



HAL
open science

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

► **To cite this version:**

Sana Jemel, Yannick Raveloarisaona, Anne-Laure Bidaud, Aicha Kallel, Jacques Guillot, et al.. In vitro and in vivo evaluation of antifungal combinations against azole-resistant *Aspergillus fumigatus* isolates. *Frontiers in Cellular and Infection Microbiology*, 2023, 12, 10.3389/fcimb.2022.1038342 . hal-03943969

HAL Id: hal-03943969

<https://hal.u-pec.fr/hal-03943969v1>

Submitted on 17 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4 Sana Jemel^{1,2,3}, Yannick Raveloarisaona^{4,5}, Anne-Laure Bidaud^{4,5}, Elie Djenontin^{1,4}, Aicha
5 Kallel^{2,3}, Jacques Guillot^{6,7}, Kalthoum Kallel^{2,3}, Françoise Botterel¹ and Eric Dannaoui^{1,4,5*}

6

7 ¹ Université Paris Est Créteil, Dynamyc, F-94010 Créteil, France;

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

9 ² Université Tunis EL Manar, Faculté de médecine de Tunis, Tunis 1007, Tunisie;

10 kallelkalthoum@gmail.com

11 ³ UR17SP03, Centre Hospitalo-Universitaire La Rabta, Jabbari, Tunis 1007, Tunisie

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

14 ⁵ Université de Paris-Cité, Faculté de Médecine, 75006 Paris, France

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

17 ⁷ 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 α 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 2.5×10^5 (CFU) mL⁻¹. 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% (LD₉₀) 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 DL₉₀ 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 log₂ 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 (Σ 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 LD₉₀ 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 LD₉₀ 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 LD₉₀ 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
296 and agreed to the published version of the manuscript.

297

298 **Funding:** This research received funding from MSD (Investigator Initiated Studies Program n°
299 57288).

300

301 **Institutional Review Board Statement:** Not applicable.

302

303 **Data Availability Statement:** The data presented in this study are available on request from
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;
309 travel grants from Gilead, MSD, Pfizer, and speaker's fee from Gilead, MSD, and Pfizer.
310 Jacques Guillot has received research grants from MSD Animal Health and Vetoquinol, and
311 speaker's fee from Boehringer Animal Health, Elanco, Gilead, Cerbavet and Virbac.

312

313 **References**

- 314 Arendrup, M.C., Meletiadis, J., Mouton, J.W., Lagrou, K., Hamal, P., Guinea, J., et al. (2017).
315 Method for the determination of broth dilution minimum inhibitory concentrations
316 of antifungal agents for conidia forming moulds. EUCAST definitive document E.Def
317 9.3.1.
- 318 Bidaud, A.L., Schwarz, P., Herbreteau, G., and Dannaoui, E. (2021). Techniques for the
319 assessment of in vitro and in vivo antifungal combinations. *J Fungi (Basel)* 7(2), 113.
320 doi: 10.3390/jof7020113.
- 321 Cuenca-Estrella, M., Gomez-Lopez, A., Garcia-Effron, G., Alcazar-Fuoli, L., Mellado, E.,
322 Buitrago, M.J., et al. (2005). Combined activity in vitro of caspofungin, amphotericin
323 B, and azole agents against itraconazole-resistant clinical isolates of *Aspergillus*
324 *fumigatus*. *Antimicrob Agents Chemother* 49(3), 1232-1235.
- 325 Dannaoui, E., Lortholary, O., and Dromer, F. (2004). In vitro evaluation of double and triple
326 combinations of antifungal drugs against *Aspergillus fumigatus* and *Aspergillus*
327 *terreus*. *Antimicrob Agents Chemother* 48(3), 970-978.

328 Dos Reis, T.F., Silva, L.P., de Castro, P.A., do Carmo, R.A., Marini, M.M., da Silveira, J.F., et al.
329 (2019). The *Aspergillus fumigatus* mismatch repair MSH2 homolog is important for
330 virulence and azole resistance. *mSphere* 4(4). doi: 10.1128/mSphere.00416-19.

331 Dudakova, A., Spiess, B., Tangwattanachuleeporn, M., Sasse, C., Buchheidt, D., Weig, M., et
332 al. (2017). Molecular tools for the detection and deduction of azole antifungal drug
333 resistance phenotypes in *Aspergillus* species. *Clin Microbiol Rev* 30(4), 1065-1091.
334 doi: 10.1128/CMR.00095-16.

335 Forastiero, A., Bernal-Martinez, L., Mellado, E., Cendejas, E., and Gomez-Lopez, A. (2015). In
336 vivo efficacy of voriconazole and posaconazole therapy in a novel invertebrate model
337 of *Aspergillus fumigatus* infection. *Int J Antimicrob Agents* 46(5), 511-517. doi:
338 10.1016/j.ijantimicag.2015.07.007.

339 Gangneux, J.P., Dannaoui, E., Fekkar, A., Luyt, C.E., Botterel, F., De Prost, N., et al. (2022).
340 Fungal infections in mechanically ventilated patients with COVID-19 during the first
341 wave: the French multicentre MYCOVID study. *Lancet Respir Med* 10(2), 180-190.
342 doi: 10.1016/S2213-2600(21)00442-2.

343 Jeans, A.R., Howard, S.J., Al-Nakeeb, Z., Goodwin, J., Gregson, L., Warn, P.A., et al. (2012).
344 Combination of voriconazole and anidulafungin for treatment of triazole-resistant
345 *aspergillus fumigatus* in an in vitro model of invasive pulmonary aspergillosis.
346 *Antimicrob Agents Chemother* 56(10), 5180-5185.

347 Jemel, S., Guillot, J., Kallel, K., Botterel, F., and Dannaoui, E. (2020). *Galleria mellonella* for
348 the evaluation of antifungal efficacy against medically important fungi, a narrative
349 review. *Microorganisms* 8(3), 390. doi: 10.3390/microorganisms8030390.

350 Jemel, S., Guillot, J., Kallel, K., Jouvion, G., Brisebard, E., Billaud, E., et al. (2021). In vivo
351 efficacy of voriconazole in a *Galleria mellonella* model of invasive infection due to
352 azole-susceptible or resistant *Aspergillus fumigatus* isolates. *J Fungi (Basel)* 7(12),
353 1012. doi: 10.3390/jof7121012.

354 Kirkpatrick, W.R., Perea, S., Coco, B.J., and Patterson, T.F. (2002). Efficacy of caspofungin
355 alone and in combination with voriconazole in a Guinea pig model of invasive
356 aspergillosis. *Antimicrob Agents Chemother* 46(8), 2564-2568.

357 Latge, J.P., and Chamilos, G. (2020). *Aspergillus fumigatus* and aspergillosis in 2019. *Clin*
358 *Microbiol Rev* 33(1), e00140-00118. doi: 10.1128/CMR.00140-18.

359 Lepak, A.J., Marchillo, K., VanHecker, J., and Andes, D.R. (2013). Impact of in vivo triazole and
360 echinocandin combination therapy for invasive pulmonary aspergillosis: enhanced
361 efficacy against Cyp51 mutant isolates. *Antimicrob Agents Chemother* 57(11), 5438-
362 5447. doi: 10.1128/AAC.00833-13.

363 Lestrade, P.P., Bentvelsen, R.G., Schauwvlieghe, A., Schalekamp, S., van der Velden, W.,
364 Kuiper, E.J., et al. (2019a). Voriconazole resistance and mortality in invasive
365 aspergillosis: a multicenter retrospective cohort study. *Clin Infect Dis* 68(9), 1463-
366 1471. doi: 10.1093/cid/ciy859.

367 Lestrade, P.P.A., Meis, J.F., Melchers, W.J.G., and Verweij, P.E. (2019b). Triazole resistance in
368 *Aspergillus fumigatus*: recent insights and challenges for patient management. *Clin*
369 *Microbiol Infect* 25(7), 799-806. doi: 10.1016/j.cmi.2018.11.027.

370 MacCallum, D.M., Whyte, J.A., and Odds, F.C. (2005). Efficacy of caspofungin and
371 voriconazole combinations in experimental aspergillosis. *Antimicrob Agents*
372 *Chemother* 49(9), 3697-3701.

373 Maurer, E., Browne, N., Surlis, C., Jukic, E., Moser, P., Kavanagh, K., et al. (2015). *Galleria*
374 *mellonella* as a host model to study *Aspergillus terreus* virulence and amphotericin B
375 resistance. *Virulence* 6(6), 591-598. doi: 10.1080/21505594.2015.1045183.

376 Mavridou, E., Meletiadiis, J., Rijs, A., Mouton, J.W., and Verweij, P.E. (2015). The strength of
377 synergistic interaction between posaconazole and caspofungin depends on the
378 underlying azole resistance mechanism of *Aspergillus fumigatus*. *Antimicrob Agents*
379 *Chemother* 59(3), 1738-1744. doi: 10.1128/AAC.04469-14.

380 Meletiadiis, J., Verweij, P.E., TeDorsthorst, D.T., Meis, J.F., and Mouton, J.W. (2005).
381 Assessing in vitro combinations of antifungal drugs against yeasts and filamentous
382 fungi: comparison of different drug interaction models. *Med Mycol* 43(2), 133-152.
383 doi: 10.1080/13693780410001731547.

384 Odds, F.C. (2003). Synergy, antagonism, and what the checkerboard puts between them. *J*
385 *Antimicrob Chemother* 52(1), 1.

386 Pasquier, G., Bounhiol, A., Robert Gangneux, F., Zahar, J.R., Gangneux, J.P., Novara, A., et al.
387 (2021). A review of significance of *Aspergillus* detection in airways of ICU COVID-19
388 patients. *Mycoses* 64(9), 980-988. doi: 10.1111/myc.13341.

389 Patterson, T.F., Thompson, G.R., 3rd, Denning, D.W., Fishman, J.A., Hadley, S., Herbrecht, R.,
390 et al. (2016). Practice guidelines for the diagnosis and management of aspergillosis:
391 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis* 63(4), e1-
392 e60. doi: 10.1093/cid/ciw326.

393 Philip, A., Odabasi, Z., Rodriguez, J., Paetznick, V.L., Chen, E., Rex, J.H., et al. (2005). In vitro
394 synergy testing of anidulafungin with itraconazole, voriconazole, and amphotericin B
395 against *Aspergillus* spp. and *Fusarium* spp. *Antimicrob Agents Chemother* 49(8), 3572-
396 3574. doi: 10.1128/AAC.49.8.3572-3574.2005.

397 Planche, V., Ducroz, S., Alanio, A., Bougnoux, M.E., Lortholary, O., and Dannaoui, E. (2012). In
398 vitro combination of anidulafungin and voriconazole against intrinsically azole-
399 susceptible and -resistant *Aspergillus* spp. *Antimicrob Agents Chemother* 56(8), 4500-
400 4503.

401 Raffetin, A., Courbin, V., Jullien, V., and Dannaoui, E. (2018). In vitro combination of
402 isavuconazole with echinocandins against azole-susceptible and -resistant *Aspergillus*
403 spp. *Antimicrob Agents Chemother* 62(1), e01382-01317. doi: 10.1128/AAC.01382-
404 17.

405 Resendiz-Sharpe, A., Mercier, T., Lestrade, P.P.A., van der Beek, M.T., von dem Borne, P.A.,
406 Cornelissen, J.J., et al. (2019). Prevalence of voriconazole-resistant invasive
407 aspergillosis and its impact on mortality in haematology patients. *J Antimicrob*
408 *Chemother* 74(9), 2759-2766. doi: 10.1093/jac/dkz258.

409 Rybak, J.M., Fortwendel, J.R., and Rogers, P.D. (2019). Emerging threat of triazole-resistant
410 *Aspergillus fumigatus*. *J Antimicrob Chemother* 74(4), 835-842. doi:
411 10.1093/jac/dky517.

412 Schauwvlieghe, A., Rijnders, B.J.A., Philips, N., Verwijs, R., Vanderbeke, L., Van Tienen, C., et
413 al. (2018). Invasive aspergillosis in patients admitted to the intensive care unit with
414 severe influenza: a retrospective cohort study. *Lancet Respir Med* 6(10), 782-792. doi:
415 10.1016/S2213-2600(18)30274-1.

416 Seyedmousavi, S., Bruggemann, R.J., Melchers, W.J., Rijs, A.J., Verweij, P.E., and Mouton,
417 J.W. (2013). Efficacy and pharmacodynamics of voriconazole combined with
418 anidulafungin in azole-resistant invasive aspergillosis. *J Antimicrob Chemother* 68(2),
419 385-393. doi: 10.1093/jac/dks402.

420 Sun, Y., Tan, L., Yao, Z., Gao, L., Yang, J., and Zeng, T. (2022). In vitro and in vivo interactions
421 of TOR inhibitor AZD8055 and azoles against pathogenic fungi. *Microbiol Spectr* 10(1),
422 e0200721. doi: 10.1128/spectrum.02007-21.

423 Thompson, G.R., 3rd, and Young, J.H. (2021). *Aspergillus* Infections. *N Engl J Med* 385(16),
424 1496-1509. doi: 10.1056/NEJMra2027424.

425 Ullmann, A.J., Aguado, J.M., Arikan-Akdagli, S., Denning, D.W., Groll, A.H., Lagrou, K., et al.
426 (2018). Diagnosis and management of *Aspergillus* diseases: executive summary of the
427 2017 ESCMID-ECMM-ERS guideline. *Clin Microbiol Infect* 24 Suppl 1, e1-e38. doi:
428 10.1016/j.cmi.2018.01.002.

429 Verweij, P.E., Ananda-Rajah, M., Andes, D., Arendrup, M.C., Bruggemann, R.J., Chowdhary,
430 A., et al. (2015). International expert opinion on the management of infection caused
431 by azole-resistant *Aspergillus fumigatus*. *Drug Resist Updat* 21-22, 30-40. doi:
432 10.1016/j.drug.2015.08.001.

433 Verweij, P.E., Rijnders, B.J.A., Bruggemann, R.J.M., Azoulay, E., Bassetti, M., Blot, S., et al.
434 (2020). Review of influenza-associated pulmonary aspergillosis in ICU patients and
435 proposal for a case definition: an expert opinion. *Intensive Care Med* 46(8), 1524-
436 1535. doi: 10.1007/s00134-020-06091-6.

437 Vitale, R.G., Afeltra, J., and Dannaoui, E. (2005). Antifungal combinations. *Methods Mol Med*
438 118, 143-152.

439 Zhang, M., Su, X., Sun, W.K., Chen, F., Xu, X.Y., and Shi, Y. (2014). Efficacy of the combination
440 of voriconazole and caspofungin in experimental pulmonary aspergillosis by different
441 *Aspergillus* species. *Mycopathologia* 177(1-2), 11-18. doi: 10.1007/s11046-013-9719-
442 z.

443
444
445

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 [®] ^a	0.25	0.5	0.25
Itraconazole			
EUCAST	0.25	>4	>4
Etest [®]	0.5	>32	32
Voriconazole			
EUCAST	0.25	0.5	2
Etest [®]	0.125	0.125	1
Posaconazole			
EUCAST	0.06	>4	0.5
Etest [®]	0.06	>32	0.5
Caspofungin			
EUCAST	0.25	0.5	0.5
Etest [®]	0.032	0.008	0.032

447 ^aGCS 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 ($\mu\text{g}/\text{mL}$) of drug alone		MIC ($\mu\text{g}/\text{mL}$) 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