Hydraphiles Enhance Antimicrobial Potency Against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*

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Abstract

Hydraphiles are synthetic amphiphiles that form ion-conducting pores in liposomal membranes. These pores exhibit open-close behavior when studied by planar bilayer conductance techniques. In previous work, we showed that when co-administered with various antibiotics to the DH5 α strain of *E. coli*, they enhanced the drug's potency. We report here potency enhancements at low concentrations of hydraphiles for the structurally and mechanistically unrelated antibiotics erythromycin, kanamycin, rifampicin, and tetracycline against Gram negative *E. coli* (DH5 α and K-12) and *Pseudomonas aeruginosa*, as well as Gram positive *Bacillus subtilis*. Earlier work suggested that potency increases correlated to ion transport function. The data presented here comport with the function hydraphiles to enhance membrane permeability in addition to, or instead of, their known function as ion conductors.

Graphical Abstract

Introduction

Since the development of the sulfa drug Prontosil in the 1930s, antibiotic therapy has produced a revolution in the treatment of infections.¹ Sulfa drugs were largely replaced in the 1940s by penicillin derivatives and a range of subsequent antibiotics such as the cephalosporins and the fluoroquinolones. By the 1960s, it was generally believed that the bacterial infection war had been won. In the interim, the emergence of antibiotic resistance has become an international crisis.² Both the Centers for Disease Control and Prevention (CDC)³ and the World Health Organization (WHO)⁴ have issued extensive advisories and discussions concerning the current problems.

The problem has been exacerbated by the lack of new antibiotic derivatives and antibiotic classes (new chemical entities). Encouraging recent results have appeared, however, with the reports of teixobactin⁵ and aspergillomarasmine A.⁶ In addition, such antibiotic peptides⁷ as magainin⁸ and derivatives along with various cationic amphiphiles⁹ have been extensively studied in recent years. Combinations of antibiotics and peptide antibiotics have been used against both Gram negative and Gram positive bacteria.¹⁰ Notwithstanding, the problem of antibiotic resistance persists and its threat to worldwide health is increasing.

In a preliminary report,¹¹ we showed that hydraphiles,¹² when co-administered with various antibiotics, enhanced the potency of the drugs against the microbes. The original hypothesis that potency enhancement was possible was based on the membrane activity of hydraphiles.¹³ It was surmised that the insertion of hydraphiles into a bacterial boundary layer would create an opening or defect that might permit other molecules to more readily penetrate it. We chose antibiotics as the test substance because bacteriostatic or bactericidal activity would serve as a useful metric to evaluate the hypothesis. We report here an extensive elaboration of our original study to a number of antibiotic/amphiphiles/organism combinations. In some cases, potency enhancements were found to be as high as 30-fold.

Results and Discussion

In the nearly half century since the discovery of crown ethers,¹⁴ there have been numerous reports of biological activity. Early studies showed, for example that exposure to 12-crown-4 caused testicular atrophy in mice.¹⁵ More extensive studies showed that the simple crowns 12-crown-4, 15-crown-5, and 18-crown-6 had LD₅₀ values (male white rats) of 3200, 1000, and 700 mg/kg, respectively.¹⁶ Antibacterial¹⁷ and antifungal¹⁸ activities of widely varied crown structures were reported in the early 1980s. Additional antimicrobial activity of various crown ether derivatives has been reported up to the present day.¹⁹ The diverse ranges of structures and organisms that have been studied make it difficult to generalize about crown toxicity in the absence of other substances. To the extent a conclusion can be articulated, it is that crown toxicity to Gram positive organisms such as *B. subtilis* is likely to be greater than to Gram negative organisms such as *E. coli*.

Our own studies of antimicrobial activity of hydraphiles showed the presence of the amphiphilic pore formers in the *E. coli* boundary layer.²⁰ A study of activity

against *E. coli* and *B. subtilis* revealed a correlation between hydrophile length and toxicity.¹³ Hydrophile length also correlates to efficacy of ion transport.²¹ Because of the correlation that we observed between ion transport activity and toxicity to *E. coli*, we examined a shorter-chained hydrophile that should be a poor channel-former to test its effect. The results of these studies are described below.

Compounds used. The work reported here used three different hydrophiles.²² They are illustrated in Figure 1 as **1-3**. The spacer chains represented in Figure 1 by $(\text{CH}_2)_n$ had values of n equal to 8, 14, and 16. In all cases, the two distal macrocycles were benzyl-terminated. We refer to these hereinafter as benzyl C₈ (**1**), benzyl C₁₄ (**2**) and benzyl C₁₆ (**3**) hydrophiles. All three compounds used have been fully characterized and all are previously known.^{22,23}

The antibiotics used were kanamycin sulfate [**4**, kanamycin D, mixture of (predominantly) A, B, and C], tetracycline (**5**), erythromycin (**6**) and rifampicin (**7**). These four antibiotics were obtained from commercial suppliers in the purest form available and used as received. They were selected because their chemical structures are very different. For each of the antibiotics, the mechanism of action is both well-established and different from any of the other compounds used in this study. Kanamycin A is an aminoglycoside that inhibits protein synthesis and cell respiration and causes potassium cation leakage to occur.²⁴ Tetracycline, which is produced in vast quantities annually, binds to the 30S ribosomal subunit, which inhibits peptide synthesis.²⁵ To the extent that any of the structures are related, it is erythromycin and rifampicin, both of which are macrolides. The ring sizes are vastly different as are the substitution patterns and modes of action. Erythromycin²⁶ inhibits protein synthesis by binding to the ribosomal 50S subunit. Rifampicin,²⁷ in contrast, inhibits RNA synthesis. Although both erythromycin and rifampicin ultimately inhibit protein synthesis, they do so by distinctly different interactions and mechanisms. Their different modes of action are reflected in their different indications. Rifampicin is a first line treatment for tuberculosis²⁸ and erythromycin is prescribed for such conditions as bronchitis and pertussis.²⁹ What all four antibiotics do have in common is that they all must infiltrate the cell cytoplasm to inhibit bacterial growth.

The activities of all compounds were assayed by determining the minimum inhibitory concentrations (MICs) under specified conditions. All MIC determinations were conducted according to the protocols provided by the Clinical and Laboratory Standards Institute: M07-A9.³⁰ We note for the reader unfamiliar with this assay that the procedure depends on bacterial growth observed in a sequence of serial dilutions. Thus, a minimum inhibitory concentration may be 4 μM in one determination, 8 μM in a second, and 2 μM in a third. In our studies, we typically conducted between 3-5 replicates to determine a reproducible MIC, which would be reported as 4 μM for the example given above.

The MIC was also determined for the commercial amphiphile Triton X-100 (**8**). This is a common neutral detergent that has been reported to be membrane active.³¹ It was included as a control to assess whether the effects observed for hydrophiles are essentially generic and would be manifested by any membrane

active amphiphile. The choice of Triton X-100 was also based on reports of channel-like function under certain conditions in liposomal membranes.^{32,33} The oligoethylene glycol chain of Triton X-100 varies from 9–10 units, giving an average molecular weight of 625 Daltons. This value was used in minimum inhibitory concentration (MIC) determinations.

Figure 1. Structures of hydraphile (**1-3**), kanamycin (**4**), tetracycline (**5**), erythromycin (**6**), rifampicin (**7**) and Triton X-100 (**8**).

Bacteria used. Four different microbial strains were used to study antibacterial activity. All of the synergy studies previously reported with benzyl C₁₄ hydraphile and antibiotics were against the DH5 α *E. coli* strain.¹¹ This non-pathogenic strain of *E. coli* has been optimized for laboratory use in molecular biology experiments. Results are recorded herein for that strain but new results are presented for *E. coli* (*Migula*) *Castellani and Chalmers* (MG1655) K-12 strain. K-12 *E. coli* is derived from a human stool sample and is generally considered to be more robust than DH5 α *E. coli*. The other two bacteria studied were Gram negative *Pseudomonas aeruginosa* and Gram positive *B. subtilis*. *Pseudomonas* is often found in biofilms³⁴ and is a common infection in patients using hospital breathing machines (i.e. ventilator-associated pneumonia). We used *B. subtilis* to assess the efficacy of our combination therapy in a Gram-positive bacterium.

Determination of hydraphile toxicity. We determined the MICs for each compound against *E. coli* (DH5 α), *E. coli* (K-12), *P. aeruginosa* and *B. subtilis*. Gram negative DH5 α *E. coli* was used as a control and to compare the synergy data of benzyl C₈ hydraphile (**1**) to that of the values previously reported for benzyl C₁₄ hydraphile (**2**).³⁵ This is important to be sure that bacterial strains kept in storage

have not been compromised. The MIC data are recorded in Table 1 for the hydraphiles (**1-3**), the antibiotics (**4-7**), and Triton X-100 (**8**).

Table 1. MICs of hydraphiles and antibiotics against *E. coli* (DH5 α and K-12), *P. aeruginosa* and *B. subtilis*.

Benzyl hydraphile used	<i>E. coli</i> (DH5α, μM)	<i>E. coli</i> (K-12, μM)	<i>P. aeruginosa</i> (μM)	<i>B. subtilis</i> (μM)
C ₈ hydraphile (1)	300	200	ND	ND
C ₁₄ hydraphile (2)	2	2.5	2	0.5
C ₁₆ hydraphile (3)	4	ND	2	ND
Kanamycin (4)	30	35	ND	ND
Tetracycline (5)	5	5	12	3
Erythromycin (6)	200	700	200	0.3
Rifampicin (7)	55	16	25	ND
Triton X-100 (8)	>512	>512	ND	ND
ND means not determined				

It is interesting to note that an extensive literature spanning many years shows that structurally diverse crown ethers are toxic to a range of microbes and higher cells. The variations in macrocycle, organism, and analytical methods used make it difficult to reach a firm conclusion about toxicity. To the extent such a conclusion can be reached for bacteria, it is that crowns of various types are generally more toxic to Gram positive than to Gram negative strains. We observe a similar situation with benzyl C₁₄ hydraphile. Note that the potency difference between benzyl C₁₆ hydraphiles against *E. coli* DH5 α and *P. aeruginosa* is a single dilution in the MIC analysis, although variation in responses of different strains of bacteria to xenobiotics is common.

Synergy against *E. coli* (DH5 α and K-12), *P. aeruginosa* and *B. subtilis*.

Three hydraphiles were tested against DH5 α *E. coli* in the preliminary report of this work.¹¹ Two of those are included here, benzyl C₁₄ (**2**) and benzyl C₁₆ hydraphiles (**3**), along with the shorter benzyl C₈ hydraphile (**1**). In the present study, we compare these three closely related benzyl-terminated hydraphiles that differ only in overall length, with the four structurally unrelated antibiotics noted above, against four different bacterial strains.

In each experiment, bacteria were infused with the indicated amount of hydraphile and the MIC was determined for the antibiotic in its presence. The amount of additive was always a fraction of the measured MIC value for that compound. The MIC value for Triton X-100 was >512 μ M against DH5 α *E. coli* and the amount used in the study was arbitrarily set at 64 μ M. The results are recorded in Table 2. Each entry reflects a minimum of 3 replicates and typically 5-10.

Table 2. Enhancement of antimicrobial activity by benzyl C₈, C₁₄, and C₁₆ hydraphiles (**1-3**) and Triton X-100 (**8**) against *E. coli* (DH5 α and K-12), *P. aeruginosa*, and *B. subtilis*.

Hydraphile used	[Hydraphile] (μM)	Antibiotic used	[Antibiotic] (μM)	Fold Increase
<i>E. coli</i> (DH5α)				
C ₈ hydraphile (1)	150 (1/2 [MIC])	Kanamycin	8	3-fold
C ₈ hydraphile (1)	75 (1/4 [MIC])	Kanamycin	12	2-fold
C ₈ hydraphile (1)	150 (1/2 [MIC])	Tetracycline	1	5-fold
C ₈ hydraphile (1)	75 (1/4 [MIC])	Tetracycline	2	2-fold
C ₈ hydraphile (1)	150 (1/2 [MIC])	Erythromycin	15	13-fold
C ₈ hydraphile (1)	75 (1/4 [MIC])	Erythromycin	25	8-fold
C ₈ hydraphile (1)	150 (1/2 [MIC])	Rifampicin	3	18-fold
C ₈ hydraphile (1)	75 (1/4 [MIC])	Rifampicin	3	18-fold
Triton X-100 (8)	64	Kanamycin	30	none
Triton X-100 (8)	64	Rifampicin	55	none
<i>E. coli</i> (K-12)				
C ₁₄ hydraphile (2)	1.25 (1/2 [MIC])	Kanamycin	15	2-fold
C ₁₄ hydraphile (2)	0.6 (1/4 [MIC])	Kanamycin	30	none
C ₁₄ hydraphile (2)	1.25 (1/2 [MIC])	Tetracycline	1	5-fold
C ₁₄ hydraphile (2)	0.6 (1/4 [MIC])	Tetracycline	3	2-fold
C ₁₄ hydraphile (2)	1.25 (1/2 [MIC])	Erythromycin	75	9-fold
C ₁₄ hydraphile (2)	0.6 (1/4 [MIC])	Erythromycin	300	2-fold
C ₁₄ hydraphile (2)	1.25 (1/2 [MIC])	Rifampicin	0.5	30-fold
C ₁₄ hydraphile (2)	0.6 (1/4 [MIC])	Rifampicin	4	4-fold
<i>P. aeruginosa</i>				
C ₁₄ hydraphile (2)	1 (1/2 [MIC])	Tetracycline	3.13	4-fold
C ₁₄ hydraphile (2)	0.5 (1/4 [MIC])	Tetracycline	6.25	2-fold
C ₁₄ hydraphile (2)	1 (1/2 [MIC])	Erythromycin	25	8-fold
C ₁₄ hydraphile (2)	0.5 (1/4 [MIC])	Erythromycin	50	4-fold
C ₁₄ hydraphile (2)	1 (1/2 [MIC])	Rifampicin	2.35	11-fold
C ₁₄ hydraphile (2)	0.5 (1/4 [MIC])	Rifampicin	4.7	5-fold
C ₁₆ hydraphile (3)	1 (1/2 [MIC])	Erythromycin	25	8-fold
C ₁₆ hydraphile (3)	0.5 (1/4 [MIC])	Erythromycin	50	4-fold
<i>B. subtilis</i>				
C ₁₄ hydraphile (2)	0.12 (1/4 [MIC])	Tetracycline	1.56	2-fold
C ₁₄ hydraphile (2)	0.12 (1/4 [MIC])	Erythromycin	0.16	2-fold

Comparison of C₈ benzyl hydraphile (**1**) to C₁₄ benzyl hydraphile (**2**).

Our initial assumption was that enhancement of antibiotic potency against bacteria was related to membrane penetration and ion transport efficacy. In our previous study,¹¹ we did not compare channel and non-channel amphiphiles. Because earlier results showed that benzyl C₈ hydraphile did not transport Na⁺ ions through liposomal membranes, we expected the antibiotic's MIC value to be indifferent to the presence of benzyl C₈ hydraphile (**1**). Surprisingly, **1** enhanced the potency of kanamycin, tetracycline, erythromycin, and rifampicin against

DH5 α *E. coli* between 2- and 18-fold, to extents dependent on the antimicrobial that was co-administered.

The bar graph in Figure 2 compares the observed enhancements of antibiotic potency by **1** and **2**. Each pair of bars reflects the potency enhancement of the indicated antibiotic, mediated by $\frac{1}{2}$ MIC of **1** or **2** against DH5 α *E. coli*. Both compounds enhanced the potency of each antibiotic to some extent. Benzyl C₈ hydrophile (**1**) appears marginally more active in each case than is **2**. We therefore infer that the increase in antibiotic potency is independent of the compound's ability to form ion channels. Since the four antibiotics are so structurally different, it is unlikely for hydrophiles to interact with (complex with, form clusters with) the antibiotics and act as a carrier to increase their efficacy.

Figure 2. Fold enhancements (indicated by numbers atop the columns) of tetracycline, kanamycin, erythromycin and rifampicin by $\frac{1}{2}$ [MIC] of benzyl C₈ (**1**) and benzyl C₁₄ (**2**) hydrophiles against DH5 α *E. coli*. Data for benzyl C₁₄ hydrophile were reported in reference 11.

Comparison of antimicrobial enhancement between K-12 *E. coli* and DH5 α *E. coli*. As noted above, our previous work was limited to the non-pathogenic DH5 α “laboratory strain” of *E. coli*. In the preliminary report of this effort, we also reported one example each of activity assayed with *B. subtilis* and *P. aeruginosa*. A comparison of the data obtained earlier with the DH5 α strain of *E. coli* with the K-12 strain was of obvious interest. The graph of Figure 3 compares the potency enhancements observed for the four antibiotics against the two bacterial strains. In all cases, benzyl C₁₄ hydrophile (**2**) was the additive or mediator. The observed potency enhancements for K-12 *E. coli* with all four antibiotics were equal to or greater than against DH5 α *E. coli*. In K-12 *E. coli*, the MIC of C₁₄ benzyl hydrophile was 2.5 μ M compared to 2.0 μ M against DH5 α .

It is interesting to note that the MICs of rifampicin were different against K-12 *E. coli* from DH5 α *E. coli*. This difference has been attributed to the absence of the O-antigen from the bacterium's lipopolysaccharide shell. This enhances the membrane permeability of bacteria to hydrophobic compounds.³⁶

Figure 3. Fold enhancement (indicated by numbers atop the columns) of tetracycline, kanamycin, erythromycin and rifampicin mediated by $\frac{1}{2}$ [MIC] of benzyl C₁₄ hydraphile (**2**) against DH5 α *E. coli* and K-12 *E. coli*. Data for C₁₄ hydraphile were reported in reference 11.

Growth curve with benzyl C₁₄ hydraphile and erythromycin. An obvious question concerning the results reported here is whether or to what extent, the bacteria under study suffer toxicity effects at the concentrations used. In order to address this question, we obtained growth curves for the combination of erythromycin and benzyl C₁₄ hydraphile (**2**) to determine the effect of the latter on *E. coli* (DH5 α). We measured the optical density of *E. coli* at 600 nm every 15 minutes for 12 h (720 min). The life cycle of *E. coli* is typically 22-23 min so the data reflect between 31 and 36 generations. By its nature, the study also revealed whether the antibiotic activity enhancement was a synergistic or additive effect. The results are shown below in Figure 4.

Figure 4. Graph showing the effect of benzyl C₁₄ hydraphile (**2**) on the potency of erythromycin administered against DH5α *E. coli*. Each point represents the average of three separate determinations (error bars not shown).

It is clear from the data graphed in Figure 4 that at half MIC (open squares), benzyl C₁₄ hydraphile (**2**) does not affect the growth of DH5α *E. coli* (filled circles). Erythromycin was administered at 25 μM (1/8 MIC) and 50 μM (1/4 MIC). At 25 μM erythromycin, the effect on growth was minor (filled squares). When the antibiotic's concentration was doubled to 50 μM (filled triangles), inhibition of growth was about twice that at the lower concentration. However, when 1/2-MIC of **2** and 25 μM erythromycin were combined, the growth of *E. coli* was dramatically reduced (open circles). This confirms that there is synergy between hydraphiles and antibiotics. It also indicates that hydraphiles are enhancing the potency of this antibiotic against the *E. coli* cells.

It is interesting to compare the antimicrobial potency of rifampicin with incremental amounts of benzyl C₈ (**1**) and benzyl C₁₄ (**2**) hydraphiles against K-12 *E. coli*. The graph of Figure 4 shows that **2** enhances the potency of erythromycin against DH5α *E. coli*. The K-12 strain of *E. coli* is not only more robust than DH5α, it is more susceptible to rifampicin than DH5α *E. coli* is to erythromycin. The graph of Figure 5 (and Table 1) show that the MIC of rifampicin is 16 μM in the absence of any hydraphile. At a concentration of 2.5 μM, the maximum concentration studied, **1** has no effect on rifampicin potency against the bacteria. Thus, fewer concentration studies were conducted with benzyl C₈ hydraphile. When **2** is co-administered in a concentration range from 0-2.5 μM, clear evidence is obtained for a synergistic interaction. The dotted line in the graph connects the maxima on the two axes and would indicate a simple additive effect if followed by the experimentally determined data.³⁷

Figure 5. Graph showing the effect of added hydraphiles **1** and **2** on the potency of rifampicin against the K-12 strain of *E. coli*. The dotted line predicts the profile of an additive, rather than a synergistic, effect.

Enhancement of potency against *B. subtilis* and *Pseudomonas aeruginosa*. Compounds **1**, **2**, and **3** all mediate potency enhancements when co-administered with erythromycin, kanamycin, rifampicin, or tetracycline against DH5 α and K-12 *E. coli*. Based on the somewhat diverse literature on crown toxicity to bacteria,¹³⁻¹⁹ we anticipated that Gram positive *B. subtilis* would be the most susceptible organism to hydraphiles and the most likely to show potency enhancements when exposed to low hydraphile doses and antibiotics. In fact, the potency of either erythromycin or tetracycline against *B. subtilis* (see Table 2) was enhanced only marginally by **2** (administered at $\frac{1}{4}$ MIC). In contrast, at the $\frac{1}{4}$ MIC concentration, the potency of erythromycin against DH5 α *E. coli* was enhanced 8-fold by benzyl C₈ hydraphile **1**. We note that Triton X-100, administered at concentrations as high as 64 μM showed no effect on DH5 α *E. coli* in the presence of either kanamycin or rifampicin (data not shown).

The most dramatic increase in potency was observed for the combination of **2** at $\frac{1}{2}$ MIC with rifampicin against K-12 *E. coli*. This is a very encouraging result because the 30-fold potency enhancement was observed when only 1.25 μM **2** was co-administered. Compound **2** also enhanced the potency of rifampicin by 11-fold against *P. aeruginosa* at the same [**2**] 1.25 μM concentration.

Potency enhancement by **1** of 13-fold and 18-fold when administered against *E. coli* at $\frac{1}{2}$ MIC with erythromycin or rifampicin, respectively, was also surprising as **1** is a poor ionophore. Of course, the $\frac{1}{2}$ -MIC concentration of **1** represents a much larger number of molecules than $\frac{1}{2}$ MIC of either **2** or **3**. We conclude that our original hypothesis that potency enhancement correlated with pore formation or ionophoretic activity is not substantiated, at least as the sole contributor to potency enhancement. The appealing hypothesis was always somewhat speculative considering the enormous difference between the boundary layers (membrane, cell wall) of Gram negative and Gram positive bacteria.

An alternate explanation for the enhancement is that a particular or selective interaction occurs between hydraphiles and antibiotics. Tetracycline is water-soluble and the H-bonding ability of such a polar solvent could easily mask any supramolecular interaction. We therefore decided to exclude solvent to the greatest extent possible and undertook a mass spectrometric analysis of the interaction between **3** and tetracycline. Electrospray ionization was used to moderate the experimental conditions. However, when excess tetracycline was added to **3**, no hydraphile-antibiotic adduct or cluster was observed. The major ion of **3** alone is the $[\mathbf{3}\cdot\text{Na}]^+$ adduct. The only change observed in the spectrum of **3** with the addition of tetracycline hydrochloride was the appearance of the tetracycline ($[\mathbf{5}\cdot\text{H}]^+$ and $[\mathbf{5}\cdot\text{DMSO}\cdot\text{H}]^+$) peaks as well as various adducts of **3** with protons, sodium cations, and chloride anions.

Figure 6. Electrospray mass spectra of the combination of benzyl C₁₄ hydraphile (**3**) alone, tetracycline (**5**) alone and **3** and **5** in combination in **3**:**5** ratios of 2 and 4.

A caveat in the consideration of mechanism is that the enhancements of potency apparent with 1/2 or 1/4 MIC of **1** involve larger amounts of compound than needed either for **2** or **3**. For example, at 1/4 MIC, the concentrations of **1** and **2** are ~90 µg/mL and ~700 ng/mL. Given the >100-fold difference in concentration, **1** could be operating differently from **2** or **3**. Further, the ability to form channels does not preclude **2** and/or **3** from interacting with the membrane to enhance permeability. There could be supramolecular interaction(s) not detected by mass spectrometry. The formation of supramolecular complexes or adducts is plausible within the low polarity regime of a bilayer membrane. Even if such interactions were detectable in a liposomal membrane where the phospholipids produce a relatively homogeneous bilayer, it is unclear that the same chemistry would occur in the more complex boundary layers of bacteria.

Conclusions

We present data that demonstrate an enhancement of antibiotic potency mediated by three hydraphiles interacting with four structurally diverse antibiotics in four different microbes. Both benzyl C₈ (**1**) and C₁₄ (**2**) hydraphiles significantly enhance the potency of antibiotics, in one case by up to 30-fold. Where significant

potency enhancements occur, the evidence for synergy is clear. The potency enhancements observed for **1** appear to discount an exclusive ion transport-related mechanism. We infer this because **1** is inactive as Na⁺-transporting ion channel in liposomal and planar bilayer experiments. A more general and nonspecific increase in bacterial membrane permeability seems to be the more likely explanation for the activity, although a combination of channel formation and enhancement membrane permeability cannot be discounted.

Several mechanisms have been suggested to account for the efficacy of antimicrobial peptides in fostering the transport of hydrophobic substrates. One is that the peptide binds to the membrane to form an ion channel.³⁸ A second postulate is that the peptides infiltrate the membrane according to the carpet model,³⁹ causing extensive membrane disruption and permeability increase. A recent report of synergy between such cell penetrating peptides as magainin and polymyxin with antibiotics such as piperacillin or clarithromycin suggests enhanced membrane permeability as an important mechanistic possibility.⁴⁰ Notwithstanding, a very recent report links polymyxin permeability to a membrane receptor.⁴¹ An increase in permeability mediated by hydraphiles seems plausible in the present case, but channel formation may also alter membrane structure or function or both. Studies are underway with structurally related amphiphiles not known to form channels to further define the scope of such enhancements.

Experimental section

Hydraphiles. Benzyl hydraphiles having spacer chains of 8- 14- and 16-methylene groups have been prepared and reported previously.^{21,22} Benzyl C₈, C₁₄ and C₁₆ hydraphiles for use in this study were synthesized according to a recently reported procedure.²⁴ All the hydraphiles were dissolved in DMSO before use. The final concentration of DMSO in each experiment was kept constant at 0.5%-by volume whether or not the co-solvent was required for solubility.

Antibiotics and bacteria used. All antibiotics were obtained from Sigma-Aldrich Chemical Company. Rifampicin and erythromycin were dissolved in DMSO before use. Tetracycline and kanamycin were dissolved in autoclaved milli-Q H₂O. The compounds purchased were the purest available and were used as received.

E. coli (DH5α and K-12), *B. subtilis* and *P. aeruginosa* were obtained from the American Type Culture Collection (ATCC) and were used for MIC and co-administration procedures. All bacteria were cultured in L.B. Miller media and used at exponential growth phase as outlined in the MIC procedure below.

Minimal inhibitory concentration (MIC). The MIC and co-administration studies were performed according to the procedure outlined in Clinical and Laboratory Standards Institute.²⁸ Bacteria were grown overnight from one colony forming unit (CFU) in L.B. Miller media. Compounds were prepared by serial dilutions in either DMSO or milli-Q H₂O. The compound or antibiotic [10 μL] was added to 1970 μL of L.B. Miller media in the test tubes. The bacteria were knocked back to O.D. (600 nm) = 0.600 before use and 20 μL of dispersion was added to

each test-tube. The test tubes were vortexed before and after adding bacteria and incubated overnight at 37 °C and 200 RPM, before collecting the results. Visual turbidity of the liquid in the test tubes was used to determine growth or no growth. The lowest concentration that inhibited the growth of bacteria completely, was considered to be the MIC. The data were reproduced at least three times at the MIC concentration.

Co-administration of hydraphiles and antibiotics. The co-administration of hydraphiles and antibiotics experiments were performed as outlined in the Clinical and Laboratory Standards Institute protocols.²⁸ The bacteria were prepared at O.D. 600 nm = 0.600, as outlined in the MIC procedure above. The MIC of each antibiotic was determined by using the serial dilution technique in the presence of an amount of hydraphile determined to be 1/2 or 1/4 of the hydraphile's MIC. In a test tube, L.B. Miller media was added followed by hydraphiles and antibiotics. The final volume of compounds added was kept constant at 0.5%-by volume (DMSO or dH₂O) as needed. The bacteria were knocked back and 20 µL of the dispersion was added to each test-tube. The test tubes were incubated overnight at 37 °C while stirring at 200 RPM, before collecting the results. Growth or no growth of the bacteria was determined by visual inspection. The MIC of antibiotics in the presence of hydraphiles was replicated a minimum of three times.

Growth curve. Growth curves were performed with benzyl C₁₄ hydraphile and erythromycin against DH5α *E. coli*. The growth curves were performed in a 2 mL culture. *E. coli* was grown overnight from one CFU in L.B. Miller media and knocked back to O.D. 600 nm = 0.600 before use. Media was added (L.B. Miller) to a test tube followed by desired concentration of hydraphile or erythromycin or a combination of both. The concentration (volume) of DMSO was kept constant at 0.5%-by volume. To each test tube, knocked back *E. coli* was added and the samples were vortexed. Contents of the test tubes were incubated at 37 °C and 200 RPM and its optical density was determined at λ = 600 nm every 15 minutes for 12 hours. The results are presented as optical density *vs.* time.

Electrospray ionization mass spectrometry. Spectra were obtained on a JEOL JMS-700 mass spectrometer. Samples were injected directly into the instrument at 1 mL/min and ionized by electrospray with a tip voltage of 2 kV. A mass range of 100 to 2400 m/z was scanned in the positive polarity mode. No significant peak was observed past 1700 m/z. Benzyl C₁₆ hydraphile **3** (in DMSO, 0.25-0.5 mM final concentration) was added to tetracycline **5** (hydrochloride salt in 18.2 MΩ H₂O, 1 mM final concentration). Controls were performed with hydraphile **3** and antibiotic **5** alone. In each case the final DMSO:H₂O ratio was 9:1 by volume.

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