Novel Polyvalent Ionizable Lipids Enable Targeted Delivery of mRNA to Immune Cells and iPSC-Derived MSCs

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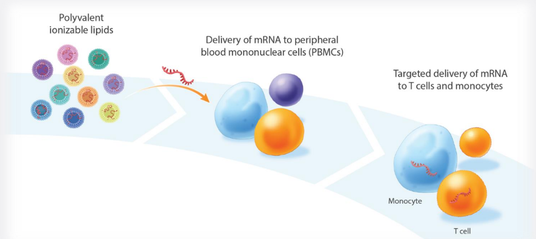
Introduction

- Since the introduction of the mRNA COVID-19 vaccines, ionizable lipid-based mRNA platforms have entered broad clinical use and have gained attention for their potential use in tissue engineering and immunotherapies
- Lipids are now considered the gold standard for in vivo mRNA delivery vehicles, but still suffer from low transfection efficiency in primary cells and stem cell derivatives
- To address this limitation, we designed a library of 4,200 lipids containing strategic modifications to the chemical structure of DLinDHS, a key component of the ToRNAdo™ Lipid Delivery System, which has effectively delivered mRNA to various cell types ex vivo and in vivo
- A panel of 82 potential candidates were synthesized and screened in iPSC-derived MSCs (iMSCs) and human peripheral blood mononuclear cells (PBMCs)
- Cells were transfected with GFP-encoding RNA complexed with each of the candidate lipids



Results

- Of the 82 lipids tested, 60 transfected iMSCs with 70-99% efficiency and 83-95% viability at 24 hours post-transfection
- 45 of these lipids induced GFP expression in primary CD14+ and CD3+ cells
- CD3+ and CD14+ PBMCs and iMSCs exhibited peak GFP fluorescence intensity at 18-24 hrs after lipoplex transfection and 24-36 hrs after LNP transfection
- Lipids containing spermine headgroups with hexyldecanoate ester tails yielded higher levels of GFP expression compared to other structural combinations
- These lipids transfected CD3+ cells with a 4- to 11-fold increase in GFP expression relative to Lipofectamine™ 3000, and transfected THP-1 monocytes at over 90% efficiency

Conclusion

- Our novel polyvalent spermine-derived ionizable lipids enable delivery of mRNA into hard-to-transfect cells, including human CD14+ and CD3+ cells
- These lipids may prove useful in future mRNA therapeutics by targeting hard-to-transfect cell types to modulate protein expression in immune cells and mediate cell host-defense responses

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1. Lipid Library Design

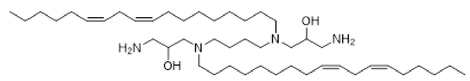

Figure 1. ToRNAdo™ lipid delivery system. The main structural elements of our group's ToRNAdo™ lipid (i.e., DLinDHS) are the dihydroxyspermine headgroup and linoleyl tails. We hypothesized that combining structural elements such as biodegradable functional groups and/or branching with polyvalent spermine-derived headgroups could lead to improved transfection efficiency and enhanced tissue targeting.

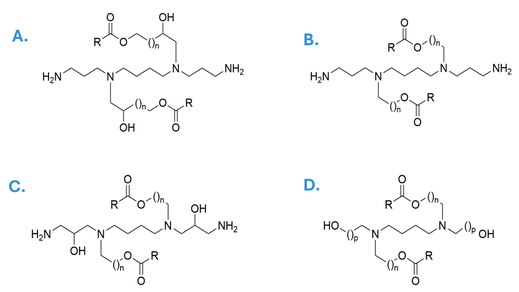

Figure 2. PBMC lipid library design. 4,200 novel ionizable lipids were computationally designed and a library was synthesized, consisting of 84 polyvalent ionizable lipids sorted into 4 major structural categories. Esters and hydroxyls were incorporated to promote in vivo biodegradability and increase lipid-mRNA formulation stability. "R" in the structures denotes alkyl or alkenyl branched lipid tails containing 10 to 24 carbon atoms. Tethered ester chains range from n=2 to n=6 and tethered primary alcohol

In designing the novel lipids, structural characteristics such as biodegradability, lipid tail length, level of branching, and branched tail symmetry were considered. Increased hydrogen bonding capacity was achieved through the introduction of hydroxyl substituents, promoting stronger intermolecular association with anionic nucleic acid phosphate groups. **A, B.** Branched ester substituted spermine analogs derived from phthalimide protected spermine. **C, D.** Branched ester substituted 1,4-diaminobutane analogs derived from bis Ns protected 1,4-diaminobutane.

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2. Method of Lipid-mRNA Delivery

Figure 3. Structure of GFP mRNA cargo. CleanCap® 5Hbb_CC, 3Hbb TT-BsaI. The mRNA cargo of the lipid delivery system consists of a 1093 base long sequence (GFP) with an HBB promoter and a GFP reporter. The GFP mRNA is 353,070 g/mol and possesses 1093 phosphates per mole.

Lipoplex Preparation

Procedure Two 1.7mL microcentrifuge tubes were labeled, one "RNA" and the other "TR". The necessary amount of OptiMEM buffer (pH 7.4) was added to each tube such that the final volume would be 15uL per tube after addition of mRNA or lipid. GFP mRNA (aqueous) was added to the "RNA" tube and ionizable lipid transfection reagent (at room temperature) was added to the tube labeled "TR". Contents of the "TR" tube were added to the "RNA" tube and immediately pipette-mixed 10x. A 2.5:1 lipid:RNA ratio (w/w) was used for all transfections. To allow for complexation, the lipid-RNA solution was incubated at RT for 5 minutes before adding dropwise into cell culture at RNA dose concentrations ranging from 0.1 to 1.33 ug RNA/mL cells.

LNP Preparation

Formulation Ionizable lipid, Cholesterol, DSPC, and PEG2000-DMG] were brought to RT and combined in a microcentrifuge tube at a molar ratio of 50 : 38.5 : 10 : 1.5 in EtoH. 170ug of mRNA was mixed in 100mM citrate buffer (pH 3.0). Microfluidic formulation was conducted with a NanoAssemblr® Benchtop using an N/P ratio of 6 and a 3:1 volume and flow rate ratio of aqueous:organic phase. Reagent amounts were determined to produce a post-formulation RNA concentration of 0.2mg/mL and total lipid concentration of 2mg/mL. LNPs were incubated at RT for 1 hr to promote full complexation.

Dialysis and Purification LNP solution was resuspended in 20 mM Tris buffer (pH 7.5) to a volume of 3mL and pipetted into a Slide-A-Lyzer G3 Dialysis Cassette. Cassettes were placed in 75mL Tris buffer with a stir bar and LNPs were dialyzed overnight with a replacement of buffer after the first 45 minutes. Dialyzed LNPs were filtered through a 0.22 µm syringe filter membrane and concentrated using Amicon Ultra filters (10 kDa MWCO) at 13xg for 20 minutes.

Characterization LNPs were characterized by DLS and Ribogreen Assay to measure size dispersion, zeta potential, and RNA encapsulation.

Transfection LNPs were diluted 1:100 – 1:500 in culture medium supplemented with FBS before adding dropwise into cell culture. Concentrations ranging from 100 to 400ng RNA/mL were used.

Cryopreservation LNPs were suspended in 20 mM Tris + 10% sucrose at a concentration of 100ug RNA/mL and snap-frozen in LN2 before storing at -80°C. LNPs left at 4°C for up to 1 week were also used for transfection.

3. PBMC Transfections

PBMC Cell Subsets	
CD3 (35-75%)	T-Cells
CD14 (10-20%)	Monocytes/Macrophages
CD19 (~15%)	B Cells
CD34 (0.1-0.2%)	HSCs and progenitor cells
CD56 (~15%)	NK Cells

Table 1. Relative PBMC sub-populations by cell surface marker expression.

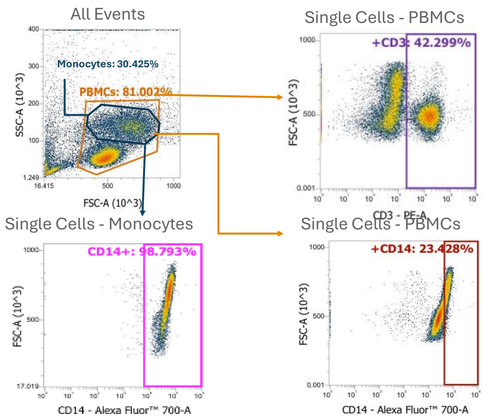

Figure 4. PBMC flow cytometric phenotyping. Expression of CD3 (a T cell marker) and CD14 (a monocyte cell marker) were evaluated using flow cytometry to validate the panel.

Primary CD3+ T Cells

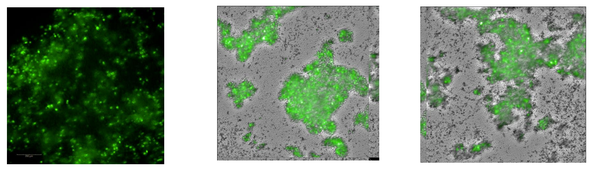

Figure 5. GFP RNA delivered to activated CD3+ PBMCs via ToRNAdo.™ CD3+ cells were isolated from PBMCs and activated for 3 days in media supplemented with CD3/28 activator and IL-2. Cells were then transfected and GFP expression was observed in aggregated T cells 24 hours later by Operetta high content imaging analysis.

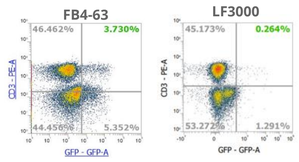

Figure 5. GFP expression in live, single CD3+ PBMCs treated with FB4-63 lipoplexes compared to Lipofectamine 3000. 7.40% of live FB4-63 treated CD3+ cells expressed GFP whereas only 0.58% of live LF3000 treated cells were GFP positive.

Primary CD14+ Cells

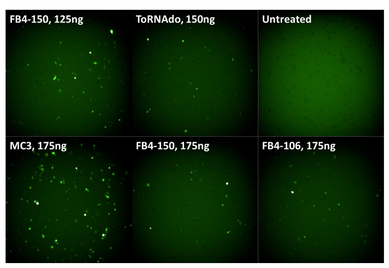

Figure 7. Isolated CD14+ PBMCs 24 hours after treatment with LNPs at their optimal RNA dose. MC3-LNPs formulated at 40:38.5:20:1.5 demonstrated higher transfection delivery efficiency than other LNPs tested.

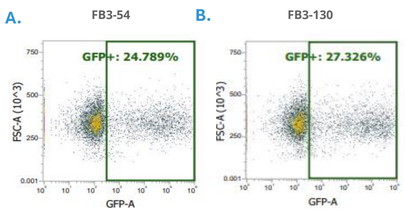

Figure 8. Isolated CD14+ PBMCs 24 hours after treatment with (A) FB3-54 and (B) FB3-130 lipoplexes at a 2.5:1 lipid:RNA dose.

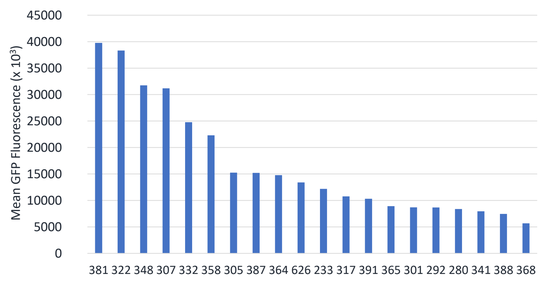

Figure 9. Mean GFP fluorescence in PBMC lipoplex library screen. The full lipid library was prepared as lipoplexes and delivered 0.2ug GFP-RNA to 100,000 PBMCs per sample. The 20 lipids exhibiting the highest mean GFP fluorescence, calculated as number of GFP+ cells in the sample multiplied by sample mean fluorescent intensity, is plotted for the peak expression time of 18 hrs post-transfection.

4. THP-1 Monocytes

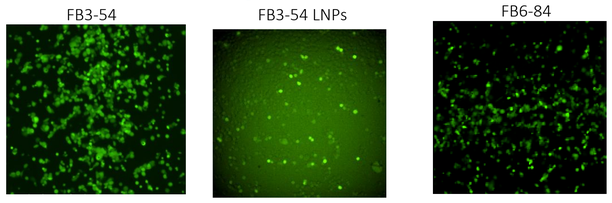

Figure 10. FB3-54 and FB6-84 lipoplexes and FB3-54 LNPs successfully transfected THP-1 cells. 0.1ug of mRNA was delivered via lipoplexes and 62.5ng via LNPs per 10,000 cells. GFP expression was imaged at 24 hrs.

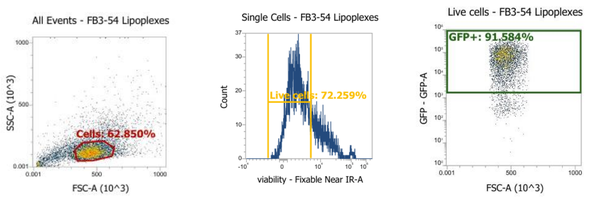

Figure 11. Flow cytometry of THP-1 cells transfected with FB3-54 lipoplexes at a 2:1 ratio. FB3-54 lipoplexes yielded over 91% transfection efficiency and cell viability over 72%.

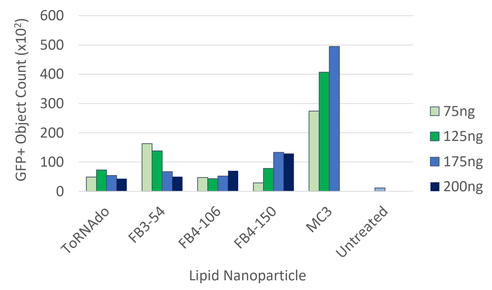

Figure 12. LNP transfection efficiency in THP-1 cells measured by flow cytometry. Optimal RNA dose varied greatly among LNP types between 75ng and 200ng per 10,000 cells.

5. iPSC-Derived Cell Transfections

iPSC-Derived MSCs

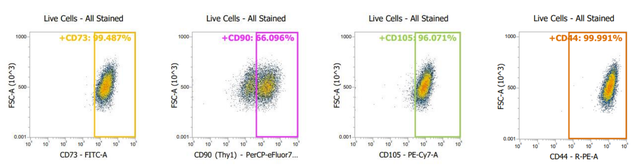

Figure 13. iMSC flow cytometric phenotyping. The iPSC-derived MSCs used for transfection were flowed for phenotypic validation. These cells possess the classical positive markers of MSCs, CD73, CD90, CD105, and CD44.

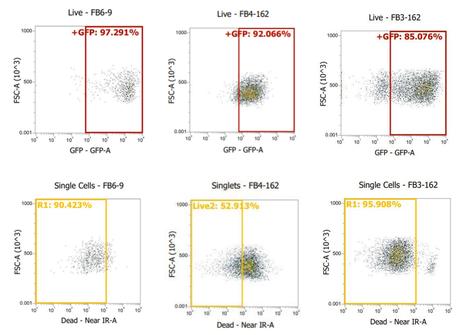

Figure 14. Flow cytometric analysis of iMSCs treated with lipoplexes at a dose of 0.2ug/mL GFP-RNA. Over 40 lipids transfected iMSCs successfully with GFP RNA - quantitative measurements of GFP (top) and viability (bottom) of the top 3 performing lipids are shown to the left.

iPSC-Derived Macrophages

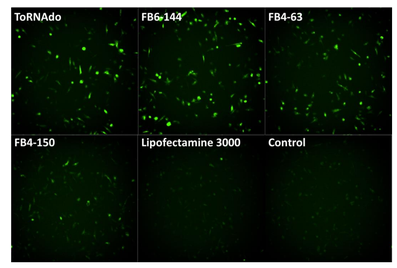

Figure 15. Lipoplexes in iPSC-derived macrophages. 0.1ug RNA was delivered per 50,000 cells. GFP channel was imaged 24 hrs post-transfection at 20x magnification.

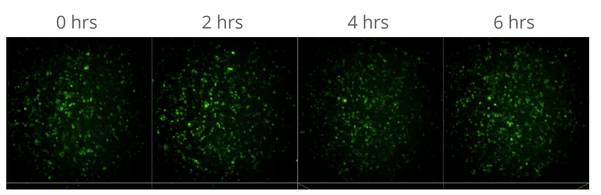

Figure 16. Six hour time course of GFP fluorescence in iPSC-derived macrophages treated with ToRNAdo LNPs at a dose of 0.96ng/ml RNA.