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Diphosphonium Ionic Liquids as Broad Spectrum Antimicrobial Agents

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Abstract

Purpose—One of the most disturbing trends in recent years is the growth of resistant strains of bacteria with the simultaneous dearth of new antimicrobial agents. Thus, new antimicrobial agents for use on the ocular surface are needed.

Methods—We synthesized a variety of ionic liquid compounds, which possess two positively charged phosphonium groups separated by ten methylene units in a “bola” type configuration. We tested these compounds for antimicrobial activity versus a variety of ocular pathogens, as well as their cytotoxicity *in vitro* in a corneal cell line and *in vivo* in mice.

Results—The ionic liquid Di-Hex C10 demonstrated broad *in vitro* antimicrobial activity at the low micromolar concentrations versus Gram-negative and Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* strains, as well as ocular fungal pathogens. Treatment with Di-Hex C10 resulted in bacterial killing in as little as 15 minutes *in vitro*. Di-Hex C10 showed little cytotoxicity at 1 μ M versus a corneal epithelial cell line or at 10 μ M in a mouse corneal wound model. We also show that this bis-phosphonium ionic liquid structure is key, as a comparable mono phosphonium ionic liquid is cytotoxic to both bacteria and corneal epithelial cells.

Conclusions—Here we report the first use of dicationic bis-phosphonium ionic liquids as antimicrobial agents. Our data suggest that diphosphonium ionic liquids may represent a new class of broad-spectrum antimicrobial agents for use on the ocular surface.

Keywords

ionic liquids; antimicrobial agents; ocular; keratitis; cornea

INTRODUCTION

One of the most disturbing trends in recent years is the growth of resistant strains of bacteria with the simultaneous dearth of new antimicrobial agents. There have been relatively few new antimicrobial agents in recent years, and those that have been approved, including

Cubicin (daptomycin) and Zyvox (linezolid), specifically target Gram-positive bacteria. Therefore, there is a need for new broad-spectrum antimicrobial agents and for new classes of antibiotics that are effective against current resistant strains. Resistant organisms such as *S. aureus* and *Enterococcus* continue to be a threat clinically^{1,2}. *S. aureus* is a well-known bacterial pathogen with a troubling propensity to develop resistance to a broad range of therapeutically important antibiotics. For example, up to 50% of *S. aureus* strains are resistant to methicillin (so-called MRSA), and even for the recently introduced Cubicin (daptomycin) and Zyvox (linezolid), reports of resistance to these compounds appeared within 6 months of their introduction to the clinic^{3–6}. Gram-negative pathogens are also on the rise with multidrug-resistant *P. aeruginosa* and *Acinetobacter* strains becoming common^{7–9}.

Antibiotic therapeutic alternatives are particularly limited in the treatment of ocular infections because compounds have to be compatible with topical use on the ocular surface. In the United States, there has been particularly strong reliance on topical fluoroquinolones since the early 1990s. However, resistance of ocular pathogens has been well documented for 2nd, 3rd and 4th generation quinolones. A recent report from the Proctor Foundations found that more than 20% of ocular *S. aureus* isolates collected between 2005 and 2008 were resistant to ciprofloxacin, moxifloxacin, and gatifloxacin¹⁰. In a Canadian endophthalmitis study, Gram-positive isolates causing endophthalmitis had “in vitro resistance to moxifloxacin (47.1%), ciprofloxacin (43.4%), gatifloxacin (36.8%), levofloxacin (29.0%)...”¹¹. Other groups have demonstrated increasing rates of resistance to ciprofloxacin among *P. aeruginosa* ocular isolates, an important cause of bacterial keratitis¹². Given ophthalmology’s reliance on a relatively small number of topical antibiotics for surgical prophylaxis and the treatment of ocular infection there is a need to develop novel broad-spectrum antimicrobials compatible with the ocular surface.

Here we report the synthesis and characterization of a new class of antimicrobial agents, called dicationic bis-phosphonium ionic liquids, which demonstrate robust antimicrobial activity versus laboratory and clinical strains of important eye pathogens, such as *S. aureus*, including MRSA strains, *P. aeruginosa*, and ocular fungal isolates, but was not cytotoxic to corneal epithelial cells. We also show that this bis-phosphonium ionic liquid structure is key, as a comparable mono phosphonium ionic liquid is cytotoxic to both bacteria and corneal epithelial cells.

MATERIALS AND METHODS

Synthesis of dicationic phosphonium

All chemicals purchased from Aldrich, Acros, Sigma or CHEM IMPEX as highest purity grade and used without further purification. All reactions were performed under nitrogen atmosphere.

All of the compounds described below were prepared using the same procedure. The alkyl phosphine is mixed with the mono or di-chloro alkane and allowed to react at 140°C for 24 hours. The mixtures were then placed under vacuum at 140°C to remove any volatile components and a clear colorless liquid was obtained in 99% yield. NMR, mass spectroscopy and elemental analysis confirmed the synthesis of these compounds.

Mono-Hex C10; Phosphonium, 1-(1-decanediyl)[1,1,1-trihexyl], chloride ($P^+ : Cl^-$) was prepared from trihexylphosphine (8.3 g, 29 mmol) and 1-chlorodecane (5.22 g, 29.6 mmol). i) ¹H NMR (CDCl₃): δ 0.78 and 0.797 (t, 12, CH₃); 1.12–1.26 (br, 24, CH₂); 1.35–1.50 (br, 16, CH₂-CH₂-P); 2.30–2.38 (br, 8, CH₂-P); ii) ¹³C NMR (CDCl₃): δ 13.71 and 13.87 (CH₃); 19.27 and 18.80 (CH₂-CH₃); 21–22 (CH₂); 28.75–31.60 (CH₂-P); iii) ³¹P NMR (CDCl₃): δ

35.46 (P^+); iv) ES MS: 427.44 m/z [MCl] $^-$ (theory: 463.20 m/z [M] $^+$), and v) Elemental analysis: (theory: C, 72.60; H, 13.20) found C, 72.53; H, 13.18.

Di-Hex C10; Phosphonium, 1,1'-(1,10-decanediyl)bis[1,1,1-trihexyl], chloride was prepared from trihexylphosphine (20 g, 70 mmol) and 1,10-dichlorodecane (7.45 g, 35.3 mmol). i) 1H NMR ($CDCl_3$): d 0.89 (m, 18, CH_3); 1.30–1.72 (br, 64, CH_2-CH_2); 2.38–2.52 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 14.52–14.60 (CH_3); 19.48–22.99 (CH_2); 30.98–31.89 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 33.92 (P^+), iv) ES MS: 747.68 m/z [MCl] $^-$ (theory: 782.65 m/z [M] $^+$); and v) Elemental analysis: (theory: C, 70.46; H, 12.60) found C, 70.34; H, 12.40.

Di-Hex C6; Phosphonium, 1,1'-(1,6-hexanediyl)bis[1,1,1-trihexyl], chloride was prepared from trihexylphosphine (20 g, 70 mmol) and 1,6-dichlorohexane (5.47 g, 35.3 mmol). i) 1H NMR ($CDCl_3$): d 0.85 (m, 18, CH_3); 1.42–1.73 (br, 56, CH_2-CH_2); 2.30–2.62 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 14.56 (CH_3); 19.45–23.09 (CH_2); 30.95–31.98 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 33.91 (P^+); iv) ES MS: 691.62 m/z [MCl] $^-$ (theory: 726.58 m/z [M] $^+$), and v) Elemental analysis: (theory: C, 69.29; H, 12.46) found C, 69.17; H, 12.50.

Di-But C10; Phosphonium, 1,1'-(1,10-decanediyl)bis[1,1,1-tributyl], chloride was prepared from tributylphosphine (20 g, 99 mmol) and 1,10-dichlorodecane (10.55 g, 50 mmol). i) 1H NMR ($CDCl_3$): d 0.86 (m, 18, CH_3); 1.17–1.25 (br, 8, CH_2-CH_2); 1.32–1.55 (br, 32, CH_2-CH_2); 2.25–2.45 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 13.60 (CH_3); 18.69–24.15 (CH_2); 28.51–30.79 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 33.97 (P^+); iv) ES MS: 579.49 m/z [MCl] $^-$ (theory: 614.46 m/z [M] $^+$); and v) Elemental analysis: (theory: C, 66.31; H, 12.11) found C, 66.24; H, 12.22.

Di-But C6; Phosphonium, 1,1'-(1,6-hexanediyl)bis[1,1,1-tributyl], chloride was prepared from tributylphosphine (20 g, 99 mmol) and 1,6-dichlorohexane (7.75 g, 50 mmol). i) 1H NMR ($CDCl_3$): d 0.87 (m, 18, CH_3); 1.50–1.75 (br, 32, CH_2-CH_2); 2.37–2.63 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 13.70 (CH_3); 19.71–24.25 (CH_2); 29.02–30.19 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 34.08 (P^+); iv) ES MS: 523.43 m/z [MCl] $^-$ (theory: 558.40 m/z [M] $^+$); v) Elemental analysis: (theory: C, 64.38; H, 11.89) found C, 64.17; H, 12.00.

Di-Oct C10; Phosphonium, 1,1'-(1,10-decanediyl)bis[1,1,1-trioctyl], chloride was prepared from trioctylphosphine (20 g, 51.8 mmol) and 1,10-dichlorodecane (5.52 g, 26.1 mmol). i) 1H NMR ($CDCl_3$): d 0.78 (m, 18, CH_3); 1.10–1.52 (br, 100, CH_2-CH_2); 2.28–2.48 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 14.23 (CH_3); 19.08–22.74 (CH_2); 29.10–31.84 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 33.93 (P^+), iv) ES MS: 915.87 m/z [MCl] $^-$ (theory: 950.84 m/z [M] $^+$), v) Elemental analysis: (theory: C, 73.14; H, 12.91) found C, 73.21; H, 12.87.

Di-Oct C6; Phosphonium, 1,1'-(1,6-hexanediyl)bis[1,1,1-trioctyl], chloride was prepared from trioctylphosphine (20 g, 51.8 mmol) and 1,6-dichlorohexane (4.05 g, 26.1 mmol). i) 1H NMR ($CDCl_3$): d 0.81 (m, 18, CH_3); 1.12–1.68 (br, 92, CH_2-CH_2); 2.28–2.58 (br, 16, CH_2-P); ii) ^{13}C NMR ($CDCl_3$): d 14.26 (CH_3); 22.01–22.79 (CH_2); 29.15–31.97 (CH_2-P); iii) ^{31}P NMR ($CDCl_3$): d 33.92 (P^+), iv) ES MS: 859.80 m/z [MCl] $^-$ (theory: 894.77 m/z [M] $^+$), v) Elemental analysis: (theory: C, 72.36; H, 12.82;) found C, 72.33; H, 12.65.

Characterization of ionic liquids

All products were characterized by: NMR spectra recorded on a Varian INOVA spectrometer (for 1H , ^{13}C , and ^{31}P at 400, 100.6, and 161 MHz, respectively). Electro Spray mass spectra were obtained on an Agilent 1100 LC/MSD Trap with ESI and APCI sources and elemental analysis was obtained from Atlantic Microlab, Inc.

Methods, MIC and MBC assays

Laboratory strains *P. aeruginosa* PA14, *Staphylococcus aureus* MZ100, *Escherichia coli* W3110, *Candida albicans* SC5314, along with clinical isolates of bacteria and fungi were from the Dartmouth-Hitchcock Medical Center and UPMC Eye Center, respectively, were used in these studies. To determine the minimal inhibitory concentration (MIC) and the minimal bacteriocidal concentration (MBC), microbial cultures plus antibiotics were incubated for ~16 hrs at 37°C without shaking. Starting inoculum was $\sim 5 \times 10^5$ CFU/ml, and all assays were performed in Mueller-Hinton medium. MIC was determined as the lowest concentration of antibiotic that resulted in an OD₆₀₀ value <0.1 after 16 hrs of incubation. For MBC determination, after incubation, using the same plates, 2–3 μ L from each well was transferred to a TSA (trypticase soy agar) or Mueller-Hinton agar plate using a multi-prong device, and the plates were incubated overnight at 37°C. Plates were then scored for presence/absence of bacteria. The lowest concentration of antibiotic that resulted in complete loss of bacterial viability was assigned the Minimal Bacteriocidal Concentration (MBC). This method was chosen because it allowed a reliable, rapid and accurate estimate of the MBC.

To determine the kinetics of killing, assays were performed as described above except 2–3 μ L from each well was transferred to a TSA (trypticase soy agar) plate using a multi-prong device at the indicated times (t = 0, before addition of the antibiotic, then at 15 min, 30 min and 1 hr, 2 hr, 4 hr, 8 hr and 24 hr after addition of the compound) instead of for the full 16 hrs.

In vitro cytotoxicity assays

Cytotoxicity was determined using a commercial reagent that measures respiration (Alamar Blue, Invitrogen #DAL1025) of epithelial cells. Human corneal epithelial (HCLE)¹³ were grown to confluence in 24 well-dishes in KSFM+ (L-glutamine-containing Keratinocyte Serum-free Medium (KSFM) supplemented CaCl₂ (3 mM), bovine pituitary extract (25 ng/ml) and human epidermal growth factor (0.2 ng/ml). The growth medium was exchanged with KSFM+ medium containing the drugs at concentrations noted in the text, with ethanol in all wells at 1.9% V/V, and then incubated at 37°C with 5% CO₂ for 2 hours. A subset of cells were disrupted using Triton-X-100 at 0.25% for a negative control, and wells with only KSFM + 1.9% ethanol were used as a positive control (called “lysis”). “Mock” treatment indicated addition of 1.9% ethanol without any added drugs. To quantitate viability, the medium with drug was removed and cells were washed with PBS (500 μ L) three times, and 500 μ L of fresh KSFM medium plus alamar blue (4%) was added and dishes were incubated at 37°C with 5% CO₂ for 3 hours. Fluorescence was read in a Biotek Synergy 2 plate reader (excitation filter: 500/27, emission filter: 620/40). The experiment was repeated twice with similar results (n=8/condition).

Animal studies

Mice were anesthetized with 1–2% isoflurane in O₂ and positioned under an operating microscope. Three scratches were made in the corneal epithelium with a 25 gauge needle with the bevel positioned up. The ionic liquid in PBS or PBS alone was delivered in a single dose to the scratched eye, and the eye scored 24 hrs later for opacity and epithelial damage. For epithelial damage, a score of 0 or 1 is assigned (0, intact corneal epithelium; 1, non-intact corneal epithelium). For opacity, scores ranging from 0–2 are assigned (0, no opacity; 1, partial opacity for 50% of the lesion; 2, complete opacity for 50% of the lesion). At the completion of the experiment, the animals were sacrificed, and treated eyes were then enucleated and fixed in formalin. Sections of the eye were stained with hematoxylin and eosin and analyzed by light microscopy. This protocol was approved by the Dartmouth IACUC (Assurance number A3259-01).

RESULTS

Synthesis and characterization of ionic liquids

The seven ionic liquids used in this study, Mono-Hex C10, Di-Hex C10, Di-Hex C6, Di-But C6, Di-But C10, Di-Oct C10 and Di-Oct C6, were synthesized as described in the Materials and Methods, and NMR, mass spectroscopy and elemental analysis were used to confirm the composition of each compound. The structures of two representative ionic liquids are shown in Figure 1.

The antimicrobial activity of ionic liquids against model Gram-positive and Gram-negative bacteria varies with their structure

To begin to establish relationships between the structure and activity of ionic liquids, we assessed the antimicrobial activity of these compounds against two model laboratory strains, the Gram-positive organism *S. aureus* and the Gram-negative organism *E. coli*. As summarized in Table 1, the MBC of the seven ionic liquids ranged from 8 µg/ml to >5000 µg/ml, indicating that there was a significant structural dependence on the antimicrobial activity of these compounds.

This analysis further illustrated that Di-Hex C10 demonstrated the most robust activity, with a MBC of 8 µg/ml (10 µM) versus *S. aureus* and 40 µg/ml (51 µM) versus *E. coli*. The broad-spectrum antibiotic ciprofloxacin is included as a control (Table 1). The measured MIC values were identical to the MBC values, indicating that these compounds killed the microbes rather than simply inhibiting their growth.

Di-Hex C10 was active in serum, showing no difference in MBC versus *S. aureus* MZ100 when the assays were performed in 10% FBS (data not shown).

Low micromolar concentrations of the ionic liquid Di-Hex C10 shows antimicrobial activity versus clinical bacterial strains, including antibiotic-resistant isolates, and fungal pathogens

To determine the microbes against which Di-Hex C10 might be active, we measured the MBC for this compound versus clinical isolates of *S. aureus* (3 isolates), methicillin-resistant *S. aureus* (MRSA, 4 isolates of the USA300 strain), *S. epidermidis* (3 isolates), *Klebsiella* (2 isolates) as well as the recent clinical isolate of *Pseudomonas aeruginosa*. As summarized in Table 2, Di-Hex C10 showed broad activity versus these clinical isolates, including activity versus MRSA and *P. aeruginosa*.

We next tested Di-Hex C10 versus several fungal pathogens, including pathogens relevant to ocular infections. The MBC of Di-Hex C10 for *C. albicans* is on par with that observed for *Staphylococcus spp.* We also assessed the efficacy of Di-Hex C10 versus ocular clinical isolates of *Fusarium* sp, *Aspergillus fumigatus*, and *Verticillium* sp. The MBC of Di-Hex C10 versus these fungal pathogens ranged from <0.5–16 µg/ml.

Taken together, these data indicate that Di-Hex C10 has broad-spectrum activity versus bacteria and fungi relevant to ocular infections. Based on its robust activity in vitro versus a broad range of ocular bacterial and fungal pathogen(s), we focused our efforts on the further characterization the ionic liquid Di-Hex C10.

Ionic liquid Di-Hex C10 shows rapid bacterial killing

A desired feature of an antimicrobial agent is the ability to rapidly kill bacteria. Thus, we assayed the kinetics of killing for Di-Hex C10 against *S. aureus* and *E. coli* at t = 0 (before addition of the antibiotic), then at 15 min, 30 min and 1 hr, 2 hr, 4 hr, 8 hr and 24 hr after

addition of the compound. We found that 30 $\mu\text{g/ml}$ Di-Hex C10 can kill *S. aureus* with an exposure time of less than 15 min, while 30 minutes of exposure was required to kill *E. coli*. Thus, for both Gram-positive and Gram-negative organisms, this ionic liquid causes rapid bacterial cell death.

Stability

Di-Hex C10 was quite stable. When stored at room temperature in ambient light, we observed no reduction in activity after 3 months (data not shown).

Ionic liquid Di-Hex C10 shows low *in vitro* cytotoxicity

To assess the impact of ILs on the host, we first performed *in vitro* cytotoxicity assays using human corneal limbal epithelial (HCLE) cells. We assessed the cytotoxicity of Di-Hex C10, as well as Mono Hex C10, a compound with a related structure but ~25–100-fold less antimicrobial activity (Table 1). We tested two different concentrations of these compounds. The higher concentration was chosen (10 μM) because it was the concentration we used in the animal experiments presented below. We also tested 1 μM , because ~90% of any compound added to the eye is typically rapidly removed by blinking^{14,15}. As described below, the animal studies used to assess *in vivo* Di-Hex C10 efficacy involves a short period of anesthesia followed by rapid waking of the animal and blinking. Thus, we anticipated that much of the compound would be rapidly removed from the eye in our animal studies. We also included a control compound, ciprofloxacin, which is an antibiotic used in the eye.

As shown in Figure 2A, 1 μM of either Mono Hex C10 (HC10) or Di-Hex C10 (DiHC10) showed no apparent cytotoxicity, as the measured alamar blue mediated fluorescence, and thus viability, for these treatment conditions was not different from the mock control, containing just the 1.9% ethanol vehicle used to solubilize the antibiotic. However, at the higher 10 μM dose, significant cytotoxicity was observed, for both the Mono Hex C10 and Di-Hex C10. Similar results were observed with A549 lung epithelial cells (data not shown). It is important to note that there was little relationship between cytotoxicity and antimicrobial activity, as Mono Hex C10 shows ~25–100-fold lower antimicrobial activity than Di-Hex C10, but similar cytotoxicity at the 10 μM dose.

For ciprofloxacin, no cytotoxicity was observed for the 10 μM dose (the concentration of Di-Hex C10 used in our studies), but was observed for the clinically-used concentration of ~10 mM (Figure 2A). The mock control showed strong alamar blue fluorescence (i.e., no loss of viability) and the lysis control suggested low cell viability, as expected.

Ionic liquid Di-Hex C10 shows no cytotoxicity in mouse corneas

As a first step to determine the efficacy of Di-Hex C10 *in vivo*, we tested whether application to the eye resulted in cytotoxicity. We applied a single dose of Di-Hex C10 using a mouse model of microbial keratitis, in the absence of added bacteria, and assessed cytotoxicity by scoring opacity and epithelial damage, as well as histopathology of the treated organ.

After scratching the mouse cornea, 5 μL of 10 μM Di-Hex C10 in PBS or PBS alone were applied, and the animals scored for corneal opacity and corneal epithelial damage at 24 hours. No corneal opacity or epithelial damage was observed for the Di-Hex C10-treated animals, and an identical finding was observed for the PBS-treated control (Fig. 2B,C). Histological studies also revealed no difference between Di-Hex C10-treated and control PBS-treated animals in this analysis (Fig. 2D). Taken together, these data show that a single treatment with Di-Hex C10 at a dose active against most microbes tested in this report does not produce any signs of corneal toxicity or impede corneal wound healing.

DISCUSSION

The studies presented here show that dicationic bis-phosphonium ionic liquids, ionic compounds which possess melting points at relatively low temperatures ($< 100\text{ }^{\circ}\text{C}$)^{16–20}, may represent a new class of broad-spectrum antimicrobial agent with potential utility on the ocular surface. Furthermore, our data show that antimicrobial activity varies with the structure and chemical properties of the compounds (Table 1). Ionic liquids have been primarily investigated for material science and industrial applications such as solvents, separation media, protein crystallization matrices, liquid crystals, batteries, and thermal fluids^{16–30}. However, earlier *in vitro* results by Seddon and colleagues showing antibacterial activity for monophosphonium ionic liquids, primarily versus Gram-positive organisms, indicated that these compounds may have utility in medicine³¹. In this work, we identified a distinct class of ionic liquid, a dicationic bis-phosphoniums, with promise as a new class of broad-spectrum antimicrobial agent with activity versus Gram-positive and -negative bacteria, as well as fungi.

We were quite encouraged to observe activity versus a number of different organisms of clinical importance. For example, Di-Hex C10 showed robust antimicrobial activity versus Gram-positive organisms such as *S. aureus* and *S. epidermidis*, as well as antibiotic resistant microbes, such as MRSA. Di-Hex C10 also showed activity versus important Gram-negative organisms such as *E. coli* and *P. aeruginosa*. Surprisingly, we also observed low micromolar activity versus several fungal organisms, including ocular pathogens, indicated that ionic liquids may be broadly useful for treating a wide range of ocular microbial infections. To further develop these compounds for clinical use will require additional structure-activity relationship studies, as well as extensive animal testing.

The mechanism of action of Di-Hex C10 or any of the dicationic bis-phosphonium ionic liquids has not yet been established. Similar compounds possessing a single cationic group, either ammonium or phosphonium (such as the monophosphonium ionic liquids³¹), are thought to act as surfactants and disrupt the cellular membrane. But the dicationic bis-phosphonium ionic liquids exhibit a bolaform shape and bolaforms are generally known not to be membrane disruptors. Bolaforms can penetrate the membrane without disruption potentially explaining why they are so much more effective versus Gram-positive organisms that lack an outer membrane. However, the fact that Di-Hex C10 is not broadly cytotoxic indicates some specificity for bacterial membranes versus host. It is also possible that these compounds are targeting a critical function in the cytoplasm. Additional studies are required to define the mechanism of action of dicationic bis-phosphonium ionic liquids and these studies are planned.

We tested Di-Hex C10 using *in vitro* cytotoxicity assays with corneal epithelial cells. We observed no cytotoxicity when the compound was applied at $1\text{ }\mu\text{M}$, but significant cytotoxicity at $10\text{ }\mu\text{M}$, which is the concentration of Di-Hex C10 used in animal studies. However, it is typical for 90% of drugs applied to the eye to be eliminated within 5 minutes of application^{14,15}, thus it is likely that only $<1\text{ }\mu\text{M}$ compound remains for a majority of the study period. Consistent with this idea, when applied directly to a scratched mouse cornea, both visual inspection of opacity and epithelial damage revealed no impact of $10\text{ }\mu\text{M}$ Di-Hex C10 treatment compared to the PBS-treated control. These observations were further substantiated by pathological studies that also detected no damage to the corneal tissue after a single dose Di-Hex C10 application. It is also important to note that there was little relationship between cytotoxicity and antimicrobial activity, as Mono Hex C10 shows ~25–100-fold less antimicrobial activity than Di-Hex C10, but the same level of cytotoxicity at the $10\text{ }\mu\text{M}$ dose. More extensive testing is required to establish both safety, as well as the *in vivo* efficacy of this class of compounds.

While we have identified a candidate compound with activity in the low micromolar range, namely Di-Hex C10, we likely have significant room for improvement in regards to antimicrobial activity and reduced cytotoxicity for this class of molecules as we have not fully explored the chemical-structure space. Moreover, these dicationic bis-phosphonium ionic liquids are prepared in one step from commercially available materials and can be readily prepared on a larger scale. In fact, ionic liquids are produced on an industrial (> kilogram) scale today³². The resulting economic incentives of reduce labor costs, materials, and time may facilitate lower cost availability of these compounds to the developing world, where costs often restrict the use of modern medicine. Given the increased resistance to front-line antimicrobial agents and the scarcity of new compounds being used in the clinic, continued development of new broad-spectrum antimicrobials is sorely needed.

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Figure 1. Chemical structures of ionic liquids

Shown are the structures for Di-Hex C10 (left) and Mono Hex C10 (right), two of the ionic liquids used in these studies.

Figure 2. Di-Hex C10 shows no toxicity in vitro or in vivo

A. Shown are in vitro cytotoxicity assays with the ionic liquids. We assessed cytotoxicity of Mono Hex C10 (MC10) and Di-Hex C10 (DiHC10) in vitro on human *cornea*-limbal epithelial (HCLE) cells using alamar blue viability assays. Decreasing fluorescence intensity is correlated with decreased host cell viability. “Mock” indicates vehicle only addition, and “lysis” indicates detergent lysed cells and represents complete loss of viability. As a control, ciprofloxacin (cipro) is added at 10 μ M (the same concentration as the IL compounds) and 10 mM, the concentration typically used for this antibiotic clinically. * $P < 0.05$ compared to mock control using a One-Way ANOVA with a Tukey’s Multicomparison Test. **B.** Shown are images of mouse eyes, which were scratched then treated with a single dose of either PBS (top) or Di-Hex C10 (bottom). Visual inspection reveals no difference in corneal or

epithelial damage between the two treatments. White circles on the eye are reflections of the light source. **C.** Mean epithelium damage score (blue) and corneal opacity score (red) serve as measures of corneal damage. Details of the scoring scale are described in the Materials and Methods. Treatment with Di-Hex C10 shows no corneal or epithelium damage (ND, no damage) as is observed with the PBS control. The maximum possible score (Max Score) for each measure is also included on the plot. **D.** Shown is a light micrograph of a representative histologic section of mouse cornea stained with hematoxylin and eosin showing pathology of sections from eyes treated with PBS (top) or Di-Hex C10 (bottom). No difference was observed in eyes treated with PBS versus Di-Hex C10.

TABLE 1

Testing ionic liquids for activity against laboratory strains.

Ionic Liquid	MBC ^a (μg/mL)	
	<i>S. aureus</i> MZ100	<i>E. coli</i> W3110
Mono Hex C10	1000	1000
Di-But C6	1000	>5000
Di-Hex C6	8	1000
Di-Oct C6	40	5000
Di-But C10	40	5000
Di-Oct C10	40	>5
Di-Hex C10	8	40
ciprofloxacin	0.16	8

^aMBC=minimal bacteriocidal concentration, measured in TSB medium after ~16 hrs of exposure to antibiotics with a starting inoculum of $\sim 1 \times 10^7$ CFU/ml.

Table 2

Testing Di-Hex C10 for activity versus clinical isolates.

Organism Tested	# isolates	MBC ^a (µg/ml)
<i>S. aureus</i> clinical isolates	5	0.8–4
MRSA(USA300)	4	0.8
<i>S. epidermidis</i> clinical isolates	3	0.8–4
<i>Klebsiella sp.</i> ^b	2	4–20
<i>P. aeruginosa</i> PA14	1	20
<i>Aspergillus fumigatus</i>	2	<0.5–2
<i>Fusarium sp.</i>	2	1–2
<i>Verticillium sp.</i>	1	16
<i>Candida albicans</i>	1	8

^aMBC, minimal bacteriocidal concentration. For all the bacterial strains, the MBC was determined using Mueller-Hinton broth and a starting inoculum of $\sim 1 \times 10^5$ CFU/ml. The MBC for *Aspergillus* and *Fusarium* was determined as reported ³³. The MBC for *Candida albicans* was determined as was described for Table 1.

^bIsolates tested were *K. pneumonia* and *K. oxytoca*.