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Synthesis and bioactivity of readily hydrolysable novel cationic lipids for potential lung delivery application of mRNAs

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ABSTRACT

Lipid nanoparticles (LNPs) mediated mRNA delivery has gained prominence due to the success of mRNA vaccines against Covid-19, without which it would not have been possible. However, there is little clinical validation of this technology for other mRNA-based therapeutic approaches. Systemic administration of LNPs predominantly targets the liver, but delivery to other organs remains a challenge. Local approaches remain a viable option for some disease indications, such as Cystic Fibrosis, where aerosolized delivery to airway epithelium is the preferred route of administration. With this in mind, novel cationic lipids (L1-L4) have been designed, synthesized and co-formulated with a proprietary ionizable lipid. These LNPs were further nebulized, along with baseline control DOTAP-based LNP (DOTAP®), and tested in vitro for mRNA integrity and encapsulation efficiency, as well as transfection efficiency and cytotoxicity in cell cultures. Improved biodegradability and potentially superior elimination profiles of L1-L4, in part due to physicochemical characteristics of putative metabolites, are thought to be advantageous for prospective therapeutic lung delivery applications using these lipids.

1. Introduction

Drug delivery to the proper cell types and tissues of interest is a major burden in any viral/nonviral therapeutic approach. In recent years, delivery of nonviral mediated therapeutic RNA (mRNA, siRNA, miRNA etc.) has proven to be successful in the clinic (MacLachlan, 2007; Semple et al., 2010; Jayaraman et al., 2012; Sabnis et al., 2018; Nabhan et al., 2016; Patel et al., 2017). One of the successful platforms of nonviral RNA delivery is using lipid nanoparticles (LNPs), as evidenced by the approval and marketing of Patisiran (ONPATTRO®), to treat transthyretin amyloidosis, (ONPATTRO FDA Label, 2021; ONPATTRO EMA, Summary of opinion, 2018) and lately by the Emergency Use Authorization (EUA) of two of the much-anticipated mRNA vaccines against Covid-19 administered intramuscularly. Much of the therapeutic success with this platform has been attributed to the efficient uptake of these LNPs in the liver through an ApoE/LDLR mediated endocytosis by the hepatocytes (Akinc et al., 2010). There is a compelling need for the therapeutic delivery of LNPs to other organs and cell types. Most recently, there has been some efforts in selectively improving tissue targeting by modifying LNP compositions and properties (Cheng et al., 2020). For example, this paper has demonstrated selective lung delivery when DOTAP was used at a high molar percentage of 50%. Yet, another recent publication has demonstrated nebulized delivery of LNPs using high C14PEG2000 molar percentage in the formulation (Lokugamage et al., 2021). In addition to these latest reports, others also have explored targeted delivery of cationic LNPs directly to airway epithelium using cationic lipids as excipients in formulating inhalable LNPs for the efficient deposition of complex RNA therapeutic modalities including, but not limited to, siRNA, miRNA, ASO, mRNA, CRISPR-sgRNA, tRNA and long non-coding RNA (Merkel et al., 2014; Jensen et al., 2012; Jensen et al., 2010; Conti et al., 2014; Kusumoto et al., 2013; Robinson et al., 2018) and for gene delivery applications (Allon et al., 2012). This approach is specifically suitable for administering mRNA encoding functional proteins whose deficit drives diseases such as, for example, Cystic Fibrosis (CF) where restoration of a deficient ion channel represents a major therapeutic development paradigm (Riordan et al., 1989; Welsh, 1990; Chu et al., 1993; Welsh et al., 1992). Therefore, considering CF as a model to further design optimal LNPs using cationic lipids L1-L4, we should consider the factors that will also influence their design such as formulation optimization, nebulization,
and physicochemical characterization. Such an approach is needed for better optimal LNPs for lung therapeutics.

LNPs principally contain an ionizable lipid that not only drives RNA encapsulation through their pH driven ability to form ion-pair interactions with the anionic phosphate backbone of RNA, but also aids in triggering endosomal escape through membrane destabilization (Kulkarni et al., 2018; Semple et al., 2001; Semple et al., 2010; Viger-Gravel et al., 2018; Lechanteur et al., 2018; Zelphati and Szoka, 1996; Sabnis et al., 2018). Other typical constituents of LNPs are a phospholipid and cholesterol to maintain structural integrity, and an outer polyethylene glycol (PEG) lipid to coat the LNP (Kulkarni et al., 2018; Semple et al., 2001; Leung et al., 2012; Crawford et al., 2011). This outer decoration protects LNPs from the host immune response, imparts serum stability and is cleavable once inside the target cell (Fig. 1 A). Typically, the ionizable lipids used in LNP formulations are neutral at physiological pH, and hence they possess the characteristics of a neutral liposome. Neutral liposomes of < 100 nm hydrodynamic size have an overwhelming bias towards hepatocytes. Previously, Yan et al. have demonstrated the plasma clearance of neutral liposomes by measuring a 3.6-fold faster rate of clearance vis-à-vis negatively charged liposomes in wild type mice versus ApoE deficient mice (Yan et al., 2005). This was further supported by Akinc et al. (Akinc et al., 2010) by showing the enhanced liver uptake of ionizable LNP (iLNP) compared to cationic LNP (cLNP) in the presence of ApoE. This necessitates alternative strategies for extrahepatic delivery of LNPs. In this regard, lung and lung airway is an organ of great therapeutic relevance that can be targeted by LNPs. An experimentally tested and potentially clinically viable approach is to use cationic LNP (cLNP), formed using cationic lipids such as DOTAP, to target lung airway epithelium. But systemic administration of cLNP has not proven to target lung for non-tumor therapeutic applications. For example, a DOTAP:Cholesterol based cationic liposome has been proven to deliver anticancer p53 gene to lung upon systemic administration (Ramesh et al., 2001). Another gene transfer study (Fletcher et al. 2006) demonstrates a 3-fold stronger gene expression in mouse lungs upon tail vein IV administration of cationic:cholesterol lipoplexes for potential antitumor therapy. Lung delivery of cationic LNPs through systemic administration is limited to lung tumors because of the enhanced permeation and

| Compound | c-LogP | (a₀) Å² | l, Å | V Å³ | CPP \* |
|----------|--------|---------|------|------|-------|
| DOTAP    | 11.77  | 52.61   | 22.92| 743.31| 0.62  |
| L1       | 11.34  | 61.84   | 24.18| 785.90| 0.52  |
| L2       | 10.85  | 52.61   | 25.44| 810.52| 0.60  |
| L3       | 10.44  | 52.61   | 24.18| 793.72| 0.62  |
| L4       | 11.46  | 52.61   | 25.44| 827.32| 0.61  |

*Calculated using ACD Labs Structure Designer v12.0. cLogP was calculated using ACD Labs version B. aCalculated using Molinspiration Chemoinformatics available on the internet. Å is the unit in Angstrom.
retention (EPR) effect of the tumor mass (Prabhakar et al. 2013). For non-tumor indications, a local cLNP mediated mRNA delivery to airway epithelial cells needs to be carefully designed taking into consideration the unique attributes of the cellular milieu and its reduced capability to metabolize various chemical components of the LNPs.

Such a design of LNP for targeting lung airway would involve three key considerations. First, packaging mRNA in a lipid composition that distributes favorably to the airway epithelium as opposed to trafficking to liver must be designed. Second, an ionizable lipid must be included to facilitate endosomal release through formation of destabilizing non-bilayer structures at acidic pH of the endosome. Third, a reliable route or method of drug administration that ensures lung deposition needs to be determined. Cationic lipids have been determined to be the lipids of choice as they distribute favorably to the airway epithelium as opposed to trafficking to liver.

We designed four DOTAP-like functional analogues (L1-L4) that are achiral, not pseudoglyceryl lipids, and may have improved biodegradability and comparability or better transfection efficiency (Fig. 1B). We surmised that the structural proximity of the ester linkages to the sterically demanding glycerol-like terminus of DOTAP would result in steric congestion that would impede the delivery of the cargo to the target epithelial cells they would need to be cleared from the lung. This clearance would likely require ester hydrolysis, and perhaps further metabolism, for all the DOTAP-like structure to be ultimately eliminated.

We designed four DOTAP-like functional analogues (L1-L4) that are achiral, not pseudoglyceryl lipids, and may have improved biodegradability and comparability or better transfection efficiency (Fig. 1B). We surmised that the structural proximity of the ester linkages to the sterically demanding glycerol-like terminus of DOTAP would result in steric congestion that would slow enzymatic hydrolysis of the ester linkages. The 19-20 atom extensions from the branch point carrying the tetra-alkylammonium head group exhibited by DOTAP would be the starting point for modification, with hydrolytically labile ester groups positioned farther from the sterically demanding quaternary carbon. Our design called for ester-transposition, relative to the structure of DOTAP, with a Z,Z-alkenone forming an ester with a polar, and symmetrical, 8-oxo-octanediolic acid derived core. The quaternary ammonium moiety was seen as being introduced as an ammonium ethyl ether and as an ammonium-alkyl entity, both derived from the ketone at the 9-position. Physicochemical parameters of the designed cationic lipids would be compared to DOTAP itself and we anticipated that a post in vivo analysis of the structure of our new lipids vs DOTAP would point us toward the preferred physicochemical space. Table 1 presents the structure of DOTAP and cationic lipids L1-L4, designed as described above and selected as potential targets along with their associated calculated LogP (c-LogP) values. DOTAP, with a 1-carbon spacer from the branch to the quaternary ammonium nitrogen is associated with a c-LogP = 11.77. Lipid L1, with symmetrical 20-atom extensions from the branch point, ester carbonyls at 8-atoms from the branch and esters formed with (Z)-2-undecenol and a 2-dimethylaminooctanol based quaternary ammonium head group is similarly lipophilic (c-LogP = 11.34). Lipids L2, L3, and L4 are also derived from symmetrical esters of 5-atoms removed from the branch point, and they differ with respect to the esterifying alcohol (L2 and L4 (Z)-2-dodecen-1-ol; 3(Z)-2-undecen-1-ol) and carbon chain length between the branch point and the quaternary ammonium head group (L2 2-C; L3 and L4 3-C). The c-LogP values for L2, L3, and L4 are 10.85, 10.44, and 11.46, respectively. While L1-L4 are very similar in size and chain extension, the presence of the ether linkage in L1 and the subtly different carbon chain lengths found in the head group and ester moieties of L2-L4 leads to c-LogP values that are similar to DOTAP (L1 and L4) and 1-1.5 log units lower (L2 and L3). The impact of these lipophilicity values will be of interest with respect to formulation into LNPs, cellular transfection, and stability. Further, we wanted to ensure that the critical packing parameters (P) of these modified cationic lipids conformed to the cylindrical shape that enabled the formation of lamellar structure. This imposed restrictions on P to be below 1 and greater than 0.5 (0.5 < P < 1). Our calculations according to the equation $P = V/aq_0$, where $V$ is the molecular volume, $aq_0$ is the polar surface area (PSA) and $l_c$ is the carbon chain length, ensured that this was indeed the case (Hsu et al., 2005). In fact, all four new cationic lipids conformed to this requirement (Fig. 1B) with the P of L1 being 0.52 while the value of L2, L3, and L4 identical to that of DOTAP. We report here on the synthesis, biodegradability, transfection efficiency, and cytotoxicity of novel cationic lipids L1-L4 that are putative functional analogs of DOTAP, in our proprietary LNP formulations for prospective lung airway delivery applications.

2. Materials and methods

2.1. Materials and methods of synthesis

2.1.1. General

Starting materials and other reagents were purchased from commercial suppliers and were used without further purification unless otherwise indicated. All reactions were performed under a positive pressure of nitrogen, argon, or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, unless otherwise indicated. The reactions were assayed by high-performance liquid chromatography (HPLC) and terminated as judged by the consumption of starting material. $^1$H NMR spectra were recorded on Varian or Bruker instruments operating at the field strength indicated. $^1$H NMR spectra are obtained as DMSO-$d_6$ or CDCl$_3$ solutions as indicated (reported in ppm), using TMS as the reference. Other NMR solvents were used as needed. When peak multiplicities are reported, the following

| Table 1 | Initial and Post Freeze-Thaw Characteristics of LNPs with DOTAP, DOTAP$^+$ and L1-L4. The physicochemical properties of the LNPs were not much impacted with freeze/thaw and remained within the expected 10% variability. DOTAP$^+$ represents formulation without DOTAP, but with ionizable lipid. Data for an initial LNP characterization without the DOTAP$^+$ control is given in Supplementary Table 1 to show the consistency of particle properties. Three independent measurements were performed for particle size and PDI. *Data represented as average±std. |
|---|---|
| Lipid | Particle Size* (nm) | PDI* | %Encap | mRNA purity |
| DOTAP$^+$ | 74.7 ± 1.4 | 0.07 ± 0.01 | 71.3 | 84 |
| DOTAP$^+$ | 74.8 ± 0.2 | 0.07 ± 0.01 | 99.8 | 89 |
| L1 | 66.3 ± 0.3 | 0.09 ± 0.03 | 100.2 | 82 |
| L2 | 70.3 ± 0.8 | 0.10 ± 0.03 | 99.9 | 87 |
| L3 | 67.8 ± 0.8 | 0.08 ± 0.01 | 99.9 | 89 |
| L4 | 68.3 ± 1.6 | 0.08 ± 0.01 | 99.8 | 83 |
abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets, q = quartet. Coupling constants, when given, are reported in hertz. NMR spectra of individual test compounds can be found in the Supporting Information.

2.1.2. Synthesis of cationic lipids L1-L4

2.1.2.1. Synthesis of common intermediate 6. Cationic lipids L1-L4 are prepared from a known common 9-oxo-heptanedioic core unit, diester 6 which was readily prepared as shown in Scheme 1. Dialkylation of TOSMIC (Mueller et al., 2004) with ethyl 8-bromo-octanoate provided crude 5, which upon acid hydrolysis, afforded keto-diester 6. For detailed synthesis protocol, please see Supplementary material.

\[ 
\text{Equation 1. Synthesis of the common intermediate, diethyl ester of 9-oxoheptanedioic acid (6).} 
\]

2.1.2.2. Synthesis of L1. The synthesis of DOTAP analog L1 is shown in Scheme 1. Reduction of the keto group of 6 provided alcohol 7 which was alkylated to afford allyl ether 8. Dihydroxylation gave a diol (VanRheenan et al., 1976) which was cleaved in situ to yield aldehyde 9. Reductive amination with dimethylamine gave amine 10 which was hydrolyzed to diacid 11. Esterification with (Z)-2-undecen-1-ol (Cerutti-Delassalle et al., 2016) provided diester 12. Finally, methylation of 12 and ion exchange (Amberlite® IRA-400(Cl)) afforded L1. For detailed synthesis protocol, please see Supplementary material.

2.1.2.3. Synthesis of L2. The synthesis of analog L2 was initiated from keto-diester, as shown in Scheme 2. Diethyl ester 6 was hydrolyzed and the resulting diacid was esterified to provide bis-ethyl butyl ester 13. Reformatsky reaction with the zincate derived from ethyl bromoacetate followed by dehydration with the Burgess reagent (Burgess et al., 1970) gave chain extended ester 14. Double bond reduction gave saturated ester 15 which suffered selective ethyl ester reduction upon exposure to LiBH4 to provide alcohol 16. Swern oxidation (Omura and Swern, 1978) and reductive amination of the resulting aldehyde afforded dimethyl amine 18. Hydrolysis of bis-ethyl butyl ester 17 and bis esterification with (Z)-2-dodecen-1-ol (Yoshida et al., 2007) yielded 18. Finally, the desired quaternary ammonium chloride analog L2 was then realized from the reaction of 18 with CH3Cl. For detailed synthesis protocol, please see Supplementary material.

2.1.2.4. Synthesis of L3 and L4. Lipids L3 and L4 were prepared from the common intermediate 6 as presented in Scheme 3. Reaction of 6 with 3-butenylmagnesium chloride gave alcohol 19 which was reduced with Et3SiH and BF3·OEt2 to afford alkene 20. Dihydroxylation and cleavage of the diol, as described for the preparation of 1 in Scheme 1, provided crude aldehyde 21 which was reductively aminated ((CH3)2NH·HCl, NaBH (OAc)3), leading to dimethyl amine 22. Ester hydrolysis provided diacid...
which was bis-esterified with either (Z)-2-undecen-1-ol or (Z)-2-dodecen-1-ol which led to diesters 24 and 25, respectively. Finally, lipids L3 and L4 were prepared upon the methylation of 24 and 25 to CH$_3$Cl. For detailed synthesis protocol, please see Supplementary material.

### Scheme 2. Synthesis of L2. Reagents and conditions: (a) LiOH, THF, H$_2$O, RT 43%; (b) i. (COCl)$_2$, cat. DMF, CH$_2$Cl$_2$, 0–5°C to RT, ii. t-BuOH, 40°C 44%; (c) Ethyl bromoacetate, Zn, TMSCl, Et$_2$O, 30°C; (d) Burgess reagent, toluene, 30°C, 61% over 2 steps; (e) H$_2$ (1 atm), Pt$_2$O, CH$_3$CO$_2$H, 40°C 95%; (f) LiBHEt$_3$, THF, –5°C 71%; (g) i. (COCl)$_2$, DMSO, CH$_2$Cl$_2$, –75°C; ii. Et$_3$N –75°C to RT; (h) (CH$_3_2$)$_2$NH-HCl, NaOAc, NaBH(OAc)$_3$, CH$_3$CO$_2$H 52% over 2 steps; (i) HCl-dioxane, CH$_2$Cl$_2$, RT; (j) (Z)-2-dodecen-1-ol, EDCl, DMAP, CH$_2$Cl$_2$, 0°C to RT 58% over 2 steps; (k) CH$_3$Cl, CH$_3$CN, RT 99%.

23 which was bis-esterified with either (Z)-2-undecen-1-ol or (Z)-2-dodecen-1-ol which led to diesters 24 and 25, respectively. Finally, lipids L3 and L4 were prepared upon the methylation of 24 and 25 to CH$_3$Cl. For detailed synthesis protocol, please see Supplementary material.

### Scheme 3. Synthesis of L3 and L4. Reagents and conditions: (a) 3-butenylmagnesium chloride, THF, –20°C; (b) Et$_3$SiH, BF$_3$Et$_2$O, CH$_2$Cl$_2$, RT, 58% over 2 steps; (c) i. K$_2$OsO$_4$, NMO, THF:H$_2$O (2:1 v/v); ii. NaOAc, rt; (d) (CH$_3$)$_2$NH-HCl, NaOAc, NaBH(OAc)$_3$, THF-CH$_3$CO$_2$H 40% over 2 steps; (e) NaOH, EtOH, RT, crude; (f) (Z)-2-undecen-1-ol, EDCl, DMAP, CH$_2$Cl$_2$, 0°C to RT, 24 41% over 2 steps; or (Z)-2-dodecen-1-ol, EDCl, DMAP, CH$_2$Cl$_2$, 0°C to RT, 25 46% over 2 steps; (g) CH$_3$Cl, CH$_3$CN, RT, 62% and 85% respectively for L3 and L4.

2.2. Methods

2.2.1. RNA Formulation into Lipid Nanoparticles and nebulization

The LNP were prepared, in a manner similar to that reported (Rajappan et al., 2020; Ramaowamy et al., 2017), by mixing appropriate volumes of lipids in ethanol with an aqueous phase containing TdTmRNA using an Arcturus proprietary mixing module, followed by downstream processing. In all these formulations a proprietary ionizable lipid was also included to facilitate endosomal release of the endocytosed particles. Briefly, lipid excipients including DOTAP or L1-L4, proprietary ionizable lipid (Rajappan et al., 2020), DSPC, cholesterol, and PEG2000-DMG were dissolved in ethanol at a molar ratio of 25:25:10:38.5:1.5. The lipids were rapidly mixed with the aqueous TdTmRNA solution prepared at pH 3.5 citrate buffer at a flow rate ratio of 1:3 (ethanol:water v/v) using a proprietary mixing module to keep the ethanol percentage constant at 25%. The formed LNPs were stabilized by sequential dilution with pH 6.0 phosphate buffer, followed by pH 8.0 HEPES buffer. To concentrate the formulation, the diluted formulations were processed with tangential flow filtration (TFF) using PES hollow fiber membrane (100 kDa MWCO, Repligen, USA), and further diafiltered with HEPES buffer. After filtering the formulation with 0.2 µm PES filter, an in-process RNA concentration determination was performed, and the formulation
concentration was adjusted to the final target concentration of 1.2 mg/mL with cryoprotectant addition. After sterile filtration, bulk formulation is aseptically filled into glass vials, and stored frozen at –70 °C. Characterization of final formulation includes particle size and polydispersity (PSD) measurement by dynamic light scattering (ZEN3600, Malvern Instrument, Malvern, United Kingdom), DNA encapsulation and quantification by a fluorometric assay using Ribogreen RNA reagent (Thermo Fisher Scientific, USA), and mRNA purity characterization by capillary electrophoresis using fragment analyzer (Advanced Analytical, USA). Briefly, mRNA was analyzed for size and integrity using a parallel capillary electrophoresis instrument Fragment Analyzer 5400. Each prepared sample is voltage injected into discrete capillaries, arranged in a parallel format. The capillaries contain a separation gel matrix infused with a fluorescent intercalating dye, that is automatically primed into the capillaries prior to each run. During electrophoresis, the nucleic acid fragments in the sample migrate and separate based upon their size, picking up dye along the way. As the separated fragments pass by the detection window, the nucleic acid bound dye is excited by a continuous light source, producing fluorescent emission which is detected by a sensitive CCD detector. The time required to pass through the detection window indicates size and the relative emission signal provides the nucleic acid concentration when compared to a calibrated ladder. ProSize analytical software automatically provides information on mRNA integrity and size distribution. In addition, to more accurately determine purity (integrity) of mRNA the data is transferred to internal Excel sheets (Purity Calculator) designed to perform mRNA peak integration according to principles used by chromatographic integration software.

All mRNA samples with initial concentration above 50 µg/mL are diluted to 50 µg/mL of mRNA with RNase-free water in a total volume of 20 µL or greater and then used for preparation of analytical samples in the 96-wells plates. Any mRNA samples with concentrations lower than 50 µg/mL are not diluted and are used as is for analytical sample preparation. When applicable, it is recommended to concentrate down the samples to meet the concentration requirement.

The prepared LNP formulations were diluted with sterile water at a ratio of 1:1 to ensure a physiological osmolality prior to nebulization with an Aerogen Solo vibrating mesh nebulizer (Aerogen, Galway, Ireland, Supplementary Figure 2) to reach 0.6 mg/mL RNA concentration. The aerosol was collected by condensation using ice-cold tube, and post-nebulized samples were subjected to quantification of mRNA encapsulation efficiency and mRNA purity/integrity evaluation. These were used in in vitro cell viability and transfection assays.

2.2.2. Mouse Plasma Stability

Lipid stock solution was prepared by dissolution of the lipid in isopropl alcohol at the concentration of 5 mg/mL. A requisite volume of the lipid-isopropyl alcohol solution was then diluted to 100 µM concentration at a total volume of 1.0 mL with 50:50 (v/v) ethanol / water. Ten microliters of this 100 µM solution was spiked into 1.0 mL of mouse plasma (BiotIVT, Cat. No.: MSE00PLHNUNN, CD-1 mouse, anticoagulant: sodium heparin, not filtered) that was pre-warmed to 37 °C and was stirred at 50 rpm with a magnetic stir bar. The starting concentration of lipids in plasma was thus 1 µM. At time points 0, 15, 30, 45, and was stirred at 50 rpm with a magnetic stir bar. The starting concentration of lipids in plasma was thus 1 µM. At time points 0, 15, 30, 45, 60 and 120 min, 0.1 mL of the plasma was withdrawn from the reaction mixture and the protein was precipitated by adding 0.9 mL of ice-cold 4:1 (v/v) acetonitrile/methanol with 1 µg/mL of a selected internal standard. After filtration through a 0.45 µm syringe filter plate, the filtrates were analyzed by LC-MS (Thermo Fisher’s Vanquish UHPLC – LTQ XL linear ion trap Mass Spectrometer); Waters XBridge BEH Shield RP18 2.5 mm (2.1×100 mm) column with its matching guard column. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in 1:1 (v/v) acetonitrile/methanol. Flow rate was 0.5 mL/min. Elution gradient was: Time 0 – 1 min: 10% B; 1–6 min: 10–95% B; 6 – 8.5 min: 95% B; 8.5–9 min: 95–10% B; 9–10 min: 10% B. Mass spectrometry was in positive scanning mode from 600 to 1100 m/z. The peak of the molecular ion of the lipids was integrated in the extracted ion chromatography (XIC) using Xcalibur software (Thermo Fisher). The relative peak area compared to T = 0, after normalization by the peak area of the internal standard, was used as the percentage of the lipid remaining at each time point. T1/2 values were calculated using the first-order decay model.

2.2.3. Cell Transfection Assay / In-Cell Western

CFBE410 cells (DF508-Human CFTR) were obtained from Fisher Scientific (Cat# SSC161) and maintained at 37 °C with 5% CO2 in complete Dulbecco’s Modified Eagle Medium (Corning Cat# 10–013-CV) supplemented with MEM Non-Essential Amino Acids Solution (Fisher Scientific Cat# 11140050) and 10% Fetal Bovine Serum (HyClone Cat# SH30071.03). For the In Cell Western assay, CFBE cells were seeded in Nunc MicroWell 96-Well Optical-Bottom Plates (Fisher Scientific Cat# 152036) at 17,000 cells/well concentration. 24 h post seeding, CFBE cells were incubated with 1, 0.5, 0.25 µg/mL of tdTomato-expressing LNP formulations, in quadruplicate for each condition. 24 h later, CFBE cells were fixed in 4% PFA for 20 min, permeabilized with 0.1% Triton X-100 and blocked in Odyssey blocking buffer (Licor Cat# 927–50000) for 45 min. Rabbit anti-RFP (Rockland Cat# 600–401–379) antibody was diluted 1:300 in Odyssey blocking buffer and incubated on cells for 1 h. IRDye 800CW Donkey anti-Rabbit IgG Secondary Antibody (Licor Cat# 926–32213) and CellTag 700 (Licor Cat# 926–41090) were diluted 1:7000 and 1:500, respectively, in Odyssey blocking buffer and incubated with CFBE cells for 1 h. Then, whole 96-Well Optical-Bottom Plates were scanned using a Licor Odyssey CLx imaging system. CellTag 700 nm signal was used as a marker of cell viability, with PBS negative samples used as a control, and reported as relative fluorescence units. tdTomato signal, obtained from the 800 nm channel, was instead normalized against the corresponding CellTag 700 nm value, and reported as relative fluorescence units. Licor Image Studio software was used for fluorescence signal analysis, while Microsoft Excel and GraphPad Prism 9 softwares were used for data analysis and visualization.

2.2.4. Flow Cytometry

CFBE410 cells were obtained from Fisher Scientific (Cat# SSC161) and maintained at 37 °C with 5% CO2 in complete Dulbecco’s Modified Eagle Medium (Corning Cat# 10–013-CV) supplemented with MEM Non-Essential Amino Acids Solution (Fisher Scientific Cat# 11140050) and 10% Fetal Bovine Serum (HyClone Cat# SH30071.03). 100k CFBE cells were plated on 24-well plates (Corning Cat# 07–200–84) and incubated with 0.5 µg/mL LNP formulations or vehicle. After 24 h, cells were harvested using TrypLE (Thermo Fisher Cat# 12605036), washed in DPBS and resuspended in FACS buffer (DPBS, 1% BSA and 2.5 mM EDTA). Quantification of tdTomato positive cells was measured using FACs analysis (ZES Cell Analyzer from Bio-Rad) and FlowJo software. Briefly, cell populations were gated on FSC-Height / SSC-Height scatter plots, and subsequently the singlets identified using FSC-Height / FSC-Width and SSC-Height /SSC-Width scatter plots. Of the singlets population, tdTomato positive cells were gated based on negative controls (vehicle and untreated).

2.2.5. Animal studies

Eight-to-twelve-old, female Balb/C mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA). Mice were housed in a pathogen-free environment in Innovive disposable IVC rodent caging system with a 12 h light/dark cycle and ad libitum access to rodent chow and water. All in vivo procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC). Balb/C mice were intra-tracheally dosed with different LNPs (DOTAP−, DOTAP+, L1-L4) carrying a TDTomato mRNA. Twenty-four hours post-dose, mice were anesthetized via 2.5% isoflurane and lungs were processed for immunohistochemistry using a TDtomato primary antibody. For detailed method, please see Supplementary Material.
3. Results and discussion

As seen in Supplementary Figure 1, DOTAP is a cationic lipid with a trimethylammonium and a 2,3-dihydroxypropane core that forms asymmetric esters with 2 molecules of oleic acid. This pseudo glyceryl form makes DOTAP chiral with unique chemical and biochemical properties. For example, the chiral nature of the molecule imposes ambiguity on the nature and rate of esterase activity on DOTAP as enzymes do have inherent preferences for cleavage of one chiral form over the other. This is further complicated by the common use of DOTAP as a racemate (vide supra). Hence, we designed cationic lipids L1-L4 which are non-chiral functional analogues of DOTAP. Further, we postulated that ester groups more distal to the branching point bearing the charged quaternary ammonium head group would be subjected to less steric congestion which would likely facilitate biodegradability, resulting in fragments which might be more readily eliminated. Such lipid configuration(s) are known to facilitate rapid elimination of LNPs (Martin et al., 2013).

As described above (vide supra), we designed the analogues of the current study to exhibit similar atom extensions from the branch point as does DOTAP. The ester carbonyl was positioned 8 atoms from the branch and similarly sized (Z)-2-alkenols formed the esters. Different head group modifications were introduced in these designs and they were not anticipated to be of source of significant problems to formulation. The single methylene spacer of DOTAP was replaced in analogs L1-L4 with 2–3 atom spacer groups, in the form of an ether (L1-L4). These changes resulted a cLogP 11.34 for L1, 10.44 for L2, 10.85 for L3, and 11.46 for L4. The importance of the lipophilicity of DOTAP with respect to formulation and performance of the LNPs containing it, and the possible impact of the range of cLogP values of cationic lipids L1-L4 on the formulation and performance of LNPs containing them would instruct us relative to the appropriateness of our initial designs and may suggest future direction(s). We combined these cationic lipids, whose function is to promote rapid elimination of LNPs (Martin et al., 2013).

Characterization of the Impact of Aerosolized LNPs on mRNA Integrity and % Encapsulated and post-nebulized LNPs were characterized for % of mRNA encapsulation efficiency and mRNA purity. These physicochemical properties were comparable across DOTAP+ and L1-L4, whereas DOTAP profile was impacted. Data was consistent across two separate repeat studies conducted with different batches of formulations. For more clarity, please see Supplementary Table 2 also.

mRNA encapsulation efficiency after nebulization.

Our next objective was to evaluate the lipids L1-L4 for their biodegradability profile in mouse plasma (Fig. 2). We also included two ionizable lipids (Lipid Control #1 and #2) with previously established mouse plasma half-lives as positive controls. In this assessment the lipids were incubated with mouse plasma for 2 h (see materials and methods, vide supra) and the percentage of intact lipid remaining at different time points was assessed. As we had expected, DOTAP was quite stable, associated with a T½ of > 120 min, while lipids L1-L4 showed much faster degradation profiles as indicated by T½ values ranging from 8.0 to 11.5 min (Fig. 2C). At 30 min, almost all L1, L3 and L4 are hydrolyzed, while ~20% L2 and 80% of DOTAP remain intact (Fig. 2B). These data support the notion that changes in the structure of DOTAP can lead to improved lipids with better biodegradability profiles, as observed for lipids L1-L4 (Fig. 2). After LNP disruption to release mRNA, individual lipid components need to be metabolized for eventual elimination. This might happen in the plasma because these smaller lipid components are often shuttled into systemic circulation, as opposed to the intact LNP. Hence, plasma stability is an appropriate measure of overall biodegradability and potential for accumulation over time. Hence, the ready plasma degradability of L1-L4 is thought to be an advantage over DOTAP.

Table 2

| Lipid   | Pre-nebulization | Post-nebulization |
|---------|------------------|-------------------|
|         | %Encap | mRNA purity | %Encap | mRNA purity (%) |
| DOTAP+  | 73.0   | 84          | 57     | 74             |
| DOTAP   | 99.7   | 91          | 97.5   | 86             |
| L1      | 100.2  | 90          | 99     | 82             |
| L2      | 100.1  | 84          | 98.3   | 84             |
| L3      | 100    | 84          | 99.3   | 82             |
| L4      | 100    | 90          | 99.2   | 84             |

After confirming the characteristics of nascent LNPs and consistency of encapsulation efficiency and mRNA integrity across multiple iterations of nebulization, our next objective was to test the efficiency of these cationic LNPs for transfection (no additional transfection reagent was used in these experiments) in Cystic Fibrosis Bronchial Epithelial (CFBE) cells. Different LNPs encapsulating a TdTomato mRNA were used to test this hypothesis. Pre- and post-nebulization fractions were collected and transfected in CFBE cells at a high (1 g/mL) and low (0.25 g/mL) dose. Transfection efficiency measured by FACS showed that DOTAP+ (DOTAP indicates DOTAP containing LNP as positive control) and L1-L4 have the highest number of cells transfected when compared to DOTAP (LNP without DOTAP, Supplementary Figure 3); which is also observed qualitatively by immunocytochemistry for TdTomato in transfected cells (Supplementary Figure 4). Cell viability was also monitored during the process, where the lowest dose (0.25 g/mL) showed minimal impact in viability comparable at both pre- and post-nebulization fractions across the tested LNPs (Supplementary Figure 5). When post-nebulization fractions are compared, we observed that lipids L3 and L4 had a larger impact on cell viability, which may not necessarily due to the chemical characteristics of these lipids, but to changes in particle characteristics post nebulization, and this warrants further exploration. Relative TdTomato protein quantitation was assessed by fluorescence intensity (Fig. 3) using In-Cell Western, (ICW) and all LNPs, except the one without DOTAP (DOTAP+), showed dose dependent changes in fluorescent intensity with pre-nebulized LNPs (Fig. 3A). The data presented by ICW (Fig. 3) and FACS (Supplementary figure 3) shows the same pre- and post-
nebulization fraction trends across all LNPs tested, independently of any sensitivity differences that may exist in each assay. Concomitantly, all nebulized LNPs also showed dose dependency (Fig. 3 B), albeit quite low intensity for DOTAP-LNP in both cases. Lipids L1-L4 performed similarly to DOTAP in terms of TdTomato protein expression in this study. All the data generated with LNP-treated cells were statistically significant at all three doses tested (two-tailed unpaired parametric t-test, Fig. 3). A heat map showing the relative percentage of TdTomato protein levels retained in the post-nebulization fraction when compared to the pre-nebulization condition. The lighter the blue color the higher the drop in protein levels, being equal or higher than 1.0 considered 100% retained.

Fig. 2. Mouse Plasma Biodegradability Profile of DOTAP Analog Lipids (L1-L4) in Comparison to DOTAP. A: Full biodegradability profile in mouse plasma. Samples were collected every 15 min for up to 2 h. B: high level overview of the biodegradability profile up to 30 min (dashed box in A). C: Half-life of the different lipids tested in mouse plasma. L1-L4 have a shorter half-life than DOTAP and, therefore, a better biodegradability profile.

Fig. 3. Relative TdTomato Protein Quantitation in CFBE cells Transduced with Pre- and Post-Nebulized Lipid Nanoparticles (LNP) Carrying a TdTomato mRNA. A-B: Quantitation of the relative levels of luminescence generated after transfection with three doses (0.25, 0.5 and 1 µg/mL) of formulations (DOTAP-, DOTAP+, L1-L4) carrying a TdTomato mRNA. Pre- (A) and post- (B) nebulization formulations were used in this study, and untransfected and vehicle (LNP buffer) were used as controls. Controls did not show any luminescence. Samples were run in quadruplicates in two independent studies and showed as mean ± SD. A dose response in protein levels was observed in all the formulations and conditions tested. Two-tailed unpaired parametric t-test was used to assess significant TdTomato expression differences (p < 0.05) between DOTAP+ and other formulations, comparing 1 ug/mL dose (**), 0.5 ug/mL dose (**) and 0.25 ug/mL dose (***) . C: Heat map showing the relative percentage of TdTomato protein levels retained in the post-nebulization fraction when compared to the pre-nebulization condition. The lighter the blue color the higher the drop in protein levels, being equal or higher than 1.0 considered 100% retained.
lipids. Lower performance of L1 may be due to the low P value of 0.52 that is at the threshold for forming stable lamellar structures. While our primary objective was to design better bio-degradable lipids and not necessarily more potent lipids, we were not surprised with these results. These results support the use of L2-L4 with cLogP values that range from 10.85 to 11.46 and P values > 0.60 in the formulation of LNPs that function equivalently to those utilizing DOTAP. These particles indeed showed excellent physicochemical properties and protected the mRNA cargo as seen from high mRNA encapsulation efficiency and mRNA purity evaluation post a freeze-thaw cycles. Our rational for exploring these lipids was an expected improvement in the biodegradation profile as well as a lack of chiral centers. In a comparison of L1-L4 with DOTAP, we desired comparable or better particle formation characteristics, mRNA release capabilities, as seen from the cognate protein expression, and comparable in vitro profile. While the plasma biodegradabilities of these lipids were demonstrated to be better than that of DOTAP, the other characteristics remained similar. In addition, the cLNPs incorporating L1-L4 were nebulized, and these aerosolized particles also showed comparable TdTomato protein expression levels in vitro, whereas L2 and L4 showed similar delivery profile than DOTAP in rodent airways. Thus, with these readily biodegradable analogues, a more extensive set of in vivo studies will be required to further validate L1-L4 for inhaled therapies using relevant animal models for respiratory diseases such as CF.

As we move forward with additional designs, we must also be mindful of the need to clear the products of ester cleavage. Hence the physicochemical space occupied by prospective new analogues and the physicochemical characteristics of the fragments of ester hydrolysis will be of importance. Moving into poor physicochemical space for the products of ester hydrolysis could greatly impact the clearance of these entities. Consider the small alteration in the structure of analog L3, as shown in L3a to be illustrative of this concern (Fig. 5). Hypothetical analogue L3a retains the ester carbonyl at a position 8 atoms from the branch point bearing the tetraalkylammonium head group, approximating the same steric environment used in the design of L3 while it moves the oxygen of the ester to a position more proximal to the tetraalkyl ammonium head group. The calculated physicochemical space occupied by prospective new analogues, the physicochemical characteristics of the fragments of ester hydrolysis will be of importance. Moving into poor physicochemical space for the products of ester hydrolysis could greatly impact the clearance of these entities. Consider the small alteration in the structure of analog L3, as shown in L3a to be illustrative of this concern (Fig. 5). Hypothetical analogue L3a retains the ester carbonyl at a position 8 atoms from the branch point bearing the tetraalkylammonium head group, approximating the same steric environment used in the design of L3 while it moves the oxygen of the ester to a position more proximal to the tetraalkyl ammonium head group. The calculated physicochemical space occupied by the fragments Metabolite 2 (c-LogP = −0.53, cLogD = −0.91) and Metabolite 4 (c-LogP = −2.08) are illustrative of this concern, as the clearance of tetraalkylammoniums of the size of Metabolite 2 and Metabolite 4 appears to be negatively impacted by lower c-LogP (Hirom et al. 1974; Neef and Meijer, 1984; Yang et al., 2009). In fact, these were part of our considerations for choosing the ester orientations as in L1-L4 as opposed to the hypothetical L3a type orientation. But further PD/PK correlation studies are warranted, and future designs will be guided by such criteria.

### 4. Conclusion

In summary, we have synthesized four novel cationic lipids that are functional analogues (L1-L4) of one of the most used cationic lipids, DOTAP, and formulated them successfully into cLNPs encapsulating an mRNA. These cLNPs show similar physical chemical characteristic as those utilizing DOTAP. These particles indeed showed excellent physicochemical properties and protected the mRNA cargo as seen from high mRNA encapsulation efficiency and mRNA purity evaluation post a freeze-thaw cycles. Our rational for exploring these lipids was an expected improvement in the biodegradation profile as well as a lack of chiral centers. In a comparison of L1-L4 with DOTAP, we desired comparable or better particle formation characteristics, mRNA release capabilities, as seen from the cognate protein expression, and comparable in vitro profile. While the plasma biodegradabilities of these lipids were demonstrated to be better than that of DOTAP, the other characteristics remained similar. In addition, the cLNPs incorporating L1-L4 were nebulized, and these aerosolized particles also showed comparable TdTomato protein expression levels in vitro, whereas L2 and L4 showed similar delivery profile than DOTAP in rodent airways. Thus, with these readily biodegradable analogues, a more extensive set of in vivo studies will be required to further validate L1-L4 for inhaled therapies using relevant animal models for respiratory diseases such as CF.

As we move forward with additional designs, we must also be mindful of the need to clear the products of ester cleavage. Hence the physicochemical space occupied by prospective new analogues and the physicochemical characteristics of the fragments of ester hydrolysis will be of importance. Moving into poor physicochemical space for the products of ester hydrolysis could greatly impact the clearance of these entities. Consider the small alteration in the structure of analog L3, as shown in L3a to be illustrative of this concern (Fig. 5). Hypothetical analogue L3a retains the ester carbonyl at a position 8 atoms from the branch point bearing the tetraalkylammonium head group, approximating the same steric environment used in the design of L3 while it moves the oxygen of the ester to a position more proximal to the tetraalkyl ammonium head group. The calculated physicochemical space occupied by the fragments Metabolite 2 (c-LogP = −0.53, cLogD = −0.91) and Metabolite 4 (c-LogP = −2.08) are illustrative of this concern, as the clearance of tetraalkylammoniums of the size of Metabolite 2 and Metabolite 4 appears to be negatively impacted by lower c-LogP (Hirom et al. 1974; Neef and Meijer, 1984; Yang et al., 2009). In fact, these were part of our considerations for choosing the ester orientations as in L1-L4 as opposed to the hypothetical L3a type orientation. But further PD/PK correlation studies are warranted, and future designs will be guided by such criteria.
Declaration of Competing Interest

The authors are all employees of Arcturus Therapeutics and may hold equities in the company. The authors declare no other competing financial interests.

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Part of the calculations in Table 1 are done using Molinspiration Property Calculation Service freely available on the internet (https://www.molinspiration.com).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemphyslip.2022.105178.

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Fig. 5. Physicochemical Space and Potential Clearance Issues. (A) Compound L3 bio-degrades into metabolite 1 and metabolite 2. (B) Hypothetical alternate design for L3 (shown as L3a) would result in metabolite 3 that is expected to have poorer clearance based on calculated cLogP of ~2.08. Calculated using ACD Labs Structure Design v12.0. cLogP was calculated using ACD Labs version B.
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