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New small-size peptides possessing antifungal activity

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Abstract

The synthesis, in vitro evaluation, and conformational study of a new series of small-size peptides acting as antifungal agents are reported. In a first step of our study we performed a conformational analysis using the molecular mechanics calculations. The electronic study was carried out using molecular electrostatic potentials (MEPs) obtained from RHF/6-31G calculations. On the basis of the theoretical predictions three small-size peptides, RQKWKWWQWR-NH₂, RQRWWQWR-NH₂, and RQRRWWQWR-NH₂ were synthesized and tested. These peptides displayed a significant antifungal activity against human pathogenic strains including Candida albicans and Cryptococcus neoformans. Our experimental and theoretical results allow the identification of a topographical template which can serve as a guide for the design of new compounds with antifungal properties for potential therapeutic applications against these pathogenic fungi.

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A B S T R A C T

1. Introduction

Fungal infections pose a continuous and serious threat to human health and life especially to immunocompromised patients.1–3 Many fungal infections are caused by opportunistic pathogens that may be endogenous (Candida infections) or acquired from the environment (Cryptococcus, Aspergillus infections). Patients with significant immunosuppression frequently develop Candida esophagitis, while cryptococcosis, caused by the encapsulated yeast Cryptococcus neoformans, has been the leading cause of fungal mortality among patients with reduced immune defence mechanisms. The latter fungal species has predilection for the central nervous system and its infection leads to severe, life-threatening meningitis. McNeil et al.4 found a dramatic increase in mortality between 1980 and 1997 due to mycoses from multiple origins, which could mainly be associated with Candida, Aspergillus, and Cryptococcus genera. However, besides these known fungal species, new emerging fungal pathogens appear every year as the cause of morbidity and life-threatening infections in the immunocompromised hosts.1,5

Although different antifungal agents are available for the treatment of fungal infections, some of them have undesirable side effects, are ineffective against new or re-emerging fungi or develop resistance mainly due to the broad use of antifungal drugs.6 Although combination therapy has emerged as a good alternative to bypass these disadvantages,7,8 there is an urgent need for a next generation of safer and more potent antifungal agents.1,8 This need has resulted in the identification of novel molecules, with a promise for future therapeutic development. Both natural and synthetic peptides have gained attention as potential new antifungal agents.9,10 These peptides proved to be able to inhibit a broad spectrum of pathogens and microorganisms11–13 and, importantly, without inducing bacterial or fungal resistance.14 Among them, some natural peptides were recently identified as antifungal compounds that showed to inhibit a broad spectrum of pathogenic microorganisms.15–18 It has been reported that a group of cationic antimicrobial peptides are major players in the innate immune response.19,20 These peptides appear to represent very ancient elements of the immune response of all living species and the induction pathways for these compounds in vertebrates, insects, and plants19–21 are highly conserved. Furthermore, it is becoming increasingly clear that cationic antimicrobial peptides have many potential roles in inflammatory responses, which represent an orchestration of the mechanisms of innate immunity.

Small cationic peptides12,22 are abundant in nature and have been described as ‘nature’s antibiotics’ or ‘cationic antimicrobial peptides’. These peptides are 12–50 amino acids long with a net
positive charge of +2 or +9, which is due to an excess of basic arginine and lysine residues, and approximately 50% hydrophobic amino acids. These molecules are folded in three dimensions so that they have both a hydrophobic face comprising non-polar amino acid side-chains, and a hydrophilic face of polar and positively charged residues: these molecules are amphipathic. Despite these two similarities these compounds vary considerably in length, amino acid sequence, and secondary structure. The different spatial orderings include small β-sheets stabilized by disulfide bridges, amphipathic α-helices and, less commonly, extended and loop structures.

Recently we reported that penetratin, a well-known cell penetrating peptide, displayed a significant antifungal effect against both Candida albicans and C. neoformans two important life-threatening infections for immunocompromised hosts. The consideration that a peptide-based antifungal agent should be as short as possible in order to reduce its production costs, prompted us to synthesize shorter derivatives of penetratin. Within that framework we synthesized shorter peptides structurally related with penetratin but those small-size peptides showed to be inactive or yielded only a marginal antifungal effects. In fact, only the tetrapeptide RQKK displayed a moderate antifungal activity against C. neoformans and was practically inactive against C. albicans. On the basis of these previous results, in the present study we aimed at developing a next generation of small-size peptides possessing antifungal properties that may be, at least, comparable to those of penetratin against C. albicans and C. neoformans. To characterize the structure–antifungal activity relationship of these compounds, in the present investigation we explored the influence of amino acid substitutions and deletions on its antifungal activity. In addition, a conformational and electronic analysis of this new series of peptides was carried out using theoretical calculations. This study was performed in order to identify a topographical and/or substructural template, which can be the starting structure for the design of new antifungal peptides.

2. Results and discussion

As stated above the principal aim of this study is to develop new antifungal peptides possessing a length as short as possible while maintaining their antifungal activity. Thus, on the basis of our previous results, the RQKK sequence was selected as the starting structure, since RQKK was the smallest peptide so far showing at least a moderate antifungal effect against C. neoformans. Therefore, we performed sequential changes on RQKK in order to obtain information on the potential role of each amino acid in the sequence.

Figure 1 shows a schematic way how the different amino acids of RQKK were replaced in a kind of ‘point mutation’ procedure. These structural changes were designed without any consideration about the possible changes in the physicochemical properties introduced with each structural modification. None of these 12 tetrapeptides obtained by this way (compounds 1–12), except the previously reported RQKK (10), displayed any significant antifungal effect against C. neoformans. They do not completely inhibit the growth of C. neoformans even at high concentrations, showing percentages of inhibition ranging from 3.72% to 78.6% at the highest concentration tested for compounds 1–9 and 11–12 thus possessing a Minimum Inhibitory Concentration (MIC) above 200 μM (Table 1). This low activity prompted us to develop new small-size peptides using a rational design based on theoretical calculations.

In our previous paper we performed a detailed conformational and electronic study for penetratin and its derivatives. These results allowed us to identify a possible ‘biologically relevant conformation’ or ‘pharmacophoric patron’ for these peptides. A particular combination of cationic and hydrophobic residues adopting a definite spatial ordering appeared to be the key parameter for the membrane transition from hydrophilic to hydrophobic phase, which could be an essential and necessary step to produce the antifungal activity. Considering these previous results we decided to synthesize a peptide, smaller than penetratin but larger than RQKK, able to adopt the pharmacophoric patron displayed by penetratin.

In our initial studies we maintained the same number of cationic amino acids (R and K) as in penetratin (compound 13), deleting Q2, I3, I5, F7, Q8, N9, and M12. This way we obtained and tested compounds 14–17 which displayed a markedly lower antifungal activity compared to compound 13 (Table 2). In fact, only peptides 14 and 16 showed a marginal effects against C. neoformans and C. albicans inhibiting 68%, 76%, and 33% at 100 μM and MICs (100% inhibition) = 200 μM, while the others may be considered as inactive compounds. Observing the MEPs previously reported for compounds 14–17 it is clear that these peptides appears to be ‘too cationic’ displaying a dominant electropositive electronic distribution. Thus, the low antifungal activity of these peptides could be attributed to the inadequate balance between cationic and hydrophobic residues in their sequences. It should be noted that a determinant role for the W residues in the membrane translocation of peptides has been proposed. The mutation of both tryptophans in penetratin was found to abolish internalization. Based on these observations, in the present new series we gradually reduced the number of cationic residues, thereby increasing the number of hydrophobic amino acids. This way, we designed a set of nine
small-size peptides possessing sequences somewhat different, but structurally much related among them (compounds 18–26, Table 2). Peptides 18 and 19 were designed as to maintain the stereo-electronic characteristics of the first four and the last three amino acids of penetratin. Note that the first four amino acids of these peptides possess as features cationic–polar–hydrophobic–cationic acids of penetratin. To better characterize the peptide spatial orientations, we plotted Edmundson wheel representations of peptides 18–26 (Fig. 3), The wheel representations obtained for peptides 18, 19, and 23 were very similar. They display two clearly differentiated faces: the ‘charged face’ (denoted in dash blue line in Fig. 3) and a ‘non-charged face’ (depicted in full green line). The first face identifies residues R1, K4, K5, and R11 (in peptide 18); R1, R4, R5, and R11 (in peptide 19) and R1, R4, and R5 in peptide 23, as those accounting for the mutual coulombic binding. The first three residues are located on the same side of the helical peptide and we call it the ‘charged face’. These positively charged residues are able to produce salt bridges with the lipids. The non-charged face is the ‘charged face’. These positively charged residues are able to produce salt bridges with the lipids.

Table 1

<table>
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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MICs</th>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>AAAA-NH₂</td>
<td>24.96 ± 11.23</td>
</tr>
<tr>
<td>2</td>
<td>AAKK-NH₂</td>
<td>70.57 ± 2.01</td>
</tr>
<tr>
<td>3</td>
<td>RAAA-NH₂</td>
<td>38.40 ± 6.1</td>
</tr>
<tr>
<td>4</td>
<td>RAAK-NH₂</td>
<td>78.60 ± 1.69</td>
</tr>
<tr>
<td>5</td>
<td>RAIR-NH₂</td>
<td>34.61 ± 4.13</td>
</tr>
<tr>
<td>6</td>
<td>RIIR-NH₂</td>
<td>65.44 ± 12.5</td>
</tr>
<tr>
<td>7</td>
<td>KQIR-NH₂</td>
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</tr>
<tr>
<td>8</td>
<td>RQIR-NH₂</td>
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<tr>
<td>9</td>
<td>RQIK-NH₂</td>
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</tr>
<tr>
<td>10</td>
<td>KQIK-NH₂</td>
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</tr>
<tr>
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<td>RIKR-NH₂</td>
<td>3.72 ± 0.41</td>
</tr>
<tr>
<td>12</td>
<td>RAIR-NH₂</td>
<td>34.61 ± 4.13</td>
</tr>
<tr>
<td>13</td>
<td>RAIR-NH₂</td>
<td>78.60 ± 1.69</td>
</tr>
<tr>
<td>14</td>
<td>RAIR-NH₂</td>
<td>34.61 ± 4.13</td>
</tr>
<tr>
<td>15</td>
<td>RAIR-NH₂</td>
<td>78.60 ± 1.69</td>
</tr>
<tr>
<td>16</td>
<td>RAIR-NH₂</td>
<td>34.61 ± 4.13</td>
</tr>
<tr>
<td>17</td>
<td>RAIR-NH₂</td>
<td>78.60 ± 1.69</td>
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<tr>
<td>18</td>
<td>RAIR-NH₂</td>
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<tr>
<td>19</td>
<td>RAIR-NH₂</td>
<td>78.60 ± 1.69</td>
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<td>21</td>
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<td>22</td>
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<td>23</td>
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</tr>
<tr>
<td>25</td>
<td>RAIR-NH₂</td>
<td>78.60 ± 1.69</td>
</tr>
<tr>
<td>26</td>
<td>RAIR-NH₂</td>
<td>34.61 ± 4.13</td>
</tr>
</tbody>
</table>

a Previously reported in Ref. 23.
b Amphotericin B.
c Ketoconazole.

It is interesting to note that the energetically and populated preferred families comprise more than 61% of the entire population for each peptide (see Table 3, last column). Thus, these families adopting an α-helix structure are the most representative forms for these peptides. This conformation is characterized by stabilizing hydrogen bonds between the carboxylic oxygen (residue i) and the NH group (residue i+4). The first and the last residues do not present a stable structure in any of the cases. A spatial image of this conformation is shown for peptide 18 in Figure 2.

The second most populated family obtained for peptides 18–20 do not demonstrate a significant percentage of population (0.44%, 2.2%, and 0.76%, respectively). The second most populated family obtained for peptides 21–26 displayed a percentage of population ranging between 4.98% and 9.18% possessing bend, turn forms or not showing any stable structures. In general these conformations showed an energy gap ranging between 4.29 and 9.50 kcal mol⁻¹ above their respective global minimum. These results suggest that α-helix forms are the highly preferred conformations for these peptides.

To better characterize the peptide spatial orientations, we plotted Edmundson wheel representations of peptides 18–26 (Fig. 3).
Table 2

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Candida albicans % inhibition (M)</th>
<th>Cryptococcus neoformans % inhibition (M)</th>
</tr>
</thead>
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<tr>
<td>M100</td>
<td>100 ± 0.2</td>
<td>100 ± 0.2</td>
</tr>
<tr>
<td>M50</td>
<td>100 ± 0.2</td>
<td>100 ± 0.2</td>
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<td>100 ± 0.2</td>
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<td>M12.5</td>
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<td>M25</td>
<td>100 ± 0.2</td>
<td>100 ± 0.2</td>
</tr>
<tr>
<td>M10</td>
<td>100 ± 0.2</td>
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<tr>
<td>M0.2</td>
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<td>100 ± 0.2</td>
</tr>
<tr>
<td>M0.1</td>
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<td>100 ± 0.2</td>
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<td>100 ± 0.2</td>
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</tr>
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<td>100 ± 0.2</td>
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<tr>
<td>M0.025</td>
<td>100 ± 0.2</td>
<td>100 ± 0.2</td>
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<tr>
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<td>M0.006</td>
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<td>M0.003</td>
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<td>15th</td>
<td>100 ± 0.2</td>
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</tr>
<tr>
<td>16th</td>
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<tr>
<td>17th</td>
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<td>100 ± 0.2</td>
</tr>
<tr>
<td>18th</td>
<td>100 ± 0.2</td>
<td>100 ± 0.2</td>
</tr>
<tr>
<td>19th</td>
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</tr>
<tr>
<td>20th</td>
<td>100 ± 0.2</td>
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</tbody>
</table>

The main difference observed in these peptides is the presence of additional charged residues in 19 and 20, which may contribute to their increased activity against Candida albicans.

2.2. Molecular electrostatic potentials (MEPs)

The MEPs of peptides 18–26 were obtained using quantum mechanics calculations (RHF/6-31G). The MEPs of peptides 18–26 were shown to provide reliable information, both on the interaction sites of molecules with point charges and on the comparative reactivities of those sites. More positive potentials reflect nucleic predominance, while less positive values represent rearrangements of electronic charges and lone pairs of electrons. The fundamental application of this study is the analysis of non-covalent interactions, mainly by investigating the electronic distribution in the molecule. Thus, this methodology was used to evaluate the electronic distribution around the molecular surface of the peptides here reported.

We evaluated and plotted the MEPs of peptides 18–26 showing only the most representative results in Figures 4–7, whereas the rest are included in Supplementary data. To better appreciate the electronic behavior of peptide 18 (Fig. 4), and considering that two different faces were signaled in Figure 3, we present the MEPs of this peptide showing both faces. Figure 4a gives the ‘charged face’ (CF) characterized by the presence of four cationic residues (R1, K4, K5, and R11). Tryptophan fluorescence studies previously reported for penetratin showed the importance of peptide positively charged residues for the initial binding to negatively charged vesicles, since double R/K (R1, K4, K5, and R11) could be responsible for the initial binding. The main positive potentials (V(r) ranging from 0.73 to 0.48 el au⁻³) are concentrated on the charged face; however it should be noted that the residue R10 is located in the hydrophobic face. Thus, this cationic residue appears to be strategically located in the middle of the non-charged face. Figure 4b displays the hydrophobic face of 18 showing four hydrophobic residues (W9, W6, W3, and W7) and a polar one (Q2). It appears that a kind of pi-staking cluster through W3/W6/W9 takes place in this portion of 18. Also peptide 18 contains two polar residues (Q2 and Q8) which are located one on each face. The MEPs displayed for peptide 19 (Fig. 1S in Supplementary data) is very similar to that obtained for 18.

The MEPs of peptides 20–22 (Figs. 2S, 3S and 5, respectively) revealed significant differences compared to those of peptides 18 and 19. They show an increasing hydrophobic zone and a systematically diminished cationic face characterized by an extended yellow and orange zone pointing to a ‘too hydrophobic’ distribution.

Although peptide 23 is smaller in size than peptides 18 and 19 the general electronic distribution of this nonapeptide is closely related to those of peptides 18 and 19 showing two clearly differentiated faces, the cationic (Fig. 6a) and the hydrophobic (Fig. 6b). In contrast the MEPs calculated for peptides 24 and 25 (Fig. 4S and 7) have an electronic distribution rather similar to those of peptides 21 and 22.
Table 3
Selected conformational search and clustering results for peptides 18–26 optimized at the EDMC/SRFOPT/ECCEP/3 level of theory

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Generated Electrost.</th>
<th>Random</th>
<th>Thermal</th>
<th>Total</th>
<th>Accepted Electrost.</th>
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<td>15</td>
<td>94.04</td>
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#F represents the total number of conformational families as result of the clustering run.

%P represents the sum of the percent relative population of #F25.

% PP percent relative population for the most populated and energetically preferred family.

* Number of conformations generated electrostatically, randomly and thermally during the conformational search.

b Number of conformations accepted from those generated electrostatically, randomly and thermally during the conformational search.

Figure 2. Spatial view of the global minimum (α-helix structure) obtained for peptide 18.

In agreement with the different wheel representation obtained for peptide 26, this peptide presented a completely different electronic distribution as well. The electronic study suggested that peptides 18, 19, and 23 have an electronic distribution in accordance with the previously proposed pharmacophoric pattern. This pattern suggests a particular combination of cationic and hydrophobic residues adopting a definite spatial ordering which appears to be the key parameter for the transition from hydrophilic to hydrophobic phase. In contrast, peptides 21, 22, 24, and 25 displayed a different electronic distribution which might be considered ‘too hydrophobic’ with respect to the more balanced electronic distributions.

2.3. Synthesis and antifungal activity

On the basis of the results obtained with the conformational and electronic studies we synthesized and tested peptides 18, 19, and 23. These peptides were thoroughly selected on the basis of their conformational and electronic behavior being closely related to that previously reported for penetratin. In addition, we synthesized and tested peptide 26 presenting an entirely different peptide spatial orientation and also a different electronic behavior not agreeing with the proposed pharmacophoric pattern and thus could act as a negative control.

The Minimum Inhibitory Concentrations (MIC) of peptide 1–26 was determined in the range of concentrations from 200 to 3.125 μM with the standardized microbroth dilution method M-27 A2 for yeasts recommended by the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical and Laboratory Standards NCCLS). At each concentration tested (200, 100, 50, 25, 12.5, 6.25, and 3.125 μM) the% of inhibition displayed by each peptide was determined. Compounds producing no inhibition of fungal growth at 200 μM were considered inactive. Table 2 gives the antifungal activity obtained for peptides 18, 19, 23, and 26 against C. albicans and C. neoformans. Results showed that the first three peptides displayed a significant antifungal activity against both fungi tested being C. neoformans the most susceptible species. Peptides 18, 19, and 23 inhibit 100% (MIC100) the growth of C. neoformans at 25 μM, but interestingly enough, they produced 66%, 51%, and 83% inhibition, respectively, at 12.5 μM. These results signify that the three compounds possess MIC50 (concentration at which the compounds produce 50% inhibition) ≤ 12.5 μM and compound 23 displayed a MIC50 ≤ 12.5 μM. The application of a less stringent end-point such as MIC30 and MIC50 has been recommended by CLSI because it showed to consistently represent the in vitro activity of compounds and many times provide a better correlation with other measurements of antifungal activity. So, the fact that these three peptides possess very low MIC30 and MIC50 values against C. neoformans is very interesting. This species remains an important 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 transplantation of solid organs. Therefore, new compounds acting against this fungus are highly welcome.

In turn, compounds 18, 19, and 23 also inhibit C. albicans, with MIC50 = 50 μM and MIC50 < 25 μM. This is also an interesting finding because candidiasis is the fourth most common nosocomial blood stream infection, representing more than 60% of all isolates from clinical infections. It should be noted that the antifungal effects obtained for these small-size peptides are slightly better to those previously reported for penetratin which displayed a MIC50 between 12.5 and 25 μM. In contrast and as we expected, peptide 26 was devoid of any significant antifungal activity. These experimental results clearly support our theoretical calculations obtained from molecular and quantum mechanics computations. In addition, these theoretical and experimental results are an additional support for the pharmacophoric pattern previously proposed for penetratin and its derivatives.

At this stage of our studies, some general conclusions may be drawn. Peptides 14–17 revealed only a marginal antifungal effect (peptide 14) or were inactive. These results indicate that such
cationic peptides could not be sufficiently hydrophobic to penetrate deeply into phospholipid model membranes.\textsuperscript{33,34} Therefore, charge neutralization is required for a deeper insertion of the peptide into the hydrophobic core of the membrane. The non-charged face possessing at least one cationic or polar residue among the hydrophobic ones observed in the MEPs of peptides 18, 19, and 23 appears to be operative in this sense. Previously reported MD simulations indicated that the aromatic residues do not contribute to the initial binding, but rather to the subsequent insertion of penetratin between the bilayer head groups, when they shield the peptide from the aqueous phase.\textsuperscript{35} The importance of hydrophobic residues seems to be crucial for the antifungal activity of these small-size peptides as well. However, our results indicate that a balanced electronic distribution (not ‘too cationic’ and not ‘too hydrophobic’) is necessary to produce the antifungal effect.

In general the toxicity of the antifungal agents is a critical aspect for their usefulness and limitations. Thus, in addition to the antifungal evaluation, the acute toxic effect of compounds 18, 19, and 23 were evaluated using a toxicity test on fish which has been previously successfully used by our group on other antifungal compounds.\textsuperscript{36–38} Our results indicated that none of these peptides displayed acute toxicity (measured as fish mortality during 96 h) at 13 $\mu$g/ml (Table S10 in Supplementary data). Although, these are preliminary results they give a promising feature about the low acute toxicity of these peptides.

In terms of bioavailability, stability, and pharmacokinetics, most peptides are as bad as proteins and, in general do not make good drugs unless chemical modifications are performed on their structure. It is clear that in general peptides possess significant limitations to be used directly as drugs; however many of these peptides are excellent starting structures to develop new drugs (generally as peptidomimetic compounds) with novel mechanisms of action and therefore developing new effective and safer therapeutic agents. It is clear that these results must be considered as preliminary results in the long way of the design of antifungal leads; however they allowed the identification of a promising 3D pharmacophore for these compounds.

\textbf{Figure 3.} Edmundson wheel representations of peptides 18–26. The number in the center of the wheel corresponds to the peptide number. The ‘charged’ and ‘non-charged’ faces are shown in blue dashed lines and full green lines, respectively. Positively charged amino acids are denoted with blue dots, the polar ones with orange, and the hydrophobic ones with yellow.
Finally, it is important to highlight that the mechanism of action of these peptides has not been determined yet. Nevertheless, as a general feature, antimicrobial cationic peptides possess a relatively non-specific mechanism of action by either acting through a detergent-like disruption of the bacterial or fungal cell membrane or by...
the formation of transmembrane pores. Therefore, due to their cationic and amphipathic structural characteristics, it is likely to be that these peptides possess the features of the general mechanisms of action of antimicrobial cationic peptides. However, we have not yet definitive results about the possible molecular mechanism for these peptides. Different bioassays are being carried out in our laboratories in order to obtain sufficient information regarding this matter.

3. Conclusions

In the present paper, we report the design, synthesis, and antifungal effects of small-size peptides. On the basis of a detailed conformational and electronic study performed on a series of compounds, we obtained a new series of small-size peptides containing 9 and 11 amino acids showing potential antifungal effects. Among the peptides tested, RQWKKWWQWRR-NH2, RQIRRWWQQWR-NH2, and RQIRRWWQQW-NH2 displayed the most potent inhibitory effect against both C. neoformans and C. albicans.

A comprehensive conformational and electronic study performed using theoretical calculations provided an additional support for the pharmacophoric pattern previously reported for penetratin and its derivatives. This pattern suggests a particular combination of cationic and hydrophobic residues adopting a definite spatial ordering which appears to be the key parameter for the antifungal activity of these selected peptides and the design of novel structurally related agents. Thus, we have identified a structural template that can serve as a 3D pharmacophore for the design of new effective antifungal compounds particularly against C. albicans and C. neoformans.

4. Experimental section

4.1. Synthetic methods

Solid phase synthesis of the peptides was carried out manually on a p-methyl benzhydrylamine resin (1 g MBHA, 0.14 mmol/g) with standard methodology using Boc-strategy. Side chain protecting groups were as follows: Arg(Tos), His(Tos), Lys(2C-Z), Cys(Mbz), Tyr(2-Bz). All protected amino acids were coupled in CH2Cl2 (5 ml) using DCC (2.5 equiv) and HOBt (2.5 equiv) until completion (3 h) judged by Kaiser et al.11 ninhydrin test. After coupling of the appropriate amino acid, Boc deprotection was effected by use of TFA/CH2Cl2 (1:1, 5 ml) for 5 min first then repeated for 25 min. Following neutralization with 10% TEA/CH2Cl2 three times (5–5 ml of each), the synthetic cycle was repeated to assemble the resin-bond protected peptide. The peptides were cleaved from the resin with simultaneous side chain deprotection by acidolysis with anhydrous hydrogen fluoride (5 ml) containing 2% anisole, 8% dimethyl sulfide and indole at 5 °C for 45 min. The crude peptides were dissolved in aqueous acetic acid and lyophilized. Preparative and analytical HPLC of the crude and the purified peptides were performed on an LKB 32264 were grown on Sabouraud-chloramphenicol agar slants for 3–4 days at 35 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid). Inocula of cell suspensions were obtained according to reported procedures and adjusted to 1–5 × 105 cells with colony forming units (CFU)/ml.

4.2. Microorganisms and media

Strains of C. albicans and C. neoformans from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. C. albicans ATCC 10231, Candida tropicalis C 131, and C. neoformans ATCC 32264 were grown on Sabouraud-chloramphenicol agar slants for 24 h at 35 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid). Inocula of cell suspensions were obtained according to reported procedures and adjusted to 1–5 × 105 cells with colony forming units (CFU)/ml.

4.3. Antifungal evaluation

The test was performed in 96 wells-microplates. Peptide test wells (PTW) were prepared with stock solutions of each peptide in DMSO (≤2%), diluted with RPMI-1640 to final concentrations 200–3,125 μM. Inoculum suspension (100 μl) was added to each well (final volume in the well = 200 μl). A growth control well (GCW) (containing medium, inoculum, the same amount of DMSO used in PTW, but compound-free) and a sterility control well (SCW) (sample, medium, and sterile water instead of inoculum) were included for each strain tested. Microtiter trays were incubated in a moist, dark chamber at 35 °C, 24 or 48 h for Candida spp. or Cryptococcus sp., respectively. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphoterin B (Sigma Chemical Co, St. Louis, MO, USA) was used as positive control (100% inhibition). Tests were performed by duplicate. Reduction of growth for each peptide concentration was calculated as follows: % of inhibition = 100 – (OD405 PTW – OD405 SCW)/OD405 GCW – OD405 SCW.

4.3.1. Statistical analysis

Data were statistically analyzed by both, one-way analysis of variance and Student’s test. A p <0.05 was considered significant.
4.4. Acute toxicity test

Toxic effect of compounds was evaluated using a toxicity test on fish. The static technique recommended by the US Fish and Wildlife Service Columbia National Fisheries Research Laboratory was modified in order to use lower amounts of tested compounds. Fish of the species *Poecilia reticulata* were born and grown in our laboratory until they reached a size of 0.7–1 cm (15 days old). In the toxicity test, 10 specimens were exposed to each of the concentration tested per drug in 2 l wide-mouthed jars containing the test solutions. Aqueous stock solutions of pure compounds diluted in DMSO were prepared and added to test chambers to get the final concentrations. The test began upon initial exposure to the peptides and continued for 96 h. The number of dead organisms in each test chamber was recorded and the dead organisms were removed every 24 h; general observations on the conditions of tested organisms were also recorded at this time; however the percentage of mortality was recorded at 96 h. Each experience was performed two times with three replicates each. We chose this technique because it is fast, economic, and easy to reproduce. This assay has previously been used by our group testing the toxicity of synthetic and natural compounds.

4.5. Computational methods

4.5.1. EDMC calculations

The conformational space was explored using the method previously employed by Livio et al. that included the electrostatically driven Monte Carlo (EDMC) method implemented in the ECEPP/3 package. Conformational energy was evaluated using the ECEPP/3 force field. Hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have been optimized using ab initio single point calculations of peptide bonds (waters = 180°) were considered. All accepted conformations were then clustered into families using the program ANALYZE by applying the minimal-tree clustering algorithm for separation, using backbone atoms, energy threshold of 30 kcal mol⁻¹, and RMSD of 0.75 Å as separation criteria. This clustering step allows a substantial reduction of the number of conformations and the elimination of repetitions. A more detailed description of the procedure used here is given in Section 4.4 Computational Methods of Ref. 23.

4.5.2. Molecular electrostatic potentials

Quantum mechanics calculations were carried out using the Gaussian 03 program. We use the most populated conformations of peptide 18–26 obtained from EDMC calculations. Subsequently, single point ab initio (RHF/6-31G) calculations were carried out. The electronic study was carried out using molecular electrostatic potentials (MEPs). These MEPs were calculated using RHF/6-31G wave functions and MEPs graphical presentations were created using the MOLEKEL program.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.009.

References and notes