Siderophage-based Monitoring of Posaconazole Therapy in a Rat Model of Invasive Pulmonary Aspergillosis

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Introduction
Invasive pulmonary aspergillosis (IPA) is a rapidly progressing opportunistic fungal infection caused by Aspergillus fumigatus in immuno-compromised patients [1]. As iron is tightly regulated within humans [2], this pathogen produces two extracellular siderophores, C. lacticiduricricivulose (C) and tetronate dehydrofumarate (D) siderophores, enabling the iron acquisition and storage [3]. Next, secreted mycotoxin gliotoxin exerts detrimental effects on several types of mammalian immune cells [4]. Most of the current diagnostic methods of IPA are time-consuming, non-specific, invasive, and can contribute to high mortality rates (30–85 %) [5]. We previously showed that siderophores could play the role of iron, and specific markers of IPA [6]. This work aims to distinguish disease progression between treated and untreated rats by simultaneous detection of siderophores, gliotoxin, antifungal posaconazole, and their metabolites (Figs. 1) from non-invasively collected urine.

Experiment
Eight female Lewis rats were infected intratracheally with the fungal strain A. fumigatus 10599 CCF (100 µL, 10^3 spores). Neutropenia was induced by two intraperitoneal doses of cyclophosphamide (75 mg/kg). Four rats received a multidose posaconazole therapy per os (4 mg/kg/day), which started three days post-inoculation. If possible, urine was collected twice a day, and lungs and blood were taken after sacrificing the rats. Samples underwent double liquid-liquid mammalian immunum extraction with ethyl acetate and consequent protein precipitation with pre-cooled methanol. The simultaneous detection of analytes was performed using a Dionex UltiMate 3000 HPLC system connected to a SolarX 12T FTICR mass spectrometer equipped with electrospray ionization positive-ion mode. Quantification was performed using a matrix-matched calibration or standard addition method.

Results
IPA monitoring in the untreated rats (Fig. 2)
Before the inoculation, there were no differences between the untreated and treated group (Fig. 2 A, B, C). Fig 2 D,E represents the urine analysis of the untreated group over the period of inoculation. After the inoculation, two peaks appeared at two different time points. In Fig. 2 D, the first peak appeared two hours after the inoculation, and the second peak appeared after two days. In Fig. 2 E, the first peak appeared two hours after the inoculation, and the second peak appeared after two days. In Fig. 2 E, the first peak appeared two hours after the inoculation, and the second peak appeared after two days.

IPA monitoring in the treated rats (Fig. 3)
Starting three days post-infection, POS was administrated every 24 hours per os. Posaconazole reaches its maximal plasma concentration after 0.5 hours and six hours [7]; therefore, in urine, its fungical effect began to be observed 24 hours after the first dose application reflected as concentration decrease of extracellular TAFB and TAFC, followed by intracellular FC. This may indicate the shutdown of siderophere biosynthesis with the consequent release of intracellular fungal hyphae content into the rat body. NA: not available.

Figures
Fig. 1 The extracellular siderophore triacetylfusarinine C (TAFC, A) forms its conjugate product triacetylfusarinine B (TAFB, B) after hydration of the ester bond (in blue). Hydroxamate groups (in red) chelate ferric cation in both TAFB, and in intracellular siderophore Terminalomycin (C). Disulfide bridge formation (in orange) in glucoticin (D, E). G. E generates deleterious reactive oxygen species. The fungal effect of posaconazole (POS, F) arises after interaction between nitrogen in triazole core (in green) and home iron in lanosterol-α-demethylase, one of the enzymes responsible for ergosterol biosynthesis.

Fig. 2 Cyclophosphamide induced neutropenia favoured the germination of A. fumigatus conidia to complete fungal hyphae, activating the secondary metabolism as reflected by urinary detection of TAFC, TAFC, FC and G. We firstly detected these biomarkers between 24–48 hours after inoculation. Further concentration increase of these biomarkers mirrored the severity of ongoing aspergillosis and, together with the lack of posaconazole intervention, all rats had to be sacrificed. NA: not available.

Fig. 3 Starting three days post-infection, POS was administered every 24 hours per os. Posaconazole reaches its maximal plasma concentration after 0.5 hours and six hours [7]; therefore, in urine, its fungical effect began to be observed 24 hours after the first dose application reflected as concentration decrease of extracellular TAFC and TAFC, followed by intracellular FC. This may indicate the shutdown of siderophere biosynthesis with the consequent release of intracellular fungal hyphae content into the rat body. NA: not available.

Fig. 4 Besides urine, we collected lungs (left) and blood (right) after rat sacrifice. POS preferably accumulates in the alveoli [7], therefore, intracellular FC represents the key metabolite showing the extent of fungal burden. The presence of FC in the treated rats points to substantial reduction but not complete eradication of A. fumigatus. Gliotoxin detection in untreated rats (representing the main circulating metabolite in the blood) may refer to the partial recovery of immune response in the alveoli, as G18 suppresses the function of alveolar macrophages. Compared to blood, neither TAFC nor TAFB was detected in the lungs. NA: not available.

Conclusions
We provided the first kinetic insight into the biosynthesis of triacetylfusarinine C, ferricin, and gliotoxin affected by multidose posaconazole extraction with ethyl acetate and consequent protein precipitation with pre-cooled methanol. The simultaneous detection of analytes was performed using a Dionex UltiMate 3000 HPLC system connected to a SolarX 12T FTICR mass spectrometer equipped with electrospray ionization positive-ion mode. Quantification was performed using a matrix-matched calibration or standard addition method.

Selective accumulation of posaconazole within the alveoli affected the presence of intracellular ferricin, indicating considerable reduction but not complete eradication of Aspergillus fumigatus.

The restored gliotoxin biosynthesis may refer to partial recovery of the alveolar immune response. The presence of gliotoxin in untreated rats, as the neutropenia was induced by two consecutive cyclophosphamide dosages five and one days before inoculation.

Our LC-FTICR-MS-based approach allows an early allocation of IPA and elucidates the treatment of pulmonary aspergillosis. Concurrent detection of antifungals and siderophores may provide an insight into the efficacy of antymycotic therapy, thus reducing the treatment time, drug-related toxicities and adverse effects.

References

Acknowledgement
• the Czech Science Foundation of the Czech Republic (21-17044S)

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Biomarkers in lungs and blood (Fig. 4)

10th AAAM, February 2nd–3rd, 2022