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Review

Design of cationic ionizable lipids for the delivery of therapeutic nucleic acids

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Ionizable cationic lipids are a critical component of lipid nanoparticles (LNPs), enabling the clinical success of nucleic acid therapeutics through effective encapsulation, delivery, and release. As the field accelerates beyond first-generation RNA medicines, the rational design of next-generation ionizable lipids has become a key area of research. In this review, we outline key design principles that guide the development of efficacious and safe ionizable lipids for nucleic acid delivery. We highlight emerging structural motifs and discuss how these features contribute to improved potency, tolerability, and endosomal escape. Representative lipid structures are used to illustrate these trends. In addition, we describe promising lipids that deviate from heuristic design principles, offering insights into alternative strategies to expand the chemical space. Together, this review provides a framework for the rational development of next-generation ionizable lipids for genetic medicines.

INTRODUCTION

Interest in the delivery of nucleic acid therapeutics (NAT) in the form of lipid nanoparticles (LNPs) has grown enormously since the 2018 approval of Onpattro and the advent of Pfizer-BioNTech and Moderna COVID-19 vaccines in 2020. A crucial component of these medications is a cationic ionizable lipid (IL), the chemical structure and physicochemical properties of which play a significant role in determining overall effectiveness. In particular, the IL must promote the formation of reasonably stable LNP formulations of nucleic acids (NAs); facilitate endosomal escape of the latter post-endocytosis of LNPs inside cells, translating into efficient expression of the NA cargo; be well tolerated; and present minimal risk of short- and long-term toxicity. Unsurprisingly, the identification of ever better-performing ILs has become an area of intense current research.

This article outlines design principles that are likely to lead to efficacious and reasonably safe cationic ILs for the formulation and delivery of NATs in LNP form. Representative lipids shown herein are drawn primarily from the literature published from January 1, 2023, to June 30, 2024. Excellent reviews of earlier work are available.[1–6] Furthermore, the cited examples are primarily lipids that have been tested *in vivo*. This is because our experience has confirmed that *in vitro* and *in vivo* results correlate poorly or not at all[7,8]; hence, *in vivo* data are especially significant.

GENERAL STRUCTURE AND PRESUMED MECHANISM OF ACTION OF A CATIONIC IL

A cationic IL consists of one or more lipophilic chains (the lipophilic domain) connected to a protonatable center, typically a tertiary amine. The protonatable center may support an accessory hydrogen bond donor moiety, which is believed to promote association with the NA. An IL thus comprises a lipophilic domain, a protonatable N atom, and an optional H-bond donor moiety arranged as shown in Figure 1. The three molecular subunits are connected through appropriate biodegradable linkers. Often, such moieties are also present within the lipophilic chains and are designed to promote rapid degradation of the IL once the LNP has been dismantled inside a cell. This prevents bioaccumulation of lipophilic matter and minimizes the likelihood of toxic effects. Ester groups are often employed for this purpose, in that they are easily hydrolyzed by endogenous esterases.

The IL in Onpattro is a compound known as D-Lin-MC3-DMA, or more simply MC3, **1.1**[9]; that in the Pfizer-BioNTech COVID vaccine is ALC-0315, **1.2**; and that in the Moderna vaccine is SM-102, **1.3**.[10,11] Figure 1 shows structures and key subunits of the three lipids. Biodegradable moieties are rendered in green.

Individually, an endogenous, zwitterionic or anionic phospholipid and the cationic (protonated) form of certain ILs can form lipid bilayer structures under appropriate conditions. The *shape hypothesis*[12,13] holds that this is because the aforementioned lipids, individually, can acquire a roughly cylindrical shape that permits bilayer formation. In contrast, the admixture of a protonated IL with a negatively charged phospholipid results in the formation of cone-shaped aggregates that are incompatible with a bilayer arrangement but tend to organize themselves in tubular structures described as a hexagonal H_{II} phase (see Figure S1). A protonated IL can thus severely disrupt

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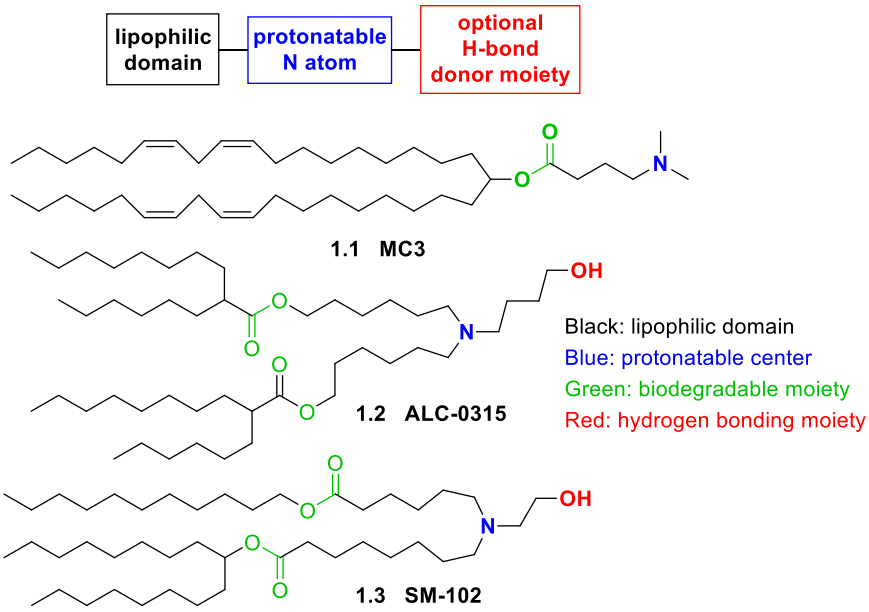

Figure 1. Structures of cationic ILs in approved medications and relevant moieties

the bilayer architecture of a biological membrane which contains net neutral and negatively charged phospholipids. This is believed to be a crucial event in the expression of the biological activity of an NA-LNP formulation. Indeed, once an LNP penetrates a cell through endocytosis, it finds itself inside an endosome, wherein the pH is gradually lowered from ca. 7.5 to about 4. Protonation of much of the IL under such conditions induces a positive charge on the LNP and promotes its close association with the negatively charged endosomal membrane. The protonated lipid is then thought to interact with the negatively charged lipids in the endosomal membrane, inducing fusion/membrane disruption via a phase change from bilayer to H_{II}. This results in liberation of the NA inside the cytoplasm, i.e., the *endosomal escape* of the therapeutic NA. Once in the cytoplasm, the NA can express its biological activity.

The shape hypothesis finds support in the observation that lipids with branched lipophilic chains, such as ALC-0315 and SM-102, are generally more efficacious than those with linear chains, viz. MC3 and KC2.[14] This is plausibly attributable to a more conical shape of phospholipid-branched IL complexes relative to complexes comprising linear ILs. The aforementioned picture also accounts for the observation that the pKa of the ionized (ammonium) form of the IL *embedded in the nanoparticle* (not in free form!)[15] is a crucial determinant of its efficacy. Too low a pKa may retard endosomal escape, to the detriment of efficacy. Conversely, too high a pKa often elicits severe toxic effects, perhaps through indiscriminate disruption of critical membranes by the positively charged nanoparticles.

HISTORICAL BACKGROUND

The field of contemporary NA-LNP therapeutics is rooted on research centering on the development of liposomal formulations of weakly basic drugs.[16] These can be loaded inside liposomes that entrap an acidic (pH ~ 4) aqueous solution of $(NH_4)_2SO_4$ through ingenious technology that relies on a chain of acid-base equilibria. Briefly, a weakly basic drug dissolved in an aqueous medium at pH ~ 7 equilibrates between its electrostatically neutral and its positively charged (protonated) forms. The neutral form of the drug, but not the charged one, can diffuse through the liposomal membrane and penetrate the liposome, where the acidic environment causes protonation and trapping of the cationic form of the drug inside the liposome.

The aforementioned technology cannot be used to entrap NAs, because they are permanently charged at biologically compatible pH's and thus unable to cross lipid membranes. Early attempts to formulate NAs in LNPs centered on co-extrusion with permanently cationic lipids such as 1,2-dioleyloxy-3-trimethylammonium propane (DOTMA, **2.1**),[17] or 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP, **2.2**)[18,19] (Figure 2), into an aqueous medium. The positively charged lipid would plausibly associate strongly with a negatively charged NA, resulting in self-assembly of nanoparticles containing NAs. This was indeed found to be the case. However, formulations so prepared were toxic at efficacious doses. It rapidly transpired that this was due to the toxicity of permanently cationic lipids in general.[20] Indeed, concerns persist to this day about the toxicity of this class of lipids,[21,22] at least when administered intravenously, despite encouraging data recently reported in the primary[23] and patent[24] literature.

The advent of *ionizable* cationic lipids, for example, 1,2-dioleoyloxy-3-dimethylamino propane (DODAP, **2.3**)[25] and 1,2-dilinoleyloxy-3-dimethylaminopropane (D-Lin-DMA, **2.4**),[26] alleviated some of the aforementioned difficulties,[27] in that these agents are only partially ionized at physiological pH, at least when embedded in an LNP.[15] However, NA-LNP formulations based on DODAP were inefficacious *in vivo*, while those based on D-Lin-DMA showed efficacy only at unacceptably toxic doses. These observations led to the hypothesis that the lack of efficacy of ester-type lipids like DODAP might have been a consequence of an excessively rapid cleavage of its ester linkages by endogenous esterases. Premature release of the oleoyl chains would clearly be catastrophic for the cohesiveness of LNPs, hence the lack of efficacy. On the other hand, the sturdy ether linkages in D-Lin-DMA may have extended the residence time of the lipid in a biological milieu to an excessive extent, thereby eliciting toxic effects. This led to the surmise that a linkage with a chemical stability intermediate between an ester and an ether might have resolved such difficulties. The corresponding author of this contribution (M.A.C.) ventured that a sensible option could be a ketal and

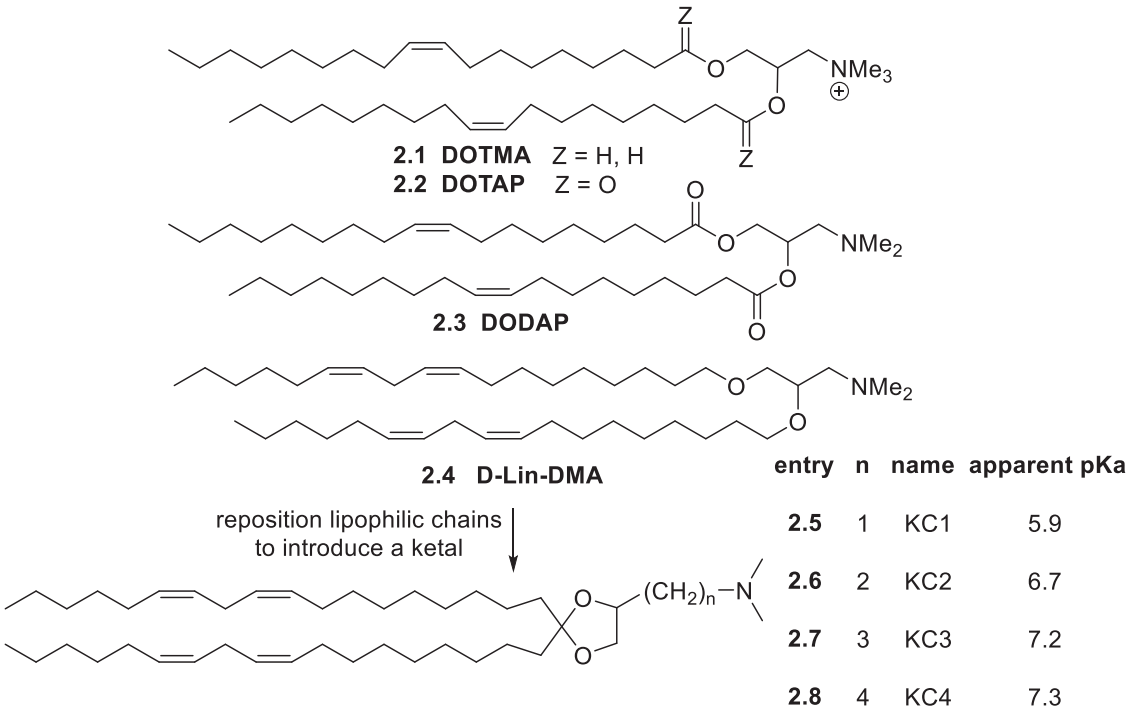

Figure 2. From early permanently cationic or ionizable lipids to the KC family

went on to conceive D-Lin-KC1, **2.5**, more simply described as KC1.[28] This terminology reflects the fact that the lipid now comprises a ketal ("K") and that the ionizable center is connected to the cyclic ketal by a C_1 ("C1") linker.

It was pleasantly surprising to observe that NA-LNP formulations comprising KC1 showed good efficacy with acceptable toxicity. On the other hand, the apparent pKa of the protonated form of the lipid in the nanoparticle[15] was 5.9. This seemed too low for efficient endosomal escape. To correct the problem, the ionizable center was repositioned progressively farther away from the dioxolane moiety, so that the diminishing (+)—inductive effect of the oxygen atoms might translate into a higher pKa. Representative lipids thus obtained are D-Lin-KC2, **2.6**; KC3, **2.7**; and KC4, **2.8**, which exhibited the anticipated trend in pKa. Among these, KC2 proved to be by far the most efficacious one, with an *in vivo* IC50 for hepatic suppression of Factor VII in mice of 0.1 mg small interfering RNA (siRNA)/kg.[28] Additional observations led to the conclusion that a competent IL for hepatic delivery of therapeutic NA should have an apparent pKa around 6.4. Further work also revealed that altering the pKa can shift LNP biodistribution toward extrahepatic tissues. For example, ILs with a higher pKa promote LNP delivery to the lungs, while a lower pKa tends to direct LNPs to the spleen.[29–31] This effect is thought to arise from charge-dependent modulation of the protein corona around an LNP, i.e., to the adsorption of particular blood proteins that promote preferential organ uptake as a function of surface charge.[29] This principle can be substantiated by the incorporation of charged alternatives into LNPs which likewise redirect biodistribution in a surface-charge-dependent manner.[31] It is important to emphasize that positive charge-driven targeting is generally not a desirable strategy. Seminal studies by Cullis and colleagues on cationic liposomes already highlighted that, although charge modulation can alter biodistribution, it comes at the cost of increased toxicity and limited translational potential.[6] Recent studies have confirmed that LNPs with high positive surface charge exhibit elevated systemic toxicity.[22]

The synthesis of KC-type lipids is lengthy (eight steps from commercial linoleyl alcohol), and it involves two problematic chemical reactions. Dr. Steven M. Ansell, a distinguished expert in the chemistry of ILs, discovered that the easier-to-make ester analog MC3, **1.1**, was about 3 times more efficacious than KC2 for hepatic siRNA delivery.[32] The new lipid ultimately became a crucial component of Onpattro.[9] This and other important results indicated that, contrary to the earlier surmise, ester groups are perfectly tolerable in ILs. Accordingly, the poor efficacy of, e.g., DODAP, cannot be ascribed to chemical or metabolic instability, but it must be due to other factors.

A HEURISTIC PRINCIPLE FOR IL DESIGN EMERGES

A considerable volume of subsequent observations suggested that the architecture of the lipid is a major determinant of its effectiveness. This led to the promulgation of the heuristic principle adumbrated in Figure 3. Thus, significant *in vivo* efficacy with acceptable toxicity is often associated with lipids wherein the lipophilic chains converge onto the same atom, A. The latter may be carbon, as in KC2

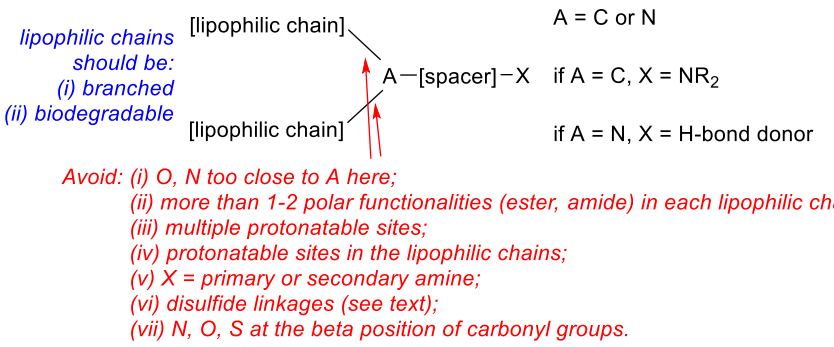

Figure 3. Guidelines for the design of efficacious and relatively safe ILs

and MC3, or nitrogen, as in ALC-0315 (also one of Dr. Ansell's inventions)[33] or SM-102.[34] In contrast, less efficacious lipids like DODAP and D-Lin-DMA exhibit lipophilic chains that converge onto adjacent atoms. If A is C, then A will also sustain the ionizable center, rendered below as X, with X equal to, e.g., NMe_2. One, but generally not both, of the small methyl groups may be replaced with a larger (C_2-C_4) alkyl possibly incorporating a hydrogen bond donor such as an OH group. If A = N, then normally, but not always (*vide infra*), A itself is the ionizable center. In the latter case, X should be a hydrogen bond donor for maximum efficacy. For example, X is an OH in ALC-0315 and SM-102, but other hydrogen bond donor moieties can be present (*vide infra*). Regardless of the nature of A, the lipophilic chains should preferentially be branched rather than linear. It is well established that chain branching enhances potency.[14] Particular forms of branching create molecular asymmetry, raising the question of whether one stereoisomer of a lipid (diastereomer or enantiomer) might be more efficacious than other(s). In some instances, this may be so,[35] but it remains unclear whether this is the case across the board. Molecular asymmetry may also be present at the level of the ionizable head group, as in KC2. Racemic and enantioenriched forms of this lipid seem to be equipotent.[28,32] The lipophilic chains should also comprise biodegradable moieties such as esters, which are easily hydrolyzed by endogenous esterases. The free lipid can thus be rapidly degraded post-disintegration of the LNPs *in vivo*, minimizing its residence time in the biological milieu, its possible bioaccumulation in fatty tissues, and the consequent likelihood of adverse effects.

Lipids in which A is an atom other than C or N have been described. Species wherein A = P were reported as early as 2019.[36] More recently, Genevant scientists have disclosed compounds in which A = Si, e.g., **S2.1** (Figure S2).[37] Unfortunately, these structurally interesting lipids tend to suffer from limited aqueous stability.[37] Lipids comprising silicon-based moieties in the lipophilic chains have also been reported, e.g., Aldexchem's **S2.2**.[38]

It seems superfluous to point out that a lipid destined to be a component of a drug for human use must be not only efficacious but also safe in the short and long term alike. For that reason, it is prudent to follow the additional guidelines in red, which are rooted in extensive experimental results. Thus, it is best to avoid (1) strongly electronegative atoms such as O or N in the immediate vicinity (1–4 atomic positions) of A in the lipophilic chains[39]; (2) more than 1–2 polar functionalities, such as ester or amide, in each lipophilic chain; (3) protonatable sites in the lipophilic chains; (4) primary or secondary amines as group X; (5) multiple protonatable sites within an IL; (6) disulfide linkages (with one possible exception—see in the following); and (7) N, O, and S centers at the beta position of carbonyl groups. Guidelines (1)–(5) are empirical and relate primarily to frequent loss of efficacy observed upon the introduction of such features. An additional concern pertains to point (5). Many recently described ILs are obtained through *N*-alkylation of polyamines such as **4.1** and **4.2** and related building blocks with appropriate electrophiles. Examples include **4.3**[40] and **4.4**[41] (Figure 4). Work by Whitehead and collaborators suggests that the nitrogen-containing subunits in such lipids tend to bind to TLR4 and CD1d receptors, thus triggering an immune response.[42] This tends to inhibit/suppress the expression of therapeutic mRNA. On the other hand, such undesirable effects may be of concern for specific applications or modes of administration, but not others.

Points (6) and (7) reflect concerns about potential long-term adverse effects. To wit, disulfide linkages (point 6) are believed to promote membrane penetration.[43] However, it is well established that disulfides are subject to exchange with endogenous thiols such as cysteine residues in polypeptides and in glutathione[44,45] and that the products of such reactions are potentially immunogenic.[46,47] Hence, it seems prudent to avoid disulfides, with the possible exception of lipoic acid. Being a disulfide, this endogenous, and thus presumably safe, enzyme cofactor does promote cellular uptake.[48,49] Perhaps for that reason, it has been incorporated into various recently described lipids, e.g., **S3.1**[50] and **S3.2**[51] (Figure S3).

Finally, and regarding point (7), a heteratomic functionality at the beta position of a carbonyl group is subject to elimination, resulting in formation of a conjugated carbonyl system. The latter is a Michael acceptor that can react with endogenous amines or thiols, again giving rise to potentially immunogenic products.[52] β-Amino- and β-alkylthio esters are especially prone to such processes, much less so amides. For example, lipid MC2, (**S4.1**, Figure S4) tends to suffer loss of Me_2NH with presumed formation of acrylate ester **S4.2**.

In most cases, the apparent pKa of the conjugate acid (=protonated) form of the IL in the LNP[15] should be between 6 and 7 for best *in vivo* efficacy, especially if the LNP formulation is to be administered intravenously. Indeed, the extent of *in vivo* mRNA expression in the liver decreases rapidly if the pKa of the formulated lipid falls outside that narrow window. This appears to be the case even for

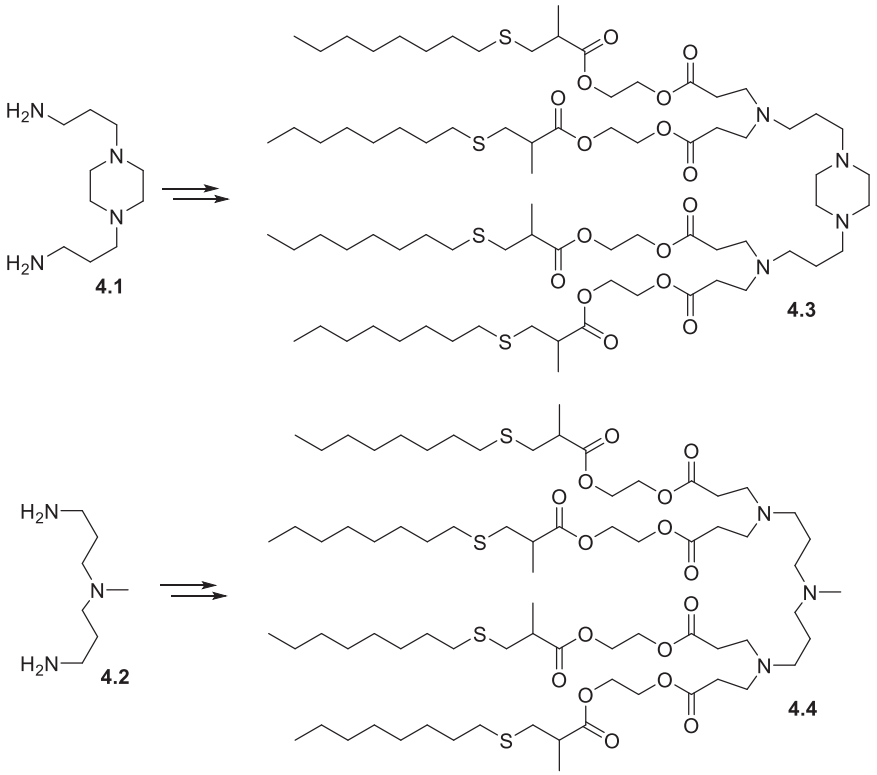

Figure 4. Potentially immunogenic polyamine moieties frequently found in ILs

in vitro experiments. A telling example is found in a recent paper that describes analogs of SM-102 obtained by "click" chemistry, all of which exhibit an unusually low pKa (Figure S5).[53] All such lipids exhibit diminished efficacy relative to **1.3**.

Severe toxic effects are likely to appear post-intravenous (i.v.) injection in rodents if the pKa of the IL in the LNP is above 7. On the other hand, the optimal value of the pKa of a lipid appears to be formulation, tissue, mode of administration, and applicationdependent, and it may well have to be slightly above 7 in certain cases that do not require i.v. administration.

Components of approved medications, such as MC3, ALC-0315, and SM-102, fully reflect the foregoing principles, as do many lipids in clinical evaluation as of 2021[54] and others that appear to be still at the stage of preclinical development. Examples wherein A = C include Genevant "trialkyl" lipids such as **5.1**[55]; Precision NanoSystems (now Cytiva) jasmonic acid-derived ones such as **5.2**[56]; Harashima lipids such as **5.3**[57]; and Li-Anderson ones, e.g., **5.4**[58] (Figure 5). A noteworthy aspect of the latter work is the use of machine learning and combinatorial chemistry for the exploration and optimization of lipid structures. This strategy represents an exciting and innovative approach. Continued validation *in vivo* will be essential to determine its ability to consistently generate highly efficacious lipids and whether such strategy can reliably match or surpass outcomes achieved through traditional rational chemistry. Notice, however, the presence of less favorable linear chains in some of the compounds of Figure 6. As an aside, the structure of **5.4** and analogs thereof, reproduced herein from reference Li et al.[58], may be incorrect based on sound chemical principles and precedent.[59]

The structures of many lipids wherein A = N are inspired by ALC-0315, **1.2**, and SM-102, **1.3**, thus conforming to the aforementioned principles. Interesting analogs of **1.2** disclosed in a Renegade patent are cyclized congeners of the original, as exemplified by **6.1**,[60] while others exhibit a range of alternative (i.e., non-OH) H-bonding groups as X. Thus, X is a squaramide in Moderna compounds such as **6.2**.[61] The squaramide appears to favor association with NAs through both H-bonding and pi stacking interactions with nucleic bases.[62,63] Some Etherna lipids, e.g., **6.3**, display a pyridoxine ester in lieu of a squaramide.[64] It seems plausible that that pyridoxine and squaramide moieties interact with NAs in a similar manner. On the other hand, certain recently described lipids in this class lack a hydrogen bond donor group. Examples include Sorrento's dimeric derivatives of ALC-0315 such as **6.4**.[65] The absence of an H-bond donor would be predicted to diminish efficacy. Notice that all such lipids exhibit branched lipophilic chains that comprise ester moieties. In some recently described lipids, the nitrogen atom upon which the lipophilic chains converge is no longer protonatable, being part of an amide or a related functionality. Instead, it functions as an anchoring point for a side chain that carries the actual ionizable center. For example, researchers at Arcturus Therapeutics have described lipids resulting from the union of an SM-102-type framework with an ionizable head group via a thiocarbamate (**6.5**, $n = 0$) or an amide (**6.5**, $n = 1$).[66] A similar theme is apparent in Sorrento Therapeutics' **6.6**, wherein a thiourea joins an ALC-0315-like framework with the ionizable moiety.[65]

Other metabolically labile groups may be present in addition to, or in lieu of, esters in the lipophilic chains. Thus, some Flagship Pioneering lipids feature phosphoramide (cf. **S6.1**, Figure S6), lactide (cf. **S6.2**), or diketopiperazine (cf. **S6.3**) moieties.[67] The H-bond donor in these compounds is an OH group, but the same patent contemplates embodiments in which an electron-deficient heterocycle (cf. **S7.1**–**S7.3,** Figure S7), a sulfamide (**S7.4**), or even an unusual amino-sulfonimidamide (**S7.5**) are present instead of an OH.[68] Heterocycles **S7.1**–**S7.3** can arguably interact with NAs similarly to squaramide or pyridoxine, while **S7.4** and **S7.5** are pure H-bond

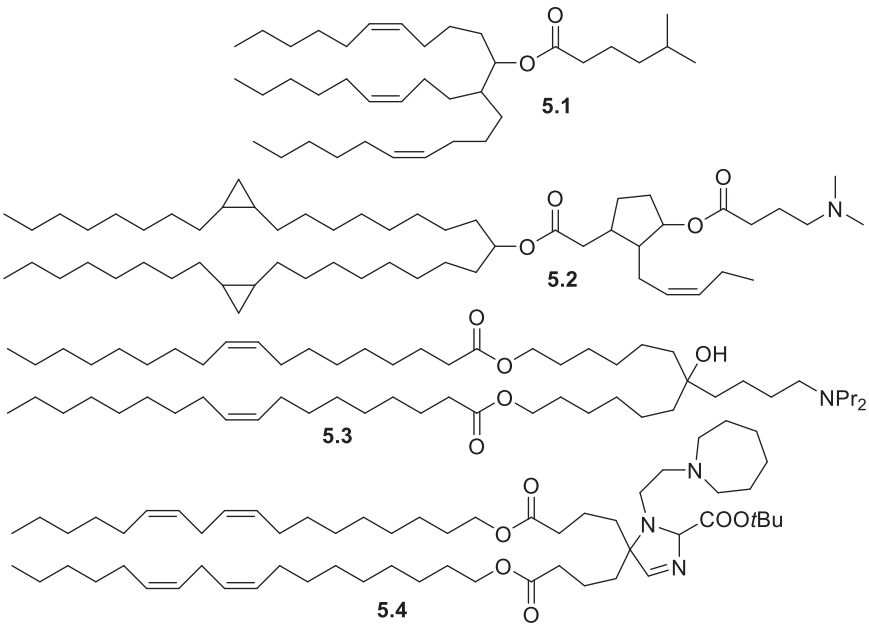

Figure 5. Representative ILs with A = C that reflect the principles of Figure 3

donors. Notice that the N atoms in **S7.1**–**S7.5** are not protonatable at physiological pH: only the nitrogen atom bonded to the lipophilic chains is. Consequently, only one protonatable center is present in these lipids, in accord with the design principles outlined earlier.

Finally, NanoVation Therapeutics' lipids[69,70] generally conform to the guidelines of Figure 3. These lipids are easy to synthesize, with demonstrated scalability to the hectogram level, and were designed to be rapidly metabolized once the LNP formulations complete their task, as confirmed through extensive postmortem analysis of rodent tissues by state-of-the-art liquid chromatography-mass spectrometry methods. More significantly, several NanoVation lipids have been validated in non-human primates and found to be safe and highly efficacious.

It is apparent from the few examples earlier that the general structure of Figure 3 can give rise to an impressive diversity of architectures, the range of which is limited only by the creativity of the lipid designer. In that respect, substantial variation is possible in the lipophilic chains, at the level of the linkers, and even in the ionizable head group.[68]

On the other hand, the foregoing design principles are guidelines, not rules. Therefore, lipids that deviate from the aforementioned standards are not necessarily poor or problematic, and, indeed, a multitude of such agents have been described in the recent literature. For instance, DODAP/D-Lin-DMA type lipids still command substantial interest, at least for particular applications, and new methods to prepare them continue to appear.[71] Representative examples include Life Technologies' **7.1**,[72] Genzyme-Tidal's **7.2**,[73] and their congeners (Figure 7). Notice that the lipophilic chains in these compounds converge on adjacent atoms, and that in **7.1** are linear instead of branched. The fatty chains in Turn Biotechnologies lipids such as **7.3**[74] do converge on the same atom but are linear. Also, electronegative O atoms are close to the point of convergence. The latter feature is also apparent in Seawolf Therapeutics lipids derived from alditols, e.g., **7.4**,[75] which nonetheless seems to be reasonably competent for mRNA delivery based on the *in vivo* expression of erythropoietin (EPO). However, the best lipid in the same patent appears to be **7.5**, which fully reflects the principles of Figure 3.

Much interest exists in lipids based on amino acid scaffolds. Such compounds tend to deviate from the standards of Figure 3 in various respects. In particular, they tend to incorporate multiple ionizable centers. Still, they may be reasonably efficacious. For example, Li's **7.6** and analogs, which are built around lysine,[76] display linear chains and two ionizable centers. The possible β-elimination of the nitrogen functionality (arrow) as per Figure S3 is less of a concern in the present case, in that the carbonyl system is part of an amide, which is much less inclined to undergo such a reaction. Certest's **7.7**, derived from homocysteine,[77] is such that the lipophilic chains connect to distinct atoms and electronegative N and O atoms are close to the point(s) of chain convergence. Beta-elimination leading to an acrylate ester could potentially occur in the lipophilic chain comprising the 7-methyloctyl ester (arrow). The same is true for lipids such as **7.8**.[78,79] The gem-dimethyl motif (arrow) in Senda's **7.9**,[80] a derivative of hydroxyproline, may have been introduced to suppress elimination of dimethylamine as per Figure S4.

The potential for β-elimination of sulfur and nitrogen functionalities is also apparent in lipids such as **8.1**[81] (Figure 8), which, in addition, exhibits 3 ionizable centers as well as disulfide linkages in the lipophilic chains. Lipids wherein disulfide functionalities are found in the ionizable head group have also been reported, e.g., **8.2**[65] and **8.3**.[82]

A considerable volume of literature centers on ILs produced by the reaction of a polyamine with alkylating agents such as epoxides, 1,4-acceptors, or a combination thereof. Such lipids inevitably exhibit multiple ionizable centers. Examples from recent patents include **8.4**,[83] **8.5**,[84] and **8.6**.[85] The nitrogen functionalities marked with asterisks in the latter are less prone to the eliminative process of Figure S6 because the carbonyl groups are now present as amides.

CONCLUSIONS AND OUTLOOK

The reader will appreciate that this review avoids comparing the efficacy of the various lipids cited. There are many reasons for

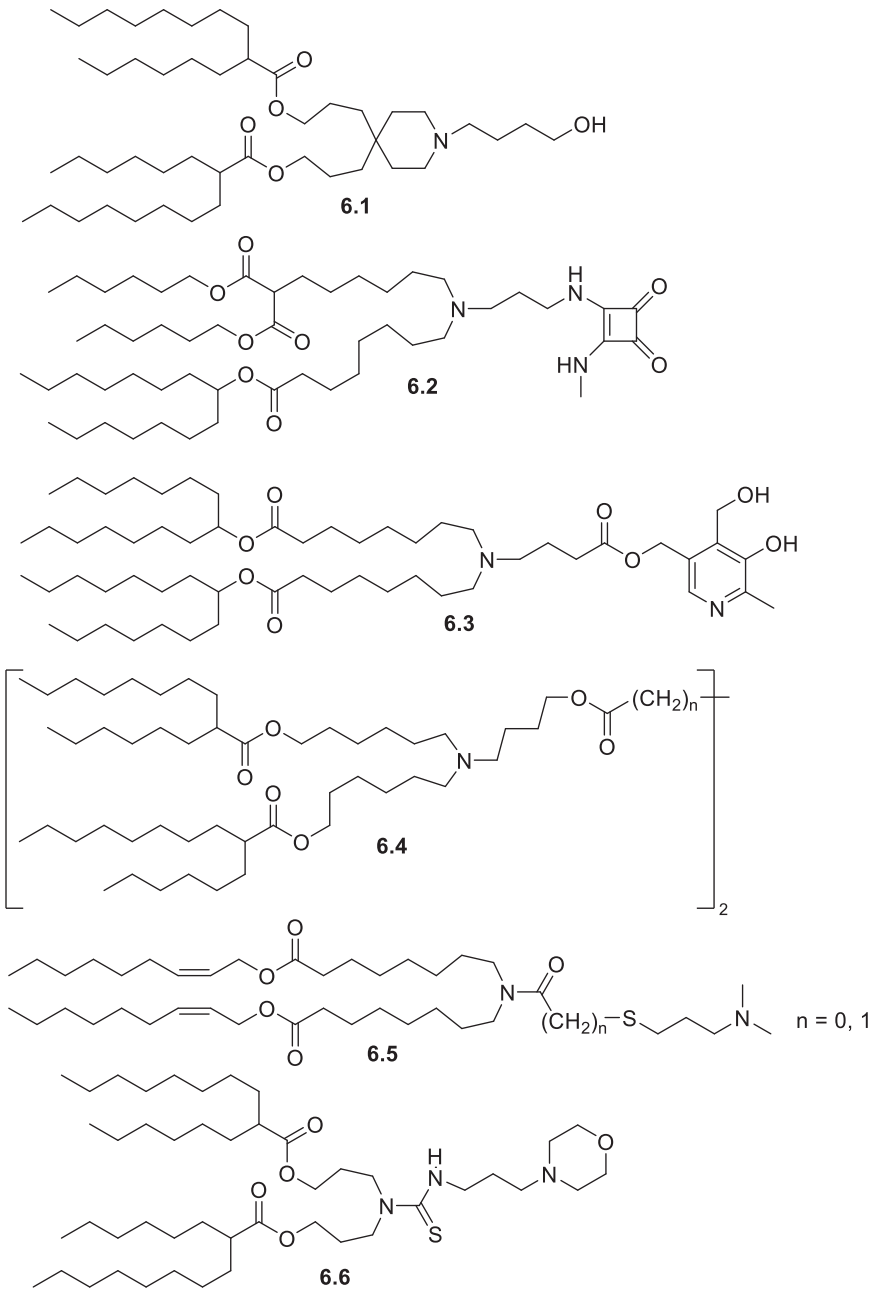

Figure 6. Representative ILs with A = N that reflect the principles of Figure 3

this. First, it is sometimes unobvious whether reported data refer to *in vivo* or *in vitro* experiments. Furthermore, meaningful comparison of *in vivo* data requires detailed information about formulation; NA sequence; nature of the mRNA modifications, including capping; administration route, type and strain of animal used; analytical methodology; quality control at all stages; and so on. Analytical rigor and operator experience as well as consistency in formulation and administration are additional non-negligible factors that further complicate meaningful benchmarking.

The principles espoused in Figure 3 provide useful guidelines, albeit not rules, for the design of ILs that combine elevated efficacy with acceptable toxicity. While at present it is difficult (impossible?) to rationalize observed correlations between structure, physicochemical properties, and biological activity, patterns are emerging, to the extent that fairly accurate predictions can be made regarding the behavior of a new lipid designed according to the foregoing principles. Still, a considerable amount of work remains to be done to unravel the subtleties of the structure-activity relationship of ILs and to optimize their properties for specific applications. Traditional medicinal chemistry approaches toward that goal have been rather successful,[86] although admittedly they are labor intensive and time consuming. Emerging technologies such as artificial intelligence (AI), machine learning (ML), and directed chemical evolution offer promising avenues to accelerate lipid discovery. A noteworthy example of AI/ML-guided design can be found in the Li-Anderson work cited earlier.[58] Even more recent contributions highlight the power of directed chemical evolution for lipid optimization.[87,88]

The successful identification of a safe and efficacious IL is but a first step toward a new therapeutic product. Indeed, the clinical translation of LNP-NA formulations constitutes a challenge of considerable magnitude. How a human subject may react in the short and the long term to the administration of an experimental medicament, whether small-molecule drug or LNP-NA and however safe and efficacious it may appear to be in Rodentia or Caniformia, remains notoriously unpredictable. For example, by the mid-2000s most of the roughly 1,000 neuroprotective agents that had been amply validated *in vitro* and in rodents in preclinical studies had failed in human trials.[89] Careful evaluation of experimental drugs in non-human primates, a costly but essential endeavor, presently remains the most reliable strategy to mitigate failure and to validate advanced products.[90]

Looking ahead, success in IL development will depend on refining design principles in concert with advances in formulation science, tissue targeting, and immunological profiling. It is worth emphasizing that the IL is the principal determinant of the therapeutic

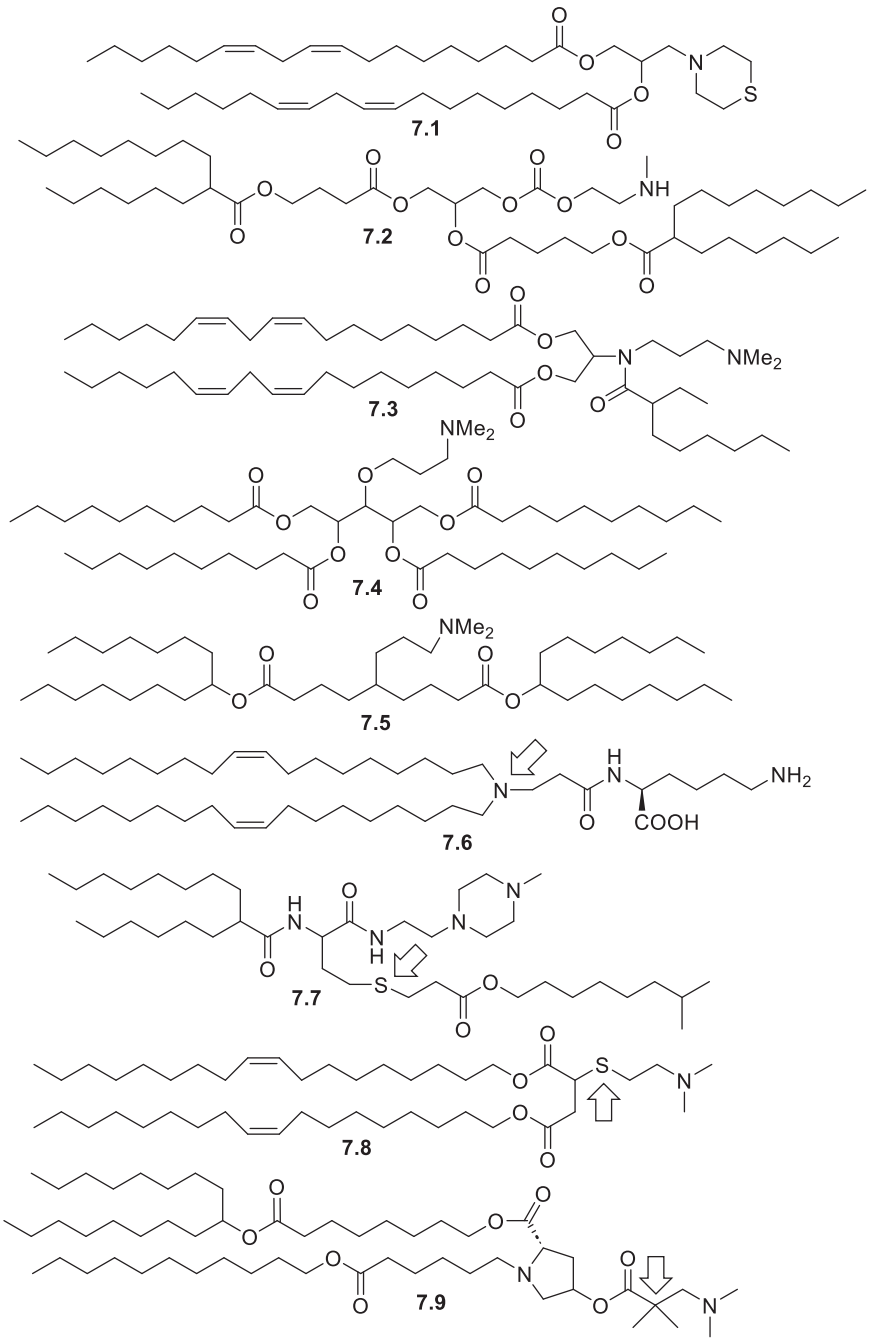

Figure 7. Representative ILs that deviate from the guidelines of Figure 3

index, i.e., governing both efficacy and tolerability. Its molecular structure dictates endosomal escape, potency, and toxicity and thus plays a central role in balancing therapeutic benefit with safety risk. In contrast, the overall LNP composition, particularly the ratio and identity of helper lipids, is a key lever for modulating biodistribution and enabling delivery to tissues beyond the liver (i.e., extrahepatic expression). Continued progress will therefore require integrated optimization of both the IL and the overall LNP formulation. As the field advances toward increasingly sophisticated delivery demands, we hope that our readers will find the guidelines outlined in this review useful, as they endeavor to conceive and develop ever more efficacious and safe ILs for genetic medicines.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to the preparation of this manuscript.

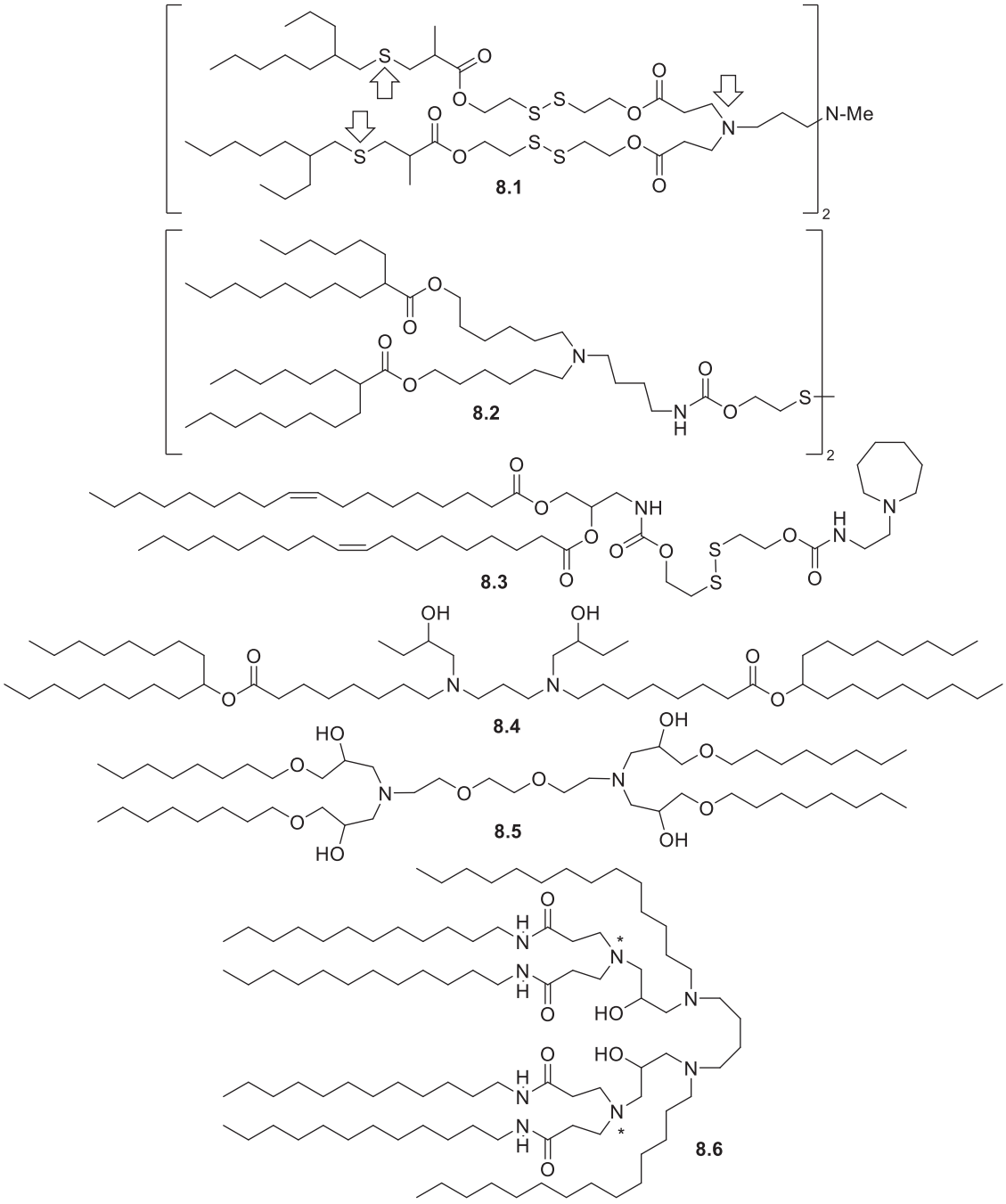

Figure 8. Representative ILs containing disulfide linkages/multiple ionizable centers

DECLARATION OF INTERESTS

J.K., D.W., P.R.C., and M.A.C. are co-founders of and have a financial interest in NanoVation Therapeutics Inc.; N.D.P.A., F.S., J.K., D.W., and M.A.C. are full-time employees of NanoVation Therapeutics Inc.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2025.101585.

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