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## **In vitro and in vivo evaluation of antifungal combinations against azole-resistant *Aspergillus fumigatus* isolates**

Sana Jemel, Yannick Raveloarisaona, Anne-Laure Bidaud, Aicha Kallel, Jacques Guillot, Kalthoum Kallel, Françoise Botterel, Eric Dannaoui

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1 ***In vitro* and *in vivo* evaluation of antifungal combinations against azole-resistant**  
2 ***Aspergillus fumigatus* isolates**

3

4 Sana Jemel<sup>1,2,3</sup>, Yannick Raveloarisaona<sup>4,5</sup>, Anne-Laure Bidaud<sup>4,5</sup>, Elie Djenontin<sup>1,4</sup>, Aicha  
5 Kallel<sup>2,3</sup>, Jacques Guillot<sup>6,7</sup>, Kalthoum Kallel<sup>2,3</sup>, Françoise Botterel<sup>1</sup> and Eric Dannaoui<sup>1,4,5\*</sup>

6

7 <sup>1</sup> Université Paris Est Créteil, Dynamyc, F-94010 Créteil, France;

8 jemelsana.benayed@gmail.com; francoise.botterel@aphp.fr; eric.dannaoui@aphp.fr

9 <sup>2</sup> Université Tunis EL Manar, Faculté de médecine de Tunis, Tunis 1007, Tunisie;

10 kallelkalthoum@gmail.com

11 <sup>3</sup> UR17SP03, Centre Hospitalo-Universitaire La Rabta, Jabbari, Tunis 1007, Tunisie

12 <sup>4</sup> Unité de Parasitologie-Mycologie, Service de Microbiologie, APHP, Hôpital Européen  
13 Georges Pompidou, Paris, France

14 <sup>5</sup> Université de Paris-Cité, Faculté de Médecine, 75006 Paris, France

15 <sup>6</sup> Dermatology-Parasitology-Mycology, Oniris, 44300 Nantes, France; jacques.guillot@oniris-  
16 nantes.fr

17 <sup>7</sup> Univ. Angers, Univ. Brest, IRF, SFR ICAT, 49933 Angers, France

18

19 **\*Correspondence:** jemelsana.benayed@gmail.com

20

21 **Abstract:**

22 Azole resistance in *Aspergillus fumigatus* (Af) has become a widespread threat and a major  
23 concern for optimal management of patients with invasive aspergillosis (IA). Combination of  
24 echinocandins with azoles is an attractive alternative option for the treatment of IA due to  
25 azole-resistant Af strains. The aim of this study was to evaluate the *in vitro* and *in vivo*  
26 combination of caspofungin (CAS) with either voriconazole (VRZ) or posaconazole (PSZ). *In*  
27 *vitro* interactions were assessed by two methods, and an animal model of IA in *Galleria*  
28 *mellonella* was used for *in vivo* evaluation. Assessment of efficacy was based on larvae  
29 mortality. Groups of 10 larvae were infected by 3 clinical strains of Af (azole susceptible, AfS;  
30 PSZ resistant, AfR1; VRZ and PSZ resistant strain, AfR2). *In vitro*, combination of CAS and  
31 azoles was indifferent against AfS, and AfR2, and a synergy was found for AfR1. When  
32 compared to VRZ monotherapy, the combination of VRZ at 4 µg/larva with CAS at 4 µg/larva  
33 improved survival of AfR2-infected larvae (p=0.0066). Combination of PSZ at 4µg/larva with  
34 CAS at 4 µg/larva improved survival of AfR1-infected larvae compared to CAS (p=0.0002) and  
35 PSZ (0.0024) monotherapy. Antagonism was never observed. In conclusion, the combination  
36 of caspofungin with azoles is a promising alternative for the treatment of azole resistant  
37 strains of Af.

38

39 **Keywords:** *Aspergillus fumigatus*; antifungal combination; *Galleria mellonella*; voriconazole;  
40 posaconazole; caspofungin; azole-resistance

41

42 **Introduction**

43 *Aspergillus fumigatus* (Af) remains one of the most potent opportunistic fungal pathogens in  
44 humans. It causes a wide range of infections including invasive aspergillosis (IA), a severe

45 condition occurring classically in immunocompromised patients. More recently, other risk  
46 factors of IA, such as severe influenza (Schauwvlieghe et al., 2018; Verweij et al., 2020) or  
47 severe COVID-19 (Pasquier et al., 2021; Gangneux et al., 2022) have been recognized. IA is  
48 associated with high mortality (Latge and Chamilos, 2020; Thompson and Young, 2021),  
49 despite effective first line treatment based on azoles (Patterson et al., 2016; Ullmann et al.,  
50 2018). Azoles are inhibitors of the sterol 14  $\alpha$  demethylase enzyme, a key step in ergosterol  
51 biosynthesis pathway. However, extensive use of azole drugs in the prevention and  
52 treatment of fungal infections, and extensive use of fungicides in agriculture, have  
53 contributed to the emergence of azole resistance in Af (Lestrade et al., 2019b). Different  
54 mechanisms of azole resistance have been reported (Dudakova et al., 2017). The most  
55 important is related to changes in the target enzyme by mutation of its gene, but drug efflux  
56 has also been reported and resistance can be multifactorial (Dudakova et al., 2017; Rybak et  
57 al., 2019). The emergence of azole resistance in Af makes the management of invasive  
58 aspergillosis more complex (Verweij et al., 2015). Azole resistance has been associated with  
59 treatment failure and excess mortality (Lestrade et al., 2019a; Resendiz-Sharpe et al., 2019).  
60 Therefore, development of alternative treatment options for IA is necessary. The  
61 combination of an azole with an echinocandin is one of the therapeutic options (Verweij et  
62 al., 2015; Ullmann et al., 2018). The complete evaluation of the efficacy of this kind of  
63 combination is an essential step for the validation of the treatment. The aim of this study  
64 was to evaluate the *in vitro* and *in vivo* activity of caspofungin (CAS) in combination with  
65 voriconazole (VRZ) or posaconazole (PSZ). For *in vivo* evaluation, we used the *Galleria*  
66 *mellonella* model that has proven its contribution to the evaluation of antifungal efficacy for  
67 the treatment of IA (Forastiero et al., 2015; Maurer et al., 2015; Jemel et al., 2020; Jemel et  
68 al., 2021).

69

## 70 **Materials and Methods**

### 71 **Strains, medium and growth conditions**

72 Three clinical strains of Af, isolated from respiratory samples, were used in the present  
73 study. Identification was confirmed by sequencing part of the gene encoding beta-tubulin.  
74 The *CYP51A* gene and its promoter had been previously sequenced to determine the  
75 mutations involved in azole-resistance (Jemel et al., 2021). We included one azole-  
76 susceptible strain (AfS) with a wild type *CYP51A* sequence, one strain (AfR1) with a G54W  
77 mutation and one strain (AfR2) with a L98H point mutation in *CYP51A* in combination with a  
78 34-bp tandem repeat in the promoter (TR34/L98H).

79 Subcultures were performed on Sabouraud dextrose agar (VWR, Fontenay-sous-bois,  
80 France) with chloramphenicol (Sigma-Aldrich, Saint Quentin-Fallavier, France). They were  
81 incubated for 7 days at 37°C to obtain sufficient sporulation.

82

### 83 ***In vitro* susceptibility and interaction between caspofungin and azoles**

84 Antifungal susceptibility testing was performed by two methods: the reference microdilution  
85 broth technique following the recommendations of the Antifungal Susceptibility Testing  
86 Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST-  
87 AFST), and a concentration gradient strip commercial method (Etest®). EUCAST was  
88 performed as recommended (Arendrup et al., 2017). For azoles and amphotericin B,  
89 minimum inhibitory concentration (MIC) values were determined after 48h of incubation by  
90 using a complete inhibition endpoint. For CAS, minimal effective concentration (MEC)  
91 endpoints were determined. Gradient concentration strip method (Etest®, Biomérieux,

92 Marcy-l'Etoile, France) was performed according to the manufacturer instructions and MICs  
93 were read after 48h of incubation.

94 *In vitro* activity of the combination of CAS with either VRZ or PSZ was first evaluated by the  
95 EUCAST reference method modified for a broth microdilution checkerboard procedure  
96 (Vitale et al., 2005; Bidaud et al., 2021). Final concentrations ranged from 0.008 to 0.5 µg/mL  
97 for CAS, 0.008 to 4 µg/mL for VRZ and PSZ. The final inoculum size in the plates was 1-  
98  $2.5 \times 10^5$  (CFU) mL<sup>-1</sup>. Microplates were incubated at 37°C and read after 48h of incubation. A  
99 growth inhibition endpoint of 50% was used both for the drugs tested alone and in  
100 combination. The experiments were performed in triplicate in each of two independent  
101 experiments. Data were first analyzed by calculation of the fractional inhibitory  
102 concentration index (FICI) interpreted as follow: synergy for FICI ≤ 0.5, no interaction for FICI  
103 between 0.5 and 4, and antagonism for FICI > 4 (Odds, 2003). A Bliss independence-based  
104 method was also used as previously described (Meletiadiis et al., 2005). Two parameters  
105 were calculated: the sum (ΣSSI) and the mean (MSSI) of percentages for all statistically  
106 significant interactions. Synergy was defined by a ΣSSI >200% and/or a 95% confidence  
107 interval of MSSI that did not include 0.

108 Activity of the combinations was also evaluated by a gradient concentration strip method  
109 (Etest®) as described previously (Vitale et al., 2005; Bidaud et al., 2021). Briefly, after  
110 inoculation of RPMI plates, one strip of VRZ or PSZ were placed on the agar surface for one  
111 hour, removed, and a strip CAS was applied exactly on the same position. MICs of the drugs  
112 alone and in combination were read after 48h of incubation.

113

114 ***Galleria mellonella* inoculation and treatment**

115 ***Galleria mellonella* infection**

116 Larvae of *G. mellonella* (Kreca® Ento-Feed BV, Ermelo, Netherlands) were used throughout  
117 the experiments. In each set of experiments, larvae were randomly distributed in groups of  
118 10 animals.

119 After culture of the three Af strains, the inoculum was prepared in phosphate-buffered  
120 saline containing 0.01% of Tween 20 (PBST), and spore suspensions were adjusted to the  
121 required concentration by counting conidia in a hemocytometer. Lethal doses 90% (LD<sub>90</sub>) of  
122 each Af strain were previously determined (Jemel et al., 2021). The injection was carried out  
123 with 10 µL in the ventral side of the last proleg by using a Hamilton® syringe.

124 **Drug preparation**

125 For treatment experiments, VRZ (Vfend® [Pfizer]) and PSZ (Noxafil® [MSD]) were dissolved in  
126 9‰ saline to obtain a stock solution at 10 mg/mL and 18 mg/mL, respectively. Required  
127 dosage was obtained by further dilutions in 9‰ saline. For CAS (Candidas® [MSD]), powder  
128 was dissolved in 10 mL of sterile distilled water to obtain a stock solution at 5 mg/mL and  
129 further dilutions were performed in 9‰ saline.

130 **Caspofungin and posaconazole monotherapy**

131 Groups of 10 larvae were infected by DL<sub>90</sub> of each Af strains. Two hours after infection,  
132 larvae were treated by injection in the ventral side of animal. CAS or PSZ were used at 1, 2, 4  
133 and 8 µg/larva. Larval survival was monitored daily for 7 days. Two control groups were  
134 used, the first group consisted of infected larvae inoculated with 9‰ saline at 2h after  
135 infection and the second group (to assess toxicity) was only inoculated with the highest  
136 doses of CAS or PSZ (8 µg/larva). All experiments were performed two times and results  
137 were pooled for analysis.

138 **Treatment combination of voriconazole or posaconazole and caspofungin**

139 Solutions of VRZ or PSZ (0.8 µg/µL) and CAS at 0.2, 0.4 and 0.8 µg/µL was obtained by  
140 carrying out dilutions in 9‰ saline. At equal volume and before inoculation to larvae, the  
141 VRZ solution was mixed with each solution of different concentration of CAS to obtain a  
142 combination of VRZ at 0.4 µg/µL and CAS at 0.1, 0.2 or 0.4 µg/µL.

143 After infection by the three strains of Af, a volume of 10 µL of each antifungal combination  
144 was injected in the haemocoel of larvae 2h after infection. Two control groups were used,  
145 the first group consisted of infected larvae inoculated with 9‰ saline at 2h after infection.  
146 The second group (to assess toxicity) was only inoculated with the highest doses of  
147 combination (CAS at 4 µg/larva combined with VRZ or PSZ at 4 µg/larva). Three groups were  
148 treated by single VRZ, PSZ or CAS at 4 µg/larva to assess the contribution of combination  
149 compared to monotherapy. All experiments were performed three times and results were  
150 pooled for analysis.

151

152 **Statistical analysis**

153 Mortality curves were generated by Kaplan Meier method and compared by the log-rank  
154 test. All analyzes were performed using GraphPad Prism V.3.0 software for Windows  
155 (GraphPad Software, San Diego, USA). A value of  $p < 0.05$  was considered to be significant.

156



## 157 **Results**

### 158 ***In vitro* antifungal susceptibility to antifungals**

159 The *in vitro* antifungal susceptibility of Af strains determined by EUCAST and Gradient  
160 Concentration Strip (GCS) is presented in Table 1. Using EUCAST, AfS with a wild type  
161 CYP51A sequence was azole-susceptible. AfR1 with a G54W mutation was resistant to PSZ  
162 and itraconazole but susceptible to VRZ. AfR2 with a L98H point mutation in *CYP51A* gene in  
163 combination with a 34-bp tandem repeat in the promoter (TR34/L98H), was resistant to the  
164 three tested azoles. Results obtained by the Etest® method were within +/- 2 log<sub>2</sub> dilutions  
165 comparable to EUCAST values (Table 1, Figure S1). For CAS, MEC for AfS, AfR1 and AfR2 was  
166 0.25, 0.5 and 0.5 µg/mL, respectively. CAS MIC values determined by Etest® was  
167 systematically lower than EUCAST MEC values.

168

### 169 ***In vitro* activity of antifungal combinations**

170 When VRZ was combined with CAS, no interaction was observed between the two drugs by  
171 FICI (Table 2). The lowest FICI for the combination was 1.01, 0.75 and 1.25 for AfS, AfR1 and  
172 AfR2, respectively. Bliss analysis showed a synergistic interaction for AfR1 ( $\Sigma$ SSI >200% and  
173 95% CI of MSSI did not include 0), but no interaction for AfS and AfR2 (Table 4). Antagonism  
174 was not detected for any of the strains.

175 When PSZ was combined with CAS, no interaction was observed between the two drugs by  
176 FICI (0.51) for AfS and AfR2 (Table 3). Due to the high level of PSZ resistance (high off-scale  
177 MIC), FICI was not computable for AfR1. By Bliss analysis, a synergistic interaction was  
178 observed for AfR1, but no interaction for AfS and AfR2 (Table 4). No antagonism was  
179 observed. Combinations were also evaluated by Etest® (Figure S2, Figure S3). Combinations  
180 were indifferent against all strains (Table S1, Table S2). There was no antagonism.

181 **Evaluation of caspofungin monotherapy in *Galleria mellonella***

182 For control groups, without treatment, the mortality was at least 95% by day 7, with a  
183 median survival time of 3 days for AfS and AfR1 and 3.5 days for AfR2 (Figure 1). . In AfS-  
184 infected groups, CAS at 2, 4, and 8 µg/larva significantly increased the survival during the 7  
185 days of experiment ( $p=0.0064$ ,  $0.017$  and  $0.0009$ , respectively). There was no difference in  
186 term of efficacy between the different doses of CAS. For AfR1-infected larvae, CAS did not  
187 provide any significant improvement in survival with a median survival time of 3 days. For  
188 AfR2-infected larvae, only CAS at 4 µg/larva significantly decreased the mortality when  
189 compared to the untreated control group ( $p=0.02$ ).

190

191 **Evaluation of posaconazole monotherapy in *Galleria mellonella***

192 For each strain, efficacy of PSZ at 1, 2, 4 and 8 µg/larva was evaluated (Figure 2). Mortality  
193 by day 7 in untreated larvae was 90%, 100% and 90% for AfS, AfR1 and AfR2, respectively.  
194 Treatment at 4 µg/larva increased survival for AfS ( $p=0.0004$ ) and AfR1 ( $p<0.0001$ ) but not  
195 for AfR2-infected larvae ( $p=0.41$ ). In AfS- and AfR1-infected larvae the rate of survival was  
196 dose dependent. Although PSZ improved survival compared to untreated controls for both  
197 AfS and AfR1-infected larvae, the drug was more effective in AfS than in AfR1-infected  
198 larvae. Survival at day 7 was 10%, 50% and 70% for AfS-infected larvae while it was 0%, 20%  
199 and 40% for AfR1-infected larvae after PSZ treatment at 2, 4 and 8 µg/larva, respectively.  
200 Moreover, median survival for AfS infected larvae and treated with PSZ at 8 and 4 µg/larva  
201 was more than >7 days and 7 days compared to 2.5 and 3 days for AfR1-infected larvae.

202

203 **Evaluation of combination of voriconazole with caspofungin in *Galleria mellonella***

204 For each isolate, larvae infected with LD<sub>90</sub> were treated with VRZ at 4 µg/larva combined  
205 with CAS at 1, 2 or 4 µg/larva at 2h post infection (Figure 3). Mortality, in untreated control  
206 groups was at least 95% at day 7 post infection. CAS monotherapy was effective only for AfS-  
207 infected larvae (p<0.0001). VRZ monotherapy significantly increased survival of AfS  
208 (p<0.0001), AfR1 (p<0.0001) and AfR2-infected groups (p=0.02) compared to untreated  
209 group. Nevertheless, the efficacy was better against AfS and AfR1 (survival of 35% and 30%,  
210 respectively) than against AfR2 (survival of 10%). The combination of VRZ (4 µg/larva) with  
211 CAS (4 µg/larva) significantly increased the survival of AfS (p<0.0001), AfR1 (p<0.0001) but  
212 not AfR2-infected larvae (p<0.25) compared to CAS monotherapy at 4 µg/larva. When  
213 compared to VRZ monotherapy, the combination (VRZ4 + CAS4) improved survival of AfR2-  
214 infected larvae (p=0.0066), but not of larvae infected by AfS (p=0.24) or AfR1 (p=0.28). At a  
215 lower concentration, CAS at 1 and 2 µg/larva combined with VRZ at 4 µg/larva did not  
216 increase the survival for any of the strain, when compared to VRZ monotherapy.

217

#### 218 **Evaluation of combination of posaconazole with caspofungin in *Galleria mellonella***

219 For each Af strain, larvae were infected by LD<sub>90</sub> and treated after 2h by PSZ at 4 µg/larva  
220 monotherapy or combined with CAS at 1, 2 or 4 µg/larva (Figure 4). At day 7 post-infection,  
221 the mortality in the untreated control groups was >90%. Treatment by CAS at 4 µg/larva  
222 significantly improved survival for AfS (p<0.0001), but not for AfR1 (p=0.02) or AfR2-infected  
223 larvae (p=0.07) compared to untreated group. PSZ at 4 µg/larva significantly improved  
224 survival only for AfS (p<0.0001) and AfR2 (p=0.0018) but not for AfR1-infected larvae  
225 (p=0.78) compared to the untreated controls. Combination of PSZ at 4 µg/larva and CAS at 4  
226 µg/larva improved survival only for AfR1-infected larvae compared to CAS (p=0.0002) and  
227 PSZ (p=0.0024) monotherapy.

228

229

230 **Discussion**

231 In the present study we found very weak in vitro interactions between caspofungin and  
232 azoles by checkerboard and by agar diffusion. A synergistic interaction was only found for  
233 one of the resistant strains (AfR1 resistant to ITZ and PSZ, susceptible to VRZ) when data  
234 were analyzed by a Bliss independence-based mathematical model. It has to be noticed  
235 that, although in vitro testing of antifungal combinations against filamentous fungi are very  
236 useful, the techniques are not well standardized, and interpretation of the results is  
237 sometimes complicated. Indeed, for azoles-echinocandins combinations, both synergistic  
238 and additive effect, depending on the study endpoint and the mathematical definitions for  
239 the drug interaction effect, have been reported (Dannaoui et al., 2004; Cuenca-Estrella et al.,  
240 2005; Meletiadis et al., 2005; Philip et al., 2005; Jeans et al., 2012; Planche et al., 2012;  
241 Seyedmousavi et al., 2013; Mavridou et al., 2015; Raffetin et al., 2018)..

242 For these reasons, in addition to in vitro studies, we used an in vivo model to assess the  
243 combinations. This model was previously used and validated for the evaluation of treatment  
244 of aspergillosis (Forastiero et al., 2015; Maurer et al., 2015; Jemel et al., 2020; Jemel et al.,  
245 2021). In a first set of experiments, monotherapies were tested at different dosages to  
246 assess their efficacy and to determine the optimal dosage for combination studies. VRZ was  
247 previously tested in the same model (Jemel et al., 2021), and it was shown that efficacy was  
248 correlated to in vitro susceptibility, and that a dosage of 4 µg/larva would be suitable for  
249 combination experiments. In the present study, we further evaluated CAS and PSZ  
250 monotherapies. For CAS monotherapy an increased survival was only observed for AfS-

251 infected larvae but without dose dependent efficacy which is in line with a previous animal  
252 study (Lepak et al., 2013).

253 For PSZ monotherapy, a dose-dependent efficacy was observed for AfS and a lower efficacy  
254 against the two PSZ-resistant strains. Nevertheless, a certain degree of efficacy was  
255 obtained against the PSZ-resistant strains with a paradoxical better efficacy against the  
256 strain with a higher MIC. Although in vitro-in vivo correlation has been reported for PSZ  
257 (Lepak et al., 2013; Forastiero et al., 2015), discrepancies between in vitro results and in vivo  
258 efficacy have also been reported previously. For example,  
259 Sun et al. (Sun et al., 2022) evaluated the *in vitro* and *in vivo* efficacy of azoles against Af and  
260 observed that PSZ improved significantly the survival of *G. mellonella* larvae infected by a  
261 PSZ-resistant strain (MIC of 2 µg/mL). Possible explanations for the efficacy of an antifungal  
262 against resistant strains could be the use of high dosages or a lower virulence (fitness-cost)  
263 of the resistant strains as shown in the study of dos Reis et al. (Dos Reis et al., 2019) in which  
264 some PSZ resistant mutants derived from a wild type strain lost their virulence.  
265 Nevertheless, in our work, the LD<sub>90</sub> was determined for the three strains and no difference in  
266 term of virulence was seen between AfS, AfR1 and AfR2 (Jemel et al., 2021).

267 Overall, in the present study, the combination of an azole with caspofungin showed both  
268 indifferent and synergistic interactions depending on the strain susceptibility.

269 When compared to VRZ monotherapy, the combination of VRZ with CAS had a better  
270 efficacy for the VRZ-resistant strain (i.e. AfR2) infected larvae. This is interesting, as  
271 combination therapy is recommended in cases of azole-resistance (Verweij et al., 2015). In  
272 previous studies, both indifferent (MacCallum et al., 2005; Zhang et al., 2014) and synergistic  
273 interactions have been reported (Kirkpatrick et al., 2002), but it has to be noticed that most  
274 of the studies have been performed with susceptible isolates.

275 In our study, combination of PSZ at 4 µg/larva and CAS at 4 µg/larva improved the rate of  
276 survival in larvae infected by AfR1 (PSZ resistant strain) when compared to CAS or PSZ alone.  
277 These observations are supported by a neutropenic murine model of pulmonary invasive  
278 aspergillosis in which efficacy was determined using quantitative PCR (Lepak et al., 2013).  
279 Combination therapy with CAS and PSZ did not enhance efficacy for PSZ-susceptible isolates.  
280 However, the drug combination produced synergistic activity against PSZ-resistant isolates.

281

## 282 **Conclusion**

283 Overall, our results showed relatively weak interactions between azoles and caspofungin  
284 against Af in vitro. In vivo, a better efficacy of the combination compared to the azole  
285 monotherapy was obtained only against the azole-resistant isolates. Antagonism was never  
286 observed.

287

288

289

290 **Supplementary Materials:** Table S1; Table S2; Figure S1, Figure S2, Figure S3

291

292 **Author Contributions:** Conceptualization, E.D.; methodology, S.J., Y.R., A-L.B., and G.J.;  
293 formal analysis, S.J. Y.R., A-L.B., and E.D.; data curation, S.J. A-L.B., and E.D.; writing—original  
294 draft preparation, S.J. and E.D.; writing—review and editing, S.J., Y.R., A-L.B., J.G., K.K., G.J.,  
295 F.B., and E.D.; supervision, E.D.; funding acquisition, F.B., E.D., and J.G. All authors have read  
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300

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302

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304 the corresponding author.

305

306 **Conflicts of Interest:** During the past 5 years, Eric Dannaoui has received research grants  
307 from MSD and Gilead; travel grants from Gilead, MSD, Pfizer, and Astellas, and speaker's fee  
308 from Gilead, MSD, and Astellas. Françoise Botterel has received research grants from MSD;  
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312

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446 **Table 1:** in vitro susceptibility of the three Af strains

Antifungal and method	MIC ( $\mu\text{g}/\text{mL}$ ) against		
	AfS	AfR1	AfR2
Amphotericin B			
EUCAST	1	1	0.5
Etest <sup>®</sup> <sup>a</sup>	0.25	0.5	0.25
Itraconazole			
EUCAST	0.25	>4	>4
Etest <sup>®</sup>	0.5	>32	32
Voriconazole			
EUCAST	0.25	0.5	2
Etest <sup>®</sup>	0.125	0.125	1
Posaconazole			
EUCAST	0.06	>4	0.5
Etest <sup>®</sup>	0.06	>32	0.5
Caspofungin			
EUCAST	0.25	0.5	0.5
Etest <sup>®</sup>	0.032	0.008	0.032

447 <sup>a</sup>GCS was determined at 48h for AfS and AfR1, and at 24h for AfR2

448

449 **Table 2:** In vitro interaction between CAS and VRZ by checkerboard

450

Isolate	MIC ( $\mu\text{g/mL}$ ) of drug alone		MIC ( $\mu\text{g/mL}$ ) of drug in combination		Lowest FICI for the combination	
	CAS	VRZ	CAS	VRZ	CAS/VRZ	Interaction
AfS	1	0.25	0.0156	0.25	1.0156	I
AfR1	1	0.5	0.25	0.25	0.75	I
AfR2	1	2	0.25	1	1.25	I

451 MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; CAS: caspofungin; VRZ:  
 452 voriconazole; I: no interaction.

453

454

455 **Table 3:** In vitro interaction between CAS and PSZ by checkerboard

456

Isolate	MIC ( $\mu\text{g/mL}$ ) of drug alone		MIC ( $\mu\text{g/mL}$ ) of drug in combination		Lowest FICI for the combination	
	CAS	PSZ	CAS	PSZ	CAS/PSZ	Interaction
AfS	1	0.125	0.0156	0.0625	0.5156	I
AfR1	1	8	ND	ND	ND	ND
AfR2	1	1	0.015625	0.5	0.5156	I

457 MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; CAS: caspofungin; PSZ:  
 458 posaconazole; I: no interaction. ND: not determined (all MICs > maximum).

459

460

461 **Table 4:** In vitro interaction between CAS and azoles evaluated by a Bliss independence-  
 462 based model

463

Isolate	CAS/VRZ combination			CAS/PSZ combination		
	ΣSSI	MSSI (95% CI)	Interaction	ΣSSI	MSSI (CI 95%)	Interaction
AfS	50.3%	3.9% (-2.3;10.0)	I	185.5%	23.2% (-2.6;49.0)	I
AfR1	747.5%	37.4% (30.1;44.6)	S	1195.3%	36.2% (30.3;42.2)	S
AfR2	89.5%	6.9% (-4.3;18.1)	I	-12.7%	-4.2% (-23.2;14.7)	I

464 ΣSSI: Sum of statistically significant interactions MSSI: Mean of statistically significant interactions; 95% CI: Confidence  
 465 interval at 95% level; CAS: caspofungin; VRZ: voriconazole; PSZ: posaconazole; S: synergy; I: no interaction.  
 466  
 467

468 **Figure legends**

469

470 **Figure 1:** Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1  
471 (middle), and AfR2 (right) and treated with caspofungin at 8, 4 or 2 µg/larva after 2h of  
472 infection. NI: non infected larvae treated with the highest doses of caspofungin (8 µg/larva).  
473 NT: infected larvae and inoculated with 10 µL of saline water. CAS: caspofungin.

474

475 **Figure 2:** Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1  
476 (middle) and AfR2 (right) and treated with posaconazole at 8, 4, 2 or 1 µg/larva after 2h of  
477 infection. NI: non infected larvae treated with the highest doses of posaconazole (8  
478 µg/larva). NT: infected larvae and inoculated with 10 µL of saline water. PSZ: posaconazole.

479

480 **Figure 3:** Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1  
481 (middle) and AfR2 (right) and treated with monotherapy of voriconazole at 4 µg/larva or  
482 combination of voriconazole 4 µg/larva and caspofungin at 4, 2 or 1 µg/larva respectively,  
483 after 2h of infection. NI: non-infected larvae treated with combination of voriconazole at 4  
484 µg/larva and caspofungin at 4 µg/larva. NT: infected larvae and inoculated with 10 µL of  
485 saline water. VRZ: voriconazole. CAS: caspofungin.

486

487 **Figure 4:** Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1  
488 (middle), and AfR2 (right) and treated with monotherapy of posaconazole at 4 µg/larva or  
489 combination of posaconazole 4 µg/larva and caspofungin at 4, 2 or 1 µg/larva respectively,  
490 after 2h of infection. NI: non infected larvae treated with combination of posaconazole at 4  
491 µg/larva and caspofungin at 4 µg/larva. NT: infected larvae and inoculated with 10 µL of  
492 saline water. PSZ: posaconazole. CAS: caspofungin.

493 **Supplementary material**

494

495 **Table S1:** In vitro interaction between CAS and VRZ by gradient concentration strips

496

Isolate	MIC (µg/mL) of drug alone		MIC (µg/mL) of drug in combination	FICI for the combination	
	CAS	VRZ	CAS+VRZ	CAS +VRZ	Interaction
AfS	0.023	0,19	0.023	1.12	I
AfR1	0.004	0.032	0.004	1.12	I
AfR2	0.016	1.5	0.016	1.01	I

497 MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; CAS: caspofungin; VRZ:  
 498 posaconazole; I: no interaction.

499



500 **Table S2:** In vitro interaction between CAS and PSZ by gradient concentration strips

501

Isolate	MIC ( $\mu\text{g/mL}$ ) of drug alone		MIC ( $\mu\text{g/mL}$ ) of drug in combination	FICI for the combination	
	CAS	PSZ	CAS+PSZ	CAS +PSZ	Interaction
AfS	0.032	0.094	0.064	2.67	I
AfR1	0.006	>32	0.006	1.00	I
AfR2	0.016	0.5	0.016	1.03	I

502 MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; CAS: caspofungin; PSZ:  
503 posaconazole; I: no interaction.

504

505

506 **Supplementary Figure legends**

507

508 **Figure S1:** In vitro determination of Minimal Inhibitory Concentrations of five antifungals by  
509 gradient concentration strip for the three stains of Af : AfS, AfR1 and AfR2. AMB:  
510 amphotericin B, ITZ: itraconazole, VRZ: voriconazole, PSZ: posaconazole and CAS:  
511 caspofungin.

512

513 **Figure S2:** In vitro determination of Minimal Inhibitory Concentrations by gradient  
514 concentration strips of voriconazole alone, caspofungin alone, and the combination of  
515 voriconazole with caspofungin for the three strains of *Aspergillus fumigatus* (AfS, AfR1 and  
516 AfR2). VRZ: voriconazole; CAS: caspofungin.

517

518 **Figure S3:** In vitro determination of Minimal Inhibitory Concentrations by gradient  
519 concentration strips for posaconazole alone, caspofungin alone and combination of  
520 posaconazole with caspofungin for the three strains of *Aspergillus fumigatus* (AfS, AfR1 and  
521 AfR2). PSZ: posaconazole; CAS: caspofungin.

522