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# Resensitization of Resistant Bacteria to Antimicrobials

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## Abstract

Hydraphiles are synthetic amphiphiles that form pores in bilayer membranes. A study was undertaken to determine if the formation of pores in liposomes would be reflected in the penetration of antibiotics into bacteria. The disruption of ion homeostasis by the pore-formers leads to microbial toxicity. Co-administration of hydraphiles at concentration ≤ ½ MIC and antimicrobials to *E. coli* or *P. aeruginosa* showed potency enhancements of up to 30-fold. A possible mechanism is the enhancement of antibiotic influx owing to membrane disruption and/or altering the ion balance within the bacterial cells.

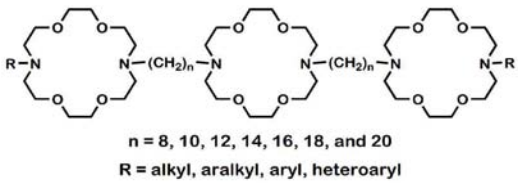
**Keywords: Erythromycin; *Escherichia coli*; Hydraphile; Ion transport; Kanamycin; Pore formation; *Pseudomonas aeruginosa;* Rifampicin; Tetracycline**

## Introduction

Beginning with the discovery of the sulfa drug Prontosil in the 1930s [1], humanity has been attacking microbes and, until recently [2], winning the antibiotic war. The emergence of antimicrobial resistance has partly reversed the situation. Certain species of bacteria are now resistant to all known antibiotics. So serious is the situation that both the Centers for Disease Control and Prevention (CDC) [3] and the World Health Organization (WHO) [4] have issued advisories. The crisis has also engendered a White House initiative [5]. The severity can be understood from statistics reported for 2013. In that year in the U.S. alone, more than 2 million people were infected by hospital acquired bacterial infections, which proved mortal to 23,000 patients.

Numerous antibacterial agents are known [6]. These function to kill bacteria by a range of mechanisms. Some interfere with protein synthesis by different interactions such as with DNA gyrase or ribosomes. Others disrupt bacterial boundary layer membranes. Still others affect the function of efflux pumps [7] that remove xenobiotics from the bacterial cytoplasm. In previous work, we developed a family of synthetic amphiphiles that form ion-conducting pores or channels within liposomal bilayers [8]. The compounds are referred to as hydraphiles and their properties have been described in a review [9]. Key features are that the hydraphiles (1) insert into bilayer membranes, (2) conduct cations in preference to anions, (3) selectively transport $Na^+$ over $K^+$ by 4:1, (5) are blocked by $Ag^+$, (6) show well-behaved open-close behavior when studied by using a planar bilayer voltage clamp apparatus [10], and (7) show length dependent [11] transport and toxicity to cellular organisms [12]. A family of hydraphiles was prepared and shown to transport ions through liposomal membranes in a fashion that depended on both the length of the amphiphile and the thickness of the membrane [13]. A general structure for hydraphiles is shown in Scheme 1.

The hydraphiles contain macrocyclic "crown" polyethers within their structure. Crown ethers have been shown to exhibit toxicity to various microbes in studies reported as long as four decades ago [14]. Such studies continue to appear [15]. Many of these studies cannot be compared owing to differences in macrocycle structure, the organism studied, and the methodology used to assess the toxic effects. In some studies, Gram-positive bacteria were exposed to various macrocycles using




**Scheme 1**: Structures of several hydraphile pore-forming synthetic amphiphiles. In the most extensively studied compounds in the family, R = benzyl ($CH_2C_6H_5$).

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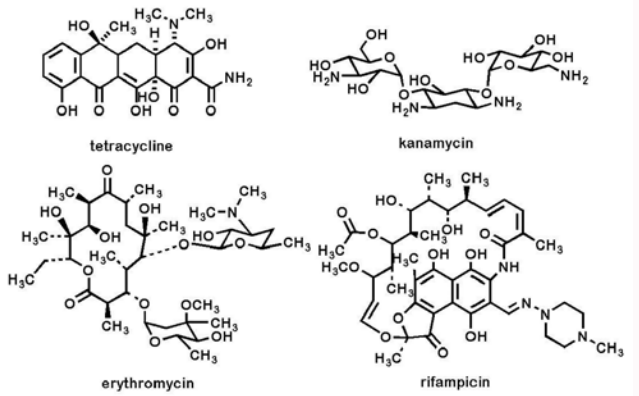



**Figure 1**: Relationship of sodium cation transport efficacy by hydraphiles to their toxicity against *E. coli* and *B. subtilis*. The transport data are for $Na^+$ in liposomal membranes. The chain length refers to "n" in Scheme 1. The

the disk diffusion method [16] and diameters were reported for the zones of inhibition [17]. In other cases, Gram negative and Gram positive bacteria and various yeasts [18] were exposed to macrocycles and minimum inhibitory concentrations were reported [19]. Such variations in experimental approaches have made comparisons difficult. To the extent that the results can be generalized, it appears that macrocycle toxicity is greater to Gram positive bacteria than to Gram negative. Of course, this selectivity is often observed for drugs having antimicrobial properties.

In our own work, we found in liposomal studies that hydraphiles having spacers (Scheme 1, "n") of 12, 14, or 16 methylene groups were the most effective pore-forming molecules [20]. Others have computationally modeled the hydraphiles and have reached similar conclusions [21]. When we compared the length dependence of transport efficacy with toxicity to either Gram negative *Escherichia coli* or to Gram positive *Bacillus subtilis*, the (inverse) correspondence shown in Figure 1 was apparent [22].

Cation transport was assessed by measuring $Na^+$ release from soybean asolectin liposomes. When the spacer chains were 8 methylenes, there was little cation release detected. The overall length of the hydraphile in this case is close to 30, which would barely span the insulator regime ("hydrocarbon slab") of a typical bilayer. The minimal transport that was observed was attributed to a carrier mechanism. When the spacer chains were decylene [$(CH_2)_{10}$], the length of an extended hydraphile increased by about 5 Å, making it marginally functional. The most efficient ion transport was observed in the 12, 14, and 16 methylene range. At longer chain lengths, function persisted but was diminished. The reduction in cation transport rate was attributed to the hydraphile being too long to form an ideal conductance pore.

The graph of Figure 1 shows two interesting features. There is a clear relationship between ion transport efficiency and potency against bacteria. The hydraphiles that are the best $Na^+$ transporters are also the most toxic to *E. coli* or *B. subtilis*. It is also the case that Gram positive *B. subtilis* is more susceptible to disruption of ion homeostasis than is Gram negative *E. coli*. As noted above, the greater susceptibility of Gram positive microbes to a range of chemical entities is a common observation.

The obvious hypothesis was that since the hydraphiles penetrate membranes and form pores, they could also assist in the passage of other species though microbial boundary layers. We therefore examined the efficacy of various antibiotics in the presence and absence of limited, nontoxic amounts of hydraphiles. The results of such studies are presented below.

## Materials and Methods

### Compounds used

The hydraphiles used to obtain the results reported here were prepared as described previously [23].

### Solvents and media used

All bacteria were grown in standard LB broth. The solubility of certain hydraphiles was such that 0.4% by volume of dimethyl sulfoxide was required. Thus, all experiments were conducted in the presence of this amount of DMSO [24], whether required or not. Controls showed that at this concentration, there was no detectable effect on any of the bacterial strains.

### Minimum inhibitory concentrations

The determination of MIC values was done in accord with the procedures published by the Clinical and Laboratory Standards Institute [25].

### Combination therapy experiments

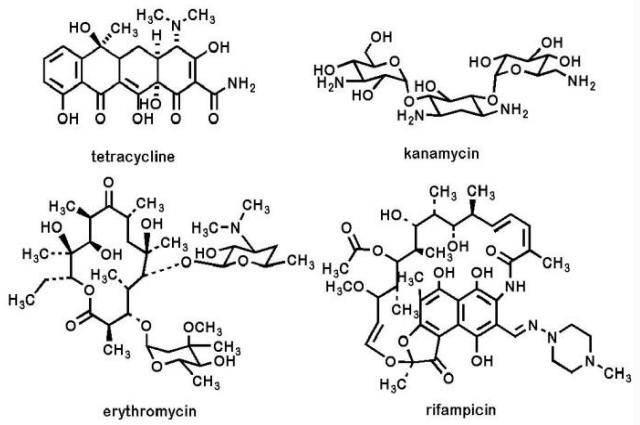
Initial experiments were conducted in test tubes. The MIC was determined using the standard protocol noted above for each bacterial strain in combination with either the hydraphile or the antibiotic. A similar experiment was conducted in the presence of either ½ or ¼ MIC in combination with the antibiotic. The MIC recorded in the presence of the hydraphile was typically lower than for the antibiotic alone and the enhancement was calculated as $MIC_{initial}/MIC_{+hydraphile}$. In a case in which the initial MIC was 100 μM and the MIC in the presence of hydraphile was 8 μM, the fold enhancement is 100/8 ≈ 12.

## Results and Discussion

The hypothesis that hydraphiles were penetrating bacterial boundary layers and creating either a pore or a local disruption in membrane organization, suggested the potential for enhancing the passage of other molecules. In liposomal studies, the metric for transport was ion release, as shown in Figure 1. The metric chosen for the studies reported here was toxicity to bacteria. We reasoned that enhancement of antibiotic potency in the presence of sub-lethal (or sub-MIC) concentrations of hydraphiles would indicate enhanced penetration. Further, the level of potency enhancement would afford a quantitative means for comparison.

Four antibiotics were chosen for the initial study. They are erythromycin, kanamycin, rifampicin, and tetracycline. These antibiotics are in different structural classes as shown in Scheme 2. To the extent that there are structural similarities, erythromycin and rifampicin are macrolides. Notwithstanding, the ring sizes and substitution patterns are significantly different.

An additional important consideration was that the modes of action for each of these compounds are well-established and different. For example, a comparison of the two macrolides reveals that erythromycin's toxic effect on bacteria results from the inhibition of protein synthesis by binding to the 50S ribosome subunit [26]. In contrast, rifampicin's toxic effect results from binding to the ⇓-subunit of RNA polymerase and thus inhibiting bacterial RNA synthesis [27]. Tetracycline, which is one of the most extensively used antibiotics in the US, interferes with peptide synthesis – prevents




**Scheme 2**: Antibiotics studied in early combination experiments.

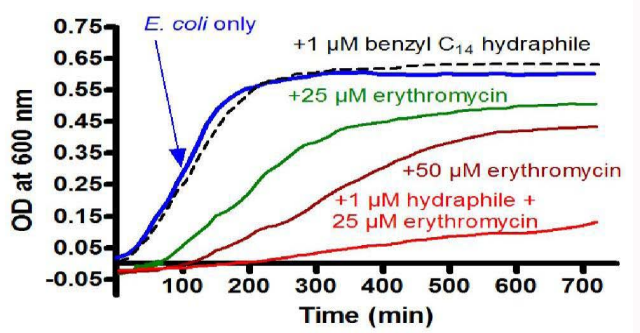



**Figure 2**: Growth of *E. coli* DH5α in the presence of 1 μM benzyl $C_{14}$ hydraphile (½ MIC) and fractional MIC quantities of erythromycin.

peptide elongation by binding to the 30S ribosome subunit [28]. Kanamycin belongs to the aminoglycoside class of antibiotics. It causes potassium leakage from cells and inhibits both cell respiration and translation [29]. We note that all four of these antibiotics must localize in the cell cytoplasm to reach/interact with its target.

Initial studies were conducted with the DH5α laboratory strain of *E. coli*. This is a non-pathogenic strain that was chosen because of its ready availability and relative safety. Minimum inhibitory concentrations were determined for this strain with the benzyl $C_{12}$ and benzyl $C_{14}$ hydraphiles (n= 12 and 14 in the structure of Scheme 1). Similarly, MICs were determined for each of the antibiotics against *E. coli*. Growth curves were conducted in the presence of fractional concentrations of hydraphiles and antibiotics. The growth after 8-16 hours did not differ from growth observed for *E. coli* alone. At fractional MIC concentrations of antibiotics, bacterial growth was diminished, approximately in accord with the MIC fraction. However, when both hydraphile and antibiotic were present at fractional concentrations, growth was significantly limited or prevented. The growth curve is illustrated in Figure 2 [30].

In order to determine if any antibiotic potency enhancement could be attributed to the presence of hydraphiles, we co-administered hydraphiles and antibiotics. First, the MIC values of the hydraphile were determined against each bacterial strain. Likewise, the MIC for each antibiotic was determined. Hydraphile was then added to the individual bacterial strain in growth media at half of its MIC. The MIC of each drug was then determined by using the standard procedure [25]. The fold enhancement is the $[MIC]_{initial}/[MIC]_{hydraphile}$. The results

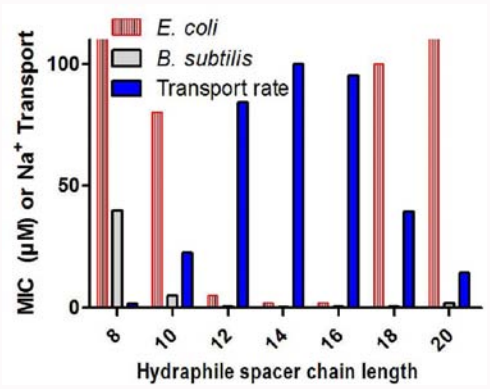
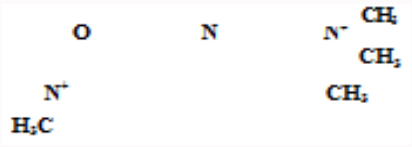



**Figure 3**: Enhancement of antimicrobial potency by benzyl $C_{14}$ hydraphile against the indicated bacterial strain. Data for DH5α *E. coli* are from reference 00 and for K-12 *E. coli* and *P. aeruginosa* are from reference 00. Data were not obtained for the combination of kanamycin with *P. aeruginosa.*



**Scheme 3**: The structure of benzoxazolium, 3-methyl-2-[[1-[3-(trimethylammonio) propyl]-4(1H)-pyridinylidene] methyl] (diiodide), called PO-PRO-1.

of these experiments are summarized in the graph of Figure 3.

Although we anticipated greater activity against Gram positive *B. subtilis* than either of the Gram negative strains, benzyl $C_{14}$ hydraphile afforded only a marginal enhancement when co-administered with antibiotics. A two-fold reduction in *B. subtilis's* MIC was observed when the antibiotic was either erythromycin or tetracycline. In either case, the hydraphile was administered at a ½ MIC concentration. These results were essentially the same as for benzyl $C_{14}$ hydraphile at ¼ MIC with either antibiotic against DH5α *E. coli*, K-12 *E. coli*, or *P. aeruginosa*.

Studies were also conducted with benzyl $C_8$ hydraphile (n = 8 in Scheme 1). The MIC of this compound against each of the bacterial strains tested was significantly higher than for the corresponding $C_{14}$ compound. For example, the MIC of benzyl $C_{14}$ hydraphile against DH5α *E. coli*, K-12 *E. coli*, or *P. aeruginosa* was approximately 2 μM in all three cases. The MICs for benzyl $C_8$ hydraphile against DH5α *E. coli* and K-12 *E. coli*, were, respectively, 300 μM and 200 μM. The MIC of benzyl $C_8$ hydraphile was not determined against *P. aeruginosa.*

Co-administration experiments were conducted with benzyl $C_8$ hydraphile only with DH5α *E. coli*. The half-MIC concentration of this hydraphile is 150 μM, substantially higher than for the corresponding $C_{14}$ compound. Even though the benzyl $C_8$ hydraphile does not function effectively as a pore-former, it enhanced antimicrobial potency at ½ MIC. The fold-enhancements were: erythromycin, 13 xs; kanamycin, 3 xs; rifampicin, 18x; and tetracycline, 5 xs. It is interesting to note that unlike most other combinations, benzyl $C_8$ hydraphile enhanced rifampicin potency by 18-fold even at ¼ MIC. More extensive studies were not conducted on this hydraphile because the high concentration required at ½ MIC made it an impractical additive or adjuvant candidate.

Control studies were conducted with the membrane penetrating

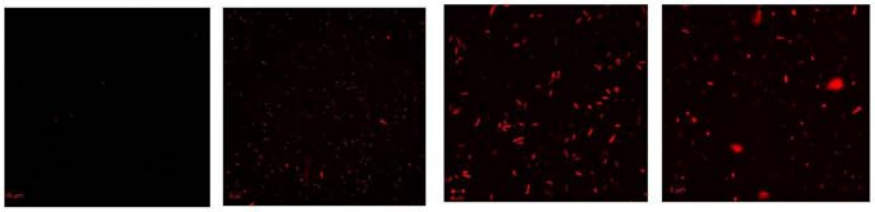


**Figure 4:** Confocal images of *E. coli* to which the fluorescent dye PO-PRO-1 has been added as follows, left to right: (Left) *E. coli* grown in the absence of any additive. (Left center) *E. coli* grown in the presence of 0.4% by volume of DMSO. (Right center) *E. coli* in the presence of 0.4% DMSO and 1 μM benzyl $C_{14}$ hydraphile. (Right) *E. coli* in the presence of 0.4% DMSO and 2 μM benzyl $C_{14}$ hydraphile. The left image was obtained at 400x magnification and all others at 640x.

amphiphile Triton X-100. This detergent has been reported to form ion-conducting pores in bilayer membranes when embedded therein [31] but not when added externally [32]. It was found that no enhancement of potency with any bacterial strain or antimicrobial noted herein was observed. At concentrations as low at 1%, membrane rupture occurred and bacterial cell death was apparent. Membrane rupture caused by this detergent was expected, but the experiment demonstrated that not any amphiphile could show enhancement effects.

Confocal microscopic studies were conducted to obtain evidence for penetration of bacterial membranes by xenobiotics. None of the antibiotics used in this study is sufficiently fluorescent to be used directly. Thus, bacteria were exposed to the fluorescent dye "PO-PRO-1." The structure of the compound and its systematic name are shown in Scheme 3.

This molecule does not ordinarily penetrate membranes. Once it is within the bacterium. Its interaction with DNA produces a significant fluorescent response. Four panels are shown in Figure 4. The left panel shows DH5α *E. coli* at 400x magnification in the absence of any additive. The second panel is similar except that 0.4% DMSO is present in the growth medium and the magnification is higher at 640 xs. In neither case is any significant fluorescence observed. The two rightmost panels show *E. coli* in the presence of both DMSO and either 1 μM or 2 μM (rightmost panel) benzyl $C_{14}$ hydraphile. In the latter two cases, the PO-PRO-1 dye has penetrated to a significant extent and there is a qualitative dependence on the hydraphile concentration.

The potential toxicity of hydraphiles - and synthetic channel-formers in general-to mammalian cells is a valid matter of concern. The fluorescent dye experiments make clear that the hydraphiles penetrate bacterial cells effectively. Preliminary data show that bacterial cells are penetrated more readily than are human embryonic kidney (HEK 293) cells. This may be due to the presence of cholesterol in the mammalian membranes. The presence of the steroid results in thickening and rigidification of the bilayer. In contrast, Gram negative *E. coli* membranes contain porins that may permit passage of the hydraphiles through the outer boundary layer.

The function, at least in part, of the hydraphile pore-formers as membrane disruptors seems reasonable in light of the antimicrobial activity of colistin. Colistin is a cyclic peptide that is approved as an antibiotic of last resort. Although it is a membrane disruptor and causes (reversible) renal damage, it is approved for clinical use despite its toxicity. The fact that antimicrobial treatments are typically short-lived, and that it is used only in extreme cases justify its level of toxic effect. Additionally, however, prodrug analogs of colistin have been introduced that ameliorate the toxicity. Other functions of the hydraphiles are possible. One mechanism by which bacteria develop resistance involves efflux pump ejection of such xenobiotics as antimicrobial agents. The compounds that are the focus of the current study may play a role as efflux pump inhibitors as well as enhancing membrane permeability. We have recently found that certain tryptophan-based amphiphiles do, indeed, function as efflux pump inhibitors. The bis (tryptophan) amphiphile had shown in scheme 4 shows a four-fold recovery of tetracycline activity against a strain of *E. coli* that incorporates the A efflux pump. The bis (tryptophan) amphiphile shows low toxicity to HEK 293 and Cos-7 cells.


**Scheme 4:** A bis (tryptophan) amphiphiles that shows low mammalian toxicity and restores tetracycline activity against a tetracycline-resistant strain of *E. coli*.

We are optimistic about the potential of the hydraphiles discussed above based on the promising results obtained with another twin-headed amphiphile. Experiments directed to understanding this and other possible rolls of the hydraphiles are underway.

## Conclusion

We have developed a family of amphiphiles that function in many ways like channel-forming proteins. These compounds penetrate bacterial membranes and also enhance either the penetration of antimicrobials into bacteria or inhibit efflux pump function, or both. In any event, they enhance the potency of a range of antibiotics against both Gram positive and Gram negative bacteria.

A number of natural cell-penetrating peptides such as colistin, daptomycin, gramicidin-S, magainin, and others are known. The hydraphiles are the first all-synthetic general class of molecules that show many of the same functions as these naturally occurring compounds. The hydraphiles show relatively low toxicity to HEK-293 cells, especially compared to colistin, which is FDA approved as an antibiotic.

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