Microbial Metabolite Inspired $\beta$-Peptide Polymers Displaying Potent and Selective Antifungal Activity

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Potent and selective antifungal agents are urgently needed due to the quick increase of serious invasive fungal infections and the limited antifungal drugs available. Microbial metabolites have been a rich source of antimicrobial agents and have inspired the authors to design and obtain potent and selective antifungal agents, poly(DL-diaminopropionic acid) (PDAP) from the ring-opening polymerization of $\beta$-amino acid N-thiocarboxyanhydrides, by mimicking $\varepsilon$-poly-lysine. PDAP kills fungal cells by penetrating the fungal cytoplasm, generating reactive oxygen, and inducing fungal apoptosis. The optimal PDAP displays potent antifungal activity with minimum inhibitory concentration as low as 0.4 $\mu$g mL$^{-1}$ against Candida albicans, negligible hemolysis and cytotoxicity, and no susceptibility to antifungal resistance. In addition, PDAP effectively inhibits the formation of fungal biofilms and eradicates the mature biofilms. In vivo studies show that PDAP is safe and effective in treating fungal keratitis, which suggests PDAPs as promising new antifungal agents.

1. Introduction

Invasive fungal infections caused 1.5 million deaths worldwide each year, of which 30–40% are Candida infections and 20–30% are through Cryptococcosis.[1] These infections are very common in immunosuppressed populations such as patients who suffer from anti-cancer chemotherapy, organ transplants, HIV infections, or long-term use of hormones.[2] Both fungi and mammalian cells are eukaryotic organisms and it is extremely difficult to find an antifungal drug with high selectivity or low toxicity. Currently, only a few classes of antifungal drugs are available but commonly with high toxicity and side effects.[3] Therefore, the quick emergence of drug-resistant pathogenic fungi has been a serious threat to human health.[4] It is in urgent need to develop potent and nontoxic antifungal agents with promising therapeutic potential.

Microbial metabolites have been a rich source to explore antimicrobial agents.[5] $\varepsilon$-poly-lysine ($\varepsilon$-PL) was discovered from the fermentation of Streptomyces albulus in 1977,[6] and has been widely used as an FDA-approved food preservative because of its antimicrobial property.[7] Although $\varepsilon$-PL is active against multiple types of bacteria, its activity against fungi is very mild and far from the requirement of an antifungal agent for therapeutic application. Cationic peptides are known to exert antimicrobial activity via initial Coulombic interaction with the negatively charged microbial cell membrane.[8] The outer membrane of fungi has lower density of negatively charged lipid than does the outer membrane of bacteria, which causes cationic peptides to have weaker interactions with fungal membranes, and therefore, weaker activities against fungi than bacteria.[9]
This explains finding of promising cationic antibacterial peptides and peptide mimics, but very few promising cationic antifungal agents. These inspired us to hypothesize that modifying the structure of e-PL by increasing the charge density (the charge density per molecular weight of the repeating unit) along the polymer could enhance the interaction between cationic peptides and fungal membrane to enable the finding of potent antifungal agents.

By reducing the carbon number on the backbone of e-PL from six to three, we design β-peptide polymer poly(DL-diaminopropionic acid) (PDAP) that has substantially increased charge density than e-PL (Figure 1a). PDAP can be easily synthesized via the moisture tolerant polymerization of β-amino acid N-thiocarboxyanhydrides (β-NTA) in our recent report. Our study indicates that optimal PDAP (a 20 mer PDAP, PDAP₂₀) is a promising antifungal agent by demonstrating potent antifungal activity against clinically isolated Candida albicans and Cryptococcus neoformans, negligible hemolysis and cytotoxicity, insusceptible to antifungal resistance, and promising therapeutic potential in vivo (Figure 1b,c).

2. PDAPs Display Potent and Selective Antifungal Activity

The β-peptide polymer PDAPs were synthesized using the water insensitive β-NTA ring-opening polymerization method, followed by deprotection under acidic condition to give the final PDAPs (Figure 2a). To explore the antifungal activity and toxicity of PDAPs, we synthesized PDAPs with five different lengths (DP = 5, 10, 20, 37, 73) and with a narrow dispersity (D) of 1.13–1.17, as characterized by proton nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) (Figure 2b,c).
Figure 2. Synthesis, characterization, and activity of PDAP. a) Synthetic scheme of PDAP. b) $^1$H NMR spectra of PDAPs at five different lengths in D$_2$O, 400 MHz. c) Summary of PDAP characterization. $^*$Average degree of polymerization (DP) and Mn were characterized by $^1$H NMR. $^k$Dispersity, characterized by GPC. Cbz-protected PDAP at variable length using DMF as the mobile phase at a flow rate of 1 mL min$^{-1}$. $^c$Not determined, the polymer cannot dissolve thoroughly to conduct accurate GPC characterization. d) Minimum inhibitory concentration (MIC) of PDAP and $\epsilon$-PL against three strains of C. albicans. The molecular weight of purchased $\epsilon$-PL is $M_w = 3.5$–$4.6$ kDa, which is similar to that of PDAP$^{20}$ ($M_w = 4.1$ kDa, $\bar{D} = 1.14$).

In the initial antifungal activity test, we evaluated PDAPs with variable chain length against C. albicans, the most common human fungal pathogen, using the minimum inhibitory concentration (MIC). All five PDAPs displayed potent antifungal activities against three strains of C. albicans with MIC in a range of 0.4–3.1 $\mu$g mL$^{-1}$; in sharp contrast, $\epsilon$-PL ($M_w = 3.5$–$4.6$ kDa) showed only mild activity against C. albicans, with MIC at 200–400 $\mu$g mL$^{-1}$ (Figure 2d). This result was consistent with the generally low antifungal activity of $\epsilon$-PL and supported our rational design of PDAPs as potent antifungal agents, which encouraged us to further explore PDAPs as new antifungal agents.

We tested PDAPs for their activities against eight strains of C. albicans and three strains of C. neoformans, using antifungal drugs amphotericin B (AmpB) and fluconazole for comparison. Among these seven fungi strains (K1, Gu5, SC5314, R01, R02, R03, R04) are clinically isolated pathogens. We found that all PDAPs showed potent antifungal activities against these fungi, and the activities increased with the increase of polymer length till reaching a plateau at 20 mer (Figure 3a). PDAP$_{20}$ (DP = 20) displayed MIC at 0.1–0.8 $\mu$g mL$^{-1}$ against all eight strains of C. albicans and C. neoformans, even superior to AmpB with MIC at 1.2–3.1 $\mu$g mL$^{-1}$. MIC$_{50}$ was used for fluconazole because it cannot inhibit 100% growth of C. albicans, therefore, a 50% inhibition of fungal growth has been widely used for fluconazole.$^{[11]}$ Notably, all strains of C. albicans still grew even at a high concentration of fluconazole (MIC $> 200$ $\mu$g mL$^{-1}$). Moreover, PDAPs were not only fungistatic but also fungicidal, with the minimum fungicidal concentration (MFC) at 0.2–1.6 $\mu$g mL$^{-1}$ against all eight strains of C. albicans and C. neoformans.

The hemolysis of PDAPs upon human red blood cells was evaluated using the minimum concentration to cause 10% hemolysis (HC$_{10}$). PDAP$_{5}$ showed HC$_{10}$ at 1000 $\mu$g mL$^{-1}$ and other PDAPs showed negligible hemolysis up to 2000 $\mu$g mL$^{-1}$, with HC$_{10}$ greater than 2000 $\mu$g mL$^{-1}$ (Figure 3a; Figure S1a, Supporting Information); whereas, AmpB is hemolytic with HC$_{10}$ at 3.1 $\mu$g mL$^{-1}$ (Figure 3a; Figure S1b, Supporting Information). We also evaluated the cytotoxicity of PDAPs upon various mammalian cells including NIH 3T3 fibroblast cells, endothelial cells (HUVEC), macrophage (RAW264.7), liver cells (LO2), kidney cells (COS7) and enterocyte (NCM-460), using the concentration to cause 50% cell death (IC$_{50}$). PDAP$_{5}$ showed IC$_{50}$ at 340–800 $\mu$g mL$^{-1}$ and other PDAPs showed low cytotoxicity up to 800 $\mu$g mL$^{-1}$, except PDAP$_{10}$ against HUVEC (IC$_{50}$ = 675 $\mu$g mL$^{-1}$) (Figure 3a; Figure S2, Supporting Information); whereas, AmpB is
Figure 3. PDAP show potent antifungal activity, low toxicity, and no susceptibility to antifungal resistance. a) Biological activity of PDAP and antifungal agents, including MIC, minimum fungicidal concentration (MFC), 10% hemolysis (HC10), 50% inhibitory concentration for cell viability (IC50), and water solubility. *MIC50 was used for fluconazole because it cannot inhibit 100% growth of *C. albicans*. b,c) Selectivity index was calculated by the ratio of b) HC10 to MIC (*C. albicans* K1 strain) and c) by the ratio of IC50 to MIC (*C. albicans* K1 strain). d) Drug resistant assay of PDAP and fluconazole against *C. albicans* (R02 strain) using continuous treatment of *C. albicans* cells with 0.5 × MIC of PDAP and 0.5 × MIC50 of fluconazole.

cytotoxic, with IC50 at 1.6 μg mL⁻¹ for fibroblast cells and 8.8–128 μg mL⁻¹ for others (Figure 3a; Figure S2, Supporting Information). All PDAPs are well soluble in water at over 200 mg mL⁻¹ compared to the poorly soluble AmpB at <0.001 mg mL⁻¹ and moderate soluble fluconazole at ≈1 mg mL⁻¹. Among all PDAPs with variable length (DP = 5, 10, 20, 37, 73), PDAP20 (DP = 20) is the minimum length to show potent antifungal activities and low toxicity, therefore, was further explored for its biological performance and therapeutic potential. The antifungal selectivity indexes, HC10/MIC and IC50/MIC, were calculated for PDAP20 as higher than 2500 and 1000, respectively, in sharp contrast to that of AmpB at 2 and 1, respectively (Figure 3b,c). These results indicated that PDAP20 has superior fungi versus mammalian cell selectivity, superior to AmpB.

In the complex physiological environment, the positively charged salt could compete with antimicrobial peptides by binding to the membrane to antagonize antimicrobial activity.[14] To examine this effect in our study, we measured the MIC values of PDAP20 under the physiological salt concentrations containing 150 × 10⁻³ m Na⁺ and 5.4 × 10⁻³ m K⁺. The results showed that PDAP20 retained potent antifungal activity (MIC = 1.6 μg mL⁻¹) under the physiological salt concentrations, only slightly attenuated compared to standard MIC test in RPMI 1640 (MIC = 0.8 μg mL⁻¹) (Table S1, Supporting Information).
increase of Na⁺ concentration from 150×10⁻³ to 300×10⁻³ m resulted in gradually attenuated but still potent antifungal activity of PDAP₂₀ (MIC = 1.6–6.3 μg mL⁻¹); whereas, the MIC value of e-PL changed from 400 to >1600 μg mL⁻¹ with the increase of Na⁺ concentration to 200×10⁻³ m (Table S1, Supporting Information). Increasing of K⁺ concentration up to 20×10⁻³ m didn’t affect the antifungal activity of either PDAP₂₀ or e-PL. These results showed that PDAP₂₀ has excellent tolerance to physiological and environmental monovalent free ions. We also performed the stability test on PDAP₂₀, at conditions of salt ions, acid and base using ¹H NMR. After PDAP₂₀ was treated with saturated NaCl, 0.1 x HCl (for pH = 1 condition) and 0.1 m NaOH (for pH = 14 condition), no significant decomposition was found by using ¹H NMR characterization (Figure S3, Supporting Information).

Resistance to antifungal drugs has been characterized in most fungal species that infect humans, which are emerging as an important clinical problem, especially azole-resistance.[13] We found that a clinically isolated C. albicans R02 strain started to adopt drug resistance after five passages of fluconazole treatment, and the MICₐ₀ value of fluconazole increased eightfold after 30 passages, from 0.2 to 1.6 μg mL⁻¹. We examined the possible antifungal resistance of PDAP₂₀ and found that fungi didn’t acquire resistance even after fungal cells were treated with PDAP₂₀ continuously over 30 passages (Figure 3d).

To figure out whether the increased charge density affect the selectivity over other microorganisms, we tested the antimicrobial activity of PDAP₂₀ against representative Gram-positive bacteria (S. aureus, S. epidermidis, B. subtilis) and Gram-negative bacteria (E. coli, A. baumannii, K. pneumoniae), comparing with e-PL. The results showed that PDAP₂₀ has low antibacterial activities to all above bacteria, with MIC values greater than 50 μg mL⁻¹ (Table S2, Supporting Information). In contrast, e-PL showed higher antibacterial activities, with MIC values in the range of 1.6–6.3 μg mL⁻¹. These results demonstrated that PDAP₂₀ has high antifungal selectivity, while e-PL has high antibacterial selectivity, indicating that polymers with high charge density may be more suitable for antifungal agents.

3. Antifungal Mechanism of PDAP

The insusceptibility of PDAP₂₀ to antifungal resistance encouraged us to explore its antifungal mechanism. We chose four folds the MIC (4×MIC) for all antifungal mechanism tests to ensure observation of the fungal killing. Different from the standard MIC test results, the MIC of the PDAP₂₀ at a high fungal cell concentration of 1.0×10⁶ CFU mL⁻¹ (used for transmission electron microscope assay) and 3.0×10⁶ CFU mL⁻¹ (used for other mechanism experiments) are 50 and 12.5 μg mL⁻¹, respectively. For these antifungal mechanism experiments, a high concentration of fungal cells were used to facilitate the sample preparation or microscopic observation. After incubation with PDAP₂₀, both C. albicans and C. neoformans showed cell lysis and large empty space in the cytosol, as well as disorganization of cytoplasm in transmission electron microscope (TEM) characterization (Figure 4a,b; Figure S4, Supporting Information). This observation is echoed by the scanning electron microscope (SEM) characterization that showed severe membrane deformation and invagination after C. albicans and C. neoformans were treated with PDAP₂₀ (Figure 4c,d).

We did the time-kill assay using 2×MFC of polymer concentration corresponding to relevant fungal cell concentration. At a fungal cell concentration of 1250 CFU mL⁻¹ (for standard MIC/MFC test), 2×MFC is 3.1 μg mL⁻¹. At the fungal cell concentration of 3.0×10⁶ CFU mL⁻¹ (used for mechanism studies), 2×MFC is 50 μg mL⁻¹. The time-kill kinetics of PDAP₂₀ against C. albicans showed that PDAP₂₀ usually takes several hours to completely kill the fungi, and the time-kill kinetics is faster at the high fungal cell concentration condition than that at the low fungal cell concentration condition (Figure S5, Supporting Information). It’s worth mentioning that at the high fungal cell concentration condition, a higher concentration of the polymer PDAP₂₀ was also used relative to that at the low fungal cell concentration condition and, therefore, reasonable to have a faster fungal cell kill kinetics. The killing efficacy is obviously dependent on the polymer concentration.

In addition, we monitored the fungicidal process of PDAP₂₀ upon C. albicans by laser scanning confocal microscope, using 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) conjugated PDAP₂₀ to track polymer in the blue channel, and propidium iodide (PI) to detect the integrity of cell membrane in the red channel.[16] The tracking started immediately after the antifungal polymer and PI were incubated with C. albicans cells (0 min), and images were collected every 10 min. We observed the antifungal polymer enriched on the cell membrane and formed a distinct blue ring within 10 min (Figure 4e). This phenomenon is consistent to our design of PDAPs that have higher density of positive charge than does e-PL, and are expected to have increased electrostatic interaction with fungal cell membrane. After 30–40 min, the antifungal polymer entered fungal cells but accompanied with little PI signal inside the cells. Starting from 50 to 60 min, obvious PI uptake was observed, indicating damage of cell membrane integrity by PDAP₂₀. These observations indicate that PDAP₂₀ enriched on fungal cell membrane first and then entered fungal cells without damaging the membrane. Notably, fungal cell membrane damage happened after PDAP₂₀ was uptaken into fungal cytoplasm.

We further explored how PDAP₂₀ undergo transmembrane into fungal cells and whether this process was energy dependent. The antifungal efficacy of PDAP₂₀ was tested at a concentration of 2×MFC in the presence of 5×10⁻³ m NaN₃ or at 4°C to keep fungal cells at an energy depletion condition.[17] The results showed about 88% of fungal cells were still killed under both conditions (Figure 5a,b), indicating that transmembrane and uptake of PDAP₂₀ can occur in an energy-independent pathway. Using confocal fluorescence microscopy to monitor the interaction between CPM-conjugated PDAP₂₀ and C. albicans, we observed that the antifungal polymer uptake by fungal cells happened even in the presence of NaN₃ (Figure 5c), which echoed energy-independent transmembrane and uptake of PDAP₂₀ as observed in above antifungal efficacy study. Precedent studies on the mechanism of cell-penetrating peptides (CPPs) provided several mechanisms for the transmembrane of CPPs,[18] As the energy independent route, the transmembrane mechanism of PDAP might follow the “inverted micelle model” or the “toroidal pore formation model”.

We further did flow cytometry experiment to investigate the antifungal mechanism using fluorescent polymer (CPM-PDAP₂₀) in the presence or absence of NaN₃. The results showed that a large proportion of fungal cells were killed in the absence of
Figure 4. Characterization of fungal cells before and after PDAP_{20} treatment. a,b) TEM micrograph of a cross-section of a) \textit{C. albicans} K1 strain and b) \textit{C. neoformans} H99 strain before and after PDAP_{20} Treatment. c,d) SEM images of c) \textit{C. albicans} K1 strain and d) \textit{C. neoformans} H99 strain before and after PDAP_{20} Treatment. e) Fluorescence confocal images demonstrate the fungicidal process. Blue and red fluorescence represent CPM-PDAP_{20} enrichment and propidium iodide (PI) uptake, respectively.
Figure 5. PDAP shows cell penetration, ROS generation and apoptosis of fungal cells. a, b) Killing efficiency assay under energy depletion state using a) $5 \times 10^{-3} \text{M}$ sodium azide or b) standing at $4^\circ \text{C}$ environment. $n = 3$, mean values $\pm$ s.d. Statistical analysis: two-tailed t-test, *$p < 0.01$, ***$p < 0.0001$. c) Colocalization of CPM-PDAP$_{20}$ (blue) and PI (red) fluorescence within C. albicans cells in the absence or presence of sodium azide. d) Flow cytometry analysis counting fluorescent fungal cells after cell incubation with $50 \mu \text{g mL}^{-1}$ CPM-PDAP$_{20}$ for 1 and 2 h in the presence or absence of NaN$_3$. A final concentration of PI at $10 \mu \text{g mL}^{-1}$ was used for two color flow cytometry. e) ROS generation over time measured by DCFH fluorescence under different PDAP$_{20}$ concentration with or without the ROS scavenger NAC. f) Colocalization of PDAP$_{20}$ and DCF fluorescence (green, generated by ROS). g) Effects of ROS scavenger NAC on the inhibition and killing of C. albicans by PDAP$_{20}$. h) Mitochondrial membrane potential detected with the fluorescent dye JC-1, which exists as aggregates (red) in normal cells and as monomers (green) in apoptotic cells. i) DNA fragmentation was visualized by green fluorescence using TUNEL staining. j) Schematic diagram of proposed fungicidal mechanism for PDAP$_{20}$. 
Figure 6. PDAP resists biofilm formation and eradicates mature biofilms. a, b) Activity of PDAP20 against a) C. albicans biofilm formation and b) mature biofilm. Embed images in (a) and (b) corresponding to biofilms that were treated with gradient concentration of PDAP20 and then were stained with MTT and dissolved in DMSO. c) Live/Dead fluorescence micrographs and d) SEM images of C. albicans mature biofilm treated with PDAP20 at concentrations of SMIC80. Untreated biofilms were used as controls.

NaN₃ (85.7% and 93.2% of total fungal cells have uptake of polymer, and 83.5% and 91.9% of total fungal cells have uptake of PI after incubation for 1 and 2 h, respectively) (Figure 5d). It is note that the polymer can still be uptaken by the fungal cells in the presence of NaN₃ (non-energy dependent conditions), though the transmembrane efficiency and fungicidal activity were weakened (only 18.3% and 49.0% uptake of polymer, and 24.7% and 53.5% uptake of PI after incubation for 1 and 2 h, respectively) (Figure 5d). These results indicated that in an energy-depletion condition, PDAP20 can still cross the fungal membrane and get into fungal cells to kill fungi but with a reduced killing kinetics; whereas, in an energy dependent condition the transmembrane and uptake of PDAP20 improved significantly to have a faster killing of fungal cells. This means both the energy dependent and energy independent mechanism exist in this system.

To figure out what happened after PDAP uptake into fungal cells and what is the primary reason for fungal membrane damage, we turned our attention to intracellular reactive oxygen species (ROS) that are usually associated with the fungicidal process and cause lipid peroxidation, membrane damage of fungal cells and apoptosis.[19] Using 2,7-dichlorofluorescein (DCF) as the ROS indicator,[20] we found ROS production and a gradual increase of ROS level over time within fungal cells after PDAP20 treatment (Figure 5e). In addition, the intracellular ROS level of PDAP20-treated fungal cells was higher than that of the hydrogen peroxide-treated fungal cells. Notably, the intracellular ROS level was dependent on PDAP20 concentration and increased incrementally with the increase of PDAP20 concentration. The intracellular ROS accumulation was further visualized and confirmed by fluorescence confocal microscopy (Figure 5f). To figure out whether ROS production is the primary reason for fungal membrane damage by PDAP20, we examined the antifungal activity of PDAP20 in the presence of 20 × 10⁻³ m NAC (antioxidant, as a ROS quencher) without affecting the growth of fungal cells (Figure 5g). We observed a remarkably reduced antifungal activity with MIC and MFC values decreased, respectively, from 0.8 to
100 μg mL⁻¹ and from 1.6 to 400 μg mL⁻¹, which indicated that intracellular ROS generation plays a significant role in the fungicidal activity of PDAP₂₀.

ROS have been regarded as primary cell death regulators and are connected to many crucial steps of the apoptotic pathway in yeast. Loss of mitochondrial membrane potential represents the early stage of apoptosis pathway, which can be detected by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidacarbocyanine iodide (JC-1) stain showing green fluorescence. Our JC-1 stain study showed that normal cells have cytosol red fluorescence; whereas, PDAP₂₀-treated fungal cells have cytosol green fluorescence, which indicates the early stage of apoptosis (Figure 5h). In addition, we analyzed DNA fragmentation of PDAP₂₀-treated fungal cells using a TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, as a late apoptosis marker. The strong green cytosol fluorescence in PDAP₂₀-treated fungal cells indicated a late stage of apoptosis (Figure 5i). These fluorescence images and aforementioned electron microscope characterization altogether imply an antifungal mechanism of the highly positively charged PDAP₂₀ that the polymer enriches onto the
negatively charged fungal membrane first and then penetrates into fungal cells, resulting in cytosol ROS production, cell apoptosis and cell death (Figure 5j).

4. PDAP Effectively Inhibits the Formation of Fungal Biofilms and Eradicates the Mature Biofilms

Fungal biofilms are frequently encountered in clinical infections and are considered as a formidable challenge to have even over 1000-folds antifungal resistance.[23] The mechanism studies on PDAP₂₀ revealed that PDAP can cross the membrane to enter fungal cells and induce apoptosis of fungi. PDAPs were not only fungistatic but also fungicidal, and didn’t induce fungi to develop antifungal resistance. Therefore, we speculated that PDAP can still be active against mature fungal biofilms. We found that PDAP₂₀ resists the formation of C. albicans biofilm at 6.3 μg mL⁻¹ (Figure 6a), and can even effectively eradicating mature C. albicans biofilms at 50 μg mL⁻¹ (Figure 6b). Live/Dead staining indicated that PDAP₂₀ effectively kill fungal cells within mature C. albicans biofilms (Figure 6c). SEM images showed that the untreated biofilm consisted of both oval planktonic and long tubular hyphae, while PDAP₂₀ treated biofilm was destroyed (Figure 6d). The effective anti-biofilm properties imply that PDAP₂₀ is promising for clinical application.

5. In Vivo Efficacy of PDAP

Toxicity of PDAP₂₀ was evaluated by an intravenous (IV) injection of PDAP₂₀ in ICR mice, using AmpB for comparison. After IV injection of a signal dose of 3 mg kg⁻¹ of AmpB, 90% of the mice died within 48 h; in sharp contrast, all mice survived after IV injection of 100 mg kg⁻¹ of PDAP₂₀ without obvious change of mice (Figure 7a), including a normal body weight compared to the saline group (Figure 7b). The main metabolic organs including kidney, liver, and spleen of the mice were sectioned and stained by H&E after IV injection of PDAP₂₀ for 14 days. All these analyses showed that IV injection of PDAP₂₀ resulted in no obvious toxicity on major organs (Figure 7c). Additional study on blood biochemistry showed that PDAP₂₀ did not cause significant changes in blood biochemical indicators including concentration of K⁺, Na⁺, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CREA) (Figure 7d), indicating the low hepatotoxicity and nephrotoxicity of PDAP₂₀. All these studies showed that PDAP₂₀ has low toxicity and is safe for in vivo application in treating fungal infections.

Encouraged by the potent antifungal activity in vitro and low toxicity of PDAP₂₀, we continued to examine the in vivo fungicidal activity and therapeutic potential of PDAP₂₀ using a biofilm-induced fungal keratitis model (Figure 7e). The eyeballs of male BALB/c mice were infected with C. albicans to have keratitis, eye ulcers were clearly observed with a dense opaque appearance. These keratitis mice were randomly grouped and treated with eye drops containing saline, AmpB and PDAP₂₀, respectively. Saline-treated mice had serious ulcers on the eyeballs, and a large amount of planktonic and filamentous fungi in the periodic acid-schiff (PAS) stained tissue slides corresponding to 5.4 log CFU per eye (Figure 7f–h). Both AmpB and PDAP₂₀ treatment remarkably alleviated eye ulcers, and significantly reduced the number of colonies by 1.8 and 1.2 log CFU per eye, respectively (Figure 7f–h). In addition, we did histological analysis on mouse corneas after PDAP₂₀ treatment at above administration concentration and found no obvious toxicity (Figure 7i). Therefore, PDAP₂₀ is a safe and effective antifungal agent in treating fungal keratitis.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

R.L. directed the whole project. D.Z. and R.L. conceived the idea, proposed the strategy, designed the experiments, evaluated the data, and wrote the manuscript together. D.Z. performed majority of the experiments. C.S. participated in vitro and in vivo antifungal experiments. Z.C. performed the keratitis experiment. Q.C. conducted the confocal experiments and cytotoxicity assay, and drew schematic diagrams. Y.B. and J.Z. participated in the in vitro antifungal experiments. K.M., S.L., X.X., and S.C. participated in the drug resistance studies. J.G. participated in the cytotoxicity assay, and drew schematic diagrams. Y.B. and J.Z. participated in the flow cytometry experiment. M.Z. participated in the polymer synthesis. X.S. and Y.D. isolated the fungi. L.Z. participated in result discussion and troubleshooting. All authors discussed the results and contributed to the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

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