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# Interaction of Platelets and Anidulafungin against *Aspergillus fumigatus*

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**The combination of platelets and anidulafungin at 0.03 μg/ml significantly ($P < 0.05$) reduced the germination rate and hyphal elongation in *Aspergillus fumigatus* compared to those with either anidulafungin only or an untreated control. Platelets decreased the expression of the *fks* gene, which plays an important role in cell wall synthesis. Our results suggest that human platelets plus anidulafungin might contribute to defense against *A. fumigatus*.**

An intact immune system is essential for the defense against fungal pathogens (1). Human platelets are known to primarily play a key role in hemostasis; however, they are considered to be part of the innate immunity (2–4). They exert antimicrobial effects against bacteria, such as *Staphylococcus aureus*, and several other microorganisms *in vitro* (4, 5). Christin et al. showed that platelets damage hyphae of *Aspergillus fumigatus* (6). Recently, we observed that platelets have the capacity to attenuate the virulence of *Aspergillus* spp. and zygomycetes *in vitro* by reducing hyphal germination and elongation (7–9). In addition, the polysaccharide galactomannan, which is released by growing and vital hyphae of *A. fumigatus*, was significantly reduced under platelet treatment (8). Previously, we have reported that human platelets act beneficially with amphotericin B against *A. fumigatus* and other *Aspergillus* spp. (10, 11). Here we investigated whether platelets and anidulafungin in combination have an added effect on fungal germination, hyphal elongation, and hyphal damage of *A. fumigatus*. In addition, we analyzed the effects of human platelets in combination with anidulafungin on expression levels of the *fks* gene, which encodes the 1,3-β-D-glucan synthase. Echinocandins interfere with cell wall synthesis by inhibiting this enzyme, which forms glucan polymers, the major component of the fungal cell wall (12).

Two clinical isolates of *A. fumigatus* were used, and the strains were obtained from patients suffering from invasive aspergillosis. Platelet concentrates (storage time < 24 h) were provided by the local Department of Immunology and Blood Transfusion at Innsbruck Medical University. The minimum effective concentration (MEC) was determined according to the Antifungal Susceptibility Testing (AFST) committee, EUCAST, broth microdilution method (13), and it was found to be 0.03 μg/ml for both isolates tested. Subsequently, 0.03 μg/ml and a subinhibitory MEC of 0.0078 μg/ml of anidulafungin were applied for further tests. The determination of germination rate and hyphal elongation was performed as described elsewhere (7–9). Conidial suspensions were treated either with platelets or anidulafungin alone or with the combination of platelets and the drug. For investigation of hyphal elongation and the germination percentage, 100 μl of platelets ($1 \times 10^8$/ml) and 100 μl of conidia ($1 \times 10^6$/ml) suspended in RPMI 1640 (Sigma-Aldrich, Vienna, Austria) were mixed in an effector-to-target-cell (E:T) ratio of 100:1 and then anidulafungin was added and the mixture was incubated at 37°C. To calculate the germination rate, the percentage of conidia that did not germinate in comparison to the percentage that germinated was evaluated. Untreated and anidulafungin-treated fungi served as controls, and each assay was assessed in triplicate.

We also determined the antifungal activity of platelets, anidulafungin, and the combination of platelets and anidulafungin by a colorimetric assay with the dye 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2-*H*-tetrazolium-5-carboxynilide sodium salt (XTT; Sigma-Aldrich) plus 40 μg/ml coenzyme Q (Sigma-Aldrich). The Northern analysis and reverse transcription-quantitative PCR (qPCR) were performed according to a standard protocol (14), and the fold increase was evaluated by using the $2^{-\Delta\Delta CT}$ method (15). Repeated-measures analysis of variance (ANOVA) was used to evaluate differences between mean values, followed by Bonferroni's multiple-comparison test. A $P$ value of <0.05 indicated statistical significance.

We found that the germination rate of *A. fumigatus* with platelets plus anidulafungin was 1.3% ± 0.5% at a concentration of 0.03 μg/ml and 2% ± 0.1% at 0.0078 μg/ml. These data revealed a significantly strong inhibitory effect on the germination rate ($P < 0.05$) compared to untreated (95.4% ± 4.3%) or exclusively platelet-treated (5.8% ± 3.0%) controls or anidulafungin treatment at 0.03 μg/ml (50.4% ± 17.2%) and at 0.0078 μg/ml (59% ± 8.9%).

Fungal hyphae interact with platelets during angioinvasion, which often results in thrombosis and tissue infarction (16). As hyphal outgrowth is responsible primarily for the invasiveness of the fungus, it can be used as an indicator of antifungal activity of drugs or human cells (16). In this study, we found a significant reduction of hyphal elongation ($P < 0.05$) by platelets, anidulafungin, and combined administration of platelets and anidulafungin, as shown in Fig. 1A; the effect was strongest applying anidulafungin at 0.03 μg/ml. This is an important finding, since the changing of morphology from conidia to filaments is essential for

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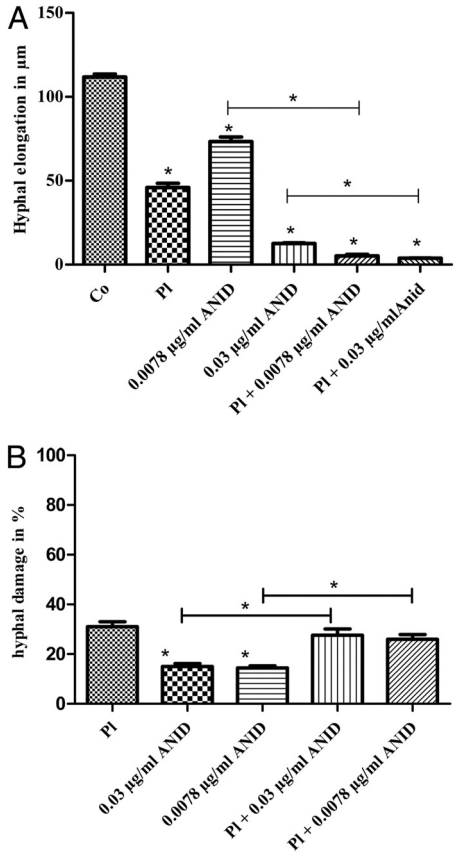


**FIG 1** (A) Effects of platelets, anidulafungin alone, and the combination of anidulafungin and platelets at an E:T ratio of 100:1 on hyphal elongation of *A. fumigatus* ($n = 2$). Aspergilli were incubated for 16 h in the absence of any platelets or drug (Co) or in the presence of platelets (Pl), 0.0078 μg/ml ANID, 0.03 μg/ml ANID, Pl and 0.0078 μg/ml ANID, or Pl and 0.03 μg/ml ANID. (B) Percentage of hyphal damage of aspergilli with platelets, anidulafungin, and the combination of anidulafungin and platelets at an E:T ratio of 100:1 by reduction of XTT. Data are representative of six independent experiments. Error bars show the standard error of the mean (SEM). An asterisk indicates a statistically significant difference with a $P$ value of $<0.05$. Co, controls, growth of untreated fungi; Pl, platelets; ANID, anidulafungin.

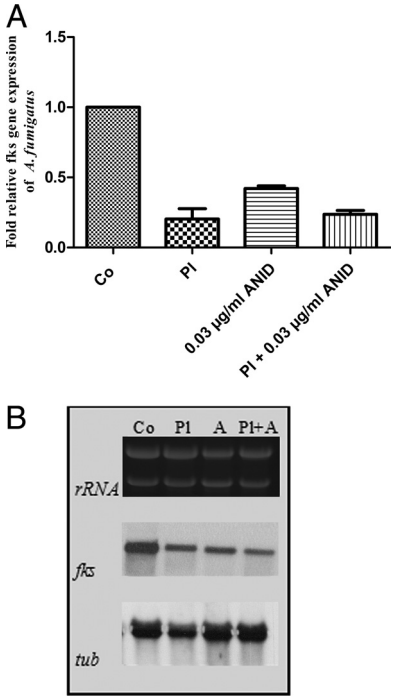


**FIG 2** (A) Relative gene expression levels of the *fks* gene of untreated, platelet-treated, anidulafungin (0.03 μg/ml)-treated, and platelet-and-anidulafungin (E:T ratio, 100:1)-treated *A. fumigatus*. (B) Northern analysis of untreated and platelet-treated *A. fumigatus fks* and *tub* gene expression levels. Following platelet, anidulafungin (0.03 μg/ml), or combination treatment for 60 min, total RNA was isolated from *A. fumigatus* and hybridized with *fks* encoding 1,3-β-D-glucan synthase for gene expression analysis. As a loading control, blots were hybridized with the β-tubulin-encoding *tub* gene of *A. fumigatus*. (A) Error bars show the SEM. Co, controls, growth of untreated fungi; Pl, platelets; ANID, anidulafungin. (B) Co, control; Pl, platelets; A, anidulafungin; Pl + A, platelets plus anidulafungin.

the fungus to become invasive. Subsequently, the fungus is capable of invading the deep tissue of the target organs (16).

Platelets damaged fungal hyphae significantly more than anidulafungin alone, as shown in Fig. 1B. Interestingly, only a modest increase in fungal injury resulted from the combination of platelets plus anidulafungin. These data support the idea that antifungal substances derived from activated platelets aid in prevention of fungal outgrowth rather than fungal impairment. So far, data on human phagocytes showed that anidulafungin supports polymorphonuclear (PMN) phagocyte-mediated hyphal damage in *A. fumigatus* (17). Also, in a recent study, micafungin was found to have an additive effect with PMN-induced damage in *A. fumigatus* hyphae (18). The exact mechanism of hyphal damage due to platelets is still unknown. However, a possible enhanced effect of immune effector cells combined with antifungal drugs against virulent fungi has been found in several studies (19–22).

Quantitative PCR and Northern blot showed that the combination of anidulafungin and human platelets induced downregulation of the target gene *fks*. Platelets were able to reduce the expression of the *fks* gene. Although platelets in combination with anidulafungin did not reveal a strong effect, platelets have the capacity to decrease the expression of *fks* (Fig. 2A). These data were confirmed with Northern analysis (Fig. 2B). The *fks* gene encodes 1,3-β-D-glucan synthase, which plays a leading role in fungal cell wall synthesis (12).

In summary, our findings demonstrate that human platelets may potentiate the antifungal properties of anidulafungin against *A. fumigatus*. We showed that platelets augment antifungal activity of anidulafungin against *A. fumigatus in vitro*, as evidenced by the reduced fungal germination rate and hyphal elongation; both processes are important in fungal invasiveness and growth. The combination of anidulafungin and human platelets induced downregulation of the target gene *fks* in *A. fumigatus*. Further research is still required to clarify the exact roles that platelets have in the immune system against fungal infections and how exactly they can help antifungal therapies.

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The authors alone are responsible for the content and writing of the paper.

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