Targeting the Homoserine Dehydrogenase of *Paracoccidioides* Species for Treatment of Systemic Fungal Infections

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ABSTRACT This work evaluated new potential inhibitors of the enzyme homoserine dehydrogenase (HSD) of *Paracoccidioides brasiliensis*, one of the etiological agents of paracoccidioidomycosis. The tertiary structure of the protein bonded to the analogue NAD, and L-homoserine was modeled by homology. The model with the best output was subjected to gradient minimization, redocking, and molecular dynamics simulation. Virtual screening simulations with 187,841 molecules purchasable from the Zinc database were performed. After the screenings, 14 molecules were selected and analyzed by the use of absorption, distribution, metabolism, excretion, and toxicity criteria, resulting in four compounds for *in vitro* assays. The molecules HS1 and HS2 were promising, exhibiting MICs of 64 and 32 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively, for the Pb18 isolate of *P. brasiliensis*, 64 $\mu\text{g} \cdot \text{ml}^{-1}$ for two isolates of *P. lutzii*, and also synergy with itraconazole. The application of these molecules to human-pathogenic fungi confirmed that the HSD enzyme may be used as a target for the development of drugs with specific action against paracoccidioidomycosis; moreover, these compounds may serve as leads in the design of new antifungals.

KEYWORDS homoserine dehydrogenase, antifungal, virtual screening, paracoccidioidomycosis

Paracoccidioidomycosis (PCM) is a systemic granulomatous mycosis and is considered the most important fungal infection in Latin America. Its etiologic agents are *Paracoccidioides brasiliensis* (1) and *P. lutzii* (2, 3). PCM is an important public health problem in Brazil and is the eighth leading cause of death from predominantly chronic or recurrent disease, falling between infectious and parasitic diseases as a leading cause of death, and among the systemic mycoses, the rate of mortality from PCM is the highest (4). The south of Brazil has shown the highest regional distribution of PCM, and the southeast is seeing a progressive decrease in the incidence of disease (5–7).

Although there are a variety of clinical forms of PCM, two main manifestations are well established: the acute or subacute form (which is seen in juveniles) and the chronic form (which is seen in adults) (8). The acute form primarily affects children and adolescents of both sexes, progresses rapidly through the lymphatic and/or hematogenous system, and has a high mortality rate as it compromises the reticuloendothelial system (spleen, liver, lymph nodes, and bone marrow) (9). The chronic form occurs in 90% of cases, especially in males, between 30 and 60 years of age, has a slow progression, and mainly affects the lungs but can be multifocal, spreading to any organ, especially mucocutaneous tissue. A patient with PCM may complain of insomnia, weakness, a lack of appetite, dysphagia, dyspnea, cough, hemoptysis, fever, weight loss,

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itching, and burning (2). Immune system alterations have been described in patients with the severe forms of PCM, including hypergammaglobulinemia, a high level of specific antibodies, eosinophilia, and depressed general and antigen-specific cellular immunity (10).

Nowadays there are no specific drugs for the treatment of PCM and treatment is limited to a few groups of antifungal agents (11). The main group includes polyenes derived from amphotericin B (AMB). They are drugs with a broad spectrum that bind to ergosterol, a component of fungal cell membranes, and thereby create pores in the cell membrane, leading to cell death (12, 13). Amphotericin B is usually effective for the treatment of systemic mycoses, but its administration is limited because of its side effects, such as fever, kidney damage, anemia, low blood pressure, headache, and nausea. Because of this, its use is restricted to the hospital environment (14, 15).

Several sulfa derivatives have been used to treat PCM since 1940 (16). However, several drawbacks to the use of sulfadiazine have been reported, such as the dosage (it must be administered more than twice a day), the prolonged treatment required, and adverse effects. Trimethoprim-sulfamethoxazole (TMP-SMZ) has frequently been used to treat PCM during the maintenance phase. Studies show that this medicine has a good distribution throughout the body; however, 5% of patients reported that this treatment does not work (17). The long duration of the treatment and the side effects are the main disadvantages of this therapy (18).

The third group of antifungal agents includes azole derivatives, which interfere with the synthesis of ergosterol, specifically, the enzyme lanosterol demethylase, leading to the accumulation of metabolites in fungal membranes and the inhibition of growth (19). This group of antifungal agents includes fluconazole, itraconazole, and, more recently, voriconazole and posaconazole. All these drugs may be administered to treat systemic mycoses (20). Fluconazole has fewer side effects than ketoconazole and may be used for the treatment of candidiasis and maintenance therapy for cryptococcal meningitis (21). Even with the advent of new azoles, such as voriconazole and posaconazole, itraconazole is still the best option for the treatment of PCM in outpatients. This is due to the high costs of the newer azoles and the lack of clinical studies capable of pointing out the advantages of using these new drugs (11).

The resistance of systemic fungi, especially *P. brasiliensis*, to the drugs currently used to treat paracoccidioidomycosis has become a public health problem, since there are already reports that *P. brasiliensis* strains are resistant to azoles (22–24). A fact that further aggravates this situation is that the drug arsenal currently available to combat the fungus is quite limited compared to the arsenal available for antibacterial chemotherapy.

The requirement for prolonged treatment is one of the main obstacles to the cure of the disease due to the high cost and the adverse effects of the available medications in the long term, which often lead patients to abandon treatment, which inevitably can lead to the emergence of more resistant varieties of *P. brasiliensis* and/or other opportunistic microorganisms (25). According to Abadio et al (26), the toxicities and drug interactions observed with contemporary antifungal therapies can be attributed to, among other causes, nonselective interactions with enzymes or cell membrane systems found in the mammalian host. Therefore, the search for new and more specific drugs that minimize the serious problem of side effects remains a major challenge (22, 26).

In this context, the enzyme homoserine dehydrogenase (HSD) belongs to the family of oxidoreductases and is essential for the biosynthesis of threonine (Thr), methionine (Met), and lysine (Lys) in the metabolic pathway of fungi and plants. Reports in the literature have described the essential nature of HSD in human-pathogenic fungi, such as *Candida albicans*, which was determined by the use of compounds that promote inhibition of this enzyme (27, 28). Evidence exists that the Met biosynthetic pathway is a proven target that should be considered in the development of antifungal agents (29). Bearing in mind that HSD is not found in humans, it could be an important target for the development of new approaches to the treatment of fungal diseases using

FIG 1 Reaction catalyzed by HSD.

drugs that exhibit a high degree of specific antifungal activity and fewer side effects (30, 31). The reaction carried out by HSD is reversible (Fig. 1) and leads to a branch point where the biosynthetic pathways diverge (32).

Therefore, the aim of this study was to evaluate theoretical and experimentally new potential inhibitors of the enzyme homoserine dehydrogenase found in the fungus *Paracoccidioides brasiliensis*. In this work, four compounds were selected from docking simulations. Two of these compounds showed antifungal activity against *Paracoccidioides* species, thereby presenting the potential to serve as leads in the design of new antifungals, especially antifungals with activity against PCM.

RESULTS

Model analysis of *P. brasiliensis* HSD. The amino acid sequence of the homoserine dehydrogenase from *P. brasiliensis* strain Pb18 deposited in UniProt (UniProt accession number [C1G1C3](#)) is not confirmed. However, this strain was chosen for use in this work because it is the most common strain encountered in Brazil and is the most virulent strain (33, 34) and because the aim of this work was to use biotechnology to detect compounds active against paracoccidioidomycosis. The amino acid sequence of *P. brasiliensis* strain Pb18 HSD shares 47.8% identity with the sequences of the templates, which is considered sufficient for homology modeling (35).

The structure of *P. brasiliensis* HSD was first modeled as a single chain. After this step, the adjacent polypeptide chain was generated by symmetry operations to produce the biological unit of the protein as a homodimer. This was necessary because a molecular dynamics (MD) study of a single chain showed that it has high structural flexibility, which made the cavity of the active site expand, making it impossible to dock L-homoserine (HSE) in redocking evaluations. The presence of the adjacent polypeptide chain restricts this motion by keeping the cavity of the active site in a more stable and less flexible configuration. The final model showed that 94.3% of the residues were in the most favorable regions of the Ramachandran plot, while the other 5.7% were in allowed regions. During the modeling process, the Modeler program copied the structure of the ligand HSE in the way in which it appears in the template protein with PDB accession number [1EBU](#). This procedure lets HSE stay in a position with seemingly unfavorable interactions in *P. brasiliensis* HSD, where its COO⁻ group was very close to the radical group COO⁻ of Glu218 (Glu208 in *Saccharomyces cerevisiae*). Furthermore, the electron density of HSE in the template protein with PDB accession number [1EBU](#) was relatively low, opening up the possibility that HSE interactions with *P. brasiliensis* HSD could occur in an alternative position. In our model, HSE was rotated 180°, so that its COO⁻ group was turned toward the deep surface of the catalytic site. After this, we observed an increase in the numbers of interactions between HSE and protein residues in our model (Fig. 2 and Table 1). For the new interactions with the protein that arose from this adjustment to become maximized, energy minimization of the system was performed as described in Materials and Methods. After these procedures, we observed that the alternative position of the ligand favors the formation of a salt bridge between the HSE NH₃⁺ group and the COO⁻ group of Asp224, as well as two new salt bridges between the HSE COO⁻ group and the N-ζ of Lys122 and Lys233. Even in the alternative position, the binding of HSE remained catalytically favorable for the transfer of the hydride, with a distance of 3.7 Å between the HSE C-γ and the nC-4 of the NAD

FIG 2 (a) Stereoview of HSE in the active site of *P. brasiliensis* HSD; (b) proposed reaction mechanisms of hydride transfer from HSE in the active site of *P. brasiliensis* HSD. wat, water.

nicotinamide ring being found (Fig. 2), whereas the distance observed in the template protein with PDB accession number 1EBU is 3.7 Å (36). In the proposed mechanism, Glu218 accepted a proton from the hydroxyl group of HSE, which donates a hydride to the *n*C-4 carbon of NAD⁺. Lys233, Lys122, and the water molecule at position 374 (Wat374) serve to bind the substrate. The details of the interactions can be seen in Table 1.

The nicotinamide ring of the cofactor NAD from *P. brasiliensis* HSD was moved an average of 2.4 Å beyond its location in the original templates. This observation was confirmed from the root mean square deviation (RMSD) calculated for this cofactor during the molecular dynamics analysis. We attribute this movement to the few spatial constraints that could anchor the nicotinamide ring in the catalytic site. However, the RMSD value is consistent with the value reported for the polypeptide chain of the protein, as it is within the expected moving average for the entire complex.

Virtual screening. The choice of a library of natural products in the virtual screening simulations had sustainability as its main motivating factor, given the fact that the production or extraction of a naturally occurring molecule would have less of an impact on the environment with sustainable management and would probably cost less than a synthetic molecule. The docking protocol established by our group takes into account the fact that a theoretical model, such as that of *P. brasiliensis* HSD, may have some structural inconsistencies compared to a crystallographic model. The virtual screening may also have inconsistencies leading to false-positive results, in which the selected

TABLE 1 Bond distances between HSE and HSD active-site residues^a

HSE atom	HSD active-site residue	Bond distance (Å)
Minimized <i>P. brasiliensis</i> HSD		
N-1	Wat374 O	2.7
C-γ	NAD <i>n</i> C-4	3.7
O-1	Lys122 N-ζ	2.6
O-2	Lys233 N-ζ	2.6
O-δ	Glu218 O-ε2	2.8
<i>S. cerevisiae</i> HSD		
N-1	Wat460 O	3.2
C-γ	NAD <i>n</i> C-4	3.7
O-δ	Lys117 N-ζ	4.8
O-δ	Lys223 N-ζ	3.8
O-δ	Asp219 O-γ2	2.8
O-2	Glu208 O-ε2	2.7

^aThe results for charge-dipole interactions are in bold.

pose of a ligand may not represent the most stable one. The sum of these inconsistencies may result in compounds that, in theory, seem promising but in *in vitro* studies may show evidence of little or no activity. In an attempt to minimize these inconsistencies, we used energy minimization techniques for maximizing the interaction of the modeled protein with the substrate to obtain a more appropriate binding site in the presence of this ligand. In addition, the substrate serves as a structural guide for the choice of the best pose of an unknown ligand. The realization of the virtual screening with the AutoDock program from 94 folders containing 2,000 ligands each, plus the structures of the substrate and the ligand 4-(4-hydroxy-3-isopropylphenylthio)-2-isopropylphenol (referred to here as REF1), led the substrate to be redocked at least 94 times, and in all cases the structures overlapped their modeled pose with an RMSD of less than 0.7 Å in all trials. The AutoDock score for L-homoserine was $-5.3 \text{ kcal} \cdot \text{mol}^{-1}$, whereas that for REF1 was $-6.0 \text{ kcal} \cdot \text{mol}^{-1}$. Only the ligands with AutoDock scores that ranked above the score for REF1 were selected from the structure and analyzed.

Once the program successfully chose the best pose for the substrate that was identical to the modeled one, it gave us more confidence that the pose for an unknown ligand with a better score could be promising. The 233 molecules selected on the first screening were again subjected to four new screenings with the same program in order to discard those compounds for which the scores were not reproducible. These reevaluations selected 216 molecules that were subjected to four new virtual screenings by means of the program Molegro (v6.0) (37), which used search and rank algorithms different from those used in the AutoDock program, culminating in 14 promising molecules that have been successfully filtered by the use of absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) criteria. A careful analysis that took into account other possible metabolic targets predicted by a server using the similarity ensemble approach (SEA) (38), plus the chemical nature of the interaction of these substances with the *P. brasiliensis* HSD enzyme, led us to identify the compounds HS2 (Zinc database accession number Zinc203432), HS3 (Zinc database accession number Zinc1531037), and HS4 (Zinc database accession number Zinc52986906). A new search of the Zinc database (39) for compounds with 80% similarity to HS2 suggested HS1 (Zinc database accession number Zinc273730). The structures of the molecules used in this work are shown in Fig. 3.

In vitro tests. Of the four compounds tested, HS3 and HS4 showed no *in vitro* antifungal activity against *Paracoccidioides* spp.; however, the activities of compounds HS1 and HS2 were more promising (Table 2). For *Candida* spp. and *Cryptococcus* spp., no compound tested showed satisfactory antifungal activity (MICs $> 512 \mu\text{g} \cdot \text{ml}^{-1}$).

HS1 and HS2 showed *in vitro* antifungal activity against two isolates of *P. lutzii* (Pb01 and 8334) and three isolates of *P. brasiliensis* (Pb18, Mg14, and Mg15). Since itraconazole (ITZ) is the reference drug used in the treatment of paracoccidioidomycosis, its MIC was also evaluated under the same conditions for comparison purposes.

The synergistic effects of compounds HS1 and HS2 with itraconazole and amphotericin B were evaluated. The results indicated that these compounds do not exhibit synergistic effects with polyene antifungals, since, as described in the literature (55, 56), the value of the fractional inhibitory concentration (FIC; which is equal to the MIC of the compound in the combination/MIC of the compound alone) index $\sum \text{FIC}$; which is equal to (MIC of itraconazole in combination with HS1 or HS2/MIC of itraconazole alone) + (MIC of HS1 or HS2 in combination with itraconazole/MIC of HS1 or HS2 alone) indicating a synergistic effect is ≤ 0.5 . However, the combined action of compounds HS1 and HS2 with itraconazole was able to reduce the MIC of itraconazole (Table 3). The $\sum \text{FIC}$ value for the combination of ITZ and HS1 was 0.475, as was that for the combination of ITZ and HS2. These values indicate that the combination of ITZ and HS1 or HS2 is synergistic.

Cytotoxicity. Cell viability (in percent) as a function of the compound concentration was determined. The *in silico* methods searched for compounds that have selective activity against the fungus but that do not cause damage to mammalian cells. Thus, the

FIG 3 Structure of ligands charged at neutral pH.

first step in the development of a new antifungal was to evaluate its *in vitro* cytotoxicity for cells of the human cell line HeLa. Cell viability was greater than 90% for all concentrations tested, and there was no statistically significant difference between the results ($P > 0.05$). This result reinforces the suggestion that these compounds are promising for development as new antifungal drugs (Fig. 4).

Molecular dynamics simulation. In order to investigate the behavior of the enzyme in the presence of the ligands, we performed 20-ns molecular dynamics simulations of *P. brasiliensis* HSD in the presence of the L-homoserine substrate, the HS1 and HS2 ligands, as well as the apo form.

The results in Fig. 5A show the RMSD of the main-chain atoms of *P. brasiliensis* HSD during the simulation. It can be seen that all structures reached thermodynamic equilibrium after 15 ns. It can also be seen from Fig. 5 that the presence of the HS1 and HS2 ligands allowed the protein to be more stable, as demonstrated by the lower value of the RMSD.

TABLE 2 MICs of the compounds tested *in vitro* for their antifungal activities against *Paracoccidioides* strains

Isolate	Compound MIC ($\mu\text{g} \cdot \text{ml}^{-1}$)			
	HS1	HS2	ITZ	AMB
<i>P. brasiliensis</i>				
Pb18	64	32	0.007	0.12
Mg14	64	64	0.007	0.12
Mg15	128	128	0.007	0.06
<i>P. lutzii</i>				
Pb01	64	64	0.007	0.06
8334	64	64	0.007	0.06
xlogP	0.11	0.49	5.3	−2.3

The interval comprising the last 5 ns of the dynamics simulation was used to evaluate the fluctuations of the alpha carbons of each residue, expressed in the form of the root mean square fluctuation (RMSF) (Fig. 5B). The regions with the greatest fluctuations were the N-terminal coil (residues 1 to 3) and the region between residues 195 and 202, which comprises a loop region in the outer surface of the protein. This loop is quite distant from the residues that form the active HSE site; therefore, its fluctuation did not affect the docking process. The high fluctuations for these two regions also contributed to an increase in the overall RMSD for the protein.

The radius of gyration (r_{gyr}) is defined as the root mean square distance of a collection of atoms from their common center of gravity. When the value of r_{gyr} remains stable during the simulation, the protein remains coiled and the presence of the ligand does not lead the protein to unfold. Figure 6 shows that all the complexes exhibited a r_{gyr} of about 27.5 Å after 2.5 ns of equilibration, suggesting that all complexes are very stable.

In this work, the active site was delimited by residues making any contact with the ligand over a distance range of 4.0 Å. In order to obtain a more accurate picture of the contribution of each residue in the delimited area of the active site, the frequency of contact of each residue with the ligand was taken as a function of the simulation time. A list of the main contact frequencies of *P. brasiliensis* HSD with the analyzed ligands is shown in Table 4.

Analysis of the results presented in Table 4 shows that the residues responsible for anchoring the ligands to the active site are Ser180, Thr182, Asp224, Asp229, and Lys233, since their contact frequencies with all ligands were greater than 80%. This active site, formed by all these residues except Lys233, is a little different from that shown in Table 1. The site displayed in Table 4, which was formed by more residues involved in anchoring the substrate than the number of residues used to form the site shown in Table 1, is much more accurate, because it represents the average number of contacts from 1,250 frames (the last 5 ns of the simulation), while the number of contacts of the

TABLE 3 Synergistic effects of HS1 and HS2 in combination with itraconazole against *P. brasiliensis*^a

Compound	MIC ($\mu\text{g}/\text{ml}$)		FIC
	Alone	Combination	
HS1	128	32	0.25
ITZ	0.008	0.0018	0.225
HS2	32	8	0.25
ITZ	0.008	0.0018	0.225

^aThe MIC values of HS1, HS2, and itraconazole alone were determined by the broth microdilution method, and the MIC values of the combinations were determined by the checkerboard assay. FIC indexes were obtained from the checkerboard assay. The ΣFIC index is equal to (MIC of itraconazole in combination with HS1 or HS2/MIC of itraconazole alone) + (MIC of HS1 or HS2 in combination with itraconazole/MIC of HS1 or HS2 alone). The ΣFIC value was 0.475 for both the combination of HS1 and ITZ and the combination of HS2 and ITZ. An ΣFIC of ≤ 0.5 is indicative of synergism.

FIG 4 *In vitro* cytotoxicity for HeLa cells. Compounds HS1 (A) and HS2 (B) were tested at concentrations of 256 to 0.5 $\mu\text{g/mL}$ (ratio 2), and cell viability was assessed by determination of mitochondrial cell activity using a CellTiter 96 AQueous One Solution cell proliferation assay kit (which contains MTS) after 48 h of incubation. The percentage of viable cells after exposure to the compounds at different concentrations was evaluated by considering the viability of untreated cells to be 100%. The experiments were performed in triplicate.

site shown in Table 1 were taken from a single frame. Table 4 also shows that each ligand makes exclusive contacts with the proteins. For example, HS1 makes important contacts with Gly215, Tyr216, Thr217, and Glu218; however, these contacts are not as important in stabilizing HS2, which, in turn, also makes an important contact with Gly351 and, in short, four more contacts than HS1. This may indicate that the affinity of HS2 for the enzyme is slightly increased compared with that of HS1, a fact that justifies the low MIC of the HS2 ligand found in *in vitro* experiments.

DISCUSSION

The results presented in Table 2 show that the MIC values determined for several isolates of two species of *Paracoccidioides* were very similar. However, the Pb18 isolate

FIG 5 Behavior of the *P. brasiliensis* HSD complexes over time in a simulation. (A) Root mean square deviation of main-chain atoms; (B) root mean square fluctuation of C- α atoms. The HSE binding site is composed of the residues of the greatest frequency from Table 1.

FIG 6 Radius of gyration calculated from the main-chain atoms of the different complexes of *P. brasiliensis* HSD during the simulation.

of *P. brasiliensis*, which was the prototype isolate used for the selection of the compounds, showed a sensitivity slightly increased over that of the other isolates tested. Two factors either individually or in combination may explain these results. The first one is the slight difference in the solubility (xlogP) of compounds HS1 and HS2, where negative values indicate that the compounds are water soluble and positive values indicate that the compounds are water insoluble. The second one is the difference in the levels of identity of the amino acid sequences of HS1 and HS2 with the amino acid sequence of HSD of *P. lutzii* Pb01 (UniProt accession number [C1GTZ6](#)), which were less than 97%. Thus, the fact that HS2 has been discovered from the structure of the *P. brasiliensis* HDS (Pb18) reinforces the capability of the structure-based virtual screening technique used to select compounds with specific activity.

Furthermore, the MICs of HS1 and HS2 were not different from the MIC values of some molecules currently under study. Abadio et al. (26) selected three molecules targeting the enzyme thioredoxin reductase by virtual screening, the same technique used in this study. These three molecules exhibited MIC values of 8 to $>256 \mu\text{g} \cdot \text{ml}^{-1}$ and 16 to $>256 \mu\text{g} \cdot \text{ml}^{-1}$ for strains of *P. brasiliensis* and *P. lutzii*, respectively. In another work with dirutheniumpentadithiocarbamate derivatives, Donnici et al. (24) found that MIC values for Pb18 strains of *P. brasiliensis* varied from 16 to $128 \mu\text{g} \cdot \text{ml}^{-1}$,

TABLE 4 Frequency of contact of the *P. brasiliensis* HSD residues with ligands^a

Residue	Contact frequency		
	HSE (substrate)	HS1	HS2
Lys122	0.50	0.99	0.62
Lys123			0.11
Phe179	0.06		0.07
Ser180	0.93	0.85	0.96
Gly181	0.98	0.73	0.93
Thr182	0.98	1.00	0.99
Gly215		0.89	0.21
Tyr216	0.47	0.92	0.52
Thr217		0.93	0.06
Glu218	0.65	0.91	0.58
Asp224	1.00	1.00	1.00
Asp229	1.00	1.00	0.99
Lys233	1.00	1.00	1.00
Gly349	0.88	0.58	0.01
Ala350	0.54	0.99	0.96
Gly351			0.74
Gly352			0.07
Nad371	1.00	0.91	1.00

^aThe results for residues with a higher frequency of contact are in bold.

depending on the substituents; however, the molecular target of these compounds is not known. Other works have identified molecules showing more promising results; for instance, a derivative of 6-quinoliny *N*-oxide chalcones showed antifungal activity similar to that of itraconazole in *in vivo* tests in mice, obtaining an MIC₅₀ and an MIC₉₀ of 7.8 and 23.4 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively (40).

Itraconazole inhibits the fungus-mediated synthesis of ergosterol via inhibition of the enzyme lanosterol 14 α -demethylase in a way that alters the permeability of the fungal cell membrane. HS1 and HS2 inhibit the enzyme homoserine dehydrogenase, which is involved in an important step in the biosynthesis of the amino acids threonine, isoleucine, and methionine. We believe that the inhibition of these two important pathways in fungal metabolism at the same time is the cause of the synergism observed in the experiments. Since HS1 and HS2 proved to be nontoxic in the model studied, their interaction with ITZ results in the ability to use lower doses of each component and, hence, less intense adverse effects and make it less likely that the microorganisms will acquire resistance.

A ClustalO alignment with the amino acid sequences of HSD from the strains evaluated in the MIC assays was performed. Although the identity between them was only 25.4%, all five key residues that bind to the HSE substrate and to the HS1 and HS2 inhibitors are conserved. Eleven other residues listed in Table 4 were conserved in all strains, except for Tyr216 (hydrophilic), which is replaced by a Phe (hydrophobic) in *P. lutzii*. According to the results presented in Table 4, Tyr216 has a significant frequency of contact with HS2. Thus, the Tyr216Phe mutation may be reflected in the affinity of *P. brasiliensis* HSD for this ligand, which justifies the higher MIC value of HS2 for *P. lutzii* that was found (Table 2). These facts suggest that these inhibitors could theoretically bind to the target enzyme in all these strains, but this was not observed in practice. However, HS1 and HS2 are considered lead molecules, and future studies that make structural modifications to these compounds that broaden their spectrum of action and also enhance their antimicrobial activity should be carried out. Since the mechanism of action of HS1 and HS2 is known, their selective toxicity could lead to the development of drugs with fewer adverse effects and, therefore, to more promising results.

In conclusion, this study describes two new amino acid derivative compounds, HS1 and HS2, that have the potential to be exploited as leads in the development of antifungal molecules and that may represent a new option in the development of antifungals against paracoccidioidomycosis. Moreover, the application of these drugs to human-pathogenic fungal strains confirmed the potential for exploitation of the homoserine dehydrogenase enzyme as a target for the development of drugs active against PCM. These results represent a direct contribution of biotechnology for the detection of compounds active against paracoccidioidomycosis.

MATERIALS AND METHODS

Model building and evaluation. The sequence of *P. brasiliensis* HSD (UniProt accession number C1G1C3) was used to search for templates, using the BLASTp program (41), by sequence alignment against sequences in the Protein Data Bank (PDB) database (42) and also by fold recognition via the pGenTHREADER method (43). The crystal structures of HSD from *Saccharomyces cerevisiae* complexed with the substrate L-homoserine and 3-aminomethyl-pyridinium-adenine-dinucleotide (36), an NAD analogue (PDB accession number 1EBU), as well as the *S. cerevisiae* HSD complexed with NAD (PDB accession number 1EBF), were selected as the best templates.

The structural model of *P. brasiliensis* HSD complexed with NAD⁺, L-homoserine, a sodium ion, and a structural water molecule in the active site was modeled by homology using Modeler (v9.11) software (44). A total of 1,300 models were built, and the best 5 were selected according to the Modeler Discrete Optimized Protein Energy (DOPE) score, with the best one being chosen by structural evaluation using ProCheck software (45).

MD simulations. The modeled structure was minimized by molecular dynamics (MD) simulation procedures to correct possible stereochemical clashes between ligands and the protein, since all ligands were copied as they appear in the templates. The NAMD2 software package (46) was used for these procedures, with a CHARMM c35b2-c36a2 force field (47) being applied to the atoms of the protein and NAD⁺. The force fields for L-homoserine and for the other ligands found by virtual screening were generated by the SwissParam server (48) in the same format. For preparation of the system, the spatial coordinates of the protein-ligand complex, as well as the enzyme in the apo form, were inserted in a periodic box with a TIP3P (transferable intermolecular potential 3P) water model. The limits lay at least

10 Å away from the outer surface of the protein under natural pressure and temperature (NPT) conditions (constants were a temperature of 300 K, a pressure of 1 atm, and the number of atoms). A sufficient amount of Cl^- counterions was added to neutralize the system charge.

The molecular dynamics simulation occurred in steps. Initially, the atoms of L-homoserine were fixed in space while all other system atoms were submitted to 30,000 steps of energy minimization by the conjugate gradient method (CG) as a way to maximize the interaction of the protein with the ligand, something that was not performed by the modeling program. In the second step, all atoms in the system were again minimized by over 10,000 CG steps. In the third step, the atoms of the protein and ligand were fixed in space while the water and ions were equilibrated for 60 ps of the MD simulation. Finally, in the fourth step, all the atoms of the system were minimized again by 20,000 new CG steps. The minimized coordinates resulting from this step were used as input parameters (reference frame) for redocking simulations, virtual screening, and long-term MD equilibrium. In the long-term molecular dynamics simulation, all atoms were equilibrated for 20 ns, which was a sufficient amount of time to observe system convergence for thermodynamic equilibrium conditions.

The analyses of the simulations were performed in terms of the root mean square deviation (RMSD) from the minimized (reference) structure. The radius of gyration (r_{gyr}) of the whole protein-ligand complex, the residues in contact with the inhibitor at distances of about 4.0 Å, and the root mean square fluctuation (RMSF) for the backbone atoms of each residue were extracted from the trajectory file.

All MD simulations were performed in four nodes on an SGI Altix ICE 8400 LX cluster, each of which was performed with two Intel six-core 5680 processors with a 3.33-GHz system (48 cores) and 36 GB of random-access memory. The cluster is available at the National Center for High Performance Computing (CENAPAD) of Unicamp.

Docking and virtual screening simulations. The docking simulations were carried out using two different programs with different search and ranking algorithms. The docking of L-homoserine was performed by both programs in the minimized protein-HSE complex (redocking) by adjusting the parameters until the RMSD obtained from the best-ranked pose stayed below 0.7 Å. All protocols allowed flexibility for the ligands. All water molecules and ions were removed from the structures.

The standard protocol for the AutoDock (v4.2.3) program (49) implemented in the Pyrx (v0.9) graphical interface (50) uses 0.375-Å grid spacing and grid centers of 62 (40 points), 25 (40 points), and -9 (40 points) at the x, y, and z axes, respectively. The protocol for the Molegro (v6.0) program (37) uses the Moldock score as a score function, the Moldock optimizer as a search algorithm, and the radius of the search sphere as 9 Å around the catalytic site. The results were ranked by the H-bond score, and all other options were set at the default settings. Redocking simulations were repeated five times with each program, and the results were reproducible.

The Library of Natural Products from the Zinc database, which has 187,841 purchasable molecules, was used for virtual screening simulations. The library was split into folders of 2,000 compounds plus L-homoserine (Zinc database accession number Zinc895146) and 4-(4-hydroxy-3-isopropylphenylthio)-2-isopropylphenol (compound REF1; PubChem identifier 448797), an inhibitor of HSD that has been described previously (28). The library was first screened by use of the AutoDock program. Visual inspection of the molecules ranked better than L-homoserine and REF1 was used to group the molecules in a new folder called "best results." The best results folder was screened four more times to eliminate false-positive results (only compounds for which the results were reproducible were considered). The molecules in the best results folder were screened another four times by use of the Molegro (v6.0) program and the protocol that had been validated earlier. The molecules chosen by both programs were then evaluated by use of a server that uses the similarity ensemble approach (SEA predictions) (38), which correlates the chemical properties of a compound with a database of activity from known molecules, thereby suggesting possible protein targets for each compound. The ADMETox properties were filtered by use of the OSIRIS property explorer (<http://www.openmolecules.org/propertyexplorer/applet.html>) and the AdmetSAR server (51), thereby showing promising features of toxicity and pharmacokinetics.

Bioassays. The molecules HS1 (Vitas-M), HS2 (Chembridge), HS3 (Sigma, Milwaukee, WI, USA), HS4 (Sigma-Aldrich, Buchs, St. Gallen, Switzerland), itraconazole (Sigma-Aldrich, St. Louis, MO, USA), and amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) were purchased for *in vitro* assays. The MICs of selected compounds were determined over a test concentration range of between 0.1 and 512 µg/ml. Stock solutions of each drug were prepared in RPMI 1640 medium without sodium bicarbonate and with L-glutamine supplemented with 2% glucose and buffered (pH 7.0) with 0.165 M morpholinepropane-sulfonic acid (MOPS; Sigma-Aldrich, St. Louis, MO, USA). Dimethyl sulfoxide (DMSO; 2% [vol/vol]) and Pluronic F-127 0.002% (wt/vol) (Sigma-Aldrich, St. Louis, MO, USA) were used to solubilize the compounds. To establish that exposure to diluent (DMSO and Pluronic F-127) did not affect the microorganisms, the fungal species were grown in the presence of diluent and their growth was compared with that of the positive control (which was grown in medium only). The concentration of amphotericin B, used as an antifungal control, ranged from 0.03 to 16 µg/ml for all species tested.

Susceptibility tests were performed with eight pathogenic yeasts: two isolates of *P. lutzii* (Pb01 and 8334), three isolates of *P. brasiliensis* (Pb18, Mg14, and Mg15), *C. albicans* (ATCC 90028), *Candida parapsilosis* (ATCC 22019), *Candida glabrata* (ATCC 90030), *Candida tropicalis* (ATCC 750), *Cryptococcus neoformans* (INCQS 40283), and *Cryptococcus gattii* (INCQS 40113). Prior to testing, each strain of *Candida* spp. was subcultured in Sabouraud dextrose agar (SDA; Becton, Dickinson and Company, Sparks, MD) and incubated at 30°C for 1 day. *C. neoformans* and *C. gattii* were subcultured in yeast extract-peptone-dextrose agar (YPD; Becton, Dickinson and Company, Sparks, MD) at 30°C for 2 days. Each isolate of *Paracoccidioides* spp. was subcultured in Fava-Netto medium (52) at 36°C for 5 days. The tests were

conducted using the broth microdilution method according to the protocol described in document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI) (54) with modifications for *Paracoccidioides* spp. and *Cryptococcus* spp.

Briefly, suspensions were prepared in sterile saline solution (0.85%) with yeast concentrations adjusted to 1×10^4 to 2×10^4 cells/ml. The MIC results for all agents were read following 7 days of incubation at 37°C. MICs were determined to be the lowest concentration of the drug able to inhibit 80% of cell growth compared with the growth of the positive control. All tests were performed three times in duplicate.

Checkerboard assays. The interaction of compounds HS1 and HS2 with amphotericin B and itraconazole was investigated by way of checkerboard tests on 96-well plates, performed according to the CLSI approved standard described in document M27-A3 (54) with modifications for *P. brasiliensis* species. In combination with itraconazole (0.00002 to 0.015 µg/ml), the concentrations of HS1 and HS2 tested with isolate Pb18 ranged from 2 to 512 µg/ml. The fractional inhibitory concentration (FIC) was calculated by taking the MIC of the compound in the combination/MIC of the compound alone. The Σ FIC index was calculated as the sum of the FICs of either drug (MIC of ITZ in the combination/MIC of ITZ alone + MIC of HS1 or HS2 in the combination/MIC of HS1 or HS2 alone). Drug interactions were classified as synergistic when the Σ FIC index value was ≤ 0.5 and antagonistic when the Σ FIC index value was > 4 (55, 56). Experiments were repeated a minimum of three times.

In vitro cytotoxicity assay. The cytotoxicity assay was performed in HeLa cells using the CellTiter 96 assay (Promega, Madison, WI, USA), which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Briefly, HeLa cells were incubated in 96-well culture plates maintained in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (5%) and treated with different concentrations of compound HS1 or HS2. After 24 or 48 h, the MTS solution was added to each well and the plate was incubated for 180 min in a CO₂ (5%) oven at 37°C. Cell viability was determined by measurement of the optical density at 490 nm in a spectrophotometer. The cytotoxicity of the compounds is presented as the average from three independent experiments with three replicates for each compound. Percent cell viability was calculated in relation to the viability of the controls, which were treated with medium only.

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