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Simplifying the Chemical Structure of Cationic Lipids for siRNA-Lipid Nanoparticles

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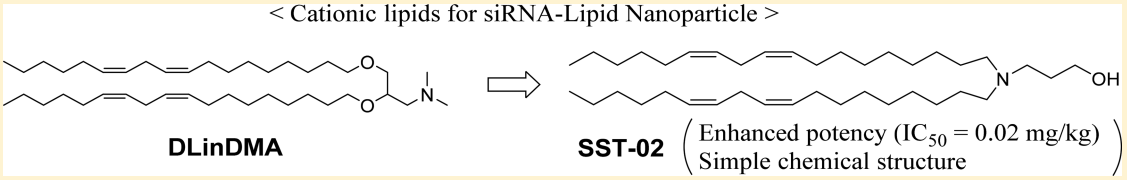
Supporting Information



ABSTRACT: We report a potent cationic lipid, SST-02 ((3-hydroxylpropyl)dilinoleylamine), which possesses a simple chemical structure and is synthesized just in one step. Cationic lipids are key components of siRNA-lipid nanoparticles (LNP), which may serve as potential therapeutic agents for various diseases. For a decade, chemists have given enhanced potency and new functions to cationic lipids along with structural complexity. In this study, we conducted a medicinal chemistry campaign pursuing chemical simplicity and found that even dilinoleylmethylamine (SST-01) and methylpalmitoleylamine could be used for the in vitro and in vivo siRNA delivery. Further optimization revealed that a hydroxyl group boosted potency, and SST-02 showed an ID_{50} of 0.02 mg/kg in the factor VII (FVII) model. Rats administered with 3 mg/kg of SST-02 LNP did not show changes in body weight, blood chemistry, or hematological parameters, while the AST level decreased at a dose of 5 mg/kg. The use of SST-02 avoids a lengthy synthetic route and may thus decrease the future cost of nucleic acid therapeutics.

KEYWORDS: *Cationic lipid, siRNA, lipid nanoparticle, LNP, drug-delivery system*

Short interfering RNAs (siRNAs) are 20−30 mer double-stranded RNAs[1,2] that form a complex with endogenous Ago2 protein in animal cells and effectively degrade the mRNAs of disease-relevant genes in a sequence-specific manner.[3] siRNAs can facilitate inhibition of biological processes that are neither targeted by small molecules nor antibodies; therefore, they are potentially useful for medicinal usage. However, due to their large molecular weight, high hydrophilicity, and enzymatic instability, in vivo delivery of siRNA into cells has been a major research topic since their discovery.

Lipid-mediated delivery vehicles such as siRNA-lipid nanoparticles (LNPs) are one of the most successful drug delivery systems for siRNA. LNPs are about 100 nm in size and are composed of siRNA, neutral lipid(s), PEG-lipid, and cationic lipid. Cationic lipids are protonated in acidic endosomes, increase the electric charge of nanoparticle surfaces, and induce siRNA migration into the cytosol. From a chemical perspective, cationic lipids used in LNPs generally have one "titrable amino group", two "aliphatic chains" (mostly unsaturated unbranched C18 hydrocarbons), and a "core" structure that connects them (Figure 1).

Since pioneering work on DLinDMA by researchers at Protiva[4] revealed that small alternation of cationic lipid chemical structures alters the whole nature of LNPs, many efforts have been made to optimize the chemical structure of

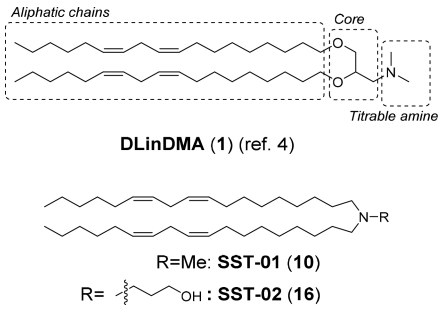


Figure 1. Structures of DLinDMA, SST-01, and SST-02.

cationic lipids (Figure S1). This "medicinal chemistry research" started with conversion of the dimethylamino moiety of DLinDMA to cyclic amines.[5] Delivery potency to the liver is boosted by DLin-KC2-DMA[6] and the U.S. Food and Drug Administration (FDA)-approved DLin-MC3-DMA,[7] wherein two aliphatic chains are connected via a carbon atom. YSK05 is an example of an acetal derivative that can be synthesized in one step.[8] A serine-derived cationic lipid is suitable for delivery to macrophages and dendritic cells.[9] Addition of a small

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portion of quaternary cationic lipids to LNPs is effective for targeting leukemia cells.[10] In some cases, asymmetric aliphatic chains work better than symmetric chains,[11] especially when one is short.[12,13] Cyclopropane is used as a bioisostere of a double bond in an unsaturated aliphatic chain.[13] Ester bonds introduced in the midst of the aliphatic chain enhance elimination from hepatocytes and thus avoid hepatotoxicity.[14,15] In this research history, medicinal chemists have provided cationic lipids with enhanced potency and new functions; however, structural complexity, lengthy-synthetic routes, and increased costs have become seemingly indispensable.

However, pursuit of the minimum components required for activity is also an important aspect of medicinal chemistry. In this research,[16] we started from DLinDMA, modified aliphatic chain and core, and successfully found the structurally simplest cationic lipid for siRNA-LNPs. We also boosted the delivery potency by adding one hydroxy functional group.

We first focused on the aliphatic chain. The chain of DLinDMA is a linoleyl ((*Z*, *Z*)-9, 12-octadecadien-1-yl) group with 18 carbons and 2 unsaturated bonds per chain. According to the literature,[4] linoleyl, compared to the one-less-unsaturated oleyl ((*Z*)-9-octadecen-1-yl) group, gives a lower melting temperature to the cationic lipid and decreases the phase-transition temperature of LNPs, resulting in potentiation of in vitro and in vivo transfection efficiency.

When designing new aliphatic chains, we considered avoiding the de novo chemical synthesis of lipid analogues because it may increase the costs and potential risks of drug metabolism. Among commercially available lipid derivatives, which are mainly derived from natural sources, we took particular note of the palmitoleyl ((*Z*)-9-hexadecen-1-yl) group. This aliphatic chain has the same unsaturation as oleyl but has two less carbon atoms (16 for palmitoleyl, 18 for oleyl). Therefore, we assumed that palmitoleyl has an effect similar to linoleyl. In fact, the melting temperatures of corresponding fatty acids is reported to be in the order of oleic acid (16.2 °C) > palmitoleic acid (0.5 °C) > linoleic acid (−6.5 °C).[17]

The synthesis of palmitoleyl congener of DLinDMA (**3**, or DPalDMA) was achieved following the method reported for DLinDMA,[4] utilizing palmitoleyl mesylate instead of linoleyl mesyate. (For the synthetic scheme, see Figure S2)

For the screening cascade, we considered that an in vitro assay was beneficial at least as the first screening before an in vivo assay. An LNP formulation incorporating ApoB-siRNA[18] for in vitro transfection experiments was prepared using a SNALP-like one-step method,[4] i.e., by mixing an ethanolic solution of lipids (cationic lipids, DSPC, cholesterol, and PEG-DMPE) and an aqueous solution of siRNA, followed by dilution and ultrafiltration. Serially diluted LNP solutions were added to HepG2 cells and after incubation for 24 h, and ApoB mRNA levels were determined using real-time quantitative polymerase chain reaction.

As shown in Table 1, consistent with the literature,[4] DLinDMA yielded better results compared to DODMA in our experiment even though we used a different PEG-lipid, siRNA, and component ratio. In addition, DPalDMA, a newly synthesized palmitoleyl congener, showed intermediate results (Table 1). These in vitro results show a good correlation with the melting temperatures of corresponding fatty acids.

ApoB-siRNA, when delivered to the liver, induces reduction of ApoB mRNA in hepatocytes and decreases the blood cholesterol level,[18] with 70 to 80% suppression as a peak. DLinDMA-LNP and DPalDMA-LNP were intravenously administered to Balb/c mice at 3 mg/kg, and the blood cholesterol levels were measured at 48 h after administration (Table 1). These in vivo results were parallel to those obtained in vitro; DPalDMA-LNP decreased blood cholesterol but to a lower extent compared to DLinDMA-LNP.

Table 1. In Vitro and in Vivo Activities of DLinDMA, DODMA, and DPalDMA LNPs[a]

cationic lipid	aliphatic chain	in vitro IC_{50} (95% C.I.)[b] [nM]	relative serum cholesterol level[c]
1 (DLinDMA)	linoleyl	0.653 (0.617−0.690)	52%
2 (DODMA)	oleyl	18.4 (17.7−19.0)	no data
3 (DPalDMA)	palmitoleyl	4.23 (4.00−4.47)	81%

[a]Each of LNPs were prepared using lipids **1**−**3** using a one-step method. ApoB-siRNA was incorporated. [b]95% C.I.; 95% confidence intervals. [c]Serum cholesterol levels relative to the saline-treated group. Dosed at 3 mg/kg.

Aiming to investigate further aspects of palmitoleyl, we compared the stability of DLinDMA and DPalDMA under UV irradiation. Thin-layer chromatography analysis showed that DLinDMA was decomposed almost completely on day 7, whereas DPalDMA showed superior stability (Figure S3). The reason for this is not completely clear; however, non-conjugated double bonds in DLinDMA may lead to photochemical instability, whereas DPalDMA has isolated double bonds. These experiments showed that the palmitoleyl group could provide increased delivery potency to cationic lipids compared to the oleyl group, and showed more stability to light compared to the linoleyl group.

Next, we proceeded to the bioisosteric conversion and simplification of the core. In DLinDMA, a 1,2-dihydroxypropane (PRO, Figure 2) unit can be regarded as the core,

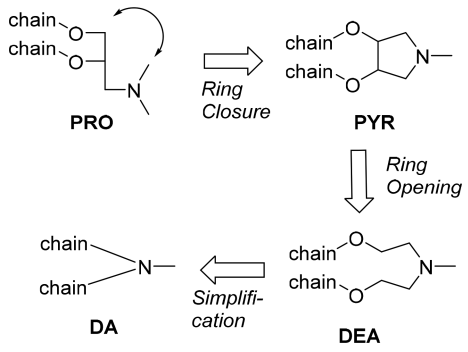

Figure 2. Design of "core" structures.

connecting its titrable amine (dimethylamino moiety) and aliphatic chains (two linoleyl moiety). First, we designed a 3,4-dihydroxypyrrolidine (PYR, Figure 2) core by connecting the *N*-methyl carbon and C1 of 1, 2-dihydroxypropane. This ring closure generated three possible stereoisomers and among them, *trans*-*R*,*R* isomer (*R*,*R*-**4**) gave the best in vitro result (Table 2). It is natural that stereoisomerism of cationic lipids affects the delivery potency of LNPs in a chiral environment of mixtures with other stereopure lipids and oligonucleotides.[19]

In the second step, the ring opening of PYR yielded a diethanolamine (DEA, Figure 2) core. An advantage of DEA is that it is achiral and, thus, simple. Interestingly, when the three

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Table 2. In Vitro and in Vivo Activities of LNPs Using Cationic Lipids with Various Cores and Aliphatic Chains[a]

no.	core	aliphatic chain	in vitro IC_{50} (95% C.I.)[b] [nM]	relative serum cholesterol level[c]
R,R-**4**	PYR	linoleyl	1.01 (0.963−1.07)	44%
cis-**4**	PYR	linoleyl	28.8 (27.9−29.6)	no data
S,S-**4**	PYR	linoleyl	5.67 (5.41−5.94)	no data
R,R-**5**	PYR	oleyl	16.4 (15.5−17.3)	no data
R,R-**6**	PYR	palmitoleyl	0.561 (0.538−0.586)	no data
7	DEA	linoleyl	25.4 (24.2−26.7)	86%
8	DEA	oleyl	0.922 (0.881−0.965)	64%
9	DEA	palmitoleyl	0.667 (0.632−0.703)	42%
10	DA	linoleyl	5.96 (5.24−6.69)	37%
11	DA	oleyl	7.30 (6.63−8.01)	82%
12	DA	palmitoleyl	0.839 (0.752−0.927)	42%

[a]Each of the LNPs were prepared using a one-step method. ApoB-siRNA was incorporated. [b]95% C.I.; 95% confidence intervals. [c]Serum cholesterol levels relative to saline-treated group. Dosed at 3 mg/kg.

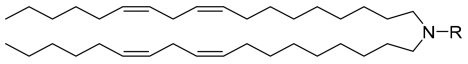
chains were attached to the DEA core, oleyl gave good result both in vitro and in vivo (Table 2). Although the reason for this inconsistency with the other "core" is unclear, some physical property of oleyl is better than that of the others, when combined with the DEA core.

In the last step, we deleted the ether functional group of DEA. This simplification resulted in di-"aliphatic chain"-amine compounds (DA, Figure 2). These can be regarded as the simplest compounds that satisfy the structural requisites of the cationic lipids in LNPs. However, to the best of our knowledge, there is no report of **10**−**12** being applied to oligonucleotide (ex. siRNA) delivery. In addition, linoleyl (**10**) and palmitoleyl (**12**) DA compounds gave better results (Table 2) compared to DLinDMA (**1**, Table 1) upon in vivo usage.

The ApoB-siRNA/blood cholesterol system has a poor dynamic range due to a plateau observed at high dose, whereas the FVII-siRNA/blood FVII protein system is reported to have a good dynamic range and is, therefore, used in many studies on LNPs.[6,7,13−15,20,21] We prepared FVII-siRNA incorporating LNPs using cationic lipid **10** in an one-step mixing process similar to the method described above. We then administered this formulation to mice and measured the FVII protein coagulation activity in blood after 48 h. At the dose of 0.3 mg/kg, 35% suppression was observed (Table 3), which was inferior to the reported potency (IC_{50} ≈ 0.03 mg/kg) of DLin-MC3-DMA.[7]

We next focused on the enhancement of delivery potency, under the condition of adding limited structural complexity.

Table 3. Effects of Various Substituents on the DA Core[a]



no.	R	relative serum factor VII protein level[b]
10	$-CH_3$	65%
13	$-CH_2CH(OH)CH_2OH$	91%
14	$-CH_2CH_2CO_2Et$	96%
15	$-CH_2CH_2CO_2NH_2$	95%
16	$-CH_2CH_2CH_2OH$	12%

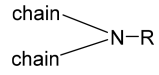
[a]Each of the LNPs were prepared using a one-step method. FVII-siRNA was incorporated. [b]Serum factor VII protein level relative to the saline-treated group. Dosed at 0.3 mg/kg.

We fixed the di-"aliphatic chain"-amine structure (DA core), and synthesized a series of dilinoleylamine derivatives with various substituents instead of the methyl group. FVII-siRNA incorporating LNPs were prepared in a one-step process using these cationic lipids and their in vivo delivery potency was measured. We found that addition of one hydroxyl group (**16**) resulted in drastic improvement (Table 3). One hypothesis for this phenomenon is that the hydroxyl group adds a hydrogen bonding interaction between the cationic lipid and siRNA. In addition, a more probable hypothesis is that a hydrophilic balance of hydroxyl and propylene groups plays an important role in the nanoparticle surface and/or cationic lipid-siRNA interaction because use of more hydrophilic **13** (two hydroxyl groups) resulted in a nearly complete loss of activity.

Formulation design is known to affect the in vivo delivery potency of LNPs. To enhance the potency of **16**-incorporating LNPs, we adopted a previously reported two-step mixing process, or Wrapsome (WS) process with minor adjustment in the amount of cationic lipid.[9,22,23] We prepared **16**-incorporating LNP using this two-step process and compared the in vivo delivery potency with that of **16**-incorporating LNP prepared using the one-step SNALP-like process described above. In the case of **16**, the former (relative serum factor VII protein level was 9%/21% for 0.1 and 0.03 mg/kg dose) showed much better results than the latter (35%/79%). Although the details are not clear, one cause may be the different average particle sizes arising from the different formulation processes. In this case, the two-step process generated slightly smaller (88 nm) particle than the one-step process (124 nm). Another cause, which likely is more dominant, may be the difference in the ratio of each lipid and the ratio of total lipid to siRNA. In this case, the LNP generated by the two-step process contained more cationic lipids and other lipids than the LNP generated by the one-step process.

Furthermore, we synthesized a series of cationic lipids in which the length between the nitrogen atom and oxygen atom in **16** was altered and the aliphatic chain were changed. LNPs incorporating these lipids and FVII-siRNA were prepared using the two-step WS process. As shown in Table 4, propylene linker (**16**) was found to be the best. Elongation of the linker (**18**, **19**) affected the potency, likely because of the high hydrophobicity. Regarding aliphatic chains, the palmitoleyl or oleyl groups reduced the delivery potency.

Table 4. Effects of N−O Linker Length and Aliphatic Chains[a]



no.	chain	-R group	relative serum factor VII protein level[b]
16	linoleyl	$-CH_2CH_2CH_2OH$	21%
17	linoleyl	$-CH_2CH_2OH$	25%
18	linoleyl	$-CH_2CH_2CH_2CH_2OH$	34%
19	linoleyl	$-CH_2CH_2CH_2CH_2CH_2OH$	65%
20	oleyl	$-CH_2CH_2CH_2OH$	82%
21	palmitolyel	$-CH_2CH_2CH_2OH$	66%

[a]Each of the LNPs were prepared using the two-step WS method. FVII-siRNA was incorporated. [b]Serum factor VII protein level relative to the saline-treated group. Dosed at 0.03 mg/kg.

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An independent experiment was conducted to calculate the IC_{50} value of **16**-LNP, which was 0.02 mg/kg (Table S1). To assess tolerability, **16**-LNP incorporating non-targeting control siRNA instead of FVII-siRNA was prepared in the same manner. For comparison, an LNP utilizing DLinDMA as a cationic lipid was also prepared with the same formulation design and process. Rats administered with 3 mg/kg of DLinDMA-LNP showed significant body weight loss, while no significant body weight loss was observed in the **16**-LNP treated groups even at 5 mg/kg dose (Figures S4). A dose of 3 mg/kg of DLinDMA-LNP resulted in a decrease in platelet and an increase in ALT and total bilirubin. In contrast, A dose of 3 mg/kg of **16**-LNP does not show these changes, and a decrease of AST was observed at 5 mg/kg dose (Table S2). Because rats are known to show lower tolerance to siRNA-LNPs than mice and humans,[24] these results suggest the favorable safety profile of **16**-LNP in humans.

In summary, we conducted a medicinal chemistry study pursuing structurally simpler cationic lipid(s) for siRNA-LNPs. Through the design and synthesis of one new "aliphatic chain" and three novel "core" structures, **10** (**SST-01**) and **12** were found to demonstrate good in vitro and in vivo effects. Although these showed inferior delivery potency, at least in delivery to the liver, compared with the cationic lipids described in latest literatures, we were satisfied with the simplest chemical structure of **10** and **12** among known cationic lipids.

Moreover, the addition of one more functional group (−OH group) into **10** yielded the surprisingly potent cationic lipid **16** (**SST-02**). This still has a simple chemical structure but showed a robust (IC_{50} ≈ 0.02 mg/kg) delivery potency to the liver, which is comparable to that of the FDA-approved DLin-MC3-DMA (IC_{50} ≈ 0.03 mg/kg).[7] Rats administered with 3 mg/kg of **16** (**SST-02**) LNP did not show changes in body weight, blood chemistry, or hematological parameters, while the AST level decreased at a dose of 5 mg/kg. Our results suggest the favorable safety and efficacy profile for application of **16** (**SST-02**) LNP.

Simple compounds can potentially be synthesized easily. In addition to the diversity-oriented medicinal chemistry route, we developed an efficiency-oriented synthetic route in which **16** (**SST-02**) can be easily synthesized from commercially available ethanolamine and linoleyl mesylate in just one step (Figure 3 and the Supporting Information).

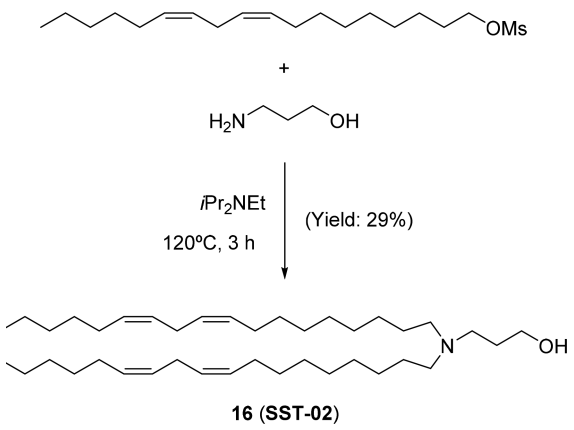

Figure 3. Synthesis of **16** (**SST-02**).

Among this series of cationic lipids, a mRNA LNP utilizing **17** was recently reported by Sabnis et al.,[25] while its potency was weaker than that of tritailed cationic lipids tailored for long single-stranded mRNA. Although more-detailed studies including pharmacology, toxicology, and manufacturing need to be conducted, these structurally simple cationic lipids may be helpful for decreasing the future costs of LNP-based nucleic acid therapeutics.[26]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00652.

Figures showing cationic lipids, a synthetic scheme, photostability analysis, and body weight change; tables showing dose−response curves and blood chemistry and hematological parameters; details on materials and methods; and ^{1}H-NMR charts (PDF)

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Author Contributions

The design and synthesis of cationic lipids were done by T.K., K.Y., T.Y., and Y.N. The design and preparation of LNP formulations were done by T.N. and N.Y. The in vitro and in vivo assay were done by F.S., H.Y., S.T., H.I., A.K.Y., and K.H. Manuscript preparation was done by T.K. The design of experiments and supervising of project were done by T.K. and A.M.

Notes

The authors declare the following competing financial interest(s): The authors are employees (for T.K., K.Y., T.N., Y.N., H.Y., F.S., H.I., A.K.Y., K.H., and A.M.) or ex-employees (for T.E., N.Y., and S.T.) of Kyowa Hakko Kirin, Co., Ltd.

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■ ABBREVIATIONS

ApoB, apolipoprotein B; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; FVII, coagulation factor VII; siRNA, short interfering RNA; linoleyl, (*Z*, *Z*)-9,12-octadecadien-1-yl; LNP, lipid nanoparticles; oleyl, (*Z*)-9-octadecen-1-yl; palmitoleyl, (*Z*)-9-hexadecen-1-yl; PEG-DMPE, 1,2-dimiristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(polyethylene glycol-2000).

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