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Antifungal activity of the cationic antimicrobial polymer-polyhexamethylene guanidine hydrochloride and its mode of action

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ABSTRACT

The antifungal activity of polyhexamethylene guanidine hydrochloride (PHMGH) was studied against various pathogenic fungi. PHMGH had more potent antifungal activity than amphotericin B, which is a commonly used antifungal drug, and also showed no hemolytic and lactate dehydrogenase release activities in the range of 1.25–40.0 μg mL^{-1}. PHMGH is a cationic polymer containing an amino group and a polymeric guanidine group. Based on its characteristics such as the cationic charge and hydrophobicity, the antifungal mechanism of PHMGH was investigated using *Candida albicans*, as a model organism. Flow cytometric contour-plot analysis and microscopy showed changes in the size and granularity of the cells after treatment with PHMGH. A membrane study using 1,6-diphenyl-1,3,5-hexatriene labelling indicated a great loss of phospholipid area in the plasma membrane following PHMGH treatment. To investigate the extent of the damage, fluorescein isothiocyanate-labelled dextran leakage from large unilamellar vesicles was observed, indicating that PHMGH acts on the fungal membranes by inducing pore formation, with the majority of pore size being between 2.3 and 3.3 nm. This mechanism was confirmed with ion transition assays using 3,3′-dipropylthiacarbocyanine iodide and an ion-selective electrode meter, which indicated that membrane depolarization involving K^+ leakage was induced. Taken together, these results show that PHMGH exerts its fungicidal effect by forming pores in the cell membrane.

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Abbreviations; AMM, Antimicrobial macromolecule; PHMGH, Polyhexamethylene guanidine hydrochloride; DMSO, Dimethyl sulfoxide; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; YPD, Yeast extract peptone dextrose; YM, Yeast malt; MIC, Minimum inhibitory concentration; PBS, Phosphate-buffered saline; FSC, Forward scatter; SSC, Side scatter; DPH, 1,6-Diphenyl-1,3,5-hexatriene; FITC, Fluorescein isothiocyanate; FD, FITC-dextran; LUV, Large unilamellar vesicles; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; $DiSC_3(5)$, 3′-Dipropylthiacarbocyanine iodide

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Introduction

Due to the increasing mortality caused by invasive mycosis and the limited arsenal of efficient and selective antifungals, fungal infections are becoming a global health threat (Akins 2005; Patterson 2005). Among various fungal pathogens, *Candida* spp. (*Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, and others) account for ~75 % of all fungal infections, and represent one of the most common causes of nosocomial infections (Rhinehart & Friedman 1999). Candidemia alone accounts for a mortality rate of ~40 %, which exceeds that of all gram-negative bacterial sepsis, indicating the medical importance of fungal diseases and the need for novel antifungal drug development (Shorr *et al.* 2009).

Antimicrobial macromolecules (AMMs) are among the most promising materials for the therapy of infectious disease. Among AMMs, microbicidal cationic polymers containing functional groups such as biguanide, quaternary ammonium salts, quaternary pyridinium salts, and phosphonium salts have received particular attention (Tashiro 2001). The cationic AMMs are divided into two classes: i) cationic poly-amino acids and ii) cationic polyelectrolytes (polymer biocides). Although AMMs mimic the biological activity of natural host-defence peptides, they have their own advantages (Gabriel *et al.* 2007). Unlike natural host-defence peptides, AMMs show low toxicity to human cells and have a low production cost. In addition, AMMs possess potent antimicrobial activity and do not elicit antimicrobial resistance. Hence, AMMs are currently used in sterile clothing, biocompatible medical materials (catheters, sutures, indwelling devices, prosthetics, etc.), air filters, and coatings that resist biofouling. For example, polyhexamethylene biguanide is a widely used environmental biocide and contact lens disinfectant owing to its antifungal activity (Messick *et al.* 1999).

Polyhexamethylene guanidine hydrochloride (PHMGH) consists of a polymeric guanidine group harbouring amino group, contributing to its activity as a cationic antimicrobial macromolecule (Oulé *et al.* 2008). Hence, it has rapid and broad-spectrum activity against both gram-positive and gram-negative bacteria. For example, PHMGH has been reported to kill methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* at concentrations as low as 0.04 % and 0.005 % (w/v), respectively, within 1.5 min (Müller & Kramer 2005). In addition, PHMGH is a water-soluble, odorless, colourless, and non-corrosive polymer, and is less toxic to humans than currently used disinfectants (Messick *et al.* 1999; Oulé *et al.* 2008). Although PHMGH is known to have potent antibacterial activity, its antifungal activity and the underlying mechanism are not well understood.

In this study, we investigated the antifungal activity of PHMGH and its mechanism by using *C. albicans* as a model organism.

Materials and methods

Preparation of PHMGH

PHMGH (Trade name: TEFLEX) was purchased from ZAO Soft Protector (St. Petersburg, Russia). TEFLEX contains very small amount of supplements such as neonol, 1-methoxy-2-propanol, hexamethylene tetramine, and carbamide. Most of these supplements are originated from the synthesis process of chemical compound. Therefore, PHMGH is main material contributing to antimicrobial activity of TEFLEX.

Preparation of amphotericin B

Amphotericin B (Sigma Chemical, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). In all experiments, the volume of DMSO treated was maintained below 10 % (v/v) in sample to block any positive effect of DMSO itself (Gerhards & Gibian 1967).

Preparation of the fungal strains

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Malassezia furfur* (KCTC 7744), *Trichosporon beigelii* (KCTC 7707), and *Trichophyton rubrum* (KCTC 6345) were purchased from the Korean Collection for Type Cultures (KCTC, Korea). The fungal strains were incubated in yeast extract peptone dextrose (YPD) broth (BD Difco, Franklin Lanes, NJ, USA) with aeration at 28 °C, and *M. furfur* was incubated in modified yeast malt (YM) broth (BD Difco) including 1 % olive oil at 32 °C.

Antifungal activity assay

The fungal cell density was adjusted to obtain standardized stock suspension (1×10^6 cells/mL) by measuring the turbidity with a spectrophotometer (DU530, Beckman, Fullerton, CA, USA). After that, a working suspension is made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with broth media, which results in 5.0×10^2 cells/mL. The resulting suspension was inoculated into microtiter plates at a volume of 0.1 mL/well. According to the Clinical and Laboratory Standards Institute guideline, test compounds were serially diluted, two-fold in the concentration range of 0.16–40.0 μg mL^{-1} (CLSI 2008a, 2008b). The minimum inhibitory concentration (MIC) values were determined in three independent assays.

Toxicity assay

A normal human blood sample was washed with phosphate-buffered saline (PBS, pH 7.4) and the plasma and buffy coat were eliminated by removal of the supernatant after centrifugation at 300× *g* for 10 min. This washing step was repeated three times, and the final concentration of the erythrocytes was adjusted to 8 %. The erythrocyte suspension was loaded into 96-well plates and treated with compounds in the concentration range of 1.25–40.0 μg mL^{-1}. After incubation at 37 °C for 1 h, the plate was centrifuged at 170× *g* for 10 min. The supernatant was collected, and then erythrocyte damage was analysed.

Hemolytic activity assay

The content of hemoglobin released in the supernatant was measured at 414 nm with an enzyme-linked immunosorbent