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Synthesis of new antifungal peptides selective against *Cryptococcus neoformans*

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ABSTRACT

Many drugs are available for the treatment of systemic or superficial mycoses, but only a limited number of them are effective antifungal drugs, devoid of toxic and undesirable side effects. Furthermore, resistance development and fungistatic rather than fungicidal activities represent limitations of current antifungal therapy. Therefore there remains an urgent need for a new generation of antifungal agents.

According to a polypharmacological approach, the present work concerns the synthesis and antifungal activity of a set of peptides designed to simultaneously target the fungal cell surface and lanosterol demethylase, a key enzyme involved in ergosterol synthesis. Our peptides include amino acid sequences characteristic of membrane-active antimicrobial peptides (AMP), and due to the presence of His residues, they carry the imidazole ring characteristic of azole compounds.

The peptides synthesized by us, were tested against different yeast species, and displayed general antifungal activity, with a therapeutically promising antifungal specificity against *Cryptococcus neoformans*.

1. Introduction

Fungal infections are a persistent major health problem, especially for immunocompromised patients. Invasive fungal infections can be life-threatening for neonates, cancer patients receiving chemotherapy, organ transplant recipients and patients with acquired immunodeficiency syndrome (AIDS). Many fungal infections are caused by opportunistic pathogens that may be endogenous or acquired from the environment, including *Candida, Cryptococcus* and *Aspergillus*. Cryptococcosis, which is caused by the encapsulated fungus *Cryptococcus neoformans*, has been lethal for HIV-infected patients. *Cryptococcus* infects pulmonary organs and can disseminate widely, most commonly to the brain and skin.[1–3]

Although it appears that many drugs are available for the treatment of systemic or superficial mycoses, there are only a limited number of effective antifungal drugs, many of them toxic and having undesirable side effects. Furthermore, resistance development and fungistatic rather than fungicidal activities represent limitations of current antifungal therapy.[4–7] Combination therapy has emerged as a good alternative to bypass these disadvantages, but there remains an urgent need for a new generation of antifungal agents.[8]

Antimicrobial peptides (AMPs) are a large class of natural peptides, structurally characterised by Trp- and Arg-rich long amino acid sequences and exhibiting activity against a broad spectrum of microbes.[9,10] The mechanisms of action for antimicrobial peptides are not completely understood, but in most cases their biological effects are believed to involve membrane disruption of the target cells.[11–21] The proposed mechanism requires positively charged aminoacids to associate with negatively charged microbial membranes, causing the peptides to adopt a globally amphiphilic helical conformation at the membrane–water interface. The ensuing membrane disruption can lead directly to cell lysis and death,[22,23] or membrane permeabilization may allow peptide molecules to reach intracellular targets.[24,25] AMPs have gained attention as potential antifungal agents because their activity is not associated with resistance phenomena. Development of resistance by sensitive microbial strains against these AMPs is less probable, because AMPs exert their action by forming multimeric pores in the cell membranes, leading to cell lysis,[26] or interaction with the RNA or DNA after penetration into the cell.[14] Models proposed to explain the mechanism of microbial cytoplasmic membrane disruption by AMPs include the 'barrel-stave' model, the 'toroid pore'

Abbreviations: AMP, membrane-active antimicrobial peptides; AIDS, acquired immunodeficiency syndrome; SPPS, solid phase peptide synthesis; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; AcOH, acetic acid; HOBt, *N*-hydroxybenzotriazole; MeOH, methanol; AcOEt, ethyl acetate; $NaHCO_3$, sodium bicarbonate; Na_2SO_4, sodium sulfate; $CHCl_3$, chloroform; TFA, trifluoroacetic acid; TIS, triisopropylsilane; HCl, hydrogen chloride; Et_2O, diethyl ether; HPLC, high performance liquid chromatography; CD, dichroism circular; MIC, minimum inhibitory concentration; SDS, sodium dodecyl sulphate; CLSI, Clinical and Laboratory Standards Institute.

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or 'wormhole' model and the 'carpet' model.[27–30] In some cases AMPs have been proved to interact with for fungi specific targets. Fungal cells are protected by cell wall and sometimes by a capsular structure. The synthetic machineries required for constructing fungal cell wall or capsular envelope are good targets for antimycotic agents, since they are unique to fungal cells and not shared by bacterial or mammalian cells. Echinocandins are the most studied natural antifungal lipopeptides acting as glucan synthase inhibitors. Theonellamides, bicyclic peptides derived from marine sponges, are recently discovered to exhibit antifungal action through a mechanistic link with 1,3-β-D-glucan synthesis.[31,32]

In light of the fact that a drugable antimicrobial/antifungal peptide should be as short as possible, synthetic combinatorial library of small microbicidal peptides, are synthesised.[33,34] Linear and cyclic RRWWRF, dubbed AMT1 and cyclo-AMT1, respectively (see Scheme 1), were recently indentified from a synthetic combinatorial library as new antimicrobial peptides,[35–37] exerting their action by inducing curvature strain, permeation or disintegration of the target membrane.[36,38] Starting from AMT1 and cyclo-AMT1 sequence, we synthesised AMT2, cyclo-AMT2, AMT3 and cyclo-AMT3 (Scheme 1) including one and two more His residues than the AMT1 analogues, respectively.

To overcome the antibiotic resistance phenomena polypharmacological strategies have been developing based on the design and synthesis of drugs characterised by single chemical entities, but modulating multiple targets simultaneously.[39] Reminiscent of a polypharmacological strategy, the imidazole ring characteristic of His side chains was designed in the attempt (intended) to confer to AMT2, cyclo-AMT2, AMT3 and cyclo-AMT3 the 14α-sterol demethylase inhibitory activity analogous to the imidazole ring of the azole antifungal compounds. The azole antifungal agents, containing either two or three nitrogens in the azole ring, are currently the most widely used and studied class of antifungal agents; they prevent synthesis of ergosterol, a major component of fungal plasma membranes, by inhibiting the cytochrome P450-dependent enzyme 14α-sterol demethylase.[40–44]

AMT1, cyclo-AMT1, AMT2, cyclo-AMT2, AMT3 and cyclo-AMT3 were tested against different yeast species, displaying general antifungal activity, with a therapeutically promising antifungal specificity against *Cryptococcus neoformans*.

2. Experimental part

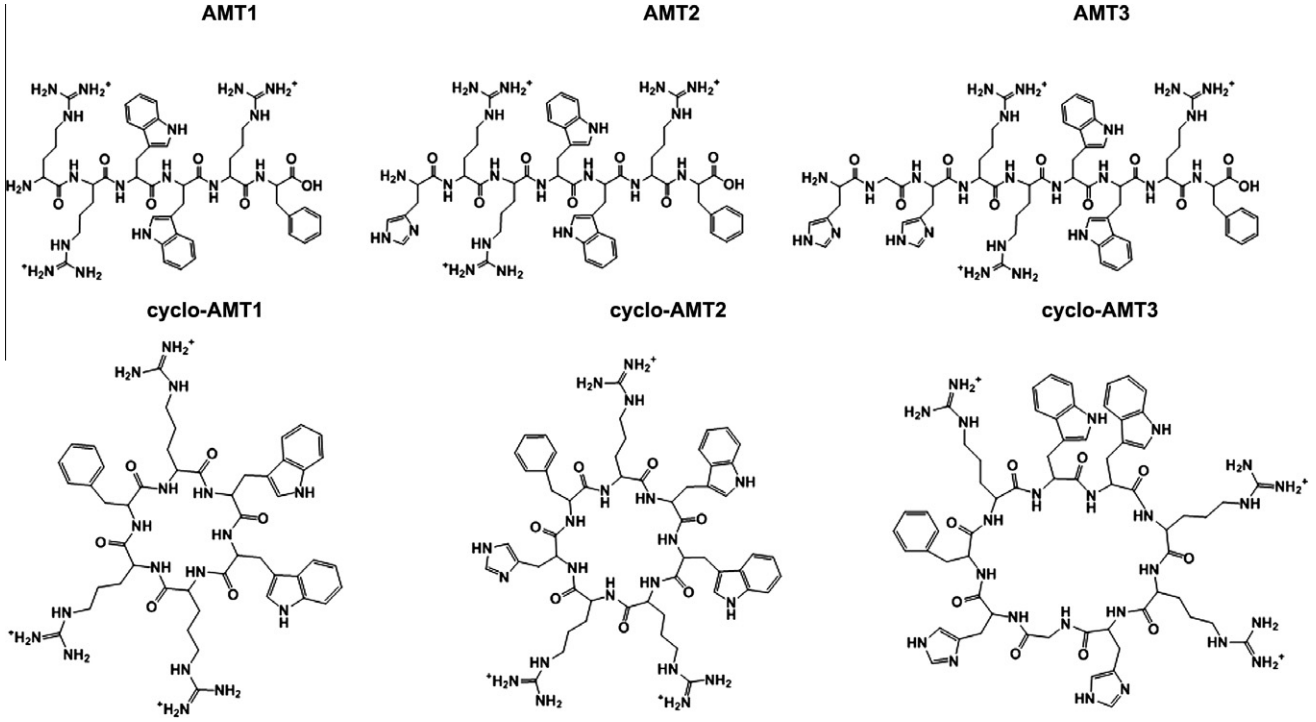
2.1. Materials

Protected amino acids and chemicals were purchased from Fluka. All other reagents and solvents were purchased from Sigma–Aldrich.

2.2. Solid-phase peptide synthesis and purification

AMT1, AMT2, AMT3, cyclo-AMT1, cyclo-AMT2 and cyclo-AMT3 were synthesized with a manual batch synthesizer (Agitatore orbitale Mod. KS 130 basic IKA, STEROGLASS, Perugia Italy) using a Teflon reactor (10 mL), applying the Fmoc/tBu solid phase peptide synthesis (SPPS) procedure, together with the following side chain protecting groups: Arg, Pbf; His, Trt; Trp, Boc.[45]

AMT1, AMT2 and AMT3 were synthesized with a Wang resin (0.6–1.0 mmol/g, 0.2 g) that was swelled with *N*,*N*-dimethylformamide (DMF) (1 mL/100 mg of resin) for 3 h before use. The Wang resin (0.6–1.0 mmol/g, 0.2 g) was treated with *N*-Fmoc amino acid derivatives (fourfold excess), which were sequentially coupled to the growing peptide chain using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (fourfold excess) in DMF and *N*,*N*-diisopropylethylamine (DIPEA) (eightfold excess). The coupling reaction time was 2 h. After deprotection of the last *N*-Fmoc group, the peptide resin was washed with methanol and dried in vacuo to yield the protected peptide-bound Wang resin. The deprotected peptide was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/H_2O/phenol/ethanedithiol/thioanisole (reagent K) (82.5:5:5:2.5:5 v/v) at a ratio of 10 mL to 0.5 g of resin at room temperature for 3 h. After filtration of the exhausted



Scheme 1.

resin, the solvent was concentrated in vacuo, and the residue was triturated with ether.

Cyclo-AMT2, cyclo-AMT1 and cyclo-AMT3 were synthesized using a 2-chlorotrityl chloride resin. The first *N*-Fmoc amino acid (0.6–1.2 equiv relative to the resin for 2-chlorotrityl resin) and DIPEA (4 equiv relative to amino acid) were dissolved in dry dichloromethane (DCM) (approx. 10 mL per gram of resin) containing, if necessary, a small amount of dry DMF (enough to facilitate dissolution of the acid). This was added to the resin and stirred for 30–120 min. After stirring, the resin was washed with 3 × DCM/MeOH/DIPEA (17:2:1), 3 × DCM, 2 × DMF and 2 × DCM. Other *N*-Fmoc amino acids (fourfold excess) were sequentially coupled to the growing peptide chain according to the Fmoc/tBu solid phase peptide synthesis (SPPS) procedure. The final cleavage with AcOH/MeOH/DCM (1:1:8) resulted in protected peptides.

2.2.1. General procedure for cyclization

A solution of the linear protected peptide (0.03 mmol) in dry DMF (6.5 mL) was added at room temperature to a reaction flask containing a solution of *N*-hydroxybenzotriazole (HOBt) (3 equiv, 12 mg, 0.09 mmol), HBTU (3 equiv, 34 mg, 0.09 mmol) and DIPEA (5 equiv, 0.26 mL, 1.5 mmol) in dry DMF (1 mL) using a syringe pump. The solution was added at rate of about 0.01 mL/min. Once the addition was complete, the mixture was stirred for 24 h at room temperature. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in ethyl acetate (AcOEt). The organic phase was washed twice with 5% aqueous sodium bicarbonate ($NaHCO_3$), dried over sodium sulfate (Na_2SO_4), and filtered. The solvent was removed by reduced pressure, and the crude residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH from 99:1 to 90:10) to yield the protected cyclic peptide as a glassy white solid.

2.2.2. Side-chain deprotection

The protected cyclopeptide (0.02 mmol) was treated with 10 mL of a solution of TFA/triisopropylsilane (TIS)/H_2O 95:2.5:2.5 at room temperature. After 24 h, the reaction mixture was evaporated in vacuo, and the residue was dissolved in 5 mL of 3 N aqueous hydrogen chloride (HCl). The aqueous phase was washed twice with diethyl ether (Et_2O) and concentrated in vacuo, yielding the side chain-deprotected cyclopeptide as a hydrochloride salt (quant.).

2.2.3. Peptide purification

All peptides were purified by preparative reversed phase high performance liquid chromatography (HPLC) using a Jupiter [Phenomenex, Anzola Emeilia (BO), Italy] C18 column (25 × 4.6 cm, 5 μ, 300 Å pore size). The column was perfused at a flow rate of 3 mL/min with a mobile phase containing solvent A (0.1% TFA in water). A linear gradient from 50% to 90% of solvent B (0.1% TFA in acetonitrile) for 40 min was adopted for peptide elution. The pure fraction was collected to yield a white powder after lyophilisation. After purification cyclo-AMT1, cyclo-AMT2 and cyclo-AMT3 were obtained in 35%, 45% and 68% overall yield, respectively.

The molecular weight of the compound was determined by mass spectral analysis.

2.2.4. Mass spectral analysis

Peptide fragments were characterised using a Finnigan LCQ-Deca ion trap instrument equipped with an electrospray source (LCQ Deca Finnigan, San Jose, CA, USA). The samples were directly infused into the ESI source using a syringe pump set at a flow rate of 5 μL/min. The data were analysed with Xcalibur software.

The calculated and measured molecular weight of each peptides are reported in Supplementary data.

2.3. Circular dichroism spectroscopy

All CD spectra were recorded using a JASCO J810 spectropolarimeter at room temperature and with a cell path length of 1 mm. CD spectra were acquired at 25 °C using a measurement range from 190 to 260 nm, 1-nm band width, four accumulations, and 10-nm/min scanning speed. Spectra were corrected for solvent contribution.

For an estimation of secondary structure content, CD spectra were analysed using the SELCONN algorithm from the DICHROWEB website.[46,47]

2.4. Yeast isolates

One-hundred and thirty-five yeast clinical isolates belonging to five *Candida* species (20 *Candida albicans*, 20 *Candida glabrata*, 20 *Candida parapsilosis*, 20 *Candida tropicalis* and 20 *Candida krusei*) and 35 isolates of *C. neoformans* were tested. The Candida isolates were obtained from clinical specimens of blood, urine, vaginal fluid and sputum, whereas the *C. neoformans* isolates were all recovered from blood and cerebrospinal fluid specimens. We also included two reference *C. neoformans* strains, H99 and the acapsular ATCC 52817. Isolates were identified to the species level by standard methods and stored as glycerol stocks at −80 °C. Prior to testing, isolates were grown on Sabouraud dextrose agar (Kima, Padua, Italy) for 48 h at 30 °C. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strain.[48]

2.5. Susceptibility testing assays

Susceptibility testing of the yeast isolates to the antifungals amphotericin B and fluconazole and to the six investigated peptides was performed by the broth microdilution method, as described in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document[48] with a final inoculum concentration of 1.5 (±1.0) × 103 cells/mL in RPMI 1640 medium buffered to pH 7.0 with morpholinepropanesulphonic acid. Standard powders of amphotericin B and fluconazole were obtained from their respective manufacturers. For each drug, trays containing 0.1 mL of the serially diluted drug solution (2× final concentration) in each well were inoculated with 0.1 mL of each diluted yeast inoculum suspension and then incubated for 48 h (*Candida* species isolates) or for 72 h (*C. neoformans* isolates) at 35 °C. The final concentrations of the standard antifungal drugs ranged from 0.03 to 16 μg/mL for amphotericin B and from 0.125 to 64 μg/mL for fluconazole, while those of the peptides ranged from 1 to 512 μg/mL. The minimum inhibitory concentration (MIC) endpoint was defined as the lowest concentration of drug that produced a prominent decrease in turbidity (∼50% reduction in growth) compared with that of the drug-free growth control.

3. Results

3.1. Antifungal activity

The Minimum Inhibitory Concentrations (MICs) of peptides AMT2, cyclo-AMT2, AMT1, cyclo-AMT1, AMT3 and cyclo-AMT3 were determined using a standardised microbroth dilution method for yeasts, as recommended by the Clinical and Laboratory Standards Institute[48] (CLSI). The application of an endpoint less than the MIC_{90} was used according to CLSI recommendations because it proved to consistently represent the in vitro activity of the compounds and often provided a better correlation with other measurements of antifungal activity. AMT1, cyclo-AMT1, AMT2, cyclo-AMT2, AMT3 and cyclo-AMT3 were tested against *C. albicans*,

Table 1
In vitro susceptibilities of *Candida* species and *Cryptococcus neoformans* isolates to six peptides[a]

Organism (n)	AMT1 (μg/mL)		cyclo-AMT1 (μg/mL)		AMT2 (μg/mL)		cyclo-AMT2 (μg/mL)		AMT3 (μg/mL)		cyclo-AMT3 (μg/mL)		AMB (μg/mL)		FLU (μg/mL)	
	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
C. albicans (20)																
48 h	128–>512	512	128–512	512	128–>512	512	64–512	256	256–>512	>512	32–128	128	0.03–0.25	0.125	0.125–0.5	0.25
C. glabrata (20)																
48 h	128–>512	512	128–512	512	128–>512	512	64–256	256	256–>512	>512	64–256	256	0.03–0.25	0.125	4–>64	64
C. krusei (20)																
48 h	128–>512	512	32–128	512	128–512	256	32–128	64	256–>512	512	32–64	64	0.03–0.125	0.06	16–>64	64
C. tropicalis (20)																
48 h	128–>512	512	128–512	512	128–512	512	64–512	256	256–>512	>512	32–128	128	0.03–0.125	0.06	0.5–4	2
C. parapsilosis (20)																
48 h	64–128	128	32–128	128	128–512	256	64–256	128	>512	512	32–128	128	0.06–0.125	0.06	1–4	2
C. neoformans (35)																
72 h	16–64	64	4–16	64	8–16	16	4–8	8	8–32	16	2–8	4	0.125–0.25	0.25	2–64	4

Abbreviations: AMB, amphotericin B; FLU, fluconazole; MIC, minimum inhibitory concentration; MIC_{90}, MIC for 90% of the isolates.
[a] As determined by the CLSI M27-A3 broth microdilution method.

C. glabrata, *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. neoformans*. The antifungal antibiotics amphotericin B and fluconazole served as positive controls for the microbes and gave the expected minimal inhibitory concentrations (MICs). For the *Candida* species isolates, MICs ranged from 64 to >512 μg/mL for the linear compounds and from 32 to 512 μg/mL for the cyclic compounds.

The MIC values shown in Table 1 indicate that all species are susceptible to the peptides tested; cyclic peptides have generally lower MIC values than their linear analogues. In particular the longest linear AMT3, having a MIC greater than 512, can be considered inactive against *C. albicans*, *C. glabrata* and *C. tropicalis*. Interestingly, all compounds are significantly more active against *C. neoformans*. *Cryptococcus* is a pathogenic fungus responsible for severe opportunistic infections. The most prominent feature of this yeast is its elaborate polysaccharide capsule, a complex structure that is required for virulence. To investigate the origin of this specificity, we tested our compounds against the acapsular *C. neoformans* strain ATCC 52817. MIC values against ATCC 52817 are quite similar to those of *C. neoformans* H99 (16 μg/mL for AMT2, 8 μg/mL for cyclo-AMT2, 16 μg/mL for AMT1, 8 μg/mL for cyclo-AMT1, 8 μg/mL for AMT3 and 4 μg/mL for cyclo-AMT3), indicating the absence of any specific interaction with the capsular structure.

3.2. CD spectroscopy

The conformational preferences of cyclic and linear AMT derivatives were screened by CD spectroscopy. All CD spectra were recorded in water and in sodium dodecyl sulphate (SDS) micelle solution. SDS micelle solution is considered a simple plasma membrane-mimicking system. CD spectra of linear and cyclic AMT derivatives were recorded in this system to explore their propensity to assume specific secondary structures in response to the presence of membrane-mimicking interfaces.

CD spectra of AMT1, AMT2 and AMT3 indicate the preponderance of disordered conformations both in water and in SDS micelles. CD spectra of cyclic peptides recorded in water and in SDS micelles are shown in Figure 1, they were quantitatively evaluated using the DICHROWEB interactive website (SELCONN algorithm); consistently with this analysis, a significant presence of turn conformation is evident in cyclo-AMT1 cyclo-AMT2 and cyclo-AMT3 in water. CD spectrum of cyclo-AMT2 in SDS micelles is similar to that exhibited in water solution; on the contrary CD spectra of cyclo-AMT1 and cyclo-AMT3 in SDS micelle solution have the shape characteristic of random coil conformation.

4. Discussion

Several short membrane-active peptides have shown antifungal activity due to their interfacial properties.[11–13] AMT1, a peptide selected from a synthetic combinatorial library, and its cyclic analogue cyclo-AMT1 were recently shown to possess antimicrobial and haemolytic activity.[36,38]

AMT2, cyclo-AMT2, AMT3 and cyclo-AMT3 were designed by adding to AMT1 and cyclo-AMT1 one and two His residues, respectively. His residues, which contain an imidazole ring in their side chains, were added in an attempt to endow AMT1 and cyclo-AMT1 analogues with additional demethylase inhibitory activity typical of the azole antifungal compounds.

Each of the peptides synthesized in our lab show even marginal antifungal activity against the selected fungal species tested; due to their sequence typical of the AMPs this fungicidal activity may be related to peptide-membrane destabilising ability.

Linear compounds are less active than their cyclic analogues. Cyclopeptides are conformationally restricted, which can allow for improved interaction specificity as well as increased metabolic stability. In agreement with CD data, which show minimal regular secondary structures in linear peptides, the conformational flexibility seems to prevent the interaction between the peptides and the fungal target. On the contrary, the higher activity exhibited by the cyclo-peptides indicates that limited peptide conformational freedom is important for antifungal activity, probably due to a required orientation of the amino acid side chain in the peptide-membrane interaction. In addition higher activity of the cyclic peptides could be also caused by the improved stability of the cyclic compound against proteolytic degradation.

As previously mentioned, our linear and cyclic AMT2 and AMT3 analogues included His residues intended to extend the membrane destabilising property of the original compounds AMT1 and cyclo-AMT1 to 14α-sterol demethylase inhibitory activity. Although data regarding the kinetics of antifungal activity (data not shown) evidenced, for AMT2 and AMT3 derivatives, a mechanism of action different than the mechanism displayed by azole compounds, thus excluding the possibility that they inhibit 14 α-demethylase, AMT2 and AMT3 including one and two His residues respectively preserve their antimicrobial activity; moreover, cyclo-AMT3, in spite of its larger size, and higher conformational flexibility, display even higher antifungal activity. Preliminary NMR conformational data show that pair staking interactions involving His and Trp rings in cyclo-AMT3 generate characteristic conformational features. These, in the hypothesis of a non aspecific mechanism of action,

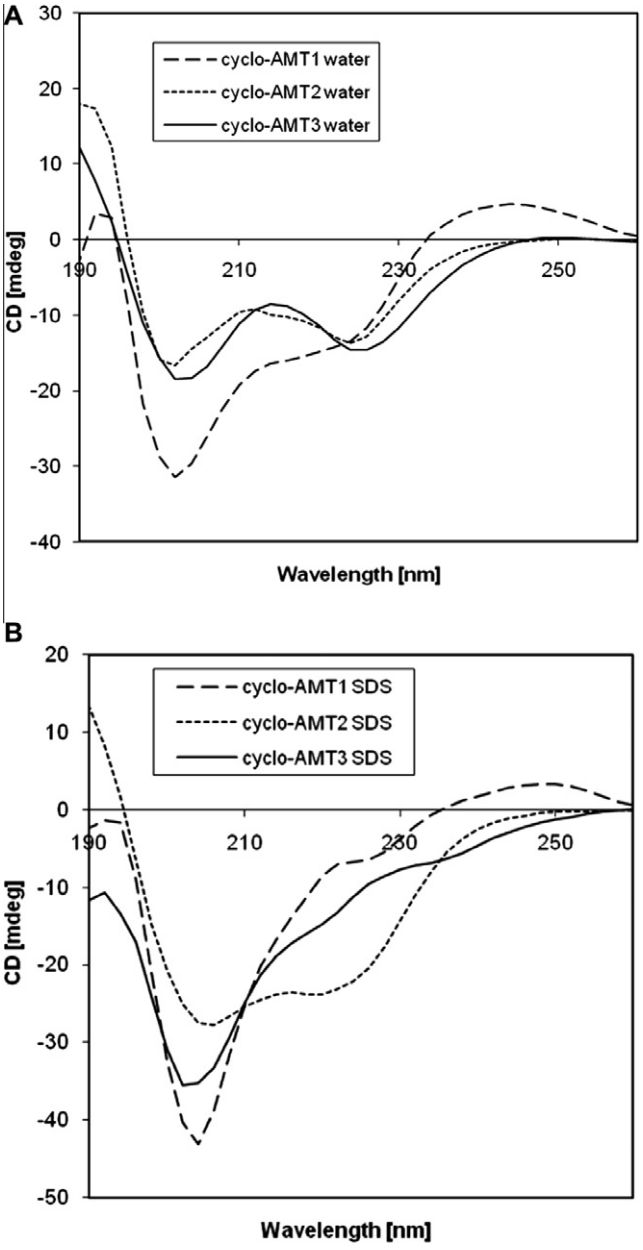


Figure 1. CD spectra of cyclo-AMT1, cyclo-AMT2 cyclo-AMT3 in water (A) and in SDS micelle solution (B).

may be at basis of an optimized interaction with the molecular target responsible for the fungicidal activity.

Our data show that all peptides tested possess very low MIC_{90} values against *C. neoformans*. These results are promising for drug development. *C. neoformans* infection is a life-threatening complication for immunocompromised hosts, being the main cause of fatal meningoencephalitis in AIDS patients and producing fatal cryptococcosis in patients who have undergone organ transplants. Thus new compounds acting against this fungus are highly desirable.

Experiments on capsular strains of *C. neoformans* excluded a selective interaction of our peptides with the most prominent feature of this yeast, the capsular structure, indicating that the specificity against *C. neoformans* depends on interaction with a *C. neoformans*-specific target. With this mind, many experiments are in progress in our lab investigating the molecular mechanisms of antifungal activity.

The newest antifungal drugs, such as the echinocandins, are highly effective against some fungal infections due to their inhibition of 1,3-β glucan synthase, an enzyme essential for cell wall biosynthesis. Growing knowledge of the biosynthetic steps regulating cell wall and capsule biosynthesis and assembly[49] lends hope that in the near future, many new biosynthetic steps can be approached as innovative targets for new effective and selective antifungal drugs.

The results of our antifungal activity studies, together with the specificity displayed by our compounds against *C. neoformans*, opens the exciting possibility that these will be lead compounds in the development of new antifungal drugs addressing specific targets responsible for fungal pathogenicity.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.033.

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