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Thermodynamic interactions of a *cis* and *trans* benzanilide with *Escherichia coli* bacterial membranes

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Abstract The activity of a membrane interactive *cis* and *trans* benzanilide against *Escherichia coli* membrane mimics was investigated using Langmuir monolayers. It was found that in the presence of *E. coli* lipid mix monolayers, *cis*-benzanilide induced maximal surface pressure changes of 1 mN m^{-1}, whereas a reduced interaction was observed with *trans*-benzanilide. Compression isotherm analysis of these monolayers showed $\Delta G_{mix} < 0$, indicating membrane stabilisation by both compounds. The binding coefficient for *trans*-benzanilide was *circa* 890 μM and for the *cis*-benzanilide was 820 μM, suggesting that both benzanilide compounds bound to the *E. coli* membranes.

Keywords Benzanilides · Binding · *Escherichia coli* membranes leakage · Phospholipid monolayers · Thermodynamic analysis

Introduction

The pharmaceutical industry has been constantly battling with multi-drug resistant (MDR) organisms, because as soon as a new antibacterial agent enters clinical practice, resistant strains are reported (Giuliani et al. 2007; Laverty et al. 2011). For example, penicillin G received Food and Drug Administration (FDA) approval in 1943, even though the first bacterial resistance due to enzymes such as penicillinase was reported 3 years earlier in 1940 (Gardener 1940). Resistance in many cases is due to a genetic mutation (Jacoby 2009) or plasmid-mediated transfer of resistance genes (Cummins et al. 2009; Pietiainen et al. 2009), as seen when changes in AmpC expression generated resistance against cefotaxime in 1981 because of the production of β-lactamase (Jacoby 2009). Another example of MDR is seen with the emergence of methicillin-resistant *S. aureus* (MRSA). In this case the glycopeptide vancomycin has been effective for the treatment of resistant bacterial infections; however, the emergence of glycopeptide resistance in *S. aureus* has highlighted concerns that strains of MRSA are also becoming resistant to vancomycin (Smith et al. 1999), and already the occurrence of vancomycin-resistant enterococci (VRE) has been reported (Cetinkaya et al. 2000). This resistance to traditional antibiotics has required the pharmaceutical industry to find effective replacements to fight against microbial infection. New interventions have included investigations into the potential use of photo-activated antibacterial chemotherapeutics such as phenothiazinium-based molecules (PhBPs) (Harris and Phoenix 2006; Phoenix et al. 2003), which research has shown are often more efficacious than conventional antibiotics (Phoenix and Harris 2003; Phoenix et al. 2003). Although having some promise, these agents have also presented a range of challenges, for example, how to ensure effective light activation. The creation of new antibacterial peptide templates derived from naturally occurring antimicrobial peptides has also been used to create novel antibiotics (Wang et al. 2009). For example, trials on the synthetically produced drug, Pexiganan™, derived from an α-helical antimicrobial peptide magainin, is particularly effective in the treatment of polymicrobic foot ulcers in diabetic patients (Yount and Yeaman 2012;

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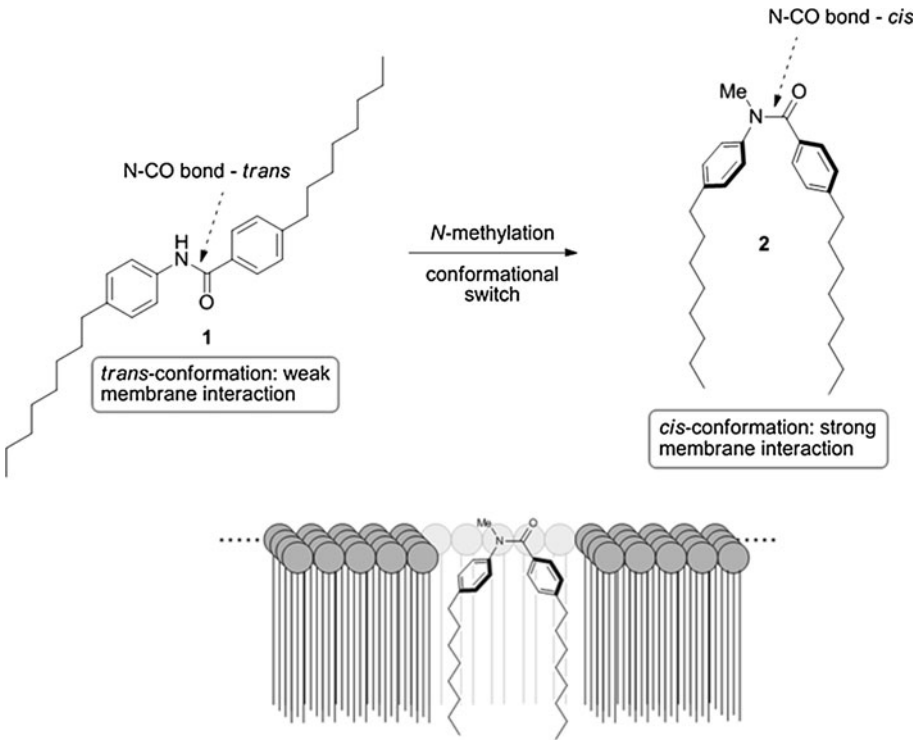

Fig. 1 Structure of *trans*-benzanilide **1** and *cis*-benzanilide **2** (*top*). Schematic depicting insertion of **2** into a lipid monolayer (*bottom*)

Zasloff 2000). Unfortunately, the high costs and difficulty of synthesis of these novel antimicrobial peptides at a commercial scale has so far prohibited widespread use by the pharmaceutical industry (Yount and Yeaman 2012). Therefore, over the last decade, there has been considerable interest in developing new classes of membrane-interactive molecules, which might provide lead compounds for the design of antimicrobial agents. In such regard, *trans*-benzanilide **1** and *cis*-benzanilide **2** (Fig. 1) were investigated to determine the nature of the interaction of the benzanilides with *E. coli* membranes and to investigate their ability to disrupt bacterial membranes in a manner that would indicate their potential to form a lead compound for the creation of a new class of antibacterial agent.

Materials and methods

Materials

The synthesis and characterisation of *trans*-benzanilide **1** and *cis*-benzanilide **2** has been previously described by Dennison et al. (2012). The phospholipids dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylglycerol (DOPG), *E. coli* cardiolipin (CL) and fluorescein-phosphatidylethanolamine (FPE) were supplied by Avanti Polar Lipids (Alabaster, Alabama) and were used without further purification. All other reagents were supplied by SIGMA (UK) and VWR (UK).

Interaction of benzanilides **1** and **2** with *E. coli* lipid monolayers at constant area

Insertion experiments were carried out at constant area to quantify the interaction of the benzanilides with a synthetic mix of *E. coli* (91 % DOPE, 3 % DOPG, 6 % CL) (Lohner and Prenner 1999) monolayers using a 601 M Langmuir Teflon trough (Biolin Scientific/KSV NIMA, Coventry, UK) by dropping phospholipid chloroform solutions onto a clean 10 mM Tris, pH 7.5 subphase. The barriers were closed at a rate of 5 cm^2 min^{-1} until a starting pressure of 30 mN m^{-1} was achieved. The monolayer was allowed to equilibrate for 30 min before either the *trans*-benzanilide (**1**) or the *cis*-benzanilide (**2**) (dissolved in 50 % DMSO in buffer) was injected into the subphase to a final subphase concentration of 400 μM (Dennison et al. 2012). Surface pressure measurements were measured using the Wilhelmy plate method previously described by Dennison et al. (2009).

Interaction of benzanilides **1** and **2** with *E. coli* lipid monolayers at constant pressure

The ability of the benzanilides to penetrate a synthetic mix of *E. coli* lipid monolayers at constant pressure was studied. Chloroform solutions (5 mM) of synthetic *E. coli* lipid mix monolayers were spread onto a clean buffer subphase as described earlier. The solvent was allowed to evaporate off for 30 min and the monolayer was compressed at a velocity of 5 cm^2 min^{-1} to give a surface pressure of

30 mN m^{-1}. The lipid monolayer was then maintained at this pressure throughout the experiment. The monolayer was allowed to equilibrate for 10 min and then either the *trans*-benzanilide (**1**) or the *cis*-benzanilide (**2**) (dissolved in 50 % DMSO in buffer) was introduced into the subphase to give a final concentration of 400 μM. The amount of either **1** or **2** inserted into the various monolayers was quantified by the relative area change $\Delta A/A$, defined as $((A_T - A_0)/A_0) \times 100$ where A_T is the area per molecule at a given time after **1** or **2** injections into the subphase and A_0 is the area per molecule before the injection of the compounds.

Isotherm measurement of benzanilides **1** and **2** with phospholipid monolayers

Stock solutions (1 mM) of DOPE, DOPG and CL were prepared in chloroform. Appropriate volumes of stock solutions were mixed to prepare a synthetic mix of *E. coli* membranes. Langmuir monolayer experiments were performed using a 601 M Langmuir Teflon trough (Biolin Scientific/KSV NIMA, Coventry, UK) at 21 °C by spreading 2.82×10^{15} molecules of lipid onto a 10 mM Tris pH 7.5 subphase (resistivity 18 ΩM cm^{-1}) containing 400 μM of either the *trans*-benzanilide (**1**) or the *cis*-benzanilide (**2**). The solvent was allowed to evaporate off for 15 min. Surface pressure (π) was monitored by the Wilhelmy method using a Whatman's CH1 filter paper plate and microbalance (Demel 1974). For isotherm measurements, the monolayer was compressed by two moveable Derlin barriers with a velocity of 10 mm/min until monolayer collapse pressure was achieved. These experiments were also repeated in the absence of either **1** or **2**.

Thermodynamic analysis of isotherms

To explore the nature of the phospholipid-benzanilide interaction, the thermodynamic stability of the mixed monolayers was investigated using Gibbs' equation, defined as follows for ternary mixtures in Eq. 1:

$$\Delta G_{\text{mix}} = \int [A_{123} - (X_1A_1 + X_2A_2 + X_3A_3)]\mathrm{d}\pi \qquad (1)$$

where $A_{1,2,3}$ is the molecular area occupied by the mixed monolayer; A_1, A_2, A_3 are the area per molecule in the pure monolayers of component 1, 2 and 3; and X_1, X_2 and X_3 are the molar fractions of the components. Negative ΔG_{mix} values indicate that the mixed film is more thermodynamically stable than its constituent single component films.

Calcein vesicle leakage of benzanilides **1** and **2**

Escherichia coli phospholipid mix (7.5 mg) was dissolved in chloroform before solvent evaporation under nitrogen gas. The lipid film was dried further overnight using a vacuum desiccator. The lipid film was hydrated using 5.0 mM HEPES (1 ml) containing 70 mM calcein and the solution then sonicated for 30 min. The solution then underwent three cycles of freeze-thawing. Liposomes were extruded 11 times through a 0.1-μm polycarbonate filter using an Avanti polar lipid min-extruder apparatus. Calcein-entrapped vesicles were separated from free calcein by gel filtration using a Sephadex G75 column (Sigma) that was rehydrated overnight in 20 mM HEPES, 150 mM NaCl and 1.0 mM EDTA. The column was eluted with 5 mM HEPES pH 7.5.

The calcein release assay was performed as previously described by Dennison and Phoenix (2011) by combining 20 mM HEPES (2 ml), 150 mM NaCl and 1.0 mM EDTA (pH 7.4), 20 μl calcein vesicles. The fluorescence intensity of calcein was measured using a FP-6500 spectrofluorometer (JASCO, Tokyo Japan), with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. To measure maximum fluorescence, 20 μl of Triton X-100 was used to dissolve the vesicles. The percentage of dye leakage was then calculated using the following equation in Eq. 2:

$$\text{Percentage leakage} = \left(\frac{F - F_0}{F_{\text{Triton}} - F_0}\right) \times 100 \qquad (2)$$

where F_0 is the fluorescence intensity of the lipid vesicles, F is the maximum fluorescence intensity in the presence of **1** and **2**, and F_{Triton} is the intensity after the addition of Triton X-100.

Binding of benzanilides **1** and **2** to lipid vesicles

Unilamellar vesicles with a mean diameter of 0.1 μm were prepared as previously described by Wall et al. (1995). In summary, the *E. coli* lipid mix containing 7.5 mg phospholipid in chloroform and 0.5 mol % of FPE was added before being dried under a stream of nitrogen. The lipid film was then hydrated with 1 ml 10 mM Tris-HCl pH 7.4; 1 mM EDTA at a total lipid concentration of 10 mg ml^{-1}, freeze-thawed 5 times and extruded 11 times using a 0.1-μm polycarbonate filter using an Avanti mini-extruder apparatus. FPE-labelled vesicles were diluted to 65 μM and fluorescence was recorded using a FP-6500 spectrofluorometer (JASCO, Tokyo Japan), with an excitation wavelength of 492 nm and an emission wavelength of 516 nm.

To investigate the binding of the benzanilides to the *E. coli* lipid mix, aliquots of either **1** or **2** (0–7 mM) were added to the FPE-labelled vesicles and the fluorescence monitored. The change in fluorescence (ΔF) of FPE-labelled vesicles with addition of the benzanilides minus FPE-labelled vesicles was plotted against benzanilide

concentration and fitted by non-linear least squares analysis to Eq. 3:

$$\Delta F = \frac{\Delta F_{\max}\,[B]}{K_d + [B]} \tag{3}$$

where $[B]$ is the benzanilide concentration, ΔF is the fluorescence change, $\Delta F_{\max}$ the maximum fluorescence change and K_d the binding constant.

Results and discussion

It is generally accepted that the killing mechanism of many antimicrobials involves the invasion of target cell membranes. This action may support translocation across the membrane in order to attack intracellular targets or lead to membrane disruption, thus causing cell death (Dennison et al. 2009). The ability of benzanilides **1** and **2** to penetrate a composite lipid mix monolayer that mimics the *E. coli* membrane was studied at constant area. An initial starting surface pressure of 30 mN m^{-1} provides a lipid-packing density, which is equivalent to that of the outer leaflet of a cell membrane, and thus serves as a model to investigate novel compounds with the potential for antimicrobial activity (Seelig 1987). Figure 2 shows that *cis*-**2** generates a 1 mN m^{-1} pressure change with *E. coli* lipid mimics whilst *trans*-**1** showed no detectable membrane perturbation. The 1 mN m^{-1} levels of interaction have been observed for other antibiotics, such as doxorubicin, which have broad-spectrum antimicrobial and antitumor activity, and are known to interchelate with bilayer structures (Hernandez et al. 1991). Furthermore, experimentation using the same lipid system described earlier but conducted at a constant surface pressure of 30 mN m^{-1} (Fig. 2b) shows no change in area for compound **1** but a ~5 % increase in area for **2**. This confirms that **1** cannot insert into monolayers at physiological pressure of 30 mN m^{-1} and implies that it has little affinity for membrane penetration. However, **2** is able to insert into the membrane, indicating that it is able to interchelate presumably because of a stronger dipole moment generated by the polar head group and aliphatic chain arrangement in the *cis* system (Fig. 1).

The thermodynamic stability of the membranes was investigated further. Figure 3a shows π-A isotherms for *E. coli* lipid mix on an air-buffer interface containing *trans*-benzanilide **1** or *cis*-benzanilide **2** in the subphase. In the presence of the *trans*-benzanilide (**1**) the isotherm film is consistent with a characteristic gaseous phase at low compression (1–5 mN m^{-1}). At higher compressions the isotherms are consistent with a liquid-expanded (LE) phase with a collapse pressure >40 mN m^{-1}. In the presence of the *cis*-benzanilide (**2**) (Fig. 3a), although at low surface

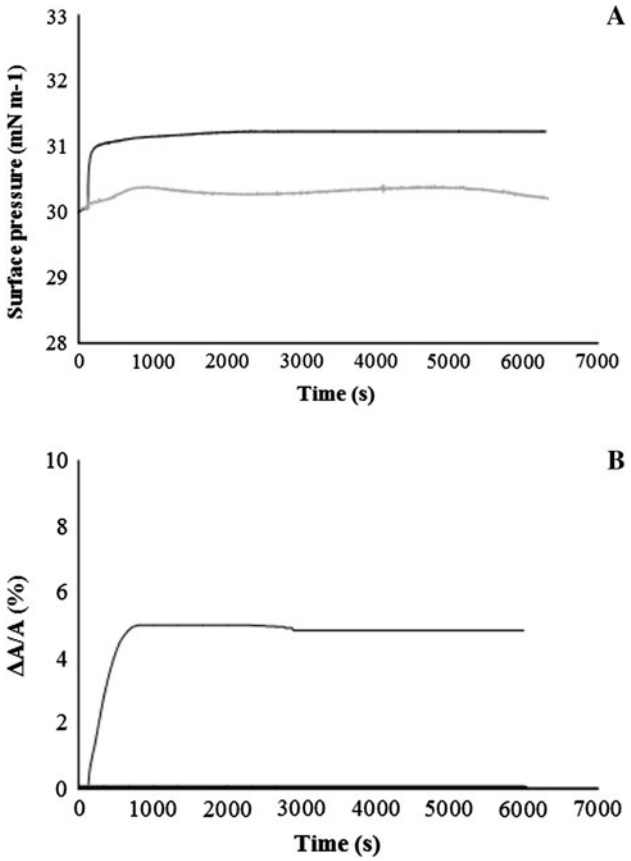

Fig. 2 Monolayer interactions of **1** (*grey*) and **2** (*black*) with the monolayer from a lipid mix that mimics the membrane of *E. coli*. **a** Monolayers were set at an initial surface pressure of 30 mN m^{-1}, mimetic of naturally occurring membranes, and maintained at constant area. After 120 s, **1** and **2** were introduced, in separate experiments, into the subphase. **b** Relative area change of **1** (*grey*) and **2** (*black*) held at a constant pressure of 30 mN m^{-1}

pressure (1–5 mN m^{-1}), the isotherms are characteristic of a gaseous phase; the isotherm progresses onto a pronounced liquid expanded state with a collapse pressure >20 mN m^{-1}. The shape of the π-A isotherms is shown to be strongly affected by the presence of the *cis*-benzanilide (**2**) in the subphase. At the phase transition observed under these conditions, the monolayer films do not undergo a transition between the expanded and condensed phases. The data would suggest that in the presence of the *cis*-benzanilide (**2**), the *E. coli* membrane is less fluid, and tighter packing of the phospholipids is observed in comparison to the presence of the *trans*-benzanilide (**1**).

The stability of the monolayers was further investigated using the Gibbs free energy of mixing (ΔG_{mix}) (Fig. 3b). Figure 3b shows that all values of ΔG_{mix} for *E. coli* lipid mix model membranes are much lower than *RT* (2,444.316 J mol^{-1}), indicating that deviations from ideal mixing behaviour are small. In the absence of compounds **1** and **2**, negative values of ΔG_{mix} were observed for the

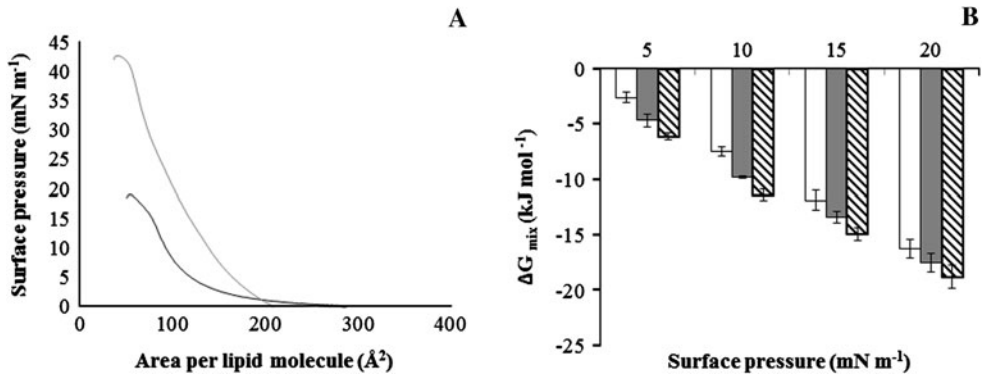

Fig. 3 **a** Compression isotherms of *E. coli* lipid mixtures in the presence of either the *trans*-benzanilide (**1**) (*grey*) or the *cis*-benzanilide (**2**) (*black*). **b** The Gibbs free energy of mixing (ΔG_{mix}) of *E. coli* monolayers at varying surface pressure in the absence (*white*) and presence of compound **1** (*grey*) and **2** (*black diagonal lines*)

E. coli lipid mix model membrane (Fig. 3b), indicating a thermodynamically stable monolayer. However, in the presence of *trans*-benzanilide **1** the monolayers were thermodynamically more stable than the control but not as stable as in the presence of *cis*-benzanilide **2**. The degree of stabilisation is greatest at lower surface pressures where the molecules would be expected to interchelate more easily. In these loosely packed arrangements, once interchelated, the benzanilides would interact with the acyl chain region of the membrane structure. The *cis* arrangement would clearly allow greater levels of hydrophobic partitioning (Fig. 2b) and lead to stabilisation of the lipid structure.

In order to further characterise the benzanilide-membrane interactions, the ability of **1** and **2** to perturb *E. coli* lipid mix vesicles was investigated using a calcein release assay. It was found that below concentrations of 400 μM, **1** induced 3.4 ± 0.3 % release of calcein and **2** induced 11.3 ± 0.2 % release of calcein in the presence of vesicles formed from an *E. coli* lipid mix. These results show that there is an increased interaction between the *cis* benzanilide and lipid systems compared to the *trans* isoform, and whilst **2** shows weak lytic properties, this has also been observed for other compounds such as doxorubicin where the drug is known to effect action by causing disordering of acyl chains rather than lysis (de Wolf et al. 1993). Lysis increases in a concentration-dependent manner for both compounds until 2 mM, after which maximal lysis is observed (36.2 ± 0.30 % for **1** and 40.5 ± 0.34 % for **2**. Figure 4). Furthermore, in experiments to determine the level at which these compounds can kill bacterial cells, it was found that whilst compounds **1** and **2** were able to kill *E. coli* cells, the concentration to do so was significantly higher (2.5 and 2 mM, respectively; Dennison et al. 2012) than the concentrations used in the membrane interaction studies mentioned above (Fig. 2), suggesting that there may be other non-specific associations between the amides and the bacterial membranes, thus preventing antibacterial

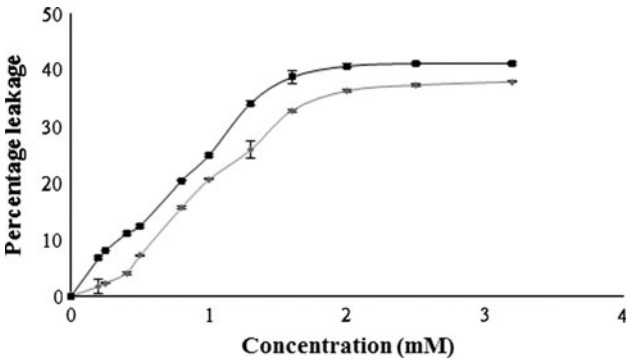

Fig. 4 Calcein release assay: percentage calcein release for a lipid mix mimetic of an *E. coli* bacterial membrane (Lohner and Prenner 1999) in the presence of either the *trans*-benzanilide (**1**) (*grey*) or the *cis*-benzanilide (**2**) (*black*). The values shown are the average and SD of four experiments

activity. It is however interesting to note that these MIC values are lower than a number of other commercial compounds tested against *E. coli*; for example, in the case of the antibiotic amoxycilin the MIC values observed were circa 7.4 mM (Kasim et al. 2011).

In order to investigate the membrane association of the benzanilides, they were assayed for their ability to bind to *E. coli* membranes using FPE-labeled vesicles, a technique that has been extensively used to model membrane binding (Hawrani et al. 2008; O'Toole et al. 2000; Wall et al. 1995). Figure 5 shows that both **1** and **2** have hyperbolic binding to FPE-labeled vesicles, which would suggest that the interaction of the benzanilides with the *E. coli* membrane was independent of each other. Further analysis of the binding coefficients with these vesicles showed that the K_d value for **1** was 890 μM and for **2** was 820 μM. The data suggest similar binding of **1** and **2**, indicating that the increased amphiphilicity of **2** does not greatly increase targeting and/or binding to the *E. coli* membrane (Dennison et al. 2012), and hence the greater efficacy of lysis and

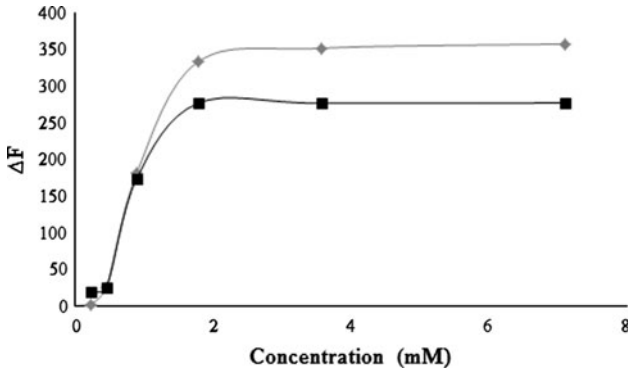

Fig. 5 The binding curve for *trans*-benzanilide **1** (*grey*) and *cis*-benzanilide **2** (*black*) with FPE labelled *E. coli* vesicles. *F* is fluorescence change in arbitrary units

insertion exhibited by **2** with these membranes is due to the more amphiphilic arrangement enabling hydrophobicity-driven penetration of the *cis*-benzanilide. Interestingly, these levels of binding are similar to doxorubicin in the presence of low levels of anionic lipid, although in that case research has shown that increasing the levels of anionic lipid enhances the binding of doxorubicin and inhibits protein translocation (Phoenix et al. 1993).

In summary, the *trans*-benzanilide (**1**) and the *cis*-benzanilide (**2**) possess limited activity against gram-negative, *E. coli* bacteria, which is supported by the MIC values, lysis and insertion into *E. coli* monolayers. However, the insertion observed for the benzanilides is consistent with other experimental results on known commercial agents, such as doxorubicin (Zorzato et al. 1985). Although both the *trans*-benzanilide (**1**) and the *cis*-benzanilide (**2**) have comparable physicochemical properties and similar binding values, the enhanced dipole of the *cis*-benzanilide (**2**) enables more effective penetration into the lipid system. Therefore, the *cis*-benzanilide (**2**) is more membrane interactive and may be a potential lead candidate for the development of novel antibiotics if analogues can be developed with more potent ability to instigate membrane disruption. For example, further studies could be undertaken to optimise the length and position of the alkyl chains for maximum membrane penetration as well as developing different linkers that also orientate the aromatic rings *cis* to each other.

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