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PROGRAMA DE PÓS-GRADUAÇÃO EM PRODUTOS
NATURAIS E SINTÉTICOS BIOATIVOS**

ELBA DOS SANTOS FERREIRA

**ATIVIDADE ANTIFÚNGICA CONTRA *Aspergillus* E
CARACTERIZAÇÃO TOXICOLÓGICA DA AMIDA SINTÉTICA 2-
CLORO-N-FENILACETAMIDA**

**JOÃO PESSOA-PB
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Orientador: Prod. Dr. Ricardo Dias de Castro

Co-orientadora: Prof. Dra. Edeltrudes de Oliveira Lima

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É justo que muito custe o que muito vale ”

Santa Teresa D'ávilla

Resumo e abstract

RESUMO

O gênero *Aspergillus* compreende um grupo de fungos saprófitos, causadores de infecções oportunistas que acometem principalmente indivíduos imunocomprometidos. As espécies *A. flavus* e *A. niger* são, respectivamente, a segunda e terceira maiores causas de aspergilose documentada na literatura. Sabendo que a resistência de *Aspergillus* spp às atuais terapias vêm crescendo e reduzindo as possibilidades de tratamento com os antifúngicos disponíveis, e que a amida sintética 2-cloro-*N*-fenilacetamida têm mostrado potencial antifúngico contra esse gênero, o objetivo desse trabalho foi avaliar o efeito da amida sintética 2-cloro-*N*-fenilacetamida sobre *Aspergillus* e determinar seu perfil toxicológico. Para tanto, foi avaliado a concentração inibitória mínima, concentração fungicida mínima, germinação de conídios, associações com antifúngicos, elucidação do provável mecanismo de ação por ensaios que envolvem parede e membrana celular fúngica, bem como predição molécula pelo docking. O perfil farmacocinético e toxicológico foi avaliado a partir de ensaios *in silico* utilizando os softwares Swiss ADMET e OSIRIS. Além disso, foi avaliado *in vitro* a citotoxicidade por meio do teste de hemólise, a capacidade protetiva por meio do teste de fragilidade osmótica e os efeitos genotóxicos *ex vivo* em células da mucosa oral. Foi observado que 2-cloro-*N*-fenilacetamida apresentou atividade antifúngica contra cepas de *A. flavus* com concentrações inibitórias mínimas entre 16 e 256 µg/mL e concentrações fungicidas mínimas que variaram de 32 a 512 µg/mL. O composto avaliado apresentou atividade antifúngica contra cepas de *A. niger* com CIMs e CFMs variando, respectivamente, de 32 e 256 µg/mL, e 64 a 1024 µg/mL, exibindo, portanto, atividade fungicida para todas as cepas utilizadas. Para ambas as espécies, 2-cloro-*N*-fenilacetamida promoveu inibição da germinação dos conídios em todas as concentrações utilizadas, e a associação com a anfotericina B ou ao voriconazol foi antagônica. A ligação ao ergosterol na membrana plasmática fúngica é o provável mecanismo de ação, junto com a possível inibição da síntese de DNA por meio da inibição da timidilato sintase. Na avaliação do perfil farmacocinético *in silico* e toxicológico *in vitro* e *ex vivo*, foi observado que a 2-cloro-*N*-fenilacetamida apresenta parâmetros físico-químicos favoráveis de acordo com a regra de Lipinski, apresentando boa biodisponibilidade por via oral, absorção pelo trato gastrointestinal, capacidade de atravessar a barreira hematoencefálica, inibição da CYP1A2 e efeitos mutagênicos, tumorigênicos e danos relacionados à reprodução. 2-cloro-*N*-fenilacetamida, apresenta baixa capacidade hemolítica nas concentrações de 50, 100 e 500 µg/mL, média toxicidade na concentração de 1000 µg/mL e mostra efeito protetivo das hemácias dos tipos sanguíneos A e O. Além disso, o composto promove poucas alterações genotóxicas, incluindo cariólise e cariorrexe em células da mucosa oral, porém em frequência menor do que o observado para o controle positivo peróxido de hidrogênio. Diante disso, pode-se concluir que 2-cloro-*N*-fenilacetamida apresenta potencial antifúngico promissor com perfil farmacocinético favorável a administração pela via oral, e baixo potencial citotóxico e genotóxico, sendo um candidato promissor para estudos de toxicidade *in vivo*.

Palavras chave: *Aspergillus*, antifúngico, acetamidas, ergosterol, toxicidade

ABSTRACT

The *Aspergillus* genus comprises a group of saprophytic fungi that cause opportunistic infections that mainly affect immunocompromised individuals. The species *A. flavus* and *A. niger* are, respectively, the second and third major causes of aspergillosis documented in the literature. Knowing that *Aspergillus* spp resistance to current therapies has been growing and reducing the possibilities of treatment with available antifungal agents, and that the synthetic amide 2-chloro-*N*-phenylacetamide has shown antifungal potential against this genus, the aim of this study was to evaluate the effect of synthetic amide 2-chloro-*N*-phenylacetamide on *Aspergillus* and to determine its toxicological profile. Therefore, the minimum inhibitory concentration, minimum fungicidal concentration, conidia germination, associations with antifungal agents, elucidation of the probable mechanism of action by assays involving fungal cell wall and membrane, as well as molecular prediction by docking, were evaluated. The pharmacokinetic and toxicological profile was evaluated from *in silico* assays using Swiss ADMET and OSIRIS software. In addition, cytotoxicity was evaluated *in vitro* through the hemolysis test, the protective capacity through the osmotic fragility test and the *ex vivo* genotoxic effects on cells of the oral mucosa. It was observed that 2-chloro-*N*-phenylacetamide showed antifungal activity against *A. flavus* strains with minimal inhibitory concentrations between 16 and 256 µg/mL and minimal fungicidal concentrations ranging from 32 to 512 µg/mL. The evaluated compound showed antifungal activity against *A. niger* strains with MICs and CFMs ranging, respectively, from 32 and 256 µg/mL, and 64 to 1024 µg/mL, thus exhibiting fungicidal activity for all strains used. For both species, 2-chloro-*N*-phenylacetamide promoted inhibition of conidia germination at all concentrations used, and the association with amphotericin B or voriconazole was antagonistic. Ergosterol binding in the fungal plasma membrane is the likely mechanism of action, together with the possible inhibition of DNA synthesis through inhibition of thymidylate synthase. In the evaluation of the *in silico* pharmacokinetic and *in vitro* and *ex vivo* toxicological profile, it was observed that 2-chloro-*N*-phenylacetamide presents favorable physicochemical parameters according to Lipinski's rule, presenting good oral bioavailability, absorption through the tract gastrointestinal, ability to cross the blood-brain barrier, inhibition of CYP1A2 and mutagenic, tumorigenic, and reproductive-related damage effects. 2-chloro-*N*-phenylacetamide, has low hemolytic capacity at concentrations of 50, 100 and 500 µg/mL, medium toxicity at a concentration of 1000 µg/mL and shows a protective effect of red blood cells of blood types A and O. The compound promotes few genotoxic changes, including karyolysis and karyorrhexis in cells of the oral mucosa, but at a lower frequency than that observed for the positive control hydrogen peroxide. Therefore, it can be concluded that 2-chloro-*N*-phenylacetamide has promising antifungal potential with a favorable pharmacokinetic profile for oral administration, and low cytotoxic and genotoxic potential, being a promising candidate for *in vivo* toxicity studies.

Keywords: *Aspergillus*, antifungal, acetamides, ergosterol, toxicity.

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A₁Cl – 2-cloro-*N*-fenilacetamida

CIM – Concentração Inibitória Mínima

DL50 – dose letal capaz de matar 50% de uma população em teste

ABPA- Aspergilose Bronco-Pulmonar Alérgica

API – Aspergilose Pulmonar Invasiva

COVID-19 – Doença do Coronavírus descoberta em 2019

SARS-CoV-2 - Coronavirus 2 da síndrome respiratória aguda grave

NK – Natural Killer

PCR – Reação em cadeia da polimerase

DNA – ácido desoxirribonucleico

CFM – Concentração Fungicida Mínima

ASD – Ágar Sabouraud Dextrose

RPMI - Roswell Park Memorial Institute

ATCC - American Type Culture Collection

DMSO – Dimetilsulfóxido

ABD – Ágar Batata Dextrose

UFC – Unidades formadoras de colônias

ICIF - Índice da Concentração Inibitória Fracionada.

CIF^A – Concentração Inibitória Fracionada de A

CIF^B – Concentração Inibitória Fracionada de B

PDB – Protein Data Banking

ADME – Absorção, distribuição, metabolismo e excreção

TPSA – área de superfície polar topológica

AMB – anfotericina B

VOR - voriconazol

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Introdução

1.0 Introdução

O gênero *Aspergillus* compreende fungos filamentosos, de fácil adaptação aos ambientes devido a não seletividade em relação às condições de crescimento, utilizando-se de uma vasta faixa de moléculas orgânicas como fonte de energia, além de crescerem em uma ampla faixa de temperatura. Além disso, possuem elevado número de conídios que quando dispersos pelo ar, percorrem longas distâncias (KRIJGSHELD et al., 2013).

Os fungos deste gênero são considerados patógenos oportunistas responsáveis por altas taxas de mortalidade (BROWN et al., 2012). *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger* e *Aspergillus terreus*, são as principais espécies responsáveis pelas infecções em humanos (MARTINS et al, 2005; MURRAY et al, 2006; JIANG et al, 2013). A contaminação por estes micro-organismos ocorre pela inalação de conídios, sendo a via aérea a principal rota de infecções. Entretanto, esses fungos podem colonizar ferimentos, como também penetrar nos tecidos por ocasião de incisões cirúrgicas (SIDRIM; MOREIRA, 1999; CHAMILOS; KONTOYIANNIS, 2005; PASQUIER et al., 2006).

As principais formas clínicas da aspergilose observadas são aspergilose cutânea (infecção na pele), otomicose aspergilar (infecção do conduto auditivo externo), onicomicose aspergilar (infecções nas unhas), aspergiloma ou bola fúngica (infecção nos pulmões), sinusite aspergilar (infecção dos seios paranasais), acometimento neurológico, e micotoxicoses, que representam intoxicações crônicas por produtos do metabolismo do fungo (SIDRIM; ROCHA, 2004).

O diagnóstico laboratorial das infecções fúngicas, de forma geral, é diretamente dependente da coleta da amostra. O exame microscópico direto de secções teciduais e amostras clínicas e o isolamento em meios de cultura específicos são geralmente considerados como o meio mais rápido para o diagnóstico destas infecções (MURRAY; ROSSENTHAL; PFALLER, 2006). Técnicas de reação em cadeia da polimerase (PCR), sequenciamento de DNA fúngico, detecção de antígenos fúngicos, bem como exames de imagem podem auxiliar no diagnóstico (DONELLI et al., 2020).

No tratamento da aspergilose, o voriconazol é o fármaco de primeira escolha (PASCUAL et al., 2008; TOMBLYN et al., 2009; LOCKHART et al., 2011), promovendo a inibição da enzima 14- α -esterol-desmetilase, promovendo a

diminuição da concentração do ergosterol na membrana fúngica, ocasionando a desestabilização da mesma (SANGLARD et al., 2009). Outras alternativas terapêuticas podem ser utilizadas em casos de pacientes que não podem fazer uso deste fármaco, seja por que as cepas estão resistentes, ou por que já não respondem a esta terapia. Nessas situações, podem ser utilizados a Anfotericina B, um fármaco da classe dos polienos que atua através da ligação ao ergosterol promovendo a formação de um poro na membrana fúngica, levando a um aumento do estresse osmótico e a perda de íons e moléculas importantes, o que ocasiona a morte do fungo. O uso desse fármaco é limitado devido a sua toxicidade (LANIADO-LABORIN; CABRALES-VARGAS, 2009).

As equinocandinas (caspofungina e micafungina) podem ser usadas como terapia de terceira escolha para o tratamento da aspergilose, atuando na parede celular fúngica através da inibição da enzima β -(1,3)-D-glucano sintase, levando à ruptura da parede celular em crescimento, o que resulta em instabilidade osmótica e morte celular (CHEN; SLAVIN; SORRELL, 2011).

Apesar da terapia antifúngica contra a aspergilose ser bem estabelecida, há relatos frequentes do aparecimento da resistência aos fármacos, onde os fungos desenvolvem mecanismos como mutações em enzimas importantes como a 14 α -esterol-desmetilase e a β -(1,3)-D-glucano sintase (KRISHNAN-NATESAN et al., 2012; HORI, SHIBUYA, 2018), superexpressão de bombas de efluxo (SANGLARD, 2016), produção de biofilme (DESAI; MITCHELL; ANDES, 2014), aumento dos níveis de ergosterol da membrana (TAYLOR et al., 2017), entre outros.

Considerando a rápida disseminação das formas de aspergilose em pacientes imunocomprometidos e a resistência das espécies de *Aspergillus* às atuais terapias, é urgente a busca por novas alternativas terapêuticas para o tratamento da aspergilose nas suas mais variadas formas. Nesse contexto, as amidas sintéticas têm sido alvo de estudos devido às suas atividades biológicas promissoras, como atividade anti-inflamatória, analgésica, antitumoral, dentre outras (KENNEDY JR, 2001).

A 2-cloro-*N*-fenilacetamida, que será referida no decorrer do trabalho como A₁Cl, é uma amida sintética ainda pouco estudada, entretanto já possui algumas atividades antimicrobianas descritas, com efeito bactericida sobre *Escherichia coli* e *Pseudomonas aeruginosa* (KATKE et al., 2011). Também apresenta ação

antifúngica, como verificado por Aschale (2012), utilizando o método de difusão de disco, onde foi possível observar atividade antifúngica contra cepas de *A. niger*.

Até o presente momento, a literatura ainda se mostra escassa no que diz respeito à atividade antifúngica da A₁Cl contra cepas do gênero *Aspergillus*. Um estudo de triagem realizado no laboratório Laboratório de Pesquisas de Atividade Antibacteriana e Antifúngica de Produtos Naturais e/ou Sintéticos Bioativos, verificou que A₁Cl apresentava atividade antifúngica em cepas de *A. flavus* e *A. niger*, com concentração inibitória mínima (CIM) de 128 µg/mL nas cepas utilizadas.

Tendo em vista a disseminação da aspergilose, principalmente em pacientes imunocomprometidos, e a dificuldade de tratamento devido à resistência as atuais terapias antifúngicas, este trabalho tem como objetivo avaliar a atividade antifúngica da amida sintética 2-cloro-*N*-fenilcetamida contra *A. flavus* e *A. niger*, bem como investigar seu perfil toxicológico, com o intuito de contribuir com a descoberta de novos compostos promissores para o tratamento da aspergilose nas suas mais variadas formas.

Referencial teórico

2.0 Referencial teórico

2.1 Gênero *Aspergillus*

O gênero *Aspergillus* pertence a família *Aspergilaceae*, ordem *Eurotiales*, classe *Eurotiomycetes* e filo *Ascomycota*, compreendendo aproximadamente 340 espécies de fungos filamentosos que desempenham importantes funções biológicas no ambiente, sendo 40 espécies consideradas patogênicas (SAMSON et al. 2014; THAKUR et al. 2015; SHISHODIA; TIWARI; SHANKAR, 2019). São fungos saprófitas, presentes no solo, no ar, na matéria em decomposição, na poeira, na água e em ambientes internos, como superfícies de edifícios e eletrodomésticos. Ganham essa nomenclatura em 1729, do padre biólogo Pier Antônio Michele, que, analisando as características microscópicas dos conidióforos dos fungos desse gênero (Figura 1), assemelhou-as aos aspersórios (*aspergere*, do latim), que são objetos utilizados por padres no catolicismo para aspersão de água benta (BENNETT, 2010).

As espécies do gênero *Aspergillus* possuem uma estrutura chamada conidióforo, que contém esporos assexuados, também chamados de conídios. O conidióforo é caracterizado por possuir em sua base uma estrutura em forma de “T” ou “L”, conhecida como “célula pé” de onde se origina uma haste que culmina na vesícula (Figura 1) (BENNETT, 2010). Uma ou duas camadas de células se originam a partir da vesícula, sendo chamadas de fiálides, e a partir das fiálides se originam milhares de esporos por conidióforo (KRIJGSHELD et al., 2013).

As espécies *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans*, *A. terreus* e *A. oryzae* são as mais estudadas desse gênero, devido à sua importância médica, agrícola e industrial (KRIJGSHELD et al., 2013), sendo *A. fumigatus* responsável pela maioria dos casos de infecção por *Aspergillus*, seguida de *A. flavus* e *A. niger* (BALAJEE et al., 2009).

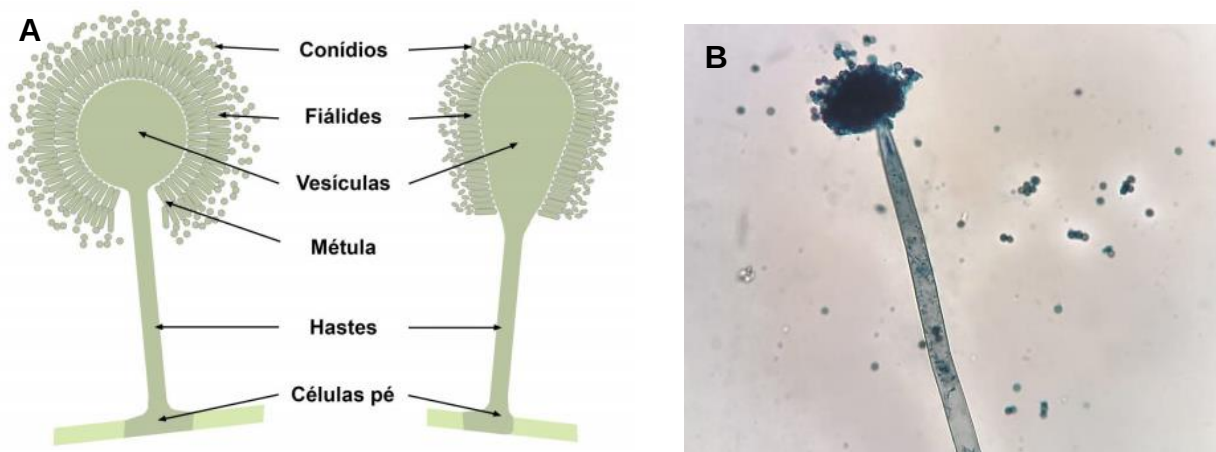


Figura 1. Representação do conidióforo, estrutura característica do gênero *Aspergillus* (A) e micrografia do conidióforo da espécie *Aspergillus flavus*, corado com azul de lactofenol, com aumento na objetiva de 100x (B).

Fonte: BRAUER, 2020 (A) e FERREIRA, 2021 (B).

Dentro das espécies do gênero *Aspergillus*, *A. flavus* é uma das mais conhecidas, sendo descrito como uma espécie em 1809, e relatado pela primeira vez como um patógeno de planta em 1920. Recentemente, *A. flavus* emergiu como um importante patógeno oportunista, sendo uma das espécies mais isoladas no âmbito da agricultura e da medicina e reconhecido como a segunda causa principal de aspergilose em humanos (BENNET, 2010). Em alguns países, essa espécie tem sido a mais comumente isolada em ambientes hospitalares e domésticos (ZEYNI; HEDAYATI 1995; HEDAYATI et al., 2005).

As colônias de *A. flavus* em ágar sabouraud dextrose cultivados por 7 dias, caracterizam-se pela textura cotonosa, plana, com sulcos radicais e coloração amarelo esverdeada (Figura 2), e em relação à micromorfologia, a espécie apresenta o conidióforo com haste longa, grossa, vesícula globosa coberta com uma camada de fiálides seguida de conídios em cadeia que formam cabeças conidiais radiadas, com 300 a 400 μm de diâmetro, as fiálides são unisseriadas ou bisseriadas, sendo os conídios globosos (Figura 2), e as hifas são hialinas, septadas e ramificadas. Apresenta rápido crescimento na faixa de temperatura de 25 a 37°C (CARVALHO, 2013).

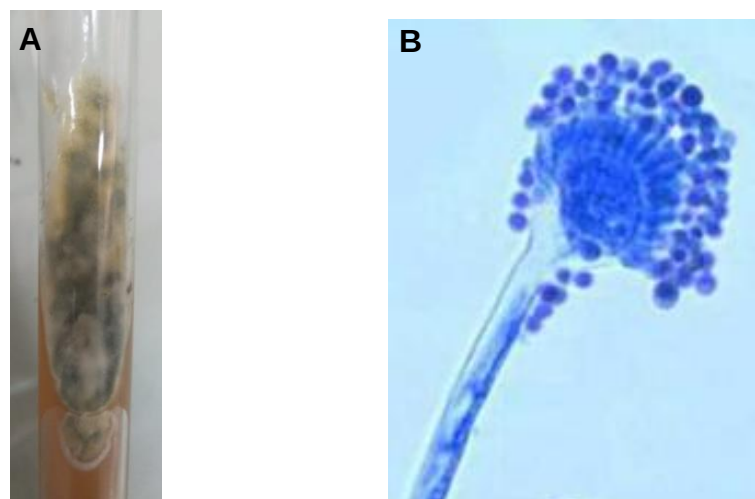


Figura 2. Cultura de *A. flavus* no meio ágar Sabouraud dextrose cultivado em tubo de ensaio inclinado (A) e aspecto micromorfológico de *A. flavus* em aumento de 400x (B).

Fonte: FERREIRA, 2021 (A) e CARVALHO, 2013 (B).

A. flavus é encontrado em regiões de clima quente e árido, como a Ásia, África, Oriente médio, e em países tropicais como o Brasil, Índia, México e Paquistão, devido à sua capacidade de sobreviver melhor nessas condições climáticas em relação a outras espécies do gênero (MIDORIKAWA et al., 2014; RUDRAMURTHY et al., 2019). Essa espécie é considerada mais virulenta do que as outras espécies do gênero em termos de tempo e inóculo inicial, e alguns estudos evidenciaram que a dose letal necessária para matar 50% (DL₅₀) dos camungongos para *A. flavus* é 100 vezes menor do que a DL₅₀ de *A. fumigatus* (MOSQUERA et al., 2001; KAMAI et al., 2002; HEDAYATI et al., 2007).

Além disso, *A. flavus* é considerado uma praga agrícola, causando prejuízos consideráveis na agricultura, infectando safras de grãos como milho, amendoim, nozes, sementes de algodão, e ainda é uma espécie produtora de aflatoxinas, que causam intoxicação alimentar (AMAIKE; KELLER 2011)

Outra espécie do gênero *Aspergillus* de importância clínica significativa é *A. niger*, que foi descrita pela primeira vez por Van Tieghen em 1867. Suas colônias se desenvolvem em menos de 7 dias e inicialmente se apresenta com aspecto de camurça branca ou amarelada e rapidamente ocorre o desenvolvimento de conídios negros (Figura 3) (MARTINS et al., 2005). Possui conidióforos longos, de paredes lisas e hialinas que apresentam coloração escura próximo à vesícula. Os conídios

são globosos, negros e as fiáldes são bisseriadas (Figura 3) (MARTINS et al., 2005; BLEICHRODT et al., 2012).

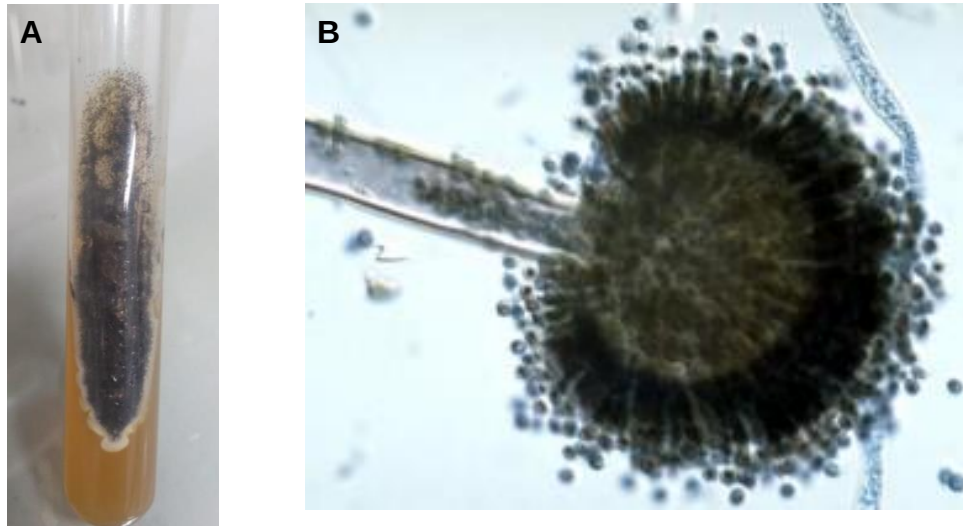


Figura 3. Cultura de *A. niger* no meio ágar Sabouraud dextrose cultivado em tubo de ensaio inclinado e aspecto micromorfológico de *A. niger* em aumento de 400x (B).

Fonte: FERREIRA, 2021 (A) e CARVALHO, 2013 (B).

A. niger é comumente encontrado no solo, lixo e na matéria em decomposição. Cresce em uma ampla faixa de temperatura, de 6–47°C com uma temperatura ótima relativamente alta de 35–37°C. Produz uma grande quantidade de conídios que se disseminam facilmente pelo ar garantindo a ocorrência ubíqua da espécie e facilitando a contaminação e colonização no ser humano (SCHUSTER et al., 2002). Clinicamente, *A. niger* é a terceira espécie do gênero *Aspergillus* mais comumente associada a casos de infecções, como otites externas (infecções no conduto auditivo externo) e aspergilose pulmonar invasiva (ARAIZA et al., 2006; PAWAR; THAKER, 2006; PERSON et al., 2010).

Além disso, essa espécie tem sido muito utilizada para a produção do ácido cítrico utilizado na indústria alimentícia como acidulante primário em alimentos como refrigerantes, sucos de frutas, geleias, entre outros produtos; na indústria farmacêutica, o ácido cítrico produzido por *A. niger* tem sido utilizado como conservante de sangue armazenado e na indústria cosmética e higiene pessoal, o ácido cítrico produzido por essa espécie é utilizado como tampão, para ajuste de pH e como antioxidante (SCHUSTER et al., 2002).

2.2 Aspergilose: formas clínicas, diagnóstico, tratamento e resistência fúngica

Aspergilose é uma micose causada por fungos do gênero *Aspergillus*, sendo considerada uma doença oportunista, afetando principalmente indivíduos imunocomprometidos, incluindo aqueles que fazem tratamento contra os diversos tipos de câncer, pacientes com doenças imunológicas congênitas, pacientes tratados para doenças autoimunes, pacientes que fazem uso crônico de corticosteroides, além de alcoolatras, diabéticos, entre outros (WALSH et al., 2008; JIANG et al., 2013; SUGUI et al., 2015; KOSMIDIS; DENNING, 2015).

As características clínicas das formas da doença são semelhantes para todas as espécies patogênicas. A principal via de contaminação é através da inalação dos conídios, devido à hidrofobicidade e a facilidade na dispersão dos mesmos, facilitam a disseminação (HEDAYATI et al., 2007). Em pacientes imunocomprometidos, o sistema imune torna-se ineficaz na eliminação dos conídios, que se instalam no órgão alvo e germinam formando hifas, que se entrelaçam para formar o micélio, como mostrado na figura 4. No início da doença, há uma resposta neutrofílica aguda em que os neutrófilos circundam as hifas, porém em indivíduos imunocomprometidos essa resposta não é eficaz e a doença se desenvolve. O crescimento da colônia fúngica pode atingir outros órgãos atuando de forma invasiva (SUGUI et al., 2015).

As apresentações clínicas da aspergilose mais frequentemente encontradas envolvem os pulmões, como aspergilose pulmonar invasiva e aspergilose broncopulmonar alérgica (ABPA). Outras infecções comuns causadas por *Aspergillus* spp. incluem sinusite invasiva ou crônica aguda, aspergilose cutânea, otomicose aspergilar, aspergiloma, aspergilose cerebral, entre outras (SUGUI et al., 2015; RUDRAMURTHY et al., 2019).

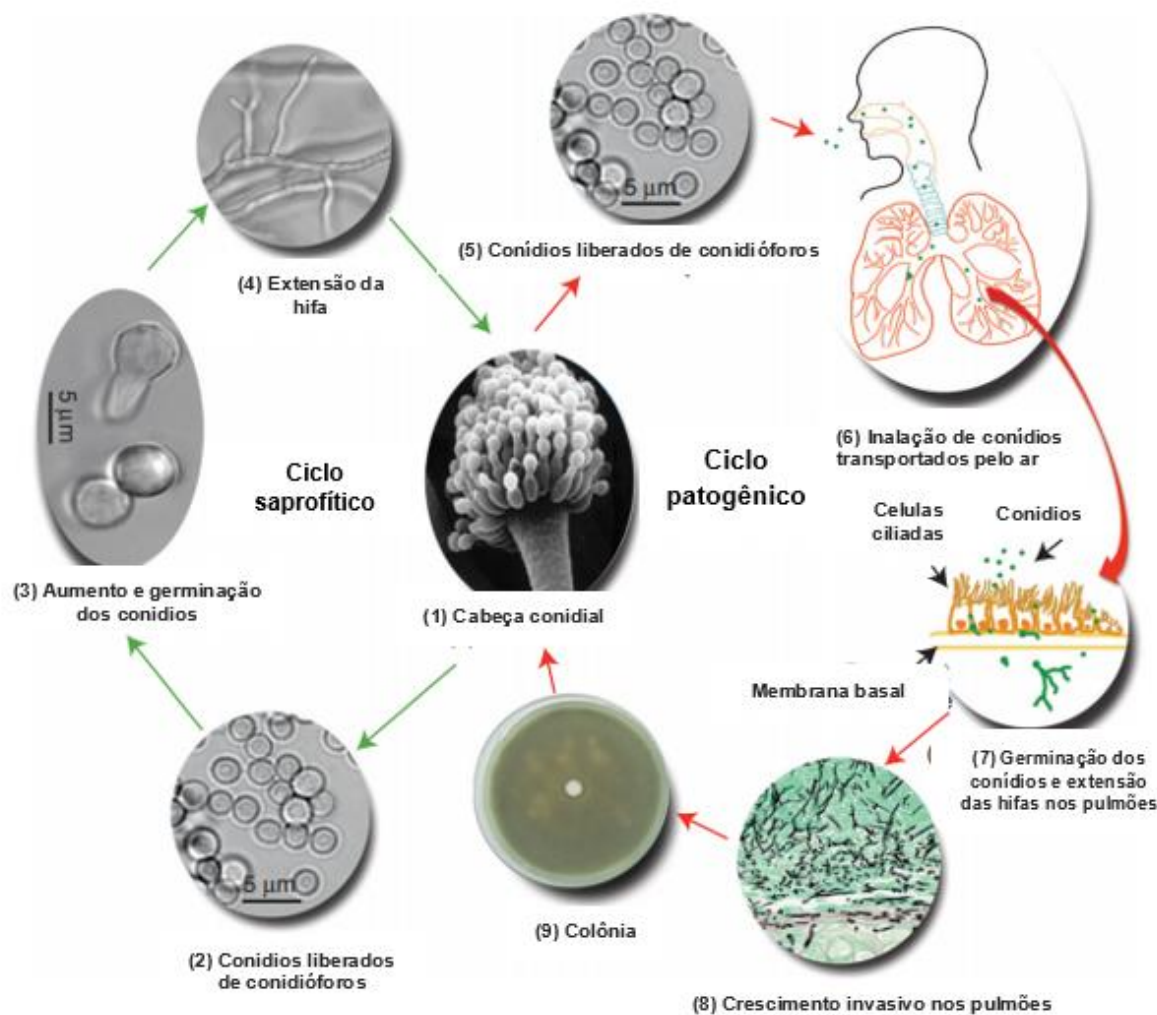


Figura 4. Ciclo saprofítico e patogênico dos fungos de *Aspergillus* spp.

Fonte: Adaptado de SUGUI et al., 2015.

Clinicamente, as formas pulmonares e das vias aéreas de aspergilose compreendem a aspergilose broncopulmonar alérgica, o aspergiloma, a aspergilose pulmonar crônica, que é composta pela aspergilose cavitária crônica, aspergilose fibrosante crônica e aspergilose necrosante crônica (semi-invasiva ou invasiva subaguda), a aspergilose pulmonar invasiva e a traqueo-bronquite (colonizante ou obstrutiva, invasiva, ulcerativa e pseudomembranosa) e a rinossinusite alérgica e invasiva (KOUSHA; TADI; SOUBANI, 2011; KOSMIDIS; DENNING, 2015).

A aspergilose pulmonar invasiva (API) é a forma mais grave de aspergilose, apresentando-se como uma infecção progressiva, aguda, severa, de mau prognóstico, podendo ser fatal (DAGENAIS; KELLER, 2009). Além disso, sabe-se que pode ocorrer disseminação fúngica dos pulmões para o trato gastrointestinal,

rins, cérebro, fígado, entre outros órgãos, originando abscessos e lesões necróticas (PERSON et al, 2010).

Dados epidemiológicos mostram que a frequência de infectados com API é em torno de 200 mil casos por ano e um total de 3 milhões de pessoas acometidas em todo o mundo, causando mais de 15% de mortes nos primeiros seis meses de doença, o que representa, aproximadamente, cerca de 450 mil óbitos somente na fase inicial (KOSMIDIS; DENNING, 2015). Além disso, quando há equívocos ou demoras no diagnóstico, a taxa de mortalidade da API pode atingir 100%, entretanto, quando o diagnóstico e o tratamento ocorrem em tempo hábil, a taxa de mortalidade pode ser inferior a 50% (GAO; SOUBANI, 2019).

Há relatos na literatura sobre a co-infecção por *Aspergillus* em pacientes com COVID-19, onde a infecção pelo SARS-COV-2 pode causar danos linfocitários, em especial nas células B, células T e células NK, podendo comprometer o sistema imunológico durante o período da doença, e a diminuição dos linfócitos e da função imunológica do hospedeiro pode favorecer as co-infecções por *Aspergillus* spp. nos pulmões, contribuindo para o aumento da mortalidade (MOJICA-CRESPO; MORALES-CRESPO, 2020).

Os sintomas mais comuns em pacientes que contraem as formas pulmonares de aspergilose incluem febre, infiltrados pulmonares acompanhados de dor torácica e hemoptise. Nas formas alérgicas observa-se também a presença de hipereosinofilia tanto no sangue como na expectoração, muco espesso amarelo-esverdeado e sinusite (CARVALHO, 2013).

As formas extrapulmonares de aspergilose incluem osteomielite aspergilar, aspergilose cutânea, otomicose aspergilar, endocardite aspergilar, aspergilose cerebral, sendo em muitas formas, resultado da infecção disseminada (CARVALHO, 2013; RUDRAMURTHY et al., 2019).

O diagnóstico da aspergilose é semelhante, independente da espécie causadora. O diagnóstico clínico é realizado com base na sintomatologia apresentada pelo paciente e o diagnóstico laboratorial envolve o exame microscópico direto, a histopatologia, a citopatologia de espécimes obtidos por punção ou biópsia para visualização de hifas septadas, hialinas e ramificadas em ângulo agudo associadas a evidência de destruição tecidual, como também a cultura do fungo. Além disso, pode-se utilizar técnicas de biologia molecular como PCR e sequenciamento do DNA fúngico, bem como a detecção de antígenos fúngicos. Para

isso podem ser utilizadas amostras como escarro, lavado brônquico alveolar, aspirado, escovamento brônquico e secreções (DONELLI et al., 2020). As técnicas de imagiologia (tomografia computadorizada de alta resolução) também são de grande valia para o diagnóstico das formas de aspergilose, em especial as formas pulmonares (CARVALHO et al., 2013).

O sucesso no tratamento da aspergilose envolve alguns fatores, não apenas a escolha da terapia antifúngica específica. Em pacientes imunocomprometidos, o conhecimento detalhado dos fatores do hospedeiro, doença subjacente, infecções concomitantes e grau e duração da imunossupressão são fundamentais para o tratamento geral. Sabe-se que a restauração imunológica é fundamental para o sucesso da terapia, enquanto a exposição contínua a agentes medicamentosos imunossupressores, como corticosteróides, pioram os quadros de aspergilose invasiva (SUGUI et al., 2015).

Atualmente, três classes de agentes antifúngicos são utilizadas para o tratamento da aspergilose: os derivados azólicos, os polienos e as equinocandinas (SHISHODIA; TIWARI; SHANKAR, 2019).

Os derivados azólicos compreendem duas grandes classes, os imidazólicos e os triazólicos, que possuem o mesmo espectro antifúngico e o mesmo mecanismo de ação. Os imidazólicos possuem dois átomos de nitrogênio no anel azólico, enquanto os triazólicos possuem três, como pode-se observar na figura 5. Os triazólicos são mais utilizados atualmente por que possuem um metabolismo lento e promovem menos efeitos adversos sobre a síntese de esteróis humanos, pois são mais específicos para a enzima fúngica (JACOBS et al., 2011).

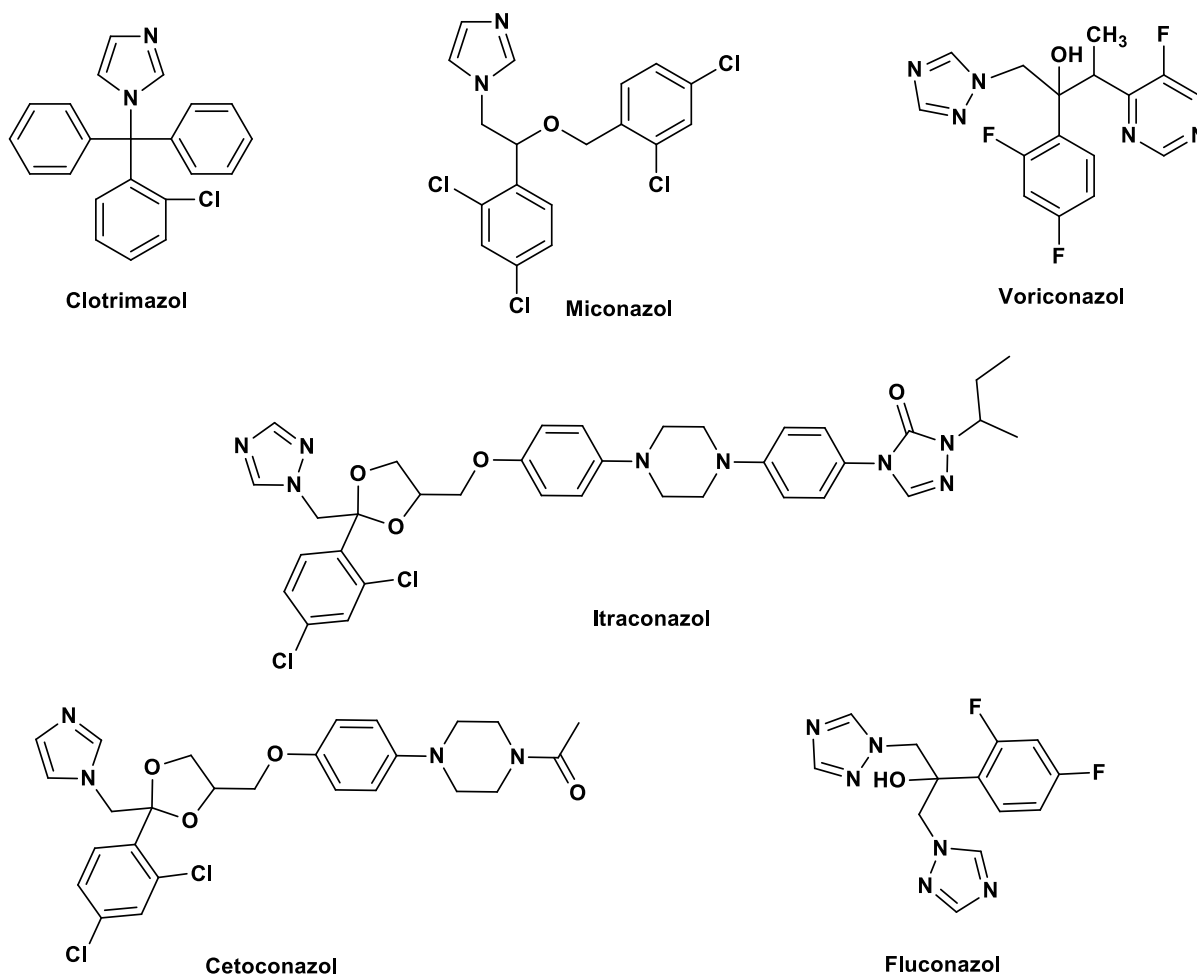


Figura 5. Estrutura química dos principais derivados imidazólicos e triazólicos.

Fonte: Adaptado de KATZUNG; TREVOR, 2017.

Os derivados azólicos utilizados para o tratamento da aspergilose incluem os triazólicos voriconazol, itraconazol, posaconazol e mais recentemente foi incluído o isavuconazol (MICELI; KAUFFMAN 2015; SUGUI et al., 2015). O mecanismo de ação dessa classe envolve a inibição da enzima 14 α -esterol-desmetilase (CYP51A), enzima pertencente ao citocromo P450 fúngico responsável pela conversão do lanosterol em ergosterol, diminuindo assim a biossíntese desse importante lipídio que compõe a membrana celular fúngica. A consequente diminuição da concentração do ergosterol e o acúmulo de 14 α -metil esteróis rompem as cadeias acil estreitamente agrupadas dos fosfolipídios nas membranas fúngicas ocasionando uma desestabilização da membrana fúngica, o que leva à disfunção das enzimas associadas à membrana, incluindo as da cadeia de transporte de elétrons, podendo

ocasionar, em última análise, morte celular (SANGLARD; ODDS, 2002; GOLAN et al., 2012).

O voriconazol é o fármaco de primeira escolha dessa classe para o tratamento da aspergilose, principalmente das formas invasivas, sendo um fármaco fúngicida para todas as espécies de *Aspergillus* e disponível para ser administrado pelas vias oral e intravenosa (JACOBS et al., 2011; GOLAN et al., 2012). Possui metabolismo hepático, inibe a CYP3A4 e por isso há a necessidade de redução da dose de inúmeros medicamentos, como a ciclosporina, fenitoína, varfarina e triazolan quando o voriconazol é iniciado. A formulação intravenosa de voriconazol não deve ser utilizada em pacientes com insuficiência renal, devido ao acúmulo do excipiente ciclodextrina, que causa toxicidade ao sistema nervoso central. Os principais efeitos adversos incluem hepatotoxicidade, elevação das enzimas hepáticas, fotofobia e exantema (GOLAN et al., 2012).

Apesar de ser utilizado como terapia de primeira escolha para o tratamento da aspergilose invasiva e outras formas de aspergilose, a resistência ao voriconazol por *Aspergillus spp.* têm sido cada vez mais relatada principalmente nas espécies *A. fumigatus*, *A. flavus* e *A. niger* (DENNING; BOWYER, 2013; HASHIMOTO et al., 2017; RUDRAMURTHY et al., 2019). Essa resistência se dá principalmente por meio de mutações genéticas na enzima 14 α -esterol-desmetilase, mais precisamente em aminoácidos presentes nessa enzima, conferindo mudanças estruturais e funcionais que interferem na afinidade de ligação à enzima (KRISHNAN-NATESAN et al., 2012). Essa mutação é conhecida em espécies de *A. fumigatus* resistentes e têm sido frequentemente observada em espécies de *A. flavus* e *A. niger* resistentes ao voriconazol (HASHIMOTO et al., 2017; RUDRAMURTHY et al., 2019).

Outros mecanismos de resistência envolvem a superexpressão de bombas de efluxo que bombeiam o fármaco para fora da célula fúngica, impedindo sua ação farmacológica (HASHIMOTO et al., 2017; PAUL et al., 2018), superexpressão do alvo, fazendo com que a concentração efetiva do medicamento também precise ser aumentada para saturar todas as moléculas alvo, o que resulta em resistência ao medicamento (SANGLARD, 2016), formação de biofilme com a capacidade de sequestrar drogas como azólicos e polienos em compartimentos extracelulares, impedindo a ação farmacológica desses fármacos (DESAI; MITCHELL; ANDES, 2014).

A anfotericina B, fármaco pertencente a classe dos polienos, é o antifúngico de maior espectro de ação, sendo fungicida e eficaz no tratamento de doenças causadas por fungos leveduriformes, filamentosos e protozoários como *Leshmania* spp, sendo a terapia de segunda escolha para o tratamento das infecções por *Aspergillus* spp, quando o voriconazol não pode ser administrado (PATTERSON et al., 2016; ULLMANN et al., 2017). Possui uma estrutura de 37 átomos de carbono formando um anel macrocíclico fechado por lactonização. Possui carácter anfipático, apresentando uma cadeia carbonada de duplas ligações conjugadas não substituídas e uma cadeia poli-hidroxilada com vários grupos hidroxilo livres, como mostrado na figura 6. Está disponível em preparações para a administração intravenosa (FILIPPIN; SOUZA, 2006; MAGINA, 2014).

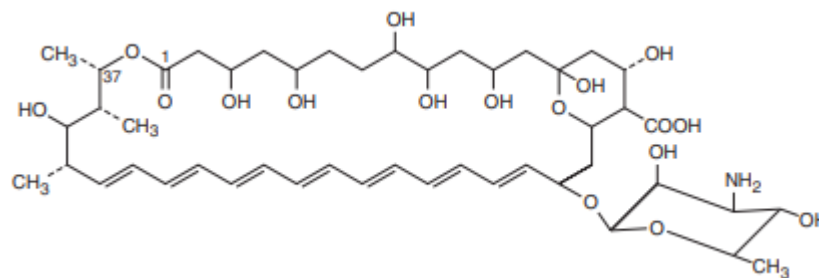


Figura 6. Estrutura química da anfotericina B.

Fonte: KATZUNG; TREVOR, 2017.

O mecanismo de ação da anfotericina B envolve a interação com esteróis da membrana celular fúngica. A anfotericina B liga-se ao ergosterol e promove alterações na permeabilidade da membrana, devido à formação de poros, que têm sua formação facilitada devido a característica anfipática da anfotericina B. As porções lipofílicas interagem ao redor do lado externo do poro e as regiões hidrofílicas revestem o lado interno, e por meio do poro formado, ocorre o extravasamento de íons, principalmente o potássio, e macromoléculas, causando a morte do fungo (STONE et al., 2016; KATZUNG; TREVOR, 2017; WIEDERHOLD et al., 2018). Além disso, a anfotericina B promove um aumento e acúmulo na célula fúngica por meio da produção de espécies reativas de oxigênio (VALIANTE et al., 2019).

Devido à ligeira afinidade para o colesterol da membrana celular do hospedeiro, a anfotericina B exibe uma toxicidade associada ao seu uso, sendo

responsável por vários efeitos adversos significativos como febre, calafrios, tremores, náusea, vômitos e dor de cabeça ocorrem freqüentemente e estão principalmente relacionadas à infusão, alterações cardiovasculares como hipotensão, arritmias cardíacas, dificuldade respiratória, hipocalemia, hipernatremia, lesão tubular renal, insuficiência renal e anemia (MORA-DUARTE et al., 2002). É praticamente insolúvel na água sendo mal absorvida no trato gastrointestinal, o que justifica a necessidade de a administrar por via intravenosa (CAMPOY E ADRIO, 2017). De todos os efeitos adversos da anfotericina B, o mais grave é a toxicidade renal, que pode acarretar em insuficiência renal, sendo constatada através de elevações séricas na concentração de creatinina, até três vezes o limite superior da normalidade (FILLIPIN; SOUZA, 2006).

Numa tentativa de diminuir os efeitos tóxicos da anfotericina B e melhor viabilizar seu uso, foram desenvolvidas formulações lipídicas que, são acumuladas preferencialmente no fígado e nos pulmões, diferentemente das formulações convencionas que são metabolizadas pelo sistema reticulo endotelial, o que pode explicar a diminuição da incidência da nefrotoxicidade e cardiotoxicidade, e pelo contrário em algumas ocasiões, um aumento da hepatotoxicidade após repetidas administrações. Entretanto, efeitos indesejáveis como febre, calafrio, rigidez, sonolência, discreta elevação dos testes de função hepática, disfunção renal e toxicidade cardiopulmonar foram documentadas em pacientes que receberam as formulações lipídicas, o que demonstra que, a toxicidade foi reduzida, mas não anulada (FILIPPIN; SOUZA, 2006; YANG et al., 2018).

A resistência à anfotericina B têm se tornado cada vez mais comum em espécies do gênero *Aspergillus*. Sabe-se que, espécies como *A. terreus* são intrinsecamente resistentes à anfotericina B (POSCH et al., 2018), e *A. flavus* têm demonstrado uma crescente resistência a esse fármaco, sendo considerado por alguns autores, uma espécie que também possui resistência intrínseca (RUDRAMURTHY et al., 2019). Devido a resistência crescente de *A. flavus* à anfotericina B, órgãos internacionais como a Sociedade Europeia de Microbiologia Clínica e Doenças Infecciosas, recomenda evitar a anfotericina B para aspergilose causada por *A. flavus* (ULLMANN et al., 2018). Os mecanismos de resistência descritos envolvem diminuição dos níveis de ergosterol na membrana, bem como aumento da atividade enzimática da peroxidase e superóxido dismutase, reduzindo a peroxidação lipídica provocada pelo fármaco (TAYLOR et al., 2017).

As equinocandinas são a classe de antifúngicos mais recente, tendo o primeiro fármaco, a caspofungina, aprovado em 2001 para uso clínico. Compreendem um grupo importante de fármacos fungistáticos para *Aspergillus*, sendo utilizadas quando o paciente não responde à terapia com azólicos e polienos (PATTERSON et al., 2016), sendo a caspofungina é o fármaco de escolha dessa classe para o tratamento da aspergilose em suas formas invasivas (CHEN; SLAVIN; SORRELL, 2011). São macromoléculas lipopeptídicas, constituídas por hexapeptídeos cíclicos com uma cadeia lateral lipídica, como mostrado na figura 7 (CHANDRASEKAR, 2010).

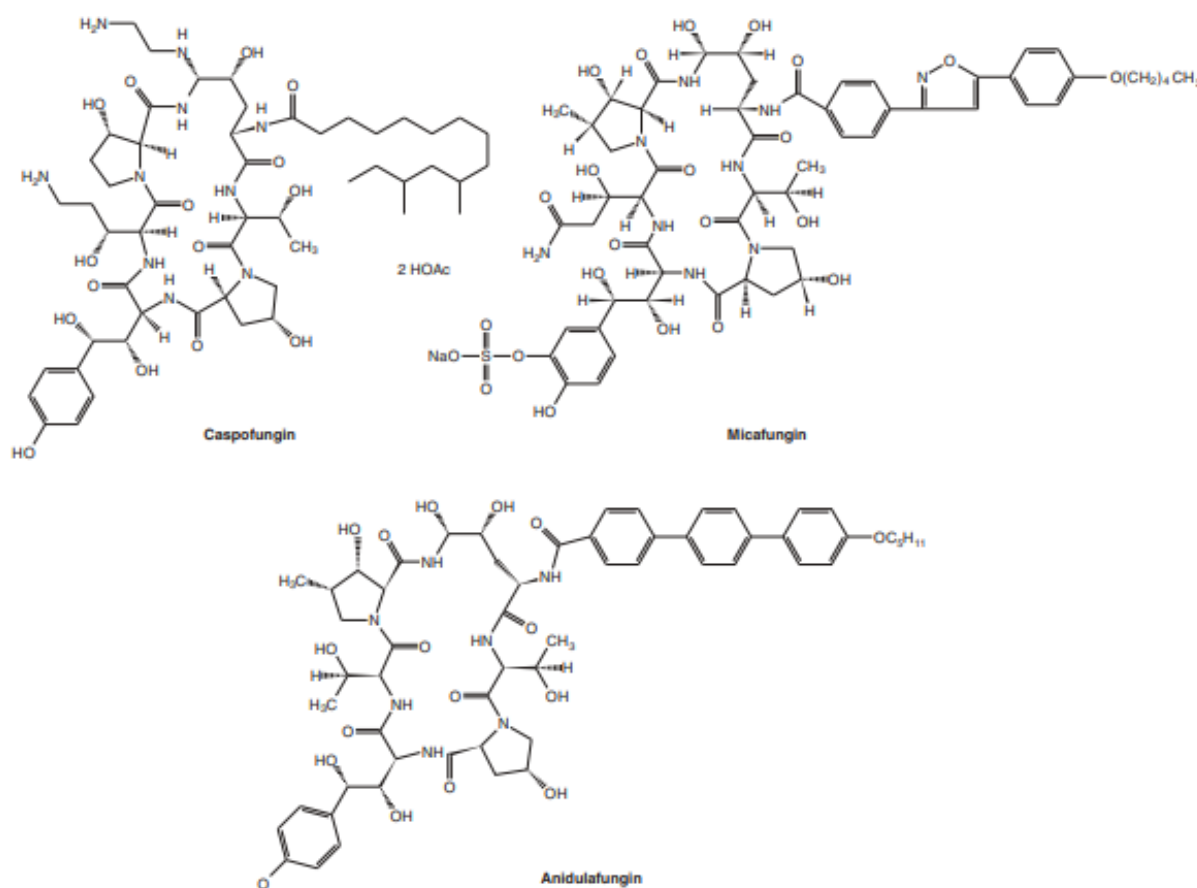


Figura 7. Estrutura química das equinocandinas: caspofungina, anidulafungina e micafungina

Fonte: CHEN; SLAVIN; SORRELL, 2011

O espectro de ação dessa classe de fármacos restringe-se apenas aos gêneros *Candida* e *Aspergillus*. Atuam inibindo de forma não-competitiva a enzima β -(1,3)-D-glucano sintase, atuando particularmente na subunidade Fks1 desta enzima, levando à ruptura da parede celular em crescimento, o que resulta em instabilidade osmótica e morte celular. A β -(1,3)-D-glucano sintase é responsável pela síntese do polissacarídeo β -(1,3)-D-glucano, principal polissacarídeo presente na parede celular fúngica, responsável por 50% da constituição da mesma, e junto com a quitina, e glicoproteínas confere integridade à parede celular (CAMPOY; ADRIO, 2017). São administradas pela via intravenosa (GOLAN et al., 2012).

Possuem baixa toxicidade, em geral os pacientes que fazem uso podem apresentar febre, urticária, náuseas e flebite no local da injeção, e pouca interação com outros medicamentos (CHEN; SLAVIN; SORRELL, 2011).

A resistência às equinocandinas podem acontecer por meio de mutações no gene *Fks1* que codifica a enzima β -(1,3)-D-glucano sintase, responsável pela síntese dos polissacarídeos β -(1,3)-D-glucanos que compõem a parede celular fúngica (HORI, SHIBUYA, 2018). É pouco comum a resistência de *Aspergillus* spp. às equinocandinas, entretanto, mais recentemente têm-se relatado o aparecimento de cepas de *A. fumigatus* com mutação no gene *Fks1*, elevando a CIM da micafungina e caspofungina para 2 ug/mL e resultou em falha terapêutica (JIMÉNEZ-ORTIGOSA et al., 2017).

Clinicamente é recomendado que o uso da caspofungina para o tratamento das formas de aspergilose seja sempre associado a um antifúngico azólico ou poliênico, tendo em vista que as equinocandinas não são fungicidas para *Aspergillus*, e o uso isolado desse fármaco poderia comprometer o tratamento farmacológico (LIMPER et al., 2011; DENNING et al., 2016). Além disso, devido à baixa penetração desses fármacos na barreira hematoencefálica, esses não podem ser utilizados no tratamento de aspergilose cerebral (ULLMANN et al., 2018; PATTERSON et al., 2016).

2.3 Toxicidade *in silico* e *in vitro*

No tocante ao desenvolvimento de novos medicamentos, além dos ensaios necessários para a comprovação da atividade farmacológica, são necessários estudos de toxicidade para caracterizar os efeitos prejudiciais dos candidatos a fármacos que podem ocorrer a partir da sua administração, para garantir a sua segurança futura (VEIGA JÚNIOR et al., 2005; SARAIVA et al., 2012). Modelos computacionais têm sido cada vez mais utilizados quando se quer prever características farmacológicas e toxicológicas de um composto, destacando-se os modelos *in silico*, que conseguem prever o comportamento farmacológico, farmacocinético e toxicológico, a partir de softwares, direcionando a pesquisa por um caminho mais curto (ANGELO; MAX; MARKUS, 2006; SRINIVAS et al., 2014).

Para a avaliação da toxicidade *in vitro*, um dos modelos mais utilizados é a avaliação da citotoxicidade em eritrócitos, útil para estimar possíveis danos que novos candidatos a fármacos possam promover no organismo quando administrados (APARICIO et al., 2005). A exposição das hemácias a um produto teste pode acarretar em ruptura dessas células, também chamada de hemólise, o que promove aumento da concentração de hemoglobina livre no plasma, podendo causar sérios danos a órgãos vitais como fígado, rins e coração (CARVALHO et al., 2007). A hemólise está diretamente correlacionada com a citotoxicidade, de forma que, quanto maior for a atividade hemolítica de uma molécula, maior é sua citotoxicidade (SCHIAR et al., 2007).

Os testes de genotoxicidade *in vitro* permitem avaliar os efeitos lesivos que as substâncias podem causar no DNA, podendo resultar em mutagênese e alterações cromossômicas, que podem atuar como biomarcadores podendo ser mensurados no organismo (SILVA et al., 2003).

O micronúcleo é considerado um biomarcador que permite avaliar alterações cromossômicas causadas por agentes genotóxicos. Ele é formado durante o processo de divisão celular, na transição das fases de metáfase e anáfase, quando há a separação de um pequeno fragmento de núcleo, que se apresenta como uma estrutura arredondada ao lado do núcleo principal (DOSI et al., 2016).

Além do micronúcleo, outras alterações celulares são formadas por meio de aberrações cromossômicas, sendo denominadas de anomalias metanucleares, podendo ser indicativo de citotoxicidade e genotoxicidade. Entre elas destacam-se a

cariólise, a cariorrexe, a binucleação, entre outras (OLIVEIRA et al., 2012; KUMAR et al., 2016). A cariólise trata-se de uma dissolução do núcleo, onde pode ser observado uma falta de coloração do núcleo, e isso se dá em resposta a lesões nucleares que ocorrem com frequência em células do epotélio não-queratinizado, estando associado a lesões celulares, reflexo de danos grosseiros que podem levar a morte celular por necrose (SCHWARZMEIER, 2018).

Já a cariorrexe é uma alteração que se caracteriza pela desintegração do núcleo formando pequenos fragmentos do material nuclear e pode acontecer durante o processo de morte celular por necrose ou apoptose, sendo a morte celular observada em estágios mais iniciais. A binucleação caracteriza-se pela formação de dois núcleos na mesma célula, que pode ocorrer durante o processo de diferenciação celular (TOLBERT et al., 1992; DUTRA et al., 2010). Essas alterações celulares podem ser observadas na figura 8.

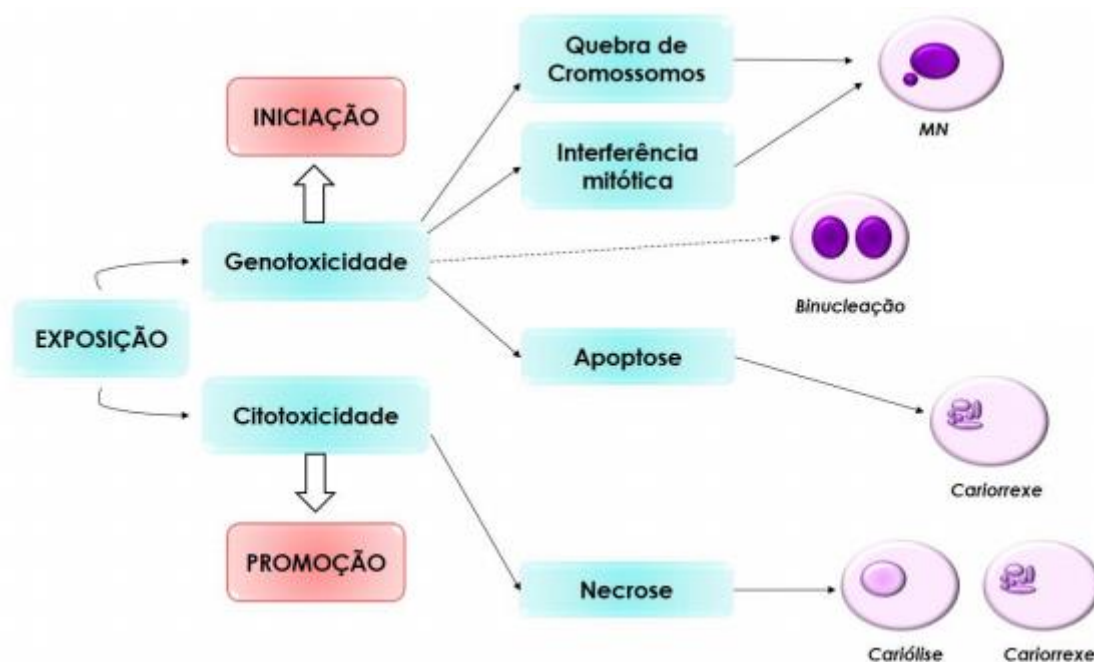


Figura 8. Esquema ilustrativo dos danos celulares causados por agentes genotóxicos e citotóxicos

Fonte: Adaptado de SCHWARZMEIER, 2018

2.4 Potencial farmacológico das amidas sintéticas

O grupamento amida é descrito na literatura como um importante grupamento químico encontrado em muitas moléculas, sendo responsável por suas atividades biológicas (RUIDER; MAULIDE, 2015). O paracetamol, por exemplo, é uma amida com atividade analgésica e antitérmica bem descrita na literatura, e amplamente utilizado no mercado (KRISTENSEN et al., 2016).

Algumas atividades biológicas de amidas sintéticas já foram descritas, como antiviral (PANDEYA et al., 1999), anti-helmíntica (MUNIRAJASEKAR; HIMAJA; SUNIL, 2011), anti-inflamatória (MATHEWS; GARDEN; FERMADES, 2007), e as atividade antibacteriana (KATKE et al., 2011; PATEL et al., 2013; JETTI et al., 2015; CORDEIRO et al., 2020) e a antifúngica (BRAVO et al., 2003; ASHALE, 2012) estão sendo cada vez mais documentadas na literatura.

2-Cloro-*N*-fenilacetamida (A_1Cl) (Figura 9), é uma amida sintética que possui semelhança estrutural com o paracetamol, sendo utilizada como intermediária de reação, obtida por meio de uma substituição nucleofílica envolvendo o cloreto de 2-cloro-acetila e anilina, utilizando apenas uma etapa reacional e com elevado rendimento. A facilidade de preparação e sua disponibilidade comercial, torna essa amida sintética um promissor candidato a fármaco.

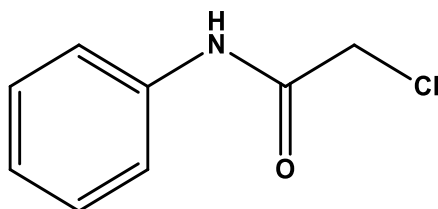


Figura 9. Estrutura química da amida sintética 2-Cloro-*N*-fenilacetamida.

Fonte: HUANG, 2016.

Por se tratar de um composto utilizado mais como intermediário de reação (PATEL et al., 2013; HUANG, 2016), A_1Cl tem sido pouco estudada em relação às suas atividades biológicas. Katke et al., (2011) em um estudo de triagem utilizando o método de difusão de discos, demonstrou a atividade antibacteriana da amida sintética em cepas de *Escherichia coli* e *Pseudomonas aeruginosa*.

Ashale (2012), sintetizou uma série de amidas substituídas e realizou um estudo de triagem utilizando o método de difusão de discos, para verificar a atividade antifúngica em bactérias, como *Staphylococcus aureus*, e em fungos como *A. niger*

e *Fusarium oxysporum*. Observou que, todas as amidas testadas mostraram atividade antifúngica nas cepas de bactérias e fungos utilizadas, destacando a atividade da A₁Cl. Além disso, esse estudo evidenciou que, as amidas substituídas independentemente da natureza e da posição do grupo substituído, mostraram maiores atividades contra *A. niger* do que o controle não substituído.

Tendo em vista o potencial antimicrobiano da A₁Cl, e a escassez e limitação dos estudos envolvendo essa molécula, esse trabalho se propõe a aprofundar os estudos farmacocinéticos, farmacodinâmicos e toxicológicos dessa amida sintética para melhor caracterizar seu potencial farmacológico e assim contribuir para estudos futuros, para viabilizar o desenvolvimento de um novo medicamento antifúngico.

Objetivos

3.0 Objetivos

3.1 Geral

Avaliar o efeito da amida sintética 2-cloro-*N*- fenilcetamida (A_1Cl) sobre *Aspergillus* e determinar seu perfil toxicológico.

3.2 Específicos

- Determinar a Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima (CFM) da A_1Cl ;
- Verificar o efeito da A_1Cl sobre a germinação dos conídios;
- Determinar o Índice de Concentração Inibitória Fracionada (método de associação – *checkerboard*) da A_1Cl com os antifúngicos padrões;
- Analisar os possíveis alvos celulares de ação antifúngica da A_1Cl através do estudo de *docking* molecular;
- Determinar o mecanismo de ação *in vitro* da A_1Cl ;
- Avaliar o perfil farmacocinético *in silico* da A_1Cl ;
- Analisar o perfil citotóxico em células sanguíneas do sistema ABO;
- Analisar o perfil genotóxico da A_1Cl em células da mucosa bucal.

Material e métodos

4.0 Material e Métodos

4.1 Locais da pesquisa

A análise da atividade antifúngica foi realizada no Laboratório de Pesquisas de Atividade Antibacteriana e Antifúngica de Produtos Naturais e/ou Sintéticos Bioativos, do Departamento de Ciências Farmacêuticas, do Centro de Ciências da Saúde, da Universidade Federal da Paraíba. A análise do perfil citotóxico foi realizada no Laboratório de Fitoterapia, Bioquímica e Microbiologia (LAFBIM), do Centro de Saúde e Tecnologia Rural, da Universidade Federal de Campina Grande, e o docking molecular foi realizado no laboratório de Quimioinformática, no Centro de Biotecnologia, da Universidade Federal da Paraíba.

4.2 Obtenção das amostras de sangue e as células da mucosa oral

As amostras de sangue (A, B e O) e esfregaços da mucosa oral foram coletados de adultos jovens saudáveis de ambos os sexos entre 18 e 40 anos de idade.

4.3 Posicionamento ético

Os ensaios de citotoxicidade e genotoxicidade foram realizados de acordo com o Código de Ética da World Medical Association e aprovados pelo Comitê de Ética do Centro Universitário de Patos (protocolo número: 3.621.284).

4.4 Meios de Cultura

Para o preparo da cultura e para avaliação da atividade antifúngica foi utilizado o meio sólido Ágar Batata Dextrose (ABD) e o meio líquido RPMI 1640-L-glutamina (Roswell Park Memorial Institute), respectivamente, ambos adquiridos da Sigma-Aldrich® (São Paulo, São Paulo, Brasil), e preparados conforme as instruções do fabricante.

4.5 Micro-organismos

Para a avaliação da atividade antifúngica foram selecionadas 14 cepas de origem clínica, pertencentes ao gênero *Aspergillus*, e duas cepas ATCC, sendo 11 da espécie *A. flavus* e 5 da espécie *A. niger* (Tabela 1), todas obtidas da coleção da Micoteca (LM) do Laboratório de Pesquisas de Atividade Antibacteriana e Antifúngica de Produtos Naturais e/ou Sintéticos Bioativos. Todas as cepas

utilizadas foram mantidas em tubos de ensaio contendo Ágar Batata dextrose por 7 dias a 35°C, e mantidas sob refrigeração (4°C).

Tabela 1. Cepas de *A. flavus* e *A. niger* de origem clínica obtidas da coleção da Micoteca Laboratório de Pesquisas de Atividade Antibacteriana e Antifúngica de Produtos Naturais e/ou Sintéticos Bioativos.

Cepas	Origem
<i>A. flavus</i> ATCC 16013	Padrão
<i>A. flavus</i> LM 12	Clínica – pulmão
<i>A. flavus</i> LM18	Clínica – pulmão
<i>A. flavus</i> LM 19	Clínica – pulmão
<i>A. flavus</i> LM 22	Clínica – pulmão
<i>A. flavus</i> LM 26	Clínica – pele (pés)
<i>A. flavus</i> LM 55	Clínica – ouvidos
<i>A. flavus</i> LM 171	Clínica – pulmão
<i>A. flavus</i> LM 210	Clínica – pele (mão direita)
<i>A. flavus</i> LM 248	Clínica – unha (mão)
<i>A. flavus</i> LM 256	Clínica – ouvidos
<i>A. niger</i> ATCC 6275	Padrão
<i>A. niger</i> LM 22	Clínica – Ouvido esquerdo
<i>A. niger</i> LM 23	Clínica – Unha (pé)
<i>A. niger</i> LM 29	Clínica – ouvidos
<i>A. niger</i> LM 66	Clínica - ouvidos

4.6 Produto teste e antifúngicos sintéticos

Para a avaliação da atividade antifúngica, foi utilizada como produto-teste a amida sintética 2-cloro-*N*-fenilcetamida, cuja síntese foi realizada no Laboratório de Pesquisa em Bioenergia e Síntese Orgânica, do Centro de Ciências Exatas e da Natureza (CCEN) da Universidade Federal da Paraíba, pelo Dr. Helivaldo Diógenes da Silva Souza, sob a supervisão do Professor Dr. Petrônio Filgueiras de Athayde Filho conforme protocolo descrito por Huang (2016).

Os antifúngicos voriconazol e anfotericina B, bem como o produto ergosterol, foram obtidos na Sigma-Aldrich® (São Paulo, São Paulo, Brasil). O sorbitol foi obtido na INLAB® (São Paulo, Brasil). Agar de dextrose de batata foi obtido de Difco Lab.

(EUA). Todas as substâncias foram dissolvidas em DMSO (dimetilsulfóxido - Sigma-Aldrich®, São Paulo, São Paulo, Brasil) e solubilizado em água destilada estéril para obter soluções na concentração de 1024 µg / mL. Para avaliação da atividade antifúngica, foi utilizado RPMI 1640 com L-glutamina (Sigma-Aldrich®, São Paulo, SP, Brasil), preparado de acordo com as instruções do fabricante.

4.7 Preparo do inóculo

A preparação do inóculo foi realizada a partir de culturas recentes de cada uma das cepas das espécies de *A. flavus* e *A. niger* previamente cultivadas em tubos estéreis contendo A.B.D inclinado, e incubados a 35°C por 7 dias. Após o período de incubação, as amostras foram retiradas de culturas crescidas, suspensas em solução salina estéril a 0,85%, transferidas para tubos estéreis e agitadas no vortex por 30 segundos para homogeneização. O inóculo foi padronizado de acordo com a concentração 0,5 da escala de McFarland (10^6 UFC/mL). A concentração foi confirmada por contagem de conídios utilizando a câmara de Neubauer (CSLI 2008; GUERRA et al., 2015).

4.8. Determinação da atividade antifúngica *in vitro*

4.8.1 Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima

A determinação da CIM da A₁Cl contra cepas de *A. flavus* e *A. niger* foi realizada usando a técnica de microdiluição em caldo recomendada pelo Clinical and Laboratory Standards Institute (CLSI 2008) com modificações. Inicialmente, 100 µL de RPMI duplamente concentrado foram adicionados aos poços, seguido por 100 µL de cada suspensão de produto (A₁Cl, voriconazol e anfotericina B) em uma concentração inicial de 1024 µg/mL na primeira linha da placa, e em seguida, para a obtenção de diferentes concentrações que variaram de 1024 até 4 µg/mL por meio de uma diluição seriada. Por último foram adicionados 10 µL de cada suspensão do micro-organismo ensaiado. Utilizou-se controles negativos (sem drogas) para avaliar a viabilidade das cepas e esterilidade do meio, bem como controles de sensibilidade (para o DMSO). Após 2 dias de incubação, a leitura visual da placa foi realizada, e a CIM foi considerada a menor concentração inibindo 100% do crescimento da cepa (CSLI 2008). Os resultados são expressos como a moda das CIMs obtidos em três testes.

Para a determinação do CFM, 100 μ L de RPMI foram adicionados a placa esterilizada de 96 poços e as amostras de 10 μ L foram removidas dos poços onde não havia crescimento de fungos nas placas da CIM e adicionadas a nova placa contendo o meio de cultura (RPMI 1640, Sigma-Aldrich®, São Paulo, SP, Brasil). As placas foram incubadas a 35°C durante 2 dias. A atividade fungicida foi considerada a menor concentração do produto capaz de inibir o crescimento visível na placa (NCUBE; AFOLAYAN; OKOH, 2007; CSLI 2008).

4.8.2 Avaliação do efeito do A₁Cl sobre a inibição da germinação dos conídios fúngicos

Para esse estudo, foram utilizadas as cepas ATCC 16013 e LM 22 da espécie *A. flavus* e as cepas ATCC 6275 e LM 66 de *A. niger*. Após a leitura das placas da CIM, foram retirados 10 μ L nas concentrações da CIM, CIM x 2 e CIM x 4 de cada produto testado e semeado em lâminas estéreis. Em seguida, foi adicionado uma gota do corante azul de lactofenol, homogeneizou-se e cobriu-se cada preparação com uma lamínula. A contagem dos conídios foi realizada em microscópio óptico em 5 campos diferentes para obter a média aritmética. Os resultados foram expressos em gráficos como a porcentagem de inibição dos conídios em função das concentrações dos produtos testados, e a interpretação dos resultados foi realizada comparando a porcentagem da germinação dos conídios em função dos produtos testados, com o controle negativo, que continha apenas o meio de cultura acrescido do inóculo (Adaptado de TRAJANO et al., 2013)

4.8.3 Avaliação da associação do A₁Cl com antifúngicos padrões frente às cepas de *A. flavus* e *A. niger*

O efeito da associação dos produtos com antifúngicos padrões foi determinado a partir da técnica de microdiluição – *checkerboard* para derivação do ICIF (Índice da Concentração Inibitória Fracionada).

Foram utilizadas soluções dos produtos testados em concentrações determinadas a partir de suas respectivas CIM. Inicialmente foi adicionado 100 μ L de RPMI nos poços da microplaca esteril contendo 96 cavidades, e em seguida adicionou-se 50 μ L de cada produto testado, nas diversas concentrações (CIM÷8, CIM÷4, CIM÷2, CIM, CIM×2, CIM×4 e CIM×8) no sentido vertical (antifúngico padrão) e horizontal (produto teste) da microplaca. Por fim, foram adicionados 20 μ L

da suspensão fúngica. O ensaio foi realizado em duplicata, sendo as microplacas incubadas a 35°C por 2 dias (ELIOPOULOS; MOELLERING, 1991; DUTTA et al., 2004).

Para o cálculo do ICIF (Índice da Concentração Inibitória Fracionada) foi utilizado o método 3 descrito por Bonapace et al., (2002) onde foi utilizada a menor CIM (onde não houve crescimento fúngico) para o cálculo, que foi realizado através da soma do $CIF^A + CIF^B$, onde A representa o produto teste e B o antifúngico padrão. O CIF^A , por sua vez, é calculado através da relação CIF^A combinado/ CIF^A sozinho, enquanto que o $CIF^B = CIF^B$ combinado/ CIM^B sozinho. A interpretação deste índice foi realizada da seguinte forma: sinergismo ($ICIF \leq 0,5$), indiferença ($0,5 < ICIF \leq 4$) e antagonismo ($ICIF > 4,0$) (ODDS, 2003).

4.8.4. Avaliação do mecanismo de ação da A₁Cl contra as cepas de *A. flavus* e de *A. niger*.

4.8.4.1 Ensaio sobre a parede celular

Para avaliar um possível mecanismo de ação da A₁Cl contra *A. flavus* e *A. niger*, envolvendo a parede celular fúngica, realizou-se a determinação da CIM na presença e na ausência do sorbitol (0,8 M) no meio, pelo método de microdiluição utilizando placas de 96 poços em “U” (Alamar, Diadema, SP, Brasil). As placas foram seladas assepticamente, incubadas a 35°C e as leituras foram realizadas após 48h. Um controle de micro-organismo e um controle de esterilidade foi realizado. O ensaio foi realizado em triplicata e expresso como a moda dos resultados (ESCALANTE et al., 2008).

4.8.4.2 Ensaio sobre a membrana celular

Para avaliar um possível mecanismo de ação da A₁Cl contra cepas de *A. flavus* e *A. niger*, envolvendo a membrana celular fúngica, realizou-se a determinação da CIM na presença e na ausência do ergosterol (400 µg/mL) (Sigma-Aldrich®) no meio, pelo método de microdiluição utilizando placas de 96 poços em um “U” (Alamar, Diadema, SP, Brasil). Um controle de micro-organismo e um controle de esterilidade foi realizado. A anfotericina B foi utilizada como controle positivo. As placas foram seladas e incubadas a 35°C por 48h para ser realizada a leitura. O ensaio foi realizado em triplicata e expresso como a moda dos resultados (ESCALANTE et al., 2008).

4.8.4.3. Docking molecular

Usando o programa ChemDraw®, a estrutura da A₁Cl foi projetada e salva como um arquivo MOL. As enzimas investigadas com seus respectivos PDB IDs foram C-14- α -desmetilase (PDB ID: 4UYL) (HARGROVE et al., 2015), esqualeno epoxidase (PDB ID: 4 MAH) (FROST et al., 1995), delta-14 -esterol redutase (PDB ID: 4QUV) (STANDARDS, 2002), 1,3- β -glucano sintase (PDB ID: 3A58) (RASOOLI; ABYANEH, 2004) e timidilato sintase (PDB ID: 5UIV) (SINHA; RULE, 2017). O ligante e seus receptores foram submetidos a acoplamento molecular usando o Molegro Virtual Docker, v. 6.0.1 (MVD) (MORRIS et al., 2009). As proteínas e estruturas de ligante foram preparadas usando as configurações de parâmetros do pacote de software padrão (pontuação de função: Pontuação de MolDock). As estruturas A₁Cl e os receptores usados foram preparados usando o software PyRx 0.9 (WOLF 2009), e os resultados são apresentados como os valores de energia da interação ligante-receptor total.

4.9. Avaliação do perfil físico-químico, farmacocinético e efeitos tóxicos *in silico* da A₁Cl

A avaliação *in silico* da toxicidade de moléculas também incluiu a análise teórica de efeitos mutagênico, tumorigênico, irritante e sobre a reprodução como descrito por Abreu, 2008. Para ser administrado pela via oral, o produto teste deve ser absorvido pelo trato gastrointestinal, e por isso, A₁Cl foi avaliada de acordo com a “Regra dos Cinco” de Lipinski, que estabelece que pelo menos três de quatro requisitos devam ser apresentados para que o composto possua uma boa biodisponibilidade oral teórica. Assim, os compostos para serem absorvidos devem possuir peso molecular 500 daltons (Da), coeficiente de partição octanol/água calculado (cLogP) 5, número de aceptores de ligação hidrogênio (nALH) 10 e número de grupos doadores de ligação hidrogênio (nDLH) 5 (LIPINSKI, 2001).

O software Swiss ADME também emprega outros filtros baseados em regras (Ghose: $180 \leq MM \leq 480$; $20 \leq N^{\circ} \text{ de átomos} \leq 70$; $40 \leq \text{Refratividade molar} \leq 130$; $-0,4 \leq \text{Log Po/w} \leq 5,6$; Veber: $N^{\circ} \text{ Ligações giratórias} \leq 10$; $TPSA \leq 14 \text{ nm}^2$; Egan: $\text{Log Po/w} \leq 5,88$; $TPSA \leq 13,16 \text{ nm}^2$) (GHOSE et al., 1999; EGAN et al., 2000; VEBER et al., 2002), que permite uma compreensão mais ampla das características físico-químicas e farmacocinéticas de um candidato a fármaco.

Para a análise da toxicidade teórica e dos parâmetros farmacocinéticos a estrutura da amida sintética A₁Cl foi submetida ao estudo *in silico* dos parâmetros ADMET (absorção, distribuição, metabolismo, excreção e toxicidade) usando o programa Osiris Property Explorer (<http://www.organic-chemistry.org/prog/peo/drugScore.html>).

4.10. Avaliação *in vitro* do perfil citotóxico da A₁Cl

4.10.1. Avaliação da atividade hemolítica da A₁Cl

Alíquotas de sangue humano (tipo A, B e O) foram misturadas com NaCl 0,9 % (na proporção de 1:30) e centrifugada durante 5 minutos a 2500 rpm para obtenção dos eritrócitos. Este procedimento foi repetido por mais duas vezes e o sedimento da última centrifugação foi ressuspenso em NaCl 0,9% para obter uma suspensão a 0,5%. Concentrações distintas (50, 100, 500 e 1000 µg/mL), da A₁Cl foram adicionadas em diferentes preparações de suspensão de eritrócitos para um volume final de 2 mL. Uma suspensão de eritrócitos não tratada foi utilizada como controle negativo (0 % de hemólise) e uma suspensão de eritrócitos acrescida de Triton X-100 a 1% como controle positivo (100 % de hemólise). Posteriormente as amostras foram incubadas por 1 hora à 22 ± 2°C sob agitação lenta e constante (100 rpm). Decorrido este tempo as amostras foram por 5 minutos centrifugadas a 2500 rpm e a hemólise foi quantificada por espectrofotometria a 540 nm (RANGEL et al., 1997).

4.10.2 Avaliação da atividade antihemolítica da A₁Cl

A avaliação da atividade antihemolítica em eritrócitos humanos foi realizada utilizando uma suspensão de eritrócitos a 0,5%. Soluções contendo concentrações diferentes (50, 100 e 500 µg/mL) da A₁Cl foram incubadas em tubos contendo 2 mL de uma suspensão de eritrócitos por 1h a 22 ± 2°C. Posteriormente, as preparações foram centrifugadas por 5 minutos a 2500 rpm e os sobrenadantes obtidos descartados. Os eritrócitos foram então ressuspenso em solução hipotônica de NaCl 0,24% e agitadas a 100 rpm, por uma hora a 22 ± 2°C. Após este período, as amostras foram centrifugadas por 5 minutos a 2500 rpm e a hemólise foi quantificada por espectrofotometria a 540 nm (DACIE, 2001). A suspensão de eritrócitos foi utilizada como controle negativo (0 % de hemólise) e uma suspensão

de eritrócitos acrescida da solução hipotônica como controle positivo (100 % de hemólise).

4.11. Avaliação *ex vivo* do perfil genotóxico da A₁Cl

4.11.1. Avaliação do efeito genotóxico da A₁Cl em células da mucosa oral

As células epiteliais foram coletadas dos lados esquerdo e direito da mucosa oral por meio de uma cytobrush (amostra endocervical/coletor de células), considerada o instrumento mais adequado para a obtenção de células esfoliadas da mucosa oral (KASSIE et al., 2001). As células foram mantidas em tubo com 5 mL de NaCl 0,9% (meio de preservação de células) até o preparo das lâminas. As células controle foram divididas em dois grupos: tratadas com peróxido de hidrogênio (0,0005%) (controle positivo) e não tratadas (controle negativo).

As células foram lavadas duas vezes em solução salina, centrifugadas por 10 min a 1.500 rpm e, a seguir, mantidas em 5 mL de solução salina. O sobrenadante foi removido. As amostras foram lavadas mais uma vez e expostas *ex-vivo* à A₁Cl (50, 100, 500 e 1000 µg/mL) por 30 min, cada teste foi realizado em triplicata. Em seguida, foram centrifugados e o sobrenadante descartado. Antes do preparo dos esfregaços, as lâminas foram pré-aquecidas a 37°C e as células homogeneizadas em um misturador vortex. Em cada concentração analisada, foram colocadas em três lâminas, secas à temperatura ambiente e fixadas em metanol: ácido acético (3:1) por 15 min (THOMAS et al., 2008). As lâminas em triplicata foram mantidas em temperatura ambiente por 12 h, após as quais foram imersas em água destilada por 1 min e coradas em Giemsa 2% para análise em microscopia óptica (GABRIEL et al., 2006).

A toxicidade celular foi avaliada por meio da presença de indicadores celulares, como micronúcleos, cariólise, cariorrexe, entre outras alterações (CARRARD et al., 2007; SPONCHIADO et al., 2016). Aproximadamente 1000 células foram analisados por lâmina, e os dados foram expressos como porcentagens que representam a média aritmética de três medições.

4.12 Análise estatística

Os resultados obtidos de CIM, CFM, mecanismo de ação em parede e membrana celular e associação foram expressos como a moda, e os resultados da porcentagem da germinação dos conídios, atividade hemolítica, anti-hemolítica e

genotoxicidade foram expressos como média $\pm$ erro padrão da média (e.p.m.), analisados estatisticamente empregando-se análise de variância (ANOVA) “one-way” seguido do pós-teste de Bonferroni para a comparação de mais de dois grupos. A hipótese nula foi rejeitada quando $p < 0,05$, e para essa análise foi utilizado o programa GraphPad Prism® versão 5.01 (GraphPad Software Inc., San Diego CA, U.S.A.). Os resultados do perfil farmacocinético e toxicológico in silico foram realizados utilizando os softwares gratuitos OSIRIS e SWISS ADMET.

Resultados e Discussão

5.0 Resultados e discussão

5.1

Antifungal activity and mechanism of action of 2-chloro-*N*-phenylacetamide: a new molecule with activity against strains of *Aspergillus flavus*

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Antifungal activity and mechanism of action of 2-chloro-*N*-phenylacetamide: a new molecule with activity against strains of *Aspergillus flavus*

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Antifungal activity of 2-chloro-*N*-phenylacetamide

Section that the article belongs to: **Biological science**

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Abstract

Aspergillus genus causes many diseases, and the species *Aspergillus flavus* is highly virulent. Treatment of aspergillosis involves azole derivatives such as voriconazole and polyenes such as amphotericin B. Due to an increase in fungal resistance, treatments are now less effective; the search for new compounds with

promising antifungal activity has gained importance. The aims of this study were to evaluate the effects of the synthetic amide 2-chloro-*N*-phenylacetamide (A₁Cl) against strains of *Aspergillus flavus* and to elucidate its mechanism of action. Thus, the minimum inhibitory concentration, minimum fungicidal concentration, conidial germination, associations with antifungal agents, cell wall activities, membrane activities and molecular docking were evaluated. A₁Cl presented antifungal activity against *Aspergillus flavus* strains with a minimum inhibitory concentration of between 16 and 256 µg/mL and a minimum fungicidal concentration between 32 and 512 µg/mL. The minimum inhibitory concentration of A₁Cl also inhibited conidial germination, but when associated with amphotericin B and voriconazole, it promoted antagonistic effects. Binding to ergosterol on the fungal plasma membrane is the likely mechanism of action, along with possible inhibition of DNA synthesis through the inhibition of thymidylate synthase. It is concluded that the amide 2-chloro-*N*-phenylacetamide has promising antifungal potential.

Keywords: 2-chloro-*N*-phenylacetamide, antifungal activity, *Aspergillus flavus*, resistance.

1. Introduction

Aspergillus genus comprises more than 250 species; it is one of the largest genera of filamentous fungi that cause human disease (Rudramurthy et al. 2019). *Aspergillus* species *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus* and *A. versicolor* are frequently involved in diseases, such as rhinosinusitis, cutaneous and subcutaneous aspergillosis, pulmonary infections, cardiac infections, otomycosis, among others (Barnes & Marr, 2006; Sugui et al. 2014; Rudramurthy et al. 2019). The genus, in general, presents low pathogenicity for humans, and it requires a large inoculum to infect immunocompromised individuals if their physiological performance is intact. However, once the infection is established, a variety of presentations are observed, with the lung being the most commonly affected organ (Castello Branco et al. 2014).

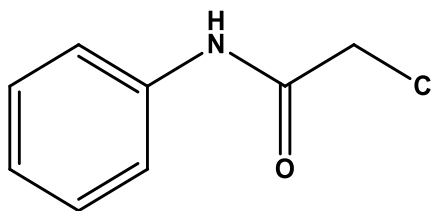
The infectious spectrum of aspergillosis is classified into four types: invasive disease, corresponding to life-threatening infections in immunocompromised patients; subacute or chronic infectious disease, in patients with structural lung abnormalities, pre-existing sinus or lung disease (or some subtle defect in innate

immunity); allergic or eosinophilic disease, manifesting in various forms, such as allergic bronchopulmonary aspergillosis, eosinophilic rhinosinusitis, and extrinsic allergic alveolitis; and locally invasive infections resulting from trauma or surgery, such as keratitis or a postoperative infection (Rudramurthy et al. 2019). *A. fumigatus* species is the agent most commonly involved in aspergillosis and it is one of the most commonly studied species.

Studies have revealed *A. flavus* as the most virulent species in terms of time and initial inoculum, which causes death in immunocompromised mice (Mosquera et al. 2001). The drugs used to treat aspergillosis include voriconazole (being the drug of choice) and amphotericin B (as a rescue therapy) when azole derivatives cannot be administered (Bellmann 2013; Patterson et al. 2016). However, *A. flavus* is resistant to azoles (Paul et al. 2015; Ukai et al. 2018) and polyenes (Taylor et al. 2017; Reichert-Lima et al. 2018), which limits the use of these drugs. New therapeutic alternatives for aspergillosis are needed.

In a previous investigation carried out by our research group, an unprecedented antifungal activity of the synthetic amide 2-chloro-*N*-phenylacetamide (A_1Cl) (Figure 1) against *A. flavus* strains was demonstrated, with MICs ranging from 16 to 256 $\mu\text{g/ml}$. However, the mechanism of action against the microorganism under study is still unknown. Thus, our objective was to further evaluate the effects of the synthetic amide 2-chloro-*N*-phenylacetamide against various strains of *Aspergillus flavus* and to elucidate its probable mechanism of action.

Figure 1: Chemical structure of the synthetic amide 2-chloro-*N*-phenylacetamide.



Fonte: Huang (2016)

2 Materials and methods

2.1 Microorganisms

To evaluate antifungal activity, eleven (11) strains of clinical origin and one (1) ATCC strain of *A. flavus* (shown in Table I) were obtained from the collection of the

Research Laboratory Micoteca for Antibacterial and Antifungal Activity of Natural and/or Bioactive Synthetic Products. The strains on *Aspergillus flavus* were isolated and grown on potato dextrose agar for 7 days at 35°C. After growth, they were stored under refrigeration at 4°C. To prepare the inoculum, samples were taken from the grown cultures, suspended in sterile 0.85% saline and transferred to sterile tubes. The inoculum was standardized according to the 0.5 concentration of the McFarland scale (10^6 CFU/mL). The concentration was confirmed by counting the conidia using a Newbauer chamber (CSLI 2008; Guerra et al. 2015).

Table I. Clinical strains of *A. flavus* and ATCC strain were obtained from the Micoteca Laboratory collection for Research on Antibacterial and Antifungal Activity of Natural and/or Synthetic Bioactive Products.

Strains of <i>A. flavus</i>	Origin
ATCC 16013	Standard
LM 12	Clinical - lungs
LM18	Clinical - lungs
LM 19	Clinical - lungs
LM 22	Clinical - lungs
LM 26	Clinical – skin (feet)
LM 55	Clinical – ears
LM 171	Clinical - lungs
LM 210	Clinical – skin (right hand)
LM 248	Clinical – nails (hand)
LM 256	Clinical – ears
LM 298	Clinical - lung

2.2 Drugs and Reagents

The tested product was the synthetic amide 2-chloro-*N*-phenylacetamide, obtained by synthesis (Huang 2016). The antifungals voriconazole and amphotericin B, and the ergosterol were obtained from Sigma-Aldrich® (São Paulo, São Paulo, Brazil). The sorbitol was obtained from INLAB® (São Paulo, Brazil). Potato dextrose agar was obtained from Difco Lab. (USA). All of the substances were dissolved in

DMSO (dimethylsulfoxide – Sigma-Aldrich®, São Paulo, São Paulo, Brazil). and solubilized in sterile distilled water to obtain solutions at a concentration of 1024 µg/mL. For evaluation of the antifungal activity, RPMI 1640 with L-glutamine was used (Sigma-Aldrich®, São Paulo, SP, Brazil), and prepared according to the manufacturer's instructions.

2.3 Determination of *in vitro* antifungal activity

2.3.1 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A₁Cl against strains of *A. flavus*.

The MIC determination of A₁Cl against strains of *A. flavus* was performed using the broth microdilution technique recommended by the Clinical and Laboratory Standards Institute (CLSI 2008). Initially, 100 µL of double-concentrated RPMI was added to the wells, followed by 100 µL of each product suspension (A₁Cl, voriconazole, and amphotericin B) at an initial concentration of 1024 µg/mL in the first row of the plate, and then the range of concentrations from 1024 to 4 µg/mL were obtained by means of serial dilution. Finally, 10 µL of each of the tested microorganism suspensions was added. Negative controls (without drugs) were used to assess the viability of the strains and the sterility of the medium, as well as the sensitivity controls (to DMSO). After 2 days of incubation, visual plate reading was performed, and the MIC was considered the lowest concentration inhibiting 100% of the strain growth (CLSI 2008). The results are expressed as the mode of the MICs obtained in three tests.

For the determination of the MFC, 100 µL of RPMI was added to the 96-well sterile plate, and 10 µL samples were removed from wells where there was no fungal growth on MIC plates and added to the new plate containing the culture medium (RPMI 1640, Sigma-Aldrich®, São Paulo, SP, Brazil). The plates were incubated at 35°C for 2 days. The fungicidal activity was considered the lowest concentration of the product capable of inhibiting visible growth on the plate (Ncube et al. 2007; CLSI 2008).

2.3.2. Evaluation of A₁Cl inhibition of conidial germination in *A. flavus* strains

The strains ATCC 16013 and LM 22 were used for this test. After reading the MIC plates, 10 µL of the MIC, MIC × 2 and MIC × 4 concentrations were removed from each product tested and seeded on sterile slides. A drop of lactophenol blue

dye was then added to each preparation, homogenized, and covered with a coverslip. Conidia counting was performed under an optical microscope in 5 different fields to obtain an arithmetic mean. The results were expressed in graphs as the percentage of conidial germination as a function of the concentrations of the tested products (Adapted from Trajano et al. 2013).

2.4. Evaluation of the mechanism of action of A₁Cl against clinical strains of *A. flavus*

2.4.1 Cell wall assay

To assess the possible mechanisms of action involving *A. flavus* fungal cell wall, the MIC of A₁Cl was determined in the presence and absence of sorbitol (INLAB®, São Paulo, Brazil) (0.8 M) in the medium using the microdilution method and 96-well “U” plates (Alamar, Diadema, SP, Brazil). The plates were aseptically sealed and incubated at 35°C. Readings were taken at 2 days. A microorganism check and a sterility check were performed. The test was performed in triplicate and expressed as the mode of the results (Escalante et al. 2008).

2.4.2 Cell membrane assay

To assess the possible mechanisms of action involving *A. flavus* fungal cell membrane, the MIC of A₁Cl was determined in the presence and absence of ergosterol (400 µg/mL) (Sigma-Aldrich®) in the medium using the microdilution method with 96-well “U” plates (Alamar, Diadema, SP, Brazil). A microorganism check and a sterility check were performed. Amphotericin B was used as a positive control. For reading, the plates were sealed and incubated at 35°C for 2 days. The test was performed in triplicate and expressed as mode of the results (Escalante et al. 2008).

2.4.3 Molecular docking

Using the ChemDraw® program, the A₁Cl structure was designed and saved as an MOL file. The enzymes investigated with their respective PDB IDs were C-14- α -demethylase (PDB ID: 4UYL) (Hargrove et al. 2015), squalene epoxidase (PDB ID: 4MAH) (Frost et al. 1995), delta-14-sterol reductase (PDB ID: 4QUV) (Standards 2002), 1,3- β -glucan synthase (PDB ID: 3A58) (Rasooli & Abyaneh 2004) and thymidylate synthase (PDB ID: 5UIV) (Sinha & Rule 2017). The ligand and its

receptors were subjected to molecular coupling using the Molegro Virtual Docker, v. 6.0.1 (MVD) (Morris et al. 2009). The proteins and ligand structures were prepared using the standard software package parameter settings (function Score: MolDock Score). The A₁Cl structures and the receptors used were prepared using PyRx 0.9 software (Wolf 2009), and the results are presented as the total ligand-receptor interaction energy values.

2.5 Assessment of A₁Cl with standard antifungal associations

The effects of the associations of the products with standard antifungals were determined using the microdilution - checkerboard technique for derivation of the FICI (Fractional Inhibitory Concentration Index). Solutions of the tested products were used in concentrations determined from their respective MICs. Initially, 100 µL of RPMI was added to the wells of a sterile 96-well microplate, and then 50 µL of each tested product was added at different concentrations ($MIC \div 8$, $MIC \div 4$, $MIC \div 2$, MIC, $MIC \times 2$, $MIC \times 4$, and $MIC \times 8$) in the vertical (standard antifungal) and horizontal (test product) directions of the microplate. Finally, 20 µL of the fungal suspension was added. The assay was performed in triplicate, with the microplates incubated at 35°C for 2 days (Eliopoulos & Moellering 1991; Dutta et al. 2004).

For calculation of the FICI (Fractional Inhibitory Concentration Index), the method described by Bonapace et al. (2002) was used, and the lowest MIC was used (where there was no fungal growth) for the calculation performed by adding the $FIC^A + FIC^B$, where A represents the test product and B the standard antifungal. The FICI, in turn, is calculated using the combined FIC^A/FIC^A ratio alone, while the FIC^B is calculated using the formula $FIC^B = combined\ FIC^B/FIC^B$ alone. The interpretation of this index was performed as follows: synergism = ($FICI \leq 0.5$), antagonism = ($FICI > 4.0$), and indifference = ($0.5 < FICI \leq 4$) (Odds et al. 2003).

2.6 Statistical analysis

The results obtained from the MIC, MFC, cell wall, and cell membrane assays are expressed as the mode. The results of the conidial germination percentage and cytotoxic profile analysis are expressed as the mean $\pm$ standard error of the mean (S.E.M.), which were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni post-test to compare more than two

groups. The null hypothesis was rejected when $p < 0.05$, and the GraphPad Prism® program version 5.01 (GraphPad Software Inc., San Diego CA, U.S.A.) was used.

3 Results

3.1 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A₁Cl against strains of *A. flavus*.

The product A₁Cl presented MICs that varied from 16 to 256 µg/mL, as shown in Table II, inhibiting visible growth of *A. flavus* strains used in this study. At concentrations below 100 µg/mL, six strains (50%) were weakly sensitive to A₁Cl, with MICs established between 16 and 64 µg/mL.

In the assessment of the minimum fungicidal concentration (MFC) of A₁Cl, a variation of the MFC between 32 and 512 µg/mL was observed (Table II).

Regarding the standard antifungal amphotericin B, it was noted that the strains presented MICs that varied between 1 and 16 µg/mL, with 8 out of 12 presenting MICs of > 2 µg/mL, while the MFC of amphotericin B for the tested strains ranged from 2 to 32 µg/mL (Table II). For voriconazole, there was a variation in MIC between 0.5 and 2 µg/mL, with its MFC being equal to 2 µg/mL for all strains tested (Table II).

Table II. Averages of the MIC ($\mu\text{g/mL}$) and MFC ($\mu\text{g/mL}$) values for A_1Cl , Amphotericin B and Voriconazole against strains of *A. flavus*.

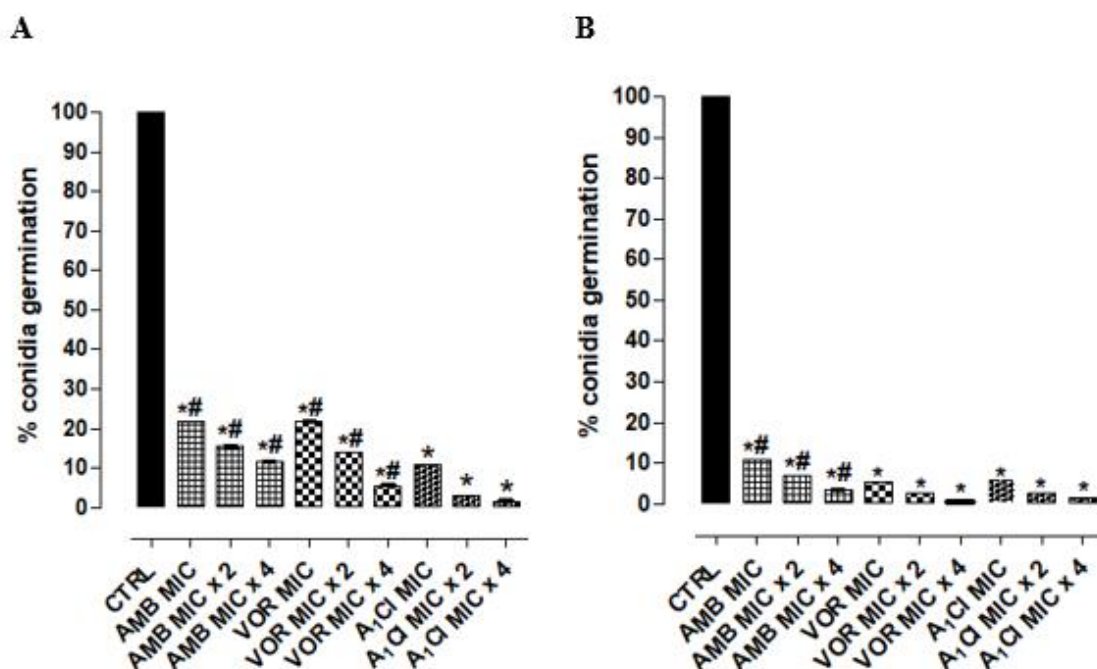
	A_1Cl			Amphotericin B			Voriconazole		
<i>Aspergillus flavus</i>	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
ATCC 16013	128	128	1	2	8	4	1	2	2
LM 12	32	32	1	4	4	1	2	2	1
LM 18	64	64	1	8	32	4	2	2	1
LM 19	16	32	2	2	8	4	2	2	1
LM 22	64	128	2	2	2	1	2	2	1
LM 26	64	64	1	16	32	2	2	2	1
LM 55	256	512	2	16	32	2	2	2	1
LM 171	128	256	2	4	8	2	2	2	1
LM 210	128	512	4	1	4	4	0,5	2	4
LM 248	128	128	1	16	16	1	1	2	2
LM 256	64	128	2	16	16	1	1	2	2
LM 298	256	256	1	8	8	1	1	2	2

MIC = Minimum Inhibitory Concentration, MFC = Minimum Fungicidal Concentration.

3.2 Inhibition of conidial germination: A_1Cl against strains of *A. flavus*

A_1Cl , when submitted to *A. flavus* strain ATCC 16013, reduced the germination of conidia at the three concentrations used, presenting results superior to those found for amphotericin B and voriconazole (Figure 2A). The clinical LM 22 strain, when exposed to A_1Cl , presented a decrease in conidial germination at all concentrations tested, similar to voriconazole, yet the conidial germination inhibition presented by amphotericin B was higher (Figure 2B).

Figure 2. Percentage germination of the conidia of *A. flavus* strains ATCC 16013 (A) and LM 22 (B) against the products amphotericin B (▨), voriconazole (▤) and A₁Cl (▧).



The columns and vertical bars represent the mean and the S.E.M. respectively, (n = 3). One-way ANOVA followed by Bonferroni's posttest. * $p < 0.05$ (compared to the negative control) # $p < 0.05$ (compared to A₁Cl). S.E.M. = standard error of the mean, CTRL (■) = Control, MIC= Minimal Inhibitory Concentration, AMB (▨) = Amphotericin B; VOR (▤) = Voriconazole; A₁Cl (▧) = 2-chloro-*N*-phenylacetamide;

3.3 Evaluation of the effect of A₁Cl on the fungal cell wall.

To verify the possible action of A₁Cl on the fungal cell wall, sorbitol was used as an osmotic protector. In this study, the MIC values of A₁Cl in the presence of sorbitol remained the same in relation to the control (RPMI), suggesting that A₁Cl does not act at the level of the cell wall, as shown in Table III.

Table III. MIC values ($\mu\text{g/mL}$) of A_1Cl in the presence and absence of sorbitol (0.8M) for the ATCC 16013 and clinical LM 22 *Aspergillus flavus* strains.

Microorganisms	<i>A. flavus</i> ATCC 16013	<i>A. flavus</i> LM 22	<i>A. flavus</i> ATCC 16013	<i>A. flavus</i> LM 22
	RPMI (Control)		Sorbitol	
A_1Cl	128 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$	128 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$

3.4 Evaluation of the effect of A_1Cl on the fungal cell membrane.

Whether A_1Cl interacts with the fungal cell membrane through complex formation with ergosterol was also investigated. An increase in the MIC of A_1Cl was observed, from 128 to 1024, related to the ATCC 16013 strain and from 64 to 1024 related to *A. flavus* LM 22 strain in the presence of ergosterol, compared to the RPMI control, indicating a potential mechanism of action against the fungal cell membrane. When amphotericin B was used as a positive control, an increase in MIC from 2 $\mu\text{g/mL}$ to 32 $\mu\text{g/mL}$ was also observed in the two strains tested in the presence of ergosterol compared to the RPMI control, confirming a mechanism of action against the fungal cell membrane as already described in the literature (Table IV).

Table IV. MIC values of A_1Cl and Amphotericin B for the ATCC 16013 and clinical LM 22 *Aspergillus flavus* strains in the presence and absence of ergosterol (400 $\mu\text{g/mL}$).

Microorganisms	<i>A. flavus</i> ATCC 16013	<i>A. flavus</i> LM 22	<i>A. flavus</i> ATCC 16013	<i>A. flavus</i> LM 22
	RPMI (Control)		Ergosterol	
A_1Cl	128	64	1024	1024
Amphotericin B	2	2	32	32

3.5 Evaluation of the mechanism of action of A₁Cl by molecular docking

In this assay, the interaction was demonstrated through binding energy, obtained by binding the A₁Cl test product with the enzymes C-14- α -demethylase, squalene epoxidase, delta-14-sterol reductase, 1,3- β -glycan synthase, and thymidylate synthase, as shown in Table V.

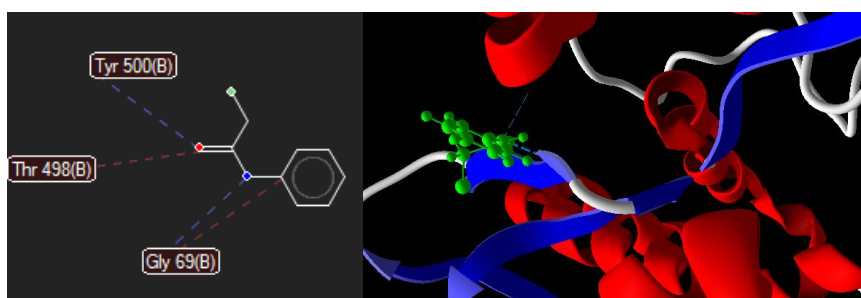
Table V. Binding energy values (Kcal/mol) for the synthetic amide A₁Cl with the molecular targets: C-14- α -demethylase (4UYL), 1,3- β -glycan synthase (3A58), squalene epoxidase (4MAH), delta-14-sterol reductase (4QUV), and thymidylate synthase (5UIV).

PDB ID	4UYL	3A58	4MAH	4QUV	5UIV
A ₁ Cl	-55.87	-58.82	-60.28	-61.92	-83.36

PDB ID: Protein code registered with Protein Data Bank (PDB)

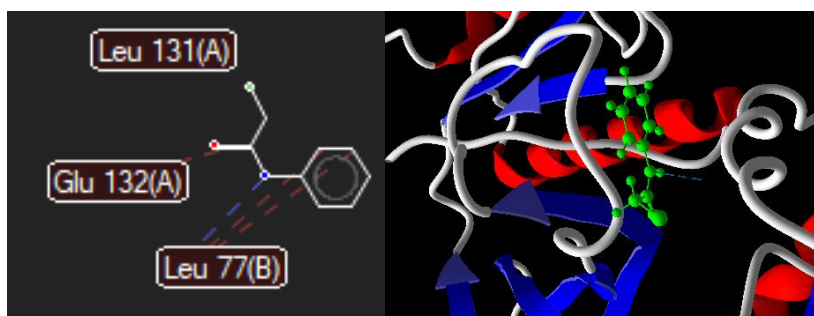
In the molecular docking assay, A₁Cl interacted with the enzyme C-14- α -demethylase through hydrogen bonds (blue dashes) between the enzyme's tyrosine amino acid and the carbonyl oxygen of A₁Cl, which also interacted through steric interactions (red dashes) with the enzyme's amino acid threonine, presenting a binding energy of -55.87 Kcal/mol. In addition, steric interactions of the enzyme's amino acid glycine with the carbon α 1 of A₁Cl, as well as hydrogen bonds with the aromatic ring, were observed, as shown in Figure 3.

Figura 3. Map of interactions of A₁Cl with the enzyme sterol-14- α -demethylase (PDB ID: 4UYL). The dashed lines in blue represent hydrogen interactions and the red ones represent steric interactions.



Regarding the interaction of A₁Cl with the enzyme 1,3- β -glucan synthase (3A58), steric interactions (red dashes) were observed between the carbonyl oxygen of A₁Cl and the amino acid glutamic acid132(A) of the enzyme, presenting an energy of -58.82 Kcal/mol. The amino acid leucine77(B) of the enzyme interacts with the aromatic ring of A₁Cl by means of steric bonds and with carbon α 1 through hydrogen bonds (blue dashes), as shown in Figure 4.

Figure 4. Map of interactions of A₁Cl with the enzyme 1,3- β -glucan synthase (PDB ID: 3A58). The dashed lines in blue represent hydrogen interactions and the red ones represent steric interactions.



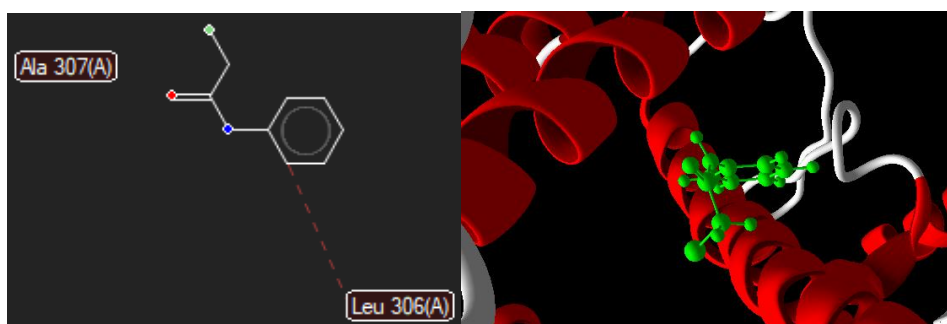
Squalene epoxidase interacted with A₁Cl by means of steric interactions (red dashes) with the amino acids asparagine194, proline173, proline193, phenylalanine192, and glutamine66 of the enzyme with the aromatic ring and the α 1 carbon of A₁Cl, presenting a binding energy of -60.28 Kcal/mol (Figure 5).

Figura 5. Map of A₁Cl interactions with the enzyme squalene epoxidase (PDB ID: 4MAH). The dashed lines in blue represent hydrogen interactions and the red ones represent steric interactions.



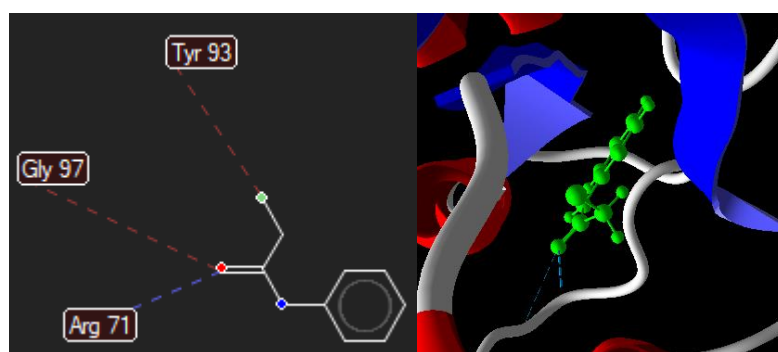
The enzyme Delta-14-sterol reductase interacted with A₁Cl by means of steric bonds (red dashes) with the aromatic ring of A₁Cl, presenting a binding energy of -61.92 through the amino acid leucine306A (Figure 6).

Figura 6. Map of A₁Cl interactions with the enzyme Δ -14-sterol reductase (PDB ID: 4QUV). The dashed lines in blue represent hydrogen interactions and the red ones represent steric interactions.



Regarding the interaction of the enzyme thymidylate synthase (5UIV) with A₁Cl, steric interactions (red dashes) were identified between glycine97 of the enzyme and the carbonyl oxygen of A₁Cl, as well as between the enzyme's tyrosine amino acid and the A₁Cl methyl, with a binding energy of -83.36 Kcal/mol. Hydrogen interactions (blue dashed lines) were also observed between amino acid arginine 71 of the enzyme and the oxygen in the A₁Cl carbonyl (Figure 7).

Figura 7. Map of interactions of A₁Cl with the enzyme thymidylate synthase (PDB ID: 4QUV). The dashed lines in blue represent hydrogen interactions and the red ones represent steric interactions.



3.6. Assessment of A₁Cl – standard antifungal associations

The effect of A₁Cl in association with amphotericin B and voriconazole (Table VI) against *A. flavus* strains was investigated. It was observed that regardless of *A. flavus* strain, the association of A₁Cl with amphotericin B or with voriconazole resulted in the same antagonistic response pattern, considering that the FICI was > 4.0; see Odds et al. (2003).

Table VI. Fractional Inhibitory Concentration Index (FICI) of the association of A₁Cl with amphotericin B or voriconazole against *Aspergillus flavus* strains.

Association	<i>A. flavus</i>	<i>A. flavus</i>
	ATCC 16013	LM 22
FIC^A	4	8
(A₁Cl)		
FIC^B	0.031	2
(Amphotericin B)		
FICI(A₁Cl+ Amphotericin B)	4.031	10
Type of interaction (A₁Cl + Amphotericin B)	Antagonism	Antagonism
FIC^B	0.031	4
(Voriconazole)		
FICI (A₁Cl + Voriconazole)	8.031	12
Type of interaction (A₁Cl + Voriconazole)	Antagonism	Antagonism

4. Discussion

The emergence of resistant *Aspergillus* strains, in addition to causing higher mortality rates, indicates the need for the identification of new compounds with promising antifungal activity (Sanglard 2016). Since A₁Cl presented promising antifungal activity in screening studies performed by our research group, this aroused our interest in investigating the molecule in depth and in verifying its antifungal potential.

MIC testing was performed for the A₁Cl compound, and a variation from 16 to 256 µg/mL was observed for *A. flavus* strain (Table II), Fifty percent of the *A. flavus* strains (Table II) which was considered in this case as the ideal concentration of a substance (Holetz et al. 2002). The strong antimicrobial activity of A₁Cl has also been demonstrated by Katke et al. (2011) against strains of *Escherichia coli* and *Pseudomonas aeruginosa* and by Aschale (2012) against strains of *A. niger*, demonstrating that substituted chloroacetamides present greater antifungal activity than unsubstituted chloroacetamides, regardless of the positions or nature of the substituent.

In the A₁Cl and MFC assays against *A. flavus* strains, a range of 32 to 512 µg/mL was observed (Table II). To explain the nature of the antimicrobial effect with regard to growth inhibition or death, Hazen (1998) established that a substance is to be considered fungicidal when the MFC/MIC ratio is ≤ 4 , and when the ratio is > 4 , the compound is to be considered fungistatic. In this study, A₁Cl was assayed as a fungicide for all strains tested, with MFC/MIC ratios ranging from 1 to 4 (Table II). The fungicidal activity of this compound is promising, since treatment of diseases that affect immunocompromised individuals, such as invasive aspergillosis, one of the prominent causes of death in these patients, requires drugs with fungicidal potential (Chowdhary et al. 2015).

It is well known that there are great difficulties involved in correlating and interpreting microdilution test results together with clinical results, and the scientific literature has not yet defined breakpoints for assessing the susceptibility or resistance of clinical species of *Aspergillus* genus. However, Borman et al. (2017) presented a study with the objective of facilitating the development of cutoff points for several fungi, including *Aspergillus*. According to these authors, strains that present MIC values above 2 µg/mL are considered resistant to amphotericin B. The authors

also observed that 13.7% of the tested *A. flavus* strains were thus considered resistant.

Reichert-Lima et al. (2018), assessing the susceptibility of *Aspergillus* strains to antifungals used in the clinic, found that of 27 strains of *A. flavus* tested, (81%) 22 obtained MICs of ≥ 2 $\mu\text{g/mL}$, considered high MIC values. Garcia-Martos et al. (2004) also evaluated the susceptibility of *Aspergillus* species against the antifungals amphotericin B, itraconazole, and voriconazole and observed that *A. flavus* presents little sensitivity to amphotericin B.

In this study, 8 of 12 *A. flavus* strains tested presented MICs of > 2 $\mu\text{g/mL}$ (Table II) and are considered resistant to amphotericin B. The literature reports that the sensitivity profile of *A. flavus* presents elevated MIC values for amphotericin B and that values above 2 $\mu\text{g/mL}$ are associated with failures in treating invasive aspergillosis (Hadrach et al. 2012; Gonçalves et al. 2013; Heo et al. 2015). The mechanisms that confer resistance to amphotericin B are still not well understood; however, according to Chamilos & Kontoyiannis (2005), two mechanisms may be involved: decreasing ergosterol in the cell membrane (target of the drug) due to mutations in its biosynthetic pathway and an increase in the production of catalases, which provide resistance to fungal cells against oxidative stress caused by the drug.

Despite being used to treat many fungal diseases, such as aspergillosis, the appearance of nephrotoxicity (caused by amphotericin B) is common, even in lipid formulations. The mechanism by which this happens is mediated at the cellular level through apoptosis (Varlam et al. 2001). The effect is common in approximately 65% of patients and can cause significant loss of renal function, even requiring dialysis (Falci & Pasqualotto 2015). The search for a substance with promising antifungal activity and low toxicity as a substitute for amphotericin B is critical.

Voriconazole presented MICs varying between 0.5 and 2 $\mu\text{g/mL}$ for strains of *A. flavus* (Table II). According to Borman et al. (2017), strains that present MICs above 2 $\mu\text{g/mL}$ are resistant to voriconazole. Wang et al. (2018), in a study verifying resistance patterns, observed that all 23 strains of *A. flavus* tested were considered sensitive to voriconazole, with MICs ranging between 0.5 and 1 $\mu\text{g/mL}$. Based on these studies, all of *A. flavus* strains used in this study are sensitive to voriconazole.

In view of the aspergillosis resistance to current antifungal therapies, especially amphotericin B, and of the need to seek new therapeutic strategies, the A₁CI molecule is promising for presenting antifungal effects. Knowing that in the field

of organic synthesis, a molecule can undergo changes in its chemical structure to improve its activity and taking into account that A₁Cl is a synthetic molecule, modifications can be made to make it more potent.

With regard to the development of new drugs with antifungal potential, a major challenge due to the similarity between fungal cells and human cells is the difficulty of selecting targets. The targets for new antifungals must be exclusive to the fungus or sufficiently different from their human cell analogs to avoid adverse pharmacodynamic effects (Martinez-Rossi et al. 2008; Guerra et al. 2015). Thus, to elucidate A₁Cl's mechanism of action, tests were performed on the cell wall and membrane of the species studied.

Ergosterol is the main component of sterol present in the fungal plasma membrane and it plays the same role in fungal membranes as cholesterol plays in mammalian cell membranes (Bowman & Free 2006), exhibiting qualitatively similar properties to cholesterol.

If A₁Cl activity is promoted through its binding to ergosterol of the fungal membrane, exogenous ergosterol, when added to the culture medium, will prevent this binding. Thus, an increase in MIC for A₁Cl in the presence of exogenous ergosterol will occur since only a higher concentration of the product in the medium will guarantee interactions with fungal membrane ergosterol (Lunde & Kubo 2000; Escarlante et al. 2008).

In this study, an increase in the MIC of A₁Cl was observed in the presence of exogenous ergosterol for the two strains tested compared to the control (RPMI) (Table IV). As a positive control, amphotericin B, which acts at the level of the fungal cell membrane, was used. An increase in MIC was observed in relation to the control (RPMI) (Table IV), which was expected, considering that its mechanism of action is already well elucidated and involves binding with ergosterol, the consequential formation of pores in the membrane, and leakage of the intracellular content, culminating in death to the fungus (Filippin & Souza 2006). Thus, it is suggested that A₁Cl exerts its mechanism of action in *A. flavus* interfering with cell functions involving the membrane ergosterol.

Computational tools, such as docking, have been reported in the literature as essential for the development of computer-aided drugs (Brooijmans & Kuntz 2003).

In the dockings performed in this work, the interactions of A₁Cl with important enzymes responsible for the synthesis of the fungal cell wall and membrane

components, as well as for nucleic acid formation, were analyzed. The interactions were demonstrated through the binding energies obtained by binding the test product to C-14- α -demethylase, squalene epoxidase, delta-14-sterol reductase, 1,3- β -glycan synthase and thymidylate synthase.

C-14- α -demethylase (4UYL) is a microsomal enzyme associated with cytochrome P-450 (CYP), encoded by the *ERG11* gene, which is involved in the final stages of ergosterol biosynthesis, the main sterol responsible for regulating fluidity and other functions of the fungal plasma membrane (Yang et al. 2015). The enzyme 1,3- β -glycan synthase (3A58) is responsible for the synthesis of β -glycans, an important component of the fungal cell wall (Cortés & Russi 2011). Squalene epoxidase (4 MAH) is a monooxygenase that participates in ergosterol biosynthesis together with delta-14-sterol reductase and is responsible for catalyzing the conversion of squalene to 2,3-oxidosqualene (Ruckenstuhl et al. 2007). Inhibition of this enzyme promotes toxicity, affecting the structure and function of the cell membrane and leading to impairment of the fungal cell's selective permeability and consequent cell lysis (Sueth-Santiago et al. 2015). The enzyme thymidylate synthase (5UIV) is responsible for DNA synthesis and protein synthesis of the fungus, and of all of the enzymes studied, thymidylate synthase presented the lowest binding energy with A₁Cl (-83.36 Kcal/mol); the lower the binding energy, the greater the ligand's affinity for the receptor (Qidwai 2017) and thus a greater affinity for thymidylate synthase (Table V). It is possible that one of the mechanisms of action of A₁Cl is interference in DNA synthesis through inhibition of this enzyme, promoting the death of the fungus.

This study elucidating the mechanism of action of A₁Cl is unprecedented, and its mechanisms of action against strains of *A. flavus* have been demonstrated in the literature for the first time.

In addition to isolated antifungal activity, many compounds can alter the effects of the antifungals on the market. In this study, for the first time, it was shown through the checkerboard trial that A₁Cl interacts antagonistically with the two antifungals used (Table VI).

In clinical practice, drug interactions are common, the definition of which is the association of two or more drugs, such that the safety or efficacy of one drug is significantly altered in the presence of the other (Gaddis et al. 2002). In this study, it was observed that one of the mechanisms of action of A₁Cl occurs through binding to

ergosterol present in the fungal cell membrane, probably leading to the formation of pores in the membrane and extravasation of cellular content, similar to amphotericin B. If associated with voriconazole, which acts by inhibiting C-14- α -demethylase and decreasing ergosterol biosynthesis, the interaction with ergosterol promoted by A₁Cl would be impaired due to the deficiency of ergosterol synthesis promoted by voriconazole, thus compromising the antifungal activity of A₁Cl, an antagonistic association (Fillipin & Souza 2006).

The association A₁Cl and amphotericin B should be better studied and detailed, since, in the trial presented, the result was antagonism. Amphotericin B is chemically incompatible with some drugs in the clinic, such as sodium chloride 0.9%, meropenem, midazolan, calcium gluconate and vancomycin (Santos et al. 2011) generating the presence of precipitates, turbidity, changes in color and chemical reactions, and in association with some antifungals such as itraconazole, terbinafine and voriconazole, has an antagonistic effect when studied in strains of the genus *Aspergillus* (Vasquez 2008). It is possible that there was chemical incompatibility between the two products, where a chemical reaction could have occurred, forming a substance different from the originals, which may not have the same pharmacological action as the initials, justifying the effect presented.

Given the above, it can be concluded that the synthetic amide 2-chloro-*N*-phenylacetamide (A₁Cl) presents antifungal activity against *A. flavus* being fungicidal. The results also suggest a mechanism of action related to the presence of ergosterol in the cell membrane and possibly action at the DNA level, yet further studies to prove this hypothesis will be required, yet further studies to prove this hypothesis will be required. This study's findings strongly suggest that 2-chloro-*N*-phenylacetamide presents antifungal potential against *A. flavus*.

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Author contributions

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Maria Barbosa-Filho, Luciana Scotti, Edeltrudes de Oliveira Lima, and Ricardo Dias de Castro are responsible for writing the article, and the individual contributions of each author to the article follow. Elba dos Santos Ferreira participated in the design of the project, collection and analysis of data, writing of the article and critical review of the intellectual content. Laisa Vilar Cordeiro and Daniele de Figueredo Silva participated in data collection, data analysis and work review. Luciana Scotti participated in data collection and article review. Helivaldo Diógenes da Silva Souza, José Maria Barbosa-Filho and Petronio Filgueiras Athayde-Filho participated in the synthesis of the synthetic amide, data analysis and article review. Edeltrudes de Oliveira Lima and Ricardo Dias de Castro directed all stages of the work and participated in the review and writing of the project and work, including final approval of the manuscript.

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5.2

Evaluation of antifungal activity and mechanisms of action of the synthetic amide 2-chloro-N-phenylacetamide against strains of *Aspergillus niger*

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Evaluation of antifungal activity and mechanisms of action of the synthetic amide 2-chloro-N-phenylacetamide against strains of *Aspergillus niger*

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Abstract

Aspergillus niger is a filamentous fungus of the *Aspergillus* genus that causes infections such as otitis and pulmonary aspergillosis in immunocompromised individuals. Treatment of aspergillosis involves either voriconazole (first choice) or amphotericin B. However due to an accelerating increase in fungal resistance to these therapies, the search for new compounds with antifungal activity has intensified. The objective of this study was to verify the antifungal activity and the mechanism of action of the synthetic amide 2-chloro-N-phenylacetamide against strains of *Aspergillus niger*. In this sense, the minimum inhibitory concentration, minimum fungicidal concentration, conidia germination, associations with antifungals and mechanisms of action on the wall and fungal cell membrane were evaluated. 2-chloro-N-phenylacetamide presented antifungal activity against varied strains of *Aspergillus niger* with minimum inhibitory concentrations between 32 and 256 µg/mL and minimum fungicidal concentrations between 64 and 1024 µg/mL. The minimum inhibitory concentration of 2-chloro-N-phenylacetamide also inhibited the germination

of conidia. However when associated with amphotericin B or voriconazole, 2-chloro-*N*-phenylacetamide presented antagonistic effects. Interaction with ergosterol in the fungal plasma membrane is the probable mechanism of action. We conclude that the synthetic amide 2-chloro-*N*-phenylacetamide presents promising antifungal potential.

Keywords: 2-chloro-*N*-phenylacetamide, *Aspergillus niger*, Resistance, Ergosterol, Antifungal activity, Aspergilosis.

1. Introduction

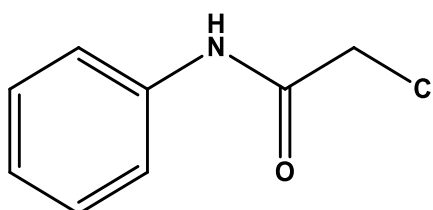
Aspergillus niger is a species of filamentous fungus belonging to the genus *Aspergillus*, and commonly used in the food industry. When the ability of the species to produce citric acid was observed for the first time in 1919, it acquired importance. This ability is being exploited industrially today in the food, cosmetic, and pharmaceutical industries, among others (Schuster et al., 2002). Its industrial importance has grown, and since 1960 it has been used to produce enzymes important for processing fruits, in baking, in starch processing, and in other foods (Cairns et al., 2018).

A. niger is a non-pathogenic fungus widely distributed in nature. However, in immunocompromised individuals, it presents opportunistically, and can cause serious local and systemic infections (Schuster et al., 2002), such as external and internal otitis (Vennewald et al., 2003; Mishra et al., 2004) or pulmonary aspergillosis (Korzeniowska et al., 1995), the most serious of *Aspergillus* infections.

Aspergillosis is treated with azole derivatives, such as voriconazole, the drug of first choice, and polyenes such as amphotericin B, used as rescue therapy or when azole derivatives cannot be administered (Bellmann, 2013; Patterson et al., 2016). There are reports in the literature of *A. niger* strains resistant to azole derivatives (Hashimoto et al., 2017). Due to nephrotoxicity (even in lipid formulations) the use of amphotericin B is limited (Varlam et al., 2001) since it can cause significant loss of renal function in approximately 65% of patients who use it (Falci and Pasqualotto, 2015). Considering the limitations of the current treatments and the recent emergence of fungal resistance, the search for new therapeutic alternatives with antifungal potential to treat aspergillosis gains new importance.

Studies carried out in our research laboratory have shown that the synthetic amide 2-chloro-*N*-phenylacetamide, known as A₁Cl (Figure 1), presents promising antifungal potential against strains of *A. flavus* (data not yet published), and Aschale et al., (2021), in a screening study, demonstrated that A₁Cl presents promising antifungal activity against strains of *A. niger*. Thus, our objective is to verify the antifungal activity and mechanism of action of the synthetic amide 2-chloro-*N*-phenylacetamide against strains of *Aspergillus niger*.

Figure 1: Chemical structure of 2-chloro-*N*-phenylacetamide (synthetic amide).



2. Materials and methods

2.1 Microorganisms

For the antifungal activity evaluations, one ATCC strain (American Type Culture Collection) and four clinical strains of the species *A. niger* (Table 1) were obtained from the Mycotech Lab collection of the Research Laboratory for Antibacterial and Antifungal Activity of Natural Products and/or Bioactive Synthetics, UFPB. Strains of *A. niger* were grown on potato dextrose agar for 7 days at 35°C. For the preparation of the inoculum, the samples were taken from grown cultures, suspended in sterile 0.85% saline, and transferred to sterile tubes. The inoculum was standardized according to the 0.5 concentration of the McFarland scale (10^6 CFU/mL), and the concentration was confirmed by counting conidia using a Neubauer chamber (CSLI, 2008; Guerra et al., 2015).

Table 1. Strains of *A. niger* obtained from the UFPB - Mycotech Lab - Laboratory for Research on Antibacterial and Antifungal Activity of Natural and/or Synthetic Bioactive Products collection.

Strains <i>A. niger</i>	Origin
ATCC 6275	Standard
LM 22	Clinic - Left ear
LM 23	Clinic - Nail (foot)
LM 29	Clinic - ears
LM 66	Clinic - ears

2.2 Drugs and Reagents

The tested product was the synthetic amide 2-chloro-*N*-phenylacetamide, obtained by synthesis (Huang, 2016). The antifungals voriconazole and amphotericin B, and the ergosterol were obtained from Sigma-Aldrich® (São Paulo, São Paulo, Brazil). The sorbitol was obtained from INLAB® (São Paulo, Brazil). Potato dextrose agar was obtained from Difco Lab. (USA). All of the substances were dissolved in DMSO (dimethylsulfoxide – Sigma-Aldrich®, São Paulo, São Paulo, Brazil). and solubilized in sterile distilled water to obtain solutions at a concentration of 1024 µg/mL. For evaluation of the antifungal activity, RPMI 1640 with L-glutamine was used (Sigma-Aldrich®, São Paulo, SP, Brazil), and prepared according to the manufacturer's instructions.

2.3.3 Determination of *in vitro* antifungal activity

2.3.1 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A₁Cl against strains of *A. niger*.

The MIC determination of A₁Cl against strains of *A. niger* was performed using the broth microdilution technique recommended by the Clinical and Laboratory Standards Institute (CLSI, 2008). Initially, 100 µL of double-concentrated RPMI was added to the wells, followed by 100 µL of each product suspension (A₁Cl, voriconazole, and amphotericin B) at an initial concentration of 1024 µg/mL in the first row of the plate, and then the range of concentrations from 1024 to 4 µg/mL were obtained by means of serial dilution. Finally, 10 µL of each of the tested microorganism suspensions was added. Negative controls (without drugs) were used

to assess the viability of the strains and the sterility of the medium, as well as the sensitivity controls (to DMSO - Dimethylsulfoxide). After 2 days of incubation, visual plate reading was performed, and the MIC was considered the lowest concentration inhibiting 100% of the strain growth (CSLI, 2008). The results are expressed as the mode the MICs obtained in three tests.

For the determination of the MFC, 100 μ L of RPMI was added to the 96-well sterile plate, and 10 μ L samples were removed from wells where there was no fungal growth on MIC plates and added to the new plate containing the culture medium (RPMI 1640, Sigma-Aldrich®, São Paulo, SP, Brazil). The plates were incubated at 35°C for 2 days. The fungicidal activity was considered the lowest concentration of the product capable of inhibiting visible growth on the plate (Ncube et al., 2007; CSLI, 2008).

2.3.2. Evaluation of A₁Cl inhibition of conidial germination in *A. niger* strains

The strains ATCC 6275 and LM 66 were used for this test. After reading the MIC plates, 10 μ L of the MIC, MIC $\times$ 2 and MIC $\times$ 4 concentrations were removed from each product tested and seeded on sterile slides. A drop of lactophenol blue dye was then added to each preparation, homogenized, and covered with a coverslip. Conidia counting was performed under an optical microscope in 5 different fields to obtain an arithmetic mean. The results were expressed in graphs as the percentage of conidial germination as a function of the concentrations of the tested products (Adapted from Trajano et al., 2013).

2.4. Evaluation of the mechanism of action of A₁Cl against clinical strains of *A. niger*

2.4.1 Cell wall assay

To assess the possible mechanisms of action involving *A. niger* fungal cell wall, the MIC of A₁Cl was determined in the presence and absence of sorbitol (INLAB®, São Paulo, Brazil) (0.8 M) in the medium using the microdilution method and 96-well “U” plates (Alamar, Diadema, SP, Brazil). The plates were aseptically sealed and incubated at 35°C. Readings were taken at 2 days. A microorganism check and a sterility check were performed. The test was performed in triplicate and expressed as the mode of the results (Escalante et al., 2008).

2.4.2 Cell membrane assay

To assess the possible mechanisms of action involving *A. niger* fungal cell membrane, the MIC of A₁CI was determined in the presence and absence of ergosterol (400 µg/mL) (Sigma-Aldrich®) in the medium using the microdilution method with 96-well “U” plates (Alamar, Diadema, SP, Brazil). A microorganism check and a sterility check were performed. Amphotericin B was used as a positive control. For reading, the plates were sealed and incubated at 35°C for 2 days. The test was performed in triplicate and expressed as the mode of the results (Escalante et al., 2008).

2.5 Assessment of A₁CI with standard antifungal associations

The effects of the associations of the products with standard antifungals were determined using the microdilution - checkerboard technique for derivation of the FICI (Fractional Inhibitory Concentration Index). Solutions of the tested products were used in concentrations determined from their respective MICs. Initially, 100 µL of RPMI was added to the wells of a sterile 96-well microplate, and then 50 µL of each tested product was added at different concentrations ($MIC \div 8$, $MIC \div 4$, $MIC \div 2$, MIC, $MIC \times 2$, $MIC \times 4$, and $MIC \times 8$) in the vertical (standard antifungal) and horizontal (test product) directions of the microplate. Finally, 20 µL of the fungal suspension was added. The assay was performed in triplicate, with the microplates incubated at 35°C for 2 days (Dutta et al., 2004).

For calculation of the FICI (Fractional Inhibitory Concentration Index), the method described by Bonapace et al., (2002) was used, and the lowest MIC was used (where there was no fungal growth) for the calculation performed by adding the $FIC^A + FIC^B$, where A represents the test product and B the standard antifungal. The FICI, in turn, is calculated using the combined FIC^A/FIC^A ratio alone, while the FIC^B is calculated using the formula $FIC^B = \text{combined } FIC^B/FIC^B$ alone. The interpretation of this index was performed as follows: synergism = ($FICI \leq 0.5$), antagonism = ($FICI > 4.0$), and indifference = ($0.5 < FICI \leq 4$) (Odds et al., 2003).

2.6 Statistical analysis

The results obtained from the MIC, MFC, cell wall, and cell membrane assays are expressed as the mode. The results of the conidial germination

percentage and cytotoxic profile analysis are expressed as the mean $\pm$ standard error of the mean (S.E.M.), which were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni post-test to compare more than two groups. The null hypothesis was rejected when $p < 0.05$, and the GraphPad Prism® program version 5.01 (GraphPad Software Inc., San Diego CA, U.S.A.) was used.

3.0 Results

3.1 Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of A₁CI against strains of *A. niger*.

A₁CI presented a minimum inhibitory concentrations (MIC) varying from 32 to 256 $\mu\text{g/mL}$ (Table 2), inhibiting visible growth of the *A. niger* strains used in this study. For minimum fungicidal concentration (MFC), A₁CI presented concentrations varying from 64 to 1024 $\mu\text{g/mL}$, as shown in table II.

As for the susceptibility of *A. niger* strains to amphotericin B, differing MICs, ranging from 0.125 to 16 $\mu\text{g/mL}$ were observed (Table 2). For voriconazole, it was observed that the A₁CI MICs ranged from 1 to 32 $\mu\text{g/mL}$, with three strains presenting an MIC > 2 , and MFCs ranging from 1 to 128 $\mu\text{g/mL}$ (Table II).

Table II. Mean MIC ($\mu\text{g/mL}$) and MFC ($\mu\text{g/mL}$) values for A₁Cl, Amphotericin B, and Voriconazole against strains of *A. niger*.

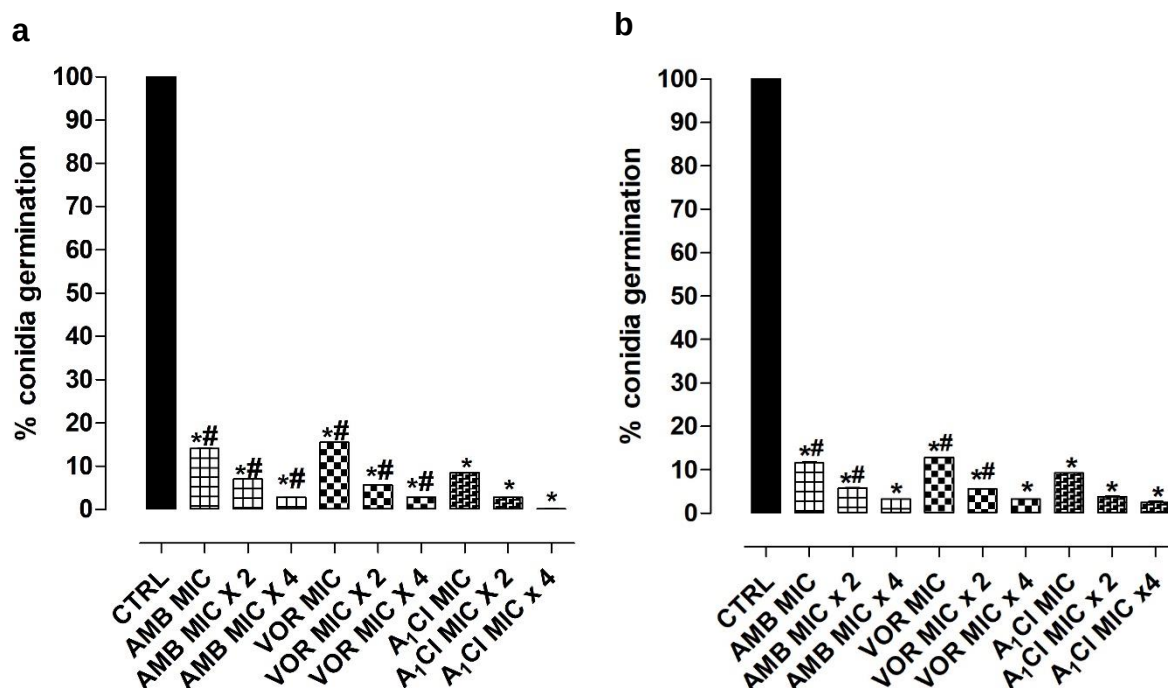
	A ₁ Cl			Amphotericin B			Voriconazole		
<i>Aspergillus niger</i>	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
ATCC 6275	128	512	4	2	4	2	2	2	1
LM 22	64	64	1	0.5	0.5	1	4	4	1
LM 23	32	128	4	0.12	0.12	1	1	1	1
				5	5				
LM 66	256	1024	4	4	16	4	32	128	4
LM 86	256	1024	4	16	64	4	32	128	4

As for the susceptibility of *A. niger* to amphotericin B, each strain presented a different MIC, which ranged from 0.125 to 16 $\mu\text{g/mL}$ (Table 2). For voriconazole, it was observed that MICs ranged from 1 to 32 $\mu\text{g/mL}$, with three strains showing MICs > 2 and MFCs ranging from 1 to 128 $\mu\text{g/mL}$ (Table II).

3.2 Evaluation of the effect of A₁Cl against *A. niger* strains - conidia germination

For these evaluations, the *A. niger* ATCC 6275 standard and LM 66 clinical strains were used. It was observed that the synthetic amide A₁Cl inhibited ATCC 6275 conidia germination in all three concentrations used, superior to results found for either voriconazole or amphotericin B (Figure 2A). For the LM 66 strain, A₁Cl also presented better results than amphotericin B and voriconazole (in two of the three concentrations used) in inhibiting conidia germination (Figure 2B).

Figure 2. Conidia germination percentages for *A. niger* strains ATCC 6275 (a) and LM 66 (b), against amphotericin B (AMB) (■), voriconazole (VOR) (▣), and A₁CI (▤).



The columns and vertical bars respectively represent the mean and S.E.M., ($n = 3$). One-way ANOVA followed by Bonferroni's posttest. * $p < 0.05$ (compared to the negative control), # $p < 0.05$ (compared to A₁CI). S.E.M. = standard error of the mean, CTRL = Control, MIC= Minimal Inhibitory Concentration, AMB = Amphotericin B, VOR = Voriconazole, A₁CI = 2-chloro-*N*-phenylacetamide

3.3 Evaluating A₁CI and standard antifungals in associations against *A. niger* strains using the checkerboard method

In this assay, for both the *A. niger* ATCC 6275 and LM 66 strains, growth was observed despite every A₁CI/amphotericin or A₁CI/voriconazole combination (Table 3), characterizing, according to Odds et al. (2003), an antagonistic association between the antifungals and the synthetic amide. This, considering that the FICI was > 4 .

Table III. Fractional Inhibitory Concentration Index (FICI) of the association of A₁Cl with amphotericin B against *A. niger* strains.

Strains	FIC ^A (A ₁ Cl)	FIC ^B (Amphotericin B)	FICI	Type of interaction
<i>A. niger</i> ATCC 6275	8	8	16	Antagonism
<i>A. niger</i> LM 66	8	8	16	Antagonism

FIC = Fractional Inhibitory Concentration, FICI = Fractional Inhibitory Concentration Index.

Table IV. Fractional Inhibitory Concentration Index (FICI) of the association of A₁Cl with voriconazole against *A. niger* strains.

Strains	FIC ^A (A ₁ Cl)	FIC ^B (Voriconazole)	FICI	Type of interaction
<i>A. niger</i> ATCC 6275	8	8	16	Antagonism
<i>A. niger</i> LM 66	8	8	16	Antagonism

FIC = Fractional Inhibitory Concentration, FICI = Fractional Inhibitory Concentration Index.

3.4 Evaluation of A₁Cl effects on the fungal cell wall.

To verify potential A₁Cl activity on the fungal cell wall, sorbitol was used as an osmotic protector. In this study, the MIC values of A₁Cl in the presence of sorbitol remained the same relative to the control (RPMI), suggesting that A₁Cl does not act at the level of the cell wall, as shown in Table V.

Table V. MIC values ($\mu\text{g/mL}$) for A_1Cl in the presence and absence of sorbitol (0.8M) for the *Aspergillus niger* ATCC 6275 and LM 66 clinical strains.

Microorganisms	<i>A. niger</i> ATCC 6275	<i>A. niger</i> LM 66	<i>A. niger</i> ATCC 6275	<i>A. niger</i> LM 66
Drugs	RPMI (Control)		Sorbitol	
A_1Cl	128 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$	128 $\mu\text{g/mL}$	64 $\mu\text{g/mL}$

3.5. Evaluation of the effect of A_1Cl on the fungal cell membrane.

Whether A_1Cl interacts with the fungal cell membrane through the formation of a complex with ergosterol was investigated. An MIC increase in the presence of ergosterol, compared to the RPMI control for A_1Cl was observed, from 128 to 1024 $\mu\text{g/mL}$, against the ATCC 6275 strain, and from 256 to 1024 $\mu\text{g/mL}$ against the LM 66 strain. This indicates a potential mechanism of action against the fungal cell membrane. When amphotericin B was used as a positive control, an MIC increase from 2 $\mu\text{g/mL}$ to 32 $\mu\text{g/mL}$ for the ATCC 6275 strain was observed. In relation to the LM 66 strain, we observed an MIC increase from 4 $\mu\text{g/mL}$ to 64 $\mu\text{g/mL}$, (RPMI control), confirming the mechanism of action (against the fungal cell membrane) already described in the literature (Table VI).

Table VI. MIC values for A_1Cl and Amphotericin B against *Aspergillus niger* ATCC 6275 and clinical LM 66 in the presence and absence of ergosterol (400 $\mu\text{g/mL}$).

Microorganisms	<i>A. niger</i> ATCC 6275	<i>A. niger</i> LM 66	<i>A. niger</i> ATCC 6275	<i>A. niger</i> LM 66
Drugs	RPMI (Control)		Ergosterol	
A_1Cl	128	256	1024	1024
Amphotericin B	2	4	32	64

4.0 Discussion

As the number of *Aspergillus* isolates presenting resistance to current therapies increases (promoting higher mortality rates), it becomes necessary to search for new therapeutic alternatives with antifungal activity (Hagiwara et al., 2016). In screening studies, A₁Cl presents demonstrated promising antifungal activity (Aschale, 2012), which has been confirmed in our research, and therefore we decided to continue our A₁Cl antifungal studies on its activity against *A. niger*.

A₁Cl MIC testing was performed using *A. niger* strains, and a range from 32 to 256 µg/mL was observed (Table II), the MIC range was considered ideal for 60% of the strains tested (Holetz et al., 2002).

Our results are similar to those found by Aschale (2012), who, using the disk diffusion method, analyzed the antifungal activity of a series of chloroacetamides, and found that A₁Cl demonstrated strong antifungal activity against strains of *A. niger*, and also demonstrated that substituted chloroacetamides present greater antifungal activity than unsubstituted chloroacetamides, regardless of the position or nature of the substituent.

The MFCs obtained for A₁Cl ranged from 64 to 1024 µg/mL. To explain the nature of the antimicrobial effect in relation to growth or death inhibition, Hazen (1998) established that a substance should be considered fungicidal when the MFC/MIC ratio is ≤ 4 , and when the ratio is > 4 , the compound must be considered fungistatic. In this study, A₁Cl was considered fungicidal for all strains tested, with MFC/MIC ratios ranging from 1 to 4 (Table II). This fungicidal activity is promising, since to avoid death in immunocompromised patients presenting invasive aspergillosis (or others), requires drugs with a fungicidal potential (Chowdhary et al., 2015).

With regard to the susceptibility of *A. niger* strains to amphotericin B, the MIC variation observed was from 0.125 to 16 µg/mL (Table II). Yet, two of the five strains presented MICs of greater than 2 µg/mL. According to Borman et al., (2017), these strains are resistant to amphotericin B.

A. niger resistance to polyenes is uncommon in the literature. To explain this resistance to polyenes, some authors suggest reductions in ergosterol concentrations in the fungal lipid membrane, making it difficult for the polyenes to interact (Blum et al., 2013).

Another important mechanism evidenced by Jukic et al., 2017, is greater superoxide dismutase activity, (the enzyme helps decrease free radical scavenging superoxide anions). Increased expression of the heat shock protein Hsp70 may also contribute to increased amphotericin B resistance since the use of Hsp70 inhibitors significantly increases the effectiveness of amphotericin B against *Aspergillus* isolates (Cowen and Lindquist 2005; Blatzer et al., 2015; Tiwari et al., 2015). It is thus possible that some of these mechanisms may be affecting the *A. niger* resistance to amphotericin B described in this work.

In relation to voriconazole, it was observed that three of the 5 tested strains presented MICs equal to or above 2 µg/mL, the MICs ranged from 1 to 32 µg/mL (Table II). Thus, according to the criteria of Borman et al., (2017), 60% of the *A. niger* strains used in this study were resistant to voriconazole.

Resistance to azoles is increasingly common for *Aspergillus*, being reported all around the world. Hashimoto et al., (2017) evaluating the susceptibility of *A. niger* to itraconazole and voriconazole, observed that 55% of *A. niger* strains tested were resistant. This resistance may be caused by both mutation or overexpression of the *cyp* gene 51 A, which encodes 14- α lanosterol demethylase, an enzyme that is involved in the ergosterol biosynthesis and found in *A. niger* cytochrome P450.

Such mutations may be associated with prolonged azole antifungal treatment (where the fungus may quickly adapt in the presence of the compound) developing resistance (Arendrup et al., 2010), or to environmental exposure (fungicides and azole herbicides used in crops) (Verweij et al., 2009). Other mechanisms of resistance to 14- α lanosterol demethylase inhibitors have also been described, such as the increased efflux pump activity, and biofilm development (Chowdhary et al., 2017).

Aspergillosis is a fungal disease, whose main form of transmission is conidia inhalation; these penetrate the organism through the respiratory system, germinate and originate hyphae in the lungs, and can also invade other tissues (Raja and Singh, 2006). Knowing that the germination mechanism of the conidia is important for the asexual reproduction of the fungus, substances that act by reducing germination, would affect the reproduction of the fungus as well as the spread of the disease.

We therefore evaluated the ability of A₁Cl to decrease *A. niger* conidia germination, and it was observed to reduce germination in all tested concentrations, presenting favorable MIC results, and for some strains (Figure 2), it was more

effective than either amphotericin B or voriconazole (clinical antifungals). The effectiveness of A₁Cl in reducing conidial germination, and affecting both the reproduction and spread of fungal spores was demonstrated and is promising for future clinical use.

Combining antimicrobial substances can provide treatment alternatives for infections which do not respond to conventional therapies, allowing greater effectiveness in the treatment, lower drug concentrations, and reduce adverse effects (Drago et al., 2007; Mitsugui et al., 2008). In this study, it was demonstrated for the first time in *A. niger* strains that A₁Cl interacts antagonically with voriconazole and amphotericin B (Table III and IV), shown by FICI > 4.0 by Odds et al., (2003).

Drug interactions, which are defined as associations between two or more drugs where one interferes with the effectiveness of the other, are common in clinical practice (Gaddis et al., 2002). In this study, it was seen that A₁Cl can act by binding to ergosterol in *A. niger* strains, and it is already well described in the literature that voriconazole acts by inhibiting C-14- α -demethylase, thus decreasing the biosynthesis of ergosterol. Thus, an association between voriconazole and A₁Cl would impair interaction with ergosterol, due to the depletion promoted by voriconazole, thus compromising the antifungal activity of A₁Cl (Fillipin and Souza 2006).

In view of the antagonistic result found in our association assay, the A₁Cl/amphotericin B association needs more in-depth study. Amphotericin B is known to be clinically incompatible with certain drugs, including meropenem and vancomycin. These incompatibilities can generate precipitates, turbidity, and color change, as well as chemical reactions (Santos et al., 2011).

When amphotericin B is associated with other antifungals such as itraconazole, voriconazole, and terbinafine in strains of the genus *Aspergillus*, the association results are antagonistic (Vasquez, 2008). Thus, the possibility of chemical incompatibility between amphotericin B and A₁Cl, where through chemical reaction the formation of a product different from the originals may have occurred with some loss or decrease in pharmacological activity is justified.

In the development of new antifungal drugs, the choice of the pharmacological target is important, because due to the similarities between human cells and the fungal cell, drugs selective only for fungal cells are difficult to find. Therefore, it is important that pharmacological targets for new antifungals are expressed exclusively in the fungus, or are significantly different from their analogs in human cells, this, to

avoid potentially negative pharmacodynamic interactions (Martinez-Rossi et al., 2008; Guerra et al., 2015). Thus, to investigate the mechanism of action of A₁Cl in strains of *A. niger*, the interaction of this molecule with the fungal cell wall, and with ergosterol in the fungal cell membrane was evaluated.

Sorbitol (an osmotic protector that stabilizes fungal protoplasts) was used to investigate the activity of A₁Cl on the cell wall. The functioning of specific fungal cell wall inhibitors is reversed in media containing sorbitol (Frost et al., 1995). Sorbitol protects cells by allowing them to grow in the presence of fungal cell wall inhibitors, growth that would be inhibited in the absence of sorbitol.

If the substance presents action on the fungal cell wall, as observed in the sorbitol medium, there will be an increase in its MIC value, as compared to its MIC value observed in a sorbitol-free medium (standard medium) (Frost et al., 1995; Svetaz et al., 2007). In this study, there was no increase in the MIC value of A₁Cl in the presence of sorbitol in *A. niger* strains (Table V), which indicates that A₁Cl does not act on the fungal cell wall.

Ergosterol is the most abundant sterol in fungal cell membranes, and regulates membrane permeability and fluidity. Ergosterol is the target of most clinical antifungals because of its crucial functions, unique structural properties, and involvement in specific biosynthetic steps (Douglas and Konopka, 2014; Dhingra and Cramer, 2017).

Exogenous ergosterol was used to study the interaction of A₁Cl with ergosterol. If A₁Cl's activity involves binding ergosterol, exogenous ergosterol added to the culture medium will reduce binding to ergosterol already in the fungal membrane, and cause an MIC increase for A₁Cl in the presence of exogenous ergosterol, since only a higher concentration of the product in the medium will guarantee interaction with ergosterol in the fungal membrane (Lunde and Kubo, 2000; Escarlante et al., 2008).

An increase in A₁Cl's MIC in the presence of exogenous ergosterol was observed in this study for the two strains tested when compared to the control (RPMI) (Table VI). Amphotericin B was used as a positive control which acts at the fungal cell membrane level, and increased the MIC when compared to the control (RPMI), this, as observed in Table VI was expected.

In view of these results, it is suggested that the mechanism of action A₁Cl against *A. niger*, involves interference in cell functions involving membrane ergosterol.

Given the above, we conclude that the synthetic amide 2-chloro-*N*-phenylacetamide (A₁Cl) presents antifungal activity against *A. niger*, being fungicidal with a mechanism of action related to ergosterol binding, interfering in cellular functions related to this sterol. The results of this study confirm the antifungal potential of 2-chloro-*N*-phenylacetamide against *A. niger*.

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Author contributions

The authors Elba dos Santos Ferreira, Laísa Vilar Cordeiro, Daniele de Figuerêdo Silva, Helivaldo Diógenes da Silva Souza, Petrônio Filgueiras Athayde-Filho, José Maria Barbosa-Filho, Edeltrudes de Oliveira Lima, and Ricardo Dias de Castro are responsible for writing the article, and the individual contributions of each author to the article follow. Elba dos Santos Ferreira participated in the design of the project, collection and analysis of data, writing of the article and critical review of the intellectual content. Laísa Vilar Cordeiro and Daniele de Figueredo Silva participated in data collection, data analysis and work review. Helivaldo Diógenes da Silva Souza, José Maria Barbosa-Filho and Petronio Filgueiras Athayde-Filho participated in the synthesis of the synthetic amide, data analysis and article review. Edeltrudes de Oliveira Lima and Ricardo Dias de Castro directed all stages of the work and participated in the review and writing of the project and work, including final approval of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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5.3

Toxicological profile of synthetic 2-chloro-*N*-phenylacetamide: *in silico*, *in vitro*, and *ex vivo* studies.

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Toxicological profile of synthetic 2-chloro-N-phenylacetamide: *in silico*, *in vitro*, and *ex vivo* studies

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Abstract

In the development of new drugs, cyto and genotoxicity assays are important because they allow cellular analysis of possible damages that a molecule may cause, and *in silico* studies allow for a prediction of pharmacokinetic properties, as well as possible toxic effects that drug candidate substances may cause. The aim of this study was to evaluate the toxicological properties of the synthetic amide 2-chloro-*N*-phenylacetamide using *in silico*, *in vitro*, and *ex vivo* studies, as well as to predict its pharmacokinetic properties. The *in silico* physicochemical and pharmacokinetic properties were evaluated using the Swiss ADME software, based on Lipinski's parameters, such as molecular weight, octanol/water partition coefficient, and the number of hydrogen donors and acceptors. Potential mutagenic and tumorigenic effects were predicted using the OSIRIS software. Both the cytotoxicity and anti-hemolytic capacity of the synthetic amide were evaluated *in vitro* through hemolysis testing with red blood cells obtained from healthy individuals (ABO system). Subsequently, the *ex vivo* genotoxic capacity of 2-chloro-*N*-phenylacetamide was evaluated in cells of the oral mucosa, revealing cell alterations such as caryolysis, karyorrhexis, and micronuclei. According to Lipinski's rule, 2-chloro-*N*-phenylacetamide presents favorable physicochemical parameters, good oral bioavailability and gastrointestinal tract absorption, and crosses the blood-brain barrier. Yet 2-chloro-*N*-phenylacetamide inhibits CYP1A2, and presents itself as mutagenic, tumorigenic, and causes reproductive effects. At concentrations of 50, 100, and 500 µg/mL, 2-chloro-*N*-phenylacetamide presents little hemolytic effect, medium toxicity at a concentration of 1000 µg/mL, and protective effect for type A and O red blood cells. In oral mucosa cells it promotes little genotoxic change, the most prevalent alterations being caryolysis and karyorrhexis. Therefore, it may be concluded that 2-chloro-*N*-phenylacetamide presents a favorable pharmacokinetic profile for oral administration, and low cytotoxic and genotoxic potential, being a promising candidate for *in vivo* toxicity studies.

Keywords: 2-chloro-*N*-phenylacetamide, hemolysis, genotoxicity, pharmacokinetics, red blood cells, oral bioavailability.

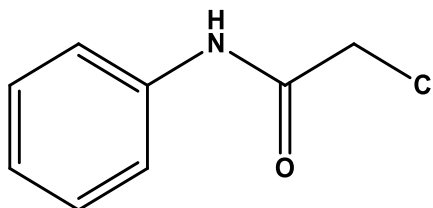
1.0 Introduction

In drug development, toxicity tests are essential because they allow determination of safety standards for the tested product, as well as enabling identification and characterizing harmful effects (Saraiva et al., 2012). Since no chemical substance is free of toxic effect, all bioactive molecules (as drug candidates) must undergo toxicity assessment tests so that they can be safely used (Veiga Júnior et al., 2005).

Cytotoxicity assays quantifying hemolysis allow investigation of both the hemolytic and protective potential of a wide variety of products, while permitting estimations of erythrocyte damage that the tested drug may produce *in vivo*, or in the body (Lexis et al., 2006). Genotoxicity tests allow analyzing harmful effects that substances can promote in DNA, and which may result in mutagenesis (Silva et al., 2003). Such harmful effects appear as nuclear alterations that form chromosomal aberrations: such as karyolysis, which (as a response to damage) is characterized by nuclear dissolution and a lack of cell nuclei coloration; karyorrhexis, the disintegration of the nucleus causing the formation of small nuclear particles during the process of necrosis and apoptosis and the micronuclei, rounded structures next to the main nucleus, formed by failures in cell division (Tolbert et al., 1992; Kumar et al., 2016).

2-chloro-*N*-phenylacetamide, presented as A₁Cl (Figure 1), is a synthetic amide obtained by means of a nucleophilic substitution involving 2-chloro-acetyl chloride and aniline (Huang, 2016), and is commonly used as a reaction intermediate (Huang, 2016; Patel et al., 2012). However, there are reports in the literature of its biological activities. Katke et al. (2011) found that A₁Cl presents promising antifungal and antibacterial activity, and in a screening study, Aschale (2012) demonstrated the favorable activity of A₁Cl against *Aspergillus niger*.

Figure 1: Chemical structure of the synthetic amide 2-chloro-*N*-phenylacetamide.



Despite demonstrating potent antimicrobial activity, little is known about the toxicity of A₁Cl, and therefore, the aim of this study was to analyze the *in*

vitro cytotoxic and *ex vivo* genotoxic potential of 2-chloro-*N*-phenylacetamide, as well as predict its pharmacokinetic properties from *in silico* tests.

2.0 Materials and Methods

2.1 Substance

The 2-chloro-*N*-phenylacetamide, obtained by synthesis (Huang 2016).

2.2 Obtaining blood samples and oral mucosa cells

The tests were performed in accordance with the Code of Ethics of the World Medical Association, and approved by the Ethics Committee of the *Centro Universitário de Patos* (protocol number: 3.621.284). Blood samples (A, B, and O) and oral mucosal smears were collected from healthy young adults of both sexes between 18 and 40 years of age.

2.3. Evaluation of the physicochemical, pharmacokinetic and *in silico* toxic effects of A1Cl

The *in silico* evaluation of the toxicity of molecules also included the theoretical analysis of mutagenic, tumorigenic, irritant and reproductive effects as described by Abreu, 2008. To be administered orally, the test product must be absorbed by the gastrointestinal tract, and by Therefore, A₁Cl was evaluated according to Lipinski's "Rule of Five", which states that at least three of four requirements must be met for the compound to have good theoretical oral bioavailability. Thus, the compounds to be absorbed must have molecular weight 500 daltons (Da), calculated octanol/water partition coefficient ((Log P_{o/w}) 5, number of hydrogen bond acceptors (nALH) 10 and number of hydrogen bond donor groups (nDLH) 5 (Lipinski, 2001).

The Swiss ADME software also employs other rules-based filters (Ghose: 180 ≤ MM ≤ 480; 20 ≤ No. of atoms ≤ 70; 40 ≤ Molar refractivity ≤ 130; -0.4 ≤ Log P_{o/w} ≤ 5.6; Veber: No. Swivel connections ≤ 10; TPSA ≤ 14 nm²; Egan: Log P_{o/w} ≤ 5.88; TPSA ≤ 13.16 nm²) (Ghose et al., 1999; Egan et al., 2000; Veber et al., 2002), which allows a broader understanding of the physicochemical and pharmacokinetic characteristics of a drug candidate.

For the analysis of theoretical toxicity and pharmacokinetic parameters, the structure of the synthetic amide A₁Cl was submitted to *in silico* study of ADMET parameters (absorption, distribution, metabolism, excretion and toxicity) using the

Osiris Property Explorer program (<http://www.organic-chemistry.org/prog/peo/drugScore.html>).

2.4. *In vitro* cytotoxic profile evaluation

2.4.1. Hemolytic activity assessment

To obtain the erythrocytes, aliquots of human blood (type A, B and O) were mixed with 0.9% NaCl (at a proportion of 1:3), and centrifuged for 5 minutes at 2500 rpm. The procedure was repeated twice, and the pellet from the last centrifugation was resuspended in 0.9% NaCl to obtain a 0.5% suspension. Different concentrations (50, 100, 500, and 1000 $\mu\text{g/ml}$) of A₁Cl were added to the different erythrocyte suspension preparations to complete final volumes of 2 ml. An untreated erythrocyte suspension was used as a negative control (0% hemolysis), and an erythrocyte suspension plus 1% Triton X-100 was used as a positive control (100% hemolysis). The samples were incubated for 1 hour at $22 \pm 2^\circ\text{C}$ under slow and constant agitation (100 rpm), and the samples were then centrifuged for 5 minutes at 2500 rpm and hemolysis was quantified by spectrophotometry at 540 nm (Rangel et al., 1997).

2.4.2 Evaluation of anti-hemolytic activity.

The evaluation of anti-hemolytic activity was performed using a 0.5% erythrocyte suspension. Solutions containing different A₁Cl concentrations (50, 100, 500, and 1000 $\mu\text{g/ml}$) were incubated in tubes containing 2 ml of an erythrocyte suspension for 1h at $22 \pm 2^\circ\text{C}$. The preparations were centrifuged for 5 minutes at 2500 rpm and the supernatants were discarded. The erythrocytes were then resuspended in a hypotonic solution (0.24% NaCl) and shaken at 100 rpm for one hour at $22 \pm 2^\circ\text{C}$. At one hour, the samples were centrifuged for 5 minutes at 2500 rpm, and hemolysis was quantified by spectrophotometry at 540 nm (Dacie, 2001). The erythrocyte suspension was used as a negative control (0% hemolysis), and an erythrocyte suspension plus the hypotonic solution as a positive control (100% hemolysis).

2.5. *Ex vivo* cytotoxic profile evaluation

2.5.1. Evaluation of genotoxicity in oral mucosa cells

Epithelial cells were collected from the left and right sides of the oral mucosa using a cytobrush, which is considered the most suitable instrument for obtaining exfoliate cells from the oral mucosa (Kassie et al., 2001). The cells were kept in a tube with 5 mL of cell preservation medium (0.9% NaCl) until the slides were prepared. Cell controls were divided into two groups: those treated with hydrogen peroxide (0.0005%) (positive control), and those left untreated (negative control).

The cells were washed twice in saline solution, centrifuged for 10 min at 1,500 rpm, and then kept in 5 mL of saline solution. The supernatant was removed, and the samples were washed once more and exposed *ex vivo* to A_1Cl (at 50, 100, 500, and 1000 $\mu\text{g/mL}$) for 30 minutes, each test was performed in triplicate. They were then centrifuged and the supernatant discarded. Prior to smear preparation, slides were pre-warmed to 37°C and the cells homogenized in a vortex mixer. At each concentration analyzed, they were placed on three slides, dried at room temperature, and fixed in methanol: acetic acid (3:1) for 15 min (Thomas et al., 2008). The slides (triplicate) were kept at room temperature for 12 h, after which they were immersed in distilled water for 1 min and stained in 2% Giemsa for analysis under optical microscopy (Gabriel et al., 2006).

Cellular toxicity can be assessed using cellular change indicators, such as micronuclei, karyolysis, and karyorrhexis, among others (Carrard et al., 2007; Sponchiado et al., 2016). Approximately 1000 cells were analyzed per slide, and data were expressed as percentages representing the arithmetic mean of three measurements.

2.6 Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (S.E.M.), and statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post-testing, to compare more than two groups. The null hypothesis was rejected when $p < 0.05$. GraphPad Prism® version 5.01 program (GraphPad Software Inc., San Diego CA, U.S.A.) was used.

3.0 Results

3.1. Evaluation of the *in silico* pharmacokinetic profile.

A₁Cl presents an octanol/water partition coefficient (Log P) equal to 1.67. According to Lipinski (Log Po/w ≤ 5), Ghose (Log Po/w ≤ 5.6), and Egan (Log Po/w ≤ 5.8) rules, the molecule is considered lipophilic. Regarding solubility in water, A₁Cl was considered to have high solubility (Table 1).

Regarding the absorption parameter, A₁Cl presented a high probability of absorption from the gastrointestinal tract, of crossing the blood-brain barrier, and not interacting with the P-glycoprotein. As for metabolism, A₁Cl is an enzyme inhibitor of CYP1A2 alone (Table 1).

Table 1. Theoretical analysis of A₁Cl pharmacokinetic parameters using the Swiss ADME software.

Parameters	A ₁ Cl
Physicochemical Properties	
Formula	C ₈ H ₈ ClNO
Molecular Weight	169.61 g/mol
N. Heavy atoms	11
Fraction Csp ³	0.12
N. Rotatable Bonds	3
N. H-bonds acceptors	2
N. H-bonds donors	1
Molar Refractivity	45.55
TPSA ^a	2.91 nm ²
Lipophilicity	
Consensus ^b Log P _{o/w} ^c	1.67
Log S (Ali)	-1.85
Water Solubility	
Class ^d	Very Soluble
Pharmacokinetic properties	
Gastrointestinal absorption	High
Blood-Brain Barrier permeation	Yes
P-glycoprotein substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 1 violation: atoms < 20
Veber	Yes; 0 violation
Egan	Yes; 0 violation
Bioavailability Score	0.55

A₁Cl: 2-chloro-*N*-phenylacetamide; TPSA: Topological polar surface area; ^bConsensus Log P_{o/w}: Average of all five predictions; ^cLog P_{o/w}: The partition coefficient between n-octanol/water; ^dSolubility Class: insoluble < -10 < poorly < -6 < moderately < -4 < soluble < -2 < very < 0 < highly.

With regard to toxicity risks, A₁Cl is mutagenic, tumorigenic, and causes reproductive effects, as shown in Table 2.

Table 2. Theoretical analysis of A₁Cl toxicity risk using Osiris software.

Parameters	A ₁ Cl
Mutagenic	Yes
Tumorigenic	Yes
Irritant	No
Reproductive Effect	Yes

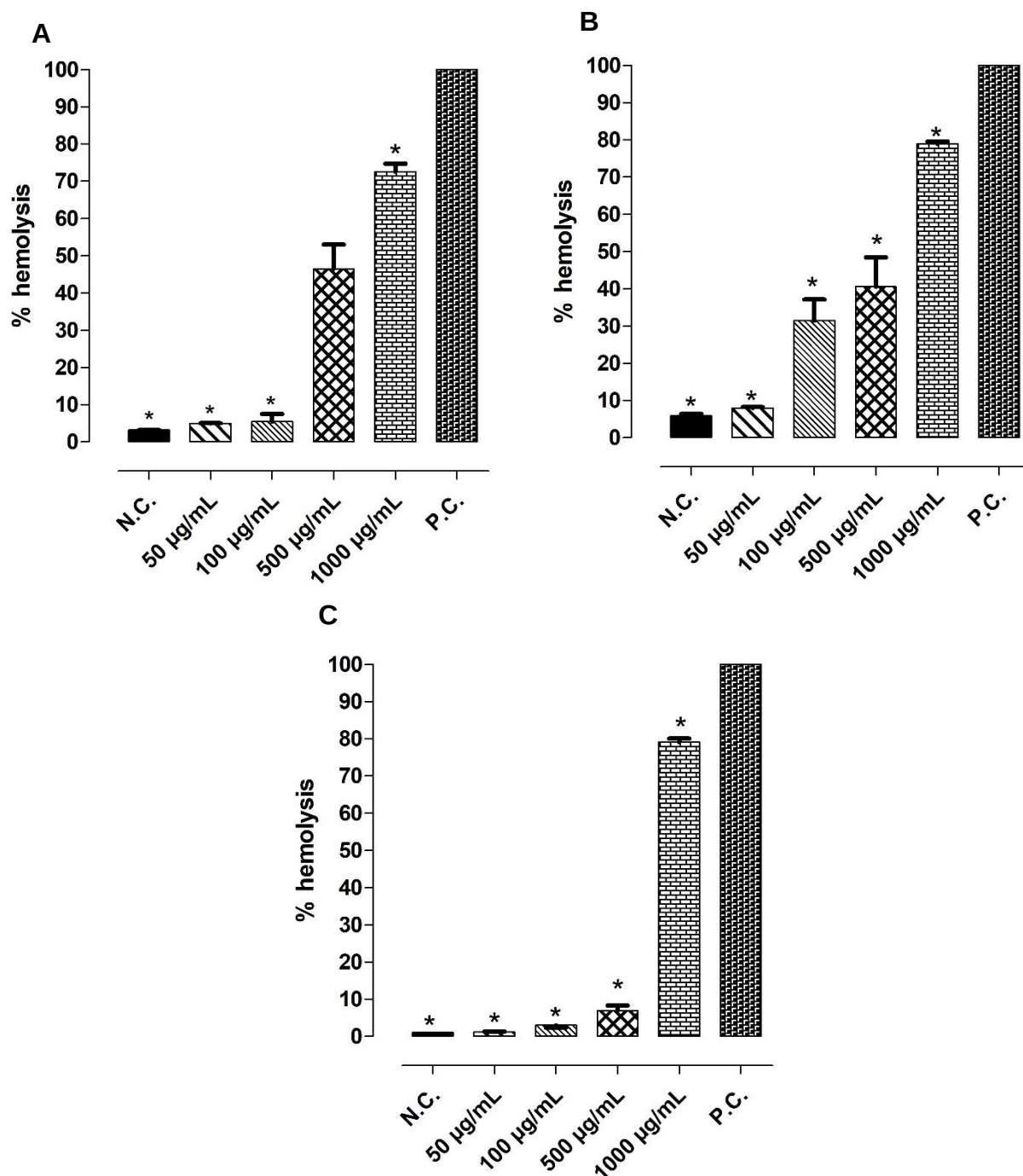
A₁Cl: 2-chloro-*N*-phenylacetamide

3.2. Cytotoxic profile evaluation.

3.2.1 Hemolytic activity.

For all blood types, the A₁Cl product presented low toxicity at concentrations 50, 100, and 500 µg/mL, and a hemolysis percentage ranging from 5 to 46% for type A (Figure 2A), 7 to 40% for type B (Figure 2B), and 1.2 to 6.9% for type O (Figure 2C). At a concentration of 1000 µg/mL, A₁Cl presented hemolysis of 72.6% for type A (Figure 2A), 78.9% for type B (Figure 2B), and 79.1% for type O (Figure 2C), being considered of medium toxicity at this concentration. All concentrations were compared to the positive control.

Figure 2: Percent hemolysis of 2-chloro-N-phenylacetamide against erythrocytes of the ABO system, type A (A), type B (B) and type O (C), ordered in the following groups: Negative control (■), concentrations of 50 , 100 , 500 , 1000  and positive control .



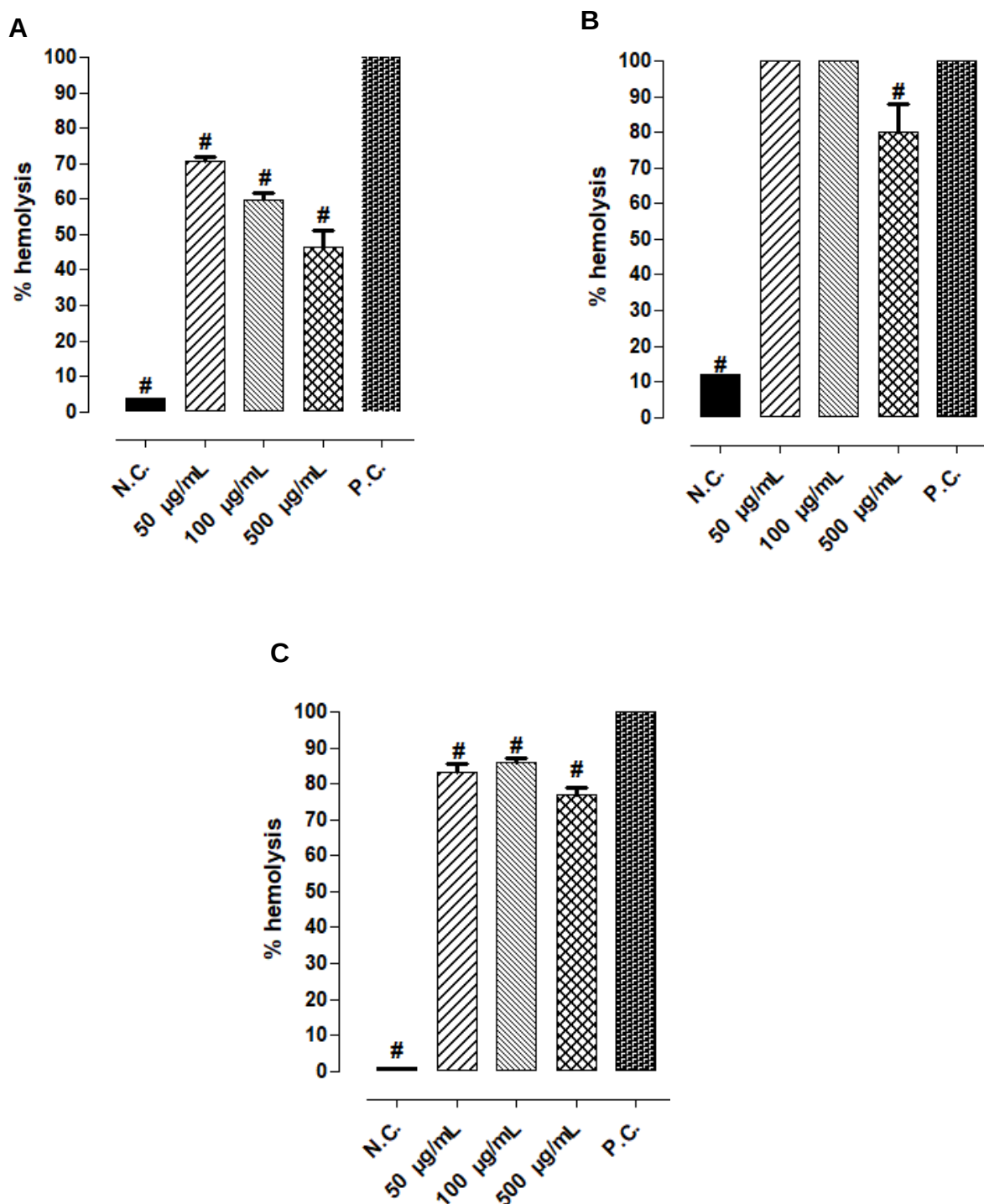
The columns and vertical bars respectively represent the mean and S.E.M., (n=3). One-way ANOVA followed by Bonferroni post-test. *p < 0.05 (compared to positive control). N.C. = negative control (erythrocytes 0.5%); P.C. = positive control 1% Triton X-100).

3.2.2. Evaluation of anti-hemolytic activity.

For blood types A and O, A₁Cl presented a protective effect, with a 20 to 47% lower degree of hemolysis than the positive control at all concentrations tested (Figure 3A, 3B and 3C).

For blood type B, it was observed that A₁Cl promoted complete hemolysis (100%) at the first two concentrations, yet at the 500 µg/mL concentration, decreased hemolysis was observed.

Figure 3: Anti-hemolytic activity of the synthetic amide 2-chloro-*N*-phenylacetamide against erythrocytes of the ABO system, type A (A), type B (B) and type O (C), ordered in the following groups: Negative control (■), concentrations of 50 μ g/mL, 100 μ g/mL, 500 μ g/mL, and positive control (▨).



The columns and vertical bars represent the mean and the S.E.M., respectively, (n=3). One-way ANOVA followed by Bonferroni post-test. * $p < 0.05$ (compared to positive control). N.C. = negative control (erythrocytes 0.5%); P.C. = positive control NaCl 0,25%).

3.3 *Ex vivo* evaluation of cytotoxic profile.

3.3.1 Evaluation of genotoxicity in oral mucosa cells.

The A₁Cl genotoxicity test with cells from the oral mucosa revealed few cellular alterations (even at 1000 µg/mL) when compared to the positive control. The percentage of normal cells was higher than 85% at all concentrations of A₁Cl used in this study, and the most prevalent alterations were caryolysis and karyorrhexis, with fewer alterations than the positive control (Table 3).

Table 3. Evaluation of A₁Cl genotoxicity in oral mucosa cells.

Group	Karyolysis	Karyorrhexis	Micronucleus	Normal
N.C.	1.01%*	1.00%*	0.33%*	92.45%*
P.C. H ₂ O ₂	6.33%	11.00%	1.70%	76.96%
A ₁ Cl 1000 µg/mL	3.00%*	1.33%*	0.44%*	88.77%*
A ₁ Cl 500 µg/mL	1.9%*	5.23%*	0.43%*	86.67%*
A ₁ Cl 100 µg/mL	5.7%	3.30%*	0.0%*	85.67%*
A ₁ Cl 50 µg/mL	3.33%*	3.33%*	0.33%*	85.33%*

One-way ANOVA followed by Bonferroni post-test. * $p < 0.05$ (compared to positive control). N.C. = negative control; PC = positive control.

4.0 Discussion

To theoretically assess the toxicological potential and pharmacokinetic properties of A₁Cl, *in silico* tests were performed using the online software Swiss ADME and Osiris.

Using online calculations, the Swiss ADME software establishes a correlation between the physicochemical properties of a selected compound and its theoretical pharmacokinetic profile. This correlation is determined according to Lipinski's Rule of Five, where oral bioavailability of a drug can be affirmed given no more than one violation of the following parameters: Molecular mass ≤ 500 Da; Log Po/w ≤ 5 ; number of H-bond donors ≤ 5 ; number of H-binding acceptors ≤ 10 (Lipinski et al., 1997; and Daina et al., 2017). The software also considers drug similarity in accordance with parameters proposed by other authors (Ghose et al., 1999; Egan et al., 2000; Veber et al., 2002).

In accordance with the rules of Lipinski, Ghose, Veber, and Egan, and from the data obtained in Table 1, it was found A₁Cl presents physicochemical parameter values with acceptable values, suggesting that A₁Cl presents good availability for oral

administration. Since it is the more convenient route, oral administration gains greater acceptance, and given fewer undesirable effects when compared to other routes of administration, it facilitates adherence to treatment (Diniz et al., 2013). For a drug to be well absorbed, it must be lipophilic to cross plasma membranes, and yet be soluble in aqueous solutions to afford access to the cell surface. In addition, low interactivity with the P-glycoprotein allows a drug not to be pumped out of the cell, thus remaining to exert its pharmacological activity (Whalen et al., 2016).

The hydrophilic and lipophilic characteristics of A₁Cl, as well as its low interaction with P-glycoprotein, make it a good drug candidate, with good absorption and distribution throughout the body, or if administered orally, good bioavailability. In addition, its lipophilicity allows it to easily cross the blood-brain barrier for use in treatment of diseases that affect the central nervous system.

Regarding the metabolic-pharmacokinetic parameter, the molecule only inhibits the CYP1A2 enzyme. Cytochrome P450 enzymes play an essential role in drug biotransformation, and inhibition of certain isoenzymes, such as CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4. Such inhibition is a main cause of drug interactions which may result in decreased metabolism of enzyme substrates. For drugs, this may lead to toxicity (Huang et al., 2008), since drugs that are metabolized by the CYP1A2 enzyme, such as selective serotonin reuptake inhibitor antidepressants and first and second generation antipsychotics, may suffer decreased metabolism and thus increased pharmacological activity, when used concomitantly with A₁Cl (Flockhart, 2007).

For toxicity, A₁Cl is mutagenic, tumorigenic, and causes reproductive effects. Yet molecular modification strategies, which are constantly employed to improve the pharmacological profiles of known drugs, may be useful to decrease the adverse effects of A₁Cl and improve its activity (Jaimes et al., 2019; Pereverzeva et al., 2020). Using A₁Cl as a structural template in new studies to obtain new derivatives with better toxicological profiles would be promising. *In silico* tests are important to make predictions, but pharmacological tests involving cells, biological systems, *in vivo* and clinical models are necessary for the knowledge to be deepened.

Determination of the cytotoxic profile in human erythrocytes is an effective *in vitro* experimental model that investigates toxicity, as well as potential protective effects for a wide variety of substances. Erythrocyte hemolysis may be directly correlated with toxic effects of a tested substance (Brandão et al., 2005). In the

literature, there are no reports involving the cytotoxicity of 2-chloro-*N*-phenylacetamide, making this work pioneer in the determining its cytotoxic activity against red blood cells.

To perform this assay, red blood cells and the ABO system were used. According to Rangel et al., (1997), a substance is considered to be of low toxicity when it presents hemolysis of between 0 and 40%; of medium toxicity when presenting hemolysis between 40 and 80%, and highly toxic when hemolysis is above 80%. In this study, it was observed that A₁Cl presents low hemolytic potential at concentrations of 50, 100, and 500 µg/mL, and medium hemolytic potential at concentrations of 1000 µg/mL.

The assay suggests that A₁Cl presents lower hemolytic potential when interacting with red blood cells that lack carbohydrates in the erythrocyte membrane, such as blood type O red cells which present neither N-acetylgalactose (serotype A) or D-galactose (serotype B). Similar results were described by Cordeiro et al., (2020), where an acetamide presenting low hemolytic potential against red blood cells of the ABO system (mainly in serotype O) was also studied.

Butta et al., (2019) synthesized a series of bi-heterocyclic acetamides, and upon evaluation observed their low hemolytic potential, these were classified as low toxicity molecules, and useful for treatment of diseases related to skin pigmentation.

Thus, it may be concluded that the synthetic amide A₁Cl presents low cytotoxicity in red blood cells in the ABO system.

To verify whether the low toxicity of the product might be related to some type of protective effect, the erythrocytes were subjected to a hypotonic medium in contact with A₁Cl. This would verify whether the product protects erythrocytes from hemolysis induced by osmotic fragility (influx of water). It was observed that A₁Cl did not present a protective effect (for type B red blood cells), except at the concentration of 500 µg/mL. For this assay, the 1000 µg/mL concentration was not used, since it had produced the highest rates of hemolysis.

Cordeiro et al., (2020), also tested the protective potential of a synthetic amide against erythrocytes of the ABO system, and reported similar results. It was observed that the molecule presents moderate hemolysis and moderate protective effect in certain concentrations, despite variations in cell lysis according to blood group, this makes it necessary to increase the concentrations used for the effect to be potentiated.

Low osmotic pressure in the extracellular medium causes erythrocytes to absorb water to achieve osmotic equilibrium, and in extreme conditions, hypotonic hemolysis occurs when excess extracellular water causes significant swelling and disruption of the erythrocyte, which releases its intracellular contents. In hypotonic conditions, reducing erythrocyte membrane fluidity can reduce hemolysis (Zhang et al., 2019).

It is possible that A₁Cl promotes an alteration in the lipid bilayer structure of the erythrocyte membrane, reducing fluidity and consequently reducing hemolysis. However, further study is needed to prove this hypothesis.

Assessment of the genotoxicity of a product is performed with the aim of identifying potential interactions with nucleic acids within the cell, or its capability of generating chromosomal and DNA alterations. This may be evidenced by the presence of micronuclei, karyorrhexis, and karyolysis, among others (Sponchiado et al., 2016). There are no reports in the literature involving the genotoxicity of 2-chloro-N-phenylacetamide, and this work is pioneer for determining the genotoxicity of this synthetic amide in oral mucosa cells.

In this study, A₁Cl presented a low toxicity threshold against oral mucosa cells. Similar results were observed by Cordeiro et al. (2020) for an acetamide, when demonstrating its low potential for inducing cellular alterations. Reinforcing the data presented in this study, Moore et al., (2019) has demonstrated the low cytotoxic and mutagenic potential of certain acetamides.

Karyolysis indicates nuclear dissolution, which can be observed as a lack of staining in the cell nucleus, a response to cell damage that frequently occurs in non-keratinized epithelium cells. The presence of karyorrhexis promotes the formation of small fragments of nuclear material, which is common during necrosis and apoptosis (Tolbet et al., 1992). In this case, the cells exposed to A₁Cl presented few changes of this type, suggesting that the synthetic amide under study has little capacity to induce lesions and/or cause cell death. Yet *in vivo* studies involved animal models are needed to complement this cytotoxic evaluation.

Given the above, it is suggested that the synthetic amide 2-chloro-N-phenylacetamide has a favorable pharmacokinetic profile for oral administration, and with its low cytotoxic and genotoxic potential, a strong candidate for future *in vivo* toxicity assays.

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Author contributions

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Conflict of interest

The authors declare that they have no conflict of interest.

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Conclusão

5.0 Conclusão

Diante do exposto, pode-se concluir que A₁Cl:

- Apresenta atividade antifúngica contra cepas de *A. flavus* e *A. niger*, sendo fungicida nas concentrações testadas;
- O mecanismo de ação contra cepas de *A. flavus* e *A. niger* envolve a interação com o ergosterol da membrana plasmática fúngica, além de uma provável atuação *in silico* a nível de DNA;
- Apresenta-se como um bom candidato a fármaco;
- Apresenta perfil farmacocinético *in silico* favorável para administração pela via oral;
- Apresenta efeito protetivo em eritrócitos do tipo A e O;
- Mostra-se com potencial citotóxico de baixo a moderado para eritrócitos e baixo potencial genotóxico em células da mucosa oral, sendo um forte candidato para estudos de toxicidade *in vivo*.

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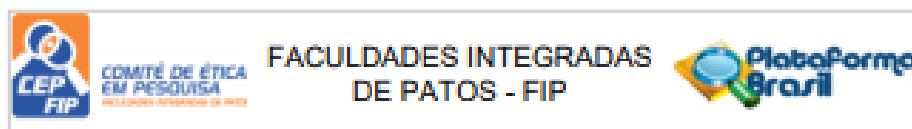
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Anexos

Parecer do comitê de ética das Faculdades Integradas de Patos para a realização dos ensaios de toxicidade *in vitro*.



PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ANÁLISE DAS ATIVIDADES ANTIMICROBIANA, CITOTÓXICA E MUTAGÊNICA DE PRODUTOS NATURAIS E SINTÉTICOS BIOATIVOS

Pesquisador: ALESON PEREIRA DE SOUSA

Área Temática:

Versão: 3

CAAE: 20419319.5.0000.5181

Instituição Proponente:

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.853.358

Apresentação do Projeto:

INTRODUÇÃO

Inúmeras doenças microbianas de origem bacteriana, viral ou fúngica são relatadas na literatura. Em muitos casos, os responsáveis pelas infecções adquirem resistência ao medicamento utilizado, e por isso grande número de antimicrobianos tem sido desenvolvido

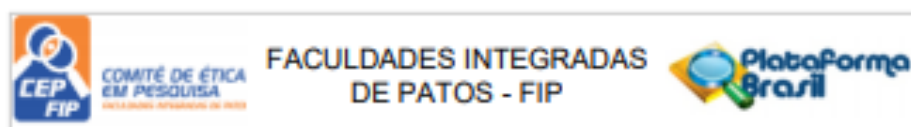
pela indústria farmacêutica (KUBO et al., 1993; KOSHI; CHERIAN, 1995; DEUS et al., 2011).

Para que um produto, seja ele natural ou sintético, venha se tornar um medicamento seguro e eficaz, é necessário a realização de estudos toxicológicos para determinação de padrões de segurança que devem ser determinados antes da utilização do medicamento na clínica. Dessa forma, o estudo da toxicologia de um produto é de grande valia, pois permite a caracterização dos efeitos deletérios das drogas ocorridos a partir da sua administração, contribuindo para decidir se um produto deve ser adotado ou não para uso clínico (SARAIVA et al., 2012).

Um dos modelos experimentais utilizados para avaliação da toxicidade *in vitro* é o ensaio de citotoxicidade em eritrócitos. Esse teste é usado como método de triagem para toxicidade de novas drogas objetivando estimar o dano que elas podem produzir *in vivo* em eritrócitos do organismo (SCHREIER et al., 1997; APARICIO et al., 2005).

A genotoxicidade é a área da genética que estuda os efeitos de determinadas substâncias sobre a

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estrutura do DNA, que pode resultar em um processo denominado mutagênese. Neste sentido, os agentes que alteram a sequência do DNA são considerados "tóxicos" para os genes e são, então, chamados de genotóxicos (SILVA et al., 2003). Assim, as pesquisas sobre genotoxicidade podem também comprovar o efeito anticarcinogênico de algumas substâncias (PAOLINI, 2003).

Dessa forma, este projeto objetiva-se avaliar o potencial antimicrobiano e citotóxico das amidas sintéticas N-(4-flúor-3-nitrofenil) acetamida, 2-Cloro-N-(4-flúor-3-nitrofenil) acetamida, 2-Bromo-N-(4-flúor-3-nitrofenil) acetamida, 2-Bromo-N-fenilacetamida, 2-cloro-N-fenilacetamida; híbridos moleculares do Triclosan: (5-cloro-2-(2,4-diclorofenoxi)fenil-2-acetoxibenzoato, 5-cloro-2-(2,4-diclorofenoxi)fenil-2-(3-benzofenil)propanoato, 5-cloro-2-(2,4-diclorofenoxi)fenil-2-(4-oxifenil)propanoato); os isômeros R - (+)-citronelol e S - (-)-citronelol, dos óleos essenciais de *Eucalyptus citriodora*, *Eucalyptus globulus*, *Lúpulo Columbus*, extratos de *Hordeum vulgare*, *Spondias mombin*, *Tracheophyta* e *Urtica dioica* L. e os flavonoides Vitexina, Tiliosídeo e Trimetoxiflavona, para contribuir com a descoberta de novas drogas com potencial farmacológico promissor.

HIPÓTESES

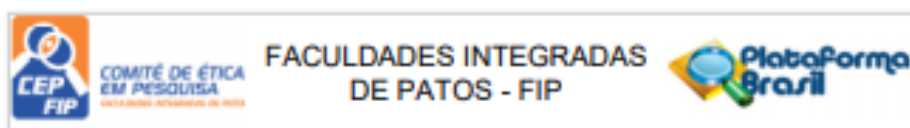
As substâncias em estudo possuem atividade antimicrobiana frente aos microrganismos de interesse clínico desse estudo, bem como possuem atividade antioxidante e toxicidade reduzida. As atividades biológicas envolvendo as substâncias em estudo não foram ainda investigadas detalhadamente em relação à sua capacidade antibacteriana e antifúngica. Este trabalho propõe-se a investigar a hipótese de que estas substâncias podem desempenhar ação antimicrobiana frente aos microrganismos de interesse clínico. Também será testada a hipótese de que estas substâncias isoladas apresentem efeito oxidante, hemolítico e potencial mutagênico reduzido.

METODOLOGIA

Os ensaios laboratoriais referentes ao estudo da atividade antibacteriana serão realizados no Laboratório de Pesquisa de Atividade Antibacteriana e Antifúngica de Produtos Naturais e/ou Sintéticos Bioativos, localizado no Centro de Ciências da Saúde (CCS) do Departamento de Ciências Farmacêuticas (DCF) da Universidade Federal da Paraíba (UFPB) / João Pessoa - PB, sob coordenação da Prof.^a Dr.^a Edeltrudes de Oliveira Lima. Os microrganismos utilizados nos ensaios serão isolados, identificados quanto à espécie e analisados sobre os seus perfis de resistência antibiótica.

Os ensaios toxicológicos e da avaliação da atividade antioxidante das substâncias serão realizados no Laboratório de Bioquímica da Unidade Acadêmica de Ciências Biológicas, localizado no Centro de Saúde e Tecnologia Rural (CSTR) da Universidade Federal de Campina

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Continuação do Parecer: 3.653.358

Grande (UFCG) / Patos – PB, sob coordenação do Prof. Dr. Abrahão Alves de Oliveira Filho.

Objetivo da Pesquisa:

Investigar o potencial antimicrobiano e a toxicidade in vitro das amidas sintéticas N-(4-flúor-3-nitrofenil) acetamida, 2-Cloro-N-(4-flúor-3-nitrofenil) acetamida, 2-Bromo-N-(4-flúor-3-nitrofenil) acetamida, 2-Bromo-N-fenilacetamida, 2-cloro-N-fenilacetamida; híbridos moleculares do Triclosan: (5-cloro-2-(2,4-diclorofenoxi)fenil-2-cetoxibenzoato, 5-cloro-2-(2,4-diclorofenoxi)fenil-2-(3-benzofenil)propanoato, 5-cloro-2-(2,4-diclorofenoxi)fenil-2-(4-isobutifenil)propanoato); os isômeros R - (+)-citronelol e S - (-)-citronelol, dos óleos essenciais de Eucalyptus citriodora, Eucalyptus globulus, Lúpulo Columbus, extratos de Hordeum vulgare, Spondias mombin, Tracheophyta e Urtica dioica L. e os flavonoides Vitexina, Tiliosídeo e Trimetoxiflavona.

Avaliação dos Riscos e Benefícios:

Os riscos e os benefícios foram descritos conforme a Resolução 466/2012 do CNS/MS.

Comentários e Considerações sobre a Pesquisa:

Verifica-se direcionamento metodológico adequado à realização de um trabalho com relevância acadêmica, científica e social.

Considerações sobre os Termos de apresentação obrigatória:

Os Termos foram apresentados de acordo com a Norma Operacional 001/2013 do CNS/MS.

Conclusões ou Pendências e Lista de Inadequações:

Cumpridas as pendências lançadas no parecer anterior, nos posicionamos de maneira FAVORÁVEL à realização do estudo.

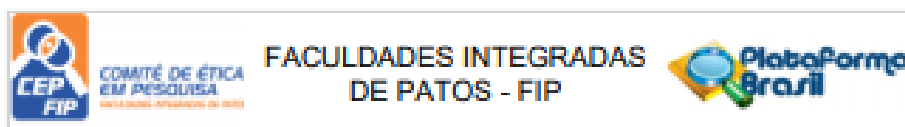
Considerações Finais a critério do CEP:

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Research Article

Effects of Redox Disturbances on Intestinal Contractile Reactivity in Rats Fed with a Hypercaloric Diet

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Few studies have associated the effects of changes in caloric intake and redox disturbances in the gastrointestinal tract. Therefore, the present study aimed at evaluating the hypercaloric diet consumption influence on the contractile reactivity of intestinal smooth muscle, morphology, and oxidative stress of rat ileum. Wistar rats were randomly divided into groups that received a standard diet and fed with a hypercaloric diet for 8 weeks. Animals were euthanized, and the ileum was isolated to isotonic contraction monitoring. Morphology was evaluated by histological staining and oxidative stress by quantification of malondialdehyde levels and total antioxidant activity. Cumulative concentration-response curves to ECI and carbachol were attenuated in rats fed with a hypercaloric diet compared to those that received a standard diet. In addition, an increase in caloric intake promotes a rise in the thickness of the longitudinal smooth muscle layer of rat ileum and tissue malondialdehyde levels, characterizing lipid peroxidation, as well as a decrease in the antioxidant activity. Thus, it was concluded that the consumption of a hypercaloric diet impairs rat intestinal contractility due to mechanisms involving modifications in the intestinal smooth muscle architecture triggered by redox disturbances.

1. Introduction

World Health Organization (WHO) defines obesity as a chronic condition characterized by an excessive accumulation of adipose tissue that causes health risk [1]. Therefore, obesity is categorized in the 10th revision of the International

Classification of Diseases (ICD-10) at endocrine, nutritional, and metabolic diseases section [2].

Currently, several models develop obesity in animals through genetic mutations. However, most cases of human obesity are considered polygenic because of several gene integration. Thus, when analyzing the genesis of obesity in

Research Article

Potential Therapeutic Role of Dietary Supplementation with *Spirulina platensis* on the Erectile Function of Obese Rats Fed a Hypercaloric Diet

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Spirulina platensis, an important source of bioactive compounds, is a multicellular, filamentous cyanobacterium rich in high-quality proteins, vitamins, minerals, and antioxidants. Due to its nutrient composition, the alga is considered a complete food and is recognized for its anti-inflammatory, antioxidant, antiobesity, and reproductive effects. All of which are important for prevention and treatment of organic and metabolic disorders such as obesity and erectile dysfunction. The aim of this study was to investigate the modulatory role of *Spirulina platensis* food supplementation and the mechanisms of action involved in reversing the damage caused by a hypercaloric diet on the erectile function of rats. The animals were divided into a standard diet group (SD, $n = 5$); a hypercaloric diet group (HCD, $n = 5$); a hypercaloric diet group supplemented with *S. platensis* at doses of 25 (HCD+SP25, $n = 5$), 50 (HCD+SP50, $n = 5$), and 100 mg/kg (HCD+SP100, $n = 5$); and a hypercaloric diet group subsequently fed a standard diet (HCD+SD, $n = 5$). In the rats fed a hypercaloric diet, dietary supplementation with *S. platensis* effectively increased the number of erections while decreasing latency to initiate penile erection. Additionally, *S. platensis* increases NO bioavailability, reduces inflammation by reducing the release of contractile prostanooids, enhances the relaxation effect promoted by acetylcholine (ACh), restores contractile reactivity damage and cavernous relaxation, reduces reactive oxygen species (ROS), and increases cavernous total antioxidant capacity (TAC). Food supplementation with *S. platensis* thus restores erectile function in obese rats, reduces production of contractile prostanooids, reduces oxidative stress, and increases NO bioavailability. Food supplementation with *S. platensis* thus emerges as a promising new therapeutic alternative for the treatment of erectile dysfunction as induced by obesity.



Article

Potential of 2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide Against *Klebsiella pneumoniae* and In Vitro Toxicity Analysis

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Abstract: *Klebsiella pneumoniae* causes a wide range of community and nosocomial infections. The high capacity of this pathogen to acquire resistance drugs makes it necessary to develop therapeutic alternatives, discovering new antibacterial molecules. Acetamides are molecules that have several biological activities. However, there are no reports on the activity of 2-chloro-N-(4-fluoro-3-nitrophenyl)acetamide. Based on this, this study aimed to investigate the in vitro antibacterial activity of this molecule on *K. pneumoniae*, evaluating whether the presence of the chloro atom improves this effect. Then, analyzing its antibacterial action more thoroughly, as well as its cytotoxic and pharmacokinetic profile, in order to contribute to future studies for the viability of a new antibacterial drug. It was shown that the substance has good potential against *K. pneumoniae* and the chloro atom is responsible for improving this activity, stabilizing the molecule in the target enzyme at the site. The substance possibly acts on penicillin-binding protein, promoting cell lysis. The analysis of cytotoxicity and mutagenicity shows favorable results for future in vivo toxicological tests to be carried out, with the aim of investigating the potential of this molecule. In addition, the substance showed an excellent pharmacokinetic profile, indicating good parameters for oral use.

Keywords: 2-chloro-N-(4-fluoro-3-nitrophenyl)acetamide; *Klebsiella pneumoniae*; antibacterial; toxicity; minimum inhibitory concentration

1. Introduction

The *Klebsiella pneumoniae* bacteria, belonging to the Enterobacteriaceae family, are Gram-negative, facultative, immobile, and encapsulated anaerobes. It is one of the opportunistic pathogens of greatest clinical relevance, known to be associated with nosocomial infections. It is estimated that this species, alone, is responsible for approximately one third of all infections caused by Gram-negative bacteria in general, causing, for example, pneumonia, urinary tract infections, bacteremia, endocarditis, and liver

Review Article

The Use of Essential Oils and Their Isolated Compounds for the Treatment of Oral Candidiasis: A Literature Review

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In this literature review, we present the main scientific findings on the antifungal activity of essential oils (EOs) applicable for a new drug formulation to treat oral candidiasis. Seven literature databases were systematically searched for eligible in vitro and clinical trials. Selected articles were screened for biological activity, botanical species, phytochemical composition, study design, and methodological quality. A total of 26 articles were included in the review, of which 21 were in vitro studies and 5 clinical trials. The most promising EOs were obtained from *Allium tuberosum*, *Cinnamomum cassia*, *Cinnamomum zeylanicum*, and *Coriandrum sativum* L. Among the phytochemicals, citral and thymol were the most active. Clinical trials indicated that the EOs from *Pelargonium graveolens* and *Zataria multiflora* are potentially effective to treat oral candidiasis. Further nonclinical and clinical studies with these EO are warranted to determine their potential use and safety for the treatment of oral candidiasis.

1. Introduction

Oral candidiasis is an infection caused by *Candida* spp. which manifests clinically as erythematous, ulcerated, sensitive white lesions, with soft consistency and easy removal, commonly affecting the palate, oral mucosa, tongue, or oropharynx [1]. Due to the opportunistic pathogenicity of yeasts, oral candidiasis is more prevalent in immunocompromised individuals [2].

Yeast colonization of complete dentures occurs mainly due to the strong adhesion of yeast cells to acrylic resin of base materials [1]. Direct adhesion of yeasts onto dental surfaces is a critical pathogenic factor for the onset of dental

stomatitis. Yeast cells can co-aggregate with various bacterial species from the oral plaque and integrated into a robust biofilm pellicle on the surface of dentures. Oral biofilms can be considered microbial reservoirs and significantly affect the oral and systemic health of denture users [1, 3].

Polyene and azole drugs, such as nystatin and miconazole, respectively, have been commonly prescribed for the treatment of oral candidiasis [2]. However, recent years have seen failures in antifungal therapy due to increasing microbial resistance rates and high drug toxicity, which have altogether contributed to rise the prevalence of morbidity and mortality indicators related to fungal infections [4].

Capítulo de livro

SUBSTÂNCIAS COM ATIVIDADE CONTRA FUNGOS DO GÊNERO *Aspergillus*

Elba dos Santos Ferreira^a
Ricardo Dias de Castro^b

INTRODUÇÃO AO TEMA

Aspergilose é uma infecção fúngica oportunista, das mais comuns em todo o mundo, e atinge principalmente indivíduos com baixa imunidade. É causada por fungos do gênero *Aspergillus*, que compreende fungos filamentosos, amplamente distribuídos na natureza, sendo encontrados no solo, no material orgânico em decomposição, podendo originar vários quadros clínicos, responsáveis por altas taxas de mortalidade¹.

Sua contaminação para o ser humano se dá por inalação de estruturas reprodutivas, chamadas esporos ou conídios, sendo a via aérea a principal via de penetração. Entretanto, esses fungos podem contaminar ferimentos, como também penetrar nos tecidos por ocasião de cirurgias. A inalação de esporos também pode causar doenças pulmonares que vão desde a inflamação local das vias aéreas a infecções pulmonares graves e com risco de vida, tais como Aspergilose broncopulmonar alérgica e Aspergilose invasiva².

Além da infecção nos pulmões, que é a forma mais grave da Aspergilose, os fungos do gênero *Aspergillus* também podem causar Aspergilose cutânea (infecção na pele), otomicose aspergilar (infecção do ouvido), onicomicose aspergilar (infecções nas unhas), sinusite aspergilar (infecção dos seios paranasais), acometimento neurológico, e micotoxicoses, que representam intoxicações por toxinas produzidas por este gênero, que contaminam alimentos³.

O medicamento mais utilizado e mais eficiente hoje para o tratamento da Aspergilose é o Voriconazol. Entretanto, em alguns casos, pode ocorrer uma “adaptação” dos fungos a esse medicamento criando mecanismos de re-

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Livro

Obesidade como determinante para o desenvolvimento da disfunção erétil

A obesidade é uma doença crônica, caracterizada pelo aumento demasiado da adiposidade corporal, que apresenta uma etiologia multifatorial abrangendo fatores relacionados à ingestão excessiva de alimentos pouco saudáveis, redução progressiva da atividade física, fatores genéticos, metabólicos, sociais, comportamentais e culturais. Recentemente, o impacto da obesidade sobre a fertilidade masculina elevou o interesse da população em entender os processos subjacentes a essa problemática e, dessa forma, minimizar as alterações nocivas à função sexual, como a disfunção erétil. Esta é definida como a incapacidade constante de alcançar e/ou manter uma ereção peniana suficiente para a satisfação sexual e, desponta entre os problemas de saúde mais temidos pelos homens. Do ponto de vista terapêutico, é possível reverter esse quadro através de procedimentos cirúrgicos, da utilização de dispositivos que promovem a ereção por vácuo, da reposição hormonal e da utilização de um arsenal de medicamentos. Nesse livro, descubra a interface fisiopatológica entre a obesidade e a disfunção erétil.



Farmacêutica pela Universidade Federal da Paraíba (UFPB), Mestre e Doutora em Farmacologia pelo Programa de Pós-graduação em Produtos Naturais e Sintéticos Bioativos (PgPNSB/UFPB). Professora Doutora de Medicina da Universidade Estadual de Roraima. Atuando com os temas de obesidade, disfunção erétil e sinalização dos processos fisiopatológicos.



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Obesidade como determinante para o desenvolvimento da disfunção erétil

Modulação negativa da obesidade sobre a função erétil: determinante primário nos problemas sexuais masculinos

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We certify that the poster "**Spirulina platensis supplementation improves rat erections in a model of erectile dysfunction induced by hypercaloric diet**" by **Souza ILL, Barros BC, Ferreira ES, Carvalho MTL, Interaminense LFL, Cavalcante FA, Silva BA** was presented at the 49th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE), held in Ribeirão Preto, São Paulo, Brazil, from October 17 to 20, 2017.

Maria Christina W. Avellar
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