J|A|C|S
COMMUNICATIONS

Published on Web 04/19/2002

Facilitated Phospholipid Flip-Flop Using Synthetic Steroid-Derived Translocases

Timothy N. Lambert,[†] J. Middleton Boon,[†] Bradley D. Smith,*[,†] M. Nieves Pérez-Payán,[‡] and Anthony P. Davis*[,‡,§]

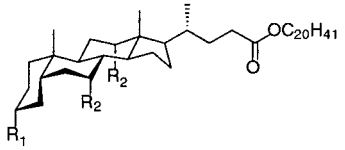
Department of Chemistry and Biochemistry and Walther Cancer Research Center, University of Notre Dame, Notre Dame, Indiana 46556, Department of Chemistry, Trinity College, Dublin 2, Ireland, and School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK

Received February 25, 2002

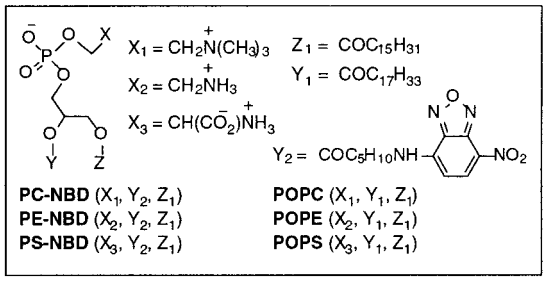
It is well-established that lateral diffusion of a phospholipid through one leaflet of a bilayer membrane is very rapid, whereas translocation of a phospholipid from one side of the bilayer to the other (a process also known as flip-flop) is quite slow.[1] In most animal cells, phospholipid translocation is controlled by membrane-bound proteins called translocases.[2] Some of these translocases are energy-consuming, active-transport systems, which means they produce transmembrane phospholipid distributions that are not symmetric. These asymmetric distributions control a range of important cellular functions such as signal transduction, membrane fusion, blood coagulation, and cell apoptosis.[3] We are attempting to develop low-molecular-weight synthetic translocases that promote phospholipid translocation and thus alter transmembrane phospholipid distributions. Synthetic translocases should be useful as pharmaceuticals or as chemical tools for biological membrane research.[4] Previously, we have reported that tris(2-aminoethyl)amine (tren)-derived translocases can facilitate phosphatidylcholine translocation across synthetic vesicle and erythrocyte membranes.[5] Mechanistic studies indicate that these synthetic translocases form hydrogen-bonded complexes with the highly polar phosphate portion of the zwitterionic phosphocholine headgroup which promotes diffusion across the lipophilic interior of the membrane.[5d] This current contribution describes the development of a new series of significantly more active synthetic translocases derived from cholic acid.[6]

Cholate derivatives **1**−**5** were prepared using previously reported methods.[7] Phospholipid translocation was monitored via the well-established 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite quenching assay which uses probe phospholipid containing an NBD group in one of its acyl chains.[5,8] Exo-labeled vesicles were 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 outer monolayer of the fluid-phase vesicles. 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. 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.

All translocation measurements were conducted in 5 mM TES/100 mM NaCl buffer at pH 7.4 and 25 °C with 120 nm unilamellar vesicles prepared by membrane extrusion.[5]



1 R_1 = O(CO)CH_3, R_2 = NH(CO)NHC_6H_5
2 R_1 = H, R_2 = NH(CO)NHC_6H_5
3 R_1 = O(CO)CH_3, R_2 = NH(CO)CH_3
4 R_1 = O(CO)CH_3, R_2 = NH_2
5 R_1 = OH, R_2 = OH



PC-NBD (X_1, Y_2, Z_1) **POPC** (X_1, Y_1, Z_1)
PE-NBD (X_2, Y_2, Z_1) **POPE** (X_2, Y_1, Z_1)
PS-NBD (X_3, Y_2, Z_1) **POPS** (X_3, Y_1, Z_1)

The inward translocation of PC-NBD across vesicle membranes composed of POPC and 5 mol % of one of the "membrane-anchored" translocase candidates **1**−**5** is illustrated in Figure 1. It is clear that the bis(phenylurea) derivatives **1** and **2** are very efficient translocases (they are approximately an order of magnitude more active than our previously reported tren-derived systems[5]) with translocation half-lives of ∼15 min and expected final equilibrium states of 60% PC-NBD probe in the outer monolayer. The failure of control compounds **3**−**5** to facilitate any phospholipid translocation suggests that **1** and **2** do not simply disrupt the bilayer structure and generate local "flip sites".[9] Moreover, vesicle leakage experiments show that large fluorescent dyes (e.g., calcein) are retained inside POPC vesicles containing compounds **1**−**5** (5 mol %) for at least 12 h. Concentration studies with bis-urea **1** indicate that observed PC-NBD translocation rate constants have a first-order dependence on translocase concentration. The membrane-anchored translocases **1**−**5** were also evaluated for their abilities to translocate PE-NBD and PS-NBD across POPC vesicle membranes. With compounds **1** or **2** (5 mol %) the order of facilitated translocation is PC-NBD ($t_{1/2} \approx 15$ min) > PE-NBD ($t_{1/2} \approx 50$ min) ≫ PS-NBD ($t_{1/2} > 180$ min), whereas compounds **3**−**5** do not improve inward translocation of any phospholipid probe.

* To whom correspondence should be addressed. E-mail: smith.115@nd.edu.
† University of Notre Dame.
‡ Trinity College Dublin.
§ Present address, University of Bristol.

10.1021/ja026022y CCC: $22.00

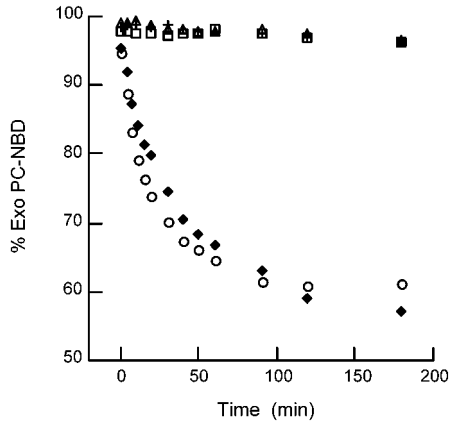
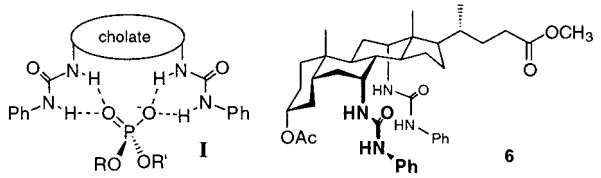


Figure 1. Percent exo PC-NBD in the outer leaflet of POPC vesicles (25 μM) containing 5 mol % of **1** (◆), **2** (○), **3** (□,), **4** (×), or **5** (△) in 5 mM TES/100 mM NaCl buffer at pH 7.4, 25 °C. Each point represents the average of three separate experiments with an uncertainty of ±4 percentage units.

In an effort to gain structural insight, a series of NMR titration studies were conducted.[5b] The binding of POPC to **1** or **2** in $CDCl_3$ is too strong ($>10^5$ M^{-1}) to be determined quantitatively by NMR; however, the complexed-induced changes in chemical shift are consistent with the 1:1 supramolecular complex **I**.[10] In the case of bis(amide) **3**, a relatively weak POPC association constant of 44 M^{-1} was obtained under the same conditions. Attempts to measure binding of phosphatidylethanolamine and phosphatidylserine by NMR titration methods were hampered by self-aggregation of these aminophospholipids, although qualitatively it appeared that binding to the translocases was quite weak. This conclusion was confirmed by a series of UV titration studies in 99:1 $CHCl_3/CH_3OH$. Addition of POPC to translocase **1** produces an increase in translocase absorbance, and an association constant of $(1.2 \pm 0.1) \times 10^5$ M^{-1} was extracted using nonlinear methods.[11,12] In contrast, addition of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) to **1** produced such slight changes in absorbance that association constants could not be determined reliably. We tentatively attribute this very weak binding to inter- or intramolecular hydrogen bonding involving the cationic ammonium and anionic phosphate (and carboxylate) residues.



For employment as a pharmaceutical or as a reagent for cell biology,[4] a synthetic translocase needs to be formulated so that it can be delivered to a biological sample. With this goal in mind we prepared the partially water-soluble, methyl cholate bis(phenylurea) **6** and evaluated its ability to promote PC-NBD translocation across erythrocyte cell membranes. As shown in the Supporting Information, cholate-derived **6** is significantly more effective as a phosphatidylcholine translocase than our previously reported tren-derived sulfonamide system.

In summary, cholate bis(phenylureas) are highly effective promoters of phosphatidylcholine translocation across vesicle and cell membranes. They are approximately an order of magnitude more active than our previously reported tren-derived systems.[5] The urea side chains are essential for strong binding of the highly polar phosphate portion of the headgroup and apparently cannot be replaced by simple amide, alcohol, or amine moieties. A future goal is to develop cholate-derived translocases with increased activity for phosphatidylserine and phosphatidylethanolamine. Our previous success with the tren-derived translocases,[5c] plus the knowledge that naturally occurring binders of phosphatidylserine[13] and phosphatidylethanolamine[14] are known, augurs well for this effort.

Acknowledgment. This work was supported by the National Institutes of Health (GM 59078), the Walther Cancer Research Center, the University of Notre Dame, the European Commission, and the EPSRC (GR/R04584/01).

Supporting Information Available: Synthetic procedures, UV titrations, and translocation data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Rothman, J. E.; Dawidowicz, E. A. *Biochemistry* **1975**, *14*, 2809−2816. (b) Moss, R. A. *Pure Appl. Chem.* **1994**, *66*, 851−858. (c) Kornberg, R. D.; McConnell, H. M. *Biochemistry* **1971**, *10*, 1111−1120.
(2) Daleke, D. L.; Lyles, J. V. *Biochim. Biophys. Acta* **2000**, *1486*, 108−127.
(3) (a) Boon, J. M.; Smith, B. D. *Med. Res. Rev.* **2002**, *22*, 251−281. (b) Fadok, V. A.; de Cathelineau, A.; Daleke, D. L.; Henson, P. M.; Bratton, D. L. *J. Biol. Chem.* **2001**, *276*, 1071−1077. (c) Bevers, E. M.; Comfurius, P.; Dekkers, D. W. C.; Harmsma, M.; Zwaal, R. F. A. *Biol. Chem.* **1998**, *379*, 973−986. (d) Bevers, E. M.; Comfurius, P.; Dekkers, D. W. C.; Harmsma, M.; Zwaal, R. F. A. *Lupus* **1998**, *7*, S126−S131. (e) Bratton, D. L.; Fadok, V. A.; Richter, D. A.; Kailey, J. M.; Guthrie, L. A.; Henson, P. M. *J. Biol. Chem.* **1997**, *272*, 26159−26165.
(4) Recently, one of our tren-derived translocases was used as a biological tool in the study of the peroxisome proliferator-activated receptor γ (PPARγ). Davies, S. S.; Pontsler, A. V.; Marathe, G. K.; Harrison, K. A.; Murphy, R. C.; Hinshaw, J. C.; Prestwich, G. D.; Hilaire, A. St.; Prescott, S. M.; Zimmerman, G. A.; McIntyre, T. M. *J. Biol. Chem.* **2001**, *276*, 16015−16023.
(5) (a) Boon, J. M.; Smith, B. D. *J. Am. Chem. Soc.* **2002**, *124*, 11924−11925. (b) Boon, J. M.; Smith, B. D. *J. Am. Chem. Soc.* **2001**, *123*, 6221−6226. (c) Boon, J. M.; Shukla, R.; Smith, B. D.; Licini, G.; Scrimin, P. *Chem. Commun.* **2002**, 260−261. (d) Boon, J. M.; Lambert, T. N.; Smith, B. D.; Beatty, A. M.; Ugrinova, V.; Brown, S. N. *J. Org. Chem.* **2000**, *67*, 2168−2174..
(6) For recent examples of cholic acid derived supramolecular devices, see: (a) Janout, V.; Staina, I. V.; Bandyopadhya, P.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 9926−9927. Bandyopadhya, P.; Janout, V.; Zhang, L.-H.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 7691−7696. (b) Goto, C.; Yamamura, M.; Satake, A.; Kobuke, Y. *J. Am. Chem. Soc.* **2001**, *123*, 12152−12159. (c) Vandenburg, Y. R.; Smith, B. D.; Pérez-Payán, M. N.; Davis, A. P. *J. Am. Chem. Soc.* **2000**, *122*, 3252−3253. De Muynck, H.; Madder, A.; Farcy, N.; De Clerq, P. J.; Pérez-Payán, M. N.; Öhberg, L. M.; Davis, A. P. *Angew. Chem., Int. Ed.* **2000**, *39*, 145−148. (d) Guan, Q.; Li, C.; Schmidt, E. J.; Boswell, J. S.; Walsh, J. P.; Allman, G. W.; Savage, P. B. *Org. Lett.* **2000**, *2*, 2837−2840. (e) Cheng, Y.; Suenaga, T.; Still, W. C. *J. Am. Chem. Soc.* **1996**, *118*, 1813−1814.
(7) (a) Davis, A. P.; Pérez-Payán, M. N. *SynLett* **1999**, 991−993. (b) Ayling, A. J.; Pérez-Payán, M. N.; Davis, A. P. *J. Am. Chem. Soc.* **2001**, *123*, 12716−12717.
(8) (a) McIntyre, J. C.; Sleight R. G. *Biochemistry* **1991**, *30*, 11819−11827. (b) Moss, R. A.; Bhattacharya, S. *J. Am. Chem. Soc.* **1995**, *117*, 8688−8689.
(9) For synthetic translocases that operate by generating local "flip sites", see: (a) Bhattacharya, S.; Moss, R. A.; Ringsdorf, H.; Simon, J. *Langmuir* **1997**, *13*, 1869−1872. (b) Kol, M. A.; De Kroon, A. I. P. M.; Rikjers, D. T. S.; Killian, J. A.; De Kruijff, B. *Biochemistry* **2001**, *40*, 10500−10506.
(10) Addition of POPC to **1** or **2** induces all four urea protons to undergo large downfield movements in chemical shift (∼2 ppm) and substantial changes in the cholate *N*-phenyl signals. The sharp titration end point clearly indicates a 1:1 stoichiometry, but we presently cannot rule out an alternative complex structure described in Bühlmann, P.; Nishizawa, S.; Xiao, K. P.; Umezawa, Y. *Tetrahedron* **1997**, *53*, 1647−1654.
(11) Xie, H.; Yi, S.; Wu, S. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2751−2754.
(12) A similar association constant has been obtained with an alternative synthetic phosphatidylcholine receptor. Magrans, J. O.; Ortiz, A. R.; Molins, M. A.; Lebouille, P. H. P.; Sánchez-Quesada, J.; Prados, P.; Pons, M.; Gago, F.; De Mendoza, J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1712−1715.
(13) (a) Williamson, P.; Van Den Eijnde, S.; Schlegel, R. A. *Methods Cell Biol.* **2001**, *66*, 339−364. (b) Rauch, J.; Janoff, A. S. *Lupus* **1996**, *5*, 498−502.
(14) Aoki, O.; Uenaka, T.; Aoki, J.; Umeda, M.; Inoue, K. *J. Biochem.* **1994**, *116*, 291−297.

JA026022Y