Supporting Information

Multi-arm ε-polylysines exhibit broad-spectrum antifungal activities against *Candida species*

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Experimental section

Materials: Liner oligo(ε-lysine)s (ε-OLs): K_3 (K^{ε}-K^{ε}-K^{ε}, 407 g/mol), K_4 (K^{ε}-K^{ε}-K^{ε}-K^{ε}, 528 g/mol), K_5 (K^{ε}-K^{ε}-K^{ε}-K^{ε}-K^{ε}, 660 g/mol), and K_7 (K^{ε}-K^{ε}-K^{ε}-K^{ε}- K^{ε}- K^{ε}- K^{ε}, 916 g/mol) were purchased from Apeptide Co., Ltd. (Shanghai, China). ε-PL (~ 4000 g/mol) was purchased from Zhejiang Silver-Elephant Bio-engineering Co., Ltd. Mouse IL-6 ELISA Kit and Rat TNF-α ELISA Kit were purchased from LMAI Bio Co., Ltd. DNA damage assay kit by γ-H2AX immunofluorescence was purchased from Beyotime.

Animals: All mice (approximately 6 weeks, 20 ± 2 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd (NO. SCXK (Liao) 2022---0001). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care and Use Committee of Jilin University (the assigned approval/accreditation number: SYXK (Ji) 2022-0018).

Fungal strains: *C. albicans* ATCC 10231, *C. tropicalis* ATCC 750, *C. krusei* ATCC 6258, *C. glabrata* ATCC 15126, *C. parapsilosis* AS 2.590, *A. niger* ATCC 16404, *A. oryzae* AS 3.802 and *Mucorales (Mucor racemosus* AS 3.2729) were obtained from Shanghai Luwei Technology Co., Ltd. Multidrug resistant *C. albicans* FCA 001 was donated by Bethune First Hospital of Jilin University, isolated from ICU.

Characterization: CD: Each polymer was dissolved in PBS buffer at a final concentration of 0.5 mg/mL, and the solution was filtered through a 0.22 μm filter. The CD spectrum of each polymer was collected at 37 ℃ with the wavelengths ranging from 180 nm to 280 nm on J-815 circular dichroism spectroscopy (JACS, Easton, MD, USA). All samples were repeated 3 cycles. ^{1}H NMR spectra were recorded on a Bruker AV300 NMR spectrometer in D_2O. The GPC curves of ε-mPLs and ε-PL were determined by gel permeation chromatography (GPC) equipped with a Waters Ultrahydrogel column and a Waters 1515 isocratic HPLC pump with a Waters 2414 refractive index detector. 0.2 M acetic acid/0.1 M sodium acetate buffer was used as the eluent at a flow rate of 1 mL/min at 35°C. The pH value of sample solution was determined by pH meter (Mettler Toledo, FE28). All tests are repeated three times and averaged. Purification of ε-mPLs was performed on an FPLC (GE healthcare, AKTA explorer 100) with a gel column (1.5 cm × 30 cm) and the UP water with 0.1% acetic acid as the mobile phase at the flow rate of 1 mL/min. The exact mass of ε-mPLs were tested by a HR-ESI-MS (Thermo Scientific™ Q Exactive™). The morphologies of the microorganisms were characterized by field emission scanning electron microscopy (FE-SEM, Zeiss, Merlin) at an accelerating voltage of 1 kV. Fluorescence imaging was performed on a confocal laser scanning microscope (CLSM, Nikon C2plus). The optical density at 600 nm (OD_{600}), 570 nm (OD_{570}), and 260 nm (OD_{260}) were tested by a multifunctional microplate scanner (TECAN).

Synthesis of the 4-arm core (4R): A 4-arm core was synthesized according to a previously reported method with slight modification[1, 2]. A 100 mL round-bottom flask with a magnetic was dried under a nitrogen atmosphere, methyl L-lysinate dihydrochloride (Lys-OMe·2HCl, 4 mmol, 0.932 g), (R)-2, 6-bis((tert-butoxycarbonyl) amino) hexanoic acid (BOC_2Lys-OH, 8.5 mmol, 2.944 g), 4-dimethylaminopyridine (DMAP, 8.5 mmol, 0.549 g), 1-Hydroxybenzotriazole (HOBt, 8.5 mmol, 0.608 g), O-benzotriazole-*N*, *N*, *N*', *N*'-tetramethyl-uronium-hexafluorophosphate (HBTU, 8.5 mmol, 1.896 g) and 20 mL ultradry DMF were added in sequence. The mixture was stirred at room temperature in a glove box for 24 hours, and then the solvent was removed under high vacuum

to obtain a yellow solid. The solid was dissolved in 2 mL ethanol and then the solution was precipitated in 100 mL aqueous $NaHSO_4$ (0.05 M, pH = 2.5) at 4 ℃, followed by filtration to obtain a white powder. The precipitate was washed with Ultra-pure water (UP water) twice to give the *N*-BOC protected intermediate product 2.8 g. The intermediate product was dissolved in TFA at a concentration of 5 mg/mL and the solution was stirred at room temperature for 2 h, precipitated into 50 times excess cold diethyl ether, centrifuged and dissolved with a little UP water, and dialyzed with100 D dialysis bags to obtain the final product, 4-arm core (4R).

Synthesis of 3R and 6R: N^{α}-BOC-L-lysine methyl ester hydrochloride (2 mmol, 0.593 g), BOC_2Lys-OH (2.5 mmol, 0.866 g), HOBt (2.5 mmol, 0.338 g), DMAP (2.5 mmol, 0.305 g), HBTU (2.5 mmol, 0.948 g) and 20 mL ultradry DMF were added to a 50 mL round-bottom flask. The mixture was stirred at room temperature in the glove box overnight. The deprotection and purification procedures were same as that for 4R to give the final product 3R (3-arm core, yellow or white). 6R was synthesized by coupling 3R with BOC_2Lys-OH.

Synthesis of G4: 4R (0.6 mmol, 0.2781 g), BOC_2Lys-OH (3 mmol, 1.0392 g), HOBt (3 mmol, 0.4 g), HBTU (3 mmol, 1.137 g) and DMAP (3 mmol, 0.366 g) were added into 20 mL of ultra-dry DMF in a 100 mL round-bottom flask. The mixture was stirred at room temperature in a glove box for 24 hours, and then the solvent was removed under high vacuum to obtain a yellow solid (0.21 g). The *N*-BOC group was deprotected with TFA, and dialyzed with a 100 Dalton dialysis bag to obtain 3rd generation dendritic polylysine G3. The 4^{th} generation dendritic polylysine (G4) was repeated in the same procedure, and by FPLC.

Synthesis of BOC protected ε-oligolysine (K_mBOC_{m+1}): A representative synthesis procedure for K_3BOC_4 was detailed. K_3 (0.5 mmol, 200 mg) was dissolved in 20 mL of UP water, and excess Et_3N (0.2 mL) was added. A solution of 20 mL di-tert-butyl decarbonate (3 mmol, 0.655 g) in 1,4-dioxane was added dropwisely to the above system over approximately 1 h. The mixture was stirred at room temperature overnight, and then extracted with ether (10 mL). The aqueous phase was adjusted to pH 4 using 10% citric acid solution, and then extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ phase was combined and washed with saturated $NaHCO_3$, water, and brine. The organic phase was dried with anhydrous Na_2SO_4 and $CHCl_3$ was removed under vacuum to obtain the final product K_3BOC_4 (white solid, 0.27 g).

Synthesis and purification of ε-mPL: A representative synthesis procedure for 4R-K3 was detailed. K_3BOC_4 (0.29 g, 0.243 mmol), HOBt (0.048 g, 0.358 mmol), DMAP (0.0437 g, 0.358 mmol), 4R (0.025 g,0.06 mmol) and HBTU (0.135 g, 0.358 mmol) were added into 30 mL of ultra-dry DMF in the glove box, and the mixture was stirred at room temperature for 24 hours. After removal of DMF under high vacuum, 3 mL of ethanol was added to dissolve the solid and the mixture was slowly added into 100 mL of cold 5% citrate solution. The precipitate was washed twice with water, and freeze dried to obtain 0.26 g of crude *N*-BOC protected 4R-K3. Deprotection of the *N*-BOC group was performed in TFA (3 mL) at room temperature, and the mixture was precipitated into 50 mL of cold diethyl ether to yield crude 4R-K3 (0.13 g). Purification was performed on an FPLC (GE healthcare, AKTA explorer 100) with a Sephadex G15 gel column (1.5 cm × 30 cm) and the UP water with 0.1% acetic acid as the mobile phase at the flow rate of 1 mL/min to give 4R-K3 (0.08 g, purity >95%). Other ε-mPLs were synthesized using the same procedure and purified on different columns, e.g. Sephadex G15 for 3R-K3, 3R-K4, and G4, Sephadex G20 for 3R-K5, 4R-K4, 4R-K5 and 6R-K3, and Sephadex G25 for 3R-K7, 4R-K7, 6R-K4 and 6R-K5, with a purity of >95%. **ESI-MS: 3R-K3** (found 286.41759 (z=5), 357.76984 (z=4);

calculated: 1427.073); **3R-K4** (found 913.68205 (z=2), 609.4548 (z=3), calculated: 1825.3641); **3R-K5** (found: 733.21571 (z=3), 1099.32043 (z=2); calculated: 2195.624); **3R-K7** (found: 371.65764 (z=8), 424.75055 (z=7), 495.20737(z=6); calculated: 2964.211); **4R-K3** (found: 391.89785 (z=5), 489.62051 (z=4); calculated: 1954.4413); **4R-K4** (found: 353.41061 (z=7), 412.14429 (z=6), 494.37151 (z=5); calculated: 2465.8217); **4R-K5** (found: 371.6576 (z=8), 424.7505 (z=7), 602.8395 (z=5), 753.2976 (z=4); calculated: 2964.197); **4R-K7** (found: 363.82069 (z=11), 400.10202 (z=10), 499.87526 (z=8), calculated: 3988.9460); **6R-K3** (found: 371.65764 (z=8), 602.83953 (z=5), 753.29769 (z=753.29769); calculated: 2964.1831); **6R-K4** (found: 415.75344 (z=9), 467.61145 (z=8), 632.12983 (z=6); calculated: 3722.781); **6R-K5** (found: 451.44017 (z=10), 501.37714 (z=9), 563.92367 (z=8), calculated: 4501.320); **G4** (found: 391.89578 (z=5), 489.62081 (z=4). calculated: 1954.482).

Synthesis of FITC-3R-K7: 3R-K7 (100 mg) and FITC (3',6'-dihydroxy-5-isothiocyanato-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one, 2.2 mg) was dissolved in 10 mL of DMSO, and the mixture was stirred in dark at room temperature for 20 hours. After removal of DMSO under high vacuum, 1 mL of UP water was added to dissolve the solid and purification was performed on an FPLC described above to give FITC-3R-K7 (45 mg, 40%). The solid was stored in cool and dark condition.

Anti-Candida susceptibility test: The anti-*Candida* susceptibility assay was conducted using the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) constitution of M27-A8. The RPMI-MOPS buffer (Sterile RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) and 3-(*N*-morpholino) propanesulfonic acid (MOPS, final concentration 0.165 mol/L for pH 7.0)) was used to prepare the stock solution of each polymer with a concentration of 6 mg/mL, and then the stock solution was sequentially diluted by 2-fold. The spores were cultured in sabouraud dextrose broth (SDB) by incubating at 35 ℃ for 24 h, and centrifuged at 5000 rpm for 6 min. The cells were washed with PBS 3 times and then diluted in the RPMI-MOPS buffer to ~10^3 CFU/mL as the working suspension. 25 μL of each dilution of the polymer was added to the 96-well microtiter plate, followed by addition of 175 μL of the inoculum to give a final inoculum of ~10^3 CFU/mL. The plate was incubated for 48 h at 35 °C. The MIC value was estimated by measuring the OD_{600} value with a microplate reader (TECAN). As the controls, the concentration ranges of 0.0313 to 16 μg/mL for amphotericin B and 0.125 to 64 μg/mL for fluconazole were used based on previous studies.

Antimould test: The anti-mold susceptibility was determined using the broth microdilution method according to the CLSI guideline M38-A2. A twofold dilution series of each polymer in the sterile medium was made according to the method described above. Molds were cultured on Potato Dextrose Agar (PDA) plates by incubating at [28 ℃, humidity > 90%] for ~14 days. PBS containing 0.5% (v/v) Tween 20 was used to wash the agar plate, and then 8-layer gauze filtration was used to obtain a spore suspension. The spore suspension was centrifuged, and followed by dilution with RPMI-MOPS buffer to afford the working suspension with a concentration of 4 × 10^4 CFU/mL (counted by blood cell counting board). 25 μL of the dilutions of each polymer was added to the 96-well microtiter plate, followed by addition of 175 μL of the inoculum to give a final inoculum of ~10^4 CFU/mL. The plates were incubated for 72 h at 35 °C. Fungal growth was examined by visual observation.

In vitro cell toxicity test: NIH 3T3 cells were cultured into the 96-well plate, and the final cell density was ~8000 cell of each well. After 12-18 h of incubation, the culture medium was removed

carefully, and 175 μL RPMI 1640 medium and 25 μL polymer solution were added sequentially. The plate was placed into the incubator (37 ℃, 5% CO_2) for another 24 h. 20 μL (5 mg/mL) of the MTT solution was added to each well in a dark environment, and the culture was incubated for another 4 h. The solution in each well was removed and 200 μL of DMSO was added. The plate was placed in a shaking bed to dissolve the crystal by gently shaking. Inoculum without polymer was used as the positive control, while the RPMI 1640 medium was used as the blank. The absorbance at 570 nm was measured by a microplate reader. All assays were repeated three times.

$$\text{Cell viability} = \frac{ODsample - ODnegetive}{ODpositive - ODnegetive} \times 100\%$$

Membrane integrity study: Mid-log phase *C. albicans* (ATCC 10231) was centrifuged and washed with PBS three times, and then diluted with the RPMI-MOPS buffer to a spore density of 10^5 CFU/mL as the working suspension. 10 μL of 3R-K5 or 4R-K7 solution was added to 990 μL of the spore suspension with a final concentration of 1×, 2× or 4× MIC in a 1.5 mL microcentrifuge tube. Fluconazole and Amp. B at a concentration of 1× MIC and PBS were used as the control groups. The tube was incubated at 35 ℃ for 4 h, and the suspension was centrifuged at 5000 rpm for 6 min. 200 μL of the supernatant was transferred to a 96-well cell plate, and the absorbance at 260 nm (OD_{260}) was measured by a microplate reader. All assays were performed in triplicates.

The killing kinetics: *C. albicans* (ATCC 10231) was cultured in SDB medium for 20-22 h, washed with sterile PBS twice and diluted with RPMI-MOPS buffer to give the working suspension with a spore density of 1×10^4 CFU/mL. 200 μL of RPMI-MOPS buffer containing polymer (3R-K7, 4R-K5 and ε-PL) or fluconazole was added to 20 mL of the spore suspension in a 50 mL centrifuge tube to give a final concentration of 1×, 2× or 4× MIC and the tube was then incubated at 35 ℃. The spore suspension was diluted for plating on SD agar plates at various time points of treatment and the plates were incubated at 35 ℃ for 20 h for CFU counting. The test was independently repeated 3 times.

Antimicrobial resistance study: Antimicrobial resistance was determined according to a previously reported method.[3] *C. albicans* was cultured in SDB medium at 35 ℃ for 20 h, and the spores were diluted with SDB to a cell density of ~10^3 CFU/mL. The fungal suspensions were treated with 3R-K7, 4R-K5 or control antibiotics fluconazole at a final concentration of 0.125 × MIC respectively followed by incubation for 24 h under shaking (130 rpm) at 35 ℃. An aliquot of 1 mL of mixture was sampled and diluted to a cell density at ~10^3 CFU/mL for a new cell-drug incubation cycle. The above cycle was repeated every 24 h. The MIC values of polymers (3R-K7, and 4R-K5) and fluconazole against *C. albicans* ATCC 10231 were examined every 2 days. Based on the MIC values determined by the test, the concentration of polymers and fluconazole throughout the resistance study was adjusted to keep at 0.125× MIC and 0.125× MFC_{50} respectively. An increased MIC value means the development of drug resistance.

Characterization of C. albicans morphology: Mid-log phase *C. albicans* cells was centrifuged and washed with PBS three times, and then diluted in RPMI-MOPS buffer to 10^8 CFU/mL. 100 μL of 3R-K7 (240 μg/mL) or 4R-K5 (480 μg/mL) in PBS was added to 900 μL of the cell suspension to reach the concentration of 4× MIC, and PBS was used as the negative control. The mixture was incubated at 35 ℃ for 6 h. The spores were collected by centrifugation at 4000 rpm for 10 min, washed with PBS twice and fixed with 2.5% glutaraldehyde in PBS at 4 ℃ overnight. The spores were further washed with PBS and dehydrated with gradient ethanol solutions (30, 50, 70, 80, 90 and then 100% ethanol) and dropped onto the silicon wafer. The cell morphologies were imaged by

SEM.

Intracellular ROS level test: Log-phase *C. albicans* cells was washed and diluted with PBS to a cell density of ~10^8 CFU/mL. 0.5 mL of 50 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) in PBS was added to an equal volume of the fungal suspension. After being incubated for 30 min in dark, fungal cells were washed with PBS twice to remove DCFH-DA outside the cells and then diluted in PBS to a cell density of 10^6 CFU/mL as the working suspension. 10 μL of 3R-K7 solution was added to 90 μL of the working suspension in a black 96-cell well plate to give a final concentration of 1×, 2× or 4× MIC, and PBS was used as the control. The DCFH-DA fluorescence intensity was recorded continuously in a microplate reader (excitation λ (Ex) = 488 nm, emission λ (Em) = 530 nm). The test was run in triplicates and was independently repeated twice.

Hemolysis and Hemagglutination Assay: Fresh blood was collected from a healthy male New Zealand White rabbit. Red blood cells (RBCs) were collected by centrifugation at 1500 rpm for 10 min, washed three times in PBS buffer and diluted to the final concentration (2%, v/v). For the hemolysis assay, 900 μL of the 2% RBCs suspension was incubated with 100 μL of polymer solutions in two-fold serial dilution (ranging from 7500 to 30 μg/mL in PBS) in a 1.5 mL microcentrifuge tube for 1 h at 37 °C. After centrifugation at 3000 rpm for 5 min, an aliquot (150 μL) of the supernatant was carefully transferred to a new 96-well plate, and the absorbance at 570 nm was determined with a microplate reader (TECAN). PBS containing 0.8% Triton-100 was used as a positive control, while PBS was used as a negative control. The percentage of hemolysis was calculated using:

$$\text{Hemolysis} = \frac{Abs_{570}\ of\ sample - Abs_{570}\ of\ negative\ control}{Abs_{570}\ of\ positive\ control - Abs_{570}\ of\ negative\ control} \times 100\%$$

All experiments were repeated three times.

The hemagglutination assay was conducted according to previously reported method with slight modification[4]. Briefly, 25 μL of polymer solutions in PBS was added to a V-shaped microtiter plate, followed by addition of 75 μL 2% (v/v) RBCs suspensions. The plate was tapped carefully for 10 times on all four sides to facilitate mixing. The plate was incubated at 25 ℃ for 1 hour. The readout was performed in a tilted plate position (90° for 25 s), and nonagglutinated RBSs started to run down, forming a tear-like shape, which was clearly different from partial or complete agglutination.

In situ time-lapse fluorescence confocal imaging of C. albicans: Time-lapse fluorescence confocal imaging was performed using a previously reported method with slight modification [5]. *C. albicans* (ATCC 10231) was cultured in SDB medium and incubated at 35 ℃ for 24 h to a mid-log phase. The suspension was centrifuged and washed with PBS twice, then diluted in RPMI-MOPS buffer to a cell density of ~10^5 CFU/mL as the working suspension. 90μL of the spore suspension was added to a 24-well plate and the plate was kept still for 5 min to allow the spore to settle and adhere to the well bottom. 10 μL of FITC-3R-K7 (4× MIC, green fluorescence) solution in PBS and 10 μL of propidium iodide (20 μM, red fluorescence) solution in PBS were then added into the well. Images were taken every 30 seconds for a total of 20 minutes and recorded with CLSM in three channels: bright field, 488 nm and 562 nm.

Mitochondrial membrane potential study: Mid-log phase *C. albicans* (ATCC 10231) was diluted with RPMI-MOPS buffer to a cell density of ~10^6 CFU/mL. 50 μL of 3R-K7 was added to 450 μL *C. albicans* suspension to reach a concentration of 2× MIC, and the mixture was cultured at 35 ℃ for 2 h. PBS was used as a control. The fungal cells were washed with PBS twice and re-

suspended in 200 μL of JC-1（5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide）solution with a final concentration of 10 μg/mL, and then cultured at 35 ℃ in dark for another 20 min. PBS was used to remove excess dye, and the fungal cells were re-suspended in PBS. 150 μL of the fungal suspension was added into a 15 mm glass bottom cell culture dish (NEST®) and scanned with CLSM in three channels: bright field, 488 nm and 562 nm.

DNA damage assay: Mid-log phase *C. albicans* (ATCC 10231) was diluted with RPMI-MOPS buffer into ~10^6 CFU/mL, then treated with 3R-K7 (2× MIC as final concentration), and cultured at 35 ℃ for 10 h. The fungal suspension was centrifuged (6000 rpm, 5 min) and the cells were washed with PBS twice and then re-suspended in PBS. DNA damage assay was using DNA Damage Assay Kit by γ-H2AX Immunofluorescence (Beyotime). All measure operation steps were strictly followed instruction.

Inhibition of C. albicans biofilms formation: *C. albicans* (ATCC 10231) was cultured in the SDB medium and the suspension was incubated at 35 ℃ for 24 h. The suspension was centrifuged and washed with PBS buffer twice, and then diluted with RPMI-MOPS buffer to a concentration of 10^5 CFU/mL. 100 μL of 3R-K7 solutions with concentrations from 1500 to 6 μg/mL and fluconazole solutions with concentrations from 64 to 0.25 μg/mL was added to the 96-well plate. 100 μL of the spore suspension was added to each well, and the plate was incubated at 35 ℃ for 48 h. The supernatant was carefully removed and washed with PBS twice, followed by addition of 100 μL of the MTT (0.5 μg/mL) solution in DMSO. The suspension was incubated at 35 ℃ for 4 h in dark. The supernatant was removed and the spores were washed with PBS once, and then 100 μL of DMSO was added. Inoculum without antifungal agents was used as the positive control, while the SDB medium was used as the blank. The absorbance at 570 nm was measured with a microplate reader. The test was performed in triplicates.

$$\text{Cell viability} = \frac{ODsample - ODblank}{ODpositive - ODblank} \times 100\%$$

C. albicans biofilms formation: Mid-log phase *C. albicans* (ATCC 10231) was centrifuged and washed with PBS twice, and then diluted with RPMI-MOPS buffer to a cell density at ~10^5 CFU/mL as the working suspension. 200 μL of the suspension was added to a 96-well plate and incubated at 35 ℃ to obtain a mature biofilm. After 48 h of culture, the supernatant was carefully removed and washed with PBS once to remove the plankton cells before further experiments.

Activity against mature biofilms[6-8] The mature biofilms in a 96-well plate were treated with 200 μL of 3R-K7 solutions with concentrations of 100 μg/mL, 200 μg/mL and 400 μg/mL in RPMI-MOPS, respectively, and 200 μL of fluconazole in RPMI-MOPS (32 μg/mL, 128× MFC_{50}) for 6 h. The supernatant was removed carefully, and the biofilm was washed with 0.9% saline once and then stained with 50 μL of acridine orange (AO, 10 μM) and 50 μL of PI (5 μM) for 10 minutes in dark. The fluorescence was recorded by CLSM using the "XYZ" scan mode in three channels: the bright field, green channel (λ (Ex) = 561 nm, λ (Em) = 585.0 nm) and red channel (λ (Ex) = 488 nm, λ (Em) = 514 nm).

The biofilm's biomass was measured using a crystal violet (CV) assay.[9] The mature biofilms were treated with 3R-K7 with concentrations of 750 μg/mL-24 μg/mL and fluconazole with concentrations of 64 μg/mL-8 μg/mL in RPMI-MOPS for 24 h. The supernatant was carefully removed and the biofilm was washed with PBS twice, fixed with methanol for 20 minutes and dried for another 20 minutes at room temperature after the removal of methanol. 100 μL (0.1%, w/w) of the CV solution was added to each well in dark and the supernatant was removed 30 minutes later.

The biofilm was washed with PBS once and 100 μL of ethanol was added to each well. After 30 minutes of destaining, 80 μL of the supernatant was transferred to a new 96-well plate. Inoculum without antifungal agents was used as the positive control, while the RPMI-MOPS medium only was used as the negative control. The absorbance at 570 nm was measured with a microplate reader. The test was performed in triplicates. The percentage of biomass within the mature biofilm was calculated from

$$\%\ \text{Biofilms biomass} = \frac{ODsample - ODnegative}{ODpositive - ODnegative} \times 100$$

The biofilm viability was determined by the MTT assay. The mature biofilms were treated with 3R-K7 with concentrations of 750 μg/mL-24 μg/mL and fluconazole with concentrations of 64 μg/mL-8 μg/mL in RPMI-MOPS for 24 h. The supernatant was carefully removed and the biofilm was washed with PBS twice. 20 μL (5 mg/mL) of the MTT solution was added to each well in a dark environment, and the plate was incubated for another 4 h. The solution in each well was removed and 200 μL of DMSO was added. The plate was placed in a shaking bed to dissolve the crystal by gently shaking. Inoculum without antifungal agents was used as the positive control, while the RPMI-MOPS medium was used as the blank. The absorbance at 570 nm was measured with a microplate reader. The test was performed in triplicates. The percentage of surviving cells within the mature biofilm was calculated from

$$\%\ \text{Cell viability} = \frac{Abs_{570}^{polymers} - Abs_{570}^{blank}}{Abs_{570}^{control} - Abs_{570}^{blank}} \times 100$$

The morphology of biofilms: The *C. albicans* (ATCC 10231) suspension (~ 10^5 CFU/mL in RPMI-MOPS buffer) was added to a 24-well plate containing silicon chips (0.6 cm × 0.6 cm) that were cultured for 48 h to obtain mature biofilms as described above. The supernatant in each well was carefully removed and the chips were washed with PBS once, treated with 500 μL of 3R-K7 (375 μg/mL, 4× MIC) or PBS (as a control) at 35 ℃ for 12 h, and then washed with 1 mL PBS twice. The fungal cells were fixed with 2.5% glutaraldehyde overnight at 4 ℃ and then dehydrated with a series of ethanol solutions (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 10 min each). The morphologies of biofilms were imaged by SEM.

In vivo chronic toxic study: Mice (20 ± 2 g, Kunming, male) were randomly divided into 4 groups (n = 8), and 200 μL of 3R-K7 or 4R-K7 solution (2000 μg/mL) was injected at the dose of 20 mg/kg through the tail every three days for 21 days. Fluconazole at the dose of 20 mg/kg and PBS were used as positive and negative controls, respectively. The body weights were recorded every 2 days. All mice were sacrificed after 23 days observation. The main organs (heart, liver, kidney, spleen, lung) were harvested, fixed with 4% paraformaldehyde, and stained with H&E. The morphologies of organs were imaged with a microscope.

Systemic murine candidiasis model: This infection model was conducted according to the previously reported method with slight modification.[3, 10] Mice (20 ± 2 g, Kunming, male) were fed for five days and then randomly divided into 5 groups (n = 10). The immune suppressed mice were obtained by intraperitoneal injection (*i.p.*) of cyclophosphamide in PBS at the dose of 100 mg/kg for three days. Log-phase *C. albicans* (ATCC 10231) was washed with saline twice and diluted to a concentration of 2 × 10^8 CFU/mL. 200 μL of the fungal suspension was intravenously injected (*i.v.*) through the tail vein for each mouse. 200 μL of 0.9% saline (the negative control), 3R-K7 (1.0 mg/mL) and fluconazole (1.0 mg/mL, the positive control) was intravenously injected 2 h post-

infection, and followed by four injections for the next four days. One group of infected mice was injected with single dose of 3R-K7 (1.0 mg/mL) and one group of mice was not infected during the experiment. The numbers of death/live mice were recorded twice a day for 18 days, and the body weights were recorded every 2 days. The main organs of dead mice and sacrificed mice on day 18 were harvested, fixed with 4% paraformaldehyde, and stained with H&E for histological analysis.

Fungal burden of organs: The immune suppressed mice were divided in 4 groups (n = 6) and inoculated with *C. albicans* (ATCC 10231) as described above. One group of mice was euthanized after 2 h post-infection, and other three groups were treated with 3R-K7 (10 mg/kg), fluconazole (10 mg/kg) and 0.9% saline (as negative control) for 24 h by intravenous injection, respectively. All mice were euthanized and their main organs (kidney and spleen, one organ from each animal) were harvested and homogenized in 1 mL of sterile 0.1% TX-100 saline solution. Serial 10-fold dilutions of the homogenates were plated on SD agar, the plates were incubated at 35 ℃ for 48 h, and the numbers of colonies on the plates were counted.

Statistics analysis: All data are shown as the means ± standard deviation of at least three samples. Statistically significant difference was evaluated by one-way analysis of variance (Tukey), and the statistical significance was considered as p (*) < 0.05, p (**) < 0.01 and p (***) < 0.001 (n = 3).

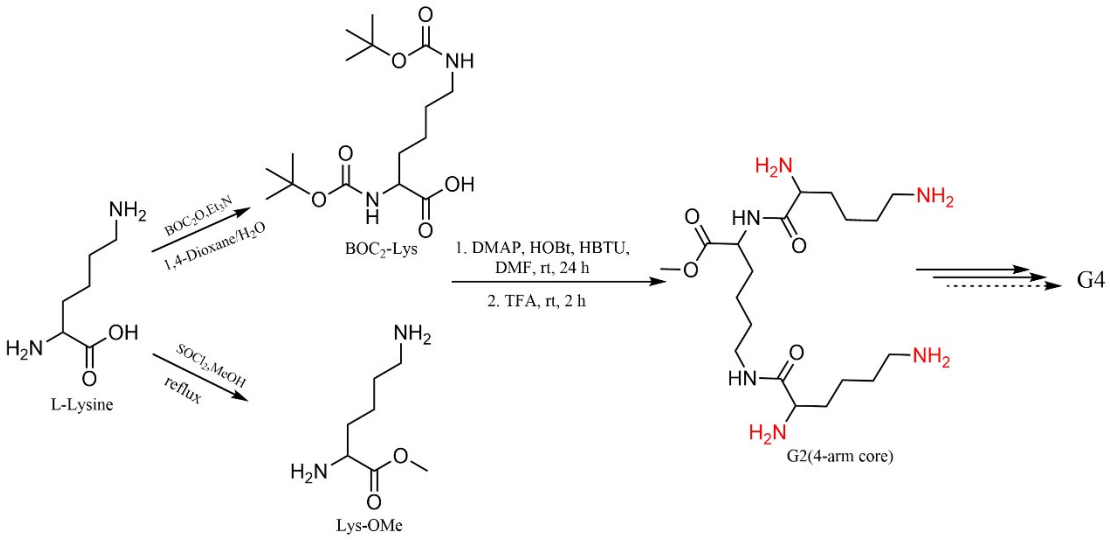


Fig. S1 Synthesis of 4R.

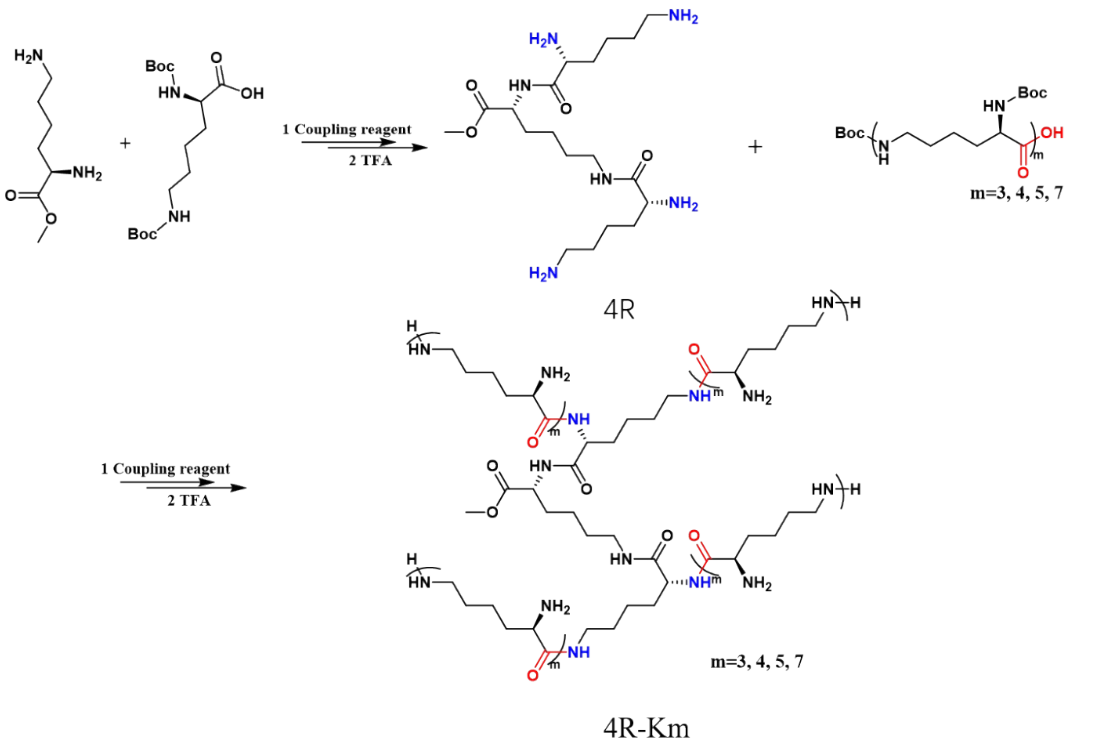
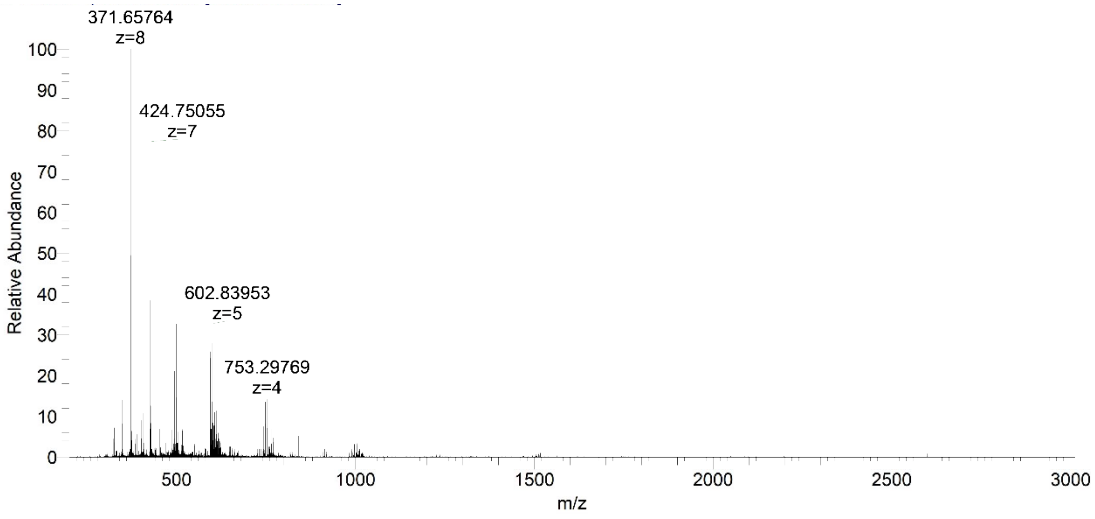


Fig. S2 Synthesis of 4R-Km.



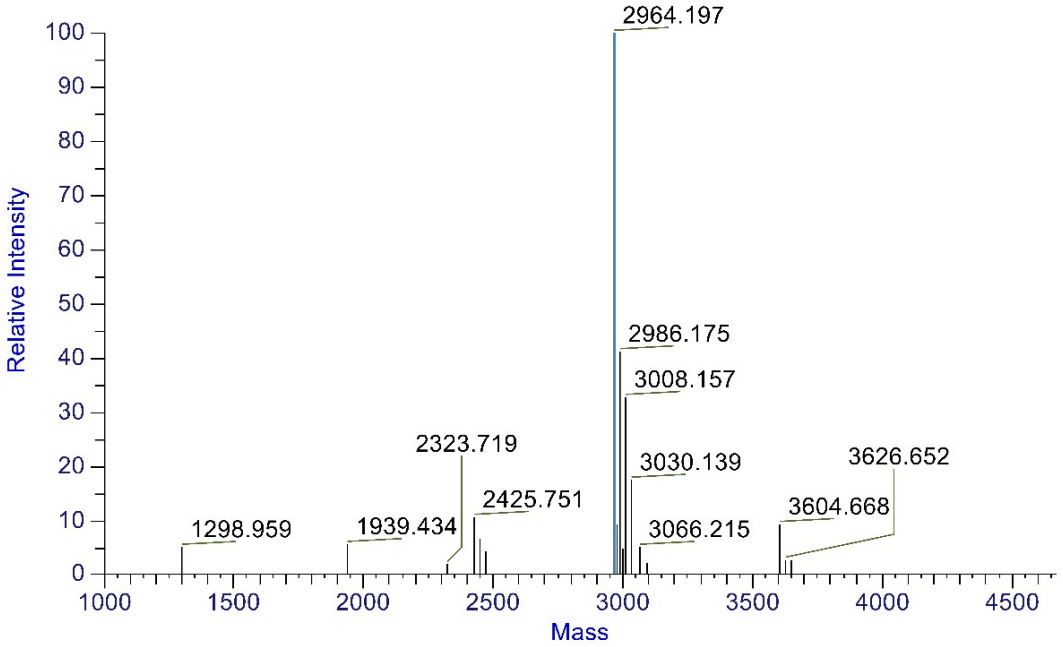


Fig. S3 Representative HR-ESI spectra of 3R-K7. ESI full mass spectrum (above) and calculated mass 2964.197 (below).

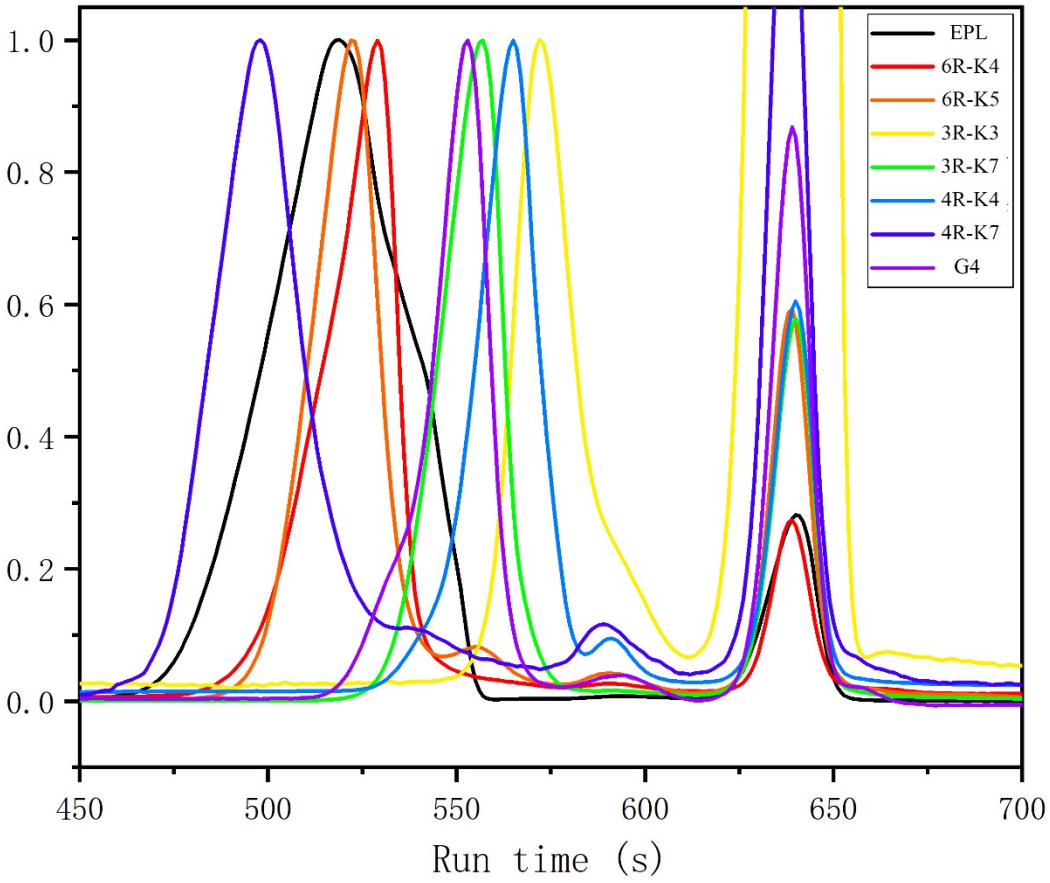


Fig. S4 GPC curves of *ε*-mPLs, EPL and G4.

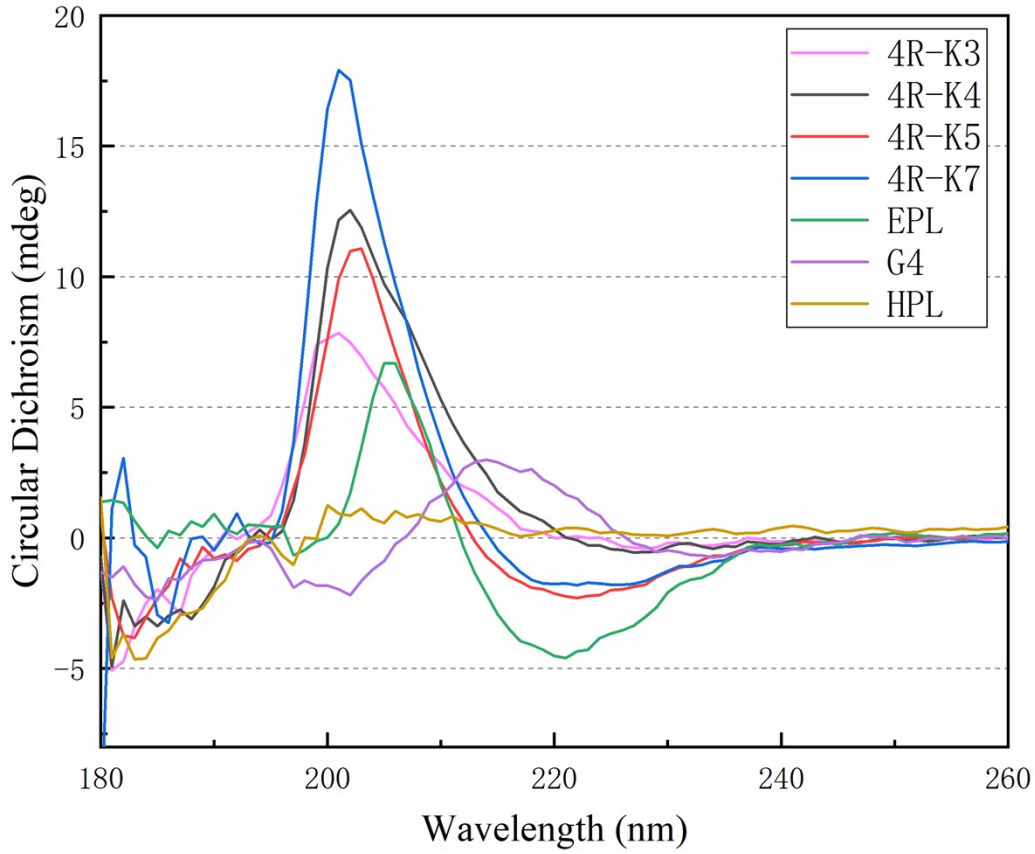


Fig. S5 The CD spectra of 4R-Km, EPL, G4, and HPL in PBS buffer.

	Numbers of Lysine	MIC (μg/mL)		
		Candida	Gram +	Gram -
		C. albicans ATCC 10231	MRSA ATCC 43300	*P. aeruginosa* ATCC 9027
3R-K3	11	>750	>750	>750
3R-K4	14	>750	>750	>750
3R-K5	17	96	188	188
3R-K7	23	24	96	96
4R-K3	15	375	188	188
4R-K4	19	375	188	188
4R-K5	23	48	48	48
4R-K7	31	48	24	24
6R-K3	23	>750	750	375
6R-K4	29	>750	750	375
6R-K5	35	188	375	96
G4	15	>750	375	96
EPL	25~35	375	24	12
K7	7	>750	750	>750

Fig. S6 Antimicrobial activities of ε-mPLs, G4, K7 and EPL.

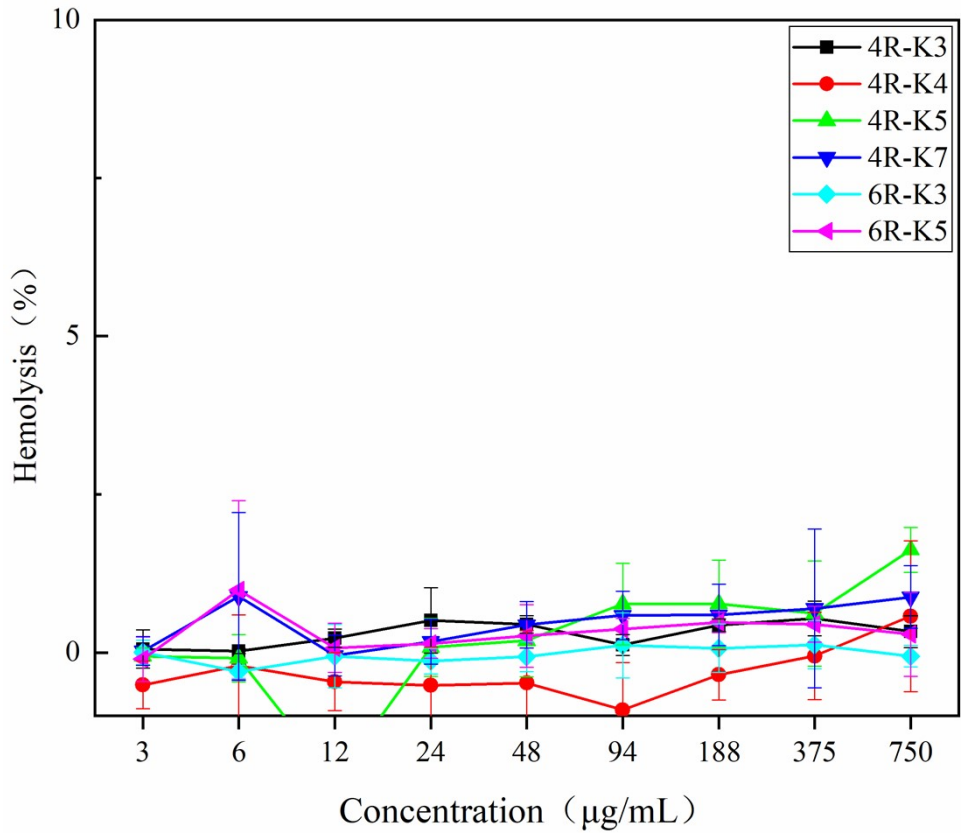


Fig. S7 Percent hemolysis ratios of 4R-Km and 6R-Km towards RBCs.

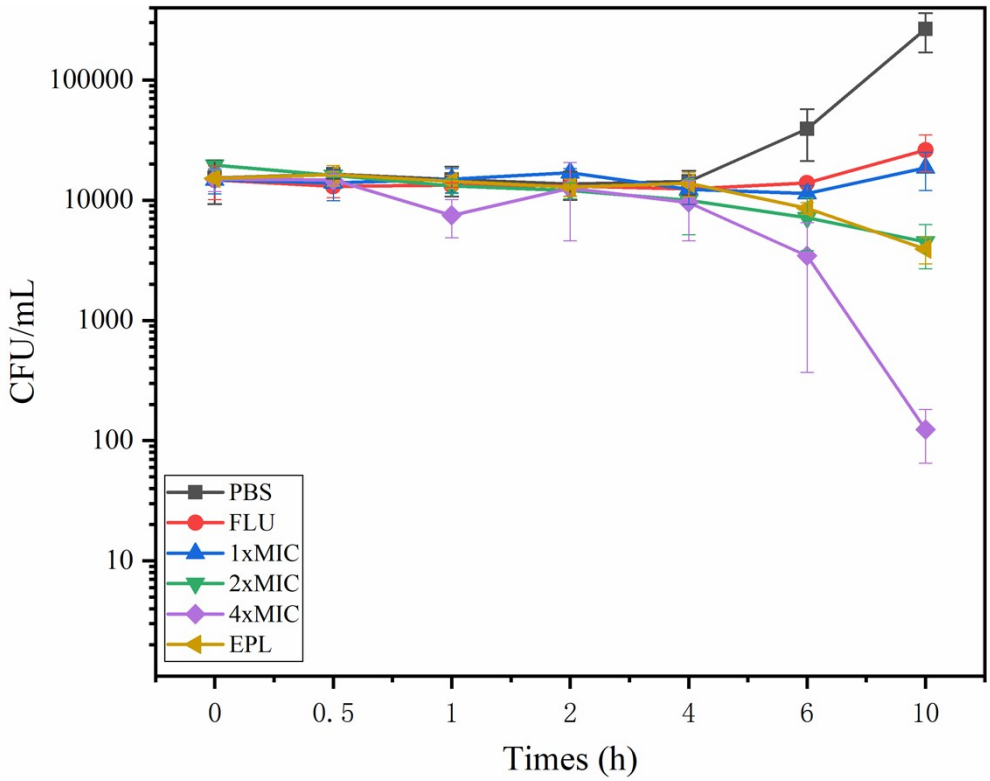


Fig. S8 Killing kinetics of 4R-K5 against *C. albicans* at 1× MIC, 2× MIC, and 4× MIC.

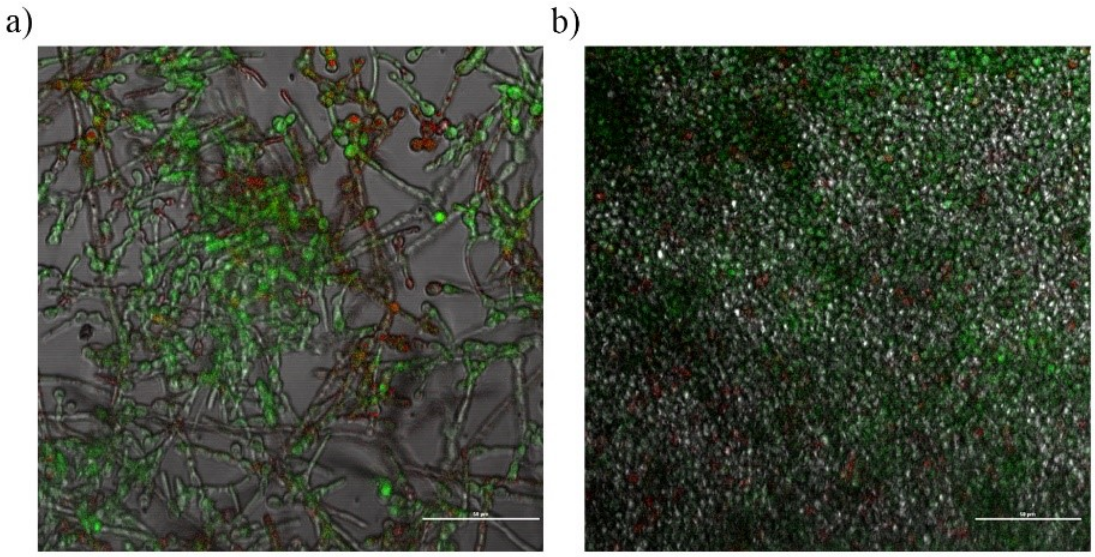


Fig. S9 AO/PI staining of mature biofilms containing both a) hyphae and b) yeast states treated with 3R-K7 (scale bar = 50 μm).

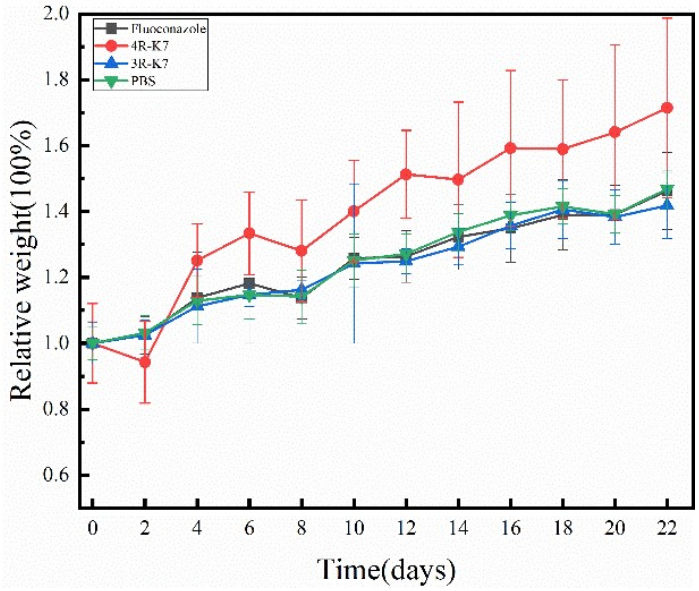


Fig. S10 Relative body weight changes of Kunming mice treated with 4R-K7, 3R-K7, PBS and fluconazole as a function of time.

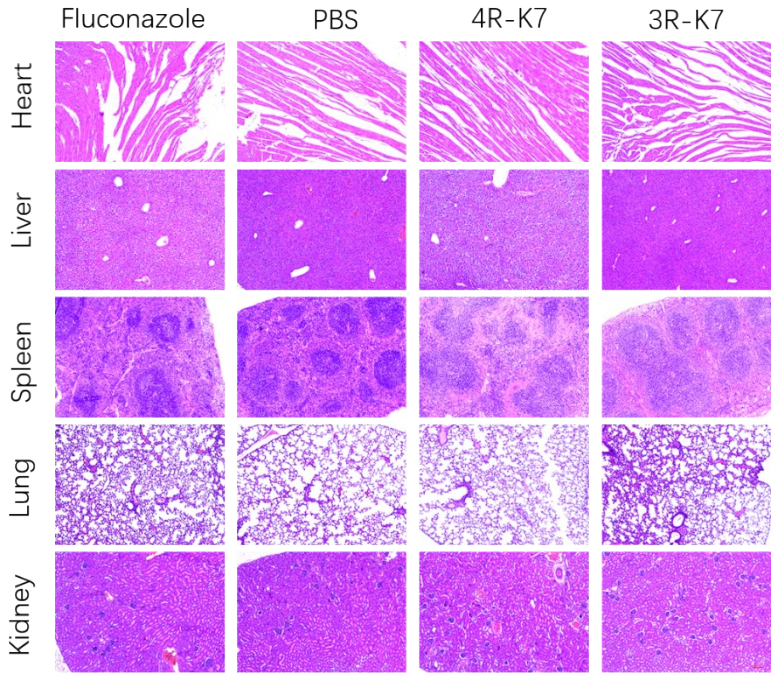


Fig. S11 Histologic studies of main organs of mice treated with fluconazole, PBS, 4R-K7 and 3R-K7.

Reference

1. Z. Kadlecova, L. Baldi, D. Hacker, F. M. Wurm and H. A. Klok, *Biomacromolecules*, 2012, **13**, 3127-3137.
2. M. Driffield, D. M. Goodall and D. K. Smith, *Org. Biomol. Chem.*, 2003, **1**, 2612-2620.
3. X. Liu, Y. Yang, M. Han, J. Guo, H. Liu, Y. Liu, J. Xu, S. Ji and X. Chen, *Adv. Healthc. Mater.*, 2022, **11**, e2201091.
4. L. Kaufmann, M. Syedbasha, D. Vogt, Y. Hollenstein, J. Hartmann, J. E. Linnik and A. Egli, *J. Vis. Exp.*, 2017, DOI: 10.3791/55833.
5. J. Xie, M. Zhou, Y. Qian, Z. Cong, S. Chen, W. Zhang, W. Jiang, C. Dai, N. Shao, Z. Ji, J. Zou, X. Xiao, L. Liu, M. Chen, J. Li and R. Liu, *Nat. Commun.*, 2021, **12**, 5898.
6. L. Su, Y. Li, Y. Liu, R. Ma, Y. Liu, F. Huang, Y. An, Y. Ren, H. C. Mei, H. J. Busscher and L. Shi, *Adv. Funct. Mater.*, 2020, **30**.
7. V. Basso, D. Q. Tran, J. B. Schaal, P. Tran, Y. Eriguchi, D. Ngole, A. E. Cabebe, A. Y. Park, P. M. Beringer, A. J. Ouellette and M. E. Selsted, *Sci. Rep.*, 2019, **9**, 16905.
8. R. M. Green and K. L. Bicker, *ACS Infect. Dis.*, 2022, **8**, 310-320.
9. X. Wang, G. Wang, J. Zhao, Z. Zhu and J. Rao, *ACS Macro Lett.*, 2021, **10**, 1643-1649.
10. A. Halperin, Y. Shadkchan, E. Pisarevsky, A. M. Szpilman, H. Sandovsky, N. Osherov and I. Benhar, *J. Med. Chem.*, 2016, **59**, 1197-1206.