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Thaís Felli Kubiça

CARACTERIZAÇÃO MOLECULAR E PROSPECÇÃO DE COMBINAÇÕES ANTIFÚNGICAS SINÉRGICAS "*IN VITRO*" FRENTE A *Trichosporon asahii*, ANTES E APÓS EXPOSIÇÃO PROLONGADA AO FLUCONAZOL

Santa Maria, RS
2016

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciências Farmacêuticas.**

Orientador: Prof. Dr. Sydney Hartz Alves

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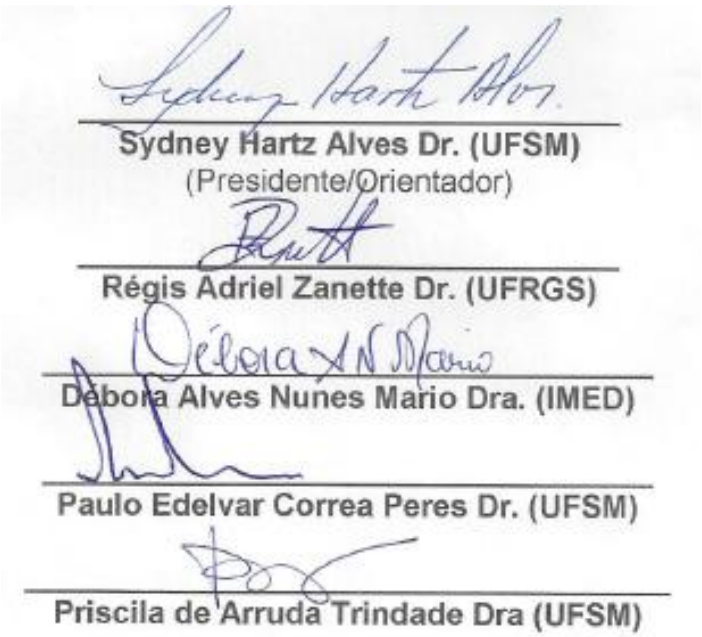

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Sem sonhos, a vida não tem brilho.
Sem metas, os sonhos não têm alicerces.
Sem prioridades, os sonhos não se tornam reais.

(Augusto Cury)

RESUMO

CARACTERIZAÇÃO MOLECULAR E PROSPECÇÃO DE COMBINAÇÕES ANTIFÚNGICAS SINÉRGICAS "*IN VITRO*" FRENTE À *Trichosporon asahii*, ANTES E APÓS EXPOSIÇÃO PROLONGADA AO FLUCONAZOL

AUTORA: Thaís Felli Kubiça
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A ocorrência de micoses invasivas causadas por patógenos fúngicos emergentes tem aumentado consideravelmente nas últimas décadas. O gênero *Trichosporon* compreende espécies relevantes neste cenário, devido à reduzida suscetibilidade à anfotericina B (AMB) e às equinocandinas, bem como o aparecimento de cepas resistentes aos antifúngicos azólicos, em especial ao fluconazol (FCZ). Dentre as estratégias para combater as falhas terapêuticas, a combinação de fármacos com distintos mecanismos de ação tem merecido atenção. Este estudo objetivou identificar genotipicamente os isolados clínicos (n=30), bem como avaliar a suscetibilidade *in vitro* de *T. asahii*, antes e após exposição prolongada ao fluconazol. Os agentes testados foram os antifúngicos AMB, FCZ, itraconazol (ITZ), voriconazol (VCZ), posaconazol (POS), caspofungina (CPF), micafungina (MCF) e anidulafungina (AND), e compostos não antifúngicos tacrolimus (FK506), disseleneto de difenila (DPDS) e ebselen (EBS); todos testes foram realizados pela determinação das concentrações inibitórias mínimas (CIMs), conforme o protocolo M27-A3 (CLSI, 2008). *T. asahii* apresentou-se como a única espécie identificada molecularmente dentre os isolados provenientes de amostras de urina e sangue caracterizados. As CIMs confirmaram a resistência intrínsica de *T. asahii* às equinocandinas (CIMs ≥ 4 µg/mL), bem como a superioridade dos compostos triazólicos frente a essa espécie. Ademais, foi possível observar que, com exceção da AMB (CIM_{90} = 1 µg/mL), os isolados FCZ-resistentes (FR) foram menos sensíveis aos azólicos do que o grupo FCZ-sensível (FS), sendo esse fenômeno de resistência cruzada mais expressivo frente ao ITZ (90%), seguido do POS (36,67%) e VCZ (10%). Os resultados das associações de antifúngicos e não antifúngicos frente aos grupos de *T. asahii* FS e FR foram avaliados pelo método de microdiluição *checkerboard.* O FK506 (CIMs > 64 µg/mL), assim como o DPDS (CIMs ≥ 8 µg/mL), não demonstraram satisfatória ação antifúngica frente *T. asahii* FS e FR. Entretanto, consideráveis percentuais de interações sinérgicas foram exibidos na associação de AMB + FK506 (96,67%), CPF + FK506 (73,33%) e AMB + DPDS (90%) frente aos isolados FS, assim como frente ao grupo de *T. asahii* FR: CAS + DPDS (96.67%), AMB + DPDS (93.33%), FCZ + DPDS (86.67%), and ITZ + DPDS (83.33%). Por outro lado, o organocomposto EBS destacou-se pelos baixos valores de CIMs (0,25 - 8 µg/mL) quando testado isoladamente, além do sinergismo na associação com AMB (90%) sobre *T. asahii* FR. Adicionalmente, foram testadas combinações AMB e equinocandinas ou FCZ, e CPF + FCZ frente aos isolados FR que, com exceção da combinação CPF + FCZ (66,67% de sinergismo), resultaram em predomínio de atividade indiferente. Interações antagônicas não foram observadas nas associações entre antifúngicos. Neste contexto, a exposição *in vitro* a concentrações crescentes de fluconazol é um fator importante para a emergência de resistência em *T. asahii*, fenômeno este que agrega consequências para o perfil de suscetibilidade desta espécie. Além disso, os sinergismos observados *in vitro*, são promissores para o desenvolvimento de estudos *in vivo*, bem como elucidar a atividade do FK506 e organocompostos de selênio contra *T. asahii.*

Palavras chave: Resistência. Suscetibilidade. Tacrolimus. Disseleneto de difenila. Ebselen.

ABSTRACT

MOLECULAR CHARACTERIZATION AND "*IN VITRO*" PROSPECTION OF SYNERGISTIC ANTIFUNGAL COMBINATIONS FOR *Trichosporon* asahii BEFORE AND AFTER PROLONGED EXPOSURE TO FLUCONAZOLE

AUTHOR: THAÍS FELLI KUBIÇA
ADVISOR: DR. SYDNEY HARTZ ALVES

The occurence of invasive fungal infections caused by emerging fungal pathogens has increased considerably in the last decades. The genus *Trichosporon* comprises species relevant in this context, due to reduced susceptibility to amphotericin B (AMB) and echinocandins, as well as the emergence of resistant strains to azole antifungals, especially to fluconazole (FCZ). Among the strategies to combat the therapeutic failures, the combination of drugs with different mechanisms of action has deserved attention. This study aimed to identify genotypically clinical isolates (n = 30), and to evaluate the susceptibility *in vitro* of *T. asahii*, before and after induction the resistance to fluconazole, to antifungal agents: AMB, FCZ, itraconazole (ITZ), voriconazole (VCZ), caspofungin (CPF), micafungin (MCF) and anidulafungin (AND), and non-antifungal compounds: tacrolimus (FK 506), diphenyl diselenide (DPDS), and ebselen (EBS), by determining the minimum inhibitory concentrations (MICs), as M27-A3 (CLSI, 2008). *T. asahii* was the most prevalent specie among isolates from urine and blood identified. The results MICs confirm the intrinsic resistance of *T. asahii* to echinocandins (MICs ≥ 4 μg mL-^{1}), and the superiority of the triazole compounds against this specie. Moreover, it was observed that, other than AMB (MIC_{90} = 1 μg mL-^{1}), the fluconazole-resistant isolates (FR) were less sensitive to azoles than fluconazole-sensitive group (FS), and this cross-resistance phenomenon is more significant forward to ITZ (90%), followed by the POS (36.67%) and VCZ (10%). The results of the antifungal associations and non-antifungal compounds *against T. asahii* FS and FR were evaluated by microdilution checkerboard method. FK506 (MICs > 64 μg mL-^{1}) and the DPDS (MICs ≥ 8 μg mL-^{1}), did not show satisfactory antifungal activity against *T. asahii* FS and FR. However, high percentages of synergistic interactions were exibited in the association of AMB + FK506 (96.67%), CPF + FK506 (73.33%) and AMB + DPDS (90%) against FS isolates, as well against the *T. asahii* FR group: CAS + DPDS (96.67%), AMB + DPDS (93.33%), FCZ + DPDS (86.67%), and ITZ + DPDS (83.33%). On the other hand, the organic compound EBS stood out by the low MIC values (0.25 to 8 μg mL-^{1}) when tested alone, and a strong synergism in combination with AMB (90%) against *T. asahii* FR. Additionally, were tested combinations of AMB and echinocandins or FCZ, and CPF + FCZ against FR isolates that, other than CPF + FCZ combination (66.67% synergistic interactions), resulted in predominantly indifferent activity. Antagonistic interactions were not observed in the associations of antifungals. In this context, the *in vitro* exposure to increasing concentrations of fluconazole is an important factor for the emergence of resistance in *T. asahii*, this phenomenon brings consequences for the susceptibility profile of this specie. Moreover, the synergism observed in vitro is promising for the development of new studies to understand the activity of FK506 and organoselenium compounds against *T. asahii* for future application as a complementary role in the treatment of tricosporonosis.

Keywords: Resistance. Susceptibility. Tacrolimus. Diphenyl diselenide. Ebselen.

LISTA DE TABELAS

ARTIGO 1
Tabela 1 - *In vitro* susceptibility of *Trichosporon asahii* isolates to antifungal agents and tacrolimus. ……..38
Tabela 2 - Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* before resistance induction……..39
Tabela 3 - Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* after resistance induction……..40
MANUSCRITO 1
Tabela 1 - Minimum inhibitory concentration (MIC) of antifungal agents and organoselenium compounds against *Trichosporon asahii* (µg/mL). ……..62
Tabela 2 - Fractional inhibitory concentration index (FICI) and geometric mean (GM) of interactions between antifungal agents and organoselenium compounds against clinical isolates of *Trichosporon asahii*……..63
MANUSCRITO 2
Tabela 1 - *In vitro* susceptibilities of *Trichosporon asahii* isolates to antifungal agents before and after exposure to fluconazole……..……83
Tabela 2 - *In vitro* combinations of antifungal agents and organoselenium compounds against fluconazole-resistant *T.asahii* isolates…….…….84

LISTA DE ABREVIATURAS E SIGLAS

AMB	anfotericina B (*amphotericin B*)
ABCL	anfotericina B complexo lipídico
ABCD	anfotericina B em dispersão coloidal
AND	anidulafungina (*anidulafungin*)
ASD	ágar Sabouraud dextrose
CIM	concentração inibitória mínima
CIM_{50}	concentração inibitória mínima para 50% dos isolados testados
CIM_{90}	concentração inibitória mínima para 90% dos isolados testados
CLSI	*Clinical and Laboratory Standards Institute*
CPF/CAS	caspofungina (*caspofungin*)
DMSO	*dimethyl sulfoxide*
DNA	*deoxyribonucleic acid*
DPDS	*diphenyl diselenide* (disseleneto de difenila)
EBS	ebselen
EMCV	*encephalomyocarditis virus* (vírus da encefalomiocardite)
EUCAST	Comitê Europeu para Testes de Suscetibilidade Antimicrobiana
5FC	5-flucitosina
FK506	tacrolimus
FICI	*fractional inhibitory concentration index*
FR	fluconazol-resistente (*fluconazole-resistant*)
FS	fluconazol-sensível (*fluconazole-susceptible*)
GM	*geometric mean*
HSV-1	*herpes simplex virus* (vírus herpes simples tipo 1)
ICIF	índice de concentração inibitória fracionária
ITZ	itraconazol (*itraconazole*)
L-AmB	anfotericina B lipossomal
MCF	micafungina *(micafungin*)
MG	média geométrica
MIC	*minimal inhibitory concentration*
MOPS	*morpholinepropanesulfonic acid*
NFAT	*nuclear factor of activated T-cells*
OS	organocomposto de selênio
PCR	*polymerase chain reaction*
POS	posaconazol (*posaconazole*)
ROS	*reactive oxygen species*
SDA	*Sabouraud dextrose agar*
SIDA	Síndrome da Imunodeficiência Adquirida
UTI	Unidade de Terapia Intensiva
VCZ	voriconazol (*voriconazole*)

LISTA DE ANEXOS

ANEXO A - CARTA DE ACEITE ARTIGO 1.....104
ANEXO B - SUBMISSÃO DO MANSUCRITO 1.....105

SUMÁRIO

1 APRESENTAÇÃO..... 14
1.2 REFERENCIAL TEÓRICO..... 16
1.2.1 O gênero *Trichosporon* e a tricosporonose..... 16
1.2.2 Agentes antifúngicos..... 19
1.2.2.1 Derivados poliênicos..... 20
1.2.2.2 Derivados azólicos..... 20
1.2.2.3 Alilaminas..... 21
1.2.2.4 Análogos da pirimidina..... 22
1.2.2.5 Equinocandinas..... 22
1.2.3 O problema terapêutico nas infecções pelo gênero *Trichosporon* os mecanismos de resistência aos agentes antifúngicos..... 23
1.2.3.1 Resistência aos poliênicos..... 24
1.2.3.2 Resistência aos azólicos..... 25
1.2.3.3 Resistência à terbinafina e flucitosina..... 26
1.2.3.4 Resistência às equinocandinas..... 26
1.2.4 A terapêutica combinada como alternativa às micoses de difícil tratamento..... 27
1.2.4.1 Associações entre antifúngicos e quimiossensibilizantes..... 28
1.2.4.1.1 Tacrolimus..... 29
1.2.4.1.2 Organocompostos de selênio..... 30
1.3 PROPOSIÇÃO..... 33
1.4 MATERIAIS E MÉTODOS..... 34
2 ARTIGO 1 – Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates…….. 35
3 MANUSCRITO 1 – *In vitro* activity of diphenyl diselenide and ebselen alone and in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates…… 43
4 MANUSCRITO 2 – *In vitro* activity of antifungal agents alone and in combination against *Trichosporon asahii* isolates before and after prolonged exposure to fluconazole…… 64
5 DISCUSSÃO..... 85
6 CONCLUSÃO..... 90
REFERÊNCIAS..... 92
ANEXO A - CARTA DE ACEITE ARTIGO 1..... 104
ANEXO B – SUBMISSÃO DO MANSUCRITO 1..... 105

1 APRESENTAÇÃO

A incidência de infecções por *Trichosporon* spp. tem aumentado proporcionalmente ao crescimento do número de pacientes com doenças hematológicas malignas ou outras condições associadas com imunossupressão, tais como procedimentos médicos invasivos, tratamento com fármacos quimioterápicos, uso de antibióticos de amplo espectro, bem como a terapia com drogas imunossupressoras na prevenção à rejeição ao transplante de órgãos (CHAGAS-NETO et al., 2009; COLOMBO; PADOVAN; CHAVES, 2011; GIRMERNIA et al., 2005; RUAN; CHIEN; HSUEH, 2009; YANG; GAO; LI, 2014). Recentemente, *Trichosporon* spp. foi considerado como a segunda ou terceira levedura mais isolada em laboratórios clínicos. Em pacientes com infecção disseminada, *T. asahii* tem sido descrito como a espécie mais prevalente, sendo classificada como patógeno oportunista emergente (COLOMBO; PADOVAN; CHAVES, 2011).

A infecção hematogênica por micro-organismos do gênero *Trichosporon* geralmente está associada a cateteres ou a disseminação a partir do trato respiratório ou gastrointestinal, rendendo hemoculturas positivas e lesões cutâneas, em um quadro similar às candidemias (CHAGAS-NETO; CHAVES; COLOMBO, 2008; WALSH et al, 2004). Em pacientes com doenças hematológicas malignas, *Trichosporon* spp. foi classificado como o segundo agente mais comum de infecções fúngicas disseminadas, conduzindo a taxas de até 80% de mortalidade (GIRMENIA et al., 2005). Em pacientes neutropênicos, esse índice chegou a atingir aproximadamente 100% (BASSETTI et al., 2004).

A terapêutica antifúngica é problemática porque, via de regra, *Trichosporon* é resistente intrinsicamente às equinocandinas (BAYRAMOGLU et al., 2008; LIAO et al., 2012; YANG; GAO, LI, 2014) e tem apresentado suscetibilidade reduzida à anfotericina B (CHAGAS-NETO et al, 2009; GIRMENIA et al, 2005; PAPHITOU et al, 2002; RASTOGI et al., 2016). Os antifúngicos azólicos parecem ter melhor atividade quando comparados a outros fármacos disponíveis comercialmente (ARIKAN et al, 2005; CHAGAS-NETO et al, 2009; MONTOYA et al., 2015; PAPHITOU et al, 2002). Todavia, a terapia prolongada com fluconazol pode desencadear resistência de *T. asahii* a múltiplos fármacos (KUSHIMA et al., 2012; RIBEIRO et al., 2008; WOLF et al., 2001). Embora o voriconazol represente a melhor opção para o tratamento da tricosporonose em situações que não respondem a outros agentes antifúngicos

(FALK et al., 2003; HARIZOLAN et al., 2013), casos de cepas resistentes já foram documentados (BASSETTI et al., 2004; KUSHIMA et al., 2012; RASTOGI et al., 2016; RIBEIRO et al., 2008). Além disso, os altos custos da terapia com esse novo triazólico muitas vezes inviabilizam seu uso na prática clínica.

A terapia combinada com fármacos antifúngicos de diferentes classes pode representar uma alternativa para a redução da resistência antifúngica de *T. asahii* e das altas taxas de mortalidade (LI et al., 2010, LI et al., 2011; SERENA et al., 2005). Organocompostos de selênio como disseleneto de difenila e ebselen, têm evidenciado elevadas taxas de sinergismo quando combinados com agentes antifúngicos convencionais *in vitro*, demonstrando potencial para uso como alternativa na terapia adjuvante de infecções causadas por *Candida glabrata* e *Fusarium* spp. (DENARDI et al., 2013, VENTURINI et al., 2016).

Ademais, estudos recentes têm demonstrado que a exploração da via da calcineurina dos fungos é promissora para o desenvolvimento de novos agentes, incluindo a terapia combinada com antifúngicos frente espécies de *Aspergillus*, *Fusarium*, *Candida* e *Cryptococcus neoformans* (DENARDI et al., 2015; KONTOYANNIS et al., 2003; SHALIT; SHADKCHAN, MIRCUS, 2009; STEINBACH et al., 2004). Além disso, visto que pacientes transplantados são submetidos à profilaxia com agentes antifúngicos concomitantemente à terapia imunossupressora (BAYRAMOGLU et al., 2008; YANG; GAO; LI, 2014), percebe-se que o conhecimento das possíveis interações dessa associação é de extrema importância a fim de garantir segurança e eficácia do tratamento. Estudos neste sentido ainda não foram desenvolvidos com espécies de *Trichosporon*.

Neste contexto, em decorrência das altas taxas de mortalidade causadas pela tricosporonose, da disponibilidade de um número reduzido de agentes antifúngicos e da redução da suscetibilidade de *T. asahii* frente aos compostos azólicos, a busca por novas estratégias, como a combinação *in vitro* entre compostos de diferentes classes, continua sendo um assunto relevante e digno de investigação. Por outro lado, a exposição prolongada ao fluconazol, *in vitro*, é um fator importante para a emergência de resistência em *T. asahii*, fenômeno este que pode agregar consequências para o perfil de suscetibilidade desta espécie na terapia isolada e em combinação a outros agentes antifúngicos.

1.2 REFERENCIAL TEÓRICO

1.2.1 O gênero *Trichosporon* e a tricosporonose

O gênero *Trichosporon* (Basidiomycota, Hymenomycetes, Tremelloidae, Trichosporonales) foi designado pela primeira vez em 1865 por Beigel, que observou esse micro-organismo causando infecção benigna em cabelos. A palavra *Trichosporon* é derivada do grego e representa a combinação das palavras "*trichos"*, que significa cabelo, e "*sporon*", que significa esporos (COLOMBO; PADOVAN; CHAVES, 2011). Dessa forma, a nomenclatura está relacionada com a presença de nódulos irregulares ao longo dos cabelos da cabeça ou pelos do corpo (CHAGAS-NETO; CHAVES; COLOMBO, 2008). A denominação inicial *T. beigelii* foi sofrendo alterações ao longo dos anos e a taxonomia do gênero foi sendo progressivamente modificada por ferramentas moleculares capazes de discriminar entre as espécies filogeneticamente relacionadas (COLOMBO; PADOVAN; CHAVES, 2011).

Atualmente, existem cerca de 50 espécies incluídas no gênero *Trichosporon*, sendo que 16 dessas são capazes de causar doença em humanos (COLOMBO; PADOVAN; CHAVES, 2011). Dentre essas, seis estão relacionadas com a maior incidência de infecções: *T. ovoides* e *T. inkin* são agentes etiológicos de *Piedra* branca em cabelos da cabeça e pelos pubianos, respectivamente; *T. asteroides* e *T. cutaneum* são encontrados em infecções superficiais, como onicomicoses por exemplo; e *T. asahii* e *T. mucoides* estão envolvidos em infecções profundas, denominadas tricosporonose invasiva (CHAGAS-NETO; CHAVES; COLOMBO, 2008; COLOMBO; PADOVAN; CHAVES, 2011; SUGITA et al., 1999).

Espécies de *Trichosporon* já foram consideradas como a segunda ou terceira levedura mais isolada em laboratórios clínicos, representando 5,5 a 10,6% de todos isolados (PFALLER et al., 2005; PFALLER et al., 2007; PFALLER et al., 2009). Em pacientes com infecção disseminada, *T. asahii*, conhecida anteriormente como *T. beigelii,* foi descrita como a espécie mais prevalente, sendo classificada como patógeno oportunista emergente (GIRMENIA et al., 2005; GUEHO; HOOG; SMITH**,** 1992; MEYER et al., 2002; WALSH et al., 2004).

Fenotipicamente, *Trichosporon* spp*.* caracteriza-se pela habilidade de formar artroconídios, blastoconídios, hifas e pseudo-hifas, sendo que todos os organismos deste gênero são capazes de assimilar diferentes carboidratos, fontes de carbono e

de degradar a ureia. Em ágar Sabouraud dextrose, as colônias crescem com aspecto de levedura, cerebriformes, de superfícies radiais, que podem se tornar secas e membranosas com o tempo. A coloração varia do branco ao creme, sendo que a diferenciação precisa das espécies de *Trichosporon* ocorre através de análise micromorfológica, aliada a ensaios bioquímicos e utilização de ferramentas moleculares (CHAGAS-NETO; CHAVES; COLOMBO, 2008; COLOMBO; PADOVAN; CHAVES, 2011; DE HOOGS et al., 2000).

O diagnóstico de tricosporonose pode ser um desafio e vários métodos moleculares têm sido desenvolvidos, incluindo aqueles baseados em PCR ("*polimerase chain reaction*"), tecnologia Luminex xMAP (uma nova técnica de citometria de fluxo com potencial para o detecção de importantes espécies de fungos na medicina) e ferramentas de proteômica (COLOMBO; PADOVAN; CHAVES, 2011). Os agentes podem ser isolados de diversos materiais biológicos, como sangue, biópsias de pele e urina (COX; PERFECT, 1998; MIRZA, 1993; TAKAMURA et al., 1999).

Organismos pertencentes ao gênero *Trichosporon* podem ser encontrados, com frequência, em substratos na natureza, como solo, madeira em decomposição, ar, rios, lagos, água do mar, besouros, excrementos de pássaros, morcegos, pombos e bovinos. No entanto, esses fungos podem também, ocasionalmente, pertencer à microbiota gastrointestinal e cavidade oral permanente dos seres humanos, bem como transitoriamente colonizar o trato respiratório e pele (COLOMBO; PADOVAN; CHAVES, 2011; GUEHO; SMITH; HOOG, 1998; WALSH et al., 2004).

Infecções por *Trichosporon* spp. estão associadas a um amplo espectro de manifestações clínicas, incluindo desde infecções cutâneas superficiais em indivíduos imunocompetentes até doença sistêmica severa em pacientes imunocomprometidos (COLOMBO; PADOVAN; CHAVES, 2011; COX & PERFECT, 1998). Na sua maioria, as espécies de *Trichosporon* são causadoras de lesões superficiais benignas, conhecidas como *Piedra* branca*.* Essas manifestações caracterizam-se pela presença de nódulos irregulares, de coloração branca ou castanha clara, que podem ser encontrados na barba, bigode, axilas e área genital (KIKEN et al., 2006; SUGITA et al., 1995). Geralmente, esse tipo de infecção apresenta resposta satisfatória após remoção do cabelo seguida por terapia antifúngica tópica e/ou oral (KIKEN et al., 2006).

Embora muitas vezes o isolamento de *Trichosporon* spp. em laboratórios esteja relacionado a episódios de colonização ou infecções superficiais, a maior relevância de *Trichosporon* spp. está como agente de micose oportunista causando infecções invasivas, principalmente em imunocomprometidos (CHAGAS-NETO; CHAVES; COLOMBO, 2008). Esse tipo de infecção causada por *Trichosporon* spp. geralmente é precedida de colonização do trato respiratório e gastrointestinal, sendo comumente associada ao uso de cateteres venosos centrais (WALSH et al., 2004).

Pacientes não imunocomprometidos portadores de próteses valvares, submetidos à diálise peritoneal ou uso de cateteres urinários também podem desenvolver tricosporonose (KRZOSSOK et al., 2004; MARTINEZ-LACASA et al., 1991; MOOTY et al., 2001; RASTOGI; NIRWAN, 2007; SOOD et al., 2006). Sendo assim, visto que infecções do trato urinário causadas por fungos podem estar associadas a dispositivos médicos, a prevalência de *Trichosporon* isolado desse tipo de paciente tem aumentado consideravelmente (RASTOGI; NIRWAN, 2007; SUN et al., 2012).

Além disso, a elevada incidência de infecção invasiva com *Trichosporon* spp. também pode estar associada com granulocitopenia e função fagocítica debilitada. Dentre os fatores de risco pode-se destacar o aumento de doenças degenerativas, o maior número de transplante de órgãos, a síndrome da imunodeficiência adquirida (SIDA), o tratamento com fármacos imunossupressores e terapias quimioterápicas, bem como o uso de antibióticos de amplo espectro e o número progressivo de procedimentos médicos invasivos (ERER et al., 2000; GIRMERNIA et al., 2005; KRCMERY, 1999; LEAF; SIMBERKOFF, 1989; MIRZA, 1993; WALSH et al, 2004; YANG; GAO; LI, 2014).

Espécies de *Trichosporon* têm sido reconhecidas como agentes causadores de fungemia, especialmente em pacientes com neutropenia e câncer, podendo a tricosporonose assemelhar-se à candidíase hematogênica (CHAGAS-NETO; CHAVES; COLOMBO, 2008). Em pacientes com doenças hematológicas malignas, este gênero foi classificado como o segundo agente mais comum de infecções fúngicas disseminadas (CHAGAS-NETO et al., 2009; COLOMBO, PADOVAN; CHAVES, 2011), conduzindo a 80% de mortalidade mesmo após tratamento com anfotericina B (GIRMENIA et al., 2005). Em pacientes com neutropenia persistente, esse índice pode atingir aproximadamente 100% (BASSETTI et al., 2004).

As manifestações clínicas descritas para a disseminação hematogênica por *Trichosporon* spp. podem incluir múltiplas lesões cutâneas, infiltrações pulmonares, danos neurológicos, corioretinite e até choque séptico, com insuficiência renal. Nesse casos, os antifúngicos triazólicos têm sido considerados os fármacos de escolha para o tratamento de infecções causadas por esse patógeno (CHAGAS-NETO et al., 2009; GUO et al., 2011; HARIZOLAN et al., 2013; JIANG et al., 2013; PAPHITOU et al., 2002; RODRIGUEZ-TUDELA et al., 2005; RUAN; CHIEN; HSUEH, 2009; TAJ-ALDEEN et al., 2009; XIA et al., 2012).

Visto que a infecção invasiva por *Trichosporon* spp. tem sido documentada principalmente em pacientes com câncer ou que são expostos a múltiplos procedimentos médicos invasivos, é possível que a capacidade de aderência do *Trichosporon* spp. e a formação de biofilmes em dispositivos implantados possam explicar o progresso da tricosporonose invasiva, uma vez que este promove a capacidade de fuga de drogas e resposta imune do hospedeiro. Além disso, a presença de glucuronoxilomanana (GXM) nas paredes celulares de *Trichosporon* spp. e a sua capacidade para produzir as proteases e lipases são todos fatores provavelmente relacionado com a virulência neste gênero (COLOMBO; PADOVAN; CHAVES, 2011).

Dessa forma, considerando que as infecções fúngicas emergentes são normalmente difíceis de diagnosticar, refratárias a antifúngicos convencionais e associadas a altas taxas de mortalidade (CHAGAS-NETO; CHAVES; COLOMBO, 2008; COLOMBO; PADOVAN; CHAVES, 2011; WALSH et al., 2004), ressalta-se que o estudo do gênero *Trichosporon* requer aprofundamento e investigações.

1.2.2 Agentes antifúngicos

Dentre as classes de agentes antifúngicos disponíveis para terapia, pode-se incluir: derivados poliênicos (anfotericina B e nistatina), azólicos (cetoconazol, fluconazol, itraconazol, voriconazol, ravuconazol, posaconazol e isavuconazol), as alilaminas (terbinafina e naftifina), análogos de pirimidina (5-flucitosina) e equinocandinas (caspofungina, anidulafungina e micafungina) (CATALÁN; MOONTEJO, 2006; DERESINSKI; STEVENS, 2003; JOHNSON et al., 2004; THOMPSON et al., 2009).

1.2.2.1 Derivados poliênicos

Os poliênicos são fármacos fungicidas produzidos por bactérias do gênero *Streptomyces*, que apresentam porções hidrofílicas e hidrofóbicas em suas moléculas. Os dois principais poliênicos disponíveis para terapia antifúngica são nistatina e anfotericina B, que têm seu mecanismo de ação baseado na desestabilização da membrana plasmática do fungo. Esses fármacos exercem sua atividade antimicótica através de sua ligação ao ergosterol, presente na membrana da célula fúngica. Desta maneira, formam-se poros ou canais com aumento na permeabilidade da membrana, alterando sua integridade e ocasionando o extravasamento de componentes citoplasmáticos vitais, levando à morte celular (GOODMAN; GILMAN, 2003).

Devido a sua toxicidade, a nistatina é usada somente para tratamento tópico. Já a anfotericina B requer administração intravenosa, intratecal ou inalatória, pois não é absorvida via oral (GUBBINS; ANAISSIE, 2002). Um dos seus principais efeitos adversos é a nefrotoxicidade, decorrente do efeito diretamente nas células tubulares renais resultando em necrose tubular aguda, vasoconstrição e redução da filtração glomerular. Mais de 80% dos pacientes que receberam anfotericina B apresentam redução da função renal e alguns permanecem com comprometimento mesmo após o término do tratamento (BRANCH, 1988).

Novas formas de apresentação da anfotericina B foram desenvolvidas pela indústria farmacêutica para tentar amenizar sua toxicidade: anfotericina B complexo lipídico (ABCL), anfotericina B em dispersão coloidal (ABCD) e a forma lipossomal (L-AmB). Estas formulações mantêm o mesmo espectro de ação da anfotericina B na forma de desoxicolato, sendo que as formas lipídicas aparentam ser mais potentes e menos nefrotóxicas (DUPONT, 2002; REX et al., 2001).

1.2.2.2 Derivados azólicos

Os azólicos são antifúngicos caracterizados por apresentarem um anel pentagonal na sua estrutura molecular, unido por uma ligação carbono-nitrogênio com outros anéis aromáticos. Estes agentes antifúngicos são classificados em imidazólicos, quando o anel pentagonal possui três átomos de carbono e dois de nitrogênio (cetoconazol, miconazol), e triazólicos quando o anel possui dois átomos

de carbono e três de nitrogênio (fluconazol, itraconazol, voriconazol, posaconazol, ravuconazol e isavuconazol) (CATALÁN; MOONTEJO, 2006; THOMPSON et al., 2009).

Seu mecanismo de ação também se faz através da alteração de permeabilidade da membrana fúngica e fluidez. Todavia, essa alteração decorre da inibição da síntese do ergosterol, pois os azólicos se ligam as enzimas do citocromo P450 do fungo (Erg11p) e inibem a 14-alfa-desmetilação do lanosterol, um precursor do ergosterol (PONTÓN; QUINDÓS, 2006; SPINOSA; GÓRNIAK; BERNARDI, 2002). A conformação exata do centro ativo difere entre as diferentes espécies fúngicas, e a ligação de cada azol com cada uma das várias classes de enzimas Erg11p determinará o seu espectro de atividade (PONTÓN; QUINDÓS, 2006).

A substituição do anel imidazol por outro triazol aumentou a especificidade de ligação com a enzima Erg11p (PONTÓN; QUINDÓS, 2006). Dessa forma, os triazólicos, diferentemente dos imidazólicos, possuem alta afinidade pelo citocromo P450 fúngico e baixa afinidade pelo citocromo P450 dos mamíferos, o que resulta em uma classe de fármacos com grande eficiência e baixa toxicidade (SPINOSA; GÓRNIAK; BERNARDI, 2002).

Todos os azólicos têm meia-vida relativamente longa, possibilitando fazer a terapia em uma única dose ou duas doses diárias. Os principais efeitos colaterais que os azólicos apresentam são hepatotoxidade, intolerância gastrintestinal e hipersensibilidade (TAVARES, 2001).

1.2.2.3 Alilaminas

A terbinafina, classificada como uma alilamina, é um agente antifúngico de uso oral e tópico que possui amplo espectro de ação *in vitro* e *in vivo* frente a fungos causadores de micoses superficiais e sistêmicas. Devido sua natureza lipofílica e queratofílica, e capacidade de acumular no tecido adiposo e queratinoso, é considerado o antifúngico de eleição para o tratamento das dermatofitoses e onicomicoses (BALFOUR; FAULDS, 1992; DARKES et al., 2003).

A ação da terbinafina decorre da capacidade de atuar na prevenção da biossíntese do ergosterol através da inibição específica e seletiva da esqualeno-epoxidase fúngica (Erg1p). Diferentemente dos azólicos, a terbinafina se liga fracamente ao citocromo P450, portanto, não interferindo na produção de hormônios

esteroides no hospedeiro, além de baixo potencial na interação com outros medicamentos. Além disso, a esqualeno epoxidase dos mamíferos é consideravelmente menos sensível do que a enzima fúngica à inibição por terbinafina. Os efeitos adversos, apesar de pouco frequentes (10,4 a 1,5%), consistem de sintomas gastrointestinais e cutâneos, cansaço e mal-estar (BALFOUR; FAULDS, 1992).

1.2.2.4 Análogos da pirimidina

A flucitosina (5FC) trata-se quimicamente de uma pirimidina fluorada, a qual dentro das células fúngicas sofre uma desaminação, responsável por sua ativação (PFALLER et al., 2002). É um agente antifúngico sintético do tipo antimetabólito, ativo contra uma gama limitada de infecções fúngicas sistêmicas, sendo principalmente eficaz nas infecções causadas por leveduras. Quando administrada isoladamente, é comum o desenvolvimento de resistência, por isso costuma ser usada em combinação com outros antifúngicos, como anfotericina B ou azólicos (CARRILLO-MUNOZ et al., 2006; DENNING et al., 2010; GOPINATHAN et al., 2013).

1.2.2.5 Equinocandinas

A mais recente classe de agentes antifúngicos é representada pelas equinocandinas: caspofungina, anidulafungina e a micafungina, as quais são constituídas de lipopeptídeos derivados de produtos naturais de fermentação fúngica. Esses fármacos atuam através da ligação e inibição da β-(1,3)–D-glucano-sintase (Fks1p), enzima responsável pela síntese da parede celular dos fungos, a qual é composta de um complexo de proteínas e policarboidratos, como glucana, manana e quitina (GUBBINS; ANAISSIE, 2002). Assim, o bloqueio dessa enzima provoca uma instabilidade osmótica comprometendo a integridade da membrana fúngica, causando morte celular (MORRIS; VILLMANN, 2006).

Devido ao distinto modo de ação, as equinocandinas evidenciam toxicidade seletiva frente a células fúngicas, uma vez que os mamíferos não possuem a molécula de glucana nas suas células (ARIKAN et al., 2005).

1.2.3 O problema terapêutico nas infecções pelo gênero *Trichosporon* e os mecanismos de resistência aos agentes antifúngicos

Resistência antifúngica é um conceito amplo que descreve as falhas da antifungicoterapia em combater uma infecção fúngica (ALEXANDER; PERFECT, 1997). Este fenômeno está relacionado a fatores como a farmacodinâmica do antifúngico, ou aqueles envolvidos com o paciente ou com a espécie causadora da infecção (PFALLER; DIEKEMA, 2007).

A resistência *in vitro* pode ser dividida em resistência primária, também conhecida como resistência intrínseca ou inata, que ocorre quando o micro-organismo é naturalmente resistente ao fármaco antifúngico, ou seja, ocorre antes da exposição a esse fármaco (ALEXANDER; PERFECT, 1997; LEWIS, 2009). Já, a resistência secundária ou adquirida, se desenvolve após a exposição aos antifúngicos devido às alterações genotípicas estáveis ou transitórias. Ocorre quando o microrganismo que está infectando o indivíduo se torna resistente após a exposição a um agente antifúngico utilizado no tratamento (ALEXANDER; PERFECT, 1997; BENNETT; IZUMIKAWA; MARR, 2004).

Um terceiro tipo de resistência antifúngica é designado "resistência clínica", que engloba a progressão ou recaída de uma infecção por um isolado de fungos que, em testes de laboratório, parecem ser totalmente suscetíveis ao antifúngico utilizado para o tratamento da infecção. Resistência clínica de fungos geralmente ocorre em pacientes imunocomprometidos (por exemplo, com Síndrome da Imunodeficiência Adquirida (SIDA), neutropenia), ou portadores de cateteres (ALEXANDER; PERFECT, 1997). Em alguns casos, as alterações na concentração da droga no sangue causadas por interações medicamentosas podem contribuir para esse tipo de resistência (KONTOYIANNIS; LEWIS, 2002).

Apesar da crescente relevância do gênero *Trichosporon* na medicina contemporânea, o tratamento de pacientes com tricosporonose continua a ser um desafio, uma vez que existem poucos dados disponíveis sobre a atividade de drogas antifúngicas *in vitro* e *in vivo* frente às espécies clinicamente relevantes. As dificuldades na identificação das espécies dentro do gênero aliada à ausência de padronização para testes de suscetibilidade frente a *Trichosporon* spp. contribuem para o limitado número de informações disponíveis sobre o tratamento dessa infecção (CHAGAS-NETO; CHAVES; COLOMBO, 2008; PAPHITOU et al., 2002).

O documento M27-A3 do "*Clinical and Laboratory Standards Institute*" (CLSI) (CLSI, 2008), o qual descreve os testes de suscetibilidade antifúngica para fungos leveduriformes, não aborda especificamente o gênero *Trichosporon*. Dessa forma, a maioria dos estudos *in vitro* de investigação da suscetibilidade de *Trichosporon* spp. a antifúngicos utilizam o método de microdiluição em caldo atualmente padronizado para *Candida* spp. e *Cryptococcus neoformans* (CLSI, 2008) ou uma adaptação do Comitê Europeu para Testes de Suscetibilidade Antimicrobiana (EUCAST) por método de microdiluição em caldo, uma recomendação originalmente proposta para o gênero *Candida* (CUENCA-ESTRELLA et al., 2003).

1.2.3.1 Resistência aos poliênicos

Dados indicam que o tratamento com anfotericina B tem atividade limitada *in vitro* e *in vivo* para espécies do gênero *Trichosporon* (CHAGAS-NETO et al., 2009; GIRMENIA et al., 2005; HOY et al., 1986; KALKANCI et al., 2010; PAPHITOU et al., 2002; RODRIGUEZ-TUDELA et al., 2005; SUZUKI et al., 2010; WOLF et al., 2001). Embora alguns isolados possam ser inibidos utilizando concentrações seguras de anfotericina B, em estudos realizados com modelos animais ou em pacientes com neutropenia, a atividade fungicida não foi observada (CHAGAS-NETO et al., 2009; GIRMENIA et al., 2005; SUZUKI et al., 2010).

Em um estudo realizado com 25 pacientes neutropênicos que desenvolveram tricosporonose sistêmica e foram tratados com anfotericina B, apenas quatro desses sobreviveram (HOY et al., 1986). Além disso, Girmenia et al. (2005) relataram uma resposta clínica de apenas 24% após o tratamento com anfotericina B em 55 pacientes com doenças hematológicas e tricosporonose disseminada (GIRMENIA et al., 2005).

O aparecimento de cepas resistentes aos poliênicos provavelmente está relacionado com alterações qualitativas e quantitativas no teor de esteróis presentes na membrana da célula fúngica. Dentre os possíveis mecanismos de resistência, pode-se destacar a susbtituição do ergosterol por outros esteróis, diminuição do conteúdo de ergosterol (DICK; MERZ; SARAL, 1980), ou ainda, aumento da atividade da catalase, que produz uma sensibilidade reduzida aos danos oxidativos (SOKOL-ANDERSON; BRAJTBURG; MEDOFF, 1986). Os principais defeitos na biossíntese do ergosterol, analisados em espécies de *Candida*, incluem as mutações

nos genes *ERG3*, *ERG5, ERG6*, *ERG11* e *ERG 25*, que acarretam na diminuição da produção ergosterol e/ou produção de esteróis anormais (BARKER; ROGERS, 2006; VANDEPUTTE et al., 2007).

1.2.3.2 Resistência aos azólicos

Quando comparados a outros antifúngicos disponíveis comercialmente, os azólicos parecem ter melhor atividade *in vitro* e *in vivo* frente *Trichosporon* spp., sendo essa característica mais acentuada na espécie de *T. asahii* do que outras espécies do gênero (ARIKAN et al., 2005; CHAGAS-NETO et al., 2009; KALKANCI et al., 2010; MONTOYA et al., 2015; PAPHITOU et al., 2002; RODRIGUEZ-TUDELA et al., 2005; RUAN; CHIEN; HSUEH, 2009; SUZUKI et al., 2010). Entretanto, o aumento do uso dessa classe de agentes antifúngicos em Unidades de Terapia Intensiva (UTIs), tem levado à seleção e isolamento de cepas mais resistentes (KUSHIMA et al., 2012; WOLF et al., 2001). Ocorrência de falhas terapêuticas com o uso de antifúngicos azólicos (KUSHIMA et al., 2012; WOLF et al., 2001), incluindo o voriconazol (BASSETTI et al., 2004; KUSHIMA et al., 2012; RASTOGI et al., 2016; RIBEIRO et al., 2008), bem como casos de cepas de *Trichosporon* spp. multirresistentes têm sido descritos (FALK et al., 2003; RASTOGI et al., 2016; RIBEIRO et al., 2008; SILVA et al., 2008; WOLF et al., 2001).

Isolados clínicos de *T. asahii* provenientes de seis pacientes não-granulocitopênicos, internados em unidades de terapia intensiva apresentaram suscetibilidade reduzida à anfotericina B, flucitosina e antifúngicos azólicos, *in vitro* (WOLF et al., 2001). Reduzida suscetibilidade a esses mesmos antifúngicos também foi relatada, posteriormente, no estudo de Silva et al. (2008) utilizando isolados clínicos de *T. asahii* recuperados da cavidade oral e urina de pacientes internados em Unidade de Terapia Intensiva.

O aparecimento de cepas de *T. asahii* multirresistentes provavelmente está relacionado com o crescente aumento do uso terapêutico dos azólicos. Kushima et al. (2012) descreveu que o uso a longo prazo de fluconazol, *in vivo*, pode induzir à susbtituição de aminoácido da *ERG11p* e, consequentemente, desencadear resistência de *T. asahii* a múltiplos fármacos (KUSHIMA et al., 2012).

1.2.3.3 Resistência à terbinafina e flucitosina

A resistência fúngica à terbinafina é pouco frequente, entretanto, quando presente pode estar relacionada com uma mutação no gene *ERG1* (KLOBUCNIKOVA et al., 2003; LEBER et al., 2003) ou superexpressão de proteínas transportadoras (bombas de efluxo), principalmente as codificadas pelo gene *CDR* (DARKES et al., 2003). Ensaios *in vitro* sugeriram que cepas de *Trichosporon* spp. podem ser menos sensíveis ou resistentes a esse antifúngico (LI et al., 2011; XIA et al., 2012). Por outro lado, Taverna et al. (2014) comprovaram a sensibilidade de *Trichosporon* spp. a essa alilamina.

Em relação à 5FC, algumas leveduras são intrinsicamente resistentes a esse agente devido a perda da permease necessária para o transporte da citosina. Por outro lado, a resistência adquirida resulta de defeitos no metabolismo da 5FC por meio de mutações na citosina desaminase ou uracil fosforribosil transferase (CARRILLO-MUNOZ et al., 2006; DENNING et al., 2010; GOPINATHAN et al., 2013).

A utilização de 5FC para o tratamento da tricosporonose é limitada, pois dados *in vitro* sugerem que cepas de *Trichosporon* podem apresentar resistência a esse antifúngico quando testados isoladamente (CHAGAS-NETO et al., 2009, MEKHA et al., 2010). No entanto, alguns autores demonstram bons resultados na combinação de 5FC com anfotericina B no tratamento da tricosporonose (GIRMENIA et al., 2005). Consequentemente, até o momento, esse agente antifúngico não tem sido recomendado com segurança para o tratamento da tricosponose invasiva.

1.2.3.4 Resistência às equinocandinas

As equinocandinas, quando administradas isoladamente, não apresentam atividade contra *Trichosporon* spp. Portanto, devido à resistência intrínsica, não são recomendadas para o tratamento tricosporonose (BAYRAMOGLU et al., 2008; SUN et al., 2008, YANG; GAO; LI, 2014).

Os casos de resistência intrínseca a essa classe de antifúngicos, descritos em *Cryptococcus* spp., *Trichosporon* spp., *Fusarium* spp. e zigomicetos estão associados com quantidades insuficientes da enzima β-(1,3)-D-glucana sintase ou uma forma mutante da enzima que impede a ligação do fármaco (CHANDRA;

MOHAMMAD; GHANNOUM, 2009). Todavia, a resistência adquirida pode estar relacionada a mutações no gene *FSK1*, que codifica uma subunidade da β-(1,3)-D-glucana sintase (CHANDRA; MOHAMMAD; GHANNOUM, 2009).

1.2.4 A terapêutica combinada como alternativa às micoses de difícil tratamento

Em resposta às falhas da terapêutica, vários autores têm buscado estratégias que garantam o sucesso da atividade antifúngica. Entre estas estratégias, a combinação de fármacos tem merecido atenção (MUKHERJEE et al., 2005). Benefícios potenciais do uso da terapia associada incluem o amplo espectro de ação, maior potência comparada à monoterapia, tolerabilidade, redução de efeitos colaterais e da resistência (LEWIS; KONTOYIANNIS, 2001). Combinações envolvendo equinocandinas e poliênicos ou equinocandinas e compostos azólicos têm demonstrado sinergismo frente a espécies de *Trichosporon* spp. (CHEN et al., 2014; BASSETTI et al., 2004; KAMBERI et al., 1998; LI et al., 2010; Li et al., 2011; SERENA et al., 2005).

A associação de micafungina e anfotericina B apresentou um índice de 78% de interações sinérgicas em um estudo com 27 isolados de basidiomicetos utilizando a técnica de *"checkerboard"* e microdiluição. Tal combinação apresentou o resultado mais significativo frente à espécie *T. asahii* (100%), a qual foi efetiva nos 10 isolados testados (SERENA et al., 2005). O mecanismo proposto para a ocorrência de interações sinérgicas entre esses fármacos é de que as equinocandinas podem aumentar a penetração da anfotericina B na membrana da célula, potencializando a atividade antifúngica desse poliênico (CHEN et al., 2014).

Da mesma forma, a terapia de combinação de caspofungina e anfotericina B foi usada com sucesso para o tratamento de paciente com tricosporonose**.** Bassetti et al. (2004) descreveram um relato de caso de fungemia causada por *T. asahii* em um paciente com neutropenia intensa e leucemia mieloide aguda. O paciente não foi curado após a administração de fluconazol, voriconazol e anfotericina B lipossomal. No entanto, a terapia teve êxito após a associação de caspofungina e anfotericina B (BASSETTI et al., 2004). Essa mesma associação, exibiu resultados satisfatórios frente *T. asahii*, apresentando efeito sinérgico expressivo (89%), em comparação às

outras combinações testadas (anfotericina B e voriconazol, e voriconazol e caspofungina) (Li et al., 2010).

Achados de interações sinérgicas de equinocandinas e outras classes de antifúngicos, além dos poliênicos, também têm sido relatados. A combinação de caspofungina com itraconazol mostrou uma alta percentagem de sinergismo (72,2%) frente a isolados clínicos de *T. asahii*, sugerindo potencial para o uso na terapia da tricosporose (Li et al., 2011). Os autores sugerem que o mecanismo de interação sinérgica entre esses antifúngicos possa ser devido à inibição simultânea de diferentes alvos na célula fúngica, tal como parede e membrana celular. Essa hipótese também pode ser aplicável na associação de equinocandina e poliênico, uma vez que anfotericina B também exerce sua ação na membrana celular (Li et al., 2011).

Desta forma, o elevado número de possíveis combinações de fármacos que podem ser testadas e as particularidades de cada espécie de fungo são desafios na descoberta de combinações sinérgicas entre os fármacos, especialmente em relação a *T. asahii* fluconazol-resistente.

1.2.4.1 Associações entre antifúngicos e quimiossensibilizantes

A resistência de *Trichosporon* spp. aos antifúngicos convencionais, especialmente às equinocandinas (BAYRAMOGLU et al., 2008; LIAO et al., 2012; KARAPINAR et al., 2015; MATSUE et al., 2006; SUN et al., 2008; YANG; GAO; Li, 2014), à anfotericina B (CHAGAS-NETO et al., 2009; KALKANCI et al., 2010; MONTOYA et al., 2015; PAPHITOU et al., 2002; RASTOGI et al., 2016; RODRIGUEZ-TUDELA et al., 2005) e, posteriormente, aos azólicos (FALK et al., 2003; RASTOGI et al., 2016; RIBEIRO et al., 2008; SILVA et al., 2008; WOLF et al., 2001) sugere a busca de novas estratégias terapêuticas, tais como a associação entre antifúngicos e compostos de diferentes classes. Esses compostos, muitas vezes, não evidenciam ação direta (intrínseca) sobre a célula fúngica, no entanto, podem interferir em alguns mecanismos celulares permitindo uma maior penetração e ação do agente antifúngico (MUKHERJEE et al., 2005).

O termo quimiossensibilização foi originalmente introduzido como uma estratégia para combater o desenvolvimento de resistência em células tumorais a agentes antineoplásicos. Envolve a utilização de um produto químico que torna as

células cancerígenas mais sensíveis a um agente terapêutico, resultando em doses menores de fármacos antineoplásicos citotóxicos e, consequentemente, superando a resistência das células cancerígenas a estes fármacos (SHABBITS; HU; MAYER, 2003). Tal fato evita a toxicidade para as células não-alvo e diminui os efeitos colaterais para o paciente associados aos fármacos antineoplásicos (CAMPBELL; CHAN; KIM, 2012).

Os mecanismos de resistência desenvolvidos por fungos patógenos frente aos agentes antifúngicos são quase que paralelos aos desenvolvidos pelas células frente aos fármacos quimioterápicos. O desenvolvimento de células cancerígenas resistentes a agentes antineoplásicos envolve mutações em genes-alvos, aumento da regulação ou expressão de genes controladores de bombas de efluxo e da produção de enzimas que detoxificam as drogas e atuam no reparo de DNA (CAMPBELL; CHAN; KIM, 2012; SHABBITS; HU; MAYER, 2003). Dessa forma, em patógenos fúngicos, os quimiossensibilizantes agem desestabilizando a integridade da membrana fúngica, inibindo bombas de efluxo ou induzindo ao estresse oxidativo (CAMPBELL; CHAN; KIM, 2012).

Quando utilizados como monoterapia, os agentes quimiossensibilizantes podem ter uma atividade antifúngica insignificante. No entanto, quando esses são empregados em associação com agentes antifúngicos podem resultar em ação sinérgica, uma vez que podem afetar o patógeno alvo tornando-o mais vulnerável ao agente antifúngico. Com a quimiossensibilização, a dose efetiva do agente antifúngico pode ser significativamente reduzida, além de possibilitar a reversão de cepas anteriormente resistentes a determinado antifúngico em cepas sensíveis (JOHNSON et al., 2004).

1.2.4.1.1 Tacrolimus

Tacrolimus (previamente conhecido como FK506) é um agente imunossupressor, produzido a partir de *Streptomyces tsukubaensis,* amplamente utilizado para prevenção à rejeição de transplante de órgãos (STEINBACH et al., 2007). Assim como a ciclosporina, esse fármaco exerce o bloqueio do sistema imune através da inibição da calcineurina, uma proteína fosfatase envolvida na ativação de linfócitos, que são responsáveis pela síntese de citocinas, e regulação do NFAT (*nuclear factor of activated T-cells*) (BAKSH; BURAKOFF, 2000).

Além disso, essa proteína pode agir na fisiologia fúngica, incluindo a regulação da progressão do ciclo celular, homeostase de cátions, biossíntese da parede celular, virulência e resistência fúngica (BLANKENSHIP; HEITMAN, 2005). Imunossupressores têm sido descritos capazes de inibir a função da calcineurina em *Candida* spp., *Cryptococcus neoformans* e *Aspergillus fumigatus,* afetando funções essenciais da célula fúngica (BLANKENSHIP; HEITMAN, 2005; STEINBACH et al., 2007, LI et al., 2015).

Estudos também têm demonstrado que inibidores da calcineurina podem exercer interação sinérgica quando combinados com caspofungina (KONTOYIANNIS et al., 2003; SHALIT et al., 2009; STEINBACH et al., 2004;) ou antifúngicos azólicos (DENARDI et al., 2015; MAESAKI et al., 1998; SUGITA et al., 2005; SUN et al., 2008; UPPULURI et al., 2008) frente a patógenos humanos, tais como *Cryptococcus neoformans*, *Fusarium* spp., *Aspergillus* spp., *Candida* spp. e *Malassezia* spp. Entretanto, até o momento, não se tem conhecimento de relatos na literatura sobre a ação de inibidores da calcineurina frente a espécies de *Trichosporon*.

1.2.4.1.2 Organocompostos de selênio

A importância biológica do selênio, e suas formas inorgânicas, levaram ao desenvolvimento de compostos orgânicos de selênio farmacologicamente ativos e com baixa toxicidade (MUGESH; DU MONT; SIES, 2001; WIRTH, 2000). O interesse na utilização dessas moléculas em bioquímica começou a partir de achados que revelaram que esses compostos são muito menos tóxicos do que espécies inorgânicas de selênio (MUGESH; DU MONT; SIES, 2001).

A crescente descrição das propriedades farmacológicas desta classe de compostos, tais como antioxidantes, inibidores enzimáticos, neuroprotetores, agentes antitumorais e anti-infecciosos, indutores de citocinas e imunomoduladores (MUGESH; DU MONT; SIES, 2001; NARAJJI; KARVEKAR; DAS, 2007; NOGUEIRA; ZENI; ROCHA, 2004) tem incentivado a pesquisa de novas propriedades terapêuticas dos organocompostos. Dentre esses, merecem destaque o disseleneto de difenila [$(PhSe)_{2;}$ DPDS] e o ebselen (2-fenil-1,2- benzisoselenazol-3(2H)-one; EBS).

O DPDS é um organocomposto de selênio simples, estável, altamente lipofílico e amplamente utilizado como intermediário em sínteses orgânicas, o qual reage de forma muito eficaz com hidroperóxidos e peróxidos orgânicos (WIRTH, 2000). Recentemente, as atividades biológicas do DPDS foram estudadas e este demonstrou ser um bom candidato para fins terapêuticos. Dentre suas principais propriedades farmacológicas, destacam-se as atividades antioxidante (PRIGOL et al., 2009; STANGHERLIN et al., 2009), antinociceptiva, anti-inflamatória (NOGUEIRA et al., 2003; SAVEGNAGO et al., 2007; SAVEGNAGO et al., 2008; ZASSO et al., 2005), antiúlcera (GHISLENI et al., 2008) e hepatoprotetora (NOGUEIRA; BORGES; SOUZA, 2009). Além disso, estudos em modelos animais demonstram que em doses com efeitos biológicos conhecidos o DPDS não apresenta efeitos tóxicos (SAVEGNAGO et al., 2007).

A atividade microbiológica do DPDS já foi evidenciada frente a fungos filamentosos (*Aspergillus* spp. e *Fusarium* spp.), *Cryptococcus neoformans*, espécies de *Candida* e *Pythium insidiosum* (DENARDI et al., 2013; LORETO et al., 2011a; LORETO et al., 2011b; LORETO et al., 2012; ROSSETI et al., 2011; ROSSETI; ROCHA; COSTA, 2015; SOTEROPOULOS et al., 2000; VENTURINI et al., 2016). Uma hipótese para o mecanismo biológico de sua ação inibitória é a interação com grupos sulfidrilas de biomoléculas presentes na célula fúngica (BILLACK; SANTORO; LAU-CAM, 2009; WOJTOWICZ et al., 2004).

O significado dos estudos de avaliação da atividade antifúngica do disseleneto de difenila não está apenas na descoberta das propriedades dos organocompostos de selênio, mas também, no seu potencial para desempenhar um papel complementar no tratamento de infecções fúngicas (LORETO et al., 2011a). Nesse sentido, posteriormente, estudos realizados por Denardi et al. (2013) e Venturini et al. (2016) foram desenvolvidos a fim de analisar a associação de DPDS com antifúngicos convencionais frente isolados clínicos de *Candida glabrata* e *Fusarium* spp., respectivamente. Elevados percentuais de sinergismo foram exibidos na associação do composto orgânico com anfotericina B (acima de 70%) evidenciando seu potencial para aplicação na terapia adjuvante frente a patógenos fúngicos humanos (DENARDI et al., 2013; VENTURINI et al., 2016).

Estudos sobre as propriedades antifúngicas do EBS, composto sintético, de baixa toxicidade que compartilha propriedades farmacológicas com o DPDS (NOGUEIRA; ZENI; ROCHA, 2004), demonstraram sua atividade contra espécies

de *Candida* e *Aspergillus* (BIEN et al., 1999; BILLACK; SANTORO; LAU-CAM, 2009; WOJTOWICZ et al., 2004), *Cryptococcus* (SOTEROPOULOS et al., 2000), *Saccharomyces* (BIEN et al., 1999; BILLACK; SANTORO; LAU-CAM, 2009; CHAN et al., 2007) e *Fusarium* (VENTURINI et al., 2016).

Além de sua atividade frente a leveduras e fungos filamentosos, o EBS e seus análogos demonstraram inibição do efeito citopático do vírus herpes simples tipo 1 (HSV-1), vírus da encefalomiocardite (EMCV), bem como ação frente a bactérias Gram-positivas (*Staphylococcus aureus* e *Bacillus*) (WOJTOWICZ et al., 2004) e ao protozoário *Trypanosoma brucei*, agente etiológico da doença do sono em humanos (JOICE et al., 2013). As propriedades antimicrobianas do EBS, decorrentes da inibição da tiorredoxina redutase de bactérias (LU et al., 2013) bem como da inativação de uma importante enzima da glicólise de *T. brucei* (TbHK1) sugerem que os derivados benzisoselenazol podem ser úteis para a investigação e, posterior desenvolvimento de novos compostos para uso terapêutico no tratamento de infecções.

Até o momento, a ação sinérgica do EBS para aplicação na terapia combinada com fármacos antifúngicos foi estudada apenas frente a espécies de *Fusarium* (VENTURINI 2016). Os mecanismos de ação antifúngica propostos para o EBS são similares aos outros organocompostos de selênio (BILLACK; SANTORO; LAU-CAM, 2009; WOJTOWICZ et al., 2004). Estudos sugerem que esse composto é capaz de interagir com grupos sulfidrilas das células (WOJTOWICZ et al., 2004) assim como inibir a H+-ATPase da membrana plasmática fúngica (BILLACK; SANTORO; LAU-CAM, 2009; BILLACK et al., 2010; CHAN et al., 2007; SOTEROPOULOS et al., 2000). Por outro lado, a atividade do EBS frente a cepas de *Candida albicans* resistentes ao fluconazol (BILLACK; SANTORO; LAU-CAM, 2009) enfatiza a necessidade de exploração de sua ação frente a micro-organismos resistentes a esse azólico.

1.3 PROPOSIÇÃO

Avaliar a suscetibilidade *in vitro* de isolados clínicos de *T. asahii* aos antifúngicos de diferentes classes e a interação de diferentes combinações de antifúngicos, bem como entre antifúngicos e não antifúngicos e mais especificamente:

- Identificar, genotipicamente, isolados clínicos identificados fenotipicamente como *Trichosporon* spp.;

- Induzir a resistência de isolados clínicos de *T. asahii* frente ao fluconazol;

- Avaliar a suscetibilidade de isolados clínicos de *T. asahii* frente aos antifúngicos: anfotericina B, fluconazol, itraconazol, voriconazol, posaconazol, caspofungina, micafungina e anidulafungina, antes e após exposição prolongada ao fluconazol;

- Avaliar o fenômeno de resistência cruzada entre antifúngicos azólicos para *T. asahii* fluconazol-resistente;

- Avaliar a atividade das combinações entre agentes antifúngicos de diferentes classes frente aos isolados fluconazol-resistentes;

- Avaliar a suscetibilidade de *T. asahii* fluconazol-sensíveis e fluconazol-resistentes frente ao tacrolimus, isoladamente, e em associação com anfotericina B, fluconazol, itraconazol e caspofungina.

- Avaliar a suscetibilidade de *T. asahii* fluconazol-sensíveis e fluconazol-resistentes frente aos organocompostos de selênio disseleneto de difenila e ebselen, isoladamente, e em associação com anfotericina B, fluconazol, itraconazol e caspofungina.

1.4 MATERIAIS E MÉTODOS

As metodologias, resultados e discussões desta tese de doutorado encontram-se descritos em três capítulos: Artigo 1: “Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates”; Manuscrito 1: *“In vitro* activity of diphenyl diselenide and ebselen alone and in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates”; Manuscrito 2: “*In vitro* activity of antifungal agents alone and in combination against *Trichosporon asahii* isolates before and after prolonged exposure to fluconazole”, e representam a íntegra deste estudo.

Os itens DISCUSSÃO e CONCLUSÕES, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre o artigo científico e manuscritos contidos neste trabalho. As REFERÊNCIAS referem-se somente às citações que aparecem nos itens REFERENCIAL TEÓRICO, DISCUSSÃO e CONCLUSÕES desta tese, uma vez que as referências utilizadas para a elaboração dos artigos estão mencionadas nos mesmos.

2 ARTIGO

Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates

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Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates

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ABSTRACT

The antifungal activity of tacrolimus in combination with antifungal agents against different fungal species has been previously reported. Here we report the *in vitro* interactions between tacrolimus and amphotericin B, fluconazole, itraconazole, and caspofungin against 30 clinical isolates of both fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii*. For these analyses, we used the broth microdilution method based on the M27-A3 technique and checkerboard microdilution method. Tacrolimus showed no activity against *T. asahii* strains (minimal inhibitory concentrations, MICs > 64.0 μg mL^{-1}). However, a larger synergistic interaction was observed by the combinations tacrolimus + amphotericin B (96.67%) and tacrolimus + caspofungin (73.33%) against fluconazole-susceptible isolates. Combinations with azole antifungal agents resulted in low rates of synergism for this group (fluconazole + tacrolimus = 40% and itraconazole + tacrolimus = 10%). Antagonistic interactions were not observed. For the fluconazole-resistant *T. asahii* group, all tested combinations showed indifferent interactions. The synergism showed against fluconazole-susceptible *T. asahii* isolates suggests that the potential antifungal activity of tacrolimus deserves *in vivo* experimental investigation, notably, the combination of tacrolimus with amphotericin B or caspofungin.

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Introduction

The incidence of invasive mycoses caused by emergent fungal pathogens has proportionally grown with the increased number of immunocompromised hosts, such as AIDS patients, transplant recipients treated with immunosuppressive drugs, and those on cancer therapy.[1–3] *Trichosporon asahii* is the most frequently involved species in disseminated and deep-seated trichosporonosis including systemic infections due to therapeutic failure in transplanted patients.[2,3]

The first-line treatment for trichosporonosis includes the use of azole antifungal agents, since *Trichosporon* spp. is resistant to amphotericin B and echinocandins.[4–6] However, the frequent exposure to azoles can lead to development of secondary resistance to azoles and sometimes to multidrug resistance, resulting in therapeutic failures[7–9] and increasing mortality rates.[1,10] Combination therapy is a rational alternative that has been studied to improve the efficacy of antimicrobial therapy for difficult-to-treat infections, including overcoming concerns of antimicrobial resistance.[11–13]

Recent studies have shown that using fungal calcineurin pathways holds great promise for the future development of novel agents, including combination therapy with antifungals against fungal pathogens.[14–17] The antifungal activity obtained by the combination of the calcineurin inhibitor tacrolimus (FK506) plus antifungal agents has not yet been evaluated against *Trichosporon* species. In this context, the aim of this study was to evaluate the *in vitro* activity of the combination of FK506 with amphotericin B, fluconazole, itraconazole, and caspofungin against fluconazole-susceptible and fluconazole-resistant *T. asahii* strains.

Material and methods

Clinical isolates and molecular identification

One group of 30 fluconazole-susceptible (FS) strains of clinical isolates *T. asahii* maintained in the collection of the Department of Microbiology and Parasitology at the Federal University of Santa Maria, Santa Maria, RS, Brazil were studied. A second group of fluconazole-resistant strains (FR) ($n=30$) was obtained from the FS group after sequential exposure to growing concentrations of fluconazole, as previously described by Fekete-Forgacs et al.,[18] with the following modifications: the final concentration of fluconazole was 128 $\mu g\,mL^{-1}$, and the incubation temperature was 35 °C with shaking for 48 h. Cells from this culture were plated on SDA plates, and a single colony was designated isolated FR. The standard strain *T. asahii* CBS 2479 was also included in the susceptibility tests.

The identity of these isolates was confirmed using standard microbiological and molecular methods. Total DNA was extracted according to the protocol described by Moller et al.[19] and Klassen et al.[20] with modifications. Amplification of the IGS1 region (rDNA intergenic) was performed by PCR using the primers 26F (5′ATCCTTTGCAGACGACTTGA-3′) and 5SR (5′AGCTTGACTTCGCAGATCGG-3′).[5] The PCR products were purified and sequenced. These sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers: KR233249, KT438844, KR872655, KR872656, KR872657, KR872660, KR872661, KR233246, KR233247, KR233248, KT365854, KT365855, KR872662, KT438839, KT438840, KR872663, KR912056, KT438842, KT365856, KT438847, KR912058, KR912064, KT365858, KR912065, KT365859, KT438846, KT365860, KR912059, KT365861, KR912066.

Chemicals

Amphotericin B (AMB) (Sigma Chemical Co. – St. Louis, MO, USA), fluconazole (FCZ) (Sigma Chemical Co. – St. Louis, MO, USA), itraconazole (ITZ) (Frangon of Brazil, Pharmaceutical, Ltd., São Paulo, Brazil), caspofungin (CAS) (Merck, Darmstadt, Germany), and tacrolimus (FK506) (Janssen-Cilag Pharmaceutica, Beerse, Belgium) were employed.

The stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co.), except for FCZ, which was diluted in sterile distilled water. FK506 was dissolved in methanol. Working solutions were prepared according to the document M27-A3 of the Clinical and Laboratory Standards Institute.[21]

In vitro susceptibility and drug interaction tests

Susceptibility assays were performed using the broth microdilution method, as described by the document M27-A3 of the Clinical and Laboratory Standards Institute.[21] The strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included in the tests as controls. The minimum inhibitory concentrations (MICs) were defined as the lowest drug concentration able to inhibit 50% (for FCZ, ITZ, and CAS) or 100% (for AMB and FK506) of fungal growth when compared to the growth of the control. The highest concentrations used were 16 $\mu g\,mL^{-1}$ for AMB, 128 $\mu g\,mL^{-1}$ for FCZ, 16 $\mu g\,mL^{-1}$ for ITZ and 64 $\mu g\,mL^{-1}$ for CAS and FK506. The MICs employed as indicators for resistance were: $\geq 2\,\mu g\,mL^{-1}$ (AMB)[5,22,23]; $\geq 64\,\mu g\,mL^{-1}$ (FCZ)[6,24]; $\geq 1\,\mu g\,mL^{-1}$ (ITZ),[23] and $\geq 2\,\mu g\,mL^{-1}$ (CAS).[5,23] After exposing the strains to growing concentrations of FLZ, the strains were separated in two groups: (a) FS group (fluconazole-susceptible group), formed by the strains not exposed to FLZ; and (b) FR group (fluconazole-resistant group) formed by the same strains after exposure to FLZ, and now showing MICs ranging from 64 to 128 $\mu g\,mL^{-1}$.

In vitro combinations of antifungal agents with FK506 against the FS and FR groups of *T. asahii* were evaluated by the microdilution checkerboard method.[25] For the calculation of the fractional inhibitory concentration index (FICI), MIC values related to 100% inhibition of growth were used. Synergism was defined as the FICI ≤ 0.5, indifference was $0.5 < \text{FICI} \leq 4.0$, and antagonism FICI > 4.0. The FICIs were calculated for all wells along the turbidity/non-turbidity interface where the lowest FICI as the final point was determined.

Statistical analysis

The statistical analysis used to test the Susceptibility of the two groups (susceptible strains *vs.* resistant strains) to treatment with individual antifungal agents was analyzed with the *t*-test. Statistical significance was set at $p<0.05$. Results

were analyzed using the software Graph Pad Prism5 (Graph Pad Software version 6.01, CA).

Results

The *in vitro* susceptibilities of 30 *T. asahii* isolates (FS) against the antifungal agents and FK506 are described in Table 1. FS strains showed low MICs range for FCZ (1.0–16.0 $\mu g\,mL^{-1}$) and ITZ (0.13–1.0 $\mu g\,mL^{-1}$) compared to FR strains: FCZ (64.0 to >128.0 $\mu g\,mL^{-1}$) and ITZ (0.5–16.0 $\mu g\,mL^{-1}$). Interestingly, for AMB, the number of resistant isolates decreased (90–3.33%) in the FR group (MIC range = 0.13–4.0 $\mu g\,mL^{-1}$); and for CAS the MICs remained similar in the two groups (MIC range for FS or FR = 4.0–16.0 $\mu g\,mL^{-1}$). FK506 did not show antifungal activity against FS and FR isolates at the highest concentration tested (MICs > 64.0 $\mu g\,mL^{-1}$). The statistical analysis showed significant differences between the susceptibility of FS *vs.* FR groups for azole antifungal agents (FLZ: $p < 0.0001$, ITZ: $p < 0.007$), as well as for AMB ($p < 0.0001$) and CAS ($p < 0.0001$).

The MICs and combinations results of antifungal agents and tacrolimus against *T. asahii* before and after resistance induction are presented in Tables 2 and 3. FK506 combined with AMB against the FS *T. asahii* isolates showed the highest percentage of synergism (96.67%), followed by the combination with CAS (73.33%). In the synergistic interactions between AMB + FK506 or CAS + FK506, the MIC values of FK506 decreased from 64 $\mu g\,mL^{-1}$ to 0.5 $\mu g\,mL^{-1}$. In contrast, in the FR group the majority of combinations with FK506 showed interactions classified as indifferent: AMB (76.67%), CAS (73.33%), FCZ (63.33%) and ITZ (50%). Antagonisms were not detected for combinations with FCZ and CAS but were showed with AMB (13.33%) and ITZ (10%).

Discussion

T. asahii is the most frequently involved species in disseminated fungal infections including cases of systemic infection in transplanted patients related to therapeutic failures.[2,3] The prognosis of trichosporonosis is very poor, showing mortality rates as high as 80%.[1,10] The lack of response to therapy is related to the relative resistance of *T. asahii* to many different antifungals.[3,4,7,9]

Moreover, the correct identification of *Trichosporon* species is important because the susceptibility is species dependent. In agreement with previous reports,[10,26,27] our results confirm that *T. asahii* clinical isolates seem to be more resistant *in vitro* to amphotericin B than to triazole compounds (Table 1).

Interestingly, our findings demonstrated that after sequential exposure to growing concentrations of fluconazole, the number of resistant isolates to AMB (MIC values $\geq 2\,\mu g\,mL^{-1}$) decreased from 90% to 3.33% (Table 1). Although this inverse relationship has been previously reported,[5,7,27,28] targeted deletion of *ERG3* or *ERG11* in *Candida albicans* and *Candida glabrata* seems to be a potential mechanism that may lead to increased susceptibility to AMB with increased resistance to azoles.[29,30]

Similarly, clinical failure and breakthrough infections with *Trichosoporon* have been reported with the use of echinocandins.[3,31–34] As expected, we found MICs $\geq 4\,\mu g\,mL^{-1}$ for caspofungin in all our isolates (Table 1). The cases of intrinsic resistance to echinocandins already described for *Cryptococcus* spp., *Trichosporon* spp., *Fusarium* spp., and zygomycetes are associated with insufficient sensitivity of the target enzyme, beta 1,3-D-glucan synthase, to the drug or a mutated form of the enzyme that precludes echinocandin binding.[35]

Regarding the azole antifungal agents, our results demonstrated that prolonged exposure of the clinical isolates to fluconazole showed an increased MIC for itraconazole characterizing cross-resistance among azoles (Table 1). Multidrug resistance to antifungal agents has been reported for *T. asahii* in previous studies.[4,7,9] *T. asahii* clinical isolates from nongranulocytopenic patients showed reduced susceptibility *in vitro* to AMB, flucytosine, fluconazole, itraconazole and ketoconazole.[7] Kushima et al.[9] described that long term use of fluconazole *in vivo* may lead to replacement of the amino acid *ERG11p* and thus trigger *T. asahii* resistance to multiple drugs. This study demonstrated that the MICs for other azole antifungal agents against *T. asahii* strains increased in parallel with the MIC for fluconazole, while the AMB MICs did not significantly change.[9]

Tacrolimus (previously known as FK506) is an effective immunosuppressant, obtained from *Streptomyces tsukubaensis*, and is widely used for prevention of transplant rejection.[36]

Table 1 – *In vitro* susceptibility of *Trichosporon asahii* isolates to antifungal agents and tacrolimus.

Agents	Group of isolates	Geometric mean	MIC range	MIC_{50}	MIC_{90}	Number (%) of resistant isolates
AMB	FS	2.24	1.0–8.0	2.0	4.0	27 (90)
	FR	0.41	0.13–4.0	0.25	1.0	1 (3.33)
FCZ	FS	2.41	1.0–16.0	2.0	8.0	0 (0)
	FR	64.00	64.0 to >128.0	64.0	64.0	30 (100)
ITZ	FS	0.27	0.13–1.0	0.25	0.50	1 (3.33)
	FR	1.52	0.5–16.0	1.0	4.0	27 (90)
CAS	FS	7.82	4.0–16.0	8.0	8.0	30 (100)
	FR	7.29	4.0–16.0	8.0	8.0	30 (100)
FK506	FS	ND	>64.0	ND	ND	ND
	FR	ND	>64.0	ND	ND	ND

MIC range, minimal inhibitory concentration range ($\mu g\,mL^{-1}$); MIC50, MIC at which 50% of isolates tested were inhibited; MIC90, MIC at which 90% of isolates tested were inhibited; AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; FS e FR, fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates; ND, not determined.

Table 2 – Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* before resistance induction.

Isolates	MICs (μg mL^{-1}) and combinations results before resistance induction											
	AMB/FK506			FCZ/FK506			ITZ/FK506			CAS/FK506		
	On its own	In the combination		On its own	In the combination		On its own	In the combination		On its own	In the combination	
06	2.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
11	2.00	0.25	S	2.00	1.00	I	1.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
13	2.00	0.50	S	4.00	1.00	S	1.00	1.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	4.00		>64.00	0.50		>64.00	0.50	
19	2.00	0.25	S	4.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
21	2.00	0.50	S	32.00	1.00	I	2.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
29	4.00	1.00	S	64.00	1.00	I	2.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
31	2.00	0.25	S	4.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.5		>64.00	0.50	
32	4.00	1.00	S	16.00	1.00	I	2.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
36	1.00	0.13	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
43	2.00	0.25	S	64.00	1.00	S	1.00	0.25	S	32.00	4.00	S
	>64.00	0.50		>64.00	4.00		>64.00	0.50		>64.00	0.50	
44	4.00	0.25	S	8.00	1.00	I	1.00	2.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
46	2.00	0.50	S	4.00	1.00	I	1.00	1.00	I	32.00	16.00	I
	.>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
47	8.00	0.50	S	32.00	1.00	I	2.00	1.00	I	64.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
48	2.00	0.50	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
49	2.00	0.25	S	16.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
50	2.00	0.25	S	8.00	1.00	S	1.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
51	4.00	0.25	S	2.00	1.00	I	1.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
53	4.00	0.25	S	4.00	1.00	S	2.00	0.50	S	16.00	16.00	I
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
54	2.00	0.50	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
56	2.00	0.50	S	8.00	1.00	I	1.00	0.50	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
58	2.00	0.50	S	16.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
59	1.00	0.50	I	16.00	1.00	S	1.00	0.25	S	64.00	8.00	S
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
60	2.00	0.25	S	8.00	1.00	S	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
61	8.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
62	2.00	0.25	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
63	2.00	0.50	S	8.00	1.00	S	1.00	0.02	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	64.00		>64.00	0.50	
64	2.00	0.25	S	4.00	1.00	S	0.50	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
65	4.00	0.25	S	8.00	1.00	I	0.50	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
66	2.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
67	1.00	0.25	S	4.00	1.00	S	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	

MICs, minimal inhibitory concentrations as the lowest concentration that showed 100% inhibition of fungal growth (μg mL^{-1}); AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; S, synergism; I, indifference.

Table 3 – Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* after resistance induction.

Isolates	MICs ($\mu g\,mL^{-1}$) and combinations results after resistance induction											
	AMB/FK506			FCZ/FK506			ITZ/FK506			CAS/FK506		
	On its own	In the combination		On its own	In the combination		On its own	In the combination		On its own	In the combination	
06	1.00	0.50	I	>128.00	32.00	S	>16.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
11	0.25	0.25	I	128.00	32.00	S	4.00	1.00	S	8.00	4.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
13	0.50	0.50	I	>128.00	64.00	I	>16.00	4.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
19	0.50	0.25	I	128.00	64.00	I	4.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
21	0.25	0.25	I	128.00	32.00	S	2.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
29	1.00	0.25	S	128.00	128.00	I	>16.00	8.00	I	16.00	32.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
31	0.50	0.13	S	128.00	128.00	I	4.00	0.50	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
32	0.25	0.50	I	128.00	128.00	I	>16.00	4.00	S	16.00	32.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
36	0.50	0.25	I	128.00	64.00	I	2.00	2.00	I	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
43	0.25	0.25	I	128.00	32.00	S	2.00	1.00	I	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
44	0.25	0.25	I	128.00	32.00	S	>16.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
46	1.00	1.00	I	128.00	128.00	I	>16.00	2.00	S	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
47	4.00	0.25	S	64.00	64.00	I	2.00	2.00	I	16.00	16.00	I
	>64.00	5.00		>64.00	0.50		>64.00	0.50		>64.00	0.50	
48	0.50	0.25	I	>128.00	32.00	S	4.00	1.00	S	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
49	0.50	0.25	I	128.00	64.00	I	8.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
50	0.50	0.25	I	128.00	32.00	S	>16.00	1.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
51	0.25	0.25	I	128.00	32.00	S	2.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
53	0.25	0.50	I	128.00	128.00	I	2.00	16.00	A	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
54	0.25	0.13	I	128.00	64.00	I	>16.00	1.00	I	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
56	0.13	0.50	A	>128.00	64.00	I	>16.00	8.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
58	0.50	2.00	A	128.00	128.00	I	8.00	32.00	A	16.00	1.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
59	0.50	0.25	I	>128.00	64.00	I	4.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
60	0.25	0.50	I	128.00	128.00	I	>16.00	32.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
61	0.25	0.50	I	>128.00	64.00	I	>16.00	4.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
62	1.00	0.50	I	>128.00	128.00	I	4.00	2.00	I	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
63	0.25	2.00	A	>128.00	8.00	S	16.00	8.00	I	16.00	2.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
64	0.25	0.13	I	128.00	64.00	I	2.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
65	0.25	1.00	A	>128.00	16.00	S	1.00	8.00	A	16.00	1.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
66	0.25	0.50	I	128.00	64.00	I	1.00	2.00	I	16.00	2.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
67	0.50	0.50	I	128.00	32.00	S	8.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	

MICs, minimal inhibitory concentrations as the lowest concentration that showed 100% inhibition of fungal growth ($\mu g\,mL^{-1}$); AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; S, synergism; I, indifference; A, antagonism.

This compound exerts its effects by blocking the immune system through inhibition of calcineurin.[37] Moreover, this protein can also affect essential functions of the fungal cell and it is intrinsically involved in the growth and pathogenesis of three major fungal species: *Cryptococcus neoformans*, *C. albicans*, and *Aspergillus fumigatus*.[36,38] In this study, FK506 showed low antifungal activity when tested alone against FS and FR *T. asahii* isolates (MICs > 64.0 $\mu g\,mL^{-1}$).

However, against the FS group, the results of our study (Table 2) demonstrate strong *in vitro* synergism of FK506 combined with drugs that were ineffective in inhibiting this group of *T. asahii* clinical isolates (Table 1) such as caspofungin (73.33%) and AMB (96.67%). High percentage synergistic interactions for caspofungin plus FK506 have been previously reported against *C. neoformans*, *Fusarium* spp., and *Aspergillus* spp.[11,14,39]

The potential enhanced antifungal activity of caspofungin in combination with other antifungal agents and anti-calcineurin drugs against clinical isolates of *Fusarium* spp. was demonstrated by Shalit et al.[11] The association of this echinocandin with FK506 appeared synergistic against all the isolates tested.[11] The antifungal effect exhibited by immunosuppressants cyclosporin A and FK506 is probably related to calmodulin activated protein phosphatase involved in fungal stress response, virulence, and antifungal resistance.[36] Steinbach et al.[14] also demonstrated a synergistic interaction of cyclosporin and FK506 with caspofungin against *A. fumigatus*. In this study, the calcineurin inhibitors were capable of causing a delay in filamentation of *A. fumigatus*, which suggested that inhibition of this pathway may potentiate the action of conventional antifungal agents in combination therapy against invasive aspergillosis.[14]

The pharmacokinetics of caspofungin is unaltered by coadministration of tacrolimus, but caspofungin may reduce tacrolimus concentrations by up to 20%.[40] Therefore, monitoring standard tacrolimus blood concentrations and appropriate tacrolimus dose adjustments are recommended for patients receiving both therapies.

On the other hand, the synergism observed with the association of FK506 and AMB can benefit the antifungal therapy regimen through the reductions in time to treatment response, dose with associated toxicity, costs, and decreased potential of microorganism-acquired resistance. Although AMB has been reported to have an insignificant effect on tacrolimus metabolism,[41] this polyene is well known to be nephrotoxic. Therefore, monitoring of serum creatinine is probably warranted for patients receiving both drugs.[42]

Studies have also shown that calcineurin inhibitors may exert a synergistic interaction when combined with antifungal azoles against *Candida* species.[15,16] However, our results demonstrated that combinations of FCZ or ITZ with FK506 produced interactions mainly indifferent emphasizing a lack of effect against FS and FR *T. asahii* isolates (Tables 2 and 3). The pharmacokinetics interactions between FK506 and azoles are well known and show that azoles inhibit the metabolism of FK506, requiring monitoring of the plasmatic concentration of FK506.[42]

In conclusion, our findings demonstrated that the combination of FK506 with AMB or CAS leads to high rates of synergism *in vitro*. These combinations against *T. asahii* isolates that presented resistance to both AMB and CAS deserve attention as candidates for *in vivo* studies focusing *T. asahii* experimental infections.

Conflicts of interest

The authors declare no conflicts of interest.

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3 MANUSCRITO 1

In vitro activity of diphenyl diselenide and ebselen alone and in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates

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Full title: *In vitro* activity of diphenyl diselenide and ebselen alone and in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates

Short title: Organoselenium compounds against *Trichosporon asahii*

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Key words: susceptibility, organoselenium compounds, fluconazole resistance, interactions, microdilution.

Abstract

This study evaluated the *in vitro* susceptibility of fluconazole-susceptible (FS, n = 30) and fluconazole-resistant (FR, n = 30) *Trichosporon asahii* isolates to diphenyl diselenide (DPDS) and ebselen (EBS) alone and in combination with amphotericin B (AMB), fluconazole (FCZ), itraconazole (ITZ), and caspofungin (CAS). For these assays, we used a broth microdilution technique based on the M27-A3 document from the Clinical and Laboratory Standards Institute (CLSI) and a checkerboard microdilution method. EBS exhibited antifungal activity against both the FS and FR *T. asahii* isolates (minimal inhibitory concentration; MIC range = 0.25–0.5 and 1–8 μg mL^{-1}, respectively). For DPDS, the MIC range values were higher: 8–64 and 16–64 μg mL^{-1} for the FS and FR groups, respectively. The combinations demonstrating the greatest synergistic activity against FR *T. asahii* strains were: CAS + DPDS (96.67%), AMB + DPDS (93.33%), FCZ + DPDS (86.67%), and ITZ + DPDS (83.33%). The combinations AMB + DPDS and AMB + EBS exhibited the highest synergistic interaction against the FS group (90%). Antagonistic effects were only observed in the following combinations: FCZ + EBS (80%) and FCZ + DPDS (13.33%) against the FS group, and ITZ + EBS (20%) against the FR group. Our findings suggest that the antimicrobial activity of DPDS and EBS against *T. asahii* and their use as an adjuvant therapy with antifungal agents warrant *in vivo* experimental investigation.

Introduction

Trichosporon spp. are basidiomycetous yeast-like fungi that are widely distributed in nature. They are occasionally isolated from the gastrointestinal tract and oral cavity microbiota; they have also been reported to transiently colonize the skin and respiratory tract.[1] Over the last few decades, the clinical significance of *T. asahii* as the etiological agent of deep-seated infections or trichosporonosis has been recognized, especially in patients with hematological malignancies or other medical conditions associated with immunosupression.[1,2] The prognosis for trichosporonosis is very poor, and crude mortality rates of patients with *Trichosporon* fungemia remain as high as 42 to 87%.[1,2]

The lack of response to therapy is related to the relative resistance of *T. asahii* to many antifungal agents. Caspofungin and amphotericin B exhibit limited activity against *Trichosporon* species, and strains resistant to multiple azole agents have been recovered from patients.[3-6]

Diphenyl diselenide (DPDS) and ebselen (EBS) are organoselenium compounds possessing several pharmacological properties,[7] including antimicrobial activity.[8-17] Moreover, high rates of synergism have been observed when combining each of these compounds with conventional antifungal agents *in vitro*. This synergism indicates their potential in controlling antimicrobial resistance in adjuvanted antifungal therapy.[11,17] The antifungal activity of combinations of DPDS or EBS plus antifungal agents has not yet been evaluated against *Trichosporon* species.

In this context, this study aimed to evaluate the *in vitro* susceptibility of fluconazole-susceptible and fluconazole-resistant *T. asahii* isolates to DPDS and

EBS alone and in combination with amphotericin B, fluconazole, itraconazole, and caspofungin, using the Clinical and Laboratory Standards Institute (CLSI) M27-A3 broth microdilution technique.

Materials and methods

Clinical isolates and molecular identification

Two groups of *T. asahii* were used; the first group included clinical fluconazole-susceptible (FS, n = 30) strains isolated from blood and urine cultures, obtained from fungal collections of the Department of Microbiology and Parasitology at the Federal University of Santa Maria, Santa Maria, RS, Brazil. The second group included fluconazole-resistant (FR, n = 30) derivatives obtained from susceptible isolates from an *in vitro* method of exposition to increasing concentrations of fluconazole based on the technique described by Fekete-Forgács et al.[18] This technique was adapted in the following manner: the final concentration of fluconazole was 128 μg mL^{-1}, and the incubation temperature was 35 °C with shaking for 48 h. Cells from this culture were plated on Sabouraud Dextrose Agar (SDA; Merck, KGaA, Darmstadt, Germany), and a single colony was designated the FR isolate.

The identities of these isolates were confirmed using standard microbiological and molecular methods. Total DNA was extracted according to the protocol described by Moller et al.[19] and Klassen et al.[20] with the following modifications: the isolates were macerated in a lytic buffer (0.15 M NaCl, 50 M Tris-HCl, 10 mM EDTA, and 2% sodium dodecyl sulfate (SDS); pH 8) before undergoing phenol extraction, and the total DNA was then resuspended in sterile TE. RNA was removed by

treatment with RNAse A (1 µL, Invitrogen) for 1 h at 37°C. The total DNA concentrations were determined using spectrophotometry. Amplification of the IGS1 region (rDNA intergenic spacer) was performed by PCR using the primers 26F (5′ATCCTTTGCAGACGACTTGA-3′) and 5SR (5′AGCTTGACTTCGCAGATCGG-3′).[21] The PCR products were purified and sequenced. These sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers: KR233249, KT438844, KR872655, KR872656, KR872657, KR872660, KR872661, KR233246, KR233247, KR233248, KT365854, KT365855, KR872662, KT438839, KT438840, KR872663, KR912056, KT438842, KT365856, KT438847, KR912058, KR912064, KT365858, KR912065, KT365859, KT438846, KT365860, KR912059, KT365861, KR912066.

Chemicals

Amphotericin B (AMB; Sigma Chemical Co., St Louis, MO, USA), fluconazole (FCZ; Sigma Chemical Co.), itraconazole (ITZ; Frangon of Brazil Pharmaceutical Ltd., São Paulo, Brazil), and caspofungin (CAS; Merck, Darmstadt, Germany) were obtained commercially as standard powders.

Diphenyl diselenide (DPDS) was synthetized as described by Paulmier.[22] Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one; EBS) was synthesized according to the method of Engman & Halberg.[23] The ^{1}H NMr and ^{13}C NMR spectra analysis recorded analytical and spectroscopic data in full agreement with their expected structures. The chemical purity of the compounds (99.9%) was determined by gas chromatography/high-performance liquid chromatography (GC/HPLC) at the

Laboratory of Synthesis, Reactivity, Toxicological and Pharmacological Evaluation of Organochalcogens, located at the Federal University of Santa Maria, Brazil.

Stock solutions of AMB, ITZ, CAS, DPDS, and EBS were prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co.), while FCZ was diluted in sterile distilled water. Working solutions were diluted in RPMI 1640 medium (Sigma Chemical Co.) and adjusted to pH 7.0 with morpholinepropanesulfonic acid buffer (MOPS; Sigma Chemical Co.) according to the M27-A3 document of the CLSI.[24] The final concentration of DMSO did not exceed 1% in any of the wells.

In Vitro Susceptibility and Drug Interaction Tests

Trichosporon asahii susceptibility tests were conducted by broth microdilution in RPMI-1640 as described in M27-A3 document (CLSI, 2008). [24] *T. asahii* CBS 2479, *Candida krusei* ATCC 6258, and *C. parapsilosis* ATCC 22019 were included in the susceptibility tests as controls. Isolates were grown on SDA and incubated at 35°C for 48 h. The minimum inhibitory concentrations (MICs) were defined as the lowest concentration of the agent that inhibited 50% (for FCZ, ITZ, and CAS) or 100% (for AMB, DPDS, and EBS) of fungal growth compared with the growth of the control. The highest concentrations used were 16 μg mL^{-1} for AMB, 128 μg mL^{-1} for FCZ, 16 μg mL^{-1} for ITZ, and 64 μg mL^{-1} for CAS, DPDS, and EBS. The MICs employed as indicators of resistance were: ≥ 2 μg mL^{-1} (AMB);[21,26] ≥ 64 μg mL^{-1} (FCZ);[27,28] ≥ 1 μg mL^{-1} (ITZ),[26] and ≥ 2 μg mL^{-1} (CAS).[21,26] After exposing the strains to increasing concentrations of FCZ, the strains were separated in two groups: a) FS (fluconazole-susceptible), formed by the strains not exposed to FCZ; and b) FR

(fluconazole-resistant), formed by the same strains after exposure to FCZ, with MICs ranging from 64 to 128 μg $mL^{-1.}$

The effects of interactions between AMB, FCZ, ITZ, CAS, and organoselenium compounds on 30 strains of both groups were evaluated using the microdilution checkerboard method.[29] For the calculation of the fractional inhibitory concentration index (FICI), MIC values related to 100% inhibition of growth were used. The FICI values were interpreted as follows: FICI ≤ 0.5, synergism; 0.5 < FICI ≤ 4, indifference; and FICI > 4.0, antagonism.[29] FICIs were calculated for all wells along the turbidity/non-turbidity interface where the lowest FICI as the final point was determined.

Statistical analysis

The differences between MICs and FICIs obtained from both strains were evaluated by nonparametric Wilcoxon paired t-test using SigmaPlot version 11.0 (Systat Software, San Jose, CA, USA). Significant differences were deemed to be those with p-values below 0.05.

Results

The results of *in vitro* susceptibility testing of *T. asahii* isolates to the antifungal and organoselenium compounds are listed in Table 1. The MIC ranges and geometric means (parenthesized, in μg mL^{-1}) of the FS/FR groups for the antifungal agents were as follows: 1–8 (2.24)/0.13–4 (0.41) for AMB, 1–16 (2.41)/64–128 (64) for FCZ, 0.13–1 (0.27)/0.5–16 (1.52) for ITZ, and 4–16 (7.82)/4–16 (7.29) for CAS.

EBS demonstrated antifungal activity against *T. asahii* isolates in both groups, with MIC ranges (GM) of 0.25–0.5 μg mL^{-1} (0.29) and 1–8 μg mL^{-1} (2.05) for FS and FR, respectively. For DPDS, the MIC range values (GM) were higher: 8–64 μg mL^{-1} (22.11) for the FS group, and 16–64 μg mL^{-1} (54.44) for the FR group. Statistical analysis revealed significant differences between the susceptibility of both groups to azole antifungal agents (FCZ and ITZ: $p < 0.001$), as well as to AMB ($p < 0.001$) and organoselenium compounds (DPDS and EBS: $p < 0.001$).

The results for each drug combination are detailed in Table 2. The percentages of synergism (FS group/FR group) observed were as follows: AMB + DPDS (90/93.33), AMB + EBS (90/0), FCZ + DPDS (20/86.67), FCZ + EBS (0/23.33), ITZ + DPDS (13.33/83.33), ITZ + EBS (6.67/3.33), CAS + DPDS (60/96.67), CAS + EBS (46.67/23.33). Antagonistic effects were observed only in the following combinations: FCZ + EBS (80%) and FCZ + DPDS (13.33%) against the FS group, and ITZ + EBS (20%) against the FR group. Statistical differences between groups FS and FR were observed for AMB + EBS, FCZ + DPDS, FCZ + EBS, ITZ + DPDS, and CAS + DPDS ($P < 0.001$).

Discussion

Based on the geometric mean, the MICs of the strains in the FR group were in general higher than those of the FS *T. asahii* strains, but statistically significant differences were not observed for CAS (Table 1). Cross-resistance between FCZ and ITZ was detected. Multidrug resistance to antifungal agents has been reported for *T. asahii* in previous studies.[4-6] Long-term use of FCZ *in vivo* may lead to replacement of the amino acid *ERG11p* and thus trigger *T. asahii* resistance to multiple drugs.[6]

Interestingly, our results demonstrated that after sequential exposure to increasing concentrations of FCZ, the number of isolates resistant to AMB decreased. Sanglard et al.[30] and Cho et al.[31] have related that the targeted deletion of *ERG3* or *ERG11* in *Candida* species seems to be a potential mechanism that may lead to increased susceptibility to AMB with increased resistance to azoles. Although we did not explore these mechanisms, they may explain our current findings.

As for the organoselenium compounds, *T. asahii* isolates were more susceptible to EBS than to DPDS, and the FS group demonstrated lower MIC values than the FR group (Table 1). However, the greatest rates of synergism (83.33% to 96.67%) were obtained for combinations of antifungal agents and DPDS against FR *T. asahii* strains (Table 2), with the exception of combinations of AMB and DPDS or EBS, with 90% synergy against the FS group observed for both organoselenium compounds.

Previous studies have reported the *in vitro* antimicrobial activity of DPDS against *Candida* spp., *Aspergillus* spp., *Fusarium* spp.,[9,11,12,17] and *Pythium insidiosum*,[10] as well as the antimicrobial activity of EBS against *Cryptococcus neoformans*, *C. albicans*, *C. parapsilosis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Penicillium chrysogenum*, *P. citrinum*, and *Fusarium* spp.[13-17] Loreto el al.[9] reported high MIC (64 µg mL^{-1}) and minimum fungicidal concentration (> 64 µg mL^{-1}) values for DPDS against *Trichosporon ovoides.* In the present study, the MIC ranges of DPDS were 8–64 µg mL^{-1} and 16–64 µg mL^{-1} against FS and FR strains, respectively.

Our findings showed that *T. asahii* strains were inhibited by lower MICs of EBS (0.25–0.5 µg mL^{-1} for FS, 1–8 µg mL^{-1} for FR) than of DPDS (8–64 µg mL^{-1} for FS, 16–64 µg mL^{-1} for FR). The ability of EBS to inhibit the growth of FR strains of *C.*

albicans seems to be related to its ability to act as an antifungal agent despite upregulation of proteins that can confer resistance to FCZ.[16]

A recent report evaluated the antifungal activity of DPDS and EBS, alone and in combination with antifungal agents, against 25 isolates of *Fusarium* spp.[17] These authors observed that all isolates recorded higher MIC values of DPDS (4–32 μg mL^{-1}) than of EBS (2–8 μg mL^{-1}). They also reported that combinations of EBS + AMB (88%) and DPDS + AMB (72%) exhibited high rates of synergism[17].

Interestingly, in the present study, after sequential exposure to increasing concentrations of FCZ, synergistic interactions against resistant isolates using FCZ + DPDS increased from 20% to 86.67%. The combination of DPDS + FCZ demonstrated a predominance of indifferent (50%) and antagonistic (40%) interactions against *C. glabrata* strains.[11] However, for the DPDS + AMB combination, a predominance of synergistic (76.66%) interaction was observed.[11]

The antifungal activity of organoselenium compounds could be due to their ability to interact with sulfhydryl groups of one or more L-cysteine residues within Pma1p that are critical for H^+ transport in fungal cells.[14,16,32] According to Rosa et al.[8], DPDS can reduce levels of cellular glutathione (GSH), the main non-enzymatic cellular antioxidant in yeasts, thus sensitizing the cell to the damaging effects of reactive oxygen species (ROS).

The potential therapeutic use of organoselenium compounds depends on more detailed toxicological studies in the coming years, but we cannot ignore the promise of DPDS and EBS as pharmaceutical agents. Nogueira et al.[7] only suggest relative safety of these compounds for humans after short-term intake schedules, and the beneficial effects and toxicity appear to be directly related to the dose. Here, the geometric means of the MICs of DPDS in combination with AMB (5.16 μg mL^{-1}),

FCZ (8.98 µg mL^{-1}), ITZ (6.35 µg mL^{-1}), and CAS (8,38 µg mL^{-1}) against FR strains, as well as the geometric means of the MICs of EBS in combination with AMB against the FS group (0.29 µg mL^{-1}), were lower than the plasma concentrations of these compounds demonstrated in previous studies.[33,34] DPDS demonstrated less toxicity when compared with EBS in a rat model after acute peritoneal treatment,[35] and hematological and biochemical parameters indicated the absence of detectable toxicity caused by DPDS.[10]

In this scenario, it is important to conduct further studies that assess the mechanisms by which the organoselenium compounds render pathogens more susceptible to antifungal agents. Combination therapy is a promising alternative when monotherapy is not effective, since synergism between two agents may reduce treatment toxicity through the administration of lower doses and may also induce resistant microorganisms to become more susceptible to antifungal agents.

In conclusion, we demonstrated that DPDS and EBS exhibit *in vitro* antifungal activity. Combination of these organoselenium compounds with AMB, FCZ, ITZ, or CAS leads to high rates of synergism *in vitro*, notably against FR *T. asahii* strains. These results suggest that DPDS and EBS compounds deserve attention as candidates for *in vivo* studies focusing on *T. asahii* experimental infections.

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Declaration of interest

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

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Table 1. Minimum inhibitory concentration (MIC) of antifungal agents and organoselenium compounds against *Trichosporon asahii* (µg/mL).

Drugs	Groups	GM	MIC Range	MIC_{50}	MIC_{90}
Antifungals					
AMB	FS	2.24*	1-8	2	4
	FR	0.41*	0.13-4	0.25	1
FCZ	FS	2.41*	1-16	2	8
	FR	64*	64->128	64	64
ITZ	FS	0.27*	0.13-1	0.25	0.5
	FR	1.52*	0.5-16	1	4
CAS	FS	7.82	4-16	8	8
	FR	7.29	4-16	8	8
Organoselenium compounds					
DPDS	FS	22.11*	8-64	32	64
	FR	54.44*	16->64	64	>64
EBS	FS	0.29*	0.25-0.5	0.25	0.5
	FR	2.05*	1-8	2	4

Groups of *Trichosporon asahii* isolates: FS, fluconazole-susceptible *Trichosporon asahii* strains, before resistance induction and FR, fluconazole-resistant *Trichosporon asahii* strains, after resistance induction; GM, MIC geometric mean; MIC_{50} and MIC_{90}, MIC at which 50% and 90% of the isolates tested were inhibited, respectively; AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; DPDS, diphenyl diselenide; EBS, ebselen; * $P < 0.05$.

Table 2. Fractional inhibitory concentration index (FICI), geometric mean (GM) of interactions between antifungal agents and organoselenium compounds against clinical isolates of *Trichosporon asahii*.

Drug combination	Before resistance induction				After resistance induction			
	FICI-GM	**Interaction (%)**			**FICI- GM**	**Interaction (%)**		
		Syn	**Ind**	**Ant**		**Syn**	**Ind**	**Ant**
AMB + DPDS	0.24	90	10	0	0.30	93.33	6.67	0
AMB + EBS	0.25*	90	10	0	1.16*	0	100	0
FCZ + DPDS	1.15*	20	66.67	13.33	0.21*	86.67	13.33	0
FCZ + EBS	8.47*	0	20	80	0.72*	23.33	76.67	0
ITZ + DPDS	0.94*	13.33	53.33	0	0.22*	83.33	16.67	0
ITZ + EBS	1.04	6.67	93.33	0	1.52	3.33	76.67	20
CAS + DPDS	0.36*	60	40	0	0.18*	96.67	3.33	0
CAS + EBS	0.42	46.67	53.33	0	0.52	23.33	76.67	0

FICI, fractional inhibitory concentration index; GM, geometric mean; Syn, synergism; Ind, indifference; Ant, antagonism; AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; DPDS, diphenyl diselenide; EBS, ebselen; *Significant difference ($P < 0.05$) between groups I (before resistance induction) and II (after resistance induction).

4 MANUSCRITO 2

In vitro activity of antifungal agents alone and in combination against *Trichosporon asahii* isolates before and after prolonged exposure to fluconazole

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Manuscrito a ser submetido

In vitro activity of antifungal agents alone and in combination against *Trichosporon asahii* isolates before and after prolonged exposure to fluconazole

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Summary

The extensive use of azole antifungals has promoted the resistance of *Trichosporon* spp. to these drugs. The combinations of drugs must be known because they can overcome this problem. In the present study we evaluated the *in vitro* activity of conventional antifungal agents against two groups of *T. asahii* strains: one containing fluconazole-susceptible (FS) clinical isolates (n = 30), and the other containing fluconazole-resistant (FR) laboratory derivative (n = 30). Our aim was to examine the changes on susceptibility accompanying the development of resistance to fluconazole. In addition, we have evaluated the combination of amphotericin (AMB) with echinocandins (caspofungin, CPF; micafungin, MCF, and anidulafungin, AND) or fluconazole (FCZ), and CPF with FCZ against FR *T. asahii* strains. For these analyses, we have used the broth microdilution method based on the M27-A3 technique and the checkerboard microdilution method. Our findings have confirmed the ability of *T. asahii* isolates to become resistant to FCZ and indicated that this resistance was crossed with itraconazole (ITZ) (90%), posaconazole (POS) (36.67%), and voriconazole (VCZ) (10%). We have also tested combinations of CAS + AMB, MCF + AMB, AND + AMB, FCZ + AMB, and CPF + FCZ against the FR *T. asahii* group, which has also showed resistance to echinocandins. Most combinations have produced indifferent interactions, and the best synergism (66.67%) has appeared when CPF and FCZ were combined against the FR *T. asahii* group. Antagonism was not detected in any combinations.

Key words: fluconazole resistance, trichosporonosis, susceptibility tests, combined therapy.

Introduction

Trichosporon spp. is a yeast-like fungus that can colonize mainly the gastrointestinal and respiratory tracts and the skin of humans. However, in the last decades *T. asahii* has become clinically important as the etiological agent of deep-seated infections or trichosporonosis.[1] This pathogen is considered an important cause of disseminated yeast infections by non-*Candida* species, specifically in immunosuppressed patients, which is associated with mortality rates that may reach up to 100% in patients with persistent neutropenia.[2-4]

Unfortunately, the most appropriate form of therapy for *Trichosporon* infections and the efficacy of treatment are uncertain. Clinical failures of amphotericin B and echinocandins have been reported.[2,5-10] There is some evidence that triazoles are more active *in vitro* and, perhaps, are more clinically effective than is amphotericin B against *T. asahii*.[11-14] However, some authors reported that the long-term use of azoles might lead to multidrug resistance.[15-17] Although voriconazole may offer a clinical solution in trichosporonosis when other antifungals have failed, the susceptibility of *T. asahii* to this antifungal agent has been decreasing.[16-19] Furthermore, the usefulness of azoles in intensive care units (ICUs) may be limited by patients' unreliable gastrointestinal function and their frequent dependence on haemodialysis.[20]

In recent years, the combinations of antifungal agents have been suggested as an alternative strategy for *Trichosporon* infections, and the combinations of amphotericin B with echinocandins or azoles, and echinocandins with azoles, have been suggested as potential therapeutic options.[3,21-26] Up to now, the *in vitro* activity

of antifungal agents in combination has not yet been evaluated against *T. asahii* isolates after prolonged exposure to fluconazole.

Therefore, this study was addressed to evaluate the *in vitro* activity of amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, micafungin, and anidulafungin against *Trichosporon asahii* isolates before and after prolonged exposure to fluconazole and to study the effects of this exposure. In addition, we have evaluated the combination of amphotericin B with echinocandins or fluconazole, and fluconazole with caspofungin against FR *T. asahii* group.

Materials and methods

Clinical isolates

We have studied two groups of *T. asahii* isolates. The first one included 30 clinical fluconazole-susceptible (FS) isolates recovered from blood culture and urine, obtained from the fungal collection of Federal University of Santa Maria's Mycological Research Laboratory (LAPEMI), Santa Maria, RS, Brazil. The second group included 30 fluconazole-resistant (FR) strains obtained from susceptible isolates through an *in vitro* method of prolonged exposure to fluconazole, previously described by Fekete-Forgács et al.[27] with the following modifications: the final concentration of fluconazole was 128 μg mL^{-1}, and the incubation temperature was 35^{o}C with shaking for 48h. Cells from this culture were plated on Sabouraud Dextrose Agar (SDA; Merck, KGaA, Darmstadt, Germany) plates, and a single colony was designated as isolated FR.

Identification of isolates

The identity of these isolates was confirmed using standard microbiological and molecular methods and was described in our previous report.[27] The sequences have been deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers: KR233249, KT438844, KR872655, KR872656, KR872657, KR872660, KR872661, KR233246, KR233247, KR233248, KT365854, KT365855, KR872662, KT438839, KT438840, KR872663, KR912056, KT438842, KT365856, KT438847, KR912058, KR912064, KT365858, KR912065, KT365859, KT438846, KT365860, KR912059, KT365861, KR912066.

Antifungal Agents

Amphotericin B (AMB; Sigma Chemical Co., St Louis, MO, USA), fluconazole (FCZ; Sigma Chemical Co.), itraconazole (ITZ; Frangon of Brazil Pharmaceutical Ltd., São Paulo, Brazil), voriconazole (VCZ) (Pfizer Pharmaceutical Group, New York, NY), posaconazole (POS; Sigma Chemical Co.), caspofungin (CPF) (Merck, Darmstadt, Germany), micafungin (MCF) (Astellas, Chuo, Tokyo, Japan), and anidulafungin (AND) (Pfizer Pharmaceutical Group, New York, NY) were obtained commercially. The stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co.), except for FCZ, which was diluted in sterile distilled water. Working solutions were prepared according to the document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI).[29]

Antifungal susceptibility and drug combination tests

Susceptibility testing was performed in accordance with the guidelines of M27-A3 broth microdilution technique approved by the CLSI,[29] using RPMI 1640 medium with L-glutamine (Sigma Chemical Co.), buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co.). Inoculums concentrations ranged from 0.5 x 10^3 to 2.5 x 10^3 CFU mL^{-1} and the microplates were, incubated aerobically at 37^oC for 48 h.

The highest concentrations used were 16 µg mL^{-1} for AMB, 128 µg mL^{-1} for FCZ, 16 µg mL^{-1} for ITZ, VCZ, and POS; and 64 µg mL^{-1} for CPF, MCF, and AND. The minimum inhibitory concentrations (MICs) were determined after 48h of incubation and were defined as the lowest drug concentration able to inhibit 50% (for azoles and echinocandins) or 100% (for AMB) of fungal growth when compared to the growth of the control. All assays were performed in duplicate. The standard strains *T. asahii* CBS 2479, *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains.

In vitro combinations of amphotericin B with echinocandins or fluconazole, and fluconazole with caspofungin against FR *T. asahii* group were evaluated by the microdilution checkerboard method.[30] In order to evaluate the interaction of agents, the fractional inhibitory concentration index (FICI) was calculated for each combination. Fractional inhibitory concentration (FIC) was calculated for each agent by dividing the inhibitory concentration of each antifungal agent, when used, by its MIC. FIC values were then added together to define the interaction of the combination. Synergy was defined as an FICI ≤ 0.5, indifference (no interaction) when 0.5 < FICI ≤ 4.0, and antagonism when FICI > 4.0.[30]

Statistical analysis

The MICs were analyzed by comparing the susceptible group and the group exposed to FCZ using the nonparametric Wilcoxon paired *t* test. The SigmaPlot Software version 11.0 was used, and the significance level was 0.05.

Results

The *in vitro* antifungal susceptibilities of the FS and FR *T. asahii* isolates are summarized in Table 1. Of note, 90% of the FS isolates tested had MICs ≥ 2 μg mL^{-1} for AMB. All isolates (FS/FR) had MICs ≥ 2 μg mL^{-1} for the three echinocandins tested. Regarding to azole antifungal agents, FS isolates were susceptible to these compounds, showing MIC_{90} of 8 μg mL^{-1} for FCZ , 0.5 μg mL^{-1} for ITZ, and 0.25 μg mL^{-1} for VCZ and POS. Comparatively FR group was less susceptible to ITZ (MIC_{90} = 4 μg mL^{-1}), VCZ (MIC_{90} = 2 μg mL^{-1}), and POS (MIC_{90} = 8 μg mL^{-1}) than sensitive isolates. In addition, the susceptibility of FR group to AMB showed AMB MIC_{90} = 1 μg mL^{-1}. The statistical analysis showed significant differences between the susceptibility of FS *vs.* FR groups for azole drugs (FCZ, ITZ, POS: $P < 0.001$, VCZ $P = 0.001$), as well as to AMB ($P < 0.001$).

Table 2 depicts the MICs and FICIs for each combination after *in vitro* prolonged exposure to fluconazole, as well as the effects of antifungal agent combinations, which demonstrated synergism, indifference or antagonism. The FICIs (geometric mean) values for each combination were: 0.56 [CPF + AMB], 0.64[MCF + AMB], 0.51[AND + AMB], 0.61[FCZ + AMB], and 0.4 [CPF + FCZ].

The majority of associations which showed indifferent interactions were: MCF + AMB (22/73.33%), AND + AMB (22/73.33%), CPF + AMB (21/70%), and FCZ +

AMB (19/63.33%) against FR *T. asahii* isolates. The best synergism was showed by the combination CPF + FCZ (66.67%) against the FR group. Antagonisms were not detected for all the combinations studied.

Discussion

The emergence of *T. asahii* with reduced susceptibility to conventional antifungal agents, notably to FCZ, is a matter of concern. In this context, we focused in the comparison of the susceptibility of *T. asahii*, before and after sequential exposure to growing concentrations of FCZ. Additionally, we analyzed the effect of the combination of antifungal agents against the FCZ resistant group.

In agreement with previous findings[11-14] FS *T. asahii* group was more resistant *in vitro* to AMB than triazole compounds (Table 1). The low MICs exhibited for azoles, especially for VCZ against *T. asahii* strains, confirm other *in vitro* studies.[13,14,19] There are two reasons that may explain why VCZ may be effective in the treatment of mycoses such as tricosporonosis that do not respond to other azoles: this triazole has high affinity for fungal 14-α-demethylase and also inhibits 24-methylene dihydrolanosterol demethylation of certain yeasts and filamentous fungi, resulting in potencies 1.6 to 160 times greater than those of FCZ in the inhibition of *C. albicans* and *Aspergillus fumigatus*, respectively. [33,34]

Although most *T. asahii* clinical isolates are susceptible to azoles, and VCZ may offer a clinical solution for trichosporonosis when other antifungal agents fail, the increasing use of azole class compounds in ICUs may lead to the selection and isolation of more resistant species. It is well established that FCZ resistance occurs after long-term contact with this antifungal agent.[15,17,35]

Here, the cross-resistance of *T. asahii* to FCZ and ITZ, VCZ, and POS was remarkable, and this phenomenon was more pronounced for ITZ (90%), followed by POS (36.67%) and VCZ (10%) (Table 1). Cross-resistance among azoles, including VCZ, is a recent phenomenon among *Trichosporon* sp. clinical isolates, scarcely documented and studied. [16-19]

The occurrence of multi-drug resistant strain of *T. asahii* recovered from skin has already been described.[16] Araujo et al.[16] showed that when MICs of FCZ achieve values ≥ 64 μg mL^{-1} against this species, there is a pattern of cross-resistance to all azole drugs, including ITZ (MIC = 16 μg mL^{-1}), VCZ (MIC = 16 μg mL^{-1}), and POS (MIC = 16 μg mL^{-1}), as well as to AMB (MIC = 32 μg mL^{-1}). Furthermore, high MICs for all tested antifungal agents (AMB >16 μg mL^{-1}; FCZ > 64 μg mL^{-1}; ITZ > 16 μg mL^{-1}; VCZ > 4 μg mL^{-1}; and POS > 4 μg mL^{-1}) were detected forward *T. asahii* isolated from blood of biliary cirrhosis patients of north India.[18]

Posteriorly, Kushima et al.[17] also showed that the MICs of *T. asahii* strains for ITZ and VCZ increased in parallel with the MICs for FCZ, although the AMB MICs did not change significantly. In the present study, the susceptibility to AMB increased with resistance to FCZ, and this finding may be related to the targeted deletion of *ERG3* or *ERG11* genes of the fungus, as described for *C. albicans* and *C. glabrata*.[36,37]

Azoles such as FCZ act on the ergosterol biosynthesis pathway by inhibiting the lanosterol 14-α-demethylase (Erg11 protein; Erg11p), a cytochrome P450-dependent enzyme encoded by the *ERG*11 gene. Recently, the single amino acid substitution G453R, and the consequent decrease in affinity for Erg11p, was reported as a resistance mechanism to FCZ in *T. asahii.* [17]

Regarding to echinocandins, high MICs of CPF (MICs ≥ 4 μg mL^{-1}), MCF, and AND (MICs ≥ 64 μg mL^{-1}) were observed against both the FS and FR groups. These *in vitro* findings are consistent with the increasing number of reports on disseminated *Trichosporon* infections in patients receiving echinocandin antifungal treatment.[6-10] A breakthrough invasive trichosporonosis in a bone marrow transplanted recipient receiving CPF as prophylaxis against *Aspergillus* infection has been described.[38]

Thus, since the antifungal monotherapy against disseminated trichosporonosis may have failed, there has been growing interest in treating these infections with combined antifungal therapy.[22,24-26] The use of azoles in combination therapies has been suggested as a potential therapeutic option.[21,25]

Although echinocandins are considered intrinsically inactive against *Trichosporon* spp., the combination of CPF + FCZ has demonstrated synergistic interaction against the FR *T. asahii* group (Table 2). In this combination, the MICs of CPF and FCZ ranged from 0.5-2 and 1-32 μg mL^{-1}, respectively, which are totally achievable in human plasma.[39,40] The synergistic interaction for ITZ combined with CPF (72.2%) against *T. asahii* has been previously reported.[25] We suggest that the mechanism by the synergistic interactions [FCZ + CPF] here reported may be similar to that proposed by those authors, which is related to simultaneous inhibition of different fungal targets, such as cell wall and cell membrane.[25]

In vivo and *in vitro* studies have demonstrated that the combination between AMB and echinocandins[3,22-26] or FCZ[21,25] shows the best synergistic effect. However, here, the results differ somewhat from these researches. The combinations [AMB+CPF], [AMB+MCF], [AMB+AND], and [AMB+FCZ] showed no interactions *in vitro* against most strains tested. The MICs of three echinocandins decreased significantly, whereas the MICs of polyene remained mostly between 0.25 and 0.5

(Table 2). This generated FICIs > 0.5, thus characterizing the indifferent interaction. This may be explained by the fact that FR *T. asahii* required low concentrations of AMB; thus, the combinations with other antifungal agents did not result in lower FICIs (≤ 0.5). Therefore, although these combinations only showed approximately 30% of synergistic interactions *in vitro*, these combined therapies might be potential options for therapy and could improve the poor outcomes in profoundly neutropenic patients with disseminated trichosporonosis.

Antifungal activity based on combined therapy is a promising alternative when monotherapy is not effective, since the achievement of synergy between two agents may enable practitioners to diminish drug dosages, expand the coverage in seriously ill patients with mixed infections, and induce the resistant microorganisms to become susceptible to antifungal agents or at least more sensitive to treatment.[41]

In conclusion, our findings demonstrated that cross-resistance of *T. asahii* among azoles as ITZ, POS, and VCZ occurs after prolonged exposure to FCZ. Since the checkerboard method is a preliminary *in vitro* test, further studies are required in order to provide clearer information on susceptibility differences between FS and FR *T. asahii* strains when exposed to echinocandin-polyene, azole-polyene or echinocandin-azole combinations. *In vivo* studies are warranted to determine the usefulness of these combinations in the combined therapy for the *T. asahii* infections.

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Conflicts of interest

There are no conflicts of interest to declare for any of the authors in the study.

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Table 1 *In vitro* susceptibilities of *Trichosporon asahii* isolates to antifungal agents before and after exposure to fluconazole.

Agents	Group of isolates	MIC Range	MIC_{50}/ MIC_{90}	Number (%) of resistant isolates	Susceptibility breakpoints (μg mL^{-1})
AMB	FS	1 – 8*	2/ 4	27 (90)	$< 2^{13,14,16}$
	FR	0.13 – 4*	0.25/ 1	1 (3,33)	
FCZ	FS	1 - 16*	2/ 8	0 (0)	$< 64^{31,32}$
	FR	64 - >128*	64/ 64	30 (100)	
ITZ	FS	0.13 – 1*	0.25/ 0.5	1 (3,33)	$< 1^{16}$
	FR	0.5 – 16*	1/ 4	27 (90)	
VCZ	FS	0.03 – 0.5*	0.03/ 0.25	0 (0)	$< 4^{16,18}$
	FR	0.13 – 16*	1/ 2	3 (10)	
POS	FS	0.13 – 0.25*	0.13/ 0.25	0 (0)	$< 4^{16,18}$
	FR	0.13 – 16*	2 / 8	11 (36,67)	
CPF	FS	4 – 16	8/ 8	30 (100)	$< 2^{13,16}$
	FR	4 – 16	8/ 8	30 (100)	
MCF	FS	>64	ND	30 (100)	$< 2^{13,16}$
	FR	>64	ND	30 (100)	
AND	FS	>64	ND	30 (100)	$< 2^{13,16}$
	FR	>64	ND	30 (100)	

MIC range, minimal inhibitory concentration range (μg mL^{-1}); MIC50, MIC at which 50% of isolates tested were inhibited; MIC90, MIC at which 90% of isolates tested were inhibited; AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; CPF, caspofungin; MCF, micafungin, AND; anidulafungin; FS, and FR, fluconazole-susceptible, and fluconazole-resistant *T. asahii* isolates; ND: not determined; * $P < 0.05$.

Table 2 *In vitro* combinations of antifungal agents and organoselenium compounds against fluconazole-resistant *T. asahii* isolates.

Isolates	Drugs in combination									
	CPF/AMB[a]		**MICA/AMB**[a]		**AND/AMB**[a]		**FCZ/AMB**[a]		**CPF/FCZ**[b]	
	MICs	**FICI (X)**	**MICs**	**FICI (X)**	**MICs**	**FICI (X)**	**MICs**	**FICI (X)**	**MICs**	**FICI (X)**
Ta1	0.5/0.5	0.53 (I)	0.5/0.5	0.51 (I)	0.5/0.5	0.51 (I)	32/0.06	0.31 (S)	8/1	1.02 (I)
Ta2	0.5/0.5	2.06 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	32/0.06	0.5 (S)	0.5/8	0.19 (S)
Ta3	0.5/0.5	1.03 (I)	0.5/0.5	1.01 (I)	0.5/0.13	0.26 (S)	1/1	2.01 (I)	8/1	1.02 (I)
Ta4	0.5/0.5	1.03 (I)	0.5/0.5	1.01 (I)	0.5/0.25	0.51 (I)	64/0.06	0.63 (I)	0.5/16	0.31 (S)
Ta5	8.0/0.13	0.75 (I)	0.5/0.13	0.51 (I)	0.5/0.13	0.51 (I)	32/0.06	0.5 (S)	0.5/8	0.16 (S)
Ta6	0.5/0.25	0.28 (S)	8/0.13	0.26 (S)	0.5/0.13	0.14 (S)	1/0.5	0.51 (I)	0.5/64	1.06 (I)
Ta7	0.5/0.13	0.28 (S)	0.5/0.25	0.51 (I)	0.5/0.13	0.26 (S)	1/0.25	0. 51 (I)	2/1	0.27 (S)
Ta8	16.0/0.06	1.25 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	1/0.5	2.01 (I)	8/1	1.02 (I)
Ta9	0.5/0.13	0.28 (S)	0.5/0.13	0.26 (S)	0.5/0.13	0.26 (S)	1/0.25	0.51 (I)	0.5/16	0.31 (S)
Ta10	0.5/0.13	0.53 (I)	0.5/0.13	0.51 (I)	0.5/0.13	0.51 (I)	1/0.25	1.01 (I)	1/1	0.15 (S)
Ta11	0.5/0.13	0.53 (I)	0.5/0.25	1.01 (I)	0.5/0.13	0.51 (I)	128/0.13	1.50 (I)	0.5/32	0.63 (I)
Ta12	0.5/0.5	0.53 (I)	0.5/0.5	0.51 (I)	0.5/0.25	0.26 (S)	1/0.5	0.51 (I)	4/1	1.02 (I)
Ta13	0.5/0.5	0.16 (S)	0.5/0.5	0.14 (S)	0.5/0.25	0.07 (S)	1/0.5	0.15 (S)	0.5/32	0.56 (I)
Ta14	0.5/0.13	0.28 (S)	0.5/0.13	0.26 (S)	0.5/0.25	0.51 (I)	8/0.06	0.19 (S)	0.5/16	0.38 (S)
Ta15	0.5/0.13	0.28 (S)	0.5/0.13	0.26 (S)	0.5/0.25	0.51 (I)	8/0.06	0.19 (S)	0.5/8	0.19 (S)
Ta16	0.5/0.13	0.28 (S)	0.5/0.13	0.26 (S)	0.5/0.25	0.51 (I)	32/0.06	0.38 (S)	0.5/8	0.19 (S)
Ta17	0.5/0.13	0.53 (I)	0.5/0.13	0.51 (I)	0.5/0.25	1.01 (I)	8/0.06	0.31 (S)	0.5/32	0.56 (I)
Ta18	0.5/0.13	0.53 (I)	0.5/0.13	0.51 (I)	0.5/0.25	1.01 (I)	1/0.25	1.01 (I)	0.5/32	0.56 (I)
Ta19	0.5/0.13	0.53 (I)	0.5/0.25	1.01 (I)	0.5/0.25	1.01 (I)	128/0.06	1.25 (I)	8/1	1.02 (I)
Ta20	0.5/0.13	1.95 (I)	0.5/0.13	1.01 (I)	0.5/0.13	1.01 (I)	128/0.06	1.50 (I)	2/1	0.26 (S)
Ta21	0.5/0.5	1.03 (I)	0.5/0.5	1.01 (I)	0.5/0.5	1.01 (I)	64/0.5	1.50 (I)	0.5/16	0.31 (S)
Ta22	0.5/0.25	0.53 (I)	0.5/0.25	0.51 (I)	0.5/0.25	0.51 (I)	32/0.25	0.75 (I)	0.5/32	0.31 (S)
Ta23	0.5/0.25	1.03 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	64/0.06	0.75 (I)	0.5/16	0.31 (S)
Ta24	0.5/0.25	1.03 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	64/0.06	0.75 (I)	0.5/16	0.31 (S)
Ta25	0.5/0.13	0.16 (S)	0.5/0.13	0.14 (S)	0.5/0.13	0.14 (S)	16/0.06	0.19 (S)	0.5/16	0.31 (S)
Ta26	0.5/0.25	1.03 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	64/0.06	0.75 (I)	0.5/16	0.38 (S)
Ta27	0.5/0.13	0.53 (I)	0.5/0.5	2.01 (I)	0.5/0.25	1.01 (I)	1/0.25	1.01 (I)	0.5/16	0.31 (S)
Ta28	0.5/0.13	0.53 (I)	0.5/0.13	0.51 (I)	0.5/0.13	0.51 (I)	64/0.06	0.75 (I)	0.5/16	0.31 (S)
Ta29	16.0/0.06	1.25 (I)	0.5/0.25	1.01 (I)	0.5/0.25	1.01 (I)	32/0.06	0.5 (S)	0.5/16	0.31 (S)
Ta30	0.5/0.13	0.28 (S)	16/0.13	0.5 (S)	0.5/0.13	0.26 (S)	32/0.06	0.38 (S)	0.5/16	0.31 (S)

CPF, caspofungin; AMB, amphotericin B; MCF, micafungin, AND; anidulafungin; FCZ, fluconazole; FICI, fractional inhibitory concentration index calculated for each agent by dividing the inhibitory concentration of each antifungal agent able to inhibit 100% of fungal growth; X, FICI interpretation; S, synergism, I, indifference; Ta, *T. asahii* fluconazole-resistant isolates.

5 DISCUSSÃO

A incidência de infecções fúngicas causadas por espécies de *Trichosporon* aumentou consideravelmente nos últimos anos, estando essas associadas com significativa morbidade, hospitalização prolongada, altas taxas de mortalidade e aumento dos custos à saúde. Estes fatores, aliados à emergência de resistência de *T. asahii* aos antifúngicos azólicos e à disponibilidade limitada de fármacos antifúngicos, evidenciam a necessidade de aprofundamento de estudos com isolados resistentes ao fluconazol para avaliar a dimensão desta resistência, bem como a exploração de possíveis alternativas para o tratamento da tricosporonose (COLOMBO et al., 2011).

Estudos recentes recomendam o uso de antifúngicos azólicos nas infecções disseminadas por *Trichosporon* spp*.*, uma vez que o perfil típico de suscetibilidade desse gênero é a resistência à anfotericina B e às equinocandinas (CHAGAS-NETO et al., 2009; RUAN; CHIEN; HSUEH, 2009; TAJ-ALDEEN et al., 2009). Entretanto, diferentes espécies podem ter diferentes padrões de respostas, uma vez que *T. asahii* tem demonstrado maior resistência, *in vitro*, à anfotericina B do que aos compostos triazólicos, enquanto espécies de *Trichosporon* não - *asahii* parecem ser mais resistentes aos antifúngicos azólicos quando comparados a *T. asahii* (ARIKAN et al., 2005; CHAGAS-NETO et al., 2009; KALKANCI et al., 2010; MONTOYA et al., 2015; PAPHITOU et al., 2002; RODRIGUEZ-TUDELA et al., 2005; RUAN; CHIEN; HSUEH, 2009; SUZUKI et al., 2010).

A avaliação, *in vitro,* da atividade de antifúngicos convencionais de diferentes classes frente a *T. asahii* FS e FR revelou que as cepas FR apresentaram diminuição da suscetibilidade para os antifúngicos azólicos, revelando a capacidade desta espécie em desenvolver resistência cruzada *in vitro* ao itraconazol (90%), posaconazol (36.67%) e voriconazol (10%) mediante exposição crescente ao fluconazol (manuscrito 2). Embora muitos isolados clínicos de *T. asahii* apresentem boa sensibilidade aos azólicos, o uso intenso de antifúngicos dessa classe em hospitais de várias partes do mundo tem levado à seleção e isolamento de cepas mais resistentes (RASTOGI et al., 2016; RIBEIRO et al., 2008; SILVA et al., 2008; WOLF et al., 2001). O uso prolongado de fluconazol pode levar à substituição do

aminoácido G453R da *ERG11p* e, consequentemente, promover o desenvolvimento de resistência de *T. asahii* a múltiplos fármacos (KUSHIMA et al., 2012).

Diferentemente do que é relatado em estudos prévios (CHAGAS-NETO et al., 2009; KALKANCI et al., 2010; RODRIGUEZ-TUDELA et al., 2005; WOLF et al., 2001), os resultados apresentados no artigo 1 e manuscritos 1 e 2, demonstram o aumento da sensibilidade de *T. asahii* à anfotericina B com o aumento da resistência ao fluconazol. Para Kushima et al. (2012), as CIMs de itraconazol e voriconazol frente a *T. asahii* aumentaram em paralelo com as CIMs de fluconazol; entretanto, as CIMs de anfotericina B não evidenciaram alterarações significativas após prolongada exposição ao azólico. A deleção de *ERG3* ou *ERG11* em espécies de *Candida* parece ser um mecanismo potencial que pode determinar maior suscetibilidade ao poliênico com aumento da resistência aos azólicos (CHO; SHIN; KIM, 2014; SANGLARD et al., 2003).

Por outro lado, a constatação de resistência de *T. asahii* às equinocandinas vai ao encontro ao que já foi reportado (BAYRAMOGLU et al., 2008; KARAPINAR et al., 2015; LIAO et al., 2012; MATSUE et al., 2006; SUN et al., 2010; YANG; GAO; LI, 2014). O fenômeno de resistência intrínseca a essa classe de antifúngicos, descrito para outras leveduras, pode estar relacionado a quantidades insuficientes da enzima β-(1,3)-D-glucana sintase ou uma forma mutante da enzima que impede a ligação do fármaco (CHANDRA; MOHAMMAD; GHANNOUM, 2009).

Sendo assim, a verificação da ocorrência de resistência cruzada entre azólicos, bem como a comprovação da resistência intrínsica da espécie em estudo à classe das equinocandinas, enfatiza a busca de alternativas para o tratamento de infecções causadas por *T. asahii;* para este momento a antifungicoterapia está despreparada. Em situações de terapêutica primária ou profilática, a combinação de fármacos antifúngicos com diferentes mecanismos de ação pode apresentar-se como uma alternativa promissora devido à capacidade de reduzir o potencial dos micro-organismos em adquirir resistência (JOHNSON et al., 2004). Por outro lado, fármacos rotineiramente utilizados na clínica para o tratamento de diversas patologias, tais como agentes imunossupressores (FK506), ou outros compostos sintéticos (DPDS e EBS), podem potencializar a atividade de agentes antifúngicos convencionais, concorrendo ao aumento do espectro de atividade, bem como redução do tempo de resposta ao tratamento, redução de doses, dos custos com antifungicoterapia e de toxicidade.

Apesar dos resultados promissores já relatados, obtidos através de algumas associações antifúngicas frente a *Trichosporon* spp. (BASSETTI et al., 2004; KAMBERI et al., 1998; Li et al., 2010, LI et al., 2011; SERENA et al., 2005, SERENA et al., 2005b), a atividade de combinações de agentes antifúngicos frente a *T. asahii* após prolongada exposição ao fluconazol ainda não havia sido relatada. A avaliação *in vitro* das combinações entre anfotericina B e equinocandinas e/ou fluconazol e anfotericina B frente a *T. asahii* FR demonstrou predomínio de interações indiferentes (manuscrito 2). Dados de estudos *in vitro* permanecem controversos; sinergismo, indiferença e antagonismo têm sido descritos por diferentes autores para combinações entre anfotericina B e equinocandinas (BASSETTI et al., 2004; Li et al., 2010, Li et al., 2011; SERENA et al., 2005, SERENA et al., 2005b) ou fluconazol (KAMBERI et al., 1998).

Por outro lado, a combinação de caspofungina e fluconazol demonstrou elevado percentual de sinergismo frente *T. asahii* FR (manuscrito 2), o que, de certa forma, confirma o sinergismo da combinação de caspofungina com itraconazol, previamente descrito (Li et al., 2011). Adimitimos que o mecanismo de interações sinérgicas entre fluconazol e caspofungina pode ser semelhante ao proposto por Li et al. (2011), que atribuiram inibição simultânea de diferentes alvos da célula fúngica, tais como a parede e membrana celular (Li et al., 2011).

Ademais, a investigação de distintas combinações entre antifúngicos com não antifúngicos tem evidenciado importantes sinergismos *in vitro*, os quais são candidatos a estudos experimentais *in vivo* (ARGENTA et al., 2012; DENARDI et al., 2013; DENARDI et al., 2015; KONTOYANNIS et al., 2003; SHALIT et al., 2009; STEINBACH et al., 2004; VENTURINI et al., 2011; VENTURINI et al., 2016). Visto que pacientes transplantados são submetidos à profilaxia com agentes antifúngicos concomitantemente à terapia imunossupressora, percebe-se que o conhecimento das possíveis interações sinérgicas ou antagônicas dessa associação é de extrema importância para garantir a segurança e eficácia do tratamento.

Os dados apresentados no artigo 1 indicam que FK506 não exibiu atividade antifúngica até a concentração máxima utilizada quando testado isoladamente frente aos isolados clínicos de *T. asahii* FS e FR (CIMs > 64 µg/mL). Entretanto, considerando que a exploração da via da calcineurina dos fungos parece ser promissora para o desenvolvimento de novos antifúngicos (BLANKENSHIP et al., 2005; DENARDI et al., 2015; KONTOYIANNIS et al., 2003; ONYEWU et al., 2003;

STEINBACH et al., 2007; SUN et al., 2008), a associação de FK506 com fármacos disponiblizados para o tratamento de infecções por *T. asahii* também foi estudada (artigo 1). A constatação de efeito sinérgico na combinação de FK506 com antifúngicos, até então, ineficazes na inibição do crescimento dos isolados clínicos de *T. asahii* FS corrobora os achados de estudos prévios (KONTOYIANNIS et al., 2003; SHALIT et al., 2009; STEINBACH et al., 2004), os quais demonstraram elevado percentual de interações sinérgicas para a associação de caspofungina e FK506, frente *Cryptococcus neoformans*, espécies de *Fusarium* e *Aspergillus*. O efeito antifúngico exibido por imunossupressores, como ciclosporina A e FK506, provavelmente está relacionado com calmodulina ativada por uma proteina fosfatase envolvida com a resposta ao estresse fúngico, virulência e resistência antifúngica (STEINBACH, et al., 2007).

Além disso, o sinergismo observado na associação do FK506 com anfotericina B pode trazer benefícios à terapia antifúngica, como a redução do tempo de resposta ao tratamento, dose e toxicidade, além da possibilidade de diminuir o potencial dos microrganismos em adquirir resistência. Embora a anfotericina B já tenha sido relatada por demonstrar efeito insignificante no metabolismo de FK506 (IWASAKI et al., 1993), sabe-se que esse poliênico é bem conhecido por seus danos renais. Até o momento, os estudos de associação entre a anfotericina B e os agentes imunossupressores voltados a investigar seu potencial antifúngico ainda são escassos. Todavia, o conhecimento das possíveis interações da coadministração desses fármacos, assim como o monitoramento diário de suas concentrações séricas e de parâmetros bioquímicos, indicadores de lesão renal, podem trazer segurança à terapia dos pacientes que recebem ambos os fármacos (PATERSON; SINGH, 1997).

Numa perspectiva ainda mais inovadora, os organocompostos de selênio DPDS e EBS também foram investigados, pois os relatos de comprovação de suas propriedades antifúngicas (CHAN et al., 2007; BIEN et al., 1999; BILLACK; SANTORO; LAU-CAM, 2009; DENARDI et al., 2013; LORETO et al., 2011a; LORETO et al., 2011b; LORETO et al., 2012; ROSSETI; ROCHA; COSTA, 2015; ROSSETI et al., 2011; SOTEROPOULOS et al., 2000; VENTURINI et al., 2016; WOJTOWICZ et al., 2004), aliados a baixa toxicidade representada por esses compostos (NOGUEIRA et al., 2004), são fatores que justificaram a inclusão neste estudo. A atividade do DPDS e EBS, isolados ou em combinação com outros

agentes antifúngicos, frente a *Trichosporon* spp., ainda não havia sido relatada. De acordo com os resultados apresentados no manuscrito 1, isolados clínicos de *T. asahii* foram mais suscetíveis ao EBS do que DPDS, e o grupo FS apresentou valores de CIMs mais baixos do que o grupo FR. A menor atividade antifúngica observada para o DPDS pode ser devido à diferenças nas interações com as células fúngicas causadas por efeitos eletrônicos e esteroquímicos deste composto. Além disso, a capacidade do EBS em inibir o crescimento de *T. asahii* após exposição prolongada ao fluconazol pode estar relacionada à sua habilidade de agir como um agente antifúngico frente à regulação da expressão de proteínas que conferem resistência a este azólico (BILLACK; SANTORO; LAU-CAM, 2009).

Por outro lado, elevados percentuais de sinergismo foram observados para as combinações de antifúngicos com DPDS frente aos isolados clínicos de *T. asahii* FR, com exceção das combinações entre anfotericina B com os organocompostos, os quais exibiram forte interação sinérgica frente ao grupo FS. A atividade antifúngica apresentada por esses organocompostos de selênio pode ser decorrente da habilidade de interação com grupos sulfidrílicos presentes na célula fúngica e/ou na habilidade de inibir a H^+-ATPase da membrana dos fungos (BILLACK; SANTORO; LAU-CAM, 2009; MUGESH; DU MONT; SIES, 2001; WOJTOWICZ et al., 2004).

Desta forma, a exposição *in vitro* a concentrações crescentes de fluconazol é um fator importante para a emergência de resistência em *T. asahii*, fenômeno este, que agrega consequências para o perfil de suscetibilidade desta espécie, não só a outros agentes antifúngicos, como também a imunossupressores e compostos sintéticos, tais como o DPDS e EBS. Estudos futuros *in vitro* podem ser realizados a fim de testar outras possibilidades de combinações entre agentes antifúngicos frente *T. asahii*, e comparar o efeito dessas combinações entre os grupos FS e FR. Tal investigação poderá melhor caracterizar a atividade das combinações antifúngicas sobre estes agentes, podendo sinalizar quanto à segurança ou não destas combinações sobre *Trichosporon* multirresistentes.

Os resultados apresentados neste trabalho podem auxiliar estudos experimentais *in vitro* ou *in vivo* a fim de contribuir para um melhor entendimento da emergência de resistência ao fluconazol, das diferenças de suscetibilidade entre cepas de *T. asahii* FS e FR, bem como os mecanismos pelos quais o FK506 e organocompostos de selênio interagem com esses patógenos, isoladamente e em associação, a fim de torná-los mais sensíveis aos agentes antifúngicos.

6 CONCLUSÃO

Os resultados obtidos no presente estudo permitem concluir que:

- *T. asahii* apresentou-se como a única espécie identificada dentre os 30 isolados clínicos selecionados para esse estudo;
- Os isolados clínicos de *T. asahii* apresentaram boa sensibilidade aos antifúngicos azólicos (fluconazol, itraconazol, voriconazol, posaconazol), sensibilidade reduzida à anfotericina B e resistência às equinocandinas (caspofungina, micafungina e anidulafingina) antes da exposição prolongada ao fluconazol;
- Resistência cruzada foi detectada entre aos antifúngicos azólicos após indução de resistência ao fluconazol, sendo esse fenômeno mais pronunciado para o itraconazol (90%), seguido do posaconazol (36,67%) e voriconazol (10%);
- Após a exposição sequencial a concentrações crescentes de fluconazol, o número de isolados resistentes à anfotericina B decresceu de 90% a 3,33%; entretanto, para as equinocandinas o percentual de resistência permaneceu o mesmo entre as cepas de *T. asahii* FS e FR (100%);
- O voriconazol apresentou a melhor atividade antifúngica frente ao micro-organismo em estudo;
- A atividade das combinações *in vitro* entre anfotericina B e equinocandinas ou fluconazol apresentou, em sua maioria, interações com ação indiferente sobre *T. asahii* FR;
- A associação de caspofungina e fluconazol apresentou o melhor percentual de sinergismo (66,67%) sobre *T. asahii* FR dentre as combinações testadas entre agentes antifúngicos;
- O tacrolimus não evidenciou atividade antifúngica quando testado isoladamente, entretanto elevados percentuais de sinergismos foram detectados nas associações do imunossupressor com anfotericina B (96,67%) e caspofungina (73,33%) frente aos isolados de *T. asahii* FS;
- As combinações de anfotericina B, fluconazol, itraconazol e caspofungina com tacrolimus resultaram em interações indiferentes frente ao grupo de isolados clínicos de *T. asahii* FR;

- Isolados clínicos de *T. asahii* foram mais sensíveis ao ebselen do que disseleneto de difenila; e o grupo de isolados FS apresentou CIMs mais baixas do que o grupo FR para ambos compostos;
- Elevados percentuais de sinergismo foram evidenciados nas combinações de disselelento de difenila com caspofungina (96,67%), anfotericina B (93,33%), fluconazol (86,67%), itraconazol (83,33%) frente aos isolados clínicos de *T. asahii* FR;
- As interações mais sinérgicas frente ao grupo de cepas FS para os organocompostos de selênio ocorreram nas combinações com anfotericina B (90%);
- O organocomposto ebselen destacou-se como o agente não antifúngico com melhor atividade antimicótica frente *T. asahii*;

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ANEXO A – CARTA DE ACEITE DO ARTIGO 1 (*Article in press:* 30-9-16)

---------- Forwarded message ----------
From: **Carlos Brites** <crbrites@gmail.com>
Date: 2016-08-05 8:49 GMT-03:00
Subject: Your Submission
To: sydneyalves.ufsm@gmail.com

Ms. Ref. No.: BJID-D-16-00185R2
Title: Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant Trichosporon asahii isolates
Brazilian Journal of Infectious Diseases

Dear Sydney,

I am pleased to inform you that your paper "Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant Trichosporon asahii isolates" has been accepted for publication in Brazilian Journal of Infectious Diseases.

Thank you for submitting your work to Brazilian Journal of Infectious Diseases.

Yours sincerely,

Carlos Brites
Editor-in-Chief
Brazilian Journal of Infectious Diseases

ANEXO B – SUBMISSÃO DO MANUSCRITO 1

21-Sep-2016

Dear Ms. Kubiça:

Your manuscript entitled "In vitro activity of diphenyl diselenide and ebselen alone and in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant Trichosporon asahii isolates" has been successfully submitted online and is presently being given full consideration for publication in Medical Mycology.

Your manuscript ID is MM-2016-0283.

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Thank you for submitting your manuscript to Medical Mycology.

Sincerely,

Editor-in-Chief