



Anion transport across varying lipid membranes – the effect of lipophilicity

Journal:	<i>ChemComm</i>
Manuscript ID:	CC-COM-01-2015-000823.R1
Article Type:	Communication
Date Submitted by the Author:	12-Feb-2015
Complete List of Authors:	Gale, Philip; University of Southampton, School of Chemistry Spooner, Michael; University of Southampton, Chemistry

COMMUNICATION

Anion transport across varying lipid membranes – the effect of lipophilicity†

Cite this: DOI: 10.1039/x0xx00000x

Michael J. Spooner^a and Philip A. Gale^{a,*}Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

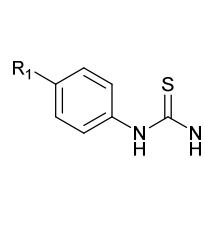
www.rsc.org/

The anion transport properties of a range of alkyl-substituted phenylthioureas were tested in vesicles of different lipid composition. Although changes in the bilayer affected the rate of transport for all compounds in the series, the ‘ideal’ log P for peak activity did not change depending on the composition of the bilayers tested.

The development of small-molecule transmembrane anion carriers has attracted significant attention recently, as these compounds have potential as future therapeutics for treatment of conditions such as cystic fibrosis in addition to being useful tools for the study of transport processes across cell membranes.^{1,2} Transport studies are often carried out using large unilamellar vesicles (LUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).¹ Biological membranes, however, are significantly more complex, containing a wide range of phospholipids, sterols, proteins and other molecules.^{3,4} Little work has been done to directly compare the performance of synthetic anion transporters in different lipid environments.

The lipophilicity of an anion carrier is known to affect its transport activity^{5,6} and calculated log P (clogP) values (the *n*-octanol/water partition coefficient) have been shown to be key in determining the transport activity of simple thioureas.⁷ Additionally, Quesada *et al.* have shown that for the tambjamine class of transporters, there exists an optimum log P for transport.⁸

It is known that energy barriers exist for solutes crossing lipid bilayers that vary in magnitude depending on lipid composition. In particular, computational studies have shown that the magnitude of these barriers affect the partitioning of solutes into the head or tail regions of the bilayer depend on the hydrophobic nature of the solute⁹. We wished to investigate whether similar effects could affect the ideal log P for anion receptors in different lipid bilayers therefore



#	R ₁	R ₂	clog P	#	R ₁	R ₂	clog P
1	H	Et	2.19	9	Pe	Pe	5.94
2	Me	Et	2.68	10	Pe	Hx	6.40
3	Et	Et	3.14	11	Hx	Hx	6.85
4	Et	Pr	3.66	12	Hx	Hp	7.31
5	Pr	Pr	4.12	13	Hp	Hp	7.77
6	Pr	Bu	4.57	14	Hp	Oc	8.22
7	Bu	Bu	5.03	15	Oc	Oc	8.68
8	Bu	Pe	5.49				

Fig. 1 General structure and calculated log P values for the thiourea series. †

we investigated how the transport activity of a series of simple phenylthiourea molecules varied between bilayers formed of different lipids.

A range of thioureas (**Fig. 1**) was synthesised with an increasing length of alkyl substituent, such that the set of compounds spanned the full range of log P values from 2 to 8.5. Thioureas were chosen as they are simple yet highly effective anion transporters.^{7,10} The length of the alkyl chains was increased in a symmetrical manner, in accordance with the recently described principle of lipophilic balance.¹⁰ The compounds were synthesised from the appropriate isothiocyanates and anilines and full synthetic details are given in the ESI†. Compounds **1**¹¹, **2**¹¹, **3**¹² & **10**⁷ have been previously reported.

The association constants for anion binding were determined for compounds **1**, **7** & **15** by ¹H NMR titration in DMSO-*d*₆/0.5% H₂O using the WINEQNMNR2 program¹³. The results are shown in Table 1. As expected the binding affinities did not vary significantly across the

Table 1 Anion binding constants for **1**, **7** & **15** and various TBA salts in DMSO-*d*₆/H₂O (0.5 %) at 298 K. Values calculated using WINEQNMNR2 assuming a 1:1 binding mode. Errors ≤ 10 %. See ESI for details†.

Guest \ Host	1	7	15
Cl ⁻	14	17	16
NO ₃ ⁻	<10	<10	<10
H ₂ PO ₄ ⁻	180	220	160
SO ₄ ⁻	200	220	250

^aChemistry, University of Southampton, Southampton SO17 1BJ, UK. Email: philip.gale@soton.ac.uk; Fax: +44 (0)2380 596805; Tel: +44 (0)2380 593332

†Electronic Supplementary Information (ESI) available: Experimental details of thiourea synthesis, transport studies and NMR titration data. See DOI: 10.1039/c000000x/

series, in line previously reported thioureas^{7,10}. Any change in relative rate is therefore not likely to be attributable to differences in anion binding.

Lipid vesicles were prepared by previously reported methods¹⁴⁻¹⁶. Chloride efflux from the vesicles was measured using a chloride ion-selective electrode. After 5 minutes, the vesicles were lysed to calibrate the readings to 100% efflux. Transport activity was quantified by a linear approximation, as described previously by Quesada *et al.*⁸

Initial transport rates were plotted against $\log P$ values obtained from the ALOGP³⁷ method (via the VCC Lab web applet^{18,19}, see Fig. 1). This model was chosen over a selection of other computational methods as it gave the best correlation with retention times obtained by RP-UHPLC (see ESI for details).²⁰

For POPC bilayers, the peak transport rate was observed for $\log P = 5-6$ (Fig. 3). The existence of the optimum $\log P$ range has been rationalised as being due to a balance between the receptor being lipophilic enough to cross the tail region of the membrane, without being so lipophilic as for delivery to the bilayer to be an issue²¹. It might be expected that if the effect were purely a partitioning effect then the optimum $\log P$ would be the same for the same bilayer composition. This result suggests otherwise as it is different from that found in studies of other sets of compounds⁸, and presumably the effect is more complex and the optimum $\log P$ range is specific to transporter class.

The experiments were also conducted in vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (Fig. 2), both having the same tail groups as POPC. PG lipids have a glycerol head-group in the place of the choline moiety in PC lipids and have an overall negative charge in the head-group region. Across the whole series, transport was slower across POPG bilayers (Fig. 3), possibly as the formation of the negatively charged chloride complex was disfavoured. The peak in activity with respect to $\log P$ remained unchanged.

POPE has a similar structure to POPC but lacks the three methyl groups appended to the nitrogen atom (Fig. 2). Vesicles of a 3:1 POPE:POPC ratio were used as POPE will not form vesicles on its own (due to the higher transition temperature for this lipid and the likelihood of forming a combination of gel and liquid crystalline phases at the concentrations used in these experiments²²). In contrast to POPG, the transport rate across the mixed lipid bilayer was significantly faster than that across the pure POPC bilayer for the whole series (Fig. 3), where it might be expected that increased head-group – head-group interactions for PE would increase the energy barrier in the polar region of the membrane⁹. Instead, the transporter molecules may be relieving some potential energy introduced by inclusion of the non-bilayer-forming lipid in the membrane³. Again, the peak in activity did not appear to change with respect to $\log P$, although 6 & 10 also matched the rate of 7, 8 & 9, suggesting that a different rate-determining step in transport may be operating in this system.

To investigate the effect of changing the lipid tail group, the screening was repeated using a fully-saturated PC lipid DPPC (Fig. 2). These experiments were carried out at higher temperature due to the higher phase transition temperature of DPPC. The rate across the series was not significantly different from the POPC control (Fig. 4). This would suggest that the rate-limiting step for transport is not the diffusion across the tail region of the bilayer.

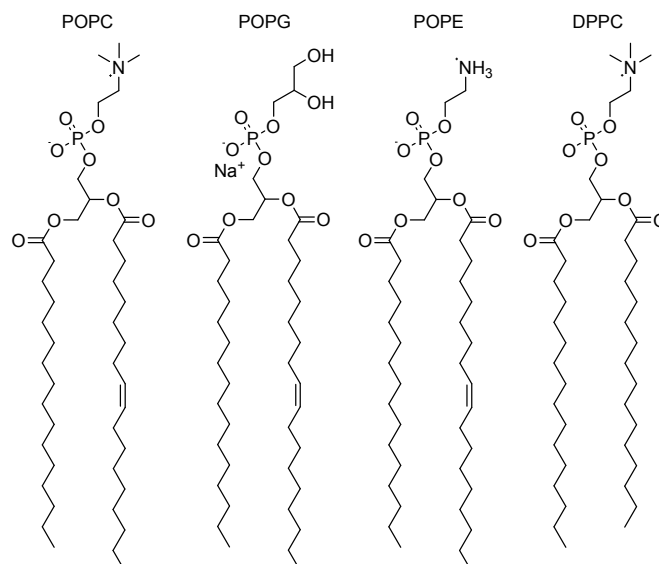


Fig. 2 Structures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC).

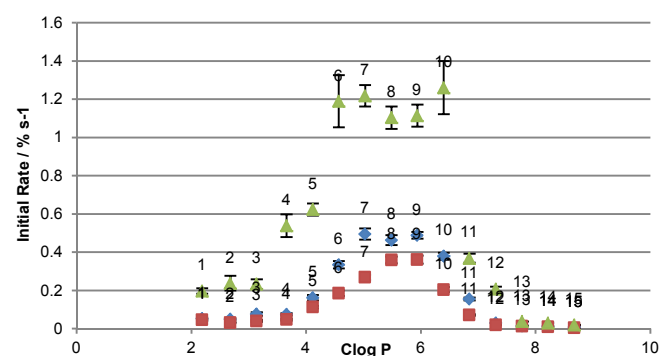


Fig. 3 Initial rate of chloride efflux by 1 – 15 (1% loading wrt. lipid) from various lipid vesicles (containing 489 mM NaCl, buffered to pH 7.2 with 5mM sodium phosphate salts), plotted against $\log P$ of the transporter molecule. Vesicles suspended in 489 mM NaNO₃, buffered to pH 7.2 with 5 mM sodium phosphate salts. Blue diamonds: 100 % POPC; red squares: 100 % POPG; green triangles: 3:1 POPE:POPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

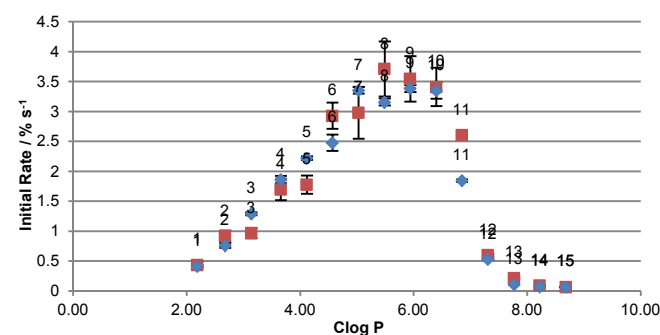


Fig. 4 Initial rate of chloride efflux by 1 – 15 (1% loading wrt. lipid) from DPPC vesicles (containing 489 mM NaCl, buffered to pH 7.2 with 5mM sodium phosphate salts) at 55°C, plotted against $\log P$ of the transporter molecule. Vesicles suspended in 489 mM NaNO₃, buffered to pH 7.2 with 5 mM sodium phosphate salts. Blue diamonds: 100 % POPC; red squares: 100 % DPPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

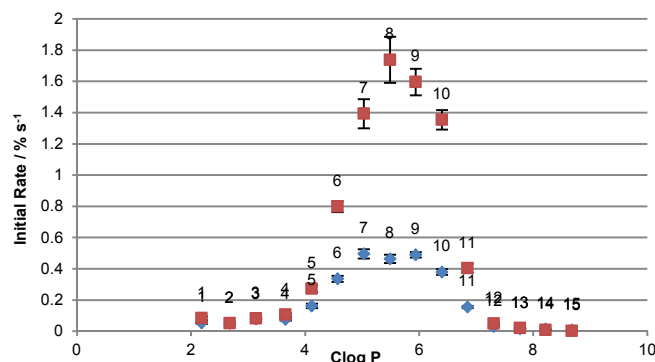


Fig. 5 Initial rate of chloride efflux by 1 – 15 (1% loading wrt. lipid) from 7:3 POPC:Chol vesicles (containing 489 mM NaCl, buffered to pH 7.2 with 5mM sodium phosphate salts), plotted against clog P of the transporter molecule. Vesicles suspended in 489 mM NaNO₃, buffered to pH 7.2 with 5 mM sodium phosphate salts. Blue diamonds: 100 % POPC; red squares: 7:3 POPC:Chol. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

Experiments were also conducted using POPC vesicles doped with cholesterol. The condensing and ordering effects of cholesterol (Chol) on the membrane are well described,²³ hence cholesterol assays have previously been used as evidence for a mobile carrier mechanism in anion transport^{5,24} (as slower diffusion across a more rigid membrane is evidence that supports a mobile carrier over a channel mechanism).

For this series of thioureas, transport rate increased ubiquitously in 7:3 POPC:Chol vesicles (**Fig. 5**). Crucially, the ideal lipophilicity range remained constant. This provides further evidence that diffusion across the tail region is not the rate-determining step, and in addition to other previous results^{23,25} that cholesterol assays are not sufficient evidence of a mobile carrier assay mechanism.

Finally, to explore any effect of the delivery of the compounds into the membrane, experiments in POPC/POPG/POPE:POPC 3:1 were repeated in the presence of external NaSO₄. The SO₄²⁻ ion has a high energy of solvation²⁶ and is generally considered not able to be transported through lipid bilayers mediated by small molecule transporters although there are compounds that have been shown to be capable of transporting this anion^{6,27}. After 2 minutes allowing the transporter to equilibrate with the SO₄²⁻ suspended vesicles, transport was initiated with the addition of a pulse of NaNO₃.

In the three systems, the relative peak in optimal log P was retained in the presence of sulfate (**Fig. 6**). In the POPG system, transport rates were dramatically reduced for all compounds. It has been proposed that glycerol –OH groups form hydrogen bonds to the lipid phosphate to screen the negative charge, packing lipid molecules closer together²⁸. It may be that the kosmotropic properties of sulfate are affecting the water lipid interface. More work needs to be done to confirm the origin of this effect.

A degree of Cl⁻ transport was observed in the 3:1 POPE:POPC system before the nitrate spike addition. In further assays, we found no direct evidence of sulfate transport or vesicle leakage. A slight dependence of chloride transport on the present cation was found however, indicating that MCl co-transport may make a minor contribution to the overall efflux of chloride. Slight de-acidification of the vesicle interior was also seen for the most active compounds in an HPTS pH assay, indicating a small amount of possible HCl transport. We suspect these two mechanisms may explain this effect in this system. This data is presented in full in the ESI†.

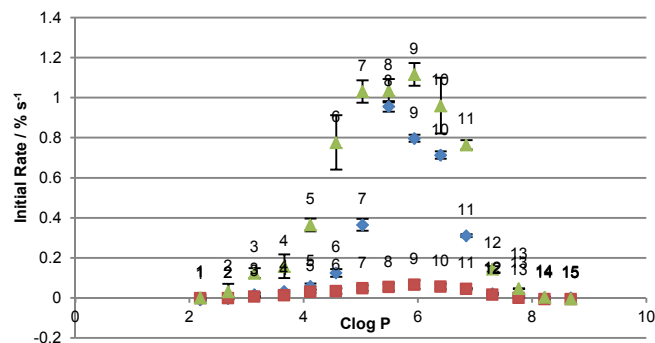


Fig. 6 Initial rate of chloride efflux by 1 – 15 (1% loading wrt. lipid) from various lipid vesicles (containing 489 mM NaCl, buffered to pH 7.2 with 5mM sodium phosphate salts) in the presence of external NaSO₄, plotted against clog P of the transporter molecule. Vesicles suspended in 162 mM Na₂SO₄ buffered to pH 7.2 with 5 mM sodium phosphate salts. Transport initiated with a pulse of NaNO₃ in external buffer, to a final concentration of 40 mM. Rate plotted against clog P of the transporter molecule. Blue diamonds: 100 % POPC; red squares: 100 % POPG; green triangles: 3:1 POPE:POPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

For the other systems, the compounds in the middle of the log P range appear to transport faster in the sulfate system. We attribute this to the longer equilibration time allowing majority of transporters to be ideally located within the membrane when transport is initiated, giving a boost to the initial rate. Conversely, those at the hydrophobic and hydrophilic extremes of the series transport more slowly. The more polar compounds may be involved in competitive binding to SO₄²⁻ at the interface, whilst the hydrophobic compounds are likely further confined to the tail region due to the strengthening of the hydrophobic effect by the sulphate ion.

Conclusions

We have shown that in the different bilayer systems tested, the ideal range of log P for maximum transport rate by this series of compounds does not change. We note that the ideal log P range appears to be a property of the transporter class and not of the membrane.

Optimising log P is still an important factor for maximising transport rate for a given compound series, although it is important to note that other design factors must determine the absolute rate in different lipid systems. We have shown that different lipid environments have a significant influence on transporter activity and it will be important in further work to identify what the limiting steps are for different receptor classes. This will aid more targeted molecular design for natural target membranes and help ensure that future model bilayers accurately represent the target cellular environment to avoid potentially misleading results.

Acknowledgements

We thank the EPSRC for a DTG studentship (MJS) and for EPSRC Core Capability Funding (EP/K039466/1). PAG thanks the Royal Society and the Wolfson Foundation for a Research Merit Award.

Notes and references

† Pe = Pentyl, Hx = Hexyl, Hp = Heptyl, Oc = Octyl

1. N. Busschaert and P. A. Gale, *Angew. Chem. Int. Ed.*, 2013, **52**, 1374-1382.
2. J. M. Tomich, U. Bukovnik, J. Layman and B. D. Schultz, *Channel Replacement Therapy for Cystic Fibrosis - Cystic Fibrosis - Renewed Hopes Through Research*, InTech, 2012.
3. D. E. Vance and J. E. Vance, *Biochemistry of Lipids, Lipoproteins and Membranes*, 2008.
4. G. van Meer, D. R. Voelker and G. W. Feigenson, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 112-124.
5. C. J. E. Haynes, S. J. Moore, J. R. Hiscock, I. Marques, P. J. Costa, V. Félix and P. A. Gale, *Chem. Sci.*, 2012, **3**, 1436-1444.
6. N. Busschaert, M. Wenzel, M. E. Light, P. Iglesias-Hernández, R. Pérez-Tomás and P. A. Gale, *J. Am. Chem. Soc.*, 2011, **133**, 14136-14148.
7. N. Busschaert, S. J. Bradberry, M. Wenzel, C. J. E. Haynes, J. R. Hiscock, I. L. Kirby, L. E. Karagiannidis, S. J. Moore, N. J. Wells, J. Herniman, G. J. Langley, P. N. Horton, M. E. Light, I. Marques, P. J. Costa, V. Félix, J. G. Frey and P. A. Gale, *Chem. Sci.*, 2013, **4**, 3036-3045.
8. V. Saggiomo, S. Otto, I. Marque, V. Félix, T. Torroba and R. Quesada, *Chem. Commun.*, 2012, **48**, 5274-5276.
9. C. L. Wennberg, D. v. d. Spoel and J. S. Hub, *J. Am. Chem. Soc.*, 2012, **134**, 5351-5361.
10. H. Valkenier, C. J. E. Haynes, J. Herniman, P. A. Gale and A. P. Davis, *Chem. Sci.*, 2014, **5**, 1128-1134.
11. W. Weith, *Berichte der deutschen chemischen Gesellschaft*, 1875, **8**, 1523-1530.
12. A. Yahyazadeh and Z. Ghasemi, *Eur. Chem. Bull.*, 2013, **2**, 573-575.
13. M. J. Hynes, *J. Chem. Soc., Dalton Trans.*, 1993, 311-312.
14. R. C. MacDonald, R. I. MacDonald, B. P. M. Menco, K. Takeshita, N. K. Subbarao and L. Hu, *Biochim. Biophys. Acta*, 1991, **1061**, 297-303.
15. B. D. Smith and T. N. Lambert, *Chem. Commun.*, 2003, 2261-2268.
16. A. V. Koulov, T. N. Lambert, R. Shukla, M. Jain, J. M. Boon, B. D. Smith, H. Li, D. N. J. Sheppard, Jean-Baptiste, J. P. Clare and A. P. Davis, *Angew. Chem. Int. Ed.*, 2003, **42**, 4931-4933.
17. V. N. Viswanadhan, A. K. Ghose, G. R. Revankar and R. K. Robins, *J. Chem. Inf. Comp. Sci.*, 1989, **29**, 163-172.
18. VCCLAB, Virtual Computational Chemistry Laboratory, <http://www.vcclab.org>.
19. I. V. Tetko, J. Gasteiger, R. Todeschini, A. Mauri, D. Livingstone, P. Ertl, V. A. Palyulin, E. V. Radchenko, N. S. Zefirov, A. S. Makarenko, V. Y. Tanchuk and V. V. Prokopenko, *J. Comput. Aided Mol. Des.*, 2005, **19**, 453-463.
20. OECD, *OECD Guidelines for the Testing of Chemicals, Section 1*, 2004.
21. C. J. E. Haynes, N. Busschaert, I. L. Kirby, J. Herniman, M. E. Light, N. J. Wells, I. Marques, V. Félix and P. A. Gale, *Org. Biomol. Chem.*, 2013.
22. R. Marinov and E. J. Dufourc, *Eur. Biophys. J.*, 1996, **24**, 423-431.
23. T. Róg, M. Pasenkiewicz-Gierula, I. Vattulainen and M. Karttunen, *Biochim. Biophys. Acta*, 2009, **1788**, 97-121.
24. P. A. Gale, *Acc. Chem. Res.*, 2011, **44**, 216-226.
25. N. Busschaert, I. L. Kirby, S. Young, S. J. Coles, P. N. Horton, M. E. Light and P. A. Gale, *Angew. Chem. Int. Ed.*, 2012, **51**, 4426-4430.
26. Y. Marcus, *J. Am. Chem. Soc., Faraday Trans.*, 1991, **87**, 2995-2999.
27. N. Busschaert, L. E. Karagiannidis, M. Wenzel, C. J. E. Haynes, N. J. Wells, P. G. Young, D. Makuc, J. Plavec, K. A. Jolliffe and P. A. Gale, *Chem. Sci.*, 2014, **5**, 1118-1127.
28. D. E. Elmore, *FEBS Lett.*, 2006, **580**, 144-148.