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Short imidazolium chains effectively clear fungal biofilm in keratitis treatment

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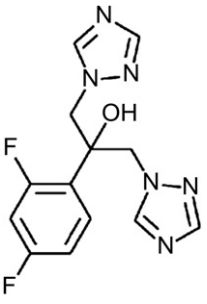
ABSTRACT

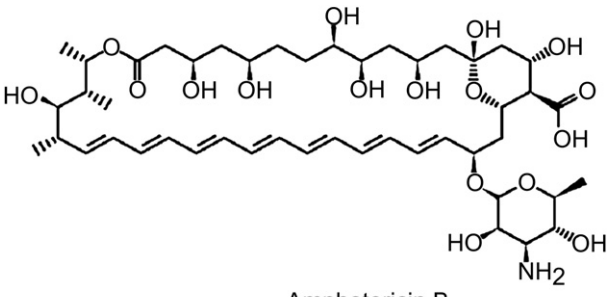
Fungal keratitis is a leading cause of ocular morbidity throughout the world. However, current therapies against fungal keratitis are often ineffective. Herein, we have developed the amphiphilic main-chain imidazolium polymer (**PIM-45**) and oligomer (**IBN-1**) materials that can efficiently inhibit the growth of fungi with low minimal inhibition concentration (MIC) values and clear the fungal biofilm, while displaying minimal hemolysis. *In vivo* keratitis treatment indicates that topical solutions of these poly-imidazolium salts (PIMSs) are safe and as effective as that of amphotericin B, the most commonly used agent for the treatment of *Candida albicans* (*C. albicans*) keratitis. Compared to the costly and unstable amphotericin B and fluconazole, **PIM-45** and **IBN-1** are easy to prepare, inexpensive and stable. They can be stored in phosphate-buffered saline (PBS) solutions with long shelf life for routine topical use.

1. Introduction

Fungal keratitis is a leading cause of ocular morbidity throughout the world, and it is also a major eye disease that leads to blindness in Asia [1,2]. Fungal keratitis is mainly caused by yeast-like fungi (such as *Candida albicans*) and filamentous fungi (such as *Aspergillus fumigatus*) [1–3]. Current therapies against fungal keratitis are often ineffective due to several reasons. (i) Fungal keratitis infection often exists as a biofilm, which is particularly difficult to clear because of its encasement in a protective and impermeable extracellular matrix (ECM) [4]. Much higher doses of antimicrobials are needed for biofilm clearance as compared to planktonic microbials. (ii) Shortage of broad-spectrum efficient antifungi drugs. Current clinical drugs for fungal keratitis include azole compounds (such as fluconazole) and polyenes (such as amphotericin B) (see below). The fungi static nature of azole compounds, which function by enzyme inhibition, requires a prolonged course of application. Azoles are also extremely unstable; their topical solutions should be kept refrigerated for no longer than 48 h and protected from light, as recommended by the manufacturer [5]. Polyenes, which function via disrupting the permeability of ions through the cell membrane, are rather expensive. Due to their poor penetration property, solubility and stability, they are limited in application [2,5,6]. These limitations, together with the development of drug resistance, have led to the low efficacy and unsatisfactory outcome associated with the current therapies for fungal keratitis.



Fluconazole



Amphotericin B

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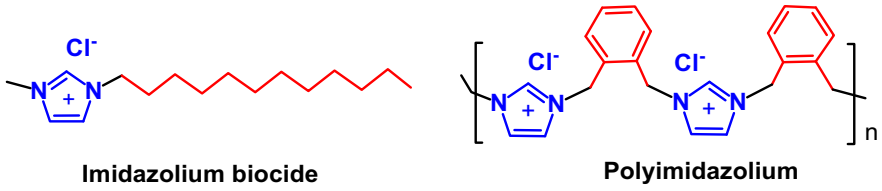


Scheme 1. Structural examples of typical biocide imidazolium salt and PIMS. Hydrophobic regions are shown in red, and hydrophilic regions are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In response to the growing health threat posed by resistant pathogenic microorganisms, the development of antibiotics with new mechanisms of action, including peptides and synthetic polymers, have attracted considerable research interests [7–9]. Amphiphilic peptides (AMPs) or synthetic polymers have unique killing mechanism that may slow down the development of drug resistance [10,11]. However, no antifungi/biofilm properties have been reported for most of these new materials [12–16]. Herein, we present a class of short-chain imidazolium polymeric materials (**IBN-1** and **PIMS-45**) with amphiphilic structures that are highly effective against yeast-like fungi *C. albicans* and filamentous fungi *Aspergillus niger* (*A. niger*). These materials can effectively clear *C. albicans* biofilm, and demonstrate high activity towards a broad spectrum of bacterial infections. MIC against Gram-positive bacteria *Bacillus subtilius* (BS) was 7.8 μg/mL for **PIM-45** and 1.5 μg/mL for **IBN-1**, and MIC against Gram-negative bacteria *Escherichia coli* (EC) was 35 μg/mL for **PIM-45** and 3.9 μg/mL for **IBN-1**. These short-chain imidazolium polymers represent a promising therapy for keratitis.

Imidazolium salts (IMSs), which are derivatives of imidazoles, are known for their biological properties, such as antioxidative, antiinflammatory, antifibrotic and anti-cancer characteristics [17,18], and as antibacterial and antifungal agents [19–25]. The key components of existing IMS-based antimicrobial agents are a hydrophilic imidazolium ring head and a long hydrophobic N-alkyl chain (C_nH_{2n+1}, $n > 10$) (Scheme 1), giving it the amphiphilic nature [26–28]. These biocidal IMSs usually have very low selectivity, and a minimal hemolytic concentration (MHC)/MIC usually in the range of 1.25–5. Recently, we have developed a short linear imidazolium oligomer, **IBN-1** (Scheme 2), which demonstrated broad-spectrum activities against various bacteria [29]. In this design, the imidazole is directly linked with single aryl or a short alkyl groups (C_nH_{2n+1}, $n < 4$) (Scheme 2), and the use of strongly hydrophobic long alkyl chain is avoided. The hydrophilic cationic imidazolium species and small hydrophobic groups sit alternatively in the backbone of this short-chain structure, and therefore may form globally amphiphilic topology like the AMPs. On the other hand, the lower positive charge on each short chain may allow this material to selectively bind to the microbial's membrane, which has a higher negative charge than red blood cell. This may impart antimicrobial properties and a high selectivity.

2. Materials and methods

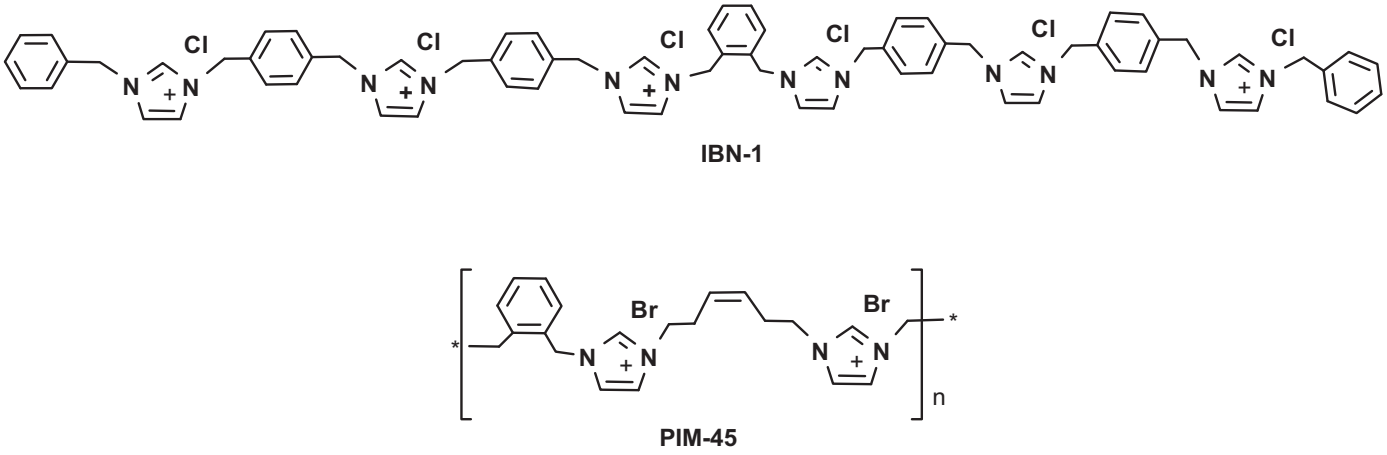
2.1. Synthesis of PIMSs

IBN-1 and **PIM-45** were synthesized based on condensation reactions as reported by us previously [29–31]. All solvents were of HPLC grade, and purchased from Aldrich or Fluka. All starting materials were commercially available and used as received, unless otherwise indicated. Nuclear magnetic resonance (NMR) spectra were obtained using a Brucker AV-400 (400 MHz) spectrometer. Chemical shifts were reported in ppm from tetramethylsilane with the solvent resonance as the internal standard.

2.2. Antifungal studies

C. albicans (American Type Culture Collection (ATCC) 10231, yeast) and *A. niger* were used as representative fungi for testing the antimicrobial functions of the PIMSs. *C. albicans* suspensions were prepared from fresh overnight cultures in Yeast Mold (YM) (Difco) broth using −80 °C-frozen stock cultures. Subsamples of these cultures were grown for another 3 h, and adjusted to an optical density of ~0.1 at 600 nm, giving a density of 10^7–10^8 CFU/mL. *A. niger* were cultured on Sabouraud dextrose agar plates in incubator at 37 °C. The spores were harvested from 96-h cultures, and suspended homogeneously in phosphate buffered saline (PBS). A homogeneous spore suspension was obtained by incubating the tube at 37 °C for 60 min with intermittent shaking. The spores in the suspension were counted, and their number was adjusted to 1×10^8 spores/ml before performing the experiments. Amphotericin B (Sigma–Aldrich, sterilized in deionized (DI) water) and fluconazole (Ningjiang Pharmaceutical & Chemical Corp., China) were examined as the commercial antifungal agents.

The MICs of fungicidal agents were determined by microdilution assay. For *C. albicans*, the yeast inoculum used was 100 μL/well for fungicidal agents (10^7–10^8 CFU/mL) in 2-fold dilutions, producing concentrations of 1–250 μg/mL in the media and giving a total amount of 200 μL/well. The 96-well microtiter plates (Nunc Microwell Plates) were incubated at 22 °C for 24 h. MIC corresponded to the minimum concentration necessary to inhibit complete cell growth. For *A. niger*, autoclaved Sabouraud



Scheme 2. Molecular structures of **IBN-1** and **PIM-45**.