

Facilitated phosphatidylserine flip-flop across vesicle and cell membranes using urea-derived synthetic translocases

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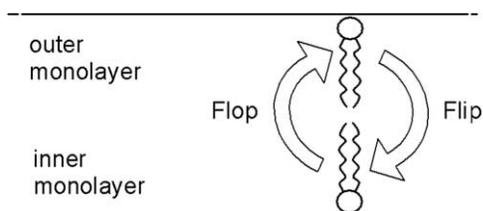
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Tris(2-aminoethyl)amine derivatives with appended urea and sulfonamide groups are shown to facilitate the translocation of fluorescent phospholipid probes and endogenous phosphatidylserine across vesicle and erythrocyte cell membranes. The synthetic translocases appear to operate by binding to the phospholipid head groups and forming lipophilic supramolecular complexes which diffuse through the non-polar interior of the bilayer membrane.

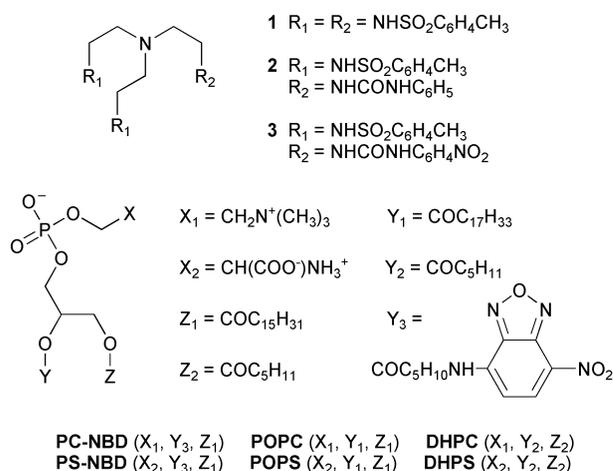
Introduction

Phospholipids are asymmetrically distributed across biological membranes; the aminophospholipids, such as phosphatidylserine (PS), are enriched on the cytoplasmic surface of animal plasma membranes, while choline-based lipids are enriched on the outer surface.^{1,2} The phospholipid asymmetry is vital not only for exocytosis and intracellular fusion processes, but also for membrane-protein interactions and signal transduction pathways.³⁻⁶ In model vesicle membranes, spontaneous membrane translocation (or flip-flop, see Scheme 1) of phospholipids is a very slow process with a typical half-life of hours to days.⁷ In cell membranes, the asymmetric distribution is generated and maintained by a family of membrane-bound transport proteins called phospholipid translocases. The best known translocase is the aminophospholipid flippase which pumps phosphatidylserine to the membrane inner monolayer.^{8,9}



Scheme 1 Phospholipid translocation or flip-flop.

Our research group is attempting to design synthetic translocases for use as pharmaceuticals or as chemical tools for biological membrane research.¹⁰⁻¹⁶ For instance, we reported that tris(2-aminoethyl)amine (tren) sulfonamide **1** (Scheme 2) facilitates phosphatidylcholine translocation across synthetic vesicle and erythrocyte membranes. Mechanistic studies indicate that **1** forms a supramolecular complex with the phosphatidylcholine head group, which diminishes head group hydrophilicity and promotes diffusion through the non-polar interior of the bilayer membrane. More recently we have focused on synthetic translocases that facilitate phosphatidylserine flip-flop. In particular, our aim is to develop PS-scramblases that raise the concentration of phosphatidylserine on the cell surface, which in turn is known to induce various biological processes such as blood coagulation and cell clearance. Our previous work has shown that the facilitated translocation of phospholipids with multiply charged head groups, like phosphatidylserine, is a difficult supramolecular challenge that requires a complementary,



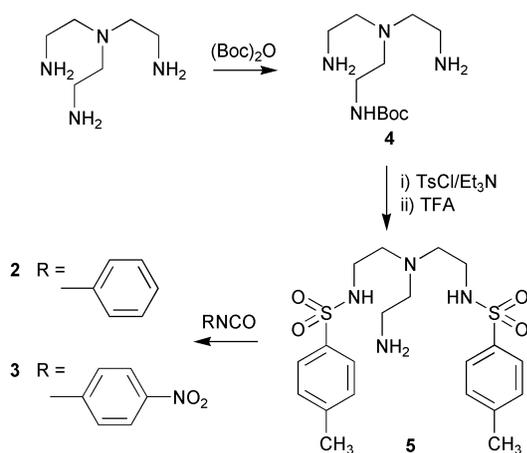
Scheme 2

multitopic synthetic receptor with appropriate charge and lipophilicity.¹⁵ While compound **1** has no PS-scramblase activity, we recently screened a library of tren-derived receptors and uncovered some active derivatives with appended urea groups.¹⁶ This unexpected observation has motivated us to prepare some simple tren-urea derivatives and evaluate them as synthetic phospholipid translocases and more specifically as PS-scramblases. Here, we report that tren derivatives **2** and **3** (Scheme 2) with appended urea and sulfonamide groups are remarkably effective at promoting the translocation of phosphatidylcholine and phosphatidylserine across vesicle and cell membranes. We also characterize the anion and proton binding properties of these synthetic translocases, and propose a translocation mechanism that is consistent with the data.

Results

Synthesis

Tren sulfonamide **1** was prepared using a previously reported method.¹⁰⁻¹² Mono-urea derivatives **2** and **3** were synthesized in three steps (Scheme 3). Treatment of tris(2-aminoethyl)amine with di-*tert*-butyl dicarbonate gave the desired mono-Boc-protected **4** in 69% yield. Reaction of **4** with toluenesulfonyl chloride and subsequent deprotection with TFA gave bisulfonamide **5**, which was reacted with the appropriate arylisocyanate to give **2** or **3**.



Scheme 3

Flip into vesicles and cells

Phospholipid translocation was monitored *via* the well established NBD/dithionite quenching assay which uses fluorescent phospholipid probes containing a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group in one of its acyl chains (Scheme 2).^{10,17,18} Exo-labeled vesicles were prepared by addition of a small aliquot of NBD-lipid (2 mol% of total phospholipid) in ethanol to a dispersion of vesicles. The NBD-lipid readily inserts into the outer monolayer of fluid-phase vesicles. Upon treatment with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), the NBD fluorescence is quenched due to reduction of the NBD nitro group. Bilayer membranes are effectively impermeable to dithionite; therefore, only NBD-phospholipid located in the outer leaflet is chemically quenched. The inward translocation experiment is initiated by adding a small aliquot of synthetic translocase candidate to the exo labeled-vesicles. At any given time, the fraction of probe located in the outer monolayer can be determined from the drop in fluorescence intensity when a portion of the vesicles is subjected to dithionite quenching.

Inward translocation of NBD-PS induced by addition of $12.5 \mu\text{M}$ of one of the synthetic translocase candidates **1**, **2** or **3** was measured using vesicles composed of POPC/cholesterol (7 : 3, $25 \mu\text{M}$ total lipid concentration). The background rate for inward translocation of NBD-PS is very slow, with a translocation half-life of much greater than 3 h. Addition of sulfonamide **1** produces no enhancement of inward translocation, whereas the presence of mono-urea derivatives **2** or **3** produces a significant rate increase with translocation half-lives of 12 min and 60 min respectively (Fig. 1). Also, compounds **2** and **3** facilitated NBD-PC translocation by approximately the same

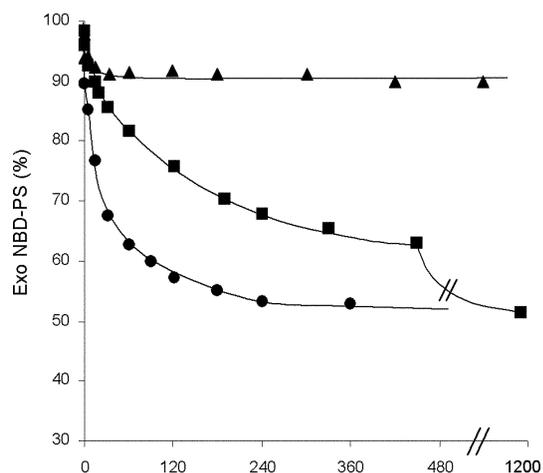


Fig. 1 Inward translocation of NBD-PS (2 mol%) across POPC/cholesterol (7 : 3) vesicles ($25 \mu\text{M}$) in the presence of $12.5 \mu\text{M}$ **1** (▲), **2** (■), or **3** (●) at pH 7.4, 25°C .

amount as NBD-PS (Table 1). Concentration studies showed that the rate of translocation increases as the concentration of **2** or **3** is raised. Control experiments verified that compounds **1–3** do not induce leakage of entrapped carboxyfluorescein from the vesicles.

In addition to enhanced phosphatidylserine translocation across vesicle membranes, mono-urea derivatives **2** and **3** can facilitate the inward-translocation of phosphatidylserine across erythrocyte cell membranes. The inward-translocation assay was initiated by treating a population of erythrocytes with a small aliquot of PS-NBD (1% of total phospholipid). The probe molecules that did not insert into the outer monolayer of the erythrocyte membrane were removed by washing. Subsequent translocation of the probe to the inner monolayer of the erythrocyte membrane was measured by removing a small sample of cells at periodic intervals and using the above mentioned NBD/dithionite assay. When the translocase candidate was added as an ethanolic solution to human erythrocytes, less than 2% of hemolysis¹⁹ occurred over a 6 h time period. Therefore, it appears that these compounds do not compromise membrane integrity. To ensure that dithionite did not enter the erythrocytes and quench the probe on the inside, it was necessary to inhibit the band 3 anion exchanger protein by pretreating the cells with 4,4'-diisothiocyanatostilbene-2,2'-disulfonate.^{20,21} In addition, the cells were pretreated with the aminophospholipid flippase inhibitor *N*-ethylmaleimide (NEM) which prevents endogenous, enzyme-mediated, translocation of phosphatidylserine to the membrane inner monolayer.⁸ The data in Fig. 2 clearly show that urea-derived **2** and **3** can significantly facilitate inward translocation of NBD-PS probe as compared to sulfonamide **1**, although the activity of **3** in erythrocytes is lower than in vesicles.

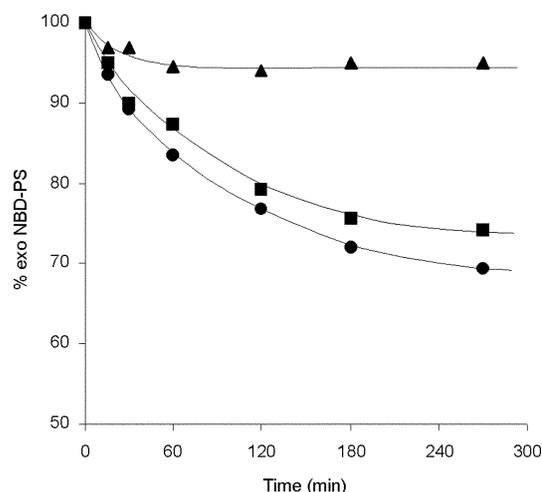


Fig. 2 Inward translocation of NBD-PS (1 mol%) across erythrocyte membranes in the presence of $40 \mu\text{M}$ **2** (■), **3** (●), or no addition (▲). The cells were pretreated with the aminophospholipid flippase inhibitor *N*-ethylmaleimide (NEM, 10 mM) which prevents endogenous translocation of phosphatidylserine.

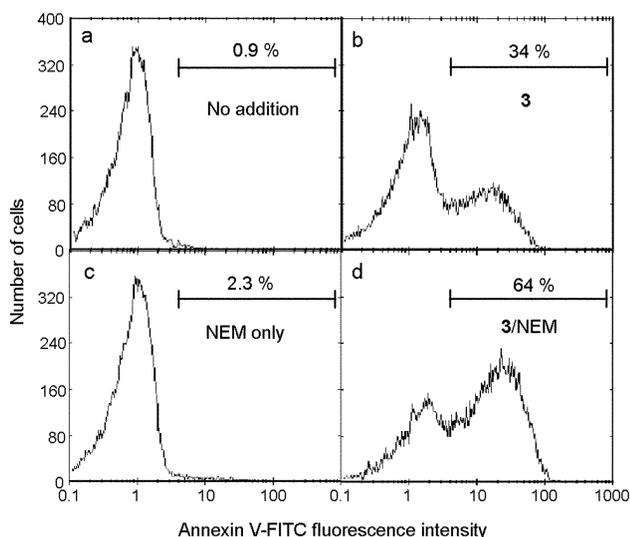
Phosphatidylserine scrambling in blood cells

Flow cytometry experiments show that compound **3** can scramble the distribution of endogenous phosphatidylserine across erythrocyte membranes. The appearance of phosphatidylserine on the cell surface following treatment with compound **3** ($10 \mu\text{M}$, 3 h, $1.5 \times 10^7 \text{ cells cm}^{-3}$) was detected using the fluorescein-labeled, PS-binding protein, annexin V (annexin V-FITC). The distribution of fluorescence intensity from a representative flow cytometry experiment is shown in Fig. 3. Results obtained with normal erythrocytes are represented in the top half of Fig. 3(a,b), while the cells in the bottom half of Fig. 3 (c,d) were pretreated with the aminophospholipid flippase inhibitor (NEM). As expected, hardly

Table 1 Half-lives for inward translocation of NBD-lipid facilitated by translocase candidates and phospholipid association constants

Lipid	Compound	Translocation half-life ^a /min	$K/10^4 \text{ M}^{-1}$
NBD-PS	1	>180	—
	2	60	$0.12 (\pm 0.02)^b$
	3	12	$1.9 (\pm 0.2)^b$
NBD-PC	1	>180	—
	2	60	—
	3	14	$2.2 (\pm 0.1)^c$

^a Inward translocation of 2 mol% NBD-lipid across POPC/Cholesterol (7 : 3) vesicles (25 μM) in the presence of 12.5 μM synthetic translocase candidates at pH 7.4, 25 °C. ^b Association with POPS in 99 : 1 $\text{CHCl}_3/\text{CH}_3\text{OH}$ at 25 °C. ^c Association with POPC in 99 : 1 $\text{CHCl}_3/\text{CH}_3\text{OH}$ at 25 °C.

**Fig. 3** Flow cytometry analysis of annexin V-FITC binding to normal (a,b) or NEM treated (c,d, 10 mM) erythrocytes. Before exposure to annexin V-FITC, the erythrocytes in (b) and (d) were incubated at 37 °C for 3 h with 10 μM of 3.

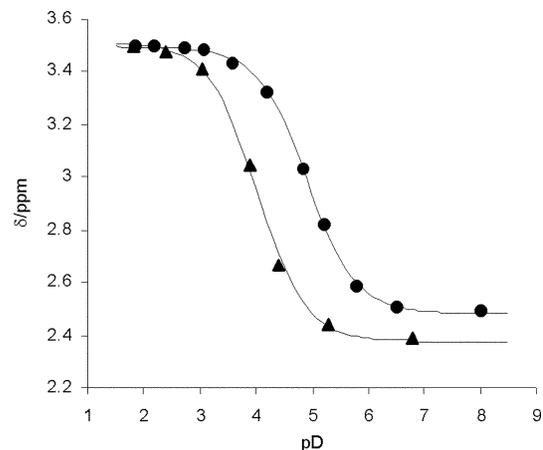
any normal cells (which sequester most of the phosphatidylserine in the inner monolayer of the membrane) were stained by annexin V-FITC (Fig. 3a), but 34% of the cells were stained after treatment with compound 3 (Fig. 3b). If the endogenous aminophospholipid flippase is inhibited with NEM, then the PS-externalization effect induced by compound 3 is approximately double (Fig. 3d). The lack of hemoglobin leakage (< 3% leakage over 3 h) indicates that the compounds do not induce nonselective membrane transport.

pK_a measurements

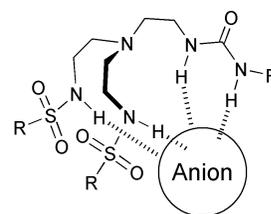
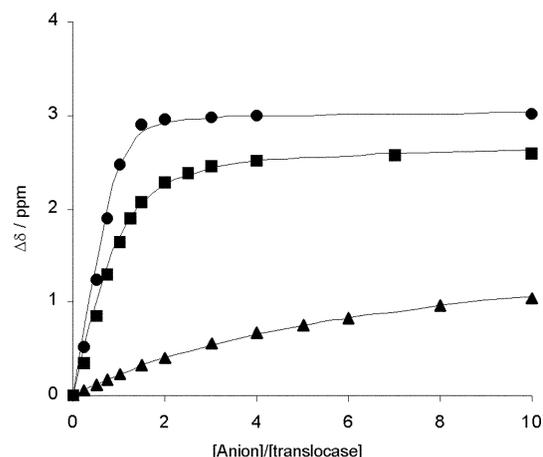
The translocase candidates are only moderately soluble in pure water. Therefore, the pK_a values of their protonated forms were determined by ^1H NMR titration method in 9 : 1 $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ (Fig. 4), and found to be $4.01 (\pm 0.04)$ for sulfonamide 1 and $4.91 (\pm 0.01)$ for mono-urea derivative 2. These values are in line with previous measurements on related systems,¹² and suggest that the translocases exist predominantly as their free bases in the bulk solution of the phospholipid translocation assay ($\text{pH} = 7.4$).

Anion and phospholipid binding

The translocation data suggest that tren-urea derivatives 2 and 3 are able to form hydrogen-bonded complexes with the phospholipid head groups. To gain structural insight into the complexes, a series of NMR titration studies were conducted. The anion binding ability of the most active translocase 3 was investigated by ^1H NMR spectroscopy in $\text{DMSO}-d_6$, a highly polar solvent that minimizes ambiguities due to inter- and intramolecular aggregation. Compound 3 was titrated with tetrabutylammonium salts of H_2PO_4^- , CH_3CO_2^- , and Cl^- . In all cases the addition of anionic guests resulted in large down

**Fig. 4** Change in ^1H NMR chemical shift (δ) for NCH_2 signal in 1 (\blacktriangle) and 2 (\bullet) in 9 : 1 $\text{CD}_3\text{OD}/\text{D}_2\text{O}$.

field shifts of aryl urea NH protons, alkyl urea NH protons, and sulfonamide NH protons, suggesting that both the urea and sulfonamide moieties are involved in the anion binding as shown in Scheme 4. Titration isotherms were generated by monitoring aryl urea NH signals (Fig. 5), and the association constants were determined by fitting the curves to a 1 : 1 binding model using iterative computer methods. In the case of CH_3CO_2^- the 1 : 1 binding stoichiometry was confirmed by a

**Scheme 4** Supramolecular complex of 3 with anion.**Fig. 5** Change in ^1H NMR chemical shift of the aryl urea NH signal for translocase 3 ($\Delta\delta$) upon addition of tetrabutylammonium salts of CH_3CO_2^- (\bullet), H_2PO_4^- (\blacksquare) and Cl^- (\blacktriangle) in $\text{DMSO}-d_6$ and at 25 °C.

Job plot (Fig. 6). The observed association constants are CH_3CO_2^- (4500 M^{-1}), H_2PO_4^- (900 M^{-1}), and Cl^- (32 M^{-1}). The relatively strong affinity for CH_3CO_2^- is attributed to its shape and relatively high basicity.

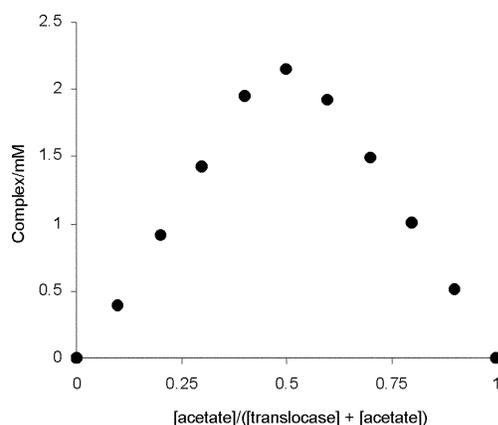


Fig. 6 Job plot for translocase **3** and tetrabutylammonium acetate by ^1H NMR in $\text{DMSO}-d_6$ at 25°C . $[\text{translocase}] + [\text{acetate}] = 5 \text{ mM}$.

Compounds **2** and **3** were also evaluated for their abilities to associate with phospholipid head groups. Attempts to measure the binding of phospholipids by ^1H NMR in weakly polar solvents were hampered by self-aggregation effects, however, the changes in chemical shifts were consistent with hydrogen-bonding between the phospholipid head groups and the urea and sulfonamide NH groups. The self-aggregation problems were overcome by conducting UV titration studies in 99 : 1 $\text{CHCl}_3/\text{CH}_3\text{OH}$ at 25°C . The typical titration isotherm shown in Fig. 7 was generated by monitoring the change in UV absorption of the *N*-aryl urea groups in **3** upon addition of POPS, and the association constant was determined by fitting the curve to a 1 : 1 binding model using an iterative computer method. Compounds **2** and **3** bind POPS with association constants of $(0.12 \pm 0.02) \times 10^4 \text{ M}^{-1}$ and $(1.9 \pm 0.2) \times 10^4 \text{ M}^{-1}$, respectively. The stronger binding exhibited by **3** is attributed to the increased acidity of its urea NH residues. The data listed in Table 1 shows that an increase in phospholipid association constant correlates with a decrease in NBD-lipid translocation half-life.

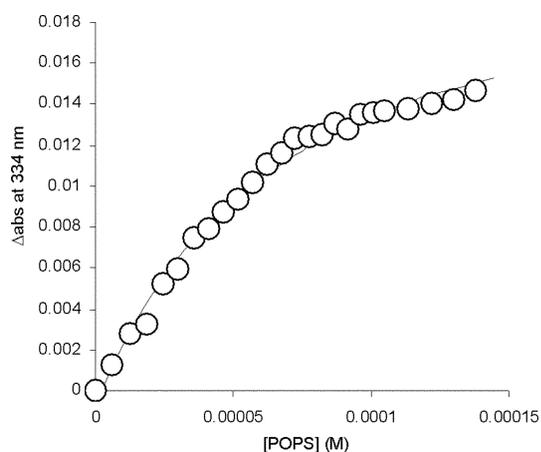


Fig. 7 Change in absorbance (Δabs) upon titration of **3** ($15 \mu\text{M}$) with POPS in 99 : 1 $\text{CHCl}_3/\text{CH}_3\text{OH}$ at 25°C and its curve fitting using a 1 : 1 binding model.

Additional structural information was obtained by using electrospray ionization mass spectrometry to analyze an equimolar mixture of **3** and DHPS, a phosphatidylserine derivative with short C_6 acyl chains (structure shown in Scheme 2). In the negative ion mode, the base peak corresponds to a 1 : 1 complex of **3** with DHPS ($[\mathbf{3} + \text{DHPS}^-]$, m/z 1072.4). In the positive ion mode, three signals are detected, whose m/z ratios correspond

to complexes of composition $[\mathbf{3} + \text{DHPS}^- + 2\text{H}^+]$ (m/z 1074.4), $[\mathbf{3} + \text{DHPS}^- + \text{H}^+ + \text{Na}^+]$ (m/z 1096.4), and $[\mathbf{3} + \text{DHPS}^- + 2\text{Na}^+]$ (m/z 1118.4). Thus, mass spectrometry shows that **3** can bind to the anionic head group of DHPS, and that this binary complex can also associate with H^+ or Na^+ ions.

Discussion

We have previously demonstrated that tren-sulfonamide **1** can facilitate the membrane flip-flop of phosphatidylcholine. Compound **1** associates with the phosphate diester residue in the zwitterionic phosphatidylcholine head group, which diminishes its hydrophilicity and promotes diffusion across the lipophilic interior of the membrane.² Compound **1** is unable to accelerate the flip-flop of anionic phosphatidylserine, presumably because it does not provide an appropriate level of lipophilicity and charge balance.

Tren-urea derivatives **2** and **3** have strong affinities for anions and it is not surprising that they can facilitate phosphatidylcholine flip-flop (Table 1). Less easily rationalized are their impressive abilities to promote phosphatidylserine flip-flop. Compound **3** with its more acidic urea NH residues, has a stronger affinity for phospholipid head groups and is a more efficient translocase. Not only can compound **3** translocate NBD-PS into vesicle and cells, it can promote phosphatidylserine flop, or translocation from inside to outside. In other words, it can act as a synthetic PS-scrambase. The evidence presented in this paper combined with the results of our previous studies suggest that compounds **2** and **3** operate by forming a supramolecular complex with the phosphatidylserine head group, and that the complex diffuses across the bilayer membrane. Charge balance demands that facilitated translocation of anionic phosphatidylserine, by **2** or **3**, must also include a cationic residue in the kinetically active complex. The mass spectrometry data suggests that this is likely to be a H^+ or Na^+ . We envision that compounds **2** and **3** promote phosphatidylserine scrambling across the membranes of red blood cells in the following way. After addition to the sample, the neutral form of **2** or **3** partitions into the cell membrane and diffuses to the inner leaflet either by itself or hydrogen bonded to a phospholipid like phosphatidylcholine. It can return to the outer leaflet as a supramolecular complex with anionic phosphatidylserine and a balancing cation like H^+ or Na^+ .

Conclusion

We have shown that tren-urea derivatives **2** and **3** can greatly facilitate the inward and outward translocation of phospholipids, including phosphatidylserine and its fluorescent derivative, NBD-PS, across vesicle and erythrocyte membranes. Furthermore, these synthetic translocases can function as PS-scrambases; that is, they can work against the inward pumping action of the endogenous aminophospholipid flippase and increase the amount of phosphatidylserine on the cell surface. The structural simplicity of these compounds makes them attractive candidates for further development as either chemical tools for biological membrane research, or perhaps eventually as chemotherapeutic agents. From a molecular design perspective, it is becoming increasingly clear that the construction of a successful synthetic translocase for phosphatidylserine should include a relatively large hydrogen-bonding pocket that can efficiently desolvate the multiply-charged phosphatidylserine head group.^{15,16}

Experimental

General

Compounds **1**,¹⁰ **4** and **5**¹² were prepared as reported previously. All lipids were purchased from Avanti Polar Lipids, Inc (USA).

Synthesis of urea, 2

A solution of 4-methylphenylisocyanate (0.020 g, 0.16 mmol) in dichloromethane (10 cm³) was added dropwise to a stirred solution of **5** (0.075 g, 0.16 mmol) in dichloromethane (30 cm³) at ice bath temperature. The mixture was allowed to stir overnight at room temperature. Removal of solvents and purification by chromatography on silica (EtOAc : hexane, 8 : 2) gave a colorless oil (0.055 g, 59%) δ_{H} (300 MHz; CDCl₃; Me₄Si) 2.35 (6H, s, ArCH₃), 2.43 (6H, m, NCH₂CH₂), 2.98 (4H, m, NCH₂CH₂-SO₂Ar), 3.21 (2H, m, NCH₂CH₂NHCO), 5.98 (1H, s, br, CH₂NHCONHAr), 6.20 (2H, s, br, CH₂NHSO₂Ar), 6.98 (1H, t, *J* 6.0, ArH), 7.21 (6H, m, ArH), 7.42 (2H, d, *J* 12.0, ArH), 7.49 (1H, s, br, CH₂NHCONHAr) and 7.76 (2H, d, *J* 11.9, ArH); δ_{C} (75 MHz; CDCl₃; Me₄Si) 21.6, 37.6, 41.3, 54.1, 119.4, 122.6, 127.2, 129.0, 130.0, 136.7, 139.7, 143.7 and 156.5; HRMS *m/z* (FAB) 574.2189 (M⁺. C₂₇H₃₅N₅O₅S₂ requires 574.2189).

Synthesis of urea, 3

The above procedure was used to prepare **3** as a colorless oil (0.058 g, 43%) δ_{H} (300 MHz; CDCl₃; Me₄Si) 2.36 (6H, s, ArCH₃), 2.54 (6H, m, NCH₂CH₂), 3.01 (4H, m, NCH₂CH₂-SO₂Ar), 3.26 (2H, m, NCH₂CH₂NHCO), 6.04 (2H, s, br, CH₂NHSO₂Ar), 6.16 (1H, s, br, CH₂NHCONHAr), 7.23 (4H, d, ArH, *J* 12.0), 7.55 (4H, d, ArH, *J* 12.0), 7.73 (2H, d, ArH, *J* 9.0), 8.01 (1H, s, CH₂NHCONHAr) and 8.05 (2H, d, ArH, *J* 9.0); δ_{C} (75 MHz; CDCl₃; Me₄Si) 22.1, 38.7, 42.0, 54.4, 118.4, 125.7, 127.7, 130.6, 144.6, 147.0 and 155.8; HRMS *m/z* (FAB) 619.1997 (M⁺. C₂₇H₃₅N₆O₇S₂ requires 619.2009).

pK_a determination

Translocase candidates were dissolved in 9 : 1 CD₃OD/D₂O (1 mM, 0.75 cm³), and 4 molar equivalents of DCl were added to fully protonate the tertiary amine. Chemical shifts of the NCH₂ and NCH₂CH₂ protons were monitored upon the addition of small aliquots of NaOD stock solution (0.1 M). A pD reading was also recorded after each addition. The changes in chemical shift were plotted against the pD values, and the pK_a values were extracted using a curve fitting procedure.¹²

Vesicle preparation

A film of the vesicle lipids was dried under vacuum for at least 1 h. Hydration was performed at room temperature with an appropriate amount of TES buffer (5 mM TES, 100 mM NaCl, pH 7.4). Multilamellar vesicles were generated using a Vortex mixer; use of a glass bead ensured complete lipid removal from the flask wall. The multilamellar vesicles were extruded to form large unilamellar vesicles with a hand-held Basic LiposoFast device purchased from Avestin, Inc., Ottawa, Canada. The vesicles were extruded 29 times through a 19 mm polycarbonate Nucleopore filter with 100 nm diameter pores.

Erythrocyte preparation

Fresh human blood was treated with EDTA solution (dipotassium salts) and erythrocytes were isolated by centrifugation at 7500 rpm for 5 min, followed by washing three times with 4 volumes of ice cold solution of 138 mM NaCl, 5 mM KCl, 6.1 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, 1 mM MgSO₄ at pH 7.4. The cells were used the same day. All incubations were carried out in the above mentioned buffer at 37 °C, 20% hematocrit in capped plastic tubes unless otherwise stated.

Inward NBD-lipid translocation assay

The inward translocation assay using phospholipids with 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) labels was adapted from the original paper by McIntyre and Sleight.^{17,18} Exo-labeled vesicles were generated upon addition of a concentrated

ethanolic solution of NBD-lipid (final NBD-lipid concentration was 0.125 μM) to a 45 cm³ solution of POPC/cholesterol (7 : 3) vesicles (final total lipid concentration was 25 μM) at room temperature. To each vesicle solution, a synthetic scramble candidate was added from a ethanolic stock solution (solvent alone does not induce translocation). Over the time course of 3 h, a series of 3 cm³ aliquots were removed and assayed for extent of translocation. The 200 s assay consisted of a dithionite injection (60 mM, 0.18 cm³ of a 1 M solution) at *t* = 50 s and a Triton X-100 injection (0.5%, 0.075 cm³ of a 20% solution) at *t* = 150 s. Data points were collected every second. The percentage of exo NBD-lipid located in the vesicle outer monolayer was calculated according to the following equation, (%exo NBD-lipid) = [(F_i - F_f)/F_i] × 100, where F_i and F_f are the intensities just prior to the addition of dithionite and Triton X-100, respectively. All % exo NBD-lipid values contain 5% error. In some cases, the translocation curves appeared to have biexponential character, but because of the uncertainty in the data a double exponential analysis was not attempted. Instead, the reported translocation half-lives simply indicate the time taken to reach 80% exo NBD-lipid, which is halfway toward an equilibrium value of 60% exo NBD-lipid.

Carboxyfluorescein leakage assay

Excitation was set at 495 nm, and fluorescence emission was measured at 520 nm using an open filter. Vesicles encapsulating carboxyfluorescein were freeze-thawed 10 times prior to extrusion. The fluorescence of a 3 cm³ sample of vesicles (25 μM) in TES buffer was monitored. After 50 s, an aliquot of translocase (40 μM) was added. Detergent (0.5% Triton X-100) was added at *t* = 250 s. Total assay time was 300 s. A constant fluorescent intensity up until the point of detergent addition indicated no vesicle leakage.

Hemolysis assay¹⁹

Absorbance was monitored from 600 to 300 nm. A series of 1 cm³ samples was prepared: 0% hemolysis (only buffer), 100% hemolysis (only H₂O), **1** (40 μM in buffer), **2** (40 μM in buffer), and **3** (40 μM in buffer). To each, 100 μL of packed red blood cells was added (10% hematocrit). Samples were incubated at 37 °C for varying time periods before removal of 100 μL aliquots for hemolysis analysis. The aliquots were centrifuged for 10 min at 1000 rpm, and the supernatant was diluted to 1 cm³ with buffer before recording the absorbance of the hemoglobin in solution (*A* at 414 nm). The percent hemolysis was calculated according to the following equation, %hemolysis = 100 - [(A_{100%} - A)/(A_{100%} - A_{0%}) × 100].

¹H NMR titration

Small aliquots of guest stock solution (0.375 M) were added to an NMR tube containing a solution of translocase in DMSO-*d*₆ (5 mM, 0.75 cm³), followed by the acquisition of a ¹H NMR spectrum. Concentrations and equivalents were adjusted to give the optimum change in Weber *p* values (0.2–0.8).²² Titration isotherms were generated for the NH protons of sulfonamide or urea residues. Fitting the data to a 1 : 1 binding model using an iterative curve-fitting method yielded association constants and maximum change in chemical shift.²³

Job plot

Job's method was used to determine the stoichiometry of the complex formed between **3** and tetrabutylammonium (TBA) acetate.²³ Stock solutions (5 mM) of **3** (host, H) and TBA acetate (guest, G) were prepared in DMSO-*d*₆. In separate NMR tubes, 10 samples of varying **3** : TBA acetate ratios were prepared. A ¹H NMR spectrum was acquired for each sample, and the concentration of the complex ([HG]_{eq}) was determined according to the following equation: [HG]_{eq} = [H]₀ × [(δ_{obs} - δ₀)/

$(\delta_{\max} - \delta_0)$], where $[H]_0$ is the pre-equilibrium host concentration, δ_{obs} is the observed chemical shift, δ_0 is the chemical shift of the free host, and δ_{\max} is the chemical shift of the complex (δ_{\max} was obtained from curve-fitting methods used to derive the association constant). The conventional Job's plot ($[HG]_{\text{eq}}$ vs. host mole fraction) was then prepared.

UV titration

UV titration experiments were conducted by adding POPC or POPS to solutions of compounds **2** or **3** in 99 : 1 CHCl_3 : CH_3OH at 295 K. Specifically, small aliquots of phospholipid stock solution (0.315 mM phospholipid in 15 μM scramblase solution) were added to a cuvette containing a solution of scramblase (15 μM , 1 cm^3), followed by the acquisition of a UV spectrum. The total volume of added phospholipid was 0.4 cm^3 or 6 molar equivalents. Titration isotherms were generated from the changes in absorbance, and the data were fitted to a 1 : 1 binding model.²⁴ An iterative curve-fitting method yielded association constant and maximum change in absorbance. The association constants listed in the text are the average of three independent measurements.

Flow cytometry

Erythrocytes were isolated by centrifugation at 7900 rpm for 5 min, washed three times with 4 volumes of ice cold 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4 (PBS), and suspended in PBS at a density of 1.5×10^8 cells cm^{-3} . A portion of the cells was pretreated with *N*-ethylmaleimide (NEM, 10 mM in PBS) for 30 min at room temperature. After 2 PBS washings, the NEM pretreated cells were suspended in PBS at a density of 1.5×10^8 cells cm^{-3} . A 10 mM translocase solution was prepared by dilution of a DMSO or ethanol stock solution into PBS, and a 0.05 cm^3 aliquot of either normal or NEM-pretreated cells was added to 0.45 cm^3 of this translocase solution (1.5×10^7 cells cm^{-3}). The resulting solutions were incubated at 37 °C for 3 h. To prepare the samples for flow cytometry analysis,²⁵ the above solutions were resuspended in 0.5 cm^3 of 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4 (binding buffer). In a 5 cm^3 culture tube, 0.015 cm^3 annexin V-FITC (BD Biosciences/Pharmingen) was added to 100 μL of the erythrocyte solution (1.5×10^6 cells), and the mixture was incubated at 37 °C for 15 min. An additional 0.6 cm^3 of binding buffer was added before measuring fluorescence intensity and size with a Beckman Coulter Epics XL flow cytometer. Control experiments indicate that ethanol or DMSO alone has no effect on the amount of bound annexin V-FITC.

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