Amlodipine and lufenuron as repurposing drugs against *Sporothrix brasiliensis* (#89950)

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Amlodipine and lufenuron as repurposing drugs against *Sporothrix brasiliensis*

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Background. Sporotrichosis caused by *Sporothrix brasiliensis* is a globally emerging infectious disease with limited therapeutic options. Thus, we aimed to evaluate the *in vitro* activity of amlodipine (AML) and lufenuron (LUF) alone and their interaction with itraconazole (ITZ), the first-choice drug against *S. brasiliensis.*

Methods. Twenty clinical isolates of *S. brasiliensis* from two hyperendemic regions were tested through a microdilution assay to evaluate the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of AML and LUF. Checkerboard assay was performed with 10 isolates for both drug interactions with ITZ.

Results. AML showed inhibitory and fungicidal activity against all isolates included, with MIC values ranging from 32 to 256 µg/mL, and MFC from 64 to 256 µg/mL. However, none of the *S. brasiliensis* isolates were inhibited by the highest soluble concentration of LUF (MIC > 64 µg/mL for all strains). Synergic interaction of AML and LUF with ITZ occurred in 50% and 40% of the isolates tested, without any antagonistic effects.

Conclusion. Both repurposing drugs evaluated in our study showed a promising *in vitro* activity, especially in synergy with ITZ against *S. brasiliensis*, warranting future investigations.

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Abstract

Background. Sporotrichosis caused by *Sporothrix brasiliensis* is a globally emerging infectious disease with limited therapeutic options. Thus, we aimed to evaluate the *in vitro* activity of amlodipine (AML) and lufenuron (LUF) alone and their interaction with itraconazole (ITZ), the first-choice drug against *S. brasiliensis*.
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KEYWORDS: antifungal, *Sporothrix* spp., zoonosis, pre-clinical tests.

Introduction

Sporotrichosis caused by *Sporothrix brasiliensis* poses a severe public health challenge in Brazil and had spread to other countries over the last decade, emerging as a global infectious disease (Rabello et al., 2022; Gómez-Gaviria et al., 2023). Distinct genotypic profiles were shown to coexist in the major Brazilian hyperendemic areas, with *S. brasiliensis* isolates from Rio de Janeiro state (RJ) differing from those from Rio Grande do Sul state (RS) (Rodrigues et al., 2013; Losada et al., 2023; Spruijtenburg et al., 2023).

One essential aspect to control sporotrichosis cases is the treatment of different hosts, mainly infected cats. Since the arsenal of approved drugs to treat sporotrichosis is limited to four antifungals (itraconazole, terbinafine, potassium iodide and amphotericin B) that are associated with many adverse effects, drug repurposing is a promising field of study, and the evaluation of new antifungal compounds for this mycosis requires considering distinct genotypic profiles of *S. brasiliensis*, to account for potential variations in the susceptibility of these different strains (de Souza et al., 2018; Poester et al., 2022).

Amlodipine is a calcium channel blocker drug known to inhibit efflux pumps, which is an interesting mechanism of action against fungi and other microorganisms (Coelho et al., 2015; Homa et al., 2017; Nakasu et al., 2022). Thus, studies that evaluate the activity of this drug against *S. brasiliensis*, both alone and in combination with itraconazole (ITZ), which is first-choice drug for sporotrichosis are requested. Similarly, lufenuron, an anti-ectoparasite drug that acts on chitin, presents potential as a topical treatment for *S. brasiliensis*, as chitin is also present in the fungal cell wall (Moriello, 2004; Rust, 2005). Pre-clinical studies with these both drugs against *S. brasiliensis* was not described, therefore, our study aims to evaluate their *in vitro* activity, alone and in combination with ITZ, against *S. brasiliensis* strains from two different genotypes.

Materials & Methods

Twenty isolates of *S. brasiliensis* were included in the study, with nine originating from RJ, 10 from RS, and type strain (CBS 120339) also isolated in RJ. All clinical isolates were stored in the mycological collections from the participants laboratories (Mycology Laboratory from *Universidade Federal do Rio Grande* - FURG and Mycology Laboratory from *Instituto Nacional de Infectologia Evandro Chagas* - *Fundação Oswaldo Cruz* - Fiocruz). They have been previously identified by a species-specific PCR (Rodrigues et al., 2015). To evaluate the genotype of isolates, eight strains were genotyped by partial sequences of the translation elongation factor-1 alpha (EF1α) and the calmodulin gene (CAL), following the PCR conditions described by Marimon et al. (2007) and Rodrigues et al. (2013), respectively. Automated sequencing was done using the FIOCRUZ Technological Platforms and the sequences were edited by Sequencher Software Package (version 4.9). Phylogenetic analyses were carried out using maximum likelihood method, and trees were constructed using MEGA 6 (Tamura et al., 2013), confidence values were performed using 1000 bootstrap replicates and they were shown next to the branches (Felsenstein, 1985). *S. brasiliensis* sequence from this study was deposited

at GenBank (numbers: OQ865503, OQ865516, KC576606, AM116899, OQ865505, OQ865518, OQ865506, OQ865519, OQ865507, OQ865520, OQ865508, OQ865521, OQ865509, OQ865522, OQ865510, OQ865523, KC576614, AM117437, KC576608, AM116908, KC576615, AM747302, KC576611, AM398393, KC576612, AM398396, MW066427, MW075142) sequences belonging to the others *Sporothrix* species deposited at GenBank were included in the phylogenetic analysis and *Ophiostoma pallidulum* was used as outgroup. The haplotype network was built with the software Network 10.2.0.0 using the Median-joining networks method (Polzin and Daneschmand, 2003), gaps and missing data were excluded from the analysis.

Drugs were obtained commercially and include ITZ (Sigma-Aldrich®, San Luis, Missouri, EUA), amlodipine (Valdequimica®, São Paulo, Brazil), and lufenuron (Copervet®, Minas Gerais, Brazil). These drugs were diluted and stored as stock solutions in dimethyl sulfoxide, 51.200 µg/mL to amlodipine and 6.400 µg/mL to lufenuron and ITZ.

The *in vitro* activities of drugs were evaluated through the microdilution assay, following the M38-A2 protocol from the Clinical and Laboratory Standards Institute (CLSI, 2008). The solubility of lufenuron and amlodipine in RPMI 1640 medium was tested to define their highest testable concentration, resulting in a range of 1 to 64 µg/mL and 8 to 512 µg/mL, respectively. Isolates from seven days on potato dextrose agar (PDA) (Kasvi®, São José dos Pinhais, Paraná, Brazil) with their concentration adjusted to 0.8×10^4 to 10^5 colony-forming units (CFU) per mL by spectrophotometry (530 nm). To confirm the inoculum concentration, the pour-plate technique was performed and colonies were counted after seven days of incubation. A standardized solution of inoculum and drug stock solutions were diluted in RPMI 1640 medium and distributed into 96-well polystyrene plates (100 µl of inoculum and 100 µl of drug dilutions). The microplates were then incubated for 72 hours at 35 °C. Visual readings were made to determine the minimal inhibitory concentration (MIC) of each drug, defined as the concentration that completely inhibited fungal growth. In addition, the minimal fungicidal concentration (MFC) was evaluated through plating 50 µl of each well without visual growth on PDA. The MIC/MFC50, MIC/MFC90 (concentration able to inhibit/kill 50 and 90% of the isolates, respectively), and geometrical means (GM) were calculated.

Ten isolates of the twenty (four from RJ, five from RS - randomly selected, and the S. brasiliensis type strain) were used for drug interaction evaluation (amlodipine + ITZ or lufenuron + ITZ) by a checkerboard assay (Eliopoulos & Moellering, 1991; Poester et al., 2020). The concentrations of repurposing drugs and test conditions were performed as described above, and ITZ was tested in concentrations from 0.03125 to 8 µg/mL. ITZ MIC values were classified as wild-type (<2 µg/ml) or non-wild-type (≥2 µg/ml) using the Epidemiological Cutoff Values (ECVs) described by Espinel-Ingroff et al., (2017). The fractional inhibitory concentration index (FICi) was determined to classify the drug associations as follows: strong synergism (SS) when FICi < 0.5, weak synergism (WS) when 0.5 < FICi <1, additive (AD) when 1 <FICI <2, indifferent (IND) when FICi = 2, and antagonistic (ANT) when FICi >2.

Results

Eight *S. brasiliensis* isolates included genotype analyses were separated into two distinct groups, according to the haplotype network constructed using the concatenated EF1α and CAL sequences. These genotypes separated isolates from RJ (genotype H1) and RS (genotype H2 and H3) (Figure 1).

Figure 1. (A) Phylogenetic relationships of *Sporothrix brasiliensis* from Rio de Janeiro (RJ) and Rio Grande do Sul (RS) states generated by maximum likelihood using partial nucleotide sequences of the translation elongation factor-1 alpha (EF1α) and the calmodulin gene (CAL). Indices of support based on 1000 bootstrap replications added to respective branches. (B) Haplotype network of *S. brasiliensis* from RJ and RS generated by Median-joining based on partial nucleotide sequences of EF1α and the CAL. The size of the circumference is proportional to the haplotype frequency. Black dots represent median vectors. The numbers around each vertex represent the amount of mutations separating each haplotype.

Figure 2 summarizes the MIC results of the three drugs herein tested. In brief, ITZ exhibited MIC values ranging from 0.125 to 1 µg/mL (MIC50 and MIC90 of 1 µg/mL), The GM of ITZ MIC values for RJ and RS isolates was 1 and 1.19 µg/mL, respectively. Additionally, two isolates (one from RJ and the other from RS) were classified as non-wild type, showing MIC values >8 µg/mL for this azole (Figure 2).

Amlodipine showed both inhibitory and fungicidal activity against all isolates (*n*=20) with MIC values ranging from 32 to 256 µg/mL (MIC50 and MIC90 of 128 µg/mL). The GM of MIC values for RJ and RS isolates was 97 and 103.97 µg/mL respectively. MFC values ranged from 64 to 256 µg/mL (MFC50 of 128 µg/mL, and MFC90 of 256 µg/mL), GM of 194.01 µg/mL and 157.59 µg/mL to RJ and RS isolates, respectively. In contrast, lufenuron did not inhibit any *S. brasiliensis* isolates, with MIC values higher than 64 µg/mL for all strains.

Figure 2. Results of the *in vitro* susceptibility of 20 *Sporothrix brasiliensis* isolates from Rio de Janeiro (RJ) and Rio Grande do Sul (RS) states to amlodipine, lufenuron and itraconazole. MIC: Minimal inhibitory concentration; MFC: Minimal fungicidal concentration.

Regarding the interaction of drugs with ITZ, when in association with amlodipine, a beneficial interaction was observed in 60% of cases (10% SS, 40% WS, and 10% AD), while 40% showed indifference. In association with lufenuron, 40% WS was found, and 60% of isolated showed indifference (Table 1).

Table 1. Results of the *in vitro* susceptibility of 10 *Sporothrix brasiliensis* isolates to amlodipine (AML) and lufenuron (LUF) in combination with itraconazole (ITZ).

Discussion

Our study showed the *in vitro* activity of two repurposing drugs, either alone or in association with ITZ, against the pathogenic species *S. brasiliensis*. These isolates were obtained from clinical cases in the two main sporotrichosis hyperendemic regions in Brazil (Gremião et al., 2020; Munhoz et al., 2022; Losada et al., 2023; Spruijtenburg et al., 2023). Sporotrichosis represents a severe public health problem in Brazil and currently it is emerging as a global concern. Repurposing drugs offer a promising area of investigation into the field of treating this disease, since they already have pharmacological information available, reducing the time needed to develop and discover new therapies, which would contribute to a better control of this disease.

Amlodipine demonstrated inhibition and killing of all included isolates, and its activity was further increased when combined with ITZ, the drug of choice for sporotrichosis. A promising antifungal activity of this drug was also showed in combination with fluconazole against *Candida albicans*, changing the resistance status of strains to the azole drug (Liu et al., 2016). Unfortunately, this was not observed with the two ITZ non-wild-strains included in this study. Similarly, amlodipine in combination with the salt besylate showed promising results in inhibiting and killing *C. albicans* and *C. glabrata*, with MIC and MFC values ranging from 8 to 512 µg/mL, and also showing activity in inhibiting the biofilm formation of these fungal pathogens (Gupta et al., 2016). Regarding toxicity of amlodipine, genotoxicity was suggested, but not conclusively proven, and cytotoxicity was observed only at higher doses (204.44 µg/mL) than the MIC90 value (128 µg/mL) found in our study (Zheng et al., 2010; Salih et al., 2022).

Lufenuron, which has been proposed as a compound to treat dermatophyte infections in animals (Moriello, 2004), did not demonstrate inhibitory activity against *S. brasiliensis* in our study. However, its topical application may complement systemic ITZ therapy for sporotrichosis, since a beneficial effect of its *in vitro* association with ITZ was shown in our study for some isolates.

While our study showed a similar susceptibility profile between the RJ and RS isolates tested regarding their susceptibility to amlodipine, lufenuron, and ITZ, it is important to highlight the necessity to include genotypically diverse *Sporothrix* isolates in all studies aiming to discover new antifungal drugs or to test susceptibility of commercial approved drugs. In fact, when comparing the treatment of human patients from RJ and RS, higher doses of ITZ were required to achieve clinical cure in patients from the RS hyperendemic area compared to those from RJ (Barros et al., 2011; Poester et al., 2022). Our selection of isolates from RJ and RS considered that the Brazilian hyperendemic originated predominantly from these two epidemiological sources, which probably underwent clonal dispersion to other states (Losada et al., 2023; Spruijtenburg et al., 2023).

Conclusions

Given the urgent need for more therapeutic options to control the high dissemination of sporotrichosis, our study is pioneering in showing the activity of two repurposing drugs alone

and/or in association with ITZ against *S. brasiliensis* from two epidemiological sources in Brazil. Therefore, it instigates further pre-clinical studies (both *in vitro* and *in vivo*) with both repurposing drugs herein evaluated. These studies hold the potential to advance the development of new treatment strategies for this challenging infectious disease.

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Table 1(on next page)

Results of the in vitro susceptibility of 10 Sporothrix brasiliensis isolates to amlodipine (AML) and lufenuron (LUF) in combination with itraconazole (ITZ).

Table 1. Results of the *in vitro* susceptibility of 10 *Sporothrix brasiliensis* isolates to amlodipine (AML) and lufenuron (LUF) in combination with itraconazole (ITZ).

		MIC*					MIC				
FURG ID	**Source**	**ANL alone**	**ANL comb**	**ITZ alone**	**ITZ comb**	**IN****	**LUF alone**	**LUF comb**	**ITZ alone**	**ITZ comb**	**IN****
716	RS	64	32	1	0.25	WS	>64	16	1	0.5	WS
1078	RS	32	8	1	0.5	WS	>64	>64	1	1	IND
1878	RS	64	8	0.5	0.125	SS	>64	>64	0.5	0.5	IND
3952	RS	64	64	>8	>8	IND	>64	>64	>8	>8	IND
5150	RS	64	32	1	0.25	WS	>64	16	1	0.5	WS
9011	RJ	64	64	0.5	0.5	IND	>64	>64	0.5	0.5	IND
9013	RJ	32	32	1	1	IND	>64	4	1	0.5	WS
9014	RJ	128	128	>8	>8	IND	>64	>64	>8	>8	IND
9015	RJ	32	8	0.5	0.25	WS	>64	1	0.5	0.25	WS
9017	ATCC	64	32	1	0.5	AD	>64	>64	1	1	IND

FURG ID: Isolate identification of *Universidade Federal do Rio Grande*; MIC: Minimal inhibitory concentration; comb: MIC of each drug when used in combination; IN: Interpretation. *MIC expressed as µg/mL. **IN: <0.5 strong synergism (SS); 0.5–<1 weak synergism (WS); 1–<2 additive (AD); 2 indifferent (IND); >2 antagonism (AN).

Figure 1

Figure 1. Phylogenetic relationships and Haplotype network of *Sporothrix brasiliensis* from Rio de Janeiro (RJ) and Rio Grande do Sul (RS) states

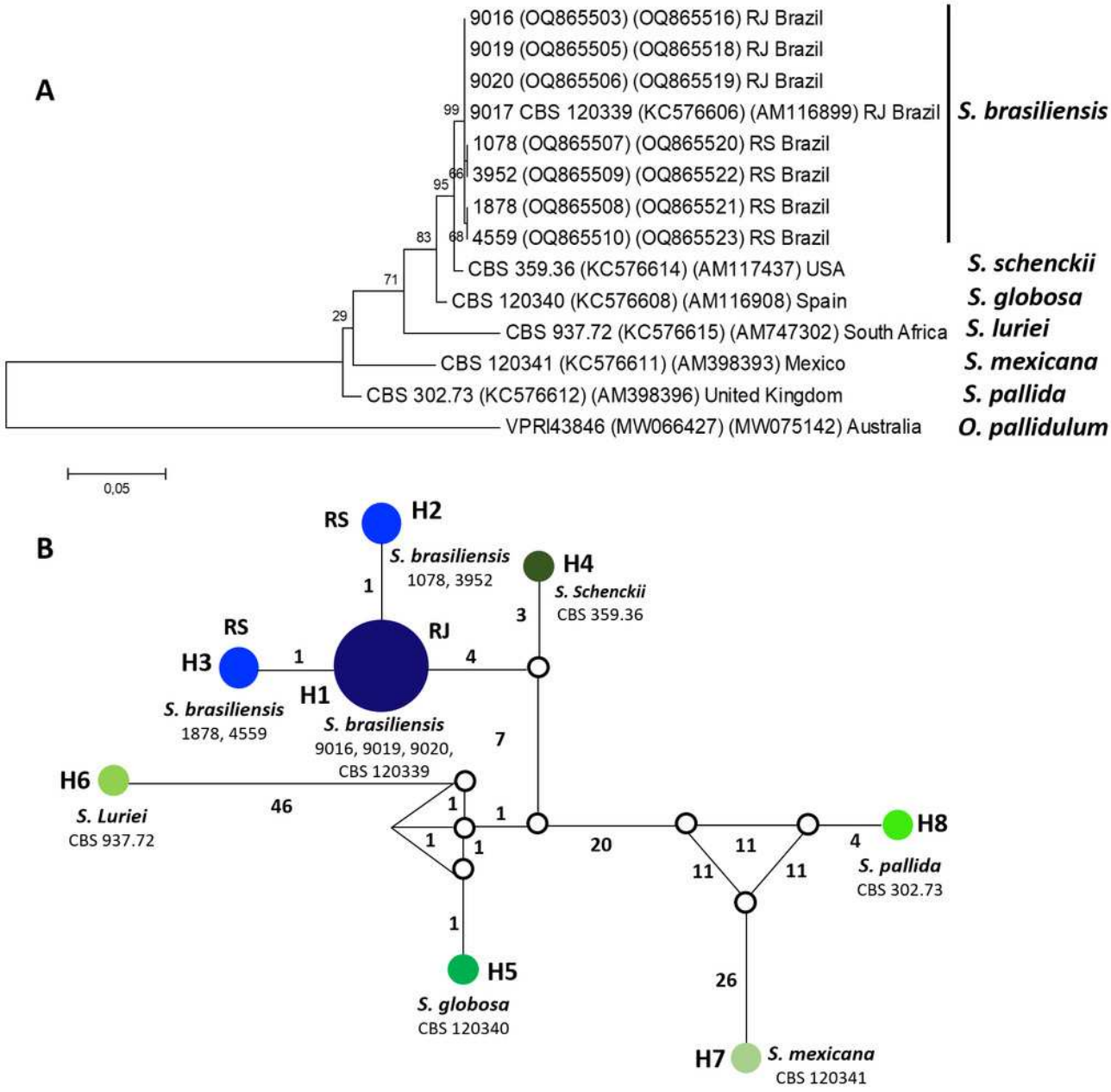

Figure 2

Figure 2. Results of the *in vitro* susceptibility of 20 *Sporothrix brasiliensis* isolates from Rio de Janeiro (RJ) and Rio Grande do Sul (RS) states to amlodipine, lufenuron and itraconazole