Hydroxycholesterol Substitution in Ionizable Lipid Nanoparticles for mRNA Delivery to T Cells

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Introduction: Delivery of nucleic acids, such as mRNA, to immune cells has become a major focus of biomaterials in the past decade with ionizable lipid nanoparticles (LNPs) emerging as a clinically validated delivery platform. These LNPs—typically composed of ionizable lipids, cholesterol, phospholipids, and polyethylene glycol (Fig. 1A)—have been designed and optimized for a variety of applications including cancer therapies, vaccines, and gene editing.[1] Despite their potency and safety, almost 70% of LNPs are recycled from cells by recycling endosomes. Previous studies have shown that cholesterols that have been modified with hydroxyl groups can alter endocytic recycling mechanisms.[2] Here, we explore the incorporation of hydroxycholesterols into LNP formulations to evaluate their impact on mRNA delivery to T cells and their effect on endosomal trafficking mechanisms.

Materials and Methods: A library of 24 LNPs was formulated using 6 X-hydroxycholesterol modifications (7α, 7β, 19, 20, 24, 25). Each of the 6 hydroxycholesterols were substituted in various ratios relative to unmodified cholesterol (12.5%, 25%, 50%, and 100% substitutions). Note that a base formulation (S2), representing a 0% cholesterol substitution, served as a control. This library was evaluated in immortalized T cells (Jurkats) for the delivery of luciferase mRNA as well as toxicity. Size, polydispersity (PDI), and zeta potential, encapsulation efficiency, and pKa were used to characterize LNPs. Confocal microscopy of T cells was used to quantify the expression of endosomal stages (early, late, and recycling) after 4 hrs of treatment with candidate particles. Average per cell (*n=50)* expression was reported (mean $\pm$ sd).

Results and Discussion: All *X*-hydroxycholesterol LNP candidates demonstrated a unimodal distribution of luciferase mRNA delivery across the various substitution ratios (12.5%, 25%, 50%, 100%) in Jurkats when compared to S2, with more delivery occurring at 25% and 50% substitutions of each hydroxycholesterol candidate. Toxicity data demonstrated that all formulations had similar cytotoxicity profiles as S2, suggesting an adequate safety profile. Size, PDI, zeta potential, and encapsulation efficiency did not differ significantly between the particle formulations and S2, indicating that the incorporation of hydroxycholesterols allowed for LNP formation. To evaluate the translatability of these modifications, the top performing hydroxycholesterol substitutes were screened *ex vivo*, during which some of the trends in luciferase mRNA delivery translated into primary human T cells. Importantly, a 25% and a 50% substitution of 7α-hydroxycholesterol (A1-25 and A1-50) demonstrated a 68% and 80% increase, respectively, in luciferase mRNA delivery compared to S2. These top performers (A1-25 and A1-50) were further evaluated in a dose response study where they demonstrated improvements in mRNA delivery at high doses with minimal changes to cell viability. Finally, in order to assess whether these modifications were impacting endosomal trafficking, confocal microscopy was used to quantify endosomal trafficking of cells treated with hydroxycholesterol-containing LNPs. It was revealed that A1-25 and A1-50 increased late endosomal expression and reduced recycling endosome expression, suggesting that these modified LNPs reside in and escape the late endosome at higher rates (Fig. 1B).

Conclusions: Hydroxycholesterol substitutions, particularly in the 7α position, at substitution ratios of 25% and 50%, demonstrate a statistically significant increase in delivery of mRNA to T cells. Imaging studies revealed that a potential mechanism for this improvement may lie in the ability of the modified LNPs to remain in the late endosome and avoid endocytic recycling. Future studies will evaluate these LNPs using therapeutic assays and explore the biodistribution of these LNPs *in vivo*. Ultimately, the present work provides a potential modification strategy for enhancing mRNA delivery to T cells via LNPs for applications in CAR T cell therapy and other cancer immunotherapies.

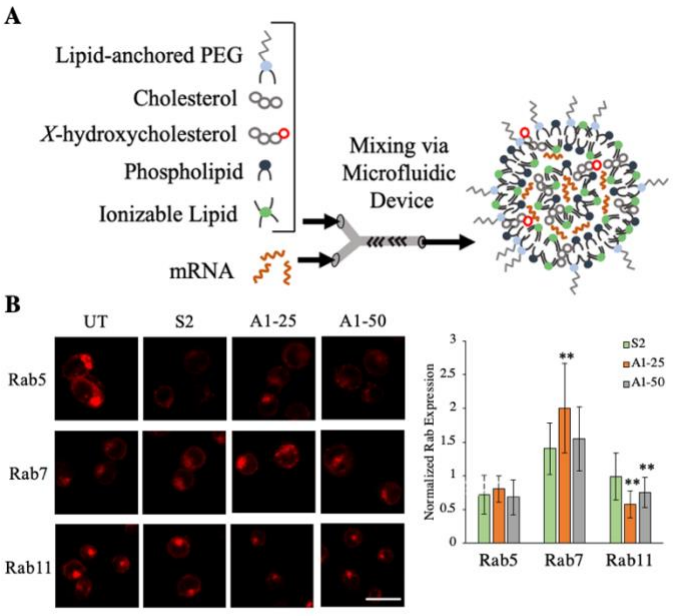

Figure 1. (A) Schematic of expected LNP formation using microfluidic mixing. (B) Representative confocal microscopy (scale bar 20 μm) of LNP-treated Jurkat. LNPs were stained for Rab5, Rab7, and Rab11 as markers of the early, late, and recycling endosomes. Per cell expression, normalized to untreated cells, is averaged across at least 50 cells per group.

References: 1. Margaret M. Billingsley, MMB, Nano Letters, 2020, 20, 1578-1589. 2. Siddharth Patel, SP, Nature Comm., 2020, 11, Article 983.