly limited by toxicity of the initial delivery vehicles. The concept of a therapeutic siRNA, developed as a drug, transformed the technology beyond a laboratory method and beyond preclinical target validation. The quest for an siRNA therapeutic platform accelerated the industry over the past 3 years toward an intense focus on the delivery of siRNA with clinically acceptable safety margins. In response to this shift in focus, biotechnology companies have

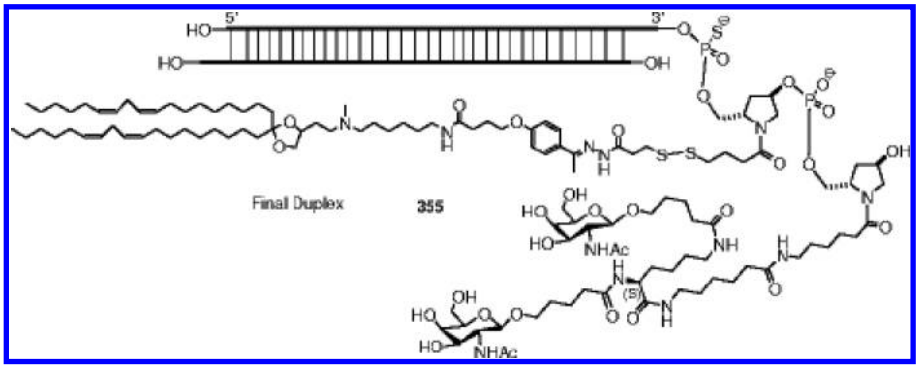

Figure 23. Example 23, WO 2009/126933.[53]

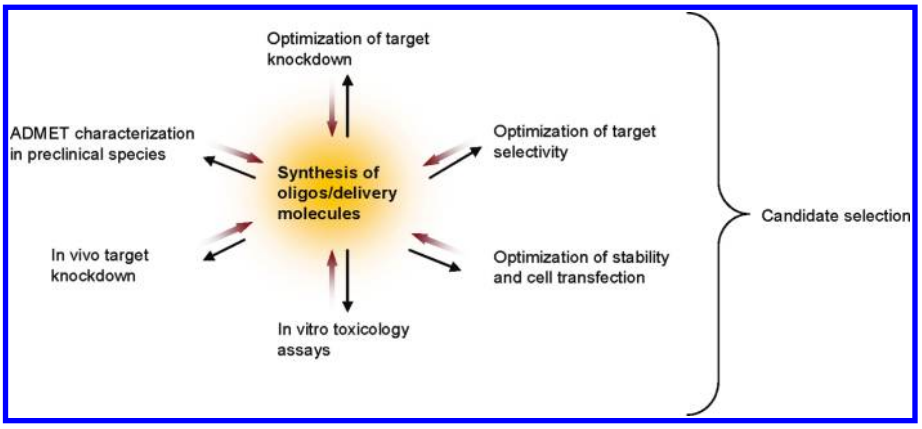

Figure 24. Proposed model for therapeutic siRNA optimization.

specialized in oligonucleotide delivery. These small delivery companies either have contracted with other small biotechs that specialize in siRNA gene sequence identification and oligonucleotide optimization or have been purchased outright by bigger firms.

The development of vehicles for siRNA delivery continues to evolve, and further optimization will require individual solutions to problems encountered by unique oligonucleotides, different target tissues, and specific indications, each dependent on the choice of delivery vehicle, whether liposome, polymer conjugate, or peptide-based systems. The medicinal chemistry of siRNA delivery is likely to require the same optimization cycles of SAR as with small molecule drug discovery. Synergies between the siRNA and the delivery vehicle are expected to be unique, in which an oligonucleotide can present different physical properties derived from both the sequence chemistry and stabilization modifications. Therefore, a considerable effort is needed to realize an siRNA therapeutic that provides potency, durability, and safety. The current specialized and fragmented approach within the industry may not be enough to sustain the efficient discovery of siRNA drugs. The discovery of siRNA therapeutics will require decades of experience in medicinal chemistry and pharmaceutical science that small molecule drug discovery has provided. The issues of enzymology, ADME, and toxicology pertaining to the delivery of siRNA are all very similar to small molecule drug discovery. However the approaches to solve these problems diverge somewhat, primarily because of the difference in size and physical properties of an oligonucleotide either complexed with or conjugated to a delivery vehicle.

In 2004, our colleagues at Merck published a Science Viewpoint article covering the intricate nature of small molecule drug discovery and how that process has evolved into a circular, nonlinear process involving varying expertise at even the earliest stages of the cycle.[55] We propose a model for the future of siRNA therapeutic design that similarly relies on a multitude of skill sets from the outset (Figure 24). In particular, we suggest that the intimate association of all aspects in oligonucleotide design with delivery optimization would yield significant improvements in efficiency. For instance, enhanced potency could result from the synergy of optimized siRNA/enzyme kinetics with improved endosomal escape features imparted by the delivery vehicle. Layered onto the delivery platform optimization, there must be specific disease franchise input. Scenarios will almost certainly emerge where, by nature of the disease and target, teams will focus on a specific delivery vehicle best suited for the specific needs of the target. Optimization of this vehicle could then occur simultaneously with oligonucleotide design and synthesis. In fact, as the industry moves further into conjugated siRNA therapeutic delivery modalities, it will become increasingly important that we do this given the inherent interplay between oligonucleotide structure and delivery vehicle physical properties.

One of the greatest differences between small molecule medicinal chemistry and siRNA delivery lies in the isolation and characterization of molecules. Unlike small molecule purification and analysis, typically normal or reverse phase chromatography followed by ^{1}H NMR and LCMS analysis, siRNA conjugates that range in size from about 14 000 to 70 000 MW, as well as liposome particles of 70−100 nm size, require additional methods. Taking advantage of size and charge differentials between reagents and products, purifications can utilize ultracentrifugation, size exclusion chromatography (SEC), strong ion exchange resins, and molecular

weight cutoff filters used in dialysis and tangential flow filtration (TFF). Analytical methods are numerous and diverse. Molecular weight may be obtained by GPC, MALLS, or MALDI-TOF. Charge may be differentiated through polyacrylamide gel electrophoresis (PAGE) or obtained via ζ potential or isoelectric point. Size is typically measured through dynamic light scattering (DLS) or observed via cryogenic electron microscopy (cryo-EM). Physical properties are also measured via sucrose density gradients, small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC), and isothermal calorimetry (ITC).

As in any small molecule program with an enzyme target, RISC/Ago2 enzyme binding kinetics and catalytic activity can potentially impact the potency and efficacy of a siRNA therapeutic. It is conceivable that the sequence itself and stabilization pattern, both of which provide unique chemistries, could impact enzyme affinity, binding kinetics, and activity. Beyond the inherent activity of the oligonucleotide at the RISC target, the potency and toxicity of a siRNA therapeutic will also be heavily influenced by the delivery vehicle. Therefore, the ADMET properties of the siRNA particle or conjugate become critical. At this juncture, two strategies can be pursued. One approach is to employ local delivery of the siRNA to compartments such as the eye, lung, joint, or mucosa, etc. Local delivery of siRNA therapeutics can evade some of the hurdles posed by in vivo efficacy, specifically biodistribution. The second approach involves systemic delivery, typically intravenous injection, although a recent report describes the potential for oral delivery as a future option.[56]

The current practice of iv injection of siRNA within a liposome or as a conjugate generally delivers the majority of the dose to the liver. This biodistribution takes advantage of the more fenestrated endothelium of this tissue for transport of macromolecules to the target hepatocytes. Beyond liver distribution, tumor tissue has been the only other major tissue compartment accessed because of the leaky vasculature in this disease state.[57] A common strategy in the oncology scenario is to utilize the enhanced permeability and retention (EPR) effect through extension of systemic circulation (e.g., increased PEG content).[58] Typically distribution within tumor tissue itself is heterogeneous because of multiple tumor microenvironments and associated barriers that impact efficient delivery. For instance, proliferating tumor cells exhibit differential extracellular pH, compressed blood and lymphatic vessels leading to interstitial hypertension, impaired convective transport, and slow interstitial diffusion. It follows that tumor vessels are characterized by heterogeneous vascular permeability and blood flow. Lower molecular weight siRNA conjugates can display some distribution to other organs, such as kidney and spleen. The long-term goal of targeting tissues other than liver and tumor will require the development of technologies that enable the passage of large molecules across tight junction cells and endothelia, a remarkably daunting challenge.

Because of the very short residence time in the blood, PK in this compartment tends to be irrelevant. With the majority of the dose sequestered to the liver, liver PK levels would be presumably relevant. But indeed it is the intracellular fraction of dose in the liver that is more relevant, more specifically the PK of RISC-loaded siRNA that becomes the most critical in assessing PK/PD relationships. This concept in part is demonstrated by the example given above of an siRNA PVE polymer conjugate that requires the covalent attachment of NAG targeting ligands for intracellular delivery of siRNA into hepatocytes.[46]

There are many successful examples of the in vivo delivery of siRNA, with regard to efficacy alone and primarily in mice. The current status in the field is moving toward greater therapeutic indices, with demonstration of efficacy and safety margins in higher species such as non-human primates. The focus on adverse effects and tolerability stems from a multitude of factors, many of which are not well understood. Some of the more documented adverse effects arising from siRNA delivery within liposomes or conjugates are mentioned here. The immune response arising from Toll-like receptor (TLR) activation by a foreign oligonucleotide is one of the earliest studied phenomena.[59,60] This pathway has been suppressed considerably by the incorporation of extensive sugar modifications to the oligonucleotide itself. The acute adverse effects of cytokine storm and coagulopathy can be triggered by a large net positive charge on the delivery particle or conjugate, as well as opsonization of foreign material in the bloodstream.[16b,c] Opsonization is the process by which antigens are altered so that they are more efficiently destroyed by immune cells. It is a process by which an antigen is coated with antibodies or a complement protein to make it identified by phagocytes. Masking of positive charge and PEG modification of the siRNA delivery construct are typically employed to suppress large net positive charge. The opsonization of liposomes may be heavily influenced by large particle sizes, as well as nonuniformity of particle surface. The aggregation of polymer and peptide conjugates into large particles can be of concern as well.

Regardless, the major adverse effect reported is typically related to liver damage. With the majority of dose distributed to the liver, potential tissue damage is often signaled by sharp increases in the liver transaminases, ALT and AST. This adverse effect is presumably derived from the lytic properties engineered into delivery vehicles to induce endosomal escape.[61] This comes in the form of cationic lipid small molecules formulated into liposomes or much larger cationic polymers and peptides which are reversibly masked. The consequence that most likely results from this amphiphilic design is a lytic species that continues to lyse healthy cells.[62] Methods to address this adverse effect could potentially be either the adjustment of pK_a related to the cationic species in an effort to improve biocompatibility via pH-dependent lysis[33,43] or the incorporation of biodegradability into the delivery vehicles to better clear any lytic species.

The current clinical landscape of siRNA therapeutics is considerably small. A handful of biotechnology companies have pursued local delivery of modified siRNA in human subjects. These include Quark, Transderm, ZaBeCor, and the RSV candidate from Alnylam. Four companies are highlighted that have Phase I programs to look at the safety and tolerability of systemic delivery of siRNA. Alnylam (ALN-VSP-02) is studying the delivery of two siRNA genes (KSP and VEGF) formulated in the SNALP liposome engineered by Tekmira (exact composition not disclosed). The indication is hepatocellular carcinoma (HCC)—primary or metastatic liver tumors. A recent press release from the ASCO 2010 meeting has noted that among the findings from the ongoing study was the suggestion of an anti-VEGF effect in the majority of treated patients. Notably, one patient in the study died from liver failure following treatment, an adverse event that was deemed possibly related to the drug (*Gene Silencing News*, May 27, 2010). Tekmira (PRO-040201) has also used its SNALP vehicle in a Phase I study to determine the safety and tolerability of ApoB siRNA delivery to liver, targeted toward

hypercholesterolemia (exact composition not disclosed). A recent press release from Tekmira disclosed that it had halted its Phase I study (*RNAi News*, February 11, 2010). Of the 23 subjects enrolled, 17 subjects received a single dose of ApoB SNALP at one of seven different dosing levels. According to the report there was no evidence of the preclinical DLT, liver toxicity, and of the two subjects treated at the highest dose level, one subject experienced flulike symptoms consistent with stimulation of the immune system caused by the siRNA oligonucleotide. Of the two subjects treated at the highest dose, the average transient reduction of ApoB protein and LDL cholesterol was 21.1% and 16.3%, respectively. The company is considering a number of slightly more modified siRNAs that will not stimulate the immune system for evaluation in a second Phase I hypercholesterolemia trial. In addition, the siRNA will be delivered using an improved second generation SNALP formulation. Silence (ATU-027) is studying the delivery of PKN3 siRNA to vascular endothelial cells, with application to solid tumors. The siRNA lipoplex (ATU-Plex) described earlier is another variant of liposome formulation (Figure 12).[20] Calando (CALAA-01) is targeting solid tumors as well, with the delivery of RRM2 siRNA within their cyclodextrin-containing polymer (RONDEL).[30] Their delivery vehicle is engineered with a targeting agent (AD-PEG-Tf, Figure 16) that targets cells overexpressing the transferrin receptor.

4. How Chemistry Can Enable Fulfillment of the siRNA Therapeutics Goal

The effective delivery of siRNA in vivo is a major challenge.[63] This can be divided into a number of major hurdles needed to overcome, with each step including a significant medicinal chemistry component (Figure 25). The first involves the chemical stability, potency, and specificity of the synthetic siRNA oligonucleotide. This challenge has seen great progress by various institutions.[64] Second is the synthetic assembly and packaging of the siRNA into a viable delivery vehicle. As outlined above, initial success has been realized across a number of delivery platforms, including lipid nanoparticle formulations, polymer polyplexes, and conjugates of various polymers, peptides, and cholesterol. A third step is modulating the clearance of the siRNA, as well as the delivery vehicle. Striking the correct balance is essential for generating a potent therapeutic that carries an appropriate safety margin, similar to small molecule drug discovery. Chemistry modification to the oligonucleotide helps to resist nuclease degradation and increase stability. The utilization of PEG to stabilize the delivery vehicle can increase systemic half-life. Yet the degradation and clearance of individual lytic components would be beneficial to decrease accumulation and reduce toxicity following siRNA transfection.

The biodistribution of siRNA to a specific tissue is an unsolved problem in the industry. Aside from local delivery, the systemic exposure of large and poorly diffusable siRNA delivery agents primarily results in distribution to the liver. This poses a great challenge to medicinal chemists who wish to engineer siRNA therapeutics that target specific organs beyond liver. An increased half-life in circulation may allow for greater partitioning into tissues, a phenomenon of which PEG derivatization has shown utility. An aspect of siRNA delivery in which chemistry can have a profound impact is the engineering of targeting ligand conjugates to a specific receptor for tissue retention. An example of this was presented above

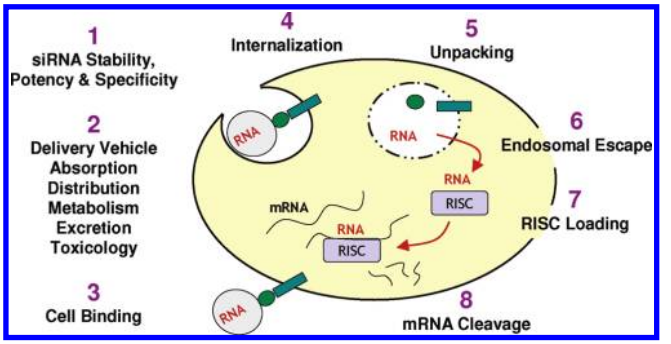

Figure 25. All steps involved in the systemic administration of RNAi therapeutics are dependent on successful medicinal chemistry strategies.

regarding the use of NAG for intracellular uptake. Targeted delivery is an entire topic of its own and a concept that is out of scope here.[63,65] Certainly the elements of targeted small molecule drug delivery and imaging agents weigh into this concept, all of which require clever design and chemistry execution.

Within the pathway of endocytosis, the acidity of the endosome increases to pH 5−6, and escape from this compartment is possible if the siRNA delivery complex induces lysis of the endosomal membrane. Endosomal escape must be chemically engineered into the delivery vehicle to avoid maturation to the lysosome from which there is no escape upon degradation of the siRNA via hydrolytic enzymes and a lower pH 4.5. This step is currently a cornerstone of many medicinal chemistry designs, the pH-dependent lysis of the endosome.[33,38,43,46,48] Now with entry into the cytoplasm, the chemistry design of oligonucleotide sequence and stabilization chemistry can impact RISC loading and mRNA cleavage efficiency. Subsequent to cellular transfection and mRNA knockdown, the disposition, clearance, and excretion of the delivery vehicle components can impact the toxicity profile. Inventing new designs for the synthesis of delivery vehicles that incorporate biodegradability and/or biocompatibility should improve toxicity profiles under chronic exposure. In addition to these medicinal chemistry challenges of siRNA delivery, the design and synthesis of fluorescent probes and radiolabeled tracers can have very big impact on the understanding of biodistribution and clearance, as well as intracellular trafficking of both the siRNA payload and the delivery vehicle components.

The perspective of approaching lead optimization for siRNA delivery as a SAR-driven exercise was presented above (Figure 24). One can imagine a future state of medicinal chemistry if RNAi therapeutics is truly successful, where the delivery of siRNA would become another platform from which to innovate and invent new drugs. There are multiple approaches within drug discovery that are available to the medicinal chemist for the prosecution of a therapeutic pathway. Typical approaches include the rational design of single molecules, small focused libraries of chemical space, PET ligand design, or the engineering of an affinity selection molecule. One could add to this list of approaches the design of an appropriate delivery vehicle for siRNA. The RNAi platform could be integrated across therapeutic areas and franchises and utilized based on a prioritization of approaches. In this future state, medicinal chemistry teams could be mobilized toward developing a delivery strategy unique to a given indication, tissue compartment, and siRNA oligonucleotide.

The potential impact of RNAi therapeutics upon medicine is great. The field could allow an expanded access to targets across the entire genome. The percentage of protein targets deemed nondruggable by small molecules can potentially be accessed within the siRNA manifold as well. The current hope and promise are that RNAi therapeutics will provide innovative and differentiated medicines at the frontier of the drug industry and address unmet medical needs. The next decade will reveal whether this hope and promise can become a reality, through clinical success in liver delivery and preclinical advances in targeting outside the liver.

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Biographies

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Steven L. Colletti received his Ph.D. in Chemistry from Boston University, followed by an NIH Postdoctoral Fellowship at The Scripps Research Institute, La Jolla, CA. In 1994 he joined the Department of Medicinal Chemistry at Merck Research Laboratories in Rahway, NJ, where he worked on small molecule drug discovery in the therapeutic areas of atherosclerosis, hypertension, diabetes, obesity, rheumatoid arthritis, asthma, and infectious diseases. During his tenure at Merck he has held positions from bench chemist to program leader and is currently Director of Medicinal Chemistry heading the Department of siRNA Delivery Chemistry in West Point, PA. Steve has coauthored over 50 research publications and been named an inventor on over 30 PCT applications.

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