

Combined Effect of Platelets and Anidulafungin against *Aspergillus fumigatus* Infections

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Abstract

Invasive aspergillosis (IA) is one of the major causes of morbidity and mortality in severely immunocompromised patients. Despite the existence of antifungal treatment IA mortality rate remains extremely high and may reach up to 80%. Previous studies have suggested important role of platelets in antifungal host defence. *In vitro* data show that platelets are able to attenuate germination, hyphal elongation and viability of *Aspergillus fumigatus*. Interaction of platelets with *Aspergillus fumigatus* induces differential expressions of fungal genes associated with stress response regulation, cellular transport and metabolism.

A new class of antifungals, echinocandins (caspofungin, micafungin and anidulafungin), have entered the market. Anidulafungin displays strong antifungal activity against *Candida* and *Aspergillus* species and has very few side effects due to its specific inhibiting effect on the fungal cell wall synthesis. Anidulafungin is currently licensed for the treatment of adult patients with invasive candidiasis. Clinical significance of anidulafungin for IA treatment has to be further evaluated.

Recently published studies have shown that efficiency of antifungal substances can be enhanced when combined with platelets. In this review we discuss the literature on the potential combined effect of platelets and anidulafungin against *Aspergillus fumigatus* infections.

Keywords: *Aspergillus*; Platelets; Anidulafungin; Infection

Introduction

Invasive aspergillosis (IA) is one of the major causes of morbidity and mortality in severely immunocompromised patients such as cancer patients with chemotherapy-induced neutropenia, hematopoietic stem cell and transplant recipients [1,2]. The number of immunocompromised patients is constantly increasing due to improved survival of cancer patients, intensive cytotoxic therapy and higher number of organ transplantation recipients. These factors render the patients at risk of acquiring opportunistic fungal infections [3].

The frequency and relative importance of these infections are on the rise in all developed countries, which is possibly related to increased numbers of immunocompromised patients, owing to improved survival from AIDS, malignancies and more intensive cytotoxic therapy, more transplantation (with immunosuppression) for organ dysfunctions, and better therapy and prophylaxis for candidal infections.

Despite the existence of antifungal treatment IA mortality rate may reach up to 80% [4]. Reported reasons for the inefficiency of the antifungal medication include late IA diagnosis, developed drug resistance and infection of sites such as central nervous system that

cannot be effectively treated with existing drugs [5]. IA has also strong impact on hospital costs. While aspergillosis is found in 0.03% off all US hospitalizations, the pharmacy costs account for 30% of total expenditures. Analyses of the hematopoietic stem cell transplant recipients sub-group displayed mean costs of \$ 442 233 per patient per year [6].

A new class of antifungals, echinocandins (caspofungin, micafungin and anidulafungin), have entered the market. These compounds are associated with fewer side effects as compared to antifungal drugs of other classes due to their exclusive activity on the fungal cell wall, a structure which is absent in mammalian cells. Currently, caspofungin is the only echinocandin licensed for the second line therapy of IA [7].

Recent publications from our group and others have shown surprising interaction of platelets with *Aspergillus* that resulted in fungal cell damage and suppressed fungal germination. Moreover, we have shown that platelets may enhance antifungal effect when combined with anidulafungin. The exact molecular mechanisms of platelet interaction with *Aspergillus* as well as the role of platelets in extended antifungal effect of anidulafungin remain obscure.

Pharmacokinetics and pharmacodynamics of Anidulafungin

Chemical structure and mechanism of action. Anidulafungin (Ecalta®, Pfizer Limited, Sandwich, Kent, UK) belongs to echinocandin class of antifungal drugs. Similar to other echinocandins,

anidulafungin is a cyclic hexa-lipo-peptide; its N-aryl side chain comprises three phenyl groups. The common mechanism of action of echinocandins is a non-competitive inhibition of 1, 3- β -D-glucan synthase. 1, 3- β -D-glucan is a polysaccharide which is essential for the stability of the inner layer of the fungal cell wall [7–9]. Lack of the β -(1, 3)-D-glucan causes abnormal morphology of the fungal cell with thinning of the cellular wall, abnormal swelling and aberrant budding [10].

Pharmacodynamics and clinical use. Anidulafungin displays strong antifungal activity against *Candida* and *Aspergillus* species. It is not active against *Cryptococcus neoformans*, *Fusarium* species, and *Zygomycetes*. Quantification of the activity of anidulafungin against *Aspergillus* is based on its effect on fungal morphology which is expressed by the minimal effective concentration (MEC), in contrast to minimal inhibitory concentration (MIC).

Anidulafungin and other echinocandins display a pharmacodynamic particularity which is termed “paradoxical pharmacodynamic effect”. Exposure of a susceptible fungus to increasing drug concentrations results in growth inhibition when the sub-inhibitory concentration is exceeded. However, when the concentration is further increased, a decline of the antifungal activity takes place. Finally, at highest concentrations, fungal growth is inhibited again [11–13]. This phenomenon was observed at therapeutic concentrations *in vitro* and *in vivo* [13], although clinical relevance and underlying cellular mechanisms remain unclear. A variety of effects has been hypothesized to contribute, such as increased synthesis of cell wall chitin and altered activity of protein kinase C, and calcineurin [12]. The pharmacokinetic/pharmacodynamic indices correlating best with antifungal activity of anidulafungin are the ratio between peak level and the MIC of pathogen (C_{max}/MIC) as well as the ratio between the area under the concentration-time curve and the MIC (AUC/MIC) [14,15]. This is based on a relevant post-antifungal effect of echinocandins [16].

Anidulafungin is licensed for treatment of adult patients with invasive candidiasis. Together with other echinocandins, anidulafungin has been recommended for treatment of candidaemia by current guidelines [17–19]. High dose echinocandin treatment is mentioned as an alternative therapeutic option for *Candida* endocarditis, but not for the *Candida* infections of the central nervous system, because of the poor penetration into brain tissue [19]. Although anidulafungin is active against *Aspergillus* *in vitro*, it is currently not used for the treatment of IA. Efficacy of anidulafungin in combination with voriconazole has been recently investigated in 277 haematological patients. This double-blind randomized trial did not reveal a significant difference in mortality between the voriconazole-anidulafungin combination and voriconazole monotherapy [20]. Therefore, the routine use of this combination for IA is discouraged by current guidelines but it can be considered in selected patients under particular clinical conditions [21]. The adverse effects of anidulafungin are similar to caspofungin and micafungin and include nausea, diarrhoea, headache, phlebitis and pruritus, leukopenia, neutropenia, anaemia, hypokalaemia and hepatotoxicity. The risk of hepatic adverse effects, however, appears to be lower for anidulafungin when compared to other antifungal medications such as micafungin [22–24].

Pharmacokinetics: Similarly to other currently licensed echinocandins, anidulafungin displays insufficient enteral absorption and has therefore to be administered by intravenous infusion [25]. The standard dose of anidulafungin recommended by the manufacturer comprises a 200-mg-loading-dose applied over 3 h once on day 1, and

a maintenance dose of 100 mg once daily (infusion time [T_{inf}]=90 min). In plasma, anidulafungin undergoes spontaneous ring opening. The respective product which is further degraded by hydrolysis and N-acetylation independently from phase I and phase II metabolism is eliminated subsequently via biliary excretion [24,26]. The degradants of anidulafungin are eliminated mainly by the faeces. Only 10% of the administered radioactivity was recovered as intact drug, 90% as degradants. Plasma pharmacokinetics of anidulafungin has been investigated under various conditions [27]. In a small study of healthy volunteers, the mean peak level was 4.1 μ g/mL, the AUC was 102 μ g·h/mL and the half-life was 28 h after administration of 90 mg of ¹⁴C-labeled anidulafungin. By population pharmacokinetics similar data were obtained. The mean half-life was 26 h, the volume of distribution at steady state was 33 L, and the anidulafungin clearance amounted to 0.9 L/h.

So far drug-drug interactions involving anidulafungin appear to be rare. Its potential for causing interactions with concomitant medications has been shown to be even lower than that of other echinocandins, as anidulafungin does not inhibit cytochrome-P-450 isoenzymes. Recently, however, anidulafungin has been reported to inhibit the ATP-binding cassette transporter breast cancer resistance protein (BCRP) *in vitro* [28]. A few clinical studies have assessed drug-drug interactions involving anidulafungin. Simultaneous administration with voriconazole did not cause an altered exposure to anidulafungin or voriconazole [29]. Administration of cyclosporine A together with anidulafungin resulted in a slight increase in anidulafungin exposure (22% increase in AUC_{0- ∞}). No relevant drug-drug interaction was observed between tacrolimus and anidulafungin [30].

As anidulafungin is degraded spontaneously, renal impairment has no influence on its elimination [24]. In patients with impaired liver function, a slightly decreased anidulafungin exposure was observed which, however, does not require increased doses [31]. This holds also true for critically ill patients, where the median AUC and the median peak concentration were slightly below those in healthy subjects or in patients in a more stable condition [32]. Extracorporeal organ support, e.g. renal replacement therapy, had no relevant effect on anidulafungin pharmacokinetics [33–37].

Data on tissue penetration of anidulafungin has been limited so far. In human pulmonary epithelial lining fluid of healthy volunteers, relatively low concentrations (mean, 0.9 μ g/mL) were achieved, whereas the drug accumulated in pulmonary alveolar macrophages (mean concentration ~100 μ g/mL) and in peripheral blood mononuclear cells and in polymorphonuclear leukocytes [38,39]. In rabbits and in rats, the highest concentrations were measured in lung and liver. The lowest amounts were recovered from the brain, the vitreous humour, the aqueous humour, and the choroid [40,41].

Antifungal activity of platelets

Function of neutrophils, dendritic cells and alveolar macrophages during innate lung defence against *Aspergillus* species is extensively studied and discussed in the literature [42–45]. Interestingly, an increasing number of publications suggests that internal communication and interactions between immune cells result in a stronger cell activation and increase response to fungal pathogens [46,47]. Moreover, a recent publication by Savers et al has demonstrated that exposure of mice deficient in T-, B- and NK-cells to a nonlethal *A. fumigatus* inoculum consequently protected mice against a lethal exposure [48]. In pre-exposed mice neutrophils and

macrophages have been recruited in higher numbers and also secreted higher amounts of specific cytokines [48]. Further intriguing aspects of “trained immunity” concept may arise in future studies and involve other cellular and molecular players.

In the raw of recent publications the surprising function of platelets in *Aspergillus* species defence has been recognized. Beyond long-established function in haemostasis regulation and thrombus formation, platelets are highly active in the host defence against pathogens [42,43]. Platelet interaction with other cell types relays on variety of surface receptors that include integrins, selectins, leucine-rich repeats receptors, tyrosine kinase receptors and others. Platelet granules and lysosomes contain extensive collection of inflammatory mediators; their rapid exocytosis is a unique feature of platelets. Moreover, platelets contain functional protein translation machinery and may also synthesize microvesicles (reviewed in [42]).

It has been previously observed that low platelet counts associate with poor outcome of IA [49]. Thrombocytopenia was shown to precede fungal infections in liver transplant recipients [50]. An early study by Christin et al. demonstrated that in the presence of *A. fumigatus* platelets express glycoprotein CD63 and supplement polymorphic neutrophils [51]. In the immunocompromised patient, inhaled *Aspergillus* conidia germinate into hyphae, the growing and invading structures of filamentous fungi. Consequently, blocking fungal germination and delaying hyphal growth is crucial in preventing invasive disease. The fungal cell wall plays an important role during growth and helps *Aspergillus* spp. to invade host tissue [52]. The fungal cell wall also protects fungi against a hostile environment and represents a target for the host immune system and antifungal substances. Hence, the maintenance of cell wall integrity and functionality is crucial for *A. fumigatus*.

We have shown a direct binding of platelets to *Aspergillus* conidia and hyphae; this interaction induced platelet activation and resulted in the fungal cell damage. The interaction with platelets also reduced hyphal germination and elongation of *Aspergillus* whereas serotonin was immediately released from dense granules of platelets [53,54]. Furthermore, the platelet treatment decreased metabolic activity of *A. fumigatus* and caused reduction of polysaccharide galactomannan [55], which is released by growing and vital hyphae [1]. The presence of platelets that were pre-activated by fungal supernatant nearly completely suppressed any germination of *A. fumigatus*, thus pointing out that platelets can be stimulated by the direct contact with *Aspergillus* and may also sense the presence of fungus-derived soluble factors [56]. We have also found a specific transcriptional response of *A. fumigatus* disposed to platelets. Fungal detoxification mechanisms were induced at early time points, while respiration and associated processes were down-regulated over the course of time. Fungal Apoptosis- and cell death-related genes were also found to be induced due to platelets contact *in vitro* [57].

Only few studies have investigated molecular mechanisms of platelet-*Aspergillus* interaction. Results of Rambach et al. [58] suggest that platelets activation involves interaction with fungal polysaccharide galactosaminogalactan (GAG). GAG is localized to fungal cell wall and can also be secreted [58]. Previous studies in two murine models of IA identified GAG as a fungal virulence factor that mediates *A. fumigatus* adhesion and suppresses inflammatory responses *in vivo* [59]. GAG was also proposed to shield hyphal β -glucan from the immune system [59]. In recent publication, Lee et al has hypothesised that protective effect of the cell wall-associated GAG is direct: GAG contributes to resistance to NADPH oxidase-dependent neutrophil damage during fungal

infection [60]. Other cell wall components, including chitin, galactomannan and β -1,3-glucan were not affected by the platelet stimulation of *Aspergillus* [58].

Recent compelling publication by Lefrancias et al. showed that production of platelets from mouse megakaryocytes takes place in lung microcirculation where more than 10 million platelets are produced per hour, thus making lung to a primary site of platelet biogenesis [61]. Furthermore, results of Lefrancias et al. suggest that the lung may contain specific signalling partners for megakaryocytes that promote platelet release and may play a role in pathogen defence mechanisms [61,62].

A variety of physiological functions taken by platelets makes it unlikely that every platelet will contribute to all these processes. The concept of platelet heterogeneity has been discussed previously [63]. However, the emerged question whether platelets in circulation represent different populations, each prone to a certain response, or whether it is the local activating environment that governs their ultimate fate remains unanswered. Up until now platelet heterogeneity was studied only in thrombus formation: even within a single thrombus platelets were shown to differ in morphology and in surface characteristics. Inter-platelet variations in expression of receptors and adhesive surface proteins were linked to response variation [64,65]. Diverse environmental factors including adherence surface and local differences in rheology were suggested to contribute to platelet heterogeneity and response variation. On the other hand, *in vitro* experiments using isolated platelets showed evidence for non-environmental factors contributed to platelet response heterogeneity [66].

Platelets enhance effect of antifungals

Interaction of platelets and fungal substances is of high interest as it might be beneficial in combating fungal infections. Our *in vitro* studies have shown that platelets in combination with polyenes and azoles antifungals exert additive effects in reducing the germination rate and hyphal elongation of *A. fumigatus*. Among tested antimycotic substances, amphotericin B in combination with human platelets revealed the best results [54]. Interestingly, exposure of *A. fumigatus* to platelets caused downregulation of several genes associated with cell wall integrity [63]. Similarly, cell wall integrity/maintenance-related genes were repressed by antifungals e.g. Afu3g08110 by amphotericin B or Ags² by voriconazole.

Platelets and anidulafungin

In our previous studies we have investigated whether platelets and anidulafungin in combination have an added antifungal effect. We have found that the antifungal *in vitro* activity of anidulafungin against *A. fumigatus* is enhanced when combined with anidulafungin. Using two clinical isolates of *A. fumigatus* we have shown that germination rate and hyphal elongation of *A. fumigatus* was significantly stronger inhibited when treated with platelets and anidulafungin as compared to exclusively platelet-treated or anidulafungin-treated fungi. In addition, the glucan synthase *FKS* gene, that encodes 1,3- β -D-glucan synthase, was downregulated.

Interestingly, mechanism of *A. fumigatus* resistance to echinocandin has been shown to involve amino acid changes in hot-spot regions of Fks subunits, which results in decreased sensitivity of the enzyme to this antimycoticum class.

Figure 1 displays a model of combined effect of platelets and anidulafungin on the *A. fumigatus*. Activation of platelet by *A. fumigatus* causes direct effect of platelet on fungal wall component(s), one of which was identified as GAG. Anidulafungin on the other hand inhibits the 1,3- β -D-glucan synthesis. As a consequence of platelet/

anidulafungin treatment fungal cell wall integrity is lost, fungal virulence is inhibited and fungus becomes more susceptible to further attack by immune cells. Exact molecular mechanisms underlying platelet activation as well as binding further partners remain to be identified.

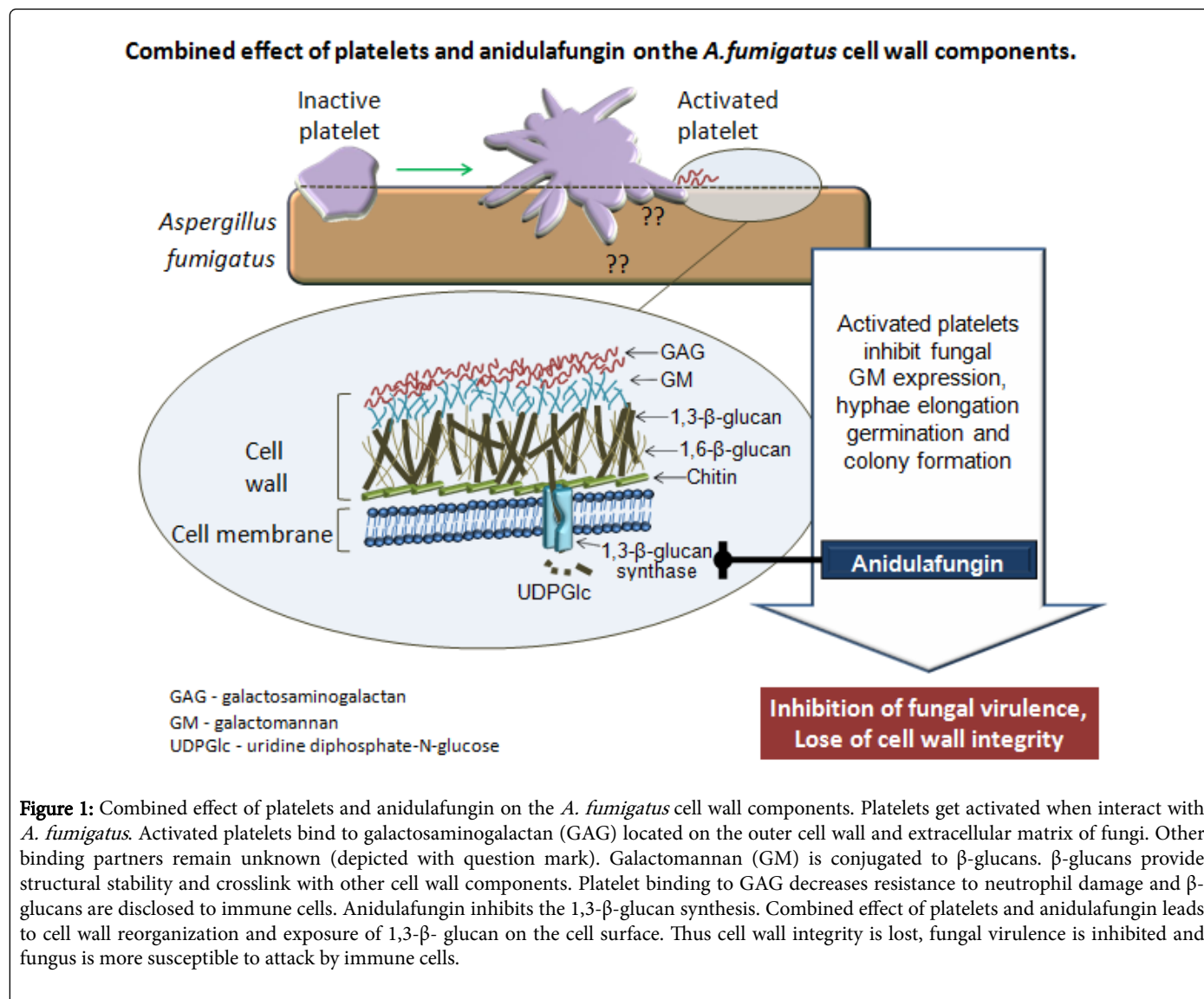


Figure 1: Combined effect of platelets and anidulafungin on the *A. fumigatus* cell wall components. Platelets get activated when interact with *A. fumigatus*. Activated platelets bind to galactosaminogalactan (GAG) located on the outer cell wall and extracellular matrix of fungi. Other binding partners remain unknown (depicted with question mark). Galactomannan (GM) is conjugated to β -glucans. β -glucans provide structural stability and crosslink with other cell wall components. Platelet binding to GAG decreases resistance to neutrophil damage and β -glucans are disclosed to immune cells. Anidulafungin inhibits the 1,3- β -glucan synthesis. Combined effect of platelets and anidulafungin leads to cell wall reorganization and exposure of 1,3- β -glucan on the cell surface. Thus cell wall integrity is lost, fungal virulence is inhibited and fungus is more susceptible to attack by immune cells.

Concluding Remarks and Outlook

Growing experimental evidence underlines important role of platelets in anti-fungal defence. In terms of platelet-*Aspergillus* interaction it is intriguing to speculate that release of fungus-specific platelets may take place in lung at the primary contact with conidia or/and with not yet identified fungus-derived soluble factors. This particular platelet population may directly bind and attack conidia to prevent hyphae growth locally, release soluble factors (e.g. serotonin) and possibly express microvesicles that will message to other immune cells thus supporting antifungal defence. Since contact of a healthy individual with *Aspergillus* conidia occurs on a daily basis one can suggest existence of fungi-specific platelet population in blood circulation. How these platelets are activated and regulated in

immunocompromised patient and whether immunosuppression setting changes platelet biogenesis in lungs remains to be investigated.

The concept of antimycotic consolidation with host immune cells has been rising over last years. Existing research data point on the platelets/anidulafungin synergetic effect in targeting fungal cell wall. Interestingly, anidulafungin accumulates in pulmonary alveolar macrophages, peripheral blood mononuclear cells and in polymorphonuclear leukocytes [38,39]. Therefore it is compelling to investigate whether anidulafungin also accumulates in platelets and whether it influences the potential production of platelets in the lung as a primary site of platelet biogenesis.

More studies are needed to undercover underlying molecular mechanisms and to improve the clinical effectiveness of anidulafungin.

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