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Impact of milbemycin oxime on fluconazole resistance in *Candida auris*

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Background: *Candida auris* is a pathogenic yeast that can develop resistance to multiple antifungals, particularly to azoles (e.g. fluconazole). Milbemycin oxime potentiates the effect of fluconazole against *Candida* spp. by inhibiting ABC transporters, such as Cdr1, which is involved in azole drug efflux.

Objectives: This study aimed to assess the interaction of milbemycin oxime and fluconazole against clinical ($n=4$) and laboratory-generated ($n=4$) *C. auris* isolates with different mechanisms of azole resistance.

Methods: Interactions of milbemycin oxime and fluconazole were assessed by chequerboard assays and defined as synergistic, indifferent or antagonistic according to the FIC index (FICI) values. The fluorescent substrate rhodamine 6 g (R6G) was used to measure ABC transporter activity in the absence or presence of milbemycin oxime.

Results: A synergistic interaction between milbemycin oxime and fluconazole was observed against most isolates, including those harbouring Cdr1-independent mechanisms of azole resistance (e.g. *ERG11* mutations). The highest synergism was observed in a laboratory-generated strain overexpressing *CDR1*, while the interaction was indifferent in a strain lacking *CDR1*. R6G experiments confirmed the inhibitory effect of milbemycin oxime on ABC transporters.

Conclusions: Milbemycin oxime could represent an interesting adjunctive therapy against azole-resistant *C. auris*, particularly those with *CDR1* overexpression.

Introduction

Candida auris (recently renamed *Candidozyma auris*) is a yeast pathogen that can cause nosocomial outbreaks of invasive candidiasis and develop resistance to all antifungal drug classes.[1] Since 2009, six genetically distinct clades of *C. auris* have emerged simultaneously across at least three continents.[1] Most clinical isolates exhibit resistance to fluconazole, the most common azole drug used for the treatment of *Candida* infections.[1]

Azole resistance in *C. auris* can occur through mutations in the azole target gene *ERG11* or through the up-regulation of efflux pumps, such as Cdr1 (an ABC transporter regulated by Tac1b) and Mdr1 (an MFS transporter regulated by Mrr1).[2–6] Milbemycin oxime is a veterinary antiparasitic drug, which also displays antifungal properties.[7–9] The main effect of milbemycin oxime consists of the inhibition of ABC transporters, including the Cdr1 transporter, which can potentiate the activity of azoles by interfering with their efflux.[7,10] Moreover, milbemycin oxime also displays some antifungal activity *per se* at relatively high concentrations, probably by reactive oxygen species formation.[11]

In this study, we investigated the *in vitro* interaction of milbemycin oxime and fluconazole in different clinical and laboratory-generated *C. auris* isolates with distinct mechanisms of azole resistance.

Materials and methods

The *C. auris* isolates of this study are described in Table 1. Four clinical isolates representing the four main clades with different levels of fluconazole susceptibility and mechanisms of resistance were selected

Table 1. Clinical and laboratory-generated isolates of this study: origin, genotypic characteristics and results of chequerboard assays

Isolate	Origin	Genotypic characteristics	FLC MIC (mg/L)	MO MIC (mg/L)	Interaction MO-FLC (FICI)
I.2	Clinical strain (clade I)	*ERG11*^{K143R} *TAC1B*^{A640V}[a]	256	16	Synergistic (0.25)
II.1	Clinical strain (clade II)	—	1	16	Indifferent (0.51)
III.8	Clinical strain (clade III)	*ERG11*^{V125A,F126L} *MRR1*^{N647T}[b]	256	16	Synergistic (0.25)
IV.1	Clinical strain (clade IV)	—	4	16	Synergistic (0.25)
TAC1B^{HA}	Laboratory strain (background IV.1)	*TAC1B* C-terminal 3×HA tag[c]	128	16	Synergistic (0.13)
MRR1^{HA}	Laboratory strain (background IV.1)	*MRR1* C-terminal 3×HA tag[d]	16	8	Synergistic (0.26)
*cdr1*Δ	Laboratory strain (background IV.1)	*CDR1* deletion	0.5	16	Indifferent (0.58)
*mdr1*Δ	Laboratory strain (background IV.1)	*MDR1* deletion	4	16	Synergistic (0.25)

FLC, fluconazole; MO, milbemycin oxime; FICI, FIC index.
[a]Gain-of-function mutation associated with *CDR1* up-regulation.[6]
[b]Gain-of-function mutation associated with *MDR1* up-regulation.[2]
[c]Genetic hyperactivation of the transcription factor Tac1b resulting in *CDR1* up-regulation.[12]
[d]Genetic hyperactivation of the transcription factor Mrr1 resulting in *MDR1* up-regulation.[12]

(I.2, II.1, III.8, IV.1).[13] In addition, we used four laboratory strains generated in the IV.1 background, in which the efflux pumps genes *CDR1* and *MDR1* were deleted (*cdr1*Δ and *mdr1*Δ, respectively) or overexpressed following hyperactivation of their respective transcription factors by addition of a 3×HA C-terminal tag (*TAC1B*^{HA} and *MRR1*^{HA}, respectively), as previously described.[2,12]

Fluconazole (Sigma–Aldrich, St. Louis, USA) and milbemycin oxime (milbemycin oxime CRS, European Pharmacopoeia, Strasbourg, France) were obtained as powders and diluted in DMSO for stock solutions of 10 mg/mL. Drug interaction between fluconazole and milbemycin oxime was tested by chequerboard assays in broth microdilution according to the EUCAST protocol in 96-well plates. (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E.Def_7.4_Yeast_definitive_revised_2023.pdf). The plates were read by a LUMIstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) to measure the absorbance (OD_{600}). The growth percentage was calculated in comparison with the control well (i.e. without drug). Considering the fungistatic effect of fluconazole and milbemycin oxime, the MIC was defined as the concentration at which 50% growth inhibition was achieved for both drugs. The drug interaction was calculated by the FIC index (FICI) and defined as synergistic (≤0.5), indifferent (>0.5 to 4) or antagonistic (>4), as previously described.[14] Experiments were performed in duplicate.

The efflux of rhodamine 6G (R6G), a fluorescent substrate of ABC transporters, was measured in the selected isolates in the absence or presence of milbemycin oxime at 5 mg/L, according to a previously described protocol.[15] Glucose 1% was added 5 min after the start of the experiment to provide energy needed for efflux pump activity. The relative fluorescence was calculated in comparison with the fluorescence at $t=0$. The slope of the curve (m) upon glucose addition was determined between 5 and 15 min of the experiment using the non-linear fit function in GraphPad (Prism 10, GraphPad Software, San Diego, CA, USA).

Results

As shown in Table 1, the chequerboard assays demonstrated a synergistic interaction between fluconazole and milbemycin oxime for all isolates, except two (II.1 and *cdr1*Δ), for which we observed indifference. Details of the chequerboard assays are represented in a two-colour heat map in Figure S1 (available as Supplementary data at *JAC-AMR* Online). Because milbemycin oxime is supposed to inhibit ABC transporters (i.e. Cdr1), we hypothesized that its interaction with fluconazole could be influenced by the underlying mechanism of azole resistance. Among clinical isolates, the interaction was synergistic (FICI=0.25) against all fluconazole-resistant isolates, including those with the presence of Cdr1-independent mechanisms of resistance (*ERG11* mutations, *MDR1* up-regulation). Synergism was achieved against one azole-susceptible (WT) isolate (IV.1) but not the other (II.1). Among laboratory-generated isolates, a highly synergistic effect (FICI=0.13) was observed in *TAC1B*^{HA}, in which *CDR1* was drastically up-regulated (23.13-fold compared with its background strain, as previously published).[12] On the contrary, the addition of milbemycin oxime to fluconazole had no significant effect (indifferent interaction) in the *cdr1*Δ strain. A moderate synergistic effect (FICI=0.25) was observed in the strains harbouring artificial variations of *MDR1* expression, but not *CDR1* (*MRR1*^{HA} and *mdr1*Δ strains).

To further analyse the link between milbemycin oxime and the Cdr1 transporter, we measured the efflux of R6G (fluorescent substrate of ABC transporters including Cdr1) in the different clinical and laboratory-generated isolates in the absence and presence of milbemycin oxime (Figure 1). Upon addition of glucose (required for efflux pump activation), milbemycin oxime could achieve a substantial reduction of fluorescence in all isolates. This effect was less pronounced in the *cdr1*Δ strain, in which basal fluorescence was considerably reduced compared with its background strain (IV.1). In *TAC1B*^{HA}, the slope of the fluorescent signal was considerably increased compared with the background strain, with a possible saturation effect over time. Milbemycin oxime was still effective to reduce R6G efflux in this strain. The *MRR1*^{HA} and *mdr1*Δ strains exhibited profiles that were similar to their background strains in the absence or presence of milbemycin oxime.

Discussion

In this study, we analysed the interaction between milbemycin oxime, an ABC transporter inhibitor, and fluconazole in different clinical and laboratory *C. auris* isolates with different levels of

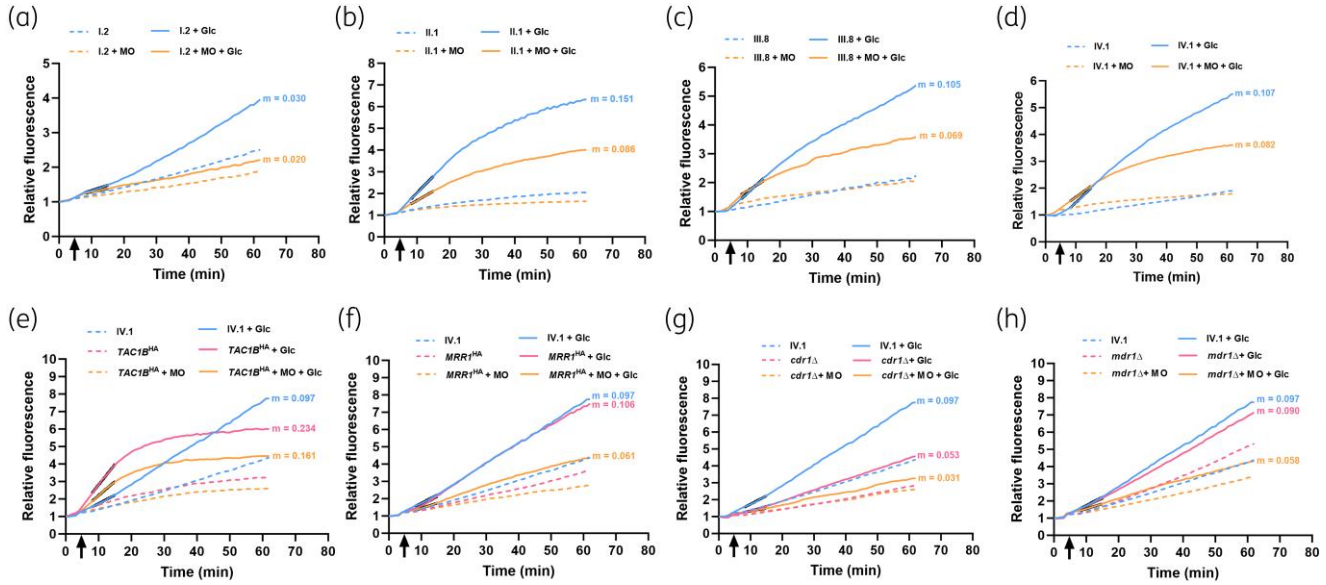

Figure 1. Measurement of the fluorescent ABC transporter substrate R6G in different *C. auris* isolates in the absence or presence of milbemycin oxime (MO). (a–d) Clinical isolates. (e–h) Laboratory-generated strains and their background strain (IV.1). Relative fluorescence was calculated from the initial fluorescence at $t=0$. The arrow indicates the timepoint of glucose 1% (Glc) addition (at 5 min from the start of measurement). The black line represents the fitted curve between 5 and 15 min of the experiment. The value of the slope (m) is indicated for each curve. MO at 5 mg/L was added at $t=0$.

fluconazole susceptibility and distinct mechanisms of azole resistance. Overall, we observed synergistic interactions against most isolates, with FICI values comparable to that reported for other *Candida* spp.[8,11] Our results allowed three levels of interactions to be distinguished. First, the highest synergistic effect was reached in the laboratory-generated strain in which fluconazole resistance mainly relied on *CDR1* up-regulation (*TAC1B*^{HA}). Second, we observed a moderate synergistic effect (FICI = 0.25) on azole-resistant isolates harbouring at least one Cdr1-independent mechanism of resistance, such as *ERG11* mutation or *MDR1* up-regulation (I.2, III.8, *MRR1*^{HA}). Third, we observed no significant interaction in a *CDR1*-null mutant. These results confirm that the effect of milbemycin oxime mainly relies on the inhibition of the Cdr1 transporter, which was shown to be crucial for azole resistance in *C. auris*.[5,16] This mechanism was confirmed by the R6G experiments showing the inhibitory effect of milbemycin oxime in relation to the different levels of *CDR1* expression in the laboratory-generated strains. Among isolates harbouring other clinically relevant mechanisms of azole resistance, such as *ERG11* mutations, the synergistic effect of milbemycin oxime suggests that its impact on Cdr1 activity was sufficient to achieve a significant reduction of azole resistance. Finally, and not surprisingly, the impact of milbemycin oxime on fluconazole-susceptible strains (II.1, IV.1, *mdr1*Δ) was modest (FICI = 0.25–0.51), probably acting on the basal level of Cdr1 production.

Further experiments, in particular using murine models, are required to assess the potential role of milbemycin oxime for the treatment of *C. auris* infections. Our *in vitro* results are encouraging. Indeed, we observed a significant reduction of MIC (i.e. below the tentative fluconazole MIC breakpoints proposed by the US CDC https://www.cdc.gov/candida-auris/hcp/laboratories/antifungal-susceptibility-testing.html) against most *C. auris* isolates, including some harbouring *ERG11* mutations (Figure S1). This effect was achieved at milbemycin oxime concentrations of 2–4 mg/L. Pharmacokinetic profiles of milbemycin oxime in dogs receiving the drug as tablets at therapeutic antiparasitic concentrations (0.25–1 mg/kg) showed low bioavailability, with peak plasma concentrations usually below 1 mg/L.[17,18] However, higher bioavailability has been achieved with novel oral formulations (i.e. nanoemulsion) or by the IV route (peak concentrations 2–10 mg/L with a half-life of about 20 h).[19] Higher doses of milbemycin oxime (i.e. 2.5 mg/kg) have been well tolerated in a murine model of *Candida glabrata* infection and were sufficient to potentiate the effect of fluconazole.[11] To our knowledge, there is currently no pharmacokinetic studies of milbemycin oxime in humans. However, a close compound from the milbemycin family, moxidectin, is approved for the treatment of onchocerciasis in humans.[20] Taken together, these data suggest that milbemycin oxime may represent an interesting drug candidate for adjunctive therapy of invasive candidiasis due to azole-resistant *Candida* spp., including *C. auris*.

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Supplementary data

Figure S1 is available as Supplementary data at *JAC-AMR* Online.

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