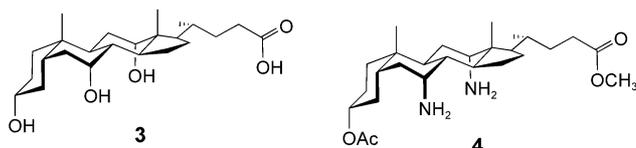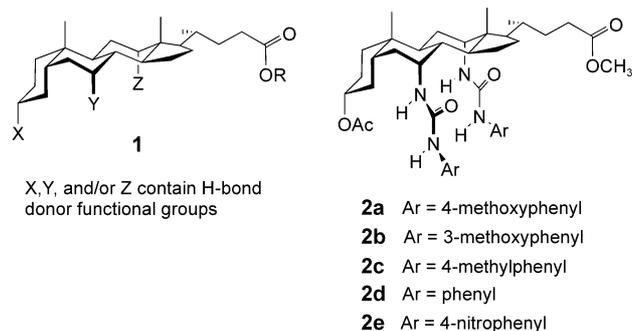**Membrane Transport**

Chloride Transport Across Vesicle and Cell Membranes by Steroid-Based Receptors**

*Atanas V. Koulov, Timothy N. Lambert,
Rameshwer Shukla, Mahim Jain, J. Middleton Boon,
Bradley D. Smith,* Hongyu Li, David N. Sheppard,*
Jean-Baptiste Joos, John P. Clare, and Anthony P. Davis*

It is well-established that molecules which transport cations across cell membranes (cationophores) can have potent biological effects.[1] Anion flux is also important to the cell and, correspondingly, anion carriers may be capable of biological activity. Indeed, chloride transporters have direct medical potential as treatments for cystic fibrosis and other diseases caused by defective channel proteins.[2] Despite this motivation, there have been relatively few reports of aniono-phore natural products[3] or of anion transport by synthetic systems.[4] Most, moreover, have involved cationic centers,[5] which can assist anion passage through the formation of an ion pair.[4a–g] Anion transport by purely electroneutral systems is still quite rare.[4j–l] The recently described "cholapod" anion receptors **1** are intrinsically lipophilic, potentially membrane-soluble, and tuneable to very high affinities.[6] Certain cholapods have proved capable of "flippase" activity, that is, they can convey polar head groups across phospholipid membranes.[7] We therefore supposed that electroneutral cholapods might transport inorganic anions by a "shuttle" mechanism, in the manner of cationophores such as valino-mycin. We now report that cholapods **2** are indeed capable of transporting chloride ions across liposomal membranes and also across live cells grown as polarized epithelia.

[*] Prof. Dr. B. D. Smith, A. V. Koulov, T. N. Lambert, R. Shukla, M. Jain,
J. M. Boon
Department of Chemistry and Biochemistry and
Walther Cancer Research Center
University of Notre Dame
Notre Dame, IN 46556 (USA)
Fax: (+1) 574-631-6652
E-mail: smith.115@nd.edu

Dr. D. N. Sheppard, Dr. H. Li
Department of Physiology
School of Medical Sciences
University of Bristol
University Walk, Bristol BS8 1TD (UK)
Fax: (+44) 117-928-8923
E-mail: d.n.sheppard@bristol.ac.uk

Prof. Dr. A. P. Davis, Dr. J.-B. Joos, J. P. Clare
School of Chemistry
University of Bristol
Cantock's Close, Bristol BS8 1TS (UK)
Fax: (+44) 117-929-8611
E-mail: anthony.davis@bristol.ac.uk

X,Y, and/or Z contain H-bond donor functional groups

2a Ar = 4-methoxyphenyl
2b Ar = 3-methoxyphenyl
2c Ar = 4-methylphenyl
2d Ar = phenyl
2e Ar = 4-nitrophenyl

Receptors **2** were prepared from cholic acid **3** by conversion into diamine **4**[8] and treatment with appropriate isocyanates. Initially, their anion-transport properties were studied by following chloride efflux from unilamellar vesicles using a chloride-selective electrode. More specifically, 30 mM unilamellar vesicles (200 nm mean diameter) were prepared by extruding a 7:3 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol[9] in aqueous NaCl (500 mM). This stock vesicle dispersion was dialyzed against aqueous $NaNO_3$ (500 mM) to replace the external chloride ions with nitrate and then diluted with 500 mM $NaNO_3$ to give a 1 mM total lipid concentration. As shown in Figure 1, addition of a solution of **2a–e** in THF (4.0 μM final cholapod concentration) caused chloride efflux from the inner phase of the vesicles. The initial rates of chloride efflux and the association constants of **2a–e** for $Et_4N^+Cl^-$ and $Et_4N^+NO_3^-$ in
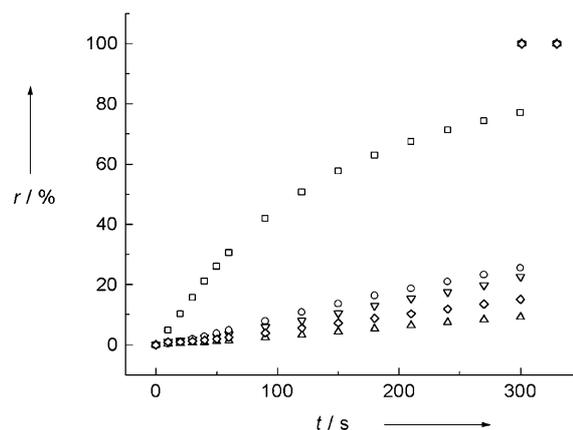

Figure 1. Chloride release (*r*) upon addition of **2e** (□), **2d** (○), **2c** (▽), **2b** (◇), and **2a** (△). At time 0 s cholapod (4 μM) was added to vesiclesn (1 mM total lipid) containing NaCl (500 mM) dispersed in $NaNO_3$ (500 mM). The detergent (polyoxythylene(8) lauryl ether) was added at time 300 s to disrupt the vesicles and release remaining chloride ions.

Table 1: Initial rates of Cl⁻ release from vesicles and affinity constants.

Compound	Initial rate of efflux [% s⁻¹][a]	K_a (Et₄N⁺Cl⁻) [M⁻¹][b]	K_a (Et₄N⁺ NO₃⁻) [M⁻¹][b]
2a	0.031	3.4×10^6	2.1×10^6
2b	0.051	1.2×10^7	8.8×10^6
2c	0.075	6.3×10^6	5.1×10^6
2d	0.085	$1.5 \times 10^{7[c]}$	$1.0 \times 10^{7[c]}$
2e	0.52	5.2×10^8	1.7×10^8

[a] For details see text and legend to Figure 1. [b] Average of two measurements. [c] Determinations made on the corresponding eicosyl ester to avoid overlap of NMR signals.

water-saturated CHCl₃, as measured by extraction, are listed in Table 1.[6b] The efflux rates correlate reasonably well with the association constants, thus indicating that a hydrogen-bonding recognition event controls the transport process. As expected, compounds **5** and **6** (Boc = *tert*-butoxycarbonyl) with very weak chloride affinity produced no measurable efflux.
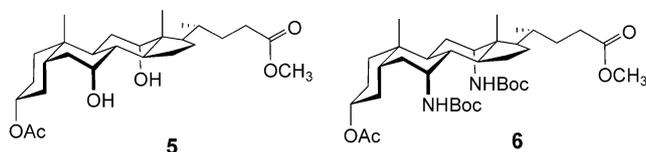
Further studies were performed on **2d** to clarify the mechanism of transport. Firstly, chloride efflux was shown to be unaltered upon changing the entrapped countercation from sodium to either potassium or caesium (see the Supporting Information), which indicates that the metal cation is not involved in the transport process. Secondly, the external anion was varied. As shown in Figure 2, the effect was substantial. For example, replacement of the external NO₃⁻ with HCO₃⁻ ions lowered transport rates by about 80%, while substitution with SO₄²⁻ ions prevented chloride
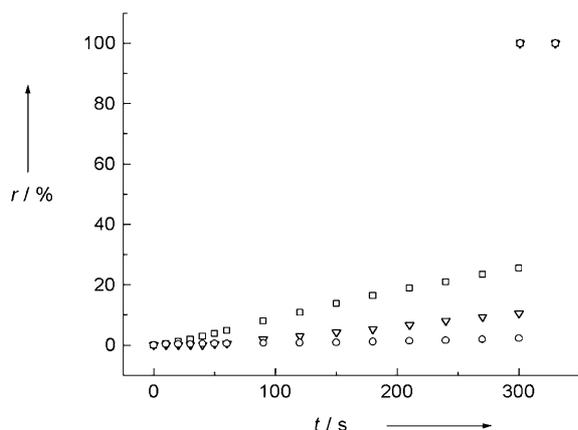
efflux altogether. Addition of external NO₃⁻ ions to this latter experiment restored transport (see the Supporting Information). These results clearly indicate an anion antiport mechanism; substantial chloride efflux can only occur if there is corresponding influx of an external anion. Cholapod **2d** transports both Cl⁻ and NO₃⁻ ions, but is less effective for the more hydrophilic HCO₃⁻ and SO₄²⁻ ions. In the case of external sulfate, a small amount of chloride transport (undetectable using the Cl⁻ electrode) creates a transmembrane potential difference, after which further efflux cannot occur. In support of this mechanism, cholapod **2d** induced an electric potential (inside negative) across vesicle membranes partitioning Na₂SO₄ (inside) and NaCl (outside), as judged by fluorescence experiments using the potential-sensitive dye safranin O (see the Supporting Information).[4l]

The ability of **2e** to transport chloride ions into vesicles was detected by using ³⁵Cl NMR spectroscopy. In this case, vesicles were formed in 300 mM NaBr solution and then dialyzed against aqueous NaCl (300 mM) containing CoCl₂ (10 mM) as a ³⁵Cl NMR shift reagent.[4a,10] Internalized chloride ions were detected as an unshifted ³⁵Cl NMR resonance after addition of **2e** (Figure 3). Separate resonance peaks for the internal and external chloride ions were observed for at least 24 h, thus showing that the vesicle membranes remain unperturbed by the cholapod.

In principle, cholapods **2** might act as mobile carriers or through formation of multicomponent channels. To date, there are two pieces of evidence suggesting the former. First,
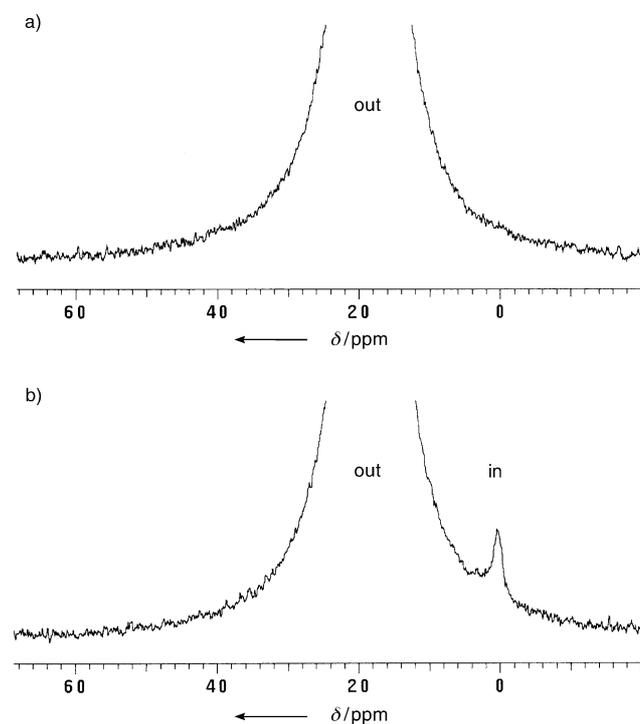

Figure 2. Dependence of the chloride release (*r*) induced by **2d** on external anions. The vesicles containing NaCl (500 mM) were dispersed in isotonic NaNO₃ (□), NaHCO₃ (▽), or Na₂SO₄ (○). See Figure 1 for other details.

Figure 3. ³⁵Cl NMR spectra of vesicles (30 mM total lipid) containing 300 mM NaBr dispersed in a solution of 300 mM NaCl and 10 mM CoCl₂. Spectra were obtained a) before and b) 24 h after addition of **2e** (4 μM). Signals corresponding to chloride ions outside and inside the vesicles are labeled accordingly.

efflux rates with **2d** increased just 3.2-fold when the receptor concentration was increased from 4 to 40 μM (see the Supporting Information). This less than first-order dependence suggests the formation of inactive, rather than active, aggregates at the higher concentration.[11] Second, there is no cholapod-mediated efflux of chloride ions from vesicles composed of dipalmitoylphosphatidylcholine (DPPC) at room temperature (gel phase), but facilitated efflux occurs above the temperature for the gel/liquid crystalline phase transition (fluid phase). This is signature behavior of a mobile carrier (for example, monensin) whose activity depends on membrane viscosity; in contrast, channel activity should not show this effect.[12]

Finally, cholapod-mediated Cl⁻ transport was demonstrated in live cells by using Madin Darby canine kidney (MDCK) epithelia and the Ussing chamber technique.[13] The method involves growing an oriented layer of cells on a filter support and placing it between current- and voltage-measuring electrodes. Endogenous active transport systems generate a potential difference across the cell membranes. Addition of a passive chloride transporter causes discharge of the electrochemical gradient, which is detected as a flow of current. Receptor **2e** (80 μM) did indeed produce this effect (see the Supporting Information). As expected, the response was anion-dependent; replacement of chloride by gluconate anions on either side of the membrane reduced the signal by about 80%. Moreover, the response was different from that induced by activators of native chloride channels found in MDCK epithelia (see the Supporting Information).

In conclusion, cholapods **2** have been shown by three techniques (electrochemical, NMR, and fluorescence) to transport chloride ions across vesicle membranes. The data favors an antiport mechanism with the cholapod more likely acting as a mobile carrier than a channel. The cholapod does not disrupt the bilayer membrane and is able to convert a chloride concentration gradient into a transmembrane electrical potential. Facilitated chloride transport has also been demonstrated in polarized epithelial cells, which augurs well for future applications in biochemistry and medicine.

[1] M. Dobler in *Comprehensive Supramolecular Chemistry, Vol. 1* (Ed.: G. W. Gokel), Pergamon, Oxford, **1996**, p. 267.
[2] J. R. Broughman, L. P. Shank, W. Takeguchi, B. D. Schultz, T. Iwamoto, K. E. Mitchell, J. M. Tomich, *Biochemistry* **2002**, *41*, 7350, and references therein.
[3] The prodigiosin family of antibiotics have been shown to be H⁺Cl⁻ cotransporters; for a leading reference, see K. Tanigaki, T. Sato, Y. Tanaka, T. Ochi, A. Nishikawa, K. Nagai, H. Kawashima, S. Ohkuma, *FEBS Lett.* **2002**, *524*, 37.
[4] Leading references: a) A. V. Koulov, J. M. Mahoney, B. D. Smith, *Org. Biomol. Chem.* **2003**, *1*, 27; b) L. A. J. Chrisstoffels, F. de Jong, D. N. Reinhoudt, *Chem. Eur. J.* **2000**, *6*, 1376; c) L. A. J. Chrisstoffels, F. de Jong, D. N. Reinhoudt, S. Sivelli, L. Gazzola, A. Casnati, R. Ungaro, *J. Am. Chem. Soc.* **1999**, *121*, 10142; d) J. L. Sessler, W. E. Allen, *Chemtech* **1999**, *29*, 16; e) M.

Merritt, M. Lanier, G. Deng, S. L. Regen, *J. Am. Chem. Soc.* **1998**, *120*, 8494; f) N. Sakai, S. Matile, *Tetrahedron Lett.* **1997**, *38*, 2613; g) B. Dietrich, T. M. Fyles, M. W. Hosseini, J.-M. Lehn, K. C. Kaye, *J. Chem. Soc. Chem. Commun.* **1988**, 691; h) D. P. Wallace, J. M. Tomich, J. W. Eppler, T. Iwamoto, J. J. Grantham, L. P. Sullivan, *Biochim. Biophys. Acta* **2000**, *1464*, 69; i) K. E. Mitchell, T. Iwamoto, J. Tomich, L. C. Freeman, *Biochim. Biophys. Acta* **2000**, *1466*, 47; j) P. H. Schlesinger, R. Ferdani, J. Liu, J. Pajewska, R. Pajewski, M. Saito, H. Shabany, G. W. Gokel, *J. Am. Chem. Soc.* **2002**, *124*, 1848; k) V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger, J. T. Davis, *J. Am. Chem. Soc.* **2002**, *124*, 2267; l) V. Sidorov, F. W. Kotch, J. L. Kuebler, Y. F. Lam, J. T. Davis, *J. Am. Chem. Soc.* **2003**, *125*, 2840.
[5] Present in the transporter itself or in cotransported counter-cations.
[6] a) A. P. Davis, J. J. Perry, R. P. Williams, *J. Am. Chem. Soc.* **1997**, *119*, 1793; A. J. Ayling, M. N. Pérez-Payán, A. P. Davis, *J. Am. Chem. Soc.* **2001**, *123*, 12716; b) A. J. Ayling, S. Broderick, J. P. Clare, A. P. Davis, M. N. Pérez-Payán, M. Lahtinen, N. J. Nissinen, K. Rissanen, *Chem. Eur. J.* **2002**, *8*, 2197.
[7] T. N. Lambert, J. M. Boon, B. D. Smith, M. N. Pérez-Payán, A. P. Davis, *J. Am. Chem. Soc.* **2002**, *124*, 5276; J. M. Boon, T. N. Lambert, A. L. Sisson, A. P. Davis, B. D. Smith, *J. Am. Chem. Soc.* **2003**, *125*, 8195.
[8] A. P. Davis, M. N. Pérez-Payán, *Synlett* **1999**, 991.
[9] The 7:3 ratio of POPC to cholesterol mimics that present in animal cell membranes. Vesicles composed only of POPC were disrupted by the receptors, as determined by carboxyfluorescein leakage assay.
[10] F. G. Riddell in *Encyclopedia of Spectroscopy and Spectrometry* (Eds.: J. C. Lindon, G. E. Tranter, J. L. Holmes), Academic Press, London, **1999**, p. 1584.
[11] NMR spectra of **2d** in CDCl₃ showed concentration dependence, which confirmed the tendency for aggregation in nonpolar media.
[12] G. Deng, T. Dewa, S. L. Regen, *J. Am. Chem. Soc.* **1996**, *118*, 8975.
[13] D. N. Sheppard, M. R. Carson, L. S. Ostedgaard, G. M. Denning, M. J. Welsh, *Am. J. Physiol.* **1994**, *266*, L405.