A structure-based approach for the discovery of inhibitors against methylcitrate synthase of \textit{Paracoccidioides lutzii}

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis, endemic in Latin America, caused by fungi of the genus \textit{Paracoccidioides}. The treatment of PCM is complex, requiring a long treatment period, which often results in serious side effects. The aim of this study was to screen for inhibitors of a specific target of the fungus that is absent in humans. Methylcitrate synthase (MCS) is a unique enzyme of microorganisms and is responsible for the synthesis of methylcitrate at the beginning of the propionate degradation pathway. This pathway is essential for several microorganisms, since the accumulation of propionyl-CoA can impair virulence and prevent the development of the pathogen. We performed the modeling and molecular dynamics of the structure of \textit{Paracoccidioides lutzii} MCS (PIMCS) and performed a virtual screening on 89,415 compounds against the active site of the enzyme. The compounds were selected according to the affinity and efficiency criteria of in vitro tests. Six compounds were able to inhibit the enzymatic activity of recombinant PIMCS but only the compound ZINC08964784 showed fungistatic and fungicidal activity against \textit{Paracoccidioides} spp. cells. The analysis of the interaction profile of this compound with PIMCS showed its effectiveness in terms of specificity and stability when compared to the substrate (propionyl-CoA) of the enzyme. In addition, this compound did not show cytotoxicity in mammalian cells, with an excellent selectivity index. Our results suggest that the compound ZINC08964784 may become a promising alternative antifungal against \textit{Paracoccidioides} spp.

Abbreviations: CS: citrate synthase; MCC: methylcitrate cycle; MCS: methylcitrate synthase; MIC: minimum inhibitor concentration; MFC: minimum fungicidal concentration; MD: molecular dynamics; NPT: constant number of particles, pressure and temperature; ns: nanoseconds; NVT: constant number of particles, volume and temperature; PIMCS: methylcitrate synthase of the \textit{Paracoccidioides lutzii} MCS; PCM: Paracoccidioidomycosis; PIMCSr: methylcitrate synthase recombinant; ps: picoseconds; PDB: Protein Data Bank

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis of high prevalence in Brazil. It is caused by fungi of the genus \textit{Paracoccidioides} (Martinez, 2017). In addition to the species \textit{Paracoccidioides lutzii} and \textit{Paracoccidioides brasiliensis}, three new species have emerged. \textit{Paracoccidioides americana}, \textit{Paracoccidioides venezuelensis} and \textit{Paracoccidioides restrepensis} recently been classified as independent species (Brummer et al., 1993; Turissini et al., 2017). Thermal dimorphism is a relevant factor of fungi of this genus. The fungus exists in the mycelial form at 26°C when growing in the environment. When infecting a mammalian host, the temperature of above 36°C provokes a transition of hyphae into the yeast form, which results in a greater multiplication capacity within the lung tissue (Carbonell & Rodriguez, 1965; Souza & Taborda, 2017).

The major problem of PCM is the treatment, which is restricted to three classes of antifungals. Due to their low costs, azoles (itraconazole) and sulfamethoxazole/trimethoprim are the drugs of first choice by the Health System in Brazil. These classical antifungal drugs are indicated for mild and moderate cases of the disease. The more expensive polvenes such as various amphotericin B formulations are restricted to more severe cases. Depending on the severity of the disease, PCM treatment lasts from 6 months to 2 years.
(Shikanai-Yasuda et al., 2006). All currently used drugs have well-described side effects, ranging from headache and nausea to nephrotoxicity and hepatotoxicity. New strategies of treatment are required to minimize side effects and sequelae (Bocca et al., 2013; da Costa & Marques da Silva, 2014). Previous studies have pointed out that the selection of drug targets that are absent in humans could potentially minimize toxic side effects (Abadio et al., 2011; Bocca et al., 2013).

Pathogens face a hostile environment within host cells. Although glucose is a preferred carbon source of bacteria and fungi, within macrophages fatty acids become the main source of carbon available during infection (Passalacqua et al., 2016; Sprenger et al., 2018). Increased fatty acid metabolism in *Mycobacterium tuberculosis* during infection leads to the accumulation of propionyl-CoA and high levels of this substrate are toxic to the pathogen (Zimmermann et al., 2017). Similarly, accumulation of propionyl-CoA in the opportunistic pathogenic fungus *Aspergillus fumigatus* (Ibrahim-Granet et al., 2007) and the protozoan parasite *Toxoplasma gondii* (Brock et al., 2002) results in decreased virulence and reduces spore viability, conidia growth, and polyketide biosynthesis in *Aspergillus* spp. (Abily-Donval et al., 2017; Luciani et al., 2020). Furthermore, the fungus *Trichoderma atroviride* that is used as a biocontrol agent showed reduced efficiency in plant root colonization and increased propionate sensitivity when the metabolism of propionyl-CoA was interrupted (Wongkittichote et al., 2017). In all cases, propionyl-CoA either derives from the degradation of odd-chain fatty acids, the direct activation of propionate or the degradation of amino acids such as isoleucine, valine and methionine. All organisms mentioned above have in common that they utilize the methylcitrate cycle (MCC) for propionyl-CoA degradation. In this cycle, propionyl-CoA is alpha-oxidized to pyruvate, which involves the key enzymes methylcitrate synthase (MCS), methylcitrate dehydratase and methylisocitrate lyse (Brock et al., 2002; Limenitakis et al., 2013; Pronk et al., 1994). The newly formed central metabolite pyruvate is subsequently used for energy metabolism and biomass formation. It is noteworthy that inefficient propionyl-CoA degradation also leads to life-threatening diseases in humans such as propionic or methylmalonic acidemia (Baumgartner et al., 2014). However, propionyl-CoA degradation in humans is carried out by the methylmalonyl-CoA pathway and involves the enzymes propionyl-CoA carboxylase, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase resulting in the final product succinate (Abily-Donval et al., 2017; Luciani et al., 2020; Wongkittichote et al., 2017). MCC appears as an attractive target to disrupt propionyl-CoA metabolism in pathogenic microorganisms without affecting the degradation of propionyl-CoA in the human host. In terms of *Paracoccidioides* species, Santos and collaborators (Santos et al., 2020) demonstrated by qPCR analysis that propionate induced the expression of all genes of the methylcitrate pathway in *P. lutzii*, with the highest expression of the gene encoding for the MCS. This first enzyme of the initiates the propionate detoxification process by irreversibly condensing propionyl-CoA with oxaloacetate, which results in the formation of methylcitrate.

Computational methods for the design of medicine, have become an ally of the pharmaceutical industry and research, as they can be applied in the initial direction of researching new drugs (Talevi, 2018). Among several computational methods, virtual screening has stood out as an important tool, reducing costs and optimizing the results (Guedes et al., 2014). The purpose of the protein-ligand adjustment is to explore the most favorable and likely conformation of the complex. The scoring function is one of the most important components in the screening process, a precious ally in finding leading compounds, also called ‘lead-like’. The combination of protein molecular dynamics (MD) and methods of interaction with the ligand are being increasingly applied to new therapeutic approaches (Nair & Miners, 2014). Predicting bioactive compounds through virtual receptor-based screening is a promising approach. This method has been used in the last few years in order to identify specific target compounds and inhibit enzymes related to the viability of *Paracoccidioides* spp., such as malate synthase, isocitrate lyase, thioredoxin reductase, homoserine dehydrogenase and chorismate synthase (Abadio et al., 2015; Bagatin et al., 2017; Costa et al., 2015; da Silva et al., 2019; Rodrigues-Vendramini et al., 2018).

Therefore, MCS appeared as a promising target for the discovery of new antifungal drugs using computational analyses. Thereby, the study presented here complements our previous successful search for compounds against *Paracoccidioides* spp. by selecting specific protein targets absent in humans in combination with natural compounds that inhibit growth and virulence of *Paracoccidioides* spp. (Costa et al., 2015; e Silva et al., 2018; Silva et al., 2018). Computational biology is a new route for the identification of such inhibitory compounds, reducing time and cost for the development of new antifungal drugs (Freitas e Silva et al., 2020). One of the main methods is based on the modeling of the three-dimensional structure of the cellular targets and the virtual screening for inhibitors of these targets (Cloete et al., 2016).

In the present work, we propose that MCS of *Paracoccidioides lutzii* (PIMCS) depicts a new antifungal drug target for treatment of PCM. Modeling and MD on the *P. lutzii* MCS were performed and compounds were selected from a docking and virtual screening approach for subsequent experimental tests. The results show that one of the selected compounds reduces the MCS enzymatic activity *in vitro* and provoked fungistatic and fungicidal effects on *Paracoccidioides* cultures.

**Methods**

**PIMCS 3-D modeling**

Molecular modeling of MCS from *Paracoccidioides lutzii* (PIMCS code PAAG 04550) was performed by the I-TASSER server (Iterative Threading ASSEmbly Refinement) available at https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The PDBePQR server (http://nbrc-222.ucsd.edu/pdb2pqr_2.0.0/) was used to predict amino acid protonation states at pH 7 before proceeding with the MD simulations of the model.
The classical MD simulation was performed through the GROMACS package (Pronk et al., 2013), AMBER ff99SB-ILDN force field and explicit solvent according to the TIP3P model. The first step is a rapid energy minimization with a cut-off of 1000 kJ/mol. The system was subjected to an NVT (constant number of particles, volume and temperature) and NPT (constant number of particles, pressure and temperature) simulation of 100 ps in order to achieve the thermodynamic equilibrium of the variables. Then, the protein was subjected to a simulation of 200 ns, temperature of 300 K, pressure of 1 atm during a time interval of 2 fs, without the restriction of any possible conformation.

Sequence and structure of PIMCS were compared with other proteins experimentally resolved and deposited in the PDB (Protein Data Bank) (https://www.rcsb.org/). The BLAST-Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/) and TM-align (https://zhanglab.ccmb.med.umich.edu/TM-align/) (Zhang & Skolnick, 2005) servers were used. The sequences and structures of the proteins were retrieved from the PDB. The Molprobsity server (http://molprobity.biochem.duke.edu/) was used to evaluate the quality of the molecule before and after MD (Chen et al., 2010). Pocket analysis of the PIMCS was performed through PockDrug (http://pockdrug.rpbs.univ-paris-diderot.fr) (Hussein et al., 2015) in order to characterize sites of ligand interaction and to identify which pocket should be used to perform the virtual screening.

Virtual screening and molecular docking simulations
The simulations were carried out at the Collaborative Nucleus of Biosystems at the Federal University of Jataí (NCBios–UFJ) in a cluster with 2 PowerEdge R610 units (32 cores), 1 PowerEdge R815 unit (64 cores), 4 OptiPlex 980 units (32 cores) and 8 OptiPlex 9010 units (64 cores), totaling 192 processing cores, on Linux platform. Molecular docking and virtual screening were performed in the active site of the enzyme. MGLtools package was used to build a 44 Å² grid to enclose the docking region (Morris et al., 2009), centered on the −7.13, 1.83 and 18.85 Å coordinates (xyz). Propionyl-CoA was anchored to PIMCS by molecular docking simulations using the AutoDock Vina (ADVina) program (Trott & Olson, 2009) with the set grid and exhaustiveness of 8. The ranking of compounds was initially defined based on the score provided by ADVina. Comparison between the mode of binding of the substrate and the compound to the pocket were done to verify the affinity of the compound ZINC08964784 and the substrate to the target protein. Natural compounds (89,415 compounds) retrieved from the ZINC database were used to perform the virtual screening. The selection of the best compounds was based on affinity and efficiency criteria. The former depends on the energy score of ADVina and the latter on the success rate (Costa et al., 2015). Subsequently, the virtual hits were filtered using an aggregator advisor tool to identify molecules that are known to aggregate in experimental assays (Irwin et al., 2015; Sink et al., 2010). In addition, the Hierarchical Clustering Analysis (HCA), the Molecular ACCess System Structural (MACCS) key descriptors and the Tanimoto coefficients were used to select the most representative compounds regarding the structural diversity. Finally, the selected virtual hits were purchased and submitted to in vitro evaluation. Three-dimensional structure analyzes and alignments were performed using the PyMOL software (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). For the interaction analysis between the ligands and the PIMCS protein, we used the software Discovery Studio Visualizer, which was used to plot the 2-D diagrams of interaction (BIOVIA: Dassault Systèmes, 2019). For the analysis of ADME properties (absorption, distribution, metabolism, excretion and toxicity) the admetSAR server was used (Cheng et al., 2012). The 2-D similarity between the most promising compound (ZINC8964784) and known MCS inhibitors was calculated by similarity maps (Riniker & Landrum, 2013) implemented in RDKit program (http://www.rdkit.org/) using CountMorgan fingerprints (radius 2, 1024 bits). Similarity map is a general approach for the visualization of fingerprint similarities between two chemical structures.

Microorganism and culture conditions
Paracoccidioides lutzii (Pb01), P. brasilienensis (Pb18), P. americana (Pb02) and P. restrepiensis (PbEPMB3) were obtained from patients with chronic PCM isolated in Goiânia (Brazil), São Paulo (Brazil), Caracas (Venezuela), Bogotá (Colombia), respectively (Muñoz et al., 2016). The manipulation of the fungus followed the safety criteria of biological risk class 2 according to Ordinance No. 2349 of the Brazilian Ministry of Health. Cells were cultivated in liquid Fava-Netto medium (1% peptone (w/v), 0.5% (w/v) meat extract, 0.3% (w/v) protease peptone, 1% (w/v) brain heart infusion, 0.5% (w/v) yeast extract, 4% (w/v) glucose, 0.5% (w/v) NaCl, 5 μg/mL gentamycin) under constant agitation at 37 °C and at pH 7.2 for 3 days (Netto et al., 1969).

Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)
MIC tests were performed according to the Clinical and Laboratory Standards Institute (CLSI 2008) with modifications to be performed on Paracoccidioides spp. (Silva et al., 2018). In order to mimic the environment where the methylcitrate cycle enzymes are induced, the experiments were carried out using RPMI 1640 medium without glucose, but supplemented with 5 mM of sodium propionate (Sigma-Aldrich, USA). We added dilutions of the compounds and fungal suspension to a final concentration of 1 × 10⁵ cells/mL in each well of the microplate. To determine the maximum fungal growth (positive control), cells were incubated in the presence of medium without the selected compounds. The plates were maintained at 37°C under agitation for 48 h. Subsequently, 20 μL of a 0.02% resazurin solution (a redox indicator) was added and incubated for 24 h under the same conditions. The MIC was determined visually, based on the reduction of resazurin (blue) to resorufin (pink), which indicates the presence of cellular metabolism. Then, a subculture
was plated by transferring 20 µL of the corresponding MIC material to a petri dish containing solid Fava-Netto medium. An aliquot of the positive control was also plated. The plates were incubated at 37 °C for 7 days and MFC was determined by colony counting. The MFC was defined as the lowest concentration at which fungal growth was no longer observed.

**Cytotoxicity against mammalian cells**

BALB/c 3T3 clone A31 (ATCC® CCL-163™) cells were cultured in Dulbecco’s Modified Eagle’s medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Nutricell, BRA). 1 × 10⁵ cells/mL were incubated with different concentrations of the compound for 48 h at 37 °C, 5% CO₂. Then, 20 µL of resazurin at 0.02% (Sigma-Aldrich, USA) was added and the plate was further incubated for 24 h. The cytotoxic concentration was visually determined by the color change of the dye. The selectivity index was determined by the ratio between cytotoxicity and MIC (Santos et al., 2020).

**Generation of the MCS expression plasmid**

The coding region of the *Paracoccidioides* spp. MCS was PCR amplified with Phusion Polymerase (Thermo Scientific) from cDNA using oligonucleotides IfpET43McsAPb_f (5'-TCA TAG CCG ATC CAT GTC GAC TGC GGA ATC TGA CCT G-3') and IfpET43McsAPb_r (5'-GTG CGG CCG CAA GCT TTA TTT CCT GGA AAG ATC AAG GAG G-3'). The PCR product was gel purified and inserted into a BamHl/NotI restricted modified pET43.1H6 vector (Hortschansky et al., 2007) by in vitro recombination using the infusion HD cloning kit (Takara), which introduced a His-tag at the N-terminus of the MCSs. The assembled plasmid was amplified in chemically competent *Escherichia coli* DH5-alpha cells and isolated using the NucleoSpin plasmid purification kit (Machery-Nagel). For protein production, the plasmid was transferred to electrocompetent *E. coli* BL21 (DE3) cells.

**Paracoccidioides lutzii MCS heterologous expression and recombinant protein purification**

The MCS recombinant (PIMCSr) production was initiated by adding 1 mM of isopropyl thio-b-β-galactoside (IPTG) (Sigma-Aldrich, USA). Electroporated BL21 cells expressing PIMCSr were incubated with lysozyme 500 µg/mL and then sonicated on ice. The nickel-nitrilotriacetic acid resin (Ni-NTA) (Invitrogen, USA) was used to purify the protein. The purified and inserted into a pET43.1H6 vector (Hortschansky et al., 2007) by in vitro recombination using the infusion HD cloning kit (Takara), which introduced a His-tag at the N-terminus of the MCSs. The assembled plasmid was amplified in chemically competent *Escherichia coli* DH5-alpha cells and isolated using the NucleoSpin plasmid purification kit (Machery-Nagel). For protein production, the plasmid was transferred to electrocompetent *E. coli* BL21 (DE3) cells.

**PIMCSr enzymatic assay and inhibition assay**

MCS activity was performed according to a DTNB (5,5'-dithiobis-2-nitrobenzoic acid) protocol described previously (Abily-Donval et al., 2017), with minor modifications. We observed that with 50 mM Tris-HCl (pH 8), 1 mM DTNB, 0.4 mM propionyl-CoA, 2 mM oxaloacetate and 0.375 µg/µL of purified PIMCSr, we obtained the best activity condition according to the linearity of the reaction. Then, we proceeded with the activity inhibition testing by adding the selected compounds. Initially, they were diluted in DMSO (dimethyl sulfoxide) in order to produce a stock solution. The compounds were added to a final concentration of 6, 100 and 200 µM. The inhibition rate was evaluated by the Vmax value of the reaction, according to the slope of the curve. The absorbance variation was measured at the wavelength of 412 nm and at 37 °C through the reaction of released Coenzyme-A (Maerker et al., 2005). The inhibition assay was performed identically to the PIMCSr enzymatic assay, except that each one of the compounds was added to the reaction mixture. Control of reaction with DMSO was performed.

**Results**

**Modeling and molecular dynamics**

PIMCS (PAAG 04550) was modeled through the I-TASSER. This server predicts the structure of proteins by threading, based on the crystals of higher homology. The generated model presented a confidence score (C-score) of −0.08, which represents the quality of the models. The TM-score (0.70) measures the structural similarity between proteins. In addition, according to the I-TASSER function prediction, the modeled protein shows citrate synthase (CS) activity, 2-MCS activity and GO-scores (gene ontology score) of 0.78 and 0.50, respectively. After the modeling process, the protonation state of the charged amino acid residues was predicted by the PDB2PQR server at pH 7, and this was the input model for the MD simulation. MD was performed with the GROMACS package to improve the quality of the generated model, since the minimization of energy and the conditions of the solvent environment favor the stabilization of the molecule.

The ratio of the mean square deviation (RMSD) of the protein is plotted in Figure 1(A). It shows the structural variation of the molecule during the simulation. In addition, the thousands of poses that the protein assumes during the simulation were grouped into clusters with a cut-off point of 0.35 Å (Figure 1(B)). The molecule equilibration started at 100 ns of simulation and from that moment on the RMSD did not change considerably. The cluster diagram showed similar results with a cluster of conformations starting around 100 ns and remained until the end of the simulation. In the analysis of RMSF we noticed that the most flexible amino acids were the residues 1–15; 56–78; 223–240; 455–469, in loop and at the extremity regions in the protein model (Figure 1(C)). This suggests that the protein has reached its natural stability and fluctuations are due to its natural flexibility required to
fulfill its function, being characterized by harmonic movements over time.

In order to have an overview of the difference between the PlMCS models before and after the MD simulation, we proceeded with a structural quality analysis using the Molprobity server. The Ramachandran diagram showed a significant decrease of amino acid residues at forbidden positions, in relation to the phi and psi angles of the backbone (Supplementary data 1). Forbidden positions mean that at a given angle value there are steric shocks between amino acid side chains and this position is not compatible with the native protein. There was a significant improvement in the value of the phi and psi angles, making the model closer to the native structure. In addition, the molprobity score before MD was 2.84 (29% of the high-quality protein crystals present this score) and after MD was 1.43, which corresponds to the 97% score of the high protein crystals quality. All these results demonstrate the importance of the dynamic molecular simulation to obtain a higher quality model. Thus, virtual screening and molecular docking can be performed with greater confidence.

**Pockets analysis and homology**

Following the MD of PlMCS, we analyzed residues involved in the enzymatic activity and the pocket where catalysis takes place. We used the PockDrug server in order to predict pockets. Pockets are classified according to hydrophobicity and volume. The probability of a certain pocket being targeted is given by the drug dependence score. The pocket 0 is the largest cavity of the protein, with a volume of 4507 Å³ and comprises atoms of 51 amino acid residues, 76% of them are hydrophobic. This cavity has a surface area of approximately 1520 Å² and presented a druggability score of 94%.

The crystallographic MCS and CS of *Salmonella typhimurium* (PDBID: 3O8J), *Aspergillus fumigatus* (PDBID: 4CTS), *Sus scrofa* (PDBID: SUZQ), *Homo sapiens* (PDBID: 5UZQ) and *Mycobacterium tuberculosis* (PDBID: 3HWK) were used for sequence and structure alignment with PlMCS (Table 1). MCS from *A. fumigatus* showed greater similarity to PlMCS (83% identity) but no detailed record of this crystal has been reported (data not yet published), so the analysis was limited to the MCS records of *Salmonella typhimurium* and CS from *Sus scrofa*.

The 6BOO sequence presented 95% coverage and 83% identity, 4CTS sequence has 92% and 52%, 3HWK exhibits 70% and 23%, respectively. The CS sequence of *H. sapiens* (5UZQ) shows 93% coverage and 52% identity, while MCS of *M. tuberculosis* (3HWK) has only 69% coverage and 23% identity, respectively. MCS and CS proteins are conserved among several species, even for phylogenetically distant species and they share similar protein motifs.

One of these motifs comprises 13 amino acids and contains a standard region for the identification of CS. Starting with a glycine and the fourth amino acid histidine is a catalytic residue (Prosite PS00480). The sequence alignment of this standard motif shows conserved amino acids among species (Supplementary data 1). To see the complete alignment, access the Supplementary data 2. Most of the amino acids are conserved in at least five of the six organisms analyzed. Interestingly, the histidine residue at position 4, which is very important for the catalytic activity, is conserved in the enzymes of all the species analyzed.

The structural analysis showed an acceptable degree of similarity between 6BOO, SUZQ and 4CTS with PlMCS. The
RMSD values were 3.42, 3.40, 3.30 Å and the alignment resulted in 78%, 51% and 48% of identity, respectively. In addition, CS of *S. scrofa* contains the amino acids HIS274, HIS320 and ASP375 that are essential for the catalysis. In *S. typhimurium* this catalytic triad corresponds to HIS235, HIS274 and ASP325. By overlapping the structures, we predicted that in *P. lutzii* those residues correspond to HIS305, HIS351 and ASP408, respectively (Figure 2(A)). The pocket 0 contains the catalytic triad and we concluded that this is the active site cavity of *PlMCS* (Figure 2(B)).

Virtual screening and molecular docking

Virtual screening was performed against the pocket 0 region and the best compounds interacting with *PlMCS* were selected according to the affinity criterion as described by Costa et al (Costa et al., 2015). Based on this criterion, the best 100 compounds were selected with the lowest energy score (range between −13.2 and −8.0) and the highest success rate. The latter refers to the percentage of interactions of a specific energy value among 1000 simulations. Figure 3 shows the energy profile of those 100 compounds and their success rate. Finally, nine compounds with structural difference were selected for the experimental validation and enzymatic assays (Figure 4).

Inhibition of enzyme activity

The compounds selected by virtual screening were tested and validated experimentally through enzymatic assays. The compounds ZINC02127034, ZINC08790776 and ZINC08790763 inhibited 9%, 12% and 20% of the enzyme activity at a concentration of 6 μM, respectively. The increase in the concentration of the compounds to 100 μM inhibited 39%, 22% and 23% of the enzymatic inhibition, respectively. In addition, compounds ZINC08964784 and ZINC08792179 that showed no inhibition at 6 μM showed an inhibition rate of 17% and 16% at a concentration of 100 μM, respectively. At a concentration of 200 and 500 μM, the compound ZINC02127034 revealed an inhibition rate of 33% and 65%, respectively, while the compound ZINC08964784 inhibited 12% and 38% of the enzyme activity at the same concentrations (Table 2).

ZINC08964784 acts as a potent antifungal inhibitor

Among the nine compounds selected by the virtual screening, four of them were able to inhibit the growth of *P. lutzii* with MIC values ranging from 6.04 to 193.28 μM. The fungicidal effect was observed at the same concentration as the MIC (Table 2). None of these four compounds showed cytotoxicity at the MIC concentration and selectivity index ranged from >10.08 to >322.83. The compound ZINC08964784 was the most efficient compound in terms of antifungal activity, with MIC and MFC of 6.04 μM and selectivity index of >322.83 (Table 2).

Due to the excellent results found for ZINC08964784, biological tests were extended to other species of *Paracoccidioides*. *P. lutzii*, *P. brasiliensis*, *P. restrepiensis* were equally sensitive to the compound with MIC and MFC of 6.04 μM, while *P. americana* presented MIC and MFC of 12.08 μM (Table 3). In addition, an ADMET analysis (absorption, distribution, metabolism, excretion and toxicity) of the compound ZINC08964784 demonstrated that it has a high chance of intestinal absorption and of crossing the blood-brain barrier. Inhibition or interaction with CYP2C9 and CYP2D6, in addition to carcinogenic and mutagenic activity are unlikely (see Supplementary data Table S1).
Figure 3. Interaction energy score graph of the 100 best PMCS ligands as well as the percentage of these connections with the protein.

Figure 4. Chemical structure of the nine compounds selected by virtual screening, with promising activity against PMCS.
Table 2. Bioactivity of the compounds.

| Sample          | MW   | 6 μM | 100 μM | 200 μM |
|-----------------|------|------|--------|--------|
| ZINC08964784    | 513.0| NO   | 17%    | 12%    |
| ZINC08764810    | 462.6| NO   | NO     | 4%     |
| ZINC08790776    | 503.0| 12%  | 22%    | NO     |
| ZINC08790763    | 512.0| 20%  | 23%    | 19%    |
| ZINC08792248    | 402.6| NO   | NO     | NO     |
| ZINC11867126    | 522.0| NO   | NO     | NO     |
| ZINC02127034    | 446.0| 9%   | 39%    | 33%    |
| ZINC08876950    | 471.0| NO   | NO     | NO     |
| ZINC08792179    | 520.0| NO   | 16%    | 21%    |

*aCalculated through Vmax, using the concentrations of 6, 100 and 200 μM of compound in the reaction. MW: molecular weight; MI: minimum inhibitory concentration (μM); MFC: minimum fungicidal concentration (μM); CC: cytotoxic concentration (μM); SI: selectivity index.

Table 3. Biological activity of ZINC08964784 against other species of the Paracoccidioides genus.

|       | MIC | MFC | SI    |
|-------|-----|-----|-------|
| P. lutzii       | 6.04  | 6.04 | >322.83 |
| P. brasiliensis | 6.04  | 6.04 | >322.83 |
| P. restrepiensis| 6.04  | 6.04 | >322.83 |
| P. americana    | 12.08 | 12.08 | >161.41 |

MIC: minimum inhibitory concentration (μM); MFC: minimum fungicidal concentration (μM); SI: selectivity index.

ZINC0896474 x propionyl-CoA

Propionyl-CoA, the substrate of the MCS, was anchored in the pocket 0 region of the active site. The binding energy score of this conformational mode was −9.3 kcal/mol. Propionyl-CoA performs several van der Waals interactions, characterized by transient dipole moment. In addition, we identified other favorable interactions, such as hydrogen bonding, saline bridge and charge attraction. ZINC08964784 had an interaction energy of −12.5 kcal/mol, performing several interactions, such as of van der Waals, hydrogen and hydrophobic groups. Most interesting are pi-type interactions that occur in three ways: pi-alkyl (aromatic ring and hydrophobic group), pi-pi (between two aromatic rings) and pi-anion (aromatic ring and an anion). These interactions greatly favor the stability of the ligand in the pocket and together they reduce the free binding energy (Figure 5).

Most of the PIMCS residues involved in the interaction with propionyl-CoA and the compound ZINC08964784 are common. PIMCS interaction with propionyl-CoA has five specific amino acids that do not participate in the interaction with ZINC08964784. In addition, there are three specific amino acids in the interaction between PIMCS and propionyl-CoA. The major difference between the two ligands is that ZINC08964784 was able to perform a greater variety of interactions, especially hydrophobic and pi-like interactions (Figure 5).

In order to determine the structural novelty of ZINC08964784, we performed a literature search for known MCS inhibitors. Through this analysis, we identified the compound V-13-009920 (IC_{50}=4.0±1.1 μM) of M. tuberculosis (MtMCS) (VanderVen et al., 2015). According to the similarity maps shown in Figure 6, ZINC08964784 and V-13-009920 are structurally dissimilar (Tanimoto coefficient = 0.1). These results indicate that our hit compound represents a new molecular scaffold for prospective studies of hit-to-lead optimization.

Discussion

The treatment of PCM is mainly based on the administration of at least three types of antifungals, including sulfonamide compounds, azole derivatives and amphotericin B (Goughenour & Rappleye, 2017). The number of people who abandon treatment and who have serious effects associated with these drugs is a public health problem (Shikanai-Yasuda et al., 2017). This reinforces the need to develop new and more efficient antifungals to improve patients’ prognosis.

The gold standard method for drug discovery is the high-throughput screen, where hundreds to thousands of compounds are experimentally tested against a target. However, the costs of such technique restrict its use only to large research centers. In this scenario, virtual screening emerges as an alternative of lower cost and time, as it direct research and optimizes experimental approaches (Shoichet, 2004). The combination of MD with protein-ligand interaction methods is extensively used in the discovery of pharmacological targets and new therapeutic approaches (Nair & Miners, 2014). One of the earliest approaches was based on minimization of energy, refinement of the structure model, MD and docking allied to conventional experiments to identify HIV protease inhibitors (Wlodawer & Vondrasek, 1998).

The rational design of an antifungal prototype with properties that specifically affect a pathogenic target that is absent in humans may lead to a new drug with minimal toxicity to host cells. The proposition of bioactive compounds using receptor-based virtual screening is promising. Through this approach, specific target compounds were proposed to inhibit enzymes related to the viability of Paracoccidioides spp. as malate synthase, thioredoxin reductase, homoserine dehydrogenase and chorismate synthase (Abadio et al., 2015; Bagatin et al., 2017; Costa et al., 2015; Rodrigues-Vendramini et al., 2018). Here, a promising new antifungal compound against Paracoccidioides spp. has been proposed was identified by a virtual docking screening approach against PIMCS, which is an enzyme absent in mammalian cells.

A conserved standard region presents in MCS and CS results in a very similar catalytic mechanism. This region comprises 13 amino acid residues and a histidine residue...
that is highly conserved among species (see Figure 2(A)) (Wiegand et al., 1984). This region is identical in *A. fumigatus* and *P. lutzii*, spanning through residues 302 to 314. The 3O9J structure of *S. typhimurium* was the first crystal structure record of an enzyme with MCS activity and the catalytic triad in the residues HIS235, HIS274 and ASP325 was confirmed (Chittori et al., 2011). The catalytic triad is another conserved region between MCS (3O8J) and CS (4CTS) proteins (see Figure 2(B)). Although *A. fumigatus* showed greater sequence and structure similarities with *Pl* MCS, there is no publication available regarding the 6BOO crystal, limiting further comparison between them.

In the present work, we showed the advantage of using MD, especially when dealing with protein structures that were modeled in silico. The stabilization of *PIMCS* was achieved in half of the simulated time and there was
improvement in the quality of the molecule after the MD simulation with significant reduction of atoms that were within unfavorable positions and under steric shocks. We also showed that pocket analysis was very useful in confirming the cavity that was formed in the docking and virtual screening. The virtual screening of compounds resulted in 100 best compounds based on the energy score of interaction and success rate in the pocket defined as the catalytic site. Although most compounds reduced enzymatic activity, only one compound was able to inhibit and prevent the fungal growth at a reasonably low concentration. The thick wall of the fungus that is composed mainly of glucans and chitin may have been an impediment to the action of some of the compounds in the culture susceptibility assays (Puccia et al., 2011; Tomazett et al., 2011).

A similar approach carried out on P. lutzii screened natural compounds against malate synthase, an enzyme of the glyoxylate cycle. In this previous work the authors found a compound that inhibit 91% of the enzymatic activity and showed a MIC value of 62 μg/mL, about threefold higher than the MIC for the compound under study (Costa et al., 2015). A different approach with the natural compound argentinolate showed promising inhibitory effect against P. lutzii growth. In this case, the authors found a MIC value of 18 μg/mL, which is similar to our results for the compound ZINC08964784 (Do Prado et al., 2014). These data reinforce the relevance of the results obtained here, since ZINC08964784 presented MIC of 6.04 μM.

ZINC08964784 had an interaction energy of $-12.5$ kcal/mol in 95% of the virtual screening simulations for its best position in the pocket. Interaction between propionyl-CoA and the PIMCS presented an energy of $-9.2$ kcal/mol. This difference between energy scores indicates that ZINC08964784 has a greater affinity to the PIMCS pocket than propionyl-CoA. We hypothesize that the compound prevented the interaction between the substrate and the pocket, which is reflected in a reduction of the enzyme activity when the inhibitor compound was added. When we compared the interaction between ZINC08964784 and PIMCS, favorable interactions could contribute energetically to the protein-ligand affinity. We observed three alkyl-type interactions between amino acids (ALA308, ALA309 and VAL345) and toluene-CH3. Pi-alkyl interactions were found between VAL345 and the benzene ring. These interactions are common among hydrophobic groups and contribute to the stabilization of the ligand within the protein active site (Bissantz et al., 2010).

The ASP408 residue is negatively charged and therefore, when it is close to an aromatic ring, a pi-anion interaction is formed. Another interesting interaction is the pi-pi (T-shaped), which occurs between the HIS351 residue and an aromatic ring of the compound ZINC08964784. This perpendicular and apolar interaction is described as a favorable intermolecular attraction (Wu et al., 2008). The amino acids HIS351 and ASP408 are part of the catalytic triad, probably acting similarly to what was described for CS of S. scrofa (Wiegand et al., 1984). A study carried out in 2009 aimed at designing a more efficient inhibitor of the CS, based on the aminopyrrolidine structure. Were tested the insertion of functional groups, including a benzene ring in the inhibitor molecule. The resultant compound was able to perform a pi-pi interaction, which along with other intermolecular interactions, improved the interaction efficacy 1000-fold (Zbinden et al., 2009). The amino acids HIS269, ASN406 and GLY352 perform conventional hydrogen interactions, which are considered essential for the high affinity between proteins and ligand. These interactions, although weak, represent an important function in molecular recognition between ligand-receptor and the stability between two molecules.

In general, all amino acids that are essential for enzymatic catalysis were involved in the interactions with the two ligands. The compound ZINC08964784 has three aromatic rings in its chain, whereas propionyl-CoA does not. ZINC08964784 was able to perform more pi-type interactions and hydrophobic interactions, which contributed significantly to the stability and maintenance of this interaction within the pocket. All of these favorable interactions may have contributed to the results achieved in the biological tests.

To our knowledge, there is only one inhibitor for MCS described so far, which according to our analyzes is dissimilar to hit compound ZINC8964784. Thus, both structures could give rise to other promising ones. It is worth mentioning that the MCS inhibitor has not been evaluated against M. tuberculosis. On the other hand, we identified that the compound ZINC8964784 inhibited the Paracoccidioides spp. growth and excellent results were obtained in ADMET analyzes.

**Conclusion**

The in silico and experimental results showed that ZINC08964784 inhibited the P. lutzii PIMCS protein. In addition, it was able to exert fungistatic and fungicidal activity on several species of the genus Paracoccidioides without toxicity to mammalian cells. This suggests that the compound may be a promising antifungal against PCM. Chemical modifications can be performed for prospective studies of hit-to-lead optimization to enhance the interaction of the compound with the enzyme and the in vivo assay.

**Disclosure statement**

No potential conflict of interest was reported by the authors.
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