A transmembrane anion transporter selective for nitrate over chloride†

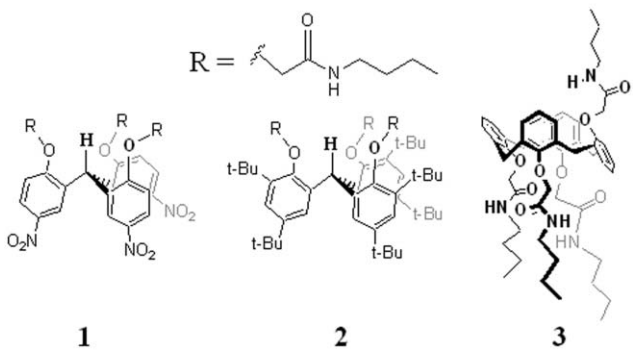
Paul V. Santacroce, Oluyomi A. Okunola, Peter Y. Zavalij and Jeffery T. Davis*

Received (in Austin, TX, USA) 23rd May 2006, Accepted 6th June 2006
First published as an Advance Article on the web 26th June 2006
DOI: 10.1039/b607221f

The C_3-symmetric triamide 1 selectively transports NO_3^- anions across lipid vesicles: this H^+–NO_3^- co-transporter alters the pH inside of liposomes experiencing a NO_3^-/Cl^- gradient.

Identification of transmembrane chloride transporters is important, given this anion's role in human health.[1] A number of compounds, including peptides, lipopeptides, sterols, and pyrroles transport Cl^- across phospholipid membranes.[2–6] During a survey of potential Cl^- transporters we discovered that tris(5-nitro-2-butylamidomethoxyphenyl) methane **1** selectively transports NO_3^-, rather than Cl^-, across phospholipid vesicles. While many neutral receptors bind the nitrate anion,[7,8] this is the first report of a compound that shows such selectivity for the transmembrane transport of NO_3^- over Cl^-.[9] Since nitrate is an environmentally important anion, for plants and animals,[10,11] compounds that selectively transport NO_3^- across membranes may find some key applications.

We previously demonstrated that amide functionalized calix[4]-arenes and acyclic oligophenoxyacetamides transport Cl^- across membranes.[12–14] The most effective of these Cl^- transporters were the partial cone calix[4]arene (paco **3**),[14] and an acyclic trimer of phenoxyacetamide.[13] To explore how the geometry of ligands with three hydrogen-bond amide NH donors influences the anion binding and transport, we turned to C_3-symmetric receptors based on the triphenoxymethane (TPM) core.



1 **2** **3**

TPM derivatives have been reported by Dinger and Scott to bind metal cations.[15–17] In addition, Böhmer's group recently showed that TPM analogs with urea groups form dimers.[18] The promising molecular recognition properties of these TPM compounds led us to prepare nitro tripod **1** and tris(3,5-di-*tert*-butyl-2-butylamidomethoxyphenyl) methane **2**. We reasoned that TPM derivatives **1** and **2**, like calixarene **3**, might transport anions across phospholipid membranes.

Tripods **1** and **2** were prepared in 2 steps. Acid-catalyzed condensation of 5-nitrosalicylaldehyde and *p*-nitrophenol gave a known triphenol,[18] which was alkylated with 2-bromo-*N*-butyl-acetamide to give nitro tripod **1**. A similar protocol gave *t*-butyl tripod **2**.[15] A single crystal X-ray analysis of nitro tripod **1** revealed that its three secondary amide chains point in the same direction, creating a binding site for anions (Fig. 1).[19] Negative mode ESI-MS showed that **1** and **2** indeed formed 1 : 1 complexes with Cl^- and NO_3^-, when the tripods were mixed with tetrabutylammonium (TBA) salts in CH_2Cl_2. ESI-MS competition experiments revealed that nitro tripod **1** was modestly selective for binding Cl^- over NO_3^-, whereas *t*-butyl tripod **2** had no Cl^-/NO_3^- selectivity.

The anion binding properties of **1** and **2** in CD_2Cl_2 solution were evaluated by ^1H NMR titration with TBACl and $TBANO_3$. No chemical shift changes occurred upon addition of TBA salts to solutions of **2**, indicating that the *t*-butyl tripod binds weakly to Cl^- and NO_3^-. In contrast, nitro tripod **1** coordinated Cl^- and NO_3^- in CD_2Cl_2, as indicated by downfield shifts for the amide NH that hydrogen bonds with the anions ($\Delta\delta$ = 2.32 ppm for Cl^- and 1.37 ppm for NO_3^-). In addition, the bridgehead CH near the anion binding site also underwent a significant downfield shift in the presence of these TBA salts. In CD_2Cl_2, nitro tripod **1** binds Cl^- (K_a = 816 ± 108 M^{-1}) more strongly than NO_3^- (K_a = 326 ± 113 M^{-1}).[20] Overall, the X-ray, ESI-MS and NMR data for tripod **1** confirmed that the TPM scaffold can be used to develop an anion receptor.

We tested whether tripods **1** and **2** could transport Cl^- or NO_3^- across EYPC vesicles.‡ In these experiments we compared the

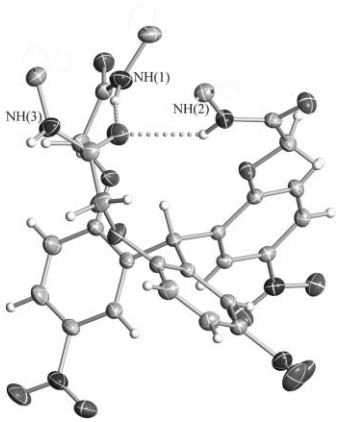


Fig. 1 Depiction of the X-ray crystal structure of nitro tripod **1**. The *n*-butyl sidechains have been removed for clarity. All three amide sidechains point in the same direction, providing a potential anion binding pocket. Two of the NH groups are hydrogen bonded to the amide C=O group of the third chain.

Dept. of Chemistry and Biochemistry, University of Maryland, College Park, MD, 20742, USA. E-mail: jdavis@umd.edu; Tel: +1 301-405-1845
† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b607221f

transport properties of **1** and **2** with those for paco **3**, a known Cl^- transporter.[14] We first used the classical "base pulse" assay to compare transmembrane transport properties for **1–3**.[12,21] In this assay, activity is measured by monitoring pH changes inside a liposome containing the HPTS dye. Addition of NaOH creates a pH gradient across the membrane. If a compound mediates transmembrane ion transport (*via* either cation influx or anion efflux), then the intravesicular pH increases as monitored by HPTS fluorescence. If the compound cannot transport ions there is no change in HPTS fluorescence.

Our standard, paco **3**,[14] changed HPTS fluorescence in solutions containing either NaCl or $NaNO_3$ (Fig. 2) indicating that **3** can transport both Cl^- and NO_3^- anions across EYPC liposomes. Fig. 2A shows that tripods **1** and **2** (at 2 : 100 molar ratio to the lipid) had little ability to transport Cl^-, a finding confirmed in experiments that used a chloride dye, lucigenin,[22] to monitor intravesicular Cl^- concentration (Fig. 5 in the ESI†).

Fig. 2B shows that nitro tripod **1** transports NO_3^- anions across EYPC membranes as effectively as paco **3**. The *t*-butyl tripod **2**, on the other hand, is not a NO_3^- transporter, as there was little change in the intravesicular pH upon addition of **2**. These base-pulse experiments provided the first indication that nitro tripod **1** might be a transmembrane NO_3^- anion transporter.

We confirmed that nitro tripod **1** transports NO_3^- across phospholipid membranes. As depicted in Fig. 3A we developed an assay that uses nitrate reductase to monitor the release of NO_3^- from EYPC vesicles.[23] Nitrate reductase uses the NADPH cofactor to reduce nitrate to nitrite. NADPH has a characteristic absorbance band at 340 nm, whereas the oxidized cofactor $NADP^+$ does not absorb in this region. Release of NO_3^- from vesicles was followed by changes in the NADPH absorbance at 340 nm. Furthermore, the enzymatically generated nitrite anion, NO_2^-, was trapped to give diazo dye **4** in the Griess reaction (Fig. 3A).[23] This dye absorbs at 543 nm. Thus, a decrease in

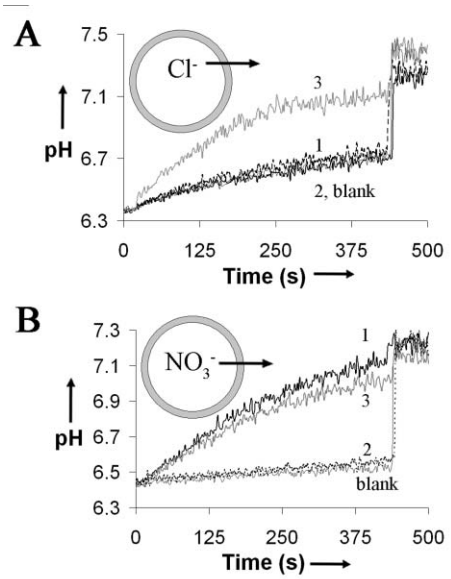


Fig. 2 Base-pulse experiments. (A) Chloride containing EYPC liposomes (pH 6.4) in Cl^- solution (pH 7.4). (B) Nitrate containing EYPC liposomes (pH 6.4) in NO_3^- solution (pH 7.4). At $t = 0$ s, DMSO solutions of **1–3** were added and the intravesicular pH was determined by monitoring changes in HPTS fluorescence ratios.

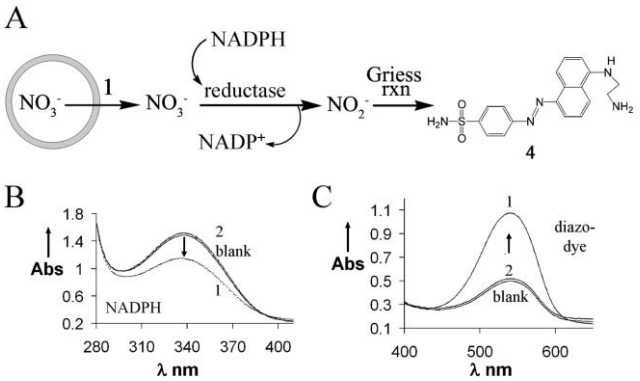


Fig. 3 Transmembrane transport of NO_3^-. (A) Tripod **1** transports NO_3^- out of EYPC vesicles suspended in NaCl solution. Nitrate reductase reduces extravesicular NO_3^- to NO_2^-. The resulting NO_2^- is then trapped to give diazo dye **4**. (B) UV spectrum of EYPC vesicle suspension after addition of **1**, **2** or a DMSO blank. The arrow indicates a decrease in NADPH absorbance at 340 nm in the presence of **1**. (C) UV spectrum of **4** after Griess reaction of enzymatically produced NO_2^-. Arrow indicates increase in absorbance for diazo dye **4** in the presence of nitro tripod **1**.

NADPH absorbance at 340 nm and a concomitant increase in the 543 nm absorbance for **4** allowed us to monitor NO_3^- release from phospholipid vesicles.

EYPC liposomes (100 nm) filled with 100 mM $NaNO_3$–10 mM sodium phosphate (pH 7.2) were eluted from a Sephadex G-25 column using a solution of 100 mM NaCl–10 mM sodium phosphate (pH 7.2). Gel filtration was done to replace the bulk of the extravesicular NO_3^- with Cl^-. The resulting EYPC liposome suspension (100 μL) was diluted into 1.9 mL of a 100 mM NaCl–10 mM sodium phosphate (pH 7.2) solution containing 0.3 units of nitrate reductase and 5 mM NADPH. Solutions of either tripod **1** or **2** in DMSO (2 : 100 molar ratio to lipid) were added to the EYPC suspensions and the resulting mixture was incubated for 3 h to ensure complete reduction of nitrate to nitrite. Subsequently, the Griess reaction of the enzymatically generated NO_2^- was done by addition of the appropriate anilines.[23]

The UV spectra in Fig. 3B show significant consumption of NADPH in the presence of the NO_3^--loaded liposomes and nitro tripod **1**. In contrast, when *t*-butyl tripod **2** was added to the same NO_3^--loaded liposomes there was little change in NADPH absorbance. As shown in Fig. 3C, the 543 nm absorbance for diazo **4** formed in the Griess reaction confirmed that significantly more nitrate was released from EYPC liposomes in the presence of nitro tripod **1**, as compared to *t*-butyl tripod **2**. These two assays, based on nitrate reduction and subsequent trapping of the extravesicular nitrite, confirm that nitro tripod **1** transports NO_3^- across EYPC vesicles.

The data in Fig. 2 and 3 suggested that nitro tripod **1** might control the selective release of nitrate from phospholipid vesicles. As such, we utilized the significant NO_3^-/Cl^- transmembrane transport selectivity shown by nitro tripod **1** to alter the pH within phospholipid vesicles experiencing a NO_3^-/Cl^- gradient. Thus, EYPC liposomes containing the pH sensitive HPTS dye in 100 mM $NaNO_3$–10 mM sodium phosphate buffer (pH 6.4) were suspended in a solution of 100 mM NaCl–10 mM sodium phosphate buffer (pH 6.4). Then, DMSO solutions of either tripods **1** or **2**, or paco **3**, were added to the EYPC suspension. As

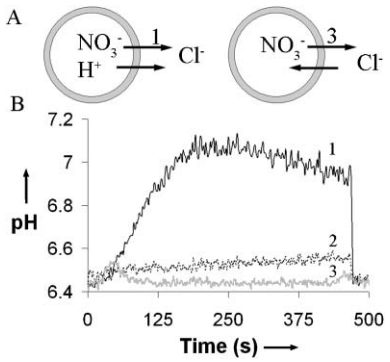


Fig. 4 Change in intravesicular pH. (A) Nitro tripod **1** functions as a H^+–NO_3^- co-transporter whereas paco **3** functions as a NO_3^-–Cl^- anion exchanger. (B) A plot of intravesicular pH *vs.* time in experiments where NO_3^- loaded EYPC liposomes (pH 6.4) suspended in a NaCl solution (pH 6.4) were treated with compounds **1–3**.

shown in Fig. 4, addition of nitro tripod **1** resulted in a rapid increase in the intravesicular pH (Δ pH = 0.7), whereas addition of *t*-butyl tripod **2** or paco **3** caused little change in the internal pH. The constant pH in a system experiencing a significant transmembrane NO_3^-/Cl^- gradient indicates that paco **3**, which can transport Cl^- and NO_3^- (Fig. 2 and ref. 14), operates *via* a NO_3^-–Cl^- anion exchange process. The nitro tripod **1**, on the other hand, readily moves NO_3^- out of the liposome along the NO_3^-/Cl^- gradient, but is unable to compensate by transporting Cl^- from the extravesicular buffer into the liposome. To maintain electroneutrality nitro tripod **1** facilitates H^+–NO_3^- co-transport (or the equivalent OH^-–NO_3^- counter-transport) across the vesicular membrane.

In summary, nitro tripod **1** selectively transports NO_3^- across liposomal membranes in what is effectively a H^+–NO_3^- co-transport process. This is, to our knowledge, the first report of a synthetic compound with such a marked selectivity for transport of NO_3^- over Cl^-. As we continue to establish structure–function relationships for synthetic anion transporters, we hope to use tripod **1** and analogs, to induce pH changes in cells experiencing a NO_3^-/Cl^- gradient.[24]

This research was supported by the US Department of Energy (BES, Separations and Analysis Program). PVS thanks the Univ. of Maryland's HHMI Program for support as a "Teacher-Scholar" Post-Doctoral Fellow. We thank Prof. Volker Böhmer for providing information regarding the synthesis of nitro tripod **1**.

Notes and references

‡ Liposome preparation. More details can be found in the ESI† of this paper and in ref. 12. Egg yolk phosphatidylcholine (EYPC) lipid (60 μL of 1 g mL^{-1} stock solution, Avanti Polar Lipids) was diluted in 5 mL of 5% MeOH–$CHCl_3$ in a rb flask. The solvent was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* for 2 h. The lipid film was hydrated with 1 mL of phosphate buffer containing HPTS fluorescent dye. After 10 freeze–thaw cycles, the liposomes were subjected to high-pressure extrusion 21 times at rt through a 0.1 μm polycarbonate membrane (Whatman). Such an extrusion procedure gives large unilamellar vesicles with an average diameter of 100 nm (see www.avantilipids.com/LUVET.html). The liposome solution was passed over a Sephadex G-25 column and the isolated liposomes were diluted to give a concentration of 25 mM in EYPC.

1 J. M. Boon and B. D. Smith, *Curr. Opin. Chem. Biol.*, 2002, **6**, 749; J. L. Sessler, P. A Gale and W.-S Cho, *Anion Receptor Chemistry*, 2006, Royal Society of Chemistry, Cambridge, UK.
2 J. R. Broughman, L. P. Shank, W. Takeguchi, B. D. Schultz, T. Iwamoto, K. E. Mitchell and J. M. Tomich, *Biochemistry*, 2002, **41**, 7350.
3 P. H. Schlesinger, R. Ferdani, J. Liu, J. Pajewska, R. Pajewki, M. Saito, H. Shabany and G. W. Gokel, *J. Am. Chem. Soc.*, 2002, **124**, 1848; P. H. Schlesinger, R. Ferdani, R. Pajewski, J. Pajewska and G. W. Gokel, *Chem. Commun.*, 2002, 840.
4 A. V. Koulov, T. N. Lambert, R. Shukla, M. Jain, J. M. Bood, B. D. Smith, H. Y. Li, D. N. Sheppard, J. B. Joos, J. P. Clare and A. P. Davis, *Angew. Chem., Int. Ed.*, 2003, **42**, 4931; J. P. Clare, A. J. Ayling, J.-B. Joos, A. L. Sisson, G. Magro, M. N. Perez-Payan, T. N. Lambert, R. Shukla, B. D. Smith and A. P. Davis, *J. Am. Chem. Soc.*, 2005, **127**, 10739.
5 P. A. Gale, M. E. Light, B. McNally, K. Navakhum, K. I. Sliwinski and B. D. Smith, *Chem. Commun.*, 2005, 3773; J. L. Sessler, L. R. Eller, W.-S. Cho, S. Nicolaou, A. Aguilar, J. T. Lee, V. M. Lynch and D. J. Magda, *Angew. Chem., Int. Ed.*, 2005, **45**, 5985.
6 J. L. Seganish and J. T. Davis, *Chem. Commun.*, 2005, 5781.
7 A. P. Bisson, V. M. Lynch, M.-K. C. Monahan and E. V. Anslyn, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2340; S. Mason, T. Clifford, L. Seib, K. Kuczera and K. Bowman-James, *J. Am. Chem. Soc.*, 1998, **120**, 8899; K. Kavallieratos, R. A. Sachleben, G. J. van Berkel and B. A. Moyer, *Chem. Commun.*, 2000, 187; K. Choi and A. D. Hamilton, *J. Am. Chem. Soc.*, 2001, **123**, 2456; R. Herges, A. Dikmans, U. Jana, F. Kohler, P. G. Jones, I. Dix, T. Fricke and B. König, *Eur. J. Org. Chem.*, 2002, 3004; J. L. Sessler, D. An, W.-S. Cho, V. Lynch and M. Marques, *Chem.–Eur. J.*, 2005, **11**, 2001; J. Mahoney, K. A. Stucker, H. Jiang, I. Carmichael, N. R. Brinkmann, A. M. Beatty, B. C. Noll and B. D. Smith, *J. Am. Chem. Soc.*, 2005, **127**, 2922.
8 Computational design of nitrate receptors: B. P. Hay, T. K. Firman and B. A. Moyer, *J. Am. Chem. Soc.*, 2005, **127**, 1810.
9 A nitrate selective electrode based on a C_3-symmetric amide: J. A. Ortuno, R. Exposito, C. Sanchez-Pedreno, M. I. Alero and A. Espinosa, *Anal. Chim. Acta*, 2004, **525**, 231.
10 B. G. Forde, *Biochim. Biophys. Acta*, 2000, **1465**, 219.
11 T. T. Mensenga, G. J. A. Speijers and J. Meulenbelt, *Toxicol. Rev.*, 2003, **22**, 41.
12 V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger and J. T. Davis, *J. Am. Chem. Soc.*, 2002, **124**, 2267.
13 V. Sidorov, F. W. Kotch, J. L. Kuebler, Y.-F. Lam and J. T. Davis, *J. Am. Chem. Soc.*, 2003, **125**, 2840.
14 J. L. Seganish, P. V. Santacroce, K. J. Salimian, J. C. Fettinger, P. Zavalij and J. T. Davis, *Angew. Chem., Int. Ed.*, 2006, **46**, 3334.
15 M. B. Dinger and M. J. Scott, *Eur. J. Org. Chem.*, 2000, 2467.
16 M. B. Dinger and M. J. Scott, *Inorg. Chem.*, 2000, **39**, 1238.
17 M. B. Dinger and M. J. Scott, *Inorg. Chem.*, 2001, **40**, 1029.
18 Y. Rudzevich, V. Rudzevich, D. Schollmeyer, I. Thondorf and V. Böhmer, *Org.Letters*, 2005, **7**, 613.
19 Crystal data for nitro tripod **1**: $C_{37}H_{46}N_6O_{12}$, M_r = 766.80, crystal dimensions 0.04 × 0.23 × 0.41 mm^3, monoclinic, space group $P2_1/n$, a = 13.1120(7) Å, b = 16.6088(9) Å, c = 18.4861(10) Å, β = 108.5200(10)°, V = 3817.3(4) $Å^3$, Z = 4, D_x = 1.334 g cm^{-3}, MoKα. Data were collected on a Bruker SMART 1000 CCD diffractometer at 223(2) K. The structure refined to convergence [$\Delta/\sigma \leqslant 0.001$] with $R(F)$ = 4.34%, w$R(F^2)$ = 8.47%, GOF = 1.00 for 8657 unique reflections. CCDC 607508. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b607221f.
20 NMR association constants were determined using the program "Associate 1.6" from the F. N. Diederich group at ETH.
21 K. Kano and J. H. Fendler, *Biochim. Biophys. Acta*, 1978, **509**, 289; N. Sakai, K. C. Brennan, L. A. Weiss and S. Matile, *J. Am. Chem. Soc.*, 1997, **119**, 8726.
22 (*a*) J. Biwersi, B. Tulk and A. S. Verkman, *Anal. Biochem.*, 1994, **219**, 139; (*b*) A. McNally, A. V. Koulov, B. D. Smith, J. B. Joos and A. P. Davis, *Chem. Commun.*, 2005, 1087.
23 D. L. Granger, R. R. Taintor, K. S. Boockvar and J. B. Hibbs, *Methods Enzymol.*, 1996, **268**, 142.
24 For a report on H^+–NO_3^- co-transporters in mammalian cells: C.-W. Chow, A. Kapus, R. Romanek and S. Grinstein, *J. Gen. Physiol.*, 1997, **110**, 185.

