Anidulafungin in Combination with Amphotericin B against Aspergillus fumigatus.

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ABSTRACT

We investigated the effects of anidulafungin alone and in combination with amphotericin B against *Aspergillus fumigatus*. Indifference was the only type of interaction observed in vitro. Anidulafungin at 1 and 5 mg/kg/day, amphotericin B at 1 mg/kg/day and combination therapy prolonged the survival of mice with invasive aspergillosis. Anidulafungin at 5 mg/kg/day, alone and in combination, reduced the kidney fungal burden. Overall, the combination was not superior to the most active single drug.
The high mortality rate of invasive aspergillosis has driven recent efforts to determine the efficacy of combination therapy in the treatment and management of those infections (1,6,7,16,17,19,20,23,29). Therefore, in this study, the in vitro and in vivo efficacy of the new echinocandin anidulafungin (AFG) was analysed alone and in combination with amphotericin B (AMB) against *Aspergillus fumigatus*.

Three clinical strains (F2, F3, F4) isolated from BALs of hematological patients were identified to species level by conventional methods (24).

AMB was used as pure powder (Sigma) for in vitro studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb) for in vivo studies. Pure powder of AFG (Pfizer) was dissolved in DMSO and further diluted in the test medium or sterile saline solution for in vitro and in vivo studies, respectively.

MICs and MECs were determined in RPMI 1640 medium by the CLSI M38-A2 broth microdilution method (10,12). Either for susceptibility and checkerboard assays, the MICs/MECs were read visually at 24- and 48- hrs (10,25). Drug interactions were classified as synergistic, indifferent, or antagonistic based on the fractional inhibitory concentration (FIC) index (16).

Minimum fungicidal concentration (MFC) was considered the concentration of antifungal agents alone or in combination that yielded no growth (27).

Metabolic activities of conidia and hyphae were assessed in RPMI 1640 medium with L-glutamine, without phenol red and NaHCO$_3$ by XTT assay (Tox-2, Sigma) (2,3,21).

An experimental CD1 mice (Charles River, Calco) model of invasive aspergillosis was performed following the previously reported procedures (4). A total of three separate in vivo studies were performed by injection of *A. fumigatus* F3 isolate. The drug treatments were started 2 hrs after the infection. AMB at 0.5, 1 mg/kg/day, AFG at 1, 5 mg/kg/day and combination regimens were administered intraperitoneally.
In survival studies, the mice were treated daily from day 0 to day 4 and observed for 10 consecutive days.

Brain and kidney fungal burdens were determined at day 4 postinfection by CFU count and qPCR based on that described by Bowman et al. (5).

Histopathology analysis was performed at day 4 postinfection (4). The number of fungal microabscesses was evaluated in 20 consecutive microscopic fields. Each section was classified based on the number of fungal microabscesses as follows: absence (-), <5 (+), ≥5 to ≤20 (++), >20 (+++).

The in vitro results were analysed by either Mann-Whitney U test or Student’s t-test considering a P value of P<0.05 as significant. Survival and tissue burden studies were analysed by log rank and Mann-Whitney U test, respectively. Due to multiple comparison, a P value of <0.016 was considered as significant.

Our in vitro results are shown in table 1. Overall, AFG MECs were significantly lower than AMB MICs and indifference was the only type of interaction among the two drugs.

MFCs values were all >16 µg/ml for AFG, while the combination values were statistically lower than those of AFG alone, but not of AMB alone.

The metabolic activity studies either on conidia or on hyphae are presented in figure 1.

AFG, AMB and the combination regimens showed a dose-dependent reduction of metabolic activity against conidia, but generally the combination was not more effective than the most active drug alone. Against the hyphae, AMB showed a decreased activity, while AFG was not active. The combination showed to be effective, but not more than AMB alone.

The in vivo results are shown in figure 2. In studies #1 and #2, all drug regimens prolonged significantly the survival over that of control animals. In both studies, combination-treated group did not increase significantly the survival time with the respect to AMB- and AFG-treated groups.

In study #3, AFG at 5 mg/kg/day and the combination regimen, but not AMB at 0.5 mg/kg/day,
significantly prolonged the survival time with respect to the control group. Combination treatment did not extend survival beyond that of AFG treated group. Kidney and brain burden results are shown in Table 2. Only AFG at 5 mg/kg/day and the respective combination with AMB were effective at reducing the CFU or CE per gram of kidney tissues. No treatments were effective at reducing the brain burdens. Consistent with these data, a decreased number of fungal microabscesses were observed in kidney tissues, but not in brain tissues, of mice treated with AFG at 5 mg/kg/day (Figure 3). Our AFG MEC values were similar to those previously reported for \textit{A. fumigatus} isolates (12,22). In agreement with a previous study conducted by Philip \textit{et al.} (28), AFG used in combination with the polyene yielded an indifferent type of interaction. Our in vivo results showed that AFG given at 1 and 5 mg/kg/day were effective at prolonging the survival. These data correspond to those already reported in other experimental models of aspergillosis (26,32). Here, we found that the combination was not more effective than the most active drug alone in all three survival experiments. In terms of kidney tissue burdens, we found that AFG given at 5 mg/kg/day, but not at 1 mg/kg/day, reduced fungal burden with respect to untreated controls. The combination was not more active than AFG alone. Several published studies have already explored the effects of echinocandins other than AFG combined with various AMB formulations against \textit{Aspergillus} spp.. With the exception of two studies suggesting beneficial effects of combined therapies (i.e.: CAS+AMB, Micafungin+AMB) over the monotherapies (11,30), the remaining experiments showed that combinations did not enhance the effects of the most active single drug (8,9,13,15,18,31).

We showed that neither single drugs nor combinations were active in brain tissues. The lack of AFG efficacy in brains, but not in kidneys, might be explained by its pharmacokinetics features. Groll \textit{et al.} (14) have studied the AFG tissue distribution in healthy rabbits and reported an undetectable cerebrospinal fluid concentration. Overall, our results showed that the new
echinocandin AFG has the potential to be used as a therapeutical treatment against invasive aspergillosis. The combination therapy of AFG with AMB did not improve the outcomes analysed in the present study, although antagonism was not observed.
REFERENCES


Table 1. In vitro susceptibility tests of anidulafungin and amphotericin B alone and in combination, against three clinical isolates of *A. fumigatus*.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Drugs</th>
<th>Susceptibility results (µg/ml) reported as</th>
<th>Type of interactions by definition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>F2</td>
<td>AFG</td>
<td>.001</td>
<td>.001-.002</td>
</tr>
<tr>
<td></td>
<td>AMB</td>
<td>.5</td>
<td>.25-.5</td>
</tr>
<tr>
<td></td>
<td>AFG/AMB</td>
<td>.001/.03</td>
<td>.001-.002/0.03-.06</td>
</tr>
<tr>
<td>F3</td>
<td>AFG</td>
<td>.001</td>
<td>.001-.002</td>
</tr>
<tr>
<td></td>
<td>AMB</td>
<td>.25</td>
<td>.25-.5</td>
</tr>
<tr>
<td></td>
<td>AFG/AMB</td>
<td>.001/.03</td>
<td>.001/0.03</td>
</tr>
<tr>
<td>F4</td>
<td>AFG</td>
<td>.002</td>
<td>.001-.002</td>
</tr>
<tr>
<td></td>
<td>AMB</td>
<td>.25</td>
<td>.125-.5</td>
</tr>
<tr>
<td></td>
<td>AFG/AMB</td>
<td>.002/0.25</td>
<td>.001-.002/0.03-.25</td>
</tr>
</tbody>
</table>

1. Each testing was run in triplicates and repeated on 2 different days.
2. MIC, minimum inhibitory concentration as the lowest concentration of AMB that prevents any discernible growth (100% inhibition); MEC, minimum effective concentration, was read as the lowest concentration of AFG that led to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well; MFC, minimum fungicidal concentration was defined as the lowest concentration of antifungal compound yielding no growth.
3. The MEC values were reported as reading end points of the checkerboard assays.
4. The interaction was defined as synergistic if the FIC (fractional inhibitory concentration) index was less than or equal to 0.50, indifferent if the FIC index was greater than 0.50 and less than or equal to 4.0, and antagonistic if the FIC index was greater than 4.0.
Table 2. Fungal burden in tissues of *A. fumigatus*-infected mice measured by CFU and qPCR assays

<table>
<thead>
<tr>
<th>Challenge dose (conidia/mice)</th>
<th>Treatment</th>
<th>Brain burden</th>
<th>Kidney burden</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean log_{10} CFU/g of tissue ± SD</td>
<td>Mean log_{10} CE/g of tissue ± SD</td>
<td>Mean log_{10} CFU/g of tissue ± SD</td>
</tr>
<tr>
<td>3.5x10^5</td>
<td>Control</td>
<td>3.17 ± 0.44</td>
<td>4.49 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>AMB 1 mg/kg/day</td>
<td>2.51 ± 0.74</td>
<td>3.73 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>AFG 1 mg/kg/day</td>
<td>3.02 ± 0.83</td>
<td>4.50 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>AMB 1 + AFG 1</td>
<td>3.06 ± 0.60</td>
<td>4.78 ± 0.47</td>
</tr>
<tr>
<td>3.2x10^6</td>
<td>Control</td>
<td>3.73 ± 0.25</td>
<td>5.60 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>AMB 0.5 mg/kg/day</td>
<td>3.82 ± 0.22</td>
<td>5.88 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>AFG 5 mg/kg/day</td>
<td>4.08 ± 0.40</td>
<td>6.27 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>AMB 0.5 + AFG 5</td>
<td>4.16 ± 0.71</td>
<td>6.58 ± 0.98</td>
</tr>
</tbody>
</table>

The animals were infected with *A. fumigatus* #3 isolate (3.5x10^5 conidia/mouse and 3.2x10^6 conidia/mouse in study #2 and #3, respectively) and euthanized 3 days later. There were seven animals per group and fungal burdens of brains and kidneys were determined by measuring colony forming unit (CFU) or conidial equivalent (CE) per gram of tissue. Values represent the mean ± standard deviation. Asterisks indicate treatment groups with reduced fungal burdens over the controls (due to multiple comparison, *P* values of < 0.016 were considered statistically significant).
Figure 1. Percentage of metabolic activity of three clinical isolates of *Aspergillus fumigatus* (F2, F3, F4) at the stage of non-germinated conidia (A, at the top) and filamentous forms (B, at the bottom) detected by XTT assay. Anidulafungin (white bars), amphotericin B (striped bars) and the combination of the two antifungal agents (black bars) were tested to a concentration of 1/4X, 1X and 4X the respective MICs/MECs. The bars represent the means in percentage of the metabolic activity in presence of the drugs with respect to the growth controls. The error bars indicate the standard deviations of the means. Letters \(^{a}\) and \(^{b}\) indicate a reduced metabolic activity of the combination versus anidulafungin and amphotericin B alone, respectively \((P < 0.05)\). Each strain was tested in triplicate.
Figure 2. Survival of mice infected intravenously with *A. fumigatus* F3 clinical isolate. In study #1, the animals were infected with $1.5 \times 10^7$ conidia/mouse and treated with AMB at doses of 1 mg/kg/day, AFG at 1 mg/kg/day and the respective combination regimen; in study #2, the mice were infected with $3.5 \times 10^5$ conidia/mouse and treated with AMB at 1 mg/kg/day, AFG at 1 mg/kg/day and the respective combination; in study #3, the animals were infected with $3.2 \times 10^6$ *A. fumigatus* conidia/mouse and treated with AMB at 0.5 mg/kg/day, AFG at 5 mg/kg/day and the corresponding combination. The therapies were started 2 hrs postinfection (day 0) and continued through day 4 postinfection (5 consecutive days). There were from 9 to 16 mice in each group. Asterisks indicate groups with prolonged survival over controls (due to multiple comparison, *P* values of < 0.016 were considered statistically significant).
Figure 3. Histopathological sections of kidney and brain tissues stained with Grocott Gomori (original magnification x25) from mice infected with $3.2 \times 10^6$ conidia of *Aspergillus fumigatus* F3 isolate. Representative histopathological sections of kidney (top) and brain (bottom) tissues from control mice (C) and from mice treated for three consecutive days with AMB at 0.5 mg/kg/day and AFG at 5 mg/kg/day.