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Platelets enhance activity of antimycotic substances against non-*Aspergillus fumigatus Aspergillus* species *in vitro*

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Platelets are known to be part of haemostasis but they are also players in innate host defense. Recently, we observed that platelets attenuate the virulence of *Aspergillus* spp. *in vitro*. However, little is known about the antifungal effects of platelets in the presence of antimycotics against non-*A. fumigatus Aspergillus* species. We therefore investigated whether platelets increase the *in vitro* activity of amphotericin B, voriconazole, posaconazole and caspofungin against two clinical isolates each of *Aspergillus flavus, Aspergillus* terreus and *Aspergillus niger*. The antifungal activity was evaluated by assessing germination percentages, hyphal elongation and hyphal damage by use of XTT. The combination of platelets plus amphotericin B significantly ($P < 0.05$) enhanced the reduction of germination percentage compared to either substance alone. Among triazoles, voriconazole exhibited significant effects with platelets for all tested aspergilli. Overall, these findings suggest that among the tested antimycotic substances, amphotericin B in combination with platelets has enhancing effects in reducing germination and hyphal elongation in the tested non-*A. fumigatus Aspergillus* species. These data indicate that platelets act beneficially with antimycotics in an early stage of fungal growth by blocking and/or delaying fungal germination and hyphal elongation; both crucial mechanisms in the development of invasive fungal disease.

Keywords platelets, non-*A. fumigatus Aspergillus*, hyphal damage, amphotericin B, caspofungin

Introduction

Due to the rising number of immunocompromised hosts, the incidence of invasive fungal diseases, such as invasive aspergillosis (IA), is increasingly recognized [1,2]. These infections are associated with a high mortality rate, mainly affecting patients with underlying corticosteroid or immunosuppressive therapy, allogeneic stem cell transplants, or patients suffering from prolonged neutropenia [3–5]. Although *Aspergillus fumigatus* is the most frequent infectious agent, non-*A. fumigatus* aspergilli species such as *Aspergillus flavus*, *Aspergillus terreus* or *Aspergillus niger* are increasingly recognized [6,7]. Host defense against fungi involves innate, humoral and cell-mediated immunity [1,8]. Cells of the innate immunity such as polymorphonuclear leucocytes and macrophages play an important role in the immune response against fungal pathogens [1]. Platelets are known to play central roles in the processes of haemostasis, inflammation and wound healing [9]. They show similarities to granulocytes in structure and function [10], contribute to innate immune defense and have the capacity, *in vitro,* to exert antimicrobial actions against fungi [11]. It was found that platelets were able to interact with several microorganisms such as *Staphylococcus aureus* [12]. Recently, we observed that they impair the viability of *Aspergillus* spp. by reducing hyphal elongation by granules

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dependent mechanism [13]. Although platelets were not able to internalize the fungi, the virulence of *Aspergillus* spp. was strongly attenuated [13]. Similar results were found with the interaction of human platelets with *Zygomycetes* spp. [14].

The therapeutic strategies for patients consist of prophylaxis in risk groups and the early application of antifungal agents in suspected or probable disease [15]. For many years, amphotericin B has been the drug of choice in the treatment of IA [1]. However, several other antifungal drugs, for example voriconazole, posaconazole and caspofungin are available for treatment options [16–18].

A possible synergistic effect of immune effector cells in combination with antimycotics against *Aspergillus* spp. has been found in several studies [19–22]. Recently, we observed potential synergistic effects of human platelets in combination with antimycotic substances against *A. fumigatus* [23]. Among the aspergilli, *A. fumigatus* is usually sensitive to the polyenes and the azoles [24]. In contrast, *A. terreus* displays, *in vitro*, high minimal inhibitory concentrations (MIC) for amphotericin B reflecting the potential for primary polyene resistance [25]. Furthermore, interspecies differential fungicidal activities are known for voriconazole and amphotericin B among these three non-*A. fumigatus Aspergillus* species [26].

We therefore investigated whether human platelets enhance the antifungal capacities of amphotericin B, voriconazole, posaconazole and caspofungin against *A. terreus*, *A. flavus* and *A. niger*, as evidenced by germination percentages, hyphal elongation and percent of hyphal damage.

Materials and methods

Fungi

In this study two clinical strains each of *A. terreus, A. flavus* and *A. niger* were used. Conidial suspensions were maintained in sterile water at room temperature. Subcultures were grown on Sabouraud 2% dextrose agar (SDA; Merck, Darmstadt, Germany) and incubated at 35°C for 4 days. For the preparation of the inocula, the plate was flooded with sterile phosphate buffered saline (PBS; Biochrom KG, Vienna, Austria). To remove the hyphae, the suspension was filtered through sterile gauze. Then, conidia were counted with a haemocytometer and suspended in PBS at 1×10^6 colony forming units (CFU)/ml. The number of conidia in the suspensions was verified by plating serial dilutions of the conidial suspension and recording the CFU counts.

Antifungal drugs

The following four antifungal agents were tested: amphotericin B (Sigma Aldrich, Vienna, Austria), voriconazole (kindly provided by Pfizer, Vienna, Austria), posaconazole (kindly provided by Schering-Plough Research Institute, Kenilworth, NJ) and caspofungin (MSD, Vienna, Austria). Each drug was prepared as described in the NCCLS document M38-A [27] and sub-inhibitory drug concentrations of 0.5 μg/ml and 1 μg/ml were used for each strain.

Platelets

Fresh platelets suspensions (Pl) were provided by the local Department of Immunology and Blood Transfusion. Platelets were collected from healthy blood donors by thrombocytapheresis using an Amicus cell separator (Baxter, Vienna, Austria) and the platelet count was 1×10^8/ml.

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. All donors gave written informed consent to the use of residual platelet concentrates for research purposes. All residual platelet concentrates were de-identified and analyzed anonymously.

Determination of germination percentage and hyphal elongation

After conidia were treated either with the combination of platelets and antifungals or with each substance alone, the morphology of the fungi was investigated by assessing both the germination percentages and hyphal elongation [28]. Therefore, 100 μl each of platelets plus 100μl conidia suspended in RPMI 1640 (Sigma-Aldrich, Vienna, Austria) were mixed in an effector:target (E:T) ratio of 100:1. Antifungal substances at 0.5 μg/ml and 1 μg/ml of each antimycotic substance were added. For controls, 100 μl of the fungal suspensions were incubated with 100 μl of PBS only. The solutions were inoculated into microwell plates (Greiner, Vienna, Austria) and incubated at 37°C. After 16 h of incubation, the platelets were lysed with ice-cold water and fungi were stained with Calcofluor White (Polysciences) to permit the microscopic examination of the morphology of the conidia. A micrometer was used for length measurement and 50 conidia were measured per sample. To calculate the germination rate, the percentage of conidiaose that did not germinate was compared to those that had germinated within this period of time. Untreated and antifungal treated fungi served as controls. Each sample was assessed in triplicate.

Hyphal damage assay

The impairment of hyphae was evaluated by a colorimetric assay with the dye (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazoilum-5-carboxynilide) sodium salt

(XTT, Sigma-Aldrich, Vienna, Austria) plus 40 μg/ml coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma-Aldrich, Vienna, Austria) [22]. To induce conidial germination, 200 μl of a suspension of 1×10^6 CFU/ml of each fungus in RPMI 1640 was inoculated into each well of a flat bottom well cell cluster and incubated for 18 h at 37°C. Platelets were added to appropriate wells at an E:T ratio of 100:1, and antifungals were added to specified concentrations. All mixtures were incubated at 37°C for 1 h after which the platelets were lysed by washing them three times with ice-cold double-distilled water. Then, 150 μl of phosphate-buffered saline containing 0.25 mg of (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazoilum-5-carboxynilide) sodium salt plus 40 μg/ml coenzyme Q was added and incubated at 37°C for 1 h. Subsequently 100 μl were transferred to a spectrophotometer. The optical density was determined at 450 nm using a reference filter with 690 nm. Antifungal activity was calculated as a percentage of hyphal damage = (1–X/C) × 100, where X is the optical density of the test wells and C is the optical density of the control wells, containing hyphae only.

Statistical analysis

All experiments were conducted with the platelets from one donor. For each condition, duplicate or quadruplicate wells were used and tests repeated six times with the platelets of six different donors. The average value for these replicate wells was taken as the value for that particular donor and experiment. Then the mean ± standard error of mean (SEM) for all experiments performed under the same conditions was calculated using the averages for the replicate wells of each experiment. Repeated-measures analysis of variance (ANOVA) was used to statistically evaluate differences between mean values, followed by Bonferroni's Multiple Comparison Test. A P value of <0.05 indicated statistical significance.

Results

Germination percentage

The germination percentages of the non-*A. fumigatus Aspergillus* strains with amphotericin B and platelets alone or in combination are shown in Fig. 1A–C. Amphotericin B alone significantly reduced the germination percentage of all tested aspergilli (Fig. 1A–C) except for the use of low amphotericin B (0.5 μg/ml) levels against *A. terreus* as shown in Fig. 1C. Amphotericin B plus human platelets revealed significantly greater inhibition of germination percentages ($P < 0.05$) in all tested aspergilli than amphotericin B or platelets alone as indicated in Fig. 1A–C.

Caspofungin significantly reduced the germination percentages of all tested aspergilli as shown in Fig. 1D–F. The combination of caspofungin at 0.5 μg/ml and 1 μg/ml plus platelets significantly reduced the germination percentage when compared to caspofungin alone for all tested aspergilli as shown in Fig. 1D–F. Posaconazole and voriconazole revealed significant reduction of germination percentages in all tested fungi as shown in Fig. 2. The combination of posaconazole with platelets did not enhance these effects as shown in Fig. 2A–C. In contrast, voriconazole exerted significantly stronger reduction of germination percentages when used in combination with platelets for all tested aspergilli as shown in Fig. 2D–F.

Hyphal elongation

Hyphal elongation of all tested non-*A. fumigatus Aspergillus* was significantly reduced by platelet treatment as shown in Fig. 3 and Fig. 4. Amphotericin B at both concentrations significantly reduced hyphal elongation in *A. flavus* and *A. niger* as shown in Fig. 3A, B. However, amphotericin B plus human platelets showed no significantly greater inhibition of hyphal elongation for these two species (Fig. 3A, B). Hyphal elongation was not significantly reduced by treatment with amphotericin B at 0.5 μg/ml as noted in Fig. 3C. However, when amphotericin B at 0.5 μg/ml was used in combination with platelets, a significant reduction in hyphal elongation could be observed with *A. terreus* (Fig. 3C). The hyphal elongation of all tested aspergilli with or without caspofungin and platelets is shown in Fig. 3D–F.

The azoles significantly reduced hyphal elongation of all tested aspergilli as indicated in Fig. 4A–F. The combination of posaconazole plus human platelets did not show significantly greater inhibition of hyphal elongation ($P < 0.05$) in all tested aspergilli as shown in Fig. 4A–C. Voriconazole plus platelets revealed significantly stronger effects in the reduction of hyphal elongation in *A. terreus* as shown in Fig. 4F.

Hyphal damage

The effects on hyphae of the tested aspergilli with amphotericin B, caspofungin, posaconazole, voriconazole and platelets alone or in combination with platelets are shown in Table 1. The ability of hyphae to reduce XTT, which is regarded to be an established indicator of fungal cell damage, was decreased by platelets for all tested non-*A. fumigatus Aspergillus* species. After an incubation period of 60 min, hyphal damage of *A. flavus, A. niger* and *A. terreus* are given in Table 1. No significant effect on hyphal damage was found with platelets plus either antimycotic substance when compared to antimycotics alone. However, significant differences between platelets and antimycotic substances in damaging hyphae could be observed as shown in Table 1.

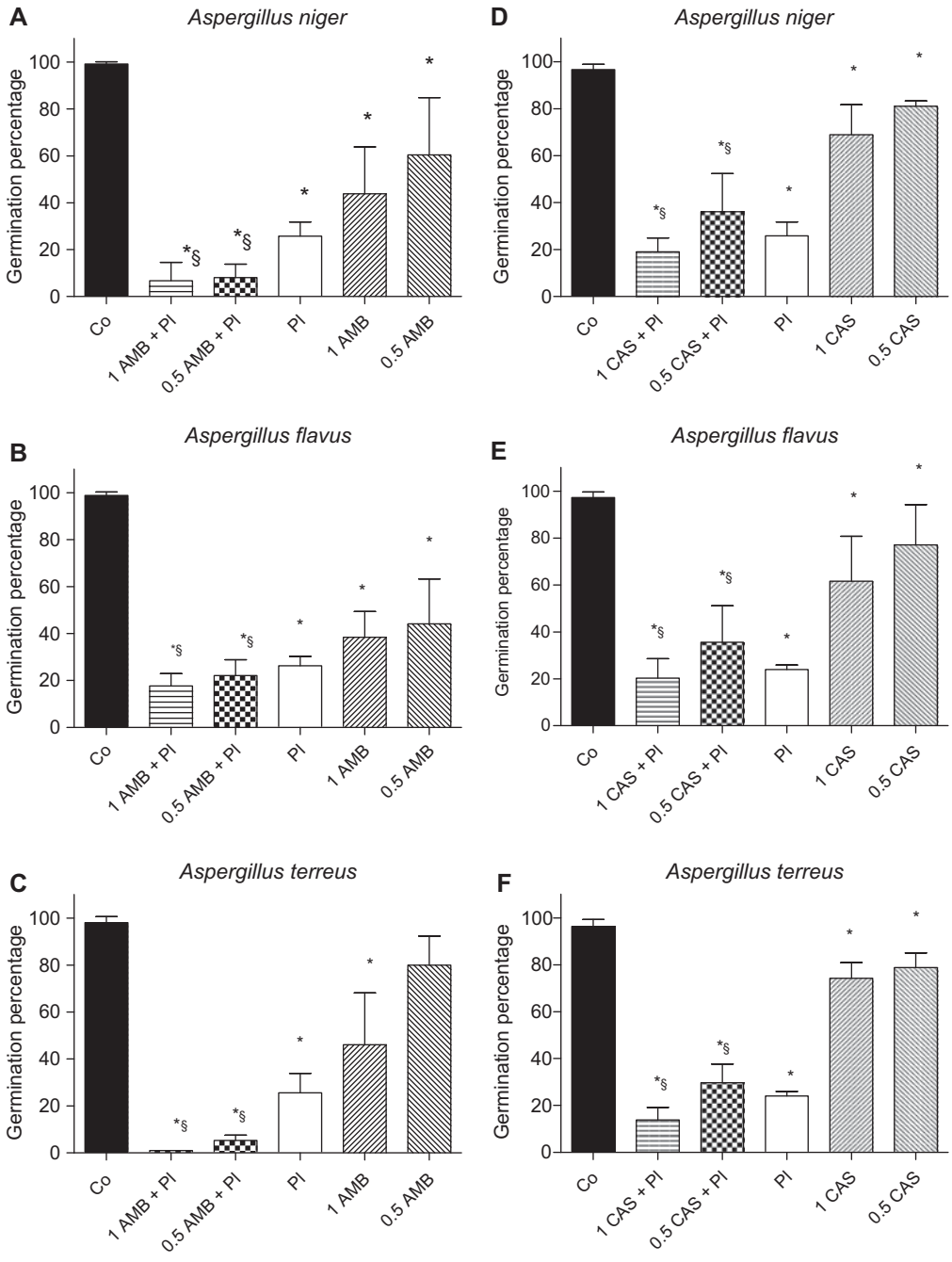

Fig. 1 Effect of platelets alone, amphotericin B (AMB) or caspofungin (CAS) alone and in combination with platelets on germination percentage of two clinical strains each of *Aspergillus flavus, A. niger* and *A. terreus*. Non-*A. fumigatusAspergillus* species were untreated (black bars), combinatory treated with Pl + either antimycotic at 0.5 μg/ml (horizontally striped bars) and 1 μg/ml (dotted bars), platelet (Pl)-treated (white bars) and either antimycotic-treated at 0.5 μg/ml or 1 μg/ml (diagonally striped bars) for 16 h at 37°C for detection of germination percentage. Mean values ± SD of $n = 12$ (six independent experiments for each of two fungal isolates). $*P < 0.05$ versus control for all samples. §$P < 0.05$ between test substance and test substance plus platelets.

Discussion

Aspergillus species remain the predominant etiological agents of invasive fungal infections among patients with hematologic malignancies and recipients of solid-organ and hematopoietic stem-cell transplants [1,2]. Although *A. fumigatus* accounts for a majority of cases of IA, non-*A. fumigatus Aspergillus* species are emerging as significant pathogens in certain medical centers [6,7].

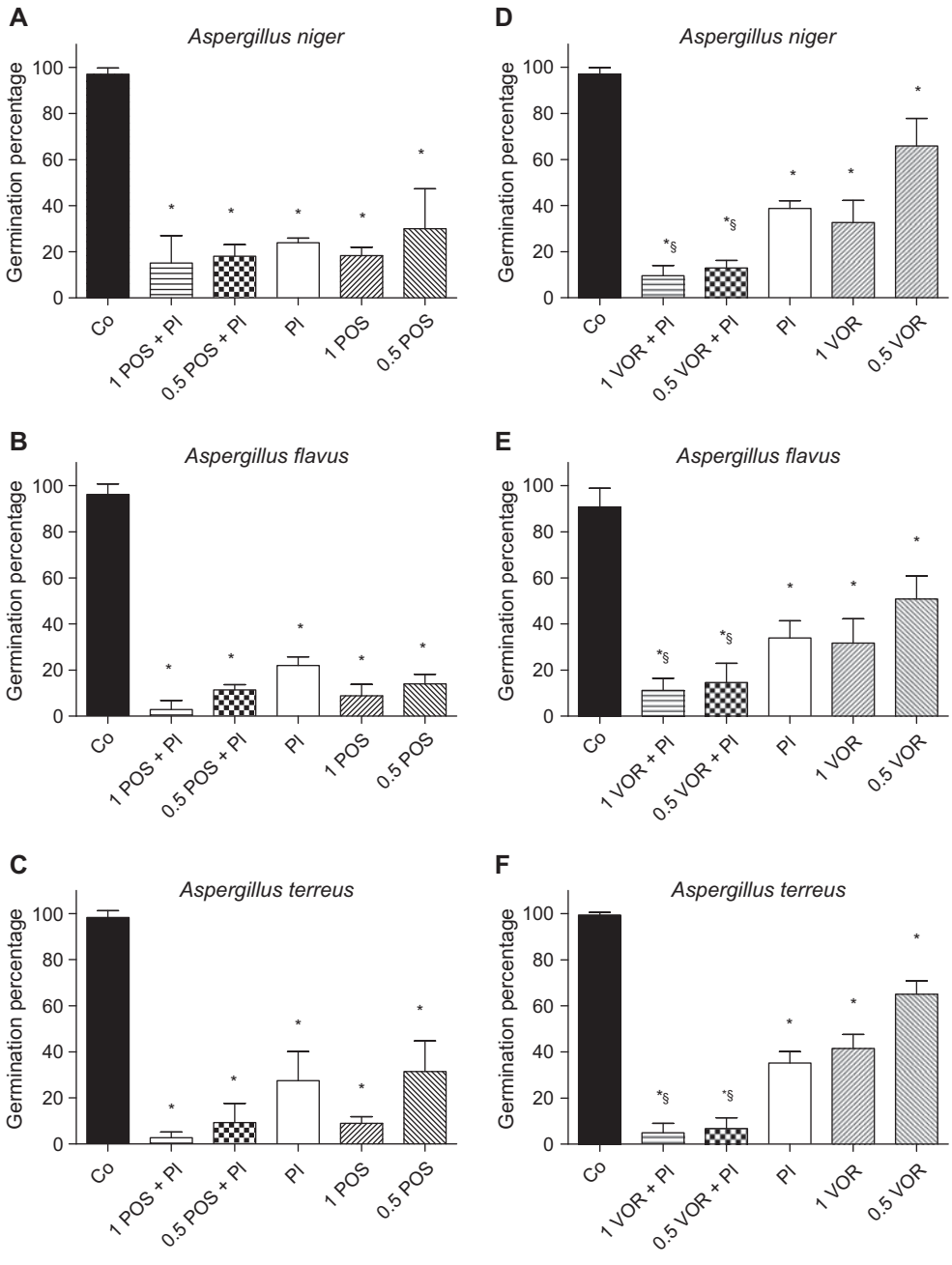

Fig. 2 Effect of platelets alone, posaconazole (POS) or voriconazole (VRZ) alone and in combination with platelets on germination percentage of two clinical strains each of *Aspergillus flavus*, *A. niger* and *A. terreus*. Non-*A. fumigatus Aspergillus* species were untreated (black bars), combinatory treated with Pl + either antimycotic at 0.5 μg/ml (horizontally striped bars) and 1 μg/ml (dotted bars), platelet (Pl)-treated (white bars) and either antimycotic-treated at 0.5 μg/ml or 1 μg/ml (diagonally striped bars) for 16 h at 37°C for detection of germination percentage. Mean values ± SD of $n = 12$ (six independent experiments for each of two fungal isolates). $*P < 0.05$ versus control for all samples. §$P < 0.05$ between test substance and test substance plus platelets.

Human platelets are able to attenuate the virulence of aspergilli probably by granule-dependent mechanisms [13]. They contain three distinct cytoplasmatic granule types: (i) dense granules, which store mediators including serotonin, adenosine nucleotide diphosphate; (ii) alpha granules that are involved in adhesion, coagulation and contain an arsenal of platelet microbicidal proteins; and (iii) lysosomal granules that contain lysosomal enzymes [28]. These distinct platelet granules are subject to discrete or synchronous release, dependent on antagonist specificity and potency [29].

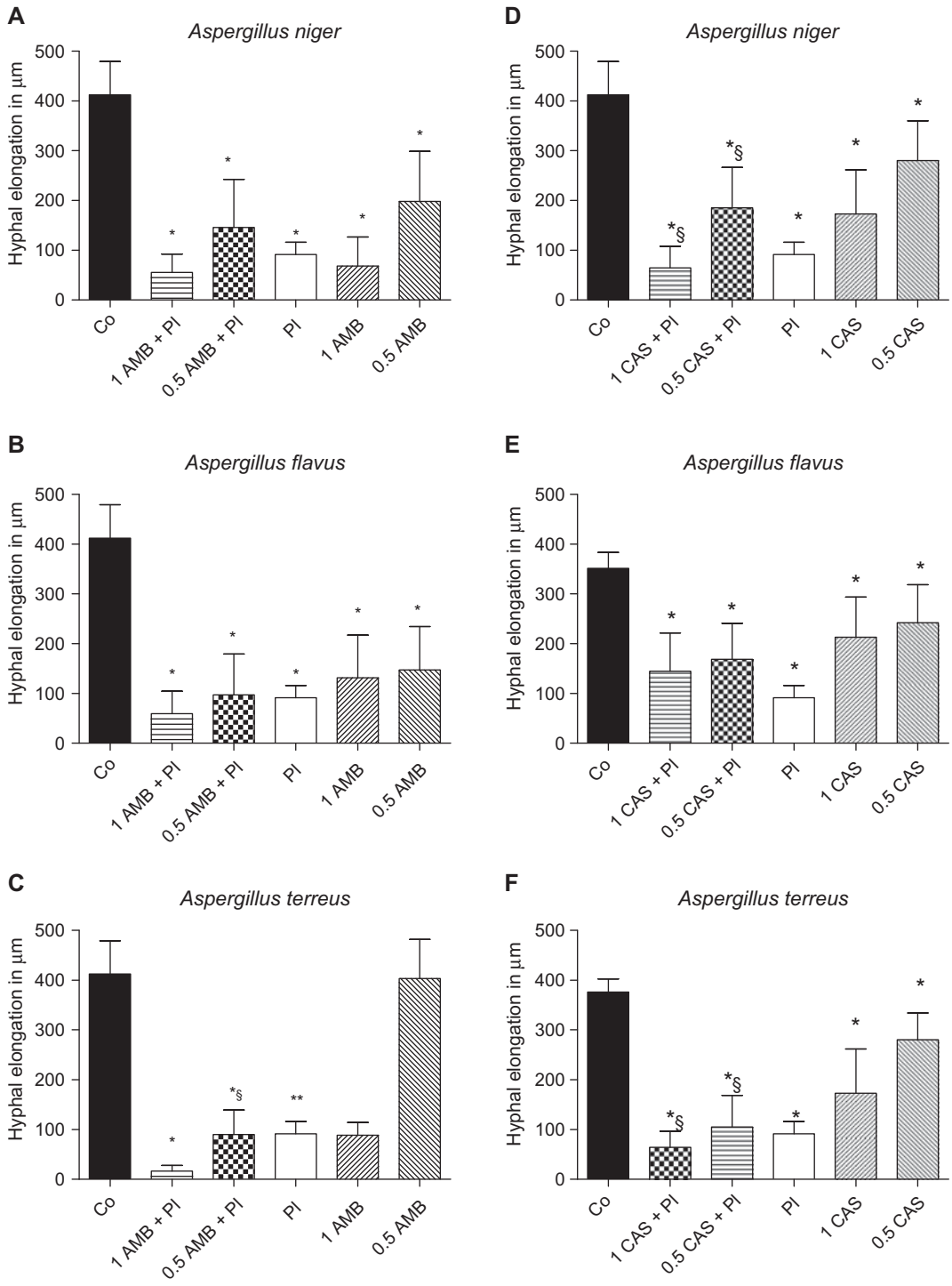

Fig. 3 Influence on hyphal elongation of two clinical isolates each of *Aspergillus flavus*, *A. niger* and *A. terreus* by treatment with platelets alone, amphotericin B (AMB) or caspofungin (CAS) alone and in combination with platelets. Non-*A. fumigatus Aspergillus* species were untreated (black bars), combinatory treated with Pl + either antimycotic at 0.5 µg/ml (horizontally striped bars) and 1 µg/ml (dotted bars), platelet (Pl)-treated (white bars) and either antimycotic-treated at 0.5 µg/ml or 1 µg/ml (diagonally striped bars) for 16 h at 37°C for detection of hyphal elongation. Mean values ± SD of $n = 12$ (six independent experiments for each of two fungal isolates). $*P < 0.05$ versus control for all samples. §$P < 0.05$ between test substance and test substance plus platelets.

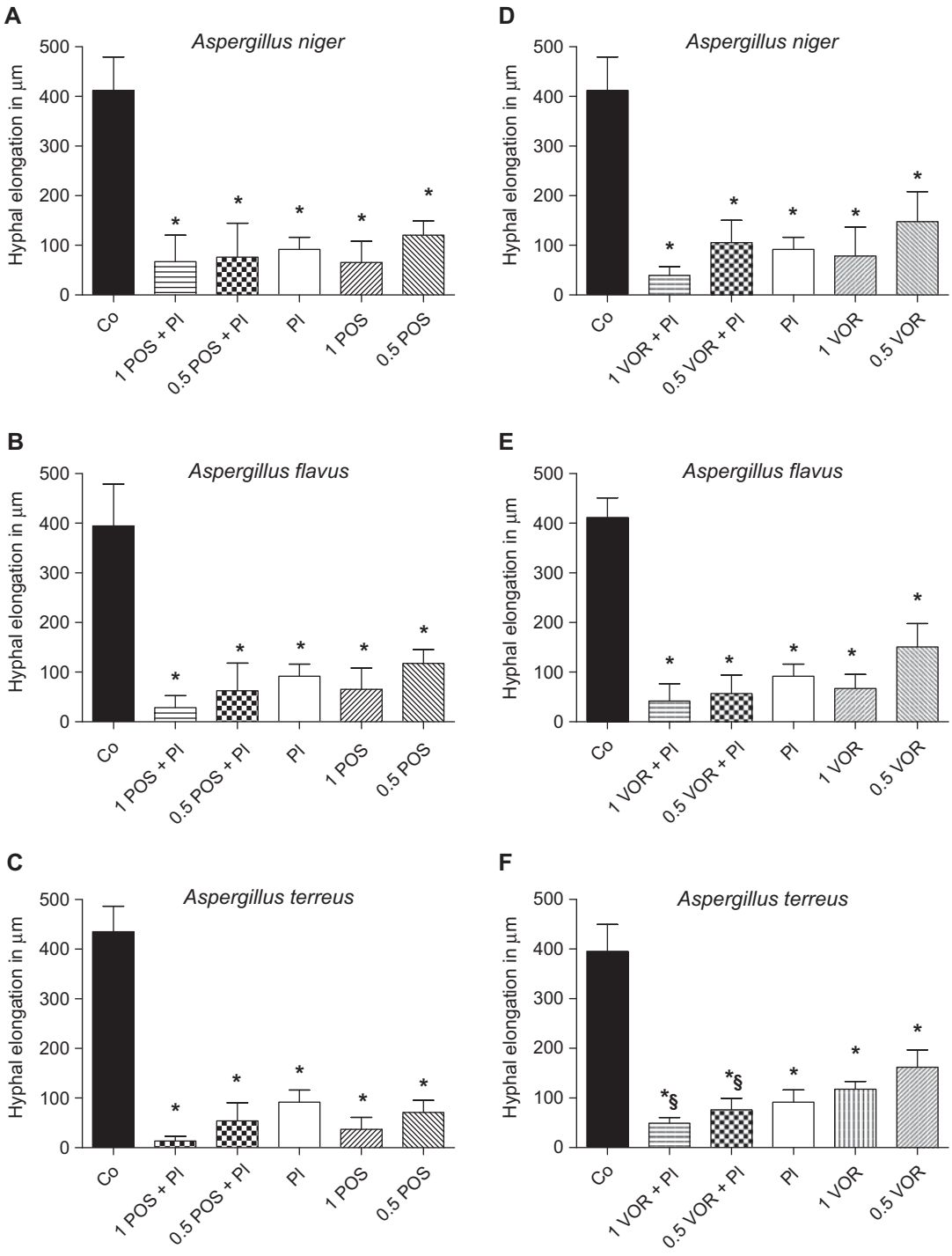

Fig. 4 Influence on hyphal elongation of two clinical isolates each of *Aspergillus flavus*, *A. niger* and *A. terreus* by treatment with platelets alone, posaconazole (POS) or voriconazole (VRZ) alone and in combination with platelets. Non-*A. fumigatus Aspergillus* species were untreated (black bars), combinatory treated with Pl + either antimycotic at 0.5 µg/ml (horizontally striped bars) and 1 µg/ml (dotted bars), platelet (Pl)-treated (white bars) and either antimycotic-treated at 0.5 µg/ml or 1 µg/ml (diagonally striped bars) for 16 h at 37°C for detection of hyphal elongation. $*P < 0.05$ versus control for all samples. §$P < 0.05$ between test substance and test substance plus platelets.

Table 1 Percentage of hyphal damage produced by platelets alone, antimycotics alone or the combination of antimycotics with platelets on hyphal damage of non-*A. fumigatus* aspergilli. Hyphal damage was assessed by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazoilum-5-carboxynilide) sodium salt assay. Columns represent the mean ± SEM hyphal damage of six experiments. An asterisk (*) indicates a significant difference of $P < 0.05$ between platelets and antimycotic substances.

	% Hyphal damage compared to 100% control*		
Condition (conc [µg/ml])	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus terreus*
Pl	16.7 ± 9.0	22.8 ± 9.4	28.9 ± 5.6
AMB (0.5)	36.9 ± 8.4	51.1 ± 8.0*	26.7 ± 5.5
AMB (1)	48.6 ± 3.6*	51.2 ± 4.4*	33.2 ± 6.4
AMB (0.5) + Pl	26.8 ± 6.9	28.8 ± 11.2	32.2 ± 5.6
AMB (1) + Pl	41.8 ± 6.7	45.1 ± 8.0	35.8 ± 7.2
CAS (0.5)	21.1 ± 2.9	56.7 ± 14.3*	38.9 ± 12.5
CAS (1)	14.0 ± 6.9	59.9 ± 6.5*	25.7 ± 8.4
CAS (0.5) + Pl	26.2 ± 3.0	37.6 ± 10.6	41.9 ± 8.7
CAS (1) + Pl	21.8 ± 4.8	56.0 ± 3.9	33.3 ± 8.1
POS (0.5)	11.1 ± 0.9	7.4 ± 1.3*	28.0 ± 5.4
POS (1)	10.3 ± 1.7	6.6 ± 2.6*	12.6 ± 9.0
POS (0.5) + Pl	12.9 ± 6.4	11.9 ± 2.3	12.6 ± 0.1
POS (1)+ Pl	15.2 ± 6.2	13.7 ± 3.2	14.9 ± 0.2
VRZ (0.5)	6.5 ± 1.4*	10.3 ± 5.7*	30.7 ± 7.3
VRZ (1)	8.6 ± 3.3*	7.7 ± 2.3*	19.5 ± 1.4
VRZ (0.5) + Pl	9.7 ± 2.3	12.1 ± 5.1	31.0 ± 6.2
VRZ (1) + Pl	12.6 ± 4.5	9.0 ± 4.4	18.3 ± 2.9

Pl, platelets; AMB, amphotericin B; CAS, caspofungin; POS, posaconazole; VRZ, voriconazole.
*Controls were set 100% (see methods).

In this study, we showed that, *in vitro* platelets in combination with antimycotics exert effects in reducing germination rate and hyphal elongation of non-*A. fumigatus Aspergillus* species. Among the tested asntimycotics, the best results were obtained with amphotericin B in combination with platelets. In contrast, platelets plus antimycotics lack any affect in damaging hyphae.

The influence of platelets themselves and in combination with antimycotics on germination rates and hyphal elongation is of great interest as fungal morphology is considered to be associated with host invasion [30]. The ability of *Aspergillus* to undergo morphological changes from conidia to filaments was not examined as only hyphae are seen in tissues [30]. Our recent studies demonstrate that platelets attenuate the virulence of aspergilli as shown by a significant reduction of fungal germination and hyphal elongation ($P < 0.05$) [13]. One of the key processes in IA is the angioinvasion of hyphae [30]. Once the hyphae have entered the bloodstream, they must penetrate the blood vessels to invade the deep tissues of the target organs. During angioinvasion, *Aspergillus* hyphae interact with human platelets and this often results in thrombosis and tissue infarction [31]. Therefore, the ability to grow is essential to establish invasive diseases [30] and germination rates and hyphal elongation are good indicators of the antifungal activity of substances and/or cells [31].

Recently, we showed that the combination of platelets plus amphotericin B potentiated the antifungal activity significantly by ($P < 0.05$) reducing the germination rate and hyphal elongation of *A. fumigatus* [23]. In this study, we found the same results for the non-*A. fumigatus Aspergillus* species including *A. terreus,* which is well known to be intrinsically resistant to amphotericin B [32]. This is an important finding, as *A. terreus* is an emerging cause of infection at our institution, the Medical University Hospital of Innsbruck [25]. Human platelets contain antifungal platelets factors and are able to synthesize reactive oxygen species [10,11] that could contribute to the effects found with amphotericin B.

The changes in the ability to reduce the tetrazolium dye XTT indicated that platelets inflicted significant damage to hyphae of *Aspergillus* spp. The induced damage due to platelets in this study is consistent with earlier findings by Christin *et al*. [33]. However, the addition of antimycotics had no beneficial effect on hyphal damage as this is known for polymorphic neutrophils or macrophages [19–22]. Previous studies have shown that voriconazole may have effects in combination with host phagocytes against *A. fumigatus* [34]. Studies by Gil-Lamaignere *et al*. [20] and Dotis *et al*. [19] revealed strong interactions on hyphal damage by amphotericin B and immune effector cells *in vitro*. Likewise, Vora *et al*. [34] demonstrated increased hyphal damage with voriconazole and phagocytic cells, which was enhanced using granulocyte-macrophage stimulating factor. Furthermore, caspofungin exerted cooperative effects with human effector cells [35]. However, the reason for the lack of effects is not known.

Human platelets are active players in the host defence as shown by many *in vitro* studies [9–13]. These *in vitro* data are supported by *in vivo* studies that showed that thrombocytopenia is associated with a greater risk of invasive fungal disease after liver transplantation [37] and for infants with very low birth weights (< 1500 g) [38]. Moreover, patients with invasive fungal disease had a significantly longer duration of thrombocytopenia compared to those without infection [37]. In cases of sepsis, the frequency of thrombocytopenia was significantly higher in cases of fungal infection than in gram positive infection. This thrombocytopenia seen in patients with sepsis episodes may be caused, at least in part, by consumption of platelets [38].

While the exact mechanism of this cooperative interaction is not known yet, it is possible that platelets act via a mechanism that is distinct from that of antimycotics and may be involved in membrane permeabilization. As contact

with *Aspergillus* spp. activates platelets [13] leading to granule release, it is also possible that platelet granule content enhances the antifungal capacities of antifungals. Several platelet secretion products are known to exhibit antibacterial activity [11]. Serotonin (5-HT), which is stored in platelets' dense granules and released after contact with *Aspergillus*, is well known to possess antifungal properties [28]. Therefore, these released substances could support the antifungal capacities of antimycotics.

In conclusion, these findings suggest that human platelets potentiate the efficacy of antimycotics, especially in inhibiting fungal outgrowth. In particular, the understanding and identification of the crucial role of platelets in combination with antimycotic substances represents an interesting target for further investigations. A better appreciation of the mechanism of these significant effects may allow for a better understanding of invasive fungal infections and may lead to better treatment for this devastating disease.

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