PEPTÍDEOS BIOINSPIRADOS NA DEFENSINA DE PLANTA *Pv*D_1: ATIVIDADE ANTIMICROBIANA E MECANISMO DE AÇÃO EM COMBINAÇÃO COM ANFOTERICINA B SOBRE LEVEDURAS

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UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

CAMPOS DOS GOYTACAZES – RJ

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Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Doutora em Biotecnologia Vegetal.

Orientadora: Profª. Drª. Valdirene Moreira Gomes

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"**É JUSTO QUE MUITO CUSTE O QUE MUITO VALE**"

(Santa Teresa d'Ávila)

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LISTA DE ABREVIATURAS

AmB- Anfotericina B

AMPs - do inglês, Antimicrobial Peptides

CMI- Concentração Mínima Inibitória

DAPI - 4',6-diamidino-2-fenilindol

DIC - do inglês, Differential Interference Contrast

DNA - Ácido Desoxirribonucleico

FIC - do inglês, Fractional Inhibitory Concentration

IC_{50} - do inglês, Inhibitory Concentration that diminishes a specified response to 50%

IP - Iodeto de propídeo

kDa - quilodaltons

MIC - do inglês, Minimal Inhibitory Concentration

OMS - Organização Mundial da Saúde

PBS - do inglês, Salina Fosfatada Tamponada

ROS - do inglês, Reactive Oxygen Species

SDS - Dodecil Sulfato de Sódio

RESUMO

SOUZA, Thaynã Amanda Melo, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro, agosto de 2024. **Peptídeos bioinspirados na defensina de planta *Pv*D$_1$: atividade antimicrobiana e mecanismo de ação em combinação com anfotericina B sobre leveduras;** Orientadora: Valdirene Moreira Gomes.

Peptídeos antimicrobianos (AMPs) são moléculas que participam da primeira linha de defesa de diversos organismos vivos com atividade inibitória sobre uma ampla gama de patógenos. Com o aumento da população mundial e mudanças climáticas a demanda de produtos para saúde humana, incluindo os fármacos, cresceram. Desta forma o aumento do interesse na busca de soluções biotecnológicas para desenvolvimento de compostos menos tóxicos e por fármacos que não induzam a resistência microbiana tornou-se ainda mais necessária. A defensina *Pv*D$_1$, isolada de sementes de *Phaseolus vulgaris* tem um padrão γ-core (GXCX3-9C) que pode estar relacionado às propriedades antimicrobianas das defensinas de plantas, sendo assim um alvo de estudos para melhor elucidação das atividades biológicas dessa molécula. O objetivo deste estudo foi avaliar a atividade antimicrobiana de peptídeos bioinspirados baseados na defensina *Pv*D$_1$ sobre o desenvolvimento de leveduras de importancia médica e o modelo *Saccharomyces cerevisiae*, e sua ação em combinação com o antifúngico convencional anfotericina B. Inicialmente os peptídeos bioinspirados utilizados foram testados sobre o desenvolvimento de leveduras e posteriormente em combinação com o antifúngico anfotericina B a fim de verificar uma possível ação sinérgica. Estudos de mecanismos de ação como permeabilização de membrana, aumento da indução de espécies reativas de oxigênio, danos na parede celular, ao DNA e as membranas vacuolares, além funcionalidade mitocondrial, foram realizados para melhor entendimento das formas de ação dos peptídeos bioinspirados em sinergia com anfotericina B. Os peptídeos utilizados neste trabalho foram escolhidos após alguns testes, dando a possibilidade de ser um protótipo para o desenvolvimento de um novo antifúngico, com custos mais baixos para a indústria farmacêutica e, ao mesmo tempo, mantendo propriedades antimicrobianas. A combinação sinérgica encontrada para a levedura *Candida albicans* foram capazes de causar danos a parede celular, ao DNA e perda de funcionalidade mitocondrial. As analises por microscopia eletrônica de varredura demostraram alterações morfológicas e as análises por microscopia eletrônica de transmissão demostraram alterações significativas em suas estruturas comprometendo parede e membrana celular e algumas organelas como núcleo e mitocôndria.

As concentrações sinérgicas encontradas para a levedura *Candida tropicalis* e o modelo *S. cerevisiae*, também foram capazes de causar danos ao DNA, a parede celular e perda da funcionalidade mitocondrial, além de irregularidades significativas em sua morfologia e ultraestrutura. Para *S. cerevisiae* é possível observar maior número de vacúolos por célula quando se comparado ao controle da mesma. Os resultados obtidos poderão contribuir para o meio científico com o objetivo de utilizar peptídeos antimicrobianos sintéticos derivados de plantas combinados com o antifúngico anfotericina B como novos biofármacos terapêuticos.

Palavras-chave: Antifúngico, Atividade sinérgica, Fungos, Terapia combinada

ABSTRACT

SOUZA, Thaynã Amanda Melo, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro, August 2024. **Peptides bioinspired by plant defensin *Pv*D$_1$: antimicrobial activity and mechanism of action in combination with amphotericin B on yeasts**; Advisor: Valdirene Moreira Gomes.

Antimicrobial peptides (AMPs) are molecules that participate in the first line of defense of several living organisms with inhibitory activity against a wide range of pathogens. With the increase in the world population and climate change, the demand for products for human health, including pharmaceuticals, has increased. Thus, increased interest in the search for biotechnological solutions for the development of less toxic compounds and for pharmaceuticals that do not induce microbial resistance has become even greater. The defensin *Pv*D$_1$, which is isolated from *Phaseolus vulgaris* seeds, has a γ-core pattern (GXCX3-9C) that may be related to the antimicrobial properties of plant defensins; thus, *Pv*D1 is a target of studies to better elucidate the biological activities of this molecule. The aim of this study was to evaluate the antimicrobial activity of synthetic peptides based on the defensin *Pv*D$_1$ on the development of medically important yeasts and the model *Saccharomyces cerevisiae* and their action in combination with the conventional antifungal amphotericin B. Initially, the synthetic peptides used were tested for their ability to develop yeasts in combination with the antifungal amphotericin B to verify their possible synergistic action. Studies of mechanisms of action, such as membrane permeabilization, increased induction of reactive oxygen species, damage to the cell wall, DNA and vacuolar membranes, and mitochondrial functionality, were performed to better understand the forms of action of the synthetic peptides in synergy with amphotericin B. The peptides used in this work were chosen after some tests, suggesting the possibility of being a prototype for the development of a new antifungal, with lower costs for the pharmaceutical industry and, at the same time, maintaining antimicrobial properties. The synergistic combination found for the yeast *Candida albicans* was capable of causing damage to the cell wall and DNA and the loss of mitochondrial functionality. Scanning electron microscopy analyses demonstrated morphological alterations, and transmission electron microscopy analyses revealed significant alterations in their structures, which compromised the cell wall and membrane and some organelles, such as the nucleus and mitochondria. The synergistic concentrations found for the yeast *Candida tropicalis* and the model *S. cerevisiae* were also capable of causing damage to the DNA and cell wall and the loss of mitochondrial

functionality, in addition to significant irregularities in their morphology and ultrastructure. For *S. cerevisiae*, it was possible to observe a greater number of vacuoles per cell than in the control. The results obtained may contribute to the scientific community with the objective of using synthetic antimicrobial peptides derived from plants combined with the antifungal amphotericin B as new therapeutic biopharmaceuticals.

Keywords: Antifungal, Synergistic activity, Fungi, Combination therapy

1. INTRODUÇÃO

De acordo com dados da Global Action Fund for Fungal Infection (GAFFI), mais de 300 milhões de pessoas sofrem de doenças graves causadas por fungos, com uma mortalidade anual que chega a 1,6 milhões. No Brasil, um estudo do Conselho Federal de Medicina revela que aproximadamente 14,5% das infecções em pacientes internados em UTIs são causadas por fungos, e essa taxa está aumentando. Nesse contexto, peptídeos antimicrobianos bioinspirados, especialmente aqueles baseados em peptídeos naturais de plantas, se destacam como promissores candidatos no combate às infecções fúngicas. Esses peptídeos podem ser modificados racionalmente para aprimorar sua eficácia terapêutica (Villanueva e Fanjul, 2017; Slezina e Odintsova, 2023).

A terapia combinada de antifúngicos convencionais com outras moléculas que potencializam seu efeito terapêutico e permitem a redução da dose aplicada, tem mostrado resultados promissores nos últimos anos. No entanto, a estabilidade biológica dos peptídeos para uso sistêmico ainda representa um desafio para sua comercialização, uma vez que esses peptídeos são suscetíveis à degradação proteolítica, o que pode diminuir sua atividade e encurtar sua meia-vida. A combinação com antifúngicos convencionais pode melhorar essas características negativas associadas ao uso isolado de peptídeos bioinspirados (Taveira et al., 2016; Konakbayeva e Karlsson, 2023).

A ação sinérgica entre antifúngicos convencionais e outras moléculas que potencializam seu efeito terapêutico e permitem a redução da dose aplicada tem gerado resultados promissores nos últimos anos (Lima et al., 2020). Para superar os desafios associados às moléculas naturais, como complexidade estrutural e a necessidade de altas concentrações para alcançar uma atividade antifúngica desejável, há um crescente interesse na modificação de peptídeos antimicrobianos sintéticos inspirados na natureza. Essas modificações visam aproveitar seu alto potencial biotecnológico e melhorar a eficácia terapêutica.

Desta forma, os peptídeos antimicrobianos sintéticos passam a ser uma excelente alternativa para superar as dificuldades associadas à, por exemplo, extração de peptídeos naturais. Na síntese de peptídeos sintéticos, as moléculas são produzidas com base em um desenho racional que relaciona estrutura e atividade, permitindo a adição sequencial de aminoácidos e a realização de modificações pontuais. Esse processo facilita a análise e otimização dessas moléculas para melhorar sua eficácia (Haney, Mansour e Hancock, 2017). Atualmente, várias defensinas estão em desenvolvimento e em fase de testes clínicos como tratamentos para infecções fúngicas, bacterianas e virais. No entanto, apenas um peptídeo

derivado de uma defensina de planta, a Pezadeftida (HXP124), figura entre os candidatos em lista para uso clínico. Isso evidencia a necessidade de avanços na descoberta de novas moléculas para aumentar a disponibilidade de antifúngicos eficazes (Hein et al., 2022).

1.1. Peptídeos antimicrobianos de plantas

Os peptídeos antimicrobianos, também conhecidos como AMPs (do inglês *Antimicrobial Peptides*), são moléculas comuns de defesa presentes em diferentes grupos de organismos desde bactérias, plantas, insetos, aves e mamíferos, entre outros (Mahlapuu et al., 2016). Estas moléculas constituem a primeira linha de defesa de qualquer espécie e são componentes conservados do sistema imune inato, que é classificada como uma resposta imediata durante o evento inicial de infecção, promovendo assim a proteção contra patógenos do ambiente (Mookherjee et al., 2020). Por serem evolutivamente antigos, esses peptídeos conseguem abranger um amplo espectro de atividade inibitória, que vai de bactérias Gram positivas e negativas, fungos, vírus, parasitas e até mesmo células de câncer (Zhu et al.,2016; Figueira et al., 2017).

Os AMPs pertencem a um grupo diverso e abundante de moléculas e são produzidos por animais e plantas, em diversos tecidos e tipos celulares (Mihajlovic e Lazaridis, 2010). Os peptídeos antimicrobianos já foram isolados em diversos órgãos e tecidos da planta, como folha, frutos e sementes, em sua maioria apresentam aproximadamente de 12 a 100 resíduos de aminoácidos, massa molecular inferior à 10 kDa, são anfipáticos e possuem carga líquida positiva em pH fisiológico Apresentam estabilidade em altas temperaturas e à degradação enzimática, devido aos resíduos de cisteínas, geralmente em números pares (4, 6 ou 8), que se encontram interconectadas formando ligações dissulfeto (Campos et al., 2018).

Os modelos de interação dos peptídeos com a membrana plasmática mais comuns e aceitos, baseados em experimentos com membranas bacterianas, são os de aduela de barril, o modelo tapete e o modelo poro toroidal. No modelo aduela de barril, os AMPs interagem com os resíduos hidrofóbicos dos peptídeos, voltados para o interior da bicamada lipídica, e os resíduos hidrofílicos, orientados para o lúmen do poro recém-formado, aglomerando-se. No modelo poro toroidal os peptídeos se reorientam na membrana durante o arrasto de agregação dos lipídios com eles (por meio de interações eletrostáticas entre grupos principais de fosfolipídios e resíduos hidrofílicos de AMPs), dessa forma a membrana é "dobrada" e as camadas unidas formam o poro toroidal. No modelo tapete, os peptídeos agem como detergentes, cobrindo a membrana de maneira eletrostática (na forma monomérica ou oligomérica). Esse “tapete” de moléculas anfipáticas causa um deslocamento fosfolipídico,

altera as propriedades da membrana e rompe a membrana (Mookherjee et al., 2020; Na Chen, Cheng Jiang, 2023). Dependendo da concentração, o AMP pode se comportar como detergente (Boto; De La Lastra; González, 2018). Exemplos dessas interações podem ser observados na Figura 1.

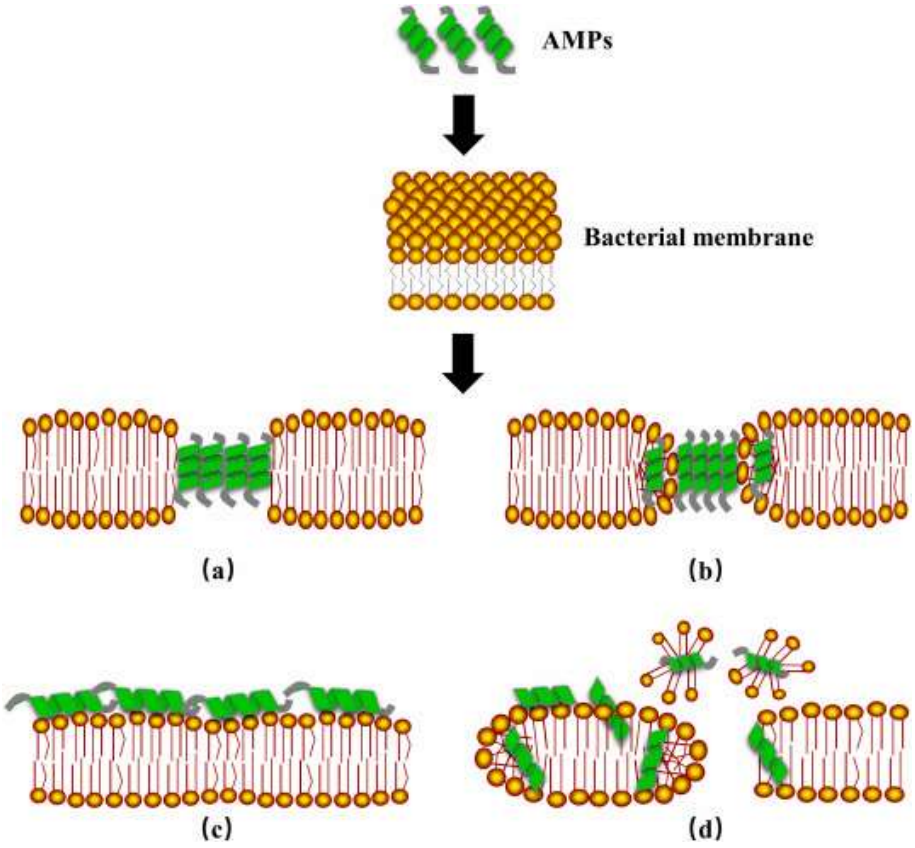


Figura 1: Exemplos dos diferentes tipos de interações entre peptídeos antimicrobianos e membranas citoplasmáticas de bactérias, (a) Modelo aduela de barril, (b) Modelo poro toroidal, (c) Modelo carpete e (d) Modelo detergente (Na Chen, Cheng Jiang, 2023).

Todas as interações dos AMPs de plantas com membrana plasmática de microrganismos causam disfunção da mesma, podendo levar consequentemente à morte celular. Alguns autores como Zhang et al., (2019), discutem acerca dos fenômenos que podem acontecer durante e após a interação do AMP com a membrana plasmática dos micro-organismos.

A presença de carga líquida positiva ajuda os AMPs a se ligarem rápida e eficientemente à membrana carregada negativamente e interferir na organização da

membrana celular (Figura 2A). Os AMPs inibem a síntese de biomoléculas intracelulares ou extracelulares, como ácidos lipoteicóicos, bloqueando a síntese da parede celular (Figura 2B). Os AMPs são capazes aumentar o efluxo de K^+ e Ca^{2+} celular no citoplasma. O acúmulo de Ca^{2+} quebra sua homeostase, resultando na geração de espécies reativas de oxigênio (ROS), levando à apoptose celular (Figura 2C). Os AMPs também causam inibição da síntese de ATP ao interromper as atividades da ATP sintase ou bloquear a cadeia transportadora de elétrons, levando a um dano do metabolismo energético. Por meio do metabolismo energético ou do dano à atividade enzimática, os processos celulares dependentes de ATP são bloqueados (Figura 2D).

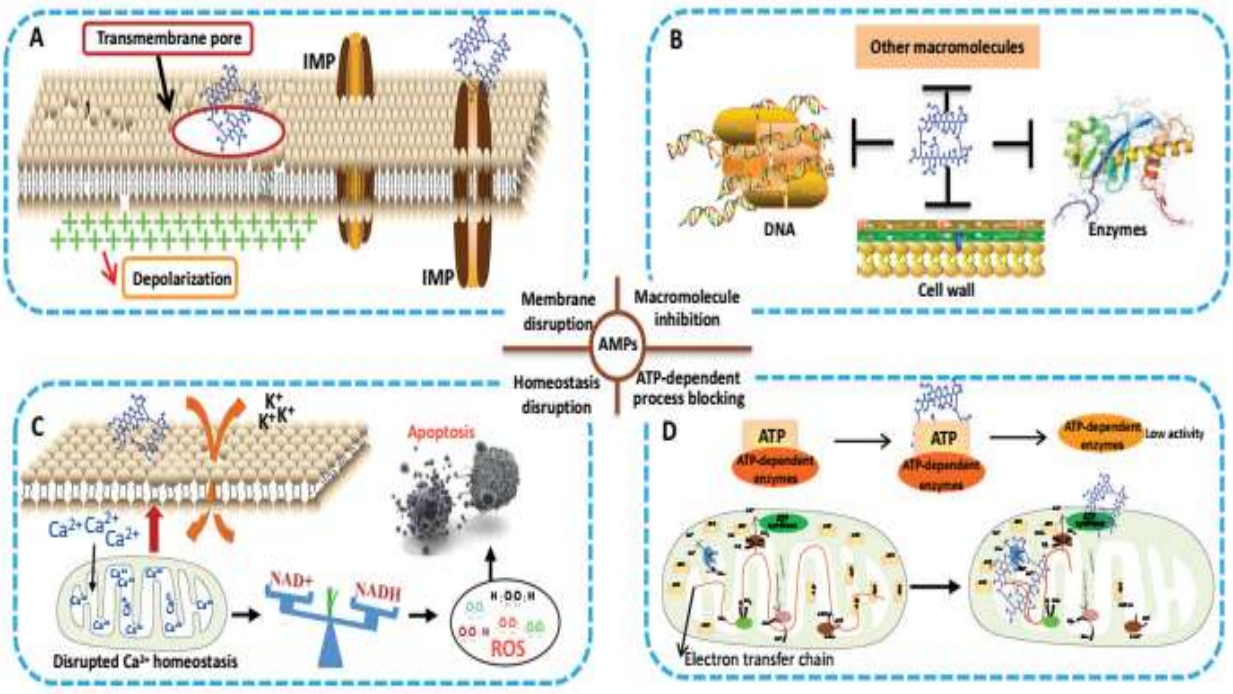


Figura 2: Mecanismo de ação dos AMPs visando patógenos microbianos. (A), os AMPs se ligam à membrana celular e levam à flutuação da membrana, despolarizando e formando grandes poros transmembrana. Os AMPs também podem interagir com as proteínas importantes da membrana como a IMP (Important Membrane Protein). (B) os AMPs inibem macromoléculas intracelulares ou extracelulares, incluindo síntese de DNA, enzimas, peptideoglicano (precursor da parede celular), e outras macromoléculas. (C) os AMPs aumentam a concentração de Ca^{2+} no citoplasma e mitocôndrias, interrompendo a homeostase do Ca^{2+}, aumentando a proporção de NAD^+/NADH, consequentemente induzindo a geração de ROS e apoptose. (D) os AMPs interagem com o ATP diretamente e assim diminuem a atividade das enzimas dependentes de ATP. Então, os AMPs bloqueiam as sínteses de ATP pela interação com a ATP sintase ou bloqueando a cadeia transportadora de elétrons, resultando no bloqueio de processos dependentes de ATP (Zhang et al., 2019).

A classificação dos peptídeos antimicrobianos de plantas é feita não considerando exclusivamente a identidade na sequência de aminoácidos, embora as regiões altamente conservadas sejam para algumas famílias como uma assinatura. Ainda assim, a principal característica que separa os AMPs em famílias são os arranjos estruturais tridimensionais, que pode ser composto por α-hélices, folhas β, serem cíclicos, lineares ou ainda, formar uma molécula composta por estruturas anteriormente citadas conectadas por loops. As principais famílias de AMPs de plantas são as tioninas, as defensinas, os peptídeos heveína-símile, as knotinas, as proteínas transferidoras de lipídeos (LTPs), as snakinas, os inibidores de proteases e os ciclotídeos (Koehbach e Craik, 2019). Dentre os principais AMPs de plantas estudados encontramos principalmente as defensinas (Broekaert et al., 1997; Reddy et al., 2004).

1.2. Defensinas de plantas

As defensinas podem ser consideradas um dos maiores grupos de AMPs já estudados. Apresentam uma grande diversidade em relação à sequência de resíduos de aminoácidos (45-54 resíduos), dado que é responsável por suas diferentes atividades biológicas *in vitro*. Porém, a estrutura tridimensional é altamente conservada em três folhas betas antiparalelas conectadas a uma alfa-hélice. Esta estrutura é sustentada por ligações dissulfeto entre os oito resíduos de cisteínas (C1-C8/C2-C5/C3-C6/C4-C7) componentes da estrutura primária (Parisi et al., 2019). Elas possuem um amplo espectro de atividade, que pode ser contra fungos (leveduriforme e filamentosos), bactérias, protozoários, vírus envelopados, bactérias gram-positivas, bactérias gram-negativas (Gebara et al., 2020), sendo que algumas já demonstraram atividade antitumoral (Figueira et al., 2017).

Estas fazem parte de uma subfamília de peptídeos de plantas que foram caracterizados por Terras et al., (1995), sendo obtidos a partir de sementes de trigo e cevada. A família de defensinas de plantas são peptídeos catiônicos. Fazem parte dos componentes importantes produzidos no sistema de defesa das plantas, e estão localizados na periferia de diferentes órgãos, como sementes e frutos (Shirakawa et al., 2014; Cools et al., 2017).

1.2.1. Características estruturais e atividades descritas para defensinas de plantas

As variações em sequências primárias são as responsáveis por diferentes funções atribuídas às defensinas de plantas, incluindo atividade antibacteriana, tolerância ao zinco, atividade inibitória de protease e α-amilase, atividade bloqueadora dos canais de íons, dentre

outras (Sagaram et al., 2011; Carvalho e Gomes, 2011; Khan et al., 2019).

A sequência de aminoácidos de diferentes defensinas de plantas pode variar em menos de 35% a mais de 90% (Lacerda et al., 2014). A maioria das defensinas de plantas possuem oito resíduos de cisteína conservados (C1 a C8) e o número de resíduos de aminoácidos entre as cisteínas C1 a C3 e C4 a C6 varia de três a dez vezes, enquanto o número de resíduos de aminoácidos entre outras cisteínas é conservado: C3-x-x-x-C4, C6-x-C7 e C7-x-x-x-C8, onde x representa qualquer aminoácido (van der Weerden, N.L.; Anderson, M.A.; 2013; Kovaleva et al., 2020). As ligações dissulfeto entre resíduos de cisteína seguem o seguinte padrão: C1-C8, C2-C5, C3-C6 e C4-C7 (Kovaleva at al., 2020) (Figura 3).

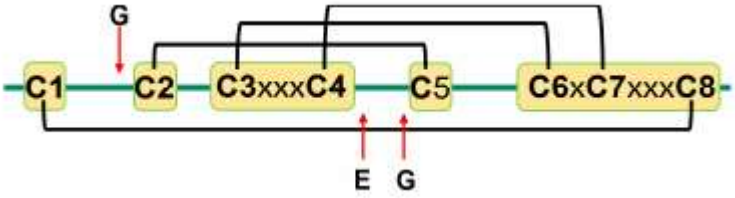


Figura 3: Representação esquemática de defensinas de plantas. Onde as letras mostram os aminoácidos conservados. O número conservado de aminoácidos (x — qualquer aminoácido) que separa as cisteínas C3 e C4 (três aminoácidos), C6 e C7 (um aminoácido) e C7 e C8 (três aminoácidos). As letras e setas vermelhas significam os aminoácidos conservados, assim como suas localizações aproximadas na sequência de aminoácidos, respectivamente. Os colchetes pretos representam ligações dissulfeto (Adaptada de Kovaleva et al., 2020).

Embora as estruturas terciárias sejam bastante conservadas (Figura 4), quando um alinhamento das estruturas primárias de várias defensinas de plantas acontece, pode-se observar que entre os resíduos de cisteína existe uma grande variação na sequência de aminoácidos originando várias atividades biológicas e mecanismos de ação para os diferentes membros da família das defensinas (Parisi et al., 2019, Sathoff et al., 2019).

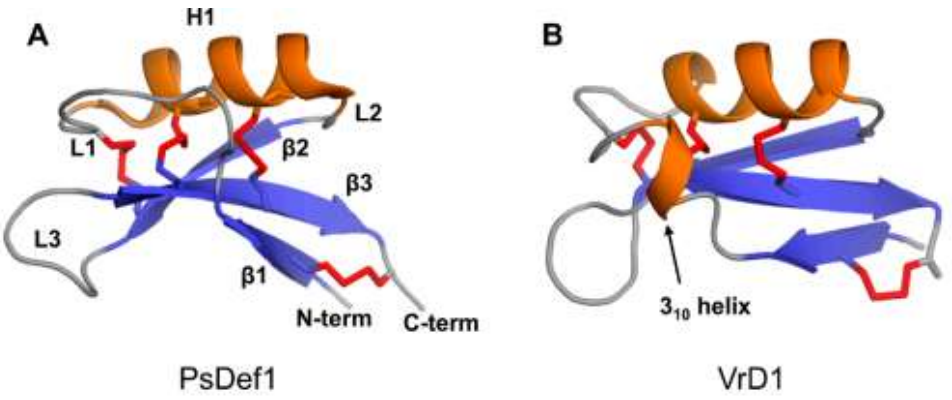


Figura 4: Estruturas tridimensionais de dois monômeros de defensina vegetal. Estruturas de (A) PsDef$_1$ e (B) VrD1. As ligações dissulfeto são mostradas usando bastões vermelhos. Letras indicam elementos estruturais secundários na estrutura de PsDef1 da seguinte forma: loops L1-L3, fitas beta β1-β3, alfa-hélice H1. (Adaptado de Kovaleva et al., 2020).

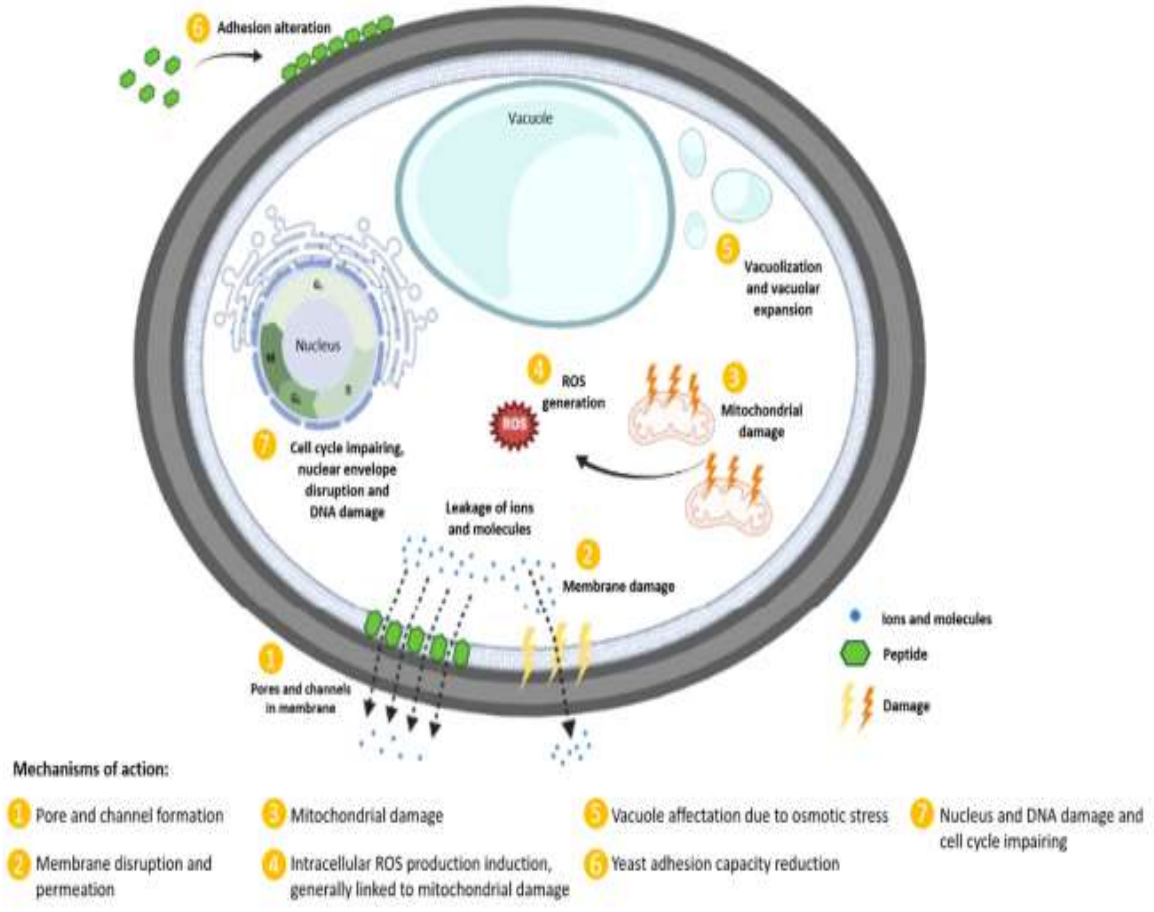
As ligações dissulfeto são conhecidas por aumentar a estabilidade termodinâmica de proteínas globulares, onde a presença de quatro ligações dissulfeto explica a importante estabilidade estrutural das defensinas de plantas em altas temperaturas e pH (Tam et al., 2015; Kovaleva et al., 2020). Alguns dados espectroscópicos de FTIR (Fourier Transform InfraRed) de Ermakova et al. (2016), demonstraram que o conteúdo de estruturas secundárias α-helicoidais e de folha beta em PsDef1 foi preservado até 80 °C, e nenhum desdobramento induzido termicamente foi detectado. Após o processo de resfriamento de 80 °C de volta para 25 °C, o espectro de FTIR mostrou-se semelhante ao observado a 25 °C antes do aquecimento, mostrando o comportamento reversível de PsDef1. Além disso, a defensina PgD5 *de Picea glauca* reteve 71% da atividade antifúngica contra *Verticillium dahlia* após 30min de tratamento a 75 °C e 61% a 100 °C (Picart et al., 2012), enquanto a defensina OsAFP1 do arroz demonstrou uma perda insignificante de atividade após aquecimento a 100 °C por 10 minutos (Ochiai et al., 2018). Já para as defensinas Rs-AFP1 e Rs-AFP2 não foram observadas influências negativas quando submetidas a exposição de 100 °C por 15min (Shwaiki et al., 2020). O peptídeo antifúngico semelhante à defensina NRBAP de *Phaseolus vulgaris*, demonstra capacidade de reter sua atividade após a exposição a 100 °C por 30 min e a índices altos de pH, variando de 0–12 (obtido pela dissolução do NRBAP em ácido clorídrico) (Chan et al., 2013).

Existe nas defensinas a ocorrência de um arranjo estrutural conservado chamado de gama core (GXC(X3-9)C, onde X corresponde a qualquer aminoácido e os números indicam

o espaçamento em aminoácidos entre os AMPs que contêm pontes dissulfeto, onde são caracterizados pela presença de duas folhas β antiparalelas com uma pequena região de volta interposta (De Coninck et al., 2013; Li et al., 2023). Ao longo dos anos pesquisadores estão estudando o motif γ-core em defensinas de plantas, como por exemplo, Samblanx e colaboradores em 1997, demonstraram que a substituição ou remoção de resíduos de aminoácidos, reduziu a atividade biológica de RsAFP2 (uma defensina de *Raphanus sativus*).

Devido à grande diversidade estrutural das defensinas de plantas, esta classe de moléculas possuem a capacidade de apresentar diversas atividades biológicas *in vitro* (Hegedüs e Marx, 2013, Parisi et al., 2019). Dentre estas, podemos citar a atividade antibacteriana, inibição de amilases de intestino de insetos, inibição de tripsina, inibição de síntese de proteínas, bloqueio de canais de sódio, bem como papeis na tolerância aos metais pesados e no desenvolvimento das plantas, além de atividade anticâncer (Melo et al., 2002; Wong et al., 2006; Carvalho e Gomes, 2009; Santos et al., 2010; Oomen et al., 2011; van der Weerden e Anderson, 2013; Parisi et al., 2019).

A atividade antimicrobiana dessas moléculas é principalmente observada contra fungos, sendo bem menos pronunciada contra bactérias, sendo que nessas últimas, é apenas observada contra bactérias Gram-positivas (Carvalho e Gomes, 2011; Li et al., 2019). Elas possuem



grande capacidade de inibir o crescimento de uma grande variedade de fungos filamentosos e leveduras, e a atividade antifúngica, bem como a concentração necessária para suas funções inibitórias, dependerão do fungo testado e da defensina em questão (Mello, 2014; Parisi et al., 2019b). Em 2022 Perez-Rodrigues e colaboradores, abordaram alguns mecanismos de ação que as defensinas de plantas apresentam especificamente em leveduras (Figura 5).

Figura 5: Representação esquemática dos mecanismos de ação de peptídeos antimicrobianos (defensinas de plantas) para leveduras (Adaptado de Perez-Rodrigues et al., 2022).

1.2.2. Mecanismo de ação das defensinas de plantas

Os processos de mecanismo de ação que levam a inibição do crescimento fúngico pelas defensinas de plantas têm sido alvo de estudo de diversos autores. Algumas evidências demonstram vários passos que envolvem um mecanismo extracelular atuando na parede celular e/ou na membrana plasmática, bem como atuando em alguns alvos intracelulares (Carvalho e Gomes, 2011; Kovaleva et al., 2020; Tetorya et al., 2023).

A primeira descoberta sobre o mecanismo de ação das defensinas de plantas foi relatada por Thevissen et al. (1996). Eles demostraram que as defensinas Dm-AMP1 e RsAFP2 causavam um influxo de Ca^{+2} e um efluxo de K^{+}, resultando em mudanças no potencial de membrana e a esperada alcalinização do meio de incubação na interação com o fungo *Neurospora crassa* (Figura 6). No ano de 1999 o mesmo autor demonstrou que a defensina Dm-AMP1 era capaz de permeabilizar a membrana do fungo filamentoso *N. Crassa*. Em 2011, Mello e colaboradores demostraram que uma defensina isolada de sementes de *P. vulgaris* denominada *Pv*D_1, foi capaz de causar a permeabilização da membrana de diversas leveduras, como *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *Pichia membranifaciens*, assim como dos fungos filamentosos *Fusarium oxysporum*, *F. solani* e *F. lateritium.*

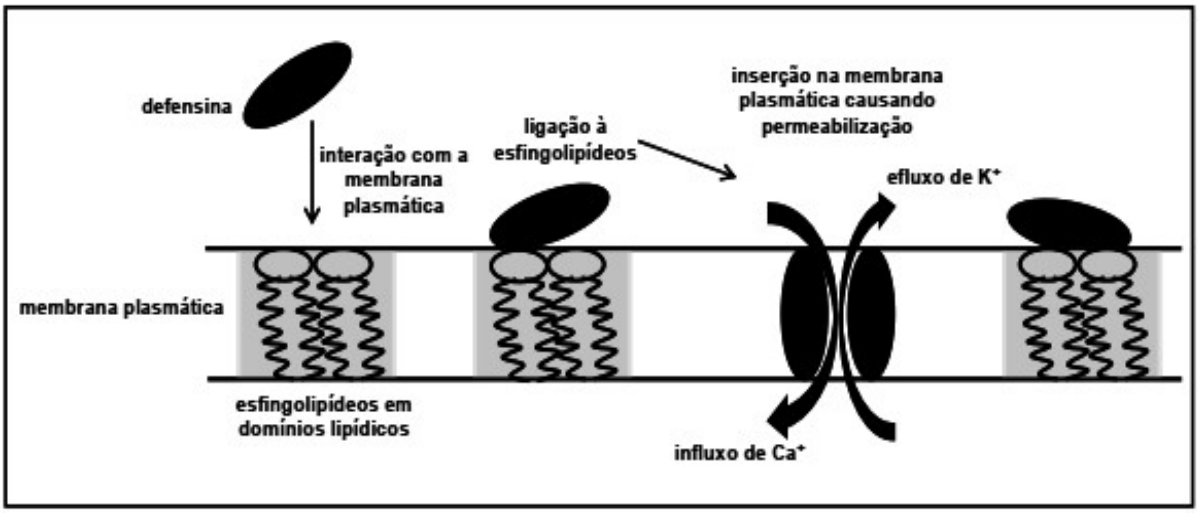


Figura 6: Representação esquemática do primeiro modo de ação descrito para as defensinas de plantas em fungos (Adaptado de Thevissen et al., 1996).

Alguns estudos excluem uma interação direta das defensinas catiônicas com os fosfolípideos carregados negativamente da membrana plasmática de fungos bem como com a formação de poros (Hegedüs e Marx, 2013). Os primeiros estudos sobre o modo de ação das defensinas de plantas realizados há muito tempo atrás, focam na existência de alguns domínios específicos de ligação na membrana plasmática de fungos, também conhecido como domínios lipídicos, que são especificamente enriquecidos em esfingolípideos e esteróis, como esfingolípideos contendo manosildiinositolfosforilceramida (M(IP)2C) e glicosilceramida (GlcCer) (Figura 7), o que permite uma alta concentração local de defensinas ligadas à membrana (Thevissen et al., 2004; Mello et al., 2019).

Thevissen e colaboradores no ano de 2004 estudaram especificamente os sítios de ligação das defensinas de plantas Dm-AMP1 e Rs-AFP2 na membrana de fungos, onde eles conseguiram identificar e caracterizar alguns grupos de esfingolipídeos. Neste estudo, o alvo da defensina Dm-AMP1 na membrana foi identificado usando cepas mutantes de *Saccharomyces cerevisiae* que eram resistentes a essa defensina, onde o gene IPT1 foi identificado como o provável fator de susceptibilidade da cepa do tipo selvagem. O gene IPT1 é responsável por codificar a enzima inositol fosfotransferase que participa da última etapa da síntese do M(IP)2C, um complexo esfingolípideo presente abundantemente na membrana de *S. cerevisiae*. O nível de M(IP)2C está diretamente relacionada com a sensibilidade antifúngica à Dm-AMP1, mostrando o papel do complexo de esfingolipídeos/defensina na atividade antifúngica de Dm-AMP1.

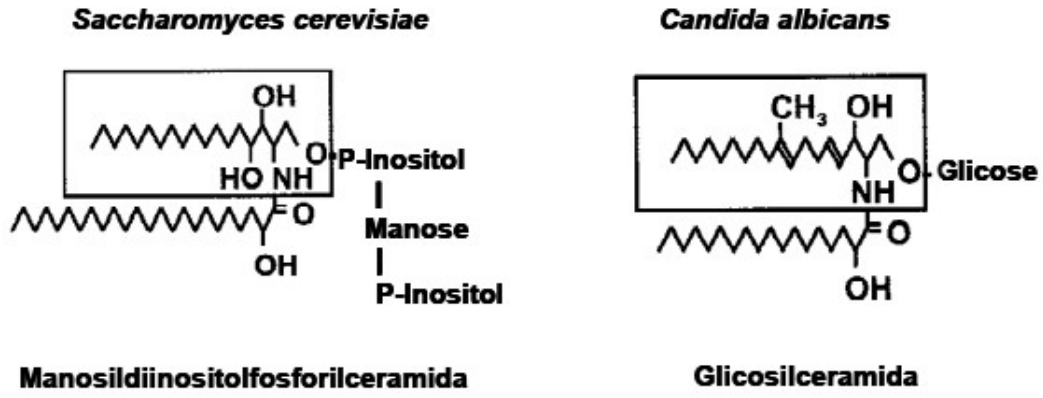


Figura 7: Representação esquemática da estrutura dos esfingolipídeos que são sítios de ligação das defensinas de plantas na membrana dos fungos *Saccharomyces cerevisiae* e *Candida albicans* (Adaptado de Thevissen et al., 2004).

No estudo de Thevissen e colaboradores em 2007 foi mostrado que as defensinas Hs-AFP1 e Rs-AFP2 tinham a capacidade de inibir o crescimento de *C. albicans* e *C. krusei*, mas não de *C. glabrata*. Os autores então atribuíram esses resultados ao fato de que *C. glabrata* não sintetiza glicosilceramidas, sendo assim possível alvo para a defensina Rs-AFP2. Semelhantemente, em 2010 Medeiros e colaboradores, apresentaram dados onde cepas de *C. albicans* mutantes (ΔGCS1), que eram incapazes de sintetizar glicosilceramidas, apresentavam mais resistência à defensina Psd1 isolada de *Pisum sativum*.

Até o momento, o mecanismo de ação mais bem caracterizado para as defensinas de plantas envolve a interação específica com componentes da membrana celular, como glicoproteínas, esfingolipídeos ou fosfolipídeos, permeabilização da membrana plasmática, produção de espécies reativas de oxigênio (ROS) e apoptose (Sher Khan et al., 2019; Tetorya et al., 2023).

Quando se fala de mecanismo de ação das defensinas de plantas, alguns estudos têm demonstrado a importância do motif γ-core para a atividade biológica desta molécula. No estudo de Sagaram e colaboradores (2011), foi testado a atividade antifúngica e morfogênica da defensina MtDef4 e de seu motif γ-core. Foi observado que peptídeos curtos derivados apenas do γ-core de MtDef4 foram capazes de inibir significativamente o crescimento do fungo *Fusarium graminearum*. Também, demonstraram que a estrutura anfipática do γ-core e os aminoácidos catiônicos e hidrofóbicos estão, direta ou indiretamente, envolvidos na interação com componentes da membrana plasmática de fungos, como a glicosilceramida. Dessa forma, esses resultados indicam que o motif γ-core possui um papel fundamental na

atividade biológica das defensinas de plantas, transformando essa região atraente para modificações direcionadas e racionais, visando a melhoria da atividade da molécula, e ainda para a síntese de novos peptídeos bioativos para aplicação terapêutica.

Desta forma, os peptídeos sintéticos baseados na estrutura primária de defensinas de plantas também têm sido bastante estudados, e a região motif γ-core é o principal alvo de estudos por ter um papel fundamental na atividade antimicrobiana dos AMPs (Yount e Yeaman, 2004). Nas defensinas de plantas, esse motif se tornou um local atraente para modificações específicas e direcionadas, pois já foi descrito que para algumas defensinas a atividade está no motif γ-core (Ramesh et al., 2016). Para defensinas de plantas, o motif γ-core está na fórmula dextromérica NH 2– [X1-3] - [GXC] - [X3-9] - [C] –COOH, sendo X qualquer aminoácido (Yount e Yeaman 2004). As maiores variações entre os resíduos de aminoácidos ocorrem nos loops das defensinas da planta, especialmente na região do loop entre as fitas β2 e β3, que compreende o próprio motif γ-core (Sarfuddin Azmi e Mohd Kamil Hussain, 2021).

1.3. Peptideos bioinspirados sintéticos

O caminho para descoberta de novos AMPs naturais é normalmente demorado, uma vez que apresenta baixo rendimento e alto custo de produção. Além disso, os AMPs naturais podem possuir algumas características indesejáveis a sua aplicação terapêutica, como instabilidade devido à degradação por proteases do hospedeiro, baixa seletividade, baixa hidrossolubilidade, atividade hemolítica, toxicidade do hospedeiro e sensibilidade ao sal (Boto; Pérez de la Lastra; González, 2018).

Buscando superar essas desvantagens, os peptídeos antimicrobianos sintéticos surgem como uma alternativa. Em sua síntese, as moléculas são produzidas baseando-se no desenho racional que relaciona a estrutura com a atividade dessas moléculas, uma vez que cada aminoácido é adicionado sequencialmente, sendo possível fazer modificações pontuais, analisando e otimizando essas moléculas (Haney; Mansour; Hancock, 2017). Dentre as estratégias aplicadas, está a utilização da sequência de AMPs de ocorrência natural, onde a mesma é utilizada para projetar uma nova molécula. Outra rota é o uso de bibliotecas de AMPs usando ferramentas de bioinformática para auxiliar na triagem e combinação de novas sequências de AMPs (Ong; Wiradharma; Yang, 2014; Ciumac *et al.*, 2019). Algumas dessas modificações incluem a adição de resíduos hidrofóbicos e catiônicos para maior atividade antimicrobiana. Outros exemplos são o uso de D-aminoácidos, ciclização, acetilação e utilização de peptidomiméticos para melhora da estabilidade *in vivo* (Kumar; Kizhakkedathu;

Straus, 2018; Torres et al., 2019).

Em 2011, Sagaram e colaboradores demonstraram que a síntese de peptídeos que se estende além do motif γ-core é capaz de melhorar a atividade antifúngica. Nesse estudo, o peptídeo sintético (GMA-4C) derivado do motif γ-core continha o C terminal da defensina MtDef4 e possuia em sua estrutura aminoácidos catiônicos e hidrofóbicos. Tais características eram importantes para sua atividade antifúngica. Em 2013, Sagaram e colaboradores demonstraram que existem mais elementos importantes para melhorar a atividade antifúngica de um peptídeo sintético, como a síntese de peptídeos estendendo além do motif γ-core e a manutenção da região C terminal do peptídeo com aminoácidos catiônicos e hidrofóbicos. Já em relação a carga dos peptídeos, Lacerda et al. (2014) conseguiram demonstrar que os aminoácidos carregados positivamente localizados no motif γ-core foram importantes para a atividade antifúngica dos peptídeos, já que a substituição de resíduos neutros por resíduos carregados positivamente aumentou a atividade inibitória sobre fungos patogênicos.

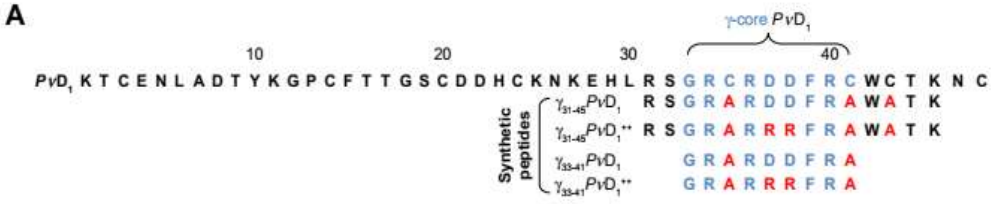
Em 2021, Toledo e colaboradores exploraram a correlação entre a atividade biológica e a estrutura das defensinas de plantas, comparando suas estruturas primárias por sobreposição com VuDef1 (uma defensina de feijão *Vigna unguiculata*) e DD (um peptídeo denominado A36,42,44γ32-46VuDef), o que indicou a posição favorável e o aminoácido a ser alterado. Três novos peptídeos com modificações de carga, hidrofobicidade (RR e WR) e quiralidade (D-RR) foram projetados e testados contra leveduras patogênicas. Os três peptídeos projetados tiveram melhor atividade inibitória contra as leveduras, com melhor potência, amplo espectro de inibição e com baixa toxicidade para células de mamíferos.

Com a finalidade de testar experimentalmente um conjunto de peptídeos de 17 resíduos de aminoácidos contendo o motif γ-core da defensina MtDef4, Tetorya et al., (2023) demostraram que o peptídeo GMA4CG_V6, exibiu uma potente atividade antifúngica contra o patógeno fúngico *Botrytis cinerea*, responsável pela doença do mofo cinzento em frutas e vegetais. Ainda neste estudo, foi identificado o mecanismo de ação do peptideo GMA4CG_V6, onde seu alvo de ação foi predominantemente na membrana plasmática, sendo capaz de causar permeabilização desta membrana, rápida internalização no vacúolo e citoplasma e afinidade pelos bioativos fosfoinositídeos fosfatidilinositol 3-fosfato (PI3P), PI4P e PI5P.

O estudo de novos peptídeos sintéticos baseados em moléculas vegetais para bioinspirar novos fármacos ativos contra fungos resistentes têm aumentado nos últimos anos. Em um recente estudo de Aguiar et al., (2023) foram observados a atividade e o mecanismo de ação de um peptídeo sintético denominado Mo-CBP3-PepII contra a levedura multirresistente *Cryptococcus neoformans*. O peptídeo causou a morte do fungo *C. neoformans*, induzindo o

acúmulo de superóxido e peróxido de hidrogênio nas células, além de causar uma redução na atividade da superóxido dismutase, ascorbato peroxidas e catalase nas células tratadas. Também foi observado que o peptideo Mo-CBP3-PepII causou inibição da atividade da biossíntese de lactato desidrogenase ergosterol e indução do desacoplamento do citocromo c da membrana mitocondrial.

Mello et al. (2019) desenharam quatro novos peptídeos baseados no motif γ-core da defensina de *Phaseolus vulgaris* (*Pv*D$_1$). Baseado nessa sequência do motif γ-core juntamente com partes das folhas β2 e β3 (de Arg31 a Lys45), foi desenhado um peptídeo de 15 resíduos de aminoácidos, que contém a sequência RSGRARDDFRAWATK, denominado $\gamma_{31\text{-}45}$*Pv*D$_1$ (Figura 8). Outro peptídeo, com base na sequência do $\gamma_{31\text{-}45}$*Pv*D$_1$, teve sua carga positiva líquida aumentada com a substituição dos dois resíduos de ácido aspártico (Asp), nas posições 37 e 38, por dois resíduos arginina (Arg), dando origem à sequência de aminoácidos RSGRARRRFRAWATK, chamado $\gamma_{31\text{-}45}$*Pv*D$_1^{++}$. Outros dois peptídeos, de 9 resíduos cada um, foram sintetizados com as sequências de aminoácidos indo da Gly33 à Cys41 (próprio γ-core da *Pv*D$_1$) tendo as sequências de aminoácidos GRARDDFRA e GRARRRFRA e sendo chamados de $\gamma_{33\text{-}41}$*Pv*D$_1$ e $\gamma_{33\text{-}15}$*Pv*D$_1^{++}$. Das características bioquímicas os peptídeos sintéticos de 15 resíduos $\gamma_{31\text{-}45}$*Pv*D$_1$ e $\gamma_{31\text{-}45}$*Pv*D$_1^{++}$ possuem carga de +3 e +7, e a massa molecular é de 1792,99 Da e 1875,19 Da, respectivamente. Para os peptídeos menores de 9 resíduos de aminoácidos $\gamma_{33\text{-}41}$*Pv*D$_1$ e $\gamma_{33\text{-}41}$*Pv*D$_1^{++}$, a carga foi de +1 e +5, sendo a massa molecular de 1063,14 Da e 1145,34 Da, respectivamente, onde foi demonstrado que o peptídeo $\gamma_{31\text{-}45}$*Pv*D$_1^{++}$ causou os efeitos mais tóxicos na levedura *Candida buinensis*.



B

Synthetic peptide names	Size in amino acids	Charge (pH 7.0)	Molecular weight (Da)
$\gamma_{31\text{-}45}$PvD$_1$	15	+3	1,792.99
$\gamma_{31\text{-}45}$PvD$_1^{++}$	15	+7	1,875.19
$\gamma_{33\text{-}41}$PvD$_1$	9	+1	1,063.14
$\gamma_{33\text{-}41}$PvD$_1^{++}$	9	+5	1,145.34

Figura 8: Desenho, alinhamento e características bioquímicas dos peptídeos sintéticos (Adaptado de Mello et al., 2019).

O avanço nas pesquisas envolvendo os peptídeos bioinspirados em peptideos antimicrobianos vegetais ocorre devido ao aumento de patógenos humanos multiresistentes e da alta resistência aos fármacos antifúngicos comerciais (Pfaller et al., 2019; Berman e Krysan, 2020).

1.4. Mecanismos de resistência antimicrobiana

Atualmente a resistência antimicrobiana é considerada um dos maiores problemas quando se pensa em saúde pública. Esta é um fenômeno que pode ocorrer de forma natural devido às características intrínsecas do microrganismo ou ser adquirida após contato imediato com algum fármaco, devido a mutações e/ou transferências de genes de resistência (Figura 9) (Sabtu et al., 2015).

Dentre os mecanismos envolvidos no processo de resistência antimicrobiana, a mais comum é a modificação do fármaco que está sendo utilizado através da ação de enzimas como por exemplo, as enzimas modificadoras de aminoglicosídeos que catalisam as reações de acetilação, fosforilação ou adenilação (Ramirez; Tolmasky, 2010). O processo de inativação de fármacos pelas das β-lactamases também é bem conhecido. Nesse caso, as β-lactamases atuam destruindo a ligação amida do anel β-lactâmico presente em antibióticos como penicilina, ampicilina, amoxicilina, imipenem, piperacilina e ceftazidima (Tooke et al., 2019). Essas enzimas são capazes de alterar os fármacos utilizados, diminuindo sua afinidade pelo seu alvo.

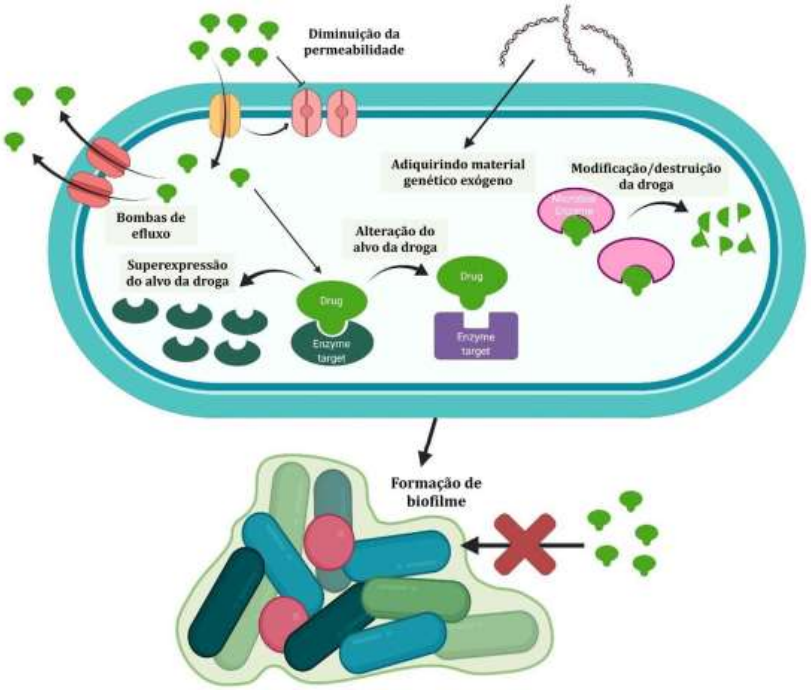


Figura 9: Representação do mecanismo de resistência antimicrobiana (Dissertação de Lima, 2021).

Ainda sobre os mecanismos de resistência, temos a alteração do alvo do fármaco, o mecanismo mais comum de resistência de fungos patogênicos (Fisher et al., 2018). Esse tipo de alteração ocorre devido à inserção de mutações pontuais nos genes que codificam o sítio alvo, modificações enzimáticas do sítio de ligação ou desvio do alvo original, o que causa a diminuição da afinidade pelo fármaco pelo alvo (Lambert, 2005).

Um bom exemplo é a lanosterol desmetilase, codificada pelo gene ERG11 em *C. albicans*, e a β-1,3 glucano sintase, codificada pelo gene FKS em diferentes espécies de fungos. Tais enzimas são os alvos para a ligação dos fármacos antifúngicos azóis e equinocandinas, respectivamente, onde qualquer alteração como a mutação, torna a cepa resistente (Sagatova et al., 2016). Ainda, o alvo do fármaco também pode ser superexpresso, resultando na resistência quando se define uma concentração efetiva mais alta a ser alcançada. (Warburton; Amodeo; Roberts, 2016).

O nível de concentração do fármaco na célula microbiana é outra estratégia desenvolvida para a resistência antimicrobiana. Ela acontece por mecanismos que envolvem as bombas de efluxo, onde as proteínas de membrana expulsam o fármaco de dentro da célula em alta velocidade, não permitindo assim que elas cheguem a uma concentração suficiente para exercerem seu efeito antimicrobiano. Essas bombas não são específicas para uma família de

antibióticos, elas conseguem expulsar uma grande variedade de fármacos, entre eles os inibidores da síntese de proteínas, fluoroquinolonas, β-lactâmicos, carbapenêmicos, polimixinas e azóis (Blanco et al., 2016). Em *Acinetobacter baumannii* é a bomba AdeABC que confere resistência a várias classes de antibióticos como os aminoglicosídeos (ABDI et al., 2020). Já em *C. albicans*, a bomba ABC Cdr1 é a principal causa da resistência aos azóis (Holmes et al., 2008).

Existe a resistência adquirida aos antimicrobianos que pode ser obtida através de mutações, afetando a expressão ou a função das porinas por perda, modificação de seu tamanho, de sua condutância ou redução da sua expressão (Christaki; Marcou; Tofarides, 2020). Temos como exemplo as mutações na proteína F da porina da membrana, que conseguem resultar no desenvolvimento de resistência em *Escherichia coli* (Chang et al., 2018).

A capacidade de formação de biofilme também está entre as estratégias de mecanismo de resistência antimicrobiana. O biofilme é um tipo de organização microbiana multicelular, que é fixada a uma superfície e formada pelo agrupamento de proteínas, polissacarídeos e DNA em uma matriz polimérica extracelular (Singh et al., 2017). A resistência ocorre devido à proteção mecânica desta matriz, que evita a difusão dos antimicrobianos ou ligação direta a alguns antibióticos. Assim, os micro-organismos que produzem essa matriz podem também secretar enzimas capazes de degradar compostos tóxicos, como os fármacos. Alguns exemplos de micro-organismos que possuem a capacidade de produção de biofilme como mecanismo de resistência aos antimicrobianos são *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus mutans*, *Aspergillus fumigatus* e *Candida* spp. (Hall; Mah, 2017; Silva et al., 2017).

1.5. Antifúngico anfotericina B

Em 1956, um antibiótico macrolídeo, chamado de anfotericina B e produzido por *Streptomyces nodosus*, foi isolado por Gold e colaboradores. A composição deste antibiótico é caracterizada pela presença de um anel de átomos com uma região hidrofílica, contendo algumas hidroxilas, e outra lipofílica contendo quatro ligações duplas conjugadas (Dollery, 1991).

A anfotercina B pertence à classe dos polienos, é insoluvel em água, fotossensível, termosensivel e apresenta atividade antifúngica em pH 6 a 7,5. Atualmente é o primeiro fármaco de escolha para infecções sistêmicas mais graves (Perea, 2021). Mediante a dose utilizada, o efeito deste polieno pode ser fungicida ou fungistático, atuando na permeabilidade da membrana plasmática. Sua atividade primeiro depende da ligação ao ergosterol da membrana.

No efeito fungistático da anfotericina B, ocorre a perda de íons intracelulares. Nesse caso, o efeito pode ser reversível quando se retira todo fármaco em contato com o microrganismo. Já no efeito fungicida da anfotericina B, ocorre a formação de poros na membrana e consequentemente perda de alguns constituintes moleculares importantes e danos oxidativos, o que resulta em uma condição irreversível levando à morte do micro-organismo (Brajtburg et al., 1990; Inmaculada Quiles-Melero, Julio García-Rodríguez; 2021).

Desta forma, a anfotericina B é uma molécula anfipática (Figura 10), que possui uma região hidrofóbica, tornando-a insolúvel em meios aquosos. Esta característica de solubilidade aquosa é adquirida através da formulação com o deoxicolato de sódio ou uma variedade de carreadores lipídicos (Mesa-Arango et al., 2012).

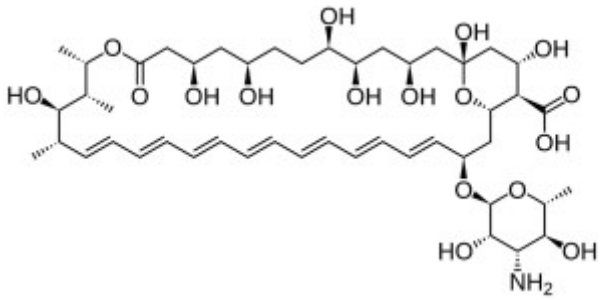


Figura 10: Representação da estrutura molecular da anfotericina B.

O mecanismo de ação dos polienos envolve ligação com um componente esteróide essencial de membrana fúngica chamado ergosterol. A estrutura poliênica da molécula consegue formar alguns complexos com o ergosterol de membrana, ocasionando a formação de poros na membrana, resultando na perda de sua integridade e rápido extravasamento de íons, levando à morte celular (Figura 11) (Lemke et al., 2005).

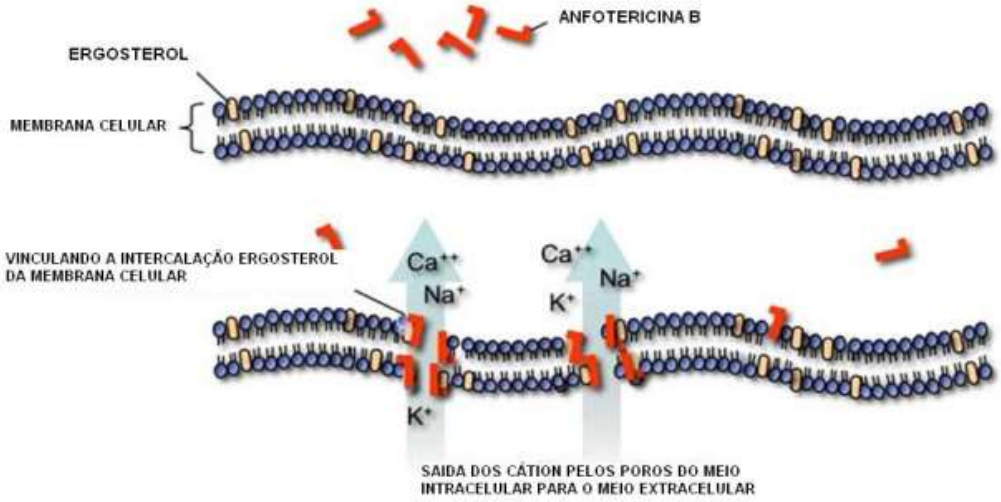


Figura 11: Representação esquemática do mecanismo de entrada da anfotericina B na membrana do fungo e formação dos poros (Anete, 2011).

A utilização desse fármaco causa efeitos colaterais adversos. O efeito mais indesejável e grave é a toxidade renal, em que a anfotericina B causa um grau de redução da função renal em mais de 80% dos pacientes que recebem o medicamento. Dessa forma, várias formulações lipídicas de anfotericina B têm sido desenvolvidas com o objetivo de reduzir sua toxicidade, entre elas podemos citar a anfotericina B convencional (C-AMB), a anfotericina B lipossomal (L-AMB), o complexo lipídico de anfotericina B (ABLC) e a dispersão coloidal de anfotericina B (Wall e Lopez-Ribot, 2020).

Existe um efeito fungicida da anfotericina B em espécies de *Candida*, porém com maior toxicidade quando comparada ao fluconazol, que se encontra nas prateleiras para venda direta (Marra e Camargo,2002).

1.6. Terapia antifúngica combinada

Os estudos envolvendo a terapia antifúngica combinada entre moléculas vêm ao longo dos anos sendo elucidado, uma vez que a utilização de substâncias antifúngicas associadas tem início com testes *in vitro* onde é possível observar se as interações moleculares agem de forma positiva no que diz respeito à inibição de microrganismos (Castro et al., 2010).

Combinando dois agentes antimicrobianos diferentes pode-se ampliar o espectro de

atividade através de diferentes mecanismos de ação que cada molécula exerce, principalmente quando se trata do início do tratamento, pois a carga elevada de microrganismo é capaz de induzir à seleção de organismos resistentes, criando um ambiente propício a sua proliferação exacerbada. Podemos citar o exemplo da anfotericina B, que é capaz de induzir uma permeabilidade da membrana celular fúngica, facilitando a penetração de outro antifúngico e com isso sua associação permite níveis intracelulares mais elevados deste fármaco (O'Kane et al.,2020).

A atividade sinérgica entre grupos de antifúngicos envolve uma série de mecanismos que justificam esta combinação, como por exemplo, a inibição de diferentes estágios nas vias bioquímicas intracelulares fúngicas em que são essenciais a sobrevivência celular (Andrade et al., 2019). A importância da interação sinérgica entre AMPs e outros agentes antimicrobianos é que a maioria dos AMPs é capaz de causar a formação de poros nas membranas microbianas, facilitando a entrada dos fármacos na célula alvo, permitindo assim que os mecanismos que levam à morte celular aconteçam (Li; Fernández-Millán; Boix, 2020).

Esta combinação pode ser eficaz para superar os mecanismos de resistência que envolvem a membrana celular fúngica, como a baixa penetrabilidade do fármaco, além de reduzir a dose efetiva minimizando o potencial de toxicidade, reduzindo os custos do tratamento (Sierra et al., 2017)). Assim, os AMPs podem ser empregados como adjuvantes, facilitando e complementando a ação dos agentes antimicrobianos convencionais (Li; Fernández-Millán; Boix, 2020; Lima et al., 2020).

Várias combinações sinérgicas antifúngicas têm sido relatadas. Podemos citar a combinação de azólicos com os poliênicos, onde a maioria desses trabalhos mostra uma interação *in vitro* diante de fungos como A*spergillus* spp, *Candida* spp., e C*ryptococcus neoformans*. O principal benefício oferecido pela combinação de antimicrobianos é a redução das doses dos medicamentos em sua forma individual, visando minimizar seus efeitos de toxicidade, bem como o surgimento de resistências fúngicas e assim, a redução da duração da terapia (Chaturvedi et al., 2011; Kibbler, 2012; Neto et al., 2022).

Há algumas metodologias que são utilizadas para mensurar os efeitos de combinação entre fármacos. Uma das formas mais conhecidas é o método de Checkerboard, no qual se baseia inicialmente nas concentrações inibitórias mínima (CIM) de forma isoladas e em combinação, sendo cada substância analisada, de maneira a avaliar a evolução da CIM individualmente e diante da presença de um segundo fármaco (De Paula et al., 2021).

No Checkerboard a interpretação dos resultados acontece através da determinação do Índice de Concentração Inibitória Fracionada- FICI (Figura 12). Para se estabelecer o critério

de efeito sinérgico, o resultado esperado entre as associações deve corresponder a um índice ≤ 0,5, assim como se uma associação entre duas ou mais substâncias resulta em uma FIC > 0,5 e ≤ 1 em relação ao resultado da CIM de cada substância separadamente, pode-se dizer que essa associação tem um efeito aditivo ou indiferente. Já nas combinações antagonistas, acontece quando o resultado da associação é capaz de aumentar o FIC em um valor ≥ 4 (Tabela 1) (Mulyaningsih et al., 2010).

CMI da droga A combinada/CMI da droga A + CMI da droga B combinada/CMI da droga B

Figura 12: Fórmula da Concentração Inibitória Fracionada- FIC. CMI-A e CMI-B: Concentração de diferentes compostos testados (Oliveira et al., 2014).

Tabela 1: Tipos de interações medicamentosas e índice de Concentração Inibitória Fracionada (FIC).

TIPOS DE INTERAÇÕES	FIC
Sinérgico	**≤ 0,5**
Aditivo e/ou Indiferente	**> 0,5 e ≤ 1**
Antagonista	**≥ 4**

Fonte: Produzida pelo próprio autor**.**

1.7. Leveduras do gênero *Candida* e *Saccharomyces cerevisiae*

As leveduras são consideradas um bom modelo de estudo por serem de fácil manipulação em laboratório, de crescimento rápido, por permitir que sequências de DNA exógeno possam interagir com seu genoma, isso tudo devido ao fato de uma grande variedade de processos intracelulares e serem conservados ao longo da evolução (Barr, 2003; Feldmann, 2005; Roque et al., 2020).

Leveduras do gênero *Candida*, principalmente a espécie *C. albicans*, são leveduras que se relacionam com o hospedeiro de forma comensal e podem ser isoladas a partir do trato gastrointestinal, mucosa oral e vaginal de muitos, se não, de todos os indivíduos saudáveis. No

entanto, podem causar infecções em indivíduos que estejam com o sistema imunológico comprometido (Millon et al.,1994; Morschhäuser, 2016; Paludo e Marin, 2018).

Várias espécies do gênero *Candida* podem causar alguma patogenicidade aos seres humanos. Podemos citar a candidíase, meningite, infecções no sangue, ou até infecções sistêmicas em pacientes imunocomprometidos, principalmente aqueles que são submetidos a quimioterapias no tratamento do câncer (Isola et al., 2009).

Uma forma mais agressiva de patogenicidade das leveduras do gênero *Candida* é a capacidade de formação de biofilmes, que se resume em uma massa fúngica que resulta da multiplicação e desenvolvimento de microrganismos, que ficam aderidos à superfície sólida, ou seja, presa em uma matriz (Stoodley et al., 2002). Essa estratégia de crescimento proporciona, aos microrganismos importantes benefícios, assim como: um aumento considerável da concentração de nutrientes nas interfaces líquido-biofilme, sabendo que a matriz polimérica favorece a absorção de moléculas de nutrientes; proteção contra condições ambientais ofensivos, como diferenças de pH, quantidades de sais, desidratação, presença de bactericidas, antibióticos, entre outros (Dorobantu et al., 2012).

O combate das infecções causadas por leveduras do gênero *Candida* é feito por antifúngicos principalmente das classes dos azóis, os quais são limitados devido à grande resistência que esses microrganismos adquirem com o uso continuo (Szweda et al., 2014; Araújo et al., 2020). A resistência primária de leveduras aos azóis, de forma especial ao Fluconazol, pode ser atribuída principalmente ao efluxo, no qual a perda do fármaco no interior celular acontece através de transporte ativo e é mediada através da glicoproteína P ou por outras proteínas (Cannon et al., 2009; Lima et al., 2020).

Ainda falando sobre levedura de importância médica, temos a *Saccharomyces cerevisiae*, que embora seja mais conhecida por suas funções na panificação e na fabricação de cerveja, é um micro-organismo encontrado em vários habitats e foi adaptada para uso em laboratório. A *S. cerevisiae* se tornou um poderoso sistema de modelo genético, pois deriva em parte de sua recombinação homóloga eficiente, o que permite que pesquisadores modifiquem seus genes com facilidade. (Karathia et al., 2011; Hanson, PK; 2018).

Por ser um organismo eucarioto, a levedura *S. cerevisiae* é usada amplamante como modelo para células de organismos mais complexos, como humanos. Esta levedura foi o primeiro eucarioto a ter seu genoma totalmente sequenciado (Goffeau et al., 1996), onde algumas análises da sequência mostraram que 31% de seus genes codificadores de proteínas tinham homólogos com o genoma humano (Botstein, Chervitz e Cherry, 1997). As características principais para que esta levedura seja um bom modelo, envolve o ciclo de vida

curto, facilidade de manipulação, genoma bem anotado, caixa de ferramentas molecular expansiva, conservação de vias biológicas e bioquímicas eucarióticas básicas, fazem da levedura um excelente organismo modelo para estudar processos celulares eucarióticos (Vanderwaeren et al., 2022).

Na área medica, a pesquisa de anticorpos anti-*Saccharomyces cerevisiae* (ASCA) é marcador importante para diagnóstico da doença chamada de Crohn. Um representante do gênero, o *Saccharomyces boulardii*, tem utilização terapêutica no tratamento do trato gastrointestinal. Em humanos, o gênero *Saccharomyces* pode estar presente como colonizante na mucosa gastrointestinal, respiratória e urinária em pacientes com doenças de base. Nos últimos anos, a incidência de infecções pelo gênero *Saccharomyces* vem aumentando, sendo a *Saccharomyces cerevisae* responsável pela maior incidência que pode chegar a 4% dos isolados de fungos em hemocultura e a associação com outros fungos (principalmente *Candida*) tem sido a causa de altas taxas de mortalidade (Richardson et al., 2008; Silva et al., 2011; Liesbeth Demuyser e Patrick Van Dijck, 2019; Vanderwaeren et al., 2022).

Após pandemia causada pelo coronavírus SARS-CoV-2, as infecções principalmente relacionadas a fungos tiveram um grande aumento, entre elas as doenças fúngicas relacionadas a uma infecção da corrente sanguínea por *Saccharomyces cerevisiae* (Ventoulis et al., 2020).

A combinação ou sinergismo de peptídeos sintéticos e antifúngicos convencionais, tem se tornado cada dia mais uma alternativa para produção de um fármaco que seja mais eficiente em baixas doses. No sinergismo entre peptídeos e antifúngicos convencionais, acontece a ação combinada, causando uma potencialização na diminuição no crescimento do microrganismo, em comparação com a inibição do crescimento usando as substâncias sozinhas (Taveira et al., 2016; Cools et al., 2017; Lima et al., 2020).

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a atividade antimicrobiana de peptídeos bioinspirados na defensina de planta *Pv*D_1 sobre o desenvolvimento de leveduras do gênero *Candida* e *Saccharomyces cerevisiae* e seu mecanismo de ação em combinação com o antifúngico convencional anfotericina B.

2.2. Objetivos específicos

A: Avaliar o efeito antifúngico dos peptídeos bioinspirados em leveduras;
B: Determinar as combinações sinérgicas entre os peptídeos bioinspirados e a anfotericina B;
C: Avaliar a atividade antifúngica dos peptídeos bioinspirados isolados e em combinação com anfotericina B sobre células de leveduras;
D: Estudar o efeito das combinações sinérgicas sobre a permeabilização de membranas, monitoramento de morte celular, integridade das membranas vacuolares, integridade da parede celular, degradação de DNA, funcionalidade mitocondrial dos microrganismos utilizados;
E: Analisar o efeito morfológico e ultraestrutural dos peptídeos bioinspirados isolados e em combinação com anfotericina B sobre leveduras através de microscopia eletrônica.

3. CAPÍTULOS

Capítulo1:

Synergistic action of synthetic peptides defensin-like and amphotericin B causes disruption of the plasma membrane and cell wall in *Candida albicans*

Published: Bioscience Reports (2024) 44 BSR20232075 https://doi.org/10.1042/BSR20232075

Capítulo 2:

Enhancing antifungal efficacy through synergistic interaction of synthetic peptide $\gamma_{31\text{-}45}$*Pv*D_1^{++} and amphotericin B against *Candida tropicalis* and *Saccharomyces cerevisiae*

To be submitted: *Bioconjugate Chemistry* - (https://pubs.acs.org/page/bcches/about.html)
2 Year Impact Factor 2023: 4.0

CAPÍTULO 1

Synergistic action of synthetic peptides defensin-like and amphotericin B causes disruption of the plasma membrane and cell wall in *Candida albicans*

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Research Article

Synergistic action of synthetic peptides and amphotericin B causes disruption of the plasma membrane and cell wall in *Candida albicans*

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The objective of this work was to evaluate the combination of synthetic peptides based on the γ-core motif of defensin PvD_1 with amphotericin B (AmB) at different concentrations against *Candida albicans*. We applied the checkerboard assay using different concentrations of the commercial drug AmB and the synthetic peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and $\gamma_{33\text{-}41}PvD_1^{++}$ against *C. albicans*, aiming to find combinations with synergistic interactions. Between these two interactions involving $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB, an additive effect was observed. One such interaction occurred at concentrations of 0.009 μM of peptide $\gamma_{31\text{-}45}PvD_1^{++}$ and 13.23 μM of AmB and another condition of 0.019 μM of peptide $\gamma_{31\text{-}45}PvD_1^{++}$ and 6.61 μM of AmB. The other two concentrations of the interaction showed a synergistic effect in the combination of synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB, where the concentrations were 1.40 μM peptide $\gamma_{31\text{-}45}PvD_1^{++}$ and 0.004 μM AmB and 0.70 μM $\gamma_{31\text{-}45}PvD_1^{++}$ peptide and 0.002 μM AmB. We proceeded with analysis of the mechanism of action involving synergistic effects. This examination unveiled a range of impactful outcomes, including the impairment of mitochondrial functionality, compromise of cell wall integrity, DNA degradation, and a consequential decline in cell viability. We also observed that both synergistic combinations were capable of causing damage to the plasma membrane and cell wall, causing leakage of intracellular components. This discovery demonstrates for the first time that the synergistic combinations found between the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB have an antifungal effect against *C. albicans*, acting on the integrity of the plasma membrane and cell wall.

Introduction

In recent decades, a drastic increase in systemic fungal infections has been observed, which has led researchers worldwide to seek the development of new effective drugs [1]. Commercial antifungals, like amphotericin B (AmB), is considered the gold standard due to its high efficacy in different types of infections, despite its severe side effects [2].

Polyenes belong to a category of antibiotics that are readily accessible on the market and are primarily employed in the treatment of fungal ailments. Notable members of this group include nystatin, natamycin, and AmB. Among these, AmB stands out as the most potent in terms of toxicity [3]. The ongoing evaluation of polyenes in clinical applications, particularly AmB, stems from certain drawbacks associated with their use as antifungal agents. The mechanism of their action entails binding to ergosterol, resulting in the creation of pores within the cell membrane of fungi. This disruption ultimately leads to oxidative damage

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and membrane rupture. Despite its status as a potent broad-spectrum fungicidal treatment characterized by a low occurrence of microbial resistance, the clinical utilization of AmB is still restricted [4,5].

AmB boasts an amphipathic character and is distinguished by its notable water solubility. Its conventional administration involves a formulation known as AmB deoxycholate (D-AMB), which uniquely forms micelles upon introduction into aqueous solutions. Nonetheless, concerns regarding potential nephrotoxicity and cardiotoxicity associated with D-AMB have ignited substantial interest within the scientific community. As a result, considerable efforts have been channeled into exploring synergistic combinations involving other compounds in this domain, as noted by Carolus and Cavassin [3,6]. Antimicrobial peptides (AMPs) naturally occur in nature and can also be engineered through rational synthesis. They exhibit broad-ranging antimicrobial activity and are particularly effective against many strains of fungi. Several research efforts have focused on investigating the synergistic combination of these peptides with commercial antifungals, with the aim of creating new therapeutic agents that not only increase efficacy but also decrease the occurrence of adverse effects [2,7,8].

The yeasts of the genus *Candida*, mainly *C. albicans*, are yeasts that interact with the host in the commensal form and can be isolated from the gastrointestinal tract and oral and vaginal mucosa of many, if not all, healthy individuals. As one of the most widespread fungi in the human microbiome, *C. albicans* is one of the main causes of fungal infections, with invasive candidiasis being the cause of the high mortality rate, which varies from 46 to 75%. The progression of *C. albicans* pathogenesis is driven by alterations in the morphology of yeast cells, transitioning into hyphae (or pseudohyphae), accompanied by an augmented generation of virulence factors during infection [9].

Drug interactions have been a path that researchers have pioneered in the search for new, more effective and less toxic drugs. The synergistic combination of caspofungin (CAS) and fluconazole was effective in decreasing *C. glabrata* resistance, and other interactions have been described [10–12]. Combinatorial therapy has emerged as a pivotal strategy in the quest for novel antifungal agents, aiming to discover fresh therapeutic modalities. Among these, AMPs present a significant alternative, highlighting their ability to yield bactericidal and antifungal effects, among other benefits, when employed in conjunction with other treatments [12–14].

AMPs have been known since the 1940s. However, in the early 1980s, their central role in the innate immunity of different organisms was revealed, such as insect cecropin, mammalian defensins and magainin in amphibians [15]. Its broad antimicrobial activity has increased interest in these molecules. In addition, AMPs have some characteristics that make them excellent therapeutic agents, such as broad antimicrobial activity, making this molecule active against different microorganisms. Its small size and high stability in insulting environments, and its ease of engineering, peptides by molecular biology or even chemical synthesis [16]. This last technique has the advantage of allowing the incorporation of uncommon amino acids, such as nonproteinogenic or D-enantiomers, into AMP primary structures, improving their activity, specificity and stability, decreasing their toxicity and protecting them from proteolytic degradation in the target organism [17].

Synthetic antimicrobial peptides thus emerge as an option for combination with already commercial drugs in the search to increase their activity and reduce side effects. The plant family of defensins are cationic peptides with a characteristic domain of eight cysteine residues forming four disulfide bonds. They are part of the important components produced in the plant defense system and are located on the periphery of different organs, such as seeds and fruits [18,19]. Synthetic peptides derived from the primary structure of plant defensins have been extensively studied, with particular emphasis on the γ-core motif region. This region is of significant interest due to its crucial role in the antimicrobial activity of AMPs [19,20]. The γ-core motif has emerged as an attractive site for targeted modifications in plant defensins, as certain defensins have demonstrated activity in this specific region. The γ-core motif in plant defensins is represented by the formula NH_2–[X_{1-3}] - [GXC] - [X_{3-9}] - [C]-COOH, with X denoting any amino acid [20].

In 2019 [21] Mello et al. designed four new peptides inspired by the γ-core region of *Phaseolus vulgaris* defensin (PvD_1). The peptides synthesized were $\gamma_{31\text{-}45}PvD_1$ (RSGRARDDFRAWATK), consisting of 15 amino acids from Arg31 to Lys45, which includes parts of the β2 and β3 sheets. The modified version, $\gamma_{31\text{-}45}PvD_1{}^{++}$, was created to enhance the positive charge by substituting two aspartic acid residues at positions 37 and 38 with arginine (RSGRARRRFRAWATK). $\gamma_{31\text{-}45}PvD_1$ and $\gamma_{31\text{-}45}PvD_1{}^{++}$ had net charges of +3 and +7 and molecular masses of 1792.99 and 1875.19 Da, respectively. Additionally, two other peptides were synthesized, each comprising 9 residues. The first one, $\gamma_{33\text{-}41}PvD_1$ (GRARDDFRA), corresponds to the native γ-core motif from Gly33 to Cys41 and exhibited a charge of +1 and a molecular mass of 1063.14 Da. The second one, $\gamma_{33\text{-}41}PvD_1{}^{++}$, is a modified version with increased positive charge achieved by replacing the two aspartic acid residues with arginine (GRARRRFRA), showing a charge of +5 and a molecular mass of 1145.34 Da. Remarkably, the peptide $\gamma_{33\text{-}41}PvD_1{}^{++}$ demonstrated the most toxic effects on the yeast *C. buinensis*, suggesting that this peptide triggers cell death via apoptosis.

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In ththe present study, our objective was to evaluate the combination of synthetic peptides based on the γ-core motif of defensin PvD_1 ($\gamma_{33\text{-}41}PvD_1^{++}$ and $\gamma_{31\text{-}45}PvD_1^{++}$) with AmB against *C. albicans* in the search for a synergistic concentration to contribute to the development of new drugs.

Materials and methods

Microorganisms

The yeast *Candida albicans* was obtained from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. Yeast was maintained on Sabouraud 2% glucose ágar (Merck) at the Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Synthetic peptides

This study employed two peptides designed by Mello et al. [21] The first peptide, $\gamma_{31\text{-}45}PvD_1^{++}$, consisted of 15 amino acid residues with a charge of +7 (RSGRARRRFRAWATK). The second peptide, $\gamma_{33\text{-}41}PvD_1^{++}$, was composed of 9 amino acid residues with a charge of +5 (GRARRRFRA). AminoTech (São Paulo, Brazil) performed the peptide synthesis and conducted quality and purity analyzes (≥ 95%) using reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. The synthetic peptides were solubilized in water and utilized in all *in vitro* assays.

Antifungal assay with synthetic peptides

For yeasts, aliquots were taken from plates containing grown colonies and placed in new Petri dishes containing Sabouraud agar (10 g/L peptone, 2 g/L glucose, 20 g/L agar) (Merck) striations on the middle. These new plates were kept in an oven at 30°C for 24 h. After growth, with a Drigalski loop, the cells were removed and homogenized in 10 ml of Sabouraud broth (10 g/L peptone, 2 g/L glucose) (Merck) for quantification in a Neubauer chamber (LaborOptik) with the aid of an optical microscope (Axiovison A2, Zeiss). Subsequently, the yeast cells (1×10^4 cell ml^{-1}) were incubated in Sabouraud broth containing preestablished concentrations of the synthetic peptides and AmB, which ranged from 1.56 to 200 μM. The assay was performed in cell culture microplates (96 wells) at 30°C for 24 h. Cell growth was determined by optical density, monitored at 24 h in a microplate reader at a wavelength of 620 nm. Each test will be performed in triplicate. The entire procedure will be performed under aseptic conditions in a laminar flow, according to the methodology adapted [22]. The MIC_{100} was determined as the minimum inhibitory concentration in μM required to completely inhibit fungal growth, with visual interpretation used for assessment. On the other hand, the MIC_{50} represents the peptide concentration in μM that resulted in 50% inhibition of fungal growth, and it was estimated using nonlinear regression analysis.

Checkerboard assay

For the evaluation of the interaction between the different concentrations of each compound, the fractional inhibitory concentration index (FIC) was calculated according to the following formula:

$$\text{A/MIC A} + \text{B/MIC B} = \text{FIC A} + \text{FIC B} = \text{FIC index}$$

where A and B are the MICs of each drug used individually. The FIC index value is then used to categorize the interaction of the two antifungals used. The combination is considered synergistic when the FIC index value is < 0.5, showing that the combination of the compounds increases the inhibitory activity of one of the compounds. When the FIC index value is between 0.5 and 4, the combination is considered indifferent or additive, meaning that the combination of compounds does not increase the inhibitory activity or slowly increases the activity due to the additive effect of both combinations. An FIC index > 4 categorizes antagonism, which occurs when the combination of compounds decreases the activity of the compounds. To evaluate the combinatorial effect of synthetic peptides and AmB against *C. albicans*, the serial microdilution technique for the checkerboard assay was performed according to the methodology described [23]. One hundred microliters of standardized *Candida* suspensions (1×10^4 cell ml^{-1}), which were further diluted one-fold, were added to each well, and plates were incubated at 30°C for 24 h. The final concentration ranged from 13.23 to 0.82 μM for peptides $\gamma_{33\text{-}41}PvD_1^{++}$, 11.25 to 0.70 μM for $\gamma_{31\text{-}45}PvD_1^{++}$, and 0.019 to 0.001 μM for AmB. Cell growth was determined by optical density in a microplate reader at a wavelength of 620 nm (EZ Read 400, Biochrom).

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Yeast viability assay

To check whether the inhibition of yeast growth was caused by fungicidal or fungistatic activity, the different groups of yeast cells (treatment and control) were washed and diluted 1,000-fold. Yeast cells were quantified in a Neubauer chamber and an aliquot with 1×10^2 cells ml^{-1} was prepared and spread with a Drigalski spatula on the surface of a Petri dish containing Sabouraud agar and grown at 30°C for 36 h. At the end of this period, the colony-forming units were counted, and the Petri dishes were photographed.[13] The experiments were carried out in triplicate, and the results are shown assuming that the control represents 100% viability.

Mitochondrial function assay

Mitochondrial functionality was evaluated by light fluorescence microscopy using the fluorescent probe MitoTracker Red FM (Thermo Fisher). After 24 h of incubation, cells from *C. albicans* were incubated with 200 nM MitoTracker Red FM for 30 min at 25°C. Then, the cells were observed under an optical microscope (Axioplan. A2, Zeiss) attached to an AxioCAM MRc5 camera (Zeiss), and the images were analyzed by Axiovision version 4.0 software (Zeiss) equipped with a 581 nm excitation filter and a 644 nm emission filter. A control with cells heated in the presence of 300 µM acetic acid for 15 min was performed. The experiment was repeated three times.

Detection of chromatin condensation in *Candida albicans*

Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI, SigmaAldrich) was performed as previously described [24]. The synergistic conditions and controls were transferred to a 96-well cell culture plate and incubated for 24 h. A higher cell density was used in this assay to allow microscopic visualization of cells. For nuclear staining, after incubation, the cells were washed with PBS, incubated with 4 µg/ml DAPI in PBS for 10 min, rinsed three times with PBS and then visualized under a DIC epifluorescence microscope (Axio Imager. A2, Zeiss) equipped with a fluorescence filter set (excitation 365 nm; emission 397 nm). The experiment was repeated three times.

Detection of wall integrity in *Candida albicans*

To assess the integrity of the cell wall, we employed the fluorescent probe Calcofluor White (SigmaAldrich) in our analysis. After subjecting the samples to synergistic conditions for 24 h, a 100 µl portion of the solution was extracted. Subsequently, this portion was mixed with 10 µl of Calcofluor White and incubated for 5 min at 30°C in the dark. Following the aforementioned duration, the cells were examined using a light microscope (Axioplan. A2, Zeiss) coupled with an AxioCAM MRc5 camera (Zeiss). The acquired images were subsequently processed using Axiovision version 4.0 software (Zeiss), which was equipped with a fluorescence filter set (excitation 365 nm; emission 397 nm). To ensure reliable results, the experiment was replicated three times.

Ultrastructure analysis

For analysis of the ultrastructure of *C. albicans* cells, the synergistic conditions of 1.40 µM peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and 0.004 µM AmB or 0.70 µM peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and 0.002 µM AmB were fixed for 1 h in a solution containing 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in sodium cacodylate buffer 0.1 mol L^{-1}, pH 7.4. After these procedures, the cells were washed twice with phosphate buffered saline (PBS) for 10 min and postfixed for 1 h in the dark with a solution containing 1% osmium tetroxide (OsO_4) and 1.6% ferrocyanide in 0.1 M sodium cacodylate buffer. Subsequently, the cells were washed in the same buffer, dehydrated in acetone (30%, 50%, 70%, 90%, 100% and 100% super dry) and embedded in Epon [25]. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Jeol JEM 1400 Plus transmission electron microscope.

Statistical analysis

Antimicrobial assays were performed in triplicate and repeated three times. Graphs were plotted as the means with standard deviation from an independent assay for antimicrobial assays. The data obtained in the tests were statistically tested by one-way ANOVA, where $p<0.05$ and $p<0.01$ were considered significant, using GraphPad Prism 8 software.

Results

Effect of synthetic peptides and amphotericin B on *Candida albicans*

Figure 1 shows the antifungal activity of the peptides $\gamma_{31\text{-}45}PvD_1^{++}$, $\gamma_{33\text{-}41}PvD_1^{++}$ and AmB. This figure shows the inhibition profile of the molecules against the growth of *C. albicans*. When performing the methodology to find the minimum inhibitory concentration (MIC) of the molecules used that inhibit 50% of cell growth, the results showed

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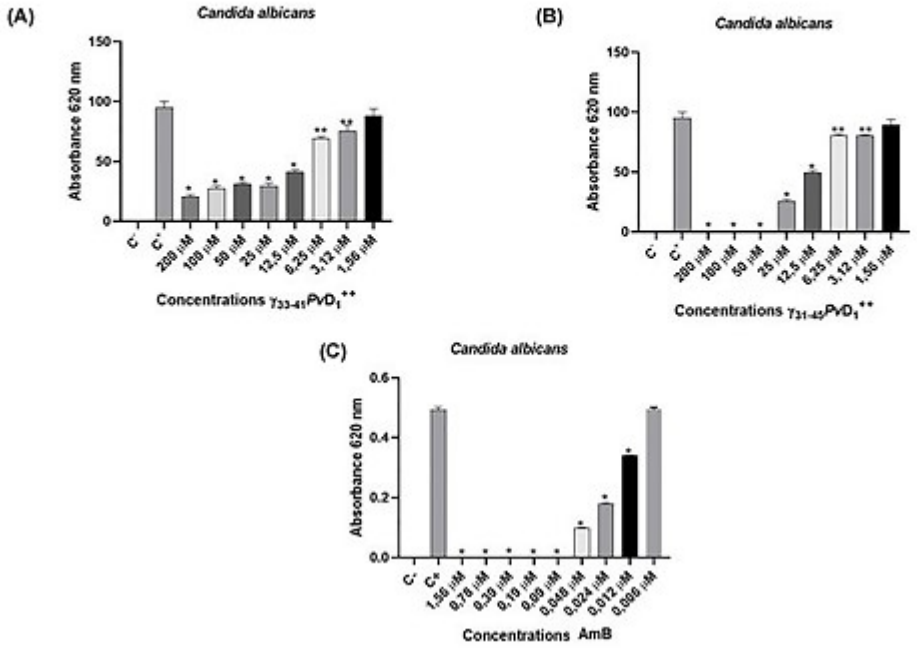

Figure 1. Effect of synthetic peptides and AmB on *C. albicans* cells

Growth of *C. albicans* incubated for 24 h in the presence of $\gamma_{33\text{-}41}PvD_1^{++}$ (A), $\gamma_{31\text{-}45}PvD_1^{++}$ (B) and AmB (C). All experiments were performed in triplicate; $^*p<0.05$; $^{**}p<0.01$.

Table 1 Minimum inhibitory concentration (μM) of synthetic peptides and AmB

Candida albicans			
AmB MIC_{100} (μM)	AmB MIC_{50} (μM)	$\gamma_{33\text{-}41}PvD_1^{++}$ MIC_{50} (μM)	$\gamma_{31\text{-}45}PvD_1^{++}$ MIC_{50} (μM)
0.09	0.019	13.23	11.25

Table 2 Combined activity between bioinspired peptides and AmB

DRUG A	DRUG B	FIC A (μM)	FIC B (μM)	Σ FIC	Action
AmB	$\gamma_{33\text{-}41}PvD_1^{++}$	0.009/0.019	13.23/13.23	1.47	Additive or Indifferent
	$\gamma_{33\text{-}41}PvD_1^{++}$	0.019/0.019	6.61/13.23	1.49	Additive or Indifferent
	$\gamma_{31\text{-}45}PvD_1^{++}$	0.004/0.019	1.40/11.25	0.33	Synergism
	$\gamma_{31\text{-}45}PvD_1^{++}$	0.002/0.019	0.70/11.25	0.16	Synergism

FIC values: ≤0.5 = Synergism; 0.5 < FIC < 1.0 = Additive or Indifferent; >1.0 = Antagonist.

that 11.25 μM $\gamma_{31\text{-}45}PvD_1^{++}$, 13.23 μM $\gamma_{33\text{-}41}PvD_1^{++}$ and 0.09 μM and 0.019 μM AmB were able to inhibit 50% of the cell growth of *C. albicans* yeast, while 0.9 μM AmB was able to inhibit 100% of the cell growth (Table 1).

Checkerboard assay

Table 2 shows that two combinations of the peptides $\gamma_{33\text{-}41}PvD_1^{++}$ and AmB were able to generate additive interactions, and two combinations of the peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB were able to generate synergistic interactions. The additive interaction was shown at 0.009 μM $\gamma_{33\text{-}41}PvD_1^{++}$ and 13.23 μM AmB and with 0.019 μM $\gamma_{33\text{-}41}PvD_1^{++}$

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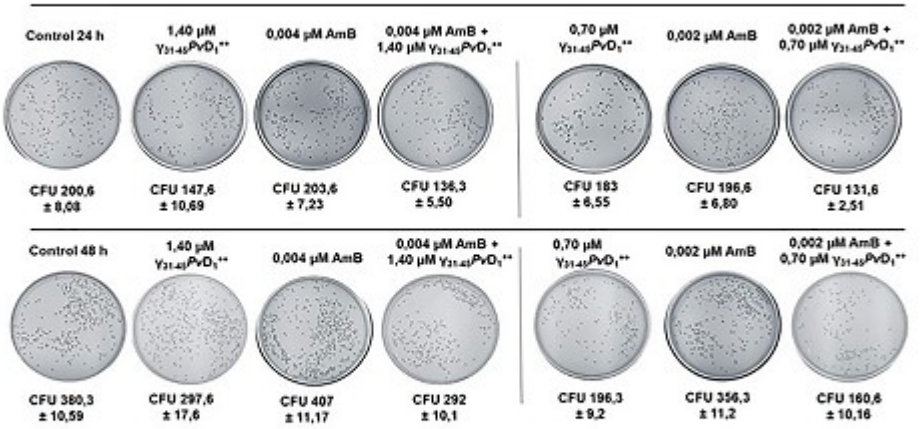

Figure 2. Growth of *C. albicans* cells incubated with peptides individually and in combination with AmB

Growth of *C. albicans* yeast cells incubated with $\gamma_{31-45}PvD_1^{++}$ individually and in combination with AmB for 24 and 48 h. All experiments were performed in triplicate.

and 6.61 μM AmB. The concentrations with synergistic interactions were 1.40 μM $\gamma_{31-45}PvD_1^{++}$ and 0.004 μM AmB and 0.70 μM $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB. From these data, we continue with the synergistic conditions in the action mechanism steps. The synergistic combinations identified in these studies demonstrate a difference when compared with the IC_{50} values of AmB and $\gamma_{31-45}PvD_1^{++}$. Specifically, the combination of 1.40 μM $\gamma_{31-45}PvD_1^{++}$ with 0.004 μM AmB concentrations was 4.5 and 8 times lower, respectively, than their individual IC_{50} values. For the combination of 0.70 μM $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB, the IC_{50} of AmB was 9.5-fold lower, and compared with the IC_{50} of the peptide $\gamma_{31-45}PvD_1^{++}$, it was 16-fold lower.

Viability assay

Figure 2 shows the cell viability assay. The synergistic combinations used individually were not able to cause a loss of viability when compared with the control. At 24 h of incubation, we observed the highest decline in viability in the combination of 0.70 μM peptide $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB, resulting in 65.60% viability. Additionally, the combination of 1.40 μM $\gamma_{31-45}PvD_1^{++}$ and 0.004 μM AmB peptides showed 67.9% viability (Table 3). After 48 h of testing, it was observed that *C. albicans* cells also exhibited susceptibility to the combination of 0.70 μM $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB peptides, resulting in an approximate viability of 42% (Table 3). These findings suggest that the inhibitory effect of the synergistic combinations is fungistatic.

Analysis of mitochondrial functionality

The results demonstrate that the combination of synthetic peptide $\gamma_{31-45}PvD_1^{++}$ and AmB at synergistic concentrations leads to a loss of mitochondrial functionality in *C. albicans* cells (Figure 3). The synergistic concentrations of 1.40 μM $\gamma_{31-45}PvD_1^{++}$ and 0.004 μM AmB, as well as 0.70 μM $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB, induced a notable loss of mitochondrial functionality. Notably, when the peptides or AmB were used individually, at the same concentrations as the synergistic combination, the mitochondria maintained their functionality, similar to the controls.

Chromatin condensation in *Candida albicans*

As observed in Figure 4, the synergistic combinations of 1.40 μM $\gamma_{31-45}PvD_1^{++}$ and 0.004 μM AmB, as well as 0.70 μM $\gamma_{31-45}PvD_1^{++}$ and 0.002 μM AmB, caused damage to the DNA of *C. albicans*. In control cells, DAPI (4′,6-diamidino-2-phenylindole) labeling exhibits intense fluorescence, indicating the intactness of cellular DNA. This characteristic is also evident in cells treated with individual concentrations of the synthetic peptide $\gamma_{31-45}PvD_1^{++}$ and AmB.

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Table 3 Viability of the *Candida albicans* cell culture after 24 h and 48 h of treatment with the synergistic conditions

Incubation time	Samples	CFU	Viability (%)	Loss of viability (%)
24h	Control	200.6	100	0
	1.40 μM $\gamma_{31\text{-}45}PvD_1^{++}$	147.6	73.57	26.43
	0.004 μM AmB	203.6	101,4	0
	0.004 μM AmB + 1.40 μM $\gamma_{31\text{-}45}PvD_1^{++}$	136.3	67.9	32.10
	0.70 μM $\gamma_{31\text{-}45}PvD_1^{++}$	183	91.22	8.78
	0.002 μM AmB	196.6	98	2
	0.002 μM AmB + 0.70 μM $\gamma_{31\text{-}45}PvD_1^{++}$	131.6	65.60	34.40
48h	Control	380.3	100	0
	1.40 μM $\gamma_{31\text{-}45}PvD_1^{++}$	297.6	78.2	21.80
	0.004 μM AmB	407	107.10	0
	0.004 μM AmB + 1.40 μM $\gamma_{31\text{-}45}PvD_1^{++}$	292	76	24
	0.70 μM $\gamma_{31\text{-}45}PvD_1^{++}$	196.3	51.56	48.39
	0.002 μM AmB	356.3	93	7
	0.002 μM AmB + 0.70 μM $\gamma_{31\text{-}45}PvD_1^{++}$	160.6	42.2	57.80

The CFU obtained in the control was assumed to be 100% of viability.

Detection of wall integrity in *Candida albicans*

We performed an analysis of the cell wall integrity of *C. albicans* following a 24 h assay in the presence of synergistic combinations of the synthetic peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB. In the control and individual concentrations of the peptide or AmB, we observed cells that were prominently stained with calcofluor White, indicating the integrity of their cell walls. The resulting blue fluorescence indicates the presence of an intact cell wall, as clearly depicted in Figure 5. However, after treatment with synergistic combinations, we no longer observed these intense cell markings but rather fragmented ones, suggesting the loss of cell wall integrity and possibly compromising cell viability.

Ultrastructural alterations in *Candida albicans*

The ultrastructural features of *C. albicans* cells, encompassing all control treatments, are depicted in Figure 6. The most notable differences are observed within the cellular components of the cell wall and plasma membrane. The control cells presented a uniform surface without apparent rupture. However, under synergistic conditions, it is evident that the treated *C. albicans* cells show severe compromise of their plasma membrane and cell wall integrity. Additionally, in some cases, cell leakage is observed, and chromatin condensation is confirmed by TEM.

Discussion

The escalating antimicrobial resistance to conventional drugs underscores the pressing demand for innovative and efficient alternatives. These alternatives should offer swift and comprehensive antifungal and antimicrobial action while minimizing potential safety-related impacts on infections [26].

In this work, we analyze the combination of synthetic peptides based on the region motif γ-core of a *V. unguiculata* seed defensin (PvD_1) and the commercially used drug AmB in developing *C. albicans*. The combination of synthetic peptides and conventional antifungals has increasingly become an alternative for producing a more efficient drug that is more efficient in low doses. In the synergism between these molecules, the combined action of the synthetic peptide with the same occurs, causing a potentiation in the decrease in the growth of the microorganism in comparison with the inhibition of the growth of the individual substances [16,18].

The γ-core region is a structural element shared by most host antimicrobial peptides (AMPs), which in some AMP families, such as defensins, contribute to their antimicrobial properties. This motif has been extensively studied in the search for new anti-infective agents [19].

We started by determining the minimum inhibitory concentration (MIC_{50}) of synthetic peptides and AmB against *C. albicans*. The MIC_{50} values for $\gamma_{33\text{-}41}PvD_1^{++}$, $\gamma_{31\text{-}45}PvD_1^{++}$, and AmB were 13.23, 11.25, and 0.019 μM, respectively (Table 1 and Figure 1). Fungal cells employ various defense mechanisms to counteract the toxicity of AmB. The

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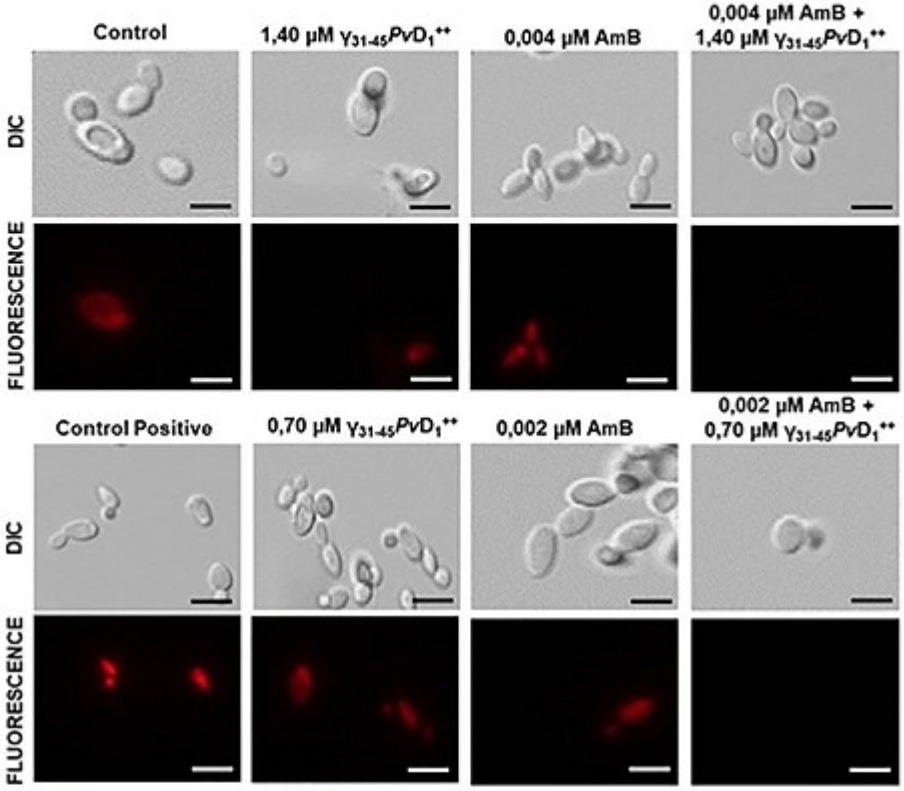

Figure 3. Mitochondrial functionality assay of *C. albicans* cells

Images of the mitochondrial functionality assay of *C. albicans* cells after treatment with $\gamma_{31\text{-}45}PvD_1^{++}$ individually and in combination with AmB for 24 h. The fluorescent probe MitoTracker was used to visualize mitochondrial activity. Positive control cells were treated with 300 mM acetic acid; bars = 20 μm.

cell wall immobilizes AmB molecules, and extracellular structures are binding sites for these molecules. Recent studies have shown the specific biological activity of AmB in *C. albicans* cells, particularly its affinity for binding to the cell membrane of young cells during the budding stage. This interaction significantly affects the membrane's structural properties and the transport of physiological ions and penetration into the cytoplasm. Consequently, various intracellular organelles and physiological processes are affected as a result [27].

Some mechanisms of action of antimicrobial peptides from plants against yeast have been elucidated, especially in the case of defensins, such as plasma membrane permeabilization, mitochondrial damage and DNA damage, impairing the cell cycle [28]. Therefore, we analyzed whether some of these events were capable of affecting the yeast *C. albicans* when treated with the synergism between AmB and the synthetic peptide.

To assess synergistic combinations, a checkerboard assay was employed. This assay evaluates the impact of a test substance in combination with an antimicrobial agent at various concentrations. Our results demonstrate two combinations with additive effects and two with synergistic effects (Table 2).

In 2023 [29] the lead compounds LP-23, DP-23, SA4, and SPO, synthesized via solid-phase peptide synthesis, exhibited synergistic effects when combined with specific antibiotics. This synergy was observed against a spectrum of bacterial and fungal strains, underscoring the potential of these compounds in enhancing antimicrobial efficacy. The

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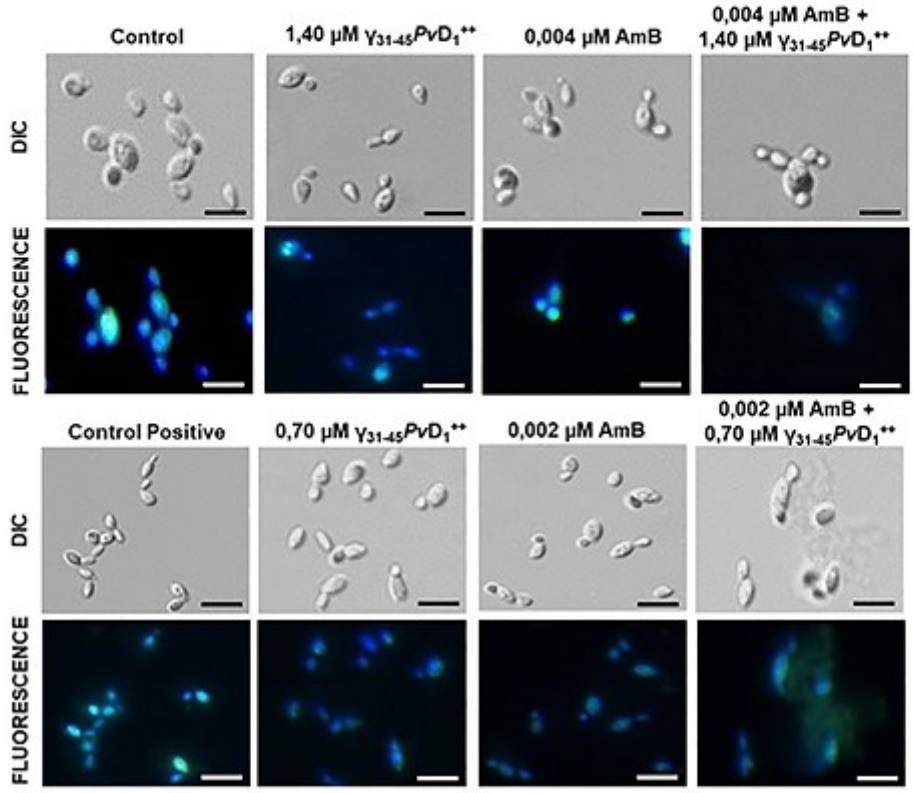

Figure 4. Chromatin condesation assay of *C. albicans* cells

Chromatin condensation assay images of *C. albicans* cells after treatment with $\gamma_{31\text{-}45}PvD_1^{++}$ individually and in combination with AmB for 24 h. To visualize the nuclei, DAPI was used. As a positive control, cells were treated at 100°C for 1 min; bars = 20 µm.

assessment of peptide-antibiotic combinations involved the utilization of the checkerboard method. The minimum inhibitory concentration (MIC) in these combinations was expressed using the fractional inhibitory concentration index (FIC). Virtually all pairings of peptides or with AmB and fluconazole exhibited potent synergy against *Aspergillus niger* and *Aspergillus flavus*. For instance, the FIC index of combinations such as LP-23 + AmB, DP-23 + AmB, SA4 + AmB, and SPO + AmB were 0.141 and 0.275, 0.290 and 0.267, 0.321 and 0.251, and 0.385 and 0.186, respectively. These findings underscore the potential of these combinations in combating these fungal strains effectively. In a recent review conducted by [14], the authors expound upon a series of synergistic peptide combinations with AmB, exhibiting efficacy against *Candida* spp. noteworthy examples encompass the peptide combination LL-37 + AmB, revealing FIC values ranging between 0.13 and 0.31 against *Candida auris*. Another finding entails the peptide combination Dq-3162 + AmB, showcasing an FIC value of 0.3125 for *C. albicans*, *C. tropicalis*, and *C. krusei*. Similarly, the interaction involving peptide Dq-2562 + AmB exhibited an FIC value of 0.3125 against the same strains: *C. albicans*, *C. tropicalis*, and *C. krusei*. These observations underscore the potential of these combined approaches in tackling these specific *Candida* strains effectively. In our study, we discovered FIC values that closely paralleled those reported in the aforementioned review. Specifically, our investigation revealed synergistic peptide and AmB combinations that yielded FIC values of 0.16 and 0.33 against *C. albicans*. In 2020 [2], made a significant discovery, identifying a synergistic effect between AmB and several drugs, including erythromycin, riluzole, nortriptyline,

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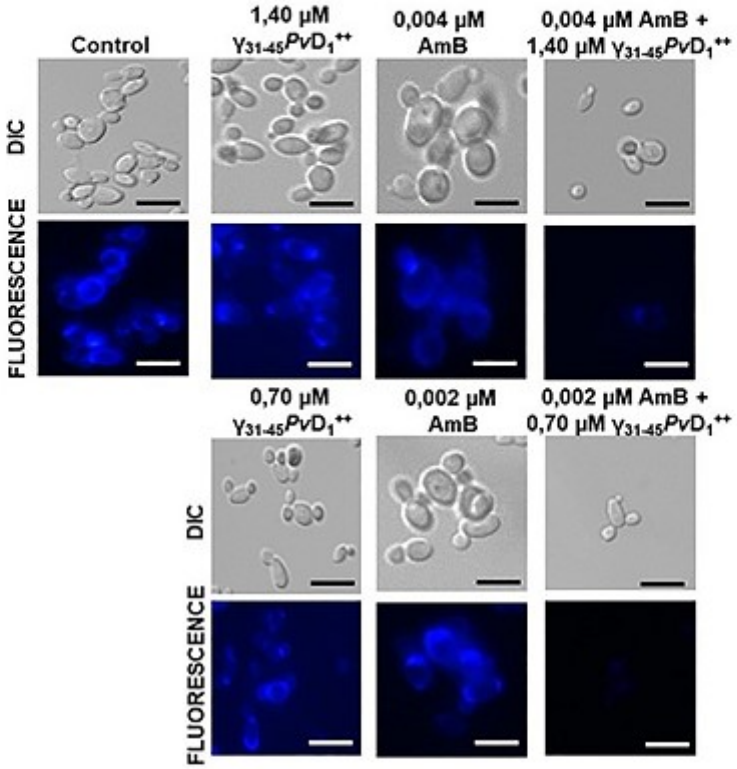

Figure 5. Cell wall integrity assay of *C. albicans* cells

Image detection of the wall integrity of *C. albicans* cells after treatment with $\gamma_{31\text{-}45}PvD_1^{++}$ individually and in combination with AmB for 24 h. The wall integrity of *C. albicans* cells was assessed using a calcofluor white probe; bars = 20 μm.

kenodiol, nisoldipine, promazine, chlorcyclizine, cloperastine, and glimepiride. Notably, all interactions with AmB demonstrated a synergistic effect, as evidenced by a low inhibitory concentration (FIC index of 0.5). This synergistic action was particularly observed in the inhibition of *C. albicans* spp. and *Cryptococcus neoformans*, highlighting the potential of this drug combination for therapeutic applications against these pathogens.

Among the mechanisms of action already described for peptides with antimicrobial activity, the interaction with the target membrane is one of the most studied. This interaction can result in pore formation or a direct interaction with a lipid domain, causing membrane permeabilization. Therefore, this mechanism of action makes antimicrobial peptides good models for understanding the mechanisms that lead to microorganism death. MitoTracker Red, a nontoxic fluorescent chemical probe containing a thiol chloromethyl-reactive fraction, is employed to visualize the distribution of mitochondria in living cells over an extended period. Additionally, it enables the quantification of mitochondrial potential through fluorescence microscopy analysis, as described by [30]. We analyzed whether synergistic combinations were capable of causing a loss of mitochondrial functionality in *C. albicans* cells, where the combinations were capable of causing disturbances in mitochondrial functionality within 24 h of the test (Figure 3).

Analyzing mitochondrial functionality serves as an effective method for investigating the mechanism of action of therapeutic molecules, including synthetic peptides. In a recent study [31], this approach was employed to examine the impact of a synthetic peptide derived from a plant defensin. The study revealed that the peptides RR (27.5 μM) and D-RR (23 μM) induced a rapid loss of mitochondrial functionality within just 1 and 6 h of incubation, respectively,

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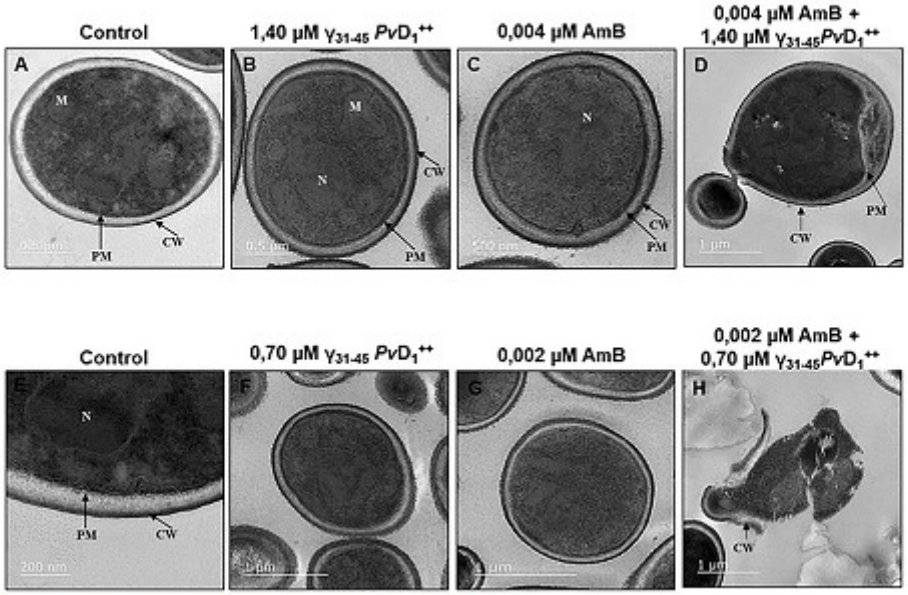

Figure 6. Transmission electron microscopy assay of *C. albicans* cells

Ultrastructural changes visualized by transmission electron microscopy in *C. albicans* yeast cells after treatment with $\gamma_{31-45}PvD_1^{++}$ individually and in combination with AmB for 24 h. **(A)** negative control; **(B-C)** Individual $\gamma_{31-45}PvD_1^{++}$ and individual AmB respectively; **(F-G)** Individual $\gamma_{31-45}PvD_1^{++}$ and individual AmB respectively. In the synergistic combination between the peptide and AmB **(D, H)**, the plasma membrane and cell wall are ruptured, which indicates cell permeabilization.

in *C. tropicalis* cells. Similarly, in the case of *C. albicans*, the synthetic peptide WR demonstrated the ability to cause a comparable effect within 1 h of incubation.

Initially, it was believed that the mechanism of action responsible for the inhibition or killing of microbial growth by antimicrobial peptides involved damage to the microbial cell membrane. In fact, it is known that some antimicrobial peptides are able to interact with components of the microbial membrane, such as phospholipids and sphingolipids, and are involved in some cellular signaling cascades that involve mitochondrial functioning and the integrity of nucleic acids [32].

In addition to the observed loss of mitochondrial functionality, our study also explored the impact of synergistic combinations on other organelles and the cell wall. Particularly noteworthy was the detection of concentrated and intense nuclear fluorescence in both the control group and individual concentrations of the synergistic combinations. This finding strongly indicates the preservation of nuclear integrity, as illustrated in Figure 4. In contrast, the absence of these nuclear markings in the case of the synergistic combinations strongly suggests significant chromatin condensation or the potential leakage of DNA along with intracellular materials. These observations indicate a cell wall and plasma membrane disruption, leading to compromised cellular integrity. To assess nuclear morphology and DNA integrity in cells, we utilized DAPI. This cell-nonpermeable DNA-binding substance selectively interacts with the minor groove of double-stranded DNA, resulting in fluorescence emission. By specifically binding to DNA, DAPI facilitates the visualization and evaluation of cellular processes involving DNA, such as chromatin condensation and potential leakage of genetic material caused by cell wall and plasma membrane disruptions. The utilization of DAPI as a tool in our study proved valuable in understanding the underlying cellular changes induced by the synergistic combinations [22]

The cell wall is a cell structure found in various organisms, including fungi, plants, and bacteria, regardless of whether it is eukaryotic or prokaryotic. It serves multiple critical functions, contributing to the overall health and

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viability of the cell. Its primary role is maintaining cell integrity and shape, providing structural support, and preventing cell collapse. Additionally, the cell wall acts as a protective barrier for the plasma membrane, shielding the cell from external stresses and potential damage [33]. In the present study, we successfully demonstrated that both synergistic combinations caused significant damage to the cell wall of *C. albicans* cells (Figure 5). The control cells and the individual components of the synergistic combinations displayed intact cell walls with marked fluorescence. In the cells treated with the synergistic combinations, fragmented fluorescent labeling was evident, strongly suggesting a loss of integrity in the cell wall of *C. albicans*. These findings hold significant promise for developing novel antifungal therapies. Understanding how these synergistic combinations bring about such pronounced effects on the cell wall can offer valuable insights into the mechanisms of action and aid in designing more targeted and effective treatments against fungal infections.

The investigation of peptides with antimicrobial activity targeting the cell wall of microorganisms has captivated the attention of numerous scientists seeking innovative therapeutic alternatives. In recent years, the rise of antibiotic-resistant pathogens has posed significant challenges in treating infectious diseases. Therefore, exploring alternative antimicrobial agents, such as peptides, has become a priority in biomedical research [34].

In 2023, Reis [35] made a significant discovery by identifying a potent host defense mimetic peptide called brilacidin (BRI). Their findings revealed that when used in combination with CAS, BRI exhibited remarkable synergistic effects against both CAS-sensitive and CAS-resistant isolates of various pathogenic fungi, including *Aspergillus fumigatus*, *C. albicans*, *C. auris*, and *Cryptococcus neoformans*, the latter being intrinsically resistant to CAS. The mode of action of BRI involves two critical mechanisms, enabling its impressive antifungal activity. First, BRI effectively affects the cell wall integrity pathway of fungal pathogens, destabilizing their cell walls. This disruption severely compromised the structural integrity of the fungal cells, making them more susceptible to further treatment by antifungal agents such as CAS.

The present study observed the impact of synergistic combinations on the morphology and ultrastructure of *C. albicans* (Figure 6). The TEM images reveal a notable difference in the structure of the plasma membrane and cell wall, thus supporting the data presented thus far in the present study. The plasma membrane and cell wall appear intact in the control cells and the individual concentrations of the synergistic combination. However, in the case of the synergistic combinations, a distinct alteration in the plasma membrane and cell wall morphology becomes evident, underscoring the disruptive effects of the combination treatment on these vital cellular components. The synergistic combinations manifest profound effects on *C. albicans* cells, as evidenced by the detachment of the plasma membrane and cell wall, abnormal cell morphology, and disruption of cytoplasmic content and organelles. These observations strongly suggest that the anti-*Candida* activity of the synergistic combinations primarily relies on their impact on membrane permeability and the cell wall. This approach targets various cellular components, including mitochondria and the nucleus, which aligns with the well-established antifungal mode of action exhibited by polyenes such as nystatin and AmB. The observed deformities in *C. albicans* cells provide further support for the disruptive effects of the synergistic combinations, leading to the impairment of essential cellular processes and ultimately inhibiting *Candida* growth.

In 2020 [36], Seyedjavadi et al. focused on the mode of action of the synthetic peptide MCh-AMP1 against the growth of *C. albicans*. Their study revealed similar outcomes, illustrating the peptide's impact on the cellular mechanisms of *C. albicans*. The research demonstrated that MCh-AMP1 targeted the membrane integrity of *C. albicans*, leading to membrane disruption, altered cell morphology, and impaired organelle function. These findings further support the notion that antimicrobial peptides, such as MCh-AMP1 and our synergistic combinations, exert their antifungal effects through multiple pathways, including membrane destabilization and disruption of vital cellular components. In 2016, Yu et al. [33] highlights the fungicidal effect of the synthetic peptide MCh-AMP1 at 32 and 64 μg/ml concentrations. The peptide targeted disrupted membrane integrity, resulting in increased permeability. Additionally, the study revealed that MCh-AMP1 treatment led to the generation of reactive oxygen species (ROS), further contributing to antimicrobial action. These findings provide valuable insights into the mode of action of MCh-AMP1, underscoring its ability to effectively combat fungal growth by targeting critical cellular components such as the membrane and inducing oxidative stress through ROS production.

In the face of an escalating global menace posed by antibiotic resistance, antimicrobial peptides (AMPs) have emerged as a promising new class of drugs in the fight against diverse infectious diseases. The 2023 [37] Safronova et al. shed light on the compelling potential of AMPs as a viable alternative to traditional antibiotics. The heightened interest in AMPs is particularly noteworthy, driven by recent outbreaks of secondary infections during the COVID-19 pandemic, which have exacerbated the urgent need for effective agents against bacterial and fungal infections. This comprehensive review serves as a timely reminder of the critical role AMPs can play in combating infectious diseases, especially when conventional antibiotics struggle against drug-resistant pathogens. With the acute shortage

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of suitable therapeutic options, the exploration of AMPs represents a promising avenue in the search for innovative treatments.

Conclusion

This discovery reveals the antifungal effect of synergistic combinations of the synthetic peptide $\gamma_{31\text{-}65}PvD_1{}^{++}$ and AmB against the yeast *C. albicans*. This combination targets the plasma membrane and cell wall. In addition, this synergism is also capable of causing loss of cellular functionality and DNA degradation. Together, these data represent a promising new therapeutic option for the treatment of fungal diseases associated with this microorganism.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Thayna Souza: Conceptualization, Resources, Data curation, Methodology, Writing—original draft. **Erica Mello:** Conceptualization, Funding acquisition, Writing—original draft. **Gabriel B. Taveira:** Data curation, Formal analysis, Funding acquisition, Writing—original draft. **Felipe Moreira:** Methodology. **Sergio Henrique Seabra:** Methodology. **André O. Carvalho:** Formal analysis, Writing—review & editing. **Valdirene M. Gomes:** Conceptualization, Supervision, Funding acquisition, Writing—original draft, Project administration, Writing—review & editing.

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Abbreviations

AmB, amphotericin B; AMP, antimicrobial peptide; BRI, brilacidin; CAS, caspofungin; CFU, colony forming units; CW, cell wall; DIC, differential interference contrast; M, mitochondria; N, nucleus; PM, plasma membrane.

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CAPÍTULO 2

Potentiating antifungal activity: Synergistic effect of synthetic peptide $\gamma_{31\text{-}45}$*Pv*D_1^{++} and amphotericin B against *Candida tropicalis* and *Saccharomyces cerevisiae*

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Potentiating antifungal activity: Synergistic effect of synthetic peptide γ_{31-45}*Pv*D_1^{++} and amphotericin B against *Candida tropicalis* and *Saccharomyces cerevisiae*

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ABSTRACT

In recent years, following the COVID-19 pandemic, the emergence of antibiotic-resistant microorganisms has increased. This surge has prompted global research efforts into new biologically active molecules, particularly those focused on combination therapies involving antifungal agents. Synthetic antimicrobial peptides have emerged as promising candidates to address this demand. Therefore, the objective of this study was to assess the synergistic therapeutic effects of the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ combined with amphotericin B (AmB) on *Candida tropicalis* and *Saccharomyces cerevisiae*. This study aimed to identify a synergistic concentration that could facilitate advancements in the development of new drugs. Here, we demonstrate synergistic combinations of synthetic peptide and amphotericin B (AmB) that effectively induce yeast cell death in *C. tropicalis* and *S. cerevisiae*. The checkerboard test revealed synergistic concentrations of 0.048 µM for AmB and 0.39 µM for the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ for *C. tropicalis* and 0.097 µM for AmB and 0.78 µM for the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ for *S. cerevisiae*. The mechanism of action underlying the death of these yeasts when exposed to these synergistic combinations includes several processes: loss of cell wall integrity, impairment of mitochondrial functionality, DNA condensation, and damage to cell morphology and ultrastructure. The data presented in this study contribute significantly to the development of new therapies involving combinations of antifungal molecules.

KEYWORDS: Bioactive molecules, Combined therapeutics, Yeasts.

1. INTRODUCTION

In recent decades, despite the emergence of new antifungal medications, including broad-spectrum triazoles and echinocandins designed for prophylaxis, empirical therapy, and targeted treatment, the mortality rates associated with candidaemia and other invasive infections have remained unchanged (Tome et al., 2018). Historically, *Candida albicans* has been the predominant pathogen; however, the incidence of invasive infections caused by *C. tropicalis* has steadily increased. Vulnerable populations include transplant recipients, individuals with HIV/AIDS or cancer, patients receiving immunosuppressive therapy, those receiving total parenteral nutrition, and premature infants. Nonalbicans *Candida* infections now account for 56.5% of candidemia cases, with *C. glabrata* (33.3%), *C. tropicalis* (20.3%), *C. parapsilosis* (1.4%), and *C. kefyr* (1.4%) being among the most common *Candida* species (Brilhante et al., 2019).

The use of the same medication repeatedly for different therapies increases the risk of developing resistant microbial strains. Consequently, escalating doses become necessary, potentially resulting in unwanted side effects (Li et al., 2015). The utilization of combined therapies is considered an excellent strategy to counteract the emergence of resistant strains. The synergistic effects between therapeutic molecules enable dose reductions, offering significant advantages (Zhou et al., 2015).

The process of discovering new natural antimicrobial peptides (AMPs) is often time-consuming because of their low yield and high production costs. In addition, natural AMPs may present undesirable characteristics for therapeutic applications, such as instability due to degradation by host proteases, low selectivity, low water solubility, hemolytic activity, toxicity to the host, and salt sensitivity (Boto; Pérez de la Lastra; González, 2018). To overcome these limitations, synthetic antimicrobial peptides have emerged as promising alternatives. The synthesis of these peptides is based on a rational design that relates the structure to the activity of the molecule. Each amino acid is added sequentially, allowing for specific modifications and the analysis and optimization of the molecules (Haney; Mansour; Hancock, 2017).

The combination of the antifungal agent amphotericin B (AmB) is endorsed by the Infectious Diseases Society of America for treating *Candida* infections (Pappas et al., 2016) owing to the increased risk of resistance. Commonly studied combinations involve pairing a commercial antifungal with a specific inhibitor targeting a protein of interest, such as antifungals combined with Hsp90 inhibitors (Veri & Cowen, 2014) or protein kinase C inhibitors (LaFayette et al., 2010).

In recent years, the study of new synthetic peptides based on plant AMP molecules in the search for new drugs that are active against resistant fungi has increased. In 2011, Sagaram and collaborators demonstrated that the synthesis of peptides extending beyond the γ-core motif is capable of improving antifungal activity and that the synthetic peptide (GMA-4C) derived from the γ-core motif that contains the C-terminus of the MtDef4 defensin, which has cationic and hydrophobic amino acids in its structure, was important for its antifungal activity. In 2021, Toledo and collaborators explored the correlation between the biological activity and structure of plant defensins. In this work, three new peptides with modifications of charge, hydrophobicity (RR and WR) and chirality (D-RR) were designed and tested against pathogenic yeasts.

In 2019, Mello and collaborators designed four new peptides on the basis of the γ-core motif of *Phaseolus vulgaris* defensin (*Pv*D_1). On the basis of this γ-core motif sequence together with parts of the β2 and β3 sheets (from Arg31 to Lys45), a 15-residue peptide was designed whose net positive charge increased by replacing the two aspartic acid (Asp) residues at positions 37 and 38 with two arginine (Arg) residues, giving rise to the amino acid sequence RSGRARRRFRAWATK, called $\gamma_{31\text{--}45}$*Pv*D_1^{++}. More recently, our group published a discovery highlighting the synergistic effects between synthetic antimicrobial peptides and amphotericin B against the yeast *C. albicans*. This combination was capable of killing this microorganism with doses of amphotericin B 100 times lower than those used commercially (Souza et al., 2024).

The main objective of this study was to evaluate the synergistic therapeutic effect of the combination of the synthetic peptide $\gamma_{31\text{-}45}$*Pv*D_1^{++} with AmB on *C. tropicalis* and *Saccharomyces cerevisiae*, as a valuable model for the study, with the goal of identifying a viable synergistic concentration that contributes to the development of new drugs.

1. MATERIALS AND METHODS

2.1 Microorganisms

The yeasts *Candida tropicalis* and *Saccharomyces cerevisiae* were obtained from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. Yeast was maintained on Sabouraud 2% glucose agar (Merck) at the Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

2.2 Synthetic peptides

This study employed two peptides designed by Mello (2019). The peptide $\gamma_{31\text{-}45}PvD_1^{++}$ consists of 15 amino acid residues with a charge of +7 (RSGRARRRFRAWATK). AminoTech (São Paulo, Brazil) performed peptide synthesis and conducted quality and purity analyses (≥ 95%) via reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. The synthetic peptides were solubilized in water and utilized in all *in vitro* assays.

2.3 Antifungal assay with synthetic peptides

For yeasts, aliquots were taken from plates containing grown colonies and placed in new Petri dishes containing Sabouraud agar (10 g/L peptone, 2 g/L glucose, 20 g/L agar) (Merck) striations in the middle. These new plates were kept in an oven at 30 °C for 24 h. After growth, with a Drigalski loop, the cells were removed and homogenized in 10 mL of Sabouraud broth (10 g/L peptone, 2 g/L glucose) (Merck) for quantification in a Neubauer chamber. (Labor Optik) with the aid of an optical microscope (Axiovison A2, Zeiss). The yeast cells (1×10^4 cells. mL^{-1}) were incubated in Sabouraud broth containing preestablished concentrations of the synthetic peptides and amphotericin B, which ranged from 1.56 μM to 200 μM. The assay was performed in cell culture microplates (96 wells) at 30 °C for 24 h. Cell growth was determined by optical density, which was monitored at 24 h in a microplate reader at a wavelength of 620 nm. Each test will be performed in triplicate. The MIC_{100} was determined as the minimum inhibitory concentration in μM required to completely inhibit fungal growth, with visual interpretation used for assessment. On the other hand, the MIC_{50} represents the peptide concentration in μM that resulted in 50% inhibition of fungal growth, and it was estimated via

nonlinear regression analysis (Taveira et al., 2022).

2.4 Checkerboard assay

To evaluate the interaction between the different concentrations of each compound, the fractional inhibitory concentration index (FIC) was calculated according to the following formula:

$$A/MIC\ A + B/MIC\ B = FIC\ A + FIC\ B = FIC\ index$$

where A and B are the MICs of each drug used individually. The FIC index value is then used to categorize the interaction of the two antifungals used. The combination is considered synergistic when the FIC index value is < 0.5, showing that the combination of the compounds increases the inhibitory activity of one of the compounds. When the FIC index value is between 0.5 and 4, the combination is considered indifferent or additive, meaning that the combination of compounds does not increase the inhibitory activity or slowly increases the activity due to the additive effect of both combinations. An FIC index > 4 categorizes antagonism, which occurs when the combination of compounds decreases the activity of the compounds. To evaluate the combined effects of synthetic peptides and AmB against *C. albicans*, the serial microdilution technique for the checkerboard assay was performed according to the methodology described. One hundred microliters of standardized yeast suspensions (1×10^4 cell. mL^{-1}), which were further diluted one-fold, were added to each well, and the plates were incubated at 30 °C for 24 h (EZ Read 400, Biochrom) (Johnson et al., 2004).

2.5 Cell death monitoring assay and membrane permeabilization

Cell death was monitored via propidium iodide (PI) staining, and plasma membrane permeabilization was assessed via SYTOX Green uptake, following the methodologies described by Deere et al. (1998) and Thevissen et al. (1999), respectively. Yeast cells were incubated with the indicated treatments for 24 h. After incubation, a 100 µL aliquot of each yeast cell suspension was treated with 1 µg mL^{-1} PI and 0.2 µM SYTOX Green for 15 min at 30 °C. The cells were then analyzed via a DIC optical microscope (Axiovision 4, Zeiss) equipped with fluorescent filters: PI detection (excitation wavelength, 561 nm; emission wavelength, 630 nm) and fluorescein detection (excitation wavelength, 450–490 nm; emission wavelength, 500 nm).

2.6 Analysis of vacuolar membranes

Vacuolar mapping of *C. tropicalis* and *S. cerevisiae* yeast cells was performed via the FM4-64 probe. Yeast cells were subjected to their respective conditions for 24 h. A 100 µL aliquot of each yeast cell suspension was subsequently incubated with 5 µL of FM4-64 for 1 h at 30 °C (Vida and Emr, 1995). The cells were then analyzed via an AxioVision version 4.0 DIC optical microscope (Zeiss) equipped with a 581 nm excitation filter and a 644 nm emission filter.

2.7 Mitochondrial function assays

Mitochondrial functionality was evaluated by light fluorescence microscopy using the fluorescent probe MitoTracker Red FM (Thermo Fisher). After 24 h of incubation, the cells from *C. tropicalis* and *S. cerevisiae* were incubated with 200 nM MitoTracker Red FM for 30 min at 25 °C. The cells were subsequently observed under an optical microscope (Axioplan. A2, Zeiss) attached to an AxioCAM MRc5 camera (Zeiss), and the images were analyzed with AxioVision version 4.0 software (Zeiss) equipped with a 581 nm excitation filter and a 644 nm emission filter. A control with cells heated in the presence of 300 µM acetic acid for 15 min was used. The experiment was repeated three times.

The activity of mitochondrial dehydrogenase enzymes was investigated. Yeasts were incubated with 10 µL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) (Sigma–Aldrich Co.) and 0.24 mM duraquinone (Sigma–Aldrich Co.) under aseptic conditions. Assays were performed in triplicate. The absorbance of the formazan crystals in the mixture was measured at 450 nm via a microplate reader (EZ Read 400) as described by Tsukatani et al. (2003).

2.8 Detection of chromatin condensation

Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) was performed as previously described Soares et al., (2017). The synergistic conditions and controls were transferred to a 96-well cell culture plate and incubated for 24 h. A relatively high cell density was used in this assay to allow microscopic visualization of the cells. For nuclear staining, after incubation, the cells were washed with PBS, incubated with 4 µg/mL DAPI in PBS for 10 min, rinsed three times with PBS and then visualized under a DIC epifluorescence

microscope (Axio Imager. A2, Zeiss) equipped with a fluorescence filter set (excitation 365 nm; emission 397 nm). The experiment was repeated three times.

2.9 Detection of wall integrity

To assess the integrity of the cell wall, we employed the fluorescent probe Calcofluor White (Sigma–Aldrich) for our analysis. After the samples were subjected to synergistic conditions for 24 h, 100 µL portions of the solution were extracted. This portion was subsequently mixed with 10 µL of Calcofluor White and incubated for 5 min at 30 °C in the dark. Following the aforementioned duration, the cells were examined via a light microscope (Axioplan. A2, Zeiss) coupled with an AxioCAM MRc5 camera (Zeiss). The acquired images were subsequently processed via AxioVision version 4.0 software (Zeiss), which was equipped with a fluorescence filter set (excitation 365 nm; emission 397 nm). To ensure reliable results, the experiment was replicated three times (Souza et al., 2024).

2.10 Analysis by scanning electron microscopy

The cells were washed three times with PBS and fixed for 48 h in a solution composed of 2.5% glutaraldehyde, 4.0% paraformaldehyde, 0.05 M sodium cacodylate buffer (pH 7.2), and 0.5 mM calcium chloride. Then, three 30 min washes were performed with the same buffer, and the cells were placed under coverslips covered with 0.1% poly-D-lysine, followed by three 15 min washes in the same buffer. The samples were postfixed in 1% osmium tetroxide for 1 h and washed three times for 15 min with the same buffer. After serial dehydration with increasing ethanol, the samples were subjected to the critical point using the Bal-Tec Critical Point Dryer CPD 030 apparatus. The dried coverslips were subsequently fixed with carbon adhesive tape on appropriate supports and metallized with a thin layer of 20 nm gold via the Bal-Tec Sputter Coater SCD 050. The samples were then observed and documented via a ZEISS DSEM 962 scanning electron microscope at a voltage of 25 kV.

2.11 Ultrastructure Analysis

For analysis of the ultrastructure, the synergistic conditions were fixed for 1 h in a solution containing 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4. After these procedures, the cells were washed twice with phosphate-

buffered saline (PBS) for 10 min and postfixed for 1 h in the dark with a solution containing 1% osmium tetroxide (OsO_4) and 1.6% ferrocyanide in 0.1 M sodium cacodylate buffer. The cells were subsequently washed in the same buffer, dehydrated in acetone (30%, 50%, 70%, 90%, 100% and 100% superdry) and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Jeol JEM 1400 Plus transmission electron microscope (Moreira et al., 2021).

2.12 Statistical analysis

Antimicrobial assays were performed in triplicate and repeated three times. Graphs were plotted as the means with standard deviations from an independent antimicrobial assay. The data obtained in the tests were statistically tested by one-way ANOVA, where $p<0.05$ was considered significant, via GraphPad Prism 8 software.

3. RESULTS

3.1 Effect of synthetic peptides and amphotericin B

Table 1 shows the concentrations of AmB required to completely inhibit yeast growth at both the 100% and 50%. Specifically, for *C. tropicalis* and *S. cerevisiae*, inhibitory concentrations of 0.58 µM, 1.17 µM, and 0.195 µM, 0.39µM respectively, were observed. Additionally, the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ impeded 50% of the fungal growth of both *C. tropicalis* and *S. cerevisiae* at concentrations of 3.12 µM and 25 µM, respectively.

Table 1. Minimum inhibitory concentration (µM) of synthetic peptides and amphotericin B (AmB).

	AmB MIC_{100} (µM)	**AmB MIC_{50} (µM)**	**$\gamma_{31\text{-}45}PvD_1^{++}$ MIC_{50} (µM)**
Candida tropicalis	**0.58**	**0.195**	**3.12**
Saccharomyces cerevisiae	**1.17**	**0.39**	**25**

3.2 Checkerboard

In our quest for synergistic therapeutic combinations via the Checkerboard assay, we identified synergistic conditions for both *C. tropicalis* and *S. cerevisiae*. Table 2 outlines the concentrations of AmB combined $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB. Notably, synergistic combinations of 0.048 µM AmB, 0.78 µM $\gamma_{31\text{-}45}PvD_1^{++}$, 0.048 µM AmB and 0.39 µM $\gamma_{31\text{-}45}PvD_1^{++}$ were observed, and synergistic concentration 0.048 µM AmB and 0.39 µM $\gamma_{31\text{-}45}PvD_1^{++}$, was used for tests in search of mechanism of action and electron microscopy.

The lowest combination obtained was used in the action mechanisms and electronic microscopy experiments. For the yeast *S. cerevisiae*, the identified combinations with either additive or indifferent effects were as follows: 0.39 µM AmB paired with 6.25 µM $\gamma_{31\text{-}45}PvD_1^{++}$, 0.39 µM AmB paired with 12.5 µM $\gamma_{31\text{-}45}PvD_1^{++}$, and 0.39 µM AmB paired with 3.12 µM $\gamma_{31\text{-}45}PvD_1^{++}$. Notably, a synergistic effect of 0.097 µM AmB and 0.78 µM $\gamma_{31\text{-}45}PvD_1^{++}$ was detected, as shown in Table 3. The combination that shows synergism was used in the action mechanisms and electronic microscopy experiments.

Table 2. Combined activity between bioinspired peptides and amphotericin B (AmB) (*Candida tropicalis*).

DRUG A	DRUG B	FIC A (μM)	FIC B (μM)	∑ FIC	ACTION
AmB	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.048/0.195	3.12/3.12	1.24	Additive or Indifferent
	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.048/0.195	1.56/3.12	0.74	Additive or Indifferent
	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.048/0.195	0.78/3.12	0.49	Synergism
	$\gamma_{31\text{-}45}$ ***Pv***D_1^{++}	0.048/0.195	0.39/3.12	0.36	Synergism

Table 3. Combined activity of the bioinspired peptide and amphotericin B (*Saccharomyces cerevisiae).*

DRUG A	DRUG B	FIC A (μM)	FIC B (μM)	∑ FIC	ACTION
AmB	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.39/0.39	12.5/25	1.5	Additive or Indifferent
	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.39/0.39	6.25/25	1.25	Additive or Indifferent
	$\gamma_{31\text{-}45}$***Pv***D_1^{++}	0.39/0.39	3.12/25	1.12	Additive or Indifferent
	$\gamma_{31\text{-}45}$ ***Pv***D_1^{++}	0.097/0.39	0.78/25	0.27	Synergism

3.3 Cell death monitoring assay and membrane permeabilization

We analyzed the mechanism of action of the synergistic combinations identified for the yeasts used in this study. Using the SYTOX Green probe, we evaluated whether the tested concentrations were capable of causing permeabilization of the cell membrane. In addition, we

employed the propidium iodide (IP) technique to verify whether the necrosis pathway was activated. In Figure 1, we present the results of these analyses for *Candida tropicalis* cells. During a follow-up of 6, 12, 18 and 24 h of incubation, only at the AmB concentration used and from 18 h of incubation was it possible to observe the labeling of both types of fluorescence. No labeling was detected under the other conditions, including the synergistic combination. These results suggest that the mechanism responsible for the death of this microorganism does not involve the necrosis pathway or the permeabilization of the plasma membrane.

The results of these analyses for the yeast *Saccharomyces cerevisiae* are presented in Figure 2. At none of the analyzed times (6, 12, 18 and 24 h) was the labeling of the probes observed. These findings suggest that none of the treatments, including the synergistic combination, affect the membrane permeabilization or activation of the necrosis pathway of this yeast.

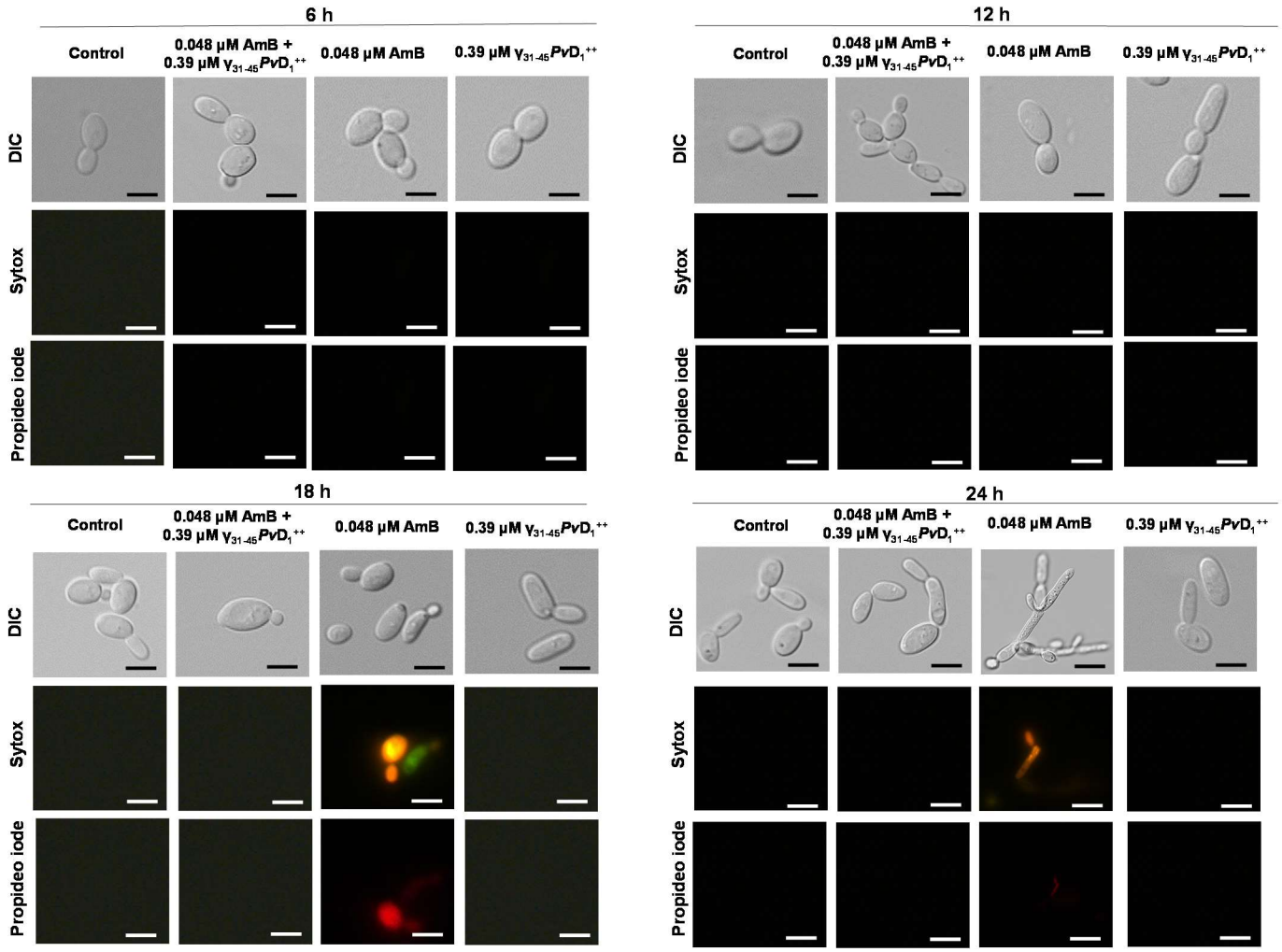

Figure 1: Cell death Monitoring assay and membrane permeabilization: Fluorescence microscopy images of *C. tropicalis* cells following a membrane permeabilization assay using the fluorescent probe SYTOX Green. The cells were exposed to the synthetic peptide and AmB alone or in combination for 6, 12, 18, or 24 h and then analyzed for membrane permeabilization. All the samples

were also treated with propidium iodide. Bars = 20 µM.

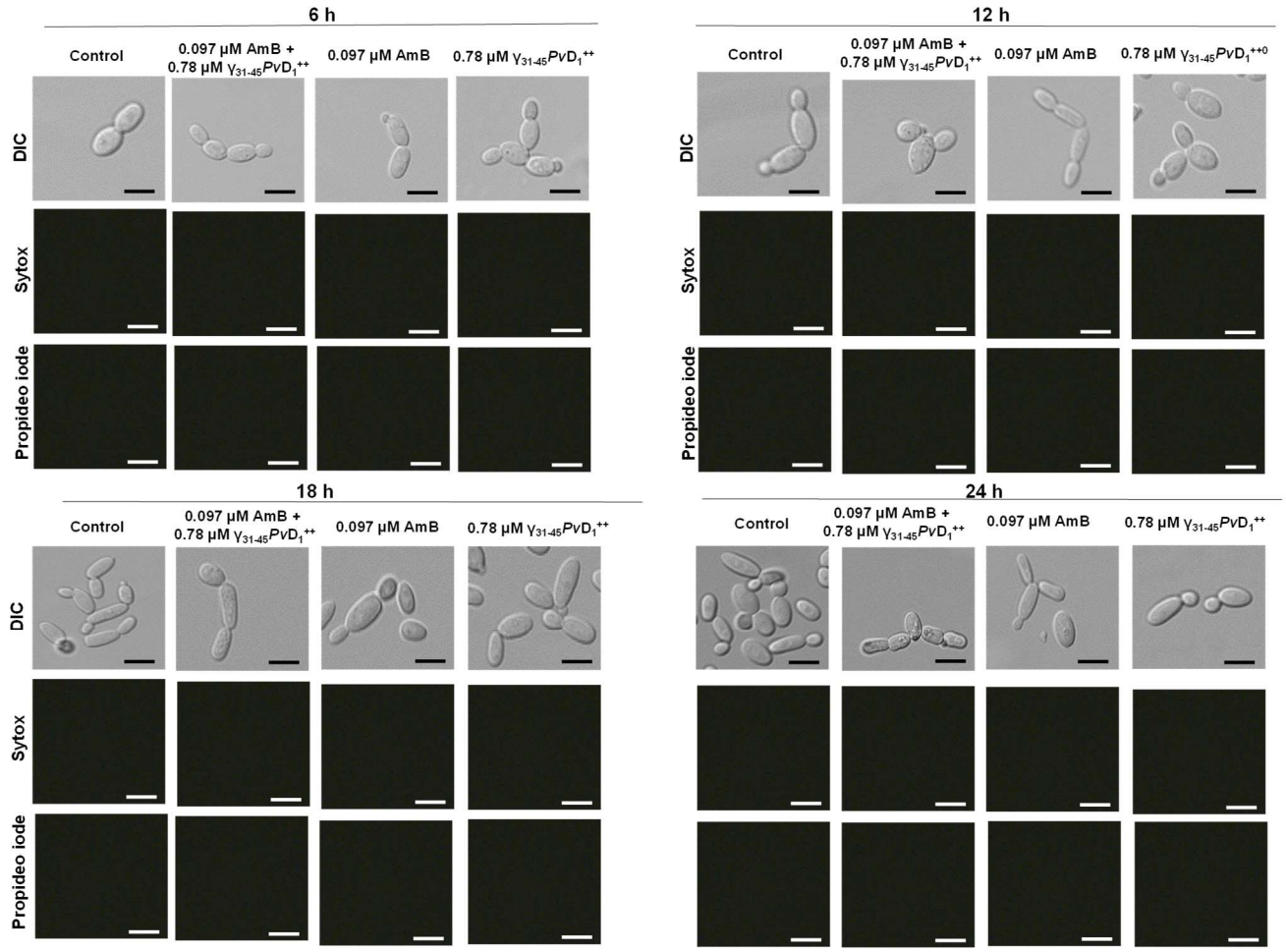

Figure 2: Cell death Monitoring assay and membrane permeabilization: Fluorescence microscopy images of *S. cerevisiae* cells following a membrane permeabilization assay using the fluorescent probe SYTOX Green. The cells were exposed to the synthetic peptide and AmB alone or in combination for 6, 12, 18, or 24 h and then analyzed for membrane permeabilization. All the samples were also treated with propidium iodide. Bars = 20 µM.

3.4 Analysis of vacuolar membranes in *C. tropicalis* and *S. cerevisiae*

The effects of the synergistic combinations of the yeasts *Candida tropicalis* and *Saccharomyces cerevisiae* on vacuolar membranes were evaluated via the FM4-64 probe. This probe is lipophilic and cannot cross cell membranes but intercalates in the plasma membrane and is internalized by cells by endocytosis, allowing the labeling of vacuolar membranes. After incubating the *C. tropicalis* and *S. cerevisiae* cells for 24 hours in the presence or absence of the synergistic combinations, the cells were reincubated with 5 µM of FM4-64 probe for 60

min at 30 °C. Figure 3 presents the results of this analysis, which revealed that the FM4-64 probe labeled the vacuole membrane, resulting in a ring staining pattern in all the cells of the control and treatment groups and a similar pattern in the *C. tropicalis* cells. However, in *S. cerevisiae* cells, a greater number of vacuoles were observed in the samples treated with the synergistic combination than in the control samples and the samples treated with both the synthetic peptide and AmB alone.

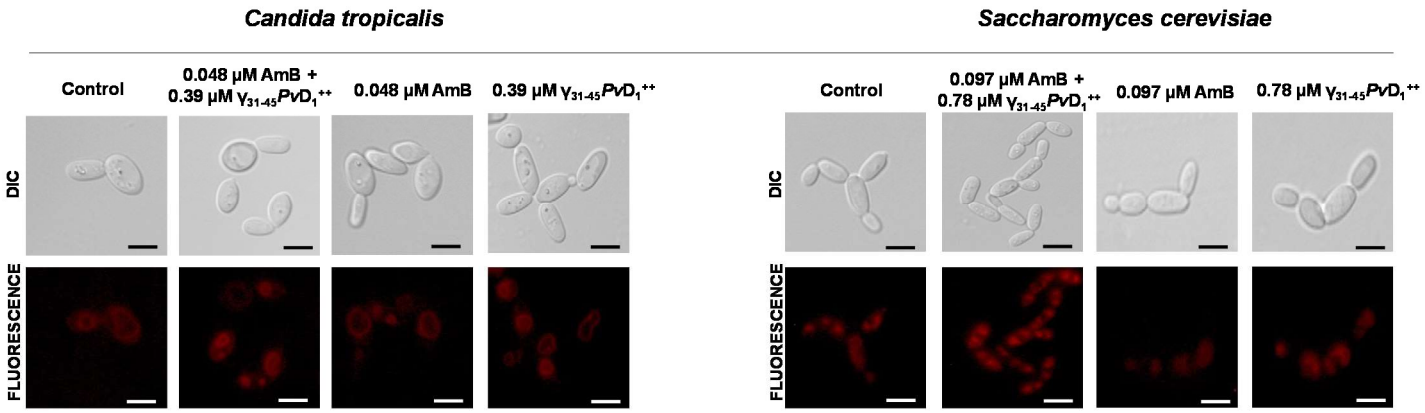

Figure 3: Vacuolar mapping using the FM4-64 probe. Control cells, AmB and the synthetic peptide alone and combined for 24 h and then treated with 5 µM FM4-64 for 60 min at 30 °C for *C. tropicalis* and *S. cerevisiae* cells. The control was treated with FM4-64 alone. Bars = 20 µM.

3.5 Mitochondrial functionality

The results demonstrated that the combination of the synthetic peptides $\gamma_{31\text{-}45}PvD_1^{++}$ and AmB at synergistic concentrations led to a loss of mitochondrial functionality in *C. tropicalis* (Figure 4A) and *S. cerevisiae* cells (Figure 4C). Synergistic concentrations of 0.39 µM $\gamma_{31\text{-}45}PvD_1^{++}$ and 0.048 µM AmB for *C. tropicalis* as well as 0.78 µM $\gamma_{31\text{-}45}PvD_1^{++}$ and 0.097 µM AmB for the yeast *S. cerevisiae* induced a notable loss of mitochondrial functionality. Notably, when the peptides or AmB were used individually at the same concentrations as the synergistic combination, the mitochondria maintained their functionality, similar to that of the controls.

We quantified mitochondrial activity by measuring the activity of mitochondrial dehydrogenase enzymes that convert WST-1 to formazan. The synergistic combination in *C. tropicalis* cells decreased dehydrogenase activity by 36% (Figure 4B), whereas in *S. cerevisiae* cells, there was a 48% decrease in dehydrogenase activity (Figure 4D). Thus, we suggest that

the synergistic conditions, in contrast to those of both *C. tropicalis* and *S. cerevisiae,* have a mode of action that involves a reduction in mitochondrial function.

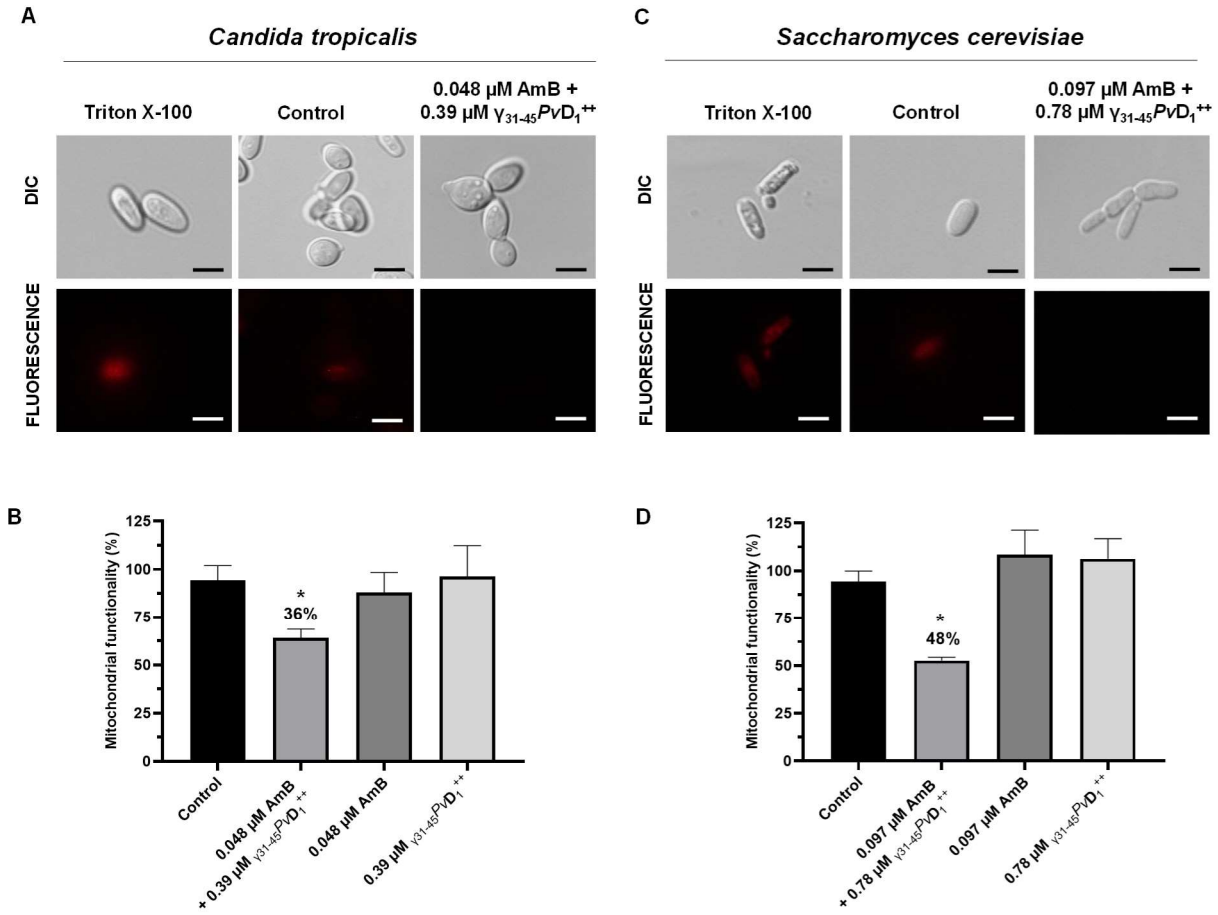

Figure 4: Mitochondrial functionality. Fluorescence microscopy images of *C. tropicalis* (A) and *S. cerevisiae* (C) cells treated with the synergistic combination of the synthetic peptide and AmB, as well as with the isolated concentrations and the control (untreated) and 1% de Triton X-100 (positive control), incubated for 24 h with 200 nM MitoTracker Red. Differential interface contrast (DIC) is used for visualization. Scale bars = 20 µM. Effects of the synergistic combination of the synthetic peptide and AmB, as well as the isolated concentrations, on the reduction of tetrazolium salt (WST-1) to formazan by mitochondrial dehydrogenases in *C. tropicalis* cells (B) and *S. cerevisiae* cells (D). Formazan formation was measured at 450 nm. The asterisk indicates statistical significance compared with the control, according to Tukey's test: * $p < 0.1$.

3.6 Chromatin condensation

The synergistic combinations of 0.39 µM $\gamma_{31\text{-}45}PvD1^{++}$ and 0.048 µM AmB for *C. tropicalis* and 0.78 µM $\gamma_{31\text{-}45}PvD1^{++}$ and 0.097 µM AmB for *S. cerevisiae* caused DNA damage. In control cells, labeling with 4',6-diamidino-2-phenylindole (DAPI) results in intense and punctate fluorescence, indicating the integrity of the cellular DNA. In the synergistic

combinations of both yeasts analyzed in this work, we observed a lack of punctual marking, thus demonstrating a loss of integrity of the cellular genetic material (Figure 5).

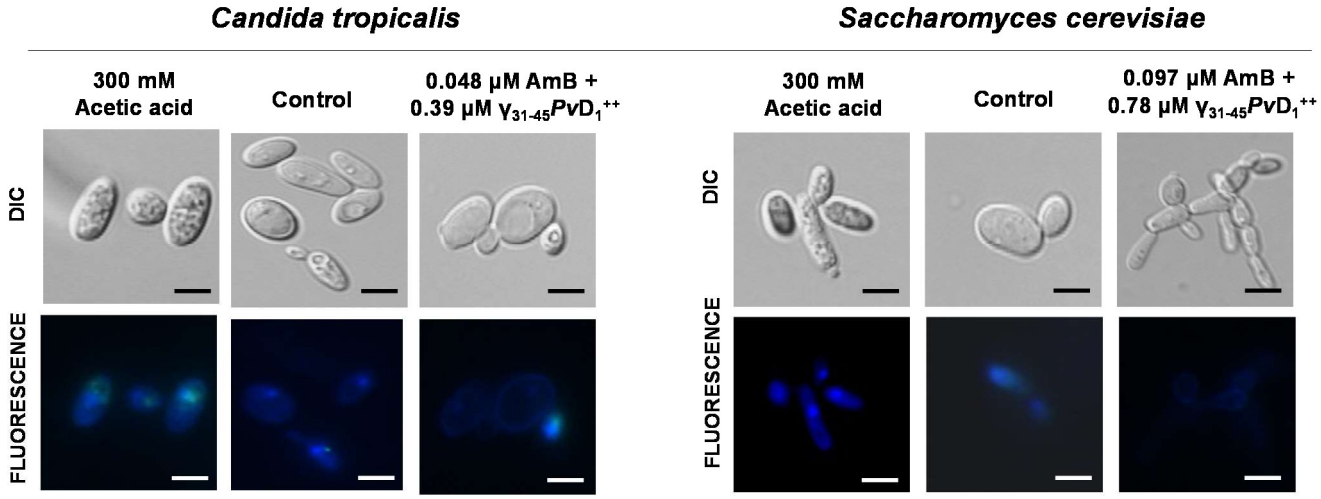

Figure 5: Chromatin condensation assay images of *C. tropicalis* and *S. cerevisiae* cells after treatment with $\gamma_{31\text{-}45}PvD1^{++}$ in combination with AmB for 24 h, control (untreated) and 300 mM acetic acid (positive control). To visualize the nuclei, DAPI was used. As a positive control, the cells were treated at 100 °C for 1 min. Bars = 20 µM

3.7 Detection of wall integrity

Analysis of the integrity of the cell walls of *C. tropicalis* and *S. cerevisiae* was carried out after 24 h in the presence of synergistic combinations of the synthetic peptides $\gamma_{31\text{-}45}PvD1^{++}$ and AmB. At control concentrations, we observed cells that were strongly stained with calcofluor white, indicating the integrity of their cell walls. The resulting blue fluorescence indicates the presence of an intact cell wall, as clearly represented in Figure 6. However, after treatment with synergistic combinations, intense labeling was not observed, suggesting the loss of cell wall integrity and possibly a decrease in cell viability, especially in *S. cerevisiae*.

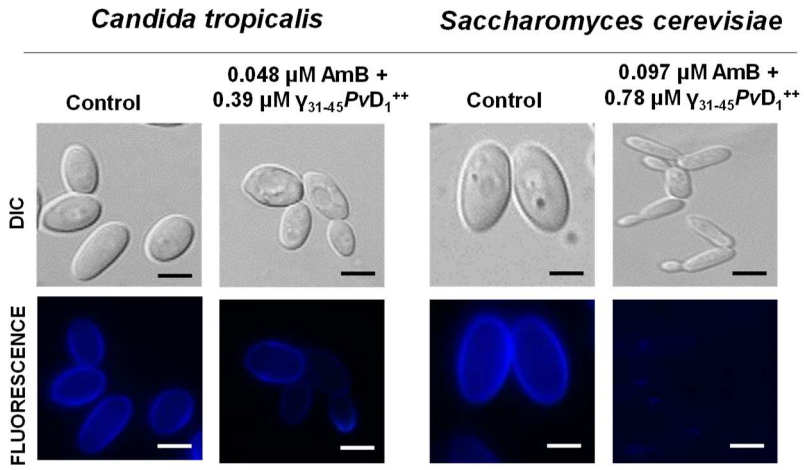

Figure 6: Images of the wall integrity of *C. tropicalis* and *S. cerevisiae* cells after treatment with $\gamma_{31\text{-}45}PvD_1^{++}$ in combination with AmB for 24 h, control (untreated). The wall integrity of the cells was assessed via a calcofluor white probe. Bars =20 µM.

3.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to evaluate surface damage to *C. tropicalis* (Figure 7A, B, C and D) and *S. cerevisiae* (Figure 7 E, F, G and H) following all the treatments. For *C. tropicalis*, the control (Figure 7A) cells did not show any changes in surface area. Treatment with AmB alone (Figure 7C) resulted in cell elongation but not major changes in the cell surface; after treatment with the individual synthetic peptides (Figure 7B), the cells were similar to those of the control. However, the combination of both molecules (Figure 7D) caused some damage to the cells, where the observed effects included pronounced roughness and evident cell lysis.

In the case of *S. cerevisiae*, control cells (Figure 7E) presented no surface damage or alterations; they appeared as oval-shaped cells without cracks or scars. Treatment with the synthetic peptide (Figure 7F) or AmB (Figure 7G) individually resulted in only minor damage, such as wrinkles and slight changes in cell morphology, which closely resembled those of the control. In contrast, the combination of both molecules (Figure 7H) caused severe damage to the cells; the observed effects included squamous and rod-shaped cells with pronounced roughness, significant morphological changes, and evident signs of cell lysis leading to cytoplasmic loss.

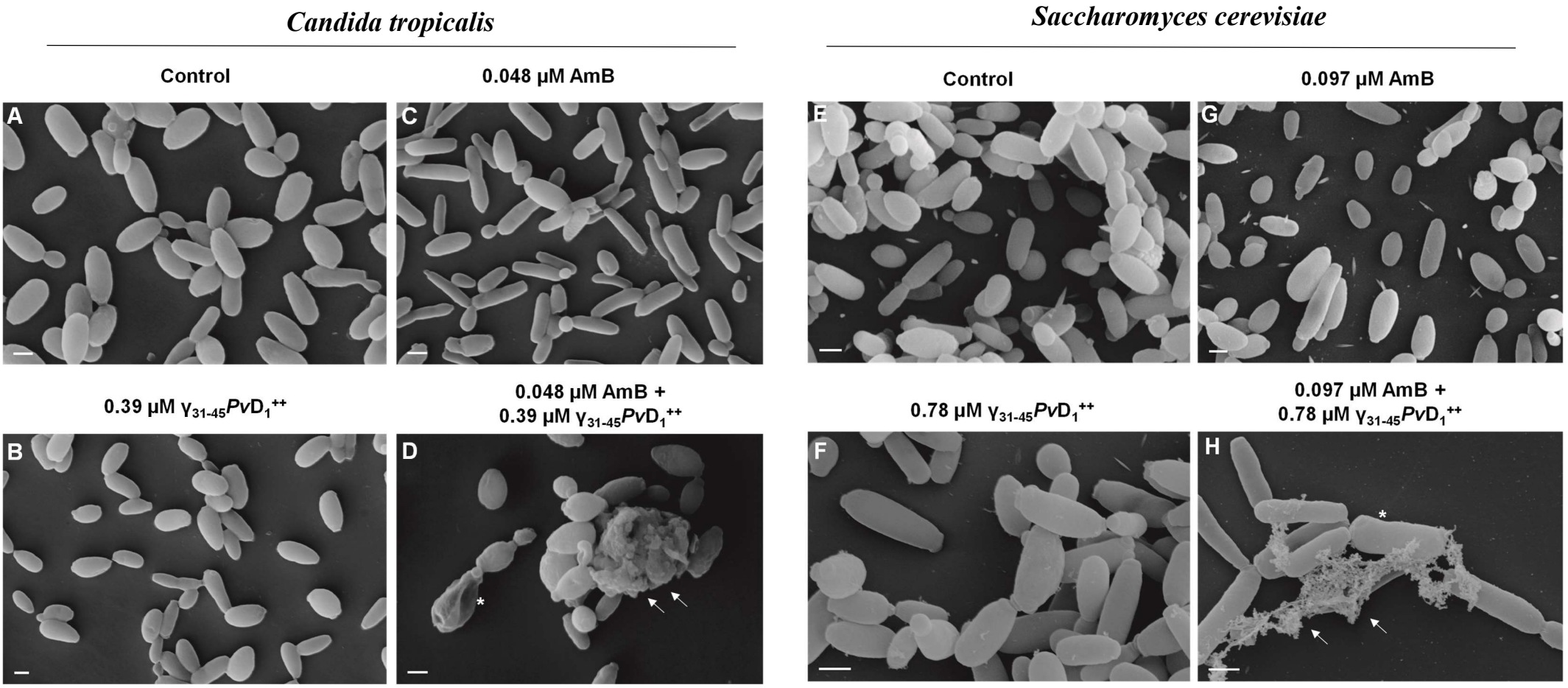

Figure 7: Scanning electron microscopy (SEM) images reveal alterations in the cell surfaces of *C. tropicalis* and *S. cerevisiae*. For *C. tropicalis*, see Figure 7A (Control), 7B (Bioinspired peptide), 7C (AmB), and 7D (Synergism). For *S. cerevisiae*, refer to Figure 7E (Control), 7F (Bioinspired peptide), 7G (Amphotericin B), and 7H (Synergism). Arrows indicate loss of cytoplasmic material, while asterisks denote changes in the membrane surface. Scale bars = 1 µM and 2 µM.

3.9 Ultraestrutural analysis

The ultrastructural characteristics of *C. tropicalis* (Figure 8A, B, C and D) and *S. cerevisiae* (Figure 8E, F, G and H) cells under all treatment and control conditions are detailed. For *C. tropicalis* cells, in the control (Figure 8A), the cell wall and plasma membrane remained intact, as did organelles such as the nucleus, mitochondria and vacuoles. After treatment with only the synthetic peptide (Figure 8C) or AmB (Figure 8B) alone, the cells and organelles remained similar to those of the control. However, in cells treated with the synergistic combination (Figure 8D), the genetic material and mitochondria were significantly damaged.

Figure 8 reveals striking differences in cellular components such as the cell wall, plasma membrane and some organelles. The control (Figure 8E) cells exhibited a uniform surface with no visible rupture. When treated with the synthetic peptide (Figure 8G) or AmB (Figure 8F) alone, the cells closely resembled the control. In particular, treatment with the synthetic peptide resulted in an increase in the number of vacuoles, indicating a possible defensive response against the bioinspired peptide in *S. cerevisiae* cells. In contrast, under synergistic conditions (Figure 8H), *S. cerevisiae* cells presented severe impairments in plasma membrane integrity, cell wall structure and cytoplasmic organization, clearly indicating cellular extravasation.

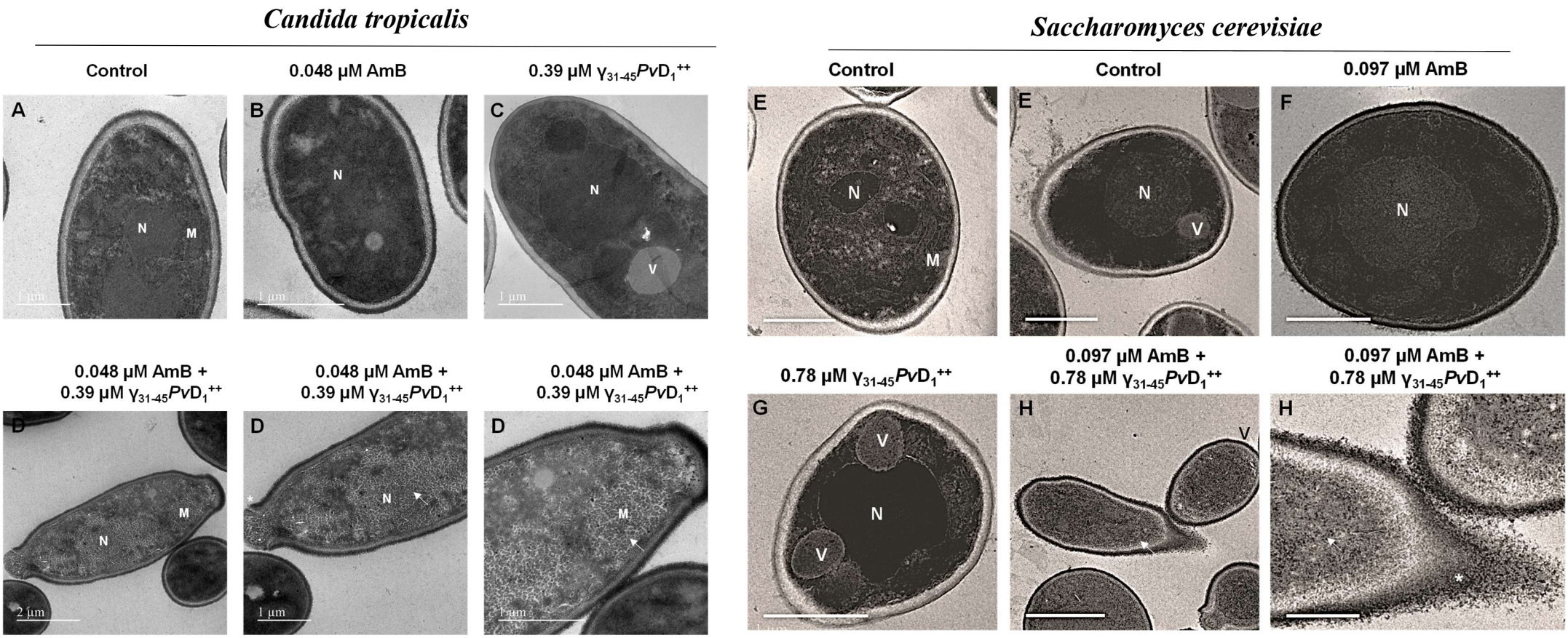

Figure 8: Ultrastructural changes visualized by transmission electron microscopy in *C. tropicalis* yeast cells are shown in Figure 8A (Control), 8B (AmB), 8C (Bioinspired peptide), and 7D (Synergism), while for *S. cerevisiae*, see Figure 8E (Control), 8F (AmB), 8G (Bioinspired peptide), and 8H (Synergism). Arrows indicate cytoplasmic disorganization and degradation, and asterisks show leakage of cytoplasmic material. (N) represents the nucleus, (M) mitochondria, and (V) vacuoles. Barr = 1 µM and 2 µM.

4 DISCUSSION

Natural plant antimicrobial peptides (AMPs) are promising as therapeutic agents for treating infections. However, these methods have drawbacks, such as susceptibility to proteolysis and high costs associated with isolation and purification. Development of synthetic antimicrobial peptides based on natural molecules offers a viable solution to overcome these limitations. Synthetic peptides can retain the antimicrobial efficacy of their natural counterparts while potentially mitigating issues such as toxicity to mammalian cells and reducing the likelihood of antimicrobial resistance due to their distinct mechanisms of action (Lima et al., 2017; Souza et al., 2020).

The rational design of synthetic peptides on the basis of sequences from natural proteins has gained traction owing to several advantages. These synthetic peptides can be meticulously crafted to increase their efficacy while minimizing potential side effects (Lata et al., 2007). Moreover, multiple functionalities can be incorporated into the same peptide sequence. Several online servers facilitate peptide design (Sharma et al., 2016), enabling the creation of peptides with characteristics that may not exist in proteins that serve as models (Collier et al., 2011).

In this study, we investigated the synergistic effects of a synthetic peptide based on the γ-core motif of *Phaseolus vulgaris* seed defensin, known as $\gamma_{31\text{-}45}$*Pv*D1^{++}, in combination with the widely used drug amphotericin B for combating *C. tropicalis* and using *S. cerevisiae* as a model. The yeast *Saccharomyces cerevisiae* is widely used as a model for cells of more complex organisms, such as humans. This yeast was the first eukaryote whose genome was completely sequenced (Goffeau et al., 1996). Its main characteristics that make it an excellent model include its short life cycle, ease of manipulation, well-annotated genome, wide range of molecular tools, and conservation of basic eukaryotic biological and biochemical pathways (Vanderwaeren et al., 2022).

Recently, our research group discovered synergistic interactions between the synthetic peptide $\gamma_{31\text{-}45}$*Pv*D1^{++} and AmB against the yeast *C. albicans*. This combination exhibited a potent fungicidal effect, characterized by mechanisms including increased membrane permeability, impaired mitochondrial function, and DNA degradation (Souza et al., 2024).

We initiated our quest for a new combined therapy option against clinically significant yeasts by determining the MIC_{50} of the synthetic peptide and AmB. Subsequently, we conducted a checkerboard test to identify combinations that exhibit synergistic effects. Table 1 shows the concentrations required to achieve the MIC_{50} for both the synthetic peptide and AmB.

For *C. tropicalis* cells, 0.195 μM AmB and 3.12 μM synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ were used to inhibit 50% of the growth of these cells. For the yeast *S. cerevisiae*, 0.39 μM AmB and 25 μM of the synthetic peptide $\gamma_{31\text{-}45}PvD_1^{++}$ inhibited 50% of its growth. From these values, it was possible to proceed to the Checkerboard test.

Synergistic combinations for developing combination therapies were identified for both yeasts via the Checkerboard method. Consequently, synergistic concentrations of 0.048 μM AmB + 0.39 μM $\gamma_{31\text{-}45}PvD_1^{++}$ for *C. tropicalis* (Table 2) and 0.097 μM AmB + 0.78 μM $\gamma_{31\text{-}45}PvD_1^{++}$ for *S. cerevisiae* (Table 3) were used to determine the mechanism of action involved in the death of each yeast studied.

We investigated whether the identified synergistic concentrations could induce membrane permeabilization, leading to the death of the tested microorganisms. To assess this, we utilized Sytox Green (5 μg/mL) and propidium iodide (0.5 μg/mL) probes, conducting analyses after 6, 12, 18, and 24 hours of incubation with the treatments. For *C. tropicalis* (Figure 1), we observed positive staining for both probes at a concentration of 0.048 μM AmB after 18 hours of incubation; however, no positive staining was detected under the other conditions. These results indicate that the identified synergistic combination for this yeast does not promote membrane permeabilization. For *S. cerevisiae* (Figure 2), we did not observe positive labeling for either probe at any of the tested time points. This indicates that the identified synergistic combination for this yeast was also unable to promote membrane permeabilization.

To investigate the mechanisms of action involved in the death of yeasts treated with the identified synergistic concentrations, we analyzed the vacuoles of both species. Cytoplasmic vacuolization is a well-documented morphological phenomenon, often seen in mammalian cells after exposure to bacterial or viral pathogens, as well as various natural and artificial low molecular weight compounds. However, the mechanisms underlying vacuolization in yeast are still under investigation for a more comprehensive understanding. While vacuolization typically accompanies cell death, its specific role in these processes remains unclear (Shubin et al., 2016). In this study, we observed significant differences in the number of vacuoles between the control and the synergistic treatment in *S. cerevisiae*, a finding that was not evident in *C. tropicalis*.

The data for S. cerevisiae are shown in Figure 3, where we used the FM4-64 probe to visualize the vacuolar membranes. We observed that the number of vacuoles in a cell increases when exposed to the synergistic condition compared to the control, suggesting an indication of the onset of cell death.

Mitochondrial functionality is crucial for cellular processes and development. We

investigated whether the synergistic combinations identified for each yeast studied could impair mitochondrial function. Both combinations were indeed capable of damaging the mitochondria of the cells (Figure 4).

In 2020, Bezerra and collaborators studied the effects of combining the synthetic peptides Mo-CBP3-PepI and Mo-CBP3-PepIII (50 μg mL) with the drugs nystatin and itraconazole on the development of *C. albicans* and *C. parapsilosis*, and their results demonstrated that the combination of these molecules significantly increased the endogenous production of reactive oxygen species (ROS), which is directly related to the loss of mitochondrial functionality.

Aguiar et al. (2023) demonstrated that combinations of the drug itraconazole and the synthetic peptides Mo-CBP3-PepII, RcAlb-PepII, RcAlb-PepIII, PepGAT and PepKAA (25 μM) induced the formation of membrane pores and the overaccumulation of ROS in *Cryptococcus neoformans* cells, which is a characteristic of ROS accumulation related to the loss of mitochondrial functionality.

In the study by Lucas et al. (2023), the mechanism of action of three bioinspired peptides RR ($A_{36,42,44}R_{37,38}\gamma_{32-46}$VuDef), D-RR (D-$A_{36,42,44}R_{37,38}\gamma_{32-46}$VuDef) and WR ($A_{42,44}R_{37,38}W_{36,39}\gamma_{32-46}$VuDef) against the yeasts *C. tropicalis* and *C. albicans* was investigated. Among the mechanisms studied, the focus was on mitochondrial functionality, with all peptides demonstrating the ability to cause mitochondrial damage. The results showed that, for *C. tropicalis*, the RR and D-RR peptides caused an increase of 305.2% and 565.1% in mitochondrial activity, respectively, after 1 hour and 30 minutes of incubation. For *C. albicans*, the WR peptide resulted in an increase of 835.9% in mitochondrial activity after 20 minutes of incubation and 376.4% after 1 hour. One of the authors' conclusions is that mitochondrial functionality is one of the main targets of bioinspired peptides, contributing to the death of the yeasts *C. tropicalis* and *C. albicans*.

Another critical aspect of the mechanism of action of effective antifungals involves targeting DNA, where the drug can induce degradation through various metabolic pathways. Therefore, this study evaluated the ability of the synergistic combinations identified for each yeast to cause DNA degradation. Intense and concentrated nuclear fluorescence was observed in both the control group and the groups treated with individual concentrations of the synergistic agents. This finding strongly suggests the preservation of nuclear integrity, as depicted in Figure 5. However, in the synergistic combinations, the absence of nuclear staining suggested significant chromatin condensation or potential leakage of DNA along with intracellular materials.

4',6'-Diamidino-2-phenylindole (DAPI) was used to evaluate DNA integrity in our study. This compound binds to DNA, is not cell permeable, and selectively interacts with the minor groove of double-stranded DNA, emitting fluorescence upon binding. By binding to DNA, DAPI facilitates the visualization and assessment of cellular processes involving DNA, such as chromatin condensation and potential leakage of genetic material due to rupture of the cell wall and plasma membrane. The use of DAPI as a tool in our study proved invaluable for understanding the cellular changes induced by synergistic combinations (Broekaert et al., 1990).

In 2017, Soares and collaborators demonstrated that the defensin ApDef1 induced cell death in *S. cerevisiae* cells with an MIC of 7.8 μM. They observed significant cell death after 18 hours of exposure to ApDef1, with 98.76% of the cell population dying within 3 hours. Incubation with ApDef1 resulted in membrane permeabilization, increased production of endogenous reactive oxygen species (ROS), chromatin condensation, and activation of caspases. Similar to our study, they utilized the DAPI probe for chromatin condensation analysis, highlighting its efficacy as a tool for such investigations.

The pursuit of combination therapy involving antifungals that target microbial cell walls has garnered significant interest among scientists seeking innovative therapeutic alternatives. In 2019, the World Health Organization (WHO) established a list aimed at combating resistant pathogens, categorizing them on the basis of the level of threat they pose to public health. Pathogens classified under "urgent threats" include *Candida auris* and certain antibiotic-resistant bacteria. The "serious threats" category encompasses pathogens whose impact on public health necessitates prompt and sustained action. Notably, this includes serious infections caused by drug-resistant species within the *Candida* genus, primarily *C. albicans*, followed by *C. tropicalis*, *C. parapsilosis*, and *C. krusei*.

In this study, the effects of synergistic combinations of *C. tropicalis* and *S. cerevisiae* on the integrity of fungal cell walls were assessed via the use of calcofluor dye. This dye exhibits affinity for components presents in the fungal cell wall, aiding in the observation of structural changes and damage caused by the treatments. Figure 6 shows the results of these analyses. Blue fluorescence signifies the presence of an intact cell wall, as observed in the control and in the individual synergistic combinations. Following treatment with synergistic combinations of both *C. tropicalis* and *S. cerevisiae*, the intense cell wall markings observed in the control and individual treatments were notably absent. Instead, fragmented markings were observed, indicating a loss of cell wall integrity and potentially compromised cell viability. Similar effects

were observed by Souza et al. (2024) after a synergistic combination of the same synthetic peptide with AmB against *C. albicans was discovered*. The synergistic concentrations were capable of inducing the loss of cell wall integrity in this fungus, resulting in cellular extravasation.

To explore the effects of the synergistic combinations on the surface structure and morphology of the yeasts studied, scanning electron microscopy was conducted. Figure 7 illustrate these findings for *C. tropicalis* (Figure 7A, B, C and D) and *S. cerevisiae* (Figure 7E, F, G and H). The control cells (Figure 7A, E) and those treated with the synthetic peptide or AmB alone (Figure 7BC, FG) displayed smooth surfaces without rigidity, furrows, or wrinkles. In contrast, cells treated with the synergistic combinations (Figure 7D, H) presented severe damage, including high levels of roughness, significant changes in morphology, and evident signs of cell lysis resulting in the loss of cytoplasm. This same effect was observed by Bezerra et al. in 2020 when strains of *C. albicans* and *C. parapsilosis* were treated with combinations of the synthetic peptides Mo-CBP3-PepI + nystatin and Mo-CBP3-PepIII + nystatin. Their study demonstrated that these synergistic combinations induce morphological changes and result in the loss of cytoplasmic components in treated cells.

Lima and collaborators (2020) demonstrated, through scanning electron microscopy analysis, that a synergistic combination of synthetic peptides bioinspired by peptides extracted from *Moringa oleifera* seeds and the antifungal nystatin showed a remarkable antifungal effect. This synergistic combination increased the anticandidal activity of nystatin, a conventional antifungal, approximately 50-fold and was able to induce significant morphological changes in *C. albicans* cells.

Wu et al. (2010), in their ultrastructure study, demonstrated that control *C. albicans* cells presented normal cell morphology, with well-defined cell walls, intact nuclei and numerous membranous organelles. In contrast, cells exposed to PLE, a macrocyclic bis(bibenzyl) with antifungal activity isolated from the liverwort *Marchantia polymorpha* L., showed extensive chromatin condensation into aggregates at the nuclear envelope, a typical sign of apoptosis.

To comprehensively understand how the identified synergistic combinations effectively kill the yeast cells studied in this research, ultrastructural analyses were conducted via transmission electron microscopy. As shown in Figure 8, the data confirmed that the plasma membrane and cell wall remained intact in the control cells and in the cells treated with individual concentrations of the synergistic combination for *C. tropicalis* (Figure 8A, B and C) and *S. cerevisiae* (Figure 8E, F and G), respectively. However, after treatment with the synthetic

peptide (Figure 8F), an increase in the number of vacuoles was observed, which we hypothesize to be a cellular defense response in *S. cerevisiae*. For the synergistic combinations of both yeasts *C. tropicalis* (Figure 8D) and *S.cerevisiae* (Figure 8H), a noticeable change in the morphology of the plasma membrane and cell wall was noted, indicating the disruptive effects of the combined treatment on these cellular components. This cellular disorganization compromises the clear visualization of organelles such as the nucleus, mitochondria, and vacuole. Specifically, for *S. cerevisiae*, the synergistic combination was able to increase the number of vacuoles compared with the control. Struyfs et al. (2020) reported the loss of vacuolar function in mutant strains when they were exposed to moderate doses of the defensin HsAFP1. They concluded that vacuolar function is a crucial determinant of lifespan in mutant *S. cerevisiae*.

The results of this study highlight the importance of understanding the innovative role of synergistic combinations of antifungal molecules, especially in view of the emergence of new fungal infections for which there are still no effective treatments. In this context, the use of synthetic peptides in combination with antifungals already on the market has emerged as a promising approach for the discovery of new drugs.

5. CONCLUSION

The discovery of a synergistic combination of a synthetic peptide and amphotericin B represents a significant advance in the development of combination therapies for fungal diseases. The synergistic combinations identified in this study not only affect the cell wall integrity of the yeasts tested but also induce the loss of mitochondrial functionality and the degradation of nuclear components. This multifaceted approach highlights its potential to combat fungal infections more effectively. The damage observed in the ultrastructure and morphology of the cells tested highlights the importance of studying the synergism between antifungal molecules to improve their activity.

In addition, the use of the *S. cerevisiae* model demonstrated the efficacy of these synergistic concentrations in model microorganisms, allowing the comparison of their activity with that of other more complex microorganisms.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

All the authors contributed to the data analysis, drafted and revised the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST

All the authors declare that there are no conflicts of interest associated with this work.

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CONCLUSÕES GERAIS

Os resultados obtidos neste trabalho, permitem concluir que:

- O peptideo bioinspirado utilizado neste trabalho foi capaz de inibir o crescimento de levedura *C. albicans* em baixas doses quando combinada com doses menores do antifúngico anfotericina B.
- Nas condições sinergicas encontradas para *C. albicans*, a concentração de AmB utilizada é cerca de 100 vezez menor que as doses do mesmo antifungico utilizado em hospitais.
- Para *C. albicans*, a combinação sinergica do peptideo bioinspirado e o antifúngico anfotericina B foi capaz de causar perda da integridade da parede celular, perda da funcionalidade mitocondrial, danos ao DNA, além de causar alterações ultraestruturais nestas células.
- Foram encontradas combinações sinérgicas para as leveduras *C. tropicalis* e *Saccharomyces cerevisia*e capazes de levar a morte destes microorganismos.
- A combinação sinérgica encontrada para *C. tropicalis* foi capaz de causar danos severos a está célula onde perda de funcionalidade mitocondrial e danos ao DNA estão envolvidos no mecanismo de morte deste microorganismo.
- Os danos causados pela combinação sinérgica encontrada para a levedura *S. cerevisiae*, além de causar danos da funcionalidade mitocondrial e ao DNA, foram capazes de alterar o número de vácuolos, indicando uma tentativa da célula de minimizar os efeitos gerados pelo tratamento.
- As descobertas destes estudos contribuem de forma positiva para o caminho de desenvolvimento de novos fármacos inspirados em peptídeos bioinspirados em combinação com AmB.

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