In vitro combination of anidulafungin and voriconazole against intrinsically azole susceptible and resistant Aspergillus spp.

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Summary
In vitro interaction of anidulafungin with voriconazole was tested by a microdilution broth checkerboard technique and an agar diffusion method against 30 Aspergillus spp. clinical isolates belonging to 5 different species. By using a complete inhibition endpoint, indifferent interactions were observed for 97% of the isolates by the checkerboard technique (FIC index from 0.5 to 2) and for 100% of the isolates by agar diffusion method (variation of -2 to +1 log₂ dilutions).
Voriconazole is the first-line therapy of invasive aspergillosis (19, 37). Nevertheless, mortality remains high due to different factors (4, 7, 21, 24, 27). Although azoles are very active in vitro against *A. fumigatus* (18), several studies have reported de novo or acquiredazole-resistance (9, 11, 15). While in selected populations of patients the frequency of these azole-resistant isolates may be low (2, 13), an emergence of azole-resistance is reported in Europe in clinical and environmental *A. fumigatus* isolates (8, 31, 34). Moreover, other pathogenic *Aspergillus* species are naturally azole-resistant (6, 33). For these emerging species (26, 35), the first-line voriconazole therapy may be not recommended (37) and therefore, combination therapy may be of interest.

Since a clinical trial evaluating the efficacy of voriconazole plus anidulafungin vs. voriconazole as first-line treatment in invasive aspergillosis has recently been completed, we aim to evaluate the in vitro interaction of voriconazole with anidulafungin against different *Aspergillus* species.

Thirty *Aspergillus* spp. clinical isolates belonging to intrinsically azole susceptible and resistant species (11 *A. fumigatus*, 5 *A. flavus*, 5 *A. terreus*, 5 *A. calidoustus*, 3 *A. nidulans*, and 1 *A. sydowii*) were tested. Species identification was performed by sequencing the betatubulin and/or calmodulin gene (1) as recommended (5). Drug combinations were tested by two different techniques: a broth microdilution checkerboard procedure based on the Clinical Laboratory Standards Institute (CLSI) M38A2-document (10) and an agar diffusion test (Etest®) (36).

For checkerboard studies, the final concentrations of voriconazole (Pfizer Inc., New York, USA), and anidulafungin (Pfizer) were 0.03 to 2 µg/ml, and 0.0001 to 0.06 µg/ml, respectively. Spore suspensions were counted in a haemocytometer and adjusted to the required concentration. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included as quality controls. MICs were determined visually after 48h of incubation at
35°C in two independent experiments. At first, MICs were determined with a complete inhibition endpoint. Alternatively, a partial inhibition endpoint (50% inhibition for voriconazole and MECs, determined as previously described (3) for anidulafungin alone or for both drugs in combination) was also used. Fractional inhibitory concentrations (FIC) indices (16) were calculated and drug interactions were defined as synergistic, additive (i.e. no interaction / indifferent) or antagonistic when the FIC index was ≤ 0.5, > 0.5 and ≤ 4, or > 4, respectively (25).

Antifungal susceptibility was also evaluated by Etest® (AB Biodisk, Solna, Sweden). RPMI agar plates were inoculated with a spore suspension adjusted to 10^6 conidia/ml. For combination studies anidulafungin Etest® strips were placed on RPMI agar, discarded after 1 hour, and voriconazole strips were placed at the same position. After incubation at 35°C for 48h, MICs were determined visually with either a complete or partial inhibition endpoint (17). Experiments were run in duplicate. Synergy and antagonism were defined respectively as a decrease or an increase of ≥ 3 dilutions of the resultant MIC (20).

The activity of voriconazole and anidulafungin either alone or in combination was first determined by checkerboard microdilution (Table 1). Voriconazole MICs ranged from 0.06 to 4 µg/ml, with differences between species. Voriconazole MICs against *A. fumigatus*, *A. flavus*, and *A. terreus* ranged from 0.25 to 1, those of *A. calidoustus* were higher, ranging from 2 to 4 µg/ml. All isolates from all species exhibited low anidulafungin MECs (range: 0.001 to 0.06 µg/ml). Overall, and whatever the triazole susceptibility, the combination of voriconazole and anidulafungin showed no interaction (FIC indices between 0.50 and 2) for 97% of the isolates by using a complete inhibition (MIC-0) endpoint. A synergistic interaction was observed for only one *A. sydowii* isolate (FIC = 0.5). When a less stringent endpoint (MEC) was used, a synergistic interaction was observed for one *A. calidoustus* (FIC = 0.28) and an antagonistic interaction was observed for 2 *A. flavus* isolates (FIC = 4.5) and one *A.
*nidulans* isolate (FIC=9) while no interaction (FIC indices between 0.75 and 2.5) was observed for 87% of the isolates. MICs of the replicates were within \( \pm 1 \log_2 \) dilution in 88% of the cases. Results of agar diffusion tests are shown in Table 2. Voriconazole MICs ranged from 0.03 to 8 µg/ml with higher MICs against *A. calidoustus* (range: 2 to 8 µg/ml) than against *A. fumigatus, A. flavus*, and *A. terreus* (range: 0.125 to 0.5 µg/ml). All isolates exhibited low anidulafungin MICs (range: 0.002 to 0.008 µg/ml). In combination, by using a complete inhibition endpoint, voriconazole and anidulafungin showed no interaction: for all isolates, the voriconazole MICs in combination were within \( \pm 2 \) Log\(_2\) dilutions of the voriconazole MICs tested alone. With a partial inhibition endpoint, no interaction was observed for 93% of the isolates and an increase of 3 \( \log_2\) dilutions was noted for two *A. calidoustus* isolates. Typical patterns observed by Etest® are shown on Figure 1. MICs were within \( \pm 1 \) Log\(_2\) dilution for all the replicates.

In the present study the combination of voriconazole and anidulafungin rarely showed an interaction. Because *in vitro* antifungal interaction against filamentous fungi remains difficult to test (23), we used two unrelated techniques. Although Etest® assesses interactions at a certain concentration ratio of drugs in combination whereas checkerboard microdilution assay evaluate interactions at different concentration ratios, the results obtained by the two techniques were globally similar. Etest® was used previously for testing antifungal combinations (12, 14, 20) but this is the first study, to our knowledge, that used Etest® to evaluate the interaction between anidulafungin and voriconazole. By including intrinsically azole-susceptible and resistant *Aspergillus* species, we demonstrated that the lack of interaction between the two drugs is not dependent on the azole susceptibility of the isolates.

Previous studies evaluating the interaction between anidulafungin and voriconazole against *Aspergillus* spp. showed conflicting results (28-30, 32). In one *in vitro* study, almost no interaction was found against different species of *Aspergillus* (28). In another study,
synergistic interactions were found (30). Differences between studies may be related to different methodological approaches. In particular, limitations of the present study may be related to the visually determined MIC endpoints which may be subjective, particularly for combination with echinocandins, and the wide range of FIC indices cutoff used to detect synergy. Recently, a randomized trial evaluating the efficacy of anidulafungin and voriconazole in combination for primary therapy of invasive aspergillosis has been completed and showed that the combination was not associated with a lower risk of early mortality compared to voriconazole alone (22).

In conclusion, our results showed that combination of anidulafungin with voriconazole is not synergistic \textit{in vitro} against various triazole-susceptible or resistant \textit{Aspergillus} species.

\textbf{Acknowledgements}

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and triple combinations of antifungal drugs against Aspergillus fumigatus and 

testing of sequential isolates of *Aspergillus fumigatus* recovered from treated patients.


Table 1: Drug interaction of voriconazole in combination with anidulafungin against 30 isolates of *Aspergillus* spp. determined by checkerboard microdilution broth technique and using two different endpoints.

<table>
<thead>
<tr>
<th>Species (number of isolates)</th>
<th>MIC/MEC range (GM) (µg/ml) of the drugs alone&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FIC index range (GM) for the combination VRZ / ANI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VRZ</td>
<td>ANI</td>
</tr>
<tr>
<td>A. fumigatus (11)</td>
<td>0.25 (0.25)</td>
<td>0.001-0.03 (0.012)</td>
</tr>
<tr>
<td>A. flavus (5)</td>
<td>0.5-1 (0.87)</td>
<td>0.008-0.06 (0.024)</td>
</tr>
<tr>
<td>A. terreus (5)</td>
<td>0.5 (0.5)</td>
<td>0.008-0.015 (0.01)</td>
</tr>
<tr>
<td>A. calidoustus (5)</td>
<td>2-4 (3.48)</td>
<td>0.008-0.06 (0.024)</td>
</tr>
<tr>
<td>A. nidulans (3)</td>
<td>0.06-0.125</td>
<td>0.002-0.008</td>
</tr>
<tr>
<td>A. sydowii (1)</td>
<td>0.25</td>
<td>0.015</td>
</tr>
<tr>
<td>All species (30)</td>
<td>0.06-4 (0.48)</td>
<td>0.001-0.06 (0.014)</td>
</tr>
</tbody>
</table>

<sup>a</sup>GM, Geometric mean; VRZ, voriconazole; ANI, anidulafungin. MIC/MEC were determined visually as the concentration that gave 100% of inhibition (MIC-0) for VRZ and abnormal hyphal growth (MEC) for ANI. <sup>b</sup>Corresponding to the lowest FIC index.
**Table 2:** Drug interaction of voriconazole in combination with anidulafungin against 30 isolates of *Aspergillus* spp. determined by Etest® and using two different endpoints.

<table>
<thead>
<tr>
<th>Species (number of isolates)</th>
<th>MIC range (GM) (µg/ml) of the drugs alone&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC range (GM) (µg/ml) of the combination VRZ / ANI</th>
<th>Variation range (median) of MIC (Log&lt;sub&gt;2&lt;/sub&gt;-dilutions)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VRZ</td>
<td>ANI</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (11)</td>
<td>0.125 (0.125)</td>
<td>0.002-0.008 (0.003)</td>
<td>0.06-0.125 (0.10)</td>
</tr>
<tr>
<td><em>A. flavus</em> (5)</td>
<td>0.25-0.5 (0.33)</td>
<td>0.002-0.004 (0.002)</td>
<td>0.125-0.25 (0.19)</td>
</tr>
<tr>
<td><em>A. terreus</em> (5)</td>
<td>0.125-0.5 (0.25)</td>
<td>0.002-0.004 (0.003)</td>
<td>0.125-0.25 (0.14)</td>
</tr>
<tr>
<td><em>A. calidoustus</em> (5)</td>
<td>2-8 (2.64)</td>
<td>0.002-0.004 (0.003)</td>
<td>0.5-4 (1)</td>
</tr>
<tr>
<td><em>A. nidulans</em> (3)</td>
<td>0.03-0.06</td>
<td>0.002-0.004</td>
<td>0.03-0.06</td>
</tr>
<tr>
<td><em>A. sydowii</em> (1)</td>
<td>0.125</td>
<td>0.002</td>
<td>0.125</td>
</tr>
<tr>
<td>All species (30)</td>
<td>0.03-8 (0.25)</td>
<td>0.002-0.008 (0.003)</td>
<td>0.003-4 (0.16)</td>
</tr>
</tbody>
</table>

<sup>a</sup> GM, geometric mean; VRZ, voriconazole; ANI, anidulafungin. MICs were determined visually as the concentration that gave 100% of inhibition for VRZ and partial inhibition for ANI. <sup>b</sup> Number of log₂-dilution differences between MIC of the drug alone and in combination.
Figure legends:

Figure 1: Agar diffusion test of the combination of voriconazole with anidulafungin against *A. fumigatus* FUM02 (panel A), *A. flavus* FLA05 (panel B), *A. terreus* TER01 (panel C), and *A. calidoustus* UST01 (panel D). For combination, an anidulafungin Etest® strip was placed on the agar surface, left for 1h, removed and a voriconazole strip was then applied.