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Synthetic oligoureas of metaphenylenediamine mimic host defence peptides in their antimicrobial behaviour

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ABSTRACT

Oligomeric ureas of *m*-phenylenediamine target anionic DMPG (dimyristoylphosphatidylglycerol) and possess promise as antimicrobial agents. Their similar size, shape and hydrophobicity to helical antimicrobial peptides (AMPs) may be important for activity to exist and the ability of these compounds to insert into a well ordered lipid environment.

Increasing bacterial resistance to conventional antibiotics has led the pharmaceutical industry to seek novel lead compounds, of which antimicrobial peptides (AMPs) have shown potential because of their wide antimicrobial specificity. However, the high costs and difficulty of synthesis of these AMPs, on a commercial scale, as well as their poor pharmacokinetic properties and high toxicity, has prohibited wide spread use by the pharmaceutical industry.[1] Current research is therefore focused on the design and activity of a number of AMP mimics to try and overcome such shortcomings; for example, peptidomimetics, including: peptoids, β-peptides, phenylene, ethynylene and arylamide oligomers.[2] It is proposed that these nonconventional antimicrobial foldamers maintain the amphiphilic and structural characteristics of the naturally occurring AMPs, but are more easily accessible, tunable and potentially more cost effective. Nevertheless, despite promising results in early-stage clinical trials, some peptidomimetics have met with difficulty securing FDA approval, which can, in part, be linked to their poor metabolic stability. As such, more stable, protease resistant mimetics of antimicrobial peptides have been suggested as new templates for antibacterial compound design.[3]

It is recognized that oligoureas consisting of *m*-phenylenediamine monomeric units possess stacked conformations both in the solid and solution state,[4–7] and these molecules have been extensively studied, for example, by using diastereotopic markers as an indication of helix length and stability in solution.[5] In addition, studies on their dynamic conformational behaviour in various solvents have been published,[6,7] but to date their potential as antimicrobial agents has not been assessed. It has been shown that helicity can be a key factor in antimicrobial activity[8] and can also enhance activity or efficacy of naturally occurring AMPs.[9] Based on precedent surrounding the antimicrobial activity of naturally occurring helical oligopeptides,[10–14] along with the fact that a stacked conformation exists in non-natural foldamers,[15] it was hoped that oligoureas of *m*-phenylenediamine, such as **3** (Fig. 1), would be able to mimic the membrane interaction and antimicrobial activity of naturally occurring oligopeptides. Herein, we describe our results showcasing the interactions between different length *m*-phenylenediamine oligoureas (**1**–**3**, Fig. 1) and different lipid membranes, at a range of concentrations, and demonstrate their potential as novel lead compounds for the treatment of bacterial infections.

Work began by identifying mono-, tetra- and hepta-ureas, **1**, **2** and **3**,[4,16] as the test compounds to ascertain the importance of oligourea length and hydrophobicity in effecting antimicrobial activity; previous work has also shown the extent of stacking of these compounds in both the solid state and in solution.[4]

In order to test the hypothesis that the oligoureas **1**, **2** and **3** had potential for interaction with a lipid surface their surface activity was investigated at an air-buffer interface (Fig. 2). The data shows that the surface activity for **3** increases in a concentration dependent manner to a maximum of 13.43 mN m^{-1} at 250 μM. Whilst lower than that usually observed for membrane interactive oligopeptides such as aurein 2.5 (25 mN m^{-1} at 4 μM, Fig. 2),[17] the data clearly indicates the potential for localization at an asymmetric air-buffer interface and shows a similar concentration dependent

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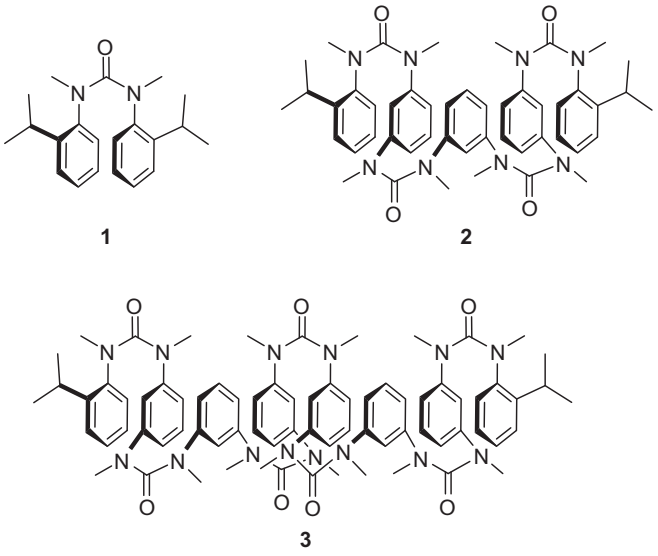

Figure 1. Structures of the ureas studied for potential antimicrobial activity.[4,16]

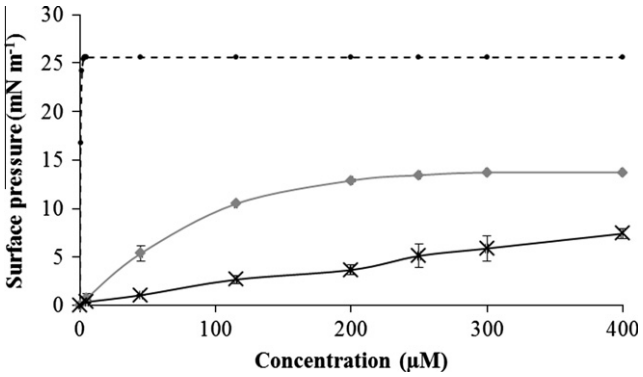

Figure 2. Surface activity of **2** (black), **3** (grey) and aurein 2.5 (dotted), at an air-buffer interface.

increase to peptides. Conversely, the surface activity of **2** (Fig. 2) does not reach its maximum over the same concentration range and only reaches 7.4 mN m^{-1} at 400 μM indicating that it has a lower interaction with the asymmetric interface compared to **3**; monourea **1** was not found to be surface active.

The stability of the interaction between these oligoureas (**1**–**3**) and phospholipid monolayers was also investigated through thermodynamic analysis. Figure 3 shows that in the presence of dimyristoylphosphatidylethanolamine (DMPE), at low urea/lipid ratios, **1** displays negative values of ΔG_{mix} at all surface pressures studied, indicating a stable interaction. However, in the presence of dimyristoylphosphatidylglycerol (DMPG) the ΔG_{mix} values for **1** are positive at all concentrations and surface pressures studied indicating that there is greater destabilization of the DMPG membrane in the presence of **1**. It is known DMPG is more densely packed than DMPE,[18] and furthermore, urea **2** and **3** with DMPE show increased levels of membrane destabilization at higher surface pressures (20 mN m^{-1}, white), which are representative of biological systems, compared to lower surface pressures (5 mN m^{-1}, black) reinforcing the view that effective interaction of the oligourea with the lipid interface requires a more ordered lipid structure.

Within each surface pressure dataset there is also an increase in destabilization as the concentration of the urea increases from 100 to 1 (black) to 10 to 1 (white) [$F_{3,18} = 1295.94$; $p = 0.00$]; such a trend is also mirrored with DMPG and urea **2** except that the level of destabilization is greater with this lipid [$T = -7.467$; $p = 0.017$]. Oligourea **3** also echoes the data obtained with **2**, but yet again the levels of destabilization of both lipids are greater than the levels seen with **1** and are greatest with DMPG. However, there is only a small difference between ureas **2** and **3** with DMPG [$T = 3.210$; $p = 0.049$] and it is not statistically significant.

The observed differences between the negative lipid DMPG and the zwitterionic lipid DMPE suggested a localized, head-group induced, pH effect may have been at play at the interface; it is believed that this may in part have been controlling their interaction with the ureas. However, this was discounted after the membrane lysis experiments were repeated over the pH range ~5–9 and pH was shown to have no effect on lysis (see Supplementary data) implying the enhanced activity with DMPG is due to more stable association with the anionic lipid interface. Such association would presumably be due to packing effects given the oligourea itself is not charged. This may be of importance if these compounds are to have potential as a membrane interactive antimicrobial lead compounds since it is known that phosphatidylglycerol (PG) and its derivatives can be found as the major components of bacterial membranes, as in the case of *Staphylococcus aureus*.[19]

Calcein leakage assays were undertaken to investigate the lytic properties of the oligoureas with DMPE and DMPG phospholipids (Fig. 4). Figure 4 shows **1**, **2** and **3** only exhibit variation in the levels of lysis against calcein-loaded vesicles made up from DMPE at high concentration (1 mM) [$F_{3,11} = 79.092$; $p = 0.00$] and that there is no significant difference seen at lower concentrations [$F_{1,2} = 2.379$; $p = 0.174$] (Fig. 4A). In comparison, for DMPG vesicles (Fig. 4B) a higher level of lysis was observed for each of the oligoureas **1**–**3** at all concentrations tested and concentration and length-dependent lysis was observed; that is, as the length of the oligourea increases from 1→4→7 urea units the percent lysis also increases, as it does as the concentration increases for each compounds studied as well. Moreover, levels as high as 61% and 69% were observed for tetraurea **2** and heptaurea **3**, respectively, both at 1 mM (grey) with DMPG. This is comparable to other antimicrobials, indicating that these oligoureas may have potential as a new class of antimicrobial agent, although oligourea **3** caused slightly higher levels of lysis than oligourea **2** [$T = -11.050$; $p = 0.002$] (Fig. 4B).

In order to confirm the oligoureas' ability to act as potential lead compounds for the treatment of bacterial infections, compounds **1**, **2** and **3** were tested for antibacterial activity against Gram negative *Escherichia coli* and Gram positive *S. aureus* where the minimum inhibitory concentration (MIC) was defined as the lowest oligourea concentration at which growth was inhibited and no bacterial regrowth was observed. At concentrations ranging from 0 to 2 mM compound **1** showed no antimicrobial activity against the bacteria tested. However, **2** and **3** were found to be effective against *E. coli* and *S. aureus*, and although both exhibited high MICs of 1 mM this correlates with the lysis data and would indicate that they may form a template for further development.

In conclusion, we have shown that oligomeric ureas of *m*-phenylenediamine target anionic DMPG and possess promise as antimicrobial agents which could potentially be optimized through analogue preparation in order to improve potency and aqueous solubility. It was postulated that the similarity of urea **3** to helical antimicrobial peptides in terms of size, hydrophobicity, and possibly helicity, may be important for activity to exist. The ability of these compounds to insert into a well ordered lipid environment has also been demonstrated with increased efficacy at higher surface pressure. The calcein leakage assay (Fig. 4) shows oligourea **2** is almost as active as urea **3** with both lipids, and that oligourea **2** was also able to destabilize ordered DMPG monolayers as effectively as oligourea **3**. There is a clear concentration dependent