Review

Clinical Lipidology

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Development and clinical applications of siRNA-encapsulated lipid nanoparticles in cancer

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Efficient delivery of siRNA to cancer cells after systemic administration poses a significant challenge. While many methods of nucleic acid delivery have been described, encapsulation of siRNA in lipid nanoparticles (LNPs) represents the most clinically advanced delivery approach. Currently, there are two siRNA-LNP-based treatments (ALN-VSP and TKM-PLK1) in clinical trials targeting solid tumors, with additional studies ongoing for noncancer diseases. The consensus from these clinical studies is that siRNA-LNP represents safe and potent silencing systems. Improvements in LNP technology through development of more potent and biocompatible ionizable cationic lipids along with targeting lipids to mediate delivery of LNPs specifically to cancer cells are on the horizon. In combination with genomic screening, it is possible that within the next 5 years the pathogenic drivers of individual cancers will be identified and siRNA-based personalized medicines will be formulated to achieve successful treatment of cancer and other genetic diseases.

Keywords: cancer • delivery • ionizable cationic lipids • lipid nanoparticle • potent • siRNA

Cancer is the leading cause of death worldwide, with the most recent available data from the International Agency for Research on Cancer reporting through the GLOBOCAN Project (2012) that 8.2 million deaths in 184 countries were caused by cancer, which rose from 7.6 million deaths in 2008 [1,2]. It is estimated that by 2025 there would be 13 million deaths accounted for by cancer. In Canada, the numbers were even higher, with approximately 30% of deaths being caused by cancer. In 2012, it was estimated that 500 new diagnoses and 200 deaths per day were due to cancer [3]. The hallmark of cancer is gene mutation(s) resulting in uncontrolled cell growth with malignant tumors invading neighboring and distant tissues if left untreated. Currently, conventional cancer treatment involves surgery, chemotherapy and/or radiation, alone or in combination. Unfortunately, significant side effects are associated with chemo and radiation therapies, and in many cases these treatments have only limited effectiveness. The poor response rates to these therapies can be due to genetic incompatibility, location of tumor, severe toxicity or some combination of these.

Cancer-causing gene mutations result in very complex modifications of cellular function that can affect multiple biological pathways. Therefore, one of the challenges in successfully treating cancer is determining and targeting the specific pathogenic mechanism(s) involved. Unfortunately, since most cancers have a unique genetic code, many mechanisms are possible, frequently rendering conventional cancer treatments ineffective [4]. As a result of this heterogeneity, there is a need to treat cancers, even those of the same tissue with the same diagnoses/classification, as unique diseases [5–7]. Therefore, to improve the specificity of treatment when dealing with genetic diseases such as cancer, it would be beneficial to take a personalized medicine approach [5,6]. This new revolution in disease treatment requires an understanding of the specific pathogenic mechanisms and preparation of tailored ther-

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apies for individual patients [5,6]. As the 'omics' revolution (genomics, proteomics, metabolomics, among others) identifies specific genes and gene products that are pathogenic drivers of cancer, there will be a need to target and regulate the expression of these crucial genes. Since many of these targets will be 'nondruggable' with conventional small molecule or antibody treatments, alternative strategies are needed to realize the potential of the personalized medicine approach for treating genetic diseases. An attractive strategy by which to treat genetic diseases is through siRNA-based therapeutics [8].

siRNA therapeutics

RNAi, a process of dsRNA-mediated gene-specific silencing, was first described in mammalian systems by Fire and colleagues [9,10]. The subsequent discovery that short synthetic dsRNA termed siRNA are capable of inducing RNAi and mediating specific silencing of virtually any target gene has been the basis for what is now a crucial tool in basic research. More recently, there has been significant and growing interest and excitement in the potential therapeutic application of siRNA, particularly against the numerous 'undruggable' targets. siRNA is a very attractive therapeutic alternative to small molecule chemotherapeutic agents or antibody drugs owing to the specificity, excellent safety profile and the potential for rapid early-stage drug development based on the availability of genomic screening/sequencing data to enable siRNA sequence selection [8]. Despite the tremendous potential of siRNA therapeutics, its use as a free drug has faced several significant obstacles including rapid clearance upon systemic administration, rapid breakdown of unmodified siRNA in biological fluids, poor accumulation at disease site, inefficient intracellular delivery and unintended stimulation of immune system.

Key modifications to siRNA structure have largely provided solutions to the issues of stability, off target effects [11,12] and immune stimulation [13,14]. For example, chemical modifications, such as phosphodiester backbone modifications, 2′OH positional modification, ribose modification and residue replacement, have overcome the increased sensitivity of the ribose backbone of siRNA to hydrolysis compared with DNA, and, thus, stabilized the molecule, leading to improved siRNA activity. Although this review will not cover advancements in siRNA design and modification, a number of recent comprehensive reviews are available [15,16]. In addition, siRNA modifications have also been used to address unintended stimulation of the immune system (for comprehensive reviews see [17,18,19]). While unmodified siRNA has shown to be effective in gene silencing, it has also been shown that these molecules can exert a nonspecific therapeutic effect that is attributable to stimulation of immune system [20]. As extensively reported, unintended immune stimulation has a significant potential to skew experimental results, particularly those from *in vivo* studies. For example, using a nonimmunostimulatory siRNA control, Robbins and colleagues were able to show that active siRNA duplexes can result in antiviral activity and suppressed gene expression through mechanisms unrelated to the specific RNAi gene silencing mechanism, but rather, due to immune stimulation [20]. Therefore, the selection of nonimmunostimulatory siRNA molecules is critical when designing target siRNA and control sequences as these may impact the therapeutic effect and its interpretation, as well as potentially mediating adverse side effects. In addition to impacting interpretation of experimental results, this phenomenon has also been found to impact therapeutic development of siRNA. Unintended immune stimulation has proven to be the dose-limiting toxicity [13] in early siRNA clinical trials.

Targeting of free siRNA

Although the issues of rapid breakdown/sensitivity to nuclease and nonspecific and unintended immune stimulation have largely been addressed through chemical modification of the siRNA, these changes have not improved the intracellular accumulation as negatively charged macromolecules do not readily diffuse across cell membranes. To overcome this hurdle, strategies to incorporate additional modifications, such as inclusion of targeting moieties, have been employed to improve the uptake of siRNA into cells. For example, Wolfrum and colleagues showed that upon conjugation to cholesterol or other lipophilic moieties, the lipophilic siRNA associated with serum components such as albumin and HDLs and LDLs [21,22]. This was found to mediate siRNA accumulation in different tissues based on the interaction of the associated serum components with their cognate receptors on the plasma membrane of various cell types [22]. However, the process was not as simple as first proposed. The import of these conjugated siRNA was still reported to be dependent on transmembrane protein systemic RNAi-deficient-1 SIDT1, suggesting a duality where association with the lipoprotein serves to dock the lipophilic-conjugated siRNA to surface of cells while SIDT1 mediates the intracellular accumulation of siRNA [22]. This process was observed in the gene specific silencing of the protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase CNPase in rat and nonhuman primate (NHP) brain by direct intraparenchymal infusion [23] that was further improved upon siRNA conjugation to cholesterol [24]. Extensive efforts were thus made in the fields

of lipophilic-conjugated siRNA, as well as conjugation to small molecules using modular click chemistry [25].

This strategy has proven so promising that it is currently undergoing evaluation in human clinical trials. In supporting preclinical studies in mice and NHP, conjugation of siRNA against transthyretin (TTR), a plasma retinol and thyroxin binding and transport protein, to the carbohydrate GalNac (*N*-acetylgalactosamine) proved to induce potent silencing following subcutaneous (s.c.) administration, resulting in 50% target gene silencing (ED_{50}) at doses of <5 mg/kg. This was mediated by efficient siRNA uptake into the liver via the cognate receptor for GalNac, the asialogycoprotein receptor. This provided an extremely wide therapeutic index (ratio of toxic dose to therapeutic dose) since five doses daily at 300 mg/kg in rats and single 300 mg/kg doses in NHPs were found to be well tolerated [26]. Currently, a Phase I clinical study in the UK is ongoing evaluating the s.c. administration of GalNac-conjugated to TTR siRNA for the treatment of TTR-mediated amyloidosis [27]. Also, this technology has been applied to silence the antithrombin (AT) gene as a treatment strategy for hemophilia. Preclinical studies using GalNac conjugated to anti-thrombin siRNA (ALN-AT3) administered by s.c. administration have demonstrated potent, titratable and reversible silencing of AT3 [28,29] and recently, this product was granted orphan drug designation by the USA FDA [27].

These are examples of siRNA conjugated to a targeting ligand with therapeutical potential in the liver following s.c. administration. While the effective doses are considerably higher (ED_{50} <5 mg/kg) than those necessary for similar silencing following intravenous administration of lipid nanoparticle (LNP) encapsulated siRNA (ED_{50} ~0.03 mg/kg) in NHP [30] (see the 'LNP formulations of siRNA' section), the advantages of such a route of administration make this strategy very attractive. The targeting of GalNac to the asailoglycoprotein receptor is very specific, mediating targeted uptake in the liver, thus providing a viable strategy for the treatment of cancers and other genetic diseases of the liver. For diseases outside the liver, however, the short plasma half-life of conjugated-siRNA (in the range of 0.5–2 h [22]) and hepatocyte affinity do not facilitate accumulation at extrahepatic sites of disease.

Delivery of siRNA using lipoplexes

First-generation lipoplex systems consisting of permanently positive charged particles were toxic. However, with synthesis of less toxic lipids various lipoplex delivery systems have been tested for siRNA delivery in various tumor models. The most promising lipoplex system in the clinic is AtuPlex employed by Silence Therapeutics (London, UK). The AtuPlex technology revolves around the complexing of siRNA with lipoplexes comprising of polycationic lipid AtuFect1 along with 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine and polyethylene glycol (PEG)–lipid [31,32]. AtuPlex delivery systems showed successful gene silencing *in vitro*. *In vivo* biodistribution studies in mice, however, reported somewhat confounding biodistribution data where delivery of siRNA to several organs, such as liver, spleen, lung, heart, pancreas and kidney was detected as early as 5 min post-administration [32]. Although siRNA was detected in several organs, the authors pinpointed the uptake of AtuPlex-siRNA and gene silencing to the endothelial cells of the organ's vasculature [32]. These findings led to the development of their lead candidate Atu027, a lipoplex formulation delivering siRNA against PKN3 to solid tumors [33]. Preclinical studies using Atu027 showed PKN3 silencing that was maintained for 144 h that inhibited tumor development on Matrigel and cell migration (of human umbilical vascular endothelial cells and human microvascular endothelial lung lymphatic cells) [33]. *In vivo* studies showed that PKN3 silencing was observed in lung and liver of mice, rats and cynomolgus monkey. When tested in mice bearing orthotopic prostate or pancreatic tumors, repeated Atu027 treatment showed inhibition of tumor growth and lymph node metastasis while causing no detectable toxicity [33]. Futhermore, since Atu027 showed successful delivery and gene silencing in lung cells owing to their highly vascularized nature, Atu027 studies were extended to several pulmonary metastatic tumor models [34]. Although metastasis was prevented by Atu027 treatment, repeated dosing did not reduce tumor volume of primary tumor [34]. Phase I clinical studies of Atu027 have been completed on 33 evaluable patients with solid tumors, indicating that Atu027 was well tolerated with a maximum tolerated dose of 0.336 mg/kg [35]. Currently, patients with pancreatic cancer are being recruited for a continuing Phase Ib/IIa study using Atu027 in combination with gemcitabine. As well, a second Phase Ib trial has been approved to look at the combination of Atu027 with cisplatin, 5-FU and cetuximab in the treatment of recurrent and metastatic head and neck squamous cell carcinoma [36].

Delivery of siRNA using neutral liposomes

In addition to cationic LNPs, neutral 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) liposomal systems have also been used to deliver siRNA. Neutral liposomal systems containing siRNA against the oncogene EphA2 (tyrosine kinase receptor) have been evaluated in orthotopic tumor models in mice bearing HeyA8 or SKOV3 ovarian cancer [37–39].

EphA2siRNA-DOPC systems mediated gene-specific silencing, as well as reduction of tumor volume upon repeated dosing [37,39]. The tumor volume was further reduced when cotreated with paclitaxel [37–39]. This formulation is currently undergoing Phase I clinical studies to establish toxicity and maximal tolerated dose profiles in patients with advanced and recurrent cancer. The initial preclinical studies showed great promise and EphA2-siRNA-DOPC systems have been further improved upon in ongoing preclinical studies. The anti-tumor effects of EphA2siRNA-DOPC systems were enhanced by loading them to biodegradable mesoporous silicon particles (S1MP) approximately 2-μm diameter with 26-nm pores [40] or co-treatment with FAK-siRNA-DOPC systems [41]. The loading of EphA2-siRNA-DOPC to S1MP resulted in sustained gene silencing over a 3-week period with a single dose in comparison to EphA2-siRNA-DOPC alone, which only lasted 5–6 days with repeated dosing. The sustained EphA2 silencing resulted in reduced tumor volume in two orthotopic ovarian tumor models, as well as reduced microvessel density and cell proliferation [40]. When cotreated with FAK-siRNA-DOPC systems, significant tumor growth reduction in conjuction with reduced cell proliferation and angiogenesis was also observed [41].

Other noncationic lipid delivery systems

Other noncationic lipid delivery systems include pH-sensitive fusonic peptide [42], cell penetrating peptide [43], cationic polymers [44], lipid–calcium phosphate systems [45–47], anionic–lipoplex bridged to siRNA through calcium [48,49], cationic polysaccharide chitosan [50] and cyclic oligomers of glucose-based delivery systems, such as cyclodextrin (for review see [51]).

LNPs

LNPs were first used for the delivery of poorly tolerated and/or soluble drugs to improve therapeutic index and overally efficacy (Figure 1). These systems were comprised of lipid bilayers surrounding a central aqueous core (for detailed review see [52–54]). Encapsulation of these drugs prolongs their lifetime in the circulation and protects the cargo from degradation, thus improving delivery to sites of disease. The enhanced delivery to target sites ultimately results in an overall improvement in drug efficacy. Therefore the application of liposomal systems to encapsulate genetic drugs (nucleic acids) was viewed as a potential strategy to achieve a safer alternative to viral systems for gene therapy. Felgner and colleagues first showed that permanently positively charged lipids can be synthesized and used for the delivery of plasmids for transfection purposes *in vitro* [55]. Unfortunately, the great expectations for *in vivo* applications ultimately led to disappointing results as lipoplex systems proved to be very toxic and have extremely rapid clearance and, thus, a short circulation half-life due to the inherent positive charge and large size (>200 nm). One challenge of encapsulating plasmid in the early days proved to be the sheer size of some plasmids. However, advances in encapsulation technology, such as cosolubilizing lipid and plasmid in the detergent octylglucopyranoside followed by the removal of detergent by dialysis against a citrate buffer [56] or using ethanol to destabilize preformed vesicles to allow encapsulation of nucleic acids by electrostatic forces [57], enabled efficient encapsulation (Figure 1B). Subsequently, the synthesis and use of ionizable cationic lipids drastically improved the toxicity of plasmid LNP. While these systems would be positively charged when presented to an acidic environment such as the buffer system for encapsulation (thus promoting interaction with the anionic nucleic acid payload and enhancing encapsulation efficiency) or an acidic milieu inside maturing endosomes (thus promoting release of the nucleic acid to the cell cytoplasm following endosomal uptake of the LNP) the LNP would be neutral at physiological conditions (such as in the circulation following intravenous administration) and, thus, possess prolonged circulation lifetimes. Structures of encapsulated plasmid or anti-sense oligo nucleotides showed bilayer systems with an aqueous core, however, further advances to encapsulation technology would provide stable solid core systems (Figure 1C).

LNP formulations of siRNA

LNPs represent one of the most clinically advanced technology currently available for siRNA delivery. The tremendous progress seen in LNP systems for delivery of siRNA has largely been driven by the successful collaborations between Alnylam Pharmaceuticals (Boston, MA, USA) and its partners in the pharmaceutical industry (Tekmira Pharmaceuticals Corporation and Acuitas Therapeutics, formerly known as AlCana Technologies Ltd., BC, Canada) and academia (University of British Columbia, BC, Canada and Massachusetts Institute of Technology, MA, USA). These collaborations have been largely responsible for the evolution of ionizable cationic lipid/lipid-like substrates capable of mediating extremely potent *in vivo* gene silencing.

The current LNP formulation process involves the spontaneous formation of LNP through the rapid mixing of lipid components (comprised of ionizable/cationic that is used for encapsulation of nucleic acids and imparting fusogenic properties to the LNP, structural lipids such as 1,2-distearoyl-*sn*-glycero-3-phosphocholine and cholesterol, and PEG-lipids for providing particle stability during formulation and prolonging cir-

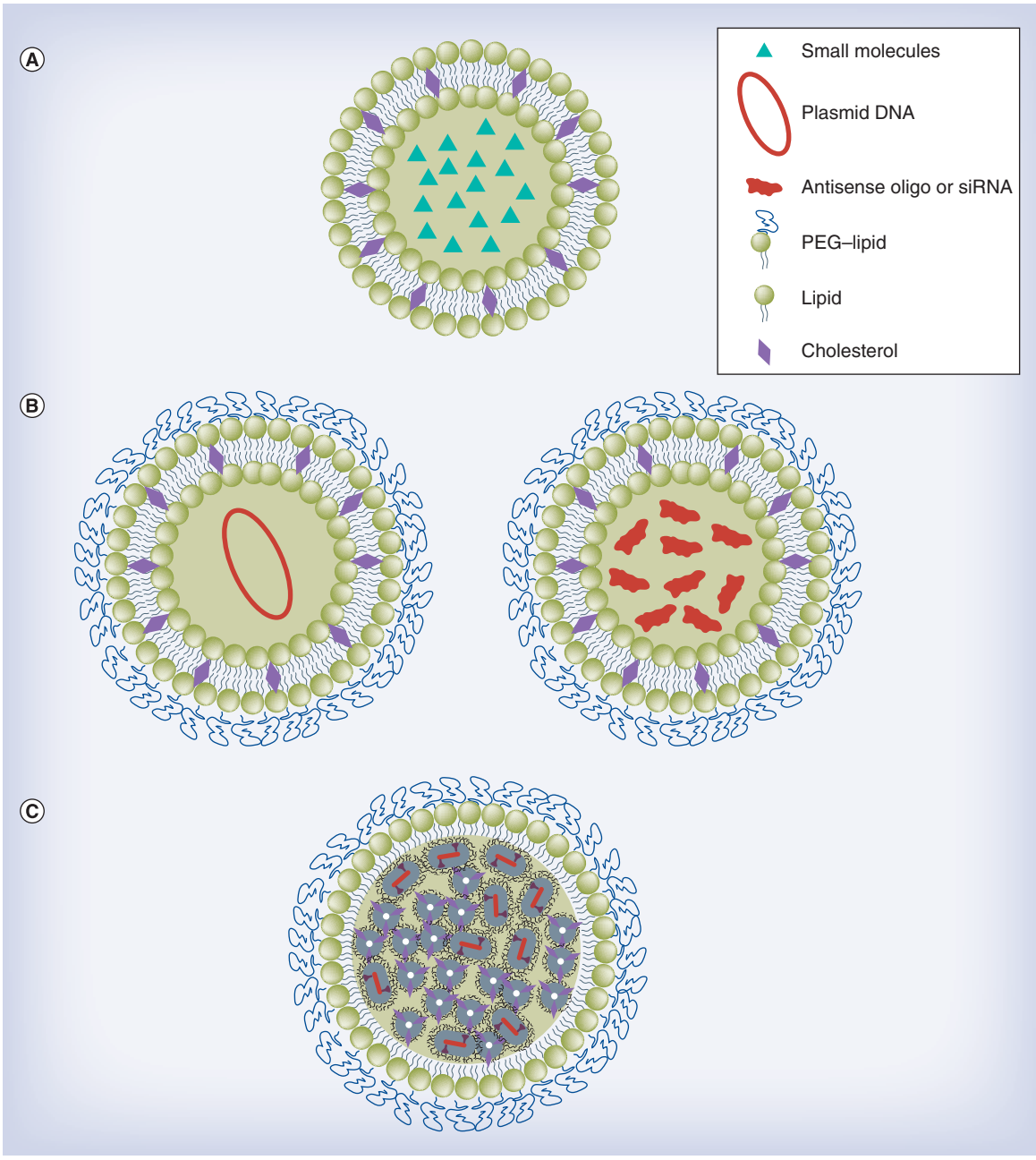

Figure 1. Lipid nanoparticle with different cargoes. (A) Encapsulation of small molecule therapeutic in a liposome. **(B)** Encapsulation of nucleic acid using the preformed vesicle method. Plasmid, antisense oligonucleotides and siRNA can be encapsulated using ethanol destabilization protocol. **(C)** Encapsulation of nucleic acids by spontaneous vesicle formation by in-line mixing protocol or microf uidics micromixer method.
PEG: Polyethylene glycol.

culation, which is further discussed in the 'Targeting lipids' section) dissolved in ethanol with genetic (i.e., nucleic acid-based such as siRNA) drugs in an acidic aqueous stream. The acidic aqueous buffer functions to protonate the ionizable cationic lipid, which electrostatically associates with negatively charged nucleic acids. This process was first demonstrated using plasmid [58,59] and antisense oligonucleotide [60–62] systems and later developed for siRNA [63].

siRNA-LNP systems have been shown to be active in a number of biological systems including NHPs where silencing of the target gene, apolipoprotein B (ApoB) was durable over a span of several days [64]. Since the development of first-generation siRNA-LNP systems, siRNA-LNP systems have undergone a rapid evolution, with progressive improvements in potency through optimization of the LNP composition, as well as its lipid components. Perhaps the most

significant advances have been achieved through the synthesis of novel cationic lipids incorporating modifications in the head group, linker and/or fatty acyl chains (Figure 2) [30,65–66]. For example, first-generation cationic lipids such as 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP) and 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) have ED_{50}s of 40–50 and 1 mg/kg, respectively in Factor VII (FVII) silencing model [66]. The difference between these two lipids is in the linker between the head group and acyl chains, where DLinDAP contains an ester bond in comparison to the ether linkage in DLinDMA. Upon applying yet another modification of the linker to a ketal linkage, along with a head group change, the development of 1,2-dilinoleyloxy-keto-N, N-dimethyl-3-aminopropane (DLinKDMA) and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[2,4]-dioxolane (DLinKC2DMA) resulted in a 2.5- and a 10-fold activity improvement, respectively, in mice compared with DLinDMA-based LNP [66]. Using a rational design approach for the ionizable cationic lipids, it has been possible to make further dramatic improvements in the potency of the LNP systems. Through this approach, it has been shown that the pKa of the cationic lipid plays a crucial role in potency [66]. As first proposed by Hafez and Cullis, a bilayer to nonbilayer membrane transition resulting from ion pairing between cationic lipids, such as those in the LNP, and anionic lipids, such as those in the endosomal membrane, could facilitate fusion of the endosomal membrane with the LNP and allow intracellular delivery of the siRNA [67,68]. Internalized LNP systems in endosomal compartments are exposed to a progressive acidification mediated by V-ATPase, resulting in the ionization of the cationic lipid to an extent dictated by the pKa of the head group. The prominent feature of lipids capable of mediating the most potent siRNA-based gene silencing was a narrow optimum pKa range of 6.2–6.5, where the lowest ED_{50} (for O-[Z,Z,Z,Z-heptatriaconta-6,9,26,29-tetraen-19-yl]-4-[*N*, *N*-dimethylamino] DLinMC3DMA) reached 0.03 mg/kg in mice (Figure 2) [30]. Ultimately, through this work, optimization of the lipid composition allowed a greater than 200-fold improvement in the ED_{50} of siRNA-LNP systems compared with those DLinDMA-LNP systems [30,66] originally described. The importance of pKa is not only a determining factor in the potency of cationic lipids but also in the potency of lipid-like substrates often referred to as lipidoids [69]. To date, the most potent cationic lipid that has been synthesized is DLinMC3DMA. DLinMC3DMA-based systems with a pKa in the optimal range (pKa = 6.44) are capable of mediating siRNA release to the cytosol as demonstrated by cryoelectron microscopy [70].

However, additional increases in potency have also been realized through changes to the LNP formulation methodology and its composition. For example, production of LNP incorporating the novel 50% DLinKC2DMA (mole:mole) lipid using an in-line process resulted in a decrease in ED_{50} (dose resulting in a 50% reduction compared with control) compared with 40% cationic lipid-containing LNP produced using preformed vesicle (PFV) methodology, from 0.1 to 0.02 mg/kg in mice when silencing the gene for the FVII clotting factor produced in the liver [66]. Importantly, these DLinKC2DMA-LNP systems were well tolerated and proved to be highly potent in NHP where these systems were capable of silencing the therapeutically relevant TTR gene in cynomolgus monkey, with ED_{50} of 0.3 mg/kg [66]. To date, six siRNA-LNP drug candidates based on LNP technology have been evaluated in human clinical trials by Alnylam and Tekmira, five of which are ongoing with one in Phase II and one in Phase III trials. While most of these clinical studies are being carried with first-generation LNPs incorporating DLinDMA, the most advanced clinical study evaluating TTR silencing utilizes the more potent DLinMC3DMA-based LNP systems [71]. Results from these TTR trials have been very promising. Patients treated with ALN-TTR01 (DLinDMA-based LNP) or ALNTTR02 (DLinMC3DMA-based LNP) demonstrated greater than 50% TTR silencing for 28 days at 1 mg/kg dose for ALN-TTR01 and 0.15–0.5 mg/kg for ALN-TTR02 [71] with little or no drug-related adverse events.

In terms of cancer, two LNP systems to treat liver cancer have been evaluated in the clinic. A Phase I clinical trial targeting vascular endothelial growth factor and kinesin spindle protein KSP (ALN-VSP) in 41 patients with hepatic and extrahepatic tumors has been recently completed and reported [72]. Repeat administration of escalating doses ranging from 0.1 to 1.5 mg/kg (average dose/patient: 6.8) was well tolerated and resulted in detectable siRNA accumulation in tumors along with target gene-specific silencing [72]. Seven patients with minimal stable disease condition continued with a Phase I extension study. Overall, the ALN-VSP clinical Phase I study demonstrated the safety of siRNA-LNP systems in humans for cancer treatment [72]. In another cancer driven clinical Phase I study using the stable nucleic acid lipid particle technology, 24 patients with solid tumors were treated with PLK1siRNA-LNP (TKM-PLK1) [73]. Consistently, siRNA-LNP systems were well tolerated and were clinically active as assayed

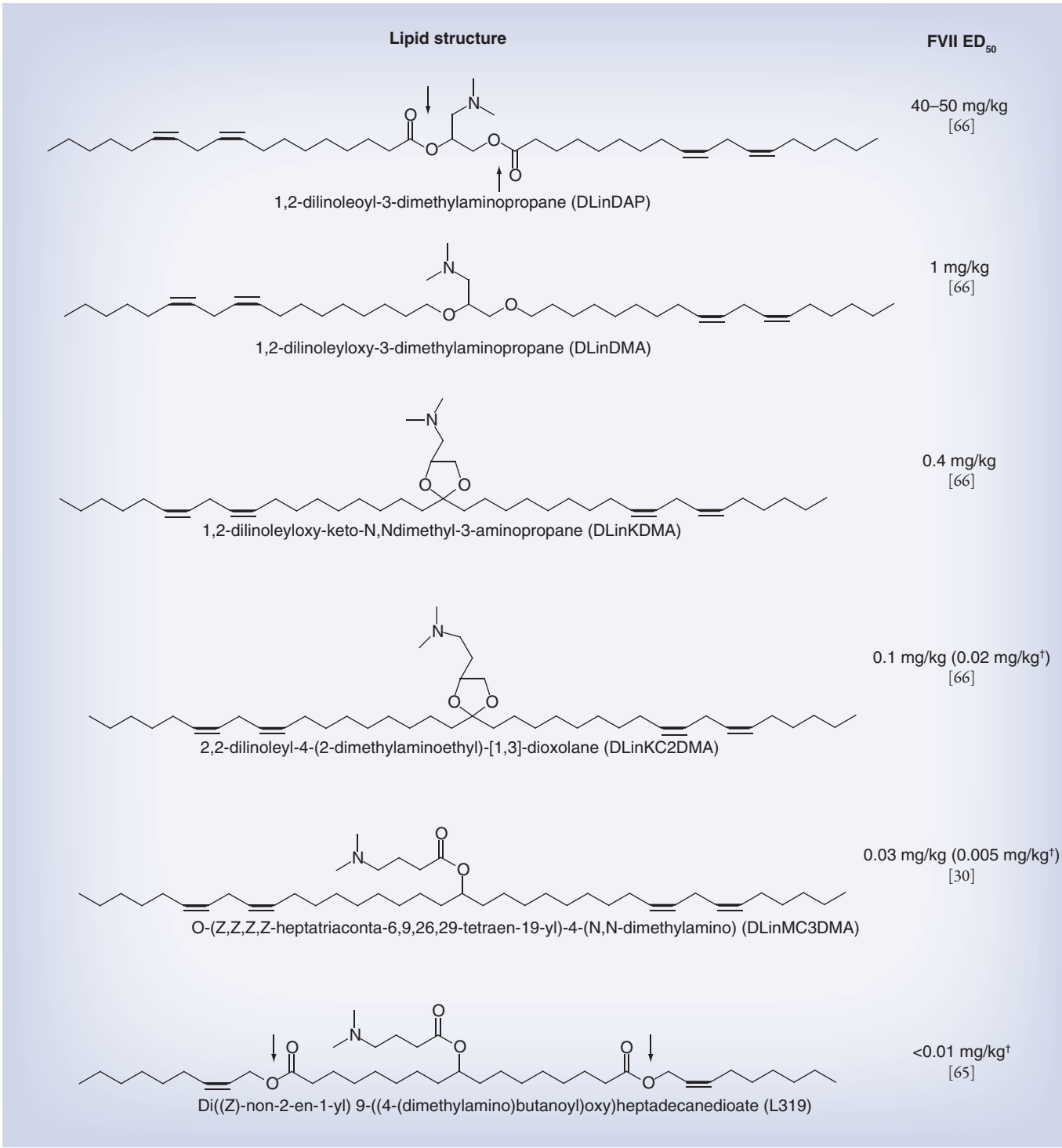

Figure 2. Structures of cationic lipids and corresponding liver Factor VII ED_{50}. ED_{50} corresponds to formulations consisting of mole ratio of 40% cationic lipid, 10% PEG-lipid, 40% cholesterol and 10% 1,2-distearoyl-*sn*-glycero-3-phosphocholine. Arrow indicates biocleavable sites.
†Indicates optimized formulation consisting of 50% cationic lipid, 1.5% PEG-lipid, 38.5% cholesterol and 10% 1,2-distearoyl-*sn*-glycero-3-phosphocholine.
FVII: Factor VII.

by tumor biopsy [73]. Enrollment to Phase I/II studies for patients with either advanced gastrointestinal neuroendocrine tumors or adrenocortical carcinoma has been initiated with additional enrollment to Phase I/II studies for patients with hepatocellular carcinoma in 2014 [74].

Delivery of siRNA using lipidoids

Other siRNA delivery modalities have employed lipid-like molecules termed 'lipidoids' for the delivery of genetic material. Lipidoids delivery systems are similar to ionizable cationic LNP systems as it uses lipid-like molecules (for encapsulation of nucleic acids and fusogenic properties to deliver siRNA to cytosol), cholesterol and PEG-lipid [75]. The key difference between LNPs that use lipidoid instead of ionizable cationic lipids is the excess positive charges associated with lipidoid nanoparticles since lipidoid molecules possess multiple protonable amines linked to various acyl chains. Similar to the work with cationic lipids, improved lipidoid systems were identified through screening programs. In this case, initial screens incorporated modular chemical synthesis in conjunction with plasmid delivery-based activity assays [76,77], which was later refocused to the delivery of siRNA [75,78–80]. These lipid-like substrates were synthesized and tested *in vitro* prior to *in vivo* testing [75,79–80]. Initial screening efforts identified $98N_{12}$-5, which showed siRNA- and miRNA-mediated gene silencing in murine liver, peritoneal macrophages and lung by different routes of administration [75]. However, pharmacodynamic studies following intravenous and intraperitoneal injection only showed significant silencing in the liver [81]. This is most likely explained by the pharmacokinetics and biodistribution characteristics of these lipidoid particles with over 95% of the administered dose accumulating in liver and spleen within 1 h with less than 3.5% injected dose detected in the blood over that same time [78]. Also, the silencing and tolerability studies were carried in both rats and NHP with the optimal formulation termed LNP-01 showing good silencing with slight increase of ALT and AST in NHP [44,75].

The most potent lipidoid molecule synthesized to date is C12-200 with ED_{50} <0.01 mg/kg for FVII silencing in mice compared with the ED_{50} of <1.5 mg/kg seen with first-generation lipidoid $98N_{12}$-5 [79]. C12-200 lipidoid nanoparticles were well tolerated at 1 mg/kg, 100-fold higher than the dose in mice, which mediated simultaneous 50% silencing of five different gene targets by siRNA delivered using a single lipidoid nanoparticle [79]. Furthermore, the gene silencing of TTR in NHP was observed with ED_{50} <0.03 mg/kg [79]. The mechanism of action of lipidoid nanoparticles, like that of LNPs, relies on the pKa [69]. However, lipidoid-mediated siRNA cellular uptake and delivery is different from that mediated by LNP systems containing the ionizable cationic lipids [70]. Intracellular accumulation and processing of LNP is achieved through clathrin-mediated endocytosis and macropinocytosis with the eventual degradation of unreleased siRNA in lysosomes [70,82] By contrast, lipidoid nanoparticle uptake is mediated by macropinocytosis with colocalization of nanoparticles with markers of the recycling endosomal compartments, suggesting the expulsion of part of the siRNA-LNP out of the cell [83].

Further advances to LNP technology

siRNA LNP developed by Alnylam Pharmaceuticals, Tekmira Technologies and Acuitas Therapeutics are the most potent systems for gene silencing in the liver and improvements to the potency and tolerability of these liver-targeted siRNA-LNP systems continue to be made. Unfortunately, similar potencies have not yet been achieved for extrahepatic targets. For example, DLinKC2DMA LNP systems in LnCaP prostate cancer mouse model required a regimen of six doses at 10 mg/kg [84] and mouse immune cells required a dose of 5 mg/kg [85] to observe appreciable gene silencing. Therefore, much work remains to achieve effective extrahepatic siRNA-LNP-mediated gene silencing at therapeutically relevant doses. However, it is envisioned that continued improvement of these systems for liver silencing will be adapted to achieve more efficient extrahepatic, tissue-specific uptake, likely through incorporation of targeting lipids, a topic that will be discussed in the 'Targeting lipids' section.

siRNA LNP formulation by microfluidics

Semple and colleagues showed that the silencing activity of LNP can be dramatically impacted by the formulation process. For example, DLinKC2DMA-based LNP particles made using in-line methodology were found to be fivefold more active than LNPs consisting of the same lipid components made using PFV methodology [66]. While both processes have their respective advantages, the limitations of the PFV method include a minimum formulation volume, variable encapsulation efficiency and a relatively more time-consuming process. In comparison, the in-line methodology is a simpler process, but is difficult to apply to small sample volumes due to the high flow rates employed, particularly when trying to achieve particles smaller than 50 nm in diameter. To address these limitations, a new technology has been developed to formulate LNP using micromixers [86,87]. The technology relies on the rapid mixing of lipids dissolved in an ethanol stream with siRNA dissolved in an aqueous stream using a staggered herringbone micromixer structure. Using this apparatus at 2 ml/min, complete mixing of the two streams is achieved in 3 ms [86]. The resultant particles are monodispersed and sizes are tunable by simply varying the PEG-lipid content. In fact, through this approach, it is possible to generate 'limit size' siRNA-LNP of approximately 30 nm diameter [86]. The activity of siRNA-LNP systems generated using the microfluidic mixer are similar or greater potency

than formulations of similar size formulated using the in-line methology [66,86]. The physical properties of the siRNA-LNP systems generated using the microfluidic mixer have been well characterized and consist of electron-dense solid core particles with the siRNA protected from external factors, such as nuclease activity (Figure 1C) [88]. The key advantages to using the microfluidic mixer are: rapid and reproducible mixing rates, tunable size, reproducibility, scalability and ease of use.

Biodegradable lipids

The current gold standard ionizable cationic lipid is DLinMC3DMA, a lipid that has been shown to be highly potent in mice, rats, nonhuman primates [30] and humans [71]. Although the tolerability of DLinMC3DMA in mice, rats or NHP have not been published, the much more potent DLinMC3DMA, with an ED_{50} of 0.005 mg/kg, would be tolerable at higher doses as its predecessor DLinKC2DMA with an ED_{50} of 0.2 mg/kg was shown to be well tolerated to doses of 3 mg/kg. This would provide a 150-fold greater therapeutic index based on the relative potency of DLinKC2DMA [66]. Most importantly, clinical studies have shown that DLinMC3DMA is safe and well tolerated in humans while maintaining high bioactivity [71]. Therefore, this generation of lipids is potent and relatively well tolerated. However, there remains a desire to develop increasingly biocompatible therapeutic reagents that are easily eliminated [65]. This next generation of ionizable cationic lipid has been achieved in the collaboration between researchers from Alnylam Pharmaceuticals, Acuitas Therapeutics and UBC. The early work in ionizable lipids showed that DLinDAP had very poor activity [66] that was later attributed to the rapid degradation of the ester linkage between the head group from the fatty acyl chains by endogenous esterases/lipases (Figure 2) [82]. In order to retain potent activity in the ionizable cationic lipids, Maier and colleagues designed biodegradable lipids based on DLinMC3DMA (Figure 2) [65], retaining key characteristics known to engender good activity, specifically, an optimal pKa of 6.2–6.5 and a conical lipid shape to induce a nonbilayer transition. To these key features, biocleavable sites were incorporated (Figure 2) [65]. From this work di([Z]-non2-en-1-yl) 9-([4-(dimethylamino) butanoyl]oxy)heptadecanedioate (L319) showed similar gene silencing activities to DLinMC3DMA in mice while remaining tolerable at doses of 10 mg/kg in rats. This was likely due, at least in part, to the rapid and complete elimination of the lipid by 72 h postadministration in rats, with excretion of 30% in the urine by 12 h and 40% in the feces within 12–24 h [65]. The complete elimination of L319 in plasma in cynomolgus monkeys was observed by 48 h [65].

Targeting lipids

Much of the research in this area has focused on siRNA-LNP geared towards hepatic delivery to treat liver diseases. This was designed to take advantage of the inherent tendency of these LNP systems to accumulate in the liver and be taken up into hepatocytes. The efficient intracellular delivery has been attributed to LNP uptake into the hepatocytes via a very robust endogenous hepatic targeting process mediated by ApoE binding and uptake via LDL receptors [89]. The short half-life of the liver-targeted siRNA-LNP systems following systemic administration (<30 min) is a direct consequence of this rapid hepatic accumulation [65]. However, design of LNP systems to reach extrahepatic targets will require significantly longer circulation lifetimes. It is well known that LNP with long (>10 h) circulation lifetimes exhibit significant accumulation of LNP at sites of disease such as in distal tumors and sites of inflammation [90,91]. It is also well known that prolonged LNP circulation can be achieved by increasing the PEG-lipid content to effectively shield the siRNA-LNP and prevent association of ApoE and other serum proteins to the LNP. This can be achieved by either incorporating a greater percentage of PEG-lipid into the LNP, using PEG-lipids with increasing lengths of acyl chains or a combination of the two. Increasing acyl chain lengths can modulate LNP circulation lifetime as these PEG-lipids remain associated with the LNP for progressively longer periods of time [92]. Specifically, C14–PEG has a desorption rate of 45%/h while C16-PEG and C18-PEG have desorption rates of 1.3 and 0.2%/h respectively [92]. This translates to LNP clearance of $t_{1/2}$ of 35 min for C14-PEG, 2 h for C16-PEG and 3.7 h for C18-PEG containing LNP at an initial PEG content of 1.5% [92]. Not surprisingly, prolonging the association of PEG-lipid with the LNP by modulation of acyl chain length resulted in a concomitant decrease in liver accumulation and a corresponding decrease in siRNA-mediated silencing activity in the liver [92]. For example, liver activity using the Factor VII silencing model showed progressive increase in ED_{50} to 0.02, 0.03 and 0.04 mg/kg for systems containing 1.5% mole C14-PEG, C16-PEG or C18-PEG, respectively. Interestingly, increasing PEG content from 1.5 to 2.5% mole C14-PEG had little effect in silencing activity while a significant reduction in potency was observed for 2.5% mole C18-PEG [92], likely due to the rapid exchange of C14-PEG from both the 1.5 and 2.5% PEG-lipid particles compared with C18-PEG-LNP. From these data, it can be concluded that the use of longer acyl chain length PEG-lipids would be advantageous in increasing both LNP circulation and delivery of LNP to extrahepatic tumor sites following systemic administration.

Unfortunately, increased PEG-lipid shielding also has the potential disadvantage of compromising intracellular uptake. The use of endogenous targeting mechanisms paralleling the ApoE-mediated LNP delivery to liver has been proposed as a strategy to achieve efficient uptake into extrahepatic tissues. Ideally, this involves the incorporation of an exogenous targeting moiety into the lipid composition that would direct LNP to receptors expressed on the surface of target cell and mediate intracellular uptake and accumulation. Proof-of-concept studies have shown that siRNA-LNP are not efficiently taken up into hepatocytes of mice lacking ApoE and as a result, were ineffective in mediating gene silencing [89]. However, upon conjugating an exogenous ligand, GalNac, to C18-PEG and incorporating this targeting lipid to the LNP composition, the siRNA activity was restored in ApoE$^{-/-}$ mice. The recovery of siRNA activity was directly correlated to the presence of GalNac since highly PEGylated stealth (to prevent ApoE association) targeted LNP systems had no activity in knockout mice lacking the cognate GalNac receptor ASGR2$^{-/-}$ [89].

A substantial body of work with regards to synthesis of targeting lipids continues to grow. The targeting ligands that have been evaluated in this regard include small molecules [93–99], peptides or antibodies (fragment or whole) [90,100–105], sugars [106–109] and aptamers [110] (for comprehensive review see [111]). Overall, it can be concluded that targeting moieties have the capacity to mediate enhanced LNP uptake and thus improve nanoparticle potencies. For example, extensive targeting studies have been carried by Huang's group including work targeting nanoparticles via anisamide to cells sigma receptors dating back to 2004 [112]. Based on earlier work targeting liposomes containing small molecule drugs, specifically doxorubicin [112], this group has reported on siRNA carriers systems targeted against different types of cancer that show an impressive ED_{50} of 0.075 mg/kg in the tumor [113–115]. In addition, screening approaches have been successfully applied to identify additional ligand–receptor pairs that can be used to effectively target siRNA-LNP [93]. For example, 800 small molecules were screened *in vitro* to identify molecules that enhance LNP uptake and a cardiac glycoside family of drugs that binds to plasmalemal Na^+/K^+ ATPase was identified to enhance the uptake of siRNA-LNP [93]. From this class of drugs, strophanthidin) was conjugated to a C18-PEG-lipid and incorporated into siRNA-LNP. LNP-strophanthidin PEG systems showed enhanced uptake in a range of cancer cell types including prostate, ovarian, pancreas, breast and liver cancer cell lines [93]. This is an example of promiscuous enhancement of uptake as Na^+/K^+ ATPase expression is observed in all tissues [93].

Conclusion

The evolution of LNP has been extremely rapid over the past 3 years as key design features of the nanoparticle, the formulation technology and ionizable cationic lipid that improve potency have been identified and exploited. While the first-generation lipid compositions incorporating DLinDMA showed promising results in the clinic, these systems have been significantly improved with the advent of second-generation lipids, such as DLinMC3DMA, which show dramatically increased potency while remaining well tolerated [71]. Available data to date all indicate that these LNP systems are safe and active at target tissues, particularly the liver [71,72]. The incorporation of newer biodegradable lipids will further improve on the safety profile of siRNA-LNP drugs while novel formulations methods along with targeting lipids will make it possible to customize siRNA-LNP for different subtypes of cancer.

Future perspective

Preclinical studies demonstrate potent DLinMC3-DMA-based LNP systems with ED_{50} in nonhuman primates to be <0.03 mg/kg in liver. Although this dose is substantially low, it must be recognized that these LNP systems readily accumulate in the liver, therefore, it is only applicable to liver cancer. For extrahepatic cancers it is estimated that few percentages of injected dose accumulate to tumor and with TTR02 clinical study showing tolerable doses at 0.5 mg/kg so it would be favorable to achieve extrahepatic tumor activity at below 0.5 mg/kg. The application of siRNA-LNP systems for cancer therapy is clearly at early stages. As stated, the greatest need is to improve the potency of currently available systems from current dose levels to those that are therapeutically relevant. As discussed, there are a number of strategies that may allow this to be achieved. First, in order to get LNP to accumulate at tumor sites, longer circulation lifetimes requiring stable PEG-lipid coatings will be necessary. In turn, this will require the use of targeting ligands to promote uptake into target cells. Second, the potency of these systems may be improved by improving release of encapsulated siRNA following uptake by endocytosis. Current indications are that even for optimized cationic lipids, less than 2% of the delivered siRNA dose escapes from the endosome and is available for gene silencing through an RNAi mechanism [70]. Therefore, there is significant room to enhance activity by improving endosomal release. Once effective delivery systems to distal tumors have been developed the possibilities are legion. As dem-

onstrated for liver targets, siRNA-LNP systems can carry siRNA cargos to silence five or more genes in target cells. One can therefore imagine personalized siRNA-LNP therapeutics containing a variety of siRNAs against causal proteins identified in individual tumors. Given the excellent toxicity profiles of current siRNA-LNP systems it may be expected that such systems would be relatively nontoxic and readily used in combination with standard chemotherapy protocols.

Executive summary

siRNA therapeutics

- Systemic administration of unmodified siRNA resulted in rapid clearance, rapid breakdown of unmodified siRNA in biological f uids, poor accumulation at disease site, inefficient intracellular delivery and unintended stimulation of immune system.
- Key modifications to siRNA structure have largely provided solutions to the issues of stability, off-target effects and immune stimulation.

Targeting of free siRNA

- siRNA conjugated to a targeting ligand improved cellular uptake of siRNA.
- Preclinical studies of GalNac-conjugated siRNA have shown successful target gene silencing. GalNac-TTRsiRNA is undergoing a clinical trial in UK.

Delivery of siRNA using lipoplexes

- Lipoplex technology has improved drastically as preclinical studies show safe and efficacious silencing systems.
- Preclinical studies show AtuPlex nanoparticles accumulating in various organs such as liver, spleen, lung, kidney, heart and pancreas. The accumulation was observed preferentially to endothelial cells of the organ's vasculature.
- The most clinically advanced lipoplex-siRNA drug candidate Atu027 showed positive Phase I results warranting two separate Phase Ib studies.

Delivery of siRNA using neutral liposomes

- Preclinical studies using neutral liposomes based on DOPC have antitumor effects. These effects include reduced tumor growth, cell proliferation and angiogenesis in ovarian cancer tumor models. A Phase I study of EphA2-siRNA-DOPC is ongoing to investigate tolerability and maximum tolerated dose in patients with advanced and recurrent cancer.

Lipid nanoparticles

- Efficient encapsulation of genetic drugs into lipid nanoparticles (LNPs) was crucial to employing LNPs as delivery vehicles.

Lipid nanoparticle formulations of siRNA

- Structure/activity relationship studies correlated the potency of ionizable cationic lipids to the pKa of ionizable cationic lipid head group.
- The gold standard lipid DLinMC3DMA in an optimized siRNA-LNP system have ED_{50} ~0.03 mg/kg in NHP.
- Six siRNA-LNP drug candidates are undergoing clinical trials with two candidates used for liver cancer.
- Phase I studies show siRNA-LNP drug candidates to be tolerable with successful target gene silencing.

Delivery of siRNA using lipidoids

- C12–200 is the most potent lipidoid molecule synthesized to date with gene silencing of TTR in nonhuman primates at ED_{50} <0.03 mg/kg. Similar to ionizable cationic lipids, the mechanism of action is dependent on the pKa.
- Lipidoid nanoparticle uptake is mediated by macropinocytosis with colocalization of nanoparticles with recycling endosomes. The siRNA is shown to be expulsed out of the cell.

Further advances to LNP technology

- siRNA LNP formulation by microf uidics:
  - Microf uidics micromixers form electron dense lipid nanoparticles with similar or greater potency than LNPs formulated by other means. The key advantages to using the microf uidic mixer are: rapid and reproducible mixing rates, tunable size, reproducibility, scalability and ease of use.
- Biodegradable lipids:
  - Potent biocompatible cationic lipids can be designed to replace existing ionizable cationic lipids. This new generation of lipids provide biodegradable LNP with improved drug tolerability and rapid clearance.
- Targeting lipids:
  - PEG-lipids dictate the lifetime circulation of LNP systems. Longer circulating systems can be achieved by increasing mole percentage of PEG or using longer acyl chains to slow the PEG-lipid exchange rate.
  - Targeting moieties such as small molecules, peptides/antibodies, sugars, and aptamers can be conjugated to PEG-lipids to generate cell specific targeting LNP systems.

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