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Ligand-Phospholipid Conjugation: a Versatile Strategy for Developing Long-Acting Ligands that Bind to Membrane Proteins by Restricting the Subcellular Localization of the Ligand

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Abstract: We hypothesized that if drug localization can be restricted to a particular subcellular domain where their target proteins reside, the drugs could bind to their target proteins without being metabolized and/or excreted, which would significantly extend the half-life of the corresponding drug-target complex. Thus, we designed ligand-phospholipid conjugates, in which the ligand is conjugated with a phospholipid through a polyethylene glycol linker, to restrict the subcellular localization of the ligand in the vicinity of the lipid bilayer. Here, we present the design, synthesis, pharmacological activity, and binding mode analysis of ligand-phospholipid conjugates with muscarinic acetylcholine receptors as the target proteins. These results demonstrate that ligand-phospholipid conjugation can be a versatile strategy for developing long-acting ligands that bind to membrane proteins in drug discovery.

Introduction

The pharmacological effects of drugs are generated by the drug-target complex, and the time-course is governed by both the drug pharmacokinetics, i.e., administration, distribution, metabolism, and excretion, and the pharmacodynamics, i.e., affinity and kinetics of the drug-target interactions.[1] The concentration of the drug-target complex must generally be maintained at a high enough level that the pharmacodynamic effects can continue to be generated throughout the treatment period.[1] Historically, drug candidates have been developed by focusing primarily on their target binding affinity, however, and therefore many of them have failed in pre-clinical and clinical trials due to their poor treatment efficacy due to an inadequate *in vivo* drug-target complex concentration, which is a major problem in drug discovery.[2] Here we present a ligand-phospholipid conjugation strategy to remarkably extend the half-life of the drug-target complex, which can be a useful approach for addressing this problem, allowing us to develop highly potent drugs with prolonged pharmacodynamic effects.

Although the optimization of the pharmacokinetic properties by chemical modification of the lead compounds is the primary approach to solving this problem,[3] extensive efforts are required on a case-by-case basis, and furthermore, a long plasma half-life is potentially promote off-target toxicity. Another approach is to use compounds that bind covalently to the target biomolecule[4] or compounds with a long target residence time.[1] Because the dissociation rate of these compounds is slow, the drug-target complex continues to be present even after the elimination of the free compounds from the systemic circulation. The development of covalent inhibitors, however, is applicable only in limited cases where the reactive amino acid residue of the targets is available,[4] and intentional optimization of the dissociation half-life is also quite difficult.[1c] Furthermore, the covalent modification or tight binding of drugs, which induces conformational change of the target biomolecules,

sometimes invokes immune responses, causing significant toxicity.[1a,4b,5] Thus, there is no versatile approach for addressing this problem and exhaustive case-by-case optimization of the pharmacokinetic properties is generally required, which significantly retards the drug discovery process.

In cellular systems, subcellular localization of biomacromolecules, such as proteins, is strictly regulated, which is of vital importance for their organized biological functions in the cell. We hypothesized that modifying a drug to induce its localization to a particular subcellular domain containing the target protein would result in the drug working for a longer time without being metabolized and/or excreted. Membrane proteins are one of the most important drug targets[6] including receptors,[7] transporters,[8] and channels,[9] which localize in the membrane by hydrophobic interactions with the lipid bilayer. Some membrane proteins are anchored to the membrane by conjugating with non-peptide hydrophobic anchors, such as glycosylphosphatidylinositol (GPI),[10] fatty acids, or isoprenoids.[11] Although a fatty acid or isoprenoid is not hydrophobic enough to stably anchor the conjugated proteins,[11d,12] a GPI anchor, whose phospholipid moiety is more hydrophobic than a fatty acid or isoprenoid, functions as a very stable anchor.[13]

Thus, we thought that effective membrane anchoring of small molecules would be possible by conjugating them to a phospholipid, and devised the ligand-phospholipid conjugation strategy as shown in Figure 1.[14] The ligand-phospholipid conjugates (LPCs) are expected to anchor the attached ligand to the membrane where they can avoid being metabolized and/or excreted, which will significantly extend the half-life of the ligand-target complex to prolong the duration of its pharmacodynamic effects. It has been reported that this kind of interaction between drugs and cell membrane often contribute to prolong ligand residence time relating to the clinical efficacy.[14] In this paper, we present the design, synthesis, pharmacological activity and binding mode analysis of

LPCs with muscarinic acetylcholine receptors as target proteins, demonstrating that the ligand-phospholipid conjugation strategy can be a versatile methodology for developing long-acting ligands that bind to membrane proteins due to restriction of the ligand subcellular localization in the vicinity of the lipid bilayer.

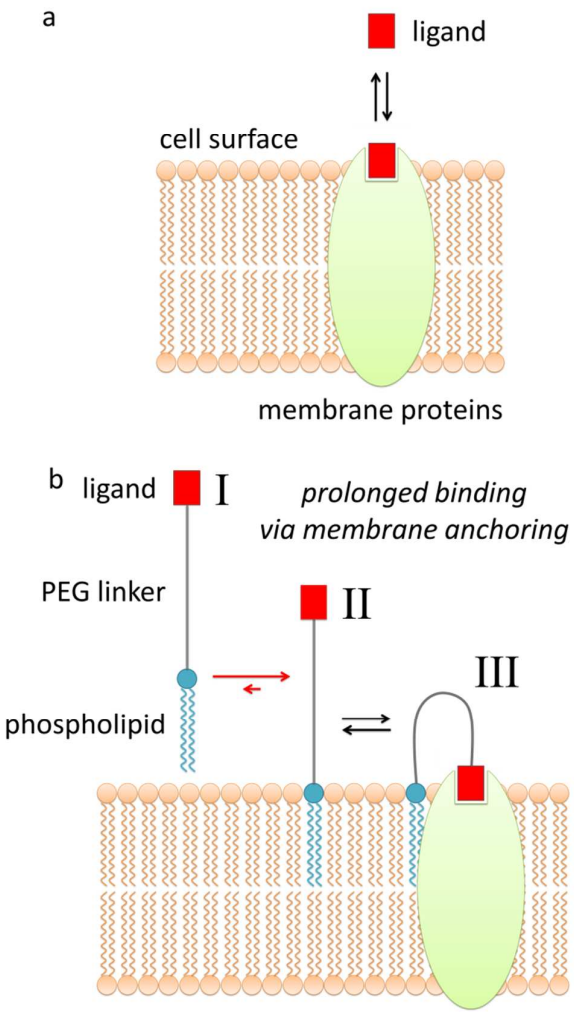


Figure 1. The ligand-phospholipid conjugation strategy for developing ligands with prolonged activity due to restriction of the ligand localization: (a) a common ligand interacting with its target membrane protein; (b) a ligand conjugated with phospholipid through a polyethylene glycol (PEG) linker.

Results and Discussion

Design of LPCs. We designed the ligand-phospholipid conjugates (LPCs), in which a ligand is associated with a phospholipid through a PEG linker as shown in Figure 2, to develop a versatile methodology, which is generally and easily applicable for various ligands that bind to target membrane proteins. A variety of LPCs could be readily synthesized by amide coupling of ligands bearing a linker moiety with carboxylic acid to the PL-PEG units with a terminal amino group, which could be prepared using a phospholipase D from *Streptomyces* sp. (PLDP) catalyzed transphosphatidylation method that we previously developed.[15] In the LPCs, the hydrophobic region of the phospholipid moiety functions as an effective anchor to the membrane, and a hydrophilic and flexible polyethylene glycol (PEG)[16] linker allows for binding of the attached ligand to the target membrane protein.

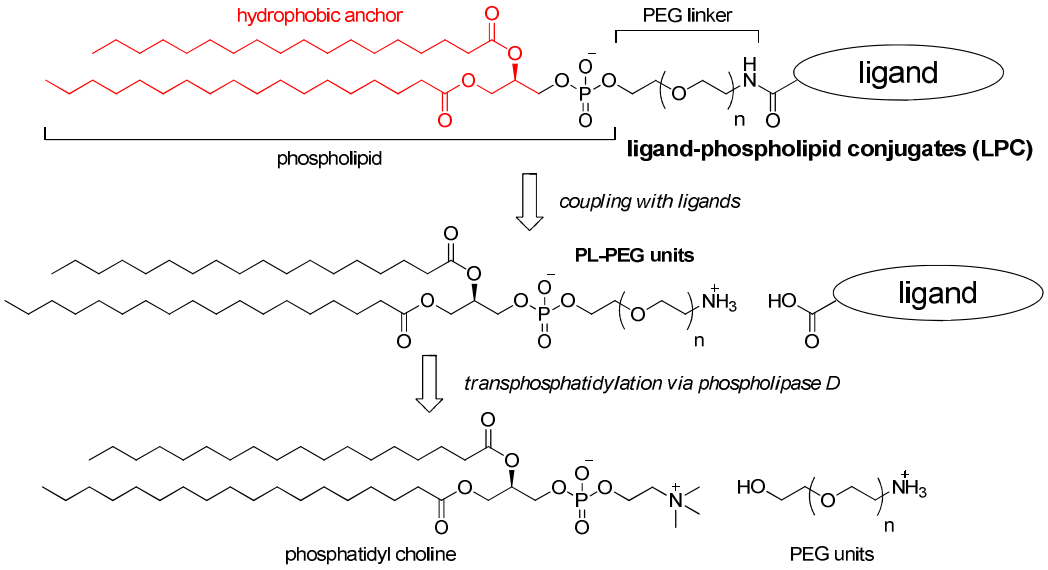
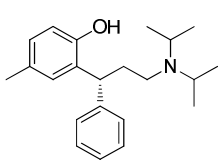


Figure 2. Design and synthetic strategy of LPCs

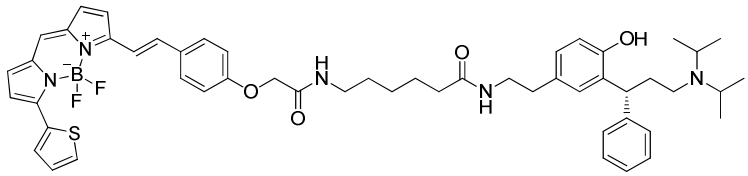
We selected the muscarinic acetylcholine receptor as a target membrane protein to demonstrate the

effectiveness of our strategy, because the X-ray crystal structures of the M2 and M3 receptors have been solved, making the rational structure-based drug design (SBDD) possible.[17] Furthermore, the highly potent antagonist with a large fluorescent group was previously reported, enabling the rational ligand-based drug design (LBDD).[18] As summarized in Table 1, the fluorescent analogue **1** derived from the muscarinic receptor antagonist tolterodine, a clinical drug used in the treatment of overactive bladder,[19] has significant binding affinity to muscarinic receptors, comparable to tolterodine,[18] suggesting that introduction of the PL (phospholipid) -PEG units to tolterodine would be achieved without a significant loss of binding affinity to the receptors.

Table 1. Binding affinity of tolterodine and its fluorescent derivative **1** to muscarinic receptors reported by Jones and co-workers.[17]



tolterodine



1

compound	K_i [nM]				
	M1	M2	M3	M4	M5
tolterodine	1.4	2.7	3.6	3.1	2.2
1	4.6	9.0	10.3	6.3	17.5

To design LPCs targeting muscarinic receptors, we performed a docking simulation of compound **2**, which is a partial structure of fluorescent compound **1** without the fluorescent group, with the X-ray analyzed structure of the

M3 muscarinic receptor.[17b] As shown in Figure 3, the terminal structure of compound **2** shown in red is apparently exposed to the solvent at the receptor surface, indicating that the terminal moiety can be modified without significantly decreasing the binding affinity. Thus, we designed a series of LPCs **3**-**8** targeting muscarinic receptors by conjugating tolterodine with a phospholipid through various lengths of PEG linkers, as shown in Figure 4. The impact of the PEG-linker length on the pharmacological activity of LPCs would be investigated by evaluating these compounds.

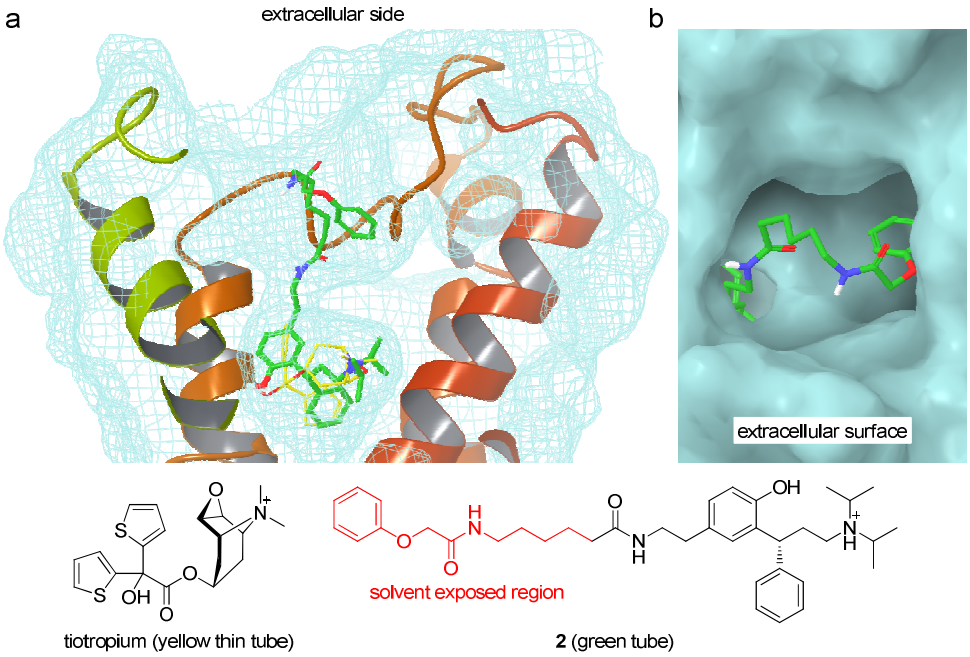


Figure 3. Plausible binding mode of compound **2** to the M3 muscarinic receptor predicted by a docking simulation: green tube, the predicted binding mode of **2**; yellow thin tube, the X-ray analyzed binding mode of tiotropium to the M3 receptor reported by Kruse and co-workers (PDB code, 4DAJ);[17b] a solvent accessible surface of the M3 receptor is shown in mesh (a) or solid (b).

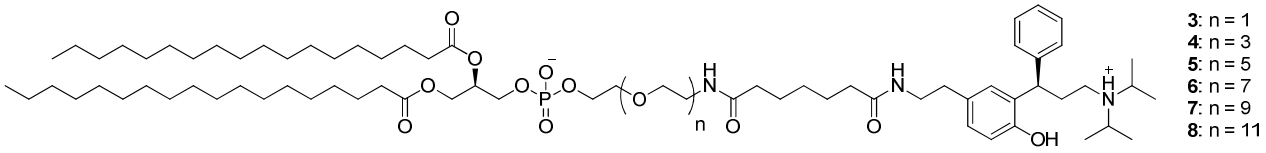
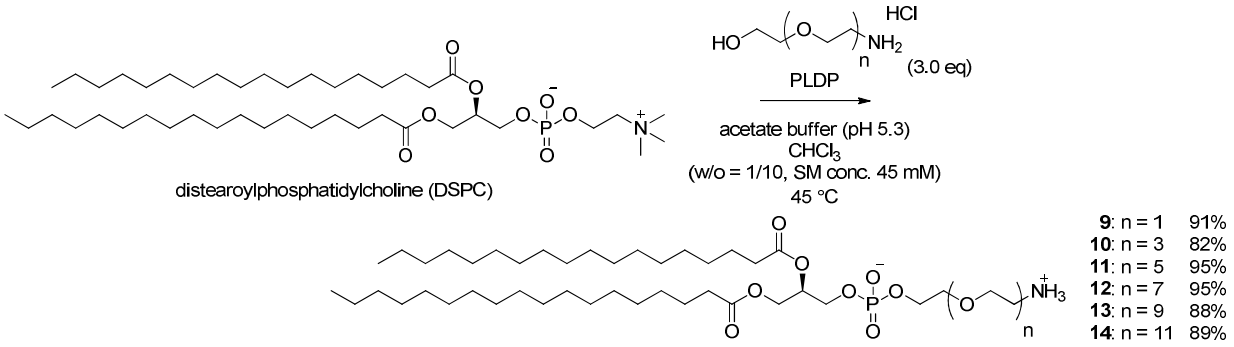


Figure 4. The designed LPCs targeting muscarinic receptors

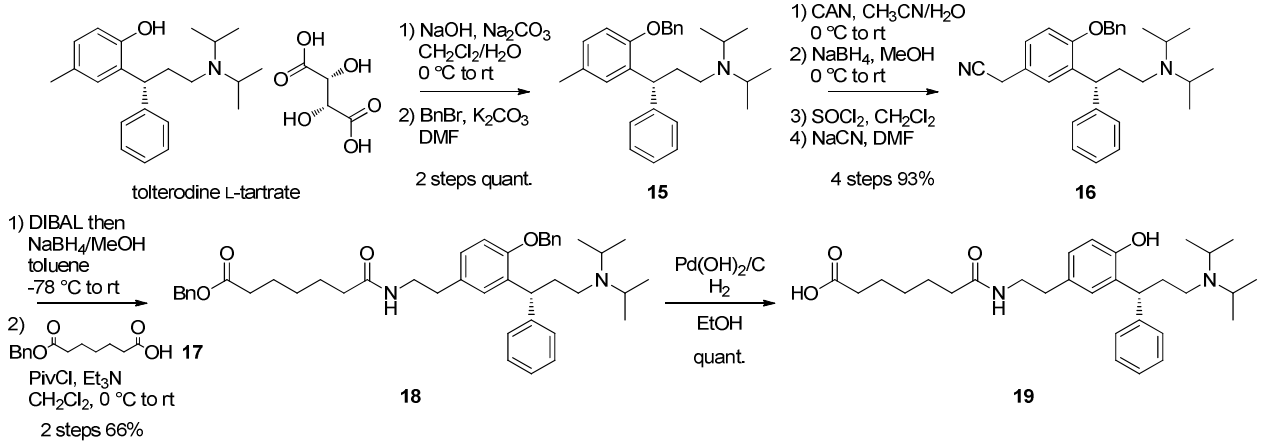
Synthesis of the PL-PEG units and LPCs. The designed PL-PEG units **9-14** were synthesized by PLDP (from *Streptomyces* sp.) catalyzed transphosphatidylation which is previously developed by us,[15] as shown in Scheme 1. The transphosphatidylation is an enzymatic phosphatidyl group transfer reaction with a two-phase system of $CHCl_3$-acetate buffer that effectively provides a variety of phospholipid derivatives from a phosphatidylcholine (phosphatidyl donor) and an alkanol (phosphatidyl acceptor).[15] The initial attempt using free amine PEG units as phosphatidyl acceptors afforded almost none of the transphosphatidylation products and most of the distearoylphosphatidylcholine (DSPC) remained intact, suggesting that the PLDP was denatured under the alkaline conditions due to the basic acceptors. Therefore, we used hydrochloride salts of the PEG units as acceptors, which gave the desired PL-PEG units **9-14** as transphosphatidylation products in excellent yields. Thus, we efficiently synthesized a series of the key PL-PEG units that could be generally used for the synthesis of various LPCs, by the PLDP-catalyzed transphosphatidylation.[15]

Scheme 1. Synthesis of PL-PEG units **9-14** by phospholipase D (PLDP) catalyzed transphosphatidylation



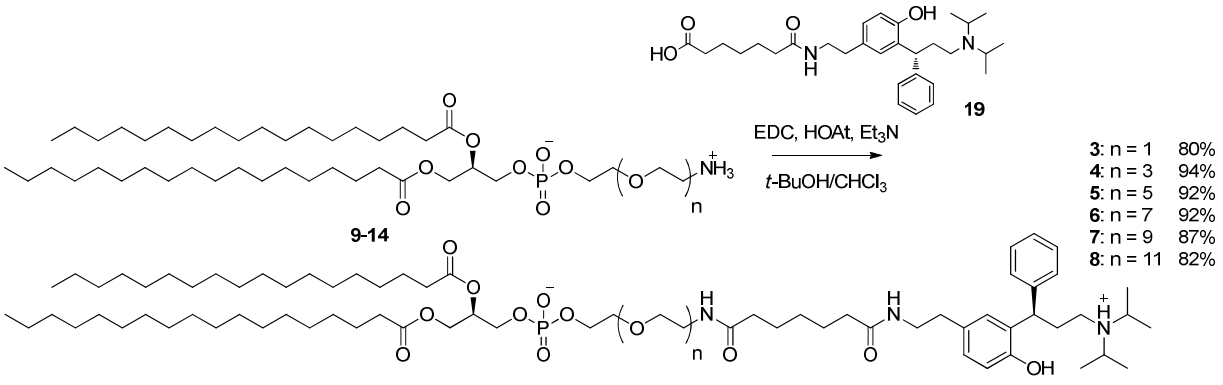
The synthesis of the tolterodine unit **19** is shown in Scheme 2. The phenolic hydroxyl group of tolterodine was protected by a benzyl group to yield compound **15**. The benzylic methyl group of **15** was oxidized with ceric ammonium nitrate (CAN) to yield the corresponding aldehyde, which was subsequently reduced to alcohol with $NaBH_4$ in methanol. The resulting alcohol was successively treated with $SOCl_2$ and NaCN to yield nitrile **16**. The cyano group of **16** was reduced with DIBAL, and the resulting amino group was condensed with carboxylic acid **17** by the mixed anhydride method to afford compound **18**. The two benzyl groups of **18** were simultaneously removed by hydrogenolysis to yield the tolterodine unit **19**.

Scheme 2. Synthesis of tolterodine unit **19**



Finally, the tolterodine unit **19** was condensed with each of the PL-PEG units **9-14**, as shown in Scheme 3. Although tolterodine unit **19** was almost insoluble in $CHCl_3$, a mixed solvent of $CHCl_3$ and *t*-BuOH effectively dissolved it for use in the reaction. The condensation effectively proceeded to afford a series of designed LPCs **3-8** in high yields. These results suggested that various ligands bearing a linker moiety with a carboxyl group can be generally and readily derivatized into the corresponding LPCs by condensation with the key PL-PEG units.

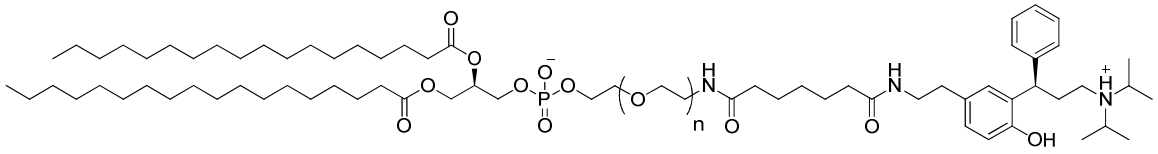
Scheme 3. Synthesis of the designed LPCs **3-8** targeting muscarinic acetylcholine receptors



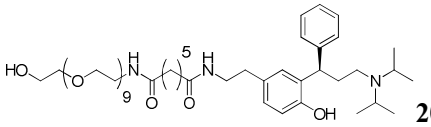
Pharmacological effects of the LPCs targeting muscarinic receptors. The binding affinity of the synthesized LPCs **3-8** to muscarinic receptors in rat brain homogenates was investigated using [^{3}H] *N*-methyl scopolamine.[20] As summarized in Table 2, the binding affinity of the LPCs to the muscarinic receptors was significantly affected by the PEG-linker length: as the PEG-linker length increased from n = 1 to n = 9 (LPCs **3-7**), the binding affinity clearly increased. The LPCs would be initially incorporated into the membrane by the strong but non-specific hydrophobic interactions between the phospholipid moiety and the membrane to anchor the attached ligand to the

membrane (Figure 1b, II). Thereafter, lateral diffusion of the anchor moiety in the membrane would effectively facilitate the attached ligand to interact with its target protein (Figure 1b, III). Therefore, if the PEG-linker was not long enough, the hydrophobic region of the phospholipid moiety would have to protrude from the membrane to place the attached ligand into the binding pocket of the target protein, which would impair the binding affinity, as clearly shown in LPC **3** (n = 1). On the other hand, LPC **8** (n = 11) showed almost the same binding affinity as LPC **7** (n = 9), suggesting that when the PEG-linker is longer than the optimal length, the impact of the linker length on the binding affinity is insignificant probably due to the highly flexible nature of the PEG-linker.[16b] Thus, in the design of LPCs, using a long PEG-linker might effectively provide potent LPCs without requiring the optimization of the linker length. We also synthesized PEG-tolterodine **20**, having the PEG linker but not the phospholipid moiety, which showed a binding affinity slightly lower than tolterodine itself, probably because of the introduced PEG-linker moiety. Importantly, LPC **7** with the same length of PEG linker as **20**, had a binding affinity much higher than **20**, and even higher than tolterodine itself, suggesting that the membrane anchoring effectively improved the binding affinity by localizing the attached ligand close to its target receptor in the membrane.x

Table 2. Binding affinity of the LPCs **3**-**8**, PEG-tolterodine **20** and tolterodine to muscarinic receptors in rat brain homogenates using [^{3}H] *N*-methyl scopolamine



compound	n	log IC_{50}[a]	[^{3}H] *N*-methyl scopolamine binding

			(% of control)[a]		
			no wash	wash × 1	wash × 2
3	1	-6.91 ± 0.065	66.4 ± 2.1	59.2 ± 2.4	
4	3	-7.74 ± 0.004	32.3 ± 3.4	15.5 ± 2.0	
5	5	-7.86 ± 0.036	14.5 ± 4.5	5.1 ± 1.1	
6	7	-8.15 ± 0.043	4.7 ± 0.6	2.1 ± 0.3	
7	9	-8.53 ± 0.076	6.0 ± 2.2	1.7 ± 0.6	2.1 ± 0.3
8	11	-8.49 ± 0.074	11.2 ± 4.4	1.9 ± 0.8	
tolterodine		-8.20 ± 0.018	9.0 ± 0.8	36.2 ± 1.7	93.2 ± 3.8
 **20**		-7.88 ± 0.082	7.1 ± 0.9	34.9 ± 0.8	63.5 ± 4.9

[a]Based on at least four experiments

Next, we conducted wash-out experiments to investigate the duration of the compound binding to the muscarinic receptors. Rat brain homogenates containing muscarinic receptors were incubated with 100 nM LPCs **3**-**8**, tolterodine, or PEG-tolterodine **20** at 25 °C, and [^{3}H] *N*-methyl scopolamine[20] binding to the muscarinic receptors was measured before and after wash-out with a ice-cold buffer solution. As shown in Figure 5, none of the LPCs showed any decrease in binding after wash-out regardless of the extent of their binding affinity, suggesting that the duration of the binding was solely due to the membrane anchoring. In contrast, the binding of tolterodine and PEG-tolterodine **20** decreased drastically after wash-out, showing drastic effects of the membrane

anchoring to extend the receptor binding period of the anchored ligand. Interestingly, all LPCs showed increased binding affinity to the receptors after the first wash-out compared with those before the wash-out, suggesting that the LPCs bind to the receptors in a time-dependent manner after anchoring into the membrane (Figure 1b, II to III). Thus, the designed LPCs had potent binding affinity to the muscarinic receptors, and importantly, the binding was not attenuated even after the wash-out, which was in stark contrast with the drastic attenuation in the parent compound tolterodine, as expected.[21]

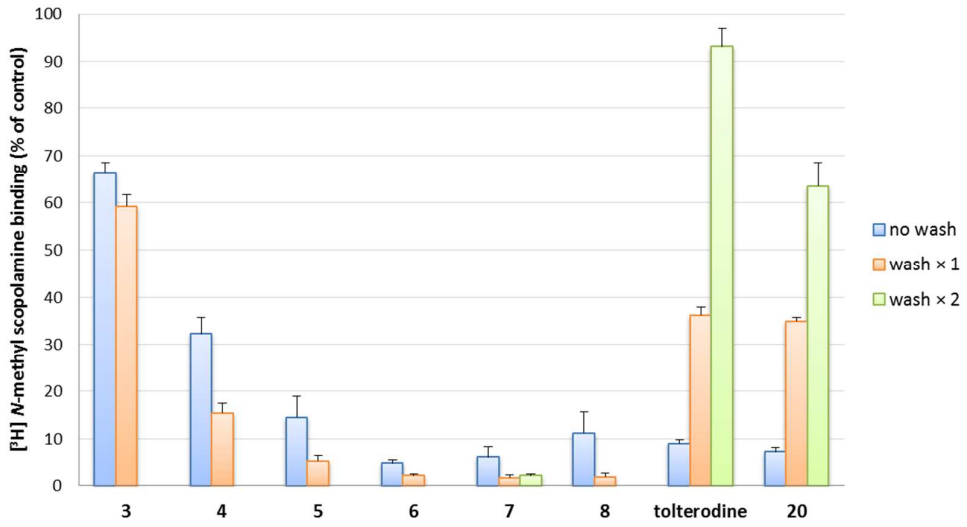


Figure 5. [^{3}H] *N*-methyl scopolamine binding to muscarinic receptors treated with LPCs **3**-**8**, PEG-tolterodine **20** and tolterodine before and after wash-out. Data are Based on at least four experiments.

Finally, we investigated the pharmacological effects of LPC **7** *in vivo*. Tolterodine or LPC **7** was intravesically instilled to rats, and [^{3}H] *N*-methyl scopolamine[20] binding to the muscarinic receptors in the bladder was directly measured by dissecting the bladder 30 min or 24 h after the instillation to calculate the apparent K_d value ($K_{d\mathrm{app}}$). At 30 min after the instillation, both tolterodine and LPC **7** increased the $K_{d\mathrm{app}}$ value of the [^{3}H] *N*-methyl

scopolamine to a similar extent, showing that both of them effectively bound to the muscarinic receptors *in vivo*. Importantly, the $K_{d\text{app}}$ value of [^{3}H] *N*-methyl scopolamine was not significantly decreased even after 24 h in rats treated with LPC **7**, while the value was significantly decreased after 24 h in rats treated with tolterodine. To the extent that an increase in $K_{d\text{app}}$ values for radioligands in drug-pretreated tissues in the radioreceptor assay refers generally to competition with the radioligand for same binding sites.[22] Therefore, these findings indicate that LPC **7** exhibits long-term binding to muscarinic receptors even *in vivo*, compared with its parent compound tolterodine.

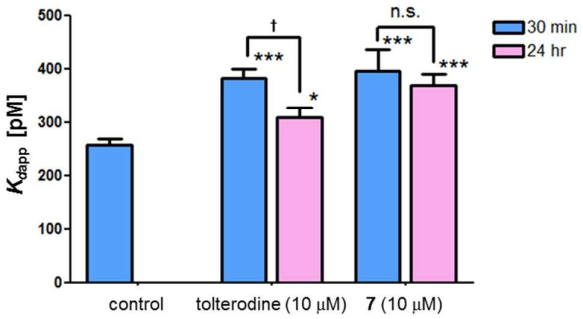


Figure 6. $K_{d\text{app}}$ value of [^{3}H] *N*-methyl scopolamine binding to the muscarinic receptors in the bladder of rats treated with tolterodine or LPC **7**. The $K_{d\text{app}}$ value was directly measured by dissecting the bladder 30 min or 24 h after the intravesical instillation of the compounds. *$p < 0.05$, ***$p < 0.001$ vs control; $^{\dagger}p < 0.05$ vs tolterodine (30 min), n.s., not significant, n = 4.

Molecular dynamics analysis of the binding mode of LPCs. The binding mode of LPC **3** and **7** were analyzed by molecular dynamics (MD) simulations using the X-ray structure of the M3 muscarinic receptor[17b] (see

supporting information for details). The z-coordinates of the phosphorous atom and ω-carbons of the phospholipid moiety during the simulation (time step = 0.1 ns, 1000 steps) were plotted for LPC **3** (n = 1) and **7** (n = 9) as shown in Figure 7a, and the snapshots of the simulations at 20, 50 and 100 ns, along with their initial model, are shown in Figure 7b (LPC **3**, n = 1) and 7c (LPC **7**, n = 9), respectively. Both of the systems appeared to reach equilibrium within 50 ns, and after that, the z-coordinates of the ω-carbons of LPC **3** ($C1_{n1}$ and $C2_{n1}$) were around 20 – 30 Å, while those of LPC **7** ($C1_{n9}$ and $C2_{n9}$) were around -5 – 5 Å. This clearly shows that the phospholipid moiety of the LPC **3** protrudes from the lipid bilayer to have the attached ligand moiety interact with the muscarinic receptor (Figure 7b), which will significantly deteriorate its binding affinity (Table 2: tolterodine, log IC_{50} = -8.20; LPC **3**, log IC_{50} = -6.91). On the other hand, the phospholipid moiety of LPC **7** is well embedded in the lipid bilayer even when the ligand moiety interacts with the muscarinic receptor (Figure 7c), which will facilitate the ligand binding rather than disrupt it by working just like a "bivalent" ligand (Table 2: tolterodine, log IC_{50} = -8.20; LPC **7**, log IC_{50} = -8.53).[16b]

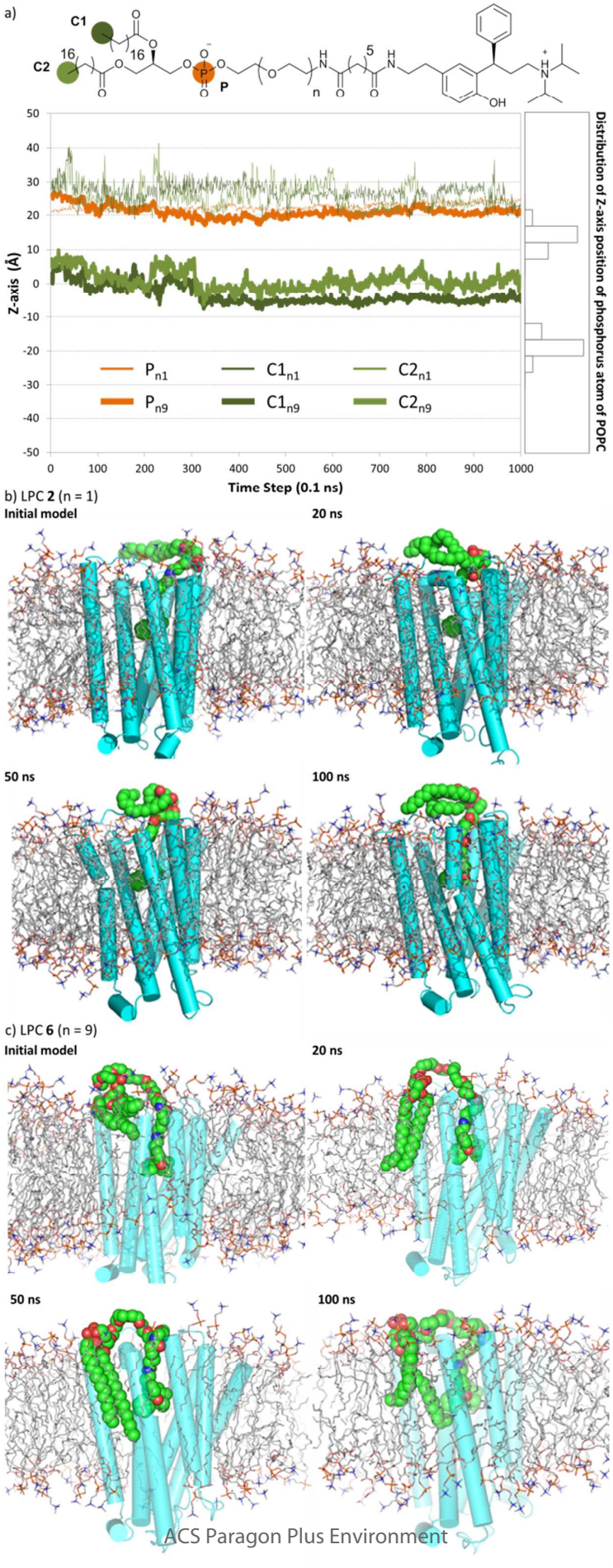


Figure 7. Design of LPCs targeting muscarinic receptors: (a) structures of tolterodine, tiotropium and compound 1; (b) and (c) plausible binding mode of compound 1 to the M3 muscarinic receptor predicted by a docking simulation (green tube, the predicted binding mode of 1; yellow thin tube, the X-ray analyzed binding mode of tiotropium to the M3 receptor reported by Kruse and co-workers (PDB code, 4DAJ);[10] a solvent accessible surface of the M3 receptor is shown in mesh (b) or solid (c)); (d) structures of the designed LPCs **2**-**7**.

Conclusions

We successfully developed a ligand-phospholipid conjugation strategy to develop long-acting ligands that bind to membrane proteins due to restriction of the subcellular localization of the conjugated ligands in the vicinity of the lipid bilayer. The designed and synthesized LPCs had significantly prolonged target binding not only *in vitro* but also *in vivo*, compared with their parent compound, suggesting that this strategy is practical for actual drug discovery research. In principle, any type of ligands can be anchored to the outer-leaflet of the lipid bilayer by derivatizing them into the corresponding LPCs, which, except for the intravesically instillation, may be used as inhalation and ointment drugs. Furthermore, LPCs can be synthesized by a simple amide coupling between ligands and the designed key PL-PEG units, which were efficiently synthesized by PLDP-catalyzed transphosphatidylation, which we previously developed,[15] showing that this is a versatile and readily available strategy. Importantly, although phospholipid conjugates of proteins or small molecules have been reported to date, which includes antibody- or small molecule-phospholipid conjugates for liposome targeting or GPI-anchored proteins or peptides expressed by genetic modification,[22] none of the phospholipid conjugates that restrict the subcellular localization of the ligand for developing long-acting ligands was known. Thus, this newly developed

ligand-phospholipid conjugation strategy based on the totally new concept "restricting the ligand subcellular localization" will be a widely applicable methodology for developing long-acting drug candidates that target membrane proteins.

EXPERIMENTAL SECTION

General methods and materials

^{1}H-NMR spectra were recorded in $CDCl_3$ at ambient temperature unless otherwise noted, at 400 or 500 MHz, with TMS as an internal standard. ^{13}C NMR spectra were recorded in $CDCl_3$ at ambient temperature unless otherwise noted, at 100 or 125 MHz. Silica gel column chromatography was performed with silica gel 60 N (spherical, neutral, 63-210 μm, Kanto Chemical Co., Inc.). Flash column chromatography was performed with silica gel 60 N (spherical, neutral, 40-50 μm, Kanto Chemical Co., Inc.). Celite 545 was purchased from Kanto Chemical Co., Inc. Pd/C (PE type) was purchased from N.E. Chemcat Co. $Pd(OH_2)/C$ was purchased from Tokyo Chemical Industry Co., Ltd. [*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 3.03 TBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Combustion analysis was performed to confirm ≥95% sample purity (within ±0.4% of the calculated value).

General Procedure for the Preparation of LPCs 3-8. To a solution of tolterodine unit **19** (20.9 mg, 0.0420 mmol, 1.0 equiv) in a mixture of $CHCl_3$ and *t*-BuOH (1.25 ml, 3:2) was added HOAt (6.86 mg, 0.0504 mmol, 1.2 equiv) and EDC·HCl (9.66 mg, 0.0504 mmol, 1.2 equiv). After 5 min, $CHCl_3$ (750 μl), triethylamine (7.02 μl, 0.0504 mmol, 1.2 equiv) and the PL-PEG unit (0.0420 mmol, 1.0 equiv) were added and the resulting

mixture was stirred until consumption of the PL-PEG unit was confirmed by TLC analysis. The reaction mixture was diluted with a mixture of $CHCl_3$ and MeOH (2:1) and the resulting mixture was washed twice with 1 N HCl. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography ($CHCl_3$/ MeOH 20:1-9:1) and the fractions containing desired product were concentrated *in vacuo*. The resulting residue was dissolved in a mixture of $CHCl_3$ and MeOH (2:1) and washed with 1 N HCl and water. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was further purified by trituration with *n*-hexane and subsequently lyophilized from water to yield the corresponding LPC as a white amorphous solid.

Preparation and Spectral and Analytical Data of LPC 3. LPC **3** (42.7 mg, 0.0336 mmol, 80%) was prepared from PL-PEG unit **9**. $[\alpha]^{22}_{D}$ -3.86 (*c* 0.56, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.60 (br, 1H, amide NH), 7.53 (br, 1H, amide NH), 7.36 (d, *J* = 7.6 Hz, 2H, aromatic), 7.29 (dd, *J* = 7.6, 7.6 Hz, 2H, aromatic), 7.20 (t, *J* = 7.6 Hz, 1H, aromatic), 7.05 (s, 1H, aromatic), 6.88 (d, *J* = 7.6 Hz, 1H, aromatic), 6.73 (d, *J* = 7.6 Hz, 1H, aromatic), 5.26-5.16 (m, 1H, glycerol CH), 4.38 (dd, *J* = 12.4, 3.8 Hz, 1H, glycerol CH_2), 4.31 (dd, *J* = 7.6, 7.6 Hz, 1H, benzyl CH), 4.16 (dd, *J* = 12.4, 6.7 Hz, 1H, glycerol CH_2), 4.05-3.92 (m, 4H, $POCH_2$ and glycerol CH_2), 3.65-3.58 (m, 2H, OCH_2), 3.58-3.47 (m, 4H, OCH_2 and NCH), 3.47-3.30 (m, 4H, $CONHC\underline{H}_2$), 2.97-2.79 (m, 2H, NCH_2), 2.69 (t, *J* = 6.7 Hz, 2H, benzyl CH_2), 2.65-2.46 (m, 2H, $NCH_2C\underline{H}_2$), 2.35-2.21 (m, 4H, $COCH_2$), 2.17-2.02 (m, 4H, $NHCOC\underline{H}_2$), 1.66-1.39 (m, 8H, $NHCOCH_2C\underline{H}_2$ and $COCH_2C\underline{H}_2$), 1.34-1.18 (m, 68H, CH_2 and isopropyl CH_3), 1.18-1.07 (m, 2H, CH_2), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.5, 174.2, 173.6, 173.2, 152.5, 142.4, 130.1, 129.3, 128.3, 127.8, 126.4, 115.4, 70.2 (d, $J_{C\text{-}P}$ = 6.7 Hz), 70.1 (d, $J_{C\text{-}P}$ = 4.8 Hz), 69.1, 64.5 (d, $J_{C\text{-}P}$ = 5.7 Hz),

63.1 (d, $J_{C\text{-}P}$ = 4.8 Hz), 62.4, 54.3, 54.3, 46.2, 41.7, 40.2, 39.0, 35.6, 35.5, 34.1, 34.0, 33.8, 31.6, 29.4, 29.4, 29.3, 29.1, 28.9, 28.8, 27.6, 24.9, 24.8, 24.6, 24.6, 22.4, 18.1, 18.0, 16.5, 16.3, 13.7; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.08; LRMS (ESI) *m/z* 1292.91 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{73}H_{128}N_3O_{12}PNa$: 1292.9128 [$(M+Na)^+$], found: 1292.9099; Anal. calcd for $C_{73}H_{128}N_3O_{12}P\cdot 3.5H_2O$: C, 65.73; H, 10.20; N, 3.15. Found: C, 65.86; H, 9.84; N, 3.13.

Preparation and Spectral and Analytical Data of LPC 4. LPC **4** (53.6 mg, 0.0394 mmol, 94%) was prepared from PL-PEG unit **10**. $[\alpha]^{20}_D$ -0.15 (*c* 0.63, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.51 (br, 1H, amide NH), 7.38 (br, 1H, amide NH), 7.36 (d, *J* = 7.6 Hz, 2H, aromatic), 7.30 (dd, *J* = 7.6 Hz, 2H, aromatic), 7.20 (t, *J* = 7.6 Hz, 1H, aromatic), 7.03 (s, 1H, aromatic), 6.87 (d, *J* = 7.6 Hz, 1H, aromatic), 6.73 (d, *J* = 7.6 Hz, 1H, aromatic), 5.25-5.17 (m, 1H, glycerol CH), 4.38 (dd, *J* = 11.5, 2.9 Hz, 1H, glycerol CH_2), 4.31 (t, *J* = 6.7 Hz, 1H, benzyl CH), 4.19-4.12 (m, 1H, glycerol CH_2), 4.03-3.90 (m, 4H, $POCH_2$ and glycerol CH_2), 3.70-3.58 (m, 10H, OCH_2), 3.58-3.47 (m, 4H, OCH_2 and NCH), 3.43-3.32 (m, 4H, $CONHC\underline{H}_2$), 2.95-2.74 (m, 2H, NCH_2), 2.68-2.60 (m, 2H, benzyl CH_2), 2.60-2.47 (m, 2H, $NCH_2C\underline{H}_2$), 2.36-2.22 (m, 4H, $COCH_2$), 2.15-2.04 (m, 4H, $NHCOC\underline{H}_2$), 1.66-1.49 (m, 8H, $COCH_2C\underline{H}_2$), 1.38-1.11 (m, 70H, CH_2 and isopropyl CH_3), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.3, 174.1, 173.5, 173.1, 152.4, 142.4, 130.0, 129.4, 128.2, 127.7, 127.6, 126.3, 115.2, 70.3 (d, $J_{C\text{-}P}$ = 7.6 Hz), 70.2, 70.1 (d, $J_{C\text{-}P}$ = 8.6 Hz), 69.9, 69.7, 69.3, 64.2 (d, $J_{C\text{-}P}$ = 5.7 Hz), 63.1 (d, $J_{C\text{-}P}$ = 4.8 Hz), 62.4, 54.2, 46.1, 41.4, 40.3, 38.9, 35.6, 35.5, 34.2, 33.9, 33.7, 31.6, 29.3, 29.2, 29.0, 28.8, 27.9, 25.0, 24.6, 24.5, 22.3, 18.0, 17.9, 16.4, 16.3, 13.6; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.03; LRMS (ESI) *m/z* 1380.96 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{77}H_{136}N_3O_{14}PNa$: 1380.9652 [$(M+Na)^+$], found: 1380.9618; Anal. calcd for $C_{77}H_{136}N_3O_{14}P\cdot$

3.3H_2O: C, 65.21; H, 10.13; N, 2.96. Found: C, 64.90; H, 9.74; N, 2.89.

Preparation and Spectral and Analytical Data of LPC 5 (56.1 mg, 0.0388 mmol, 92%) was prepared from PL-PEG unit **11**. $[\alpha]^{20}_D$ 2.58 (*c* 0.51, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.51 (br, 1H, amide NH), 7.37 (br, 1H, amide NH), 7.37 (d, *J* = 7.6 Hz, 2H, aromatic), 7.30 (dd, *J* = 7.6, 7.6 Hz, 2H, aromatic), 7.20 (t, *J* = 7.6 Hz, 1H, aromatic), 7.03 (d, *J* = 1.9 Hz, 1H, aromatic), 6.89 (dd, *J* = 7.6, 1.9 Hz, 1H, aromatic), 6.74 (d, *J* = 7.6 Hz, 1H, aromatic), 5.26-5.18 (m, 1H, glycerol CH), 4.39 (dd, *J* = 12.4, 3.8 Hz, 1H, glycerol CH_2), 4.35-4.25 (m, 1H, benzyl CH), 4.16 (dd, *J* = 12.4, 7.6 Hz, 1H, glycerol CH_2), 4.02-3.91 (m, 4H, $POCH_2$ and glycerol CH_2), 3.70-3.57 (m, 18H, OCH_2), 3.57-3.48 (m, 4H, OCH_2 and NCH), 3.43-3.30 (m, 4H, CONHC$\underline{H}_2$), 2.94-2.79 (m, 2H, NCH_2), 2.73-2.64 (m, 2H, benzyl CH_2), 2.64-2.50 (m, 2H, $NCH_2C\underline{H}_2$), 2.35-2.23 (m, 4H, $COCH_2$), 2.15-2.04 (m, 4H, NHCOC$\underline{H}_2$), 1.66-1.39 (m, 8H, $COCH_2C\underline{H}_2$), 1.39-1.14 (m, 70H, CH_2 and isopropyl CH_3), 0.89 (t, *J* = 6.7 Hz, 3H, CH_3), 0.89 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (125 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.2, 173.9, 173.5, 173.1, 152.4, 142.4, 130.1, 129.4, 128.2, 127.8, 127.5, 126.3, 115.3, 70.4 (d, *J* = 8.4 Hz), 70.2, 70.1, 70.1, 70.0, 70.0, 69.7, 69.3, 64.2 (d, *J* = 6.0 Hz), 63.1 (d, *J* = 6.0 Hz), 62.4, 54.1, 46.1, 41.5, 40.3, 38.9, 35.7, 35.6, 34.2, 33.9, 33.8, 31.6, 29.4, 29.3, 29.2, 29.2, 29.0, 29.0, 28.8, 28.8, 28.1, 25.0, 24.6, 24.5, 22.3, 18.0, 17.9, 16.4, 16.3, 13.7; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.03; LRMS (ESI) *m/z* 1469.01 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{81}H_{144}N_3O_{16}PNa$: 1469.0176 [$(M+Na)^+$], found: 1469.0144; Anal. calcd for $C_{81}H_{144}N_3O_{16}P\cdot 3.0H_2O$: C, 64.81; H, 10.07; N, 2.80. Found: C, 64.94; H, 9.82; N, 2.76.

Preparation and Spectral and Analytical Data of LPC 6. LPC **6** (59.2 mg, 0.0386 mmol, 92%) was prepared from PL-PEG unit **12**. $[\alpha]^{20}_D$ 3.97 (*c* 0.87, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.45 (br, 1H, amide NH), 7.36 (d, *J* = 7.6 Hz, 2H, aromatic), 7.30 (dd, *J* = 7.6, 7.6 Hz, 2H, aromatic), 7.28 (br, 1H,

amide NH), 7.20 (t, *J* = 7.6 Hz, 1H, aromatic), 6.99 (d, *J* = 1.9 Hz, 1H, aromatic), 6.88 (dd, *J* = 8.6, 1.9 Hz, 1H, aromatic), 6.74 (d, *J* = 8.6 Hz, 1H, aromatic), 5.25-5.17 (m, 1H, glycerol CH), 4.38 (dd, *J* = 12.4, 2.9 Hz, 1H, glycerol CH_2), 4.31 (t, *J* = 6.7 Hz, 1H, benzyl CH), 4.15 (dd, *J* = 12.4, 7.6 Hz, 1H, glycerol CH_2), 4.03-3.91 (m, 4H, $POCH_2$ and glycerol CH_2), 3.71-3.57 (m, 26H, OCH_2), 3.57-3.47 (m, 4H, OCH_2 and NCH), 3.43-3.30 (m, 4H, $CONHC\underline{H}_2$), 2.94-2.78 (m, 2H, NCH_2), 2.67 (t, *J* = 6.7 Hz, 2H, benzyl CH_2), 2.63-2.47 (m, 2H, $NCH_2C\underline{H}_2$), 2.36-2.23 (m, 4H, $COCH_2$), 2.17-2.02 (m, 4H, $NHCOC\underline{H}_2$), 1.66 (m, 8H, $COCH_2C\underline{H}_2$), 1.37-1.17 (m, 70H, CH_2 and isopropyl CH_3), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.1, 173.9, 173.5, 173.1, 152.5, 142.4, 130.0, 129.4, 128.2, 127.8, 127.8, 127.5, 126.3, 115.4, 70.4 (d, *J* = 8.5 Hz), 70.1, 70.0, 69.7, 69.3, 64.2 (d, *J* = 5.6 Hz), 63.1 (d, *J* = 4.7 Hz), 62.4, 54.1, 46.1, 41.6, 40.4, 38.9, 35.6, 35.6, 34.3, 33.9, 33.8, 31.6, 29.4, 29.3, 29.2, 29.2, 29.0, 29.0, 28.8, 28.8, 28.2, 25.0, 24.6, 24.5, 22.3, 18.0, 16.4, 13.7; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.02; LRMS (ESI) *m/z* 1557.07 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{85}H_{152}N_3O_{18}PNa$: 1557.0701 [$(M+Na)^+$], found: 1557.0691; Anal. calcd for $C_{85}H_{152}N_3O_{18}P\cdot 3.0H_2O$: C, 64.24; H, 10.02; N, 2.64. Found: C, 64.33; H, 9.77; N, 2.61.

Preparation and Spectral and Analytical Data of LPC 7. LPC **7** (59.3 mg, 0.0365 mmol, 87%) was prepared from PL-PEG unit **13**. $[\alpha]^{20}_D$ 5.06 (*c* 0.98, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.44 (br, 1H, amide NH), 7.36 (d, *J* = 7.6 Hz, 2H, aromatic), 7.30 (dd, *J* = 7.6, 7.6 Hz, 2H, aromatic), 7.26 (br, 1H, amide NH), 7.20 (t, *J* = 7.6 Hz, 1H, aromatic), 6.98 (d, *J* = 1.9 Hz, 1H, aromatic), 6.88 (dd, *J* = 7.6, 1.9 Hz, 1H, aromatic), 6.74 (d, *J* = 7.6 Hz, 1H, aromatic), 5.25-5.17 (m, 1H, glycerol CH), 4.38 (dd, *J* = 11.5, 2.9 Hz, 1H, glycerol CH_2), 4.31 (dd, *J* = 7.6, 7.6 Hz, 1H, benzyl CH), 4.15 (dd, *J* = 11.5, 6.7 Hz, 1H, glycerol CH_2), 4.03-3.89 (m, 4H, $POCH_2$ and glycerol CH_2), 3.71-3.57 (m, 34H, OCH_2), 3.57-3.48 (m, 4H, OCH_2 and NCH),

3.43-3.29 (m, 4H, CONHC$\underline{H}_2$), 2.93-2.81 (m, 2H, NCH_2), 2.71-2.63 (m, 2H, benzyl CH_2), 2.63-2.47 (m, 2H, $NCH_2C\underline{H}_2$), 2.35-2.24 (m, 4H, $COCH_2$), 2.17-2.03 (m, 4H, $NHCOC\underline{H}_2$), 1.66-1.45 (m, 8H, $COCH_2C\underline{H}_2$), 1.40-1.17 (m, 70H, CH_2 and isopropyl CH_3), 0.88 (t, J = 6.7 Hz, 3H, CH_3), 0.88 (t, J = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.1, 173.8, 173.5, 173.0, 152.5, 142.4, 130.0, 129.3, 128.2, 127.9, 127.7, 127.5, 126.3, 115.4, 70.3 (d, J = 8.5 Hz), 70.1, 70.0, 70.0, 69.8, 69.7, 69.3, 64.2 (d, J = 5.6 Hz), 63.1 (d, J = 4.7 Hz), 62.4, 54.1, 54.1, 46.1, 41.7, 40.4, 38.8, 35.6, 35.6, 34.3, 33.9, 33.7, 31.6, 29.4, 29.3, 29.2, 29.2, 29.0, 29.0, 28.8, 28.8, 28.2, 25.0, 24.6, 24.5, 22.3, 18.0, 17.9, 16.3, 13.6; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.15; LRMS (ESI) *m/z* 1646.12 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{89}H_{160}N_3O_{20}PNa$: 1645.1225 [$(M+Na)^+$], found: 1645.1204; Anal. calcd for $C_{89}H_{160}N_3O_{20}P\cdot 2.5H_2O$: C, 64.08; H, 9.97; N, 2.52. Found: C, 64.07; H, 9.81; N, 2.45.

Preparation and Spectral and Analytical Data of LPC 8. LPC **8** (58.8 mg, 0.0344 mmol, 82%) was prepared from PL-PEG unit **14**. $[\alpha]^{20}_D$ 4.79 (*c* 0.89, $CHCl_3$/ MeOH 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$/ CD_3OD 3:1) δ 7.43 (br, 1H, amide NH), 7.36 (d, J = 7.6 Hz, 2H, aromatic), 7.30 (dd, J = 7.6, 7.6 Hz, 2H, aromatic), 7.25 (br, 1H, amide NH), 7.20 (t, J = 7.6 Hz, 1H, aromatic), 6.97 (d, J = 1.9 Hz, 1H, aromatic), 6.88 (dd, J = 7.6, 1.9 Hz, 1H, aromatic), 6.74 (d, J = 7.6 Hz, 1H, aromatic), 5.26-5.16 (m, 1H, glycerol CH), 4.38 (dd, J = 12.4, 2.9 Hz, 1H, glycerol CH_2), 4.31 (dd, J = 7.6, 7.6 Hz, 1H, benzyl CH), 4.15 (dd, J = 12.4, 6.7 Hz, 1H, glycerol CH_2), 4.03-3.90 (m, 4H, $POCH_2$ and glycerol CH_2), 3.72-3.58 (m, 42H, OCH_2), 3.58-3.47 (m, 4H, OCH_2 and NCH), 3.43-3.29 (m, 4H, CONHC$\underline{H}_2$), 2.93-2.83 (m, 2H, NCH_2), 2.66 (t, J = 7.6 Hz, 2H, benzyl CH_2), 2.63-2.47 (m, 2H, $NCH_2C\underline{H}_2$), 2.29 (t, J = 7.6 Hz, 2H, $COCH_2$), 2.29 (t, J = 7.6 Hz, 2H, $COCH_2$), 2.17-2.04 (m, 4H, $NHCOC\underline{H}_2$), 1.65-1.49 (m, 8H, $COCH_2C\underline{H}_2$), 1.38-1.18 (m, 70H, CH_2 and isopropyl CH_3), 0.88 (t, J = 6.7 Hz,

3H, CH_3), 0.88 (t, J = 6.7 Hz, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$/ CD_3OD 3:1) δ 174.1, 173.8, 173.5, 173.0, 152.5, 142.4, 130.0, 129.3, 128.2, 127.9, 127.7, 127.5, 126.3, 115.4, 70.4, 70.3, 70.1, 70.1, 70.0, 70.0, 69.7, 69.3, 64.2 (d, J = 5.6 Hz), 63.1 (d, J = 4.7 Hz), 62.4, 54.1, 54.1, 46.1, 41.7, 40.4, 38.8, 35.6, 35.6, 34.3, 33.9, 33.7, 31.6, 29.4, 29.3, 29.2, 29.2, 29.0, 29.0, 28.8, 28.8, 28.2, 25.0, 24.9, 24.6, 24.5, 22.3, 18.0, 16.4, 13.7; ^{31}P-NMR (202 MHz, $CDCl_3$/ CD_3OD 3:1) δ 3.15; LRMS (ESI) *m/z* 1734.18 [$(M+Na)^+$]; HRMS (ESI) calcd for $C_{93}H_{168}N_3O_{22}PNa$: 1733.1749 [$(M+Na)^+$], found: 1733.1732; Anal. calcd for $C_{93}H_{168}N_3O_{22}P\cdot 1.5H_2O$: C, 64.26; H, 9.91; N, 2.42. Found: C, 64.16; H, 9.83; N, 2.41.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details of synthesis except for final compounds, biological evaluations, computational simulations, a table listing combustion analysis data for final compounds (PDF)

Molecular formula strings (CSV)

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The authors declare no competing financial interest.

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ABBREVIATIONS USED

CAN, ceric ammonium nitrate; DSPC, distearoylphosphatidylcholine; GPI, glycosylphosphatidylinositol; LBDD, ligand-based drug design; LPC, ligand-phospholipid conjugates; MD, molecular dynamics; PEG, polyethylene glycol; PLDP, phospholipase D from *Streptomyces* sp; PL, phospholipid; SBDD, structure-based drug design,

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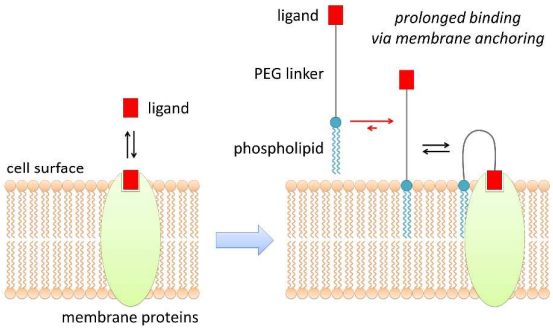
Table of Contents Graphic.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