Improved smallest peptides based on positive charge increase of the $\gamma$-core motif from $PvD_1$ and their mechanism of action against Candida species

Introducción

Plant defensins are peptides identified in different plant tissues and organs, and several studies demonstrated the important role they played in the innate immune system of plants. The participation of plant defensins in the defense responses is supported by reports of their strong antimicrobial activity in vitro, not only against plant pathogenic fungi, but also against human ones, along with the demonstration that transformed plants engineering to constitutively express defensin genes, is resistant to fungal diseases.

Plant defensins present a diversity of primary structures coded in 45–54 amino acid residues. The amino acid composition of plant defensins make them highly basic, giving the molecules a net positive charge at neutral pH. Several amino acid residues are conserved among the plant defensin primary structures, such as the strictly conserved $\gamma$-core motif (GXCX$_3\gamma$C) that is related to their antimicrobial properties. The aim of this work was to design synthetic peptides based on the region corresponding to the $PvD_1$ defensin $\gamma$-core that are the smallest amino acid sequences that bear the strongest biological activity.

Methods: We made rational substitutions of negatively charged amino acid residues with positively charged ones, and the reduction in length in the selected $PvD_1$ $\gamma$-core sequence to verify whether the increased net positive charges and shortened length are related to the increase in antifungal activity. Herein, we opted to evaluate the action mechanism of $\gamma$$_{13-4}$-$PvD_1^{\gamma\gamma}$ peptide due to its significant inhibitory effect on tested yeasts. In addition, it is the smallest construct comprising only nine amino acid residues, giving it a better possibility to be a prototype for designing a new antifungal drug, with lower costs to the pharmaceutical industry while still maintaining the strongest antimicrobial properties.

Results: The $\gamma$$_{13-4}$-$PvD_1^{\gamma\gamma}$ peptide caused the most toxic effects in the yeast Candida buinensis, leading to membrane permeabilization, viability loss, endogenous reactive oxygen species increase, the activation of metacaspase, and the loss of mitochondrial functionality, suggesting that this peptide triggers cell death via apoptosis.

Conclusion: We observed that the antifungal activity of $PvD_1$ is not strictly localized in the structural domain, which comprises the $\gamma$-core region and that the increase in the net positive charge is directly related to the increase in antifungal activity.

Keywords: defensin, antimicrobial peptide, cationic, mechanism of action
their three-dimensional structure, allowing this family of plant peptides to resist harsh environmental conditions, such as protease degradation and pH and temperature extremes. Despite the variation in the primary structure, a completely opposite effect is observed at the tertiary structure level, which is well defined and conserved, generally consisting of one α-helix and three antiparallel β sheets. The diversity of plant defensins at their primary structure level is responsible for their different biological activities in vitro. Among the activities already described, the best characterized is their growth inhibition of a large variety of filamentous fungi and yeasts, including those pathogenic to humans.

The mechanism of action involved in the fungal growth inhibition by plant defensins has been investigated by several authors, and the evidence demonstrates several steps involving an extracellular mechanism acting on the cell wall and/or plasma membrane, further acting on intracellular targets. In addition to this whole membrane permeabilization process involving plant defensins, some defensins have been shown to induce apoptosis or programmed cell death in susceptible yeast and fungal species.

In regard to the plant defensin structures, studies have demonstrated that two disulfide bridges, those formed between the Cysα and Cysβ located in the α-helix and the Cysγ, and Cysγβ, located in the last β-sheet, form a structural arrangement named the cysteine-stabilized αβ motif (CSEβ motif), characteristic of peptides with antimicrobial activity. In addition to this CSEβ motif, plant defensins also have another conserved framework region characterized, called the γ-core, which has the conserved sequence (GXCXγ,C, where X may be any amino acid residue). Some studies have shown that the region responsible for the biological activity of plant defensins resides in the γ-core region. De Samblanx et al. showed that the substitution or removal of the amino acid residues located in this region, which encompasses β2 and β3 sheets, reduced the biological activity of RsAFPγ (a defensin from Raphanus sativus). Sagaram et al. demonstrated that the main determinants of the antifungal activity of MsDefγ and MtDefγ (defensins from Medicago sativa and Medicago truncatula, respectively) reside in their γ-core motifs. In another study, it was shown that the interaction of PoDγ defensin from Pisum sativum with the membrane is mediated, in part, by the amino acid residues that compose the γ-core region.

Knowing that the region comprising the plant defensin γ-core is related to the antimicrobial properties of these peptides, the principal aim of this work was to design synthetic peptides based on the region corresponding to the PoDγ defensin γ-core that are the smallest amino acid sequences that bear the strongest biological activity. PoDγ is an isolated defensin from Phaseolus vulgaris seeds with antifungal properties, anti-Leishmania activity, and anticancer activity. Additionally, knowing that the charge, length, and hydrophobicity influence the activity of antimicrobial peptides, we made rational substitutions of negatively charged amino acid residues with positively charged ones, as well as the reduction in length in the selected PoDγ defensin γ-core sequence to verify whether the increased net positive charge and the shortened length are related to the increase in antifungal activity. In addition, some tests were carried out to better understand the mechanism of action of the peptides derived from the γ-core region of PoDγ defensin.

Materials and methods

Microorganisms

The yeast species Candida albicans (CE022) and Candida buinensis (3982) were maintained in Sabouraud agar (1% peptone, 2% glucose, and 1.7% agar; Merck). The yeast cells were provided by Laboratório de Fisiologia e Bioquímica de Micorganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Analysis of amino acid sequence of PoDγ defensin

The amino acid sequence of the PoDγ defensin from P. vulgaris was obtained as previously described by Games et al. In brief, the first 21 amino acid residues of PoDγ were obtained by Edman degradation of the purified peptide. Based on this 21 amino acid sequence, a degenerated primer was designed and used in an RT-PCR together with an oligo-dT primer. The amplicon was cloned into the pTZ57R/T vector (InStAclone™ PCR Cloning Kit, Fermentas) and then submitted to sequencing by Big Dye1 Terminator v3.1 Kit (Applied Biosystems) in an ABI 3110 Genetic Analyzer (Applied Biosystems).

Synthetic peptide design

By comparison analysis of the primary structure of PoDγ defensin with other defensin primary structures and by superimposing onto these sequences the sequence of the γ-core, as defined by Yount and Yeaman, we identified and selected the region corresponding to the PoDγ γ-core. This region encompasses the γ-core itself and amino acid residues that are part of the flanking β2 and β3 sheets. Based on these comparative analyses, two peptides with 15 amino acid residues and two peptides with 9 amino acid residues each were then chemically synthesized by the GenOne Soluções em Biotecnologia (Rio de Janeiro, Brazil). The purity level of the synthetic peptides was >95% as determined by...
high-performance liquid chromatography (4.6×250 mm PLRP-S 100A), using a solution A (0.1% trifluoroacetic acid in 100% acetonitrile) and a solution B (0.1% trifluoroacetic acid in 100% water) with the following gradient: 0%–15% of solution A from 0 to 25 minutes; 40%–100% of solution A from 25 to 30 minutes. The Cys (C) residues of these peptides were replaced by Ala (A) residues. Additionally, two Asp (D) residues were substituted by two Arg (R) residues in two peptides. All these peptides were resuspended in ultrapure water. Their molecular masses and isoelectric points were determined by the Expasy Compute pi/Mw tool.28–30

Effect of synthetic peptides on yeast growth

Yeast cell inocula from C. albicans and C. buinensis were removed from stock tubes containing Sabouraud agar and transferred to Petri dishes containing Sabouraud agar. Cells were grown at 30°C for 2 days. After this period, each cell aliquot was added to 10 mL sterile culture medium (Sabouraud broth, 1% peptone, 2% glucose, Merck). Yeast cells were quantified in a Neubauer chamber (LaborOptik). Initially, the C. albicans and C. buinensis yeast cells (1×10⁴ cells mL⁻¹) were incubated in 100 µL of Sabouraud broth containing the selected synthetic peptides at increasing concentrations from 18.35 to 293.6 µM.

The assay was performed on cell culture microplates (96 wells; Nunc) at 30°C for 24 hours. Optical readings at 620 nm (EZ Read 400, Biochrom) were collected 24 hours after the start of assay. Control cells were grown in the absence of synthetic peptides. The assay was done three times according to a methodology adapted from Broekaert et al.31 The yeast growth inhibition data were evaluated by the one-way ANOVA, and the differences of the mean at P<0.05 were considered significant. All statistical analyses were performed using the GraphPad Prism software (version 6.0 for Windows).

Cell viability

To verify whether the growth inhibition of C. buinensis cells was caused by the fungicidal or fungistatic effect of the smallest active peptide, the control cells (without the peptide) were washed and diluted 1,000-fold. A 100 µL aliquot of the dilution was spread with a Drigalski spatula on the surface of a Petri dish containing Sabouraud agar and grown at 30°C for 48 hours. At the end of this period, the colony forming units were determined, and Petri dishes were photographed.32 The same procedure was repeated with the yeasts treated with 36.7 and 73.4 µM of the smallest and the strongest active peptide for 24 hours. The experiments were performed in triplicate, and the results are shown assuming that the control represents 100% cell viability. These data were evaluated by one-way ANOVA, and the differences of the mean at P<0.05 were considered significant. All statistical analyses were performed using the GraphPad Prism software (version 6.0 for Windows).

Effect of a synthetic peptide on plasma membrane permeabilization

The plasma membrane permeabilization of C. buinensis cells treated with 25 µM of the smallest and the strongest active peptide was evaluated using Sytox green fluorescent dye (Invitrogen), according to the methodology described by Thevissen et al.33 Sytox green is a dye that has high affinity for nucleic acids and penetrates the cell only when its membrane is compromised, emitting a strong green fluorescent. After the growth inhibition assay, C. buinensis yeast cells grown in the absence (control) and in the presence of the smallest and the strongest active peptide were incubated with Sytox green at a final concentration of 0.2 µM (dissolved in dimethyl sulfoxide), according to instructions provided by the manufacturer. A positive control with 300 mM ethanol was done. After 15 minutes incubation at 25°C with constant agitation at 500 rpm, the cells were observed under an optical microscope (Axioplan.A2, Zeiss) coupled to an AxioCAM MRc5 (Zeiss) camera and the images were analyzed by the Axiovision software version 4.0 (Zeiss). The microscope was equipped with a set of fluorescent filters for fluorescein detection (excitation wavelength between 450 and 490 nm and emission of 500 nm). The results represent triplicate experiments.

Determining the induction of intracellular oxygen reactive species

To evaluate whether the mechanism of action of the smallest and the strongest active peptide involves the induction of oxidative stress, the fluorescent probe 2’,7’-dichloro-fluorescein diacetate (H₂DCFDA) was used according to the methodology described by Mello et al.35 This dye enters the cell passively, is deacetylated by intracellular esterases and after being oxidized by reactive oxygen species (ROS), becomes fluorescent. After 24 hours incubation with 25 µM of the smallest and the strongest active peptide, 50 µL of C. buinensis cells grown in the absence (control) and in the presence of the peptide were incubated with 20 µM of the H₂DCFDA probe (Calbiochem), for 2 hours at 25°C with constant agitation at 500 rpm. A positive control was done with 300 mM hydrogen peroxide. After this period, the cells were observed under an optical microscope (Axioplan. A2, Zeiss) coupled to an AxioCAM MRc5 (Zeiss) camera, and the images were analyzed by the Axiovision software version 4.0 (Zeiss). The microscope was equipped with a
set of fluorescent filters for fluorescein detection (excitation wavelength between 450 and 490 nm and emission of 500 nm). The results represent triplicate experiments.

Analysis of mitochondrial functionality
Mitochondrial functionality was assessed by fluorescent dye Rhodamine 123 (Sigma). Rhodamine 123 is a cationic fluorescent dye that has a high affinity to the electrical potential of membranes; thus, it marks active mitochondria in living cells resulting in a bright red fluorescence. In contrast, the treated samples present a weak or absent fluorescent signal. This test was done as described in the section “Effect of synthetic peptides on yeast growth” with the following differences: after incubation with 25 µM of the smallest and the strongest active peptide for 24 hours, the C. buinensis cells were resuspended, washed once in 500 µL PBS (10 mM NaH₂PO₄, 0.15 M NaCl) with pH 7.4 and resuspended in 50 µL of the staining solution (supplied by the kit) containing 50 µM FITC-VAD-FMK marker. After incubation for 20 minutes at 30°C with constant agitation at 500 rpm, the cells were again washed in 500 µL PBS and resuspended in 20 µL PBS. Negative control (in the absence of the peptide) and positive control cells (incubated with 300 mM acetic acid) had the same treatment as cells treated with the peptide. The cells were analyzed by DIC on the optical microscope equipped with a fluorescence filter for fluorescein detection (excitation wavelength 450–490 nm and emission wavelength 500 nm).

Results
Synthetic peptide design
After the analysis of the primary structure of PνD₁, previously obtained by Games et al.,¹² and other defensin peptides,⁷ the region corresponding to the PνD₁ γ-core was selected and is highlighted in blue (Figure 1A). Based on the PνD₁ γ-core sequence, together with parts of the β2 and β3 sheets (from Arg₂ to Lys₂₆), an amino acid stretch giving rise to

![Figure 1](https://www.dovepress.com/)

**Figure 1** Design, alignment and biochemical characteristics of the synthetic peptides.

**Notes:** (A) Alignment of the primary structures of PνD₁ and the four synthetic peptides in amino acid one-letter code. Numbers above the PνD₁ sequence indicate the peptide size in amino acids. The amino acid residues in blue represent the PνD₁ γ-core region. The amino acid residues in red represent the replaced residues in the original PνD₁ sequence as follows: C residues were replaced by A, and D residues were replaced by R. Amino acid residues in black are not part of the γ-core region and were not changed. Numbers in the synthetic peptide names stand for the amino acid position in the original PνD₁. Double-plus (++) indicates the double replacement from the original D to R amino acid residues to increase the synthetic peptide positive net charge. (B) Biochemical characteristics of the synthetic peptides.

<table>
<thead>
<tr>
<th>Synthetic peptide names</th>
<th>Size in amino acids</th>
<th>Charge (pH 7.0)</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ₁₃₄₋₃₋₅₋₁₅ PνD₁</td>
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<td>+3</td>
<td>1,792.99</td>
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<tr>
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<tr>
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<tr>
<td>γ₁₋₅₋₁₅ PνD₁</td>
<td>9</td>
<td>+5</td>
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</tr>
</tbody>
</table>
the peptide of 15 residues with the following amino acid sequence was chosen: RSGRARDDFRAWATK, which was called $\gamma_{31-45}^{PV}D_1$ (Figure 1A). Another peptide, based on the $\gamma_{31-45}^{PV}D_1$ design, had its net positive charge increased with the replacement of the two Asp residues, at positions 37 and 38, by two Arg residues, giving rise to the following amino acid sequence: RSGRARRRFRAWATK, which was called $\gamma_{31-45}^{PV}D_1^{++}$ (Figure 1A). Two other peptides of nine residues each, smaller than $\gamma_{31-45}^{PV}D_1$ and $\gamma_{31-45}^{PV}D_1^{++}$, and had the sequences comprising Gly$_{33}$ to Cys$_{41}$ ($\gamma_{33-41}^{PV}D_1$-core itself) with the amino acid sequences GRARDDFRA and GRARRRFRA, which were called $\gamma_{33-41}^{PV}D_1$ and $\gamma_{33-41}^{PV}D_1^{++}$, respectively, were also synthesized (Figure 1A). All the synthesized peptides had their Cys residues replaced by Ala residues (Figure 1A). These substitutions were made to prevent the formation of disulfide bridges. Similarly, Schaaper et al$^{34}$ replaced the Cys residues by $\alpha$-aminobutyric acid, to avoid the formation of such undesired bridges. The biochemical characteristics of synthesized peptides are shown in Figure 1B.

**Growth inhibition assay**

To analyze the effect of the synthetic peptides on the growth of yeasts, *C. albicans* and *C. buinensis*, $\gamma_{31-45}^{PV}D_1$, $\gamma_{31-45}^{PV}D_1^{++}$, $\gamma_{33-41}^{PV}D_1$, and $\gamma_{33-41}^{PV}D_1^{++}$ peptides were used at increasing concentrations from 18.35 to 293.6 µM (Figures 2 and 3).

With the $\gamma_{31-45}^{PV}D_1$ peptide, we observed that all used concentrations inhibited the growth of yeast *C. albicans*, reaching

![Antifungal effects of $\gamma_{31-45}^{PV}D_1$ and $\gamma_{31-45}^{PV}D_1^{++}$ incubated for 24 hours at different concentrations on Candida albicans and Candida buinensis. *Indicates significance by the one-way analysis of variance (ANOVA) (P<0.05).](image-url)
40% inhibition at the concentration of 293.6 µM. The yeast *C. buinensis* had its growth inhibited starting from the 73.4 µM concentration, and it was possible to observe the 100% inhibition, when the peptide concentration was 293.6 µM (Figure 2). When the positive net charge of this peptide was increased by the synthesis of \( \gamma_{33-45}^{\mathrm{PvD}_1} \), the peptide concentration of 18.35 µM inhibited 60% growth of the yeast *C. albicans*, and starting from the concentration of 73.4 µM, the yeast growth was completely inhibited (Figure 2). For yeast *C. buinensis*, the lowest used peptide concentration of 18.35 µM completely inhibited yeast growth (Figure 2).

When we used the smallest peptides, we observed that \( \gamma_{33-41}^{\mathrm{PvD}_1} \) was not able to inhibit the growth of *C. albicans* at any concentrations tested (Figure 3). For *C. buinensis*, this peptide inhibits growth by 15% and 17% at the two highest used concentrations, respectively (Figure 3). However, the peptide \( \gamma_{33-41}^{\mathrm{PvD}_1}^{++} \) that had its positive net charge increased showed a greater inhibition potential, being able to inhibit the growth of *C. albicans* at all concentrations tested and reaching 63% inhibition at the concentration of 293.6 µM (Figure 3). For *C. buinensis*, this peptide had an even greater inhibitory potential, being able to inhibit 100% of its growth starting from the concentration of 36.7 µM (Figure 3).

Based on the results from Figures 2 and 3, the designed smallest peptide that had the strongest antimicrobial activity

\[
\gamma_{33-41}^{\mathrm{PvD}_1}^{++}
\]

Figure 3 Antifungal effect of \( \gamma_{33-41}^{\mathrm{PvD}_1} \) and \( \gamma_{33-41}^{\mathrm{PvD}_1}^{++} \) incubated for 24 hours at different concentrations on *Candida albicans* and *Candida buinensis*. *Indicates significance by the one-way analysis of variance (ANOVA) \( P < 0.05 \).
was $\gamma_{33-41}PvD_1^{++}$ when incubated with $C. buinensis$. Because of this outcome, this peptide and this yeast species were chosen for the next experiments.

**Cell viability**

Based on the results obtained in the growth inhibition assay, we found that the minimal inhibitory concentration (MIC) of $\gamma_{33-41}PvD_1^{++}$ peptide was 36.7 $\mu$M for $C. buinensis$ cells (Figure 4A). The $\gamma_{33-41}PvD_1^{++}$ peptide at 36.7 and 73.4 $\mu$M induced the viability loss in $C. buinensis$ cells. The cells treated with 36.7 $\mu$M of this peptide had an 84% loss of viability and the cells treated with 73.4 $\mu$M had a 100% loss of viability, showing that the effect of this peptide is fungicidal to $C. buinensis$ (Figure 4B and C). Starting from these experiments, all following assays were performed with yeast $C. buinensis$ with the $\gamma_{33-41}PvD_1^{++}$ at 25 $\mu$M, because at this concentration, below the determined MIC, we were able to obtain reasonable amounts of cells for visualization by optical microscopy.

**Effect of $\gamma_{33-41}PvD_1^{++}$ on plasma membrane permeabilization**

The ability of the $\gamma_{33-41}PvD_1^{++}$ to permeabilize the $C. buinensis$ plasma membrane was analyzed using Sytox green. The analysis by fluorescence microscopy revealed that $C. buinensis$ cells were labeled by the dye, when treated with $\gamma_{33-41}PvD_1^{++}$ at 25 $\mu$M; thus, these data suggest that $\gamma_{33-41}PvD_1^{++}$ acts on the plasma membrane of $C. buinensis$, structurally compromising it and allowing its permeabilization for the labeling dye. In the control (in the absence of peptide),

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**Figure 4** MIC determination and cell viability loss.

**Notes:** (A) Images of the plate wells at the end of the growth inhibition assay (at 24 hours) showing the growth pattern of $Candida buinensis$ cells at the bottom of the wells in the absence (control) and in the presence of different $\gamma_{33-41}PvD_1^{++}$ concentrations. (B) Table showing the number of CFU and the percentage of viability loss of $C. buinensis$ cells after treatment with 36.7 and 73.4 $\mu$M of $\gamma_{33-41}PvD_1^{++}$ for 24 hours, respectively. *Indicates significance by the one-way analysis of variance (ANOVA) (P<0.05). (C) Images of Petri dishes showing the CFU as described in (B). The experiments were carried out in triplicate.

**Abbreviations:** CFU, colony forming units; MIC, minimal inhibitory concentration.
no fluorescence was observed and in the positive control (300 mM ethanol), fluorescent cells were observed (Figure 5).

**Reactive oxygen species assay detection**

After the growth inhibition assay, *C. buinensis* cells were incubated with the H$_2$DCFDA probe for the detection of endogenous ROS production. When incubated in the presence of 25 µM of $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$, the cells showed ROS labeling (Figure 6), suggesting that a $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$-induced increase in oxidative stress may underlie the growth inhibitory effect on this yeast. In the control (in the absence of peptide), no fluorescence was observed and in the positive control (300 mM hydrogen peroxide), fluorescent cells were observed (Figure 6).

**Analysis of mitochondrial functionality**

In *C. buinensis* cells treated with $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$ at 25 µM for 12 hours is observed a diminished mitochondrial activity as indicated by the weaker red fluorescent signal of Rhodamine 123 (Figure 7) in comparison to the untreated cells, and after additional 24 hours in the presence of $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$ it is also observed a shrinkage and condensation of cell cytoplasm. A similar weak fluorescent signal was observed for the 300 mM ethanol-treated samples (positive control). Control cells with functional mitochondria evinced a strong signal of Rhodamine 123 fluorescence (Figure 7). This result indicated that after treatment with $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$, the *C. buinensis* cells lost their electrical mitochondrial membrane potential, causing the dysfunction of their mitochondria.

**Detection of caspase activity induced by $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$**

The detection of caspase activity was performed using the CaspACE FITC-VAD-FMK in situ marker. The *C. buinensis* yeast cells grown in the absence (control) and in the presence of 25 µM of $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$ were subsequently incubated with the FITC-VAD-FMK marker. It can be observed that the cell treatment with $\gamma_{\text{33-41}}$, $\text{PvD}_1^{++}$ resulted in the activation of metacaspases, making it possible to observe a strong labeling of these cells. Positive control cells, treated with 300 mM acetic acid, a known apoptosis inducer in yeast, also showed labeling. However, the untreated cells (negative control) did not show metacaspase activity (Figure 8). These data suggest a programmed cell death that occurs by an apoptotic pathway.

**Discussion**

Plant defensins are peptides whose activity and mechanisms of action are primarily described against a wide variety of filamentous fungi and yeasts; however, their mechanism of
Antimicrobial activity of smallest peptides derived from defensin $P\nu D_1$

Action has not yet been fully elucidated. The defensins have a structure composed of an $\alpha$-helix and two $\beta$-sheets connected by four disulfide bridges. Yount and Yeaman showed that the peptides stabilized by disulfide bridges possess amino acid residues that are important for antimicrobial activity in the $\gamma$-core region that encompasses $\beta_2$ to $\beta_3$ sheets. Many studies have already shown that the activity of plant defensins is related to this structural domain.

**Figure 6** Images of reactive oxygen species assay detection in Candida buinensis cells after treatment with $\gamma_{33-41}P\nu D_1$ (25 $\mu$M) for 24 hours. Control cells were treated only with the 2',7'-dichlorofluoresceindiacetate probe and positive control cells were treated with 300 mM hydrogen peroxide. Bars =20 $\mu$m.

Abbreviation: DIC, differential interference contrast.

**Figure 7** Images of mitochondrial functionality assay of Candida buinensis cells after treatment with $\gamma_{33-41}P\nu D_1$ (25 $\mu$M) for 12 and 24 hours. Control cells were treated only with Rhodamine 123 probe and positive control cells were treated with 300 mM ethanol. Bars =20 $\mu$m.

Abbreviation: DIC, differential interference contrast.
In the previous work performed by our group, the PvD_{1} defensin isolated from P. vulgaris seeds inhibited growth of various yeasts.\textsuperscript{14,15,16} In this study, we designed synthetic peptides based on the PvD_{1} γ-core. Initially, we designed two peptides of 15 residues that comprised the region from Arg_{31} to Lys_{45}, called γ_{31-45}PvD_{1} and γ_{31-45}PvD_{1} ++ , the latter having its positive net charge increased (Figure 1). Some studies have identified charge, length, and hydrophobicity as the parameters that influence the activity of antimicrobial peptides.\textsuperscript{26,27} Regarding the length, it has already been reported that larger peptides that cover the entire β2 and β3 sheets, where the γ-core region is inserted, have higher activity.\textsuperscript{14} Sagaram et al.\textsuperscript{25,26} also demonstrated that synthesis of peptides extending beyond the γ-core region improves antifungal activity and that the synthetic peptide (GMA-4C) derived from the γ-core region also contained the C-terminus of MtDef_{1} defensin with cationic and hydrophobic amino acids that were important for its antifungal activity. Regarding the charge, Lacerda et al.\textsuperscript{19} demonstrated that the positively charged amino acids located in the γ-core were essential for the antifungal activity of peptides, since the substitution of neutral residues by positively charged amino acid residues in the γ-core region increased their inhibitory activity against pathogenic fungi. The mechanism behind this interconnection between positive charge increasing and antimicrobial inhibition increasing is based mainly in the opposite charge attraction.\textsuperscript{38} Fungal cells have negatively charged structures in their cell walls, such as phospholipids,\textsuperscript{39} that may serve as primary anchor sites for the positively charged peptides. Then the peptides became able to interact with the membrane. The interaction with the membrane is possible because of the amphipathic character of antimicrobial peptides which turning them able to interact with the negative charge of phospholipids hear groups and also with the hydrophobic core of the membrane.\textsuperscript{11,33} This interaction explains the ability of antimicrobial peptide to cause membrane permeabilization. Similarly, the His and Arg residues present in the γ-core were important for the oligomerization and antifungal activity of the peptide derived from MtDef_{1} defensin against the filamentous fungi Neurospora crassa and Fusarium graminearum.\textsuperscript{36} Thus, we designed the peptide γ_{31-45}PvD_{1}, which had additional amino acids of the flanking β2 and β3 sheets, beside the amino acids that compose the γ-core and increased the charge of γ_{31-45}PvD_{1} ++ by replacing two Asp by two Arg. When we tested the antifungal activity of γ_{31-45}PvD_{1}, it inhibited the growth of C. albicans at all concentrations tested. For C. buinensis, we observed inhibition only starting from the concentration of 73.4 μM, with MIC being 293.6 μM concentration. When we increased the net positive charge of γ_{31-45}PvD_{1} ++ , it exhibited higher antifungal activity. All tested concentrations of this

![Figure 8](https://www.dovepress.com/)

**Figure 8** Images of detection of metacaspase activity assay of Candida buinensis cells after treatment with γ_{31-45}PvD_{1} ++ (25 μM) for 24 hours. Control cells and cells treated with γ_{31-45}PvD_{1} ++ were incubated with CaspACE FITC-VAD-FMK probe. Positive control cells were treated with 300 mM acetic acid and analyzed by fluorescence microscopy. Bars =20 μm.

**Abbreviation:** DIC, differential interference contrast.
peptide inhibited the *C. albicans* growth, with MIC being 73.4 µM. For yeast *C. buinensis*, MIC was determined at the lowest concentration used, at 18.35 µM (Figure 2).

Although some previous works have shown that the peptide length influenced its antifungal activity, if a smaller molecule obtained from the native sequence of a peptide still had biological activity, it would be more commercially interesting due to the low cost of its synthesis and could be seen as a promising molecule for the control of fungal diseases. To find an even smaller molecule with antifungal potential that could be used as a novel drug, we designed two peptides of nine amino acid residues each that comprised the γ-core region from Gly33 to Cys41, called γ33-41*PvD*1 and γ33-41*PvD*4++, where the latter also had its positive net charge increased as discussed above (Figure 1). Our choice of the smallest sequence with the strongest biological activity is based on requirements of desired characteristics for the pharmaceutical industry to alleviate problems like toxicity, drug stability, specificity, and less immunogenicity. For these reasons, we chose to work with the γ33-41*PvD*4++ peptide.

When we tested the activity of these peptides, the γ33-41*PvD*1 peptide at different concentrations did not inhibit the growth of *C. albicans* cells, whereas for yeast *C. buinensis*, it showed low activity at high concentrations (Figure 3). These results are consistent with the works of Schaaper et al14 and Sagaram et al21 in the sense that the smallest peptides, only encompassing the γ-core, as in our study, did not bear strong biological activity, at least for *C. albicans* and *C. buinensis*. Additionally, as in another example of Sagaram et al23 the γ-core alone may or may not present biological activity similar to the entire defensin. These authors tested the activity of two synthetic peptides called GMA1 and GMA2, derived, respectively, from the γ-core of the defensins MsDef1 and MtDef1, against the filamentous fungus *F. graminearum*. The peptide GMA1, even at the concentration of 96 µM, had no antifungal activity. In contrast, the peptide GMA2 exhibited antifungal activity at 6 µM, and when used at 12 µM, was able to completely inhibit the conidial germination of the tested fungus. These results make it evident that only the γ-core region of the MtDef1 defensin is sufficient for antifungal activity; however, the γ-core of the MsDef1 defensin by itself is not sufficient for antifungal activity, similar to the γ-core (γ33-41*PvD*1) of the *PvD*1 defensin.

On the other hand, when we tested the antifungal activity of γ33-41*PvD*4++, with the positive net charge increased by replacing two Asp37,38 residues by two Arg residues, we observed that it inhibited the growth of *C. albicans* and *C. buinensis*, with the MIC for *C. buinensis* of 36.7 µM. Previous studies showed that higher the net positive charge, the higher will be the antifungal activity of peptides, and the positively charged amino acids, such as Arg and Lys, were very important for antifungal activity. The synthetic peptide MBGo1, derived from *R. AtpF*2 defensin, which had its net positive charge increased due to the substitution of some amino acid residues by Arg residues, showed higher activity against the filamentous fungus *Fusarium culmorum*.34 Sagaram et al23 showed that MtDef1 defensin had five basic amino acids in its γ-core, and MsDef1 defensin had two basic amino acids and two acidic amino acids. After an exchange of the γ-core region between these two defensins, MsDef1 had a two fold higher antifungal activity, thus showing the importance of positively charged amino acid residues for the higher antifungal activity of this peptide.

In the growth inhibition assays, γ33-41*PvD*4++ peptide showed higher activity against *C. buinensis* cells, and this inhibitory effect on yeast growth was fungicidal (Figure 4). Taveira et al41,42 found that another cationic peptide isolated from *Capsicum anuum* fruits, belonging to thiophin family, called CaThi, had a fungicidal effect against six species of *Candida* genus and *Fusarium solani*. It is worth emphasizing that the fungicidal characteristic is very important for the development of new therapeutic drugs, since substances that present fungistatic effect can contribute to the development of resistant microorganisms.43,44

Considering the results obtained, we began to investigate the mechanism of action of γ33-41*PvD*4++, responsible for the inhibition of *C. buinensis* growth. One characteristic of the cationic antimicrobial peptides is their ability to permeabilize the plasma membrane of microorganisms.15,36 Initially, we used Sytox green, a dye that has high affinity for nucleic acids and penetrates cells only when their plasma membrane is compromised. We observed that the *C. buinensis* cells, when treated with the γ33-41*PvD*4++ peptide, were marked by the dye (Figure 5). Sagaram et al23 showed that MsDef1γ4 peptide was also able to permeabilize the membrane; however, the tested fungus was *F. graminearum*. In another work, Islam et al36 found that the cationic amino acid residues, present in the γ-core of MtDef1 defensin, were responsible for its antifungal activity and that at micromolar concentrations, MtDef1 caused the membrane permeability of the filamentous fungi *F. graminearum* and *N. crassa*. These same authors also observed that there was an increase in the production of intracellular ROS in *F. graminearum* and *N. crassa* hyphae after these cells were treated with MtDef1γ.

ROS have been considered as primary regulators of cell death and are linked to many crucial apoptotic pathways...
in yeasts. Studies have shown that an increase of ROS in the medium can be toxic to the organisms, leading to the destruction of several cell types through apoptotic pathways. Previously, our research group showed that 

\[ \gamma_{33-41} \text{PvD}_1 \]  

induced ROS in \textit{C. albicans} and \textit{Fusarium oxysporum} cells. In this study, we observed that \textit{C. buinensis} cells were labeled by the 2,7-dichlorofluorescein diacetate probe when they were treated with \[ \gamma_{33-41} \text{PvD}_1 \] , thus indicating that it caused an increase in endogenous ROS production in these cells (Figure 6). Due to the increased ROS production in the presence of \[ \gamma_{33-41} \text{PvD}_1 \] , our next objective was to verify if the apoptotic process could be taking place when the \textit{C. buinensis} cells were treated with \[ \gamma_{33-41} \text{PvD}_1 \] . For this process, we verified the presence of active metacaspases and the dissipation of mitochondrial membrane potential in these cells. Caspases play a central role in signaling death by apoptosis. These are described as specific cysteine-containing aspartate proteases that are among the key markers of the apoptotic pathway, including yeasts. In these two assays, it was possible to observe that \[ \gamma_{33-41} \text{PvD}_1 \] activated the metacaspases in \textit{C. buinensis} cells and caused a collapse of mitochondrial membrane potential in these cells (Figures 7 and 8). Taveira et al found that \textit{CaThi} caused an activation of metacaspase in \textit{F. solani}, indicating that programmed cell death could be triggered by \textit{CaThi} in this fungus. In another study, Soares et al showed that \textit{ApDef}, defensin, isolated from the seeds of \textit{Adenanthera pavonina}, caused an increase in the ROS production and accumulation that led to permeabilization of the plasma membrane and, consequently, the death of \textit{Saccharomyces cerevisiae} cells through a metacaspase-dependent apoptotic process. It has also been observed by Vieira et al that \textit{C. albicans} cells lost mitochondrial functionality when treated with the \textit{Lp-Def}1 defensin, isolated from \textit{Lecythis pisonis} seeds.

**Conclusion**

In conclusion, taken together, our results suggest that the antifungal activity of 

\[ \textit{PvD}_1 \]  

is not strictly localized in the structural domain, which comprises the \( \gamma \)-core region; however, we can state that the addition of amino acid residues beyond the \( \gamma \)-core region, which comprises parts of the \( \beta_2 \) and \( \beta_3 \) sheets, is important for antifungal activity. Additionally, we observed that the increase in the net positive charge is directly related to the increase in antifungal activity. In this work, we opted to evaluate the mechanism of action of the \[ \gamma_{33-41} \text{PvD}_1 \] peptide due to its significant inhibitory effect on tested yeast. In addition, \[ \gamma_{33-41} \text{PvD}_1 \] is the smallest construct comprising only nine amino acid residues, which gives it a better possibility to be the basis for a design of a new antifungal drug, with lower costs to the pharmaceutical industry, but still maintaining its antimicrobial properties. In relation to the antifungal activity of \[ \gamma_{33-41} \text{PvD}_1 \] , it was fungicidal, and its effects on the growth inhibition of \textit{C. buinensis} are related to membrane permeabilization, an endogenous increase in ROS, the loss of mitochondrial functionality and caspase activation, suggesting that \[ \gamma_{33-41} \text{PvD}_1 \] triggers \textit{C. buinensis} cell death via apoptosis, as demonstrated by the key markers of this pathway.

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**Author contributions**

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

**Disclosure**

The authors report no conflicts of interest in this work.

**References**


