

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
CENTRO DE BIOTECNOLOGIA  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

**ANÁLISE *EX VIVO* DO POTENCIAL ANTIFÚNGICO DA  
MOLÉCULA PLUMIERIDINA EM *Cryptococcus neoformans***

TESE DE DOUTORADO

Vanessa de Abreu Barcellos

Porto Alegre, abril de 2018

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do título de Doutor em Ciências.

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## LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

5-FUTP	trifosfato de 5- fluorouridina
5-FdUMP	5-fluoro-2' desoxiuridina-5' -monofosfato
5- FC	5-fluorocitosina
° C	grau Celsius
AFLP	polimorfismo de comprimento de fragmento amplificado
AIDS	do inglês, <i>acquired immunodeficiency syndrome</i>
AMB	anfotericina B
Ab 18B7	anticorpo 18B7
BHE	barreira hematoencefálica
cDNA	DNA complementar
COBEA	Colégio Brasileiro de Experimentação Animal
CONCEA	Conselho Nacional de Controle de Experimentação Animal
DMEM	do Inglês, <i>Dulbecco's Modified Eagle Medium</i>
DNA	ácido desoxirribonucleico
FDA	do inglês, <i>Food and Drug Administration</i>
FLZ	fluconazol
GFP	do inglês, <i>green fluorescent protein</i>
HIV	do inglês, <i>human immunodeficiency vírus</i>
IDSA	do inglês, <i>Infectious Diseases Society of America</i>
mg	miligrama (s)
ml	mililitro (s)
mAb18B7	anticorpo monoclonal anti - cápsula 18B7
MLST	tipagem de sequência multilocus
MIC	concentração mínima inibitória
ng	nanograma (s)
KAT	Katushka
OMS	Organização Mundial da Saúde
ORF	do inglês, <i>open reading frame</i>
PBS	do inglês, <i>phosphate buffered solution</i>
PLD	plumieiridina
RNA	ácido ribonucleico
RT-qPCR	pcr em tempo real
r.p.m	rotação por minuto
SNC	sistema nervoso central
SOD	superóxido dismutase
UFC	unidades formadoras de colônia
VLZ	voriconazol
YPD	do inglês, <i>yeast peptone dextrose</i>
µg	micrograma (s)
µl	microlitro (s)

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## RESUMO

Criptococose é uma infecção fúngica invasiva causada pelas espécies patogênicas *Cryptococcus neoformans* e *Cryptococcus gattii*. Esta doença afeta aproximadamente 200.000 indivíduos mundialmente. A alta incidência de mortalidade por meningoencefalite criptococócica associada a problemas de toxicidade, resistência e escassez de terapias antifúngicas torna necessária a pesquisa por novos fármacos. Nosso grupo identificou o potencial terapêutico de um iridóide, plumieridina, isolado do extrato aquoso de sementes de *Allamanda polyantha* (apocinácea). Sendo assim, o objetivo desse estudo é avaliar as atividades biológicas do composto plumieridina contra *C. neoformans*. Nós propusemos o uso de um modelo prático para avaliar a eficácia desse composto como tratamento para a criptococose. Nossa estratégia aborda a infecção de camundongos com uma linhagem fluorescente de *C. neoformans*, seguida de uma análise de órgãos colonizados baseada na fluorescência. Camundongos foram infectados intranasalmente com linhagens fluorescentes de *C. neoformans* e tratados com plumieridina ou fluconazol durante 14 dias. Os pulmões e o cérebro foram excisados e examinados no equipamento IVIS LUMINA II, para quantificação da fluorescência e determinação de unidades formadoras de colônia (UFC). A análise por imagem demonstrou uma redução da carga fúngica cerebral e pulmonar de camundongos infectados com *C. neoformans*. Além disso, para identificar possíveis alvos de plumieridina, estudos *in silico* foram realizados utilizando o servidor pharmMapper. Entre os alvos preditos, três proteínas envolvidas na via das pirimidinas foram identificadas, timidilato-sintase, orotidine-5'-fosfato-descarboxilase e diidroorotato-desidrogenase. As estruturas de timidilato-sintase e orotidina-5'-fosfato-descarboxilase de *C. neoformans* foram modeladas e submetidas à técnica de *docking* molecular com a molécula plumieridina, para melhor entender os mecanismos de interação com *C. neoformans*. Plumieridina interage com os aminoácidos no centro catalítico das enzimas. Além disso, o perfil de expressão de genes de *C. neoformans* relacionados à via das pirimidinas na presença de plumieridina também foi avaliado por PCR em tempo real (RT-qPCR). Uma diminuição significativa nos níveis de transcrição dos genes da timidilato sintase e da orotidine-5'-fosfato-descarboxilase foi detectada quando as células foram cultivadas na presença de plumieridina. Nossos resultados podem contribuir para descrição e desenvolvimento de novos antifúngicos para o tratamento da criptococose.



## ABSTRACT

Cryptococcosis is an invasive fungal infection caused mainly by the pathogenic species *Cryptococcus neoformans* and *Cryptococcus gattii*. This disease affects approximately 200,000 individuals annually worldwide. Currently, antifungal drugs show some limitations related to toxicity and resistance, which emphasizes the importance of search for new drugs. Our group identified the antifungal potential of one iridoid - Plumieridine - isolated from the aqueous extract of *Allamanda polyantha* (Apocynaceae) seeds. Therefore, the objective of this study is to evaluate the biological activities of plumieridine compound against *C. neoformans*. We propose the use of a practical model to evaluate the treatment efficacy of this compound against *C. neoformans*. Our approach involves mice infection with *C. neoformans* fluorescent strain, followed by fluorescence-based imaging analysis of the colonized organs. Mice were infected intranasally with the fluorescent *C. neoformans* strain and treated with plumieridine or fluconazole for 14 days. The lung and brain were excised and examined in an IVIS Lumina II instrument to determine fluorescence intensity and for CFU determination. Imaging analysis showed that the treatment with plumieridine reduced the pulmonary and cerebral fungal burden of mice infected with *C. neoformans*. Moreover, in order to identify a possible target of plumieridine, studies *in silico* were performed using PharmMapper server. Among the predicted targets, three proteins from the route of pyrimidines were identified, thymidylate synthase, orotidine-5'-phosphate decarboxylase and dihydroorotate dehydrogenase. The structures of thymidylate synthase and orotidine-5'-phosphate decarboxylase were modeled to *C. neoformans* and docked with plumieridine. We found that plumieridine interact with the amino acids at the catalytic center of the enzymes. Furthermore, the gene expression profile of *C. neoformans* related to route of pyrimidines in the presence of plumieridine was evaluated by RT-qPCR. A significant decreased in the transcript levels of thymidylate synthase and orotidine-5'-phosphate decarboxylase genes was detected when cells were cultured in the presence of plumieridine. Our results may contribute for description and development of new antifungal compounds for cryptococcosis treatment.

## 1. JUSTIFICATIVA

De acordo com as estimativas, existem cerca de 5 milhões de espécies fúngicas em nível mundial, mas somente uma algumas dessas espécies causam infecções em humanos (O'brien *et al.*, 2005; Blackwell, 2011; Kim, 2016). No Brasil, um recente estudo demonstra que doenças fúngicas afetam mais de 3,8 milhões da população (Giacomazzi *et al.*, 2016). A alta incidência de mortalidade se deve geralmente a infecções fúngicas invasivas, como candidíase, aspergilose, histoplasmose e criptococose que ocorrem principalmente em indivíduos imunocomprometidos (Ostrosky-Zeichner *et al.*, 2010; Martín-Peña *et al.*, 2014). A criptococose é uma infecção causada majoritariamente pelas espécies *Cryptococcus neoformans* e *Cryptococcus gattii* (Skolnik *et al.*, 2017). Essas duas espécies têm gerado 220.000 casos anualmente, causando mais de 181.100 mortes, sendo a segunda maior causa de mortalidade em indivíduos acometidos pelo HIV, ficando abaixo somente da tuberculose (Rajasingham *et al.*, 2017). No entanto, a doença não é reconhecida pela Organização Mundial da Saúde como uma doença tropical negligenciada (Molloy *et al.* 2017).

Criptococose é fatal sem tratamento efetivo (Coelho e Casadevall, 2016). A Sociedade Americana de Doenças Infecciosas (IDSA) recomenda como tratamento efetivo anfotericina B (AMB) em combinação com 5-fluorocitosina (5-FC) nas primeiras duas semanas de infecção, posteriormente a continuidade do tratamento é com fluconazol (FLZ) (Perfect *et al.*, 2010). Entretanto, esses fármacos utilizados apresentam algumas limitações como, resistência, toxicidade e alto custo. AMB pode gerar efeitos adversos como nefrotoxicidade devido ao uso prolongado da terapia medicamentosa (Lewis, 2011; Williamson 2017). A resistência gerada por fluconazol é um problema emergente e a medicação prolongada pode ocasionar outras infecções

fúngicas (Sheehan *et al.*, 1999). Na África, a resistência a antifúngicos pertencentes à classe dos azóis possui um alto índice (Mpoza *et al.* 2017). Além disso, não há disponibilidade da 5-FC na África e na Ásia onde o índice de meningite criptocócica é altíssimo (Loyse *et al.*, 2013). Portanto, existe uma necessidade premente de desenvolvimento de novos antifúngicos eficientes frente à meningite criptocócica. Tecnologias por imagem têm contribuído significativamente no desenvolvimento de potenciais fármacos para o tratamento de doenças e infecções (Andreu *et al.*, 2013). A utilização de marcadores fluorescentes permite avaliar em tempo real a distribuição do patógeno no hospedeiro, bem como a visualização *in vivo* e *ex vivo* da ação dos tratamentos terapêuticos sobre a infecção (Kong *et al.*, 2016).

Plantas são fontes importantes para a descoberta de novos medicamentos. De 1.453 novos fármacos aprovados pela agência reguladora norte americana (FDA) 40 % são provenientes de compostos naturais ou derivados (Katz e Baltz, 2016). Em estudos anteriores, nosso grupo identificou o potencial antifúngico do extrato bruto e do iridóide, plumieridina, de *Allamanda polyantha* (Apocynaceae), contra *C. neoformans* e *C. gattii* (Bresciani, 2013).

A emergência de microrganismos multi-resistentes associado à toxicidade de fármacos atuais reforça a necessidade do desenvolvimento de novas estratégias para controle e tratamento da criptococose (Rodrigues, 2016). Nesse contexto, o presente trabalho propõe utilizar um modelo de monitoramento por bioimagem baseado na emissão de fluorescência, a fim de monitorar a ação do composto natural plumieridina sobre a infecção de *C. neoformans*.

## 2. REVISÃO DA LITERATURA

### 2.1 *Cryptococcus neoformans* e *Cryptococcus gattii*

O gênero *Cryptococcus* é conhecido há mais de 100 anos, aproximadamente mais de 70 espécies estão incluídas nele. *C. neoformans* e *C. gattii* são os agentes causadores da criptococose, doença que acomete humanos e animais (Kwon-Chung *et al.*, 2014). Embora essas duas espécies apresentem uma similaridade de 80-90 % do genoma, características específicas e distintas quanto à patogenicidade e ecologia são descritas (Kavanaugh *et al.*, 2006; Chang *et al.*, 2015). *C. neoformans* acomete indivíduos imunocomprometidos, transplantados, com câncer, em tratamento com imunossupressores e infectados com HIV, apresentando uma ampla distribuição global (Lin, 2009). Já *C. gattii* é considerado um patógeno primário, acomete também indivíduos imunocompetentes e é comumente isolado de árvores, especialmente *Eucalyptus* spp., e também já foi relatado seu isolamento no ar e no solo (Ngamskulrunroj *et al.*, 2012). Como existe uma grande quantidade de partículas infectantes no ambiente, o fungo poderá entrar em contato com diferentes hospedeiros. Alguns estudos relataram que *C. neoformans* e *C. gattii* podem sobreviver e se replicar no interior de amebas e nematódeos no solo, supondo que esses hospedeiros alternativos possam ter um papel importante na distribuição e virulência de diferentes linhagens (Steenbergen *et al.*, 2001; Steenbergen e Casadevall, 2003; Fu e Casadevall, 2018).

A levedura *C. neoformans* foi isolada pela primeira vez em 1894, em amostras de suco de frutas, pelo cientista italiano Francesco Sanfelice (Staib *et al.*, 1973). Devido ao aumento no índice de indivíduos portadores de HIV com criptococose causada por *C. neoformans*, esta levedura passou a ser considerada um importante patógeno humano oportunista. *C. neoformans* é considerado a espécie modelo do complexo (Maziarz e Perfect, 2016). O número de casos de criptococose causado por *C. gattii* é

significativamente menos frequente mundialmente (Kwon-Chung *et al.*, 2014). A distribuição de *C. gattii* era considerada restrita a regiões de clima tropical e subtropical, contudo, ganhou mais atenção da comunidade científica após ter ocasionado um surto de criptococose na ilha de Vancouver, na Columbia Britânica, Canadá (Kidd *et al.*, 2004). Por muito tempo a criptococose foi considerada como uma doença causada apenas pela espécie *C. neoformans*. Posteriormente, após diversos estudos moleculares, *C. neoformans* variedade *gattii* foi categorizada como uma nova espécie: *Cryptococcus gattii* (Boekhout *et al.*, 1997). Estima-se que *C. gattii* tenha divergido de *C. neoformans* aproximadamente há 37,5 milhões de anos. *C. neoformans* e *C. gattii* são leveduras encapsuladas classificadas em cinco sorotipos (A, B, C, D e AD) de acordo com as características antigênicas da cápsula polissacarídica (Franzot *et al.*, 1999). Os sorotipos A e D correspondem a *C. neoformans* var. *grubii* e *C. neoformans* var. *neoformans*. Já os sorotipos B e C correspondem a *C. gattii* (anteriormente classificado como *C. neoformans* var. *gattii*) (Kwon-Chung *et al.*, 1982). Por sua vez, as duas espécies são subdividas em quatro tipos moleculares de acordo com padrões obtidos por técnicas como PCR *fingerprint*, Análise de polimorfismo de tamanho fragmento amplificado (AFLP) e Tipagem de sequência multilocus (MLST): VNI, VNII, VNIII, VNIV e VNB correspondem aos tipos moleculares de *C. neoformans* e VGI, VGII, VGIII e VGIV a *C. gattii*. Recentemente, pela utilização de metodologias de sequenciamento foi proposta a divisão de *C. neoformans* em duas espécies (*C. neoformans* e *Cryptococcus denoformans*) e a divisão de *C. gattii* em 5 espécies (*C. gattii*, *Cryptococcus bacillisporus*, *Cryptococcus deuterogattii*, *Cryptococcus tetragattii* e *Cryptococcus decagattii*) (Hagen *et al.*, 2015). Apesar da nova proposta, não existe ainda uma caracterização biológica detalhada de cada uma das sete espécies propostas.

## **2.2 PRINCIPAIS DETERMINANTES DE VIRULÊNCIA**

Microrganismos patogênicos estabelecem uma relação complexa com o hospedeiro infectado. Após a infecção, o patógeno reconhece o ambiente do hospedeiro e responde com mudanças celulares adaptativas. Esta resposta frequentemente inclui a indução de atributos que proporcionam ao microrganismo uma melhor sobrevivência e proliferação em seu novo ambiente (Hernández-Chávez *et al.*, 2017). Entre os principais atributos relacionados à virulência, destacam-se a presença de uma cápsula polissacarídica, a produção de melanina e a capacidade de desenvolvimento a 37°C (Dylag, 2015; Taylor-Smith e May, 2016).

### **2.2.1 Cápsula polissacarídica**

A cápsula polissacarídica é o principal fator associado à virulência de *C. neoformans* e *C. gattii* (Zaragoza *et al.*, 2009). Sua importância é evidenciada quando mutantes que apresentam defeitos na formação capsular são avirulentos no modelo de infecção murino (Chang e Kwon-Chung, 1994; Zaragoza *et al.*, 2008).

A cápsula polissacarídica envolve a parede celular do fungo formando uma barreira física e é constituída por dois polissacarídeos: o principal polissacarídeo é a glucoronoxilomanana (GXM) que corresponde cerca de 90% da massa dessa estrutura; além da GXM, a cápsula também é constituída de galactoxilomanana (GalXM) em menor quantidade (5%) e de 1% de manoproteínas (Zaragoza *et al.*, 2009). Vários estudos comprovaram que esses polissacarídeos são sintetizados intracelularmente e transportados por vesículas através da parede celular, e posteriormente são ligados não covalentemente à parede da célula (O'meara e Alspaugh, 2012).

A cápsula de *C. neoformans* pode alterar de tamanho e essa variação pode estar relacionada com alguns estímulos ambientais, como pressão osmótica e quantidade de glicose. Isolados ambientais apresentam tamanho de cápsula reduzido, e o mesmo ocorre em condições padrão de laboratório, com cultivo em meio rico em nutrientes (Zaragoza e Casadevall, 2004). Porém, *in vivo*, as células de *Cryptococcus* spp. apresentam grande aumento no tamanho da cápsula, e ainda observa-se diferença de tamanho conforme o órgão analisado no hospedeiro (Crabtree *et al.*, 2012). As células isoladas de pulmão apresentam cápsulas mais proeminentes do que as de células isoladas de cérebro (Zaragoza e Casadevall, 2004). O aumento da cápsula ocorre principalmente devido a uma resposta a espécies reativas de oxigênio, peptídeos antimicrobianos e ao antifúngico AMB. Esse aumento capsular auxilia o patógeno a sobreviver dentro do fagossomo (Bojarczuk *et al.*, 2016). Nos pulmões, a presença de baixas quantidades de ferro e altos níveis de CO<sub>2</sub> promove o aumento da cápsula, também verificado durante os períodos de infecção aguda. *In vitro*, também pode ser induzida por altas concentrações de CO<sub>2</sub>, pH, nitrogênio, limitação de glicose, limitação de ferro e por soro de mamíferos em baixos níveis de CO<sub>2</sub> (Doering, 2009). Sendo assim, a cápsula polissacarídica é uma estrutura dinâmica regulada em resposta a vários estímulos (Doering, 2009).

Durante a infecção a cápsula polissacarídica modula a resposta imune do hospedeiro, pois tem ação antifagocítica, inibindo a ação de macrófagos, células dendríticas e neutrófilos (Bojarczuk *et al.*, 2016). Já foi observado que mutantes acapsulados de *C. neoformans* não se replicam no interior de macrófagos; além disso, foi demonstrado que macrófagos fagocitam preferencialmente células com cápsulas menores (Feldmesser *et al.*, 2000; Bojarczuk *et al.*, 2016). Quando internalizada por macrófagos, a levedura pode liberar polissacarídeos da cápsula, em vesículas, no

citoplasma dos macrófagos alterando sua função ou causando lise, podendo inibir a liberação de citocinas e a migração de leucócitos para os sítios de inflamação; atua também, como proteção frente a espécies reativas de oxigênio (Leopold Wager *et al.*, 2016). Além disso, no meio ambiente a cápsula é responsável por conferir proteção contra a desidratação e predadores naturais (Alspaugh, 2015).

### **2.2.2 Produção do pigmento melanina**

A melanina é produzida por uma ampla variedade de fungos e também se mostra importante para infecção causada por leveduras patogênicas do complexo *Cryptococcus* spp. (Casadevall *et al.*, 2000). Encontra-se normalmente na parede celular, desempenhando importante papel na proteção a variações de temperatura e predadores naturais, como as amebas (Malliaris *et al.*, 2004; Nosanchuk e Casadevall, 2006). Este pigmento é hidrofóbico, negativamente carregado, de alta massa molecular. Ele é formado pela polimerização oxidativa de compostos fenólicos e/ou indólicos (Nosanchuk e Casadevall, 2006). Foi demonstrado que a deleção de genes envolvidos na melanização de *Cryptococcus* spp. causa uma drástica redução na letalidade (Salas *et al.*, 1996) e disseminação no hospedeiro (Noverr *et al.*, 2004).

A síntese de melanina é catalisada pela enzima lacase a partir de fontes exógenas de 3-4 dihidroxifenil alanina (L-DOPA), norepinefrina, epinefrina e dopamina (Casadevall *et al.*, 2000; Nosanchuk *et al.*, 2015). Devido à disponibilidade desses compostos como neurotransmissores, é possível o tropismo do complexo *Cryptococcus* spp. para o SNC. Regiões do cérebro ricas em catecolaminas, como o núcleo basal, são as mais afetadas (Nosanchuk *et al.*, 2000).

Durante a infecção, a enzima lacase também está intimamente associada à proteção contra macrófagos alveolares, reduzindo a ação antifagocítica (Zhu e



Williamson, 2004). Alguns estudos também demonstraram que alta atividade da lacase aumenta a sobrevivência de *C. neoformans* no interior dos macrófagos (Sabiiti *et al.*, 2014). Além disso, a expressão do gene LAC1 A está associada à disseminação da infecção dos pulmões para outros órgãos (Noverr *et al.*, 2004). Estudos *in vitro* demonstraram que a presença do pigmento protege as células de oxidação e do antifúngico anfotericina B (Jacobson e Tinnell, 1993). A terapia antifúngica se mostrou menos efetiva em isolados clínicos de *C. neoformans* que apresentaram uma maior atividade da lacase associado com a produção de melanina (Nosanchuk e Casadevall, 2006). Em relação à secreção do pigmento, a atividade da lacase é associada com vesículas extracelulares secretadas por *C. neoformans*, sugerindo uma possível participação dessas estruturas na síntese e secreção de melanina (Rodrigues *et al.*, 2008).

### **2.2.3 Capacidade de desenvolvimento a 37°C**

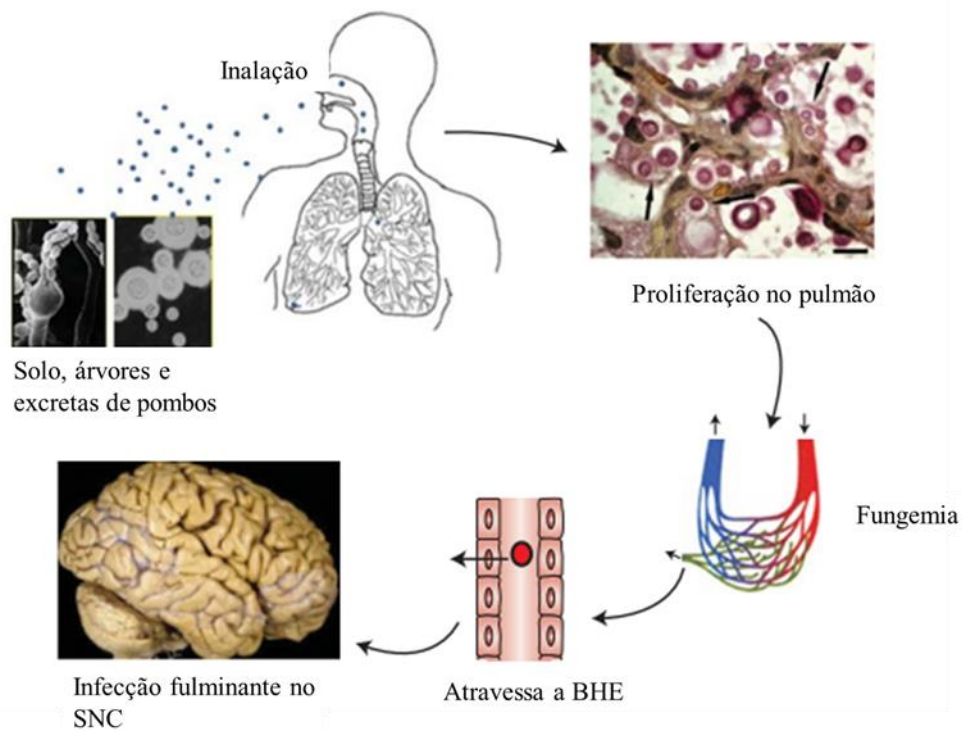
A capacidade de desenvolvimento a temperatura fisiológica é essencial para que ocorra o estabelecimento da infecção por *C. neoformans* e *C. gattii*, sendo que as demais espécies do complexo *Cryptococcus* são incapazes de estabelecer a infecção e raramente possuem a capacidade de desenvolvimento a 37°C *in vitro*. Estes atributos estão diretamente relacionados à ação de uma variedade de produtos gênicos. Um dos primeiros genes associados ao desenvolvimento a 37°C foi *CNA1* que codifica calcineurina A, a subunidade catalítica da proteína calcineurina (Odom *et al.*, 1997). Com a construção de um mutante para o gene *CNA1*, foi comprovado que calcineurina é necessária para virulência de *C. neoformans*. Estudos demonstraram que mutantes de *C. gattii* para *CNA1* também apresentam deficiência de desenvolvimento a 37°C e virulência atenuada ou ausente (Odom *et al.*, 1997). Além disso, foi observado que tanto em *C. neoformans* quanto em *C. gattii* mutantes para calcineurina apresentam disrupção

da membrana plasmática a 37°C. Contudo, diferentemente de *C. neoformans*, *C. gattii* requer calcineurina para o desenvolvimento na presença de Ca<sup>2+</sup>, sugerindo diferenças no papel da proteína para homeostase deste íon (Chen *et al.*, 2013). Análise do perfil transcricional de *C. neoformans* a temperatura fisiológica demonstrou indução da expressão de 49 genes. Rosa e Silva e colaboradores identificaram 29 genes diferencialmente expressos a 37°C, relacionados à sinalização, montagem da parede celular, estresse e integridade da membrana (Rosa e Silva *et al.*, 2008).

## **2.3 CRIPTOCOCOSE**

### **2.3.1 Patogênese da criptococose**

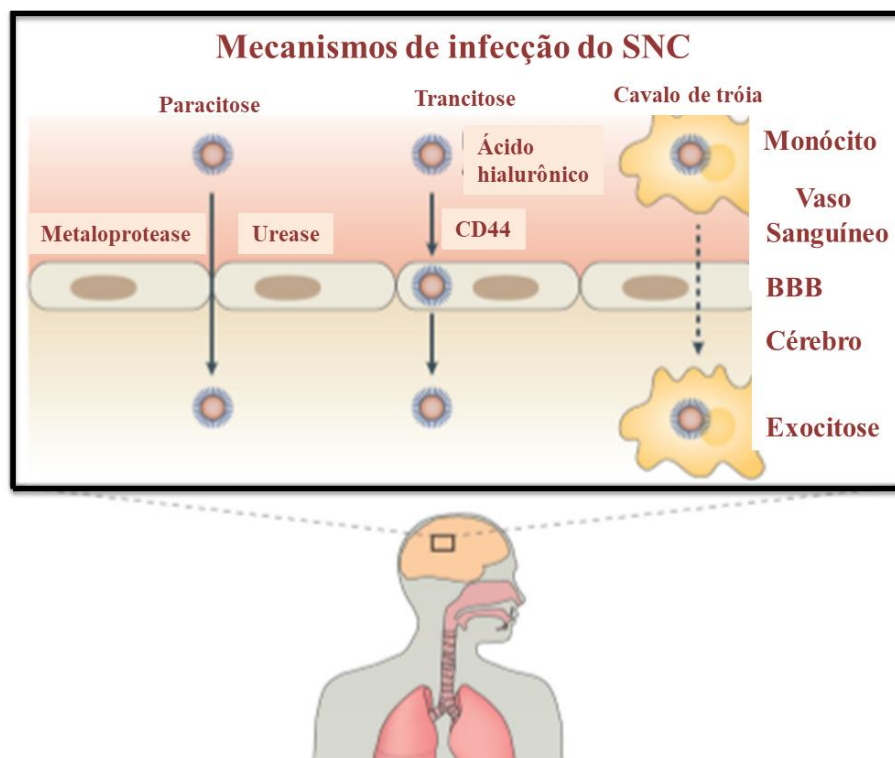
A meningite criptocócica (MC) é a causa mais comum de meningite em adultos que pode levar a óbito (Taylor-Smith e May, 2016). A prevalência da infecção é em pacientes imunocomprometidos, porém o número de infecção em indivíduos imunocompetentes tem aumentado (Bielska e May, 2016; May *et al.*, 2016). Acredita-se que a via de infecção envolva a inalação de células leveduriformes desidratadas ou basidiósporos do ambiente, com tamanho aproximado de 1-3 µm, podendo gerar uma infecção aguda ou ainda assintomática que pode ser eliminada pelo sistema imune ou permanecer como forma latente da infecção (Velagapudi *et al.*, 2009). O quadro da infecção pode restabelecer quando a imunidade do hospedeiro estiver comprometida, e o patógeno pode se disseminar pela via hematogênica e atingir diversos órgãos, como ossos, pele, olhos, trato urinário, entre outros, até o sistema nervoso central (SNC), ocasionando o quadro mais grave da doença, a meningite (**Figura 1**) (Sabiiti e May, 2012; Colombo e Rodrigues 2015).



**Figura 1.** Modelo de disseminação de *Cryptococcus* spp. do ambiente ao SNC. Figura adaptada de (Kwon-Chung *et al.*, 2014).

As células criptocócicas se proliferam extra e intracelularmente no trato respiratório, podendo ou não interagir com as células fagocíticas, como exemplo os macrófagos alveolares, onde podem rapidamente se desenvolver e proliferar após a internalização (Gordon e Read, 2002). Além da capacidade de se replicar no interior de macrófagos, as células podem ser transferidas de um macrófago para o outro, de maneira não destrutiva. Esse mecanismo facilita o transporte desse patógeno para diferentes órgãos, inclusive a travessia da barreira hematoencefálica (BHE), pelo qual o fungo ultrapassa o endotélio no interior do monócito. Essa via de disseminação da infecção é conhecida como “cavalo de Tróia” (**Figura 2**) (Kim, 2008). Além desse mecanismo, é possível a entrada do patógeno no SNC, através da transcitose, quando a célula leveduriforme, inicialmente esférica, atinge uma forma ovoide possibilitando interações com as células endoteliais (Chang *et al.*, 2011). Esse sistema envolve a

captação das células fúngicas pelas células endoteliais, que também se modificam, e a transmigração carrega o fungo pelo citoplasma até atingir o cérebro (Vu *et al.*, 2009). Recentemente, foi demonstrado que a produção e liberação da metaloprotease Mpr1 por *C. neoformans* é essencial para o remodelamento da membrana celular endotelial. Experimentos de infecção de camundongos com linhagens mutantes para Mpr1 apresentaram diminuição na quantidade de unidades formadoras de colônias no cérebro (Vu *et al.*, 2014; Na Pombejra *et al.*, 2017). Outro mecanismo pelo qual o fungo consegue alcançar o SNC é denominado paracitose e ocorre quando a célula fúngica atravessa a BHE entre as junções celulares, dessa forma alterando a integridade da BHE. Estudos associados aos mecanismos de transmigração comprovam que a inibição da enzima urease é capaz de impedir a infecção do SNC por *C. neoformans*, demonstrando a importância da participação dessa enzima para o patógeno atravessar capilares cerebrais (Shi *et al.*, 2010).



**Figura 2. Mecanismos de infecção do SNC.** Após a replicação no pulmão, *Cryptococcus* spp. é capaz de se disseminar a outros tecidos, incluindo o SNC. A entrada no SNC pode ocorrer por três vias: O mecanismo mais conhecido é o “cavalo de tróia”, onde *Cryptococcus sp.* ultrapassa a BHE por meio de macrófagos infectados. Paracitose, a célula fúngica ultrapassa a barreira pelas junções celulares enquanto a trancitose ocorre por meio das células endoteliais. Figura adaptada de May *et al.*, (2016).

### 2.3.2 Manifestações clínicas

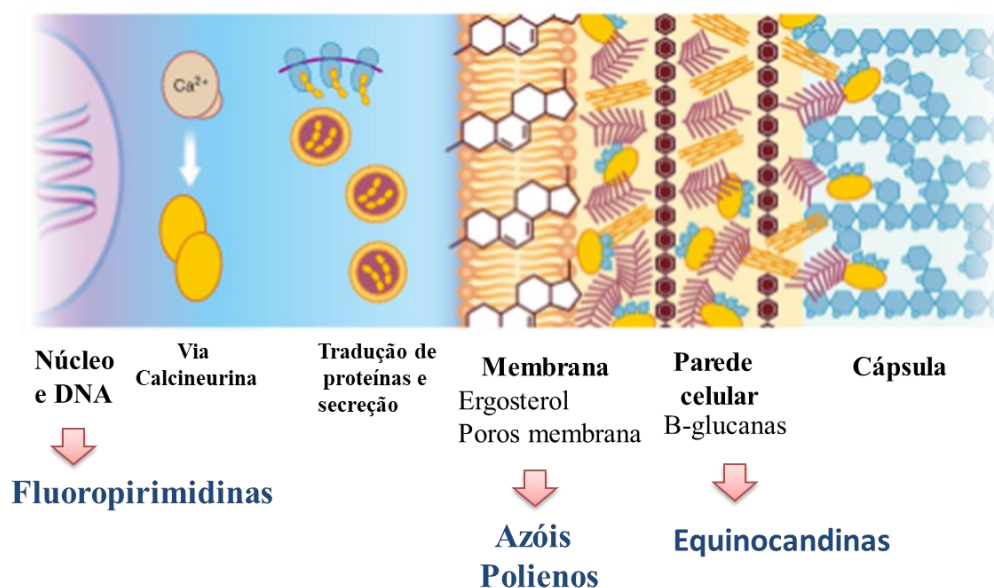
A criptococose atinge principalmente os pulmões e o SNC. *C. gattii* produz criptococomas nos pulmões e no cérebro, os quais podem estar associados com complicações no SNC (La Hoz e Pappas, 2013). Geralmente, o tratamento requer intervenção cirúrgica e/ou terapia antifúngica prolongada, muitas vezes havendo sequelas neurológicas. Na infecção por *C. neoformans* as lesões pulmonares e cerebrais se apresentam na forma de infiltrados difusos e o índice de mortalidade dos indivíduos infectados é muito maior (Chen *et al.*, 2000). Diferentes estudos comparando a progressão da doença mostraram que *C. gattii* causa frequentemente infecção pulmonar em indivíduos imunocompetentes, com menor número de casos de disseminação ao SNC (Espinel-Ingroff e Kidd, 2015). Além disso, foi observada maior carga fúngica nos pulmões de camundongos infectados por via intranasal com *C. gattii* e não foram observadas células no cérebro até a terceira semana de infecção (Ngamskulrunroj *et al.*, 2012). Já os camundongos infectados por via intranasal com *C. neoformans* apresentaram meningoencefalite grave, e as leveduras foram detectadas no cérebro na primeira semana de infecção. Entretanto, camundongos infectados por via intravenosa, tanto *C. gattii* quanto *C. neoformans* causaram infecção cerebral grave (Ngamskulrunroj *et al.*, 2012).

As manifestações clínicas da criptococose podem ocasionar diferentes sintomas no paciente. Dentre eles, podem ser relatados dor de cabeça, letargia, febre,

irritabilidade e perda de memória (Sabiiti e May, 2012; Maziarz e Perfect, 2016). Esses sintomas podem permanecer por um período longo. Entretanto, em pacientes com debilidade no sistema imunológico, o quadro da meningoencefalite é mais grave. Ocorre um aumento de células fúngicas no SNC, e, por consequência, uma maior concentração de conteúdo polissacarídico no líquido cefalorraquidiano, ocasionando um aumento na pressão intracraniana, que pode gerar neuropatia craniana (Maziarz e Perfect, 2016).

## 2.4 AGENTES ANTIFÚNGICOS

Os agentes antifúngicos, com base em seus mecanismos de ação, podem ser classificados nos seguintes grupos: azóis, polienos fluoripirimidinas e equinocandinas (Figura 3) (Roemer e Krysan, 2014).



**Figura 3.** Esquema demonstrativo de alvos antifúngicos em *Cryptococcus* spp. Figura adaptada de Coelho e Casadevall (2016).

### 2.4.1 Inibidores da biossíntese do ergosterol - azóis

Ergosterol é o principal componente da membrana celular e contribui para uma variedade de funções celulares como a fluidez e integridade da membrana (Tatsumi *et al.*, 2013). Azóis são os antifúngicos mais utilizados no uso clínico devido ao amplo

espectro de atividade que possuem. Seu principal alvo é a enzima citocromo P450 lanosterol 14 $\alpha$ -demetilase codificada pelo gene ERG11, que catalisa a remoção do grupo 14 $\alpha$ -metil do lanosterol, convertendo-o em ergosterol na membrana celular (Kathiravan *et al.*, 2012). Esta enzima contém porfirina de ferro em sua atividade ativa e causa bloqueio da via de biossíntese de ergosterol resultando acúmulo de 14 $\alpha$ -metil-3,6-diol que é tóxico e inibe a replicação e o desenvolvimento fúngico (Kanafani e Perfect, 2008). A conformação exata do sítio ativo difere entre espécies diferentes de fungos e P450 mono-oxigenases mamíferas. O tipo de interação entre cada molécula azol e cada tipo de P450 determina a característica do antifúngico e seu efeito (Kanafani e Perfect, 2008).

Os azóis são moléculas orgânicas cíclicas que podem ser classificados em dois grupos: imidazóis e triazóis. Os imidazóis (cetoconazol, miconazol, clotrimazol e econazol) foram os primeiros a serem desenvolvidos. No entanto, devido à alta toxicidade e interação com outros medicamentos foram substituídos pelos triazóis (Roemer e Krysan, 2014). A primeira geração de triazóis (itraconazol e fluconazol) apresentou um amplo espectro de atividade antifúngica quando comparado com imidazol. Fluconazol é ativo contra *Candida* spp., *C. neoformans*, *Histoplasma* spp., e *Blastomyces* spp (Garrelts *et al.*, 2004). Itraconazol apresenta um amplo espectro de atividade contra leveduras. No entanto, uma limitação clínica é sua ineficácia contra alguns patógenos emergentes como *Scedosporium* spp., *Fusarium* spp., e *Mucorales* spp. (Denning e Hope, 2010).

O uso de imidazóis restringe-se ao tratamento de micoses superficiais e os triazóis podem causar hepatotoxicidade e interagir com outros medicamentos, o que pode causar problemas no tratamento de pacientes que recebem medicação diversificada

(Chen e Sorrell, 2007). Com isso, uma segunda geração de triazóis foi desenvolvida: voriconazol e posaconazol foram aprovados pelo órgão regulador americano (FDA) em 2002 e 2006, respectivamente (Kwon e Mylonakis, 2007). Ambos são considerados fungicidas e também tem um amplo espectro de atividade.

#### **2.4.2 Disruptores da membrana fúngica - polienos**

Polienos são moléculas orgânicas macrolíticas conhecidas como macrolídeos. Nistatina, natamicina e AMB são os três polienos de uso clínico. Eles são produtos naturais e foram isolados de cultivos de *Streptomyces noursei*, *Streptomyces natalensis* e *Streptomyces nodosum* (Hossain e Ghannoum, 2000). Devido a sua estrutura anfifílica essas moléculas se ligam a bicamada lipídica e formam um complexo com o ergosterol produzindo poros. Com isso causam desestabilização da estrutura, formando áreas de tensão na membrana, aumentando a permeabilidade a cátions, e por fim, causando morte celular (Kontoyiannis e Lewis, 2002). O fármaco AMB está incluído na classe dos poliênicos, é eficiente no tratamento de um amplo espectro de infecções fúngicas e é considerado fungicida, porém é tóxico a células mamíferas, apresentando alta frequência de nefrotoxicidade e hepatotoxicidade. Apresenta uma excelente absorção pelos tecidos, incluindo o sistema nervoso central. Contudo, apresenta uma resposta lenta ao tratamento, que o torna ideal para tratamentos de longa duração (Ostrosky-Zeichner *et al.*, 2010).

Nistatina e natamicina são ativos contra *Cryptococcus* spp., *Candida* spp., *Aspergillus* spp., e *Fusarium* spp. Nistatina é utilizada para o tratamento de candidíase vaginal, cutânea e esofágica. Natamicina pode ser utilizada para o tratamento de ceratose fúngica e infecções de córnea (Zotchev, 2003). AMB é recomendada para o



tratamento de infecções causadas por *Candida* spp., *Aspergillus* spp., *Fusarium* spp., e *Cryptococcus* spp. entre outros (Laniado-Laborín e Cabrales-Vargas, 2009).

Polienos têm uma afinidade por colesterol. Esta afinidade explica a alta toxicidade associada a estes antifúngicos e numerosos efeitos adversos (Prasad *et al.*, 2016). AMB é o polieno mais utilizado para o tratamento de infecções sistêmicas. Devido a sua hidrofobicidade e pouca absorção do trato gastrointestinal, é administrada intravenosamente causando efeitos colaterais no fígado e nos rins (Goldstein *et al.*, 2017).

Recentemente alguns estudos têm buscado novas moléculas que minimizem a toxicidade desses compostos poliênicos. Novos polienos semi-sintéticos estão sendo desenvolvidos apresentando uma melhor solubilidade em água, menor toxicidade e alta atividade contra *S. cerevisiae* e linhagens de *C. albicans* resistentes a AMB (Chandrasekar, 2011). As formulações lipídicas de AMB têm permitido que este polieno seja mais efetivo, reduzindo sua toxicidade no hospedeiro (Pound *et al.*, 2011; Miceli e Chandrasekar, 2012). Todavia o problema não foi eliminado e essas formulações têm um custo maior que a forma convencional

### **2.4.3 Inibidores da síntese de ácidos nucleicos - fluoropirimidinas**

Flucitosina está incluída no grupo das fluoropirimidinas e atua inibindo a síntese de DNA e de proteínas. Sabe-se que flucitosina é transportada para o interior das células fúngicas pela citosina permease e convertida por citosina desaminase (enzima ausente em tecidos humanos) a 5-fluorouracil (Loyse *et al.*, 2013). Após a conversão em 5-fluorouracil, duas estratégias de atividade antifúngica podem ocorrer: 5-fluorouracil pode ser convertido a 5-fluorouridina trifosfato (FUTP), que é incorporado ao RNA, levando a inibição da síntese proteica, ou, pode ser convertido a 5-fluorodeoxiuridina

monofosfato (FdUMP) pela enzima uridina monofosfato pirofosforilase, levando à inibição da síntese de DNA pela inibição da enzima timidilato sintase, importante para síntese de timidina (Vermees *et al.*, 2000). Muitos fungos filamentosos não possuem as enzimas citosina permease e citosina desaminase, o que impede o uso da droga, porém 5-FC é ativa contra *Candida* spp. e *Cryptococcus* spp. (Hospenthal e Bennett, 1998). O surgimento recorrente de resistência em *Candida* spp. e *Cryptococcus* spp., contudo, restringe seu uso como monoterapia (Kanafani e Perfect, 2008). Assim, seu papel torna-se limitado ao uso combinado com outras drogas antifúngicas, como AMB, para o tratamento de meningite causada por *Cryptococcus* spp. (Chen e Sorrell, 2007).

#### **2.4.4 Inibidores da $\beta$ -glucana- sintase- equinocandinas**

As equinocandinas são lipopeptídeos semi-sintéticos derivados de produtos naturais fúngicos, sendo eficazes contra diversas infecções fúngicas. Atuam como inibidores da  $\beta$ -(1-3)glucan-sintase da parede celular que são polissacarídeos que consistem em monómeros de D-glicose unidos um ao outro por ligações  $\beta$ -(1,3) ou  $\beta$ -(1,6)glucana (Eschenauer *et al.*, 2007). Atualmente são três equinocandinas utilizadas no tratamento de infecções fúngicas: caspofungina, micafungina e anidulafungina (Cappelletty e Eiselstein-Mckitrick, 2007). No entanto, são relativamente ineficazes contra *Cryptococcus* spp. (Feldmesser *et al.*, 2000; Maligie e Selitrennikoff, 2005) e, portanto, não são utilizadas clinicamente no tratamento da criptococose (Maligie e Selitrennikoff, 2005). Mutantes de *C. neoformans* para o gene da glucana-sintase, são sensíveis ao estresse, e apresentaram deficiências na arquitetura da parede celular. Além disso, esses mutantes foram incapazes de causar virulência em camundongos (Reese *et al.*, 2007). Uma possível explicação para a falta de eficiência das equinocandinas em *criptococcus* spp. é a melanização fúngica no cérebro do hospedeiro. A melanização associada à parede celular pode impedir que o fármaco atinja seu alvo enzimático na

levedura. Em outros trabalhos já foi observado que a melanização aumentou a rigidez da parede celular, possivelmente isso esteja contribuindo para a diminuição da suscetibilidade a equinocandinas *in vivo* (Horcajada *et al.*, 2007).

Atualmente, o número de novos antifúngicos desenvolvidos é limitado quando comparado com o desenvolvimento de novos fármacos antibacterianos, visto que, na maioria das vezes, os potenciais alvos para terapia antifúngica apresentam um alto risco de toxicidade, pois fungos e mamíferos apresentarem similaridade na organização celular (Roemer e Krysan, 2014; Denning e Bromley, 2015).

A OMS e a IDSA reconheceram como melhor tratamento a combinação de AMB e 5-FC no combate à criptococose (Day *et al.*, 2013). Embora esta combinação tenha sido utilizada por muitos anos, apenas recentemente ensaios clínicos demonstraram que a utilização combinada dos fármacos é mais eficaz do que AMB em monoterapia (Day *et al.*, 2013). Contudo, no Brasil e em vários outros países em desenvolvimento na Ásia e na África, a 5-FC não está disponível comercialmente (Yamauti *et al.*, 2017). Não está claro o motivo exato de a 5-FC não estar disponível no Brasil, porém, a utilização da 5-FC com a AMB ainda é a melhor opção terapêutica, devido a menor taxa de mortalidade gerada (Govender *et al.*, 2014). Nos países desenvolvidos, com maior acesso a AMB e 5-FC, a mortalidade anual devido à criptococose é de aproximadamente 25%, enquanto nas regiões de recursos limitados, onde o fluconazol (FLZ) é a única terapia disponível a mortalidade é muito maior (Roemer e Krysan, 2014).

A doença causada por *C. gattii* e *C. neoformans* apresenta aspectos clínicos fundamentalmente diferentes. No entanto, as estratégias estabelecidas pela atual diretriz da IDSA são similares para o tratamento da criptococose causada por ambas as

espécies devido à escassez de estudos que comparam os efeitos da terapêutica para cada uma delas. Além disso, os indivíduos que desenvolvem criptococose podem ser agrupados em diferentes categorias: aqueles que estão infectados com HIV, pacientes transplantados que utilizam terapias imunossupressoras e indivíduos imunocompetentes. Embora existam diferenças na suscetibilidade desses indivíduos, a terapia antifúngica é similar para todos os pacientes (Coelho e Casadevall, 2016).

## **2.5 CARACTERÍSTICAS DE UM FÁRMACO IDEAL**

Além de ter uma atividade antifúngica direcionada a *Cryptococcus* spp., um agente eficaz anticriptococócico deve ter uma série de propriedades farmacológicas que não são necessariamente essenciais para o tratamento de outras infecções fúngicas invasivas (Krysan, 2015). Fármacos com atividade fungicida apresentaram resultados mais eficazes contra a criptococose em relação a fármacos com atividade fungistática (Bicanic *et al.*, 2009). Atualmente, o único medicamento utilizado clinicamente com atividade fungicida em relação à *Cryptococcus* spp. é a AMB (Krysan, 2015). Devido à manifestação mais comum da criptococose ser a meningoencefalite, qualquer terapia eficaz deve ultrapassar a BHE do SNC (Chayakulkeeree e Perfect, 2006). A alta incidência da mortalidade, em diferentes lugares do mundo, causada pela criptococose ocorre principalmente em indivíduos infectados com HIV. Muitos desses locais possuem infraestrutura e recursos médicos limitados. Portanto, as novas terapias precisam ser de baixo custo e facilmente administráveis (Rajasingham *et al.*, 2017b). A biodisponibilidade oral do fluconazol é uma das principais características que impulsionam seu uso como terapia alternativa em regiões com recursos limitados (Sloan *et al.*, 2009) e, inversamente, uma limitação significativa para a utilização da AMB que

necessita de internação hospitalar para administração intravenosa. Portanto, o custo e a biodisponibilidade do fármaco são outros fatores que devem ser considerados (Sloan *et al.*, 2009).

Nenhum dos medicamentos atuais utilizados para o tratamento da criptococose atende os critérios para um agente anticriptocócico ideal. A AMB possui administração intravenosa e requer monitoramento laboratorial devido a sua toxicidade (Sloan *et al.*, 2009). Fluconazol é fungistático e não fungicida, e a 5-FC é apenas adjunta e também requer monitoramento laboratorial por ser tóxica (Krysan, 2015). Finalmente, apenas o fluconazol está disponível em regiões com recursos limitados e é a única terapia administrada por via oral. Certamente, uma droga que atenda todos os requisitos de uma terapia criptocócica ideal seria de grande importância clínica para a saúde da população.

## **2.6 A UTILIZAÇÃO DE COMPOSTOS NATURAIS COMO FÁRMACOS**

Produtos naturais representam uma grande família de diversas entidades químicas com uma ampla variedade de atividades biológicas com múltiplo uso, principalmente na agricultura, medicina veterinária e humana (Katz e Baltz, 2016). Eles são originários de bactérias, fungos, plantas e fontes marinhas animais. A produção de novos agentes farmacêuticos, herbicidas e inseticidas tem impulsionado a investigação de produtos naturais que apresentem moléculas biologicamente ativas (Katz e Baltz, 2016; Silva *et al.*, 2016). Desde a descoberta da penicilina, há mais de 75 anos, mais de 23.000 produtos naturais já foram caracterizados, sendo a grande maioria deles produzida por bactérias, principalmente pela família Actinomycetaceae (Bérdy, 2012).

O uso de produtos naturais na medicina são mais utilizados no tratamento de infecções, principalmente na terapia antibacteriana (Katz e Baltz, 2016). Duas

diferentes famílias de produtos naturais são utilizadas para tratar infecções fúngicas como AMB e nistatina (Katz e Baltz, 2016). Portanto, produtos naturais são uma importante fonte de novos protótipos para o desenvolvimento de fármacos (Cragg e Pezzuto, 2016).

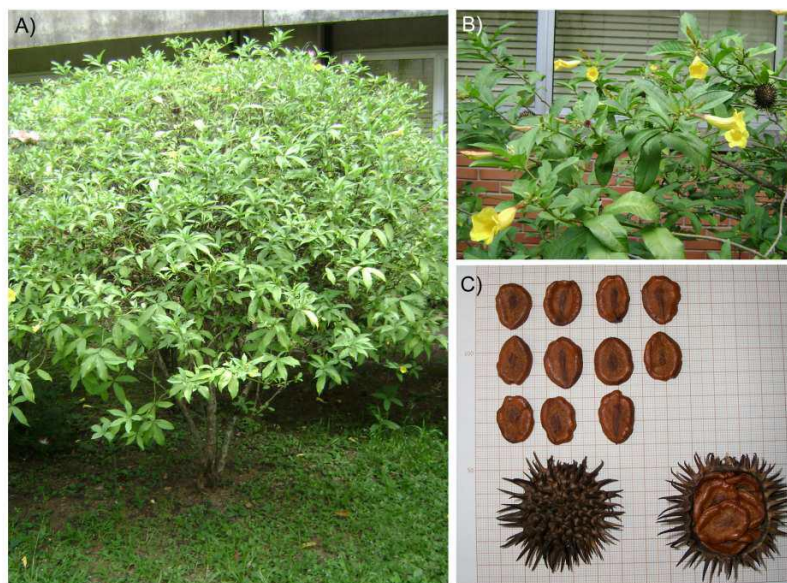
Diante da problemática atual das terapias antifúngicas, como falta de biodisponibilidade e problemas relacionados à toxicidade e resistência, se torna necessária a busca por novos tratamentos para a criptococose. Neste contexto, produtos naturais assumem um papel relevante como fonte para busca por novos antifúngicos. Dos fármacos aprovados em 2013, metade foi considerada produto natural, ou derivado de produto natural, mostrando a importância desses produtos para a indústria farmacêutica (Demain, 2014).

### **2.6.1 Iridóides**

Iridóides são metabólitos secundários produzidos por plantas e animais e possuem funções de defesa contra predadores (Dinda *et al.*, 2007). Considerando uma classificação geral, iridóides podem ser divididos em dois grandes grupos: iridóides carbocíclicos e seco-iridóides. Estruturalmente são classificados como monoterpênoides ciclopentano-[c]-pirano, ocorrendo comumente na forma glicosilada (Habtemariam, 2018). Estão presentes em diversas formulações utilizadas na medicina popular como sedativo antipirético e cicatrizante; com isso inúmeros estudos foram realizados para investigar suas propriedades biológicas (Singh e Sharma, 2015). Dentro da classe dos iridóides podem ser encontrados compostos com amplo espectro de atividades biológicas como: protetor cardiovascular, hepatoprotetor, hipoglicêmico e hipolipidêmico, antiinflamatório, antiespasmódico, antitumoral, antiviral, imunomodulador, antibiótico, antifúngico, entre outros (Dinda *et al.*, 2007). Entre 1994

a 2005, mais de 418 moléculas foram descobertas. Nos anos seguintes (de 2005 a 2008), foram catalogados mais 172 compostos e, em sua última revisão (2008 - 2010) foram descritos mais 129 novos compostos (Dinda *et al.*, 2007). Em média são descobertas aproximadamente 45 novas moléculas iridoides por ano, baseado no registro dos últimos 16 anos (sem considerar as moléculas anteriormente descritas - aproximadamente 600 moléculas). Esses dados mostram que os iridoides podem ser uma grande fonte de moléculas biologicamente ativas.

Bresciani (2013) avaliou o efeito antifúngico do extrato aquoso das sementes da planta *Allamanda polyantha* (**Figura 4**) que apresenta um alto teor de iridóides. *A. polyantha*, conhecida popularmente como alamanda-ereta ou alamanda-de-cerca, é um arbusto semilenhoso comum no litoral brasileiro principalmente nas regiões norte e nordeste, e pertence à família Apocynaceae (Abdel-Kader *et al.*, 1997). Floresce durante grande parte do ano, principalmente na primavera, com flores amarelas. Possui frutos capsulares espinhacentos contendo numerosas sementes aladas. O extrato aquoso de sementes de *A. polyantha* apresentou atividade antifúngica contra *C. gattii* (MIC = 70 µg/mL) e *C. neoformans* (MIC = 563 µg/mL) (Bresciani, 2013). Foram identificados neste extrato aquoso dois compostos com atividade antifúngica, sendo eles: o composto plumierídeo (isolado pela primeira vez em 1897 pela empresa Merck) (Boros *et al.*, 1991) e o composto plumieridina (Boros *et al.*, 1991). Plumieridina foi isolada pela primeira vez há 100 anos, no entanto teve sua atividade antifúngica recentemente descrita (Bresciani, 2013). Anteriormente foi demonstrado que plumieridina foi obtida pela hidrólise enzimática de plumierídeo (Schliemann, 1982), e foi isolada de *Plumeria obtusa* e *Allamanda nerifolia* (Saleem *et al.*, 2011).



**Figura 4.** Imagens de *Allamanda polyantha*. A) *Allamanda polyantha* com porte de arbusto semelhante ereto; B) Ramo com flores e um fruto; C) Fruto e sementes de *A. polyantha*: Imagem adaptada de Bresciani, 2013.

## 2.7 BIOIMAGEM E O MONITORAMENTO DE TRATAMENTOS TERAPÊUTICOS

Atualmente a bioimagem representa uma inovação em ensaios pré-clínicos no desenvolvimento de drogas. O monitoramento de doenças em animais baseado na fluorescência possibilita o entendimento da progressão de processos infecciosos em tempo real e o desenvolvimento de novos tratamentos terapêuticos (Kong *et al.*, 2016). Zelmer e colaboradores (2012) desenvolveram um método para testar a eficácia de novos fármacos contra a tuberculose *in vivo* a partir da construção de linhagens repórter fluorescentes (Zelmer *et al.*, 2012). Recentemente, Kong e colaboradores (2016) avaliaram a eficácia de rifampicina e isionazida *in vivo* e *in vitro* por bioimagem, após a expressão da proteína fluorescente td tomato em *Mycobacterium bovis* (Kong *et al.*, 2016). Assim como a fluorescência, o sistema por imagem baseado na bioluminescência também tem se destacado. Brock e colaboradores (2008) desenvolveram uma estratégia baseada na bioluminescência para avaliar a eficácia de diferentes drogas no tratamento da doença causada por *Aspergillus fumigatus*, a partir da construção de uma linhagem



expressando constitutivamente o gene repórter da luciferase de vagalume (Brock *et al.*, 2008). Vocat e colaboradores (2015), construíram uma linhagem bioluminescente denominada 18b-lux, por transformação de *Mycobacterium bovis* com o vetor expressando o operon luxCDABE que foi isolado de *Photorhabdus luminescens*. Esta linhagem foi utilizada para testar potenciais fármacos contra o bacilo não replicativo de *Mycobacterium bovis in vitro* e *in vivo* (Vocat *et al.*, 2015). Recentemente foi realizada uma revisão de estudos que monitoraram doenças infecciosas *in vivo* utilizando a bioimagem através da bioluminescência. A dinâmica da infecção de diferentes patógenos, como infecções bacterianas (biofilmes, meningite, otite, osteomielite, salmonelose, tuberculose), infecções fúngicas causadas por *Candida spp.* e *Aspergillus spp.*, infecções parasitárias por *Plasmodium spp.*, *Leishmania spp.*, *Trypanosoma spp.*, e *Toxoplasma spp.* e infecções virais foram avaliadas pelo método (Avci *et al.*, 2017). Gupta e colaboradores construíram dois genes repórteres, um fluorescente e outro luminescente, com o intuito de identificar novos antimicrobianos *contra Mycobacterium abscessus* através de um *screening* de uma biblioteca de compostos (Avci *et al.*, 2017). Com isso, o sistema por bioimagem tem contribuído para a compreensão da interação patógeno–hospedeiro, a localização do patógeno, assim como, a visualização *in vivo* do efeito de tratamentos terapêuticos para doenças infecciosas.

### **2.7.1 Marcadores fluorescentes**

John Frederick William Herschel observou na luz do sol, um brilho azul superficial em uma solução de quinina e descreveu pela primeira vez a fluorescência, em 1845. O fenômeno da fluorescência consiste na absorção de energia por um elétron, passando do estado fundamental (S0) para o estado excitado (S1) (Chudakov *et al.*, 2010). O retorno deste elétron ao estado fundamental é acompanhado pela liberação de energia em excesso através da emissão de luz.

A utilização de marcadores fluorescentes em estudos de bioimagem é fundamental para a compreensão de inúmeras doenças. Osamu Shimomura descreveu a proteína verde fluorescente (GFP) em 1962, que foi isolada da água-viva *Aequorea victoria* (Chudakov *et al.*, 2005). Osamu ganhou o Prêmio Nobel de Química em 2008 pela descoberta, juntamente com Roger Tsien e Martin Chalfie. Martin Chalfie, pelo pioneirismo na incorporação da GFP em organismos vivos e sua utilização como marcador biológico, e para Roger Tsein, pela ampliação de seu uso em processos biotecnológicos e biomédicos. Chalfie foi o primeiro pesquisador a utilizar GFP como marcador da expressão gênica, o modelo utilizado foi o nematódeo *C. elegans* (Chapman *et al.*, 2005). Homólogos da proteína fluorescente verde de *A. victoria* (GFP) são amplamente utilizadas na biotecnologia em diversas aplicações. Inúmeras proteínas fluorescentes de diferentes cores foram descobertas e desenvolvidas (Hoffman, 2005).

Diversos fatores podem influenciar a sensibilidade da detecção para a obtenção de uma imagem de alta qualidade, como a propagação da luz pelo tecido, o brilho, o sinal de autofluorescência dos tecidos e a disponibilidade de oxigênio (Andreu *et al.*, 2011). A propagação da luz através do tecido é influenciada pela absorção e espalhamento de luz. Em contato com o tecido os fótons emitidos em um comprimento de onda visível e infravermelho sofrem um espalhamento resultando na difusão dos fótons. Além disso, a intensidade da luz é reduzida após passar pelo tecido. Existe uma perda de aproximadamente dez vezes do valor da intensidade dos fótons a cada centímetro de profundidade do tecido (Contag *et al.*, 1995). Outro fator que interfere na qualidade da imagem é o brilho, a proteína fluorescente deve ser expressa eficientemente e possuir brilho suficiente para fornecer um sinal significativamente acima da autofluorescência tecidual para a análise adequada da imagem (Gurskaya *et al.*, 2001). O brilho é determinado por uma variedade de fatores incluindo as

características intrínsecas da proteína (velocidade de maturação, eficiência de maturação e fotoestabilidade); as propriedades ópticas do sistema de aquisição de imagens (comprimento e intensidade da iluminação, o espectro de filtros); e além dessas a sensibilidade da câmera e do olho humano ao espectro de emissão (Shaner *et al.*, 2005). O marcador deve também possuir uma fotoestabilidade adequada que possibilite a visualização até a finalização do experimento.

A proteína vermelha Katushka, recentemente desenvolvida e utilizada para estudos *in vivo*, apresenta características superiores quando comparada a outros biomarcadores (Shcherbo *et al.*, 2009). Katushka é uma proteína far-vermelha mutante derivada de uma proteína vermelha fluorescente da anêmona *Entacmaea quadricolor* (Shcherbo *et al.*, 2007). Esse fluoróforo possui comprimento de onda de excitação de 588 nm e de emissão 635 nm, apresenta mais brilho que outros biomarcadores, além disso, possui tempo de maturação mais rápido, e alta estabilidade ao PH e maior fotoestabilidade (Andreu *et al.*, 2011), tornando-o um excelente biomarcador para análises *ex vivo* e *in vivo*.

### **2.7.2 IVIS LUMINA II e a captação de imagem**

O sistema de imagem óptica inclui a imagem biofotônica, uma técnica altamente sensível e não tóxica, baseada na detecção da luz visível produzida por moléculas bioluminescentes ou fluorescentes (Andreu *et al.*, 2011). A bioluminescência é produzida através de reações de oxidação catalisada pela luciferase, e a fluorescência é obtida a partir da excitação de moléculas fluorescentes (Zelmer *et al.*, 2012; Andreu *et al.*, 2013). O equipamento IVIS LUMINA II é capaz de detectar o sinal fluorescente e luminescente, sendo um sistema sensível de imageamento *in vivo* e *ex vivo*. A luz emitida é detectada externamente através da câmera de carga acoplada (CCD),

extremamente sensível, associada a um compartimento isolado de luz com mais de 21 filtros, incluindo filtros de emissão e excitação. Assim é possível observar e quantificar a distribuição espacial e temporal da produção da luz no interior de animais vivos (Leblond *et al.*, 2010).

### 3. OBJETIVOS

O presente trabalho teve por objetivo avaliar as atividades biológicas do composto plumieridina em *Cryptococcus neoformans*.

#### 3.1 Objetivos específicos

- ✓ Avaliar se o composto plumieridina altera os principais fatores de virulência de *C. neoformans*.
- ✓ Avaliar a toxicidade de plumieridina.
- ✓ Utilizar um modelo prático *ex vivo* para monitorar a eficácia do composto plumieridina sobre a infecção de *C. neoformans*.
- ✓ Investigar o mecanismo de ação do composto plumieridina.

#### 4. MANUSCRITO CIENTÍFICO

### **PLUMIERIDINE: A MOLECULE WITH ANTIFUNGAL POTENTIAL FOR TREATMENT OF CRYPTOCOCCAL MENINGITIS**

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**Keywords:** Plumieridine, *C. neoformans*, thymidylate synthase, imaging analysis, orotidine-5'-phosphate decarboxylase

#### **Authors contributions**

Vanessa de A, Barcellos, Charley C, Staats, Augusto Schrank, Gilsane L, von Poser, Lívia Kmetzsch, Marilene H, Vainstein prepared the experimental design.

Claudia L, Fernandes, Renato K, Corá conducted bioinformatics analysis.

Vanessa Barcellos, Adriana C, Silva, Júlia C, Reuwsaat, Eamim D, Squizani, Natalia K, Oliveira conducted CFU analysis and animal experimentation.

Vanessa Barcellos, Glauber de S. Araújo, Susana Frases conducted scanning electron microscopy experiments.

## ABSTRACT

Cryptococcosis is an invasive fungal infection caused by the pathogenic species *Cryptococcus neoformans* and *Cryptococcus gattii*. This disease affects approximately 200,000 individuals annually worldwide. Currently, antifungal drugs show some limitations related to toxicity and resistance, which emphasizes the importance of searching for new drugs. Our group previously identified the antifungal potential of plumieridine, an iridoid isolated from aqueous extracts of *Allamanda polyantha* (Apocynaceae) seeds. Here we sought to determine the biological activities of plumieridine to control *C. neoformans* using a murine model of cryptococcosis. The test was based on mice infection with a *C. neoformans* strain expressing a Katushka reporter, followed by fluorescence-based imaging and CFU analysis of the mice colonized organs. Imaging analysis showed that plumieridine treatment reduced pulmonary and cerebral fungal burden of infected mice. Three proteins from the route of pyrimidines metabolism were identified as putative targets of plumieridine: thymidylate synthase, orotidine-5'-phosphate decarboxylase and dihydroorotate dehydrogenase. The structures of thymidylate synthase and orotidine-5'-phosphate decarboxylase from *C. neoformans* were modeled and the docking of plumieridine was analyzed. According to the models, plumieridine interacts with the amino acids at the catalytic center of the enzymes. In addition, a significant decrease in the transcript levels of thymidylate synthase and orotidine-5'-phosphate decarboxylase genes was detected when *C. neoformans* cells were cultured in the presence of plumieridine. These results support the potential of plumieridine as an antifungal effective against *Cryptococcus* spp.

**Keywords:** plumieridine, *C. neoformans*, thymidylate synthase, imaging analysis, orotidine-5'-phosphate decarboxylase.

## INTRODUCTION

Cryptococcosis is a systemic fungal disease caused by *Cryptococcus neoformans* and *Cryptococcus gattii*. This disease affects the lungs and brain of humans and animals (Kwon-Chung *et al.*, 2014; Colombo and Rodrigues, 2015). It has been estimated that human cryptococcosis affects nearly 200,000 individuals each year, and it is the second leading cause of mortality associated with AIDS (Kim, 2016; Rajasingham *et al.*, 2017). In Brazil, cryptococcosis is the main fungal systemic disease in patients with HIV/AIDS (De Oliveira *et al.*, 2014). In addition, *C. gattii* is considered endemic in the North and Northeast of Brazil, presenting significant mortality and lethality (Souto *et al.*, 2016; Barcellos *et al.*, 2018).

The World Health Organization (WHO) recommend that the appropriate treatment on the onset cryptococcosis is based on the combination of amphotericin B (AMB) and 5-flucytosine (5FC). However, in Brazil and several other developing countries, 5FC is not licensed, available, or affordable (Yamauti *et al.*, 2017). Current treatment in such countries is then based on combination of AMB and fluconazole (Perfect *et al.*, 2010). However, AMB is not available in most of the African continent and antifungal resistance to azoles has reached alarming numbers (Mpoza *et al.*, 2017). Therefore, development of antifungal resistance, toxicity to multiple organs, including liver and kidney, and limitations of the activity spectrum of these antifungals hampers this treatment scheme (Williamson, 2017; Mpoza, 2017; Rodrigues, 2016). Moreover, a steady increase in susceptible populations was observed in the last years (Engelthaler *et al.*, 2014) pointing to the importance to develop new therapies.

Among 1,453 new chemicals approved by the US Food and Drug Administration as of 2013, 40 % were natural products or derivate of natural products (Katz e Baltz, 2016). Iridoids are secondary metabolites produced by plants and animals (Tanahashi, 2017) and



recent studies have also shown their role in treatment of neurodegenerative diseases and as wound-healing compounds (Barreto *et al.*, 2014; Habtemariam, 2018). Two iridoids (plumericin and isoplumericin) were described as having *in vitro* antifungal activity for *Candida* species and *C. neoformans* (Singh *et al.*, 2011).

Here, we propose the use of a practical model *ex vivo* to monitor the treatment efficacy of plumieridine against *C. neoformans*. Experimental models for determination of infection progression and *in vivo* antifungal susceptibility are essential for the development of efficient therapeutic protocols (Avci *et al.*, 2017; Krafft *et al.*, 2017). The progress of bioimaging of bioluminescent or fluorescent fungal strains has driven a great improvement in the understanding of biological systems, especially related to pathological aspects (Andreu *et al.*, 2011). Bioluminescent *A. fumigatus* strains expressing a codon-optimized version of firefly luciferase was used to describe the infection process of aspergillosis (Galiger *et al.*, 2013). However, studies using fluorescence imaging to evaluate drug efficacy against fungal pathogens are limited. In this context, our approach involves mice infection with a *C. neoformans* fluorescent strain and treatment with plumieridine, followed by fluorescence-based imaging analysis of the colonized organs.

## **RESULTS**

### **Determination of minimal inhibitory concentration (MIC)**

To evaluate the antifungal activity of plumieridine to cryptococcal species, the minimal inhibitory (MIC) and fungicide (MFC) concentrations were determined according to the NCCLS standards. We found that plumieridine is fungicidal to cryptococcal cells, and that *C. neoformans* H99 strain is more sensitive to this compound compared to *C. gattii* (Table 1).

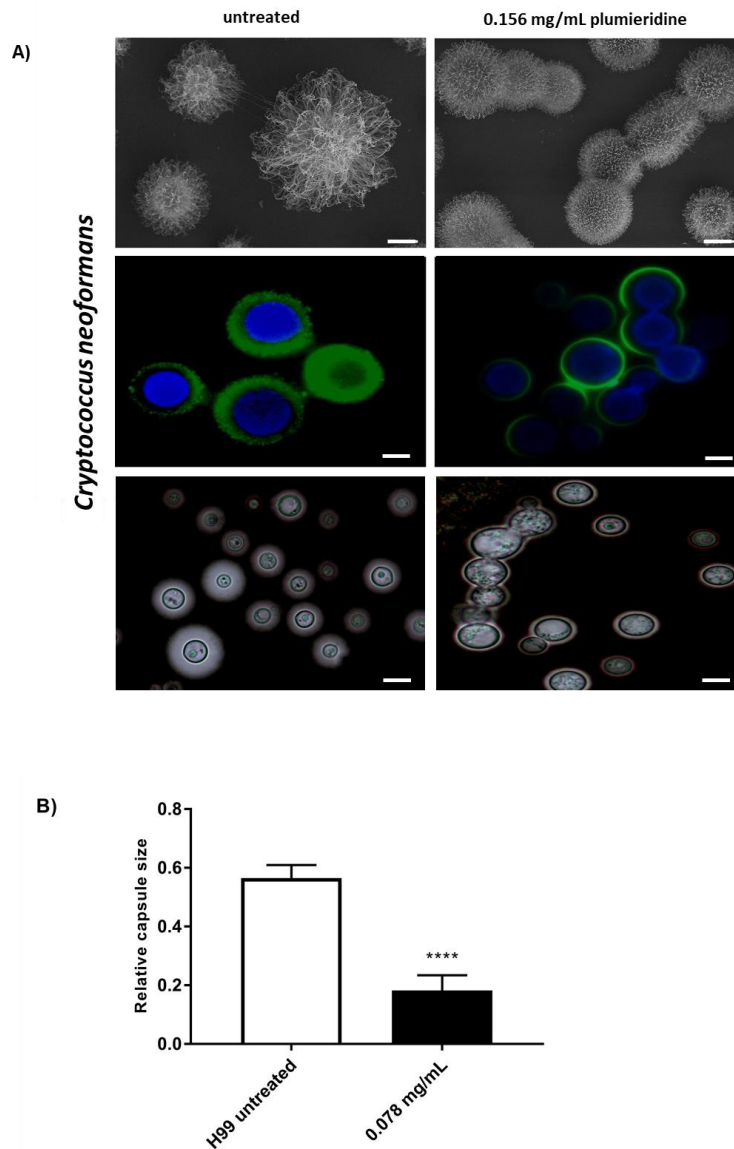
A higher inhibitory activity of fluconazole was detected when compared to the MIC and MFC of plumieridine (Table 1).

**Table 1.** Minimal inhibitory concentration (MIC) and minimal fungicide concentration (MFC) of plumieridine and fluconazole on *Cryptococcus* pathogenic species.

Species	Strains	Plumieridine (mg/mL)		Fluconazole (mg/mL)	
		MIC	MFC	MIC	MFC
<i>Cryptococcus neoformans</i>	H99	0.156	0.312	0.0016	0.0032
<i>Cryptococcus gattii</i>	R265	0.312	0.624	0.008	0.0016

### Plumieridine affects capsule size and fungal morphology

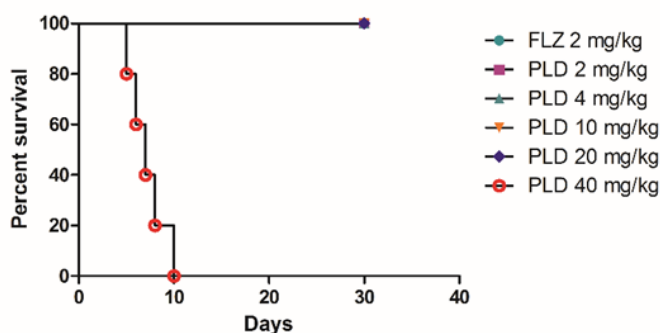
To assess whether plumieridine affects cellular structures of *C. neoformans*, we evaluated the cell morphology and capsule size and using India ink counterstaining, fluorescence microscopy, and scanning electron microscopy. Capsule formation by *C. neoformans* H99 was inhibited by plumieridine in the presence of a sub-inhibitory concentrations (78 µg/mL) (Figure 1A). Fungal cells cultivated in the presence of plumieridine showed marked morphological alterations, as revealed by SEM analysis. The cells formed clusters, resembling incomplete cell separation (Figure 1A). However, these cell clusters present recognition by the anti-GXM antibody 18-B7 (Figure 1A), suggesting that the participation of the capsular components in this phenotype is less important.



**Figure 1. Plumieridine alters *C. neoformans* H99 cell morphology and capsule.** A) *C. neoformans* cells were incubated for seventy-two hours with plumieridine (0.156 mg/ml) and analyzed by optical microscopy, scanning electron microscopy and immunofluorescence microscopy. Cell wall was stained with calcofluor white (blue), and capsule was stained with anti-GXM antibody 18-B7 (10  $\mu$ g/mL) (green). Scale bar = 5  $\mu$ m. B) Relative capsule size was determined as the ratio between the diameter of the total cell and the cell body diameter. At least 100 cells were measured for each growth condition. Scale bar = 5  $\mu$ m. Error bars indicating SD. One-Way ANOVA was performed with Tukey's post hoc test \*\*\*\* $p < 0.0001$ .

### Oral toxicity study

To test the *in vivo* activity of plumieridine as therapeutic agent, and its possible toxic effects on the host, we carried experiments in a mice model. Five distinct concentrations of plumieridine were daily administered to mice using oral gavage and clinical symptoms were evaluated. Only the higher concentration (40 mg/kg) displayed clear signals of toxicity to mice (Figure 2). After the fifth day of administration of plumieridine (40 mg/kg) the mice started diarrhea and become lethargic. No symptoms were observed in mice treated with the lower concentrations analyzed.

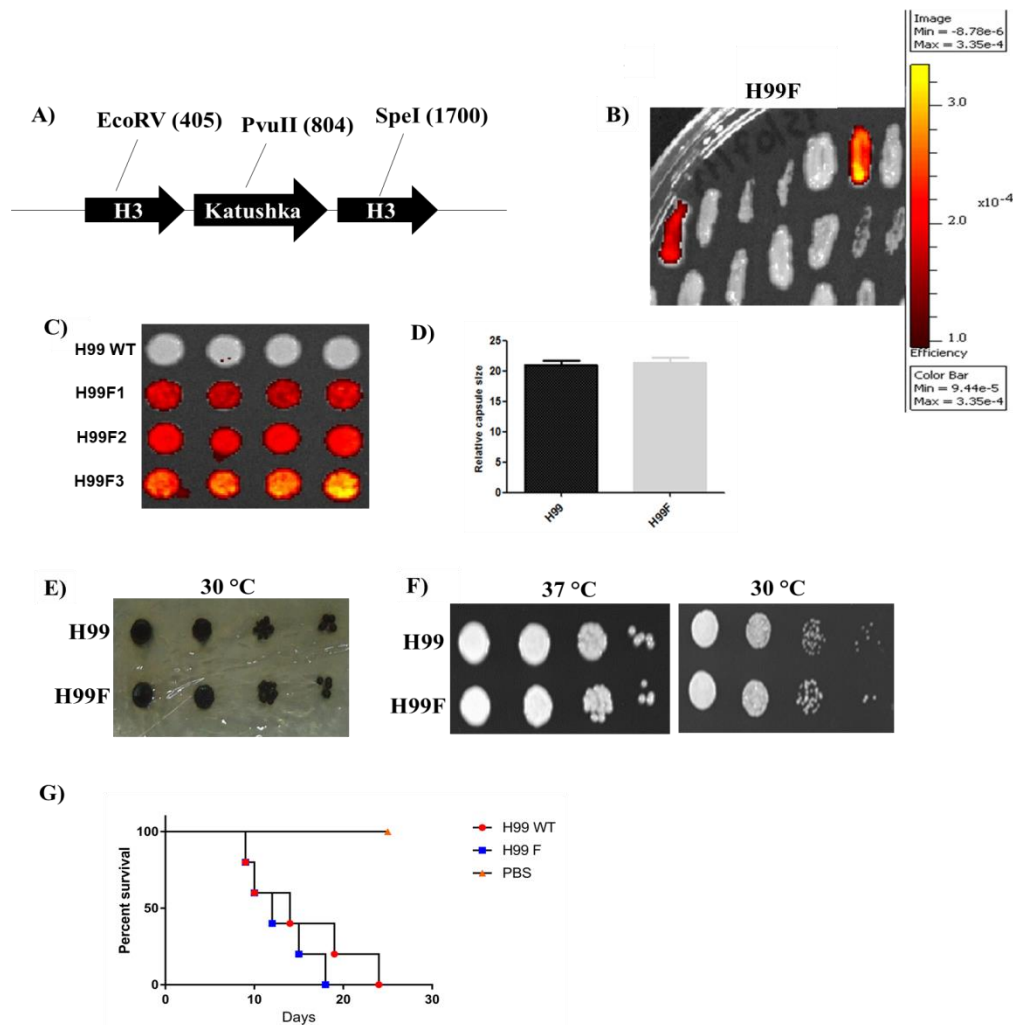


**Figure 2. Toxicity *in vivo* assay.** Five concentrations of PLD were tested in mice. The doses were applied daily for 15 days by oral gavage. FLZ was administered as control (2 mg/kg/day). Log-rank (Mantel-Cox) Test was performed  $p = 0,1386$ .

### **Construction of a cryptococcal strain expressing the far red fluorescence protein *Katushka***

To analyze the fungal burden *in vivo* during treatment with plumieridine, we constructed a *C. neoformans* strain expressing the far-red fluorescent protein KAT. The strains were constructed by introduction of a cassette encompassing the KAT, whose expression was driven by the histone 3 putative promoter and terminator sequences (Figure 3A). Resulting colonies (H99F1, H99F2 and H99F3) were then evaluated by their fluorescence emission in an imaging system (Figure 3B). To ensure that the selected strains had no alterations in virulence traits, we evaluated phenotypic alterations in the canonic cryptococcal virulence determinants. The KAT expression strain (H99F3) did not presented

differences in capsule size (Figure 3C), melanin production (Figure 3D), neither growth at 37 °C (Figure 3E), compared to the WT H99 strain. In addition, virulence assays using a murine model of cryptococcosis did not revealed differences in the median lethal times of H99F when compared to WT H99 (Figure 3F). Therefore, the insertion of the KAT expression cassette did not lead to major virulence alterations in H99F strain.

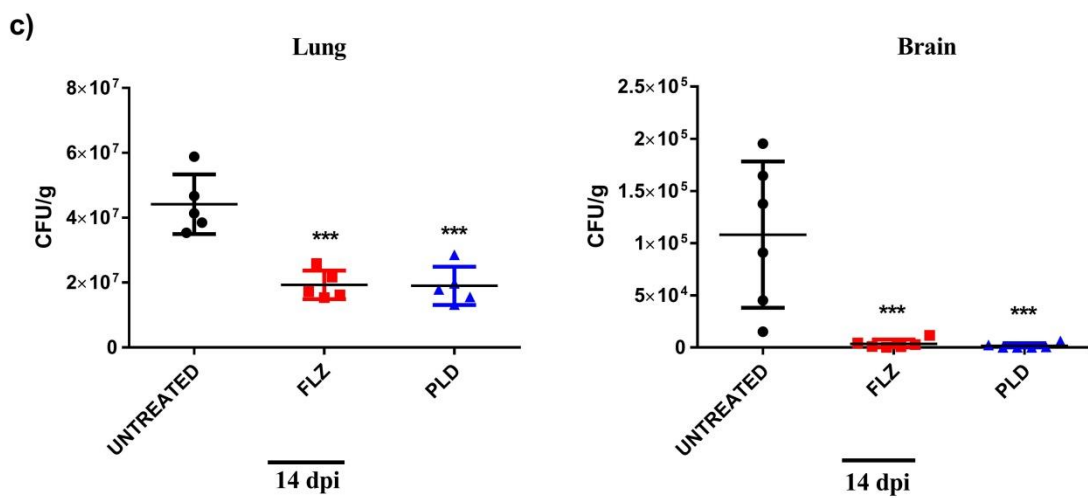
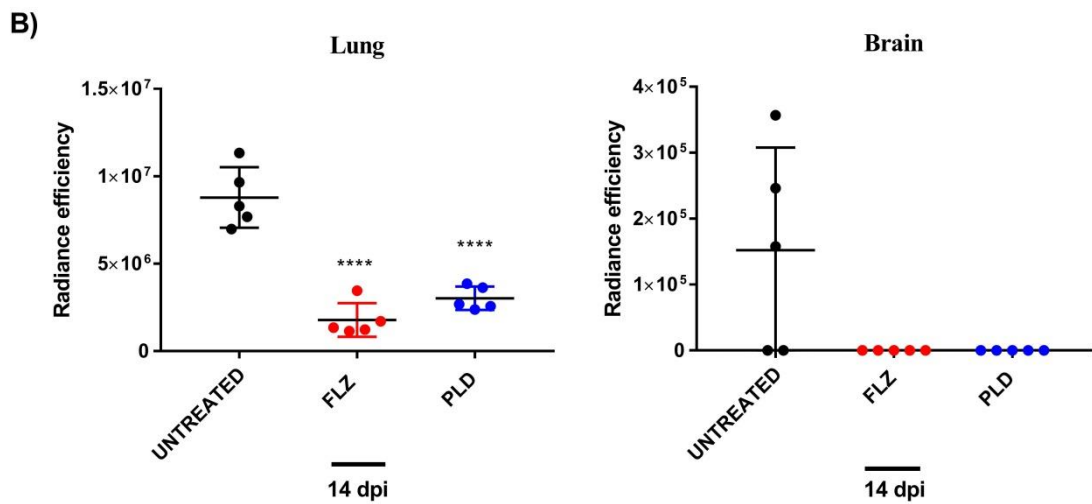
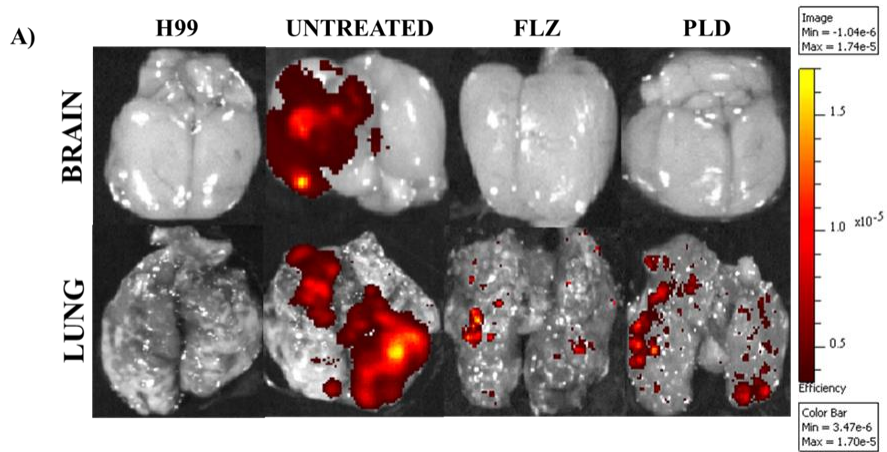


**Figure 3. Construction and characterization of *C. neoformans* strains constitutively expressing the Katushka reporter cassette.** A) Fluorescent strains of *C. neoformans* (H99F) constitutively expressing the Katushka reporter gene. B) Fluorescence levels were determined with Living Image and IVIS Lumina (Xenogen) software. Parameters used: f/stop: 4, binning: 4 and 10 seconds exposure. C) Selection of *C. neoformans* fluorescent strains (H99F1, H99F2, H99F3) D) Relative capsule sizes of H99 and H99F strains. Determination of capsular dimensions revealed similar values for both strains. E) Visual analysis of melanin

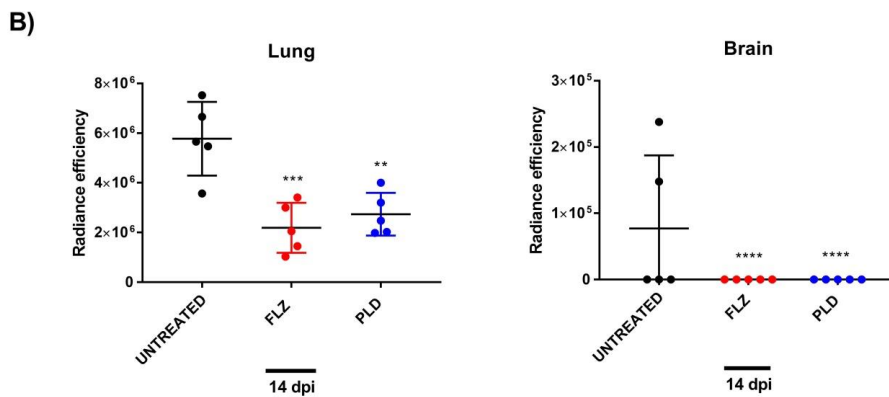
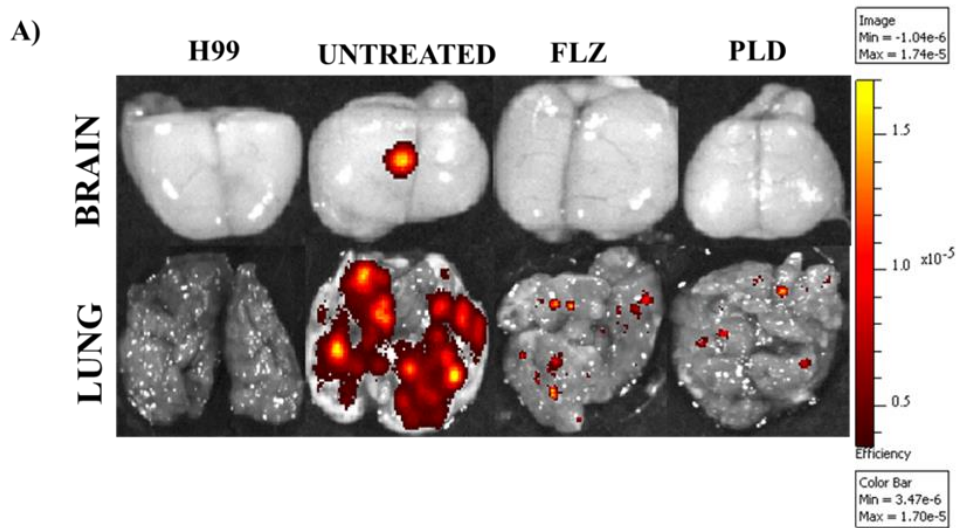
production after fungal growth in solid media supplemented with L-DOPA in different temperatures. F) H99F and H99 WT had similar growth rates, without interference in fluorescence emission by the H99F cells. G) Virulence assay of H99 and H99F strains in an intranasal inhalation infection model with BALB/c mice. Log-rank (Mantel-Cox) Test was performed  $p= 0,1568$

### **Plumieridine reduced fungal burden in lungs and brain of *C. neoformans* infected mice**

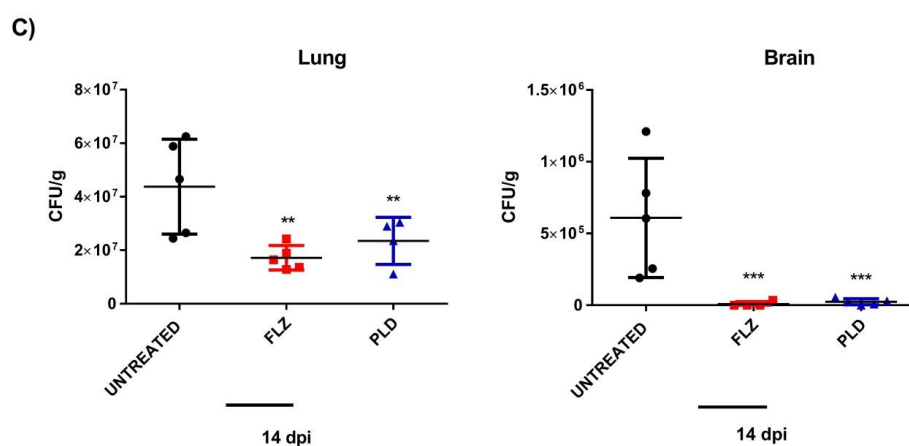
Mice were infected with *C. neoformans* H99 or H99F strains and monitored for organ colonization by CFU counting and fluorescence of the KAT expression cassette. After 15 days of infection, *ex vivo* analyses demonstrated a high fungal load in the lungs and brain of untreated mice. Orally treated mice with plumieridine (4 mg/kg/day) displayed significant reduced fungal loads in the lungs and brain after 15 days post-infection (dpi) (Figure 4A). This reduction was similar to that of the group treated with FLZ. These findings were confirmed by quantification of fluorescence (Figure 4B) and CFU counting tests (Figure 4C). In order to evaluate the effects of an alternative via of administration, mice were infected with H99 or H99F and treated with daily intraperitoneal injections of plumieridine or fluconazol. After 15 days of infection, mice treated with plumieridine (4 mg/kg/day) displayed reduced fungal loads in the lungs and brain when compared to the untreated group (Figure 5A), also confirmed by fluorescence (Figure 5B) and CFU (Figure 5C) quantification. Collectively, these data indicate that the effect of plumieridine in cryptococcosis was independent of the administration routes.



**Figure 4. Determination of fungal loads in lungs and brain of mice treated with plumieridine or fluconazole by oral gavage.** Analyses of fluorescence detection (A and B) and CFU determination (C) of infected organs were conducted after 14 days post-infection (dpi). Infected mice with H99F were treated by oral gavage for 14 days with 4 mg/kg/day of PLD or 2 mg/kg/day FLZ. Error bars indicating SD mean values were compared with untreated group using Two-Way ANOVA with Bonferroni pos-hoc test \*\*\*  $p < 0.001$







**Figure 5. Determination of fungal loads in lungs and brain of mice intraperitoneally treated with plumieridine or fluconazole.** Analyses of fluorescence detection (A and B) and CFU determination (C) of infected organs were conducted after 14 days post-infection (dpi). Infected mice with H99F were intraperitoneally treated for 14 days with 4 mg/kg/day of PLD or 2 mg/kg/day FLZ. Error bars indicating SD mean values were compared with untreated group using Two-Way ANOVA with Bonferroni pos-hoc test \*\*\*  $p < 0.001$

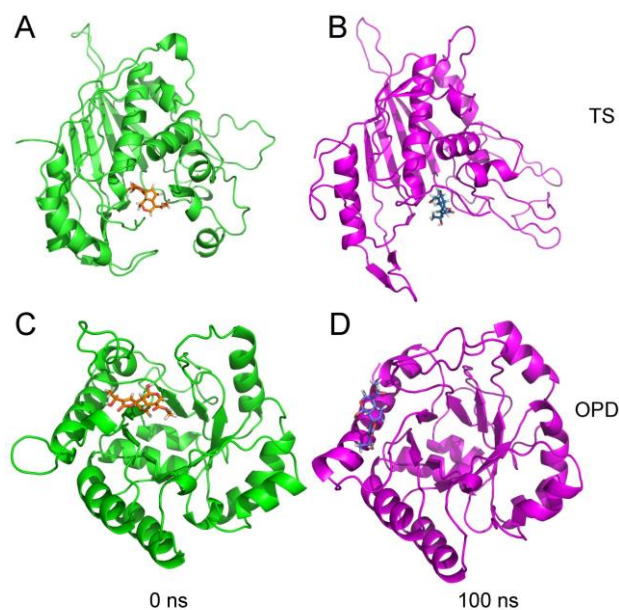
### The pyrimidine pathway as a possible target of plumieridine

Possible targets of plumieridine were investigated by *in silico* analysis using PharmMapper server (Liu *et al.*, 2010). Among the predicted targets, three proteins from the route of pyrimidines were identified: thymidylate synthase, orotidine-5'-phosphate decarboxylase and dihydroorotate dehydrogenase. Both thymidylate synthase and orotidine-5'-phosphate decarboxylase had templates to use for comparative modeling, using the sequence from *C. neoformans* H99 strain with the structures of 2AAZ (Finer-Moore *et al.*, 2005) to thymidylate synthase and 3GDK (Chan *et al.*, 2009) to orotidine-5'-phosphate decarboxylase, respectively. Ten models were generated to each protein by using Modeller 9.15 (Sali e Blundell, 1993) and were analyzed according to Modeller protocol by stereochemistry (Chen *et al.*, 2010) and theoretical analysis (Benkert *et al.*, 2009).

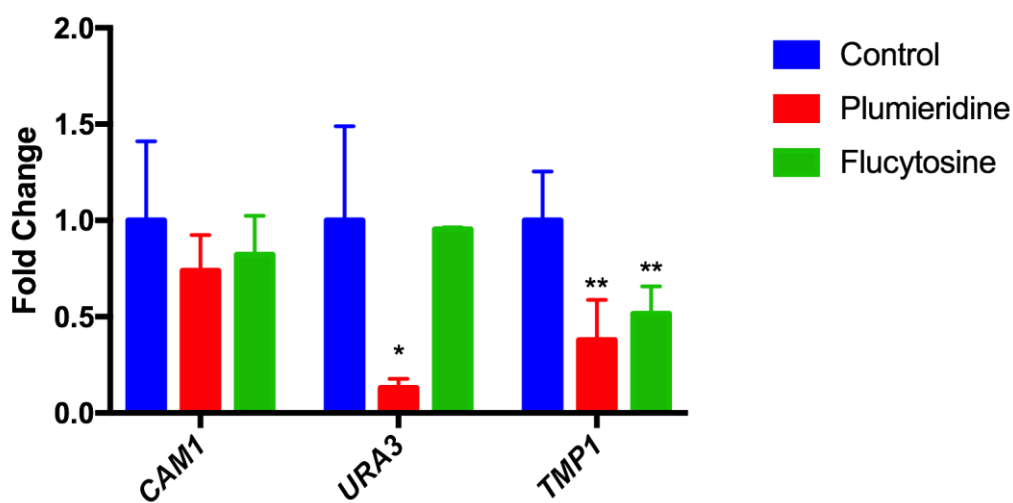
By using SUCH criteria, the better results of each protein were submitted to docking servers SwissDock (Grosdidier *et al.*, 2011) and PatchDock (Schneidman-Duhovny *et al.*,

2005). The data was clustered based in ranking and active site localization. Considering the thymidylate synthase, the positions with better ranks were near to the active site and were used for molecular dynamics assays. Also, the orotidine-5'-phosphate decarboxylase interaction with plumieridine occurs possibly through the active site, which possess better docking ranks. To obtain further details of the interaction, we performed molecular dynamics simulation of the proteins in the presence and absence of docked plumieridine. Such analysis suggests that, in both cases, the plumieridine interacts with the amino acids of the catalytic center (Figure 6). In the simulations with thymidylate synthase, plumieridine interacts with the amino acids involved with the substrate and cofactor (folate) sites, and throughout the simulation the ligand was in the active site region, demonstrating the potential to act as inhibitor to this enzyme. In the simulations with orotidine-5'-phosphate decarboxylase, the plumieridine maintained a position in the region of the active site, however the residues that interact with the ligand had variations when compared to thymidylate synthase simulations.

In order to evaluate the effects of plumieridine exposure in the expression profile of *C. neoformans* genes related to pyrimidines synthesis pathway, we performed RT-qPCR analysis (Figure 7). A significant decreased in the transcript levels of thymidylate synthase and orotidine-5'-phosphate decarboxylase was detected when cells were cultured in the presence of plumieridine. Also, 5-FC exposure led to a decrease in the transcript levels of thymidylate synthase (Figure 7). As control, the expression levels of calmodulin were tested to evaluate the possibility of a more generalized effect of plumieridine or flucytosine in *C. neoformans* cells (Figure 7), which was not observed.



**Figure 6. Molecular structures of plumieridine with thymidylate synthase (A and B) and orotidine-5'-phosphate decarboxylase (C and D).** The images represent the initial point of molecular dynamics (green structures) and the final point of molecular dynamics (magenta structures). We can observe that plumieridine (orange in A and blue in B) linked to thymidylate synthase preserved a stable orientation during the simulation. The plumieridine linked to orotidine-5'-phosphate decarboxylase simulation (orange in C and blue in D) maintained a near orientation during simulation, but not the same, as observed in thymidylate synthase simulation.



**Figure 7. The relative transcript levels of genes related to the pyrimidines synthesis route are affected by plumieridine.** Transcript levels of orotidine-5'-phosphate

decarboxylase (*URA3*), thymidylate synthase (*TMPI*), and calmodulin (*CAMI*) were quantified by RT-qPCR. *C. neoformans* cells were cultured in the absence (control) or in the presence of PMD and 5-FC. The data were normalized using actin transcript levels from cDNA samples. Error bars indicate SD. One-Way ANOVA was performed with Brown-Forsythe test for statistical analysis. \* $p < 0.05$ . \*\* $p < 0.01$ .

## DISCUSSION

Cryptococcal meningitis is still a problem in countries with few resources, along with a high HIV prevalence and limited access to health services (Rodrigues, 2016). The use of AMB and FLZ, although effective, is linked to resistance development and toxicity to some organs (Nooney *et al.*, 2005; Coelho e Casadevall, 2016; Nett e Andes, 2016). Furthermore, the cost of treatment is high and its availability is limited to hospitals in urban centers (Perlin *et al.*, 2017). So, these facts emphasize the need for new options for cryptococcosis treatment.

In this study we evaluated the antifungal potential of plumieridine using an imaging detection model of cryptococcosis (Krafft *et al.*, 2017). Fluorescence imaging is a rapid and efficient method for evaluation of disease progression, as well as the treatment efficacy analysis of drug candidates in mice (Zelmer *et al.*, 2012; Kong *et al.*, 2016). Usually fungal burden is determined by CFU recovered from organ homogenates, but almost 3 days are necessary for *C. neoformans* growth on agar plates (Ngamskulrungrroj *et al.*, 2012) (Krockenberger *et al.*, 2010). We performed *ex vivo* imaging analysis using a *C. neoformans* strain expressing the katushka reporter protein (Shcherbo *et al.*, 2007) to compare with the CFU counting traditionally used to determine fungal loads. The |KAT reporter protein has a higher emission wavelength (635 nm), avoiding the interference of endogenous chromophores. Furthermore, this fluorophore presents greater photostability, faster maturation time and is 1.4 times brighter than mCherry reporter protein, representing an efficient biomarker for *in vivo* analysis (Andreu *et al.*, 2011).

Both imaging analysis and CFU counting demonstrated that plumieridine has significant effect on controlling *in vivo* growth of *C. neoformans*. The imaging analysis revealed reduced pathogen burden in lungs and brain of infected mice after fourteen days of PLD administration either orally or intraperitoneally. Our group recently described the antifungal activity of this compound (Bresciani, data not published), isolated from *Allamanda polyantha* extracts. Plumieride, an iridoid also isolated from this plant extract, enhances the release of TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 in immune compromised mice (Singh *et al.*, 2017). Recently, an antidepressant-like effect of plumieride was also demonstrated (Bonomini *et al.*, 2017). In our study, plumieridine reduced the fungal burden in the brain and lungs of infected BALB/c mice. One of the most significant aspects of potential anti-cryptococcal drugs is their ability to accumulate in the CNS (Zhai *et al.*, 2012), since the fungus specially proliferates in brain tissue (Shi e Mody, 2016). Plumieridine reduced *C. neoformans* capsule formation, effect also reported in cells treated with antifungal drugs such as AMB, VLZ and FLZ *in vitro* and *in vivo* (Khan *et al.*, 2009; Vitale *et al.*, 2012). We hypothesize that the herein described impact of plumieridine on capsular architecture may affect *C. neoformans* growth.

Alterations in *C. neoformans* cell morphology were observed in the presence of plumieridine. The interaction between  $\beta$ -1,4-N-acetylglucosamine (GlcNAc) oligomers and capsular components could be a mechanism involved in capsule dynamics during *C. neoformans* growth (Rodrigues *et al.*, 2008). Previously, our group observed that GlcNAc oligomers from *C. neoformans* cells exposed to an *A. polyantha* crude seed extract were not recognized by WGA lectin (Bresciani, data not published). Then, it is feasible to assume that plumieridine may affect the capsule separation during *C. neoformans* bud detachment. However, the molecular mechanisms involved in these processes are not yet fully understood (Zaragoza & Casadevall, 2004; Rodrigues *et al.*, 2008).

Several studies have indicated that metabolic pathways are potentially good targets to develop therapies with anticryptococcal effects (Song *et al.*, 2012; Mazu *et al.*, 2016; Nett e Andes, 2016). Flucytosine, a fluorinated analogue of cytosine, is known to be effective in the treatment of candidiasis and cryptococcosis (Loyse *et al.*, 2013; Asadzadeh *et al.*, 2017). However, 5-FC is toxic and is frequently associated to resistance in *Candida* and *Cryptococcus* species, which restrict its use in monotherapy protocols (Hospenthal e Bennett, 1998; Vermes *et al.*, 2000; Nett e Andes, 2016). 5-FC is transported into the cell, converted to 5-FU and incorporated into RNA leading to miscoding and disruption of protein synthesis. Additionally, phosphorylated 5-FU blocks DNA synthesis by inhibiting thymidylate synthase (Loyse *et al.*, 2013). Here, by *in silico* studies, we also found possible targets of plumieridine in three proteins from the pyrimidines synthesis pathway: thymidylate synthase, orotidine-5'-phosphate decarboxylase and dihydroorotate dehydrogenase. Furthermore, we showed a significant decrease in transcript levels of thymidylate synthase and orotidine-5'-phosphate decarboxylase genes in cell cultures in the presence of plumieridine. The molecular dynamic analysis demonstrated the possibility of plumieridine to interact with both enzymes in the active site. Taken together, our results revealed a potential anticryptococcal activity of plumieridine *in vitro* and *in vivo* and suggests a possible mechanism of action by inhibiting pyrimidines synthesis pathway.

## **METHODS**

### **Ethics statement**

The animals were cared for according to the Brazilian National Council for Animal Experimentation Control (CONCEA) and Brazilian College of Animal Experimentation (COBEA) guidelines. Mice were housed in groups of five in filtered top ventilated cages, and provided with food and water *ad libitum*. All efforts to minimize animal suffering were made.

Before mortality analysis, mice were intraperitoneally anesthetized with 100 mg/kg ketamine and 16 mg/kg xylazine. Mice were analyzed twice daily for any signs of suffering, defined by weight loss, weakness or the inability to eat or drink. Mice were sacrificed following the first signs of suffering. The Universidade Federal do Rio Grande do Sul Ethics Committee for Use of Animals approved the use of animals in the present work.

### **Preparation of aqueous crude seed extract of *Allamanda polyantha* and isolation of plumieridine**

Crushed seeds were placed in contact with ultrapure water (10 g/20 mL) for 4 h, under agitation. The liquid suspension was centrifuged (for 10 min at 8,000 rpm). The resulting supernatant was filtered in filter paper and polypropylene prefilter (AP 25, Millipore). The aqueous extract was completely lyophilized at -50 °C and 0.040 mbar (Christ Alpha 1-4 LD plus, Germany) and stored at -80 °C. Lyophilized crude extract was subjected to silica gel column chromatography (70-320 mesh, Merck), using a gradient elution of dichloromethane: methanol (95:5 to 80:20) as the mobile phase to obtain plumieridine (90:10, 200 mg). The fractions were chromatographed over preparative TLC (20 cm × 20 cm, 0.5 mm layer, SiO<sub>2</sub> F254 plates – Merck) using a mixture of dichloromethane:methanol (80:20) as eluent.

### **Oral Toxicity Study**

An initial repeated dose study was prepared following OECD guidelines N°. 407. The plumieridine compound was administered at different doses to determine no observed adverse effect level (NOAEL) in female BALB/c mice, in groups of 6 animals. Five concentrations (2 mg/kg, 4 mg/kg, 10 mg/kg and 20 mg/kg, 40mg/kg) of plumieridine were tested in mice, with daily administration for 14 days. The animals were observed for any change in general behavior/or untoward symptom and mortality over a four-week period.

## **Strains and growth conditions**

The wild-type *C. neoformans* H99 strain, *C. gattii* R265 strain and a recombinant strain constitutively expressing Katushka fluorescent gene (*H99F*) were used in this study. These strains were cultivated overnight in YPD medium (2% dextrose, 2% peptone and 1% yeast extract), at 30°C, and 200 rpm.

## **Construction of a *C. neoformans* strain that constitutively expresses the Katushka reporter gene**

The 532 bp and 619 bp fragments corresponding to the histone H3 promoter region and terminator region of *C. neoformans* were amplified by PCR. The Katushka coding sequence plasmid pTURBO-FP635-N was also amplified. The PCR products were analyzed in an agarose gel (0.8%) and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). These fragments were fused by PCR and cloned in to the plasmid pJAF15. The product was transformed into *E. coli* TG-2 cells. Approximately 10 µg of the resulting recombinant vector were used to perform *C. neoformans* biolistic transformation (Toffaletti *et al.*, 1993). Transformants were selected on YPD agar medium supplemented with 200 µg/ml of hygromycin.

## **Fluorescence detection in transformed cells**

The fluorescence levels in *C. neoformans* recombinant cells were determined in the IVIS Lumina. The IVIS Lumina system settings used included an f/stop of 8, a medium binning and a 10 second exposure for cell detection.

## **Analysis of virulence determinants**



The *C. neoformans* H99F strains were evaluated for the key virulence determinants in the *Cryptococcus* model: pigmentation, capsule formation and ability to grow at 37°C. Melanin production was determined visually after the cells were spotted on solid minimal medium supplemented with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) and cultivated for 72 h either at 30 °C or 37 °C (Zhu e Williamson, 2004). For the analysis of capsule formation, the strains were initially cultivated in YPD for 18 h. After this time, the culture was centrifuged for 10 min at 3,522 x g and the cells were washed twice with phosphate-buffered saline (PBS). Aliquots of 10<sup>4</sup> cells were incubated in RPMI-1640 medium (GIBCO, USA) in a final volume of 500 µl in 24-well plate for 72 h at 37°C and 5 % CO<sub>2</sub>. Cells were mixed with India ink and analyzed under microscope as described above. Cell and capsule sizes were measured using ImageJ software (NIH, <http://rsb.info.nih.gov/ij/>). Total cell size was defined as the diameter of the complete cell including the capsule. Capsule size was calculated as the difference between the diameter of the total cell and the cell body diameter, defined by the cell wall. At least 100 cells were measured for each growth condition.

### **Analysis of antifungal susceptibility**

Minimum inhibitory concentrations (MICs) of plumieridine were determined against *C. neoformans* H99 and *C. gattii* R265 strains using the methods proposed by the Clinical and Laboratory Standards Institute M27-A2. Plumieridine was suspended in MilliQ water with 10% DMSO, and filtered prior to use (polyvinylidene difluoride filter, 0.22 µm pore size, Millipore). In 96-well plates, plumieridine was serially diluted (4 mg/mL – 0.003 mg/mL) in RPMI 1640 (pH 7; 2% glucose) buffered with MOPS. Plates were maintained at 37 °C for 72 h. MIC was defined as the lowest concentration at which the yeasts did not demonstrate visible growth. The inoculum of *C. neoformans* and *C. gattii* were prepared following the M27-A2 protocol. To determine the fungicide or fungistatic effect, 10 µL of cell suspensions

from wells without visible growth were spotted onto YPD plate and incubated for 3 days at 37 °C. No colony formation indicated the fungicidal effect.

### **Analysis of Capsular Size and Morphology**

*C. neoformans* was grown in YPD for 24 h at 30 °C and 150 rpm. After this time, the culture was centrifuged for 10 min at 5,000 rpm and the cells were washed twice with PBS. Aliquots of  $10^6$  cells were incubated in RPMI-1640 medium (GIBCO, USA) with different concentrations of plumieridine, in a final volume of 500  $\mu$ l in 24-well plate for 72 h at 37 °C and 5% CO<sub>2</sub>. Cells were mixed with India ink and analyzed by microscopy approaches. Capsule size, calculated with the ImageJ Software, was defined as the difference between the diameter of the total cell and the cell body diameter, defined by the cell wall. At least 100 cells were measured for each growth condition. For additional analysis of capsular morphology, cellular suspensions were processed for fluorescence microscopy. Briefly, fungal cells were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 1 hour followed by incubation with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (Sigma, St. Louis, MO). Cell structures were then stained using calcofluor white (5  $\mu$ g/mL) (Sigma, St. Louis, MO) and anti-GXM antibody 18-B7 (10  $\mu$ g/mL) (kindly provided by Dr. Arturo Casadevall) followed by anti-murine IgG Alexa Fluor 488 conjugated antibody (Invitrogen, Carlsbad, CA) for 30 min at 37°C. Images were acquired using an Axyoplan 2 microscope (Carl Zeiss, Germany). Cell surface structures were also observed by scanning electron microscopy (SEM) as described elsewhere (Frases *et al.*, 2009).

### ***In vivo* drug administration**

Intranasal infection was performed as previously described (Kmetzsch *et al.*, 2010). For treatment, 4 to 6 week old female BALB/c were arranged in groups of twelve animals.

The control groups were infected with wild-type *C. neoformans* or the mutant strain (H99F). Initially, infected mice were treated by oral gavage every 14 days with 4 mg/kg/day of PLD (the first dose was administered 1 day before intranasal infection), or the mice were treated daily with FLZ (2 mg/kg/day) for control. The animals were euthanized, and lungs and brain were excised. The fluorescence levels of infected organs were analyzed in the IVIS Lumina System. Fluorescence measurements are expressed as radiance efficiency values, which correspond to surface radiance normalized to the illumination intensity incident on the object. After fluorescence analysis, colony-forming units (CFU) were determined.

### ***In silico* prediction and modelling of potential cellular targets for plumieridine**

To identify a possible target of plumieridine, *in silico* studies were performed using PharmMapper server (Liu *et al.*, 2010). Among the predicted targets, three proteins from the route of pyrimidines were identified: thymidylate synthase, orotidine-5'-phosphate decarboxylase and dihydroorotate dehydrogenase. The structures of thymidylate synthase and orotidine-5'-phosphate decarboxylase were modeled and docked with plumieridine. The Modeller 9v15 program (Sali e Blundell, 1993) was used to model the proteins. The thymidylate synthase and orotidine-5-phosphate decarboxylase were modeled according comparative modeling technique. The templates used were the structures of thymidylate synthase from *C. neoformans* (PDBID 2AAZ) (Finer-Moore *et al.*, 2005) with 97% identity to sequence target, and the orotidine-5-phosphate decarboxylase from *Saccharomyces cerevisiae* (PDBID 3GDK) with identity of 52% with target sequence. PatchDock (Schneidman-Duhovny *et al.*, 2005) and SwissDock (Grosdidier *et al.*, 2011) were used to docking plumieridine. The better results of docking were refined by molecular dynamics using GROMACS package (Berendsen *et al.* 1995) with the GROMOS 53a6 force field (Oostenbrink *et al.*, 2005).

The expression profile of *C. neoformans* genes related to the route of pyrimidines in the presence of plumieridine was evaluated by RT-qPCR. Cells were cultured initially for 18 h at 30°C and 200 rpm in YPD medium. Subsequently,  $5 \times 10^7$  cells of *C. neoformans* was grown in 20 mL YPD supplemented or not with 156 µg/ml PMD or 1,25 µg/ml FLC for 4 h. Three independent RNA samples were prepared using TRIzol (Invitrogen) according to the manufacturer's protocol. After DNase treatment, reverse transcriptase reactions were performed. Reactions of RT-qPCR were performed using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Platinum SYBR green qPCR Supermix (Invitrogen) supplemented with 5 pmol of each primer and 2 µL of cDNA in a final volume of 20 µL were used as reaction mix. Each cDNA sample was analyzed in triplicate with each primer pair. Data were normalized using actin transcript levels. The  $2^{-\Delta Ct}$  method was used to determine the relative expression (Navidshad *et al.*, 2012).

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## 5. DISCUSSÃO

No presente trabalho, propusemos avaliar a atividade antifúngica *in vivo* do composto natural plumieridina, através da bioimagem baseada na emissão de fluorescência. O sistema de análise por imagem permitiu monitorar a localização do patógeno, bem como avaliar os efeitos de diferentes tratamentos antifúngicos durante a infecção (Andreu *et al.*, 2011). O uso de marcadores fluorescentes se destaca, pois, a imagem é adquirida rapidamente através da excitação de uma proteína fluorescente pela luz UV (Zelmer *et al.*, 2012). Esse processo difere da bioimagem obtida por bioluminescência, a qual necessita da adição de um substrato para a atividade da enzima luciferase (Andreu *et al.*, 2013). Em virtude disso, construímos e caracterizamos uma linhagem de *C. neoformans* fluorescente expressando constitutivamente o gene repórter TURBOFP635 (codificador da proteína Katushka). A proteína Katushka possui características mais promissoras que outros marcadores (Gurskaya *et al.*, 2016). A utilização desse marcador permite análises experimentais de longa duração, devido à elevada foto estabilidade, maior brilho, e comprimento de emissão mais elevado (635 nm), além disso, foi desenvolvida para ser utilizada em estudos *in vivo* (Shcherbo *et al.*, 2007).

O sistema de análise por imagem possibilitou avaliar a disseminação da levedura patogênica *C. neoformans* e sua resposta ao tratamento com plumieridina. A análise por imagem demonstrou a redução da carga fúngica no pulmão e no cérebro de camundongos após 14 dias de administração do composto plumieridina. As observações por imagem foram consistentes, quando comparadas com os resultados do método tradicional de contagem de UFC em órgãos infectados. A plumieridina foi isolada pela primeira vez há 100 anos, mas teve sua atividade antifúngica apenas recentemente descrita (Bresciani, 2013). Anteriormente foi demonstrado que plumieridina foi obtida pela hidrólise enzimática de plumierídeo (Schliemann, 1982), e foi isolada de *Plumeria obtusa* e *Alamanda nerifolia* (Saleem *et al.*, 2011). A

Plumieridina e o plumierídeo apresentam estrutura similar. Estudos mostraram que plumierídeo aumentou significativamente a resposta imunológica Th-1 em camundongos imunocomprometidos Balb/c (Singh *et al.*, 2017). Recentemente, também foi demonstrado o efeito antidepressivo de plumierídeo *in vivo* (Singh *et al.*, 2017). Um dos aspectos mais importante da plumieridina como potencial fármaco anticriptococus é a capacidade de acumular no SNC, visto que *C. neoformans* prolifera principalmente para o cérebro (Zhai *et al.*, 2012). O plumierídeo e a plumieridina já foram descritos como inibidores de crescimento de mudas de trigos, além disso, plumierídeo possui atividade antidermatófito (Adams *et al.*, 1979) (Tiwari *et al.*, 2002). Recentemente, foi investigada sua atuação como agente antioxidante natural contra danos peroxidativos no fígado de mamíferos. Plumierídeo demonstrou potencial antioxidante, estimulando a regeneração hepática (Singh *et al.*, 2014). Também já foi testada sua atividade contra o câncer (Dobhal *et al.*, 2004). Diferentemente do plumierídeo, há poucos relatos na literatura sobre as atividades biológicas da plumieridina. A atividade antifúngica *in vivo* desse composto foi avaliada pela primeira vez no presente trabalho.

*C. neoformans* e *C. gattii* apresentaram diferença de suscetibilidade *in vitro* na presença de plumieridina, sendo que *C. neoformans* foi mais suscetível a plumieridina em comparação à *C. gattii*. Essa diferença já foi também observada para outros fármacos. Por exemplo, *C. gattii* foi mais suscetível a fluconazol e voriconazol do que isolados de *C. neoformans* (Khan *et al.*, 2009). Além disso, também observamos alterações morfológicas distintas em *C. neoformans* na presença de plumieridina. As células apresentaram divisão celular incompleta, e a célula filha permaneceu ligada a célula mãe. Oligômeros de quitina estão associados com a parede celular de *C. neoformans* e *C. gattii*, formando uma estrutura em forma de anel ao redor da junção de brotamento entre célula filha e mãe. Esta estrutura se associa as junções de brotamento nos estágios finais de divisão celular e são estruturas

importantes para a conexão da parede celular com componentes da cápsula (Rodrigues *et al.*, 2008). Bresciani (2013), observou que WGA lectina não reconhece a formação de cadeias de células de *C. neoformans* tratadas com o extrato de sementes de *A. polyantha* (Bresciane, 2013). Portanto, sugerimos que plumieridina esteja inibindo de alguma forma a produção de oligômeros de quitina, fazendo com que a célula filha permaneça ligada a célula mãe formando cadeias de células lineares. No entanto, são necessárias mais análises para confirmar essa hipótese. Além desses dados, demonstramos que plumieridina tem a capacidade de reduzir a cápsula polissacarídica de *C. neoformans*. A formação da cápsula em *Cryptococcus* spp. é um importante fator de virulência, uma vez que mutantes com diminuição ou defeitos na cápsula são avirulentos (Zaragoza *et al.*, 2009). Recentemente, foi observado que inibindo os oligômeros de quitina, a secreção de GXM é afetada para o meio extracelular, inibindo a formação de cápsula. Contudo essa inibição da formação da cápsula não foi observada em *C. gattii* (Fonseca *et al.*, 2013).

Vias metabólicas podem ser potenciais alvos para o desenvolvimento de terapias anticriptocócicas (Fujihashi *et al.*, 2015). Estudos recentes mostraram que defeitos na rota de síntese de novo de purinas resultaram em problemas no desenvolvimento e atenuação da virulência em *C. neoformans* (Morrow *et al.*, 2012). Também foi descrita uma proteína envolvida na síntese de guanina, com características muito destoantes entre fungos e mamíferos. Isso outorga às rotas de síntese de ácidos nucleicos um alvo potencial no estudo de novos fármacos para terapia da criptococose (De Gontijo *et al.*, 2014).

Paralelamente aos experimentos realizados neste trabalho utilizamos métodos *in silico* para prever potenciais proteínas alvo para ação da plumieridina, baseando-se nas características químicas do ligante (Liu *et al.*, 2010). Entre os alvos preditos, três proteínas da rota das pirimidinas foram identificadas: timidilato sintase, orotidina-5'-fosfato descarboxilase e diidroorotato desidrogenase. A enzima timidilato sintase é alvo do

antineoplásico 5-FU e do antifúngico 5-FC (Vermes *et al.*, 2000). 5-FC é um fármaco já conhecido por ser eficaz no tratamento da criptococose e candidíase. Contudo, 5-FC é considerada tóxica, e causa resistência principalmente nas espécies de *Cryptococcus* e *Candida* quando utilizada em monoterapia (Vermes *et al.*, 2000). A toxicidade ocorre pela conversão de 5-FC em alguns metabólitos, principalmente em 5-FU. 5-FU pode exercer atividade antifúngica por dois mecanismos (Vermes *et al.*, 2000). Primeiramente 5-FU pode ser convertido a FUTP e se incorporar no RNA, inibindo a síntese de proteínas. No outro mecanismo 5-FU é convertido a FdUMP inibindo a síntese de DNA através da inibição da timidilato sintase (Vermes *et al.*, 2000). Portanto, sugerimos que a timidilato-sintase é um alvo promissor da plumieridina. Já orotidina 5'-fosfato descarboxilase é uma enzima envolvida na biossíntese de pirimidinas, catalisa a descarboxilação da orotidina-5'-monofosfato para formar uridina-5'-monofosfato (Fujihashi *et al.*, 2015). Não há relatos na literatura da orotidina 5'-fosfato descarboxilase como alvo antifúngico, no entanto é uma enzima com potencial terapêutico, uma vez que, tem sido investigada como potencial alvo de drogas para várias doenças, incluindo, malária, leishmaniose, infecções bacterianas e virais (Fujihashi *et al.*, 2015). Orotidina 5'-fosfato descarboxilase frequentemente é utilizada como marcador de seleção em leveduras (Hisatomi *et al.*, 1998).

Após a identificação dos alvos, avaliamos o perfil de expressão dos genes da timidilato-sintase (TMP1) e da orotidina-5'-fosfato-decarboxilase (URA3) por PCR em tempo real (RT-qPCR). Os resultados sugerem que plumieridina está modulando a expressão de TMP1 e URA3, pois ocorre uma diminuição nos níveis de transcritos de ambas as enzimas. Essa diminuição nos níveis dos transcritos de TMP1 também foi detectada quando as células foram incubadas com flucitosina, porém URA3 não foi regulada em resposta a flucitosina.

Também foi possível modelarmos as estruturas da timidilato-sintase e da orotidina-5'-fosfato-decarboxilase de *C. neoformans*. Os modelos foram submetidos à técnica de *docking*

molecular com a molécula plumieridina, para melhor entender os mecanismos de interação com *C. neoformans*. Os dois modelos demonstraram que ambas as enzimas interagiram no sítio ativo. A interação de plumieridina-timidilato sintase durante todo tempo de simulação ocorreu no sítio ativo da enzima. A interação de plumieridina-orotidina-5'-fosfato descarboxilase nos estudos de *docking* e dinâmica molecular também ocorreu no sítio ativo, mas durante essa simulação plumieridina migrou para uma região adjacente próxima ao sítio ativo. Desta forma, os resultados computacionais sugerem que as duas enzimas da via das pirimidinas podem ser alvos da plumieridina, validando os dados experimentais. Estes dados estão de acordo com os resultados anteriores que sugerem a diminuição nos níveis de transcritos de ambas às enzimas na presença da plumieridina.

De acordo com os testes de suscetibilidade fúngica *in vivo* por bioimagem, a molécula plumieridina demonstrou potente eficácia, em comparação com fluconazol, no tratamento da criptococose. O mecanismo de ação dessa molécula também foi investigado e sugerimos dois potenciais alvos para plumieridina que estão envolvidos na via das pirimidinas, a timidilato-sintase e a orotidine-5'-fosfato-decarboxilase. Existem poucos estudos referentes à plumieridina na literatura. Por tanto, estes dados podem ser a base para futuros testes clínicos e o desenvolvimento de um novo tratamento para a criptococose

## 6. CONCLUSÃO

Por meio de diferentes experimentos, podemos afirmar que a plumieridina demonstrou atividade antifúngica frente a *C. neoformans*, em modelo de infecção murino. Também demonstramos que plumieridina inibe a formação de um dos principais fatores de virulência de *C. neoformans*, a cápsula polissacarídica. Tal estudo é de extrema importância, visto que, atualmente diversos problemas são enfrentados na terapia antifúngica. Além disso, inovamos no método para avaliar a suscetibilidade antifúngica ao utilizarmos a bioimagem baseada na

fluorescência. Pouquíssimos trabalhos são encontrados na literatura referentes à *Cryptococcus* spp. utilizando essa estratégia. Outro fator que deve ser considerado quando se estuda o potencial de uma droga é o mecanismo de ação. No presente estudo sugerimos alvos para a plumieridina, através de estudos *in silico* e *in vitro*. Como citado anteriormente, timidilato-sintase é alvo do antineoplásico 5-FU e do antifúngico 5-FC. Os dados nos sugerem que PMD tem alvo similar ao da 5-FC, porém plumieridina poderá não ser tão tóxica quanto 5-FC. Portanto, a plumieridina é um candidato promissor para futuros estudos de desenvolvimento de drogas terapêuticas.

## 7. PERSPECTIVAS

Em virtude dos resultados obtidos, em experimentos futuros avaliaremos a atividade mutagênica da plumieridina e continuaremos investigando seu mecanismo de ação.

I. Construção de um mutante de superexpressão do gene codificador da proteína timidilato sintase.

II. Avaliação da sensibilidade da linhagem selvagem (H99) e mutante *TMP1* de *C. neoformans* a plumieridina e determinação da concentração inibitória da plumieridina e flucitosina para *C. neoformans*.

III. Determinação do perfil de expressão de genes de *C. neoformans* H99 e mutante *TMP1* relacionados à via das pirimidinas em presença da plumieridina e flucitosina.

IV. Avaliação da atividade citotóxica e genotóxica da plumieridina em células mamíferas através dos ensaios cometa e de micronúcleo.



## 8. ANEXOS

## 9. BIBLIOGRAFIAS

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# Genotypic and Phenotypic Diversity of *Cryptococcus gattii* VGII Clinical Isolates and Its Impact on Virulence

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The *Cryptococcus gattii* species complex harbors the main etiological agents of cryptococcosis in immunocompetent patients. *C. gattii* molecular type VGII predominates in the north and northeastern regions of Brazil, leading to high morbidity and mortality rates. *C. gattii* VGII isolates have a strong clinical relevance and phenotypic variations. These phenotypic variations among *C. gattii* species complex isolates suggest that some strains are more virulent than others, but little information is available related to the pathogenic properties of those strains. In this study, we analyzed some virulence determinants of *C. gattii* VGII strains (CG01, CG02, and CG03) isolated from patients in the state of Piauí, Brazil. The *C. gattii* R265 VGIIa strain, which was isolated from the Vancouver outbreak, differed from *C. gattii* CG01, CG02 and CG03 isolates (also classified as VGII) when analyzed the capsular dimensions, melanin production, urease activity, as well as the glucuronoxylomannan (GXM) secretion. Those differences directly reflected in their virulence potential. In addition, CG02 displayed higher virulence compared to R265 (VGIIa) strain in a cryptococcal murine model of infection. Lastly, we examined the genotypic diversity of these strains through Multilocus Sequence Type (MLST) and one new subtype was described for the CG02 isolate. This study confirms the presence and the phenotypic and genotypic diversity of highly virulent strains in the Northeast region of Brazil.

**Keywords:** *Cryptococcus gattii*, molecular type VGII, MLST, differential pathogenicity, virulence factors

## INTRODUCTION

Isolates from the *Cryptococcus neoformans*–*Cryptococcus gattii* complex are the etiological agents of cryptococcosis, a life-threatening disease that affects the lungs and brains of humans and animals (Kwon-Chung et al., 2014). It has been estimated that each year human cryptococcosis affects nearly 220,000 individuals, of which 181,100 cases result in death (Rajasingham et al., 2017). Although members of the *C. neoformans* complex are the major agents of the cryptococcosis cases worldwide, *C. gattii* species complex isolates have emerged as primary pathogens due to their

increasing geographical expansion and the high lethality rate of their infections (Chen et al., 2014; Espinel-Ingroff and Kidd, 2015; Amburgy et al., 2016; Farrer et al., 2016; Lockhart et al., 2016; Souto et al., 2016; Acheson et al., 2017; da Silva et al., 2017; Noguera et al., 2017; Velez and Escandon, 2017).

By means of serology, *C. gattii* isolates are classified into serotypes B and C. Multilocus sequence typing (MLST) of the species revealed five distinct molecular types: VGI, VGII, VGIII, VGIV, and VGIV/VGIIIc (Meyer et al., 2009; Springer et al., 2014). Efforts to recognize the five distinct genotypes into seven species were already proposed (Hagen et al., 2015). However, there is no consensus in the scientific community (Hagen et al., 2017; Kwon-Chung et al., 2017). All the molecular types can be found globally, but isolates of the VGII genotype are predominant (Chen et al., 2014). Cryptococcosis caused by VGII lineages is endemic in the north and northeastern regions of Brazil, and predominates in immunocompetent individuals. This leads to high morbidity and mortality rates, which can range from 37 to 49%, including children and young adults (Trilles et al., 2008; Martins et al., 2011; Souto et al., 2016).

Areas with the highest cryptococcal infection incidence include Vancouver Island, Canada, the US Pacific Northwest, and in some tropical and subtropical regions. Previous cryptococcosis outbreaks in these areas were predominantly caused by *C. gattii* of molecular type VGII (Kidd et al., 2007). Three distinct clonal lineages (subtypes) within the VGII molecular type (VGIIa, VGIIb, and VGIIc) were identified through PCR-fingerprinting, Amplification fragment length polymorphism (AFLP) analysis and MLST. Recent studies have shown that the *C. gattii* lineages involved in those outbreaks emerged as a result of recombination in the native rainforest of Northern Brazil and were then dispersed onto temperate regions (Hagen et al., 2013; Engelthaler et al., 2014). *C. gattii* VGII isolates have a high clinical relevance and phenotypic variations that directly affect its pathogenicity in distinct models of cryptococcosis, as observed in pivotal studies (Cheng et al., 2009; Ngamskulrungraj et al., 2012; Firacative et al., 2014; Farrer et al., 2015).

Several factors that contribute to *C. neoformans*–*C. gattii* species complex virulence have been established, such as the polysaccharide capsule, the melanin within the cell wall, urease and laccase enzymatic activities the ability to grow at a temperature of 37°C, and the mating type (Steenbergen and Casadevall, 2003; Kronstad et al., 2011; Kwon-Chung et al., 2014; Bielska and May, 2016). Although all pathogenic cryptococci present these features, there are some important differences among them which can influence the hypervirulence of *C. gattii* VGII isolates specifically (O'Meara and Alspaugh, 2012).

The fundamental roles of the polysaccharide capsule in cryptococcal virulence are well defined. For instance, acapsular strains of *C. neoformans* are less virulent than encapsulated strains, as acapsular strains strongly induce host immune responses (Buchanan and Murphy, 1998). Glucuronoxylomannan (GXM) is the major polysaccharide in the composition of the capsule, and is abundantly secreted in culture fluids and infected tissues; GXM is another contributing factor toward the virulence of *C. neoformans*. On the other hand, Zaragoza and coworkers found clear evidence that *in vitro*

capsule size has no correlation with virulence in *C. neoformans* (Cherniak and Sundstrom, 1994; Zaragoza et al., 2009; Fonseca et al., 2010). Another important factor affecting cryptococcal virulence is melanin production. Previous studies have suggested that high laccase activity in *C. neoformans* increases cryptococcal intracellular survival within macrophages and confers resistance to antifungal drugs (Sabiiti et al., 2014). In addition, the mating type also influence *C. gattii* virulence. Considering VGII genotypes, the mating type alpha is more prevalent worldwide and a correlation between fertility and virulence could be draw (Ngamskulrungraj et al., 2008).

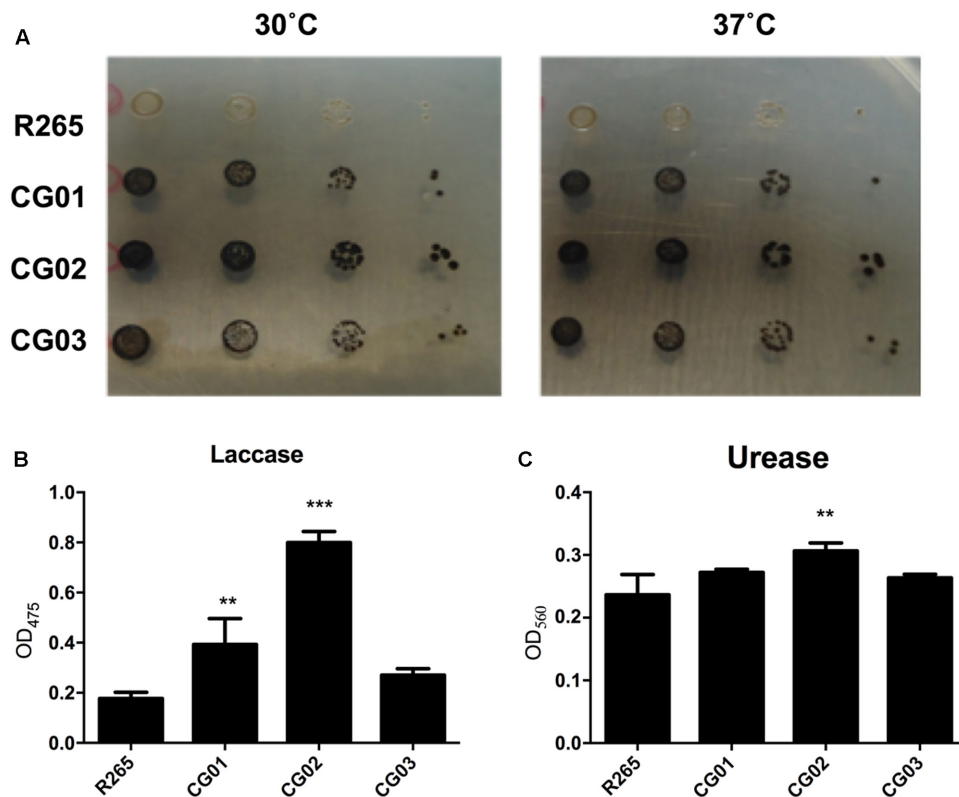
The aim of this study is to investigate the impact of phenotype and genotype on the virulence of three clinical VGII isolates (CG01, CG02, and CG03) obtained from Piauí, Brazil.

## RESULTS

### CG Isolates Displayed Higher Production of Melanin and Urease

To evaluate possible differences regarding the virulence between the isolates, *in vitro* assays were performed to measure the best characterized cryptococcal virulence factors. The production of melanin by *C. gattii* VGII isolates was assessed employing both spot plate and spectrophotometric assays. After 72 h of incubation of cryptococcal isolates serially diluted onto minimal media containing L-DOPA, we noticed that CG01, CG02, and CG03 isolates displayed increased rates of pigmentation when compared to R265 (**Figure 1A**). In addition, we evaluated the laccase activity, by the quantification of melanin-like pigment upon exposure to L-DOPA; after 72 h of incubation, the supernatant was collected and the amount of melanin-like pigment was detected spectrophotometrically (OD<sub>405</sub>). In comparison to the R265 strain, CG02 cultures showed a fourfold increase in laccase activity, the CG01 cultures showed a twofold increase in laccase activity, and the CG03 cultures showed no significant differences in laccase activity (**Figure 1B**). Urease activity was also analyzed for CG01, CG02, and CG03 isolates. Following the incubation of each strain in the Roberts urea broth, the amount of urease was quantified spectrophotometrically in the supernatant (OD<sub>560</sub>). The urease activity of CG01 and CG03 cultures were similar to that of R265, whereas the CG02 isolate displayed a slight increase in urease activity compared to R265 (**Figure 1C**).

Capsule enlargement and GXM secretion are essential factors for cryptococcal pathogenesis (Zaragoza et al., 2009); these factors were compared between our isolated strains and R265 (O'Meara and Alspaugh, 2012). After culturing in minimal media for 72 h, yeast cells were incubated with 18B7 anti-GXM monoclonal antibody and evaluated through fluorescence microscopy. A higher labeling by 18B7 antibody was found in the CG03 strain in comparison to the R265 strain (**Figure 2A**). Determination of the capsule diameter ratio showed that all three strains had a larger capsule size compared to the R265 strain (**Figure 2B**). The amount of secreted GXM was evaluated by ELISA. All three strains presented higher amounts of polysaccharide content in the culture supernatant compared



**FIGURE 1** | Phenotypic analysis of *C. gattii* isolates. **(A)** Visual analysis of melanin production after fungal growth in solid media supplemented with L-DOPA at 30°C and 37°C. **(B)** Laccase activity measured in liquid media containing L-DOPA. **(C)** Urease activity assay, cells were incubated in Roberts urea broth. Urease activity was measured by optical density at 560 nm. Error bars indicate SD. Mean values were compared using two-way ANOVA with Tukey's *post hoc* test. \*\* $p < 0.005$ , and \*\*\* $p < 0.001$ .

to R265 strain (Figure 2C). Capsular morphological analysis by scanning electron microscopy revealed a clear difference among capsular fibers of the isolate cells (Figure 2A). Although the secretion of capsular components, such as GXM, is required for capsule assembly, capsule enlargement also requires polysaccharide molecules with higher effective diameters (Frases et al., 2009). We then analyzed this parameter in GXM fractions from R265, CG01, CG02, and CG03 cells. We detect some differences on the diameter distribution profiles of purified GXM from culture supernatants of the four strains. The CG03 and CG01 isolates showed a longer polysaccharide size distribution with a maximum size of 10.000 nm, R265 and CG02 cells presented a polysaccharide size distribution shorter than CG01 and CG03 cells, with maximal range size of 6.000 nm (Figure 3).

### CG02 Is Less Sensitive to Phagocytes

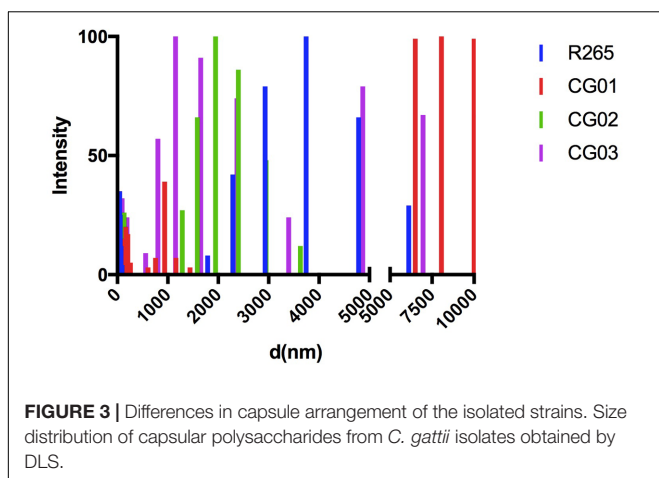
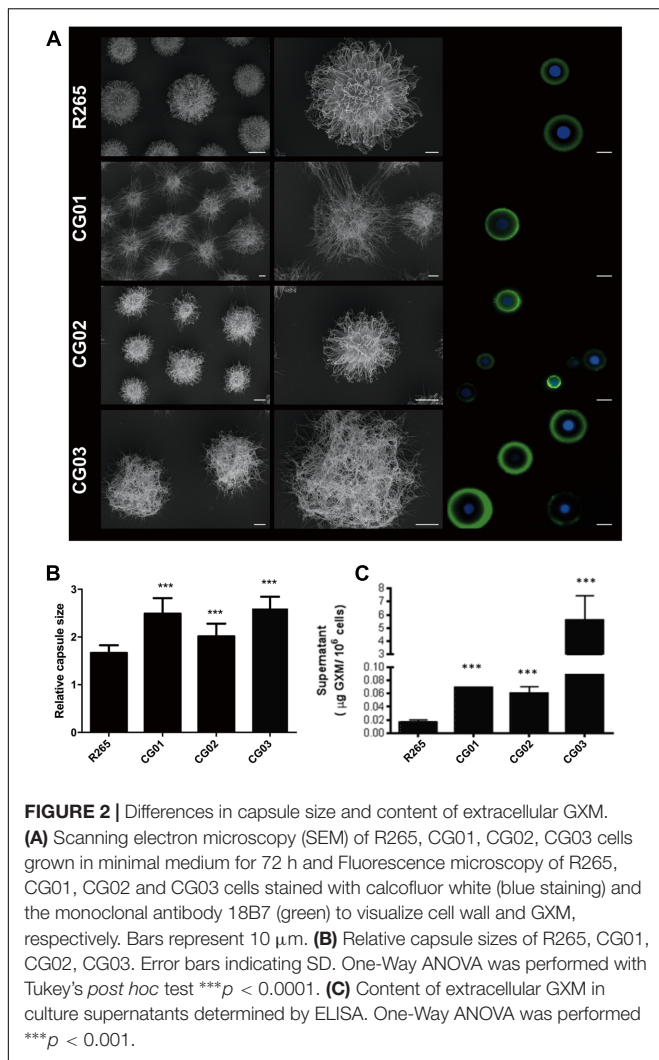
Members of the *C. neoformans-C. gattii* species complex are facultative intracellular pathogens that can survive in macrophages (Feldmesser et al., 2000). Since cryptococcal interaction with the host's cells is a limiting factor which influences the outcome of the disease (Leopold Wager et al., 2016), we assessed the ability of the yeast isolates to survive to the antifungal activity of macrophage cells. Afterward, the susceptibility of CG01, CG02, and CG03 isolates to the antifungal

activity of J774.16 macrophage-like cells was evaluated *in vitro*. Yeast cells were opsonized with antibody 18B7 and incubated with J774.16 cells for 2 and 24 h. The number of associated cells was then determined by CFU analysis following supernatant removal and washing off of non-associated cells. In comparison to R265 cells, CG03 showed lower rates of association/survival after either 2 or 24 h of interaction with J774.16 macrophage-like cells; while the CG01 and CG02 strains displayed increased association/survival rate after 2 h of incubation. No differences were detected between CG01 and CG02 strains after 24 h of incubation (Figure 4).

### Phenotypic Associations and Virulence

Phenotypic variations linked to virulence factors were observed for the investigated isolates and these were compared to R265 strain. To evaluate whether these phenotypes are directly related to the isolates virulence, mice were infected mice ( $n = 5$  animals/isolate) and compared host survival and fungal burden. Differences in virulence profile were observed for all investigated isolates. Mortality curves revealed that the CG02 isolate displayed a hypervirulent profile in the intratracheal model of infection ( $LT_{50} = 21$  days) (Figure 5A) and the intranasal inhalation model (Figure 5B). In fact, the CG02 strain produced a higher pulmonary fungal burden overall after 10





and 15 days post-infection (**Figure 5C**) and was the only isolate detected in the brain after 10 days of infection (**Figure 5D**). Based on prior studies, the VGIIa R265 strain was chosen as a positive control for virulence (median survival = 31.6

(Kidd et al., 2004). CG01 had a similar lethality compared to R265 (median survival = 31.8 days); furthermore, both CG01 and R265 strains produced a similar pulmonary fungal burden (**Figure 5C**). The CG03 had an attenuated virulence profile then compared to R265 (median survival = 41.6 days). Both CG01 and CG03 were unable to reach the brain (**Figure 5E**). Lungs infected with R265, CG01, CG02 and CG03 isolates (**Figure 5E**) all comprised mostly of cryptococcal cells (cryptococcomas).

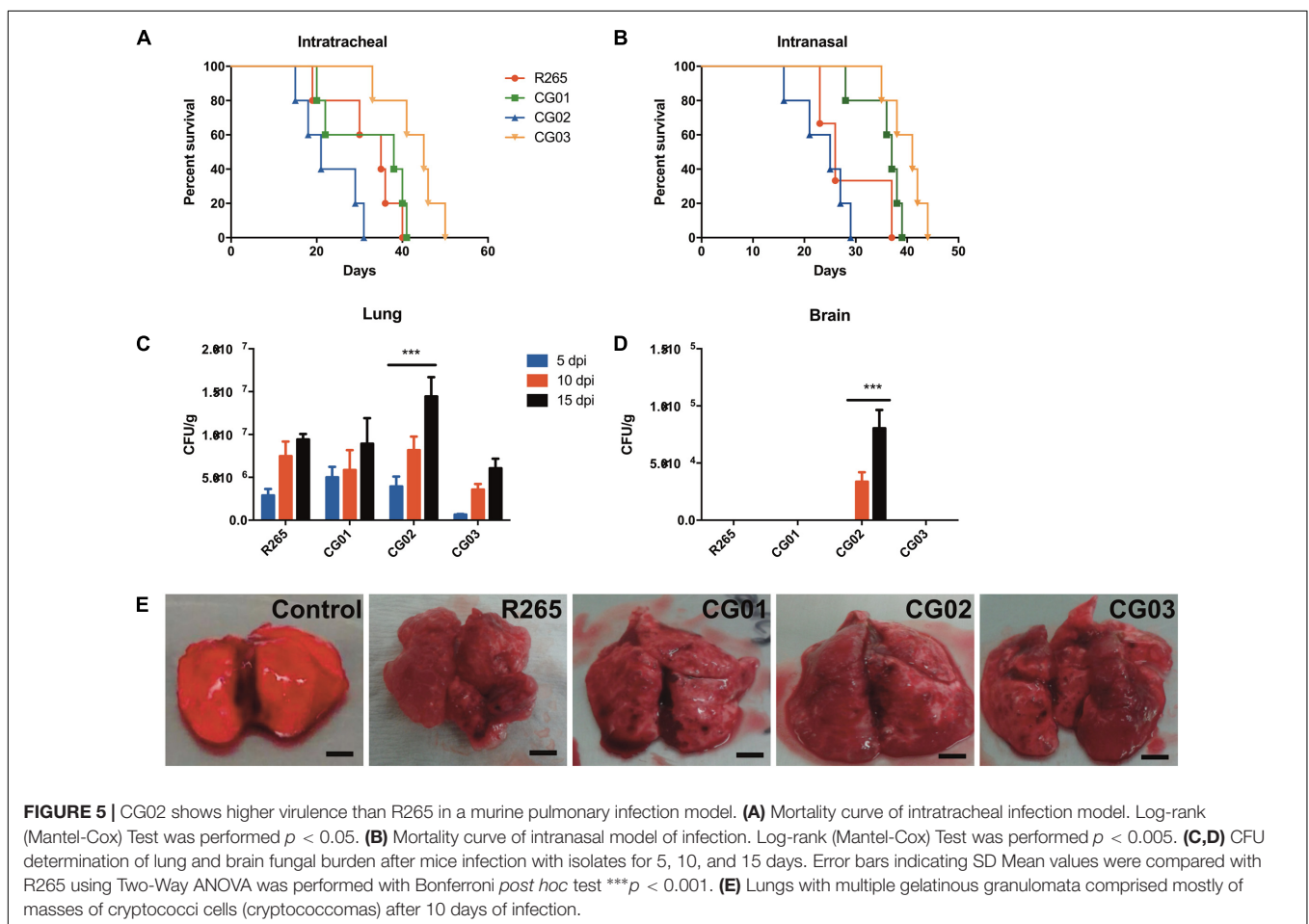
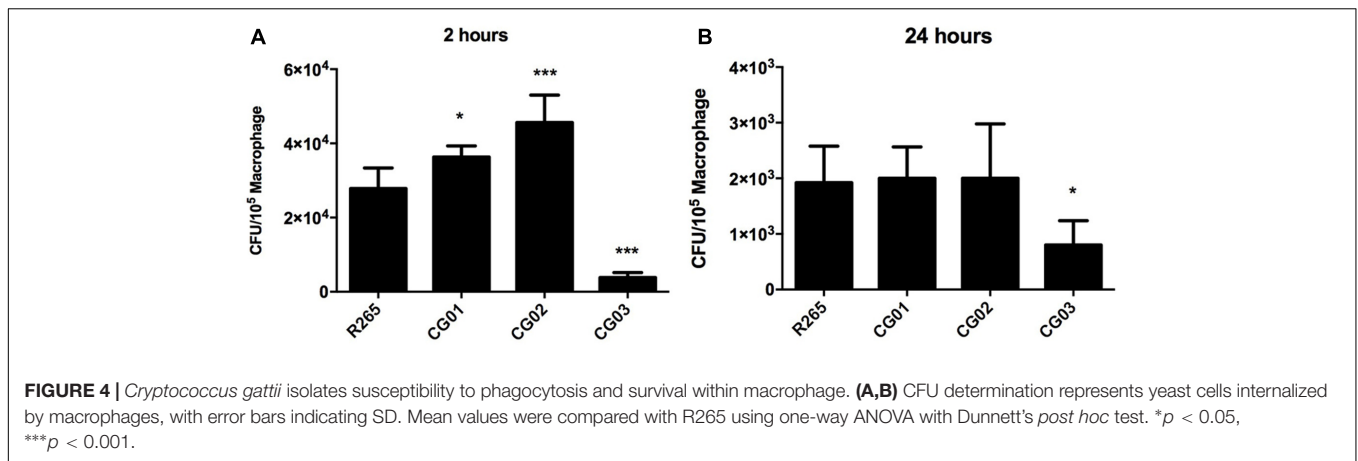
### MLST Analysis Revealed a New Sequence Type (ST)

With the intent of evaluating the genetic diversity of the three *C. gattii* VGII isolates in our study, we performed the ISHAM MLST consensus typing scheme. DNA samples were sequenced for all seven loci according to the ISHAM typing scheme (Meyer et al., 2009). The sequences (Supplementary File 1) were used for MLST genotypes assignment (**Table 1**). The MLST profiling of the isolates revealed a clear genetic diversity. CG01 was classified as the sequence type 125 (ST 125), while CG03 belongs to the ST 127. A new subtype was described for CG02 isolate, which was named as ST 454.

### DISCUSSION

The *C. gattii* species complex has attracted attention as a public health issue (Springer et al., 2012). Some studies have highlighted the importance of understanding the pathogenesis and virulence of VGII molecular type, as it was the cause of the Vancouver outbreak in 1999 (Byrnes et al., 2010; Bartlett et al., 2011; Ngamskulrungrroj et al., 2011, 2012; Chen et al., 2014; Springer et al., 2014; Farrer et al., 2016). In the North and Northeast regions on Brazil, the *C. gattii* species complex harbors isolates that can be considered as endemic fungal pathogen that infects otherwise healthy individuals classified as immunocompetent (Martins et al., 2013). Different virulence profiles of *C. gattii* species complex isolates have been detected (Ngamskulrungrroj et al., 2011; Thompson et al., 2014; Rodrigues et al., 2015), as a recent study with globally selected *C. gattii* species complex strains (including all molecular types) demonstrated that virulence is related to the distinct characteristics of individual strains and is not specifically associated with a particular *C. gattii* species complex molecular type (Firacative et al., 2014).

The results obtained in this study indicate remarkable differences in the pathogenicity of three *C. gattii* species complex isolates belonging to the same molecular type (VGII) from Piauí, Brazil. The pathogenic factors linked to these differences were mostly related to the surface architecture of *C. gattii*, including melanin synthesis, capsular structure and urease activity. The pathogenicity analyses were based on assays performed *in vitro* and *in vivo* with macrophages and mice, respectively. Additionally, it was demonstrated that the CG02 strain is hypervirulent in comparison to the R265 (VGIIa) strain; CG02 also has the ability to penetrate into the central nervous system (CNS), leading to the development of meningoencephalitis, which is not a common characteristic of *C. gattii* VGII species.



The ability of CG02 strain to transpose the blood brain barrier must be one of the reasons as to why it displays a higher virulence profile when compared to R265 and other CG strains. Despite no information about the MAT locus identity of the isolates here described, a higher proportion of MAT alpha strains are present in Brazil (Trilles et al., 2008; Souto et al., 2016). Considering that *C. gattii* MAT alpha strains are usually more virulent compared to MAT a strains (Ngamskulrungraj et al., 2008), the observed

differences in virulence of CG strains compared to R265 strains (MAT alpha) could not be assumed to be driven by differences in the mating type.

Melanin is necessary for the protection of fungal cells against the antimicrobial strategies produced by the host (Doering et al., 1999; Nosanchuk and Casadevall, 2006). Our findings showed that the hypervirulent isolate CG02 had increased rates of pigmentation at 37°C and released higher amounts of these

**TABLE 1** | Assignment of MLST alleles.

Strain	Alleles							ST
	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	
R265	1	1	4	4	1	14	7	20
CG01	2	27	52	4	2	27	32	125
CG02	33	6	87	29	2	1	1	454*
CG03	2	16	54	4	26	15	7	127

Alleles and Sequence Types (ST) found in *C. gattii* VGII isolates from patients in Piauí, Brazil. \*, New Sequence Type.

pigment molecules in culture. Melanin synthesis is related with neurotropism in *C. neoformans* (Nosanchuk et al., 2000). Previous studies have demonstrated that higher laccase activity in *C. neoformans* increased its survival within macrophages (Sabiiti et al., 2014). In this study, the association of CG02 cells to macrophages-like cells was significantly higher than that of CG01, CG03, and R265. Furthermore, CG02 isolate displayed increased levels of urease activity. The urease activity is related to the paracellular transmigration mechanism, which can cause damage to the brain microvasculature endothelial cell (BMEC) tight junctions, thereby facilitating *C. neoformans* blood-to-brain invasion (Shi et al., 2010; Feder et al., 2015). Interestingly, the CG02 was the only among the studied isolates which was detected in the brain after 10 days of murine infection. Cryptococcal cells that are more susceptible to phagocytosis have a higher intracellular survival rate within phagocytes and thus a more efficient dissemination to the brain (Sabiiti et al., 2014). Cryptococci can usually survive, replicate intracellularly and laterally, and then transfer from one macrophage to another, eventually invading tissues and organs (Leopold Wager et al., 2016). They can use macrophages as trafficking vehicles (Trojan horse mechanism) for dissemination, allowing the yeast to cross the blood-brain barrier into the central nervous system (Charlier et al., 2009). We hypothesized that the CG02's higher association to macrophages enhanced the Trojan horse mechanism, resulting in more severe cryptococcosis *in vivo*; whereas CG03's lower phagocytic susceptibility would have an attenuated virulence profile when compared to R265. Altogether, those results suggest that the CG02 isolate was detected in the brain due to increased laccase and urease activity, and to enhanced dissemination, possibly through the Trojan horse mechanism.

One significant virulence factor and determinant feature in *C. neoformans* and *C. gattii* species complex, which instigates disease, is their ability to produce a polysaccharide capsule (Zaragoza et al., 2009). Mutants with defective capsule formation are avirulent in murine models of infection (Chang and Kwon-Chung, 1994). In this study, the least virulent isolate displayed the largest capsular dimensions. The CG03 isolate displayed the largest quantities of GXM detected in cell surface and showed the highest polysaccharide content in culture supernatant. This phenotype was accompanied by lower susceptibility of association to murine macrophages. This lower CFU count could be due to an increased polysaccharide capsule size, which prevents macrophage phagocytosis. Previous studies

demonstrate that VGIIa strains have a small polysaccharide capsule size *in vitro*, evidence that correlates capsule size with the virulence profile (Ngamskulrungraj et al., 2011). It has also been shown in previous works that the formation of a capsular network with reduced dimensions can facilitate the host defense through several mechanisms. Fonseca et al. (2010) suggested that synthesis of capsular structure with reduced dimensions could increase susceptibility to phagocytosis.

Altogether, our results points to a higher production of virulence factors for the CG strains compared to R265. It is known that continued sub culturing affects phenotypic and genotypic characteristics of cryptococcal cells (Cavalcante et al., 2007). However, the assessment of virulence profiles of the R265 strain by our group over the last decade did not revealed major differences (Schneider et al., 2012; Godinho et al., 2014; Feder et al., 2015; Schneider Rde et al., 2015; Joffe et al., 2017; Ribeiro et al., 2017). In this way, the differences of virulence properties between CG strains and R265 could not be caused by sub culturing and should be considered due to inherent genotypic and phenotypic characteristics.

Determination of specific genotypes and their correlations with virulence is an important epidemiological tool for precise and efficient vigilance (Alanio et al., 2017). *C. gattii* VGII isolates can be grouped into three genotypes: VGIIa, VGIIb, and VGIIc, which belongs to ST 20, ST 7, and ST 6, respectively. In this study, we compared the *in vitro* phenotypic assays and presence of different virulence factors to the virulence *in vivo*. We also correlated genotype with virulence. The genetic diversity of three *C. gattii* VGII isolates from Piauí, Brazil was evaluated using the ISHAM MLST consensus typing scheme. All three detected STs exhibited phenotypic and virulent differences. Analysis of the subtypes ST125 (CG01), ST127 (CG03), and ST452 (CG02) indicated significant differences in the colonization of the lungs and brains of mice. ST125 (CG01) and ST127 (CG03) displayed a dominant pulmonary infection in mice and some phenotypic similarity with the isolate R265 (ST20-VGIIa). In comparison to members of the *C. neoformans* species complex, isolates of the *C. gattii* species complex usually cause lung infections, and their VGII strains are less efficient in spreading to the human brain (Ngamskulrungraj et al., 2012). On the other hand, the new ST454 (CG02) strain was found in both the lungs and brains of mice, and was hypervirulent in comparison to ST125 (CG01), ST127 (CG03), and ST20 (R265) isolates. Previous studies have correlated specific genotypes with virulence within a larger number

of global VGIIa and VGIIb isolates and demonstrated that genotype (VGIIa) was more virulent than the genotype (VGIIb) (Ngamskulrungraj et al., 2011). Moreover, VGIIa and VGIIc subtypes presented different phenotypes, as well as displayed predominance in pulmonary infections and an increased *in vivo* virulence (Harris et al., 2011). Only the VGIIb presented the clinical features of neurotropism, already described in a rat model of cryptococcosis (Krockenberger et al., 2010). Due to a combination of phenotypic and genotypic properties as well as geographic proximity, it has been proposed that the VGIIa and VGIIc subtypes have only recently originated from a common ancestor (Harris et al., 2011). Thus, we suggest that the genetic and phenotypic profile is a determinant of pathogenic potential. This was anticipated in a seminal work that described that the Amazon rainforest is the source of hypervirulent strains (Hagen et al., 2013). However, broader analysis now suggests that the semi-arid desert in the Northeast of Brazil can represent the origin of cryptococcal hypervirulent strains (Souto et al., 2016).

Our work supports the notion that members of the *C. gattii* species complex are phenotypically heterogeneous pathogens; they presented significant genotypic diversity, which resulted in a different pattern of clinical disease. In fact, this follows the sense of a new nomenclature scheme (Hagen et al., 2015). This has been the first study to establish a correlation between genotype, virulence and phenotype using clinical isolates from Brazil. Notably, it has been recently demonstrated that the Brazilian *C. gattii* VGII subtypes have great genetic variability. This diversity is due to the presence of both mating types in clinical and environmental samples, which generates recombination from sexual reproduction (Souto et al., 2016). Our results demonstrate the emergence of hypervirulent genotypes from Northeastern regions of Brazil, those of which may expand and provide insights about the origins of the outbreak. Furthermore, we propose that differences in the melanin production, capsular structure and urease activity are involved in the pathogenic profile of *C. gattii* VGII isolates.

## MATERIALS AND METHODS

### Ethics Statement

The animals were taken care according to the Brazilian National Council for Animal Experimentation Control (CONCEA) guideline. Mice were housed in groups of five in filtered top ventilated cages and were provided with food and water ad libitum. All efforts to minimize animal suffering were made. Before mortality analysis, mice were intraperitoneally anesthetized with 100 mg/kg ketamine and 16 mg/kg xylazine. The mice were analyzed twice daily for any signs of suffering, defined by weight loss, weakness, or the inability to eat or drink; they were sacrificed following any signs of suffering. The Universidade Federal do Rio Grande do Sul (UFRGS) Ethics Committee for Use of Animals (CEUA – 19801) approved the use of animals in the present work.

## Fungal Strains and Media

Four different *C. gattii* strains were used in the study. The first strain, R265 (ATCC MYA 4093), was previously identified (Kidd et al., 2004). The three other *C. gattii* strains (CG01, CG02 and CG03) of molecular type/genotype VGII involved in this study were isolated from immunocompetent patients who had cryptococcal meningitis (two children and one adult, from Piauí State, Brazil) (Martins et al., 2013). Such strains are available under request in the Collection of Pathogenic Fungi from the Instituto de Pesquisa Evandro Chagas – FIOCRUZ<sup>1</sup> with the following accession codes: Cg01 – CFP255; Cg02 – CFP258; Cg03 – CFP386. Cells were kept in YPD medium (2% glucose, 2% peptone, and 1% yeast extract). For subsequent experiments, minimal medium was used (15 mM glucose, 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 29.4 mM KH<sub>2</sub>PO<sub>4</sub>, 13 mM glycine and 3 μM thiamine-HCl, pH 5.5).

## Phenotypic Characterization

Pigmentation, urease activity, capsule formation and ability to grow at 37°C were evaluated for each isolate. Melanin production was visually determined following the growth of cryptococcal cells, which had been serially diluted in solid minimal medium supplemented with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) and incubated for 72 h either at 30°C or 37°C (Zhu and Williamson, 2004). Laccase activity was quantified as previously described (Pukkila-Worley et al., 2005). Urease activity was evaluated according as previously (Singh et al., 2013). The measurements (OD<sub>560nm</sub>) in urea broth were performed after a 4 h interval. All phenotypic assays were performed thrice. For capsule measurements, R265, CG01, CG02, and CG03 were grown in YPD for 24 h at 30°C and 200 rpm. The culture was then centrifuged for 10 min at 3,522 × g and the cells were washed twice with phosphate-buffered saline (PBS). Aliquots of 10<sup>4</sup> cells were incubated in RPMI-1640 medium (GIBCO, United States) in a final volume of 500 μL in 24-well plate for 72 h at 37°C and 5% CO<sub>2</sub>. Cells were mixed with India ink and analyzed under microscope as described above. Cell and capsule sizes were measured using ImageJ software (NIH<sup>2</sup>). Total cell size was defined as the total diameter of the cell, including the capsule. Capsule size was calculated as the difference between the diameter of the total cell and the cell body diameter, defined by the cell wall. At least 100 cells were measured for each growth condition.

## Fluorescence-Based and Scanning Electron Microscopy Analysis of the Cell Surface

Staining of the cryptococcal surface components (chitin and GXM) was performed as described (Kmetzsch et al., 2011). Fungal cells were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, United States) for 1 h, and were then incubated

<sup>1</sup><http://cfp.fiocruz.br/index>

<sup>2</sup><http://rsb.info.nih.gov/ij/>



with PBS supplemented with 1% bovine serum albumin (Sigma, St. Louis, MO, United States). Cell structures were then stained using calcofluor white (5  $\mu\text{g}/\text{mL}$ ) (Sigma, St. Louis, MO, United States) and incubated with 18B7 anti-GXM monoclonal antibody (10  $\mu\text{g}/\text{mL}$ ) (kindly provided by Dr. Arturo Casadevall) followed by anti-murine IgG Alexa Fluor 488 conjugated antibody (Invitrogen, Carlsbad, CA, United States) for 30 min at 37°C. Images were acquired using an Axyoplan 2 microscope (Carl Zeiss, Germany). Cell surface structures were also observed by scanning electron microscopy (SEM) (Frasers et al., 2009).

## GXM Effective Diameter

For polysaccharide effective diameter determination, extracellular GXM was isolated from culture supernatants as previously described (Nimrichter et al., 2007). Yeast cells were cultivated in a minimal medium for 2 days at room temperature with shaking and were separated from culture supernatants by centrifugation at 4,000  $\times g$  (15 min, 4°C). The supernatant fluids were collected and again centrifuged at 15,000  $\times g$  (15 min, 4°C) to remove smaller debris. The pellets were discarded and the resulting supernatant was concentrated approximately 20-fold using an Amicon (Millipore, Danvers, MA, United States) ultrafiltration cell (Nimrichter et al., 2007). After supernatant concentration, the viscous layer formed was collected using a cell scraper and transferred to graduated plastic tubes for GXM determinations. The effective diameter of GXM in these samples was determined by Quasi elastic light scattering in a 90Plus/BIMAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY, United States), using minor modifications of the method described previously (Frasers et al., 2009).

## Macrophage Assays

Assays were conducted to evaluate the susceptibility of the *C. gattii* strains cells to the antifungal action of phagocytes. Macrophage-like J774.16 cells were seeded at a density of 1  $\times 10^5$  cells/100  $\mu\text{L}$  of DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS – Sigma) in each well of the 96-well culture plates (TPP). After 24 h of incubation (37°C and 5% CO<sub>2</sub>), the medium was replaced with fresh medium containing 1  $\times 10^6$  cells of each fungal strain, obtained after a 18 h of growth in YPD and extensive washing in PBS and opsonization with anti-GXM antibody 18B7 (final concentration of 1  $\mu\text{g}/\text{mL}$ ). The plates were then further incubated (2 and 24 h at 37°C and 5% CO<sub>2</sub>). Yeast cells that were not internalized by the macrophages were removed with PBS washes. Fungal survival was evaluated after macrophage lysis with sterile ice-cold water and subsequent plating on YPD for Colony Forming Units (CFU) determination. This assay was performed in thrice for each strain.

## Virulence Assay

Virulence studies were conducted as previously described (Steenbergen and Casadevall, 2003). Fungal cells were cultured in 50 mL YPD medium at 30°C overnight with shaking, washed twice and suspended in PBS. Groups of five female BALB/c mice

(~5 weeks old) were intraperitoneal anesthetized with Ketamine (100 mg/kg) and Xylazine (16 mg/kg) and then infected with 1  $\times 10^5$  yeast cells using an intranasal inhalation infection model. We also performed intratracheal infection of mice with 2  $\times 10^6$  yeast cells/mL (groups of 5 female BALB/c mice). The infection was performed and monitored twice daily for moribund signals. The median survival values were calculated by the Kaplan–Meier survival analysis. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee.

## Organ Fungal Burden

Fungal cells were cultured in 50 mL YPD medium at 30°C overnight with shaking, washed twice and resuspended in PBS. Groups of 5 female BALB/c mice (~5 weeks old) were intraperitoneal anesthetized with Ketamine (100 mg/kg) and Xylazine (16 mg/kg) and then infected with 1  $\times 10^5$  yeast cells and monitored twice daily for moribund signals. Mice were euthanized by CO<sub>2</sub> inhalation. The lungs and brain were homogenized in PBS, diluted and plated on YPD for CFU determination at each time point for fungal load determination (5, 10, and 15 days post infection).

## Multilocus Sequence Typing (MLST)

Isolates were subtyped using MLST using partial sequence analysis of six housekeeping genes (*URA5*, *CAP59*, *LAC1*, *GPD1*, *PLB1*, *SOD1*) and the *IGS1* region. For each locus studied, different genetic sequences present within a species are assigned as distinct alleles. The combination of the identified alleles at each of the loci defines the allelic profile or sequence type for each isolate. The data generated can be used to determine whether the fungal isolates are clonal or have undergone recombination. The *URA5*, *IGS1*, *CAP59*, *LAC1*, *GPD1*, *PLB1*, and *SOD1* gene fragments were amplified using the published PCR conditions for all seven loci (Meyer et al., 2009). PCR products of the six housekeeping genes and the *IGS1* region were purified and sequenced by Ludwig Biotec, Porto Alegre. Sequences were analyzed using the Electropherogram quality analysis<sup>3</sup> and aligned using Muscle algorithm available in MEGA 6.06. Allele numbers and sequence types (ST) were determined using the online *C. gattii* MLST database<sup>4</sup>. The new allele was submitted to the ISHAM MLST database for inclusion.

## AUTHOR CONTRIBUTIONS

VB, LM, AF, JR, ES, GdSA, SF, CS, AS, LK, and MV prepared the experimental design. VB, LM, AF, JR, and ES conducted the phenotyping, CFU analysis, and animal experimentation. VB, GdSA, and SF performed the MEV, immunofluorescence and LS measurements. SF, CS, AS, LK, and MV provided reagents

<sup>3</sup><http://asparagin.cenargen.embrapa.br/phph/>

<sup>4</sup><http://mlst.mycologylab.org/DefaultInfo.aspx?Page=Cgattii>

and equipment. VB, LM, AF, JR, ES, GdSA, SF, CS, AS, LK, and MV discussed the results and wrote and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.00132/full#supplementary-material>

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