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Repurposing approach identifies new treatment options for invasive fungal disease

Isis Regina Grenier Capoci[a,*], Daniella Renata Faria[a], Karina Mayumi Sakita[a], Franciele Abigail Vilugron Rodrigues-Vendramini[a], Patricia de Souza Bonfim-Mendonça[a], Tania Cristina Alexandrino Becker[a], Érika Seki Kioshima[a], Terezinha Inez Estivalet Svidzinski[a], Bernard Maigret[b]

[a] Department of Clinical Analysis and Biomedicine, State University of Maringá, Maringá, Paraná, Brazil
[b] LORIA, Lorraine University, Nancy, France

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ABSTRACT

Drug repositioning is the process of discovery, validation and marketing of previously approved drugs for new indications. Our aim was drug repositioning, using ligand-based and structure-based computational methods, of compounds that are similar to two hit compounds previously selected by our group that show promising antifungal activity. Through the ligand-based method, 100 compounds from each of three databases (MDDR, DrugBank and TargetMol) were selected by the Tanimoto coefficient, as similar to LMM5 or LMM11. These compounds were analyzed by the scaffold trees, and up to 10 compounds from each database were selected. The structure-based method (molecular docking) using thioredoxin reductase as the target drug was performed as a complementary approach, resulting in six compounds that were tested in an *in vitro* assay. All compounds, particularly raltegravir, showed antifungal activity against the genus *Paracoccidioides*. Raltegravir, an antiviral drug, showed promising antifungal activity against the experimental murine paracoccidioidomycosis, with significant reduction of the fungal burden and decreased alterations in the lung structure of mice treated with 1 mg/kg of raltegravir. In conclusion, the combination of two *in silico* methods for drug repositioning was able to select an antiviral drug with promising antifungal activity for treatment of paracoccidioidomycosis.

1. Introduction

Invasive fungal disease (IFD) is a major cause of morbidity and mortality in patients with compromised immunity [1,2]. *Candida* spp., *Cryptococcus* spp. and *Paracoccidioides* spp. are three important fungal genera that can cause IFD. All are of high clinical importance, as shown in several recent studies [3–5]. The IFD can be caused by endemic fungi, usually in cases of fungal reactivation from a previous contact. However, especially in immunocompromised patients, this disease can also be caused by universally distributed opportunistic fungi [1,2]. Various factors contribute to immunosuppression, such as transplantation, autoimmune, oncological and hematological diseases and the use of broad-spectrum drugs [6–9]. Due to the limited currently available antifungal drugs, and the limitations on their use because of drug interactions and toxicity [10,11], new search methods are increasingly needed in order to find new compounds for treating IFD.

The recent approach of the rational drug design has already been explored by the group, especially against the target thioredoxin reductase (Trr1) [12,13]. This important flavoenzyme is involved in essentials cellular processes, as the cells protection against oxidative stress [14,15]. Therefore, this enzyme whose isoform of the low molecular mass (~35 kDa) is present only in plants, some parasites, prokaryotes and fungi [16], is a promising target for the new therapeutic options development for IFD treatment.

In a recent study by our group, two hit compounds that interact with the enzyme Trr1 from *Candida albicans* were selected through molecular modeling and virtual screening. These compounds showed promising antifungal activity against these three important pathogenic fungi,

Abbreviations: TRR1, thioredoxin reductase; LMM5, 4-(N-benzyl-N-methylsulfamoyl-N-(5-(4-methoxybenzyl-1,3,4-oxadiazol-2-yl benzamide; LMM11, 4-(N-cyclohexyl-N-ethylsulfamoyl-N-(5-(furan-2-yl-1,3,4-oxadiazol-2-yl benzamide; DMSO, dimethyl sulfoxide

* Corresponding author at: Universidade Estadual de Maringá, Departamento de Análises Clínicas, Divisão de Micologia Médica, Laboratório de Ensino e Pesquisa em Análises Clínicas, Av. Colombo 5790, 87020-900 Maringá, PR, Brazil.
E-mail address: isiscapoci@hotmail.com (I.R.G. Capoci).

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culminating in a patent application for this new antifungal class: 1,3,4-oxadiazoles, termed LMM5 and LMM11 [17]. However, these compounds are still hits that should be optimized and tested in several stages of evaluation, until a new drug can be made available on the market. Given the urgency of new options for the treatment of fungal infections, drug repositioning using virtual screening appears to be a viable alternative to accelerate this process.

Virtual screening of chemical libraries has been widely used in drug discovery, as a rapid and inexpensive alternative [18,19], facilitated by the rapid growth of public biological databases and web resources that provide bioactivity data for compounds and their targets available for research [20,21]. Computational methods for virtual screening are classified as (1) ligand-based drug design (e.g., ligand similarity) and (2) structure-based drug design (ligand docking) [22]. The principle of ligand-based drug design is the use of known active ligands as a template to search for other active ligands, assuming that chemically similar compounds would have similar biological activities [23]. An advantage of ligand-based design is that regardless of the 3D structure of the target, the descriptor calculations are relatively rapid, expediting the availability of new ligands [24]. In this approach, the structural, physical and chemical properties of the ligands are meticulously studied and correlated with the desired pharmacological activity [25,26].

Structure-based drug design techniques can only be applied to compounds for which the 3D structure is known, either experimentally or through computational homology modeling [27]. One of the most frequently used techniques is molecular docking, which has become an important tool for the discovery of new drugs [28]. This useful approach has been possible since the first algorithms were developed in the 1980s, and can predict with some accuracy the best conformation of a compound that will bind to its appropriate target [29].

Drug repositioning or repurposing is the strategy of developing new indications for previously approved drugs, advanced clinical candidates, or drugs for which the targets have already been discovered [30,31]. The field of drug repositioning is growing rapidly, shortening the time for identification, characterization and structural optimization for novel drug candidates, with economic benefits and expedited approval schedules [32–34] due to the reduced risk and shorter time to market made possible by the availability of preclinical data [35]. This method has been a viable alternative for the discovery of new drugs to treat neglected diseases [36,37]. Successful examples of drug repositioning have been reported [38–41]. The objective of this study was drug repositioning using virtual-screening computational methods and validation of antifungal activity, from compounds selected against important pathogens that cause IFD.

2. Methods

2.1. In silico approach

2.1.1. LMM5 and LMM11

The files in SMILE format (.smi) of the hit compounds: F2368-0617 (LMM5) and F2832-0099 (LMM11) were provided by the company Life Chemicals (http://www.lifechemicals.com), and the Spatial Data File (.sdf) files for PoseView and LigPlot were from the ZINC15 (ZINC000008923378 = LMM5) and ChEMBL (2093327 = LMM11) databases.

2.1.2. Databases

For drug repositioning, three databases were used: MDL Drug Data Report – MDDR (jointly produced by BIOVIA and Thomson Reuters), DrugBank (available at https://www.drugbank.ca/) and TargetMol provider (http://targetmol.com/).

2.1.3. Ligand-based drug repositioning

The Tanimoto coefficient (Tc) was used for similarity determination, in which Tc = 0 was attributed to compounds with least similarity and Tc = 1 for those with most similarity. The first step was to search in each database for the 100 compounds with greatest similarity to LMM5 or LMM11. Next, the scaffold trees were constructed using the Scaffold Hunter program [42] (http://scaffoldhunter.sourceforge.net/) to select up to ten of the best compounds from each database. Based on the Scaffold definition: the part of a structure which remains after all terminal chains have been removed [43]. A single scaffold was defined for the compounds (LMM5 and LMM11) and the program's algorithm searched in each 100 compounds, from the database, those compounds that shared the minimum chemical structure (scaffold). The set of compounds were organized into a unique tree of hierarchy, where the scaffolds are the nodes of that tree [44]. Therefore, the compounds selected were those that within the hierarchy tree shared scaffolds with LMM5 or LMM11.

Table 1
Six sub-libraries with the Tanimoto coefficient range of selected compounds.

Compounds	Tanimoto coefficient (Tc)
Compounds similar to LMM5 from DrugBank (100)	0.30–0.43
Compounds similar to LMM5 from MDDR (100)	0.43–0.48
Compounds similar to LMM5 from Targetmol (100)	0.28–0.42
Compounds similar to LMM11 from DrugBank (100)	0.30–0.40
Compounds similar to LMM11 from MDDR (100)	0.41–0.60
Compounds similar to LMM11 from Targetmol (100)	0.26–0.38

2.1.4. Structure-based drug repositioning

To complement the first method, the 600 most similar compounds to LMM5 and LMM11, 300 of each, were submitted to the docking simulation by the GOLD software [45] against thioredoxin reductase from *Candida albicans* (CaTrr1), in its reduced conformation. The CaTrr1 model was obtained by homology modeling. The compound structures were converted to three-dimensional models by CORINA [46]. For each compound, a maximum of 50 docking runs were performed; if the top 5 conformations converged within a 1.5 Å RMSD range, the docking process was suspended. To select the compounds for testing *in vitro*, a table was created for each database, containing the compounds selected by the ligand-based method, the 5 compounds with the best GOLD values, and the 5 compounds with the highest Tc values (Supplementary material). Compounds with GOLD values less than 60 were excluded. The interactions of the remaining compounds with the amino acids at the *Ca*Trr1 catalytic site were analyzed by the PoseView tool (http://proteinsplus.zbh.uni-hamburg.de/). These results were compared with the hit compound interactions (LMM5 or LMM11). The last analysis was performed to identify the compounds available for purchase.

The interaction of compounds selected with the *Ca*Trr1 protein was also visualized with the Visual Molecular Dynamics (VMD) program, available at http://www.ks.uiuc.edu/Research/vmd/ and the LigPlot program [47], available at https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

2.2. In vitro approach

2.2.1. Compounds

The compounds glyburide, gliquidone, zafirlukast, raltegravir, venetoclax and SR9243 were obtained from the company Targetmol (http://targetmol.com/). The stock solution of these compounds were prepared with dimethyl sulfoxide (DMSO) in accordance with the manufacturer's recommendations. The highest DMSO concentration used in the experiments did not exceed 0.5% (an concentration non-toxic, as described in CLSI document [48]).

2.2.2. Organisms

In vitro susceptibility tests were performed for eight pathogenic yeasts: *P. lutzii* (Pb01), *P. brasiliensis* (Pb18), *C. albicans* (ATCC 90028),

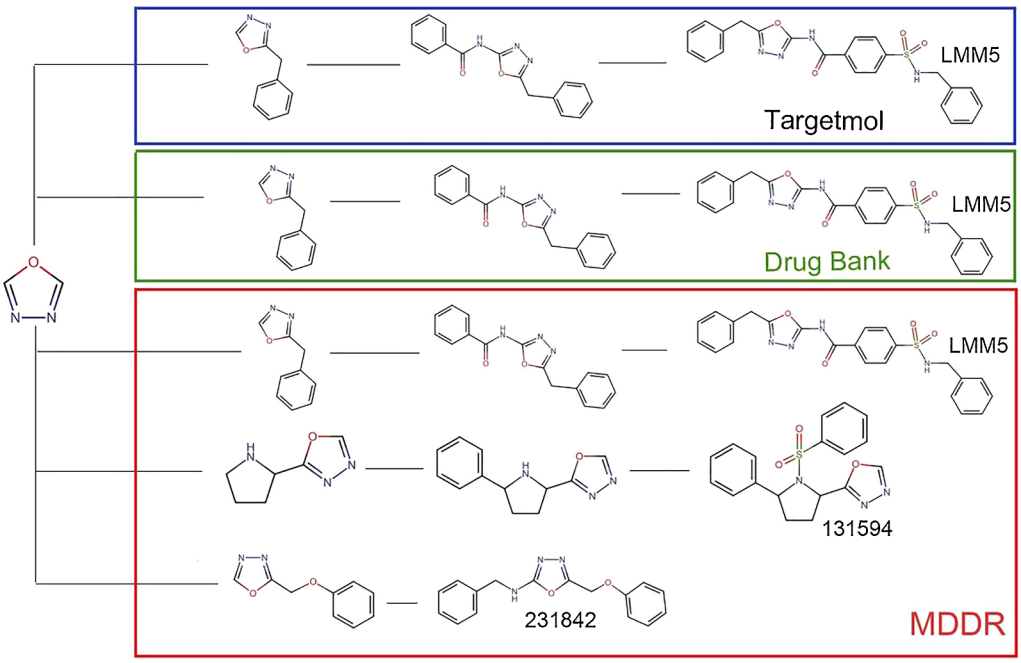

Fig. 1. Scaffold tree from the TargetMol, DrugBank and MDDR databases, for similar compounds to LMM5.
Two compounds from MDDR (131594 and 231842) were selected; for DrugBank and TargetMol, no scaffolds were identified. The scaffold trees were constructed with the Scaffold Hunter program (http://scaffoldhunter.sourceforge.net/).

C. parapsilosis (ATCC 22019), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258) and *C. neoformans* (INCQS). Prior to testing, each strain of *Candida* spp. was subcultured in Sabouraud dextrose agar (SDA, Difco™, Detroit, MI, USA) and incubated at 35 °C for 24 h. *C. neoformans* was subcultured in Yeast Extract Peptone Dextrose Agar (YPD; Becton, Dickinson and Company, Sparks, MD, USA) at 25 °C for 48 h. *Paracoccidioides* spp. isolates were subcultured in Fava-Netto medium at 36 °C and used on the 5th day of culture.

2.2.3. Antifungal susceptibility assays

The Minimum Inhibitory Concentrations (MICs) of the six virtually selected compounds were determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute M27-A3 (CLSI) [48], against *Candida* isolates. The method was adapted for *Paracoccidioides* spp. and *C. neoformans* conditions as described by Abadio et al. [18]. The tests were performed in flat-bottom 96-well microtiter plates (Techno Plastic Products, Switzerland) and the final concentrations of the compounds studied ranged from 0.5 to 256 μg/ml. Endpoint determination readings were performed visually considering 100% inhibition of growth for either *Candida*, *Cryptococcus* or *Paracoccidioides*.

2.3. In vivo approach

The compound raltegravir (isentress®) was used for the preliminary antifungal activity *in vivo* test. Fifteen male 6-week-old BALB/c mice were divided in 3 groups (n = 5), treated with raltegravir (1 mg/kg), fluconazole (5 mg/kg) and with vehicle (PBS, DMSO). The experimental paracoccidioidomycosis model was established by administering 1×10^6 yeast cells *(P. brasiliensis)* in 50 μl of phosphate buffered saline (PBS) by the lateral tail vein. After 24 h of infection, the respective treatments were administered intraperitoneally according to group, once a day for a period of 15 days. The mice were euthanized and the lungs were aseptically removed for histopathological evaluation, with Gomori & Grocott combined with hematoxylin and eosin (H&E). The histopathological samples were observed and photographed using a binocular light microscope (Motic BA310, Moticam 5 camera), at ×400 and ×600 magnifications. To quantify the fungal cells present in the histological sections, the lung tissue was cut sequentially, and the lung area from four sections was measured and the *P. brasiliensis* yeast cells were counted. The mean number of fungal cells found in each animal group (control or treated) was divided by the total lung area in the histological section.

2.4. Ethical approval

All procedures were approved by the Institutional Ethics Committee for Animal Experimentation of the Universidade Estadual de Maringá, Paraná, Brazil (Approval No. CEUA 9810191015, 04/22/2016). The mice were treated according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

3. Results

3.1. Drug repositioning

Initially, the ligand-based approaches were used to select compounds similar to LMM5 and LMM11, which are hits and showed promising antifungal activity [17], indicating that the identification of chemically related compounds would be a promising way to develop new antifungals. Accordingly, three drug databases (MDDR, DrugBank and TargetMol) were used to identify a total of 600 compounds by Tc analysis, 100 compounds from each database. Six sub-libraries containing 100 compounds were created: three for each of the hits (LMM5 or LMM11), and one from each database. The Tc values ranged from 0.26 to 0.60 (Table 1). The highest Tc values were found for compounds from the MDDR database and similar to LMM11 (0.41–0.60).

Each of the sub-libraries was submitted to scaffold tree analysis. Among the compounds similar to LMM5, only two compounds were selected from MDDR. For DrugBank and TargetMol sub-libraries, no scaffolds were identified (Fig. 1). However, for similar compounds to LMM11, six compounds were chosen from DrugBank, ten from MDDR, and one from TargetMol (Fig. 2). Tables listing these compounds are shown in Supplementary materials. The compounds with the largest number of scaffolds were selected. Therefore, the scaffold tree was decisive for the choice of candidates.

The results of the ligand-based method were complemented with the docking simulation, using the sub-libraries. For the structure-based method, the target used as the three-dimensional model was

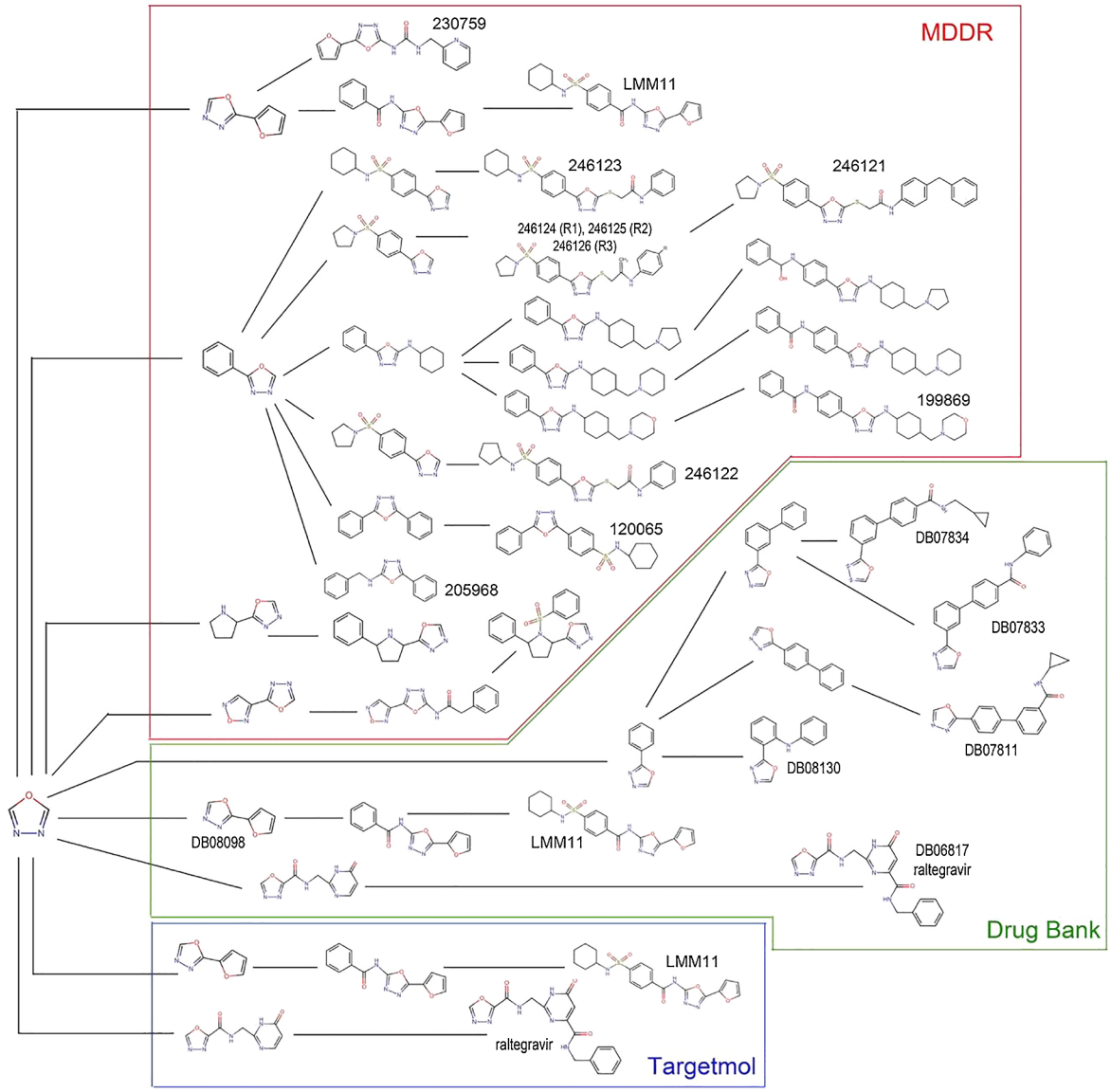

Fig. 2. Scaffold tree from TargetMol, DrugBank and MDDR databases for similar compounds to LMM11.
Six compounds from DrugBank (DB07834, DB07833, DB07811, DB08130, DB08098 and DB06817), ten from MDDR (230759, 246123, 246124 (R1:CH3), 246125 (R2:CH2CH3), 246126 (R3:OCH3), 199869, 246122, 120065, 205968 and 246121) and one from TargetMol (raltegravir) were selected. The scaffold trees were constructed with the Scaffold Hunter program (http://scaffoldhunter.sourceforge.net/).

Table 2
Indication, GOLD values and Tanimoto coefficients of six compounds selected by drug repositioning for antifungal activity *in vitro*.

Compound	Indication	GOLD value	Tc with LMM5	Tc with LMM11
Raltegravir	For the treatment of HIV-1 infection in conjunction with other antiretrovirals	72.20	0.278388	0.306338
Gliquidone	Used in the treatment of diabetes mellitus type 2	82.96	0.387387	0.346939
Zafirlukast	For the prophylaxis and chronic treatment of asthma	80.20	0.418803	0.340909
Venetoclax (ABT-199)	For the treatment of patients with chronic lymphocytic leukemia (CLL) with 17p deletion, as detected by an FDA approved test, who have received at least one prior therapy	100.37	0.320755	0.30383
Glyburide	Indicated as an adjunct to diet to lower the blood glucose in patients with NIDDM whose hyperglycemia cannot be satisfactorily controlled by diet alone	83.78	0.425743	0.347826
SR9243	Is a LXR inverse agonist that induces LXR-corepressor interaction; shows anticancer activity and selectively targets the warburg effect and lipogenesis	85.17	0.370730	0.306034

thioredoxin reductase from *Candida albicans* (*Ca*Trr1), previously constructed by homology modeling. This approach was able to select the 63 best compounds that interacted with *Ca*Trr1; 22 of them were similar to LMM5 and 41 similar to LMM11 (Supplementary materials). Interestingly, some compounds were selected from more than one database, for example raltegravir was selected by both DrugBank and TargetMol. This was important for the choice of compounds to be acquired. Six compounds were selected for the *in vitro* determination of antifungal activity (Table 2); availability for purchase was crucial for this choice. The two-dimensional and three-dimensional interaction of these compounds with *Ca*Trr1 was analyzed by the Ligplot and VMD program, respectively. As shown in Fig. 3, all compounds interacted

with the cysteines of the *Ca*Trr1 catalytic site (Cys142 and/or Cys145).

3.2. The in vitro antifungal activity

The six compounds were tested against three important fungal pathogens, *Candida* spp., *Cryptococcus neoformans* and *Paracoccidioides* spp. All compounds tested showed antifungal activity against *P. brasiliensis* (Pb18), with MIC values ranging from 16 to 64 μg/ml. *P. lutzii* appeared to be less sensitive to the compounds selected, since only four compounds showed antifungal activity, with MIC values higher than those observed for *P. brasiliensis* (Table 3). Therefore, the most active compounds against the genus *Paracoccidioides* were the antiviral and

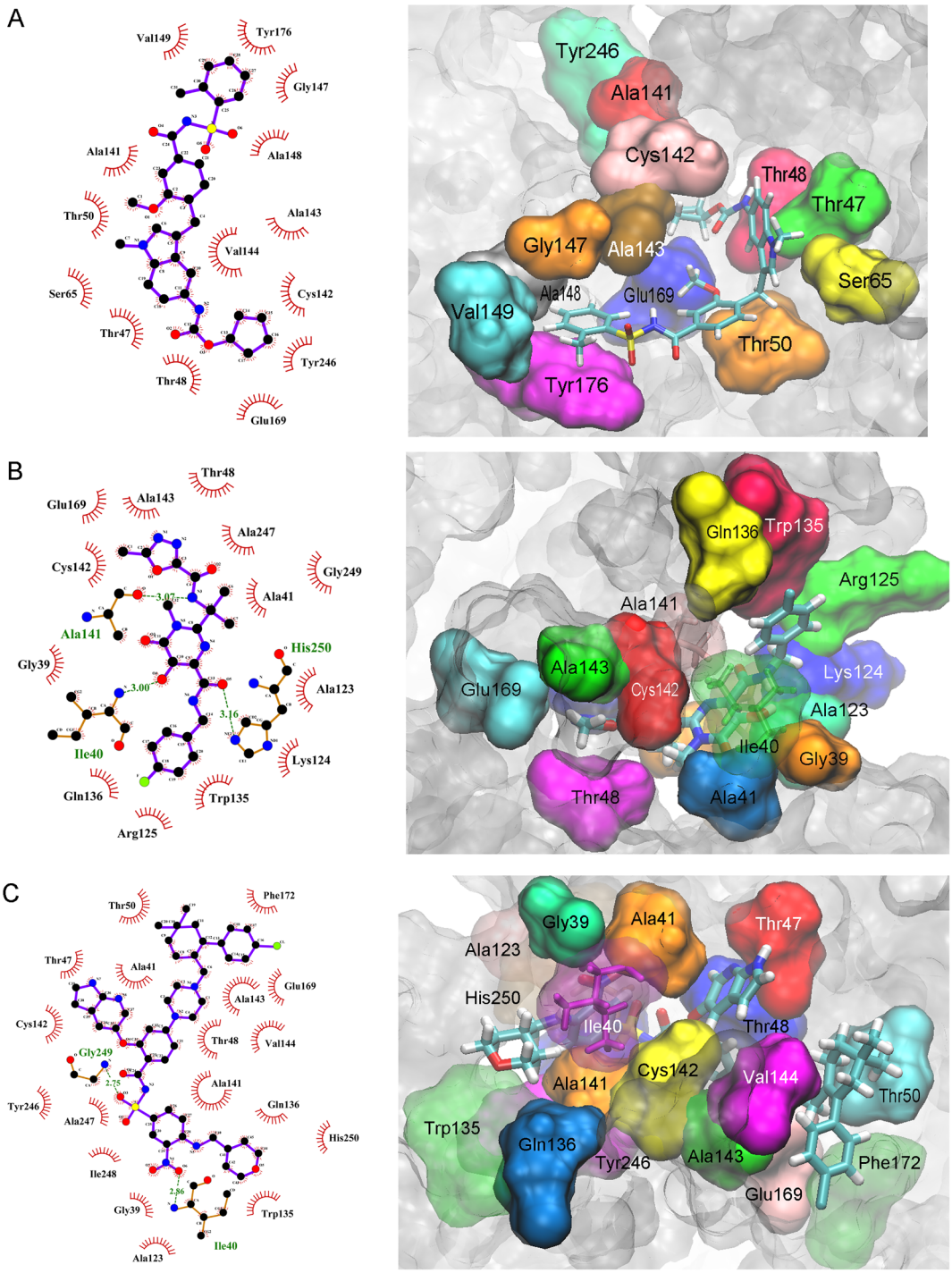

Fig. 3. Interaction thioredoxin reductase from *Candida albicans* (CaTrr1) with the six compounds selected by repurposing approach. The 2D-plot of interactions between amino acid residues of CaTrr1 and compound analyzed by LigPlot and the schematic drawing of tridimensional interaction obtained by VMD program. (A) zafirlukast; (B) raltegravir; (C) venetoclax (ABT-199); (D) glyburide; (E) gliquidone; (F) SR9243. All compounds interacted with the cysteines of the CaTrr1 catalytic site (Cys142 or Cys145). In tridimensional plot obtained by VMD, in the interactions mediated by hydrogen bonds the amino acid from CaTrr1 were presented by surface combinate with licorice representation. LigPlot: The interactions shown are those mediated by hydrogen bonds and by hydrophobic contacts. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

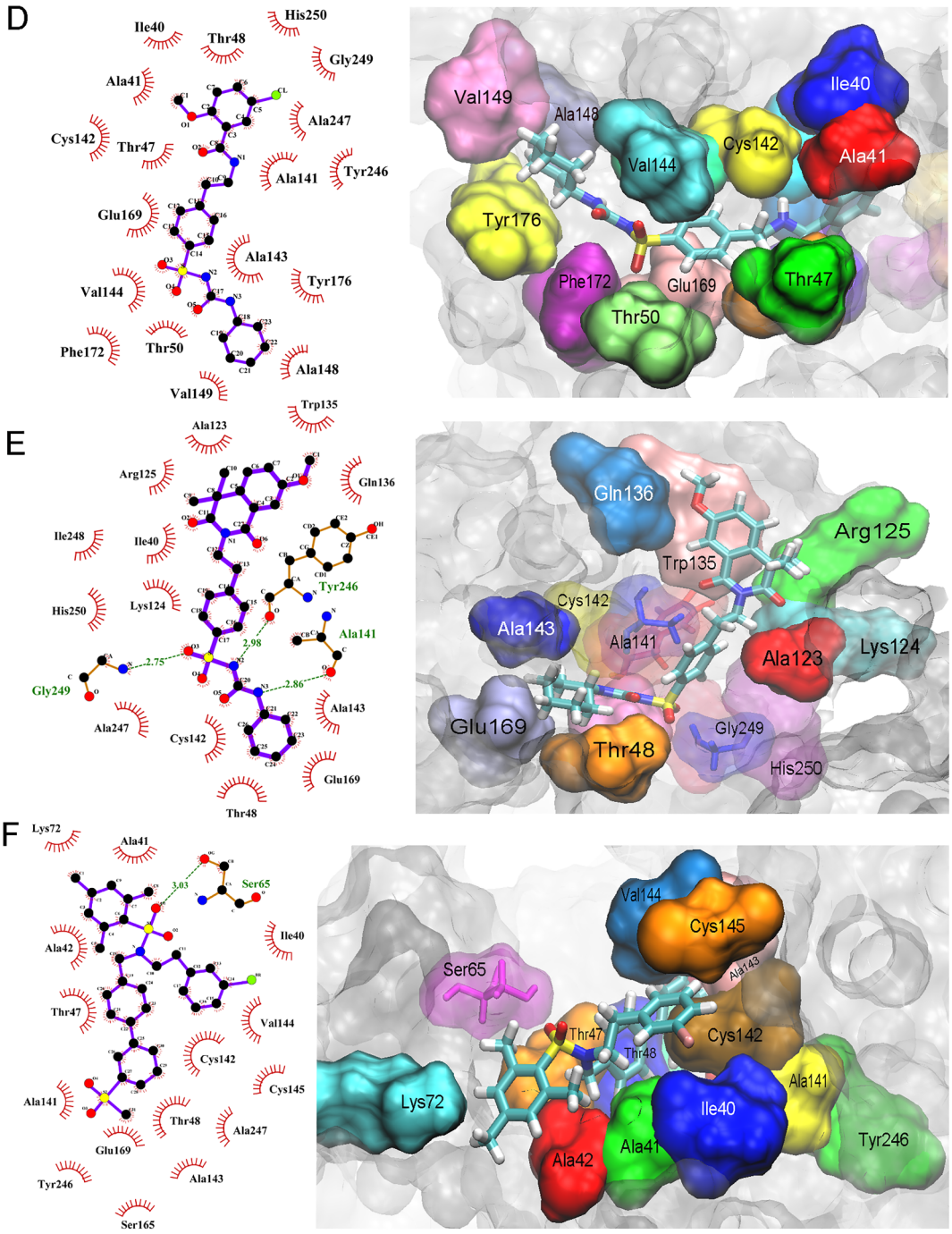

Fig. 3. (*continued*)

Table 3
In vitro antifungal activity of six compounds from drug repositioning.

Isolate	MIC (μg/ml)					
	Glyburide	Gliquidone	Zafirlukast	Raltegravir	Venetoclax (ABT-199)	SR9243
Candida albicans	> 256	> 256	> 256	128	> 256	> 256
Candida glabrata	> 256	> 256	> 256	256	> 256	> 256
Candida krusei	> 256	> 256	> 256	> 256	> 256	> 256
Candida tropicalis	> 256	> 256	> 256	256	> 256	> 256
C. parapsilosis	> 256	> 256	> 256	> 256	> 256	> 256
C. neoformans	> 256	> 256	> 256	> 256	> 256	> 256
P. brasiliensis	64	32	16	16	16	64
P. lutzii	> 256	64	32	32	64	> 256

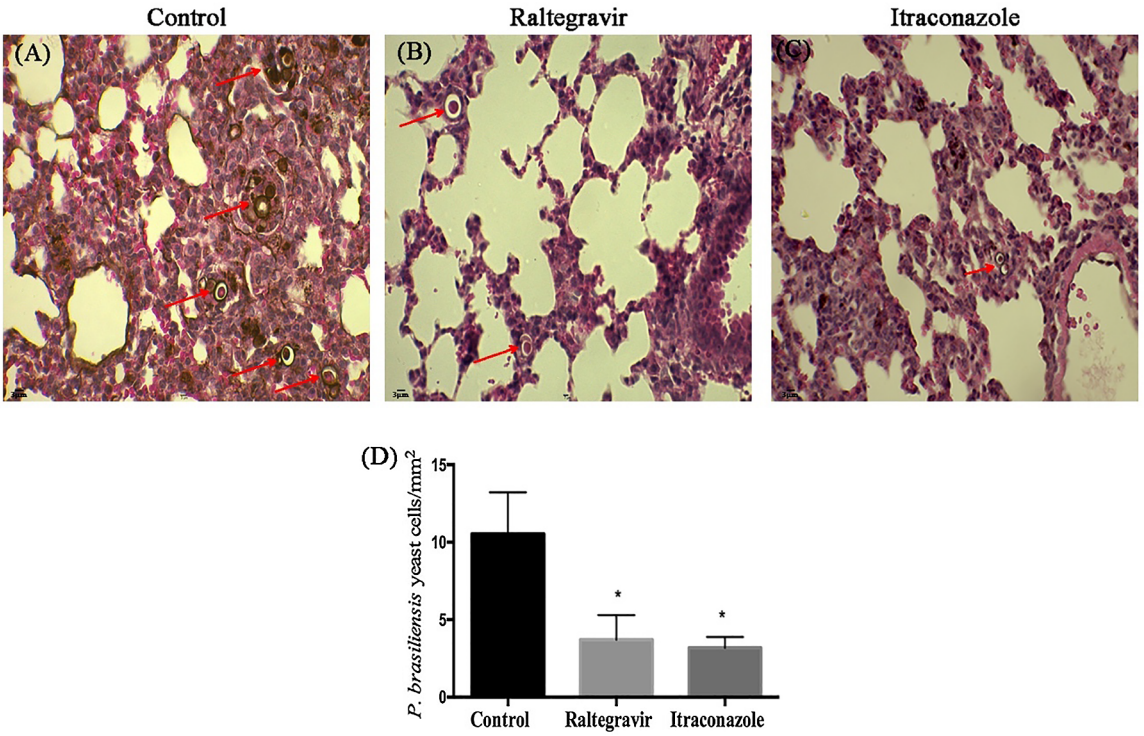

Fig. 4. Raltegravir as the efficient Paracoccidioidomycosis treatment. The mice were infected with yeast cells from *P. brasiliensis* (Pb18) by the lateral tail vein, and divided into three groups for treatment: Control (vehicle), itraconazole (5 mg/kg) and raltegravir (1 mg/kg). (A) Histological section of lung stained with Gomori & Grocott plus H&E. The control group showed many *P. brasiliensis* yeast cells (red arrow) and many changes in the pulmonary architecture. (B) The group treated with raltegravir showed few *P. brasiliensis* yeast cells (red arrow) and minor changes in the lung. (C) The group treated with itraconazole showed few *P. brasiliensis* yeast cells (red arrow) and minor changes in the lung (D) Yeast quantification in histological sections. The result shows the mean of the fungal cells found divided by the total lung area of the histological section (*P. brasiliensis* yeast cells/mm^2). $^*p < 0.05$ (Student's *t*-test). The samples were observed and photographed using a binocular light microscope (Motic BA310, Moticam 5 camera) at ×400 magnification.

Table 4
Interactions and GOLD values of three compounds with CaTrr1 and PlTrr1.

Compound	*C. albicans* model			*P. lutzii* model		
	GOLD	Cys/Cys	Loop 1	GOLD	Cys/Cys	Loop 1
Raltegravir	72.20	1/2	2*/10	67.34	2*/2	5/10
Zafirlukast	80.78	1/2	0/10	75.74	2/2	3*/10
Venetoclax	100.37	2/2	3*/10	90.99	2/2	4*/10

Cys/Cys: The number of interaction of the compound with cysteine from catalytic site. Loop 1: The number of interaction of the compound with amino acids from loop1, an important site for interaction with thioredoxin (substrate). * indicates that one of the interactions is the hydrogen bonding type.

asthma-treatment drugs, raltegravir and zafirlukast. Raltegravir also showed antifungal activity against *C. albicans* (MIC 128 μg/ml) and *C. glabrata* (MIC 256 μg/ml). None of the compounds showed antifungal activity against *Cryptococcus neoformans*.

3.3. Raltegravir as the promising PCM treatment

Due to the promising *in vitro* antifungal activity of raltegravir against fungi of the genus *Paracoccidioides*, this compound was selected for *in vivo* testing in the experimental model of systemic paracoccidioidomycosis. In this model, mice were infected with *P. brasiliensis* and treated for 15 days. After the treatment, the lungs of the mice were evaluated by histopathological analysis. As shown in Fig. 4A, the lungs of control animals clearly showed larger numbers of *P. brasiliensis* cells (red arrow) compared with the animals treated with raltegravir (1 mg/kg) or itraconazole (5 mg/kg) (Fig. 4B and C). The lung architecture of the animals treated with raltegravir or itraconazole was preserved, with few inflammatory infiltrates and few yeast cells distributed through the lung tissue (Fig. 4B). On the other hand, the control group, treated only with the vehicle, showed several inflammatory infiltrates and yeast cells, easily identified by impregnation with silver (Fig. 4A). As observed in Fig. 4D, raltegravir has similar behavior to itraconazole and was efficient in significantly reducing the yeast number in the lung, compared to the control group ($p < 0.05$).

3.4. Docking simulation against thioredoxin reductase from *Paracoccidioides lutzii (PlTrr1)*

Considering the better antifungal activity *in vitro* found for raltegravir, venetoclax and zafirlukast against *Paracoccidioides*, we performed the docking simulation using the three-dimensional model thioredoxin reductase from *P. lutzii*, previously constructed by homology modeling. Table 4 compares the interaction these compounds with two thioredoxin reductase models from *C. albicans* (CaTrr1) and *P. lutzii* (PlTrr1).

As observed, the GOLD values of the interaction with PlTrr1 were lower than with CaTrr1, but the three compounds made strong interactions (hydrogen bonds) both at the catalytic site and in the loop region of PlTrr1 (Supplementary materials). Figs. 5 and 6 in detail shows the interactions of raltegravir with CaTrr1 and PlTrr1, respectively, by ligplot and VMD. Interestingly, we can observe that raltegravir present interaction mediated by hydrogen bonds (strongly) with the Cys148 residue in catalytic site of PlTrr1 (Fig. 6C).

4. Discussion

Drug repositioning offers many benefits over *de novo* drug development; it significantly accelerates the process and reduces costs in the

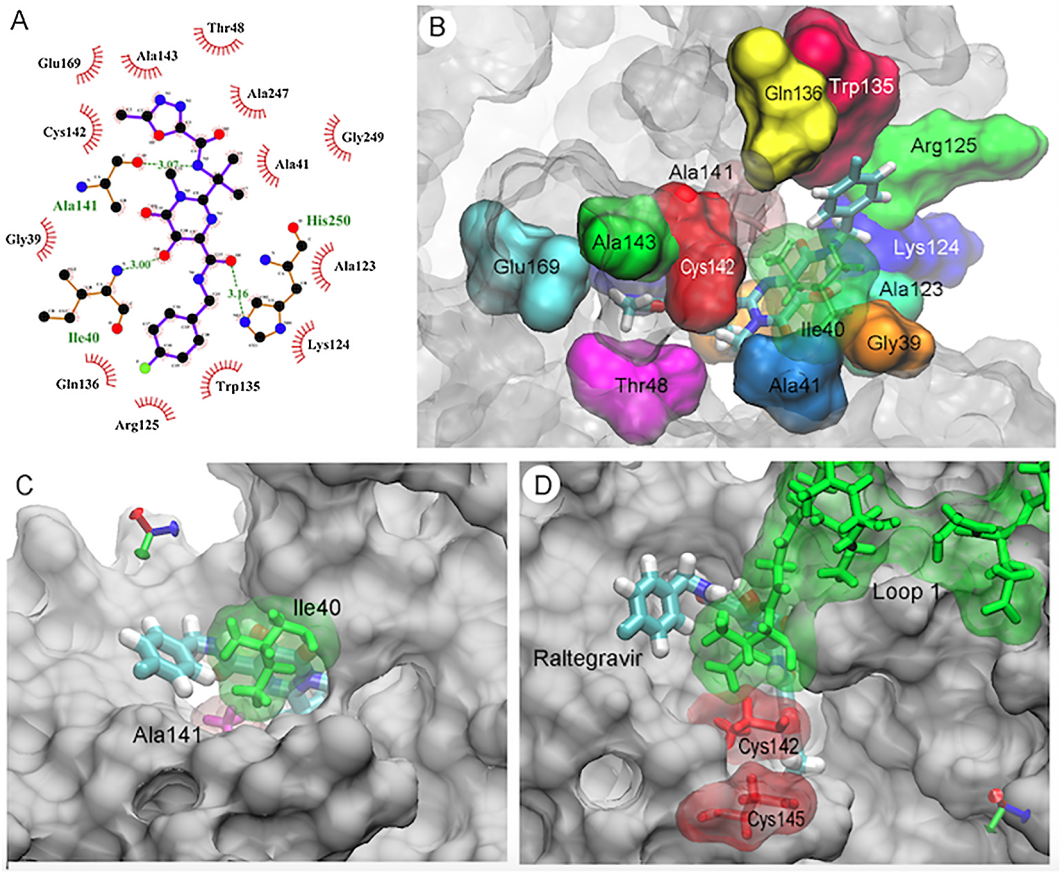

Fig. 5. Docking interaction of CaTrr1 with raltegravir. (A and B) Schematic drawing of interactions between active site residues of CaTrr1 and raltegravir, using LigPlot (A) and VMD (B). (C) The amino acids that interact with raltegravir through hydrogen bonds were designed by surface and licorice representation. (D) The raltegravir compound was able to interact with Cys142 from the CaTrr1 catalytic site (red) and the two amino acids from the loop1 region, important for interaction with the substrate: Gly39 and Ile40 (green). LigPlot: The interactions shown are those mediated by hydrogen bonds and by hydrophobic contacts. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

development pipeline, and is of great interest to academia and industry [35,49]. This approach has been a viable alternative for the discovery of new drugs against neglected diseases [37], such as paracoccidioidomycosis [50–52]. In this study, combined ligand-based and structure-based methods for drug repositioning were used.

The compounds available in three drug databases were evaluated for chemical similarity with two compounds, LMM5 and LMM11, previously identified as hit compounds for the development of new antifungals [17]. First, 100 compounds from each database were selected using the Tanimoto coefficient, a parameter frequently used in chemoinformatics, which has the advantages of conceptual simplicity, computational efficiency, and applicability to large compound databases [53–56]. We did not find a published standard value for the threshold for an acceptable or non-Tc value. Martin et al. [57] previously questioned the use of a value ≥ 0.85 of Tanimoto similarity for screening compound libraries. Since few compounds that are very active are above that threshold, these authors [57] suggested a rethinking of strategies for compound acquisition and design of combinatorial libraries. Vogt and Bajorath [58] concluded that there are generally no threshold values for similarity as an indication of the relationship between the reference compound and the database compounds. Some authors have used Tc ≥ 0.6 [59], others Tc ≥ 0.7 [60]. Given this lack of standardization, we chose not to define a threshold value for Tc, and to use the scaffold trees to filter the 100 compounds from each database, to select up to 10 that were most similar to LMM5 and LMM11.

The scaffold tree allows one to aggregate sets of molecules with a similar scaffold, and delineates the relationships among them based on the structural inclusion relationship [61,62]. In combinatorial libraries, the molecules are based on the common scaffolds, and moreover, the same synthesis route can be shared by compounds with the same scaffold [61]. Langdon et al. [63] used a scaffold tree for virtual screening of compound libraries, for the discovery of mitotic kinase inhibitors. A total of 17 compounds similar to LMM11 were selected by the scaffold trees, from the three databases. However, for the compound LMM5, this method was able to select similar compounds only in the MDDR database (two compounds).

The results of the ligand-based method were complemented with the use of the structure-based approach. Molecular docking has been used successfully in the search for new therapeutic options [18,64]. Although more virtual screening structure-based methods have been published than ligand-based, this latter approach identifies hits that are on average more potent [65]. Therefore, the combined use of these methods seems to be a promising approach for developing new drugs. Accordingly, the compounds that are chemically similar to the hits compounds and with affinity to the drug target (Trr1) were ranked by four parameters: the GOLD value, Tc, the ligand-target interaction, and availability for purchase. Six compounds were purchased for evaluation of *in vitro* antifungal activity. These compounds had four previously established therapeutic indications: anti-diabetic, anti-viral, anti-asthmatic and anti-tumor.

Holbrook et al. [66], showed that repurposed bromperidol derivatives inhibited *C. albicans* at MIC values > 32 μg/ml. Atorvastatin showed an MIC value of 256 μg/ml or higher against *C. gattii* [67]. Flubendazole, a benzimidazole, has potent *in vitro* activity against *C.*

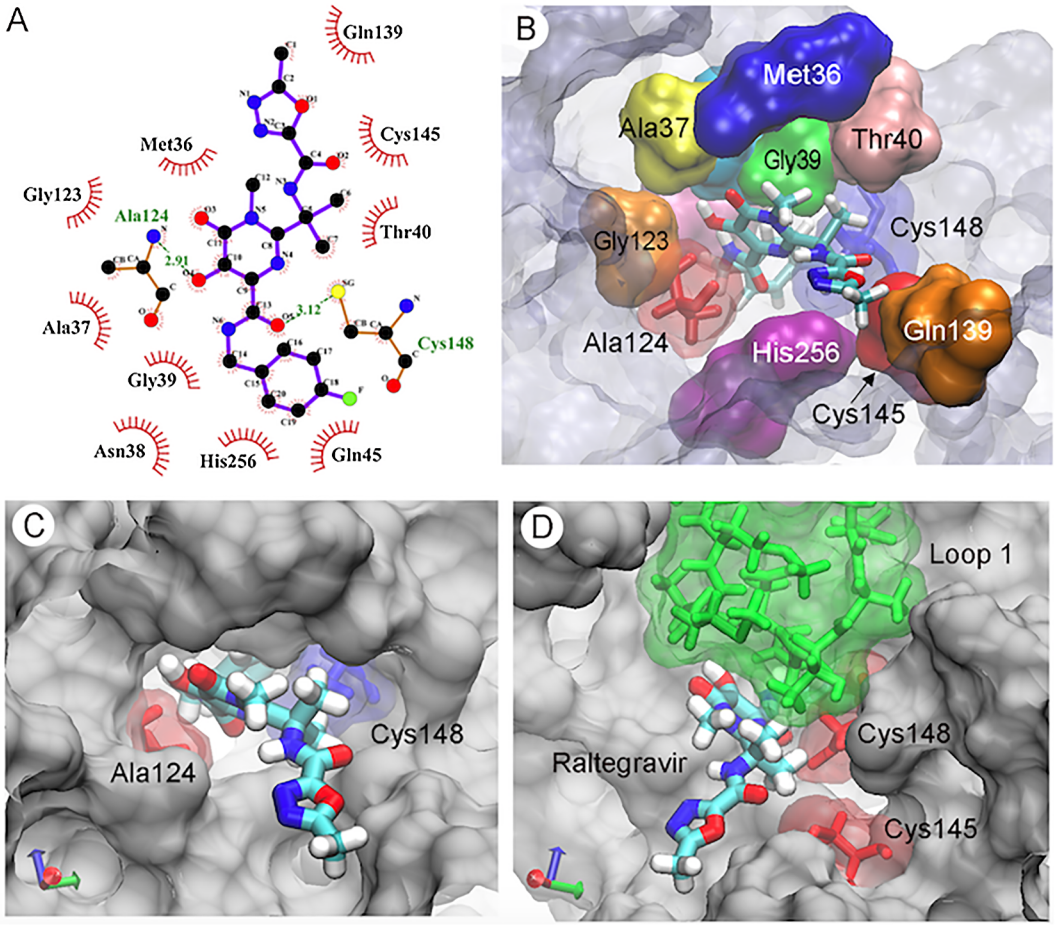

Fig. 6. Docking interaction of PlTrr1 with raltegravir. (A and B) Schematic drawing of interactions between active site residues of PlTrr1 and raltegravir, using LigPlot (A) and VMD (B). (C) The amino acids that interact with raltegravir through hydrogen bonds were designed by surface and licorice representation. Interestingly, raltegravir present interaction mediated by hydrogen bonds with the Cys148 catalytic site. (D) The compound was able to interact with Cys145 and Cys148 from the PlTrr1 catalytic site (red) and the five amino acids from the loop1 region (green), important for interaction with the substrate: Met36, Ala37, Gly39, Asn38 and Thr40. LigPlot: The interactions shown are those mediated by hydrogen bonds and by hydrophobic contacts. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating toward the ligand atoms they contact. The contacted atoms are shown with spokes radiating back (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

neoformans, with a modal MIC of 0.125 μg/ml [68]. In our *in vitro* evaluation of antifungal activity, the genus *Paracoccidioides* was inhibited by the six compounds evaluated, with low MIC values (16–64 μg/ml) (Table 3). Interestingly, the compound with antiviral activity showed the most promising results for antifungal activity, based on the *in vitro* susceptibility test. Structure-based techniques were able to select compounds with promising antifungal activity against fungi of the genus *Paracoccidioides*, with MIC values of 8–32 μg/ml [18]. These compounds interact with Trr from *P. brasiliensis* in amino-acid residues at the active site, such as Cys145 and/or Cys148. In our study, the six compounds also interacted with CaTrr1 in amino-acid residues at the active site, Cys142 and/or Cys145 (Fig. 3). The results obtained through the docking simulation using thioredoxin reductase from *P. lutzii* corroborated the antifungal activity of raltegravir, venetoclax and zafirlukast. Even with GOLD values lower than with CaTrr1, the strong interactions that the three compounds make with the catalytic site and region loop of PlTrr1 contribute to justify the better activity of these compounds in *Paracoccidioides*. Since, this flavoenzyme participates in the protection against oxidative damage [14,15], we believe that Trr1 blockade by compound could cause the accumulation of the reactive oxygen species in the fungal cell and consequently an important damage cellular.

This promising approach was able to select raltegravir for the preliminary *in vivo* validation tests against systemic paracoccidioidomycosis. The number of antifungal agents for paracoccidioidomycosis is limited and the search for new compounds has intensified; however, few studies with compounds identified from drug repositioning for the treatment of this disease are found in the literature [50,52]. On October 16, 2007, the US Food and Drug Administration (FDA) approved raltegravir (isentress®), a new class of antiretroviral drugs developed by Merck & Co., Inc., which functions as an inhibitor of HIV-1 integrase, a viral enzyme that catalyzes an essential process in the viral replication cycle, i.e., the insertion of HIV-1 proviral DNA into the host genome [69].

Raltegravir, as well as LMM5 and LMM11, is a member of the class of 1,3,4-oxadiazoles [70] and, curiously, was also discovered using *in silico* methods through simulation of molecular dynamics [71]. The *in vivo* antifungal activity demonstrated that raltegravir has similar behavior to itraconazole and significant reduction of the fungal burden in lung as well as only a few discrete changes in lung structure compared with the non-treated control. Compounds, as well as the raltegravir in this study, that share antiviral and antifungal activity have previously been reported. Studies have shown that antifungal agents available on the market, itraconazole [72,73], posaconazole [74] and micafungin [75], also have antiviral activity. Tamoxifen, used in treatment of estrogen receptor-positive breast cancer, showed antifungal activity against *C. neoformans* and antiviral activity against the hepatitis C virus [76].

5. Conclusions

Virtual screening using the complementary ligand-based and structure-based methods was effective, and can be used in drug repositioning through similarity with hits compounds, to search for drugs with antifungal activity. Our results suggest that raltegravir may be of interest for paracoccidioidomycosis treatment.

6. Contributors

I.R.G.C. E.S.K. T.I.E.S. B.M. were involved in the study design presented in this manuscript. Performed the experiments: I.R.G.C. D.R.F. K.M.S. F.A.V.R. P.S.B.M. E.S.K. Analyzed the data: I.R.G.C. T.C.A.B. E.S.K. T.I.E.S. B.M. Contributed reagents/materials/analysis tools: E.S.K. T.C.A.B. T.I.E.S. B.M. The manuscript and all associated documents were produced by I.R.G.C. E.S.K. T.I.E.S. B.M. with the assistance of all other co-authors listed.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.11.019.

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