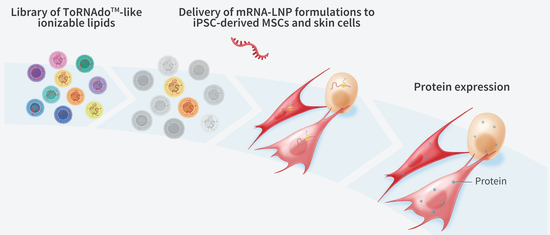
Novel Ionizable Lipids Derived from 2-Hydroxypropylamine and Spermine for mRNA-LNP Delivery

Ariadna Lubinus[1], Joseph Pisano[1], Elizabeth Belcher[2], Christopher B. Rohde[1], Matthew Angel[1,2]
[1]Factor Bioscience Inc., Cambridge MA [2]Eterna Therapeutics Inc., Cambridge MA
This work is protected by one more pending patents.

Abstract

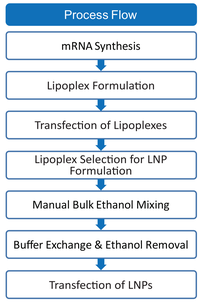
- Clinical use of lipid nanoparticles (LNPs) for mRNA delivery is expanding with the introduction of COVID-19 mRNA vaccines and in treatments for genetic diseases. Limitations of current LNPs as nucleic acid delivery systems include poor targeting, insufficient cellular uptake, and low endosomal release.
- To address these limitations, we formulated a library of 31 novel ionizable lipids and screened each lipid's efficiency in delivering mRNA to various cell types. Our goal is to identify ionizable lipids that produce LNPs with low mean particle sizes, high loading efficiencies, and enhanced mRNA gene expression comparable to or better than LNPs incorporating clinically available ionizable lipids, such as ALC0315 and DLin-MC3-DMA.
- Each of these lipids were synthesized with lipid tails containing hexyl hexyldecanoate and hydrophilic headgroups derived from either spermine, an element of ToRNAdo™ or 2-hydroxypropylamine, an element of ALC0315 (major lipid component of the Pfizer COVID-19 vaccine). LNPs were administered to iPSC-derived MSCs, primary human fibroblasts, and keratinocytes.



Conclusions

- Ionizable lipids containing variations of spermine or 2-hydroxypropylamine headgroups and hexyldecanoate-derived lipid tails were shown to bind to and deliver mRNA into various cell types with favorable particle size, encapsulation, and stability characteristics.
- This study illustrates that ionizable lipids with combinations of a bis 2-hydroxy-*n*-hexyldecanoate or bis n-hexyldecanoate lipid tail and spermine headgroup are promising components of next-generation gene therapies.
- Trends in protein expression and lipid-RNA complexation suggest that short, unsaturated carbon chains in the lipid tail region may improve RNA binding ability without compromising cell viability and should be considered in the rational design of ionizable lipids for mRNA delivery.

1 Method of Nanoparticle Formulation & Transfection



Analysis Equipment

Qubit 2.0 Fluorometer
E-Gel® Electrophoresis with ChemiDoC™ MP Imaging System
CellTiter-Glo® Luminescent Cell Viability Assay
EVOS™ M5000 Imaging System
Operetta® CLS™ High Content Analysis System with Harmony® Software
Invitrogen™ Quant-it™ RiboGreen RNA Assay Kit
Horiba nanoPartica SZ-100 Nanoparticle Analyzer
Agilent BioTek Synergy Neo Multi-Mode Microplate Reader
Oakton™ PC 700 pH/Conductivity Meter

Procedure

Part I. Formulation

1. mRNA was prepared at a ratio of 20% Cy5-labeled RNA and 80% CleanCap GFP-TT1-RNA.
2. Powder lipid stocks were desiccated for 2hrs before diluting in EtOH to a concentration of 10mg/mL.
3. Required volumes of lipids, mRNA, and citrate buffer were calculated for an N/P ratio of 6 and molar ratio of 50:38.5:10:1.5 (ionizable lipid: cholesterol: DSPC/DOPE: DMG-PEG2000). For MC3-LNPs, a ratio of 30:30:38.5:1.5 was used with pegylated lipid DMPE-PEG2000.
4. Citrate buffer was heated for 10min in a 65° water bath. The warmed citrate buffer and RNA were combined in a glass vial and stirred with a magnetic stir bar for 5min.
5. Meanwhile, lipid solutions were heated for 5min to allow full dissolution. The required volumes were combined in a glass vial such that the final LNP volume was between 200uL-300uL.
6. Dropwise, the lipid-EtOH solution was added to the RNA-citrate solution. The combined solution was mixed at RT for 1hr.

Part II. Filtration

1. 1000 MWCO Amicon Ultra filters were placed in collection tubes. Filters were primed by adding 400uL DPBS and spinning for 10min at 8,000g. Flow-through was discarded.
2. After 1hr, the stir bar was removed and the volume of each LNP solution was brought up to 400uL using DPBS.
3. Filtration was conducted 3-4 times with the Amicon filters. After each filtration, a 10uL sample was pipetted onto a pH strip. Filtrations were repeated until measured pH was above pH 6.5.
4. After the last filtration, LNPs were diluted in DPBS to a concentration of about 1000ug/mL.

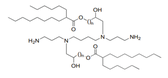
Part III. Transfection

1. 10,000 cells/well in a 96-well plate or 20,000 cells/well in a 6-well plate were seeded 24 hours before transfection.
2. LNPs were stored at 4° for up to 1 week before transfection. When ready to transfect, 20uL treatment volumes were prepared by mixing the necessary amount of LNPs with OptiMEM under a sterile environment.
3. Dropwise, LNP-OptiMEM solution was added to each well and the plate was shaken lightly to mix.

2 Classification of Ionizable Lipids

Family 1: Spermine & Bis 2-hydroxy-n-hexyldecanoate

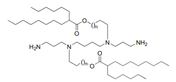
Lipid ID	Lipid Tail	Hydrophilic Headgroup	MW (g/mol)	Molecular Formula	Carbon Tether Length (n=2)
FB4-55	Bis 2-hydroxypropyl hexyldecanoate	Spermine	827.33	$C_{49}H_{98}N_4O_5$	3
FB4-84	Bis 2-hydroxybutyl hexyldecanoate	Spermine	855.39	$C_{51}H_{102}N_4$	4
FB4-116	Bis 2-hydroxypentyl hexyldecanoate	Spermine	883.44	$C_{53}H_{106}N_4O_5$	5
FB4-106	Bis 2-hydroxyhexyl hexyldecanoate	Spermine	911.5	$C_{55}H_{110}N_4O_5$	6



n = 1, 2, 3, 4, 5

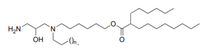
Family 2: Spermine & Bis n-hexyldecanoate

Lipid ID	Lipid Tail	Hydrophilic Headgroup	MW (g/mol)	Molecular Formula	Carbon Tether Length (n=1)
FB3-131	Bis Propyl hexyldecanoate	Spermine	795.34	$C_{49}H_{98}N_4O_4$	3
FB4-28	Bis Butyl hexyldecanoate	Spermine	823.39	$C_{51}H_{102}N_4O_4$	4
FB3-130	Bis Pentyl hexyldecanoate	Spermine	851.44	$C_{53}H_{106}N_4O_4$	5
FB3-54	Bis Hexyl hexyldecanoate	Spermine	879.50	$C_{55}H_{110}N_4O_4$	6
FB3-162	Bis Heptyl hexyldecanoate	Spermine	907.55	$C_{57}H_{114}N_4O_4$	7



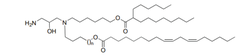
Family 3: 2-Hydroxypropylamine & Hexyl hexyldecanoate

Lipid ID	Lipid Tail	Hydrophilic Headgroup	MW (g/mol)	Molecular Formula	Carbon Tether Length (n=2)
FB1-134	Hexyl hexyldecanoate/Butyl	2-Hydroxypropylamine	484.81	$C_{29}H_{61}NO_3$	4
FB2-122	Hexyl hexyldecanoate/Octyl	2-Hydroxypropylamine	540.92	$C_{33}H_{69}NO_3$	8
FB2-124	Hexyl hexyldecanoate/Lauryl	2-Hydroxypropylamine	597.03	$C_{37}H_{77}NO_3$	12
FB2-112	Hexyl hexyldecanoate/Stearyl	2-Hydroxypropylamine	681.19	$C_{45}H_{93}NO_3$	18:0
FB2-92	Hexyl hexyldecanoate/Oleyl	2-Hydroxypropylamine	679.17	$C_{45}H_{91}NO_3$	18:1
FB2-110	Hexyl hexyldecanoate/Linoleyl	2-Hydroxypropylamine	677.16	$C_{45}H_{89}NO_3$	18:2



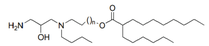
Family 4: Hydroxypropylamine & Bis *n*-hexyldecanoate

Lipid ID	Lipid Tail	Hydrophilic Headgroup	MW (g/mol)	Molecular Formula	Carbon Tether Length (n=2)
FB3-56	Hexyl hexyldecanoate/Butyl linoleate	2-Hydroxypropylamine	763.25	$C_{49}H_{97}NO_5$	4
FB3-82	Hexyl hexyldecanoate/Pentyl linoleate	2-Hydroxypropylamine	777.27	$C_{50}H_{99}NO_5$	5
FB3-1	Hexyl hexyldecanoate/hexyl linoleate	2-Hydroxypropylamine	791.3	$C_{51}H_{101}NO_5$	6
FB2-88	Bis Hexyl hexyldecanoate	2-Hydroxypropylamine	767.28	$C_{51}H_{101}NO_3$	6
FB3-2	Bis hexyl linoleate	2-Hydroxypropylamine	815.32	$C_{57}H_{105}NO_5$	6



Family 5: 2-hydroxypropylamine & m-Hexyldecanoate/Butyl

Lipid ID	Lipid Tail	Hydrophilic Headgroup	MW (g/mol)	Molecular Formula	Tail Linkage Length (y=2)	Carbon Tether Length (n=2)
FB4-8	Propyl hexyldecanoate/Butyl	2-Hydroxypropylamine	442.73	$C_{29}H_{58}NO_3$	3	4
FB4-30	Butyl hexyldecanoate/Butyl	2-Hydroxypropylamine	456.76	$C_{30}H_{60}NO_3$	4	4
FB4-10	Pentyl hexyldecanoate/Butyl	2-Hydroxypropylamine	470.78	$C_{31}H_{62}NO_3$	5	4
FB4-24	Heptyl hexyldecanoate/Butyl	2-Hydroxypropylamine	498.84	$C_{33}H_{66}NO_3$	7	4



3 Novel Lipids Complex with RNA at Low Weight Ratios

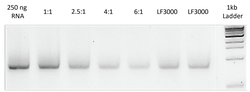


Figure 1. Gel electrophoresis of FB2-110 complexation with RNA. At a 2.5:1 ratio, the fraction of RNA complexed (43.1%) is comparable to Lipofectamine 3000 (47.6%).

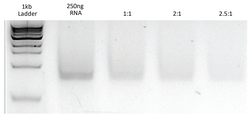


Figure 2. Gel electrophoresis of ToRNAdo-RNA complexation. ToRNAdo displays highly efficient RNA binding ability, exhibiting 82.4% complexation at a 2.5:1 lipid:RNA weight ratio.

			Lipid: RNA Weight Ratio				
Lipid ID	Lipid Family	Tether Length	1:1	2:1	2.5:1	3:1	4:1
FB455	1	3	0.587	0.684	0.711	0.769	0.899
FB484	1	4	0.52	0.837	0.876	0.918	0.997
FB4-106	1	5	0.185	0.406	0.74	0.917	0.975
FB4-116	1	6	0.488	0.499	0.541	0.841	0.906
FB3-131	2	3	0.617	0.708	0.761	0.903	1
FB4-28	2	4	0.729	0.526	0.551	0.661	0.694
FB2-124	3	12	0	0.1661	0.31	0.335	0.45
FB2-88	4	6	0.056	0.0876	0.124	0.133	0.183
ToRNAdo	N/A	N/A	0.552	0.715	0.824	0.856	0.937

Table 2. **Lipid-RNA Complexation Efficiency vs. Weight Ratio.** Gel electrophoresis was used to measure the fraction of total RNA complexed at different lipid:RNA weight ratios. Lipid tether regions less than 6 carbons long performed better on average than those with longer tethers. Family 1 lipids and FB3-131, both displaying 80% complexation at a 3:1 ratio, showed high complexation efficiencies comparable to ToRNAdo.

4 Lipoplexes and LNPs Deliver mRNA to iMSCs, HEKn, and Fibroblasts

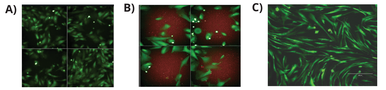


Figure 3. Lipoplexes were delivered to three cell types. Operetta images of four nonadjacent regions within one well have been combined in each figure. **A)** 20,000 iMSCs at 14 hours post-treatment with lipoplexes comprising FB3-54 and ccGFPTT1 RNA at 1 mg lipid/mg RNA. **B)** 20,000 human epidermal keratinocytes (neonatal) treated with FB3-54 and 20% Cy5-GFP-RNA at 2:1 lipid:RNA. **C)** 20,000 human dermal fibroblasts treated with ToRNAdo™ lipoplexes at 2:1 lipid:RNA.

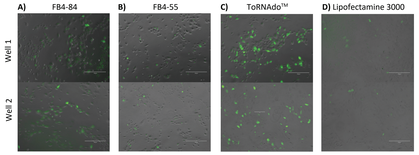


Figure 4. GFP expression in human epidermal keratinocytes (neonatal) 24 hours post-transfection. Keratinocytes were treated with lipoplexes comprising CleanCap® GFP-TT1 mRNA and a Family 4 lipid, FB4-84 or FB4-55. ToRNAdo and Lipofectamine treatments were positive controls. Lipoplexes were delivered in duplicate at a dose of 500ng RNA per 20,000 HEKn. Two wells are shown as stacked images in Figure 2(a-d).

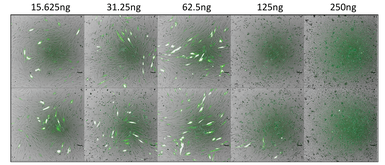


Figure 5. iMSCs treated with ToRNAdo™ LNPs (50:30:38.5:1.5 ToRNAdo:Cholesterol: DSPC:DMG-PEG2000) exhibit maximum GFP expression with low toxicity at a dose of 62.5ng RNA per 20,000 cells.

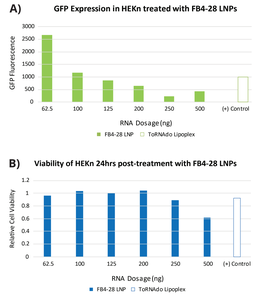


Figure 6. Microplate imaging analysis of FB4-28 LNP-treated HEKn. **A)** Peak GFP fluorescence occurred at a 62.5ng RNA dose/20K cells with 2.5x fluorescence of ToRNAdo lipoplexes. The slight increase in GFP fluorescence at 500ng represents the autofluorescence of dead iMSCs. **B)** HEKn viability measured >80% relative to untreated cells for doses up to 250ng/20K cells and dropped to 60% at the 500ng dose.

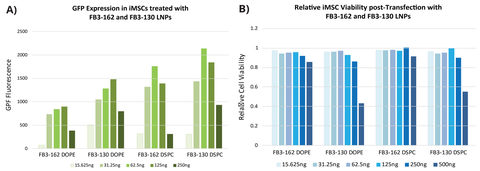


Figure 7. **A)** JP3-162 and JP3-130 LNPs formulated with DSPC and DOPE exhibited peak fluorescence at 62.5ng. Fluorescence values are background subtracted. **B)** iMSCs treated with JP3-162 LNPs maintained >80% cell viability at all doses whereas JP3-130 LNP treatments exhibited a reduction of >40% at the 500ng dose.

Ionizable Lipid	Helper Lipid	Mean Size	Mean Size S.D.	Mode Size	Mode Size S.D.	PDI	PDI S.D.	RNA Encapsulation Efficiency (%)	N/P
FB4-28	DSPC	121.5	17.7	110.5	7.9	0.70	0.08	95.6	6
ToRNAdo	DSPC	168.7	15.1	112.2	13.6	0.34	0.09	60.2	4
ToRNAdo	DSPC	151.7	53.3	131.1	9.0	0.47	0.15	76.2	4
ToRNAdo	DSPC	171.2	28.2	149.3	14.5	0.55	0.15	83.3	4
FB3-162	DSPC	130.7	14.3	119.3	12.6	0.26	0.06	100.0	6
FB3-162	DOPE	111.7	15.6	107.5	10.8	0.25	0.07	100.0	6
FB3-130	DSPC	112.3	12.0	111.9	12.6	0.61	0.29	100.0	6
FB3-130	DSPC	192.6	60.8	168.2	23.7	0.37	0.02	100.0	6
FB3-130	DOPE	139.1	12.0	127.8	9.7	0.31	0.08	100.0	6
FB2-88	DSPC	177.6	58.1	149.0	10.5	0.35	0.70	78.8	4
FB4-24	DOPE	94.4	20.8	87.5	17.6	0.42	0.23	80.5	6
FB1-134	DSPC	156.2	34.3	143.9	16.6	0.35	0.16	90.8	4
FB1-134	DSPC	194.9	51.0	175.0	11.0	0.22	0.02	100.0	4
FB1-134	DSPC	114.8	18.9	111.3	18.1	0.37	0.16	34.3	4
MC3-MB	DSPC	152.5	48.1	130.5	6.7	0.25	0.06	95.0	4
MC3-MD	DSPC	167.9	49.7	143.3	0.75	0.26	0.03	72.7	4
ALC0315	DSPC	203.0	72.9	161.8	0.2	0.16	0.03	34.2	4

Table 3. **Characterization of Lipid Nanoparticles.** LNPs were formulated with N:P ratios of 4:1 or 6:1. A sample of the formulated LNPs were diluted 1:50 in DPBS and characterized for particle size analysis. Mean and mode diameters for all LNPs formulated with novel lipids measured between 100 and 200nm, and polydispersity index measured below 0.5 for most LNPs. RNA encapsulation efficiency measured 60% for all but 1 novel lipid formulation.