In partnership with HELIX BIOTECH

Application Note

Ionizable Lipid Composition Influences Lipid Nanoparticle Efficacy in Multiple Cell Types *In Vitro*

Graham Taylor, Ph.D.[†], Ryan Markewicz[†], J. Paige Gronevelt[§], and Julie Rumble, Ph.D.[§]
[†]Helix Biotech, Knoxville, TN; [§]Cayman Chemical, Ann Arbor, MI

Key Features

- Cell lines derived from different tissues have distinct reporter expression patterns in response to a variety of lipid nanoparticles (LNPs).
- Serum requirement for LNP uptake and cargo expression *in vitro* vary with cell type and ionizable lipid.
- Experimental design is of utmost importance in assessing the efficacy of any given LNP preparation.

Introduction

Lipid nanoparticles (LNPs) represent an extremely promising technology for delivery of gene products for a host of potential health applications, from immunization against emerging pathogens to personalized neoantigen cancer therapy and gene therapy for many diseases.[1] The clinical use of two of the COVID-19 vaccines (BNT162b2 and mRNA-1273) has been a driving force for recognizing the potential of this technology for its safety and efficacy. LNPs are usually composed of four lipid types, a cationic/ionizable lipid, a helper phospholipid, a PEGylated lipid, and cholesterol, which together encapsulate and protect nucleic acid cargo. These LNPs are taken up by cells into the endolysosomal system, where a decrease in pH induces the release of the nucleic acid into the cytoplasm where it can exert its effect, either on other RNAs in the case of siRNA cargo, or producing protein, in the case of mRNA or DNA cargos.

In order to utilize LNPs most effectively for the variety of potential uses, many different aspects of these tools can be varied to change cell and/or tissue tropism, increase the ability to evade the immune system, or effectively release cargo to the cytoplasm.[2] One of these variable aspects is the cationic/ionizable lipid itself. There are currently dozens of different published lipids, and literature suggests that they can have a significant effect on cargo delivery to different tissues. Here, we examine a common cationic lipid (DOTAP) as well as three well-known ionizable lipids: SM-102, ALC-0315, and DLin-MC3-DMA (MC3). SM-102 and ALC-0315 are the key ionizable lipids in Moderna and Pfizer COVID-19 vaccines, respectively. MC3 is the ionizable lipid in Alnylam's approved siRNA drug, Onpattro®.

When formulating and testing LNPs, it is important to carefully consider the model system that will most effectively assess the quality of the particles made. While using cell lines as *in vitro* model systems can have the advantage of elucidating particular cell types that an LNP can target, there are factors that may be missing from *in vitro* experiments that may alter the efficacy of an LNP preparation. For instance, ApoE from serum has been published to form part of a protein corona that facilitates the uptake of LNP by hepatocytes, and other serum proteins may be important for uptake by other cell types.[3] Establishing best practices for *in vitro* testing of LNPs is an area of ongoing research.

In this study, four different formulations of LNPs were generated by Helix Biotech (Helix), varying primarily in the cationic/ionizable lipid included. Those were DOTAP, ALC-0315, SM-102, and MC3. These LNPs encapsulated mRNA encoding eGFP as a reporter. Cayman tested the expression of GFP induced by each of these LNPs in four different cell models: Huh7 hepatocytes, A549 lung epithelial cells, primary monocyte-derived macrophages, and PMA-differentiated THP-1 cells. Finally, all cell models were assayed both in the presence and absence of fetal bovine serum (FBS) to test the dependence on ApoE and other serum factors.

Methods

Reagent Preparation and LNP Formulation

All lipids used in this study were supplied by Cayman Chemical and prepared and formulated into LNPs at Helix. Lipid mixes were prepared in ethanol with fixed molar ratios of 50:10:38.5:1.5 for the cationic/ionizable lipid, DSPC, cholesterol, and DMG-PEG, respectively. The cationic lipids were either DOTAP, ALC-0315, SM-102, or MC3. The final lipid concentration was 25 mM in ethanol. Payload consisted of 300 µg of eGFP-mRNA (GenScript), diluted using 100 mM sodium acetate buffer (pH 4, ThermoFisher) to 116.67 µg/ml.

A Nova™ Benchtop nanoparticle production system with impinged jet mixers (IJM) was first primed with ethanol. Nuclease-free water was then used to prime the mRNA channel. eGFP-mRNA and the lipid mixture in ethanol were loaded into separate syringes and inserted into syringe pumps. Total flow rate was 15 ml/min with a flow rate ratio of 5:1 (mRNA:lipid). Resulting LNPs were transferred to a 100 kDa dialysis filter (Spectra/Por®) and placed in 1 L of 1X PBS (pH 7.4, ThermoFisher), for overnight dialysis to remove residual ethanol and neutralize pH. LNPs were collected, filter-sterilized with 0.2 µm filters (Pall), and then stored at 4°C until use.

Particle Analysis

Post-dialysis, LNPs were transferred to cuvettes and diluted 100X using 1X PBS. Hydrodynamic particle size and polydispersity index (PDI) were measured by dynamic light scattering (Anton Paar Litesizer™ 500). The RNA encapsulation efficiency was measured *via* Quant-iT™ RiboGreen RNA assay kit (Invitrogen) and RNA reagent (Invitrogen). This assay begins by measuring the quantity of free unencapsulated RNA (U). The total RNA quantity (T) was then measured by solubilizing the LNPs using Triton™ X-100 (Sigma Aldrich) to free encapsulated RNA. The encapsulation efficiency (EE) was calculated as follows:

$$EE\% = 100 \times (T-U)/T$$

Table 1. Particle characteristics of LNPs used in this study.

Ionizable Lipid	Size (nm)	PDI	Encapsulated RNA (µg/ml)	EE%
DOTAP	58.6	0.203	83.8	90.5
ALC-0315	60.8	0.031	91.1	86.4
SM-102	63.7	0.173	80.4	85.9
MC3	63.1	0.012	65.4	84.8

Cell Culture

All cell culture and imaging studies were performed at Cayman Chemical. Huh7 hepatocytes were cultured in DMEM + 10% FBS, A549 lung epithelial cells were cultured in F12K + 10% FBS, and THP-1 monocytes were cultured in RPMI-1640 + 10% FBS + 50 µM β-mercaptoethanol. THP-1 cells were seeded into a 96-well fluorescent imaging plate 48 hours before addition of LNPs. Media supplemented with 100 nM PMA was added to differentiate THP-1 cells into macrophage-like cells. Before adding LNPs, media containing PMA was removed and unsupplemented media was added. Huh7 and A549 cells were seeded into 96-well fluorescent imaging plates 24 hours before the addition of LNPs. Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor *via* density gradient centrifugation using Histopaque®-1077 (Millipore Sigma). Cells were resuspended in RPMI-1640 + 10% FBS + 1% L-glutamine. Monocytes were enriched by adhesion to fluorescent imaging plates for two hours at 37°C and nonadherent cells were washed off with PBS. Human monocyte-derived macrophages (HMDMs) were differentiated in media containing 50 ng/ml M-CSF for seven days, refreshing media with M-CSF every other day until addition of LNPs. All cells were cultured in the presence of 1% penicillin and streptomycin.

LNP Addition and Imaging

LNPs were diluted to 500 ng/ml (mRNA concentration) in media with and without FBS and four 2-fold serial dilutions were made. Cells undergoing treatment of LNPs in the absence of serum were washed twice with PBS before addition of particles in serum-free media. Diluted LNPs in media were added to cells and plates were transferred to a BioSpa™ 8 automated cell incubator (Agilent) and imaged using brightfield and GFP LED/filter on a Cytation™ 5 cell imaging multi-mode plate reader (Agilent) every four hours for 48 hours total. After 48 hours, media was removed, cells were stained with Hoechst 33342 dye for nuclei, and imaged using brightfield, GFP LED/filter and DAPI LED/filter. Images were analyzed in Gen5 software and GraphPad Prism was used to generate graphs.

Results

GFP Expression in the Presence of Serum

To evaluate expression differences between LNPs based on different ionizable lipids, each of the LNPs generated was added to cells in the presence of 10% FBS. As shown in **Figure 1** (top left), Huh7 hepatocytes were effectively transfected with several of the particles. SM-102 and MC3 both showed initial expression of GFP within eight hours of treatment, though SM-102 continued to increase throughout the course of the experiment, while MC3 nearly leveled off. ALC-0315 showed substantially later induction of expression, while DOTAP did not induce measurable GFP expression.

A lung epithelial cell line, A549, was successfully transfected with the same particles as the Huh7 cells in the presence of serum, with SM-102 inducing the most widespread expression of GFP and least toxicity as measured by cell loss (data not shown). Induction of GFP was similar for the first 12 hours with MC3 but was largely lost after 24 hours as cells detached. ALC-0315 induced late expression of GFP, but it plateaued at a low level as compared to SM-102. In THP-1 monocytes differentiated to macrophage-like cells with PMA, both SM-102- and MC3- induced GFP expression but resulted in toxicity, observable as cell detachment, within 48 hours. GFP induced by ALC-0315 was barely detectable in THP-1 macrophages and DOTAP-induced GFP expression was undetectable.

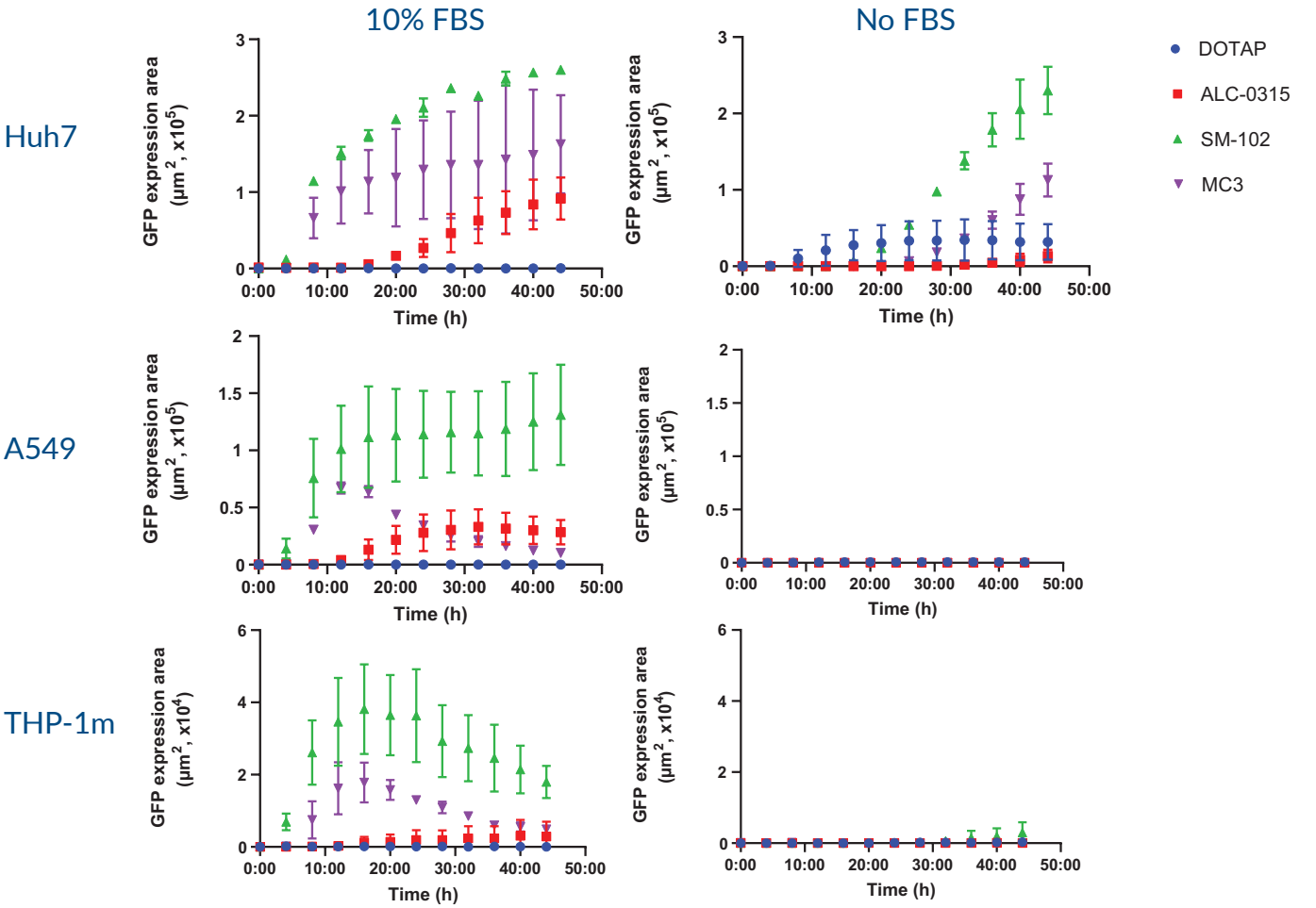

Figure 1. Kinetic analysis of LNP-induced expression of GFP in hepatocytes (Huh7), lung epithelial cells (A549) and macrophages (THP-1m) treated with 500 ng/ml of the indicated LNP formulations with (left) or without (right) FBS. A single image was captured from each well in a 96-well plate every four hours and the total area per image was calculated by Gen5 image analysis software.

Serum Dependence of LNP-mediated GFP Expression

Literature reports have highlighted the effect that serum-derived corona proteins may have on the uptake of LNPs into cells, but this has not been systematically characterized for all cell types or LNP formulations. We tested all four formulations in all four cell lines in the absence of FBS, shown in the right side of **Figure 1**. Huh7 hepatocytes were still able to take up LNPs and express GFP without serum, but for most of the LNPs tested, the level of expression was attenuated and timing was later. Perhaps most interestingly, DOTAP was taken up in the absence of serum where it had not been taken up in the presence of serum, albeit at a low level. Neither A549 cells nor THP-1 macrophages were able to take up the LNPs and express GFP to any degree without serum present. We hypothesize that Huh7 hepatocytes produce some of the corona proteins for uptake, including ApoE, and as such do not have as much of a requirement for exogenously supplied serum. Indeed, as demonstrated by DOTAP treatment of Huh7 cells, endogenous proteins may be more efficacious than bovine serum proteins.

Primary Macrophages

HMDMs were also tested with each of the LNP preparations (**Figure 2**). Some cell loss was observed at higher doses (data not shown), so 125 ng/ml treatment in the presence of serum was chosen as the representative dose for these cells. SM-102 and MC3 were again the preparations that induced expression of GFP in these cells, but SM-102 induced at least 10-fold greater intensity of GFP expression than MC3. In addition, the expression was strongly visible with SM-102 within four hours of treatment and reached a plateau by hour 16. MC3 expression of GFP was not detectable until 12 hours and did not peak until 36 to 40 hours after treatment. Area under the curve (AUC) calculation (**Figure 2, right**) was used to quantitatively compare all of the formulations at all concentrations over the complete time course. For HMDMs, this calculation further highlights the high efficacy of SM-102 in these cells, but also shows that ALC-0315 did transfect at the highest concentration tested. The data from serum-free transfection in HMDMs were uninterpretable due to high background and thus, are not shown here.

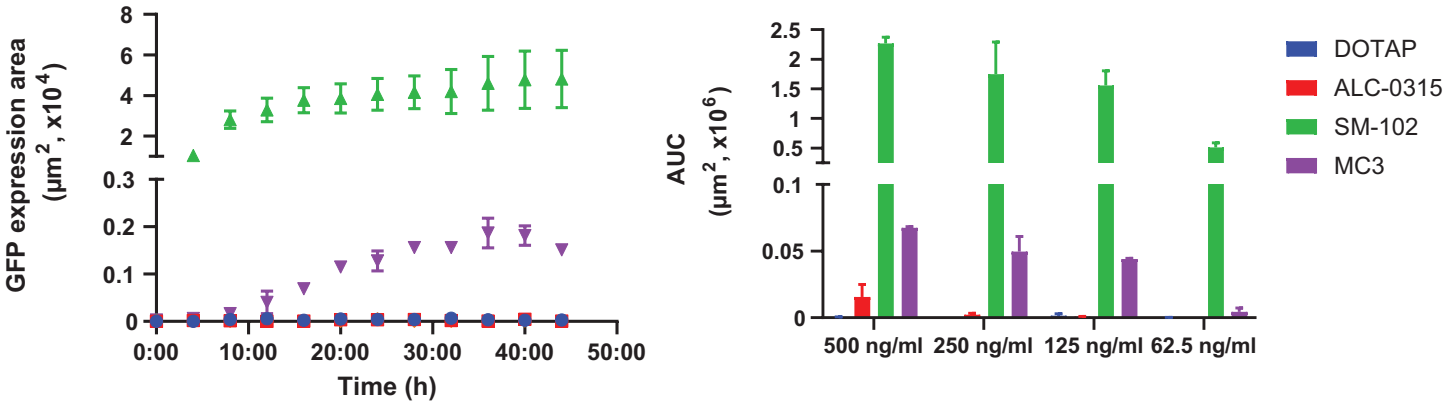

Figure 2. Analysis of human monocyte-derived macrophage transfection with GFP-encoding LNPs. Fresh monocytes were differentiated to macrophages by incubation with M-CSF for seven days, then treated with varying concentrations of LNPs in the presence of serum and imaged periodically over two days. Total area of green fluorescence was calculated for each image (left), and area under the curve (AUC, right) was calculated for the 48-hour experiment.

Transfection Efficiency

Finally, 48 hours after the start of treatment with LNPs, media was removed from all wells and cells were stained with Hoechst 33342 in order to visualize nuclei. All wells were imaged for GFP and Hoechst 33342, and images were used to determine cell counts and percentage of cells transfected by the LNPs (**Figure 3**). At the 125 ng/ml dose of each LNP, minimal cytotoxicity (based on cell loss) was observed (data not shown), so this dose was chosen for analysis. The percentage of the nuclei in each image which were also GFP positive was calculated, and data are shown in the bottom panel of **Figure 3**. SM-102 was the most effective of these LNPs, but MC3 also induced GFP expression in a more cell-type dependent manner.

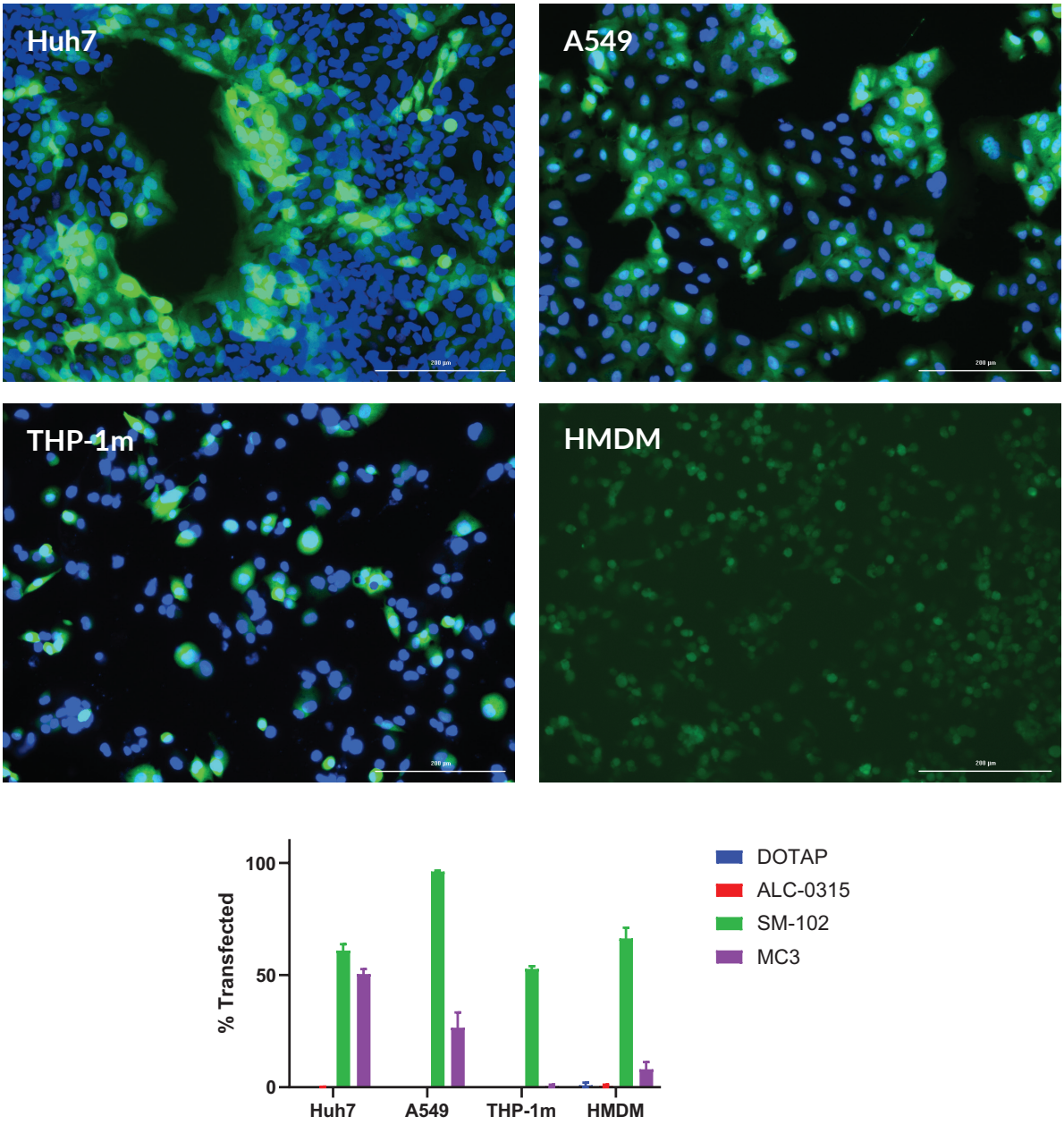

Figure 3. Transfection efficiency of LNPs in multiple cell lines. 48 hours after treatment, cell supernatant was removed and replaced with Hoechst in PBS for nuclei visualization. A single image was captured from each well (top, representative images), using GFP (green) and DAPI (blue) LED/filter sets. Percentage of nuclei associated with GFP expression was calculated (bottom, 125 ng/ml only).

Table 2. Summary of experimental results.

		Huh7	A549	THP-1m	HMDM
DOTAP	with serum	-	-	-	-
	without serum	+	-	-	N/A
ALC-0315	with serum	+	+	-	+/-
	without serum	-	-	-	N/A
SM-102	with serum	+++	+++	+++	++++
	without serum	++	-	-	N/A
MC3	with serum	++	++	++	++
	without serum	+	-	-	N/A

Conclusions

As summarized in **Table 2**, in this study we have demonstrated production of multiple high-quality LNP formulations and quantitative assays to evaluate the cellular effects of treatment with those LNPs. We found that hepatocytes were generally the most transfectable cell type for the LNPs tested, which is perhaps unsurprising given that much of literature describes the liver as the primary target of most LNPs. Of the LNP formulations tested, SM-102 was the most efficacious, broadly inducing high levels of GFP expression in all cell types tested. MC3-based particles also had broad efficacy for the cell types tested but did not overall induce as high expression of GFP as SM-102 particles. It is possible that as the ionizable lipid in the siRNA therapy Onpattro®, MC3 is better at delivering shorter sequences than the longer GFP sequence included here. The data shown here emphasizes the importance of choosing the best ionizable lipid for the target and payload.

We also shed light on the use of *in vitro* models for testing LNP function. Notably, inclusion of serum with the LNP was absolutely critical in all cell types tested except for hepatocytes. Hepatocytes likely produce the requisite proteins and thus do not rely on supplemental serum. Further, DOTAP-based particles only worked in hepatocytes in the absence of serum, suggesting that bovine serum components could exert a negative effect on uptake of some LNPs (such as DOTAP in this experiment) *in vitro*.

It is no surprise that SM-102 and MC3 performed the best in terms of inducing GFP expression, considering the present formulation resembles that of Moderna's mRNA-1273 and Alnylam's Onpattro® formulations (notably the PEGylated lipid, DMG-PEG). ALC-0315, the ionizable lipid from the Pfizer's COVID-19 vaccine, did not induce high levels of GFP expression in any model tested. This may be due to the difference in PEGylated lipid used in the Pfizer's COVID-19 vaccine and the present work, though further studies will be required to elucidate these differences. The data here highlight the care with which formulations must be tested in order to best understand the efficacy of any given LNP preparation.

References

1. Hou, X., Zaks, T., Langer, R., *et al.* Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater.* **6(12)**, 1078-1094 (2021).
2. Han, X., Zhang, H., Butowska, K., *et al.* An ionizable lipid toolbox for RNA delivery. *Nat. Commun.* **12(1)**, 7233 (2021).
3. Francia, V., Schiffelers, R.M., Cullis, P.R., *et al.* The biomolecular corona of lipid nanoparticles for gene therapy. *Bioconjug. Chem.* **31(9)**, 2046-2059 (2020).