Chem Biol Drug Des 2011; **78:** 33–41

doi: 10.1111/j.1747-0285.2011.01114.x

Research Article

Design, Synthesis, and Biological Evaluation of β-Lactam Antibiotic-Based Imidazolium- and Pyridinium-Type Ionic Liquids

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We herein report the preparation and investigation of antibacterial activity of biocidal ionic liquids (ILs) consisting of cationic imidazolium or pyridinium and an anionic β-lactam antibiotic. The antibacterial properties were quantified by measuring the minimum inhibitory concentration and minimum bactericidal concentration against *Escherichia coli O157:H7*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecium*. In general, the ILs had improved antibacterial activity than their parent materials, whether individually or in combination. In 83% of the experiments, the ampicillin ILs (Amp-ILs) had better antibacterial activities than their quaternary halide parent materials, whereas in 92% of the experiments, Amp-ILs outperformed the commercially available sodium ampicillin salt. Amp-ILs had up to 43 times improved antibacterial activity than sodium ampicillin. Overall, when normalized for ampicillin content, ILs had greater antimicrobial activity against *E. coli O157:H7*, *K. pneumoniae*, *S. aureus*, and *E. faecium* than sodium ampicillin alone.

Key words: antibacterial, cetylpyridinium, imidazolium, ionic liquid, quaternary ammonium compounds, sodium ampicillin, β-lactam

Received 8 October 2010, revised 25 January 2011 and accepted for publication 6 March 2011

Data accumulated over the last decade show that there is an association between antimicrobial resistance in *Staphylococcus aureus*, *enterococci*, and Gram-negative *bacilli* and increases in mortality, morbidity, length of hospitalization, and cost of healthcare (1,2). Patients infected with antimicrobial-resistant organisms have higher associated treatment costs (US$6000–$30 000) than do patients with infections of antimicrobial-susceptible organisms (3). Consequently, many antibiotics, particularly β-lactam drugs, are becoming less effective treatment options (1,4–7). This challenge necessitates the development of more effective antibacterial agents. The development of ionic liquids (ILs) composed of antibiotics fortified with other antibacterial compounds was investigated in this study as a promising strategy to address antibiotic resistance.

Ionic liquids are low-melting (i.e. <100 °C) salts that result from the pairing of various organic or inorganic cations and anions (8). They are considered to be 'designer' salts as their individual physical and chemical properties can be tuned to a variety of desirable features. Recently, studies have explored the use of ILs in many applications such as chemical synthesis (9,10), catalysis (11), separation science (12,13), and electrochemistry (14). For example, active pharmaceutical compounds have been paired with typical IL cations (i.e. imidazolium-, pyridinium-, or phosphonium-type cations) to resolve problems with drug polymorphism, purity, and intestinal absorption (15). ILs have also been applied to microbiological studies as antibacterial agents (16,17). The use of quaternary ammonium compounds (QACs) in combination with non-nutritive sweeteners has led to a class of sweet and antibacterial ILs with potential use in the food industry (18,19). These examples demonstrate the tunability of ILs and their potential to improve the efficacy of antibiotics.

The synthesis of antibacterial β-lactam ILs is a facile process using anion metathesis, making these compounds cost-effective alternatives to designing new β-lactamase-resistant antibiotics (15). To date, reported antibacterial ILs have been composed of heterocyclic nitrogenous cations such as QACs [e.g. benzalkonium chloride, cetyltrimethylammonium bromide (CTAB), and cetylpyridinium bromide (CPB)] and anions from halogenated sources (15,20). Initially, the antibacterial properties of an IL were investigated by only modifying the structure of the cation. Enhanced antimicrobial activity was observed with increasing alkyl length in both *n*-alkylimidazolium and *n*-alkylpyridinium structures (16,17). However, the cytotoxicity of many halogenated ILs has been shown to interfere with cell signaling, hence limiting their use in biological applications (21,22).

Many strategies have been proposed to extend the efficacy and antibacterial spectrum of current antibiotics. As alternatives to *de novo* drug synthesis, methods that employ organized media as potential delivery agents to improve the absorption of various pharmaceutical agents have met with some clinical success (23–25). Examples include

the use of QACs to improve the absorption, transport, and efficacy of various drugs. Specifically, dihydropyridinium salt delivery systems have successfully achieved targeted penicillin delivery to the central nervous system (26). Other pyridinium salts have been used to selectively facilitate drug transport across the brain–blood barrier and dermis via enzymatic hydrolysis (27–30). CTAB and related surfactants have enhanced drug absorption by various strains of Gram-negative and Gram-positive bacteria (31). These formulation strategies have been shown to facilitate the uptake of various antibiotics and improve their treatment properties. An additional technique that has recently been employed is the use of combination drug therapy. This method uses the individual properties of two or more pharmaceutical agents in combination to improve or enhance the properties of either drug. An example of this approach is the drug Unasyn® (Pfizer, New York, NY, USA) which is composed of the β-lactam class antibiotic, ampicillin, and the β-lactamase inhibitor, sulbactam. Other examples of drug combination therapy includes the combination of piperacillin and tazobactam, amoxicillin and clavulanate, and trimethoprim and sulfamethoxazole (32). In this study, a new set of ampicillin salts with quaternary ammonium cations such as 1-hexadecyl-3-methylimidazolium, 1-hexadecyl-2,3-dimethylimidazolium, and cetylpyridinium were synthesized and found effective on Gram-negative bacteria *Escherichia coli O157:H7* and *Klebsiella pneumoniae*, as well as on the Gram-positive *S. aureus* and *Enterococcus faecium*. By taking advantage of the antibacterial nature of both the cationic and anionic components of the IL, we demonstrate the antibacterial utility of ILs for various biological and medical applications.

Materials and Methods

Reagents

1-Methylimidazole and 1,2-dimethylimidazole, 1-bromohexadecane, CPB, CTAB, sodium ampicillin ([Na][Amp]), sterile disks, brain heart infusion broth (BHI broth), Petri dishes (100 × 20 and 150 × 20 mm), 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), chloroform, and diethyl ether were purchased from Sigma Aldrich (St Louis, MO, USA) and used without further purification; isopropanol and acetone were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA), and ethanol was purchased from Pharmco-Aaper and Commercial Alcohols (Brookfield, CT, USA). All solvents purchased were of analytical grade. Agar technical grade was purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ, USA).

Culture preparation

Test organisms, *E. coli O157:H7* (*ATCC 43895*), *S. aureus* (*ATCC 6538*), *E. faecium* (*ATCC 49474*), and *K. pneumoniae* (*ATCC 4352*), were grown individually on BHI agar for 24 h at 37 °C (33).

General procedure for quaternization reactions

Equimolar amounts of 1-methylimidazole or 1,2-dimethylimidazole and 1-bromohexadecane were stirred in anhydrous ethanol under reflux for 48 h in an argon atmosphere. Ethanol was removed and the product was washed with diethyl ether. A white solid was obtained after filtration. The product was dried and purified in acetone using the recrystallization method.

General procedure for anion metathesis

A typical anion-metathesis reaction procedure is as follows. [CP][Br] (1 equiv.) was dissolved in chloroform with the slow addition of aqueous [Na][Amp] (1.1 equiv.) into the solution. The chloroform/water mixture (4:1 v/v) was stirred for 48 h at room temperature. The upper aqueous solution was separated and washed with fresh chloroform to obtain all exchanged product. The removal of chloroform *in vacuo* yielded the white product, [CP][Amp], which was further freeze-dried on a lyophilizer. Other ILs (i.e. [$C_{16}M_1$Im][Amp] and [$C_{16}M_2$Im][Amp]) were synthesized in the same manner.

Ionic liquid characterization

These compounds were characterized using ^{1}H-NMR (Bruker Avance-250, 250 MHz) with deuterated dimethylsulfoxide (DMSO) as solvent. The elemental composition was determined using *Leco 932* CHNS Analyzer (Atlantic Microlab, Inc. Norcross, GA, USA). The thermal properties including melting points were determined using a differential scanning calorimetry (DSC Q100; TA Instruments, Wilmington, DE, USA).

Critical micelle concentration

The critical micelle concentration of the Amp-ILs was determined by measuring the surface tension with a Sigma 703 tensiometer at 298 K. Several half-fold dilutions were made from a 2 mM stock solution of both QAC and Amp-ILs. This method used a DuNuoy ring with a circumference of 5.992 cm.

Solubility

The solubility of Amp-ILs was determined using a Shimadzu UV-3101 PC scanning spectrophotometer. Briefly, this included measuring the absorbance of half-fold dilution series ranging up to 2 mg/mL of sodium ampicillin in water. Because sodium ampicillin has three characteristic absorption bands (i.e. 257, 262, and 268 nm) (34), these bands were used to confirm and quantify the solubilities of the Amp-ILs via the construction of a calibration curve (R = 0.99). Two milligram per milliliter of each Amp-IL was suspended in water with 1 min of high mixing and 30 min of sonication at room temperature. The suspension was filtered with a 0.45 μm filter (Whatman, Piscataway, NJ, USA) and the filtrate was analyzed using absorbance spectroscopy to quantify maximum solubility. The Amp-IL-acquired absorbance was converted into concentration using a Beer's law relationship from the sodium ampicillin slope. It was noted that the imidazolium bromide absorbs at lower energies and therefore does not interfere with the absorbance intensities of the ampicillin anion (35–37). However, CPB does absorb within this same range. To compensate for the cross-absorbance in [CP][Amp], the absorbance of CPB at equal concentrations of [CP][Amp] was subtracted to only obtain the absorbance of the anionic portion of the Amp-IL.

Amp-IL characterization

Cetylpyridinium ampicillin ([CP][Amp]), yellow solid, yield, 92%. Mp = 56 °C [CP][Amp]. CMC = 24 μM [CP][Amp]; 690 μM CPB. Water solubility: 348 μg/mL. 1H NMR (250 MHz, DMSO) δ 8.73 (tt, 5H), 7.24 (m, 5H), 2.09 (s, 14H), 1.91 (s, 4H), 1.49 (d, 6H), 1.24 (s,

20H), 0.86 (s, 2H). Anal. Cacld for $C_{37}H_{56}N_4O_4S$: C, 68.06; H, 8.64; N, 8.58; S,4.91. Found: C,67.97; H, 8.57; N, 8.55S, 4.84.

1-hexadecyl-3-methylimidazolium ampicillin ([$C_{16}M_1$Im][Amp]), off-white solid, yield, 91%. Mp = 55 °C [$C_{16}M_1$Im][Amp].CMC = 24 μM [$C_{16}M_1$Im][Amp]; 430 μM [$C_{16}M_1$Im][Br]. Water solubility: 379 μg/mL. 1H NMR (250 MHz, DMSO) δ 9.11 (s, 1H), 8.31 (s, 1H), 7.72 (d, 2H), 7.59 – 7.12 (m, 5H), 5.36 (s, 2H), 4.14 (t, 2H), 3.85 (s, 2H), 2.09 (s, 3H), 1.77 (t, 2H), 1.47 (dd, 10.0 Hz, 6H), 1.24 (s, 28H), 1.09 (s, 3H), 0.85 (d, 2H).Anal. Cald for $C_{36}H_{57}N_5O_4S$: C, 65.92; H, 8.76; N, 10.68; S, 4.89. Found: C, 65.79; H, 8.81; N, 10.42; S, 4.87.

1-hexadecyl-2,3-dimethylimidazolium ampicillin ([$C_{16}M_2$Im][Amp]), off-white solid, yield, 94%. Mp = 65 °C [$C_{16}M_2$Im][Amp]. CMC = 92 μM [$C_{16}M_2$Im][Amp]; 450 μM [$C_{16}M_2$Im][Br]. Water solubility: 475 μg/mL. 1H NMR (250 MHz, DMSO) δ 8.27 (s, 1H), 7.60 (dd, 2H), 7.53 – 6.95 (m,5H), 5.70 (s, 2H), 5.30 (d, 4H), 4.07 (t, 2H), 3.73 (s, 3H), 2.09 (s, 1H), 1.68 (s, 6H), 1.22 (s, 30H), 0.83 (d, 3H).Anal. Cald for $C_{37}H_{59}N_5O_4S$: C, 66.33; H, 8.88; N, 10.45; S, 4.79. Found: C, 66.21; H, 8.84; N, 10.44; S, 4.72.

Antibacterial activity

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC values were determined in triplicate by the broth dilution method in a 96-well microtiter plate using Mueller-Hinton Broth (38). The test organisms were grown individually on BHI agar for 24 h at 37 °C prior to each antibacterial test. The growth was adjusted using colony plate counts. Bacteria of 10^5 CFU/mL concentrations were exposed to an Amp-IL concentration range of 0.8–0.2 mM. The MIC for each Amp-IL was recorded as the lowest concentration that showed no turbidity after 24 h of incubation at 37 °C. Turbidity is an indication of microbial growth and if present, the corresponding concentration of antibacterial agent is considered ineffective. To determine whether the Amp-ILs inhibited growth or killed the bacteria, twenty microliters of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT (1 mg/mL) was added to the non-turbid wells of the MIC assay plate and incubated for 2 h at 37 °C for the bacteriostatic/bacteriocidal status determination (39,40). In the case of viable cells with inhibited growth, the tetrazolium dye (i.e. yellow solution) would be metabolically reduced to aqueous soluble formazan crystals (i.e. purple solution); however, a solution containing dead bacterial cells would remain yellow (40).

Results and Discussion

Synthesis and characterization

The synthesis of 1-alkyl-3-methylimidazolium ILs involved the quaternization of 1-methylimidazole or 1,2-dimethylimidazole with 1-bromohexadecane followed by anion exchange. Quaternization was carried out for 48 h under reflux in anhydrous ethanol under argon atmosphere. Amp-ILs (Table 1) were synthesized by anion-exchange reactions between the synthesized imidazolium bromides [Im][Br] or commercially available QAC, CPB [CP][Br], and excess sodium ampicillin [Na][Amp] in a chloroform/water (4:1 v/v) mixture. The resulting products, 1-hexadecyl-3-methylimidazolium ampicillin [$C_{16}M_1$Im][Amp], 1-hexadecyl-2,3-dimethylimidazolium ampicillin [$C_{16}M_2$Im][Amp], and cetylpyridinium ampicillin [CP][Amp], were isolated as solids at room temperature and purified by washing with anhydrous diethyl ether. These salts have limited solubility in water but are soluble in ethanol, isopropanol, dimethylsulfoxide, and chloroform.

The solubility of Amp-ILs in water was characterized using UV/vis spectroscopy. We observed that the solubilities of Amp-ILs were 475, 379, and 348 μg/mL for [$C_{16}M_2$Im][Amp], [$C_{16}M_1$Im][Amp], and [CP][Amp], respectively. A 100- to 150-fold reduction (R^2 = 0.99) in the aqueous solubility of ampicillin was observed once the sodium was replaced with a quaternary ammonium group. A positive control consisting of Amp-ILs in isopropanol confirmed that the anionic ampicillin was an intact part of the IL structure as evidenced by absorption bands at 257, 262, and 268 nm.

Amp-ILs were also characterized using ^{1}H-NMR and elemental analysis. All Amp-ILs contained the chemical shifts of the ampicillin anion and the respective cations. In the case of [$C_{16}M_1$Im][Amp], a singlet peak was observed with a chemical shift at 9.11 ppm, which was attributed to the acidic proton in the C2 position. However, this acidic peak is absent in the spectra for [C16M_2Im][Amp] because of the methyl group substituted on C2. In addition, the chemical shifts between the hydrogen on the C4 and C5 positions of the imidazolium rings decreased upon successful metathesis. A secondary set of multiplets ranging from 8.27 to 9.19 ppm present in the ^{1}H-NMR of [CP][Amp] are attributed to the more electron-withdrawing nitrogen in the pyridinium ring. The anion exchange from bromide to ampicillin anion was confirmed by examining the multiplet ranging from 7.11 to 7.52 ppm that was directly contributed by the benzyl group in the ampicillin structure. Lastly, a strong singlet at approximately 1.22 ppm validated the existence of the long alkyl chain in the cation moiety for each Amp-IL.

Evaluation of antibacterial activity

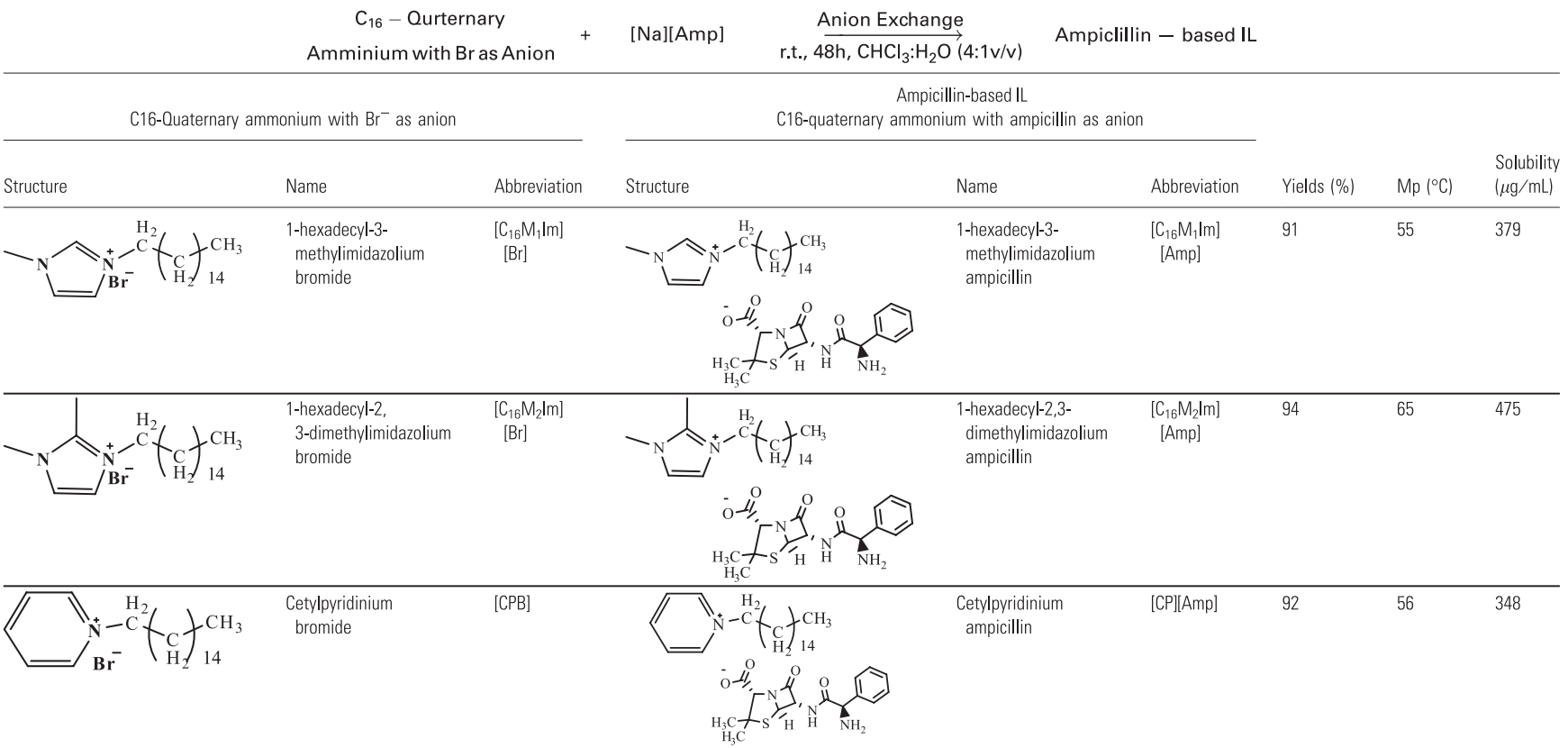
MIC results

The trend of increasing toxicity with long alkyl chain lengths paired with ampicillin as an anion was observed in both ampicillin-type, pyridinium- and imidazolium-based ILs. To quantify their antibacterial activities, the MICs and MBC were determined. As controls, the components of the ILs were evaluated for antimicrobial activity to determine the difference in activity between the molecular and IL forms of the compounds. The MIC results demonstrate that each Amp-IL exhibited similar or improved activity in the Amp-IL form against both Gram-positive and Gram-negative bacteria (Table 2). Overall increases in activity ranged from 2 to 43 times compared with [Na][Amp].

Imidazolium-based ampicillin ionic liquids

The imidazolium-type ampicillin salts were tested against *E. coli O157:H7*, *K. pneumoniae*, *S. aureus*, and *E. faecium* to investigate structure and activity relationships. These compounds are composed of three parts: the head imidazole, the C16-alkyl chain, and the

Table 1: Synthesis of four ampicillin-based ionic liquids by anion-exchange reactions



C16-Quaternary ammonium with Br^- as anion			Ampicillin-based IL C16-quaternary ammonium with ampicillin as anion					
Structure	Name	Abbreviation	Structure	Name	Abbreviation	Yields (%)	Mp (°C)	Solubility ($\mu g/mL$)
	1-hexadecyl-3-methylimidazolium bromide	$[C_{16}M_1Im][Br]$		1-hexadecyl-3-methylimidazolium ampicillin	$[C_{16}M_1Im][Amp]$	91	55	379
	1-hexadecyl-2,3-dimethylimidazolium bromide	$[C_{16}M_2Im][Br]$		1-hexadecyl-2,3-dimethylimidazolium ampicillin	$[C_{16}M_2Im][Amp]$	94	65	475
	Cetylpyridinium bromide	[CPB]		Cetylpyridinium ampicillin	[CP][Amp]	92	56	348

Table 2: Minimum inhibitory concentration (μM) of imidazolium- and pyridinium-type ampicillin ionic liquids

	Escherichia coli O157:H7	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Enterococcus faecium*
[$C_{16}M_1Im$][Br]	10	15	15	1
[$C_{16}M_1Im$][Amp]	9	15	30	13
[$C_{16}M_2Im$][Br]	12	15	23	9
[$C_{16}M_2Im$][Amp]	9	15	14	0.4
[CPB]	13	13	15	2
[CP][Amp]	6	9	8	0.4
[Na][Amp]	12	20	27	17
[Na][Br]	na	na	na	na
[K][Cl]	na	na	na	na
[Na][Cl]	na	na	na	na

*na denotes that no antibacterial activity occurred within the concentration range tested.
CPB, cetylpyridinium bromide.

anion (bromide or ampicillin). The MICs of 1-hexadecyl-3-methylimidazolium and 1-hexadecyl-2,3-dimethylimidazolium bromides (Table 2) against *E. coli O157:H7* showed marginal difference; however, the former had greater activity. In the case of *K. pneumoniae*, the 1-hexadecyl-3-methylimidazolium bromide was more effective. The antimicrobial mechanism of these types of long alkyl imidazolium bromides against bacteria is still not known in detail but is thought to involve a general perturbation of the lipid bilayer in bacterial membranes (16,17,41). Ahlström *et al.* (42) previously reported that QACs with a C16 hydrophobic chain affected the outer membrane of Gram-negative bacteria more extensively than shorter-chain compounds, leading to the leakage of cytoplasmic material and eventual death of the bacterial cell. Previous studies have also demonstrated that imidazolium halides with hydrophobic groups in the C1 and C3 positions of the imidazolium ring demonstrated higher antibacterial activity than 1,2,3-trisubstituted imidazoles (17). This demonstrates that the relative antibacterial activity of these types of compounds can be attributed to the alkyl chain length and head group substitutions, but not the imidazole ring structure.

In the case of the Gram-positive bacterium *E. faecium*, [$C_{16}M_1Im$][Br] required nine times less material than [$C_{16}M_2Im$][Br] to inhibit its growth (Table 2). This result can be attributed to the lack of a lipopolysaccharide layer and agrees with previous published results in which Gram-positive bacteria were more susceptible than Gram-negative bacteria to permeation by the long alkyl chain present on the imidazolium ring. Again, it is suggested that monoalkyl QACs bind by ionic and hydrophobic interactions to microbial membrane surfaces arranged with the hydrophobic tails inserted into the lipid bilayer, resulting in the rearrangement of the membrane and subsequent leakage of intracellular contents (41). In our study, *S. aureus* required greater concentrations for inhibition but this result is attributed to the acquired resistance developed in this strain, as determined using the disk-diffusion assay (data not shown).

After undergoing anion exchange from bromide to the antibiotic, ampicillin, our results demonstrate a reduction in the amount of ampicillin required to inhibit the growth of the challenge pathogens. As literature has suggested that there are minimal effects on antibacterial activity from the variation of the anion within imidazolium- and pyridinium-type ILs, any changes in antibacterial activity for our Amp-ILs will be attributed to the antibiotic ampicillin (16,43,44). Similar to the investigation conducted by Docherty *et al.*, (45) we also investigated the antibacterial activity of the halide salts, sodium bromide, potassium chloride, and sodium chloride in which no antibacterial activity was observed within the concentration range studied in this investigation (Table 2). When comparing the antibacterial activities of the ampicillin imidazoliums with the halide imidazoliums, reductions in antibacterial activity were evident (Table 2). There were minimal changes in the antibacterial activity of *E. coli O157:H7* and *K. pneumoniae* for [$C_{16}M_1Im$][Amp] and [$C_{16}M_2Im$][Amp]. This could be explained by the known activities of penicillin and its analogs against Gram-negative bacteria. First, the β-lactam must be able to penetrate the outer lipopolysaccharide (LPS) envelope and intrinsically bind to the different target proteins within the bacteria cell wall (46). However, the hydrophilic nature of the LPS protects the cell from large, hydrophobic perturbation agents such as certain antibiotics, detergents (47), or in this case Amp-ILs. In addition, the large masses of the ILs may inhibit the drugs from passing through pores located in the LPS layer. Despite being specific to the types of bacteria, the pores located in *E. coli* do not allow molecules with masses larger than 600 Da to enter the cell(48) and our Amp-ILs have masses >650 Da on average. Therefore, the antibacterial activity of Amp-ILs against *E. coli O157:H7* and *K. pneumoniae* may be solely a result from the cationic portion of the ionic liquid structure. This is further supported in the results outlined in Table 2 where it is evident that neither halide nor ampicillin imidazolium produced substantial changes in antibacterial activity compared with [Na][Amp]. When comparing [$C_{16}M_1Im$][Amp] and [$C_{16}M_2Im$][Amp] with [Na][Amp] activities for *E. coli O157:H7* and *K. pneumonia*e, respectively, the MIC was improved by 25% for both bacteria. In comparison between Amp-ILs and the halide imidazolium counterparts, a 10% improvement in MIC for the two pathogens was observed.

It is commonly recognized that penicillin and its analogs are more effective on Gram-positive than on Gram-negative bacteria because of the absence of the LPS in Gram-positive bacteria (49). In Table 2, it can be seen that the antibacterial activities for ampicillin imidazoliums significantly improved requiring 13 and 0.4 μM for [$C_{16}M_1Im$][Amp] and [$C_{16}M_2Im$][Amp], respectively, to inhibit *E. faecium* compared with [Na][Amp]. Similar activity is not seen with *S. aureus* for both ampicillin-type imidazoliums. When compared with [Na][Amp], [$C_{16}M_2Im$][Amp] required 50% lower concentration to inhibit the growth of resistant *S. aureus*. It is believed that the inherent resistance shown by *S. aureus* in this study could be attributed to its development into a mucoid strain which is less susceptible to long alkyl disinfectants. If this is the case, we attribute the [$C_{16}M_2Im$][Amp]'s improvement in antibacterial activity to hydrophobicity and its ability to transport through the slime layer of *S. aureus*.

Pyridinium-based ampicillin ionic liquids

The antibacterial activity of the pyridinium-type ampicillin IL was also investigated against *E. coli O157:H7*, *K. pneumonia*, *S. aureus*, and *E. faecium*. These compounds are also composed of three

Table 3: Minimum inhibitory concentrations based on ampicillin content for ampicillin-based ILs and sodium ampicillin in μM and μg/mL (values in the parentheses)

	% Ampicillin	*Escherichia coli O157:H7*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Enterococcus*
[$C_{16}M_1$Im][Amp]	53	4.7 (1.91)	7.9 (3.19)	7.9 (6.38)	6.9 (2.73)
[$C_{16}M_2$Im][Amp]	52	4.7 (2.96)	7.8 (5.22)	7.3 (4.64)	0.2 (0.14)
[CP][Amp]	53	3.2 (2.01)	4.7 (2.99)	4.2 (2.53)	0.2 (0.29)
[Na][Amp]	93	11.1 (4.03)	18.6 (6.90)	25.1 (9.21)	15.8 (5.75)

parts: the head pyridinium ring, the C16-alkyl chain, and the anion (bromide or ampicillin). Similar to imidazolium halides, pyridinium halides have been extensively investigated to determine the structural contributions to their disinfecting properties (50). The addition of a long alkyl chain to C1 on the pyridinium ring results in increased IL toxicity against *V. fischeri* (45). It has also been demonstrated that C16-alkyl chains are the most effective portion of the ionic liquid structure when reducing the growth of bacteria (17,51). Therefore, it is suggested that the hydrophobic chain on the pyridinium ring helps to perturb the cell wall. For the IL [CP][Amp], this cell wall permeation would improve the access of the ampicillin anion to the cell wall.

The MICs of [CP][Amp] against both *E. coli O157:H7* and *K. pneumonia* showed improvement in the antibacterial activities when converted into an Amp-IL (Table 2). After exchange of the bromide with the ampicillin anion, improvements were observed up to seven times for both Gram-negative and Gram-positive bacteria. These improvements are attributed to a combination of the long alkyl pyridinium and ampicillin moieties. In comparison with the antibacterial activity of [Na][Amp], the MIC was improved up to 43 times. The most significant improvement was observed when *E. faecium* was tested.

As the MIC value was equal to MBC for each Amp-ILs, the antibacterial activities of these compounds were considered to be bactericidal. Therefore, we hypothesize that the antibacterial behavior of the investigated Amp-ILs is because of the combination of a cell wall permeant with a transpeptidase inhibitor.

Effect of ampicillin content in ILs

The activity of the IL compounds is more clearly described by normalizing the concentrations based on the percentage of ampicillin content within the Amp-ILs and [Na][Amp] (Equation 1).

$$\frac{Mw_{ampicillinoate}}{Mw_{Amp-IL}} \times MIC \quad (1)$$

For all Amp-ILs, it was observed that the reduced concentration of ampicillin anion in Amp-ILs was required to inhibit bacterial growth compared with [Na][Amp] (Table 3). Depending on the bacterial species, the ampicillin content required for inhibition was in the microbe susceptible range for ampicillin activity (i.e. 0.1–20 μg/mL) (33) The average concentrations of ampicillin, in Amp-ILs, required to kill *E. coli O157:H7*, *K. pneumoniae*, *S. aureus*, and *E. faecium* were found to be 2.29, 3.8, 4.51, and 1.05 μg/mL, whereas the MIC in terms of ampicillin content for [Na][Amp] were 4.03, 6.90, 9.21, and 5.75 μg/mL, respectively. This is a two- to sixfold reduction in ampicillin content required for inhibitory activity compared with [Na][Amp]. These results demonstrate that lower amounts or fewer moles of Amp-ILs are required to inhibit the growth of the tested inoculums, which in turn could lower dosage amounts if applied in pharmaceutical systems.

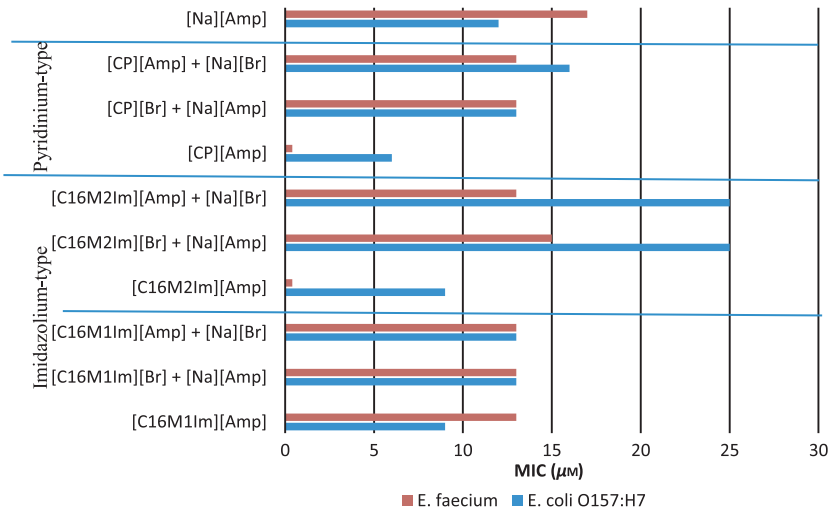


Figure 1: Comparison of minimum inhibitory concentration between ampicillin ionic liquids and their starting materials tested in combination.

Combinatorial effect of QAC and sodium ampicillin co-activity

It has been reported that the effect of a pharmaceutical agent on both resistant and sensitive bacterial strains can be enhanced with surfactants. For example, Suling and O'Leary (52) observed that the addition of CTAB increased the activity of penicillin G on *E. coli*, *P. mirabilis*, *K. pneumoniae*, and various *Staphylococci* strains. However, this was not completely evident in our study. It was observed that the use of both QAC and [Na][Amp] tested in combination did not outperform the antibacterial activities of the Amp-ILs. For example, in Figure 1, the antibacterial activities of $[C_{16}M_1Im][Br]$ + [Na][Amp], and [CP][Br] + [Na][Amp] were 30% less, in comparison with $[C_{16}M_1Im][Amp]$ and [CP][Amp] for *E. coli O157:H7*. Similarly, the antibacterial activity of $[C_{16}M_2Im][Br]$ + [Na][Amp] was reduced by 80%, compared with the IL form, when two components were combined against *E. coli O157:H7*. Our results demonstrate that the use of either QAC, $[C_{16}M_1Im][Br]$ + [Na][Amp], or $[C_{16}M_1Im][Amp]$ could equally inhibit the growth of *E. faecium*, whereas both [CP][Amp] and $[C_{16}M_2Im][Amp]$ required 73% reduction in concentration compared to the starting materials (Figure 1). As controls, [Na][Br] was added to each Amp-IL to understand the effect of the salt as a by-product in these studies, and it was observed that it did not enhance the antibacterial activity of the Amp-ILs. In fact, the antibacterial activities of Amp-IL were reduced with the addition of equal moles of [Na][Br]. It is hypothesized that this result is related to the change in the isotonic environment of the bacteria, resulting in a reduction in bacteria size and steric inhibition of the antimicrobial molecule absorption.

Overall, the Amp-ILs have an increased antibacterial activity for the investigated pathogens. In 83% of the experiments, we demonstrate that Amp-ILs are more effective antibacterial agents than each salt individually, or in combination, for the tested bacteria. Further investigation into this behavior will be conducted in future studies.

Conclusion

We have successfully demonstrated the synthesis and antibacterial application of a novel class of antibiotic-based ILs composed of either imidazolium or pyridinium cations. Significant improvements in the bactericidal activity of the ampicillin anion were obtained when the anion was combined with a QAC as the cation. The results indicate that Amp-ILs may be effective alternatives as antibacterial agents in lieu of the use of either individual ionic parent compounds (i.e. [QAC] or [Na][Amp]) or the combination in solution (i.e. [QAC] + [Na][Amp]).

The advantages by use of antibiotic-based ILs are numerous. Aside from the possibility of extending the clinical usage of antibacterial agents that are associated with bacterial resistance, there is potential to improve the half-life, reduce dosage rates, tailor the bioavailability of the drug, reduce costs associated with new drug testing and formulation, and expand the therapeutic activity of the antibiotic by pairing it with other biological ions. Although these specific types of ampicillin ionic liquids may be considered toxic and not cleared for systemic use, the potential to apply these antibacterial materials to biomedical sterilization, food processing, and wound care therapy is promising.

Acknowledgments

The authors thank Shreya Datta and Reshani Senevirathne for their assistance in the microbiological studies. Marsha Cole thanks the Louis Stokes-Louisiana Alliance for Minority Participation (LS-LAMP) Bridge-to-Doctorate and UNCFSP Harriet Jenkins Fellowships for financial support. Isiah M. Warner acknowledges the National Science Foundation (Grant no. CHE-0911118) for support of this research.

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