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Synthetic Ion Channels: A New Class of Membrane Disruptor and Efflux Pump Inhibitor for the Recovery of Antibiotic Potency

by

Mohit B. Patel

M. Sc., Biochemistry & Biotechnology, University of Missouri St. Louis, 2012
B. Sc., Biochemistry & Biotechnology, University of Missouri St. Louis, 2011

A Dissertation Submitted to The Graduate School of the

University of Missouri - St. Louis

in partial fulfillment of the requirements for the degree

Doctor of Philosophy

in

Cell and Molecular Biology

December 2016

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ABSTRACT OF THE DISSERATION

Synthetic Ion Channels: A New Class of Membrane Disruptor and Efflux Pump Inhibitor for the Recovery of Antibiotic Potency

by

Mohit B. Patel

Doctor of Philosophy in Biology
University of Missouri-St. Louis, 2016
Dr. George W. Gokel, Advisor

Antibiotic resistance has become a world-wide health care crisis. In 2013 there were 50,000 deaths in U.S. and EU, associated with hospital acquired bacterial infections. This problem is exacerbated by the lack of new antibiotics in development. There was only one combination antibiotic approved by the FDA in 2015. Here, we report that synthetic amphiphiles represent a new class of adjuvants that rescue antibiotic potency against multidrug resistant bacteria.

Ion channel proteins maintain ion homeostasis through the cellular membrane. Hydraphiles are amphiphiles designed and synthesized in the Gokel lab that show many of the same properties as protein ion channels. Hydraphiles were previously shown to have antimicrobial property against *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. The antimicrobial property of hydraphile was correlated to its ability to span the bacterial membrane and transport cations such as Na^+ and K^+. We report that hydraphiles can be used to inhibit antibiotic efflux pumps and increase the bacterial membrane permeability.

Most of the studies involving this class of molecules have been focused on developing new structures, and assessing their effect on ion transport and antibiotic potency. This is the first report that shows that hydraphiles recover the activity of tetracycline and fluoroquinolones (ciprofloxacin, norfloxacin) against two Gram negative (*E. coli* and *Klebsiella pneumoniae*) and one Gram positive (*Staphylococcus aureus*) bacteria. Out of the four hydraphiles tested (C_8-C_{14}), benzyl C_{14} hydraphiles is most efficient as an adjuvant. At sub-lethal concentrations, hydraphiles do not inhibit bacterial growth, show synergy with

existing antibiotics and transport K^+ ions. Controls confirmed that the structure and function of hydraphiles are critical for its activity.

The outer membranes of Gram negative bacteria provide for an attractive target for antibiotic development. We showed that benzyl C_{14} hydraphile localize in the *E. coli* and human embryonic kidney (HEK-293) cell membranes. At one-half the minimal inhibitory concentration hydraphiles can increase the permeability of bacterial membranes. However, they did not alter the membrane permeability of mammalian cells. Scanning electron microscopy confirmed that benzyl C_{14} hydraphiles form 100-200 nm aggregates that attach to the bacterial surface, induce osmotic stress and cause disruption of the cytoplasmic membrane. An advantage of this approach is that bacteria cannot readily develop resistance to membrane-active amphiphiles as observed with benzyl C_{14} hydraphiles.

Efflux pumps provide resistance to multiple different classes of antibiotics and its activity depends on existing cation gradients. Here we show that hydraphiles can transport K^+ from bacteria and inhibit the activity of norA efflux pump in *S. aureus*. As a result, the accumulation of the substrate/antibiotic increases in the *S. aureus* cytoplasm. This increases the antibiotic potency. We observed that at sub-lethal concentrations of benzyl C_{14} hydraphile the survival of three different mammalian cells was 80-100%. Cytotoxicity from hydraphiles was lower than CCCP, a known efflux pump inhibitor, but its efficacy to inhibit the efflux pump and recover antibiotic potency was far superior.

Overall, we report a novel adjuvant platform that could be used to rescue the efficacy of existing antibiotics for the treatment of life-threatening bacterial infections.

Summary and Contributions

This dissertation consists of ideas and results, the majority of which are the original work of the author. Many collaborators and lab members have helped me along the way. Details of all the contributions are outlined below.

Chapter 1. This is an introductory chapter. It does not contain any experimental results. I wrote this chapter.

Chapter 2. This chapter was published in Bioorganic and Medicinal Chemistry. There are eight authors listed on the publication. I determined the minimal inhibitory concentration and performed combination studies with benzyl C_8 and C_{14} hydraphiles against K-12 and DH5α *E. coli*, *B. subtilis* and *P. aeruginosa* which are included in tables 2.1 and 2.2 and, figures 2.2, 2.3 and 2.5. Dr. Saeedeh Negin and Michael R. Gokel performed MIC and combination studies with Triton X-100. Screening with benzyl C_{16} hydraphile and growth curve was performed by Dr. Zachary Cusumano. Dr. Joseph W. Meisel conducted the high resolution mass spectrometry shown in figure 2.6.

Chapter 3. Hydraphiles used in chapter 3 were prepared by Michael Hayes. I performed all the MIC screening, combination studies, growth curves and checkboard experiment with benzyl C_8-C_{14} hydraphiles and controls against Tet^R *E. coli* and *K. pneumoniae*. Evan C. Garrad performed some of the MIC and combination studies with Tet^R *E. coli*. Dr. Saeedeh Negin and Michael R. Gokel helped with the combination studies between ciprofloxacin and benzyl C_8 and C_{14} hydraphile or lariat ethers and against *K. penumoniae*. Dr. Joseph W. Meisel helped me with reviewing the data and determining appropriate controls.

Chapter 4. The dansyl labeled C_{14} hydraphile used in chapter 4 were prepared by Michael Hayes. I performed all the localization studies, membrane permeability studies, nucleic acid release and SEM microscopy. I prepared the samples for SEM studies. Dr. David Osborn and I acquired the SEM image.

Chapter 5. Hydraphiles used in chapter 5 were prepared by Michael Hayes. I performed the MIC screening, combination studies, ethidium bromide release and accumulation studies, resistance development and cytotoxicity studies. I performed the K^+ release study with benzyl C_{14} hydraphile. Evan C. Garrad performed K^+ release studies with benzyl C_8, C_{10} and C_{12} hydraphiles. Dr. Joseph W. Meisel helped me with reviewing data and determining appropriate controls.

Mohit. B. Patel
Professional Publications

1. Atkins, J. L., Patel, M. B., Cusumano, Z., Gokel, G. W., 'Enhancement of antimicrobial activity by synthetic ion channel synergy.' *Chem. Commun.*, **2010**, 46, 8166-8167.
2. Atkins, J. L., Patel, M. B., Daschbach, M. M., Meisel, J. W., Gokel, G. W., 'Anion complexation and transport by isophthalamide and dipicolinamide derivatives: DNA plasmid transformation in *E. coli*'. *J. Am. Chem. Soc.*, **2012**, 134, 13546-13549.
3. Gokel, G. W., Curvey, N. S., Gokel, M. R., Lindsey, M. E., Meisel, J., Negin, S., Patel, M. B., Sedinkin, S. L., 'Fluorescent pore-former suggests an approach for the enhancement of the antibiotic efficacy.' *Biosens. J.*, **2013**, 1, 103.
4. Patel, M. B., Stavri, A., Curvey, N., Gokel, G. W., 'Hydraphile synthetic ion channels alter root architecture in Arabidopsis thaliana.' *Chem. Commun.*, **2014**, 50, 11562-4.
5. Negin, S., Gokel, M. R., Patel, M. B., Sedinkin, S. L., Gokel, G. W., 'The aqueous medium dimethylsulfoxide conundrum in supramolecular biological studies.' *RSC Adv.,* **2015**, *5,* 8088-8093.
6. Cantwell, R., Garrad, E. C., Gokel, M. R., Hayes, M. J., Meisel, J. W., Negin, S., Patel M. B., Gokel, G. W., 'Biological activity of macrocyclic cationic transporters.' *Curr. Org. Chem.,* **2015**, 19, 2278-2285.
7. Cantwell, R., Gokel, M. R., Meisel, J. W., Negin, S., Patel, M. B., and Gokel G. W., "Cation binders, amphiphiles, and membrane active transporters," Chapter 17 in R. M. Izatt, *Macrocyclic and Supramolecular Chemistry: How Izatt-Christensen Award Winners Shaped the Field*, J. Wiley and Sons, 2015, submitted May 30, **2015**, *in press*.
8. Patel, M. B., Garrad, E. C., Stavri , E., Gokel, M. R., Negin, S., Meisel, J. W., Cusumano, Z., Gokel, G. W., 'Hydraphile enhance antimicrobial potency against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.' *Bioorg. Med. Chem.*, **2016**, 24, 2864-2870.
9. Meisel, J. W., Patel, M. B., Gokel, G. W., 'Condensation of plasmid DNA by benzyl hydraphiles and lariat ethers: dependance on pH and chain length' *Supramol. Chem.*, **2016**, http://dx.doi.org/10.1080/10610278.2016.1192170.
10. Meisel, J. W., Patel, M. B., Garrad, E. C., Gokel, G. W., Novel *bis*(tryptophan) antimicrobials reverse tetracycline resistance in *Escherichia coli*.' *J. Am. Chem. Soc.*, **2016**, *138*, 10571-10577.

11. Negin, S.; Patel, M. B.; Gokel, M. R.; Gokel, G. W., Antibiotic Potency against *E. coli* is Enhanced by Channel-forming Alkyl Lariat Ethers. *Chem. BioChem*, **2016**, DOI: 10.1002/cbic.201600428.

Patent Applications

1. George W. Gokel, Michael R. Gokel, Saeedeh Negin, and Mohit B. Patel, International Application Number: PCT/US2015/034550; Title of Invention: Enhancement of Antibiotic Efficacy, filed June 5, **2015**, published December 15, **2015**.
2. George W. Gokel and Mohit B. Patel, International Application Number PCT/US15/44353, Title of the invention: "Compositions And Methods For Synthetic Amphiphile Induced Changes In Plant Root Morphology,"filed August 7, **2015,** published February 11, **2016.**
3. George W. Gokel, Michael R. Gokel, Saeedeh Negin and Mohit B. Patel, Continuation in part, Provisional Application Number 15/186,070 (US2015/034550), Title of the invention: "Molecules that inhibit efflux pumps in multidrug resistant bacteria and uses thereof," filed June 16, **2016.**
4. George W. Gokel, Joseph W. Meisel and Mohit B. Patel, Provisional Application Number 62/352,983, Title of the invention: "Bis-amino acid based compounds and use thereof," filed June 21, **2016.**

Presentations

1. Nanofrontiers Symposium, Research Poster Presentation, Washington University St. Louis, **2010**, St. Louis, MO.
2. Disruptive Diner, Nanopossibilities, Research Presentation, **2013**, St. Louis.
3. Washington University in St. Louis, Skandalaris Center. Panelist for "Technological advances in health care.", **2013**.
4. Missouri Technology Expo, Missouri University, Research Presentation, **2013**, Columbia, Missouri.
5. Fast Track I Grant Award, G. W. Gokel Lab., Research and commercialization presentation, University of Missouri-Columbia, **2013**.
6. Fast Track I Grant Award, G. W. Gokel Lab., Research and commercialization presentation, University of Missouri-Columbia, **2014**.
7. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Research Poster Presentation, University of Missouri St. Louis, **2013**, St. Louis, MO.

8. Graduate Research Symposium, Research Poster Presentation, University of Missouri-St. Louis, **2014**.
9. Real World Career Experiences Exploring, Research Presentation, Center for Nanoscience, University of Missouri-St. Louis, **2014**.
10. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Research Poster Presentation, St. Louis University, **2014**, St. Louis, MO.
11. Biology Graduate Student Association Research Symposium, Research Poster Presentation, University of Missouri-St. Louis, **2015**.
12. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Research Poster Presentation, University of Missouri St. Louis, **2015**, St. Louis, MO.
13. American Society of Microbiology, Microbe, Research presentation 'Hydraphile synthetic ion channels inhibit efflux pump activity' June **2016**, Boston, MA.

Distinctions

1. Poster presentation Award, Missouri NanoFrontiers Symposium, **2010**.
2. Dean's List, University of Missouri-St. Louis, **2011**.
3. Dean's List, University of Missouri-St. Louis, **2012**.
4. Student Inventor of the Year, University of Missouri-St. Louis, **2013**.
5. Honorary Member, National Academy of Inventors, **2013**.
6. Arch Grants Global Business Planning Competition, St. Louis, MO, **2013**.
7. Investment Winner, Olin Cup Business Planning Competition, Washington University in St. Louis, **2013**.
8. Top 30 Under 30 Business Leaders of Tomorrow, St. Louis Business Journal, **2013**.
9. Fast Track Grant-I Award, G. W. Gokel Laboratory, University of Missouri-Columbia, **2013**.
10. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Poster Award, Runner-up, **2013**.
11. Fast Track Grant-I Award, G. W. Gokel Laboratory, University of Missouri-Columbia, **2014**.
12. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Poster Award, **2014**.
13. St. Louis Institute of Nanoscience and Nanomedicine (SLINN), Poster Award, **2015**.
14. Raju Mehra, Outstanding Biology Graduate Student of the Year, Department of Biology, University of Missouri-St. Louis, **2016**.

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I thank my committee for their endless support. Dr. Wendy M. Olivas has provided me invaluable advice and guidance throughout my time here. I highly appreciate that she believed in me to succeed. This will always motivate me to do better. Dr. Sam Wang has provided advice for numerous studies through our many scientific discussions. He motivates me to be a better scientist. I am grateful to Dr. Lon Chubiz for allowing me to access his lab and his guidance every time I needed it. The access to plate reader in his lab allowed me to conduct high-throughput studies. He also provided guidance regarding my experiments, controls, publications, and career decisions. He truly cares for his student success, not only in sciences but also in their career. I am grateful to have a distinguished and valuable committee.

I dedicate this Ph.D. dissertation to my parents. The courage and sacrifice it took to send their young son to a different country and their trust in me to succeed is beyond description. It will inspire me for the rest of my life. I thank my brother and my grandparents who have always loved, supported and encouraged me. A special thanks to my uncle and my aunt for providing me a home away from home. Lastly, I would thank my friends who have not only believed in me but also supported me equally through all the ups and downs.

Many graduate and undergraduate colleagues have helped me and contributed to this process. I have made lifelong friends with whom I have had many valuable discussions. I am grateful to my coworkers Dr. Joseph W. Meisel, Dr. Saeedeh Negin, Dr. Sergey Sedinkin, Evan C. Garrad, Ariel Stavri, Michael R. Gokel and Ryan Cantwell. I am also thankful to Dr. David Osborn in microscopy

and Dr. Michael Nichols in the cell culture facility. They have provided me with invaluable assistance and knowledge in their respective field of expertise. At last, I would like to thank the University of Missouri-St. Louis and the department of biology, for providing me this opportunity. There are many more people who have helped, motivated and made this journey possible. Thank you to all.

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CHAPTER 1

Introduction to the Antimicrobial Resistance Problem and Hydraphile Synthetic Ion Channels

1.1 Antimicrobial resistance (AMR). Antibiotics have been used to treat bacterial infections and to save lives since the 1940s.[1] Due to their widespread use, bacteria have acquired resistance to all known classes of antibiotics (figure 1.1).[2] We are now in the post-antibiotic era, where an infection could prove to be fatal due to lack of an effective antibiotic treatment. In 2013, there were 23,000 deaths and 2 million illnesses caused by antibiotic resistant infections in the US alone.[3] The problem is worse in developing countries where *Mycobacterium tuberculosis* has become untreatable due to AMR.[4] Patients in the hospital are at a greater risk of infections from multi-drug resistant (MDR) bacterial infections, due to the widespread use of antibiotics in hospitals.[5] In particular, immunocompromised patients are at greater risk of being affected by MDR bacteria.[5]

This problem is exacerbated by a lack of new antibiotics in development. Whereas the period from 1951 through 2000 witnessed an average of three new antibiotics per year, only four antibiotics have been approved since 2011.[6] Only one adjuvant-antibiotic was approved by the FDA in 2015.[7] No new class of antibiotics has been developed in the last four decades that targets Gram negative infections.[8] Absent new solutions, the number of global deaths is estimated to reach 10 million by 2050 with productivity losses exceeding $100 trillion.[9] This is equivalent to the absence of any economic contribution by UK, to the global economy, for over 35 years. It is clear that there is not only a need for new antibiotics, but new classes of antibiotics are desperately needed as well.

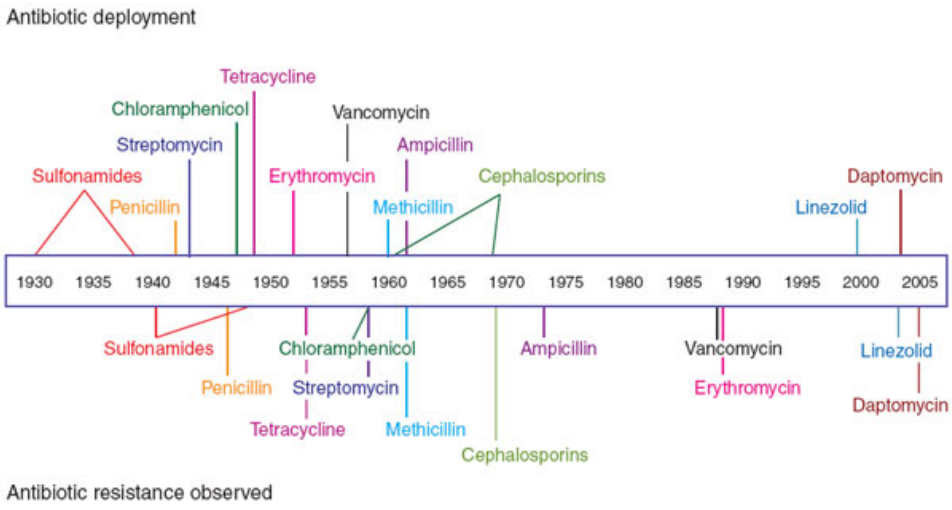

Figure 1.1. Timeline of antibiotic discovery (top) and development of resistance to antibiotics (bottom) from reference 2.[2]

The ability to transfer multiple resistance genes on the same plasmid, between bacteria, allows for the rapid spread of antibiotic resistance.[10] Antibiotics that target DNA gyrase, cell wall synthesis, ribosomal subunits, *etc*. have been deployed. Currently the antibiotic classes commonly used in clinics are carbapenem, cephalosporin and linezolid. Similar to penicillin, carbapenem and cephalosporin are also members of the β-lactam family that targets cell wall synthesis. However, several known β-lactamase enzymes, such as New Delhi β-metallo lactamase-1 (NDM-1)[11], provide resistance to carbapenems, cephalosporin and other β-lactams.[2] Therefore, antibiotics such as polymyxins have made a comeback, after 50 years.[12] Polymyxin binds to the lipid A component (figure 1.3) of Gram negative bacteria and disrupts bacterial membranes causing cell death. They are, however, associated with renal toxicity, and hence used as a drug of last resort.[13] An *E. coli* carrying a plasmid with a resistance gene to polymyxin (mcr-1) in the US, was recently reported as well.[14] This emphasizes the worldwide need for a solution to AMR.

1.2 Antibiotic resistance mechanisms. The generally accepted mechanisms of bacterial resistance are either intrinsic (chromosomal) or acquired (plasmid). There are three known resistance mechanisms (figure 1.2 below). **1.** Bacteria can secrete enzymes that target and degrade antibiotics. For example, β-lactamase targets the β-lactam ring of penicillins. **2.** A mutation could occur in the binding site of the antibiotic target. For example, mutation in the DNA gyrase-A results in resistance to fluoroquinolones such as ciprofloxacin.[15] **3.** Efflux pumps remove antibiotics from the cellular cytoplasm either to the periplasm or to the medium surrounding the cells.[16] For example, AcrAB, MdfA, and NorE type efflux pumps provide resistance to multiple classes of antibiotics in a range of Gram negative bacteria.[17] The efflux pump mechanism is usually manifested in combination with decreased permeability of the outer membrane in Gram negative bacteria.[18] The additional semi-permeable bilayer membrane decreases the antibiotic penetration in to the cell. ESKAPE pathogens are bacteria that escape the effect of antibiotics and cause serious health concerns.[19] It is an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter species*. The majority of the ESKAPE pathogens are Gram negative bacteria. Bacteria usually manifest different combinations of the above mentioned resistance mechanisms to provide resistance to multiple different antibiotics. *Our focus is going to be increasing the membrane permeability and inhibiting efflux pump activity. More details about these are outlined below.*

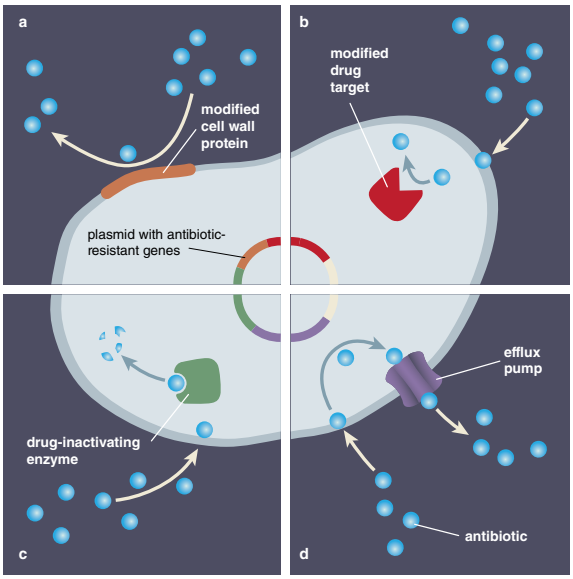

Figure 1.2. Mechanisms through which bacteria develop resistance to antibiotics (illustration from reference 20).[20]

1.3 Membrane permeability. Membranes pose a major barrier for transport of molecules into the cells. Cell membranes are fluid structures made up of mixtures of various lipids and proteins ("the fluid-mosaic model").[21] Small hydrophobic species such as O_2, N_2 and uncharged polar molecules such as water and urea can diffuse through the membrane.[22] However, large uncharged polar molecules such as glucose and sucrose, and charged ions such as H^+, Na^+, K^+, Cl^- and HCO^{3-} cannot diffuse through the membranes.[21] Transport of ions and other molecules through the membranes is attained by transmembrane channel proteins. The majority of antibiotics need to pass through the membrane and bind to its cytoplasmic target to inhibit bacterial growth. Some of the antibiotics diffuse through the membranes, whereas some antibiotics pass through porins (*e.g.* Outer Membrane Proteins, OMPs) found in the membranes of bacteria (see figure 1.4).[23]

In Gram negative bacteria, the outer membrane (OM) provides an additional challenge for entry into the cell. The OM consists of lipopolysaccharides (LPS) and phospholipids (figure 1.3 below).[18] LPS consists of a lipid A structure, core polysaccharide, and O-antigen repeats. The LPS is functionalized with anionic charges, is highly crosslinked and varies among different species in the types of fatty acids and acyl groups that are present.[18]

This prevents the entry of toxic molecules, such as antibiotics. Additionally, LPS acts as an antigen and triggers an immune response in humans. Since the OMs serve as a significant, primary barriers to antibiotic entry, their disruption could serve as a strategy to increase antibiotic delivery/efficacy. In recent years, there has been an increasing interest in this area of research. Membranes provide a previously under-explored opportunity for the development of new antibiotics.

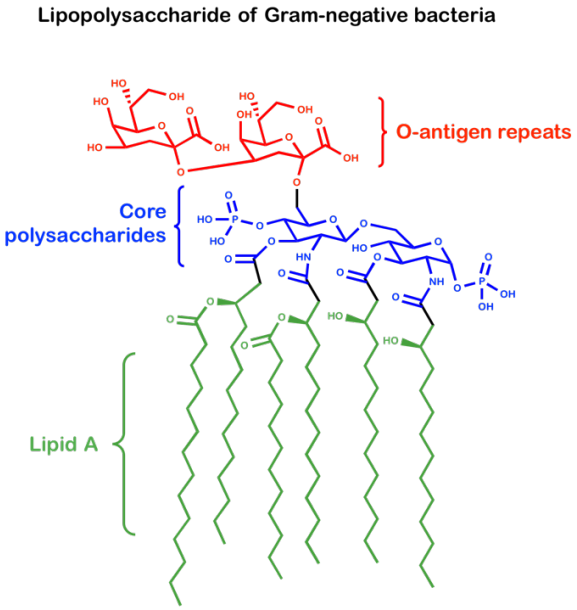

Figure 1.3. Structure of lipopolysaccharide (LPS) in the outer membrane of Gram negative bacteria.[24]

1.4 Efflux pumps. In addition to OM, efflux pumps decrease the cytoplasmic concentration of antibiotics. Overexpression of porins increases efflux of toxic and unwanted molecules from the cells.[23] However, efflux pumps are specialized transmembrane proteins that identify and export antibiotics. Certain efflux pumps provide multi-drug resistance.[16,17,18] The multi drug resistance conferred by efflux pumps can eventually cause acquisition of other types of resistance such as antibiotic degrading enzymes or mutation of antibiotic target sites.[25] There are five known types of efflux pumps. They are: the major facilitator superfamily (MFS), the ATP binding cassette (ABC), the small multidrug resistance (SMR), the resistance nodulation cell division (RND), and the multi and toxic compound extrusion (MATE).[26] The efflux pumps are classified based on phylogenetic grouping.[27] All the efflux pumps except ABC utilize either a proton or sodium gradient as an antibiotic-cation antiport. ABC hydrolyzes an ATP molecule to provide energy for antibiotic transport.

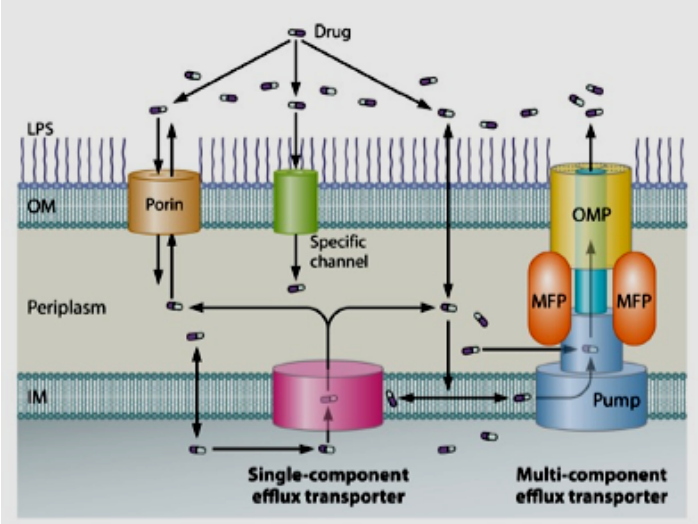

Figure 1.4. Resistance developed by single component and tripartite efflux pumps in Gram negative bacteria (illustration from reference 26).[26]

Efflux pumps exist as either a single-component pump in the cytoplasmic membrane (CM) or as a tripartite system that spans the CM, periplasmic space, and the outer membrane (OM). See figure 1.4 above. Some of the efflux pumps that span only the CM produce minimal resistance.[27] The MFS and SMR type efflux pumps serve as examples. Here, the lipophilic antibiotics that are effluxed from CM can easily accumulate in and diffuse back from the periplasm into the cytoplasm (figure 1.4). Some of these single-component efflux pumps have strong kinetics and still produce a high level of resistance.[28] For example, tetA and AcrB efflux pumps localize in the CM and produce resistance to tetracyclines.[8] The RND type efflux pumps transport antibiotics from the cell cytoplasm directly to the medium surrounding the cell. The RND pumps are also located in the CM but they interact with outer membrane channels (TolC or OprM).[28] These porins are used to transport toxic molecules in all bacteria. The periplasmic adaptor proteins or membrane fusion proteins (AcrAB or MexAB) connect the channels in the CM to those of the OM, making a tripartite complex that spans the CM, periplasm and OM. Some MATE, ABC, and MFS may also function as a tripartite complex.[28]

The efflux pumps in Gram negative and –positive bacteria are highly effective at keeping a low cytoplasmic concentration of antibiotics. Along with OM, efflux pumps create a general mechanism of resistance that spans across

various classes of antibiotics. Gram negative strains that consist of both efflux pumps and antibiotic target site mutations do exist. However, such mechanisms contribute independently to resistance development.[29] For example, a 64-fold recovery in the Minimal Inhibitory Concentration (MIC) of fluoroquinolones resulted from deletion of MexAB-OprM efflux pump in a fluoroquinolone resistant *Pseudomonas aeruginosa* strain that had also acquired resistance by a mutation in DNA gyrase.[30] *Hence, the inhibition of efflux pump activity could significantly increase the sensitivity of antibiotics against bacteria.*

1.5 Efflux Pump Inhibitors (EPI). Five different approaches have been reported for increasing antibiotic concentration in the cell cytoplasm of efflux pump expressing Gram negative bacteria.[30]

1) Stealth. Modifying the structure of existing antibiotics decreases their affinity for the binding site of efflux pumps. This results in an increase in the cytoplasmic concentration of the antibiotics. For example, efflux pumps do not recognize the tetracycline analog tigecycline.[31]

2) Competitive inhibition. An EPI that acts as a substrate for an efflux pump binding site, competes for the antibiotic efflux and recovers the antibiotic efficacy. For example, phenylalanine arginyl β-naphthylamide (PAβN) acts as a competitive inhibitor to restore the activity of macrolides.[32]

3) Steric hindrance. EPI that binds strongly in the cavity of efflux pump, creates a steric hindrance for the transport of antibiotics by the efflux pumps. PAβN has an affinity for the binding pocket in AcrB and MexB efflux pumps. It recovers levofloxacin efficacy against *Pseudomonas aeruginosa*.[11] Bacteria may develop a mutation that prevents the identification and binding of PAβN in the efflux pumps, rendering them useless.

4) Uncoupling. As noted above, efflux pumps depend on a proton or sodium gradient or on an ATP molecule for antibiotic antiport. Collapse of such ion gradients or ATP production could inhibit efflux pump activity. For example, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibits the activity of efflux pumps by dissipating the proton motive force required for ATP production. Problems due to the cytotoxicity of CCCP have prevented its development for clinical use.[18]

5) Membrane permeability. Antibiotic influx could be increased by increasing the OM permeability of Gram negative bacteria. For example, colistin, a mixture of closely related polymyxin molecules, disrupts the cell membrane and increases the permeability of antibiotics. Polymyxin is considered an "antibiotic of last resort" for the treatment of MDR infections due to its cytotoxicity.[13] All of these methods are still under development. *There is no EPI available as a treatment for MDR Gram negative infections*.

1.6 Our Strategy. Our focus is to develop a combination antibiotic using strategies 4 and 5 outlined in section **1.4**. The global use of Augmentin® is a successful example for the clinical use of a combination antibiotic. Augmentin® is a combination of amoxicillin (a β-lactam antibiotic) and potassium clavulanate, a β-lactamase inhibitor.[33] This combination defeats the β-lactamase resistance mechanism and restores the potency of amoxicillin. We hypothesize the use of synthetic amphiphiles as drug delivery agents and efflux pump inhibitor.

Drugs are available that targets bacterial membranes and cell walls. β-Lactams are not optimal for increasing membrane permeability and they suffer from wide-spread resistance from β-lactamases. Polymyxin, which was isolated from *Bacillus polymyxa*, targets lipid A in Gram negative bacteria.[24] Its lipophilic tail could protrude in the hydrophic region of the membrane that induces cell membrane disruption.[24] It has shown synergy with known antibiotics, including carbapenems. However, polymyxins also induce kidney damage that limits its clinical use. Much of antibiotic discovery around membrane disruptors is focused on developing structural analogues of polymyxins that are less toxic.[24] There has not been much success owing to the difficulty in manipulating structures and predicting nephrotoxicity. A more recent antibiotic that targets membrane is daptomycin. Daptomycin is indicated for use against Gram positive bacteria. Daptomycin and polymyxin are both cyclic peptides that could be classified as natural amphiphiles. Resistance from bacteria is another challenge that plagues the development of polymyxin and daptomycin analogues.[14]

We outline a strategy to use synthetic amphiphiles to disrupt bacterial cell membrane that would allow for penetration of antibiotics into the more problematic Gram negative bacteria. The synthetic amphiphiles outlined here could also disrupt cation gradients with the resulting inhibition of efflux pump activity. An increase of antibiotic concentrations in the cell cytoplasm would result in increased efficacy of antibiotics. Research in our lab has focused on the design, synthesis, and activity of such synthetic ion transporters. The synthetic

nature of our molecules would allow for development of numerous structural analogues and a molecule that disrupts cell membrane with minimal cytotoxicity.

1.7 Synthetic amphiphiles and ion channel. Amphiphiles are molecules that consist of both hydrophobic (water-hating) and hydrophilic (water-loving) components. Amphiphiles with a hydrophilic headgroup and a hydrophobic tail are known to self-assemble.[34] Different assemblies could be formed based on the structure and geometry of amphiphiles.[34] For example, bilayers are formed by amphiphiles that consist of one hydrophilic head group and two long hydrophobic chains. Cell membranes are made up of such amphiphiles called phospholipids. Phospholipids typically comprise phosphorylated diacylglycerol.[21]

Cellular membranes separate and insulate cells from their surroundings. Membrane proteins such as receptors, transporters, and ion channel proteins comprise 20%-30% of the human genome.[35] Ion channel proteins maintain ion homeostasis by transporting ions through the membrane's ~30 Å hydrocarbon or insulator regime.[36] Natural channels exhibit features such as voltage gating, rectification, ion selectivity, and open-close behavior.[36] There has been an increasing interest in designing and synthesizing molecules that show many of the properties as amphiphiles and ion channels.[37]

Since the early work by Tabushi *et al.* in the 1980s, there has been a dramatic increase in the number and variety of synthetic ion channels.[38] Synthetic ion channels designed and developed in the Gokel lab mimic some features of the well-known KcSA protein channel.[36,39] These synthetic channels have an entry and exit portal and a hydrated "central relay." They are referred to in our lab as "hydraphiles."

1.8 Hydraphile ion channel structure. Hydraphiles typically consist of *tris*(macrocyclic) structures known as crown ethers. Figure 1.5 shows 18-crown-6 (top). The units within the hydraphiles are diaza-18-crown-6 modules, where diaza represents two nitrogen substitutions that replace oxygen atoms. The number 18 is the total number of atoms in the ring and 6 is the number of heteroatoms (*i.e.*, O or N rather than C). Numerous relatively simple crown ether derivatives are known to transport cations through bulk liquid membranes.[39] In contrast to these, hydraphiles function as channels rather than carrier

transporters. Hydraphiles typically consist of three crown-ethers connected by two alkyl chains (figure 1.5).

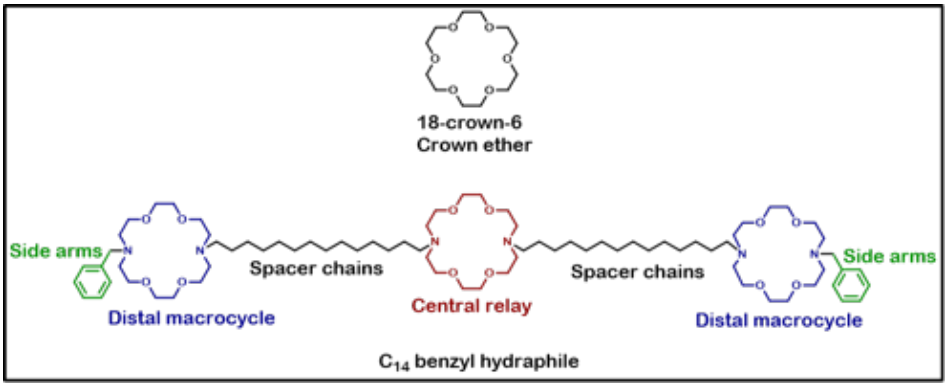

Figure 1.5. Structure of a crown ether molecule and benzyl C_{14} hydraphile.

Hydraphiles have the following essential features.

1) The two distal diaza-18-crown-6 groups function as entry and exit portals. These moieties also serve as amphiphilic head groups.

2) These distal crown ethers are connected to a hydrophobic covalent spacer chain. The length of the compound is dependent on the length of the spacer chains. Each methylene (CH_2) group adds ~1.25 Å to the overall length of a hydraphile.

3) The spacer chains are linked to the central diaza-18-crown-6 macrocycle. This central macrocycle acts as a central relay (polar regime) that stabilizes the cation in transit through the bilayer's hydrophobic regime. This feature is similar to that of the water-filled capsule in the KcSA channel.[36]

4) The distal macrocycles are linked to the side arms that stabilize the pore by interacting with the phospholipid head groups. This is important for the ion conductance ability of hydraphiles. The hydraphile shown in figure 1.5 has benzyl group side arms and hence the named "benzyl hydraphile." The number of carbon atoms in the spacer chains is also included in the nomenclature. For example, the compound illustrated has twin 14-carbon spacer chains and is called "benzyl C_{14} hydraphile."

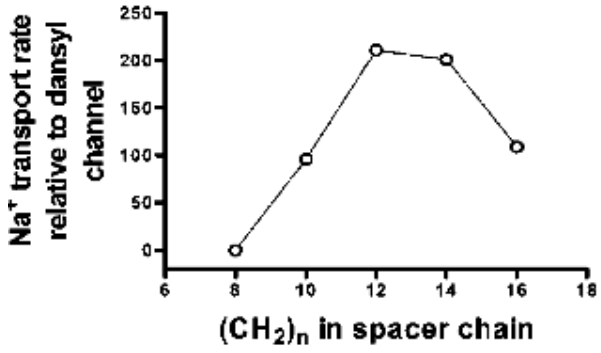

Figure 1.6. Sodium transport by benzyl C_8-C_{16} hydraphiles in liposomes (illustration from reference 25).

1.9 Hydraphile function. Hydraphiles have the following functions. Planar bilayer studies confirm that hydraphiles transport cations (Na^+, K^+, H^+) and show open close behavior similar to that observed with known channel proteins.[40] Successful ion transport studies were also conducted in liposomes[25] and in whole cells (patch clamp with HEK-293 cells).[41] *We rationalized that since hydraphiles transport ions from liposomes and in mammalian cells, they would also transport cations (Na^+ and K^+) from bacteria.* The channels formed by hydraphiles are unimolecular and non-rectifying.[40] Hydraphiles are cation channels that are four-fold selective for Na^+ over K^+. Hydraphiles show length dependent activity, *i.e.,* benzyl C_{12}-C_{16} hydraphiles are better ion transporters than shorter or longer analogs. Fully extended benzyl C_{12}-C_{16} hydraphiles are estimated to span 30-40 Å. This is the approximate thickness of natural lipid bilayers. Benzyl C_8 hydraphile, which is 14 Å shorter than benzyl C_{14} hydraphile, is not long enough to span the bilayer and fails to form channels. Longer hydraphiles are poorer transporters, probably owing to a less organized conductance state (figure **1.6**).[40]

The benzyl C_{14} hydraphile was the most efficient ion transporter tested. It is unknown if the ions are transported through the pore formed by hydraphile or through a disruption caused by the hydraphiles. Computational studies by others suggest the former.[42] Upon addition of membrane thickening agents such as cholesterol to liposomes, it was observed that the spectrum of activity (ion transport) of hydraphiles shifted towards the longer spacer chain lengths.[43] This study showed that changes in membrane composition, such as addition of cholesterol, could change the activity of hydraphiles. *We rationalize that since the membrane compositions of bacteria and mammalian cells vary widely, hydraphiles could have different ion transport and toxic effects in different cell types.*

1.10 Hydraphile biological activity. Amphiphilic hydraphiles localize in the bilayer membrane and mimic some ion-conducting properties of protein channels. A fluorescently tagged (dansyl) hydraphile localizes in the periphery of *Escherichia coli* (figure 1.7).[43]

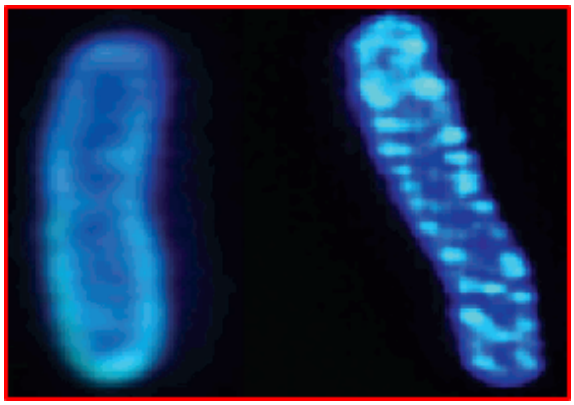

Figure 1.7. Localization of dansyl C_{12} hydraphile in the membrane of *E. coli* cells. Aggregation is observed after 5 minutes (right) (illustration from reference 43).

Sodium and potassium are the most common cations *in vivo*. Altering ion homeostasis of these two cations would have a detrimental effect on the viability of cells. Hydraphiles showed antimicrobial activity against *E. coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* as confirmed with the Kirby Bauer and the Minimal Inhibitory Concentration (MIC) experiments.[43] Hydraphiles had greater antimicrobial activity against Gram positive than Gram negative bacteria. The MICs of benzyl C_{12}, C_{14}, and C_{16} hydraphiles against *E. coli* and *B. subtilis* were lower than those of benzyl C_8, C_{10}, C_{18} and C_{20} hydraphiles.[44] A correlation between the spacer chain length of hydraphile, ion transport and antibiotic efficacy was apparent. If the hydraphile was too short, it failed to transport ion and inhibit bacterial growth. Hydraphiles with spacer chains longer than 16-carbons atoms are inefficient in ion transport and bacterial growth inhibition.[43] As seen in the figure **1.8**, the hydraphiles that showed greatest sodium release from liposomes (C_{12}, C_{14} and C_{16}) also showed the lowest MICs.[45]

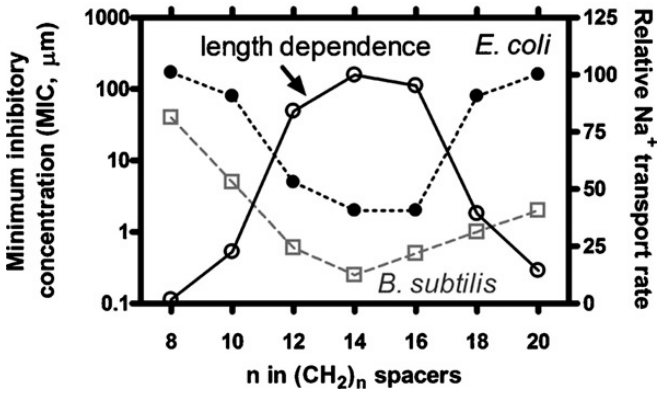

Figure 1.8. Correlation between antibiotic activity and ion transport by hydraphiles. The graph represents sodium transport (open circles) mediated by hydraphiles from liposomes. The filled circles are the MIC of hydraphiles against *E. coli* and the open squares are the MIC against *B. subtilis* (illustration from reference 45).[45]

A structure activity relationship study showed that replacing the -NH with oxygen in the distal macrocycles resulted in the loss of both antimicrobial and ion transport activity.[44] Numerous studies showed that the antimicrobial activity of hydraphiles correlates to their ability to localize in membranes and to transport ions.[43,44,45] At their bactericidal concentrations, the benzyl C_{12} and C_{14} hydraphiles killed half of *E. coli* populations in 8.5 and 9.1 minutes.[44] For comparison, kanamycin kills half the *E. coli* population in 44.8 minutes. This shows that hydraphiles rapidly cause cell death, most likely by the disruption of ion homeostasis.

An important preliminary study and rationale for this research is reported in Chemical Communications **2010**.[46] There, hydraphiles were administered at ½ [MIC] to DH5α *E. coli* cells, along with four different antibiotics. The antibiotics used for this study were kanamycin, tetracycline, erythromycin and rifampicin. These antibiotics are different in structure and have different cellular targets. It was reported there that hydraphiles, at sub-lethal concentrations, could increase the efficacy of four structurally and functionally different antibiotics against *E. coli* (DH5α) and *B. subtilis*.[46] For example, in the presence of 1 μM benzyl C_{16} hydraphile, the MIC of rifampicin decreased from 50 μM to 3 μM (figure 1.9).[5] This exhibited an enhancement of rifampicin activity by 16-fold. Activity of erythromycin was recovered by 8-fold, tetracycline by 4-fold and kanamycin by 2-fold. Similar results were observed with benzyl C_{14} hydraphile and alkyl substituted C_{12} hydraphile.[46] Since enhancement was observed with four

different antibiotics, a more general mechanism of enhancement was hypothesized. *This warrants the investigation of hydraphiles as membrane permeabilizing agents and for drug delivery.*

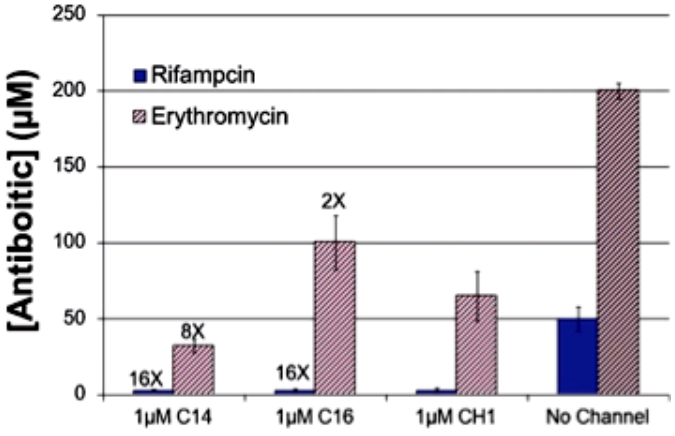

Figure 1.9. Enhancement of erythromycin and rifampicin by C_{12}, C_{14} and C_{16} hydraphiles against DH5α *E. coli* (illustration from reference 46).[46]

We hypothesize that if hydraphiles localize in the membrane of E. coli and span the bilayer, they will increase the membrane permeability of Gram negative bacteria. Altered OM permeability would allow for increased influx of antibiotics into the periplasm and the cytoplasm.

Hydraphiles have been reported to show activity in a variety of cell types. In colon carcinoma cell lines, the benzyl C_{12} hydraphiles induced apoptosis at 80 μM.[47] The benzyl C_{14} hydraphile was shown to induce blebbing in mammalian cells, a property attributed to apoptosis.[47] Another study showed that hydraphiles could be used for direct injection chemotherapy. Hydraphiles induced tumor tissue damage in mice.[48] This damage was localized to the tumor tissue and did not spread to the surrounding tissue.[47] The concentration of hydraphile used for this study was much greater (40X) than its MIC observed against *E. coli* and *B. subtilis*. It is critical for any potential drug application to determine the selectivity of hydraphiles between bacteria and mammalian cells at lower concentrations.

We recently reported that hydraphiles show a chain length dependence on plants. In this study, benzyl C_{14} hydraphile at 20 μM and 50 μM decreases the primary root length and increases the lateral root density of *Arabidopsis thaliana*

roots.[49] Shorter, inefficient benzyl C_8 hydraphile, showed no effect on the root architecture at 50 μM. No effect of hydraphiles was observed on the germination of *A. thaliana* seeds. Auxin transport and transcription repressor mutants were used to rule out their involvement in the auxin pathway. It is hypothesized that hydraphiles alter the potassium ion homeostasis in plant roots, which is known to have a dramatic effect on root development in *A. thaliana*.[49]

Overall, hydraphile ion channels affect multiple different types of cells, likely due to disruption of cation (Na^+ and K^+) gradients. However, a detailed and systematic study of hydraphile's application as an antimicrobial or an antibiotic-adjuvant has not been conducted. In addition, such study is lacking not only with hydraphiles but also with any of the reported synthetic amphiphiles or synthetic ion channels. We explore the use of hydraphiles as membrane permeabilizing agents and as efflux pump inhibitors. This will allow for antibiotic accumulation in the cell cytoplasm and rescue antibiotic activity against both antibiotic-resistant and -sensitive bacteria.

1.11 Rationale and hypothesis. Efflux pumps depend on cation antiport to transport the antibiotics from the periplasm or the cell cytoplasm. *We therefore rationalize that since hydraphiles can transport cations across a variety of membranes, they could also disrupt the sodium or proton gradient required by the efflux pumps for antibiotic antiport*. This would result in the uncoupling of efflux pumps from the energy source and decrease the efflux of antibiotics from the cell cytoplasm. *We hypothesize that if hydraphiles can form channels and transport ions in bacteria, then they would result in uncoupling of efflux pump activity.* Hence, a combination of hydraphiles and antibiotic could be used to rescue the activity of antibiotics against resistant, efflux pump expressing Gram negative bacteria.

The amphiphilic nature of hydraphiles could also disrupt the phospholipids at the site of hydraphile insertion in the membrane. This would allow for an increase in membrane permeability. We rationalize that since hydraphiles could increase antibiotic efficacy against DH5α *E. coli*, they could increase the bacterial cell membrane permeability. *We hypothesize that if hydraphiles localize in the cell membrane of bacteria, then they could increase the cell membrane permeability to antibiotics.* Ion carriers transport ions through membranes by diffusion of the carrier●ion complex through the bilayer. The difference in structures and functions of channels and carriers may result in different effects on membrane permeability, efflux pump inhibition, and rescue

of antimicrobial potency. We will therefore use lariat ethers, a structural analogue of hydraphile, which is known to function as ion carrier, as a control.

Antibiotics can selectively inhibit bacterial cell growth. There are few antibiotics, such as polymyxin, that are also toxic to human cells. It is therefore critical to distinguish between the effect of hydraphiles as membrane permeabilizing agents in bacteria and mammalian cells. The cytotoxicity of hydraphiles to various mammalian epithelial cell lines was also be surveyed. One of the major concerns today in new antibiotic development is the development of resistance. Antibiotic stewardship programs are being implemented in hospitals to limit the use of antibiotics and to prevent resistance development. Bacteria would have to change the cell membrane composition or the membrane biosynthesis pathway in order to develop resistance to amphiphilic compounds. *We therefore hypothesize that if hydraphiles are membrane active molecules, then bacteria would take much longer to develop resistance to hydraphiles than an antibiotic with a specific cytoplasmic target.*

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CHAPTER 2

Hydraphiles Increase the Potency of Tetracycline, Kanamycin, Erythromycin, and Rifampicin Against Antibiotic-Sensitive *Escherichia Coli*, *Pseudomonas Aeruginosa,* and *Bacillus Subtilis*

This chapter has minor changes to the published version:

Patel, M. B.; Garrad, E. C.; Stavri, A.; Gokel, M. R.; Negin, S.; Meisel, J W.; Cusumano, Z.; Gokel, G. W., Hydraphiles enhances antibiotic potency against *Escherichia Coli*, *Pseudomonas Aeruginosa* and *Bacillus Subtilis, Bioorganic and Medicinal Chemistry*, **2016**, *24*, 2864-2870.

Following changes were made to the published version:

1. Figure 2.3 has been updated.
2. The description of figures 2.2, 2.3, 2.4 and 2.5 have been updated to add the number of trials and statistical analysis.
3. The paragraph preceding the figures has number of trials and description of standard deviation.
4. One new paragraph has been added to the introduction.

2.1. 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 or "NCE"). Encouraging recent results have appeared, however, with the reports of teixobactin[5] and aspergillomarasmine A.[6] In addition, such antibiotic peptides[7] as magainin[8] and derivatives along with various cationic amphiphiles[9] 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.

Hydraphiles have been reported to inserted in to the lipid bilayer and formed non-rectifying cation channels. Since hydraphiles insert in the bilayer membranes, we hypothesized that they could increase the membrane permeability. This could allow penetration of antibiotics through the bacterial membranes and increase in their potency. To test this hypothesis, we determined the potency of antibiotics in the presence of hydraphiles.

In a preliminary report,[11] we showed that hydraphiles,[12] when co-administered with various antibiotics, enhanced the potency of drugs against 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/amphiphile/organism combinations. In some cases, potency enhancements were found to be as high as 30-fold.

2.2. Compounds used.

The work reported here used three different hydraphiles.[14] They are illustrated in Figure 2.1 as **1**-**3**. The spacer chains represented in Figure 2.1 by $(CH_2)_n$ 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 C_8 (**1**), benzyl C_{14} (**2**) and benzyl C_{16} (**3**) hydraphiles. All three compounds used have been fully characterized and all are previously known.[15,22]

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.[15] Tetracycline, which is produced in vast quantities annually, binds to the 30S ribosomal subunit, which inhibits peptide synthesis.[16] To the extent that any of the structures are related, it is erythromycin and rifampicin, both of which are macrolides. The ring sizes are different as are the substitution patterns and modes of action. Erythromycin[17] inhibits protein synthesis by binding to the ribosomal 50S subunit. Rifampicin,[18] 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 tuberculosis[19] and erythromycin is prescribed for such conditions as bronchitis and pertussis.[20] What all four antibiotics do have in common is that they all must infiltrate the cell cytoplasm to inhibit bacterial growth.

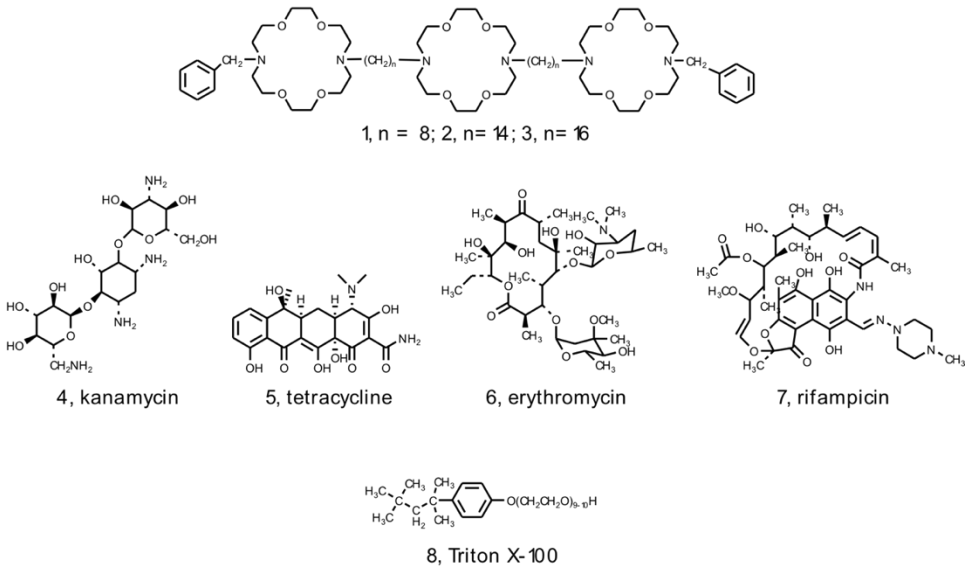

Figure 2.1. Structures of benzyl C_8, C_{14} and C_{16} hydraphile (**1**-**3**), kanamycin (**4**), tetracycline (**5**), erythromycin (**6**), rifampicin (**7**) and Triton X-100 (**8**).

2.3. Bacteria used.

Four different microbial strains were used to study antibacterial activity. All of the synergy studies previously reported with benzyl C_{14} hydraphile and antibiotics were against the DH5α *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 DH5α *E. coli*. The other two bacteria studied were Gram negative *Pseudomonas aeruginosa* and Gram positive *B. subtilis*. *Pseudomonas* is often found in biofilms[21] 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.

2.4. Results and Discussion.

In the nearly half century since the discovery of crown ethers,[22] there have been numerous reports of biological activity. Early studies showed, for example that exposure to 12-crown-4 caused testicular atrophy in mice.[23] More extensive studies showed that the simple crowns 12-crown-4, 15-crown-5, and 18-crown-6 had LD_{50} values (male white rats) of 3200, 1000, and 700 mg/kg, respectively.[24] Antibacterial[25] and antifungal[26] 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.[27] 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*. Indeed, this trend is often observed with antimicrobials.

Our own studies of antimicrobial activity of hydraphiles showed the presence of the amphiphilic pore formers in the *E. coli* boundary layer.[28] A study of activity against *E. coli* and *B. subtilis* revealed a correlation between hydraphile length and toxicity.[13] Hydraphile length also correlates to efficacy of ion transport.[29] Because of the correlation that we observed between ion transport activity and toxicity to *E. coli*, we examined a shorter-chained hydraphile that should be a poor channel-former to test its effect. The results of these studies are described below.

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 hydraphiles 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.

2.4.1 Determination of hydraphile MIC. We determined the MICs for each compound against *E. coli* (DH5α), *E. coli* (K-12), *P. aeruginosa* and *B. subtilis.* Gram negative DH5α *E. coli* was used as a control and to compare the synergy data of benzyl C_8 hydraphile (**1**) to that of the values previously reported for benzyl C_{14} hydraphile (**2**).[34] This is important to be sure that bacterial strains kept in storage have not been compromised. The MIC data are recorded in Table 2.1 for the hydraphiles (**1**-**3**), the antibiotics (**4**-**7**), and Triton X-100 (**8**).

Table 2.1. MICs of hydraphiles and antibiotics against *E. coli* (DH5α and K-12), *P. aeruginosa* and *B. subtilis*.

Benzyl hydraphile used	*E. coli* (DH5α, μM)	*E. coli* (K-12, μM)	*P. aeruginosa* (μM)	*B. subtilis* (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				

The results reported in table 2.1, were accumulated from atleast three replicates. The MIC experiments were conducted using serial dilution concentrations. Hence, the errors in the results could by 2-fold dilution. However, the results were reproduced three times to confirm the reported MIC above.

To the extent a conclusion can be reached from the MIC screening, it is that benzyl C_{14} hydraphile is generally more toxic to Gram positive than to Gram negative strains. Note that the potency difference between benzyl C_{16} hydraphiles against *E. coli DH5a* and *P. aeruginosa* is a single dilution in the MIC analysis, although variation in responses of different strains of bacteria to xenobiotics is common.

2.4.2. Synergy against *E. coli* (DH5α and K-12), *P. aeruginosa* and *B. subtilis*. Three hydraphiles were tested against DH5α *E. coli* in the preliminary report of

this work.[11] Two of those are included here, benzyl C_{14} (**2**) and benzyl C_{16} hydraphiles (**3**), along with the shorter benzyl C_8 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 μM against DH5α *E. coli* and the amount used in the study was arbitrarily set at 64 μM. The results are recorded in Table 2.2. Each entry reflects a minimum of 3 replicates and typically is 5-10.

The results reported in table 2.2, were accumulated from five replicates. The MIC experiments were conducted using serial dilution concentrations. Hence, the errors in the results could by 2-fold dilution. However, the results were reproduced three times to confirm the reported MIC above.

Table 2.2. Enhancement of antimicrobial activity by benzyl C_8, C_{14}, and C_{16} hydraphiles (**1**-**3**) and Triton X-100 (**8**) against *E. coli* (DH5α and K-12), *P. aeruginosa*, and *B. subtilis*.

Hydraphile used	[Hydraphile] (μM)	Antibiotic used	[Antibiotic] (μM)	Fold Increase
		***E. coli* (DH5α)**		
C_8 hydraphile (**1**)	150 (½ [MIC])	Kanamycin	8	3-fold
C_8 hydraphile (**1**)	75 (¼ [MIC])	Kanamycin	12	2-fold
C_8 hydraphile (**1**)	150 (½ [MIC])	Tetracycline	1	5-fold
C_8 hydraphile (**1**)	75 (¼ [MIC])	Tetracycline	2	2-fold
C_8 hydraphile (**1**)	150 (½ [MIC])	Erythromycin	15	13-fold
C_8 hydraphile (**1**)	75 (¼ [MIC])	Erythromycin	25	8-fold
C_8 hydraphile (**1**)	150 (½ [MIC])	Rifampicin	3	18-fold
C_8 hydraphile (**1**)	75 (¼ [MIC])	Rifampicin	3	18-fold
Triton X-100 (**8**)	64	Kanamycin	30	none
Triton X-100 (**8**)	64	Rifampicin	55	none
		***E. coli* (K-12)**		
C_{14} hydraphile (**2**)	1.25 (½ [MIC])	Kanamycin	15	2-fold
C_{14} hydraphile (**2**)	0.6 (¼ [MIC])	Kanamycin	30	none
C_{14} hydraphile (**2**)	1.25 (½ [MIC])	Tetracycline	1	5-fold
C_{14} hydraphile (**2**)	0.6 (¼ [MIC])	Tetracycline	3	2-fold
C_{14} hydraphile (**2**)	1.25 (½ [MIC])	Erythromycin	75	9-fold
C_{14} hydraphile (**2**)	0.6 (¼ [MIC])	Erythromycin	300	2-fold
C_{14} hydraphile (**2**)	1.25 (½ [MIC])	Rifampicin	0.5	30-fold
C_{14} hydraphile (**2**)	0.6 (¼ [MIC])	Rifampicin	4	4-fold
		P. aeruginosa		
C_{14} hydraphile (**2**)	1 (½ [MIC])	Tetracycline	3.13	4-fold
C_{14} hydraphile (**2**)	0.5 (¼ [MIC])	Tetracycline	6.25	2-fold
C_{14} hydraphile (**2**)	1 (½ [MIC])	Erythromycin	25	8-fold
C_{14} hydraphile (**2**)	0.5 (¼ [MIC])	Erythromycin	50	4-fold
C_{14} hydraphile (**2**)	1 (½ [MIC])	Rifampicin	2.35	11-fold
C_{14} hydraphile (**2**)	0.5 (¼ [MIC])	Rifampicin	4.7	5-fold
C_{16} hydraphile (**3**)	1 (½ [MIC])	Erythromycin	25	8-fold
C_{16} hydraphile (**3**)	0.5 (¼ [MIC])	Erythromycin	50	4-fold
		B. subtilis		
C_{14} hydraphile (**2**)	0.12 (¼ [MIC])	Tetracycline	1.56	2-fold
C_{14} hydraphile (**2**)	0.12 (¼ [MIC])	Erythromycin	0.16	2-fold

2.4.3. Comparison of benzyl C_8 hydraphile (1) to benzyl C_{14} 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 C_8 hydraphile did not transport Na^+ ions through liposomal membranes, we expected the antibiotic's MIC value to be indifferent to the presence of benzyl C_8 hydraphile (**1**). Surprisingly, **1** enhanced the potency of kanamycin, tetracycline, erythromycin, and rifampicin against DH5α *E. coli* between 2- and 18-fold, to extents dependent on the antimicrobial that was co-administered.

The bar graph in Figure 2.2 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 ½ [MIC] of **1** or **2** against DH5α *E. coli*. The error bars represent the standard deviation in the MICs determined over five replicates. Results were considered significantly different only if the error bars did not overlap. Both compounds enhanced the potency of each antibiotic to some extent. Benzyl C_8 hydraphile (**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 hydraphiles to interact with (complex with, form clusters with) the antibiotics and act as a carrier to increase their efficacy.

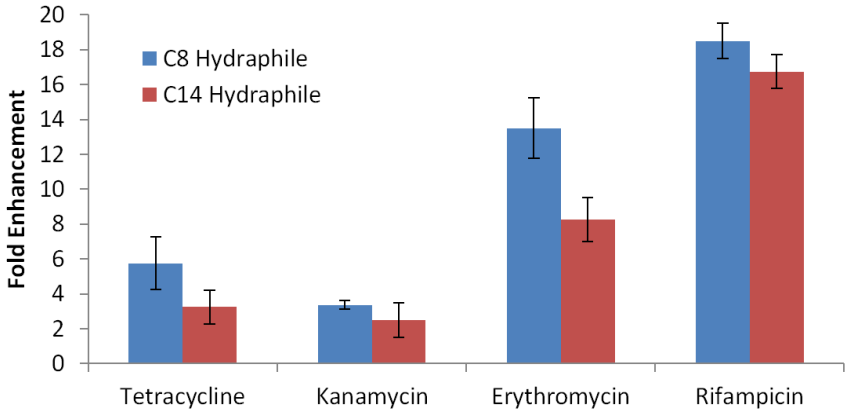

Figure 2.2. Fold enhancements of tetracycline, kanamycin, erythromycin and rifampicin by ½ [MIC] of benzyl C_8 (**1**) and benzyl C_{14} (**2**) hydraphiles against DH5α *E. coli*. The error bars

represent the standard deviation in the MICs determined over five replicates. Data for benzyl C_{14} hydraphile were reported in reference 11.

2.4.4. Comparison of antimicrobial enhancement between K-12 *E. coli* and DH5α *E. coli*. As noted above, our previous work was limited to the non-pathogenic DH5α "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 DH5α strain of *E. coli* with the K-12 strain was of obvious interest. The graph of Figure 2.3 compares the potency enhancements observed for the four antibiotics against the two bacterial strains. The error bars represent the standard deviation in the MICs determined over five replicates. Results were considered significantly different only if the error bars did not overlap. In all cases, benzyl C_{14} hydraphile (**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 DH5α *E. coli*. In K-12 *E. coli*, the MIC of benzyl C_{14} hydraphile was 2.5 μM compared to 2.0 μM against DH5α.

It is interesting to note that the MICs of rifampicin were different against K-12 *E. coli* from DH5α *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.[35]

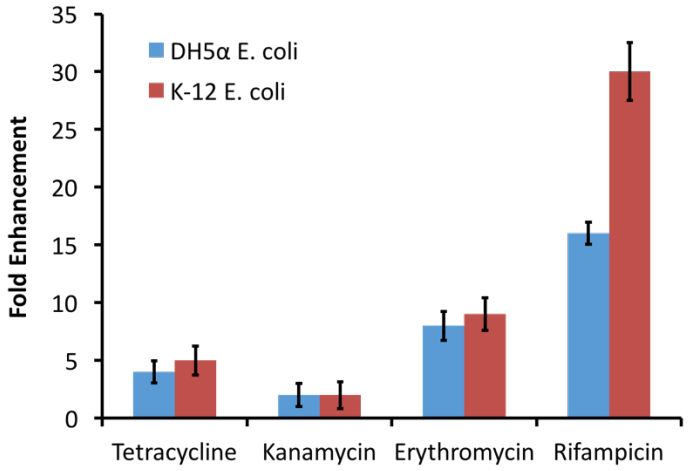

Figure 2.3. Fold enhancement (indicated by numbers atop the columns) of tetracycline, kanamycin, erythromycin and rifampicin mediated by ½ [MIC] of benzyl C_{14} hydraphile (**2**) against DH5α *E.*

coli and K-12 *E. coli*. The error bars represent the standard deviation in the MICs determined over five replicates. Results were considered significantly different only if the error bars did not overlap. Data for C_{14} hydraphile were reported in reference 11.

2.4.5. Growth curve with benzyl C_{14} 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 C_{14} hydraphile (**2**) to determine the effect of the latter on *E. coli* (DH5α). 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 2.4.

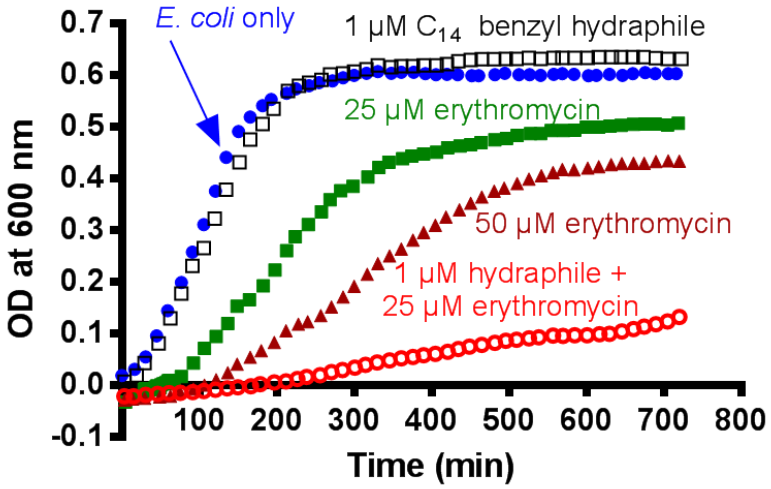

Figure 2.4. Graph showing the effect of benzyl C_{14} hydraphile (**2**) on the potency of erythromycin administered against DH5α *E. coli*. Each point represents the average of three separate determinations. Error bars indicating standard deviations did not overlap, hence have been omitted for clarification.

It is clear from the data graphed in Figure 2.4 that at ½ [MIC] (open squares), benzyl C_{14} 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 (¼ [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 ½ [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 C_8 (**1**) and benzyl C_{14} (**2**) hydraphiles against K-12 *E. coli*. The graph of Figure 2.4 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 2.5 (and Table 2.1) shows that the MIC of rifampicin is 16 μM in the absence of any hydraphile. Each point represents the average of three separate determinations. Error bars indicating standard deviations did not overlap, hence have been omitted for clarification. 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 C_8 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.[36]

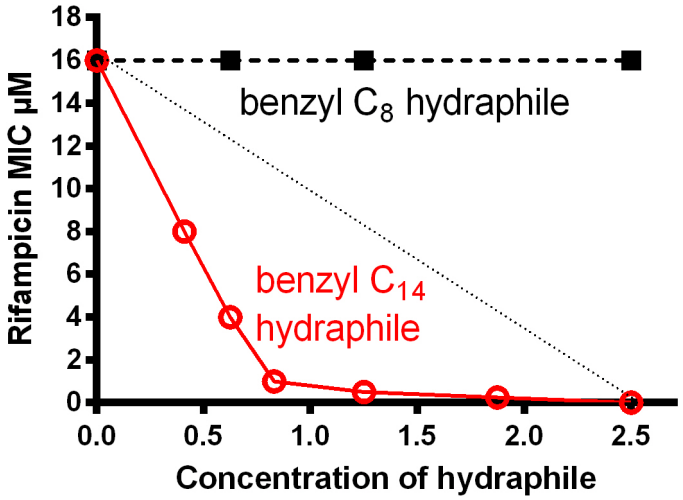

Figure 2.5. Graph showing the effect of added hydraphiles **1** and **2** on the potency of rifampicin against the K-12 strain of *E. coli*. The

dotted line predicts the profile of an additive, rather than a synergistic, effect.

2.4.6. 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 DH5α 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 2.2) was enhanced only marginally by **2** (administered at ¼ [MIC]). In contrast, at the ¼ [MIC] concentration, the potency of erythromycin against DH5α *E. coli* was enhanced 8-fold by benzyl C_8 hydraphile **1**. We note that Triton X-100, administered at concentrations as high as 64 μM showed no effect on DH5α *E. coli* in the presence of either kanamycin or rifampicin.

The most dramatic increase in potency was observed for the combination of **2** at ½ [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 ½ [MIC] with erythromycin or rifampicin, respectively, was also surprising as **1** is a poor ionophore. Of course, the ½-[MIC] concentration of **1** represents a much larger number of molecules than ½ [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 (figure 2.6). Electrospray ionization was used to moderate the experimental conditions. The experiment was conducted in a duplicated, where the same outcome was observed. When excess tetracycline was added to **3**, no hydraphile-antibiotic adduct or cluster was observed. The major ion of **3** alone is the $[\mathbf{3}\bullet Na]^+$ adduct. The only change observed in the spectrum of **3** with the addition of tetracycline hydrochloride was the appearance of the tetracycline ($[\mathbf{5}\bullet H]^+$ and $[\mathbf{5}\bullet DMSO\bullet H]^+$) peaks as well as various adducts of **3** with protons, sodium cations, and chloride anions.

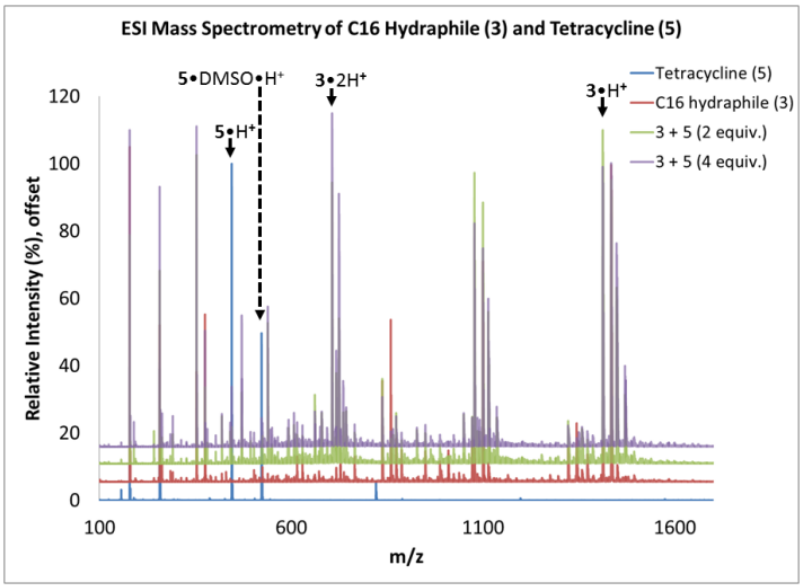

Figure 2.6. Electrospray mass spectra of the combination of benzyl C_{14} hydraphile (**3**) alone, tetracycline (**5**) alone and **3** and **5** in combination in **3**:**5** ratios of 2 and 4.

A caveat in the consideration of mechanism is that the enhancements of potency apparent with ½ or ¼ [MIC] of **1** involve larger amounts of compound than needed either for **2** or **3**. For example, at ¼ [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.

2.5. 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 C_8 (**1**) and C_{14} (**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.[37] A second postulate is that the peptides infiltrate the membrane according to the carpet model,[38] 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.[39] Notwithstanding, a very recent report links polymyxin permeability to a membrane receptor.[4041] 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.

2.6. Experimental section.

2.6.1. Hydraphiles. Benzyl hydraphiles having spacer chains of 8- 14- and 16-methylene groups have been prepared and reported previously.[14] Benzyl C_8, C_{14} and C_{16} 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.

2.6.2. 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 H_2O. 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.

2.6.3. Minimal inhibitory concentration (MIC). The MIC and co-administration studies were performed according to the procedure outlined in Clinical and Laboratory Standards Institute.[30] 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 H_2O. 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.

2.6.4. 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 ½ or ¼ 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 dH_20) 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.

2.6.5. Growth curve. Growth curves were performed with benzyl C_{14} 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.

2.6.6. 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 C_{16} hydraphile **3** (in DMSO, 0.25-0.5 mM final concentration) was added to tetracycline **5** (hydrochloride salt in 18.2 MΩ H_2O, 1 mM final concentration). Controls were performed with hydraphile **3** and antibiotic **5** alone. In each case the final DMSO:H_2O ratio was 9:1 by volume.

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CHAPTER 3

Hydraphile Synthetic Ion Channels Rescue Antimicrobial Potency Against Efflux Pump Expressing Antibiotic Resistant Gram Negative Bacteria

The data reported in this chapter has not been published yet but is covered under two patent applications:

PCT Patent Application Number PCT/US2015/034550
Provisional Application Number 15/186,070 (CIP US2015/034550)

3.1. Introduction.

Antibiotic resistance has become a world-wide crisis.[1] With the emergence of multi-drug resistant (MDR) bacteria, we have entered the post-antibiotic era.[1] With only one antibiotic approved in 2015, the rate of antibiotic discovery is at an all-time low.[2] A patient infected with MDR bacteria has few treatment options including surgery and amputations. In some cases, due to lack of an effective antibiotic treatment, the infection proves to be fatal. In 2013, in U.S. alone, there were 2 million hospital acquired infections. These infections proved to be fatal for 23,000 patients.[3]

Two fatal diseases caused by MDR bacteria are pneumonia and urinary tract infections (UTI). In about 1 million people that are hospitalized with pneumonia, 50,000 people die.[4] In addition, hospital associated pneumonia (HAP) and ventilator associated pneumonia (VAP) are on the rise.[5] UTI affects 150 million people per year around the world and 10.5 million people per year in U.S.A.[6] Increased mortality is associated with catheter related UTIs. The primary causative agents for UTI and pneumonia are *Escherichia coli* and *Klebsiella pneumoniae*.[7] Both of these are Gram negative bacteria and their treatment has become increasingly difficult owing to antibiotic resistance.

Antibiotics commonly used for the treatment of UTI and pneumonia are ciprofloxacin (fluoroquinolones), third and fourth generation cephalosporins and carbapenems.[8] Treatment with these antibiotics has become difficult due to the acquisition of extended spectrum β-lactamase (ESBLs) enzymes and efflux pumps.[9] Plasmids encoding ESBLs and efflux pumps are easily acquired by the bacteria, which allows for a rapid spread of antibiotic resistance.[10] In addition to the resistance mechanisms, Gram negative bacteria have an outer membrane (OM). This OM acts as a barrier for the antibiotic's entry in to the cell. The lipopolysaccharide (LPS) in the OM is made up of a lipidA structure, core polysaccharides, and O-antigen repeats.[11] This not only prevents the passage of antibiotics, but also acts as an antigen. The majority of virulent ESKAPE pathogens[12] are Gram negative pathogens.[7] Polymyxin targets lipidA of LPS and disrupts the OM.[13] It is associated with renal toxicity, hence it is used as a drug of last resort.[13] Recently, an *Escherichia coli* strain was found in the U.S., carrying a polymyxin resistance gene, *mcr-1*.[14] There are no other antibiotics available that could target the membrane of Gram negative bacteria.

Due to increasing resistance, research and clinical use of combination therapy has increased dramatically. The only antibiotic approved in 2015 was

Avycaz, a combination of avibactam (a β-lactamase inhibitor) and a ceftazidime (a third generation cephalosporin).[15] It is one of the very few combination drugs that is indicated for use against Gram negative infections such as *K. pneumoniae*. Augmentin, the first combination antibiotic, is another successful example of this approach. Combination antibiotics provide the ability to use the existing arsenal of antibiotics for the treatment of MDR infections. However, bacterial enzymes (NDM-1) that can evade the effect of all known β-lactamase inhibitors have been reported.[3,15] We therefore investigated the use of hydraphile synthetic ion channels as adjuvants to rescue the efficacy of existing antibiotics. In particular, the ability of hydraphiles to rescue antibiotic potency against efflux pump expressing, MDR Gram negative bacteria was explored.

Crown ethers of different sizes have been known to form stable and selective complexes with metal and organic cations.[16,17,18] Numerous structural variations of these macrocyclic compounds have been shown to transport ions through bilayer membranes, either as carriers or as channels.[19] Most of the reported studies focused on developing new crown-ether based molecules that could mimic natural ion transport proteins such as valinomycin and gramicidin.[20] Valinomycin is a K^+ carrier[21] whereas gramicidin forms a dimerized cation selective protein channel in the membrane.[21] Biological activity for some of the macrocyclic compounds has also been reported.[22] These studies focus on their application either as an antimicrobial[23] or chemotherapeutic drug.[24] The antimicrobial studies were limiting in the fact that they typically could only conclude that the crown ether based molecules were more active against Gram positive rather than Gram negative bacteria. There was no mechanism determined or further studies reported.

We have developed and extensively studied a family of crown ether based synthetic ion channels that we have called hydraphiles (figure 3.1).[25] Hydraphiles typically consist of three diaza-18-crown-6 residues connected by alkyl spacer chains of appropriate length.[27] Numerous studies have confirmed that the two distal macrocyclic crown ethers of hydraphiles align in the mid-polar region of the bilayer membrane and the central ring is in the region of lowest polarity of the membrane (the midplane). The central macrocycle (central relay) acts as the energy lowering portal for the transfer of cations through the hydrophobic region.[27] We have confirmed that hydraphiles having appropriate spacer chain lengths csan form a channel that conducts cations, specifically Na^+ and K^+ ions.[26] They form non-rectifying channels, hence transporting ions based on cation

gradients. The ability of hydraphiles to transport cations depends on the concentration, length and composition of the bilayer membrane.[27]

Hydraphiles have been known to show antimicrobial potency against *Escherichia coli, Bacillus subtilis* and *Saccharomyces cerevisiae*.[28] Hydraphiles with spacer chain lengths of $[(CH_2)_n]$ = 12, 14 or 16 are excellent ion transporters and have antimicrobial properties.[29] However, when the spacer chain length was as short as octylene (n = 8), they were not efficient ion transporters or bactericidal. Hence, the toxicity of hydraphiles to bacteria has been related to their chain length and ion transport capability. It was therefore hypothesized that hydraphiles channels disrupted cation homeostasis in bacteria, causing cell death. Studies have shown that the hydraphiles also induce apoptosis in cancerous cells[30] and increase lateral root density of *Arabidopsis thaliana* plant[31].

We recently reported that C_{14} benzyl hydraphile, C_{16} benzyl hydraphile and alkyl substituted C_{12} hydraphiles could rescue the activity of rifampicin, erythromycin, kanamycin, and tetracycline against both DH5α and K-12 *E. coli*.[32] The greatest recovery was observed with rifampicin (16-fold) and erythromycin (8-fold). Rifampicin[33] and erythromycin[34] are both hydrophobic antibiotics that cannot effectively penetrate through the outer membrane (OM) of Gram negative infections. We also reported that the activity of these antibiotics was enhanced against *Pseudomonas aeruginosa* and *B. subtilis*.[35] When C_8 benzyl hydraphile was used as a control, we observed that rifampicin activity was not recovered at concentrations similar to that of C_{14} benzyl hydraphile, *i.e.* 2.5 μM. However, at 150 μM of C_8 hydraphile (1/2 [MIC]), the activity of rifampicin was enhanced by 18-fold. Again, a length dependent effect of hydraphiles was apparent. At sub-lethal concentration of Benzyl C14 hydraphile, growth of DH5α *E. coli* was similar to *E. coli* alone control. There was no interaction observed in mass spectrometry studies between hydraphiles and tetracycline. We therefore hypothesized that hydraphiles increase permeability of Gram negative bacteria, allowing for antibiotic diffusion into the bacterial cytoplasm.

So far no study has been reported about hydraphile's activity against multi-drug resistant bacterial strains. Specifically, no studies have investigated the ability of hydraphile or synthetic ion transporters as an antibiotic adjuvant or an efflux pump inhibitor. Bacterial efflux pumps provide a resistance mechanism that affects multiple classes of antibiotics.[36] Acquisition of efflux pump-based resistance usually leads to the acquisition of other types of resistance mechanisms (*e.g.* target mutation and antibiotic-degrading enzymes). Such

mechanisms contribute independently to resistance development.[37] All efflux pumps utilize either a cation gradient (proton or sodium) or hydrolysis of an ATP molecule for active antibiotic efflux.[38,39] Antimicrobial resistance in Gram negative bacteria is a combination of second membrane diminished influx and increased efflux of antibiotics.[40] Numerous approaches have been reported for increasing antibiotic concentration in the cell cytoplasm of efflux pump expressing bacteria.[41] For example, phenylalanine arginyl β-naphthylamide (PAβN) recovers levofloxacin efficacy against *P. aeruginosa*.[41] Here we report a novel approach that recovers antimicrobial potency against efflux pump expressing Gram negative bacteria.

We hypothesized that if hydraphiles increase the membrane permeability and inhibit efflux pump activity, then the activity of antibiotics could be recovered by hydraphiles against efflux pump expressing resistant Gram negative bacteria. Here, we report that at sub-lethal concentrations, hydraphile synthetic ion channels recover the activity of tetracycline and ciprofloxacin against resistant *E. coli* and *K. pneumoniae*. Both strains were confirmed to have efflux pumps specific to tetracycline. Ciprofloxacin resistance occurred from both efflux pump and target site mutation (DNA Gyrase A). A synergy between hydraphiles and antibiotics was confirmed. Growth curve studies showed that ½ [MIC] of Benzyl C14 hydraphile extended the lag phase but did not inhibit the growth of *E. coli*. These results were similar to that of colistin (polymyxin E) but different than the common detergent triton X-100. Hydraphile analogue lariat ethers, which were thought to be ion carriers, also recovered tetracycline activity by 16-fold. A chain length dependent effect of hydraphiles on antibiotic recovery was observed.

3.2 **Compounds used**.

This study included four hydraphiles that varied in the spacer chain lengths: benzyl C_8-C_{14} hydraphiles (compounds **1 – 4**). The benzyl C_{12} and C_{14} hydraphiles are known to span the membrane and efficiently transport cations.[33] Benzyl C_8 and C_{10} hydraphile are not optimal to span the membrane and inefficient at ion transport as observed by liposomal release experiments.[33] Hence, benzyl C_8 and C_{10} hydraphile are used as controls for this study.

Lariat ethers are synthetic ionophores that act as carriers. They have a central 18-crown-6 macrocycle connected to two alkyl chains (figure 3.1). Compared to the hydraphile structure, lariat ethers lack the two distal

macrocycles present in hydraphiles. We therefore used C_8-C_{16} lariat ethers (**5 - 9**), expecting them to be ion carriers that were structurally similar to the hydraphiles. However, very recent results show that lariat ether may also act as pore formers.[42]

The rescue of tetracycline (**10**) efficacy was examined in the presence of hydraphiles. Tetracycline binds to the 30S subunit of the ribosomes and inhibits peptide elongation.[43] Tetracycline has a broad spectrum of activity against both Gram positive and –negative bacteria. Due to increasing resistance, their use in humans has been limited.[48] Regardless, their use in the animal feed market has been increasing. This further contributes to the resistance development by bacteria to tetracyclines. Efflux pumps are the primary reason for resistance development to tetracycline.[48] Successful recovery of tetracycline could bring this effective antibiotic back to the clinic.

Another antibiotic included in this study was ciprofloxacin (**11**). Ciprofloxacin targets the DNA gyrase A that belongs to the fluoroquinolone class of antibiotics.[44] The activity of ciprofloxacin was determined against *K. pneumoniae*. Clinical use of ciprofloxacin has decreased due to the mutations in DNA gyrase and the resistance-nodulation division (RND) type efflux pumps.[49] The OM of *K. pneumoniae* also decreases the permeability of ciprofloxacin. The efficacy of ciprofloxacin in the presence of hydraphiles and control was determined.

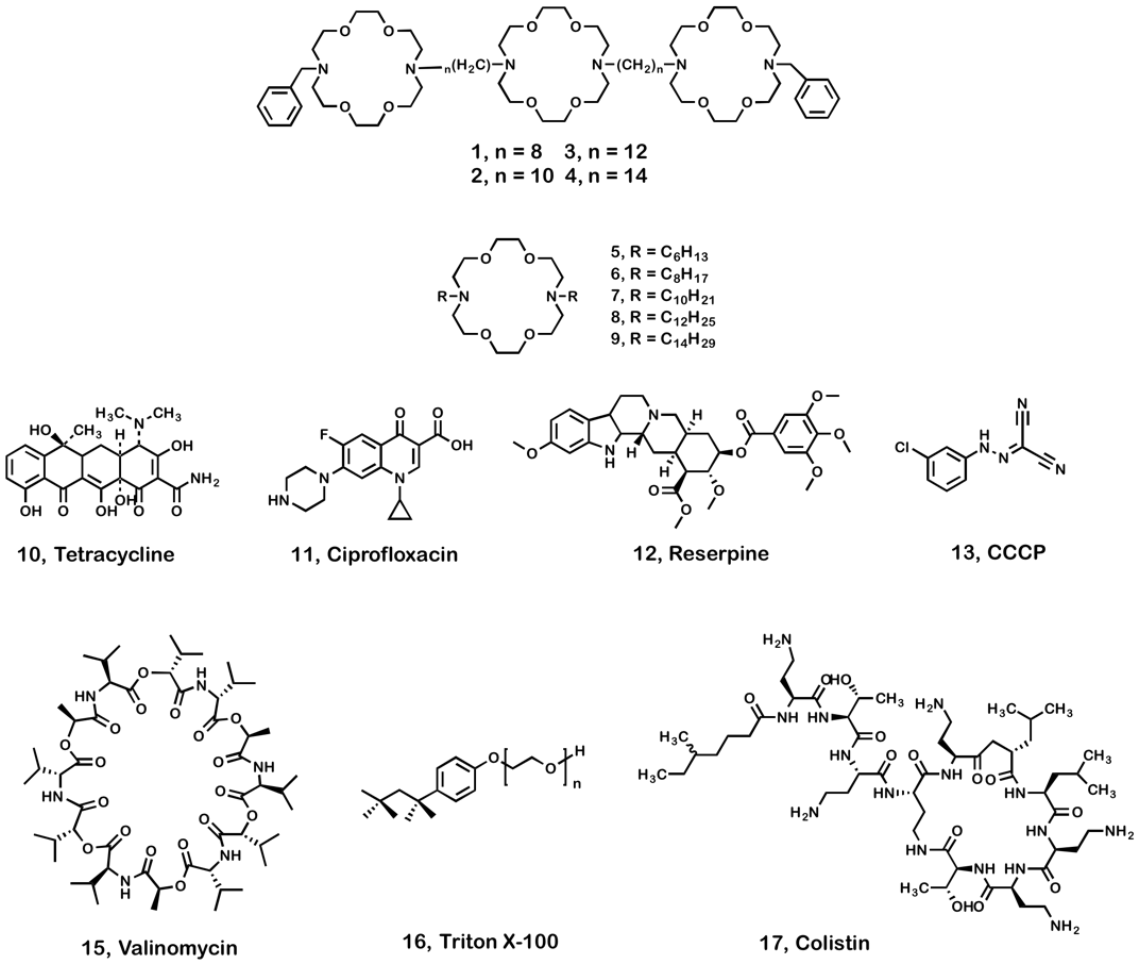


Figure 3.1. Structures of compounds used for the study. C_8-C_{14} hydraphiles (**1**-**4**), lariat ethers (**5**-**9**), tetracycline (**10**), ciprofloxacin (**11**), reserpine (**12**), CCCP (**13**), valinomycin (**15**), triton X-100 (**16**) and colistin (**17**).

Known efflux pump inhibitors[45] reserpine (**12**) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP, **13**) were used as controls. Reserpine blocks the efflux pumps preventing substrate transport where as CCCP dissipates proton motive force in bacteria resulting in uncoupling of efflux pumps from their energy source. Protein ionophores such as gramicidin-D (**14**) and valinomycin (**15**) were used as controls. Triton X-100 (**16**) was used as control for its known detergent like effect. Colistin (**17**), a Gram negative membrane disruptor was also used a control.

3.3. Results and Discussion.

3.3.1. Minimal Inhibitory Concentrations (MICs). Our aim was to determine if the activity of antibiotics could be rescued against Gram negative bacteria in the presence of synthetic amphiphiles. For this purpose, we first determined the MICs on all the compounds mentioned above against *K. pneumoniae* (ATCC BAA 2146) and *E. coli* (tetracycline resistant). Both the strains used have efflux pumps.

3.3.1.1. Bacteria used.

Tet^R *E. coli*. Tet^R *E. coli* was made by transforming competent JM109 *E. coli* with pBR322 plasmid. This plasmid consists of the tetA [class C] gene[46] that expresses the tetA efflux pump. The tetA efflux pump localizes in the cytoplasmic membrane (CM).[51] It provides tetracycline resistance by transporting tetracycline from the cell cytoplasm into the periplasmic space. It utilizes the bacterial proton gradient that exists across CM, to efflux tetracycline.[47] This plasmid also expresses the β-lactamase gene. The MIC of tetracycline against Tet^R *E. coli* was 900 μM and that of ampicillin was > 1000 μM. The MIC of tetracycline against non-resistant K-12 *E. coli* was 6 μM. This confirms that the pBR322 plasmid is expressed and the Tet^R *E. coli* is resistant to tetracycline and ampicillin. The tetA efflux pump is highly specific and efficient at exporting tetracycline from the cell cytoplasm. It does not even recognize the tetracycline analogue-minocycline as its substrate. The MIC of minocycline against Tet^R *E. coli* was 15 μM, which is similar to the sensitive K-12 *E. coli*.

Bacteria isolated from patients usually possess multiple different types of efflux pumps and other resistance mechanisms targeted towards the same antibiotic. It is therefore difficult to study the effect of hydraphiles on one efflux pump in patient isolated strains. The Tet^R *E. coli* strain provides a control strain with one efflux pump targeting tetracycline, which is used to test the effect of hydraphiles.

***K. pneumoniae*.** The *K. pneumoniae* strain used in these studies was acquired from ATCC (ATCC BAA 2146). This bacterium was isolated from a patient with UTI and is resistant to more than 30 different antibiotics.[48] It expressed the tetA (MFS type), ABC and RND type efflux pumps. In addition, it also has binding site mutations and New Delhi β Metallo-lactamase (NDM-1) enzyme that provides resistance to multiple different classes of antibiotics.[53] This *K. pneumoniae* is a robust strain as it has already developed resistance to range of antibiotics and was isolated from a patient. Infections caused by *K. pneumoniae*

are difficult to treat with existing antibiotics, even with adjuvants (β-lactamase inhibitors). No NDM-1 inhibitors have yet been developed. The MIC of ciprofloxacin was 700 μM in this strain. The resistance to ciprofloxacin can be attributed to a combination of efflux pumps and mutations in DNA gyrase A. The MIC of tetracycline was 1000 μM, which is a result of tetA efflux pump. Development of a new antibiotic therapy against *K. pneumoniae* is of clinical significance against this and related strains. The results of MIC are shown in Table 3.1 below. The MICs reported in table 3.1 are the average of 5 trials. The standard deviation was calculated and represented as '±' the average MIC.

Table 3.1: Minimal Inhibitory Concentration (MIC) in μM

Compounds used	***K. pneumoniae***	***E. coli* (Tet^R)**
C_8 hydraphile (**1**)	200 ± 10	250 ± 10
C_{10} hydraphile (**2**)	56 ± 5	35 ± 3
C_{12} hydraphile (**3**)	35 ± 3	5 ± 1
C_{14} hydraphile (**4**)	10 ± 2	2 ± 1
Tetracycline (**10**)	1000 ± 100	900 ± 100
Ciprofloxacin (**11**)	700 ± 100	0.5 ± 0.25
Colistin (**17**)	0.5 ± 0.25	0.25 ± 0.25
CCCP (**13**)	N.D.	56 ± 5
Reserpine (**12**)	N.D.	> 128
N.D. = not determined		

We first determined the MIC of benzyl C_8-C_{14} hydraphiles (**1**-**4**) against the both Gram negative bacteria. The MIC of Benzyl C14 hydraphile against Tet^R *E. coli* was 2 μM and against *K. pneumoniae* it was 10 μM. For comparison, the MIC of tetracycline against *E. coli* was 6 μM. Benzyl C_{12} hydraphile and C_{14} hydraphile were equally active. However, C_{10} and C_8 hydraphiles (compounds **3** and **4**) were less active. This trend follows the ion transport and membrane spanning ability of hydraphiles: C_{14} hydraphile ≥ C_{12} hydraphile > C_{10} hydraphile > C_8 hydraphile.

Colistin, a membrane disruptor, was more active than Benzyl C14 hydraphile. Colistin is also associated with renal toxicity. The MIC of colistin against *E. coli* was 0.25 μM and against *K. pneumoniae* it was 0.5 μM. The efflux pump inhibitors CCCP (**12**) and reserpine (**13**) are not efficient antimicrobials as

both of their MICs were > 56 μM. CCCP is known to be more active against Gram positive bacteria than Gram negative bacteria. Compared to the Tet^R *E. coli, K. pneumoniae* was less susceptible to the hydraphiles and antibiotics. The differences might be attributed to the differences in the cell membrane compositions among the two strains. *K. pneumoniae* also has a greater number of resistance mechanisms and genes that decrease the antibiotic efficacy.

3.3.2. Combination studies. Through the MIC screening, we determined the antimicrobial properties of each compound. Antibiotic (tetracycline and ciprofloxacin) MICs were greater than those of hydraphiles. If hydraphiles rescue antibiotic potency, then the MIC of antibiotics would decrease in the presence of sub-lethal (< MIC) concentrations of hydraphiles. We therefore determined the MIC of antibiotics, in the presence of different fractional MIC concentrations of hydraphiles.

Tet^R *E. coli*. Tet^R *E. coli* expresses the tetA efflux pump. The tetA efflux pump belongs to the MFS class of efflux pumps.[51] It localizes in the inner membrane of the *E. coli*. TetA efflux pumps selectively transport tetracycline molecules in exchange for a proton. The tetA efflux pump is highly selective. Hydraphiles and tetracycline are structurally and functionally distinct molecules. It is unlikely that the hydraphiles could be a substrate of the tetA efflux pump. The hydraphiles used for this study were: benzyl C_8-C_{14} hydraphiles. If hydraphile disrupted the cell membrane and inhibited the efflux pump activity, the activity of tetracycline would be recovered. To prevent any toxic effect from hydraphiles, we determined the MIC of tetracycline in the presence of either 1/2 or 1/4 the [MIC] of hydraphiles. The results of three replicates are shown below in table 3.2. The MICs reported in table 3.2 are the average of 3 trials. The standard deviation was calculated and represented as '±' the average MIC. A decrease in the MIC was considered significant only in the error in the data did not overlap. The fold enhancement was determined using the average MICs shown in column 3, hence no errors are shown. However, enhancement greater 2-fold were only considered significant.

Table 3.2: Recovery of tetracycline activity against Tet^R *E. coli* by hydraphiles

Amphiphile used	[Amphiphile] μM	[Antibiotic] μM	Fold enhancement	FIC index[a]
No amphiphile	-	900 ± 100	n/a	n/a
C_8 hydraphile	1	600 ± 100	1.5-fold	0.73
C_8 hydraphile	62.5 (¼[MIC])	82 ± 15	11-fold	0.34
C_8 hydraphile	125 (½[MIC])	30 ± 8	30-fold	0.53
C_{10} hydraphile	1	600 ± 100	1.5-fold	0.69
C_{10} hydraphile	8.75 (¼[MIC])	200 ± 20	5-fold	0.69
C_{10} hydraphile	17.5 (½[MIC])	40 ± 5	23-fold	0.54
C_{12} hydraphile	1	300 ± 75	3-fold	0.53
C_{12} hydraphile	1.25 (¼[MIC])	400 ± 50	2-fold	0.69
C_{12} hydraphile	2.5 (½[MIC])	55 ± 5	16-fold	0.56
C_{14} hydraphile	0.5 (¼[MIC])	360 ± 40	3-fold	0.65
C_{14} hydraphile	1 (½[MIC])	220 ± 25	4-fold	0.74
C_{14} hydraphile	1 (½[MIC])	> 1000 (Ampicillin)	0-fold	n/a
a, see below for a discussion of the FIC index				

As seen in table 3.2 above, the activity of tetracycline was recovered in the presence of benzyl C_8-C_{14} hydraphiles against Tet^R *E. coli*. In the presence of 1 μM benzyl C_{14} hydraphile, the MIC of tetracycline decreased from 900 ± 100 μM to 250 ± 25 μM. This documents the recovery of tetracycline activity by 4-fold. Here, the fold-enhancement was calculated by dividing the MIC of antibiotic alone, by the MIC of antibiotic determined in the presence of hydraphiles. In the presence of ½ [MIC] of benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles tetracycline activity was recovered by 30, 23, 16 and 4-fold, respectively. At ¼ [MIC] of benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles tetracycline activity was recovered by 11, 5, 2 and 3-fold, respectively. In the presence of 125 μM benzyl C_8 hydraphile, the tetracycline MIC was as low as 30 μM. It may appear that benzyl C_8 hydraphile is the most efficient hydraphile at recovering the tetracycline activity. However, it is important to note that the MIC of benzyl C_8 hydraphile is much greater than benzyl C_{14} hydraphile. So a large amount of compound was required at the same fraction of MIC (one-half).

To compare the efficacy of hydraphiles with each other, an experiment was performed at the lowest ½ [MIC] value of all the hydraphiles used, *i.e.* 1 μM.

At 1 μM, benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles recovered tetracycline activity by 1.5, 1.5, 3 and 4-fold, respectively. From this experiment it was revealed that C_{14} hydraphile, the most potent ion transporter, was also the most efficient at recovering tetracycline activity against Tet^R *E. coli*.

There was no recovery of ampicillin activity in the presence of benzyl C_{14} hydraphiles. Ampicillin resistance in the Tet^R *E. coli* was a result of β-lactamase enzymes. The recovery of antibiotic activity observed in the presence of hydraphiles appears to be limited to efflux pump inhibition. However, the disruption of ion gradients by hydraphiles could affect the secretion or biosynthesis of β-lactamase enzymes. The failure of ampicillin recovery by the benzyl C_{14} hydraphiles, the most potent ion transporter in this series, shows that the inhibition of any pathway involving β-lactamase is unlikely.

The fractional inhibitory concentration (FIC) index[49] was calculated for each combination tested and the values are reported in table 3.2. The FIC index is a theoretical means of determining synergy, additive, or antagonist effects between two compounds. It is calculated by adding the fractional MICs of the two compounds used. A FIC index of 0.5 or lower is conservatively defined as synergism. However, a FIC index of 1 or lower is also considered a more general definition of synergy. In a broader sense, all the hydraphiles showed some synergy with tetracycline. Most of the hydraphiles tested had FIC index of approximately 0.5. The FIC data show that only benzyl C_8 hydraphile at ¼ [MIC] (11-fold recovery) fits the narrow definition of synergy. FIC > 1 is considered antagonism, which was not observed with any hydraphile tested.

3.3.3. Growth curve. The FIC index discussed above showed that hydraphiles may only show moderate synergy with tetracycline. We wanted to determine if sub-lethal concentrations of hydraphiles affected the growth of Tet^R *E. coli*. We therefore performed growth curve studies shown in figure 3.2. The compounds used for growth curve study were C_{14} hydraphile and tetracycline against the Tet^R *E. coli*. The Tet^R *E. coli* was treated with either 1 μM C_{14} hydraphile (1/2 [MIC]), 2 μM C_{14} hydraphile (MIC), 220 μM tetracycline (1/4 [MIC]), 900 μM tetracycline (MIC) and the combination of 1 μM C_{14} hydraphile + 220 μM tetracycline. Untreated Tet^R *E. coli* and Tet^R *E. coli* treated with 0.5% (v/v) DMSO were used as controls. The growth of Tet^R *E. coli* was monitored every 30 minutes by measuring the optical density (O.D.) of the cultures at λ = 600 nm.

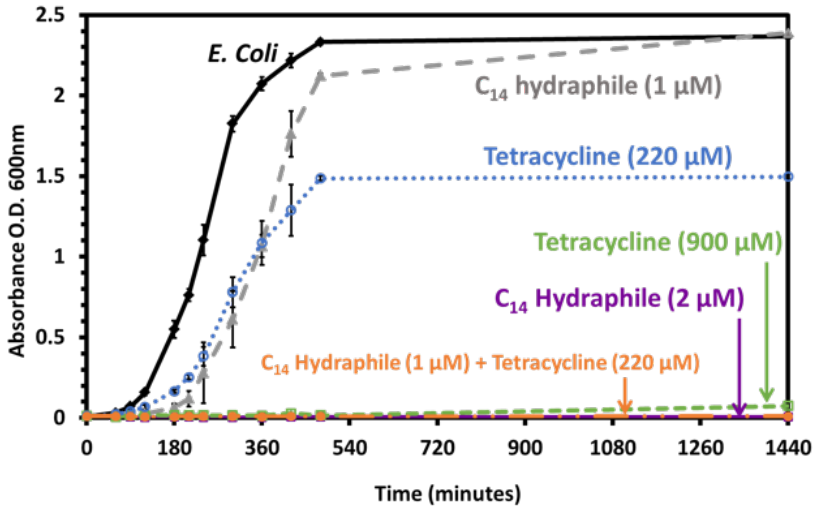


Figure 3.2. Growth curve of Tet^R *E. coli* in the presence of C_{14} hydraphiles and tetracycline. Growth of Tet^R *E. coli* was monitored in the presence of 1 µM and 2 µM C_{14} hydraphile, 220 µM and 900 µM tetracycline and the combination of 1 µM C_{14} hydraphile + 220 µM tetracycline. Error bars represent the standard deviation in 3 replicates. Results were considered significant if the error bars did not overlap.

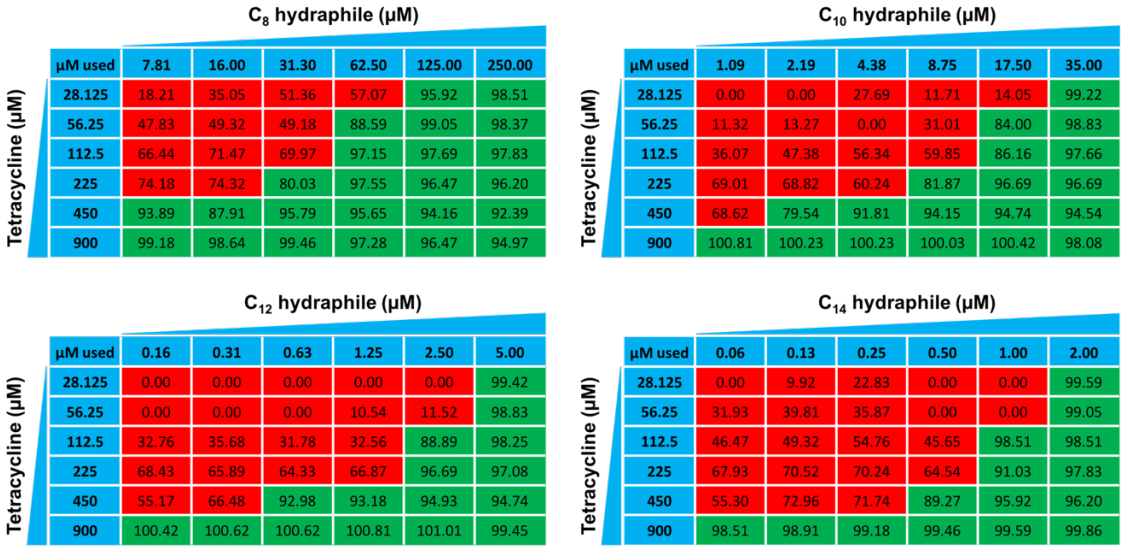
As seen in figure 3.2, the Tet^R *E. coli* has a lag phase (~2 hours), exponential growth phase and a stationary phase. When Tet^R *E. coli* is treated with benzyl C_{14} hydraphile at 2 µM (MIC; purple line), the growth of Tet^R *E. coli* is completely inhibited. However, when the Tet^R *E. coli* is treated with ½ [MIC] of benzyl C_{14} hydraphile (1 µM), the lag phase is extended to ~3 hours. After 3 hours, the growth of Tet^R *E. coli* treated with ½ [MIC] hydraphile recovers and parallels that of untreated Tet^R *E. coli*. During the exponential phase, the growth rate of *E. coli* alone was calculated at 0.692/hr, which is within experimental error of 0.658/hr in the presence of 1 µM benzyl C_{14} hydraphile. The lag phase could have been extended because of the death in the Tet^R *E. coli* population caused by half-[MIC] benzyl C_{14} hydraphile. It is also likely that the Tet^R *E. coli* took longer to prepare for the exponential phase due to osmotic stress caused by non-rectifying hydraphile channels. Similar growth rates during the exponential growth phase and complete recovery of Tet^R *E. coli* growth support the osmotic stress hypothesis.

In the presence of tetracycline at 220 µM, Tet^R *E. coli* growth was inhibited. This was not surprising because it has been previously reported that *Salmonella*

typhimurium expressing the tetA efflux pump had increasing inhibition of growth with increasing concentration of tetracycline.[50] In the presence of 900 μM tetracycline, Tet^R *E. coli* growth was completely inhibited. However, when the combination of ½ [MIC] of benzyl C_{14} hydraphile and ¼ [MIC] of tetracycline was tested, completely inhibition of Tet^R *E. coli* growth was observed. This proves a synergistic activity between benzyl C_{14} hydraphile and tetracycline, regardless of the FIC index > 0.5. Two key observations are made here. First that the half-[MIC] of benzyl C_{14} hydraphile increases lag phase by 1 hour but does not inhibit the bacteria growth. Second, there is synergy between half-[MIC] benzyl C_{14} hydraphile and tetracycline against the tetA efflux pump expressing *E. coli*.

3.3.4. Checkerboard. To investigate the recovery of tetracycline activity by hydraphiles in detail, we performed checkerboard experiments. The checkerboard experiment is a detailed combination study in which numerous concentrations (serial dilutions) of one compound are tested against numerous concentrations (serial dilutions) of the antibiotic of interest. For example, six concentrations of benzyl C_{14} hydraphile (1, 1/2, 1/4, 1/8, 1/16 and 1/32 [MIC]) were tested against tetracycline at 1, 1/2, 1/4, 1/8, 1/16 and 1/32 [MIC]. The results are presented as heat maps in figure 3.3. The results could further be analyzed to draw isobolograms. We performed checkerboard experiments with benzyl C_8-C_{14} hydraphiles against tetracycline in Tet^R *E. coli*.

The results of checkerboard experiments with benzyl C_8-C_{14} hydraphiles against tetracycline in Tet^R *E. coli* are shown in figure 3.3 below. The checkerboard experiment was conducted in duplicates. Each cell in figure 3.3 (red/green) shows percent inhibition of cell growth in the presence of respective concentrations of tetracycline and hydraphiles. Any growth inhibition of greater than 80% was considered as the MIC and were highlighted in green. Any MIC lower than ½ [MIC] concentration of both the compounds was considered as synergistic. With benzyl C_8 hydraphile, the recovery of tetracycline activity is observed with concentrations as low as ~ 8 μM and the synergy is observed at a concentration as low as 31 μM (1/8 [MIC]).



C_8 hydraphile (μM)

Tetracycline (μM) \ μM used	7.81	16.00	31.30	62.50	125.00	250.00
28.125	18.21	35.05	51.36	57.07	95.92	98.51
56.25	47.83	49.32	49.18	88.59	99.05	98.37
112.5	66.44	71.47	69.97	97.15	97.69	97.83
225	74.18	74.32	80.03	97.55	96.47	96.20
450	93.89	87.91	95.79	95.65	94.16	92.39
900	99.18	98.64	99.46	97.28	96.47	94.97

C_{10} hydraphile (μM)

Tetracycline (μM) \ μM used	1.09	2.19	4.38	8.75	17.50	35.00
28.125	0.00	0.00	27.69	11.71	14.05	99.22
56.25	11.32	13.27	0.00	31.01	84.00	98.83
112.5	36.07	47.38	56.34	59.85	86.16	97.66
225	69.01	68.82	60.24	81.87	96.69	96.69
450	68.62	79.54	91.81	94.15	94.74	94.54
900	100.81	100.23	100.23	100.03	100.42	98.08

C_{12} hydraphile (μM)

Tetracycline (μM) \ μM used	0.16	0.31	0.63	1.25	2.50	5.00
28.125	0.00	0.00	0.00	0.00	0.00	99.42
56.25	0.00	0.00	0.00	10.54	11.52	98.83
112.5	32.76	35.68	31.78	32.56	88.89	98.25
225	68.43	65.89	64.33	66.87	96.69	97.08
450	55.17	66.48	92.98	93.18	94.93	94.74
900	100.42	100.62	100.62	100.81	101.01	99.45

C_{14} hydraphile (μM)

Tetracycline (μM) \ μM used	0.06	0.13	0.25	0.50	1.00	2.00
28.125	0.00	9.92	22.83	0.00	0.00	99.59
56.25	31.93	39.81	35.87	0.00	0.00	99.05
112.5	46.47	49.32	54.76	45.65	98.51	98.51
225	67.93	70.52	70.24	64.54	91.03	97.83
450	55.30	72.96	71.74	89.27	95.92	96.20
900	98.51	98.91	99.18	99.46	99.59	99.86

Figure 3.3. Checkerboard experiment with benzyl C_8-C_{14} hydraphile and tetracycline against Tet^R *E. coli*. Values show percent inhibition of bacterial growth in the presence of respective concentrations of hydraphile+tetracycline shown. Inhibition on bacterial growth considered at MIC are shown in green and others are shown in red. Hydraphiles and tetracyclines were used at 1/32, 1/16, 1/8, 1/4, 1/2 and 1x [MIC].

The checkerboard experiment revealed that a recovery of tetracycline activity is observed with 2 μM, 0.625 μM and 0.500 μM concentrations of benzyl C_{10}, C_{12} and C_{14} hydraphiles, respectively. Synergy with benzyl C_{10} hydraphile at 8.75 μM was clear. Antibiotic recovery was apparent with all four hydraphiles at lower concentrations as well. As observed in the growth curve experiments, benzyl C_{14} and benzyl C_{12} hydraphiles may not affect the growth of Tet^R *E. coli* at sub-lethal concentrations. Hence, it could be concluded that by the measure of both the growth curves and the FIC index, benzyl C_8-C_{14} hydraphiles show synergy.

3.3.5. Isobologram. Results from the checkerboard experiment were used to draw comparisons between hydraphiles. An isobologram was drawn (figure 3.4) that shows the concentrations of hydraphiles used on the x-axis and the concentration of tetracycline used on the y-axis. Each data point represents the MIC of tetracycline in the presence of the corresponding MIC of the hydraphile used. A comparison could be made between hydraphiles for their ability to recover the tetracycline potency. To recover the tetracycline efficacy 8-fold from

900 µM to 112.5 µM in Tet^R *E. coli*, 1 µM benzyl C_{14} hydraphile is required. However, to observe similar recovery with benzyl C_8 hydraphile, a concentration > 50 µM would be necessary. The isobologram revealed the following trend regarding the hydraphile's ability to rescue the tetracycline potency (figure 3.4): benzyl C_{14} hydraphile ≥ benzyl C_{12} hyraphile > benzyl C_{10} hydraphile > benzyl C_8 hydraphile.

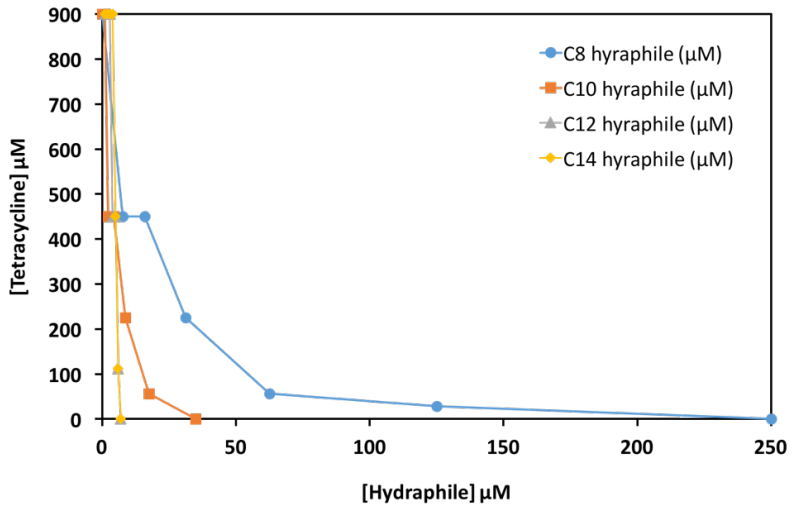


Figure 3.4. Isobolograms of C_8-Benzyl C14 hydraphiles in Tet^R *E. coli*. The x-axis represents the concentration of hydraphiles used in µM and the y-axis represents the concentrations of tetracycline in µM. Each data point represents the MIC of tetracycline in the presence of the corresponding hydraphile used. This is a theoretical representation of checkerboard and hence no error bars shown.

Overall, the checkerboard experiment showed the limit and efficacy of each hydraphile for the recovery of tetracycline activity against Tet^R *E. coli*. We had previously reported a trend for the ability of benzyl C_8- C_{14} hydraphiles to transport sodium from liposomes.[30] The trend for sodium ion transport from liposomes for benzyl hydraphiles was C_{14} ≥ C_{12} > C_{10} >C_8 hydraphile.[30] A direct correlation between the spacer chain length, liposomal sodium transport and the recovery of tetracycline activity against resistant strains could be observed. It was hypothesized that benzyl C_{14} and C_{12} hydraphiles are optimal for spanning the bacterial membrane and transporting ions, which affects both the efflux pump activity and transport of antibiotics into the cell cytoplasm. These hydraphiles may also increase membrane permeability, further affecting antibiotic transport. On the other hand, benzyl C_{10} and C_8 hydraphile are poor

ion transporters and may not span the bilayer. For this reason, a higher concentration of benzyl C_8 and C_{10} hydraphile is required to observe the same effect on tetracycline recovery. Different concentrations of hydraphiles may also affect the aggregation properties of hydraphiles. We have addressed the mechanistic questions in subsequent chapters. Below are the results of various controls that represent our attempt to confirm our conclusions. We have also determined the extent of antimicrobial recovery to different strains and antibiotics.

3.3.6. Controls. We first used various compounds to determine if a specific function and structure of hydraphile is critical to have an effect on tetracycline activity in Tet^R *E. coli*. Hydraphiles form ion channels in the cell membrane, rather than functioning as carriers. The well-known ion carrier, valinomycin, and ion channel, gramicidin-D, were used to determine if antimicrobial recovery was a function of ion transport. The activity of known efflux pump inhibitors such as CCCP and reserpine was also compared to hydraphiles. The activities of the detergent, triton X-100, and a specific membrane disruptor, colistin, were also compared to those of hydraphiles.

Functional controls. Hydraphiles were designed to and shown to perform many of the same functions as a protein ion channels. Hydraphiles insert in the membranes, selectively transport cations, specifically Na^+ and K^+ and show open-close behavior.[30] We wanted to compare the activity of hydraphiles to the compounds that were structurally different, but had a similar function. An ion carrier, ion channel, and a detergent were used. As seen in table 3.3 below, we first tested valinomycin, gramicidin-D, and triton X-100. Valinomycin and gramicidin-D are both peptides that transport potassium. Triton X-100 is an oligomer detergent. Hydraphile is neither a peptide nor a polymer.

The combination studies with valinomycin and gramicidin-D were conducted at 20 μM. The concentrations of some of the compounds were limited due to low solubility in dH_2O or DMSO. The MICs of gramicidin-D and valinomycin were > 128 μM, where as that of triton X-100 was > 1 mM. At 20 μM, valinomycin and triton X-100 recovered the efficacy of tetracycline by 2-fold. Valinomycin, an ion carrier, binds potassium and transports it across membranes. We did not expect a high antimicrobial recovery by valinomycin, as it is an ion carrier. It does not localize in the membrane to cause membrane disruption. Gramicidin-D needs to dimerize in the membrane to form a channel and transport ions. Therefore, the antimicrobial recovery by gramicidin-D

depends on its ability to dimerize in the bacterial membrane to form a channel. Gramicidin-D did not recover tetracycline potency. These results showed that hydraphile did not act similar to a dimerized ion channel, an ion carrier, or a simple detergent. None of them had FIC < 1. In this regard, the hydraphile structure has a unique function. We do note that a known ion channel that does not require to dimerize in the membrane of bacteria would be a better control. We partly addressed this issue with the use of colistin. However, such channel formers are also considered as membrane disruptors. The functional control data described as summarized in table 3.3. The results of three replicates are shown below in table 3.3. The MICs reported in table 3.3 are the average of 3 trials. The standard deviation was calculated and represented as '±' the average MIC. A decrease in the MIC was considered significant only in the error in the data did not overlap. The fold enhancement was determined using the average MICs shown in column 3, hence no errors are shown. However, enhancement greater 2-fold were only considered significant.

Table 3.3: Recovery of tetracycline activity by controls against Tet^R *E. coli*

Amphiphile used	[Amphiphile] μM	[Tetracycline] μM	Fold enhancement	FIC index
No amphiphile	-	900 ± 100	n/a	
CCCP	1	900 ± 100	1-fold	1
CCCP	21	450 ± 50	2-fold	0.75
CCCP	42	225 ± 25	4-fold	0.75
Reserpine	64	450 ± 50	2-fold	1
Reserpine	128	225 ± 25	4-fold	1
Colistin	0.03125	225 ± 25	4-fold	0.52
Colistin	0.0625	56 ± 5	16-fold	0.31
Gramicidin-D	20	900 ± 100	1-fold	1
Valinomycin	20	450 ± 100	2-fold	1
Triton X-100	20	450 ± 100	2-fold	1
Triton X-100	1700 (0.1%)	450 ± 100	2-fold	1

We compared hydraphiles with the known efflux pump inhibitors, reserpine and CCCP. Reserpine blocks the efflux pump channel and CCCP dissipates the proton gradient required for antibiotic efflux. CCCP is a better

control for hydraphiles, since both of them disrupt ion gradients. Both reserpine and CCCP recovered tetracycline activity by 4-fold (table 3.3). The FIC index of reserpine was 1, whereas CCCP was 0.75. CCCP may show some synergy, but its use limited by high cytotoxicity. It has been reported that CCCP and reserpine are more effective against Gram positive than Gram negative bacteria. These results are comparable to that of benzyl C_{14} hydraphile. The benzyl C_{14} hydraphile showed 4-fold tetracycline recovery at 1 μM. Hence, benzyl C_{14} hydraphile affords the same results as known EPIs, but at ~ 100-fold lower concentration.

Colistin, a Gram negative specific membrane disruptor was also used as a control. In the presence of ¼ [MIC] (0.0625 μM) of colistin, the activity of tetracycline was recovered by 16-32 fold. Colistin was tested at various concentrations to draw the isobologram shown in figure 3.5 below. Any data point that falls on the line connecting the two MICs is considered as additive. Any data point below this line is synergistic, whereas above the line is considered antagonism. Colistin shows synergy with tetracycline at nanomolar (< 100 nM) concentrations. It could be concluded that in efflux pump expressing Gram negative bacteria, membrane disruption results in greater recovery of antimicrobial activity rather than EPIs that disrupt ion gradients. Benzyl C_8 hydraphile at half-[MIC] recovered tetracycline activity by 30-fold. We speculate that C_8 hydraphile acts primarily as a membrane disruptor at ½ [MIC] rather than as an ion transporter. This result of tetracycline recovery by benzyl C_8 hydraphile is similar to that of colistin, albeit at a much higher concentration. The efficient ion transporter benzyl C_{14} hydraphile, recovers activity by 4-fold, which is similar to that of CCCP, an EPI. However, both the hydraphiles could be functioning as a combination of membrane disruptor and an indirect inhibitor of efflux pump activity.

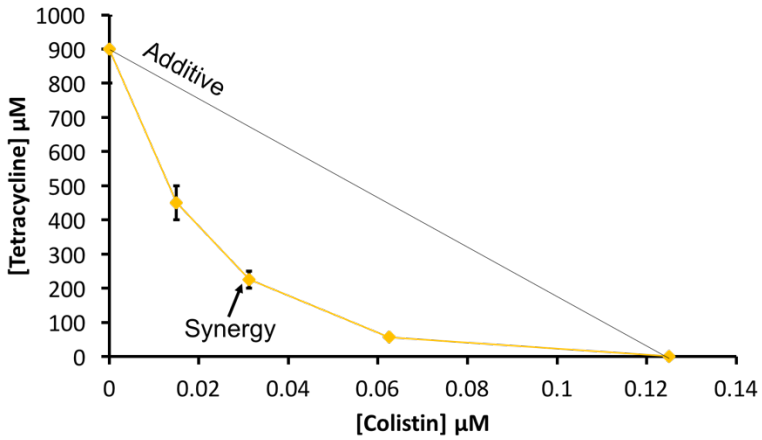
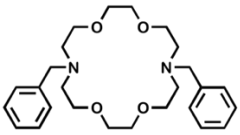
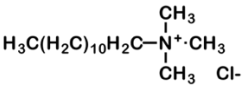


Figure 3.5. Isobologram of colistin against Tet^R *E. coli*. This x-axis represents the concentration of colistin used (in µM) whereas the y-axis represents the concentrations of tetracycline (in µM). Each data point represents the average of three replicates of the MIC of tetracycline in the presence of the corresponding hydraphile used. The error bars represent the standard deviation in the MICs of tetracycline.

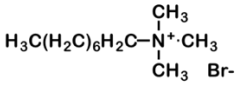
Structural controls. We next tested to determine if hydraphile structure was important to observe the recovery of antimicrobial efficacy. For this purpose, we determined the recovery of tetracycline activity against Tet^R *E. coli* using lariat ethers, dibenzyl diazacrown, and quaternary ammonium compounds such as C_8 and C_{12} trimethyl ammonium salts (figure 3.6 below). The results of three MIC replicates are shown below in table 3.4. The standard deviation was calculated and represented as '±' the average MIC. A decrease in the MIC was considered significant only in the error in the data did not overlap. The fold enhancement was determined using the average MICs shown in column 3, hence no errors are shown. However, enhancement greater 2-fold were only considered significant.



Dibenzyl di-aza-crown



C_{12} trimethylammonium chloride



C_8 trimethylammonium bromide

Figure 3.6. Structures of dibenzyl diazacrown and C_8 and C_{12} trimethyl ammonium salts used as structural controls.

Table 3.4: Activity of lariat ethers, di benzyl di-aza-crown and trimethyl ammonium salts against Tet^R *E. coli*

Amphiphile used	[Amphiphile] μM	[Antibiotic] μM	Fold enhancement	FIC index
No amphiphile	-	900 ± 100	n/a	n/a
C_8 lariat ether	60 (½[MIC])	175 ± 25	5-fold	0.7
C_8 lariat ether	40 (¼[MIC])	233 ± 25	4-fold	0.5
C_{10} lariat ether	9 (½[MIC])	56 ± 5	16-fold	0.56
C_{10} lariat ether	6 (¼[MIC])	225 ± 25	4-fold	0.5
C_{12} lariat ether	128 (½[MIC])	450 ± 50	2-fold	1
Dibenzyl diaza crown	128	900 ± 100	1-fold	1
$CH_3(CH_2)_7NMe_3Br$	128	450 ± 50	2-fold	1
$CH_3(CH_2)_{11}NMe_3Cl$	128	225 ± 25	4-fold	1

Lariat ethers have one central macrocycle connected to two alkyl chains. Lariat ethers lack the two distal macrocycles that are found in hydraphiles. Numerous derivatives of lariat ethers have been studied and reported to transport cations, specifically Na^+ and K^+. They have been report to transport cations by a carrier mechanism. In addition to ion transport, lariat ethers also possess biological activity. The reports of their applications include antimicrobial activity,[51] chemotherapy,[52] and recently as modulators of the plant root architecture (data not published). Lariat ethers are not only the structural

analogues of hydraphiles, they also provide a control for an ion carrier that is structurally similar to hydraphiles. As shown in table 3.4 above, C_8, C_{10}, and C_{12} lariat ethers recovered tetracycline activity by 5, 16 and 4-fold, respectively. The FIC index indicated synergy with both C_8 and C_{10} lariat ethers.

Another structural analogue that was used was dibenzyl diazacrown (compound **12**). Compound **12** has one macrocycle and no alkyl chain linkers (see figure 3.6 above). It shows no ion transport activity or antimicrobial property. Compound **12** showed no recovery of tetracycline activity.

It is well known that the positive charge and hydrophobic tail of colistin and other antimicrobials are critical for activity.[15] Loss of the positive charges or the hydrophobic tails results in loss of colistin antimicrobial property.[15] We therefore hypothesized that the nitrogen atom linked to the alkyl chains was important for hydraphile activity. To test this hypothesis, we utilized trimethylammonium bromide salts: $CH_3(CH_2)_7NMe_3Br^-$ and $CH_3(CH_2)_{11}NMe_3Cl^-$. Such quaternary ammonium compounds with long alkyl chains ($\geq C_{16}$) are used as sterilizing agents in clinics.[53] The MICs of C_8 and C_{12} trimethylammonium salts were > 128 μM. Tetracycline recovery with C_8 and C_{12} trimethylammonium bromides was only 2-4 fold at 128 μM (table 3.4), whereas C_8 and C_{12} hydraphiles showed 30- and 16-fold recovery at ½ [MIC] concentrations. Hence, hydraphiles are more active as antimicrobials and adjuvants compared to the lariat ethers, dibenzyl diazacrown and quaternary ammonium compounds.

These data for structural controls show that the combined elements of hydraphile structure are important to observe the recovery of antimicrobial activity. Their function is different than that of the quaternary ammonium compounds. Amongst all the structural variations of hydraphiles studied, benzyl C_8-C_{14} hydraphiles (**1-4**) and colistin were the most effective compounds.

3.3.7. Activity against *Klebsiella pneumoniae*. We used a *K. pneumoniae* (ATCC BAA 2146) strain to investigate the extent and applicability of hydraphiles as adjuvants. This strain of *K. pneumoniae* was isolated from a patient, is resistant to > 30 antibiotics and expresses genes for tetA, RND, and ABC type efflux pumps. It is also resistant to carbapenems (penicillins) due to the presence of the NDM-1 gene. We wished to determine if hydraphiles could recover tetracycline activity in a robust Gram negative strain that expresses multiple different types of efflux pumps including tetA.

As seen in table 3.5 below, benzyl C_8-C_{14} hydraphile recovered tetracycline activity against MDR *K. pneumoniae*. The MICs of benzyl C_8-C_{14} hydraphiles against *K. pneumoniae* are reported in table 3.1 above. The MICs of hydraphiles against *K. pneumoniae* were greater than those observed against Tet^R *E. coli*. The results of three MIC replicates are shown in the table below. The standard deviation was calculated and represented as '±' the average MIC. A decrease in the MIC was considered significant only in the error in the data did not overlap. The fold enhancement was determined using the average MICs shown in column 3, hence no errors are shown. However, enhancement greater 2-fold were only considered significant. In the presence of ½ and ¼ [MIC] of benzyl C_8 hydraphile, tetracycline's MIC reduced from 1000 μM to 25 μM and 250 μM, respectively. This represents a recovery of 40- and 4-fold. Similarly, half-[MIC] benzyl C_{10}, C_{12} and C_{14} hydraphiles recovered the tetracycline activity by 8, 8 and 16-fold. Benzyl C_{14} hydraphile recovered tetracycline activity by 4-fold against Tet^R *E. coli* and 16-fold against *K. penumoniae*. This change might be due to the higher concentration of benzyl C_{14} hydraphiles used against *K. pneumoniae i.e.* 5 μM. In either case, it was apparent that hydraphiles recover the activity of tetracycline against tetA efflux pump expressing *E. coli* and *K. pneumoniae*. The results of three MIC replicates are shown below in table 3.5. The standard deviation was calculated and represented as '±' the average MIC. A decrease in the MIC was considered significant only in the error in the data did not overlap. The fold enhancement was determined using the average MICs shown in column 3, hence no errors are shown. However, enhancement greater 2-fold were only considered significant.

Table 3.5: Recovery of tetracycline activity against *K. pneumoniae* by hydraphiles.

Amphiphile used	[Amphiphile] μM	[Antibiotic] μM	Fold enhancement	FIC index
Antibiotic used: Tetracycline				
No amphiphile	-	1000 ± 100	n/a	n/a
C_8 hydraphile	2.5	1000 ± 100	1-fold	1
C_8 hydraphile	50 (¼[MIC])	250 ± 50	4-fold	0.5
C_8 hydraphile	100 (½[MIC])	25 ± 10	40-fold	0.53
C_{10} hydraphile	2.5	900 ± 100	1.1-fold	1
C_{10} hydraphile	14 (¼[MIC])	300 ± 50	3-fold	0.58
C_{10} hydraphile	28 (½[MIC])	125 ± 25	8-fold	0.63
C_{12} hydraphile	2.5	500 ± 50	2-fold	0.57
C_{12} hydraphile	8.75 (¼[MIC])	300 ± 25	3-fold	0.58
C_{12} hydraphile	17.5 (½[MIC])	125 ± 25	8-fold	0.63
C_{14} hydraphile	2.5 (¼[MIC])	350 ± 50	3-fold	0.58
C_{14} hydraphile	5 (½ [MIC])	62.5 ± 25	16-fold	0.56
Antibiotic used: Ampicillin				
C_{14} hydraphile	5 (½[MIC])	> 1000	0-fold	n/a
Antibiotic used: Ciprofloxacin				
No amphiphile	-	700 ± 100	n/a	n/a
C_8 hydraphile	100 (½[MIC])	70 ± 20	10-fold	0.6
C_{12} hydraphile	8.75 (¼[MIC])	300 ± 25	2-fold	0.67
C_{12} hydraphile	17.5 (½[MIC])	175 ± 25	4-fold	0.75
C_{14} hydraphile	2.5 (¼[MIC])	400 ± 50	2-fold	0.82
C_{14} hydraphile	5 (½ [MIC])	250 ± 25	3-fold	0.85

We also determined if the recovery of antimicrobial potency was extensible to a different class of antibiotic or if it was specific to tetracyclines. The RND type of efflux pump is a tripartite system that spans both the bacterial membranes and is capable of transporting multiple different classes of antibiotics.[40] The *K. pneumoniae* strain was reported to be resistant to ciprofloxacin, a fluoroquinolone class of antibiotic.[53] Resistance to ciprofloxacin could be resulting from both target site mutation and RND type efflux pump. It has been reported that resistance to an antibiotic from two different mechanisms are independent and not synergistic.[47] We determined if hydraphiles could recover ciprofloxacin activity against *K. pneumoniae*. Table 3.5 shows the recovery of ciprofloxacin activity by benzyl C_8, C_{12} and C_{14} hydraphile. Benzyl C_{10}

hydraphile was not tested. All three hydraphiles recovered ciprofloxacin activity. The greatest ciprofloxacin recovery of 10-fold was observed with benzyl C_8 hydraphile. Benzyl C_{14} hydraphile showed a recovery of 3-fold at ½ [MIC]. The recovery of ciprofloxacin activity (10-fold) was much lower than that observed against tetracycline (40-fold). Ciprofloxacin and tetracycline are different in structures and have different cytoplasmic targets. Both of the antibiotics are hydrophilic and need to localize in the cell cytoplasm in order to bind to their targets. We hypothesize that the additional ciprofloxacin resistance could be a result of a mutation in the DNA gyrase A. Hydraphiles may not be able to recover resistance caused by target site mutation. A direct comparison between antibiotic recovery of two Gram negative strains is shown in figure 3.7. This is a theoretical representation of the MIC values reported in table 3.5 above. Hence no error bars are shown.

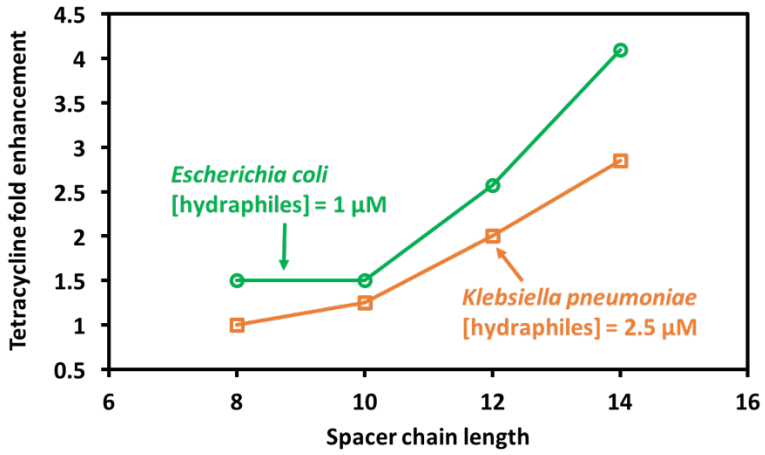


Figure 3.7. A comparison of recovery of tetracycline activity against Tet^R *E. coli* and *K. pneumoniae* (ATCC BAA 2146) by benzyl C_8-C_{14} hydraphiles. Benzyl C_8-C_{14} hydraphiles were used at 1 µM against Tet^R *E. coli* (open circles) and 2.5 µM against *K. pneumoniae* (open squares).

3.3.8. Hydraphile activity against *K. pneumoniae* and *E. coli*. To compare the activity of each hydraphile against *K. pneumoniae*, a study was conducted at the lowest ¼ [MIC] of any hydraphile used, which was 2.5 µM. These data were also compared to the controlled concentrations study performed with benzyl C_8-C_{14} hydraphiles against Tet^R *E. coli* at 1 µM. The results of the tetracycline recovery study are represented in the graphical format in figure 3.6 above. In the *K. pneumoniae* strain, when the concentration was kept constant at 2.5 µM, the

following trend was apparent: benzyl C_{14} hydraphile > C_{12} hydraphile > C_{10} hydraphile > C_8 hydraphile. This trend paralleled that of hydraphiles against Tet^R *E. coli*. However, the efficacy of hydraphiles to recover tetracycline activity against Tet^R *E. coli* was greater, at lower concentration, than against *K. pneumoniae*. This feature could be due to the fact that the *E. coli* strain used for study was a genetically modified laboratory strain. The membranes of the two strains have different compositions. The more robust MDR *K. pneumoniae* may already have some mutations that alter the phospholipids and lipid A in the cell membrane. These changes could decrease the efficacy of the amphiphilic compounds such as hydraphiles and colistin. Resistance mechanisms other than efflux pump may also provide additional tetracycline resistance in *K. pneumoniae*. In either case, tetracycline activity was clearly recovered in both Gram negative bacteria.

3.4. Summary and conclusions.

Synthetic amphiphiles have been studied for their ability to transport ions either as a channel or a carrier.[19-21] Hydraphiles have been studied and determined for their ability to form cation channels.[29] It was hypothesized that the disruption of ion homeostasis in bacteria caused by hydraphiles resulted in cell death and this accounted for the antimicrobial property. Regardless of the numerous structural and biological studies with crown ether-based molecules, no previous systematic study addresses their use as antimicrobial or antibiotic adjuvants. We recently reported that hydraphiles enhance the potency of four different classes of antibiotics against sensitive strains of Gram positive and Gram negative bacteria.[36,39] Out of four antibiotics tested, the potency of two hydrophobic antibiotics, rifampicin and erythromycin, was enhanced the most.[36,39] This observation was hypothesized to be an effect of membrane disruption caused by hydraphiles localizing in the membrane of bacteria.

A greater healthcare concern is caused by the antibiotic resistant Gram negative bacteria.[1-9] Multi drug resistant Gram negative infections are the primary cause of diseases such as urinary tract infection and pneumonia.[7,8] The resistance is primarily caused by the presence of secondary outer membrane and efflux pumps that decreases the cytoplasmic concentration of antibiotics.[13] There are no efflux pump inhibitors developed for clinical use. There is no study reported about the activity of synthetic amphiphiles against multi-drug resistant bacteria. Our extensive data reported here supports the statement that hydraphiles of appropriate spacer chain length can recover the activity of

tetracycline and fluoroquinolone against efflux pump expressing resistant *E. coli* and *K. pneumoniae*.

All four hydraphiles studied, benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles rescued antimicrobial potency. TetA efflux pump expressed in the Tet^R *E. coli* are highly selective and effective at effluxing tetracycline molecules. Recovery of tetracycline activity in Tet^R *E. coli* is only possible if the cytosolic concentration of tetracycline is increased to an effective level. Hence, it could be concluded that hydraphiles successfully rescue tetracycline activity, possibly by increasing the cytosolic concentrations of tetracycline. It may appear that benzyl C_8 hydraphile is most effective at recovering antibiotic activity. This was an attribute of the high concentration of C_8 hydraphile used. Upon further investigation, following trend was confirmed for the efficacy of antimicrobial recovery by benzyl hydraphiles: C_{14} hydraphile > C_{12} hydraphile > C_{10} hydraphile > C_8 hydraphile (figures 3.4 and 3.7). However, tetracycline activity recovery was observed with benzyl C_8 and C_{10} hydraphile at concentrations as low as 8 μM and 3 μM, respectively (figure 3.3).

Synergy between hydraphiles and tetracyclines was confirmed using the FIC index and growth curves. Growth curves showed no inhibition of Tet^R *E. coli* growth by the highest concentration of benzyl C_{14} hydraphile used for combination studies, *i.e.* 1 μM (½ [MIC]). Here, the increase in lag-phase observed could be due to the ion transport ability of hydraphiles causing an osmotic stress. The extent of the antibiotic recovery by hydraphiles was successfully extended to patient isolated MDR *K. pneumoniae* and a different class of antibiotic, fluoroquinolones.

Comparisons to structural variations showed that hydraphiles were most effective amongst lariat ether, trimethylammonium salts and dibenzyl diazacrown at recovering antimicrobial potency. Lariat ethers, previously characterized as ion carrier, did show remarkable recovery of tetracycline activity against Tet^R *E. coli* (table 3.4). However, *K. pneumoniae* was completely resistant to all lariat ethers. Lariat ethers may only increase antibiotic potency by disrupting membrane integrity. Lariat ethers were previously shown to form aggregates in aqueous solution, of 100-200 nm in size. It is possible that lariat ethers organize in the membrane to form a pore, a previously uncharacterized property.

Amongst all the functional controls tested, the activity of hydraphiles was found to be comparable to that of CCCP and colistin. Both CCCP and colistin are known to affect ion gradients and transport in bacteria.[13,45] CCCP is commonly used as an efflux pump inhibitor in laboratory studies but its use in clinic is limited due to cytotoxicity.[45] This prompts the need for study of hydraphiles on mammalian cells, which is addressed in chapter 4. These controls show that hydraphiles either disrupt ion gradients in bacteria or disrupts membrane integrity providing the antibiotic access to the bacterial cytosol. Mechanistic studies are addressed in Chapter 5. A combination of two mechanisms is also possible, but would be difficult to distinguish from the individual effect.

Colistin (polymyxins) is used as a drug of last resort for the treatment of MDR infections. A recent study reported the first colistin resistant *E. coli* in US.[14] This shows the need to preserve colistin as an antibiotic for future use. Our results for tetracycline recovery by colistin (16-32 fold) against Tet^R *E. coli* shows that natural amphiphiles such as polymyxins, not only synthetic amphiphiles, could be used as adjuvants or EPI. A new use of natural amphiphiles as efflux pump inhibitors is contemplated based on the results reported here.

3.5 Experimental Methods

3.5.1. Compounds and antibiotics used. Two classes of synthetic amphiphiles were used in this study. First class of compounds are called hydraphiles. Hydraphiles were synthesized in the Gokel lab and purity was confirmed by NMR and mass spectrometry. Four hydraphiles differing in its spacer chain lengths were used. We used benzyl C_8, C_{10}, C_{12} and C_{14} hydraphile. Similarly, five lariat ethers were used for this study, benzyl C_8-C_{16} lariat ethers. Dibenzyl diazacrown was also used as a control. The antibiotics of choice were tetracycline, ampicillin, ciprofloxacin, and colistin. CCCP and reserpine, known efflux pump inhibitors were also used as controls. All the antibiotics and known EPI were received from Sigma-Aldrich and used as received.

3.5.2. Bacteria used. There were two strains of bacteria used for this study. Tet^R *E. coli* was prepared by transforming the competent JM109 *E. coli* with pBR322 plasmid (Carolina Biological). The transformation was performed using heat-shock method as outlined by the manufacturer (Promega). The resulting *E. coli* cells were tetracycline and ampicillin resistant and designated Tet^R *E. coli*. The Tet^R *E. coli* expressed tetA efflux pump and β-lactamase enzyme. The *Klebsiella pneumoniae* (ATCC BAA 2146) strain was acquired from ATCC. It is a clinical

strain (BSL-2) isolated from a patient's urine sample. The *K. pneumoniae* is reported to be resistant to more than 30 different antibiotics including classes such as carbapenems, fluoroquinolones, cephalosporins, macrolides and tetracyclines. It expresses efflux pumps from three different classes: RND, ABC and MFS. TetA is the MFS type efflux pump expressed in the *K. pneumoniae*, which is used for the combination study.

3.5.3. Minimal Inhibitory Concentrations (MIC). Tet^R *E. coli* was grown in L.B. Miller media containing 100 µg/mL Ampicillin. *K. pneumoniae* was grown in the cation adjusted Mueller Hinton II (MHII) media. The cells were grown overnight from one colony forming unit (CFU) in 2 mL media. On the day of the experiment, bacteria were knocked back to $O.D._{600}$ = 0.100 and incubated at 37 °C until the $O.D._{600}$ reached 0.500 (4 x 10^8 CFU/mL). These cells were diluted 100-fold by adding 20 µL cells in 1980 µL MH-II media (without antibiotics) to get 4 x 10^6 CFU/mL. The 20 µL diluted cells were added to each well (final volume/well = 200 µL) after the addition of antibiotics or synthetic amphiphiles. The final cell concentration per well was approximately 4 x 10^5 CFU/mL (or 8 x 10^4 CFU/well).

Compounds were either dissolved in DMSO or dH_2O. In sterile 1.5 mL micro-centrifuge tubes, compounds were serially diluted to form stock concentrations. Dilution(s) of compounds added to each well. Compounds were always administered at a constant volume such that the final DMSO concentration in each well was 0.5% volume/volume (1 µL of final 200 µL).

In a 96-well plate, first the media was added followed by addition of the compounds. The final volume of each well was 200 µL. For combination studies, after the addition of amphiphiles, antibiotics were added. The compounds or antibiotics that were dissolved in dH_2O, 10 µL/well was used. In the case of DMSO alone control, 1µL of DMSO was added to each well. For dH_2O control, 10 µL of dH_2O was added to each well. No compound or solvent were added for cells alone and media alone control. Contents of the well were mixed well by pipetting up and down three times. After mixing, 20 µL cells at 4 x 10^6 CFU/mL were added to each well. Contents were mixed again by pipetting up and down 3 to 4 times. Empty wells were filled with 200 µL Phosphate Buffered Saline (PBS) to minimize evaporation. The plates were incubated at 37°C for 18-20 hours. Results were collected by determining the O.D. at λ = 600 nm using a plate reader (BioTek Cytation 3) in Dr. Lon Chubiz's lab. Each compound was tested in triplicate per plate. Percent inhibition was calculated by comparing to the cell

alone control. Growth inhibited of ≥ 90% was considered as the MIC. The data was reproduced 2 more times on two separate plates.

3.5.4. Checkerboard. In a checkerboard experiment, each column of the plate had different concentration of the amphiphile increasing by serial dilution. Two amphiphiles were tested per plate. Each row of the checkerboard experiment had different concentrations of antibiotics increasing by serial dilutions. The concentrations of amphiphile and antibiotic tested were 1/2, 1/4, 1/8, 1/16 and 1/32 the [MIC]. *E. coli* and *K. pneumoniae* were tested using the checkboard experiment. The MIC procedure used for the checkerboard experiment was the same as outlined above. Cells and compound alone controls were also used. The checkerboard experiment did not have a biological replicate but the data was reproduced three times before reporting. The data was represented as a heat map and as a isobologram.

3.5.5. Growth Curve. The growth curve experiment was performed using Tet^R *E. coli*. The compounds studied using growth curves were benzyl C_{14} hydraphile and tetracycline. Here, the *E. coli* was grown overnight from one CFU in L.B. Miller media containing 100 μg/mL Ampicillin. On the day of the experiment the cells were knocked back in L.B. Miller media containing 100 μg/mL Ampicillin, to O.D. 600 = 0.550 before use. In a sterile 250 mL flask, 50 mL L.B. media was added, followed by benzyl C_{14} hydraphile and tetracycline. The concentrations of benzyl C_{14} hydraphile tested were 1 and 2 μM. The concentrations of tetracycline tested were 220 and 900 μM. In addition, a growth curve containing a combination of benzyl C_{14} hydraphile (1 μM) and tetracycline (220 μM) was also tested. Cells only and DMSO (0.5% v/v) alone controls were also performed. Compounds were mixed by swirling the flask. To each flask, knocked back cells were added so that the final cell concentration in each flask was approximately 4 x 10^5 CFU/mL. The flasks were incubated at 37 °C and 200 RPM. A 2 mL sample was taken every 30 minutes (for next 24 hours) to determine the O.D. at λ = 600 nm. A graph was plotted for O.D. *vs.* time to generate a growth curve.

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CHAPTER 4

Hydraphiles Localizes in the Bacterial Cell Membrane and Selectively Increases Bacterial Membrane Permeability

The data reported in this chapter have not been published.

4.1. Introduction

Antibiotic resistance is a world-wide crisis. The Center for Disease Control and Prevention (CDC)[1], the World Health Organization (WHO)[2], the Wellcome trust[3], the White House[4], and similar organizations around the world have issued reports outlining the threat from antibiotic resistant bacteria. We have now entered a post-antibiotic era, in which the antibiotics that were once life-saving treatments, have now become ineffective. In 2013, in the U.S. alone, there were more than 2 million illnesses caused by antibiotic resistant infections acquired in hospitals.[1] Due to the lack of effective antibiotics, bacterial infections proved to be fatal for 23,000 patients.[1] World-wide bacterial infections cause 700,000 deaths each year.[3,5] It has been estimated that in the absence of any solution, this number may increase to 10 million deaths per year by 2050.[3] It is clear that there is an urgent need to address the antibiotic resistant bacterial infections.

The problem of antibiotic resistance has been exacerbated by the lack of antibiotics in development. Whereas the period from 1951 through 2000 witnessed an average of three new antibiotics per year, only four antibiotics have been approved since 2011.[6] Soon after the introduction of a new antibiotic in clinical use, bacterial resistance to the antibiotic has been reported.[7] The spread of resistance between different strains of bacteria is rapid. This is facilitated by the plasmids encoding these resistance genes.[8] Development of new antibiotics is critical to keep up with the development and the spread of antibiotic resistance. However, only one adjuvant-antibiotic was approved by the FDA in 2015.[9] No new class of antibiotic has been developed in the last four decades that targets Gram negative infections.[10] The last class of antibiotics developed for targeting Gram negative bacteria was tetracycline. Today, wide spread resistance to tetracycline has limited its use in clinic.

There are three mechanisms of antibiotic resistance: antibiotic degrading enzymes, binding site mutation, and efflux pumps.[11] Bacteria are known to produce enzymes such as β-lactamase that degrade β-lactam ring of the penicillin antibiotics. Currently, the majority of new antibiotic research is based on developing β-lactamase inhibitors. Antibiotics such as penicillin, ampicillin, and first and second generation cephalosporins have lost their efficacy due to the development of new types of β-lactamases such as New Delhi Metallo β-lactamase (NDM-1).[12] Mutations in the DNA gyrase or ribosomes prevent binding of fluoroquinolones, macrolides, and tetracycline to its binding site.[13] So far, only one class of antibiotic resistance inhibitor is available on the market: β-

lactamase inhibitors. Our interest is to inhibit efflux pumps and increase membrane permeability.

Bacterial efflux pumps decrease the cytoplasmic concentration of toxic substances including antibiotics.[14] Overexpression of porins increases efflux of toxic and unwanted molecules from cells.[15] However, efflux pumps are specialized transmembrane proteins that may specifically export antibiotics. Certain efflux pumps provide multi-drug resistance.[16] The multidrug resistance conferred by efflux pumps can eventually cause acquisition of other types of resistance such as antibiotic degrading enzymes or mutation of antibiotic target sites.[17] All the efflux pumps except ATP binding cassette (ABC) utilize either a proton or sodium gradient as an antibiotic-cation antiport.[16] ABC hydrolyzes an ATP molecule to provide energy for antibiotic transport. The efflux pumps in Gram negative and –positive bacteria are highly effective at keeping a low cytoplasmic concentration of antibiotics.[18] Along with the outer membrane of Gram negative bacteria, efflux pumps create a general mechanism of resistance that spans across various classes of antibiotics.

The outer membranes of Gram negative bacteria provide for an attractive target for antibiotic development. The majority of antibiotics need to pass through the membrane and bind to its cytoplasmic target to inhibit bacterial growth. Some of the antibiotics diffuse through the membranes, others pass through porins (*e.g.* outer membrane proteins, OMPs) found in membranes.[19] The outer membrane consists of lipopolysaccharide (LPS) and phospholipids.[20] LPS consists of a lipid A structure, core polysaccharide and O-antigen repeats. The LPS is replete with anionic charges, is highly crosslinked and varies among different species in the types of fatty acids and acyl groups that are present.[20] This prevents the entry of toxic molecules, such as antibiotics. Additionally, LPS acts as an antigen and triggers an immune response in humans. Pathways involved in the outer membrane protein trafficking and assembly; and localization and transport of lipoproteins have been used as molecular targets for antibiotic development.[10] Since the OM serves as a significant and primary barrier to antibiotic entry, their disruption could serve as a strategy to increase antibiotic delivery/efficacy. However, there has been little success in developing antibiotics that target bacterial membranes.

Colistin (polymyxin) is the only antibiotic available on the market that targets and disrupts the outer membrane of Gram negative bacteria.[21] Polymyxin, isolated from *Bacillus polymyxa*, binds to the lipid A component of the

Gram negative bacteria. This cyclic peptide could be considered a natural amphiphile that disrupts bacterial cell membranes inducing cell death. They are used as a last line of defense in the treatment of multidrug resistant infections.[10] Polymyxins are associated with renal toxicity.[22] For effective *Pseudomonas aeruginosa* killing, 0.5 μg/mL (0.28 μM) - 2 μg/mL (1.14 μM) plasma concentration of colistin is required.[23] Studies have shown that trough concentrations greater than 2.2 μg/mL (1.25 μM) results in 65-85% nephrotoxicity.[24]

It has been hypothesized that the hydrophobic tail of polymyxin results in mammalian cell toxicity. Replacement of the long hydrophobic tail with N-phenyl pyridone and Cl-phenyl urea has resulted in a greater therapeutic margin but decreased its antimicrobial efficacy.[25] A polymyxin analogue with a decreased number of positive charges resulted in lower cytotoxicity but maintained MIC_{90} of 1 μg/mL. The majority of the antibiotic studies in this area focuses on either developing safer polymyxin analogs or new peptides. No new structure or approach has been developed to target Gram negative membranes since polymyxins. Bacterial resistance to polymyxins has been negligible. An *Escherichia coli* carrying a plasmid with resistance gene to polymyxin (mcr-1), was recently reported in the U.S.[26] There is a need to identify novel membrane disruptors before wide-spread resistance to colistin is developed. Here, we report that hydraphiles disrupt *E. coli* membrane integrity and increase membrane permeability.

Hydraphiles are synthetic ion channels that conduct cations, specifically Na^+ and K^+ ions.[27] Since the early work by Tabushi *et al.* in the 1980s, there has been a dramatic increase in the number and variety of synthetic ion channels.[28] Hydraphile synthetic ion channels designed and developed in the Gokel lab mimic some features of the well-known KcsA protein channel.[29,30] These synthetic channels have an entry and exit portal and a hydrated "central relay." Computational and biophysical studies suggested that hydraphiles are membrane bound.[31] Planar bilayer studies confirm that hydraphiles transport cations (Na^+, K^+, H^+) and show open-close behavior similar to that observed with known channel proteins.[32,33] Hydraphiles form non-rectifying channels, hence transporting ions based on cation gradients.[34] Hydraphiles show length dependent ion transport[35] and antimicrobial activity[36]. Benzyl C_{12}-C_{16} hydraphiles are better ion transporters than shorter or longer analogs. Fully extended C_{12}-C_{16} hydraphiles are estimated to span 30-40 Å. This is the approximate thickness of natural lipid bilayers. C_8 hydraphile, which is 14 Å

shorter than C_{14} hydraphile, is not long enough to span the bilayer and fails to form channels. Longer hydraphiles are poorer transporters, probably owing to a less organized conductance state.

A dimethyl aminonapthalenesulfonyl (dansyl) substituted C_{12} hydraphile was previously used to visualize the localization of hydraphiles in *E. coli*.[36] Three key observations were made in this experiment. First, dansyl C_{12} hydraphile was primarily localized in the membrane or on the membrane surface (figure 4.1). The cytoplasmic localization was also observed but it was much lower. It was however unclear if the hydraphiles were localized in the membrane or on the surface of the bacteria. Second, the membrane localization was observed at 12 μM of dansyl C_{12} hydraphile, a value closer to its minimal inhibitory concentration (MIC). Third, after 5 minutes of treatment time with dansyl C_{12} hydraphile, an increased fluorescence was localized to certain regions/spots in the membrane. This increased fluorescence could indicate aggregation of hydraphile in the membrane. This hypothesis has not been tested. The membrane localization of hydraphiles in *E. coli* has not been confirmed with co-localization of a known membrane stain. Localization of hydraphiles in the mammalian cells has also not been investigated.

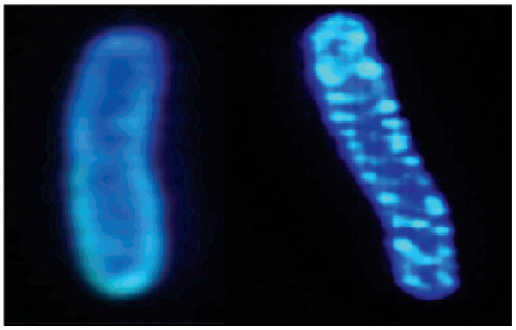

Figure 4.1. Localization of dansyl C_{12} hydraphile in *E. coli* cell membrane at 12 μM. Initially a uniform localization was observed (left). After 5 minutes, the fluorescence in the membranes was localized to certain regions (right). Figure reproduced from reference 36.

In two previous chapters, we showed that benzyl C_8-C_{14} hydraphiles increase antibiotic potency against sensitive[37] and resistant bacteria[38]. The activity of rifampicin and erythromycin were enhanced by more than 15-fold against Gram negative bacteria such as *Escherichia coli* and *Pseudomonas*

aeruginosa. These antibiotics are hydrophobic and cannot penetrate the Gram negative cell membrane. It was therefore hypothesized that the hydraphiles increase the outer membrane permeability to antibiotics. In the following study, we showed that hydraphiles rescue tetracycline and ciprofloxacin activity against efflux pump expressing resistant *E. coli* and *Klebsiella pneumoniae*. A synergy between hydraphiles and antibiotics was confirmed. The rescue of antibiotic potency by hydraphiles was chain length dependent; benzyl C_{14} hydraphile was the most potent adjuvant. It was hypothesized that hydraphiles increase cytoplasmic concentration of antibiotics by uncoupling efflux pumps from the ion gradients and increasing the membrane permeability of the Gram negative bacteria. We have tested the 'increase in membrane permeability' hypothesis here.

It was hypothesized that if hydraphiles localize in the cell membrane of the bacteria, then they could disrupt the membrane integrity and increase membrane permeability. Here, we report that dansyl labeled C_{14} hydraphile localizes in the membrane of Tet^R *E. coli* and human embryonic kidney (HEK-293) cells. The membrane localization was similar to that of well-known membrane localizing stain FM4-64 FX. Higher concentration of dansyl C_{14} hydraphile (32 μM) was used for microscopy, however membrane localization was confirmed at 2 μM. Cytoplasmic localization of dansyl C_{14} hydraphile was also observed in both the Tet^R *E. coli* and HEK-293 cells. However, this could be the effect of cell death or increase in membrane permeability caused by hydraphile itself. After 5 minutes of treatment, hydraphile aggregates were observed in the *E. coli* membrane. This was confirmed using scanning electron microscopy (SEM). SEM also revealed membrane disruption of Tet^R *E. coli* cells caused by hydraphiles. An increase in the membrane permeability and membrane disruption of Tet^R *E. coli* was observed with benzyl C_8-C_{14} hydraphiles at half-[MIC]. Membrane permeability was confirmed with potassium and nucleic acid release. No increase in the permeability of HEK-293 membranes was observed at 2x [MIC] of benzyl C_8-C_{14} hydraphiles.

4.2. Compounds used.

In the combination studies, benzyl C_{14} hydraphile was the most potent compound. We therefore used C_{14} hydraphile for localization studies. Our previous success[36] with dansyl tagged hydraphiles, rationalized the use of the dansyl-tag for the membrane localization experiments outlined below. The localization of amphiphiles was determined by preparing fluorescent C_{14} hydraphiles. The benzyl side arms in the benzyl-C_{14} hydraphile was substituted

with the fluorescent dansyl group. The structures of dansyl C_{14} hydraphile (**1**) is shown in figure 4.2. Dansyl group is fluorescent itself, and hence provides dansyl C_{14} hydraphile an ability to fluoresce that could be observed under a confocal microscope. A well-known membrane localizing stain FM4-64 FX (**6**) was used as a control.[39] FM4-64 FX stains were acquired from Thermo-Fischer. These compounds were used to study membrane localization of hydraphiles.

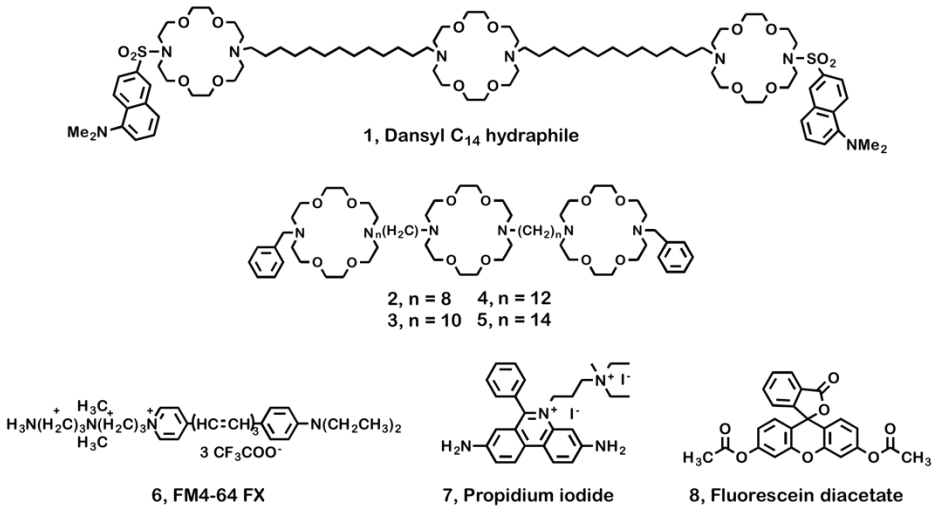

Figure 4.2. Structures of the dansyl C_{14} hydraphile (**1**), benzyl C8-C14 hydraphiles (**2**-**5**), FM4-64 FX (**6**), propidium iodide (**7**) and fluorescein diacetate (**8**).

The membrane permeability was studied with four hydraphiles: benzyl C_8 – benzyl C_{14} (compounds **2**-**5**). We previously showed that benzyl C_8-C_{14} hydraphiles recovered antimicrobial potency against tetracycline resistant *E. coli* and multi-drug resistant *K. pneumoniae*. We therefore tested compounds **2**-**5** for their ability to increase membrane permeability. Propidium iodide, a membrane impermeable stain (**7**), and fluorescein diacetate (**8**), a cell viability stain were used to study membrane permeability. Scanning electron microscopy was performed to determine the effect of hydraphiles on cell membrane surface.

4.3. Bacteria used.

All the studies reported here were conducted with a genetically modified strain of *E. coli*. The competent JM109 *E. coli* was transformed with pBR322 plasmid that expressed the tetA efflux pump and the β-lactamase enzyme. The resulting strain was labeled Tet^R *E. coli* and was resistant to tetracycline and ampicillin. We previously proved that hydraphiles rescue tetracycline activity

against Tet^R *E. coli* and show synergy with tetracycline. The same strain is used here to test the hypothesis that hydraphiles localize in the bacterial cell membrane and increase membrane permeability.

4.4. Results and Discussion.

4.4.1. Membrane localization. It was previously reported that dansyl C_{12} hydraphile localized in the periphery of DH5α *E. coli* (figure 4.1 above). However, it was unclear if the hydraphiles were in the membrane or on the surface of *E. coli*. A single image of the bacteria was produced. It was unclear if the majority of the *E. coli* cell population exhibited similar membrane localization of hydraphile. To date the localization of C_{14} hydraphile, our most potent adjuvant, has never been studied. We therefore synthesized dansyl labeled C_{14} hydraphiles. Co-localization of these compounds were studied in Tet^R *E. coli* and HEK-293 mammalian cells. Here, we test the hypothesis that if hydraphiles localize in the membrane of bacteria, then they disrupt the membrane integrity and increase the membrane permeability.

4.4.1.1. Localization in bacterial cells. Dansyl labeled hydraphile was added to Tet^R *E. coli* along with a known membrane localizing stain. A lipophilic molecule, FM4-64 FX, was used to conduct co-localization studies (figure 4.2). [39] FM4-64 FX exhibits low fluorescence when in dissolved water. However, when it localizes in the outer leaflet of the bilayer membrane, its fluorescence intensity increases. Its excitation and emission peaks are 515 nm and 640 nm.[39] We contemplated using DAPI as a cytoplasmic stain as well. DAPI fluoresces after it binds to the AT region of the DNA.[40] It is commonly used as a counterstain to mark cell cytoplasm in the bacteria. The excitation and emission peaks of DAPI are 358 nm and 461 nm, respectively. Its peaks are distinct from FM4-64 FX but overlap with those of the dansyl groups of compound **1**. The excitation and emission peaks of dansyl group were ~ 335 nm and 500 nm, respectively. Hence, DAPI and dansyl labeled hydraphiles could not be used for a co-localization study.

Controls. We first performed the control with Tet^R *E. coli* individually treated with either FM4-64 FX or dansyl C_{14} hydraphile. Here, the exponential phase *E. coli* were concentrated at O.D.600 ~ 1.3 and suspended in phosphate buffered saline (PBS). A desired concentration of either FM4-64 FX or dansyl C_{14} hydraphile was added to the bacteria. The cells treated with FM4-64 FX were incubated for 10 minutes, washed and observed under a confocal microscope. *E. coli* treated with dansyl C_{14} hydraphile were observed under the microscope,

immediately after adding the hydraphile. The results are reported as the unaltered images acquired under the Zeiss LSM 700 confocal microscope with 640X magnification (figure 4.3.1). Under magnification greater than 640X, the image did not focus well due to the small size of *E. coli* cells. Instead we digitally magnified the images and added as inserts with yellow boundary in the figure 4.3 to show clear localization of the stains. The top row of figure 4.3.1 indicates bright field image of the cells, fluorescence in the middle row and overlay of these two in the third row.

A clear membrane localization of FM4-64 FX (5 μg/mL, red) is observed 10 minutes after incubation with Tet^R *E. coli* (figure 4.3.1). This confirms that the membrane localization in bacteria could be observed under a confocal microscope. Next we determined the localization of dansyl C_{14} hydraphile at 2, 8 and 32 μM. The high concentration of the hydraphile used corresponds to the high concentration ($O.D._{600}$ ~ 1.3) of cells required for this experiment. We confirmed that the MIC of benzyl C_{14} hydraphile was 8 μM at this *E. coli* cell concentration. At 2 μM of compound **1**, only 2 to 3 cells/frame were observed with the dansyl fluorescence. Only one *E. coli* was observed with clear membrane localization (data not shown). We therefore tested higher concentrations of 8 and 32 μM.

At 8 μM of dansyl C_{14} hydraphile, an increased number of the Tet^R *E. coli* had dansyl fluorescence. These cells showed clear membrane localization (middle column, figure 4.3.1). Cytoplasmic localization was also observed. However, no aggregation of hydraphiles was apparent at 8 μM. When manually counted, 38% of the fluorescent cells had membrane localization with hydraphiles. In comparison 100% of fluorescent cells had membrane localization with FM4-64 FX. This could be due to the lower fluorescence intensity of dansyl as compared to the FM4-64 FX. We therefore determined localization at 32 μM of dansyl C_{14} hydraphile. Two key observation were made. First, greater number of cells were fluorescent with dansyl labeled C_{14} hydraphile. Second, membrane and cytoplasmic localization was observed clearly (right column, figure 4.3.1). When manually counted, 55% of the fluorescent cells had membrane localization with hydraphiles at 32 μM. Similarly, images from three different trials (data not shown) were analyzed and a standard deviation of 10% was observed. We therefore used 32 μM for the co-localization studies reported in figure 4.3.2.

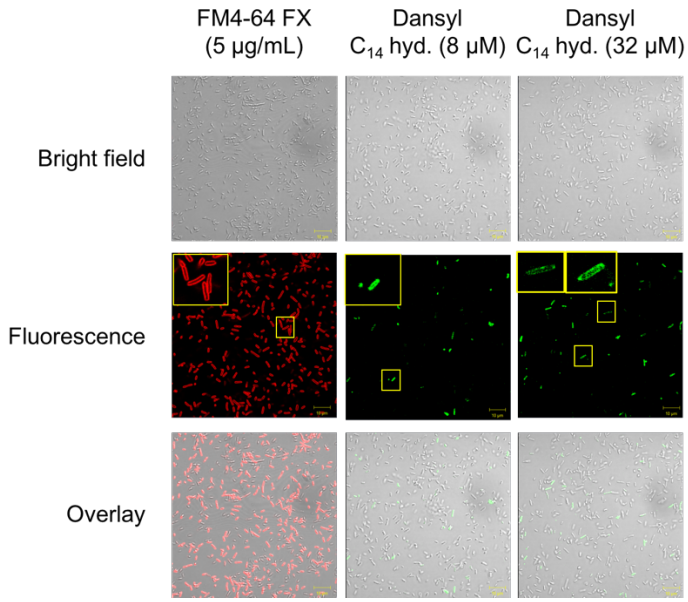

Figure 4.3.1. Independent localization of FM4-64 FX (5 µg/mL) and dansyl C_{14} hydraphile (8 µM and 32 µM) in Tet^R *E. coli*. Images were acquired on confocal microscope immediately after treating the cells. The scale is 10 µm.

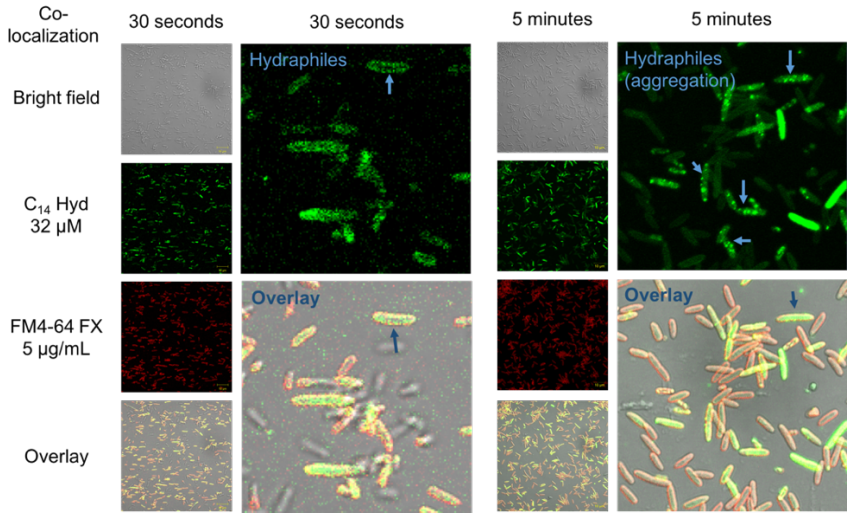

Figure 4.3.2. Co-localization of dansyl C_{14} hydraphile (32 µM) with FM4-64FX (5 µg/ml) in Tet^R *E. coli*. FM4-64 FX and dansyl C_{14} hydraphile were fixed after 30 seconds and 5 minutes with 4% (v/v) formaldehyde. In 30 seconds membrane localization and in 5 minutes, aggregates and, membrane and cytoplasmic localization was observed. The scale is 10 µm.

Co-localization. For co-localization studies Tet^R *E. coli* cells were first treated with FM4-64 FX (5 μg/mL) for 10 minutes and fixed with ice cold 4% (v/v) formaldehyde. These cells were washed and treated with 32 μM dansyl C_{14} hydraphile for either 30 seconds or 5 minutes. The stain was fixed with formaldehyde, washed with phosphate buffered saline and observed under a confocal microscope. One of the images from a Z-stack of 30 seconds and 5-minute treatment is shown in figure 4.3.2 above. The larger images are digitally magnified to clearly indicate localization of the stains. At 30 seconds, membrane localization of both dansyl C_{14} hydraphile (green) and FM4-64 FX (red) was observed. In the overlay image for 30 seconds, a yellow (red + green) color in the membrane confirms membrane localization of hydraphiles. Cytoplasmic localization could also be observed in these cells.

After 5-minute the fluorescence the image was clear, without any background. Three observations were made after 5 minutes of treatment with hydraphiles. First, membrane localization is further confirmed in the overlay image with yellow color in the periphery of the bacteria. Second, some cells had cytoplasmic localization of dansyl C_{14} hydraphile. This could be an effect of cell death or increase in membrane permeability caused by hydraphiles itself. Third, as reported previously, aggregates of hydraphiles were clear in the membrane of the Tet^R *E. coli*. These aggregates were either on the surface of the bacteria or in the membrane. We further analyzed these aggregates in the SEM study below under membrane disruption. Co-localization studies confirmed that the dansyl C_{14} hydraphile localizes in the membrane of *E. coli*, as early as 30 seconds after treatment. Dansyl C_{14} hydraphile aggregates in or on the membrane around 5 minutes after treatment. *E. coli* with cytoplasmic localization of dansyl C_{14} hydraphiles was also observed.

Overall, dansyl C_{14} hydraphile clearly showed localization in the periphery of the Tet^R *E. coli*. It is possible that this localization of hydraphile depends on the concentration and time of the treatment. We therefore observe a mixture of cell population with either membrane localization, cytoplasmic localization and both.

4.4.1.2. Localization in mammalian cells. Hydraphiles have been previously shown to form channels even in mammalian cells.[33] For hydraphiles to form channels in the mammalian cell membrane, they have to localize in the membrane. Eukaryotic cells have multiple organelles that are surrounded by

membranes, such as mitochondria, endoplasmic reticulum, nucleus, *etc*. If hydraphiles do localize in the cytoplasm of mammalian cells, they could also insert in to the membranes of any of the membrane covered organelles. Hydraphiles could also affect the endocytosis pathway. We hypothesized that if hydraphiles localize in the cytoplasmic membrane of mammalian cells, then they could form ion channels in the mammalian cells. Benzyl C_{14} hydraphile was shown to form channels in HEK-293 cells.[33] To test the localization in mammalian cells, we used dansyl labeled C_{14} hydraphile. FM4-64 FX was used to tag membranes.

Controls. We first performed controls with HEK-293 cells either treated with no stain, FM4-64FX or dansyl C_{14} hydraphile. The results of the controls are shown in figure 4.4 below. Here, each stain was studied as a separate experiment and does not represent a co-localization study. The results are divided into three rows, bright field, fluorescence and overlay. The 'bright field' shows the image of the HEK-293 cells without any excitation of the fluorescent tags. The 'fluorescent' image shows the localization of the fluorescent tags whereas the 'overlay' images are the overlay of bright field and the fluorescent image. In each case the cells in the bright field image appears healthy, except maybe for dansyl C_{14} hydraphile. No study was performed here to confirm viability of the cells. Higher concentrations dansyl C_{14} hydraphile (32 μM) were required to observe optimal fluorescence from the dansyl groups. These conditions might not be optimal for the viability of the cells. The FM4-64 FX shows localization in the cell membrane of HEK-293 cells, as seen with fluorescent and overlay images.

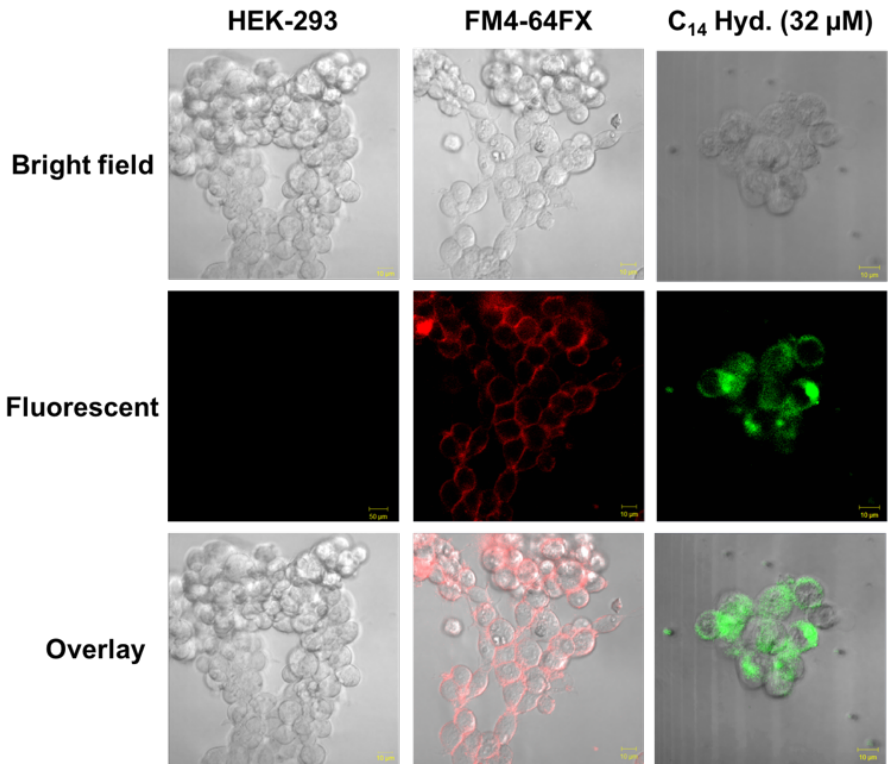

Figure 4.4. Localization of FM4-64FX (red), and dansyl labeled C_{14} hydraphile (green) in mammalian cells. The magnification is 400-X and the scale is 10 µm.

When dansyl tagged C_{14} hydraphile was added to the HEK-293 cells, two observations were made. First, all the cells showed either membrane or nucleus localization. In a few cells, only membrane localization was observed. Second, in at least two cells localization in the nucleus was observed. These cells might be undergoing cell death allowing hydraphiles to enter the cytoplasm and the nucleus. Overall, dansyl C_{14} hydraphile was observed in the cytoplasmic membrane of HEK-293 cells. In some cases, cytoplasmic or nuclear localization is also possible. Membrane localization is confirmed with co-localization with FM4-64 FX below.

Co-localization of FM4-64 FX and dansyl C_{14} hydraphile. To confirm that hydraphiles were in the cell membrane of HEK-293 cells, a co-localization study was performed. Here, the optimally cultured HEK-293 cells were suspended in PBS containing FM4-64 FX and dansyl C_{14} hydraphile (32 µM). The localization of cell membrane stain FM4-64 FX along with dansyl C_{14} hydraphile would confirm plasma membrane localization. It is possible that localization of FM4-64

FX in the membrane could affect the localization or the fluorescence properties of the dansyl C_{14} hydraphile. The FM4-64 FX localizes only in the outer leaflet of the bilayer. It only fluoresces in the hydrophobic regime. Dansyl C_{14} hydraphile spans the lipid bilayer. The distal crowns and the attached fluorescent dansyl groups localize in the midpolar region of the bilayer, in both leaflets. The dansyl fluorescence however may or may not be effected by the polarity. If hydraphiles disrupt the membrane structure, the fluorescence of FM4-64 FX may be affected and *vice versa*. The results of these experiments are shown below in figure 4.5.

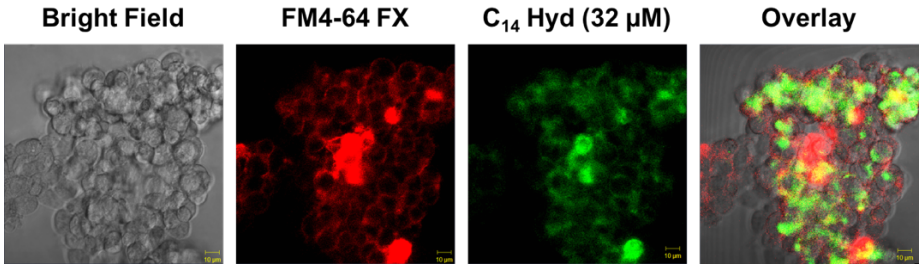

Figure 4.5. Co-localization of dansyl C_{14} hydraphile (green) with FM4-64 FX (red).

When FM4-64 FX was added along with dansyl C_{14} hydraphile, both stains were found in the plasma membrane (figure 4.5). Dansyl C_{14} hydraphile was observed in the cell membrane. Some hydraphiles were also found in the cell cytoplasm and nucleus. The nuclear staining could be caused by cell death. Few of the cells' membranes that were fluorescent with FM4-64 FX were not fluorescent with dansyl C_{14} hydraphile. It was also observed that in the overlay, only a few cells were observed with both FM4-64 FX and dansyl C_{14} hydraphile (yellow staining). These results confirm that hydraphiles do localize in the cytoplasmic membranes of mammalian cells. It is also likely that hydraphiles can be found in the cell cytoplasm and nucleus. This staining by hydraphile was similar to that observed in the bacteria. The concentration of hydraphiles required in HEK-293 and Tet^R *E. coli* was 32 µM. Membrane localization in *E. coli* was also observed at concentration as low as 2 µM. This shows that there should be some selectivity in membrane localization between bacteria and mammalian cells at the concentration used for synergy, *i.e.* 1 µM.

Hydraphiles do localize in the cellular membranes. They could also localize in the cell cytoplasm. However, the cytoplasmic localization could be an artifact of cell death induced by hydraphiles localized in the membrane.

4.4.2. *E. coli* membrane disruption. The combination studies showed that all four benzyl hydraphiles (C_8 - C_{14}) rescued antibiotic potency against efflux pump expressing resistant *E. coli* and *K. pneumoniae*. The activity of hydraphiles was similar to that of CCCP and colistin. Colistin is a well-known membrane disruptor and it showed 16- to 32-fold tetracycline recovery against Tet^R *E. coli*. We hypothesized that if hydraphiles localize in the membrane of *E. coli* cells, then it could disrupt membrane integrity and increase the permeability of bacterial membranes. Localization studies showed that hydraphiles do localize in the membranes of mammalian and bacterial cells. They were also found in the cell cytoplasm. We next wanted to observe if hydraphiles caused disruption of membrane integrity in Tet^R *E. coli*. We hypothesized that if ½ [MIC] hydraphiles caused disruption of *E. coli* cell membrane, then the changes in the surface (membrane) of *E. coli* would be observed by SEM analysis.

To confirm if benzyl C_8 and C_{14} hydraphile both affect the membrane integrity of individual bacterial cells, we used scanning electron microscopy. Here the Tet^R *E. coli* cells were treated with ½ [MIC] of benzyl C_8 and C_{14} hydraphiles, loaded on to a membrane, fixed and stained before observing by SEM (figure 4.6). The samples were observed at 10,000X to 50,000X magnification.

Under the 'amphiphile alone' column of figure 4.6, membrane background alone (top) and an aggregate formed by benzyl C_{14} hydraphile (bottom) in the absence of bacteria was observed. Similar aggregates were observed with benzyl C_8 hydraphiles. In an untreated Tet^R *E. coli* cell, the membrane is corrugated and no membrane disruption or aggregates is apparent. When the Tet^R *E. coli* was treated with either hydraphiles or hydraphile + tetracycline, three key features were observed with both benzyl C_8 and C_{14} hydraphiles (figure 4.7).

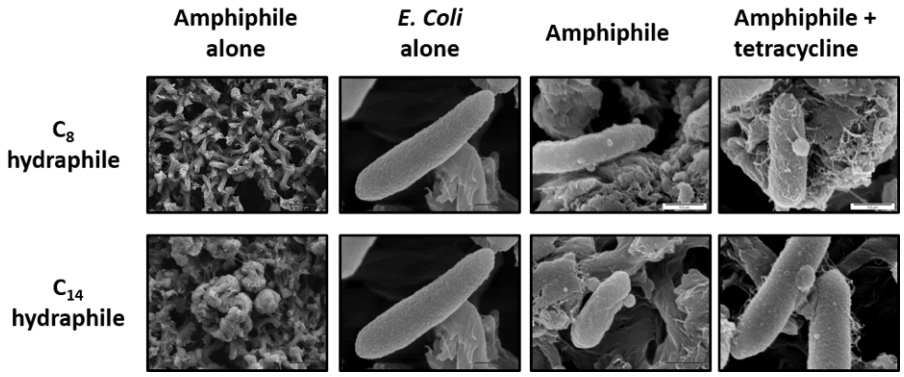

Figure 4.6. Scanning electron microscopy images of Tet^R *E. coli* treated with benzyl C_8 hydraphile (top panel) and benzyl C_{14} hydraphile (bottom panel). The column labeled 'amphiphiles alone' shows the nylon membrane (top) and benzyl C_{14} hydraphile aggregate (bottom).

First, we observed uniform, well-formed aggregates of hydraphiles of approximately 100-120 nm on the surface of *E. coli* (figure 4.8, a). Hydraphiles may form uniform 100-200 nm aggregates before attaching and inserting to the *E. coli* membranes. Alternatively, these aggregates may have also formed after the disruption of *E. coli* membrane. In such a case these aggregates may comprise a mixture of *E. coli* membrane lipids and hydraphiles.

Secondly, we observed irregular blisters on the surface of *E. coli* membranes. These blisters were distinct from the hydraphile aggregates observed. In figure 4.8, b, blisters and aggregates are both observed. It is known that if cytoplasmic membrane is disrupted, the cytosolic content is released in the periplasmic space, forming a blister/bulge of the outer membrane.[41] Hence, hydraphiles that disrupted the inner membrane could have formed the blisters from the outer membrane.

Lastly, we observed some bacteria with membrane smoothening (figure 4.8, c). It is known that under osmotic stress, water uptake by bacteria cause swelling of the bacterial cell.[41] This swelling would cause the corrugated membrane to stretch and become smooth. Taken together, these images show that hydraphiles form aggregates that attach to the bacterial surface. These hydraphiles could both transport ions as observed with membrane smoothening and disrupt membranes as observed with membrane blisters. These results also confirm that hydraphiles are membrane active compounds in bacterial cells. We

are currently quantifying the number of cells that have the properties outline above.

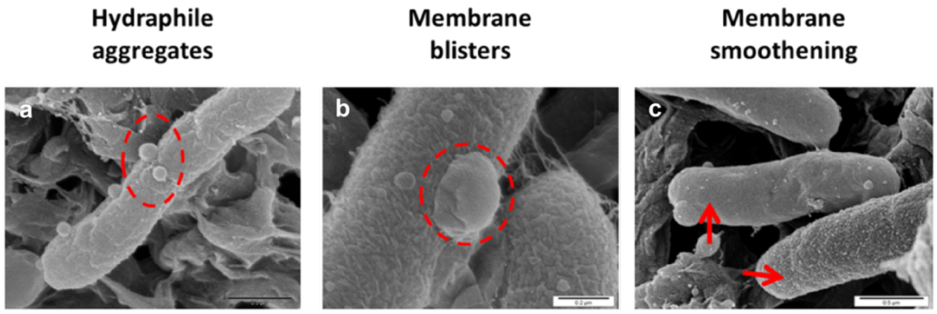

Figure 4.7. Scanning electron microscopy images of common traits observed with Tet^R *E. coli* was treated with either benzyl C_8 hydraphile and benzyl C_{14} hydraphile. The image on the left shows a hydraphile aggregate (a), a membrane blister in the middle (b) and the membrane smoothening on the right (c).

4.4.3. Increase in membrane permeability. We have confirmed that when hydraphiles are added to Tet^R *E. coli*, it localizes in the cell membrane and disrupts membrane integrity. This should result in an increase in membrane permeability. Here we test if hydraphiles could increase the permeability of *E. coli*. We also determined if hydraphiles could increase the permeability of mammalian cells (HEK-293). In these studies, we used propidium iodide localization and nucleic acid release to show that benzyl C_8 - C_{14} hydraphiles increase membrane permeability. Potassium release is also considered to be an evidence of increased membrane permeability. Potassium transport is a known function of hydraphiles. We have addressed K^+ transport in chapter 5. Below is the evidence for an increase in membrane permeability caused by hydraphiles.

4.4.3.1. Bacterial cell membrane permeability. We first tested the permeability of Tet^R *E. coli* using a membrane impermeable stain propidium iodide (PI) and cell viability stain fluorescein diacetate (FDA). PI does not permeate viable cells. However, it is known to enter the cells when membrane integrity is compromised or as a result of cell dead. Once PI localizes in the bacterial cytoplasm, it intercalates with nucleic acids and its fluorescence intensity increases dramatically. Hence, PI serves as a stain to probe cell membrane permeability. However, cells that are PI positive may also be dead. We therefore used FDA, a cell viability stain. Fluorescein diacetate is permeable to cell

membrane but not fluorescent. Once it enters the cytoplasm of viable cells, FDA is converted to fluorescein by endogenous esterase enzymes. The fluorescence from fluorescein indicates viable cells.

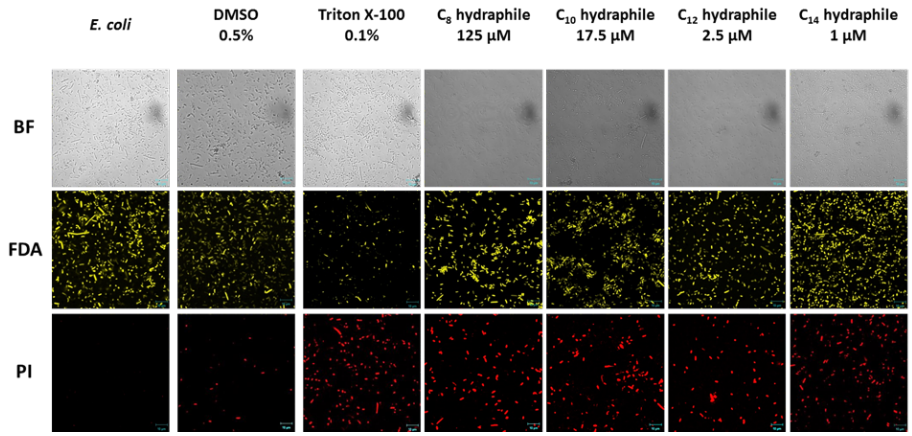

Figure 4.8. Benzyl C_8-C_{14} hydraphiles (1/2 [MIC]) mediated permeability of propidium iodide and fluorescein diacetate in Tet^R *E. coli*. *E. coli* alone, DMSO (0.5% v/v) and triton X-100 (0.1% v/v) were used as controls.

We used Tet^R *E. coli* alone and Tet^R *E. coli* treated with 0.5% (v/v) DMSO and 0.1 % (v/v) triton X-100 as controls. As expected, all the cell in *E. coli* alone control were alive and showed fluorescence for FDA (yellow) and none for PI (red). This shows that the membranes of live cells were not permeable to PI when the Tet^R *E. coli* was untreated. DMSO was used as a solvent for hydraphiles. It was necessary to confirm that the amount of DMSO added was inconsequential because DMSO affects membrane permeability[42,43] and biological activity.[44] The presence of DMSO in some cases mimics the action of water channels at sufficiently high concentrations.[45] After treating the Tet^R *E. coli* cells with 0.5% DMSO, the results were similar to that of Tet^R *E. coli* alone control. We observed only 2-3 cells that showed PI fluorescence. Triton X-100 is a well-known and characterized detergent. When used at 0.1% v/v (1.6 mM), Triton X-100 disrupts bacterial and mammalian cell membranes. We therefore used, Triton X-100 as a control. In the presence of triton X-100, there was PI fluorescence increase and cell viability decreased dramatically.

The highest concentration of hydraphile that showed synergy was at ½ [MIC]. A growth curve confirmed that at ½ [MIC] of benzyl C_{14} hydraphile there was no inhibition of growth. We therefore tested all the hydraphiles at one-half

the MIC concentration for their ability to increase Tet^R *E. coli* membrane permeability. If any increase in permeability would be observed with ½ [MIC] hydraphiles, it would be an effect of membrane localization rather than cell death. FDA would qualitatively confirm cell viability as well. Previously reported kinetics studies showed that hydraphiles at bactericidal concentration could kill half the *E. coli* cell population in 9 minutes.[36] Here, the Tet^R *E. coli* cells were treated with hydraphiles, PI and FDA for 10 minutes.

The ½ [MIC] of benzyl C_8, C_{10}, C_{12}, and C_{14} hydraphile, used here were 125 μM, 17.5 μM, 2.5 μM and 1 μM, respectively. As seen in figure 4.9, in the presence of these concentrations of benzyl C_8-C_{14} hydraphiles, the cell viability (FDA panel, yellow) was comparable to that of *E. coli* alone controls. This confirms that the ½ [MIC] concentration does not result in a decrease in cell viability. After treatment with the hydraphiles, PI permeability (PI panel, red) was clearly increased. The increase in PI staining was comparable to that of Triton X-100 at 0.1% or 1600 μM. It could be argued that the images do not confirm that the viable cells are the same cells with increased cell membrane permeability. The increase in cell membrane permeability could be attributed to the toxicity of hydraphiles. On the contrary, the results from growth curves did not show any inhibition of *E. coli* growth in presence of ½ [MIC] benzyl C_{14} hydraphile. We thus confirmed the membrane disruption and increase in membrane permeability using SEM and nucleic acid release, respectively.

4.4.3.2. Nucleic acid release data. An increase in bacterial membrane permeability causes the release of the cytosolic content. The potassium concentration in *E. coli* cytosol is ~200 mM and that of media is ~10 mM. Another major component of bacterial cytoplasm are nucleic acids. Hence, when the cytosolic membrane is disrupted, it causes leakage of both potassium ions and nucleic acids. Hydraphiles are known to form cation channels that could transport K^+ ions. Ion transport by hydraphiles is not necessarily related to membrane disruption and leakage. Benzyl C_8-C_{14} hydraphiles do transport K^+ ions and the analysis of ion transport is outlined in chapter 5.

We determined if 260 nm absorbing material (DNA and RNA) was released from Tet^R *E. coli* after treatment with benzyl C_{14} hydraphile for 10 minutes. The results are outlined in figure 4.9 below. A UV/Vis spectrophotometer was used to measure absorbance at 260 nm. The cell concentration of Tet^R *E. coli* required for this experiment was 5.6×10^8 cells/mL. The MIC of benzyl C_{14} hydraphile at this high cell concentration was 8 μM. We

therefore determined nucleic acid release at 1, 2, 5 and 8 μM of benzyl C_{14} hydraphile concentrations. DMSO at 0.25% (v/v) was used as 0 μM data point. The MIC concentration of benzyl C_{14} hydraphile induces cell death. At this MIC concentration, nucleic acids are released into the cell surrounding. When sub-lethal concentration of benzyl C_{14} hydraphile (5 μM) was used, nucleic acid release was still observed. The amount of nucleic acids released in the presence of 5 μM benzyl C_{14} hydraphiles were half as much as observed with 8 μM C_{14} hydraphile. No nucleic acids were released at 1 or 2 μM of benzyl C_{14} hydraphile. This study confirms that nucleic acid release correlates to increasing, but sub-lethal, concentration of benzyl C_{14} hydraphile.

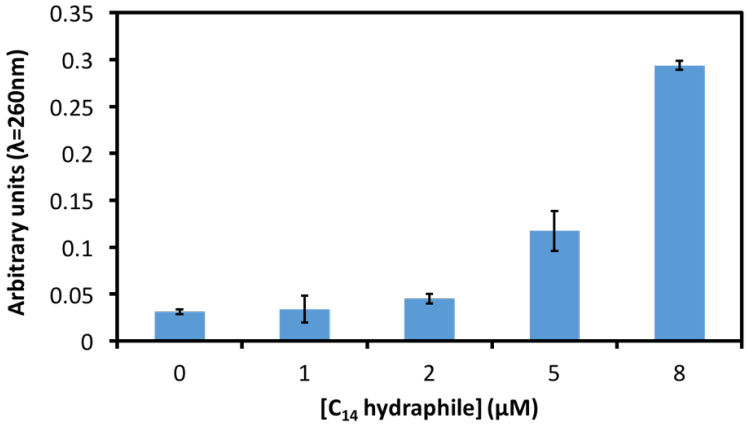

Figure 4.9. Nucleic acid release from Tet^R *E. coli* after treatment with benzyl C_{14} hydraphile for 10 minutes. The x-axis represents the concentration of benzyl C_{14} hydraphile used and the y-axis represents the absorbance at 260 nm. Experiment represents the average of three trials and the error bar represents the standard deviation in the results.

4.4.3.3. Permeability of HEK-293 cells. We previously confirmed that hydraphiles could localize in the membrane of HEK-293 cells. It could be argued that hydraphiles also localize in and disrupt mammalian cell membranes. We therefore tested the effect of benzyl C_8-C_{14} hydraphiles on the membrane integrity of mammalian HEK-293 cells (figure 4.11). We used PI and FDA to study the *E. coli* membrane permeability. The HEK-293 cells were cultured to optimal conditions and treated with hydraphiles, PI and FDA for 2 hours before confocal microscopic examination.

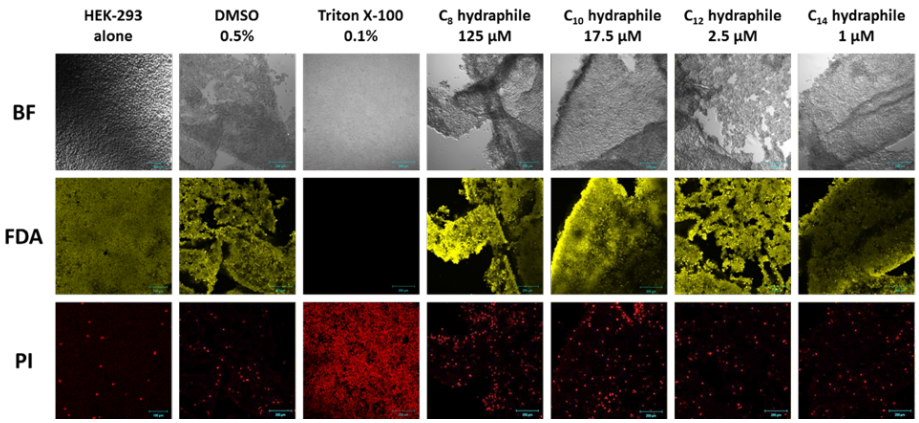

Figure 4.10. Benzyl C_8-C_{14} hydraphiles (1/2 [MIC]) mediated permeability of propidium iodide and fluorescein diacetate in HEK-293 cells. HEK-293 alone, DMSO (0.5%) and Triton X-100 (0.1%) were used as controls.

HEK-293, DMSO (0.5%) and Triton X-100 (0.1%) were used as controls. For HEK-293 alone and DMSO, there was high cell viability and membranes were impermeable. At 0.1% or 1.6 mM Triton X-100 (a detergent), it killed all the HEK-293 cells and disrupted membranes showing high PI fluorescence. No FDA fluorescence was observed in the presence of Triton X-100. However, the viability was high and minimal PI fluorescence was observed with ½ [MIC] of benzyl C_8-C_{14} hydraphiles (figure 4.10). This shows that at ½ [MIC] hydraphiles disrupt bacterial membrane but does not affect mammalian membranes. We confirmed that even at 2 X [MIC] concentrations, hydraphiles do not affect HEK-293 mammalian cell membrane integrity. High cell viability and no increase in cell membrane permeability was observed for HEK-293 cells treated with at 2 X [MIC] of benzyl C_8-C_{14} hydraphiles for 2 hours (data not shown). A selectivity in the ability of hydraphiles to increase the bacterial membrane permeability was observed. A change in mammalian cell membrane permeability may be possible, but at a much higher concentration of hydraphiles.

4.5. Summary and conclusions.

Hydraphiles have been known to have antimicrobial properties that correlates to their ability to transport cations. We recently reported that hydraphiles could be used to increase antimicrobial potency against sensitive strains of bacteria. Against Gram negative bacteria, the activity of hydrophobic antibiotics (rifampicin and erythromycin) was enhanced more than hydrophilic antibiotics. This suggests a mechanism involving increased membrane

permeability. Hydraphiles also rescued antimicrobials potency against multi-drug resistant Gram negative bacteria. The hypothesis involved inhibition of efflux pump activity and increased membrane permeability by hydraphiles. To our knowledge, no mechanistic studies for antimicrobial properties of synthetic amphiphiles have been reported to date.

As reported here, hydraphiles localize in the membranes of both bacterial and mammalian cells. Hydraphiles were also found in the cytoplasm of the bacterial cells and the nucleus of the mammalian cell. This could be an effect of cell death or an increase in the membrane permeability. The localization studies do require higher magnification and resolution than is currently available to confirm the localization in mammalian cells. We propose the use of high-resolution microscopy to observe localization at different concentrations and duration of treatment. As observed in the SEM study, hydraphiles clearly disrupt the membrane of *E. coli* cells. Hydraphiles were observed to form aggregates of 100 – 200 nm in size. These aggregates fuse with the bacterial membrane allowing for localization of hydraphiles in the membrane. This effect of hydraphile on bacteria was previously undocumented. Once hydraphiles insert in the membrane of bacteria, it exerts osmotic stress due to disruption of ion gradient. Eventually the cytoplasmic membrane disrupts forming blisters observed on the outer membrane of bacteria. TEM studies of a cross-section of a hydraphile treated *E. coli* could be used to confirm these observations.

Hydraphiles increase the membrane permeability of *E. coli* cells. At ½ [MIC] of benzyl C_8-C_{14} hydraphiles, PI fluorescence increased as was that of fluorescein. This shows that hydraphiles at sub-lethal concentrations could increase membrane permeability without decreasing the overall cell viability. The increase in membrane permeability was confirmed by the leakage of potassium ions and nucleic acids (260 nm absorbing material) from the cells. In both of these experiments, only partial leakage of cytosolic content was observed at ½ [MIC] of the hydraphiles used. This could account for the increase in the lag phase observed for the *E. coli* growth curve (chapters 2 and 3). However, bacterial growth was not ultimately inhibited. Increased membrane permeability could allow for increased transport of antibiotics through the cell membrane. This would increase antibiotic concentration in the cell cytoplasm that could be observed as an increase in the antibiotic potency. Efflux pumps may still affect antibiotic accumulation in cell cytoplasm. The effect of hydraphiles on efflux pumps and the accumulation of antibiotic in the cell cytoplasm is addressed in chapter 5.

Interestingly, hydraphiles did not increase the permeability of HEK-293 mammalian cells, even at twice the MIC concentrations. Clearly there is a difference in the hydraphile activity observed between bacterial and mammalian cells. The composition of mammalian cell membrane and *E. coli* cell membranes are dramatically different. The phospholipids such as phosphatidylglycerols found in the bacterial membrane are acidic.[46] The LPS of the Gram negative bacteria also consists of anionic molecules.[46] In Gram positive bacteria, the teichoic acids or lipoteichoic acids are anionic in nature.[46] It is well-known known that many of the antimicrobial peptides, including colistin, interact with bacteria because they are both amphiphilic and cationic.[47] The cationic property allows for selectivity between bacteria and mammalian cells. The mammalian cells have zwitterionic phosphatidylcholine in the outer leaflet of the membrane.[48] They could have hydrophobic interactions with hydraphiles, exerting cytotoxicity. However, since the acidic phospholipids are localized in the inner leaflet of the mammalian membranes, some selectivity of hydraphiles seems possible. Mammalian membranes contain cholesterol. In liposomal studies, the addition of cholesterol to liposomes exhibited a change in hydraphile activity related to changes in membrane thickness.[34] Hence, it is not surprising that hydraphiles show some selectivity in increasing cell membrane permeability. The cytotoxicity of hydraphiles are addressed in chapter 5.

Colistin is currently considered as the antibiotic of last resort due to its cytotoxicity. Resistance development to colistin has been recently reported. Colistin binds to the lipidA component of Gram negative bacteria and disrupts membrane integrity. It could be considered as a natural amphiphiles. Another natural amphiphile, daptomycin disrupts the membranes of Gram positive bacteria. Here, we have shown that hydraphiles disrupts membranes of Gram negative bacteria. Hydraphiles may hold potential not only as an adjuvant but also as antibiotics. The amphiphilic and cationic nature of hydraphiles is similar to many of the cyclic antimicrobial peptides. Hydraphiles are completely synthetic molecules. Its structure could be modified to understand the role of hydrophobicity and charge to make it highly selective to bacteria. Studies with synthetic amphiphiles with permanent positive charges are underway.

4.6. Experimental procedure.

4.6.1. Compounds and bacteria used. Benzyl C_8 – C_{14} benzyl hydraphiles were prepared by Gokel lab. To conduct the localization two new amphiphiles were designed and prepared. Benzyl side arms were substituted for dansyl side arms in dansyl C_{14} hydraphile. The purity of compounds were confirmed by the NMR and mass spectrometry. FM4-64 FX and propidium iodide were acquired from Thermo-Fischer. Fluorescein diacetate was acquired from Sigma-Aldrich.

The Tet^R *E. coli* strain used here was made by transforming competent *E. coli* cells with pBR322 plasmid. This strain was used for combination studies in chapter 3 and for localization, permeability, nucleic acid release and scanning electron microscopy studies conducted here. The Tet^R *E. coli* was cultured in L. B. Miller media containing 100 µg/mL Ampicillin.

4.6.2. Localization in bacterial cells. Tet^R *E. coli* was grown overnight from one CFU in L.B. miller media containing 100 µg/mL Ampicillin. *E. coli* was knocked back and grown to O.D. (600 nm) = 1.300 before use. Cells were centrifuged at 6500 RM (3000Xg) for 5 minutes and resuspended in PBS. Dansyl labeled C_{14} dansyl hydraphile (C_f = 2 µM) was added to 1990 µL of cells. For co-localization study, FM4-64 FX (C_f = 10 µg/mL) were also added. The cells were incubated for 5 minutes at 200 RPM and 37°C. Cells were washed again at 6500 RPM (3000X g) for 5 minutes and re-suspend the cells in fresh PBS. A 20 µL sample was loaded on to clean glass slide and covered with coverslip. The slide was observed using Zeiss LSM 700 microscope.

4.6.3. *E. coli* Membrane permeability using PI and FDA. To test the membrane permeability of the Tet^R *E. coli,* the bacteria was first grown overnight from one CFU in media containing 100 µg/mL Ampicillin at 37°C and 200RPM. Tet^R *E. coli* was then knocked back to O.D. 600 nm = 0.550 before use. In a sterile test tube, cells were added followed by either triton X-100, DMSO or compounds **1-4** at half-MIC concentrations and incubated at 37°C and 200 RPM. The concentration of DMSO was kept constant at 0.5% -by volume in each case. After 30 minutes of incubation, the cells were washed by centrifugation at 3000xg for 5 minutes and re-suspended in sterile phosphate buffered saline (PBS). Propidium iodide (30 µM, Thermo-Fischer) and fluorescein diacetate (60 µM, Sigma-Aldrich) were added to the Tet^R *E. coli* cells in the PBS, mixed by vortexing and incubated at 37°C and 200 RPM. After 30 minutes, the cells were washed again by centrifugation at 3000xg for 5 minutes. The pellet was suspended in a fresh PBS,

loaded onto a clean glass slide, covered with a cover slip and observed under Zeiss LSM 700 confocal microscope.

4.6.4. Localization and permeability of HEK-293 cells. HEK-293 cells were donated by Dr. Michael Nichols. Cell lines were regularly maintained in growth media containing DMEM (ATCC), 10% fetal bovine serum (FBS, ATCC) and 1% penicillin-streptomycin solution (ATCC). Adherent HEK-293 cells were trypsinized using 0.25% (w/v) trypsin-EDTA (Sigma-Aldrich), suspended in a fresh media and diluted to get a concentration of 3×10^5 cells/ml. Cells were seeded in a 96-well plate (100 µL/well) to get 3×10^4 cells/well. The plates were incubated for 24 hours at 5% CO_2 and 37°C to reach a confluency of 80-90%.

In a sterile 1.5 mL micro-centrifuge tube, dansyl C_{14} hydraphile and dansyl C_{16} lariat ether (0.5% DMSO) were mixed with PBS. FM4-64 FX, DAPI and PI were also added to PBS for co-localization study. The spent media in the plate was replaced with 100 µL media containing dansyl labeled amphiphiles and other stains. The cells were incubated at 37°C and 5% CO_2 for 30 minutes before observing under Zeiss LSM 700.

For permeability study, the cells were cultured as outlined above. HEK-293 (90% confluent) were then seeded in a 96-well plate to get 30,000 cells/well. After 24 hours of incubation at 37°C and 5% CO_2, the spent media was replaced with media (DMEM and 10% FBS) containing compound **1-4** at ½ X or 2 X [MIC]. Triton X-100 at 0.1%-by volume (1670 µM) and DMSO 0.5% -by volume were also used as controls. After 2 hours of incubation, spent media was replaced with PBS containing propidium iodide (30 µM) and fluorescein diacetate (60 µM) and incubated at 37°C and 5% CO_2. After 2 hour of incubation, the spend media was replaced with fresh PBS and the cells were observed under Zeiss LSM 700 confocal microscope. The images were reported without any alterations. The gain and the intensity in all the images were kept constant.

4.6.5. Scanning electron microscopy. To perform scanning electron microscopy Tet^R *E. coli* was grown overnight from one CFU in L.B. miller media containing 100 µg/mL Ampicillin. *E. coli* was knocked back and grown to O.D. (600 nm) = 1.000 before use. For *E. coli* alone control, the filter membrane (0.45 µm) was dipped in the *E. coli* culture. *E. coli* was treated with various concentration of C_8 and C_{14} hydraphiles for 10 minutes before loading on to filter membrane. The cells were fixed by transferring the filter membrane containing *E. coli* cells to 2.5% (v/v) glutaraldehyde (stock 25%) for 60 min. Cells were washed for 15

minutes by transferring the membrane in PBS. The cells were stained by transferring the membrane to 1%(v/v) OsO_4 for 1 hour. Serial dehydration of the sample was performed with 30%, 50%, 70%, 80%, 90%, 100%, 100% (v/v) ethanol. Sample was dipped in each ethanol concentration for 10 minutes. The cells were critical-point dried and sputter coated with gold before observing under JOEL 6320F SEM. The images were acquired and reports without any modifications.

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CHAPTER 5

Hydraphiles: Non-Resistant Molecules that Indirectly Inhibit Efflux Pump Activity with Minimal Cytotoxicity

The data reported in this chapter has not been published yet but is covered under two patent applications.

5.1 Introduction.

Antimicrobial resistance is a major health concern.[1] The urgency of developing new antibiotics and approaches for the treatment of multi-drug resistant bacterial infections cannot be overstated.[2] This problem could be addressed by the development of new antibiotics or by rescuing the efficacy of existing antibiotics.[3] To our knowledge Teixobactin is the only new class of antibiotic currently in development.[4] The interest in adjuvant for current antibiotics has been growing with many new adjuvants in development. Only one antibiotic was approved in 2015,[5] and it was an antibiotic adjuvant (β-lactamase inhibitor).[6] Searching for and developing new antibiotics has proven to be a daunting task.[7] This is due to our inability to isolate and culture bacteria from different sources such as soil. Therefore, antibiotic adjuvants provide a unique opportunity. Adjuvants are molecules that inhibit the known resistance mechanisms in bacteria and restore antibiotic potency. This circumvents the need to discover new antibiotics and addresses the antibiotic resistance problem.

One of the three mechanisms[8] through which bacteria develop resistance is called efflux pumps.[9] Efflux pumps are transmembrane proteins that export antibiotics from the cell cytoplasm to its surrounding.[10] This decreases the accumulation of antibiotics in the cell cytoplasm and hence decreases their potency. This allows for bacteria to develop other types of resistance, *i.e.* mutation in target sites.[11] Efflux pumps may also span only the cytoplasmic membrane. Single-component efflux pumps export the antibiotic to the periplasmic space of the Gram negative bacterial membrane. The concentration of antibiotic in the periplasm increases until it matches the concentration of antibiotic in the external media. Antibiotics could diffuse back into the cytoplasm from the periplasmic space. However, the kinetics of these efflux pumps (for example, tetA) favor a low cytoplasmic concentration of antibiotic.[12] Efflux pumps,[13] along with bacterial membranes,[14] present an attractive target for development of adjuvant antibiotics.

Antibiotic efficacy in bacteria could be affected by multiple different resistance mechanisms. However, such mechanisms work independently. Inhibition of any one resistance mechanism could result in a dramatic recovery of antimicrobial activity. For example, a 64-fold recovery in the Minimal Inhibitory Concentration (MIC) of fluoroquinolones resulted from deletion of the MexAB-OprM efflux pump in a fluoroquinolone resistant *Pseudomonas aeruginosa* strain that had also acquired resistance by a mutation in DNA gyrase.[15] Hence, the

inhibition of efflux pump activity and/or increasing permeability of antibiotic to Gram negative bacteria could significantly increase the sensitivity of antibiotics.[16]

Five different approaches have been reported for increasing antibiotic concentrations in the cell cytoplasm of efflux pump expressing Gram negative bacteria.[17] These are described in detail in Chapter 1 (page 7).[18,19,20,21,22] Efflux pump inhibitors (EPI) could be classified either as indirect or direct inhibitors. Direct inhibitors are molecules that interact with the efflux pumps.[23] The only direct EPI that has reached clinical trials is PAβN.[19] It is indicated for the treatment of *P. aeruginosa* infection in the lungs. It recovers levofloxacin activity. The majority of research focuses on direct efflux pump inhibitors revolves around developing structural analogues of PAβN. *To our knowledge, there is no direct EPI available as a treatment for multidrug resistant Gram negative infections*. Compounds that cause a decrease in efflux pump activity, without directly interacting with the efflux pumps, are called indirect EPIs. Compounds that increase membrane permeability (polymyxin)[24] and disrupt ion gradients (CCCP) are considered indirect inhibitors of efflux pumps.[21] However, resistance to polymyxin has been recently reported in the U.S.[25] Here, we report that hydraphile synthetic ion channels cause indirect inhibition of efflux pumps.

Our lab has extensively studied and developed amphiphilic compounds called hydraphiles.[26] Hydraphiles are synthetic ion channels that exhibit many of the same properties as protein channels.[27] Numerous biophysical and computational studies have confirmed that the hydraphiles of appropriate lengths[28] form non-rectifying channels that could transport cations, specifically Na^+ and K^+.[29] Planar bilayer studies confirmed open-close behavior similar to that observed with known channel proteins.[30,31] The antimicrobial properties of hydraphiles are length dependent and associated with the disruption of ion homeostasis in bacteria.[32] Benzyl C_{12}-C_{16} hydraphiles are better ion transporters than shorter or longer analogs. Hydraphiles form channels in certain mammalian cells (*e.g.* HEK-293).[31] Their use in chemotherapy[33] and altering plant root morphology[34] has also been reported.

In previous chapters we reported that hydraphiles, when used at sub-lethal concentrations, recover the potency of FDA approved antibiotics. Antimicrobial potency recovery was observed with both sensitive[35] and multidrug resistant bacteria.[36] More interestingly, efficacy was enhanced against more problematic Gram negative bacteria. In particular, tetracycline and ciprofloxacin activity was recovered against *E. coli* and *K. pneumoniae*. The *E. coli*

expressed the tetA efflux pump providing tetracycline resistance, whereas the patient-isolated *K. pneumoniae* incorporated multiple different efflux pumps including tetA, RND and ABC efflux pumps. The recovery of antibiotic potency was again dependent on the length of the hydraphiles used. The benzyl C_{14} hydraphile was the most efficient of the benzyl C_8-C_{14} hydraphiles tested. We showed that the hydraphiles could localize in the bacterial membrane and cause membrane disruption. This increases membrane permeability. Hydraphiles did localize in the membrane of mammalian cells, but membrane permeability did not increase. Here, we test our hypothesis associated with efflux pump inhibition. We also test for its applicability by determining the cytotoxicity of the compounds and the ability of bacteria to develop resistance to hydraphiles.

Efflux pumps depend on cation antiport to transport antibiotics from the periplasm or the cell cytoplasm. Hydraphiles have been shown to transport cations through liposomes and mammalian membranes. *We therefore rationalize that since hydraphiles transport cations across a variety of membranes, they could also transport cations in bacteria.* This would disrupt the cation gradient required by the efflux pumps for antibiotic antiport in bacteria. We previously reported that the crown ether based lariat ethers caused depolarization of *Bacillus subtilis* membranes.[37] Hence, disruption of bacterial cation gradients by hydraphiles is possible. *We hypothesize that if hydraphiles form channels and transport ions in bacteria, then efflux pump activity will be uncoupled from the cation gradient.* Here, we have tested this hypothesis.

We report that benzyl C_8-C_{14} hydraphiles mediate the export of potassium ions from bacteria. This potassium transport was dependent both on the concentration and the length of hydraphiles used. A correlation was observed between cation transport in bacteria, hydraphile chain length and the ability of hydraphile to recover antibiotic potency. This correlation indicates an effect on efflux pumps. We show that hydraphiles, at sub-lethal concentrations, decrease the norA efflux pump activity and increase the accumulation of antibiotics in the cell cytoplasm. Here, benzyl C_{14} and C_{12} hydraphiles were the most effective compounds. The activity of these hydraphiles was comparable to known EPIs CCCP (100 μM) and reserpine (41 μM), but at the much lower concentration of 4 μM.

E. coli was unable to develop resistance to benzyl C_{14} hydraphile beyond 4 μM, over 15 days of serial culturing. However, it did develop resistance to C_{11} lariat ether and to minocycline. Cytotoxicity studies showed that benzyl C_8, C_{10},

and C_{12} hydraphiles at ½ [MIC] decrease survival of HEK-293 cells but did not affect HeLa and Cos-7 cell lines. The benzyl C_{14} hydraphile, at ½ [MIC] (1 μM), showed approx. 100% survival with all the three cell lines used. Completion of this study has allowed us to develop a platform for testing *in vitro* antimicrobial activity, membrane permeability, efflux pump activity and cytotoxicity of synthetic amphiphiles. We will use this platform to further study structural derivatives that have greater efficacy and lower cytotoxicity. We are also currently investigating *in vivo* toxicity and efficacy of the benzyl C_{14} hydraphile.

5.2 Bacteria used.

Two strains of bacteria were used to perform the studies described below. We developed a strain of *E. coli* expressing the tetA efflux pump, called Tet^R *E. coli*. The Tet^R *E. coli* was prepared by transforming competent *E. coli* cells with the pBR322 plasmid[38] using the heat shock technique. The resulting strain expressed both the tetA efflux pump and the β-lactamase genes, providing resistance to tetracycline and ampicillin. The tetA efflux pump provides resistance only to tetracycline. Minocycline, a close tetracycline analog, was still active against Tet^R *E. coli*. We have studied the activity of numerous classes of amphiphiles against Tet^R *E. coli*. We recently reported the activity of bis-amino acid based compounds against Tet^R *E. coli*.[39] As reported in chapters 3 and 4, the Tet^R *E. coli* strain was used to conduct studies with hydraphiles. At ½ [MIC] of benzyl C_8-C_{14} hydraphiles, the activity of tetracycline was recovered against tet^R *E. coli*. Here, we have used tet^R *E. coli* to perform cation transport and sequential culturing experiments to determine resistance development.

Another strain of bacteria used in this report was *Staphylococcus aureus* 1199B. This bacteria was a donation from Dr. Glenn Kaatz at Wayne State University. The *S. aureus* 1199B was initially isolated from a patient. *S. aureus* is a Gram positive pathogen that is resistant to multiple antibiotics. The *S. aureus* 1199B overexpresses the norA efflux pump.[40] Ethidium bromide and norfloxacin are substrates for the norA efflux pump.[41] This strain is also resistant to ciprofloxacin due to a mutation in DNA gyraseA (A116E).[40] We used the fluorescence of the ethidium bromide-DNA complex to measure the activity of the norA efflux pump in the presence of benzyl C_8-C_{14} hydraphile and other EPI. Numerous studies have used this approach to measure the activity of efflux pumps in the presence or absence of EPIs.[42] Combination studies were also conducted with norfloxacin and hydraphiles against *S. aureus* 1199B.

5.3 Compounds used.

The study outlined below uses four different hydraphiles: benzyl C_8 – benzyl C_{14} (compounds **1**-**4**). The benzyl C_8 and C_{10} hydraphiles are known to be poor ion transporters as they may not span the bilayer membrane. However, benzyl C_{12} and C_{14} hydraphiles are optimal to span the phospholipid bilayer and have shown the superior ion transport from liposomes as compared to benzyl C_8 and C_{10} hydraphiles. In combination studies against Tet^R *E. coli* and *Klebsiella pneumoniae* the following trend was observed for their ability to recover tetracycline activity: benzyl C_{14} hydraphile ≥ benzyl C_{12} hydraphile > benzyl C_{10} hydraphile > benzyl C_8 hydraphile. All four hydraphiles were used to determine ion transport (K^+), efflux pump inhibition, antibiotic accumulation and cytotoxicity. For the resistance development study only the benzyl C_{14} hydraphile was used. Hydraphiles were prepared in the Gokel lab and their purity was confirmed using NMR and high resolution mass spectrometry.

In earlier work, the ion transport activity of hydraphile channels in liposomes was standardized to gramicidin-D (compound **5**), which was used as control. We therefore used it as a control for the K^+ transport experiments from bacteria. The activity of gramicidin-D could be different in bacteria and liposomes due to its requirement to dimerize in the membrane.[43] In combination studies gramicidin-D did not show recovery of tetracycline recovery against Tet^R *E. coli*.

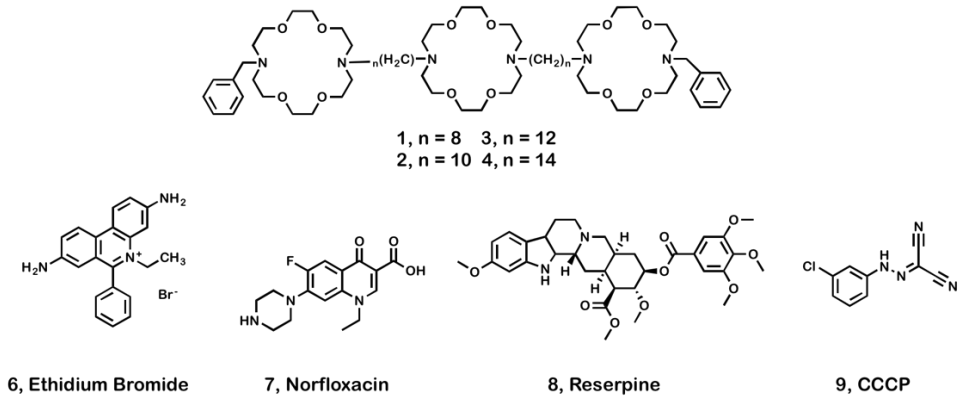

Figure 5.1. Structures of benzyl C_8-C_{14} hydraphiles (**1**-**4**), ethidium bromide (**6**), norfloxacin (**7**), reserpine (**8**) and CCCP (**9**).

S. aureus 1199B overexpresses the norA efflux pump. Ethidium bromide (**6**) and norfloxacin (**7**) are substrates of the norA efflux pump.[40,41] Compounds **6** and **7** were used for combination studies and efflux pump inhibition studies.

Reserpine (**8**) and CCCP (**9**) are known efflux pump inhibitors. We used these EPIs as controls to determine the effect of known EPIs on the norA efflux pump. Minocycline is an antibiotic control used for probing resistance development by Tet^R *E. coli*. CCCP, reserpine, daptomycin, gramicidin-D, minocycline, ethidium bromide, and norfloxacin were acquired from Sigma-Aldrich and used as received. DMSO was used as a solvent for all the compounds except for ethidium bromide. Ethidium bromide was dissolved in dH_2O.

5.4 Results and Discussion.

5.4.1. Hydraphiles transport potassium from bacteria. We have previously reported that ion transport by hydraphiles (from liposomes) depended not only on the concentration of the hydraphiles used but also on the length of spacer chains.[29,30] For example, at 12 μM, benzyl C_{12} and C_{14} hydraphiles exhibited better sodium transport from DOPC liposomes than benzyl C_8 and C_{10} hydraphiles. Notwithstanding, hydraphiles have never been shown to transport cations from bacterial cells. An increase in membrane permeability may cause release of cytosolic content including cations. It is therefore difficult to distinguish membrane permeability from ion transport. Here, we have not distinguished between these two possibilities, as their effect on bacterial cell viability, ion concentration, and antimicrobial properties is indistinguishable. However, various computational studies by others have shown that ions do flow through the channels formed by hydraphiles.[44]

We determined the ability of benzyl C_8-C_{14} hydraphiles (**1**-**4**) to transport potassium ions from Tet^R *E. coli*. The potassium concentration of an *E. coli* cell cytoplasm is 200 mM and that of phosphate buffer saline (PBS) is ~4.15 mM. When hydraphiles are added to bacteria, non-rectifying channels form in the cell membrane. As seen in the SEM study, the inner membranes are disrupted by hydraphiles forming blisters (figure 4.7b, page 90). Hydraphiles also exert osmotic stress on the bacteria causing them to swell and exhibit smoothening of the outer membrane (figure 4.7c, page 90). This indicates that hydraphile channels insert in both the outer membrane and inner membranes of *E. coli*. The proton and potassium gradients in Gram negative bacteria are maintained across the inner membrane.[45] Hydraphiles may transport potassium ions from the cell cytoplasm into the periplasmic space. When the potassium ion concentration increases in the periplasmic space, ions could leak into the cell surrounding. Hydraphiles in the outer membranes would also cause leakage of K^+ into the cell surrounding. As a result, the potassium concentration of the media (PBS)

surrounding the tet^R *E. coli* cells would increase. These change in the extracellular potassium ion concentrations ($\Delta[K^+]$ mM) were measured using a potassium selective electrode. Similar studies have been reported that measure K^+ release from bacteria using an ion selective electrode.[46,47]

Total potassium content of the Tet^R *E. coli* cells was determined by boiling the bacteria at 100 °C. The total K^+ content of Tet^R *E. coli* was used to determine the percent of potassium released in the presence of hydraphiles or controls. This was represented as 'K^+ efflux (% - total pool)'. Gramicidin-D and valinomycin were used as controls.[43] However, the requirement of gramicidin-D to dimerize in the bacterial membrane may make it ineffective method for ion transport from bacteria. Valinomycin acts as an ion carrier rather than a channel. We did not expect to observe a change in K^+ transport in the presence of valinomycin. The results of potassium transport in the presence of hydraphiles and controls are presented below in the format change in extracellular potassium concentration ($\Delta[K^+]$ mM) and increase in potassium efflux (% - total pool).

A small change in potassium efflux (~5%) was observed when tet^R *E. coli* was untreated. Since the hydraphiles were dissolved in DMSO, we tested the effect of 0.1% (v/v) DMSO on *E. coli*. In the presence of DMSO (0.1% v/v), the change in the extracellular [K^+] concentration was within experimental error of the *E. coli* alone control. The volume of DMSO was limited at 0.1% v/v as higher concentrations affected the activity of the electrode.

As expected, no change in K^+ transport was observed in the presence of a range of concentrations of valinomycin, (data not shown). In the presence of gramicidin-D, at 30 μM, potassium transport was observed. Higher concentrations of the ionophore were not tested owing to poor solubility. Gramicidin-D (30 μM) causes an efflux of 50-60% of total potassium content of *E. coli* cells (figure 5.2). In the liposomal experiments reported previously, the activity of gramicidin-D was comparable to that of benzyl C_{14} hydraphile. In Tet^R *E. coli*, the activity of gramicidin-D at 30 μM was similar to the benzyl C_{14} hydraphiles used at a concentration of 4-6 μM.

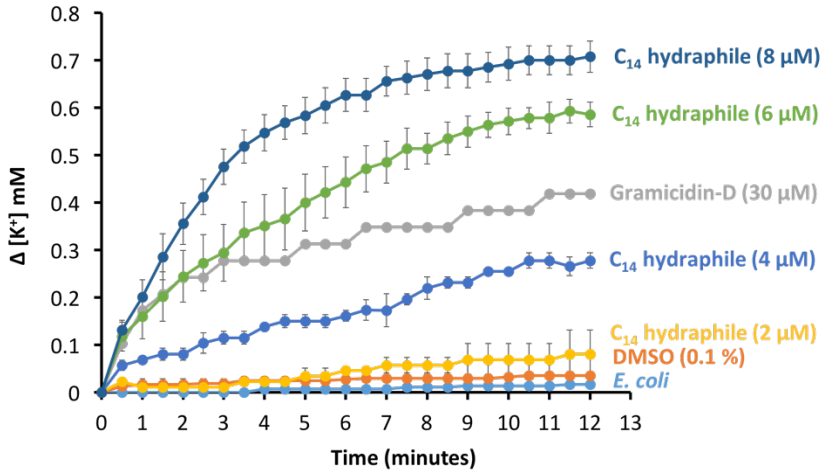

Figure 5.2 (a). Potassium ion transport by benzyl C_{14} hydraphile at 2 µM, 4 µM, 6 µM and 8 µM. The x-axis represents time in minutes and the change in extracellular potassium concentration is shown on the y-axis. Tet^R *E. coli* alone, DMSO (0.1% v/v) and gramicidin-D 30 µM are used as controls. Each data point, except for gramicidin-D, represents an average of five trials. The error bars represent the standard deviation in the results. Gramicidin-D control was tested only once to confirm its well-known property.

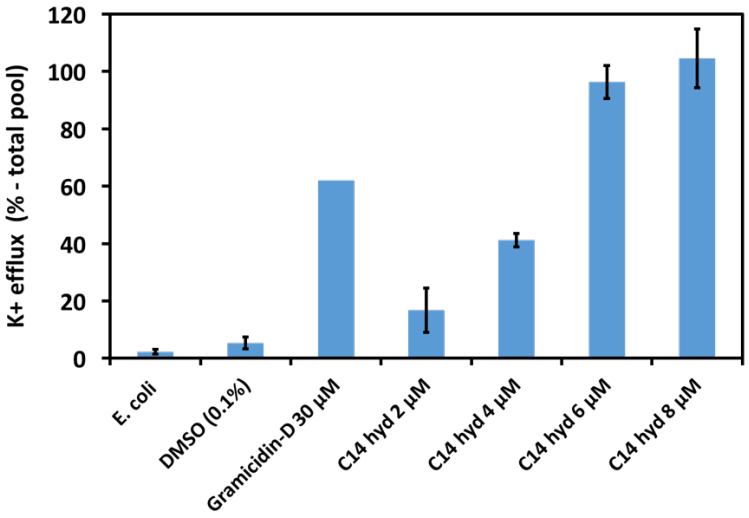

Figure 5.2 (b). Potassium ion transport by benzyl C_{14} hydraphile at 2 µM, 4 µM, 6 µM and 8 µM. The bar graph represents the percent of total potassium ions released from Tet^R *E. coli* cells after

treatment with benzyl C_{14} hydraphile, and controls Tet^R *E. coli* alone, DMSO and gramicidin-D. Each data point, except for gramicidin-D, represents an average of five trials. The error bars represent the standard deviation in the results. Gramicidin-D control was tested only once to confirm its well-known property.

The experiment requires use of high concentrations of bacteria to observe a change in electrical potential by the electrode. We therefore concentrated the exponential stage bacteria to $O.D._{600}$ ~ 1.3 before use. At this concentration of bacteria, the MIC of benzyl C_{14} hydraphile against *E. coli* was 8 μM. We therefore tested the potassium transport ability of benzyl C_8-C_{14} hydraphiles at 4 μM.

Concentration curves were determined for benzyl C_8 hydraphile and benzyl C_{14} hydraphile. When benzyl C_{14} hydraphile was added at 2 μM (1/4 [MIC]), approx. 10% K^+ was released from the *E. coli* cytoplasm. This small change in the extracellular [K^+] was within the experimental error of *E. coli* alone and DMSO controls. Benzyl C_{14} hydraphile transports potassium ion from Tet^R *E. coli* cells at MIC (8 μM) and ½ [MIC] (4 μM) concentrations. Extracellular potassium increased in the presence of benzyl C_{14} hydraphile at 8 μM, 6 μM and 4 μM (figure 5.2 a). At 8 μM of the benzyl C_{14} hydraphile, caused almost 100% of the K^+ content of *E. coli* to be release into the surrounding media (figure 5.2 b). The ion release at this concentration was stabilized after 8 minutes (figure 5.2 a). This is possibly due to the cell death or lysis caused by benzyl C_{14} hydraphile at MIC concentrations. At 4 μM of the benzyl C_{14} hydraphile (1/2 [MIC]), approx. 40% of total potassium content of Tet^R *E. coli* was released. This was lower than the known potassium ion channel, gramicidin-D at 30 μM. Potassium transport changed little after addition of benzyl C_{14} hydraphile at 2 μM. However, the change in extracellular K^+ seemed to increase over 12 minutes.

Figure 5.3 shows the ability of benzyl C_8 hydraphile to transport K^+ from *E. coli* at various concentrations. It has been reported that benzyl C_8 hydraphile is a poorer ion transporter compared to benzyl C_{14} hydraphile. The benzyl C_8 hydraphile transports potassium ion from Tet^R *E. coli* and increased extracellular K^+ concentration at 250 μM, 125 μM and 62.5 μM (figure 5.3a). These concentrations compare to the MIC, ½ [MIC] and ¼ [MIC] of benzyl C_8 hydraphile used for combination studies. At 250 μM benzyl C_8 hydraphile, caused almost ~70 % of K^+ content of Tet^R *E. coli* to be released into the surrounding media (figure 5.3b) and the ion release was still increasing after 10 minutes (figure 5.3a). In comparison, benzyl C_{14} hydraphile released 100% of

potassium content of Tet^R *E. coli* at the much lower concentration of 8 µM by 8 minutes. At 125 µM and 62.5 µM benzyl C_8 hydraphile, ~50% and 40% of total potassium content was released, respectively. This was comparable to the known potassium ion channel gramicidin-D at 30 µM. This confirms that benzyl C_8 hydraphile transports potassium ion at concentrations that showed the recovery of tetracycline activity.

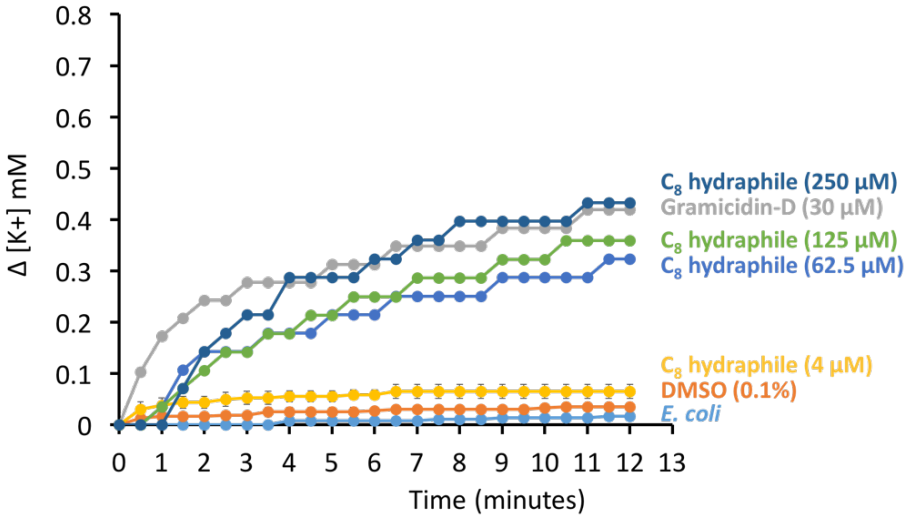

Figure 5.3 (a). Potassium ion transport by benzyl C_8 hydraphile at 4 µM, 62.5 µM, 125 µM and 250 µM. Change in extracellular potassium gradient (y-axis) over 12 minutes (x-axis) is shown in graph. Tet^R *E. coli* alone, DMSO (0.1%) and gramicidin-D are used as controls. Each data point, except for gramicidin-D, represents an average of five trials. Error bars have been omitted for clarity. Gramicidin-D control was tested only once to confirm its well-known property.

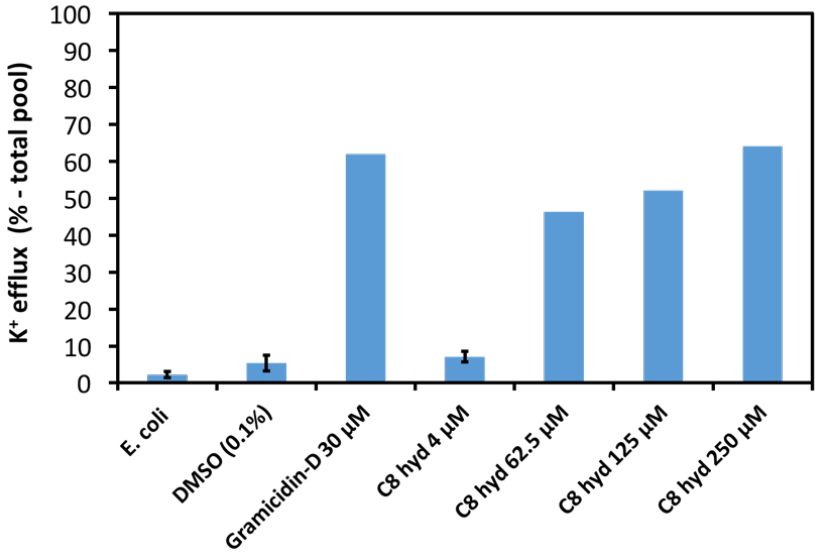

Figure 5.3 (b). The bar graph represents the percent of total potassium ions released from *E. coli* cells after treatment with benzyl C_8 hydraphile at 4 µM, 62.5 µM, 125 µM and 250 µM. Tet^R *E. coli* alone, DMSO (0.1% v/v) and gramicidin-D at 30 µM were used as controls. Each data point, except for gramicidin-D, represents an average of five trials.

When benzyl C_8 hydraphile was added at 4 µM (1/4 [MIC]), the change in K^+ concentration was comparable to that of DMSO (0.1% v/v) control. The benzyl C_{14} hydraphile at 4 µM released ~ 40% of cytosolic potassium from *E. coli*. This shows that the benzyl C_{14} hydraphiles is a more potent ion transporter that benzyl C_8 hydraphile. A comparison among benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles for ion transport from bacteria is shown below in figure 5.4. The graph also shows a comparison of ion transport by hydraphiles to the antibiotic recovery by hydraphiles, observed against the same strain of *E. coli*.

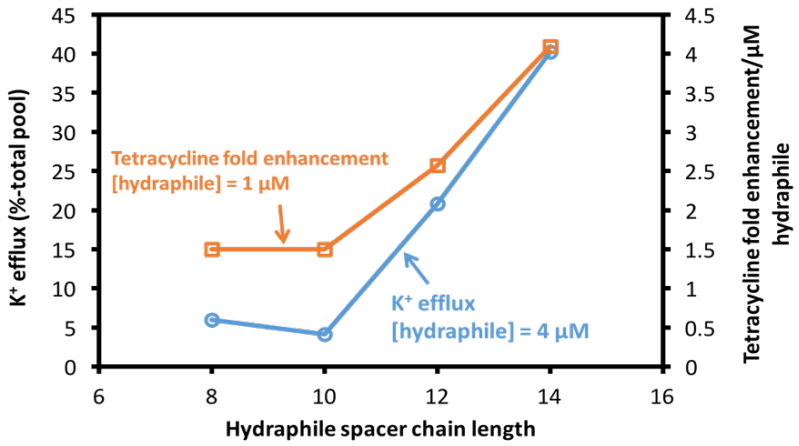

Figure 5.4. Comparison of benzyl C_8-C_{14} hydraphiles to transport potassium ions (open circles) and recovery of tetracycline activity (open squares) against Tet^R *E. coli*. Potassium release is expressed in percent of total potassium content of Tet^R E. coli released (y-axis, left) and tetracycline recovery in fold enhancement (y-axis, right). The x-axis shows the number of carbon atoms in the spacer chains of hydraphiles. This is a theoretical comparison of two sets of data, hence no error bars are indicated.

We compared the ion transport ability of hydraphiles to tetracycline efficacy recovery against Tet^R *E. coli*. A chain length dependence was observed for both potassium transport and tetracycline recovery (figure 5.4). We used a sub-lethal concentration of the most active compound (benzyl C_{14} hydraphile) for comparison of tetracycline recovery and potassium ion transport. We have previously shown that at ½ [MIC] of benzyl C_{14} hydraphile there was no inhibition of *E. coli* growth (figure 3. 2, page 53). At 4 μM, the benzyl C_{14} and C_{12} hydraphiles released approximately 40% and 25% of the total *E. coli* potassium ion content from the cell cytoplasm to the cell surrounding, respectively (figure 5.4). These are the most efficient compounds at both release of potassium ions and recovery of tetracycline activity. It also became clear that the ion transport from bacteria and the increase in antibiotic potency by hydraphiles is dependent on the spacer chain length: $C_{14} > C_{12} > C_{10} \geq C_8$. It is possible that benzyl C_{14} hydraphile is optimal for hydraphiles to span a bilayer membrane of *E. coli* to perform its function of ion transport and membrane disruption. Using shorter spacer chain lengths would fail to span the membrane, transport ions or disrupt membranes efficiently. This activity is also dependent on the concentration of amphiphile used as observed with benzyl C_8 and C_{14} hydraphiles (figures 5.2 and

5.3). Use of higher concentration of benzyl C_8 and C_{10} hydraphiles could result in ion transport and tetracycline recovery. However, these would likely be an effect of membrane disruption caused by aggregation of hydraphiles in the membrane rather than formation of an ion selective pore. Hydraphile aggregation was observed in membrane localization and SEM studies.

It is also known that potassium ion is released when the membrane integrity of bacteria is affected. However, a range of studies have been reported that demonstrate the ability of hydraphiles to form channels and transport ions. We cannot distinguish channel formation by hydraphiles from membrane disruption in bacteria. Both of these could occur as is known for natural amphiphiles such as colistin and daptomycin.[48] Hydraphiles could similarly transport sodium ions and protons.

5.4.2. Efflux pump inhibition. Hydraphiles have recovered the activity of tetracycline and ciprofloxacin against efflux pump expressing Tet^R *E. coli* and *K. pneumoniae*. If the cation gradients are disrupted by hydraphile as proved above, then the activity of efflux pumps should also be inhibited. This is indirect inhibition of efflux pumps. For direct inhibition the hydraphile would have to interact with the efflux pump protein. This seems less likely because efflux pumps are highly selective for their substrates. For example, the tetA efflux pump is selective for only tetracycline. Minocycline, a close tetracycline analog is neither recognized nor effluxed by the tetA pump. We therefore hypothesized that if hydraphile inhibit the activity of efflux pumps, then the efflux pump substrate would accumulate in the cell cytoplasm.

To test our hypothesis, we used the *S. aureus* 1199B strain overexpressing the norA efflux pump. Ethidium bromide (EtBr) is one of the substrates of the norA efflux pump. EtBr could intercalate with DNA once it enters and accumulates in the bacterial cell cytoplasm. The EtBr-DNA complex has a fluorescence intensity 10,000-fold greater than EtBr alone. We therefore utilized fluorescence intensity from DNA-EtBr complex as an indication of the localization of EtBr. Transport and localization of EtBr could be used to measure the effect of hydraphiles on the norA efflux pump.

Before we determined the effect of hydraphiles on the norA efflux pump of *S. aureus* 1199B, we determined the MICs of benzyl C_8-C_{14} hydraphiles, reserpine and CCCP. Surprisingly, benzyl C_{12} hydraphile (MIC ≤ 1 μM) activity was similar to that of benzyl C_{14} hydraphile (MIC = 1 μM). Both benzyl C_{12} and

C_{14} hydraphiles were more active than benzyl C_8 and C_{10} hydraphiles. MIC of EtBr, CCCP and reserpine were 16 μM, ≤ 4 μM and > 128 μM, respectively. We next performed combination studies to observe if benzyl C_8-C_{14} hydraphiles recovered ethidium bromide activity against *S. aureus* 1199B. The results are presented in Table 5.1 below. The MICs were conducted in 2-fold serial dilutions. The results reported below were replicated in three different experiments.

Table 5.1. Recovery of ethidium bromide activity against *S. aureus* 1199B

Amphiphile used	**[Amphiphile] μM**	**[EtBr] μM**	**Fold enhancement**
No amphiphile	-	16	n/a
C_8 hydraphile	32 (¼[MIC])	16	1-fold
C_8 hydraphile	64 (½[MIC])	8	2-fold
C_{10} hydraphile	4 (¼[MIC])	16	1-fold
C_{10} hydraphile	8 (½[MIC])	1	16-fold
C_{12} hydraphile	0.25 (¼[MIC])	16	1-fold
C_{12} hydraphile	0.5 (½[MIC])	2	8-fold
C_{14} hydraphile	0.25 (¼[MIC])	4	4-fold
C_{14} hydraphile	0.5 (½[MIC])	0.5	32-fold

In the presence of ½ [MIC] of benzyl C_8, C_{10}, C_{12} and C_{14} hydraphiles, the activity of EtBr was recovered by 2, 16, 8 and 32-fold, respectively. In contrast to the recovery of tetracycline activity against Tet^R *E. coli*, benzyl C_{14} hydraphile showed the highest recovery of EtBr in *S. aureus* 1199B. *S. aureus* is a Gram positive bacteria with only one membrane and thick cell wall. It has been reported that hydraphiles are more active against Gram positive bacteria. Hence, it is not surprising that a greater recovery of EtBr by benzyl C_{14} hydraphile is observed against *S. aureus* 1199B. At ¼ [MIC] of benzyl C_8-C_{14} hydraphiles, only 1- to 4-fold recovery of EtBr was observed. Studies with a combination of hydraphiles and norfloxacin are under way.

Combination studies proved that benzyl C_8-C_{14} hydraphiles could recover antibiotic potency against norA efflux pump expressing *S. aureus* 1199B. Next we determined the ability of hydraphiles to inhibit the activity of norA efflux pumps. Here, the *S. aureus* 1199B cells were preloaded with EtBr using 100 μM CCCP. CCCP is a known efflux pump inhibitor. Addition of CCCP dissipates proton motive force, which inhibits the activity of norA pumps and allow accumulation of EtBr in the cell cytoplasm. The cells were then washed to

remove extracellular EtBr and CCCP. The EtBr loaded cells were re-suspended in Mueller-Hinton II (MHII) media, which contains dextrose hydrolyzed from starch. Dextrose acts as an energy source for the regeneration of proton motive force. The cells were then treated with 4 µM of benzyl C_8-C_{14} hydraphiles. The MIC of benzyl C_{14} hydraphile was observed at ~ 8 µM against *S. aureus* 1199B at $O.D._{600}$ ~ 0.800 used for this experiment. If hydraphiles inhibit the activity of efflux pumps, then there should be a no or only small change in the fluorescence intensity of EtBr-DNA complex. The experiment was performed in triplicates and the error bars in figure 5.5 and 5.6 represents standard deviation.

As seen in figure 5.5 below, in the presence of benzyl C_{10}, C_{12} and C_{14} hydraphiles at 4 µM, there is only minor change in the fluorescence of EtBr. This indicates an inhibition of efflux pump activity. This inhibition was similar to that of known efflux pump inhibitors such as CCCP (100 µM) and reserpine (41 µM). When the concentration of CCCP and reserpine was decreased to 4 µM, inhibition of norA activity by known EPIs was ~30% lower than that of hydraphiles. If the efflux pump activity is not inhibited, EtBr would be released or effluxed from the cell cytoplasm, resulting in a decrease in the fluorescence intensity. The benzyl C_8 hydraphile had only a minor effect on the efflux pump activity at 4 µM. These results confirm the inhibition of efflux pump activity by hydraphiles.

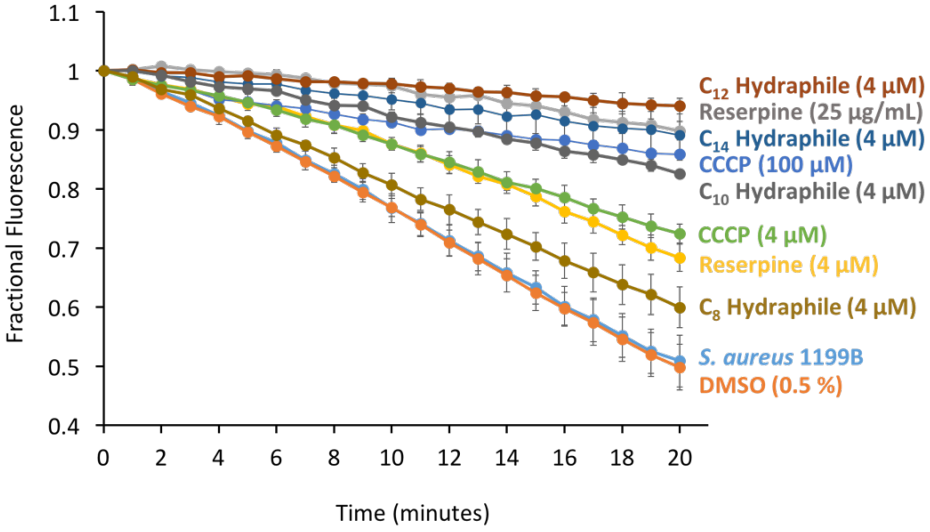

Figure 5.5. Hydraphiles inhibit the activity of efflux pumps. Release of ethidium bromide from *S. aureus* 1199B after treatment with reserpine, CCCP, benzyl C_8-C_{14} hydraphiles (4 µM). The x-axis represents time in minutes and the y-axis represents fractional

fluorescence. The results represent the average of three independent trials and the error bars represent the standard deviation.

Increase in membrane permeability and inhibition of efflux pump activity in bacteria should result in cytoplasmic accumulation of antibiotics. We used *S. aureus* 1199B to determine if, in the presence of hydraphiles, EtBr accumulation in the cell cytoplasm increased. EtBr was added to the *S. aureus* 1199B cells followed by hydraphiles or the controls. The change in fluorescence was observed for 20 minutes using a fluorimeter. If the hydraphiles allow for EtBr accumulation in the cell cytoplasm, then the fluorescence intensity would increase.

As seen in figure 5.6 below, after the addition of benzyl C_{14} and C_{12} hydraphile (4 μM) the accumulation of EtBr increased in the cell cytoplasm of *S. aureus* 1199B. Note that the EtBr accumulation by benzyl C_{12} hydraphile at 4 μM was similar to that of known efflux pump inhibitors CCCP (100 μM) and reserpine (25 μg/mL or 41 μM). The accumulation of EtBr in the presence of benzyl C_{14} hydraphile (4 μM) was greater than twice as much observed with CCCP and reserpine. However, at 4 μM the activity of CCCP and reserpine was much lower than either benzyl C_{14} or C_{12} hydraphiles. There was no change in the EtBr fluorescence intensity observed in the presence of benzyl C_8 and C_{10} hydraphiles (4 μM). The hydraphiles with shorter spacer chain lengths do not span the membrane. As indicated by their higher MICs, these compounds might also inhibit the efflux pump activity at higher concentrations. The studies conducted with *S. aureus* 1199B data show that hydraphiles indirectly inhibit the efflux pump activity and cause antibiotics to accumulate in the cell cytoplasm. Hydraphiles are at least comparable to, and better in some cases, than well-studied and reported efflux pump inhibitors such as CCCP and reserpine.

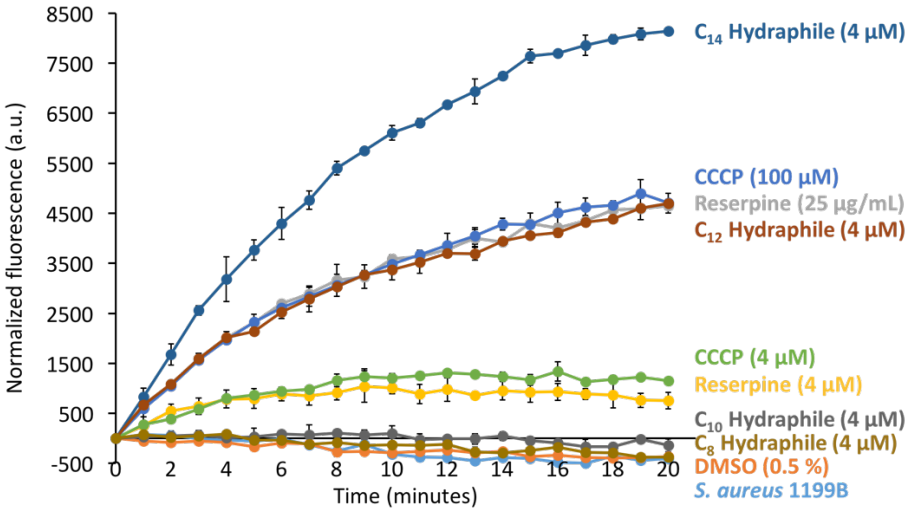

Figure 5.6. Hydraphiles increase substrate accumulation in bacteria. Accumulation of ethidium bromide in the presence of reserpine, CCCP, benzyl C_8-C_{14} hydraphiles (4 µM) in *S. aureus* 1199B expressing norA efflux pump. The x-axis represents time in minutes and y-axis represents normalized fluorescence. The results represent the average of three independent trials and the error bars represent the standard deviation.

Both the accumulation and release of EtBr from *S. aureus* 1199B in the presence of hydraphiles could be affected by (1) the disruption of membrane integrity, which allows for greater EtBr accumulation and/or (2) uncoupling of the norA efflux pump from ion gradient caused by non-rectifying hydraphile channels. We reported that the membrane permeability of bacterial cells alone was increased by sub-lethal concentrations of hydraphiles. The results in figure 5.4 show that hydraphiles could transport potassium ions from bacteria in a chain length dependent manner. Hence, hydraphiles increase bacterial membrane permeability and disrupt cation gradients, causing inhibition of efflux pumps and increased accumulation of antibiotics in the cell cytoplasm. This results in increased efficacy of antibiotics against resistant bacteria.

5.4.3. Resistance to hydraphiles. One of the major concerns with antibiotic development is the emergence of resistance by bacteria to new molecules. As a result, newer antibiotics are usually limited in use and saved for emergency purposes only. Compounds that target membranes are usually less prone to resistance development by bacteria. For example, polymyxins (colistin) has been developed and in clinical use for > 50 years. However, resistance to polymyxin

was only identified recently.[25] It is known that developing resistance to a membrane active compound would require multiple changes in membrane composition/synthesis pathways and is energetically unfavorable.[49] The antibiotic resistance problem could be addressed by developing antibiotics that are less prone to resistance development. It is obvious that hydraphiles are membrane targeting (localizing) molecules. However, it is unknown if bacteria could develop resistance to synthetic molecules, specifically synthetic amphiphiles. We therefore determined if Tet^R *E. coli* could develop resistance to benzyl C_{14} hydraphile, our most potent efflux pump inhibitor and adjuvant. We used minocycline as a positive control. Lariat ether are structurally similar to hydraphile and also showed antimicrobial recovery. We therefore used C_{11} lariat ether as a control.

We first used the mutant prevention concentration (MPC) to test resistance development. To determine the MPC, L.B. Agar plates containing different concentrations of either benzyl C_{14} hydraphile (8, 4, 2, 1, 0.5, 0.25 and 0.125 μM) or minocycline were prepared. A culture of Tet^R *E. coli* was incubated over the plate for 3 days at 37 ^oC. The highest concentration that prevents the growth of bacteria (colony forming units) is considered the MPC. Growth was observed with hydraphiles at concentrations as high as 8 μM. However, the same cells failed to grow in the L.B. media containing the same or lower concentration (4 μM) of hydraphile. This shows that the *E. coli* was not resistant to benzyl C_{14} hydraphile but the MPC was not a good technique for determination of resistance development to synthetic amphiphiles.

The technique known as the sequential culturing method was used to determine if bacteria would develop resistance to benzyl C_{14} hydraphile, C_{11} lariat ether, or minocycline. In this experiment, the bacteria were treated with increasing fractional MIC concentrations of compounds tested, for 15 days. Each day, the highest concentration of the compound that showed growth were used to determine MICs. A sample of the bacteria grown in the presence of amphiphiles were preserved for future studies. The results from the sequential culturing method are shown in figure 5.7 below.

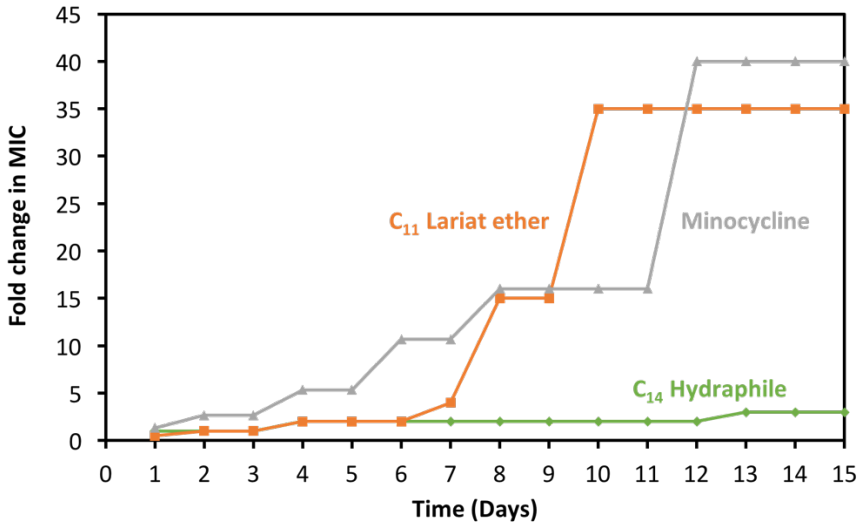

Figure 5.7. Resistance development by Tet^R *E. coli* to benzyl C_{14} hydraphile (diamonds), C_{11} lariat ether (squares) and minocycline (triangles). The x-axis represents time in days and the y-axis represents increase in MIC (in folds). The results were reproduced independently in 2 trials and the average of the MIC on each day is indicated in the graph above. MICs were conducted in serial dilutions; hence no error bars are represented. The errors were within the single dilution.

As seen in figure 5.7, Tet^R *E. coli* readily developed resistance to minocycline between 4-6 days. Resistance increase during the 15-day experiment and the MIC of minocycline MIC is eventually increased by 40-fold. This experiment does not distinguish between efflux pump or a mutation in target binding site as the resistance mechanism.

The bacteria did not develop resistance past 4 μM to benzyl C_{14} hydraphile during 15 days. Hydraphiles are synthetic molecules and bacteria lack enzymes to catalyze its break-down. Further, hydraphiles target membrane, altering the membrane composition to prevent hydraphile binding or insertion and therefore would require major changes in biochemical pathways. This would be energetically unfavorable. However, such membrane active compounds are associated with cytotoxicity or mutagenicity. The issue of ctotoxicity is addressed below.

The development of resistance by *E. coli* to C_{11} lariat ether was surprising. Lariat ether still contains one macrocycle, but might be active as a carrier or might aggregate in the membrane to function as a channel. It is possible that if lariat ethers require to form an aggregate in the membrane, then a small change in the membrane composition could inhibit lariat ether activity. If lariat ethers have a cytoplasmic target, the target site could be mutated to recognize and export lariat ethers. In either case, resistance development to C_{11} lariat ethers by Tet^R *E. coli* indicates a complete different mechanism of action or membrane targets than benzyl C_{14} hydraphile.

5.4.4. Cytotoxicity to hydraphiles. Membrane active compounds are known to have cytotoxicity to mammalian cells. The use of either colistin and CCCP is limited due to its cytotoxicity.[50] The presumed membrane penetration mechanism of action of hydraphiles may be similar to that of colistin and CCCP. Thus, toxicity is an issue. It was previously reported that LD_{50} of benzyl C_{14} hydraphile against CaCO2 cells was 2.6 μM. Other hydraphiles were not tested and their effect on renal epithelial cell lines was not established. In particular, renal toxicity is a common issue with amphiphilic molecules.[51] For our hydraphiles to be an effective adjuvant antibiotic, selectivity between bacterial and mammalian cells should be as high as possible. *We hypothesize that since hydraphiles are amphiphilic molecules, cytotoxicity to kidney cells may be observed.* At the concentration used for combination studies, *i.e.* sub-MIC, the toxicity should be limited. Some membrane active compounds are also associated with mutagenicity. We recently reported DNA gel electrophoresis data that showed hydraphiles at MIC concentrations does not bind DNA.[52] Hydraphile-DNA complexation was observed, but at much higher concentrations than its MIC against *E. coli*.

To assess the cytotoxicity of benzyl C_8-C_{14} hydraphiles, we used three mammalian epithelial cell lines: HEK-293, HeLa and Cos-7. HEK-293 and Cos-7 are human embryonic kidney and monkey kidney cells, respectively. HeLa is a cervix epithelial cell line. Previous studies determined that the activity of hydraphiles was similar to that of CCCP, an EPI, and colistin, a membrane disruptor. We therefore used colistin and CCCP as controls. We determined the viability of all three cell lines in the presence of benzyl C_8-C_{14} hydraphiles (**1**-**4**). Figure 5.8 and figure 5.9 shows the percent survival of HEK-293, HeLa and Cos-7 cells in the presence of ½ [MIC] and MIC concentrations of benzyl C_8-C_{14} hydraphiles, colistin and CCCP observed against Tet^R *E. coli*. The commercial XTT assay (Sigma-Aldrich) was used to determine the viability of mammalian

cells in the presence or absence of hydraphiles. The XTT assay depends on the enzymatic conversion of a tetrazolium dye in a vital cell to a colored formazan product. The absorbance of the colored formazan derivative is then used to quantitate the number of live cells. Untreated cells were used as controls. Cells alone were considered as 100% survival and viability in the presence of hydraphiles was calculated as percent survival.

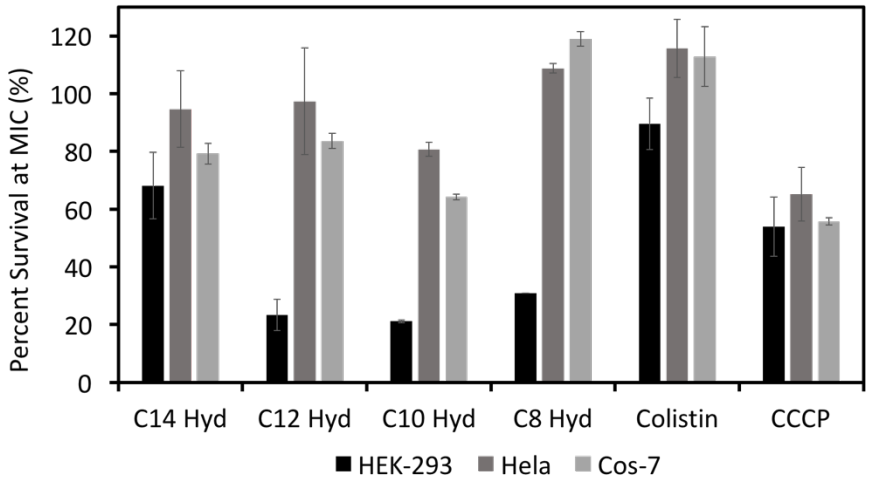

Figure 5.8. Cytotoxicity of benzyl C_8-C_{14} hydraphile, colistin and CCCP at MIC concentrations against HEK-293, HeLa and Cos-7 cells. The x-axis represents the compounds used and the y-axis represents the percent of cells survived in presence of the respective compounds used. The percent survival is the average of three independent trials that contained three replicates per trial. This indicates a total of 9 data points. The error bars represent standard deviation in three trials.

As seen in figure 5.8, benzyl C_{14} hydraphile had almost 70% survival against HEK-293 cells at MIC concentration (figure 5.8). At MIC concentrations, benzyl C_8-C_{12} hydraphiles had 20-30% survival against HEK-293 cells. HeLa and Cos-7 showed 80-100% survival against all the hydraphiles used at MIC concentrations. Hence, at MIC concentrations hydraphiles showed toxicity to one out of three epithelial cell lines tested. At MIC concentration, benzyl C_{14} hydraphile and colistin showed similar toxicity to HEK-293 cells. CCCP was found to be toxic to all cell lines and showed 50-80% survival. The concentrations used for our synergy studies were at ½ [MIC] and lower. We therefore assessed the survival of mammalian cells at ½ [MIC] of hydraphiles.

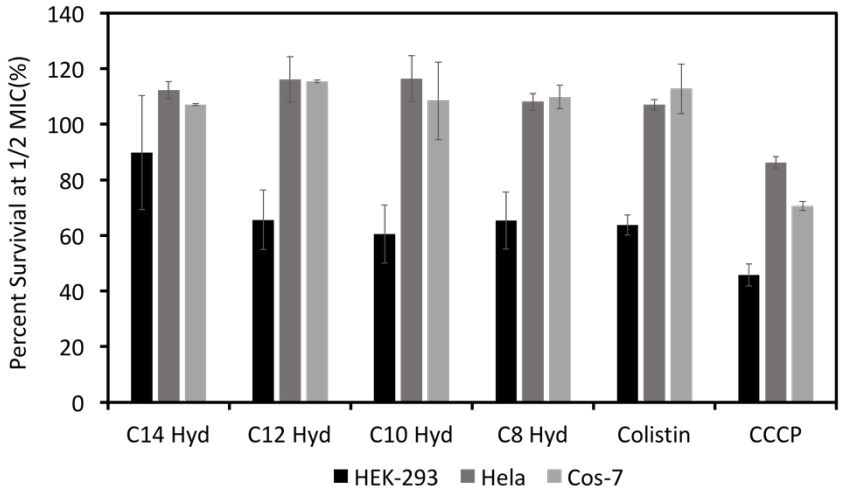

Figure 5.9. Cytotoxicity of benzyl C_8-C_{14} hydraphile, Colistin and CCCP at ½ [MIC] concentrations against HEK-293, HeLa and Cos-7 cells. The x-axis represents the compounds used and the y-axis represents the percent of cells that survived in the presence of the respective compounds. The percent survival is the average of three independent trials that contained three replicates per trial. This indicates a total of 9 data points. The error bars represent the standard deviation for three trials.

Again, cytotoxicity from hydraphiles was only observed against HEK-293 cells. At the ½ [MIC] value of benzyl C_8-C_{14} hydraphiles used for the combination studies, we observed cytotoxicity to the HEK-293 cells (figure 5.9). Survival of HEK-293 cells in the presence of ½ [MIC] of benzyl C_{14} hydraphile was 85-100%. The survival of HEK-293 cells in the presence of ½ [MIC] of benzyl C_{12}, C_{10} and C_8 hydraphile increased to 60-80% (figure 5.9) from 20-30% (figure 5.8) survival at the MIC concentration. Cos-7 and HeLa cells showed 100% survival in the presence of hydraphiles. CCCP, a known EPI, was cytotoxic to all three cell lines. Cytotoxicity of colistin to HEK-293 cells was also observed. At the low concentrations of hydraphiles and colistin used for the recovery of tetracycline activity, there was minimal to no cytotoxicity observed to the mammalian epithelial cell lines. The benzyl C_{14} hydraphile at ¼ [MIC] concentration showed an average of 96% HEK-293 survival and recovered the tetracycline efficacy by 3-fold against resistant bacteria. The data show that benzyl C_{14} hydraphile, when used at 500 nM, is non-cytotoxic to HEK-293 cells and also recovers the antibiotic activity in resistant bacteria. Further studies with

other hydraphile derivatives could reveal structures with even lower cytotoxicity.

5.5. Summary and conclusions

Hydraphiles and other synthetic amphiphiles have been known for decades. Most of the studies involving this class of molecules have been focused on developing new structures[53] and assessing their effect on ion transport.[54] Many studies have also reported their activity as antibiotics. These studies use diverse methods and very large range of bacterial strains and some yeast. None of these studies goes beyond simple survey. To the extent that conclusions can be drawn, it appears that activity is greater against Gram positive bacteria than Gram negative bacteria.[55] This is the first report that shows synthetic amphiphiles can be used to recover the efficacy of antibiotics against efflux pump expressing multidrug resistant bacteria or 'Superbugs'. We have shown that hydraphiles can recover the activity of tetracycline and fluoroquinolones (ciprofloxacin, norfloxacin) against two Gram negative and one Gram positive bacteria. One of these bacteria is *K. pneumoniae*, which was isolated from a patient, and is an urgent threat to public health. This bacterium is resistant to almost all known classes of antibiotics and the treatment of last resort is colistin. However, colistin has cytotoxicity issues. The recovery of antimicrobial efficacy by hydraphiles could make this infection treatable. In addition, a new use of colistin at sub-lethal concentrations, in combination with other antibiotics, could circumvent the cytotoxicity issue.

We report here that hydraphiles increase membrane permeability and inhibit efflux pump activity that allows for the accumulation of antibiotics in the cell cytoplasm. We first reported that hydraphiles could localize in the cell membrane of bacteria and mammalian cells. Hydraphiles were also found in the cell cytoplasm of bacteria and the nuclei of mammalian cells. This could be an effect of cell death or increased membrane permeability. Localization in the membrane and cation transport by hydraphiles resulted in both membrane disruption and osmotic stress as observed with SEM images. This function allows for an increase in the permeability of bacterial cell membranes. In contrast, the permeability of mammalian cell membranes did not change even at 4 times greater than the concentration used for the antibiotic combination studies. This could be due to the differences between the membrane composition of bacterial and mammalian cells.

Hydraphiles were shown to transport potassium ions through *E. coli* cell membranes. At ½ [MIC], all four benzyl C_8-C_{14} hydraphiles could transport cations, causing disruption of the cation gradient. A correlation between ion transport, recovery of antimicrobial potency and spacer chain length was observed. Changes in the cation gradient are expected to alter the activity of efflux pumps. NorA efflux pump activity was inhibited and the substrate accumulation was increased in the presence of hydraphiles. The efflux pump inhibition by hydraphiles is indirect as observed with CCCP rather than direct inhibition as observed with PAβN or reserpine. An advantage of the membrane-acting amphiphile approach is that bacteria cannot easily develop resistance to amphiphiles that transport ions and disrupt membranes. Of course, bacteria may still develop resistance to hydraphiles, but this may be a relatively slow process. We have shown that *E. coli* cannot develop resistance to hydraphiles for over 15 days.

The inhibition of efflux pumps by hydraphiles and other natural amphiphiles is caused by disruption of ion gradients and/or membrane integrity. However, this raises the issue of cytotoxicity and bioavailability. Our preliminary results show that hydraphiles are bioavailable through IV administration for over 2 hours. The cytotoxicity of the most effective hydraphiles at sub-MIC concentrations was minimal. Cytotoxicity was observed against only one cell line, HEK-293. When ½ [MIC] concentrations of hydraphiles were tested, the survival of all three cell lines increased. More structural studies, currently underway in the lab, could reveal compounds with greater bioavailability and even lower cytotoxicity. Cytotoxicity from hydraphiles was lower than CCCP but its efficacy to inhibit efflux pump and recover antibiotic potency was far superior.

Overall, we report a non-resistant adjuvant platform that could be used with both existing and novel molecules to recover antimicrobial potency against life-threatening bacterial infections. As a result of these studies, we have identified benzyl C_{14} hydraphile as a lead compound that acts a membrane disruptor and an efflux pump inhibitor. At ½ [MIC], it recovers antimicrobial potency and shows 80-100% survival of three different mammalian cell lines. We are currently performing *in vivo* cytotoxicity and efficacy studies with these molecules.

In vivo toxicity and efficacy studies in mouse model are already underway. The preliminary data are promising. We are working to increase the

in vitro efficacy of hydraphiles to recover antimicrobial potency and further decrease the mammalian cytotoxicity. Changes in the number of positive charges and/or alkyl chain lengths may provide for compounds with greater selectivity. Lariat ethers also provide an attractive opportunity for developing antibiotic adjuvants. Lariat ethers are easier to synthesize and show lower cytotoxicity than hydraphiles (data not shown). A methyl iodide derivative of lariat ether that has permanent positively charges showed almost 40-fold recovery at 15 μM (data not shown). These studies are still underway.

All the studies reported in this dissertation establish a platform to conduct details mechanistic and antimicrobial studies in the Gokel lab. The lab already has the ability to conduct organic synthesis. Hence, a structure activity relationship could be easily performed. One such study was recently published by our group using bis-amino acid based compounds.[39] These compounds were non-cytotoxic and also rescued tetracycline activity against Tet^R *E. coli*. These compounds are also amphiphilic. Hence, synthetic amphiphiles represent a promising new approach for developing antibiotics, efflux pump inhibitors and membrane disruptors.

5.6. Experimental procedure.

5.6.1. Compounds used. Benzyl C_8 – C_{14} hydraphiles were prepared by the Gokel laboratory as reported before. The purity of each compounds were confirmed by the NMR and high resolution mass spectrometry. Reserpine, CCCP, colistin and ethidium bromide were acquired from Sigma-Aldrich and used as received.

5.6.2. Bacteria used. Two strains of bacteria were used for experiments outlined in this chapter. The Tet^R *E. coli* strain used here was made by transforming competent JM109 *E. coli* cells with pBR322 plasmid. This strain was used for ion transport and resistance development studies conducted here. The Tet^R *E. coli* was cultured in L. B. Miller media containing 100 μg/mL Ampicillin.

S. aureus 1199B was provided by Dr. Glenn Kaatz at Wayne State University. This strain was used for efflux pump inhibition and substrate accumulation studies. The *S. aureus* 1199B was cultured in cation adjusted MHII media containing 10 μg/mL ethidium bromide.

5.6.3. Potassium transport. A calibration curve was first established using known concentration of potassium chloride. Calibration curve was used to acquire the equation for the slope and $R^2 = 0.998$. The experiment was conducted with Tet^R *E. coli*. *E. coli* was grown overnight from one CFU. Cells were knocked back to O.D. = 0.600 before use. Cells were centrifuged at 2000xg and resuspended in sterile PBS. The O.D. of cells was adjusted to 1.300. To measure the K^+ leakage, 1998 µL *E. coli* suspended in PBS were added to a plastic cup containing magnetic stirrer. The stir plate was turned on to mix the sample and the potassium selective electrode (Orion, Thermo Scientific) was immersed in the sample so that just the membrane was covered in the sample. The voltage (mV) was stabilized for 6-8 minutes. The temperature of the sample was kept constant at 25°C. The electrode was lifted and 2 µL of the either of the hydraphiles or Gramicidin-D were added. The DMSO concentration was kept constant at 0.1% (v/v). The electrode was immersed back in the solution. Multiple concentrations were tested for each compound. The mV reading was recorded every 30 seconds for 15 minutes. The concentration of potassium ion released (mM) and $\Delta [K^+]$ mM were calculated using the equation from the calibration curve.

To determine the total potassium pool of Tet^R *E. coli*, 2 mL of cells were heated at 100°C for 30 minutes on a heat block. After 30 minutes the sample was cooled down to room temperature for 60 minutes. After 60 minutes the mV reading was recorded and $\Delta [K^+]$ mM was calculated. The experiment was performed in triplicates and standard deviation in the experiment was calculated.

5.6.4. Efflux pump inhibition. Efflux pump inhibition studies were conducted with *S. aureus* 1199B in a 96-well microtiter plate with black wells and glass bottom. *S. aureus 1199B* were grown overnight from one CFU. Cells were knocked back to $O.D._{600} = 0.550$ before use. In a 1.5 mL micro-centrifuge tube, cell were spun down at 17,000 x g for 3 minutes. Cell were re-suspended in fresh MHII media containing 10 µg/mL ethidium bromide and 100 µM CCCP. The $O.D._{600}$ was adjusted to 1.000. The cells were vortexed and incubated at the room temperature for 20 minutes to load cells with ethidium bromide. After 20 minutes, the cells were centrifuged at 17,000 xg for 3 minutes. The supernatant was discarded and the cells were stored on ice. The tubes were warmed to room temperature for 5 minutes and MHII media was added to the tubes and $O.D._{600}$ was adjusted to 0.800. 100 µL of ethidium bromide loaded cells were added to each well containing either C_8-C_{14} hydraphiles, CCCP or reserpine. The content were mixed by pipetting up and down once and the fluorescence was recorded

immediately using Biotek Cytation 3 plate reader (Dr. Lon Chubiz lab). Excitation of 530 nm and emission of 600 nm was used. Reading were collected every minute for 20 minutes. The results were reproduced two more times and standard deviation was calculated. The results were graphed against time.

5.6.5. Ethidium bromide accumulation. Efflux pump inhibition studies were conducted with *S. aureus* 1199B in a 96-well microtiter plate with black wells and glass bottom. *S. aureus 1199B* were grown overnight from one CFU. Cells were knocked back to $O.D._{600}$ = 0.550 before use. While the cells grew to the optimal conditions, hydraphiles, CCCP and reserpine stock concentrations were prepared. Mid-log phase cells were centrifuged at 17,000 xg for 3 minutes and re-suspended in fresh MHII media. The $O.D._{600}$ was adjusted to 0.800. In each well 200 µL of cells were added followed by 10 µg/mL of ethidium bromide and 1 µL of C_8-C_{14} hydraphiles, reserpine or CCCP. The contents of the well were mixed by pipetting up and down once. Immediately fluorescence was measured using the Biotek Cytation 3 plate reader (Dr. Lon Chubiz lab). Excitation of 530 nm and emission of 600 nm was used. Reading were collected every minute for 20 minutes. The results were reproduced two more times and standard deviation was calculated. The results were graphed against time.

5.6.6. MIC and Synergy. MIC experiments were performed as described in the Clinical and Laboratory Standards Institute (CLSI) standard microdilution protocols. Bacteria was grown overnight from one CFU in media without antibiotics. *S. aureus* 1199B was grown in MHII media containing 10 µg/mL ethidium bromide. On the day of experiment, bacteria were knocked back to O.D. 600 nm = 0.550 in the same media. These exponential phase bacteria were then diluted in antibiotics free media to get 4 x 10^8 CFU/mL. In a 96-well, plate either L.B. Miller or MHII media was added followed by serially diluted compounds or ethidium bromide. All the hydraphiles were dissolved in DMSO and the final concentration of DMSO in each well was kept constant at 0.5% (v/v). For the combination experiments, first the hydraphile or control was added to the media in the well followed by the antibiotics. The contents of the well were mixed before adding 20 µL of bacteria to get 4 x 10^5 CFU/mL per well. The plates were incubated at 37 °C, 200RPM for 24 hours before collecting results on the Biotek Cytation 3 plate reader. No more than three plates were stacked on top of each other at a time. Optical density of the wells was determined at λ=600 nm. Media alone control was considered as 100% inhibition. Any inhibition greater than 80% was considered as the MIC. The results were reproduced three times before reporting.

5.6.7. Resistance development to hydraphiles. The ability of Tet^R *E. coli* to develop resistance to benzyl C_{14} hydraphiles and minocycline was determined using sequential culturing method. This method tests the ability of bacteria to develop resistance by inducing constant selective pressure of sub-lethal dose of antibiotics over 15 days. This procedure also accounts for only point mutations. The Tet^R *E. coli* was grown overnight from one colony in L.B. media. *E. coli* was knocked back to O.D. 600 nm = 0.100 in a 2 mL L.B. media and grown to O.D. = 0.550. L.B. media was added to the test tubes, followed by the antibiotic and then the *E. coli* and incubated at 37 °C, 200 RPM for 24 hours. Five different concentrations of hydraphiles and minocycline were set up: 0.25x, 0.5x, 1x, 2x, and 4x [MIC]. Any cultures that grew at higher than the MIC levels were passaged on antibiotic-free L.B. Agar plates and the MIC is determined. The samples were also stored for future use. The cells were diluted from the second highest concentration that allowed growth at 1:100 in fresh media containing 0.25x, 0.5x, 1x, 2x, 2.5x and 4x [MIC]. Test tubes were incubated at 37 °C, 200 RPM for 24 hours. Any cultures that grew at higher than the MIC levels were passaged on antibiotic-free L.B. Agar plates and the MIC was determined. The procedure was continued for 15 days. The results are represented in graphical format of MIC *vs.* days.

5.6.8. Mammalian cell cytotoxicity. HeLa (ATCC CCL-2) cells were acquired from ATCC. Cos-7 (ATCC CRL-1651) cells were donated by Dr. C. Dupureur and HEK-293 cells were donated by Dr. M. Nichols. Cell lines were regularly maintained in growth media containing DMEM (ATCC), 10% fetal bovine serum (FBS, ATCC) and 1% penicillin-streptomycin solution (ATCC). Adherent HEK-293, HeLa and Cos-7 cells were trypsinized using 0.25% (w/v) trypsin-EDTA (Sigma-Aldrich), suspended in a fresh media and diluted to get a concentration of 3×10^5 cells/ml. Cells were seeded in a 96-well plate (100 µL/well) to get 3×10^4 cells/well. The plates were incubated for 24 hours at 5% CO_2 and 37°C to reach a confluency of 80-90%.

In a sterile 1.5 mL micro-centrifuge tube, benzyl C_8-C_{14} hydraphile, CCCP and colistin (0.5% DMSO) were mixed with assay media (DMEM + 10% FBS) and serially diluted by 2-fold each to get 2[MIC], [MIC], ½[MIC] and ¼[MIC] concentrations. A control containing 0.5% DMSO was also prepared. After 24 hours, the spent media in the 96-well plate containing HEK-293, HeLa and Cos-7 cells (90% confluency) was replaced with 100 µL media containing the benzyl C_8-C_{14} hydraphile, CCCP and colistin at various concentrations. The cells were

incubated at 37°C and 5% CO_2 for 24 hours before performing XTT assay (Sigma-Aldrich). The XTT assay was performed according to the manufacturer's protocol. After 24 hours of treatment with compounds, the media was replaced with PBS and 25 µL XTT was added to each well. The XTT assay works by the reduction of tetrazolium compound by alive cells to the colored soluble formazan product. The absorbance of the product was measured at 450 nm (XTT) and 690 nm (background). Percent survival was calculated by comparing the average absorbance of cells treated with benzyl C_8-C_{14} hydraphile, CCCP and colistin to that of cells alone. Three replicates were performed for each treatment. Average percent survival and standard deviation were calculated.

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