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ORIGINAL ARTICLE

Design of improved synthetic antifungal peptides with targeted variations in charge, hydrophobicity and chirality based on a correlation study between biological activity and primary structure of plant defensin γ-cores

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Abstract

Microbial resistance to available drugs is a growing health threat imposing the need for the development of new drugs. The scaffold of plant defensins, including their γ-cores, are particularly good candidates for drug design. This work aimed to improve the antifungal activity of a previous design peptide, named $A_{36,42,44}\gamma_{32-46}$*Vu*Def (for short DD) against yeasts by altering its biochemical parameters. We explore the correlation of the biological activity and structure of plant defensins and compared their primary structures by superimposition with *Vu*Def$_1$ and DD which indicated us the favorable position and the amino acid to be changed. Three new peptides with modifications in charge, hydrophobicity (RR and WR) and chirality (D-RR) were designed and tested against pathogenic yeasts. Inhibition was determined by absorbance. Viability of mammalian cells was determined by MTT. The three designed peptides had better inhibitory activity against the yeasts with better potency and spectrum of yeast species inhibition, with low toxicity to mammalian cells. WR, the most hydrophobic and cationic, exhibited better antifungal activity and lower toxicity. Our study provides experimental evidence that targeted changes in the primary structure of peptides based on plant defensins γ-core primary structures prove to be a good tool for the synthesis of new compounds that may be useful as alternative antifungal drugs. The method described did not have the drawback of synthesis of several peptides, because alterations are guided. When compared to other methods, the design process described is efficient and viable to those with scarce resources.

Keywords Antifungal activity · Minimal inhibitory concentration · Lethal dose · Pathogenic yeasts

Abbreviations

DD	$A_{36,42,44}\gamma_{32-46}$*Vu*Def
RR	$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def
D-RR	D-$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def
WR	$A_{42,44}R_{37,38}W_{36,39}\gamma_{32-46}$*Vu*Def
AMP	Antimicrobial peptides

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CC_{50}	50% Cytotoxic concentration
DL_{100}	Lethal dose
GlcCer	Glucosylceramide
PA	Phosphatidic acid
$PI(4,5)P_2$	Phosphatidylinositol 4,5 bisphosphate
$M(IP)_2C$	Mannosyldiinositol phosphorylceramide
MIC_{100}	Minimal inhibitory concentration

Introduction

Microbial resistance to available drugs is a growing public health threat and fungal infections, such as candidiasis and aspergillosis, caused by resistant fungi are among the most worrying (Jensen 2016; Fisher et al. 2018). In addition, infections caused by multi-resistant *Candida* to commercial antifungals have been increasing in recent decades (Bongomin et al. 2017; Fisher et al. 2018). Especially those caused by *C. auris* that is an emerging yeast species, which has a phenotype of multiple resistance to the main classes of antifungals clinically available, and has caused serious invasive infections in hospitalized patients (Forsberg et al. 2019). In addition to the multi-resistance problem that severely limits its treatment, *C. auris* also spreads very easily in hospital environment aggravating the problem (Forsberg et al. 2019). Therefore, the need for the research and development of new substances that act as an alternative to existing antifungals become evident, to overcome the problem of widespread resistance among infectious agents, to be new options for the treatment of fungal diseases in view of the scarcity of antifungals compared to antibacterial agents (Costa-de-Oliveira and Rodrigues 2020), and to combat new emerging pathogens such as *C. auris*. Among candidate substances are the antimicrobial peptides (AMPs).

All extant organisms, ranging from bacteria to mammals, encode and express AMPs. AMPs are peptides composed of L-amino acids in chains of up to 100 units, in a wide variety of primary structures, organized in amphiphilic positively charged three-dimensional structures (Koehbach and Craik 2019). The amphiphilicity property enables them to be soluble in aqueous environment and to interact with lipid membranes, explaining their antimicrobial activity (Brogden 2005; Ghosh et al. 2018; Ciumac et al. 2019). AMPs are known since the 40s' (Balls and Hale 1940), but only at the beginning of the 80s' had become clear their pivotal role in innate immunity of different organisms like cecropin from insects (Steiner et al. 1981), defensins of mammals (Selsted et al. 1985) and magainin in amphibians (Zasloff 1987). The antimicrobial activity of AMPs was demonstrated against different microorganisms such as viruses, bacteria, fungi, and protozoa, and extends beyond antimicrobial activity being also able to inhibit the growth of cancer cells (Deslouches and Di 2017). The recognition of the broad spectrum of antimicrobial activity along with their clear participation in innate immunity in metazoan boosted the interest on AMPs, which culminated in the discovery of many AMPs in different organisms (Koehbach and Craik 2019). Several of them with specific biological activities (Whittington and Belov 2007; Sunagar et al. 2013). Moreover, AMPs have some characteristics that classify them as possible therapeutic agents: (1) their broad antimicrobial activity which means that one AMP inhibits different microorganisms (Greco et al. 2019); (2) their small size and high stability to harsh environments (e.g. pH extremes, high temperature, and protease resistance) (Beer and Vivier 2008; Colgrave and Craik 2004); (3) amenability to peptide engineering by molecular biology or chemical synthesis (De Samblanx et al. 1997). This last technique has the advantage to allow the incorporation into primary structures of AMP of unusual amino acids, like non-proteinogenic or D-enantiomers (Ding et al. 2020; Greco et al. 2019), improving their activity, specificity and stability, decreasing their toxicity and protecting them from proteolytic degradation in the target organism (Li et al. 2016). Based on these characteristics, some authors claimed that AMPs could be interesting scaffolds for the development of antimicrobial substances for therapeutic use in humans to refrain the upsurge of microbial resistance (Zasloff 1987; Zhang et al. 2019). Thus, many AMPs from different sources are in clinical trials (Ghosh et al. 2018).

Plants produce several AMPs that have the potential to be used in the development of new antimicrobial therapeutics. Plant AMPs participate, as in other organisms, in their innate immunity, protecting them from viruses, bacteria and fungi attacks (Campos et al. 2018). One of the best characterized plant AMPs are the defensins which are known since the 90′s (Colilla et al. 1990; Méndez et al. 1990). Since then, the characterization of many peptides belonging to this family had given great knowledge about their biology. These molecules are synthesized as a protein precursor processed in the endoplasmic reticulum yielding mature peptides of 45–54 L-amino acid residues in their primary structure that are arranged in a tertiary structure composed of three antiparallel β-strands and one α-helix (Carvalho and Gomes 2011). This tertiary structure is highly conserved among the family (Carvalho and Gomes 2011) with some known exceptions like *Vr*D1 (from *Vigna radiata*, mung bean) that has a 3_{10} type helix between the β_1 strand and the α-helix (Liu et al. 2006), and Sd_5 (from sugarcane) that has an unstructured C-terminal extension (De Paula et al. 2011). Amidst the amino acids that constitute the primary structure are eight strictly positional conserved cysteine residues that bind in specific pairs to form four disulfide bridges with C_1–C_8, C_2–C_5, C_3–C_6, and C_4–C_7 arrangement (Thomma et al. 2002; Shafee et al. 2017) with the exception of *Ph*D_1 and *Ph*D_2 (from *Petunia hybrida*, ornamental petunia), which have five

disulfide bridges (Lay et al. 2003). These bridges turn the molecule globular and compact by holding tight together the secondary structural elements and are responsible for the high physicochemical stability of plant defensins, especially the C_1–C_8 bridge turn the molecule pseudocyclic (Carvalho and Gomes 2011). Two of the disulfide bridges, the C_3–C_6 and C_4–C_7 that bind the α-helix to the β_3 strand, are part of the structural arrangement denominated cysteine stabilized αβ (CSαβ) motif (Thomma et al. 2002; Carvalho and Gomes 2011). Plant defensins were included in the superfamily of *cis*-defensins, because the disposition of those two disulfide bridges, i.e. between the α-helix and the β_3 strand, that bind the same secondary structural elements and also because they are organized at the same side of their three-dimensional structures (Shafee et al. 2017). Another amino acid residue that is well conserved in the primary structure of plant defensins is a glycine at approximately position 32 [all numbers cited in the text refer to the position of the amino acid in the primary structure of the molecule, and considering the alignment made in the Supplementary Table 1 the corresponding position is 38 including the gaps (−)]. This glycine is part of another well conserved motif, the γ-core, which occurs in other AMPs as well Yount and Yeaman (2004). For plant defensins, the γ-core is in the dextromeric formula NH_2–[X_{1-3}]–[GXC]–[X_{3-9}]–[C]–COOH, X being any amino acid (Yount and Yeaman 2004).

With the exception of those conserved residues (e.g. C and G) in the primary structure of plant defensins, other amino acids that compose it show high variation. The greatest variations between amino acid residues occur in the loops of the plant defensins, especially in the loop region between the β_2 and β_3 strands, which comprises the γ-core itself (Supplementary Table 1; Carvalho and Gomes 2011). This amino acid diversity in the loop regions drives variations in the three-dimensional position and length of the loops and additionally many amino acids that compose them are exposed to the molecule surface (Liu et al. 2006; Sagaram et al. 2013; Machado et al. 2018). For these reasons, they are free to interact with other proteins or targets and this may be the explanation for the broad biological activities already described for plant defensins. In fact, for plant defensins, their inhibitory activities on protein translation (Méndez et al. 1990, 1996) and α-amylases (Bloch and Richardson 1991; Lin et al. 2007; Pelegrini et al. 2008; Dos Santos et al. 2010), tolerance to heavy metal (Mirouze et al. 2006), blockage of ion channels (Spelbrink et al. 2004), antimicrobial inhibitory spectra (Carvalho and Gomes 2011), their capacity to bind to membrane lipids (phospholipids and sphingolipids) (Baxter et al. 2015; Gonçalves et al. 2012; De Paula et al. 2011; Poon et al. 2014), dimerization (Lay et al. 2012; Song et al. 2011) and antifungal inhibitory mechanisms (Coninck et al. 2013; Parisi et al. 2019) are well described. Also, it is startling that many of these abilities are linked to the amino acid stretch that compose the γ-core which is a desirable characteristic for drug design as explained in the follow paragraph (Supplementary Table 1).

Since the γ-core has a fundamental role in the antimicrobial activity of AMPs (Yount and Yeaman 2004) including plant defensins, it has become an attractive region for targeted modifications. Because the restriction of the biological activity to a minimal stretch of amino acids, such as the γ-core in plant defensins, is important for miniaturization of the biologically active sequence that is a desirable characteristic for drug development, which might be further manipulated to improve stability, decrease toxicity toward host and, moreover, lower the production costs (Ramesh et al. 2016). Foremost, plant defensins present another desirable characteristic for drug development as these peptides are, in general, not active against mammalian cells, at least at the concentrations that causes inhibition of microorganisms (Carvalho and Gomes 2011; Vriens et al. 2015). This safety to mammalian cells is reinforced by in vivo tests in murine models where *Rs*-AFP_2 (defensin from *Raphanus sativus*, radish) was as active as fluconazole in reducing candidiasis caused by *C. albicans* (Tavares et al. 2008) and *No*D173 (defensin from *Nicotiana occidentalis*, tobacco) that inhibited the growth of solid B16-F1 melanoma tumor (Lay et al. 2019). Both defensins presented no toxicity to the animal hosts. Taking the aforementioned data together, the scaffold of plant defensins, including their γ-cores, are particularly good candidates for drug design.

Our research group is focused on the *Vu*Def_1 (this peptide is now renamed from *Vu*-Def to *Vu*Def_1 following the nomenclature rule proposed for plant defensins by Sathoff et al. 2019), a plant defensin isolated from *Vigna unguiculata* (cowpea) seeds, that presented in combination with another AMP, a lipid transfer protein, inhibitory activities against filamentous fungi (Carvalho et al. 2001), α-amylases from insects (Dos Santos et al. 2010), and against *Leishmania amazonesis* (Souza et al. 2018). We had also synthesized a peptide containing *Vu*Def_1 γ-core, named $A_{36,42,44}\gamma_{32-46}$*Vu*Def (in which $A_{36,42,44}$ denotes the positions where the three C were replaced by A; γ_{32-46} denotes the position of amino acids in the primary sequence of *Vu*Def_1 where the γ-core is found, and for short, this peptide will be called in the following text DD, because it has two adjacent aspartic acids in its primary structure) which also presented activity against *L. amazonesis* as *Vu*Def_1 (Souza et al. 2019). The strong activity of DD against *L. amazonesis* impelled us to test the peptide against pathogenic yeasts. Nonetheless, DD showed no significant activity against them. This result further prompted us to improve its activity against yeasts by altering its biochemical parameters through amino acid substitutions. There are some approaches to design new peptides (Fjell et al. 2012). However, these methods present the drawback of the necessity of production of several peptides

that need to be tested, and in many cases, they present lower activity than the original peptide (Schaaper et al. 2001; Misawa et al. 2017) turning the process inefficient and imposes a limiting factor to those with scarce resources. In this work, we explore the correlation of the biological activity and primary structures of plant defensins for design new peptides with improved antifungal activity. Accordingly, we search for articles correlating the structure and activity of plant defensins, compiled them in a table, analyzed their primary structures and their biological activities. Based on this analysis and to test our hypothesis that this correlation study could indicate both the favorable position and the amino acid to be changed, we designed three new peptides with targeted variations in charge, hydrophobicity and chirality. The new synthetic peptides were tested against the same yeasts as the original DD. Our results indicated that the three new designed peptides based on this approach had better inhibitory activity against the pathogenic yeasts and one of them was the best in potency and in the spectrum of yeast species inhibition. Additionally, all three new peptides had low toxicity to mammalian cells.

Materials and methods

Database analysis, peptide and biophysical properties analysis

We searched for articles on data banks at National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/pubmed), Science Direct (https://www.sciencedirect.com/), and Google Scholar (https://scholar.google.com.br/) using a combination of the following keywords as search parameters: "defensin", "plant", "improved", "variant", "motif", "mutational", "domain", and "mechanism of action". The retrieved articles had the primary structures of plant defensins or their derived peptides analyzed. Their primary structures were first aligned by Clustal Omega multiple sequence alignment with default settings (https://www.ebi.ac.uk/Tools/msa/clustalo/; Sievers et al. 2011) and the amino acid alterations were marked in the aligned sequences, as well as the effect resulting from the performed changes on the biological activity. These data are presented in Supplementary Table 1.

We also analyzed the hydrophobicity and net charge of the modified defensins or their derived peptides (Supplementary Table 1). Hydrophobicity was calculated by PepDraw which uses the hydrophobicity Wimley-White scale (http://pepdraw.com/), and net charge was calculated by PepCalc (https://pepcalc.com/).

To compare the correlation between the potency of the inhibitory activity on fungi with charge and hydrophobicity of plant defensins and their derived peptides, we selected articles and extract the pieces of information: charge and hydrophobicity (from Supplementary Table 1) and the concentration in μM that inhibits 50% of the tested fungus (IC_{50}). To give a unifying parameter in these correlation analyzes we chose the same fungus. The data were organized in graphics with the parameters of charge and hydrophobicity classified in increasing order using Excell.

Based on the analysis of the Supplementary Table 1, we generated three new peptides derived from the previous peptide DD (Souza et al. 2019) altering its net charge, hydrophobicity and chirality.

Peptide chemical synthesis

DD and the three new designed peptides were acquired commercially by Aminotech. All peptides were dissolved in pure sterilized water at 2 μg/mL (approximately 1000 μM, depending on the peptide molecular weight) and stored in aliquots at – 70 ºC. Peptides purity was assured as ≥ 95% as determined by reversed-phase high-pressure liquid chromatography and mass spectrometry analyzes (Supplementary Figs. 1, 2, 3, and 4).

Yeasts and antimicrobial assay

The yeasts *Candida albicans* (CE022), *Candida buinensis* (4674), *Candida parapsilosis* (CE002), *Candida pelliculosa* (3974), *Candida tropicalis* (CE017) and *Saccharomyces cerevisiae* (1038) were grown in Sabouraud agar (10 g/L peptone, 20 g/L D(+)glucose, 17 g/L agar, Merck) at 30 ºC for 24 h and then stocks of each yeast were maintained at 4 °C and transferred to a new medium every 3 months.

The yeasts were grown on new Sabouraud agar at 30 ºC for 24 h. After the growth period, a colony was resuspended in Sabouraud broth (5 g/L peptone from meat, 5 g/L peptone from casein, 20 g/L D(+)glucose, 17 g/L agar, Merck) and the cells were counted in a Neubauer chamber (Laboroptik) in an optical microscope (Axio Imager.A2, Zeiss). The assay was performed on a polystyrene 96-well microplate (Nunc, Thermo Scientific) and was composed of 2000 cells/mL, 18.5 μM of each peptide filter-sterilized (0.22 μm, Millex-GV, Millipore) and 100 μL (final volume) of Sabouraud broth. Yeast growth was determined after incubation for 24 h at 30 °C by absorbance at 620 nm (EZ Read 400, Biochrom) as described by Broekaert et al. (1990). Samples in which no peptides were added were considered as controls (100% of growth). Wells containing only Sabouraud broth were considered blanks. Gray scale images of the bottom of the wells were obtained by Galaxy Note 9 camera (Samsung) at 24 h.

Determination of minimal inhibitory concentration

After determining the inhibitory activity against the yeasts for each peptide at the fixed concentration of 18.5 µM, we chose the combination of the most sensitive yeast and the strongest peptide and determined their minimal inhibitory concentration (MIC_{100}). The assay was performed as described in item *Yeasts and antimicrobial assay*, with the modification that different concentrations of the peptides were used: 14, 18.5, 23, 27.5, 32, and 36.5 µM, depending on the original inhibition of each peptide determined at the first antimicrobial assay. MIC_{100} was defined as visually the lowest tested concentration of peptides in µM that caused the complete yeast growth inhibition under the conditions the assay was done (30 ºC for 24 h in Sabouraud broth) (Broekaert et al. 1990; Wiegand et al. 2008).

Determination of yeast cell viability and lethal dose

After the MIC_{100} determination assay, the content of the wells were washed once in Sabouraud broth and evenly spread with a Drigalski spatula on a Petri dish containing Sabouraud agar and incubated at 30 ºC for 24 h to allow the development of colonies. After the formation of the colonies, they were analyzed in relation to the control sample, which was considered 100% viable. Gray scale images were acquired as described in item *Yeasts and antimicrobial assay*. Viability was defined as the ability of the yeasts cells to divide and thereof to forming colonies in appropriated conditions (30 ºC for 24 h in Sabouraud broth). This peptide concentration in µM that causes the death of all cell population in the original assay was defined as the lethal dose (LD_{100}).

After LD_{100} determination for each peptide, a control with fluconazole (Sigma-Aldrich) with the same LD_{100} concentration determined for the peptides was tested. Fluconazole was resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 2 µg/µL (stock). The control, without adding fluconazole, was done with 0.4% DMSO, the same final concentration of DMSO in the fluconazole treated samples. After 24 h, images of the well bottom were acquired as described in item *Yeasts and antimicrobial assay*.

Mammalian cell viability assay of design peptides

RAW 264.7 murine macrophages (American Type Culture Collection, ATCC TIB-71) and THP-1 human monocytes (ATCC TIB-202) were cultured in DMEM-F12 supplemented with 10% fetal bovine serum and gentamicin (50 µg/mL) in 5% CO_2 at 37 ºC in the LBR, from CBB, UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil. Cells (5×10^5 cells/mL) were separately seeded in 96-well tissue culture plates and incubated for 24 h at 37 °C in 5% CO_2. RAW 264.7 reached 60–80% confluence and THP-1 reached 7×10^5 cells/mL. Then, the designed peptides were added at 14, 18.5, 23, 27.5, 36.5 and 50 µM and incubated for further 24 h at same conditions. After 24 h of incubation with peptides, 10 µL of MTT reagent (5 mg/mL, Sigma-Aldrich) was added and incubated for an additional 2 h at 37 °C in 5% CO_2. The MTT solution was removed and 100 µL of acidified isopropanol was added to solubilize the formazan crystals formed. The absorbance was measured at 570 nm and absorbance for background correction was determined at 620 nm. Non-treated cells were used as a positive control (optical density (O.D.) 1.94 ± 0.05, cell viability—98.8 ± 2.1%) and 1% Triton X-100 detergent-treated cells as a negative control (O.D. 0.14 ± 0.03, cell viability—0%). The percentage of cell survival was calculated as follows: % Cell viability = 100 × (experimental well ABS – negative viability control) / (positive viability control). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration (µM) required for the reduction of cell viability by 50%, which were calculated by regression analysis (Moodley et al. 2014).

Statistical analysis

The antimicrobial assays were done in triplicate and repeated three times and the mammalian viability assay was done in duplicate and repeated three times. Graphics were depicted as means with standard deviation of one independent assay for antimicrobial assays or by mean values obtained over three experiments for mammalian viability assay. The data obtained in the assays were statistically tested by the one-way ANOVA test, where $P < 0.05$ was considered significant, using the GraphPad Prism 8 software.

Results and discussion

Previously, we had synthesized a peptide based on the *Vu*Def_1 γ-core, namely $A_{36,42,44}\gamma_{32-46}$*Vu*Def (DD), which has the primary structure of *Vu*Def_1 γ-core except by the exchange of three C residues by A ones (Souza et al. 2019). The A substitutions turn this peptide slightly less hydrophobic, from + 21.98 to + 20.42 kcal/mol (according to the hydrophobicity scale used, that the more positive the value, the more hydrophilic it is) but it retained the same + 2 net charge comparing the original primary structure of *Vu*Def_1 γ-core. Additionally, the A substitutions made disulfide bridges formation impossible and avoided a free C to remain (Table 1). To solve this problem, some authors substituted the C by aminoisobutyric acid (Schaaper et al. 2001). DD had inhibitory activity against *L. amazonensis* as the entire *Vu*Def_1 indicating that the inhibitory activity on *L. amazonensis* is located within *Vu*Def_1 γ-core (Souza et al. 2019).

Table 1 Primary structure, biophysical properties and biological activity of the synthetic peptides based on the *Vu*Def$_1$ γ-core which were designed according to the information of Supplementary Table 1

Defensin/peptide name (peptide name abbreviation)	Primary structure (one letter code) 1 11 21 31 41 51	Net charge (at pH 7.0)	Hydrophobicity (Kcal/mol)	Exchaged amino acids and their effect in the biological activity	References
*Vu*Def$_1$	--MKTCENL- ADTYRGPCFT TGSCDDHCKN KEHLL-SGRC R-----DDVR -CWCTRNC	+0.7	+54.73	Obs: inhibits *Leishmania amazonensis*.	Souza et al. 2013; 2019; this work
$A_{36,42,44}\gamma_{32\text{-}46}$*Vu*Def (DD)	L-SGRA R-----DDVR -AWATR	+2	+21.98 (+20.42)	Obs: $C_{36}A$; $C_{42}A$; $C_{44}A$, inhibits *L. amazonensis* as *Vu*Def$_1$ and was slightly active against yeasts.	
$A_{36,42,44}R_{37,38}\gamma_{32\text{-}46}$*Vu*Def (RR)	L-SGRA R-----RRVR -AWATR	+6	+18.32 (+21.98)	$D_{37}R$; $D_{38}R$; increase activity on yeasts *Candida albicans* and *Candida tropicalis*;	This work
D-$A_{36,42,44}R_{37,38}\gamma_{32\text{-}46}$*Vu*Def (D-RR)	L-SGRA R-----RRVR -AWATR	+6	+18.32 (+21.98)	Obs: same sequence as $A_{36,42,44},R_{37,38}\gamma_{32\text{-}46}$*Vu*-Def but all amino acids are D-stereoisomers;	
$A_{42,44}R_{37,38}W_{36,39}\gamma_{32\text{-}46}$*Vu*Def (WR)	L-SGRW R-----RRWR -AWATR	+6	+14.10 (+21.98)	$A_{36}W$; $V_{40}W$; $C_{44}A$; $D_{37}R$; $D_{38}R$; increase activity on yeasts *C. albicans*, *Saccharomyces cerevisiae* and *C. tropicalis*.	

The number of the amino acids was kept as the original article and the numbers above the sequences are only for guidance of the amino acid position. When necessary gaps (–) were introduced to improve the alignment. Numbers in parenthesis indicated the net charge or the hydrophobicity of the correspondent peptide sequence in the defensin or in the peptide from which it was derived. In bold are the amino acids that were exchanged in the sequence the synthetic peptides

The γ-core as defined by Yount and Yeaman (2004) is indicated by a box but instead to box it as NH_2–[$X_{1\text{-}3}$]–[GXC]–[$X_{3\text{-}9}$]–[C]–COOH we boxed it as NH_2–[GXC]–[$X_{3\text{-}9}$]–[C]–COOH because most of the article used this amino acid stretch as the γ-core. In the primary structure of *Vu*Def$_1$ is shown an initial methionin (in bold and italicized) that was included as a requirement for its expression using the pET-32 EK/LIC vector as described in Santos et al. (2010).

Because of the satisfactory inhibitory activity of DD against *L. amazonensis*, in this work, we tested it against five yeast species of medical importance and to which therapeutic substances are required, and also against the model yeast *S. cerevisiae*. We used the same incubation time (24 h) and concentration (18.5 μM) determined for *L. amazonensis* (Souza et al. 2019) in the beginning of our tests. But DD did not present significant activity (Fig. 1).

Because of DD low activity against the tested yeasts, we planned to improve its activity against yeasts by altering its biochemical properties. Many synthetic peptides had been generated by different methods (Fjell et al. 2012), and for example, in the linguistic model described by Loose et al. (2006), the design had been done based on AMPs of natural occurrence that was interpreted as a grammar, the authors had to analyzed a set of 3 million possible sequences from this grammar from which they choose 42 to be synthesized. From the 42 designed peptides, 2 were insoluble. In addition to these 42 peptides, a further 42 randomized sequences were made as controls so that they bear no resemblance to any grammar, and 4 were insoluble. Two groups of peptides were also synthesized, one comprising eight peptides from the Antimicrobial Peptide Database as positive controls and the other composed of six peptides from non-antimicrobial proteins as negative controls. To optimize resources and time, we do the Supplementary Table 1 (which is supplied as Word.doc to facilitate comparison) that compiled literature analysis of articles that correlate plant defensin primary structures and biological activities. Based on this Supplementary Table 1, we designed three new peptides to test our hypothesis that the correlation of primary structures and biological activities of plant defensins could be used to design new peptides with the clear indication the position and also what type of amino acid should be better choice for substitution. We found 27 articles that correlate the primary structure and biological activity of 19 plant defensins and derived peptides (Supplementary Table 1). Changed amino acids were indicated in the primary structure and also the correlated aftermaths of the change had on the biological activity, *i.e.* if it increased, decreased or was neutral. It is interesting to note that main changes that influenced the biological activity were in the region that is responsible for plant defensins biological activity, the γ-core (see 4th paragraph of introduction; Supplementary Table 1). Before we start the discussion about the design, we drew important pieces of information about the Supplementary Table 1 that will be discussed below.

First about the correlation of length in amino acid residues and inhibitory activity of plant defensin-derived peptides against fungi. We note, like our DD, the ineffectiveness of very short peptides based on the plant defensin γ-cores against fungi. Peptides encompassing only the γ-core amino acid residues, or smaller, did not have the correspondent inhibitory potency against microorganisms like the original defensin did or have no activity. For example, GMA$_1$, GMA$_1$-L, and GMA$_4$, GMA$_4$-L derived from *Ms*Def$_1$ (defensin from *Medicago sativa*, alfalfa) and *Mt*Def$_4$ (defensin from *Medicago truncatula*, lucerne), respectively (Muñoz et al. 2014; Sagaram et al. 2011); C_{36}–C_{45}, derived from *Rs*-AFP$_2$ (De Samblanx et al. 1996); $\gamma_{33\text{-}41}$*Pv*D$_1$ derived from *Pv*D$_1$ (defensin from *Phaseolus vulgaris*, common bean) (Mello et al. 2019) (Supplementary Table 1). Also, smaller peptides had lower inhibitory activity when compared to their bigger counterparts that covers the entire γ-core region alongside some residues of the β_2 and β_3 strands. For example, GMA$_1$ have no antifungal activity and GMA$_1$-C have antifungal activity; GMA$_4$ and $\gamma_{33\text{-}41}$*Pv*D1^{++} has lower antifungal activity than GMA$_4$-C and $\gamma_{33\text{-}45}$*Pv*D1^{++} (derived from the γ-core of the defensin *Pv*D$_1$) (Supplementary Table 1). Rekdal et al. (1999) provided similar results for lactoferricin analogs (peptides with antimicrobial activity derived from lactoferrin obtained by gastric digestion), in which the derived peptides should not be shorter than 15 amino acid residues to maintain their antimicrobial activity. According to our analysis, the minimum size for these peptides derived from plant defensins to be active is about nine amino acid residues. In fact, this size limit is observed in nature, the smallest known peptides with broad antimicrobial

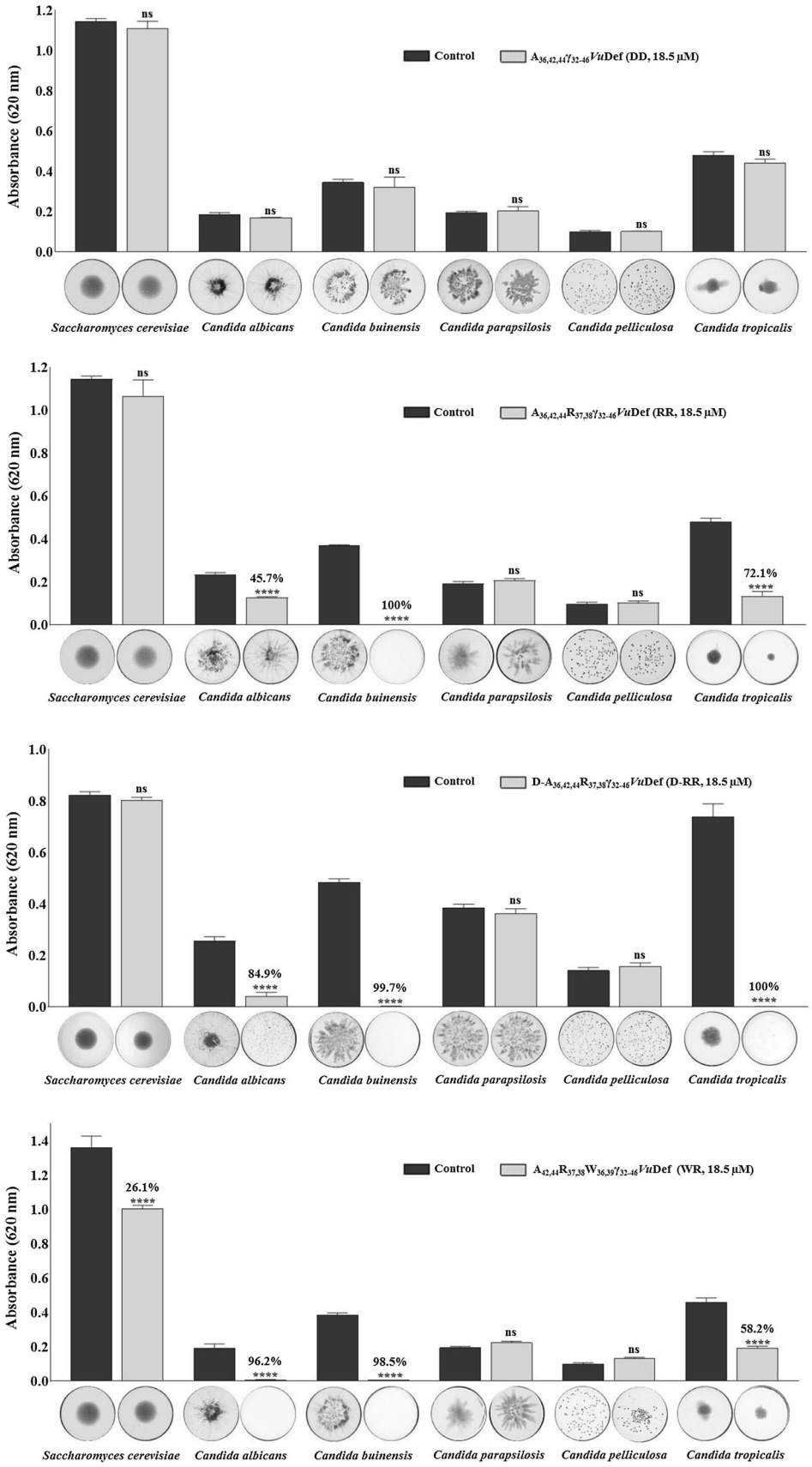


Fig. 1 Yeast growth in the absence (control) and in the presence of the four synthetic peptides. The percentage of inhibition of fungal growth is shown above the test bars and the image of the bottom wells with the growth pattern of each yeast species is shown below their correspondent bars. The image is representative of one assay out of three. Asterisks indicate statistical significance (****$P < 0.0001$) and *ns* non significant from control

activity against different microorganisms are the temporins, which have about 10–13 amino acid residues long, obtained from the skin secretion of anuran amphibian *Rana temporaria* (European common frog) (Mishra et al. 2018) and bactenecin with 13 amino acid residues from bovine neutrophils (Cherkasov et al. 2009).

The reason for the weaker or ineffectiveness of antifungal activity of short peptides is not well understood. One possible explanation for the activity of γ-core derived peptides that does not match the activity of defensin as a whole could be the observations that for some plant defensins there are amino acids important for biological activity tracked outside this motif. For example, the K_4 (position 7 in the Supplementary Table 1) in *Na*D_1 (defensin from *Nicotiana alata*, ornamental tobacco) and the K_6 (position 7 in the Supplementary Table 1) in TPP3 (defensin from *Lycopersicon esculentum*, tomato) are outside those defensin γ-cores. They are essential for binding to the fungal target and dimerization as demonstrated by their replacement by A, which turns both defensins ineffective in their ability to form dimers, which is important for lipid biding, antifungal and antitumor activities (Lay et al. 2012; Baxter et al. 2015). It is also observed that the microorganism species is relevant to the inhibition process, because despite being inactive against yeasts, DD was active against *L. amanzonensis* (Souza et al. 2019). Corroborating with this suggestion are studies with fungi demonstrating that lipids, especially the negatively charged ones, that are present in the fungal membrane, such as mannosyldiinositol phosphorylceramide ($M(IP)_2C$), phosphatidic acid (PA) and phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$), may be the targets of plant defensins and the cationic and hydrophobic amino acids of the γ-core region are important for this interaction (Parisi et al. 2019). *Mt*Def_4, for example, had different mechanism of action on the fungi *Neurospora crassa* and *Fusarium graminearum*, because the differential concentration of sphingolipids in those fungi, as suggested by the authors (El-Mounadi et al. 2016). Ramamoorthy et al. (2007) observed that the inhibition of the fungus *F. graminearum* by the defensin *Ms*Def_1 depended on the sphingolipid glucosylceramide (GlcCer), but not the inhibition caused by *Mt*Def_4. Likewise, *S. cerevisiae* that does not have GlcCer in its membrane is resistant to *Rs*-AFP_2, while *Pichia pastoris* and *C. albicans* yeasts that have GlcCer are sensitive to *Rs*-AFP_2. Additionally, *P. pastoris* and *C. albicans* mutants, without GlcCer in their membranes, exhibited resistance to *Rs*-AFP_2 (Thevissen et al. 2004). The higher affinity and capacity of protegrin-1 (AMP of the cathelicidin class obtained from pig neutrophils) to lyse erythrocyte membranes from mice, rabbits and humans whereas is ineffective to bovine, sheep and porcine erythrocytes (Bellm et al. 2000) are supposedly explained by the differential lipid composition of erythrocyte membranes of those organisms (Nouri-Sorkhabi et al. 1996a, b). Therefore, perhaps the yeasts tested in this work might not have the specific target of DD, which would explain its lack of activity. Besides that, the two negative charged amino acids (D_{37} and D_{38}; positions 47 and 48 in the Supplementary Table 1, respectively) of DD may result in repulsion from negative charged structures in the fungal cell wall, e.g. manophosphoproteins, or membrane constituents, as explained above, and demonstrated to other peptides where some interaction positions with microbial membrane constituents are not favorable by charge repulsion (Haney et al. 2007), which may resulted in a weak interaction and consequently antifungal activity. This characteristic seems to be more important for the peptide than to the *Vu*Def_1 itself, probably because in the entire defensin there are other amino acid interactions that compensate this repulsion.

The second observation is about charge and hydrophobicity. Supplementary Table 1 shows that the alterations that add positively charged and hydrophobic amino acid residues were the most positively influential on the biological activity of plant defensins (Supplementary Figs. 5, 6 and 7). Studies that correlate the structure and mechanism of action of plant defensins show that the attraction of opposite charges between positively charged defensins with the negatively charged membranes of microorganisms ensures the initial interaction. Then, the hydrophobic region of the defensin interacts with the hydrophobic part of the membrane, which can cause its destabilization (Giuliani et al. 2007) as explained above. This oppose charge interaction is supported by the abrogation of plant defensins antifungal effect caused by the addition of divalent cations, especially Mg^{+2} and Ca^{+2}, in the culture medium which screen electrostatic charges disrupting the initial attraction (Terras et al. 1993; Van der Weerden et al. 2008). Our observation that the more cationic peptides have stronger inhibitory activity is supported by studies with plant defensins isoforms that the more cationic ones had stronger inhibitory activity. For example, *Rs*-AFP_1 and *Rs*-AFP_2 are two natural defensin isoforms from *R. sativus* seeds, which are nearly identical in their primary structure except by the substitutions of E_5 to Q and N_{27} to R in *Rs*-AFP_2 (position 7 and 29 in Supplementary Table 1). The first substitution removes one negative charge from *Rs*-AFP_2 and the second adds one positive charge to it. Therefore, *Rs*-AFP_2 is more cationic (net charge from + 3.6 to + 5.6) and hydrophobic (from + 36.68 to + 34.78 kcal/mol) and these increments in cationicity and hydrophobicity were correlated with the *Rs*-AFP_2 stronger inhibitory active against fungi than *Rs*-AFP_1, even in media with a high ionic strength that inactivate most of AMPs (Terras et al. 1993; Tam et al. 2002). The isoforms *Ph*$_1$ and *Ph*$_2$ (Lay et al. 2003) and *Vr*D_1 and *Vr*D_2 (Lin et al. 2007) are other examples. Beside these observations, we can mention a chimera of *Ms*Def_1, called *Ms*Def_1-γ_4 in which the DDFQ amino acids of *Ms*Def_1 have been replaced by

the amino acids that determine *Ms*Def_4 antifungal activity, GFRRR. This substitution increased the net charge (from + 2.6 in *Ms*Def_1 to + 6.6 in *Ms*Def_1-γ_4) and hydrophobicity (from + 51.68 kcal/mol *Ms*Def_1 to + 49.17 kcal/mol in *Ms*Def_1-γ_4), resulting in a significant increase in antifungal activity of the chimera when compared to *Ms*Def_1 and it was as potent as *Ms*Def_4 (Supplementary Table 1). The *Oef*Def1.1_V5 variant also showed an improved antifungal activity when compared to the original *Oef*Def1.1 (defensin from *Olea europaea*, olive). In this case, replacing the KHYG residues to AAAA decreased the positive charge (from + 8.1 in *Oef*Def1.1 to + 7.0 in *Oef*Def1.1_V5), but increased the hydrophobicity (from + 50.31 kcal/mol in *Oef*Def1.1 to + 46.74 kcal/mol in *Oef*Def1.1_V5). Also, the chimera *Vr*D_2c produced by the exchange of RDDFR from *Vr*D_2 to GMTRT from *Vr*D_1 that increased its net charge (from + 2.7 to + 3.7) and its hydrophobicity (from + 54.42 to + 48.02 kcal/mol) resulted in an increased inhibitory potency against α-amylase. Not only alterations in stretches of amino acids resulted in this effect, but also point changes. For example, to *Rs*-AFP_2 the $N_{36}R$ change (position 41 in Supplementary Table 1) increased the modified peptide net charge from + 5.6 to + 6.6 (although the hydrophobicity decreased from + 34.78 to + 35.74 kcal/mol), this net charge increasing augmented its antimicrobial activity. A similar alteration, $Q_{40}R$ (position 50 in Supplementary Table 1) (increased net charge from -1.1 to -0.1, although a slight decreased in hydrophobicity from + 46.84 to + 47.88 kcal/mol) in *Mt*Def_2 had the same effect. Likewise, amino acid exchanges that decreased the positive charge worsened the biological activity of defensins. For example, *Rs*-AFP_2 has K at position 43 (position 50 in Supplementary Table 1), and when it was replaced by Q (decrease net charge from + 5.6 to + 4.6 and hydrophobicity from + 34.78 to + 32.75 kcal/mol) the inhibitory activity of the defensin decreased; the same is observed for $R_{38}Q$ (position 50 in Supplementary Table 1) (which decrease net charge from + 2.6 to + 1.6 and increased hydrophobicity from + 51.68 to + 50.64 kcal/mol) in *Ms*Def_1, and RR to AA (position 49 and 50 in Supplementary Table 1) in *Mt*$Def_4^{RGFRRR/RGFAA}$ (with decrease net charge from + 5.8 to + 3.8 and increased hydrophobicity from + 49.16 to + 46.54 kcal/mol) (Supplementary Table 1). Still in regard to charge, it is important to mention that the position of charge insertion is important for activity. For example, the inclusion of positively charged amino acids by substitution of $V_{38}R$ and $A_{41}R$ (positions 43 and 48 in Supplementary Table 1, respectively) in *Rs*-AFP_2 decreased its antimicrobial activity (Supplementary Table 1). Other substitution outside the γ-core, such as $S_{12}R$, $I_{26}R$, $L_{28}R$, $I_{46}R$ (positions 15, 29, 31, 53 in Supplementary Table 1, respectively) also decreased its antimicrobial activity. In addition, the removal of those hydrophobic residues, concomitantly reduced hydrophobicity (+ 36.13, + 37.71, + 37.84 and + 37.71 kcal/mol, respectively) compared to the original *Rs*-AFP_2 (+ 34.78 kcal/mol), which may also have contributed to decreased antifungal activity. These observations reinforce that not only positive charge increase is important, but also the position where the positive charge is inserted, probably to avoid charge repulsion as explained in the 4th paragraph of the Results and discussion section, along with the hydrophobicity for biological activity.

Based in all pieces of information aforementioned, we specifically compared the previous sequence of *Vu*Def_1 and DD with other defensins and their derived peptides and acknowledged that in the γ-core region, highlighted in a red box in Supplementary Fig. 1, there are some amino acids exchanged in some plant defensins or derived peptides that resulted in variation in the biological activity that were considered interesting to change in the original sequence of DD. The positive charges at positions 41 and 50 (reference position indicated in the Supplementary Table 1) that are conserved and important for the biological activity of plant defensins are of special interest (Supplementary Fig. 8). Please refer to the 5th paragraph of the Results and discussion section for the discussion of these charges in *Rs*-AFP_2, *Ms*Def_1, *Mt*Def_2, *Mt*$Def_4^{RGFRRR/RGFRAA}$. Also *Os*AFP_1 (defensin from *Oryza sativa*, rice) has a K_{35} and K_{42} (positions 41 and 50 in the Supplementary Table 1) that were replaced by A (which decreased the antifungal activity in both variants, and also increased hydrophobicity from + 51.24 to + 48.94 and + 48.94 kcal/mol, respectively). At those correspondent positions DD has positively charged amino acids, R, therefore, we maintained them in our design approach. In relation to the substitution of negative charges, *Ms*Def_1 has the sequence in its γ-core $RDD_{36}FR$ (position 48 in the Supplementary Table 1) which was replaced by the sequence of the *Mt*Def_4 γ-core $RGFR_{39}RR$ (position 48 in the Supplementary Table 1) generating the peptide *Ms*Def_1-γ_4, with increased antifungal activity (and hydrophobicity increased from + 51.68 to + 49.17 kcal/mol), and DD has a very similar sequence to *Ms*Def_1, $RDD_{38}VR$ (position 48 in the Supplementary Table 1). Also in peptides derived from the γ-core of defensin *Pv*D_1 where the replacement of two negative residues in $\gamma_{31\text{-}45}$*Pv*D_1 (RSGRARD$_{37}$D$_{38}$FRAWATK) (position 47 and 48 in the Supplementary Table 1) by two positive residues in $\gamma_{31\text{-}45}$*Pv*D_1^{++} (RSGRARR$_{37}$R$_{38}$FRAWATK, which also increased its hydrophobicity from + 24.78 to + 21.12 kcal/mol), improved its antifungal activity. The same was observed with the defensin *Mt*Def_4, which lost its antifungal activity when the $F_{38}R_{39}$ residues, corresponding to our D_{37} and D_{38} (positions 48 and 49 in the Supplementary Table 1) were replaced by A. In this case, a decrease in both net charge (from + 5.8 to + 4.8) and hydrophobicity (from + 49.16 to + 50.06 kcal/mol) were observed (Supplementary Table 1). Therefore, we selected D_{37} and D_{38}

(positions 48 and 49 in Supplementary Table 1) to be substituted by R. Based on this charge analysis, we designed two new peptides. In the first, the R_{36} and R_{40} (positions 46 and 50 in Supplementary Table 1), flanking the *Vu*Def$_1$ γ-core sequence, were kept and D_{37} and D_{38} were replaced by R, and it was called $A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def ($R_{37,38}$ indicate the position of the change of DD for RR, positions 48 and 49 in Table 1. For short, we will call this peptide RR because of the two R that replaced the two D). The second peptide had the exact RR sequence except all amino acids are D-enantiomers, thus it was named D-$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def (D-RR to indicate the change in chirality), and both peptides had its net charge increased from + 2 to + 6 and also its hydrophobicity was increased from + 21.98 to + 18.32 kcal/mol in regard to DD (Table 1). We choose to synthesize a peptide composed of D-amino acids, because studies had shown that they are more resistant to degradation by proteases while retaining antimicrobial activity and are in general less toxic to mammalian cells (Hamamoto et al. 2002; Braunstein et al. 2004).

The third new designed peptide we maintained the charge (+ 6) but has increased its hydrophobicity since we realized that in the region of the γ-core in *Rs*-AFP$_2$ the change of $Y_{38}G$ (position 42 in Supplementary Table 1) decreased its hydrophobicity from + 34.78 to + 36.64 kcal/mol (without charge variation) as well as its antimicrobial activity. The same is observed for SPE10 (defensin from *Pachyrhizus erosus*), the change of $D_{38}F_{39}$ to ND (position 48 and 49 in Supplementary Table 1) the hydrophobicity drop from + 59.01 to + 61.57 kcal/mol (both retained + 0.7 charge) and the peptide C_{36}-C_{45}(Y_{38}A) (the change of Y_{38}A, position 42 in Supplementary Table 1) derived from *Rs*-AFP$_2$ the hydrophobicity drop from + 11.60 to + 12.81 kcal/mol. It is interesting to note the presence of an aromatic amino acid residue in this region (Y_{38} *Rs*-AFP$_2$ and F_{39} in SPE10) (position 42 and 49 in Supplementary Table 1, respectively) that seemingly contributes to both hydrophobicity and antimicrobial activity. DD γ-core region has V_{39} in correspondent position (Supplementary Table 1), thus it was selected to be substituted. Another position was the A_{36} (position 40 in Supplementary Table 1) which replaced a C in that position in the original *Vu*Def$_1$ γ-core sequence. As it is a hydrophobic amino acid it was also chosen to be substituted. For the substitutions, we chose an aromatic residue, and W was chosen. We decided to keep the positive charges conserved at positions 41 and 50 (Supplementary Table 1), as described in the 5th paragraph of the Results and discussion section, and the peptide was called $A_{42,44}R_{37,38}W_{36,39}\gamma_{32-46}$*Vu*Def (where $W_{36,39}$ indicate the position of the change of A_{36} and V_{39} for W, for short WR to summarize the two amino acids exchanged) (Table 1). The substitutions A_{36}W and V_{39}W ensured greater hydrophobicity to WR (+ 14.10 kcal/mol) when compared to RR and D-RR (+ 18.32 kcal/mol) and DD (+ 21.98 kcal/mol). And the same + 6 net charge of RR and D-RR. Higher hydrophobic character of all three new synthesized peptides was confirmed by the increased retention time in reversed-phase C-18 column in HPLC, especially for WR (Supplementary Figs. 1, 2, 3 and 4). W was chosen, because it is a neutral amino acid found in high proportion in short biologically active AMPs such as indolicidin (AMP from bovine neutrophil) and tritrpticin (AMP from pig bone marrow) (Mishra et al. 2018). Its uncharged aromatic side chain has the ability to form hydrogen bonds and insert itself uniquely in microbial membranes (Strøm et al. 2000; Bi et al. 2013). In addition, tryptophan-rich peptides with biological activity also contain arginine residues. The positive charge of this amino acid assists in the initial electrostatic attraction and formation of hydrogen bonds between the peptide and the target membrane. Then, W is anchored to the membrane with high affinity (Chan et al. 2006). Studies carried out with indolicidin, tritrpticin, bovine lactoferricin and temporins provided evidences that the unique properties of the side chains of the W and R residues render short peptides highly active, with antibacterial, antifungal, antiviral and antitumor, albeit with low hemolytic activity (Lawyer et al. 1996; Strøm et al. 2000; Bi et al. 2013; Shagaghi et al. 2016).

Next, the antifungal activity of the three new synthetic peptides was tested using the same conditions as DD. We found that RR inhibited 47.5, 100, and 72.1% of the growth of *C. albicans*, *C. buinensis*, and *C. tropicalis*, respectively, however, it was unable to inhibit the growth of *S. cerevisiae*, *C. parapsilosis* and *C. pelliculosa* as DD (Fig. 1). This inhibition, obtained by measuring cell cultures optical density, can also be observed visually, since there was a clear decrease in the mass of yeast cells at the microplate well bottom and there was a change in the fungal growth pattern for *C. albicans* and also for *C. parapsilosis*, albeit they were not inhibited, when compared to their respective controls (Fig. 1). As expected, only the increase in the positive net charge from + 2 to + 6 and in specific positions in RR was sufficient for an improvement in its antifungal activity when compared to the original DD. D-RR, which has the same biochemical characteristics of RR but in D configuration, inhibited the same yeasts as RR, 84.9, 99.7, and 100% of the growth of *C. albicans*, *C. buinensis*, and *C. tropicalis*, respectively, but with more potency, and was unable to inhibit the growth of *S. cerevisiae*, *C. parapsilosis* and *C. pelliculosa* (Fig. 1) as RR. Corroborating the high inhibition activity, there was no visible mass of cells at the well bottom of the inhibited yeasts captured by the camera (Fig. 1). Since both RR and D-RR had inhibitory activity against the same yeasts suggests that their mechanism of action is independent of chirality. The higher potency of D-RR against the inhibited yeast may be related to protease resistance (Hamamoto et al. 2002; Braunstein et al. 2004) that will be further investigated. WR inhibited 26.1, 96.2, 98.5, and

58.2% of the growth of *S. cerevisiae*, *C. albicans*, *C. buinensis*, and *C. tropicalis*, respectively, and was unable to inhibit the growth of *C. parapsilosis* and *C. pelliculosa* (Fig. 1). However, to *C. parapsilosis* and *C. pelliculosa*, there was a change in the fungal growth pattern, despite they were not inhibited, when compared to their respective controls (Fig. 1). The increase in positive net charge (from + 2 to + 6) and hydrophobicity (from + 21.98 to + 14.1 kcal/mol) made WR with the best antifungal activity when compared to the other three peptides tested. Our results clearly indicated that augmented charge and hydrophobicity by targeted modification of amino acids in the designed peptides improved their antimicrobial activity. All designed peptides inhibited more yeast species and with greater potency than DD, although *C. parapsilosis* and *C. pelliculosa* were not inhibited by all tested peptides in our conditions (Fig. 1 and Table 2).

Based on the first antimicrobial assays, the combinations of the most sensitive and medically relevant yeast and the most active synthetic peptide were chosen for MIC_{100} and cell viability determinations (LD_{100}). Depending on the percentage of inhibition observed for the 18.5 µM concentration of the first assays, we decreased or increased it in 4.5 µM increments to find the MIC_{100} and LD_{100} for each chosen peptide-yeast combination. For RR and *C. tropicalis* the initial 18.5 µM concentration was increased until 36.5 µM. At the 23 µM, there was a small mass of yeast cells visible at the bottom of the microplate well that was not captured by the camera. At the concentration from 27.5 to 36.5 µM, there was no visible growth at the bottom of the well, and as we had defined our MIC_{100} as the concentration that completely inhibits the visible growth, 27.5 µM was the MIC_{100} (Fig. 2a). In cell viability assay, no colony developed indicating that this concentration was also the lethal dose (LD_{100}) (Fig. 2b). For D-RR and *C. albicans*, we rose the initial 18.5 µM concentration up to 36.5 µM and already at 23 µM, there was no visible growth at the well bottom, and it was determined as the MIC_{100} (Fig. 2c). The viability test indicated that no colony developed at 36.5 µM and it was determined as the LD_{100} (Fig. 2d). For the other lower concentrations, few colonies developed (Supplementary Fig. 9). For D-RR and *C. tropicalis* we decreased the initial concentration to 14 µM, and there was no visible growth at the well bottom, and this concentration was determined as the MIC_{100} (Fig. 2e). However, for the viability assay, few colonies developed at this concentration (Supplemented Fig. 9). Therefore, to determine the LD_{100}, we seeded the yeast cells treated with the higher concentrations into a new fresh medium, at 18 µM few colonies developed (Supplementary Fig. 9), and from 23 to 36.5 µM no colony developed, therefore, the LD_{100} was 23 µM (Fig. 2f). For WR and *C. albicans*, we decreased the concentration for 14 µM since 18.5, 23, 27.5 and 36.5 µM no growth could be observed at the bottom of the wells, although they were not captured by the camera (Fig. 2g). However, at 14 µM, a small mass of yeast cells was observed which was not captured by the camera, and therefore, 18.5 µM was determined as the MIC_{100}. In the viability assay, for the 14, 18.5 and 23 µM concentrations, few colonies developed, and for the 27.5 µM, no colony developed, indicating this concentration as the LD_{100} (Fig. 2h). The effect of the synthetic peptides on the viability of the yeasts species was fungicide and their effect were confronted to a widely used fungice, fluconazole (Sapampinato and Leonardi, 2013), used at corresponding concentration of peptides DL_{100}. For all tested yeasts, the inhibitory effect of fluconazole was weaker than that the synthetic peptides because was observed a cell mass at the bottom of the wells (Fig. 2b, d, f and h). As consequence of this partial inhibition, to all tested yeasts the antifungal effect of fluconazole was fungistatic (Fig. 2b, d, f and h). Thevissen et al. (2004) demonstrated in vitro that the defensins *Hs*-AMP_1 (defensin from *Heuchera sanguinea*, coralbell), *Rs*-AFP_2 and *Dm*-AMP_1 (defensin from *Dahlia merki*, dahlia) were fungicidal for *Candida* species tested and more efficient in killing yeasts when compared to commercial antifungals, among them fluconazole. Our peptides showed fungicidal action, which reduces the chances of selection of resistant strains, an important characteristic for new drugs (Levy and Marshall 2004; Thevissen et al. 2004).

Additionally, we tested the toxicity of the three new peptides toward two lineages of mammalian cells. All three peptides had low toxicity (Fig. 3a, b) as indicated by the low reduction of metabolic activity. For RR, at the LD_{100} determined for *C. tropicalis* (27.5 µM), murine macrophages and human monocytes were metabolically inhibited by 14.7 and 14.9%, respectively. For D-RR, at the LD_{100} determined for *C. albicans* (36.5 µM) and *C. tropicalis* (23 µM), murine macrophages and human monocytes were metabolically inhibited by 17.2, 14.9, 20.5 and 14.6%, respectively. For WR, at the LD_{100} determined for *C. albicans* (27.5 µM), murine macrophages and human monocytes were metabolically inhibited by 3.6 and 10.2%, respectively. Even at the highest tested concentration of 50 µM, which is much higher than the LD_{100} for the tested yeasts, the most toxic peptide, D-RR, only lowered the metabolic activity of murine macrophages and human monocytes by 23.9 and 25.1%, respectively (Fig. 3a, b). As seen in Fig. 3, the non-adherent cell line (human monocytes) was slightly more sensitive than the adherent cell line (murine macrophages). The reason for that observation is because the surface area exposed to peptides is greater in the non-adherent cell line than in the adherent one. By testing toxicity with adherent and non-adherent cell lines we have a more real view of toxicity to tissues and blood cells. Additionally, both cell lines are important immune cells that act as the first defense line in the immune response to pathogens, and for this reason,

Table 2 Summary of the biological effects of the four synthetic designed peptides against different yeasts species and mammalian cells

Yeast species	Peptide name (peptide name abbreviation) and biological effects											
	$A_{36,42,44}\gamma_{32-46}$*Vu*Def (DD)			$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def (RR)			D-$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def (D-RR)			$A_{42,44}R_{37,38}W_{36,39}\gamma_{32-46}$*Vu*Def (WR)		
	Growth inhibition (%) at 18.5 μM	MIC_{100} (μM)	LD_{100} (fungicide effect, μM)	Growth inhibition (%) at 18.5 μM	MIC_{100} (μM)	LD_{100} (fungicide effect, μM)	Growth inhibition (%) at 18.5 μM	MIC_{100} (μM)	LD_{100} (fungicide effect, μM)	Growth inhibition (%) at 18.5 μM	MIC_{100} (μM)	LD_{100} (fungicide effect, μM)
Saccharomyces cerevisiae	ni	–	–	ni	–	–	ni	–	–	26.1	–	–
Candida albicans	ni	–	–	45.7	–	–	84.9	23	36.5	96.2	18.5	27.5
Candida buinensis	ni	–	–	100	–	–	99.7	–	–	98.5	–	–
Candida parapsilosis	ni	–	–	ni	–	–	ni	–	–	ni	–	–
Candida peliculosa	ni	–	–	ni	–	–	ni	–	–	ni	–	–
Candida tropicalis	ni	–	–	72.1	27.5	27.5	100	14	23	58.2	–	–
Mammalian cells	Reduction of metabolic activity (%)											
	-			At the same LD_{100} for *C. tropicalis*			At the same LD_{100} for *C. albicans*		At the same LD_{100} for *C. tropicalis*	At the same LD_{100} for *C. tropicalis*		
RAW 264.7 murine macrophage	–			14.7			17.2		14.9	3.6		
THP-1 human monocite	–			14.9			20.5		14.6	10.2		

The MIC_{100} and viability (LD_{100}) were tested only against the combination of the most sensible yeast species and the strongest peptide determined by the first antimicrobial screen at 18.5 μM

ni yeast was not inhibited at the condition tested, (−) not determined

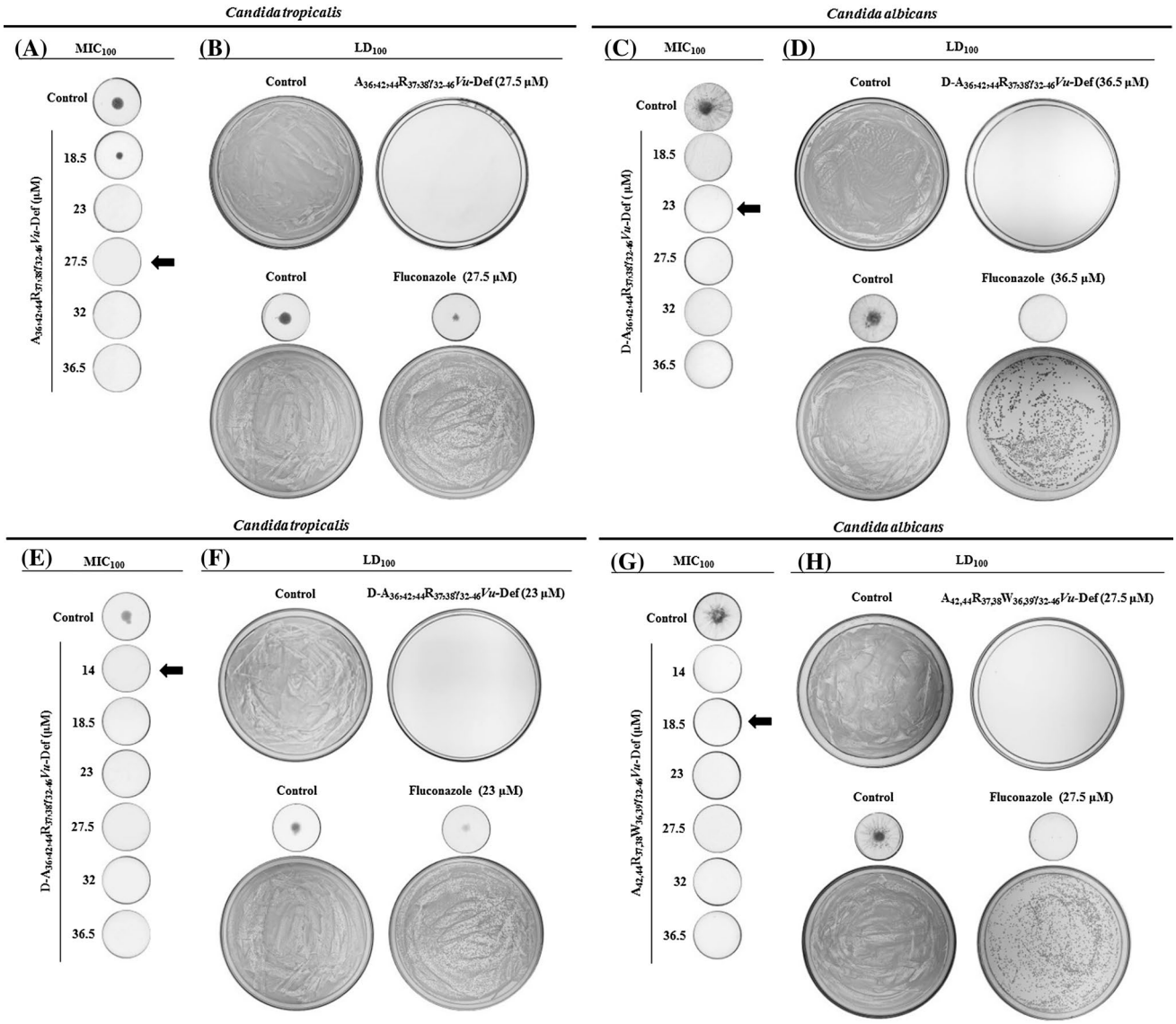
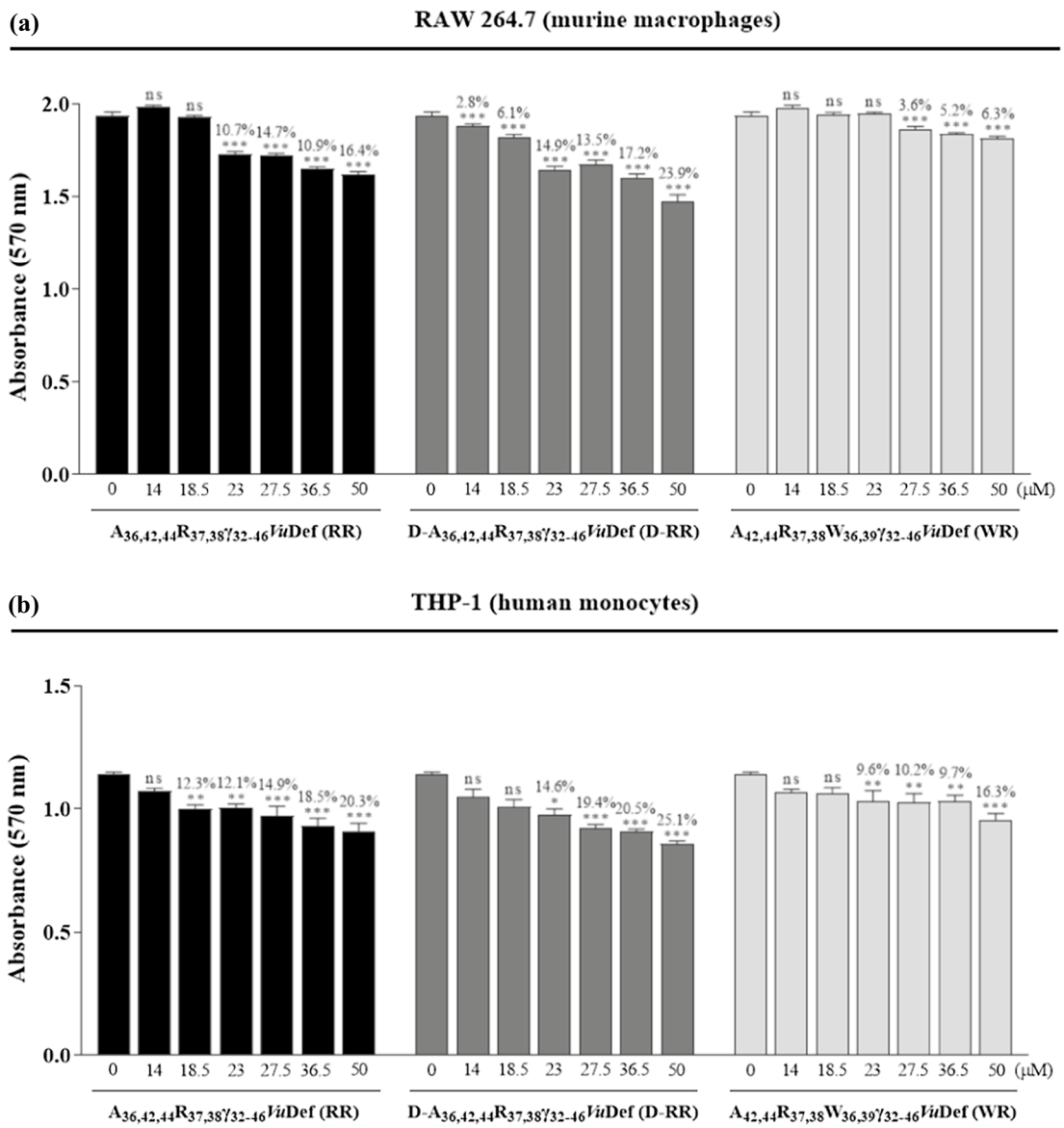


Fig. 2 Determination of the minimal inhibitory concentration (MIC_{100}) (**a**, **c**, **e** and **g**) and cell viability (LD_{100}) (**b**, **d**, **f** and **h**) assays for the combination of the chosen peptides and yeasts. **a**, **c**, **e** and **g** Images of the bottom of the wells at the end of the assay (24 h) and the arrow points to MIC_{100}. Peptide concentration above this one pointed by the arrow presents a small mass of cells growth which was not captured by the camera and below this indicated concentration no growth is observed. **b**, **d**, **f** and **h** In the viability assay is indicated the concentration of the MIC_{100} assay that kills all initial cell population and therefore, indicating a fungicide action at this concentration and also the LD_{100}. In the well treated with fluconazole, at the same LD_{100} concentration determined to the peptides, it was possible to see a small fungal growth and, after plating, there was the growth of colonies, demonstrating fungistatic action. The images are representative of one assay out of three

they should not be seriously affected by the peptides to fulfill their primordial defense function. The 50% cytotoxic concentration (CC_{50}) for all three peptides for the two cell lineages were higher than 50 µM (Fig. 3c) indicating a discriminating capability between yeasts and mammalian cells by the peptides which is a good feature for a drug candidate. The low toxicity of our peptides is in agreement of studies that showed low toxicity of plant defensins to mammalian cells (Tavares et al. 2008; Carvalho and Gomes 2011; Vriens et al. 2015; Lay et al. 2019). Altogether, our results are summarized in Table 2.

As predicted, the increase in net positive charge and hydrophobicity in the WR resulted in the best antifungal activity when compared to the other three peptides tested. However, only the increase in the positive charge in RR was enough for an improvement in its antifungal activity when compared to DD (Fig. 1). A similar result was obtained by Mello et al. (2019) with peptides derived from the γ-core



(c)

Peptide name (peptide name abbreviation)	CC_{50} (μM)	
	RAW 264.7 murine macrophages	THP-1 human monocytes
$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def (RR)	> 50	> 50
D-$A_{36,42,44}R_{37,38}\gamma_{32-46}$*Vu*Def (D-RR)	> 50	> 50
$A_{42,44}R_{37,38}W_{36,39}\gamma_{32-46}$*Vu*Def (WR)	> 50	> 50

CC_{50} – 50% cytotoxic concentration

Fig. 3 Viability of RAW 264.7 murine macrophage (**a**) and THP-1 human monocite (**b**) cells in response to the synthetic peptides after 24 h treatment. Cell viability was assayed by the colorimetric MTT based assay. Cell viability percentage was calculated in relation to the positive control (O.D. 1.94 ± 0.05, cell viability—98.8 ± 2.1%), untreated macrophages, and to the negative control, macrophages culture treated with 1% (v/v) Triton X-100 (0.14 ± 0.03, cell viability—0%). The bars for each sample refer to concentrations tested in ascending order. The results presented are mean values obtained over three experiments, each done in duplicate. **c** 50% cytotoxic concentration (CC_{50}) required for the reduction of cell viability by 50%, which were calculated by regression analysis. ***$P < 0.001$ compared to untreated group (0 μM) determined by Tukey test

of the PvD_1 as explained in the 6th paragraph of the Results and discussion section about the replacement of two negative residues (*DD*) in $\gamma_{31\text{-}45}PvD_1$ (RSGRAR*DD*FRAWATK) by two positive residues (*RR*) in $\gamma_{31\text{-}45}PvD_1^{++}$ (RSGRAR*RR*FRAWATK). The $\gamma_{31\text{-}45}PvD_1^{++}$ also has a higher hydrophobicity (+17.39 kcal/mol) than $\gamma_{31\text{-}45}PvD_1$ (+21.12 kcal/mol), which may have contributed to its antifungal activity improvement (Supplementary Table 1). In regard to the antimicrobial spectra of plant defensins, some fungal species are inhibited while others are not (Fig. 1 and Table 2). This can be partially explained by the peptide sequence and structure. Furthermore, the opposite charge attraction can also partially explain the plant defensins and their derived peptides capability of interact and inhibit microorganisms, because the difference in the composition of the fungal membrane, as explained in the 4th paragraph of the Results and discussion section. Additionally, the RGFRRR-positive amino acid sequence present in the γ-core of $MtDef_4$ is essential for the binding of the defensin to PA in the target membrane, cell entry and induction the fungal death. Mutants in which the positive sequence was exchanged to AAARR and RGFRAA lost their ability to bind to the PA, were unable to enter in the fungal cell and kill the fungus *F. graminearum* (Sagaram et al. 2013). Such results indicate that the fungal inhibition caused by defensins is species dependent, that is, they depend on a specific target presented by the fungal species. It is likely that the directional amino acid changes made to the WR peptide reinforced its interaction with the target membrane and consequently increased its antifungal activity. This was also observed by Saravanan et al. (2014) where the authors synthesized seven peptides from ten residues derived from HBD-28 C-terminal (human β-defensin), with modifications that increased net charge, by adding R, and hydrophobicity, by adding W. Their results showed that peptides with a higher positive net charge and higher hydrophobicity, such as RWKRWWRRKK-NH_2 (charge + 7.1 and hydrophobicity + 20.45 kcal/mol) and RKKRWWRRKK-NH_2 (charge + 8.1 and hydrophobicity + 25.34 kcal/mol) had the best antimicrobial activity, without increasing cytotoxicity for mammalian cells. In addition, it was shown that these peptides interacted strongly with the microbial membrane, destabilized it and caused its permeabilization. Such results show the importance of positively charged and hydrophobic amino acids in the AMP interaction with microorganisms, not only for plant defensins, but also for other AMPs and/or peptides derived from them as well.

Conclusion

Despite plant defensins huge primary structure variation which difficult a straightforward analysis (Lacerda et al. 2014), our data clearly demonstrated that the correlation of biological activity and primary structure of plant defensins that some position of preference for certain amino acid residues, especially R in positions 41 and 50, and aromatic residue approximately in position 42. Thus, we explored the correlation of the biological activity and structure of plant defensins and design new peptides with improved antifungal activity. Importantly, as this approach in mainly focused on the γ-core that plant defensins share with many other AMPs (Yount and Yeman 2004), it has the potential to be applied to other γ-core containing AMPs. This approach has the advantages of: (1) being fast, once the table presented in the Supplementary Fig. 1 is ready and primary structures contained in it can be easily compare with other AMPs, (2) there is no need to synthesize peptide libraries, because the modifications are guided by studies that demonstrate activity or loss of activity due to specific amino acid exchange. Our results indicate that the targeted modifications in the RR, D-RR and WR peptides resulted in improved antifungal activity by inhibiting more yeast species with high potency, when compared to the original DD, with WR, the most hydrophobic and cationic peptide, exhibited better antifungal activity among the four and also was the less toxic to mammalian cells in our conditions. So far, we showed that the three new peptides have antifungal activity, depending on charge, hydrophobicity and yeast species. However, more research is still needed to understand the mechanisms of action of these molecules and to identify their possible cellular targets, which is of fundamental importance for the design and development of new peptides with further improved biological activity. Therefore, our study provides experimental evidence that targeted changes in the primary structure of peptides based on plant defensins γ-core primary structures prove to be a good tool for the synthesis of new compounds that may be useful as alternative antifungal drugs.

Author contributions EBT participated in the peptide design, antifungal assays, and contributed to the writing of the manuscript, DRL participated in the peptide design and antifungal assays, TLBVS and SDC performed the mammalian viability assay and data analysis, EL and MFM participated in mammalian viability assay experimental design and data analysis, FZD performed statistical analyses, VMG performed experimental design, AOC participated in conception, experimental design and data analysis, contributed to the writing of the manuscript, and revised the manuscript.

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Compliance with ethical standards

Conflicts of interest The authors declare any conflicts of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

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