Albumin Enhances Caspofungin Activity against *Aspergillus* Species by Facilitating Drug Delivery to Germinating Hyphae

Petros Ioannou,^a Aggeliki Andrianaki,^a Tonia Akoumianaki,^a Irene Kyrmizi,^a Nathaniel Albert,^c David Perlin,^b George Samonis,^a Dimitrios P. Kontoyiannis,^c Georgios Chamilos^a

Department of Medicine, University of Crete, Heraklion, Crete, Greece^a; Public Health Research Institute, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, New Jersey, USA^b; Department of Infectious Diseases, The University of Texas, MD Anderson Cancer Center, Houston, Texas, USA^c

The modest *in vitro* activity of echinocandins against *Aspergillus* implies that host-related factors augment the action of these antifungal agents *in vivo*. We found that, in contrast to the other antifungal agents (voriconazole, amphotericin B) tested, caspofungin exhibited a profound increase in activity against various *Aspergillus* species under conditions of cell culture growth, as evidenced by a ≥ 4 -fold decrease in minimum effective concentrations (MECs) ($P = 0.0005$). Importantly, the enhanced activity of caspofungin against *Aspergillus* spp. under cell culture conditions was strictly dependent on serum albumin and was not observed with the other two echinocandins, micafungin and anidulafungin. Of interest, fluorescently labeled albumin bound preferentially on the surface of germinating *Aspergillus* hyphae, and this interaction was further enhanced upon treatment with caspofungin. In addition, supplementation of cell culture medium with albumin resulted in a significant, 5-fold increase in association of fluorescently labeled caspofungin with *Aspergillus* hyphae ($P < 0.0001$). Collectively, we found a novel synergistic interaction between albumin and caspofungin, with albumin acting as a potential carrier molecule to facilitate antifungal drug delivery to *Aspergillus* hyphae.

Echinocandins comprise a group of cell wall-active antifungal agents targeting β -1,3 glucan synthase that display potent antifungal activity against *Candida* and *Aspergillus* species (1–4). Caspofungin is the first member of this class of antifungal compounds that has been approved as first-line therapy for invasive candidiasis and as salvage therapy for invasive aspergillosis. Importantly, clinically effective concentrations of echinocandins cause extensive hyphal damage but fail to inhibit *Aspergillus* growth *in vitro*, an effect that implies a differential mode of action of these antifungal agents *in vivo* (5–7). In addition, this lag in *in vitro* activity of echinocandins creates problems in the development of reliable susceptibility methods to evaluate the activity of these compounds against *Aspergillus* spp. Thus, instead of the MIC, the minimum effective concentration (MEC), defined as the lowest concentration that leads to significant morphological changes resulting in the formation of short, stubby, and highly branched hyphae under microscopy (8, 9), was used. The MEC has been introduced as the endpoint of *in vitro* activity of echinocandins against *Aspergillus* spp. (8, 9).

Immunomodulation has been proposed as a supplemental mechanism of action of echinocandins to explain the increased efficacy of these antifungal agents *in vivo* (10, 11). Indeed, previous studies have reported that subinhibitory concentrations of echinocandins cause extensive cell wall remodeling and result in high levels of β -1,3 glucan surface exposure in germinating hyphae of *Candida* and *Aspergillus*. In particular, echinocandin-induced β -1,3 glucan surface exposure triggers a potent activation of dectin-1 signaling and results in the increased release of proinflammatory cytokines (11) and enhanced antifungal activity of phagocytes against *Aspergillus* hyphae *ex vivo* (10).

Furthermore, the tissue microenvironment at the site of infection may also account for the enhanced *in vivo* activity of echinocandins against *Aspergillus* spp. For example, *in vitro* simulation of tissue hypoxia results in increased antifungal activity of echinocandins via poorly characterized mechanisms (12). Here, we

tested the effect of cell culture growth, a more physiologically relevant environment than standard *in vitro* susceptibility testing conditions, in the activity of echinocandins compared to other antifungal agents against *Aspergillus* spp. In contrast to all antifungal agents tested, including the other echinocandins, we found a caspofungin-specific increase in anti-*Aspergillus* activity under conditions of cell culture growth, mediated by albumin. At a molecular level, we discovered a novel synergistic interaction of albumin and caspofungin resulting in increased delivery of both molecules to *Aspergillus* hyphae.

MATERIALS AND METHODS

Reagents. XTT [(2,3)-bis (2-methoxy 4-nitro 5-sulfonyl)-2H-tetrazolium carboxanilide; Sigma-Aldrich] was used as previously published (13). 6-Carboxyfluorescein diacetate (CFDA; Sigma-Aldrich) was diluted in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml and kept at -20°C until use. Menadione (Sigma-Aldrich) was dissolved in acetone at a stock concentration of 1 M, filter sterilized, and kept at 4°C until use. DMSO, bovine serum albumin (BSA), and fluorescein isothiocyanate (FITC)-conjugated BSA (FITC-BSA) were all purchased from Sigma-

Received 21 August 2015 Returned for modification 27 September 2015

Accepted 29 November 2015

Accepted manuscript posted online 7 December 2015

Citation Ioannou P, Andrianaki A, Akoumianaki T, Kyrmizi I, Albert N, Perlin D, Samonis G, Kontoyiannis DP, Chamilos G. 2016. Albumin enhances caspofungin activity against *Aspergillus* species by facilitating drug delivery to germinating hyphae. *Antimicrob Agents Chemother* 60:1226–1233. doi:10.1128/AAC.02026-15.

Address correspondence to Dimitrios P. Kontoyiannis, dkontoyi@mdanderson.org, or Georgios Chamilos, hamilos@imbb.forth.gr.

T.A. and I.K. contributed equally to this work.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02026-15>.

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FIG 1 Cell culture conditions potentiate the activity of caspofungin against *Aspergillus* spp. Inhibitory dose-response curves of caspofungin (CAS) (A) or voriconazole (VRC) (B) under reference testing conditions according to the CLSI M38 A2 document (closed symbols, RPMI with MOPS) or under conditions of cell culture growth (open symbols, RPMI with 10% FCS) against clinical isolates of different *Aspergillus* species. Percentages of metabolic activity under the condition without antifungal based on the mean absorbance of the formazan product and standard errors of the means (represented by error bars) were plotted in relation to increasing concentrations of each antifungal agent. Lines are the regression lines of a four-parameter logistic model (Hill equation).

Aldrich. Fluorescent Alexa Fluor 488-conjugated secondary rabbit anti-mouse antibody was purchased from Molecular Probes (Invitrogen).

Fungal isolates. *Aspergillus* clinical isolates, all collected from patients with invasive aspergillosis, were from the laboratory collection at the Mycology Research Laboratories at The University of Texas M. D. Anderson Cancer Center. *Aspergillus* conidia were harvested in phosphate-buffered saline (PBS) under sterile conditions, passed through a 40- μ m nylon cell strainer, washed twice in sterile PBS, and adjusted to a concentration of 1×10^6 to 5×10^6 conidia/ml. *Candida parapsilosis*, isolate ATCC 22019, served as a quality control for susceptibility studies.

Antifungal agents. Stock solutions from pure powders were prepared in DMSO (Sigma-Aldrich) for voriconazole, anidulafungin, and amphotericin B or in sterile water for caspofungin and micafungin, all at a stock concentration of 5 mg/ml, and were stored at -70°C until use.

Culture media and susceptibility testing studies. Antifungal susceptibility testing was performed using broth microdilution methods according to the CLSI M38-A2 document (14), with some modifications. Briefly, conidial suspensions were diluted in the appropriate RPMI growth medium and dispensed (100 μ l) at a final concentration of 0.4×10^4 to 5×10^4 /ml into wells of a flat-bottom 96-well microtiter plate containing serial 2-fold dilutions of antifungals. Plates were then incubated for 24 or

48 h at 37°C , and voriconazole and amphotericin B MICs were read as the lowest drug concentrations that showed absence of growth or complete growth inhibition (100% inhibition). For echinocandins, the MEC, defined as the minimal drug concentration producing short, stubby, hyperacutely branching hyphae, was determined by microscopic examination of the microdilution plates. *Candida parapsilosis* ATCC 22019 served as a quality control isolate for each experimental run, with susceptibility testing performed according to the CLSI M27-A3 document (15).

For susceptibility testing under cell culture conditions, we used medium containing RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, and 10 mM HEPES at pH 7.4 and performed experiments in a CO_2 incubator with humidity control at a temperature of 37°C and 5% CO_2 concentration. In subsequent studies, several parameters of cell culture growth were modified, including the presence of FCS and albumin and the type of buffer (MOPS [morpholinepropanesulfonic acid] instead of HEPES) used.

The XTT reduction assay was performed using the methods reported by Meletiadis et al. (16). After 24 h of incubation, plates were removed from the incubator, and 100 μ l PBS containing tetrazolium salt XTT and menadione was added to each well (final concentrations for XTT and menadione, 0.25 mg/ml and 25 μ M, respectively), and the tray

TABLE 1 Effect of cell culture conditions on inhibitory activities of caspofungin and voriconazole against different *Aspergillus* species based on EC_{50} values obtained from the nonlinear regression analysis or the MIC (or MEC for caspofungin) values^a

Strain	Caspofungin						Voriconazole					
	EC_{50} (ng/ml)		Slope		MEC (ng/ml)		EC_{50} (ng/ml)		Slope		MIC (ng/ml)	
	MOPS	FCS	MOPS	FCS	MOPS	FCS	MOPS	FCS	MOPS	FCS	MOPS	FCS
<i>A. fumigatus</i> 293	NA	16.06	NA	-2.04	500	125	127.90	60.05*	-2.91	-2.43	250	250
<i>A. fumigatus</i> ATCC 46645	243.40	12.85*	-0.79	-3.78*	500	125	104.90	36.48*	-1.29	-2.13	250	250
<i>A. flavus</i> clinical isolate 1	160.20	17.43*	-6.25	-2.83	500	250	58.95	43.11	-1.25	-1.45	500	125
<i>A. flavus</i> clinical isolate 2	64.83	22.43	-1.74	-1.80	250	125	71.16	22.18*	-2.50	-3.15	500	63
<i>A. terreus</i> clinical isolate 1	793.90	11.25*	-3.21	-1.62	1000	16	87.97	31.23*	-2.09	-2.09	250	125
<i>A. terreus</i> clinical isolate 2	175.20	0.08	-6.02	-22.52	500	31	73.81	54.40	-2.61	-1.53	500	500

^a Symbol and abbreviation: *, $P < 0.05$ compared to the MOPS condition; NA, not applicable due to incomplete growth inhibition.

was incubated for an additional 2 h. Trays were then removed from the incubator, and absorbance of the XTT reduction product, formazan, was read at 450 nm and 655 nm using a microplate spectrophotometer (Bio-Rad model 680). The percentage of the metabolic activity was expressed with the following formula: $\% \text{ MA} = 100\% \times (\text{OD}_{450} - \text{OD}_{655})_{\text{experiment}} / (\text{OD}_{450} - \text{OD}_{655})_{\text{control}}$, where MA is metabolic activity and OD_{450} and OD_{655} are the optical densities at 450 and 655 nm, respectively.

CFDA fluorometric viability assay. The CFDA fluorescent viability assay was performed as previously described (17). Briefly, after 24 h, CFDA was added to the medium at a final concentration of 50 $\mu\text{g}/\text{ml}$, incubated at 37°C and 5% CO_2 for an additional 45 min, and washed twice with PBS. The fluorescence intensity of the wells was measured with a Biotek fluorescence plate reader with excitation at 460 nm and emission at 528 nm.

Fluorescence imaging studies. For the experiments with FITC-BSA, 10^4 conidia of *Aspergillus* were grown for 17 h on sterile coverslips with or without caspofungin. Then, the medium was removed, and FITC-BSA or regular BSA was added for 1 h at the indicated concentration. In some experiments, FITC-BSA or BSA was added from the beginning of incubation. In other experiments, resting conidia, swollen conidia, or mature hyphae were incubated for 1 h with FITC-BSA. To test the specificity of albumin binding to *Aspergillus* hyphae in the FITC-BSA assay, we performed competition studies by blocking hyphae with 150 mg/ml BSA for 1 h before incubation with FITC-BSA. For assessment of caspofungin binding to *Aspergillus* hyphae, we used fluorescence-labeled caspofungin (covalently linked caspofungin to the fluorescent molecule 7-amino-91f-(L3-dichloro-9;9-dimethyl-acridm-2-one) (7-amino DDAO), as described previously (18). Briefly, DDAO-caspofungin (final concentration, 1 $\mu\text{g}/\text{ml}$) was incubated for 1 h with *Aspergillus* hyphae, with or without the presence of 3 mg/ml BSA. For all fluorescence imaging studies, coverslips were always washed twice with PBS to remove the unbound compound, mounted on slides with Mowiol, and immediately assessed by confocal microscopy. All images were acquired with a Leica TCS SP2 confocal microscope and analyzed with the use of Leica software LAS AF version 2.6.0. Per-pixel quantification of the membrane fluorescence was performed in images acquired with single scans.

SEM studies. Samples grown on 12-mm round coverslips were fixed with 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3), washed with 0.1 M cacodylate buffer (pH 7.3) for 3×10 min, postfixed with 1% cacodylate-buffered osmium tetroxide for 1 h, and washed with 0.1 M cacodylate buffer 3 times and distilled water 2 times. The samples were sequentially treated with Millipore-filtered 1% aqueous tannic acid for 30 min in the dark, washed 3 times in distilled water, and incubated in Millipore-filtered 1% aqueous uranyl acetate for 1 h in the dark. The samples were rinsed thoroughly with distilled water 2 times for 5 min each. The samples were then dehydrated with a graded series of increasing concentrations of ethanol for 5 min each. The samples were then transferred to graded series of increasing concentrations of hexamethyldisilazane (HMDS) for 5 min each and air dried overnight. Samples were mounted onto double-stick carbon tabs (Ted Pella, Inc., Redding, CA). Samples were examined in a JSM-5910 scanning electron microscope (SEM; JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 5 kV.

Data analysis. A four-parameter logistic model (Hill equation) using computer curve-fitting software (Prism 4; GraphPad Software, Inc., San Diego, CA, USA) was fitted to the experimental data performed in triplicate to derive the 50% effective concentration (EC_{50}) and the steepness of the inhibitory dose-response curve (Hill slope or coefficient). Significant changes in the antifungal activity of caspofungin and voriconazole between different culture conditions were assessed by comparison of the inhibitory dose-response curves. Unpaired *t* test or one-way analysis of variance (ANOVA) with Dunnett's posttest where applicable were used to assess statistical differences in mean fluorescence intensity per pixel in the fluorescence studies. In all experiments, statistical significance was set at a

FIG 2 The increased anti-*Aspergillus* activity of caspofungin under conditions of cell culture depends on the presence of serum. Inhibitory dose-response curves of caspofungin (CAS) under conditions of cell culture growth (RPMI with HEPES and 5% CO_2) (A), reference testing conditions according to the CLSI M38 A2 document with 5% CO_2 (RPMI with MOPS and 5% CO_2) (B), and reference testing conditions according to the CLSI M38 A2 document (RPMI with MOPS and 0.03% CO_2) (C) against *Aspergillus fumigatus* strain 293, in the presence of different contents of FCS (vol/vol). Percentages of metabolic activity under the condition without caspofungin based on the mean absorbance of the formazan product and standard errors of the means (represented by error bars) were plotted in relation to increasing concentrations of caspofungin (CAS). Lines are the regression lines of a four-parameter logistic model (Hill equation).

P value of <0.05. All of the experiments were performed at least twice in triplicate.

RESULTS

Conditions of cell culture growth potentiate caspofungin activity against *Aspergillus* species. In order to explore host-derived factors that enhance *in vivo* activity of echinocandins against *Aspergillus* spp., we performed susceptibility studies under conditions of cell culture. We initially assessed the activity of caspofungin and voriconazole against different *Aspergillus* spe-

TABLE 2 Effect of FCS and albumin (BSA) on inhibitory activities of caspofungin against *Aspergillus fumigatus* strain 293 under different culture conditions based on EC₅₀ values obtained from the nonlinear regression analysis or the MEC values^a

Serum and concn	RPMI HEPES with 5% CO ₂		RPMI MOPS			
			5% CO ₂		0.03% CO ₂	
	EC ₅₀	MEC	EC ₅₀	MEC	EC ₅₀	MEC
FCS (%)						
0	414.0	500	199.9	500	212.0	500
1	55.0*	250	20.3*	250	30.1	250
5	30.4	125	12.7*	250	12.9*	250
10	9.9*	63	5.9*	250	0.9*	63
20	24.9*	63	7.9	125	2.8*	31
BSA (mg/ml)						
0			177.5	500	98.4	500
0.15			22.9**	250	16.2**	250
0.75			21.4**	250	16.0	250
1.5			18.1**	125	16.1**	125
3			16.4**	125	17.6**	125

^a Symbols: *, $P < 0.05$ compared to 0% FCS; **, $P < 0.05$ compared to 0 mg/ml BSA. Caspofungin concentrations are in nanograms per milliliter.

cies using standard broth microdilution methods (14) in (i) reference medium (RPMI with MOPS) and growth in an ambient air incubator or (ii) in cell culture medium (RPMI with 10% FCS) and growth in a CO₂ incubator (5% CO₂) with humidity control.

We found that susceptibility testing under conditions of cell culture growth resulted in a significant increase in activity of caspofungin against all *Aspergillus* isolates tested as evidenced by (i) a significant reduction in MEC (2- to 64-fold) and a profound decrease in EC₅₀s (2- to 1,000-fold), determined by the XTT assay (Fig. 1 and Table 1). In contrast, susceptibility testing under conditions of cell culture showed no significant effect on the activity of voriconazole against all the *Aspergillus* isolates tested (Fig. 1 and Table 1). Of interest, there was a partial reduction in the MICs of amphotericin B against 50% (2/4) *Aspergillus* strains tested under cell culture conditions (see Table S1 in the supplemental material).

In addition, we assessed the effect of cell culture conditions in the anti-*Aspergillus* activity of caspofungin by employing the CFDA fluorescence viability assay. In agreement with the results of the XTT colorimetric assay, we found that caspofungin induced a significant decrease in CFDA fluorescence under conditions of cell culture in comparison to susceptibility testing under reference

FIG 3 The serum-dependent increase in anti-*Aspergillus* activity of caspofungin is mediated by albumin. Inhibitory dose-response curves of caspofungin (CAS) against *A. fumigatus* strain 293 under reference testing conditions in 5% CO₂ (A) or 0.03% CO₂ (B) and in the presence of different contents of albumin. Percentages of metabolic activity under the condition without caspofungin based on the mean absorbance of the formazan product and standard errors of the means (represented by error bars) were plotted in relation to increasing concentrations of caspofungin (CAS). Lines are the regression lines of a four-parameter logistic model (Hill equation). (C) SEM images of *A. fumigatus* strain 293 grown overnight in standard culture medium alone or in the presence of albumin at sub-MEC of caspofungin or in albumin plus sub-MEC of caspofungin.

FIG 4 Preferential binding of albumin to germinating forms of *Aspergillus*. *A. fumigatus* strain 293 resting conidia, swollen conidia (resting conidia allowed 5 h to grow in RPMI-HEPES at 37°C and 5% CO₂), and mature hyphae (resting conidia allowed 17 h to grow to hyphae in RPMI-HEPES at 37°C and 5% CO₂) were incubated with 1.5 mg/ml FITC-BSA for 1 h in RPMI-HEPES at 37°C and 5% CO₂, washed, and immediately assessed with a confocal microscope.

conditions (see Fig. S1 in the supplemental material). Collectively, our studies reveal a significant increase in the antifungal activity of caspofungin against *Aspergillus* under conditions of cell culture growth.

The increased anti-*Aspergillus* activity of caspofungin under conditions of cell culture depends on serum. Next, we analyzed the effects of different parameters of cell culture growth on anti-*Aspergillus* activity of caspofungin. We initially performed susceptibility testing in the presence of serum and found a significant dose-dependent increase in the activity of caspofungin against *Aspergillus* upon the addition of different concentrations of serum in the culture media (Fig. 2 and Table 2). Importantly, the augmenting effect of serum in caspofungin activity against *Aspergillus* was independent of medium composition (Fig. 2A to C) and CO₂ concentration (Fig. 2B and C). Collectively, these studies reveal that the increase in the activity of caspofungin against *Aspergillus* under cell culture conditions is dependent on serum.

The serum-dependent increase in the anti-*Aspergillus* activity of caspofungin is mediated by albumin. Next, we sought to identify the serum component(s) that accounted for the increased anti-*Aspergillus* activity of caspofungin. We initially evaluated the effect of albumin, the major protein component of serum, in caspofungin activity against *Aspergillus*. We found that the presence of physiologically relevant concentrations of albumin resulted in an up to 10-fold increase in caspofungin activity, in a dose-dependent way (Fig. 3A and B and Table 2). Furthermore, scanning electron microscopy studies revealed that sub-MEC concentrations of caspofungin caused significant *Aspergillus* hyphal damage in the presence of albumin (Fig. 3C). Importantly,

albumin did not enhance the activity of the other two echinocandins, micafungin and anidulafungin, against various *Aspergillus* strains tested (see Table S2 in the supplemental material). Finally, we confirmed that albumin had no effect in fungal growth at the concentrations used for *in vitro* studies (see Fig. S2 in the supplemental material).

Albumin binds selectively to germinating forms of *Aspergillus*. Albumin is a large protein that binds with high affinity to many antimicrobial agents, including echinocandins (19). Therefore, we hypothesized that albumin binding to caspofungin could facilitate the delivery of antifungal molecules to the fungal cell wall. Of interest, we found a high degree of association of albumin with the surface of germinating hyphae of *Aspergillus* that was not observed with resting and swollen conidia (Fig. 4). The specificity of binding of FITC-albumin with *Aspergillus* hyphae was verified by competition studies with unlabeled albumin (see Fig. S3 and S4 in the supplemental material).

In addition, we noticed a significant increase in binding of FITC-albumin to caspofungin-treated versus control, untreated hyphae following 18 h of exposure to caspofungin ($P < 0.0001$) (see Fig. S5 in the supplemental material). Collectively, these studies demonstrate a preferential binding of albumin on the surface of germinating hyphae of *Aspergillus* that is further enhanced upon treatment with caspofungin.

Albumin acts as a carrier molecule to increase caspofungin delivery to *Aspergillus* hyphae. Next, we generated fluorescence-labeled caspofungin covalently linked to 7-amino DDAO (DDAO-caspofungin) to directly evaluate whether albumin acts as a carrier molecule to increase delivery of caspofungin to *Aspergillus*

hyphae. The appropriate concentration of DDAO-caspofungin with or without albumin was added to wells containing mature *Aspergillus* hyphae for 1 h, after which the cultures were washed twice with PBS to remove the unbound drug and immediately assessed by confocal microscopy. We found that addition of albumin in the culture medium resulted in a significant (~ 4.5 -fold) increase in association of DDAO-caspofungin with *Aspergillus* hyphae (Fig. 5). Collectively, our studies demonstrate the ability of albumin to act as a carrier that facilitates delivery of caspofungin to *Aspergillus* hyphae.

DISCUSSION

In this study, we examined whether host-related factors associated with the tissue microenvironment at the site of infection are implicated in improved *in vivo* activity of echinocandins against *Aspergillus* by performing susceptibility testing under conditions of cell culture growth that simulate several aspects of physiological growth in tissue. Of interest, we found a profound and selective increase in the activity of caspofungin under conditions of cell culture growth that was consistent against all *Aspergillus* species tested and was not observed with the other classes of antifungal agents tested.

We next focused our studies on the effect of serum in the anti-*Aspergillus* activity of caspofungin, because of the major effects of serum components in the activity of many antimicrobial agents, including the echinocandins (19–28). Our findings demonstrated a profound enhancement of caspofungin activity in the presence of serum against all *Aspergillus* strains tested. Previous studies have evaluated the effect of serum on the activity of echinocandins against *Aspergillus* with discordant results (19, 27, 28). In agreement with our findings, Chiller et al. demonstrated that serum increased the activity of caspofungin against *Aspergillus* (27). In a recent study by Elefanti et al., the effects of a high serum concentration in the activity of various antifungal agents against different *Aspergillus* spp. were analyzed. The authors found that echinocandin activity was generally reduced in the presence of serum; however, a positive effect of serum was noticed in the activity of all three echinocandins against *Aspergillus* at concentrations above the MEC (29). Importantly, in contrast to previous studies (19, 30), we opted to use a lower concentration of serum (10%), which could be physiologically more relevant, since the interaction of caspofungin with *Aspergillus* occurs in the infected lung, where the concentration of serum is low (31).

Of interest, in contrast to the free-drug hypothesis, caspofungin, an antifungal compound that is more than 97% bound to serum proteins (1, 32), displayed a profound increase in antifungal activity in the presence of serum. In order to get mechanistic insight on the interaction of serum with caspofungin, we focused on albumin, the major serum protein component that binds highly to caspofungin (1, 32, 33). Indeed, we found that the positive interaction of serum with caspofungin was mediated almost exclusively by albumin. Previous studies reported on the negative effect of serum on the sensitivity of β -1,3 glucan synthase to echinocandins (19); we prioritized our research on the property of albumin to act as a carrier molecule. Importantly, we found a selective binding of fluorescence-labeled albumin on germinating forms of *Aspergillus*, which was further enhanced upon treatment with caspofungin and could be related to a charge-mediated effect (11, 34, 35) or to a more specialized mechanism of interaction (36). Furthermore, confocal imaging studies using fluorescence-

FIG 5 Enhanced caspofungin association with *Aspergillus* hyphae in the presence of albumin. Resting conidia of *A. fumigatus* strain 293 were grown in RPMI-HEPES at 37°C and 5% CO₂ for 17 h to allow germination to mature hyphae, and then they were incubated with fluorescent DDAO-caspofungin at a concentration of 1 μ g/ml with (albumin condition) or without (control condition) the presence of 3 mg/ml BSA for 1 h at 37°C and 5% CO₂. Hyphae were then immediately assessed with a confocal microscope. Shown are representative images from both conditions (A) and per-pixel quantification of fluorescence, expressed as mean fluorescence intensity (MFI), normalized to the control condition (B). Horizontal lines show the mean of each condition. ***, $P < 0.001$ compared to the control condition.

labeled caspofungin provided direct evidence on the ability of albumin to facilitate caspofungin delivery to *Aspergillus* hyphae. Notably, the synergistic interaction of caspofungin with serum albumin was not observed with the other two echinocandins, micafungin and anidulafungin.

Collectively, our studies discover a novel mechanism of synergistic interaction between caspofungin and albumin against *Aspergillus* that might have physiologic and therapeutic implications. Of interest, the enhancement of the activity of an antimicrobial drug is not unique to echinocandins, since there is evidence that components of serum enhance the activity of other antimicrobial regimens, including macrolides, although the mechanism of this interaction was not evaluated (21, 23–25). Defining the structural requirements that allow albumin to selectively improve delivery and enhance anti-*Aspergillus* activity of caspofungin compared to other antifungal agents could pave the way for the design of novel carrier molecules that enhance the delivery of other compounds to their therapeutic targets. Finally, further studies on a larger collection of *Aspergillus* strains across

different laboratories are needed to confirm that the synergistic interaction of albumin with caspofungin should be taken into account in *in vitro* susceptibility studies.

FUNDING INFORMATION

This work was supported by a Marie Curie International Reintegration grant (IRG-260210) to G.C., an M. D. Anderson Cancer Center core grant (CA16672) from the University of Texas to D.P.K., NIH grant R01AI069397 to D.S.P., and CCSG grant NIH P30CA016672 to the High Resolution Electron Microscopy Facility at the M. D. Anderson Cancer Center.

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