

Ionic liquids as a class of materials for transdermal delivery and pathogen neutralization

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Biofilm-protected microbial infections in skin are a serious health risk that remains to be adequately addressed. The lack of progress in developing effective treatment strategies is largely due to the transport barriers posed by the stratum corneum of the skin and the biofilm. In this work, we report on the use of Ionic Liquids (ILs) for biofilm disruption and enhanced antibiotic delivery across skin layers. We outline the syntheses of ILs, analysis of relevant physicochemical properties, and subsequent neutralization effects on two biofilm-forming pathogens: *Pseudomonas aeruginosa* and *Salmonella enterica*. Further, the ILs were also examined for cytotoxicity, skin irritation, delivery of antibiotics through the skin, and treatment of biofilms in a wound model. Of the materials examined, choline-geranate emerged as a multipurpose IL with excellent antimicrobial activity, minimal toxicity to epithelial cells as well as skin, and effective permeation enhancement for drug delivery. Specifically, choline-geranate was comparable with, or more effective than, bleach treatment against established biofilms of *S. enterica* and *P. aeruginosa*, respectively. In addition, choline-geranate increased delivery of cefadroxil, an antibiotic, by >16-fold into the deep tissue layers of the skin without inducing skin irritation. The in vivo efficacy of choline-geranate was validated using a biofilm-infected wound model (>95% bacterial death after 2-h treatment). This work establishes the use of ILs for simultaneous enhancement of topical drug delivery and antibiotic activity.

antibacterial | antimicrobial agents | antibiotic resistance | ion-pairing | formulation

Microorganisms that are protected in a biofilm pose a significant health risk due to their antibiotic resistance and recalcitrance to treatment. Biofilm-protected bacteria account for ~80% of total bacterial infections in humans and are 50–1,000 times more resistant to antibiotics than their planktonic counterparts (1). The antibiotic resistance of many biofilms originates in a layer of extracellular polymeric substances (EPSs) comprising polysaccharides, humic acids, nucleic acids, and peptides (2) that serve as a transport barrier. Further, biofilms are able to grow and persist on living as well as nonliving surfaces and are routinely found in hospital settings where patients are at high risk (3, 4). For example, the opportunistic Gram-negative pathogen, *Pseudomonas aeruginosa*, is commonly associated with high rates of morbidity and mortality, including urinary tract infections, septicemia, and endocarditis (5).

Biofilms are especially problematic when they establish on human skin because their presence serves to escalate the severity of pathology and slow wound healing (6). In addition, the sustained presence of dermal biofilms, which increases the severity and duration of bacterial infections, elevates the risk of contact transfer in home and hospital settings. Several common dermatological diseases, including atopic dermatitis, bullous impetigo, diabetic ulcers, and acne, routinely manifest biofilms of *Staphylococcus aureus* and other bacteria (7, 8).

Effective treatment of skin-based bacterial biofilms has been identified as a serious and unmet medical need (1, 9). However, biofilms established on skin are inherently difficult to treat, largely due to the outermost layer of the skin, the stratum corneum (SC), being a natural barrier for drug delivery. The SC is an effective transport barrier and severely limits diffusion of drugs (10). Although the SC may sometimes be compromised at the site of the skin biofilm due to the presence of a wound, an effective treatment must deliver antibiotics to all areas of infection. Biofilms often persist in the periphery of the actual wound, beneath an intact, healthy skin barrier because they exhibit local deep-tissue invasion (11, 12). To address this pathological concern, several formulation- and device-based methods were developed to increase drug delivery into skin (10) and biofilms (13, 14); however, their utility is limited. Specifically, device-based methods are difficult to use on large skin areas. Further, the presence of wounds at the site of biofilms poses a challenge in the use of devices. Formulation-based approaches make use of penetration enhancers such as fatty acids and alcohols, which often disrupt the skin lipid bilayer, thus enhancing transport (15). This approach often suffers from the inability to simultaneously enhance transport across both the SC and the biofilm. In addition, biofilm formation often leads to

Significance

Effective treatment of skin-based bacterial biofilms has been identified as a serious and unmet medical need. Biofilm-protected bacteria account for ~80% of bacterial infections in humans and are 50–1,000 times more resistant to antibiotics than their planktonic counterparts. Biofilms in skin are further protected by the outermost layer of skin, the stratum corneum, which serves as a natural barrier to most therapeutics. Here, we present compelling evidence for exploiting ionic liquids (ILs) as an arsenal of materials both in a concerted effort to combat antibiotic-resistant bacterial biofilms in skin as well as for topical transdermal drug delivery. Our comprehensive strategy resulted in the identification of ILs that are effective at disrupting biofilms, neutralizing pathogens, and enhancing delivery of antibiotic into skin. Moreover, ILs did not show skin irritation that is typically associated with topical formulations.

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skin inflammation, leading to keratinocyte apoptosis and inhibition of reepithelialization (16). Formulations that are designed to enhance transdermal transport often contain strong penetration enhancers that can further inflame the wound site and adversely affect wound healing (17).

Here, we report the use of both ionic liquids (ILs) and deep eutectic solvents (DESs) as a dual-purpose strategy for both disruption and neutralization of biofilm-forming pathogens, as well as drug delivery into and across the skin. Briefly, ILs are organic salts comprised of an organic cation and an organic or inorganic anion that, when mixed in 1:1 molar ratio (true ionic liquid), give rise to a room-temperature ionic liquid (RTIL) (18). Conversely, DESs are broadly defined as a mixture of charged and neutral species, either in equimolar or imbalanced ratios, that, when combined, have a much lower melting point than the individual component. Taken together, both ILs and DESs have gained acceptance for their beneficial antimicrobial and antifungal properties as demonstrated in several reports over the last decade (19–22). More recently, both ILs and DESs were found to increase drug solubility and serve as a carrier for topical drug delivery (23–26). Our comprehensive approach is unique in that we examine a panel of in-house synthesized ILs and DESs (henceforth referred to as ILs for simplicity) for all of the following applications: (i) antimicrobial properties on two biofilm forming Gram-negative pathogenic bacteria, *Pseudomonas aeruginosa* ATCC15692 and *Salmonella enterica* serovar Typhimurium LT2; (ii) cytotoxicity effects on mammalian cell lines; (iii) skin irritation potential; (iv) transdermal drug delivery properties; and (v) treatment of biofilm on a skin-wound model. This comprehensive screening strategy enabled the discovery of one IL, choline-geranate (11), which emerged as a multipurpose formulation with excellent antimicrobial activity, minimal toxicity to epithelial cells as well as skin, and effective permeation enhancement for drug delivery.

Results

Selection and Synthesis of ILs. The ionic liquids used in this study were prepared from a variety of organic and inorganic ions with the goal of producing fluid materials that would both have biofilm disruption and pathogen neutralization activity and facilitate transdermal drug delivery (Table S1). Of the materials studied, 1, 2, 5, 6, 9, 10, and 12 are true ionic liquids comprised of an equimolar ratio of cationic and anionic species. The DESs, 3, 4, 7, 8, and 11, comprise two carboxylate or urea equivalents and one cation equivalent, which form eutectics with a melting point lower than that of the original components. Representative ¹H and ¹³C NMR spectra for ILs 2, 5, and 6, and DESs 3 and 11 are found in Fig. S1. For the purpose of clarity, the entire panel is referred to as ionic liquids (ILs) throughout this text; however, specific identification as IL/DES is also provided (Table S1). Components in 3, 4, 17, 8, and 11 are generally recognized as safe (GRAS) materials. ILs 4, 7, 8, 9, 10, 11, and 12 were expected to enhance transdermal drug transport because at least one of their components (the anion) is a known chemical penetration enhancer (27) (Table S2). ILs possessed a composition-dependent viscosity (Table S3). Viscosity impacts the ease of handling in terms of admixing drugs and placement on skin. The densities of ILs ranged from 0.88 g/mL to 1.54 g/mL. Lipophilicities, measured in terms of octanol-water partition coefficient (log *P*_{o/w}), also varied depending on the choice of component (Table S3). With the exception of 1, 3, and 4, the log *P*_{o/w} values were slightly positive (~0.04–1.34).

IL Antibiofilm Activity. Most ILs in our panel exhibited strong antimicrobial activity against the biofilm-forming Gram-negative pathogens, *Pseudomonas aeruginosa* ATCC15692 and *Salmonella enterica* serovar Typhimurium LT2 (28, 29). Initially, an IL exposure time of 6 h was chosen for the *P. aeruginosa* biofilm, which resulted in complete bacterial neutralization. Therefore,

a shorter 2-h treatment was used to resolve differences between ILs [Fig. 1 (biofilms grown for 72 h) and Fig. S2 (biofilms grown for 24 h)]. ILs 3, 7, and 11 were most effective on the 72-h *S. enterica* biofilm (100% bacterial death) (Fig. 1A; the *Inset* shows data log scale for ILs with high potency). With the exceptions of 1, 4, and 6 (<10-fold bacterial death), the remaining ILs, 2, 5, 8, 9, and 10, were moderately effective (1,000- to 10,000-fold reduction in cell viability). IL 12 had no apparent effect on cell viability (*P* > 0.4). High efficacy of ILs was also observed on the 72-h *P. aeruginosa* biofilm although some differences were noted compared with the *S. enterica* biofilm. ILs 7, 9, and 11 in addition to 2, 10, and 12 reduced bacterial viability within the biofilm by ~5 log₁₀. The remaining ILs, 1, 3–6, and 8, had a nominal effect (<10- to 100-fold reduction in viability) whereas 1 resulted in no significant reduction (*P* > 0.06) (Fig. 1B). The biofilms grown for 24 h were more susceptible to neutralization than the 72-h biofilms when exposed to neat ILs over the 2-h period (Fig. S2).

IL Cytotoxicity in Primary Human Cells. The cytotoxicity of dilute ILs was tested in normal human bronchial epithelial (NHBE)

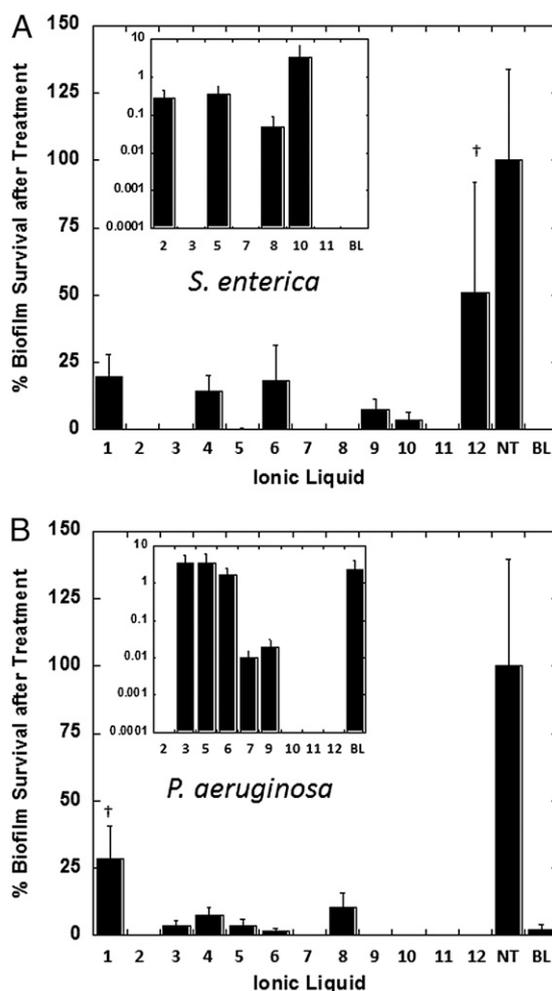


Fig. 1. Effect of ILs on 72-h bacterial biofilms. Percent biofilm survival after 2-h application for IL panel, NT (no treatment), and BL (bleach treatment) on (A) *S. enterica* biofilms or (B) *P. aeruginosa* biofilms. Biofilms were grown for 72 h before formulation treatment. Error bars represent mean \pm SE for *n* = 6. All conditions were significantly different from the untreated control (NT, *P* < 0.05) except where noted determined by Student *t* test. [†]*P* > 0.05. Percent biofilm survival after treatment is shown in the *Inset* on a log₁₀ scale to show differences between the most efficacious ILs.

primary cells. NHBE cells were used as a model nonskin cell to assess potential toxicity in the event of systemic absorption. ILs exhibited composition-dependent toxicity, and the IC_{50} values ranged from 0.001 mM (2) to 10 mM (4) (Table S4). The toxicity behavior was not entirely determined by a single component. Specifically, choline-based ILs exhibited both high (8, IC_{50} = 0.034 mM) and low (11, IC_{50} >> 2 mM) cytotoxicity effects. Choline-based ILs were generally less toxic to NHBE cells: for example, the IC_{50} of choline-geranate (11) is at least an order of magnitude higher than that of tetraalkylphosphonium-geranate (12) (Table S4). In total, 8 of the 12 ILs, at 0.82 mM (a concentration at which all ILs were readily soluble), exhibited <50% cytotoxicity on the NHBE cells (compared with 1% Triton X-100, which resulted in complete NHBE cell death). Of the ILs tested, 11 and 7 had both remarkable antimicrobial activity and minimal cytotoxicity effects whereas 11 was essentially nontoxic (<5%) to the primary cells (Fig. 2).

IL-Mediated Enhancement of Drug Delivery into Skin. The ability of ILs to enhance skin penetration was assessed using mannitol as a model drug (Fig. 3 A and B and Fig. S3). Mannitol was chosen because it is a small hydrophilic molecule with low permeability into skin and is not enzymatically degraded during transport through skin. ILs 7 and 11 were of particular interest for transdermal transport enhancement in view of their high antibacterial activity and low cytotoxicity in human primary cells. Other ILs, especially those prepared using known skin-penetration enhancers were added to skin-permeation studies. The effect of ILs on skin permeability varied with composition. Surprisingly, 4 and 7 reduced mannitol permeation into skin compared with saline control (Fig. 3A). The oleate-based formulations (8 and 9) did not significantly enhance total permeation compared with the control; however, transport into deep skin was enhanced by 9 as much as fivefold (Fig. 3B). IL 11 led to the highest enhancement of penetration, with nearly fivefold enhancement of total delivery (Fig. 3A) and >10-fold more drug into deep tissue layers in the skin (Fig. 3B). Penetration across the skin into the receiver compartment was also enhanced by twofold (Fig. 3B). Depth of penetration could potentially be controlled by choosing the IL. IL 9 resulted in enhanced delivery to the superficial layer of the skin. Alternatively, 10 and 11 exhibited delivery primarily in the deep tissue layers, with 10 showing the most preference for enhancement in the epidermis. IL 11 showed the highest preference for enhancement in the dermis.

The ability of ILs to enhance skin penetration of a model antibiotic, cefadroxil, was also assessed (Fig. 3 C and D and Fig. S4). Only ILs that showed enhancement of mannitol delivery

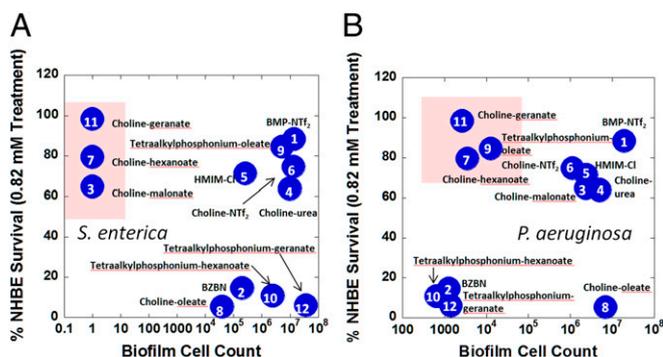


Fig. 2. IL cytotoxicity in primary human cells. Percent NHBE survival plotted versus antibiofilm activity against (A) *S. enterica* or (B) *P. aeruginosa* 72-h biofilms. High NHBE survivability coupled with low biofilm cell count (highlighted by red rectangle) indicates potential for use in applications where absorption into the bloodstream is expected to occur.

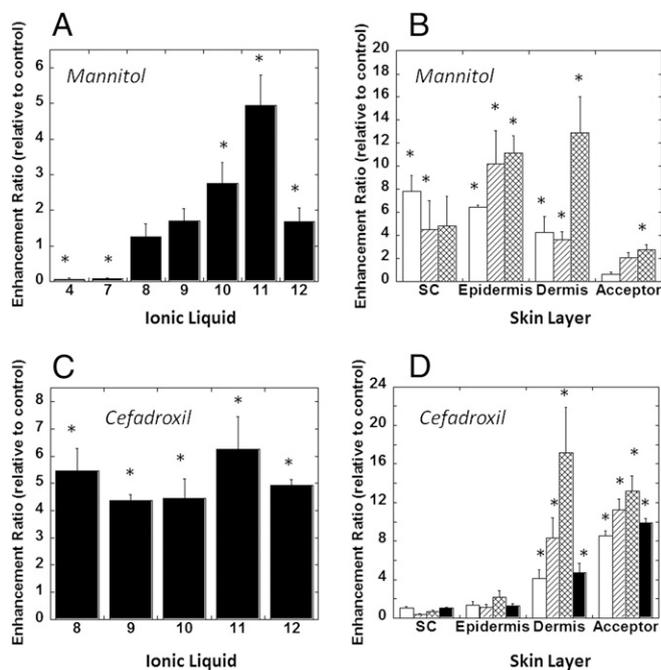


Fig. 3. Skin penetration of model drugs was enhanced by ILs. (A) Enhancement of total mannitol delivery into the skin relative to PBS (control) solution. (B) Mannitol penetration into individual layers in the skin. Depth increases from left to right: 9 (open bars), 10 (hatched bars), 11 (crosshatched bars). (C) Enhancement of total cefadroxil delivery into the skin relative to PBS (control) solution. (D) Cefadroxil penetration into individual layers in the skin. Depth increases from left to right: 9 (open bars), 10 (hatched bars), 11 (crosshatched bars), 12 (closed bars). PBS control was performed for each skin piece used to account for variability between skin pieces. Error bars represent mean \pm SE for $n = 3$. * $P < 0.05$ compared with control formulation using Student *t* test.

were tested for cefadroxil delivery. All ILs delivered 15–20% of the applied dose, which translates to approximately fivefold enhancement of cefadroxil delivery into the skin (Fig. 3C). IL 11 led to the highest enhancement of cefadroxil delivery into dermis (~16-fold compared with aqueous solution) (Fig. 3D). Other ILs also led to varied degrees of enhanced transport. IL 11 arose as the leading formulation, with optimal antibacterial, cytotoxicity, and skin-permeation properties.

Skin Irritation Assessment. ILs 9–12 were assessed for skin irritation potential because of their high dermal-transport enhancement properties. This task was first performed using Fourier transform infrared spectroscopy (FTIR) because changes in the integrated absorbance of the SC spectrum between 1,650 cm^{-1} and 1,660 cm^{-1} , which corresponds to alpha helix content in the SC, have been shown to correlate with skin irritation (27). Individual components of ILs including geranic acid and choline showed significant irritation potential (Fig. 4A). Geranic acid (applied neat) exhibited the most irritation potential, with a $\Delta(1,650-1,660)$ of ~ 32 a.u. $\cdot cm^{-1}$, compared with 16 a.u. $\cdot cm^{-1}$ for the positive control (5% SDS). The cationic components of ILs also exhibited significant irritation potentials of ~ 25 a.u. $\cdot cm^{-1}$ and 22 a.u. $\cdot cm^{-1}$ for choline (2.26 M in PBS) and tetraalkylphosphonium (applied neat), respectively. In contrast to their individual components, ILs 9–12 showed no significant change in $\Delta(1,650-1,660)$ (Fig. 4A). This striking observation was further confirmed for the lead IL (11) by measurements in reconstructed human epidermal cultures (EpiDerm). Release of interleukin-1 α from EpiDerm was previously established as an indicator for irritation (30). The difference between the irritation potential of

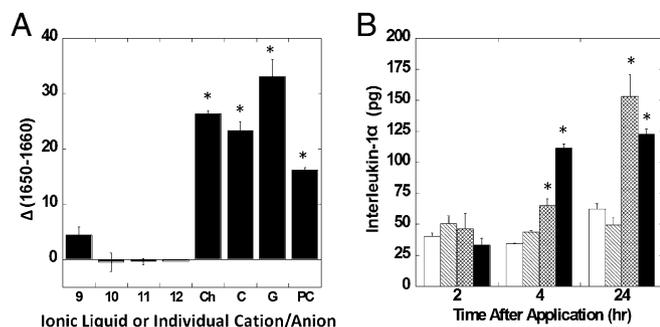


Fig. 4. Skin irritation was reduced by ILs compared with the individual components. (A) Irritation potential was first determined by measuring the change in the integrated absorbance of the FTIR spectra between 1,650 cm^{-1} and 1,660 cm^{-1} due to application of ILs or individual components choline (Ch), tetraalkylphosphonium-chloride (CYPHOS 101, further abbreviated as C in the figure), and geranic acid (G), or 5% SDS (positive control, PC) for 24 h. Error bars represent mean \pm SD for $n = 3$, $*P < 0.01$. (B) Irritation potential determined by FTIR analysis was further confirmed by measuring interleukin-1 α secretion due to application of **11** or its anionic component, geranic acid, on a MatTek Epiderm human skin equivalent model. PBS (negative control, open bars), **11** (hatched bars), geranic acid (cross-hatched bars), 5% SDS (positive control, filled bars). Error bars represent mean \pm SD for $n = 3$. $*P < 0.05$ compared with both the negative control and **11** determined by ANOVA and post hoc test in Origin Pro.

the IL and its components was resolved within 24 h (Fig. 4B). Whereas the positive control (5% SDS) and geranic acid (applied neat) resulted in a significant increase in interleukin-1 α release, the effect of **11** was comparable with that of the negative control. This observation is a unique feature of **11**; the individual components have a high irritation potential, but, when geranic acid and choline are combined, only nominal irritation was observed.

Validation Against Biofilm-Infected Wounds. To assess the potential clinical efficacy of **11** and the corresponding ceftazidime (cefadroxil structural analog) formulation, a *P. aeruginosa* biofilm was grown on a wounded MatTek Epiderm FT human skin equivalent tissue. Ceftazidime is a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria and has proven activity against *Pseudomonas* sp. We determined that the minimum inhibitory concentration (MIC) for ceftazidime against planktonic *P. aeruginosa* was 1 $\mu\text{g}/\text{mL}$, which is in good agreement with the MIC values listed in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) database (31). The corresponding *P. aeruginosa* biofilm in the wound model, however, saw only a marginal decrease ($\sim 20\%$) in bacterial viability after a 2-h exposure to 1 $\mu\text{g}/\text{mL}$ ceftazidime in a saline solution (Fig. 5). In contrast, both **11** and **11** formulated with 1 $\mu\text{g}/\text{mL}$ ceftazidime reduced bacterial viability within the biofilm by $>95\%$ ($P < 0.05$) and $>98\%$ ($P < 0.05$), respectively (Fig. 5), thus confirming the ability of **11** to both neutralize pathogens and deliver antibiotics.

Discussion

ILs are organic, nonvolatile salts with improved thermal properties compared with standard organics, and they are tunable by facile modification of their cation and anion components or as parts of mixtures (32). Further, the ability to modulate the individual components presents an advantageous framework for tuning secondary and tertiary physicochemical characteristics without sacrificing the primary function of the IL (23). There are numerous reports in the literature for utilization of ILs in biological applications, as outlined in the Introduction. However, this study provides, for the first time, to our knowledge, compelling evidence for utilization of neat ILs for concerted pathogen neutralization and transdermal and topical drug delivery

across skin layers. In addition, several of the ILs in our panel proved effective for disrupting biofilms produced by the pathogenic bacteria *P. aeruginosa* and *S. enterica*. ILs were previously adopted as antimicrobial agents against multiple Gram-negative and Gram-positive biofilm-forming pathogens and fungi (19, 21) and, thus, provided inspiration for our selection of the cationic (e.g., cholinium) and anionic (e.g., organic acids) components in our formulations (22). More recently, utilization of ILs in both drug formulations as active pharmaceutical ingredients (APIs) (24, 33) and as carriers in topical drug delivery systems (25) has proven a promising alternative to more traditional approaches (10). To our knowledge, there are no reports in the literature that combine the antibiofilm behavior of ILs with cytotoxicity effects on mammalian cell lines in addition to IL-mediated transdermal drug delivery across multiple skin layers in a single, concerted study.

The biological outcome of ILs, including transdermal delivery, antibacterial activity, and cytotoxicity, is related to the chemical properties of ILs and their components. FTIR studies showed that **11** produced a marked effect on the SC structure (Fig. S5). Specifically, **11** reduced the area of the 2,850 cm^{-1} peak, an indicator of the SC lipid phase (34), which indicates that **11** extracted the SC lipids. This observation is a significant shift from the behavior of its individual components in dilute solutions, especially geranic acid and its derivatives, which have been known to fluidize the SC lipids (27). ILs are well-recognized for their solubilizing ability of amphipathic molecules, and, thus, it would not be surprising if ILs excel in solubilizing the SC lipids as well. Direct assessment of lipid extraction by IL and its detailed mechanisms warrants further studies.

The lack of skin irritation potential for **11** and other ILs is a particularly interesting finding. It likely originates from the ability of the ILs to shield the charge due to ion pairing, thus exposing their hydrophobic moieties. Because high molecular charge is usually associated with irritation, pairing and shielding of charges should lead to reduced irritation. Low conductivity is one indication of ion pair association, and **11** has a very low conductivity relative to most other ILs studied (Table S3). The reduced levels of acidic proton in the IL, compared with neat geranic acid, may also contribute to the reduced irritation. Skin

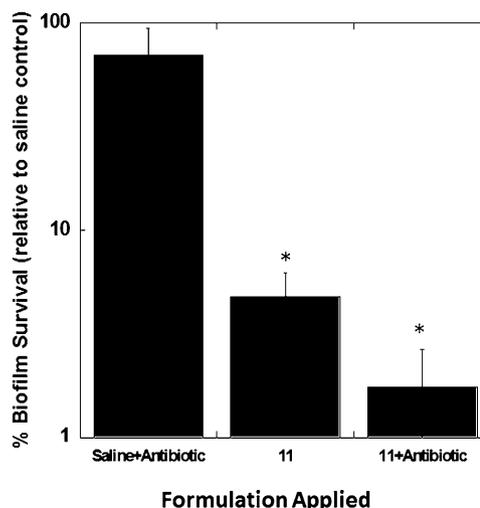


Fig. 5. Proof-of-concept validation of formulation efficacy against biofilm-infected wounds. IL **11** with or without 1 $\mu\text{g}/\text{mL}$ ceftazidime was applied neat on wounded, *P. aeruginosa*-infected, MatTek Epiderm human skin equivalent tissues for 2 h. Efficacy was compared with saline plus antibiotic, and % biofilm survival was determined based on saline-only control solution. Error bars represent mean \pm SE for $n = 3$. $*P < 0.05$ compared with saline plus 1 $\mu\text{g}/\text{mL}$ ceftazidime determined by Student *t* test.

exposed to geranic acid experiences a pH in the range of 5–6 whereas skin exposed to the IL encounters a more neutral environment. This finding provides an exciting opportunity for the use of penetration enhancers that cannot be otherwise used due to high toxicity. Specifically, formulating chemicals as ILs may provide a new strategy to mitigate the toxicity of penetration enhancers.

The relationships between the antibacterial efficacy of neat ILs and key molecular properties, including lipophilicity, viscosity, ionic strength, density, solubility in LB, and conductivity, were assessed. A weak, but statistically significant, trend (t test, $P = 0.02$) was found between biofilm survival (\log_{10} biofilm cell count) and conductivity (mS/cm). Lower conductivity, or tighter association of ion pairs, therefore correlates with high efficacy against biofilms. High efficacy of **11** is consistent with this trend. High lipophilicity was correlated to cytotoxicity of aqueous, diluted solutions of ILs to NHBE cells (% survival at 0.82 mM, t test, $P = 0.0072$). Lipophilicity (as assessed by the ALOGPS 2.1 algorithm) (35) of aqueous preparations of the individual components of ILs was also predictive of biofilm toxicity. The breakdown in this relationship for the toxicity of neat ILs in biofilms (see for example, the high biofilm toxicity of choline-malonate, **3**, and the low lipophilicity) suggests that phase-transfer properties from water to octanol do not approximate the interaction between neat ILs and the cell membrane. Such trends indicate the presence of differing mechanisms that explain antibacterial activity and cytotoxicity due to ILs and their individual components.

Ionic liquids are increasingly recognized as unique solvents because of their tunable physical, chemical, and biological characteristics. In this manuscript, we provide, to our knowledge, the first example for use of neat ILs as concerted antimicrobial agents and transdermal drug-delivery agents. Several of the ILs examined in these studies comprise GRAS materials to facilitate their future use in the clinic. The experimental approach outlined in this manuscript provides a generalized framework for designing novel IL-based formulations for topical treatment of a variety of skin-related diseases. Applications of ILs for the treatment of skin-based biofilms are particularly appealing. The total economic burden of skin disease was estimated to be ~\$96 billion in 2004, and the prevalence and healthcare costs for skin disease have been increasing over the last three decades (36). Bacterial infections in the skin are among the most common diagnoses in hospital patients, accounting for ~10% of all hospital visits (37). *S. aureus* infections acquired in hospitals, which account for only ~16% of nosocomial infections, are estimated to result in \$9.5 billion in extra patient costs and ~12,000 deaths annually (9). This burden is not restricted to hospitals alone and spreads to other locations (38). Of greater concern, biofilms are a major cause of chronic wounds and wound degeneration (39). Wounds from infected surgical incisions result in ~1 million additional hospital days (40). Additional causes of bacterial infected wounds include traumatic injuries, as well as diabetic foot ulcers, venous leg ulcers, and pressure ulcers. Alarming, 14–24% of diabetic ulcers require amputation (8). See *SI Materials and Methods* and *Table S5* for a more detailed description of biofilms' roles in skin disease.

This study provides compelling evidence in favor of exploiting ionic liquids for dermal delivery of antibiotics for the treatment of biofilm-infected wounds. In this regard, we find that ceftazidime may particularly benefit from an IL application. Ceftazidime is a third-generation cephalosporin that has broad-spectrum activity against both Gram-negative and Gram-positive bacteria (41), but also has limited skin permeability, poor water solubility, and a short shelf life. The best IL candidate, **11**, delivered ceftazidime through topical application for the treatment of a biofilm-infected wound (*P. aeruginosa*) (Fig. 5). *P. aeruginosa* are typically present on human skin and in chronically infected wounds (42, 43).

ILs offer an ideal solvent system for topical and transdermal delivery of drugs. Compared with traditional organic solvents such as ethanol, ILs can be formulated to be less toxic to cells (44). These findings address a major issue associated with solvent-induced skin irritation typical with many skin formulations. Further, the unique physicochemical properties of ILs can be exploited to solubilize different classes of drugs and additional penetration enhancers to further increase formulation potency. Finally, the ILs possess innate antibacterial activity, likely by disruption of both the charged and hydrogen bonding networks typically found within polypeptides (proteins, e.g., keratin), polynucleotides, and polysaccharides (e.g., cellulose) (3, 45, 46), and algae cell walls (47). Once the biofilm is disrupted, delivery of antibiotics is greatly enhanced, and any dispersed pathogens are generally restored to normal antibiotic susceptibility.

In summary, this study provides a detailed account of IL syntheses, their evaluation on the biofilm-forming pathogens *P. aeruginosa* and *S. enterica*, and an account of transdermal drug delivery properties. Further, we studied the ILs for their cytotoxicity to primary human cell lines. Overall, of the 12 materials examined, **11** (choline-geranate) proved to be the most efficacious. Future efforts should focus on assessing the efficacy of **11** against Gram-positive bacteria and in clinical models.

Materials and Methods

Synthesis and Characterization of ILs. Detailed methods describing synthesis of ILs and characterization of their lipophilicity, viscosity, density, and conductivity are described in *SI Materials and Methods*.

Measurement of Skin Transport. ^3H -labeled Mannitol and Cefadroxil were obtained from American Radiolabeled Chemicals and Moravek, respectively. Franz diffusion cells (FDCs) were used to assess the transport enhancement of ionic liquids using a previously established protocol (48). See *SI Materials and Methods* for details.

Fourier Transform Infrared Spectroscopy Measurements. FTIR of isolated porcine skin exposed to ILs was performed using methods described in Karande et al. (27). See *SI Materials and Methods* for further details.

Measurement of Skin Irritation. Skin irritation was further assessed by measuring interleukin-1 α release from MatTek Epiderm FT human skin equivalent tissues (MatTek Corporation). Experiments were performed according to the manufacturer's recommended protocol.

Strains and Biofilm Cultivation. The Gram-negative pathogenic bacteria *Pseudomonas aeruginosa* ATCC15692 and *Salmonella enterica* serovar typhimurium LT2 were used for the biofilm growth studies.

IL Challenge on the Biofilms. IL challenge on the biofilms was performed using a modified MBEC plate assay as described in Ceri et al. (49). See *SI Materials and Methods* for further details.

Mammalian Cell Culture and Exposure. Toxicity of ILs against normal human bronchial epithelial (NHBE) primary cells was tested using standard cell-culture protocols. See *SI Materials and Methods* for further details.

Antimicrobial Studies. A 5,120- $\mu\text{g}/\text{mL}$ working stock of ceftazidime (Sigma Aldrich) in water buffered with sodium bicarbonate was freshly prepared for each experiment. The broth microdilution method as outlined in the Clinical and Laboratory Standards Institute (CLSI) was used to determine antimicrobial behavior of ceftazidime on each pathogen. See *SI Materials and Methods* for further details.

Biofilm-Infected Wound Model. Experiments were performed by an independent testing facility (BioScience Laboratories) specializing in FDA-approved testing of topical formulations for the treatment of biofilms. Briefly, *P. aeruginosa* ATCC15692 was cultured in sterile Tryptic Soy Broth for 24 h at 35 °C. After 24 h, this culture was inoculated onto the surface of Tryptic Soy Agar and incubated at 35 °C until sufficient growth was observed. Immediately before inoculation of wounded tissues, the challenge suspension was prepared by transferring the microorganism from the

agar plate to sterile saline solution at $\sim 1 \times 10^7$ cfu/mL. The 24-h biofilm was chosen because extending biofilm to longer times compromised tissue integrity.

Twenty-four hours before testing, wounded tissues, prepared using MatTek Epiderm FT human skin equivalent tissues (MatTek Corporation) according to the manufacturer's recommended protocol, were inoculated with 0.01-mL aliquots of the challenge suspension and incubated for 24 h at 37 °C and 5% CO₂. After 24 h, the tissues were rinsed in PBS to remove any planktonic bacteria. Then, 200 μ L of saline, saline plus antibiotic (1 μ g/mL), 11, or 11 plus antibiotic (1 μ g/mL) was applied to the contaminated wounded tissues for 2 h. Untampered wounded tissue was used as a negative control, and formulations with antibiotic were prepared using a 100 μ g/mL ceftazidime in water stock. Each formulation was tested in triplicate. Following exposure, each wounded tissue was transferred to a separate tissue grinder, homogenized in Tryptic Soy Broth, and sonicated for 5 min to

thoroughly break up cells. Cell viability was determined by enumeration on Tryptic Soy Agar plates.

Statistical Analysis. Data reported are mean \pm SE except where otherwise noted. Where appropriate, statistical significance was confirmed by one way-ANOVA or two way-ANOVA and post hoc test using Origin Pro, or by the two-tailed, unpaired Student *t* test in Microsoft Excel. The level of significance was set at $P < 0.05$.

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