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TÂNIA PEREIRA SALCI

Novos alvos para o desenvolvimento de antifúngicos direcionados à candidíase invasiva

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Tese apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutora em Biociências e Fisiopatologia
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O conhecimento é uma aventura em aberto. O que significa que aquilo que saberemos amanhã é algo que desconhecemos hoje; e esse algo pode mudar as verdades de ontem.

Karl Popper

Novos alvos para o desenvolvimento de antifúngicos direcionados à candidíase invasiva

RESUMO

Infecções fúngicas invasivas (IFI) são a terceira causa de infecções hospitalares. O gênero *Candida* é o agente etiológico responsável por pelo menos 80% dos casos de IFI. A espécie *C. albicans* ainda é mais prevalente na maioria dos países; no entanto, as espécies *Candida* não-*C. albicans* (CNCA) têm emergido, destancando-se *C. parapsilosis*. Apesar da importância clínica das leveduras, para tratamento dessas infecções contamos com um arsenal terapêutico restrito e com alta ocorrência de reações adversas. Além disso, mesmo quando a terapia antifúngica é instituída a taxa de mortalidade permanece elevada, o que torna a busca por novas opções terapêuticas imperativa. Dessa forma, o presente estudo teve por objetivos explorar os possíveis alvos para o desenvolvimento de novos antifúngicos direcionados à candidíase invasiva; e avaliar a atividade antifúngica de uma molécula previamente selecionada *in silico*. O primeiro artigo "Targeting *Candida* spp. to develop antifungal agents" se trata de uma revisão sobre os possíveis alvos de *Candida* spp. para novas opções terapêuticas. Reúne informações sobre mutantes nulos, virulência e papel na estrutura celular, citolocalização, genes de co-regulação e compostos capazes de anular a expressão dessas proteínas. Esses dados servirão de base para futuras orientações de pesquisas para a seleção de novos compostos com potencial ação antifúngica. No segundo artigo "A new small molecule KRE2 inhibitor against invasive *Candida parapsilosis* infection" foi avaliada uma nova *small molecule* MOL3, previamente selecionada por modelagem molecular e varredura virtual em uma quimioteca. Nesse estudo foram realizados testes *in vitro* e *in vivo* os quais provaram o efeito antifúngico promissor de MOL3 além de não apresentar toxicidade em animais.

Palavras-chave: *Candida*. Infecção hospitalar. Agentes antifúngicos. Alvos para medicamentos. Manosiltransferases.

New targets for the development of antifungal agents against invasive candidiasis

ABSTRACT

Invasive fungal infections (IFIs) are the third cause of hospital-acquired infections. The genus *Candida* is the etiological agent responsible for at least 80% of cases of IFIs. The specie *C. albicans* is still more prevalent in most countries; however, the species *Candida* non-*C. albicans* (CNCA) have emerged, highlighting *C. parapsilosis*. Despite the clinical importance of yeasts, there is a restricted therapeutic arsenal for treatment of these infections, with high occurrence of adverse reactions. Moreover, the mortality rate remains high even when the antifungal therapy is instituted, which makes the search for new therapeutic options imperative. Thus, the present thesis aims to explore the possible targeting *Candida* spp. to develop antifungal agents; and evaluate the antifungal activity of a molecule previously selected *in silico*. The first article "Targeting *Candid*a spp. to develop antifungal agents" is a review that provide a holistic view of the main gene targets of *Candida* spp., with a focus on null mutants, virulence, cytolocalization, co-regulatory genes, and compounds already described capable of overriding the expression of proteins. These data will serve as a basis for future *in silico* research on antifungal agents. In the second article "A new small molecule inhibitor against KRE2 invasive *Candida parapsilosis* infection" was evaluated the antifungal activity and toxicity of MOL3, a new small molecule previously selected by molecular modeling and virtual screening. The tests were done in both *in vitro* and animal models. The selection of MOL3 was successful, revealing a promising antifungal candidate.

Keywords: *Candida*. Cross infection. Antifungal Agents. *Drug Targeting*. Mannosyltransferases.

Tese elaborada e formatada conforme as normas das publicações científicas: Drug Discovery Today (artigo 1) disponível em: <http://csmres.co.uk/cs.public.upd/article-downloads/Instructions-to-Authors-June-2014.pdf> e Future Microbiology (artigo 2) disponível em: <http://www.futuremedicine.com/page/authors.jsp#Guidelines>*

SUMÁRIO

CAPÍTULO I

INFECÇÕES FÚNGICAS INVASIVAS

Infecções fúngicas invasivas (IFI) são infecções severas causadas por fungos, ocorrem quando esses micro-organismos atingem a corrente sanguínea ou se disseminam pelo organismo humano causando infecção grave como as candidemias e infecções em órgãos profundos [1]. As IFIs são associadas a maior mortalidade em pacientes internados em unidades de terapia intensiva (UTI), em uso de agentes antimicrobianos de amplo espectro, imunodepressores, extremos de idade, doenças malignas, exposição a procedimentos invasivos, insuficiência renal, alto tempo de permanência hospitalar, entre outros fatores, alguns frutos do próprio avanço tecnológico da medicina moderna como a quimioterapia e transplante de órgãos [2].

A incidência de infecções hospitalares por fungos tem aumentado substancialmente nas últimas décadas [3]. O gênero *Candida* é o agente etiológico responsável por pelo menos 80% dos casos de IFI [4-6] e está elencado entre os principais micro-organismos causadores de infecções hospitalares [7]. *C. albicans* foi, até alguns anos, a espécie que mereceu maior atenção clínica. No entanto, em paralelo com o aumento global das infecções fúngicas tem sido observado que as infecções causadas por *Candida* não-*C. albicans* (CNCA), tais como *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. lusitaniae* e *C. guillermondii* estão emergindo [8-11].

A taxa hospitalar brasileira de incidência de candidemia varia de 1,20 a 2,49 episódios por 1.000 admissões [12-15]. O que é semelhante à observada em países como o Peru, Índia, Taiwan e Tailândia [11, 16] e superior a países como Bélgica, China e Hong Kong [16, 17]. Entretanto, quando se relaciona incidência de candidemia a pacientes adultos críticos, mesmo em países desenvolvidos, como a França e os EUA, essa taxa pode chegar a 3,3 casos por 1000 admissões [18, 19] e aumenta ainda mais quando envolve malignidades hematológicas (6.5‰) [19]. Dados recentes de um estudo com pacientes críticos no Brasil identificou uma incidência de 6,0‰ [20].

Globalmente, a taxa de mortalidade em pacientes críticos com infecções fúngicas invasivas por *Candida* varia de 40 a 70% [21, 22]. O estudo multicêntrico epidemiológico brasileiro mais recente revelou alarmante taxa de mortalidade por candidemia de 72,2% [23]. Mesmo em pacientes que recebem tratamento antifúngico, a taxa de mortalidade pode chegar a 67% [11, 24, 25]. Essa alta taxa de insucesso terapêutico pode estar relacionada à demora

para iniciar o tratamento antifúngico e à habilidade das leveduras em aderir e formar biofilme em diferentes superfícies, contribuindo para a resistência [19]. Além disso, o arsenal terapêutico disponível ainda é restrito e a incidência de cepas resistentes está aumentando [3, 26].

Para controle dessas infecções, os medicamentos constituem elementos importantes, pois são responsáveis pela mudança no quadro de mortalidade das doenças transmissíveis, aumento da expectativa de vida e erradicação de doenças, trazendo benefícios sociais e econômicos à população. Assim, busca-se o desenvolvimento de antimicrobianos ideais para o tratamento dessas infecções, com amplo espectro de ação, baixa toxicidade e perfil farmacocinético favorável.

EMERGÊNCIA DA ESPÉCIE *Candida parapsilosis*

C. parapsilosis tem sido um patógeno frequentemente associado a infecções em neonatos[27, 28]. Especialmente em neonatos prematuros e de baixo peso, infecções causadas por essa espécie são responsáveis pelo aumento do tempo de permanência hospitalar, altas taxas de mortalidade e prejuízo do desenvolvimento neurológico [29-31].

Os fatores de risco para desenvolvimento de candidemia em neonatos inclui extrema prematuridade, parto vaginal, colonização por *Candida*, Apgar inferior a cinco em cinco minutos, choque, uso de antimicrobianos, nutrição parenteral por mais de cinco dias, permanência hospitalar por mais de sete dias, procedimentos invasivos, entre outros [32, 33].

Apesar da associação de *C. parapsilosis* à pediatria, essa espécie vem emergindo como causadora de infecções também em adultos. Atualmente, é descrita como a segunda causa de IFI tanto em países emergentes [11, 23, 34] como também em desenvolvidos [19]. Na Costa Rica, foi recentemente reconhecida como o principal agente de candidemia em um hospital terciário [35].

Mesmo que a mortalidade por IFI causada por *C. parapsilosis* em adultos seja menor quando comparada a causada por outras espécies e a causada por essa mesma espécie em neonatos [17, 23], diversos autores têm destacado o aumento da resistência de *C. parapsilosis* ao tratamento com antifúngicos. São descritas cepas resistentes aos azóis [20, 33, 36], anfotericina B [37] e às equinocandinas [38, 39]. É comprovado que a múltipla exposição de *C. parapsilosis* às equinocandinas tem tornado resistentes cepas inicialmente consideradas suscetíveis [40].

Outro aspecto dessa espécie que chama atenção é a capacidade de adesão e formação de biofilme [37]. Entre as principais espécies de *Candida* clinicamente relevantes, *C.*

parapsilosis é a que apresenta maior capacidade de desenvolver esse perfil de virulência [41]. Biofilmes são responsáveis pelo aumento da capacidade de colonizar e invadir tecidos e pela persistência fúngica na superfície. Essa característica torna as infecções mais difíceis de serem tratadas e contribui para a ocorrência de maiores taxas de mortalidade [42, 43].

HISTÓRIA DOS AGENTES ANTIFÚNGICOS

(Este tópico é parte de um artigo publicado, escrito pela autora da tese - Molecules 2014, 19, 2925-2956; doi:10.3390/molecules19032925)

Para tratamento de IFIs causadas por *Candida* spp. as opções terapêuticas atualmente disponíveis são limitadas e estão restritas a quatro principais classes de antifúngicos: polienos (anfotericina B), os azóis de primeira geração (fluconazol), azóis de segunda geração (voriconazol, posaconazol, ravuconazol) e as equinocandinas (caspofungina, anidulafungina e micafungina).

Historicamente, nas primeiras décadas do século XX, iniciou a preocupação com dermatófitos e candidíase oral. Consequentemente, as tentativas terapêuticas eram direcionadas ao tratamento dessas infecções. Em 1935, Wieder [44] reconheceu que o número de dermatoses estava aumentando, necessitando de compostos mais específicos com essa finalidade. A partir desse momento, o interesse pela terapia antifúngica clínica aumentou gradualmente.

Nesse período, as terapias eram limitadas a medicamentos não específicos e descobertos de forma empírica. Assim como iodeto; mercúrio; ácidos benzóico e salicílico; derivados de fenol; ácido undecilênico; violeta de metila; derivados de sulfonamida; e outros agentes tóxicos, incluindo bromo, permanganato de potássio e óleo de terebintina com azeite de oliva [45-47].

Griseofulvina, um composto derivado de *Penicillium griseofulvum*, foi o primeiro composto químico com ação inibitória seletiva contra fungos e foi amplamente utilizado para tratar infecções fúngicas superficiais [48, 49].

Nistatina, inicialmente chamada de fungicina, foi o primeiro composto poliênico com atividade antifúngica conhecida [50] e provada [51, 52].Um importante avanço foi a descoberta da anfotericina B, que até hoje é reconhecida como o antifúngico mais efetivo, apesar de seus efeitos adversos e toxicidade dose dependente, incluindo prejuízos renais e hipocalemia [53]. Formulações lipídicas desse composto têm um amplo espectro de atividade contra fungos patogênicos, com menor taxa de nefrotoxicidade. Entretanto, uma importante desvantagem dessas formulações lipídicas é seu alto custo[54].

Flucitosina (5-flucitosina), uma pirimidina halogenada, foi reportada como um antifúngico em 1961 [55] e descrita como um agente promissor para o tratamento de micoses sistêmicas causadas por *Cryptococcus neoformans* e *C. albicans*. Todavia, atualmente a limitação de sua efetividade é reconhecida e é utilizada para o tratamento de criptococose em associação a outros antifúngicos [56].

A descoberta dos azóis, compostos com o sufixo genérico "conazol", foi um marco na história do desenvolvimento dos antifúngicos [57]. O cetoconazol foi introduzido no mercado em 1981 e representou um avanço na pesquisa pela busca de novos agentes seguros e efetivos para o tratamento oral de infecções fúngicas sistêmicas. Em um programa de triagem, mais de 100 derivados triazólicos do 2,4-diclorofenil foram testados em modelo animal de infecções fúngicas, dos quais apenas alguns compostos foram comercializados [57].

Fluconazol, um triazol primeiramente descrito em 1985 por Richardson et al. [58], tornou-se o principal antifúngico prescrito em apenas alguns anos depois do início de seu uso clínico [59, 60]. Nessa mesma década, itraconazol se mostrou efetivo contra *Aspergillus* spp. e *Paracoccidioides* spp. Esse composto também apresenta alta afinidade por tecidos queratinizados, assim também é indicada para o tratamento de onicomicoses e dermatofitoses [61].

Um aumento substancial no número de antifúngicos foi observado no início deste século com a comercialização dos novos azóis e equinocandinas. Voriconazol foi o primeiro azol introduzido como um composto aprovado como primeira escolha para tratamento de aspergilose invasiva e com atividade também contra *Fusarium* e *Scedosporium* [62], que eram difíceis de tratar. Outros novos triazóis, como ravuconazol e posaconazol não resultaram em ganhos terapêuticos significativos [61]. Estruturalmente, voriconazol e ravuconazol são mais parecidos com o fluconazol, enquanto que o posaconazol se assemelha ao itraconazol [49].

No início dos anos 2000, três equinocandinas foram aprovadas para uso em seres humanos, caspofungina, anidulafungina e micafungina [63-65]. O complexo pneumocandina foi produzido como um metabólito secundário da fermentação do fungo *Glarea lozoyensis*, que é precursor do lipopeptídeo sintético caspofungina [66]. Esses fármacos são geralmente bem tolerados; além disso, apresentam interação medicamentosa mínima e perfil farmacocinético favorável [67-69]. Protocolos de tratamento recomendam o uso desses compostos como primeira linha de escolha para tratamento de candidemia e candidíase invasiva; e como alternativa terapêutica para tratamento de aspergilose, devido sua atividade contra fungos resistentes ao fluconazol[70].

Outros compostos promissores não têm sido utilizados ou caíram em desuso devido a toxicidade, custo, espectro de ação ou farmacocinética não favorável. Muitas possibilidades de elucidação de compostos antifúngicos foram exauridas nos últimos anos com algum fracasso [57].

A NECESSIDADE DE NOVOS ANTIFÚNGICOS É IMPERATIVA

A similaridade do ergosterol com o colesterol humano permite a ocorrência de múltiplas reações adversas. Além disso, os azóis são inibidores do complexo enzimático citocromo P450 e são relacionados a mais de 100 relevantes interações medicamentosas [71]. Os polienos, representados pela anfotericina B, são bem conhecidos acerca de sua nefrotoxicidade, o que torna seu uso inapropriado em muitos casos [72]. Somente as equinocandinas atuam especificamente em enzimas da parede celular dos fungos. Entretanto, todos os agentes antifúngicos são relacionados a hepatotoxicidade [73].

Além dos fatores inerentes aos compostos com ação antifúngica, o desenvolvimento da resistência fúngica tem sido cada vez mais descrito. O uso muito difundido e prolongado dos azóis tem levado ao desenvolvimento do fenômeno de multirresistência a drogas, o que representa um obstáculo para o sucesso terapêutico. Muitos mecanismos de resistência são propostos em relação aos azóis [74, 75]; outros antifúngicos, como os novos azóis, equinocandinas, e mesmo a anfotericina têm sido relacionados à resistência de cepas [76-78].

A taxa de insucesso terapêutico, já descrita anteriormente, é alarmante. A resistência geral de *Candida* spp. ao fluconazol é de 7,6% [17]. O voriconazol apresenta, por vezes, resistência cruzada aos outros azóis [79, 80]. O uso de equinocandinas tem sido relacionado ao aumento da concentração inibitória mínima (CIM) em cepas de *Candida* spp. [81], e é comprovado que a múltipla exposição de leveduras a esses agentes antifúngicos contribui para alteração do perfil de suscetibilidade [40]. A anfotericina B, mesmo quando apresenta suscetibilidade em testes *in vitro,* em 50% dos casos clínicos resulta em falha terapêutica [82].

Este cenário mostra a necessidade de atenção dos profissionais de saúde a respeito do tratamento precoce e mais efetivo. Assim, pesquisas por alternativas terapêuticas mais específicas são cada vez mais imperativas. Novos antifúngicos devem possuir espectro de atividade prolongado contra fungos, perfil farmacocinético aprimorado, de modo a reduzir a frequência de dosagem, possibilidade de administração oral e parenteral, poucos efeitos adversos e interações medicamentosas. A necessidade de drogas mais específicas é um fato, a identificação de alvos nos patógenos oportunistas tem contribuído para entender a maquinaria fúngica e direcionar o desenvolvimento de novos fármacos.

GENÔMICA COMPARATIVA COMO UMA ALIADA PARA O DESENVOLVIMENTO DE NOVOS ANTIFÚNGICOS

Esforços têm sido realizados no sentido de elucidar os genomas das principais espécies patogênicas de *Candida*. Todas pertencem à família *Saccharomycetaceae*, entretanto há uma grande diversidade entre elas. *C. albicans* é diploide e sexuada [83]; *Candida parapsilosis* e *C. tropicalis* são diploides e não apresentam um ciclo sexual conhecido [84, 85]; essas três espécies apresentam dimorfismo e pertencem ao clado *Candida* [86]. Entretanto, *C. glabrata* é haploide, monomórfica, assexuada e filogeneticamente mais próximas da espécie *Saccharomyces* [86, 87] (Figura 1). A espécie *C. krusei* foi pouco explorada até o momento, sabe-se que é uma levedura haploide, e na base de dados NCBI encontra-se informações sobre sua fase sexuada que é denominada de *Pichia kudriavzevii.*

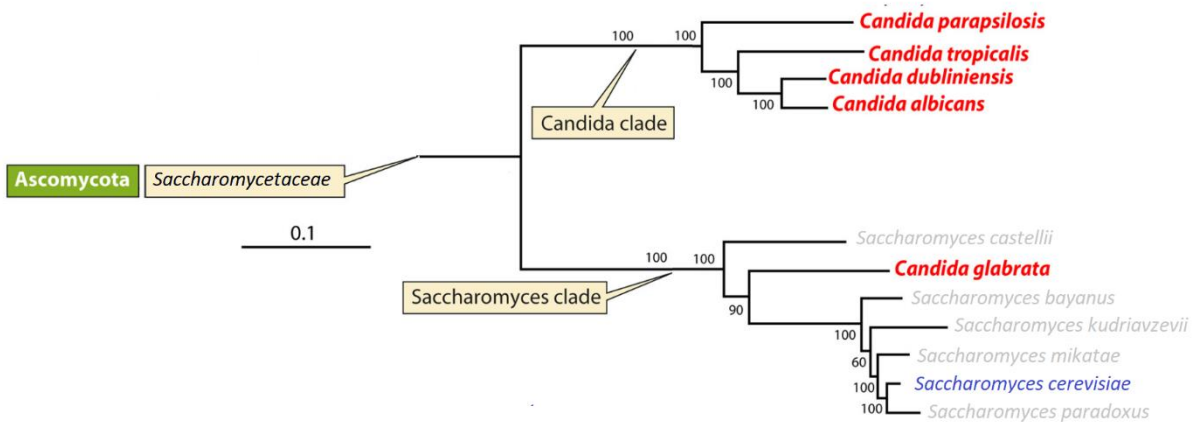

Figura 1. Árvore filogenética das principais espécies de *Candida* patogênicas ao homem (Adaptado de Butler, 2010).

O genoma de *C. albicans* está bem descrito, porém para as espécies CNCA ainda muitos estudos devem ser realizados. Este fato deve-se principalmente não pela característica do genoma, mas sim por sua maior frequência frente as outras espécies. Sabe-se que o genoma de *C. albicans* é de aproximadamente 16Mb organizados em 8 pares de cromossomos. Apesar de *C. dubliniensis* ser uma CNCA, devido a sua similaridade com o genoma de *C. albicans*, é uma espécie bastante descrita e, por muitas vezes, confundida com a mesma. Outra espécie muito frequente e, não menos importante é *C. tropicalis*, destaca-se principalmente por sua virulência e alta letalidade causando candidemia em pacientes oncogênicos, o tamanho exato do seu genoma e número de cromossomos não são totalmente conhecidos. Estima-se que o tamanho do genoma haploide seja de 15 Mb organizados em 5 a 6 cromossomos. O genoma de *C. parapsilosis* é de aproximadamente 26 Mb, organizados em 14 cromossomos. O genoma de *C. glabrata* é de aproximadamente 12.3 Mb, organizados em 13 cromossomos [88].

Além da caracterização funcional, o conhecimento do genoma de células patogênicas viabiliza processos de pesquisa e desenvolvimento de novas drogas, a partir da identificação de possíveis alvos. Estes podem ainda ser mais bem selecionados utilizando-se da genômica comparativa, o que permite o direcionamento para moléculas inócuas ao homem e que ajam contra o maior número de fungos.

Esses estudos dependem da definição prévia dos possíveis alvos, com base na pesquisa de genes específicos. Contudo, as informações sobre genes de *Candida*, apesar de comuns, encontram-se fragmentadas na literatura. Frequentemente a nomenclatura é confusa e as informações muitas vezes são controversas. Esses fatores dificultam a busca do conhecimento e mostram a necessidade de um documento que concentre as principais publicações e resultados disponíveis até o momento uniformizando a diversidade de dados. Essa lacuna deu origem ao primeiro artigo apresentado nesta tese "Targeting *Candida* spp. to develop antifungal agents" (Capítulo 2), os genes discutidos são os presentes em *Candida* spp. e ausentes no genoma humano mais explorados na literatura.

SELEÇÃO DE NOVOS COMPOSTOS COM AÇÃO ANTIFÚNGICA POR *HIGH-THROUGHPUT SCREENING*

Os fármacos com ação antifúngica comercialmente disponibilizados até o momento foram desenvolvidos com base na triagem empírica de compostos naturais, derivados principalmente de micro-organismos, seguido de seu isolamento e purificação e/ou síntese. Entretanto, a triagem empírica é cada vez menos usada pela indústria farmacêutica para desenvolvimento de novos compostos, uma vez que a introdução de um novo fármaco no mercado por esses métodos pode demorar em média de 12 a 15 anos e custar mais de um bilhão de dólares. Além disso, a chance de comercialização é baixa, devido a toxicidade observada em fases mais avançadas da pesquisa e que inviabiliza uma gama de compostos [89].

Com os avanços na indústria farmacêutica e química e a expansão do número de moléculas, quimiotecas foram criadas para organizar o fornecimento desses insumos [90]. No ano 2000, havia milhares de compostos químicos disponíveis, dos quais apenas aproximadamente 10.000 apresentavam características *drug-like*. Iniciou-se uma discussão sobre a necessidade de as informações sobre esses compostos químicos serem uniformes. Além disso, falava-se da necessidade da "*drugability*" preceder à triagem para ligação em receptores biológicos [91].

Assim, a partir da observação de mais de 2.000 compostos biodisponíveis por via oral, com solubilidade e permeabilidade adequados, Lipinski et al [92] determinaram o que foi chamado de a "Regra dos cinco". Essa teoria apresenta preditores de permeabilidade e absorção, especialmente para compostos peptídicos, como não ter mais que cinco H-ligantes doadores, dez H-ligantes receptores, massa molecular de até 500 Da e, em relação à lipofilicidade, Log P < 5.

Atualmente, as quimiotecas oferecem informações sobre os preditores estabelecidos por Lipinski et al [93], além de outros filtros que aumentam a possibilidade de absorção e biodisponibilidade, como os discutidos em Huggins et al. [94]. O quadro 1 mostra informações sobre algumas quimiotecas que incluem *small molecules* em seu arsenal e que foram utilizadas em estudos para a busca de potenciais antifúngicos.

Quadro 1. Quimiotecas de compostos químicos usadas em estudos para obtenção de potenciais agentes antifúngicos

Biblioteca	Nº de compostos disponibilizados	Url	Localização
Life Chemicals	1.350.000	http://www.lifechemicals.com	Canadá
ChemBridge	1.100.000	http://www.chembridge.com	EUA
BioAscent	206.000	http://www.bioascent.com	Reino Unido
National Cancer Institute (NCI)	140.000	http://scs.illinois.edu/htsf/compound_collection/nci.php	EUA
EMC Microcollections	30.000	http://www.microcollections.de/	Alemanha
LOPAC	1.280	http://www.sigmaaldrich.com/life-science/cell-biology/bioactive-small-molecules/lopac1280-navigator.html	EUA

A emergência de compostos químicos disponíveis tornou necessária a evolução com que esses compostos fossem testados. Para isso, a triagem de alta performance (HTS - *High-throughput screening*) pode integrar automação e/ou os avanços na área computacional com o conhecimento biológico para avaliar milhares e até milhões de compostos com propriedade para se tornarem fármacos [90]. A HTS pode ser realizada a partir de métodos *in silico* [95] ou pela realização de experimentos *in vitro* [96]. Assim, testes mais complexos e envolvendo

animais são aplicados somente após a seleção prévia de estruturas capazes de interagir com alvos celulares.

Nos experimentos *in vitro*, a automação tem sido uma aliada para varrer grandes coleções de compostos químicos em busca de novas possibilidades para o tratamento antifúngico. Nesses casos, são utilizadas placas miniaturizadas para testar a ação de milhares de compostos contra determinados micro-organismos, de forma adaptada ao que é realizado em testes de microdiluição em caldo. Depois de uma triagem inicial, compostos com melhores perfis são selecionados para testes mais específicos, chegando-se aos melhores *hits*, como nos estudos de Tsukahara et al [97] e Toenjes et al [98], por exemplo.

Nos modelos *in silico,* as técnicas computacionais como a modelagem molecular e os estudos de encaixe molecular (*molecular docking*) têm sido aplicados em varredura virtual para triagem de moléculas em quimiotecas para a seleção de *hits* [99]. Estudos realizados por Abadio et al. [95, 100] levaram em consideração a especificidade de alvos presentes em fungos e ausentes em humanos. Modelos em 3D de algumas dessas proteínas, como KRE2, foram geradas por modelagem molecular com auxílio de programa computacional. Compostos químicos foram selecionados da quimioteca Life Chemicals para realização de simulações de encaixe (*docking simulations*), com o intuito de bloquear a ação da enzima. Alguns compostos foram selecionados e um deles foi testado *in vitro* e *in vivo* apresentando resultados promissores. A eficácia da triagem realizada por esse método foi comprovada no artigo aceito para publicação na revista Future Microbiology constante nesta tese, intitulado "A new small molecule KRE2 inhibitor against invasive *Candida parapsilosis* infection" (Capítulo 2).

Os *hits* podem ser selecionados a partir da varredura de quimiotecas com compostos novos, ainda não comercializados, ou coleções de compostos previamente aprovados para outros fins. A busca por moléculas ainda não testadas torna mais provável a chance de descoberta e patenteamento de compostos, embora seja maior o risco de não se conseguir bons resultados. De outra forma, os fármacos já patenteados podem ser redirecionados para fins diferentes dos já estabelecidos [90]. Um exemplo disso está descrito no estudo realizado por Rabjohns et al [96], os quais utilizaram a coleção LOPAC$^{®1280}$, que é composta de fármacos já aprovados para tratamento de outras patologias, para buscar uma molécula com ação antifúngica contra *Cryptococcus neoformans.*

Assim, pesquisadores têm concentrado esforços para o desenvolvimento de agentes antifúngicos a partir de moléculas selecionadas por HTS. O composto E1210, derivado de 1-(4-butilbenzil)isoquinolina tem apresentado potencial ação antifúngica tanto *in vitro* como *in*

vivo [101], foi selecionado por HTS em quimioteca e seu alvo molecular é a proteína Gwt1, que é requerida para a acilação do inositol em um passo inicial da via biossintética de glicosilfosfatidilinositol (GPI), necessária para a montagem da parede celular fúngica [97]. Outras *small molecules* com potencial antifúngico são descritas na literatura, algumas com alvos bem definidos e testes de suscetibilidade *in vivo* e *in vitro* realizados (Tabela 1).

Tabela 1. Novas *small molecules* com potencial ação antifúngica selecionadas por *high-throughput screening*

COMPOSTO	ALVO	QUIMIOTECA	SELEÇÃO DO HIT	AÇÃO CONTRA FUNGOS PATOGÊNICOS *in vitro*	*in vivo*	REF.
E1210	GWT1	Não especificado	*In vitro*	*Candida* spp. *Aspergillus fumigatus* *Fusarium solani* *Pseudallescheria boydii* *Scedosporium prolificans* *Paecilomyces lilacinus*	*Candida* spp. *Aspergillus* spp. *Fusarium solani*	[97, 102, 103]
SM21	HWP1	ChemBridge	*In vitro*	*Candida* spp.	*C. albicans*	[98, 104]
D75-4590	Kre6p	Daiichi Sankyo	*In vitro*	*Candida* spp.	Não obteve sucesso	[105, 106]
D11-2040 **(Derivado do D75-4590)**				*Candida* spp. *Cryptococcus neoformans* *Trichosporon* spp. *Aspergillus* spp. *Paecilomyces variotii* *Pseudallescheria boydii*	Não foi testado	[107]
D21-6076 **(Derivado do D75-4590)**				*Candida* spp.	*C. albicans* *C. glabrata*	[106]
GSI578	Glucan synthase	Alanex Corp	*In vitro*	*Candida* spp. *A. fumigatus*	Não foi testado	[108]
EMC120B12	Erg11p	EMC Microcollections		*Candida* spp. *Issatchenki orientalis* *C. neoformans*	Não foi testado	[109-111]
SCH A **SCH B** **SCH C** **SCH D**	β-1,3-glucan synthase	Schering-Plough	*In vitro*	*Candida* spp. *A. fumigatus*	*C. glabrata*	[112]

Tabela 1. *Cont.*

COMPOSTO	ALVO	QUIMIOTECA	SELEÇÃO DO HIT	AÇÃO CONTRA FUNGOS PATOGÊNICOS *in vitro*	*in vivo*	REF.
F0876-0030 F1806-0122 F3307-0100	TRR1	Life Chemicals	*In silico*	*Paracoccidioides* spp. *Candida albicans*	Não foram testados	[95]
61894700 80527891	Não definido	ChemBridge	*In vitro*	*C. albicans*	*C. albicans*	[113]
41F5	Purinoma		*In silico* *In vitro*	*Histoplasma capsulatum* *Cryptococcus neoformans*	Não foi testado	[114, 115]
10058-F4	Não definido	LOPAC	*In vitro*	*C. neoformans*		[96]
NSC 1520	CYP51	National Cancer Institute (NCI)	*In silico*	*Candida* spp. *Aspergillus niger*	Não foi testado	[116]
MOL3	KRE2	Life Chemicals	*In silico*	*Paracoccidioides* spp. *Candida* spp.	*C. parapsilosis*	[117-119]

OBJETIVO GERAL

Explorar os possíveis alvos para o desenvolvimento de antifúngicos direcionados para o tratamento de candidíase invasiva. E avaliar a atividade antifúngica de uma molécula previamente selecionada *in silico.*

OBJETIVOS ESPECÍFICOS

Investigar os principais alvos de *Candida* descritos na literatura para gerar informações que auxiliem a seleção de novos compostos com ação antifúngica.

Validar e confirmar a atividade de um novo composto selecionado por varredura virtual, MOL3, a respeito de sua ação antifúngica contra as principais espécies de *Candida* causadoras de infecções, especialmente contra a espécie emergente *C. parapsilosis*.

Comparar a ação de MOL3 com as obtidas com os antifúngicos clássicos.

Analisar a toxicidade de MOL3 por meio de estudos *in vitro* e *in vivo*.

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CAPÍTULO II

Artigo: “TARGETING *Candida* spp. TO DEVELOP ANTIFUNGAL AGENTS”

TARGETING *Candida* spp. TO DEVELOP ANTIFUNGAL AGENTS

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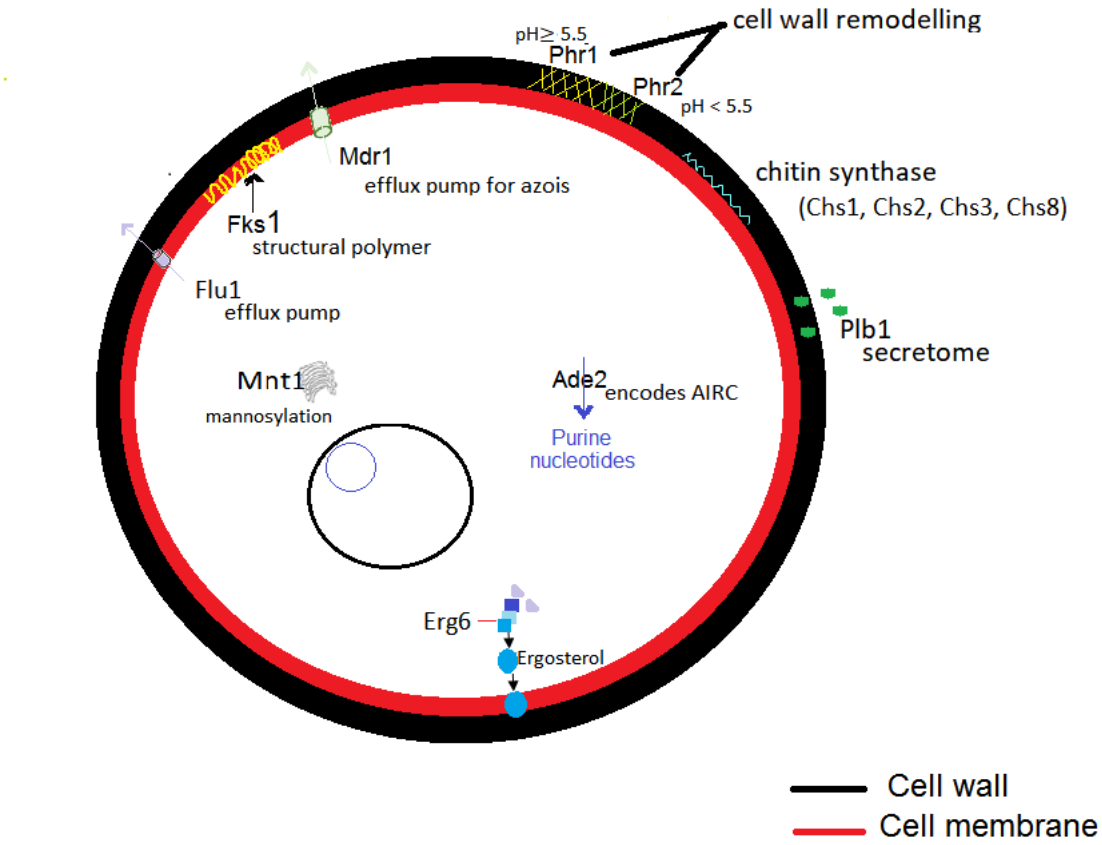
Abstract

Invasive fungal infections (IFI) correspond to 20% of all microbial infections, and the most common etiologic agent is *Candida* sp. There are few established drugs to treat *Candida* IFI, so search for more specific antifungal therapies has become necessary. Studies to explore the interaction of compounds with specific target receptors in order to select agents with antifungal action have been increasingly. The present review shows a holistic view about the main targeted genes from *Candida* spp., gathers information about null mutants, virulence and role in the cellular structure, cytolocalization, co-regulatory genes, and compounds capable of overriding the expression of proteins. These data will serve as a basis for future research directions for development of antifungals.

Graphical abstract



Summary

- *Candida* is the most prevalent invasive fungal pathogen
- Antifungal agents are restricted and need to be more directed to the exclusively fungal structure
- Comparative genomics as ally for development of new antifungals
- Fungal targeted components
    - Cell wall
        - pH-controlled Wall-Protein-Encoding genes
            - pH-regulated protein 1 - Phr1p
            - PH-regulated protein 2 - Phr2p
        - Chitin Synthase Enzymes
            - Chitin synthase 1 - Chs1p
            - Chitin synthase 2 - Chs2p
            - Chitin synthase 3 - Chs3p
            - Chitin synthase 8 - Chs8p
        - Phospholipase B - Plb1p
    - Plasma membrane
        - Drug:H+ antiporter family 1 (DHA1)
            - Multidrug resistance protein - Mdr1p
            - Fluconazole resistance protein - Flu1p
        - β-1,3-glucan synthase catalytic subunit - Fks1p
        - Sterol 24-C-methyltransferase - Erg6p
    - Cytoplasm
        - Phosphoribosylaminoimidazole carboxylase - Ade2p
    - Endoplasmic Reticulum and Golgi complex
        - Alpha-1,2-mannosyltransferase – Mnt1p
    - Proteins with unknown cytolocalization
        - Mitogen-activated protein kinase kinase kinase - Ssk1p
        - Trehalose synthesis genes
            - Alpha-trehalose-phosphate synthase (UDP-forming) - Tps1p
            - Trehalose-phosphatase - Tps2p

Candida is the most prevalent invasive fungal pathogen

The most common invasive fungal infections include the *Candida* spp.[1]. *Candida albicans* has been described for years as the main species causing infections by this genus. However, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and others *Candida* non-*albicans Candida* (CNAC) species have been increasingly described [2]. The mortality rates in critically ill patients suffering from *Candida* invasive fungal infections vary between 40 and 70% [3, 4]. Even among those who receive antifungal treatment, the mortality may reach 67% [2].

Antifungal agents are restricted and need to be more directed to the exclusively fungal structure

To treat fungal infection, there are few medicines available. Originated in 1955, the amphotericin B was the pioneer to treat invasive mycoses, and is considered the most effective antifungal drug until nowadays. In the course of more than 60 years, other antifungal agents available for the treatment of systemic candidiasis include azoles (fluconazole, itraconazole, ketoconazole), new azoles (ravuconazole, posaconazole and voriconazole), and echinocandins (caspofungin, anidulafungin, and micafungin). The last antifungals hit the market around 2001 and after that no other drug has been approved [5].

Despite the complexity of fungal cells, most patented compounds target ergosterol biosynthesis, just echinocandins act in cell wall enzymes. The similarity of ergosterol with human cholesterol allows the occurrence of multiple adverse reactions [6]. Moreover, the development of fungal resistance has been increasingly described for all therapeutic classes [7].

Thus, search for more specific alternative therapies has become increasingly necessary. New antifungal drugs should possess extended spectrum of activity against fungi, improved pharmacokinetic profile so as to reduce dosing frequency, possibility of oral and parenteral administration, low adverse effects, and few drug-drug interactions.

Comparative genomics as an ally for development of new antifungals

The identification of new antifungal targets in opportunistic pathogenic fungi has been contributed to understand the fungal machinery and has been an important tool for new developing drugs. Efforts have been made to elucidate the genetic sequencing of the main pathogenic *Candida* species.

In addition to the functional characterization, the knowledge of the genome of pathogenic cells enables processes of research and development of new drugs, from the identification of possible targets. These targets can also be better selected using comparative genomics, which allows the targeting of molecules that are harmless to humans and that act against the greatest number of fungi.

The urgency of finding new drugs is peaking interest in the use of *in silico* studies to explore the interaction of compounds with target receptors. Molecular docking has been applied in virtual screening of small molecule libraries for hit identification and optimization. Chemical probes that form a covalent bond with a protein target often show enhanced selectivity, potency and utility for biological studies. So, *in vitro* and *in vivo* tests can occur only after selection of structures that are highly likely to interact with cellular targets. This economic and cost-effective methodology holds great promise for drug discovery projects [8].

Articles Selection

A list of 228 genes with homology to other fungal species absent in the genome of *Homo sapiens* and *M. musculus*, described by Braun et al [9], served as a basis for searching for material to compose this study. A PubMed search was made for publications involving each of these genes and their aliases described in Candida Genome Database (CGD), which were related to the term Mesh "*Candida*".

All manuscripts were evaluated and selected for essential virulence-related genes, with great impact in the literature, and included in the review the following information, when available: function and cellular location of each protein expressed by these genes, virulence attributes, co-regulators as well as compounds already described with the ability to negate the expression of these targets. The genes with the highest number of publications (15) were selected and the articles reviewed (Figure 1).

CLUSTAL Omega analysis was performed by optimal alignment of the amino acid sequences using CLUSTAL2.1 software to compare proteins with synonymous nomenclatures in the literature, and was used Blastp to determine protein identity between the main *Candida* species (Table 1).

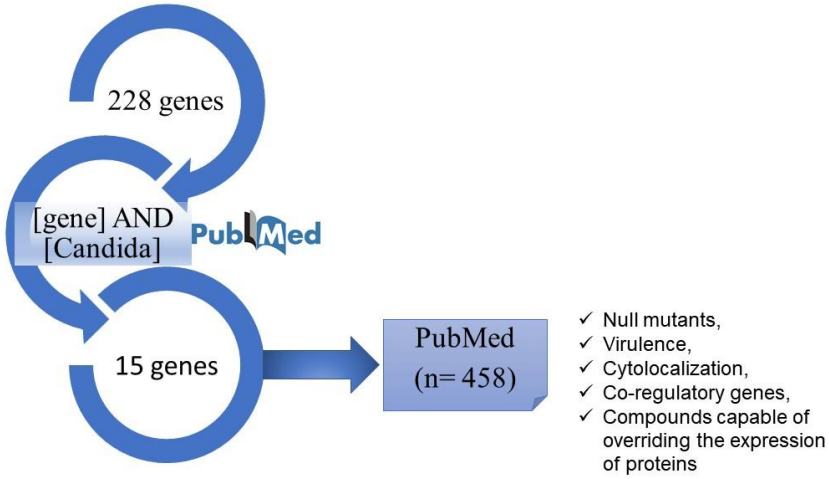


Figure 1. Articles selection

Table1. Selected proteins (targeted componentes) with most relevant publications that have a very strong identity fungal genomes but no significant sequence similarity to genes in the genomes of either humans or mice. The *C. albicans* percentage identity with other *Candida* especies was calculated by Blastp.

Protein	Synonymous (CGD)	Citolocalization	Nº of articles in Pubmed (gene) AND (*Candida*)	***C.albicans* % identity**			
				Cd	*Ct*	*Cp*	*Cg*
Phr1	NA	Cell wall	44	90	75	61	58
Phr2	NA	Cell wall	24	92	80	74	64
Chs1	NA	Cell wall	13	98	83	74	NA
Chs2	NA	Cell wall	18	96	80	75	NA
Chs8	Chs3 Chs21	Cell wall	16	95	81	71	NA
Plb1	NA	Cell wall	18	92	61	60	NA
Mdr1	Gyp2	Plasma membrane	128	95	79	65	NA
Flu1 (Tpo1)	Flu1 Tpo2	Plasma membrane	15	95	77	69	NA
Fks1	Gsc1 Gsl1 Gsl21 Gsl2 Gsl22	Plasma membrane	113	99	95	90	73
Erg6	NA	Plasma membrane	15	97	88	90	64
Mnt1 (Kre2)	Mnt1 Mnt2	Golgi complex	12	89	72	64	NA
Ssk1	NA	NA	19	86	55	48	NA
Tps1	NA	NA	15	99	95	87	74
Tps2	NA	NA	8	97	83	76	54

Cd: *C. dubliniensis;* Cp: *C. parapsilosis*, Ct: *C. tropicalis*, Cg: *C. glabrata*, NA: not available

Fungal targeted components

Cell wall

The cell wall (CW) of a fungal cell is responsible for its form and provides a number of essential functions, including mechanical strength, plasticity, maintenance of cell shape and integrity, besides mediating the adhesion and function as signaling to activate transduction pathways within the cell involved in virulence mechanisms [10]. In fungi the structural and infrastructural components of the CW, are composed specially by polysaccharides and glycoproteins [11].

pH-controlled Wall-Protein-Encoding genes

C. albicans has a family of five GH72-encoding genes: PHR1, PHR2, PHR3, PGA4 and PGA5. Phr1p and Phr2p are highly similar to *Saccharomyces cerevisiae* Gas1p, they share the same activity *in vitro* and PHR1 complements gas1Δ mutation [12].

PHR1 and PHR2 are a classical example of pH-controlled wall-protein-encoding genes. The predicted amino acid sequence of Phr2p, was 54.9% identical to that of Phr1p, belong to the same family and share a similar function.

• pH-regulated protein 1 - Phr1p
Synonymous: there is no

PHR1 is a pH-regulated gene, and is expressed when the ambient pH is at 5.5 or higher [13]. This enzyme belong to family GH72 of the beta-(1,3)-glucanosyltransferases which acts as a cell wall remodelling [14]. Under a cell wall stress the gene is induced which is required for cell wall cross-linking and integrity [15]. Furthermore, it is an important component of the fungal response to immune cell attack [16]. Phr1p also labelled the hyphal septa, where it colocalized with chitin [17].

PHR1 null mutant results in growth and morphological defects at neutral to alkaline pHs. It was avirulent in a mouse model of systemic infection (pH ~ 7.0), but uncompromised in its ability to cause vaginal infection (pH ~ 4.5) in rats [13]. PHR1 null mutants also exhibited a marked reduction in adhesion, was unable to penetrate and invade reconstituted human epithelia, and it is of fundamental importance in the maintenance of the morphological state, since PHR1 plays a key role in the maintenance of hyphal growth, like germ tubes elongation [14, 18, 19]. Furthermore, when PHR1 is deleted there is a decreased caspofungin sensitivity [20].

When the PHR1 gene is suppressed, there is an upregulation in other genes that compensate for the absence of production of the corresponding protein, which does not make the pathogen unfeasible. However, the addition of mutations may contribute to the unviability of yeast, the deletion of CHS3 or CHS8 at pH greater than 7.5 impairs the cell integrity during the yeast-hypha (Y-H) transition [12]. Rim101p is also associated with PHR1 regulation, it acts as a transcription factor and directly regulates pH-conditional gene expression [21]. This fungal flexibility in maintaining cell wall integrity contribute to assessments of glucan remodeling and should be through as a target for therapy.

Sosinska et al. [22] suggest Phr1 as a wall protein target from which immunogenic peptides could be selected as candidate for a combinatorial vaccine development. Taff et al. [23] concur that the inhibitors for this enzyme would provide promising anti-biofilm therapeutics, because Phr1p play specifics roles in the wall proteome and the secretome, which thrive in multiple niches of the human body. One of the major changes in apparent abundance in the secretome of fluconazole-treated cells was the increase in Phr1 putative wall repair-related protein [15, 24], it would also be interesting to think of an inhibitor to act in conjunction with available therapies.

• ***pH-regulated protein 2 - Phr2p***
Synonymous: there is no

PHR2 was considered a functional homolog of PHR1 by Mühlschlegel and Fonzi [25]. However, PHR2 is expressed at an ambient pH below 5.5, and the growth and morphology of the null mutant is compromised below this pH [13].

All studies addressing PHR2, present PHR1 data together. Only two publications address PHR2 without mentioning his counterpart. One is about the proteome of *Candida albicans* grown under vagina-simulative conditions [26]. The other, demonstrated the Phr2p involving in cell wall biogenesis and integrity and is important for *C. albicans* cell surface proteins during biofilm formation [27] appears induced during late steps of development (i.e. mature biofilms) [28].

Chitin Synthase Enzymes

Chitin is an essential structural polysaccharides of the fungal cell wall. There are four genes encoding chitin synthase enzymes, CHS1 [29], CHS2 [30], CHS3 [31] and CHS8 [32]. The predicted amino acid sequence regarding to Chs1p is 40.2% to Chs2p, 18.8% to Chs3p and 37.2% to Chs8p.

There are two distinct forms of chitin microfibrils, the long-chitin and short-chitin rodlets [33]. At least three pathways co-ordinately regulate chitin synthesis and activation of chitin synthesis operates at both transcriptional and post-transcriptional levels, PKC, HOG MAP kinase, and Ca^{2+}/calcineurin [32].

The deletion of these genes together showed changes in chitin synthase activity. However, homozygous chs2Δchs3Δ null mutant cells were slightly attenuated the virulence [34], and chs2Δchs8Δ mutant are important for maintaining cell integrity during early polarized growth in the yeast cell cycle, but less so in later phases of the cell cycle [35]. When in the absence of both Chs1 and Chs2, Chs3 and Chs8 formed a functional salvage septum, showing redundant strategies to enable septation and cell division to be maintained [36]. Therefore, it seems important to devise strategies to concomitantly inhibit all chitin synthesis enzymes.

All four genes encoding chitin synthases were upregulated by *Candida* biofilms micafungin treatment [37], a possibility to increase the efficacy of the treatment is to test a drug capable of inhibiting the action of chitin synthases associated with micafungin may be. However, just plagiochin E (PLE), an antifungal macrocyclic bis(bibenzyl) isolated from liverwort *Marchantia polymorpha* L, was investigate about its effect on the expression of Chs genes (CHS), presenting downregulated the expression of CHS1, and upregulated the expression of CHS2 and CHS3 [38].

• *Chitin synthase 1 - Chs1p*
Synonymous: there is no

Chs1 of *C. albicans* is an essential chitin synthase required for synthesis of the septum and for cell integrity [34]. It is expressed in both yeast and hyphae at a lower level. The Δchs1 conditional mutant was avirulent in an experimental model of systemic infection [39].

• *Chitin synthase 2 - Chs2p*
Synonymous: there is no

Gene identical to others reported:

In *C. albicans* CHS2 gene encodes a chitin synthase that is expressed preferentially in the hyphal form [30] and is not essential for growth, dimorphism, or virulence [34, 40].

• Chitin synthase 3 - Chs3p
Synonymous: there is no

CHS3 is required for synthesis of the chitin rings found on the surface of yeast cells, but not formation of septa in either yeast cells or germ tubes. CHS3 null mutants are significantly less virulent than the parental strain [41]. Short-chitin rodlets were absent in chs3 null mutants [33].

• Chitin synthase 8 - Chs8p
Synonymous: Chs3p (24.43% identity to Chs8p) and Chs21p (the sequence was not found)

CHS8 is a chitin synthase gene expressed in both yeast and hyphal cells of *Candida albicans*, the null mutant strains had a 25% reduction in chitin synthase activity and is dispensable for growth [32], and is particularly related to long-chitin microfibrils reduction [33].

• Phospholipase B - Plb1p
Synonymous: there is no

Phospholipase B (PLB1) gene is involved in the pathogenicity of *Candida albicans*. Plb1 is described as part of the secretoma of *Candida* being important for nutrient acquisition from lipid degradation. Extracellular enzymes transit the cell wall and may be found in the wall [24, 42].

Plb1 null mutant is viable, and in a murine model for hematogenously disseminated candidiasis, its virulence was significantly attenuated compared with the isogenic wild parental strain [43]. Plb1 secreted by *C. albicans* enhances the ability of this organism to cross the gastrointestinal tract and disseminate hematogenously [44].

Blastospores and pseudohyphae expressed higher levels of PLB1 mRNA than germ-tube-forming cells [45]. Isolates high biofilm formers were correlated with high levels of Plb1 [46]. TUP1 is a general transcriptional repressor [45], and disruptions in TOP2 significantly upregulated PLB1 [47].

Plasma membrane

The plasma membrane is essential for pathogenesis because this important barrier mediates morphogenesis and invasive growth, as well as secretion of virulence factors, cell wall synthesis, nutrient import, and other processes [48].

Drug:H+ antiporter family 1 (DHA1)

Candida spp. can develop resistance to antifungals through active drug export mediated by multidrug efflux pumps, in particular by members of the drug:H+ antiporter Family 1 (DHA1), here we will present two members Mdr1 and Flu1.

• Multidrug resistance protein - Mdr1p
Synonymous: Gyp2p, BENr [49-52] bmrP [49]

The MDR1 gene, encoding a membrane transport protein of the major facilitator superfamily, which actively exports azoles out of the cel [51, 53]. The homozygous mdr1/mdr1 null mutants from fluconazole-resistant clinical *Candida* spp. isolates, resulting in enhanced susceptibility [54, 55]. Structurally, the Mdr1p has two transmembrane domains, each comprising six transmembrane helices, interconnected with extracellular and intracellular loops. The intracellular loop, ICL3, is an essential structural element of the protein, once sequence deletions and insertions in this domain yield a non-functional protein [56].

There seems to be a preference of the MDR1 efflux pump for fluconazole [51, 53, 57-60] and voriconazole [60, 61]. High levels of Mdr1p is related with development of gradual resistance, inclusively in previous susceptible strain [57, 62, 63]. Otherwise, azoles like ravuconazole, isavuconazole, posaconazole, itraconazole, ketoconazole suffer minimal effect from the overexpression of MDR1 [55, 64-67]; MDR1 does not produce significant changes in echinocandin susceptibility [68] nor in amphotericin B [69].

In addition to the action of MDR1, most of the action of other genes is described with parallel actions that result in the resistance of *Candida* spp., like the ABC transporter (CDR1 and CDR2), together they are major mediators of azole resistance; and ERG11, involved in ergosterol biosynthesis [58, 67, 70]. The cross-resistance observed between the azois is associated with CDR1/2 and ERG11 overexpression [71, 72].

Other genes were studied in order to explain the regulation of MDR1 expression. Znaidi et al [73] believe that Upc2p, a key regulator of ergosterol metabolism, may contribute to azole resistance by regulating the expression of drug efflux pump-encoding genes. This relationship was not described in other studies, and years later Schubert et al [74] showed that the induction of MDR1 expression by toxic chemicals occurred independently of Upc2.

Researches done by Morschhäuser et al. [75] and Dunkel et al. [59] proved that mutations in the MRR1 gene, followed by loss of heterozygosity, results in a constitutive MDR1 upregulation, which plays a central role in the development of drug resistance in *C.*

albicans. Thereafter, Schubert et al. [74] and Silva et al. [60] also described the MRR1 as a major cause of MDR1 upregulation in *C. dubliniensis* and *C. parapsilosis* respectively.

High-level over-expression of MDR1 is commonly mediated by mutation in MRR1, a trans-acting factor, that is mutated by gain-of-function [76, 77]. A P683H point mutation in MRR1 gene, overexpressed the Mdr1 pump in a fluconazole-resistant strain was described by Kalkandelen et al [78]; homozygous mutation in MRR1 genes (T2957C/T2957C), with the amino acid exchange L986P was described by Zhang [79]; and Eddouzi et al [80] report a gain-of-function mutation V877F in MRR1.

CAP1 was initially described in involvement of with the MDR1 overexpression [81], however disruption of CAP1 increased the levels of MDR1 expression instead of suppress. Further studies showed that Cap1p, which has been previously implicated in cellular responses to oxidative stress, could be a potent activator of MDR1 expression [73, 82], described that the induction of MDR1 by toxic drugs is partially dependent on Cap1. Moreover, was described Ada2 as a subunit of the SAGA/ADA coactivator complex, which is recruited for Cap1 to promote the MDR1 activity in strains that do not contain Mrr1 gain-of-function mutations [83].

Another described regulator of MDR1 expression is Mcm1p, which binds directly to an Mcm1p binding motif found in the promoter, the MRR1 [84]. When activated by oxidative stress or a gain-of-function mutation, Mrr1 requires either Mcm1 or an active Cap1 to cause overexpression of the MDR1 efflux pump [85].

In vivo, the Fluco-S strains appear to be more virulent than strains Fluco-R, once the mortality of the FLU-S isolate (100%) was higher than that of the FLU-R isolate (60%), when FLU was absent [86]. Lohberger et al [87] demonstrated that the transcription factor MRR1 had no significant effect on *C. albicans* virulence.

Cowen et al. [88], Rogers et al. [89], and Karababa [90] demonstrated that GRP2 and IFD5 (oxido-reductive functions), IPF5987 and SNZ1 (potentially involved in pyridoxine synthesis) are genes commonly regulated in MDR1 upregulation. The common regulation of these genes constitutes a group of genes necessary for the adaptation of *C. albicans* to drug exposure and compose clusters of genes needed for resistance development [90].

A positive correlation between SAP2 and MDR1 also was described. Patients infected with *C. albicans* and treated with suboptimal doses of fluconazole may experience enhanced in virulence. Amongst the overexpression of MDR1, it was observed a increased production of secreted aspartyl proteinase (Sap) [91].

Negative regulator of MDR1 have also been described. In *C. albicans*, Regulator of Efflux Pump 1 (REP1) null mutations decreased the susceptibility to antifungal drugs, resulting from increased expression of MDR1 mRNA [92]. And, null mutation of CPH1 increased the expression of MDR1 as well as decreased susceptibility to fluconazole and voriconazole, proving that Cph1p negatively regulates MDR1 [69].

Sessile cells display phenotypic traits dramatically different from those of their free-living, planktonic counterparts, sessile cells overexpressed MDR1 and also ERG11 [93]. Mukherjee et al [94] stated that the MDR1 gene, as well as CDR1, is mainly expressed in the early stage of biofilm formation.

However, Perumal et al. [95] suggested that drug efflux pumps did not contribute to resistance, that cell density has a role in the phenotypic resistance of biofilm. And Watamoto et al. [96] showed that transcriptional expression of MDR1 and others resistance genes was lower in recently adhered *C. albicans* than in the stationary phase planktonic cultures, they also affirmed that the expression of MDR1 in biofilms greatly increased on challenge with amphotericin B but not with the other drugs tested. *In vitro* studies showed pronounced growth rates of the Fluconazole-S isolate and a more intense biofilm-building activity compared with the Fluconazole-R isolate [86].

Given the importance of MDR1 for *Candida* viability, studies have described new compounds with antifungal potential based on inhibition of expression of MDR1. Natural derivative compounds like, a antifungal plant peptide purified from fruits of *Trapa natans* showed the inhibition of *C. tropicalis* growth *in vitro* and disrupted the biofilm formation [97]; tetrandrine, a natural, bis-benzylisoquinoline alkaloid, exhibits synergistic effects with fluconazole [98, 99]; kaempferol, an active flavonoid, induced reversion in fluconazole resistant *C. albicans* [100].

Retigeric acid B, a lichen derived pentacyclic triterpenoid [101]; two sulfated sterols extract of *Topsentia* sp. (geodisterol-3-O-sulfite and 29-demethylgeodisterol-3-O-sulfite) [102]; and thymol and carvacrol, the principal chemical components of thyme oil [103] displayed synergistic antifungal activity with azoles in *C. albicans* known to overexpress MDR1. The fatty acid synthesis inhibitor cerulenin derivatives [104], and farnesol an endogenous quorum sensing molecule [105] decreased the resistance of the Mdr1 efflux pump-overexpressing *C. albicans* isolates and biofilms, respectively.

Antimicrobial derivatives was also tested against biofilms of *C. albicans* and their activity was associated with decreasing efflux pump-encoding genes like MDR1. Levofloxacin derivatives [106], and doxycycline in combination with fluconazole [107]

presented good results. Finally, a single novel small molecule has been described so far with a highly potentiated activity against a collection of clinical isolates and lab strains that overexpress MDR1, bis(1,6-a:5',6'-g)quinolizinium 8-methyl-salt (BQM) was reported as a potent and broad antifungal activity [108].

• ***Fluconazole resistance protein - Flu1p***
Synonymous: Tpo2, Tpo1

C. albicans FLU1 is also annotated in CGD as TPO1 and TPO2. The rule of TPO genes in *S. cerevisiae* is well known, they are involved in polyamine efflux. However, TPO1 denomination was did not encountered in association with *Candida* studies. In a phylogenetic and syntenic analyses of the evolution of FLU1 gene, Dias and Sá-Correia [109] showed that TPO1 is also described to *C. glabrata*, however in *C. albicans* its homolog is denominated FLU1, they have 44% of similarity. To *C. tropicalis* is considered the hypothetical protein CTRG_04977, which has 62% of identity of CaFlu1p. And, to *C. parapsilosis*, in NCBI-Protein was shown hypothetical protein CPAR2_300750 the identity 44% about CaFlu1p. TPO2, on the other hand, there seems not to be a different name for the same protein [109, 110]; however, the identity between CaTpo2p and CaFlu1p is 63%.

All articles found associate FLU1 with resistance to fluconazole due to the transport of the drug out of the cells. It is always related to the other genes classified as efflux pump MDR1, CDR1, CDR2. Only one article searched about TPO2.

• ***β-1,3-glucan synthase catalytic subunit - Fks1p***
Synonymous: Gsc1p, Gsl1p, Gsl2p, Gsl21p, Gsl22p, Etg1p [111], Cwh53p [112], Pbr1p [113], and Cnd1p [114].

A major structural polymer of yeast and fungal cell walls, 1,3-beta-D-Glucan, is synthesized from UDP-glucose by the multisubunit enzyme 1,3-beta-D-glucan synthase (GS). Yeast GS is composed of a catalytic subunit encoded by FKS1 and FKS2 and a regulatory subunit encoded by RHO1 [115-117]. FKS1 gene encodes a 215-kDa integral membrane protein (Fks1p) which is predicted to contain 10 to 12 transmembrane domains with a structure resembling integral membrane transporter proteins [118, 119]. Deletion of the FKS1 gene leads to a significant reduction in GS activity, which reduces the synthesis of the major structural component of the cell wall, with impact in fitness and virulence of yeast [120]. This GS, would be one of the promising targets for antifungal agents. However, the several

transmembrane domains impossible the crystallization of this protein and makes the molecular modeling difficult.

Aside from *in vitro* studies, a research evidenced the FSK1 essentiality in the *Candida* survival and virulence in murine model. Thus, the most recent antifungals that won the market were the echinocandins, fungicidal antibiotics that inhibit GS in several human fungal pathogens. CaFks1p was described as the target of the echinocandins [116], however, echinocandin resistance associated with mutations in FKS1 began to be described even before its commercial availability[121]. Over the years, some studies have been conducted to prove the association of FKS1 mutations with resistance to this drug class in different *Candida* species [122-126]. The substitution of serine for proline, tyrosine or phenylalanine in position 645 has been the most commonly described, in addition to other mutations that also confer the loss of sensitivity to the newest antifungals [125-128].

In addition to mutations in the target drug, another aspect to be considered is the upregulation of parallel genes. García-Rodriguez et al. [129] demonstrated that deletion of the fks1 gene increases chitin synthesis due an activation of the CSIII activity (Chs3p and Chs4p) mediated by an unusual localization of the active CSIII complex. The authors concluded that the change would be lethal if a second mutation in the CHS3 or CHS4 gene was performed, responsible for the synthesis of cell wall chitin [120]. Other authors have also proved the increase chitin in FKS mutant strains [36, 130, 131]. Drakulovski et al. [132] showed a *C. albicans* strain with high MIC for caspofungin and no FKS1 mutations exhibits a high chitin content and mutations in two chitinase genes, this fact allowing the strain to compensate for its low 1,3-beta-D-Glucan content and the effect of caspofungin. Despite this, no studies were found that invest in blocking different genes with related functions.

In order to identify novel chemical classes of GS inhibitors, Kondoh et al. [133] screened a chemical library monitoring inhibition of the *Candida albicans* GS activity. They described piperazine propanol derivative GSI578 which was identified as a potent inhibitor against GS *in vitro*. Other studies with potential GS inhibitors targeting *Candida* spp. have been carried out, such as piperazinyl-pyridazinone compounds [134] papulacandin D [135]; AMG-148, an oxathiolone-fused chalcone derivative [136]; caffeic acid derivatives [137].

Biofilm *C. albicans* was more resistant to antifungals than recently adhered cells and stationary-phase planktonic cultures. The expression of FKS1 in biofilms greatly increased on challenge with caspofungin and amphotericin B ($P<0.01$) [96].

The biofilm mass and metabolic activities were reduced in most of the fks1 mutants as compared with reference strain. Structural analyses revealed that the fks1 mutant biofilms

were generally less dense and had a clear predominance of yeast and pseudohyphae, with unusual "pit"-like cell surface structures [138].

• ***Sterol 24-C-methyltransferase - Erg6p***
Synonymous: there is no

ERG6 is a sterol methyltransferase gene, involved in ergosterol biosynthesis, it is not essential for viability of yeast [139]. However, *C. lusitaniae* erg6 delta strain resulted in resistance to amphotericin B [140]. In *C. glabrata*, a mutation in the ERG6 gene led to a reduced susceptibility to polyenes and to a pseudohyphal growth due to the subsequent changes in sterol content of the plasma membrane [141, 142]. In *C. parapsilosis*, there is not a relevant pattern of expression of this gene in antifungal resistant isolates [143].

The ERG6 availability to use as a screen for new antifungals targeted was first indicated by Jensen-Pergakes [139] who exalted the fact that it is not found in humans. More recently, ERG6 was pointed out by comparative genomics *in silico* analysis as a potential candidate to drug targets by Abadio et al [144]. However, until now, no compound with expression inhibitory activity has been described.

Cytoplasm

• ***Phosphoribosylaminoimidazole carboxylase - Ade2p***
Synonymous: there is no

ADE2 gene encodes phosphoribosylaminoimidazole carboxylase (AIRC) which participates in the biosynthesis of purine nucleotides. The nucleotide sequence of the *Candida albicans* ADE2 gene was determined for the sequence possesses an uninterrupted open reading frame of 1704 nucleotides corresponding to 568 amino acid residues [145].

Studies on ADE2 has a larger focus on gene manipulation techniques. It is a nonessential loci, mutants deficient in Ade2p are adenine auxotrophs [146] and leads to the accumulation of a red pigment when incubated in media containing a limited amount of adenine [147]. In Becker's model [148], there were between 0 and 60% survival in mice infected with nulls mutants ADE2. Strengthening the "not essential" aspect in virulence of *Candida* species.

The gene for the secreted acid protease (ACP), a potential virulence factor of *Candida* species was inactivated by cotransformation of an ade2 *C. tropicalis* mutant with a linear

DNA fragment carrying a deletion in ACP, and the replicative vector pMK16 which carries a selectable ADE2 gene marker [149].

Endoplasmic Reticulum and Golgi complex

Endoplasmic reticulum (ER) is crucial for protein folding, glycosylation and secretion in eukaryotic organisms. The synthesis of eukaryotic N-linked glycans is initiated at the luminal side of the ER. Moreover, the glycosylation of cell wall proteins involves the activities of families of endoplasmic reticulum and Golgi-located glycosyl transferases [150].

• ***Alpha-1,2-mannosyltransferase – Mnt1p***
Synonymous: Kre2, Mnt2, Ktr1

The KRE2/MNT1 family encode a set of multifunctional mannosyltransferases that participate in encoding the α-1,2 mannosyltransferase protein [151]. KRE2 is described in *Paracoccidioides lutzii* and is considered the MNT1 ortholog in *Candida* spp.

Mora-Montes et al. [150], concluded that MNT1/KRE2 gene family participates in three types of protein mannosylation in *C. albicans*, and these modifications play vital roles in fungal cell wall structure and cell surface recognition by the innate immune system. Compounds that are able to block the production of these mannoproteins may be important candidates for the development of new antifungal agents [144].

Proteins with unknown cytolocalization

• ***Mitogen-activated protein kinase kinase kinase - Ssk1p***
Synonymous: there is no

In *C. albicans* SSK1 have a regulatory function in some aspects of cell wall biosynthesis, stress adaptation [152, 153], and adaptive functions for the survival against human neutrophils [154]. Ssk1p is not described as wall protein, but regulates adhesins.

Study with Δssk1 null strains in *C. albicans* shows defective hyphal development, and virulence studies indicated that SSK1 is essential for the pathogenesis [155]. *C. albicans* without the ssk1 response is less able to adhere to human esophageal cells [156] probability by the difficult to hyphal formation and a down regulation of ALS1 [132, 156].

Trehalose synthesis genes

In yeasts, accumulation of trehalose is one of the principal defense mechanisms under stress conditions [157, 158]. There are two trehalose synthesis genes described TPS1 and TPS2. Tps2p has 33.7% identity of tps1p.

Although some authors cite TPS1 and TPS2 potentiality as target for antifungal drugs [159, 160], so far no studies were found that sought compounds with this porpouse.

• ***Alpha-trehalose-phosphate synthase (UDP-forming) - Tps1p***
Synonymous: there is no

TPS1 gene from *C. albicans* encodes trehalose-6-phosphate synthase. Double disruptant tps1/tps1 mutant failed to produce germ tubes in some conditions and is avirulent in mouse model of infection [161].

Presence of trehalose is a contributory factor that protects the cell from injury caused by macrophages[158]. However, lack of trehalose is compensated by an over-glycosylation of the cell wall components in the tps1Δ/tps1Δ mutant, which reduces susceptibility to killing [162].

Trehalose was related to a protective role in the resistance of *C. albicans* to AmB. Due to the transitory accumulation of TPS1 mRNA or to the activation of trehalose synthase (Tps1p) with the simultaneous deactivation of neutral trehalase (Ntc1p). That made the tps1Δ/tps1Δ mutant more susceptible to AmB than parental strain [160].

• ***Trehalose-phosphatase - Tps2p***
Synonymous: there is no

TPS2 gene encoding the trehalose-6-phosphate phosphatase activity in *C. albicans* [163]. TPS2 is a gene not required for growth under standard conditions but required for pathogenicity in a host. Double disruptant tps2/tps2 mutant showed reduced growth at high temperatures, a marked sensitivity to heat shock and severe oxidative exposure and virulence is strongly reduced in a mouse model of systemic infection [163-165].

Concluding remarks

The need for new antifungals is imperative, advances in comparative genomics and in the methods for screening of molecules are options for search of new agents. The essential genes for *Candida* and absent in humans are possible targets for development and selection of new compounds with antifungal action. Some of these targets, like the Mdr1 and Fks1, are

extensively explored in literature and feature studies on molecules selected for high-throughput screening derived from natural substances with ability to block genes expression. Other targets must still be explored, for this *in silico* tests have been promising, because they are able to reduce time and cost of research. The joint use of compounds able to block different targets is also something to be experienced, as they are observed in the presence of upregulated gene or even overexpression due antifungals already available commercially. To *in silico* methods can be applied successfully, it is important to know the possible targets.

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Artigo: "A NEW SMALL MOLECULE KRE2 INHIBITOR AGAINST INVASIVE *Candida parapsilosis* INFECTION"

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A NEW SMALL MOLECULE KRE2 INHIBITOR AGAINST INVASIVE *Candida parapsilosis* INFECTION

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Running title: KRE2 inhibitor against invasive *C. parapsilosis*

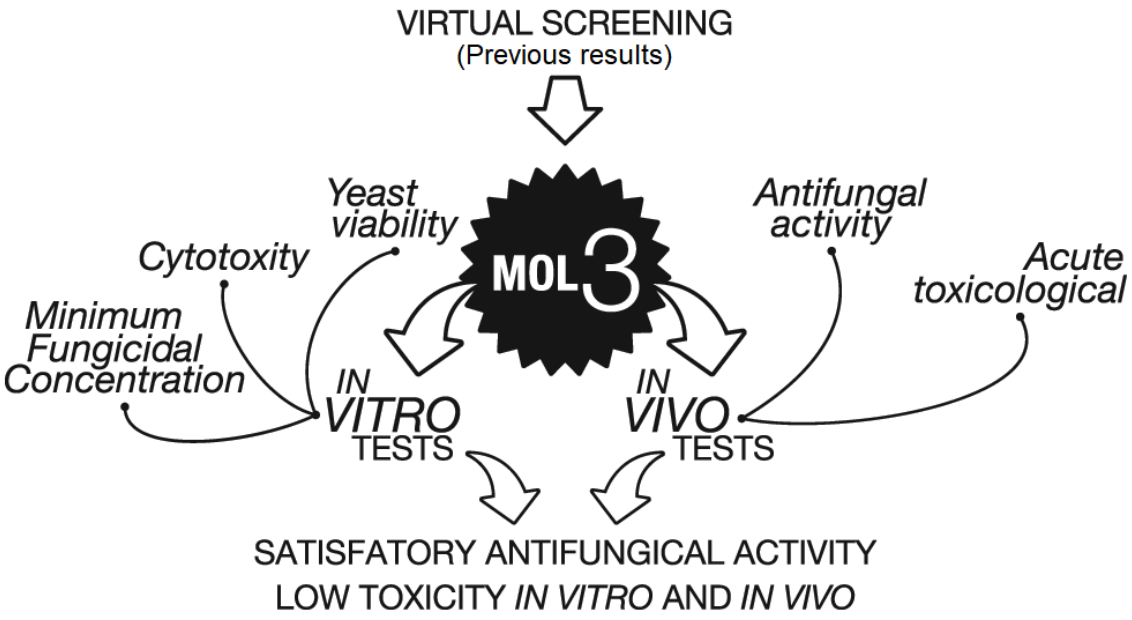
Summary Points

MOL3 is a small molecule that was selected by virtual screening.

The MFC of MOL3 against *Candida* spp. ranged from 16 to 128 mg/L.

MOL3 was toxicologically inert in an animal model.

MOL3 significantly reduced experimental murine systemic infection by *C. parapsilosis*.



ABSTRACT

Aim: To investigate the antifungal activity of MOL3, a small molecule that was selected by virtual screening, against *Candida* spp.

Materials & Methods: The antifungal activity of MOL3 was evaluated using standard strains and clinical isolates. Activity was evaluated in both *in vitro* tests and animal models.

Results: The minimum fungicidal concentration (MFC) of MOL3 against *Candida* spp. ranged from 16 to 128 mg/L. MOL3 at the sub-MFC inhibited hyphal elongation. The remaining yeast cells presented morphological changes and were metabolically inactive. MOL3 was toxicologically inert both *in vitro* and in the animal model. MOL3 also reduced experimental systemic infection by *C. parapsilosis* in mice.

Conclusion: The selection of MOL3 by virtual screening was successful, revealing a promising antifungal candidate.

Keywords: Virtual screening; Invasive Candidiasis; KRE2 protein

1. INTRODUCTION

Invasive fungal infections (IFIs) have presented a high incidence worldwide [1], [2]. The genus *Candida* is the most common infectious agent of IFIs, accounting for 80% of fungal infections of the circulatory bloodstream [2]. The species most frequently involved with IFIs are *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata* and *C. krusei* [1]. The *C. parapsilosis* complex has emerged as important opportunistic pathogens that are involved in IFIs [3, 4]. It is currently the main species that is isolated in pediatric intensive care patients [5]. Infections by *C. parapsilosis* have caused concern because of changes in their patterns of susceptibility and high rate of mortality, especially among newborns [6], which has led to the continual search for new therapeutic options.

The treatment of IFIs currently relies on a restricted therapeutic arsenal [7]. Greater resistance to antifungal agents by emerging species has also been observed, which can be primarily attributed to the prior exposure of fungi to these therapeutic agents [8]. Mutations of *C. parapsilosis* that are responsible for the development of resistance have been attributed to the use of the newest antifungals agents, especially echinocandins, which favor amino acid substitutions in the FKS1 and FKS2 genes [9]. Moreover, treatment with conventional antifungals is associated with numerous drug interactions [10], toxicity [11], and treatment failure [12].

New drugs with antifungal action need to be developed. The conventional research and development (R&D) process for new drugs is expensive and time-consuming [13]. Advances in computational techniques and hardware have enabled *in silico* methods that are able to considerably accelerate this process [14]. Rational drug design and high-throughput screening have reduced the timeframe of the hit-to-drug schedule [15], lowered the risk of toxicity and ineffectiveness, and increased the overall efficiency of the drug discovery process [16]. Our research group has been working with *in silico* methods, including homology modeling and virtual screening, to allow the identification of new potential drug targets and antifungal agents. Recently, potential drug targets for human fungal pathogens, such as α-1,2 mannosyltransferase (KRE2), have been identified [17].

MOL3 is a small molecule that was selected by virtual screening, which is currently undergoing the patent process. Preliminary studies showed that MOL3 inhibited the activity of KRE2 in *Paracoccidioides lutzii* and Mnt1p in *C. albicans* (unpublished data). The KRE2 gene is an ortholog of MNT1 in *Candida*, in which the active site domains in corresponding proteins are conserved [17]. The sequence identity between the KRE2 and MNT1 proteins is 50% *C. albicans* and *C. tropicalis*, 48% in *C. parapsilosis*, and 46% in *C. glabrata* (the

genome of *C. krusei* has not yet been sequenced). KRE2 participates in encoding the α-1,2 mannosyltransferase protein [18], which is involved in cell wall stability and confers virulence characteristics, such as interactions with host tissues [19]. Compounds that are able to block the production of these mannoproteins may be important candidates for the development of new antifungal agents [20] because this class of proteins is absent in humans [17, 21]. The aim of the present study was to evaluate the antifungal activity of MOL3 against five pathogenic species of *Candida* that are frequently identified in clinical infections, including clinical isolates of *Candida parapsilosis* complex species.

2. MATERIAL AND METHODS

All of the procedures were approved by the Ethics Committee of the Universidade Estadual de Maringá (opinion no. 160/2013).

2.1. Microorganisms

The screening included reference strains of five pathogenic, frequently identified species of *Candida* (*C. albicans*, ATCC 90028; *C. parapsilosis*, ATCC 22019; *C. tropicalis*, ATCC 40042; *C. glabrata*, ATCC 90030; *C. krusei*, ATCC 40147). The study included 14 clinical isolates of *C. parapsilosis* complex because it is an especially relevant epidemiological species in our environment. These strains were obtained from patients with nosocomial infection (all from the mycology collection of the Medical Mycology Laboratory, Universidade Estadual de Maringá, Paraná, Brazil).

2.2. Chemical compounds

The KRE2 protein of *Paracoccidioides lutzii* has approximately 50% sequence homology to the *Candida* spp. template in the Protein Data Bank (ID: 1S4N). Therefore, homology modeling was performed, which allowed the construction of the predicted tridimensional structure of this target. Recent studies performed virtual screening to select the main small molecules that interact with KRE2 of *P. lutzii* (unpublished data).

In a currently unpublished study by our group, the bank of commercially available compounds from the database of Life Chemicals Company (Niagara-on-the-Lake, Canada) provided an initial selection of 750,000 compounds that were docked to the KRE2 model by virtual screening simulations. A filtering process resulted in 3,000 compounds with drug-like features and certain chemical diversity. Docking simulations were performed using the GOLD 3.2 program [22], which allows partial flexibility of the binder and amino acid residues of the

active site of the target protein, resulting in identification of the 17 best hits as potential inhibitors of KRE2. MOL3 presented the best results with regard to *in vitro* antifungal activity against six pathogenic fungi, including *P. lutzii* and *C. parapsilosis*. Therefore, MOL3 was chosen for further studies. To facilitate the complete solubility of MOL3, the following diluents were added: 0.02% non-ionic surfactant Pluronic F-127 (P/F-127; Sigma) and 0.5% dimethylsulfoxide (DMSO). This combination of diluents was included as the control in all of the experiments.

The other drugs were acquired commercially: amphotericin B (Bristol-Myers Squibb, Bedford, OH, USA), voriconazole (Pfizer Ireland Pharmaceuticals, Dun Laoghaire, Dublin, Ireland), fluconazole (Pfizer), and micafungin (Astellas, Takaoka, Toyama, Japan).

2.3. Animals

Male, immunocompetent BALB/c mice, six weeks of age and weighing an average of 22 g, were obtained from the central vivarium of the Universidade Estadual de Maringá under specific pathogen-free conditions.

2.4. *In vitro* studies

2.4.1. Broth microdilution tests

All of the isolates were tested against amphotericin B, fluconazole, voriconazole, micafungin, and MOL3 according to Clinical and Laboratory Standards Institute protocol M27-A3 [23]. Three independent assays were performed with MOL3 at concentrations of 1-256 mg/L.

The minimum inhibitory concentration (MIC) was interpreted according M27-S4 [24] for established antifungal agents. Visual and spectrophotometric point readings were not possible for MOL3, because the final product presented turbidity. Therefore, the minimum fungicidal concentration (MFC) was determined as described below.

After 48 h of incubation, 2 µl aliquots of the homogenized content of each plate-well were subcultivated in Petri dishes that contained Sabouraud agar (SDA) and then incubated at 37°C for 24 h. The MFC of MOL3 was considered the concentration that reduced cell growth by 90%.

2.4.2. Evaluation of yeast viability

Standard yeast strains and clinical isolates of *C. parapsilosis* species complex that were exposed to the MFC and sub-MFC were evaluated by transmission electron microscopy

(TEM) and scanning electron microscopy (SEM). After the microdilution test, the contents of three plate-wells for each concentration were transferred to microtubes. The yeast cells were washed three times with 0.1 M cacodylate (pH 7.4) buffer and fixed in 2.5% glutaraldehyde. Scanning electron microscopy and TEM were performed as described previously [25].

The LIVE/DEAD Yeast Viability Kit (L7009, Molecular Probes, Leiden, The Netherlands) was used to determine the metabolic activity of *C. parapsilosis* in MOL3 susceptibility testing. An inoculum that contained 2-3 × 10^3 yeast/ml of *C. parapsilosis* ATCC plus the sub-MFC (16 mg/L) or MFC (32 mg/L) of MOL3 to a final volume of 1 ml was incubated at 37°C for 48 h. We also tested fluconazole, control live cells, and control dead cells (heat-killed at 60ºC for 1 h). Staining and the interpretation of fluorescence were performed according to the manufacturer's instructions.

2.4.3. Cytotoxicity test

HeLa lineage cells were cultivated at 37ºC in a 5% CO2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM; Gibco, St. Louis, MO, USA) that contained 10% fetal bovine serum (Invitrogen, São Paulo, São Paulo, Brazil) and 1% penicillin/streptomycin (Gibco). After reaching 80% confluence in a cell culture flask (TPP, Trasadingen, Switzerland), the cells were suspended in a solution that contained 25% trypsin-ethylenediaminetetraacetic acid (Gibco). The cell concentration was adjusted to 2 × 10^5 cells/ml in DMEM without penicillin/streptomycin, and the suspension was added to 96-well plates (TPP). Prior to cytotoxicity testing, the wells were washed twice with phosphate-buffered saline (PBS), and the cells were exposed to MOL3 at concentrations of 1-256 mg/L (corresponded to the concentration that were used for susceptibility testing). As controls, the cells were exposed to the respective amounts of diluent (PBS, DMSO, and P/F-127) without MOL3. The test was performed after 24 h of incubation at 37°C in a 5% CO2 atmosphere. Cytotoxicity was measured as the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboximetoxyfenyl)-2-(4-sulfofenyl)-2H-tetrazolium (MTS; Promega, Madison, WI, USA) in DMEM without phenol red. After 3 h of incubation in the dark, the absorbance of formazan (i.e., a product of the bioreduction of epithelial cells that is soluble in tissue culture medium) was read at 490 nm using an ASYS apparatus (Biochrom, Holliston, MA, USA). The percentage of cell viability (%CV) was calculated by the formula %CV = (At/Ac) × 100, where At and Ac refer to the absorbance of test substance and control (untreated cells), respectively [26]. Cytotoxicity of the compounds was evaluated as the mean of three independent experiments.

2.5. *In vivo* studies

2.5.1. Acute toxicological evaluation of MOL3

The mice were divided into three groups: normal untreated animals (tGN group), control animals treated with 200 µl of vehicle (PBS, DMSO, and P/F-127; tGC group), and animals treated with 0.05 g/kg MOL3 in a single dose (tGT group). The animals were evaluated over 14 days, anesthetized, and sacrificed.

2.5.1.1. Hematological examination

After 14 days and prior to treatment, we performed a hematologic evaluation of all of the animals. An aliquot of blood was taken from the distal end tail of the mice to determine total white blood cell count and white blood cell differentials.

2.5.1.2. Hippocratic screening

Hippocratic screening of the *in vivo* toxicity of MOL3 was performed according to Malone and Robichaud [27]. After MOL3 administration, the animals were individually observed at 0, 15, 30, 60, 120, 240, and 480 min daily for 14 days. During this period, behavioral parameters, weight change, and survival were evaluated.

2.5.1.3. Biochemical examination

On the last day, aspartate transaminase (AST), alanine transaminase (ALT), creatinine, and glucose (Gold Analisa, Belo Horizonte, MG, Brazil) were evaluated. The biochemical analyses were performed using blood samples that were collected with heparin from the abdominal vena cava on day 14.

2.5.1.4. Organ weigth and histopathological examination

During necropsy, the liver and kidneys were harvested, macromorphologically assessed, and weighed.

2.5.2. Evaluation of antifungal activity of MOL3

The systemic candidiasis model was established in 15 mice by administering 100 µl of a solution that contained 1×10^7 viable *C. parapsilosis* yeast cells (ATCC 22019) through the lateral tail vein according to Conti et al [28], with modifications. The animals were divided into three groups (n = 5). Three hours after infection, the treatments were administered (100

µl, intraperitoneally, every 12 h for 5 days) according to group. The test group (TG) was treated with 0.025 g/kg MOL3 in PBS and 0.02% P/F-127. The standard group (SG) was treated with 0.005 g/kg fluconazole according to Wu et al. [29]. The control group (CG) was treated with PBS and 0.02% P/F-127. All of the animals were sacrificed on day 5 of treatment. The kidneys were then collected. One kidney was weighed, steeped, homogenized in PBS, serially diluted, and plated on SDA. The plates were incubated at 37°C for 24 h to count colony-forming units (CFUs), with the final value adjusted according to body weight (CFU/g). The other kidney was fixed in 10% formalin, maintained in 70% ethanol, and sliced into histological sections that were stained with Gomori & Grocott and hematoxylin and eosin (H&E) in three independent experiments.

2.6. Statistical analysis

The data are expressed as mean ± standard deviation (SD). Significant differences between means were analyzed using Student's t-test. Analysis of variance (ANOVA) was performed to analyze the toxicity data, followed by the Tukey test. The statistical analyses were performed using Prism 6.0 software (GraphPad, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. *In vitro* results

3.1.1. Susceptibility testing

Fig. 1 shows the antifungal activity of MOL3 against the five principal *Candida* ATCC species. The MFC of MOL3 against *C. albicans*, *C. parapsilosis*, and *C. krusei* was 32 mg/L. The MFC against *C. tropicalis* was 64 mg/L. *C. glabrata* presented a reduction of growth at the highest MOL3 concentration tested (256 mg/L). Considering the positive effect of MOL3 against *C. parapsilosis* and because of the increasing nosocomial importance of this species, especially with regard to neonatal infection, clinical isolates of this species were selected from the yeast bank of the Medical Mycology Laboratory, Universidade Estadual de Maringá, for susceptibility testing.

—Fig. 1—

Table 1 shows the susceptibility profile of 14 *C. parapsilosis* clinical isolates for established antifungal agents (MIC) and MOL3 (MFC). All of the isolates were susceptible to

amphotericin B. One isolate (CpHU199) was dose-dependently susceptible to fluconazole, with intermediate susceptibility to micafungin. Two isolates (CpHU313 and CpHU359) presented intermediate susceptibility to voriconazole. Eight clinical isolates (HUM91, CpHU141, CpHU199, CpHU201, CpHU325, CpHU357, CpHU358, and CpHU359) presented intermediate susceptibility to micafungin. CpHU141 presented intermediate susceptibility to voriconazole. Only three clinical isolates were susceptible to all of the antifungal agents tested. MOL3 inhibited the growth of these clinical isolates at concentrations of 16-128 mg/L. The MFC50 and MFC90 were 64 and 128 mg/L, respectively.

—Table 1—

3.1.2. Evaluation of yeast viability

Scanning electron microscopy was used to observe possible morphological changes in yeast that were treated with MOL3 at the MFC (Fig. 2G, H) and sub-MFC (Fig. 2D, E). The untreated control underwent the same test conditions but without MOL3 exposure (Fig. 2A, B). All of the *C. parapsilosis* cells in the control group presented a smooth wall surface, an oval shape, elongated structures, and abundant pseudohyphae (Fig. 2A). The sub-MFC of MOL3 (16 mg/L) significantly reduced elongated structures. The oval form predominated (Fig. 2D), with slight surface changes in some cells that were characterized by mild convolution (Fig. 2E) and fewer, although still frequent, yeast cells. The MFC of MOL3 (32 mg/L) significantly reduced the number of yeast cells (Fig. 2G). The few remaining cells presented an oval shape and surface changes (Fig. 2H), such as convolutions (arrow in figure) and retractions (open arrow in figure).

Transmission electron microscopy revealed that untreated cells presented the typical morphology of yeast with good survival conditions, with uniform-density bounded organelles that were surrounded by a regular intact plasma membrane and cell wall (Fig. 2C). The sub-MFC of MOL3 caused some cells to lose the cellular organelle contour, reflecting necrosis (Fig. 2F). The MFC of MOL3 had more pronounced effects than the sub-MFC, resulting in a significant loss of cytoplasmic content and cell death (Fig. 2I).

—Fig. 2—

Fluorescent staining (Fig. 3) showed that the sub-MFC of MOL3 (16 mg/L) caused a reduction of filamentation and the loss of fluorescence that was intense in the control group,

indicating living cells. The MFC of MOL3 (32 mg/L) resulted in a dramatic reduction of the number of cells and the total absence of filamentation. Most of the remaining cells were metabolically inactive and presented a reduction of the cell wall, indicated by Calcofluor White M2R (CFW). MOL3 was apparently more effective than fluconazole in reducing filamentation, but the proportion of dead/living cells appeared to be similar between both compounds.

—Fig. 3—

3.1.3. Cytotoxicity

HeLa cells presented approximately 70% viability after 24 h exposure to the higher concentration of MOL3 that was used for susceptibility testing (256 mg/L). At lower concentrations, cell viability was even higher, indicating that the compound had no *in vitro* cytotoxicity. No significant differences were found between the MOL3-treated groups and the groups that were exposed only to the diluent and the control group.

3.2. *In vivo* results

3.2.1. Acute toxicity

We tested the acute toxicity of MOL3 using the Hippocratic test. The group that was treated with 0.05 g/kg MOL3 in a single dose (tGT group) presented abdominal contortion and piloerection within the first 30 min, after which no changes were observed in the parameters that were analyzed. The tGC group also presented no changes in behavioral parameters, similar to normal untreated mice (tGN group). Weight gain also progressed normally in all groups, with no statistically significant differences between them (Fig. 4A). The weight of the liver (Fig. 4B) and kidneys (Fig. 4C) was similar between groups ($p > 0.05$). None of the animals died within the follow-up period.

With regard to biochemical parameters, no changes in AST (Fig. 4D), creatinine (Fig. 4F), or glucose (Fig. 4G) were observed between groups. Alanine aminotransferase activity significantly increased in the tGT group compared with the tGN group (Fig. 4E), which also occurred in mice that were treated with 200 µl of vehicle (PBS, DMSO, and P/F-127; tGC group). Treatment with MOL3 or the diluent only was not associated with changes in blood profiles.

—Fig. 4—

3.2.2. Antifungal activity in a murine model of invasive fungal infection

MOL3 significantly reduced experimental murine infection by *C. parapsilosis*. On day 5, CFUs that were detected in the kidneys significantly decreased in the standard group that was treated with fluconazole (SG) and test group that was treated with MOL3 (TG) compared with the control group (GC; $p < 0.001$; Fig. 5B). Gomori & Grocott staining clearly revealed fungal yeast structures only in the control group (Fig. 5A), which did not occur in the groups that were treated with either fluconazole or MOL3. The histology of the kidneys, revealed by H&E staining, in mice in the GC (Fig. 5C), GP (Fig. 5D), and GT (Fig. 5E) groups indicated no significant histopathological changes, with no differences in histomorphology.

—Fig. 5—

4. DISCUSSION

To our knowledge, MOL3 is the first compound that has been proven to have antifungal activity both *in vitro* and *in vivo* against protein expressed by KRE2/MNT1. Through genomic screening, the KRE2 protein was identified as a potential target for new antifungal development [17]. Although not essential, it is important for fungus survival in the host. The KRE2 model was constructed and validated [30]. MOL3 was selected by virtual screening as a potential chemical compound that is able to inhibit KRE2, which is currently undergoing the patent process. Detailed information about the MOL3 screening strategy and its chemical structure cannot be published at this time because of the ungoing patent process, but they will be disclosed in a follow-up study. In preliminary tests, MOL3 presented *in vitro* antifungal activity against three pathogenic fungi of clinical interest, including *Paracoccidioide*s spp., *Candida* spp., and *Cryptococcus* sp. (data not shown). Despite the heterogeneity of the clinical isolates with regard to various susceptibility to commercially available antifungal agents, MOL3 maintained its fungicidal effects against all yeasts tested compared with their respective controls. The significant reduction of fungal load and the proven toxicological inertia of the compound in the murine model make this small molecule a promising therapeutic candidate.

Researchers have concentrated efforts on developing small-molecule antifungal agents [31]. The compound E1210 is derived from 1-(4-butylbenzyl)isoquinoline and has been shown to have potent antifungal actions both *in vitro* and *in vivo*, in which it inhibited the biosynthesis of fungal glycosylphosphatidylinositol [32, 33]. Another small molecule, SM21,

inhibited the yeast-hyphae transition in C. *albicans* [34]. D75-4590 and its derivatives inhibited ß-1,6-glucan synthesis in different fungal species [35]. ASP2397, whose mode of action has not yet been elucidated, presented antifungal actions mainly against *Aspergillus* and *C. glabrata* [36].

In the present study, MOL3 prevented the growth of the main *Candida* species at a concentration of 32 mg/L. This effect appeared to be attributable to its action on KRE2/MNT1. At higher concentrations, the fungicidal activity of MOL3 increased, with the complete inhibition of yeast growth. *In vitro* tests further revealed its spectrum of action against the principal *Candida* species, thus demonstrating the success of our target selection. Among the different species evaluated, encouraging data were generated for *C. krusei*. Despite the lower incidence, this fungal species currently has only restricted treatment options and is difficult to control because of its intrinsic resistance to azoles [37]. *C. parapsilosis* is an epidemiologically emerging yeast and important pathogen among neonatal infections [4, 5]. In the present study, MOL3 was effective against clinical isolates of *C. parapsilosis* that presented diverse intermediate and resistant susceptibility to standard antifungal agents.

The effects of MOL3 against *C. parapsilosis* are promising and demonstrate the inhibitory actions of α-1,2-mannosyltransferase against this species, although the KRE2/MNT1 protein had been described for *C. albicans* [21]. In addition to the proven antifungal actions both *in vitro* and *in vivo*, SEM of cells that were exposed to MOL3 revealed important structural alterations that indicated cellular destruction, the loss of cellular contours, and pseudo-filamentation. KRE2-deficient strains present alterations in N-glycosylation and O-mannosylation, leading to structural impairments of the cell wall [20] and reducing virulence potential by reducing the ability of yeast to form hyphae-like structures. This is an important characteristic of MOL3's actions against *Candida* species [38].

In addition to alterations of morphological aspects of *C. parapsilosis* isolates that were exposed to MOL3, we also observed a reduction of the number of yeast cells. Taking into account that for the accomplishment of the technique the inoculum was identical, although SEM is inappropriate for cellular quantification, we observed a substantial reduction of the number of cells that were treated with MOL3 compared with the control. Fig. 2 shows representative images of the fields that were evaluated (> 50 fields). Fewer cells were also confirmed by LIVE/DEAD fluorescent staining, which also revealed cell wall alterations and metabolically inactive yeast cells that were exposed to the MFC of MOL3. Comparisons with the control indicated that the sub-MFC of MOL3 also caused these effects, but they were less pronounced. The reductions of both the cell wall and cell metabolism that were induced by

the MFC of MOL3, together with antifungal activity (Fig. 1), demonstrate the promising fungicidal profile of MOL3. Moreover, the decrease in CFW-stained cells suggests that MOL3 may damage or disrupt the cellular structure of yeast, resulting in fewer cells that retain metabolic activity (Fig. 3).

We used comparative genomics to aid in the selection of MOL3 [17, 30]. This compound presented direct actions on drug targets in yeast that are not present in humans, thus providing a broader spectrum of antifungal activity. This fact likely makes MOL3 superior to other available antifungals. The currently available drug arsenal is associated with toxicity [11], drug interactions [10], a narrow spectrum of action [12], and cross-resistance [39], which justifies the need for new compounds with antifungal properties.

MOL3 presented satisfactory results *in vivo*. The 0.025 g/kg dose-dependently reduced the number of CFUs compared with the control group, similar to the results of fluconazole treatment (Fig. 5). Treatment began 4 h after infection. Considering that MOL3 is a pure chemical compound and not a pharmaceutical formulation, we chose to use a methodology that has already been employed for small molecules [29, 34].

Toxicological inertia is another factor that should be considered. The 2×MFC did not produce significant toxicity in animals, reflected by the stability of weight gain, the preservation of renal and hepatic characteristics, and the stability of biochemical parameters (Fig. 4). Abdominal contortions and piloerection that were initially observed in the first intervals after administration are related to the intraperitoneal route of administration. The only change that was observed was in ALT activity, which may be explained by the action of the diluent. This change in ALT activity was observed in both the MOL3-treated group and control group that received only P/F-127 and DMSO.

One limitation of the present study was our difficulty in solubilizing MOL3, which prevented toxicity testing at higher concentrations. Even with the addition of chemical solvents, the concentration that we used corresponded to the safe limit. We should further caution that our results are preliminary. Another challenge was the visual and spectrophotometric MIC readings; therefore, we instead calculated the MFC. We are planning further chemical enhancements of this molecule to increase water solubility in an effort to either negate or minimize the necessity of diluents.

We also seek to perform studies to determine the pharmacokinetic profile of this new antifungal compound. The present results elucidate the mechanism of action of MOL3 and indicate that the virtual screening was successful. Nonetheless, the present findings need to be confirmed by other research strategies, such as those that evaluate the effects of MOL3 on

macromolecular synthesis (e.g., experiments performed by Kitamura [40]) and effects on KRE2 mutants. Further studies should also seek to develop derivatives that have better antifungal activity.

5. CONCLUSION

In summary, MOL3 exerted promising antifungal effects. We found both inhibitory and fungicide effects of MOL3 against *Candida* spp. *in vitro*, specifically against *C. parapsilosis*. We also observed a significant reduction of yeast in a murine model of systemic infection *in vivo*. MOL3 was toxicologically inert in HeLa cells and in an animal model. Our findings may contribute to the development of new treatment options for IFI, with a principal focus on the treatment of *Candida parapsilosis*.

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Transparency declarations

The authors declare that there is no conflict of interest regarding the publication of this paper.

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FIGURE LEGENDS

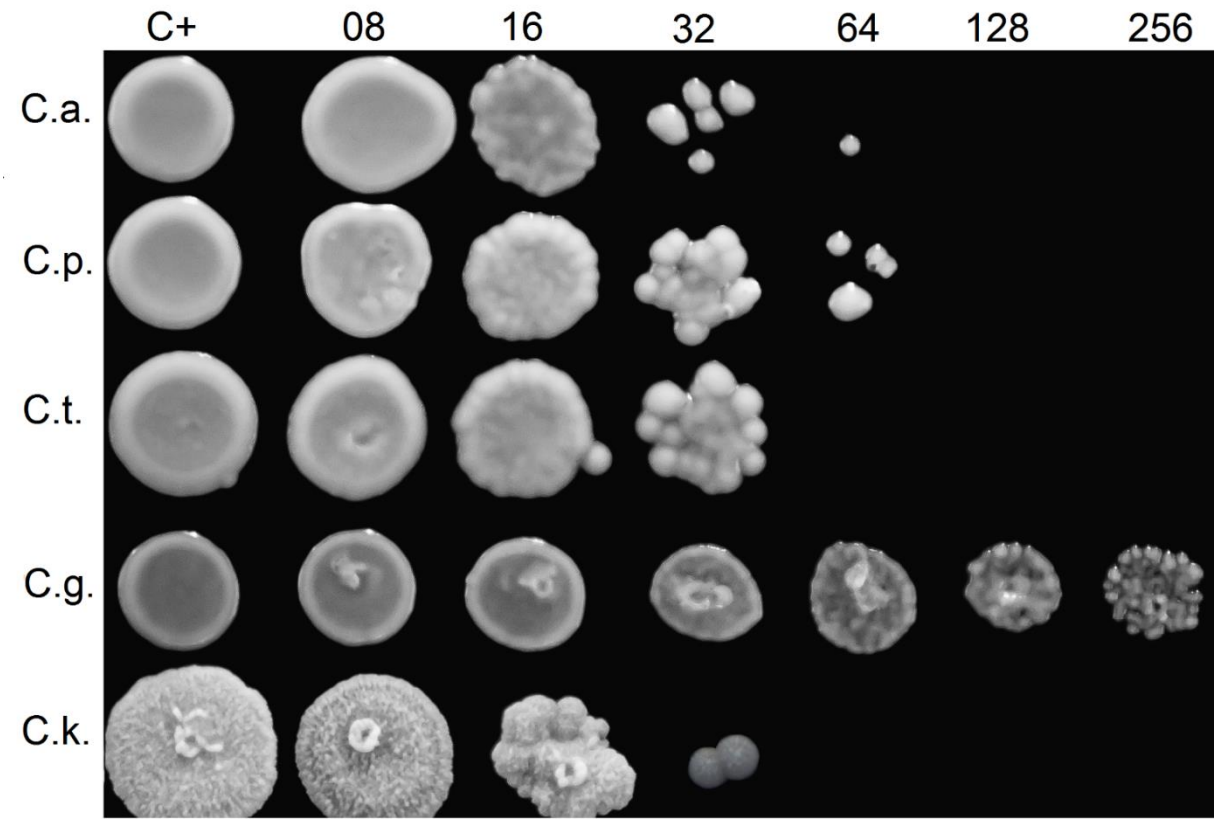

Fig. 1. Determination of minimum fungicidal concentration (MFC) of MOL3 (mg/L) against reference species of *Candida* spp. (C.a.: *C. albicans* ATCC90028; C.p.: *C. parapsilosis* ATCC22019; C.t.: *C. tropicalis* ATCC40042; C.g.: *C. glabrata* ATCC90030; C.k.: *C. krusei* ATCC40147) that were exposed to various concentrations of MOL3 (08-256 mg/L). C+, positive control (inoculum under the same conditions but without MOL3, including the diluents).

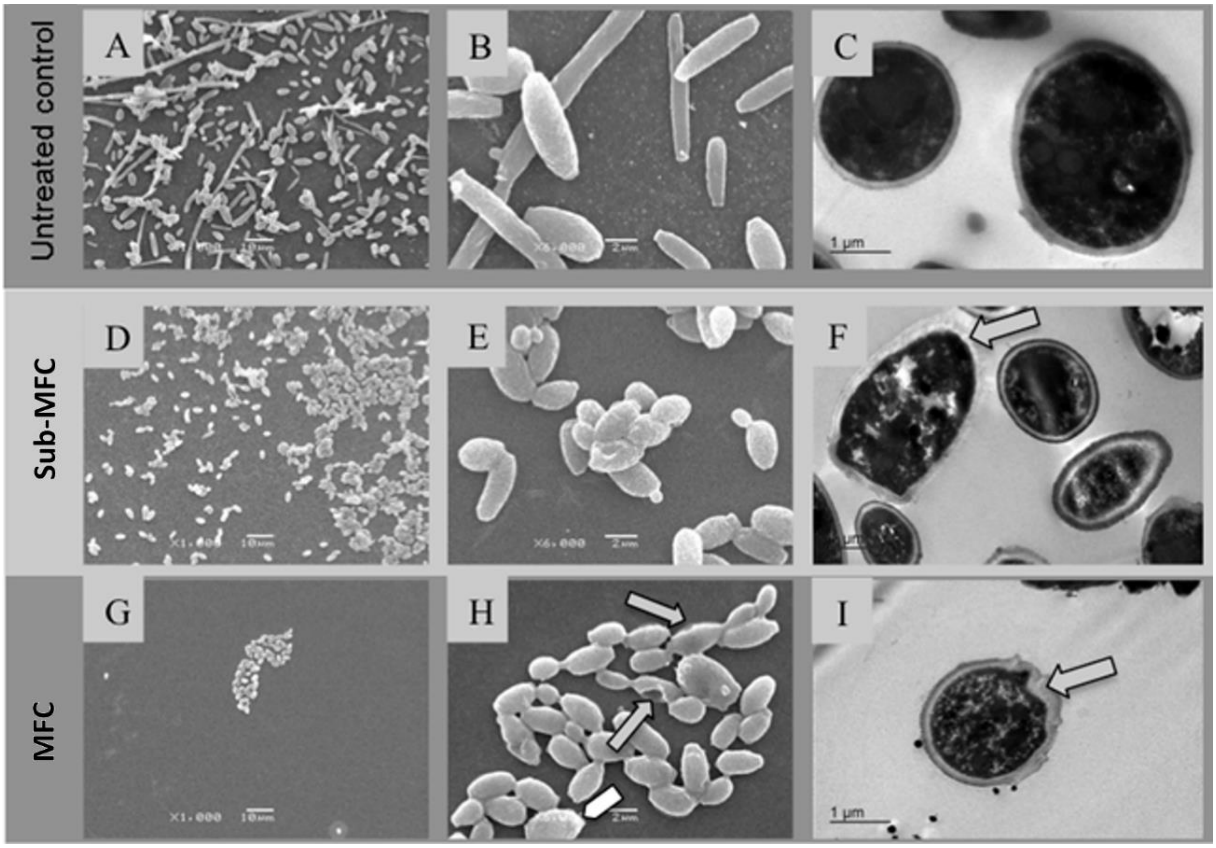

Fig. 2. Effect of MOL3 on *C. parapsilosis* cells (ATCC22019) observed by TEM and SEM (in approximately 50 fields). (A-C) Positive control (inoculum under the same conditions but without MOL3, including the diluents). (D-F) Sub-MFC of MOL3 (16 mg/L). (G-I) MFC of MOL3 (32 mg/L). Scanning electron microscopy revealed abundant blastoconidia, elongated oval cells (pseudohyphae), and a smooth and regular structure in untreated control cells (A, B). The sub-MFC of MOL3 reduced the number of MOL3 pseudohyphae and resulted in cells with a slightly irregular surface (D, E). The MFC of MOL3 caused a dramatic reduction of the number of yeast cells (G), with marked surface irregularities, convolution, and retraction (H). Transmission electron microscopy revealed cells with preserved morphological characteristics in untreated cells (C), the loss of cellular organelle outlines and necrosis in cells that were treated with the sub-MFC of MOL3 (F), and the loss of cytoplasmic structures, cell membrane invagination, and cell wall deformities in cells that were treated with the MFC of MOL3 (I).

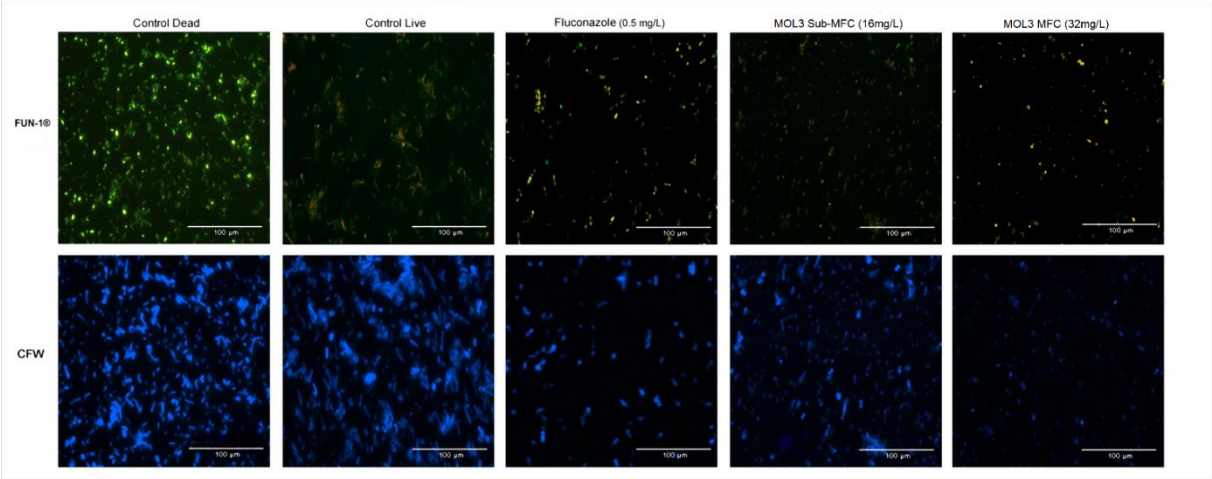

Fig. 3. Effect of MOL3 on *C. parapsilosis* cells (ATCC22019), revealed by the LIVE/DEAD Yeast Viability Kit (L7009). The fourth and fifth columns show cells from an inoculum that contained 2-3 × 10^3 yeast/ml that were exposed to the sub-MFC (16 mg/L) and MFC (32 mg/L) of MOL3, respectively. The assay was conducted according to CLSI M27-A3. The cells were incubated at 37°C for 48 h. The sub-MFC caused a slight decrease in the number of yeast, but the pattern of cellular metabolism was similar to the live control. The MFC caused a marked decrease in the number of cells, and most of the cells died. The MFC of fluconazole (0.5 mg/L) under the same conditions resulted in some dead yeast cells but also live cells.

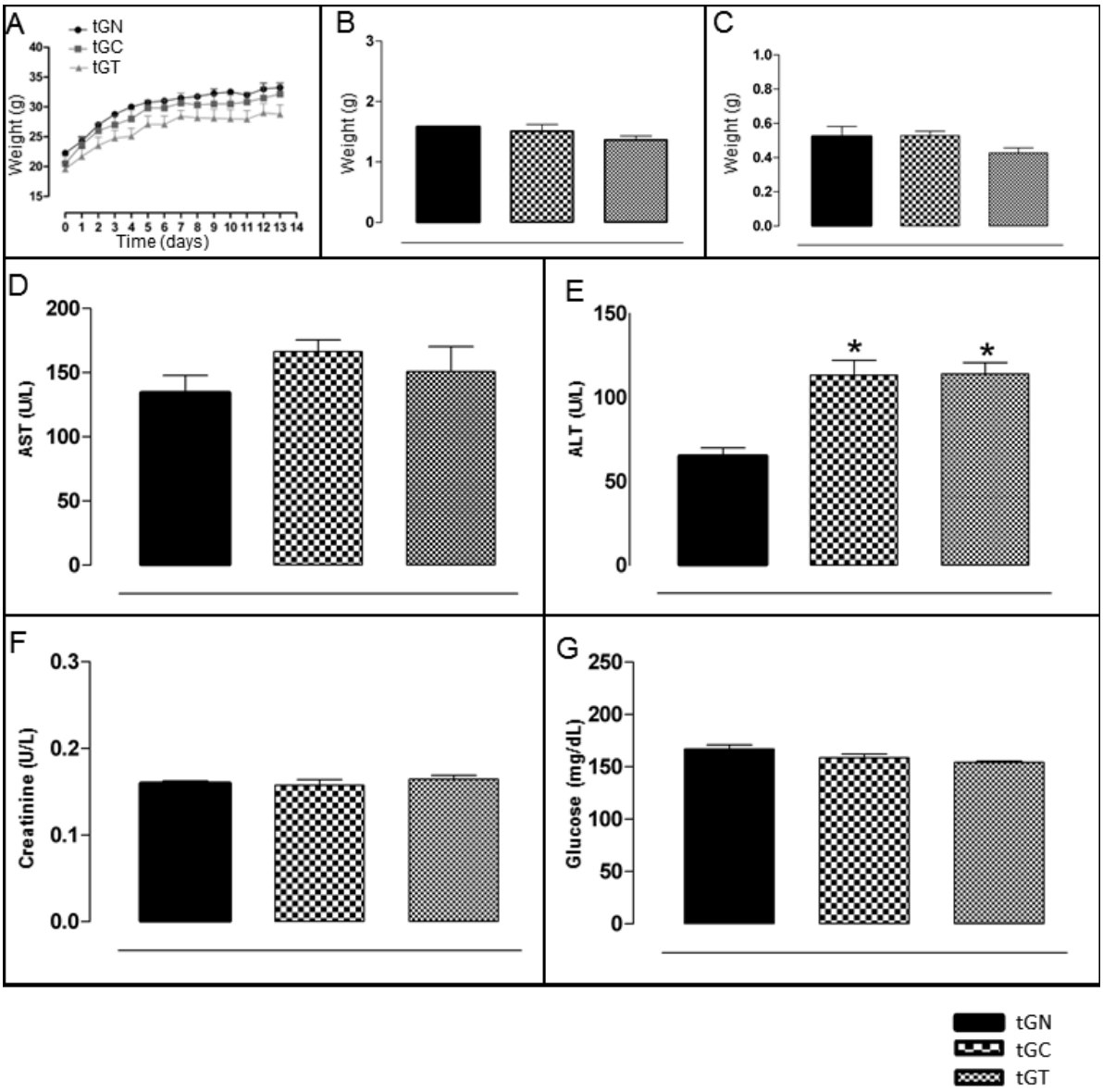

Fig.4. Acute toxicity test in mice. (A) Progressive weight evolution in the three groups. (B) Liver weight. (C) Kidney weight. (D-G) Biochemical parameters in plasma: aspartate aminotransferase (AST) activity (D), alanine aminotransferase (ALT) activity (E), creatinine (F), glucose (G). tGN, normal group; tGC, control group treated with diluent; tGT, group treated with MOL3. *$p < 0.05$ (ANOVA followed by Tukey test).

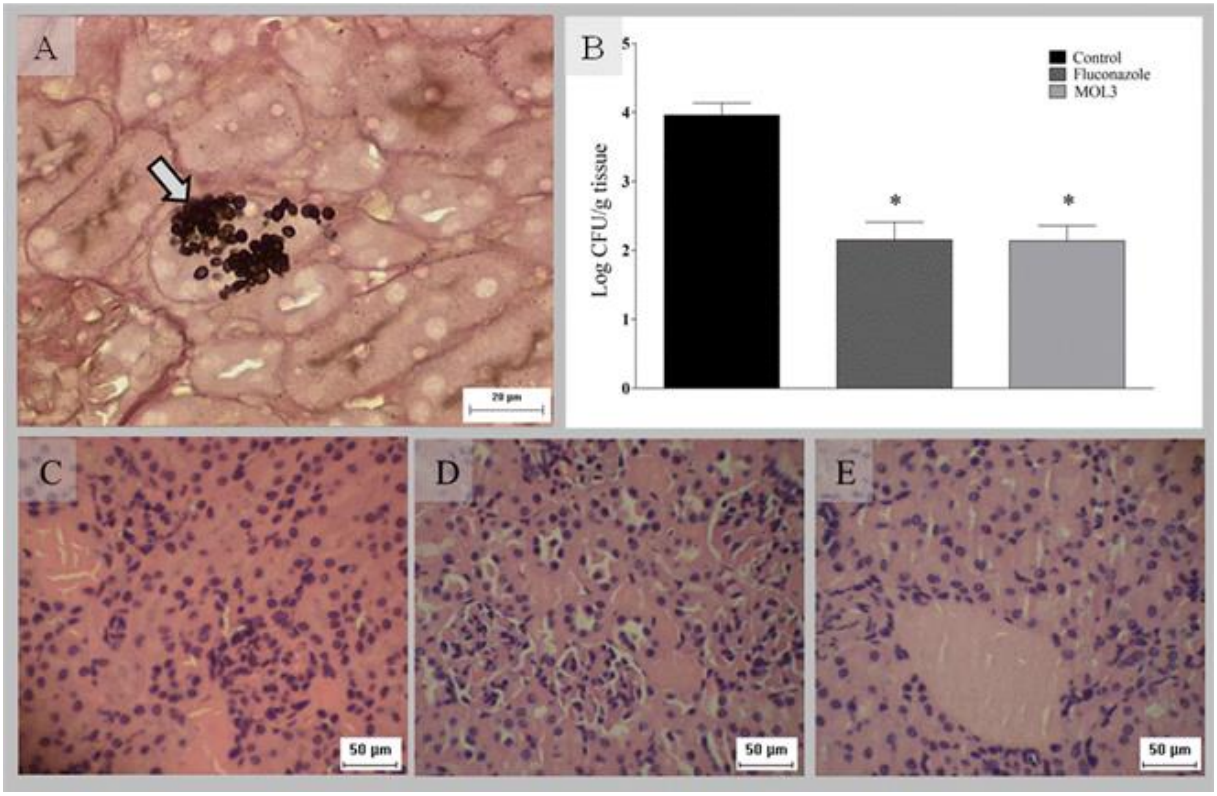

Fig. 5. Effect of systemic treatment with MOL3 on candidiasis in a murine model. Fifteen mice were infected with 1×10^7 yeast cells of C. parapsilosis (ATCC22019) and divided into three groups: TG, SG, and CG. (A) Histological section stained with Gomori & Grocott and H&E in the control group, showing the presence of yeast in the cortical region of the kidney. (B) On day 5, the number of colony forming units (CFU) in the kidneys significantly decreased in the SG and TG groups compared with the CG group (*$p < 0.05$). (C-E) Histological sections stained with H&E, showing no significant histopathological changes in the three groups: CG (C), SG (D), CG (E). CG, control group treated with PBS; SG, standard group treated with fluconazole (0.005 g/kg); TG, test group treated with MOL3 (0.025 g/kg). *$p < 0.05$ (Student's t-test).

Table 1. Susceptibility profile of 14 clinical isolates of *C. parapsilosis* against established antifungal agents and MOL3.

	MIC				MFC (mg/L)
Strains	VRC	FLC	AMB	MFG	MOL3
HUM90b	I	S	S	S	64
HUM91	S	S	S	I	128
CpHU141	I	S	S	I	64
CpHU199	S	SDD	S	I	32
CpHU201	S	S	S	I	128
CpHU302	S	S	S	S	64
CpHU307	S	S	S	S	64
CpHU313	I	S	S	S	128
CpHU325	S	S	S	I	64
CpHU335	S	S	S	S	16
CpHU357	S	S	S	I	64
CpHU358	S	S	S	I	64
CpHU359	S	S	S	I	64
CpHU363	S	S	S	R	64

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; VRC, voriconazole; FLC, fluconazole; AMB, amphotericin B; MFG, micafungin; S, susceptible; I, intermediate; SDD, susceptible dose-dependent; R, resistant [23, 24]. The MFC_{50} and MFC_{90} of MOL3 were 64 and 128 mg/L, respectively.

CAPÍTULO III

CONCLUSÕES

As publicações referentes aos possíveis alvos de *Candida* mais descritos na literatura foram revisadas para permitir uma visão do conhecimento gerado até o presente momento. Esse compêndio apoiará o direcionamento de novas pesquisas sobre opções terapêuticas com ação antifúngica.

O composto MOL3 apresentou efeito antifúngico promissor contra as principais espécies de *Candida* patogênicas, incluindo a ação sobre cepas de *C. parapsilosis* isoladas de pacientes hospitalizados.

Os estudos *in vivo* provaram que MOL3 é capaz de solucionar infecção sistêmica por *C. parapsilosis* ao tratar com sucesso camundongos infectados por *C. parapsilosis*.

MOL3 apresentou biocompatibilidade, o que foi comprovado pela ação antifúngica *in vivo* e também pela ausência de toxicidade nos estudos *in vitro* e *in vivo*.

Estudos de simulações de encaixe molecular por varredura virtual mostraram-se promissores para o desenvolvimento de novos antifúngicos, devido sua eficácia em selecionar compostos capazes de bloquear alvos específicos. Além disso, apresentam custo e tempo reduzidos quando comparados aos métodos clássicos de seleção de drogas. Nesse estudo, foi comprovada a eficácia da seleção de composto (MOL3) capaz de bloquear alfa-1,2-manosiltranferase como um promissor agente para tratamento de candidíase invasiva, especialmente as causadas pela espécie emergente *C. parapsilosis.*

PERSPECTIVAS FUTURAS

Frente aos promissores resultados obtidos com MOL3, os próximos passos da investigação contemplarão as possíveis modificações moleculares para otimização do hit, visando melhorar as questões de solubilidade, aprimoramento da ação antifúngica e do perfil farmacocinético. Além de estudos para avaliar a atividade antifúngica de MOL3 frente a outros fungos patogênicos como os gêneros *Paracoccidioides* e *Cryptococcus*, por exemplo.

Além disso, novas possibilidades de compostos poderão ser selecionadas tendo como modelo o sucesso da busca pela MOL3. Buscar, por reposicionamento *in silico* de fármacos aprovados para o tratamento de diversas doenças, que possam inibir a atividade da alfa-1,2-manosiltransferase para o tratamento de infecções por *Candida*.

Explorar alvos e aspectos que não tenham sido contemplados na literatura utilizando o modelo empregado para a seleção de MOL3 para bloqueio da ação de alfa-1,2-manosiltransferase.