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# Bactericidal urea crown ethers target phosphatidylethanolamine membrane lipids<sup>†</sup>

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An increasing number of people are infected with antibiotic-resistant bacteria each year, sometimes with fatal consequences. In this manuscript, we report a novel urea-functionalized crown ether that can bind to the bacterial lipid phosphatidylethanolamine (PE), facilitate PE flip-flop and displays antibacterial activity against the Gram-positive bacterium *Bacillus cereus* with a minimum inhibitory concentration comparable to that of the known PE-targeting lantibiotic duramycin.

Infectious diseases represent a leading cause of death worldwide. While the advent of antibacterial agents has led to much improvement, most antibiotics in clinical use today were developed during the 1940s to 1960s.<sup>1</sup> This lack of novel drugs has given bacteria time to develop numerous resistance mechanisms against the most commonly used antibiotics. There is thus an urgent need for the development of new antibiotics with a low chance of inducing resistance. One drug target that has become increasingly popular in this regard is the bacterial membrane. It contains one third of the proteins in the bacterium and is the site for crucial biological processes which could be disrupted with membrane-binding antibiotics.<sup>2</sup> Resistance is thought to be less likely due to the rapid bactericidal effect of membrane disruption, and the fact that lipid mutations are less trivial than protein mutations. Unsurprisingly, there are many natural products with antibacterial activity that function by targeting the membrane, most notably antimicrobial peptides (AMPs).<sup>3</sup> AMPs are a large family of naturally occurring peptides that are usually poly-cationic and amphiphilic in nature.<sup>3</sup> It is generally believed that the cationic charge is responsible for membrane binding and the selectivity towards bacterial cells, which display larger amounts of negatively charged phospholipids than mammalian cells.<sup>4,5</sup> However, the use of AMPs for the systemic treatment of bacterial infections is hindered by their poor pharmacokinetics, high production cost, high dosage requirements, and risk for resistance due to proteases.<sup>6</sup> It is therefore beneficial to develop non-peptidic molecules that can bind to bacterial lipids and exert antibacterial activity in a similar fashion to AMPs.

Supramolecular chemists have started to develop small molecules that bind to lipid headgroups, but the focus has been on mammalian lipids such as phosphatidylserine (PS),<sup>7-10</sup> phosphoinositides (PI),<sup>11,12</sup> and phosphatidylcholine (PC).<sup>13,14</sup> Where bacterial membranes have been targeted, it has been limited to polycationic species that bind to the negatively charged lipids in bacteria (e.g., cardiolipin and phosphatidylglycerol).<sup>15-24</sup> However, the extensive use of nonselective coulombic interactions creates a risk for off-target effects and human toxicity. In contrast, here we describe the development of neutral non-peptidic compounds that target the bacterial lipid phosphatidylethanolamine (PE). PE is a zwitterionic phospholipid found in most Gram-negative bacteria<sup>25,26</sup> and in certain Gram-positive bacteria.<sup>5</sup> In mammalian cells, the most common zwitterionic lipid is phosphatidylcholine (PC).<sup>4</sup> PE and PC have a similar headgroup but differ in their degree of methylation of the ammonium group (Scheme 1a), allowing selective binding of PE over PC. Two AMPs, duramycin and cinnamycin, have been shown to selectively target PE in bacterial membranes and apoptotic cells.<sup>27-29</sup> Yet, the number of nonpeptidic small molecules that target PE is very limited,<sup>30–37</sup> and it is thus desirable to develop novel small molecules with high PE selectivity and antibacterial potency.

Our approach in developing a PE targeting receptor utilizes a urea functionality to bind to the phosphate moiety in PE,<sup>38</sup> 18-crown-6 to bind to the ammonium group of PE,<sup>39</sup> and a trifluoromethyl-substituted phenyl substituent as a lipophilic membrane anchor<sup>40</sup> (Scheme 1b). The urea and crown ether functionality are linked together through either a rigid linker (**1a**) or a flexible linker (**1b**) to determine the geometry that

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(a) lipid headgroups



Scheme 1 (a) Structure of the head group of the targeted bacterial lipid PE and the mammalian lipid PC. (b) Structure of the putative PEbinding compounds 1a and 1b and the proposed complex of 1a with PE. (c) Structure of control compounds 2a and 2b.

allows the best binding to the head group. Control compounds **2a**, **2b**, and 18-crown-6 (**18C6**) were also synthesized to investigate the importance of the urea or crown ether moieties in the molecular recognition of PE lipids (Scheme 1c). Synthetic details and characterization are provided in the ESI.<sup>†</sup> Crown ether derivatives have previously been reported as antimicrobial agents due to their ability to function as ionophores for K<sup>+</sup> ions.<sup>41</sup> However, the low membrane selectivity of ionophores has impaired their clinical usefulness in most cases. In contrast, our design takes advantage of **18C6**'s known ability to selectively bind to primary ammonium cations over more substituted ammonium cations.<sup>39</sup> Combined with the phosphate binding unit and membrane anchor, the crown ether derivatives become PE-selective membrane-active agents with improved antibacterial potency.

To assess the selectivity of 1a and 1b for PE over PC lipids, we initially performed a set of <sup>1</sup>H NMR titrations in organic solvents. Under these conditions the lipids do not form membranes but are free in solution. While this is not a perfect mimic of biological conditions, it allows an accurate determination of association constants and a good indication of the inherent head group selectivity of each compound. The titrations were performed in 0.5% Milli-Q H<sub>2</sub>O, 24.5% DMSO-d<sub>6</sub> and 75% CDCl<sub>3</sub> for solubility reasons and using either POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) or POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) as guest (ESI<sup>†</sup>). The data was fitted using Bindfit<sup>42</sup> and the obtained association constants ( $K_a$ ) are given in Table 1. The <sup>1</sup>H NMR titrations confirmed that rigid compound 1a binds more strongly to POPE ( $K_a = 531 \text{ M}^{-1}$ ) than POPC ( $K_a = 72 \text{ M}^{-1}$ ). Surprisingly, the flexible crown ether analog 1b showed no

Table 1Overview of the PE-binding and antibacterial ability of hosts1a-2b and 18-crown-6. All data is the average of at least 3 independentrepeats and errors represent standard deviations

	$K_{\rm a}$ (M <sup>-1</sup> ), NMR <sup><i>a</i></sup>		$K_{\rm SV}$ (M <sup>-1</sup> ), fluorescence <sup>b</sup>		MIC <sup>c</sup>
Host	POPE	POPC	NBD-PE	NBD-PC	μM)
1a 1b 2a 2b 18C6	$531 \pm 56$ Weak <sup>d</sup> $263 \pm 18$ Weak <sup>d</sup> $180 \pm 45$	$72 \pm 6$ Weak <sup>d</sup> $129 \pm 9$ $22 \pm 3$ n.d. <sup>g</sup>	$\begin{array}{l} (6.3\pm0.8)\times10^4\\ \mathrm{Weak}^e\\ \mathrm{n.d.}^f\\ \mathrm{n.d.}^f\\ \mathrm{Weak}^e \end{array}$	$\begin{array}{l} (1.3\pm0.1)\times10^4\\ \mathrm{Weak}^e\\ \mathrm{n.d.}^f\\ \mathrm{n.d.}^f\\ \mathrm{Weak}^e \end{array}$	25-30 >100 >100 6.25 >100

<sup>*a*</sup> Association constant ( $K_a$ ,  $M^{-1}$ ) obtained through <sup>1</sup>H NMR titrations in 0.5% H<sub>2</sub>O:24.5% DMSO-d<sub>6</sub>:75% CDCl<sub>3</sub> at 298 K. <sup>*b*</sup> Stern–Volmer constant ( $K_{SV}$ ,  $M^{-1}$ ) obtained through titrations of the hosts into POPC or 1:1 POPE:POPC liposomes containing NBD-labelled lipids. <sup>*c*</sup> Minimum inhibitory concentration (MIC) obtained using broth microdilution methods. <sup>*d*</sup> No significant change in chemical shift was observed. <sup>*e*</sup> Not significant change in fluorescence intensity was observed. <sup>*f*</sup> Not determined (n.d.) due to insolubility. <sup>*g*</sup> Not determined (n.d.) due to lack of protons capable of H-bonding.

measurable interactions with either lipid. Computational modelling using Molecular Operating Environment (MOE) suggests that the flexible linker allows an intramolecular H-bond between the urea NHs and crown ether oxygens, thereby blocking the binding site (ESI<sup>†</sup>). The control compounds 2a, 2b and 18C6 did not bind as effectively as 1a either. The "rigid control" 2a showed non-selective binding to both lipids, while the "flexible control" 2b only showed minimal binding to POPC (Table 1). The stronger interaction of POPE with 2a versus 2b is probably due to hydrogen bonding between the methoxy substituents of 2a and the ammonium group of POPE. Association constants with 18-crown-6 could only be determined for POPE due to the lack of protons capable of H-bonding in both POPC and 18C6. However, binding studies clearly showed that the crown ether was able to complex POPE  $(K_a = 180 \text{ M}^{-1})$ , but to a smaller extent than compound **1a** which can coordinate both the ammonium and phosphate groups of POPE.

After observing selective binding in organic solvents, we investigated the interaction of the compounds with lipids that are part of phospholipid membranes using fluorescence titrations. Due to the inability of pure POPE to form stable liposomes,<sup>43</sup> the titrations were performed with either POPC liposomes containing 1 mol% 18:1-06:0 NBD-PC, or 1:1 POPE: POPC liposomes containing 1 mol% 18:1-06:0 NBD-PE (ESI<sup>†</sup>). The NBD fluorophore in the labelled lipids is attached to the acyl chain of the lipids, but is known to loop up to the polar membrane-water interface.44 As such, NBD-labelled lipids function as surface probes and have been used to monitor lipid phase separation,45 and lipid binding and partitioning.<sup>46–48</sup> The addition of 1a to PE-containing liposomes caused complete quenching of the NBD fluorophore (Fig. 1). Fluorescence quenching was less pronounced when 1a was added to PC liposomes, suggesting selective binding of PE over PC. Quenching of the NBD-labelled lipids by compound 1a showed a Stern-Volmer relationship (Fig. 1, inset), and the



**Fig. 1** Binding of **1a** to liposomes. Fluorescence spectra obtained through the titration of **1a** into a solution of **1**:1 POPC : POPE liposomes containing 1 mol% NBD-labelled PE. Spectra were normalized based on the maximum intensity of the spectrum before the addition of **1a**. The inset shows the Stern–Volmer plots of the fluorescence titrations of compound **1a** into either **1**:1 POPC : POPE liposomes containing 1 mol% NBD-labelled PE ('PE' green squares) or pure POPC liposomes containing 1 mol% NBD-labelled PC ('PC', purple open circles). Plots are the average of at least 4 independent Stern–Volmer plots, and error bars represent standard deviations.

obtained Stern–Volmer constants further confirmed the selectivity of **1a** for PE over PC (Table 1). None of the other compounds tested showed any fluorescence quenching with either PE or PC lipids (ESI†). Overall, this data supports our previous finding that **1a** can bind strongly and selectively to PE.

To provide further evidence of favourable binding to PE in lipid bilayers, we performed other liposome-based experiments such as calcein leakage assays<sup>49</sup> and lipid flip-flop assays.<sup>50</sup> The calcein leakage assays did not indicate membrane disruption or pore formation by any of the crown ether derivatives (ESI<sup>†</sup>). In contrast, the lipid flip-flop studies showed more promising results. In this assay, 100 nm unilamellar DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) liposomes were prepared containing 1 mol% NBD-PE or NBD-PC in the outer leaflet of the membrane. Translocation or 'flip-flop' of phospholipids across a lipid bilayer is normally a very slow process with a half-life of a few hours.<sup>51</sup> In the presence of molecules that can bind to the lipid head group, the polarity of the head group can be reduced and lipid translocation can be facilitated.<sup>50,52</sup> To detect flip-flop, the NBD group of lipids in the outer leaflet can be selectively quenched via reduction with membrane-impermeable dithionite.53 Residual fluorescence will be the result of flip-flop of the NBD-labelled lipid from the outer leaflet of the membrane to the inner leaflet. The results for the PE flip-flop assay are given in Fig. 2. Only compound 1a is able to facilitate PE translocation, in agreement with the



Fig. 2 Lipid flip-flop induced by 1a-2b. (Top) Experimental set-up: 100 nm DOPC liposomes (25  $\mu$ M) containing fluorescent NBD-PE in the outer leaflet of the membrane are incubated with 25  $\mu$ M 1a-2b, 18-crown-6 or DMSO to induce lipid flip-flop. At certain time intervals, the fluorescence of the NBD-PE lipids in the outer leaflet is quenched by the addition of dithionite to calculate the % of NBD-PE that has been flipped by the compounds. (Bottom) Percent of NBD-PE flipped by 25  $\mu$ M 1a-2b, 18-crown-6 or DMSO over a time scale of 60 minutes. Plots are the average of at least 4 independent repeats, and error bars represent standard deviations.

stronger PE binding ability for **1a** observed in the <sup>1</sup>H NMR and fluorescence titrations. In fact, facilitated PE flip-flop could be observed for **1a** at concentrations as low as 3.125  $\mu$ M (ESI<sup>†</sup>), which is a significant improvement on a previously reported synthetic crown ether sulfonamide that could only mediate modest PE flip-flop at high concentrations (100  $\mu$ M).<sup>52</sup> Flipflop of PC lipids was not observed for any of the compounds, further confirming the high selectivity of **1a** for PE over PC (ESI<sup>†</sup>).

Lastly, we wanted to determine if the PE-targeting compounds possess antibacterial activity. PE is found in the inner membrane and the inner leaflet of the outer membrane of Gram-negative bacteria, rendering access to PE in Gram-negative bacteria challenging.<sup>25,26</sup> On the other hand, most Grampositive bacteria lack PE, except for species of *Bacillus* and *Clostridium*. Any compound targeting PE is therefore expected to function as a narrow-spectrum antibacterial agent against these bacterial species. Narrow-spectrum antibiotics are gaining popularity because they do not select for resistance in non-pathogenic bacteria and do not impact the human microbiome.<sup>54</sup> With this in mind, we performed a screening assay where the compounds were incorporated into a Müller-Hinton

agar medium, and the agar was subsequently inoculated with the bacterial species S. simulans (0% PE),<sup>55</sup> B. subtilis (20-30% PE),<sup>56</sup> and *B. cereus*  $(40-50\% \text{ PE})^{57}$  (ESI<sup>†</sup>). Compounds **1b**, **2a** and 18-crown-6 did not inhibit the growth of any of the bacteria, consistent with their lack of activity in the assays described above. In contrast, 2b showed antibacterial activity against all bacteria tested, regardless of their PE content. This indicates that 2b exerts its antibacterial activity through a mechanism that does not involve PE binding, consistent with its lack of PE binding observed in the <sup>1</sup>H NMR titrations and flip-flop assays. More interestingly, compound 1a had no effect on the growth of S. simulans, caused a significant delay in the growth of B. subtilis and complete inhibition of bacterial growth of B. cereus. The correlation with the PE-content of these bacterial species suggests that the mechanism of 1a involves binding to PE lipids.

The antibacterial activity of 1a against B. cereus was subsequently investigated in more detail. B. cereus is a common cause of foodborne illness,<sup>58</sup> is closely related to the bioterrorism agent B. anthracis59 and is therefore a pathogen of interest. The minimum inhibitory concentration (MIC) of compounds 1a-2b against B. cereus was determined using standard broth microdilution methods<sup>60</sup> (Table 1 and ESI<sup>†</sup>). Compound 1a showed an MIC value of 25-30 µM, comparable to the MIC value obtained for the known PE-targeting peptide duramycin (MIC ~32 µM, ESI†). Membrane-active antibiotics are normally bactericidal rather than bacteriostatic.61,62 The minimum bactericidal concentration (MBC), defined as the lowest concentration needed to kill 99.9% of bacteria, was 35-40 µM for 1a, which is only slightly higher than its MIC value (25-30 µM). This suggests that compound 1a has bactericidal activity and points towards a mode of action that involves the bacterial membrane. Further evidence of a membrane-based mechanism came from live cell imaging and Gram staining of the B. cereus bacteria after 24 h incubation with compound 1a (ESI<sup>†</sup>). This revealed a pronounced elongation of the bacterial cells, which is a morphological change that has been observed for other Bacillus species upon alteration of their membrane composition.<sup>63</sup> In addition, we investigated the ability of 1a to cause membrane depolarization of B. cereus cells using the voltage-sensitive dye Disc<sub>3</sub>(5).<sup>64</sup> This cationic membrane-permeable fluorophore accumulates in polarized cells, where it self-quenches. When the membrane potential is dissipated, the dye is released into the medium and de-quenched, which can be followed by a fluorometric assay (Fig. 3a). Alternatively, the depolarization event can also be studied using fluorescence imaging (Fig. 3b). In this case, cells that are polarized show a pronounced red fluorescence due to the accumulation of  $Disc_3(5)$ , whereas depolarized cells do not show fluorescence. Gramicidin was used as a positive control known to cause membrane depolarization,65 and clindamycin was used as a negative control because it targets the ribosome rather than the bacterial membrane.<sup>66</sup> At 25  $\mu$ M (1× MIC) 1a caused partial depolarization of B. cereus, while full depolarization was seen at 40  $\mu$ M (1.6 $\times$  MIC) and 250  $\mu$ M (10 $\times$  MIC). These



**Fig. 3** Membrane depolarization of *B. cereus* by **1a** measured using  $\text{Disc}_3(5)$ . (a) Fraction of  $\text{Disc}_3(5)$  released after 15 minutes incubation with clindamycin (negative control, 1 µg mL<sup>-1</sup>, 1× MIC), 25 µM **1a** (1× MIC), 40 µM **1a** (1.6× MIC), 250 µM **1a** (10× MIC) or gramicidin (positive control, 1.25 µM, 1× MIC). Data is the average of 2 biological × 2 technical repeats and error bars represent standard deviations. (b) Brightfield and fluorescence imaging of *B. cereus* incubated for 15 minutes with 4% DMSO (blank) or **1a** (250 µM, 10× MIC). Absence of fluorescence indicates that the cells are depolarized. Scale bars represent 10 µm.

results confirm that **1a** functions as a bactericidal agent against *B. cereus* due to its ability to interact with the bacterial membrane.

#### Conclusions

In this manuscript, we have identified a new crown ether urea derivative **1a** that is able to selectively bind to the bacterial lipid PE over the mammalian lipid PC in both solution and in liposomes. Furthermore, the compound functions as a bactericidal agent against *B. cereus* with an MIC value of 25–30  $\mu$ M and causes membrane depolarization in this bacterium. The other urea and crown ether compounds in this manuscript did not have the same affinity for PE, demonstrating that the 18-crown-6 and urea group are both required for strong PE head group binding and a rigid linker is needed between the two to achieve the right conformation. We are currently synthesizing a series of analogs of this promising lead compound to optimize its antibacterial activity.

### Conflicts of interest

There are no conflicts to declare.

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