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Novel Antifungal Peptaibols Emericellipsins A-E with Anticancer and Antibiofilm Potential from an alkalophilic fungus Emericellopsis alkalina

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Abstract: A novel complex of antimicrobial peptides with antifungal and cytotoxic activity was derived from the alkalophilic fungus Emericellopsis alkalina VKPM F-1428 isolated from soda soil. Novel peptides were assigned "Emericellipsins", so-called because of their source fungus. The complete primary structure and detailed biological activity were determined for the five of them. The dominant peptaibol - emericellipsin A (EmiA) showed strong antifungal, cytotoxic properties. The inhibitory activity of the major compound, EmiA against azole-resistant pathogenic Aspergillus spp., Candida spp. and Cryptococcus spp. was similar to amphotericin B. In addition, EmiA demonstrated low cytotoxic activity to the normal cell line (human postnatal fibroblasts, HPF) but possessed cancer selectivity to human myelogenous leukaemia (K-562) and human colon cancer (HCT-116) cell lines. Emericellipsin A revealed negligible hemolytic activity at concentrations of 0–20 μM, making it a low-toxicity compound regarding normal human cells, but with a potentially high therapeutic index. The inhibited effect of EmiA on biofilm formation of clinical pathogens Staphylococcus aureus and Candida albicans was determined. The results obtained suggest EmiA as a natural compound with a prospect for using it to develop a promising antifungal agent for invasive mycoses therapy, especially for treatment multi-drug resistant aspergillosis and cryptococcosis.

Keywords: peptaibols; emericellipsin A; antifungal drugs; invasive mycoses; cytotoxic activity

1. Introduction

The largest group of AMPs, the peptaibols, consist of a peptide core approximately 2–21 amino acids in size, attached to a nonpeptidic component. Peptaibols might own promising potential to be designed as peptide-based drugs because they act against cell membranes rather than a specific target, thus lowering the possibility of the onset of multi-drug resistance [1 - 3]. A majority of them summarized in the offline version of "Comprehensive Peptaibiotics Database" [2], online Norine database https://bioinfo.cristal.univ-lille.fr/norine/index.jsp; Protein databases https://www.rcsb.org/ and DBAASP https://dbaasp.org/home. Peptaibols are produced by fungi, especially of the Emericellopsis, Trichoderma, Acremonium genera. In growing microbial resistance to marketed antibiotics, peptaibols are of interest in the search's context for alternative sources of "next-generation" antibiotics. From the pharmacological perspective, peptaibols exhibit a variety of bioactivities, including antibacterial, antifungal, anticancer, immunosuppressive, antimycoplasmic and wound healing properties [1 -4].

A new species E. alkalina was isolated from halophilic environments in 2013. Its isolates grew well in a wide pH range (from 4.0 to 11.2) but with a slight optimum at 9-11, showing an alkaliphilic phenotype. Primary screening among 65 isolates of this species revealed E. alkalina strain VKPM F1428 the presence of antimicrobial activity against pathogenic micromycetes [3]. Further was carried out the isolation and structural and functional characterization of a novel, previously undescribed, secreted antimicrobial peptides copmplex, called emericellipsin A - E, which is a product of nonribosomal synthesis and belongs to the group of peptaiboles [3]. Here we focus on its antifungal, cytotoxic and antibiofilm activities.

2. Materials and Methods

Fungi were cultivated according to the previous protocol on special alkaline medium at 26 0C in Erlenmeyer flask in stationary conditions [2]. To isolate the antibiotic substances, the CF of the producers was extracted three times with ethyl acetate in a ratio of organic solvent: CF 1: 5. Chromatographic separation was carried out using a Phenomenex Jupiter C4 column (150× 2 mm,2 μm) (Phenomenex, USA) in a linear gradient of acetonitrile in water from 5% to 70% with addition of 0.1% formic acid. A typical profile was obtained, which contains a number of peaks eluted from the column from 60.0 to 66.5 min. All dominant peaks were collected manually to provide characterization at the structural and functional levels.

Experiments for determining cytotoxic activity of the compound in MTT assays were performed as previously described [4]. The following tumor cell lines were used for this study: HCT-116 (colon cancer cell line), B16 (mouse melanoma cell line), K-562 (leukemia cell line), MDA-MV-231 (human breast cancer line). Human postnatal fibroblasts were used as a normal cell line, and doxorubicin only was used as a positive control.

The spectrum of the antifungal action was also evaluated in clinical isolates of filamentous fungi and yeast with multiple resistance; these were obtained from the collection of the Moscow Government Health Department Scientific and Clinical Antituberculosis Center (Russia). Aspergillus niger 1133 m, A. terreus 497, A. fumigatus 390m, C.albicans 1582, C. glabrata 1402, C. tropicalis 156, C. krusei 1447, C. parapsilosis 571, Cryptococcus neoformans 297 and Cr. laurentii 325m were isolated from patients having invasive pulmonary aspergillosis and oropharyngeal HIV-positive patients with cryptococcosis. All clinical isolates demonstrated azole-resistance in vitro to commercial azoles. MICs and MFCs were measured as previously described [2].

Clinical bacterial isolates Staphylococcus aureus, Enterococcus faecalis and E. faecium were taken from the collection of cultures of the laboratory for infections related to the provision of medical care, Central Research Institute of Epidemiology (Moscow, Russia). MICs and the MBCs of antibiotics against the bacterial strains were determined by dilution method outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Staphylococci were characterized by sensitivity to aminoglycosides, fluoroquinolones, glycopeptides, and β-lactams, with the exception of penicillins; enterococci were characterized by sensitivity to ampicillin, vancomycin, linezolid, and nitrofurantoin (except resistance E. faecium to ampicillin). Bacterial isolates were incubated at 37°C for 24 h in the presence of various concentrations of emericellipsin A in 96-well plates with Teflon cubes. The cubes containing the biofilms were then transferred into wells of 96-well microtiter plate containing two hundred microliters of serial two-fold dilutions of antimicrobial peptide and were incubated for 18 hours at 37 °C. After biofilm treatment the teflon cubes were transferred into the neutralizer, and then sonicated for 30 minutes to dislodge the biofilm. The MBECb is defined as the minimum concentration of antimicrobial peptide that eradicates the biofilm. To determine the MBECb values, sonicated biofilms were recultivated in Mueller-Hinton broth and visually checked for turbidity in the wells. Count viable cells from biofilms were determined on plates. To determine the MICb values of peptides, a visual check for turbidity in the wells of the plate was performed. The MBCb value represents the lowest concentration of antimicrobial drug which kills the population of the dispersed cells from the biofilm [6].

3. Results

All compounds obtained were initially characterized and identified by HPLC/ESI-MS as a previously described. According to previously obtained data, the structure of the main antibiotic, emericellipsin A as determined by hetero nuclear NMR spectroscopy, has the following form: Methyldecanoyl-MePro-AHMOD-Ala-Aib-Ile-Iva-bAla-Alaol-Glyol. Furthermore, it contains nine amino acid residues with modification of the N-terminal amino group and C-terminal hydroxyl. The results made it possible to identify all four compounds as close homologues of emericellipsin A with single substitutions: from isovaline to α-aminoisobutiric acid ("B" form), from alanine to serine at position 3 ("C" form), from α-aminoisobutiric acid to isovaline at position 4 ("D" form), and from alaninol to α-aminoisobutiric at position 8 ("E" form) Table 1 [2]

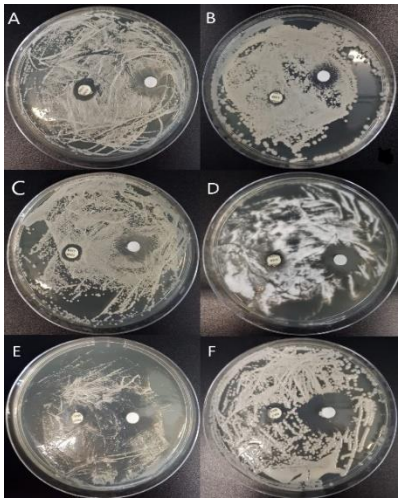
Table 1. Structural characterization of emericillipsins A-E.

Peptaibol	M+H, Da	Amino acid sequence
EmiA	1050.69	Methyldecanoyl-MePro-AHMOD-Ala-Aib-Ile-Iva-βAla-Alaol-Glyol
EmiB	1036.77	Methyldecanoyl-MePro-AHMOD-Ala-Aib-Ile-Aib-βAla-Alaol-Glyol
EmiC	1068.76	Methyldecanoyl-MePro-AHMOD-Ser-Aib-Ile-Iva-βAla-Alaol-Glyol
EmiD	1064.77	Methyldecanoyl-MePro-AHMOD-Ala-Iva-Ile-Iva-βAla-Alaol-Glyol
EmiE	1078.75	Methyldecanoyl-MePro-AHMOD-Ala-Aib-Ile-Iva-βAla-Aib-Glyol

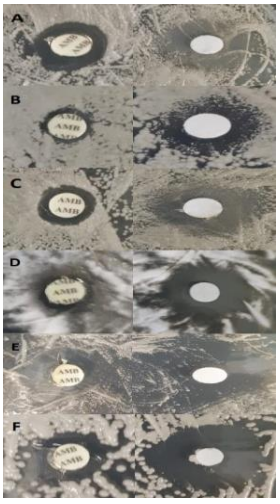
*all substitutions according to the EmiA sequence are inserted by red color. The reference structure of EmiA is framed. Methyldecanoyl – Methyldecanoyl;MePro - methylproline; AHMOD - 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid; Ala – alanine; Aib - α-aminoisobutiric acid; Ile - isoleucine; Iva – isovaline; βAla – β-alanine; Alaol - alaninol ; Glyol -glycinol; Ser - serine.

Each of individual compounds, EmiA – EmiE was tested on antifungal activity on opportunistic and clinical isolate's mold and yeast pathogen tests.

The lead compound EmiA inhibits the whole panel of opportunistic and clinical isolates in agar dilution assays (Figure 1). EmiD and EmiE were totally inactive against all reference and clinical strains of Aspergillus.



AmpB



EmiA

Figure 1. Antifungal activity of EmiA in disk diffusion assays against clinical pathogenic isolates: A - *Candida albicans* 1402, B - *Candida krusei* 1447, C - *Candida tropicalis* 156, D - *Aspergillus niger* 1133 m, E - *Aspergillus fumigatus* 390m, F - *Cryptococcus neoformans* 297.

EmiB and EmiC displayed low activity only against the A. niger strains, whereas both of these peptides were inactive against another Aspergillus spp. The MIC values for EmiA were in 0.5 – 4 μg/ml range and confirmed the higher activity of against clinical isolates.

The results of the quantitative analysis of antifungal activity against pathogenic multidrug yeast strains obtained with the MIC and MFC are shown in Table 2. EmiA was active against the isolates of C. albicans, C. glabrata, and C. neoformans with MIC/MFC in the range 0.25–4 μg/ml. In agreement with the preliminary results, the lowest activity (MIC = 2 μg/mL) was observed against C. albicans 1402. Fluconazole (FZ) showed little or no activity against the whole panel of Candida isolates, in accordance with previous findings that these species are resistant to commercial azoles. Interestingly, EmiA *exerted strong* antifungal *activity similar to that of AmpB* toward both species of Cryptococcus isolates that had intrinsic resistance to caspofungin (CAS) and FZ.

Table 2. MIC and MFC of emericellipsin A against clinical yeast isolates.

	Minimal Inhibitory/Fungicidal Concentration (MIC/MFC) (μg/mL)			
	Compound			
Strain	EmiA	AmpB	CAS	FZ
Candida albicans 1402	2/4	0,5/0,5	0,06/0,5	64/128
C. glabrata 1402	0,25/0,5	1/1	0,06/1	128/>256
C. krusei 1447	0,5/2	2/4	0,25/1	R
C. tropicalis 156	1/2	0,5/1	0,06/0,5	64/128
C. parapsilosis 571	1/1	1/1	1/1	128/>256
Cryptococcus neoformans 297	0,5/0,5	0.5/1	>64	16/128
Cr. laurentii 325m	0,5/0,5	0.25/0,5	16	32/128

FZ – fluconazole, CAS – caspofungin, AmpB – amphotericin B, EmiA – emericellipsin A.

We discovered cytotoxic action of major compound EmiA towards a spectrum of tumor cell lines in vitro. Normal cell line HPF was also tested. Thus, we determined the IC50 values for a wide range of active concentrations (1-16 μM) that could potentially result in different resistance among cancer cell lines to antibiotic action. Moreover, comparison of dose-dependent curves of EmiA and doxorubicin could reveal that they are different overall. As a result, the application of doxorubicin to cells at a low concentration even led to high mortality, whereas incubation with EmiA was strictly dose-dependent (Table 3).

Table 3. IC50 values of emericellipsin A compared with a control antitumor antibiotic (doxorubicin).

	$IC_{50,\ \mu g/mL}$					
Compound	**HCT-116**	**MCF-7**	**HPF**	**K-562**	**B16**	**MDA-MB-231**
EmiA	2.4±0,3	11.54±1,26	12.06±1,60	1,049±0,15	16,78±2,18	8,39±1,09
Doxorubicin	0.116±0,02	0.29±0,04	0.116±0,02	0.145±0,02	0.35±0,04	0.46±0,05

Emi A also inhibited the biofilm formation of S. aureus clinical isolates. The maximum effect was observed at peptide concentrations corresponding to 1/2 MBC and ranged from 73.2 to 97.8%, depending on the isolate (Table 4). At a concentration of 1.5 μg/mL, biofilm formation significantly decreased. MBEC (the minimum concentration of antimicrobial peptide that eradicates the biofilm) against S.aureus 2 was 50 μg/mL.

Table 4. MIC, MBC, and MBEC of emericellipsin A against clinical bacterial isolates.

Clinical isolates	**MICb,**	**MBCb,**	**MBECb,**

	μg/mL	(μg/mL)	(μg/mL)
S. aureus 1	12,5	25	100
S. aureus 2	12,5	25	50
S. aureus 3	25	100	100
S. aureus 4	12,5	25	100
E. faecium 5	25	100	200
E. faecalis 6	50	100	200
E. faecalis 7	50	100	200
E. faecalis 8	25	100	200
E. faecalis 9	25	100	200

4. Conclusion

Novel antimicrobial lipopeptaibols emericillipsins A- E from the alkaliphilic fungus E. alkalina promise treatment alternatives to licensed antifungal drugs for invasive mycosis therapy for multidrug-resistant aspergillosis and cryptococcosis. Emericellipsin A was like that of amphotericin B against drug-resistant pathogenic fungi. Also, it showed low cytotoxic activity towards the normal HPF line but possessed cancer selectivity to the K-562 and HCT-116 cell lines. It was shown for the first time that antimicrobial metabolites of fungi from the peptaibol group can inhibit pathogenic forms of bacteria by affecting biofilm formation, a key factor of antibiotic resistance.

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Conflicts of Interest: The author declares no conflict of interest.

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