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The combination of manogepix and itraconazole is synergistic and inhibits the growth of *Madurella mycetomatis in vitro* but not *in vivo*

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Abstract

Mycetoma is a neglected tropical disease commonly caused by the fungus *Madurella mycetomatis*. Standard treatment consists of extensive treatment with itraconazole in combination with surgical excision of the infected tissue, but has a low success rate. To improve treatment outcomes, novel treatment strategies are needed. Here, we determined the potential of manogepix, a novel antifungal agent that targets the GPI-anchor biosynthesis pathway by inhibition of the GWT1 enzyme. Manogepix was evaluated by determining the minimal inhibitory concentrations (MICs) according to the CLSI-based *in vitro* susceptibility assay for 22 *M. mycetomatis* strains and by *in silico* protein comparison of the target protein. The synergy between manogepix and itraconazole was determined using a checkerboard assay. The efficacy of clinically relevant dosages was assessed in an *in vivo* grain model in *Galleria mellonella* larvae. MICs for manogepix ranged from <0.008 to >8 mg/l and 16/22 *M. mycetomatis* strains had an MIC ≥4 mg/ml. Differences in MICs were not related to differences observed in the GWT1 protein sequence. For 70% of the tested isolates, synergism was found between manogepix and itraconazole *in vitro*. *In vivo*, enhanced survival was not observed upon admission of 8.6 mg/kg manogepix, nor in combination treatment with 5.7 mg/kg itraconazole. MICs of manogepix were high, but the *in vitro* antifungal activity of itraconazole was enhanced in combination therapy. However, no efficacy of manogepix was found in an *in vivo* grain model using clinically relevant dosages. Therefore, the therapeutic potential of manogepix in mycetoma caused by *M. mycetomatis* seems limited.

Lay summary

Treatment of *Madurella mycetomatis*-caused mycetoma consists of extensive exposure to antifungals and surgery. To improve therapy, we evaluated manogepix, a novel antifungal agent, as a therapeutic option against *M. mycetomatis*. Our findings suggest limited therapeutic potential for manogepix.

Key words: mycetoma, manogepix, *Madurella*, *in vitro* susceptibility, synergism, *Galleria mellonella*.

Introduction

Recognized as a neglected tropical disease by the World Health Organization in 2016, mycetoma remains a major health concern in regions of Africa, Latin America, and Asia.[1,2] Mycetoma can be of either bacterial (actinomycetoma) or fungal (eumycetoma) origin. Globally, the fungus *Madurella mycetomatis* is the most common causative agent and is reported in over 70% of all eumycetoma cases.[3] Eumycetoma is considered an implantation mycosis, in which the causative agent is implanted into the subcutaneous tissue via a minor trauma. The disease starts as a localized infection in the subcutaneous tissue, eventually forming debilitating masses, draining sinuses, and grains harboring the infectious agent. Mycetoma generally affects the feet, legs, and hands.[4]

Treatment of fungal eumycetoma remains challenging due to the resilient nature of fungal infections and the limited therapeutic options associated with the close resemblance of the fungal and human cells.[5,6] The recommended treatment relies on treatment with antifungal agents belonging to the class of azoles, such as itraconazole, fosravuconazole, posaconazole, voriconazole, or terbinafine.[7–11] At the moment, itraconazole is considered the drug of choice for mycetoma. Generally, antifungal treatment is started 6 months prior to surgical intervention. Then the lesion is surgically removed, and at least another 6 months of post-operative antifungal treatment are given. However, the post-operative recurrence rate varies from 25% to 50%.[12] In addition to high recurrence rates, loss of follow-up frequently occurs due to dissatisfaction with the therapeutic outcome, side effects, and the severe financial burden of the therapy on an average household.[6] Therefore, to improve the *M. mycetomatis* therapy outcome, other promising antifungal agents should be explored.

Manogepix, the active moiety of the prodrug fosmanogepix, is a new first-in-class antifungal agent currently in phase II clinical trials for the treatment of invasive fungal infections.[13,14] Manogepix targets the glycosylphosphatidyli-

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Table 1. Overview of included *Madurella mycetomatis* isolates and the respective minimum inhibitory concentrations of manogepix and itraconazole.

Isolate	ITS ascension number	GWT1 ascension number	Origin	Itraconazole (mg/l)	Manogepix 50% (mg/l)	Manogepix 80% (mg/l)
MM55	JN573181.1	LCTW02000523.1	Sudan	0.125	4	4
SO1	MW493233	OR134903	Somalia	0.063	4	4
MM13	JX280866.1		Sudan	0.125	4	4
MM14	MW513510		Sudan	0.063	8	8
MM25	MW494400		Sudan	0.016	4	4
MM30	MW520456		Sudan	0.063	4	4
MM35	MW513693		Sudan	0.125	2	4
MM36	MW520454		Sudan	0.25	4	4
MM41	MW520455		Sudan	0.063	≤0.008	≤0.008
MM52	JN573179.1		Sudan	0.25	4	8
MM54	JN573180.1		Sudan	0.063	8	8
MM71	ON319059.1		Sudan	0.125	0.5	8
MM83	ON319061.1		Sudan	0.125	0.25	2
I1	JX280864.1		India	0.063	0.5	8
I11	MW541890	OR134900	India	0.25	≤0.008	0.031
CBS116298	MW542679		Ivory Coast	0.063	8	8
Peru72012	ON319062.1	OR134902	Peru	0.063	0.5	1
P1	MW520453	OR134901	Mali	0.063	≤0.008	≤0.008
AL1	MW541888		Algeria	0.016	2	2
T606931	MW541889		Unknown	0.031	8	8
CBS247.48	JX280745.1		Unknown	0.063	4	4
Pasteur 10.757	OR095764		France	0.25	8	16
MIC range				0.016–0.250	≤0.008–8	≤0.008–16
MIC_{50}				0.063	4	4
MIC_{90}				0.25	8	8

nositol (GPI) anchor biosynthesis pathway by inhibition of the GWT1 protein, a conserved enzyme that catalyzes the inositol acylation.[15] In turn, the maturation of GPI-anchored proteins is prevented, compromising the cell wall integrity, germ tube formation, and biofilm formation.[15] Studies have shown that manogepix is active against different clinically relevant moulds, including azole- and echinocandin-resistant *Aspergillus* spp. and *Candida* spp.[14,16,17] Given the potential of manogepix, the aim of our study was threefold. First, we wanted to establish whether the growth of *M. mycetomatis* could be inhibited. Second, we tried to determine if manogepix could enhance the activity of itraconazole. Third, we wanted to determine if manogepix showed *in vivo* efficacy in a *Galleria mellonella* mycetoma model.

Methods

Drugs and fungal isolates

Manogepix (APX001A) was provided by Pfizer (formerly Amplyx Pharmaceuticals, Inc.), and itraconazole was obtained from Janssen Pharmaceutical Products, Belgium. A total of 22 clinical isolates of *M. mycetomatis* were included in this study. The isolates originated from Sudan, Mali, Peru, Somalia, Ivory Coast, Algeria, and France. The origin of three isolates is unknown (Table 1). All isolates were previously identified based on morphology, PCR, and sequencing of the internal transcribed spacer (ITS).[18,19] Furthermore, genetic variability was determined by *Mmy*STR analysis, as described by Nyuykonge and associates.[20]

GWT1 protein comparison

The GWT1 encoding sequence was extracted from GenBank from the annotated *M. mycetomatis* MM55 genome (BioProject: PRJNA267680, Accession: LCTW00000000.2).[21] The reference sequence (Accession: LCTW02000523.1) was used to retrieve the *GWT1* sequences of four *M. mycetomatis* isolates (SO1, Peru72012, P1, and I11) from whole genome sequence data (data not published). The coding sequences were used to generate protein sequences using the built-in translation function in Molecular Evolutionary Genetics Analysis (MEGA, version X), and a multiple sequence alignment (MSA) was constructed of the five *M. mycetomatis* GWT1 sequences using Clustal Omega V1.2.4.[22,23]

In vitro susceptibility testing

The antifungal susceptibility of *M. mycetomatis* against manogepix was determined according to a modified CLSI method using resazurin as a viability dye, as previously described.[24,25] Within this modification, we generate hyphal fragments as inoculum instead of using conidia as stated in the guideline. In short, all isolates were cultured on Sabouraud Dextrose Agar (SDA) at 37°C for 2–3 weeks prior to susceptibility testing. Mycelium was harvested and sonicated at 10 microns for 10 s (Soniprep 150 Plus, MSE), transferred into RPMI 1640 medium containing 0.35 gr/l L-glutamine and 1.98 mM 4-Morpholinepropanesulfonic acid (MOPS), and further incubated for 7 days at 37°C. Mycelium was harvested by centrifugation, washed, and sonicated using the same settings described above. A standardized hyphal suspension of 70% ± 2% was prepared in RPMI 1640 medium containing 0.35 gr/l L-glutamine and 1.98 mM MOPS. A twofold dilution series for both manogepix and itraconazole was prepared in sterile dimethyl sulfoxide (DMSO) (Merck, Germany). The drugs were transferred to round bottom plates (Corning Fisher, the Netherlands) and further diluted in standardized hyphal suspension and resazurin. Final concentrations ranged from 0.008 mg/l to 16 mg/l for manogepix, 0.008 mg/l to 4 mg/l for itraconazole, and a final concentration of 37.5 mg/l for resazurin. The cultures were incubated for 7 days at 37°C under 5% CO_2 conditions. After incubation, the absorbance of the supernatant was determined spectrophotometrically using the EPOCH 2 microplate reader (BioTek, USA), and the MIC was calculated according to the formula below, in which the reduction of

resazurin to resorufin is determined by measuring the decrease in absorbance of resazurin. The MIC was determined as the lowest concentration where the metabolic activity was ≥80%.

$$\text{Percentage Metabolic activity} = \left(\frac{[\text{Absorbance of negative control}] - [\text{Absorbance of test}]}{[\text{Absorbance of negative control}] - [\text{Absorbance of growth control}]}\right) * 100).$$

The minimal effective concentration (MEC) could not be determined because a hyphal inoculum was used as a starting inoculum. Instead, the recently reported surrogate maker of ≥50% was used.[26]

Analysis of synergism between manogepix and itraconazole

Synergism between manogepix and itraconazole was evaluated by checkerboard microdilution as previously described, using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) as a viability dye.[27] Manogepix and itraconazole concentrations ranged from 0.0025 to 8 mg/l and 0.002 to 0.5 mg/l, respectively. The MIC was determined by calculating the percentage of metabolic inhibition using the formula below:

$$\text{Percentage Metabolic activity} = \left(\frac{[\text{Absorbance of test}] - [\text{Absorbance of negative control}]}{[\text{Absorbance of growth control}] - [\text{Absorbance of negative control}]}\right) * 100).$$

The MIC was considered the first value in each row and column in which ≥80% reduction of fungal metabolic activity was found. To determine synergism between manogepix and itraconazole, the fractional inhibitory concentration (FIC) was determined. The FIC was calculated according to the formula below, determining up to three decimal places:

$$\text{FIC} = \left(\frac{[\text{MIC of manogepix in combination}]}{[\text{MIC of manogepix}]}\right) + \left(\frac{[\text{MIC of itraconazole in combination}]}{[\text{MIC of itraconazole}]}\right).$$

The FIC was determined for all wells of the microtitration plates that correspond to an MIC in combinations, reporting both the minimum and maximum FIC values.[28] A minimum FIC value <0.5 indicates synergism, a maximum FIC >4 indicates antagonism, and an FIC between 0.5 and 4 is interpreted as indifferent. Three biological replicates were performed for each respective isolate, and the median FIC min and FIC max were used to determine synergism or antagonism.

Galleria mellonella grain model

The *in vivo* efficacy of manogepix monotherapy and in combination with itraconazole was determined as described by Eadie and associates.[29] In brief, *M. mycetomatis* strain MM55 was cultured on SDA at 37°C for 3 weeks. Mycelium was harvested and sonicated at 10 microns for 30 s, transferred into RPMI 1640 medium containing 350 mg/l L-glutamine, 1.98 mM MOPS, and 100 mg/l chloramphenicol, and further incubated for 2 weeks at 37°C. The fungal mycelium was harvested, sonicated at 10 microns for 2 min, and diluted to an inoculum size of 4 mg per larvae. Next, the larvae were injected with the fungal inoculum in the left lower proleg using an insulin 29-G U-100 needle (BD Diagnostics, Sparks, USA). At 4 h, 28 h, and 52 h after infection, larvae were treated with 20 μl of 0.21 mg/ml manogepix, 0.14 mg/ml itraconazole, or a combination of both. To reach this concentration, manogepix and itraconazole were first dissolved in DMSO and further diluted in PBS, so that the final concentration of DMSO did not exceed 5%, a concentration well tolerated by the larvae. This resulted in a final concentration of 8.57 mg/kg manogepix and 5.71 mg/kg itraconazole in the larvae. Dosages were based on clinically relevant dosages of 600 mg and 400 mg of manogepix and itraconazole, respectively, based on an average of 70 kg person.[30] The survival of the larvae was monitored for 10 days after infection. The toxicity of the compounds was assessed separately by administering treatment to healthy, uninfected larvae equal to the infected groups. The toxicity of the drugs was monitored for up to 5 days after the initial treatment. Pupa formed during the monitored period were excluded from the analysis. Three biological replicates were performed.

Results

Manogepix inhibits *M. Mycetomatis* growth *in vitro*

We determined the *in vitro* susceptibility of itraconazole and manogepix against 22 *M. mycetomatis* isolates. As shown in Figure 1, the MIC of itraconazole ranged between 0.016 and 0.25 mg/l and of manogepix between <0.008 and 16 mg/l. No minimal effective concentration could be observed due to the use of a hyphal inoculum as the starting method; therefore, a 50% reduction in metabolic activity was used as a surrogate marker (Table 1). Using this surrogate marker, the same range of inhibitory concentrations was noted for manogepix, and also the same MIC50 was obtained. A complete overview of the included isolates and the corresponding MIC values for both manogepix and itraconazole are provided in Table 1. As can be seen in this table, 16 of the 22 isolates had MICs for manogepix of 4 mg/l or higher.

No genetic link between high and low MICs for manogepix

To determine if there was a genetic link for the high diversity in MICs obtained for manogepix, the 22 included *M. mycetomatis* isolates were typed with the *Mmy*STR assay, and the translated DNA sequence of *GWT1* of five isolates with diverse MICs was compared. All 22 isolates had a unique *Mmy*STR profile (Fig. 2A). No apparent clusters are observed between the genotype and the susceptibility to manogepix. For five *M. mycetomatis* isolates for which whole genome sequence data was available, the GWT1 protein sequence was compared. As shown in Figure 2B, the GWT1 protein was conserved among these different isolates, and no differences in amino acids were observed. Of these five isolates, I11 and P1 had a very low MIC of <0.008 mg/l, Peru72012 had an MIC of 1 mg/l, and MM55 and SO1 had a relatively high MIC of 4 mg/l. The Val-168 residue corresponding to the valine residue that has been linked to resistance in different isolates of *C. albicans*, *C. glabrata*, and *S. cerevisiae* is conserved among all isolates[31] (Fig. 2B).

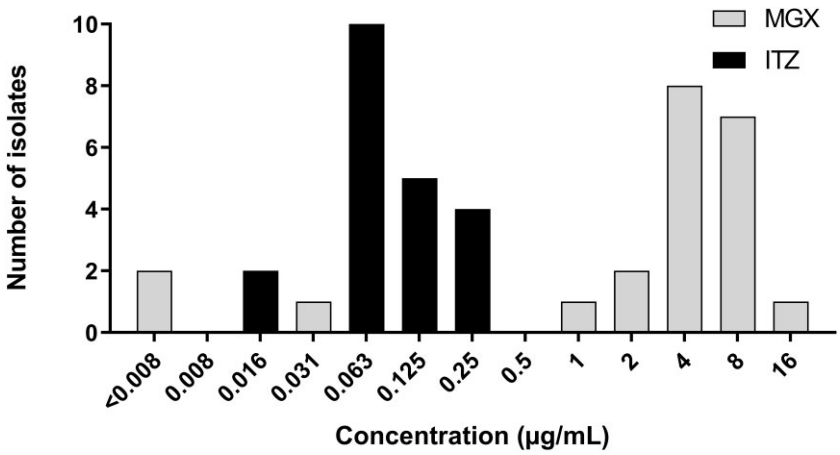


Figure 1. Antifungal susceptibility, expressed as MICs, of 22 *Madurella mycetomatis* isolates to manogepix and itraconazole. MIC distribution as determined by the resazurin assay for manogepix (MGX) and itraconazole (ITZ). The MIC is depicted as calculated using an 80% inhibition cutoff.

Manogepix and itraconazole are synergistic against *M. Mycetomatis*

To determine if manogepix and itraconazole act synergistically and could potentially be combined in therapy, a checkerboard assay was performed on a smaller subset of 10 different *M. mycetomatis* isolates. As shown in Table 2, combined exposure of manogepix and itraconazole for MM14, MM25, and P1 was found to be indifferent, with median FIC min values of 0.501, 0.547, and 0.563, respectively. This indicates that against these three isolates, manogepix and itraconazole do not act synergistically. For the other seven isolates, median FIC min values of ≤0.5 were found, indicating that manogepix and itraconazole act synergistically.

Manogepix in mono- and combination therapy does not enhance larval survival

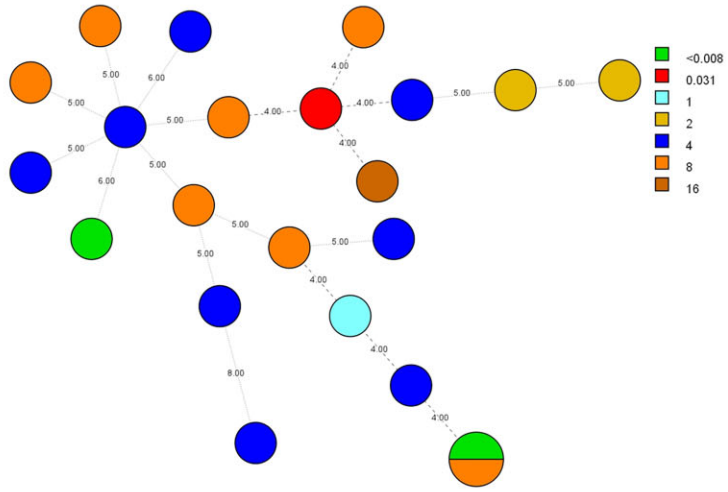
Although relatively high MICs were obtained for manogepix, synergy was obtained with itraconazole. We, therefore, determined the *in vivo* efficacy of a clinically relevant dosage of manogepix as monotherapy and in combination with itraconazole, the current drug of choice for mycetoma therapy, in our *M. mycetomatis G. mellonella* grain model. For these selected dosages, no toxicity was observed in *G. mellonella* larvae (data not shown). As shown in Figure 3, both 8.57 mg/kg manogepix as monotherapy and in combination with 5.71 mg/kg itraconazole did not significantly prolong the survival of *M. mycetomatis*-infected larvae.

Discussion

With the discovery of manogepix, numerous studies have been conducted on its effectiveness in treating invasive fungal diseases. In this study, we demonstrate growth inhibition of *M. mycetomatis* upon exposure to manogepix. According to the established method for susceptibility testing for *M. mycetomatis*, we report a colorimetric MIC_{50} of 4 mg/l and an MIC_{90} of 8 mg/l for manogepix, respectively, using both a 50% and an 80% inhibition cutoff vs. an itraconazole MIC_{50} and MIC_{90} of 0.063 mg/l and 0.25 mg/l. The broad range of MICs ranging from ≤0.008 to >4 mg/l could not be explained by differences in the drug target enzyme, as this was conserved within the *M. mycetomatis* isolates analyzed.

Manogepix has been reported to be effective in inhibiting the yeasts *Candida* spp. and *Cryptococcus* spp.[30–32] For *Candida* spp., reported MIC_{90} values ranged from 0.008 mg/l (for *C. albicans*) to 0.5 mg/l (for *C. kefyr*), with the exception of *C. krusei* (MIC_{90} > 0.5 mg/l reported).[31,32] The higher MICs observed for *M. mycetomatis* were in the same range as those reported for other filamentous fungi. For *Aspergillus* spp., MIC values for manogepix were above 8 mg/l when read with the standard CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods for *in vitro* susceptibility testing of filamentous fungi.[33] Using 2,3-Bis(2-Methoxy-4-Nitro-5- Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H- Tetrazolium Hydroxide (XTT) as a viability dye, MICs >0.5 mg/l were obtained for *A. fumigatus*. However, similar to the echinocandins, alteration in growth for filamentous fungi was observed at a much lower concentration, and for *A. fumigatus*, only 0.06 mg/l manogepix was needed to alter the fungal growth.[26] Therefore, similar to the echinocandins, for filamentous fungi, it is recommended to determine the concentration at which growth is visibly altered, defined as the MEC. The MEC_{90} values for manogepix against *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* ranged between 0.03 mg/l and 0.125 mg/l.[16] For other rare moulds, among which the genetically more closely related *Fusarium* spp. and *Scedosporium* spp., similar MEC_{90} values between 0.03 mg/l and 0.25 mg/l are reported.[34–38] MEC values for filamentous fungi are determined visually as the first concentration where growth is altered. This is clearly seen when conidia or spores are used as a starting inoculum, although lab-to-lab differences in interpretation are noted. Since *M. mycetomatis* only sporulates on rare occasions, hyphal fragments are used as starting inoculum in standard *in vitro* susceptibility assays.[25] Unfortunately, when hyphal fragments are used, it is not possible to reliably determine an MEC since no clear morphological differences are noted for manogepix and echinocandins.[39] Recently, a colorimetric alternative for visual MEC determination was described for *Aspergillus* spp. In this alternative method, a lower starting inoculum and the viability dye XTT are used, and a 50% reduction in metabolic activity corresponded to the visual MEC.[26] Upon implementing this 50% reduction threshold in our methodology, a 50% reduction in metabolic activity with our standard hyphal starting inoculum did not result in a significantly lower MIC value. A possible explanation may be

(A)



(B)

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Mmy_mm55              MASTEAAANNATAQSYKQLKEDFVSNLSGGSVTEIAQVCAVAPVVALLWSALQARQSFFK    60
Mmy_SO1_GWT1          MASTEAAANNATAQSYKQLKEDFVSNLSGGSVTEIAQVCAVAPVVALLWSALQARQSFFK    60
Mmy_Peru72012_GWT1    MASTEAAANNATAQSYKQLKEDFVSNLSGGSVTEIAQVCAVAPVVALLWSALQARQSFFK    60
Mmy_P1_GWT1           MASTEAAANNATAQSYKQLKEDFVSNLSGGSVTEIAQVCAVAPVVALLWSALQARQSFFK    60
Mmy_I11_GWT1          MASTEAAANNATAQSYKQLKEDFVSNLSGGSVTEIAQVCAVAPVVALLWSALQARQSFFK    60
                      ************************************************************

Mmy_mm55              PYTTLAFAVDFLLNVGALLLSVTLYSSAPLLLNILLLTAAAFVSAIPPNVSAPRKRPRLP    120
Mmy_SO1_GWT1          PYTTLAFAVDFLLNVGALLLSVTLYSSAPLLLNILLLTAAAFVSAIPPNVSAPRKRPRLP    120
Mmy_Peru72012_GWT1    PYTTLAFAVDFLLNVGALLLSVTLYSSAPLLLNILLLTAAAFVSAIPPNVSAPRKRPRLP    120
Mmy_P1_GWT1           PYTTLAFAVDFLLNVGALLLSVTLYSSAPLLLNILLLTAAAFVSAIPPNVSAPRKRPRLP    120
Mmy_I11_GWT1          PYTTLAFAVDFLLNVGALLLSVTLYSSAPLLLNILLLTAAAFVSAIPPNVSAPRKRPRLP    120
                      ************************************************************

Mmy_mm55              PNAEPKTPSAPLGALSTKPFLTNYRGNMMVVTCICILAVDFRLFPRRFAKVETWGTSLMD    180
Mmy_SO1_GWT1          PNAEPKTPSAPLGALSTKPFLTNYRGNMMVVTCICILAVDFRLFPRRFAKVETWGTSLMD    180
Mmy_Peru72012_GWT1    PNAEPKTPSAPLGALSTKPFLTNYRGNMMVVTCICILAVDFRLFPRRFAKVETWGTSLMD    180
Mmy_P1_GWT1           PNAEPKTPSAPLGALSTKPFLTNYRGNMMVVTCICILAVDFRLFPRRFAKVETWGTSLMD    180
Mmy_I11_GWT1          PNAEPKTPSAPLGALSTKPFLTNYRGNMMVVTCICILAVDFRLFPRRFAKVETWGTSLMD    180
                      ************************************************************

Mmy_mm55              MGVGSFVFSAGVVASRPVLKEQADGRTTPLGARLVRSLRHSLPLLALGVVRLLSVKGLDY    240
Mmy_SO1_GWT1          MGVGSFVFSAGVVASRPVLKEQADGRTTPLGARLVRSLRHSLPLLALGVVRLLSVKGLDY    240
Mmy_Peru72012_GWT1    MGVGSFVFSAGVVASRPVLKEQADGRTTPLGARLVRSLRHSLPLLALGVVRLLSVKGLDY    240
Mmy_P1_GWT1           MGVGSFVFSAGVVASRPVLKEQADGRTTPLGARLVRSLRHSLPLLALGVVRLLSVKGLDY    240
Mmy_I11_GWT1          MGVGSFVFSAGVVASRPVLKEQADGRTTPLGARLVRSLRHSLPLLALGVVRLLSVKGLDY    240
                      ************************************************************

Mmy_mm55              AEHVTEYGVHWNFFFTLGFLPPFVALFQSALKVVPSYAGLAILLGVLYQVVLESTALKAY    300
Mmy_SO1_GWT1          AEHVTEYGVHWNFFFTLGFLPPFVALFQSALKVVPSYAGLAILLGVLYQVVLESTALKAY    300
Mmy_Peru72012_GWT1    AEHVTEYGVHWNFFFTLGFLPPFVALFQSALKVVPSYAGLAILLGVLYQVVLESTALKAY    300
Mmy_P1_GWT1           AEHVTEYGVHWNFFFTLGFLPPFVALFQSALKVVPSYAGLAILLGVLYQVVLESTALKAY    300
Mmy_I11_GWT1          AEHVTEYGVHWNFFFTLGFLPPFVALFQSALKVVPSYAGLAILLGVLYQVVLESTALKAY    300
                      ************************************************************

Mmy_mm55              ILVGPRDNLLSMNREGIFSFWGYLAIFLAGQDTGMFVLPRNLNPRGIASSRRTALPLTMA    360
Mmy_SO1_GWT1          ILVGPRDNLLSMNREGIFSFWGYLAIFLAGQDTGMFVLPRNLNPRGIASSRRTALPLTMA    360
Mmy_Peru72012_GWT1    ILVGPRDNLLSMNREGIFSFWGYLAIFLAGQDTGMFVLPRNLNPRGIASSRRTALPLTMA    360
Mmy_P1_GWT1           ILVGPRDNLLSMNREGIFSFWGYLAIFLAGQDTGMFVLPRNLNPRGIASSRRTALPLTMA    360
Mmy_I11_GWT1          ILVGPRDNLLSMNREGIFSFWGYLAIFLAGQDTGMFVLPRNLNPRGIASSRRTALPLTMA    360
                      ************************************************************

Mmy_mm55              AWSAVWIALYFLCTDYTYGAGLTVSRRLANLPYMLWVVAFNTTLVLAFCLVDTIFFPAFY    420
Mmy_SO1_GWT1          AWSAVWIALYFLCTDYTYGAGLTVSRRLANLPYMLWVVAFNTTLVLAFCLVDTIFFPAFY    420
Mmy_Peru72012_GWT1    AWSAVWIALYFLCTDYTYGAGLTVSRRLANLPYMLWVVAFNTTLVLAFCLVDTIFFPAFY    420
Mmy_P1_GWT1           AWSAVWIALYFLCTDYTYGAGLTVSRRLANLPYMLWVVAFNTTLVLAFCLVDTIFFPAFY    420
Mmy_I11_GWT1          AWSAVWIALYFLCTDYTYGAGLTVSRRLANLPYMLWVVAFNTTLVLAFCLVDTIFFPAFY    420
                      ************************************************************

Mmy_mm55              KAQDTKAEKEAYSTATSKVLRAYNRNGLAVFLLANLLTGLVNMTVPTLDVGRIATIGILV    480
Mmy_SO1_GWT1          KAQDTKAEKEAYSTATSKVLRAYNRNGLAVFLLANLLTGLVNMTVPTLDVGRIATIGILV    480
Mmy_Peru72012_GWT1    KAQDTKAEKEAYSTATSKVLRAYNRNGLAVFLLANLLTGLVNMTVPTLDVGRIATIGILV    480
Mmy_P1_GWT1           KAQDTKAEKEAYSTATSKVLRAYNRNGLAVFLLANLLTGLVNMTVPTLDVGRIATIGILV    480
Mmy_I11_GWT1          KAQDTKAEKEAYSTATSKVLRAYNRNGLAVFLLANLLTGLVNMTVPTLDVGRIATIGILV    480
                      ************************************************************

Mmy_mm55              AYMGTLTAVAVGLDMYDITIKL* 502
Mmy_SO1_GWT1          AYMGTLTAVAVGLDMYDITIKL* 502
Mmy_Peru72012_GWT1    AYMGTLTAVAVGLDMYDITIKL* 502
Mmy_P1_GWT1           AYMGTLTAVAVGLDMYDITIKL* 502
Mmy_I11_GWT1          AYMGTLTAVAVGLDMYDITIKL* 502
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Figure 2. (A) Minimum spanning tree (MST) showing the genetic diversity of the panel of *Madurella mycetomatis* isolates included for *in vitro* susceptibility testing. Each circle represents a unique genotype, and the size is proportional to the number of isolates of the respective genotype. The numbers on the connecting lines represent the number of different STR markers between the genotypes. Each color represents the MIC of manogepix against the respective isolate in mg/l. (B) Multiple sequence alignment of the GWT1 protein from *M. mycetomatis* isolates MM55, SO1, Peru72012, P1, and I11. The Val-168 residue previously identified in resistant isolates of *C. albicans*, *C. glabrata*, and *S. cerevisiae* is highlighted by the red box[30].

Table 2. Overview checkerboard outcome. Synergism is indicated by Synergy (S) or Indifferent (I).

Isolate	Median FIC min	FIC min range	Median FIC max	FIC max range	Synergism
MM14	0.501	0.500–0.750	0.750	0.625–1.000	I
MM25	0.547	0.504–1.000	1.141	1.031–2.001	I
MM54	0.126	0.070–0.127	0.625	0.563–0.625	S
MM55	0.258	0.156–0.258	0.750	0.620–0.750	S
MM83	0.344	0.250–0.375	0.816	0.563–1.016	S
SO1	0.141	0.127–0.188	0.594	0.563–0.625	S
AL1	0.195	0.133–0.250	0.688	0.625–0.750	S
P1	0.563	0.500–1.063	1.500	0.750–2.063	I
CBS247.48	0.227	0.188–1.000	0.625	0.625–1.000	S
Peru72012	0.174	0.078–0.504	1.377	0.563–2.500	S

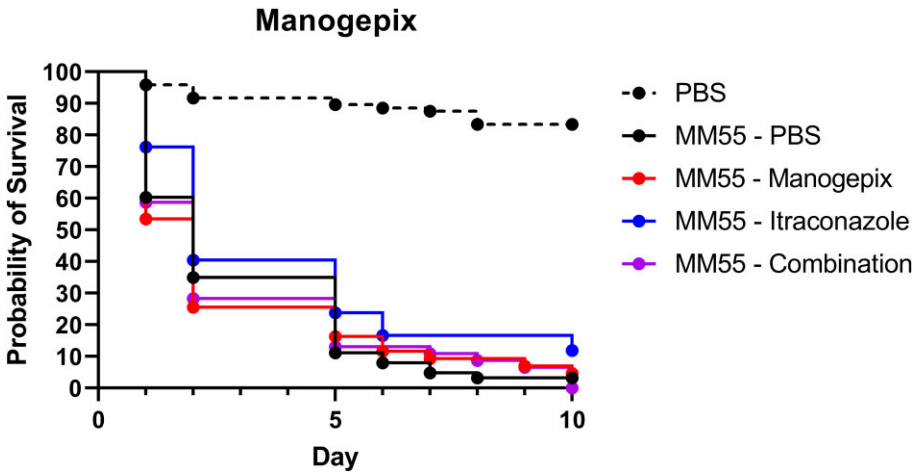

Figure 3. *In vivo* efficacy of 8.57 mg/kg manogepix, 5.71 mg/kg itraconazole, a combination of manogepix and itraconazole (8.57 and 5.71 mg/kg, respectively), or a 5% DMSO in PBS control within *Galleria mellonella* larvae. The full lines indicated infected larvae treated with the corresponding dosage at 4-, 28-, and 52 h post-infection. Dotted lines indicate uninfected larvae treated with a 5% DMSO in PBS, which were treated at the same time points as the infected groups. All larvae were followed up until 10 days after infection, and no significant difference was observed between any of the test groups compared to the infected PBS (5% DMSO) control.

the number of fungal cells within the starting inoculum, as this effect for *Aspergillus* was also only observed when a 1:1000 diluted starting inoculum was used or because hyphal fragments were used instead of conidia.[16] Altogether, the determination of the MIC, and especially the MEC, of manogepix against *M. mycetomatis* provides limited insights due to the starting conditions used in this methodology, regardless of the inhibition threshold, and should be interpreted with care.

Although for most strains, a concentration of 4 mg/l manogepix was needed to inhibit *M. mycetomatis* growth, we did observe synergy when manogepix was combined with itraconazole. This synergy was also observed for *Candida*, *Cryptococcus*, and *Aspergillus*.[40,41] We, therefore, evaluated the *in vivo* efficacy of manogepix alone and in combination in an *M. mycetomatis G. mellonella* grain model. Unfortunately, neither for manogepix alone nor for the combination of manogepix and itraconazole prolonged larval survival was noted. This was in contrast to the efficacy shown for other fungal infections. In murine models of invasive candidiasis, 78 and 104 mg/kg fosmanogepix significantly reduced the fungal burden in the kidney, lung, and brain, resulting in enhanced murine survival.[42,43] The same was found when mice with invasive pulmonary Aspergillosis, Scedosporiosis, Mucormycosis, or disseminated Fusariosis were treated with fosmanogepix.[44–46] Fosmanogepix alone was ineffective in a *C. neoformans* meningitis mice model, but in combination with fluconazole, the fungal burden in the brain was significantly reduced.[40] This difference in efficacy for manogepix against *M. mycetomatis* might be due to a difference in formulation or dosage used. In most studies, the prodrug fosmanogepix is used instead of manogepix. Fosmanogepix is cleaved into manogepix by the activity of host phosphatases. Since *G. mellonella* is not a mammalian model and there was no data on the activity of the *G. mellonella* phosphatases in comparison to those in mouse models, we used the active moiety manogepix in the *G. mellonella* model instead of the prodrug. Furthermore, the concentration we used in our study, 8.57 mg/kg, was much lower than the concentrations used in other studies in mice. In mice, the concentrations ranged from 26 mg/kg to 264 mg/kg. Dosages used in humans in ongoing clinical trials are 600 mg/dosage, which, based on an average 70 kg adult, translates to 8.57 mg/kg.[30] Another factor may be the difference in virulence and biofilm formation, as manogepix inhibits glycophosphatidylinositol biosynthesis. This pathway is directly linked to *A. fumigatus* morphogenesis and virulence and has been described to suppress biofilm formation in *C. albicans*.[15,47] For *M. mycetomatis*, grains (biofilm-like structures), in particular, are challenging as these remain viable for extended periods of time, even upon prolonged treatment with itraconazole.[11] In our grain model, grains are observed after 4 h, at which time point the first dosage is administered, meaning that inhibition of grain for-

mation could not be assessed.[48] Altogether, both the treatment time, selected dosage, and additional unknown factors on the clearance and bioavailability of manogepix could contribute to the contradictory efficacy results compared to other studies and thus be seen as shortcomings in our study. For a thorough *in vivo* evaluation of fosmanogepix in the treatment of eumycetoma, additional studies are needed, including dose-finding studies as well as studies in mammalian models.

To conclude, we determined the potential of manogepix as a treatment for eumycetoma caused by *M. mycetomatis*. We found that relatively high concentrations of manogepix are needed to completely inhibit the growth of *M. mycetomatis*, which was similar to other filamentous fungi. *In vitro*, combining manogepix and itraconazole was synergistic, a finding not documented *in vivo*. Based on these findings, manogepix seems inferior to itraconazole in mycetoma treatment.

Author contributions

Mickey Konings (Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Kimberly Eadie (Data curation, Investigation, Validation, Writing – review & editing), Nikolaos Strepis (Data curation, Formal analysis, Investigation, Software, Validation, Writing – review & editing), Bertrand Nyuykonge (Formal analysis, Investigation, Writing – review & editing), Ahmed H. Fahal (Resources, Writing – review & editing), Annelies Verbon (Supervision, Writing – review & editing), and Wendy W.J. van de Sande (Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing)

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Declaration of interest

None of the authors had any potential conflicts of interest.

Ethical approval

Not required.

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