UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

ANÁLOGOS DA 8-HIDROXIQUINOLINA COMO CANDIDATOS A AGENTES ANTIMICÓTICOS: ESTUDO DA ATIVIDADE ANTIFÚNGICA, MECANISMOS DE AÇÃO E PARÂMETROS TOXICOLÓGICOS

BRUNA PIPPI

Orientador: Prof. Dr. Alexandre Meneghello Fuentefria
Coorientador: Prof. Dr. Saulo Fernandes de Andrade

Porto Alegre
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DAS UTOPIAS

"Se as coisas são inatingíveis... ora!

Não é motivo para não querê-las...

Que tristes os caminhos, se não fora

A presença distante das estrelas! "

Mário Quintana

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Dedico este trabalho aos meus maiores incentivadores:

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"Não é sobre chegar no topo do mundo e saber que venceu. É sobre escalar e sentir que o caminho te fortaleceu. É sobre ser abrigo e também ter morada em outros corações. E assim ter amigos contigo em todas as situações... A gente não pode ter tudo, qual seria a graça do mundo se fosse assim? Por isso eu prefiro sorrisos e os presentes que a vida trouxe pra perto de mim" (Ana Vilela)

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RESUMO

A incidência e gravidade de doenças fúngicas têm aumentado significativamente, apresentando elevados índices de morbidade e mortalidade. Além disso, a emergência de fungos resistentes aos fármacos disponíveis e a escassez de agentes antifúngicos seguros tem tornado ainda mais crítico o controle desses micro-organismos. Portanto, as atuais circunstâncias justificam a nossa pesquisa destinada a caracterizar a atividade anti-*Candida* e antidermatofítica, obter informações sobre o mecanismo de ação antifúngico e avaliar parâmetros toxicológicos de análogos de 8-hidroxiquinolina. Cinco derivados da 8-hidroxiquinolinas foram avaliados: clioquinol (composto **1**), ácido 8-hidroxiquinolinil-5-sulfônico (composto **2**), ácido 7-iodo-8-hidroxiquinolinil-5-sulfônico (composto **3**), 8-hidroxiquinolinil-5-(N-4-clorofenil)sulfonamida (PH151) e 8-hidroxiquinolinil-5-(N-4-metoxifenil)sulfonamida (PH153). As 8-hidroxiquinolinas mostraram atividade antifúngica para todos os isolados de *Candida* e dermatófitos testados. Clioquinol **1** foi o composto que apresentou menores valores de CIM, seguido das sulfonamidas (PH151 e PH153) e ácidos sulfônicos (composto **2** e **3**). Os compostos foram fungistáticos para *C. albicans* e fungicidas para dermatófitos (exceto PH151). A modelagem PK/PD mostrou EC_{50} variando entre 0,181 - 0,306 µg/mL para clioquinol **1**, 0,873 - 1,329 µg/mL para PH151 e 7,105 - 8,865 µg/mL para PH153. Em relação aos mecanismos de ação envolvidos, todos os derivados mostraram efeito sobre a parede celular fúngica. Os ácidos sulfônicos **2** e **3** comprometeram, também, a integridade funcional da membrana plasmática; e clioquinol **1**, PH151 e PH153 inibiram a formação de pseudo-hifas em *C. albicans*. Ainda, clioquinol **1** exibiu ação sobre biofilme de *Candida* formado em microplaca de poliestireno e em dispositivo intrauterino. Clioquinol **1,** PH151 e PH153 mostraram ótimos efeitos antifúngicos via oral em *D. melanogaster* imunodeficientes infectadas com *C. albicans*. Os derivados da 8-hidroxiquinolina apresentaram baixa toxicidade em modelos alternativos como pele de orelha de porco, *D. melanogaster* e embriões de galinha. O composto **3** foi o único que demonstrou toxicidade nesses modelos, uma vez que foi letal para o embrião de galinha. Os estudos com embriões e larvas de *zebrafish* foram realizados apenas com os derivados PH151 e PH153 e mostraram mortalidade dependente da dose. A aparente toxicidade foi detectada nas fases embrio-larval, logo, não se pode inferir sobre potencial toxicidade em adultos; mas podem servir de alerta para uma possível embriotoxicidade. Em conjunto, os dados deste estudo apoiam o potencial do clioquinol **1**, PH151 e PH153 para tratar candidíase sistêmica e dermatomicose. Por fim, estes compostos demonstram ser candidatos antimicóticos apropriados para avançar em estudos com roedores.

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ANALOGUES OF 8-HYDROXYQUINOLINE AS CANDIDATE TO ANTIMYCOTIC AGENTS: STUDY OF ANTIFUNGAL ACTIVITY, ACTION MECHANISMS AND TOXICOLOGICAL PARAMETERS

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ABSTRACT

The incidence and severity of fungal diseases have significantly increased, presenting high morbidity and mortality rates. In addition, the emergence of drug-resistant fungi and the scarcity of safe antifungal agents have become the control of these microorganisms even more critical. Therefore, the current circumstances justify our research designed to characterize anti-*Candida* and antidermatophytic activity, gain insights into the antifungal action mechanism and to evaluate toxicological parameter of 8-hydroxyquinoline analogues. Five 8-hydroxyquinoline derivatives were evaluated: clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**), 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**) 8-hydroxyquinoline-5-(N-4-chlorophenyl)sulfonamide (PH151) and 8-hydroxyquinoline-5-(N-4-methoxyphenyl)sulfonamide (PH153). The 8-hydroxyquinolines showed antifungal activity for all *Candida* and dermatophytes tested. Clioquinol **1** was the compound that presented lower MIC values, followed by sulfonamides (PH151 and PH153) and sulfonic acids (compounds **2** and **3**). The compounds were fungistatic for *C. albicans* and fungicides for dermatophytes (except PH151). PK/PD modeling showed EC_{50} ranging from 0.181 - 0.306 µg/mL for clioquinol **1**, 0.873 - 1.329 µg/mL for PH151 and 7.105 - 8.865 µg/mL for PH153. Regarding the mechanisms of action involved, all derivatives showed effect on the fungal cell wall. Sulphonic acids **2** and **3** also compromised the functional integrity of the cytoplasmic membrane; and clioquinol **1**, PH151 and PH153 inhibited the formation of pseudohyphas in *C. albicans*. Further, clioquinol **1** exhibited action on *Candida* biofilm formed on polystyrene microplate and intrauterine device. Clioquinol 1, PH151 and PH153 showed excellent oral antifungal effects in immunodeficients *D. melanogaster* infected with C. albicans. The 8-hydroxyquinoline derivatives showed low toxicity in alternative models such as pig ear skin, *D. melanogaster* and chicken embryos. Compound **3** was the only one that demonstrated toxicity in these models, since it was lethal to the chicken embryo. Studies with zebrafish embryos and larvae were performed only with the PH151 and PH153 derivatives and showed dose-dependent mortality.The apparent toxicity was detected in the embryo-larval stages, therefore, it is not possible to infer about potencial toxicity in adults; but may be an alert for possible embryotoxicity.Taken together, data from this study support the potential of clioquinol 1, PH151 and PH153 to treat systemic candidiasis and dermatomycosis. Finally, these compounds demonstrate to be appropriate antimycotic candidates to advance in rodent models.

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LISTA DE ABREVIATURAS E SIGLAS

ATCC	“American Type Culture Collection”
BDA	Batata Dextrose Ágar
CIM	Concentração Inibitória Mínima
CIMS	Concentração Inibitória Mínima séssil
CLSI	“Clinical Laboratory Standards Institute”
DIU	Dispositivo Intrauterino
DL_{50}	Dose que mata 50%
DNA	Ácido Desoxirribonucleico
FDA	“Food and Drug Administration”
GAIN	“Generating Antibiotic Incentives Now”
HET-CAM	“Hens Egg Test-Chorion Allantoic Membrane”
IC_{50}	Concentração que Inibe 50%
ICBS	Instituto de Ciência Básicas da Saúde
IFB	Inibição da Formação do Biofilme
IFC	Instituto Federal Catarinense
IFIs	Infecções Fúngicas Invasivas
MEV	Microscopia Eletrônica de Varredura
MOPS	Ácido Morfolinopropanosulfônico
MTT	Brometo de 3 - (4,5-dimetiltiazol-2-il) -2,5 difenil-tetrazólio
PBS	Tampão Fosfato-Salino
PK/PD	Farmacocinética/Farmacodinâmica
RB	Remoção do Biofilme
RNA	Ácido Ribonucleico
RPMI	“Roswell Park Memorial Institute “
RS	Rio Grande do Sul
SDA	Ágar Sabouraud Dextrose
SDS	Dodecilsulfato de Sódio
SMON	Neuropatia Mielo-Óptica Subaguda
UFC	Unidades Formadoras de Colônias
UFRGS	Universidade Federal do Rio Grande do Sul
UNIPAMPA	Universidade Federal do Pampa

CVV	Candidíase Vulvovaginal
XTT	2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazol-5-carboxanilida

1 INTRODUÇÃO

Nos últimos anos, a incidência e gravidade de doenças fúngicas têm aumentado significativamente, com índices elevados de morbidade e mortalidade, especialmente em pacientes com o sistema imunológico debilitado. Além disso, a emergência de fungos resistentes aos antimicrobianos tem tornado ainda mais crítico o controle desses micro-organismos. A resistência aos antifúngicos traz alto risco ao paciente com infecções sistêmicas e também prolonga o tempo de internação gerando custos elevados para o sistema de saúde. Ainda, os antifúngicos têm diversas outras limitações relacionadas à toxicidade. Perfis de segurança restringem o uso clínico e, portanto, o número de fármacos disponíveis para tratar tais infecções permanece pequeno. As limitadas opções terapêuticas tornam o fenótipo de resistência um problema grave na quimioterapia de infecções fúngicas e exigem soluções rápidas.

Diante desse cenário preocupante, existe uma clara necessidade de revalidação dos antifúngicos e introdução de novas estratégias farmacêuticas. Nesse contexto, derivados da 8-hidroxiquinolina podem vir a ter um importante papel na terapia fúngica. Com alta versatilidade sintética e grande variedade de efeitos biológicos, a 8-hidroxiquinolina e seus derivados apresentam estruturas químicas interessantes para o desenvolvimento de novos candidatos a fármacos.

O clioquinol é um derivado 5,7-dihalogenado da 8-hidroxiquinolina que foi usado entre 1950 e 1970 no tratamento de doenças intestinais e infecções de pele. Em 1970 suas formulações orais foram retiradas do mercado devido a relatos de neurotoxicidade. No entanto, ainda há controvérsia sobre o tema. Atualmente, o clioquinol é usado como antibiótico tópico. Além de seu uso como antimicrobiano, o clioquinol e alguns análogos têm mostrado potencial efeito para o tratamento das patologias não-infecciosas como câncer, doença de Alzheimer, Parkison e Huntington.

Uma vez que pesquisas terapêuticas com clioquinol têm sido bastante exploradas, o seu uso sistêmico recebeu nova atenção. Dado o potencial de reintrodução de formulações orais para novas indicações, uma maior compreensão de sua toxicologia é importante, bem como avaliações biológicas e toxicológicas de seus análogos. Além disso, embora a atividade antifúngica do clioquinol já seja

bastante conhecida, ainda não existem estudos consistentes sobre o modo de ação deste fármaco.

Em suma, a síntese de novos derivados antifúngicos utilizando como protótipos agentes antifúngicos com conhecida eficácia, pode levar a obtenção de novos candidatos a fármacos antimicóticos. Nestas condições, as suscetibilidades celulares e a toxicidade frente a células humanas podem ser controladas pelas modificações estruturais aplicadas, fornecendo antifúngicos seletivos e eficazes para o tratamento de infecções causadas por patógenos resistentes. Portanto, as atuais circunstâncias justificam a nossa pesquisa destinada à caracterização e avaliação antifúngica de novos análogos da 8-hidroxiquinolina, bem como o estudo de propriedades que possam compreender o mecanismo de ação e toxicidade de análogos já existentes, especialmente o clioquinol.

2 OBJETIVOS

2.1 Objetivo Geral

Caracterizar a atividade antifúngica, obter informações sobre o mecanismo de ação e avaliar parâmetros toxicológicos de análogos de 8-hidroxiquinolina.

2.2 Objetivos Específicos

2.2.1 Avaliar a atividade anti-*Candida* e antidermatofítica *in vitro* de cinco derivados da 8-hidroxiquinolina: clioquinol, ácido 8-hidroxiquinolinil-5-sulfônico, ácido 7-iodo-8-hidroxiquinolinil-5-sulfônico, 8-hidroxiquinolinil-5-(*N*-4-clorofenil)sulfonamida (ou PH151) e 8-hidroxiquinolinil-5-(*N*-4-metoxifenil)sulfonamida (ou PH153), sendo os dois últimos sintetizados pelo nosso grupo de pesquisa;

2.2.2 Avaliar se os derivados da 8-hidroxiquinolina apresentam propriedades fungicidas ou fungistáticas;

2.2.3 Obter informações sobre a dinâmica de interação do clioquinol, PH151 e PH153 com fungos;

2.2.4 Estudar características que possam auxiliar na compreensão do mecanismo de ação antifúngico de derivados da 8-hidroxiquinolina;

2.2.5 Investigar a ação do clioquinol, do ácido 8-hidroxiquinolinil-5-sulfônico e do ácido 7-iodo-8-hidroxiquinolinil-5-sulfônico sobre biofilmes de *Candida* spp. em microplacas de poliestireno;

2.2.6 Averiguar a ação do clioquinol sobre biofilmes de *Candida* spp. pré-formados em dispositivos intrauterinos;

2.2.7 Avaliar a influência da ligação da proteína plasmática albumina sobre a atividade antifúngica de derivados de 8-hidroxiquinolinas;

2.2.8 Verificar o efeito protetor dos derivados da 8-hidroxiquinolina em *Drosophila melanogaster* imunodeficientes infectadas sistemicamente por *Candida albicans;*

2.2.9 Analisar o potencial irritativo dos derivados da 8-hidroxiquinolina

2.2.10 Verificar a toxicidade dos derivados da 8-hidroxiquinolina em modelo *ex vivo* e em diferentes modelos animais alternativos, tais como *Drosophila*

melanogaster imunodeficientes, embriões de galinha e embriões e larvas de *zebrafish.*

3 REVISÃO DA LITERATURA

3.1 Infecções fúngicas

No início do século XX, epidemias bacterianas eram uma causa global e importante de mortalidade. Em contraste, as infecções fúngicas eram menosprezadas. Na década de 60, terapias antibacterianas foram desenvolvidas e, em paralelo, observou-se um aumento drástico na ocorrência de infecções fúngicas, as quais atualmente representam um problema de saúde pública mundial (Vandeputte et al., 2012).

Dados sugerem que várias doenças fúngicas são sub-documentadas (Calderone et al., 2014). Ainda assim, a incidência delas supera as expectativas, acometendo aproximadamente 1,2 bilhão de indivíduos (Chang et al., 2017). Esta crescente incidência é influenciada pelos atuais avanços médicos, os quais melhoram a sobrevida de pacientes em situação de risco e, consequentemente, aumentam a população de imunodeprimidos (Clark e Hajjeh, 2002). Casos relacionados à imunodeficiência, tais como pacientes com AIDS, câncer, transplantados ou que necessitam de terapia imunossupressora ou procedimentos cirúrgicos invasivos são os principais acometidos por tais infecções (Vandeputte et al., 2012; Chang et al., 2017).

Entre os casos mais severos de doenças fúngicas, estão as infecções fúngicas invasivas (IFIs) que podem afetar a circulação sanguínea, sistema nervoso central, bem como órgãos específicos, incluindo pulmão, fígado e rins (Roemer e Krysan, 2014; Chang et al., 2017). A maioria dos fungos que causam IFIs é oportunista e pode estabelecer a doença em pessoas com comprometimento da resposta imunológica (Roemer e Krysan, 2014). Infecções fúngicas invasivas causam pelo menos 1,5 milhão de mortes a cada ano. A taxa de mortalidade, perturbadoramente elevada, excede a malária e é semelhante à tuberculose resistente a medicamentos (Calderone et al., 2014). *Candida*, *Aspergillus*, *Cryptococcus*, *Pneumocystis*, *Mucor* e *Rhizopus* são os agentes etiológicos mais frequentemente envolvidos e responsáveis por 90% dessas mortes (Pianalto e Alspaugh, 2016; Campoy e Adrio, 2017). Esses dados têm importância relevante para profissionais da área da saúde e pesquisadores com interesse em fungos patogênicos humanos (Calderone et al., 2014).

As IFIs representam apenas uma parte da magnitude das doenças fúngicas. Os fungos também causam infecções de mucosas e micoses superficiais, cutâneas e subcutâneas. Exemplos específicos incluem candidíase vaginal, esofágica e orofaríngea, bem como dermatofitoses em diversas regiões do corpo. Embora pessoas com o sistema imune deficiente possam apresentar elevadas taxas e maior gravidade da doença, essas micoses são frequentes entre imunocompetentes (Roemer e Krysan, 2014). Apesar da mortalidade praticamente nula (Calderone et al., 2014), elas têm maior incidência do que as IFIs e diminuem a qualidade de vida dos indivíduos afetados (Campoy e Adrio 2017); além disso, podem ser persistentes e exigir tratamento contínuo e, por vezes, não ocorre completa erradicação (Scorzoni et al., 2017).

3.1.1 Infecções por dermatófitos

Os dermatófitos são um grupo de fungos filamentosos hialinos e septados que causam micoses cutâneas em indivíduos saudáveis e em imunocomprometidos (Mukherjee et al., 2003). Eles têm a capacidade de digerir a queratina da pele, cabelo e unha de humanos e outros mamíferos, utilizando como nutriente durante a instalação da infecção (Achterman e White, 2013). Essas infecções geralmente são restritas à camada córnea da epiderme e tecidos queratinizados (Achterman e White, 2012). Entretanto, embora raras, dermatofitoses disseminadas podem ocorrer, acometendo especialmente imunocomprometidos (Galhardo et al., 2004; Marconi et al. 2010) e apresentam taxa de falha de tratamento particularmente alta (30,8%) (Achterman e White, 2012).

As micoses causadas por dermatófitos estão entre as infecções mais frequentes e são denominadas de *tineas* ou dermatofitoses (White et al, 2014; Dalla Lana et al., 2016). Elas podem ser classificadas de acordo com a localização anatômica da lesão. As mais relevantes são: *tinea capitis* (couro cabeludo), *tinea corporis* (ombros, tronco e braços), *tinea cruri* (virilha), *tinea unguium* (unhas; também chamada de onicomicose), *tinea barbae* (barba), *tinea manuum* (mão) e *tinea pedis* (pés) (Dalla Lana et al., 2016), demonstradas na figura 1. O quadro clínico mais comum de dermatofitose inclui placas anulares, despigmentação, prurido e perda de cabelo. Desenvolvem-se do centro da lesão para as bordas, com intensa descamação geralmente associada à resposta inflamatória resultante da

atividade queratinolítica. As lesões são contagiosas e impactantes na qualidade de vida dos pacientes (Moraes et al., 2001).

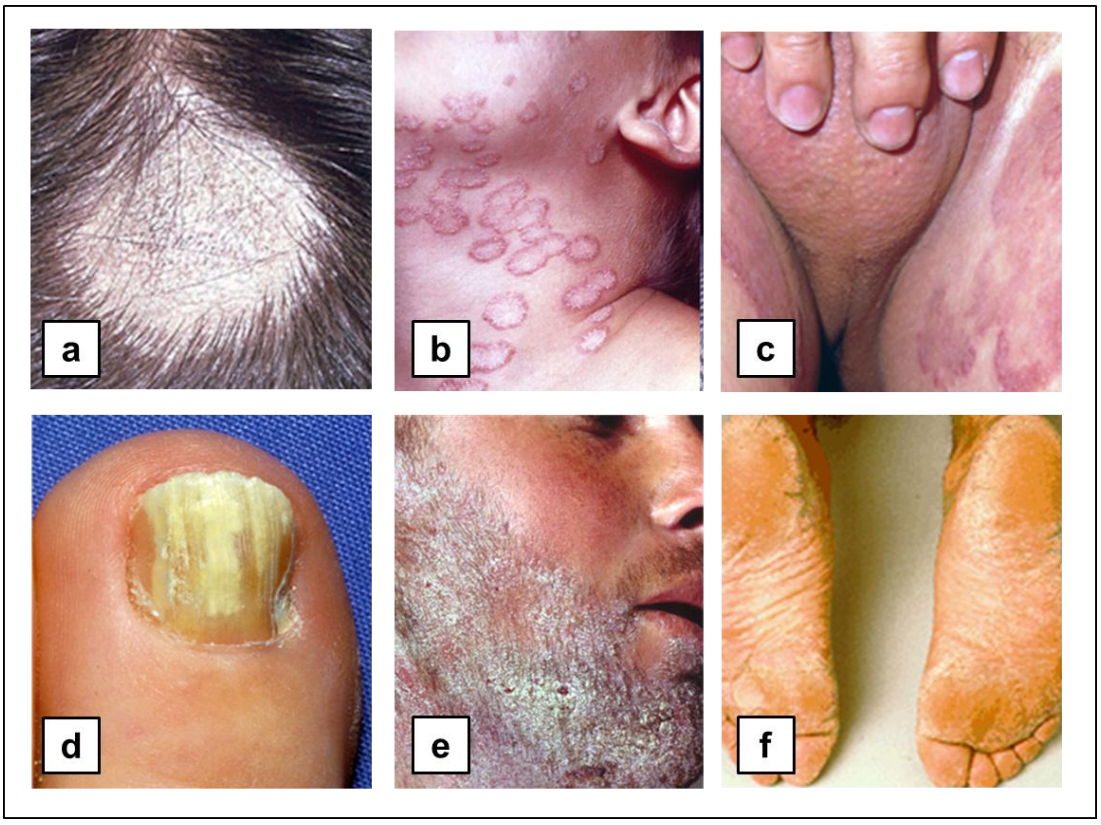

Figura 1. Imagens de dermatofitoses: *tinea capitis* (a), *tinea corporis* (b), *tinea cruri* (c), *tinea unguium* (d), *tinea barbae* (e) e *tinea pedis* (f) (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

Existem pelo menos 40 espécies dos dermatófitos que infectam humanos, e muitos desses fungos podem causar doenças em mais de um local do corpo. Epidemiologicamente, três gêneros fúngicos predominam nas dermatofitoses: *Trichophyton, Microsporum* e *Epidermophyton* (White et al, 2014). Entre as espécies de maior relevância clínica estão *M. canis*, *M. gypseum*, *M. nanum*, *T. mentagrophytes*, *T. rubrum*, *T. tonsurans*, *T. violaceum*, *T. shoenlini* e *E. flocosum* (Dalla Lana et al., 2016). *T. rubrum, T. mentagrophytes, M. canis* e *M. gypseu*m são destacados no texto que segue, uma vez que são foco de nossa pesquisa.

3.1.1.1 *Trichophyton rubrum*

T. rubrum é considerado um fungo antropofílico e a transmissão ocorre de pessoa para pessoa (Nenoff et al., 2014; White et al, 2014). *T. rubrum* é o principal agente envolvido na maioria das dermatofitoses, especialmente onicomicoses, representando em torno de 70% dos casos. A elevada prevalência está associada a esta espécie ser refratária aos tratamentos usuais devido a sua grande adaptação ao hospedeiro humano (Dalla Lana et al., 2016). Este dermatófito está associado a infecções crônicas com inflamação leve (White et al, 2014). A cultura e a micromorfologia de *T. rubrum* estão ilustradas na figura 2.

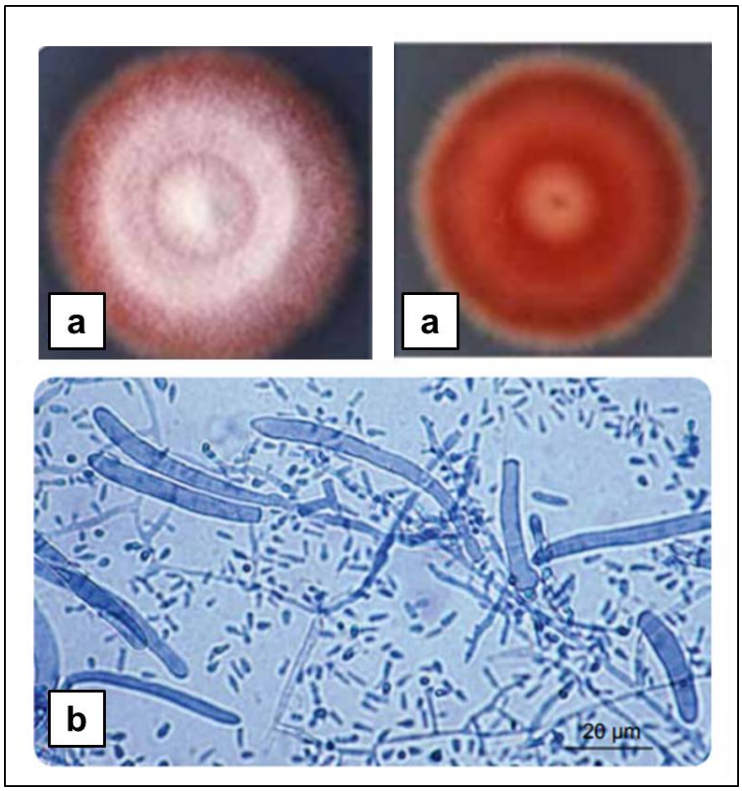

Figura 2. Imagens de cultura (a) e micromorfologia (b) de *Trichophyton rubrum* (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

3.1.1.2 *Trichophyton mentagrophytes*

T. mentagrophytes é um fungo zoofílico, sendo o segundo dermatófito mais comum em infecções fúngicas e tende a causar *tinea unguium*, *tinea pedis* e,

com menor frequência, *tinea corporis* (Nenoff et al., 2014). Este fungo parasita um número elevado de animais tais como coelhos, cavalos e porcos, e por isso, é mais frequente em meio rural e causa lesões com intermediária à intensa reação inflamatória (Dalla Lana et al., 2016). A cultura e a micromorfologia deste dermatófito estão ilustradas na figura 3.

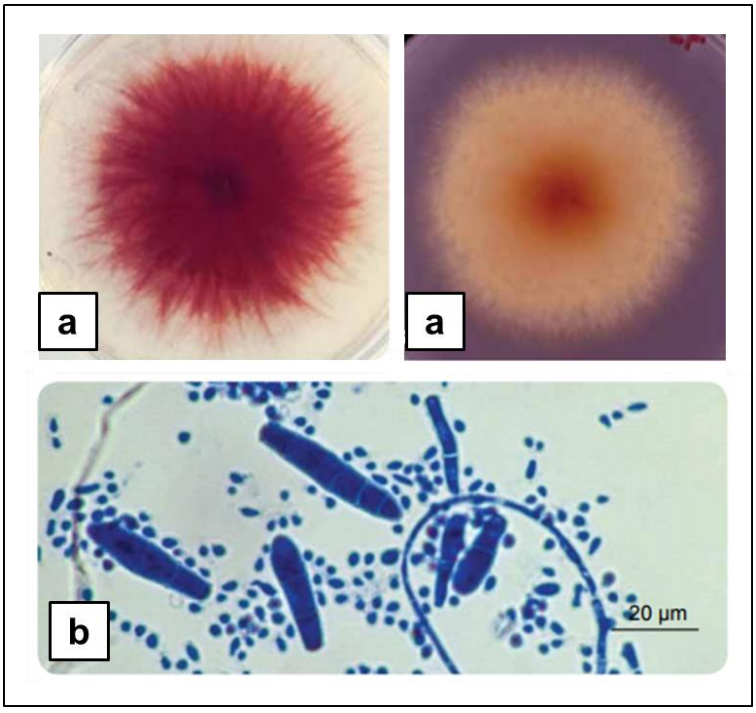

Figura 3. Imagens de cultura (a) e micromorfologia (b) de *Trichophyton mentagrophytes* (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

3.1.1.3 *Microsporum canis*

M. canis também é considerado um patógeno zoofílico. *Este fungo* é o agente mais isolado nos casos de *tinea capitis* sendo responsável por 67% das infecções dermatofíticas do couro cabeludo (Dalla Lana et al., 2016). Além disso, *M. canis* também é comumente isolado de indivíduos com *tinea corporis* (Nenoff et al., 2014). A infecção por esse micro-organismo é frequente em crianças e está associada ao contato com cães e, principalmente, gatos infectados (Nenoff et al., 2014). A cultura e a micromorfologia de *M. canis* estão ilustradas na figura 4.

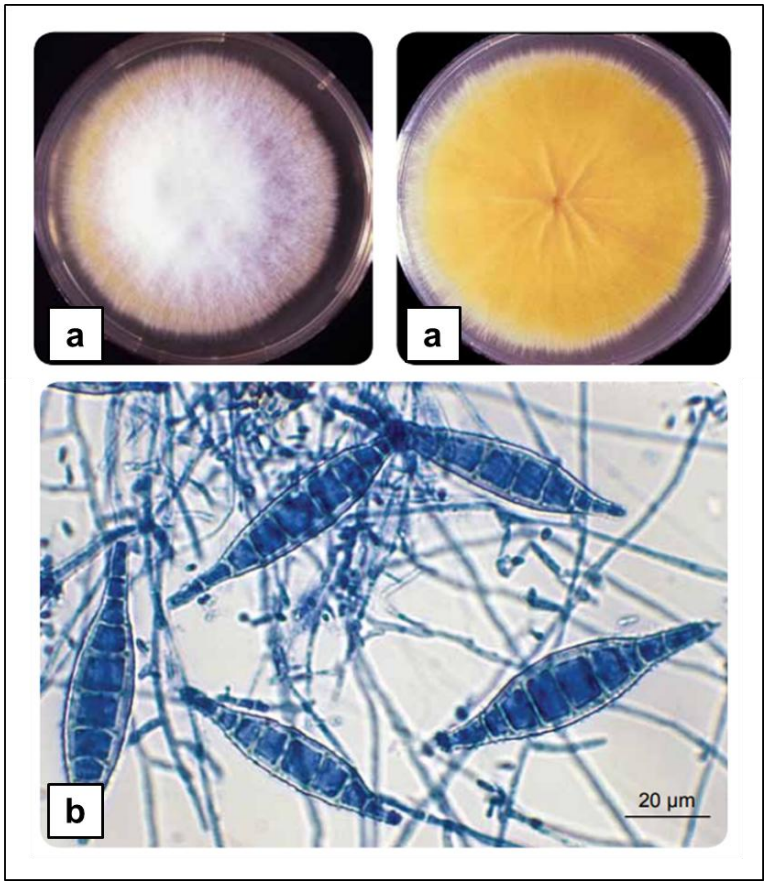

Figura 4. Imagens de cultura (a) e micromorfologia (b) de *Microsporum canis* (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

3.1.1.4 *Microsporum gypseum*

M. gypseum é um fungo geofilico uma vez que é encontrado no solo e ocasionalmente infecta animais (Laniosz e Wetter, 2014; Segal e Frenkel, 2015). A transmissão desse dermatófito ocorre através do contato direto com o solo; assim, crianças que brincam ao ar livre e jardineiros são os principais acometidos (Nenoff et al., 2014). *M gypseum* tipicamente se manifesta como *tinea corporis*, *tinea barbae* e *tinea capitis* (Fike et al. 2018). Este fungo causa lesões clínicas agudas de intensa reação inflamatória (White et al, 2014) e há relatos de que pode provocar dermatites graves e crônicas em pacientes com imunocomprometimento (Dalla Lana et al., 2016). A cultura e a micromorfologia deste dermatófito estão ilustradas na figura 5.

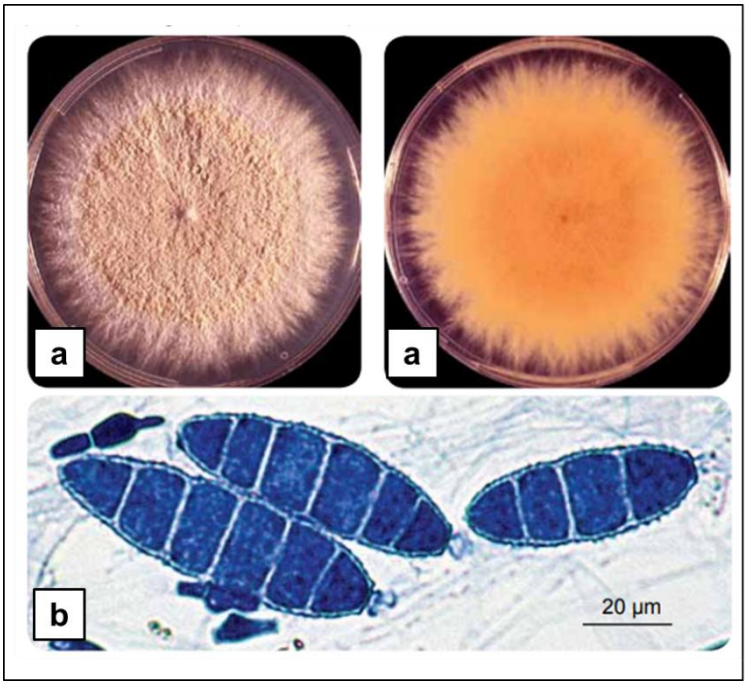

Figura 5. Imagens de cultura (a) e micromorfologia (b) de *Microsporum gypseum* (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

Embora os dermatófitos sejam prevalentes em todo o mundo, esses fungos apresentam epidemiologia que varia em relação a fatores regionais, sazonais, de faixa etária, frequência de contato com animais, condições higiênico-sanitárias e exposição a locais públicos (Aquino et al., 2007). Os climas quentes e úmidos são fatores que predispõem as populações a infecções por *tinea*. As baixas condições socioeconômicas também estão fortemente ligadas a taxas de prevalência de tais infecções. Os países em desenvolvimento têm altas taxas de *tinea capitis*, enquanto os países desenvolvidos têm taxas elevadas de *tinea pedis* e onicomicose (Achterman e White, 2012).

Estima-se que pelo menos meio bilhão de dólares por ano sejam gastos em tratamento de dermatofitoses (Achterman e White, 2012). O tratamento pode ser tópico ou sistêmico, com uma variedade de fármacos antifúngicos. Os mais comuns são os imidazóis, a terbinafina (White et al, 2014) e o ciclopirox olamina (Pires et al, 2014). As infecções por dermatófitos são difíceis de tratar e, frequentemente, se recidivam no mesmo local de infecção inicial (White et al, 2014). Atualmente,

desconhece-se se isso se deve à eliminação incompleta do fungo durante o tratamento com posterior recidiva ou se representam novas infecções (Achterman e White, 2012).

Pesquisas com dermatófitos e atividade antfúngica são raras e o conhecimento sobre o tema é limitado. Isso ocorre porque muitos microbiologistas não consideram esse grupo de fungos tão importantes como outros micro-organismos infecciosos. Assim, há um pequeno número de pesquisadores que trabalham no problema, além dos financiamentos que também são restritos. No entanto, embora a mortalidade por dermatofitos seja muito baixa, há uma morbidade significativa associada a essas infecções, com custos bastante elevados para o sistema de saúde (Achterman e White, 2012; White et al, 2014).

3.1.2 Infecções por *Candida*

Candida spp. residem como micro-organismos comensais na pele e membranas mucosas de indivíduos saudáveis. Todavia, as espécies desse gênero tendem a expressar sua virulência e desenvolver infecção (candidíase) quando critérios específicos, tais como imunossupressão, são atendidos (Sardi et al., 2013). As infecções causadas por espécies de *Candida* variam. Elas podem ser autolimitantes como também podem se apresentar em forma de lesões superficiais da mucosa, pele e unhas, muitas vezes recorrentes e crônicas; ou ainda, podem ser invasivas ou disseminadas com alto risco de vida (MacCallum 2012). A figura 6 ilustra lesões ocasionadas por esse gênero, bem como a colônia e sua micromorfologia.

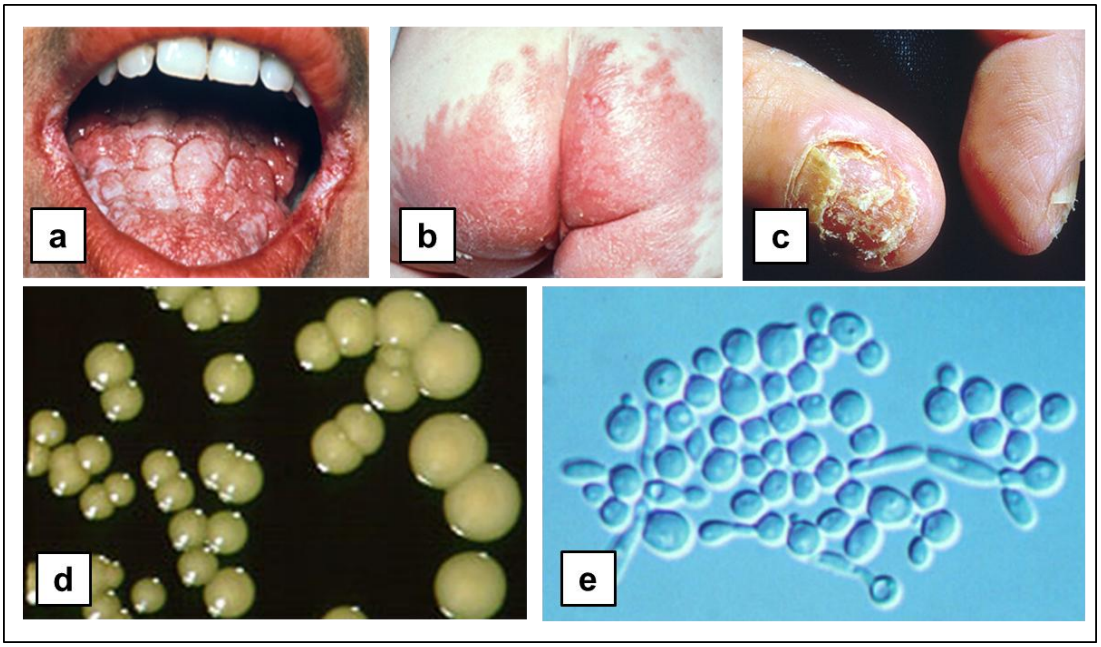

Figura 6. Imagens de infecções por *Candida* sp.: candidíase de mucosa (a), candidíase de pele (b) e candidíase de unha (c); e colônia (d) e micromorfologia (e) (Fonte: Mycology Online – National Mycology Reference Centre, 2017).

Candidemia é um problema crescente nos hospitais com relevantes implicações clínicas (Doi et al., 2016). Espécies de *Candida* são a quarta causa mais comum de infecções nosocomiais da corrente sanguínea (Chandra e Mukherjee, 2015) e estão associadas a uma taxa de mortalidade de 30% em crianças e até 50% em adultos (Moran et al., 2009). Com alto grau de severidade, tais infecções aumentam o tempo de permanência do paciente no hospital e geram custos elevados ao sistema de saúde (Lockhart, 2014; Doi et al., 2016).

Embora a distribuição das espécies tenha mudado ao longo das últimas três décadas, infecções invasivas por *Candida* spp. são atribuídas principalmente a cinco espécies: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* e *C. krusei* (Pfaller et al., 2007; Lockhart et al., 2012). A epidemiologia dessas infecções mostra padrões de incidência distintos variando entre diferentes regiões geográficas e até mesmo entre os centros médicos dentro da mesma região (Pfaller et al., 2004a; Colombo et al., 2013). *C albicans* ainda é considerada a espécie mais frequente associada à candidemia, entretanto, espécies não-*albicans* estão emergindo (Lortholary et al., 2014). No Brasil, um recente estudo envolvendo 16 hospitais

distribuídos nas cinco regiões do país constatou que *C. parapsilosis* e *C. tropicalis* são as espécies não-*albicans* mais prevalentes, representando 24,1% e 15,3% dos casos, respectivamente (Doi et al., 2016).

3.1.2.1 *Candida albicans*

C. albicans é a mais estudada espécie do gênero *Candida e* é uma das leveduras mais patogênicas para o ser humano (Pappas et al., 2006). Esta espécie possui fatores de virulência que auxiliam na infecção, tal como alteração da forma levedurifome para a forma filamentosa de crescimento. Essa transição dimórfica está associada a propriedades importantes para a interação com o hospedeiro, tais como: adesão a células epiteliais e endoteliais, escape de macrófagos e evasão imune; além de ser um processo biológico fundamental para a formação do biofilme (Lu et al., 2014; Nobile e Johnson 2015). *C. albicans* também secreta diversos tipos de enzimas, como proteinases e fosfolipases, que também influenciam na virulência.

Os fatores de risco para a infecção por *C albicans* incluem uso de antibióticos de amplo espectro, presença de um cateter venoso central, procedimentos gastrointestinais e nutrição parenteral (Moran et al., 2010).

3.1.2.2 *Candida glabrata*

Candida glabrata é a espécie não-*albicans* mais frequente nos EUA e sua incidência tem aumentado ao longo da última década (Baddley et al., 2001; Malani et al., 2005; Lockhart, 2014). A razão para este aumento é desconhecida, mas há especulações de que é devido ao uso generalizado do fluconazol (a que *C. glabrata* tem susceptibilidade diminuída) como terapia profilática (Lockhart, 2014).

Além disso, a resistência cruzada entre fluconazol e outros triazóis pode ocorrer e está bem descrita entre os isolados dessa espécie (Pfaller et al., 2004b; Pfaller et al., 2005a; Sanguinetti et al., 2005). Tal resistência tem se manifestado clinicamente, demonstrando que pacientes com fungemia por *C. glabrata* e exposição prévia ao fluconazol falharam no tratamento com voriconazol (Magill et al., 2006).

Devido à alta incidência de resistência aos azóis, o uso de equinocandinas tornou-se uma constante opção para o tratamento de infecção por *C. glabrata*. Coincidindo com este aumento do uso, a resistência de *C. glabrata* a

esta classe tem sido relatada (Pfaller et al. 2012). Em adição, um fator preocupante é a frequência de isolados de *C. glabrata* resistentes tanto a equinocandinas quanto ao fluconazol (Lockhart, 2014).

3.1.2.3 *Candida parapsilosis*

Em comparação com outras espécies, *C. parapsilosis* mostra uma série de características distintas, como a alta habilidade de desenvolver biofilmes em dispositivos intravasculares e alta afinidade pela nutrição parenteral (Fernández-Ruiz et al., 2014). Além disso, esta espécie é frequentemente isolada das mãos dos profissionais de saúde, o que contribui para a transmissão horizontal deste micro-organismo em unidades de terapia intensiva (Trofa et al., 2008; Pammi et al., 2013). Adicionalmente, infecção por *C. parapsilosis* é um problema relevante em recém-nascidos prematuros e contribui significativamente para a mortalidade neonatal (Pammi et al., 2013).

Mais uma questão de preocupação reside no seu perfil de suscetibilidade às equinocandinas, uma vez que antifúngicos desta classe apresentam concentrações inibitórias mínimas (CIM) elevadas para *C. parapsilosis*. Essa informação leva a questionamentos sobre equinocandinas serem apropriadas ou não para o tratamento de doença invasiva causada por essa espécie fúngica (Fernández-Ruiz et al., 2014).

3.1.2.4 *Candida tropicalis*

A incidência de infecções na corrente sanguínea por *C. tropicalis* aumentou nos últimos anos, especialmente em pacientes idosos com neoplasia hematológica e comorbidades respiratórias crônicas (Fernández-Ruiz et al., 2015). Sua importância é ainda maior no sul da Europa (Pemán et al., 2012) e países latino-americanos (Corzo-Leon et al., 2014).

C. tropicalis apresenta várias características peculiares que aumentam sua patogenicidade, tais como: capacidade de secreção de enzimas hidrolíticas extracelulares (coagulase, fosfolipase e proteinase), formação de biofilmes e mudança fenotípica (Fernández-Ruiz et al., 2015; Zuza-Alves et al., 2016). De acordo com os dados recentes encontrados na literatura, *C. tropicalis* foi descrita como a espécie não-*albicans* mais aderente às células epiteliais (Biasoli et al., 2010;

Zuza-Alves et al., 2016). Além disso, resistência de isolados clínicos de *C. tropicalis* aos antifúngicos azólicos tem sido amplamente relatada (Liu et al., 2014; Zuza-Alves et al., 2016).

3.1.2.5 *Candida krusei*

C. krusei tem sido associada a pacientes com câncer e àqueles que receberam terapia antifúngica profilática (Schuster et al., 2013). Essa espécie tem sido reconhecida como um patógeno fúngico potencialmente multirresistente devido à sua resistência intrínseca ao fluconazol combinada com relatos de suscetibilidade diminuída tanto à flucitosina quanto à anfotericina B (Pfaller et al., 2008). O voriconazol é usado com sucesso em infecções por *C. krusei* (Kullberg et al., 2005), e equinocandinas têm demonstrado excelente atividade *in vitro* (Pfaller et al. 2005b). Todavia, autores também vêm relatando uma diminuição da sensibilidade para esses fármacos (Pelletier et al. 2005; Hakki et al., 2006; Pfaller et al., 2008)

O fenótipo multirresistente exibido por *C. krusei* traz um dilema terapêutico quando se está considerando escolhas de tratamento para pacientes neutropênicos e criticamente doentes, especialmente para aqueles com exposição prévia ao fluconazol (Pfaller et al., 2008; Schuster et al., 2013). Dessa maneira, tal como *C. glabrata*, *C. krusei* deve ser considerada uma espécie importante para ser monitorada em relação ao desenvolvimento da resistência antifúngica (Pfaller et al., 2008).

A candidíase invasiva é uma infecção frequente e grave para pacientes em UTI, associada a fracos resultados clínicos após tratamento. Atualmente existe uma grande discussão sobre a intervenção profilática antifúngica em candidíase invasiva. Esse procedimento pode ser indicado para pacientes com maior risco, mas, em geral, não deve ser recomendada. Entre os motivos para a não indicação é a dificuldade da escolha empírica do antifúngico em vista a tantos relatos de micro-organismos resistentes e a alta possibilidade de ocorrer pressão seletiva destes. Se um fármaco antifúngico mais eficiente estivesse disponível, talvez, a profilaxia poderia ser recomendada em mais situações e evitaria os altos índices de mortalidade associados a tais infecções (Playford et al., 2010).

3.1.2.6 Candidíase vulvovaginal associada à formação de biofilmes em dispositivos intrauterinos (DIU)

A candidíase vulvovaginal (CVV) é uma infecção que afeta milhões de mulheres e é a causa mais comum de vaginite aguda na Europa, nos Estados Unidos e em alguns países tropicais (Paiva et al., 2012). Estas infecções são causadas pelo crescimento anormal de leveduras do gênero *Candida* na mucosa do trato genital feminino (Paiva et al., 2010). Estima-se que aproximadamente 75% das mulheres sofrem pelo menos um episódio de CVV durante a vida e aproximadamente 40-50% delas apresentam episódios adicionais (Mayer et al., 2013). Ainda, 5% desenvolvem episódios repetitivos (3 a 4 vezes ao ano) - uma condição conhecida como CVV recorrente (Paiva et al., 2012).

Os fatores de risco para CVV incluem o uso de hormônios reprodutivos, tratamento antibiótico, uso de contraceptivos orais, gravidez e diabetes mellitus (Mayer et al., 2013). O uso do dispositivo intrauterino (DIU) como contraceptivo ainda não foi reconhecido como um fator predisponente (Paiva et al., 2010), entretanto, estudos prévios têm demonstrado que mulheres usuárias de DIU apresentam mais infecção CVV que aquelas que não utilizam o dispositivo (Chassot et al., 2008). Além disso, morte fetal intrauterina e sepse materna por *Candida* em mulheres com DIU também foi previamente relatado (Segal et al., 2001; Barth et al., 2002). Complementando tais evidências, DIUs removidos de mulheres demonstraram estar altamente contaminados com *C. albicans*, além de diversos outros micro-organismos, tais como Staphylococcus epidermidis, enterococos e lactobacilos anaeróbicos.

As infecções associadas aos dispositivos médicos, como DIU, são comumente relacionadas ao biofilme. Os micro-organismos originários da microbiota normal do trato genital feminino, incluindo *Candida* spp., podem aderir e colonizar estes dispositivos formando comunidades microbianas altamente estruturadas e complexas que são encapsuladas dentro de uma matriz extracelular rica em polissacarídeos (Pál et al., 2005). Esta comunidade microbiana é chamada de biofilme e sua formação está esquematizada na figura 7 (Ramage et al., 2012; Scorzoni et al., 2017). O ambiente celular proporcionado pelo biofilme oferece muitos benefícios aos agentes patogênicos microbianos, tais como proteção contra

defesas imunes do hospedeiro, resistência a estresses físicos e químicos e disponibilidade de nutrientes (Vipulanandan et al., 2018).

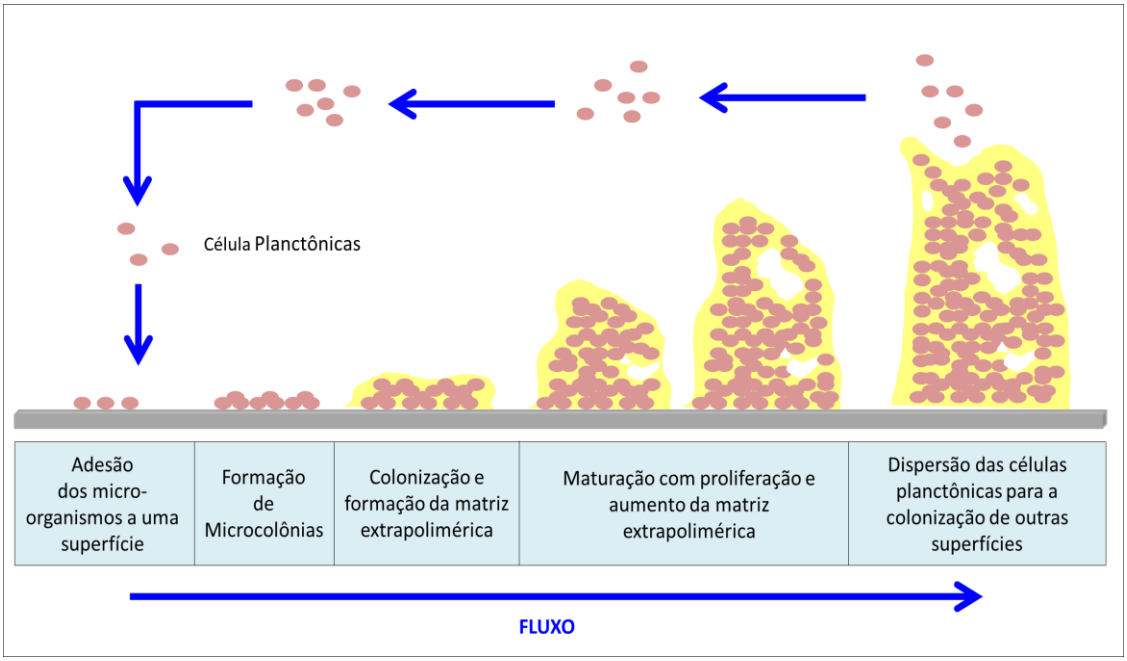

Figura 7. Etapas da formação do biofilme (Pippi 2018).

Curiosamente, o biofilme fúngico em DIU tem sido pouco estudado. Essa falta de interesse é preocupante porque os DIUs são implantados por longos períodos na mucosa uterina (Paiva et al., 2010). Mais de 80 milhões de mulheres usam DIU como método contraceptivo (Paiva et al., 2010).Os mais utilizados são aqueles que possuem cobre ou progesterona como substância quimicamente ativa. O modelo de cobre é feito de polietileno, com filamentos de cobre enrolados em suas hastes e apresenta uma cauda que facilita a localização do dispositivo para remoção, conforme ilustrado na figura 8 (Oster e Salgo, 1975). Esta cauda é composta por um monofilamento de plástico rodeado por uma bainha de nylon (Donlan e Costerton, 2002).

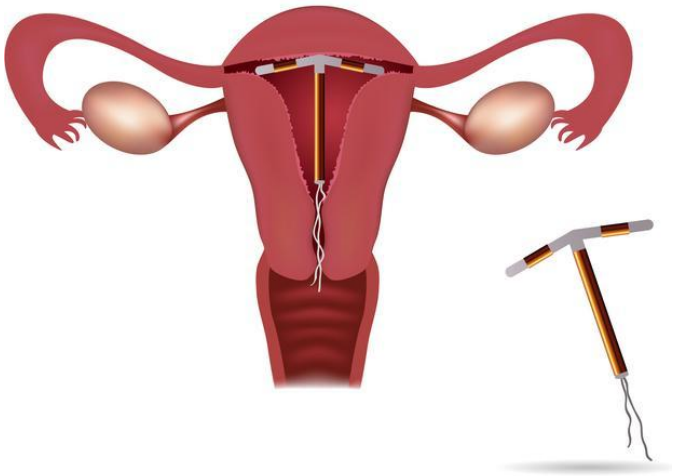

Figura 8. Dispositivo intrauterino (DIU) de cobre
(Fonte: http://www.newkidscenter.com/Copper-Iud-Side-Effects.html).

Estudos propuseram que a cauda do DIU pode ser determinante para que os micro-organismos migrem até a cavidade endometrial (Donlan e Costerton, 2002), visto que ela é uma ponte entre o ambiente externo, a vagina colonizada por leveduras e o trato genital superior (Chassot et al., 2008). Uma vez que as leveduras alcançam a cavidade endometrial, as partes cobertas de cobre parecem contribuir para a retenção do micro-organismo no local (Chassot et al., 2008; Auler et al., 2010; Paiva et al., 2010).

Os antifúngicos azólicos, como fluconazol e albaconazol, são bastante utilizados para tratar CVV (Kim and Sudbery, 2011). Entretanto, cabe destacar que os biofilmes fúngicos aumentam a resistência aos antifúngicos em até 1000 vezes (Scorzoni et al., 2017). Assim, a presença do biofilme na superfície do DIU contribui para proteger as leveduras da ação dos antifúngicos, ao mesmo tempo que contribui para a persistência do micro-organismo no local levando a infecções recorrentes (Auler et al., 2010). Esta é uma razão pela qual as infecções causadas por biofilmes são difíceis de tratar sem remoção dos dispositivos (Pál et al., 2005). Cada vez mais é necessária uma compreensão mais clara da formação de bioflimes em DIU, bem como é preciso avaliar tratamentos que possam controlar esse processo.

3.2 Terapia antifúngica e Mecanismo de Ação

Atualmente, cinco principais classes de agentes antifúngicos (azóis, equinocandinas, poliênios, análogos de pirimidina e alilaminas) são usadas por via oral, tópica ou intravenosa para o tratamento de infecções fúngicas (Campoy e Adrio, 2017). Em relação ao sítio de atuação, a terapia antifúngica está baseada em fármacos que atuam principalmente na membrana citoplasmática, parede celular ou síntese de DNA (ácido desoxirribonucleico) e proteínas (figura 9). A seguir serão discutidas as principais classes de antifúngicos com seus respectivos mecanismos de ação (Campoy e Adrio, 2017).

3.2.1 Azóis

Os azóis são antifúngicos de amplo espectro e são os fármacos mais comuns para o tratamento e prevenção de doenças fúngicas (Vandeputte et al., 2012). Os fármacos desta classe inibem a enzima a lanosterol 14α-desmetilase, a qual é responsável pela remoção oxidativa da metila do lanosterol na rota biossintética do ergosterol. Assim, a ligação dos azóis a esta enzima resulta em interrupção da biossíntese do ergosterol, o qual é essencial para manter a integridade da membrana fúngica (Pianalto et al., 2016; Chang et al 2017). Como consequência disso, há acúmulo de esteróis 14-metilados (precursores do ergosterol) que conduzem à síntese de 14α-metil-3,6-diol - o qual é tóxico para as células fúngicas (Vandeputte et al., 2012; Campoy e Adrio, 2017).

Os primeiros azóis que surgiram foram os imidazóis, os quais apresentam dois átomos de nitrogênio no anel azólico, tais como cetoconazol, econazol, miconazol e clotrimazol. Os derivados modificados no anel ativo, apresentando três átomos de nitrogênio, são denominados triazóis e apresentam maior espectro de ação e melhor perfil de segurança em relação aos imidazóis. Itraconazol e fluconazol são os triazóis de primeira geração (Denning e Hope, 2010; Campoy e Adrio, 2017). No entanto, ambos estão associados ao aumento da resistência devido, principalmente, à sua natureza fungistática. Em vista disso, uma segunda geração de triazois, representada por voricinazol e posaconazol, foi desenvolvida com efeito fungicida (Campoy e Adrio, 2017).

3.2.2 Equinocandinas

As equinocandinas representam a mais nova classe de antifúngicos (Chang et al., 2017). Atualmente, três representantes são aprovadas para uso clínico: caspofungina, micafungina e anidulafungina (Pianalto et al., 2016). Estes agentes antifúngicos são inibidores não competitivos de β-1,3-D-glicana sintase, uma das principais enzimas envolvidas na biossíntese da parede celular fúngica (Vandeputte et al., 2012; Pianalto et al., 2016; Chang et al., 2017). Sua inibição leva à desestabilização da parede celular, com instabilidade osmótica e lise das células fúngicas (Campoy e Adrio, 2017).

As equinocandinas apresentam baixa toxicidade para o hospedeiro (Pianalto et al., 2016; Campoy e Adrio, 2017), entretanto, não são biodisponíveis oralmente, o que limita seu uso a nível hospitalar sob a forma intravenosa (Spampinato e Leonardi; 2013). Esta classe de antifúngicos não é ativa contra *Cryptococcus*, *Trichophyton*, *Fusarium*, fungos dimórficos ou Zygomycetes (Vandeputte et al., 2012; Chang et al., 2017). Além disso, isolados de *Candida* resistentes à equinocandina estão sendo relatados com maior frequência, em especial *C. glabrata* (Pfaller et al., 2015).

3.2.3 Polienos

Os polienos são fungicidas de amplo espectro. Os fármacos que pertencem a este grupo têm alta afinidade pelo ergosterol da membrana fúngica, se complexando e formando pequenos canais transmembranares, os quais alteram a permeabilidade da membrana. Esse efeito destrói o gradiente de prótons e permite a saída de componentes citoplasmáticos vitais, o que leva ao rompimento da membrana e morte celular (Denning e Hope, 2010; Flevari et al., 2013). Estudos recentes também relatam que esses medicamentos atuam como "esponjas", ligando e removendo o ergosterol da membrana plasmática, o que leva à redução da integridade membranosa (Anderson et al., 2014).

Os polienos, entretanto, apresentam afinidade pelo colesterol, ainda que menor que pelo ergosterol, explicando os vários efeitos colaterais e a alta toxicidade, especialmente nefrotoxicidade (Shukla et al.; 2016, Chang et al., 2017). Nistatina, natamicina e anfotericina B são os medicamentos poliênicos em uso (Shukla et al., 2016; Campoy e Adrio, 2017). Nistatina e natamicina estão disponíveis apenas como formulações tópicas. Já anfotericina B é usada apenas como formulações

intravenosas e foi o primeiro antifúngico utilizado para o tratamento de IFIs (Chang et al., 2017) (Pianalto et al. 2016). É relevante destacar aqui que *C. glabrata* é intrinsecamente pouco suscetível a esses antifúngicos e outras espécies de *Candida*, como *C. krusei*, são mais propensas a adquirir resistência aos polienos (Vandeputte et al., 2012).

3.2.4 Análogo da pirimidina

Flucitosina (5-fluorocitosina) é um análogo de pirimidina fluorada com propriedade fungistática. É um antifúngico sintético ativo contra uma gama limitada de infecções fúngicas sistêmicas, sendo principalmente eficaz naquelas causadas por leveduras do gênero *Candida* e *Cryptococcus* (Shukla et al., 2016). Este antifúngico inibe a síntese de DNA e RNA (ácido ribonucleico) (Pianalto et al. 2016). A flucitosina é transportada para o interior das células fúngicas pela citosina permease, onde é convertida a 5- fluorouracil pela enzima citosina desaminase. 5-Fluorouracil pode sofrer fosforilações, produzindo nucleotídeos fluorados tóxicos e ser incorporando ao RNA, o que resulta na interrupção da síntese proteica (Carrillo-Muñoz et al., 2006; Denning e Hope, 2010). 5-Fluorouracil também pode ser convertido em 5-fluoro-basculante, que é um inibidor da timidilato sintase, uma enzima essencial para a síntese de DNA (Vandeputte et al., 2012; Campoy et al., 2017).

Flucitosina é seletivamente tóxico para fungos, pois há pouca ou nenhuma citosina desaminase em células de mamíferos, levando a efeitos colaterais insignificantes (Shukla et al., 2016). Além disso, esse fármaco apresenta alta hidrossolubilidade e tamanho pequeno, difundindo rapidamente em todo o corpo mesmo quando administrado por via oral (Vandeputte et al., 2012).

3.2.5 Alilaminas

As alilaminas são agentes fungicidas representados por terbinafina e naftifina. O mecanismo de ação desses antifúngicos consiste no bloqueio dos primeiros passos da biossíntese de ergosterol através da inibição alostérica da enzima fúngica esqualeno epoxidase (Shukla et al., 2016, Campoy e Adrio, 2017). A inibição dessa enzima leva ao acúmulo de quantidades tóxicas de esqualeno e ausência de outros derivados de esteróis, tais como ergosterol. Como consequência,

há aumento da permeabilidade da membrana e desorganização celular (Campoy e Adrio, 2017). Posto que alilaminas e azóis atuam em dois pontos diferentes da mesma via, há, em geral, sinergismo entre essas classes (Cantón et al., 2005).

As alilaminas são empregadas para tratar dermatofitoses, uma vez que acumulam mais na pele e nas unhas em relação ao plasma sanguíneo, provavelmente devido à sua lipofilicidade (Ngo et al., 2016).

3.2.6 Outros antifúngicos

Griseofulvina é um dos agentes antifúngicos mais antigos. Seu mecanismo de ação baseia-se na inibição da divisão celular ao interagir com a tubulina, interferindo na montagem de microtúbulos fúngicos e rompendo o fuso mitótico (Campoy e Adrio, 2017). Além disso, esse fármaco também atua como um inibidor da síntese do ácido nucleico (Lacaz et al., 2002). Griseofulfina é disponível na forma oral e tópica (Lacaz et al., 2002). Entretanto, este fármaco apresenta hepatotoxicidade e o espectro de ação é restrito aos dermatófitos (Campoy e Adrio, 2017).

A amorolfina é um antifúngico derivado da morfolina e atua inibindo duas enzimas envolvidas na biossíntese de ergosterol: Δ-7-8-isomerase e Δ-14-redutase, impedindo a formação da membrana celular fúngica (Campoy e Adrio, 2017). Apesar de seu amplo espectro de atividade, esses agentes antifúngicos são restritos ao tratamento tópico de infecções dermatofíticas, especialmente onicomicose, visto que apresentam numerosos efeitos colaterais (Vandeputte et al., 2012; Campoy e Adrio, 2017).

O ciclopirox é um derivado da hidroxipiridona e apresenta vasto espectro de ação antifúngica, sendo efetivo contra a maioria dos fungos patogênicos responsáveis por onicomicoses (Gupta et al., 2000). É um fármaco usado como agente antifúngico tópico, na forma de creme e solução, e ainda disponível em forma de esmalte (Leem et al., 2003). Seu mecanismo de ação ainda não foi totalmente elucidado, mas sabe-se que este antifúngico quela cátions polivalentes, tais como o Fe^{3+}, inibindo enzimas metais-dependentes - catalase e peroxidase - responsáveis pela degradação de radicais peróxidos no interior da célula fúngica. Além disso, ciclopirox também afeta a produção de energia do transporte mitocondrial de

elétrons, a captação de nutrientes e a síntese de proteínas e ácidos nucleicos (Gupta et al., 2013).

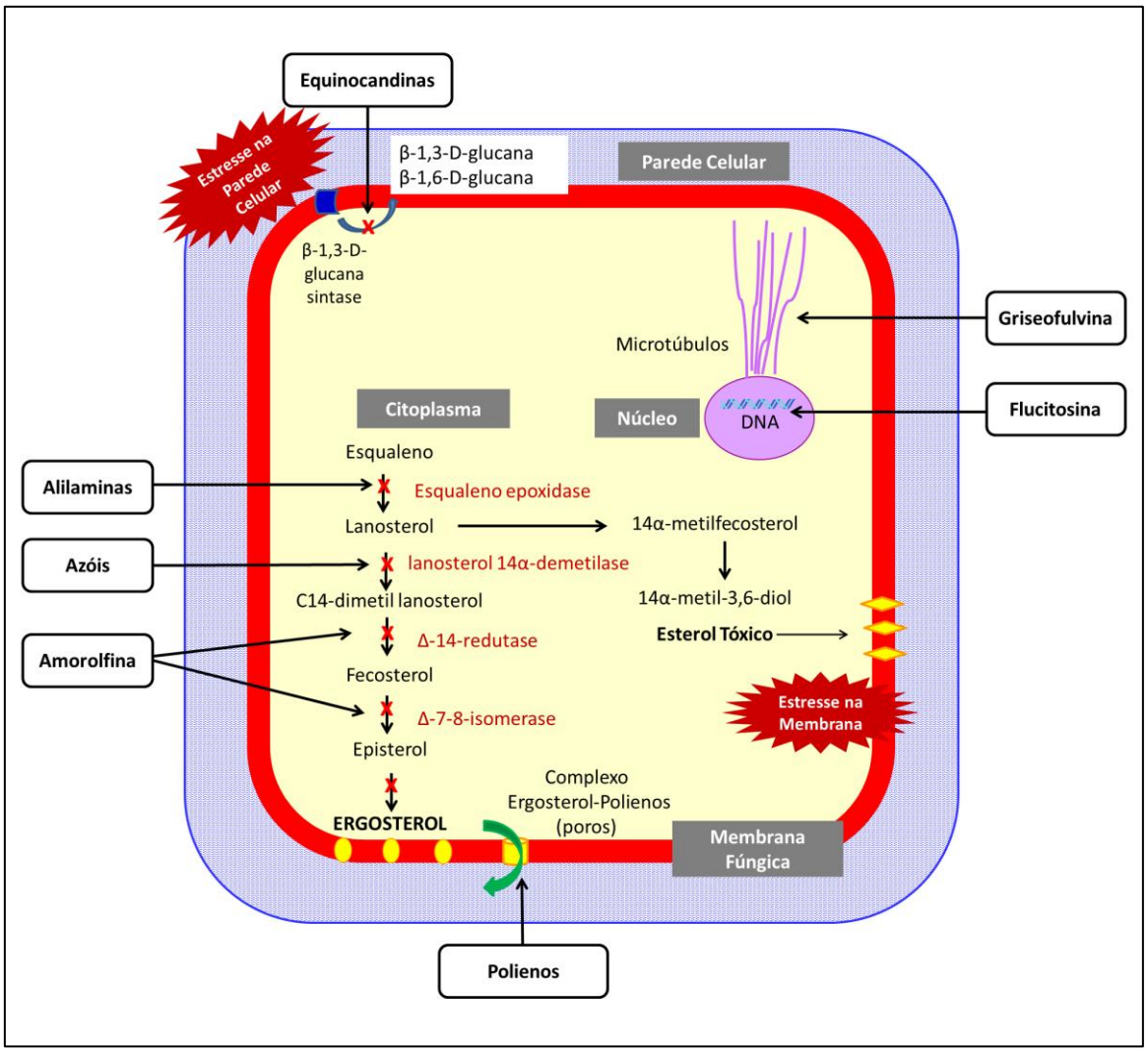

Figura 9. Alvo dos principais antifúngicos (Pippi 2018).

3.3 Desafios na terapia antifúngica

Como visto, há poucos medicamentos no mercado farmacêutico para tratar infecções fúngicas. Além disso, o tratamento de tais infecções apresenta diversas complicações devido à alta toxicidade de alguns fármacos utilizados, baixa tolerância a altas doses de antifúngicos, efeitos colaterais indesejados e espectro de atividade estreito. Os antifúngicos disponíveis também podem apresentar várias outras desvantagens relacionadas à eficácia e propriedades farmacocinéticas, tais

como baixa penetração de drogas, problemas com a biodisponibilidade do fármaco e interações medicamentosas. Em adição, custo elevado e longos períodos de terapia são requeridos, o que diminui a adesão do paciente ao tratamento completo. (Campoy e Adrio, 2017; Chang et al., 2017, Fuentefria et al.; 2018).

O pequeno número de medicamentos antifúngicos disponíveis não seria um problema se as respostas às terapias fossem satisfatórias. Entretanto, geralmente, esse não é o caso (Roemer e Krysan, 2014). Além dos desafios citados acima, o gerenciamento clínico de doenças fúngicas é ainda comprometido pelo surgimento de isolados resistentes à terapia antifúngica, que restringem ainda mais as opções de tratamento. Ainda, mais ameaçadora, é a evolução dos organismos multirresistentes, os quais apresentam baixa suscetibilidade a várias classes de agentes antifúngicos (Cowen et al., 2014).

3.3.1 Resistência aos antifúngicos

A incapacidade de responder à terapia antifúngica é complexa e depende de fatores associados ao micro-organismo (resistência microbiológica) e ao hospedeiro (resistência clínica).

A resistência microbiológica é a falta de suscetibilidade do micro-organismo ao antifúngico, a qual pode ser detectada pela avaliação da concentração inibitória mínima (CIM) através de testes *in vitro* (Fuentefria et al., 2018). A resistência microbiológica pode ser descrita como intrínseca ou extrínseca. A primeira é quando o fungo é inerentemente menos suscetível a um determinado antifúngico; a segunda é o desenvolvimento de resistência em resposta à exposição a um antifúngico (Chang et al., 2017; Fuentefria et al., 2018).

A resistência clínica é quando um paciente não responde ao tratamento após administração de uma dose padrão de antifúngico (Chang et al., 2017). A resposta clínica bem sucedida depende não só da suscetibilidade do micro-organismo, mas também do sistema imunológico do hospedeiro, da penetração e distribuição do fármaco e da adesão do paciente à terapia (Fuentefria et al. 2018). A presença de cateteres internos, válvulas cardíacas artificiais e outros dispositivos cirúrgicos também podem contribuir para infecções refratárias, pois os organismos infectantes podem estabelecer biofilmes que resistem à ação dos antifúngicos (Chang et al., 2017).

Vários mecanismos de resistência desenvolvidos por micro-organismos já foram relatados. Os mecanismos de resistência aos azóis são os mais estudados e incluem: superexpressão de bombas de efluxo (aumenta expulsão do fármaco da célula reduzindo a sua concentração no local de ação), mutação da enzima alvo (reduz afinidade entre fármaco e enzima), superexpressão da enzima alvo (acúmulo de moléculas alvo torna a biossíntese do ergosterol possível mesmo na presença do fármaco) e alteração de outras enzimas da via de biossintese do ergosterol (resulta em acúmulo de esteróis não tóxicos que podem substituir o ergosterol da célula) (Pfaller 2012; Shukla et al. 2016).

Já a resistência às equinocandinas acontece principalmente devido a substituições de aminoácidos que levam a alterações na enzima alvo, o que diminui a sensibilidade ao fármaco (Perlin 2011; Spampinato e Leonardi; 2013; Cowen et al., 2014).

Falhas clínicas em casos de tratamento de alilaminas também já foram relatadas, mas seus mecanismos de resistência ainda são mal compreendidos (Shukla et al., 2016). Entretanto, parece que bombas de efluxo em dermatófitos estão relacionadas à resistência à terbinafina (Scorzoni et al., 2017).

Em relação à flucitosina, numerosos mecanismos podem levar à resistência, uma vez que várias enzimas estão envolvidas no modo de ação deste medicamento (Spampinato e Leonardi, 2013). Devido à alta frequência de resistência, a flucitosina raramente é utilizada como monoterapia e seu uso é associado ao fluconazol e anfotericina B (Vandeputte et al., 2012; Campitelli et al., 2017; Chang et al., 2017).

Ao contrário da flucitosina, o desenvolvimento da resistência adquirida aos antifúngicos poliênicos ainda é bastante raro (Hughes e Andersson et al., 2015). Esse grupo não requer internalização nas células fúngicas para exercer seu efeito, escapando das enzimas metabolizadoras e dos sistemas de efluxo. A resistência a esta classe está associada a uma diminuição ou ausência de ergosterol na membrana plasmática. As principais alterações envolvidas são mutações em enzimas não essenciais na via de biossíntese do ergosterol, o que leva a substituição do ergoterol por outros esteróis na membrana. Essa alteração pode, ainda, conferir resistência cruzada entre azóis e polienos (Vandeputte et al., 2012).

Recentemente houve um importante reconhecimento sobre a importância de fungos patogênicos resistentes e a necessidade de novos antifúngicos. Nos EUA, o “Generating Antibiotic Incentives Now” (GAIN) incentiva a redução da incidência de bactérias resistentes através de descoberta de novos antimicrobianos destinados a tratar infecções graves e expandiu a lista de bactérias resistentes para incluir fungos resistentes. A Lei GAIN visa aumentar o valor comercial dos antimicrobianos, estendendo por mais 5 anos o prazo de exclusividade concedido aos medicamentos inovadores pela “Food and Drug Administration” (FDA). Este período adicional de exclusividade aumenta o potencial de lucros de novos antimicrobianos, dando às empresas inovadoras mais tempo para recuperar seus custos em investimento (Calderone et al., 2014).

3.4 Descoberta e desenvolvimento de novos antifúngicos

Ao longo dos últimos 30 anos, a importância dos medicamentos antifúngicos para a prática da medicina moderna tem aumentado drasticamente (Roemer e Krysan, 2014). A terapia antifúngica tem avançado, entretanto, o número de casos de infecção e resistência ainda são alarmantes e o controle dessas doenças está longe de ser alcançado (Scorzoni et al., 2017).

A farmacoterapia de infecções fúngicas é desafiadora devido a semelhança celular entre o patógeno fúngico e o hospedeiro mamífero, ambos organismos eucarióticos. A conservação da biologia celular dificulta o desenvolvimento de antifúngicos seguros, uma vez que é raro encontrar uma substância que tenha toxicidade seletiva apenas para o fungo e não cause danos ao paciente (Campoy e Adrio, 2017, Fuentefria et al.; 2018). Por isso, o número de agentes antifúngicos é limitado em comparação aos medicamentos antibacterianos (Campoy e Adrio, 2017).

Além dos desafios científicos que afetam a identificação de novos compostos antifúngicos, como o perfil de toxicidade citado, a avaliação de novos antimicrobianos também apresenta uma série de desafios econômicos (Roemer e Krysan, 2014). Pesquisas focadas na terapia de infecções fúngicas são subfinanciadas em relação a outras doenças. Isso ocorre porque o tamanho do mercado e, portanto, o lucro, é considerado muito pequeno comparado ao custo do desenvolvimento de novos medicamentos. Ainda, terapias antimicrobianas são

utilizadas por aproximadamente 2-3 semanas, enquanto doenças crônicas podem exigir tratamento contínuo durante toda a vida (Calderone et al., 2014).

Para finalizar, após o novo antifúngico ser inserido no mercado, ainda pode ocorrer o desenvolvimento de resistência (Calderone et al., 2014). Tomados em conjunto, talvez não seja surpreendente que o ritmo atual do desenvolvimento de fármacos antifúngicos seja muito lento e não acompanhe as necessidades clínicas. Atualmente, apenas algumas moléculas promissoras estão em desenvolvimento pré-clínico ou nos primeiros estágios de desenvolvimento clínico; o que não nos traz a certeza que essas moléculas serão um novo antifúngico (Campoy e Adrio, 2017). Portanto, há uma necessidade emergente de preencher o *pipeline* com novos medicamentos antifúngicos (Roemer e Krysan, 2014; Campoy e Adrio, 2017).

Para enfrentar os desafios impostos na terapia antifúngica, é necessário um compromisso renovado e decidido da indústria farmacêutica em parceria com laboratórios acadêmicos, combinando estratégias inovadoras de triagem de novas compostos para alcançar o sucesso (Roemer e Krysan, 2014).

3.4.1 Estratégias de triagem de novos compostos biologicamente ativos

A síntese e semi-síntese química, rastreamento de produtos naturais e a reutilização de medicamentos aprovados (fora da patente) são algumas das abordagens a serem seguidas para a descoberta de novas moléculas antifúngicas (Campoy e Adrio, 2017).

O *design* de fármacos é baseado no aperfeiçoamento de estruturas de uma série química (ou *hit*) a fim de otimizar a atividade e seletividade usando, inicialmente, ensaios *in vitro* para confirmação da ação biológica. Além disso, o perfil toxicológico é realizado desde o início, uma vez que a toxicidade é característica limitante para desenvolvimento de fármacos antifúngicos. Ainda, a capacidade de modificações sintéticas, propriedades físico-químicas e a farmacocinética da série são também avaliadas, resultando na seleção de um candidato a fármaco adequado para avançar. O composto principal (*lead compound*) que dá origem à série pode ser um fármaco já utilizado e que precisa ser otimizado para uma nova indicação. Cabe ressaltar aqui que a escolha de um fármaco como protótipo pode ser um ponto de partida mais imediato (Calderone et al., 2014).

3.4.2 Pré-seleção de compostos biologicamente ativos em modelos animais alternativos

Como já mencionado, a avaliação da atividade antifúngica *in vitro* e a citotoxicidade são os primeiros passos na triagem para o desenvolvimento de um novo antifúngico. No entanto, após determinar a atividade antifúngica e toxicidade, é necessário entender se o composto tem o mesmo comportamento quando é submetido a sistemas complexos. Considerando isso, experimentos *in vivo* usando modelos animais convencionais e alternativos são cruciais para a confirmação dos dados *in vitro* (Scorzoni et al., 2016).

Classicamente, os modelos animais mamíferos são considerados o padrão-ouro para estudos de descoberta de fármacos, virulência e resposta imune. Entretanto, o uso destes animais é altamente regulado, com muita dependência de comitês de ética e boas práticas laboratoriais (Scorzoni et al., 2017). Assim, com o objetivo de conscientização desse problema científico, em 1959, Russell e Burch descreveram o conceito de "modelo animal alternativo" através na teoria dos 3Rs: Redução, Reposição e Refinamento (Russell e Burch, 1959).

Nas últimas décadas, o uso de modelos animais alternativos tem sido incentivado para testes *in vivo*. Amebas, insetos, nematoides, peixes e embriões de galinha são usados porque os sistemas neurais destes animais são pouco desenvolvidos e, portanto, quase não apresentam dor. Além disso, apresentam origens genéticas bem definidas, são fáceis de manipular, a manutenção tem baixo custo e um grande número de animais pode ser usado em cada experiência; e para finalizar, existe uma boa correlação entre animais alternativos e mamíferos (Dolganiuc e Szabo, 2009; Trevijano-Contador e Zaragoza, 2014). Portanto, tais modelos apresentam uma aplicação adequada para a pré-seleção de compostos biologicamente ativos (Scorzoni et al., 2016). A seguir serão discutidos três modelos alternativos *in vivo*, os quais foram abordados em nossa pesquisa experimental.

3.4.2.1 *Drosophila melanogaster*

Os invertebrados, como insetos, podem ser usados como modelo animal alternativo. A *Drosophila melanogaster* (mosca da fruta) é descrita como um modelo para investigar a virulência de agentes patogênicos humanos e para verificar a eficácia de novos compostos antifúngicos (Scorzoni et al., 2017). A infecção pode

ser realizada por injeção, contato de rolamento ou ingestão (Lionakis e Kontoyiannis, 2012). Já o tratamento antifúngico é realizado por ingestão, misturando o composto ao alimento (Zanette e Kontoyiannis, 2013).

Drosophila está desprovida de um sistema imune adaptativo, mas possui uma resposta imune inata (primeira linha de defesa contra *C. albicans*) com mecanismos de defesa altamente semelhantes aos mamíferos (Alarco et al., 2004). Além disso, as pesquisas demonstraram que as moscas de *D. melanogaster* possuem duas vias de sinalização conservadas que são ativadas durante as respostas imunes: imd e Toll. A via Toll é crítica para a proteção de *D. melanogaster* contra fungos e as mutações neste via conduzem à morte das moscas rapidamente. Assim, *D. melanogaster* mutantes toll-deficientes são bastante interessantes em modelos de infecção por fungos, uma vez que mimetizam a imunodeficiência, a qual é uma condição para o estabelecimento de diversas doenças fúngicas (Lionakis et al., 2005).

3.4.2.2 *Zebrafish*

Vertebrados inferiores, como embriões, larvas e adultos de *Danio rerio (zebrafish)* têm sido bastante utilizados como modelo de toxicidade e teratogênese. O desenvolvimento do *zebrafish* é rápido, uma vez que a maioria dos órgãos é formada durante o desenvolvimento precoce do embrião (Racz et al., 2017). Essa é uma vantagem interessante, porque os embriões de *zebrafish* até 5 dias pós-fertilização não são considerados animais e, portanto, experimentos realizados durante esta etapa de desenvolvimento não requerem regulamentos éticos (Racz et al., 2017; Scorzoni et al., 2017).

Eles compartilham alta homologia genética com o homem (~70%) e mostram respostas moleculares biologicamente conservadas (como desenvolvimento de órgãos e sinalização de células e tecidos) (Racz et al., 2017), o que torna esse sistema frequentemente utilizado na pesquisa médica (Ordas et al., 2015; Phillips e Westerfield, 2014). Uma vez que *zebrafish* são pequenos animais, de boa transparência óptica e com alta capacidade reprodutiva, esses organismos têm potencial para rastreio de alto rendimento (Racz et al., 2017).

O modelo já foi útil para estudar a virulência de *C. albicans* (Chen et al., 2015) e a patogênese de *C. neoformans* (Tenor et al., 2015). Apesar da importância

deste modelo, até à data, experiências de eficácia antifúngica não foram validadas (Scorzoni et al., 2017).

3.4.2.3 Embriões de Galinha

Outro modelo não convencional amplamente utilizado para testes *in vivo* é embriões de galinha. Este é um modelo biológico utilizado em uma ampla gama de estudos toxicológicos devido a sua facilidade de execução, desenvolvimento rápido, embriogênese precoce e sensibilidade a substâncias. Os embriões crescem sob condições controladas e pequenas variações resultam em desenvolvimento anormal ou morte. Essa característica os torna altamente apreciados em estudos de toxicidade, uma vez que qualquer alteração no seu ambiente oferece respostas imediatas aos efeitos tóxicos das substâncias em estudo (Betsabee et al., 2017). Ainda, semelhante ao *zebrafish*, os embriões de galinha não são considerados organismos vivos e questões éticas são menos complicadas (Scorzoni et al., 2017). Além disso, embriões de galinha também são utilizados para estudos de virulência fúngica (Kaerger et al., 2015). Entretanto, ainda não existe uma descrição da avaliação da eficácia antifúngica usando embriões de galinha (Scorzoni et al., 2017).

Embora três tipos de modelos animais tenham sido discutidos aqui, é importante avaliar qual é o melhor sistema para cada estudo. Independentemente dos benefícios, os modelos de mamíferos ainda são necessários. Mas, cabe destacar, que os animais alternativos são adequados para rastreio antes de estudos em mamíferos (Scorzoni et al., 2017).

3.5 Derivados da 8-Hidroxiquinolina

É claro que a descoberta de novos antifúngicos é essencial, porém pesquisas mais detalhadas de moléculas com atividade antimicótica conhecida e modificações estruturais para melhorar a ação parecem ser alternativas mais rápidas para esta finalidade (Ngo et al., 2016). Neste contexto, os derivados de 8-hidroxiquinolina podem desempenhar um papel importante. A 8-hidroxiquinolina apresenta um anel heterocíclico de seis membros fundido a um anel benzênico, conforme ilustrado na figura 10 (Sashidhara et al., 2009).

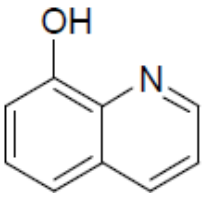

Figura 10. Representação da estrutura química da 8-hidroxiquinolina.

8-Hidroxiquinolina e seus derivados são uma subclasse de quinolinas com uma grande variedade de atividades biológicas. Elas têm sido utilizadas como fungicida na agricultura e conservante nas indústrias têxtil, madeireira e papelaria (Oliveri e Vecchio, 2016).

O interesse em 8-hidroxiquinolinas cresceu exponencialmente nas últimas duas décadas por apresentarem uma estrutura privilegiada que por modificações em posições específicas pode levar a obtenção de novos candidatos a fármacos para diversas enfermidades (Oliveri e Vecchio, 2016). A reatividade da 8-hidroxiquinolina permite a geração e otimização de um grande número de derivados que são amplamente explorados para efeitos biológicos, tais como efeito neuroprotetor, anticancerígeno, anti-séptico, antimalárico, antituberculotico, anti-HIV, antibacteriano e antifúngico (Dixit et al., 2010; Oliveri e Vecchio, 2016).

O membro mais conhecido dessa família é o clioquinol (5-cloro-7-iodo-8-hidroxiquinolina). O clioquinol é um derivado 5,7-dihalogenado da 8-hidroxiquinolina que foi amplamente usado no período entre 1950 e 1970 como um agente antimicrobiano oral para o tratamento de doenças intestinais e infecções de pele (Mao e Schimmer, 2008; Wehbe et al., 2017).

No entanto, em 1970, as formas orais foram retiradas do mercado devido a relatos de neurotoxicidade em pacientes japoneses, especificamente por ter sido considerado um agente causador de uma síndrome denominado neuropatia mielo-óptica subaguda (síndrome SMON) (Nakae et al., 1973). Esta síndrome envolve o aparecimento subagudo de distúrbios sensoriais, motores dos membros inferiores e comprometimento visual (Oliveri e Vecchio, 2016). Mais de 10.000 japoneses foram afetados pela síndrome e após a proibição da venda do clioquinol houve um desaparecimento drástico de novos casos (Konagaya et al., 2004).

Entretanto ainda há controvérsia da associação entre o uso de clioquinol e SMON. Curiosamente, os relatórios epidemiológicos sugerem que o clioquinol não era responsável por SMON e nenhuma outra população apresentou uma resposta adversa semelhante (Wehbe et al., 2017). Alguns estudos relatam que muitos destes casos podem ter sido relacionados com a deficiência de vitamina B12 concomitante (Helmuth, 2000); ou também devido às diferentes formulações de clioquinol usadas no Japão, além da influência da suscetibilidade genética para este efeito secundário, visto que a taxa de incidência da doença em outros países era quase nula (Nakae et al., 1973; Helmuth, 2000).

As formulações tópicas para o tratamento de infecções cutâneas continuaram disponíveis (Mao e Schimmer, 2008; Oliveri e Vecchio, 2016) e atualmente clioquinol é usado como antibiótico tópico sob o nome comercial Vioform® (Wehbe et al., 2017). Associação de clioquinol com outros fármacos também é utilizada topicamente (POLIDERMS®: valerato de betametasona + sulfato de gentamicina + tolnaftato + clioquinol). Além de seu uso como antimicrobiano, o clioquinol se tornou um protótipo para o desenvolvimento de compostos antitumorais e, recentemente, também tem mostrado potencial efeito para o tratamento das doenças de Alzheimer, Parkison e Huntington (Franklin et al. 2016; Zhang et al., 2013; Finkelstein et al., 2016; Huntington Study Group Reach2HD Investigators, 2015). Assim, a possibilidade de reintrodução de formulações orais do clioquinol para novas indicações também estimula a reutilização deste agente como antifúngico sistêmico. Porém, ainda exige-se maior compreensão sobre suas propriedades tóxicas e seu modo de ação. Da mesma maneira, é importante otimizar derivados da 8-hidroxiquinolina a fim de encontrar um melhor efeito ou menor toxicidade.

4 MATERIAL E MÉTODOS

4.1 Local da realização da pesquisa

Os ensaios e avaliações microbiológicas *in vitro* de derivados da 8-hidroxiquinolina foram realizados nas dependências do Laboratório de Micologia Aplicada, na Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul (UFRGS). Experimentos relacionados à síntese de compostos antifúngicos foram feitos pelo “Pharmaceutical Synthesis Group” da mesma instituição. Os estudos de modelagem matemática foram aplicados no Centro Bioanalítico de Medicamentos também da Faculdade de Farmácia da UFRGS. Os testes de atividade antifúngica em modelo alternativo de *D. melanogaster* foram realizados no DrosoLab localizado no Instituto de Ciência Básicas da Saúde (ICBS, UFRGS). Ainda, testes de toxicidade dérmica, permeação cutânea, e estudos comportamentais e de irritabilidade em embriões de galinha foram conduzidos no Laboratório de Farmacologia pertencente ao Instituto Federal Catarinense (IFC) de Concórdia, RS. Por fim, estudos toxicológicos em *zebrafish* foram elaborados no Grupo de Pesquisa Estresse Oxidativo e Sinalização Celular da Universidade Federal do Pampa, localizado no campus de São Gabriel, Rio Grande do Sul (UNIPAMPA, RS).

4.2 Isolados fúngicos

Um total de 73 isolados fúngicos foi incluído neste estudo: *C. albicans* (11), *C. glabrata* (10), *C. krusei* (9), *C. parapsilosis* (11), *C. tropicalis* (11), *M. canis* (6), *M. gypseum* (5), *T. mentagrophytes* (5) e *T. rubrum* (5). Todos os isolados estão depositados na Coleção Micológica da Universidade Federal do Rio Grande do Sul (Porto Alegre, Brasil) e são originários do Programa Nacional de Controle de Qualidade do Brasil, raspados de unhas e pele, hemocultura, secreções vaginais, urina, aspirado traqueal e ponta de cateter. Todos foram identificados fenotipicamente através do sistema automatizado *Vitek Yeast Biochemical Card* (BioMerieuxVitek,. Hazelwood, Mo) ou tiveram a sua identificação confirmada por sequenciamento das regiões ITS e D1/D2 do gene 26S ribossomal. Cepas padrão de *C. albicans* (ATCC 18804), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019) e *C. tropicalis* (ATCC 750) foram obtidas da ATCC (American Type Culture Collection, Manassas, VA, EUA) e incluídas como controles. Os isolados

permaneceram armazenados em geladeira cultivados em SDA (ágar Sabouraud dextrose; HiMedia, Índia).

4.3 Agentes antifúngicos

Os derivados da 8-hidroxiquinolina inicialmente avaliados foram clioquinol (5-cloro-7-iodo-8-hidroxiquinolina, composto 1) e dois derivados ácidos sulfônicos: ácido 8-hidroxiquinolinil-5-sulfônico (composto 2) e ácido 7-iodo-8-hidroxiquinolinil-5-sulfônico (composto 3). As três 8-hidroxiquinolinas citadas acima foram adquiridas comercialmente da Sigma-Aldrich (EUA).

Subsequentemente, a conversão do ácido sulfônico em sulfonamidas foi realizada pelo nosso grupo de pesquisa, como descrito por Joaquim et al. (2018), resultando em outros dois derivados de 8-hidroxiquinolina também incluídos neste estudo: 8-hidroxiquinolinil-5-(*N*-4-clorofenil)sulfonamida (PH151) e 8-hidroxiquinolinil-5-(*N*-4-metoxifenil)sulfonamida (PH153). As estruturas químicas das 8-hidroxiquinolinas avaliadas neste estudo são ilustradas na figura 11.

Anidulafungina, anfotericina B e fluconazol foram utilizados como controles positivos de alguns experimentos. Soluções-estoque de fluconazol (Sigma-Aldrich) e anidulafungina (Pfizer®, Nova York, EUA) foram preparadas em água destilada estéril. As soluções-estoque de todos os outros agentes antifúngicos avaliados foram preparadas em DMSO (Sigma-Aldrich). As soluções de trabalho foram diluídas no momento do uso em meio de ensaio, a fim de obter uma concentração máxima de 2% de DMSO nos experimentos.

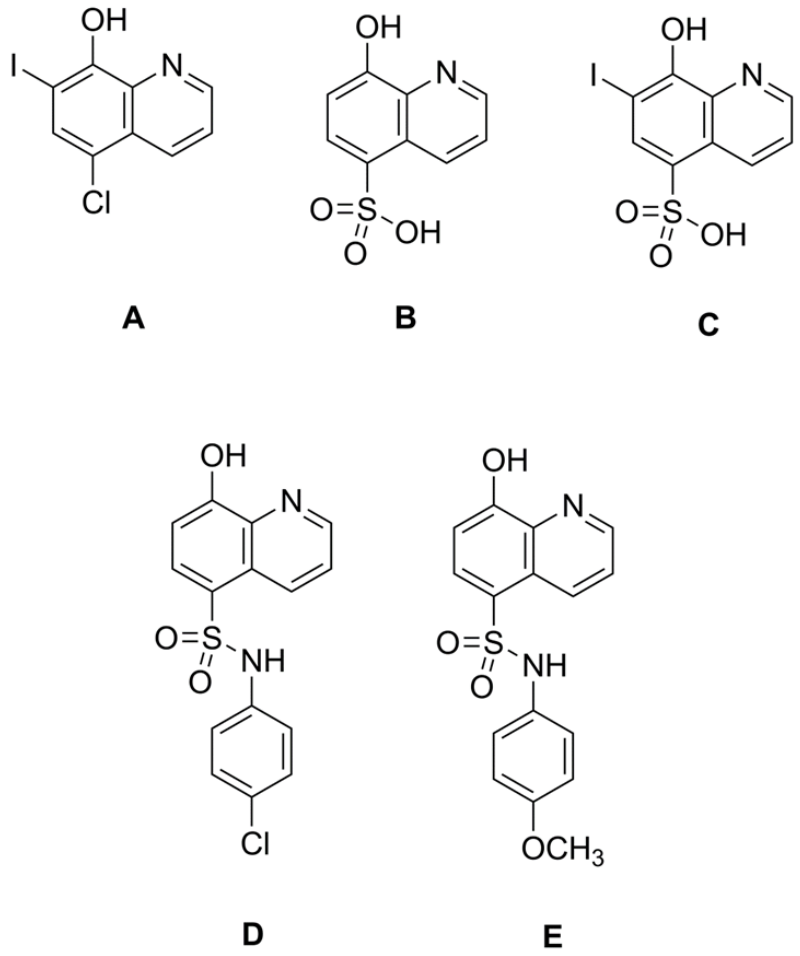

Figura 11. Estrutura química dos derivados da 8-hidroxiquinolina.
(A) Clioquinol (composto 1);
(B) Ácido 8-hidroxiquinolinil-5-sulfônico (composto 2);
(C) Ácido 7-iodo-8-hidroxiquinolinil-5-sulfônico (composto 3);
(D) 8-Hidroxiquinolinil-5-(*N*-4-clorofenil)sulfonamida (PH151);
(E) 8-Hidroxiquinolinil-5-(*N*-4-metoxifenil)sulfonamida (PH153).

4.4 Teste de suscetibilidade fúngica aos derivados da 8-hidroxiquinolina

As concentrações inibitórias mínimas (CIMs) dos análogos da 8-hidroxiquinolina foram determinadas para todos os isolados incluídos neste estudo

através do método de microdiluição em caldo de acordo com as normas de padronização publicadas em documentos da *Clinical Laboratory Standards Institute* (CLSI). Os protocolos M27-A3 e M38-A2 foram utilizados para testar a suscetibilidade das espécies de *Candida* e fungos filamentosos (*Microsporum* spp. e *Trichophyton* spp.), respectivamente (CLSI, 2008).

Para obtenção de células viáveis utilizadas nos experimentos, espécies de *Candida* foram crescidas em SDA (HiMedia) por 24 h a 35 °C. A seguir as células foram suspensas em solução salina 0,85% estéril (Dinâmica; Diadema, SP) e ajustadas em espectrofotômetro (GT220, Global Trade Tecnology) em comprimento de onda de 530 nm, a fim de se obter transmitância equivalente a escala 0,5 de McFarland ($1x10^6$ a $5x10^6$ unidades formadoras de colônias por mL ou UFC/mL). A suspensão fúngica de trabalho foi preparada fazendo-se uma diluição 1:50 seguida de uma diluição 1:20 em caldo RPMI 1640 (*Roswell Park Memorial Institute* 1640; Gibco, Nova York) - sem bicarbonato de sódio, tamponado com MOPS (ácido morfolinopropanosulfônico; Sigma-Aldrich) e ajustado para pH 7,0 - resultando em concentração 1 a $5x10^3$ UFC/mL.

Os inóculos de fungos dermatofíticos foram procedentes de subcultivo em placas contendo BDA (agar de dextrose de batata, HiMedia, Índia), as quais foram incubadas por 7 dias em temperatura de 30 °C. As colônias foram cobertas com solução salina 0,85% estéril e suspensão fúngica resultante foi quantificada em câmara de Neubauer e ajustada com RPMI 1640 para obter 2 a 6 x 10^3 UFC /mL.

A microdiluição em caldo foi realizada em microplacas de poliestireno estéreis com 96 poços. Diluições 1:2 em série dos agentes antifúngicos foram feitas em meio PRMI com posterior adição do inóculo fúngico. O experimento final resultou em diferentes concentrações dos compostos testados frente a 0,5 a $2,5x10^3$ UFC /mL de *Candida* e 1 a $3x10^3$ UFC /mL de dermatófitos.

Os experimentos foram realizados em quadruplicata e incubados a 35 °C para *Candida* spp. e 30 °C para dermatófitos. As CIMs foram definidas como a menor concentração das 8-hidroxiquinolinas em que os micro-organismos testados não demonstraram crescimento visível após 48 h (*Candida* sp.) ou 96 h (para *Microsporum* spp. e *Trichophyton* spp.) de incubação.

4.5 Avaliação de dano celular

Após o tempo de incubação e avaliação visual dos testes de susceptibilidade antifúngica, a leitura colorimétrica foi realizada com MTT [brometo de 3-(4,5-dimetiltiazol-2-il)-2,5 difeniltetrazólio] (Sigma-Aldrich) (Chiou et al., 2001). Para isso, o sobrenadante foi descartado das microplacas e as células fúngicas foram incubadas a 35 ° C em uma solução aquosa de MTT (0,05 mg/mL) durante 3 h (*Candida* spp.) ou 24 h (*Microsporum* spp. e *Trichophyton* spp.). MTT é um sal tetrazólico amarelo reduzido por enzimas desidrogenases mitocondriais que formam cristais de formazana azul (detectado espectrofotometricamente a 570 nm) quando as células estão ativas. Após o tempo de incubação, a solução de MTT foi retirada das microplacas e as leveduras coradas permaneceram no fundo. Os cristais de formazana azul foram extraídos das células com 150 µL de álcool isopropílico (Vetec, Brasil) e 100 µL da cada poço foram transferidos para uma nova microplaca.

As absorbâncias (A) dos cristais de formazana resultantes das células tratadas com diferentes concentrações de derivados de 8-hidroxiquinolinas e das células não tratadas foram medidas (SpectraMax-M2; Molecular Devices®, EUA) em dois comprimentos de ondas (570 e 690 nm). A porcentagem de dano celular foi calculada pela equação 1:

$$Dano\ celular\ (\%) = \left[1 - \frac{(\mathrm{A570}\ -\ \mathrm{A690}_{células\ tratadas}\ )}{(\mathrm{A570}\ -\ \mathrm{A690}_{células\ não\ tratadas}\ )}\right] x\ 100 \qquad (1)$$

Os dados foram analisados estatisticamente por One-Way ANOVA e teste Tukey de comparações múltiplas. $P \leq 0{,}05$ foi considerado significativo.

4.6 Ensaio de tempo de morte

Os ensaios de tempo de morte foram realizados com um isolado representativo de cada gênero estudado e a inclusão de uma cepa padrão foi priorizada. Os procedimentos foram conduzidos como descrito anteriormente por Klepser et al. (1998) e Ghannoum et al. (2013).

Para a levedura (C*andida albicans*) preparou-se uma suspensão fúngica a partir de uma cultura de 24 h a 35 °C em SDA (HiMedia), e a turbidez foi ajustada para o padrão 0,5 de McFarland (1 a 5 x 10^6 UFC / mL). Uma diluição 1:10 desta

suspensão foi preparada adicionando 1 mL de suspensão fúngica em 9 mL de RPMI 1640 sem (controle sem tratamento) ou com a quantidade desejada de derivados da 8-hidroxiquinolina. Esta diluição resultou em uma solução com um inóculo de aproximadamente 1 a $5x10^5$ UFC/mL (Klepser et al., 1998).

Para os fungos filamentosos, preparou-se uma suspensão de cada fungo após 7 dias de cultura em BDA (HiMedia) a 30°C, ajustado para 1 a 3 x 10^3 UFC/mL em RPMI 1640 sem (controle) ou com a quantidade desejada de 8-hidroxiquinolinas (Ghannoum et al., 2013).

As concentrações utilizadas para cada composto foram: CIM, CIMx2, CIMx4 e CIMx8. Os experimentos foram realizados em triplicatas e incubados a 35 °C para *Candida* e 30 °C para dermatófitos. Em tempos predeterminados (0, 3, 6, 12, 24 e 48 h para *Candida;* e 0, 3, 6, 12, 24, 48 e 96 h para dermatófitos), alíquotas de 100 µl foram assepticamente removidas de cada tubo e diluídas em série de 10 (até 10^{-3}) com água estéril. Em seguida, 15 µl diretamente das soluções testes e de cada diluição foram semeadas em placa contendo SDA (HiMedia) com o auxílio de uma alça de Drigalski. As placas foram incubadas a 35 °C/ 48 h para *Candida* e 30 °C/ 96 h para dermatófitos. Após este período, a contagem das colônias foi realizada.

O fundamento da técnica é determinar a cinética dos compostos testes, avaliando-se a capacidade fungicida dos mesmos em função do tempo. As curvas de tempo de morte foram plotadas em função da média do $\log_{10}$ UFC/mL contra o tempo de exposição das células fúngicas a várias concentrações das 8-hidroxiquinolinas, incluindo os desvios padrão. O efeito fungicida foi considerado quando houve diminuição de ≥ 99,9% no $\log_{10}$ do número de UFC/mL em comparação com o inóculo inicial (tempo zero) sem tratamento.

4.7 Modelagem Matemática PK/PD

Os dados obtidos no ensaio de tempo de morte foram utilizados para aplicar a modelagem matemática. Modelos Emax adaptados descritos por Li et al. (2009) e Treyaprasert et al. (2007) foram utilizados na modelagem de *C. albicans* (equação 2), *M. canis* (equação 3) e *T. mentagrophytes* (equação 4 e 5).

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C^h}{EC_{50} + C^h}\right)\right] N \quad (2)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)(1 - exp^{-zt})\right] N \quad (3)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)\right] N \quad (4)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right) - \left(\frac{K_{max}C}{EC_{50} + C}\right)(1 - exp^{-zt})\right] N \quad (5)$$

Onde d*N* / d*t* é a variação do número de células fúngicas em função do tempo; k_0 (h^{-1}) é a constante de velocidade de geração fúngica na ausência de agente antifúngico; k_{max} (h^{-1}) é constante de velocidade de morte máxima (efeito máximo); EC_{50} (µg/mL) é a concentração de agente antifúngico necessária para obter 50% do efeito máximo; *C* (µg / mL) é a concentração de agente antifúngico em determinado tempo (t); *N* (log_{10} UFC / mL) é o número de células fúngicas viáveis; e N_{max} é o número máximo de células. Os modelos levam em consideração *delay* de crescimento (*x*) *delay* de morte (*z*), e incorporam um fator de Hill (*h*) que modifica a inclinação e suaviza as curvas.

A modelagem matemática dos dados da curva de morte foi realizada com o software de regressão não-linear Scientist® 3.0 (Micromath, Salt Lake, EUA). Os gráficos foram inspecionados visualmente quanto à qualidade do ajuste, critério de seleção do modelo (MSC), coeficiente de determinação (R^2) e correlação entre os pontos medidos e calculados.

Após, no intuito de comparar os níveis de concentração necessários para o efeito definidos na modelagem PK/PD com os valores possíveis de serem alcançados *in vivo*, os níveis de concentração plasmática por tempo de um estudo disponível na literatura para dosagem oral de clioquinol (Jack e Riess, 1973) foram

modelados no software Scientist v3 através do modelo de dois compartimentos. Três regimes de dosagem orais foram simulados: i) uma dosem preditiva de 25 mg a cada 12 h; ii) uma dosagem preditiva de 50 mg a cada 12 h; iii) uma dosagem preditiva de 100 mg a cada 24 h. Ambas analisadas por 6 dias.

4.8 Ensaio de proteção de sorbitol

O efeito dos derivados da 8-hidroxiquinolinas sobre a integridade da parede celular fúngica foi avaliado pelo ensaio de proteção de sorbitol usando todas as espécies fúngicas estudadas. Os fungos sem parede celular ou com parede celular defeituosa são incapazes de crescer em condições normais. No entanto, segundo Frost et al. (1995), a parede celular é dispensável se os fungos estão protegidos por barreira osmótica. Assim, o crescimento dos fungos com integridade da parede celular comprometida é possível se sorbitol for suplementado em meio de cultura, pois este atua como um protetor osmótico (Junior et al. 2009).

Baseado nisso, as CIMs dos derivados da 8-hidroxiquinolina foram determinadas pela técnica de microdiluição em caldo (CLSI M27-A3 para *Candida* spp., CLSI M38-A2 para dermatófitos) na ausência e presença de sorbitol 0,8 M (Sigma-Aldrich) adicionado ao meio de cultura do ensaio como um osmoprotetor. Os experimentos foram realizados em duplicata. Anidulafungina (Pfizer®) foi utilizada como controle positivo, uma vez que este antifúngico tem ação sobre a parede celular. As CIMs foram avaliadas após 2 e 7 dias de incubação a 35 °C para *Candida* spp.; e após 4 e 7 dias de incubação a 30 °C para dermatófitos (Escalante et al., 2008). Os agentes antifúngicos que inibem a parede celular fúngica foram identificados quando os valores de CIM obtidos na presença de sorbitol foram mais elevados do que na sua ausência após sete dias de incubação (Junior et al. 2009).

4.9 Ensaio de ligação ao ergosterol

A capacidade dos derivados da 8-hidroxiquinolina se complexarem ao ergosterol presente na membrana fúngica foi avaliada pelo ensaio de ligação ao ergosterol usando todas as espécies fúngicas estudadas. Os agentes antifúngicos podem se ligar diretamente ao ergosterol da membrana, formando poros que alteram a permeabilidade. Como consequência, há perda de conteúdo intracelular e ruptura da membrana fúngica (Denning e Hope, 2010; Flevari et al., 2013). O agente

antifúngico se liga rapidamente ao ergosterol externo quando adicionado ao mieo de cultura, evitando a ligação ao ergosterol da membrana fúngica; como consequência, atividade reduzida do agente é observada (Escalante 2008).

Baseado nisso, as CIMs dos derivados da 8-hidroxiquinolina foram determinadas pela técnica de microdiluição em caldo (CLSI M27-A3 para *Candida* spp., CLSI M38-A2 para dermatófitos) na ausência e presença de diferentes concentrações (50-250 µg/mL) de ergosterol externo (Sigma-Aldrich, St. Louis , MO, EUA) adicionado ao meio de ensaio. O ergosterol comercial foi dissolvido em dimetilformamida (Sigma-Aldrich) e diluído no meio de cultura RPMI 1640, de forma que a concentração final do solvente fosse de 0.1%. Os experimentos foram realizados em duplicata. Anfotericina B (União Química, São Paulo, Brasil) foi utilizada como controle positivo, uma vez que este antifúngico tem afinidade pelo ergosterol da membrana e age pelo mecanismo citado acima. As CIMs foram avaliadas após 2 dias de incubação a 35 °C para *Candida* spp.; e após 4 dias de incubação a 30 °C para dermatófitos (Escalante et al., 2008). Os agentes antifúngicos que se complexam ao ergosterol da membrana foram identificados quando os valores de CIM obtidos na presença de ergosterol exógeno foram mais elevados do que na sua ausência (Junior et al. 2009).

4.10 Efeito de extravasamento celular

Dano na membrana fúngica pode ser detectado medindo a liberação de componentes intracelulares (Escalante et al. 2008). Assim, o possível extravasamento celular ocasionado por derivados da 8-hidroxiquinolina foi avaliado pela medição de materiais absorventes a 260 nm liberados para o meio, especialmente nucleotídeos, dos quais uracil apresenta maior absorbância (Lunde e Kubo, 2000). O ensaio foi realizado com um isolado representativo de cada gênero/espécie estudado e os procedimentos foram conduzidos como descrito anteriormente (Escalante et al. 2008; Lunde e Kubo, 2000).

Células de *Candida* cultivadas a 35 °C durante 48 h em SDA (HiMedia) foram lavadas três vezes (3000 rpm por 10 min, Spinlab SL-5M, Ribeirão Preto, Brasil) e ajustadas para aproximadamente 1 a $5x10^6$ UFC/mL com tampão MOPS gelado (0,16 M pH 7; Sigma-Aldrich). Uma diluição 1:10 de suspensão fúngica foi feita em soluções de 8-hidroxiquinolinas preparadas em tampão MOPS gelado,

resultando em uma suspensão com inóculo fúngico de 0,1 a 0,5x10^6 UFC/mL. Para dermatófitos, foram preparadas suspensões fúngicas com células cultivadas a 30 °C durante 10 dias em BDA (HiMedia) e ajustadas para 1-3 x 10^3 UFC/mL em soluções de 8-hidroxiquinolinas preparadas em tampão MOPS gelado.

As suspensões fúngicas foram tratadas com MIC de 8-hidroxiquinolinas. Células não tratadas e incubadas com tampão MOPS foram utilizadas como controle negativo. As células tratadas com anfotericina B (6,25 µg/mL) e SDS (2%) (Dodecilsulfato de sódio, Neon, São Paulo, Brasil) foram utilizadas como controle positivo. Os experimentos foram realizados em triplicata e a incubados a 35 °C para *Candida* spp. e 30 °C para dermatófitos. Alíquotas foram assepticamente removidas de cada tubo em intervalos diferentes (6, 24 e 48 h para *Candida* spp., 6, 24, 48 e 96 h para dermatófitos) e centrifugadas a 10000 rpm durante 10 min (Biosystems MCD2000, Curitiba, Brasil). Os sobrenadantes foram coletados para análise de absorbância a 260 nm em um espectrofotômetro Agilent Technologies 8453 (Santa Clara, EUA). Soluções de SDS (2%), anfotericina B (6,25 µg/mL) e 8-hidroxiquinolinas (MIC) sem inóculo fúngico foram utilizadas como controles (branco) para as leituras de absorbância.

Os resultados foram expressos como médias ± desvio padrão. A absorção obtida para 8-hidroxiquinolinas, SDS e anfotericina B foi comparada à absorção do controle não tratado e analisada por One-Way ANOVA, bem como o teste de comparações múltiplas de Dunnett. $P \leq 0,05$ foi considerado estatisticamente significante.

4.11 Microscopia eletrônica de varredura (MEV)

As alterações morfológicas nas células fúngicas cultivadas na presença de 8-hidroxiquinolinas foram observadas utilizando o método de macrodiluição em caldo (CLSI, 2008) seguido de MEV, conforme descrito abaixo. Após o período de incubação necessário para o método de macrodiluição em caldo (*Candida*: 48 h a 35 °C, *dermatófitos*: 96 h a 30 °C), células fúngicas tratadas com concentrações subinibitórias de 8-hidroxiquinolinas (MIC/2) e células não tratadas (controle) foram lavadas três vezes com PBS (tampão fosfato-salino) (3000 rpm durante 5 min, Biosystems MCD2000, Curitiba, Brasil). Após lavagem, as células foram fixadas em 1 mL de fixador de Karnovsky modificado, adaptado de Joubert et al. (2015). Então,

os poços foram lavados três vezes (3000 rpm durante 5 minutos) com cacodilato de sódio 0,1 M tamponado a pH 7,2 contendo sacarose 0,2 M e $MgCl_2$ 2 mM com o auxílio de duas pipetas, que foram utilizadas para adição e remoção simultânea para evitar exposição das células fúngicas ao ar. As células foram aderidas em lamínulas anteriormente funcionalizadas com poli-L-lisina durante 1 h. As células aderidas foram desidratadas em uma série graduada de soluções de acetona recentemente preparadas: 30, 50, 70, 95 (5 min) e 100% (10 min). As amostras foram então submetidas à secagem por ponto crítico (EM CPD 300, Leica), montadas em *stubs* metálicos, revestidas por pulverização com uma camada de ouro e paládio de 15-20 nm e visualizadas em um microscópio eletrônico de varredura (Carl Zeiss EVO® MA10 Carl, Oberkochen , Alemanha) operando a 10kV.

4.12 Efeito de derivados de 8-hidroxiquinolina sobre a formação de biofilme de *Candida* spp.

Os poços de uma placa de microdiluição foram pré-revestidos com derivados de 8-hidroxiquinolinas para investigar a capacidade dos compostos para prevenir a formação de biofilme de *Candida* (Bachmann et al., 2002). Resumidamente, foram adicionados 100 µL de volumes de diluições em série de derivados da 8-hidroxiquinolina em poços selecionados de uma placa de microdiluição e incubados a 4 °C por 48 h. Após este período, o excesso de derivados de 8-hidroxiquinolinas foi aspirado e as microplacas foram lavadas com solução salina estéril. Células de *Candida* cultivadas 24 h em SDA (HiMedia) foram ressuspensas a uma concentração de 10^6 UFC/mL em RPMI 1640 e, em seguida, adicionadas a cada poço da placa de microdiluição contendo 180 µL de RPMI 1640 (diluição 1:10). As placas foram incubadas durante 48 h a 35 °C para permitir a formação do biofilme.

A biomassa do biofilme formado foi medida de acordo com Stepanović et al. (2007), com modificações. Para remover células não aderentes, o conteúdo dos poços foi aspirado e lavado três vezes com solução salina estéril. As células aderidas foram fixadas com 150 µL de metanol durante 20 min. O solvente foi retirado e as microplacas permaneceram durante 30 min à temperatura ambiente para secagem. Após, as células aderidas foram coradas com 150 µL de cristal violeta 0,5% (p/v) (Synth, São Paulo, Brasil) por 15 min. A solução foi então

removida lavando cuidadosamente os biofilmes sob água corrente até o excesso de corante ser removido. A coloração do biofilme foi extraída com 150 µL de etanol 95% (Merck, Alemanha) durante 30 min sem agitação; e 100 µL deste foram transferidos para uma placa de microdiluição limpa. O ensaio foi realizado em triplicada.

As absorbâncias (A) resultantes das células tratadas com diferentes concentrações de 8-hidroxiquinolinas e das células não tratadas foram medidas (SpectraMax®) a 450 nm e os valores são proporcionais à quantidade de biomassa total do biofilme (maior a quantidade de material biológico, maior o nível de coloração e absorção).

O efeito percentual inibitório dos derivados de 8-hidroxiquinolina sobre a formação de biofilme de *Candida* spp. foi expresso como a porcentagem da densidade óptica média dos poços tratados com derivados de 8-hidroxiquinolina em comparação com o controle não tratado. Esta porcentagem foi calculada pela equação 6:

$$\%\ de\ Inibição\ da\ formação\ do\ biofilme\ (IFB) = \left[1 - \frac{(\mathrm{A450}_{poço\ tratado}\ )}{(\mathrm{A450}_{poço\ não\ tratado}\ )}\right] x\ 10 \quad (6)$$

A redução da absorção em 50% e 90% foi relatada como IFB_{50} e IFB_{90}, respectivamente. A análise estatística foi realizada por One-Way ANOVA, bem como o teste de comparações múltiplas de Tukey. $P \leq 0{,}05$ foi considerado estatisticamente significante. As análises foram realizadas usando o Minitab versão 17.0 (Minitab Inc., State College, PA).

4.13 Suscetibilidade de biofilme de *Candida* spp. aos derivados da 8-hidroxiquinolina

Para testar a suscetibilidade dos biofilmes de *Candida* aos derivados de 8-hidroxiquinolina, a CIM séssil (CIMS) foi determinada de acordo com a literatura (Shuford et al., 2007; Mowat et al., 2007; Ramage et al . 2001) com algumas modificações. Células de *Candida* cultivadas 24 h em SDA foram ressuspensas a uma concentração de 10^6 UFC/mL em RPMI 1640 e, em seguida, adicionadas a cada poço da placa de microdiluição contendo 180 µL de RPMI 1640 (diluição 1:10). As placas foram incubadas durante 48 h a 35 °C para permitir a formação do biofilme. Após este período, o conteúdo dos poços foi aspirado e lavado três vezes

com solução salina estéril para remover as células não aderentes. As células aderidas foram expostas a diluições seriadas de derivados de 8-hidroxiquinolina e incubadas durante 48 h a 35 °C. Após incubação, o excesso de derivados de 8-hidroxiquinolina foi aspirado e as microplacas foram lavadas três vezes com solução salina estéril a fim de remover as células não aderentes.

O efeito dos derivados de 8-hidroxiquinolina em biofilmes pré-formados foi estimado utilizando o ensaio de redução do XTT [2,3-bis (2-metoxi-4-nitro-5-sulfo-fenil) -2H-tetrazol-5-carboxanilida]. O ensaio do XTT é uma técnica de quantificação baseada na atividade metabólica de células viáveis e a redução do corante XTT para formazana solúvel em água são medidos (Ramage et al., 2001).

Resumidamente, XTT (Sigma-Aldrich) foi preparado como uma solução saturada a uma concentração de 0,5 mg/mL em tampão MOPS e armazenado a -70 ° C até à sua utilização. Antes do uso, uma alíquota da solução estoque de XTT foi descongelada e menadiona (preparada 10 mM em acetona, Sigma-Aldrich) foi adicionada a fim de obter uma concentração final de 1 mM. Então, uma alíquota de 100 µL de XTT-menadiona foi adicionada a cada poço. As microplacas foram incubadas no escuro durante 2 h a 37 °C e 75 µL de cada poço foram transferidos para uma placa de microdiuição limpa. O ensaio foi realizado em triplicata. A variação colorimétrica a 490 nm (reflexo da atividade metabólica do biofilme) foi medida com um leitor multiplacas (SpectraMax®) e a absorbância é proporcional ao número de células microbianas metabolicamente ativas. O efeito antifúngico dos derivados de 8-hidroxiquinolina sobre biofilme de *Candida* spp. foi expresso como a porcentagem da densidade óptica média dos poços tratados com derivados de 8-hidroxiquinolina em comparação com o controle não tratado. Esta porcentagem foi calculada pela equação 7:

$$\%\ de\ Inibição\ do\ biofilme\ pré-formado\ = \left[1 - \frac{\left(\mathrm{A490}_{Biofilme\ tratado}\right)}{\left(\mathrm{A490}_{\ Biofilme\ não\ tratado}\right)}\right] x\ 100 \qquad (7)$$

A redução da absorção (ou CIM séssil) em 50% e 90% foi relatada como $CIMS_{50}$ e $CIMS_{90}$, respectivamente. A análise estatística foi realizada por One-Way ANOVA, bem como o teste de comparações múltiplas de Tukey. $P \leq 0,05$ foi considerado estatisticamente significante. As análises foram realizadas usando o Minitab versão 17.0 (Minitab Inc., State College, PA).

4.14 Efeito de derivados de 8-hidroxiquinolina sobre remoção de biofilme de *Candida* spp.

Para testar o efeito de derivados de 8-hidroxiquinolina sobre a remoção de biofilme, o método de Ramage et al. (2001) foi aplicado com algumas modificações. Células de *Candida* cultivadas por 24 h em SDA foram ressuspensas a uma concentração de 10^6 UFC/ mL em RPMI 1640 e, em seguida, adicionadas a cada poço da placa de microdiluição contendo 180 µL de RPMI 1640 (diluição 1:10). As placas foram incubadas durante 48 h a 35 °C para permitir a formação do biofilme. Após este período, o conteúdo dos poços foi aspirado e lavado três vezes com solução salina estéril para remover as células não aderentes. As células aderidas foram expostas a diluições seriadas de derivados de 8-hidroxiquinolina e incubadas durante 48 h a 35 °C. Após incubação, o excesso de derivados de 8-hidroxiquinolina foi aspirado e as microplacas foram lavadas três vezes com solução salina estéril a fim de remover as células não aderentes.

As células aderidas foram fixadas com metanol e o efeito da remoção de biofilme dos derivados de 8-hidroxiquinolina foi determinado pela adição de cristal violeta 0,5% (Synth) como descrito acima (Stepanović et al., 2007). O ensaio foi realizado em triplicada.

As absorbâncias (A) resultantes das células tratadas com diferentes concentrações de 8-hidroxiquinolinas e das células não tratadas foram medidas (SpectraMax®) a 450 nm e os valores são proporcionais à quantidade de biomassa total do biofilme. Cristal violeta é adequado para medir a quantidade de biofilme, mas não a sua atividade, de modo que a coloração com cristal violeta pode ser usada para medir a remoção, mas não a desinfecção (Pitts et al., 2003).

O efeito percentual da remoção de biofilme de *Candida* spp. por derivados de 8-hidroxiquinolina foi expresso como a percentagem da densidade óptica média dos poços tratados com derivados de 8-hidroxiquinolina em comparação com o controle não tratado.

Esta porcentagem foi calculada pela equação 8:

$$\%\ de\ Remoção\ de\ biofilme\ (RB) = \left[1 - \frac{\left(\mathrm{A450}_{Biofilme\ tratado}\right)}{\left(\mathrm{A450}_{\ Biofilme\ não\ tratado}\right)}\right] x\ 100 \qquad (8)$$

A redução da absorção em 50% e 90% foi relatada como RB_{50} e RB_{90}, respectivamente. A análise estatística foi realizada por One-Way ANOVA, bem como o teste de comparações múltiplas de Tukey. $P \leq 0,05$ foi considerado estatisticamente significante. As análises foram realizadas usando o Minitab versão 17.0 (Minitab Inc., State College, PA).

4.15 Ensaio de formação de biofilme de *Candida* spp. sobre DIU e ação do clioquinol

Células de *Candida* cultivadas por 24 h em SDA foram ressuspensas a uma concentração de 10^6 UFC/mL em água peptonada (pH = 4,5; condições vaginais) (HiMedia, Índia). Uma diluição 1:10 desta suspensão foi feita por adição de 3 mL de suspensão fúngica em 27 mL de água de peptona. Os DIUs (Modelo TCu 380A, Furp - adotados pelo Ministério da Saúde do Brasil para pacientes no Sistema de Saúde Pública) foram colocados nesta suspensão de leveduras durante 48 h a 35 °C para permitir a formação de biofilme. Subsequentemente ao período de incubação, os dispositivos foram lavados três vezes com água estéril para remoção das células não aderidas. Então, duas etapas foram realizadas como descrito abaixo e ilustrado na figura 12:

Etapa 1: os DIUs contendo células aderidas foram expostos durante 8 h (tempo aproximado em que um creme vaginal permanece em contato) a uma temperatura de 35 °C aos agentes antifúngicos clioquinol e fluconazol, ambos preparados em 30 mL de água peptonada a uma concentração de 64 µg/mL. O mesmo procedimento foi realizado com um DIU exposto à água peptonada sem adição de agente antifúngico (controle não tratado). Após o período de incubação, alíquotas de 10 µL foram assepticamente removidas de cada frasco e diluídas em série de 10 vezes (até 10^{-3}) com água estéril. Em seguida, 10 µL diretamente das soluções testes e de cada diluição foram semeados em SDA para permitir a determinação das UFC removidas do dispositivo pelos agentes antifúngicos, mas que permaneceram vivas.

Etapa 2: Após a etapa 1, os DIUs foram retirados das soluções testes e colocados em frascos contendo 30 mL de água. Então, as células aderidas foram removidas do dispositivo por sonicação a uma frequência de 40 KHz durante 10 min. Esta água sonicada foi diluída em série de 10 vezes (até 10^{-3}) com água estéril e

semeada em SDA para determinação das UFC que permaneceram vivas e aderidas ao DIUs após contato com os agentes antifúngicos.

Todas as diluições e contagens foram feitas em triplicada. As placas foram incubadas durante 24 h a 35 °C, e as UFC por DIU foram determinadas. A quantificação das células de biofilme vivas no DIU foi determinada pela soma das UFC encontradas na etapa 1 e UFC encontradas na etapa 2. Assim, as células de biofilme vivas do DIU não tratadas e tratadas foram calculadas pela equação 9:

$$UFC_{Biofilme} = UFC_{Etapa\,1} + UFC_{Etapa\,2} \quad (9)$$

A porcentagem de erradicação do biofilme por clioquinol e fluconazol foi expressa como a porcentagem da UFC do DIU tratado em comparação com o controle não tratado. Esta percentagem foi calculada pela equação 10:

$$Erradicação\ do\ Biofilme\ (\%) = \left[\frac{\left(UFC_{Biofilme\ não\ tratato} - UFC_{Biofilme\ tratado}\right)}{UFC_{\ Biofilme\ não\ tratado}}\right] x\ 100 \quad (10)$$

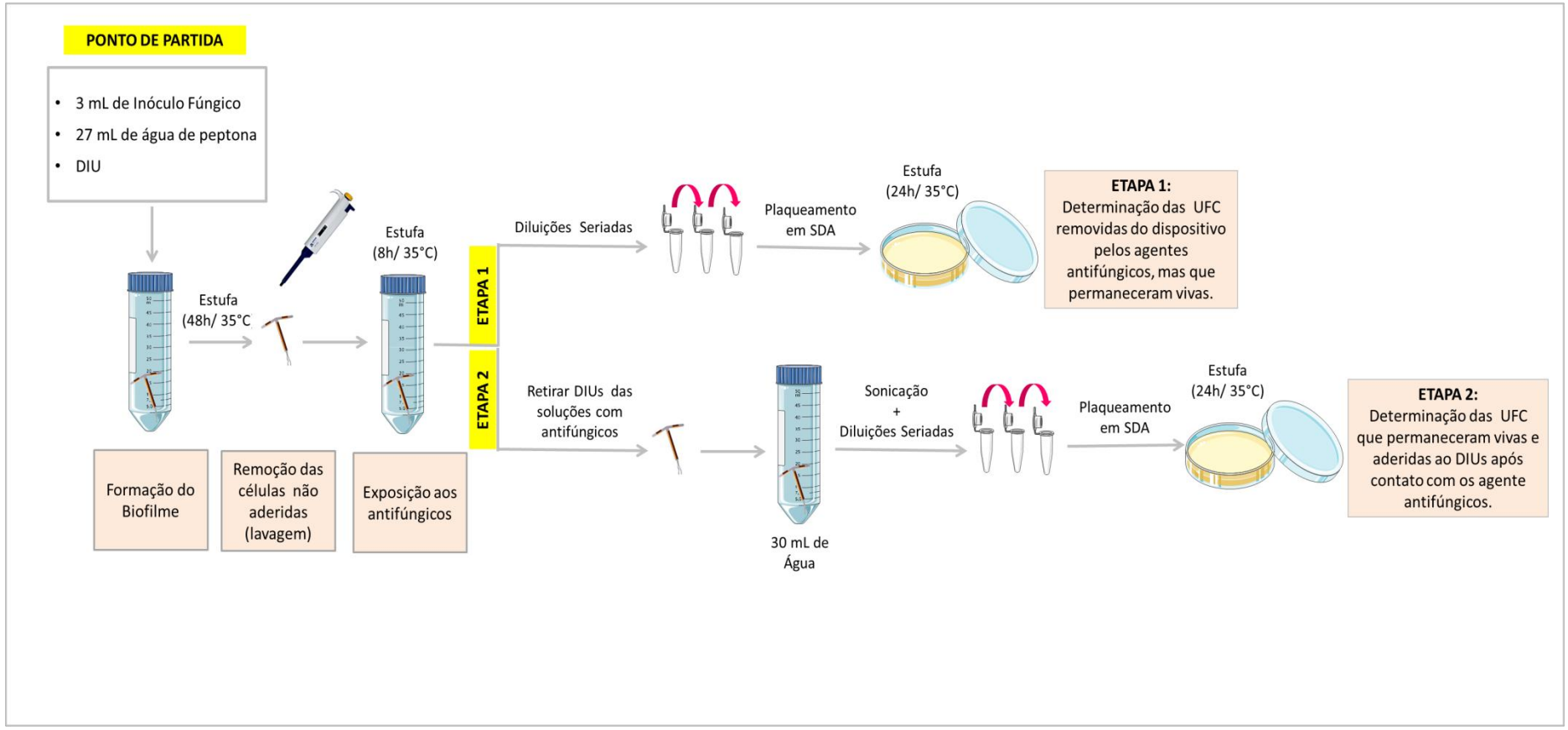

Figura 12. Esquema das etapas do teste de ação antifúngica sobre biofilme formado em dispositivo intrauterino (DIU) (Pippi 2018).

4.16 Efeito protetor de derivados da 8-hidroxiquinolina em moscas *D. melanogaster* infectadas com *Candida albicans*

Experimentos em modelo alternativo *in vivo* utilizando como hospedeiro *D. melanogaster* imunodeficientes foram conduzidos a fim de verificar o efeito protetor de derivados de 8-hidroxiquinolinas em infecção sistêmica por *C. albicans*.

Fêmeas de *D. melanogaster* Oregon^{R} Tl-deficientes (presente de Dr. Dimitrios Kontoyiannis da University of Texas MD Anderson Cancer Center) foram utilizadas nos ensaios. Mutantes de transheterozigotos Tl-deficientes foram gerados através do cruzamento de moscas portadoras de um alelo termossensível de Tl (Tlr632) com moscas portadoras de um alelo nulo de Tl (TlI-RXA) (Zanette et al., 2013). As moscas são armazenadas em garrafas de poliestireno (24x90mm) no laboratório DrosoLab da Universidade Federal do Rio Grande do Sul (Porto Alegre, Brasil). Procedimentos padrão foram utilizados para manipulação, alimentação e habitação de moscas em todos os experimentos (Chamilos et al 2008).

Para a realização do ensaio, as fêmeas de *D. melanogaster* Tl-deficiente (grupos de 10 a 15 moscas) foram colocadas em frascos vazios por 6-8 h em jejum, e depois transferidas para frascos com alimentos contendo 100 µL de diferentes concentrações de derivados de 8-hidroxiquininas (MIC, MICx2, MICx4, MICx8 e 1mg/mL), como previamente descrito (Chamilos et al., 2006). Após 24 h, as moscas foram infectadas, por injeção, com *Candida albicans* ATCC 18804 (10^8 células/mL preparadas em solução salina 0,85%) e mantidas a 29 °C nos frascos contendo os agentes antifúngicos. A sobrevivência das moscas foi avaliada diariamente até o sétimo dia após a injeção. As moscas que morreram no período de 3 h após injeção foram consideradas mortas como consequência do procedimento e foram excluídas da análise de sobrevivência.

O mesmo procedimento foi realizado para outro grupo de moscas, mas sem expor as moscas às 8-hidroxiquinolinas (controle não tratado). Um grupo com moscas não infectadas (controle não infectado) também foi avaliado concomitantemente. Além disso, antes das experiências de proteção, as moscas foram expostas a alimentos contendo 8-hidroxiquinolinas (1 mg /mL) para verificar a toxicidade dos compostos. Os experimentos foram realizados em triplicata em dias diferentes usando um total de 30-45 moscas por grupo testado.

As curvas de sobrevivência foram traçadas usando a análise de Kaplan-Meier e as diferenças nas taxas de sobrevivência entre os grupos foram analisadas usando o teste log-rank. As análises estatísticas foram realizadas com o software GraphPad Prism (versão 6.0; software GraphPad). P ≤ 0,05 foi considerado estatisticamente significativo.

4.17 Avaliação da carga fúngica no tecido de *D. melanogaster* tratadas com derivados da 8-hidroxiquinolina

A quantificação de células de *Candida* nos tecidos das moscas foi realizada após 7 dias de infecção. As moscas de cada grupo foram coletadas e separadas entre vivas e mortas, e moídas em 1 mL de solução salina 0,85%. Diluições em série (até 10^{-6}) foram feitas em triplicata e 10 µl diretamente das soluções contendo as moscas e de cada diluição foram semeadas em placa contendo SDA com o auxílio de uma alça de Drigalski. O número de UFC foi contado após 48 h de incubação a 35 °C.

Os testes de Kruskal-Wallis e post-hoc Dunn (análise de variância não paramétrica) foram utilizados para determinar diferenças estatisticamente significativas na carga fúngica entre os grupos. As análises estatísticas foram realizadas com o software GraphPad Prism (versão 6.0; software GraphPad). P ≤ 0,05 foi considerado estatisticamente significativo.

4.18 Ligação dos derivados de 8-hidroxiquinolina à proteína plasmática

A ligação à proteína plasmática ocorre predominantemente com albumina. Para avaliar a influência da ligação de proteínas sobre a atividade de derivados de 8-hidroxiquinolinas, avaliamos o crescimento fúngico em meio de cultura livre de proteína e meio contendo albumina de soro bovino. Assim, CIMs dos derivados da 8-hidroxiquinolina foram determinadas contra *C. albicans* ATCC 18804 pelo método de microdiluição em caldo padrão (CLSI M27-A3, 2008) usando meio RPMI 1640 suplementado com 40 g/litro de albumina de soro bovino (INLAB Confiança, São Paulo, Brasil) (Schafer-Korting et al., 1991). Os experimentos foram realizados em duplicata e anfotericina B (União Química, São Paulo, Brasil) foi utilizada como controle positivo (Bekersky et al., 2002).

Os agentes antifúngicos que se ligam a proteínas plasmáticas foram identificados quando os valores de CIM obtidos na presença de albumina foram mais elevados do que na sua ausência após dois dias de incubação.

4.19 Avaliação histopatológica de pele de porco exposta aos derivados da 8-hidroxiquinolina

Amostras de tecido de porcos machos adultos, recentemente abatidos no Instituto Federal de Santa Catarina (campus Concordia), foram utilizadas para avaliar o dano tecidual devido à ação de derivados da 8-hidroxiquinolina. Os porcos foram abatidos de acordo com as regras do Ministério da Agricultura, Pecuária e Abastecimento, respeitando o bem-estar dos animais (MAPA, 2013), e a pele da orelha do porco foi utilizada aqui. Os tecidos foram removidos dentro de um período de 5 minutos após o abate; os pelos foram cuidadosamente removidos por um aparador elétrico e os tecidos foram transportados para o laboratório em tampão Krebs-Hepes gelado (INLAB, Brasil). As amostras de pele foram montadas em células de difusão de Franz (Logan Instrument Corp, NJ, EUA) com área de difusão de aproximadamente 1,75 cm^2. O lado epidérmico da pele foi exposto às 8-hidroxiquinolinas na concentração 1 mg/mL (dissolvidas em PBS pH 7,0) por um período de 6 h (tempo em que o perfil de permeação é mais intenso). O mesmo procedimento foi realizado utilizando PBS pH 7,0 como controle negativo e a uma solução de NaOH 0,1 M como controle positivo.

Fragmentos desses tecidos foram coletados, fixados em formalina tamponada neutra a 10%, processados rotineiramente e corados com hematoxilina e eosina. Por fim, foram examinados sob microscopia óptica. Os experimentos foram realizados em triplicata.

4.20 Ensaio da membrana cório-alantoide de ovo embrionado de galinha ou HET-CAM

O uso do método alternativo HET-CAM (Hens Egg Test-Chorion Allantoic Membrane) ou ensaio da membrana cório-alantoide tem sido utilizado como modelo alternativo para verificar o potencial irritativo de compostos. O ensaio HET-CAM baseia-se na determinação de alterações macroscópicas que podem

ocorrer na membrana cório-alantoide de ovo embrionado de galinha em decorrência da aplicação de ingredientes com potencial irritante (Oliveira et al. 2012).

Para tal avaliação, ovos brancos férteis e frescos, do tipo comercial Lohmann (Lohmann selected Leghorn, LSL, Brasil) foram utilizados. Os ovos foram mantidos em condições de incubação otimizadas (temperatura entre 38 - 39 ° C e umidade entre 55 – 60 %, durante 10 dias). No décimo dia, a casca de ovo, ao redor do espaço aéreo, foi cuidadosamente removida com uma ferramenta rotativa (Dremel, Racine WI). Posteriormente, 0,3 mL dos derivados da 8-hidroxiquinolina a uma concentração de 1 mg/mL foram adicionados sobre cada ovo. Durante 5 minutos, a membrana cório-alantoide foi examinada e as reações fisiológicas observadas foram graduadas em função de seu tempo de aparecimento. O efeito de irritação foi avaliado nos períodos de 30 s, 2 min e 5 min após a aplicação de cada composto. O experimento foi realizado em triplicata. O mesmo procedimento foi realizado utilizando solução salina 0,9% como controle negativo e solução de NaOH 0,1 M como controle positivo do teste. O resultado do índice de irritação (IS-" irritation score") foi dado de acordo com a equação 11, em uma escala de 0 a 4,9 indicando não irritante (ou praticamente sem irritação) e 5,0 a 21 denotando irritante (irritação moderada / grave ou extrema) (ICCVMA, 2010).

A comparação de IS foi analisada por One-Way ANOVA e posterior teste de comparações múltiplas de Tukey. P ≤ 0,05 foi considerado estatisticamente significante. As análises foram realizadas usando o Minitab versão 17.0 (Minitab Inc., State College, PA).

$$IS = \left(\left(\frac{(301 - Tempo\ de\ Hemorragia)}{300}\right)x5\right) + \left(\left(\frac{(301 - Tempo\ de\ Lise)}{300}\right)x7\right) + \left(\left(\frac{(301 - Tempo\ de\ Coagulação)}{300}\right)x9\right) \quad (11)$$

4.21 Teste comportamental em embrião de galinha

Ovos brancos férteis e frescos do tipo comercial Lohmann (*Gallus gallus domesticus*) foram utilizados neste teste. Os ovos foram mantidos a 37,6 ± 0,4 °C e 50 % de umidade relativa, e permaneceram girando automaticamente até o dia 12.

No décimo segundo dia, os ovos foram abertos e 100 µl de 8-hidroxiquinolinas (1 mg/mL ou 0.25 mg/mL) foram adicionados a cada ovo. O mesmo procedimento foi realizado utilizando solução salina 0,9% e epinefrina (1 mg/mL) como controle negativo e positivo, respectivamente. Os seguintes parâmetros dos embriões de galinha foram observados durante dois minutos: pulsação arterial, abertura do bico e movimento do embrião.

4.22 Manutenção e reprodução de *zebrafish*

Zebrafish adulto de tipo selvagem (*Danio rerio*) foram obtidos de um fornecedor local e mantidos em um sistema aquático recirculante (Zebtec®) em condições adequadas de água (pH 7.2, 400 µS de condutividade e 28 °C). Os peixes foram alimentados duas vezes por dia com alimentos de peixe comercial e suplementados com camarão salmoura (*Artemia salina*) uma vez por dia (Westerfield, 2000). Os peixes foram mantidos com fotoperíodo de 14h/10h (14h de luz e 12 h de escuro) e os valores de amônia, nitrito e nitrato eram menores que 0,2 ppm, 0,05 ppm e 0,05 ppm, respectivamente. Machos e fêmeas (2: 1) de z*ebrafish* adultos foram colocados em pares durante a noite e na manhã seguinte a reprodução foi induzida por irradiação luminosa. Os testes foram conduzidos com embriões (até 48 hpf; horas pós-fertilização) e larvas (de 48 até 168 hpf) de *zebrafish* expostos ao PH151 e PH153. Os protocolos experimentais utilizados neste trabalho foram aprovados pelo comitê de Ética local (CEUA - UNIPAMPA: protocolo 003-2016).

4.23 Exposição de embriões de *zebrafish* aos derivados da 8-hidroxiquinolina e avaliação da mortalidade

Após a reprodução, os ovos fertilizados foram coletados, lavados com água do sistema e estocados aleatoriamente em grupos. Ovos 3 hpf foram incubados em placas de Petri de vidro contendo 20 mL de soluções de PH151 e de PH153 preparadas em água do sistema.

Os estudos de exposição foram realizados em triplicata com 20 embriões por grupo e os experimentos foram repetidos pelo menos três vezes independentemente. As soluções de trabalho foram preparadas em DMSO e diluídas em água do sistema a fim de obter uma concentração máxima de 0,005% de DMSO

nos experimentos. Foram utilizadas duas curvas de exposição diferentes. Primeiramente, os embriões (3 hpf) foram expostos a 0,25 - 16 µg/mL de PH151 e 1 - 64 µg/mL de PH153 durante 24h. Em 2 e 24h os embriões viáveis foram quantificados como parâmetro de sobrevivência e mortalidade. Após, os embriões (3 hpf) também foram expostos estaticamente a 0,06 - 0,25 µg/mL de PH151 e 0,25 - 1 µg/mL de PH153 durante 7 dias (168 hpf). DMSO 0,005% foi usado como controle do veículo. Para o grupo de controle, apenas a água do sistema foi utilizada.

4.24 Estimulação ao toque e resposta natatória de larvas de *zebrafish* expostas aos derivados da 8-hidroxiquinolina

Larvas de *zebrafish* com 72 hpf (3 dias) expostas a concentrações da segunda curva de exposição (0,06 - 0,25 µg/mL de PH151 e 0,25 - 1 µg/mL de PH153) foram submetidos ao teste comportamental de resposta ao toque (Saint-Amant e Drapeau, 1998). Nesta fase de desenvolvimento, as larvas permanecem inertes no ambiente, respondendo apenas a estímulos, tal como o toque. Resumidamente, as larvas expostas ao PH151 e PH153, bem como os grupos controles, foram colocados individualmente no centro de uma placa de Petri contendo 20 mL de água do sistema e cada larva foi tocada por uma pinça (estímulo) utilizando teste duplo cego. Foi observado o número de estímulos necessários para o primeiro deslocamento e a capacidade da larva executar um escape padrão a fim de verificar parâmetros motores e sensitivos. A tigmotaxia foi determinada pela porcentagem de larvas que escaparam para a periferia do aparato.

4.25 Comportamento exploratório das larvas de *zebrafish* (“Open Field”) expostas aos derivados da 8-hidroxiquinolina

Após o tempo final de exposição aos compostos (7 dias ou 168 hpf), as larvas de *zebrafish* foram submetidas ao teste de comportamento exploratório (Colwill e Creton, 2011; Altenhofen et al., 2017). Resumidamente, cada larva foi colocada individualmente em um dos poços (2 mL) de placas de cultura celular de 24 poços. O local de natação da larva foi gravado durante 300s após 60s de habituação usando um estereomicroscópio digital conectado a um laptop com uma taxa de 30 frames/s e um software de rastreamento de vídeo apropriado (ANY-mazeTM, Stoelting CO, EUA). O aparelho foi virtualmente dividido em duas seções

circulares (meio e periferia) para avaliar a exploração espacial. A distância percorrida (m), o ângulo de rotação absoluto (°) e o tempo médio por visita na área central foram utilizados para medir parâmetros motores e locomotores.

4.26 Avaliação de processo apoptóticos por laranja de acridina em larvas de *zebrafish* expostas aos derivados da 8-hidroxiquinolina

O corante fluorescente laranja de acridina foi utilizado para a detecção de células no processo de apoptose. Após o tempo final de exposição aos compostos (7 dias ou 168 hpf), cinco larvas de *zebrafish* foram colocadas em eppendorf contendo 2 ml de água do sistema e laranja de acridina na concentração final de 5 µg/mL. Os eppendorf com as larvas foram incubados no escuro durante 30 min. Após, as larvas foram lavadas três vezes na água do sistema e, então, fixadas em lâminas para microscopia com 1,5% de metilcelulose. As imagens foram obtidas pelo microscópio de fluorescência Olympus IX71 com câmera acoplada (Tilton et al., 2008).

4.27 Análise estatística dos ensaios com embriões e larvas de *zebrafish*

A normalidade dos dados e a homogeneidade das variâncias foram analisadas pelos testes de Kolmogorov-Smirnov e Bartletts, respectivamente. Os resultados dos parâmetros comportamentais e apoptóticos foram expressos como média ± erro padrão (S.E.M.) e analisados por análise de variância unidirecional (ANOVA), seguido de Tukey. Devido à sua distribuição não paramétrica, o tempo de imobilização (ou tempo na área central) foi expresso como intervalo interquartil médio e analisado por Kruskal-Wallis seguido do teste de comparação múltipla de Dunn. $P \leq 0,05$ foi considerado estatisticamente significativo.

5 ARTIGOS

5.1 Artigo 1

O artigo intitulado “Evaluation of 8-Hydroxyquinoline Derivatives as Hits for Antifungal Drug Design” foi publicado na “Medical Mycology”.

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Full Title: Evaluation of 8-Hydroxyquinoline Derivatives as Hits for Antifungal Drug Design

Short Title: Antifungal 8-Hydroxyquinoline Derivatives

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Keywords: clioquinol, 8-hydroxyquinoline derivatives, *Candida*, dermatophyte, fungicidal effect.

Abstract

Clioquinol is an 8-hydroxyquinoline derivative that was widely used from the 1950s to 1970s as an oral anti-parasitic agent. In 1970, the oral forms were withdrawn from the market due to reports of toxicity, but topical formulations for antifungal treatment remained available. Thus, the purpose of this study was to evaluate the toxicity, anti-*Candida* and anti-dermatophyte activity and to determine pharmacodynamic characteristics of clioquinol and other 8-hydroxyquinoline derivatives (8-hydroxy-5-quinolinesulfonic acid and 8-hydroxy-7-iodo-5-quinolinesulfonic acid). Antifungal activity was tested by broth microdilution and the fungicidal or fungistatic effect was checked by a time-kill assay. Permeation and histopathological evaluation were performed in Franz diffusion cells with ear skin of pigs and examined under light microscopy. An HET-CAM test was used to determine the potential irritancy. The three compounds were active against all isolates showing anti-*Candida* and anti-dermatophyte activity, with MIC ranges of 0.031–2 µg/ml, 1–512 µg/ml and 2–1024 µg/ml for clioquinol, 8-hydroxy-5-quinolinesulfonic acid and 8-hydroxy-7-iodo-5-quinolinesulfonic acid, respectively. All compounds showed fungistatic effect for *Candida*. 8-hydroxy-5-quinolinesulfonic acid, 8-hydroxy-7-iodo-5-quinolinesulfonic acid showed a fungicidal effect for *M. canis* and *T. mentagrophytes,* and clioquinol showed fungicidal effect only for *T. mentagrophytes*. Furthermore, they presented a fungicidal effect depending on the time and concentration. The absence of lesions was observed in histopathological evaluation and no compound was irritating. Moreover, clioquinol and 8-hydroxy-5-quinolinesulfonic acid accumulated in the epithelial tissue, and 8-hydroxy-7-iodo-5-quinolinesulfonic acid had a high degree of permeation. In conclusion, 8-hydroxyquinoline derivatives showed antifungal activity and 8-hydroxy-5-quinolinesulfonic acid demonstrated the potential for antifungal drug design.

Introduction

Fungal infections represent a threat to human health. They can be classified according to the extent of infected tissue as: superficial, cutaneous, subcutaneous and systemic mycoses. Such infections cause conditions that range from athlete's foot to severe disseminated disease. Dermatophytes—mainly represented by the genera *Trichophyton, Microsporum* and *Epidermophyton*—are quite widespread worldwide and are naturally able to cause an infection in the healthy population. These fungi affect keratinised structures of the body and can cause chronic and uncontrolled infections.[1,2]

In contrast, mucosal infections are mostly caused by opportunistic yeasts, mainly species of *Candida.* These yeasts can develop infections especially when the immune system of the patient is compromised. *Candida* spp. have also been associated with systemic infections, and with high rates of morbidity and mortality.[2]

In addition, the incidence and severity of fungal diseases has increased in recent years, and the widespread use of antifungal agents has serious implications for antifungal therapy due to the emergence of resistant strains leading to treatment failures. This scenario represents a public health problem.[3,4] Besides problems related to resistance, many antifungal drugs have several limitations associated with toxicity. Safety profiles of the antifungals have limited their clinical use and have restricted the number of medicines available to treat such infections.[5] In view of this situation, there is a clear need for a reevaluation of antifungals and introduction of new pharmaceutical strategies. In this context, 8-hydroxyquinoline derivatives may come to play an important role in antifungal therapy.

Clioquinol (compound **1**; 5-chloro-7-iodo-8-hydroxyquinoline) is a dihalogenated 8-hydroxyquinoline that was widely used from the 1950s to 1970s as an oral anti-parasitic agent.[6] In 1970, the oral formulation compounds were withdrawn from the market due to reports of sub-acute myelo-optic neuropathy (SMON) in Japanese patients.[7] However, these reports are controversial because the clioquinol had been used for 20 years prior to these events without incident. Moreover, these effects could be associated with formulations used in Japan and/or with genetic susceptibility (inherited increase in the risk of developing a disease)[6,7] since they were rarely observed in other countries.[8]

Topical formulations for the treatment of fungal infections have remained available in many countries.[6] The use of topical clioquinol in high concentrations can cause skin irritation, but it is not a common contact allergen and has not been associated with development of skin malignancies.[9,10] Furthermore, there are few reports of antimicrobial resistance against clioquinol.[10] Recently compound **1** had a renewed interest as a lead compound in the design of anticancer agents and a chelating agent with the potential effect for the treatment of Alzheimer's, Parkinson's and Huntington's disease.[11-14]

There is already great potential for reintroduction of compound **1** for new indications, but its mode of action on the fungal cells has not yet been elucidated. Researchers have already proposed other 8-hydroxyquinoline derivatives with antifungal potential, but have used outdated methodologies and without toxicological information on human cells.[15,16]

Thus, the purpose of this study was to evaluate the toxicity, anti-*Candida* and anti-dermatophyte activity (n= 73) and to determine pharmacodynamic characteristics of clioquinol (compound **1)** and two other 8-hydroxyquinoline derivatives: compound **2** (8-hydroxy-5-quinolinesulfonic acid) and compound **3** (8-hydroxy-7-iodo-5-quinolinesulfonic acid) in order to find novel antifungals and to gain some insights into their pharmacodynamic characteristics. Sulfonic acid derivatives have the advantage of being easily modified while clioquinol is less reactive. Thus, it is possible to obtain a great structural diversity using these compounds. Furthermore, they have fewer halogen atoms than compound **1**, which can be considered an important and favourable structural characteristic to reduce the toxicity.[17]

Materials and methods

Fungal Strains: A total of 73 strains were included in this study: *C. albicans* (11), *C. glabrata* (10), *C. krusei* (9), *C. parapsilosis* (11), *C. tropicalis* (11), *M. canis* (6), *M. gypseum* (5), *T. mentagrophytes* (5), and *T. rubrum* (5). All strains were deposited in the Mycology Collection of Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil) and originated from the National Programme of Quality Control of Brazil: nail and skin scrapes, hemoculture, vaginal secretions, urine, tracheal aspirate and catheter tip. Standard strains of *C. albicans* (ATCC 18804), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750)

were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and were included as controls.

Antifungal Compounds: Clioquinol (compound **1**; MW= 305.50 g/mol), 8-hydroxy-5-quinolinesulfonic acid (compound **2**; MW= 225.22 g/mol) and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**; MW= 351.12 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the experiments, these compounds were dissolved in DMSO (Sigma-Aldrich) and after being diluted in RMPI 1640 (Gibco; Grand Island, NB, USA), buffered with MOPS (Sigma-Aldrich) to obtain maximum concentration of 2% DMSO.

Antifungal Susceptibility Testing: Minimum inhibitory concentrations (MICs) of analogues of 8-hydroxyquinoline were determined by the broth microdilution method according to M27-A3 protocol for the *Candida* species and M38-A2 for the filamentous fungi (*Microsporum* spp. and *Trichophyton* spp.).[18,19] Serial two-fold dilutions were made in RPMI 1640 medium and the final test range of concentrations was: 0.016–8 µg/ml of compound **1,** 0.25–128 µg/ml (for *Candida* spp.) and 2–1024 µg/ml (for dermatophytes) of compound **2** and **3**. The experiments were carried out in quadruplicate. MICs were defined as the lowest concentration of compounds at which the microorganisms tested did not demonstrate visible growth in 48 h (*Candida* spp.) or 96 h (for *Microsporum* spp. and *Trichophyton* spp.), and the values of MIC_{50} and MIC_{90} were determined for each tested species. *C. albicans* ATCC 18804, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. tropicalis* (ATCC 750) were used as quality control.

Cell damage evaluation by the MTT-based assay: After the incubation time and visual evaluation in the antifungal susceptibility testing, colorimetric reading was performed with MTT suspension [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) for all tested concentrations of the compounds. For this, the supernatant was discarded and the fungal cells were incubated for 3 h (*Candida* spp.) and 24 h (*Microsporum* spp. and *Trichophyton* spp.) at 35 °C in a suspension containing 0.05 mg/ml MTT. MTT is a tetrazolium salt

reduced by dehydrogenase enzymes that form blue formazan crystals, which were then extracted with isopropanol.[20]

Absorbances (A) were measured on a multiplate reader SpectraMax-M2; (Molecular Devices®, Silicon Valley, CA, USA) in two wavelengths (570 and 690 nm). The percent cell damage was calculated by the following equation: % cell damage = [1 – (A570 – A690 with drugs) /(A570 – A690 without drugs)] x 100.[20]

Time-Kill Assay: A time-kill assay was carried out with one representative strain of each genus studied (*C. albicans* ATCC 18804, *M. canis* MCA 01 and *T. mentagrophytes* TME 40) and the procedures were conducted as previously described by Klepser et al. (1998) and Ghannoum et al. (2013), for *Candida* spp. and dermatophytes, respectively.[21,22] Each isolate was tested for compounds **1**, **2** and **3**.

For the yeast (*C. albicans* ATCC 18804), a fungal suspension was prepared from 24 h culture at 35 °C on SDA (Sabouraud Dextrose Agar; HiMedia; India) with chloramphenicol and it was adjusted to a 0.5 McFarland turbidity standard (1–5 x 10^6 CFU/ml). A 1:10 dilution of this suspension was made by adding 1 ml of fungal suspension to 9 ml of RPMI 1640 with or without (growth control) the desired amount of antifungal compounds. This dilution resulted in a solution with an inoculum of approximately 1–5 x 10^5 CFU/ml.[21] For the filamentous fungi (*M. canis* MCA 01 and *T. mentagrophytes* TME 40), a suspension of each fungus was prepared from a 7-day culture at 30 °C on potato dextrose agar (HiMedia) and it was adjusted to 1–3 x 10^3 CFU/ml in RPMI 1640 with or without (control) the desired amount of antifungal compounds.[22]

When used to assess an antimicrobial agent, kill curves were constructed by testing several multiples of the MIC. The concentrations used for each compound were: MIC, MICx2, MICx4 and MICx8. All test solutions and controls were made in triplicate and they were incubated at 35 °C (*Candida*) or 30 °C (dermatophytes). At predetermined time points (0, 3, 6, 12, 24, and 48 h for *Candida* and 0, 3, 6, 12, 24, 48 and 96 h for dermatophytes), 100 µl samples were aseptically removed from each tube and serially diluted 10-fold (until 10^{-3}) with sterile water. Then, 15 µl directly from the test solution and each dilution solution were plated onto a SDA plate, using a Drigalski spreader.[22] Plates were incubated at 35 °C for 48 h for *Candida* and 30 °C for 96 h for dermatophytes. After this period, the colony count was performed.

Time kill curves were constructed by plotting mean $\log_{10}$ CFU/ml against the exposure time of fungal cells to various concentrations of the antifungal agents, including the standard deviations. Fungicidal effect was considered when there was a decrease of ≥99.9% in the $\log_{10}$ of the number of CFU/ml compared with starting inoculum.

Determination of permeation and histopathological evaluation of 8-hydroxyquinoline derivatives: Tissue samples from adult male pigs, freshly slaughtered at the Institute Federal of Santa Catarina, Concordia campus, were used to evaluate the formation of tissue damage due to the action of the compounds **1**, **2** and **3**. Pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture, respecting animal welfare.[23] Ear skin of pigs was used in this study. The tissues were removed within a period of 5 min after the slaughter; the hairs were carefully removed by an electric trimmer and transported to the laboratory in an ice-cold Krebs-Hepes buffer (INLAB, São Paulo, SP, Brazil). Skin samples were mounted in Franz diffusion cells (Logan Instrument Corp., Somerset, NJ, USA) with the diffusion area of approximately 1.75 cm^2. The epidermal side of the skin was exposed to a PBS pH 7.0 (negative control) and to 0.1 M NaOH solution (positive control) for a period of 6 h (time that the permeation profile is more intense). The 8-hydroxyquinoline derivatives were dissolved in a PBS pH 7.0 at a concentration of 1 mg/ml (a higher concentration than MIC). The epidermal side of the skin was subjected to extract contact with this solution for a period of 6 h. Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely and stained with hematoxylin and eosin, and examined under light microscopy. The experiments were carried out in triplicate.

Hens' Egg Test on the Chorioallantoic Membrane (HET-CAM): White fertile eggs of fresh Lohmann commercial breed (Lohmann selected Leghorn, LSL, São José do Rio Preto, SP, Brazil) were used in the HET-CAM test. The eggs were kept at optimised incubation conditions (temperature between 38 to 39 °C and humidity between 55 and 60% for 10 d). On the 10^{th} day, the egg shell, around the airspace, was carefully removed with a rotary tool (Dremel, Racine, WI). Afterwards, 0.3 ml of each compound at a concentration of 1 mg/ml was added to each egg,

respectively (negative control–0.9% saline solution; positive control–0.1 M NaOH solution; test substances–compounds **1**, **2** and **3**). The irritation effect was evaluated at 30 s, 2 min, and 5 min after the application of each substance. The experiment was performed in triplicate. The result of the irritation score (IS) was given according to the equation below, on a scale from 0 to 4.9 denoting nonirritant (or practically no irritation) and 5.0 to 21 denoting irritant (moderate/severe or extreme irritation).[24] Comparison of IS was analysed by one-Way ANOVA and Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant.

$$IS = \left(\left(\frac{(301 - Hemorrhage\ Time)}{300}\right)x5\right) + \left(\left(\frac{(301 - Lysis\ Time)}{300}\right)x7\right) + \left(\left(\frac{(301 - Coagulation\ Time)}{300}\right)x9\right)$$

Results

All 8-hydroxyquinoline derivatives were able to inhibit the in vitro growth of the tested *Candida* spp., *Microsporum* spp. and *Trichophyton* spp. The MIC values ranged from 0.031–2 µg/mL, 1–512 µg/mL and 2–1024 µg/mL for compounds 1, compounds 2 and compounds 3, respectively; the cell damage in the MIC exceeded 90% for all isolates. The antifungal data and of the three compounds against *Candida* spp. (n= 52) and dermatophytes (n= 21) are summarised in Table 1. The results are expressed as ranges of variation of minimum and maximum MIC values obtained for each species as well as cell damage caused by each compound at its MIC (presented as average ± standard deviation). Also, the values of MIC_{50} (MIC value which inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates) were determined for each tested species. MIC values of the compounds against all isolates are available in the supplementary material (Table S1 and Table S2).

Regarding the fungicidal kinetics, graphical representations of the $\log_{10}$ of the numbers of CFU/ml versus time for different concentrations (MIC, MICx2, MICx4 and MICx8) of-compounds **1**, **2** and **3** are presented in Figure 1.

Compound **1** exhibited fungistatic (< 99.9% decrease in the $\log_{10}$ of the number of CFU/ml compared with starting inoculum) activity against *C. albicans* and *M. canis* at all test concentrations. At concentrations ranging from MIC to MICx4, slight growth was observed for *C. albicans*, but the growth was less than that observed with the control. For *M. canis*, the number of CFU/ml remained constant or with a small decrease over time, and the greatest reduction was perceptible in MICx8 within 48 h. In contrast, compound **1** exhibited fungicidal (≥99.9% decrease in the $\log_{10}$ of the number of CFU/ml compared with starting inoculum) activity for the *T. mentagrophytes* isolate. However, this effect was concentration and time dependent. For this isolate, all test concentrations showed a fungicidal effect when evaluated at 96 h, and the MICx8 had the same effect in less time—48 h. In none of the test concentrations was there an increase in the number of CFU/ml, independent of time.

Compound **2** showed a similar inhibition profile to compound **1** for *C. albicans*, presenting a fungistatic effect in all test concentrations. However, growth of the *C. albicans* strain was also observed at MICx8 within 6 h. After this time, at this concentration, a stabilisation or slight decline in growth was observed. This exhibited a fungicidal effect for the two dermatophyte strains. Although the fungicidal activity varied among test isolates, it was observed that fungicidal capacity increased with increasing concentrations of compound **2** and exposure time to this compound. For *M. canis*, the number of CFU/ml remained constant or with a small decrease in MIC. At the concentrations equal to MICx2 and MICx4, a fungicidal effect was observed at 96 h. At MICx8, this effect was observed at 24 h and it remained so until 96 h. Regarding *T. mentagrophytes,* the fungicidal effect was only seen at 96 h for MICx4 and MICx8.

As with the other two compounds, compound **3** exhibited fungistatic activity for *C. albicans*. On the other hand, when analysing the results of this compound, a different effect was observed. Until 24 h, a better inhibitory effect was observed at the MIC followed by MICx2, MICx4 and MICx8, respectively. At 48 h, a better inhibitory effect was seen in MICx2 followed by MICx4, MIC and MICx8. In general, at all concentrations, cell growth was observed, but growth was less than in the control. This effect was not observed for the filamentous fungi tested in this study. At the concentrations equal to MIC and MICx2, a slight decrease in the number of CFU/ml was noted for *M. canis* and *T. mentagrophytes.* MICx4 demonstrated

fungicidal activity in 96 h for both dermatophytes; and MICx8 was fungicidal at 24 h and 12 h for *M. canis* and *T. mentagrophytes,* respectively.

The histopathological evaluation by microscopic analysis of the ear skin incubated with compounds **1**, **2** and **3** showed no lesions (Figure 2), demonstrating a low topical toxicity of the compounds. This allowed continuing the permeation studies in order to determine the site of accumulation of the compounds that showed a different profile. Compound **3** accumulated on the receptor compartment of the Franz cell, suggesting that there is permeation through the skin and muscle, while compounds **1** and **2** were retained in epithelial tissue. The relationship between irritation score (IS) and the logarithm of the concentrations of 8-hydroxyquinoline derivatives is represented by equation 1. Compounds **1**, **2** and **3** showed IS of 2.56, 4.53 and 2.69, respectively, showing a statistically significant difference when compared with the positive control (IS= 19.81). Compounds **1** and **3** were significantly less irritant than compound **2**, however, all 8-hydroxyquinoline derivatives tested were classified as nonirritant (NI) when tested according to this methodology.

Discussion

Susceptibility tests with 8-hydroxyquinoline derivatives were carried out with a wide variety of fungi, including yeast and filamentous fungi. Currently, among the major drugs used in the treatment of *Candida* spp. are azoles, echinocandins and amphotericin B.[25] Antifungals usually used were not effective in inhibiting some of these isolates in vitro. A total of 21 of the 52 *Candida* isolates were resistant or showed a lower susceptibility to more than one antifungal azole.[26] Furthermore, the CG 40039, CK 01 and CK 02 isolates presented resistance or showed a lower susceptibility to at least one azole antifungal and to anidulafungin. Terbinafine and griseofulvin are among the drugs commonly used for dermatophytosis treatment[27], and it was observed that among the 21 dermatophytes evaluated, 5 of them (MCA 36, MCA 40, MGY 58, TME16 and TME 34) showed a lower susceptibility to these two drugs and to ketoconazole as well.[28]

Microbiological resistance is a determining factor for the occurrence of success or failure in therapy, and in vitro tests are important for assessment.[4] The three 8-hydroxyquinoline derivatives show in vitro activity for all isolates tested.

These findings are very important, since some of these isolates have multiresistance to drugs used in therapy.

The mode of action of clioquinol and other 8-hydroxyquinoline derivatives on the fungal cells are not well understood. It is known that the group of 8-hydroxyquinoline inhibits certain enzymes related to DNA replication in viruses and 8-hydroxyquinoline inhibits RNA synthesis in yeast. However, studies on the mechanism of action of this group are scarce. Still, it is important to highlight that modifications in the molecule structure can provide additional mechanisms.[29,30]

The performance of the three compounds against dermatophytes indicated greater efficacy for compound **1**, followed by compound **2** and compound **3**. This same order of effectiveness of the compounds was also observed against yeasts. Compound **1** remained the most active and MIC values of all tested compounds were lower for *Candida* spp. than dermatophytes.

The literature has shown that the biological activity depends on the nature of the groups attached to the 5- and 7-positions of quinoline ring. Previous studies demonstrated that the presence of chlorine and bromine halogens in the 5- or 7-positions of substances, generally, was related to an increased antifungal activity, which was not observed with the fluorinated compounds. The presence of iodine in 7-position has also led to an improvement in the activity against some species.[16] This evidence corroborates the results found by our group, in which compound 1 (clioquinol) was the most active molecule. This finding may be related to the presence of iodine in the 7- position and chlorine in 5- position of its structure.[16] Although it was verified that the presence of halogens may be linked to the relevant activity observed, it is known that they can influence the increase in toxicity of compounds. Compound **2** presented antifungal activity even without the presence of halogen in its structure. The MIC values were similar or slightly lower than the values of compound **3**.

Gershon et al. evaluated a series of 8-hydroxyquinoline derivatives (including compounds **2** and **3**) containing a sulfonic acid against six fungal species: *Aspergillus niger, A. oryzae, Myrothecium verrucaria, Trichoderma viride, Mucor circinelloides* and *T. mentagrophytes.*[16] In contrast to results found in our study, these researchers found no antifungal activity for compound **2** against the species tested; however, tests with yeasts were not performed, and *M. canis*, *M. gypseum*

and *T. rubrum* were also not evaluated. Moreover, 100 µg/ml was the highest tested concentration of the compound and, as we have shown in this study, filamentous fungi were less susceptible. The advantage of compound **2** in relation to other 8-hydroxyquinoline derivatives tested is the absence of a halogenated substituent, which could decrease the toxicity of the molecule. Furthermore, the modification of the 8-hydroxyquinoline with sulfonic acids improves the water solubility, since this is a polar group, and this property is important for drugs. [31]

Antifungal activity of the 8-hydroxyquinoline derivatives against different genera was confirmed by a time-kill assay that is concentration and/or time dependent.[32] Compound **1** is a known antifungal and the literature describes the 8-hydroxyquinoline derivatives as fungistatic agents.[33,34] However, we were unable to find consistent information in the literature with appropriate methodologies demonstrating this effect. Although compound **1** has been used for many years, studies about the time-kill have not yet been performed. Therefore, it is extremely important to make a better assessment of the subject in order to have a greater pharmacodynamic understanding of the 8-hydroxyquinoline derivatives. In fact, our results showed a fungistatic effect for *C. albicans* and *M. canis*. A reduction greater than 50% of CFU/ml compared to the *C. albicans* starting inoculum was achieved in just 12 h at a concentration equal to MICx8. For *M. canis*, this reduction was achieved in 24 h at MICx8 and in 48 h at MICx4. However, contrary to the literature reports,[33,34] this compound was fungicidal for *T. mentagrophytes*. All tested concentrations had 100% reduction of CFU/ml in 96 h. The time-kill assays for filamentous fungi were evaluated until the maximum time of 48 h[22,35]. We opted to evaluate at 96 h because it is the incubation time in susceptibility tests. Surprisingly, this time gave us the best result for *T. mentagrophytes.* In addition, at MICx8, with only 6 h of contact, there was already >50% reduction of CFU/ml and also for 12 h at MICx4. At 24 h, all concentrations reached >50% reduction of CFU/ml compared to the starting inoculum for this fungus.

The fungicidal effect of compounds **2** and **3** has not yet been reported. With regard to dermatophytes, an exposure time of 96 h was also important for the effect of these two compounds. In 96 h, they had a fungicidal effect for dermatophytes at the highest concentrations and reduction > 50% in CFU/ml compared to the initial inoculum for the lowest concentrations. Since the time-kill

assay showed that the fungi are not immediately killed (Figure 1), the exposure time can play a significant role in the fungicidal action of these compounds.

Compound **2** had a fungistatic effect for *C. albicans* and the maximum reduction in CFU/ml compared to the initial inoculum was achieved immediately after exposure, reaching from 33.33% to 42.86% reduction, depending on the compound concentration.

The fungistatic effect for *C. albicans* was also observed for compound 3. Additionally, a paradoxical effect seems to be involved. We postulate that *C. albicans* showed higher growth in elevated concentrations (MICx8 and MICx4) when compared to the MIC; and the concentrations equal to MIC and MICx2, at time 0 h, were able to reduce more than 50% of CFU/ml. However, the paradoxical growth phenomenon is still only a supposition since it is necessary to test more isolates to confirm this effect. Some authors have already reported this effect with echinocandin. Some yeasts and filamentous fungi are able to grow in elevated caspofungin concentrations much higher than the MICs.[36] This phenomenon can be associated with the metabolic adaptation of microorganisms and activation of resistance mechanisms, or it can be related to aggregation of the drug at high concentrations.[2,37] However, the abilities of subpopulations to survive at high drug concentrations can be only an in vitro effect. Moreover, current data suggest that the clinical relevance of the paradoxical growth effect is not likely to be significant.[37]

It was noted that none of the compounds showed fungicidal activity on *C. albicans*, although the MIC values observed in broth microdilution tests were lower than for dermatophytes. The differences between the forms of preparation of fungal inoculum for the time-kill assay may have contributed to this. For dermatophytes, the starting inoculum used for time-kill tests was similar to that recommended for in vitro susceptibility determinations, approximately 1–3 x 10^3 CFU/ml.[22] Using the same starting inoculum facilitates comparisons between MIC and time-kill data by precluding potential discrepancies resulting from an inoculum effect. The inoculum of yeasts for the time-kill requires certain changes due to the limit of quantitation. The starting inoculum recommended for in vitro antifungal susceptibility testing (0.5–2.5 x 10^3 CFU/ml)[18] for *Candida* spp. is much lower than the inoculum recommendations for time-kill testing (1–5 x 10^5 CFU/ml) for the same species.[21]

Clinically, pharmacodynamic differences may influence the selection of optimal dose regimens for antifungal therapies. Although numerous factors may influence the in vivo activity of an agent, our data suggest that anti-dermatophyte activity of compounds **2** and **3** can be maximised by administering high doses. Agents in which the antifungal activity improves with increasing concentration (e.g., amphotericin B) may be optimised by the administration of prolonged therapeutic regimens. In contrast, the efficacy of tested compounds against *Candida* spp. does not improve with increasing concentration. This occurs because their fungistatic effects are concentration-independent. Fluconazole, one of the most used antifungals, acts in this way, requiring continuous administration of the drug.[38]

Fungicidal results for dermatophytes were a stimulus to investigate the *ex vivo* toxic effects of 8-hydroxyquinoline derivatives. The low toxicity in histopathological evaluation and the low level of irritability presented by compounds are interesting properties for the development of new drugs for the treatment of dermatophytosis. Besides these features, the compounds should also have low skin permeability because dermatophytes only reach the level of the epidermis and the action of antifungals should be local. The accumulation of compounds **1** and **2** in epithelial tissue shows that they could have local action, and also shows the possibility of their use in this type of infection. Compound **3** showed a high degree of permeation suggesting a systemic action.

When used topically, clioquinol can produce skin irritation, pruritis, erythema, swelling or pain.[39] However clioquinol is a rare sensitiser and has not been associated with the development of skin malignancies.[10] Lazarov et al. (2004) found that only 3 of 2156 patients in a contact dermatitis clinic were patch test positive to clioquinol 5%.[40] Similarly, Morris et al. (2002) found 8 of 1119 patients;[41] and Agner and Menne (1993) reported that 21 of 4556 patients were patch test positive to clioquinol.[42]

Katsarou et al. (1999) reported cases of both type I and type IV hypersensitivities to topical clioquinol. Of the 664 patients studied with suspected contact dermatitis, 13 had an immediate patch test reaction (wheal and flare) to clioquinol, while 6 had a delayed patch test reaction.[43] Despite the clioquinol be little sensitiser, modification of the chemical structure can result in cytotoxic activity. Thus, it is important to carry out toxicological studies using models such as HET-CAM,

which allow to discriminate various levels of toxicity by means of the IS calculation. The HET-CAM is a very sensitive test to determine the irritation effect associated with topical use. In this way, this methodology becomes an alternative to other in vivo tests.[24,44,45] The sulfonic acid derivatives showed an appropriate score confirming their potential.

Finally, 8-hydroxyquinoline derivatives showed antifungal activity of in vitro susceptibility testing. These compounds showed a fungicidal effect depending on the species, time and concentration. Compound 2 had an intermediate activity without permeating the skin. Moreover, it was nonirritant and did not possess dermatologic toxicity. Although this compound presents a high MIC, it is easily modified using classical reactions. Thus, it is possible to obtain a great structural diversity using this compound for antifungal drug design. Fungi susceptibility and toxicity against human cells could be optimised by appropriate structural modifications, providing selective antifungal agents that can become effective alternatives for the treatment of infections caused by resistant pathogens. However, more studies on pharmacokinetics, mechanism of action, and verification of in vivo toxicity are needed. Work in this direction is underway and the results will be reported in due course.

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Conflict of interest

No conflict of interest declared.

Tables

Table 1. Ranges of variation of minimum and maximum MIC values (MIC ranges), cell damage in the MIC (average ± standard deviation), MIC_{50} (MIC value that inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates) of 8-hydroxyquinoline derivatives [clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**)**,** and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**)] obtained for each species.

Species	**Compound 1**				**Compound 2**				**Compound 3**			
	MIC ranges	Cell Damage	MIC_{50}	MIC_{90}	MIC ranges	Cell Damage	MIC_{50}	MIC_{90}	MIC ranges	Cell Damage	MIC_{50}	MIC_{90}
C. albicans (n= 11)	0.063 – 0.25	96.9 ± 1.0	0.25	0.25	1 – 128	96.4 ± 1.1	64	128	4 – 128	96.1 ± 1.0	64	128
C. glabrata (n= 10)	0.031 – 0.125	93.6 ± 1.5	0.063	0.125	1 – 64	92.6 ± 1.2	8	32	2 – 128	95.4 ± 1.0	16	64
C. krusei (n= 9)	0.063 – 1	94.9 ± 1.5	0.25	0.25	2 – 128	96.6 ± 1.2	8	64	2 -128	96.4 ± 1.0	16	64
C. parapsilosis (n= 11)	0.125 – 0.5	96.1 ± 1.3	0.25	0.25	32 – 64	98.5 ± 0.5	64	64	32 – 128	97.1 ± 0.8	64	128
C. tropicalis (n= 11)	0.063 – 0.25	95.3 ± 0.9	0.125	0.125	16 – 128	93.6 ± 0.9	64	64	32 – 128	95.9 ± 0.9	64	128
M. canis (n= 6)	0.25 – 1	95.3 ± 0.9	0.25	0.25	128 – 256	95.5 ± 1.3	256	256	256 – 512	95.4 ± 1.1	256	512
M. gypseum (n= 5)	0.5 – 1	97.0 ± 0.7	0.5	1	256	96.7 ± 0.8	256	256	256 – 1024	94.9 ± 1.3	512	1024
T. mentagrophytes (n= 5)	0.25 – 2	95.1 ± 1.1	0.5	2	64 – 512	94.0 ± 1.0	256	512	128 – 1024	94.2 ± 1.4	256	1024
T. rubrum (n= 5)	0.125 – 1	95.8 ± 2.0	0.5	1	128 – 256	94,7 ± 1.6	128	256	128- 256	96.8 ± 0.6	256	256

n = the number of fungal isolates of each species;
MIC ranges, MIC_{50} and MIC_{90} are expressed in µg/ml and cell damage in %.

Figures

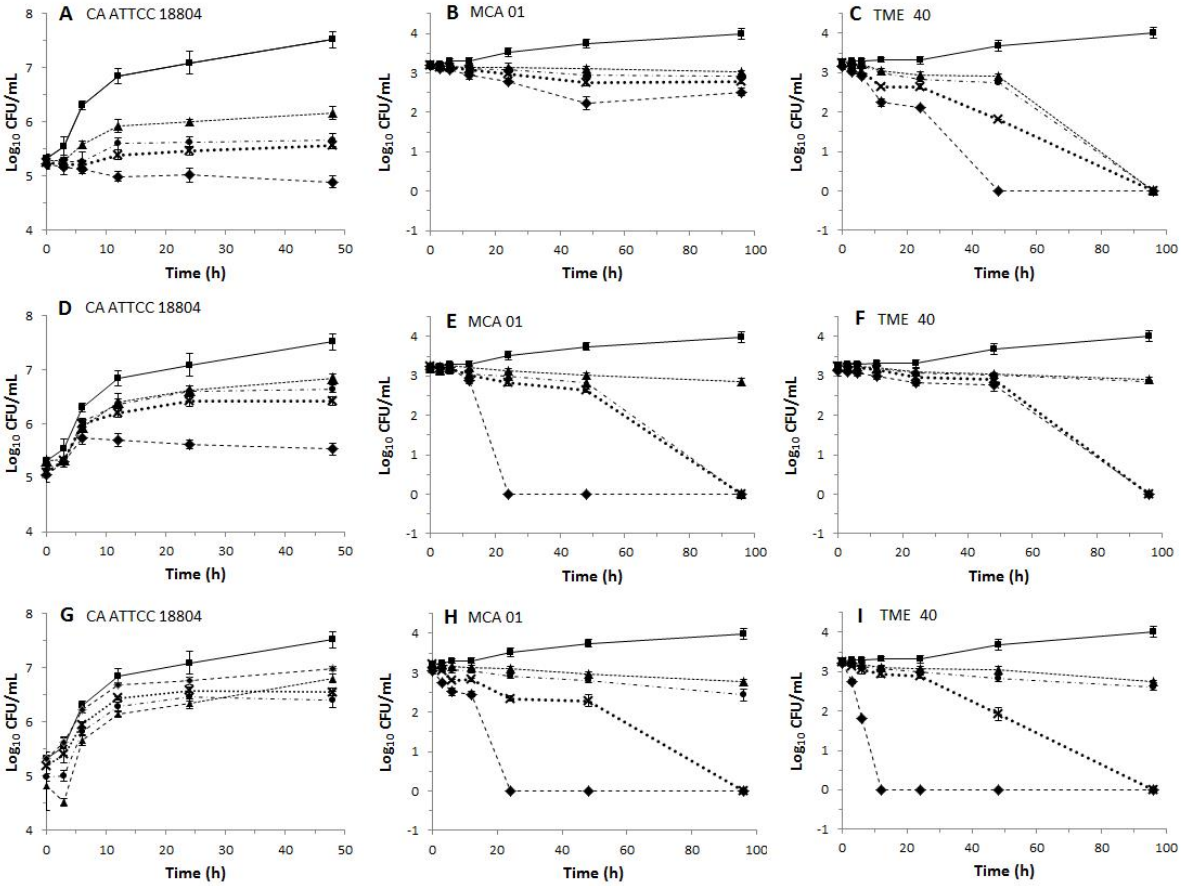

Figure 1. Log plots of killing kinetics of clioquinol (compound **1;** A, B and C)*****, 8-hydroxy-5-quinolinesulfonic acid (compound **2;** D, E and F)[#] and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3;** G, H and I)[+] against *C. albicans* (CA ATCC 18804), *M. canis* (MCA 01) and *T. mentagrophytes* (TME 40) isolates at the following concentrations: MIC (▲), MICx2 (●), MICx4 (**x**) MICx8 (♦) and control (■).

* clioquinol (compound **1**) MIC: 0.250 µg/ml (CA ATCC 18804), 0.250 µg/ml (MCA 01) and 0.500 µg/ml (TME 40);

[#] 8-hydroxy-5-quinolinesulfonic acid (compound **2**) MIC: 64 µg/ml (CA ATCC 18804), 128 µg/ml (MCA 01) and 64 µg/ml (TME 40);

[+] 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**) MIC: 32 µg/ml (CA ATCC 18804), 256 µg/ml (MCA 01) and 256 µg/ml (TME 40).

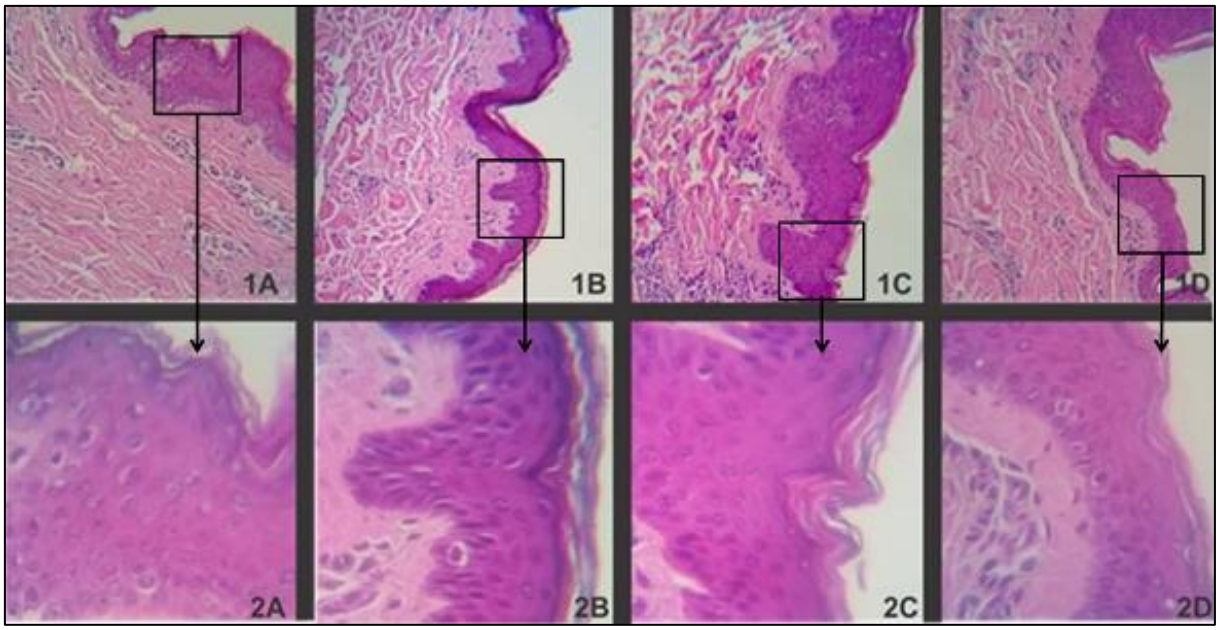

Figure 2. Histopathological evaluation of porcine cells treated with 8-hydroxyquinoline derivatives [clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**)**,** and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**)] and negative control at 100 and 400 times magnification. The concentration of each 8-hydroxyquinoline derivative used in the test was 1 mg/ml.
1A. Swine epidermal cells treated with PBS pH 7.0 at 100 times magnification;
1B. Swine epidermal cells treated with compound **1** at 100 times magnification;
1C. Swine epidermal cells treated with compound **2** acid at 100 times magnification;
1D. Swine epidermal cells treated with compound **3** at 100 times magnification;
2A. Swine epidermal cells treated with PBS pH 7.0 at 400 times magnification;
2B. Swine epidermal cells treated with compound **1** at 400 times magnification;
2C. Swine epidermal cells treated with compound **2** at 400 times magnification;
2D. Swine epidermal cells treated with compound **3** at 400 times magnification.

Supporting Information

Table S1. Minimum inhibitory concentration (MIC) and cell damage in the MIC of 8-hydroxyquinoline derivatives [clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**) and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**)] against *Candida* sp.

Species	Isolate	Compound 1		Compound 2		Compound 3	
		MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)
C. albicans (n=11)	CA ATCC18804*+	0.250	98.0 ± 0.3	64.0	99.1 ± 0.1	32.0	98.6 ± 0.2
	CA 01+	0.125	97.6 ± 1.6	16.0	98.6 ± 0.2	32.0	98.7 ± 0.2
	CA 04	0.125	98.9 ± 0.2	16.0	97.8 ± 1.4	32.0	98.1 ± 0.2
	CA DEB05	0.125	98.0 ± 0.4	128.0	98.7 ± 0.6	128.0	96.8 ± 2.0
	CA DEB06+	0.063	97.4 ± 4.1	1.0	89.3 ± 2.8	4.0	89.8 ± 0.8
	CA DEB09	0.125	92.6 ± 1.1	64.0	95.4 ± 1.2	64.0	94.5 ± 0.9
	CA Den02	0.500	98.0 ± 0.6	64.0	97.1 ± 0.6	64.0	98.1 ± 0.3
	CA Den05	0.250	94.3 ± 0.8	64.0	95.8 ± 2.5	64.0	96.7 ± 3.2
	CA Den06	0.250	95.4 ± 0.6	64.0	94.9 ± 0.8	64.0	91.8 ± 1.5
	CA Den09	0.250	98.0 ± 1.0	128.0	97.6 ± 1.1	128.0	98.0 ± 0.6
	CA Den10	0.250	97.3 ± 0.8	64.0	95.8 ± 0.4	64.0	95.5 ± 0.9
	M	**0.210**	**96.9 ± 1.0**	**61.2**	**96.4 ± 1.1**	**61.5**	**96.1 ± 1.0**
	Mdn	**0.250**	**97.6 ± 0.8**	**64.0**	**97.1 ± 0.8**	**64.0**	**96.8 ± 0.8**
C. glabrata (n=10)	CG 40039#	0.063	90.6 ± 3.9	8.0	97. 8 ± 0.6	16.0	97.3 ± 0.5
	CG 06	0.125	96.4 ± 0.6	16.0	92.7 ± 1.8	128.0	98.5 ± 0.2
	CG 09+	0.125	90.8 ± 0.7	32.0	95.5 ± 1.9	64.0	98.8 ± 0.2
	CG RL09m	0.063	91.7 ± 1.3	2.0	88.7 ± 1.3	4.0	91.0 ± 2.2
	CG RL12m+	0.031	97.8 ± 0.6	1.0	89.0 ± 2.0	16.0	95.0 ± 1.4
	CG RL22+	0.063	98.8 ± 0.4	64.0	98.6 ± 0.4	64.0	98.5 ± 0.1
	CG RL24	0.063	94.6 ± 4.9	16.0	93.3 ± 1.1	16.0	96.1 ± 1.4
	CG RL34m+	0.125	91.4 ± 0.8	1.0	91.2 ± 0.6	8.0	95.1 ± 0.7
	CG RL37m+	0.063	91.4 ± 1.1	32.0	91.4 ± 1.1	8.0	92.3 ± 2.6
	CG RL49	0.063	92.3 ± 0.7	2.0	93.3 ± 0.7	2.0	91.0 ± 0.8
	M	**0.078**	**93.6 ± 1.5**	**17.4**	**92.6 ± 1.2**	**32.6**	**95.4 ± 1.0**
	Mdn	**0.063**	**92.0 ± 0.8**	**12.0**	**92.7 ± 1.1**	**16.0**	**95.6 ± 0.8**
C. krusei (n=9)	CK ATCC6258+	0.250	97.4 ± 1.8	8.0	98.4 ± 0.5	16.0	97.7 ± 1.1
	CK 01+,#	0.125	97.0 ± 0.4	8.0	97.3 ± 1.2	16.0	97.2 ± 0.4
	CK 02+,#	0.250	98.8 ± 0.5	64.0	98.9 ± 0.3	64.0	98.0 ± 0.5
	CK 03+	0.125	93.1 ± 5.3	8.0	94.9 ± 0.7	16.0	92.1 ± 3.3
	CK CVB42	0.063	93.0 ± 0.4	64.0	96.2 ± 1.3	64.0	97.2 ± 1.2
	CK CVB44	0.125	95.2 ± 1.9	64.0	97.2 ± 0.7	64.0	98.4 ± 0.4
	CK Den33	0.250	94.6 ± 1.5	2.0	91.7 ± 4.2	2.0	90.5 ± 0.7
	CK Den43	1.000	85.3 ± 0.9	128.0	95.9 ± 1.6	128.0	97.2 ± 1.1
	CK RL52+	0.250	98.4 ± 0.4	8.0	98.5 ± 0.6	16.0	98.9 ± 0.1
	M	**0.271**	**94.8 ± 1.5**	**39.3**	**96.6 ± 1.2**	**42.9**	**96.4 ± 1.0**

Species	Isolate	Compound 1		Compound 2		Compound 3	
		MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)
	Mdn	**0.250**	**95.2 ± 0.9**	**8.0**	**97.2 ± 0.7**	**16.0**	**97.2 ± 0.7**
C. parapsilosis (n=11)	CP ATCC22019	0.250	99.0 ± 0.1	64.0	98.5 ± 0.3	128.0	97.6 ± 0.4
	CP RL01m	0.250	98.1 ± 0.4	64.0	99.1 ± 0.2	64.0	95.1 ± 1.8
	CPR RL05	0.125	96.6 ± 3.9	64.0	98.7 ± 0.5	64.0	98.5 ± 0.3
	CP RL11m	0.125	94.5 ± 1.3	32.0	98.6 ± 0.6	64.0	98.5 ± 0.1
	CP RL13m	0.125	95.8 ± 0.2	32.0	98.1 ± 0.7	32.0	98.7 ± 0.3
	CP RL20	0.250	97.8 ± 1.2	32.0	98.4 ± 0.3	64.0	94.7 ± 1.2
	CP RL27m^{+}	0.250	98.9 ± 1.4	32.0	98.9 ± 0.1	64.0	99.3 ± 0.1
	CP RL32	0.500	88.3 ± 2.1	64.0	97.6 ± 0.5	64.0	96.3 ± 0.4
	CP RL33	0.125	95.6 ± 2.3	64.0	98.7 ± 0.9	64.0	95.9 ± 1.9
	CP RL38^{+}	0.250	97.4 ± 0.2	64.0	98.6 ± 0.6	64.0	95.7 ± 1.3
	CP RL47^{+}	0.250	95.2 ± 0.9	64.0	98.3 ± 0.3	128.0	98.3 ± 0.6
	M	**0.227**	**96.1 ± 1.3**	**52.4**	**98.5 ± 0.5**	**72.7**	**97.1 ± 0.8**
	Mdn	**0.250**	**96.6 ± 1.2**	**64.0**	**98.6 ± 0.5**	**64.0**	**97.6 ± 0.4**
C. tropicalis (n=11)	CT ATCC750	0.125	96.0 ± 0.4	64.0	95.7 ± 0.9	128.0	96.8 ± 0.9
	CT 06	0.125	96.1 ± 0.8	16.0	97.1 ± 1.4	32.0	96.1 ± 1.9
	CT 56^{+}	0.125	95.8 ± 0.4	128.0	95.3 ± 0.3	128.0	94.2 ± 0.6
	CT 57A	0.063	94.3 ± 1.1	32.0	95.1 ± 0.7	32.0	98.7 ± 0.2
	CT 72A^{+}	0.250	98.9 ± 0.4	32.0	98.5 ± 0.3	64.0	93.8 ± 0.9
	CT 72P^{+}	0.125	92.3 ± 1.6	64.0	95.0 ± 0.5	128.0	96.7 ± 1.1
	CT 94P^{+}	0.125	94.7 ± 0.6	64.0	95.7 ± 0.3	64.0	95.8 ± 1.0
	CT 102A^{+}	0.125	94.7 ± 1.9	64.0	96.5 ± 0.4	128.0	96.5 ± 0.2
	CR RL06	0.125	92.9 ± 0.5	64.0	95.9 ± 2.2	64.0	95.3 ± 3.0
	CT RL10	0.125	97.3 ± 2.3	64.0	98.3 ± 0.2	64.0	98.0 ± 0.2
	CT RL14	0.125	95.4 ± 0.3	64.0	95.8 ± 2.5	64.0	93.3 ± 0.3
	M	**0.131**	**95.3 ± 0.9**	**59.6**	**96.3 ± 0.9**	**81.5**	**95.9 ± 0.9**
	Mdn	**0.125**	**95.4 ± 0.6**	**64.0**	**95.8 ± 0.5**	**64.0**	**96.1 ± 0.9**

Geometric mean (M) and median (Mdn) of MIC and cell damage are expressed below each species; n = the number of fungal isolates of *Candida albicans* (CA), *Candida glabrata* (CG), *Candida krusei* (CK), *Candida parapsilosis* (CP) and *Candida tropicalis* (CT);
* Isolates used in the study of time kill.
$^{+}$Resistant isolates to more than one azole antifungal (fluconazole, itraconazole, miconazole, ketoconazole, voriconazole).
$^{\#}$Resistant isolates to azole antifungal and anidulafungin.

Table S2. Minimum inhibitory concentration (MIC) and cell damage in the MIC of 8-hydroxyquinoline derivatives [clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**) and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**)] against dermatophytes.

Species	Isolate	Compound 1		Compound 2		Compound 3	
		MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)	MIC (µg/ml)	Cell Damage (%)
M. canis (n=6)	MCA 01*	0.250	96.0 ± 1.1	128.0	93.1 ± 0.5	256.0	96.2 ± 0.5
	MCA 29	0.250	95.5 ± 1.3	128.0	96.5 ± 1.0	256.0	97.6 ± 1.1
	MCA 32	0.250	96.4 ± 0.5	256.0	92.6 ± 1.3	512.0	92.8 ± 1.5
	MCA 36#	1.000	95.8 ± 0.9	256.0	96.7 ± 0.5	512.0	93.4 ± 1.3
	MCA 38	0.250	93.1 ± 0.3	256.0	96.5 ± 2.1	256.0	96.2 ± 1.1
	MCA 40#	1.000	95.2 ± 1.0	256.0	97.4 ± 2.5	256.0	96.3 ± 1.3
	M	**0.500**	**95.3 ± 0.9**	**213.3**	**95.5 ± 1.3**	**341.3**	**95.4 ± 1.1**
	Mdn	**0.250**	**95.7 ± 1.0**	**256.0**	**96.5 ± 1.2**	**256.0**	**96.2 ± 1.2**
M. gypseum (n=5)	MGY 01	0.500	99.2 ± 0.2	256.0	98.0 ± 0.2	512.0	94.8 ± 1.1
	MGY 33	0.500	98.7 ± 1.0	256.0	97.0 ± 1.1	512.0	95.9 ± 1.9
	MGY 42	0.500	98.3 ± 1.0	256.0	97.8 ± 0.2	256.0	95.0 ± 0.8
	MGY 50	0.500	96.7 ± 0.7	256.0	95.9 ± 1.1	256.0	93.8 ± 1.7
	MGY 58#	1.000	92.3 ± 0.5	256.0	94.8 ± 1.2	1024.0	95.1 ± 1.0
	M	**0.600**	**97.0 ± 0.7**	**256.0**	**96.7 ± 0.8**	**512.0**	**94.9 ± 1.3**
	Md	**0.500**	**98.3 ± 0.7**	**256.0**	**97.0 ± 1.1**	**512.0**	**95.0 ± 1.1**
T. mentagrophytes (n=5)	TME 16#	0.250	90.4 ± 0.2	64.0	91.7 ± 0.8	128.0	95.3 ± 1.5
	TME 32	0.500	97.8 ± 0.3	256.0	92.6 ± 0.8	256.0	93.3 ± 0.4
	TME 33	1.000	96.5 ± 0.3	256.0	95.7 ± 0.5	512.0	94.2 ± 1.1
	TME 34#	2.000	91.2 ± 3.6	512.0	93.1 ± 0.9	1024.0	92.4 ± 2.4
	TME 40*	0.500	99.36 ± 1.3	64.0	96.8 ± 1.8	256.0	95.9 ± 1.4
	M	**0.850**	**95.1 ± 1.1**	**230.4**	**94.0 ± 1.0**	**435.2**	**94.2 ± 1.4**
	Mdn	**0.500**	**96.5 ± 0.3**	**256.0**	**93.1 ± 0.8**	**256.0**	**94.2 ± 1.4**
T. rubrum (n=5)	TRU 43	0.125	95.1 ± 1.9	128.0	98.6 ± 0.6	128.0	98.3 ± 0.6
	TRU 45	1.000	97.5 ± 0.9	128.0	94.4 ± 2.1	256.0	97.0 ± 0.5
	TRU 47	1.000	95.2 ± 2.8	256.0	92.2 ± 1.8	256.0	91.2 ± 1.0
	TRU 50	0.250	93.6 ± 3.8	128.0	94.3 ± 1.9	128.0	98.5 ± 0.4
	TRU 51	0.500	97.6 ± 0.4	128.0	94.0 ± 1.6	256.0	99.0 ± 0.25
	M	**0.575**	**95.8 ± 2.0**	**153.6**	**94.7 ± 1.6**	**204.8**	**96.8 ± 0.6**
	Mdn	**0.500**	**95.2 ± 1.9**	**128.0**	**94.3 ± 1.8**	**256.0**	**98.3 ± 0.5**

Geometric mean (M) and median (Mdn) of MIC and cell damage are expressed below each species; n = the number of fungal isolates of *Microsporum canis* (MCA), *Microsporum gypseum* (MGY), *Trichophyton mentagrophytes* (TME) and *Trichophyton rubrum* (TRU).
* Isolates used in the study of time kill.
#Resistant isolates to ketoconazole, griseofulvin and terbinafine.

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5.2 Artigo 2

O artigo intitulado “New insights into the mechanism of antifungal action of 8-hydroxyquinolines” foi submetido no “Saudi Pharmaceutical Journal”.

Title: New insights into the mechanism of antifungal action of 8-hydroxyquinolines

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Abstract

The 8-hydroxyquinoline core is a privileged scaffold for drug design explored to afford novel derivatives endowed with biological activity. Our research aimed at clarifying the antifungal mechanism of action of clioquinol, 8-hydroxy-5-quinolinesulfonic acid, and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (three 8-hydroxyquinoline derivatives). The antifungal mode of action of these compounds on *Candida* spp. and dermatophytes was investigated using sorbitol protection assay, cellular leakage effect, ergosterol binding assay, and scanning electron microscopy. Clioquinol damaged the cell wall and inhibited the formation of pseudohyphae by *C. albicans*. The 8-hydroxy-5-quinolinesulfonic acid derivatives compromised the functional integrity of cytoplasmic membranes. To date no similar report was found about the antifungal mechanism of 8-hydroxyquinolines. These results, combined with the broad antifungal spectrum already demonstrated previously, reinforce the potential of 8-hydroxyquinolines for the development of new drugs.

Keywords: clioquinol, 8-hydroxyquinoline derivatives, mechanism of action, *Candida* spp., dermatophytes.

1. Introduction

The discovery of new antifungal agents is modest when compared to antibacterial drugs. Similarities between fungi and mammals eukaryotic cells limit their development. Besides, the traditionally smaller number of fungal compared to bacterial infections also slowed the discovery of antifungals (Vandeputte et al., 2012; Scorzoni et al., 2017). However, in recent decades, a dramatic increase of fungal infections has been noticed and diseases related to them are now considered a global public health problem (Vandeputte et al., 2012).

Fungi are significantly affecting the growing population of patients with weakened immune system and the systemic fungal infections are increasingly important causes of high morbidity and mortality. Additionally to life-threatening systemic infection, such as those caused by *Candida* spp., superficial and cutaneous mycoses can persist requiring continuous treatment (Scorzoni et al., 2017). In addition, resistance to antifungal drugs has become an important problem and further intensifies the need for new treatment strategies (Lukaszuk et al., 2017).

It is clear that the discovery of new antimicrobials is essential nevertheless more detailed research of molecules with known antifungal activity and structural modifications to improve the action seem to be faster alternatives to this end (Ngo et al., 2016). 8-Hydroxyquinoline derivatives are a subclass of quinolines with a wide variety of biological effects. The 8-hydroxyquinoline derivatives emerged as a privileged scaffold for drug design being widely explored for several biological functions such as: neuroprotection, anticancer, anti-HIV, and antifungal effects (Oliveri and Vecchio, 2016).

The most known member of this family is clioquinol (5-chloro-7-iodo-8-hidroxyquinoline, **1**) (Oliveri and Vecchio, 2016). Clioquinol was marketed in 1934 by Ciba-Geigy (now Novartis) as an antimicrobial agent for the treatment of a wide range of intestinal diseases. In the 1970s, it was withdrawn from the market as an oral agent due to association with a neurodegenerative syndrome known as subacute myelooptic neuropathy (SMON) (Mao and Schimmer, 2008; Nakae, 1973; Oliveri and Vecchio, 2016). Clioquinol is currently available as a topical formulation for the treatment of skin infections(Mao and Schimmer, 2008), and in the 2000s the interest in this drug reappeared as a potential treatment of non-infectious pathologies such as: Alzheimer’s, Parkinson’s and Huntington’s disease (Finkelstein et al., 2016;

Franklin et al. 2016; Huntington Study Group Reach2HD Investigators, 2015; Zhang et al., 2013).

Although the antifungal activity of clioquinol is well known and presents rare reports of resistance (Alsterholm et al., 2010), its mechanism of action into fungal cells are poorly understood. Some groups have proposed 8-hydroxyquinoline derivatives as new antifungals but without mentioning how these compounds act on fungal cells (Gershon et al. 2001; Pippi et al., 2017).

Thus, herein it is presented some insights into the mechanism of action of clioquinol (compound **1**) and two other 8-hydroxyquinoline derivatives: 8-hydroxy-5-quinolinesulfonic acid (compound **2**) and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**) (Figure 1). In vitro methods were employed to analyze alterations of fungal morphology, cell wall damage, cellular leakage, and ergosterol complexation. Since modifying and/or repurposing current treatments represent valid ways of seeking new antifungal agents, our research aimed to gain information about the mode of action in order to rationalize the design of new antifungal derivatives.

2. Material and methods

2.1. Fungal Strains

A total of 27 isolates were included in this study: three strains of *Candida albicans* (ATCC 18804, CA 01, CA DEB 05), *C. glabrata* (CG RL12m, CG RL24, CG RL49), *C. krusei* (CK 03, CK CVB 42, CK Den 43), *C. parapsilosis* (CP RL11m, CP RL27m, CP RL 38), *C. tropicalis* (ATCC 750, CT 57A, CT 72A), *Microsporum canis* (MCA 01, MCA 29, MCA 40), *M. gypseum* (MGY 42, MGY 50, MGY 58), *Trichophytum mentagrophytes* (TME 16, TME 32, TME 40) and *T. rubrum* (TRU 43, TRU 50, TRU 51). All isolates are deposited into the Mycology Collection of Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil) and were obtained from the National Program of Quality Control of Brazil and clinical samples. Standard strains of *C. albicans* (ATCC 18804) and *C. tropicalis* (ATCC 750) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and were included as controls.

2.2. Antifungal Compounds

Clioquinol (**1**), 8-hydroxy-5-quinolinesulfonic acid (**2**), and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (**3**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were dissolved in DMSO (Sigma-Aldrich) and diluted in a medium assay to obtain a maximum concentration of 2% DMSO in the experiments.

2.3. Sorbitol Protection Assay

The effect of the 8-hydroxyquinoline derivatives on the integrity of the fungal cell wall was evaluated by sorbitol protection assay. Minimum inhibitory concentrations (MICs) of 8-hydroxyquinoline derivatives **1-3** were determined by the standard broth microdilution (CLSI M27-A3 for *Candida* spp.; CLSI M38-A2 for dermatophytes) (CLSI, 2008) in the absence and presence of 0.8 M sorbitol (Sigma-Aldrich) added to the RPMI 1640 growth medium (Gibco) as an osmoprotectant. Microplates were incubated at 35 °C and 30 °C for *Candida* spp. and dermatophytes, respectively. Experiments were carried out in duplicate for all three strains of each species. Anidulafungin (Pfizer, New York, USA) was used as positive control. MICs were measured after 2 and 7 days for *Candida* spp.; and after 4 and 7 days for dermatophytes (Escalante et al., 2008).

2.4. Cellular leakage effect

The cell leakage was assessed by measuring 260-nm-absorbing materials released to the medium, primarily representing nucleotides of which uracil-containing compounds exhibited the strongest absorbance (Lunde and Kubo, 2000). The measurement of cellular leakage was carried out with one representative strain of each species studied (*C. albicans* ATCC 18804, *C. glabrata* CG RL24, *C. krusei* CK Den 43, *C. parapsilosis* CP RL38, *C. tropicalis* ATCC 750, *M. canis* MCA 01, *M. gypseum* MGY 50, *T. mentagrophytes* TME 40 and *T. rubrum* TRU 43) and the procedures were conducted as previously described with some modifications (Escalante et al., 2008; Lunde and Kubo, 2000). *Candida* spp. were cultured on SDA (Sabouraud Dextrose Agar; HiMedia; India) at 35 °C for 48 h. After incubation, the cells were washed three times (3000 rpm for 10 min, Spinlab SL-5M, Ribeirão Preto, Brazil), suspended and adjusted to approximately 1-5x10^6 cells/ml with cold 0.16 M MOPS buffered at pH 7. A 1:10 dilution of fungal suspension was made in cold MOPS buffer with the desired amount of 8-hydroxyquinoline derivatives 1-3 in order

to obtain the final concentration equal to MIC. This dilution resulted in a suspension with fungal inoculum of 0.1-0.5 x 10^6 cells/ml. For dermatophytes, a suspension of each fungus was prepared after 10 days at 30 °C on PDA (potato dextrose agar; HiMedia; India) and adjusted to 1–3 x 10^3 cells/ml with cold MOPS buffer and compounds **1-3**. Untreated cells incubated with MOPS buffer were prepared as negative control and cells treated with amphotericin B (6.25 µg/ml) and SDS (2%) (Sodium dodecyl sulfate; Neon, São Paulo, Brazil) were used as positive control. Solutions and controls were tested in triplicate. Incubation was performed at 35 °C (*Candida* spp.) or 30 °C (dermatophytes). Aliquots were taken at time intervals (6, 24 and 48 h for *Candida* spp.*;* 6, 24, 48 and 96 h for dermatophytes) and centrifuged at 10000 rpm for 10 min (Biosystems MCD2000, Curitiba, Brazil). The absorbance of supernatants was analyzed at 260 nm in a spectrophotometer (Agilent Technologies 8453, Santa Clara, EUA). Solutions of SDS (2%), amphotericin B (6.25 µg/ml), and 8-hydroxyquinoline derivatives **1-3** (MIC) without fungal inoculum were used as blank for absorbance readings. The results were expressed as means ± standard deviation. Absorption obtained for derivatives **1-3**, SDS and amphotericin B were compared to the absorption of the untreated control and analyzed by one-Way ANOVA as well as Dunnett's multiple comparisons test. $P \leq 0.05$ was considered statistically significant.

2.5. Ergosterol binding assay

The ability of the 8-hydroxyquinoline derivatives to complex with ergosterol in the fungal membrane was evaluated by ergosterol binding assay. The MICs of 8-hydroxyquinoline derivatives **1-3** were determined by the standard broth microdilution (CLSI M27-A3, 2008.; CLSI M38-A2, 2008) in the absence and presence of different concentrations (50-250 µg/ml) of external ergosterol (Sigma-Aldrich, St. Louis, MO, USA) added to the RPMI 1640 growth medium. The plates were incubated at 35 °C and 30 °C for *Candida* spp. and dermatophytes, respectively. Experiments were carried out in triplicate and tested for all three strains of each species. Amphotericin B (União Química, São Paulo, Brazil) was used as positive control. MICs were measured after 48 h of incubation for *Candida* spp. and 96 h for dermatophytes (Escalanete et al., 2008).

2.6. Scanning electron microscopy (SEM)

Morphological changes in *C. albicans* ATCC 18804, *T. mentagrophytes* TME 40 and *M. canis* MCA 01 grown in the presence of 8-hydroxyquinoline derivatives **1-3** were determined using the macro broth dilution method (CLSI M27-A3; CLSI M38-A2).[15,16] Treated and untreated (control) cells were analyzed by SEM. After the incubation period (*C. albicans*: 48 h at 35 °C; *M. canis* and *T. mentagrophytes*: 96 h at 30 °C), fungal cells treated with the derivatives **1-3** (MIC/2) and untreated cells were washed three times with PBS (3000 rpm for 5 min. Biosystems MCD2000, Curitiba, Brazil). After washing, cells were fixed in 1 ml of modified Karnovsky's fixative adapted from Joubert et al. (2015). Then, the wells were washed three times (3000 rpm for 5 min) in 0.1 M sodium cacodylate buffered at pH 7.2 containing 0.2 M sucrose, and 2 mM $MgCl_2$ with the aid of two pipettes, which were used for addition and concurrent removal to avoid air exposure. Cells were adhered in coverslips previously functionalized with poly-L-lysine for 1 h. Adhered cells were dehydrated in a series of freshly made solutions of graded acetone 30, 50, 70, 95 (5 min) and 100% (10 min). Samples were then subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10 Carl, Oberkochen, Germany) operating at 10kV.

3. Results

3.1. Sorbitol Protection Assay

When *Candida* spp. were treated with 8-hydroxyquinoline derivatives **1-3** in a medium supplemented with sorbitol, MICs increased after seven days of incubation compared to MIC in medium without sorbitol. MICs of clioquinol **1** increased for all dermatophytes tested. MICs to the compound **2** also increased for dermatophytes, except for *T. rubrum*. Compound **3** had the MIC increased for *M. canis* and *M. gypseum* isolates. However, the MICs for *T. rubrum* and *T. mentagrophytes* remained constant. After addition of sorbitol into the medium, the MIC values of anidulafungin (positive control) increased 2 to 128-fold, 2 to 16-fold for clioquinol **1**, and until 8 and 4-fold for the compounds **2** and **3**, respectively (Tables S1 and S2).

3.2. Cellular leakage effect

It was noted that clioquinol **1** did not cause nucleic acid leakage from fungal cells, while the compounds **2** and **3** occasioned it to all *Candida* spp. (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*) and dermatophytes (*M. canis*, *M. gypseum*, *T. mentagrophytes* and *T. rubrum*).

Significant ($P \leq 0.05$) release of cellular material was observed for compounds **2** and **3**, as well as positive controls (SDS and amphotericin B). All results were compared to untreated control. On the other hand, clioquinol **1** was not statistically different from the untreated control. Figure 2 shows leakage from cells over 48h (*Candida albicans*; figure 2A) and 96 h (*M. canis*, figure 2B; *T. mentagrophytes*; figure 2C) period due to 8-hydroxyquinoline derivatives **1-3**, SDS, amphotericin B, and untreated control. A representative isolate of each genus studied was selected to compose figure 2, but all other isolates had the same effect (data not shown).

3.3. Ergosterol binding assay

MICs of 8-hydroxyquinoline derivatives **1-3** against *Candida* spp. (Table S3) and dermatophytes (Table S4) did not increase after adding different concentrations of ergosterol. As expected, a 16 to 128-fold increase of MIC values was observed for amphotericin B (positive control). All isolates tested behaved similarly.

3.4. Scanning electron microscopy (SEM)

SEM images of *C. albicans* (ATCC 18804) depict important morphological changes (Figure 3) in the presence of the 8-hydroxyquinoline derivatives **1-3**. Untreated cells present its natural smooth oval-shape and polar bud scars bearing blastoconidia as well as pseudohyphae. Addition of clioquinol **1** shrinks the cell surface modifying its format to circular. Cell clusters and blastoconidia are still observed, although without pseudohyphae. *C. albicans* grown in the presence of compound **2** revealed the shrinkage of cell and the presence of pseudohyphae but with less blastoconidia and a few chlamydospores. Similarly, cells treated with compound **3** demonstrate pseudohyphae with less blastoconidia. Larger round-cells and non-polar bud scars are widely noted.

Both *M. canis* MCA 01 (Figure 4) and *T. mentagrophytes* TME 40 (Figure 5) incubated with 8-hydroxyquinoline derivatives **1-3** show irregular, rough and groove cell wall. Some hyphal cells of *M. canis* treated with compound **3** exhibited extensive degeneration. The fungal wall displays notable pitting and tears with apparent extravasation of the intracellular material.

4. Discussion

Based on the results of the sorbitol protection assay, we observed that the 8-hydroxyquinoline derivatives **1-3** could target the fungal cell wall. After adding sorbitol, increased MICs were observed, indicating some effect of the compounds **1-3** on the fungal cell wall. This effect was species dependent. *Candida* spp. and dermatophytes exhibit damage to the wall when treated with clioquinol **1**. In the same way, compound **2** showed action on the wall, except for *T. rubrum* cells. Compound **3** also damages the cell wall, although *T. rubrum* and *T. mentagrophytes* appear not to suffer this action.

Further additional level of evidence on the mode of action was assessed by the measurement of cellular leakage for detection of cell membrane as a possible target site. Data obtained by measuring these UV-absorbing materials revealed that the sulfonic quinolines (compounds **2** and **3**) enhanced the cell permeability of *Candida* spp. and dermatophytes (Figure 2). The high absorbance observed suggests that nucleic acids were lost through cytoplasmic membrane irreversible damage (Khan et al., 2013). This effect was also observed for amphotericin B and SDS (positive controls) which are membrane disruptive-agents that affect cell permeability and show lethal action against fungal cells. These observations indicate that the fungal membrane is a possible site of action for 8-hydroxyquinoline-sulfonic acids. In contrast, small values of absorbance were read when fungi were treated with clioquinol **1** indicating that cells were not lysed. Therefore, clioquinol **1** does not appear to target the fungal-cell membrane.

Due to the acidity of sulfonic acids, 8-quinolinol sulfonic acids **2** and **3** are deprotonated at the culture medium (pH = 7). Thus, active species of these compounds present ionic characters which penetrate the fungal cells in the same manner (Gershon et al, 2001). Clioquinol **1** does not bear a sulfonic acid group, still it presents a phenolic proton with pKa around 7.4 which can be removed at the pH of

the culture medium. However, less than half of the clioquinol molecules also will be at the ionic form (Völgyi et al., 2007).

Antifungal agents exhibiting membrane-damaging effects can target ergosterol which is a structural component of the membrane, forming transmembrane pores that lead to alterations on the membrane permeability with loss of intracellular content and consequent fungal membrane disruption. If the activity of the antifungal agent is a consequence of binding to ergosterol, external ergosterol would prevent the binding, consequently, the MIC of the antifungal agent would increase (Escalante et al, 2008). Amphotericin B is a well-known membrane-disruptive agent lethal to fungi. This drug complex with membrane-ergosterol forming stable pores into the membrane allowing permeability of ions such as potassium and proton (Flevari et al., 2013; Lunde and Kubo, 2000; Scorzoni et al., 2017). Thus, the possible binding of 8-hydroxyquinoline derivatives **1-3** to ergosterol was verified. However, unchanged MICs for derivatives **1-3** in the presence of exogenous ergosterol suggested that they do not act by binding to the membrane ergosterol. Therefore, the destabilization of the membrane that occurs by the action of compound **2** and **3** is not by direct binding to the plasma membrane. Thus, other targets as enzyme of the ergosterol biosynthesis pathway may be involved.

Next, morphological changes in fungal cells after treatment with 8-hydroxyquinoline derivatives **1-3** were evaluated by SEM. Fungal cells were subjected to sub-minimal inhibitory concentration (sub-MIC) of **1-3** since malformations were previously detected at this dilution (Escalante et al., 2008).

The demonstration that clioquinol **1** inhibits pseudohyphae formation of *C. albicans* (Figure 3 – B1 and B2) is an important finding. Studies suggest that pseudohyphae are an intermediate state between yeast cells and hyphae (Lu et al., 2014). The ability to switch between yeast, pseudohyphal, and hyphal growth forms is one of the most investigated virulence factors of *C. albicans* (Albertin and Marullo, 2012; Whiteway and Bachewich, 2007). The morphological transition from yeast to filamentous form represents an essential aspect of the pathogen's biology and is linked to important properties for interaction with the host, such as: adhesion to epithelial and endothelial cells, escape from phagocytes and immune evasion. The capacity of hyphal growth is believed to be essential for pathogenicity at both superficial and systemic levels (Lu et al., 2014). Although the commercially available

antifungals have the plasma membrane and the cell wall as main targets (Ngo et al., 2016), recent studies have focused on the inhibition of virulence factors to develop new therapies (Lu et al., 2014; Scorzoni et al., 2017).

The cell envelope of *Candida* sp. appeared to be damaged by the 8-hydroxyquinoline derivatives **1-3** as evidenced by the shrinkage of the cell surfaces. Chlamydospores that were present when *Candida* cells were treated with compound **2** are often formed when the fungus is under extreme growth conditions (Figure 3 – C1). Indeed, the formation of chlamydospore is associated with the microorganism mechanism of defense under adverse conditions (Whiteway and Bachewich, 2007). Numerous non-polar bud scars noted on cells treated with compound **3** (Figure 3 – D2) suggest that the normal yeast division process may have been affected resulting in single cells with multiple attempts to divide but not increasing the viable number of cells (Khan et al., 2013). The presence of the large round cells (Figure 3 – D1 and D2) reinforce this hypothesis and can indicate variation in their genome size. The endopolyploidy phenomenon (somatic polyploidy) can occur in *C. albicans*, through the fusion of their diploid cells resulting in tetraploids cells. Endopolyploidy arises by recurrent cycles of DNA replication without cellular division via either endoreduplication or endomitosis processes. Environmental factors, such as antifungal treatments, have been shown to induce variation of genome content and chromosomal complement in various fungal species (Albertin and Marullo, 2012). In addition, the presence of the large round cells could also indicate change in cell permeability

Similarly, irregular and rough dermatophytes cell walls are seen after treatment with 8-hydroxyquinoline derivatives **1-3** (Figure 4 and Figure 5). These modifications may also be a consequence of membrane permeability. The damage caused by compound **3** on the *M. canis* wall was more clearly identified being pitting and tears easily visualized (Figure 4 - D2).

5. Conclusion

Clioquinol **1** inhibits the formation of pseudohyphae in *C. albicans* cells and appears to act more effectively on the cell wall than 8-hydroxyquinolines derivatives containing sulfonic acid. On the other hand, 8-hydroxyquinoline-sulfonic acids **2** and **3** compromised the functional integrity of cytoplasmic membranes

suggesting that their antifungal mechanism occurs mainly due to membrane damage. This demonstrates that by changing the substitution pattern of the nucleus of 8-hydroxyquinoline it is possible to obtain different mechanisms for antifungal activity. Rational drug design can accelerate the development of new antifungal agents by optimizing activity through appropriate structural modifications. 8-Hydroxyquinolines are strong candidates for this purpose. This study is very important because without adequate identification of the mechanism of action we cannot design new drugs effectively. In addition, we emphasize that to date no similar report was found about the antifungal mechanism of 8-hydroxyquinolines. However, additional studies including a larger number of isolates should be performed in order to confirm the data found here. These results, combined with the broad antifungal spectrum already demonstrated previously by us, reinforce the potential of this class of substances to develop antimicrobial drugs.

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Conflict of interest

No conflict of interest declared.

Figures

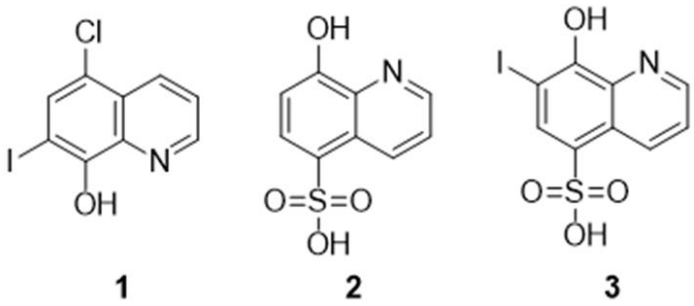

Figure 1. Chemical structures of 8- hidroxyquinoline derivatives **1-3**.

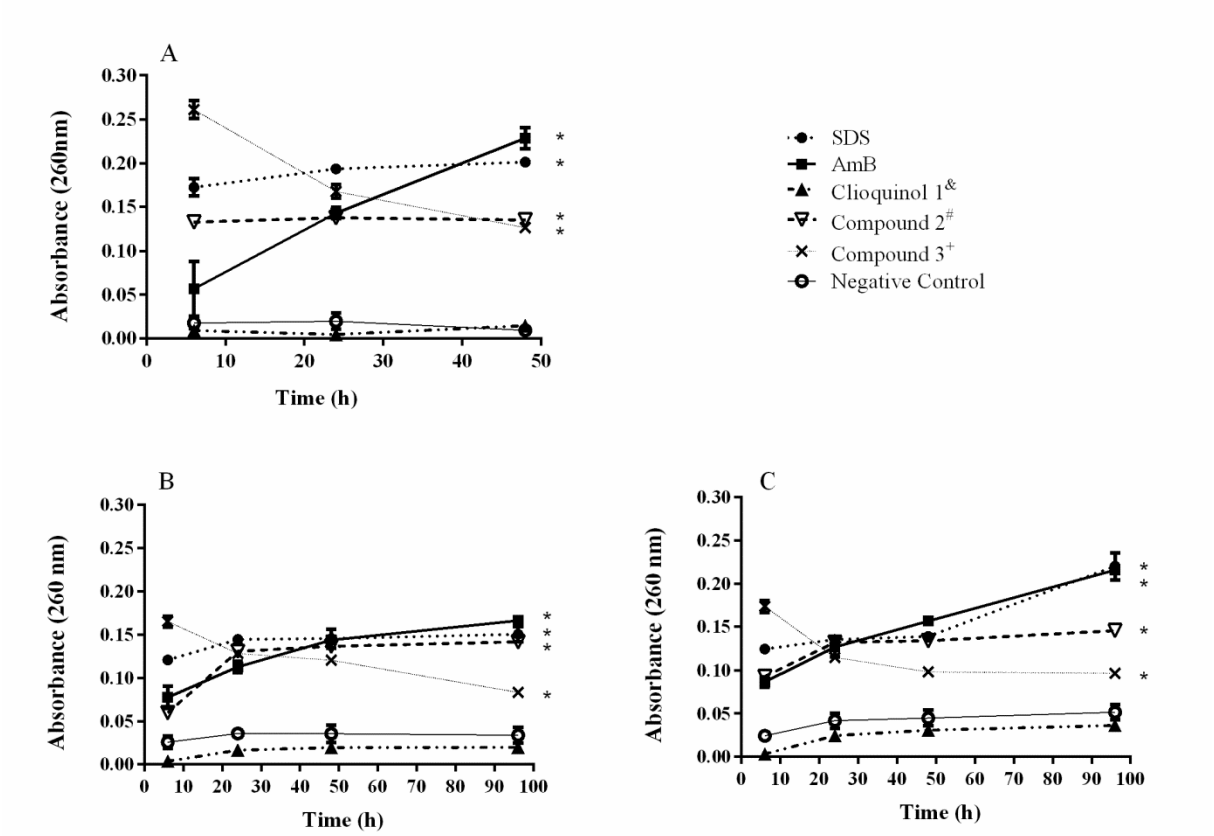

Figure 2. Cellular leakage of 260-nm-absorbing materials of *Candida albicans* ATCC 18804 (A), *Microsporum canis* MCA 01 (B) and *Trichophyton mentagrophytes* TME 40 (C) treated with MIC of 8-hydroxyquinoline derivatives 1-3 by time intervals (6, 24 and 48 h for *C. albicans*; 6, 24, 48 and 96 h for dermatophytes). Amphotericin B (6.25 µg/ml) and SDS (2%) are the positive controls. Untreated cells are negative control. The asterisks indicate statistical difference compared to the negative control (* $p < 0.05$).

[&] (1) clioquinol MIC: 0.250 µg/ml (ATCC 18804), 0.250 µg/ml (MCA 01) and 0.500 µg/ml (TME 40);

[#] (2) 8-hydroxy-5-quinolinesulfonic MIC: 64 µg/ml (ATCC 18804), 128 µg/ml (MCA 01) and 64 µg/ml (TME 40);

[+] (3) 8-hydroxy-7-iodo-5-quinolinesulfonic acid MIC: 32 µg/ml (ATCC 18804), 256 µg/ml (MCA 01) and 256 µg/ml (TME 40).

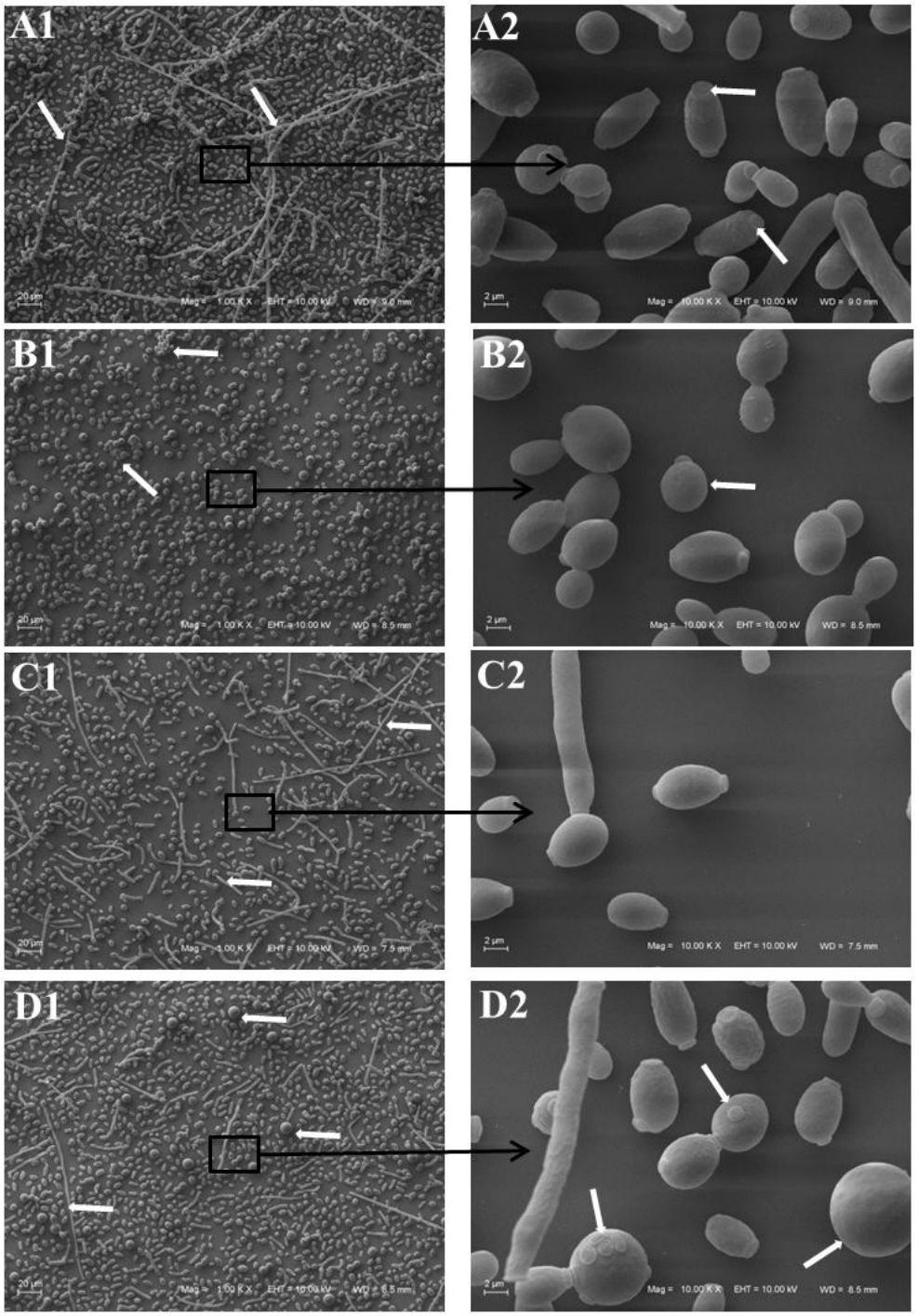

Figure 3. Scanning electron microscopy of *Candida albicans* ATCC 18804 treated with sub-inhibitory concentration of 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) and untreated cells. **(A)** Untreated cells; A1 (Bar = 20 µm): white arrows indicate pseudohyphae; A2 (Bar = 2 µm): white arrows indicate polar bud scars. **(B)** Hyphal cells treated with clioquinol **1**; B1 (Bar = 20 µm): white arrows indicate cell clusters; B2 (Bar = 2 µm): white arrow indicates rounded cells.**(C)** Hyphal cells treated with compound **2**; C1 (Bar = 20 µm): white arrows indicate pseudohyphae with few blastoconidia and chlamydospore; C2 (Bar = 2 µm). **(D)** Hyphal cells treated with compound **3**; D1 (Bar = 20 µm): white arrows indicate pseudohyphae with few blastoconidia and larger round cells; D2 (Bar = 2 µm): white arrows indicate pseudohyphae with few blastoconidia and larger round cells.

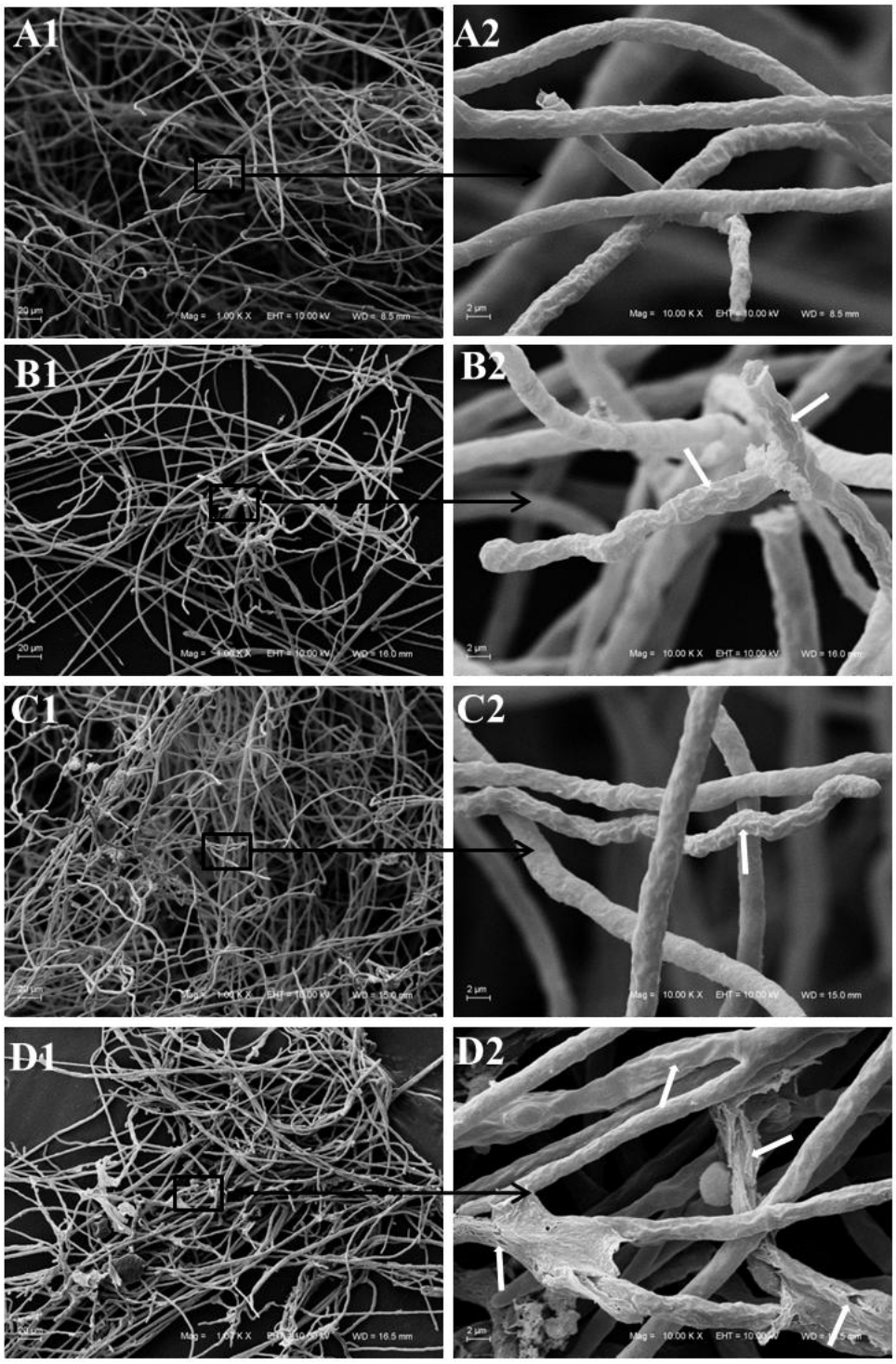

Figure 4. Scanning electron microscopy of *Microsporum canis* MCA 01 treated with sub-inhibitory concentration of 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) and untreated cells. **(A)** Untreated cells; A1 (Bar = 20 µm); A2 (Bar = 2 µm). **(B)** Hyphal cells treated with clioquinol **1**; B1 (Bar = 20 µm); B2 (Bar = 2 µm): white arrow indicates irregular and rough cell walls with grooves. **(C)** Hyphal cells treated with compound **2**; C1 (Bar = 20 µm); C2 (Bar = 2 µm): white arrow indicates irregular and rough cell walls with grooves. **(D)** Hyphal cells treated with compound **3**; D1 (Bar = 20 µm); D2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears.

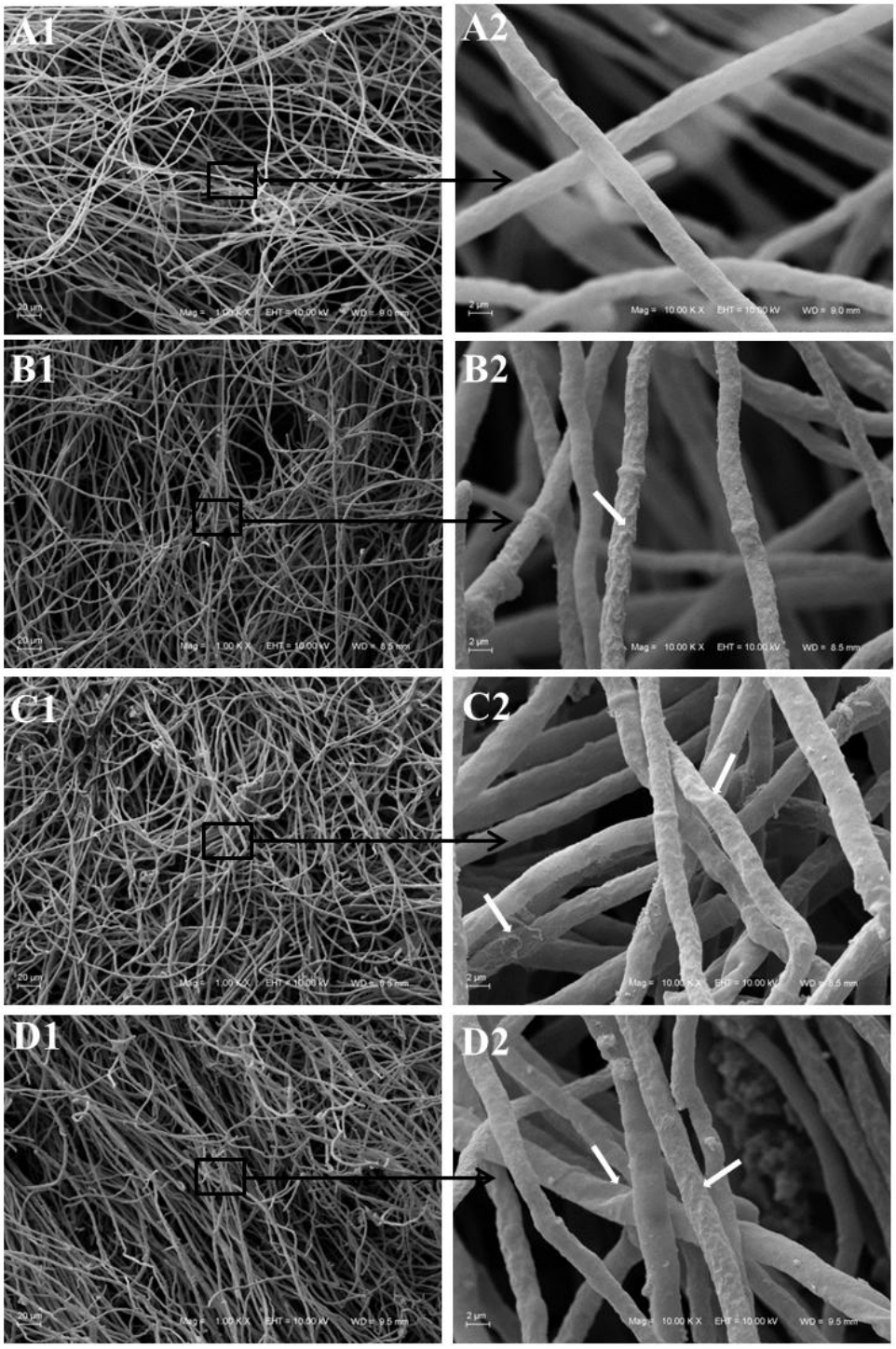

Figure 5. Scanning electron microscopy of *Trichophyton mentagrophytes* TME 40 treated with sub-inhibitory concentration of 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) and untreated cells. **(A)** Untreated cells; A1 (Bar = 20 µm); A2 (Bar = 2 µm). **(B)** Hyphal cells treated with clioquinol **1**; B1 (Bar = 20 µm); B2 (Bar = 2 µm): white arrow indicates irregular and rough cell walls with grooves. **(C)** Hyphal cells treated with compound **2**; C1 (Bar = 20 µm); C2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves. **(D)** Hyphal cells treated with compound **3**; D1 (Bar = 20 µm); D2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears.

Supporting Information

Table S1. Effect of sorbitol on the minimum inhibitory concentration (MIC; µg/ml) of anidulafungin and 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) against *Candida* sp.

Isolate	MIC Anidulafungin				MIC Compound 1				MIC Compound 2				MIC Compound 3			
	Day 2		Day 7		Day 2		Day 7		Day 2		Day 7		Day 2		Day 7	
	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)
ATCC 18804 (CA)	0.031	> 4.0	0.063	> 4.0	0.250	0.250	0.250	0.500	64.0	64.0	64.0	256.0	32.0	64.0	64.0	256.0
CA 01	0.063	0.125	0.063	0.25	0.125	0.250	0.125	0.500	16.0	32.0	16.0	32.0	32.0	32.0	32.0	128.0
CA DEB 05	0.007	0.250	0.015	0.500	0.125	8.0	1.0	16.0	128.0	256.0	128.0	256.0	128.0	256.0	128.0	256.0
CG RL12m	0.015	0.015	0.031	0.500	0.031	0.125	0.031	0.125	1.0	1.0	1.0	4.0	16.0	16.0	64.0	128.0
CG RL24	0.015	0.015	0.015	0.063	0.063	0.125	0.063	0.125	16.0	16.0	16.0	64.0	16.0	16.0	32.0	64.0
CG RL49	0.015	0.063	0.015	0.063	0.063	0.250	0.125	0.250	2.0	8.0	4.0	16.0	2.0	8.0	8.0	16.0
CK 03	0.007	0.008	0.063	> 4.0	0.125	0.500	0.250	0.500	8.0	32.0	16.0	64.0	16.0	16.0	32.0	64.0
CK CVB 42	0.007	0.008	0.063	4.0	0.063	0.125	0.063	0.250	64.0	64.0	64.0	256.0	64.0	64.0	64.0	128.0
CK Den43	0.500	0.500	1.0	> 4.0	1.0	2.0	2.0	4.0	128.0	128.0	256.0	512.0	128.0	128.0	256.0	512.0
CP RL11m	0.015	0.063	0.031	0.063	0.125	0.500	0.250	0.500	32.0	64.0	32.0	256.0	64.0	64.0	128.0	256.0
CP RL27m	0.125	0.125	0.125	0.250	0.250	0.250	0.500	8.0	32.0	32.0	128.0	256.0	64.0	64.0	128.0	256.0
CP RL 38	0.250	0.500	1.0	4.0	0.250	0.500	1.0	2.0	64.0	64.0	128.0	256.0	64.0	128.0	128.0	256.0
ATCC 750 (CT)	0.008	4.0	0.063	4.0	0.125	0.125	0.250	0.500	64.0	256.0	64.0	512.0	128.0	256.0	256.0	1024.0
CT 57A	0.031	4.0	0.031	4.0	0.063	0.063	0.063	0.125	32.0	64.0	32.0	64.0	32.0	64.0	32.0	128.0
CT 72A	0.016	0.250	0.063	> 4.0	0.250	0.250	0.250	0.500	32.0	32.0	32.0	64.0	64.0	64.0	128.0	256.0

CA: *Candida albicans*; CG: *Candida glabrata*; CK: *Candida krusei*, CP: *Candida parapsilosis*; CT: *Candida tropicalis*.
S (-): growth medium without sorbitol; S (+): growth medium with sorbitol.

Table S2. Effect of sorbitol on the minimum effective concentration (MEC; µg/ml) of anidulafungin and minimum inhibitory concentration (MIC) of 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) against dermatophytes.

Isolate	**MEC Anidulafungin**				**MIC Compound 1**				**MIC Compound 2**				**MIC Compound 3**			
	Day 4		Day 7		Day 4		Day 7		Day 4		Day 7		Day 4		Day 7	
	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)
MCA 01	0.25	0.500	0.250	1.0	0.250	2.0	0.250	2.0	128.0	128.0	128.0	256.0	256.0	256.0	256.0	512.0
MCA 29	0.125	0.500	0.250	1.0	0.250	0.500	0.250	0.500	128.0	128.0	128.0	256.0	256.0	512.0	512.0	1024.0
MCA 40	0.500	1.0	0.500	1.0	1.0	4.0	2.0	4.0	256.0	512.0	256.0	512.0	256.0	256.0	256.0	512.0
MGY 42	0.250	1.0	0.500	1.0	0.500	1.0	0.500	1.0	256.0	512.0	256.0	512.0	256.0	256.0	256.0	512.0
MGY 50	0.250	0.500	0.500	2.0	0.500	1.0	0.500	1.0	256.0	256.0	256.0	512.0	256.0	256.0	256.0	512.0
MGY 58	0.125	0.500	0.500	1.0	1.0	2.0	1.0	2.0	256.0	512.0	256.0	512.0	1024.0	1024.0	1024.0	2048.0
TME 32	0.250	2.0	0.500	2.0	0.500	1.0	0.500	1.0	256.0	256.0	256.0	512.0	256.0	256.0	256.0	256.0
TME 40	0.250	0.500	0.500	1.0	0.500	1.0	0.500	1.0	64.0	64.0	64.0	128.0	256.0	256.0	512.0	512.0
TME 16	0.500	1.0	0.500	2.0	0.250	1.0	0.250	2.0	64.0	64.0	64.0	128.0	128.0	128.0	256.0	256.0
TRU 43	1.0	2.0	1.0	2.0	0.125	0.250	0.125	0.250	128.0	128.0	128.0	128.0	128.0	128.0	256.0	256.0
TRU 50	0.500	1.0	0.500	2.0	0.250	0.500	0.250	1.0	128.0	128.0	128.0	128.0	128.0	256.0	256.0	256.0
TRU 51	0.500	1.0	0.500	2.0	0.500	2.0	1.0	2.0	128.0	128.0	128.0	128.0	256.0	256.0	256.0	256.0

MCA: *Microsporum canis*; MGY: *Microsporum gypseum*, TME: *Trichophyton mentagrophytes*; TRU: *Trichophyton rubrum*.
S (-): growth medium without sorbitol; S (+): growth medium with sorbitol.

Table S3. Effect of different concentrations of external ergosterol (50-250 µg/ml) on the minimum inhibitory concentration (MIC; µg/ml) of amphotericin B and 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) against *Candida* sp.

Isolate	MIC Amphotericin B						MIC Compound 1						MIC Compound 2						MIC Compound 3					
	Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)					
	0	50	100	150	200	250	0	50	100	150	200	250	0	50	100	150	200	250	0	50	100	150	200	250
ATCC 18804 (CA)	0.500	2.0	4.0	8.0	16.0	32.0	0.250	0.250	0.250	0.250	0.250	0.250	64.0	64.0	64.0	64.0	64.0	64.0	32.0	32.0	32.0	32.0	32.0	32.0
CA 01	1.0	2.0	4.0	8.0	32.0	32.0	0.125	0.125	0.125	0.125	0.125	0.125	16.0	16.0	16.0	16.0	16.0	16.0	32.0	32.0	32.0	32.0	32.0	32.0
CA DEB 05	1.0	4.0	8.0	16.0	32.0	32.0	0.125	0.125	0.125	0.125	0.125	0.125	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0
CG RL12m	0.250	1.0	2.0	4.0	8.0	16.0	0.031	0.031	0.031	0.031	0.031	0.031	1.0	1.0	1.0	1.0	1.0	1.0	16.0	16.0	16.0	16.0	16.0	16.0
CG RL24	0.500	2.0	4.0	8.0	32.0	32.0	0.063	0.063	0.063	0.063	0.063	0.063	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
CG RL49	0.500	1.0	2.0	4.0	8.0	16.0	0.063	0.063	0.063	0.063	0.063	0.063	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
CK 03	0.500	2.0	4.0	8.0	8.0	16.0	0.125	0.125	0.125	0.125	0.125	0.125	8.0	8.0	8.0	8.0	8.0	8.0	16.0	16.0	16.0	16.0	16.0	16.0
CK CVB 42	0.500	2.0	4.0	4.0	8.0	16.0	0.063	0.063	0.063	0.063	0.063	0.063	64.0	64.0	64.0	64.0	32.0	32.0	64.0	64.0	64.0	64.0	64.0	64.0
CK Den43	0.250	2.0	4.0	8.0	16.0	32.0	1.0	1.0	1.0	1.0	1.0	1.0	128.0	128.0	128.0	128.0	64.0	64.0	128.0	128.0	128.0	128.0	128.0	128.0
CP RL11m	1.0	2.0	4.0	4.0	8.0	16.0	0.125	0.125	0.125	0.125	0.125	0.125	32.0	32.0	32.0	32.0	32.0	32.0	64.0	64.0	64.0	64.0	64.0	64.0
CP RL27m	0.500	2.0	4.0	8.0	8.0	16.0	0.250	0.250	0.250	0.250	0.250	0.250	32.0	32.0	32.0	32.0	32.0	16.0	64.0	64.0	64.0	64.0	64.0	64.0
CP RL 38	0.500	1.0	4.0	8.0	32.0	32.0	0.250	0.250	0.250	0.250	0.250	0.250	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0	64.0
ATCC 750 (CT)	0.063	0.250	1.0	2.0	4.0	8.0	0.125	0.125	0.125	0.125	0.125	0.125	64.0	64.0	64.0	64.0	64.0	64.0	128.0	128.0	128.0	128.0	128.0	128.0
CT 57A	0.500	4.0	8.0	16.0	16.0	32.0	0.063	0.063	0.063	0.063	0.063	0.063	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0
CT 72A	0.250	2.0	8.0	16.0	16.0	32.0	0.250	0.250	0.250	0.250	0.250	0.250	32.0	32.0	32.0	32.0	32.0	32.0	64.0	64.0	64.0	64.0	64.0	64.0

CA: *Candida albicans*; CG: *Candida glabrata*; CK: *Candida krusei*, CP: *Candida parapsilosis*; CT: *Candida tropicalis*.

Table S4. Effect of different concentrations of external ergosterol (50-250 µg/ml) on the minimum inhibitory concentration (MIC; µg/ml) of Amphotericin B and 8-hydroxyquinoline derivatives [clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**) and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**)] against dermatophytes.

Isolate	**MIC Amphotericin B**						**MIC Compound 1**						**MIC Compound 2**						**MIC Compound 3**					
	Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)						Ergosterol Concentration (µg/ml)					
	0	50	100	150	200	250	0	50	100	150	200	250	0	50	100	150	200	250	0	50	100	150	200	250
MCA 01	2.0	4.0	4.0	8.0	16.0	32.0	0.250	0.250	0.250	0.250	0.250	0.250	128.0	64.0	64.0	64.0	64.0	64.0	256.0	256.0	256.0	256.0	256.0	256.0
MCA 29	0.250	2.0	4.0	8.0	16.0	16.0	0.250	0.250	0.250	0.250	0.250	0.250	128.0	128.0	128.0	128.0	128.0	128.0	256.0	256.0	256.0	256.0	128.0	128.0
MCA 40	0.250	1.0	2.0	4.0	8.0	16.0	1.0	1.0	1.0	1.0	1.0	1.0	256.0	256.0	256.0	256.0	128.0	128.0	256.0	128.0	128.0	128.0	128.0	128.0
MGY 42	1.0	4.0	8.0	16.0	32.0	64.0	0.500	0.500	0.500	0.500	0.500	0.500	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0
MGY 50	1.0	4.0	8.0	16.0	32.0	64.0	0.500	0.500	0.500	0.500	0.500	0.500	256.0	128.0	128.0	128.0	128.0	128.0	256.0	256.0	256.0	256.0	256.0	256.0
MGY 58	2.0	8.0	16.0	32.0	64.0	64.0	1.0	1.0	1.0	1.0	1.0	1.0	256.0	256.0	256.0	256.0	256.0	256.0	1024.0	512.0	512.0	512.0	512.0	512.0
TME 32	2.0	8.0	8.0	16.0	32.0	64.0	0.500	0.500	0.500	0.500	0.500	0.500	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0	256.0
TME 40	2.0	8.0	8.0	16.0	32.0	64.0	0.500	0.500	0.500	0.500	0.500	0.500	64.0	64.0	64.0	64.0	64.0	64.0	256.0	256.0	256.0	256.0	256.0	256.0
TME 16	0.500	2.0	4.0	8.0	16.0	32.0	0.250	0.250	0.250	0.250	0.250	0.250	64.0	64.0	64.0	64.0	64.0	64.0	128.0	128.0	128.0	128.0	128.0	128.0
TRU 43	2.0	4.0	8.0	16.0	32.0	64.0	0.125	0.125	0.125	0.125	0.125	0.125	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0
TRU 50	1.0	8.0	16.0	32.0	32.0	64.0	0.250	0.250	0.250	0.250	0.250	0.250	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0	128.0
TRU 51	0.500	2.0	4.0	8.0	8.0	16.0	0.500	0.500	0.500	0.500	0.500	0.500	128.0	128.0	128.0	128.0	128.0	128.0	256.0	256.0	256.0	256.0	256.0	128.0

MCA: *Microsporum canis*; MGY: *Microsporum gypseum*, TME: *Trichophyton mentagrophytes*; TRU: *Trichophyton rubrum*.

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5.3 Artigo 3

O artigo intitulado “Clioquinol is a promising preventive morphological switching compound to treat *Candida* infections linked to use of intrauterine device” foi submetido no “Journal of Medical Microbiology”.

Title: Clioquinol is a promising preventive morphological switching compound to treat *Candida* infections linked to use of intrauterine device

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Keywords: clioquinol, 8-hydroxyquinoline derivatives, antifungal agents, *Candida* spp., biofilm, intrauterine device.

Subject category: Prevention and Therapy

Abstract

Purpose: *Candida* biofilm infections are frequently linked to use of biomaterials and present clinical significance because they are commonly resistant to antifungals. Clioquinol is antiseptic drug and is effective against multidrug-resistant *Candida*. We investigated the effect of clioquinol and two other 8-hydroxyquinoline derivatives on *Candida* biofilm. **Methodology:** The ability to inhibit the biofilm formation, inhibit preformed biofilm and remove established biofilms were evaluated using in vitro assays on microtiter plate. The action of clioquinol on biofilm in intrauterine device (IUD) was also investigated describing the first protocol that quantifies the inhibitory action of compounds on biofilms formed on IUD. **Results:** Clioquinol is the most effective 8-hydroxyquinoline derivative. It prevents more than 90% of biofilm formation and this can be attributed to blockade of hyphae development. Clioquinol has also reduced the metabolic activity of sessile *Candida* but the susceptibility was lower compared to planktonic cells (0.031-05 µg ml^{-1} to inhibit 50% planktonic cells; and 4-16 µg ml^{-1} to inhibit 50% preformed biofilms). On the other hand, almost complete removal of biofilms was not achieved for most of the isolates. *Candida* spp. have also showed ability to form biofilm on copper IUD and clioquinol eradicated 80-100% of these biofilms. **Conclusion:** Results indicate a potential application in terms of biomaterials for 8-hydroxyquinoline derivatives. Clioquinol might be used as coatings to prevent morphological switching and avoid biofilm formation. Furthermore, clioquinol may have future applications in the treatment of *Candida* infections linked to the use of IUD.

Introduction

Vulvovaginal candidiasis (VVC) affects millions of women and is a very common cause of acute vaginitis [1]. It is estimated that approximately 75% of women experience at least one episode of CVV during life and approximately 40-50% of them have additional episodes [2]. Furthermore, 5% of them develop repetitive episodes (3-4 times in a period of 12 months) - a condition known as recurrent VVC [1]. The use of intrauterine device (IUD) as contraceptive has not yet been recognized as a predisposing factor [3], however, previous studies have shown that women who use IUDs have more VCV infection than those who do not use the device [4]. Auler et al. (2010) described two cases of patients with signs and symptoms of recurrent VVC who used IUDs as a contraceptive method. They indicated the presence of the biofilm on the surface of the IUD and verified that the absence of this source of microorganisms was essential to avoid the relapses in these patients [5]. In addition, intrauterine fetal death and maternal sepsis by *Candida* in women with IUDs has also been previously reported [6,7].

Device-associated infections are commonly related to biofilm: complex structured community of microorganisms that are adhered at a surface or interface and encased within a polysaccharide-rich extracellular matrix [8]. This environment offers many benefits to microbial pathogens such as protection from host immune defenses, resistance to physical and chemical stresses and nutrient availability [9]. *Candida* biofilms present important clinical and economic significance because they are commonly resistant to antifungals, including azoles, polyenes and echinocandins [9,10]. The presence of biofilm on the surface of the IUD helps to protect yeast from the action of antifungal agents contributing to the persistence of the microorganism in the site and leading to recurrent infections [5]. So, the eradication of a biofilm usually involves treatment with toxic concentrations of drugs, and the recommended procedure includes removal of the contaminated device [11]. Therefore, biofilm formation in IUDs and treatments that can control this process require further investigation.

Clioquinol (5-chloro-7-iodo-8-hidroxyquinoline) is an 8-hydroxyquinoline derivative available as topical medicine for antiseptic treatments [12]. In 1970, their oral formulations were withdrawn from the market due to reports of sub-acute myelo-optic neuropathy (SMON) in Japanese patients [13]. However there is still

controversy of the association of the use of clioquinol and SMON. Interestingly, epidemiological reports suggest that clioquinol was not responsible for SMON and no other population had a similar adverse response [14]. Recently the interest in this drug reappeared as a potential treatment for several pathologies [15-18]. Clioquinol **1** has proven to be very effective against different clinically important fungi, especially *Candida* spp. and dermatophytes, and it is also active against those isolates displaying multidrug resistance [19]. Furthermore, there are few reports of antimicrobial resistance against clioquinol [20].

Considering such properties and the lack of studies of clioquinol in fungal biofilm, the aim of this study was to investigate the action of clioquinol **1** and two other 8-hydroxyquinoline derivatives (8-hydroxy-5-quinolinesulfonic acid **2** and 8-hydroxy-7-iodo-5-quinolinesulfonic acid **3**) (Fig. 1) on *Candida* biofilms. So, the capacity to inhibit the biofilm formation, to inhibit preformed biofilm and to remove established biofilms were evaluated in microtiter plate. The ability of *Candida* spp. to form biofilm in intrauterine copper device (copper IUD) and the action of clioquinol on these was also investigated.

Methods

Fungal strains: Biofilm assays was carried out with one strain of each *Candida* sp. studied (*C. albicans* ATCC 18804, *C. glabrata* CG RL24, *C. krusei* CK Den 43, *C. parapsilosis* CP RL38 and *C. tropicalis* ATCC 750). All isolates are deposited in the Mycology Collection of Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil) and were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and clinical samples.

Antifungal compounds: Clioquinol (compound **1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**), and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were solved in DMSO (Sigma-Aldrich) and diluted in medium assay (RPMI 1640) (Gibco; Nova York USA) to obtain a maximum concentration of 2% DMSO to the experiments.

Susceptibility of planktonic *Candida* cells to 8-hydroxyquinoline derivatives: planktonic minimum inhibitory concentrations (PMICs) of analogues of 8-hydroxyquinoline were determined by the broth microdilution method according to M27-A3 protocol [21]. Serial two-fold dilutions were made in RPMI 1640 medium and the final test range of concentrations were 0.016-8 µg ml^{-1} of clioquinol **1** and 0.25-128 µg ml^{-1} of compound **2** and **3**. The experiments were carried out in quadruplicate. MICs were defined as the lowest concentration of compounds at which the microorganisms tested did not demonstrate visible growth in 48 h.

Cell viability was assessed according Pippi et al. (2017): the supernatant was discarded and the fungal cells were incubated for 3 h at 35°C in a suspension containing 0.05 mg ml^{-1} MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) (Sigma-Aldrich) [19]. MTT is a tetrazolium salt reduced by dehydrogenase enzymes that form blue formazan crystals, which were then extracted with isopropanol [22].

Absorbances (A) were measured on a multiplate reader SpectraMax-M2; (Molecular Devices®, USA) in two wavelengths (570 and 690 nm) and the percent cell damage was calculated by the following equation: % planktonic cell damage = [1 – (A570 – A690 with drugs) /(A570 – A690 without drugs)] x 100 [19].

Statistical analysis was performed with one-Way ANOVA as well as Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. The analyses were performed by using Minitab version 17.0 for software (Minitab Inc., State College, PA).

Effect of coating the wells of a microtiter plate with 8-hydroxyquinoline derivatives on *Candida* sp. biofilm formation: A modified assay was used in which the wells of a microtiter plate were directly precoated with 8-hydroxyquinoline derivatives in order to investigate the compounds ability to prevent *Candida* biofilm formation [23]. Briefly, 100 µl volumes of serial dilutions of 8-hydroxyquinoline derivatives (Clioquinol **1**: 0.125-64 µg ml^{-1}; Compound **2** and **3**: 1-512 µg ml^{-1}) were added to selected wells of a microtiter plate and incubated at 4°C for 48 h. After incubation, excess 8-hydroxyquinoline derivatives were aspirated and the plates were washed once in sterile saline. *Candida* cells cultured 24h on Sabouraud dextrose agar (SDA) (HiMedia; Mumbai, India) were resuspended at a

concentration of 10^6 CFU ml^{-1} in RPMI 1640 and then 20 µl of these fungal inoculums were added to each well of microtiter plate and the wells were filled with 180 µl of RPMI 1640 (1:10 dilution). The plates are incubated for 48 h at 35°C to allow biofilm formation.

Biofilm biomass was assessed according Stepanović et al. (2007), with modifications [24]. To remove non-adherent cells, the contents of the wells were aspirated and washed three times with sterile saline. The attached cells were fixed with 150 µl of methanol (Merck; Darmstadt, Germany) for 20 min and dried for 30 min at room temperature. A volume of 150 µl of crystal violet 0.5% (w/v) (Synth, São Paulo, Brasil) was used to stain the fungal for 15 min. The solution was then removed by carefully rinsing the biofilms under running water until excess stain was removed. The biofilm was eluted with 150 µl of 95% ethanol (Merck) for 30 min without shaking and 100 µl of this was transferred to a clean 96-well microtitre plate. The assay was performed in triplicate.

Absorbances (A) were measured on a multiplate reader (SpectraMax®) at 450 nm and the values are proportional to the quantity of total biofilm biomass (the greater the quantity of biological material, the higher the level of staining and absorbance). The percent inhibitory effect of 8-hydroxyquinoline derivatives on *Candida* sp. biofilm formation was expressed as the percentage of the mean optical density (OD) of 8-hydroxyquinoline derivatives-treated wells compared to the untreated control. This percent was calculated by the following equation: % Inhibition of biofilm formation (IBF) = [1 – (A450 treated well) /(A450 untreated well)] x 100.

The reduction of absorption by 50% and 90% was reported as the IBF_{50} and IBF_{90}, respectively.Statistical analysis was performed with one-Way ANOVA as well as Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. The analyses were performed by using Minitab version 17.0 for software (Minitab Inc., State College, PA).

Susceptibility of *Candida* biofilms to 8-hydroxyquinoline derivatives: For testing the susceptibility of *Candida* biofilms to 8-hydroxyquinoline derivatives the sessile MIC (SMIC) was determinate according to the literature with some modifications [25-27]. *Candida* cells cultured 24h on SDA were resuspended at a concentration of 10^6 CFU ml^{-1} in RPMI 1640 and then 20 µl of these fungal inoculums

were added to each well of microtiter plate and the wells were filled with 180 µl of RPMI 1640. The plates are incubated for 48 h at 35°C to allow biofilm formation. To remove non-adherent cells, the contents of the wells were aspirated and washed three times with sterile saline. The remaining attached fungi were exposed of serial dilutions of 8-hydroxyquinoline derivatives (Clioquinol **1**: 0.125-64 µg ml^{-1}; Compound **2** and **3**: 1-512 µg ml^{-1}) and incubated for 48 h at 35 °C. After incubation, excess 8-hydroxyquinoline derivatives were aspirated and the plates were washed three times with sterile saline to remove non-adherent cells.

The effect of 8-hydroxyquinoline derivatives on preformed biofilms was then estimated by using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay. The XTT assay is a quantification technique based on the metabolic activity of viable cells and the reduction of the XTT dye to a water-soluble formazan are measured [27]. Briefly, XTT (Sigma-Aldrich) was prepared as a saturated solution at a concentration of 0.5 mg ml^{-1} in MOPS buffer and stored at -70°C until use. Before use an aliquot of the XTT stock solution was thawed, and menadione (10 mM prepared in acetone; Sigma-Aldrich) was added to a final concentration of 1 mM. A 100 µl aliquot of XTT-menadione was then added to each well. The plates were then incubated in the dark for 2 h at 37°C and 75 µl of each well was transferred to a clean 96-well microtitre plate. The assay was performed in triplicate. The colorimetric change at 490 nm (a reflection of the metabolic activity of the biofilm) was measured with a multiplate reader (SpectraMax®) and the absorbance is proportional to the number of metabolically active microbial cells. The antifungal effect of 8-hydroxyquinoline derivatives on *Candida* sp. biofilm was expressed as the percentage of the mean optical density (OD) of 8-hydroxyquinoline derivatives-treated wells compared to the untreated control. This percent was calculated by the following equation: % Inhibition of preformed biofilm = [1 – (A490 treated biofilm) /(A490 untreated biofilm)] x 100.

The reduction of absorption by 50% and 90% was reported as the $SMIC_{50}$ and $SMIC_{90}$, respectively. Statistical analysis was performed with one-Way ANOVA as well as Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. The analyses were performed by using Minitab version 17.0 for software (Minitab Inc., State College, PA).

Effect of biofilm removal of 8-hydroxyquinoline derivatives: For testing the effect of biofilm removal of 8-hydroxyquinoline derivatives, the method of Ramage et al. (2001) was applied with some modifications [27]. *Candida* cells cultured 24h on SDA were resuspended at a concentration of 10^6 CFU ml^{-1} in RPMI 1640 and then 20 µl of these fungal inoculums were added to each well of microtiter plate and the wells were filled with 180 µl of RPMI 1640. The plates are incubated for 48 h at 35°C to allow biofilm formation. To remove non-adherent cells, the contents of the wells were aspirated and washed three times with sterile saline. The remaining attached fungi were exposed to serial dilutions of 8-hydroxyquinoline derivatives (Clioquinol **1**: 0.125-64 µg ml^{-1}; Compound **2** and **3**: 1-512 µg ml^{-1}) and incubated for 48 h at 35°C. After incubation, excess 8-hydroxyquinoline derivatives were aspirated and the plates were washed three times with sterile saline to remove non-adherent cells. The attached cells were fixed with methanol and the effect of biofilm removal of 8-hydroxyquinoline derivatives was determined by addition of the 0.5% crystal violet as previously described above [24]. The assay was performed in triplicate.

Absorbances (A) were measured on a multiplate reader (SpectraMax®) at 450 nm and the values are proportional do the quantity of total biofilm biomass. Crystal violet is suitable for measuring the amount of biofilm, but not its activity, so crystal violet staining can be used to measure removal but not disinfection [28]. The percent removal effect of 8-hydroxyquinoline derivatives on *Candida* sp. biofilm was expressed as the percentage of the mean optical density (OD) of 8-hydroxyquinoline derivatives-treated wells compared to the untreated control. This percent was calculated by the following equation: % biofilm removal (BR) = [1 – (A450 treated biofilm) /(A450 untreated biofilm)] x 100.

The reduction of absorption by 50% and 90% was reported as the BR_{50} and BR_{90}, respectively. Statistical analysis was performed with one-Way ANOVA as well as Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. The analyses were performed by using Minitab version 17.0 for software (Minitab Inc., State College, PA).

Biofilm formation assay on IUD and action of clioquinol: *Candida* cells cultured 24h on SDA were resuspended at a concentration of 10^6 CFU ml^{-1} in peptone water (pH=4.5, vagina-like conditions) (HiMedia). A 1:10 dilution of this

suspension was made by adding 3 ml of fungal suspension to 27 ml of peptone water. The IUDs (Model TCu 380A, Furp - adopted by the Brazilian Ministry of Health for patients in the public health system) were placed in this yeast suspension for 48 h at 35°C to allow biofilm formation. Subsequent to the incubation period, the devices were washed three times with sterile water for removal of the non-adhered cells. Then, two steps were performed:

Step 1: IUDs containing adhered cells were exposed for 8 h (approximate time that a vaginal cream stays in contact) at 35°C to 64 µg ml^{-1} of clioquinol and fluconazole (Sigma-Aldrich) prepared in 30 ml of peptone water. The same procedure was performed with IUD exposed to peptone water without addition of antifungal agent (untreated control). After the incubation period, 10 µl were aseptically pipetted from each flask and serially diluted 10-fold (until 10^{-3}) with sterile water. Then, 10 µl directly from flask and 10 µl of each dilution were seeded in SDA to allow the determination of the CFU removed from the device by antifungal agents, but that remained alive.

Step 2: After step 1, the IUDs were added to another flask with 30 ml of sterile water, and the adhered cells were removed from the device by sonication at a frequency of 40 KHz for 10 min. The sonicated water phase was serially diluted 10-fold (until 10^{-3}) with sterile water and seeded in SDA to determine the CFU that remained alive and adhered to the IUD after contact with the antifungal agent.

All dilutions and counts were made in triplicate. The plates were incubated for 24 h at 35°C, and the CFU per IUD were determined. The quantification of the live biofilm cells on the IUD was determined by the sum of CFU found in step 1 and CFU found in step 2. So, the live biofilm cells of untreated and treated IUD were calculated by the following equation: $CFU_{Biofilme} = CFU_{step\ 1} + CFU_{step\ 2}$

The percentage of biofilm eradication by clioquinol and fluconazole was expressed as the percentage of the CFU of treated IUD compared to the untreated control. This percent was calculated by the following equation: % biofilm eradication = $[(CFU_{Untreated\ Biofilm} - UFC_{Treated\ Biofilm}) / UFC_{Untreated\ Biofilm}] \times 100$.

Results

The biofilm forming capacity of different *Candida* species on microtiter plate was confirmed in our study once the untreated control was evaluated in parallel

in the crystal violet assay and all strains showed being biofilm formers. The 8-hydroxyquinoline derivatives were used to prevent biofilm formation and to treat preformed biofilm in order to inhibit or remove it. The results of planktonic MICs (PMICs), inhibition of biofilm formation, sessile MICs (SMICs) and biofilm removal of 8-hydroxyquinoline derivatives are shown in Table 1. The action of different concentrations of clioquinol **1**, compound **2** and compound **3** on planktonic cell and biofilm of *C. albicans* is schematically represented in Fig. 2.

We have observed that precoating the microplates with clioquinol has prevented successful the adhrerence and germination of the yeast cells, resulting in scant biofilms. Cliquinol at 16 and 32 μg ml^{-1} has inhibited more than 90% of biofilm formation, except for *C. krusei* CK Den43 that the tested concentrations of clioquinol inhibited only 50%. However, the 8-hydroxyquinoline derivatives **2** and **3** have shown only 50% inhibition on some strains.

In addition, a sharp reduction of the metabolic activity of cells within the biofilm as assessed by the XTT reduction assay was demonstrated when preformed biofilms of *C. albicans* ATCC 18804, *C. glabrata* CG RL24 and *C. parapsilosisi* CP RL 38 were exposed to the clioquinol **1**. The 8-hydroxyquinoline derivatives **2** and **3** reduced more than 50% of biofilm metabolic activity of some strains only.

On the other hand, the complete removal of the biofilms was not achieved by treatment with 8-hydroxyquinoline derivatives. Clioquinol **1** has removed more than 90% only for the biofilm of *C. glabrata* CG RL24. However, the 8-hydroxyquinoline derivatives-treated biofilms have less biomass compared to untreated control, presenting 50% removal in some cases.

Candida spp. has showed ability to form biofilm on copper IUD. The IUD presented between 113000 and 47200000 colony-forming units (CFU), depending on the *Candida* species. *Candida albicans* was the strongest biofilm-forming species on IUD, followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei*, respectively. The data of CFU per treated and untreated IUD as well as the percentage of biofilm eradication by clioquinol and fluconazole are shown in Table 2. The percentage of biofilm eradication presented by clioquinol varied from 80.19 (for isolate CP RL38) to 100% (for isolate CG RL24). The eradication of biofilm by fluconazole varied from 48.67 (for isolate CK Den 43) to 98.65% (for strain *C. tropicalis* ATCC 750).

Discussion

Biofilms are communities of highly organized cells that represent the foundation of multicellular life [8]. The strategy of cell-cell communication controls unnecessary overpopulation and competition for nutrients and has important implications for the infectious process [27]. So, biofilms have unique developmental characteristics and are in general much more difficult to treat [8,9]. In this era of widespread antimicrobial resistance, it is crucial the evaluation of current and new antifungal agents against cells in biofilms [8]. Research on the antifungal activity of 8-hydroxyquinoline derivatives has been developed for planktonic fungal cells; however, the consideration of a sessile microbial lifestyle has been neglected.

An attractive option to the control of biofilm formation is to coat biomaterials with antifungal agents in order to prevent the colonization of cells. Thus, different concentrations of 8-hydroxyquinoline derivatives were used to coat the wells of a microtiter plate, and the effect on subsequent biofilm formation was assessed with crystal violet. As shown in Fig. 2B, coating with clioquinol resulted in a reduction of adherent cells and preventing more than 90% of biofilm formation. Compounds 2 and 3 have shown more than 50% reduction in the biofilm biomass, however they were less potent than clioquinol (Fig. 2F and 2J).

We have previously demonstrated that clioquinol **1** inhibits pseudohyphae formation of *C. albicans* and this finding is possibly related to the results found here (unpublished results). The dimorphic transition from a yeast form to a hyphal form represents an essential aspect of the pathogen's biology. It is linked to essential properties for interaction with the host, such as adhesion to epithelial and endothelial cells and appears to be a pivotal biological process required for biofilm formation [29, 30]. Ramage et al. (2002) demonstrated that *C. albicans* mutant strains that cannot germinate were unable to form biofilms [31]. Studies conducted by Baillie et al. (1999) concluded that hyphae are essential elements for providing structural integrity and the multi-layered architecture that is characteristic of mature biofilms [32]. So, our results indicate that the drastic reduction of *C. albicans* biofilm density is likely attributed to a direct consequence of the inhibitory effect of clioquinol on the morphogenetic process. Similar results were found by Ramage et al. (2007). They showed that EDTA blocks hyphal development in *C. albicans* and this chelating agent may also affect biofilm formation [33].

In addition to the importance in biomaterials, biofilm formation is also an important virulence factor of *Candida* sp. because it protects cells from host immune responses [34]. Thus, clioquinol can also contribute for reducing the virulence factor which helps pathogenesis, because it inhibits dimorphic transition (unpublished results) and reduce the biofilm formation.

When the infection by *Candida* is established, antifungal treatment without implant removal is rarely effective. So, device removal is considered a prerequisite to a successful therapy [30,35]. The susceptibility studies to 8-hydroxyquinoline derivatives demonstrated a rise from the $PMIC_{50\text{ and }90}$ to the corresponding $SMIC_{50\text{ and }90}$, a characteristic of *Candida* biofilms previously described by other authors [25,36,37]. Although our study evidences the efficacy of clioquinol in reducing the metabolic activity of sessile *Candida* (ATCC 18804, CG RL32 and CP RL38), the SMIC90 values of clioquinol were increased 128 to 512-fold over the PMIC90. Similarly, Khan and Ahmad (2012) observed that the SMIC of amphotericin B and fluconazole was increased 16 to 512 and 8 to 1024 -fold over, respectively [37]. Shuford et al. (2007) found increased SMIC for amphotericin, caspofungin, and voriconazole [25]. Hawser and Douglas (1995) also found that biofilms were more resistant to amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole [36]. A sharp reduction of the metabolic activity of cells within the biofilm can also be observed when preformed *Candida* biofilms were exposed to 8-hydroxyquinoline-sulfonic acids. However, contrary to clioquinol, these derivatives reduced less than 90% of the metabolic activity of sessile *Candida* within mature biofilms, even at high concentrations. Based on these results, we noted a clear structure-activity relationship. It seems that the presence of the appropriate substituents at the phenyl ring (5-chloro and 7-iodo) are essential for the efficacy in reducing the metabolic activity of sessile *Candida*.

We have also evaluated the ability of 8-hydroxyquinoline derivatives to remove the biofilms from microtiter plate. It was observed that the derivatives were less effective in removing the preformed biofilm, since the concentrations required for this action were higher than those required inhibition of biofilm formation and for inhibition of the metabolic activity of the preformed biofilm. The 50% removal of biofilm was achieved at high concentrations of clioquinol (16 - 64 $\mu g\ ml^{-1}$) and almost complete removal of biofilm was only obtained for *C. glabrata* RL24. In addition, the

compound **2** and **3** removed only 50% of the biofilm biomass of one and two strains, respectively. Polyethylene is a material that has been shown to be an excellent model in the study of microorganisms' biofilm formation. It has the advantage of being commercially available as sterilized microplates permitting the analysis of several samples simultaneously [4]. However it is not used in the production of medical devices. So, after observing the results obtained on a microplate, we chose clioquinol as representative of 8-hydroxyquinoline derivatives to evaluate the effect on preformed biofilm in IUD. Initially, the biofilm forming capacity of different *Candida* species was confirmed by counts of cells grown on the IUD surface. Chassot et al. (2008) also described the ability of *C. albicans* to form biofilm in cupper IUD [4] but studies with non-*albicans* species have not yet been reported to date. They noted that the cells adhered strongly to the three parts of the IUD (copper-covered part of the IUD, part of the IUD without copper and IUD tail) [4]. In addition, studies have proposed that the IUD tail can be decisive for the microorganisms of the vagina to migrate into the endometrial cavity [4,38]. Once the yeast reaches the endometrial cavity, the copper-covered parts appear to contribute to the retention of the microorganism at the site [3-5].

Interestingly, the fungal biofilm in IUDs has been rarely studied. This lack of interest is worrying because IUDs are extraneous surfaces implanted for long periods in the uterine mucosa [3]. More than 80 million women use IUDs as a contraceptive method [3] and IUDs removed from women demonstrated to be highly contaminated with *C. albicans* [38]. Demirezen et al. (2005) showed that IUD users had more *Candida* infection then nonusers [39]. Besides that, IUD retention may cause serious clinical complications and VVC has been resolved with the removal of device [4,40]. Therefore, understanding the action of antifungal agents on IUDs is necessary.

Here we find that clioquinol was very effective, especially for *C. glabrata*, since total biofilm eradication was evidenced in the device. *C. glabrata* is known to be resistant to antifungals, therefore eradicate biofilms of this species is a very important characteristic [41,42]. Clioquinol was also extremely effective for *C. albicans* and *C. tropicalis*, the two species that formed biofilm more strongly. In addition, the data showed that clioquinol resulted in greater biofilm eradication than fluconazole, regardless of species. Fluconazole has showed low eradication (< 70%) of *C. krusei*

and *C. parapsilosis* biofilm. Such findings are very relevant because azole antifungals, especially fluconazole, are widely used to treat VVC [43]. So, the high eradication of biofilms in IUDs associated with rare reports of fungal resistance to clioquinol may make this drug an important option for treating recurrent CCV associated with IUD use.

In conclusion, our data showed a varying level of attenuation of formation, reduction of metabolic activity and removal of *Candida* biofilm in the presence of 8-hydroxyquinoline derivatives in a concentration-dependent manner. Finally, the results indicate a potential application regarding biomaterials for 8-hydroxyquinoline derivatives, especially clioquinol. This drug might be used as coatings to prevent morphological switching from yeast to hyphae and avoid biofilm formation. Furthermore, clioquinol may still have future applications in the treatment of *Candida* infections linked to the use of IUD. Finally, this is the first protocol that quantifies the inhibitory action of compounds on biofilms formed on surfaces IUD.

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Conflict of interest

No conflict of interest declared.

Tables

Table 1. Effects of 8-hydroxyquinoline derivatives **1-3** (**1:** clioquinol; **2:** 8-hydroxy-5-quinolinesulfonic acid; **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid) on planktonic cells *and Candida* biofilms.

Assay	Isolate	Concentration (μg ml^{-1})					
		Clioquinol 1		Compound 2		Compound 3	
		$PMIC_{50}$	**$PMIC_{90}$**	**PMIC50**	**$PMIC_{90}$**	**$PMIC_{50}$**	**$PMIC_{90}$**
Susceptibility of Planktonic Cells	ATCC 18804 (CA)	0.063	0.25	32	64	16	32
	CG RL24	0.031	0.063	8	16	8	16
	CK Den 43	0.5	1	64	128	64	128
	CP RL 38	0.125	0.25	32	64	32	64
	ATCC 750 (CT)	0.063	0.125	16	64	64	128
		$IBFC_{50}$	**$IBFC_{90}$**	**$IBFC_{50}$**	**$IBFC_{90}$**	**$IBFC_{50}$**	**$IBFC_{90}$**
Inhibition of Biofilm Formation	ATCC 18804 (CA)	2	16	256	> 512	512	> 512
	CG RL24	4	16	256	> 512	256	> 512
	CK Den 43	8	> 64	512	> 512	512	> 512
	CP RL 38	8	32	> 512	> 512	> 512	> 512
	ATCC 750 (CT)	4	32	512	> 512	> 512	> 512
		$SMIC_{50}$	**$SMIC_{90}$**	**$SMIC_{50}$**	**$SMIC_{90}$**	**$SMIC_{50}$**	**$SMIC_{90}$**
Susceptibility of Biofilms	ATCC 18804 (CA)	8	64	256	> 512	256	> 512
	CG RL24	4	32	256	> 512	256	> 512
	CK Den 43	8	> 64	> 512	> 512	512	> 512
	CP RL 38	4	32	256	> 512	512	> 512
	ATCC 750 (CT)	16	> 64	512	> 512	> 512	> 512
		BRC_{50}	**BRC_{90}**	**BRC_{50}**	**BRC_{90}**	**BRC_{50}**	**BRC_{90}**
Biofilm Removal	ATCC 18804 (CA)	64	> 64	512	> 512	> 512	> 512
	CG RL24	16	64	256	> 512	512	> 512
	CK Den 43	64	> 64	> 512	> 512	> 512	> 512
	CP RL 38	32	> 64	> 512	> 512	> 512	> 512
	ATCC 750 (CT)	64	> 64	> 512	> 512	> 512	> 512

CA: *C. albicans*; CG: *C. glabrata*; CK: *C. krusei*, CP: *C. parapsilosis;* CT: *C. tropicalis.*
$PMIC_{50}$ and $PMIC_{90}$: minimum inhibitory concentration that inhibits 50% and 90% of the planktonic cells, respectively;
$IBFC_{50}$ and $IBFC_{90}$: concentration that inhibits the formation of 50% and 90% of the biofilm, respectively;
$SMIC_{50}$ and $SMIC_{90}$: minimum inhibitory concentration that inhibits 50% and 90% of the sessile cells, respectively;
CBR_{50} and CBR_{90}: concentration that removes 50% and 90% of the biofilm, respectively.

Table 2. Effects of clioquinol **1** and fluconazole on *Candida* biofilms formed in intrauterine device (IUD)

Isolate	CFU per IUD			Biofilm Eradication (%)	
	Untreated	Clioquinol 1	Fluconazole	Clioquinol 1	Fluconazole
ATCC 18804 (CA)	47200000	980000	2670000	97.92	94.34
CG RL24	191000	0	8000	100.00	85.65
CK Den 43	113000	18000	31000	84.07	48.67
CP RL38	1060000	208000	390000	80.19	62.14
ATCC 750 (CT)	11244000	6000	45000	99.95	98.65

CA: *C. albicans*; CG: *C. glabrata*; CK: *C. krusei*, CP: *C. parapsilosis;* CT: *C. tropicalis;*
CFU: colony-forming units.

Figures

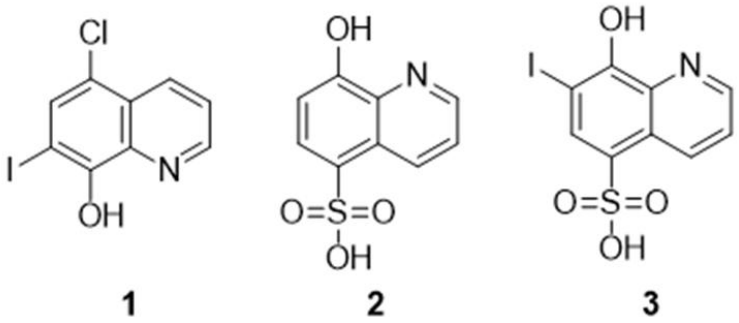

Figure 1. Chemical structures of 8- hydroxyquinoline derivatives **1-3**: (1) clioquinol, (2) 8-hydroxy-5-quinolinesulfonic acid and (3) 8-hydroxy-7-iodo-5-quinolinesulfonic acid.

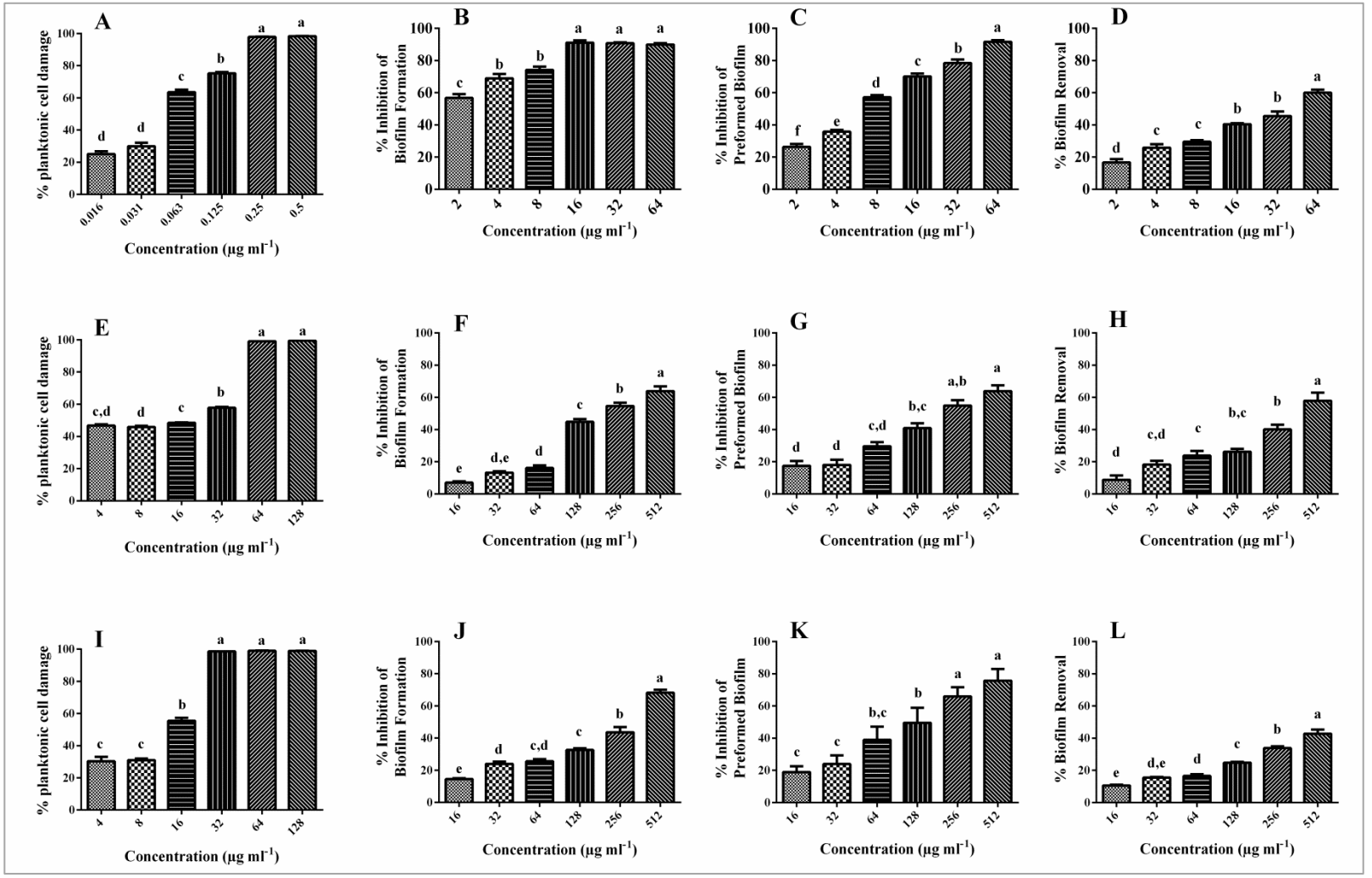

Figure 2. Action of different concentrations of 8-hydroxyquinoline derivatives **1-3** [**1:** clioquinol (A, B, C, D); **2:** 8-hydroxy-5-quinolinesulfonic acid (E, F, G, H); **3:** 8-hydroxy-7-iodo-5-quinolinesulfonic acid (I, J, K, L)] on planktonic cells and biofilm of *Candida albicans* ATCC 18804. Statistically significant differences ($p < 0.05$) are indicated with different letters

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5.4 Artigo 4

O artigo intitulado “Oral clioquinol is effective in the treatment of Toll-deficient *Drosophila* flies infected with *Candida albicans*” será submetido na “Mycoses”.

Title: Oral clioquinol is effective in the treatment of Toll-deficient *Drosophila* flies infected with *Candida albicans*

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Keywords: 8-hydroxyquinolines, clioquinol, antifungal, PK/PD modeling, *Candida* spp., *Drosophila melanogaster.*

Abstract:

Clioquinol **(1)** is a 8-hydroxyquinoline widely used in the 1950s–1970s as antimicrobial agent but its oral formulations were withdrawn from the market due to neurotoxicity reports. Currently there is possibility of reintroduction of oral formulations to new indications and this stimulates the reuse of clioquinol as a systemic antifungal agent. The aim of this study was to evaluate the antifungal activity and toxicological parameters of clioquinol and other 8-hydroxyquinoline derivatives [8-hydroxy-5-quinolinesulfonic acid **(2)** and 8-hydroxy-7-iodo-5-quinolinesulfonic acid **(3)**] using alternative animal models and to study the interaction dynamic of clioquinol with *Candida albicans* and dermatophytes (*Microsporum canis* and *Trichophyton mentagrophytes)*. We used Toll-deficient *Drosophila melanogaster* flies to test the protective effect of 8-hydroxyquinolines against *Candida albicans* infection. Toxicological parameters (arterial pulsation, number of opening of the beak and movement of the embryo) were investigated in chicken embryo. Application of mathematical modeling (optimized Emax models) on time-kill data of clioquinol against *C. albicans* and dermatophytes was performed to obtain pharmacodynamic characteristics. Clioquinol fully protected *D. melanogaster* flies from the infection, whereas compounds **2** and **3** protected flies but the survival did not exceed 63%. The three compounds did not cause significant changes in opening of the beak and movement of the chicken embryo; however clioquinol and compound **2** increased arterial pulsation. Compound **3** did not show changes in these parameters but it was lethal at 1 mg/mL. Effective concentration (EC_{50}) found in modeling indicated that clioquinol was highly effective against *C. albicans* (0.306 µg/mL), *M. canis* (0.181 µg/mL) and *T. mentagrophytes* (0.434 µg/mL) in easily achievable serum levels; and clioquinol achieved maximum effect most rapidly for *C. albicans*. Taken together, these results support the potential of clioquinol to be used as a systemic antifungal agent.

Introduction

Candida species were recently considered the fourth most common pathogen associated to nosocomial blood infections.[1] *Candida* bloodstream infections are a particular concern for patients with immunosuppressive disorders, in intensive care settings, with central venous catheter, and patients receiving parenteral nutrition and/or prolonged broad-spectrum antibiotics.[1,2] Mortality rates can reach up to 40-50% despite prophylactic antifungal use and appropriate treatment methods.[3] Furthermore, candidemia are associated with significant costs and prolonged hospital stay.[2]

The commonly used antifungal drugs such as polyenes and azoles present problems of host toxicity and either intrinsic or acquired resistance by fungi. Current treatments are effective, but the emergence of multidrug-resistant fungi has concerned researchers and health professionals.[4-6]

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, **1**) was widely used in the 1950s – 1970s as an oral antiparasitic agent and has proven antifungal activity.[7,8] The oral formulations were withdrawn from the market in 1970 due to reports of subacute myelo-optic neuropathy (SMON).[9] However, topical formulations for the treatment of skin infections remained available in many countries.[7] In addition to its use as an antimicrobial agent, clioquinol has become a prototype for the development of antitumor compounds and has also recently shown potential effect for the treatment of Alzheimer's, Parkinson and Huntington diseases.[10-12] Thus, the possibility of reintroduction of oral formulations of clioquinol to new indications also stimulates the reuse of this agent as a systemic antifungal agent. In parallel, the interest in others 8-hydroxyquinolines has also increased significantly once they are privileged structures for the design of new drugs with biological effects on various targets.[8,13]

Here, we used Toll-deficient *D. melanogaster* flies as a host model to test the protective effect of 8-hydroxyquinoline derivatives against systemic infection by the human opportunistic fungal pathogen *Candida albicans*. We also assessed toxicological parameters using chicken embryos and applied mathematical modeling on time-kill data of *C. albicans*, *Microsporum canis* and

Trichophyton mentagrophytes to obtain pharmacodynamic characteristics of clioquinol.

Materials and methods

***D. melanogaster* stocks:** Females *D. melanogaster* Oregon^{R} toll-deficient were used in the experiments. Toll-deficient transheterozygote mutants were generated by crossing flies carrying a thermosensitive allele of Tl (Tl^{r632}) with flies carrying a null allele of Tl ($Tl^{l\text{-}RXA}$), as previously described.[14] Fly strains were a gift from Dimitrios P. Kontoyiannis (University of Texas MD Anderson Cancer Center). Standard procedures were used for manipulation, feeding, and housing of flies in all experiments.[14,15]

Fungal strain and antifungal compounds: *C. albicans* ATCC18804 was used as reference strain in the present study. Mathematical modeling was also performed with the dermatophytes *M. canis* MCA 01 (National Program of Quality Control of Brazil) and *T. mentagrophytes* TME 40 (clinical isolate). Clioquinol (**1**), 8-hydroxy-5-quinolinesulfonic acid (compound **2**), and 8-hydroxy-7-iodo-5-quinolinesulfonic acid (compound **3**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were solved in dimethylsulfoxide (DMSO) and diluted in distilled water to obtain a maximum concentration of 2% DMSO to the experiments.

8-Hydroxyquinolines protection experiments: To determine whether the 8-hydroxyquinolines treatment protects flies against candidiasis, groups of 12 Toll-deficient female flies were housed in empty vials for 6–8 h to starve them and then transferred to vials with food containing 100 µL the following concentrations of 8-hydroxyquinolines derivatives: clioquinol **1**, 0.25 µg/mL (MIC), 0.5 µg/mL (2xMIC), 1 µg/mL (4xMIC), 2 µg/mL (8xMIC) and 1 mg/mL; Compound **2**, 64 µg/mL (MIC), 128 µg/mL (2xMIC), 256 µg/mL (4xMIC), 512 µg/mL (8xMIC) and 1 mg/mL; Compound **3**, 32 µg/mL (MIC), 64 µg/mL (2xMIC), 128 µg/mL (4xMIC), 256 µg/mL (8xMIC) and 1 mg/mL. After 24 h, flies were injected in the thorax with a needle that had been dipped into a 0.85% NaCl solution containing 10^{8} *C. albicans* cells/mL. After infection, flies returned

to vials containing antifungal drugs and were maintained at 29 °C. Survival of the flies was assessed daily for seven days after infection. Flies that died within 3 h of injection were considered to have died as a result of the procedure and were excluded from the survival analysis.[14,15] Positive control was provided by infecting flies that were not exposed to antifungal compounds, whereas for negative control flies were punctured with a needle that was dipped into sterile saline. Furthermore, prior to the protection experiments, the flies were exposed to food containing 8-hydroxyquinolines (1 mg/mL) to verify the toxicity of the compounds. Each experiment was performed in triplicate on different days.

Survival curves were plotted using Kaplan-Meier analysis, and differences in survival rates between the groups were analyzed using the log-rank test (GraphPad Prism software; version 6.0;). $P \leq 0.05$ was considered to be statistically significant.

Assessment of tissue fungal burden: Quantification of *Candida* cells in the tissues of flies was performed after seven days of infection. Flies from each group were collected and separated in live and dead flies and ground in 1 mL of 0.85% NaCl solution. Serial dilutions were done in triplicate and plated onto Sabouraud dextrose medium. The number of colony-forming units (CFU) was counted after 48 h of incubation at 35 °C.

The nonparametric Kruskal-Wallis test and post-hoc Dunn's test were used to determine statistically significant differences in fungal burden between the groups. Statistical analyses were performed using the GraphPad Prism software. $P \leq 0.05$ was considered to be statistically significant.

Plasma protein binding: Plasma protein binding occurs predominantly with albumin. To evaluate the influence of protein binding on 8-hydroxyquinoline derivatives activity, we evaluated the fungal growth in protein-free nutrient broth and nutrient broth containing bovine serum albumin. Thereafter, minimum inhibitory concentrations (MIC) of clioquinol **1**, compound **2** and compound **3** were determined against *C. albicans* ATCC 18804 by the standard broth microdilution M27-A3 protocol (CLSI, 2008)[16], using RPMI 1640 supplemented with 40 g/liter bovine serum albumin (INLAB Confiança, São

Paulo, Brazil).[17] Experiments were carried out in duplicate and amphotericin B (União Química, São Paulo, Brazil) was used as positive control.[18]

Pharmacokinetic/Pharmacodynamic (PK/PD) modeling: Time-kill curve data of clioquinol for *C. albicans, M. canis* and *T. mentagrophytes* were previously published by our group and were used here to apply the mathematical modeling.[8] An in vitro kinetic model was used to investigate the antifungal efficacy of constant drug concentrations during 48 h for *C. albicans* (inoculum of 1 – 5 x 10^5 CFU/mL) and 96 h for dermatophytes (inoculum of 1-3 x 10^3 CFU/mL). The selection of clioquinol concentrations tested in each fungal strain was based on their MIC values (MIC, 2xMIC, 4xMIC and 8xMIC) and samples were taken at 0, 3, 6, 12, 24, and 48 h for *Candida* and 0, 3, 12, 24, 48 and 96 h for dermatophytes in order to determine the $\log_{10}$ CFU/mL. A control experiment with untreated fungi was run simultaneously.

Adapted Emax models described by Li et al. (2009)[19] and Treyaprasert et al. (2007)[20] were used in the *C. albicans* (equation 1) and dermatophytes (*M. canis:* equation 2; *T. mentagrophytes:* equation 3) modeling, respectively:

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C^h}{EC_{50} + C^h}\right)\right] N \qquad (1)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)(1 - exp^{-zt})\right] N \qquad (2)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)\right] N \qquad (3)$$

Where d*N*/d*t* is the variation in number of fungi as a function of time, k_0 (h^{-1}) is the fungal growth rate constant in the absence of clioquinol, *k*max (h^{-1}) the maximum killing rate constant (maximum effect), EC_{50} (µg/mL) the

concentration of clioquinol necessary to produce 50% of maximum effect, *C* (µg/mL) the concentration of clioquinol at any time (*t*), *N* (log_{10} CFU/mL) the number of viable fungal cells and N_{max} is the maximum number of cells. The models account for delays in growth (x) and onset of kill (*z*), and incorporate a Hill factor (*h*) that modifies the steepness of the slopes and smoothes the curves.

The mathematical modeling of the kill curve data were performed with the non-linear regression software program Scientist® 3.0 (Micromath, Salt Lake, UT, USA). Graphs were visually inspected for quality of fit and model selection criterion (MSC), coefficient of determination (R^2) and correlation between measured and calculated data points.

Then, in order to compare the concentration levels required for the effect defined in the PK/PD modeling with the possible values to be achieved in vivo, the plasma concentration levels by time of a study in humans available in the literature for oral dose os clioquinol[21] were modeled using the two-compartment model (analysis by Scientist v3). Three oral dosing regimens were simulated: i) a predictive dose of 25 mg every 12 h; ii) a predictive dose of 50 mg every 12 h; iii) a predictive dose of 100 mg every 24 h.

Behavioral test on chicken embryo:

White Lohmann fertile eggs (Lohmann selected Leghorn, São José do Rio Preto, SP, Brazil) were used in this test. The eggs were maintained at 37.6 ± 0.4 °C and 50% relative humidity while being turned automatically until day 12. On day 12, the eggs were opened and 100 µl of clioquinol **1**, compound **2** and compound **3** at 1 mg/mL were added to each egg. Compound **3** was also tested at 0.25 mg/mL. Epinephrine (1 mg/mL) and saline solution (0.85%) were used as positive and untreated controls, respectively. The following parameters were observed for two minutes in the chicken embryos: arterial pulsation, number of opening of the beak and movement of the embryo.

The results were analyzed by one-way ANOVA as well as Dunnett's multiple comparisons test using GraphPad Prism software. $P \leq 0.05$ was considered to be statistically significant.

Results

Pilot experiments showed that all 8-hydroxyquinolines were found to be nontoxic to Toll-deficient *D. melanogaster* flies at 1 mg/mL (data not shown). The efficacy of 8-hydroxyquinolines treatment on Toll-deficient flies infected with *C. albicans* is displayed in Figure 1.

Flies fed with food containing all concentrations of clioquinol, except 0.25 µg/mL, had a significantly higher survival rate than untreated control flies. Indeed, flies were fully protected when exposed to 1 mg/mL of clioquinol **1** since this group did not present statistical difference when compared to the uninfected group control (Figure 1A). Flies treated with compound **2** (512 µg/mL and 1 mg/mL) and **3** (128 and 256 µg/mL; and 1 mg/mL) also had a significantly higher survival rate than untreated control flies (compound **2**: $P < 0.01$; compound **3**: $P < 0.001$) (Figure 1B and 1C, respectively). Furthermore, both live and dead flies treated with 1 mg/mL of 8-hydroxyquinolines had significantly lower fungal burdens than did untreated control flies after seven days of infection (clioquinol **1** and compound **3**, $P < 0.01$; compound **2**, $P < 0.05$) (Figure 2). Flies fed with food containing 512 µg/mL of compound **2** also had significantly lower fungal burdens than did untreated control flies ($P < 0.05$).

The effects of compounds on fungal growth in medium containing 40g/L bovine serum albumin was similar to the effects of amphotericin B. MICs of clioquinol 1 against *C. albicans* increased from 0.25 to > 8 µg/mL after adding of bovine serum albumin. MICs of compound **2** and **3** increased from 64 and 32 to 256 and 128 µg/mL, respectively. When the strain was treated with amphotericin B in a medium supplemented with bovine serum albumin, MICs increased from 0.25 to 2 µg/mL.

Clioquinol **1** and compound **2** at 1 mg/mL showed to be not lethal for chicken embryo. These compounds did not cause significant changes in the number of opening of the beak and movement of the embryo (Figure 3B and 3C), but significantly increased arterial pulsation ($P \leq 0.05$) (Figure 3A). Compound **3** at 1 mg/mL was lethal for the chicken embryo. Thereby, a new sublethal concentration was defined to evaluate the behavioral parameters. The 0.25 mg/mL concentration of this compound did not show significant changes in all parameters evaluated. As expected, a significant increase ($P \leq 0.05$) of the number of opening of the beak, in movement of the embryo and in arterial

pulsation was observed for chicken embryos treated with epinephrine (positive control) (Figure 3).

The fitted curves of clioquinol against three different fungal species are shown in Figure 4 and the determined pharmacodynamics parameters are listed in Table 1. Data showed that clioquinol is highly effective against *C. albicans*, *M. canis* and *T. mentagrophytes* as EC_{50} were 0.306 µg/mL and 0.181 µg/mL and 0.434 µg/mL, respectively. In addition, it can be verified that EC_{50} values for dermatophytes were lower than MIC value. To simulate the plasma levels expected for different oral dosing regimens of clioquinol, pharmacokinetic data were modeled and are showed in the figure 5.

Discussion

Based on the results of 8-hydroxyquinolines protection experiments, we observed that the derivatives had a significant concentration-dependent protective effect in Toll-deficient *D. melanogaster* infected with *C. albicans*. The paradoxical growth previously reported for compound **3** during time-kill studies was not noticed during in vivo studies with *D. melanogaster.*[8] The highest concentrations evaluated were those in which greater protection of the flies were obtained. These results showed that the abilities of subpopulations to survive at high drug concentrations (paradoxical growth) may be only an in vitro effect as suggested earlier.[8,22,23] Although treatment with compounds **2** and **3** showed increased survival of infected flies, these derivatives protected only 45% and 63% of them, respectively.

Clioquinol **1** (1 mg/mL) was the only 8-hydroxyquinoline derivative that fully protected the infected flies. Similar results were found by Chamilos et al. (2006) using fluconazole.[24] They found that fluconazole (1 mg/mL) also had a significant protective effect in flies infected with a susceptible *C. albicans* strain, but this drug did not fully protect the infected flies since survival did not exceed 80%. Previous studies conducted by our research group has shown that clioquinol **1** inhibits pseudohyphae formation of *C. albicans*[25], and this this finding is possibly related to the results found here. Noble et al. (2017) showed that *C. albicans* mutants that grow only as yeasts or filaments are both defective in bloodstream infection models, suggesting that the ability to interconvert

between different cell types is required for virulence.[26] So, our results indicate that the protection of the flies may be attributed to a direct consequence of the inhibitory effect of clioquinol on the morphogenetic process.

Clioquinol has been shown high affinity to albumin and this may be indicative of low bioavailability and compromised efficacy.[27] However this property apparently did not block the in vivo action in *D. melanogaster*. Several antifungal drugs, such as amphotericin B, ketoconazole and itraconazole, also exhibit this property.[17,18]

Behavioral parameters of chicken embryos were used to evaluate toxicological responses. The results showed that high concentrations of clioquinol **1** and compound **2** did not cause interruption of embryogenesis. The low toxicity found here corroborates with the results found in *D. melanogaster* and suggeste safety during pregnancy. The significant increase in the arterial pulsation presented by the chicken embryo exposed to clioquinol **1** and compound **2** indicates the stimulating action as probable side effect of these compounds. On the other hand, it was possible to observe high toxicity of compound **3** in chicken embryos once 1 mg/mL was lethal for this animal model. However, animals during embryonic development are more sensitive and vulnerable than during the other stages of life.[28]

PK/PD modeling is applied to predict tested and untested doses, to improved dosing regimens of currently available antibiotics and to facilitate development of new antimicrobial agents.[29] Optimized Emax models are useful to describe the relationship between concentration and effect and have been shown to be successfully applied in our study with clioquinol.[19,30,31] It is important to note that it was necessary to apply different PK/PD models for each individual strain in order to obtain a better description of the antifungal activity.

Clioquinol rapidly achieved kill rates for *C. albicans* reaching the maximum effect after 13h. On the other hand, the maximum effect for *M. canis* and *T. mentagrophytes* was reached after 49h and 24h, respectively. So, the data show that clioquinol achieved highest kill rates against the isolate that proliferated most rapidly. Previous studies with voriconazole also showed a faster effect for strains that grow rapidly.[19,32] In addition, effective concentration (EC_{50}) values indicated that clioquinol was highly effective against *C. albicans*

and dermatophytes in easily achievable serum levels (Figure 5). It's possible predict that during 6 days the three dosing regimens tested are sufficient to maintain plasma concentrations above the effective antifungal concentration, suggesting that the lowest dose (25 mg every 12 h) could be used to treat fungal diseases. A phase I study of clioquinol in patients with advanced hematologic malignancies showed that dose-limiting neurotoxicity and abdominal pain were observed at a dose of 1600 mg twice daily.[33] Before being withdrawn from the market, the antimicrobial therapeutic dose of clioquinol varied among 250 to 3500 mg per day. Nonetheless, most patients were treated with 750 to 1000 mg per day.[34-36] This approach of combining in vitro time–kill data with existing in vivo PK data might serve to delineate a model for future studies in animals in order to define optimal antifungal regimens of clioquinol. Despite the several advantages of using in vitro experiments combined with modeling, the growth and killing behavior assessed in vitro usually represents the antimicrobial effect imposed by the drug without considering the host immune system.[29]

Finally, we found that 8-hydroxiquinoline derivatives, especially clioquinol, successfully protected Toll-deficient *Drosophila* flies infected with *C. albicans*, demonstrating the antifungal action of compounds when administered orally. The PK/PD modeling also showed high effectiveness of clioquinol against different fungal strains including *C. albicans* and dermatophytes. Since the possibility of reintroduction of clioquinol oral formulations is imminent, these findings are important for conducting future experiments of systemic infection and pharmacokinetics in mammalian animal models. Simulations based on previous trials can also predict the outcome and help to come up with dose recommendations.

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Conflict of interest

No conflict of interest declared.

Tables

Table 1. Pharmacodynamic parameters determined for clioquinol and goodness of fit criteria.

Parameters	Fungi		
	C. albicans (ATCC 18804)	*M. canis* (MCA 01)	*T. mentagrophytes* (TME 40)
MIC* (µg/mL)	0.250	0.250	0.5
K_0 (h^{-1})	0.152	0.259	0.552
K_{max} (h^{-1})	0.051	0.014	0.029
EC_{50} (µg/mL)	0.306	0.181	0.434
N_{max} ($\log_{10}$ CFU/mL)	7.366	3.977	4.010
x (h^{-1})	0.957	0.031	0.002
z (h^{-1})	-	0.282	-
h	2.078	-	-
MSC/R^2 (range)	2.449- 3.640/0.999	2.307 - 4.166/0.999	2.460 – 4.659/0.999

* Previously determined by broth microdilution.[8]

Figures

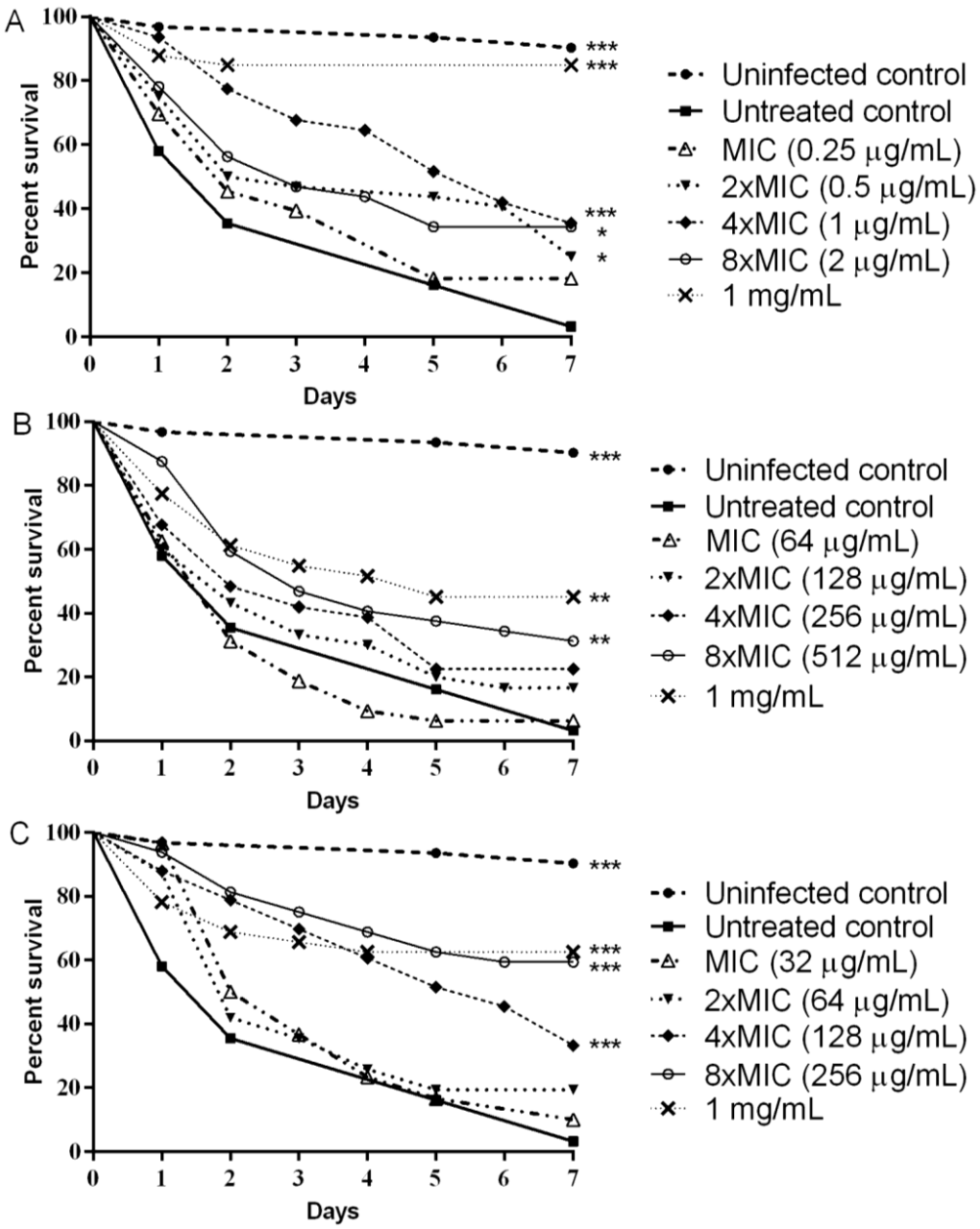

Figure 1. Percent survival of Toll-mutant *Drosophila melanogaster* injected with *Candida albicans* and treated with clioquinol **1** (A), compound **2** (B), compound **3** (C). The asterisks indicate statistical difference compared to the untreated control (*** p< 0.0001; **p< 0.001; *p<0.05).

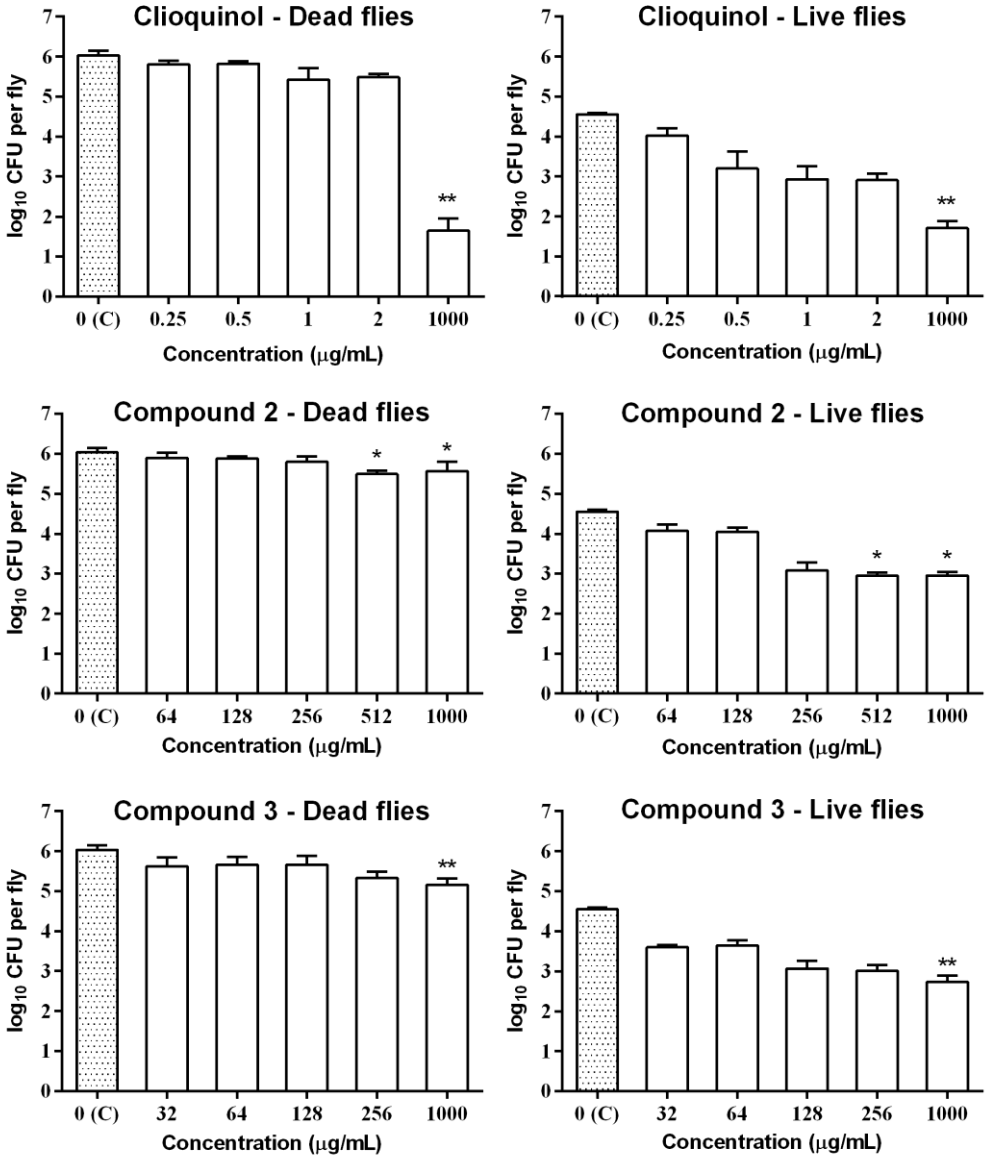

Figure 2. Fungal burdens of live and dead flies after seven days of infection and treatment with different concentrations of 8-hydroxyquinoline derivatives. The asterisks indicate statistical difference compared to the untreated control (C) (**p< 0.001; *p<0.05).

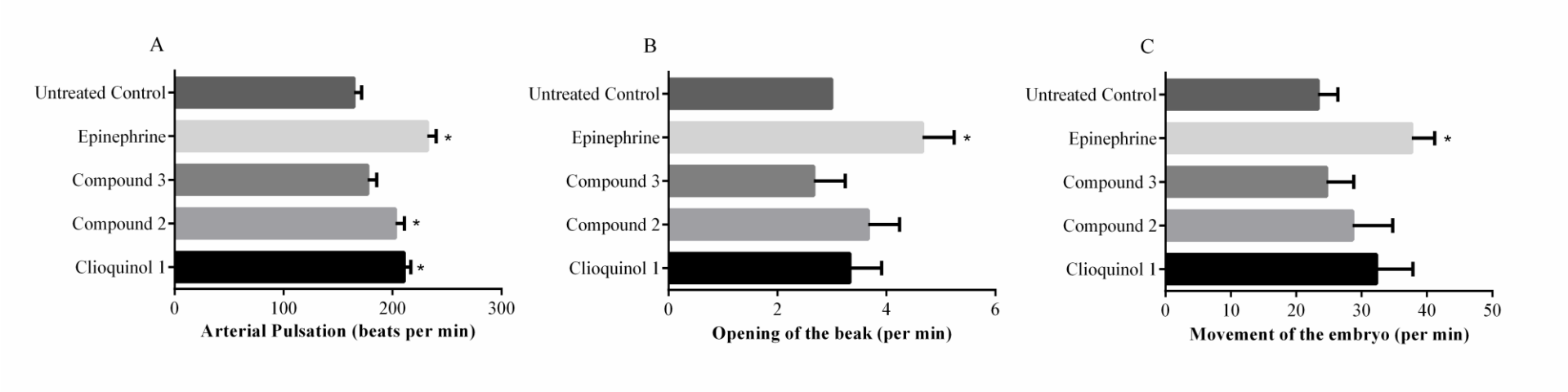

Figure 3. Behavioral testing in chicken embryos exposed to clioquinol **1** (1 mg/mL), compound 2 (1 mg/mL), compound **3** (0.25 mg/mL), epinephrine (positive control; 1 mg/mL) and saline solution (untreated control) with observation of arterial pulsation (A), opening of the beak (B) and movement of the embryo (C). The asterisks indicate statistical difference compared to the untreated control (* $p < 0.05$).

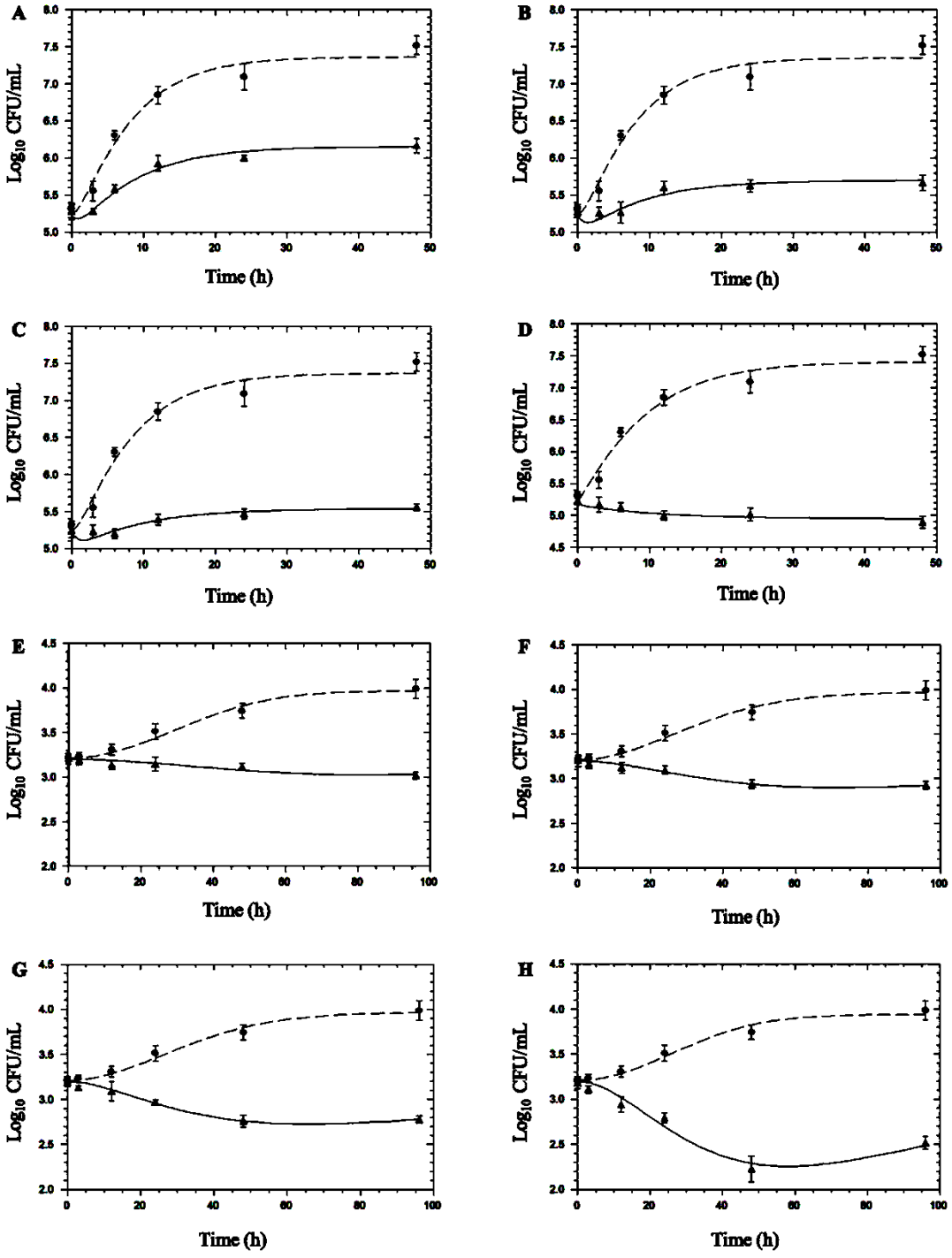

Figure 4. Fitted curves resulting from modeling fungal growth (dashed line) and kill for clioquinol (full line) against *C. albicans* ATCC 18804 (A-D) (A: MIC; B: 2xMIC; C: 4xMIC; D: 8xMIC); *M. canis* MCA 01(E-H) (E: MIC; F: 2xMIC; G: 4xMIC; H: 8xMIC) and *T. mentagrophytes* TME 40 (I-L) (I: MIC; J:2xMIC; K: 4xMIC; L: 8xMIC) isolates.
* MIC: minimum inhibitory concentration; CFU: colony-forming units.

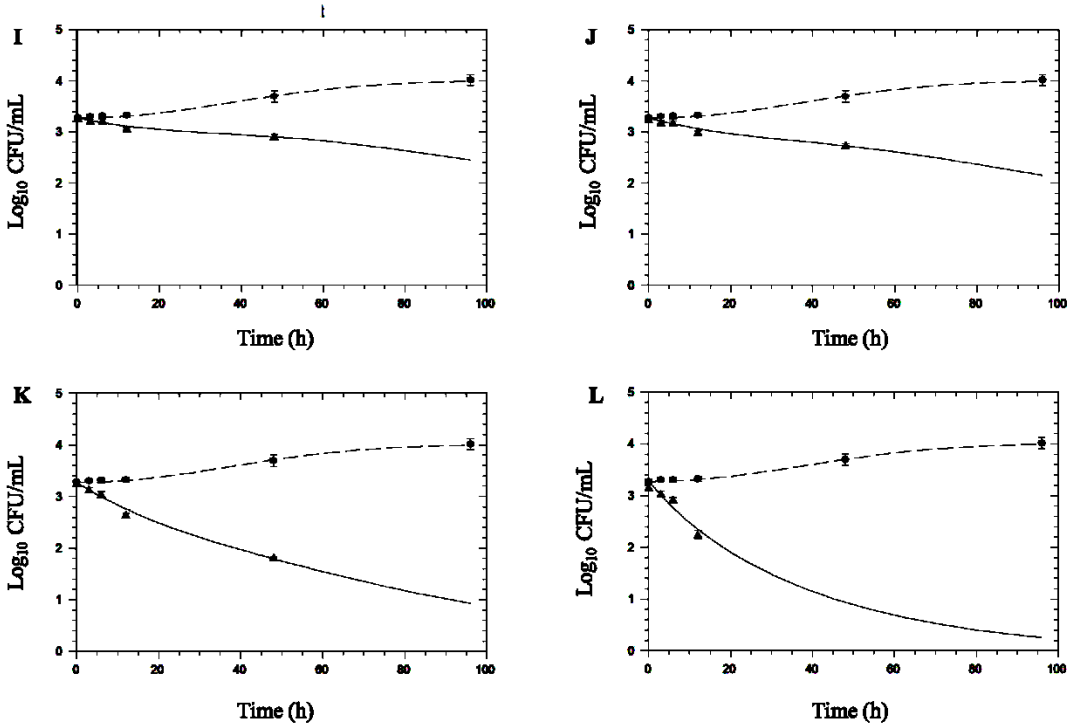

Figure 3. (*Continued*).

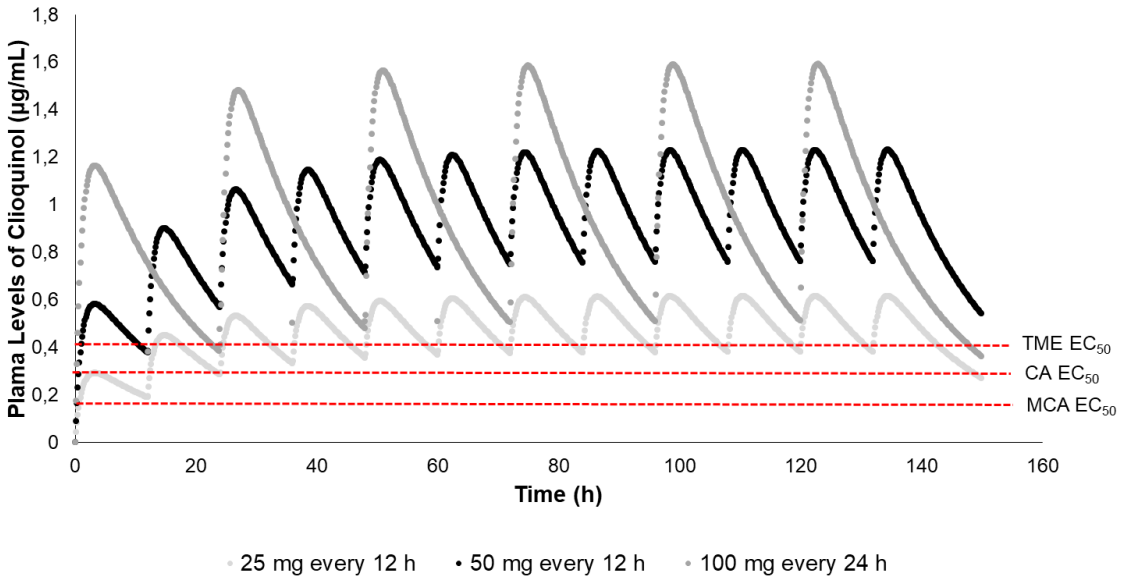

Figure 5. Plasma profile modeling of clioquinol in humans using three predictive oral dosage regimens (25 mg every 12 h; 50 mg every 12 h; and 100 mg every 24 h). The effective concentrations (EC_{50}) of clioquinol for *C.albicans* (CA), *M. canis* and *T. mentagrophytes* are indicated with the red dash.

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5.5 Artigo 5

O artigo intitulado “8-Hydroxyquinoline-5-sulfonamides are promising antifungal candidates for topical treatment of dermatomycosis” será submetido na “Bioorganic & Medicinal Chemistry”.

Title: 8-Hydroxyquinoline-5-sulfonamides are promising antifungal candidates for topical treatment of dermatomycosis

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Abstract

The antifungal therapy currently available presents several complications as high toxicity, side effects, narrow activity spectrum and evolution of multidrug-resistant fungi. Therefore, there is an emerging need to fill the pipeline with new antifungal drugs. The interest in hydroxyquinolines has exponentially grown in the last two decades due to the high synthetic versatility. Recent study conducted by our group has explored the antifungal potential of 8-hydroxyquinoline derivatives; and 8-hydroxyquinoline-5-sulfonamides show promising results. With the future prospect of developing a topical drug to treat candidiasis and dermatophytosis, the purpose of this study was to evaluate the potential for topical toxicity of 8-hydroxiquinoline-5-(N-4-chlorophenyl)sulfonamide (PH151) and 8-hydroxiquinoline-5-(N-4-methoxyphenyl)sulfonamide (PH153), as well as to explore pharmacodynamic characteristics by time-kill studies with mathematical modeling and some insights into the mechanism of action. Data from this study showed that PH151 and PH153 sulfonamide derivatives are nonirritant molecules with absence of topical toxicity. They act on the cell wall of *Candida* sp. and dermatophytes and inhibit the pseudohyphae of *C. albicans*. PH151 exhibited fungistatic activity; and PH153 was fungistatic for *C. albicans* and fungicidal for dermatophytes (concentration and time dependent). The EC_{50} ranged from 0.873 to 1.329 µg/mL for PH151 and 7.105 to 8.865 µg/mL for PH153; and PH153 achieved maximum effect more rapid than PH151. Taken together, these results support the potential of these molecules as a promising antifungal candidate for the treatment of candidiasis and dermatophytosis.

Keywords: 8-hydroxyquinoline derivatives, topical antifungal agent, mechanism of action, PK/PD modeling *Candida* spp., dermatophytes.

1. Introduction

Data suggest that several fungal diseases are under-documented.[1] Even so, their incidence exceeds expectations affecting approximately 1.2 billion individuals.[2] Fungi can cause serious invasive infections with a high mortality rate.[2,3] Moreover, these microorganisms also cause mucosal infections and superficial, cutaneous and subcutaneous mycoses. Specific examples include vaginal, esophageal and oropharyngeal candidiasis, as well as dermatophytosis in various regions of the body.[3] In these cases, mortality is practically null, but they have high incidence and decrease the quality of life of the affected individuals; in addition, they may be persistent and require continuous treatment.[4]

There are few drugs in the pharmaceutical market to treat fungal infections. Besides that, the antifungal therapy presents several complications due to the high toxicity, low tolerance to high doses, unwanted side effects and narrow activity spectrum.[5,6] The small number of antifungal drugs available would not be a problem if responses to therapies were satisfactory. However, usually this is not the case.[3] In addition to the challenges mentioned above, clinical management of fungal diseases is further compromised by the emergence of isolates resistant to antifungal agents, which restricts the treatment options. Even more threatening is the evolution of multidrug-resistant organisms, which have low susceptibility to various classes of antifungal agents.[7]

Currently, only a few promising molecules are in preclinical development or in the early stages of clinical development; which does not bring us the certainty that these molecules will be a new antifungal agent.[5] Therefore, there is an emerging need to fill the pipeline with new antifungal drugs.[3,5]

The interest in hydroxyquinolines has exponentially grown in the last two decades due to the high synthetic versatility, which allows the generation and optimization of a large number of derivatives that are widely exploited for biological purposes.[8,9] Recent studies conducted by our group have explored the antifungal potential of 8-hydroxyquinoline derivatives, showing relevant action of sulfonic acid derivatives against *Candida* spp. and dermatophytes.[10] Furthermore, the conversion of the sulfonic acid into a sulfonamide has shown very interesting advantages in relation to the increase of the antifungal effect,

highlighting the 8-hydroxiquinoline-5-(N-4-chlorophenyl)sulfonamide (PH151) and 8-hydroxiquinoline-5-(N-4-methoxyphenyl)sulfonamide (PH153) molecules (figure 1).[11]

With the future prospect of developing a topical drug to treat candidiasis and dermatophytosis, the purpose of this study was to evaluate the potential for topical toxicity of PH151 and PH153. Still, the time-kill studies with subsequent application of mathematical modeling were conducted in order to determine pharmacodynamic characteristics, resulting in the selection of an appropriate drug candidate to advance. Some insights into the mechanism of action of these compounds are also presented here.

2. Materials and methods

2.1. Fungal Strains

Eight isolates were included in this study: *Candida albicans* ATCC 18804, *C. glabrata* CG RL24, *C. krusei* CK Den 43, *C. parapsilosis* CP RL 38, *C. tropicalis* ATCC 750, *Microsporum canis* MCA 01, *M. gypseum* MGY 50, *Trichophytum mentagrophytes* TME 40 and *T. rubrum* TRU 43. All isolates are deposited into the Mycology Collection of Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil) and were obtained from the National Program of Quality Control of Brazil and clinical samples. Reference strains of *C. albicans* (ATCC 18804) and *C. tropicalis* (ATCC 750) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and were included as controls.

2.2. Antifungal Agents

8-Hydroxiquinoline-5-(N-4-chlorophenyl)sulfonamide (compound PH151) and 8-hydroxiquinoline-5-(N-4-methoxyphenyl)sulfonamide (compound PH153) were synthesized as described by Joaquim et al.[11] These compounds were solved in DMSO (Sigma-Aldrich) and diluted in the assay medium to obtain a maximum concentration of 2% DMSO to the experiments.

2.3. Histopathological evaluation of pig ear skin exposed to of 8-Hydroxyquinoline-5-sulfonamides

Tissue samples from adult male pigs, freshly slaughtered at the Institute Federal of Santa Catarina – campus Concordia, were used to evaluate the formation of tissue damage due to the action of PH151 and PH1513. Pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture, Livestock and food supply, respecting animal welfare.[12] Ear skin of pigs was used in this study. The tissues were removed within a period of 5 min after the slaughter; the hairs were carefully removed by an electric trimmer and transported to the laboratory in ice-cold Krebs-Hepes buffer (INLAB, Brazil). Skin samples were mounted in Franz diffusion cells (Logan Instrument Corp., NJ, USA) with the diffusion area of approximately 1.75 cm^2. The epidermal side of the skin was exposed to PBS pH 7.0 (negative control) and to 0.1 M NaOH solution (positive control) for a period of 6 h. The 8-hydroxyquinoline-5-sulfonamides were dissolved in a PBS pH 7.0 at a concentration of 1mg/mL (a higher concentration than MIC). The epidermal side of the skin was subjected to contact with this solution for a period of 6 h. Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely and stained with hematoxylin and eosin (HE), and examined under light microscopy. The experiments were carried out in triplicate.

2.4. Hens' Egg Test on the Chorioallantoic Membrane (HET-CAM)

Fresh white fertile eggs Lohmann commercial breed (Lohmann selected Leghorn, LSL, Brazil) were used in the HET-CAM test. The eggs were kept at optimized incubation conditions (temperature between 38 to 39°C and humidity between 55 and 60% for 10 d). On the 10^{th} day, the egg shell, around the airspace, was carefully removed with a rotary tool (Dremel, Racine WI). Afterwards, 0.3 ml of each substance was added to each egg, respectively (negative control – 0.9% saline solution; positive control – 0.1 M NaOH solution; test substances – PH151 and PH153). Irritation effect was evaluated in times of 30 s, 2 min, and 5 min after the application of each substance. The experiment was performed in triplicate. The result of the irritation score (IS) was given according to the equation below, on a scale from 0 to 4.9 denoting nonirritantion (or practically no irritation) and 5.0 to 21 denoting irritantion (moderate/severe or extreme irritation).[13] Comparison of IS was analyzed by one-Way ANOVA and

Tukey's multiple comparisons test. P ≤ 0.05 was considered statistically significant.

$$IS = \left(\left(\frac{(301 - Hemorrhage\ Time)}{300}\right)x5\right) + \left(\left(\frac{(301 - Lysis\ Time)}{300}\right)x7\right) + \left(\left(\frac{(301 - Coagulation\ Time)}{300}\right)x9\right) \quad (1)$$

2.5. Time-Kill Assay

Time-kill assay was performed for PH151 and PH153 against *C. albicans* ATCC 18804, *M. canis* MCA 01 and *T. mentagrophytes* TME 40. The procedures were conducted as previously described by Klepser et al. (1998) and Ghannoum et al. (2013).[14,15]

For *C. albicans*, a yeast cell suspension was prepared from 24 h culture at 35°C on SDA (Sabouraud dextrose agar; Himedia; India) with chloramphenicol and it was adjusted to a 0.5 McFarland turbidity standard (1 – 5 x 10^6 CFU/ml). A 1:10 dilution of this suspension was made by adding 1 ml of fungal suspension to 9 ml of RPMI 1640 with or without (growth control) the desired amount of antifungal agents. This dilution resulted in a solution with an inoculum of approximately 1 – 5 x 10^5 CFU/ml.[14] For the filamentous fungi (*M. canis* MCA 01 and *T. mentagrophytes* TME 40), a suspension of each fungus was prepared from 7 days culture on potato dextrose agar (Himedia) and it was adjusted to 1-3 x 10^3 CFU/ml in RPMI 1640 with or without (control) the desired amount of antifungal agents.[15]

The concentrations used for each compound were: MIC, MICx2 and MICx4. All test solutions and controls were made in triplicate and they were incubated at 35 °C (*Candida*) or 30 °C (dermatophytes). At predetermined time points (0, 3, 6, 12, 24, and 48 h for *Candida;* and 0, 3, 6, 12, 24, 48 and 96 h for dermatophytes), 100 µl samples were aseptically removed from each tube and serially diluted 10-fold (until 10^{-3}) with sterile water. Then, 15 µl directly from the test solution and each dilution solution were spread onto a SDA plate. Plates were incubated at 35 °C/48 h for *Candida* and 30 °C/96 h for dermatophytes.

After this period, the colony count was performed for determination of $\log_{10}$ CFU/ml.

Fungicidal effect was considered when there was ≥99.9% decrease in the $\log_{10}$ of the number of CFU/ml compared with starting inoculum.

2.6. PK/PD modeling

Kill curve data were used to apply the mathematical modeling. Adapted Emax models described by Li et al. (2009) and Treyaprasert et al. (2007) were used for *C. albicans* (equation 2)[16], *M. canis* (equation 3)[17] and *T. mentagrophytes* (equation 4 for PH151; and equation 5 for PH153)[17] modeling:

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C^h}{EC_{50} + C^h}\right)\right] N \quad (2)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)(1 - exp^{-zt})\right] N \quad (3)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - exp^{-xt}) - \left(\frac{K_{max}C}{EC_{50} + C}\right)\right] N \quad (4)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right) - \left(\frac{K_{max}C}{EC_{50} + C}\right)(1 - exp^{-zt})\right] N \quad (5)$$

Were d*N*/d*t* is the variation in number of fungal cells as a function of time, k_0 (h^{-1}) is the fungal growth rate constant in the absence of clioquinol, *k*max (h^{-1}) the maximum killing rate constant (maximum effect), EC_{50} (µg/mL) the concentration of clioquinol necessary to produce 50% of maximum effect, *C* (µg/mL) the concentration of clioquinol at any time (*t*), *N* ($\log_{10}$ CFU/mL) the number of viable fungal cells and N_{max} is the maximum number of cells. The models account for delays in growth (x) and onset of kill (*z*), and incorporate a Hill factor (*h*) that modifies the steepness of the slopes and smoothes the curves.

The mathematical modeling of the kill curve data were performed with the non-linear regression software program Scientist® 3.0 (Micromath, Salt

Lake, UT, USA). Graphs were visually inspected for quality of fit and model selection criterion (MSC), coefficient of determination (R^2) and correlation between measured and calculated data points.

2.7. Sorbitol Protection Assay

The effect of 8-hydroxyquinoline-5-sulfonamides on the integrity of the fungal cell wall was evaluated by sorbitol protection assay using one strain of each species (*C. albicans* ATCC 18804, *C. glabrata* CG RL24, *C. krusei* CK Den 43, *C. parapsilosis* CP RL 38, *C. tropicalis* ATCC 750, *M. canis* MCA 01, *M. gypseum* MGY 50, *T. mentagrophytes* TME 40 and *T. rubrum* TRU 43). Minimum inhibitory concentrations (MICs) of PH151 and PH153 were determined by the standard broth microdilution (CLSI M27-A3 for *Candida* spp.; CLSI M38-A2 for dermatophytes)[18,19] in the absence and presence of 0.8 M sorbitol (Sigma-Aldrich) added to the assay medium (RPMI 1640; Gibco) as an osmoprotectant. Experiments were performed in duplicate and anidulafungin (Pfizer, New York, USA) was used as positive control. MICs were measured after 2 and 7 days of incubation at 35 °C for *Candida* spp.; and after 4 and 7 days of incubation at 30 °C for dermatophytes.[20]

2.8. Cellular leakage effect

The cell leakage was assessed by measuring 260-nm-absorbing materials released to the medium, especially uracil nucleotide, which exhibit the strongest absorbance.[21] The assay was carried out with one representative strain of each genus studied (*C. albicans* ATCC 18804, *M. canis* MCA 01 and *T. mentagrophytes* TME 40) and the procedures were conducted as previously described.[22] *Candida* cells cultured at 35 °C for 48 h on SDA (Sabouraud Dextrose Agar; HiMedia; India) were washed three times (3000 rpm for 10 min, Spinlab SL-5M, Ribeirão Preto, Brazil) and diluted to approximately 1-5x10^6 cells/ml with cold 0.16 M MOPS buffer, pH 7. A 1:10 dilution of fungal suspension was made in cold MOPS buffer with each 8-hydroxyquinoline-5-sulfonamide, resulting in a suspension with fungal inoculum of 0.1-0.5 x 10^6 cells/ml. For dermatophytes, fungal suspensions were prepared with cells cultured at 30 °C for 10 days on PDA (potato dextrose agar; HiMedia; India) and adjusted to 1–3 x 10^3 cells/ml in cold MOPS buffer with addition of PH151.

Fungal suspensions were treated with MIC of PH151 and PH153. Untreated cells incubated with MOPS buffer were used as negative control. Cells treated with amphotericin B (6.25 µg/ml) and SDS (2%) (Sodium dodecyl sulfate; Neon, São Paulo, Brazil) were prepared as positive control. The experiments were carried out in triplicate and incubation was performed at 35 °C for *Candida* spp. and 30 °C for dermatophytes. Aliquots were taken at different intervals (6, 24 and 48 h for *Candida* spp.*;* 6, 24, 48 and 96 h for dermatophytes) and centrifuged at 10000 rpm for 10 min (Biosystems MCD2000, Curitiba, Brazil). The supernatants were collected for absorbance analysis at 260 nm in an Agilent Technologies 8453 spectrophotometer (Santa Clara, EUA). Solutions of SDS (2%), amphotericin B (6.25 µg/ml), and PH151 (MIC and 8xMIC) without fungal inoculum were used as blank for absorbance readings. The results were expressed as means ± standard deviation. Absorption obtained for PH151, PH153, SDS and amphotericin B were compared to the absorption of the untreated control and analyzed by one-Way ANOVA as well as Dunnett's multiple comparisons test. $P \leq 0.05$ was considered statistically significant.

2.9. Ergosterol binding assay

The ability of the 8-hydroxyquinoline-5-sulfonamides to complex with ergosterol in the fungal membrane was evaluated by ergosterol binding assay for one strain of each species (*C. albicans* ATCC 18804, *C. glabrata* CG RL24, *C. krusei* CK Den 43, *C. parapsilosis* CP RL 38, *C. tropicalis* ATCC 750, *M. canis* MCA 01, *M. gypseum* MGY 50, *T. mentagrophytes* TME 40 and *T. rubrum* TRU 43). The MICs of PH151 and PH153 were determined by the standard broth microdilution[18,19] in the absence and presence of different concentrations (50-250 µg/ml) of external ergosterol (Sigma-Aldrich, St. Louis, MO, USA) added to the assay medium (RPMI 1640). Experiments were performed in duplicate and amphotericin B (União Química, São Paulo, Brazil) was used as positive control. MICs were measured after 48 h of incubation at 35 °C for *Candida* spp. and 96 h of incubation at 30 °C for dermatophytes.[20]

2.10. Scanning electron microscopy (SEM)

Morphological changes in fungal cells grown in the presence of PH151 and PH153 were determined using the broth macrodilution method[18,19] followed by SEM, as described below. After the incubation period required for the broth macrodilution method (*C. albicans*: 48 h at 35 °C; *M. canis* and *T. mentagrophytes*: 96 h at 30 °C), fungal cells treated with PH 151 (MIC/2) and untreated cells (control) were washed three times with PBS (3000 rpm for 5 min; Biosystems MCD2000, Curitiba, Brazil) and fixed in 1 ml of modified Karnovsky's fixative adapted from Joubert et al. (2015).[23] After, the wells were washed three times (3000 rpm for 5 min) in 0.1 M sodium cacodylate buffered at pH 7.2 containing 0.2 M sucrose, and 2 mM $MgCl_2$ with the aid of two pipettes, which were used for addition and concurrent removal to avoid air exposure. Cells were adhered in coverslips previously functionalized with poly-L-lysine for 1 h. Adhered cells were dehydrated in a series of freshly made solutions of graded acetone 30, 50, 70, 95 (5 min) and 100% (10 min). Samples were then subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10 Carl, Oberkochen, Germany) operating at 10kV.

3. Results

3.1 Histopathological evaluation of pig ear skin exposed to of 8-Hydroxyquinoline-5-sulfonamides

The histopathological evaluation by microscopic analysis of the pig ear skin exposed to PH151 and PH153 demonstrated no lesions (Figure 2) as well as PBS (negative control). This result confirmed a low topical toxicity of 8-hydroxyquinoline-5-sulfonamides.

3.2. Hens' Egg Test on the Chorioallantoic Membrane (HET-CAM)

The irritation score (IS) of 8-hydroxyquinoline-5-sulfonamides was represented by equation 1. PH151 and PH153 showed IS of 3.15 and 2.91, respectively, showing statistically significant difference ($p \leq 0.05$) when compared with the positive control (IS= 19.83). PH153 presents IS significantly

lower than PH151, however, both were classified as nonirritant according to tested methodology.

3.3. Time-Kill Assay and PK/PD modeling

The PH151 compound exhibited fungistatic activity for *C. albicans* and dermatophytes. The PH153 compound showed fungistatic effect for *C. albicans* and exhibited fungicidal activity (≥99.9% reduction in CFU) for *M. canis* and *T. mentagrophytes*. However, this effect was concentration and time dependent. PH153 showed fungicidal effect at 96 h in the concentrations equal to MICx2 and MICx4. The fitted curves resulting from modeling of PH151 and PH153 against the three different fungal species are shown in figure 3 and 4, respectively, and the determined PD parameters are listed in Table 1.

3.4. Sorbitol Protection Assay

The MICs of PH151, PH153 and anidulafungin (positive control) for *Candida* spp. and dermatophytes increased when the medium was supplemented with sorbitol. The increased MIC was observed in 2/4 and 7 days of incubation for all isolates, as showed in table 2.

3.5. Ergosterol binding assay

MICs of PH151 and PH153 for *Candida* spp. and dermatophytes remained unchanged in the presence of different concentrations of external ergosterol. In contrast, a 16 to 128-fold increase of amphotericin B MIC values was observed (data not shown)..

3.6. Cellular leakage effect

It was noted that PH151 and PH153 did not cause nucleic acid leakage from fungal cells, since all tested isolates showed no statistical difference compared to the untreated control. As expected, significant ($P \leq 0.05$) release of cellular material was observed for SDS and amphotericin B (positive controls) (Figure 5).

3.7. Scanning electron microscopy (SEM)

The images obtained by SEM depict morphological alterations of fungi exposed to PH151 and PH153. The untreated *C. albicans* ATCC 18804 cells have an oval-shape as well as a wide presence of pseudohyphae (Figure 6A). Treatment with PH151 and PH153 modifies its shape to round cells. Pseudohyphas have not been identified (Figure 6B and 6C). Furthermore, some larger round-cells and formation of cell clusters are observed when *C. albicans* is treated with PH151. The untreated dermatophytes cells have regular and intact hyphae (Figure 7A and 8A). Dermatophytes treated with PH151 and PH153 show irregular, rough and groove cell wall. *M. canis* MCA 01 (Figure 7B and 7C) and *T. mentagrophytes* TME 40 (Figure 8B and 8C) treated with PH151 and PH153 display fungal wall with notable pitting and tears and extensive degeneration.

4. Discussion

Topical treatment of fungal skin infections has several benefits such as direct action at the site of infection, increase of treatment efficacy and minimized entering of drugs to systemic circulation with reduction of side effects.[24] The low toxicity in histopathological evaluation and the low level of irritability presented by PH151 and PH153 are important properties for the development of new drugs for the topical treatment of skin mycoses.

The MIC has been the major pharmacodynamics parameter to check the susceptibility of microorganisms to antimicrobial agents since it is a relatively simple test and thereby can be applied easily by laboratories.[25] However, MIC value do not provide a very detailed characterization of antimicrobial activity because it is a measure evaluated at a single time-point and does not report the rate of microbial kill. So, the dynamic changes in growth and susceptibility over the studied time period are not taken into account.[17,25] On the other hand, time–kill curves are increasingly used to offer detailed information about the antimicrobial efficacy as a function of both time and concentration.[15,26] In addition, pharmacokinetic/pharmacodynamic (PK/PD) modeling has become a powerful tool to evaluate the antimicrobial effect. [17,27,28]

With the use of time-kill assays we showed that by changing the substituent in the phenyl ring of 8-hydroxyquinolines it is possible to obtain

different inhibition patterns of fungal cells. The methoxy presence in PH153 appears to be important for the fungicidal activity against dermatophytes. On the other hand, the chlorine present in PH151 favor the fungistatic activity. However, no substituent has led to the fungicidal activity for *C. albicans*. In this regard, our data were in agreement with previously reported in vitro results of the fungistatic and fungicidal activity of others 8-hydroxyquinoline derivatives against *C. albicans* and dermatophytes, respectively.[10]

To model our time kill data it was necessary to apply different PK/PD models for each individual strain in order to obtain a better description of the antifungal activity. Summarizing, the data showed that PH151 and PH153 are effective against *C. albicans*, *M. canis* and *T. mentagrophytes* in low concentrations. The EC_{50} ranged from 0.873 to 1.329 µg/mL for PH151 and 7.105 to 8.865 µg/mL for PH153 (concentrations similar to the MIC values). Although the EC_{50} were higher for PH153, this compound achieved maximum effect more rapidly than PH151 for all fungi studied. PH153 reached the maximum effect against *C. albicans*, *M. canis* and *T. mentagrophytes* after 7 h, 17 h and 0.25 h, respectively; while the estimated time for maximum effect of PH151 was 34 h, 25 h and 12 h. This approach of combining in vitro time–kill data with PK/PD modeling might serve to delineate future studies in animals in order to define the dosage of the compounds to be applied to the animal. However, in this case it is still necessary to measure the permeation profile in tissues of PH151 and PH153 to better delineate in vivo studies.

PK/PD modeling can be more efficiently applied to optimize the use of currently available antimicrobial agents and to facilitate development of new antimicrobial agents. Although the experimental data of candidate drugs can be sparse, the modeling still allow for the drug-effect parameters to be successfully estimated and make drug development faster at a lower cost. Furthermore, the model can be constantly updated during drug development once the parameters can be re-estimated to improve and extend the predictive capacity when new findings are obtained.[25]

To gain insight into the mode of action of PH151 and PH153, the integrity of the cell wall was verified. Fungi without cell wall or with defective cell wall cannot grow in the absence of sorbitol. However, the growth is possible if sorbitol is supplemented in growth medium, since it acts as an osmotic

protector. So, the antifungal agents that inhibit the fungal cell wall can be identified when the MIC values obtained in the presence of sorbitol are higher than in its absence after seven days of incubation.[29] The results of the sorbitol protection assay indicate that these compounds act on the cell wall of *Candida* spp. and dermatophytes and the effect was species independent. It is important to note that the cell wall represents a very interesting target because the human cells do not have this structure and, therefore, the toxicity to the host tends to be smaller.[30]

Previous studies conducted by our group have demonstrated effect on the fungal wall of other 8-hydroxyquinoline derivatives, such as clioquinol and derivatives containing sulfonic acid (8-hydroxy-5-quinolinesulfonic acid and 8-hydroxy-7-iodo-5-quinolinesulfonic acid). However, the effect of sulfonic acids was species dependent.[22] Furthermore, 8-hydroxy-5-quinolinesulfonic acid derivatives also showed action on the fungal cell membrane, as alteration of the cell permeability of *Candida* spp. and dermatophytes was detected by cellular leakage assay.[22] In contrast, data obtained by measuring of UV-absorbing materials revealed that PH151 and PH153 did not show such effect. So, the low absorbance observed suggests that these compounds do not alter the fungal cytoplasmic membrane indicating a different mode of action.

Antifungal agents can also bind directly to membrane-ergosterol, forming pores that alter membrane permeability. As consequence, there is loss of intracellular content and fungal membrane rupture.[31,32] In this case, the addition of an external ergosterol to the growth medium prevents the binding of the antifungal agent to the membrane-ergosterol and the MIC increases.[20] However, MICs unchanged to PH151 and PH153 in the presence of external ergosterol indicated that they do not act by binding to membrane ergosterol. The other 8-hydroxyquinoline derivatives studied by our group showed similar results.[22]

In addition, morphological changes in fungal cells after treatment with PH151 and PH153 were observed by SEM. *C. albicans* usually grows as oval yeast and branched pseudohyphae (Sudbery et al. 2004). When *C. albicans* was treated with PH151 and PH153, the pseudohyphal growth was inhibited and the fungus developed as minor yeast cells. Pseudohyphae are an intermediate state between yeast cells and hyphae.[33] Hyphal growth of *C.*

albicans is clinically relevant, once is a critical factor for pathogenesis in symptomatic mucosal infections such as oral thrush, and vaginal candidiasis, as well as fatal systemic infections. In these infections, the infected tissues often contain invading hyphae. Besides that, hyphal growth also increases adherence, biofilm formation and cellular invasion.[34] So, phenotypic switching clearly plays a role in virulence.[35] We observed that the application of even sublethal doses of PH151 (1 µg/mL) and PH153 (4 µg/mL) inhibit the pseudohyphae. These findings demonstrate a strong effect of these compounds on *C. albicans* morphogenesis, which can be considered a potential mode of action for this molecule. Clioquinol also present these characteristics, however, the derivatives containing sulfonic acid previously studied did not show the pseudohyphae inhibitory effect.[22] Furthermore, the presence of the large round cells when *C. albicans* was treated with PH151 was also observed in the SEM of cells exposed to 8-hydroxy-7-iodo-5-quinolinesulfonic acid.[22] This may indicate endopolyploidy phenomenon (somatic polyploidy) due to recurrent cycles of DNA replication without cellular division. Previous studies have demonstrated that antifungal treatment is associated with this phenomenon in several fungal species.[36]

On the other hand, irregular and rough cell wall with pitting and tears are seen in *M. canis* and *T. mentagrophytes* after treatment with PH151 and PH153. Even at sub-inhibitory doses of PH151 (0.5 µg/mL) and PH153 (4 µg/mL), fungal cell wall damage was easily visualized in dermatophytes, confirming the results obtained in the sorbitol protection assay. These modifications were also observed on the *M. canis* wall treated with 8-hydroxy-5-quinolinesulfonic.[22]

5. Conclusion

Finally, PH151 and PH153 acts on the cell wall of *Candida* sp. and dermatophytes. Thus, the conversion of the sulfonic acid into a sulfonamide was an interesting strategy for optimizing antifungal activity and target specification, since the action of sulfonic acids on the wall was species dependent and they compromised the cell membrane too. In addition, PH151 and PH153 inhibit the pseudohyphae in *C. albicans* cells. Future analyses of the effect of these compounds on known transcriptional regulators involved in the

control of the yeast-to-hypha transition are necessary, as they should be able to identify the transcription factors and signaling pathway(s) involved in the PH151 and PH153 response. Taken together, data from this study confirmed that PH151 and PH153 are highly effective, nonirritant molecules and do not exhibit topical toxicity, supporting the potential of these molecules as promising antifungal candidates for the treatment of candidiasis and dermatophytosis. Future studies in animal models are warranted to support this premise.

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Conflict of interest

No conflict of interest declared.

Tables

Table 1. Pharmacodynamic parameters determined for PH151 and PH153, and goodness of fit criteria.

Parameters	PH151			PH153		
	C. albicans (ATCC 18804)	*M. canis* (MCA 01)	*T. mentagrophytes* (TME 40)	*C. albicans* (ATCC 18804)	*M. canis* (MCA 01)	*T. mentagrophytes* (TME 40)
MIC* (µg/mL)	2	1	1	8	8	8
K_0 (h^{-1})	0.320	0.684	0.107	0.153	1.042	0.028
K_{max} (h^{-1})	0.020	0.027	0.056	0.097	0.039	2.786
EC_{50} (µg/mL)	1.329	0.923	0.873	7.280	8.865	7.105
N_{max} (log_{10} CFU/mL)	6.651	4.222	4.350	7.945	4.234	4.310
x (h^{-1})	0.130	0.002	0.060	0.742	0.001	-
z (h^{-1})	-	0.021	-	-	0.010	0.015
h	0.612	-	-	0.874	-	-
MSC (range)	2.580 – 3.938	2.194 – 3.731	1.566 – 2.284	2.209 – 2.721	2.289 – 2.282	1.065 – 3.380
R^2(range)	0.999	0.999	0.998 – 0.999	0.996 – 0.999	0.999	0.998 – 0.999

* Previously determined by broth microdilution.[11]

Table 2. Effect of sorbitol on the minimum inhibitory concentration (MIC; µg/mL) of anidulafungin, PH151 and PH153 against *Candida* spp. *and* dermatophytes.

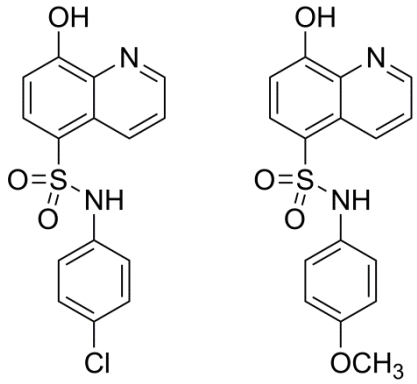
Isolate	MIC Anidulafungin				MIC PH151				MIC PH153			
	Day 2/ 4*		Day 7		Day 2/ 4*		Day 7		Day 2/ 4*		Day 7	
	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)	S (-)	S (+)
ATCC 18804 (CA)	0.031	> 4.0	0.063	> 4.0	2.0	8.0	4.0	8.0	8.0	16.0	8.0	16.0
CG RL24	0.015	0.031	0.015	0.125	2.0	8.0	2.0	8.0	8.0	16.0	8.0	16.0
CK Den43	0.500	2.0	1.0	> 4.0	1.0	2.0	1.0	4.0	8.0	16.0	16.0	32.0
CP RL38	0.250	2.0	0.5	> 4.0	2.0	8.0	4.0	16.0	16.0	32.0	16.0	32.0
ATCC 750 (CT)	0.008	0.016	0.008	> 4.0	2.0	4.0	2.0	8.0	8.0	16.0	8.0	16.0
MCA 01	0.250	1.0	0.5	>4.0	1.0	8.0	1.0	8.0	8.0	32.0	16.0	64.0
MGY 50	0.250	1.0	0.250	2.0	1.0	4.0	2.0	8.0	8.0	16.0	8.0	16.0
TME 40	0.031	4.0	0.031	4.0	1.0	2.0	1.0	4.0	8.0	32.0	8.0	32.0
TRU 51	0.5	2.0	0.5	4.0	1.0	2.0	1.0	4.0	8.0	32.0	32.0	64.0

* MICs were measured after 2 days of incubation for *Candida* spp. and after 4 days of incubation for dermatophytes.

S(-):medium without sorbitol; S (+):medium with sorbitol.

CA: *Candida albicans*; CG: *Candida glabrata*; CK: *Candida krusei*, CP: *Candida parapsilosis*; CT: *Candida tropicalis*; MCA: *Microsporum canis*; MGY: *Microsporum gypseum*, TME: *Trichophyton mentagrophytes*; TRU: *Trichophyton rubrum*.

Figures



A **B**

Figure 1. Chemical structures of 8-hydroxiquinoline-5-(N-4-chlorophenyl)sulfonamide (PH151; A) and 8-hydroxiquinoline-5-(N-4-methoxyphenyl)sulfonamide (PH153; B).

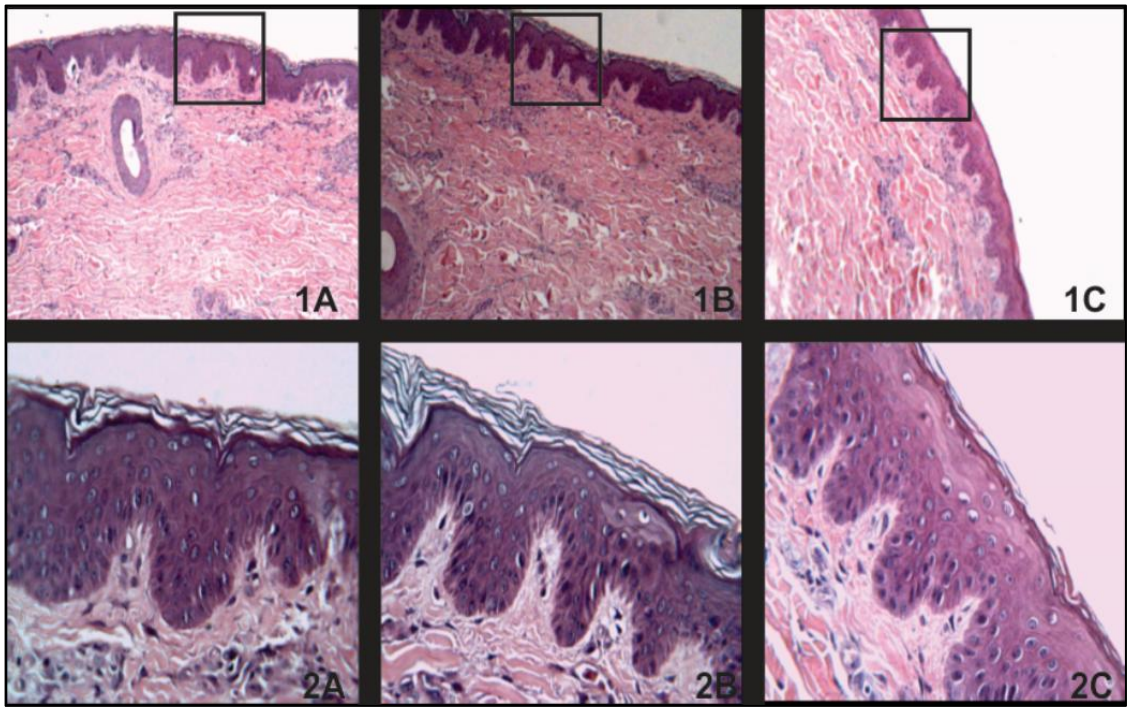


Figure 2. Histopathological evaluation of porcine cells treated with 8-hydroxyquinoline-5-sulfonamides and negative control at 100 and 400 times magnification. The concentration of each 8-hydroxyquinoline derivative used in the test was 1 mg/mL. **(1A)** Swine epidermal cells treated with PH151 at 100 times magnification; **(1B)** Swine epidermal cells treated with PH153 at 100 times magnification; **(1C)** Swine epidermal cells treated with PBS pH 7.0 at 100 times magnification; **(2A)** Swine epidermal cells treated with PH151 at 400 times magnification; **(2B)** Swine epidermal cells treated with PH153at 400 times magnification; **(2C)** Swine epidermal cells treated with PBS pH 7.0 at 400 times magnification.

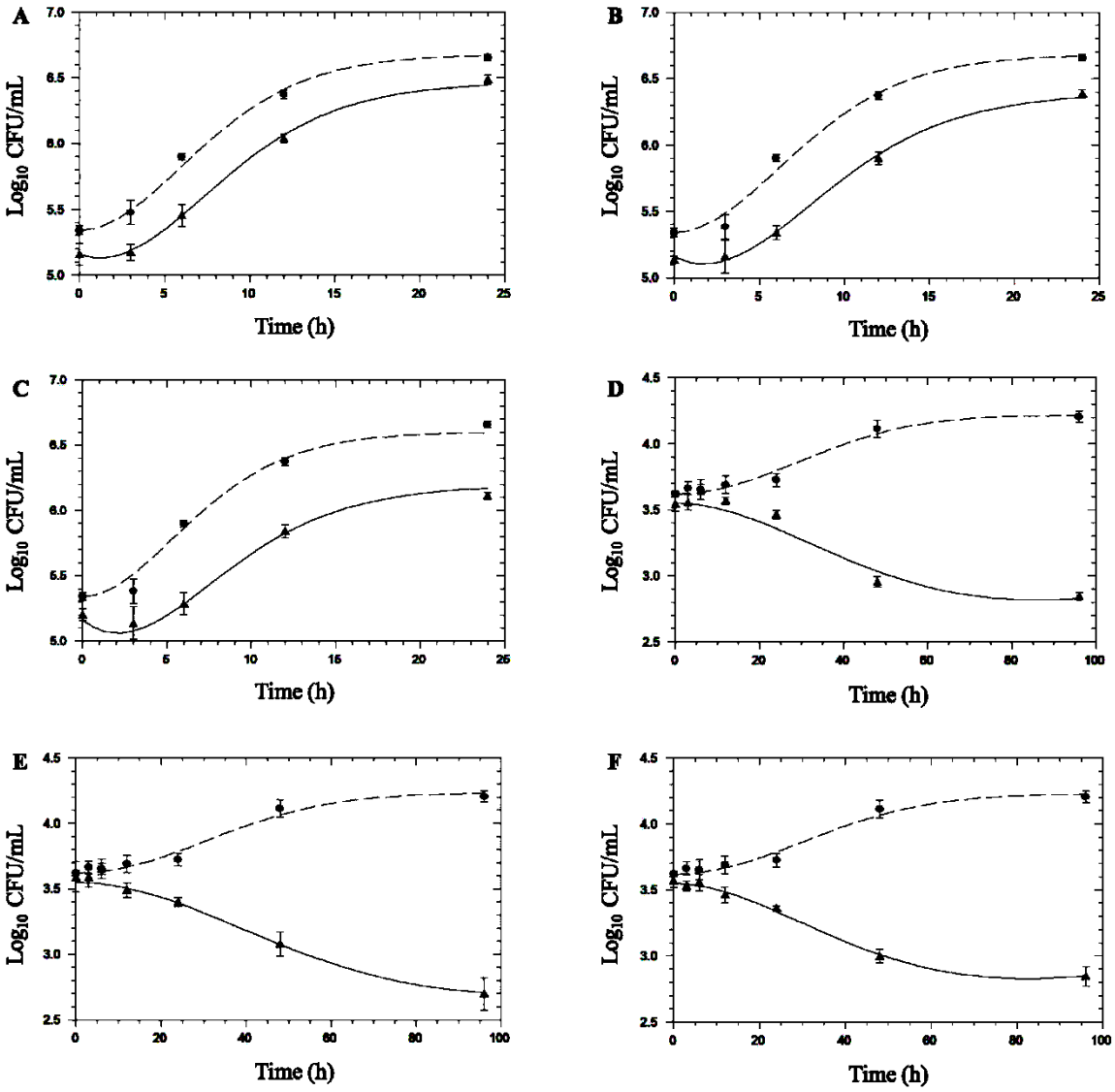


Figure 3. Fitted curves resulting from modeling of fungal growth (dashed line) and kill for PH151 (full line) against *C. albicans* ATCC 18804 (A-C) (A: MIC; B: 2xMIC; C: 4xMIC), *M. canis* MCA 01(D-F) (D: MIC; E: 2xMIC; F: 4xMIC) and *T. mentagrophytes* TME 40(G-I) (G: MIC; H:2xMIC; I: 4xMIC) isolates.
* MIC: minimum inhibitory concentration; CFU: colony-forming units.

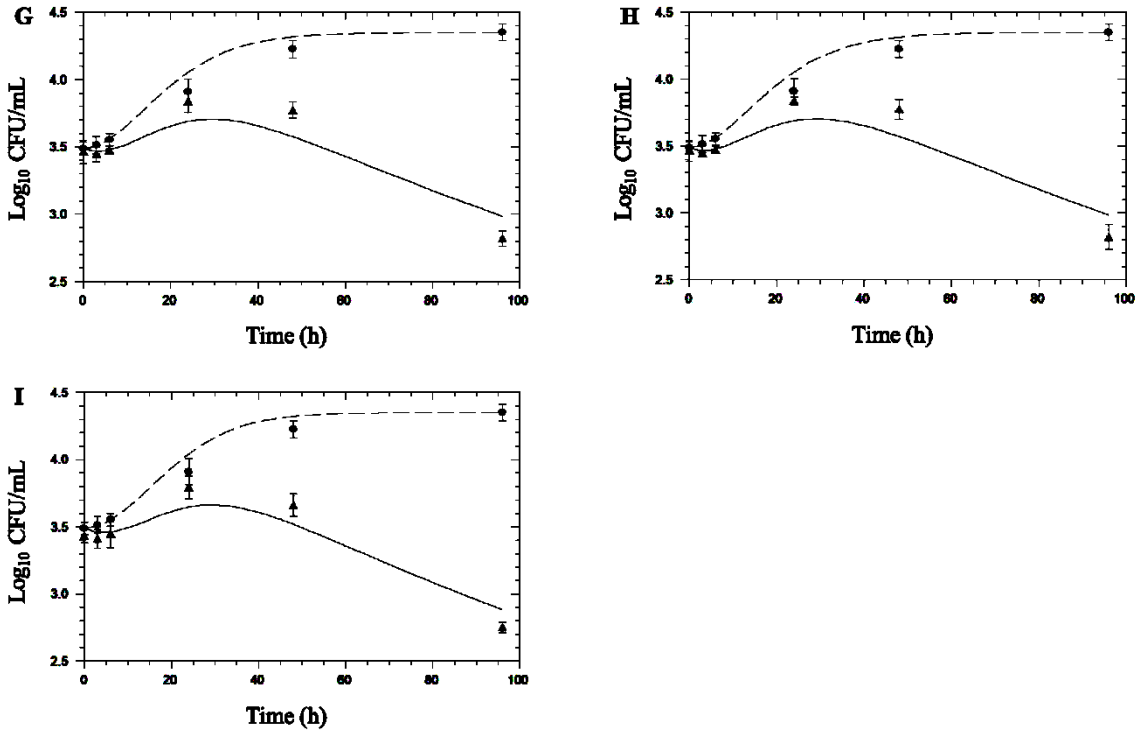


Figure 3. (*Continued*).

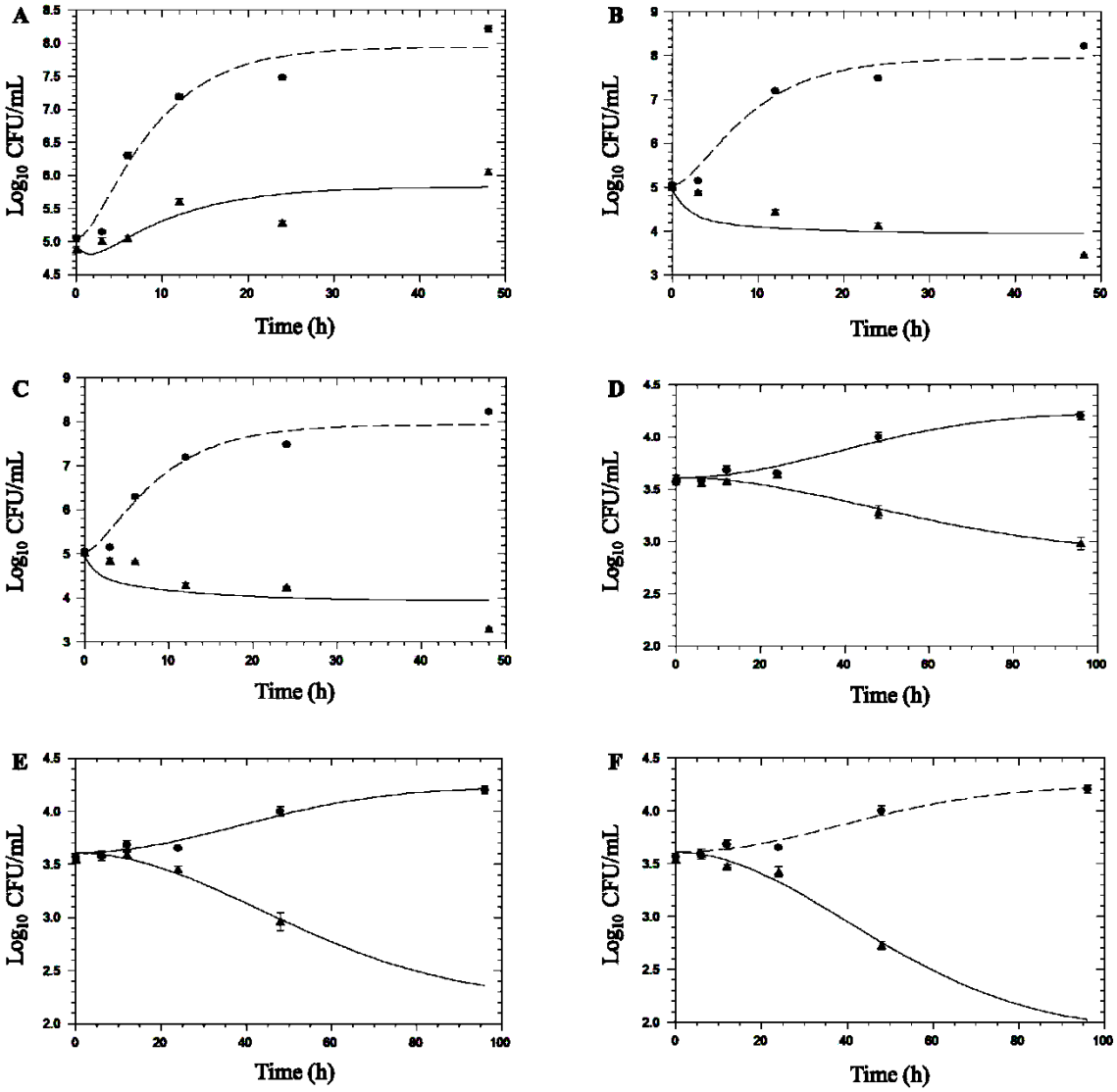


Figure 4. Fitted curves resulting from modeling of fungal growth (dashed line) and kill for PH153 (full line) against *C. albicans* ATCC 18804 (A-C) (A: MIC; B: 2xMIC; C: 4xMIC), *M. canis* MCA 01 (D-F) (D: MIC; E: 2xMIC; F: 4xMIC) and *T. mentagrophytes* TME 40 (G-I) (G: MIC; H:2xMIC; I: 4xMIC) isolates.
* MIC: minimum inhibitory concentration; CFU: colony-forming units.

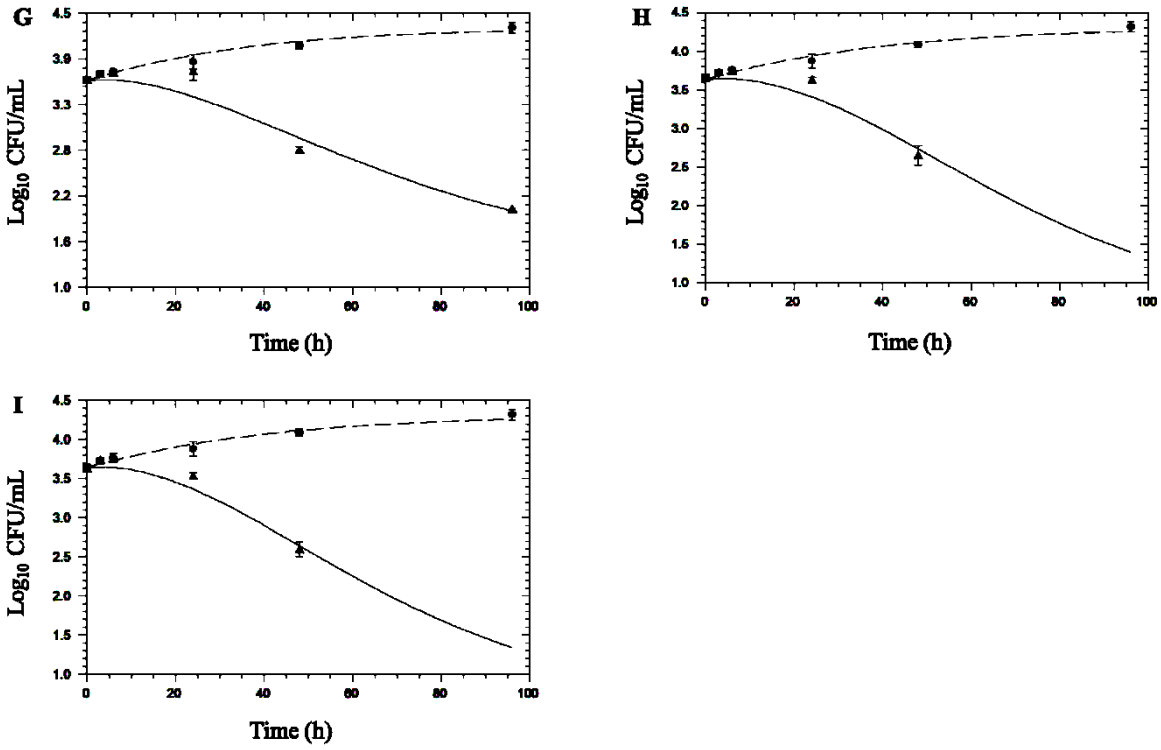


Figure 4. (*Continued*).

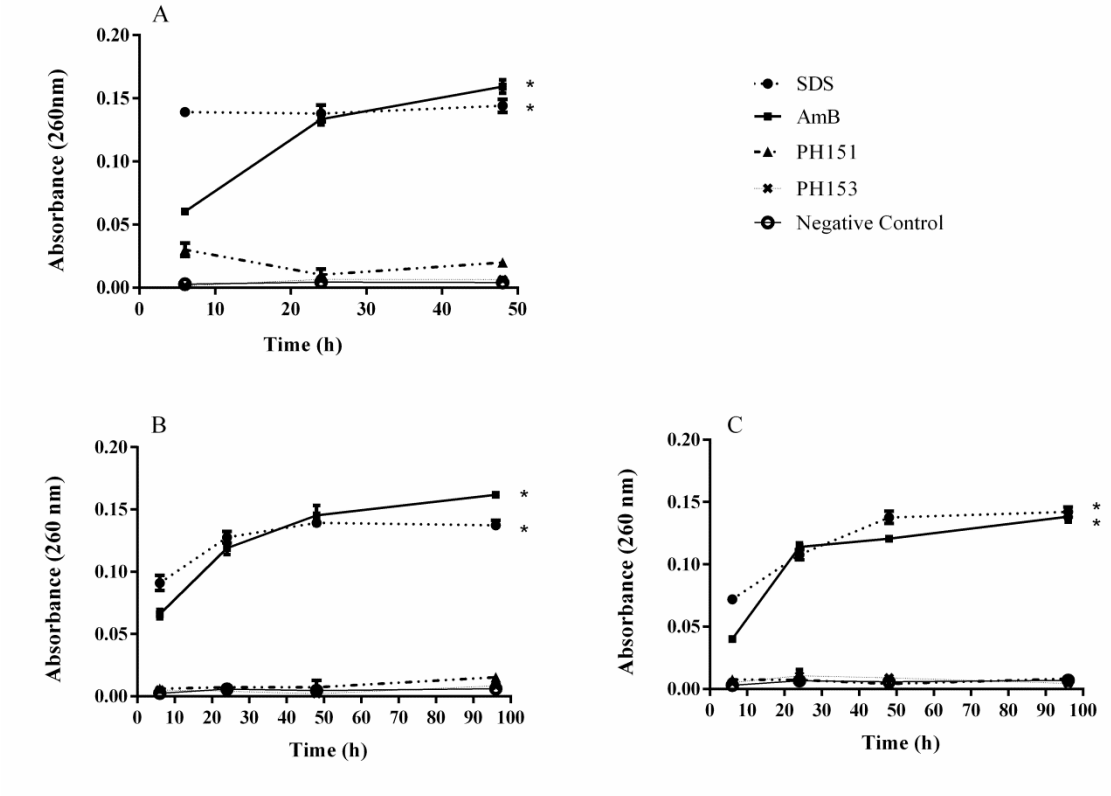


Figure 5. Cellular leakage of 260-nm-absorbing materials of *Candida albicans* ATCC 18804 (A), *Microsporum canis* MCA 01 (B) and *Trichophyton mentagrophytes* TME 40 (C) treated with MIC of PH151 and PH153. Amphotericin B (6.25 µg/ml) and SDS (2%) are the positive controls. Untreated cells are negative control.
PH151 MIC: 2 µg/ml (ATCC 18804) and 1 µg/ml (MCA 01 and TME 40).
PH153 MIC: 8 µg/ml (ATCC 18804, MCA 01 and TME 40).

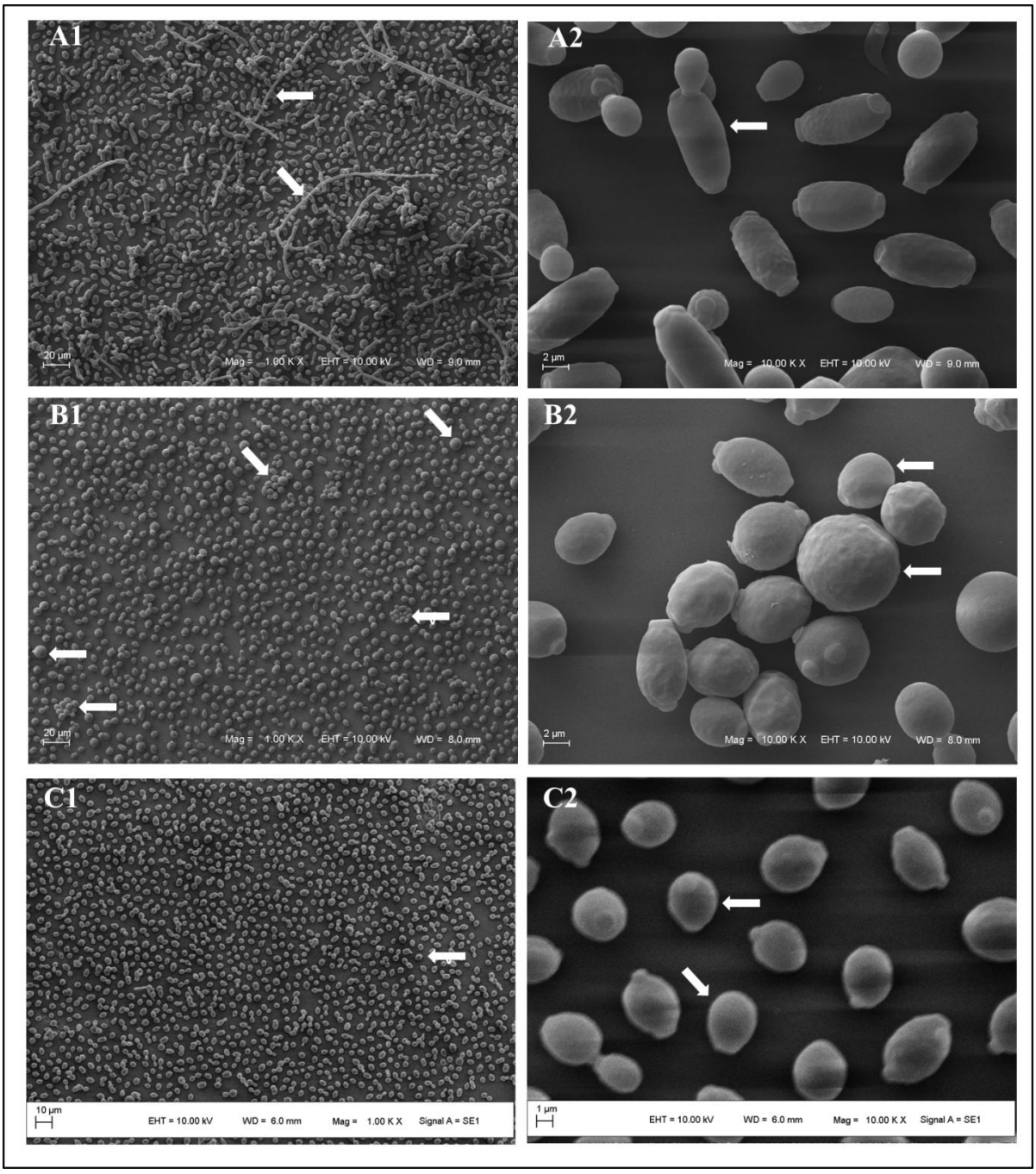


Figure 6. Scanning electron microscopy of *Candida albicans* ATCC 18804 treated with sub-inhibitory concentration of PH151 and PH153, and untreated cells. **(A)** Untreated cells; A1 (Bar = 20 µm): white arrows indicate pseudohyphae; A2 (Bar = 2 µm): white arrows indicate oval-shape. **(B)** Cells treated with PH151; B1 (Bar = 20 µm): white arrows indicate cell clusters and larger round-cells; B2 (Bar = 2 µm): white arrow indicates circular cells and larger round-cells. **(C)** Cells treated with PH153; C1 (Bar = 20 µm): white arrows indicate circular cells; C2 (Bar = 2 µm): white arrows indicate circular cells.

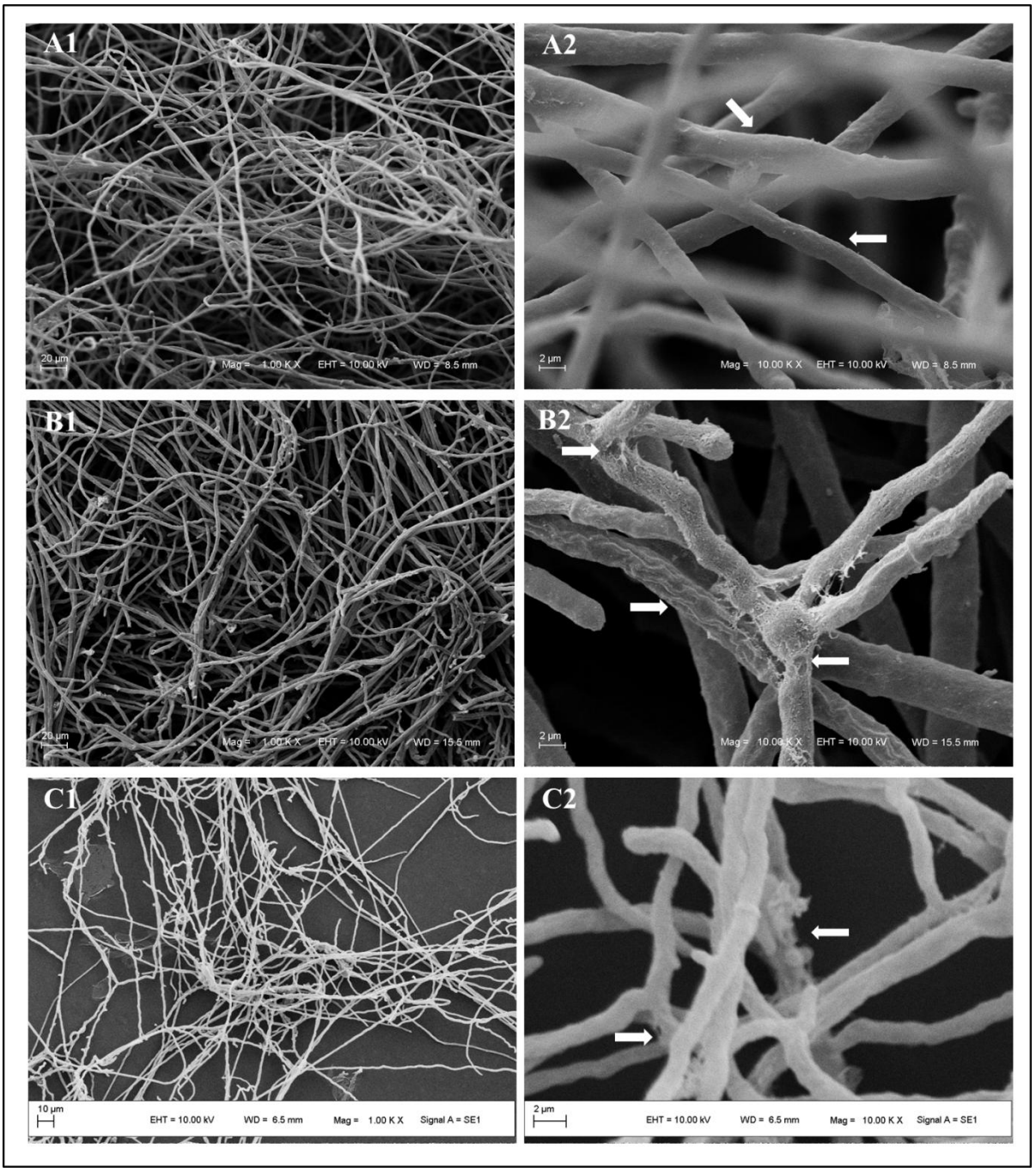


Figure 7. Scanning electron microscopy of *Microsporum canis* MCA 01 treated with sub-inhibitory concentration of PH151 and untreated cells. **(A)** Untreated cells; A1 (Bar = 20 µm); A2 (Bar = 2 µm): white arrows indicate regular and intact hyphae. **(B)** Hyphal cells treated with PH151; B1 (Bar = 20 µm); B2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears. **(C)** Hyphal cells treated with PH153; C1 (Bar = 20 µm); C2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears.

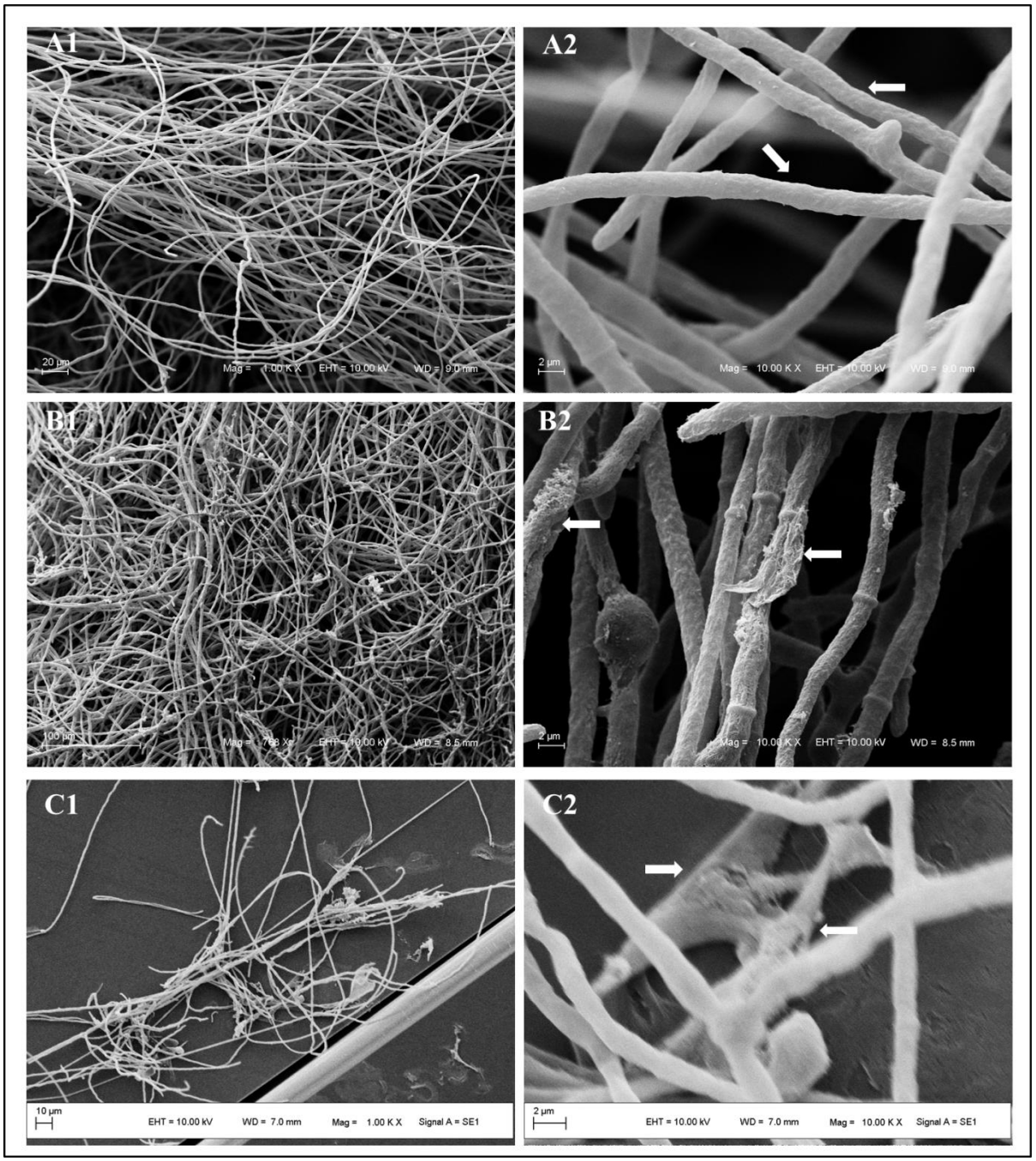


Figure 8. Scanning electron microscopy of *Trichophyton mentagrophytes* TME 40 treated with sub-inhibitory concentration of PH151 and untreated cells.**(A)** Untreated cells; A1 (Bar = 20 µm); A2 (Bar = 2 µm): white arrows indicate regular and intact hyphae.**(B)** Hyphal cells treated with PH151; B1 (Bar = 20 µm); B2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears. **(C)** Hyphal cells treated with PH151; C1 (Bar = 20 µm); C2 (Bar = 2 µm): white arrows indicate irregular and rough cell walls with grooves, pitting and tears.

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5.6 Artigo 6

O artigo intitulado “Antifungal activity and toxicological parameters of 8-hydroxyquinoline-5-sulfonamides using alternative animal models” será submetido na “Biomedicine & Pharmacotherapy”.

Title: Antifungal activity and toxicological parameters of 8-hydroxyquinoline-5-sulfonamides using alternative animal models

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Keywords: 8-hydroxyquinoline derivatives, *Candida* spp., antifungal agents, *Drosophila melanogaster*, zebrafish, chicken embryo

Abstract:

The aim of this study was to evaluate the antifungal activity and toxicological parameters of 8-hydroxyquinoline-5-(*N*-4-chlorophenyl)sulfonamide (PH151) and 8-hydroxyquinoline-5-(*N*-4-methoxyphenyl)sulfonamide (PH153) using alternative animal models. PH151 and PH153 showed low toxicity and high potential to treat candidiasis in *Drosophila melanogaster* flies. The compounds did not cause interruption of chicken embryogenesis; but PH151 increased arterial pulsation and opening of the beak. Zebrafish embryos exposed to compounds showed a dose-dependent toxicity. The sublethal concentrations 0.25 µg/mL of PH151 and 1 µg/mL of PH153 decreased the sensibility in the touch stimulation; the decreased of thigmotaxis behavior was observed only for PH153 in this concentration. In exploratory behavior test were observed an increase in swimming activity in larvae exposed to 1 µg/mL of PH153 and in time spent in central zone in larvae exposed to 0.25 µg/mL of PH151. Turn angle and apoptotic areas were not affected. The results found in zebrafish embryos differ from the other species evaluated, but they may be an alert for possible embryotoxicity. Therefore, cautious indication for pregnant women should be considered. The data support the potential of PH151 and PH153 for treatment of systemic candidiasis and demonstrated to be appropriate drug candidates for further studies using mammalian models.

Introduction

Antifungal therapy has advanced over the last decades, but the number of cases of infection and resistance rates are still alarming, and the control of these diseases is far from being achieved [1]. Immunocompromised individuals are highly susceptible to invasive infections caused by opportunistic fungi, especially *Candida* spp. [2]. With a high severity and mortality, such infections increase the patient's stay in the hospital resulting in high costs to the health system [3,4].

Currently available antifungal drugs have disadvantages related to efficacy and pharmacokinetic properties such as low penetration, problems with drug bioavailability, and drug interactions [5,6]. In addition, the pharmacotherapy of fungal infections is challenging due to cellular similarity between the fungal pathogen and the mammalian host, which hinders the development of safe antifungal drugs [6,7].

Recently, the interest in 8-hydroxyquinolines has grown because of their privileged chemical structures for the design of new drug candidates [8,9]. Previous studies carried out by our group have explored the synthesis, in vitro antifungal properties and in vitro toxicity of 8-hydroxyquinoline derivatives with sulfonamide groups. The sulfonamide modification resulted in two potent lead compounds: 8-hydroxyquinoline-5-(N-4-chlorophenyl)sulfonamide (PH151) and 8-hydroxyquinoline-5-(N-4-methoxyphenyl)sulfonamide (PH153) [10]. However, it is necessary to understand whether the compounds have the same behavior when subjected to complex systems. Considering this, in vivo experiments are crucial for confirmation of in vitro data [11].

The use of alternative animal models has been encouraged for in vivo testing due to their well-defined genetic origins, simplicity of use, low cost of maintenance and a large number of animals can be used in each experiment. Most importantly, there is a good correlation between alternative animals and mammals [12,13]. *Drosophila melanogaster* is described as a model to investigate the virulence of human pathogens and to verify the efficacy of novel antifungal agents [14]; and zebrafish and chicken embryos have been widely used for toxicity assays [15,16]. Therefore, such models present an adequate application for the pre-selection of biologically active compounds [11].

The aim of this study was to evaluate the antifungal activity and toxicological parameters of PH151 and PH153 using alternative animal models. Here, we used Toll-deficient (Tl-deficient) *D. melanogaster* flies as a host model to

test the protective effect of these compounds against *Candida albicans* infection. Toxicological parameters were evaluated in chicken and zebrafish embryos.

Materials and methods

Fungal strain and antifungal compounds: A reference *C. albicans* strain (ATCC18804) was used in the present study. 8-Hydroxyquinoline-5-(N-4-chlorophenyl)sulfonamide (compound PH151) and 8-hydroxyquinoline-5-(N-4-methoxyphenyl)sulfonamide (compound PH153) were synthesized as described by Joaquim et al. [10]. These compounds were dissolved in dimethylsulfoxide (DMSO) and diluted to obtain a maximum concentration of 2% DMSO in the experiments.

***D. melanogaster* Stocks:** Tl-deficient transheterozygote mutants were generated by crossing flies carrying a thermosensitive allele of Tl (Tl^{r632}) with flies carrying a null allele of Tl ($Tl^{l\text{-}RXA}$), as previously described [17]. Fly strains were a gift from Dr. Dimitrios Kontoyiannis from University of Texas MD Anderson Cancer Center. Standard procedures were used for manipulation, feeding, and housing of flies in all experiments [17,18].

8-Hydroxyquinolines protection experiments: To determine whether the 8-hydroxyquinoline-5-sulfonamides treatment protects flies against candidiasis, groups of 15 Tl-deficient female flies were housed in empty vials for 6–8 h to starve them and then transferred to vials with food containing 100 µL of PH151 and PH153 at the concentration of 1 mg/mL, as previously described [17]. After 24 h, a needle that had been dipped into a 0.85% NaCl solution containing 10^8 *C. albicans* yeast cells/mL was injected in the thorax of the flies. After infection, flies returned to vials containing antifungal drugs and were maintained at 29 °C. Survival of the flies was assessed daily until day 7 after injection. Flies that died within 3 h of injection were considered to have died as a result of the procedure and were excluded from the survival analysis [17,18]. Positive control was provided by infecting flies that were not exposed to antifungal compounds, whereas for negative control flies were punctured with a needle that was dipped into sterile saline. Furthermore, prior to the protection experiments, the flies were exposed to food containing 8-hydroxyquinoline-5-

sulfonamides (1 mg/mL) to verify the toxicity of the compounds. Each experiment was performed in triplicate on different days.

Survival curves were plotted using Kaplan-Meier analysis, and differences in survival rates between the groups were analyzed using the log-rank test (GraphPad Prism software; version 6.0; GraphPad Software). $P \leq 0.05$ was considered to be statistically significant.

Assessment of tissue fungal burden: Quantification of *Candida* yeast cells in the tissues of flies was performed after seven days of infection. Flies from each group were collected and separated in live and dead flies and ground in 1 mL of 0.85% NaCl solution. Serial dilutions were done in triplicate and plated onto SDA medium (Sabouraud Dextrose Agar; HiMedia; India). The number of colony-forming units (CFU) was counted after 48 h of incubation at 35 °C.

The nonparametric Mann-Whitney test was used to determine statistically significant differences in fungal burden between PH151 and PH153 treatments in comparison to the untreated control groups. Statistical analyses were performed using the GraphPad Prism software. $P \leq 0.05$ was considered to be statistically significant.

Plasma protein binding: Plasma protein binding occurs predominantly with albumin. To evaluate the influence of protein binding on 8-hydroxyquinoline derivatives activity, we evaluated the fungal growth in protein-free nutrient broth and nutrient broth containing bovine serum albumin. Thereafter, minimum inhibitory concentrations (MIC) of PH151 and PH153 were determined against *C. albicans* ATCC 18804 by the standard broth microdilution test [19] using RPMI 1640 medium supplemented with 40 g/L of bovine serum albumin (INLAB Confiança, São Paulo, Brazil) [20]. Experiments were carried out in duplicate and amphotericin B (União Química, São Paulo, Brazil) was used as positive control [21].

Behavioral test on chicken embryo: White Lohmann fertile eggs (Lohmann selected Leghorn, São José do Rio Preto, SP, Brazil) were used in this test. The eggs were maintained at 37.6 ± 0.4 °C and 50% relative humidity while being turned automatically until day 12. On day 12, the eggs were opened and 100 µl of PH151 and PH153 at concentration of 1 mg/mL were added to each egg.

Epinephrine (1 mg/mL) and saline solution (0.85%) were used as positive control and untreated control, respectively. The following parameters were observed for two minutes in the chicken embryos: arterial pulsation, opening of the beak and movement of the embryo. The results were analyzed by one-way ANOVA as well as Dunnett's multiple comparisons test using GraphPad Prism software. $P \leq 0.05$ was considered to be statistically significant.

Zebrafish maintenance and reproduction: Adult wild-type zebrafish (*Danio rerio*) were obtained from a local supplier and were kept in a recirculating aquatic system (Zebtec®) under appropriate water conditions (pH 7.2, 400 µS conductivity and 28 °C). Fishes were fed twice a day with commercial flocked fish food and supplemented with brine shrimp (*Artemia salina*) once a day. Fishes were maintained on a 14:10h photoperiod and values for ammonia, nitrite and nitrate were less than 0.2 ppm, 0.05 ppm and 0.05 ppm, respectively. The experimental protocols used in this study were approved by the local ethics committee (CEUA – UNIPAMPA: protocol 003-2016). Male and female adult zebrafish (2:1) were placed in pairs overnight, and on the next morning the reproduction was induced by light irritation.

Zebrafish embryo exposure and mortality: After reproduction, fertilized eggs were collected, washed and randomly sorted into groups. Briefly, fertilized eggs were incubated in glass Petri dishes (20 mL) in exposure solutions beginning at 3-h postfertilization (hpf). Exposure studies were conducted in triplicate with 20 embryos per dose group and experiments were repeated at least three times independently. Stock solutions were stored in complete darkness at -20 ºC and diluted to working concentrations in DMSO (0.005 % final concentration). Two different exposure curves based on MICs of the compounds on fungal cells [10] were used in this study. In the first curve, 3-hpf embryos were exposed to 0.25 – 16 µg/mL of PH151 and 1 – 64 µg/mL of PH153 for 24 h. Survival was analyzed at 2 and 24 h. In the second curve, 3-hpf embryos were exposed statically to 0.06 – 0.25 µg/mL of PH151 and 0.25 – 1 µg/mL of PH153 during seven days (168 hpf). DMSO 0.005% (v/v) was used as vehicle control. Water system was used for the control group.

Touch stimulation and swim response: Zebrafish larvae with 72 hpf exposed to concentrations of 0.06 – 0.25 µg/mL of PH151 and 0.25 – 1 µg/mL of

PH153 were submitted to the behavioral touch response test [22]. At this stage of development, the larvae remain inert in the environment, responding only to stimuli, such as touch. Each larva was gently placed in the center of a Petri dish containing 20 mL of system water and was touched by a double-blind forceps (stimulus). The number of stimuli needed for first displacement was evaluated as motor and sensitive parameters. The thigmotaxis was determined by percentage of larvae that is capable of performing standard escape towards the periphery of the apparatus.

Exploratory behavior in the open field test: At day 7 of exposure (168 hpf), zebrafish larvae exposed to the second curve concentrations were submitted to exploratory behavior, as described by Colwill & Creton (2011) and Altenhofen et al. (2017) [23,24]. Briefly, larvae were individually placed in wells of 24-well culture plates. The swimming behavior of the larva was recorded during 300 s after 60 s of habituation using a digital stereomicroscope connected to a laptop at a rate of 30 frames/s using appropriate video-tracking software (ANY-maze^{TM}, Stoelting CO, USA). The apparatus was virtually divided in two circular sections (center and periphery) to assess the spatial exploration. Distance travelled (meters), absolute turn angle (degree) and mean time per visit in central area were used to measure locomotor, motor and anxiolytic parameters.

Acridine orange in vivo: Acridine orange fluorescent dye was used for the detection of cells in the process of apoptosis. At day 7 of exposure (168 hpf), five zebrafish larvae were placed in 2-mL Eppendorf tubes, along with system water and the reagent at the final concentration of 5 µg/mL. Tubes were incubated in the dark for 30 min; thereafter larvae were washed three times in system water and fixed in slides for microscopy with 1.5% methylcellulose. Images were obtained by using Olympus IX71 fluorescence microscope with coupled camera [25].

Statistical analysis of zebrafish assay: Normality of data and homogeneity of the variances were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. The results of behavioral (endpoint) and apoptotic parameters were expressed as mean ± standard error of the mean (S.E.M.) and analyzed by one-way ANOVA followed by Tukey test. Owing to its nonparametric distribution, the immobile time was expressed as median ± interquartile range and analyzed by

Kruskal–Wallis followed by Dunn's multiple comparison test. $P \leq 0.05$ was considered to be statistically significant.

Results

Pilot experiments showed that PH151 and PH153 at 1 mg/mL were not toxic to *D. melanogaster* Tl-deficient flies. The efficacy of PH151 and PH153 treatment on Tl-deficient flies infected with *C. albicans* is displayed in Figure 1. Flies fed with food containing these compounds had a significantly higher survival rate than untreated control flies ($P < 0.001$) and did not present statistical difference when compared to the uninfected control group. Furthermore, both live and dead flies treated with PH151 and PH153 had significantly lower fungal burdens than did untreated control flies after seven days of infection, as determined by counts of colony-forming units ($P < 0.05$) (date not shown).

The effects of compounds on fungal growth in medium containing 40 g/L bovine serum albumin were similar to the effects of amphotericin B. MICs of PH151 and PH153 against *C. albicans* increased from 2 to 64 µg/mL and 8 to 16 µg/mL after adding bovine serum albumin, respectively; while MIC of amphotericin B increased from 0.25 to 2 µg/mL.

PH151 and PH153 were not lethal for chicken embryo. PH151 did not cause significant changes in the movement of the embryo (Figure 2C), but significantly increased arterial pulsation and number of opening of the beak ($P \leq 0.05$) (Figure 2A and 2 B). Conversely, PH153 did not show significant changes in all parameters evaluated. As expected, a significant increase ($P \leq 0.05$) in the number of opening of the beak, in movement of the embryo and in arterial pulsation was observed for chicken embryos treated with epinephrine (positive control) (Figure 2).

Zebrafish embryos were more sensitive to compounds when compared to *D. melanogaster* and chicken embryo. The first exposure of zebrafish embryos to PH151 (0.25 – 16 µg/mL) and PH153 (1 – 64 µg/mL) resulted in 100% mortality in the first 24 h of exposure. Thereby, new concentrations were defined to determine the sublethal concentrations of the compounds. The second curve of exposure (0.06 – 0.25 µg/mL of PH151 and 0.25 – 1 µg/mL of PH153) did not show statistical difference in embryo mortality compared to the control group, which allowed the continuity of toxicity studies (data not shown). The responses of zebrafish larvae submitted to touch stimulus are shown in Figure 3. The highest concentrations of

PH151 and PH153 demonstrated a significant increase in number of stimuli for first swimming activity of the larvae in relation to control group (Figure 3A). The group treated with the highest concentration of PH153 also showed a significant decrease in percent number of larvae *with* shelter-seeking behavior in peripheral zone after touch stimulation (Figure 3B).

Figure 4 shows the results obtained for the larval exploratory behavior test. Only the group treated with 1 µg/mL of PH153 showed a significant alteration in motor parameter in exploratory test, observed by increase in distance travelled in relation to other groups; however, no significant differences in total turn angle was observed between groups. Changes in non-motor parameters observed in exploratory test were observed only in group treated with 0.25 µg/mL of PH151, that showed a significant increase in average time spent in central zone.

The acridine orange test showed that the number of apoptotic cells did not differ in both treated groups in comparison to the control group (Figure 6).

Discussion

The increased incidence of *Candida* infections resistant to antifungals currently available requires acceleration of the discovery of new agents with properties of inhibiting this fungal pathogen [6]. *D. melanogaster* is a simpler and less laborious model than are conventional animal models and it can be appropriate for screening of compounds with cellular targets in *Candida* species [14]. The results found in the *D. melanogaster* protection experiment demonstrated that the novel 8-hydroxyquinoline derivatives synthesized by our research group have potential to treat systemic candidiasis in immunocompromised patients. Oral administration of PH151 and PH153 protected *D. melanogaster* flies from *C. albicans* infection and avoided the dissemination of the microorganism in the tissue as verified by fungal burden of the flies after seven days of infection.

We have previously found similar results using other 8-hydroxyquinoline derivatives. Clioquinol (5-chloro-7-iodo-8-hidroxyquinoline) has also fully protected *C. albicans* infected flies [26]. Such as clioquinol, PH151 and PH153 has inhibited pseudohyphae formation in *C. albicans* [27,28] and the protective effect in *D. melanogaster* can be associated to action of these compounds on the morphogenetic process. The capacity to switch between yeast and filamentous growth is the most investigated virulence factor of *C. albicans* and is critical for pathogenicity in fungal

infections [29,30]. Therefore, factors that are associated in this conversion process represent promising therapeutic targets. In addition, the survival of flies exposed to high concentrations of PH151 and PH153 indicated low toxicity of the compounds when administered orally. This result is in agreement with a previous study that verified low in vitro cytotoxicity of these compounds [10].

PH151 and PH153 bind to albumin as well as other commercially available antifungal agents such as amphotericin B, ketoconazole, and itraconazole [20,21]. The drugs with high affinity to plasma proteins, such as albumin, may have less bioavailability and compromised efficacy [31].Other 8-hydroxyquinoline derivatives also showed affinity for albumin [28], but this property apparently did not prevent the in vivo action of the compounds on *D. melanogaster*.

Behavioral parameters of chicken embryos were used to evaluate toxicological responses. Despite obvious differences between birds and mammals, these two classes share similar patterns of embryogenesis, organogenesis, cardiovascular biology, neurobiology, and many other features. Perhaps most importantly is that embryonic development can be studied independently of maternal exposure to toxic compounds [32]. The results showed that high concentrations of PH151 and PH153 did not cause interruption of embryogenesis. The low toxicity found here corroborates with the models discussed above and may indicate safety during embryogenesis. The significant increase in the arterial pulsation and in the number of opening of the beak presented by the chicken embryo exposed to PH151 can indicate the stimulating action as probable side effect of this compound. Although statistically significant, these increases were discrete and we must consider that animals during embryonic development are more sensitive and vulnerable than during the other life stages [32].

The zebrafish embryos are a promising alternative model in some fields of biomedical research, such as drug screening, safety pharmacology, and developmental toxicity assessment [33,34]. The zebrafish embryos exposed to PH151 and PH153 showed dose-dependent toxicity under laboratory conditions. The lethal dose was also dependent on the time of exposition to compounds, and was found to vary from 0.25 – 8 µg/mL for PH151 and 4 – 32 µg/mL for PH153. The lowest doses showed no difference compared to the control. Similar results were showed by Wang et al. (2010) with amphotericin B, where the lethal dose (LD_{50}) in zebrafish embryos was 5 µg/mL [35]. In addition, in vitro cytotoxicity studies

conducted by our research group demonstrated that the IC_{50} (concentration that inhibits 50%) for PH151 and PH153 on vero cells are 18.3 µg/mL and 22.1 µg/mL, respectively [10].

Tactile stimuli resulted in rapid tail coils. The touch response is mediated by sensory inputs that result in activation of the primary motoneurons. Behaviorally, touch causes the reorientation of the fish away from the stimulus, followed by swimming and escape [22]. In general, there was no motor incapacity in the group exposed to lower concentrations of compounds, once larvae responded normally to touch. On the other hand, higher concentrations required more stimuli and lower capacity of performing standard escape (thigmotaxis), which are related to low sensitivity and consequent decrease in motor activity [36]. These data are consistent with the literature showing that exposures to chemicals and pharmaceuticals products may alter levels of neurotransmitters in the brain and cause decreased motor activity [37, 38].

Locomotor behaviors play an essential role in the feeding, social, and defensive activities in zebrafish [23]. The open field test is now one of the most popular procedure in animal psychology [39] and has been successfully used in zebrafish model [23,40]. The animal is placed in the apparatus and behavioral items are recorded for a time period. In such a situation, animals spontaneously prefer the periphery of the apparatus. Increase of time spent in the central part is related to a less anxious behavior [39]. So, the highest tested concentration of PH151 displayed an increased time in the central area, which could be interpreted as anxiolytic or sedative effect, contradicting the results found in chicken embryos trials. However, they are different animal models and, therefore, these data should not be compared. The distance travelled increased when larvae were exposed to the highest concentration of PH153. Increased locomotor activity can be considered a stimulant effect [41,42]. In addition, our study demonstrated that exposure to compounds was unable to alter the absolute turn angle of zebrafish larvae. Turning angle is a behavioral motor parameter that analyzes the angle of each change of direction of the animal's body during the test. A decrease in the angle of rotation is related to a lower motor activity, which is a feature that was not detected here [24, 43].

The acridine orange staining test showed that there was only physiological apoptosis occurring in the embryos exposed to PH151 and PH153 since all tested

concentrations of compounds resulted in no differences or increase in apoptotic areas compared to control.

Apparent toxicity of PH151 and PH153 was observed on zebrafish in the embryo-larval stages; therefore, it is not possible to affirm about potential toxicity in adults. The data found in zebrafish embryos differ from the other species evaluated, but they may be an alert for possible embryotoxicity. Therefore, cautious indication for pregnant women should be considered. However, it is valid to evaluate later on a mammalian animal to verify which alternative model has more similarity.

Finally, we found that PH51 and PH153 protected Tl-deficient *Drosophila* flies infected with *C. albicans* demonstrating the antifungal effect of compounds when orally absorbed. The compounds showed low toxicity in flies and chicken embryos, and dose-dependent mortality in the zebrafish model. Furthermore, the compounds showed to alter behavioral patterns only at the highest concentrations tested. Taken together, data from this study support the potential of PH151 and PH153 for the treatment of systemic candidiasis in immunocompromised patients and they demonstrate to be appropriate drug candidates to advance in mammalian studies.

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Conflict of interest

No conflict of interest declared.

Figures

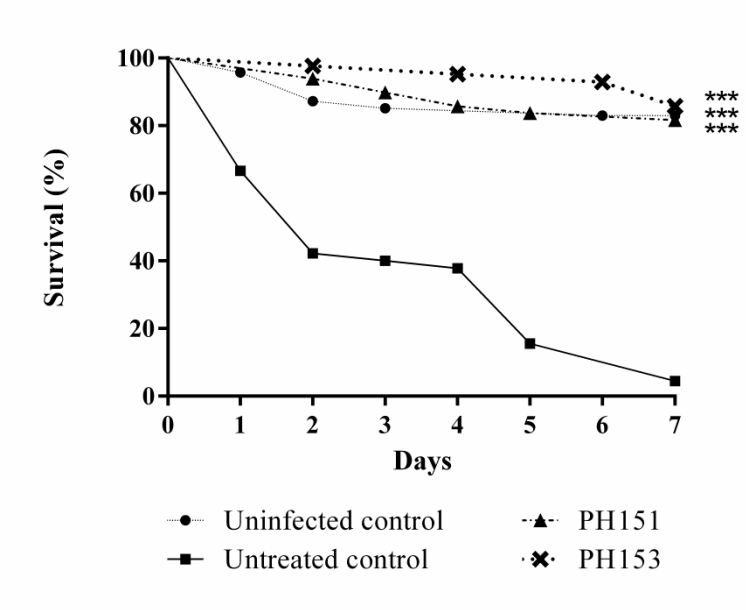

Figure 1. Percent survival of Toll mutant *Drosophila melanogaster* flies infected with *C. albicans* and treated with PH151 and PH153. No statistical difference was observed between the treatments (1 mg/ml) and uninfected control. The asterisks indicate statistical difference compared to the untreated control (***p< 0.001).

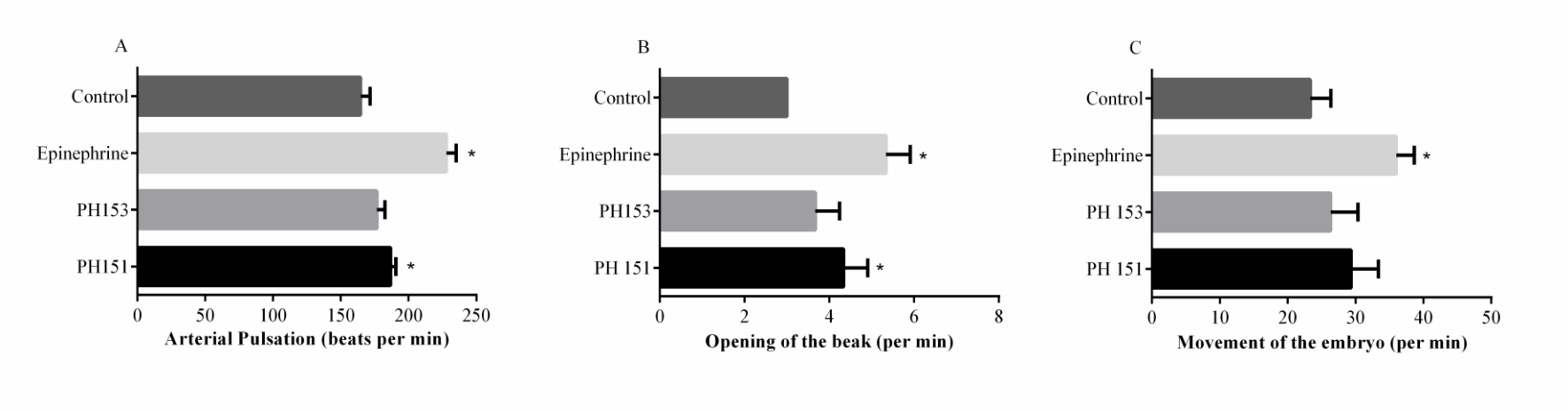

Figure 2. Behavioral testing in chicken embryos exposed to PH151, PH153, epinephrine (positive control) and saline solution (untreated control) with observation of arterial pulsation (A), opening of the beak (B) and movement of the embryo. The asterisks indicate statistical difference compared to the untreated control (*p< 0.05).

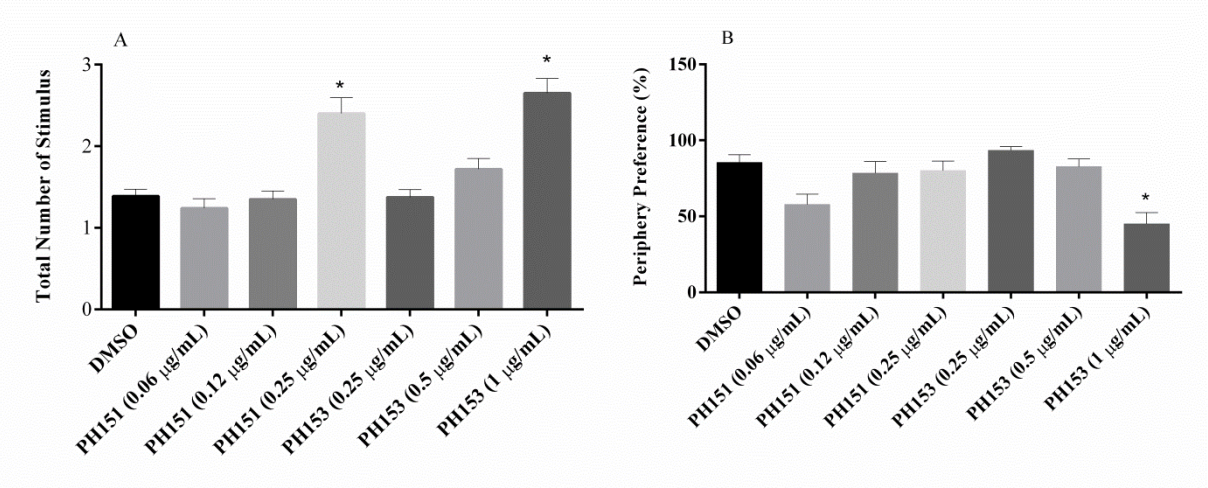

Figure 3. Responses of zebrafish larvae submitted to touch stimulus after exposition to PH151 and PH153. The number of touch stimuli required for the first displacement of the larvae (A) and the preference for the periphery of the apparatus (B) were observed. The asterisks indicate statistical difference compared to DMSO control (*p< 0.05).

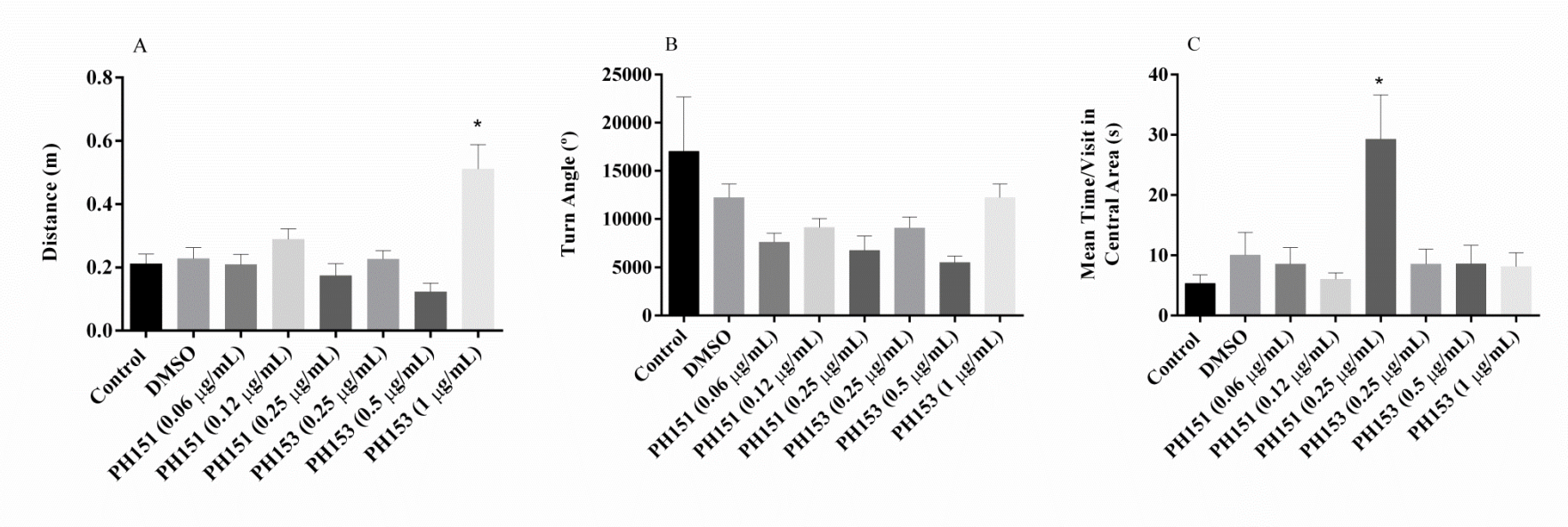

Figure 4. Exploratory behavior test (open field) in the larvae zebrafish exposed to PH151 and PH153. The distance travelled (A), absolute turn angle (B) and immobility (C) were evaluated. The asterisks indicate statistical difference compared to control (*p< 0.05).

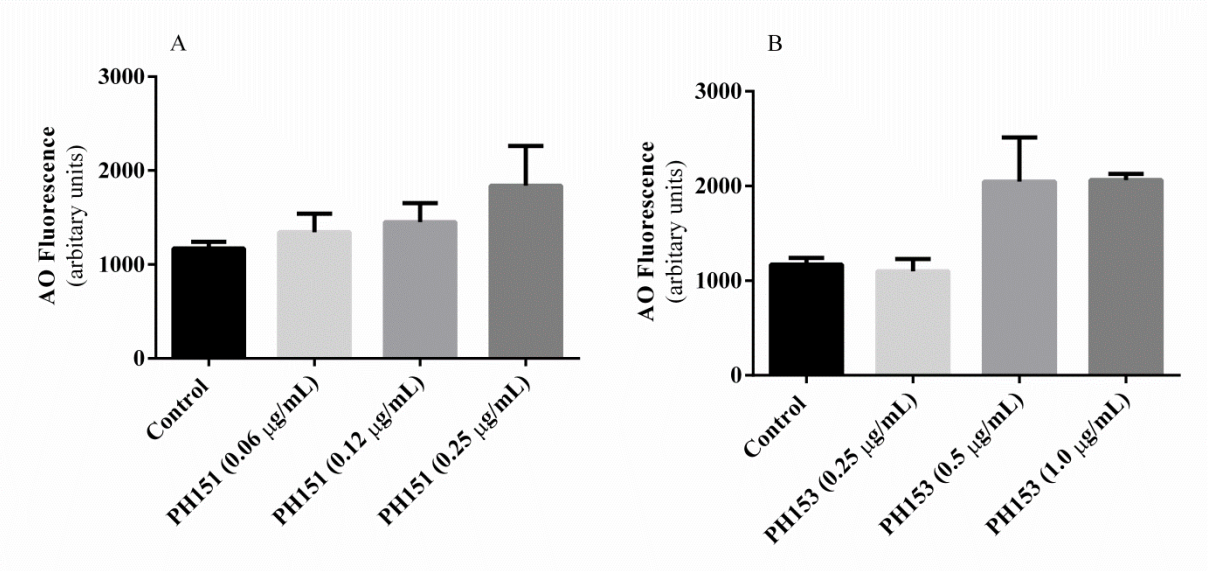

Figure 5. Detection of cells in the apoptosis process in the larvae zebrafish exposed to PH151(A) and PH153(B) by the acridine orange test. The measures did not present statistical difference compared to control.

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6 DISCUSSÃO GERAL

Os fungos têm uma evidente capacidade para desenvolverem resistência a agentes antifúngicos, ameaçando a eficácia dos tratamentos disponíveis e gerando uma crítica crise na saúde pública. A resistência fúngica à terapia torna-se um problema ainda mais grave em vista da escassez de antifúngicos utilizados na clínica e da dificuldade de encontrar novos agentes terapêuticos seguros para a saúde humana (Revie et al., 2018). Associado a esse panorama, poucos medicamentos nesta área estão sendo desenvolvidos, especialmente porque não há previsão que os antifúngicos gerem um retorno financeiro suficientemente grande para empresas farmacêuticas (Calderone et al., 2014; Polvi et a., 2015). Em vista disso, a contínua busca por estratégias terapêuticas inovadoras para combater infeções fúngicas se faz necessária para melhorar o prognóstico dos pacientes que apresentam estas doenças (Polvi et al., 2015).

Com a resistência a medicamentos antimicóticos em ascensão, nosso grupo de pesquisa defende a descoberta e desenvolvimento de novos agentes antifúngicos a fim de suprir a deficiência encontrada no mercado farmacêutico. Neste trabalho visou-se avaliar o potencial de derivados de 8-hidroxiquinolina como candidatos a agentes antimicóticos (principais resultados estão esquematizados na figura 13 e 14). Para esta finalidade, inicialmente avaliamos a atividade antifúngica do clioquinol **1**, o qual já é disponível como antisséptico na forma tópica, e de dois derivados ácidos sulfônicos da 8-hidroxiquinolina (aqui chamados de composto **2** e composto **3)**. A conversão de derivado ácido sulfônico em sulfonamidas foi realizada por Joaquim et al. (2018) e resultou em outros dois derivados com alta atividade antifúngica e que foram incluídos neste estudo (PH151 e PH153).

Testes iniciais de suscetibilidade com derivados de 8-hidroxiquinolina foram realizados com uma grande variedade de fungos, incluindo leveduras do gênero *Candida* e fungos filamentosos dermatofíticos. Os cinco derivados de 8-hidroxiquinolina avaliados neste estudo mostram atividade in vitro para todos os isolados testados. Essas descobertas são muito relevantes, visto que alguns destes isolados apresentam fenótipo multirresistente aos medicamentos utilizados na terapia, tais como azóis, equinocandinas, grisofulvina e terbinafina.

Os resultados do teste de suscetibilidade fúngica aos derivados da 8-hidroxiquinolina indicaram menores valores de CIMs para o clioquinol **1** (0.0031 - 2 µg/mL). Em geral, os derivados ácidos sulfônicos apresentaram valores moderados de CIMs, variando de 1 - 512 µg/mL para o composto **2** e de 2 - 1024 µg/mL para o composto **3**. Entretanto, a conversão dos ácidos sulfônicos em sulfonamidas favoreceu a atividade antifúngica, uma vez que os valores de CIMs variaram de 1 - 4 µg/mL para PH151 e de 4 - 16 µg/mL para PH153, como previamente descrito por Joaquim et al. (2018).

A literatura tem demonstrado que a atividade biológica é modulada pela natureza dos substituintes nas posições 5 e 7 do anel quinolina. Em estudo anterior foi verificado que a presença dos halogênios cloro e bromo nas posições 5 ou 7, em geral, estava relacionada a um aumento da atividade antifúngica, o que não foi observada com os compostos fluorados. A presença de iodo na posição 7 também levou a um incremento na atividade contra algumas espécies (Gershon et al., 2001). Esta evidência corrobora com os resultados encontrados aqui, uma vez que clioquinol **1** foi a molécula mais ativa. Este achado pode estar relacionado à presença de iodo na posição 7 e ao cloro na posição 5 da sua estrutura (Gershon et al., 2001). Embora tenha sido verificado que a presença de halogênios pode estar associada à atividade relevante observada, é de conhecimento que eles podem influenciar no aumento da toxicidade, especialmente o iodo. Por isso, é importante destacar a atividade antifúngica encontrada para o composto **2**, mesmo na ausência de halogênio em sua estrutura.

Gershon et al. (2001) avaliaram uma série de derivados de 8-hidroxiquinolina (incluindo os compostos **2** e **3**) contendo um ácido sulfônico contra seis espécies de fungos filamentosos: *A. niger*, *A. oryzae*, *Myrothecium verrucaria*, *Trichoderma viride*, *Mucor circinelloides* e *T. mentagrophytes*. Ao contrário dos nossos resultados, esses pesquisadores não encontraram atividade antifúngica para o composto **2**. No entanto, testes com leveduras não foram realizados. Além disso, 100 µg/mL foi a maior concentração testada do composto e, como mostramos aqui, os fungos filamentosos foram menos suscetíveis. Como mencionado, a vantagem do composto **2** é a ausência do iodo no anel quinolina, o que poderia diminuir a toxicidade da molécula. No entanto, os ácidos sulfônicos podem ter inconvenientes relacionados à penetração na célula fúngica, uma vez que poderiam ionizar na faixa de pH biológico devido à sua natureza ácida, dificultando sua ação. Assim, a

conversão do ácido sulfônico em uma sulfonamida é uma estratégia interessante, uma vez que a sulfonamida é um ácido muito fraco e não se ioniza (Joaquim et al., 2018). De fato, os resultados obtidos por Joaquim et al. (2018) para as sulfonamidas PH151 e PH153 apoiam essa evidência, uma vez que os valores de CIMs encontrados foram bastante inferiores que para os ácidos sulfônicos.

A CIM tem sido o principal parâmetro de farmacodinâmica para verificar a suscetibilidade de micro-organismos a agentes antimicrobianos, pois é um teste relativamente simples e, portanto, pode ser aplicado facilmente por laboratórios (Nielsen e Friberg 2013). No entanto, o valor de CIM não fornece uma caracterização muito detalhada da atividade porque é uma medida avaliada em um único ponto de tempo e não relata a taxa de morte microbiana. Assim, as mudanças dinâmicas no crescimento e suscetibilidade durante o período de tempo estudado não são levadas em consideração (Treyaprasert et al., 2007; Nielsen e Friberg 2013). Por outro lado, as curvas de tempo de morte são cada vez mais utilizadas para oferecer informações detalhadas sobre a eficácia antimicrobiana em função do tempo e da concentração (Gupta et al. 2009; Ghannoum et al., 2013).

A atividade antifúngica dos derivados da 8-hidroxiquinolina foi confirmada pelo ensaio de tempo de morte, no qual foi observado que os distintos substituintes no anel quinolina levaram a diferentes efeitos sobre as células fúngicas. O clioquinol **1** é um antifúngico conhecido e a literatura o descreve como agente fungistático (Hugo 1971; Holt 1980). No entanto, não conseguimos encontrar informações consistentes na literatura com metodologias apropriadas demonstrando esse efeito. Embora clioquinol **1** tenha sido utilizado por muitos anos, estudos de tempo de morte ainda não tinham tido realizados até então. Nossos resultados mostraram um efeito fungistático do clioquinol **1** para *C. albicans* e *M. canis*. Contrariando os relatos da literatura, este composto foi fungicida para *T. mentagrophytes*. Os ácidos sulfônicos (compostos **2** e **3**) apresentaram efeito fungistático para *C. albicans* e efeito fungicida para dermatófitos. Em relação às sulfonamidas, PH151 exibiu propriedades fungistáticas para todos os isolados testados, enquanto que PH153 apresentou efeito fungistático para *C. albicans*, mas fungicida para dermatófitos.

Além de espécie-dependente, o efeito fungicida das 8-hidroxiquinolinas avaliadas aqui também foi dependente da concentração e do tempo. Embora numerosos fatores possam influenciar na atividade *in vivo* de um agente, nossos dados sugerem que, em geral, a ação fungicida dos compostos contra dermatófitos

pode ser alcançada pela administração de doses elevadas. Agentes que melhoram a atividade antifúngica com concentrações crescentes (por exemplo, anfotericina B) podem ser otimizados pela administração de regimes terapêuticos prolongados. Em contraste, os efeitos fungistáticos contra *Candida* sp. foram independentes da concentração. O fluconazol, um dos antifúngicos mais utilizados, atua dessa forma, exigindo a administração contínua da droga (Klepser et al., 19997).

Em associação com os ensaios de tempo e morte, a modelagem PK/PD tornou-se uma ferramenta poderosa para avaliar o efeito antimicrobiano de fármacos (Nielsen et al., 2007; Treyaprasert et al., 2007; Bulitta et al., 2010). A modelagem PK/PD é aplicada para prever doses testadas e não testadas, para melhorar os esquemas de dosagem de antibióticos e antifúngicos atualmente disponíveis e para facilitar o desenvolvimento de novos agentes antimicrobianos (Nielsen e Friberg, 2013). A fim de obter informações farmacodinâmicas adicionais, a modelagem foi aplicada para os três derivados de 8-hidroxiquinolina mais ativos (clioquinol **1**, PH151 e PH153). Os modelos Emax otimizados são úteis para descrever a relação entre concentração e efeito em antimicrobianos (Dalla Costa et al., 1997; de la Pena et al., 2004; Li et. Al., 2009) e foram aplicados com sucesso em nosso estudo. Entretanto, para modelar os dados de tempo de morte, foi necessário aplicar diferentes modelos de PK/PD para cada isolado a fim de obter uma melhor descrição da atividade antifúngica.

Os dados mostraram que os derivados da 8-hidroxiquinolina são eficazes contra *C. albicans*, *M. canis* e *T. mentagrophytes* em baixas concentrações, apresentando valores de EC_{50} semelhantes aos valores de CIM. A EC_{50} do clioquinol **1** foi 0,181 µg/mL para *C. albicans*, 0,306 µg/mL para *M. canis* e 0,434 µg/mL para *T. mentagrophytes.* Em relação às sulfonamidas, a EC_{50} variou entre 0,873 - 1,329 µg/mL para PH151 e entre 7,105 - 8,865 µg/mL para PH153. Embora a EC_{50} tenha sido maior para o PH153, este composto alcançou o efeito máximo mais rápido que os outros dois compostos. O tempo estimado para o efeito máximo do PH153 contra *C. albicans*, *M. canis* e *T. mentagrophytes* foi 7h, 17h e 0.25h, respectivamente; enquanto que para PH151 foi 34h, 25h e 12h; e para o clioquinol **1** foi 13h, 48 h e 24h. É possível observar que clioquinol **1** alcançou o efeito máximo mais rápido para o isolado que apresenta a proliferação mais acelerada (*C. albicans*), tal como já evidenciado por Li et al. (2007 e 2009) em estudos com voriconazol. Ao contrário,

PH151 alcançou efeito máximo mais rapido para os dermatófitos, os quais apresentam crescimento lento.

Além disso, nós extraímos dados farmacocinéticos da literatura, encontrados em humanos, e simulamos os níveis plasmáticos esperados para diferentes regimes de dosagens orais de clioquinol **1**. Os valores de EC_{50} indicaram que o clioquinol **1** foi altamente eficaz contra os fungos testados em níveis séricos facilmente alcançáveis (Jack e Riess, 1973). Ainda, nós pudemos prever que, durante o período de 6 dias, o regime de dosagem de 25 mg a cada 12 horas é suficiente para manter as concentrações plasmáticas acima da concentração antifúngica efetiva, sugerindo que esta dose poderia ser usada para tratar doenças fúngicas e, eventualmente, minimizar ou abolir possíveis efeitos tóxicos.

Na fase I de um estudo clínico do clioquinol **1** em pacientes com neoplasia hematológica avançada, observou-se que 1600 mg duas vezes ao dia (3200 mg de dose total) é a dose limitante de neurotoxicidade e dor abdominal (Schimmer et al., 2012). Quando utilizado como antimicrobiano antes de ser retirado do mercado, a dose terapêutica de clioquinol **1** variava entre 250 - 3500 mg por dia; mas, a maioria dos pacientes eram tratados com 750 - 1000 mg por dia (Woodward e Rahman, 1969; Richards, 1971; Tsubaki et al., 1971). Esta abordagem de combinação de dados de tempo de morte *in vitro* com dados farmacocinéticos *in vivo* existentes podem servir para delinear um modelo para futuros estudos em mamíferos, a fim de definir regimes antifúngicos ideais. No entanto, características farmacocinéticas e perfil de permeação das novas 8-hidroxiquinolinas (PH151 e PH153) ainda precisam ser investigados para melhor aplicabilidade dos dados obtidos aqui.

O mecanismo de ação do clioquinol **1** e outros derivados de 8-hidroxiquinolina sobre as células fúngicas não são bem compreendidos. Sabe-se que as 8-hidroxiquinolinas podem inibir certas enzimas relacionadas à replicação do DNA em vírus e que a 8-hidroxiquinolina inibe a síntese de RNA em leveduras (Auld et al. 1974; Fraser e Creanor, 1974). No entanto, estudos sobre o mecanismo de ação antifúngico deste grupo são escassos. Assim, o modo de ação dos derivados da 8-hidroxiquinolinas foi investigado a fim de obter informações mais consistentes sobre a interação dinâmica desses compostos com os fungos. Os resultados obtidos no ensaio de proteção do sorbitol indicaram que os cinco derivados estudados aqui atuam sobre a parede celular fúngica. No entanto, o efeito dos ácidos sulfônicos

(composto **2** e **3**) sobre a parede foi dependente da espécie. Corroborando com os resultados encontrados no ensaio de proteção do sorbitol, a parede celular irregular foi facilmente visualizada na MEV de dermatófitos tratados com os derivados. Rupturas, mesmo em doses subinibitórias, foram claramente identificadas quando os dermatófitos foram tratados com PH151 e PH153; e também quando *M. canis* foi tratado com o composto **3**. A parede celular representa um alvo muito interessante porque as células humanas não possuem essa estrutura e, portanto, a toxicidade para o hospedeiro tende a ser menor (Morace et al., 2014).

Os ácidos sulfônicos **2** e **3** também mostraram ação sobre a membrana celular fúngica, uma vez que alteração da permeabilidade celular de *Candida* spp. e dermatófitos foi detectada pelo ensaio de extravasamento celular; entretanto, essa ação não foi resultante da ligação direta ao ergosterol da membrana. Além disso, na MEV foram detectadas numerosas cicatrizes de brotamento não-polares quando *C. albicans* foi tratada com o composto **3**. Tais achados podem indicar que o processo de divisão celular normal foi afetado, resultando em células únicas com múltiplas tentativas de divisão (Khan et al. , 2013). A presença das células circulares maiores (visualizadas no tratamento com composto **3** e PH151) reforça essa hipótese e pode indicar o fenômeno da endopoliploidia (poliploidia somática) devido a ciclos recorrentes de replicação de DNA sem divisão celular. Estudos anteriores demonstraram que fatores ambientais, tais como tratamentos antifúngicos, podem estar associados a esse fenômeno em várias espécies de fungos (Albertin e Marullo, 2012). A presença das células circulares grandes também poderia indicar alteração na permeabilidade celular. Entretanto, tais discussões são ainda especulativas e devem ser melhores compreendidas em estudos futuros.

Complementarmente, clioquinol **1**, PH151 e PH153 inibiram a formação de pseudo-hifas em *C. albicans*. Estudos sugerem que as pseudo-hifas são um estado intermediário entre células leveduriformes e hifas (Lu et al., 2014). A presença dessas estruturas é essencial para a virulência de *Candida* (Sudbery et al., 2004), uma vez que são uma barreira para a fagocitose, facilitam a penetração nos tecidos epiteliais e endoteliais, aumentam a aderência e contribuem para a formação de biofilme (Lu et al., 2014; Leite et al., 2015; Desai 2018). Ainda, acredita-se que a transição morfológica de levedura para a forma filamentosa é um fator crítico para a patogenicidade de infecções fúngicas em níveis superficiais e sistêmicos (Desai 2018; Cleary et al., 2016). Embora os antifúngicos comercialmente

disponíveis tenham a membrana plasmática e a parede celular como principais alvos (Ngo et al., 2016), estudos recentes se concentraram na inibição de fatores de virulência para desenvolver novas terapias (Lu et al., 2014; Scorzoni et al. ., 2017).

De fato, a inibição de pseudo-hifas parece ter um papel importante na atividade *in vivo*. Os três compostos inibidores de pseudo-hifas (Clioquinol **1**, PH151 e PH153) demonstraram ótimos efeitos antifúngicos quando administrados oralmente (1mg/mL) por *D. melanogaster* Tl-deficientes infectadas com *C. albicans*; sem aparente presença de toxicidade para este animal. Tais achados podem estar relacionados à atenuação da virulência e patogenicidade em consequência da inibição das psudo-hifas, como discutido anteriormente. Por outro lado, os ácidos sulfônicos **2** e **3** (1mg/mL) protegeram apenas 45% e 63% das moscas infectadas, respectivamente. Estudo prévio conduzido por Chamilos et al. (2006) mostrou que fluconazol (1mg/mL) também aumentou a sobrevivência das moscas infectadas com *C. albicans*, contudo a sobrevida não excedeu 80%. Assim, nossos resultados indicam que fatores associados no processo de conversão de diferentes morfologias representam mecanismos de ação promissores.

Ainda, é importante destacar que embora as 8-hidroxiquinolinas tenham se ligado à albumina bovina, o efeito de proteção destes compostos permaneceu nas experiências realizadas *in vivo*. A ligação reversível com proteínas plasmáticas ocorre quando a maioria dos fármacos atinge a circulação sistêmica e pode ser um indicativo de biodisponibilidade comprometida (Wasan et al. 2008). No entanto, vários antifúngicos exibem essa propriedade, tal como anfotericina B, cetoconazol e itraconazol B (Schafer-Korting et al., 1991; Bekersky et al. 2002).

A inibição de pseudo-hifas também pode ter uma função essencial na ação do clioquinol **1** sobre biofilme fúngico. As infecções por biofilme de *Candida* spp. são frequentemente associadas ao uso de biomateriais e apresentam significância clínica por serem comumente resistentes aos antifúngicos (Fanning e Mitchell, 2012; Vipulanandan et al., 2018). Uma opção atraente para controlar a formação de biofilme é aplicar agentes antifúngicos sobre a superfície de biomateriais para, assim, evitar a colonização de células. O revestimento dos poços de uma placa de microdiluição com clioquinol **1** resultou em redução das células aderentes evitando mais de 90% da formação do biofilme. Entretanto, uma vez que a infecção por *Candida* for estabelecida, o tratamento antifúngico sem remoção do dispositivo raramente é efetivo (Mermel et al., 2013; Nobile e Johnson, 2015). O

estudo de susceptibilidade do biofilme ao clioquinol **1** demonstrou ação deste composto sobre a atividade metabólica do biofilme, porém aumento da CIM das células sésseis em às células planctônicas foi detectado. O aumento da CIM de diversos agentes antifúngicos, tais como anfotericina B, caspofungina, flucitosina e azóis, tem sido frequentemente documentado para biofilme de *Candida* (Hawser e Douglas, 1995; Shuford et al., 2007, Khan e Ahmad, 2012). Ainda, nós observamos que clioquinol **1** foi capaz de remover biofilme pré-formado em placa de microdiluição, contudo, essa ação foi menos eficaz quando comparada à inibição da formação e inibição da atividade metabólica do biofilme.

Propriedades de inibição da formação, redução da atividade metabólica e remoção do biofilme também foram verificadas por exposição aos ácidos sulfônicos **2** e **3**. Estes compostos foram menos potentes que o clioquinol **1**, uma vez que não alcançaram redução de 90% do biofilme em todas as metodologias testadas. Como mencionado acima, a inibição das pseudo-hifas possivelmente está relacionada aos resultados encontrados aqui. Ramage et al. (2002) demonstraram que cepas mutantes de *C. albicans* incapazes de germinar não conseguiram formar biofilmes. Baillie e Douglas (1999) concluíram que as hifas são essenciais para proporcionar a integridade estrutural e a arquitetura multicamadas dos biofilmes maduros. Assim, nossos resultados indicam que a redução do biofilme de *C. albicans* provavelmente é atribuída a uma consequência direta do efeito inibitório do clioquinol **1** no processo morfogenético. Resultados semelhantes foram encontrados por Ramage et al. (2007). Eles mostraram que o EDTA bloqueia o desenvolvimento de hifas em *C. albicans* e este agente quelante também pode afetar a formação de biofilmes.

O poliestireno, material usado na fabricação de microplacas, mostrou ser um excelente modelo para estudos de biofilme (Chassot et al., 2008). No entanto, este não é utilizado na produção de dispositivos médicos. Assim, após observação dos resultados obtidos em microplaca, escolhemos o clioquinol **1** para avaliar o efeito sobre o biofilme pré-formado em DIU e comparar ao fluconazol. Este é o primeiro protocolo que quantifica a ação inibitória de compostos sobre biofilmes formados na superfície desse dispositivo. Inicialmente, a capacidade de formação de biofilme de diferentes espécies de *Candida* foi confirmada pela contagem de células na superfície do DIU. Chassot et al. (2008) também descreveram a capacidade de *C. albicans* formar biofilme no DIU de cobre, mas estudos com espécies não *albicans* ainda não foram relatados até o momento. Curiosamente, o biofilme fúngico

em DIU raramente foi estudado. Esta falta de interesse é preocupante porque este disposto parece estar relacionado à CVV e pode levar a complicações clínicas graves (Donlan e Costerton, 2002; Chassot et al., 2008).

Clioquinol **1** foi eficaz na erradicação de biofilme em DIU, especialmente para *C. glabrata*, uma vez que erradicação total foi evidenciada. *C. glabrata* é conhecida por ser resistente a antifúngicos, portanto, erradicar biofilme desta espécie é uma característica importante (Pfaller 2012; Lockhart 2014). Clioquinol **1** também foi eficaz contra biofilme de *C. albicans* e *C. tropicalis*, as duas espécies que formaram mais biofilme no dispositivo. Além disso, os dados mostraram que clioquinol **1** teve maior eficiência em erradicar biofilme que fluconazol, independentemente da espécie. Fluconazol mostrou baixa erradicação (< 70%) do biofilme de *C. krusei* e *C. parapsilosis*. Tais achados são muito relevantes porque os antifúngicos azólicos, especialmente fluconazol, são amplamente utilizados para tratar CVV (Kim e Sudbery, 2011). Assim, a alta erradicação de biofilme em DIU associado a raros relatos de resistência fúngica ao clioquinol **1**, pode tornar este composto uma opção importante para o tratamento de CVV recorrente relacionada ao uso desse dispositivo. Portanto, o desenvolvimento de um creme ou pomada vaginal se faz necessário para avaliar tal efeito, visto que as formulações comercializadas contendo clioquinol são apenas para uso dermatológico.

A ação antifúngica dos derivados da 8-hidroxiquinolina contra *Candida* spp. e dermatófitos foi um estímulo para investigar seus potenciais efeitos tóxicos a fim de desenvolver formulações farmacêuticas futuras. A ausência de lesão verificada na avaliação histopatológica e os baixos níveis de irritabilidade encontrados no HET-CAM demonstram a baixa toxicidade dérmica dos compostos. Tais resultados estão de acordo com a literatura. Clioquinol **1** tem demonstrado ser raro sensibilizante e não tem sido associado ao desenvolvimento de malignidades cutâneas (Lazarov, 2006; Alsterholm et al., 2010). Apesar de já existirem estudos demonstrando tais características do clioquinol **1**, modificações químicas estruturais poderiam resultar em diferentes padrões de toxicidade. Entretanto, isso não ocorreu. Clioquinol **1**, Composto **2**, composto **3**, PH151 e PH153 mostraram resultados similares. As características encontradas aqui são motivadoras para o desenvolvimento de novos medicamentos tópicos para tratar micoses superficiais e cutâneas, bem como infecções de mucosa. A escolha dessa via de administração tem vários benefícios, tais como: ação direta no local da infecção, aumento da

eficácia do tratamento e redução de efeitos colaterais devido à minimização da entrada do fármaco na circulação sistêmica (Güngör et al., 2013).

Além disso, parâmetros toxicológicos em embriões de galinha e *zebrafish* também foram avaliados no intuito de verificar possíveis efeitos indesejados relacionados à administração sistêmica das 8-hidroxiquinolinas. Apesar das diferenças óbvias entre aves e mamíferos, essas duas classes compartilham padrões semelhantes de embriogênese, organogênese, biologia cardiovascular, neurobiologia, entre outras características. Talvez o mais importante seja que o desenvolvimento embrionário pode ser estudado independentemente da exposição materna a compostos tóxicos (Ballatori e Villalobos, 2002; Betsabee et al., 2017). Os resultados mostraram que altas concentrações (1mg/mL) de clioquinol **1**, composto **2**, PH151 e PH153 não causaram interrupção da embriogênese. A baixa toxicidade corrobora com os resultados encontrados no modelo de *D. melanogaster* e pode indicar segurança durante a embriogênese. Por outro lado, o composto **3** mostrou toxicidade relevante uma vez que foi letal para o embrião na concentração de 1 mg/mL, o que pode ser um alerta para possível embriotoxicidade.

O aumento significativo da pulsação arterial apresentada pelos embriões de galinha expostos ao clioquinol **1**, composto **2** e PH151 pode indicar ação estimulante como um possível efeito colateral desses três compostos. O aumento do número de abertura do bico do embrião exposto ao PH151 reforça essa hipótese. Embora estatisticamente significativos, esses aumentos foram discretos. Ainda, deve-se levar em consideração que os animais durante o desenvolvimento embrionário são mais sensíveis e vulneráveis do que durante os demais estágios da vida (Ballatori e Villalobos, 2002).

Zebrafish é outro modelo alternativo explorado em algumas áreas da pesquisa biomédica, como triagem de novos fármacos, toxicidade e segurança farmacológica (Phillips et al., 2014; Ordas et al., 2015). Com o intuito de avançar nossa pesquisa em um modelo animal mamífero, os dois derivados de 8-hidroxiquinolina sintetizados pelo grupo (PH151 e PH153) foram submetidos a testes toxicológicos em *zebrafish* na fase embrio-larval. Os embriões de *zebrafish* foram mais sensíveis aos compostos quando comparados aos outros modelos animais aqui estudados. Foi possível observar toxicidade de PH151 e PH153 em estágio inicial de desenvolvimento em concentrações até 10^3 vezes menores que *D. melanogaster* e embriões de galinha.

A toxicidade encontrada foi dependente da concentração dos compostos. A dose letal de PH151 variou entre 0,25 - 8 µg/mL para PH151 e entre 4 - 32 µg/mL para PH153, dependendo do tempo de exposição dos embriões de *zebrafish*. Resultados semelhantes foram encontrados para anfotericina B no mesmo modelo animal. A DL_{50} (dose que mata 50%) em embriões de *zebrafish* para este antifúngico foi 5 µg/mL (Wang et al. 2010). Contudo, estudo prévio de citotoxicidade *in vitro* realizado por nosso grupo demonstrou que as IC_{50} (concentração que inibe 50%) em células VERO foram bem mais altas, sendo 18,3 µg/mL para PH151 (4 a 16 vezes superiores às CIMs) e 22,1 µg/mL para PH153 (cerca de 3 vezes superiores às CIMs) (Joaquim et al., 2018).

Além da mortalidade, parâmetros comportamentais também foram observados. A resposta ao toque é mediada por entradas sensoriais que resultam na ativação dos motoneurônios primários. O toque leva a reorientação do peixe para que ele se afaste do estímulo, seguido de natação e fuga (Saint-Amant e Drapeau, 1998). Em geral, não houve incapacidade motora nos grupos expostos às menores concentrações dos compostos, uma vez que as larvas responderam normalmente ao toque. Por outro lado, a maior concentração de PH151 (0,25 µg/mL) e PH153 (1 µg/mL) exigiu mais estímulos e menor capacidade de realizar o escape padrão em direção à periferia (tigmotaxia). Os resultados podem estar associados à baixa sensibilidade e consequente diminuição da atividade motora (Powers et al. 2010). Esses dados são consistentes com a literatura, a qual relata que exposições a produtos químicos e farmacêuticos podem alterar os níveis de neurotransmissores no cérebro e causar diminuição da atividade motora (Thirumalai, 2008; Eddins, 2010).

Os comportamentos locomotores desempenham um papel essencial nas atividades de alimentação, sociais e defensivas do *zebrafish* (Colwill e Creton, 2011). O teste de comportamento exploratório é um dos procedimentos mais populares em psicologia animal (Prut e Belzung, 2003) e tem sido usado com sucesso em modelos de *zebrafish* (Colwill e Creton, 2011; Ahmad e Richardson, 2013). As larvas são colocadas no aparelho e os itens comportamentais são registrados por um período de tempo. Em tal situação, os animais preferem espontaneamente a periferia do aparelho. Foi observado aumento do tempo gasto na zona central em larvas expostas a 0,25 µg/mL de PH151. Esse comportamento pode ser interpretado como efeito ansiolítico ou sedativo desse composto,

contrariando os resultados encontrados em embriões de galinha. Estes resultados contraditórios podem ser explicados devido aos diferentes modelos animais utilizados.

Por outro lado, a distância percorrida aumentou quando as larvas foram expostas à 1 µg/mL de PH153. O aumento da atividade natatória pode ser considerado um efeito estimulante (Feng et al., 2014; Shontz et al., 2018). Além disso, nosso estudo demonstrou que a exposição aos compostos foi incapaz de alterar o ângulo de giro absoluto. O ângulo de giro é um parâmetro motor comportamental que analisa o ângulo de cada mudança de direção do corpo da larva durante o teste. Uma diminuição no ângulo de rotação está relacionada a uma menor atividade motora - característica não detectada aqui (Budick e O'Malley, 2000; Altenhofen et al., 2017).

O teste de coloração com laranja de acridina demonstrou apenas apoptose fisiológica, visto que exposições a diferentes concentrações de PH151 e PH153 não resultaram em diferenças ou aumento nas áreas apoptóticas em comparação ao controle sem tratamento.

Por fim, a aparente toxicidade de PH151 e PH153 foi observada sobre *zebrafish* nas fases embrio-larval, logo, não se pode afirmar sobre uma potencial toxicidade em adultos. Os dados encontrados em embriões e larvas de *zebrafish* diferem das outras espécies avaliadas, mas podem ser um alerta para uma possível embriotoxicidade. Portanto, indicação cautelosa para mulheres grávidas deve ser considerada. Ainda, avaliações futuras em um animal mamífero para verificar qual modelo alternativo tem mais semelhança é imprescindível.

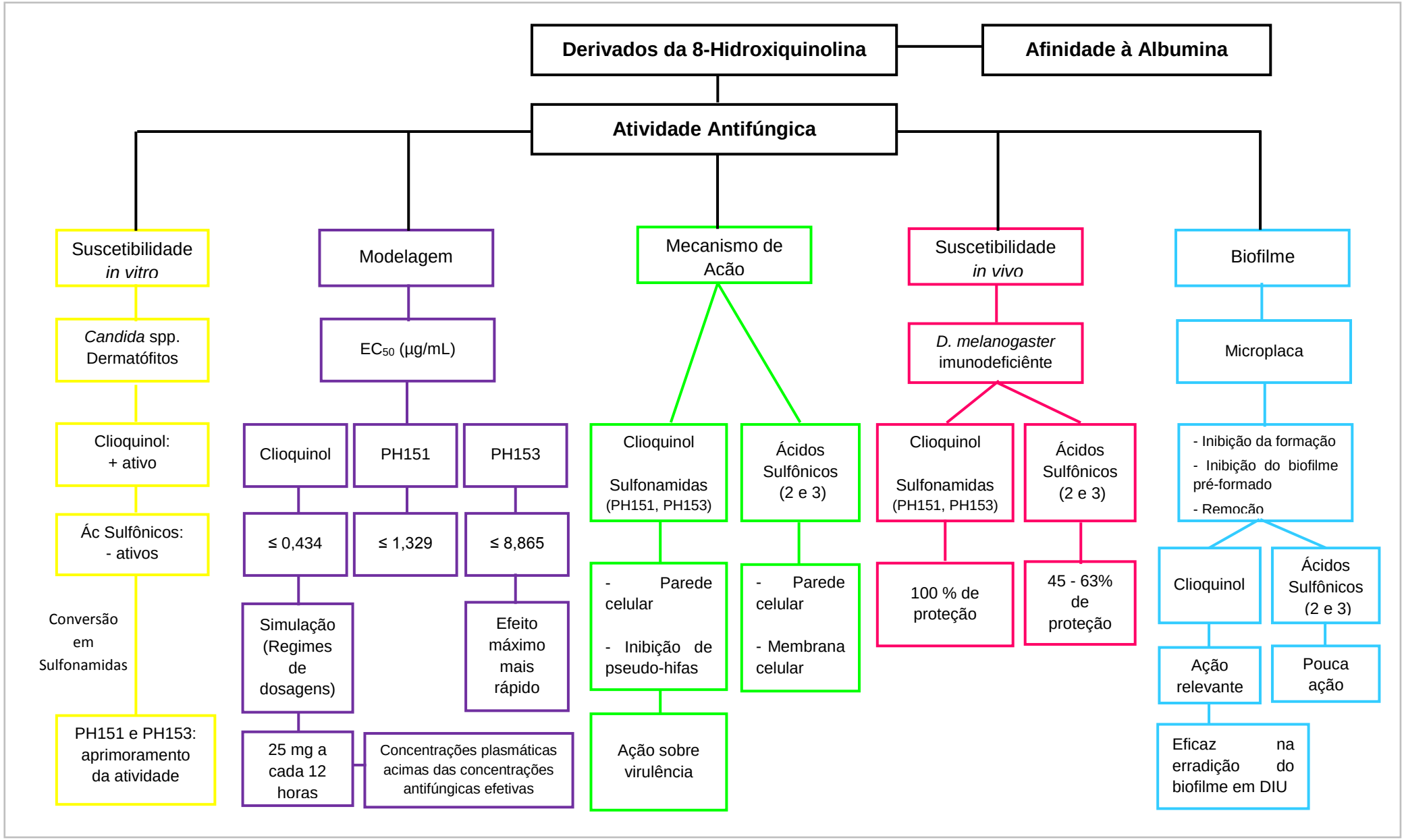

Figura 13. Representação esquemática dos principais resultados obtidos em relação à atividade antifúngica dos derivados da 8-hidroxiquinolina.

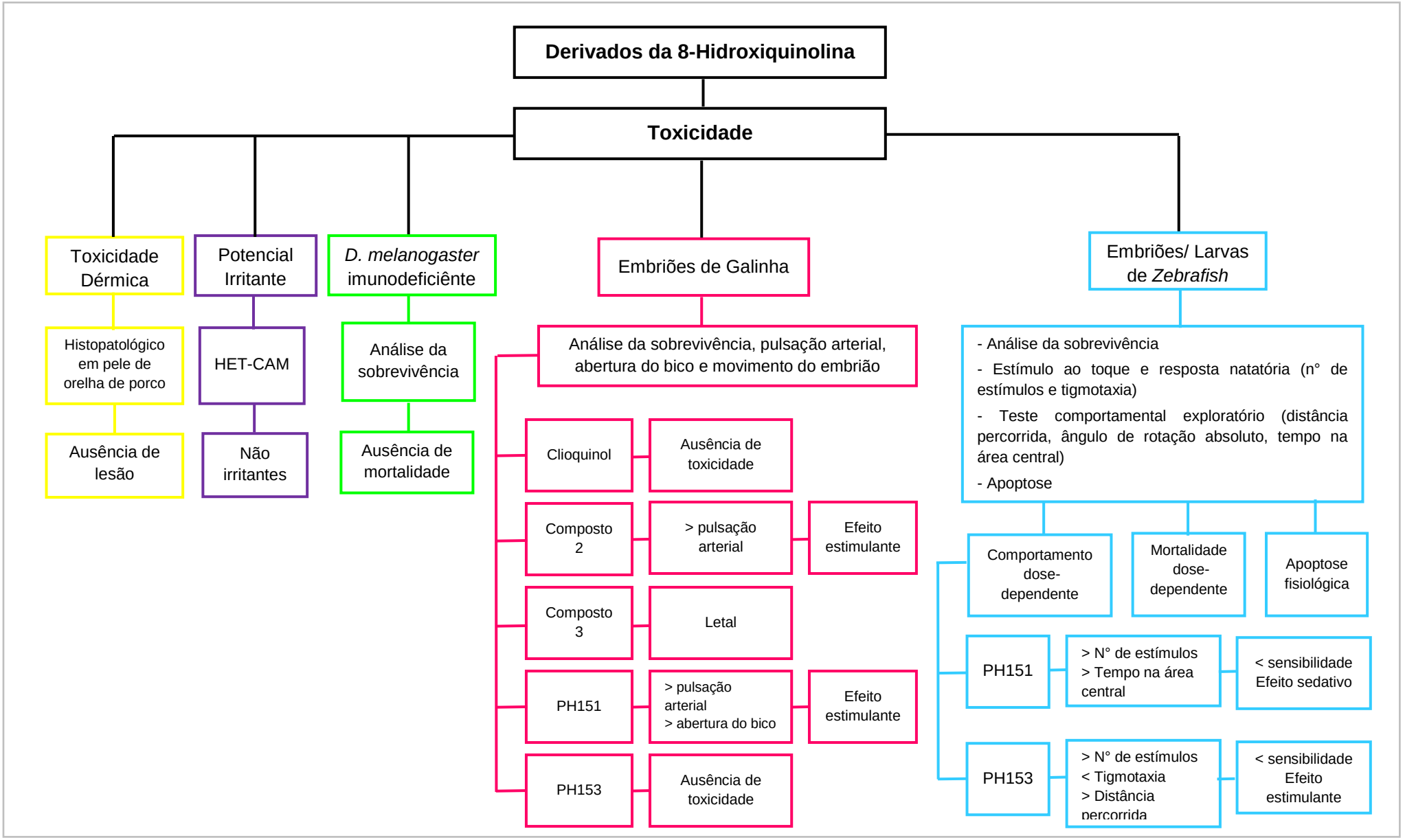

Figura 14. Representação esquemática dos principais resultados obtidos em relação à toxicidade dos derivados da 8-hidroxiquinolina.

7 CONCLUSÃO

A partir dos resultados obtidos foi possível inferir que:

✓ Os cinco derivados de 8-hidroxiquinolina avaliados neste estudo mostraram atividade *in vitro* para todos os isolados de *Candida* e dermatófitos testados. Em geral, clioquinol **1** foi o composto mais ativo, seguido das sulfonamidas (PH151 e PH153) e ácidos sulfônicos (composto **2** e **3**);

✓ De modo geral, os derivados de 8-hidroxiquinolina apresentaram efeito fungistático para *C. albicans* e efeito fungicida para dermatófitos (exceto PH151). Porém, o efeito fungicida apresentado foi dependente da concentração dos compostos e do tempo de tratamento;

✓ A modelagem PK/PD mostrou eficácia antifúngica do clioquinol **1**, PH151 e PH153 em baixas concentrações. Clioquinol **1** foi o agente antifúngico que apresentou menor EC_{50}, entretanto PH153 alcançou efeito máximo mais rapidamente;

✓ A modelagem nos permitiu prever que, durante o período de 6 dias, o regime de dosagem de 25 mg a cada 12 horas é suficiente para manter as concentrações plasmáticas acima da concentração antifúngica efetiva. Isso sugere que esta dose poderia ser usada para tratar doenças fúngicas. Uma vez que a possibilidade de reintrodução de formulações orais de clioquinol **1** é iminente, esses achados são importantes para definir dosagens antifúngicas em futuros estudos com mamíferos;

✓ Em geral, os derivados da 8-hidroxiquinolina avaliados neste estudo mostraram efeito sobre a parede celular fúngica, porém, clioquinol **1**, PH151 e PH153 pareceram ter ação mais eficaz nessa estrutura do que derivados da 8-hidroxiquinolina contendo ácido sulfônico. Os ácidos sulfônicos (compostos **2** e **3**) comprometeram, também, a integridade funcional das membranas citoplasmáticas. Adicionalmente, clioquinol **1**, PH151 e PH153 inibiram a formação de pseudo-hifas em *C. albicans*. Isso demonstra que, alterando o padrão de substituição do núcleo da 8-hidroxiquinolina, é possível obter diferentes mecanismos para a atividade antifúngica;

✓ Clioquinol **1** e os ácidos sulfônicos **2** e **3** inibiram a formação de biofilme, reduziram sua atividade metabólica e removeram biofilmes de *Candida* pré-formados em microplaca de poliestireno de uma maneira dependente da concentração.

Entretanto, os ácidos sulfônicos **2** e **3** foram menos eficazes que clioquinol **1**. A inibição das pseudo-hifas pelo clioquinol **1** pode ter um papel importante nos resultados encontrados aqui;

✓ Clioquinol **1** teve eficácia superior ao fluconazol na erradicação de biofilme formado em DIU e, no futuro, pode ser uma opção farmacológica importante para o tratamento de CVV recorrente ligada ao uso desse dispositivo. Além disso, nós padronizamos e descrevemos com sucesso, até onde sabemos, o primeiro protocolo que quantifica a ação inibitória de compostos sobre biofilmes pré-formados na superfície de DIU;

✓ As cinco 8-hidroxiquinolinas estudadas têm afinidade à albumina bovina. Entretanto, diversos antifúngicos apresentam esta propriedade e, mesmo assim, são efetivos *in vivo*;

✓ Os três compostos inibidores de pseudo-hifas (Clioquinol **1**, PH151 e PH153) demonstraram ótimos efeitos antifúngicos quando administrados oralmente por *D. melanogaster* imunodeficientes infectadas com *C. albicans.* Esses dados confirmam o potencial desses compostos para tratar candidíase sistêmica em pacientes imunocomprometidos. Por outro lado, os ácidos sulfônicos **2** e **3** foram menos efetivos. Tais achados podem estar relacionados à atenuação da virulência e patogenicidade em consequência da inibição das pseudo-hifas;

✓ Altas concentrações de derivados da 8-hidroxiquinolina não foram tóxicos para *D. melanogaster* imunodeficientes quando administradas por via oral;

✓ Os derivados da 8-hidroxiquinolina não causaram lesão na pele de orelha de porco e apresentaram baixos índices irritativos no HET-CAM, indicando baixa toxicidade dérmica desses compostos;

✓ Altas concentrações de clioquinol **1**, composto **2**, PH151 e PH153 não foram letais para embriões de galinha, o que pode indicar segurança durante a embriogênese. Por outro lado, o composto **3** foi letal para o embrião, o que pode ser um alerta para possível embriotoxicidade;

✓ A observação do comportamento de embriões de galinha expostos ao clioquinol **1**, composto **2** e PH151 indicou possível efeito estimulante desses compostos. Ao contrário, PH153 e composto **3** não demonstraram tal efeito. Entretanto, deve-se levar em consideração que os animais durante o desenvolvimento embrionário são mais sensíveis e vulneráveis do que durante os demais estágios da vida;

✓ A observação do comportamento de embriões de *zebrafish* mostrou que as maiores concentrações testadas de PH151 e PH153 podem diminuir a sensibilidade das larvas e consequentemente diminuir a atividade motora. Ainda, pôde ser sugerido um possível efeito ansiolítico para PH151 e um efeito estimulante para PH153, contrariando os resultados encontrados em embriões de galinha;

✓ Os embriões de *zebrafish* apresentaram mortalidade dependente da dose de PH151 e PH153. A toxicidade foi detectada nas fases embrio-larval, logo, não se pode inferir sobre toxicidade em adultos. Entretanto, embora esses dados divirjam das outras espécies avaliadas, podem servir de alerta para uma possível embriotoxicidade;

✓ Finalmente, nossos resultados indicam que fatores associados no processo de conversão de diferentes morfologias representam alvos terapêuticos promissores. Os dados confirmam o alto potencial do clioquinol **1**, PH151 e PH153 para tratar candidíase sistêmica e dermatomicoses. A reintrodução de formulações orais de clioquinol **1** pode ser uma estratégia mais rápida para tal finalidade. Ainda, PH151 e PH153 demonstraram ser candidatos a fármacos antimicóticos apropriados para avançar em estudos adicionais usando animais mamíferos.

8 PERSPECTIVAS FUTURAS

Com o propósito de desenvolver um novo medicamento antifúngico, os resultados obtidos aqui guiam para uma série de outros estudos que podem e devem ser desenvolvidos. Tendo como base todo o contexto apresentado, temos como perspectivas futuras continuar investigando os efeitos do clioquonol **1**, PH151 e PH153 sobre as células fúngicas, bem como seus possíveis efeitos tóxicos sobre as células humanas. Paralelamente, nosso grupo pretende seguir, através de planejamento racional, o processo de modificações estruturais da 8-hidroxiquinolina com o intuito de otimizar a suscetibilidade fúngica e a toxicidade do hospedeiro.

Como perspectivas eminentes tem-se a investigação da atividade antifúngica dos derivados da 8-hidroxiquinolina sobre *Candida auris*, visto que esta espécie tem preocupado autoridades devido à alta mortalidade e multirresistência. Ainda, propõe-se avaliar a ação de PH151 e PH153 sobre biofilmes fúngicos formados em biomateriais. Também pretendemos dar continuidade aos estudos de mecanismo de ação antifúngica a fim de identificar ações inibitórias sobre enzimas responsáveis pela síntese de estruturas essenciais da membrana e parede celular fúngica, bem como verificar possível dano ao DNA e RNA. Uma vez que clioquinol, PH151 e PH153 atuam por inibição de pseudo-hifas, nós também propomos identificar mecanismos de regulação da expressão gênica de fatores envolvidos na virulência, tais como pseudo-hifas, proteinases e fosfolipases.

Por fim, estudos em mamíferos são necessários e devem ser conduzidos pelo grupo no futuro. Tais estudos devem caracterizar a farmacocinética dos compostos, avaliar a ação antifúngica *in vivo* e verificar os efeitos tóxicos a fim de escolher regimes de dosagens adequados para administração sistêmica. Ainda, investigar o perfil de permeação também é imprescindível para definir uma dosagem ideal em uma formulação de uso tópico. Todas essas pesquisas propostas complementarão as já realizadas, a fim de certificar o uso seguro e efetivo das 8-hidroxiquinolinas para o tratamento de infecções fúngicas.

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