UNIVERSIDADE FEDERAL DO CEARÁ

CENTRO DE CIÊNCIAS

DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

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ATIVIDADE ANTIBIOFILME DE PEPTÍDEOS SINTÉTICOS: MECANISMOS DE AÇÃO E APLICAÇÕES NA SAÚDE

FORTALEZA

2022

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Dissertação apresentada à coordenação do Programa de Pós-graduação em Bioquímica da Universidade Federal do Ceará – UFC como requisito parcial para a obtenção do título de Mestre em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof°. Dr. Cléverson Diniz Teixeira de Freitas.

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FORTALEZA

2022

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

B469a Bezerra, Leandro de Paula.
Atividade antibiofilme de peptídeos sintéticos: mecanismos de ação e aplicações na saúde / Leandro de Paula Bezerra. – 2022.
112 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2022.
Orientação: Prof. Dr. Cléverson Diniz Teixeira de Freitas.
Coorientação: Prof. Dr. Pedro Filho Noronha de Souza.

1. Peptídeos. I. Título.

CDD 572

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AGRADECIMENTOS

Aos meus orientadores, Prof. Dr. Cléverson Diniz Teixeira de Freitas e o Prof. Dr. Pedro Filho Noronha de Souza pelos ensinamentos, conselhos, troca de conhecimentos e por todo o auxílio prestado ao longo dessa trajetória. Aos membros da banca examinadora por aceitarem o convite para colaborar com este trabalho.

À FUNCAP e ao governo do estado do Ceará por financiarem a minha pesquisa e permitido a minha permanência neste Programa de Pós-graduação.

A todos que, de alguma forma, contribuíram para a realização deste trabalho. Meu muito obrigado!

RESUMO

Biofilmes formados por leveduras do gênero *Candida* são um grande problema de saúde pública devido à sua alta resistência aos agentes antimicrobianos. Dentre estes, *Candida albicans*, *C. krusei* e *C. parapsilosis* são fungos resistentes a agentes antimicrobianos que são responsáveis por causar graves infecções da corrente sanguínea podendo levar os pacientes à morte. Para superar esse cenário, peptídeos antimicrobianos sintéticos (PAMs) são considerados novas ferramentas para combater infecções, tendo em vista que seu principal mecanismo de ação está relacionado com o ataque e desestabilização da membrana celular do patógeno. Estas moléculas podem ser usadas tanto isoladamente quanto em sinergismo com drogas que já apresentam tolerância pelos microrganismos. Neste trabalho, cinco PAMs sintéticos denominados *Mo*-CBP3-PepI, *Mo*-CBP3-PepII, *Mo*-CBP3-PepIII obtidos da sequência proteica de *Mo*-CBP3, uma proteína ligante à quitina purificada de sementes de *Moringa oleífera* e PepGAT e PepKAA, obtidos com base na sequência de uma quitinase de *Arabidopsis thaliana* foram testados sozinhos e em combinação com Nistatina (NYS) e Itraconazol (ITR) contra biofilmes formados por *C. albicans, C. krusei* e *C. parapsilosis*. Além disso, os mecanismos por trás da atividade tal atividade foram avaliados por microscopia de fluorescência e microscopia eletrônica de varredura (MEV). Os resultados revelaram uma melhora de 2 a 4 vezes na atividade antibiofilme dos fármacos NYS e ITR quando combinado com peptídeos. Análises microscópicas mostraram que os mecanismos de ação são baseados na degradação da parede celular, superprodução de espécies reativas de oxigênio (ROS) e formação de poros da membrana causando extravasamento de conteúdo celular e levando as células do biofilme à morte. Ao aumentar a atividade de NYS e ITR, os peptídeos apresentaram grande potencial como adjuvantes. Em conjunto, os resultados sugerem o potencial de *Mo*-CBP3-PepI, *Mo*-CBP3-PepII *Mo*-CBP3-PepIII, PepGAT e PepKAA como novas drogas e/ou adjuvantes para aumentar a atividade de drogas convencionais para o tratamento de infecções clínicas causada por biofilmes resistentes do gênero *Candida*.

Palavras-chave: atividade antibiofilme; sinergismo; *Candida*; peptídeos sintéticos; aplicação clínica.

ABSTRACT

Biofilm-forming yeasts are major public health problem because its high resistance to microbial agents. Among those, *Candida albicans, C. krusei* and *C. parapsilosis* are drug-resistant yeasts responsible for bloodstream infections leading individuals to death. To overcome this scenario, synthetic antimicrobial peptides (SAMPs) are considered new weapons to combat infections either alone or conjugated with drugs that are losing their activity because its capacity to cause a disruption of pathogen cell membrane. Here, five SAMPs named *Mo*-CBP3-PepI, *Mo*-CBP3-PepII, *Mo*-CBP3-PepIII, were designed based in the protein sequence of a chitin-binding protein from *Moringa oleifera* seeds and PepGAT and PepKAA, obtained by a protein sequence from a *Arabidopsis thaliana* chitinase were tested alone or in combination with Nystatin (NYS) and Itraconazole (ITR) against biofilms formed by *C. albicans, C. krusei* and *C. parapsilosis*. Furthermore, the mechanisms behind the antibiofilm activity was evaluated by fluorescence and scanning electron microscopies. The results revealed an improvement of 2 up to 4-fold in NYS and ITR antibiofilm activity when combined with peptides. Microscopic analyses showed that mechanisms of action is based on cell wall degradation, overproduction of reactive oxygen species (ROS) and membrane pore formation causing leakage of internal content and leading biofilm cells to death. By increasing the activity of NYS and ITR, peptides exhibited a great potential as adjuvants. Altogether, results suggest the potential of *Mo*-CBP_3-PepI, *Mo*-CBP_3-PepIII, PepGAT and PepKAA as new drugs and/or adjuvants to increase the activity of conventional drugs for treatment of clinical infection caused by resistant biofilms of *Candida* specie.

Keywords: antibiofilm activity; synergism; *Candida*; synthetic peptides; clinical application.

SUMÁRIO

1. INTRODUÇÃO

Um dos principais problemas de saúde pública mundial no século XXI tem sido a ascensão da microrganismos resistentes às drogas antimicrobianas (HILLER et al, 2019). Estudos estimam que o número de mortes causadas pela resistência microbiana ultrapasse os 10 milhões até 2050 (DODDS, 2017) onde mais de 2,7 milhões de pessoas poderão entrar em óbito a cada ano devido às infecções causadas por microrganismos em todos os continentes do globo (STROLLO et al, 2016).

Muito dessa problemática está relacionada com a resistência às drogas antifúngicas, onde espécies do gênero *Candida* tem se destacado nos últimos anos, sendo responsáveis pelos infecções mais recorrentes, levando ao quadro clínico de infecção na corrente sanguínea, conhecido como candidíase invasiva (PAPPAS et al, 2018). Mais de 700.000 casos de candidíase são relatados anualmente, sendo a taxa de mortalidade superior a 40%, gerando despesas aos cofres públicos de mais de US$ 50.000 por paciente (DHINGRA et al, 2020). Aliado a isso, está a capacidade de tais microrganismos se associarem em comunidades mais estáveis conhecidas como biofilmes, o que garante uma maior resistência à administração de drogas no combate à patogenia (RIVIE et al, 2018).

Com o aumento da resistência a muitas drogas já disponíveis no mercado, os peptídeos antimicrobianos (PAMs) surgem como uma alternativa promissora na prevenção e combate a biofilmes de alto potencial patológico. PAMs são pequenas sequências de aminoácidos que compõem o sistema imune inato de quase todos os organismos vivos, desde microrganismos até plantas, insetos e animais, incluindo o ser humano (BORARAI et al, 2020). O amplo espectro de atividade antimicrobiana demonstrado pelos PAMs desfavorecem o desenvolvimento de mecanismos de resistência pelos microrganismos, tendo em vista que o seu principal mecanismo de ação envolve o ataque a membrana celular do patógeno (LIMA et al, 2021), inviabilizando o reparo de tal estrutura celular pelas células afetadas. Entretanto, algumas desvantagens são encontradas em PAMs naturais como instabilidade físico-química, elevados níveis de toxicidade bem como elevada possibilidade de proteólise, o que dificulta o seu uso (OLIVEIRA et al, 2019). Felizmente, estes problemas podem ser contornados com o desenho racional de peptídeos sintéticos cujas sequências selecionadas apresentam o menor grau de toxicidade e podem ser mais promissoras.

Nosso grupo de pesquisa já tem purificado inúmeras proteínas com potencial antimicrobiano comprovado, dentre as quais podem ser destacas *Mo*-CBP3, uma proteína

ligante à quitina purificada de sementes de *Moringa oleífera* e *Rc*-2S-Alb, purificada da torta de mamona de *Ricinus communis*, ambas pertencentes à família das albuminas 2S. No entanto, o baixo rendimento após o processo de purificação torna o trabalho com tais moléculas inviável. Com isso, tem-se empenhado esforços no desenho de peptídeos sintéticos bioinspirados nas sequencias de proteínas anteriormente identificadas, gerando peptídeos biologicamente ativos denominados *Mo*-CBP3-PepI, *Mo*-CBP3-PepII e *Mo*-CBP3-PepIII os quais apresentam baixa ou nenhuma toxicidade às células humanas (OLIVEIRA et al, 2019). Um outro trabalho também desenvolvido pelo nosso grupo focou no desenho de dois peptídeos sintéticos baseados na sequência proteica de uma quitinase identificada em *Arabidopsis thaliana*, gerando PepGAT e PepKAA (SOUZA et al, 2020). Tais peptídeos tem demonstrado atividade contra células planctônicas de algumas espécies de bactérias e leveduras. Faz-se necessário, no entanto, a realização de estudos no tocante ao potencial de inibição e redução da biomassa de biofilmes antimicrobianos de interesse clínico (tendo em vista que a associação de microrganismos em biofilmes aumenta a resistência contra as drogas utilizadas), a elucidação dos mecanismos de ação por trás de tais atividades e, ainda, explorar os efeitos de sinergismo de tais moléculas com drogas antifúngicas convencionais.

2. CAPITULO 1 - REVISÃO DE LITERATURA

2.1 Histórico dos agentes antimicrobianos

Em 1928, com o descoberta da penicilina e posteriormente o seu uso como antibiótico em 1942 deram início a uma fase progressista no campo da medicina (STEKEL, 2018). A década seguinte ficou conhecida como aquela responsável por iniciar a chamada era dos antibióticos (1950 – 1970), a qual foi marcada pela descoberta de muitos fármacos com atividade antimicrobiana como tetraciclina (1950), eritromicina (1953), gentamicina (1967) e vancomicina (1972) (RIBEIRO DA CUNHA et al, 2019). Com isso, houve uma drástica redução no número de mortes causadas por infecções bacterianas, cuja expectativa de vida humana à época foi elevada para até 79 anos (BARREIRO et al, 2021).

Por outro lado, a descoberta e desenvolvimento de agentes antifúngicos não ocorria na mesma velocidade que àqueles fármacos antibacterianos, sendo estes últimos mais rapidamente desenvolvidos tendo em vista o grande número de infecções bacterianas registradas, quando comparadas àquelas causadas por fungos (BARTELL et al, 2019). Desta forma, o primeiro agente antifúngico, anfotericina B, apenas foi introduzido em 1958, após

aumento da taxa de crescimento no número de infecções e mortes causadas por fungos patogênicos (KEANE et al, 2018).

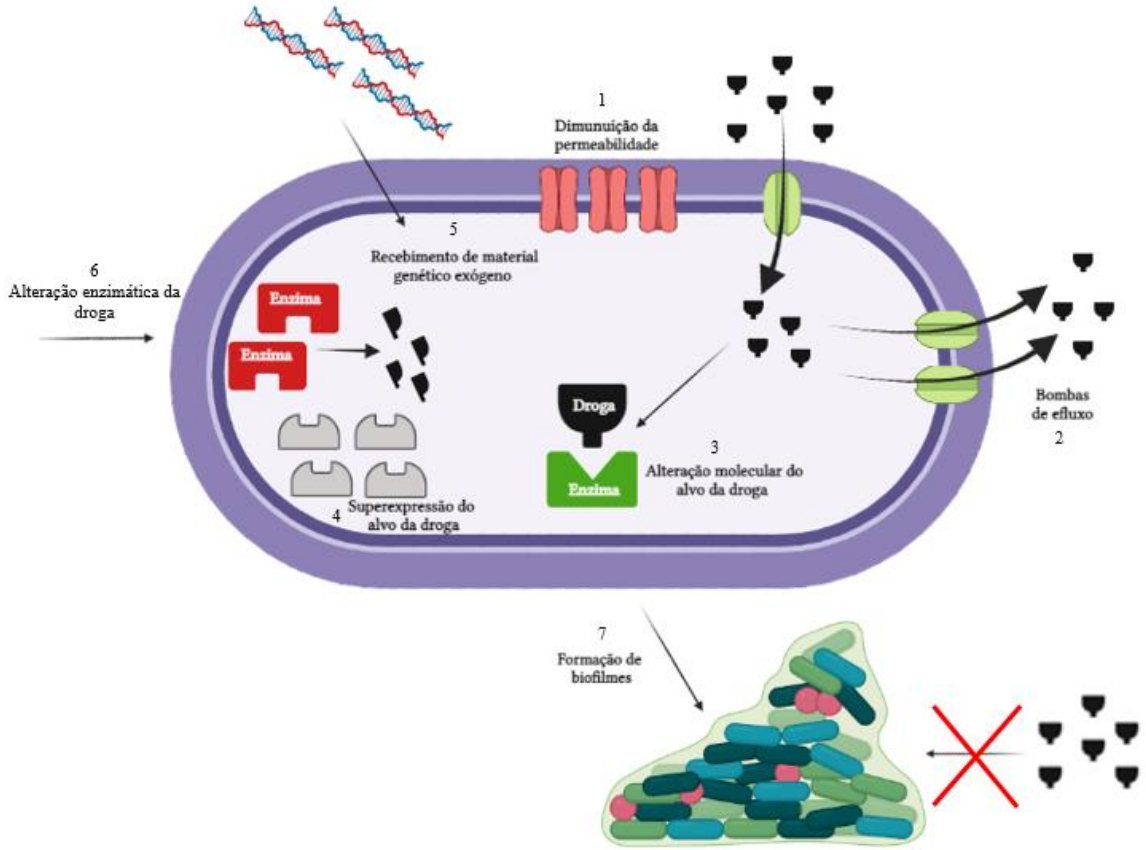
Apesar de todos os esforços, a eficiência dessas drogas não permaneceu a longo prazo. Microrganismos encontraram formas de driblar os mecanismos de ação dos antibióticos e se proliferarem continuamente, sem danos aparentes. Um fato interessante é que a resistência à penicilina (1940) foi observada antes mesmo desta droga ser disponibilizada comercialmente em 1942, o que vem se estendendo até os dias atuais (CHAN, 2020). Desde então, inúmeros microrganismos desenvolveram mecanismos de resistência e, inclusive, multirresistência a drogas, tal como *Staphylococcus aureus*, uma das bactérias mais estudadas e que apresenta resistência à vancomicina (classe dos glicopeptídeos) e meticilina, pertencente ao grupo das penicilinas (GUO et al, 2020). Outra espécie que tem recebido atenção é a *Pseudomonas aeruginosa*, na qual já existem dados na literatura relatando sua resistência a antibióticos da classe dos aminoglicosídicos, quinolonas e β-lactâmicos (PANG et al, 2019). Associado a isso, algumas espécies de fungos filamentosos e leveduriformes têm apresentado mecanismos de resistência às drogas antifúngicas disponíveis, incluindo espécies do gênero *Candida*, muitas delas com mecanismos de resposta aos antifúngicos da classe dos azóis (PRISTOV et al, 2019).

2.2 Evolução dos mecanismos de resistência

Considerado um processo evolutivo natural, o desenvolvimento de mecanismos de resistência tem sido acelerado pela pressão seletiva ocasionada pelo uso indiscriminado de fármacos antimicrobianos, como mostrados na Figura 1 (KLUMPER et al, 2019). Tal uso não se dá apenas pela má utilização ou interrupção antecipada dos tratamentos pela população, mas também pela prescrição inadequada desses medicamentos por parte de profissionais da saúde. Outrossim, está a aplicação de antimicrobianos em setores não convencionais como a agricultura, o que também contribui para o desenvolvimento da resistência microbiana (TAYLOR et al, 2020).

Com a fundamentação de que a aquisição dos mecanismos de resistência têm caráter natural e evolutivo, tais mecanismos são desenvolvidos levando em conta um conjunto de características próprias de cada microrganismo associado a fatores mutagênicos e/ou de transferência de genes de resistência entre indivíduos numa determinada população. Além disso, muitos microrganismos conseguem desenvolver mecanismos de resistência muito rapidamente em pouco tempo após exposição à droga (HILLER et al, 2019).

Figura 1 – Mecanismos de resistência aos antimicrobianos



Fonte: elaborada pelo autor

Dentre os principais mecanismos de resistência conhecidos até o momento, a redução da concentração do droga dentro da célula tem sido um ponto chave no processo de resistência microbiana. As células microbianas se especializaram na expressão de genes responsáveis pela síntese de proteínas transmembrana denominadas bombas de efluxo, cuja atividade consiste em lançar a droga recém assimilada no espaço extracelular, impedindo não apenas o acúmulo da droga no citoplasma do microrganismo, mas também sua ação farmacológica intracelular no alvo desejado (LIMA et al, 2021). Um fato curioso é que essas estruturas foram desenvolvidas por fungos e bactérias para não serem específicas para apenas uma classe de antibióticos, mas para uma grande variedade de moléculas incluindo àquelas responsáveis pela inibição da síntese da parede celular (carbapenêmicos e β-lactâmicos), inibição da produção do ergosterol de membrana (azóis), desestabilização da parede celular (polimixinas), inibição da enzima topoisomerase [ocasionando problemas na replicação do DNA (fluoroquinolonas)], entre outros. Muitos patógenos não possuem apenas um mecanismo, mas vários destes, o que resulta no surgimento de microrganismos multirresistentes (RUSS, et al 2020, BHATNAGAR et al, 2019).

Além disso, a síntese de enzimas capazes de modificar o fármaco e impedir sua ação farmacológica se configura como um dos mecanismos de resistência mais sofisticados já identificados. Dentre estas, podem ser destacas as β-lactamases cujo mecanismo consiste em desfazer a ligação amida do anel β-lactâmico, fazendo com que fármacos como penicilina, amoxicilina e ampilicina percam suas atividades farmacológicas (BEHZADI et al, 2020). A modificação do alvo celular chama a atenção dos pesquisadores devido a presença de mutações pontuais em determinadas regiões dos genes que codificam moléculas-alvo (YELIN et al, 2018). Nesse sentido, enzimas intracelulares podem ter os aminoácidos do seu sítio ativo modificado ou substituídos por outros que fazem o fármaco perder a sua afinidade pela enzima-alvo, resultando em pouca ou nenhuma atividade farmacológica. Como exemplo, o gene ERG11 em *Candida albicans* possui 217 mutações, sendo 185 destas silenciosas (que não alteram o funcionamento ou função da proteína resultante) e outras 32 mutações ocasionadas por substituição dos aminoácidos (HEALEY et al, 2018). O resultado dessas mutações faz com que a enzima lanesterol 14α-desmetilase não seja inibida pelas drogas antifúngicas da classe dos azóis e equinocandinas, garantindo que a enzima sintetize normalmente o ergosterol de membrana, tornando a cepa resistente. Ademais, o alvo farmacológico pode ser superexpresso, de modo que a concentração do fármaco disponível intracelularmente seja insuficiente para exercer uma atividade favorável (VILLASMIL et al, 2020).

A parede celular também é uma das estruturas celulares que possuem características de resistência já relatadas, destacando a redução da sua permeabilidade que culmina na redução da entrada do fármaco na célula (GHAI et al, 2018). Antimicrobianos cujas estruturas possuem um caráter hidrofílico têm como principal meio de acesso ao espaço intracelular proteínas canais responsáveis pela captação de água pelas células, as aquaporinas. Os mecanismos desenvolvidos pelos patógenos consistem em redução da expressão de aquaporinas na membrana celular, alteração da estrutura molecular ou nos índices de condutância das porinas e, inclusive, a cessação da síntese de tais canais (CHRISTAKI et al, 2020). Cepas clinicas de *Klebsiela pneumoeae* apresentam mutações no gene que codifica a aquaporina Ompk36, resultando na não passagem de fármacos hidrofílicos para o interior da célula (LIMA et al, 2021). A subunidade F da aquaporina OmpF de *Escherichia coli* também sofre mutações pontuais na sua sequência nucleotídica, implicando na redução de acesso do fármaco ao meio intracelular (LAUXEN et al, 2021).

A aquisição de genes de resistência por bactérias susceptíveis também tem se configurado um mecanismo de resistência importante (LERMINIAUX et al, 2019). Cepas mais resistentes acabam repassando (via conjugação, transformação ou transdução) genes com

capacidade para sintetizar moléculas resistentes em outras bactérias, reforçando a dificuldade de se combater bactérias que atuam em conjunto, como àquelas que se organizam em estruturas conhecidas como biofilmes (MCINNES et al, 2020).

2.3 Resistência às drogas antifúngicas e saúde humana

Dentre os microrganismos causadores de doenças em humanos, os fungos são responsáveis por uma grande parcela nos índices de infecção relatados. De acordo com o Fundo de Ação Global para Doenças Fúngicas (GAFFI, da sigla em inglês) os efeitos da contaminação por alguns fungos patogênicos deixam sequelas irreparáveis até mesmo em indivíduos imunologicamente saudáveis (RODRIGUES et al, 2021). Além disso, com o aumento do número de infecções causadas por bactérias e vírus e consequente enfraquecimento do sistema imunológico na tentativa de superar as infecções, muitos fungos oportunistas tem ganhado espaço para colonizarem superfícies do corpo humano e instalarem doenças (SALEHI et al, 2020). Como exemplo, pacientes acometidos com a Síndroma da Imunodeficiência Adquirida (AIDS) são constantemente diagnosticados com infecções oportunistas envolvendo fungos. Além disso, pacientes sob tratamento oncológico, quimioterapia e terapias imunossupressoras são especialmente atacados por tais patógenos (ARMSTRONG et al, 2019). Devido a capacidade de muitos fungos aderirem às superfícies (além dos tecidos), indivíduos portadores de dispositivos médicos como marca-passos, cateteres e válvulas cardíacas frequentemente apresentam deposição de células nesses aparelhos (RAMSTEDT et al, 2022).

Diferentemente dos antibióticos, que desde 1942 vêm sendo produzidos em larga escala e são mais fáceis de serem sintetizados, os antifúngicos apresentam algumas limitações na sua síntese. Isso acontece pelo fato dos fungos terem uma maior proximidade filogenética com os humanos, compartilhando características como seres eucariotos (VAN DAELE et al, 2019). Essa semelhança dificulta o processo de descoberta de novos fármacos pois teme-se que os novos antifúngicos desenvolvidos afetem não apenas a célula patogênica, mas também os tecidos saudáveis do hospedeiro. Dessa forma, o número de alvos moleculares inerentes apenas aos fungos se torna mais escasso (LIU et al, 2018). Assim como os antibióticos, esperasse que os antifúngicos tenham uma alta especificidade molecular, amplo espectro de atuação, mecanismos de ação variados e, sobretudo, não favoreçam a resistência cruzada entre as drogas atualmente disponíveis (HASAN et al, 2020).

Desde a década de 2000 não são lançados novos fármacos com alvos moleculares específicos apenas para os fungos (PERFECT et al, 2017). Os antifúngicos disponíveis tem um espectro de atividade limitada, atuando sobre uma pequena parcela de alvos celulares

conhecidos diferentes daqueles compartilhados entre fungos e seres humanos. Por exemplo, antifúngicos da classe dos azóis e triazóis como fluconazol, cetoconazol e voriconazol atuam na inibição da enzima lanesterol 14α-desmetilase (enzima chave na biossíntese do ergosterol) (VILLASMIL et al, 2020). Os polienos, por sua vez, atuam na desestabilização da membrana celular do microrganismo, como é o caso dos fármacos nistatina e anfotericina B via ligação com o ergosterol de membrana (ESCADÓN et al, 2019). A inibição da β-glucano sintase, por sua vez, é promovida por fármacos da classe das equinocandinas que atuam na interrupção da síntese e organização da parede celular (PRISTOV ET AL, 2019).

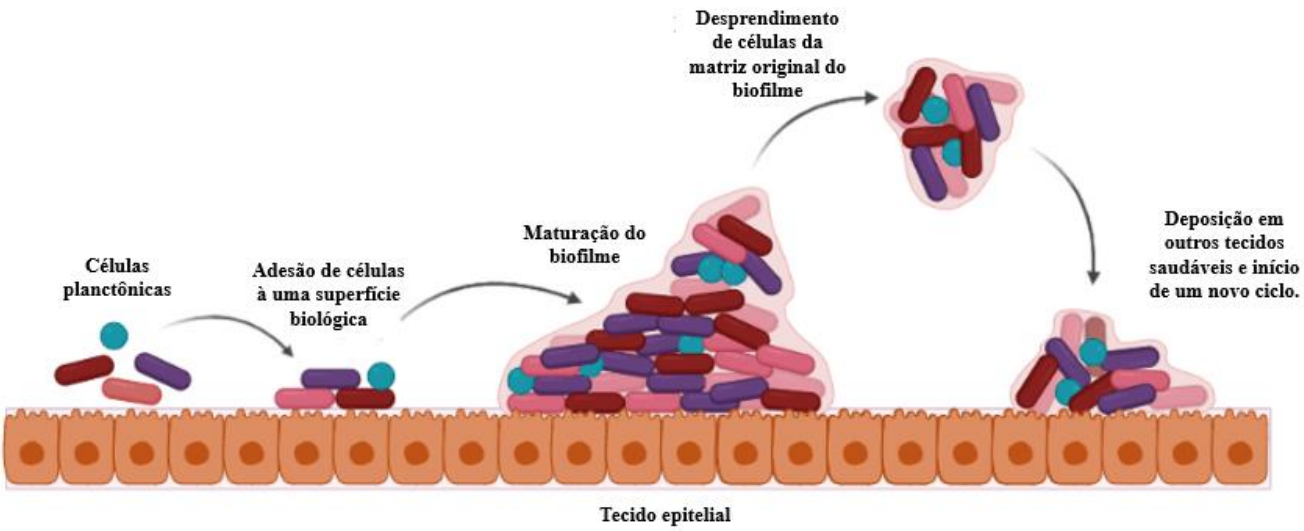
Tendo em vista essa limitação no número de alvos farmacológicos e a evolução natural dos mecanismos de resistência antimicrobianos, é urgente o desenvolvimento de alternativas que sejam capazes de interagir com alvos alternativos da maquinaria celular dos fungos e prevenir ou combater as infecções por estes causadas.

2.4 Biofilmes

Os biofilmes são estruturas formadas pelo aglomerado de células com capacidade de se aderirem à uma superfície e se desenvolverem sobre esta. A estrutura de um biofilme é imersa em uma matriz complexa que é composta por polissacarídeos, lipídeos, proteínas e ácidos nucleicos que, juntos, conferem resistência à estrutura (KHATOON et al, 2018). Esta matriz pode ser modificada pelos microrganismos a depender dos estados do ambiente no qual estão inseridos levando em consideração fatores como o tipo de organismo invadido, forças mecânicas do tecido, disponibilidade de nutrientes e substratos e condições de temperatura do local infectado (DRAGOS et al, 2018).

A formação de uma estrutura complexa como o biofilme é dividida em três estágios: (1) deposição de células planctônicas, que pode ser reversível ou irreversível com o auxílio de proteínas conhecidas como adesinas que auxiliam na adesão à superfície, (2) maturação das micro colônias depositadas e (3) dispersão de células do aglomerado pela corrente sanguínea tendo acesso a outros órgãos e tecidos e iniciando um novo ciclo (KHATOON et al, 2018). A figura 2 exemplifica as fases da formação de um biofilme em uma superfície biológica.

Figura 2 – Fases da formação e desenvolvimento de biofilmes microbianos



Fonte: elaborada pelo autor

As células constituintes do biofilme trabalham de modo a garantir estratégias de sobrevivência e eficiência na invasão dos tecidos do hospedeiro, bem como resistir a uma gama de agentes antimicrobianos aos quais são submetidas (NETT et al, 2020). Ademais, a capacidade da estrutura de ser eficiente na produção de barreiras físicas e químicas dificulta a ação do sistema imunológico dos pacientes em controlar o crescimento e desenvolvimento de tais estruturas. Por exemplo, os exopolissacarídeos constituintes da matriz extracelular atuam como agentes quelantes de drogas antimicrobianas, impedindo sua ação. Outrossim, é a síntese de enzimas extracelulares com capacidade para degradar ligações específicas de fármacos e inviabilizar sua atividade farmacológica (KAVANAUGH et al, 2019).

Ao adentrar nos tecidos, os biofilmes podem causar lesão tecidual e levar a quadros de infecção aguda. Algumas células de um aglomerado inicial podem se desprender da estrutura, invadir a corrente sanguínea e gerar novos biofilmes em tecidos saudáveis, levando a quadros de sepse generalizada (MOTTA et al, 2021). Outro fator que preocupa é que essas estruturas conseguem se formar não apenas sobre as superfícies biológicas, mas também àquelas implantadas no organismo com o intuito de prolongar o tempo de vida do paciente, como cateteres, marca-passos e válvulas cardíacas, inviabilizando seu uso em até pouco tempo após sua implantação (KURMOO et al, 2020).

Cerca de 40-50% de válvulas cardíacas e até 70% de cateteres implantados em pacientes contaminados com *Enterococcus faecalis*, *Staphylococcus Aureus* e *pseudomonas aeruginosa* são trocados devido a deposição de biofilmes microbianos nesses dispositivos

(KHATOON et al, 2018). Muitas outras doenças são desencadeadas no organismo com a deposição de biofilmes, destacando-se endocardite, fibrose cística, periodontites, sinusites e infecções renais (POURNAJAF et al, 2018; XI et al, 2019; ALONSO et al, 2021; WU et al, 2018; LEE et al, 2019). Associada a facilidade de se aderirem às superfícies biológicas e dispositivos médicos, os biofilmes contam ainda com a resiliência frente às drogas antimicrobianas, dificultando seu tratamento e erradicação (VESTBY et al, 2020). Nesse sentido, a necessidade do desenvolvimento de drogas alternativas às quais os microrganismos não sejam capazes de tolerar e que possam auxiliar no tratamento e prevenção de doenças é urgente.

2.5 Peptídeos antimicrobianos (PAMs) naturais e suas características

A prospecção de moléculas com alto potencial terapêutico e farmacológico tem se configurado como uma das áreas mais crescentes nas áreas de biologia, medicina e biotecnologia. Dentre as alternativas exploradas, os produtos naturais são fontes de novas moléculas biologicamente ativas como carboidratos, lipídios, proteínas e seus derivados. Entre estas, os peptídeos antimicrobianos naturais ganham um destaque especial pelo fato de serem encontrados em quase todos os organismos vivos, constituindo a primeira linha de defesa contra patógenos através da sua capacidade moduladora da resposta imunológica (LIMA et al, 2021).

O amplo espectro de atividade que essas moléculas possuem permite que sejam ativas contra uma gama de microrganismos incluindo bactérias, fungos, parasitas e vírus. Alguns trabalhos tem relatado também a atividade inseticida de peptídeos antimicrobianos, expandindo sua potencial aplicação também na agricultura (SOUZA et al, 2020). Tais atividades relatadas evidenciam uma forte relação com o principal mecanismo de ação desses peptídeos que envolve o ataque e desestabilização de membranas celulares, sendo capaz de atenuar ou impedir as probabilidades de desenvolvimento de mecanismos de resistência (LIMA et al, 2021).

Por serem moléculas globalmente difundidas, os peptídeos antimicrobianos possuem características em comum. Pode-se citar que estes peptídeos são formados por sequências que variam de 5 a 50 resíduos de aminoácidos e possuem carga peculiarmente positiva, a qual está relacionada com a presença de aminoácidos carregados positivamente como Lisina e Arginina, que garantem a interação com a membrana celular do microrganismo negativamente carregada do lado externo (OLIVEIRA et al, 2019). Associado a isso, o teor de aminoácidos hidrofóbicos varia de 40 a 50% entre as estruturas conhecidas e essa característica permite que os PAMs sejam capazes de interagir com as membranas biológicas, configurando-

lhes seu mecanismo de ação principal que consiste no ataque às membranas celulares. Porém, PAMs podem ser altamente tóxicos às células de mamíferos devido à presença em demasia de aminoácidos hidrofóbicos, o que causa lise celular inclusive nas células saudáveis do organismo hospedeiro (SOUZA et al, 2020).

Os resíduos de aminoácidos de caráter hidrofóbico e positivamente carregados permitem que os PAMs interajam especificamente com as membranas biológicas, desestabilizando-as. Essa atividade associa-se também à conformação adquirida pelos peptídeos em contato com o meio aquoso, sendo a estrutura em α-hélice a mais estudada devido as características anfipáticas que essa organização confere. PAMs com estrutura em folha β apresentam resíduos de Cisteína bem conservados que se responsabilizam pela estabilização da estrutura através de pontes dissulfeto (LIMA et al, 2021). Algumas sequências ricas em conteúdo de Arginina, Triptofano e Prolina contribuem na determinação de uma estrutura estendida, sem formação de estrutura secundária aparente.

2.6 Mecanismos de ação dos PAMs

Os componentes de estruturas celulares como membrana plasmática e parede celular devem ser as primeiras barreiras físicas superadas pelos PAMs para que se possa alcançar o interior da célula microbiana e desenvolver mecanismos alternativos de combate (LIMA et al, 2021). No entanto, essa superação deve levar em consideração inicialmente os componentes da parede celular como β-glucanos e polímeros de N-acetil-D-glicosamina que, em muitas vezes, são alvos de interação dos peptídeos, iniciando a desestabilização da estrutura celular ainda no primeiro contato (MAGANA et al, 2020). Alguns trabalhos, inclusive, relatam a existência de peptídeos com capacidade de interação com a quitina e outras estruturas da parede celular (AMARAL et al, 2021).

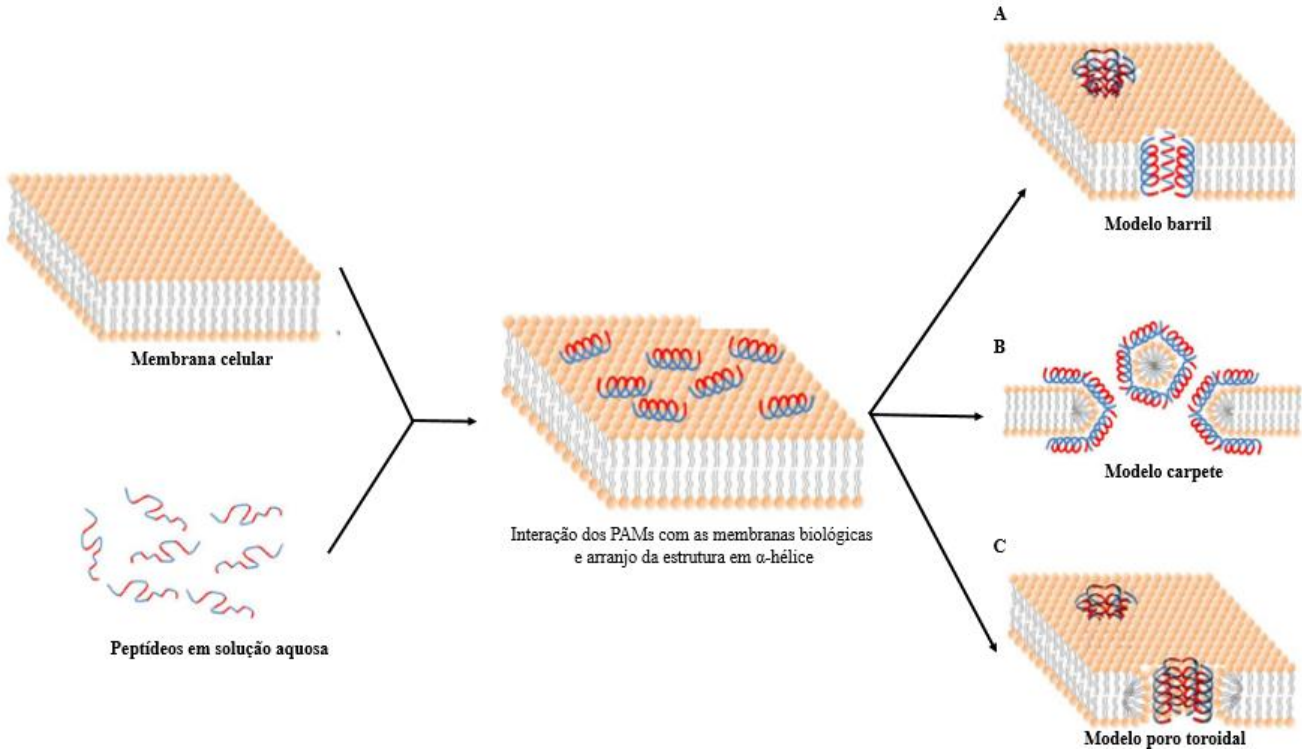
O rompimento da membrana e o acesso ao meio intracelular está intrinsecamente ligado à interação dos PAMs com a membrana celular que é garantida pelas características anteriormente descritas como carga líquida positiva (responsável por interagir com a carga negativa das porções polares dos lipídeos estruturais de membrana) e grau de hidrofobicidade (garantindo a interação com os fosfolipídios estruturais da bicamada lipídica) (AKBARI et al, 2018).

Estudos revelam os possíveis modos de interação com as membranas celulares dos microrganismos (Figura 3). Entre eles, o modelo de barril é responsável pela formação de um poro através da interação dos aminoácidos hidrofóbicos com os lipídeos de membrana. Em

determinada concentração, as unidades monoméricas peptídicas se organizam e interagem entre si de modo a atenuar a exibição dos resíduos hidrofóbicos formando poros inteiramente inseridos na porção lipídica da membrana celular (LIMA et al, 2021). Um outro mecanismo proposto e denominado modelo de poro toroidal explica que diferentemente do mecanismo anterior, os peptídeos não interagem entre si formando agregados peptídicos, mas permitem a interação dos seus grupos polares com as porções carregadas dos fosfolipídios de membrana (LIMA et al, 2021).

Adicionalmente, o modelo de carpete propõe que os peptídeos não promovem a formação de poros, mas agem de maneira aleatória sobre a membrana, desestabilizando-a com ação similar àquela apresentada pelas substâncias surfactantes. Esse mecanismo leva em consideração a interação entre as porções positivas e negativas dos peptídeos e dos fosfolipídios de membrana, respectivamente. Uma concentração elevada de PAMs depositados sobre a membrana leva a desestabilização da estrutura e, consequentemente, à formação de micelas (BOGDANOVA et al, 2020).

Figura 3: Exemplificação de alguns mecanismos de ação dos PAMs contra membranas



Fonte: LIMA et al, 2020 (com adaptações)

Modelo barril (A), Modelo carpete (B) e Modelo poro toroidal (C). Em azul, destacam-se as regiões hidrofílicas e em vermelho as regiões hidrofóbicas dos aminoácidos constituintes das PAMs

Apesar de ser o principal, a desestabilização e rompimento da membrana celular do patógeno não é o único mecanismo de ação desenvolvido pelos PAMs (CORRÊA et al, 2019). Muitos peptídeos conseguem atravessar as membranas biológicas sem causar dano algum, alcançam a matriz celular e interagem com alvos intracelulares específicos, impedindo o correto funcionamento de rotas metabólicas importantes ao desenvolvimento e manutenção das atividades biológicas dos microrganismos. Além disso, há estudos que evidenciam a participação dos PAMs em eventos de inibição da síntese de parede celular, replicação, transcrição, inibição de enzimas importantes e, inclusive, indução da morte celular programada (MORAVEJ et al, 2018). Alguns trabalhos relatam ainda a capacidade de PAMs desencadearem uma superprodução de espécies reativas de oxigênio que leva a desestabilização de proteínas, carboidratos e ácidos nucleicos, gerando a desregulação de rotas metabólicas celulares, embora os fatores que iniciam esse processo ainda sejam desconhecidos (LIMA et al, 2020).

2.7 Peptídeos antimicrobianos sintéticos

Embora os mecanismos de ação apresentados pelos PAMs naturais sejam promissores em atacar a membrana do microrganismo, desestabiliza-la e, assim, desenvolver seu potencial terapêutico, essas moléculas apresentam algumas características indesejáveis que acabam inviabilizando o seu uso (LIMA et al, 2021). Estudos mostram que os PAMs naturais são facilmente degradados por proteases do hospedeiro, apresentando ainda baixa seletividade e hidrossolubilidade. Além disso, estas moléculas podem ser altamente tóxicas para o organismo humano bem como causar hemólise em células sanguíneas saudáveis (CAPECCHI et al, 2021). Sua obtenção direta de produtos naturais também se configura como um problema pois os processos de purificação e obtenção dessas moléculas geralmente apresentam um baixo rendimento, inviabilizando sua produção de forma escalonada e acarretando em alto custo para sua obtenção (OLIVEIRA et al, 2019).

Com o intuito de superar tais adversidades, o desenho racional de peptídeos sintéticos tem se sobressaído como uma alternativa promissora na utilização de tais agentes como potentes mediadores farmacológicos (SOUZA et al, 2020). O desenho desses peptídeos consiste numa relação direta entre a estrutura dos peptídeos gerados com sua atividade, tendo em vista a possibilidade de manipulação dos aminoácidos constituintes da sequência peptídica afim de melhorar seu potencial terapêutico (TAN et al, 2021).

Dentre as ferramentas aplicadas para obtenção das sequencias desejadas está a possibilidade de uso de uma proteína natural com atividade antimicrobiana conhecida para o desenho de peptídeos com características semelhantes. As ferramentas de bioinformática disponíveis também podem auxiliar na seleção das melhores sequências proteicas sugerindo quais fragmentos destas proteínas poderiam gerar peptídeos com atividade antimicrobiana (SOUZA et al, 2020). Softwares disponíveis gratuitamente garantem a realização de ensaios *in sílico* para verificação do potencial antimicrobiano dos peptídeos gerados como também sugerem possíveis modificações afim de otimizar suas atividades (SOUZA et al, 2020). Características adicionais podem ser adotadas como aumento da concentração de resíduos de aminoácidos hidrofóbicos ou positivamente carregados, garantindo uma melhor interação com a membrana dos microrganismos (KUMAR et al, 2021; LIMA et al, 2021; SOUZA et al, 2020).

2.8 Peptídeos inspirados em *Mo*-CBP3 e uma quitinase de *A. thaliana.*

Nosso grupo de pesquisa identificou, purificou e caracterizou uma proteína ligante à quitina de sementes de *Moringa oleífera*, a qual foi denominada *Mo*-CBP3. Tal proteína foi capaz de inibir a germinação e crescimento micelial dos fungos fitopatogênicos *Fusarium solani*, *Fusarium oxysporum, Colletotrichum musae* e *Colletotrichum gloeosporioides* mesmo em baixas concentrações (2,8 μM). Apesar dos bons resultados, a proteína não mostrou uma atividade eficiente contra patógenos humanos como fungos leveduriformes do gênero *Candida*, inclusive na sua concentração mais elevada de 299,30 μM. Ademais, o baixo rendimento durante o processo de purificação demonstrou ser um processo desfavorável (BATISTA et al, 2014; GIFONI et al, 2012; NETO et al, 2017).

Com isso, peptídeos sintéticos bioinspirados na sequência primária de *Mo*-CBP3 foram desenhados, com o objetivo de melhorar a atividade dessa proteína e os peptídeos gerados foram denominados *Mo*-CBP3-PepI, *Mo*-CBP3-PepII e *Mo*-CBP3-PepIII, de acordo com as metodologias descrita por OLIVEIRA et al, 2019 e DIAS et al, 2020. Além disso, um estudo inédito realizado também pelo nosso grupo de pesquisa visou o estabelecimento de um protocolo para determinação de sequências peptídicas com alto potencial antimicrobiano. Tal trabalho culminou no desenho de dois peptídeos baseados na sequência de uma quitinase de *Arabidopsis thaliana*, denominados PepGAT e PepKAA que mostraram ser eficientes contra células planctônicas de *Candida albicans, Bacillus subtilis*, *Staphylococcus aureus* e *Salmonella enterica*, bem como contra a germinação de esporos de fungos dermatófitos do gênero *Trichophyton* (SOUZA et al, 2020). A realização deste trabalho foi possível graças a

utilização de servidores disponíveis gratuitamente e de alta eficiência no campo da bioinformática.

A sequência de *Mo*-CBP3 de *M. oleifera* bem como a de uma quitinase de *A. thaliana* disponível no NCBI (número de acesso: NP_181885.1) foram empregadas no desenho dos peptídeos através do servidor http://bioserver-3.bioacademy.gr/Bioserver/CPAmP/Peptide_index.php, disponível gratuitamente. Além disso, foram estabelecidos alguns parâmetros (Tabela 1) como carga líquida positiva, massa molecular variando de 600 a 1200 Da, índice de Bowman ≤ 2,5 e conteúdo de aminoácidos hidrofóbicos de, no mínimo, 40%. O Software Banco de Dados de Peptídeos (http://aps.unmc.edu/AP/) foi utilizando para avaliar os parâmetros estabelecidos e as pontuações Tm e sOPEP obtidas pelo servidor PEP-FOLD. (SOUZA et al, 2020)

Tabela 1 – Propriedades físico-química dos peptídeos sintéticos Mo-CBP3-PepI, Mo-CBP3-PepII, Mo-CBP3-PepIII, PepGAT e PepKAA

Propriedades físico-químicas	Peptídeos sintéticos				
	Mo-CBP3-PepI	*Mo*-CBP3-PepII	*Mo*-CBP3-PepIII	PepGAT	PepKAA
Sequência	CPAIQRCC	NIQPPCRCC	AIQRCC	GATIRAVNSR	KAANRIKYFQ
[1]Massa molecular calculada (Da)	893.12	1033.26	692.86	1044.18	1238.44
[2]Massa molecular experimental (Da)	893.10	1033.23	692.85	1044.90	1239.00
[1]Ponto Isoelétrico (pI)	7.97	7.97	8.11	12.00	10.29
[3]Índice de Bowman	1.23	2.03	1.86	2.19	2.28
[3]Razão Hidrofóbica Total (%)	62	44	66	40	40
[3]Carga líquida (+)	1	1	1	2	2
[4]Gráfico de Ramachandran (%)	100	67,7	83,3	98	75
[5]Tm	0,984	0,428	1,00	0,360	0,414
[5]sOPEP	-9.31	-8.16	-3.68	-7.39	-18.18

Fonte: OLIVEIRA et al, 2019; SOUZA et al, 2020.
[1]Obtido em ProtParam (https://web.expasy.org/protparam/)
[2]Resultados obtidos em função de espectrometria de massa conduzida.
[3]Obtido por APD3 (http://aps.unmc. edu/AP/prediction/prediction_main.php)
[4]Obtido por Rampage ((http://mordred.bioc.cam.ac.uk/~rapper/rampage.php)
[5]Obtido por PepFOLD 3.0 (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms;PEP-FOLD).

Para determinar as características antimicrobianas dos peptídeos gerados, as sequências peptídicas foram analisadas em alguns servidores disponíveis gratuitamente (Tabelas 2). A capacidade de cada peptídeo em interagir com membranas biológicas foi determinada através do servidor Cell Penetrating Peptides

(http://crdd.osdd.net/raghava/cellppd/multi_pep.php); O potencial antimicrobiano foi verificado utilizando o software iAMPpred (http://cabgrid.res.in:8080/amppred/); A previsão do potencial alergênico foi permitida com o auxílio da plataforma Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl); A capacidade de resistência dos peptídeos gerados frente às enzimas proteolíticas foi determinada por Peptide Cutter (http://imed.med.ucm.es/Tools/antigenic.pl) e a meia-vida bem como a estabilidade de cada molécula em ambiente semelhante ao intestino foram determinadas usando o servidor HLP (http://crdd.osdd.net/raghava/hlp/help.html) (SOUZA et al, 2020).

Tabela 2 – Determinação do potencial antimicrobiano, resistência a proteólise e estabilidade de peptídeos sintéticos

	Peptídeos sintéticos				
Propriedades analisadas	*Mo*-CBP3-PepI	*Mo*-CBP3-PepII	*Mo*-CBP3-PepIII	PepGAT	PepKAA
[1]CPP	Sim	Sim	Sim	Sim	Sim
[2]Potencial alérgico	Não	Não	Não	Não	Não
[3]Potencial antimicrobiano (%)					
Antibacteriano	83	71	89	77	60
Antifúngico	87	80	92	60	89
[4]Sítios de clivagem					
Tripsina (pH)	1	1	1	Sim	Sim
Pepsina (pH 1.3)	0	0	0	Sim	Não
Pepsina (pH > 2)	0	0	0	Sim	Não
[5]Tempo de meia-vida	1.265	1.209	1.309	0,879	1.115
[6]Estabilidade	Alta	Alta	Alta	Normal	Alta

Fonte: OLIVEIRA et al, 2019; SOUZA et al, 2020.
[1]Obtido por Cell PPD (http://crdd.osdd.net/raghava/cellppd/multi_pep.php)
[2]Obtido por Antigenic Prediction tool (http:// imed.med.ucm.es/Tools/antigenic.pl)
[3]Obtido por iAMPpred (http:// cabgrid.res.in:8080/amppred/)
[4]Obtido por Peptide Cutter (http://web.expasy. org/peptide_cutter/)
[5,6]Obtido por Half Life Prediction (http:// crdd.osdd.net/raghava/hlp/help.html)

Resultados preliminares mostraram a atividade de *Mo*-CBP3-PepI, *Mo*-CBP3-PepII e *Mo*-CBP3-PepIII contra as formas planctônicas das espécies *Staphylococcus aureus, Candida parapsilosis* e *Candida albicans*, bem como contra fungos dermatófitos do gênero *Trichophyton* (OLIVEIRA et al, 2019; LIMA et al, 2020). PepGAT e PepKAA apresentou atividade contra o fungo fitopatogênico *Penicillium digitatum* (LIMA et al, 2021) e contra as formas livres das bactérias patogênicas *S. aureus, B. subtilis, S. enterica* e *E. aerogenes*, bem como contra a levedura *C. albicans* (SOUZA et al, 2020). No entanto, mais estudos são necessários utilizando tais peptídeos contra as formação de biofilmes antimicrobianos, levando

em consideração que tais estruturas apresentam maiores chances de resistência às drogas atualmente disponíveis.

2.9 Sinergismo

O uso de fármacos antimicrobianos com mecanismos de ação iguais ou diferentes combinados entre sí é conhecido por sinergismo. Essa atividade sinérgica ocorre quando o uso consoante de pelo menos dois fármacos diferentes resulta em uma maior atividade do que quando ambos atuam de maneira separada (PATRA et al, 2021). PAMs são capazes de interagir com drogas antifúngicas e antibióticos levando à formação de poros na membrana e promovendo a entrada desses agentes dentro da célula, facilitando a execução do seu potencial farmacológico que pode atuar sobre a replicação do DNA, inibição da síntese proteica e síntese os componentes da parede celular (GUCWA et al, 2018). Adicionalmente, essa combinação pode ser um indicativo de quebra da resistência desenvolvida pelos patógenos que conseguem impedir a entrada da droga no seu meio intracelular, resultando em baixa ou nenhuma atividade. Outro ponto importante é que o sinergismo é responsável por diminuir as concentrações de drogas específica que apresentam potencial tóxico para as células do hospedeiro, minimizando os efeitos deletérios ao organismo (LI et al, 2020). Dessa forma, além de haver uma redução no custo do tratamento, há a possibilidade de prevenir o desenvolvimento de mecanismos de resistência pelos microrganismos, permitindo que os PAMs atuem como moléculas adjuvantes às drogas já utilizadas para tratamento (LIMA et al, 2021).

Para determinar o potencial sinérgico de tais moléculas é usado o Índice de Concentração Inibitória Fracionada (ICIF), que é obtido com base na concentração inibitória média de cada composto em combinação dividido pela concentração inibitória média de cada composto atuando sozinho, conforme a formula: [ICIF = CIFfármaco+ CIFpeptídeos = (CIfármacocomb/CIfármacosozinho) + (CIpeptídeoscomb/CIpeptídeossozinho)]. Com isso, os resultado obtidos podem ser classificados em antagônico (ICIF $\geq$ 4,0), indiferente (cuja atividade não foi alterada por nenhuma combinação) (ICIF 0,5 -4,0) e efeito sinérgico (ICIF $\leq$ 0,5) (SOUZA et al, 2020).

3. HIPÓTESE

Mo-CBP3-PepI, *Mo*-CBP3-PepII, *Mo*-CBP3-PepIII, PepGAT e PepKAA são peptídeos antimicrobianos sintéticos que possuem atividade antibiofilme, atuando na inibição e na redução da biomassa de biofilmes de *Candida* spp. através da formação de poros na membrana celular e indução da superprodução de espécies reativas de oxigênio.

4. OBJETIVOS

4.1 Objetivo geral

- Determinar o potencial antibiofilme dos peptídeos *Mo*-CBP3-PepI, *Mo*-CBP3-PepII, *Mo*-CBP3-PepIII, PepGAT e PepKAA *in vitro* e determinar os mecanismos de ação por trás de tal atividade.

4.2 Objetivos específicos

- Avaliar as propriedades físico-químicas dos peptídeos sintéticos;
- Determinar a atividade antibiofilme de peptídeos contra biofilmes formados por espécies do gênero *Candida*;
- Elucidar os possíveis mecanismos de ação envolvidos em tal atividade através de técnicas de microscopia eletrônica e microscopia de fluorescência;
- Analisar o efeito de sinergismo estabelecido com antifúngicos comerciais como Nistatina e Itraconazol;
- Determinar os níveis de toxicidade dos peptídeos contra células humanas.

5. CAPÍTULO II – Artigo científico 1

Para as sessões Metodologia, Resultados e Discussão foram utilizado artigos científicos submetidos em revistas científicas internacionais. O artigo Combined Antibiofilm Activity of Synthetic Peptides and antifungal drugs against *Candida spp*: Action mechanisms and clinical application to overcome the resistance towards antifungal drugs mostrou os índices de inibição bem como mecanismos de ação da atividade antifúngica de PepGAT e PepKAA contra biofilmes formados por leveduras das espécies *Candida albicans* e *C. krusei*.

5.1 Artigo científico 1

Artigo científico submetido à revista *Frontiers in Microbiology* fator de impacto 5.76 (Qualis A1), status *under review*.

Artigo científico 1

Combined Antibiofilm Activity of Synthetic Peptides and antifungal drugs against *Candida spp*: Action mechanisms and clinical application to overcome the resistance towards antifungal drugs

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Abstract

Yeasts belonging to the *Candida* genus are important human pathogens. *Candida* biofilm is the most common resistance mechanism, increasing 1,000 times the resistance to antifungal drugs. This study aimed to evaluate the antibiofilm activity of synthetic peptides, as well as action mechanisms and synergistic effect with Nystatin (NYS) and Itraconazole (ITR) by Scanning Electron Microscopy (SEM) and Fluorescence Microscopy (FM). ITR (1000 μg. mL^{-1}) inhibited 10% biofilm formation of *C. krusei* and NYS (1000 μg. mL^{-1}) 40% of *C. albicans*. Regarding synergistic effect, peptides enhance 7-fold the action of ITR to inhibit the biofilm formation of *C. krusei* and *C. albicans* and the degradation of formed biofilm of *C. krusei*. In combination with antifungal drugs, peptides' action mechanism involves cell wall damage, membrane pore formation, loss of cytoplasmic content, and overproduction of reactive oxygen species (ROS). Docking analysis revealed ionic and hydrophobic interactions between peptides and both drugs, which may explain the synergistic effect. Altogether, our results suggest the synthetic peptides enhance the antibiofilm activity of NYS and ITR have some potential to be employed as adjuvants and decrease the toxicity of drugs.

Keywords: Antibiofilm activity; Candidiasis; Synergism; *Candida*; Synthetic peptides; Antifungal drugs; Nystatin and Itraconazole; Action mechanisms; Clinical application.

Introduction

The fast increase of antimicrobial resistance to drugs globally is driving humanity to a "post-antibiotic era" where the available drugs will no longer work (Lima et al., 2021). In this scenario, infections caused by fungi have gained attention. Estimates suggest 200 million new cases caused by resistant fungi every year of infections with approximately 1 million deaths(M et al., 2015). Among the fungi that affect humans, those belonging to *Candida* genus 6 have a higher prevalence, 7 cases per 100 patients (Morgan et al., 2005; GD et al., 2012; Sanguinetti et al., 2015).

Candida genera affect debilitated patients, as such transplanted, cancer patients, and immune-suppressed, leading to a bloodstream infection, prolonged hospital stays, and high mortality rates (Morgan et al., 2005; GD et al., 2012; Sanguinetti et al., 2015). Until 2010, *C. albicans* carrying resistance to multiple drugs was responsible for at least 60% of clinical infections (Sanguinetti et al., 2015). Nowadays, this scenario has changed. Today, infections caused by non-*C. albicans* reached 56.5% of *Candida* infections. The new cases are divided *into C. glabrata* (33.3%), *C. tropicalis* (20.3%), *C. krusei* (1.4%), and *C. kefyr* (1.4%) (Fu et al., 2017). Another important non-albicans pathogenic yeast is *C. auris*, first described in 2009 in Japan. Today, *C. auris* is considered a Pan-resistant yeast, presenting higher resistance rates to azoles, polyenes, and echinocandins(Ademe and Girma, 2020). Even though this change *C. albicans* still holds the higher number of cases, 43.5% (Fu et al., 2017).

Many pathogenic yeasts like *Candida* spp. have developed large drug spectra resistance mechanisms. Nevertheless, one of the most important is the ability to form biofilms. The production of biofilms could increase drug resistance up 1000-fold to conventional drugs compared to free cells (De La Fuente-Núñez et al., 2016). Besides protecting by altering the pH and

osmolarity, preventing the nutrients scarcity, and alleviating mechanical, and shear forces, the matrix of biofilms boosts the resistance by protecting yeasts cells from drugs and the host´s immune response (De La Fuente-Núñez et al., 2016; Cavalheiro and Teixeira, 2018; Marak and Dhanashree, 2018; Costa-de-Oliveira and Rodrigues, 2020). It is not entirely clear the triggers to the *quorum sense* gene expression, but the composition of the matrix is known, which is rich in carbohydrates, proteins, lipids, nucleic acids, and water (Cavalheiro and Teixeira, 2018).

Biofilm development to a high level of fungal resistance with clinical implications worldwide given the small groups of antifungal agents bringing the concept of "post-antifungal" era (Chowdhary et al., 2017). In this context, the antimicrobial peptides active against biofilm could be an alternative to cope with this problem. Over the years, many researcher groups have sought antimicrobial peptides' active fungal biofilms alone or in combination with conventional drugs (Fjell et al., 2012). However, biofilm-active peptides have not yet achieved clinical trials and even commercial use. Therefore, such molecules' development, optimization, or design is needed to fight back fungal biofilms (Duncan and O'Neil, 2013).

To overcome the threat imposed by yeast's resistance to drugs, synthetic antimicrobial peptides (after that called synthetic AMPs) are an alternative either as a new drug to work alone or even as adjuvants enhancing the activity of commercial drugs(Mohamed et al., 2016; Lima et al., 2021). Synthetic AMPs were designed to have a positive charge (at least +1), amphipathic properties, acceptable hydrophobic rates (40 to 66%) (Oliveira et al., 2019; Dias et al., 2020; Souza et al., 2020b), and no-host toxicity. Breakthroughs in chemical synthesis technologies led to reduced costs for synthesis, high purity, and high amount of synthetic AMPs (Lima et al., 2021). The most advantageous technology developed allows the recovery and recycling of solvents used

during the synthesis leading to a reduced cost of synthesis, allowing the clinical application of synthetic peptides. For example, Fuzeon and Rybelsus are synthetic peptides already used to treat HIV and diabetes (Buckley et al., 2018; Pennington et al., 2021).

Recently, two synthetic peptides (PepGAT and PepKKA) were reported to strongly inhibit, at low concentrations, the growth of species of the *Candida* genus (Souza et al., 2020b). Thus, we reasoned that both peptides could be effective against biofilm from the *Candida* genus and develop synergistic effects with drugs that lost activity. The antibiofilm activity and action mechanisms PepGAT and PepKKA against *C. albicans* and *C. krusei* are described and characterized, employing advanced microscopy techniques. Furthermore, the results revealed that the synergistic effects of peptides enhanced the antibiofilm activity of commercial drugs, Nystatin (NYS) and Itraconazole (ITR), which were no longer active towards *Candida* biofilm.

Methods

Ethical Statement

Not applied for this study.

Biological materials

The clinical isolates of *C. albicans*, *C. krusei*, and *C. parapsilosis* were from the laboratory of Plant Toxins at the Department of biochemistry and molecular biology of the Federal University of Ceará (UFC) Fortaleza, Brazil.

Peptide synthesis

The synthetic peptides PepGAT and PepKAA (Souza et al., 2020b) were chemically synthesized by the company GenOne (São Paulo, Brazil), which analyzed their quality and purity (≥ 95%) by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (Souza et al., 2020b).

Biological activity

Antibiofilm and Combined effect between peptides and drugs assays

The effect of PepGAT and PepKAA on the *Candida* spp. biofilm formation was evaluated in polystyrene flat-bottom 96-well microtiter plates (Dias et al., 2020) and as indicated by the Clinical and Laboratory Standards Institute (CLSI) M38-A2 method (M38-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard-Second Edition, 2008). The cell suspensions were prepared from yeasts cultured for 18 h at 37 °C in the Sabouraud broth (Kasvi, Brazil). The cell concentration was diluted to 10^6 cells mL^{-1} in the growth medium. One hundred microliters of the cell suspensions (10^6 cells mL^{-1}) were incubated at 37 °C for 48 h in the dark with 100 μL of the synthetic peptides at concentration (50 μg mL^{-1}) in a solution composed of 5% DMSO (Dimethyl sulfoxide) in 0.15 M NaCl (DMSO-NaCl). After that, the supernatant was discarded, and the wells were washed with sterile 0.15 M NaCl, air-dried for 30 min, and the biofilm formed was stained with an aqueous solution of 0.1% (m/v) crystal violet (Sigma Aldrich, São Paulo, Brazil), for 15 min at room temperature (24 ± 2 °C). The excess of crystal violet was removed by washing three times with sterile 0.15 M NaCl (Dias et al., 2020). Finally, 250 μL of 95% (v/v) ethanol was added to solubilize the bound crystal violet, and absorbance was taken in a microplate reader (BioTekTM ELx800TM, BioTek Instruments, Inc.,

USA) at a wavelength of 570 nm. The inhibition of biofilm formation was calculated by comparing the absorbance readings of cells treated with synthetic peptides and those obtained from cells treated with DMSO-NaCl solution (negative control), ITR (1000 μg mL^{-1}), and NYS (1000 μg mL^{-1}) both used as positive controls (Dias et al., 2020).

A second experiment was carried out to evaluate the effect of the peptides on the degradation of the preformed biofilms (Dias et al., 2020). Two hundred microliters of the cell suspensions (10^6 cells mL^{-1}, prepared as described above) were incubated at 37 °C for 24 h in the dark to form the biofilm. Then, the supernatant was gently aspirated with a micropipette to remove the planktonic cells. In each well, 100 μL of each synthetic peptide (50 μg mL^{-1}) or controls plus 100 μL growth medium (Sabouraud broth, Kasvi, Brazil) were added. The plates were incubated for 24 h at 37 °C, in the dark. The culture medium was again discarded, and the same procedure described above was used to quantify the biofilm biomass remaining after incubation with the peptide (Dias et al., 2020). The controls use were the same from inhibition of biofilm formation.

The synergism assays were performed between peptides with either antifungals NYS or ITR: The combinations were constituted of each peptide (50 μg mL^{-1}) + NYS or ITR (1000 μg mL^{-1}) (Souza et al., 2020a). The control to evaluate the effectiveness of synergism was the activity presented by peptides or drugs alone. After the formulation of combinations, the antibiofilm assays for synergism analyses for *Candida* spp. were the same as described above.

Study of mechanisms of action of peptides against biofilms

Biofilm integrity by scanning electron microscopy (SEM)

Morphological changes in the biofilm of *C. albicans* and *C. krusei* were evaluated by SEM, as previously described by Staniszewska et al. (2013) with modification. The assay was performed as described above (see the Antibiofilm section), but, this time, the biofilm was grown on the

coverslip (previously treated with 0.1% gelatin [Sigma Aldrich, São Paulo, Brazil]) inside a well of six-well plates. After, the biofilm was fixed with fixation buffer [in 1% (v/v) glutaraldehyde + 4% (v/v) formaldehyde in 0.15 M sodium phosphate buffer at pH 7.0]. Next, treated with 0.2% (m/v) osmium tetroxide (Sigma Aldrich, São Paulo, Brazil) for 30 min in the dark, and successively dehydrated with ethanol concentration (30, 70, 100, 100, and 100% [v/v]) for 10 min each, after each centrifugation wash the ethanol was aspirated with an automatic pipette. Finally, final dehydration was carried out with 50/50 ethanol/hexamethyldisilazane (HMDS) for 10 min and lastly with 100% HMDS (Sigma Aldrich, São Paulo, Brazil). Then, each coverslip was removed from each well and assembled on stubs and coated with a 20 nm gold layer using a positron-emission tomography (PET) coating machine (Emitech-Q150TES, Quorum Technologies, England). Images were made in an FEI Inspect^{TM} 50 scanning electron microscope (Oregon, USA), equipped with a low energy detector (Everhart-Thornley) using acceleration beam voltage of 20,000 kV and 20,000x detector magnification (Souza et al., 2020a). The treatments for this assay were peptides alone, drugs alone, and solutions made by peptides and drugs.

Biofilm integrity determined by propidium iodide (PI) uptake

The biofilms were produced as described in the biofilm assays and the SEM microscopy sections. After, peptide-, controls-, and drugs-peptides mixed solutions treated biofilm were incubated with 10^{-3} M of propidium iodide (PI, Sigma Aldrich, São Paulo, Brazil), in the dark, at 37 ºC for 30 min (Souza et al., 2020a). Then, the biofilm-containing coverslips were washed with 0.15 M of NaCl three times to remove the unbound PI and observed under a fluorescent microscope (Olympus System BX 60, Japan) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Overproduction of reactive oxygen species (ROS) by biofilm

The overproduction of ROS was evaluated as described by Dias et al. (2020) with modifications. The biofilms were produced as described in the biofilm assays and the SEM microscopy sections. After, peptide-, controls-, and drugs-peptides mixed solution treated biofilm were incubated with 10 μM of 2',7' dichlorofluorescein diacetate (DCFH-DA, Sigma Aldrich, St., Louis, USA) at 37 °C in the dark for 30 min (Souza et al., 2020a). Then, the biofilm-containing coverslips were washed with 0.15 M of NaCl three times to remove the unbound PI and observed under a fluorescent microscope (Olympus System BX 60, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Hemolytic assay

The hemolytic activity of synthetic peptides, NYS, ITR, and combination by then was tested in A, B, and O-types of human red blood cells (HRBC), according to Souza et al. (2020). The combined solutions of peptides and either NYS or ITR were the same as used in synergism assays. The HRBCs from A, B, and O were provided by the Ceará Hematology and Hemotherapy Center (Ceará, Brazil).

The blood was collected in the presence of heparin (5 IU mL^{-1}, Sigma Aldrich, São Paulo, Brazil), centrifuged at 300 g for 5 min at 4 °C, and gently dissolved in sterile 0.15 M NaCl. The blood was washed three times with 0.15 M NaCl and diluted to a concentration of 2.5% in 0.15 M NaCl used in the assay. One hundred microliters of each blood type were incubated, individually, with solutions of synthetic peptides (50 μg mL^{-1}), NYS (1000 μg mL^{-1}), ITR (1000 μg mL^{-1}), the solution made by peptides and drugs, DMSO-NaCl, and 0.1% (v/v) Triton X-100 (the positive control for hemolysis) for 30 min at 37 °C, followed by centrifugation (300 g for 5 min at 4 °C, in centrifuge Eppendorf 5810, Germany). Then, the supernatants were collected and transferred to

96-well microtiter plates. Hemolysis (%) was calculated by measuring the supernatant absorbance at 414 nm using an automated absorbance microplate reader. Negative (0%) and positive (100%) hemolysis were determined by treating HRBCs with 5% DMSO in 0.15 M NaCl (vehicle for peptides) and 0.1% (v/v) Triton X-100, respectively. The equation calculated the hemolysis: [(Abs_{414nm} of HRBC treated with peptides-Abs_{414nm} HRBCs treated with 0.15 M NaCl) / [(Abs_{414nm} of HRBCs treated with 0.1% TritonX-100-Abs_{414nm} of HRBCs treated with 0.15 M NaCl)] x 100.

Statistical analysis

All the experiments were performed three times, and the statistics were expressed as the mean ± standard error. The data were submitted to one-way analysis of variance (ANOVA) followed by the Tukey test, using GraphPad Prisma 5.01 (GraphPad Software company, California, USA), with the significance of $p < 0.05$.

RESULTS

Antibiofilm activity of peptides and synergistic effect with antifungal drugs

The peptides (50 µg. mL^{-1}) presented different behaviors toward the biofilm of *Candida* ssp (Fig. 1). Either the peptides, antifungal drugs, or combinations had any activity against the biofilm of *C. parapsilosis* or *C. tropicalis* (Supplementary Fig. 1C and D). The first assay was done to evaluate the ability of peptides to inhibit the biofilm formation of *C. krusei* and *C. albicans* (Fig. 1A and B). To *C. krusei*, both peptides barely reach 10% of inhibition. ITR and NYS reached 20 and 0% of inhibition of biofilm formation by *C. krusei* (Fig. 1A). In contrast, the combinations of both PepGAT (50 µg mL^{-1}) + ITR (1000 µg mL^{-1}) and PepKAA (50 µg mL^{-1}) + ITR (1000 µg mL^{-1}) led to an inhibition, respectively, of 80 and 76% of *C. krusei* biofilm (Fig. 1A). The combination of peptides with NYS was not effective. Regarding *C. albicans*, NYS, PepGAT, and PepKAA

inhibited biofilm formation, 40, 10, and 20%. An interesting result was the synergism found in the combinations made by PepGAT + ITR, PepGAT + NYS, and PepKAA + NYS that inhibited, respectively, 40%, 95%, and 98% the biofilm formation of *C. albicans* (Fig. 1B).

Regarding the degradation of formed biofilm, no results were found to *C. albicans, C. parapsilosis*, and *C. tropicalis* (Supplementary Fig. 1A and B), only toward *C. krusei* (Fig. 1C). ITR, PepGAT, and PepKAA alone did not reduce biomass of preformed biofilm of *C. krusei* (Fig. 1C). In contrast, NYS was able to reduce in 20% the biomass of *C. albicans* preformed biofilm (Fig. 1C). Except for the PepGAT + NYS combination that did not significantly decrease biomass reduction, PepGAT + ITR, PepKAA + ITR, and PepKAA + NYS reduced in, respectively, 50%, 30%, and 15% the preformed biofilm of *C. albicans* (Fig. 1C).

Biofilm integrity and ROS overproduction

The evaluation of peptides alone and the combination with drugs affect the membrane of the biofilm cells. Fluorescence microscopy was employed (Figs. 2-5). Propidium iodide (PI) is a fluorophore that binds to DNA, releasing red fluorescence. However, PI only crosses the damaged cell membrane, and a healthy membrane blocks the movement of PI, leading to no fluorescence at all. As expected, all controls made by DMSO-NaCl solution (the vehicle of peptides) presented no fluorescence, suggesting the membranes in control cells have no type of pore formed (Figs. 2-5). An interesting result was the absence of fluorescence in biofilms treated with drugs (NYS and ITR) (Figs. 2-5).

Regarding the synthetic peptides, PepGAT did induce fluorescence in all treatments either in the inhibition of biofilm formation although at different intensities (Figs. 2-4) and degradation of preformed biofilm (Fig. 5). The PepKAA peptides presented a different behavior. In the case of the inhibition of biofilm formation by *C. krusei*, PepKAA alone could not induce damage in the

membrane to allow PI to pass by the membrane and emit fluorescence (Fig. 2). In contrast, PepKAA induced the fluorescence in inhibiting biofilm formation by *C. albicans* (Fig. 4). In the case of combinations made by peptides and antifungal drugs, all presented fluorescence (Figs. 2-5). This is an exciting result for three reasons: 1) drugs alone did not show any fluorescence (Figs. 2-5); 2) in the case of PepKAA, which did not show fluorescence, the combination with ITR led to a release of fluorescence (Figs. 2 and 3) in some cases, such as PepGAT+ITR (Figs. 3 and 5) fluorescence produced by the combination was higher than that produced by drugs alone, suggesting a bigger number of cell damage in the combination.

The experiments to evaluate the ROS overproduction in the biofilms revealed a different pattern than PI experiments. All positive or negative controls did not produce any fluorescence indicating no ROS production (Figs. 2-5). In inhibiting the biofilm formation of *C. krusei*, both peptides induced ROS production at different intensities, as revealed by fluorescence. In this case, the cells treated with PepGAT were brighter than those produced by PepKAA. Following this pattern, the combinations made to inhibit the biofilm formation by *C. krusei* produced only a slight fluorescence, indicating a low ROS level (Fig. 2). In the inhibition of biofilm formation by *C. albicans* to ROS production, only a slight production of ROS was indicated by a faint fluorescence in the treatment with PepKAA (Fig. 4). In contrast, brighter fluorescence indicates a higher production of ROS in both PepGAT alone and combined with ITR in the degradation of preformed biofilm of *C. krusei* (Fig. 5), suggesting that the ROS overproduction is, indeed, an important mechanism to degrade the biofilm.

Scanning electron microscopy (SEM) analyses of biofilm

SEM images revealed severe that all control made with DMSO-NaCl solution the biofilm was in an excellent spherical shape, with no cracks or damage, and cells were seen with no visible

damage to membrane or cell wall (Figs. 6-8). The SEM analysis revealed that the cells involved in biofilm formation (Figs. 6 and 7) and preformed biofilm (Fig. 8) were not affected by the treatment with either ITR or NYS, both at 1000 μg mL^{-1}.

SEM analysis strength the damages revealed by fluorescence microscopy in *Candida* cells (Figs. 2-5), which were confirmed by SEM analysis (Fig. 6-8). To inhibit the formation of *C. krusei*, PepGAT induced several damages to cells. It was possible to see a depression-like cavity on cells (Fig. 6, PepGAT and PepKAA Panels, white dashed circle), indicating damage to the cell wall. Also, tiny blebs, new buds, scars on new buds and cells, and rings of truncated bud scars were present in the treatment with both peptides (Fig. 6, white arrowheads). In both treatments with peptides, cells present wrinkles and scars all over the structure. Additionally, in both treatments with peptides alone is possible to see a lack of constriction in the solid point of the septum (Fig. 6, white open arrows).

In contrast to what was seen in the treatment with drugs alone, the combination of peptides and drugs lead to several damages to cells and thus inhibit biofilm formation. In both cases, many cells presented depression-like cavities (Fig. 6, PepGAT+ITR and PepKAA+ITR panels, white dashed circle), indicating damage to the cell wall. In addition, were noticed alterations in cell shape, wrinkles and scars all over the structure, small blebs, new buds, scars on new buds and cells, and rings of truncated bud scars in the treatment with both peptides (Fig. 6, white arrowheads), and no presence of a solid point in septum junction (Fig. 6, white open arrows).

SEM analysis revealed a different pattern in inhibiting biofilm formation of *C. albicans* (Fig. 7). The controls DMSO-NaCl solution, ITR, and NYS at 1000 μg mL^{-1} showed no significant alteration on *C. albicans* cells (Fig. 7). Treatment with PepGAT induced small blebs, new buds,

scars on new buds and cells (Fig. 7, white arrowheads). In contrast, the treatment with PepKAA is possible to damage all cells. Cells stick together with all the present bad conformation and pieces of other cells on top of them (Fig. 7, panel PepKAA). SEM analysis revealed that the combination of PepGAT + ITR killed almost all cells (Fig. 7), and the leftovers are completely damaged unable to form biofilm. The combination of PepKAA + ITR is far less effective than PepGAT + ITR, causing scars and wrinkles on cells and abnormal shape. It is also possible to see the presence of small blebs, new buds, scars on new buds and cells, and rings of truncated bud scars in the treatment with both peptides (Fig. 7, white arrowheads). The combinations of both peptides + NYS were most efficient than with combination with ITR. In both cases, all cells were dead. It was only found isolated cell wholly damaged, with the signal of loss of internal content and with no ability to form biofilm at all (Fig. 7, PepGAT and PepKAA panels).

In the degradation of *C. krusei* preformed biofilm, the SEM analysis of ITR-treated biofilm revealed no damage on the biofilm (Fig. 8). In the treatment with peptides alone or combination with ITR, the biofilm constituting cells presented damage to the cell wall, loss of internal content, tiny blebs, new buds, many scars, and wrinkles on new buds and cells (Fig. 8 white arrowheads), and rings of truncated bud scars in the treatment with both peptides (Fig. 8, white open arrows).

Hemolytic Assay

A previous study (Souza et al., 2020) showed that the synthetic peptides had no hemolytic activity against any human blood type tested (Table 1), even at 50 μg. mL^{-1}. In contrast, NYS at 1000 μg. mL^{-1} caused 100% hemolysis in all human blood types and ITR at 1000 μg. mL^{-1} caused 80, 75, and 69% of hemolysis, respectively, to Type-A, B, and O of red blood cells (Table 1).

In general, synthetic peptides with antifungal drugs decreased their hemolytic effect (Table 1). PepGAT with NYS resulted in a reduced hemolytic effect of 54, 43, and 12%, respectively, to Type-A, B, and O of red blood cells. PepGAT with ITR caused 17, 45, and 43% of hemolysis, respectively, to Type-A, B, and O of red blood cells (Table 1). The combination of PepKAA with NYS hemolyzed 15, 10, and 21%, respectively, of Type-A, B, and O of red blood cells, whereas the combination of PepKAA with ITR 21, 34, 12%, respectively, of Type-A, B, and O of red blood cells (Table 1).

Discussion

Fungal infection caused by yeasts from *Candida* species poses a critical problem in the healthcare field worldwide, leading to elevated mortality rates and high costs with medical care for hospitalized patients and governments (Pristov and Ghannoum, 2019; S et al., 2019). The drug-resistant *C. albicans* is the most severe threat to human health worldwide. It is responsible for infections caused in immunocompromised, HIV-positive, and intensive care unit patients (S et al., 2016; Costa-de-Oliveira and Rodrigues, 2020).

C. albicans and *C. krusei* have developed resistance to many antifungal drugs such as azoles (ITR), echinocandins, polyenes (NYS), among others (AT et al., 2021). Overall, the mechanisms of resistance developed by yeasts involve overexpression or alteration of the target, development of efflux pumps to remove the excess of drugs of cytoplasm, modification of the drug, and biofilm production (Orozco et al., 1998; Guinea et al., 2006; AT et al., 2021; Lima et al., 2021).

Biofilm is an excellent resistance structure and all yeasts on *Candida* genera could produce it (Cavalheiro and Teixeira, 2018). The biofilm production by *Candida* provides a different

behavior compared to planktonic life. For example, cells living in the biofilm community can attach irreversibly to any surface, inert material, living tissue, medical devices (e.i., protheses and cardioverter defibrillators), in addition to increasing the expression of virulent factors and higher resistance to antifungal drugs (Cavalheiro and Teixeira, 2018; Lima et al., 2021). Based on that, biofilm formation is a serious threat to overcome.

The effect of synthetic peptides has already been tested toward *Candida* biofilms. For instance, the synthetic peptides KU4 (96 μg mL^{-1}), uperin 3.6 (96 μg mL^{-1}), upn-lys4 (96 μg mL^{-1}), upn-lys5 (192 μg mL^{-1}), and upn-lys6 (96 μg mL^{-1}) reduced the viability of the biofilm of *C. albicans* only in 20, 35, 15, 40, and 30%, respectively (KY et al., 2015). In the case of *C. krusei*, the synthetic peptide VLL-28 at 72 μg mL^{-1} reduced the viability of *C. krusei* biofilms by 35% (Roscetto et al., 2018). Paulone et al. (2017) reported a peptide that presented an IC_{50} value against *C. albicans* biofilm of 126 μg mL^{-1} (Paulone et al., 2017).

Despite those activities of synthetic peptides against *Candida* biofilms, there also some studies reporting the synergism of synthetic peptides with antifungal drugs such as fluconazole and Amphotericin B but only against candida planktonic cells of (MacCallum et al., 2013; Park et al., 2017; do Nascimento Dias et al., 2020; Czechowicz et al., 2021; Maione et al., 2022). For example, the combination of synthetic peptide WMR (110 μg mL^{-1}) + flucanozole (10 μg mL^{-1}) inhibit in 50% the growth of *C. albicans* (Maione et al., 2022). Regarding the synergistic effect of peptides and antifungal drugs toward candida biofilm the studies are a little scare and this number is even lower with synthetic peptides. The combination of peptide HsLin06_18 (100 μg mL^{-1}) with caspofungin (10 μg mL^{-1}) inhibit in 50% the biofilm formation of *C. albicans* (Cools et al., 2017). The analysis of mechanism of action revealed that only the combination lead to membrane permeabilization of *C. albicans* cells on biofilm (Cools et al., 2017).

In another study, the synergistic action of the lipopeptide AC7BS (1000 µg mL^{-1}) + flucanozole (1000 µg mL^{-1}) reduced in 60% the preformed biofilm of *C. albicans* (Ceresa et al., 2017). Mora-Navarro et al. (2015) showed that a β-synthetic peptide (200 µg mL^{-1}) + ketoconazole (200 µg mL^{-1}) inhibit in 60% the biofilm formation. In this study, the authors did not report any mechanism of action of the combination. Compared to those above-mentioned results, here was found exciting results about synergic effect of peptides + antifungal drugs to inhibiti the biofilm formation and reduced the mass of preformed biofilm (Fig. 1) toward *Candida* biofilms. PepKAA + NYS inhibited in 98% the biofilm formation of *C. albicans* and PepGAT + ITR reduced in 55% the biomass of preformed biofilm of *C. krusei* (Fig. 1). It is important to notice that none of those above-mentioned work showed any result about the reduction of biomass of preformed biofilms. This is the great novelty of this work.

Regarding the mechanism of action only Cools et al. (2017) and Maione et al. (2022) provided a clue about how the combination between peptides and drugs act on *C. albicans* biofilm. In both studie the authors just the membrane permeabilization. Here, fluorescence (Figs. 2-5) and SEM (Figs. 5-9) analysis revealed the mechanisms behind revealed lethal damage of those combinations to *Candida* biofilms. Here, additionally, fluorescence analyses revealed an exciting result. In some cases, the combinations of peptides with antifungal drugs lead to ROS overproduction (Fig. 2 panels PepGAT + ITR and PepKAA + ITR and Fig. 3 PepGAT + ITR). Interestingly, ROS overproduction was all in the combinations of either PepGAT or PepKAA with ITR and presented the best results even to inhibit the formation of biofilm (Fig. 1A) or degrade preformed biofilm (Fig. 1C). ROS is essential to biofilm biogenesis, development, formation, the genetic variability of cells (Čáp et al., 2012). However, the line of benefits and lethal effects are tiny and easy to cross. A slight unbalance in ROS levels could lead to ROS accumulation, which

is lethal because they inactivate vital molecules such as carbohydrates, nucleic acids, proteins, and lipids, triggering programmed cell death (PCD) (Maurya et al., 2011).

Our results clear suggest peptides enhance the antibiofilm activity of NYS and ITR. There are two hypotheses to explain the improvement of antifungal drugs by peptides. The first one is directly related to the mechanism of action ITR and NYS. Although being of different classes, ITR and NYS have the same target. ITR interacts with the enzyme lanosterol 14α-demethylase to inhibit the ergosterol biosynthesis, and NYS interacts with the ergosterol in the membrane, causing an unbalance in the membrane (Borgers and Ven, 1989; AG et al., 2017). By interacting with chitin in the cell wall, both PepGAT and PepKAA cause a rupture in the cell wall (Figs. 6-8), facilitating the access of lanosterol 14α-demethylase and ergosterol, respectively, by ITR and NYS and thus enhancing their activity. The second explanation is based on the peptides attacking the membrane directly, as revealed by fluorescence microscopy (Figs. 2-5). During the treatment of yeasts with combinations made by peptides and antifungal drugs, membranes are double attacked. Peptides attack the membrane to form pores, and drugs attack their target in the membranes.

Both ITR and NYS have many collateral effects. ITR may cause vomiting, diarrhea, headache, and dizziness. Besides these collateral effects, cardiotoxicity and hypertension were attributed to ITR use(F et al., 2001). Regarding NYS, the collateral effects are poor taste (the incidence was 61.5% in one study) and gastrointestinal adverse reactions, including vomiting, nausea, diarrhea, anorexia, and abdominal pain (Macesic and Wingard, 2020). The interesting result was that the association of peptides with antifungal drugs reduced their toxicity in human erythrocytes (Table 1). In all treatments made with combinations, were found a reduction in toxicity of antifungal drugs, at least 2-fold reaching up to 8.3-fold (Table 1).

Conclusion

Here is a pioneer study showing the activity, action mechanism, and synergistic effects of two synthetic peptides (PepGAT and PepKAA) with NYS and ITR against biofilms of *C. albicans* and *C. krusei*. In the best combinations, peptides enhanced the antibiofilm activity of both drugs at least five-fold and reduced up to ten-fold their toxic effect against red blood cells. Therefore, the ability to improve the antibiofilm activity of NYS and ITR suggests that synthetic peptides can be employed as adjuvants and decrease the toxicity of these drugs.

Funding and Acknowledgments

This work was supported by grants from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP). A special thanks to CAPES for providing the grant (grant number 88887.318820/2019-00) for postdoctoral position for Pedro F. N. Souza. We are also grateful to the central analytical facilities of UFC, Brazil.

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Conflict of interest

All authors declare no conflict of interest.

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Figure Legends

Figure 1. Antibiofilm activity and synergistic effect with Nystatin and Itraconazole of synthetic peptides PepGAT and PepKAA. (A and B) Inhibition of biofilm formation, respectively, of *C. krusei* and *C. albicans*. **(B)** Biofilm degradation of *C. krusei*. All peptides were used at 50 μg mL^{-1}. Nystatin and Itraconazole at 1000 μg mL^{-1}. DMSO-NaCl as a negative control. The letters represent the mean ± standard deviation of three replicates. *Different lowercase letters indicate statically significant differences compared to DMSO by analysis of variance ($p < 0.05$).

Figure 2. Fluorescence images showing membrane pore formation and ROS overproduction as a mechanism of action to inhibit biofilm formation of *C. krusei*. Control solution of DMSO-NaCl, treated with ITR alone at 1000 μg mL^{-1}, PepGAT and PepKAA both alone at 50 μg mL^{-1}, and synergistic activity of PepGAT and PepKAA with Itraconazole. The propidium iodide uptake assay measured the membrane pore formation and ROS overproduction using 2', 7' dichlorofluorescein diacetate (DCFH-DA). Bars: 100 μm.

Figure 3. Fluorescence images showing membrane pore formation and ROS overproduction as mechanisms of action to inhibit biofilm formation of *C. albicans*. Control solution of DMSO-NaCl, treated with ITR alone at 1000 μg mL^{-1}, PepGAT and PepKAA both alone at 50 μg mL^{-1}, and synergistic activity of PepGAT and PepKAA with ITR. The propidium iodide uptake assay

measured the membrane pore formation and ROS overproduction using 2', 7' dichlorofluorescein diacetate (DCFH-DA). Bars: 100 µm.

Figure 4. Fluorescence images showing membrane pore formation and ROS overproduction as mechanisms of action to inhibit biofilm formation of *C. albicans*. Control solution of DMSO-NaCl, treated with NYS alone at 1000 µg mL^{-1}, PepGAT and PepKAA both alone at 50 µg mL^{-1}, and synergistic activity of PepGAT and PepKAA with NYS. The propidium iodide uptake assay measured the membrane pore formation and ROS overproduction using 2',7' dichlorofluorescein diacetate (DCFH-DA). Bars: 100 µm.

Figure 5. Fluorescence images showing membrane pore formation and ROS overproduction as mechanisms of action to degrade biofilm formation of *C. krusei*. Control solution of DMSO-NaCl, treated with ITR alone at 1000 µg mL^{-1}, PepGAT and PepKAA both alone at 50 µg mL^{-1}, and synergistic activity of PepGAT and PepKAA with ITR. The propidium iodide uptake assay measured the membrane pore formation and ROS overproduction using 2',7' dichlorofluorescein diacetate (DCFH-DA). Bars: 100 µm.

Figure 6. SEM images showing alterations in the biofilm of *C. krusei* cells after incubation with synthetic peptides, antifungal drugs, and combination by them. The surface of biofilm control (DMSO-NaCl panel) cells is covered by well-defined and organized structures. Biofilm cells exposed to ITR show few alterations in the cell surface. PepGAT and PepKAA-treated cells, showing some alterations in cell structure, such as scars, buds scars, and distortion in the shape of the cells. Biofilm cells were incubated with PepGAT and PepKAA in contact with Itraconazole, resulting in strong alterations in the cell membrane, scars, bud scars, and deformation of the cells.

Figure 7. SEM images showing alterations in the biofilm of *C. albicans* cells after incubation with synthetic peptides, antifungal drugs, and combination by them. The surface of biofilm control (DMSO-NaCl panel) cells is covered by well-defined structures. No relevant changes were seen in the biofilm exposed to ITR and NYS. PepGAT and PepKAA-treated biofilm, showing alterations in the cell membrane, scars, bud scars, and distortion in the biofilm structure. Biofilm exposed to combinations made by PepGAT and PepKAA in a synergistic action with ITR, showing strong alterations in the shape of cells and buds scars. Biofilm incubated with PepGAT and PepKAA in contact with NYS, what is seen is no biofilm anymore, are only the remaining dead cells.

Figure 8. SEM images show alterations in the preformed biofilm of C. krusei cells after incubation with synthetic peptides, antifungal drugs, and combination. The surface of biofilm

control (DMSO-NaCl solution) is well-defined and organized. Biofilm exposed to ITR, showing few alterations in the cell surface. PepGAT and PepKAA-treated biofilm, showing alterations in cell structure, such as scars, bud scars, and distortion in the shape of the cells. Biofilm incubated with a combination of PepGAT or PepKAA and ITR, resulting in strong alterations in the cell membrane, scars, bud scars, deformation of the cells structures, and leakage of internal content.

Supplementary Figure 1. Antibiofilm activity and synergistic effect with Nystatin and Itraconazole of synthetic peptides PepGAT and PepKAA. (A and B) Inhibition of biofilm formation, respectively, of *C. parapsilosis* and *C. tropicalis*. **(C and D)** Biofilm degradation of *C. parapsilosis* and *C. tropicalis*. All peptides were used at 50 μg mL^{-1}. Nystatin and Itraconazole at 1000 μg mL^{-1}. DMSO-NaCl as a negative control. The letters represent the mean ± standard deviation of three replicates. *Different lowercase letters indicate statically significant differences compared to DMSO by analysis of variance ($p < 0.05$).

Figures

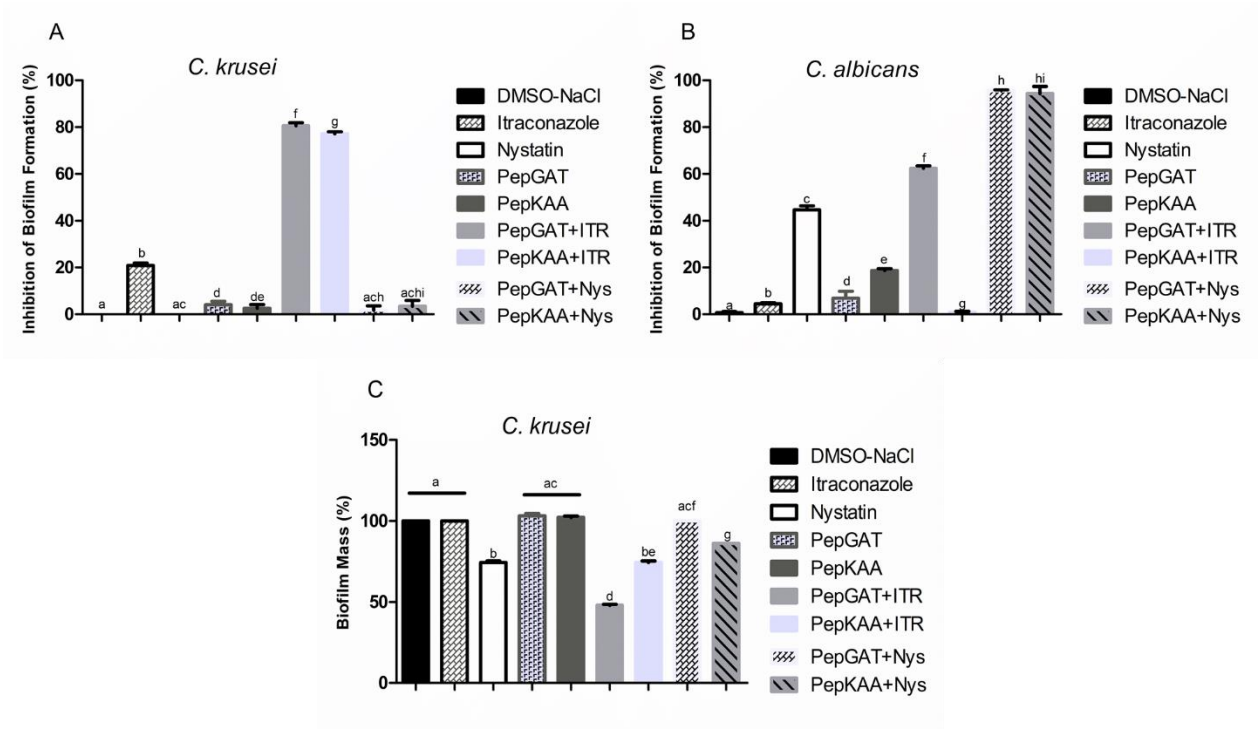


Fig. 1

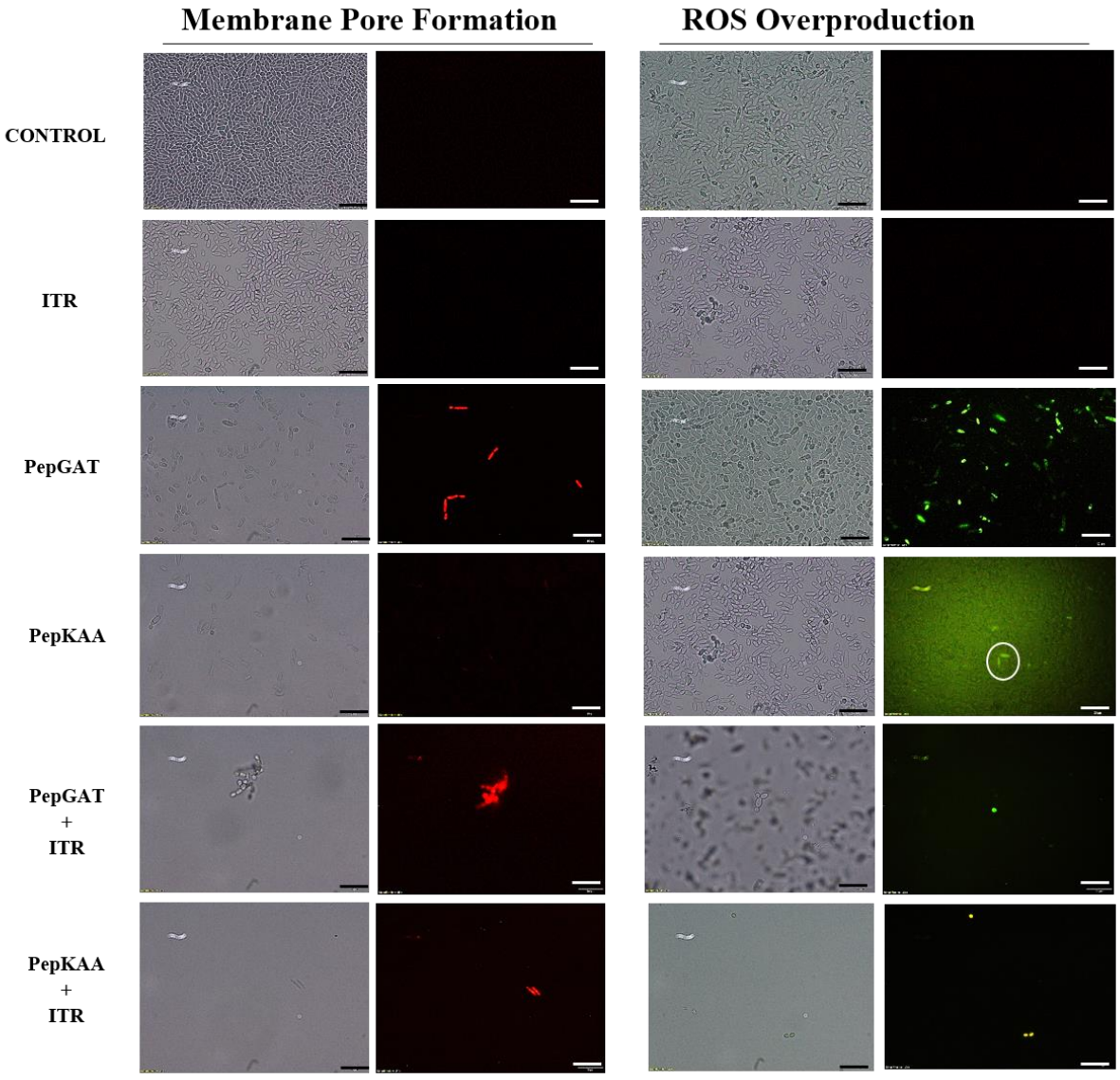

Membrane Pore Formation
ROS Overproduction
CONTROL
ITR
PepGAT
PepKAA
PepGAT + ITR
PepKAA + ITR

Fig. 2

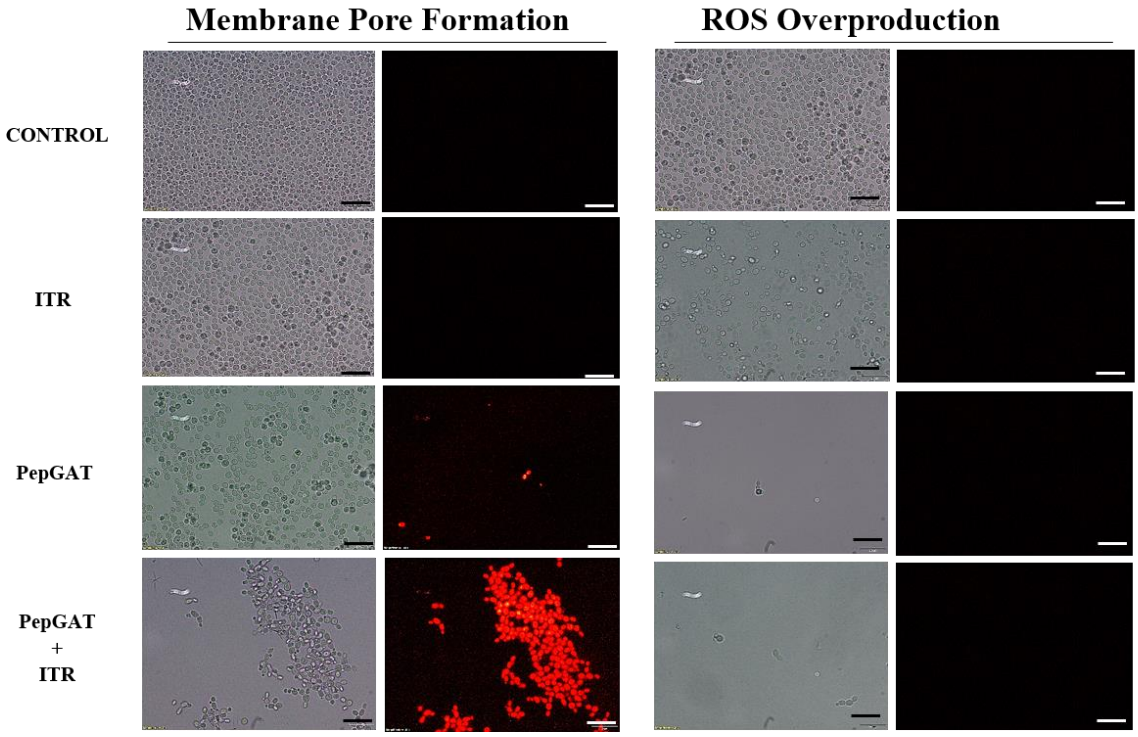

Membrane Pore Formation
ROS Overproduction
CONTROL
ITR
PepGAT
PepGAT
+
ITR

Fig. 3

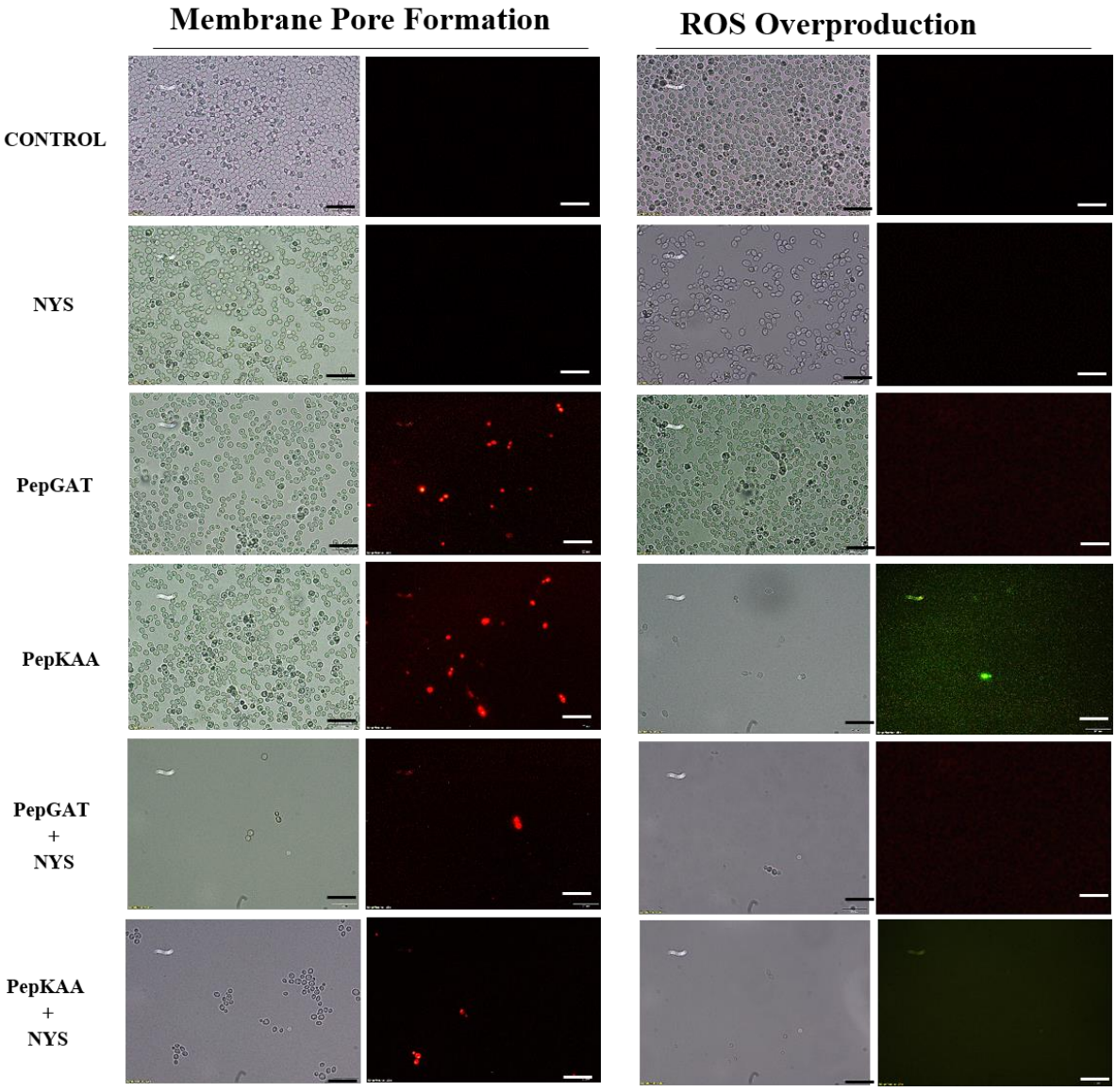

Membrane Pore Formation
ROS Overproduction
CONTROL
NYS
PepGAT
PepKAA
PepGAT + NYS
PepKAA + NYS

Fig. 4

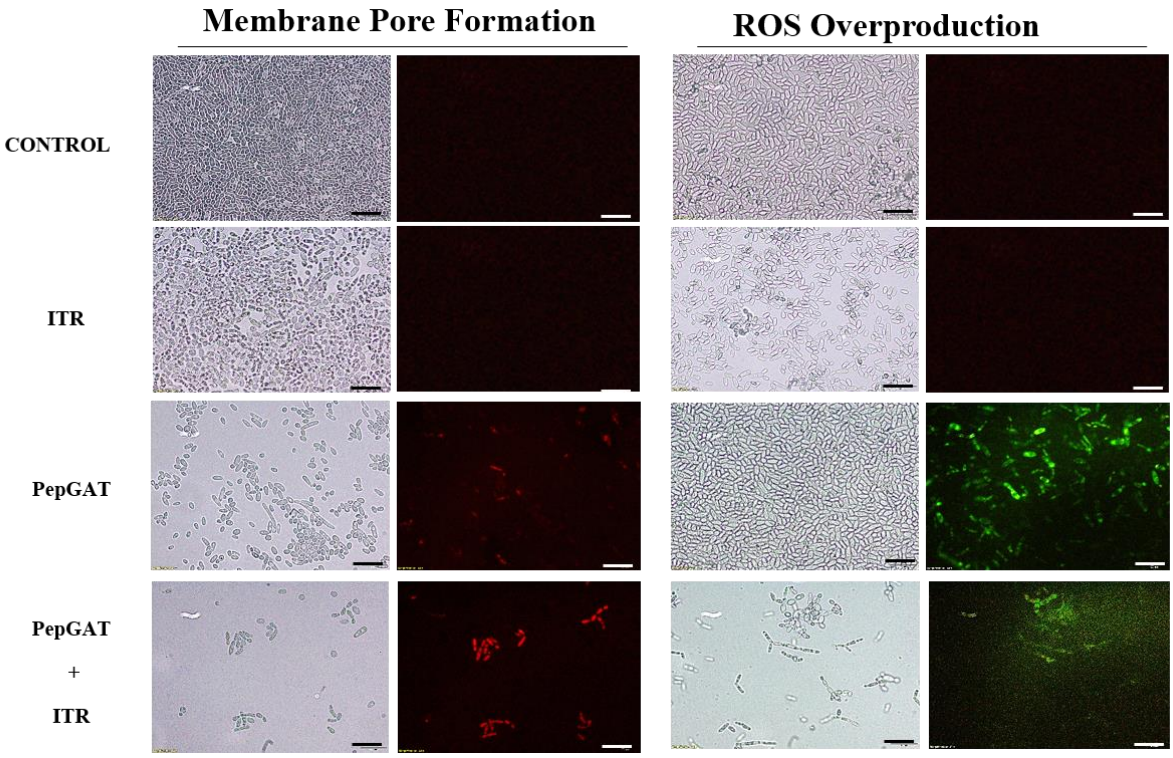

Membrane Pore Formation
ROS Overproduction
CONTROL
ITR
PepGAT
PepGAT
+
ITR

Fig. 5

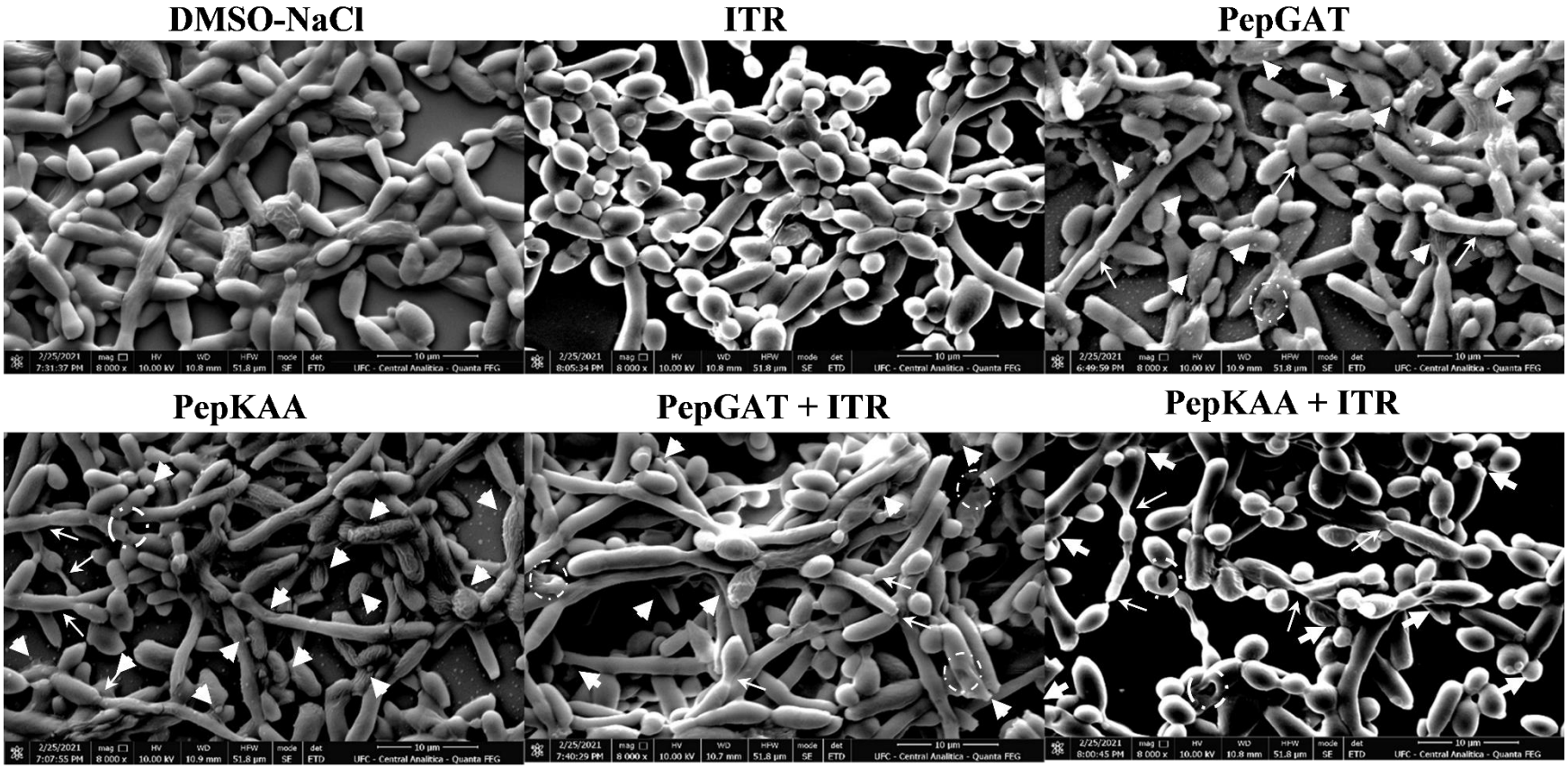


Fig. 6

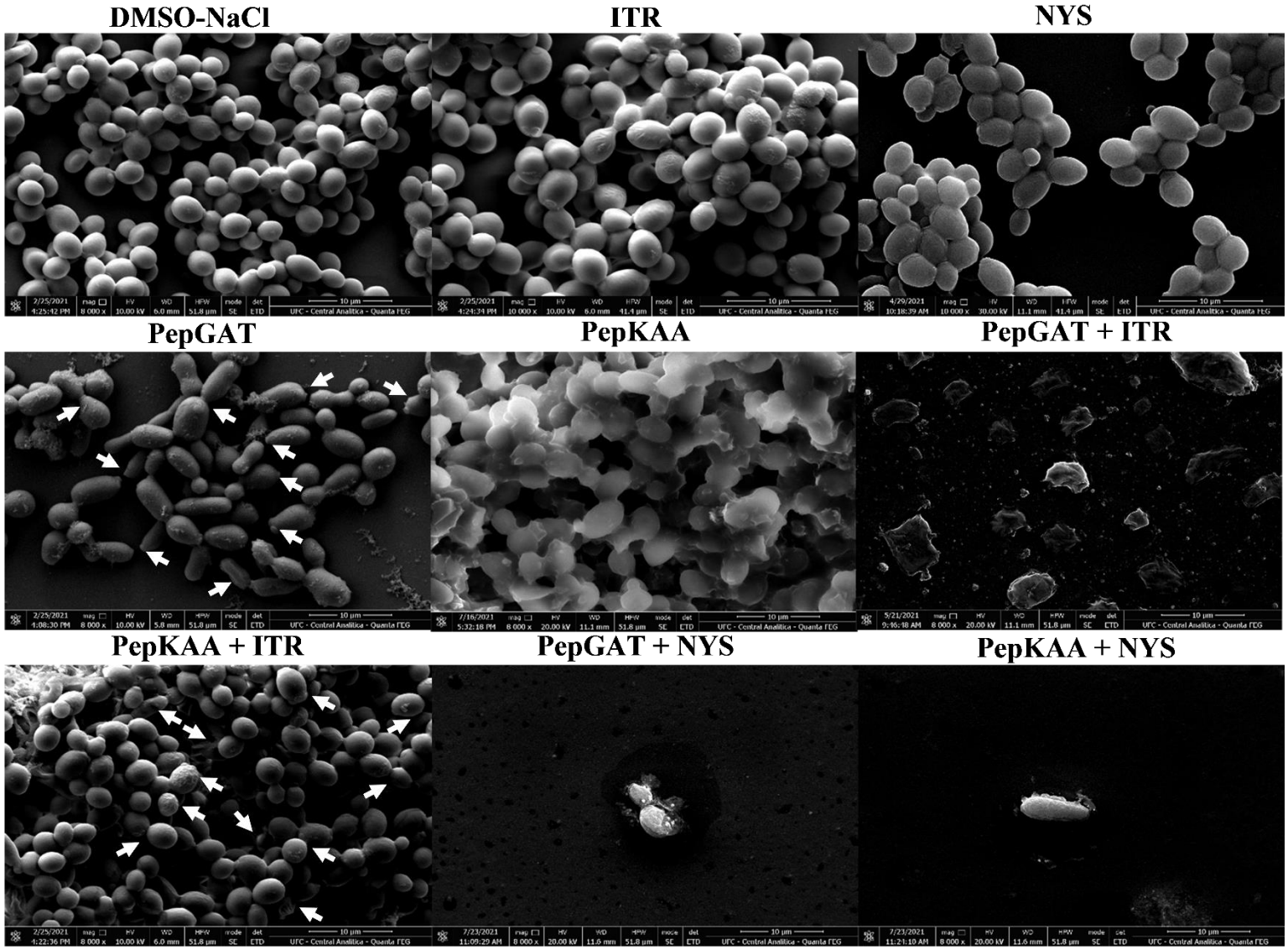

DMSO-NaCl
ITR
NYS
PepGAT
PepKAA
PepGAT + ITR
PepKAA + ITR
PepGAT + NYS
PepKAA + NYS

Fig. 7

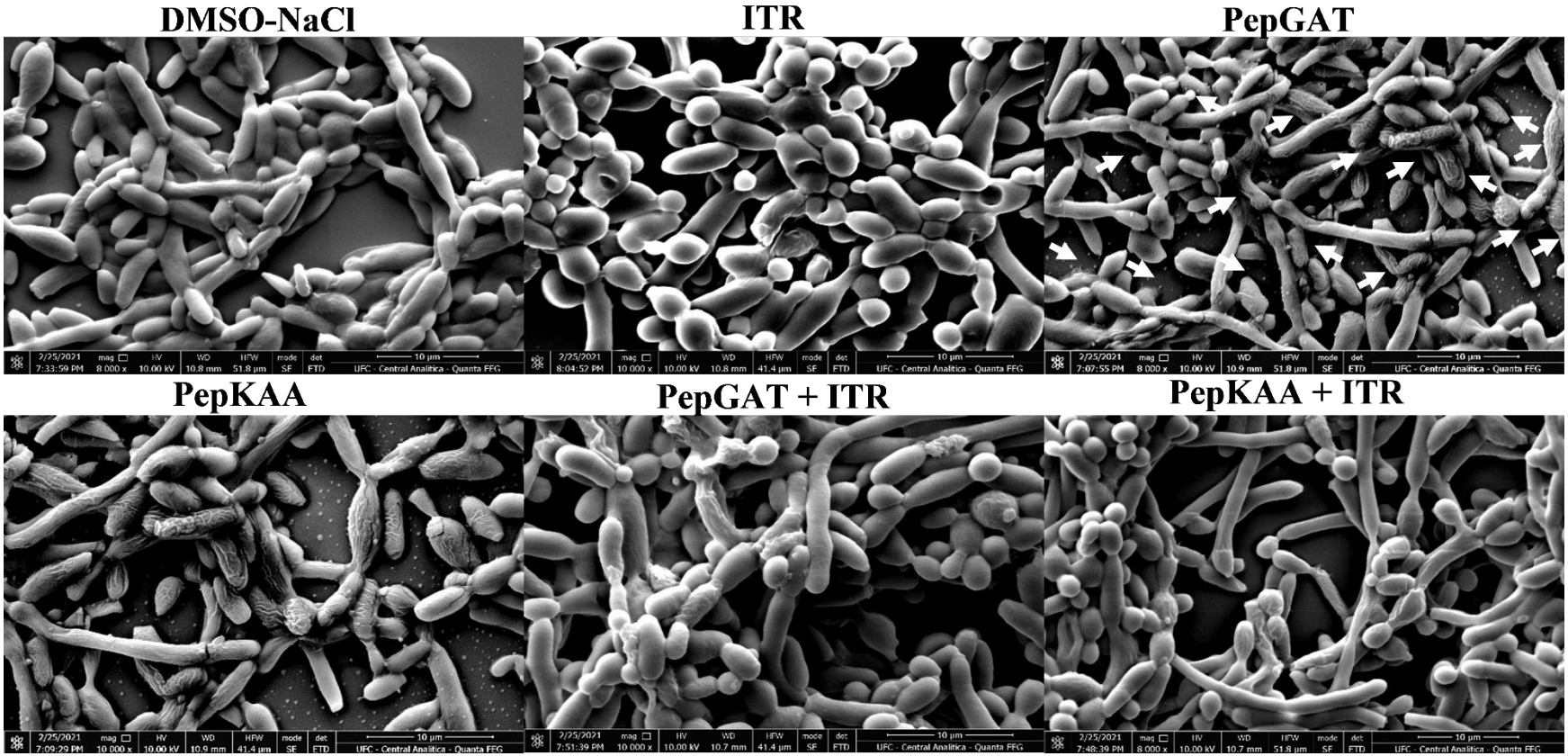
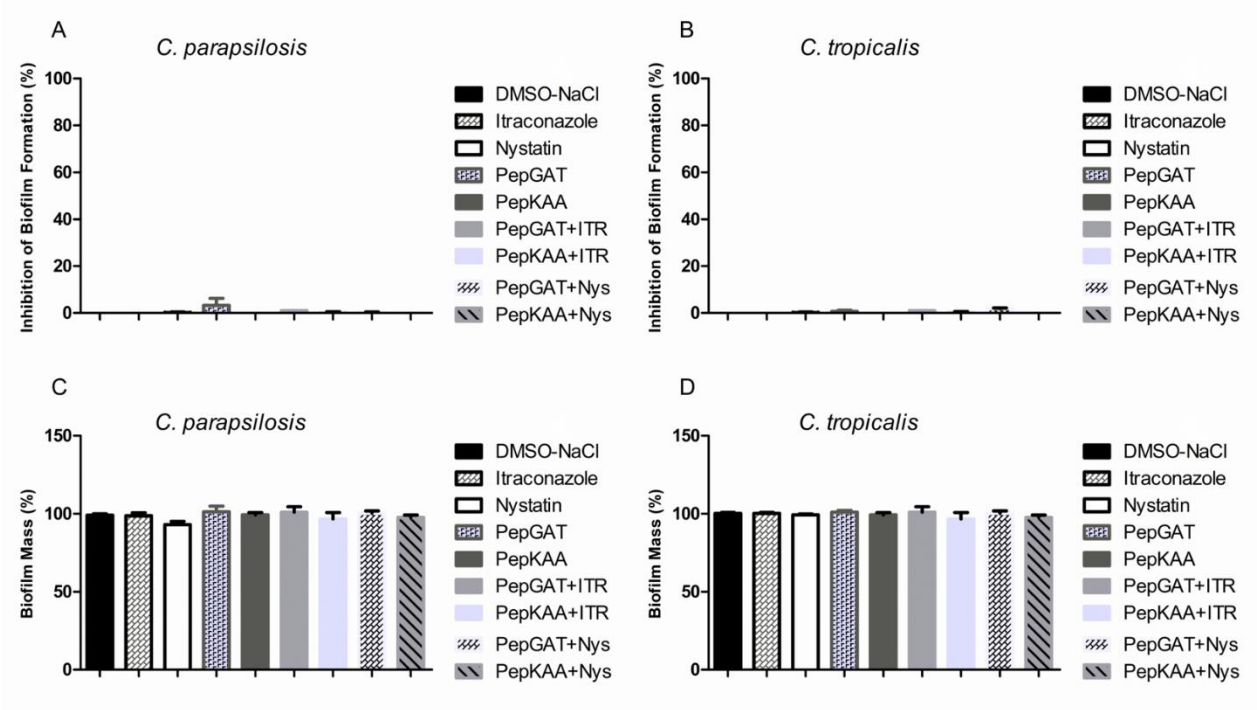


Fig. 8

Table 1. Hemolytic activity of synthetic peptides, antifungal drugs, and their combination toward human red blood cells

Peptides/Combinations	% Hemolysis		
	Type-A Blood	**Type-B Blood**	**Type-O Blood**
0.1% Triton X-100	100	100	100
DMSO-NaCl Solution	0	0	0
NYS (1000 µg mL^{-1})	100	100	100
ITR (1000 µg mL^{-1})	80	75	69
PepGAT (50 µg mL^{-1})	0	0	0
PepKAA (50 µg mL^{-1})	0	0	0
PepGAT (50 µg mL^{-1}) + NYS (1000 µg mL^{-1})	54	43	12
PepGAT (50 µg mL^{-1}) + ITR (1000 µg mL^{-1})	17	45	43
PepKAA (50 µg mL^{-1}) + NYS (1000 µg mL^{-1})	15	10	21
PepKAA (50 µg mL^{-1}) + ITR (1000 µg mL^{-1})	21	34	12


A
C. parapsilosis
Inhibition of Biofilm Formation (%)
100
80
60
40
20
0
DMSO-NaCl
Itraconazole
Nystatin
PepGAT
PepKAA
PepGAT+ITR
PepKAA+ITR
PepGAT+Nys
PepKAA+Nys
B
C. tropicalis
Inhibition of Biofilm Formation (%)
100
80
60
40
20
0
DMSO-NaCl
Itraconazole
Nystatin
PepGAT
PepKAA
PepGAT+ITR
PepKAA+ITR
PepGAT+Nys
PepKAA+Nys
C
C. parapsilosis
Biofilm Mass (%)
150
100
50
0
DMSO-NaCl
Itraconazole
Nystatin
PepGAT
PepKAA
PepGAT+ITR
PepKAA+ITR
PepGAT+Nys
PepKAA+Nys
D
C. tropicalis
Biofilm Mass (%)
150
100
50
0
DMSO-NaCl
Itraconazole
Nystatin
PepGAT
PepKAA
PepGAT+ITR
PepKAA+ITR
PepGAT+Nys
PepKAA+Nys

Supl. Fig. 1

CAPÍTULO III – Artigo científico 2

As sessões Metodologia, Resultados e Discussão serão apresentadas na forma de um artigo científico submetido em uma revista científica internacional. Sucintamente, os resultados mostram que os peptídeos sintéticos Mo-CBP3-PepI e Mo-CBP3-PepIII apresentaram atividade contra biofilmes formados por espécies de *C. albicans* e *C. parapsilosis*, levando à formação de poros na membrana e superprodução de espécies reativas de oxigênio, inibindo a formação de novos biofilmes e inviabilizando as estruturas pré-formadas.

Artigo científico 2

Artigo científico submetido à revista *Biofouling* fator de impacto 3.2 (Qualis A1) status *under review*.

Artigo científico 2

Synergistic antifungal activity of synthetic peptides and antifungal drugs against *Candida ssp* biofilms

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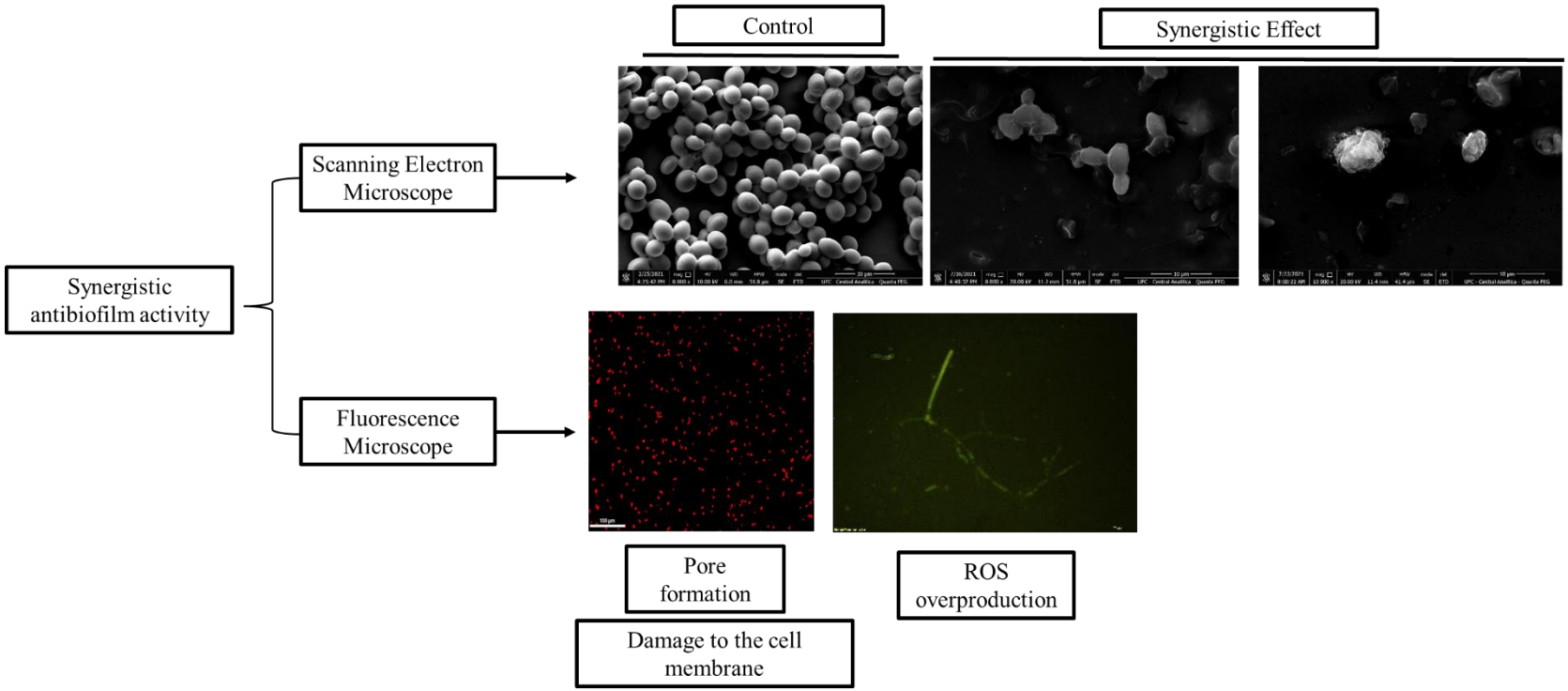
Graphical Abstract



Abstract

Biofilm-forming *Candida albicans* and *C. parapsilosis* are responsible for bloodstream infections leading individuals to death. Synthetic antimicrobial peptides (SAMPs) are considered new weapons to combat infections alone or conjugated with drugs. Here, two SAMPs named *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII were tested alone or combined with Nystatin (NYS) and Itraconazole (ITR) against *C. albicans* and *C. parapsilosis* biofilms. Furthermore, the mechanism of antibiofilm activity was evaluated by fluorescence and scanning electron microscopies. When combined with peptides, the results revealed an improvement of 2 up to 4-fold in NYS and ITR antibiofilm activity. Microscopic analyses showed cell wall degradation, ROS overproduction, and membrane pore formation causing leakage of internal content and leading biofilm cells to death. Taken together, these results suggest the potential of *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII as new drugs and adjuvants to increase the activity of conventional drugs for the treatment of clinical infections caused by *C. albicans* and *C. parapsilosis*.

Keywords: Antibiofilm activity; Candidiasis; Synergism; Synthetic peptides; Antifungal drugs;

Introduction

Candida species are the primary fungus responsible for up to 15% of hospital-acquired cases of sepsis. Biofilms are established by microbial cells on an inert or living surface, promoting the development of microcolonies with polymeric matrices, enhancing the resistance to various antimicrobial agents (Kumar et al. 2017; Lima et al. 2021). A mature biofilm formed by *Candida spp*. is coated by an extracellular matrix composed of glycoproteins (55%), carbohydrates (25%), lipids (15%), and nucleic acids (5%) that protect the microbial cells (Zarnowski et al. 2014). The National Institutes of Health (NIH) in the USA considered biofilms a public health problem and estimates biofilms are responsible for 80% of the difficulties to cure human infections (Fox & Nobile 2012; Zarnowski et al. 2014; Kumar et al. 2017; Lima et al. 2021). The most susceptible are immunocompromised patients, $AIDS^{+}$ patients, patients under chemotherapy treatment or immunosuppressive therapies, and patients carrying medical devices (catheters, pacemakers, and heart valves) (Weig 1998; Kullberg & Oude Lashof 2002).

C. albicans and *C. parapsilosis* are common opportunistic fungal pathogens asymptomatically colonizing mucosal surfaces and skin of healthy individuals leading to an infection called candidiasis (Baillie 2000). In addition, *C. albicans* and *C. parapsilosis* are responsible for bloodstream infections called candidemia in immunocompromised patients, including those in intensive care units (Sasso et al. 2017). Currently, the treatment of *C albicans* and *C. parapsilosis* infections involves applying antifungals agents that can interrupt different metabolic pathways of the cell. However, many studies have reported *Candida* resistance to these antifungal molecules (Ramage 2002; LaFleur et al. 2006; Arendrup & Patterson 2017). A study developed by Katiyar and collaborators (Katiyar et al. 2006) reported clinical isolates of *Candida sp* to carry genes conferring

resistance to all antifungal agents making those drugs useless (Katiyar et al. 2006). To overcome this situation, synthetic antimicrobial peptides (SAMPs) could be an alternative either alone or combined with antifungal drugs to control *Candida* infection and fight these pathogens' resistance (Lima et al. 2021). SAMPs have some important antimicrobial characteristics found in natural antimicrobial peptides, such as positive net charge, α-helix structure, low molecular weight (600-1200 Da), high hydrophobic ratio (40 – 60%), and amphipathicity (Pedro F.N. Souza et al. 2020; Lima et al. 2021).

Recently, our research group designed, characterized, and evaluated the antimicrobial activity of three synthetic peptides *Mo*-CBP3-PepI (CPIAQRCC, positive charge of +1 and hydrophobic ratio of 66%), *Mo*-CBP3-PepII (NIQPPCRCC, positive charge of +1, and hydrophobic ratio of 44%), and *Mo*-CBP3-PepIII (AIQRCC, positive charge of +1, and hydrophobic ratio of 44%). These peptides were designed based on the structure of *Mo*-CBP_3, a chitin-binding protein purified from *Moringa oleifera* seeds (Oliveira et al. 2019; Lima et al. 2020). The anticandidal activity and mechanism of action of these peptides were evaluated by Oliveira et al. (2019) and Lima et al. (2020).

Here, we advanced these studies by investigating the antifungal effects of *Mo*-CBP_3-PepI, and *Mo*-CBP_3-PepIII tested alone or combined with Nystatin (NYS) and Itraconazole (ITR) against *C. albicans* and *C. parapsilosis* biofilms. We demonstrated the potential of *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII as new drugs and adjuvants to increase the activity of conventional drugs for the treatment of *C. albicans* and *C. parapsilosis* biofilm formation. At the same time, it reduces drugs´ toxicity to human erythrocytes.

Materials and methods

Ethics Statement

Not applied for this study.

Biological materials

C. albicans (ATCC 10231) and *C. parapsilosis* (ATCC 22019) were obtained from the Laboratory of Plant Toxins at the Department of Biochemistry and Molecular Biology at the Federal University of Ceará. All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA).

Peptide synthesis

The Synthetic peptides *Mo*-CBP3-PepI, *Mo*-CBP3-PepII, and *Mo*-CBP3-PepIII (Oliveira et al. 2019; Lima et al. 2020) were chemically synthesized by GenOne, which had their quality and purity (≥95%) analyzed by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) and mass spectrometry.

Biological activity

Antibiofilm assay

The antibiofilm assays against *C. albicans* and *C. parapsilosis* were performed following the methodology described by Dias et al.(Dias et al. 2020), with some modifications. To evaluate the inhibition of the biofilm formation, 100 µL of *C. albicans* and *C. parapsilosis* suspension (2.5 x 10^3 CFU/mL) in Sabouraud broth were incubated in a flat bottom of 96-well plate with 100 µL of *Mo*-CBP3-PepI, *Mo*-CBP3-PepII, *Mo*-CBP3-PepIII (50 µg. mL^{-1}), at 37 °C for 48 hours. The supernatant was removed to quantify the biofilm formed, and wells were washed three times with sterile 0.15 M NaCl solution. Next, the cells were fixed with 100 µL of methanol for 15 min at 37 °C and plates were air-dried under the same conditions. Then, 200 µL of an aqueous solution of

0.1% of crystal violet was added and incubated for 30 min at room temperature (± 22 °C). To remove the excess stain, the plates were washed three times with distilled water and finally added 100 μL of 33% of acetic acid to solubilize the dye bound in the biofilm. After 15 min, the absorbance was measured at 600 nm using an automated microplate reader (Epoch, Biotek).

To perform the degradation of preformed biofilm assay, in a flat-bottom 96-well plate, 100 μL of the cell suspensions of both yeasts were incubated at 37 °C for 24 hours. Then, the supernatant was removed, and 100 μL of the Sabouraud broth media and 100 μL of each peptide were added and incubated again for 24 hours. The culture medium was again discarded, and the same procedure described above was used to quantify the biofilm mass after incubation with each peptide. In both experiments, DMSO-NaCl was used as a negative control. NYS (1000 μg mL^{-1}) and ITR (1000 μg mL^{-1}) as a positive control.

The synergism assays were carried out by combining peptides with either antifungals NYS or ITR: The combinations were constituted of each peptide (50 μg mL^{-1}) + NYS or ITR (1000 μg mL^{-1}). The control to evaluate the effectiveness of synergism was the activity presented by peptides or drugs alone. After the formulation of combinations, the antibiofilm assays for synergism analyses for *Candida* ssp. were the same as described above.

Overproduction of reactive oxygen species (ROS)

To evaluate ROS overproduction, it followed the methodology described by Dias et al.(Dias et al. 2020) with some modifications. *C. albicans* and *C. parapsilosis* were incubated with peptides for 24 hours under the same conditions as described above (Inhibition of the biofilm formation and degradation of preformed biofilm assays). Then, 50 μL of cell suspension (2.5 x 10^3 CFU/mL in contact with 50 μL of each peptide placed

in a coverslip with its respective positive and negative controls. Next, the formed biofilm in the coverslip was washed with 0.15 M NaCl three times to remove the Sabouraud media. Thus, 20 µL of 2',7' dichlorofluorescein diacetate (DCFH-DA) were added and incubated in the dark for 30 min at room temperature (± 22 °C). The coverslips were rewashed with 0.15 M NaCl to remove the excess stain and observed under a fluorescence microscope (Olympus System BX 41, at Plant Cell Biology Lab) with an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Cell membrane integrity assay

C. albicans and *C. parapsilosis*, the experiment to evaluate pore formation, were done as described by Dias et al.(Dias et al. 2020) with modifications. The biofilms were treated as the same described for ROS overproduction analysis. Thus, 20 µL of Propidium Iodide (PI) was added to the coverslip and incubated in a dark place for 30 min at room temperature (± 22 °C). Then, the samples were washed three times with 0.15 M of NaCl to remove the excess of PI and observed in a fluorescence microscope (Olympus System BX 41, at Plant Cell Biology Lab) with an excitation wavelength of 548 nm and emission wavelength of 650 nm.

Scanning electron microscopy (SEM) analysis

The morphological changes in the cells of *C. albicans* and *C. parapsilosis* were evaluated by SEM, using the method described by Staniszewska et al.(Staniszewska et al. 2013). Biofilms were fixed with 1% (v/v) glutaraldehyde in 0.15 M of sodium phosphate buffer at pH 7.0 for 16 hours. Then, the coverslips were washed with 0.15 M sodium phosphate buffer pH 7.0 three times. Next, 0.2% (v/v) osmium tetroxide was added to the samples and incubated for 30 min at 37 °C and washed again under the same conditions described above. Samples were successively dehydrated with increased ethanol

concentration (30%, 50%, 70% 100% and 100% [v/v]) for 10 minutes each at room temperature. At last, the final dehydration was realized with 50% hexamethyldisilane (HMDS) diluted in ethanol for 10 min and then 100% HDMS. The coverslips were placed on stubs and coated with a 20 nm gold layer using positron-emission tomography (PET) coating machine (Emitech-Q150TES, Quorum Technologies, England). The images were obtained with an FEI inspect^{TM}50 scanning electron microscopy, equipped with a low energy detector (Everhart-Thornley), and the acceleration used was 20.000 kV and 20.000x detector magnification.

Obtaining, files preparation, and Molecular Docking

The *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII three-dimensional (3D) structures were predicted using the PepFold server 3 (Lamiable et al. 2016). The amino acid protonation of the peptides was adjusted to pH 7.4 in ProteinPrepare (Martínez-Rosell et al. 2017). NYS (accession number CID 16219709) and ITR (accession number CID 55283) 3D structures were obtained from the database PubChem (Kim et al. 2019). The protonation of the ligands was adjusted using the software Marvin Sketch version 15.6.15. The energy minimization of the peptide hydrogens and the ligand was conducted in Discovery Studio v. 20.1 and Open Babel version 2.4.0.

Molecular docking assays were carried out in Autodock Vina, version 1.1.2 (Trott & Olson 2009). Additionally, the Autodock graphical interface version 1.5.6 was used to maintain polar hydrogens and provide charges to peptides and drugs using the Kollman united charges (Morris et al. 2009). The *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII were considered rigid molecules, and NYS and ITR were docked as flexible molecules. The grid box was defined as a 24 Å x 24 Å x 24 Å cube with the peptides in the center. The exhaustiveness was set up to 16, and all other parameters were used as default. The

163 software Discovery Studio v. 20.1 and the 3D interaction representations were realized in

164 Pymol v. 1.3

Hemolytic assay

The hemolytic activity of *Mo*-CBP_3-PepI, *Mo*-CBP_3-PepIII, NYS, ITR, and combination by then was assessed in A, B, and O-types of erythrocytes as described by Souza et al.(Pedro F.N. Souza et al. 2020). The concentrations of all solutions were the same as used in synergism assays. The HRBCs from A, B, and O were provided by the Ceará Hematology and Hemotherapy Center (Brazil).

The blood was collected in a tube with heparin (5 IU mL^{-1}), centrifuged at 300 g for 5 min at 4 °C, dissolved, washed, and diluted to a concentration of 2.5% in sterile 0.15 M NaCl. Each blood type was incubated (100 µL), with solutions of *Mo*-CBP_3-PepI, *Mo*-CBP_3-PepIII (50 µg mL^{-1}), NYS (1000 µg mL^{-1}), ITR (1000 µg mL^{-1}), the solution made by peptides and drugs, DMSO-NaCl, and 0.1% (v/v) Triton X-100 (the positive control for hemolysis) for 30 min at 37 °C, followed by centrifugation (300 g for 5 min at 4 °C), supernatants were collected and transferred to 96-well microtiter plates. Hemolysis (%) was calculated by taking absorbance at 414 nm using an automated absorbance microplate reader. Negative (0%) and positive (100%) hemolysis were determined by treating HRBCs with 5% DMSO in 0.15 M NaCl (vehicle for peptides) and 0.1% (v/v) Triton X-100, respectively. The hemolysis was calculated by the equation: [(Abs_{414nm} of HRBC treated with Solution-Abs_{414nm} HRBCs treated with 0.15 M NaCl) / [(Abs_{414nm} of HRBCs treated with 0.1% TritonX-100-Abs_{414nm} of HRBCs treated with 0.15 M NaCl)] x 100.

Statistical analysis

All the assays were performed individually three times with the statistics expressed as the mean ± standard error. The data were submitted to ANOVA software followed by the Tukey test. GraphPad Prism 5.01. was used to perform all graphics, with a significance of $P<0.05$.

Results

Antibiofilm activity of synthetic peptides and two commercial drugs

The antibiofilm activity of *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII, both at 50 μg mL^{-1} against *C. albicans* and *C. parapsilosis*, are shown in Figure 1. ITR and NYS alone inhibited *C. albicans*7% and 40% biofilm formation, respectively (Fig. 1A). *Mo*-CBP$_3$-PepI alone inhibited 10% of the biofilm formation of *C. albicans*. Regarding the synergistic effect, *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII enhanced the ITR biofilm inhibition formation in *C. albicans* about 3.5- and 4.0-fold, respectively (Fig. 1A). Combining both peptides with NYS increased the inhibition of *C. albicans* biofilm formation from 40% (NYS alone) to 90% (Fig. 1).

ITR or NYS alone inhibited, respectively, 45 and 43% of *C. parapsilosis* biofilm formation of (Fig. 1B) while combinations of *Mo*-CBP$_3$-PepI + ITR, *Mo*-CBP$_3$-PepIII + ITR, *Mo*-CBP$_3$-PepI + NYS, and *Mo*-CBP$_3$-PepIII + NYS inhibited 98%, 96%, 79%, and 82%, respectively, *C. parapsilosis* biofilm formation (Fig. 1B). In contrast, *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII inhibited 15% and 25%, respectively, *C. parapsilosis* biofilm formation (Fig. 1B). The combinations of peptides and antifungal drugs showed higher biofilm inhibition than conventional drugs. This combination was more efficient in inhibiting biofilm formation in *C. parapsilosis* than in *C. albicans*.

Regarding the degradation of preformed *C. albicans* biofilm, ITR and NYS degraded the biofilm by about 50% and 30%, respectively (Fig. 1C). *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII showed a stronger action by degrading 60% and 30% of preformed *C. albicans* biofilm. The combinations *Mo*-CBP$_3$-PepI + ITR and *Mo*-CBP$_3$-PepIII + ITR did not present any effect (Fig. 1C). However, the combinations *Mo*-CBP$_3$-PepI + NYS showed 85% degradation of *C. albicans* preformed biofilm while *Mo*-CBP$_3$-PepIII + NYS degraded 50% of *C. albicans* preformed biofilm (Fig. 1C). Regarding the degradation of

C. parapsilosis preformed biofilm, only the combination *Mo*-CBP_3-PepI + NYS reduced 50% of the biofilm biomass (Fig. 1D). Any other treatments were effective against the *C. parapsilosis* preformed biofilm (Fig. 1D).

Analysis of Candida biofilm morphology

Scanning Electron Microscopy (SEM) is a powerful tool to assess the damage of antifungal treatment on *C. albicans* and *C. parapsilosis* biofilm formation (Figs. 2 and 3). Regarding inhibition of *C. albicans* and *C. parapsilosis* biofilm formation, control cells did not show any damage or alterations on the cell surface since spherical cellular shape can be observed without cracks or scars (Figs. 2A and 3A). The treatment with peptides and drugs alone presented only mild damage such as wrinkles and slight changes on morphology of cells but nonlethal effects showing a very similar appearance to the control in the number of cells (Fig. 2B-E).

The combination *Mo*-CBP_3-PepI + ITR and *Mo*-CBP_3-PepIII + ITR showed a significant reduction in the biofilm formed compared to control (Fig. 2F and G). In these treatments, it was possible to see damage such as small blebs, new buds, scars on new buds and cells, and rings of truncated bud scars (Fig. 2F and G). The *Mo*-CBP_3-PepI + NYS and *Mo*-CBP_3-PepIII + NYS are by far the most lethal to *C. albicans* (Fig. 2F and G). In those treatments, the cells are entirely damaged with high roughness levels, severe alterations in morphology, and a clear indication of cellular lysis leading to loss of cytoplasm.

In the inhibition of *C. parapsilosis* biofilm formation, SEM analysis revealed the treatment with drugs alone (Fig. 3B and C) did not prevent the biofilm formation of *C. parapsilosis*. Although the treatment with peptides alone caused severe damage to cells, such as cell morphology and lysis alteration, this was not sufficient to inhibit the *C. parapsilosis* biofilm formation (Fig. 3D and E). SEM images revealed that all peptides

241 and drug combinations were lethal to *C. parapsilosis* (Fig. 3F-I). The synergistic effect
242 led to severe injuries such as distortions and alterations on the cell surface and shape, cell
243 wall damage, distortion of the cell membrane, and internal content loss, without any
244 possibility for *C. parapsilosis* to form a biofilm (Fig. 3F-I).

245 SEM analysis revealed *Mo*-CBP$_3$-PepI + NYS was the most efficient treatment in
246 degrading the *C. albicans* and *C. parapsilosis* preformed biofilms (Figs. 4 and 5). The
247 control biofilm (treated with DMSO) did not present any damage (Figs. 4A and 5A). The
248 biofilms treated alone with NYS (Figs. 4B and 5B) and *Mo*-CBP$_3$-PepI (Figs. 4C and 5C)
249 presented mild damage such as altered morphology and wrinkles, distortion and apparent
250 reduction in biomass compared to controls. The *Mo*-CBP$_3$-PepI + NYS presented higher
251 lethality to *C. albicans*, and *C. parapsilosis* preformed biofilms (Figs. 4D and 5D). After
252 the treatment, biofilms presented a high reduction in the biomass of both biofilms in
253 addition to severe damage such as cell depression-like cavities and damage to the cell
254 wall, in addition to alterations in cell shape, wrinkles and scars all over the structure, and
255 loss of internal content (Figs. 4D and 5D).

256 ***Membrane pore formation***

257 PI incorporation was used to evaluate the pore formation on the yeast membrane.
258 PI interacts with DNA releasing red fluorescence, but this is only possible in membranes
259 that are damaged. Healthy membranes block the PI from moving into the cell. The
260 negative control DMSO did not damage the membranes, and thus no fluorescence was
261 detected (Figs. 6-10). NYS and ITR tested alone did not show fluorescence in any
262 treatment (Figs. 6-10). However, *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII alone and in
263 combination with NYS induced red fluorescence in *C. albicans* cells, indicating these
264 cells are damaged and could present challenges to form a biofilm (Fig. 6). Similar results
265 were found with *C. parapsilosis* cells (Figs. 7 and 8). Both peptides, either alone or

combined with ITR or NYS, induced pore formation in the *C. parapsilosis* cell membrane, thus inhibiting biofilm formation (Figs. 7 and 8). In preformed biofilms, *Mo*-CBP_3-PepI alone or combined with NYS induced pore formation in the *C. albicans* and *C. parapsilosis* cell membranes, as revealed by red fluorescence (Figs. 9 and 10).

ROS production

The analysis of ROS production revealed this is a mechanism employed by peptides alone and in synergism with drugs to damage the biofilms of *Candida* species (Figs. 6-10). The results showed that in *C. albicans* cells NYS did not induce ROS overproduction. Nevertheless, *Mo*-CBP_3-PepIII, *Mo*-CBP_3-PepI + NYS, and *Mo*-CBP_3-PepIII + NYS induced a slight production of ROS (Fig. 6). None of the treatments induced ROS production during the inhibition of biofilm formation of *C. parapsilosis* (Figs. 7 and 8).

Regarding degradation of preformed biofilm, no ROS overproduction was detected with any treatment towards *C. albicans* biofilm (Fig. 9). The fluorescence images obtained from the degradation of *C. parapsilosis* preformed biofilm indicated that NYS alone is ineffective in promoting ROS generation. However, *Mo*-CBP_3-PepI alone or combined with NYS induced a strong ROS production (Fig. 10).

Molecular docking

We performed molecular docking to evaluate and understand the possible interactions of the peptides with the antifungal drugs. *Mo*-CBP_3-PepI interacts with ITR and NYS with the lowest binding interaction energy (LBIE) of -4.5 and -4.2 $kcal.mol^{-1}$, respectively (Fig. S1A and B). The amino acid residues Pro^2 and Ile^4 of the *Mo*-CBP_3-PepI peptide show Pi-Alkyl interactions with the phenyl (4.5 Å), piperazine (4.2 Å), and dichlorophenyl (4.5 Å) groups, respectively, with ITR. Cys^8 has a Pi-Anion (3.4 Å,

290 triazole group) and a Pi-Sulfur (5.1 Å, dichlorophenyl group) interaction from ITR, and
291 Arg^{6} presents only van der Waals interaction (Fig. S1A and C). The *Mo*-CBP_3-PepI
292 interacts with NYS by van der Waals interactions of Cys^{8}, Gln^{5}, Cys^{1}. An Alkyl (5.0 Å)
293 interaction with Pro^{2} and an unfavorable donor-donor (1.3 Å) with Arg^{6} are also
294 established (Fig. S1B and D).

295 *Mo*-CBP_3-PepIII presents a docking score of -4.0 and -4.1 $kcal.mol^{-1}$ with ITR
296 and NYS, respectively (Fig. S1E and F). *Mo*-CBP_3-PepIII interacts through van der Waals
297 interactions by residues Ala^{1}, Gln^{3}, and Cys^{6} with ITR. Cys5 interacts through an Amide-
298 Pi stacked (3.8 Å) with the phenyl group of ITR. The Arg^{4} of the *Mo*-CBP_3-PepIII
299 establishes a Pi-Cation interaction with the dichlorophenyl group (3.8 Å) and a Pi-Alkyl
300 interaction (4.7 Å) with the methoxyphenyl group of itraconazole (Fig. S1E and G). The
301 interaction between *Mo*-CBP_3-PepIII and NYS is supported by hydrogen bonds between
302 residues Arg^{4} (2.0 Å) and Cys^{6} (1.9 Å), as well as through van der Waals interactions
303 through residues Gln^{3} and Cys^{5} (Fig. S1F and H). Taken together, these results suggest
304 possible direct interactions between the peptides and NYS and ITR.

305 **Hemolytic Assay**

306 As shown in a previous study (Oliveira et al. 2019), the *Mo*-CBP_3-PepI and *Mo*-
307 CBP_3-PepIII had no hemolytic activity against any human blood type tested (Table S1),
308 even at 50 μg. mL^{-1}. In contrast, NYS at 1000 μg. mL^{-1} caused 100% hemolysis in all
309 human blood types and ITR at 1000 μg. mL^{-1} caused 75, 68, and 58% of hemolysis,
310 respectively, to Type-A, B, and O of red blood cells (Table S1).

311 In general, the combination of synthetic peptides with antifungal drugs decreased
312 their hemolytic effect (Table S1). The combination of *Mo*-CBP_3-PepI with NYS resulted
313 in a hemolytic effect of 14, 23, and 2%, respectively, to Type-A, B, and O of red blood

cells, and combination of *Mo*-CBP_3-PepI with ITR caused in 0, 4, and 8% of hemolysis, respectively, to Type-A, B, and O of red blood cells (Table S1). The combination of *Mo*-CBP_3-PepIII with NYS hemolyzed 45, 30, and 18%, respectively, of Type-A, B, and O of red blood cells, whereas the combination of *Mo*-CBP_3-PepIII with ITR 50, 15, 2%, respectively, of Type-A, B, and O of red blood cells (Table S1).

Discussion

Candida spp yeasts represent a serious threat to human health worldwide. The infections by these fungi include bloodstream and systemic infections leading patients to death. Some immunocompromised patients (e.g., HIV+ and diabetics) develop an untreatable infection by *Candida spp* (Weig 1998; Kullberg & Oude Lashof 2002). These opportunistic pathogens have also established resistance to several antifungal agents (Baillie 2000; Ramage 2002; Arendrup & Patterson 2017; Sasso et al. 2017). Nevertheless, these microorganisms can organize themselves into compact microcolonies called biofilms enhancing their drug resistance and complicating the treatment. Additionally, higher concentrations of drugs employed in treatments could lead to collateral effects on patients (Baillie 2000; LaFleur et al. 2006; Kumar et al. 2017). Therefore, it is urgent to seek new alternative drugs to face this scenario and help patients to recover and survive.

Natural Antimicrobial Peptides (AMPs) are promising molecules to act as substitutes or adjuvants to treat infections. However, they present some disadvantages, such as high toxicity, low resistance to proteolysis, and high cost of isolation and purification processes. The development of SAMPs is an alternative solution to bypass this problem (Pedro F.N. Souza et al. 2020; Lima et al. 2021). SAMPs are potentially promising molecules to be used as novel therapeutic drugs against pathogens, in addition to other characteristics such as low or absence of toxicity to mammalian cells, low chance

339 to develop antimicrobial resistance based on their mechanism of action (Pedro F.N. Souza
340 et al. 2020; Lima et al. 2021).

341 Bioinspired SAMPs based on natural AMPs can offer attributes that are not
342 present in the natural molecule (Mason et al. 2006; Mason et al. 2009). A good example
343 is the synthetic peptide LAH4 designed based on Magainin 2 sequence that presented a
344 potent activity against *Escherichia coli* and *Staphylococcus aureus* compared with the
345 natural peptide Magainin 2 (Mason et al. 2006; Mason et al. 2009). Recently, our research
346 group designed three peptides derived from *Mo*-CBP_3 and antifungal Chitin-binding
347 protein from *M. oleifera* seeds. *Mo*-CBP_3-PepI, *Mo*-CBP_3-pepII, and *Mo*-CBP_3-pepIII
348 inhibited the growth of *C. albicans* and *C. parapsilosis* planktonic cells by the stimulation
349 of ROS production, cell wall damage, and membrane pore formation, leading to death
350 (Oliveira et al. 2019; Lima et al. 2020). It is essential to notice that *Mo*-CBP_3 does not
351 present anticandidal activity. Those cited are two examples of gain of function presented
352 by SAMPs related to NAMPs. Based on that, we decided to evaluate the potential of *Mo*-
353 CBP3-PepI, *Mo*-CBP3-pepII, and *Mo*-CBP3-pepIII to inhibit biofilm formation and its
354 capacity to promote degradation of preformed biofilm in *C. albicans* and *C. parapsilosis*
355 cells. This study evaluated the mechanisms of action to understand how these molecules
356 work and their possible applications.

357 Regarding degradation of preformed biofilm of *C. albicans* cells, *Mo*-CBP_3-PepI
358 and *Mo*-CBP_3-PepIII were active by 40% and 70%, respectively (Fig. 1). These results
359 corroborate with Galdiero et al. (Galdiero et al. 2020) for gH625, analog from gH625-M,
360 which reduced by 61% the biomass of preformed biofilm of *C. albicans*. SEM analysis
361 of *C. albicans* and *C. parapsilosis* treated with *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII
362 showed that biofilm suffered severe damage leading biofilm structure to disorder.
363 Furthermore, SEM analysis suggests all peptides induce rupture of the cell wall and

membrane-pore formation, leading the cell to internal content loss and death (Figs. 2-5). The images also showed the presence of scars, buds scars, and cracks in biofilm-treated cells. These results corroborate the work developed by Belmadani et al. (Belmadani et al. 2018) observed that Dermaseptin-S1, an antimicrobial peptide from *Phyllomedusa sauvagii*, decreased *C. albicans* biofilm formation by alterations on the cell wall structure, membrane pore formation, and leakage of internal content (Belmadani et al. 2018).

The effects of *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII on *C. albicans* and *C. parapsilosis* biofilm growth were either cytolysis or cell membrane disruption, leading to cell death. A previous study of our research group demonstrated that *Mo*-CBP$_3$-PepIII presented a potent activity against *C. parapsilosis* planktonic cells, causing severe damage to the cell wall and membrane and leading to death through leakage of internal content (Oliveira et al. 2019). Similar behavior was observed by Sierra et al., where *C. albicans* biofilm suffered severe damage by the antimicrobial peptide Histatin-5 (Sierra et al. 2017).

These severe damages observed in the cell wall of both cells via SEM analysis can be explained considering that *Mo*-CBP3-PepI and *Mo*-CBP3-PepIII are designed based on the sequence of *Mo*-CBP3, a chitin-binding protein from *M. oleifera* seeds (Oliveira et al. 2019). Both peptides can interact with chitin present in the fungal cell wall and cause destabilization of the cell, leading to rupture, electrolyte imbalance, and thus cell death.

Unlike many commercial drugs with specific targets in enzymes from the metabolism or inactivation of protein synthesis, SAMPs target the cell membrane leading to pore formation and damage of the cell wall (Pedro F.N. Souza et al. 2020). The ability of SAMPs to alter the microbial membrane permeabilization is considered the most common mechanism of action of these molecules, making the development of resistance mechanisms by microorganisms (Pedro F.N. Souza et al. 2020; Lima et al. 2021).

Fluorescence microscopy analyses were performed to evaluate if our peptides could induce membrane-pore formation (Figs. 6-10). *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII induced PI uptake in *C. albicans* and *C. parapsilosis* biofilm, suggesting pore formation and cell membrane damage (Figs. 6-10). The fluorescence microscopy corroborates the data observed by SEM analysis, strongly indicating damage in the cell membrane. Peptides enhanced the activity of NYS and ITR towards *C. albicans* and *C. parapsilosis* biofilms. In some cases, alone, neither peptides nor drugs had any action against biofilms; however, the combinations between them were much more efficient. It is known that *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII form pores, respectively, of 6 and 20 kDa on *C. albicans* and *C. parapsilosis* membrane (Oliveira et al. 2019; Lima et al. 2020). NYS and ITR have molecular weights, respectively, of 926.1 and 705 Da. So, it is feasible to suggest that both NYS and ITR are passing through the pores formed by peptides on the membrane and somehow displaying damage in components of the cytoplasm.

Although the major mechanisms of action of synthetic peptides are to attack the cell membrane and the cell wall, these molecules can also be internalized by the cell and induce different responses, such as inhibition of cell wall synthesis or its maintenance, protein synthesis, and folding (Huang et al. 2010). Similar behavior was described by Maurya et al. [29], which showed that the peptides VS2 and VS3 can induce pore formation in *C. albicans* cells and develop distinct inhibitory activities in different targets inside the cell (Maurya et al. 2011).

Furthermore, *Mo*-CBP_3-PepI induced ROS overproduction in *C. parapsilosis* biofilm in both degradation and inhibition of biofilm. *Mo*-CBP_3-PepIII induced slight ROS generation in the inhibition of *C. albicans* biofilm formation (Fig. 6). A similar profile was observed using the killer peptides KP and MCh-AMP1, which are synthetic peptides able to induce ROS overproduction in *C. albicans* biofilm, leading to cell death

(Seyedjavadi et al. 2020). ROS are involved in the damage of essential molecules such as proteins, lipids, and DNA.

Additionally, we performed a pioneer molecular docking study to evaluate if our peptides could interact with NYS and ITR predominantly used to treat infections caused by *Candida spp* (Fig. S1). Similar behavior was detected by Souza et al. (Pedro F N Souza et al. 2020) where *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII interacted with griseofulvin by weak interactions, such as hydrogen bonds and hydrophobic interactions. The interaction of peptides with griseofulvin enhanced its activity against dermatophytes and reduced the toxicity of the drug. The interactions between both peptides and each drug can explain the great synergistic activity obtained in our results, where both peptides enhanced the activity of both drugs.

A possible clinical application of synthetic peptides is that these molecules could act as adjuvants to drugs becoming less active due to the development of antifungal clinical resistance. *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII improved the activity of NYS and ITR by up to 50% on inhibition of biofilm formation of *C. albicans* and *C. parapsilosis*. Moreover, the results showed that *Mo*-CBP$_3$-PepI enhanced NYS activity up to 60% to degrade the preformed biofilm of both yeasts. NYS (polyene) very likely promotes an antibiofilm activity by interacting with ergosterol in the fungal cell membrane, making it an efficient drug to treat skin infections caused by *Candida* species. Nevertheless, NYS presents a high hemolytic activity to human blood cells, limiting its application.

Besides improve the antibiofilm activity of NYS and ITR, peptides also reduced the hemolytic effect (Table S1). However, ITR and NYS presented undesired effects, such as vomiting, nausea, diarrhea, anorexia, abdominal pain, and dizziness. Besides these collateral effects, cardiotoxicity and hypertension were attributed to ITR usage. An

unexpected and interesting result was that the association of peptides with antifungal drugs reduced their toxicity in human erythrocytes (Table S1). For example, NYS alone caused hemolysis of 100% on type-A erythrocytes. The *Mo*-CBP_3-PepI + NYS and *Mo*-CBP_3-PepIII + NYS induced hemolysis of 0 and 45%, respectively, of type-A blood. This is a reduction of 100 and 55% in the toxicity of NYS to type-A blood cells. All treatments combining peptides with antifungal drugs were able to reduce the hemolytic effect of drugs. These results make peptides potential molecules to be applied as adjuvants of these drugs because they enhance their activity toward biofilm and, at the same, reduce their toxicity.

Molecular docking analysis between peptides and drugs revealed a clue about how peptides reduced the hemolytic effect of drugs. It seems that all rely on hydrophobicity. The membrane of erythrocytes has neutral phospholipids, which means that any interaction with those membranes must be driven by hydrophobic interactions (Huang et al. 2010). It is known that NYS and ITR are hydrophobic drugs (B et al. 2016; C et al. 2019). Hydrophobic interactions with membranes of erythrocytes may drive the hemolytic activity of NYS and ITR. The molecular docking experiments revealed that peptides performed hydrophobic interactions with NYS and ITR. Our results suggest that the hydrophobic interactions between peptides and both drugs prevent the interaction with the erythrocyte membranes reducing their hemolytic effect.

Conclusion

Antibiofilm activity, no toxicity, and synergistic effect enhancing the activity of NYS and ITR, strongly indicate that *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII are promising antibiofilm peptides to act as new antimicrobial agents. We also highlight their use for clinical application or adjuvants to conventional drugs to overcome resistance developed by *Candida species*.

Acknowledgments

This work was supported by grants from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (process numbers 308107/2013-6 and 306202/2017-4); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Instituto Nacional de Ciências e Tecnologia de Bioinspiração (Process Number: 465507/2014-0) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP). A special thanks to CAPES for providing the grant for postdoctoral position for Pedro F. N. Souza. We are also grateful to the central analytical facilities of UFC, Brazil.

Credit Author Statement:

All authors made substantial contributions. The conception and design of the study and acquisition of data, analysis, docking analysis, and interpretation were performed by LPB, CDTF, AFBS, NASN, ALCP, JTAO and PFNS. Microscopic analyses were carried out by RGGS and AFBS. Writing or revising this article was done by LPB, ALCP, GHG, and PFNS. PFNS did final approval of the version to be submitted.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Data Availability

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

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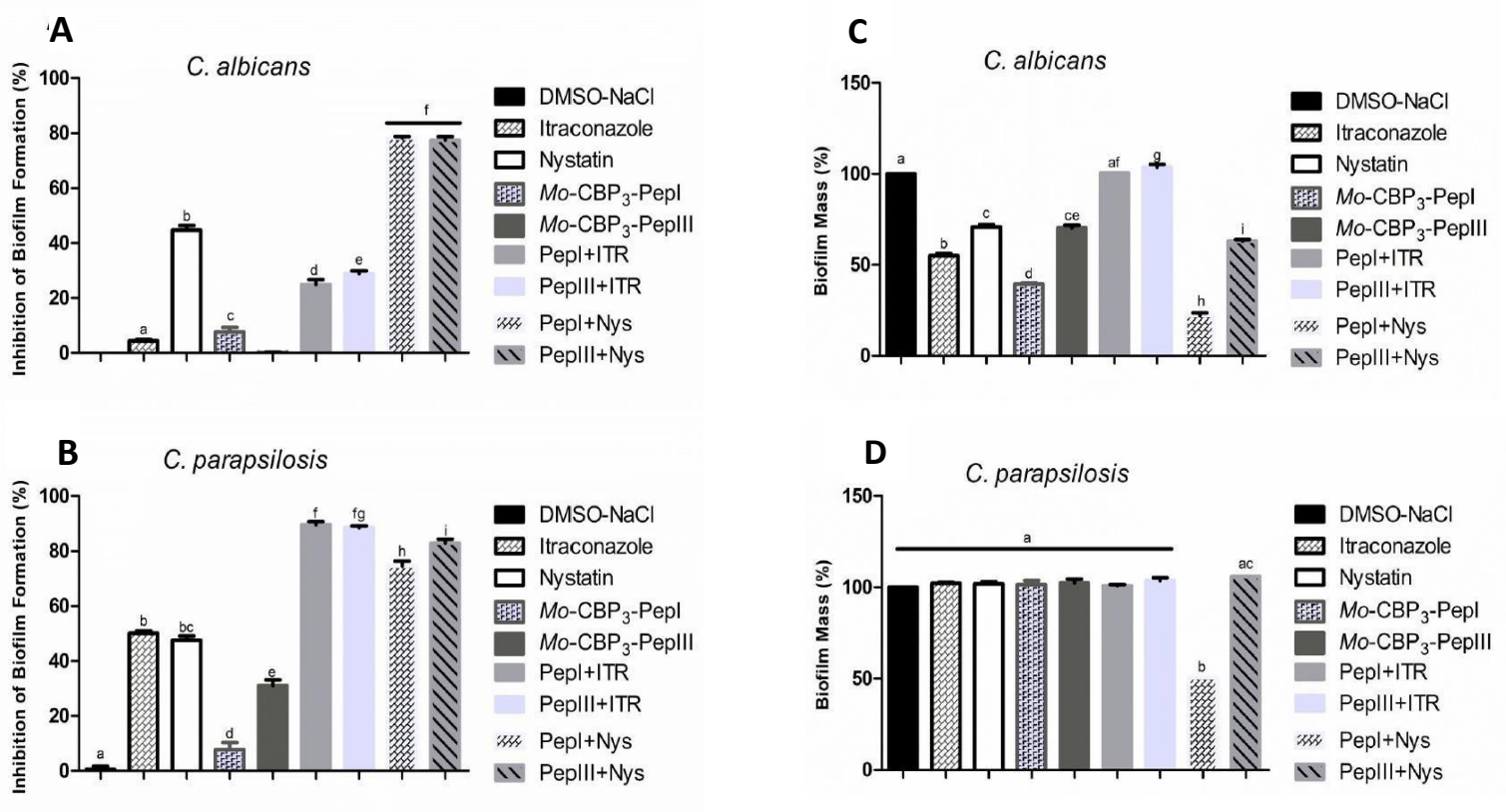


Figure 1. (**A** and **B**) Inhibitory activity of biofilm formation exhibited by Mo-CBP3-PepI and Mo-CBP3-PepIII against *C. albicans* and *C. parapsilosis*. (**C** and **D**) Degradation of preformed biofilm of *C. albicans* and *C. parapsilosis* exerted by Mo-CBP3-PepI and Mo-CBP3-PepIII. DMSO-NaCl was used as a negative control and ITR and NYS as a positive control. The letters represent the mean ± standard deviation of three replicates. *Different lowercase letters indicate statically significant difference compared to DMSO by analysis of variance ($p < 0.05$).

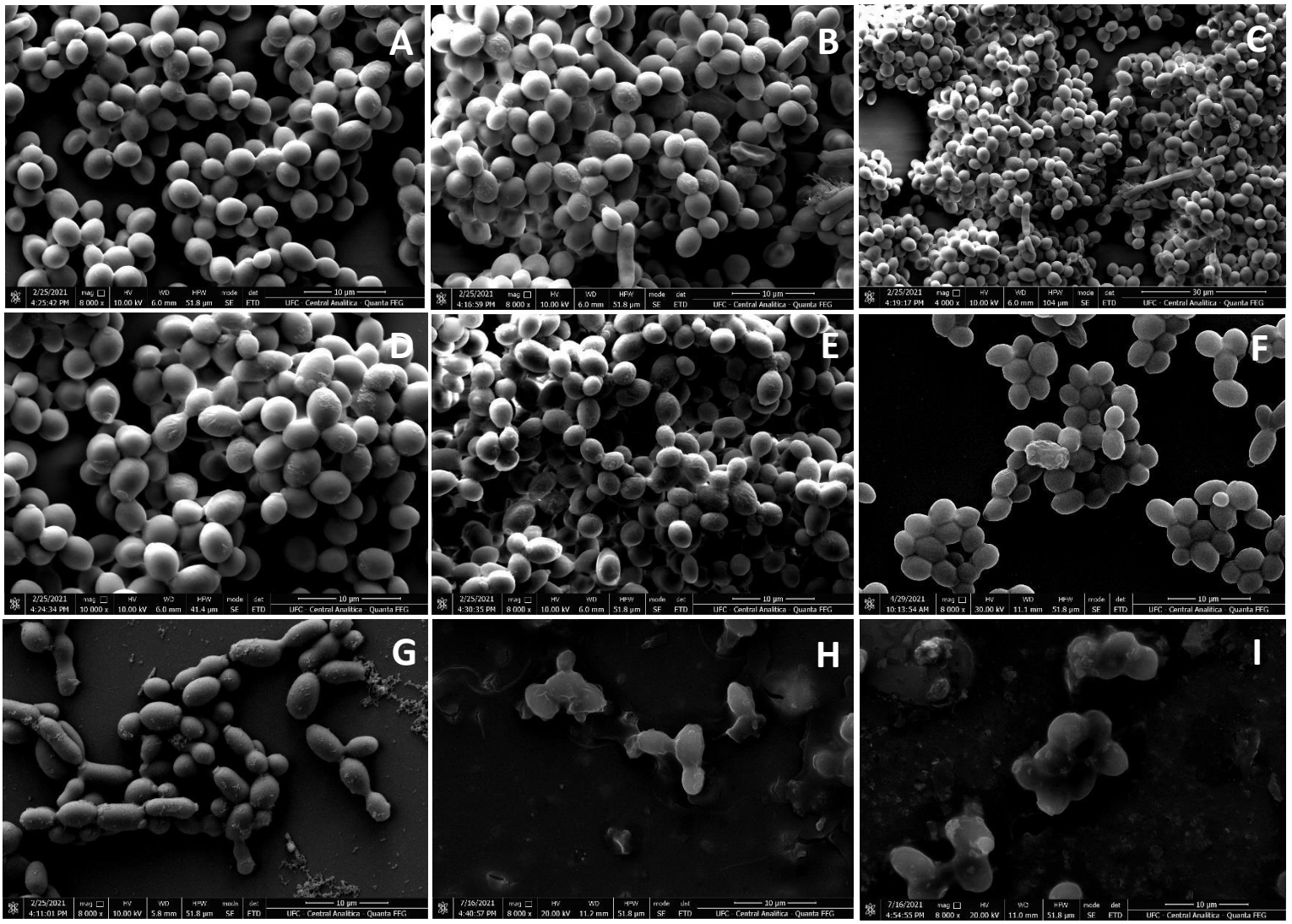


Figure 2. Scanning Electron Microscopy (SEM) images showing alterations on biofilm of *C. albicans* cells after incubation with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII. (**A**) The surface of biofilm control cells is covered by well-defined and organized structures. (**B**- **C**) Biofilm cells exposed to ITR and NYS, without major changes in the cell surface. (**D**-**E**) *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII-treated cells, showing alterations in cell membrane, scars, and buds scars. (**F-G**) Biofilm cells in contact with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII in a synergistic action with ITR, showing considerable changes in the shape of cells and buds scars. (**H-I**) Biofilm cells incubated with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII in contact with NYS, resulting into strong alterations in the shape of the cells, cell wall damage and leakage of internal content.

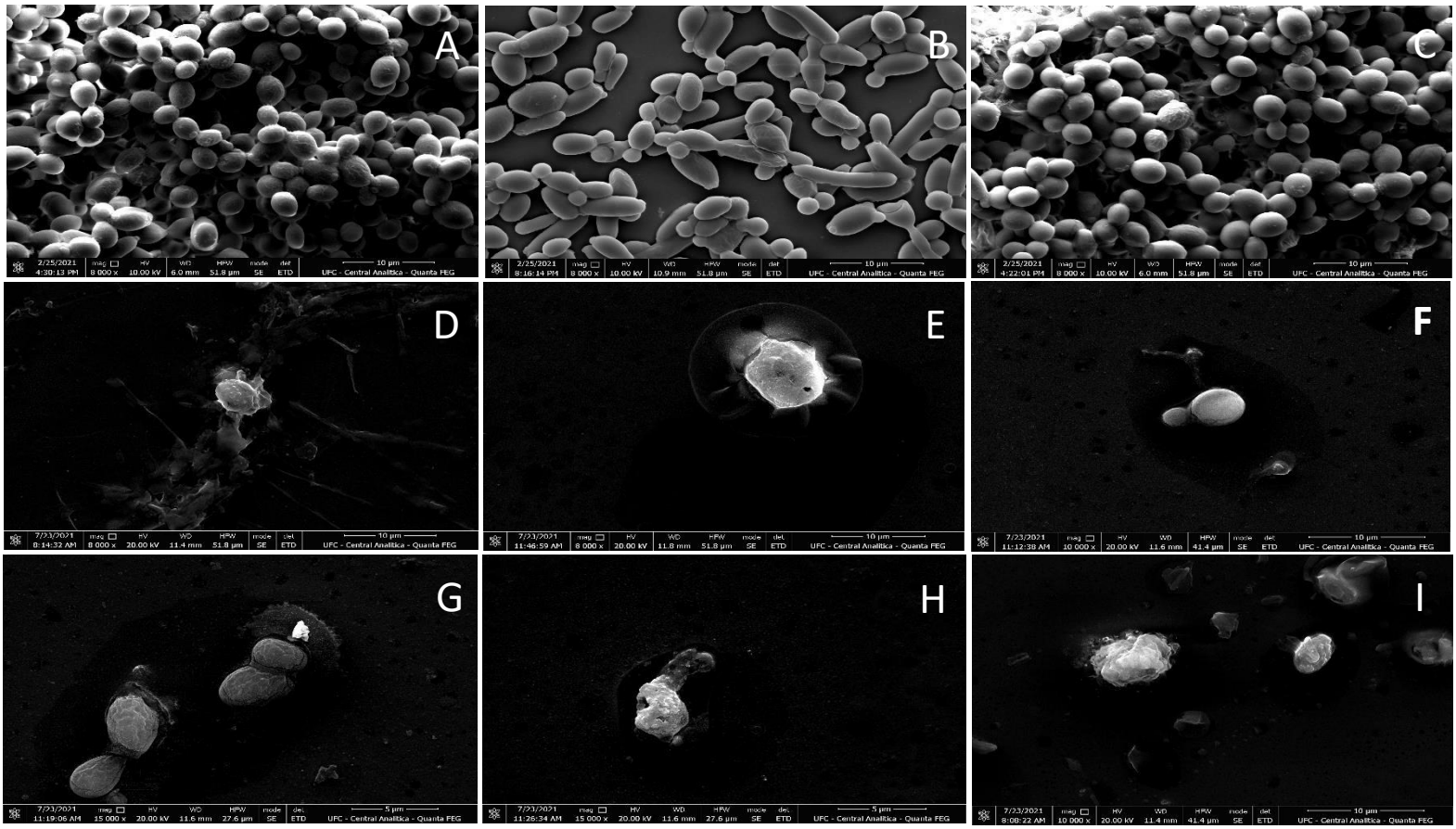


Figure 3. Scanning Electron Microscopy (SEM) images showing alterations on biofilm of *C. parapsilosis* cells after incubation with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII. (**A**) The surface of biofilm control cells is covered by well-defined and organized structures. (**B-C**) Biofilm cells exposed to ITR and NYS, without major changes in the cell surface. (**D-E**) *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII-treated cells, showing major alterations in cell membrane, such as deformation, cell wall damage and internal content loss. (**F-G**) Biofilm cells in contact with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII in a synergistic action with ITR, showing considerable changes in the shape of cells and internal content loss. (**H-I**) Biofilm cells incubated with *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII in contact with NYS, resulting into strong alterations in the membrane cell surface, cell wall damage and internal content loss.

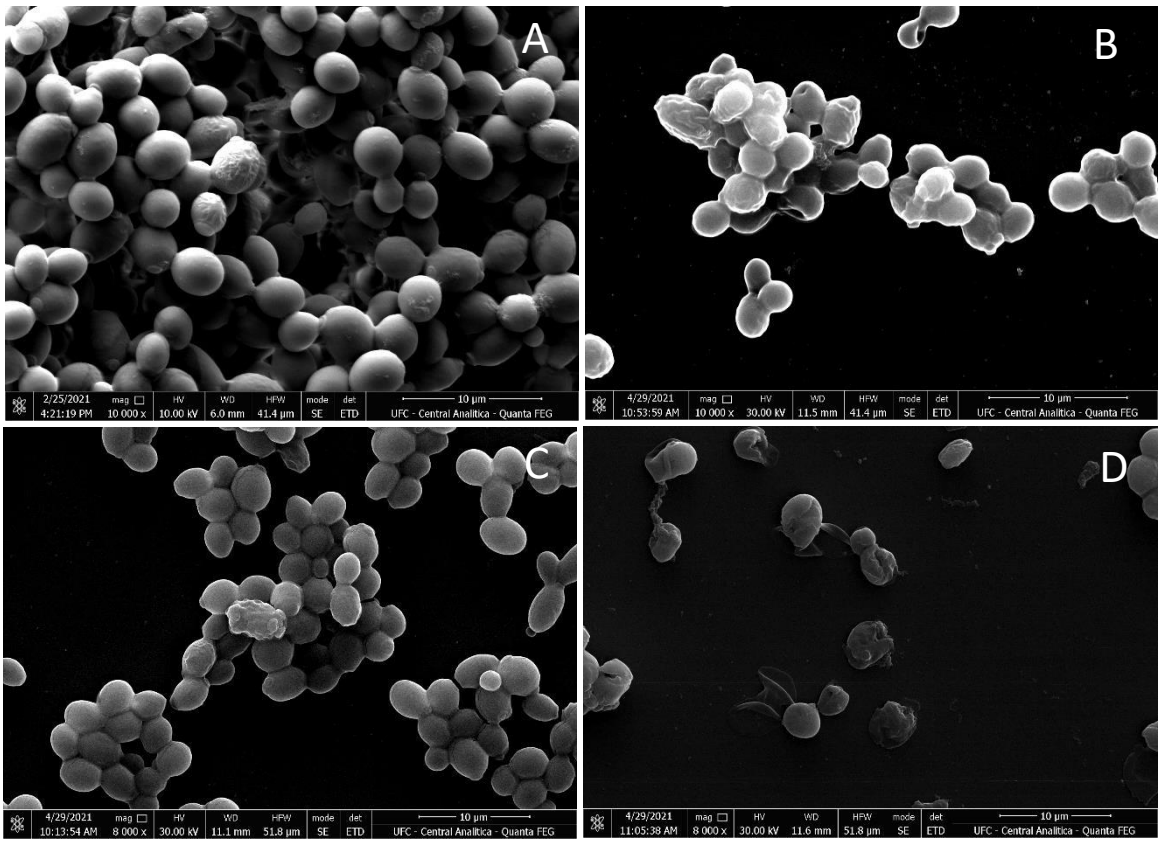


Figure 4. Scanning Electron Microscopy (SEM) images showing alterations on preformed biofilm of *C. albicans* cells after incubation with *Mo*-CBP$_3$-PepI. (**A**) The surface of biofilm control cells is covered by well-defined and organized structures. (**B**) Biofilm cells exposed to NYS, showing few alterations in the cell surface. (**C**) *Mo*-CBP$_3$-PepI-treated cells, showing some alterations in cell membrane, such as scars and cell wall damage. (**D**) Biofilm cells incubated with *Mo*-CBP$_3$-PepI in contact with NYS, resulting into strong alterations in the shape of the cells, cell wall damage and leakage of internal content.

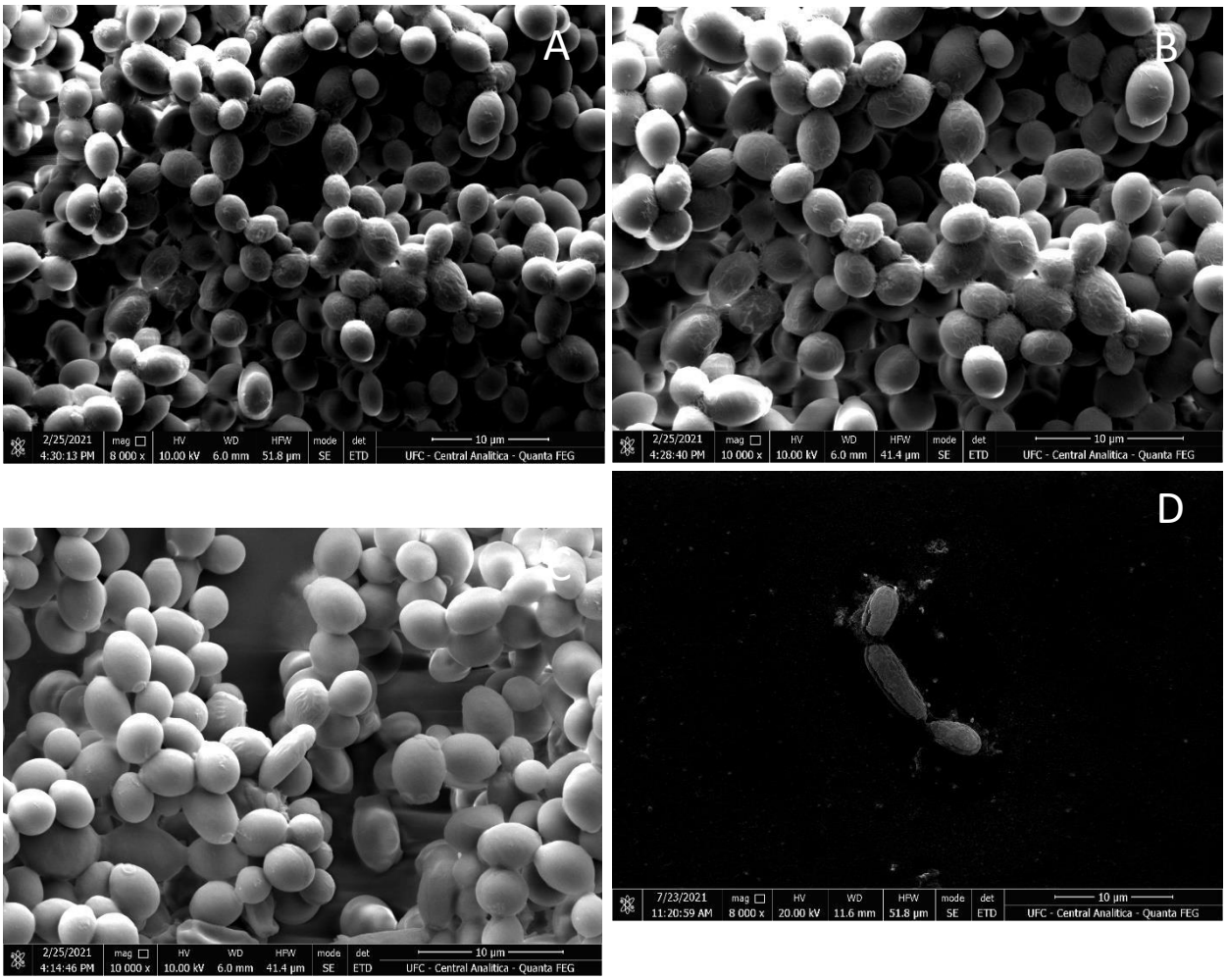


Figure 5. Scanning Electron Microscopy (SEM) images showing alterations on preformed biofilm of *C. parapsilosis* cells after incubation with *Mo*-CBP$_3$-PepI. (**A**) Thesurface of biofilm control cells is covered by well-defined and organized structures. (**B**) Biofilm cells exposed to NYS, showing few alterations in the cell surface. (**C**) *Mo*-CBP$_3$-PepI-treated cells, showing some alterations in cell membrane, such as scars and cell walldamage. (**D**) Biofilm cells incubated with *Mo*-CBP$_3$-PepI in contact with NYS, leading to strong alterations in the shape of the cells, deformation, cell wall damage and leakageof internal content.

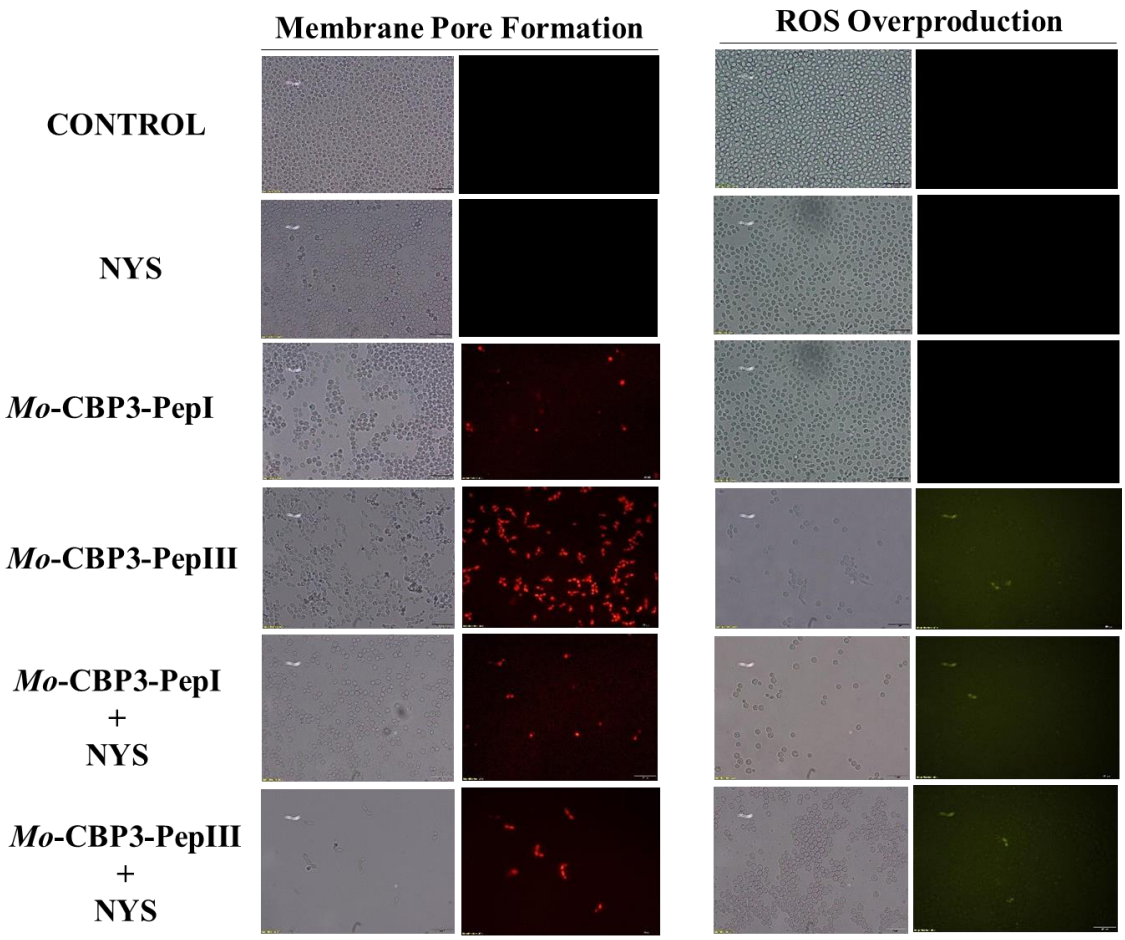


Figure 6. Fluorescence images showing membrane pore formation and ROS overproduction on inhibition of the biofilm of *C. albicans* cells. Control solution of DMSO-NaCl, treated with *Mo*-CBP3-PepI and *Mo*-CBP3-PepIII at 50 μg mL^{-1} and synergistic activity of both peptides with NYS. Membrane pore formation was measured my propidium iodide uptake assay and ROS overproduction was detected using 2', 7' dichlorofluorescein diacetate (DCFH-DA).

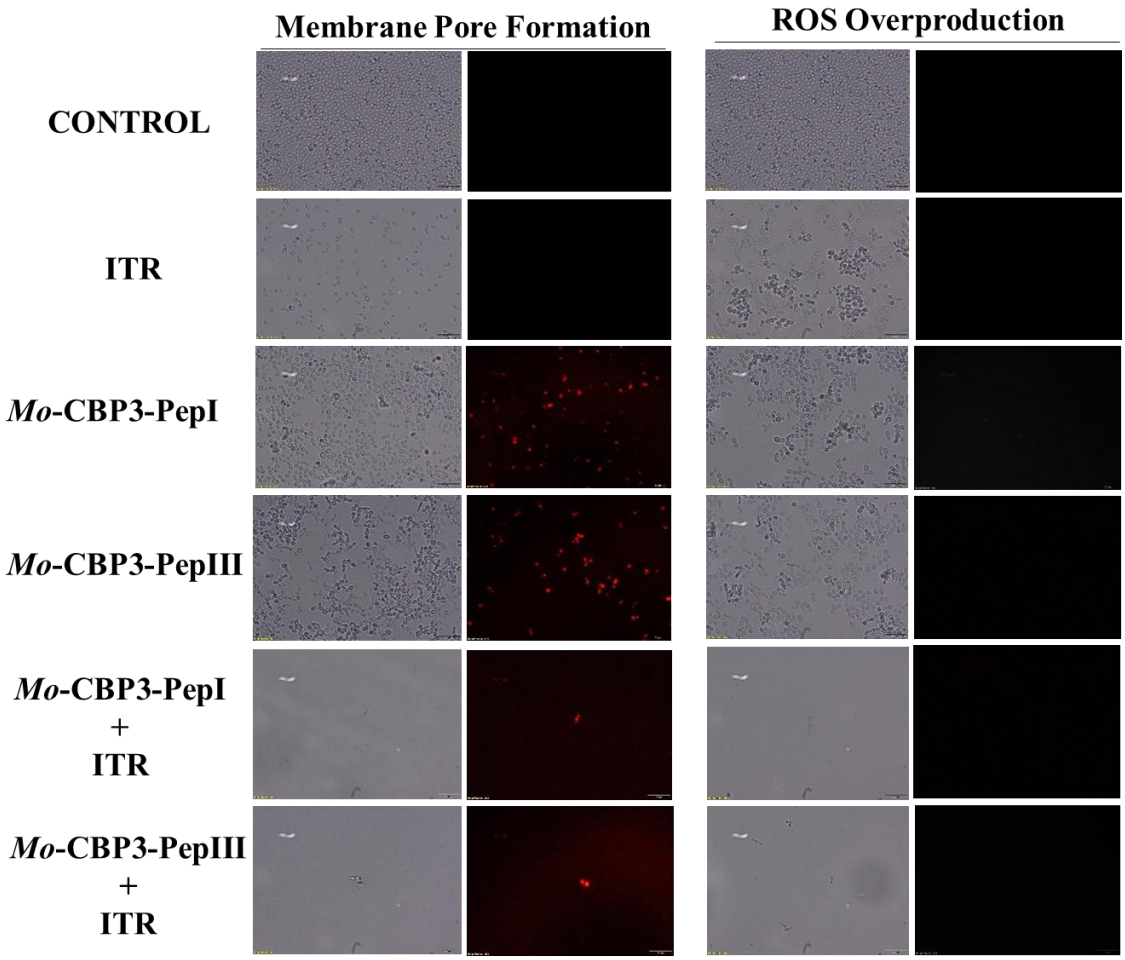


Figure 7. Fluorescence images showing membrane pore formation and ROS overproduction on inhibition of the biofilm of *C. parapsilosis* cells. Control solution of DMSO-NaCl, treated with *Mo*-CBP3-PepI and *Mo*-CBP3-PepIII at 50 μg mL^{-1} and synergistic activity of both peptides with ITR. Membrane pore formation was measured my propidium iodide uptake assay and ROS overproduction was detected using 2', 7' dichlorofluorescein diacetate (DCFH-DA).

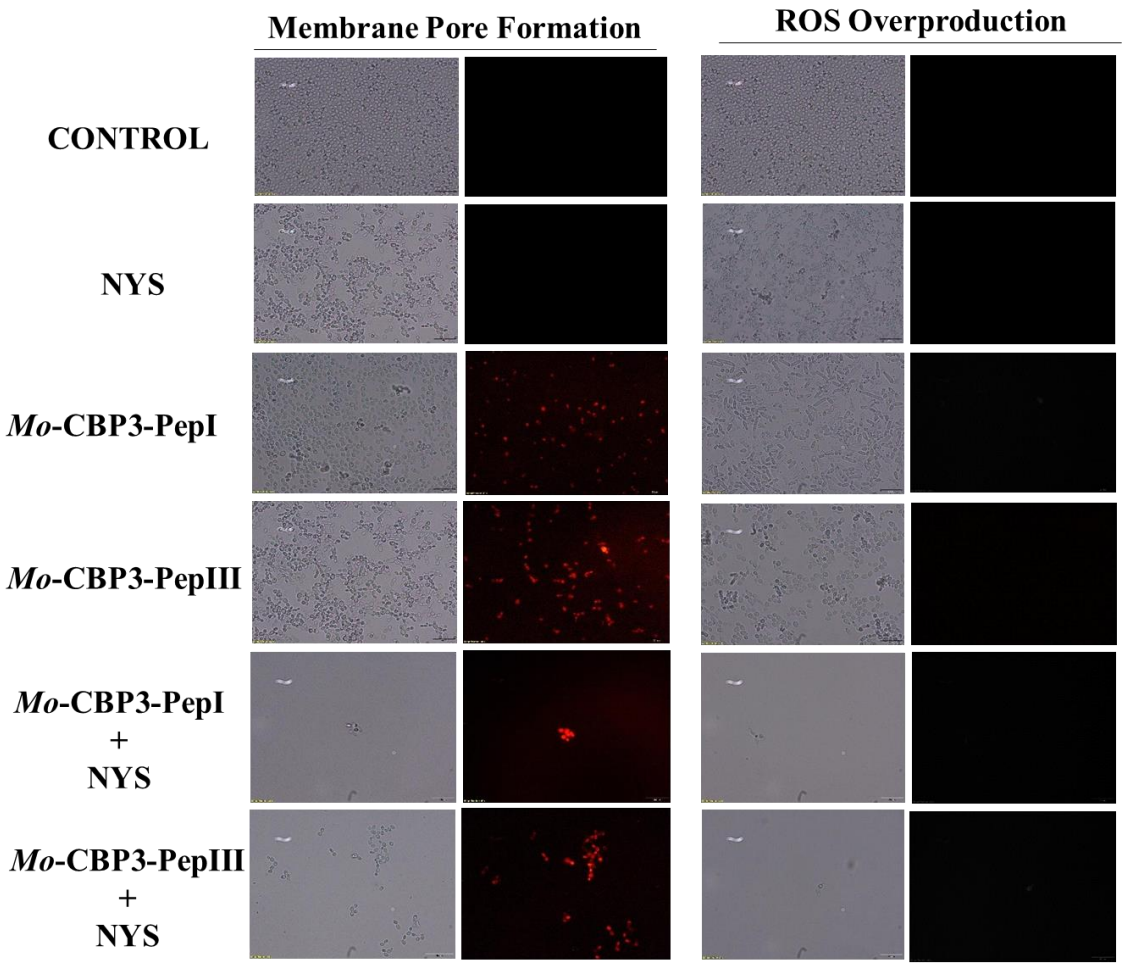


Figure 8. Fluorescence images showing membrane pore formation and ROS overproduction on inhibition of the biofilm of *C. parapsilosis* cells. Control solution of DMSO-NaCl, treated with *Mo*-CBP3-PepI and *Mo*-CBP3-PepIII at 50 μg mL^{-1} and synergistic activity of both peptides with NYS. Membrane pore formation was measured my propidium iodide uptake assay and ROS overproduction was detected using 2', 7' dichlorofluorescein diacetate (DCFH-DA).

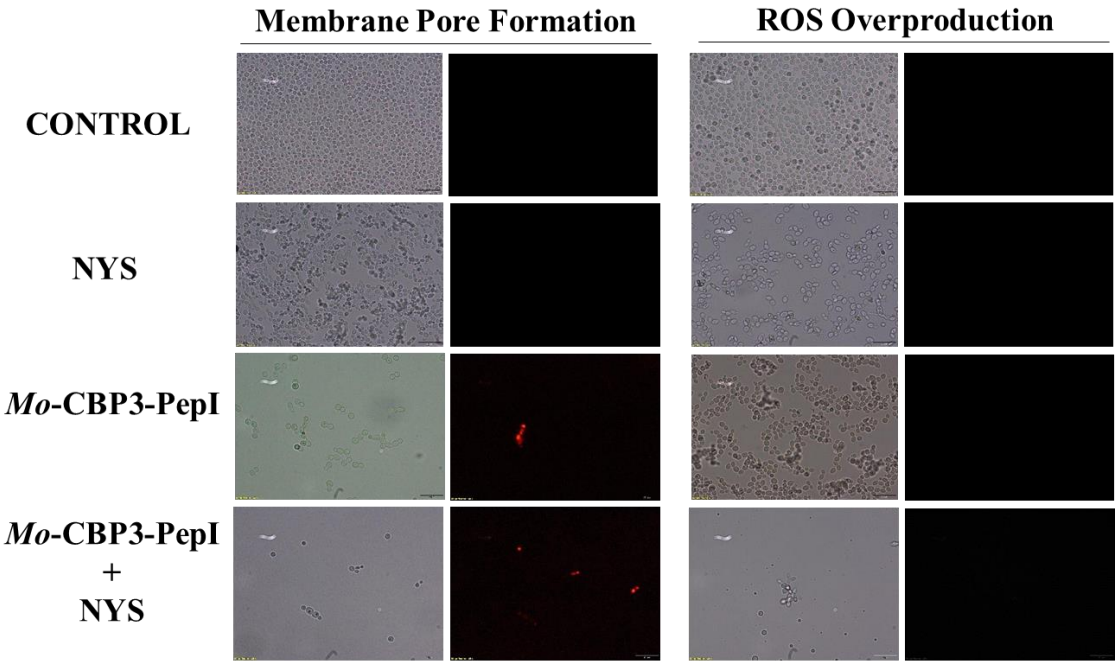


Figure 9. Fluorescence images showing membrane pore formation and ROS overproduction on degradation of the biofilm of *C. albicans* cells. Control solution of DMSO-NaCl, treated with *Mo*-CBP3-PepI at 50 μg mL^{-1} and synergistic activity with NYS. Membrane pore formation was measured my propidium iodide uptake assay and ROS overproduction was detected using 2', 7' dichlorofluorescein diacetate (DCFH-DA).

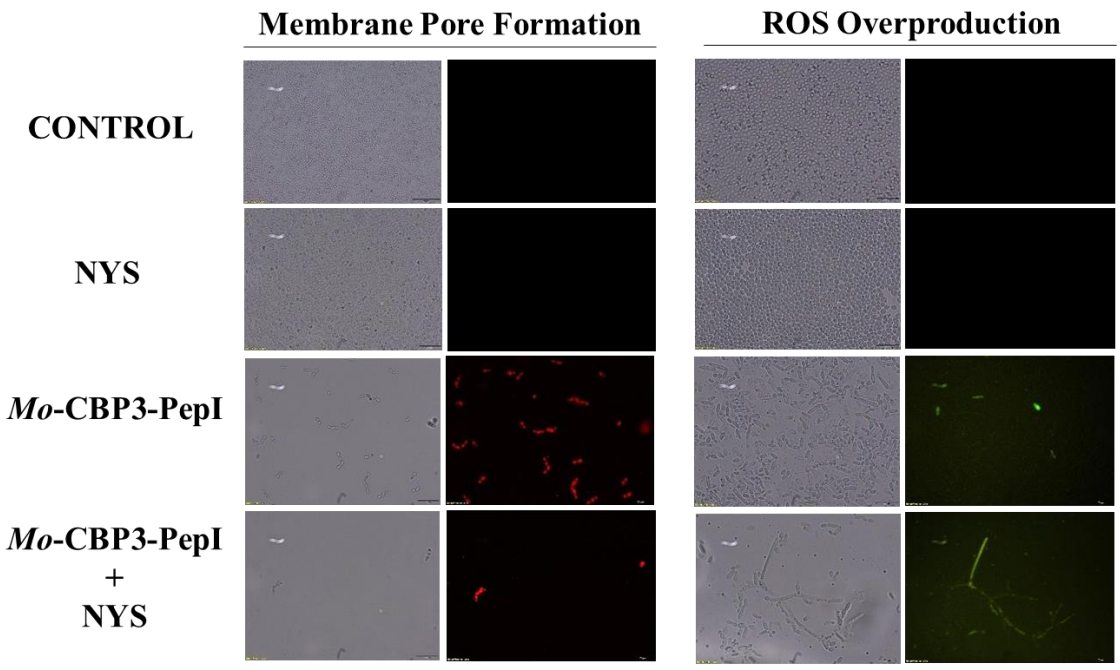
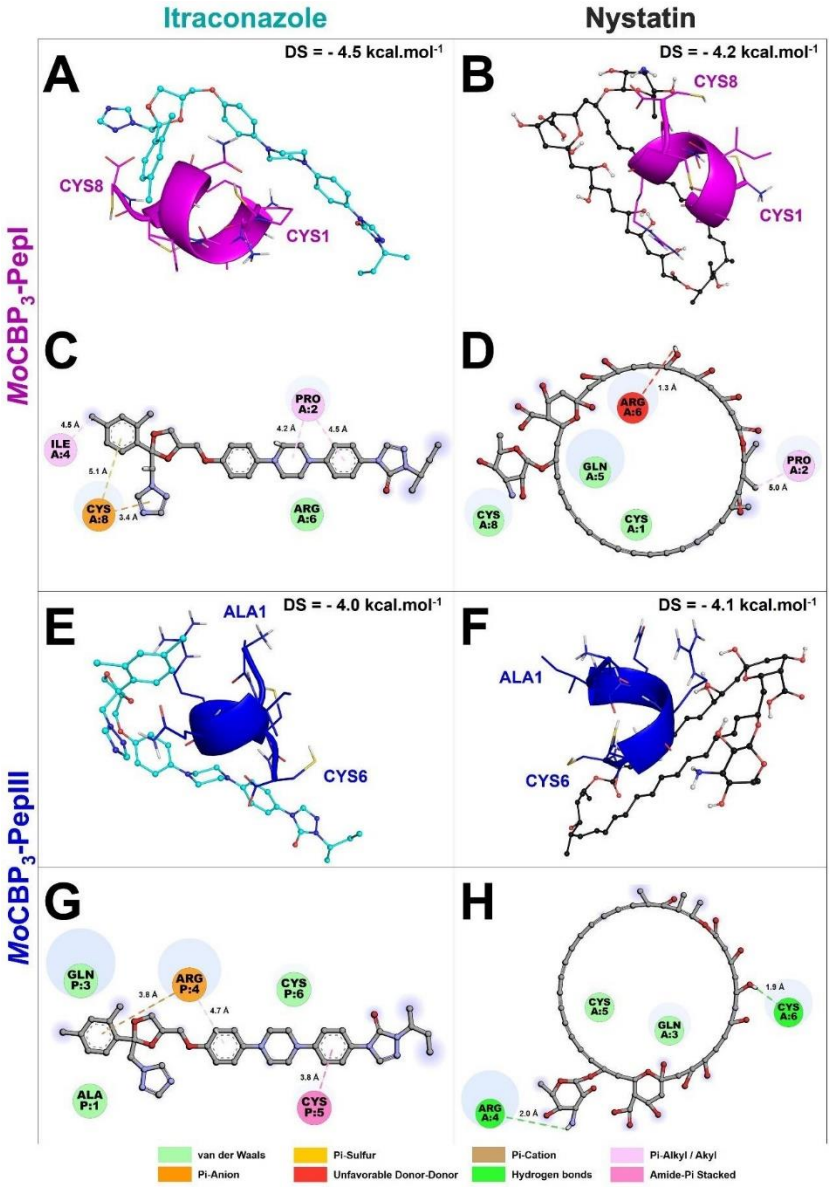


Figure 10. Fluorescence images showing membrane pore formation and ROS overproduction on degradation of the biofilm of *C. parapsilosis* cells. Control solution ofDMSO-NaCl, treated with *Mo*-CBP_3-PepI at 50 µg mL^{-1} and synergistic activity with NYS. Membrane pore formation was measured my propidium iodide uptake assay and ROS overproduction was detected using 2', 7' dichlorofluorescein diacetate (DCFH-DA).



Supplementary Figure 1. Molecular docking revealed that *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII interacting with ITR and NYS. *Mo*-CBP$_3$-PepI is represented in pink (**A** and **B**) and *Mo*- CBP$_3$-PepIII in blue (**E** and **F**). **C**, **D**, **G** and **H** show the binding sites of *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII with ITR and NYS.

Table 1. Hemolytic activity of *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII, antifungal drugs, and combined solution toward human red blood cells

Peptides/Combinations	% Hemolysis		
	Type-A Blood	**Type-B Blood**	**Type-O Blood**
0.1% Triton X-100	100	100	100
DMSO-NaCl Solution	0	0	0
NYS (1000 µg mL^{-1})	100	100	100
ITR (1000 µg mL^{-1})	75	68	58
Mo-CBP$_3$-PepI (50 µg mL^{-1})	0	0	0
Mo-CBP$_3$-PepIII (50 µg mL^{-1})	0	0	0
Mo-CBP$_3$-PepI (50 µg mL^{-1}) + NYS (1000 µg mL^{-1})	14	23	2
Mo-CBP$_3$-PepI (50 µg mL^{-1}) + ITR (1000 µg mL^{-1})	0	4	8
Mo-CBP$_3$-PepIII (50 µg mL^{-1}) + NYS (1000 µg mL^{-1})	45	30	18
Mo-CBP$_3$-PepIII (50 µg mL^{-1}) + ITR (1000 µg mL^{-1})	50	15	2

6. CONCLUSÃO

Os resultados obtidos mostram o potencial de peptídeos sintéticos bioinspirados em proteínas anteriormente identificadas contra biofilmes de microrganismos resistentes. Os peptídeos *Mo*-CBP3-PepI, *Mo*-CBP3-Pep3, PepGAT e PepKAA possuem atividade contra biofilmes de *C. albicans, C. krusei* e *C. parapsilosis* atuando tanto na inibição da formação do biofilme como na redução da biomassa de biofilmes pré-formados. Foi constatado que os mecanismos pelos quais os peptídeos desempenham sua atividade se dá por meio da formação de poros na membrana e indução da superprodução de espécies reativas de oxigênio, desestabilizando a membrana celular e causando extravasamento do conteúdo citoplasmático. Ademais, os resultados mostram que tais peptídeos podem ser capazes de se ligar a estruturas presentes na parede celular desestabilizando-a, bem como possuir alvos intracelulares específicos, levando as células à morte.

Os resultados obtidos com os ensaios de sinergismo sugerem que essas novas moléculas podem melhorar a atividade de antifúngicos já utilizados no tratamento de pacientes acometidos bem como reduzir a toxicidade de drogas comuns como nistatina e itraconazol. É válido salientar que os peptídeos não possuem efeitos tóxicos para as células do hospedeiro e não possuem atividade hemolítica contra eritrócitos humanos.

Desta forma, pode-se concluir que *Mo*-CBP3-PepI, *Mo*-CBP3-Pep3, PepGAT e PepKAA são peptídeos promissores no combate a biofilmes microbianos de interesse clínico bem como podem atuar como adjuvantes ás drogas já utilizadas, melhorando sua atividade farmacológica e, sobretudo, contribuem na superação aos mecanismos de resistência microbiana.

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APÊNDICE A - ARTIGO CIENTÍFICO 3

Artigo publicado na revista Life Sciences – Fator de impacto: 5.037

Acesso disponível em: (https://doi.org/10.1016/j.lfs.2020.118803)

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Synthetic peptides against *Trichophyton mentagrophytes* and *T. rubrum*: Mechanisms of action and efficiency compared to griseofulvin and itraconazole

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ARTICLE INFO

Keywords:
Synthetic antifungal peptides
Dermatophytes
Synthetic peptides
Griseofulvin
Itraconazole

ABSTRACT

Aims: According to the WHO, 20–25% of people worldwide are affected by skin infections caused by dermatophytes, such as those of the *Trichophyton* genus. Additionally, several dermatophytes have developed resistance to drugs such as griseofulvin and itraconazole. This study tested 28 albumins-derived antimicrobial peptides (AMPs) as alternative antidermatophytic molecules.

Main methods: Membrane pore formation assays, tests to detect overproduction of ROS, scanning electron microscopy (SEM) and fluorescence microscopy (FM) were carried out to provide insight into the mechanisms of antidermatophytic action.

Key findings: All AMPs (at 50 μg mL^{-1}) tested reduced the mycelial growth of *T. mentagrophytes* and *T. rubrum* by up to 95%. In contrast, using a concentration 20-fold higher, griseofulvin only inhibited *T. mentagrophytes* by 35%, while itraconazole was not active against both dermatophytes. Scanning electron and fluorescence microscopies revealed that the six AMPs caused severe damage to hyphal morphology by inducing cell wall rupture, hyphal content leakage, and death. Peptides also induced membrane pore formation and oxidative stress by overproduction of ROS. Based on the stronger activity of peptides than the commercial drugs and the mechanism of action, all six peptides have the potential to be either employed as models to develop new antidermatophytic drugs or as adjuvants to existing ones.

Significance: The synthetic peptides are more efficient than conventional drug to treat infection caused by dermatophytes being potential molecules to develop new drugs.

APÊNDICE B – ARTIGO CIENTÍFICO 4

Artigo publicado na revista Journal of Biomolecular Structures and Dinamics – Fator de impacto: 3.31

Acesso disponível em: (https://doi.org/10.1080/07391102.2020.1871415)

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
https://doi.org/10.1080/07391102.2020.1871415

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ACE2-derived peptides interact with the RBD domain of SARS-CoV-2 spike glycoprotein, disrupting the interaction with the human ACE2 receptor

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Communicated by Ramaswamy H. Sarma

ABSTRACT

Vaccines could be the solution to the current SARS-CoV-2 outbreak. However, some studies have shown that the immunological memory only lasts three months. Thus, it is imperative to develop pharmacological treatments to cope with COVID-19. Here, the *in silico* approach by molecular docking, dynamic simulations and quantum biochemistry revealed that ACE2-derived peptides strongly interact with the SARS-CoV-2 RBD domain of spike glycoprotein (S-RBD). ACE2-Dev-PepI, ACE2-Dev-PepII, ACE2-Dev-PepIII and ACE2-Dev-PepIV complexed with S-RBD provoked alterations in the 3D structure of S-RBD, leading to disruption of the correct interaction with the ACE2 receptor, a pivotal step for SARS-CoV-2 infection. This wrong interaction between S-RBD and ACE2 could inhibit the entry of SARS-CoV-2 in cells, and thus virus replication and the establishment of COVID-19 disease. Therefore, we suggest that ACE2-derived peptides can interfere with recognition of ACE2 in human cells by SARS-CoV-2 *in vivo*. Bioinformatic prediction showed that these peptides have no toxicity or allergenic potential. By using ACE2-derived peptides against SARS-CoV-2, this study points to opportunities for further *in vivo* research on these peptides, seeking to discover new drugs and entirely new perspectives to treat COVID-19.

ARTICLE HISTORY
Received 13 October 2020
Accepted 29 December 2020

KEYWORDS
SARS-CoV-2 RBD; COVID-19; ACE2 receptor; ACE2-derived peptides

ANEXO A – ARTIGO CIENTÍFICO 5

Artigo publicado na revista Food Chemistry – Fator de impacto: 7.514

Acesso disponível em: (https://doi.org/10.1016/j.foodchem.2021.131410)

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Latex peptidases produce peptides capable of delaying fungal growth in bread

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ARTICLE INFO

Keywords:
Antimicrobial peptides
Calotropis procera
Carica papaya
Cryptostegia grandiflora
Gluten peptides
Penicillium

ABSTRACT

Antimicrobial peptides (AMPs) have been reported to be promising alternatives to chemical preservatives. Thus, this study aimed to characterise AMPs generated from the hydrolysis of wheat gluten proteins using latex peptidases of *Calotropis procera*, *Cryptostegia grandiflora*, and *Carica papaya*. The three hydrolysates (obtained after 16 h at 37 °C, using a 1:25 enzyme: substrate ratio) inhibited the growth of *Aspergillus niger*, *A. chevalieri*, *Trichoderma reesei*, *Pythium oligandrum*, *Penicillium* sp., and *Lasiodiplodia* sp. by 60–90%, and delayed fungal growth on bread by 3 days when used at 0.3 g/kg. Moreover, the specific volume and expansion factor of bread were not affected by the hydrolysates. Of 28 peptides identified, four were synthesised and exhibited activity against *Penicillium* sp. Fluorescence and scanning electron microscopy suggested that the peptides damaged the fungal plasma membrane. Bioinformatics analysis showed that no peptide was toxic and that the antigenic ones had cleavage sites for trypsin or pepsin.