Noncovalent Conjugates of Ionic Liquid with Antibacterial Peptide Melittin: An Efficient Combination against Bacterial Cells

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ABSTRACT: Growing antibiotic resistance has become a major health problem and has encouraged many researchers to find an alternative class of antibiotics. Combination therapy (covalent/noncovalent) is supposed to increase antibacterial activity leading to a decrease in administration dosage, thus lowering the risk of adverse side effects. The covalent coupling sometimes leads to instability and loss in the structure of AMPs. Therefore, herein, we have reported innovative research involving the noncovalent coupling of melittin (MEL), an antimicrobial peptide with a series of synthesized less toxic pyrrolidinium-based ionic liquids (ILs) for which MTT assay was performed. The antibacterial results of conjugates showed remarkable improvement in the MIC value as compared to MEL and ILs alone against Escherichia coli and Staphylococcus aureus. In addition, hemocompatibility results suggested good selectivity of the noncovalent conjugate as a potential antibiotic agent. Further, the docking study was employed to acquire the most favorable conformation of MEL in the presence of ILs. The best possible complex was further studied using various spectroscopic techniques, which showed appreciable binding and stability of the complex.

INTRODUCTION

The emergence of antibiotic resistance has become a challenge for the modern medicine world and has an adverse effect on public health.1 Accelerated growth of antibiotic resistance has been formulated due to overuse of antibiotic drugs including direct or indirect intake via medicine, agriculture, and aquaculture.2 Bacterial infection diseases have become a major problem worldwide especially in hospitals. The increased mortality rate in hospitals is observed with the reason being severe bacterial infections due to their suppressed immunity, advanced modern medical practice, and generation of new multiresistance strains.3 Despite several known antibiotics and technologies, studying the inhibition of bacterial resistance as well as control remains a challenge for researchers. Therefore, the development of effective antibiotics is still a major concern for researchers to prevent resistance-borne diseases.

Antimicrobial peptides (AMPs) are natural compounds considered as a potential therapeutic source of future antibiotics because of their broad spectrum activity and proposed mode of action, which is different from those of conventional antibiotics.4 They are effective against a wide range of bacteria, fungi, viruses, etc.5 AMPs are natural endogenous polypeptides produced by multicellular organisms to protect a host from pathogenic microbes. AMPs are also defined as host defense peptides because of their essential role in constituting the innate immunity system.6 Melittin (MEL) is found to be a pharmacological component of bee venom consisting of 26 amino acids and to exhibit water solubility. Hydrophobic characteristics and a total charge of +6 at physiological pH are the origin of the antibacterial property of MEL.7 Previous studies suggested that MEL binds with the negatively charged membrane of prokaryotic and eukaryotic cells leading to the disruption of the cell membrane by pore formation.8 There is an advantage to choosing AMPs as an alternate of antibiotics as bacteria has a lower propensity to develop resistance against AMPs as compared to conventional antibiotics since it would necessitate fundamental alterations in the lipid composition of the bacterial membranes.9

It is well proven that the combination of antibiotics works well against bacterial resistance. Various groups of researchers have tried several combinations of the peptide with drug or inorganic molecules to enhance the antibacterial activity of AMPs.10 Modulation of AMPs includes coupling of antibiotics covalently or noncovalently to increase their synergistic effect. Such combinations lead to an increase in the efficacy of AMPs and reduction of other adverse side effects associated with them. A recent study of covalent conjugation of peptides with...
imidazole has shown remarkable improvement in the MIC value and emerged as a promising antimicrobial agent.\textsuperscript{14} For example, vancomycin was coupled via a CuAAC (copper-mediated coupling between an azide and alkyne) reaction to various short peptides such as magainin 2 and its analogs. Interestingly, the improved MIC value was observed as compared to vancomycin and magainin 2 alone. The dosage observed was in micromolar, which showed improved antibacterial activity and was found to be effective against vancomycin-resistant \textit{Enterococci}. The MIC value of the conjugate was less than that of vancomycin alone.\textsuperscript{12} The covalent conjugate of the broad-spectrum antibiotic is levofloxacin with indolicidin, a hydrophobic peptide, with an amide linkage. No remarkable improvement in the activity was observed as compared to levofloxacin and indolicidin alone.\textsuperscript{13}

Another study involves ubiquicidin (UBI), a cationic peptide covalently coupled to the typical antibiotic chloramphenicol (CAP). The obtained combination of the drug showed increased antibacterial activity as compared to CAP alone, and also the cytotoxicity against human cells was decreased as compared to CAP.\textsuperscript{14}

Previous studies showed that combination therapy is supposed to increase the antibacterial activity of therapeutic drugs leading to a decrease in the intake dosage consequently minimizing the adverse side effects. Despite covalent conjugation, another technique of combining two or more antibiotics could be noncovalent interactions. Noncovalent interactions involve hydrophobic interactions, van der Waal interactions, \( \pi - \pi \) interactions, etc., and have great importance in drug delivery and drug designing.\textsuperscript{15} The beauty of noncovalent conjugates over covalent conjugates is the easy release of a therapeutic agent without any special physical and thermodynamic environment.\textsuperscript{16} Therefore, to improve the antibiotic potential of MEL, the noncovalent conjugates of pyrrolidinium-based ILs and MEL were prepared, which may lead to better therapeutic agents against bacterial resistance.

Ionic liquids (ILs) are molten salt offering the possibility to design their structure according to application. The properties of ILs such as viscosity, miscibility, hydrophobicity/hydrophilicity, polarity, and, most importantly, their antimicrobial property can be tuned by changing or modifying the ion substituents or composition.\textsuperscript{17} These solvents are, therefore, often named “designer solvents”. During the last decade, ILs have revealed some promising applications in many areas, including biotechnology and biological sciences. Their fascinating physicochemical properties made them a good candidate for developing new therapeutic agents. Several positive effects of ILs have been found such as in terms of enhanced protein refolding,\textsuperscript{18} increased thermal stability,\textsuperscript{19} drug-delivery vehicles,\textsuperscript{20} and catalysts in many organic reactions.\textsuperscript{21} The recent findings showed that ILs based on imidazolium, pyridine, choline, pyrrole, etc. are extensively studied as therapeutic agents as they possess antibacterial and antifungal properties because of the high charge and hydrophobicity in ILs. The toxic effect of ILs, imidazolium, pyridinium, ammonium-based ILs, etc., limits their use as therapeutic agents.\textsuperscript{22} Inspired by the current literature, if imidazole containing quaternary ammonium cations exhibits biological activity reported to be toxic,\textsuperscript{22} we wonder of synthesizing less toxic ILs and their application in drug development. Aimed at the improvement of biological activity of AMPs, herein, we disclose the synthesis of pyrrolidinium-based ILs, which excludes the toxicity factor as it was earlier reported that pyrrolidinium-based ILs are less toxic as compared to imidazolium, pyridinium, and ammonium-based ILs.\textsuperscript{23} Also, the anionic counterpart plays an important role in the stability and activity; hence, it is paired with stable and weakly coordinating anions (e.g., bis(trifluoromethane)-sulfonylimide, (CF\(_3\)SO\(_2\))\(_2\)N\(^-\), or NTf\(_3\)^-). The antibacterial property of the series of ILs and with MEL (noncovalent conjugates) was examined against a couple of clinically relevant microorganisms. To the best of our knowledge, the study of the above-discussed interplay and its biological activity is not reported yet.

\section*{RESULTS AND DISCUSSION}

\textbf{Synthesis of Pyrrolidinum-Based ILs}. The ILs investigated in this study consist of a 1-alkyl-1-methylpyrrolidinium-based cation (where alkyl = butyl, hexyl, octyl, decyl, and dodecyl) and lithiumbis(trifluoromethane)sulfonylimide as an anion, [PyrC\(_n\)NTf\(_3\)]\(^-\) (detailed synthesis is given in the Experimental Procedure). The synthesized ILs were well characterized by \(^1\)H NMR, \(^13\)C NMR, FT-IR, and mass spectroscopies (detailed spectra are shown in the Supporting Information).

\textbf{Cytotoxicity Assay}. The synthesized ILs were subjected to cell toxicity on HEK 293 cell lines using various concentrations (10–320 \(\mu\)M) as shown in Figure 1. All the ILs showed a good response in terms of the cell viability as it was evident that, at the lowest concentration, all the five test samples did not show marked variability in terms of viability (values range between 70 and 95%), while at higher concentrations, the viability followed the same trend, and at 160 \(\mu\)M, the viability for all five ILs was very close to each other (between 70 and 95%); however, compounds [PyrC\(_{16}\)NTf\(_3\)]\(^-\) and [PyrC\(_{12}\)NTf\(_3\)]\(^-\) showed decreasing cell viability at the highest concentration of 320 \(\mu\)M as shown in Figure 1. This shows the dosage safety of the ILs to be used for further \textit{in vitro} and/or \textit{in vivo} studies. The MTT assay results obtained were compared with the literature, and it was found that the synthesized ILs possess lesser toxicity as compared to imidazolium-based and pyridinium-based ILs, which are extensively studied nowadays as antimicrobial agents in pharmaceutical industries.\textsuperscript{23}

\textbf{Antibacterial Activity}. Antimicrobial activity of synthesized ILs and MEL was assessed against clinically relevant microorganisms, including \textit{E. coli} as Gram negative and \textit{S. aureus} as Gram positive bacterial strains. Toxicity against microbes was assessed by determining the minimum inhibition concentration (MIC) values that are summarized in Table 2. It
was observed that the MIC value against Gram negative *E. coli* was higher than that of Gram positive *S. aureus*. The difference in the toxicity of ILs against microbes can be justified based on the structural difference of cell membranes. As already mentioned, the cell wall of Gram-negative bacteria *E. coli* comprises two layers, the outer membrane of about 7–8 nm, rich in negative charge and mainly of lipopolysaccharides, is relatively thinner as compared to the inner layer of approximately 2–7 nm made up of peptidoglycans. Likewise, the cell wall of Gram-positive *S. aureus* is made up of a porous peptidoglycan layer of approximately 20–80 nm, which is interconnected with negatively charged teichoic acid. Hence, it is expected that the increased hydrophobicity and high positive charge are more likely to insert into the porous cell wall and disturb the peptidoglycan layer leading to cell death. The synthesized ILs are structurally similar to the reported imidazolium-based ILs containing an ammonium moiety with a positive charge and known for its antibacterial property.\(^\text{25}\) ILs tend to get adsorbed on the surface due to its positive charge and hydrophobic characteristics. It gets absorbed and enters the cell wall by electrostatic interactions, leading to cell death. Obtained results show that ILs with longer alkyl chain lengths, [PyrC\(_{12}\)NTf\(_3\)]\(^{−}\), showed greater efficiency as an antimicrobial agent as compared to the ILs with shorter alkyl chains. The mean MIC value of each IL is shown in Figure 2 against both microbes. The antibacterial property of ILs of varying chain lengths follows the trend as reported in the case of imidazolium-based ILs.\(^\text{11,26}\)

Antibacterial efficiency of ILs collectively depends on the adsorption subsequent to the penetration power leading to the alteration in the permeability of the cell membrane and ultimately cell death. As discussed above, the bacterial activity increases with lengthening of the alkyl chain. Therefore, the biological effect of ILs is also related with surface parameters such as the critical micellar concentration (CMC) and free energy of adsorption \(\Delta G^\circ_{\text{ad}}\) as listed in Table 1 and Table S1. The critical micellar concentration (CMC), a physicochemical parameter, shows an opposite trend when compared with antibacterial activity. Table 1 shows the CMC value that was found to be decreasing with increasing alkyl chain length obtained from surface tension measurement; therefore, it can be concluded from the CMC results that the monomer concentration of ILs at the surface becomes lower with higher analogues.\(^\text{27}\) On the other hand, the value of free energy of adsorption, \(\Delta G^\circ_{\text{ad}}\), was calculated using eqs S8 and S9 in the Supporting Information where the negative value of \(\Delta G^\circ_{\text{ad}}\) was found to be increasing with increasing alkyl chain length (Table 1). The negative value of \(\Delta G^\circ_{\text{ad}}\) favors the spontaneity of adsorption. The negative increasing value of \(\Delta G^\circ_{\text{ad}}\) with increasing alkyl chain length suggests the increasing migration rate of ILs to the cell wall with increasing alkyl chain length. The maximum antibacterial efficiency against Gram positive and Gram negative bacteria was found for larger-alkyl-chain IL, [PyrC\(_{12}\)NTf\(_3\)]\(^{−}\). Thus, the rate of migration is related to the ability of ILs to easily get adsorb on the surface of the bacterial cell wall resulting into the increased antibacterial efficacy of that IL, [PyrC\(_{12}\)NTf\(_3\)]\(^{−}\).\(^\text{24}\)

**Effect of ILs on the Antibacterial Property of MEL.** For rapid inhibition of the resistant strain, the effect of ILs on the antibacterial property of MEL was studied. For each IL, there

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**Table 1. Critical Micellar Concentration (CMC) and Free Energy of Adsorption (\(\Delta G^\circ_{\text{ad}}\)) Values of ILs at 298 K**

| S. no. | ILs            | CMC (mM) ST | CMC (mM) Flr | \(\Delta G^\circ_{\text{ad}}\) (kJ) |
|-------|----------------|-------------|--------------|----------------------------------|
| 1     | [PyrC\(_4\)NTf\(_3\)]\(^{−}\) | 3.66        | 3.39         | -23.86                           |
| 2     | [PyrC\(_6\)NTf\(_3\)]\(^{−}\) | 2.26        | 2.34         | -25.05                           |
| 3     | [PyrC\(_7\)NTf\(_3\)]\(^{−}\) | 1.43        | 1.87         | -26.19                           |
| 4     | [PyrC\(_8\)NTf\(_3\)]\(^{−}\) | 0.41        | 0.46         | -29.28                           |
| 5     | [PyrC\(_9\)NTf\(_3\)]\(^{−}\) | 0.22        | 0.21         | -30.72                           |

\(\text{ST} = \text{surface tension} \quad \text{Flr = fluorescence spectroscopy}\)

**Table 2. Showing the MIC Value of MEL (5 μM), [PyrC\(_4\)NTf\(_3\)]\(^{−}\) and Ampicillin (Std Drug) against *E. coli* and *S. aureus* in mM**

| S. no. | compounds       | MIC (mM) *E. coli* | MIC (mM) *S. aureus* |
|-------|-----------------|--------------------|---------------------|
| 1     | [PyrC\(_4\)NTf\(_3\)]\(^{−}\) | 2.63               | 2.00                |
| 2     | [PyrC\(_6\)NTf\(_3\)]\(^{−}\) | 1.94               | 1.34                |
| 3     | [PyrC\(_7\)NTf\(_3\)]\(^{−}\) | 1.36               | 0.68                |
| 4     | [PyrC\(_8\)NTf\(_3\)]\(^{−}\) | 0.86               | 0.42                |
| 5     | [PyrC\(_9\)NTf\(_3\)]\(^{−}\) | 0.48               | 0.23                |
| 6     | MEL             | 0.0026             | 0.00207             |
| 7     | Ampicillin      | 0.00035            | 0.00017             |

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**Figure 2.** Showing the MIC values of [PyrC\(_4\)NTf\(_3\)]\(^{−}\), [PyrC\(_6\)NTf\(_3\)]\(^{−}\), [PyrC\(_7\)NTf\(_3\)]\(^{−}\), [PyrC\(_8\)NTf\(_3\)]\(^{−}\), [PyrC\(_9\)NTf\(_3\)]\(^{−}\), MEL, and ampicillin (std drug) against *E. coli* and *S. aureus*.
was a “critical” concentration needed for maximum microbial inactivation. The effect of selected concentrations of each ILs, namely, [PyrC4NTf3−], [PyrC6NTf3−], [PyrC8NTf3−], [PyrC10NTf3−], and [PyrC12NTf3−], on the antibacterial properties of MEL was examined by monitoring the inactivation of E. coli and S. aureus. The in vitro study of MEL alone and in combination with each IL was performed. The addition of [PyrC4NTf3−], [PyrC6NTf3−], [PyrC8NTf3−], [PyrC10NTf3−], and [PyrC12NTf3−] to MEL considerably improved in vitro antibacterial activity against E. coli and S. aureus strains compared with MEL alone.

The detectable bacterial effect on E. coli and S. aureus was achieved at an elevated concentration of MEL, 2.60 and 2.07 μM, respectively, which involves the maximum possibilities of side effects and toxic effects more prominently playing. The combination of 1.20 μM MEL and 50 μM [PyrC4NTf3−] against E. coli as shown in Figure 3a and 150 μM [PyrC4NTf3−] combined with 1.30 μM MEL against S. aureus in Figure 3b were tested and showed good results in terms of inhibition of growth. Similarly, a combination of MEL and [PyrC4NTf3−] was tested, and much improved MIC values were obtained as compared to the combination of [PyrC4NTf3−] with MEL and MEL alone. Much growth inhibition was observed in treatment with the combination of 20 μM [PyrC4NTf3−] and 1.31 μM MEL against E. coli as shown in Figure 4a as well as with 200 μM [PyrC4NTf3−] when combined with 1.29 μM MEL against S. aureus as shown in Figure 4b.

However, another combination of MEL with [PyrC4NTf3−] was tested, which further showed very good results in terms of MIC values (50 μM solution of [PyrC4NTf3−]) combined with 1.23 μM MEL against E. coli and 20 μM solution of
combined with 1.36 μM MEL against S. aureus as shown in Figure 5a,b. The combination of 10 μM [PyrC10NTf3−] and 1.22 μM MEL against E. coli and the combination of 20 μM [PyrC10NTf3−] with 1.29 μM MEL against E. coli and S. aureus as shown in Figure 6a,b, respectively, show good results as compared to the combination tried with butyl, hexyl, and octyl. Although, all combinations of ILs showed remarkable results in terms of reduction of bacterial burden. The best response in terms of the reduction of bacterial burden as shown in Figure 7a,b was observed at a 10^5–10^6 CFU/mL inoculum when susceptible E. coli was treated with a combination of 0.53 MEL and 2 μM [PyrC12NTf3−] and S. aureus strains with a combination of 0.51 MEL and 10 μM [PyrC12NTf3−] for 16–18 h.

The obtained results show that, at a much higher concentration, ILs comprising shorter alkyl chains caused maximum reduction of the bacterial burden of E. coli and S. aureus strains when treated for 16–18 h. In contrast, a much higher concentration of ILs was needed for the inhibition of S. aureus. This may be due to the tendency of S. aureus to form biofilms; however, it is the main reason for the development of multidrug resistance (MDR). Surface tension parameters help in reaching the effective mechanism taking place when E. coli and S. aureus are treated with MEL-IL noncovalent conjugates.

The unique characteristic of ILs to get adsorbed on the surface made them a good candidate to be used as a substitute in antibacterial drug development as discussed previously based on parameters listed in Table 1. The combination of ILs with MEL is one way to overcome MDR strains. In previous studies, it has been demonstrated that the combination of MEL with antibiotics helps in the inhibition of bacterial infection occurring due to MDR. The reported combination of MEL with doripenem (antibiotic) decreased the risk of cytotoxicity up to 61%. However, in the present study, the antibacterial activity results showed the improved antibacterial efficiency of MEL in the presence of all the ILs specially [PyrC12NTf3−] as a decrease in the bacterial burden of up to 95% was observed for [PyrC12NTf3−]. The combination of MEL-IL will lead to a decrease in MEL consumption and may decrease the side effects, which results in it being a good candidate for treatment of infections from E. coli and S. aureus. The increased antibacterial activity of the MEL-IL conjugate is most likely related to their site of action on a bacterial cell wall. The MEL-IL conjugate adsorbs more and could create pores inside the outer membrane of E. coli and S. aureus due to increased hydrophobicity and a more dispersed charge. This mode of action of ILs likely facilitates the penetration of MEL to reach the cell wall of bacteria, and at the next step, the MEL-IL conjugate causes the death of bacteria through inhibition of growth of bacterial cell.
membranes. The resistance arises due to the gene exchange between resistive cells and non-resistive cells.31 Herein, ILs have a property to get adsorbed over the surface. Therefore, it can be concluded that ILs binding with MEL noncovalently increases their ability to be more adsorbed on the outer membrane of the cell as compared to MEL and ILs alone leading to faster inhibition of bacterial cells. Due to increased adsorption efficiency, they also stop the exchange of gene and plasma between the cells leading to a decrease in the MDR as shown in Scheme 1. Our results have some implications for the applied use of AMPs as drugs. The production of AMPs is currently expensive.32 The broad synergy observed in our experiment means that combined applications of AMPs could also reduce the consumption of total AMPs just as in the immune system, which could eventually reduce the costs of treatment and toxicity.33 Our finding showed the enhanced activity of MEL in the presence of all ILs; however, the best combination was obtained with [PyrC12NTf3−], and it can be considered that it can be helpful as a drug for treatment of MDR.

**Hemocompatibility Assay.** Intrinsic fluorescence is generally used to study the change in the conformation of proteins on the addition of ligands. Therefore, to investigate the hemocompatibility of synthesized ILs and their conjugate with MEL, the interaction of MEL and the MEL-IL complex with human serum albumin (HSA) was performed using absorption, fluorescence, and CD spectroscopies.34

The fluorescence spectra of pure HSA (5 μM), HSA-MEL, and HSA-MEL with ILs were recorded as shown in Figure 8b. The fluorescence peak of HSA at 342 nm is a characteristic peak of tryptophan (Trp) shown in Figure 8b. The intensity increased on the addition of MEL whereas decreased in the presence of ILs. The change in fluorescence intensity indicates the interaction taking place between HSA and the MEL-IL complex. Also, the decrease in fluorescence intensity refers to the quenching process because of the various interactions taking place such as complex formation, energy transfer, excited-state reactions, and collisional quenching. According to the observation, decreased intensity of HSA may be due to the interaction of HSA with ILs, most likely by electrostatic interaction, which changes the environment around the Trp peak of tryptophan (Trp) shown in Figure 8b. The intensity of HSA-MEL and HSA-MEL with ILs decreased significantly as shown in Figure 8b. The absorption band in the range of 200–400 nm is the characteristic band of the α-helix structure of HSA as shown in Figure 8a. The obtained absorption peak at 280 nm did not shift in the presence of MEL and ILs at very low concentrations, [PyrC12NTf3−]: 50 μM, [PyrC10NTf3−]: 25 μM, [PyrC8NTf3−]: 20 μM, [PyrC6NTf3−]: 10 μM, and [PyrC4NTf3−]: 5 μM. This suggests that neither MEL nor ILs affected the structure of HSA58 and the MEL-IL complex is hemocompatible, which might help in manufacturing the therapeutic drug.

In addition to this, the far-UV CD spectra of HSA in the presence of MEL and ILs were recorded as shown in Figure 8c. The figure clearly shows an increase in the negative ellipticity of HSA in the presence of MEL both at 208 and 222 nm, which suggests that MEL stabilizes the secondary structure of

### Table 3. Showing the Effect of Synthesized Pyrrolidinium-Based ILs on the Antibacterial Activity of MEL

| microorganisms | MIC (μM) |
|----------------|----------|
|                | in the presence of [PyrC10NTf3−] |
| E. coli (MTCC 40) | 2.60 1.20 2.50 2.60 2.60 2.50 |
| S. aureus (MTCC 87) | 2.07 2.57 2.50 1.30 1.30 1.30 |

| microorganisms | MIC (μM) |
|----------------|----------|
|                | in the presence of [PyrC8NTf3−] |
| E. coli (MTCC 40) | 2.60 2.56 1.30 1.23 2.28 2.56 |
| S. aureus (MTCC 87) | 2.07 2.50 2.50 1.29 2.50 2.50 |

| microorganisms | MIC (μM) |
|----------------|----------|
|                | in the presence of [PyrC6NTf3−] |
| E. coli (MTCC 40) | 2.60 2.56 1.30 1.23 2.28 2.56 |
| S. aureus (MTCC 87) | 2.07 2.50 2.50 1.29 2.50 2.50 |

| microorganisms | MIC (μM) |
|----------------|----------|
|                | in the presence of [PyrC4NTf3−] |
| E. coli (MTCC 40) | 2.60 2.06 0.53 2.08 2.07 0.03 |
| S. aureus (MTCC 87) | 2.07 1.03 1.03 0.51 0.24 |

*Standard deviation error calculated: ±0.02*

### Scheme 1. Effect of the MEL-IL Conjugate on the Bacterial Membrane
Further, the effect of different ILs on the secondary structure of HSA in the presence of MEL was studied. It was observed that all the MEL-ILs stabilize the secondary structure of HSA. The results obtained from CD spectra comply with the results obtained from the other spectroscopic techniques.

**Effect of ILs on the Secondary Structure of MEL.** CD spectroscopy is an extensively used technique to investigate the secondary structure change in MEL. The far-UV CD spectrum of MEL in the presence and absence of ILs was recorded to see if there is any influence of synthesized ILs on the secondary structure of MEL as shown in Figure 9(a-e). The far-UV CD spectrum showed two minima at approximately 208 nm ($\pi - \pi^*$) and 222 nm ($n - \pi^*$). The two minima of 208 and 222 nm correspond to the presence of an $\alpha$-helical conformation of MEL. It is evident from Figure 9 that, in the presence of ILs, the CD spectra of MEL get shifted toward lower wavelengths and the depth of the minima also increases. This indicates that MEL undergoes toward the more folded state with a more helical conformation of MEL. It is evident from Figure 9 that, in the presence of ILs, the CD spectra of MEL get shifted toward lower wavelengths and the depth of the minima also increases. This indicates that MEL undergoes toward the more folded state with a more helical conformation of MEL. The value of $\alpha$-helical content calculated from far-UV CD spectra results showed an increase, which suggests that the ILs stabilize the secondary structure of MEL (Table 4). Further, the effect of the varying chain lengths of ILs on the formation of the $\alpha$-helical structure of MEL was analyzed using the far-UV CD spectra. The far-UV CD spectra were used to calculate the ratio of molar ellipticity at 222 and 208 nm ($R = [\theta]_{222}/[\theta]_{208}$). The value of $R$ of the MEL-IL complex was found to be approximately equal to the $R$ value associated with MEL alone, which suggests that the ILs with varied chain lengths have no significant effect on the formation of the $\alpha$-helical structure of MEL.

**Binding Study.** Binding studies were performed to investigate the complex formation (noncovalent conjugate) and the strength of the complex between ILs and MEL. First, molecular docking was performed to examine the interactions between ILs and MEL. Like the in vitro study, the computational study also helps in understanding the interaction taking place between ligands and biomolecules. Initially, we used the molecular docking technique to predict various binding sites and the mode of binding. The crystal structure of MEL (PDB ID: 1BH1) is available online. The blind docking was performed using AutoDock Tools-1.5.6 to examine the interactions taking place between ILs and MEL. The docking results suggest the involvement of van der Waals and $\pi$–cation interactions in the binding process where the amino acids Lys23, Arg22, and Ser18 were found to be responsible for van der Waals interactions, whereas Trp19 is for $\pi$–cation interactions. After the complete run, all the ILs were ranked based on their most possible lowest energy. A representative conformation of MEL-[PyrC12NTf3$^-]$ is shown in Figure 10a,b. The obtained binding energies between MEL and ILs from molecular docking are listed in Table S2, which appeared to be negative and increased on increasing alkyl chain length in ILs. The maximum negative energy was obtained when MEL binds with the IL having a dodecyl-carbon chain length, which depicts the maximum spontaneity of the complex formation. Thus, according to the binding energy and orientation, the most favorable compound selected for binding...
study with MEL was [PyrC12NTf3\(^-\)] among all synthesized ILs that show the highest binding energy. Further, various spectroscopic techniques were employed to study the conformational change in MEL in the presence of [PyrC12NTf3\(^-\)].

Fluorescence spectroscopy is the most versatile technique to study the microenvironment change in the vicinity of any fluorophore; therefore, this technique is widely used to investigate the interaction of the ligand with biomolecules.\(^{40,41}\) MEL has one aromatic fluorophore (tryptophan), which plays an important role in the generation of the fluorescence spectrum due to the presence of \(\pi\) electrons.\(^{42}\) The fluorescence emission was recorded with the excitation wavelength \(\lambda_{exc} = 280\) nm. The concentration of MEL (5 \(\mu\)M) was kept constant with varied concentrations of [PyrC12NTf3\(^-\)] (from 1.66 to 21.20 \(\mu\)M), Figure S24a shows the maximum fluorescence intensity \(\lambda_{max}\) of MEL observed at 353 nm. The fluorescence intensity shows a progressive decrease with the increasing concentration of [PyrC12NTf3\(^-\)].

Table 4. \(\alpha\)-Helical Content (%) and Ratio of Molar Ellipticity, \(R\) of MEL in the Absence and Presence of ILs at 298 K and pH 7.2

| S. no. | ILs          | \(\alpha\)-helical content (%) | \(R\) value |
|-------|--------------|-------------------------------|-------------|
| 1     | MEL          | 6.70                          | 0.72        |
| 2     | [PyrC4NTf3\(^-\)]-MEL | 9.50                          | 0.83        |
| 3     | [PyrC6NTf3\(^-\)]-MEL | 8.82                          | 0.68        |
| 4     | [PyrC8NTf3\(^-\)]-MEL | 9.43                          | 0.86        |
| 5     | [PyrC10NTf3\(^-\)]-MEL | 10.25                         | 0.76        |
| 6     | [PyrC12NTf3\(^-\)]-MEL | 8.70                          | 0.78        |

Figure 9. CD spectra of MEL (20 \(\mu\)M) in the presence and absence of different concentrations of (a) [PyrC4NTf3\(^-\)], (b) [PyrC6NTf3\(^-\)], (c) [PyrC8NTf3\(^-\)], (d) [PyrC10NTf3\(^-\)], and (e) [PyrC12NTf3\(^-\)] in 10 mM tris buffer and \(R\) value at 298 K and pH 7.2.
Further, no shift in the maximum emission at 298, 303, and 308 K was observed; this indicates that the binding of [PyrC12NTf3\textsuperscript{−}] components may not accomplish a conformational change in a particular concentration range.\textsuperscript{34} Moreover, the Stern–Volmer quenching constant, binding constant and number of binding sites were calculated using eqs S10 and S11 given in the Supporting Information (Table S3) using Figure 11a,b. The magnitude of $K_a$ indicates the strong interaction of [PyrC12NTf3\textsuperscript{−}] with MEL, whereas the value of $n$ depicts the 1:1 binding between MEL and [PyrC12NTf3\textsuperscript{−}]. With increasing temperature, the value of the binding constant decreases, which suggests that the stability of the complex decreases with increasing the temperature.

Further, to ascertain the binding mode, the thermodynamic parameters such as free energy change ($\Delta G$), enthalpy change ($\Delta H$), and entropy change ($\Delta S$) were determined.\textsuperscript{43} The thermodynamic parameters were calculated using the van’t Hoff equations (eq S12 in the Supporting Information). Figure S24b shows the van’t Hoff plot of ln $K_a$ versus 1/T for the MEL/[PyrC12NTf3\textsuperscript{−}] system. The values of the slope and intercept obtained from the van’t Hoff plot were employed to determine the values of $\Delta H$ and $\Delta S$. Further, the free energy of binding, $\Delta G$, was calculated using eq S13 (given in the Supporting Information). The values of thermodynamic parameters are listed in Table S2. In Table S2, and the negative values of $\Delta H$ and $\Delta S$ suggest that the hydrogen bonding and van der Waal forces are involved in complex formation.\textsuperscript{44} The negative value suggests that the binding process was spontaneous. The experimental results were found to be following the docking results where the binding energy was in a negative value, which confirms the spontaneity of the complex formation. With an increase in temperature, the negative value of free energy showed a decrease, which suggests that, on increasing the temperature, the stability of the complex decreases.\textsuperscript{44}

Also, the UV–vis spectrum was recorded as it assists in collecting the insightful information regarding structural change and complex formation.\textsuperscript{45} The UV–vis spectra of MEL in the absence and presence of [PyrC12NTf3\textsuperscript{−}] were recorded (Figure S25a). MEL possessed a peak of approximately 273 nm. The prominent absorption spectrum of MEL signifies the presence of tryptophan (chromophore) residues.\textsuperscript{41} The increase in the absorption intensity of MEL with increasing concentrations of [PyrC12NTf3\textsuperscript{−}] indicates the complex formation between MEL and [PyrC12NTf3\textsuperscript{−}].\textsuperscript{46} The binding constant $K_a$ was also calculated by a double reciprocal plot between $1/A_0−A$ versus $1/[\text{PyrC12NTf3}^\text{−}]$ using eq S19 given in the Supporting Information by the method described earlier.\textsuperscript{47} The value of the binding constant was in the order of $S$, which further validates the fluorescence results.

**CONCLUSIONS**

The study concludes with a successful formulation of noncovalent conjugates of all synthesized pyrrolidinium based-ILs with MEL. The improvement of the MIC value of MEL in the presence of ILs was observed against both microorganisms. In this work, we have reported the best novel antibacterial composition as MEL and [PyrC12NTf3\textsuperscript{−}], which comprises a combination of 0.53 μM MEL + 2 μM [PyrC12NTf3\textsuperscript{−}] against E. coli and 0.51 μM + 10 μM MEL.
[PyrC_{12}NTf_{3}^{-}] against S. aureus, which did not show any growth after 24 h. The MIC value of noncovalent conjugates of MEL with [PyrC_{10}NTf_{3}^{-}] and [PyrC_{12}NTf_{3}^{-}] was improved as compared to pure MEL and ILs. The noncovalent conjugate suppresses the dose intake of MEL consequently lowering the side effects associated with the higher dose and stopping the chances of getting resistant. We hereby report that the combination of pyrrolidinium-based ILs within the tested range with MEL will lead to a highly efficient antibacterial composition possessing a promising antibacterial property. Furthermore, based on the cytotoxicity results, a lesser toxicity composition possessing a promising antibacterial property.

**EXPERIMENTAL PROCEDURE**

**Materials.** Melittin (purity ≥65%), lithium bis(trifluoromethane)sulfonyl imide (purity ≥95%), tris buffer, and ampicillin salt were purchased from Sigma Aldrich. 1-Methylpyrrolidine, 1-bromobutane, 1-bromohexane, 1-bromoctane, 1-bromodecane, 1-bromododecane, dichloromethane, CDCl₃, TMS, MgSO₄, neutral aluminium oxide, ethyl acetate, ethanol, and methanol of analytical grade were used without any further purification. For antibacterial assay, two of the quality control MTCC strains, namely, Gram-positive Staphylococcus aureus (S. aureus) (MTCC 87) and Gram-negative Escherichia coli (E. coli) (MTCC 40), were obtained from the microbial-type culture collection and gene bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. MilliQ water was used throughout the experiments.

**Methods.** *Synthesis of Pyrrolidinium-Based ILs.* Pyrrolidinium-based ionic liquids [PyrC_{x}NTf_{3}^{-}], x = 4, 6, 8, 10, and 12 alkyl chains containing lithium bis(trifluoromethane)-sulfonylimide (NTf_{3}^{-}) as a counterion were synthesized through a systematic protocol. The synthesis process includes a two-pot synthesis as shown in [Schemes 2 and 3](#).

**Scheme 2. Synthesis of Intermediate ILs with Br⁻ as a Counterion**

\[
\text{1-methylpyrrolidine} \quad \text{1-alkyl-1-methylpyrrolidinium bromide (Intermediate product)}
\]

for the preparation of intermediate ILs followed by metathesis as a second step to obtain the desired ILs. All reactions were carried out under a normal environment. Thin-layer chromatography (TLC) analysis was carried out at each step to monitor the progress of the reaction, which was visualized under UV (254 nm) light. \(^1\)H NMR (400 MHz) and \(^{13}\)C NMR (100 MHz) results were recorded in parts per million relative to the signal of standard tetramethyilsilane (TMS). CDCl₃ was used as a solvent in \(^1\)H NMR (shown in the Supporting Information as Figures S1, S5, S9, S13, and S17) and \(^{13}\)C NMR (shown in the Supporting Information as Figures S2, S6, S10, S14, and S18). Fourier transform infrared (FT-IR) (shown in the Supporting Information as Figures S3, S7, S11, S15, and S19) and mass spectra (shown in the Supporting Information as Figures S4, S8, S12, S16, and S20) were also recorded to further confirm the structure. The melting point of all solvents used was uncorrected and used as it is.

**Step 1: Synthesis of Pyrrolidinium-Based-ILs (Intermediate).** A series of pyrrolidinium-based ILs were synthesized, [PyrC_{x}Br] (x = 4, 6, 8, 10, and 12) as shown in [Scheme 2](#). These ILs were synthesized by mixing bromoalkane (5.48 g, 0.04 mol) with 1-methylpyrrolidine (3.4 g, 0.04 mol) followed by the addition of ethyl acetate (15 mL) in a round-bottom flask. The reaction mixture was then stirred and refluxed for 48 h at room temperature. After refluxing, the solvent was evaporated. The synthesized product obtained after solvent evaporation was further dried under high vacuum at room temperature to remove any moisture.

**Step 2: Anion Metathesis Step (to Change the Counterion).** [Scheme 3](#) shows the metathesis step where the obtained product [PyrC_{x}Br] (2 g, 0.04 mol) was dissolved in distilled water (70 mL) to change the counterion followed by the addition of lithium bis(trifluoromethane)sulfonylimide (2 g, 0.006 mol). The reaction mixture was stirred for 24 h at 80 °C in a round-bottom flask. After completion of the reaction, the reaction mixture was allowed to cool at room temperature followed by the addition of distilled water (50 mL), and then the mixture was obtained by adding a dichloromethane solvent (100 mL). The obtained organic layer was separated and washed three times by distilled water and dried using anhydrous CaSO₄. The crude product was filtered through neutral aluminium oxide to remove yellow coloration and precipitation. To obtain the desired product, the solvent was evaporated using a rotary evaporator. The obtained product was kept in high vacuum at room temperature to avoid moisture content.

**Cytotoxicity Assay.** Cell viability testing was done using MTT on HEK293 (human embryonic kidney cells). Briefly, 10,000 cells/well were seeded into flat-bottom 96-well plates (150 μL/well) in triplicate and allowed to attach and grow. The cells were incubated for 24 h and subsequently treated with varying concentrations of the compounds ranging from 10 to 320 μM. After 48 h of treatment, the medium was removed, and cells were incubated with 20 μL of MTT (5 mg/mL in PBS) in fresh medium for 4 h at 37 °C. Formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in DMSO (150 μL/well), and quantification was performed by reading the absorbance at 540 nm after an incubation period of 15 min on the iMark microplate reader (Bio-Rad).
■ ANTIBIOTIC ACTIVITY

**Bacterial Strains.** In brief, each culture was freshly prepared and grown to the logarithmic phase in LBB under aerobic conditions at 37 °C in an orbital shaker for 16–18 h. The growth at 600 nm was monitored using Analytikjena 210 (Germany). The optical density of the culture was observed at 600 nm. The working concentration of the inoculum was approximately 10^6–10^8 CFU/mL.

**Minimum Inhibition Concentration (MIC) Determination.** Antibacterial assay was performed to determine the MIC value of MEL, ILs, and standard drug ampicillin by a twofold serial broth microdilution method against *E. coli* and *S. aureus*. 50 Twofold dilution of ILs in LBB were prepared keeping the concentration range at 5.23 to 0.005 mM for \([\text{PyrC}_12\text{NTf}_3^{-}]\), 5.32 to 0.005 mM for \([\text{PyrC}_10\text{NTf}_3^{-}]\), 2.705 to 0.002 mM for \([\text{PyrC}_8\text{NTf}_3^{-}]\), 3.32 to 0.003 mM for \([\text{PyrC}_6\text{NTf}_3^{-}]\), and 1.9 to 0.001 mM for \([\text{PyrC}_4\text{NTf}_3^{-}]\) and also from 10 to 0.019 μM for standard drug ampicillin horizontally in 96-well plates followed by the addition of 10 μL of bacterial suspension whose working concentration was approximately 10^5 of bacterial suspension whose working concentration was adjusted to 10^5–10^8 CFU/mL. All tests were performed in triplicate for each experiment for MIC determination.

**Effect of ILs on the Antibacterial Activity of MEL.** The *in vitro* study was performed to evaluate the effect of ILs on the antimicrobial activity of MEL. The testing procedure consists of various concentration combinations of ILs with MEL where 10 μM MEL was twofold serially diluted vertically in a 96-well plate ranging from 5 to 0.004 μM vertically against *E. coli*. Likewise, 8.3 μM MEL was twofold serially diluted vertically in a 96-well plate ranging from 4.15 to 0.03 μM vertically against *S. aureus*. Thereafter, \([\text{PyrC}_12\text{NTf}_3^{-}]\) (50, 100, 150, 200, and 250 μM) was added vertically. Subsequently, the experiment was repeated for \([\text{PyrC}_10\text{NTf}_3^{-}]\) (20, 50, 100, 200, and 250 μM), \([\text{PyrC}_8\text{NTf}_3^{-}]\) (10, 20, 50, 100, and 200 μM), \([\text{PyrC}_6\text{NTf}_3^{-}]\) (10, 20, 50, 100, and 150 μM), \([\text{PyrC}_4\text{NTf}_3^{-}]\) (1, 2, 5, 10, and 20 μM). Further, 10 μL of inoculum of *E. coli* and *S. aureus* was added into the separate plate including GC and SC, respectively. The plate was incubated at 37 °C for 16–18 h. Thereafter, the optical density at 600 nm was recorded by iMark microplate reader (Bio-Rad) for each plate. All tests were performed in triplicate for each experiment for MIC determination.

**Hemocompatibility Assay.** The mixture of MEL (10 μM) was prepared by mixing it with 5 μM HSA. The mixture was incubated for 30 min at 300 K. Another mixture of ILs-HSA was prepared by keeping the concentration of MEL and HSA same and adding varied concentrations of ILs, \([\text{PyrC}_12\text{NTf}_3^{-}]\) (50 μM), \([\text{PyrC}_10\text{NTf}_3^{-}]\) (25 μM), \([\text{PyrC}_8\text{NTf}_3^{-}]\) (20 μM), \([\text{PyrC}_6\text{NTf}_3^{-}]\) (10 μM), and \([\text{PyrC}_4\text{NTf}_3^{-}]\) (5 μM). The fluorescence spectra of pure HSA, pure MEL, and the mixtures were recorded at room temperature by using a Cary Eclipse spectrophotometer (Varian, U.S.A.) with a 150 W xenon lamp equipped with a Peltier control temperature device. The excitation wavelength was 280 nm, and emission was measured from 290 to 500 nm. The slit width was kept at 5 nm for all the spectral scans. Also, the absorption was recorded by using an Analytik Jena Specord-250 spectrophotometer (U.S.A.) for the same set of samples between the absorption range of 200–400 nm keeping the slit width at 2 nm using a quartz cuvette of a 1 cm path length. Also, CD spectra of the same samples were recorded on a Jasco-715spectroelrophotometer, equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All the CD spectra were recorded at room temperature, 298 K, using a thermodynamically controlled cell holder attached to a water bath with an accuracy of ±0.1 °C. 19

■ PHYSICOCHEMICAL CHARACTERIZATION

**Surface Tension Measurement.** The synthesized series of novel pyrrolidinium-based ILs were physicochemically characterized by surface tension techniques using DeltaPi-4 (Kibron, Helsinki, Finland), equipped with four parallel microbalances having a small diameter (0.51 mm) special alloy wire (probe, cleaned by blazer piezo micro torch) to analyze the formation of micelles in water. 52 The CMC of each synthesized IL was determined using surface tension at 298 K. Before measurement, the glass vessel was thoroughly rinsed with chronic acid and deionized water. The aluminum base plate used was washed thoroughly with distilled water followed by heating through an alcoholic flame. 53 The system was calibrated using water whose surface tension was 72.8 mN/m at 298 K for three consecutive trials. To determine the CMC of each IL, the stock solution was prepared and added progressively in a known volume of water (1 mL). The values of surface tension (γ) were measured by mixing and then analyzing simultaneously. The same experiment was performed for each IL. There was no minimum region or hump observed that reflects the presence of impurities in the solution. 52

**Fluorescence Spectroscopy.** The steady-state fluorescence experiments were performed using the same spectrophotometer as described in the previous section at 298 K. Pyrene, being the most sensitive fluorescent probe to analyze the change in the microenvironment in the solution, was used to further confirm the CMC value obtained from tensiometry. The value of CMC was determined by a steady-state fluorescence method by using pyrene as a probe. 54 The plot of the ratio of I/I0 against the concentration ranged from 0.41 to 3.66 mM for \([\text{PyrC}_12\text{NTf}_3^{-}]\), 0.11 to 1.54 mM for \([\text{PyrC}_10\text{NTf}_3^{-}]\), 0.27 to 2.22 mM for \([\text{PyrC}_8\text{NTf}_3^{-}]\), 0.04 to 0.63 mM for \([\text{PyrC}_6\text{NTf}_3^{-}]\), and 0.017 to 0.27 mM for \([\text{PyrC}_4\text{NTf}_3^{-}]\).

■ BINDING STUDY

Molecular docking was performed to study the binding site and mode of binding between MEL and synthesized ILs. After the complete run, according to the binding energy and orientation, the best combination was selected. The maximum binding energy was found between MEL and \([\text{PyrC}_12\text{NTf}_3^{-}]\), and the most favorable docking conformation was taken to further study the binding using various spectroscopic techniques such as UV–vis spectroscopy, fluorescence spectroscopy, and CD spectroscopy. The UV–vis and fluorescence spectra of 5 μM MEL was recorded in the absence and presence of different concentrations of \([\text{PyrC}_12\text{NTf}_3^{-}]\) (1.66 to 13.15 μM) using the same UV–vis spectrophotometer and fluorescence spectrophotometer as described in the previous section. Further, secondary structural change in MEL was studied using CD spectroscopy on the same instrument as used earlier, and various parameters were calculated. For the detailed methodology, refer to the Supporting Information.
ASSOCIATED CONTENT
1 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03777.

1H NMR, 13C NMR, FT-IR, and mass spectra of ILs, fluorescence spectroscopy, and surface tension measurement (PDF)

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Notes
The authors declare no competing financial interest.

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