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nor-MC3 and *nor*-KC2: cationic ionizable lipids for the delivery of therapeutic nucleic acids

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***nor*-MC3 and *nor*-KC2, analogues of D-Lin-MC3-DMA (cationic ionizable lipid in Onpattro®) and D-Lin-KC2-DMA (valuable research tool) wherein C_{17} lipophilic chains replace C_{18} ones, are at least as efficacious as the originals, but more economical and safer to produce.**

A recent paper describes a new type of lipid nanoparticles (LNPs) for the extrahepatic delivery of mRNA.[1] A key component of such LNPs is a cationic ionizable lipid termed *nor*-MC3, **1**. The significance of the above paper and the intense interest in LNP technology for the delivery of therapeutic nucleic acids[2] prompt us to disclose details of the synthesis and properties of **1** and its congener, *nor*-KC2, **2** (Fig. 1).

The new compounds are lower homologues of D-Lin-MC3-DMA,[3] or more simply MC3 (**3**, the ionizable lipid component of Onpattro®),[4] and D-Lin-KC2-DMA, or more simply KC2, **4**.[5] While the latter is not a component of any yet-approved medication, it is a valuable research tool that is especially effective, *inter alia*, for the delivery of plasmid DNA.[6] Notice that in **1** and **2** C_{17} lipophilic chains replace the C_{18} chains present in **3** and **4**, hence the designation *nor*-MC3/*nor*-KC2.

The synthesis of **1** and **2** (ref. 7) started with a Claisen condensation of methyl linoleate, **5**, under Tanabe–Mukaiyama conditions,[8] resulting in the formation of beta-ketoester **6** in 96% yield (Fig. 2).[9] Notice that **5** (*ca.* USD 3 per g) is only 60% of the cost of linoleyl alcohol (*ca.* USD 5 per g) and considerably cheaper that linoleyl bromide (*ca.* USD 81 per g): the starting materials for the original syntheses of **3** and **4**. Crude **6** exists as a mixture of keto- (major) and enol tautomers in variable proportions, typically about 2 : 1. The compound can be purified by normal phase medium pressure liquid chromatography (MPLC) for full characterization, in which case the keto and enol forms are separable. However, it is expedient to convert crude **6** directly into ketone **7** by ester saponification (aq. NaOH) followed by acidification and rotary evaporation of all volatiles at a bath temperature of 60 °C (decarboxylation). Crude **7** was thus obtained in just over 90% overall yield from **5**. Very pure **7** can be obtained by MPLC (see SI).

It should be noted that the Claisen condensation of **5** carried out under customary basic conditions, *e.g.*, with NaH in refluxing xylenes, as reported in a patent,[10] promotes variable degrees of double bond isomerization, as inferred from the appearance of new signals in the olefinic region of NMR spectra.[11] There seems to be no mention of the problem in said patent. We were unable to separate double bond isomers of **6**, **7**, or derived lipids, precluding the use of the latter in biological experiments. In contrast, no evidence of isomerization was apparent from the NMR spectra of crude **6** prepared by the Tanabe–Mukaiyama method, or from spectra of derived products.

Ketone **7** was advanced to *nor*-MC3 by $NaBH_4$ reduction to **8** and esterification thereof with 4-(dimethylamino)butanoic acid hydrochloride (78% yield over 2 steps), and to *nor*-KC2 by ketalization with chlorodiol **9**[11] followed by halide displacement with dimethylamine (80% over 2 steps; Fig. 3). Lipids **1** and **2** are thus available from economical **5** in only 4 steps.

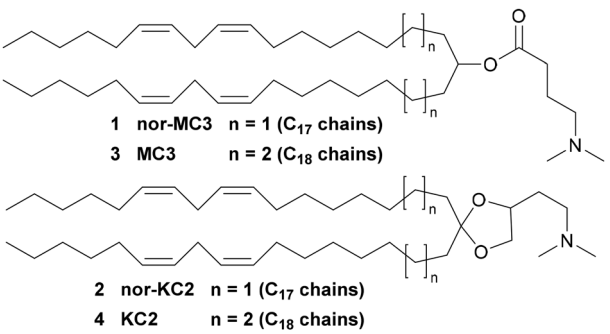

Fig. 1 Structures of *nor*-MC3, **1**, *nor*-KC2, **2**, and of the parent lipids, MC3, **3**, and KC2, **4**. See SI for details.

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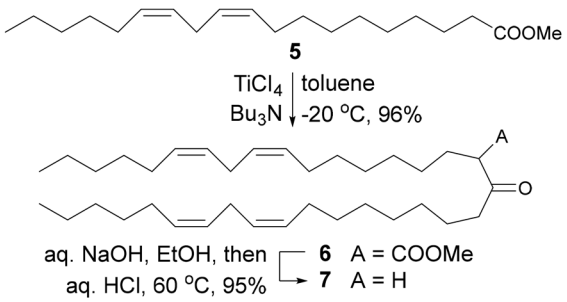

Fig. 2 Preparation of ketone **7** by Claisen condensation of methyl linoleate under Tanabe–Mukaiyama conditions. See SI for details.

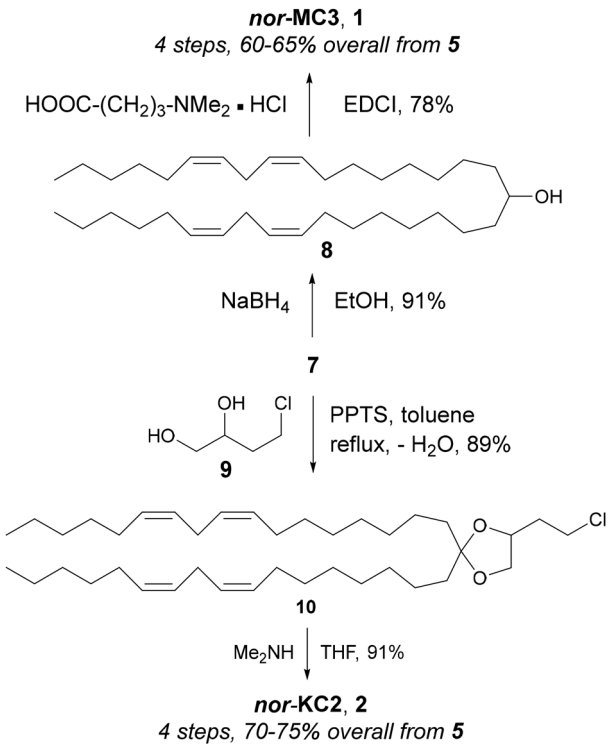

Fig. 3 Conversion of **7** into *nor*-MC3 and *nor*-KC2. See SI for details.

Key physical properties of LNP formulations of anti-firefly luciferase siRNA and firefly luciferase mRNA based on **1** and **2** were virtually identical to those of LNPs produced from MC3 and KC2 (Fig. 4), with a particle size of *ca.* 40–45 nm, high encapsulation (>90%), low polydispersity (<0.1), and identical apparent pK_a in the LNPs (~6.4).

Lipids **1** and **2** proved to be at least as efficacious as the benchmark MC3 in their ability to deliver siRNA and mRNA both *in vitro* and *in vivo*. An *in vitro* siRNA luciferase suppression assay with LNPs based on either *nor*-lipid revealed an EC50 of about 0.1 μg siRNA per mL: equivalent to that of particles based on MC3. Likewise, the *in vitro* efficacy of *nor*-MC3-based LNP formulations of firefly luciferase mRNA was practically identical to that of MC3-containing ones, but interestingly, *nor*-KC2-centered formulations were significantly more

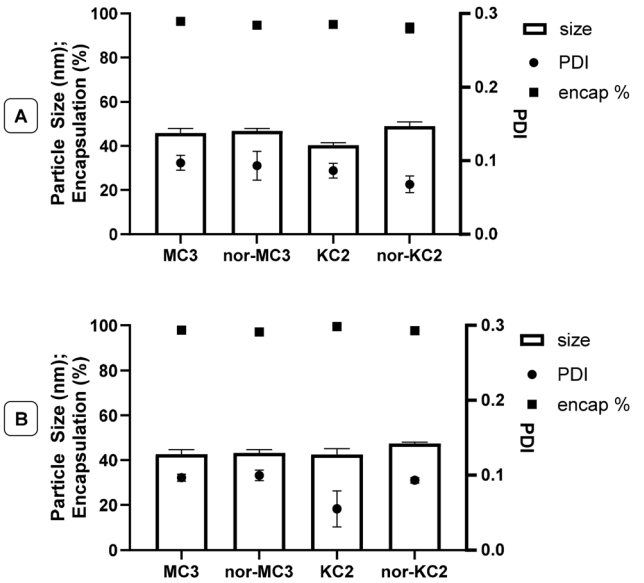

Fig. 4 Panel A: properties of LNP formulations of anti-firefly luciferase siRNA based on MC3, *nor*-MC3, KC2, and *nor*-KC2. Panel B: properties of LNP formulations of-firefly luciferase mRNA based on MC3, *nor*-MC3, KC2, and *nor*-KC2. See SI for details.

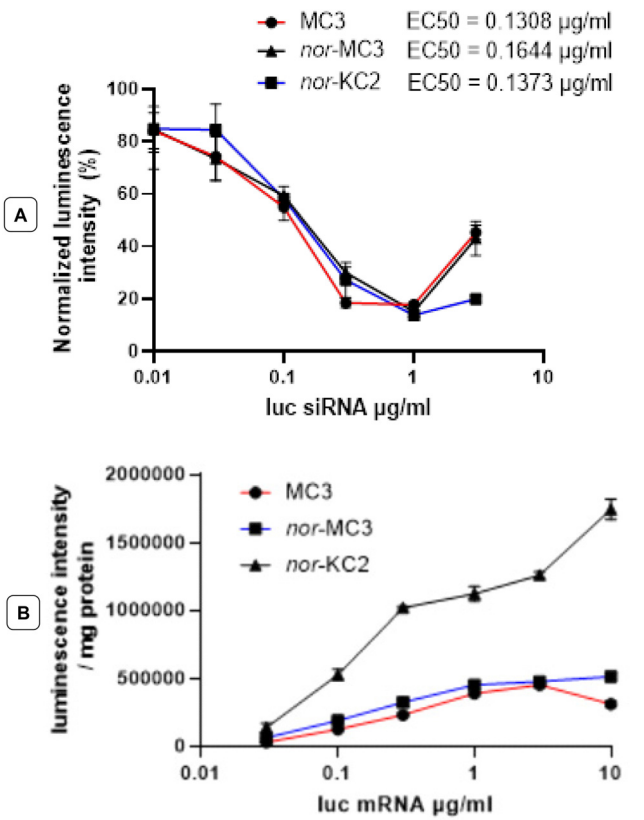

Fig. 5 Panel A: *in vitro* suppression of firefly luciferase activity with LNP formulations of siRNA based on MC3, *nor*-MC3, and *nor*-KC2. Panel B: *in vitro* expression of firefly luciferase with LNP formulations of mRNA based on MC3, *nor*-MC3, and *nor*-KC2. See SI for details.

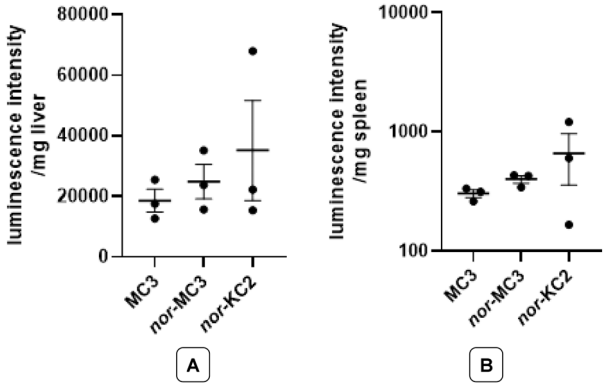

Fig. 6 Panel A: *in vivo* (mice) expression of firefly luciferase activity in the liver with LNP formulations of mRNA based on MC3, *nor*-MC3, and *nor*-KC2. Panel B: *in vivo* (mice) expression of firefly luciferase in the spleen with LNP formulations of mRNA based on MC3, *nor*-MC3, and *nor*-KC2. See SI for details.

efficacious, consistent with previous reports of delivery with KC2-containing formulations *in vitro* (Fig. 5).[12]

The difference in efficacy among the three lipids was attenuated *in vivo* (mice, Fig. 6). In all cases, no obvious adverse effects were observed in mice receiving formulations containing *nor*-MC3 or *nor*-KC2, suggesting that **1** and **2** probably are as safe as MC3. Formulations containing KC2 itself were excluded from *in vivo* studies, as MC3 is considered the gold standard for intravenous mRNA-LNP delivery and a clinically approved lipid.

The efficacy and safety of *nor*-MC3 in non-human primates was evaluated in *Macaca fascicularis* (cynomolgus monkeys) following injection of human erythropoietin (hEPO) mRNA-LNP at a dose of 0.4 mg kg^{-1}. High levels of plasma hEPO and no changes in hematology, clinical chemistry, or pro-inflammatory cytokine induction were observed compared to untreated control animals (Fig. 7). Due to the scale and ethical considerations of running studies in non-human primates, we chose to assess only *nor*-MC3, as MC3 has been reported on extensively.[13]

In summary, *nor*-MC3 and *nor*-KC2 show favorable *in vivo* delivery of nucleic acids relative to the benchmark MC3 and KC2 LNPs. Their chemical synthesis (4 steps from methyl linoleate in either case) is more concise than that of MC3 (5 steps from linoleyl alcohol[3] or 6 from methyl linoleate[3,11]) or KC2 (8 (ref. 5) or 5 (ref. 11) steps from linoleyl alcohol), relative to which it bypasses hazardous Grignard[14] and PCC oxidation[15] reactions, which are best avoided in pharmaceutical manufacturing. Furthermore, the present route to **1** and **2** affords synthetic intermediates and final compounds that are easier to purify. All this translates into significant economies in terms of reagents, solvents, chromatographic supports, operator time, and waste disposal costs.

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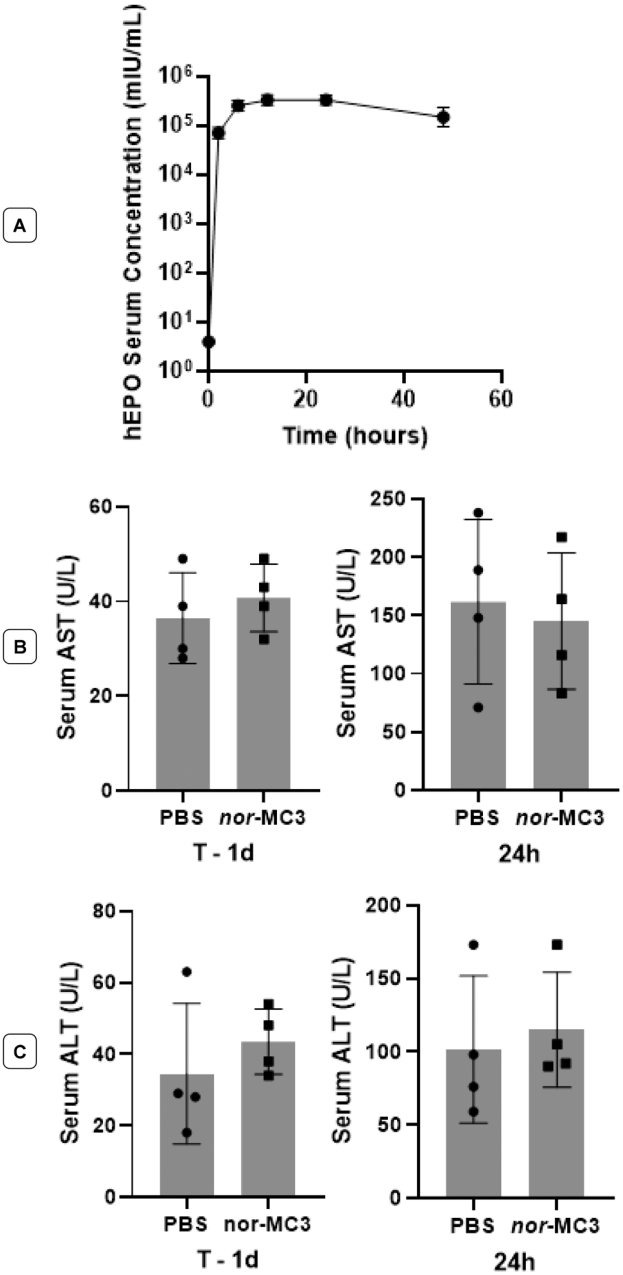

Fig. 7 *In vivo* expression of hEPO mRNA in cynomolgus monkeys. Panel A: hEPO serum concentration after delivery of 0.4 mg kg^{-1} hEPO mRNA-containing *nor*-MC3 LNPs, i.v. 20 min infusion, n = 4, mean ± SD. Panel B: serum alanine aminotransferase levels 24 hours post-dose, 0.4 mg kg^{-1} i.v. 20 min infusion, n = 4, mean ± SD, no statistical difference between PBS and *nor*-MC3. Panel C: serum aspartate aminotransferase levels 24 hours post-dose, 0.4 mg kg^{-1} i.v. 20 min infusion, n = 4, mean ± SD, no statistical difference between PBS and *nor*-MC3. See SI for details.

Author contributions

MAC conceived the *nor*-lipids. DW, PRC, JK conceived all biological work. DA, NDPA, FS, AN, TA, synthesized the compounds described herein and/or optimized the synthetic routes. DZK designed LNP formulations based on **1** and **2** and

directed their preparation. AT designed and directed the biological studies. GS acted as TA's academic supervisor.

Conflicts of interest

DW, PRC, JK, MAC are co-founders of NanoVation Therapeutics, Inc., and hold a financial interest in the company. DA was a full-time employee of NanoVation Therapeutics, Inc., during the conduct of the research described herein. NDPA, FS, AN, DZK, AT, DW, JK, MAC are full-time employees of NanoVation Therapeutics, Inc. TA, GS have no conflicts to declare.

Ethical statement

All murine procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals approved by the University of British Columbia Animal Care Committee. The cynomolgus monkey study was run by JOINN Laboratories Co. in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility using non-naïve cynomolgus monkeys of Asian origin. Animal Care was compliant with the SOPs of JOINN Laboratories (Suzhou) Co., Ltd, the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council; National Academy Press; Washington, D. C., 2010), and the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198). The sample sizes for experimental groups were chosen based on ethical principles to use the fewest number of animals possible, consistent with the study objective.

Data availability

Supplementary information (SI): procedures for the preparation, characterization, and evaluation of LNP formulations of nucleic acids; synthetic procedure, characterization data, and NMR spectra (^{1}H and ^{13}C) for all compounds. See DOI: https://doi.org/10.1039/d5pm00350d.

Additional data may be requested from the authors.

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