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ISIS REGINA GRENIER CAPOCI

Otimização de compostos inibidores da tioredoxina redutase de *Candida albicans* para o desenvolvimento de novos antifúngicos

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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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Otimização de compostos inibidores da tioredoxina redutase de *Candida albicans* para o desenvolvimento de novos antifúngicos

RESUMO

Nas últimas décadas as doenças fúngicas invasivas (DFI) destacaram-se mundialmente como uma das principais causas de infecção em humanos, principalmente em pacientes imunocomprometidos. Neste contexto, as infecções por *Candida* spp. tornaram-se uma das etiologias mais comuns com quadros de alta gravidade e *Candida albicans* continua sendo a espécie mais prevalente. Porém, outros gêneros de fungos como *Paracoccidioides* e *Cryptococcus* também podem ser responsabilizados pelas DFI. Considerando as restritas opções de antifúngicos comercialmente disponíveis, toxicidade/efeitos colaterais e o aumento de cepas resistentes, o desenvolvimento de novos fármacos se faz necessário. Estratégias objetivando diminuir custos e tempo na produção de novos medicamentos, comparado com as tradicionais, têm sido amplamente investigadas, e avanços nas técnicas computacionais e *hardware* têm habilitado métodos *in silico* para acelerar a identificação e otimização de possíveis novos fármacos. Recentemente em nosso grupo, utilizando o modelo da tioredoxina redutase de *C. albicans* e a varredura virtual em quimiotecas, foram selecionados onze compostos que interagem com esse alvo. De acordo com o primeiro *screening in vitro* de atividade antifúngica, dois deles (LMM5 e LMM11) foram selecionados com melhor atividade contra *C. albicans*. O presente trabalho objetivou utilizar técnicas *in vitro* e *in vivo* para caracterização da atividade antifúngica em *C. albicans* desses dois compostos inéditos. Além disso, foi realizada a otimização *in silico* de LMM5 e LMM11 para a obtenção de novos candidatos ao desenvolvimento de drogas antifúngicas direcionadas para o tratamento de doenças fúngicas invasivas. Com os resultados obtidos através dessas metodologias, três artigos foram gerados. O primeiro demonstrando a eficiente atividade fungistática de LMM5 e LMM11 contra *C. albicans*, apresentando baixa citotoxicidade e diminuição da carga fúngica em modelo experimental de candidíase sistêmica murina. O segundo trabalho utilizou abordagem *in silico* de reposicionamento de drogas complementados por ensaios *in vitro* e *in vivo*, resultando no antiviral raltegravir com promissora atividade antifúngica para tratamento de paracoccidioidomicose. Também com atividade anti-*Paracoccidioides*, no terceiro artigo, apresentamos os compostos ebselen e butein, que foram obtidos *in silico* por semelhança de sitio de ligação com a tioredoxina redutase de *P. lutzii* e confirmados através de ensaios *in vitro*.

Palavras-chave: Doenças fúngicas invasivas. Tioredoxina redutase. Antifúngicos. Metodologias *in silico*. Ensaios *in vitro*. Ensaios *in vivo.*

Optimization of the thioredoxin reductase inhibitor compounds from *Candida albicans* for the new antifungal agents development

ABSTRACT

Over the past decades, invasive fungal diseases (IFD) have emerged as one of the main causes of human infection worldwide, mainly in immunocompromised patients. In this context, *Candida* spp. have become of the most common etiologies in more severe cases, and *Candida albicans* remains the most prevalent species. Despite, other genera of fungi such as *Paracoccidioides* and *Cryptococcus* also have caused IFD. Considering the restricted options of commercially available antifungal, toxicity/side effects and the increase of resistant strains, the development of new drugs is necessary. Strategies aimed at reducing costs and time in the production of new drugs, compared to traditional methodologies, have been widely investigated. Advances in computer techniques and hardware have allowed *in silico* methods to accelerate the identification and optimization of possible new drugs. Recently in our group, using the thioredoxin reductase model of *C. albicans* and virtual screening of chemical library, were selected eleven compounds that interact with this target. According to the first *in vitro* screening of antifungal activity, two of them (LMM5 and LMM11) were selected with better activity against *C. albicans*. In this sense, the present study aimed, through *in vitro* and *in vivo* assays to characterize the antifungal activity against *C. albicans* of these two unpublished compounds. In addition, *in silico* optimization of LMM5 and LMM11 to obtain new candidates for the development of antifungal drugs addressed the treatment of invasive fungal diseases.

In accordance with the results obtained through these methodologies, three articles were generated. The first study demonstrated the efficient fungistatic activity of LMM5 and LMM11 against *C. albicans*, presenting low cytotoxicity and decreased fungal burden in a murine model of systemic candidiasis. The second, used *in silico* approach of drug repositioning supplemented by *in vitro* and *in vivo* assays, resulting in antiviral raltegravir with promising antifungal activity for treatment of paracoccidioidomycosis. Also with anti-*Paracoccidioides* activity, in the third article, we present the compounds ebselen and butein, which were obtained *in silico* by similarity of ligand binding site with thioredoxin reductase from *P. lutzii* and confirmed by *in vitro* assays.

Key-words: Invasive fungal diseases, Thioredoxin reductase, Antifungals, *In silico* methodologies, *In vitro* assays, *In vivo* assays

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SUMÁRIO

CAPÍTULO I

1 Introdução

1.1 Doenças Fúngicas Invasivas

As infecções causadas por fungos variam em sintomatologia e sítio de acometimento desde lesões superficiais assintomáticas localizadas até formas disseminadas que podem levar ao óbito[1]. Dentre essas infecções, destacamos as Doenças Fúngicas Invasivas (DFI). Nas últimas décadas as DFI têm despontado no cenário mundial como uma das principais causas de infecção em humanos, principalmente em pacientes imunocomprometidos ou hospitalizados, com doenças subjacentes graves[2-5], além do preocupante acometimento de pacientes pediátricos[6]. Desta forma, caracteriza um problema de saúde pública grave, associado com aumento do tempo de internação, maior morbidade e, consequentemente, altos custos para os sistemas de saúde[7,8].

As DFI podem ser divididas em duas grandes categorias: endêmicas e oportunistas[9]. As endêmicas, usualmente ocorrem em casos de reativação oriunda de contato prévio com o fungo que tem potencial patogênico, onde a suscetibilidade para a infecção é adquirida pelo ser humano ao viver em uma região geográfica que constitui o habitat natural do fungo[9]. Nesse contexto, a Paracoccidioidomicose (PCM) é umas das representantes desse grupo. Causada por fungos do gênero *Paracoccidioides* spp., infelizmente não é uma doença de notificação obrigatória, por consequência classificada como negligenciada[10], está entre as mais importantes micoses sistêmicas da América Latina, onde admite-se que 10 milhões de pessoas que vivem em regiões endêmicas rurais já entraram em contato com o fungo[11]. A PCM acomete principalmente trabalhadores rurais de baixa renda com acesso limitado aos serviços de saúde, em sua fase mais produtiva da vida, o que aliado aos efeitos indesejáveis causados pela doença que acabam por limitar o trabalhador, os impactos sociais e econômicos da doença são negativos, representando um problema de saúde pública[12,13]. O Brasil é o país com o maior número de casos reportados e tem se destacado como a oitava causa de mortalidade por doença predominantemente crônica ou por reativação, entre as infecciosas e parasitárias, e a mais elevada taxa de mortalidade entre as micoses sistêmicas[14].

O indivíduo que se encontra com estado imunológico comprometido, originado, por exemplo, por transplantes, doenças autoimunes, oncológicas e hematológicas, uso de drogas de amplo espectro, quimioterapia, infecção por HIV, procedimentos médicos

invasivos, queimaduras graves, permanência prolongada em unidades de terapia intensiva, estão mais suscetíveis a infecções caracterizadas como DFI oportunista[15]. Consequentemente ao aumento e a diversidade desses fatores que levam a imunossupressão, a DFI do tipo oportunista tem aumentado significativamente nos últimos anos[15,16]. As manifestações clínicas são variáveis, inespecíficas e estão diretamente relacionadas com o estado imunológico do hospedeiro[15].

O gênero *Candida* spp., responsável majoritariamente por este tipo de infecção, é composto por leveduras comensais que fazem parte da microbiota humana da pele e mucosas. Essa colonização pode ser considerada pré-requisito para uma infecção invasiva subsequente, que ocorre geralmente devido um aumento dos microrganismos presentes em colonização favorecida por uma debilidade do sistema imune do hospedeiro[17]. A candidíase invasiva (CI) é considerada umas das causas mais comuns de DFI com elevadas taxas de morbidade e mortalidade (atingindo em alguns casos 70%) e altos custos de internação[18]. A CI não apresenta um padrão estabelecido de manifestações ou sítio de acometimento, uma vez que cada espécie de *Candida* apresenta suas próprias características de virulência, susceptibilidade antifúngica e potencial invasivo[17]. Globalmente, a espécie *C. albicans* é a mais isolada[17,19] e o patógeno fúngico oportunista mais difundido no corpo humano, capaz de causar desde infecções da mucosa até sistêmicas[20]. *C. albicans* é polimórfica e capaz de sofrer transições morfológicas reversíveis entre as formas de crescimento de levedura, pseudo-hifa e hifa. A forma de hifa desempenha papel fundamental no processo de infecção e está associada aos fatores de virulência como aderência e secreção de hidrolases[21]. A produção de biofilme é outro fator de virulência que favorece a infecção por *C. albicans*, uma vez que no hospedeiro pode se desenvolver facilmente nos tecidos, próteses e dispositivos médicos internos como cateteres urinários, e nesses casos, está diretamente relacionado a um alto nível de resistência ao tratamento antifúngico convencional[22,23].

A criptococose é classificada também como DFI de caráter oportunista comum e grave, principalmente para pacientes em estágio avançado da infecção pelo vírus da imunodeficiência adquirida (HIV). Estimativas sugerem que há cerca de 1 milhão de novos casos de criptococose e, pelo menos 500.000 mortes anualmente em todo o mundo devido à criptococose associada ao HIV[24]. Entre os pacientes infectados pelo HIV, especialmente em países em desenvolvimento, a criptococose tem sido uma das mais comuns infecções oportunistas e causa de mortalidade. A doença pode ser causada por

duas espécies principais de patógenos, *Cryptococcus neoformans* e *C. gattii*. Esses fungos apresentam tropismo para o sistema nervoso central causando o quadro mais grave e conhecido da doença, a meningoencefalite fúngica. A incidência global dessa manifestação foi estimada em aproximadamente 220.000 novos casos por ano, sendo que em pacientes imunocomprometidos o risco é aumentado[26]. A introdução da terapia anti-retroviral para HIV (HAART) tem contribuído para diminuir as taxas de AIDS, e por consequência, de criptococose nesses pacientes. No entanto, outros fatores predisponentes para a imunossupressão, como transplantes e o uso de drogas imunossupressoras, tem mantido elevada a casuística da infecção causada por *Cryptococcus*[27]. Além disso, *C. gattii* tem sido historicamente considerado um patógeno de regiões tropicais e subtropicais e acomete inclusive indivíduos com o sistema imunológico aparentemente normal (imunocompetente)[28-30].

1.2 Tratamento das DFI

Apesar da crescente e preocupante ocorrência das DFI comprovadas pelos estudos epidemiológicos[6,9,31], as opções terapêuticas para o tratamento efetivo dessas doenças são limitadas. Dentre as poucas classes de antifúngicos disponíveis, os azólicos são os mais frequentemente utilizados, porém tem se observado a emergência de microrganismos resistentes inclusive aos novos triazólicos de segunda geração como o voriconazol[32]. Adicionalmente existem falhas quanto ao esquema de administração destes fármacos que contribuem para o aumento nas taxas de mortalidade[33].

O antifúngico mais eficiente, que ainda é o fármaco de escolha para tratamento de infeccões fúngicas invasivas e graves, é um polieno (anfotericina B). Porém, apesar do amplo espectro de atividade, seu uso se torna restrito ao ambiente hospitalar e aos casos graves com alto risco de morte devido a sua potencial nefrotoxicidade[34,35] e toxicidade hematológica descrita desde o início de seu uso[36]. Na tentativa de amenizar esses efeitos, outras formas farmacêuticas de anfotericina B foram desenvolvidas (anfotericina B lipossomal (L-Amb), complexo lipídico de anfotericina B (ABLC))[35], porém o binômio custo/benefício não foi totalmente favorável.

Por outro lado, a classe mais recente disponível no mercado de antifúngicos, as equinocandinas (caspofungina, anidulafungina, micafungina), que atuam sobre a síntese da parede celular, sem toxicidade para o homem, apresentam boa atividade contra *Candida* spp., mas também têm sido relacionadas à resistência[37]. Assim, o surgimento de

cepas resistentes[37-39] e uma gama de efeitos adversos, fazem com que a busca por novos fármacos para o tratamento das DFI seja uma urgência.

1.3 Novas estratégias para o desenvolvimento de fármacos

No geral, o processo de desenvolvimento de novos fármacos, desde a pesquisa até a comercialização é complexo, necessita de longo tempo e os custos são altos[40]. A maioria dos medicamentos disponíveis foram descobertos a partir de observações ao acaso e/ou da busca de compostos sintéticos ou naturais e, mais recentemente por meio de técnicas de *High throughput screening* (HTS), que requer alto investimento financeiro[41,42]. Metodologias visando otimizar o processo e contornar essas dificuldades no desenvolvimento de novas drogas é de grande interesse na academia e na indústria[43].

Na busca de novas estratégias para o desenvolvimento de antifúngicos, os avanços nas técnicas computacionais e *hardware* tem habilitado métodos *in silico* para acelerar a identificação e otimização de possíveis novos fármacos[44]. Desta forma, estratégias como as baseadas em ligantes e alvos, têm se mostrado um excelente complemento às técnicas experimentais na busca de novas opções terapêuticas. A estratégia de varredura virtual de compostos ativos depositados em quimiotecas têm sido de grande interesse no aprimoramento dos processos de desenvolvimento de novos fármcos, pois reduz o tempo e investimento inicial, quando comparado aos custos de um programa de varredura HTS *in vitro*[45,46]. A principal vantagem desses estudos *in silico* é que eles permitem a rápida varredura de grandes bibliotecas de pequenas moléculas (*small molecules*), para identificar as de melhores *hits*, que podem ser compradas e/ou sintetizadas e avaliadas experimentalmente[47].

Recentemente, a estratégia de reposicionamento de drogas (*drug repositioning* ou *drug repurposing*), na qual encontra-se uma nova aplicação para drogas já aprovadas, candidatos clínicos que falharam ou que foram abandonados, ou ainda cujos alvos já foram descobertos[48,49], vem trazendo vários benefícios em relação ao desenvolvimento de um medicamento totalmente inédito para a mesma indicação[50-52]. Primeiramente, o risco de falha e do tempo para o desenvolvimento é menor devido aos estudos de segurança e eficácia em modelos pré-clínicos e/ou em humanos já estarem disponíveis, além disso, em alguns casos, o desenvolvimento de formulações já podem estar concluídos[52]. Em segundo lugar, o reposicionamento de drogas também é vantajoso no quesito de custos, onde menos investimentos são necessários, podendo variar,

dependendo do estágio e do processo de desenvolvimento que o candidato a reposicionamento se encontre[53]. Os custos regulatórios e de fase III podem permanecer mais ou menos os mesmos para um medicamento de reposicionamento e para um novo medicamento na mesma indicação. Porém, pode ocorrer economia substancial nos custos dos ensaios pré-clínicos e nas fases I e II[52]. De acordo com Nosengo[54], os custos estimados de uma droga de reposicionamento foi estimado em US $ 300 milhões, em média, em comparação com um valor estimado de US $ 2 a 3 bilhões para uma nova droga. Finalmente, as drogas de reposicionamento podem revelar novos alvos e caminhos interessantes na pesquisa que podem ser explorados[52].

Unindo a varredura virtual de quimiotecas de compostos e o reposicionamento de drogas podemos utilizar técnicas baseadas no ligante e na estrutura do alvo para a busca de novos compostos com atividade antifúngica. O princípio da técnica baseada no ligante se dá pela utilização de ligantes ativos conhecidos como molde para procurar outros ligantes ativos, assumindo que compostos quimicamente semelhantes teriam atividades biológicas semelhantes[55]. Uma vantagem desta técnica é que, independentemente da estrutura 3D do alvo, os cálculos do descritor são relativamente rápidos, acelerando a disponibilidade de novos ligantes[56].

Quando a estrutura 3D do alvo é conhecida, seja experimentalmente ou através de modelagem computacional por homologia[57], uma das técnicas mais utilizadas é a baseada na estrutura chamada *docking* molecular, que vem se tornando uma importante ferramenta para a descoberta de novos fármacos[58]. Desde o desenvolvimento dos primeiros algoritmos em 1980, a técnica de *docking* é capaz de prever com alguma precisão a melhor conformação de um composto que se ligará ao seu alvo apropriado[59].

Para que uma proteína exerça sua função em uma célula a interação com seus ligantes é de extrema importância, e geralmente esses ligantes são pequenas moléculas (*small molecules*) que se ligam a bolsas côncavas (*concave pockets*) na superfície ou cavidades das proteínas[60]. Essa ligação é diretamente importante quando envolve estudos de metabolismo, descoberta de novas drogas e reposicionamento de drogas[61]. Assim, ainda em metodologias *in silico*, o conhecimento do sítio de ligação entre a proteína e o ligante permite que novas técnicas sejam desenvolvidas no rastreio de compostos que possam ser fármacos ativos[62,63].

Todas estas metodologias têm contribuído com o processo de desenvolvimento de fármacos principalmente para doenças raras ou negligenciadas, para as quais não há

muitos investimentos das indústrias farmacêuticas. Uma vez que permite conectar dados sobre drogas, proteínas e doenças, essa variada base de dados, redes e métodos computacionais pode ser útil não só para a compreensão e identificação promíscua, polifarmacologia e mecanismos de toxicidade, mas também potencialmente para moléculas que podem ter novos usos que poderá incidir e acelerar os esforços de rastreio *in vitro* de novos fármacos[50,64].

1.4 Novo alvo seletivo para fungos: Tioredoxina redutase (Trr1)

As metodologias *in silico* tem contribuído com o processo de desenvolvimento de fármacos utilizando como alvo, preferencialmente, enzimas que fazem parte apenas de rotas metabólicas dos patógenos, o que as confere a propriedade de toxicidade seletiva[65]. Nesse sentido, a flavoenzima tioredoxina redutase (Trr1), apresenta como principal função a manutenção do estado redox da célula[66]. Esta enzima faz parte de um complexo conhecido por sistema tioredoxina (**Figura 1**), um dos principais representantes dos sistemas tiol antioxidante[67], no qual a tioredoxina (Trx) é reduzida pela flavoenzima, tioredoxina redutase (EC 1.6.4.5), utilizando o NADPH e protegendo as células contra o estresse oxidativo[68,69]. Além de sua importância na defesa contra o estresse oxidativo, o sistema de tioredoxina também está envolvido em outras importantes funções nas células, como na regulação da síntese de DNA, transcrição gênica, crescimento celular e apoptose[68].

Duas isoformas de tioredoxina redutase foram caracterizadas. A isoforma de alto peso molecular (~ 55 kDa) está presente em mamíferos e alguns parasitas, enquanto a isoforma de baixo peso molecular (~ 35 kDa) é encontrada na maioria das bactérias, plantas e fungos[70]. Mesmo diante da semelhança no que diz respeito a função das duas classes, elas apresentam estruturas protéicas distintas, consequentemente a Trr1 torna-se um alvo seletivo de fármacos para os patógenos portadores da forma de baixo peso molecular[71].

Diversos estudos têm demonstrado a importância da Trr1 como alvo para desenvolvimento de novos fármacos direcionados aos patógenos de DFI. Godoy et al.[72] descreveram a utilização da Trr1 de *C. albicans* como potencial alvo para vacina e desenvolvimento de novas drogas. Em 2015, Abadio et al.[73] realizaram a modelagem molecular da Trr1 de *P. lutzii* e através da varredura virtual de biblioteca de compostos, selecionaram compostos ativos contra a flavoenzima. Em *C. neoformans* a tioredoxina

redutase se mostrou essencial para a viabilidade do fungo, indicando também seu potencial como alvo para desenvolvimento de novos fármacos contra essa espécie fúngica.

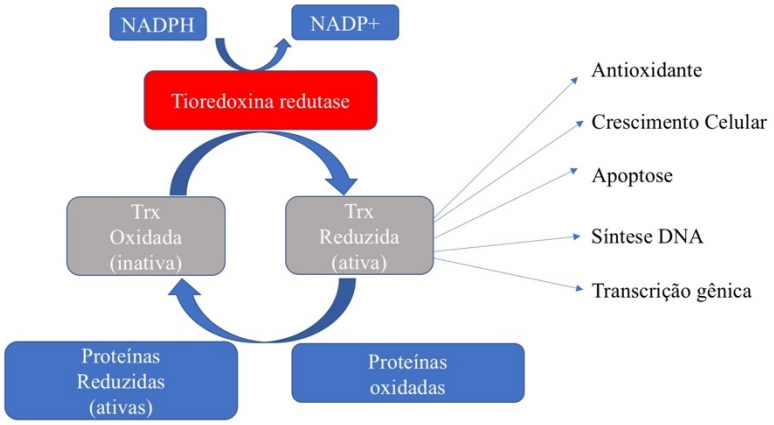

Figura 1. **Sistema tioredoxina**. A tioredoxina redutase utiliza o NADPH para catalisar a conversão da tioredoxina (Trx) da forma oxidada para a forma reduzida. A tioredoxina ativa está envolvida em diversas atividade na célula, como antioxidante, crescimento celular, apoptose, síntese de DNA e transcrição gênica. Além disso, a Trx reduzida doa elétrons para outras proteínas que se tornam ativas. Fonte: adaptado de Mustacich and Powis (2000), com modificações.

2. Justificativa

Devido à alta relevância em saúde pública e importante contribuição para pesquisa e indústrias farmacêuticas, os projetos que envolvem o desenvolvimento de novas drogas, cada vez mais, são focados na obtenção de resultados para o tratamento de espécies patogênicas humanas. A micologia médica vem ganhando cada vez mais importância no cenário mundial em reflexo ao aumento do número de doenças fúngicas invasivas. Essas doenças se tornaram um problema de saúde pública, decorrente do aumento da morbidade e mortalidade. A situação vem sendo agravada pelas mudanças no que diz respeito aos fatores que acarretam o estado de imunossupressão nos seres humanos, e que levam a uma maior chance de acometimento por esse tipo de infecção. E neste contexto, as infecções por *Candida* spp. tornaram-se uma das etiologias mais comuns com quadros de alta gravidade. Porém, outros gêneros como *Paracoccidioides* e *Cryptococcus* também podem ser responsabilizados pelas DFI. A problemática mundial de resistência aos antifúngicos comercialmente disponíveis, a incapacidade destes atuarem em alguns fungos patogênicos, bem como o grave problema de toxicidade/efeitos colaterais causados pelas drogas existentes justificam a necessidade de desenvolvimento de novos fármacos. Aliado a isso, as metodologias disponíveis para pesquisa e desenvolvimento de novas drogas são de alto custo e requerem oneroso tempo. Desta forma, alternativas para redução de custos e tempo na produção de novos medicamentos têm sido amplamente investigadas, sendo que os avanços nas técnicas computacionais e *hardware* tem habilitado métodos *in silico* para acelerar a identificação e otimização de possíveis novas drogas. Recentemente em nosso grupo, por meio de modelagem molecular a Trr1 de *C. albicans* e de *P. lutzii* foram obtidas. Através de varredura virtual em bancos de compostos químicos foram selecionados onze compostos que interagem com a Trr1 de *C. albicans*. Através de um primeiro *screening in vitro* de atividade antifúngica desses compostos, dois deles foram selecionados com melhor atividade nesse alvo. Desta forma, o presente trabalho tem como objetivo principal utilizar técnicas *in vitro* e *in vivo* para caracterização da atividade antifúngica em *C. albicans* desses dois compostos inéditos que pertencem a classe dos 1,3,4-oxadiazólicos: 4 - (N-benzil-N-metilsulfamoil) -N- (5- (4-metoxibenzil) - 1,3,4-oxadiazol-2-il) benzamida e 4- (N-ciclo-hexil-N-etilsulfamoil) - N - (5- (furan-2-il) -1,3,4-oxadiazol-2-il) benzamida, denominados LMM5 e LMM11, respectivamente, de acordo com o pedido de patente depositado em 2018 (BR 10 2018 009020 8). Além disso, otimização *in silico* desses compostos para a obtenção de novos

candidatos ao desenvolvimento de drogas antifúngicas direcionadas para o tratamento de doenças fúngicas invasivas.

3. Objetivos

3.1 Geral

Seleção de potenciais compostos com atividade antifúngica direcionados para o tratamento de micoses invasivas de relevância mundial, utilizando técnicas *in vitro*, *in vivo* e *in silico* de planejamento e otimização de fármacos.

3.2 Específicos

- Avaliar a susceptibilidade antifúngica *in vitro* de LMM5 e LMM11, previamente selecionados *in silico* contra o alvo tioredoxina redutase de *Candida albicans*, frente a cepa padrão e isolados clínicos de *C. albicans*;
- Avaliar a toxicidade *in vitro* e *in vivo* de LMM5 e LMM11;
- Determinar a curva de morte da cepa padrão de *C. albicans* frente aos compostos LMM5 e LMM11;
- Avaliar as alterações morfológicas e estruturais de *C. albicans* após exposição a LMM5 e LMM11, através da Microscopia Eletrônica de Varredura (MEV) e Microscopia Eletrônica de Transmissão (MET);
- Avaliar *in vivo* a atividade antifúngica de LMM5 e LMM11 em modelo murino de candidíase sistêmica;
- Otimizar *in silico* os dois compostos, através de técnicas baseada nos ligantes LMM5 e LMM11 e baseada na estrutura da tioredoxina redutase de *C. albicans,* com intuito de selecionar novos compostos que sejam análogos estruturais ou de reposicionamento, presentes em bibliotecas específicas de compostos;
- Utilizar técnicas *in silico* de similaridade do sítio de ligação de proteínas (modelos de Trr1 de *C. albicans* e de *P. lutzii*) para a busca de potenciais compostos com atividade antifúngica contra patógenos de DFI;
- Avaliar a susceptibilidade antifúngica *in vitro* dos novos compostos selecionados frente a fungos importantes causadores de micoses invasivas;
- Realizar testes *in vivo* com os melhores compostos selecionados.

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CAPÍTULO II

Artigo 1

Two new 1,3,4-oxadiazoles with effective antifungal activity against *Candida albicans*

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Two new 1,3,4-oxadiazoles with effective antifungal activity against *Candida albicans*

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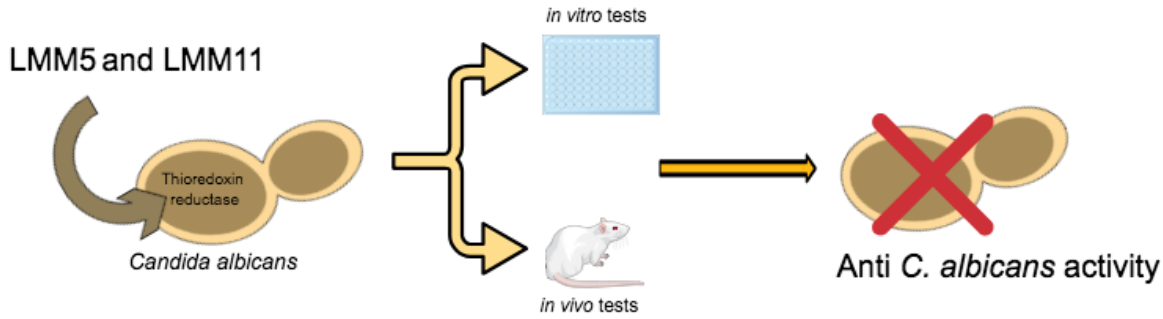
Abstract

The *Candida* infections have become a serious public health problem with high mortality rates, especially in immunocompromised patients, and the yeast *C. albicans* is the major opportunistic pathogen responsible for systemic or invasive candidiasis. Commercially available antifungal agents are restricted and fungal resistance to such drugs has increased, therefore, the development of antifungal more specific is necessary. Using assays for antifungal activity, here we report that two new compounds of 1,3,4-oxadiazoles class (LMM5 and LMM11), which were discovered by *in silico* methodologies as possible thioredoxin reductase inhibitors, were effective against *C. albicans*. Both compounds had *in vitro* antifungal activity with MIC 32 μg/mL. Cytotoxicity *in vitro* demonstrated that in MIC LMM5 and LMM11 were non-toxic in the three cell lines evaluated and *in vivo* minor alterations in parameters evaluated were observed. The kinetic of time-kill curve suggest a fungistatic profile and showed that from 12 hours the inhibitory effect of LMM5 and LMM11 can be observed, which continues at the 24 and 28 hour, being better than fluconazole. In the murine systemic candidiasis model by *C. albicans* the two compounds (5 mg/kg twice daily, intraperitoneally)

beginning 3 h post-infection significantly reduced the renal and spleen fungal burden. According to the SEM and TEM images, we hypothesized that the mechanism of action of LMM5 and LMM11 is directly related to the inhibition of the enzyme Trr1 and its effects internally in the fungal cell. In view of all the *in vitro* and *in vivo* results, LMM5 and LMM11 are effective therapeutic candidates for the development of new antifungal drugs addressed the treatment of human infections caused by *C. albicans*.

Key-words: *Candida albicans*; thioredoxin reductase; *in vitro*; *in vivo*; antifungal activity.

Graphical abstract



Highlights

- LMM5 and LMM11 have *in vitro* antifungal activity against *C. albicans*
- LMM5 and LMM11 are non-toxic for three cell lines evaluated
- Minimal cytotoxic alterations were observed in animals
- LMM5 and LMM11 reduced significantly the fungal burden in murine systemic candidiasis model
- LMM5 and LMM11 are *hits* to a future antifungal agent.

1 Introduction

Despite *Candida albicans* is found as commensal microbiota in the healthy population, this species is classified as an opportunistic fungus. Under propitious conditions, the yeast can enter the bloodstream leading to deep-tissue infections [1]. Many types of candidiasis, including systemic and invasive, have increased in last decades with high incidence in immunocompromised patients [2-4]. In retrospective analysis of the Extended Prevalence of Infection in the Intensive Care Unit Study (EPIC II), the global prevalence of candidemia was reported to be 6.9 cases per 1000 patients [5]. In Latin America, *C. albicans* is still the most commonly found species causing candidemia [6]. Therefore, infections by *C. albicans* have become a serious public health problem with high mortality rates [3,5,6].

In general, the treatment of invasive fungal infections is restricted to three classes of major antifungal agents: azoles, polyenes and echinocandins. However, these drugs present disadvantages such as toxicity, the emergence of resistance, complex drug interactions and significant limitations in spectrum of activity [6-9]. Therefore, this worrying scenario, the development of more specific antifungals is necessary.

The rational drug design has allowed the *in silico* methodologies application, to drug discovery as cost-effective alternatives [10-12]. For these approaches a promising drug target is usually an enzyme essential for the pathogen, but are absent in humans, conferring selective toxicity against infectious agent [10].

Thioredoxin reductase (Trr1) is an important flavoenzyme, which participates in important processes for cellular maintenance, protecting cells against oxidative stress [13,14]. Two isoforms of Trr1 were characterized, however, low molecular weight isoform is present only in prokaryotes, plants, some parasites and fungi [15], thus confirming a selective target for the development of new drugs.

In previous work addressed by our research group, *in silico* methodologies allowed to select two new and unpublished compounds that could be used against Trr1: 4 - (N-benzyl-N-methylsulfamoyl) -N- (5-(4-methoxybenzyl)-1,3,4-oxadiazol-2-yl) benzamide and 4-(N- cyclohexyl-N-ethylsulfamoyl) -N- (5-(furan-2-yl)-1,3,4-oxadiazol-2-yl) benzamide, that belong to the class of 1,3,4-oxadiazoles which were denominated LMM5 and LMM11, respectively [16]. Using assays for antifungal activity here, we report that these two new compounds of 1,3,4-oxadiazoles class, which were discovered by *in silico*

methodologies as possible thioredoxin reductase inhibitors, were effective against *C. albicans*.

2 Materials and Methods

2.1 Chemical Compounds

The 4 - (N-benzyl-N-methylsulfamoyl) -N- (5-(4-methoxybenzyl)-1,3,4-oxadiazol-2-yl) benzamide (LMM5) and 4-(N- cyclohexyl-N-ethylsulfamoyl) -N- (5-(furan-2-yl)-1,3,4-oxadiazol-2-yl) benzamide (LMM11) compounds were purchased from Life Chemicals (F2368-0617 and F2832-0099) and solubilized in 0.5% dimethylsulfoxide (DMSO) with addition of 0.02% nonionic surfactant Pluronic F-127 (P/F-127; Sigma). These diluents, at same concentrations were included as the control in all the experiments. Fluconazole was acquired commercially from Pfizer.

2.2 Fungal strains and growth conditions

The antifungal activity of LMM5 and LMM11 compounds was evaluated against reference strain *C. albicans* American Type Culture Collection (ATCC) 90028 and 15 clinical isolates from mycology collection of the Medical Mycology Laboratory, Universidade Estadual de Maringa, Parana, Brazil. Isolates were recovered from blood (n = 4), urine (n = 8) and catheters (n = 3). Others experiments were performed with the reference strain of *C. albicans* ATCC 90028. For each experiment, the yeasts were previously subcultured on Sabouraud Dextrose Agar (SDA, Difco^{tm}, Detroit, USA) at 35°C for 24 hours.

2.3 Antimicrobial susceptibility testing

All the isolates were tested against fluconazole and the compounds LMM5 and LMM11 according to Clinical and Laboratory Standards Institute protocol M27-A3 [17] with modifications. The LMM5 and LMM11 concentrations ranged from 0.5 to 256 µg/mL. The Minimum Inhibitory Concentration (MIC) of compounds and fluconazole was considered as the lowest concentration at which reduced 50% cell growth in relation to the positive control, through of visual and spectrophotometric readings. Three independent assays were performed.

2.4 Qualitative and quantitative analysis

For qualitative analysis of antifungal activity of LMM5 and LMM11, the minimum fungicidal concentration (MFC) was determined by inoculating of each concentration from the MIC assay into SDA plates. The plates were then incubated at 35°C for 24 h. The MFC was defined as the lowest concentration of compounds that reduced significantly yeast growth. In addition, for quantitative analysis, each compounds concentration tested were also determined the colony forming units per milliliter (CFU/mL).

2.5 Assessment of LMM5 and LMM11 cytotoxicity

2.5.1 *In vitro* cytotoxicity determination

The lineage cells, HeLa, Vero and HUVEC, were cultivated at 37ºC in a 5% CO_2 and 95% air atmosphere. For HeLa and Vero was used complete Dulbecco's modified Eagle medium (DMEM; Gibco, MO, USA) and for HUVEC, complete RPMI 1640 with HEPES buffer (Roswell Park Memorial Institute, Gibco). After confluence > 80%, the cells were trypsinized (Gibco) and the cells concentration was adjusted to 2×10^5 cells/mL in its respect medium, and the suspension was added to 96-well plates (TPP) and incubated overnight. After 24 h, the wells were washed with phosphate-buffered saline (PBS), and the cells were exposed to different concentrations of LMM5 and LMM11 (0.5–256 µg/mL) in RPMI 1640 for 24 h. The control was incubated only with RPMI 1640 or DMEM. After that, cells were washed with PBS and cytotoxicity test was performed using the Cell Titer 96 assay (Promega, Madison, WI, USA), based on the reduction of MTS in DMEM without phenol red [18]. Cytotoxicity of the compounds was evaluated as the mean of three independent experiments. The percentage of cell viability (%CV) was calculated by the following equation: $\%CV = (A_{\text{sample}}/A_{\text{blank}}) \times 100$, where blank is the medium with cells and MTS. The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (µg/mL) required for the reduction of cell viability by 50%.

2.5.2 *In vivo* cytotoxicity determination

Male BALB/c mice at 6 weeks of age were divided into four groups: Control (untreated), Diluent (treated with the PBS, DMSO and P/F-127) and treated intraperitoneally with 50 mg/kg of LMM5 and treated intraperitoneally with 50 mg/kg of LMM11. The animals were evaluated over 14 days, anesthetized and euthanized. The hematological and

biochemical examination, hippocratic screening and liver and kidneys weight and histopathological examination, were performed in accordance with Salci et al [12].

2.6 Antifungal time–kill curve

C. albicans ATCC 90028 (2-3 x 10^3 yeast/mL) was grown in RPMI 1640 medium and exposure to follow concentrations of LMM5 or LMM11: Sub-MIC (16 µg/mL), MIC (32 µg/mL) and 2xMIC (64 µg/mL). Two controls were prepared, one with only culture medium and other with fluconazole (0.25 µg/mL). At predetermined time points (0, 2, 4, 6, 8, 12, 24, 28 and 36 h), aliquots of 100 µL from each cultures were withdrawn, diluted and plated in SDA plates and were incubated at 35ºC for 24 h, and the CFU/mL was determined. Fungistatic and fungicidal activities were defined as a reduction in the number of CFU/mL, from the starting inoculum of <99.9% and ≥99.9%, respectively [19].

2.7 Ultrastructural analysis

2.7.1 Scanning Electron Microscopy (SEM)

To observe the *C. albicans* morphological changes caused by compounds, yeasts were grown (2-3 x 10^3 yeast/mL) in RPMI medium at exposure to three LMM5 or LMM11 concentrations (Sub-MIC, MIC and 2xMIC) for 24h at 35°C. Samples were adhered to the glass coverslips pre-coated with a thin layer of poly-L-lysine (Sigma Chemical Co, USA) and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, (4ºC, pH 7.0). After 24 h, the samples were dehydrated in an ethanol series (30, 50, 70, 90, and 100ºGL), critical-point dried with CO_2 (BALTEC CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a scanning electron microscope (FEI Quanta 200, Netherlands) [20].

2.7.2 Transmission Electron Microscopy (TEM)

C. albicans ATCC 90028 (2-3 x 10^3 yeast/mL) was treated with LMM5/LMM11 at MIC concentration for 24 h and then processed for transmission electron microscopy. Yeast cells were harvested, washed twice with PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Next, the cells were post-fixed in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in an increasing acetone gradient and embedded in Spurr resin (Low Viscosity

Embedding Media Spurr's Kit - #14300). Next, ultrathin sections were stained with uranyl acetate and lead citrate and images were obtained on a Zeiss 900 TEM.

2.8 Murine model of systemic candidiasis

Mouse model of systemic candidiasis was established according to a previously described method by Wong et al. [21], with modifications. For each compound twenty female BALB/c mice, at 6 weeks of age were divided in 4 groups (n=5) treated with LMM5 (5 mg/kg), LMM11 (5 mg/kg), fluconazole (5 mg/kg) or diluent (PBS, DMSO and 0.02% F-127). The systemic candidiasis model by *C. albicans* was established by administering of $5x10^5$ yeast cells (ATCC 90028) by the lateral tail vein. After three hours of infection, the respective treatments were intraperitoneally administered according to group, twice a day for a period of 5 days. The mice were euthanized and kidney and spleen were aseptically removed, weighed and then homogenized in lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol v/v, pH 8.30) and the homogenates were serially diluted before plating on SDA plates. The plates were incubated at 35ºC for 24 h, and fungal burden was expressed as the ratio of CFU/g of organs.

2.9 Histopathological analyses

The kidney and spleen were fixed in 10% formalin and then processed, preserved in paraffin, cut in 5-µm serial sections. The sections were stained by hematoxylin and eosin (H&E) and Gomori & Grocott and viewed and photographed using a binocular light microscope (Motic BA310- camera Moticam 5), at 400X and 600X magnification.

2.10 Statistical Analysis

The data were analyzed using Prism 6.0 software (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) with the Bonferroni test was used. All of the tests were performed with 95% confidence level, and $p \leq 0.05$ were considered statistically significant.

3 Results

3.1 Both LMM5 and LMM11 are promising antifungal against *Candida albicans*

Table 1 shows that the MIC values for two compounds were 32 µg/mL for the most of the *C. albicans* isolates tested. However, LMM11 exhibited more homogeneous

antifungal activity with 75% (12/16) of isolates with the same MIC value. Fluconazole had MIC of 0.125 µg/mL (12/15) and 0.25 µg/mL (3/15), and 0.25 µg/mL for reference strain. According to qualitative analysis for *C. albicans,* LMM5 (**Figure 1A**) and LMM11 (**Figure 1B**) presented fungicide a activity with MFC values starting 256 µg/mL and 64 µg/mL, respectively. Moreover, through the quantitative analysis it was possible to observe a significant reduction ($p < 0.05$) of fungal growth in MIC concentrations, correlating with the MIC values showed in **Table 1**, and for LMM11 it was possible to observe this reduction since in the subinhibitory concentration (16 µg/mL).

Table 1. Inhibitory effect of LMM5 and LMM11 and standard antifungal fluconazole against several clinical isolates of *Candida albicans*.

***Candida albicans* (16)**	**MIC (µg/mL)**		
	LMM5	**LMM11**	**Fluconazole**
10	64	32	0.125
22	8	64	0.125
36	32	32	0.125
38	32	16	0.125
40	32	32	0.125
42	16	32	0.125
43	8	16	0.250
44	16	32	0.125
45	32	32	0.125
47	32	32	0.125
50	64	32	0.250
55	16	32	0.125
58	32	32	0.250
103	16	32	0.125
107	16	64	0.125
90028[a]	32	32	0.250

Candida albicans: number of identification for clinical isolates
[a]*C. albicans* reference strain ATCC 90028
MIC: Minimum Inhibitory Concentration values are expressed as µg/mL obtained from three independent experiments.

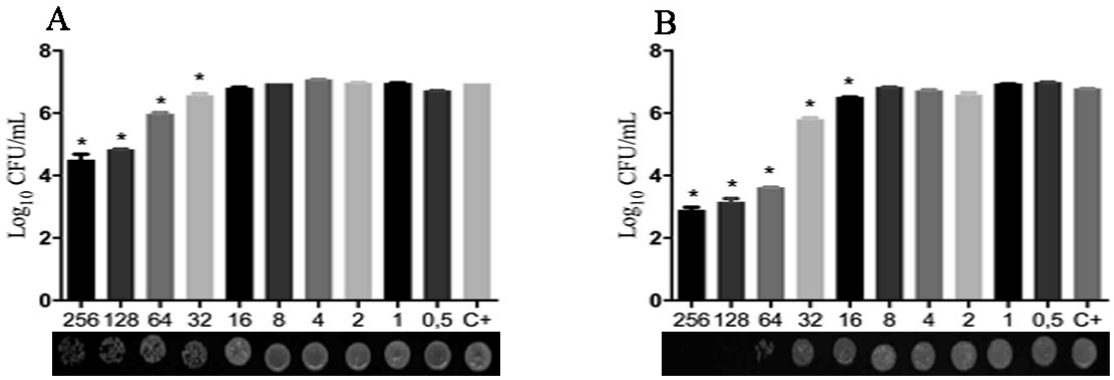


Figure 1. Quantitative and qualitative fungicide evaluation of LMM5 and LMM11 compounds against *Candida albicans.* Logarithm reduction of colony forming units and minimum fungicidal concentration (MFC) after exposure for 24 h with LMM5 (A) and LMM11 (B). C+ (Control): inoculum under the same conditions but without compounds, including the diluents. *Values of $p < 0.05$ were considered statistically significant compared with control (C+).

3.2 LMM5 and LMM11 not presented *in vitro* toxicity in three different cell lines and displays low toxicity in murine model

The *in vitro* cytotoxicity (CC_{50}) evaluation of LMM5 and LMM11 against three cell lineage is shown in **Table 2**. LMM5 was able to reduce only the HeLa viability at the highest concentration tested 256 ug/mL. LMM11 reduced at least 50% cell viability in concentrations of 256 ug/mL (Vero) and 128 ug/mL (HeLa and HUVEC). Considering that the mean MIC of these compounds was 32 ug/mL, the CC_{50} is 4- to 5- fold greater than the MIC. Diluent (DMSO + P/F-127) showed no significant toxicity in any of the evaluated parameters (data not shown).

Regarding the *in vivo* cytotoxicity assays, mild behavioral changes were observed up to 30 minutes in all groups, being more evident abdominal contortion and motor impairment. After this period, no changes were observed in any analyzed parameters. It is important to note that there was no change in the weight evolution of the animals, in the hematological profile and in the macroscopic analysis of the organs for both compounds. Regarding to biochemical parameters the values of ALT liver enzyme (**Figure 2A**) presented an increased profile in both groups of animals in comparing to control ($p < 0.05$). The activity of AST enzyme (**Figure 2B**) only increased when exposed to LMM5. According to **Figure 2E** there was change in liver weight of all treated animals,

independently of compounds or diluent ($p < 0.05$) and also in kidney of animals receiving two compounds (**Figure 2F**). There were no significant changes in the heart weight (**Figure 2G**).

Table 2. Cytotoxic concentration of LMM5 and LMM11 against three cell lines (HeLa, Vero and HUVEC) *in vitro*.

	Cytotoxic concentration (µg/mL)		
Compounds	HeLa	Vero	HUVEC
LMM5	256	*	*
LMM11	128	256	128

* : There was no significant reduction in metabolic activity at the tested concentrations.

The experiment was carried out in with three cell lines by a colorimetric assay (MTS) at 24 h, evaluating concentration range 0.5 to 256 µg/mL. The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (µg/mL) required for the reduction of cell viability by 50%.

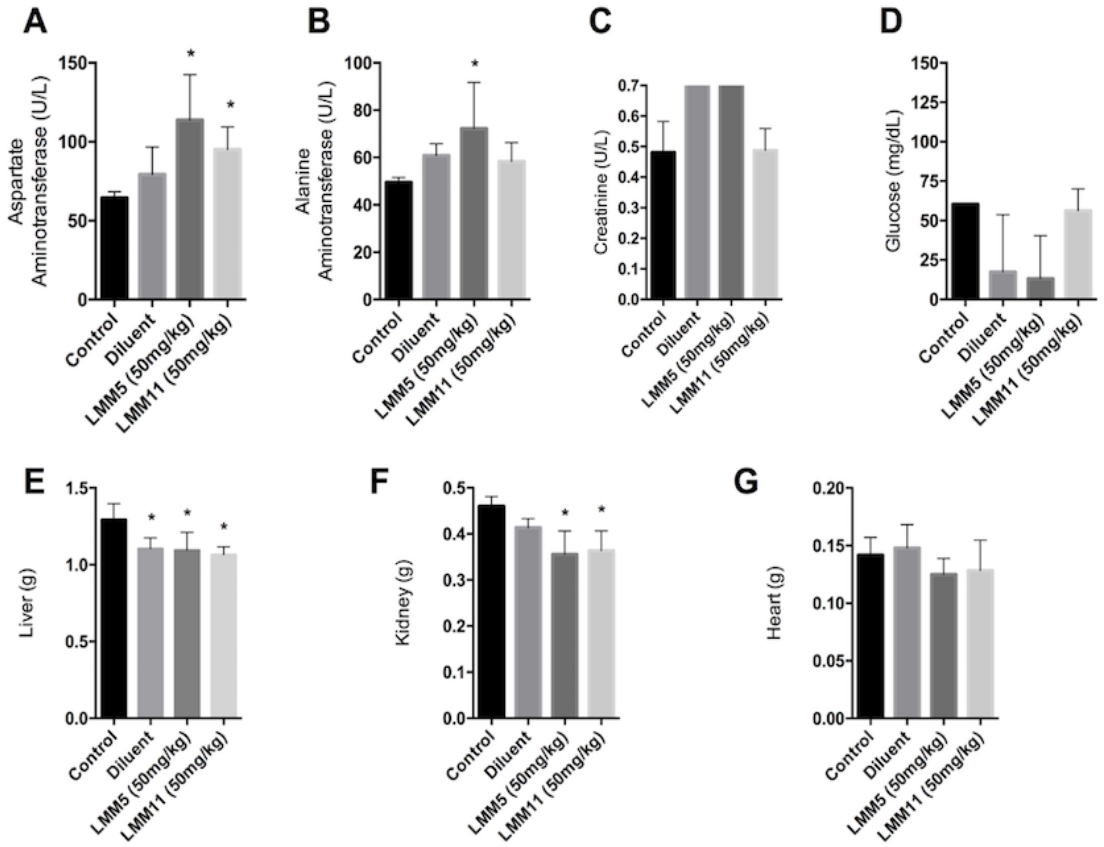


Figure 2. *In vivo* cytotoxicity of LMM5 and LMM11. This assay was performed with BALB/c male mice, infected with $5x10^5$ yeast cells (ATCC 90028) by the lateral tail vein, which were divided into four groups: Control (untreated), Diluent (treated with the diluent - PBS, DMSO and P/F-127) and one group for each compound treated with LMM5 or LMM11 (50 mg/kg) intraperitoneally. The biochemical parameters evaluated were the activity the enzymes Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Creatinine and Glucose. The weight of the liver, kidneys and heart were determined. *Values of $p < 0.05$ were considered statistically significant compared with control.

3.3 LMM5 and LMM11 are fungistatic against *Candida albicans*

The inhibitory effect of LMM5 on *C. albicans* growth was observed 12 hours after the start of incubation with the yeast comparing to the control (**Figure 3A**). This effect continued until 24 and 28 hours. For LMM11, according to **Figure 3B**, the inhibitory effect also starts with 12 hours and continue at the 28 hours, but its effect was higher than observed to LMM5, especially at the concentration of 2xMIC with reduction of

approximately two logs (log_{10}) at times 24 and 28 hours. The best effect of commercial antifungal fluconazole was observed in 24 hours in relation to the control, but the fungistatic effect was lost after 24 hours. Otherwise, the compounds appear to maintain their effect until the final time of observation. The fungistatic effect of LMM11 was better than observed to fluconazole independent of compound concentration at 28 h and 36 h. Therefore, it was possible to observe that both compounds had a better effect than fluconazole at times of 28 and 36 hours. *C. albicans* treated with LMM5 and LMM11 demonstrated endpoint activity (<99.9% reduction in numbers of CFU/ml from the starting inoculum) at different time points, suggesting fungistatic activity, as well as fluconazole.

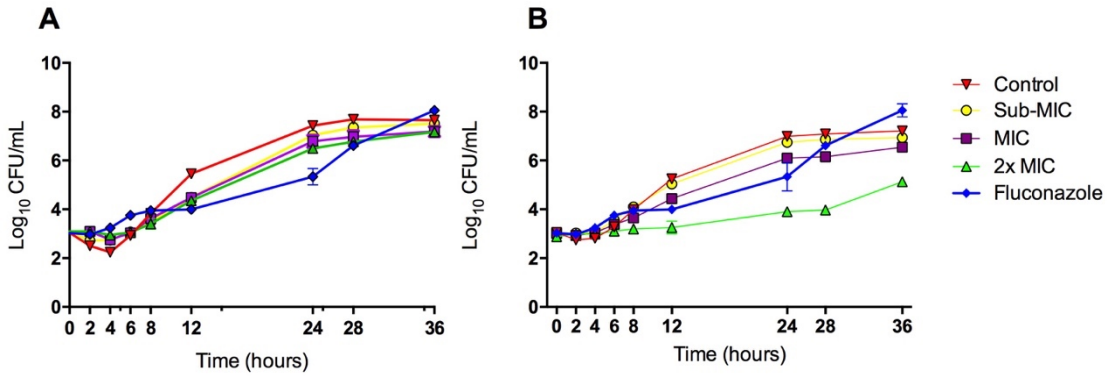


Figure 3. Killing kinetics of the LMM5 and LMM11 against *C. albicans*. Time-Kill curve of compounds: (A) LMM5 and (B) LMM11. The killing ability of the compounds was plotted from log_{10} CFU/mL versus time points. Standardized yeast cells suspensions were exposed to Sub-MIC (16 µg/mL), MIC (32 µg/mL) and 2xMIC (64 µg/mL) of LMM5 or LMM11. In time points 0, 2, 4, 6, 8, 12, 24, 28 and 36 h aliquots were diluted and plated on SDA for CFU/mL determination. Absence of compounds was used as positive control and the commercial antifungal fluconazole (0.25 µg/mL) was used for comparison. Data are representative of three independent experiments and each data point represents the mean ± standard deviation (error bars)

3.4 Ultrastructural analyzes demonstrate effective activity of LMM5 and LMM11 in *Candida albicans*

Results of SEM showed that after 24h in the presence of LMM5 or LMM11 at Sub-MIC, MIC and 2xMIC concentrations, the population of *C. albicans* declined (**Figure 4**). It was

not observed external structural changes on *C. albicans*, though proportional yeast decreased according to the increase of the compounds concentrations, clearly visualized, we suggest that the antifungal action of LMM5 and LMM11 compounds is not related to mechanisms that cause structural morphological changes. Corroborating with this observation, TEM photomicrographs (**Figure 5**) of the longitudinal and transverse sections of the untreated control cells of *C. albicans* showed that cytoplasm appeared homogeneous with a nucleus, mitochondria, surrounded by a defined cell membrane and regular cell wall. After 24 h of exposure to LMM5 and LMM11 in minimal inhibitory concentration, it was possible to observe a large number of membranous bodies, notable alterations in the cell membrane and dysfunctions of the organelles. A difference in electrodensity at the nucleus was also observed, suggesting changes in the genetic material. However, no change in cell wall was observed.

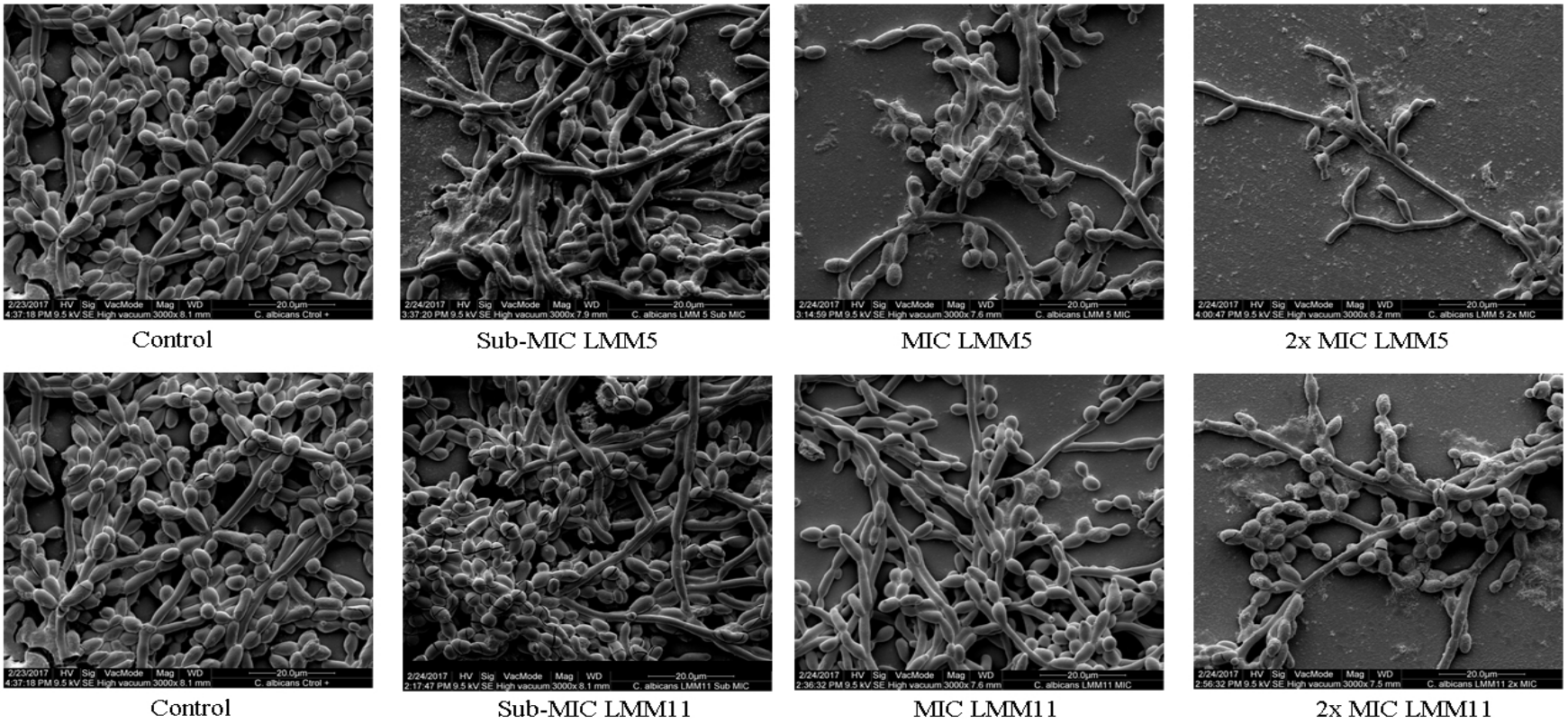


Figure 4. Scanning Electron Microscopy of *Candida albicans* reference strain after exposure to LMM5 and LMM11. Standardized yeast cells suspensions (2-3 x 10^3 yeast/mL) were exposed to the LMM5 or LMM11 for 24h/35°C in concentrations of Sub-MIC (16µg/mL), MIC (32µg/mL) and 2xMIC (64µg/mL). Control: Absence of compounds. Magnification: 3000X.

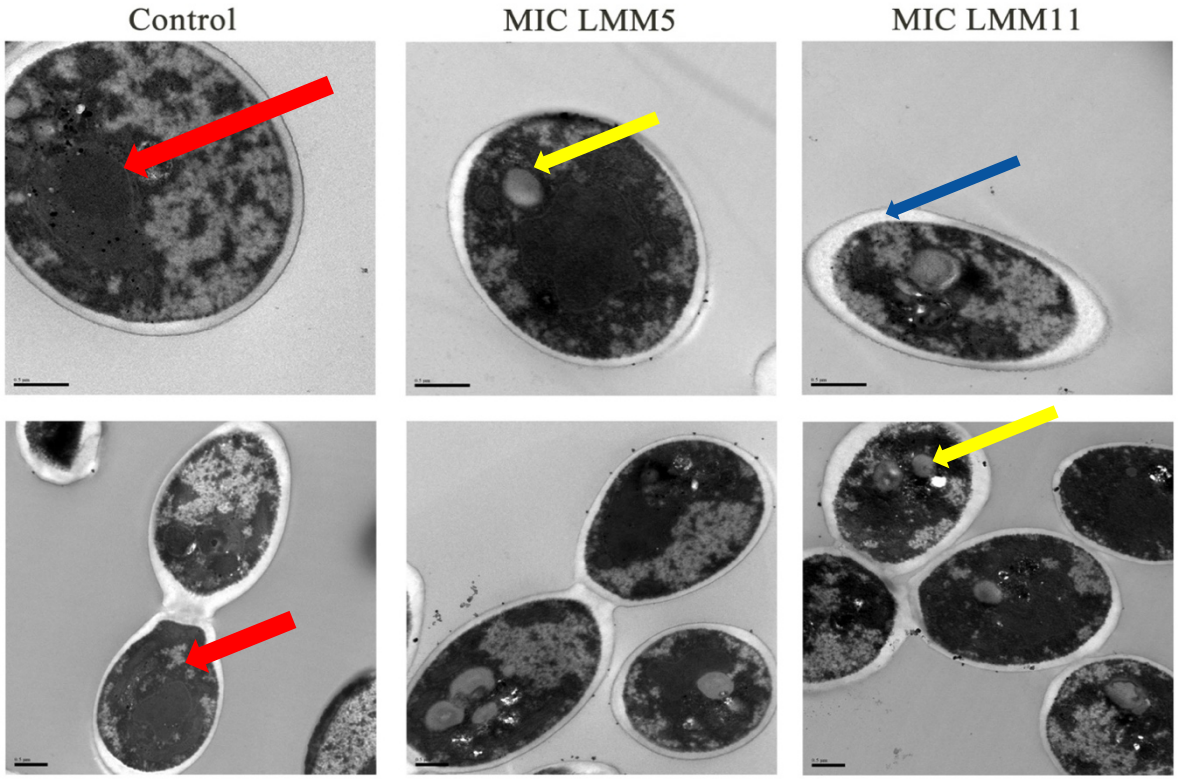


Figure 5. Transmission Electron Microscopy of *Candida albicans* reference strain after exposure to LMM5 and LMM11. Standardized yeast cells suspensions were treated with LMM5/LMM11 at 32 µg/mL (MIC) for 24 h and then processed for transmission electron microscopy. Images were obtained on a Zeiss 900 TEM. Control: Absence of compounds. Magnification: 15000x and 30000x. Red arrow: cytoplasm appeared homogeneous with a nucleus, mitochondria, surrounded by a defined cell membrane and regular cell wall; yellow arrow: large number of membranous bodies; blue arrow: alterations in the cell membrane and dysfunctions of the organelles.

3.5 LMM5 and LMM11 decreased fungal burden in murine experimental systemic candidiasis

The systemic candidiasis murine model by *C. albicans* was used for *in vivo* evaluation of antifungal activity. The treatment with LMM5 and LMM11 was able to reduce significantly the renal (**Figure 6A**) and spleen (**Figure 6B**) fungal burden in relation to control ($p < 0.05$), as well as fluconazole. In comparison between the compounds, LMM11 had a better action on inhibition of fungal burden on the kidney than LMM5 (0.5 log). The **Figure 7** shows the histopathological sections of the kidney treated with compounds and fluconazole. Analyzing histological sections stained with Gomori & Grocott, only the control group exhibited abundant presence of yeasts, as shown in **Figure 7A** and in insert with larger magnification. In the animals from groups treated with LMM5 (**Figure 7B**) or LMM11 (**Figure 7C**) and also fluconazole (**Figure 7D**), rare or none yeasts were found. In addition, the H&E staining noticed an exacerbated inflammatory infiltrate in the equivalent region where the yeasts were found in the control group (**Figure 7E**). On the other hand, no histological alterations were found on renal tissue of the animals from other groups (**Figures 7F, G and H**). Some infiltration foci were also found in other groups, but in less quantity and extent (data no show). As can be seen in **Figure 6**, the fungal burden on the spleen is less than that found in the kidney, so the histology of the spleen was not demonstrated because it did not find fungal cells in significant quantities in the histological sections.

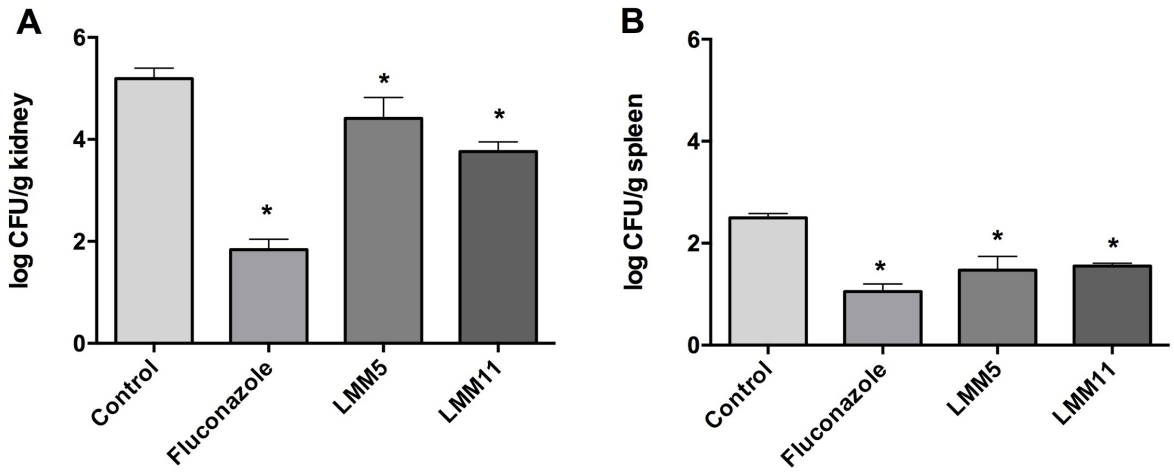


Figure 6. ***In vivo*** **evaluation of antifungal activity of LMM5 and LMM11 compounds in murine systemic candidiasis by** ***Candida albicans***. For each compound twenty female BALB/c mice, at 6 weeks of age were divided in 4 groups (n=5) treated with

LMM5 (5 mg/kg), LMM11 (5 mg/kg), fluconazole (5 mg/kg) or diluent (PBS, DMSO and 0.02% F-127). The systemic candidiasis model by *C. albicans* was established by administering of $5x10^5$ yeast cells (ATCC 90028) by the lateral tail vein. After three hours of infection, the respective treatments were administered intraperitoneally according to group, maintaining twice a day for 5 days. (A) Colony Forming Units (Log_{10} CFU) per gram of kidney. (B) Colony Forming Units (Log_{10} CFU) per gram of spleen. The bars indicate the standard deviation. *Values of $p \leq 0.05$ were considered statistically significant compared with control.

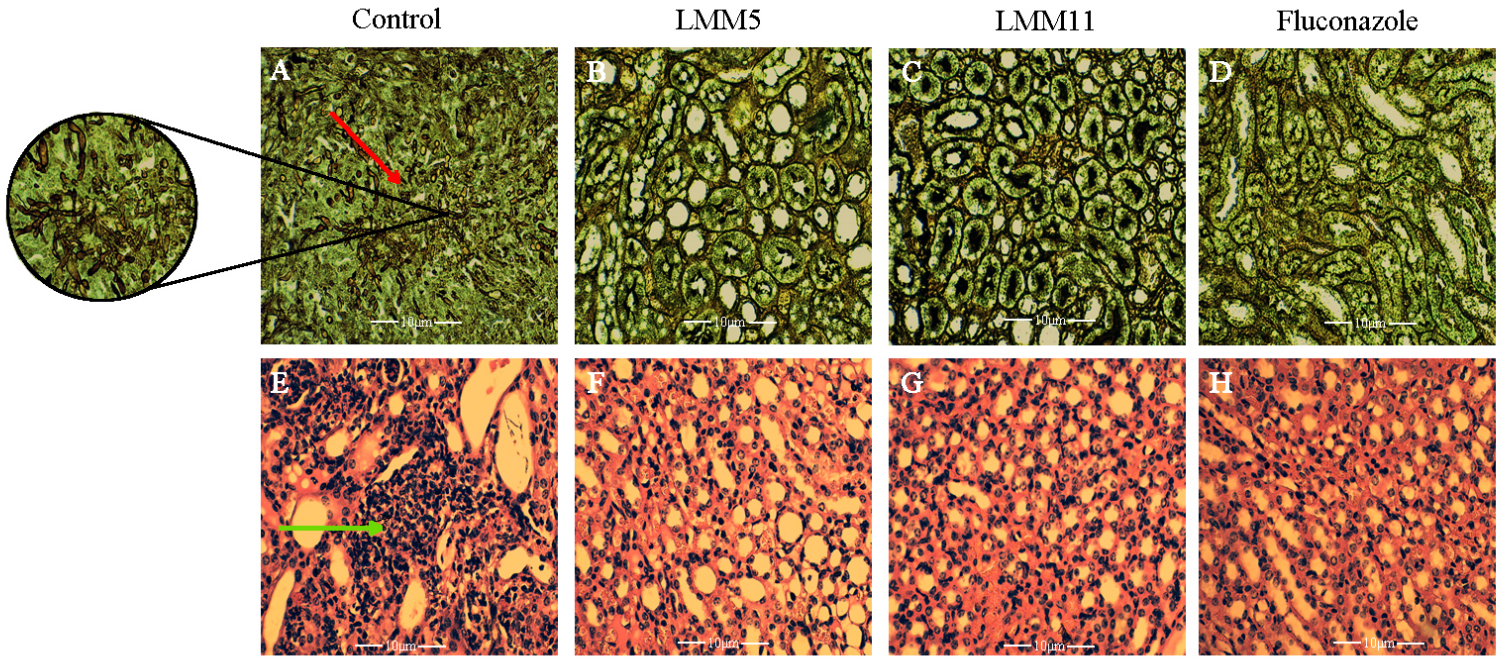


Figure 7. Histological sections of kidney of mice treated with LMM5, LMM11 and fluconazole and stained with Gomori & Grocott and hematoxylin and eosin (H&E). A-D: Gomori & Grocott; E-H: Hematoxylin and Eosin; A and E: Histological sections of kidney treated with diluent (control group) and in larger magnification the agglomerate of yeasts (red arrow) and exacerbated inflammatory infiltrate in the equivalent region where the yeasts were found in the control group (green arrow); B and F: Histological sections of kidney treated with LMM5; C and G: Histological sections of kidney treated with LMM11; D and H: Histological sections of kidney treated with fluconazole. The histopathological samples were observed and photographed using a binocular light microscope (Motic BA310- camera Moticam 5), at x400 and x600 magnification.

4 Discussion

Considering the frequency of *C. albicans* as a pathogen responsible for systemic or invasive infections and the diversity of its virulence factors, the search for new compounds and targets addressed to this species is frequent. With action in virulence factors, LaFleur et al. [22] identified small molecules with activity against *C. albicans* biofilms and Toenjes et al. [23] based in ability to inhibit the budded-to-hyphal-form transition. Acting on other mechanisms of action, Menzel et al. [24] reported satisfactory *in vitro* and *in vivo* activity of a small molecule, for treatment of invasive candidiasis. The study of Wong et al. [21] characterized novel antifungal small molecule for treating local and systemic candidiasis.

In the search for specific targets, new compounds for candidiasis treatment has emerged in patent database, as inhibitors of Chitin synthase, Glucan synthase, Mannosyl transferase and Secreted aspartyl proteases (SAPs) [25]. In this sense, Abadio et al. [10] identified potential drug targets in human fungal pathogens, among them, the Trr1, that encodes an important flavoenzyme which participates in the redox state maintenance of the cell, and the low molecular weight isoform is present only in prokaryotes, plants, some parasites and fungi [15], thus confirming a selective target for the development of new drugs.

Using an set of *in vitro* and *in vivo* assays for antifungal activity, in this study we report that LMM5 and LMM11, two inedit compounds of 1,3,4-oxadiazoles class [16], which were discovered by *in silico* methodologies as possible Trr1 inhibitors, were effective against *C. albicans*. The 1,3,4-oxadiazole is a heterocyclic compound containing an oxygen atom and two nitrogen atoms in a five-membered ring [26]. The compounds which contain this heterocyclic are widely studied by researchers because have a broad spectrum of pharmacological activities including antifungal [27], antiviral [28] and antibacterial [29].

Fungal cells present similarities with mammalian cells including cytoplasmic organelles and biosynthetic pathways making it difficult to target antifungal drugs that do not cause toxicity in humans [30,31]. Interestingly, we show that the CC_{50} values for the two compounds are at least 4- to 5-fold than the MIC found (32 μg/mL), indicating that LMM5 and LMM11 can be used as antifungal agents without causing significant toxicity in human cells at concentrations that exceed the MIC. Corroborating this result, the *in vivo* cytotoxicity in murine model with an higher dose of compounds showed only minor

alterations in biochemical parameters as well as on organ weight. These few alterations were observed in study with *C. parapsilosis* which also induced small biochemicals alterations [12].

Fluconazole is the fungistatic antifungal drug commonly used in the treatment of *Candida* infections, presenting an effective spectrum of activity against these pathogens [32], however due to its frequent use resistant strains have emerged [33,34]. A time-kill assay was employed to investigate the killing kinetics of both compounds comparing with fluconazole. LMM5 and LMM11 exhibited also fungistatic activity against *C. albicans*, nevertheless at 28 and 36 hours the compounds had better effect than fluconazole. In view of this, it is interesting to think that the activity of the compounds LMM5 and LMM11 through the inhibition of Trr1 is more efficient for the control of fungal growth than ergosterol biosynthesis pathway that fluconazole uses.

The fungistatic profile of the compounds can also be observed through the quantitative and qualitative analysis of MCF (**Figure 1**). Significant reductions in antifungal growth were found with sub-MIC and MIC concentrations, for LMM11 and LMM5, respectively.

Still demonstrating the effective and similar activity of both compounds with fluconazole, LMM5 and LMM11 administered in same dose that fluconazole [35], twice daily beginning 3 h post-infection, they were able to significantly reduced the renal and spleen fungal burden. In addition, the histological sections of kidneys showed abundant presence of yeasts only in the control group and an exacerbated inflammatory infiltrate in the same region. The models developed in mice with the direct fungus inoculation by the tail vein, mimicking the systemic infection, are widely used and considered "the Gold Standard" [12,36-38]. However, Wong et al. [21] showed the satisfactory effect of new small molecule treatment on a systemic candidiasis mouse model and the authors conclude that it is complicated to compare effective doses in different studies because there is not a standard protocol and several parameters can be altered, such as inoculum, mouse immune status, the start and interval of antifungal therapy and mouse strain. In fact, in the next works with LMM5 and LMM11, pharmacokinetic and pharmacodynamic studies should be performed to establish more appropriate doses for *in vivo* treatment, relating drug time/concentration at sites of infection. Nevertheless, as shown in the time-kill curve we can suggest a dose with effect initial from 12 hours.

Several studies have demonstrated the importance of Trr1 in pathogenic fungi of invasive diseases. Godoy et al. [39] described the use of *C. albicans* Trr1 as a potential

vaccine target and development of new drugs. In 2015, Abadio et al [11] performed the molecular modeling of Trr1 from *P. lutzii* and through virtual library scanning of compounds, selected active compounds against flavoenzyme. Thioredoxin reductase has been shown to be essential for the viability of *C. neoformans* [40]. The *in silico* methodology for inhibitors discovery of Trr1, LMM5 and LMM11, were the virtual screening, this method has been widely used to search for new compounds through a known target for various diseases [41-43]. According to the SEM and TEM images, exposure of *C. albicans* to compounds LMM5 and LMM11 are not related to structural morphological alterations, so we hypothesized that the mechanism of action of LMM5 and LMM11 is directly related to the inhibition of the enzyme Trr1 and its effects internally in the fungal cell.

This is the first screening of *in vitro* and *in vivo* assays performed for these compounds against *C. albicans*. In general, the both compounds had effective activity, however, LMM11 showed more uniform MIC values, fungistatic action with lower concentrations and greater inhibition of the fungal burden in the *in vivo* assay, so we consider that at the moment it has characteristics that make it more effective for the treatment of systemic candidiasis than LMM5. But, considering that LMM5 also presented significant results and since these compounds are still *hits*, they can be extensively modified and improved until a marketable drug is obtained.

5 Conclusion

In view of comprehensive set of *in vitro* and *in vivo* assays, LMM5 and LMM11 were active fungistatic against *C. albicans* and displays low cytotoxicity and decreased fungal burden in murine experimental systemic candidiasis. Therefore, the two compounds are effective therapeutic candidates for the development of new antifungal drugs addressed the treatment of human infections caused by *C. albicans.*

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Competing interests: None declared.

Ethical approval: All animal procedures were carried out in accordance with national regulations on animal experimentation adopted by the Brazilian Society of Laboratory Animal Science and they were approved by the Institutional Ethics Committee for Animal Experimentation of the Universidade Estadual de Maringá, Paraná, Brazil (Approval No. CEUA 9810191015, 04/22/2016)

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Artigo 2

Repurposing Approach Identifies New Treatment Options for Invasive Fungal Disease

Bioorganic Chemistry - JCR 3.92 (A2)

Repurposing Approach Identifies New Treatment Options for Invasive Fungal Disease

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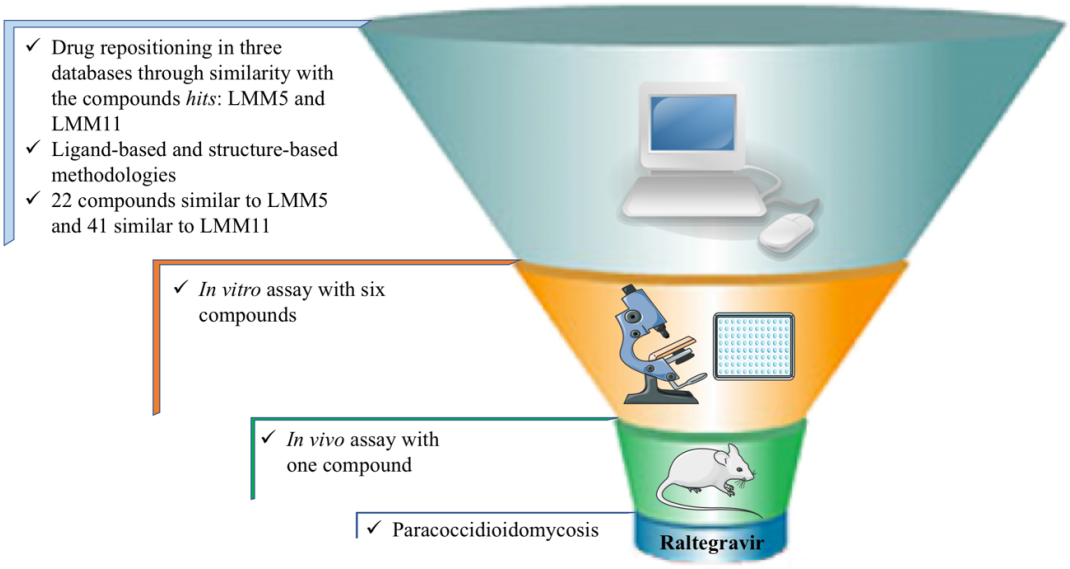
Highlights

- MDDR, DrugBank and TargetMol databases were consulted for drug repositioning.
- Combined ligand-based and structure-based methods were effective strategies for VS.
- The six compounds selected showed antifungal activity against *Paracoccidioides* spp.
- The antiviral agent raltegravir showed promising *in vivo* antifungal activity.
- The *in vivo* assay suggested that raltegravir may be an agent for PCM treatment.

Graphical Abstract



Abstract

Drug repositioning is the process of discovery, validation and marketing of previously approved drugs for new indications. Our aim was drug repositioning, using ligand-based and structure-based computational methods, of compounds that are similar to two hit compounds previously selected by our group that show promising antifungal activity. Through the ligand-based method, 100 compounds from each of three databases (MDDR, DrugBank and TargetMol) were selected by the Tanimoto coefficient, as similar to LMM5 or LMM11. These compounds were analyzed by the scaffold trees, and up to 10 compounds from each database were selected. The structure-based method (molecular docking) using thioredoxin reductase as the target drug was performed as a complementary approach, resulting in six compounds that were tested in an *in vitro* assay. All compounds, particularly raltegravir, showed antifungal activity against the genus *Paracoccidioides*. Raltegravir, an antiviral drug, showed promising antifungal activity against the experimental murine paracoccidioidomycosis, with significant reduction of the fungal burden and decreased alterations in the lung structure of mice treated with 1 mg/kg of raltegravir. In conclusion, the combination of two *in silico* methods for drug repositioning was able to select an antiviral drug with promising antifungal activity for treatment of paracoccidioidomycosis.

Keywords

drug repositioning; antifungal activity; ligand-based; structure-based; paracoccidioidomycosis; raltegravir**.**

Abbreviations

TRR1, thioredoxin reductase; LMM5, 4-(N-benzyl-N-methylsulfamoyl) -N-(5-(4-methoxybenzyl)-1,3,4-oxadiazol-2-yl)benzamide; LMM11, 4-(N- cyclohexyl-N-ethylsulfamoyl)-N-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)benzamide; DMSO, Dimethyl sulfoxide.

1. Introduction

Invasive fungal disease (IFD) is a major cause of morbidity and mortality in patients with compromised immunity [1,2]. *Candida* spp., *Cryptococcus* spp. and *Paracoccidioides* spp. are three important fungal genera that can cause IFD. All are of high clinical importance, as shown in several recent studies [3-5]. The IFD can be caused by endemic fungi, usually in cases of fungal reactivation from a previous contact.

However, especially in immunocompromised patients, this disease can also be caused by universally distributed opportunistic fungi [1,2]. Various factors contribute to immunosuppression, such as transplantation, autoimmune, oncological and hematological diseases and the use of broad-spectrum drugs [6-9]. Due to the limited currently available antifungal drugs, and the limitations on their use because of drug interactions and toxicity [10,11], new search methods are increasingly needed in order to find new compounds for treating IFD.

The recent approach of the rational drug design has already been explored by the group, especially against the target thioredoxin reductase (Trr1) [12,13]. This important flavoenzyme is involved in essentials cellular processes, as the cells protection against oxidative stress [14,15]. Therefore, this enzyme whose isoform of the low molecular mass (~ 35 kDa) is present only in plants, some parasites, prokaryotes and fungi [16], is a promising target for the new therapeutic options development for IFD treatment.

In a recent study by our group, two hit compounds that interact with the enzyme Trr1 from *Candida albicans* were selected through molecular modeling and virtual screening. These compounds showed promising antifungal activity against these three important pathogenic fungi, culminating in a patent application for this new antifungal class: 1,3,4-oxadiazoles, termed LMM5 and LMM11 [17]. However, these compounds are still hits that should be optimized and tested in several stages of evaluation, until a new drug can be made available on the market. Given the urgency of new options for the treatment of fungal infections, drug repositioning using virtual screening appears to be a viable alternative to accelerate this process.

Virtual screening of chemical libraries has been widely used in drug discovery, as a rapid and inexpensive alternative [18,19], facilitated by the rapid growth of public biological databases and web resources that provide bioactivity data for compounds and their targets available for research [20,21]. Computational methods for virtual screening are classified as (1) ligand-based drug design (e.g., ligand similarity) and (2) structure-based drug design (ligand docking) [22]. The principle of ligand-based drug design is the use of known active ligands as a template to search for other active ligands, assuming that chemically similar compounds would have similar biological activities [23]. An advantage of ligand-based design is that regardless of the 3D structure of the target, the descriptor calculations are relatively rapid, expediting the availability of new ligands [24]. In this approach, the structural, physical and chemical properties of the ligands are meticulously studied and correlated with the desired pharmacological activity [25,26].

Structure-based drug design techniques can only be applied to compounds for which the 3D structure is known, either experimentally or through computational homology modeling [27]. One of the most frequently used techniques is molecular docking, which has become an important tool for the discovery of new drugs [28]. This useful approach has been possible since the first algorithms were developed in the 1980s, and can predict with some accuracy the best conformation of a compound that will bind to its appropriate target [29].

Drug repositioning or repurposing is the strategy of developing new indications for previously approved drugs, advanced clinical candidates, or drugs for which the targets have already been discovered [30,31]. The field of drug repositioning is growing rapidly, shortening the time for identification, characterization and structural optimization for novel drug candidates, with economic benefits and expedited approval schedules [32-34] due to the reduced risk and shorter time to market made possible by the availability of preclinical data [35]. This method has been a viable alternative for the discovery of new drugs to treat neglected diseases [36,37]. Successful examples of drug repositioning have been reported [38-41]. The objective of this study was drug repositioning using virtual-screening computational methods and validation of antifungal activity, from compounds selected against important pathogens that cause IFD.

2. Methods

2.1 *In silico* approach

2.1.1 LMM5 and LMM11

The files in SMILE format (.smi) of the hit compounds: F2368-0617 (LMM5) and F2832-0099 (LMM11) were provided by the company Life Chemicals (http://www.lifechemicals.com), and the Spatial Data File (.sdf) files for PoseView and LigPlot were from the ZINC15 (ZINC000008923378 = LMM5) and ChEMBL (2093327 = LMM11) databases.

2.1.2 Databases

For drug repositioning, three databases were used: MDL Drug Data Report – MDDR (jointly produced by BIOVIA and Thomson Reuters), DrugBank (available at https://www.drugbank.ca/) and TargetMol provider (http://targetmol.com/).

2.1.3 Ligand-based drug repositioning

The Tanimoto coefficient (Tc) was used for similarity determination, in which Tc = 0 was attributed to compounds with least similarity and Tc = 1 for those with most similarity. The first step was to search in each database for the 100 compounds with greatest similarity to LMM5 or LMM11. Next, the scaffold trees were constructed using the Scaffold Hunter program [42] (http://scaffoldhunter.sourceforg e.net/) to select up to ten of the best compounds from each database. Based on the Scaffold definition: the part of a structure which remains after all terminal chains have been removed [43]. A single scaffold was defined for the compounds (LMM5 and LMM11) and the program's algorithm searched in each 100 compounds, from the database, those compounds that shared the minimum chemical structure (scaffold). The set of compounds were organized into a unique tree of hierarchy, where the scaffolds are the nodes of that tree [44]. Therefore, the compounds selected were those that within the hierarchy tree shared scaffolds with LMM5 or LMM11.

2.1.4 Structure-based drug repositioning

To complement the first method, the 600 most similar compounds to LMM5 and LMM11, 300 of each, were submitted to the docking simulation by the GOLD software [45] against thioredoxin reductase from *Candida albicans* (CaTrr1), in its reduced conformation. The CaTrr1 model was obtained by homology modeling. The compound structures were converted to three-dimensional models by CORINA [46]. For each compound, a maximum of 50 docking runs were performed; if the top 5 conformations converged within a 1.5 Å RMSD range, the docking process was suspended. To select the compounds for testing *in vitro*, a table was created for each database, containing the compounds selected by the ligand-based method, the 5 compounds with the best GOLD values, and the 5 compounds with the highest Tc values (**Supplementary material**). Compounds with GOLD values less than 60 were excluded. The interactions of the remaining compounds with the amino acids at the *Ca*Trr1 catalytic site were analyzed by the PoseView tool (http://proteinsplus.zbh.uni-hamburg.de/). These results were compared with the hit compound interactions (LMM5 or LMM11). The last analysis was performed to identify the compounds available for purchase.

The interaction of compounds selected with the *Ca*Trr1 protein was also visualized with the Visual Molecular Dynamics (VMD) program, available at http://www.ks.uiuc.edu/Research/vmd/ and the LigPlot program [47], available at https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

2.2 *In vitro* approach

2.2.1 Compounds

The compounds glyburide, gliquidone, zafirlukast, raltegravir, venetoclax and SR9243 were obtained from the company Targetmol (http://targetmol.com/). The stock solution of these compounds were prepared with dimethyl sulfoxide (DMSO) in accordance with the manufacturer's recommendations. The highest DMSO concentration used in the experiments did not exceed 0.5% (an concentration non-toxic, as described in CLSI document [48]).

2.2.2 Organisms

In vitro susceptibility tests were performed for eight pathogenic yeasts: *P. lutzii* (Pb01), *P. brasiliensis* (Pb18), *C. albicans* (ATCC 90028), *C. parapsilosis* (ATCC 22019), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258) and *C. neoformans* (INCQS). Prior to testing, each strain of *Candida* spp. was subcultured in Sabouraud dextrose agar (SDA, Difco™, Detroit, MI, USA) and incubated at 35°C for 24 h. *C. neoformans* was subcultured in Yeast Extract Peptone Dextrose Agar (YPD; Becton, Dickinson and Company, Sparks, MD, USA) at 25°C for 48 h. *Paracoccidioides* spp. isolates were subcultured in Fava-Netto medium at 36°C and used on the 5th day of culture.

2.2.3 Antifungal susceptibility assays

The Minimum Inhibitory Concentrations (MICs) of the six virtually selected compounds were determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute M27-A3 (CLSI) [48], against *Candida* isolates. The method was adapted for *Paracoccidioides* spp. and *C. neoformans* conditions as described by [18]. The tests were performed in flat-bottom 96-well microtiter plates (Techno Plastic Products, Switzerland) and the final concentrations of the compounds studied ranged from 0.5 to 256 µg/ml. Endpoint determination readings were performed visually considering 100% inhibition of growth for either *Candida*, *Cryptococcus* or *Paracoccidioides.*

2.3 *In vivo* approach

The compound raltegravir (isentress®) was used for the preliminary antifungal activity *in vivo* test. Fifteen male 6-week-old BALB/c mice were divided in 3 groups (n=5), treated with raltegravir (1 mg/kg), itraconazole (5 mg/kg) and with vehicle (PBS, DMSO). The experimental paracoccidioidomycosis model was established by

administering 1×10^6 yeast cells *(P. brasiliensis*) in 50 µl of phosphate buffered saline (PBS) by the lateral tail vein. After 24 h of infection, the respective treatments were administered intraperitoneally according to group, once a day for a period of 15 days. The mice were euthanized and the lungs were aseptically removed for histopathological evaluation, with Gomori & Grocott combined with hematoxylin and eosin (H&E). The histopathological samples were observed and photographed using a binocular light microscope (Motic BA310, Moticam 5 camera), at ×400 and ×600 magnifications. To quantify the fungal cells present in the histological sections, the lung tissue was cut sequentially, and the lung area from four sections was measured and the *P. brasiliensis* yeast cells were counted. The mean number of fungal cells found in each animal group (control or treated) was divided by the total lung area in the histological section.

2.4 Ethical approval: All procedures were approved by the Institutional Ethics Committee for Animal Experimentation of the Universidade Estadual de Maringá, Paraná, Brazil (Approval No. CEUA 9810191015, 04/22/2016). The mice were treated according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

3. Results

3.1 *Drug repositioning*

Initially, the ligand-based approaches were used to select compounds similar to LMM5 and LMM11, which are hits and showed promising antifungal activity [17], indicating that the identification of chemically related compounds would be a promising way to develop new antifungals. Accordingly, three drug databases (MDDR, DrugBank and TargetMol) were used to identify a total of 600 compounds by Tc analysis, 100 compounds from each database. Six sub-libraries containing 100 compounds were created: three for each of the hits (LMM5 or LMM11), and one from each database. The Tc values ranged from 0.26 to 0.60 (**Table 1)**. The highest Tc values were found for compounds from the MDDR database and similar to LMM11 (0.41– 0.60).

Table 1. Six sub-libraries with the Tanimoto coefficient range of selected compounds.

Compounds	Tanimoto coefficient (Tc)
Compounds similar to LMM5 from DrugBank (100)	0.30 – 0.43
Compounds similar to LMM5 from MDDR (100)	0.43 – 0.48
Compounds similar to LMM5 from Targetmol (100)	0.28 – 0.42
Compounds similar to LMM11 from DrugBank (100)	0.30 – 0.40
Compounds similar to LMM11 from MDDR (100)	0.41 – 0.60
Compounds similar to LMM11 from Targetmol (100)	0.26 – 0.38

Each of the sub-libraries was submitted to scaffold tree analysis. Among the compounds similar to LMM5, only two compounds were selected from MDDR. For DrugBank and TargetMol sub-libraries, no scaffolds were identified (**Fig. 1**). However, for similar compounds to LMM11, six compounds were chosen from DrugBank, ten from MDDR, and one from TargetMol (**Fig. 2**). Tables listing these compounds are shown in **Supplementary materials**. The compounds with the largest number of scaffolds were selected. Therefore, the scaffold tree was decisive for the choice of candidates.

The results of the ligand-based method were complemented with the docking simulation, using the sub-libraries. For the structure-based method, the target used as the three-dimensional model was thioredoxin reductase from *Candida albicans* (*Ca*Trr1), previously constructed by homology modeling. This approach was able to select the 63 best compounds that interacted with *Ca*Trr1; 22 of them were similar to LMM5 and 41 similar to LMM11 (**Supplementary materials)**. Interestingly, some compounds were selected from more than one database, for example raltegravir was selected by both DrugBank and TargetMol. This was important for the choice of compounds to be acquired. Six compounds were selected for the *in vitro* determination of antifungal activity (**Table 2**); availability for purchase was crucial for this choice. The two-dimensional and three-dimensional interaction of these compounds with *Ca*Trr1 was analyzed by the Ligplot and VMD program, respectively. As shown in **Fig. 3**, all compounds interacted with the cysteines of the *Ca*Trr1 catalytic site (Cys142 and/or Cys145).

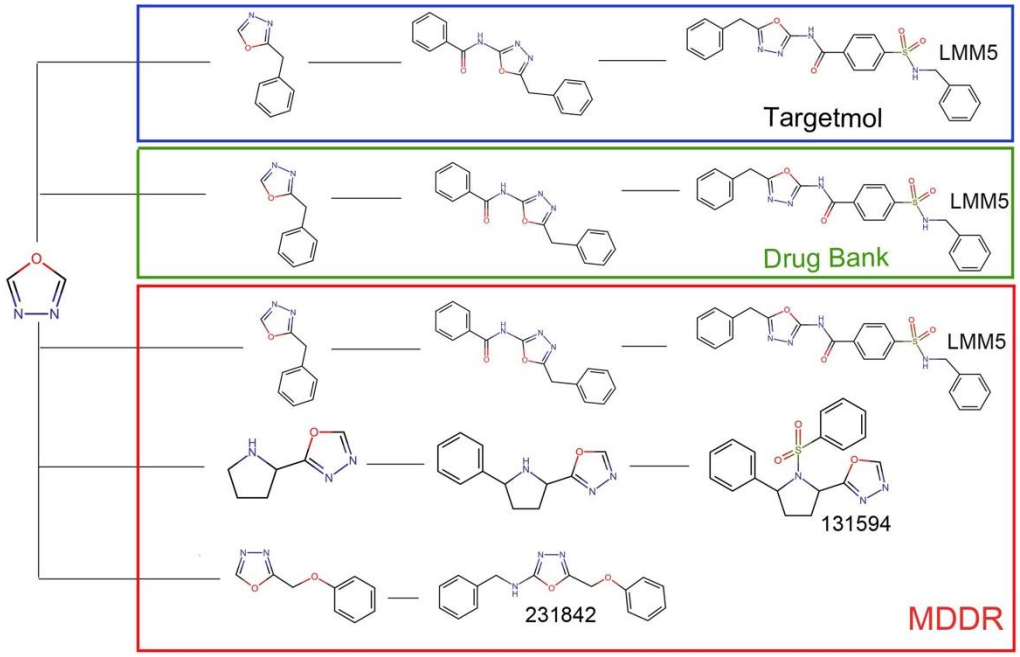


Fig. 1. Scaffold tree from the TargetMol, DrugBank and MDDR databases, for similar compounds to LMM5.

Two compounds from MDDR (131594 and 231842) were selected; for DrugBank and TargetMol, no scaffolds were identified. The scaffold trees were constructed with the Scaffold Hunter program (http://scaffoldhunter.sourceforg e.net/).

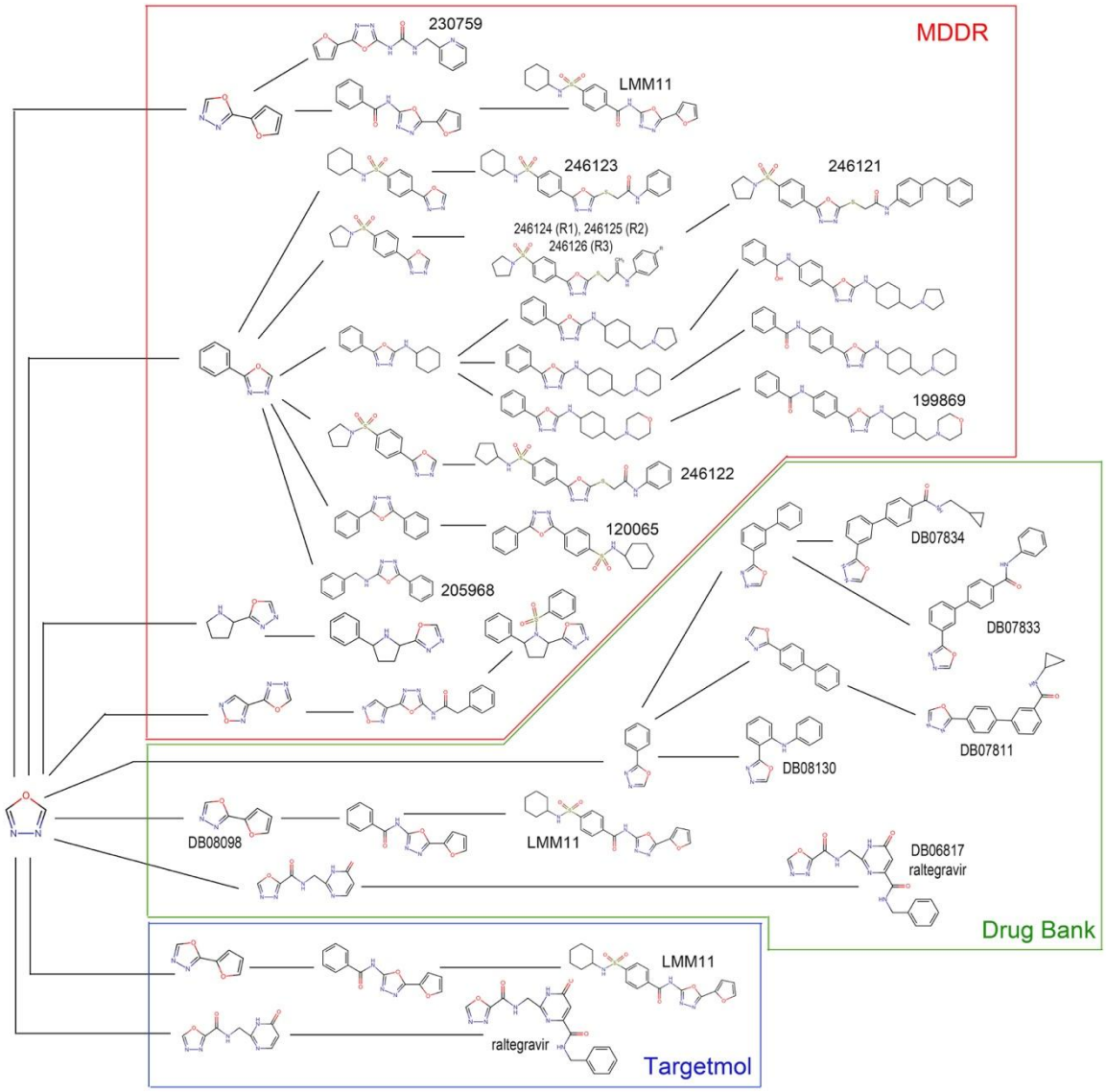


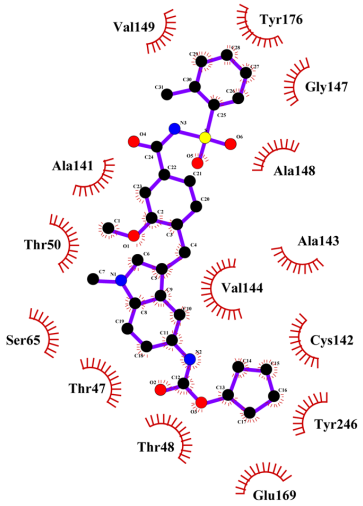
Fig. 2. Scaffold tree from TargetMol, DrugBank and MDDR databases for similar compounds to LMM11.

Six compounds from DrugBank (DB07834, DB07833, DB07811, DB08130, DB08098 and DB06817), ten from MDDR (230759, 246123, 246124 (R1:CH3), 246125 (R2:CH2CH3), 246126 (R3:OCH3), 199869, 246122, 120065, 205968 and 246121) and one from TargetMol (raltegravir) were selected. The scaffold trees were constructed with the Scaffold Hunter program (http://scaffoldhunter.sourceforg e.net/).

Table 2. Indication, GOLD values and Tanimoto coefficients of six compounds selected by drug repositioning for antifungal activity *in vitro.*

Compound	Indication	GOLD value	Tc with LMM5	Tc with LMM11
Raltegravir	For the treatment of HIV-1 infection in conjunction with other antiretrovirals	72.20	0.278388	0.306338
Gliquidone	Used in the treatment of diabetes mellitus type 2	82.96	0.387387	0.346939
Zafirlukast	For the prophylaxis and chronic treatment of asthma	80.20	0.418803	0.340909
Venetoclax (ABT-199)	For the treatment of patients with chronic lymphocytic leukemia (CLL) with 17p deletion, as detected by an FDA approved test, who have received at least one prior therapy	100.37	0.320755	0.30383
Glyburide	Indicated as an adjunct to diet to lower the blood glucose in patients with NIDDM whose hyperglycemia cannot be satisfactorily controlled by diet alone	83.78	0.425743	0.347826
SR9243	Is a LXR inverse agonist that induces LXR-corepressor interaction; shows anticancer activity and selectively targets the warburg effect and lipogenesis.	85.17	0.370730	0.306034

A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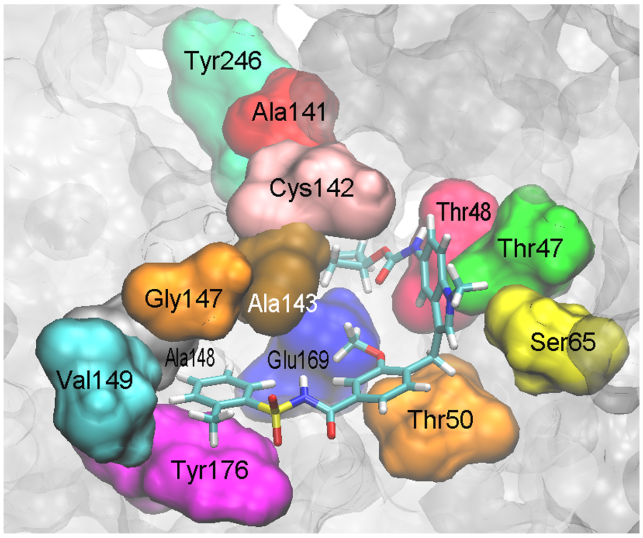
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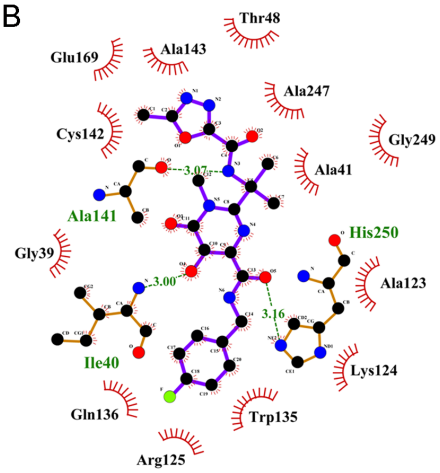
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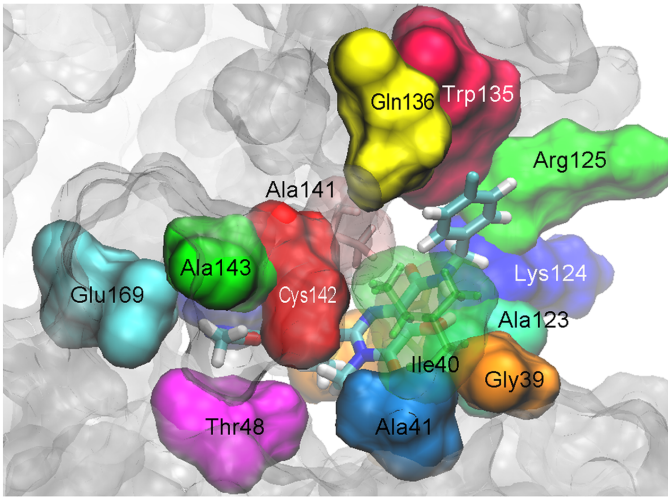
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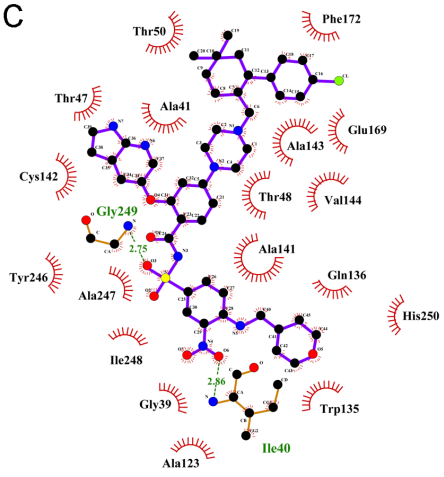
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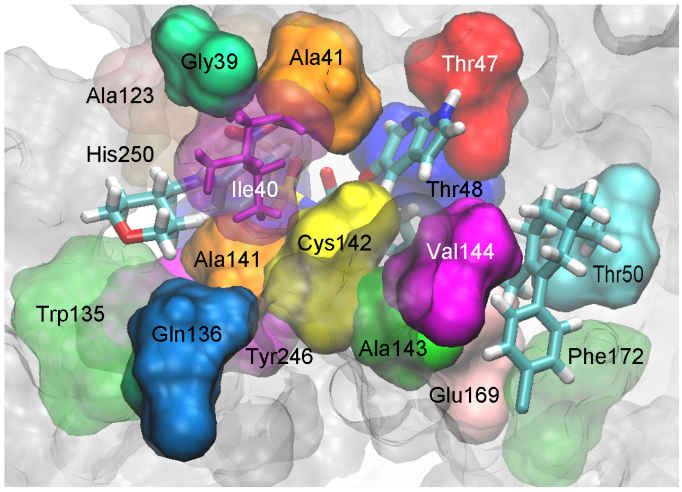
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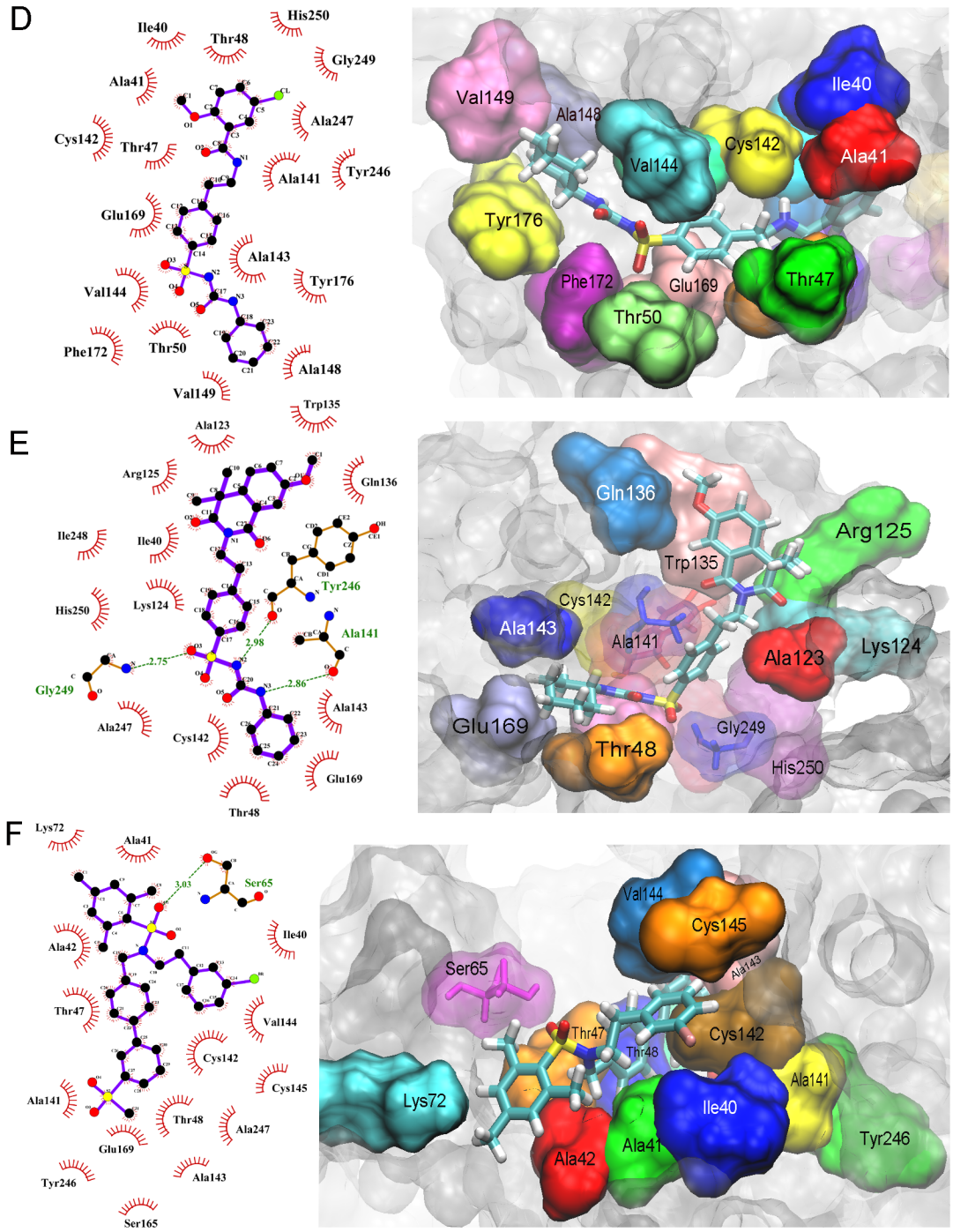

Fig. 3. Interaction thioredoxin reductase from *Candida albicans* (CaTrr1) with the six compounds selected by repurposing approach. The 2D-plot of interactions between amino acid residues of CaTrr1 and compound analyzed by LigPlot and the schematic drawing of tridimensional interaction obtained by VMD program. A: zafirlukast; B: raltegravir; C: venetoclax (ABT-199); D: glyburide; E: gliquidone; F: SR9243. All compounds interacted with the cysteines of the CaTrr1 catalytic site (Cys142 or Cys145). In tridimensional plot obtained by VMD, in the interactions mediated by hydrogen bonds

the amino acid from CaTrr1 were presented by surface combinate with licorice representation. .

LigPlot: The interactions shown are those mediated by **hydrogen bonds** and by **hydrophobic contacts**. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

3.2 *The in vitro antifungal activity*

The six compounds were tested against three important fungal pathogens, *Candida* spp., *Cryptococcus neoformans* and *Paracoccidioides* spp. All compounds tested showed antifungal activity against *P. brasiliensis* (Pb18), with MIC values ranging from 16 to 64 µg/ml. *P. lutzii* appeared to be less sensitive to the compounds selected, since only four compounds showed antifungal activity, with MIC values higher than those observed for *P. brasiliensis* (**Table 3**). Therefore, the most active compounds against the genus *Paracoccidioides* were the antiviral and asthma-treatment drugs, raltegravir and zafirlukast. Raltegravir also showed antifungal activity against *C. albicans* (MIC 128 µg/ml) and *C. glabrata* (MIC 256 µg/ml). None of the compounds showed antifungal activity against *Cryptococcus neoformans*.

Table 3. *In vitro* antifungal activity of six compounds from drug repositioning.

Isolate	**MIC (µg/ml)**					
	Glyburide	Gliquidone	Zafirlukast	Raltegravir	Venetoclax (ABT-199)	SR9243
Candida albicans	>256	>256	>256	128	>256	>256
Candida glabrata	>256	>256	>256	256	>256	>256
Candida krusei	>256	>256	>256	>256	>256	>256
Candida tropicalis	>256	>256	>256	256	>256	>256
C. parapsilosis	>256	>256	>256	>256	>256	>256
C. neoformans	>256	>256	>256	>256	>256	>256
P. brasiliensis	64	32	16	16	16	64
P. lutzii	>256	64	32	32	64	>256

3.3 Raltegravir as the promising PCM treatment

Due to the promising *in vitro* antifungal activity of raltegravir against fungi of the genus *Paracoccidioides*, this compound was selected for *in vivo* testing in the experimental model of systemic paracoccidioidomycosis. In this model, mice were infected with *P. brasiliensis* and treated for 15 days. After the treatment, the lungs of the mice were evaluated by histopathological analysis. As shown in **Fig. 4A**, the lungs of control animals clearly showed larger numbers of *P. brasiliensis* cells (red arrow) compared with the animals treated with raltegravir (1 mg/kg) or itraconazole (5 mg/kg) (**Fig. 4B e 4C**). The

lung architecture of the animals treated with raltegravir or itraconazole was preserved, with few inflammatory infiltrates and few yeast cells distributed through the lung tissue (**Fig. 4B**). On the other hand, the control group, treated only with the vehicle, showed several inflammatory infiltrates and yeast cells, easily identified by impregnation with silver (**Fig. 4A**). As observed in **Fig. 4D**, raltegravir has similar behavior to itraconazole and was efficient in significantly reducing the yeast number in the lung, compared to the control group ($p<0.05$).

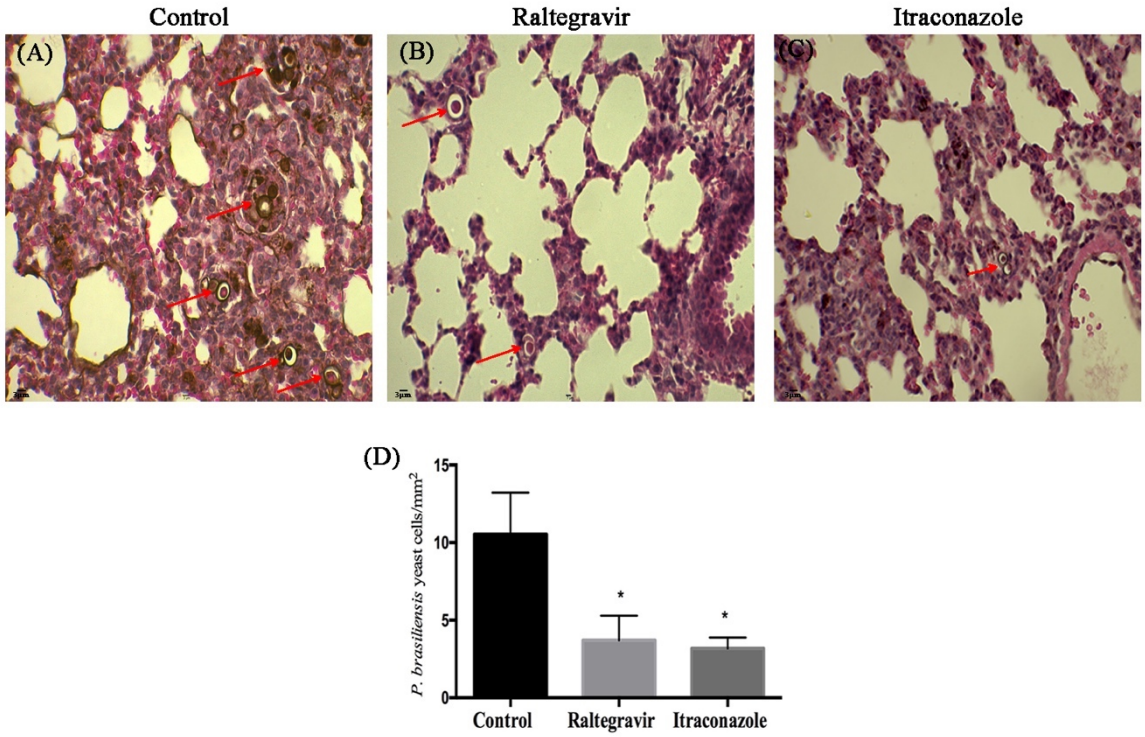

Fig. 4. Raltegravir as the efficient Paracoccidioidomycosis treatment. The mice were infected with yeast cells from *P. brasiliensis* (Pb18) by the lateral tail vein, and divided into three groups for treatment: Control (vehicle), itraconazole (5 mg/kg) and raltegravir (1 mg/kg). (A) Histological section of lung stained with Gomori & Grocott plus H&E. The control group showed many *P. brasiliensis* yeast cells (red arrow) and many changes in the pulmonary architecture. (B) The group treated with raltegravir showed few *P. brasiliensis* yeast cells (red arrow) and minor changes in the lung. (C) The group treated with itraconazole showed few *P. brasiliensis* yeast cells (red arrow) and minor changes in the lung (D) Yeast quantification in histological sections. The result shows the mean of the fungal cells found divided by the total lung area of the histological section (*P. brasiliensis* yeast cells/mm^2). *p < 0.05 (Student's *t*-test). The samples were observed and

photographed using a binocular light microscope (Motic BA310, Moticam 5 camera) at ×400 magnification.

3.4 Docking simulation against thioredoxin reductase from *Paracoccidioides lutzii* (PlTrr1)

Considering the better antifungal activity *in vitro* found for raltegravir, venetoclax and zafirlukast against *Paracoccidioides*, we performed the docking simulation using the three-dimensional model thioredoxin reductase from *P. lutzii*, previously constructed by homology modeling. **Table 4** compares the interaction these compounds with two thioredoxin reductase models from *C. albicans* (CaTrr1) and *P. lutzii* (PlTrr1).

As observed, the GOLD values of the interaction with PlTrr1 were lower than with CaTrr1, but the three compounds made strong interactions (hydrogen bonds) both at the catalytic site and in the loop region of PlTrr1 (**Supplementary materials)**. The **Fig. 5** and **Fig. 6** in detail shows the interactions of raltegravir with CaTrr1 and PlTrr1, respectively, by ligplot and VMD. Interestingly, we can observe that raltegravir present interaction mediated by hydrogen bonds (strongly) with the Cys148 residue in catalytic site of PlTrr1 (**Fig. 6C**).

Table 4. Interactions and GOLD values of three compounds with CaTrr1 and PlTrr1.

Compound	*C. albicans* model			*P. lutzii* model		
	GOLD	**Cys/Cys**	**Loop 1**	**GOLD**	**Cys/Cys**	**Loop 1**
Raltegravir	72.20	1/2	2*/10	67.34	2*/ 2	5/10
Zafirlukast	80.78	1/2	0/10	75.74	2/2	3*/10
Venetoclax	100.37	2/2	3*/10	90.99	2/2	4*/10

Cys/Cys: The number of interaction of the compound with cysteine from catalytic site. **Loop 1**: The number of interaction of the compound with amino acids from loop1, an important site for interaction with thioredoxin (substrate). * indicates that one of the interactions is the hydrogen bonding type.

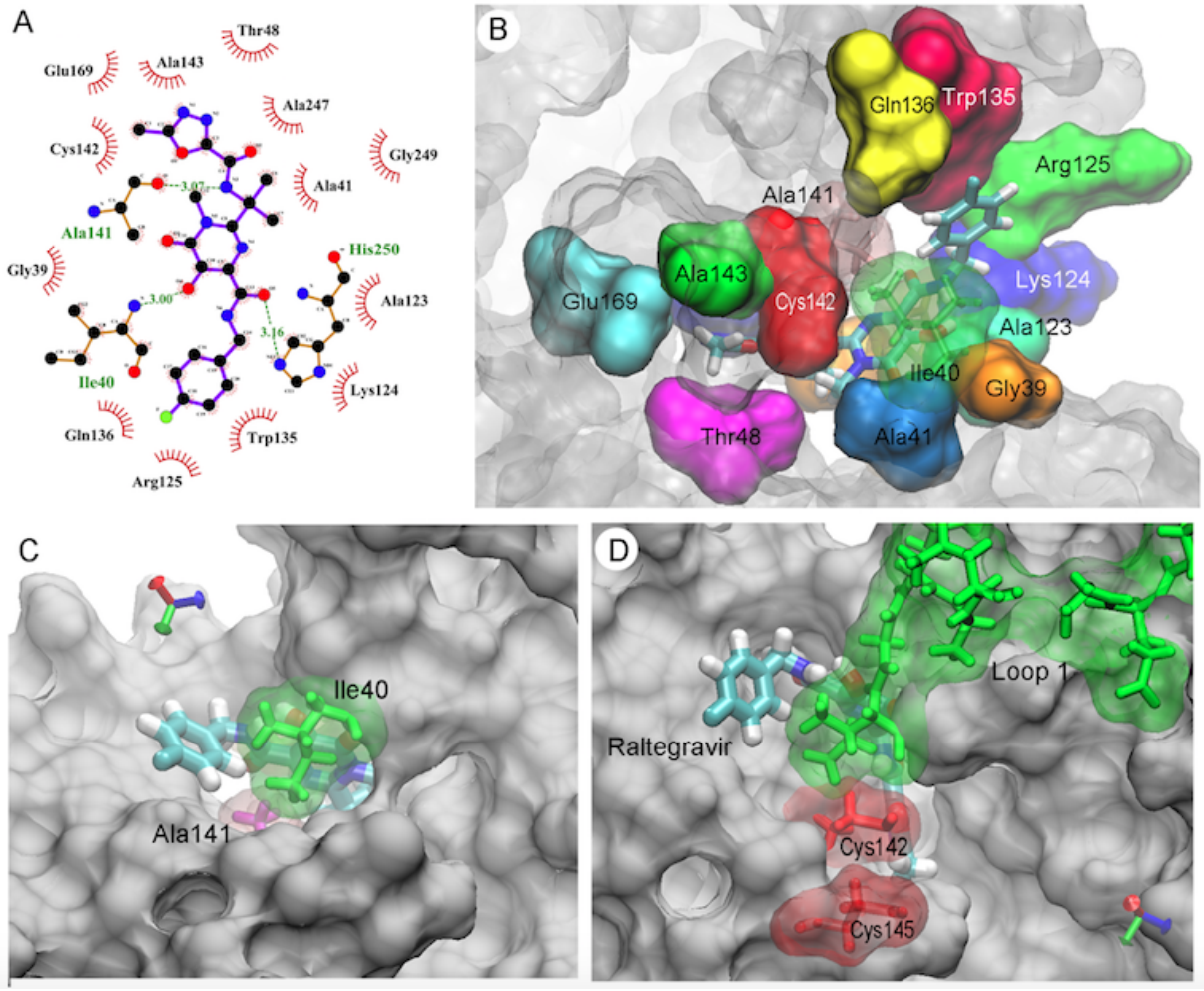

Fig. 5. Docking interaction of CaTrr1 with raltegravir. A and B: Schematic drawing of interactions between active site residues of CaTrr1 and raltegravir, using LigPlot (A) and VMD (B). C: The amino acids that interact with raltegravir through hydrogen bonds were designed by surface and licorice representation. D: The raltegravir compound was able to interact with Cys142 from the CaTrr1 catalytic site (red) and the two amino acids from the loop1 region, important for interaction with the substrate: Gly39 and Ile40 (green).

LigPlot: The interactions shown are those mediated by **hydrogen bonds** and by **hydrophobic contacts**. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

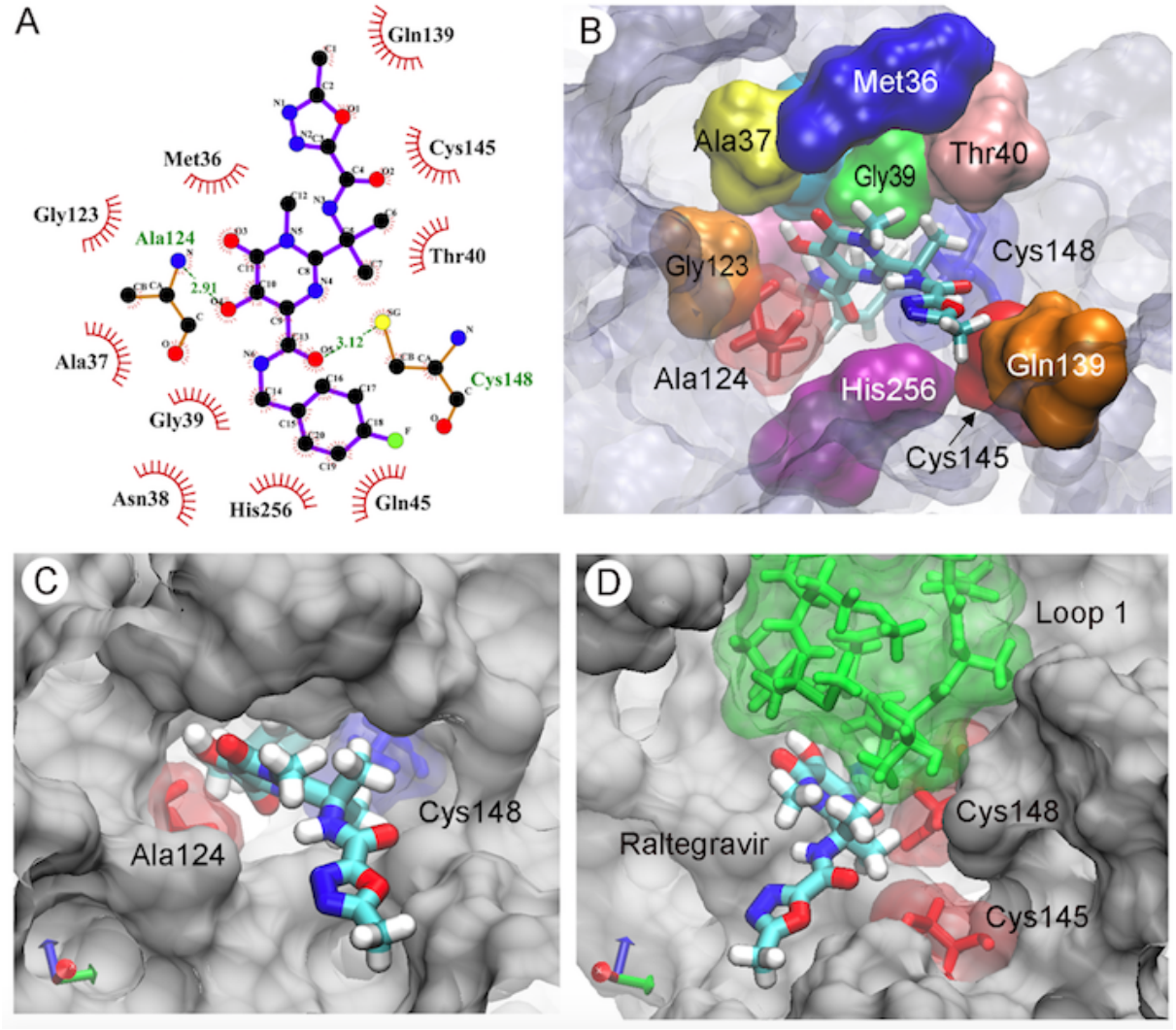

Fig. 6. Docking interaction of PlTrr1 with raltegravir. A and B: Schematic drawing of interactions between active site residues of PlTrr1 and raltegravir, using LigPlot (A) and VMD (B). C: The amino acids that interact with raltegravir through hydrogen bonds were designed by surface and licorice representation. Interestingly, raltegravir present interaction mediated by hydrogen bonds with the Cys148 catalytic site. D: The compound was able to interact with Cys145 and Cys148 from the PlTrr1 catalytic site (red) and the five amino acids from the loop1 region (green), important for interaction with the substrate: Met36, Ala37, Gly39, Asn38 and Thr40.

LigPlot: The interactions shown are those mediated by **hydrogen bonds** and by **hydrophobic contacts**. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

4. Discussion

Drug repositioning offers many benefits over *de novo* drug development; it significantly accelerates the process and reduces costs in the development pipeline, and is of great interest to academia and industry [49,50]. This approach has been a viable alternative for the discovery of new drugs against neglected diseases [37], such as paracoccidioidomycosis [51-53]. In this study, combined ligand-based and structure-based methods for drug repositioning were used.

The compounds available in three drug databases were evaluated for chemical similarity with two compounds, LMM5 and LMM11, previously identified as hit compounds for the development of new antifungals [17]. First, 100 compounds from each database were selected using the Tanimoto coefficient, a parameter frequently used in chemoinformatics, which has the advantages of conceptual simplicity, computational efficiency, and applicability to large compound databases [54-57]. We did not find a published standard value for the threshold for an acceptable or non-Tc value. Martin et al. [58] previously questioned the use of a value ≥0.85 of Tanimoto similarity for screening compound libraries. Since few compounds that are very active are above that threshold, these authors [58] suggested a rethinking of strategies for compound acquisition and design of combinatorial libraries. Vogt and Bajorath [59] concluded that there are generally no threshold values for similarity as an indication of the relationship between the reference compound and the database compounds. Some authors have used Tc ≥ 0.6 [60], others Tc ≥ 0.7 [61]. Given this lack of standardization, we chose not to define a threshold value for Tc, and to use the scaffold trees to filter the 100 compounds from each database, to select up to 10 that were most similar to LMM5 and LMM11.

The scaffold tree allows one to aggregate sets of molecules with a similar scaffold, and delineates the relationships among them based on the structural inclusion relationship [62,63]. In combinatorial libraries, the molecules are based on the common scaffolds, and moreover, the same synthesis route can be shared by compounds with the same scaffold [62]. Langdon et al. [64] used a scaffold tree for virtual screening of compound libraries, for the discovery of mitotic kinase inhibitors. A total of 17 compounds similar to LMM11 were selected by the scaffold trees, from the three databases. However, for the compound LMM5, this method was able to select similar compounds only in the MDDR database (two compounds).

The results of the ligand-based method were complemented with the use of the structure-based approach. Molecular docking has been used successfully in the search for new therapeutic options [18,65]. Although more virtual screening structure-based methods

have been published than ligand-based, this latter approach identifies hits that are on average more potent [66]. Therefore, the combined use of these methods seems to be a promising approach for developing new drugs. Accordingly, the compounds that are chemically similar to the hit compounds and with affinity to the drug target (Trr1) were ranked by four parameters: the GOLD value, Tc, the ligand-target interaction, and availability for purchase. Six compounds were purchased for evaluation of *in vitro* antifungal activity. These compounds had four previously established therapeutic indications: anti-diabetic, anti-viral, anti-asthmatic and anti-tumor.

Holbrook et al. [67], showed that repurposed bromperidol derivatives inhibited *C. albicans* at MIC values >32 μg/ml. Atorvastatin showed an MIC value of 256 μg/ml or higher against *C. gattii* [68]. Flubendazole, a benzimidazole, has potent *in vitro* activity against *C. neoformans*, with a modal MIC of 0.125 μg/ml [69]. In our *in vitro* evaluation of antifungal activity, the genus *Paracoccidioides* was inhibited by the six compounds evaluated, with low MIC values (16–64 μg/ml) (**Table 3**). Interestingly, the compound with antiviral activity showed the most promising results for antifungal activity, based on the *in vitro* susceptibility test. Structure-based techniques were able to select compounds with promising antifungal activity against fungi of the genus *Paracoccidioides*, with MIC values of 8–32 μg/ml [18]. These compounds interact with Trr from *P. brasiliensis* in amino-acid residues at the active site, such as Cys145 and/or Cys148. In our study, the six compounds also interacted with CaTrr1 in amino-acid residues at the active site, Cys142 and/or Cys145 (**Fig. 3**). The results obtained through the docking simulation using thioredoxin reductase from *P. lutzii* corroborated the antifungal activity of raltegravir, venetoclax and zafirlukast. Even with GOLD values lower than with CaTrr1, the strong interactions that the three compounds make with the catalytic site and region loop of PlTrr1 contribute to justify the better activity of these compounds in *Paracoccidioides*. Since, this flavoenzyme participates in the protection against oxidative damage [14,15], we believe that Trr1 blockade by compound could cause the accumulation of the reactive oxygen species in the fungal cell and consequently an important damage cellular.

This promising approach was able to select raltegravir for the preliminary *in vivo* validation tests against systemic paracoccidioidomycosis. The number of antifungal agents for paracoccidioidomycosis is limited and the search for new compounds has intensified; however, few studies with compounds identified from drug repositioning for the treatment of this disease are found in the literature [51,53]. On October 16, 2007, the US Food and Drug Administration (FDA) approved raltegravir (isentress®), a new class

of antiretroviral drugs developed by Merck & Co., Inc., which functions as an inhibitor of HIV-1 integrase, a viral enzyme that catalyzes an essential process in the viral replication cycle, i.e., the insertion of HIV-1 proviral DNA into the host genome [70]. Raltegravir, as well as LMM5 and LMM11, is a member of the class of 1,3,4-oxadiazoles [71] and, curiously, was also discovered using *in silico* methods through simulation of molecular dynamics [72]. The *in vivo* antifungal activity demonstrated that raltegravir has similar behavior to itraconazole and significant reduction of the fungal burden in lung as well as only a few discrete changes in lung structure compared with the non-treated control. Compounds, as well as the raltegravir in this study, that share antiviral and antifungal activity have previously been reported. Studies have shown that antifungal agents available on the market, itraconazole [73,74], posaconazole [75] and micafungin [76], also have antiviral activity. Tamoxifen, used in treatment of estrogen receptor-positive breast cancer, showed antifungal activity against *C. neoformans* and antiviral activity against the hepatitis C virus [77].

5. Conclusions

Virtual screening using the complementary ligand-based and structure-based methods was effective, and can be used in drug repositioning through similarity with hits compounds, to search for drugs with antifungal activity. Our results suggest that raltegravir may be of interest for paracoccidioidomycosis treatment.

Contributors

I.R.G.C. E.S.K. T.I.E.S. B.M. were involved in the study design presented in this manuscript. Performed the experiments: I.R.G.C. D.R.F. K.M.S. F.A.V.R. P.S.B.M. E.S.K. Analyzed the data: I.R.G.C. T.C.A.B. E.S.K. T.I.E.S. B.M. Contributed reagents/materials/analysis tools: E.S.K. T.C.A.B. T.I.E.S. B.M. The manuscript and all associated documents were produced by I.R.G.C. E.S.K. T.I.E.S. B.M. with the assistance of all other co-authors listed.

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Artigo 3

A novel, simple and efficient tool for research of new antifungal agents

Molecular Diversity - JCR 2.22 (B1)

Short Communication

A novel, simple and efficient tool for research of new antifungal agents

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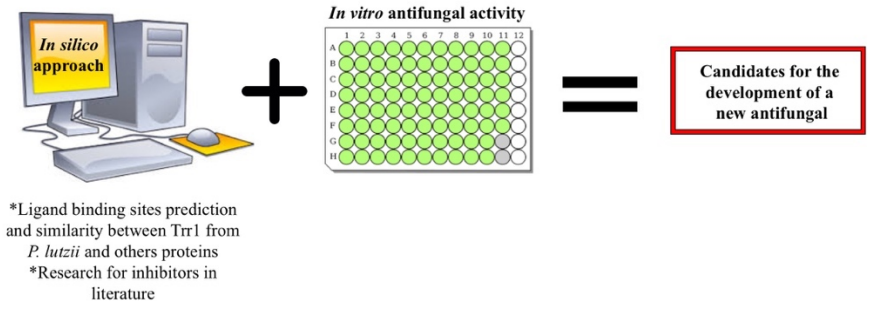
Abstract

A novel, simple and efficient tool for research antifungal candidate compounds was performed through of *in silico* approach of ligand binding sites prediction and similarity between Trr1 from *P. lutzii* and others proteins, additionally *in vitro* antifungal activity against pathogens of invasive fungal infections. This tool resulted at ebselen and butein, suggesting an efficient antifungal activity and possible candidates for the development of a new drug. In addition, this is the first study that shows the antifungal activity of ebselen and butein against *Paracoccidioides*.

Graphic abstract



Keywords antifungal; ligand binding sites; proteins; tools; similarity; *in silico*; *in vitro*

Introduction

Systemic mycoses are an emerging problem worldwide with high rates of mortality, especially in the large population of immunocompromised patients and/or those hospitalized with serious underlying diseases [2-4]. On the other hand, in comparison to the large number of antibacterial drugs, the number of clinically available antifungal agents is restricted. In addition, the emergence of resistant strains [5,6] and significant adverse events, it is urgent to develop new antifungal agents.

Aiming advantages of cost and time compared to traditional methods, computational techniques have been widely employed in the development of new drugs [7]. Efforts have been made to employ the *in silico* approaches for facilitating the search and design of selective target [8,9]. The identification and validation of appropriate targets are critical steps for designing new drugs, since this approach allows the search of selective inhibitors targeting the specific target [10,11].

Proteins play a fundamental role in almost all biological processes and at the molecular level, its functions in a cell are determined or regulated by interactions with other molecules [12,13]. In general, these are small molecules ligands such as metabolites or compounds, that bind to proteins through the binding surfaces formed by concave shapes or "pockets" [14-16]. Consequently, the identification of which residues participate in these interactions and comparing and/or classifying ligand-binding sites of small molecules can to aiding the functional elucidation of proteins, to guide the design of inhibitors and antagonists, provide a scaffold for targeted mutations and identifying cavities that are either biologically relevant or 'druggable' [13,17,18].

In this context, over the years, many *in silico* approaches based on analysis of protein sequences or structures have been developed to predict a variety of protein functional sites, including ligand binding sites [16,19,20]. The structural methods use geometric and energetic parameters to find concave regions on the protein surface that are probably binding sites for the ligands [16,21,22]. On the other hand, methods based on the sequences of the proteins, have largely exploited the conservation thereof, or the tendency of functionally or structurally important sites to accept fewer mutations relative to the remainder of the protein sequence [23].

The resulting ligand binding sites detected with any of the methods above can be used as input for the detection of similarities between ligand binding sites of others proteins [18]. Along with that, the principle defined by Klabunde [24]: "similar receptors bind similar ligands, or in other words, for a receptor as drug target of interest, known drugs and ligands of similar receptors, as well as compounds similar to these ligands, serve as a

starting point for drug discovery". Therefore, in this short communication we presented a novel, simple and efficient tool to search for new compounds with antifungal activity through the similarity between binding sites of proteins complementing with *in vitro* assay.

Results and discussion

Our research group has been studying the thioredoxin system as a promising target for the new antifungal agents development [10,25,26]. In this sense, **t**he ligand binding sites in the Trr1 from *Paracoccidioides lutzii* were predicted by the COFACTOR server [27]. This web server is a unified platform for structure-based multiple-level protein function predictions [28] and its algorithm has been extensively benchmarked by large-scale benchmarking tests and demonstrated significant advantages compared to traditional sequence-based methods. In community-wide CASP9 experiment, COFACTOR was ranked as the best method for protein–ligand binding site predictions [27]. The mandatory input for the web server is a single-chain protein structure file for the query protein in PDB format [28].

The 3D structure of PlTrr1 was obtained by homology modeling in a previous study and the conformer nº 3 (PlTrr1-3), from model of Trr1 reduced and complexed with its cofactor (FAD) of molecular dynamics simulation, was used as input of the COFACTOR server in PDB format. The output files consists of four major panels, including structural analogies, Gene Ontology (GO) terms, Enzyme Commission (EC) numbers and template of proteins with similar ligand-binding sites [28]. The COFACTOR server found three proteins that had their binding sites similar to PlTrr1-3: thioredoxin reductase of *Escherichia coli* (1f6mA) and glutathione reductase of *Homo sapiens* (1graA and 2gh5B) **(Fig. 1**). In according with values of $Cscore^{LB}$, IDEN and TM-score, the bacterial protein had a ligand binding site more similar to PlTrr1-3, than *Homo sapiens* protein (**Table 1**) Therefore, the next step of approach was to search in literature sources (e.g. PubMed), ligands databases such as ChEMBL and chemical providers (e.g. Life Chemicals), if there were known bioactives ligands for these proteins that share PlTrr1-3-binding site similarity. The **table 2** shows the ligands. As inhibitor of thioredoxin reductase of *E. coli* (1f6m) was found in the literature the ebselen [29,30]. In the ChEMBL database, 26 analogs of ebselen were obtained with 80% similarity. For glutathione reductase of *Homo sapiens*, was found in literature the butein [31-33]. In the ChEMBL database 12 bioactive ligands were found and 76 analogs of butein (80% similarity). However, only ebselen and butein were available for purchase, so the *in vitro* antifungal activity using the broth

microdilution method was performed against *Candida* spp., *Cryptococcus neoformans* and *Paracoccidioides* spp., in order to validate the *in silico* methodology.

The ebselen showed antifungal activity against all fungal species tested (**Table 3**). In general, the MIC for ebselen were low (1-8 μg/mL), suggesting an efficient and promising antifungal activity, corroborating with others studies against the genus *Candida* and *Cryptococcus* [34,35]. Ebselen, is an organoselenium compound and a clinically safe molecule, has been reported in several biological activities [36] including antifungal activity [34,35,37].

The butein is an important chalcone polyphenol first isolated from Rhus verniciflua Stokes with pharmacological and biological effects, including antioxidant, anti-inflammatory, anticancer, antidiabetic, hypotensive and neuroprotective effects [38]. The activity of butein against *Paracoccidioides* also was significant (8-32 μg/mL).

According to Skolnick [39], on average, a single domain protein contains from three to five pockets with characteristics sufficient to bind to a small ligand. However, the number of pocket shapes is much lower than the known bioactive ligands [40,41], consequently, this fact implies that one pocket shape can accommodate more than one type of ligand which generates the diversity of the biochemical function of the proteins [42]. Thus, the same ligand may bind to similar shaped pockets, but located in different proteins [43].

Interestingly, the literature shows that glutathione reductase and thioredoxin reductase are reducing thiol enzymes, which can coexist in the same organism, involved with the oxidation-reduction process and there may be overlapping functions with cross-talk between the two systems [44]. Therefore, we believe that both ebselen and butein can bind to these two enzymes and exert their antifungal activity in *Paracoccidioides*, indicating that the same ligand may bind to similar binding sites located in different proteins.

To better understand the interaction of these compounds in PlTrr1, a docking simulation was performed. The docking analysis indicates that there is an interaction of the two compounds with catalytic site residues, such as Cys148 and several amino acids of loop1 of Trr1 from *P. lutzii* (**Figure 2**). It is important to note the interaction of the aromatic rings of both compounds with the Cys148 from catalytic site (**Figure 3**) denominated π stacking. It is possible that this important interaction could be responsible for blocking the binding at the catalytic site.

As for the difference in antifungal activity between the compounds, the smaller size of the ebselen (274.17 g/mol) and polarity, in relation to the butein (272.25 g/mol), can lead to a better permeation in the fungal cell, which results in a more spectrum of activity.

Therefore, the next steps should be the enzymatic assays of PlTrr1 with the compounds, aiming to elucidate the activity.

The results of ebselen and butein open new insights for the analogs development aiming to improve even better the activity, in addition, this is the first study that shows the antifungal activity of this compounds against *Paracoccidioides* spp.

Table 1. Template proteins with similar binding site with Trr1 from *P. lutzii*. This template was generated by web server COFACTOR.

Rank	$Cscore^{LB}$	PDB hit	TM-score	$RMSD^{a}$	$IDEN^{a}$	Cov.	BS-score	Lig. Name	Predicted binding site residues
1	0.28	1f6mA	0.715	4.27	0.350	0.929	1.00	FAD	10,11,12,13,14,33,34,36,37,86,87,121, 122,256,259,262,293,294,301, 302,303,306
2	0.16	1graA	0.683	4.28	0.141	0.880	1.03	NDP	9,10,11,12,34,35,121
3	0.06	2gh5B	0.686	4.26	0.144	0.880	0.88	ELI	13,17,20,71,303,311

a) $Cscore^{LB}$ is the confidence score of predicted binding site. $Cscore^{LB}$ values range in between [0-1]; where a higher score indicates a more reliable ligand-binding site prediction.

(b) BS-score is a measure of local similarity (sequence & structure) between template binding site and predicted binding site in the query structure. Based on large scale benchmarking analysis, we have observed that a BS-score >1 reflects a significant local match between the predicted and template binding site.

(c) TM-score is a measure of global structural similarity between query and template protein.

(d) $RMSD^{a}$ the RMSD between residues that are structurally aligned by TM-align.

(e) $IDEN^{a}$ is the percentage sequence identity in the structurally aligned region.

(f) Cov. represents the coverage of global structural alignment and is equal to the number of structurally aligned residues divided by length of the query protein.

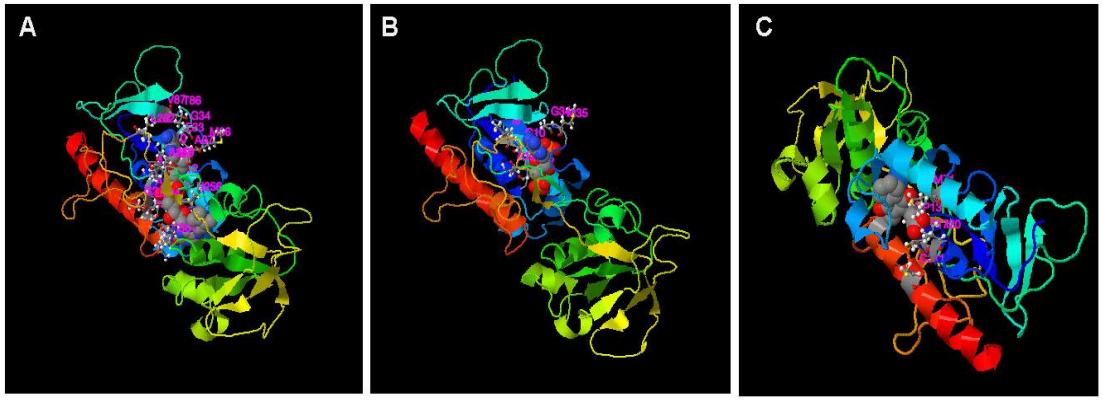


Fig. 1. Proteins with similar binding site with Trr1 from *P. lutzii*. A: 1f6mA B: 1graA C: 2gh5B

Table 2. Bioactives ligands of proteins with similar binding site to Trr1 from *P. lutzii*

PDB Identification	Result COFACTOR - Proteins with similar binding site to PlTrr1	Bioactives ligands
1F6M	Thioredoxin reductase - *Escherichia coli*	**1)** Ebselen* and 26 analogs
1GRA	Glutathione reductase - *Homo sapiens*	**1)** 12 bioactive ligands **2)** Butein* and 76 analogs**
2GH5	Glutathione reductase - *Homo sapiens*	**1)** 12 bioactive ligands **2)** Butein* and 76 analogs**
Total		**116**

*Ebselen and Butein were found in the literature as inhibitor of Thioredoxin reductase – *E. coli* and Glutathione reductase - Homo sapiens, respectively.
**The 27 analogs of ebselen and 76 analogs of butein were obtained from the ChEMBL, defined 80% of similarity.
PDB identification: https://www.rcsb.org/

Table 3. MIC of ebselen and butein against *Candida*, *Cryptococcus* and *Paracoccidioides* strains

Strain	MIC (µg/mL)	
	Ebselen	Butein
Candida albicans (ATCC 90028)	2	+256
Candida glabrata (ATCC 90030)	4	16
Candida krusei (ATCC 6258)	4	+256
Candida tropicalis (ATCC 750)	4	+256
Candida parapsilosis (ATCC 22019)	1	+256
Cryptococcus neoformans (INCQS)	2	+256
Paracoccidioides brasiliensis (Pb18)	8	8
Paracoccidioides lutzii (Pb01)	8	32

MIC: Minimum Inhibitory Concentrations of ebselen was determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute M27-A3 (CLSI). The final concentrations of the ebselen and butein ranged from 0.5 to 256 µg/mL.

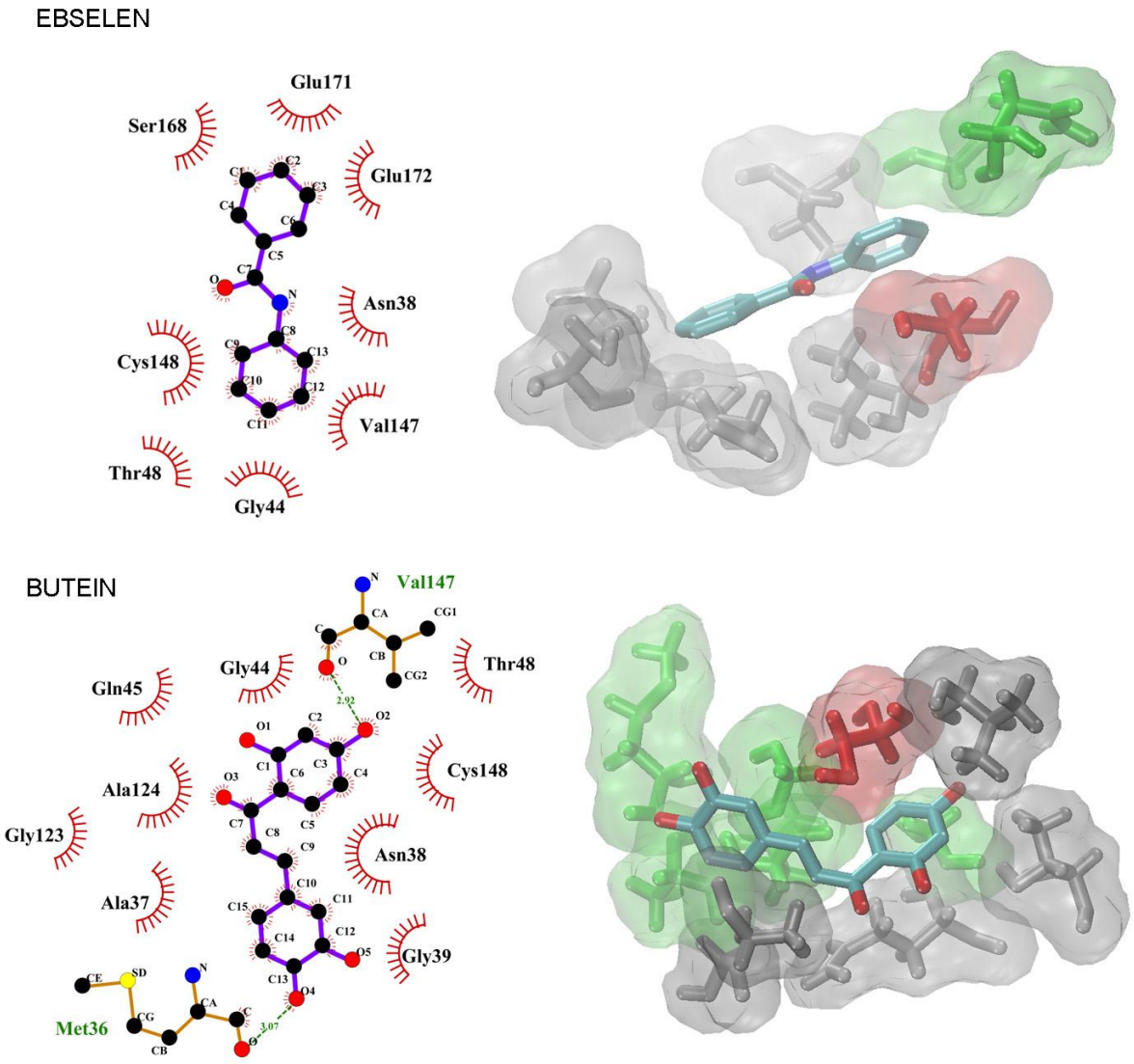


Figure 2. Interaction thioredoxin reductase from *Paracoccioides lutzii* (PlTrr1-3) with ebselen and butein. The 2D-plot of interactions between amino acid residues of PlTrr1-3 and compound analysed by LigPlot and the schematic drawing of tridimensional interaction obtained by VMD program. Both compounds interacted with the cysteine of the PlTrr1-3 catalytic site (Cys148). In tridimensional plot obtained by VMD, in the interactions between catalytic site of PlTrr1-3 and compounds were presented by surface combinated with licorice representation. Green: amino acids of loop1 of Trr1 from *P. lutzii*. Red: Cys148. Grey: others amino acids.

LigPlot: The interactions shown are those mediated by **hydrogen bonds** and by **hydrophobic contacts**. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

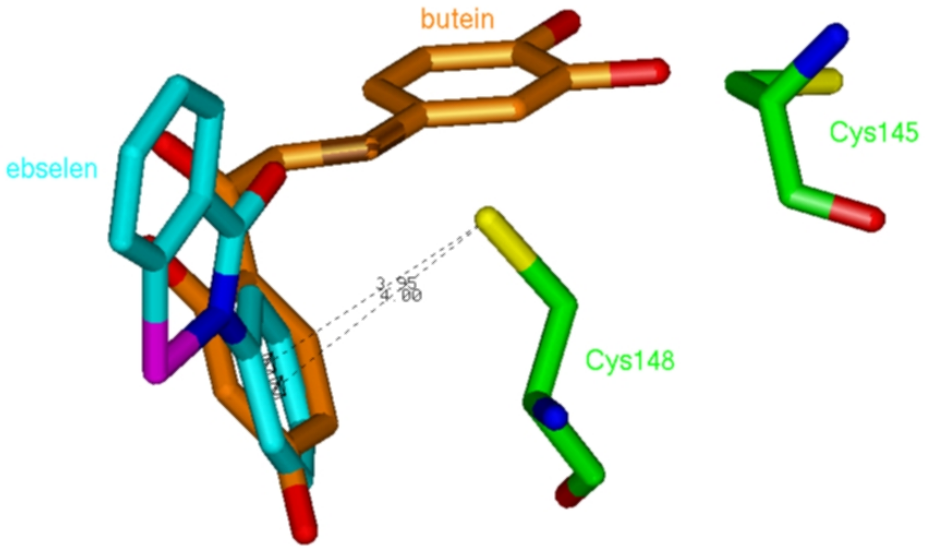


Figure 3. Interaction π stacking of the aromatic rings of ebselen and butein with the Cys148 from catalytic site.

Conclusion

In conclusion, we have demonstrated a novel, simples and efficient tool for research antifungal candidate compounds linking *in silico* and *in vitro* approaches. Ebselen and butein are possible candidate for the development of a new drug and this is the first study that shows the antifungal activity of these compounds against *Paracoccidioides* spp.

Experimental procedure

Ligand binding site prediction

Ligand binding site prediction was performed by COFACTOR web server. It is freely available at http://zhanglab.ccmb.med.umich.edu/COFACTOR [27,28].

Server input

Due to the continuity of the in silico studies of our research group, the protein chosen to perform this protocol was thioredoxin reductase from fungi *Paracoccidioides lutzii* (PlTrr1). The 3D structure of PlTrr1 was obtained by homology modeling in a previous study and the enzyme model in reduced conformation and complexed with its cofactor was the more active form. This model contains 5 families of conformation, however, one of these families (PlTrr1-3) stood out in *in silico* activity and was used as input of the COFACTOR server in PDB format.

Server output

Upon job completion, the user will be notified by email with a link to the result page on the COFACTOR server web-site. The result page consists of four major panels, including structural analogies, Gene Ontology (GO) terms, Enzyme Commission (EC) numbers and template of proteins with similar ligand-binding sites [28].

Search for bioactives ligands

According to template of proteins with similar binding site generated by COFACTOR, the next step was to search bioactives ligands of the proteins that had the binding site similar to Trr1. Literature sources (e.g. PubMed), databases ligands such as ChEMBL and chemical providers, were consulted.

For search in the literature the keywords used were: thioredoxin reductase, *E. coli*, inhibitors, glutathione reductase and ligand.

In the ChEMBL (https://www.ebi.ac.uk/chembl/) the search was performed in Target parameter (thioredoxin reductase or glutathione reductase) and the analogs of ebselen and butein were defined with 80% similarity in Ligands parameter.

Docking analysis

Ebselen and butein were submitted to the docking simulation by the GOLD software [45] against thioredoxin reductase from *P. lutzii* (PlTrr1-3), in its reduced conformation. The interaction of compounds selected with the PlTrr1-3 protein was visualized with the Visual Molecular Dynamics (VMD) program, available at http://www.ks.uiuc.edu/Research/vmd/ and the LigPlot program, available at https://www.ebi.ac.uk/thornton-srv/software/LIGPL

***In vitro* assay**

Compounds

The compounds ebselen and butein were obtained from the company Targetmol (http://targetmol.com/). The stock solution of these compounds were prepared with dimethyl sulfoxide (DMSO) in accordance with the manufacturer's recommendations. The highest DMSO concentration used in the experiments did not exceed 0.5% (an concentration non-toxic, as described in CLSI document).

Organisms

In vitro susceptibility tests were performed for pathogenic yeasts (reference strains): *P. brasiliensis* (Pb18), *P. lutzii* (Pb01), *C. albicans* (ATCC 90028), *C. parapsilosis* (ATCC

22019), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258) and *C. neoformans* (INCQS). The Minimum Inhibitory Concentrations (MICs) of selected inhibitors were determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute M27-A3 (CLSI) [46]. The final concentrations of the inhibitors ranged from 0.5 to 256 μg/mL. Endpoint determination readings were performed visually considering 100% inhibition of growth for either *Candida*, *Cryptococcus* or *Paracoccidioides.*

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Conflict of Interest: The authors declare that they have no conflict of interest.

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ANEXOS

LMM6: análogo estrutural de LMM11 com promissora atividade contra patógenos de DFI

A fim de complementar a descrição do trabalho desenvolvido durante o doutorado que originou essa tese, porém, que ainda não foi transformado em artigo científico, apresentamos nesse anexo a descoberta e resultados *in vitro* preliminares de um novo composto o qual denominamos de LMM6 (Laboratório de Micologia Médica nº6).

LMM6 foi encontrado de acordo com a mesma metodologia *in silico* empregada no artigo 2 (reposicionamento de drogas), porém, efetuando a varredura virtual em um banco de dados que possui compostos *hits*, o ChEMBL. Este composto é um análogo estrutural quimicamente muito relacionado com LMM11, que por motivos de adição no pedido de patente já requerido, sua estrutura química não será apresentada nesse trabalho.

O que nos chamou atenção e interesse para esse composto é que de acordo com os resultados de Concentração Inibitória Mínima (CIM) da **Tabela 1**, LMM6 é mais eficaz do que LMM1 contra os patógenos de doenças fúngicas invasivas avaliados.

Portanto, estudos explorando a atividade antifúngica de LMM6, assim como a investigação de seu mecanismo de ação são objetivos de estudos futuros em nosso laboratório.

Tabela 1. Concentração inibitória mínima do composto LMM6 e LMM11 frente a isolados de *Candida* spp., *Cryptococcus neoformans* e *Paracoccidioides brasiliensis*

Isolados	LMM6 (CIM μg/ml)	LMM11 (CIM μg/ml)
Candida albicans	16	32
Candida glabrata	2	16
Candida krusei	16	32
Candida tropicalis	32	128
Candida parapsilosis	32	128
Cryptococcus neoformans	16	32
Paracoccidioides brasiliensis	0.5	16

O ensaio de susceptibilidade antifúngica foi realizado de acordo com o método de microdiluição em caldo, segundo o documento M27-A3 do CLSI, com algumas modificações.
O valor de CIM considerado para *Candida* spp. e *C. neoformans* foi a menor concentração do composto que inibiu 50% do crescimento fúngico.
O valor de CIM considerado para *P. brasiliensis* foi a menor concentração do composto que inibiu 100% do crescimento fúngico.

CAPÍTULO III

Conclusões

- LMM5 e LMM11 apresentaram efetiva atividade antifúngica contra *C. albicans* com CIM de 32 μg/mL.

- Ambos compostos não foram tóxicos nas três linhagens celulares avaliadas *in vitro*, e *in vivo* apenas menores alterações nos parâmetros avaliados foram observados.

- A cinética da curva de morte demonstrou que frente a *C. albicans*, LMM5 e LMM11 são fungistáticos, com melhor atividade que o fluconazol nos tempos de 24 e 48 horas.

- De acordo com as imagens de MEV e MET, hipotetizamos que o mecanismo de ação de LMM5 e LMM11 está diretamente relacionado à inibição da enzima Trr1 e seus efeitos internamente na célula fúngica.

- Em modelo experimental murino de candidíase sistêmica, ambos compostos reduziram significativamente a carga fúngica no rim e baço de camundongos Balb/c, comprovado pelas análises histológicas coradas com H&E e Gomori e Grocott.

- Metodologias *in vitro* e *in vivo* comprovaram a atividade antifúngica contra *C. albicans* dos dois compostos previamente selecionados por varredura virtual contra o alvo tioredoxina redutase, LMM5 e LMM11, gerando o depósito de um pedido de patente (BR 10 2018 009020 8, 3 de maio de 2018).

- Através de técnica de reposicionamento de drogas, unindo metodologias baseada nos ligantes LMM5 e LMM11 e na estrutura da tioredoxina redutase, a varredura virtual de bancos de dados de compostos culminou na aquisição de 12 compostos que foram testados *in vitro* contra *Candida* spp., *Paracoccidioides* spp. e *Cryptococcus neoformans*.

- Dentre os compostos avaliados que já possuíam atividade conhecida contra alguma doença, raltegravir (utilizado comercialmente para tratamento de HIV) foi o que apresentou melhor atividade antifúngica, destacando sua atividade contra o gênero *Paracoccidioides*;

- O composto selecionado o qual denominamos LMM6, foi um análogo estrutural de LMM11 e apresentou melhor atividade em *Candida* spp. do que LMM11;

- Utilizando a metodologia *in silico* de similaridade entre o sítio de ligação de proteínas, através do modelo da Trr1 de *Paracoccidioides lutzii*, 2 compostos foram adquiridos para testes *in vitro* de atividade antifúngica contra patógenos de DFI;

- Ebselen e butein, selecionados pela metodologia anteriormente descrita, apresentaram atividade significativa e inédita contra *Paracoccidioides* spp.

Perspectivas futuras

Este trabalho proporcionará a continuidade da linha de pesquisa de desenvolvimento de novas opções terapêuticas para o tratamento de micoses do Laboratório de Micologia Médica, uma vez que os compostos adquiridos poderão ser investigados quanto a atividade antifúngica, utilizando variadas técnicas *in vitro* e *in vivo*, contra outros gêneros/espécies de fungos patogênicos humanos que não foram abordados neste estudo.

O composto denominado LMM6, será adicionado ao pedido de patente já existente assim que seus estudos forem finalizados. Ensaios contra espécies de *Candida* spp. e de *Cryptococcus* spp. serão realizados, envolvendo desde fatores de virulência até a busca do mecanismo de ação envolvido com a atividade do composto.

Ebselen será avaliado quanto sua atividade em fungos filamentosos, iniciando por *Aspergillus fumigatus*, patógeno que também se destaca no cenário das DFI, com ensaios *in vitro* e *in vivo*. Em adição, em parceria com o departamento de química da UEM, objetivamos o desenvolvimento de análogos do ebselen e butein na tentativa de que mudanças estruturais possam melhorar ainda mais a atividade do composto. Além disso, ensaios enzimáticos serão desenvolvidos.

No que diz respeito às metodologias *in silico*, todo o expertise obtido poderá ser repassado para outros alunos que desejarem desenvolvê-las, consequentemente, proporcionando a continuidade da parceria internacional entre o Laboratório de Micologia Médica da UEM e o Prof. Dr. Bernard Maigret do LORIA, França.