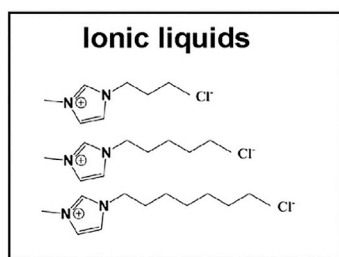


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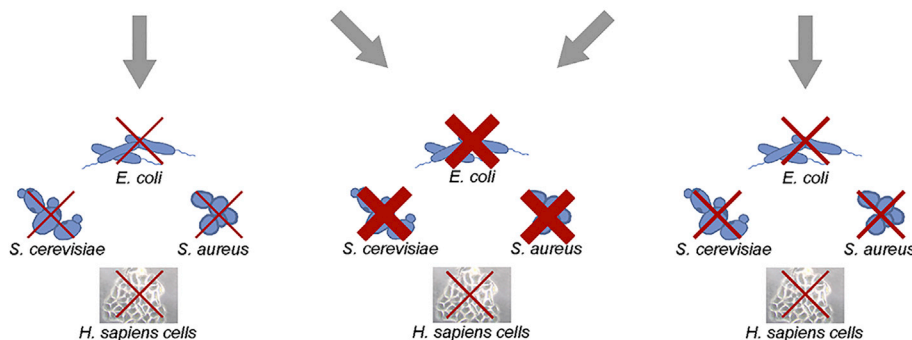
Synergistic interactions of ionic liquids and antimicrobials improve drug efficacy

Combined treatment leads to synergistic effect and enhanced specificity



Antimicrobials compounds

<i>Tetracyclin</i>	<i>Chloroamphenicol</i>
<i>Kanamycin</i>	<i>Ketoconazole</i>
<i>Doxycyclin</i>	<i>Fluconazole</i>
<i>Ampicillin</i>	<i>Linezolid</i>
<i>Polymyxin B</i>	<i>Melitten</i>



Low toxicity

Combined effect enhanced specificity

Moderate specificity

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HIGHLIGHTS

1-alkyl-3-methylimidazolium RTILs have antimicrobial properties

Alkyl chain length correlates with lethality and permeability

Combinations of RTIL + antimicrobial enhance antimicrobial effects

Combinatorial effect is not evident in human cell lines

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Article

Synergistic interactions of ionic liquids and antimicrobials improve drug efficacy

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Summary

Combinations of ionic liquids (ILs) with antimicrobial compounds have been shown to produce synergistic activities in model liposomes. In this study, imidazolium chloride-based ILs with alkyl tail length variations are combined with commercially available, small-molecule antimicrobials to examine the potential for combinatorial and synergistic antimicrobial effects on *P. aeruginosa*, *E. coli*, *S. aureus*, and *S. cerevisiae*. The effects of these treatments in a human cell culture model indicate the cytotoxic limits of ILs paired with antimicrobials. The analysis of these ILs demonstrates that the length of the alkyl chain on the IL molecule is proportional to both antimicrobial activity and cytotoxicity. Moreover, the ILs which exhibit synergy with small-molecule antibiotics appear to be acting in a membrane permeabilizing manner. Collectively, results from these experiments demonstrate an increase in antimicrobial efficacy with specific IL + antimicrobial combinations on microbial cultures while maintaining low cytotoxicity in a mammalian cell culture model.

Introduction

Antibiotic molecules are considered integral components of modern medicine in that the World Health Organization (WHO) recommends 38 different antibiotics on their list of essential medicines (Who, 2019b). Similarly, the WHO has labeled ~200 antibiotic molecules and formulations in the AWaRe classification for use and application, in the attempt to minimize the development of antibiotic resistance while preserving the usefulness of these molecules in clinical settings (Who, 2019a). In the United States, methicillin-resistant strains of *Staphylococcus epidermidis* and *Staphylococcus aureus* have been a recent cause for concern, while totally drug-resistant strains of tuberculosis have been found in Italy and Iran (Klevens et al., 2006; Mohajeri et al., 2014). Worldwide, the prevalence of antibiotic resistant pathogens found in infections has been on the increase in both community and nosocomial infections (Founou et al., 2017). Therefore, the discovery of new antimicrobial molecules or formulations is necessary to help combat the threat of drug-resistant strains of bacteria.

Strategies for combating antibiotic resistant bacteria include a number of different approaches. The scientific literature is replete with reports on novel molecules as antimicrobials including peptides, biomimetic polymers, metals, quorum signaling inhibitors, enzyme inhibitors, and non-specific biocidal molecules (Ashby et al., 2017; Capilato et al., 2017; Mosaei and Harbottle, 2019; Kamaruzzaman et al., 2019; Rozman et al., 2019; Takahashi et al., 2017; Fordham et al., 2014; D'souza et al., 2020; Zhou et al., 2019; Shirley et al., 2018). In parallel, many groups have focused on the understanding of how bacteria develop resistance to antimicrobials. These studies have led to a deeper understanding of bacterial efflux pumps, bacterial response and regulation of membrane composition, modification of the polysaccharide lipopolysaccharide/lipoteichoic acid layer, and formation of biofilms (Li et al., 2015; Lazar et al., 2018; Lee et al., 2019b; Schroeder and Stephens, 2016). One promising avenue to enhance antimicrobial efficacy is the use of existing antimicrobial compounds in combination with secondary compounds that can increase efficacy. Antimicrobial efficacy can be enhanced by both (1) combinatorial interactions in which antibiotic plus secondary compound improves antimicrobial activity and even further (2) synergistic interactions in which the combination of antibiotic plus secondary compound improves antimicrobial activity with a combined effect greater than the sum effect of either compound acting independently.

In practice, this approach has been applied to a very limited degree clinically and continues to be explored experimentally in the laboratory. One of the most widely used combinatorial formulations in clinical

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practice is amoxicillin with clavulanic acid, where amoxicillin is an inhibitor of peptidoglycan synthesis while clavulanic acid inhibits the β -lactamase enzyme which degrades amoxicillin (Huttner et al., 2019). Similar approaches combine two molecules which each independently exhibit antimicrobial activity and combine the dosing for treatment (Montelongo-Peralta et al., 2019; Opalski et al., 2020). Many of these combinations are designed such that the two antimicrobials act via different mechanisms, ideally each enhancing the activity of the other. Despite these efforts, no clear best clinical practice has emerged globally, only very narrow spectrum, case-specific protocols. In the laboratory, several studies have begun to show promise in combining compounds that enhance the ability of antimicrobials. Significant progress has been made demonstrating synergistic interactions between the antibiotic tetracycline and a number of different secondary compounds including 2-aminoimidazoles, quercetin, and baicalin (Rogers et al., 2010; Qu et al., 2019; Novy et al., 2011). Interestingly, all of these compounds' primary synergist effect is likely due to the ability of the compound to enhance antimicrobial entry into the cell.

One such class of molecules that have been shown to destabilize membranes with limited cytotoxic effects is room temperature ionic liquids (RTILs) which are salts that exist in the liquid form, absent any solvent, at room temperature (Welton, 1999; Yu et al., 2013). These molecules represent a special class of ionic liquid (IL) which has generated recent significant interest in a wide range of potential applications. In general, ILs are composed of an anion and cation pair which can often be mixed and matched, creating a vast array of possible IL species. These compounds can often exhibit chemical properties such as low melting points, negligible vapor pressure, and exceptional solvation potential. Furthermore, ILs have been extensively studied in electrochemical applications, most frequently as components for energy storage devices (Watanabe et al., 2017). ILs have received attention as potential biomaterials that can be tuned (via changing their molecular cations and anions) for various biomedical applications (Saha and Mukherjee, 2018; Schroder, 2017; Fiebig et al., 2014). Moreover, ILs have been shown to both stabilize and destabilize protein structures, enhance enzymatic activity, impact DNA structure, and exhibit sensitive protein interactions (Itoh, 2017; Schroder, 2017; Jumbri et al., 2014). However, evidence in the literature indicates that not all ILs behave similarly, and, not surprisingly, the effects on biomolecules are dependent on the molecular identity of the components of the ILs.

The interactions between ILs in aqueous solution and lipid bilayer membranes have guided the development of IL-based drug formulation and drug delivery applications (Egorova et al., 2017; Hanna et al., 2017; Pendleton and Gilmore, 2015; Singh et al., 2015, 2016; Singh and Kang, 2015; Alves et al., 2013; Weaver et al., 2010; Riduan and Zhang, 2013; Zakrewski et al., 2014; Zhang et al., 2009) and opened research avenues for IL-based antibiotics and antibiotic-IL combinations. There have also been a number of reports highlighting both experimental and computational studies on how ILs interact with lipid bilayers (Jing et al., 2016; Yoo et al., 2014). Hydrophobic ILs can insert into lipid bilayers (Yoo et al., 2016; Gal et al., 2012; Benedetto et al., 2014; Mikkola et al., 2015; Jeong et al., 2012; Gayet et al., 2011; Alves et al., 2013; Rengstl et al., 2014; Zakrewski et al., 2014; Lim et al., 2015), leading to a bilayer reorganization (Yoo et al., 2016; Benedetto et al., 2014; Kontro et al., 2016). ILs have also been incorporated with enzyme-hydrolyzing groups, alkyl chains, and stable anions with reported antibacterial effects (Cole, 2012). Additionally, a tetracycline-based IL has been shown to insert into liposome membranes with important drug delivery and antibiotic implications (Alves et al., 2013). The highly recombinant nature of ILs makes amenable to formulating "designer" reagents to treat specific cell types: rearranging both the anion and cation can allow for highly specific, tailored applications in different organisms (Pendleton and Gilmore, 2015). Previous studies demonstrated that ILs with imidazolium-based cations were successful in permeating the membranes of bacteria, yeast, and red blood cells (Cook et al., 2019). Other ILs have been shown to exhibit antimicrobial activity alone or impact the manner in which antimicrobials interact with lipid bilayers (Raucci et al., 2018; Hanna et al., 2017). Additionally, micelle formation can occur in these molecules through the interactions of the IL alkyl chains, which can influence the interactions with cellular membranes (Jungnickel et al., 2008).

Future treatment for drug-resistant microbes might entail pairing existing antimicrobials with existing or novel reagents to overall bolster drug efficacy. This could involve the pairing of two species in which one molecule enhances the delivery of the other, one species prevents degradation/breakdown of the other, or both acting on different targets in the bacterial cell. ILs are potential candidates for this combinatorial approach due to the mix-and-match compatibility of IL components and the increasing understanding of their significant ability to impact biomolecules in a tunable manner. A critical parameter in

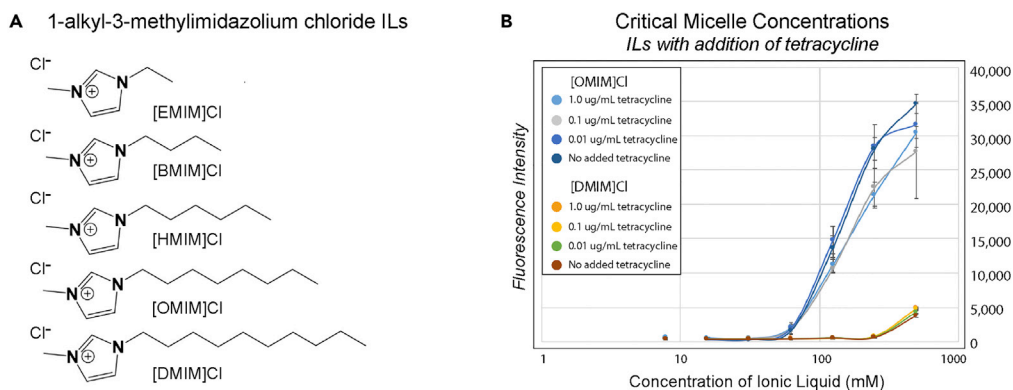


Figure 1. Ionic liquids with various chain lengths determine critical micelle concentration

1-alkyl-3-methylimidazolium chloride ILs and their effect on critical micelle formation in the presence of antibiotics. (A) Imidazolium chloride ILs with various alkyl chain lengths used to evaluate the (B) critical micelle concentrations of imidazolium chloride-based ILs in combination with a broad range of tetracycline concentrations from 0-1 μ g/mL. Assays are performed in triplicate, error bars .05 confidence intervals for relative fluorescence intensity are used.

See also [Figure S1](#)

utilizing these combinatorial approaches will be to identify the ability of ILs to enhance antimicrobial activity without increasing the cytotoxicity of combined treatments to the host. The work presented herein investigates the ability to pair specific room temperature imidazolium-based ionic liquids (RTILs) that contain variable length alkyl chains with a panel of traditional small-molecule antimicrobials and antimicrobial peptides. The data presented below highlight a subset of well-studied class of RTILs, 1-alkyl-3-methylimidazolium, and all ILs used in this study are liquid at room temperature. Finally, the combinations of these RTILs + antimicrobial compound were screened for their combined and synergistic antimicrobial efficacy against gram-negative and gram-positive bacteria, yeast, and for toxicity against mammalian cells.

Results

Critical micelle concentrations (CMCs) of ILs in combination with tetracycline

Previous work has demonstrated antimicrobial effects of ILs and a strong correlation between increased alkyl chain length and minimal inhibitory concentration (MIC) ([Cook et al., 2019](#); [Ghanem et al., 2015](#)). In this study, we evaluated both the magnitude of this antimicrobial effect, as well the potential mechanism of actions for 1-alkyl-3-methylimidazolium RTILs of differing in alkyl chain length ([Figure 1A](#)).

Possible candidate mechanisms for the antimicrobial effects of ILs include the ability of the IL to act as a potential detergent and destabilize the cell membrane while an alternative hypothesis is that the amphipathic nature of ILs may directly impact an associated antimicrobial compound through the formation of micelles. Micelles are roughly spherical structures formed by amphiphilic molecules in which the nonpolar portion of the molecule is buried at the interior of the structure while the polar portions are exposed, favorably interacting with the aqueous milieu. Previous work has shown that some of these imidazolium ILs can form micelles, and thus if antimicrobials affect the critical micelle concentration (CMC), it would inherently impact the antimicrobial activity ([Lee et al., 2019a](#)). Using a fluorescence-based assay with the environmentally sensitive fluorophore 1,6-Diphenyl-1,3,5-hexatriene (DPH), CMC of [OMIM]Cl and [DMIM]Cl was measured in the absence or presence of various concentrations of tetracycline ([Figures 1B](#) and [S1](#)). In this assay, DPH fluorescence intensity increases dramatically upon formation of micelles due to DPH partitioning into the nonpolar core of the micelle ([Chattopadhyay and London, 1984](#)). The data show that tetracycline has minimal effect on the formation of micelles over a 100-fold concentration range tested compared to the no tetracycline control for both RTILs examined. Thus, any effects of tetracycline in combination with RTILs are likely due to true synergistic behavior rather than influence on micellar properties.

Variation in alkyl chain length correlates to microbial inhibition and permeability

Microbial inhibition and cell membrane permeabilizing effects were evaluated using 3-methylimidazolium chloride RTILs of differing in alkyl chain length with four microbial strains (*P. aeruginosa*, *E. coli*, *S. aureus*,

Ionic liquid	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>A. baumannii</i>
[EMIM]Cl	>100	>100	>100	>100	>100	>100
[BMIM]Cl	100	>100	>100	>100	100	100
[HMIM]Cl	12.5	50	25	25	6.25	12.5
[OMIM]Cl	1.25	5	10	5	1.25	5
[DMIM]Cl	.31	.625	5	2.5	.31	1.25

Table 1. Inhibition of bacterial growth when treated with ILs is dependent on alkyl chain length

1-alkyl-3-methylimidazolium ILs with various alkyl chain lengths were used to evaluate the minimum inhibitory concentration (MIC) of these compounds on a variety of bacteria including *S. aureus*, *B. subtilis*, *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *A. baumannii*. Increasing alkyl chain length correlates with microbial lethality.

and *S. cerevisiae*). In agreement with other studies, baseline MIC experiments suggest that alkyl chain lengths of 6, 8, and 10 exhibit independent antimicrobial effects with longer chain lengths exhibiting the greatest inhibition across a variety of microorganisms (Table 1).

While the surfactant properties of ILs have been proposed to destabilize cellular membranes and thus elicit antimicrobial properties, we specifically tested this in the case of 1-alkyl-3-methylimidazolium RTILs to identify potential mechanisms of IL-induced microbial inhibition. Flow cytometry was used to measure the microbial cell wall permeability in the presence of short duration IL exposure (30 min) through infiltration of propidium iodide into the cell. Propidium iodide, a DNA stain, will only enter into cells in which the cell membrane is destabilized, and thus, bacterial or yeast cells exhibiting an increased fluorescent signal in the PE channel (Ex. 488nm/Em. 575nm) indicate membranes which have been permeabilized. Comparing the fluorescent signals between our positive control, cetrimonium bromide, and our sample of IL and cells, we were able to calculate the percentage of permeability. Similar to the correlation with MIC values, there is a direct correlation between longer alkyl chain lengths and greater bacterial cell permeability (Figure 2) (Figures S1A and S1B). For those ILs which presented with an antimicrobial effect, [HMIM]Cl, [OMIM]Cl, and [DMIM]Cl demonstrated a direct correlation between IL concentration, ability to inhibit bacterial growth, and the ability to permeabilize cell membranes.

The combination of ILs with antimicrobials acts to lower the minimum inhibitory concentration of a broad set of antimicrobial compounds

After establishing MIC and permeabilizing effects of ILs of varying (2-10 C) chain lengths, ILs were tested which were shown to have a partial antimicrobial effect; [HMIM]Cl and [OMIM]Cl were both subsequently evaluated for the combinatorial effects of ILs with addition of antimicrobials. Using a 96-well plate format, *P. aeruginosa*, *E. coli*, *S. aureus*, and *S. cerevisiae* in liquid culture were treated with varying concentrations of IL, antimicrobial, or both for 24 hr and then evaluated for growth by a plate reader at OD600 (Figure 3A). In total, two ILs, [HMIM]Cl and [OMIM]Cl, four microorganisms, and six antimicrobials were evaluated. Evidence of a combined effect was considered positive when the addition of both IL and antimicrobial demonstrated decreased bacteria cell growth in comparison to having IL or antimicrobial only. In one representative example, *S. cerevisiae* was treated with chloramphenicol +/- [OMIM]Cl, and reduced cell growth as seen by OD600 values was evident with both [OMIM]Cl + chloramphenicol when compared to the effect of either independently (Figure 3B).

A broad range of screening experiments for combined effects of IL + antimicrobial were completed by creating a matrix plate for each possible combination; data from each individual experiment were compiled and analyzed to look for combined effects (Table 2). From the number of possible wells that could potentially show combined effect, we calculated the percentage of those wells that showed the effect. In general, a combined effect was present in many of the IL + antimicrobial combinations for both [HMIM]Cl and [OMIM]Cl, in all four microorganisms, and with a wide range of antimicrobials. Out of the 48 possible combinations, 33 (69%) plate assays evidenced at least one combination of IL + antimicrobial that demonstrated enhanced antimicrobial efficacy in combination. Some combinations exhibited organism specificity, such as [HMIM]Cl and [OMIM]Cl + tetracycline yielding a strong combined effect in all three bacterial strains tested while [HMIM]Cl and [OMIM]Cl + kanamycin dramatically enhanced efficacy in *S. cerevisiae* only.

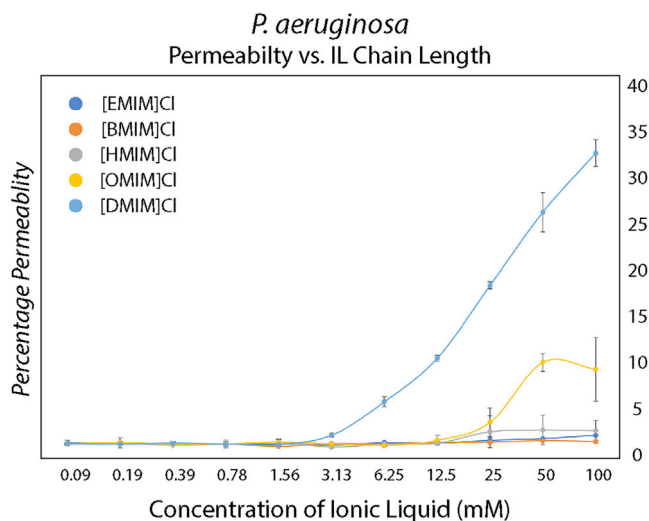


Figure 2. Increasing alkyl chain length correlates with microbial permeability

Permeabilizing effects of ILs at increasing concentrations on *P. aeruginosa* as measured by flow cytometry following a 30-min IL exposure and PI staining. Percentage of cells positive for PI staining indicated on the y axis, error bars .05 confidence intervals for triplicate treatments are used.

Compiled results from this low-resolution screen of combinatorial effects of IL + antimicrobial indicate combinatorial interactions are highly concentration dependent, suggesting that a potential synergistic effect is only present at specific concentrations of ILs or antimicrobials. In order to both validate these results, as well as reproduce these findings at higher resolution, candidate combinations which elicited strong combinatorial interactions were explored for the evidence of synergistic interactions at a finer range of IL + antimicrobial concentrations. Specifically, the hypothesis was tested that there exist concentrations of ILs which have no antimicrobial effect yet can still enhance the antimicrobial effectiveness of specific antibiotics.

Targeted analysis of candidate IL + antimicrobial combinations at refined concentrations to identify synergistic interactions

Several combinations of IL + antimicrobial demonstrated a synergistic interaction in microbial growth inhibition. Specifically, the combination of both [HMIM]Cl and [OMIM]Cl + tetracycline demonstrated a strong synergistic effect for all three bacterial strains tested. Expanding on this result, *P. aeruginosa* and *S. aureus* were treated with [HMIM]Cl and [OMIM]Cl + tetracycline for 24 hr in 96-well liquid culture plates and evaluated for bacterial growth. All four treatments resulted in evident synergistic interactions of IL + antimicrobial at multiple defined concentrations (Figures 4A–4D). While high concentrations of ILs or antimicrobial compounds were sufficient to inhibit bacterial cell growth, at lower concentrations, the presence of both IL + antimicrobial in combination yielded synergistic effect and a decrease in bacterial cell growth as indicated by the boxed regions. Specifically, synergistic interactions are evident when IL has no apparent antimicrobial effect, yet in combination with antimicrobial compound, a significant decrease in cell proliferation is evident (Boxed regions Figures 4A–4D). Furthermore, the concentration-dependent effect of IL on bacterial growth was additionally alkyl chain length dependent. The addition of [OMIM]Cl (Figures 4B and 4D) elicited a decrease in the MIC of tetracycline at approximately 4-fold lower concentration for both *P. aeruginosa* and *S. aureus* when compared to [HMIM]Cl (Figures 4A and 4C).

Combination of ionic liquid and antimicrobial in bactericidal disc assay

To further assess the combinatorial efficacy of these treatments, a bactericidal disc assay was performed, and the results reaffirmed trends observed in liquid culture assays for an expanded set of antimicrobial compounds. Addition of 6-mm cellulose discs soaked in solubilized antimicrobials to the plates allowed for the creation of zones of inhibition (ZOIs) for the ten antimicrobials evaluated: kanamycin, tetracycline, chloramphenicol, ampicillin, polymyxin B, doxycycline hyclate, clindamycin, azithromycin, melittin, and linezolid. In the assay, four increasing concentrations of [HMIM]Cl (0, 0.0025, 0.00625, 0.025 M) were chosen

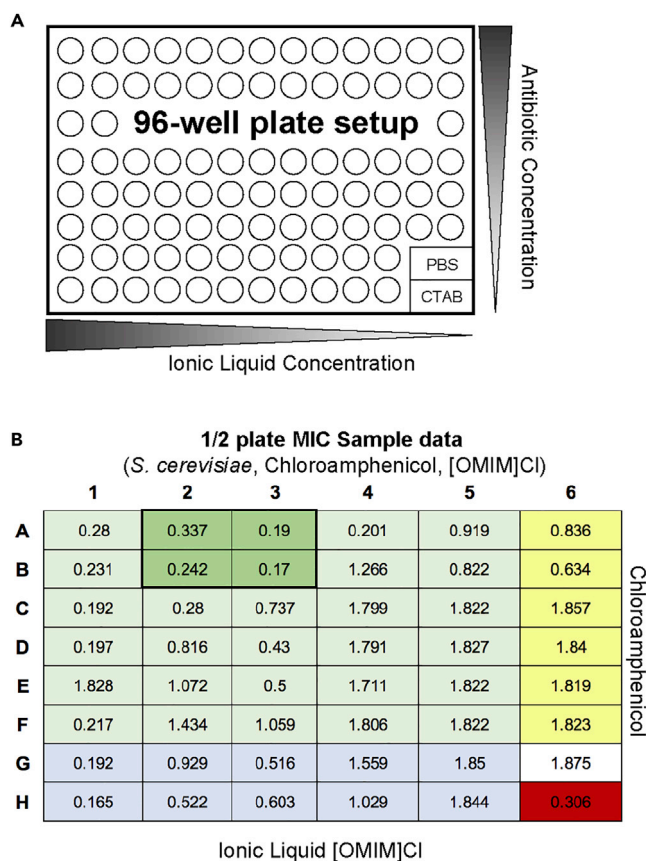


Figure 3. Combination of antimicrobial compounds with IL leads to enhanced lethality in a wide range of microbes and antimicrobials

Combinations of antimicrobials with IL of various alkyl chain lengths on 4 different microorganisms demonstrate increased antimicrobial effects with different classes of antimicrobials and ILs

(A) 96-well MIC plate setup with decreasing IL concentration from left to right and decreasing antibiotic concentrations from top to bottom.

(B) Representative initial screen conducted with 1/2 plate setup *S. cerevisiae*, chloramphenicol, and [OMIM]Cl. Yellow (antibiotic only), blue (IL only), green (combination IL + antibiotic), dark green (combination IL + antibiotic) indicating decreased bacterial growth due to combined interaction. Numerical values presented are (OD600 readings) from 96-well plate of 24-hr liquid bacterial culture indicating growth (high values) or limited growth (low values)

based on 96-well MIC liquid assays and added to LB agar plates prior pouring. After solidification, *S. aureus* was plated onto the plates, antimicrobial soaked discs were added, and plates were grown overnight before recording ZOI. Digital pictures were acquired of all plates, and measurements of ZOI diameter were performed using Adobe Illustrator (Figure 5A). The negative control phosphate buffered saline (PBS) demonstrated no zone of inhibition, while the antibiotics diffused from the antimicrobial disc into the surrounding media and inhibited bacterial cell growth in a surrounding ring. Digital pictures were taken for all combinations of antimicrobial and [HMIM]Cl concentration (0, 0.0025, 0.00625, 0.025 M). Notably, 0.025M [HMIM]Cl was completely lethal to all *S. aureus* which was evidenced as a complete lack of growth on plates even after several days in the incubator. Using the formula ((ZOI-PBS)/total plate diameter), we calculated the relative ZOI score for each antimicrobial for all different [HMIM]Cl concentrations. The data represented both numerically, as well as by shading, demonstrate a similar pattern to the liquid MIC screen on 96-well plates (Figure 5B). Out of the six antimicrobials evaluated by both methods, only ampicillin demonstrated conflicting results with a demonstrated synergistic interaction on plates and only limited potential synergistic interaction with [OMIM]Cl in liquid culture. Overall, results with ILs + tetracycline demonstrate strong and reproducible synergistic interactions. The combination of tetracycline plus [HMIM]Cl yielded larger diameter ZOI with an initial score of 22.7 at OM and increasing to 23.9 and 28.3 with 0.0025M and 0.00625M [HMIM]Cl suggesting an increase of 5.3% and 24.7%, respectively, in

Antibiotic	Organism	[HMIM]Cl	[OMIM]Cl
Chloramphenicol	<i>S. cerevisiae</i>	7.14	14.29
	<i>S. aureus</i>	14.29	0.00
	<i>E. coli</i>	0.00	14.29
	<i>P. aeruginosa</i>	10.71	7.14
Ampicillin	<i>S. cerevisiae</i>	7.14	14.29
	<i>S. aureus</i>	0.00	10.71
	<i>E. coli</i>	0.00	7.14
	<i>P. aeruginosa</i>	0.00	7.14
Kanamycin	<i>S. cerevisiae</i>	32.14	28.57
	<i>S. aureus</i>	0.00	0.00
	<i>E. coli</i>	0.00	0.00
	<i>P. aeruginosa</i>	14.29	0.00
Polymyxin B	<i>S. cerevisiae</i>	7.14	7.14
	<i>S. aureus</i>	14.29	7.14
	<i>E. coli</i>	0.00	21.43
	<i>P. aeruginosa</i>	0.00	7.14
Tetracycline	<i>S. cerevisiae</i>	10.71	0.00
	<i>S. aureus</i>	14.29	21.43
	<i>E. coli</i>	10.71	7.14
	<i>P. aeruginosa</i>	21.43	21.43
Ketoconazole	<i>S. cerevisiae</i>	7.14	0.00
	<i>S. aureus</i>	0.00	21.43
	<i>E. coli</i>	21.43	21.43
	<i>P. aeruginosa</i>	10.71	14.29

Table 2. Bacterial growth in liquid culture was evaluated for 6 different antimicrobial compounds in combination with 2 different ILs in 4 different microorganisms

Results presented indicate the percentage of wells in which a combined interaction between IL + antimicrobial was evident as decreased OD₆₀₀ compared to IL or antimicrobial only (dark green in Figure 3B), cells highlighted darker blue with higher percentage of cells displaying combined interaction. The percentage of wells yield reduced cell growth with combinations of [HMIM]Cl or [OMIM]Cl + antimicrobial.

antimicrobial potency. These data are further reflected when looking at IL + doxycycline, a close relative of tetracycline, with an effective increase of 7.3% and 17.0% increase in 0.0025M and 0.00625M [HMIM]Cl, respectively (Figure 5B).

Ionic liquid and antimicrobial cytotoxic effects on human cells

In order to understand the degree to which 1-alkyl-3-methylimidazolium of varying chain length is toxic to human cells, we treated HeLa cells with ILs or ILs + tetracycline for 24 hr and evaluated cytotoxicity. Following 24 hr of exposure in liquid culture at defined concentrations in a 96-well plate format, cells were evaluated for viability using CellTiter-Blue assay in which living cells are able to metabolize resazurin into resorufin, a fluorescent reporter molecule detected by plate spectroscopy. Consistent with findings for bacteria and yeast microorganisms, high concentrations of imidazolium chloride-based ILs are toxic to

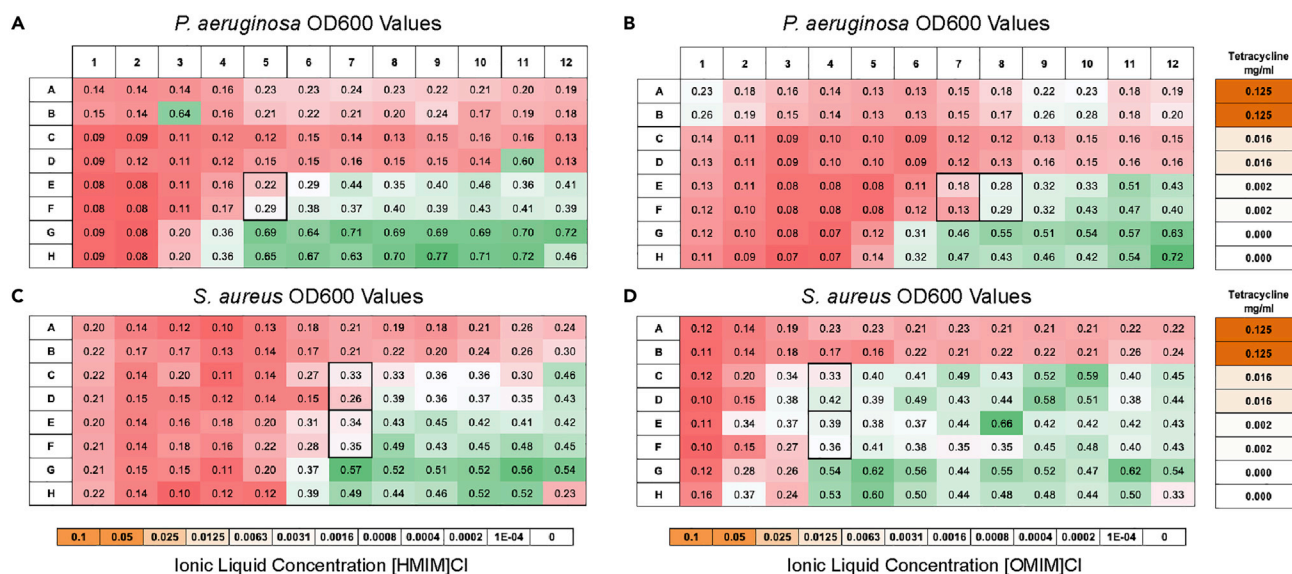


Figure 4. Synergistic response with combinations of [HMIM]Cl or [OMIM]Cl + tetracycline on microbial lethality

Targeted analysis of synergistic interactions in combinations of antimicrobials with ILs. Conditional highlighting of MIC values (OD600 readings) from 96-well plates of 24-hr liquid bacterial culture. Red represents low growth and green represents high growth.

(A–D)(A) *P. aeruginosa* treated with [HMIM]Cl + tetracycline, (B) *P. aeruginosa* treated with [OMIM]Cl + tetracycline, (C) *S. aureus* treated with [HMIM]Cl + tetracycline, (D) *S. aureus* treated with [OMIM]Cl + tetracycline. Boxes with thick borders represent wells demonstrating synergistic interactions. Regions with dark highlighted borders indicate synergism as bacterial growth limited to a greater degree than the combined effect of either IL or antimicrobial.

HeLa cells (Figure 6A). Moreover, HeLa cells treated with ILs with longer alkyl chain lengths [OMIM]Cl are less viable than when treated with [HMIM]Cl at the same final concentration.

When looking at the lethality of [HMIM]Cl or tetracycline independently on *P. aeruginosa*, low concentrations of either show no lethality while at high concentrations, treatment is highly lethal to cells (Figure 6B). Exposure to either [HMIM]Cl or tetracycline independently at concentrations of 0.05–0.025M or .01M, respectively, yields approximately 50% lethality in *P. aeruginosa*. However, when combined at significantly reduced concentrations of both molecules, similar 50% lethality is achieved. *P. aeruginosa* exhibits approximately 50% lethality at tetracycline concentrations of < .002M with the addition of 0.0031M [HMIM]Cl.

The same combinatorial treatments were given to HeLa cells using [HMIM]Cl + tetracycline, and cells were assayed for viability as previously described. As expected, in the absence of IL, the minimum concentration of tetracycline required for cytotoxic effects was higher in HeLa cells (.05 mg/mL) compared to *P. aeruginosa* (.002 mg/mL) (Figures 6B and 6C). Independently, treatment with [HMIM]Cl exhibited similar cytotoxic and inhibitory effects at the same concentration in both HeLa cells and *P. aeruginosa*, demonstrating 50% lethality at 0.05–0.025M [HMIM]Cl. However, in combination, [HMIM]Cl + tetracycline reduced the amount of tetracycline required to reach 50% lethality in *P. aeruginosa* (Figure 6A) while at the same time causing little to no increase in toxicity to human cells (Figures 6C and 6D). HeLa cells were treated in triplicates with defined, non-cytotoxic concentrations of [HMIM]Cl, 0.0031M, 0.0062M, and 0.0125M, in combination with a range of tetracycline concentrations. At concentrations of tetracycline below .02 mg/mL, HeLa cells were not affected by the combination of the both IL + antibiotic. In contrast, a synergistic interaction between the IL + antibiotic reduces the concentration of antibiotic required to inhibit *P. aeruginosa* at the same concentrations (Figure 6D).

Discussion

The potential for ILs to act as a highly tunable antimicrobial agent has gained recent attention with a number of studies demonstrating antimicrobial activity in a variety of microorganisms (Pendleton and Gilmore, 2015). However, while ILs have been shown to elicit antimicrobial activity and the mechanism of action has begun to be explored, several key challenges must be overcome to identify whether ILs have potential as therapeutic agents. In this study, we expanded on the potential mechanism of IL's antimicrobial activity

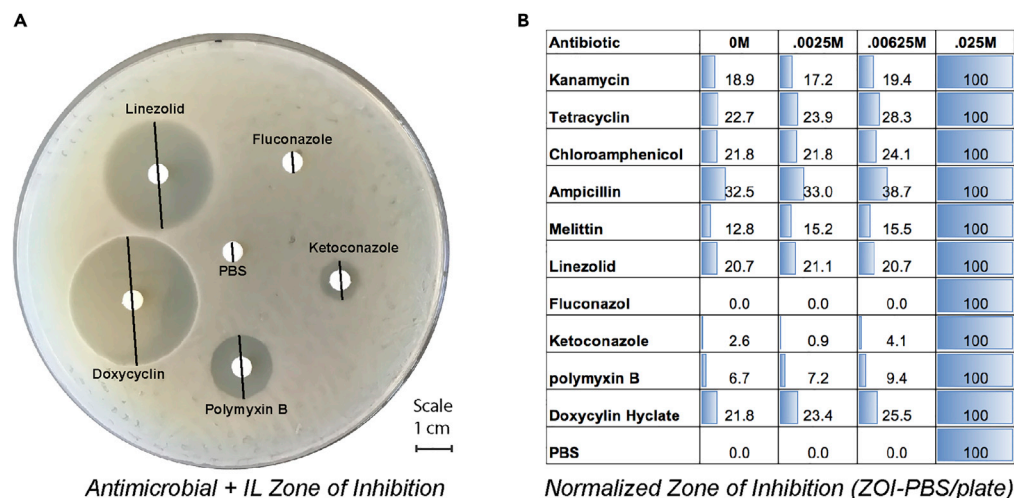


Figure 5. Bactericidal disc assays demonstrate combined effect of [HMIM]Cl or [OMIM]Cl + antimicrobial on *S. aureus* growth

Bactericidal disc assays of *S. aureus* with addition of [HMIM]Cl in combination with antimicrobials.

(A) Baseline representative growth plate – 150mm LB plate with *S. aureus* + 0M [HMIM]Cl and 6-mm discs plus 15uL indicated antimicrobial. Overlaid lines indicate the measured radius of the bacterial zone of inhibition (ZOI).

(B) Compiled results of various concentrations of IL + plus antimicrobial. The value represents the normalized radius of the zone of inhibition relative total plate radius. Blue bars indicate percentage inhibition compared to 100% inhibition (no growth) on .025M [HMIM]Cl plate.

and importantly identified previously unknown combinatorial and synergistic interactions between imidazolium chloride-based ILs and a broad class of antimicrobial agents in a subset of microorganisms. Finally, our results indicate that the enhanced toxic activity of combined IL + antimicrobial is only present in the bacterial and yeast species we investigated and not in human cell lines which points to a potential use as an adjuvant for antimicrobial treatments that may increase the efficacy of established medications by expanding the therapeutic window of existing antimicrobials.

The combination of imidazolium chloride-based ILs with 6 and 8 carbon alkyl tails exhibit combined and synergistic interactions with a wide range of antimicrobials. In two separate complementary assays, 96-well liquid culture and zone of inhibition disc assays, the combination of ILs + antimicrobial was effective in reducing the concentration of antimicrobial compound necessary to inhibit microbial growth (Figures 3, 4, and 5). Interestingly, while a surprisingly large number of different antimicrobials were enhanced by the addition of IL, several trends point to specific modes of action for IL + antimicrobial synergy. One of the strongest examples of combined effect was seen with the addition of IL + tetracycline which was evident in multiple different assays. Moreover, the efficacy of doxycycline was also strongly enhanced in our ZOI experiments. Both of these antibiotics are in the tetracycline family, an antibiotic class that inhibits protein synthesis by targeting the 30S ribosomal subunit (Connell et al., 2003). Interestingly, it has been suggested that the limited efficacy of tetracycline, as well as the mechanism for antibiotic resistance, revolves around limited uptake and retention by bacterial strains (Reynard et al., 1971). Given the potential membrane destabilizing effect of long alkyl chain ILs, it is reasonable to hypothesize that the addition of IL enhances the ability of this class of antibiotics to enter and maintain higher concentrations within the bacterial cell. Alternatively, several of the ILs in this study have been demonstrated to induce stability changes in some soluble proteins which could affect the ribosome stability or efflux pump activity (Kohn et al., 2018), although studies on those specific protein systems have not been reported.

Similarly, we found complimentary results with the improved efficacy of kanamycin in *S. cerevisiae*. It is typical practice in molecular biology to use the antibiotic kanamycin with bacteria and its analog geneticin in yeast species despite the fact that the same neomycin resistance gene confers resistance to both antimicrobials. Moreover, the primary structural difference between geneticin and kanamycin is the presence of 2 additional methyl groups that confer increased hydrophobicity (Kaplan et al., 2016). Furthermore, recent work indicates that the ability of kanamycin to enter cells is different in bacteria vs. eukaryotic cells

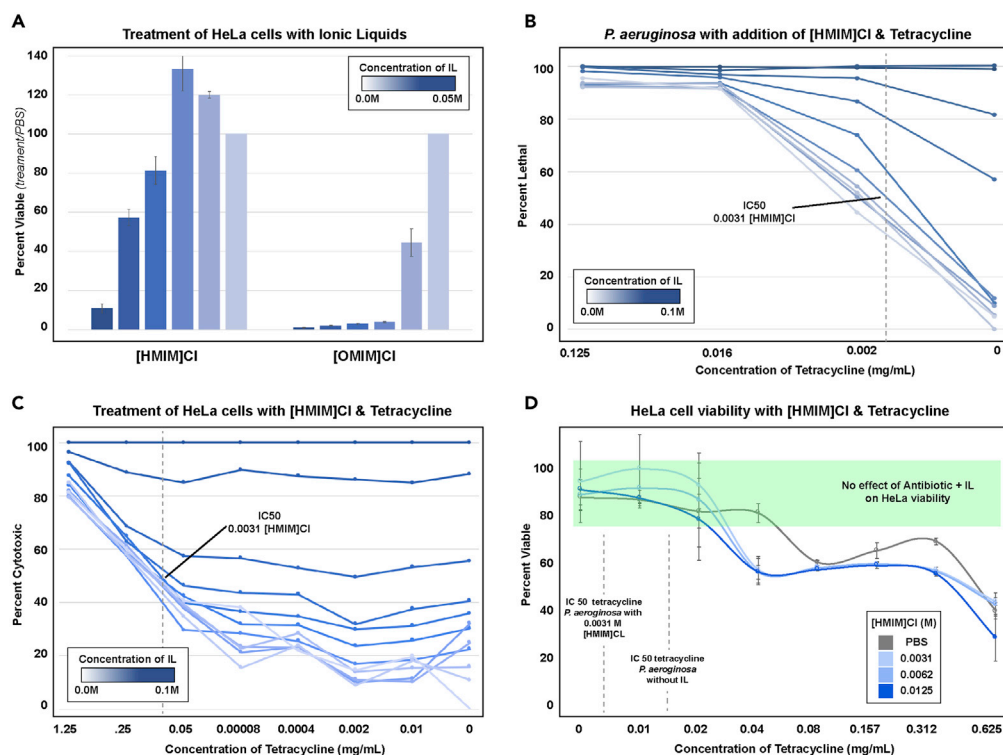


Figure 6. Treatment of with ILs + tetracycline indicates specificity of combined effect for bacteria but not human cell lines

The effects of ILs + tetracycline on human cell lines. HeLa cell lines grown in 96-well plates were exposed to IL only or IL + tetracycline for 24 hr, and cell viability was assayed with Cell-titer blue

- (A) HeLa cells treated with either [HMIM]Cl or [OMIM]Cl (no antibiotic) exhibit increasing cytotoxicity with increasing concentration of IL (n = 3) .05 confidence intervals.
- (B) Percentage lethality from 96-well plate assay *P. aeruginosa* grown with combined treatment of tetracycline + [HMIM] Cl. IC50 of .0031M [HMIM]Cl + tetracycline indicated on chart by dashed line.
- (C) Percentage cytotoxicity from 96-well plate assay HeLa cells grown with combined treatment of tetracycline + [HMIM] Cl. IC50 of .0031M [HMIM]Cl + tetracycline indicated on chart by dashed line.
- (D) Cell viability of HeLa cells with targeted concentrations of IL + tetracycline performed in triplicates. Green shading indicates concentrations of tetracycline + [HMIM]Cl with limited cytotoxicity to HeLa cells. *P. aeruginosa* IC50 tetracycline only or *P. aeruginosa* IC50 tetracycline + .0031M [HMIM]Cl indicated on chart by dashed line.

and ultimately dependent on direct interactions of kanamycin with the cell membrane (John et al., 2017). We posit that the historically evidenced reduced efficacy of kanamycin in *S. cerevisiae* may be the result of inability of kanamycin to enter the cell, which in our hands, is significantly reduced when kanamycin is combined with IL.

Amphiphilic molecules, such as ILs in this study, can act as antimicrobials in their own right, interacting with the bacterial membrane in a detergent-like manner causing destabilization of the membrane(s) (Laatiris et al., 2008). The activity of these molecules is linked to the monomer-micelle equilibrium, which dramatically changes the physical and chemical properties of the molecule(s). Several lines of experiments point to the ability of imidazolium chloride-based ILs to permeabilize cell membranes. In this study, we expand on this concept by demonstrating that even short-term exposure of microbial cell populations leads to enhanced uptake of propidium iodide (PI) into bacteria and yeast cells (Figure 2). Furthermore, previous work has shown that some of these imidazolium ILs can form micelles, and thus, if tetracycline affects the CMC, it would inherently impact the antimicrobial activity (Lee et al., 2019a). Micelle formation is primarily governed by concentration, and the concentration at which micelles form is known as the CMC. CMC has been shown to be influenced by salt concentration and other additives in solution (Palladino and Ragone, 2011). Our results point to the fact that tetracycline does not significantly affect the ability of [OMIM]Cl or [DMIM]Cl to form micelles (Figure 1B). One possible interpretation of this finding is that

tetracycline does not enhance the detergent-like properties of IL action on the cellular membrane but rather the addition of [OMIM]Cl or [DMIM]Cl to microbial populations acts independently of antimicrobial compound to elicit membrane destabilizing effects to enhance the efficacy of the antimicrobial compound applied.

RTILs are often touted as biocompatible, non-toxic, and a green alternative; however, this classification is likely only realistically applied in limited concentrations as some recent reports highlight the toxicity of these classes of molecules (Pham et al., 2010). In an effort to understand how imidazolium chloride-based ILs of varying chain length may be toxic to mammalian cells, we applied the combinatorial treatment of IL + tetracycline to mammalian cells. Though a limited approximation, these assays with HeLa human cell lines are a first step in identifying the potential human response to treatment using ILs + antibiotics (Stepnowski et al., 2004). Principle among our findings was that while the combination of [HMIM]Cl + tetracycline demonstrated a clear synergistic response as seen in the reduced amount of tetracycline required to reach 50% lethality in *P. aeruginosa* (Figure 6B), the addition of [HMIM]Cl to HeLa cells did not increase the toxicity of tetracycline in culture (Figure 6C). Taken together, our results suggest that the combination of imidazolium chloride-based ILs with antimicrobials is capable of reducing the amount of antibiotic necessary to combat bacterial growth while at the same time having little to no additional toxic effect in human cell lines. The mechanism of action for why the apparent enhancement of antibiotic delivery is effective in microbial cell populations but not mammalian cells is still unknown. One potential biochemical explanation is that antibiotic action is regulated by internal cellular concentration and that at typical treatment concentrations, microbes have a baseline ability to exclude or remove antibiotics. The addition of ILs can enhance the delivery and/or retention of antimicrobials to both microbes and mammalian cells but due to the lack of specificity of antimicrobial targets in mammalian cells, the toxicity of the antimicrobial is not increased. Future investigations will need to be performed to assess the underlying mechanism that protects the mammalian cells from higher concentrations of antibiotics while simultaneously eliciting a synergistic response in improving the efficacy of antimicrobial compounds for combating bacterial growth.

Limitations of the study

The primary limitations of this study are due to the depth of comprehensive screens in the study to infer relationships between efficacy of antimicrobial compound, IL, and microorganism being evaluated. In this study, we tested 1-alkyl-3-methylimidazolium ILs with 5 different alkyl chain lengths in combination with 10 different antimicrobial compounds for 4 different microorganisms. We are able to identify synergistic interactions with 2 of the ILs with several different antimicrobials depending on the microorganism being evaluated. While specific trends are identified that suggest that the degree of synergism may be enhanced for specific IL + antimicrobial compounds such as tetracycline + [HMIM]Cl, future work targeted at identifying statistically significant correlations with broad classes of antimicrobials or microorganisms will further aid understanding specific mechanisms of synergistic interactions.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Benjamin R Carone (carone@rowan.edu)

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data used for generation of figures in the current study are included in this publication; additional data sets and original flow cytometry files that are not included and are supporting the current study have not been deposited in a public repository and are available from the corresponding author on request.

Methods

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101853>.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.C., T.V., and G.C.; Execution of Experiments D.Y., N.P., A.S., K.C., P.T., and B.C.; Writing – Original Draft, D.Y., N.P., and B.C.; Writing – Review & Editing, T.V. and G.C.; Supervision, B.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Synergistic interactions of ionic liquids and antimicrobials improve drug efficacy

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Figure S1 - (Related to Figure 1b) - Permeabilization of *P. aeruginosa* cell lines correlates with increasing concentrations of IL

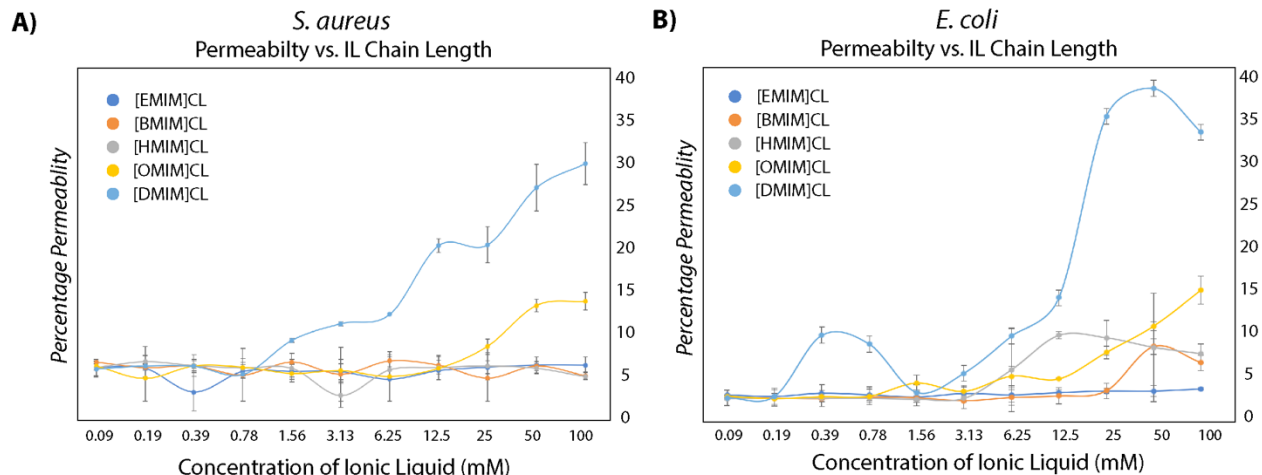


Figure S1 (Related to Figure 1b)

Permeabilizing effects of ILs at increasing concentrations on A) *S. aureus* and B) *E. coli* as measured by flow cytometry following a 30 min IL exposure and PI staining. Percentage of cells positive for PI staining indicated on the y-axis, error bars .05 confidence intervals for triplicate treatments.

Transparent Methods

Ionic Liquids (IL)

Ionic liquids used in this study: 1-ethyl-3-methylimidazolium chloride, [EMIM]Cl, Sigma Cat# 272841; 1-butyl-3-methylimidazolium chloride, [BMIM]Cl, Sigma Cat# 94128; 1-hexyl-3-methylimidazolium chloride, [HMIM]Cl, Alfa Aesar Cat # H27178; 1-octyl-3-methylimidazolium chloride, [OMIM]Cl, Alfa Aesar Cat # H59534; 1-octyl-3-methylimidazolium chloride, ([DMIM]Cl), TCI Cat #D5351. Solutions of IL at indicated concentration created with either PBS (Phosphate Buffer System) or water solvent as indicated by experiment.

Critical Micelle Concentration (CMC)

Stocks of 2 mM sodium phosphate buffer, 10 µg/mL Tetracycline, and 1.0 M {HMIM}Cl, [OMIM]Cl, and [DMIM]Cl were prepared. DPH concentration was determined by absorbance spectroscopy using $\epsilon_{350} = 88,000$ (Chattopadhyay and London, 1984). In a black, clear flat bottom 96-well plate, 50 µL of serially diluted IL was placed in each well. Then, calculated amount of buffer was added to each well. Once all wells contained buffer and IL, appropriate amounts of tetracycline for final concentrations of 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, or 0 µg/mL) was added to the wells. Right before measurement, DPH (final concentration 2.5 µM) was added with gentle mixing by pipetting to ensure no bubbles were formed. Samples were measured using $\lambda_{ex} = 358$ nm and $\lambda_{em} = 430$ nm. Experiments were performed in triplicate from treatment to measurement.

Minimal Inhibitory Concentration (MIC)

Bacteria were freshly streaked from frozen glycerol stock (-80°C) onto LB-Miller agar plates (*E. coli* D31 (Burman et al., 1968), *S. aureus* ATCC 35556, *P. aeruginosa* PA-01 (Capitato et al., 2017), *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 700603, *B. subtilis* ATCC: 6051). A single colony was taken from each streak to prepare an overnight in fresh LB broth and placed into a shaking incubator set at 37°C and 250 rpm for at least 18 hours. After 18 hours, a fresh dilution in LB Broth (1:200) was made and used for the experiment. Minimum Inhibitory Concentration (MIC) was performed using mid-log phase bacteria subsequently diluted to 5×10^5 CFU/mL in the assay plate. The diluted culture was added to each well of a sterile 96-well plate containing serially diluted aliquots of each RTIL for a final volume of 100 µL. The plate was incubated at 37°C overnight for about 18 hours. After 18 hours, the OD₆₀₀ was measured using a Spectramax M5 multimode plate reader.

Permeability Assay Flow Cytometry

Microbial cultures (*P. aeruginosa*, *E. coli*, *S. aureus*,) were grown to mid-log phase, and 90 µL of the bacterial culture added to 96-well. Prior to addition of IL, propidium iodide (PI) was added to

each well at a final concentration of 2ug/mL. Ionic liquids solutions in water ([EMIM]Cl, [BMIM]Cl, [HMIM]Cl, [OMIM]Cl, and [DMIM]Cl) were used in this assay. 10µL of ionic liquid solution was added to the 90µL of cells in each well for a total volume of 100µL, meeting the desired ionic liquid concentration (0.097, 0.196, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100mM) for each well. For the positive control, cetyl-trimethyl-ammonium-bromide (CTAB) was used at a final concentration of 0.3M. Cells were exposed to IL for 30 mins and then analyzed for internal fluorescence using a BD FACSCelesta instrument using PE-594 channel. >10,000 per well were counted for each sample.

$$\frac{\text{Ionic Liquid Sample}}{\text{CTAB Sample}} = \text{Degree of Permeabilization.}$$

Bactericidal Disc Assay

Assessment of bacterial growth on mixed media plates served as a clear visualization for the efficacy of the combinatory treatment of IL + antimicrobial. Standard LB agar plates were prepared with the addition of increasing concentrations of [HMIM]Cl: 0, 0.0025, 0.00625, and 0.025 M. Plates were allowed to solidify and dry on bench for 24 hours before use. 300µL of *S. aureus* liquid culture at mid-log phase was plated evenly on each plate with sterile glass beads. Following addition of culture the plates allowed to dry for 30 minutes before the addition of antibiotic disc. During this period the discs were prepared from the stock solutions of solubilized antibiotics; 15µL of each of the 10 antibiotics used was pipetted onto a sterile 6mm cellulose disc (BD). Antibiotics used in this study, kanamycin (50 mg/mL), tetracycline (12.5 mg/mL), chloramphenicol (34 mg/mL), ampicillin (10mg/mL), polymyxin B (23 mg/mL), doxycycline hyclate (10 mg/mL), clindamycin (5 mg/mL), azithromycin (2.5 mg/mL), melittin (100 uM), and linezolid (5 mg/mL), fluconazole (5 mg/mL), ketoconazole (1 mg/mL). After plate drying was complete, sterile forceps were used to transfer the discs to the media and the plate was grown upright in an incubator overnight at 30°C. The following day, photographs were taken of the plates using iPhone 6 to visualize the zone of inhibition (ZOI) created by the combined treatment. Final analysis of diameter of ZOI measures using Adobe Illustrator.

Treatment with HeLa cells with ILs + tetracycline

HeLa cells were grown and maintained in DMEM with 4.5 g/L glucose + Sodium Pyruvate + L-Glutamine (VWRV02-0101) plus the addition of 10% FBS. Growth media additionally contains Pen/Strep but prior to treatment with ILs, media was changed to exclude any antibiotics besides those being tested. HeLa cells were grown to confluency in a T75 dish, dissociated with 3mL Trypsin/EDTA .05M for 3 min, and resuspended in 25 mL DMEM without antibiotics, 180µL cells/media were added to each of the 96 wells on the assay plate and incubated overnight. Solutions of [HMIM]Cl and [OMIM]Cl and tetracycline were created using PBS as a solvent and 20 µL of IL or IL + tetracycline as added to 96-well plate and incubated for 24 hours. All treatments on 96-well plates were performed in triplicate. Cytotoxicity was evaluated using Promega CellTiter-Blue Cell Viability reagent according to manufacturer's guidelines. 20µL of CellTiter-Blue was added per well, then the plates were incubated for either 1, 2, or 4 hours read at in a Synergy HT fluorometer Ex/EM 485/590nm.

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