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Cathelicidin-inspired antimicrobial peptides as novel antifungal compounds

Martin van Eijk[1], Stephanie Boerefijn[1,3], Lida Cen[2], Marisela Rosa[1], Marnix J. H. Morren[1], Cornelis K. van der Ent[4], Bart Kraak[3], Jan Dijksterhuis[3], Ivan D. Valdes[2], Henk P. Haagsman[1,*] and Hans de Cock[2]

[1]Division of Molecular Host Defence, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, [2]Molecular Microbiology, Department of Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands, [3]Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands and [4]Department of Pediatric Pulmonology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

*To whom correspondence should be addressed. Prof. H. P. Haagsman, PhD. Tel: +31 30 253 5354; E-mail: H.P.Haagsman@uu.nl

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Abstract

Fungal infections in humans are increasing worldwide and are currently mostly treated with a relative limited set of antifungals. Resistance to antifungals is increasing, for example, in *Aspergillus fumigatus* and *Candida auris*, and expected to increase for many medically relevant fungal species in the near future. We have developed and patented a set of cathelicidin-inspired antimicrobial peptides termed 'PepBiotics'. These peptides were initially selected for their bactericidal activity against clinically relevant *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates derived from patients with cystic fibrosis and are active against a wide range of bacteria (ESKAPE pathogens). We now report results from studies that were designed to investigate the antifungal activity of PepBiotics against a set of medically relevant species encompassing species of *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium, Malassezia,* and *Talaromyces*. We characterized a subset of PepBiotics and show that these peptides strongly affected metabolic activity and/or growth of a set of medically relevant fungal species, including azole-resistant *A. fumigatus* isolates. PepBiotics showed a strong inhibitory activity against a large variety of filamentous fungi and yeasts species at low concentrations ($\leq$1 μM) and were fungicidal for at least a subset of these fungal species. Interestingly, the concentration of PepBiotics required to interfere with growth or metabolic activity varied between different fungal species or even between isolates of the same fungal species. This study shows that PepBiotics display strong potential for use as novel antifungal compounds to fight a large variety of clinically relevant fungal species.

Key words: antifungal peptide, cathelicidin, fungal infections, antifungal resistance, antimicrobial peptide.

Introduction

Fungal infections in humans and animals are currently treated with a relatively limited set of antifungals.[1] However, resistance to antifungals is increasing with an unprecedented rate, among others due to their extensive use in the environment outside medical care facilities.[2] Consequently, extended exposure of humans and animals to resistant pathogens is expected to lead to a rise in incurable infections. Resistance to antifungals is frequently reported for important fungal pathogens.[1,3] In addition, the global increase in immunocompromised patients, for example, due to higher use of immunoblockers results in more fungal infections. Clearly, these developments urge for new therapeutic

compounds to fight and prevent resistance and suboptimal treatments. Further, restricted or even exclusive use in medical settings will be necessary to prevent rapid resistance development in the environment.[4]

Antimicrobial peptides (AMPs, also known as host-defense peptides) exhibit strong potential to fight microbial infections. AMPs are peptides with a broad spectrum of antimicrobial activity, are produced by all eukaryotic organisms, including fungi,[5] and are an important part of the innate immune system of vertebrates.[6] They vary in length between 12 and 50 amino acids and are generally positively charged and amphipathic allowing strong interactions with hydrophobic surfaces and membranes. AMPs are very diverse with respect to sequence and structure and are generally classified based on their conformation: α-helical, β-sheet (disulfide bridges), cyclic or peptides with extended structure/random-coil.[7,8] AMPs kill target cells through diverse mechanisms primarily by disrupting the membrane. It has also been found that AMPs target key cellular processes, including DNA and protein synthesis, protein folding, cell wall synthesis and enzymatic activity.[9,10] Clearly, AMPs have the advantage of a less specific but efficient mechanism of action as compared to traditional antifungals, a feature expected to limit resistance development.

In this paper we focus on the AMPs belonging to the cathelicidin family that are synthesized as prepropeptide and share a highly conserved N-terminal cathelin domain.[11] However, the antimicrobial mature peptide varies in amino acid sequence, structure, size and activity between species.[6,11–13] Cathelicidins can be constitutively produced or induced in response to a variety of microbial infections and inflammation.[6,11,14,15] The only human cathelicidin LL-37 is produced by neutrophils, macrophages, epithelial cells, and natural killer (NK) cells.[6,14] Chicken genomes encode four cathelicidins, CATH-B1, CATH-1, CATH-2, and CATH-3,[15] the latter three were shown to possess strong antibacterial activity without inducing notable resistance.[6,16] Previously, CATH-2 and LL-37 were reported to have candidacidal activities.[17] Here we compared the antifungal activity of LL-37 and L-CATH-2 with a set of cathelicidin-inspired variants called 'PepBiotics' against several medically relevant species encompassing species belonging to the fungal genera *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium*, *Malassezia,* and *Talaromyces*. These fungal pathogens are responsible for an annual high number of human infections globally and have been extensively described in numerous overviews.[18,19] Just four fungal species causing invasive infections—*A. fumigatus*, *Cryptococcus neoformans*, *Candida albicans,* and *Histoplasma capsulatum*—kill 1.5 million people annually.[20] The rise in resistance frequency to the azole antifungal compounds in the case of *A. fumigatus* is worrying, especially due to the development of multi-azole resistance and difficulties to treat patients infected with tri-azole resistant fungi.[21,22] Azoles target the ergosterol biosynthesis pathway and inhibit the demethylation of precursor sterols of 14-α-demethylase encoded by the *cyp51A* gene[23] and a variety of mutations, including TR34/L98H and TR46/Y121F/T289, are detected in a large number of azole-resistant *A. fumigatus* strains in the Netherlands.[24] *A. fumigatus* is globally responsible for over 14 million cases per annum causing invasive and non-invasive infections in immunocompromised and competent humans, respectively.[25] Aspergillosis is a lung-associated infection and patients with for example COPD or cystic fibrosis are frequently infected. A recent survey in five Dutch CF centers revealed that *A. fumigatus* is isolated most frequently with a mean prevalence of 31.7% and 7.1% of these strains were azole-resistant strains.[26] The PepBiotics used in this study vary in their primary sequence and were initially screened for their activity against gram-negative and -positive bacteria (manuscript in preparation) and a subset was now used to study antifungal activity. Here we show that PepBiotics interfere with metabolic activity, fungal growth, and/or viability of several clinically relevant fungal strains. These results illustrate the strong potential of PepBiotics as a promising novel class of antifungal drugs to treat infections caused by clinically relevant fungal species in humans.

Methods

Strains and culture conditions

Tables 1 and 2 summarize the fungal species and strains used. New clinical isolates of *A. fumigatus* from human patients were typed and resistance profile to azoles was determined as previously described.[27] For the initial experiments we tested a

Table 1. *Aspergillus fumigatus* strains.

A. fumigatus	Source	Reference
DTO 342-B2[α]	Canine patient with SNA	27
DTO 327-A8[2]	Human ICU patient; Resistant to ITC (>16), POS (0.25), VRC (>16)	27
DTO 326-I1[β]	Human patient from hematology; Resistant to ITC (>16), POS (1), Intermediate for VRC (2)	27
CEA10Δ*KU80*	Derivative of CEA10; human patient with invasive aspergillosis	49
DTO 327-D4 (Af293)	Human patient with invasive aspergillosis	50

[α]DTO is the code used for strains present in the working collection of the Department of Applied and Industrial Mycology (DTO).

[β]clinical isolates from ICU or hematology patients at Utrecht Medical Center and suspected of invasive aspergillosis were obtained from sputum. MIC concentrations (mg/l) according to EUCAST for the azoles are indicated between brackets. ITC, itraconazole; POS, posaconazole; VRC, voriconazole. More information is available in the indicated reference.

Table 2. Fungal species.

Species	DTO number	CBS number[α]
Aspergillus fumigatus	368-D5	CBS 113.26
	368-D6	CBS 113.37
Candida albicans	262-G7	n.d.
Candida auris	384-D4	CBS 12777
Candida dubliniensis	384-D3	CBS 11947
Candida glabrata	384-C9	CBS 12544
Pichia kudriavzevii (*Candida krusei*)	105-F6	CBS 573
Candida parapsilosis	384-D2	CBS 12541
Candida tropicalis	384-D1	CBS 13079
Cryptococcus bacillisporus	384-D9	CBS 10081
Cryptococcus deneoformans	384-D7	CBS 10511
Cryptococcus deuterogattii	384-D8	CBS 10082
Cryptococcus neoformans	384-D6	CBS 9172
Cryptococcus tetragattii	384-E2	CBS 10101
Fusarium oxysporum	363-B8	n.d.
Neocosmospora (Fusarium) solani	321-I6	n.d.
Malassezia furfur	383-D8	CBS 1878
	383-D9	CBS 7019
Talaromyces marneffei	299-F6	CBS 389.87
Meyerozyma guillermondii	384-D5	CBS 12072

[α]Strains with CBS code are from the fungal collection of the Westerdijk Fungal Biodiversity Center, Utrecht, The Netherlands; n.d., only present in the working collection of the Department of Applied and Industrial Mycology (DTO).

set of *A. fumigatus* strains, including resistant ones, indicated in Table 1 for their sensitivity to PepBiotics. These strains were standardly cultured for 4 days on PDA (Difco) at 37°C. Conidia were harvested from fresh cultures by scraping the surface gently using 10 ml saline solution with 0.002% Tween 20 (Sigma). For the second set of experiments we tested a larger variety of fungal species and strains indicated in Table 2, for their sensitivity for two PepBiotics (CR172 and CR184). These fungi were cultured for one week on oatmeal agar (Quakers and Oxoid) at 25°C except for *Malassezia* spp., which were cultured on modified Dixon agar (Sigma, Difco, Fluka, Baker, and Oxoid) at 30°C. Conidia or yeast cells were gently removed from the plates with ice-cold 10 mM ACES-buffer, pH 6.8 (N-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80) and filtered using glass wool. After centrifugation for 5 minutes at 1811 RCF, cells or conidia were resuspended in 20 ml ice-cold ACES buffer and centrifuged again. Finally, cells or conidia were resuspended in 5 ml ice-cold ACES buffer. The number of isolated yeast cells or conidia was determined using a Bürker-Turk haemocytometer and appropriate dilutions were prepared as indicated in the corresponding experiments. Stock solutions of yeast cells or conidia were stored at −25°C in 30% glycerol in 0.5× saline solutions.

For metabolic assays, growth or viability experiments *Aspergillus*-minimal medium (MM) was used containing per liter: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 200 μl trace element solution (10 g EDTA/l, 4.4 g $ZnSO_4 \cdot 7H_2O$/l, 1.01 g $MnCl_2 \cdot 4H_2O$/l, 0.32 g $CoCl_2 \cdot 6H_2O$/l, 0.315 g $ZuSO_4 \cdot 5H_2O$/l, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$/l, 1.47 g $CaCl_2 \cdot 2H_2O$/l and 1.0 g $FeSO_4 \cdot 7H_2O$/l), pH 6.0 (NaOH), and 2% w/v glucose for all fungal species (except *Malassezia*). For metabolic assays and growth/viability assays we used 0.125×MM and 1× MM, respectively. Culturing was performed at 37°C or 25°C as described in the experiments. For *Malassezia* spp. viability assays MM with 0.4% v/v glycerol (instead of glucose) and supplemented with 1% v/v Tween 60 (Sigma-Aldrich), 0.4% v/v olive oil, (MMM) was used at 30°C. The specific time points corresponding to specific results are indicated in the figures and/or their legends.

Peptides

This study includes the use of 2 natural cathelicidins, human LL-37 and chicken L-CATH-2, and 9 cathelicidin-inspired therapeutic peptides denoted as PepBiotics and coded as 'CR###'. Peptide codes and sequences are listed in Table 3. All peptides used in this study were synthesized by ChinaPeptides Co., Ltd. (Chuanhong Road, Shanghai, China) using classical solid phase peptide

Table 3. Antimicrobial peptides used in this study.

Code	Amino acid sequence	Number of amino acids
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH2	37
L-CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH2	26
CR170	**RRWVQRWIRRWR**PKRIVQRIKDFLRNLV-NH2	28
CR171	RRWGRFLRK**IRRFR**PKFKRIVQRIKDFLRNLV-NH2	32
CR172	**RRWVQRWIRRWR**PKVAAA**RRWVQRWIRRWR**PKV-NH2	33
CR173	**RRWVQRWIRRWR**KVAAA**RRWVQRWIRRWR**PKV-NH2	32
CR175	**RRWVQRWIRRWR**PKVLQKKGI	21
CR181	APKAMR**WVQRWIRRWR**PKLQKKGI-NH2	24
CR182	APKAM**WVQRWIRRWR**PLQKKGI-NH2	22
CR183	APKAM**RRWVQRWIRRWR**PKVLQKNNYL-NH2	27
CR184	APKAM**RRWVQRWIRRWR**PKVFQVTGSSA-NH2	28

The patented sequence 'RRWVQRWIRRWR' as present in most peptides used in this study is displayed in bold characters (truncated in CR181 and CR182). All peptides were aligned to maximize overlap of the residues present in those peptides that only have part of this sequence present. CR172 and CR173 contain a tandem repeat of the patented sequence, separated by six (CR172) or five (CR173) spacer residues.

synthesis (SPPS) and 9-fluorenylmethoxycarbonyl (Fmoc) as a protective group at the N-terminus.[28] After synthesis, the peptides were purified by HPLC with a linear gradient system (gradient: 5–95% B in 6 minutes, flow: 1 ml/min, eluent A: 100% H_2O + 0.05% TFA; eluent B: 100% CH_3CN + 0.05% (v/v) TFA) using a C18 RP-HPLC column (i.e. C18, 3 μm, 20 × 2 mm) and detection at 220 nm. All peptides used were ≥95% pure as verified by electrospray ionization Mass Spectrometry. To enhance stability and activity, all peptides were amidated at their C-terminus (-NH_2) except CR175. After purification, stock solutions of 1.6 mM were prepared by dissolving in sterile H_2O and aliquots were stored at −80°C. Working solutions were prepared by diluting stocks with sterile H_2O or in buffer solutions as indicated, depending on experimental conditions. A patent application has been filed with regard to the unique and novel core amino acid sequence shared by PepBiotics and use of PepBiotics for a wide variety of infection-related applications (patent pending, PCT/EP2018/060402; "antimicrobial peptides and their use").

Metabolic assay

Minimal medium (MM or dilutions of MM, 50 μl) of the appropriate concentration was mixed with 30 μl milliQ water, 5 μl glucose (final 2%), 5 μl conidia or yeast suspension (10^6 cfu/ml, 5×10^3 spores or yeasts), 5 μl resazurin stock of 2.1 mM (final is 105 μM) or 210 μM (final 10.5 μM), Sigma, Cat. No. R7017-5 G) and 5 μl AMPs (diluted in water to obtain the final concentration) and were added to a well of a nonbinding ELISA plate (Greiner Bio-One, Cat. No. 655180; 96 well cell culture plate). The final volume was 100 μl and either 105 μM (incubations at 37°C) or 10.5 μM (incubations at 25°C) resazurin was used. Resazurin (blue) is an oxidation-reduction indicator and conversion into resorufin (pink) is a measure of metabolic activity.[29,30] It can be further reduced to a colorless dihydroresorufin mainly occurring upon total consumption. This latter compound can be converted back to the pink resorufin requiring atmospheric oxygen.[29] Only a limited set of compounds was described that can give false positive reactions.[31] Conversion to pink or colorless is regarded as a positive result indicating metabolic activity, but after conversion to the colorless dihydroresorufin no quantification of metabolic activity can be made. We therefore determined for the different culturing conditions the optimal amount of MM and resazurin as well as the incubation time to enable quantification of metabolic activity. During further experiments we always used 1:8 diluted MM (0.125×MM) which provided optimal measure of metabolic activity. The absorbance of the samples was measured at 570 nm using plate readers (Thermo Scientific Multiskan EX or a Spectrostar^{nano} from BMG-Labtech) and routinely corrected for the absorbance of the negative control (no fungal cells). Plates were covered with a lid and were repeatedly measured during 24 hours to max 48 hours for incubations at 37°C and up to 72 hours at 25°C, in the latter case plates were incubated while shaking at 60 rpm. Positive (no AMPs) and negative controls (no fungal cells) were routinely incorporated and the wells at the edges of the plate were filled with water and not with samples to reduce evaporation effects at 37°C. Occasionally, plates were measured at 405 nm to determine cell density.[32] No metabolic assays were possible for *Malassezia* spp. due to false negative results; no conversion of resazurin was observed while growth was detected by microscopic analysis. All fungal species (except Malassezia) used in this study did grow in 1/8 diluted MM at 25°C or in case of *Malassezia* 1/8 diluted MMM medium at 30°C in absence of antimicrobial peptides.

Viability and growth assay

Fifty μl of a solution of AMPs (diluted in 1×MM to obtain a final concentration which ranged between 0.1 and 5 μM) containing conidia/yeast cells (1500 cfu/ml) in 1×MM or 1×MMM (in case of *Malassezia* spp.) was mixed with 50 μl sterile water in a well of a 96-well plate (Costar 3879, Corning Incorporated, Corning, NY, USA) and incubated at 25°C or 30°C for *Malassezia* spp. The amount of cfu/ml was determined after 3, 24, and 48 hours by the addition of Triton X-100 (final concentration 1% v/v) to a well and the complete content was plated and incubated on a malt extract agar (MEA) plate at 25°C or mDixon plate and culturing for 7 days at 30°C (for *Malassezia*) followed by colony counting. Positive controls lacked AMPs and negative controls lacked fungal cells but contained 0.16 μM AMP. At the start of the experiment an additional sample was directly plated to determine the exact starting amount of cfu/ml at t = 0. The viability assay by direct plating worked only for *Malassezia* yeast cells but not for filamentous fungi (e.g., *Aspergillus, Fusarium,* and *Talaromyces* spp.). In the latter case fungal material was not plated but was inspected with a stereomicroscope (Nikon AZ100) to determine the extent of growth by mycelium formation. In addition, the metabolic assay was used for all filamentous but only for all yeast species (except *Malassezia* spp.).

Live-dead staining with propidium iodide

To investigate viability of *A. fumigatus,* staining with propidium iodide (PI) was performed. As positive control, 10^6 conidia of *A. fumigatus* CEA10 in 100 μl 0.125×MM in an Eppendorf tube were heat-inactivated for 20 min at 90°C. After centrifugation for 60 seconds in an Eppendorf centrifuge at 13.200 g, the conidia were resuspended in 10 μl PI (15 μM final concentration, Sigma-Aldrich). After a 15 minutes incubation at 37°C, conidia were microscopically analyzed by fluorescence microscopy with a TRITC filter and an Axioskop 2 plus fluorescent microscope at 100× magnification. To determine the effect of AMPs on viability of fungal cells using PI, mixtures of conidia in MM were prepared as described above in the absence or presence of AMPs (1 or 5 μM) and incubated at 37°C up to 48 hours, similar as described for the metabolic assays but without resazurin. After

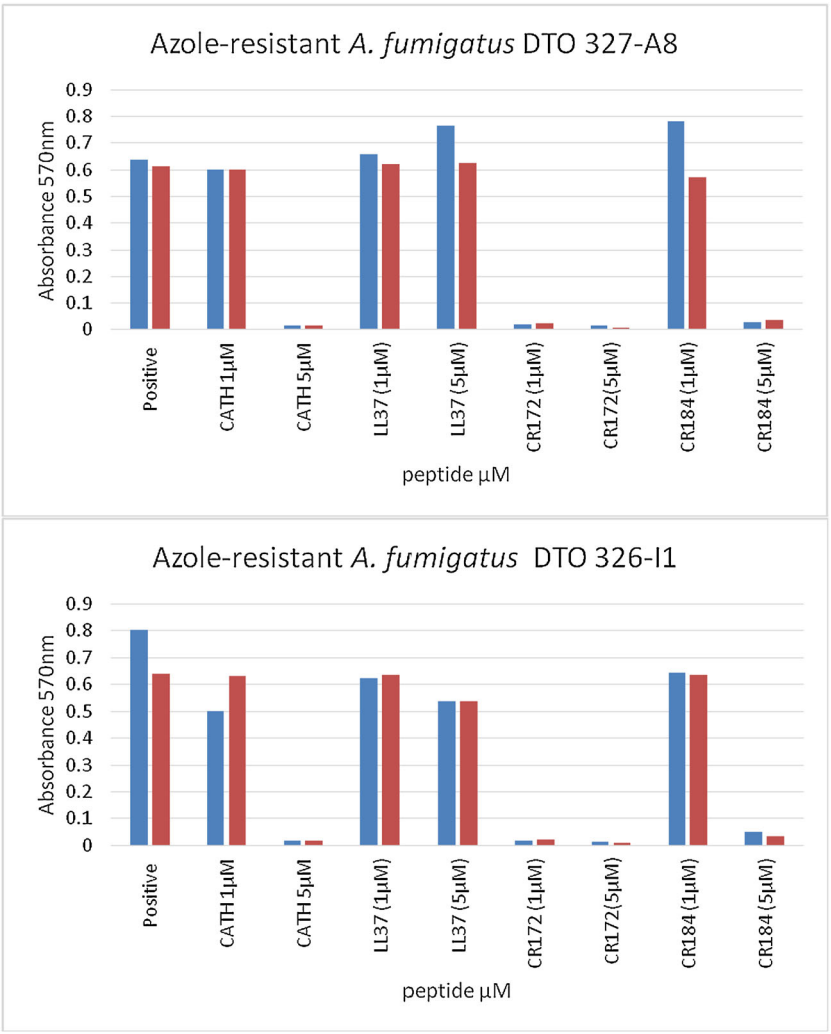

Figure 1. Azole-resistant *A. fumigatus* strains are sensitive for PepBiotics. The indicated azole-resistant strains were used in the metabolic assay using 0.125×MM and 105 μM resazurin at 37°C for 27 h in absence (positive) or presence of either L-CATH-2, LL-37, CR172 or CR184 at 1 or 5 μM final concentration. Results of two experiments are shown (blue and red bar).

various time intervals samples were taken, centrifuged, stained with PI and analyzed using fluorescent microscopy as described above. We initially tested a large set of PepBiotics in duplo experiments to obtain a more qualitative view of the effects on *A. fumigatus* strains. In the subsequent experiments with a subset of PepBiotics we tested their effects in three independent experiments on a large variety of different fungal species.

Cytotoxicity test

Cytotoxicity of PepBiotics was determined using primary human nasal epithelial cells, isolated and cultivated at UMCU to confluency in Bronchial Epithelical Cell Medium (Sciencell Research Laboratories, Sanbio, Uden, Netherlands).[33] Cells were exposed to 5 μM of peptide for 3 hours at 37°C followed by three washings with phosphate-buffered saline (PBS). WST-1 (10% v/v in cell medium) was used for the quantification of cell viability using colorimetric analysis as previously described.[34] Controls included no peptide (100% viability) and 70% ethanol (total loss of cell viability). Experiments were performed in triplo. Statistical analysis peptide-treatment versus 'no peptide' by two-tailed paired Student *t* test.

Results

Effects of AMPs on metabolic activity of *A. fumigatus*

We first compared the effect of L-CATH-2 and a set of PepBiotics on the metabolic activity of *A. fumigatus* strains Af293, CEA10 and DTO342-B2 at 1 μM. We observed in these initial experiments that a subset of the PepBiotics was markedly more active against these *A. fumigatus* strains as compared to L-CATH-2 at this concentration. PepBiotics CR170, 171, 172, 173 and 183 inhibited the metabolic activity strongly at 1 μM whereas the other

PepBiotics did not and results were comparable to L-CATH-2. (results not shown).

We next tested the activities of AMPs LL-37 and L-CATH-2 with CR172 and CR184 in more detail at 1 μM and 5 μM against two azole-resistant *A. fumigatus* strains, DTO 327-A8 and 326-I1. Both azole-resistant isolates were sensitive for PepBiotics (Fig. 1). CR172 inhibited metabolic activity strongly at 1 μM whereas CR184 and L-CATH-2 inhibited metabolic activity at 5 μM. The AMP LL-37 did not inhibit metabolic activity under these conditions. Similar results were obtained for Af293 and CEA10 (results not shown).

Killing potency of PepBiotics against *A. fumigatus* using PI staining

We next investigated whether the PepBiotics were also able to kill *A. fumigatus* using the live-dead propidium iodide staining. This stain is used to assess cell viability since it cannot pass the cytoplasmic membrane of living cells. Indeed, more than 95% of the conidia of *A. fumigatus* were fluorescently labeled when heat-inactivated for 20 minutes at 90°C (Fig. S1F). We subsequently incubated *A. fumigatus* CEA10Δ*KU80* in absence (negative control) or presence of 5 μM L-CATH-2 in 0.125×MM at 37°C and tested the viability of fungal cells by PI staining up to 20 hours. No PI positive staining of conidia, swollen conidia or hyphal forms was detected in the presence of L-CATH-2 (Fig. S1B). We repeated these experiments with the more active PepBiotic CR172 at 5 μM (Fig. S2), but although this concentration is strongly inhibiting metabolic activity, no PI positive staining of fungal cells was observed (Fig. S2D). In addition, no PI-positive staining of CEA10Δ*KU80* was observed when we added this peptide to a 23 hour culture followed by an incubation of 30 minutes (Fig. S2F). Remarkably, we did observe killing with two variant PepBiotics on a different *A. fumigatus* strain. Addition of 1 μM CR173 or CR183 to an 18-hour culture of a different *A. fumigatus* strain DTO 342-B2 followed by a 15-minute incubation did result in PI-positive staining (Fig. 2). Only hyphal structures were stained whereas dormant and swollen conidia were not PI positive. We observed that germination in the MM was much lower as compared to rich medium and preliminary data indicated that approximately 6% of conidia were germinated in 8 hours, whereas around 60% were swollen. This explains the relative low amount of hyphal structures observed (Figs. S1 and S2). Overall, these results indicate that L-CATH-2 and PepBiotics CR172, CR173, CR183, and CR184 strongly inhibit the metabolic activity or viability of *A. fumigatus* strains.

Antifungal effects of PepBiotics against clinically relevant fungi

We next investigated whether the PepBiotics CR172 and CR184 are able to inhibit metabolic activity, growth and viability of a large set of clinically relevant fungal species (Table 2) using in-

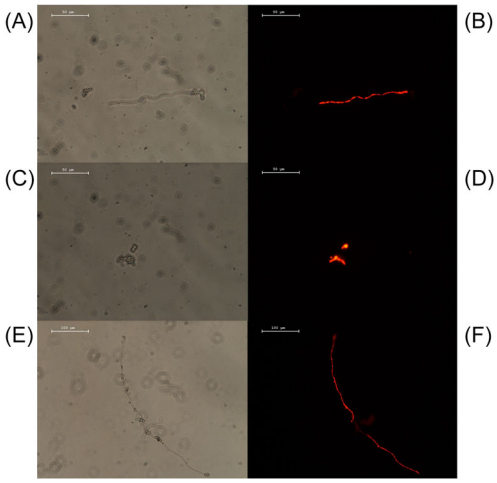

Figure 2. Propidium iodide staining of *A. fumigatus* with PepBiotics CR173 and CR183. (A) and (C) (normal filter), (B) and (D) (TRITC filter) shows an 18-h culture of *A. fumigatus* DTO 342-B2 which was subsequently incubated for 15 min with 1μM CR-173, washed and stained with PI (400x). (E) (normal filter) and (F) (TRITC filter): similar strain and culture conditions but a 15-min incubation with 1 μM CR183 (200×). (A–D), size bar 50 μm; (E–F), size bar 100 μm.

cubations in MM but at lower growth temperatures. This lower temperature was chosen to reduce the evaporation of medium during prolonged culturing in ELISA plates. All strains indicated in Table 2 were able to grow in the same *Aspergillus* MM at 25°C, except *Malassezia*, which required 30°C and addition of the lipidic compounds (1% Tween-60 v/v and 0.4% olive oil v/v) due to its inability to synthesize fatty acids *de novo*.[35] We first optimized the metabolic activity assay using *A. fumigatus* DTO 368-D5 and D6 for these new conditions in order to be able to follow activity over a prolonged period. The metabolic assay showed a steady increase in absorbance at 570 nm between 25 hours and 72 hours at 25°C using 0.125×MM and 10.5 μM resazurin (results not shown). The viability of *Malassezia* yeast cells was determined by plating of cells after washing the wells with 1% TX-100 and is expressed as cfu/ml. The growth of yeasts and filamentous fungal species was also followed microscopically (indicated as GR). The viability of filamentous fungal species could not be determined by plating since these fungal species could not be quantitatively removed from the wells by washing with TX-100. Table 4 summarizes the results of three independent experiments of metabolic assays (MA) and growth assays (GR) of a set of filamentous fungal species and representative images can be found in the supplementary Fig. S3-S7. In agreement with our findings at 37°C, *A. fumigatus* is strongly inhibited by CR172 (Fig. 3) whereas CR184 does not inhibit at 25°C (Fig. S3). Sometimes, a single spore germinated and formed a hyphal structure (see 400× magnification in Fig. 3). Similarly, the metabolic activity and growth of *Fusarium* and *Talaromyces* species tested were both more sensitive for PepBiotic CR172 and were inhibited at 0.625 μM (Table 4).

In the growth assay, two strains of *Malassezia furfur* (DTO 383-D8 and DTO 383-D9) showed different sensitivity for the

Table 4. Sensitivity of clinically relevant filamentous fungal species for PepBiotics.

Spp	DTO	Pep	5 µM GR	5 µM MA	2.5 µM GR	2.5 µM MA	1.3 µM GR	1.3 µM MA	0.6 µM GR	0.6 µM MA	0.3 µM GR	0.3 µM MA
A.fumigatus	368-D5	CR-172										
A.fumigatus	368-D5	CR-184										
A.fumigatus	368-D6	CR-172										
A.fumigatus	368-D6	CR-184										
F.oxysporum	363-B8	CR-172										
F.oxysporum	363-B8	CR-184										
N.solani	321-I6	CR-172										
N.solani	321-I6	CR-184										
T.marneffei	299-F6	CR-172										
T.marneffei	299-F6	CR-184										

Metabolic activity (MA after 72 h) and growth (GR after 8 days) of filamentous fungal species (Spp) in absence or presence of serial dilutions of two types of PepBiotics (Pep) at 25°C. Red, no growth/metabolic activity; yellow, partial growth/metabolic activity; green, no inhibition of growth/metabolic activity observed (as compared to control without peptide). Supplemental Figure S8 provides the numerical and graphical data representing average and standard deviations of the metabolic data obtained after 48 and 72 h.

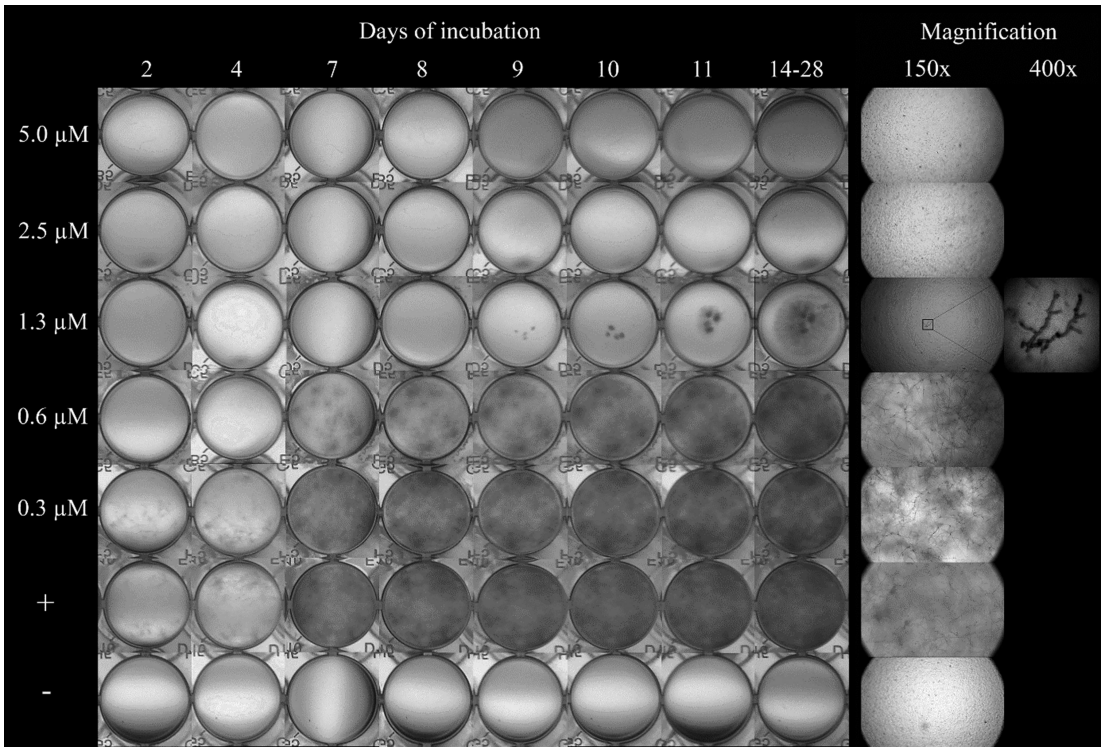

Figure 3. Growth of *A. fumigatus* (DTO 368-D5) in absence or presence of the antifungal peptide CR-172. Growth was evaluated up to 28 days at 25°C. Details of growth after 7 days of incubation as observed with a stereomicroscope at 150× and 400× magnification are shown. Incubation with conidia in absence of CR172 (indicated as +) served as positive control, culture medium in absence of conidia but presence of 0.16 μM CR172 (indicated as −) served as negative control.

peptides tested. Strain DTO 383-D8, was moderately susceptible for inhibition by CR-184 (1.3 μM) and CR-172 (5 μM) within the 48-hour incubation period (Table 5). In contrast, strain DTO 383-D9 was highly sensitive for both PepBiotics (growth assay, Table 5) and the lowest concentration tested of both CR184 and CR172 (0.3 μM) resulted in undetectable growth. Both strains were also plated after incubations with the PepBiotics to determine the viability expressed as the amount of cfu/ml after 3-, 24-, and 48-hour incubations with the PepBiotics (Fig. S7). *M. furfur* (strain DTO383-D8) strain lost viability after a 48-hour incubation with 2.5 μM CR172 (Fig. S7A) and after 24-hour incubation with 0.6 μM CR184 (Fig. S7B). In contrast, *M. furfur* (strain DTO 383-D9) lost viability already after a 3-hour incubation with 0.6 μM CR172 (Fig.S7 panel C) while no viable cells were recovered after a 48-hour incubation with 0.3 μM CR184 (Fig. S7D). These results underscore a

Table 5. Effects of PepBiotics on growth of *Malassezia furfur*.

	DTO	Peptide	5 µM	2.5 µM	1.3 µM	0.6 µM	0.3 µM
M. furfur	383-D8	CR-172					
M. furfur	383-D8	CR-184					
M. furfur	383-D9	CR-172					
M. furfur	383-D9	CR-184					

Results were obtained after 48 h of incubations in the presence of the indicated amount and type of PepBiotics. Red, no growth observed; green, growth is similar as control without peptide.

remarkable difference in sensitivity for PepBiotics between *Malassezia* strains.

Metabolic activity assays with a set of yeasts strains indicated that all tested species were highly sensitive to both PepBiotics. Concentrations of <1 μM PepBiotics abolished metabolic activity in most cases as determined after 72 hours (Table 6A,B). These included five *Cryptococcus* and six *Candida* strains, among them *C. auris* (which did not show activity at 0.6 μM, CR-172). *Cryptococcus gattii* did not grow in the MM used and sensitivity for PepBiotics to this important emerging pathogen could not yet be tested.[36]

Cytotoxicity of 5 μM PepBiotics was determined using primary human nasal epithelial cells and compared to positive (ethanol) and negative control (no peptide) (Table 7). The PepBiotics CR170, CR171, CR172 CR 182, CR184 showed no significant cytotoxic effect ($P < 0.05$), whereas L-CATH-2 and ethanol did show increased cytotoxic activity to the epithelial cells.

Discussion

An increase in prevalence of fungal infections is observed worldwide, amongst others due to new medical technologies and possibilities to treat critically ill patients. Especially, the increase in immunocompromised patients is expected to increase the prevalence of invasive and noninvasive fungal infections.[36] In addition, this category of patients is also vulnerable to infections by less common fungal species resulting in emerging fungal infections and species of the *Mucorales* group already belong to that group.[37] In addition, increased outbreaks of fungal infections associated with travel or relocation, occupation and contacts in new environmental niches contribute.[38] Among the species responsible for such outbreaks are *Fusarium* sp. causing keratitis, endophthalmitis, and fungemia and *Candida auris* infections.[39,40] The current increase in outbreaks of the multidrug resistant *C. auris* is very worrying. No established *C. auris*-specific susceptibility breakpoints have been determined and the currently used breakpoints are based on closely related *Candida* species.[1] The *C. auris* strain used in this study is the type strain CBS 12777 (also known as CBS10913, DSM21092 or JCM15448)[40,41] and antimicrobial resistance testing showed reduced susceptibility to amphotericin B, which is also observed in a large group of *C. auris* isolates.[42] The increased use of antifungals in society and the accompanied increase in antifungal resistance is expected to limit antifungal therapy in the near future even further. Development of new antifungals based on natural and synthetic antifungal peptides has increased accordingly.[36] Recent developments in the design of LL-37 based peptides with a minimal core region resulted in various peptides with different biological activities.[43]

Based on the current knowledge of the antimicrobial properties of the cathelicidin family, a set of cathelicidin-inspired PepBiotics was developed with strong antibacterial activity. These PepBiotics are based on a variety of sequences of cathelicidins and have a unique core sequence (see Table 3). PepBiotics containing the full-length patented sequence either once or twice showed in general antifungal activity at 1 μM. We show that a subset of PepBiotics has broad range antifungal activity against clinically relevant fungal species, including azole-resistant *A. fumigatus* and *C. auris* with a reduced susceptibility for mphotericin B. PepBiotics strongly interfere with metabolic activity, growth and/or viability at a submicromolar level, among others of emerging pathogens like *Fusarium* spp. and *Candida auris*. All yeasts and filamentous fungi tested in this study were inhibited by two PepBiotics, CR172 and CR184, although 5 μM CR184 was required at 37°C to inhibit *A. fumigatus*. It is remarkable that the PepBiotics CR172 and CR184 did inhibit metabolic activity of *A. fumigatus* strains at 37°C more effectively as compared to lower temperature of 25°C. Whether strain-specific differences are involved needs further investigation including the killing potential of different PepBiotics on different *A. fumigatus* strains. Strain-specific differences for these two PepBiotics were observed for two *M. furfur* strains and marked differences in the efficiency of killing were observed. It is unclear why strain-specific sensitivities occur, but it is tempting to speculate that binding to target sites but also to other binding sites at the fungal surface might be involved but more research is required to address this. Alternatively, differences in target-site composition might be responsible for observed strain-specific differences. We

[1] https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html.

Table 6 A. Sensitivity of clinically relevant yeast strains for PepBiotics.

Species	DTO	Peptide	5 µM	2.5 µM	1.3 µM	0.6 µM	0.3 µM
Pichia kudriavzevii (*Candida krusei*)	105-F6	CR-172					
Pichia kudriavzevii (*Candida krusei*)	105-F6	CR-184					
Candida albicans	262-G7	CR-172					
Candida albicans	262-G7	CR-184					
Candida glabrata	384-C9	CR-172					
Candida glabrata	384-C9	CR-184					
Candida tropicalis	384-D1	CR-172					
Candida tropicalis	384-D1	CR-184					
Candida parapsilosis	384-D2	CR-172					
Candida parapsilosis	384-D2	CR-184					
Candida dubliniensis	384-D3	CR-172					
Candida dubliniensis	384-D3	CR-184					
Candida auris	384-D4	CR-172					
Candida auris	384-D4	CR-184					
Meyerozyma guilliermondii	384-D5	CR-172					
Meyerozyma guilliermondii	384-D5	CR-184					

The indicated yeast strains were incubated with the indicated amount and type of PepBiotics for 72 h at 25°C. Red, no metabolic activity; yellow, partial, metabolic activity; green, no inhibition of metabolic activity observed (as compared to control without peptide). Supplemental Figure S8 provides the numerical and graphical data representing average and standard deviations of the metabolic data obtained after 48 and 72 h.

do not know the targets of the PepBiotics used but it cannot be excluded that intracellular targets like mitochondria are involved as was shown for histatin 5.[44] Interestingly, we also observed differences between PepBiotics in their ability to affect viability of *A. fumigatus*. PepBiotics CR173 and CR183 were able to inactivate hyphae of this fungal species (in this case DTO 342-B2) as observed with PI-staining in contrast to CR172. However, CR172 inhibits growth and metabolic activity. In the presence of the PepBiotics CR172 and CR184 metabolic activity was inhibited but probably not completely or below the detection level of the metabolic assay since germination was still observed. This might be explained by a specific effect of these PepBiotics on mitochondria and possibly comparable to histatin 5,[44] but more research is required to address this possibility. Furthermore, the difference in activity between the different PepBiotics (CR173 and CR183 vs CR172) on *A. fumigatus* might be related to a difference in target sites or efficiency of disrupting the target sites. The metabolic activity assay was not used for *M. furfur* since no activity was observed despite the observed visible inhibition of growth. We do not have a clear explanation for this observation. Possibly, the mitochondrial activity is already very low of these species in this assay and below the detection level of the assay. An alternative explanation may be that it has been reported that resazurin is trapped in the lipid-rich cell wall layer of *Malassezia*.[45] The PepBiotics CR172 and CR184 affected the viability of *M. furfur* strains indicating fungicidal activity. Both PepBiotics also affected the growth and metabolic activity of *F. oxysporum*, *F. solani*, and *T. marneffei*, indicative of at least

Table 6 B. Sensitivity of *Cryptococcus* spp. for PepBiotics.

Species	DTO	Peptide	5 µM	2.5 µM	1.3 µM	0.6 µM	0.3 µM
Cryptococcus neoformans	384-D6	CR-172					
Cryptococcus neoformans	384-D6	CR-184					
Cryptococcus deneoformans	384-D7	CR-172					
Cryptococcus deneoformans	384-D7	CR-184					
Cryptococcus deuterogattii	384-D8	CR-172					
Cryptococcus deuterogattii	384-D8	CR-184					
Cryptococcus bacillisporus	384-D9	CR-172					
Cryptococcus bacillisporus	384-D9	CR-184					
Cryptococcus tetragattii	384-E2	CR-172					
Cryptococcus tetragattii	384-E2	CR-184					

Cryptococcus spp. were incubated for 72 h at 25°C with the indicated amount and type of PepBiotics. Red, no metabolic activity; yellow, partial, metabolic activity; green, no inhibition of metabolic activity observed (as compared to control without peptide). Supplemental Figure S8 provides the numerical and graphical data representing average and standard deviations of the metabolic data obtained after 48 and 72 h.

Table 7. Cytotoxicity tests with a set of PepBiotics.

Peptide	Average (%)	SD	*P*-value
No Peptide	100.0		
EtOH	17.5	2.9	.00045
CR-170	94.7	16.9	.58
CR-171	91.0	21.6	.46
CR-172	66.7	24.8	.074
CR-182	104.3	24.0	.79
CR-184	95.8	13.6	.58
CATH-2	45.8	9.3	.010

To determine cytotoxic effects of PepBiotics cells were exposed to 5 μM of the indicated peptide. Primary human nasal epithelial cells were used that were isolated and cultivated to confluency in Bronchial Epithelical Cell medium as described in Methods. Controls included no peptide (100% viability) and ethanol (total loss of cell viability). Experiments were performed in triplo.

fungistatic activity. The latter species is a thermally dimorphic species causing systemic infections in human immunodeficiency virus (HIV) and non-HIV infected patients and is an emerging pathogen amongst others due to delayed diagnostics.[19]

In summary, our results show that PepBiotics are very promising antifungals. We showed fungicidal activity for the two *Mallasezia furfur* strains and fungistatic activity for all yeast species tested and, depending on strain and type of PepBiotic also fungicidal or fungistatic activity for the filamentous fungus *A. fumigatus*. Since *T. marneffei* is a thermally dimorphic species and experiments were performed on the filamentous form only (at 25°C), further investigations are required to determine if the pathogenic yeast form is also affected by PepBiotics. As a next step, the use of synthetic peptides enables us to design and further develop species-specific antifungals and to improve the growth inhibiting and/or killing properties against fungal cells. It should be mentioned that reducing growth without killing can already be an advantage in medical treatments as the innate or adaptive immune response, or treatment in combination with antifungals, can work in concert. Future research requires additional testing of PepBiotics on fungal biofilms since these are notoriously more resistant to antifungals.[46,47] In this respect, it might also[45] be worthwhile to investigate combinations of PepBiotics with other known biofilm-reducing agents which could result in synergistic anti-biofilm activity. Nevertheless, fungal cells escape mature biofilms in the process of generating systemic infections and these can be direct targets for PepBiotics as well.[48] In addition, natural biofilms are often fungal-bacterial mixtures and developing PepBiotics with both antifungal and antibacterial activity is clearly an advantage. Other studies have demonstrated the strong and

broad range potential of PepBiotics against a wide range of gram-negative and gram-positive pathogens, including multiresistant bacterial isolates derived from CF-patients (manuscript in preparation). Resistance development studies have provided preliminary evidence that PepBiotics do not induce bacterial resistance. In addition, several PepBiotics exhibit anti-biofilm activity against preformed *Pseudomonas aeroginosa* biofilms, an important pathogen that contributes importantly to persistence of chronic lung infections and frequently develops in co-infection with fungal pathogens. Since the antimicrobial activity of PepBiotics is preserved at physiological conditions (0.9% NaCl) and most PepBiotics possess only mild cytotoxic properties, the data presented in this study indicate that PepBiotics have potential to be an important novel class of antimicrobial drugs to fight several clinically important fungal species effectively.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Author contributions

M.v.E., H.H., H.d.C., I.V., J.D., B.K., and C.v.d.E. designed experiments. M.v.E., H.H., H.d.C., and J.D. wrote the article. L.C., I.V., S.B., M.R., M.M., B.K., and C.v.d.E. read the article and approved it. L.C., S.B., M.R., M.M., and B.K, performed the experiments

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