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Search for fungi-specific metabolites of four model drugs in postmortem blood as potential indicators of postmortem fungal metabolism☆

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ABSTRACT

Fungi colonizing cadavers are capable of drug metabolism and may thus change the metabolite pattern or concentration of drugs in forensic postmortem samples. The purpose of this study was to check for the presence of such changes by searching fungi-specific metabolites of four model drugs (amitriptyline, metoprolol, mirtazapine, and zolpidem) in decomposed postmortem blood samples from 33 cases involving these drugs. After isolation and identification of fungal strains present in the samples, each isolate was incubated in Sabouraud medium at 25 °C for up to 120 h with each model drug. One part of the supernatants was directly analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS), another after liquid–liquid extraction with chlorobutane and concentration. From 21 out of 33 decomposed postmortem blood samples (64%) a total of 30 different strains could be isolated, one from the class of Ascomycete and the rest belonging to 15 species from 8 different genera (number of species): *Aspergillus* (2), *Botrytis* (1), *Candida* (8), *Fusarium* (1), *Mucor* (1), *Penicillium* (1), and *Rodothorula* (1). In the *in vitro* studies, these microorganisms were found capable of *N*-demethylation and *N*-oxidation of amitriptyline and mirtazapine, *O*-demethylation followed by side chain oxidation of metoprolol as well as hydroxylation of all four-model drugs. In two of the postmortem blood samples, from which the fungi *Aspergillus jensenii*, *Candida parapsilosis.* and *Mucor circinelloides* had been isolated, a fungi-specific hydroxy zolpidem metabolite was detected. The presence of this metabolite in postmortem samples likely indicates postmortem fungal biodegradation.

1. Introduction

Many species of microorganisms better known as a "normal microflora" occur in the human body. Most of them are native bacteria localized mainly in the gastrointestinal tract, cavities open to the environment (oral cavity, respiratory tract), and skin surface [1]. According to Simon and Sears [2,3] the digestive tract can contain between 300 and 1000 different species of bacteria whose main functions are metabolic reactions, control of epithelial cell proliferation and barrier effect against pathogens [4]. In addition to bacteria, albeit in less quantity, different kind of fungi of the genera *Candida* [5], *Geotrichum*, *Malassezia* [6], *Trichosporon*, and *Trichophyton* can also be found in specific areas such as skin, hair, nasal mucosa, urogenital and gastrointestinal tract [7–13]. In immunocompromised patients, fungi of the genera *Aspergillus*, *Fusarium*, *Mucor*, *Curvularia* and some species of *Candida* can further be found, whose spores enter the human body through the upper respiratory tract [14–16]. In a recently published paper [17] by the authors of the present work, decomposing postmortem specimens were also found to be colonized by fungi of the genera of *Bjerkandera*, *Botrytis*, *Chaetomium*, *Circinella*, *Coroliopsis*, *Enterocarpus*, *Penicillium*, *Rhodotorula*, and *Trichoderma*.

Immediately after death, the decomposition process sets in, partly mediated by microorganisms present in the body and the surroundings of the corpse [12,18–21]. Different authors have shown that the enzymes of these microbes contribute to metabolism and degradation of xenobiotics present in postmortem

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samples [22–26]. In most of these studies, phase II metabolites (glucuronides) were degraded by β-glucuronidases present in the bacteria leading to increased concentrations of the free drugs or phase I metabolites in postmortem samples. Recently Butzbach et al. [27] showed the degradation of the antipsychotic drugs risperidone and paliperidone to their respective 2-hydroxybenzoyl benzisoxazoles by different strains of bacteria inoculated in porcine blood. Likewise, Martinez et al. [28] carried out a systematic *in vitro* study of metabolism of five model drugs using fungi isolated from decomposed human bodies [17]. The results showed the ability of some fungi to produce phase I metabolites resulting from hydroxylation, *N*- and *O*-dealkylation, and *N*-oxidation of the model drugs. Most of these metabolites were identical to those produced in humans, but some fungi produced metabolites not previously described in mammals. These new fungal metabolites (NFM) could potentially be used as markers of fungal colonization indicating postmortem fungal metabolism.

Therefore the aim of the present study was to conduct a systematic search for such fungi-specific metabolites in authentic decomposed human blood samples positive for one or more of the four model drugs in the previous studies [29]: amitriptyline (AT), metoprolol (MET), mirtazapine (MRT) and zolpidem (ZOL).

2. Materials and methods

2.1. Chemicals, reagents and fungal strains

Dextrose, protease-peptone, yeast extract, and agar were obtained from Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and magnesium sulfate were purchased from neoLab (Heidelberg, Germany). Hydrochlorides of amitriptyline (AT) and the internal standard cyproheptadine (IS), tartrate of metoprolol (MET), as well as free bases of mirtazapine (MRT), and zolpidem (ZOL) were purchased from Merck (Darmstadt, Germany). Acetonitrile (mass spec grade), dimethyl sulfoxide (DMSO), 1-chlorobutane, ammonium formate and methanol, were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade or higher. The strain CBS 167.53 of *Cunninghamella elegans* (*C. elegans*) was provided by the CBS-KNAW Fungal Biodiversity Centre of Utrecht, Netherlands. This strain was grown on Sabouraud agar plates (glucose 1%) at 25 °C and transferred to fresh plates every month. Fresh cultures were used for all the assay controls.

2.2. Solutions

Working solutions (1 mmol/L, free base) of the model drugs were prepared in sterile water with exception of the MRT solution, which was prepared in sterile water–DMSO (9:1, v/v). Working solution of IS (20 mg/L) were prepared in methanol. All solutions were stored at 4 °C for up to 1 month.

2.3. Postmortem material

A total of 33 postmortem blood samples (PBS, five heart blood and 28 femoral blood samples) from the same number of decomposed bodies found in different cities in the federal state of Thuringia, Germany and collected for routine toxicological analysis during 2010 and 2012 were used for a retrospective microbiological analysis and for a metabolism studies. The selected cases were those in which one or more of the models drugs previously studied by Martínez et al. [28] (6 for amitriptyline, 10 for metoprolol, 8 for mirtazapine, 4 for zolpidem and 5 for more than one of these drugs) had been detected during routine analysis. These postmortem materials were collected according to Martinez et al. [17] following the standard operating procedure for all full autopsies in the Institute of Forensic Medicine of Jena University Hospital and stored at −20 °C prior to and after routine toxicological analysis. At the time of the present study they had been stored for at least one year and thawed once for routine toxicological analysis and refrozen.

2.4. Isolation and identification of fungi

The isolation and identification of fungi was performed as reported previously [17]. Briefly, the PBS were thawed at room temperature and subsequently streaked on Petri dishes with Sabouraud agar and incubated at 25 °C for up to 20 days in darkness. The dishes were daily checked until fungal growth was visible. The observed colonies were picked and transferred to fresh dishes up to three times to reach pure cultures. These latter cultures were used for morphological and molecular identification.

2.5. In vitro *biotransformation procedures*

One strain of each isolated genus from postmortem material was used to carry out the *in vitro* biotransformation procedure in triplicate. The set up was similar as described previously [28]. Briefly, fungal mycelia or yeast cells of each strain were incubated in 9 mL Sabouraud liquid medium (SM) for three days (12 h for yeast) at 25 °C. Then 1 mL of AT, MET, MRT, or ZOL solution (1 mmol/L) was added to each flask and incubation was continued for another five days (three days for yeast). Positive controls (*C. elegans*, strain CBS 167.53), blank controls (drug-free cultures of the respective strains), and negative controls (solutions of drugs in SM) were run with all test incubations. From each incubation mixture, a 800 μL sample was taken after 120 h (after 72 h for yeast) and immediately centrifuged. The supernatants were used for analysis by LC–ESI–MS/MS.

2.6. Fungal incubation sample and PBS preparation for LC–ESI–MS/MS

Incubation supernatants and PBS were worked up following the procedures described by Martinez et al. [29] and Saar et al. [30], respectively. Briefly, a portion of incubation supernatant (50 μL) plus 10 μL of IS solution was diluted with 940 μL of aqueous ammonium formate solution (50 mmoL/L, pH 3.0) and 25 μL of this solution were analyzed by LC–ESI–MS/MS. PBS (500 μL) were spiked with 20 μL of IS solution and 200 μL of phosphate buffer (pH 8–9) and then extracted using liquid–liquid extraction with 1 mL of 1-chlorobutane for 5 min on a shaker at 1500 rpm. After centrifugation at 9600 × *g* for 1 min, the separated solvent layer was transferred to an autosampler vial and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 0.5 mL of aqueous ammonium formate solution (50 mmol/L, pH 3.0) and 25 μL of this solution were analyzed by LC–ESI–MS/MS.

Table 1.

2.7. LC–ESI–MS/MS analysis

The LC–ESI–MS/MS experiments were performed with a LC-20AD HPLC system (Shimadzu, Jena, Germany) interfaced with a 4000 QTrap® mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a TurboIonSpray ESI source operated in the positive mode. The system was controlled by Analyst 1.5.1 software which was also used for data analysis. All the parameters used for separation, mass spectrometric analysis and data acquisition are shown in Table 2.

3. Results and discussion

Recent *in vitro* studies have shown that a considerable percentage of decomposing human cadavers are colonized by