COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Selective phosphatidylethanolamine translocation across vesicle membranes using synthetic translocases

J. Middleton Boon,[a] Rameshwer Shukla,[a] Bradley D. Smith,*[a] Giulia Licini[b] and Paolo Scrimin[b]

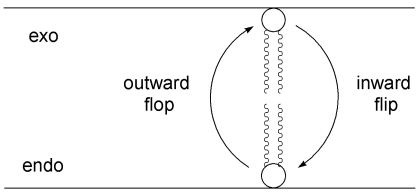
[a] *Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA. E-mail: smith.115@nd.edu*

[b] *Department of Organic Chemistry, CNR-CMRO, University of Padova, Padova, Italy*

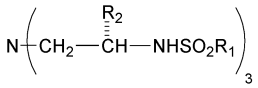
Received (in Columbia, MO, USA) 12th October 2001, Accepted 6th December 2001
First published as an Advance Article on the web 14th January 2002

Two sulfonamide derivatives of tris(aminoethyl)amine selectively facilitate the translocation of a fluorescent phospholipid probe containing the phosphoethanolamine head-group across vesicle membranes.

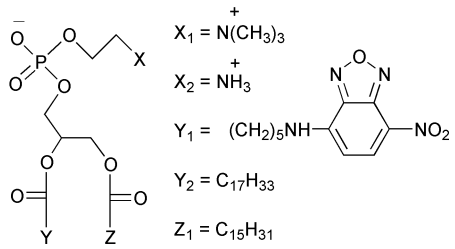
Phospholipids are asymmetrically distributed between the outer and inner monolayers of biological plasma membranes. For example, most of the phosphatidylserine and phosphatidylethanolamine is sequestered in the inner monolayer of human erythrocytes, while the outer monolayer contains most of the phosphatidylcholine and sphingomyelin.[1] Phospholipid translocation or 'flip-flop' across a bilayer membrane is inherently a slow process with a typical half-life of many hours. The asymmetric distribution is maintained by enzymes known generally as 'translocases' that facilitate translocation; however, there is little mechanistic understanding of how these enzymes work,[2] or how translocation in general occurs on a molecular level.[3] We are interested in designing synthetic translocases,[4] which can be employed as pharmaceuticals or as chemical tools for biological membrane research.[5] Recently, we reported that tren sulfonamide derivative **1** facilitates the translocation of the fluorescent probe PC-NBD across vesicle[6] and cellular[7] membranes. Compound **1** is thought to form a hydrogen-bonded complex with the phosphocholine head-group at the surface of the membrane, effectively reducing the head-group polarity and lowering the barrier to diffusion across the lipophilic interior of the membrane. In this paper, we describe two second-generation tren derivatives (compounds **2** and **3**) that selectively facilitate the translocation of PE-NBD, a fluorescent phospholipid probe that contains the phosphoethanolamine head-group.



Phospholipid translocation is monitored *via* the well-established 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite quenching assay[6–8] which uses phospholipid probes that contain an NBD group in one of the acyl chains. The assay starts with surface-differentiated vesicles which are prepared with NBD-phospholipids in either the membrane outer monolayer (exo-labeled) or inner monolayer (endo-labeled). Upon treatment with sodium dithionite ($Na_2S_2O_4$), the NBD fluorescence is quenched due to reduction of the NBD nitro group. Vesicle membranes are effectively impermeable to dithionite, therefore, only NBD-phospholipid located in the outer leaflet is chemically quenched. Exo-labeled vesicles, used to measure inward translocation (flip), are prepared by addition of a small aliquot of NBD-lipid (0.5 mol% of total phospholipid) in ethanol to a solution of unlabeled 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles. The NBD-lipid readily inserts into the vesicle outer monolayer. Endo-labeled vesicles, used for outward translocation (flop) experiments, are produced by



1 $R_1 = C_6H_4CH_3$, $R_2 = H$
2 R_1 = benzo-18-crown-6, $R_2 = H$
3 $R_1 = CF_3$, $R_2 = CH(CH_3)_2$



reacting symmetrically-labeled vesicles with dithionite, followed by vesicle filtration. At any given time, the percentage of exo NBD-phospholipid 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. The system progresses to an equilibrated state with the outer monolayer containing about 60% NBD-phospholipid.[6] All translocation measurements were conducted at pH 7.4 and 25 °C with 120 nm unilamellar vesicles that were prepared by membrane extrusion as previously described.[6,7]

The goal of this study was to find synthetic translocases that select for phosphatidylethanolamine over phosphatidylcholine. As a starting point, first-generation sulfonamide **1** was evaluated for its ability to translocate PC-NBD and PE-NBD across pure POPC vesicle membranes (25 μM phospholipid). In the absence of synthetic translocase, the inward translocation of both probes is very slow with half-lives of more than several hours. In the presence of sulfonamide **1** (38 μM), inward translocation is greatly accelerated but by similar amounts (translocation half-lives of both PC-NBD and PE-NBD are reduced to *ca.* 6 min). In other words, sulfonamide **1** does not apparently differentiate between the two phospholipid head-groups.

The first attempt to design a phosphatidylethanolamine-selective synthetic translocase was the sulfonamide crown ether **2**.† The benzo-18-crown-6 ring is well-known to associate strongly with ammonium cations in organic solvents and to associate weakly in aqueous solution.[9] Thus, it was reasoned that the sulfonamide NH residues in **2** would form hydrogen bonds with the phosphoethanolamine phosphate residue, while the crown ethers in **2** would associate with the ammonium moiety. As shown in Fig. 1, the presence of sulfonamide **2** (100 μM) has very little effect on inward PC-NBD translocation across POPC vesicle membranes (25 μM phospholipid), whereas the inward translocation of PE-NBD is decreased

DOI: 10.1039/b110357c

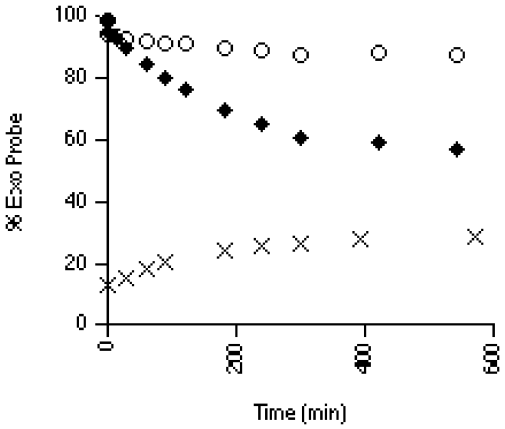


Fig. 1 Percent of probe in the exo (outer) leaflet of POPC vesicles (25 μM phospholipid) at 25 °C, pH 7.4. Inward translocation of PE-NBD (◆) and PC-NBD (○) induced by addition of **2** (100 μM) to exo-labeled vesicles. Outward translocation of PE-NBD (×) induced by addition of **2** (100 μM) to predominantly endo-labeled vesicles. Uncertainty for each point is ±3 percentage units.

significantly to a half-life of 100 min. Thus, compound **2** is able to discriminate between the phosphatidylethanolamine and phosphatidylcholine head-groups, although the phosphatidylethanolamine translocation half-life is still relatively long compared with that induced by **1**. Compared with **1**, the sulfonamide crown ether **2** is a larger molecule with increased amphiphilicity which means it probably takes longer to diffuse through the hydrophobic interior of the vesicle membrane. This also explains why the rate of outward translocation induced by **2** is about five times slower than inward translocation (Fig. 1) – compound **2** must first migrate through the membrane before it can translocate phosphatidylethanolamine to the outer surface. Control experiments performed with **2** indicate that it does not induce vesicle leakage of entrapped calcein or carboxyfluorescein, nor does it influence vesicle size as monitored by dynamic light scattering.

A much more effective synthetic phosphatidylethanolamine translocase is the tren sulfonamide **3**.[10] As shown in Fig. 2, the presence of only 8 μM of **3** reduces the half-life for inward and outward translocation of PE-NBD across POPC vesicle membranes (25 μM phospholipid) to approximately 5 min, whereas there is virtually no inward translocation of PC-NBD.‡ Control experiments show that **3** does not induce any vesicle leakage of aqueous dye contents.

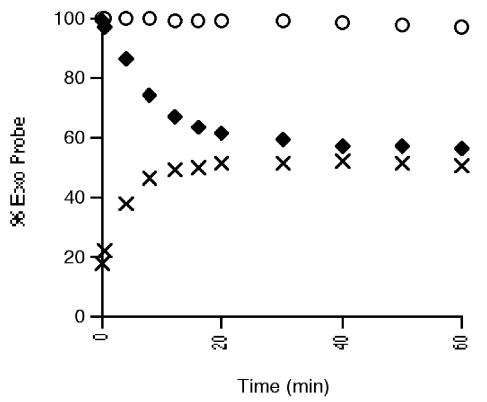


Fig. 2 Percent probe located in the exo (outer) leaflet of POPC vesicles (25 μM phospholipid) at 25 °C, pH 7.4. Inward translocation of PE-NBD (◆) and PC-NBD (○) induced by addition of **3** (8 μM) to exo-labeled vesicles. Outward translocation of PE-NBD (×) induced by addition of **3** (8 μM) to predominantly endo-labeled vesicles. Each point represents the average of three separate experiments, with uncertainty of ±3 percentage units.

The extraordinary effectiveness of **3** to act as a phosphatidylethanolamine-selective translocase is likely due to a combination of structural features. Not only are the sulfonamide hydrogens quite acidic due to the electron withdrawing effect of the trifluoromethyl groups, but the isopropyl chains located on the backbone enhance lipophilicity. The selectivity cannot be attributed to a binding preference for phosphatidylethanolamine over phosphatidylcholine by the neutral form of **3**. In fact, the reverse is true. NMR titration experiments show that **3** binds to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) in $CDCl_3$ at 25 °C with an association constant of 20 M^{-1}, whereas it binds to POPC with an association constant of 2500 M^{-1}. A plausible alternative explanation is that **3** exists as a zwitterionic species on the polar membrane surface due to proton transfer from one of the acidic trifluoromethylsulfonamide NH groups to the central tertiary amine.§ Molecular models suggest that this flexible zwitterionic version of **3** can adopt conformations that nicely complement the structure of the phosphatidylethanolamine head-group. Future studies will attempt to confirm this binding hypothesis.

In summary, the tren sulfonamides **2** and **3** selectively facilitate transbilayer migration of phosphatidylethanolamine over phosphatidylcholine. This differs from compound **1** which translocates both phospholipid head-groups equally. Compounds **2** and **3** may have potential uses in biomembrane research as synthetic phosphatidylethanolamine translocases.[5,7]

This work was supported by the National Institutes of Health and the University of Notre Dame.

Notes and references

† Compound **2** was prepared in 60% yield from tris(aminoethyl)amine and chlorosulfonylbenzo-18-crown-6; mp 168–172 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.57 (d, 3H, *J* 8.1 Hz), 7.50 (s, 3H), 6.99 (d, 3H, *J* 8.2 Hz), 6.21 (s, br, 3H), 4.25 (m, 12H), 3.93 (m, 6H), 3.71 (m, 42H), 2.91 (s, br, 6H), 2.57 (s, br, 6H); ^{13}C NMR (75MHz, $CDCl_3$): δ 151.9, 148.5, 131.5, 121.2, 112.1, 111.3, 70.6, 70.5, 70.4, 69.2, 69.1, 68.7, 68.5, 53.9, 40.9; HRMS *m/z* calc. for $C_{54}H_{85}N_4O_{24}S_3$ 1269.4716, found 1270.4793.

‡ As previously observed with sulfonamide **1**,[6] the crown derivative **2** (100 μM) is unable to facilitate inward translocation of PE-NBD across vesicle membranes composed of 1 : 1 POPC : POPE (25 μM). This inhibitory effect is attributed to the presence of intermolecular hydrogen bonds between the POPE ammonium and neighboring phosphate head-groups. Sulfonamide **3** (8 μM), however, is still able to translocate PE-NBD effectively across 1 : 1 POPC : POPE vesicle membranes although the half-life is five times slower than in pure POPC vesicles.

§ The pK_a of compound **3** is not yet known but the pK_a of trifluoromethylsulfonamide in water is 6.3.[11]

1 A. Zachowski, *Biochem. J*., 1993, **294**, 1.
2 D. L. Daleke and J. V. Lyles, *Biochim. Biophys. Acta*, 2000, **1486**, 108.
3 E. Fattal, S. Nir, R. A. Parente and F. C. Szoka, *Biochemistry*, 1994, **33**, 6721; K. Matsuzaki, O. Murase, N. Fuji and K. Miyajima, *Biochemistry*, 1996, **35**, 11 361; S. Frickenhous and R. Heinrich, *J. Theor. Biol*., 1999, **197**, 175.
4 S. Bhattacharya, R. A. Moss, H. Ringsdorf and J. Simon, *J. Am. Chem. Soc*., 1993, **115**, 13 812; S. Bhattacharya, R. A. Moss, H. Ringsdorf and J. Simon, *Langmuir*, 1997, **13**, 1869.
5 S. S. Davies, A. V. Pontsler, G. K. Marathe, G. K. Harrison, K. A. Murphy, J. C. Hinshaw, G. D. Prestwich, A. St Hilaire, S. M. Prescott, G. A. Zimmerman and T. M. McIntyre, *J. Biol. Chem*., 2001, **276**, 16 015; M. Umeda and K. Emoto, *Chem. Phys. Lipids*, 1999, **101**, 81.
6 J. M. Boon and B. D. Smith, *J. Am. Chem. Soc*., 1999, **121**, 11 924.
7 J. M. Boon and B. D. Smith, *J. Am. Chem. Soc*., 2001, **123**, 6221.
8 J. C. McIntyre and R. G. Sleight, *Biochemistry*, 1991, **30**, 11 819; C. Balch, R. Morris, E. Brooks and R. G. Sleight, *Chem. Phys. Lipids*, 1994, **70**, 205.
9 R. M. Izatt, K. Pawlak, J. S. Bradshaw and R. L. Bruening, *Chem. Rev*., 1991, **91**, 1721.
10 M. Cernerud, H. Adolfsson and C. Moberg, *Tetrahedron: Asymmetry*, 1997, **8**, 2655.
11 F. G. Bordwell, *Acc. Chem. Res*., 1988, **21**, 456.