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Effect of protein binding on the activity of voriconazole alone or combined with anidulafungin against *Aspergillus* spp. using a time-kill methodology

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ABSTRACT

Objectives: the aims of the study were to explore the activity of total and free (according to protein binding) maximal concentrations achieved in serum after multiple doses of voriconazole 400/200 mg and anidulafungin 200/100 mg against *Aspergillus fumigatus* and *Aspergillus flavus* and the human albumin or serum effects on antifungal activity.

Material and methods: Time-kill curves were performed with two *A. fumigatus* and two *A. flavus* strains at voriconazole and anidulafungin Cmax concentrations using different media: a) RPMI broth (Cmax-RPMI); b) RPMI with human serum (Cmax-HS), and c) RPMI with human albumin (Cmax-HAlb). In parallel, free-drug (*f*Cmax) concentrations considering theoretical protein binding were performed in RPMI broth. *Aspergillus* metabolic activity was measured by the XTT reduction assay.

Results: Voriconazol or voriconazole plus anidulafungin reduced >88.4% the metabolic activity of *Aspergillus* sp. at Cmax-RPMI and *f*Cmax after 48 h of exposition. Anidulafungin alone showed poor metabolic reductions (<80.1% at Cmax-RPMI and <15% at *f*Cmax). Anidulafungin activity, but not voriconazole activity alone or combined decreased in presence of HS or HAlb (more pronounced in *A. flavus* strains and HAlb). However, anidulafungin Cmax-HS or Cmax-HAlb against *A. fumigatus* strains were significantly more active ($p<0.05$) than *f*Cmax in RPMI. These species and culture medium-dependent impact of human protein binding in the activity of anidulafungin was related to macroscopic and microscopic differences among mycelial mat grown in RPMI, HS or HAlb in whose XTT retention was different.

Conclusions: Synergism could not be demonstrated due to the high activity showed by voriconazole. Protein binding has not impact on voriconazole activity and this impact is considerably less than predicted by free concentration extrapolated from theoretical binding rate on anidulafungin. The XTT colorimetric assay needs to be standardized for use with *Aspergillus* spp. since without DMSO extraction the activity of echinocandins in a free-human protein RPMI medium could be overestimated.

KEYWORDS: serum, albumin, time-kill, voriconazole, anidulafungin.

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Efecto de la unión a proteínas en la actividad de voriconazol solo o combinado con anidulafungina frente *Aspergillus* spp. mediante curvas de letalidad

RESUMEN

Objetivos: los objetivos del estudio fueron valorar la actividad de las concentraciones máximas totales y libres (de acuerdo a la unión a proteínas) alcanzadas en suero después de dosis múltiples de voriconazol 400/200 mg y anidulafungina 200/100 mg frente *Aspergillus fumigatus* y *Aspergillus flavus* y el efecto del suero y albúmina humana en la actividad antifúngica.

Material y métodos: las curvas de letalidad fueron realizadas con 2 cepas de *A. fumigatus* y 2 cepas de *A. flavus* a las concentraciones Cmax de voriconazol y anidulafungina empleando diferentes medios: a) caldo RPMI (Cmax-RPMI); b) RPMI con suero humano (Cmax-SH) y c) RPMI con albúmina humana (Cmax-AlbH). Se compararon con las concentraciones libres (*f*Cmax) en caldo RPMI teniendo en cuenta la unión a proteínas. La actividad metabólica de las cepas de *Aspergillus* fue medida por la técnica de reducción de XTT.

Resultados: voriconazol o voriconazol con anidulafungina redujeron la actividad metabólica de las cepas de *Aspergillus* >88.4% a las concentraciones Cmax-RPMI y *f*Cmax después de 48 h de exposición. Anidulafungina sola mostró bajas reducciones metabólicas (<80.1% a Cmax-RPMI y <15% a *f*Cmax). La actividad de anidulafungina, pero no la de voriconazol solo o combinado, disminuyó en presencia de suero o albúmina (más pronun-

ciado para las cepas de *A. flavus* y con albúmina). Sin embargo, las concentraciones de anidulafungina Cmax-HS o Cmax-AlbH frente las cepas de *A. fumigatus* fueron significativamente más activas ($p<0.05$) que la concentración *f*Cmax en RPMI. Este impacto de la unión a proteínas en la actividad de anidulafungina dependiente de la especie y el medio de cultivo fue relacionada con diferencias macroscópicas y microscópicas del crecimiento miceliar en RPMI, SH o AlbH en los cuales la retención fue diferente.

Conclusiones: el sinergismo entre ambos antifúngicos no pudo ser demostrado probablemente por alta actividad mostrada por voriconazol. La unión a proteínas plasmáticas no tiene impacto en la actividad de voriconazol y su efecto es considerablemente menor sobre anidulafungina que el pronosticado por la concentración libre teórica extrapolada de la tasa de unión a proteínas. El método colorimétrico del XTT necesita ser estandarizado para ser empleado con *Aspergillus* sp. ya que sin la extracción con DMSO la actividad de las equinocandinas en el medio RPMI sin proteínas humanas podría ser sobreestimada.

Palabras clave: suero, albúmina, curvas de letalidad, voriconazol, anidulafungina.

INTRODUCTION

Aspergillus spp. produces high morbidity and mortality in patients with intensive immunosuppressive therapy[1,2]. Invasive aspergillosis still presents a high mortality even in patients receiving antifungal agents active against *Aspergillus* spp. Clinical guidelines recommend voriconazole as primary treatment for invasive pulmonary aspergillosis in most patients[2], but denote a significant gap to guide the management of patients in whose aspergillosis is refractory to the treatment[2,3]. Therapeutic options for salvage therapy have demonstrated a relative success, emphasizing the importance of effective primary therapies to improve outcome[2,4] and the need for more effective salvage strategies. One approach to improve salvage therapy is antifungal combination that although it is recognizes by guidelines in this context, data for well-controlled prospective clinical trial are needed to confirm its effectiveness.

Echinocandins are potential alternatives by their distinct mechanism of action have the possibility to be used in combination with currently available standard antifungal agents[2,4-5]. Anidulafungin has shown to be active in an experimental model of pulmonary aspergillosis, but synergistic effects with voriconazole against *Aspergillus* spp. remains unclear[6-9].

Antifungal efficacies of the echinocandin drugs are reduced significantly in the presence of 50% human serum[10]. The relative low rate of protein binding of voriconazole has not impact on its in vitro activity against *Aspergillus spp.*[11], but available data support a pronounced shift of the minimal effective concentration (MEC) of echinocandins ranging from 8- to 64,000- fold in presence of human serum[10,12, 13]. The primary effect of serum binding to echinocandins is related with a concomitant decrease in inhibition of glucan synthase[10] consistent with the serum-induced shifts in the MEC.

According to free drug hypothesis only unbound fraction of an antifungal agent is pharmacologically active *in vitro* (and presumably *in vivo*)[14]. Anidulafungin (Eraxis™ package insert; Pfizer) has a protein binding rates of 99% (free fraction of 1%) and so MECs would be increased (in theory) 100-fold in presence of human serum. However anidulafungin MECs increase does not appear to be consistent with the rate of protein binding[10,12,13] and then any presumed limitations on activity due to protein binding may be far from absolute as previously have been demonstrated in antibacterial highly bound to proteins[15-16].

The objectives of this study were to explore the activity of total and free (according to protein binding) maximal concentrations achieved in serum after multiple doses of voriconazole 400/200 mg and anidulafungin 200/100 mg against *Aspergillus fumigatus* and *Aspergillus flavus* and the human albumin and serum effects on their antifungal activity.

MATERIAL AND METHODS

Strains. Two clinical isolates of *A. fumigatus* (AF5 and AF8 strains) and two isolates of *A. flavus* (AFL9 and AFL10 strains) were selected. Conidia were harvested after isolates were subcultured on potato dextrose agar slants at 35°C for 5 to 7 days and were suspended in 5 mL of 0.85% normal saline containing 0.1% Tween 20, followed by 15 s of vortexing. Conidial suspensions were adjusted for microscopic enumeration with a cell counting hemacytometer to 1×10^6 conidia/ml.

Media. RPMI 1640 medium with L-glutamine but without bicarbonate buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid (Sigma, St Louis, MO, USA) and supplemented with 2% glucose, RPMI broth containing 75% human serum (Lonza Group Ltd, Basel, Switzerland), and RPMI broth with 3.75 g/dL human albumin (A-1653; Sigma Aldrich, St Louis, MO, USA) were used for susceptibility testing and time-kill experiments. Human serum was inactivated by heat for 30 min at 56ºC before addition to RPMI. An albumin concentration of 3.75 ± 0.10 g/dl was obtained in RPMI broth plus 75% human serum by the bromcresol green method. Both protein supplemented media showed similar albumin concentrations.

Antifungal agents and susceptibility testing. Drug stock solutions of voriconazole and anidulafungin (both from Pfizer Inc, NY, USA) were prepared from pure powders. Voriconazole and anidulafungin were dissolved in 100% dimethyl sulfoxide (DMSO). Antifungal activity was determined using the EUCAST broth dilution method for voriconazole[17] and with XTT colorimetric assay for anidulafungin[18] in the three described media. Twofold serial dilutions were prepared in order to obtain final concentrations ranging from 0.015 to 16 mg/L for voriconazole and 0.06 to 64 mg/L for anidulafungin after inoculation with a conidial suspensions diluted 1:10 (1×10^5 conidia/mL) in sterile water, 100% human serum or RPMI plus human albumin.

MICs of voriconazole were read at 48 h as the lowest drug concentrations that showed complete growth inhibition. For

anidulafungin, the MEC was determined by the XTT assay (X-MEC)[18]. The X-MEC endpoint was defined as the lowest anidulafungin concentration showing a percent metabolic activity equal to or less than the cut-off level of 60% for *A. fumigatus* and 40% for *A. flavus*[18]. Susceptibility tests were repeated in at least three occasions. Paradoxical effect was considered significant if it exceeded the metabolic activity at minimum metabolic activity by at least 40%[18].

XTT assay. XTT reduction assay was performed as previously described[18,19]. Conidial suspensions were exposed to antifungal concentrations in the different culture media at a total volume of 1 ml. Additional wells containing RPMI, RPMI plus human serum, and RPMI plus human albumin only were included to correct for background absorbance. After 48h of incubation, 50 µL of tetrazolium salt XTT stock solution (5.75 mg/mL) prepared in saline containing 6.25 µM menadione (Sigma, St Louis, MO, USA) was added to each well and were incubated at 37°C for an additional 2 h. Plates were centrifuged for 5 minutes at 13.000 rpm to pellet cells. Formazan product in the supernatant was measured in terms of optical density at 460 nm (OD_{460}) using a spectrophotometer (Cintra 101, GBC, Dandenong, VIC 3175 Australia). The percentage of metabolic activity for each well with drug in relation to that of the drug-free control well (media controls) was calculated, after subtraction of the background absorbance, as $[(A_{drug\ well} - A_{background\ drug\ well})/A_{control} - A_{background\ control})] \times 100$. To determine whether a substantial proportion of formazan product was retained in cell pellets of the experiments, the pellets were resuspended in 1 ml of DMSO and centrifuged and the OD_{460} of the supernatant was determined[19].

Time-kill curves. Killing curves were performed (total volume of 1-ml) with an inoculum of 10^5 spore/ml. A final voriconazole and anidulafungin concentration of 2.08 mg/L and 8.60 mg/L, corresponding to the steady-state peak serum concentrations (Cmax) achieved after multiple 400/200 mg doses of voriconazole (VfendTM package insert, Pfizer) and 200/100 mg doses of anidulafungin (EraxisTM package insert; Pfizer), respectively, were tested in the three media described above: (i) RPMI medium (Cmax-RPMI); (ii) RPMI broth plus human serum (Cmax-HS); and (iii) RPMI broth plus human albumin (Cmax-HAlb). In parallel, killing curves with a final voriconazole and anidulafungin concentration of 0.87 and 0.086 mg/L (fCmax), corresponding to theoretical free-drug Cmax considering a 58% of protein binding for voriconazole (Vfend™ package insert, Pfizer) and a 99% for anidulafungin (EraxisTM package insert; Pfizer), were performed in RPMI medium. Control growth curves were performed in all media tested without antifungal agents. Cultures were incubated at 35°C and samples were tested for XTT assay at 6, 10, 24 and 48 h. All experiments were performed in triplicate.

Anidulafungin effects on fungal structure. Fungal structures of AF8 and AFL10 formed in time-kill experiments (control and anidulafungin curves) after 48 h incubation were extracted from wells, fixed in 4% buffered formalin for 24 hours and embedded in paraffin by routine histological methods. To visualize fungal structures by optical microscopy, 4 µm paraffin sections were stained with PAS-Alcian blue.

Statistical analysis. Differences between metabolic activities obtained in different media with Cmax and free-drug concentrations were determined by ANOVA with the Tukey test for multiple comparisons.

RESULTS

Susceptibility testing. Modal values of voriconazol MICs and anidulafungin X-MECs are summarized in the table 1. Voriconazol displayed equivalent MIC values against all strains in presence or absence of human albumin or human serum. The human protein-induced effects were observed with anidulafungin where X-MECs values shifted higher in presence of both human serum and human albumin. Serum increased anidulafungin X-MECs in 64-folds for *A. fumigatus* and >256-folds for *A. flavus*, while it was increased in 32- and 64- folds for *A. fumigatus* and >256 for *A. flavus* in presence of human albumin. None of the strains showed paradoxical effect at the range of concentrations tested.

Antifungal activity of voriconazole and/or anidulafungin in time-kill experiments. In antifungal-free wells the XTT formazan signal (measured from supernatant at 460 nm) produced by *A. fumigatus* or *A. flavus* strains increased (according to the increase of the metabolic activity in the well) from 0 to 48h. The increase of XTT formazan signal in human serum was linear with maximal measurements of absorbance at 48 h, while in RPMI and human albumin medium a decline in the trend of slope growth was observed between 24 to 48 h. In terms of absorbance units, the *Aspergillus* growth was higher in RPMI medium and human albumin than in human serum at any time-points. The presence of human albumin delayed the growth of all strains at early times respect to RPMI medium, reaching similar growth rates from 10 h and even growth rates higher for *A. flavus* at 48 h. Signals were higher in *A. flavus* at 48 h (A_{460} mean: 1.9, 2.3 and 1.7 units in RPMI, human albumin, and human serum, respectively) than in *A. fumigatus* (A_{460} mean: 1.8, 1.8 and 1.2 units in RPMI, human albumin, and human serum) in all media tested. The relative growth of *Aspergillus* strains in presence of human albumin or human serum respect to RPMI medium at 48 h are shown in the table 2. Lower absorbance values were detected in wells with antifungal agents (according to an inhibition of fungal cell metabolic activity). The effect of voriconazole, anidulafungin and voriconazole plus anidulafungin on the metabolic activity of *Aspergillus* at Cmax and *f*Cmax concentrations over study period is showed in table 3 and figure 1. Voriconazol exhibited a rapid decline in metabolic activities for all strains at both concentrations tested in RPMI medium from early times. The metabolic activity decreased from 18-38% and 22-36% at 6h to 0.01-1.1% (a reduction in the metabolic activity of >98.4%) and 2.9-10.3% (a reduction in the metabolic activity of >89.7%) at 48h for Cmax-RPMI and *f*Cmax, respectively. Voriconazole Cmax-RPMI was significantly more active than *f*Cmax against AF8 and both *A. flavus* strains at 48 h (table 3). In contrast, the

Table 1 **Antifungal activities of voriconazole and anidulafungin against *A. fumigatus* and *A. flavus* strains in the different media tested.**

Strain	Voriconazole MIC (mg/L)			Anidulafungin X-MEC (mg/L)		
	RPMI	RPMI + 75% serum	RPMI + albumin	RPMI	RPMI + 75% serum	RPMI + albumin
AF5	1	1	1	0.25	16	16
AF8	1	1	1	0.25	16	8
AFL9	2	2	2	0.25	>64	>64
AFL10	2	2	2	0.5	>64	>64

reduction in the metabolic activity was slower and less pronounced with anidulafungin for all *Aspergillus* strains showing metabolic activities from 60 to 100% at 6 and 10h for Cmax-RPMI and *f*Cmax concentrations. Substantial decreases were observed at later times but only with Cmax-RPMI. Metabolic activities in all *Aspergillus* ranged from 18 to 52% at 24h and 19 to 38% (a reduction in the metabolic activity of <80.1%) at 48h with the Cmax-RPMI concentration. Higher metabolic activities ranged from 79 to 100% at 24h and 84 to 100% (a reduction in the metabolic activity of < 15%) at 48h ($p<0.01$) were observed with *f*Cmax in all strains. Voriconazole Cmax and *f*Cmax were significantly more active than anidulafungin Cmax in RPMI medium at 48 h ($p<0.001$ and $p<0.05$). No differences were observed between voriconazole and voriconazole plus anidulafungin at these concentrations in RPMI medium at any time point.

Effect of human albumin and serum on the antifungal activity. As expected, anidulafungin activity decreased in presence of human serum or albumin at Cmax concentration, while voriconazole and voriconazole plus anidulafungin showed a pattern of fungicidal activity similar to that described at Cmax-RPMI (figure 1). Human serum or albumin effects on the anidulafungin activity were only patent at 48 h. being more pronounced with *A. flavus* strains and human albumin. As shown in table 3, reductions in the metabolic activity in RPMI medium (range of 62.2 to 80.9%) were significantly lower for both *A. fumigatus* strains (44.5-48.8%, $p<0.05$) and for AFL9 strain (32.24%, $p<0.01$) in presence of serum, and for all strains (4.7-45.1%, $p<0.01$) in presence of human albumin at 48h. However, the metabolic activity of *A. flavus* and *A. fumigatus* at Cmax-HS and *A. fumigatus* at Cmax-HAlb were significantly lower ($p<0.05$) than those measured at *f*Cmax concentration (that represent a corrected Cmax according to theoretical protein binding in humans tested in RPMI medium). Anidulafungin activity at Cmax-HAlb was higher against *A. fumigatus* than *A. flavus* ($p=0.0003$) while the efficacy of Cmax-RPMI, Cmax-HS, and *f*Cmax were similar in both species. Metabolism in presence of Cmax-HS was significantly lower than measured with Cmax-Alb against *A. flavus* strains.

Growth in different culture media. The growth of *A. fumigatus* and *A. flavus* mycelia was consistent with a liquid submerged spheroid mass of hyphae in antifungal-free wells containing RPMI medium while a dense aerial mycelial mat with abundant sporulation was common in wells with human serum or albumin. An extracellular matrix around hyphae mass was observed in section of mycelium growing in human albumin medium (figure 3C and 4C). In wells with antifungal the mass of hyphae only was noticeable in presence of anidulafungin regardless of culture medium used. Typical branched, shortened hyphae, and dilatations along the hyphal elements were observed for AF8 (figure 3B, 3D, and 3F) and AFL10 (figure 4B, 4D, and 4F). The mycelium was totally devoid of extracellular matrix in wells containing human albumin (figure 3D and 4D) and sporulation was not present in both media with human proteins. Mycelial growths were denser in hyphae content in free and anidulafungin wells with RPMI medium than in wells with human serum or albumin. Larger number of hyphae was also observed in the mycelium of *A. flavus* strains than *A. fumigatus* strains in the three media tested and with or without the antifungal agent.

Limitations of the XTT assay for antifungal activity comparison when human proteins supplemented media are used. A residual orange dye compatible with retained XTT formazan product were observed in pellets after supernatant removal. The intensity of the signal was substantial in pellets from antifungal-free wells and wells containing anidulafungin after DMSO extraction, with maximal levels at 48h. Signals in pellets from wells containing voriconazole (alone or combined) were insignificant by comparison. As show in figure 2, pellet signals from anidulafungin Cmax-RPMI and *f*Cmax wells (mean: 0.41 and 0.53 units) and antifungal free-wells containing RPMI medium (mean: 0.41 units) for *A. fumigatus* strains were similar at 48h. Signals detected in pellets and supernatant (mean: 0.46 units) for Cmax-RPMI were not significantly different. Pellet signals from antifungal free-wells containing human serum or albumin (mean: 0.11 and 0.23, respectively) were lower and negligible in wells containing anidulafungin (mean: 0.04 and 0.07, respectively). The amount of retained product in controls containing RPMI or albumin for *A. flavus* was higher than those detected for *A. fumigatus* strains, while similar values were observed when human serum was tested in both species. Pellets from anidulafungin wells (mean: 1.36 for Cmax and 1.12 for *f*Cmax) doubled the signals observed in an-

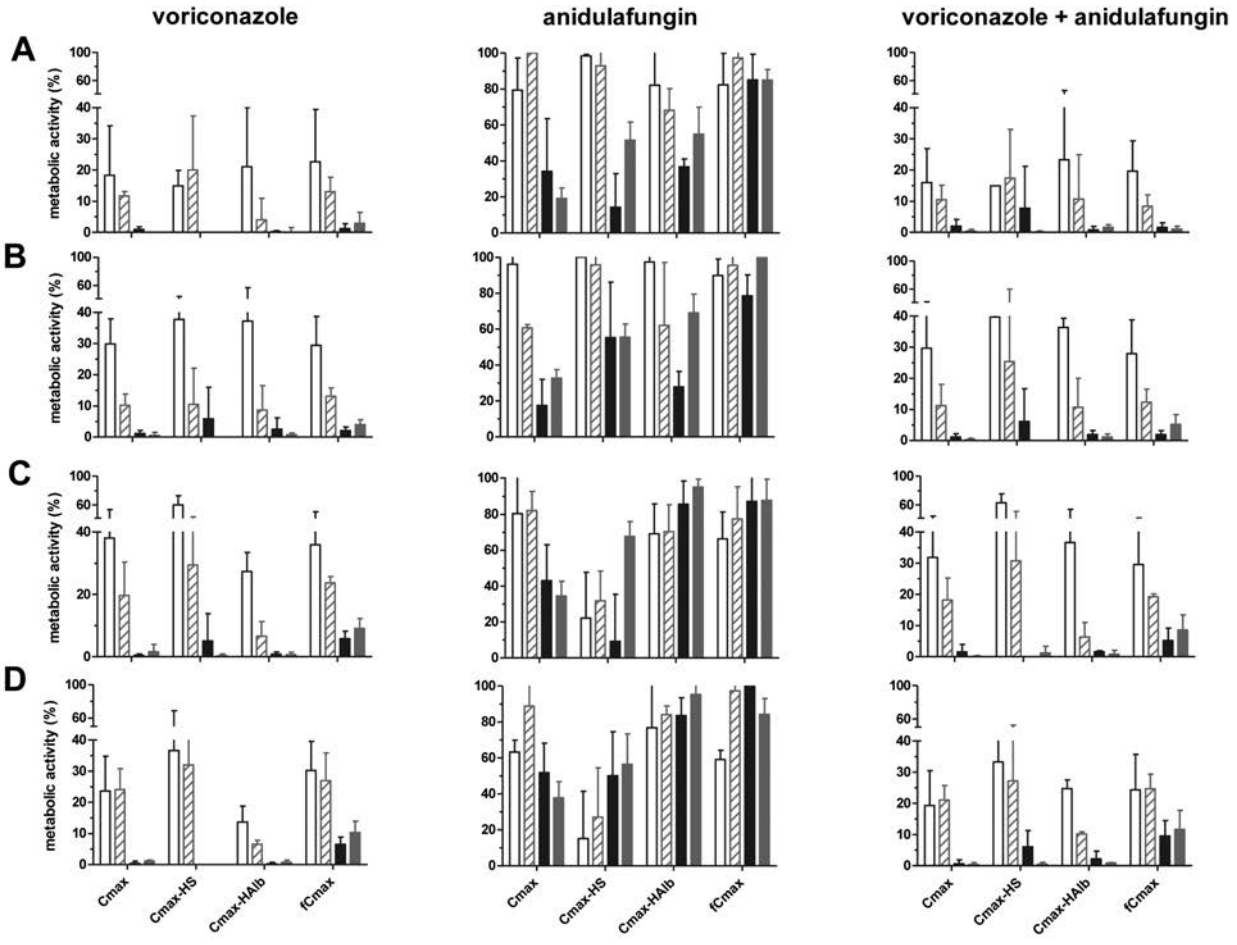

Figure 1 **Metabolic activity of AF5 (A), AF8 (B), AFL9 (C), and AFL10 (D) at 6h (white bars), 10h (striped bars), 24h (black bars) and 48h (grey bars) in presence of voriconazole and/or anidulafungin at Cmax concentrations in RPMI (Cmax-RPMI), RPMI plus serum (Cmax-HS) and RPMI plus albumin (Cmax-ALB) and at free-drug Cmax concentrations (*f*Cmax).**

tifungal-free wells containing RPMI medium (mean: 0.69) and doubled or was comparable to the supernatant signal measured at Cmax and *f*Cmax (0.69 and 1.67, respectively). Signal intensity remained very low in pellets from anidulafungin wells containing human serum or albumin (mean: 0.08 and 0.19) as in *A. fumigatus* strains.

DISCUSSION

The rapid and pronounced decrease of fungal metabolic activities in presence of voriconazole, even when the lowest concentration (*f*Cmax) was tested, makes difficult to assess the potential contribution of anidulafungin in the effect of the combination. However, the proposed voriconazole-anidulafungin combination does not appear to produce antagonistic effect against *Aspergillus* spp. and suggest its role as candidate for treatment of strains with high MICs to azoles. Lower sub-MICs concentrations of voriconazole may be more relevant to discern the contribution of anidulafungin in the combination effect.

In agreement with previous reports voriconazole demonstrated a time-dependent slow fungicidal effect on *A. fumigatus* and *A. flavus* strains by time-kill curves, showing that at least 24 hours of exposure are needed to reach fungicidal activity[20,21,22]. A slightly concentration-dependent effect was also found at the concentrations tested (Cmax; 2.08 mg/L and *f*Cmax; 0.9 mg/L) once fungal killing was significant, suggesting that AUC/MIC is, as in *Candida* spp, the critical pharmacokinetic/pharmacodynamic parameter associated with voriconazole efficacy[23]. Anidulafungin exerted a fungistatic rather fungicidal effect against both *Aspergillus* species[24] showing low metabolic activity reductions (60 to 80%) after 48 h of exposure even at Cmax concentrations exceeding the X-MEC values.

Table 2 **Relative growth (mean ± SD) of *Aspergillus* strains in the different media tested respect to RPMI broth at 48 h.**

Strain	RPMI + 75% serum			RPMI + albumin		
AF5	73.6	±	23.9	102.5	±	41.3
AF8	63.8	±	10.8	98.9	±	17.1
AFL9	89.1	±	2.1	122.4	±	2.7
AFL10	89.3	±	5.28	122.0	±	17.8

Percentages were calculated as: (Abs 460 nm in RPMI plus serum or albumin) x 100/Abs 460 nm in RPMI broth.

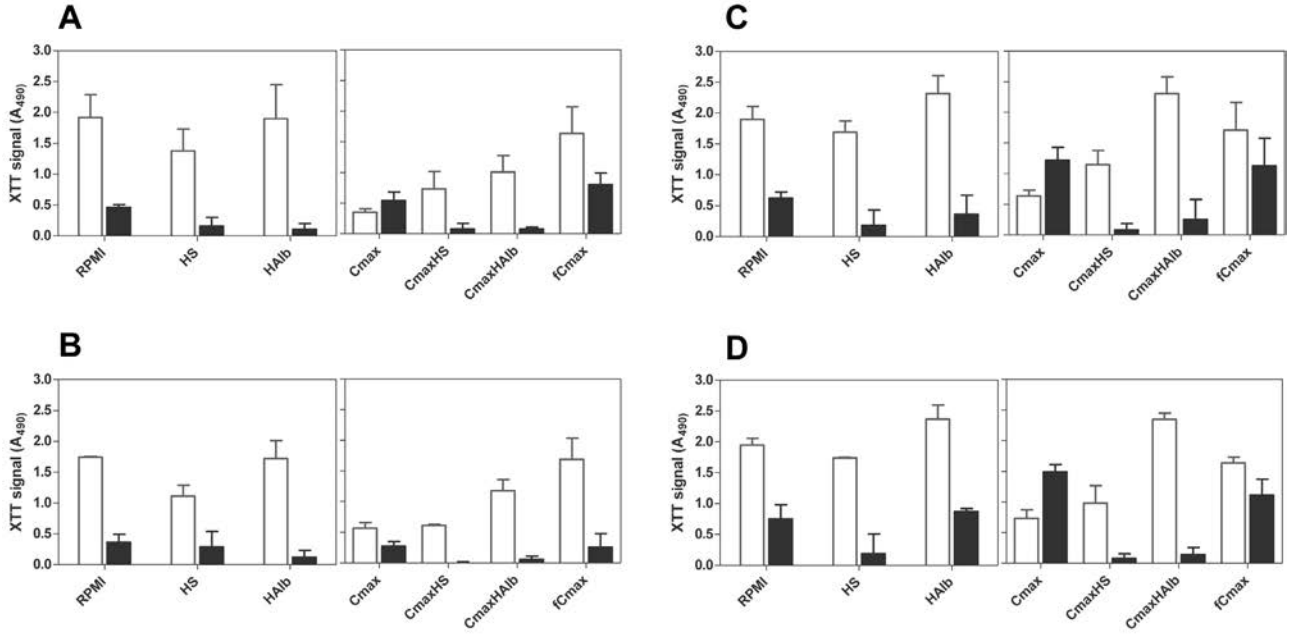

Figure 2 **XTT signal measured in supernatant (white bars) and pellets (black bars) of antifungal-free (RPMI, HS and HAlb) and anidulafungin wells at Cmax concentrations in RPMI (Cmax-RPMI), RPMI plus serum (Cmax-HS) and RPMI plus albumin (Cmax-ALB) and at free-drug Cmax concentrations (fCmax) for *A. fumigatus* 5 (A), *A. fumigatus* 8 (B), *A. flavus* 9 (C), and *A. flavus* 10 (D) after 48 hours of exposition.**

Our attempt to clarify the impact of protein binding in the efficacy of voriconazole and/or anidulafungin by time kill experiments showed, as expected by susceptibility test results, that the pattern of activity of voriconazole (and voriconazole in combination with anidulafungin) does not change when a 75% of human serum or 4 g/dl of human albumin is added to the RPMI medium. Reductions in the metabolic activity in human serum, albumin, and RPMI medium at Cmax concentration were similar despite of a theoretic 58% of drug bound to proteins (only a 42% of total drug, i.e. 0.87 mg/L, is available to exert antifungal activity) in the human serum and albumin media. In addition, the activity of voriconazole in both supplemented media was greater (significantly greater in 3/4 strains tested) than that observed at *f*Cmax (free Cmax in RPMI medium without proteins) after 48 hours of exposition. In contrast, in the case of anidulafungin, with an unbound percentage of drug of 1% (protein binding of 99%), the addition of human albumin or serum affect and reduce its activity (table 3) respect to the metabolic activity of all *Aspergillus* strains showed in RPMI medium at Cmax concentration after 48 hours. However, as occur with voriconazole, the activity of anidulafungin in supplemented media with proteins (mainly in human serum) favourably differ from the activity predicted by the theoretical *f*Cmax indicating that drug protein binding might be reversible and weak as previously it has been hypothesized for other echinocandin[25]. This hypothesis is consistent

Table 3 **Mean reductions in the metabolic activity measured for *A. fumigatus* and *A. flavus* strains after 48 h of exposure to voriconazole, anidulafungin or voriconazole plus anidulafungin.**

	Cmax	Cmax-HS	Cmax-HAlb	*f*Cmax
Voriconazole				
AF5	99.99	>99.99	99.57	97.10
AF8	99.46**	>99.99**	99.31*	96.02
AFL9	98.41*	99.67**	99.41**	90.92
AFL10	98.90**	>99.99***	99.28**	89.71
Anidulafungin				
AF5	80.87***	48.45*	45.09*	15.01
AF8	67.20***	44.50***	30.96**	0.00
AFL9	65.50***	32.24	5.23	12.22
AFL10	62.18**	43.63	4.69	15.83
Voriconazole + anidulafungin				
AF5	99.61	99.81	98.42	98.92
AF8	99.72*	>99.99*	98.87	94.83
AFL9	99.87*	98.76*	99.16*	91.42
AFL10	99.61**	99.47**	99.10*	88.36

Differences vs. fCmax: *(p<0.001), **(p<0.01), and *(p<0.05).**

with the free-drug hypothesis (only the unbound drug, under equilibrium conditions, contributes to its pharmacological effect) since it propose a dynamic interaction setting in which a non-restrictive protein binding process is present due to a rapid and continuous re-equilibrium among free-drug, protein-bound fraction and fungal targets.

Moreover, the impact of human protein binding in the activity of anidulafungin, but not voriconazole, appears be species and culture medium-dependent. Anidulafungin exhibit greater activity in serum than in presence of human albumin supplement against both species, but differences were particularly accentuated for *A. flavus*, in which a 27-39% higher metabolic reductions in presence of serum were observed ($p<0.05$). Clearly, the assumed reversibility of drug binding process would be challenged for *A. flavus* in a medium supplemented with human albumin. Since in theory extend of drug protein binding should be equivalent in both supplemented media, other additional factors must contribute to the differences observed between both protein supplemented culture medium. First, we used heat inactivated serum to avoid the activity of serum complement but recently it has been proposed an inhibitory action of serum due to its ability to chelate iron, which deprives the invading fungal pathogens of this essential nutrient[26]. In addition, previous studies have shown the ability of *Aspergillus* strains, unlike other fungal pathogens, to grow in presence of human serum up to 80%[13,14,26]. In our study, all strains grew in RPMI with 75% human serum but as is shown in table 2 the growth in serum was a 70% of this reported for RPMI broth. All strains showed similar (*A. fumigatus*) o higher (*A. flavus*) growth in RPMI plus human albumin than RPMI broth. Then some serum compound, not albumin, seems to slightly inhibit mycelial growth. In addition, the nonspecific binding to other serum proteins besides albumin, as alpha and gamma-globulins or lipoproteins, might influence the reversibility of process among anidulafungin, proteins and fungal targets, since the same albumin concentration were used in both media for a better comparison[25]. Finally the method used in this work to obtain a quantitative measurement of the antifungal activity could be contributed to explain these differences. The standard XTT method is widely used to study antifungal activity and consequently any potential limitation of the method has to be reported in everyone´s interest. Khun et al.[19] reported that while XTT method is useful for antifungal comparisons involving one strain, its use may be more problematic to compare different strains and species since different strains metabolize substrate with different capabilities. Of concern was the significant amount of retained intracellular product which only becomes soluble after cell treatment with DMSO previously observed in a study involves *Candida* spp. As we shown in this work, retention also occurs in *Aspergillus* cells. The amount of retained dye in pellets after supernatant removal varies between species and was culture medium- and incubation time- dependent. The higher incubation time the higher the XTT retention. Degree of product retained in pellet is proportional to the mycelial mass developed in wells, which is high in antifungal free-wells and in wells with presence of a fungistatic agent as anidulafungin. However, the macroscopic and microscopic detected differences among mycelial mat grown

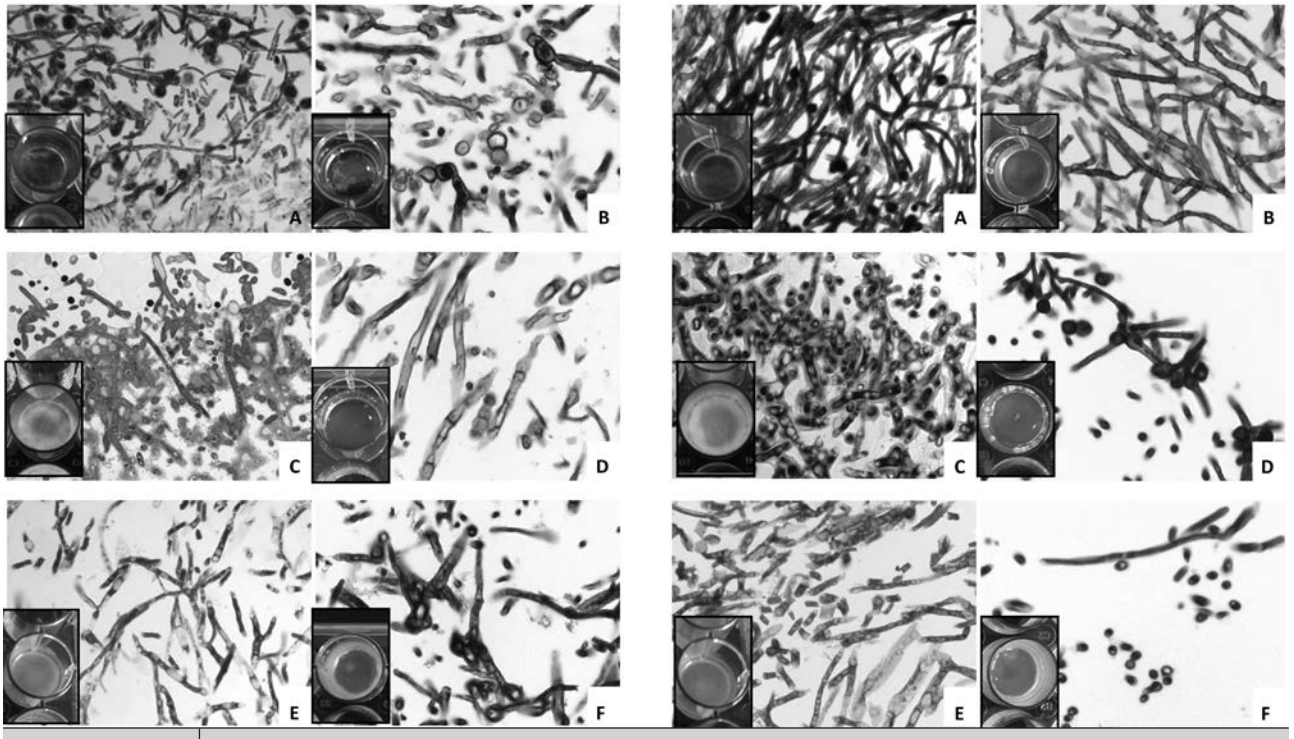

Figure 3 and 4 | **Changes in hyphal structure of *A. fumigatus* 8 (left, figure 3) and *A. flavus* 10 (right, figure 4) with Cmax anidulafungin concentration after 48 h of incubation: (A) Drug-free control in RPMI; (B) Cmax-RPMI; (C) Drug-free control in RPMI plus albumin; (D) Cmax-ALB; (E) Drug-free control in serum; (F) Cmax-HS (magnification x1000).**

in RPMI, human serum or albumin media at different time-points are also closely related with the different XTT retention. Composition and/or structural disposition of mycelial growth in RPMI medium leads to retain higher XTT product than mycelial growth under serum or human albumin presence even when the mycelial mass is similar. In summary, the antifungal potency of anidulafungin against *Aspergillus* is undervalue when metabolic activities are measured from XTT supernatant in supplemented media with human proteins while it is overestimated in free protein media as RPMI, especially with *A. flavus* (figure 4).

In conclusion, synergism could not be demonstrated due to the high activity showed by voriconazole against *Aspergillus* sp. The relative low rate of protein binding of voriconazole has no effect on its activity. Surprisingly, slight changes were observed with anidulafungin, despite its high protein binding, indicating that the impact of protein binding on antifungal activity is considerably less than predicted based on theoretical free fraction. The XXT colorimetric assay needs to be standardized for use with *Aspergillus* spp. since without DMSO extraction the activity of echinocandins in a free-human protein RPMI medium could be overestimated.

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