In vitro interaction of isavuconazole and anidulafungin against azole-susceptible and azole-resistant *Aspergillus fumigatus* isolates

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Background: The voriconazole and echinocandin combination has been found to be synergistic *in vitro* and *in vivo* against most *Aspergillus fumigatus* isolates, both with a WT azole phenotype and an azole-resistant phenotype. The interaction between isavuconazole and echinocandins is less well studied. This is especially true for azole-resistant isolates.

Objectives: We investigated the *in vitro* interaction between isavuconazole and anidulafungin for 30 *A. fumigatus* isolates including 18 azole-resistant isolates with various isavuconazole resistance phenotypes.

Methods: The isavuconazole/anidulafungin interaction was studied by using an adapted EUCAST-based 2D (12×8) chequerboard broth microdilution colorimetric assay using XTT. The interaction was analysed by FIC index (FICi) analysis and Bliss independence (BI) interaction analysis.

Results: Both the FICi analysis and the BI analysis showed synergistic interaction between isavuconazole and anidulafungin for the majority of WT and azole-resistant isolates. As we did not see significant beneficial effects of combination therapy in TR$_{46}$/Y121F/T289A isolates at clinically achievable drug concentrations, it is unlikely that TR$_{46}$/Y121F/T289A infections would benefit from isavuconazole and anidulafungin combination therapy.

Conclusions: In regions with high azole resistance rates this combination may benefit patients with WT disease, azole-resistant invasive aspergillosis and those with mixed azole-susceptible and azole-resistant infection, but may not be beneficial for aspergillosis due to isolates with high isavuconazole resistance, such as TR$_{46}$/Y121F/T289A isolates.

Introduction

It is estimated that worldwide approximately 300 000 people suffer from invasive aspergillosis annually.[1] Voriconazole and isavuconazole are currently recommended as first-line agents for the treatment of patients with invasive aspergillosis.[2–4] However, the emergence of azole resistance in *Aspergillus fumigatus* greatly challenges the management of invasive aspergillosis. As resistance selection may take place in the environment, any patient is at risk and may present with azole-resistant disease. Environmental resistance is dominated by isolates that harbour a tandem repeat (TR) in the promoter region of the *cyp51A* target gene combined with amino acid alterations, i.e. TR$_{34}$/L98H and

TR$_{46}$/Y121F/T289A. Although these mutations alter the activity of all medical triazoles, TR$_{34}$ isolates are highly resistant to itraconazole, whereas TR$_{46}$ isolates are highly resistant to voriconazole.[5,6]

In regions with endemic resistance levels exceeding 10%, an international expert panel recommended to move away from azole first-line therapy and treat with liposomal amphotericin B, or to use voriconazole combined with an echinocandin.[7] The voriconazole and echinocandin combination was found to be synergistic *in vitro* and *in vivo* against most *A. fumigatus* isolates with a WT azole phenotype.[8–19] Importantly, similar beneficial effects of this combination were also seen in most azole-resistant *A. fumigatus* isolates.[8–12,18] However, these studies involved isolates with

voriconazole EUCAST MICs of 0.5–8 mg/L and it remains unclear whether an azole and echinocandin combination can be beneficially used in isolates with high resistance to voriconazole (MICs >8 mg/L). There is a risk that in patients with infection due to highly voriconazole- or isavuconazole-resistant isolates the benefit of combination therapy is lost and therapy is based on the echinocandin efficacy, which is suboptimal in (neutropenic) patients with invasive aspergillosis.[20,21]

Isavuconazole was shown to be non-inferior to voriconazole for the treatment of invasive aspergillosis and thus received an A-I recommendation in most guidelines, similar to that of voriconazole.[2–4,22] Given the favourable pharmacokinetics and toxicity profile of isavuconazole, the drug is increasingly used in patients with invasive aspergillosis. There is currently limited evidence to support isavuconazole and echinocandin combination therapy. To the best of our knowledge, only two studies to date have investigated the interaction of isavuconazole with an echinocandin and only five azole-resistant *A. fumigatus* isolates (three with TR$_{34}$/L98H mutation, one with G54W and one with P216L) were tested.[23,24] Therefore, we investigated the *in vitro* interaction between isavuconazole and anidulafungin for 30 *A. fumigatus* isolates including 18 azole-resistant isolates with various isavuconazole resistance phenotypes.

Materials and methods

Isolates

A total of 30 *A. fumigatus* isolates were used in the study, of which 18 were azole-resistant with a known *cyp51A* mutation [6 isolates with TR$_{34}$/L98H, 2 with TR$_{46}$/Y121F/T289A, 3 with G54 (G54R, G54E, G54E), 4 with M220 (M220I, M220K, M220R, M220V), 1 with P216L, 1 with G448S and 1 with G138C] and 12 phenotypically azole WT isolates.

Antifungal agents and XTT/menadione

Isavuconazole (Sigma–Aldrich, St Louis, MO, USA) and anidulafungin (Sigma–Aldrich) were dissolved in DMSO at a concentration of 3200 mg/L. Antifungals were further diluted in RPMI 1640 to obtain the desired concentrations (described below) by serial dilutions according to the ISO scheme for preparing antifungal dilution series.[25,26] Final antifungal concentrations ranged from 0.008 to 8 mg/L for isavuconazole and 0.008 to 0.5 mg/L for anidulafungin.

XTT (Sigma–Aldrich) was dissolved in sterile saline to produce a concentration of 500 mg/L. Menadione (Sigma–Aldrich) was first diluted in absolute ethanol at a concentration of 10 000 mg/L and was then added to the XTT solution to produce an XTT/menadione solution of 31.25 μM menadione and 500 mg/L XTT. Fifty microlitres of XTT/menadione solution was added to the wells after 48 h of incubation, resulting in final concentrations of 100 mg/L XTT and 6.25 μM menadione. The latter concentrations of XTT and menadione were chosen as these were previously found to be optimal for caspofungin.[27]

MIC determination and chequerboard XTT assay

The isavuconazole/anidulafungin interaction was studied by using an adapted EUCAST-based 2D (12×8) chequerboard broth microdilution colorimetric assay using XTT, as described previously.[28] Four-fold the final concentration of isavuconazole or anidulafungin was diluted in 2-fold concentrated medium. Fifty microlitres of both isavuconazole and anidulafungin concentrations were added to 96-well microtitre plates to obtain 2-fold final concentrated medium and antifungal

combinations. Prepared microtitre plates were stored at −70°C for a maximum of 4 weeks. Spore suspensions were prepared spectrophotometrically (Spectronic GENESYS 20) in sterile saline supplemented with 0.1% Tween 20 (Sigma–Aldrich) and further diluted in sterile saline to obtain a dilution of 1×10^6–4.2×10^6 conidia. One hundred microlitres of inoculum was added to the 96-well plates and the plates were incubated at 35–37°C. Microtitre plates were then incubated for 48 h. The activities of isavuconazole and anidulafungin were determined in accordance with the EUCAST reference methods.[25] The anidulafungin minimal effective concentration (MEC) was read visually after 24 h and the isavuconazole MIC was read visually after 48 h. After 48 h, 50 μL of prepared XTT/menadione solution (to produce final concentrations of 6.25 μM menadione and 100 mg/L XTT) was added to the wells and incubated for a further 2 h. Conversion of XTT to formazan was measured as OD with a microtitre plate reader (Anthos Labtec Instruments HT3 Microtiter Plate Reader) at 450 nm with 630 nm as reference. Background OD from a similar incubated microtitre plate with added XTT/menadione solution but without inoculum was subtracted from the measurements.[11,28] Measurements were performed in triplicate. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls.

Interaction models

The interaction was analysed using two interaction models. The first was the FIC index (FICi) with inhibition endpoints based on XTT metabolism, where an FICi of ≤0.5 indicates synergism and an FICi of >4.0 indicates antagonism. An FICi between 0.5 and 4 indicates indifferent interaction. Two different endpoints were used. FIC1 was defined as 90% inhibition of formazan production compared with growth control. This endpoint, based on inhibition of formazan production, corresponded well to the isavuconazole visual MIC endpoint. FIC2 was defined as 75% inhibition compared with the growth control. Offscale MICs were adjusted to the next 2-fold higher or 2-fold lower concentration. FIC1 and FIC2 were calculated for each replicate individually. When only 2/3 samples were in accordance, the interaction conclusion of the majority was used for the isolate.

The second model used was the 3D surface interaction model based on the Bliss independence (BI) theory as previously described.[11,29] The expected growth was calculated based on the BI model using Excel (2010) and GraphPad Prism (5.03). The expected growth was subtracted from observed growth for all three replicates and the mean was used as the BI result. BI results were tested for significance using a *t*-test. The sum of all significant BI results was used to calculate Bliss interaction (ΔE), where a positive ΔE indicates synergism and a negative ΔE indicates antagonism. The zero surface indicates no interaction.

Results

The interaction between isavuconazole and anidulafungin was synergistic for the majority of isolates (Table 1). As the MEC endpoint for anidulafungin without isavuconazole, which corresponded to 50% inhibition, was out of range for most isolates, this endpoint was excluded from the analysis. The FICi could not be determined for TR$_{46}$/Y121F/T289A isolates as all defined FIC endpoints were out of the tested range. For all other isolates combined, the FIC1 was between 0.27 and 1.02. The geometric mean (GM) of FIC1 was between 0.5 and 4 when isolates were analysed in subgroups (WT, isolates with TR$_{34}$/L98H and isolates with *cyp51A* point mutations, respectively). The interaction was synergistic for 2 isolates and indifferent for 26 isolates using the FIC1 endpoints. No antagonism was observed. The FIC2 endpoint ranged between 0.002 and 1.02. The FIC2 GMs of subgroups (WT, isolates with TR$_{34}$/L98H and isolates with *cyp51A*

point mutations) were <0.5. The interaction was synergistic for 7/12 WT isolates, 6/6 isolates with TR$_{34}$/L98H and 7/10 isolates with *cyp51A* point mutations. No antagonism was observed (Table 1). TR$_{46}$/Y121F/T289A isolates were excluded as all FIC endpoints were offscale and interaction based on FIC analyses could not be calculated. The results of the FIC analysis were confirmed with BI analysis, with a mean ΔE of 138%, indicating synergism (Table 1). Five isolates had an antagonistic interaction according to the BI analysis, while 24 had a synergistic interaction (Table S1, available as Supplementary data at *JAC* Online). The 3D surface diagram is displayed for the WT isolate AZN8196 with an isavuconazole MIC of 0.5 mg/L (Figure 1). The mean ΔE was higher for WT isolates compared with TR$_{34}$/L98H isolates and isolates with *cyp51A* point mutations, but not significantly different. Only minor interaction was seen for TR$_{46}$/Y121F/T289A isolates. At the range of concentrations used in the experiment, no favourable results were observed. The ΔE was 0% for one TR$_{46}$ isolate while only minor antagonistic interaction was observed for the other isolate (ΔE of −19%).

Discussion

We found predominantly synergistic *in vitro* interaction between isavuconazole and anidulafungin for most azole-susceptible and azole-resistant *A. fumigatus* isolates. Both statistical methods, FIC analyses and BI analyses were in concordance. However, the FIC results varied between the FIC endpoints that were defined.

In vitro and *in vivo* data already support a beneficial interaction between voriconazole and anidulafungin for invasive aspergillosis.[8–17,19] Similar results were found for posaconazole or itraconazole with an echinocandin.[29–33] This study indicates a favourable

effect of combination therapy with isavuconazole and anidulafungin for *A. fumigatus* for both azole-susceptible and -resistant isolates with isavuconazole MICs between 1 and 8 mg/L.

Importantly, the favourable effects of combination therapy were not observed in our interaction model for isolates with TR$_{46}$/Y121F/T289A mutations having isavuconazole MICs >8 mg/L. To investigate drug interactions, the range of drug combinations used in the interaction model should be around the MIC of the drug. However, the precise isavuconazole MICs for TR$_{46}$/Y121F/T289A isolates are not known as the standard susceptibility testing range is truncated at 8 or 16 mg/L for isavuconazole and the exposure needed to treat isolates with such high MICs is not achievable in humans. Therefore, we did not extend the range of isavuconazole drug concentrations tested in our *in vitro* model. Thus, we cannot exclude a synergistic interaction between isavuconazole and anidulafungin in isavuconazole highly resistant *A. fumigatus* isolates. As we did not see significant beneficial effects of combination therapy at clinically achievable drug concentrations, it is unlikely that TR$_{46}$/Y121F/T289A infections would benefit from isavuconazole and anidulafungin combination therapy. This implies that when using isavuconazole plus anidulafungin for infections with TR$_{46}$/Y121F/T289A, only the efficacy of the echinocandin remains, which is suboptimal.[20,21] Thus, in geographic regions with a high incidence of TR$_{46}$/Y121F/T289A or in cases with documented TR$_{46}$/Y121F/T289A infection, liposomal amphotericin B alone, or in combination with an azole, should be considered.

The *in vitro* results of FIC2 and the BI analysis of this study are comparable to the *in vitro* results of the combination of voriconazole and anidulafungin that was tested with the same methodology[11] and confirmed in a mouse model of invasive
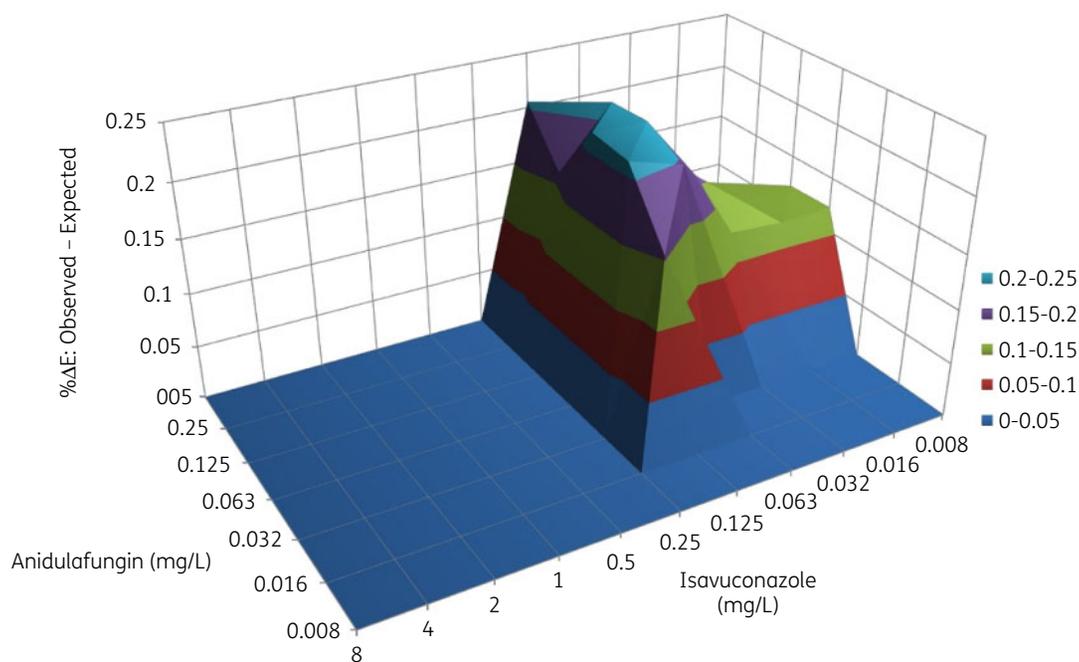

Figure 1. BI surface diagram for the combination of isavuconazole and anidulafungin for *A. fumigatus* isolate AZN8196. The 0 surface indicates no interaction whereas the surface above 0 indicates synergistic interaction. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. FIC and BI analysis of 30 azole-susceptible or -resistant *A. fumigatus* isolates

No. of isolates	Resistance genotype	ISA MIC (mg/L)[a]	FIC1			FIC2			BI, sum ΔE, mean (range)
			synergism (≤0.5)	indifference (0.5–4)	antagonism (>4)	synergism (≤0.5)	indifference (0.5–4)	antagonism (>4)	
6	TR$_{34}$/L98H	7.12 (4–>8)		6		6			89.33 (−24 to 408)
2	TR$_{46}$/Y121F/T289A	>8 (>8)		OOR			OOR		−9.5 (−19 to 0)
10	*cyp51A* point mutations	0.63 (0.25–8)	2	8		7	3		89 (−109 to 362)
12	WT isolates	0.59 (0.25–4)		12		7	5		201.83 (−112 to 522)

ISA, isavuconazole; OOR, out of range.
[a]Values shown are GM (range).

aspergillosis.[10] However, when we used the FIC1 endpoint the interactions were predominantly indifferent whereas most interactions of voriconazole and anidulafungin remained synergistic at the FIC1 endpoint. However, the difference in mean FICi was only small (0.6 for isavuconazole/anidulafungin versus 0.4 for voriconazole/anidulafungin), which challenges the clinical significance of the observed difference.[11] Therefore, we anticipate similar beneficial effects of isavuconazole/anidulafungin when used *in vivo* compared with voriconazole/anidulafungin.

Similar interactions of isavuconazole/anidulafungin were found in an *in vitro* study that evaluated the combination of isavuconazole and anidulafungin for five azole-resistant *A. fumigatus* isolates.[24] That study also found that the interaction was predominantly indifferent when complete inhibition was used as the endpoint (e.g. FIC1 in the current study), while 2/5 isolates showed synergistic interaction when incomplete inhibition (e.g. FIC2) was used as the FIC endpoint. This indicates that addition of anidulafungin does not reduce the MIC of isavuconazole, but does add to the inhibitory effect at subMIC levels.

Although the combination of voriconazole with anidulafungin did not result in significantly better efficacy in a randomized control trial, *post hoc* analysis indicated that the combination may be beneficial for a subgroup population with *Aspergillus* galactomannan indices between 0.5 and 1.5.[34] Thus, in regions with high azole resistance rates the combination may benefit patients with WT disease, azole-resistant invasive aspergillosis and those with mixed azole-susceptible and azole-resistant infection, but may not be beneficial for aspergillosis due to isolates with high isavuconazole resistance such as isolates with TR$_{46}$/Y121F/T289A. As similar *in vitro* interactions were found for isavuconazole and anidulafungin compared with the voriconazole and anidulafungin combination, the results of this study indicate that voriconazole can be replaced by isavuconazole when used in combination with an echinocandin.

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Transparency declarations

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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