Small Molecule UCP Mimics: Synthetic Anion Receptors as Fatty Acid-Activated Proton Transporters

Xin Wua,b and Philip A Galea,b\*

a Chemistry, University of Southampton, Southampton, SO17 1BJ, UK

b Present address: School of Chemistry (F11), The University of Sydney, 2006 NSW, Australia.

ABSTRACT: Uncoupling proteins (UCPs) regulate energy expenditure in living cells by inducing proton leakage across the mitochondrial inner membrane, thereby uncoupling ADP phosphorylation from nutrient oxidation. The proton transport activity of UCP1 and UCP2 requires activation by fatty acids. We report here the first examples of synthetic small molecules performing this biologically important fatty acid-activated function. We have shown that a tripodal thiourea possesses poor H+/OH− transport activity without fatty acids, but in the presence of long-chain fatty acids is “switched on” as a proton transporter with an activity close to a commonly used protonophore. The fatty acid-enhanced proton transport was also observed for other hydrogen and halogen bond-based synthetic anion transporters. We propose that these compounds induce proton permeability by catalyzing transbilayer movement (“flip-flop”) of anionic fatty acids, so allowing the fatty acids to complete a proton transport cycle. Several lines of evidence have been provided to support such a fatty acid cycling mechanism. Our findings open up new applications of anion receptor chemistry, and provide important clues for understanding biological activities of synthetic anion transporters and potentially the uncoupling mechanism of naturally occurring membrane proteins.

**Introduction**

In respiring mitochondria, the electron transport chain pumps protons from the matrix into the intermembrane space, thus converting energy from nutrient oxidation into a high membrane potential across the inner membrane. This proton gradient is primarily used to drive the energy consuming process of ATP synthesis.[1](#_ENREF_1) An increase of proton permeability in the mitochondrial inner membrane, known as uncoupling, allows the proton gradient to be dissipated bypassing ATP synthesis, leading to energy loss as heat.[2](#_ENREF_2) Although complete uncoupling is detrimental to the organism, mild and regulated uncoupling is crucial for many processes, which is performed by several mitochondria membrane proteins known as UCPs.[3](#_ENREF_3) The most extensively studied uncoupling protein UCP1 was identified in the mitochondria of brown adipose tissue and is responsible for non-shivering thermogenesis.[4](#_ENREF_4) Other homologues of UCPs may also play important biological roles including prevention of mitochondrial release of reactive oxygen species (UCP2)[5](#_ENREF_5) and regulation of fatty acid metabolism (UCP3).[6](#_ENREF_6) An intriguing characteristic of UCP1[7](#_ENREF_7) and UCP2[8](#_ENREF_8) is that their proton transport activity requires activation by free (unesterified) fatty acids. This allows UCP1 to remain in an inactive resting state until being triggered by a fatty acid-releasing cascade in response to cold exposure.[9](#_ENREF_9) The mechanism of fatty acid activation remains a controversial issue.[3](#_ENREF_3) Several mechanisms have been proposed, including H+ uniporter[4](#_ENREF_4) or OH− uniporter[9](#_ENREF_9) activated by allosteric binding of fatty acids, a fatty acid cycling model[10](#_ENREF_10), and a fatty acid shuttling model.[11](#_ENREF_11) The fatty acid cycling model postulates that UCPs indirectly facilitate proton transport by assisting the transbilayer movement (“flip-flop”) of anionic fatty acids, allowing the fatty acid proton transport cycle to complete (this is shown in Figure 1c for a synthetic anion transporter but is analogous to the process that was supposed to be facilitated by UCP). Without UCP assistance, the flip-flop of anionic forms of fatty acids is extremely slow (several orders of magnitude slower than that of the neutral forms)[12](#_ENREF_12) and thus presents a “missing step” in the fatty acid proton transport cycle (Figure 1c). We propose that the existence of the fatty acid cycling mechanism would gain further support if structurally simple synthetic anion transporters can function similarly.

Synthetic anion transporters[13](#_ENREF_13) are small lipophilic molecules that can form reversible noncovalent[14](#_ENREF_15) or covalent[15](#_ENREF_18) bonds with anionic species such as Cl−, NO3−, HCO3− and lipid phosphate head groups,[16](#_ENREF_19) spreading their negative charge over a larger surface and thus facilitating their passive transport across phospholipid bilayers. This area has attracted significant research efforts due to potential biomedical applications. These include: (1) treating cystic fibrosis by restoring the Cl− and HCO3− permeability in epithelia, thus replacing the function of the genetically impaired anion channel cystic fibrosis transmembrane regulator (CFTR);[17](#_ENREF_20) (2) inducing cancer cell apoptosis by disrupting their pH[18](#_ENREF_22) or ionic[19](#_ENREF_23) gradients. We here unravel a previously unknown function of synthetic anion transporters. We demonstrate that they can mimic the function of UCPs in facilitating H+ transport upon activation by fatty acids. We attribute this to the ability of synthetic anion transporters to catalyze the flip-flop of anionic fatty acids, which has been supported by several lines of evidence. This fatty acid-activated function of synthetic anion transporters is interesting also from the perspective of ligand-gating in membrane transport. Examples of ligand-gating in synthetic membrane transport systems include the activation of cell-penetrating peptides by amphiphilic counterions,[20](#_ENREF_24) channel assembly or opening induced by ligand binding,[21](#_ENREF_27) and sequestering of transporters by host-guest chemistry.[22](#_ENREF_30)

In this paper, we examined the fatty acid dependence of the H+/OH− transport facilitated by several synthetic anion transporters and a commonly used protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, Figure 2).[23](#_ENREF_31) The library of anion transporters studied contains representative examples of anion transporters functioning via hydrogen bonds (compounds **1**–**4**)[24](#_ENREF_32) and halogen bonds (compound **5**).[25](#_ENREF_35) In our recent study of H+/OH− transport facilitated by anion transporters, we proposed that transporter deprotonation (in the case of hydrogen bond-based transporters, Figure 1a) and OH− transport via reversible binding of OH− (Figure 1b) are two mechanisms that explain the ability of anion transporters to dissipate an electrochemical proton gradient.[26](#_ENREF_36) With the identification of fatty acid anion flippase as a third mechanism (Figure 1c, this mechanism is physiologically relevant as free fatty acids are present in biological membranes), we also examined the influence of fatty acids on the Cl− > H+/OH− selectivity of compound **6**, which was previously reported to show a high selectivity for Cl− transport over H+/OH− transport.[26](#_ENREF_36)

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**Figure 1.** Mechanisms of synthetic anion transporters facilitating electrogenic (electrophoretic) H+/OH− transport, leading to dissipation of an electrochemical proton gradient. (a) H+ transport via deprotonation and re-protonation; (b) OH− transport via reversible binding of OH−; (c) Fatty acid cycling model, in which the synthetic anion transporter catalyzes the flip-flop of the anionic form of a fatty acid. Without the anion transporter, the flip-flop of the anionic fatty acid is slow and therefore the fatty acid by itself cannot complete the proton transport cycle.

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**Figure 2.** Structures of synthetic anion transporters **1**–**6** (red), protonophore CCCP (purple) and fatty acids (olive) used in this paper. Anion binding sites of **1**–**6** are shown in blue.

**Results and discussion**

**Anion binding studies.** The affinities of **1**–**4** for Cl− and AcO− ions were assessed by UV-Vis absorption binding studies in CH3CN, using tetrabutylammonium (TBA+) salts of the anions (Table 1). Here AcO− was used as a model for fatty acid anions. In all cases except for compound **4** (whose AcO− affinity cannot be determined due to receptor deprotonation), AcO− binds more strongly than Cl−, as consistent with the more charge dense nature of AcO−. The anion affinity of different receptors follows the order **1** > **3** > **2** >> **4**, showing a positive correlation with the acidity and the number of hydrogen bond donors present. The observation of strong AcO− binding to **1**–**3** points to the possibility that these compounds facilitating the membrane transport of AcO− ions and the carboxylate head group of anionic fatty acids.

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| Table 1. Binding constants (*K* / M-1) of compounds 1–4 for Cl− and AcO− in CH3CN at 293K, determined by UV-Vis absorption titrations using TBA+ salts of the anions. | | | | |
| Anions | **1** | **2** | **3** | **4** |
| Cl− | 1.9 × 106 | 2.4 × 102 | 2.4 × 104 | 47 |
| AcO− | 2.6 × 107 | 8.9 × 103 | 9.9 × 106 a | –*b* |

*a*HOAc (5 mM) was used to suppress receptor deprotonation by AcO−. The binding constant was obtained after correction of AcO− binding to HOAc.[27](#_ENREF_37)

*b*Receptor deprotonation occurred even in the presence of HOAc. Therefore the AcO− binding constant could not be determined.

**Oleic acid flip-flop.** The flip-flop of both neutral and anionic forms of oleic acid (OA) across phospholipid bilayers was investigated using a pH-based assay.[28](#_ENREF_38) Large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with a mean diameter of 200 nm were loaded with and suspended in a potassium gluconate (KGlc, 100 mM) solution buffered to pH 7.0 with 10 mM HEPES. The change of pH inside vesicles upon the addition of OA was monitored using the pH sensitive fluorescence probe 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). As shown in Figure 3a, addition of a DMSO solution of OA (2 μM, 2 mol% with respect to lipid concentration) to the external solution caused a rapid drop of the internal pH within seconds. This phenomena has been reported by Kamp and Hamilton,[28](#_ENREF_38) and was ascribed to the rapid flip-flop of neutral OA from the outer leaflet to the inner leaflet,[29](#_ENREF_39) creating a small pH gradient with lower pH inside than outside. At this stage, the outer leaflet contains more oleate ions than the inner leaflet. Dissipation of the pH gradient would occur if oleate ions can flip-flop along their concentration gradient to symmetrize their distribution in two leaflets. Subsequently, the K+ ionophore valinomycin (0.05 mol% with respect to lipid) was added to provide counterion transport, in order to collapse the charge accumulation from an electrogenic transport process (*e.g.* oleate flip-flop). With valinomycin addition, the internal pH increases very slowly, reflecting the extremely slow flip-flop rate of oleate ions. The addition of the K+/H+ exchanger monensin at 10 min equilibrated the internal pH to the external value. When the vesicles were treated with compound **1** (0.01 mol% with respect to lipid) after valinomycin addition, the increase of internal pH greatly accelerated and was complete within minutes. The results demonstrate that compound **1** likely catalyzed the flip-flop of oleate ions. Although an alternative cause for the acceleration of pH gradient dissipation is compound **1** itself facilitating H+/OH− transport, this is not a likely option as compound **1** in the absence of fatty acids could not facilitate observable H+/OH− transport at 0.01 mol% (Figure 4b blue).

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**Figure 3.** (a) Intra-vesicular pH (measured by HPTS fluorescence) in response to the addition of oleic acid (OA, at 1 min), valinomycin (Vln, at 2 min), compound **1** (at 2.5 min, pink line only) and monensin (Mon, at 10 min). The POPC LUVs (mean diameter 200 nm) were loaded with and suspended in a potassium gluconate (KGlc, 100 mM) solution buffered at pH 7.0 with 10 mM HEPES. All compounds were added as DMSO solutions. The vertical arrows indicate the addition of compounds and the tilted arrows indicate the assignment of HPTS response to the flip-flop of neutral OA or oleate ions. Compound concentrations are shown as compound to lipid molar ratios. (b) Schematic representation of processes that led to acidification or basification of vesicles. The numberings of the processes in (b) correspond to those in (a).

**HPTS proton transport assay.** We then explored the ability of a combination of an anion transporter and OA to facilitate electrogenic (electrophoretic) H+/OH− transport to dissipate an electrochemical proton gradient, using the HPTS assay shown in Figure 4a. A base pulse of NaOH (5 mM) was added to vesicle suspensions in a lightly buffered KGlc medium, to create a pH gradient with pH 7 inside and pH ~8 outside.[30](#_ENREF_40) Here we use the untransportable anion gluconate, in order to measure solely the H+/OH− uniport activity and exclude the occurrence of H+/anion symport (or OH−/anion antiport) if a transportable anion such as Cl− was used.[31](#_ENREF_41) H+/OH− uniport facilitated by an anion transporter was measured *via* the rate of pH gradient dissipation in the presence of valinomycin that provides counterion (K+) transport. Figure 4b shows that the rate of H+/OH− transport induced by **1** (0.01 mol% with respect to lipid) dramatically accelerated when OA (2 mol% with respect to lipid) was present. OA itself gave negligible H+/OH− transport within the experimental time scale, consistent with the slow flip-flop rate of its conjugate base preventing OA itself to function as protonophores (Figure 1c). It should be noted that commercially available POPC contains fatty acid impurities (palmitic acid, PA, and OA).[32](#_ENREF_42) Strikingly, the H+/OH− transport induced by 0.01 mol% of **1** was almost completely suppressed after the vesicles were treated with bovine serum albumin (BSA, 1 mol% with respect to lipid) to remove fatty acid impurities from the vesicle membranes (Figure 4b blue). Neither OA nor BSA affected the Cl− transport activity of **1** (Figure S34), and therefore their effects observed were confirmed to arise from OA dramatically enhancing H+/OH− transport activity of **1**.

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**Figure 4.** (a) Schematic representation of the electrogenic H+/OH− transport assay based on following the rate of pH gradient dissipation. POPC LUVs with a mean diameter of ~200 nm were used. (b) H+/OH− transport induced by **1** (or OA as a control) measured by the assay shown in (a) under various conditions indicated in the figure. Valinomycin (0.05 mol%) was used in all cases. The vesicles were treated with monensin (0.1 mol%) at 200 s to collapse the pH gradient for calibration of HPTS fluorescence. Compound concentrations are shown as compound to lipid molar ratios.

To quantify the extent of OA acceleration of H+/OH− transport, dose-dependent Hill plot analyses were performed for compounds **1**–**5** and CCCP in the presence of BSA (1 mol%) or in the presence of both BSA (1 mol%) and OA (10 mol%, corresponding to ~4 mol% free concentration after BSA binding). An effective concentration to reach 50% of maximal transport at 200 s (EC50) and a Hill coefficient (*n*) were determined for each compound (Table 2) under the two conditions. The EC50 value is used to quantify the H+/OH− transport activity, while the *n* value is an indication of the stoichiometry of the complex formed to mediate the transport.[30](#_ENREF_40) Table 2 demonstrates that OA accelerated H+/OH− transport induced by all compounds except for **4** and CCCP. Compound **1** underwent a dramatic 370-fold enhancement of H+/OH− transport activity and became almost as active as CCCP upon activation by OA. For compound **3**, OA acceleration was observable but to a much lesser extent compared with less acidic thioureas **1** and **2**. This is likely due to the high acidity of **3** (p*K*a = 8.9 in 9:1 CH3CN/H2O)[26](#_ENREF_36), allowing it to function as an active protonophore via thiourea NH deprotonation (Figure 1a) in the absence of fatty acids. The OA acceleration was absent in *N*-methyl thiourea **4**. This is consistent with the poor anion binding affinity (therefore poor oleate flippase activity) and high acidity (p*K*a = 10.7 in 9:1 CH3CN/H2O)[26](#_ENREF_36) of **4**, rendering it as a strong protonophore devoid of fatty acid activation. In fact, the proton transport activity of **4** even decreased in the presence of OA, possibly resulting from the presence of negatively charged oleate ions obstructing the transmembrane movement of deprotonated **4**. The loss of fatty acid activation with a decrease of anion binding affinity from **3** to **4** supports the role of anion transporters as flippases for fatty acid anions (Figure 1c). Interestingly, the halogen bond-based transporter **5** also exhibited fatty acid activation in H+/OH− transport. Therefore, this effect appears to be universal for synthetic anion transporters that can bind carboxylate ions with sufficient affinity.

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| Table 2. Summary of H+/OH− transport data of compounds 1–5 and CCCP (based on the assay shown in Figure 4a) in the presence of BSA (1 mol%) or in the presence of both BSA (1 mol%) and OA (10 mol%). | | | | | |
| Compound | BSA | | BSA + OA | | Activation  factor*a* |
| *n* | EC50  /mol% | *n* | EC50  /mol% |
| **1** | 0.94 | 0.78 | 1.1 | 0.0021 | 370 |
| **2** | 2.7 | 5.9 | 2.2 | 0.74 | 8.0 |
| **3** | 1.1 | 0.0035 | 1.4 | 0.0013 | 2.8 |
| **4** | 1.1 | 0.0071 | 1.2 | 0.022 | 0.32 |
| **5** | –*b* | >25*b* | 2.5 | 9.0 | –*b* |
| CCCP | 0.97 | 0.0013 | 0.99 | 0.0014 | 0.93 |

*a* EC50 in the presence of BSA divided by EC50 in the presence of both BSA and OA.

*b* Too inactive for Hill analysis.

It should be noted that even after BSA treatment of the vesicles, the H+/OH− transport activities of **1** and **2** still contain a significant contribution from fatty acid-dependent pathway due to the traces of fatty acids that remain in the membrane after BSA treatment (Figure S25). However, the low activity of compound **5** obtained with 1 mol% BSA was essentially fatty acid independent (Figure S25c). This activity cannot arise from a deprotonation mechanism (Figure 1a) as **5** contains no protons. Furthermore, facilitation of buffering agent (HEPES) transport has been excluded (Figure S27) as replacing HEPES with the more hydrophilic POPSO as the buffering agent did not affect the activity of **5** in the H+/OH− transport assay. Thus the activity of **5** without fatty acids likely originates from an OH− transport mechanism (Figure 1b), presumably operating via halogen bonding to OH− ions in the case of **5**.[25](#_ENREF_35) The results in Table 2, however, imply that the putative OH− transport is a less important process to consider compared with deprotonation (Figure 1a) and fatty acid cycling (Figure 1c).

We also performed Hill plot analysis to quantify the efficacy of different fatty acids as activators of H+/OH− transport, using compound **1** at 0.01 mol% (Table 3). The results show that the saturated fatty acid PA has almost the same activity as OA in activating **1**, whereas lauric acid (LA) is about 10 times less active. To examine whether the decreased activity of LA is more likely due to the shortened alkyl chain length or reduced lipophilicity, 2-hexyldecanonic acid (2HDA) was also tested, which turned out be even more active than OA and PA. Therefore shortening the alkyl chain (while keeping lipophilicity similar) appears to be beneficial for activation. This likely arises from the less surfactant-like shape of 2HDA reducing the energy barrier of flip-flop, compared with OA and PA that align more stably with the lipid molecules within the membrane. Such an effect is reminiscent of the “lipophilicity balance” reported for anion transporters.[33](#_ENREF_43) The lower activity of LA is thus likely due to decreased lipophilicity of fatty acid anion-transporter complex resulting in a slower flip-flop rate.[34](#_ENREF_44) Other potential activators, including 1-octanoic acid, 1-hexadecanesulfonate, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) failed to show significant activation even at 10 mol% (Figure S33).

Table 3. Hill analyses of different fatty acids as activators of H+/OH− transport for compound 1 at 0.01 mol%

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| --- | --- | --- |
| Fatty acid | *n* | EC50 / mol% |
| OA | 1.2 | 0.16 |
| PA | 1.1 | 0.19 |
| LA | 0.88 | 1.8 |
| 2HDA | 1.1 | 0.023 |

**Osmotic response assay.** We used an osmotic response assay[35](#_ENREF_45) to measure the activity of Cl− uniport, H+/Cl− symport (or Cl−/OH− antiport), and AcO− uniport for compounds **1**, **2** and **5**. Note that the H+/Cl− symport activity reflects H+ transport activity if H+ transport rate-limits the overall process. These tests were conducted for two purposes: (1) to re-confirm the fatty acid activation of /OH− transport using a different assay (the abovementioned HPTS assay measures a H+/OH− flux of 5 mM and requires the use of valinomycin, whereas this osmotic assay measures a higher H+/OH− flux of 300 mM and does not require valinomycin for studying H+/OH− transport[36](#_ENREF_46)); (2) to seek possible correlation between AcO− transport and fatty acid-dependent H+/OH− transport. AcO− transport is a function closely related to the proposed fatty acid anion flippase mechanism, because both processes involve facilitated translocation of a carboxylate group across lipid bilayers. Previously, AcO− transport was studied indirectly using an HPTS assay.[37](#_ENREF_47) Note that the strong protonophores **3** and **4** cannot be tested for AcO− transport, because they would produce formal AcO− flux (via HOAc simple diffusion plus H+ transport in the opposite direction) even if they do not actually facilitate AcO− transport (Figure S41).[38](#_ENREF_48)

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**Figure 5.** (a) Schematic illustration of the assays used for measuring Cl− uniport, AcO− uniport, and H+/Cl− symport (the functionally equivalent Cl−/OH− antiport is omitted). POPC LUVs with a mean diameter of ~400 nm were used. (b) Results of the osmotic response assays for compound **1** at 2.5 m0l% in the presence of BSA (1 mol%) or both BSA (1 mol%) and OA (10 mol%). The salt efflux was initiated by addition of compound **1** at o min (cation transporters were added before)**,** and terminated at 5 min by monensin/tributyltin chloride combination for KCl efflux or monensin for KOAc efflux. Compound concentrations are shown as compound to lipid molar ratios.

POPC LUVs (mean diameter ~400 nm) loaded with KCl or KOAc were treated with a combination of the anion transporter of interest and a cation transporter (K+ uniporter valinomycin or K+/H+ antiporter monensin), and the salt efflux was followed via increase of 90° light scattering due to osmotic shrinkage of vesicles. The rationale of using valinomycin and monensin to investigate different transport processes has been described previously[26](#_ENREF_36) and is schematically shown in Figure 5a. The initial rates of different processes determined by exponential fitting of the data are shown in Table 4 (See Figures 5b and S42–S46 for original kinetic data). For compounds **1**, **2** and **5** at tested concentrations, the H+/Cl− symport (or Cl−/OH− antiport) activity is negligible without fatty acids but greatly enhanced in the presence of OA, whereas their Cl− uniport activity was unaffected by fatty acids (Figures 5b, S42 and S43). These results agree with the data from the abovementioned HPTS proton transport assay and confirm fatty acid activation of H+/OH− transport under valinomycin-free conditions. Compounds **1**, **2** and **5** facilitate AcO− transport slower than Cl− transport.[39](#_ENREF_49) Importantly, halogen-bond based transporter **5** turned out to be a rather poor AcO− transporter, with an initial rate about 20-fold slower than for Cl− transport. In contrast, both **1** and **2** show less than 5-fold differences between AcO− transport and Cl− transport. This matches with their activities in fatty acid-dependent H+/OH− transport (relative to their Cl− transport activity). Compound **5** also exhibited poor activity in fatty acid-dependent H+ transport, in contrast to **1** and **2** that are both active H+ transporters in the presence of OA. The good correlation between AcO− transport activity and fatty acid-dependent H+/OH− transport activity (relative to Cl− transport activity) provides further evidence for the fatty acid cycling mechanism.

Table 4. Initial rates (% s-1) of Cl− uniport, AcO− uniport, and H+/Cl− symport induced by 1, 2, or 5, measured with an osmotic response assay using POPC LUVs with a mean diameter of ~400 nm.

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| --- | --- | --- | --- | --- | --- | --- |
| Compound | Cl− uniport*a* | AcO− uniport*a* | H+/Cl− symport | | | |
| BSAa | BSA + OA*b* | | |
| **1** (2.5 mol%) | 5.5 | 0.98 | 0.026 | | 1.5 | |
| **2** (10 mol%) | 1.7 | 0.61 | 0.057 | | 1.2 |
| **5** (100 mol%) | 0.61 | 0.030 | 0.0051 | | 0.020 | |

*a* Measured in the presence of BSA (1 mol%). The addition of OA (10 mol%) did not significantly affect the rate of Cl− uniport (Figures S41 and S42). AcO− uniport cannot be measured in the presence of OA because OA activates H+ transport and the enhanced HOAc/H+ antiport pathway (Figure S41) will interfere with the quantification of AcO− uniport.

*b* Measured in the presence of both BSA (1 mol%) and OA (10 mol%).

Using the osmotic assay, we also found that perfluorolauric acid (PFLA, p*K*a ~0)[40](#_ENREF_50" \o "Goss, 2008 #3788) failed to activate **1** for H+/OH− transport (Figure 6), in contrast to LA (p*K*a ~ 7.5 in aggregates)[41](#_ENREF_51) which showed significant activation. Since the amount of neutral PFLA in equilibrium with its anionic form is minimal at pH 7-8, the inability of PFLA to function as an activator is consistent with the fatty acid cycling model (Figure 1c) that requires involvement of the neutral species, although the unique physicochemical properties of perfluorinated compounds[20a](#_ENREF_24) could complicate interpretation of the results.

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**Figure 6.** KCl efflux facilitated by a combination of monensin (0.5 mol%) and compound **1** (2.5 mol%) with and without the addition of lauric acid (LA) or perfluorolauric acid (PFLA), measured by light scattering using POPC LUVs with a mean diameter of 400 nm. The vesicles were loaded with KCl (300 mM) and suspended in K-gluconate (300 mM), both internal and external solutions buffered at pH 7.4 with 5 mM HEPES. This assay measured the activity of H+/Cl− symport (or Cl−/OH− antiport) facilitated by compound **1** and fatty acids. The KCl efflux was initiated by addition of compound **1** at o min (fatty acids and cation transporters were added before)**,** and terminated at 5 min by tributyltin chloride (5 mol%). The vesicles were not treated with BSA and therefore contained fatty acid impurities that were responsible for the response without fatty acid addition. Compound concentrations are shown as compound to lipid molar ratios.

**Cl− > H+/OH− selectivity.** Finally, we re-examined the Cl− > H+/OH− selectivity of tripodal anion transporters **1** and **6** under “fatty acid-free” and fatty acid-rich conditions, using our recently reported HPTS assay based on the use of proton channel gramicidin D.[26](#_ENREF_36) The results (Table S1) demonstrated that the Cl− > H+/OH− selectivity of both compounds decreased in the presence of 2 mol% of OA (because of OA acceleration of H+/OH− transport), and increased compared with our previous results after fatty acid removal by BSA. These data further confirm fatty acid activation of H+/OH− transport, obtained using a third assay that does not involve valinomycin or monensin. Although **6** still shows a significant Cl− > H+/OH− selectivity with 2 mol% of OA, new anion transporters that can maintain a higher selectivity under fatty acid-rich conditions are desired to be used as Cl− transport agents that do not disrupt cellular pH and proton gradients. Nevertheless, the almost perfect Cl− > H+/OH− selectivity of **6** under “fatty acid-free” conditions indicates that this compound can be used as a “valinomycin for Cl−“ to set the membrane potential in vesicle-based experiments after BSA-treatment of vesicles.

**Conclusions**

In summary, we have demonstrated that several synthetic anion transporters serve as functional mimics of UCPs in that they facilitate proton leak upon activation by long-chain fatty acids. The most notable example, tripodal thiourea **1**, displays a several hundred-fold difference in H+/OH− transport activity in the absence and presence of OA. This activation requires only sub-micromolar concentrations of OA and is significant even with the traces of fatty acid impurities in commercial lipid samples. We provided several lines of evidence to support a fatty acid cycling mechanism (Figure 1c), in which an anion transporter serves as a flippase for fatty acid anions. These include: (1) observation of strong AcO− binding in CH3CN for several anion transporters that show fatty acid activation; (2) absence of fatty acid activation in Cl− transport; (3) loss of fatty acid activation after *N*-methylation of a thiourea that leads to weakened anion binding; (4) strong correlation between AcO− transport activity and fatty acid-dependent H+/OH− transport activity (relative to Cl− transport), with the halogen bond donor **5** being an inactive AcO− transporter also showing a poor activity in fatty acid-activated proton transporter; (5) inability of “unprotonatable” anions including 1-hexadecanesulfonate and perfluorolaurate to function as activators. The demonstration of the fatty acid cycling mechanism with small molecules implies that the same mechanism might operate in some naturally occurring membrane proteins. The results also disclose fatty acid anion flippase as a new mechanism in which synthetic anion transporters can dissipate a pH or proton gradient in vesicle-models and cells,[42](#_ENREF_52) in addition to the previously proposed H+ and OH− transport mechanisms.[26](#_ENREF_36) This leads to a better understanding of the often observed *in vitro* toxicities of synthetic anion transporters, and provides guidelines on designing transporters with low proton transport activities (by weakening fatty acid binding) to be used as chloride ionophores for “channelopathy” treatment, or high proton transport activities (by improving fatty acid binding) to disrupt pH gradients in cancer cells. Furthermore, the work also shows the potential of using synthetic anion transporters as a new class of chemical uncouplers subject to fatty acid activation (unlike commonly used uncouplers such as CCCP that are fatty acid-independent), which could be of important utility in physiological research and treatment of obesity (by increasing energy expenditure) and age-related diseases (by reducing reactive oxygen species production).[3](#_ENREF_3)

ASSOCIATED CONTENT

UV-Vis absorption spectra, membrane transport assay details, original kinetic data, control experiments, and Cl− > H+/OH− selectivity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

\*philip.gale@sydney.edu.au

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