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Divalent Metal Ion Triggered Activity of a Synthetic Antimicrobial in Cardiolipin Membranes

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Antimicrobial peptides (AMPs) have broad-spectrum killing activity against prokaryotes but not against eukaryotes and consequently are considered to be promising antibiotic candidates. However, these peptides are typically large (up to 80 amino acid residues), structurally complex, and expensive to produce. As a result, much effort has been focused on developing synthetic analogues,[1] known as antimicrobial oligomers (AMOs). Recent human clinical data indicate that this is a promising approach.[1h,1i] There is general agreement that these antimicrobial molecules interact with the cell membrane, which can have complex distributions of lipids,[2a−e] including lipids with nonzero intrinsic curvature,[2f] such as unsaturated phosphatidylethanolamine (PE)[2g] and cardiolipin (CL).[2g−i] Bacterial membranes have a significantly higher population of negative intrinsic curvature (NIC) lipids than mammalian membranes, and it was recently shown that these differences are one mechanism by which AMOs can differentially kill bacteria over mammalian cells.[2d,e,j−l]

The specific relationship between PE and AMO **2** was recently described in detail.[2d,j] While this readily explained the potent activity against Gram-negative bacteria (minimum inhibitory concentration of 0.1 μg/mL against *Escherichia coli*), it did not explain the activity of this AMO against Gram-positive bacteria such as *Staphylococcus aureus* (0.2 μg/mL), since this organism is known to contain no PE lipid.[3] In addition, it was demonstrated that although antimicrobial activity is largely ablated in PE-knockout strains of *E. coli*,[4] residual antimicrobial activity still remains. Moreover, the previous results specifically implicated PE lipid, although it was hypothesized that NIC lipids were more important than any specific binding between the AMO and PE.

Here we examine how a prototypical synthetic AMO interacts with CL, which is the major lipid component in most Gram-positive bacteria. We show that the structural requirement needed for membrane activity of AMO **2** is the existence of NIC lipids, in this case CL. A central feature of CL is that its intrinsic curvature is influenced by the bound cations and can be tuned between zero intrinsic curvature ($C_0 \approx 0$) in the presence of monovalent salts and negative intrinsic curvature ($C_0 < 0$) when CL is bound to divalent cations such as the biologically prevalent Ca^{2+} or also Mg^{2+} [Figure S1 in the Supporting Information (SI)]. The presence of NIC lipids is one of the ingredients that facilitate pore formation.[2d,e,j−l] AMO **2** activity against CL membranes, with and without divalent metal ions (Ca^{2+}, Mg^{2+}) was evaluated using both calcein dye leakage assays (DLAs) and small-angle X-ray scattering (SAXS).

The ability of AMO **2** to induce calcein dye leakage from CL vesicles depends strongly on the presence of divalent cations. For example, leakage increased from 5% without divalent cations to 60% in the presence of 2.0 mM Mg^{2+} or 1.0 mM Ca^{2+} (Figure 1A,B). Importantly, the presence of 2.0 mM Ca^{2+} alone caused no leakage of entrapped calcein dye (Figure S2 in the SI). Furthermore, the presence of Ca^{2+} had no influence on the leakage activity of 20:80 phosphatidylglycerol (PG)/PE vesicles caused by AMO **2** (Figure 1C). These three data sets highlight the necessity of NIC lipids for AMO **2** to induce calcein release. For PG/PE vesicles that already have a large volume fraction of NIC lipids, the addition of Ca^{2+} does not enhance leakage. In contrast, AMO **2** has limited activity toward CL vesicles until a reasonable concentration of divalent cations is added to the solution. Because the plasma membranes of most Gram-positive bacteria are not composed solely of CL lipid, we also examined the dependence of AMO **2**-induced leakage in PG/CL vesicles. Again, leakage was dependent on the presence of divalent cations, as shown in Figure 1D. Not surprisingly, a higher concentration of divalent cation was required for dye leakage to occur in this PG/CL system than in pure CL vesicles (see discussion below).

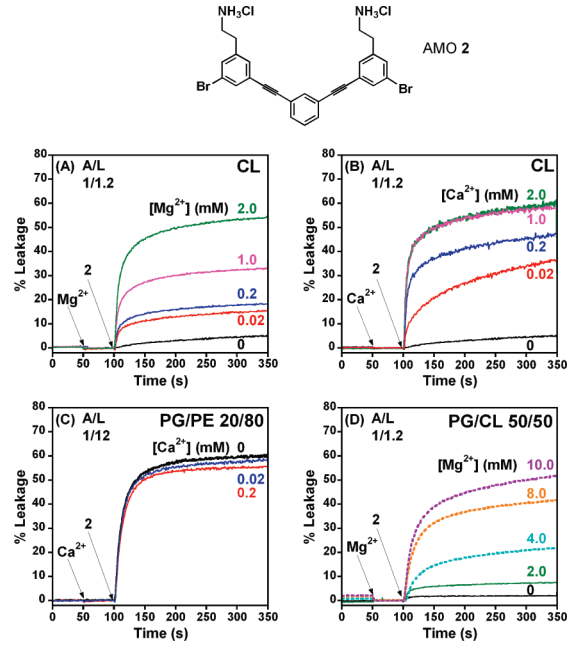

Figure 1. Calcein dye leakage induced by AMO **2** (2.5 μg/mL for A, B, and D; 0.25 μg/mL for C) from (A, B) 100% CL vesicles, (C) 20:80 PG/PE vesicles, and (D) 50:50 PG/CL vesicles with 0, 0.02, 0.2, 1.0, 2.0, 4.0, 8.0, and 10.0 mM (A, D) Mg^{2+} and (B, C) Ca^{2+}. The final lipid concentration was 5.0 μM for assay.

To further explore the relationships between AMO **2** and CL-rich membranes, we employed SAXS. Recent work showed that the induction of an inverted hexagonal H_{II} phase in PE-rich

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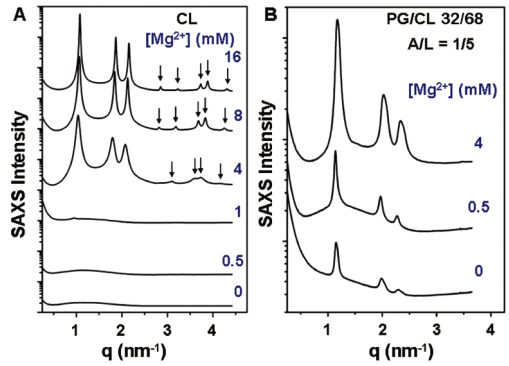

Figure 2. (A) Synchrotron SAXS data show that at sufficiently high [Mg^{2+}] (1−4 mM), Mg^{2+} ions can induce small unilamellar vesicles (SUVs) composed of CL to form an inverted hexagonal structure. (B) Synchrotron SAXS data show that AMO **2** induces SUVs composed of 32:68 DOPG/CL to form a hexagonal structure at reduced concentrations of divalent Mg^{2+} ions.

membranes by AMOs is correlated with dye leakage and bacterial growth inhibition.[2d,j] Here we mapped the structural tendency of CL-rich membranes to reorganize in the presence of AMO **2** and Mg^{2+}. Figure 2A shows SAXS data for pure CL in the presence of increasing [Mg^{2+}]. Consistent with previous results,[2i,5] CL can form an inverted hexagonal phase at sufficiently high [Mg^{2+}] concentrations (1−4 mM) in the absence of the AMO.

As Gram-positive bacterial membranes contain a mixture of CL and PG lipids, SAXS experiments were also performed on mixed-lipid compositions. The addition of anionic PG lipids to a CL membrane is expected to decrease the tendency to form negative curvature by reducing the local concentration of NIC CL. The SAXS data (Figure S5 in the SI; SAXS data without AMOs and other control experiments are described in the SI) is consistent with this expectation, as DOPG/CL vesicles with 4.0 mM [Mg^{2+}] showed no peaks corresponding to the inverted hexagonal phase, in contrast to Figure 2A. Meanwhile, Figure 2B shows SAXS data from complexes formed by AMO **2** and 32:68 DOPG/CL vesicles in the presence of different [Mg^{2+}] concentrations (0, 0.5, and 4.0 mM), with AMO/lipid (A/L) molar ratios fixed at 1:5. Consistent with the DLA results that showed some leakage (see Figure 1D), AMO **2** can induce a phase with hexagonal symmetry without Mg^{2+}, as indicated by peak positions at the characteristic ratio of $1:\sqrt{3}:2$. However, the presence of divalent Mg^{2+} increases the structural tendency to form this phase, as evidenced by stronger diffraction peaks. Also consistent with this observation, the phase with hexagonal symmetry forms at significantly lower [Mg^{2+}] in the presence of AMO **2** than for pure CL vesicles (compare panels A and B of Figure 2, specifically [Mg^{2+}] = 0.5 mM).

Electron density reconstructions (Figure S6 in the SI) from the above SAXS data confirmed that an inverted hexagonal H_{II} phase was formed and also allowed the number of AMO and Mg^{2+} ions in the H_{II} water channels to be estimated. From the reconstructed electron density profiles, we found the calibrated average electron densities at the hydrophilic surface to be 0.58, 0.57, and 0.55 e/Å^3 for complexes formed at [Mg^{2+}] = 0, 0.5, and 4.0 mM, respectively. For complexes with a high A/L ratio of 1:5 and without Mg^{2+} ions, we used a previously described procedure[2j] to estimate that approximately four AMO **2** molecules per 4 nm length of the water channel (thickness of a lipid bilayer) are embedded near the hydrophilic region of the membrane. From the reconstructed electron density profiles of complexes formed at high [Mg^{2+}] from pure CL vesicles with no added AMO, we estimate that each CL molecule binds ~0.6 Mg^{2+} ions (see the SI).

This information was used to examine the self-assembly of AMO **2** with PG/CL membranes in the presence of Mg^{2+}. With the working hypothesis that the number of bound Mg^{2+} ions per CL for 32:68 DOPG/CL is the same as that of CL vesicles, it was estimated that the numbers of AMO **2** molecules per 4 nm of channel length are ~3.6 and ~3.2 for complexes at [Mg^{2+}] = 0.5 and 4 mM, respectively. Interestingly, these observed channel occupancies of AMO **2** molecules are quite close to that found in 20:80 DOPG/DOPE at high A/L ratios.[2j] These results suggest that with the help of curvature-generating divalent Mg^{2+} ions, CL-rich membranes have approximately three AMO **2** molecules embedded in their hydrophilic surface lining per 4 nm length of water channel. These results are also consistent with recent work showing that bacterial mutants with CL-rich membranes can be killed by AMO **2** in the presence of Mg^{2+}.[2j] This suggests that CL, in the presence of Mg^{2+}, acts as an NIC lipid and functions as a structural substitute for PE during the membrane interaction by AMO **2**. It is likely that both are able to play a structural role in forming the negative curvature necessary for the circumferential barrel of the transmembrane pore.[2d]

These results indicate that the presence of NIC lipids is important for pore formation by this class of AMO molecules: In Gram-positive bacteria, CL and divalent metal cations such as Ca^{2+} and Mg^{2+} are needed. This is completely consistent with the role of PE lipid in Gram-negative bacteria, where AMO antimicrobial activity is dependent on the NIC of PE rather than a specific interaction with PE. These results lead to the conclusion that influencing curvature directly, as well as indirectly by targeting NIC lipids, can be a new approach for antibiotic design.

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Supporting Information Available: Experimental procedures and supporting figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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