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Eumycetoma causative agents are inhibited in vitro by luliconazole, lanoconazole and ravuconazole

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Abstract

Introduction: Eumycetoma is a subcutaneous mutilating disease that can be caused by many different fungi. Current treatment consists of prolonged itraconazole administration in combination with surgery. In many centres, due to their slow growth rate, the treatment for eumycetoma is often started before the causative agent is identified. This harbours the risk that the causative fungus is not susceptible to the given empirical therapy. In the open-source drug program MycetOS, ravuconazole and luliconazole were promising antifungal agents that were able to inhibit the growth of *Madurella mycetomatis*, the most common causative agent of mycetoma. However, it is currently not known whether these drugs inhibit the growth of other eumycetoma causative agents.

Materials and methods: Here, we determined the in vitro activity of luliconazole, lanoconazole and ravuconazole against commonly encountered eumycetoma causative agents. MICs were determined for lanoconazole, luliconazole and ravuconazole against 37 fungal isolates which included *Madurella* species, *Falciformispora senegalensis*, *Medicopsis romeroi* and *Trematosphaeria grisea* and compared to those of itraconazole.

Results: Ravuconazole, luliconazole and lanoconazole showed high activity against all eumycetoma causative agents tested with median minimal inhibitory concentrations (MICs) ranging from 0.008–2 μg/ml, 0.001–0.064 μg/ml and 0.001–0.064 μg/ml, respectively. Even *Ma. fahalii* and *Me. romeroi*, which are not inhibited in growth by itraconazole at a concentration of 4 μg/ml, were inhibited by these azoles.

Conclusion: The commonly encountered eumycetoma causative agents are inhibited by lanoconazole, luliconazole and ravuconazole. These drugs are promising candidates for further evaluation as potential treatment for eumycetoma.

KEYWORDS
Eumycetoma, in vitro susceptibility, itraconazole, lanoconazole, luliconazole, ravuconazole

1 | INTRODUCTION

Eumycetoma is a progressive destructive inflammatory neglected tropical infection, characterised by large painless tumorous subcutaneous lesions, the formation of multiple sinuses and the discharge of grains. It is caused by fungi mainly belonging to the fungal orders *Sordariales* and *Pleosporales*.[1–3] The most common eumycetoma causative agent, *Madurella mycetomatis*, belongs to the order of the *Sordariales*. *Falciformispora senegalensis*, *Trematosphaeria grisea* and *Medicopsis romeroi* belong to the order *Pleosporales*.[3–5]

In endemic areas, the diagnosis of mycetoma is often performed clinically which often results in misdiagnosis.[6,7] Only in larger hospitals or reference centres, identification of the causative agent to the species level may be possible.[4] Identification is usually based on histological features of the mycetoma grain and the morphology of the cultured fungal isolate. Due to the slow growth rate, it takes at least 4–6 weeks till identification.[7] Therefore, in some centres, treatment is often started before the causative agent has been identified. Thus, susceptibility of most or all causative agents for the empirically prescribed drug would be a big step forward.

Currently, the recommended therapy for eumycetoma consists of antifungal therapy combined with surgery.[8] Itraconazole is currently used as the standard antifungal agent,[4] a drug for which *Ma. mycetomatis* is susceptible but for which *Me. romeroi* and *Madurella fahalii* were found to be intrinsically resistant.[9] This standard treatment has a poor success rate (<30%) and a high recurrence rate (27%), resulting in amputations in 2.8% of cases.[10] Furthermore, due to the costs and side effects, more than half of the patients (54%) are lost to follow-up.[4,11,12]

In the open-source drug discovery program MycetOS, we recently demonstrated that two novel azoles, ravuconazole and luliconazole, were able to inhibit the growth of *Ma. mycetomatis* at very low concentrations and more importantly, were also able to prolong the survival of *Ma. mycetomatis* infected *Galleria mellonella* larvae.[13,14] Ravuconazole is a broad-spectrum triazole that showed potent activity against a wide range of fungal species, including *Aspergillus* spp., *Candida* spp. and *Ma. mycetomatis*.[15] Luliconazole is a newly FDA approved topical imidazole for the treatment of superficial mycoses such as tinea pedis, tinea cruris, tinea corporis and onychomycosis.[16–21] It also has broad in vitro activity against other non-dermatophyte fungal pathogens such as *Candida spp*., *Cryptococcus neoformans*, *Malassezia spp*, *Fusarium spp*. and *Aspergillus spp*. with MICs often lower than the standard drug of choice.[18,19,22–24] An optically related compound of luliconazole is lanoconazole,[25] which only differs in being racemic while luliconazole is an R-enantiomer. It is, therefore, most likely also active against *Ma. mycetomatis*. Due to the high potency of ravuconazole against *Ma. mycetomatis*,[15] fosravuconazole, the prodrug of ravuconazole, is currently in a clinical trial in eumycetoma patients infected by *Ma. mycetomatis* (https://clinicaltrials.gov/ct2/show/NCT03086226) in Sudan.[26]

Based on the in vivo efficacy of ravuconazole and luliconazole against *Ma. mycetomatis* in the *Galleria mellonella* larvae model, the suspected high potency of lanoconazole, and the fact that treatment for eumycetoma is often started without knowing the causative agent, it is important to establish whether other eumycetoma causative agents are also susceptible for these antifungal agents. Therefore, in this study, we evaluated the in vitro activity of ravuconazole, luliconazole and lanoconazole against *Ma. mycetomatis*, *Madurella pseudomycetomatis*, *Madurella tropicana* and *Ma. fahalii* as representatives of eumycetoma causative agents from the order *Sordariales* and *F. senegalensis*, *T. grisea* and *Me. romeroi* from the order *Pleosporales*.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates and growth conditions

Isolates consisting of *Ma. mycetomatis* ($n = 10$), *Ma. pseudomycetomatis* ($n = 6$), *Ma. tropicana* ($n = 3$), *Ma. fahalii* ($n = 2$), *F. senegalensis* ($n = 6$), *Me. romeroi* ($n = 6$) and *T. grisea* ($n = 4$) were included in this study. The isolates were originally obtained from the Mycetoma Research Center (Khartoum, Sudan), Hospital General de México Dr Eduardo Liceaga (Mexico City, Mexico), the Westerdijk Fungal Biodiversity Centre (CBS) (Utrecht, the Netherlands) and maintained in the ErasmusMC University Medical Center (Rotterdam, the Netherlands). The isolates were previously identified by sequencing the internal transcribed spacer (ITS rDNA) region, ribosomal binding protein II (RBPII) and β-tubulin sequence.[27,28] Fungal isolates were grown in Sabouraud's dextrose agar (SDA) for 3 weeks at 37°C for the *Madurella* species and room temperature (RT) for the other species.

2.2 | Antifungal agents

The following antifungal agents were used: itraconazole (Janssen Pharmaceutical Beerse, Belgium), lanoconazole (Sigma Aldrich), luliconazole (Sigma Aldrich) and ravuconazole (Eisai Co., Ltd). Prior to susceptibility testing, all antifungal agents were dissolved in DMSO with a twofold dilution range of 0.001–0.5 μg/ml for luliconazole and lanoconazole, and 0.002–4 μg/ml for itraconazole and ravuconazole.

2.3 | In vitro susceptibility testing

For in vitro susceptibility testing, experiments were performed as previously described.[29] Briefly, approximately 3 cm fungal mycelia were transferred to 15 ml RMPI 1640 medium (Lonza) supplemented with 0.35 g/L L-glutamine and 1.98 mM 4-morpholinepropanesulfonic acid (MOPS). This was followed by sonication of the fungal mycelium for 10 s at 10 microns (Beun de Ronde), after which 10 ml of RMPI medium was added. *Madurella* species and *F. senegalensis* isolates were incubated at 35°C with 5% carbon dioxide for 7 days while *T. grisea* and *Me. romeroi* isolates were incubated at 30°C for 7 days.

TABLE 1 Antifungal activity of luliconazole, lanoconazole and ravuconazle against commonly encountered eumycetoma agents

	Itraconazole			Ravuconazole			Luliconazole			Lanoconazole		
	Median[a]	GM MIC[a]	Range[a]	Median[a]	GM MIC[a]	Range[a]	Median[a]	GM MIC[a]	Range[a]	Median[a]	GM MIC[a]	Range[a]
Sordariales	0.032	0.050	0.008–≥4	0.016	0.014	0.004–0.032	0.001	0.001	0.001–0.004	0.001	0.001	0.001–0.008
Ma. mycetomatis ($n = 10$)	0.064	0.044	0.016–0.125	0.016	0.016	0.004–0.032	0.001	0.001	0.001–0.004	0.002	0.002	0.001–0.008
Ma pseudomycetomatis($n = 6$)	0.016	0.020	0.008–0.032	0.016	0.012	0.008–0.032	0.001	0.001	0.001	0.001	0.001	0.001–0.002
Ma tropicana($n = 3$)	0.032	0.032	0.016–0.064	0.016	0.020	0.016–0.032	0.001	0.001	0.001	0.001	0.001	0.001–0.002
Ma fahalii($n = 2$)	≥4	4	≥4, ≥4	0.008	0.008	0.004, 0.016	0.001	0.001	0.001, 0.001	0.001	0.001	0.001, 0.001
Pleosporales	1	0.44	0.016–≥4	0.125	0.311	0.008–8	0.008	0.010	0.004–0.064	0.008	0.012	0.008–0.125
F. senegalensis($n = 6$)	0.064	0.050	0.016–0.125	0.25	0.089	0.008–0.25	0.008	0.009	0.004–0.016	0.008	0.009	0.004–0.016
T. grisea($n = 4$)	1	0.420	0.5–2	2	1	0.125–8	0.064	0.019	0.004–0.064	0.064	0.032	0.008–0.125
Ma. romeroi($n = 6$)	≥4	≥4	≥4	0.5	0.5	0.25–1	0.008	0.008	0.008	0.008	0.009	0.00–0.016

After incubation, the mycelia were washed by centrifuging for 5 min at 2158 *g* and supplemented with fresh RMPI medium. Additional sonication for 10 s at 10 microns was performed, and a final inoculum was obtained with transmissions ranging from 68%–72% using a spectrophotometer (Novaspec II, Pharmacia Biotech). One hundred twenty microliters of fungal suspension was transferred to each well of 96-well round-bottom plates followed by 30 µl of resazurin (0.1%w/v) then 1.5 µl of respective antifungal agents. For each isolate, a drug-free control (positive control) and negative control (RMPI 1640 working solution) were included. The plates were sealed to prevent evaporation and incubated for 4 days for *Me. romeroi* and 7 days for the other species, respectively. *Trematosphaeria grisea* and *Me. romeroi* were incubated at 30°C and the other fungi at 35°C. After incubation, the supernatant was transferred to a flat bottom 96-well plate and read spectrophotometrically at 620 nm. MICs were defined as the lowest concentration with ≥75% inhibition in growth. All experiments were performed in duplicate or triplicate when the first and second values differed.

2.4 | Statistical analyses

The MICs for lanoconazole, luliconazole and ravuconazole were compared to those of itraconazole using a Mann–Whitney test with GraphPad Prism Version 8.4.3. The results were considered significant when *p*-value was ≤.05. The median MIC was considered the concentration at which 50% of the isolates were inhibited and when it was between two values, it was rounded up to the next higher concentration. The overall MICs for *Sordariales* and *Pleosporales* were obtained by adding all MICs for species belonging to each of the order.

3 | RESULTS

3.1 | Eumycetoma causative agents belonging to the order *Sordariales* are highly susceptible for luliconazole, lanoconazole and ravuconazole

As shown in Table 1, *Ma. mycetomatis*, *Ma. pseudomycetomatis* and *Ma. tropicana* had low MICs for itraconazole, with medians ranging from 0.016 to 0.064 µg/ml. However, *Ma. fahalii* was not inhibited by itraconazole. Growth was still detected at 4 µg/ml itraconazole, the highest concentration tested. In contrast to itraconazole, all species, including *Ma. fahalii*, were inhibited by low concentrations of ravuconazole, luliconazole and lanoconazole. The lowest median MICs were obtained for luliconazole, with all *Madurella* species having a median of 0.001 µg/ml, followed by lanoconazole (ranging from 0.001 to 0.002 µg/ml) and ravuconazole (Median MIC ranging from 0.008 to 0.016 µg/ml). The MICs obtained for luliconazole, lanoconazole and ravuconazole were significantly lower than those obtained for itraconazole with $P < .0001$ for both luliconazole and lanoconazole and $P = .006$ for ravuconazole.

3.2 | *Pleosporales*are susceptible for luliconazole and lanoconazole

Of the eumycetoma causative agents belonging to the order *Pleosporales*, *F. senegalensis* was susceptible for itraconazole, with a median MIC of 0.064 μg/ml. High medians were found for *T. grisea* (MIC_{50} of 1 μg/ml) and *Me. romeroi* (median > 4 μg/ml). For *F. senegalensis* and *T. grisea*, slightly higher medians were obtained for ravuconazole than for itraconazole (Table 1), but a lower median was obtained for *Me. romeroi* (median of 0.5 μg/ml). Higher potency was noted for luliconazole and lanoconazole, with medians of 0.008 μg/ml for *F. senegalensis* and *Me. romeroi* for both drugs and 0.064 μg/ml for *T. grisea*. The MICs obtained for luliconazole, lanoconazole and ravuconazole were significantly lower than those obtained for itraconazole with $P = .004$ for both luliconazole and lanoconazole and $P = .008$ for ravuconazole. Only for *T. grisea*, MICs for ravuconazole, luliconazole and lanoconazole were comparable to those of itraconazole and no significant difference was noted ($P > .05$).

As shown in Table 1, the overall MICs obtained for the eumycetoma causative agents belonging to the order of the *Sordariales* are several dilution steps lower than those obtained for the eumycetoma causative agents belonging to the order *Pleosporales*. For itraconazole, this difference was 5 twofold dilution steps, while for ravuconazole, luliconazole and lanoconazole, 3 twofold dilution steps were observed.

4 | DISCUSSION

In this study, we show that *Ma. mycetomatis*, *Ma. pseudomycetomatis*, *Ma. tropicana*, *Ma. fahalii*, *F. senegalensis*, *T. grisea* and *Me. romeroi* are all susceptible for ravuconazole, luliconazole and lanoconazole. The lowest MICs were obtained for luliconazole and lanoconazole. Our data indicate that these drugs may hold promise for the treatment of eumycetoma.

Eumycetoma treatment is usually started before the causative agent is properly identified. For the prescribing clinician, it would be ideal to know the chance that the causative agent is susceptible to the therapy given. This study confirmed that *Ma. fahalii* and *Me. sromeroi* were not inhibited by 4 μg/ml itraconazole, the highest concentration tested and higher than the attainable levels in serum, with peak concentrations up to 2.28 μg/ml.[30] Therefore, it is doubtful that itraconazole is an effective therapy for these fungi. In contrast, much lower MICs were obtained for ravuconazole, with MIC_{50}s ranging from 0.008 μg/ml to 2 μg/μl. The median MIC for *Ma. mycetomatis*, was comparable to the MIC_{50} reported in our earlier study, which was based on the MICs of 23 different *Ma. mycetomatis* isolates.[15] Interestingly, *Ma. fahalii* and *Me. romeroi* were also inhibited by ravuconazole, with median MICs of 0.25 and 0.5 μg/ml, respectively. Only *T. grisea* had a higher median of 2 μg/ml. All median MICs were lower than the maximum serum concentration (C_{max}) of 10.84 μg/ml ravuconazole observed after administering a 100 mg daily therapeutic dose.[31] Due to the high potency of ravuconazole towards *Ma. mycetomatis* and the long half-life of fosravuconazole compared to itraconazole,[15] a phase II clinical study was started. In this study, the efficacy of fosravuconazole in combination with surgery for the treatment of eumycetoma is investigated in Sudan. In the study, only patients with mycetoma caused by *Ma. mycetomatis* can be included. Here, we demonstrated that patients infected by *Ma. pseudomycetomatis*, *Ma. tropicana*, *Ma. fahalii*, *F. senegalensis* and *Me. romeroi* are also highly susceptible to ravuconazole. Therefore, new clinical studies with this drug may also include patients with eumycetoma caused by these fungi that could potentially also respond to this treatment are needed.

In this study, we also demonstrated that the new imidazoles luliconazole and lanoconazole were potent in inhibiting the eumycetoma causative agents, with median MICs ranging from 0.001 to 0.064 μg/ml. Others found median MICs of 0.008–0.064 μg/ml for *Fusarium* species, also able to cause eumycetoma.[23,24] Overall, luliconazole had a higher antifungal activity than lanoconazole which has also been reported in other studies.[22,32] This is likely due to it being strictly an R-enantiomer compared to racemic lanoconazole.[33] Furthermore, luliconazole also prolonged the survival of *Ma. mycetomatis* infected *Galleria mellonella* larvae on Day 4.[13,14] However, the large drawback of both luliconazole and lanoconazole is that these drugs are only approved for topical use in the treatment of skin infections such as athlete's feet and ringworm due to good skin pharmacokinetics.[34,35] Mycetoma is a deep-seated infection, where the bone is often involved. It is questionable if these drugs will be able to reach the site of infection. Lanoconazole and luliconazole are weak bases that are poorly soluble and are therefore retained in the skin. When 10% luliconazole solution is topically applied, only 0.063 to 0.090 ng/ml luliconazole is found back in plasma.[36] Although an oral solution is not available for clinical use, during its developmental stage the clinical efficacy of an oral suspension of luliconazole in 0.5% (w/v) carboxymethylcellulose was evaluated in murine models of systemic candidiasis and systemic aspergillosis.[37] The current unavailability of an oral solution may make treating a deep-seated infection such as mycetoma challenging. To improve drug penetration and bioavailability, several novel drug delivery systems for luliconazole and lanoconazole have been described. These include colloidal carriers such as Particle-stabilised Emulsions (PEs), hydrogels, microemulsion formulations, nanostructured lipid carriers and nanosuspension-based gels.[38–41] Most of these novel drug delivery systems increased the skin permeability compared to the currently available luliconazole cream and increased antifungal efficacy.[38] On top of that they also showed good tolerability and no systemic or local side effects in animal studies and clinical trials, this option may be worthwhile to be further investigated.[42–44] In conclusion, we demonstrated that ravuconazole, luliconazole and lanoconazole exhibited good in vitro activity against *Ma. mycetomatis*, *Ma. pseudomycetomatis*, *Ma. tropicana*, *Ma. fahalii*, *F. senegalensis*, *T. grisea* and *Me. romeroi*. Even *Ma. fahalii* and *Me. romeroi*, which are resistant against itraconazole, were inhibited by these drugs. Therefore, we suggest these drugs be further explored for the broad-based treatment of eumycetoma.

CONFLICT OF INTEREST

None to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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