

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

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1	In v	vitro	and	in	vivo	evaluation	of	antifungal	combinations	against	azole-resistant
2	Aspe	ergillu	us fun	nigc	<i>atus</i> is	olates					

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#### 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  $\mu$ g/larva with CAS at 4  $\mu$ g/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.

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

41

## 42 Introduction

Aspergillus fumigatus (Af) remains one of the most potent opportunistic fungal pathogens in
 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).

#### 70 Materials and Methods

#### 71 Strains, medium and growth conditions

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

Subcultures were performed on Sabouraud dextrose agar (VWR, Fontenay-sous-bois,
France) with chloramphenicol (Sigma-Aldrich, Saint Quentin-Fallavier, France). They were
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 performed as recommended (Arendrup et al., 2017). For azoles and amphotericin B, 88 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, Marcy-l'Etoile, France) was performed according to the manufacturer instructions and MICs
 were read after 48h of incubation.

In vitro activity of the combination of CAS with either VRZ or PSZ was first evaluated by the 94 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 1-2.5x10<sup>5</sup> (CFU) mL<sup>-1</sup>. Microplates were incubated at 37°C and read after 48h of incubation. A 98 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  $\leq$  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 (Meletiadis et al., 2005). Two parameters 105 were calculated: the sum (SSSI) 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.

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

113

#### 114 Galleria mellonella inoculation and treatment

#### 115 Galleria mellonella infection

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

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

## 124 **Drug preparation**

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

#### 130 Caspofungin and posaconazole monotherapy

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

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

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

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.

#### 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<sup>®</sup> method were within  $+/-2 \log_2$  dilutions 165 comparable to EUCAST values (Table 1, Figure S1). For CAS, MEC for AfS, AfR1 and AfR2 was 0.25, 0.5 and 0.5 µg/mL, respectively. CAS MIC values determined by Etest<sup>®</sup> was 166 167 systematically lower than EUCAST MEC values.

168

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

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

When PSZ was combined with CAS, no interaction was observed between the two drugs by FICI (0.51) for AfS and AfR2 (Table 3). Due to the high level of PSZ resistance (high off-scale MIC), FICI was not computable for AfR1. By Bliss analysis, a synergistic interaction was observed for AfR1, but no interaction for AfS and AfR2 (Table 4). No antagonism was observed. Combinations were also evaluated by Etest<sup>®</sup> (Figure S2, Figure S3). Combinations 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  $\mu$ g/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  $LD_{90}$  were treated with VRZ at 4  $\mu$ g/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  $\mu$ g/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  $\mu$ g/larva combined with VRZ at 4  $\mu$ g/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  $LD_{90}$  and treated after 2h by PSZ at 4  $\mu$ g/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  $\mu$ g/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.

#### 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  $\mu$ g/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- infected larvae but without dose dependent efficacy which is in line with a previous animalstudy (Lepak et al., 2013).

For PSZ monotherapy, a dose-dependent efficacy was observed for AfS and a lower efficacy against the two PSZ-resistant strains. Nevertheless, a certain degree of efficacy was obtained against the PSZ-resistant strains with a paradoxical better efficacy against the strain with a higher MIC. Although in vitro-in vivo correlation has been reported for PSZ (Lepak et al., 2013; Forastiero et al., 2015), discrepancies between in vitro results and in vivo 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.

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

275	In our study, combination of PSZ at 4 $\mu g/larva$ and CAS at 4 $\mu g/larva$ 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.
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290	Supplementary Materials: Table S1; Table S2; Figure S1, Figure S2, Figure S3
291	
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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.,
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- 442

		MIC (µg/mL) agains	t
Antifungal and method —	AfS	AfR1	AfR2
Amphotericin B			
EUCAST	1	1	0.5
Etest <sup>®a</sup>	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 <sup>®</sup>	0.032	0.008	0.032

#### Table 1: in vitro susceptibility of the three Af strains

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

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

Isolate	MIC (μg/mL) of drug alone		MIC (μg/mL) combina	of drug in ation	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.

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

Isolate	MIC (μg/mL) of drug alone		MIC (μg/mL) combin	of drug in ation	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).

#### Table 4: In vitro interaction between CAS and azoles evaluated by a Bliss independence-461

- 462 based model
- 463

laalata		CAS/VRZ combinat	tion		CAS/PSZ combination			
Isolate	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		

ΣSSI: Sum of statistically significant interactions MSSI: Mean of statistically significant interactions; 95% CI: Confidence

interval at 95% level; CAS: caspofungin; VRZ: voriconazole; PSZ: posaconazole; S: synergy; I: no interaction.

- 468 **Figure legends**
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#### Figure legenus

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  $\mu$ g/larva after 2h of 477 infection. NI: non infected larvae treated with the highest doses of posaconazole (8 478  $\mu$ g/larva). NT: infected larvae and inoculated with 10  $\mu$ L of saline water. PSZ: posaconazole.

479

Figure 3: Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1 (middle) and AfR2 (right) and treated with monotherapy of voriconazole at 4  $\mu$ g/larva or combination of voriconazole 4  $\mu$ g/larva and caspofungin at 4, 2 or 1  $\mu$ g/larva respectively, after 2h of infection. NI: non-infected larvae treated with combination of voriconazole at 4  $\mu$ g/larva and caspofungin at 4  $\mu$ g/larva. NT: infected larvae and inoculated with 10  $\mu$ L of saline water. VRZ: voriconazole. CAS: caspofungin.

486

Figure 4: Survival curves of groups of *G. mellonella* larvae inoculated with AfS (left), AfR1 (middle), and AfR2 (right) and treated with monotherapy of posaconazole at 4  $\mu$ g/larva or combination of posaconazole 4  $\mu$ g/larva and caspofungin at 4, 2 or 1  $\mu$ g/larva respectively, after 2h of infection. NI: non infected larvae treated with combination of posaconazole at 4  $\mu$ g/larva and caspofungin at 4  $\mu$ g/larva. NT: infected larvae and inoculated with 10  $\mu$ L of saline water. PSZ: posaconazole. CAS: caspofungin.

## 493 Supplementary material

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

Isolate	MIC (μg drug a	VIC (µg/mL) ofMIC (µg/mL) of drugdrug alonein 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: posaconazole; I: no interaction.

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

Isolate	MIC (μg drug a	MIC (μg/mL) of drug aloneMIC (μg/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.

- 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

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

517

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