Ionizable lipids with reduced numbers of tails direct lipid nanoparticle tropism to the spleen

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Statement of Purpose: Lipid nanoparticles (LNPs) are the most clinically advanced platform for mRNA delivery and consist of an ionizable lipid (IL), phospholipid, cholesterol, and PEGylated lipid. Among these components, ILs play a critical role in facilitating mRNA delivery. When exposed to the acidic environment of the endosome, ILs become protonated, prompting cargo release into the cytosol. While it is known that IL structure impacts mRNA transfection efficiency and organ tropism, the specific structure-function relationships that clarify how particular IL structural features influence LNP performance remain understudied.[1] Consequently, IL structure is not fully leveraged when designing LNPs with precise *in vivo* targeting abilities. To achieve organ-specific delivery, alternative strategies like adding a fifth permanently charged lipid component are often employed instead.[2] There is growing evidence that IL tail structure is a key determinant of LNP transfection efficiency and biocompatibility *in vivo*.[3] This project aims to explore whether altering IL tail structure can modulate LNP organ tropism. Notably, we found that LNP biodistribution can be altered by adjusting the number of IL tails. We envision the results of this project could inform rational design criteria for the next generation of LNPs.

Methods: An IL library of varying tail lengths and numbers of tails was synthesized (**Fig. 1A**). The polyamine lipid cores 200 and 494 were reacted with a varying molar ratio of the corresponding epoxide-terminated alkanes of length C10 to C14 (**Fig. 1B**). The reaction mixture was stirred for 48 h at 80 °C in ethanol. The resulting product was purified using flash chromatography and analyzed using ^{1}H NMR and LC-MS. To formulate LNPs, ILs were combined with excipient lipids and luciferase mRNA using microfluidic mixing as previously described.[4] LNPs were characterized for encapsulation efficiency (RiboGreen), hydrodynamic diameter (dynamic light scattering), and relative pK_a (TNS assay). BALB/cJ mice (n=3) were injected with LNPs at a dose of 0.1 mg/kg via the lateral tail vein. After 6 h, luciferin was injected intraperitoneally and organ luminescence was imaged and quantified using IVIS (**Fig. 1C**).

Results: Here, we developed a simple synthetic scheme for varying the number of IL tails using a stoichiometric approach. IL synthesis was confirmed to be successful based on structural characterization and purity determination. C12-200 analogues with differing numbers of tails resulted in different organ biodistribution patterns (**Fig. 1D**). While C12-200 primarily targeted the liver, its 3- and 4-tailed analogues resulted in increased delivery to the spleen and lungs. C12-200 resulted in 98% of mRNA transfection in the liver, whereas 59% and 23% of mRNA transfection occurred in the spleen for C12,4-200 and C12,3-200 respectively. Similar biodistribution results were observed with C10 and C14 tails and the 494 core, demonstrating that increased spleen tropism with fewer tails is consistent across different tail lengths and core structures. C12,4-200, with one fewer tail than C12-200, demonstrated a significant increase in spleen delivery and a significant reduction in liver delivery compared to C12-200, indicating its potential for selective delivery to the spleen. Notably, C12,4-200 performed similarly to a control formulation containing a fifth negatively charged lipid for spleen targeting, suggesting comparable targeting can be achieved by modulating only IL structure.

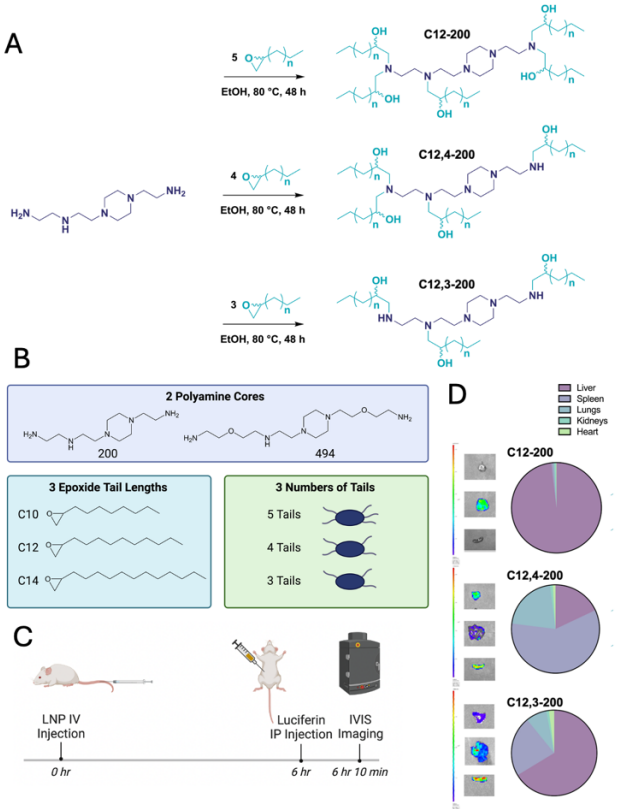

Fig. 1: (a) Representative scheme of ionizable lipid synthesis using the 200 core. The number of epoxide tails added to the lipid core was controlled by varying the reaction stoichiometry. (b) Overview of LNP library composition. (c) *In vivo* luciferase mRNA experiment schedule. (d) Overview of LNP organ biodistribution.

Conclusions: Our findings demonstrate that reducing the number of tails on a single IL structure can significantly alter LNP organ tropism, presenting a novel strategy for modulating organ specificity without requiring additional modifications to LNP formulation. Future work will examine transfection of specific spleen cell types to clarify the underlying mechanisms driving these results. Additionally, while our current structures feature tails positioned randomly on the core, future studies will investigate regiospecific tail placement. Ultimately, our work aims to establish clear and generalizable guidelines for IL design, advancing targeted LNP delivery strategies.

References: (1) Mrksich, K. Adv. Drug Deliv. Rev. 2024, 214, 115446. (2) Cheng, Q. Nat. Nanotechnol. 2020, 15, 313-320. (3) Liu, C. Int. J. Pharm. 2024, 667, 124868. (4) Chen, D. J. Am. Chem. Soc. 2012, 134, 6948-6951.