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MICROBIOLOGY

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

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Abstract: *Aspergillus* genus causes many diseases, and the species *Aspergillus flavus* is highly virulent. Treatment of aspergillosis involves azole derivatives such as voriconazole and polyenes such as amphotericin B. Due to an increase in fungal resistance, treatments are now less effective; the search for new compounds with promising antifungal activity has gained importance. The aims of this study were to evaluate the effects of the synthetic amide 2-chloro-*N*-phenylacetamide (A_1Cl) against strains of *Aspergillus flavus* and to elucidate its mechanism of action. Thus, the minimum inhibitory concentration, minimum fungicidal concentration, conidial germination, associations with antifungal agents, cell wall activities, membrane activities and molecular docking were evaluated. A_1Cl presented antifungal activity against *Aspergillus flavus* strains with a minimum inhibitory concentration of between 16 and 256 µg/mL and a minimum fungicidal concentration between 32 and 512 µg/mL. The minimum inhibitory concentration of A_1Cl also inhibited conidial germination, but when associated with amphotericin B and voriconazole, it promoted antagonistic effects. Binding to ergosterol on the fungal plasma membrane is the likely mechanism of action, along with possible inhibition of DNA synthesis through the inhibition of thymidylate synthase. It is concluded that the amide 2-chloro-*N*-phenylacetamide has promising antifungal potential.

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

INTRODUCTION

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

The infectious spectrum of aspergillosis is classified into four types: invasive disease, corresponding to life-threatening infections in immunocompromised patients; subacute or chronic infectious disease, in patients with

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

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

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

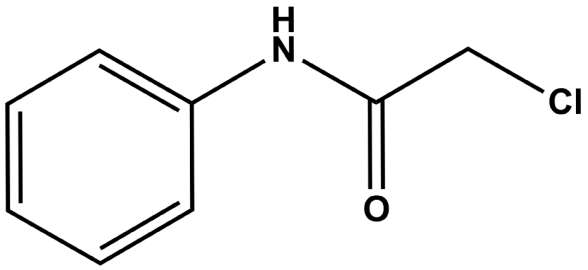

Figure 1. Chemical structure of the synthetic amide 2-chloro-*N*-phenylacetamide (A_1Cl).

MATERIALS AND METHODS

Microorganisms

To evaluate antifungal activity, eleven (11) strains of clinical origin and one (1) ATCC strain of *A. flavus* (shown in Table I) were obtained from the collection of the Research Laboratory Micoteca for Antibacterial and Antifungal Activity of Natural and/or Bioactive Synthetic Products. The strains on *Aspergillus flavus* were isolated and grown on potato dextrose agar for 7 days at 35°C. After growth, they were stored under refrigeration at 4°C. To prepare the inoculum, samples were taken from the grown cultures, suspended in sterile 0.85% saline and transferred to sterile tubes. The inoculum was standardized according to the 0.5 concentration of the McFarland scale (10^6 CFU/mL). The concentration was confirmed by counting the conidia using a Newbauer chamber (CLSI 2008, Guerra et al. 2015).

Drugs and Reagents

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

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

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

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

Determination of *in vitro* antifungal activity

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A_1Cl against strains of A. flavus

The MIC determination of A_1Cl against strains of *A. flavus* was performed using the broth microdilution technique recommended by the Clinical and Laboratory Standards Institute (CLSI 2008). Initially, 100 µL of double-concentrated RPMI was added to the wells, followed by 100 µL of each product suspension (A_1Cl, voriconazole, and amphotericin B) at an initial concentration of 1024 µg/mL in the first row of the plate, and then the range of concentrations from 1024 to 4 µg/mL were obtained by means of serial dilution. Finally, 10 µL of each of the tested microorganism suspensions was added. Negative controls (without drugs) were used to assess the viability of the strains and the sterility of the medium, as well as the sensitivity controls (to DMSO). After 2 days of incubation, visual plate reading was performed, and the MIC was considered the lowest concentration inhibiting 100% of the strain growth (CLSI 2008). The results are expressed as the arithmetic mean of the MICs obtained in three tests.

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

Evaluation of A_1Cl inhibition of conidial germination in *A. flavus* strains

The strains ATCC 16013 and LM 22 were used for this test. After reading the MIC plates, 10 µL of the MIC, MIC × 2 and MIC × 4 concentrations were removed from each product tested and seeded on sterile slides. A drop of lactophenol blue dye was then added to each preparation, homogenized, and covered with a coverslip. Conidia counting was performed under an optical microscope in 5 different fields to obtain an arithmetic mean. The results were expressed in graphs as the percentage of conidial germination as a function of the concentrations of the tested products (Adapted from Trajano et al. 2013).

Evaluation of the mechanism of action of A_1Cl against clinical strains of *A. flavus*

Cell wall assay

To assess the possible mechanisms of action involving *A. flavus* fungal cell wall, the MIC of A_1Cl was determined in the presence and absence of sorbitol (INLAB®, São Paulo, Brazil) (0.8 M) in the medium using the microdilution method and 96-well "U" plates (Alamar, Diadema, SP, Brazil). The plates were aseptically sealed and incubated at 35°C. Readings were taken at 2 days. A microorganism check and a sterility check were performed. The test was performed in triplicate and expressed as the arithmetic mean of the results (Escalante et al. 2008).

Cell membrane assay

To assess the possible mechanisms of action involving *A. flavus* fungal cell membrane, the MIC of A_1Cl was determined in the presence and absence of ergosterol (400 µg/mL) (Sigma-Aldrich®) in the medium using the microdilution method with 96-well "U" plates (Alamar, Diadema, SP, Brazil). A microorganism check and a sterility check were performed. Amphotericin B was used as a positive control. For reading, the plates were sealed and incubated at 35°C for 2 days. The test was performed in triplicate and expressed as the arithmetic mean of the results (Escalante et al. 2008).

Molecular docking

Using the ChemDraw® program, the A_1Cl structure was designed and saved as an MOL file. The enzymes investigated with their respective PDB IDs were C-14-α-demethylase (PDB ID: 4UYL) (Hargrove et al.2015), squalene epoxidase (PDB ID: 4 MAH) (Frost et al. 1995), delta-14-sterol reductase (PDB ID: 4QUV) (Standards 2002), 1,3-β-glucan synthase (PDB ID: 3A58) (RasooI & Abyaneh 2004) and thymidylate synthase (PDB ID: 5UIV) (Sinha & Rule 2017). The ligand and its receptors were subjected to molecular coupling using the Molegro Virtual Docker, v. 6.0.1 (MVD) (Morris et al. 2009). The proteins and ligand structures were prepared using the standard software package parameter settings (function Score: MolDock Score). The A_1Cl structures and the receptors used were prepared using PyRx 0.9 software (Wolf 2009), and the results are presented as the total ligand-receptor interaction energy values.

Assessment of A_1Cl with standard antifungal associations

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

For calculation of the FICI (Fractional Inhibitory Concentration Index), the method described by Bonapace et al. (2002) was used, and the lowest MIC was used (where there was no fungal growth) for the calculation performed by adding the FIC^A + FIC^B, where A represents the test product and B the standard antifungal. The FICI, in turn, is calculated using the combined FIC^A/FIC^A ratio alone, while the FIC^B is calculated

using the formula FIC^B = combined FIC^B/FIC^B alone. The interpretation of this index was performed as follows: synergism = (FICI ≤ 0.5), antagonism = (FICI > 4.0), and indifference = (0.5 < FICI ≤ 4) (Odds et al. 2003).

Statistical analysis

The results obtained from the MIC, MFC, cell wall, and cell membrane assays are expressed as the arithmetic means. The results of the conidial germination percentage are expressed as the mean ± standard error of the mean (S.E.M.), which were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni post-test to compare more than two groups. The null hypothesis was rejected when $p < 0.05$, and the GraphPad Prism® program version 5.01 (GraphPad Software Inc., San Diego CA, U.S.A.) was used.

RESULTS

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A_1Cl against strains of *A. flavus*

The product A_1Cl presented MICs that varied from 16 to 256 µg/mL, as shown in Table II, inhibiting visible growth of *A. flavus* strains used in this study. At concentrations below 100 µg/mL, six strains (50%) were weakly sensitive to A_1Cl, with MICs established between 16 and 64 µg/mL.

In the assessment of the minimum fungicidal concentration (MFC) of A_1Cl, a variation of the MFC between 32 and 512 µg/mL was observed (Table II).

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

Table II. Averages of the MIC (µg/mL) and MFC (µg/mL) values for A_1Cl, Amphotericin B and Voriconazole against strains of *A. flavus*. MIC = Minimum Inhibitory Concentration, MFC = Minimum Fungicidal Concentration.

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

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

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

Evaluation of the effect of A_1Cl on the fungal cell wall

To verify the possible action of A_1Cl on the fungal cell wall, sorbitol was used as an osmotic protector. In this study, the MIC values of A_1Cl in the presence of sorbitol remained the same in relation to the control (RPMI), suggesting that A_1Cl does not act at the level of the cell wall, as shown in Table III.

Evaluation of the effect of A_1Cl on the fungal cell membrane

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

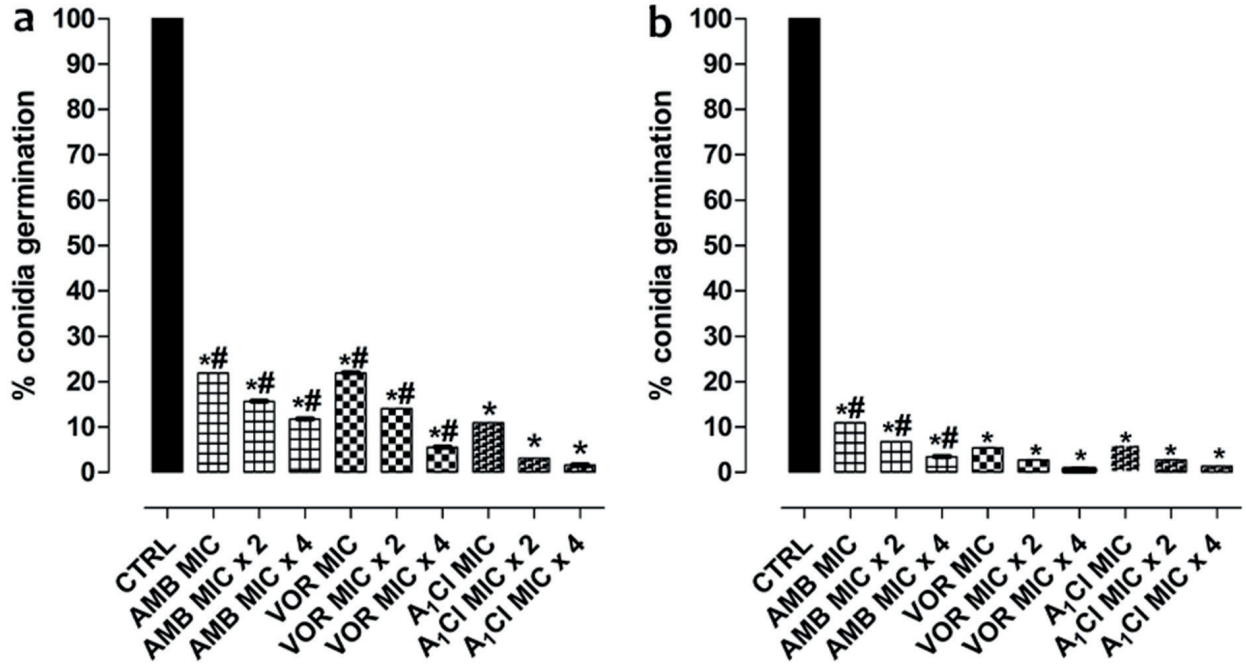

Figure 2. Percentage germination of the conidia of *A. flavus* strains ATCC 16013 (a) and LM 22 (b) against the products amphotericin B (▦), voriconazole (▩) and A_1Cl (▩). The columns and vertical bars represent the mean and the S.E.M. respectively, (n = 3). One-way ANOVA followed by Bonferroni's posttest. * p <0.05 (compared to the negative control) # p <0.05 (compared to A_1Cl). S.E.M. = standard error of the mean, CTRL = Control, MIC= Minimal Inhibitory Concentration, AMB = Amphotericin B; VOR = Voriconazole; A_1Cl = 2-chloro-*N*-phenylacetamide.

Evaluation of the mechanism of action of A_1Cl by molecular docking

In this assay, the interaction was demonstrated through binding energy, obtained by binding the A_1Cl test product with the enzymes C-14-α-demethylase, squalene epoxidase, delta-14-sterol reductase, 1,3-β-glycan synthase, and thymidylate synthase, as shown in Table V.

In the molecular docking assay, A_1Cl interacted with the enzyme C-14-α-demethylase through hydrogen bonds (blue dashes) between the enzyme's tyrosine amino acid and the carbonyl oxygen of A_1Cl, which also interacted through steric interactions (red dashes) with the enzyme's amino acid threonine, presenting a binding energy of −55.87 Kcal/mol. In addition, steric interactions of the enzyme's amino acid glycine with the carbon α1 of A_1Cl, as well as hydrogen bonds with the aromatic ring, were observed, as shown in Figure 3a.

Regarding the interaction of A_1Cl with the enzyme 1,3-β-glycan synthase (3A58), steric interactions (red dashes) were observed between the carbonyl oxygen of A_1Cl and the amino acid glutamic acid132(A) of the enzyme, presenting an energy of −58.82 Kcal/mol. The amino acid leucine77(B) of the enzyme interacts with the aromatic ring of A_1Cl by means of steric bonds and with carbon α1 through hydrogen bonds (blue dashes), as shown in Figure 3b.

Squalene epoxidase interacted with A_1Cl by means of steric interactions (red dashes) with the amino acids asparagine194, proline173, proline193, phenylalanine192, and glutamine66 of the enzyme with the aromatic ring and the α1 carbon of A_1Cl, presenting a binding energy of −60.28 Kcal/mol (Figure 3c).

The enzyme Delta-14-sterol reductase interacted with A_1Cl by means of steric bonds (red dashes) with the aromatic ring of A_1Cl,

Table III. MIC values (µg/mL) of A_1Cl in the presence and absence of sorbitol (0.8M) for the ATCC 16013 and clinical LM 22 *Aspergillus flavus* strains.

Microorganisms	*A. flavus* ATCC 16013	*A. flavus* LM 22	*A. flavus* ATCC 16013	*A. flavus* LM 22
Drugs	**RPMI (Control)**		**Sorbitol**	
A_1Cl	128 µg/mL	64 µg/mL	128 µg/mL	64 µg/mL

Table IV. MIC values of A_1Cl and Amphotericin B for the ATCC 16013 and clinical LM 22 *Aspergillus flavus* strains in the presence and absence of ergosterol (400 µg/mL).

Microorganisms	*A. flavus* ATCC 16013	*A. flavus* LM 22	*A. flavus* ATCC 16013	*A. flavus* LM 22
Drugs	**RPMI (Control)**		**Ergosterol**	
A_1Cl	128	64	1024	1024
Amphotericin B	2	2	32	32

Table V. Binding energy values (Kcal/mol) for the synthetic amide A_1Cl with the molecular targets: C-14-α-demethylase (4UYL), 1,3-β-glycan synthase (3A58), squalene epoxidase (4MAH), delta-14-sterol reductase (4QUV), and thymidylate synthase (5UIV). PDB ID: Protein code registered with Protein Data Bank (PDB).

PDB ID	4UYL	3A58	4MAH	4QUV	5UIV
A_1Cl	-55.87	-58.82	-60.28	-61.92	-83.36

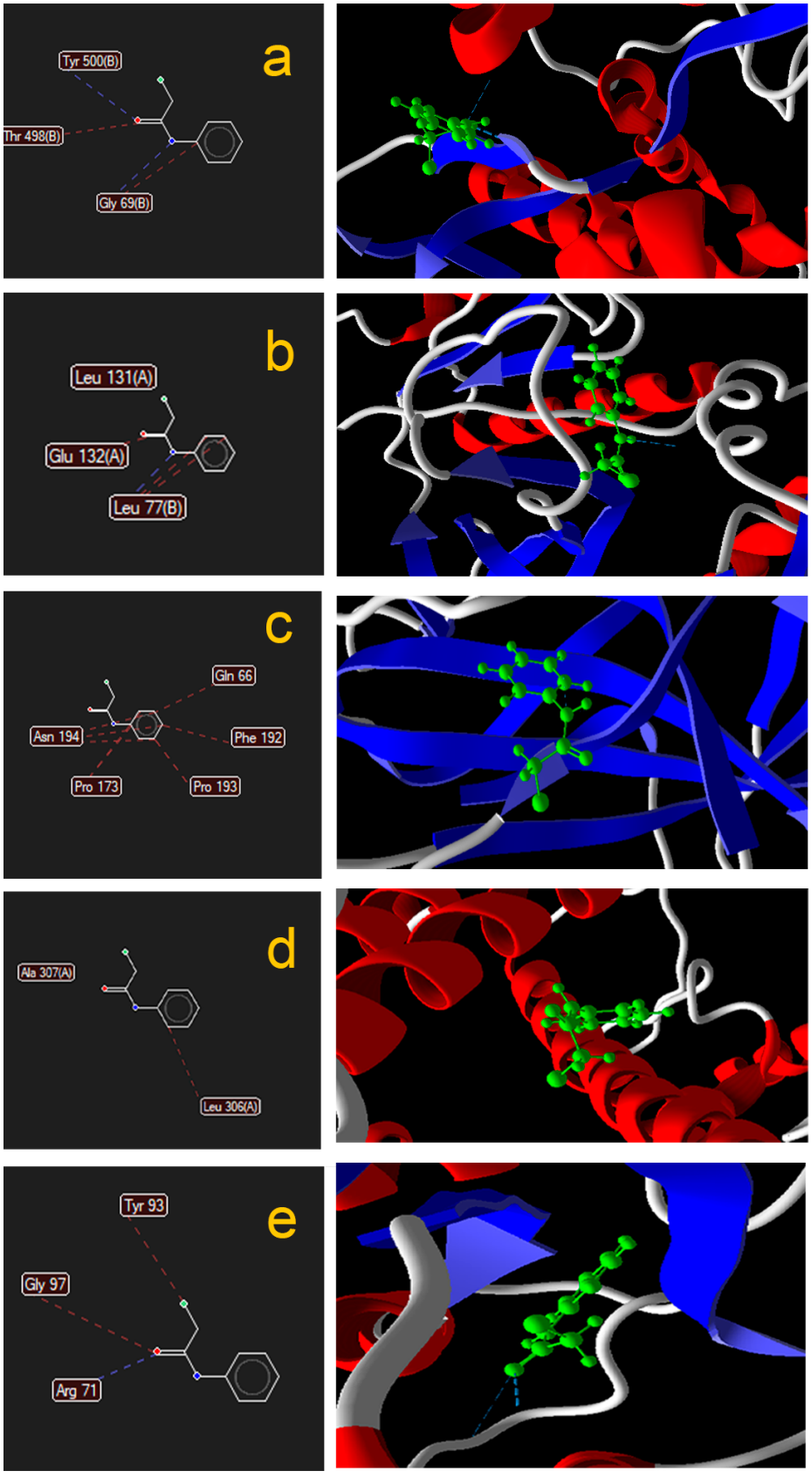

Figure 3. (a). Map of interactions of A_1Cl with the enzyme sterol-14-α-demethylase (PDB ID: 4UYL); (b) Map of interactions of A_1Cl with the enzyme 1,3-β-glucan synthase (PDB ID: 3A58); (c) Map of A_1Cl interactions with the enzyme squalene epoxidase (PDB ID: 4MAH); (d) Map of A_1Cl interactions with the enzyme Δ-14-sterol reductase (PDB ID: 4QUV) and (e) Map of interactions of A_1Cl with the enzyme thymidylate synthase (PDB ID: 5UIV). The blue dashed lines represent hydrogen interactions, and the red lines represent steric interactions. Tyr = Tyrosine, Thr = Threonine, Gly = Glycine, Leu = Leucine, Glu = Glutamate, Gln = Glutamine, Asn = Asparagine, Pro = Proline, Phe = Phenylalanine; Ala = Alanine and Arg = Arginine.

presenting a binding energy of −61.92 through the amino acid leucine306A (Figure 3d).

Regarding the interaction of the enzyme thymidylate synthase (5UIV) with A_1Cl, steric interactions (red dashes) were identified between glycine97 of the enzyme and the carbonyl oxygen of A_1Cl, as well as between the enzyme's tyrosine amino acid and the A_1Cl methyl, with a binding energy of −83.36 Kcal/mol. Hydrogen interactions (blue dashed lines) were also observed between amino acid arginine 71 of the enzyme and the oxygen in the A_1Cl carbonyl (Figure 3e).

Assessment of A_1Cl – standard antifungal associations

The effect of A_1Cl in association with amphotericin B and voriconazole (Table VI) against *A. flavus* strains was investigated. It was observed that regardless of *A. flavus* strain, the association of A_1Cl with amphotericin B or with voriconazole resulted in the same antagonistic response pattern, considering that the FICI was > 4.0; see Odds et al. (2003).

DISCUSSION

The emergence of resistant *Aspergillus* strains, in addition to causing higher mortality rates, indicates the need for the identification of new compounds with promising antifungal activity (Sanglard 2016). Since A_1Cl presented promising antifungal activity in screening studies performed by our research group, this aroused our interest in investigating the molecule in depth and in verifying its antifungal potential.

MIC testing was performed for the A_1Cl compound, and a variation from 16 to 256 µg/mL was observed for *A. flavus strain* (Table II), Fifty percent of the *A. flavus* strains (Table II) which was considered in this case as the ideal concentration of a substance (Holetz et al. 2002). The strong antimicrobial activity of A_1Cl has also been demonstrated by Katke et al. (2011) against strains of *Escherichia coli* and *Pseudomonas aeruginosa* and by Aschale (2012) against strains of *A. niger*, demonstrating that substituted chloroacetamides present greater antifungal activity than unsubstituted chloroacetamides, regardless of the positions or nature of the substituent.

Table VI. Fractional Inhibitory Concentration Index (FICI) of the association of A_1Cl with amphotericin B or voriconazole against *Aspergillus flavus* strains.

Association	*A.flavus* ATCC 16013	*A. flavus* LM 22
FIC^A (A_1Cl)	4	8
FIC^B (Amphotericin B)	0.031	2
FICI (A_1Cl+ Amphotericin B)	4.031	10
Type of interaction (A_1Cl + Amphotericin B)	Antagonism	Antagonism
FIC^B (Voriconazole)	0.031	4
FICI (A_1Cl + Voriconazole)	8.031	12
Type of interaction (A_1Cl + Voriconazole)	Antagonism	Antagonism

In the A_1Cl and MFC assays against *A. flavus* strains, a range of 32 to 512 µg/mL was observed (Table II). To explain the nature of the antimicrobial effect with regard to growth inhibition or death, Hazen (1998) established that a substance is to be considered fungicidal when the MFC/MIC ratio is ≤ 4, and when the ratio is > 4, the compound is to be considered fungistatic. In this study, A_1Cl was assayed as a fungicide for all strains tested, with MFC/MIC ratios ranging from 1 to 4 (Table II). The fungicidal activity of this compound is promising, since treatment of diseases that affect immunocompromised individuals, such as invasive aspergillosis, one of the prominent causes of death in these patients, requires drugs with fungicidal potential (Chowdhary et al.2015).

It is well known that there are great difficulties involved in correlating and interpreting microdilution test results together with clinical results, and the scientific literature has not yet defined breakpoints for assessing the susceptibility or resistance of clinical species of *Aspergillus* genus. However, Borman et al. (2017) presented a study with the objective of facilitating the development of cutoff points for several fungi, including *Aspergillus*. According to these authors, strains that present MIC values above 2 µg/mL are considered resistant to amphotericin B. The authors also observed that 13.7% of the tested *A. flavus* strains were thus considered resistant.

Reichert-Lima et al. (2018), assessing the susceptibility of *Aspergillus* strains to antifungals used in the clinic, found that of 27 strains of *A. flavus* tested, (81%) 22 obtained MICs of ≥ 2 µg/mL, considered high MIC values. Garcia-Martos et al. (2005) also evaluated the susceptibility of *Aspergillus* species against the antifungals amphotericin B, itraconazole, and voriconazole and observed that *A. flavus* presents little sensitivity to amphotericin B.

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

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

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

In view of the aspergillosis resistance to current antifungal therapies, especially amphotericin B, and of the need to seek new therapeutic strategies, the A_1Cl molecule is promising for presenting antifungal effects. Knowing that in the field of organic synthesis, a molecule can undergo changes in its chemical structure to improve its activity and taking into account that A_1Cl is a synthetic molecule, modifications can be made to make it more potent.

With regard to the development of new drugs with antifungal potential, a major challenge due to the similarity between fungal cells and human cells is the difficulty of selecting targets. The targets for new antifungals must be exclusive to the fungus or sufficiently different from their human cell analogs to avoid adverse pharmacodynamic effects (Martinez-Rossi et al. 2008, Guerra et al. 2015). Thus, to elucidate A_1Cl's mechanism of action, tests were performed on the cell wall and membrane of the species studied.

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

If A_1Cl activity is promoted through its binding to ergosterol of the fungal membrane, exogenous ergosterol, when added to the culture medium, will prevent this binding. Thus, an increase in MIC for A_1Cl in the presence of exogenous ergosterol will occur since only a higher concentration of the product in the medium will guarantee interactions with fungal membrane ergosterol (Lunde & Kubo 2000, Escalante et al. 2008).

In this study, an increase in the MIC of A_1Cl was observed in the presence of exogenous ergosterol for the two strains tested compared to the control (RPMI) (Table IV). As a positive control, amphotericin B, which acts at the level of the fungal cell membrane, was used. An increase in MIC was observed in relation to the control (RPMI) (Table IV), which was expected, considering that its mechanism of action is already well elucidated and involves binding with ergosterol, the consequential formation of pores in the membrane, and leakage of the intracellular content, culminating in death to the fungus (Filippin & Souza 2006). Thus, it is suggested that A_1Cl exerts its mechanism of action in *A. flavus* interfering with cell functions involving the membrane ergosterol.

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

In the dockings performed in this work, the interactions of A_1Cl with important enzymes responsible for the synthesis of the fungal cell wall and membrane components, as well as for nucleic acid formation, were analyzed. The interactions were demonstrated through the binding energies obtained by binding the test product to C-14-α-demethylase, squalene epoxidase, delta-14-sterol reductase, 1,3-β-glycan synthase and thymidylate synthase.

C-14-α-demethylase (4UYL) is a microsomal enzyme associated with cytochrome P-450 (CYP), encoded by the *ERG11* gene, which is involved in the final stages of ergosterol biosynthesis, the main sterol responsible for regulating fluidity and other functions of the fungal plasma membrane (Yang et al. 2015). The enzyme 1,3-β-glycan synthase (3A58) is responsible for the synthesis of β-glycans, an important component of the fungal cell wall (Cortés & Russi 2011). Squalene epoxidase (4 MAH) is a monooxygenase that participates in ergosterol biosynthesis together with delta-14-sterol reductase and is responsible for catalyzing the

conversion of squalene to 2,3-oxidosqualene (Ruckenstuhl et al. 2007). Inhibition of this enzyme promotes toxicity, affecting the structure and function of the cell membrane and leading to impairment of the fungal cell's selective permeability and consequent cell lysis (Sueth-Santiago et al. 2015). The enzyme thymidylate synthase (5UIV) is responsible for DNA synthesis and protein synthesis of the fungus, and of all of the enzymes studied, thymidylate synthase presented the lowest binding energy with A_1Cl (–83.36 Kcal/mol); the lower the binding energy, the greater the ligand's affinity for the receptor (Qidwai 2017) and thus a greater affinity for thymidylate synthase (Table V). It is possible that one of the mechanisms of action of A_1Cl is interference in DNA synthesis through inhibition of this enzyme, promoting the death of the fungus.

This study elucidating the mechanism of action of A_1Cl is unprecedented, and its mechanisms of action against strains of *A. flavus* have been demonstrated in the literature for the first time.

In addition to isolated antifungal activity, many compounds can alter the effects of the antifungals on the market. In this study, for the first time, it was shown through the checkerboard trial that A_1Cl interacts antagonistically with the two antifungals used (Table VI).

In clinical practice, drug interactions are common, the definition of which is the association of two or more drugs, such that the safety or efficacy of one drug is significantly altered in the presence of the other (Gaddis et al. 2002). In this study, it was observed that one of the mechanisms of action of A_1Cl occurs through binding to ergosterol present in the fungal cell membrane, probably leading to the formation of pores in the membrane and extravasation of cellular content, similar to amphotericin B. If associated with voriconazole, which acts by inhibiting C-14-α-demethylase and decreasing ergosterol biosynthesis, the interaction with ergosterol promoted by A_1Cl would be impaired due to the deficiency of ergosterol synthesis promoted by voriconazole, thus compromising the antifungal activity of A_1Cl, an antagonistic association (Fillipin & Souza 2006).

The association A_1Cl and amphotericin B should be better studied and detailed, since, in the trial presented, the result was antagonism. Amphotericin B is chemically incompatible with some drugs in the clinic, such as sodium chloride 0.9%, meropenem, midazolan, calcium gluconate and vancomycin (Santos et al. 2011) generating the presence of precipitates, turbidity, changes in color and chemical reactions, and in association with some antifungals such as itraconazole, terbinafine and voriconazole, has an antagonistic effect when studied in strains of the genus *Aspergillus* (Vazquez 2008). It is possible that there was chemical incompatibility between the two products, where a chemical reaction could have occurred, forming a substance different from the originals, which may not have the same pharmacological action as the initials, justifying the effect presented.

Given the above, it can be concluded that the synthetic amide 2-chloro-*N*-phenylacetamide (A_1Cl) presents antifungal activity against *A. flavus* being fungicidal. The results also suggest a mechanism of action related to the presence of ergosterol in the cell membrane and possibly action at the DNA level, yet further studies to prove this hypothesis will be required, yet further studies to prove this hypothesis will be required. This study's findings strongly suggest that 2-chloro-*N*-phenylacetamide presents antifungal potential against *A. flavus*.

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