ORIGINAL ARTICLE

The *in vitro* effects of new D186 dendrimer on virulence factors of *Candida albicans*

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The emergence of drug-resistant *Candida albicans* strains necessitates identifying new antimycotics along with studying their modes of action. The influence of a new rationally designed dendrimer D186 containing N,N-dioctyl tail and four tryptophane residues on inhibition of planktonic cells, aspartic protease *SAP5* expression and adhesion to epithelial cells was investigated. *In vitro* anti-*Candida* activities were determined against wild types, Δsap mutants and morphogenesis mutants: $\Delta efg1$, $\Delta cph1$ and $\Delta cph1/\Delta efg1$. MICs of D186 determined with M27-A3 protocol were in the range 2–16 $\mu\text{g ml}^{-1}$. Adherence assay of *C. albicans* to Caco-2 was performed in 24-well plate. Group I (MIC = 8 $\mu\text{g ml}^{-1}$, inhibition = 82.05–100%) was the most frequent followed by Group II (MIC = 4 $\mu\text{g ml}^{-1}$, inhibition = 99.64–100%) and Group III (MIC = 2 $\mu\text{g ml}^{-1}$, inhibition = 96.47–96.98%). *SAP5* expression was analyzed using RT-PCR; relative quantification was normalized against *ACT1* in cells after 18-h growth on Caco-2 cell line. D186 exhibited more potent inhibition activity (statistically significant $P \leq 0.05$) against $\Delta sap10$ and $\Delta sap9/\Delta sap10$ (MIC = 2 $\mu\text{g ml}^{-1}$) than the remaining strains tested. Pretreating cells with D186 significantly inhibited adhesion of all *Candida* strains compared with their non-treated counterparts ($P \leq 0.05$). D186 affected *SAP5* expression of all strains suggesting that this gene is controlled by environmental conditions. A hypothesis can be formulated that the hydrophobicity of D186 and presence of four Trp residues favors its accumulation in the membrane causing membrane disruption, especially facilitated in mutants perturbed in the cell wall compositions. The D186 mode of action was attributed to reduced virulence in terms of adhesiveness and pathogenic potential related to *SAP5* expression and morphogenesis.

The Journal of Antibiotics (2014) 67, 425–432; doi:10.1038/ja.2014.25; published online 2 April 2014

Keywords: antifungal activity; *Candida albicans*; virulence factors

INTRODUCTION

Candida albicans is the most common causative agent and accounts for more than half of the cases of fungal infections.¹ Furthermore, this microorganism is developing azole resistance, which is the most relevant medical problem.^{2–4} For these reasons, designing more effective antifungals is an important aspect of any anti-candidal therapy.

The secretion of proteases has been suggested as attractive targets for antimycotic agents.^{5–7} Genes belonging to the *SAP* family (*SAP1–10*) encoding secreted aspartyl proteases are expressed at various stages of the infection and specific Saps might have a special role in tissue invasion.^{8–15} To date, the *SAP4–SAP6* expression has been detected in *C. albicans* undergoing a transition from yeast to hyphae at neutral pH with *SAP5* as the predominant transcript.^{12,16–20} Owing to *SAP5* involvement in numerous events of the interaction of fungal cells with host structures, it is a promising target in the search for new antifungal drugs.

Adherence to host tissues as well as morphological versatility are thought to be important in the *C. albicans* virulence; several excellent reviews have been published recently.^{15,21,22} *C. albicans* contains the

hypha-associated genes *EFG1* and *CPH1* that mediate adhesion of *C. albicans* to mucosal surfaces.^{23–25} In particular, *Efg1* is a substrate for mammalian transglutaminase that mediates stable attachment of hyphae to epithelial cells.²³ *Efg1* has a major role in promoting filamentous growth and regulates the expression of several genes with a crucial function in the invasion of host cells or in biofilm formation.^{23,25} Another notable function of the *EFG1* gene is its direct impact on the upregulation of the *SAP4–6* expression, which are involved in the host cell invasion during hyphal growth *in vitro*.²⁵ As adhesion and morphogenesis are crucial for biofilm formation but are difficult to eradicate with conventional antifungal therapy, it is fundamental to develop new approaches to manage the factors (*EFG1* and *CPH1*) associated with morphogenesis and adhesion.

As the *SAP* expression was correlated with infections and Pepstatin A (N-acylpentapeptide isolated from *Streptomyces testaceus*) as well as with HIV-protease inhibitors (PIs) acting directly on Saps have been described,^{14,18,21,26–30} the interest in developing synthetic inhibitors of Sap and adhesion factors has increased considerably. The aspartyl PI inhibitors (ritonavir, saquinavir, indinavir, nelfinavir and amprenavir) used in HIV chemotherapy act directly on Saps1–3, inhibiting also

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Received 16 December 2013; revised 6 February 2014; accepted 17 February 2014; published online 2 April 2014

several relevant biological processes of *C. albicans*, such as growth and adhesion to mammalian cells.^{18,27–30} On the other hand, the inhibitory effect of HIV PIs in *in vitro* experimental models of candidiasis was observed at concentrations (μM range) much higher than those needed for HIV protease inhibition (nM range).

X-ray structures of aspartic proteinases with Pepstatin A, peptide-origin natural Sap inhibitor^{31,32} or with two bis-trifluoromethyl Pepstatin A analogs³³ emphasized importance of hydrophobic domains in inhibitor molecules. On the other side, several reports evidenced that natural tryptophan-rich peptides, such as indolicidin³⁴ or ApoEdpL-W—18-peptide derived from human ApoE apolipoprotein³⁵—have been shown to have antifungal activity against the *Candida* genus, related to their membranotropic properties. Moreover, substitution of hydrophobic amino acids in PMAP-23, anti-microbial peptide isolated from porcine myeloid with Trp, gave a series of peptides with enhanced antimycotic activity.³⁶

In view of these data in the present study, we synthesized novel amphiphilic dendrimer named **D186**, designed as branched analog of natural antimicrobial peptides (Scheme 1).³³ The molecule contains four tryptophan residues that in natural peptides (for example, indolicidin) facilitate high affinity to microbial membranes. For enhancement of hydrophobicity, dendrimer was supplemented with lipophilic N-dioctyl tail located at the C-terminus. The molecule is highly cationic due to protonation of the six primary and ternary amino groups.

The goal of the present study was to investigate the *in vitro* susceptibility of planktonic cells of *C. albicans* wild-type strains, the *Asap* and morphogenesis mutants: *Δefg1*, *Δcph1*, *Δcph1/Δefg1* against the new **D186** dendrimer. Furthermore, it was of interest to examine whether the expression of *SAP5* is inhibited by the compound during adhesion to a monolayer cell culture of colorectal carcinoma of Caco-2 (ATCC). Therefore, this study aimed to investigate the relation of *SAPs* and the transcriptional regulators (*EFG1* and *CPH1*) to antifungal resistance of *C. albicans* during two distinct modes of growth, namely the planktonic and adhesion.

MATERIALS AND METHODS

Strains and media

C. albicans strains^{20,23,37–41} used in the current study are listed in Table 1. *C. albicans* (strain 82) was isolated from blood samples from patient treated for *Ependymoma anaplasticum* (Staniszewska, 2009 unpublished data). The clinical isolate had been previously identified according to colony color on CHROMagar *Candida* medium and evaluated following API 20C AUX carbohydrate assimilation patterns (Staniszewska, 2009 unpublished data).¹⁷ Additionally, the identification procedure was confirmed with genetic methods using rDNA sequencing as previously described (Staniszewska, 2009 unpublished data).¹⁷ All the strains used in the present study were stored on ceramic beads in Microbank tube (Prolab Diagnostics, Richmond Hill, ON, Canada) at -70°C . Before the respective examinations, routine culturing of strains for growth was conducted at 30°C for 18 h in YEPD.⁴² L-Lysine and L-Trp were purchased from IRIS Biotech GMBH. *N,N'*-dicyclohexylcarbodiimide, hydroxybenzotriazol, chloroform, *N,N*-dimethylformamide, methyl alcohol (MeOH), ethylenediamine and sodium chloride (NaCl) were purchased from Sigma-Aldrich Inc (St Louis, MO, USA).

^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 500 or a Varian DRX 500 spectrometer, using deuterated solvents and TMS as an internal standard. Chemical shifts are reported as δ values in p.p.m. and coupling constants are in Hertz. The following abbreviations are used to describe NMR spectra: s—singlet, d—doublet, t—triplet, m—multiplet, en—ethylenediamine residue, doa—dioctylamine residue. Mass spectra were recorded with a Mariner ESI time-of-flight mass spectrometer (PerSeptive Biosystems, Ramsey, MN, USA) or a Synapt G2-S HDMS (Waters) mass spectrometer. Thin layer chromatography was performed on Merck aluminium sheet Silica Gel 60 F₂₅₄. Column chromatography was carried out using Merck silica gel (230–400 mesh) or Sephadex LH20. All solvents were reagent grade or HPLC grade and were used without any further purification.

Synthesis of dendrimer D186

As shown in Scheme 1, dendrimer **D186** was prepared by the divergent method by functionalization of the N^{α} - and N^{ϵ} atoms of L-lysine with methyl acrylate (**R4**) and the subsequent coupling of the C-terminus with *N,N'*-dioctylamine that yielded tetrabranch core molecule **D176**. Further extension of the four arms by reaction with ethylenediamine (**D179**) followed and

Scheme 1 Reagents and conditions for preparation of dendrimer **D186**.

Table 1 *Candida albicans* strains used in this study

Strain	Parental strain	Relevant characteristics or genotype	Reference
<i>Reference strains</i> ^a			
SC5314	None	Prototrophic wild-type strain	37
82	None	Clinical wild-type strain	(Staniszewska, 2009 unpublished data)
CAI4	SC5314	<i>ura3Δ::imm434/ura3Δ::imm434</i>	38
CAF2-1	SC5314	<i>ura3Δ::imm434/URA3</i>	39
<i>Δsap4 single mutant</i> ^b			
SAP4MS4B	SAP4MS3B	<i>sap4-1Δ::FRT/sap4-2Δ::FRT</i>	20
<i>sap5Δ single mutants</i> ^a			
SAP5MS4B	SAP5MS3B	<i>sap5-1Δ::FRT/sap5-2Δ::FRT</i>	20
<i>sap4Δ sap5Δ sap6Δ triple mutants</i> ^a			
SAP456MS4B	SAP456MS3B	<i>sap4-1Δ::FRT/sap4-2Δ::FRT; sap5-1Δ::FRT/sap5-2Δ::FRT; sap6-1Δ::FRT/sap6-2Δ::FRT</i>	20
<i>sap8Δ, sap9Δ, sap10Δ and sap9/10Δ mutants</i> ^b			
<i>sap8Δ</i>		<i>Δsap8::hisG/Δsap8::hisG-URA3-hisG</i>	39
<i>sap9Δ</i> + pClp10	CAI4	CAI4, <i>sap9Δ::hisG/sap9Δ::hisG</i> + pClp10 (integration)	40
<i>sap10Δ</i> + pClp10	CAI4	CAI4, <i>sap10Δ::hisG/sap10Δ::hisG</i> + pClp10 (integration)	40
<i>sap9/10Δ</i> + pClp10	CAI4	CAI4, <i>sap10Δ::hisG/sap10Δ::hisG sap9Δ::hisG/sap9Δ::hisG</i> + pClp10 (integration)	40
<i>efg1Δ and cph1Δ mutants</i> ^b			
Can16	CAI4	<i>ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG-URA3-hisG</i>	41
YLO323	CAI4	<i>ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG(CPH1)</i>	23
HLC52	CAI4	<i>ura3::1imm434/ura3::1imm434 efg1::hisG/efg1::hisG-URA3-hisG</i>	23
HLC74	CAI4	<i>ura3::1imm434/ura3::1imm434 efg1::hisG/efg1::hisG (EFG1)</i>	23
HLC84	CAI4	<i>ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG (EFG1)</i>	23

^aApart from indicated features all strains are identical to their parental strain.^bFull genotype.

subsequent coupling with Boc-L-Trp yielded dendrimer **D186**. Dendrimer was prepared as water-soluble hexahydrochloride.

N,N'-tetrakis(methoxycarbonyl)ethyl-L-lysine (**R4**). L-Lys (5 mmol) was suspended in 7.5 ml of MeOH, 0.2 g (5 mmol) NaOH in MeOH and 2.7 ml (30 mmol) of methyl acrylate was added. The reaction was carried out at the boiling point for 48 h, then cooled, the solvent evaporated, and the oily residue was shaken with 15 ml of acetone and 5 ml of 1 M HCl in MeOH. The solution was filtered and evaporated to dryness. The residue was purified using chromatography on silica gel with the eluent MeOH/CHCl₃, to yield 1.30 g (53%) *N,N'*-tetrakis(methoxycarbonyl)ethyl-L-lysine as colorless oil.

MSLR (ESI, MeOH): 491 [M + H]⁺, 513 [M + Na]⁺.

¹H NMR (500 MHz, CD₃OD) δ 3.67 (2 s, 12 H, OCH₃); 3.35–3.32 (m, 1 H, α-Lys); 3.15–2.96 (2 m, 4 H, α-N-CH₂-C); 2.95–2.91 (m, 4 H, ε-N-CH₂-C); 2.64–2.59 (m, 2 H, ε-Lys); 2.59–2.53 (m, 9 H, α, ε-CH₂COOMe), 1.79–1.59 (2 m, 2 H, β-Lys); 1.58–1.51 (m, 2 H, δ-Lys); 1.44–1.25 (m, 2 H, γ-Lys).

¹³C NMR δ 175.9 (COOH), 172.6, 172.7 (COOMe), 63.8 (α-C), 53.1 (ε-C), 51.5, 51.6 (OCH₃), 47.0, 48.6 (α, ε-N-(CH₂)₂), 31.6, 33.7 (α, ε-CH₂COOMe), 28.6 (β-C), 26.1 (δ-C), 24.6 (γ-C).

N,N'-tetrakis(methoxycarbonyl)ethyl-L-lysine diethylamide (**D176**). One gram (2.04 mmol) of *N,N'*-tetrakis(methoxycarbonyl)ethyl-L-lysine was dissolved in 10 ml of *N,N*-dimethylformamide, 0.493 g (2.04 mmol) of diethylamine, 0.312 g of hydroxybenzotriazol (2.04 mmol, monohydrate) and 0.462 g of *N,N'*-dicyclohexylcarbodiimide (2.24 mmol) were added, and the mixture was stirred at room temperature for 48 h. The DCU precipitate was then filtered off and the solvent was evaporated. The oily residue was dissolved in 30 ml of AcOEt and washed with 30 ml of 10% aqueous Na₂CO₃, 1% citric acid and brine. The organic layer was dried over anhydrous MgSO₄, filtered and concentrated in

vacuo. Purification by chromatography on silica gel with the eluent MeOH/CHCl₃ affords 1.049 g of the compound as colorless oil. Yield is 72%.

MSLR (ESI, MeOH): 714.6 [M + H]⁺, 736.7 [M + Na]⁺.

¹H NMR (CD₃OD) 3.65, 3.69 (2 s, 12 H, OCH₃); 3.60–3.50 (m, 2 H, α-Lys, C¹H₂-doa); 3.41–3.35 (m, 1 H, C¹H₂-doa); 3.22–3.12 (m, 2 H, C¹H₂-doa); 2.94–2.82 (m, 4 H, ε-N-CH₂-C); 2.78–2.71 (m, 4 H, α-N-CH₂-C); 2.48–2.36 (m, 10 H, ε-Lys, α, ε-N-CH₂-CH₂-C), 1.80–1.50 (m, 6 H, β-Lys, C²H₂-doa); 1.48–1.40 (m, 2 H, δ-Lys); 1.39–1.25 (m, 20 H, C³H₂-C⁷H₂-doa), 1.22–1.10 (m, 2 H, γ-Lys); 0.94–0.87 (m, 6 H, C⁸H₂-doa).

¹³C NMR (CD₃OD) 174.6, 174.5, 173.8 (CONH), 62.4 (α-C-Lys), 54.7 (ε-C-Lys), 52.8 (OCH₃), 50.3–47.6 (α, ε-N-CH₂), 48.3, 47.6 (C¹-doa), 35.2, 33.1 (CH₂-CONH-Lys), 33.0, 32.9 (C⁶-doa), 30.6 (β-C-Lys), 30.5, 30.4, 28.7, 28.1, 27.9 (C^{2,4,5}-doa), 28.1 (δ-C-Lys), 27.1 (C³-doa), 25.6 (γ-C-Lys), 23.7 (C⁷-doa), 14.4 (C⁸-doa).

N,N'-tetrakis(aminoethylaminocarbonyl)ethyl-L-lysine diethylamide (**D179**). A total of 0.630 g (0.882 mmol) of the amide was dissolved in 6 ml of MeOH and added very slowly dropwise to a mixture of 7 ml (106 mmol) of ethylenediamine and 14 ml of MeOH cooled to 0 °C. The reaction was then stirred at the room temperature for 6 days, the solvent was evaporated to dryness, 10 ml of 1-butanol was added to the residue and evaporated again. The prepared core compound, after being dried on a rotary evaporator for 4 h, was purified by molecular filtration on the Sephadex LH-20 packing in MeOH, to yield 0.707 g (97%) of the pure compound as yellow oil.

MSLR (ESI, MeOH): 826.9 [M + H]⁺.

¹H NMR (CD₃OD) 3.65–3.45 (m, 2 H, α-Lys, C¹H₂-doa); 3.44–3.15 (m, 11 H, C¹H₂-doa, CH₂NH); 3.04–2.70 (m, 12 H, α, ε-N-CH₂-C, CH₂NH₂); 2.78–2.71 (m, 4 H, α-N-CH₂-C); 2.52–2.30 (m, 10 H, ε-Lys, α, ε-N-CH₂-CH₂-

C), 1.78–1.40 (m, 8 H, β , δ -Lys, C²H₂-doa); 1.37–1.23 (m, 20 H, C³H₂-C⁷H₂-doa), 1.22–1.11 (m, 2 H, γ -Lys); 0.93–0.86 (m, 6 H, C⁸H₂-doa).

¹³C NMR (CD₃OD) 175.4, 175.3, 175.0, 174.0 (CONH), 62.2 (α -C-Lys), 54.5 (ϵ -C-Lys), 50.8–47.7 (α , ϵ -N-CH₂), 48.3, 47.7 (C¹-doa), 42.3, 42.1 (CH₂NH₂), 41.8, 41.6 (NH-CH₂(en)), 36.4, 34.4 (CH₂-CONH-Lys), 33.0 (C⁶-doa), 30.7 (β -C-Lys), 30.6, 30.5, 30.4, 28.7, 28.5, 28.0 (C^{2,4,5}-doa), 27.9 (δ -C-Lys), 27.8 (C³-doa), 25.7 (γ -C-Lys), 23.7 (C⁷-doa), 14.4 (C⁸-doa).

Dendrimer D186. A total of 0.289 g (0.948 mmol) of Boc-L-Trp was dissolved in 4 ml of *N,N*-dimethylformamide and cooled to 0 °C, 0.178 g (0.215 mmol) of *N,N'*-tetrakis(aminoethylaminocarbonyl)ethyl-L-lysine dioctylamide, 0.145 g of hydroxybenzotriazol (0.948 mmol, monohydrate) and 0.196 g of *N,N'*-dicyclohexylcarbodiimide (0.948 mmol) were added. After 60 min at 0 °C the mixture was stirred at room temperature for 48 h. The DCU precipitate was then filtered off and the solvent evaporated. The raw dendrimeric compound was initially purified by molecular filtration on the Sephadex LH-20 packing in MeOH, and then using chromatography on silica gel with the eluent MeOH/CHCl₃, to yield 0.263 g (62%) of the pure compound as a white solid. Then the dendrimer was converted to its hexahydrochloride by deprotection of Boc groups with HCl-saturated AcOEt. The crude was purified by RP-HPLC (Kromasil column: 100–5-C18, 21.2 × 250 mm) using a linear gradient of 40–90% methanol in water (containing 0.5% HCl), at a flow rate of 15 ml min⁻¹, to yield D186 as a lightly hygroscopic, white amorphous foam.

MSLR (ESI, MeOH): 1571.0 [M + H]⁺, 1592.9 [M + Na]⁺, 786.0 [M + 2 H]²⁺, 812.9 [M + H + Na + MeOH main peak]²⁺, 542.2 [M + 2 H + Na + MeOH]³⁺.

¹H NMR (CD₃OD) 7.73–7.64 (m, 4 H, C⁴H-Trp); 7.39 (d, 4 H, *J* = 8.08 Hz, C⁷H-Trp); 7.24 (m, 4 H, C²H-Trp); 7.13 (t, 4 H, C⁶H-Trp); 7.05 (m, 4 H, C⁵H-Trp); 4.67 (t, 1 H, α -Lys); 4.20–4.13 (m, 4 H, α -Trp); 3.75–3.70 (m, 1 H, C¹H₂-doa); 3.50–3.05 (m, 36 H, β -Trp, NH-(CH₂)₂-NH, C¹H₂-doa, α , ϵ -N-CH₂-C, ϵ -Lys); 2.90–2.65 (m, 9 H, CH₂CONH, C¹H₂-doa); 2.00–1.90 (m, 2 H, β -Lys); 1.75–1.65 (m, 2 H, δ -Lys); 1.65–1.45 (m, 4 H, C²H₂-doa); 1.44–1.20 (m, 22 H, γ -Lys, C³H₂-C⁷H₂-doa); 0.92–0.84 (m, 6 H, C⁸H₃-doa).

¹³C NMR (CD₃OD) 172.5, 170.7, 167.8 (CONH-Lys, CONH-Trp), 138.2 (C^{7a}-Trp), 128.4 (C^{3a}-Trp), 125.8 (C²-Trp), 123.0 (C⁶-Trp), 120.3 (C⁵-Trp), 119.4 (C⁴-Trp), 112.7 (C⁷-Trp), 108.2 (C³-Trp), 64.4 (α -C-Lys), 55.3 (α -C-Trp), 53.9 (ϵ -C-Lys), 50.9 (α , ϵ -N-CH₂), 47.9 (C¹-doa), 40.1–40.0 (NH-CH₂(en)), 33.0 (C⁶-doa), 30.5, 30.4 (C^{4,5}-doa), 30.3, 30.0 (CH₂-CONH-Lys), 29.2 (β -C-Lys), 28.8 (β -C-Trp), 28.1, 28.0 (C^{3,4}-doa), 24.5 (δ -C-Lys), 23.7 (C⁷-doa), 22.5 (γ -C-Lys), 14.5 (C⁸-doa).

Anti-candidal activity against planktonic growth

Antifungal susceptibility of the compound was determined for each strain using the broth microdilution reference method as recommended by the Clinical and Laboratory Standards Institute (CLSI) document M27-A3.⁴² *C. albicans* SC5314 strain was used as a control. The final inoculum of fungal suspension from 0.5×10^2 to 2.5×10^3 cells (ml saline)⁻¹ was prepared in synthetic RPMI medium (Sigma-Aldrich Inc) following the CLSI document M27-A3. The compound test wells were prepared with stock solution of the compound (1600 µg ml⁻¹) dissolved in water with 0.9% (v/v) addition of dimethylsulfoxide (DMSO). Subsequently, serial twofold dilutions were made, using RPMI as a solvent. Then, the fungal blastoconidial suspension and the antifungal agent (final dilution 1:100) were dispensed into 96-well microplates. The compound was tested at seven concentrations that ranged from 0.25 to 16 µg ml⁻¹. The DMSO concentration was maintained at 0.009% (v/v) in all the experiments, including the control ones. At this concentration, DMSO was not able to inhibit the growth of *C. albicans*. Growth control wells (containing medium, inoculum, the same amount of DMSO used in compound test well, but compound-free) and sterility control wells (sample, medium and sterile water replacing inoculum) were included for each strain tested. MIC, namely the lowest compound concentration for which the well was optically clear, was determined visually after 48 h of incubation at 35 °C. Moreover, the microplates were read with the Infinite M200 PRO NANOQuant microplate reader (Tecan Group Ltd, Männedorf, Switzerland). Additionally, MIC was read spectrophotometrically (OD₄₀₅) and the end point was calculated as a

50% reduction in OD₄₀₅ as compared with the growth in the control well. Amphotericin B (Sigma-Aldrich Inc) was used as a positive control (100% inhibition). Growth reduction for each compound concentration was calculated as follows: % of inhibition: 100—(OD₄₀₅ compound test well—OD₄₀₅ sterility control well)/(OD₄₀₅ growth control well—OD₄₀₅ sterility control well).⁴³ In order to validate our experiments, MIC value of 1 µg ml⁻¹ was obtained for Amphotericin B (Sigma-Aldrich Inc). Amphotericin B was diluted in DMSO (1600 µg ml⁻¹) to be subsequently used in the assay as a reference antifungal at the concentration of 1 µg ml⁻¹.

Inhibition of *C. albicans* adhesion to Caco-2 cell line (ATCC HTB27, LGC, Poland): macrowell-based assay

Following the supplier's guidelines, monolayers of the colon adenocarcinoma-derived cell line were cultured in the Eagle's Minimum Essential Medium (EMEM) containing fetal calf serum 10% (v/v) and pyruvic acid 1 mM, without antibiotics or antifungal agents and maintained in a humidified incubator at 37 °C in 5% (v/v) CO₂. For the experiment 1.2×10^5 of Caco-2 cells (ml EMEM)⁻¹ were seeded into 24-well plates (Corning, New York, NY, USA) and cultured up to 18 h. Subsequently, the blastoconidia were grown overnight in the YEPD medium at 30 °C. Then 200 µl of 10^4 blastoconidial cells (ml saline)⁻¹ were pre-incubated with 1800 µl of the RPMI medium (containing selected concentration of D186) for 2 h on a shaker at 35 °C. Following the pre-incubation, the cells were washed twice with phosphate-buffered saline and resuspended in 2 ml of fresh RPMI medium. The wells of the plate containing adherent endothelial Caco-2 cells were washed twice with phosphate-buffered saline and then incubated with the blastoconidial suspension (2000 µl) for 2 h at 37 °C at 5% (v/v) CO₂. Next, the non-adherent cells were removed by standard rinsing. The Caco-2 was lysed by adding sterile water resulting with *C. albicans* cells' recovery. After 18-h growth on the Sabouraud dextrose agar plates at 30 °C, the number of adherent cells was determined by colony counting and compared with the controls. Adherence was expressed as a percentage of the total number of cells added (control cells). The adhesion of the control cells was assessed as described above (but without antifungal compound). Additionally, the adhesion assay was performed in the presence of Amphotericin B at the concentration ranging from 0.25 to 2 µg ml⁻¹ as well as pepstatin A at the concentration of 15 µM. Pepstatin A stock solution was dissolved in DMSO (1 mg ml⁻¹) and heated at 60 °C for complete dissolution.

RT-PCR analyses to assess the effect of the D186 peptide on the SAP5 gene expression

Total RNA from *C. albicans* cells non- and pre-incubated with D186 (as described above) was extracted.⁴⁴ Before further examinations, total RNA of *C. albicans* was stored at -20 °C. First-strand complementary DNA synthesis was performed using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich Inc) according to the manufacturer's instructions. One microliter of total RNA, and 1 µl of oligo (dT)₂₃ (3.5 µM), and 1 µl of dNTP mix (500 µM each dNTP) were added to each tube to obtain 10 µl volume. Priming was carried out at 50 °C for 10 min. Then subsequently, 1 µl of Enhanced avian AMV-RT (1 U µl⁻¹), 1 µl of 10 × buffer for AMV-RT were added to each tube to obtain 20 µl volume. The RT reaction was carried out at 50 °C for 50 min. Appropriate Taq-Man primer set was designed for SAP5 (F-5'-CATGTGCAAGTAAGTCAACAG-3' and R-5'-CAGAAATTTCCCGTCGATGAGA-3') and ACT1 (F-5'-GACAATTTCTCTTCAGCACTAGTAGTGA-3' and R-5'-GCTGGTAGAGACTTGACCAACCA-3') as described previously.¹⁹ Complementary DNA was quantified using the FastStart Essential DNA Green Master (Roche, Germany) according to the manufacturer's instructions. Each reaction mixture (15 µl) contained FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, 2 µl (600 nm) of each forward primer and reverse primer, water PCR grade and 5 µl of template 60 ng complementary DNA.¹⁹ For reliable normalization of the SAP5 gene expression data in *C. albicans* cells grown for 18 h in both media, we used the housekeeping gene ACT1 as a reference gene. The real-time PCR reactions were performed¹⁹ at 95 °C for 15 min, followed by 45 cycles for 15 s at 94 °C and 1 min at 60 °C with the Rotor Gene 6000 (RoCorbett, Qiagen, Germany). The C_t values were provided from RT-PCR instrumentation and were imported into a spreadsheet Microsoft Excel 2010. The relative

quantification was calculated using Eq.,⁴⁵ where $\Delta C_t = \text{Avg. SAP5 } C_t - \text{Avg. ACT1 } C_t$ and $\Delta\Delta C_t = \Delta C_t - \Delta C_{\text{reference strain}}$.

Statistical analysis

Each experiment was performed in triplicate on three separate occasions. The percentage of cell growth inhibition as well as inhibition of adhesion and the SAP5 expression were formulated as a mean $\pm$ s.d. Statistical differences were evaluated through comparison with the Wilcoxon test, P -values ≤ 0.05 were considered significant.

RESULTS

Antifungal activity

At each concentration tested, the percentage of inhibition displayed by **D186** was determined. The antifungal potency of **D186** was compared with Amphotericin B and their MIC values were summarized in Table 2. A close survey of the MIC values indicated that the compound has a good antifungal effect against all the tested *Candida* strains at concentrations from 2 to 16 $\mu\text{g ml}^{-1}$. According to spectrophotometric assessment at 405 nm after 48 h, **D186** showed percentages of inhibition ranging from $98.77\% \pm 1.61$ to $100\% \pm 0.02$ for the highest concentration tested, thus possessing a $\text{MIC}_{90} \leq 16 \mu\text{g ml}^{-1}$ (Table 2). At $8 \mu\text{g ml}^{-1}$, **D186** more efficiently affected the wild-type strains (SC5314, CAF2-1), the single mutants (Δcph1 , Δefg1), Δefg1 reverted with one copy of *EFG1*, and the mutants $\Delta\text{sap4}/\Delta\text{sap5}/\Delta\text{sap6}$ decreasing the growth by 100%. The compound completely inhibits the growth of Δefg1 ($\% = 100$) at concentration of $\text{MIC} = 4 \mu\text{g ml}^{-1}$. Meanwhile, the activity of **D186** gave a twofold increase against Δsap10 and $\Delta\text{sap9}/\Delta\text{sap10}$ with MIC value of $2 \mu\text{g ml}^{-1}$. Thus, Table 2 shows three different groups, based on the MIC values of the tested antifungal substance at a concentration of $2\text{--}16 \mu\text{g ml}^{-1}$. The groups differed among their response to the compound. Group I ($\text{MIC} = 8 \mu\text{g ml}^{-1}$, inhibition = $82.05\text{--}100\%$) was the most frequent one followed by Group II ($\text{MIC} = 4 \mu\text{g ml}^{-1}$, inhibition = $99.64\text{--}100\%$) and Group III ($\text{MIC} = 2 \mu\text{g ml}^{-1}$, inhibition = $96.47\text{--}96.98\%$). To evaluate whether the differences between the wild-type strain SC5314 and mutant strains were significant, we used the non-parametric Wilcoxon matched pair test. At a concentration of

$4 \mu\text{g ml}^{-1}$, statistically significant differences in the susceptibilities to **D186** were found between SC5314 and the remaining strains ($P \leq 0.05$).

Effect of the D186 peptide dendrimer on the adhesive properties of the *C. albicans* species

Pretreating the cells with **D186** ($4\text{--}16 \mu\text{g ml}^{-1}$) inhibited significantly the adhesion of all *Candida* strains tested compared with their non-treated counterparts ($P \leq 0.05$, Table 3). Although lowest concentration of the compound (range $1\text{--}2 \mu\text{g ml}^{-1}$) was used, the strains SC5314 and Δsap8 showed an increase in adhesion. On the other hand, the increase in adhesion was not statistically significant ($P \geq 0.05$). The inhibition of adhesion exhibited by **D186** was concentration-dependent. The compound shared remarkable anti-adherence properties compared with Pepstatin A; however, these features were eightfold lower in comparison with Amphotericin B.

D186 modulates SAP5 mRNA expression

The results indicated that the SAP5 gene expression was highly induced in all the strains tested during adhesion to Caco-2 cell line and decreased upon treatment of *C. albicans* with **D186** (Figure 1). The compound significantly affected the SAP5 expression at all of the tested concentrations ($2\text{--}8 \mu\text{g ml}^{-1}$), showing a decreased SAP5 mRNA expression in *C. albicans* cells during the contact with Caco-2 cell line. No significant differences between the SAP9- and SAP10-deficient strains and the wild types were observed after 2 and 18 h, respectively. In the mutants tested during the growth in epithelial cells, the lack of one or two SAP9 or/and SAP10 gene activity was compensated by an increased expression of SAP5. The mutant lacking SAP9 displayed a significant downregulation of SAP5 in the presence of **D186** at the concentration of $2 \mu\text{g ml}^{-1}$ after a 2-h treatment.

DISCUSSION

In our study, the dendrimeric compound **D186** was first tested for its activity against planktonic cells of *C. albicans*. While the therapy for *C. albicans* infections has become a challenge, it is worth noting that

Table 2 Percentage of *Candida albicans* cells inhibited (mean $\pm$ s.d.) by the D186 synthetic peptide

<i>Candida albicans</i>	Group	MICs (μg ml ⁻¹)							
		16	8	4	2	1	0.5	0.25	1 ^a
SC5314	II	100±0.19	100±0.26	99.64±0.25	99.18±0.28	99.06±0.03	98.58±0.18	98.93±0.06	100
CAF2-1	I	100±0.52	100±0.16	98.74±0.25	99.24±0.14	98.72±0.47	98.80±0.09	98.87±0.07	100
CAI4	I	99.93±0.14	99.71±0.03	99.65±0.03	98.63±0.25	98.81±0.23	98.93±0.08	99.21±0.11	100
Δsap4	I	100±0.04	99.55±0.25	98.66±0.24	98.86±0.1	98.07±0.51	98.91±0.12	98.91±0.02	100
Δsap5	I	100±0.02	99.62±0.16	98.99±0.19	98.97±0.16	98.28±0.36	98.99±0.13	98.99±0.14	100
Δsap456	I	100±0.4	100±0.24	99.09±0.06	99.18±0.17	98.97±0.25	99.22±0.08	99.11±0.07	100
Δsap10	III	99.97±0.26	99.87±0.41	98.88±1.02	98.98±2.84	98.19±0.72	98.15±0.95	98.21±1.23	100
Δsap9/10	III	98.77±1.61	98.77±1.37	98.67±0.98	96.47±3.19	94.88±6.07	98.03±1.2	96.82±2.41	100
Δsap9	I	100.33±0.08	99.99±0.04	99.58±0.39	99.14±0.22	98.43±0.43	98.70±0.04	98.84±0.00	100
Δsap8	I	99.78±1.52	99.70±0.28	95.58±18.23	99.99±1.31	99.89±3.74	98.54±18.59	100±3.41	100
Δcph1/Δefg1 (EFG1)	I	99.91±21.96	82.05±24.92	81.99±24.87	81.73±24.68	81.67±24.63	81.76±24.70	81.77±24.70	100
Δefg1 (EFG1)	II	99.89±0.11	99.98±0.21	99.85±0.19	99.44±0.38	99.52±0.49	99.13±0.64	99.32±0.45	100
Δcph1 (CPH1)	I	100±7.76	100±3.03	98.63±3.92	98.64±3.31	100±7.24	98.79±1.35	99.93±0.24	100
Δcph1	I	100±0.27	100±0.13	99.88±0.14	99.86±0.78	99.85±0.96	99.85±0.96	99.80±0.76	100
Δefg1	II	100±0.21	100±0.09	100±0.03	99.73±1.01	99.79±1.22	99.77±1.08	99.77±1.09	100

At concentration of $16 \mu\text{g ml}^{-1}$ the following were noted: Group I, cell inhibition = $82.05\text{--}100\%$; Group II, inhibition = $99.64\text{--}100\%$; Group III, inhibition = $96.47\text{--}96.98\%$.

^aAmphotericin B in concentration of $1 \mu\text{g ml}^{-1}$ was used as a control (inhibition = 100%). The MICs assessed visually are denoted in bold. Statistically significant differences ($P \leq 0.05$) in reduction were observed between SC5314 and the remaining strains at $4 \mu\text{g ml}^{-1}$.

Table 3 The percentage of adhesion of *C. albicans* cells to the Caco-2 cell line after pretreatment with the D186 peptide

Concentration of compounds ($\mu\text{g ml}^{-1}$)		SC5314		Δsap8		Δsap9		Δsap10	
		Adherent cells	R	Adherent cells	R	Adherent cells	R	Adherent cells	R
D186	16	0.02 $\pm$ 0.02	95.0	0.10 $\pm$ 0.10	96.0	3.72 $\pm$ 6.30	62.50	0.36 $\pm$ 0.40	97.0
	8	0.06 $\pm$ 0.03	88.7	1.10 $\pm$ 1.27	40.0	5.51 $\pm$ 7.80	44.50	0.24 $\pm$ 0.05	97.9
	4	0.51 $\pm$ 0.4	4.30	1.03 $\pm$ 1.37	43.8	3.00 $\pm$ 4.98	69.70	0.80 $\pm$ 0.53	93.2
	2	1.10 $\pm$ 1.32	I	16.0 $\pm$ 14.1	I	4.87 $\pm$ 6.82	50.93	3.10 $\pm$ 4.01	73.7
	1	0.84	I	22.03 $\pm$ 31.06	I	5.52 $\pm$ 7.74	44.38	Nt	Nt
Amphotericin B	2	0.10 $\pm$ 0.02	75.0	0.20 $\pm$ 0.03	87.7	0.01 $\pm$ 0.01	99.85	0.03	99.7
	1	0.20 $\pm$ 0.25	70.0	Nt	Nt	0.12 $\pm$ 0.10	98.8	0.04	99.7
	0.25	Nt	Nt	0.34 $\pm$ 0.15	81.4	1.16 $\pm$ 1.94	88.3	0.07	99.4
Pepstatin A	15	0.05	90.6	0.136	92.6	0.12	98.7	0.10	99.1
Non-treated control cells		0.533 $\pm$ 0.321	Nt	1.83 $\pm$ 1.55	Nt	9.92 $\pm$ 8.10	Nt	11.80 $\pm$ 7.53	Nt

Abbreviations: I, stands for increased adhesion %; Nt, stands for not tested; R, adherent cell reduction %.

Significant reduction of adhesive properties ($P \leq 0.05$) in bold (compared with non-treated control cells).

Adhesion data calculated for cells grown on Sabouraud agar of 24-well plate. Adherence was expressed as a percentage of the total number of cells added (control cells non-treated).

Data are expressed as the mean $\pm$ s.d. of three independent experiments.

Figure 1 Analysis of the *SAP5* gene relative expression in *C. albicans* cells after treatment with different concentration of the D186 peptide. Pretreated cells were grown for 18 h on the Caco-2 monolayer. Data are the means from three independent experiments.

D186 displayed low MIC₉₀ value ($4 \mu\text{g ml}^{-1}$) against the *C. albicans* SC5314 reference strain. In the study, the disruption of the *SAP9–10* genes in the *C. albicans* strains influenced the increase in the tested compound's activity (MIC = $2 \mu\text{g ml}^{-1}$, $P \leq 0.05$). Moreover, it should be noted that the antifungal effects against the strains that had been deleted for *EFG1* (MIC = $4 \mu\text{g ml}^{-1}$) and *CPH1* (MIC = $8 \mu\text{g ml}^{-1}$) are slightly lower. The transcriptional factors *Efg1* and *Cph1* strongly have an impact on cell wall thickness as well as polysaccharide composition, even if only one copy of the *EFG1* or *CPH1* gene is removed.⁴⁶ In our study (Table 2), we showed that the Δefg1 null mutant is highly sensitive to D186 (cell inhibition = 100% at MIC = $4 \mu\text{g ml}^{-1}$), whereas such sensitivity was slightly reduced in the strain into which one copy of *EFG1* was reintroduced (cell inhibition = 99.85%). In addition, this compound was able to promote a comparable cell inhibition (100%) of Δcph1 at twofold increased concentration of $8 \mu\text{g ml}^{-1}$. Thus, chitin and glucan structures perturbed in the *EFG1*- and/or *CPH1*-deleted strains as well as the $\Delta\text{sap9}/\Delta\text{sap10}$ mutants^{40,46} significantly increase the fungicidal potential of the compound.

We tested sensitivity of the wild-type strains, the Δsap and morphogenesis mutants against D186 in the spectrophotometrically and visually read assays (Table 2). In the former analysis at 405 nm after 48 h of incubation, we did not observe a clear end point (inhibition = 100%) in the case of Δsap10 , $\Delta\text{sap9}/\Delta\text{sap10}$, Δsap8 , the wild-type strain CAI4 and the mutants reverted with one copy of *EFG1*. We thus assessed MIC₉₀ $\leq 16 \mu\text{g ml}^{-1}$ as a highest MIC concentration (the highest concentration with incomplete killing). On the other hand, the observable results showed MIC values with clear end point at concentration $\geq 2 \mu\text{g ml}^{-1}$ (in the case of Δsap10 and $\Delta\text{sap9}/\Delta\text{sap10}$), $\geq 4 \mu\text{g ml}^{-1}$ (SC5314, Δefg1 and its reverted strain) and $\geq 8 \mu\text{g ml}^{-1}$ (the remaining strains tested).

Interestingly, a 90-min pretreatment of planktonic cells of all the strains tested with the compound significantly affected attachment to Caco-2 (Table 3). The number of adherent cells on the surface of monolayer was reduced by 62.5–97.0% after 90-min treatment with D186 at $16 \mu\text{g ml}^{-1}$ compared with the compound-free control. In the case of the reference strain SC5314, in the presence of 16, 8 and $4 \mu\text{g ml}^{-1}$ of the compound, adherence of fungal cells was reduced by

95.0, 88.7 and 4.3%, respectively. Thus, the results showed that attachment of SC5314 to Caco-2 was inhibited by the compound in a concentration-dependent manner. Moreover, the data suggested that Sap9–10 may interact with inhibitory potency of **D186** in adhesion to the Caco-2 monolayer. The antifungal compound **D186** remarkably inhibited the adhesiveness of the *SAP9* and *SAP10* null mutants to intestinal cells (enterocytes) even at low concentration of $2\text{ }\mu\text{g ml}^{-1}$. In turn, the wild-type strain displayed increased attachment to the Caco-2 monolayer, suggesting a possible inhibition of **D186** activity by upregulation of *SAP9* and/ or *SAP10* at a concentration of $2\text{ }\mu\text{g ml}^{-1}$. Thus, those genes indirectly contributed to the adhesive phenotype by conferring resistance to **D186**. According to the earlier observations⁴⁰ Sap10 exhibits decreased sensitivity to Pepstatin A. In the light of these developments, **D186** exhibits comparable or higher inhibitory potency ($\text{MIC}_{90} = 8\text{ }\mu\text{M}$) than Pepstatin A and PIs ($10\text{--}100\text{ }\mu\text{M}$).²⁹

As the hyphae formation and *SAP5* expression are the predominant mechanisms by which *C. albicans* invades tissue damage,^{19,47} we tested whether the expression of *SAP5* is inhibited by **D186** during adhesion to the Caco-2 monolayer. Real-time PCR provides novel insight into the *SAP5* expression in *C. albicans* cells treated with **D186**. Our transcriptional data showed an upregulation of *SAP5* in strains tested and this upregulation was reversed by **D186** (Figure 1). These differences obtained could reflect the mechanism of action of this compound with respect to Sap5 inhibition. We demonstrated that **D186** influenced on the reduction of *C. albicans* pathogenesis through decreased *SAP5* mRNA expression. Our results demonstrated that RT–PCR assay is capable of differentiating **D186**-treated from non-treated cells. It is well known that *C. albicans* pathogenesis is reached through yeast-to-hyphae morphogenesis.⁴⁸ The specific gene *SAP5* was found to be preferentially expressed by *Candida* hypha forms.¹⁹ Thus, the reduction of *SAP* expression may in fact reduce the *C. albicans* virulence/pathogenesis.^{47,49} Moreover, the activity of **D186** against *C. albicans* is associated with tryptophan content (four Trp) that maximized bioactivity. As mentioned previously,^{50–52} Trp has a pivotal role in the membrane-directed antimicrobial activity. Therefore, in our study genetic changes either in *SAP9–10* or *EFG1* or *CPH1* appeared to be necessary for **D186** to gain the access to intracellular targets, facilitating its membrane penetration and increasing its contact with intracellular targets.

This is the first time to our knowledge to study the influence of compound with branched structure, enriched with tryptophan—known membrane affinity-enhancer—on virulence factors of *C. albicans*. Our data provide evidence that **D186** inhibitory mode of action is associated with a reduced virulence arsenal in terms of adhesive ability and pathogenic potential related to the expression of the *SAP5* gene as well as morphogenesis factors. The genes *SAP9* and/ or *SAP10* enhanced resistance to **D186**. Despite the fact that *in vitro* studies cannot be directly extrapolated to *in vivo* effects, the results suggested that application of the **D186** or its structural analogs in combination with antifungal agents could be a viable alternative for therapeutic and/or preventive purposes against candidiasis. These results indicate that rationally designed **D186** might represent a novel inhibitor of the family of Saps isoenzymes as well as Efg1/Cph1 inhibitors with fungicidal activity.

ACKNOWLEDGEMENTS

We are extremely grateful to Professor Hsiu-Jung Lo from the National Health Research Institute in Zhunan (Taiwan) for providing us with the following strains: Can16, YLO323, HLC52, HLC74 and HLC84. We wish to thank Professor B Hube for the strains CAF2-1, CAI4, Δsap9 , Δsap10 , $\Delta\text{sap9-10}$ and

Δsap8 . We thank Professor J Morschhäuser for providing the mutants as follows: Δsap4 , Δsap5 and $\Delta\text{sap4-6}$. We are very grateful to the Laboratory of Miniaturized Chemical System (Warsaw University of Technology) for access to electron microscopes. This work was supported by grant NN 404 113639 of the Polish Ministry of Science and Higher Education. MS and MB were also supported by funding from the National Science Centre (No. DEC-2011/03/D/ NZ7/06198). PZ acknowledges the National Centre for Research and Development, Project NR13-0153-10/2010 for covering the cost of synthesis and analysis of compound **D186**.

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