Systematic investigations on the metabolism of drugs by microorganisms colonizing corpses

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This work is dedicated in love and gratitude

to

my parents and my wife

Gutachter

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1 ABBREVIATIONS

AT	Amitriptyline
C. elegans	*Cunninghamella elegans*
C. albicans	*Candida albicans*
CI	Chemical ionization
C/P	Cardiac to peripheral ratio
CYP450	Cytochrome P450 hepatic microsomal enzyme system
DPS	Decomposed postmortem samples
EI	Electron impact
ESI	Electrospray ionization
HB	Heart blood
IFS	Isolated fungal strains
K	Control
LC	Liquid chromatography
LLE	Liquid-liquid extraction
L/P	Liver to peripheral ratio
M	Metabolite
MET	Metoprolol
MRT	Mirtazapine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NDM	Number of detected metabolites
NFM	New fungal metabolites
PBS	Postmortem blood samples
PMR	Postmortem redistribution
PMZ	Promethazine
%RPD	Percentage of remaining parent drug
SDA	Sabouraud dextrose agar
ZOL	Zolpidem

2 ZUSAMMENFASSUNG

Nach dem Tod werden Leichen von der normalen mikrobiellen Flora aus der Umgebung der Leiche besiedelt, die überwiegend aus Bakterien, fakultativ pathogenen und anderen Pilzen besteht. Diese Besiedlung ist insofern von Bedeutung, als die am Verwesungsprozess organischer Materie beteiligten Enzyme von Mikroben auch zum Metabolismus und Abbau von Wirkstoffen beitragen, die in postmortalen Proben vorkommen, und so deren Konzentration oder Metabolitenmuster verändern können. Einige dieser Veränderungen, die mit bestimmten Bakterien (*Escherichia coli, Clostridium perfringes, Clostridium sordellii, Corynebacterium sp, Enterococcus sp, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus saprophyticus, Streptomyces faecalis*) und Pilzen (*Candida albicans, Candida parapsilosis, Candida tropicalis*) zusammenhängen, sind bereits beschrieben und betreffen hauptsächlich Konzentrationsänderungen freier Wirkstoffe infolge Hydrolose ihrer Konjugate wie im Fall von Morphin. Diese bei Bakterien und einigen Hefepilzen dokumentierten Veränderungen könnten zu postmortem-toxikologischen Fehlinterpretationen führen.

Systematische Studien zu Veränderungen von Wirkstoffkonzentrationen und Metabolitenmustern durch Leichen besiedelnde Pilze liegen bislang nicht vor. Als Eukaryoten weisen Pilze jedoch Gemeinsamkeiten mit Säugetierzellen und folglich auch mit dem Stoffwechsel von Säugetieren auf. Entsprechend wurden in den hier vorgestellten Studien Pilze von menschlichem Postmortem-Material isoliert und morphologisch und molekulargenetisch identifiziert. Das in dieser Studie benutzte Postmortem-Material bestand aus 252 Proben, darunter Herzblut (HB) 96, Nieren- (69), Leber- (68) und Lungenproben (19), die im Zeitraum zwischen 2010 und 2012 bei Obduktionen von 105 fäulnisveränderten Leichen aus Thüringen entnommen worden waren. Ein Teil der Proben wurde unmittelbar nach der Obduktion bearbeitet, während der andere Teil vor der Bearbeitung mindestens ein Jahr lang bei -20°C aufbewahrt worden war. Fast alle (98%) dieser Isolate (156 Pilzstämme aus 62% des postmortalen Materials) wurden mittels DNA-Sequenzierung mit Calmodulin, ß-Tubulin, translation elongation factor (TEF) 1α und intergenic

transcribed spacer (ITS) als spezifische Primer-Sets von *Aspergillus, Penicillium, Fusarium* bzw. anderen Gattungen bis zur Spezies-Ebene identifiziert. Die vorherrschende Gattung war *Candida* (58% - sechs Arten) gefolgt von den Gattungen *Penicillium* (10,3% - zwei Arten) und *Rhodotorula* (7,1% - eine Art).

Mit den isolierten Stämmen und dem als Positivkontrolle verwendeten Pilz *Cunninghamella elegans (C. Elegans)* wurden Untersuchungen zum *in vitro* Metabolismus von fünf Modellwirkstoffen - *Amitriptylin (AT), Metoprolol (MET), Mirtazapin (MRT), Promethazin (PMT) and Zolpidem (ZOL)* - durchgeführt. Diese Stoffe werden im Menschen extensiv metabolisiert und stellen häufig verschriebene Vertreter verschiedener pharmakologischer Wirkstoffgruppen dar. Die verwendeten analytischen Techniken waren Gaschromatographie mit Massenspektrometrie-Kopplung (GC-MS) und Flüssigchromatographie Elektrosprayionisation Massenspektrometrie-Kopplung (LC-ESI-MS/MS), wobei die gleichen Trennungs- und Detektionsysteme für alle bei den fünf Modellsubstanzen produzierten Metaboliten eingesetzt wurden.

Alle isolierten Pilzstämme waren in der Lage die Modellwirkstoffe mehr oder weniger stark zu metabolisieren. Einige Pilze waren sowohl in der Lage, *in vitro* Metaboliten der Phase I bilden, die auch beim Menschen produziert werden, als auch pilzspezifische Metaboliten, die bei Säugetieren nicht beschrieben sind. Letztere könnten potentiell als Marker für eine entsprechende Pilzbesiedlung mit postmortalem Metabolismus dienen. Deshalb wurde eine systematische *in vitro* und *in mortuo* Untersuchung auf solche pilzspezifischen Metaboliten an 33 authentischen fäulnisveränderten postmortalen Blutproben (PBS) durchgeführt, die zuvor positiv auf einen der oben beschriebenen Modellwirkstoffe (AT, MET, PMZ, ZOL) getestet worden waren. Ein neuer *in mortuo* und *in vitro* Hydroxyl ZOL Metabolit wurde in PBS gefunden, die von den Pilzen *Aspergillus jensenii* und *Mucor circillenoides* besiedelt waren. Dieser Fund legt nahe, dass postmortaler Pilzstoffwechsel von Pilzen, die in fäulnisveränderten postmortalen Proben (DPS) vorkommen, in forensischen Fallproben auftreten können.

3 SUMMARY

After death cadavers are colonized by a normal flora predominant of bacteria, facultative pathogenic species, and other fungi present from the place where the corpse is found. This colonization is important because in the decomposition process of organic matter the enzymes of microbes may contribute to the metabolism and degradation of drugs present in postmortem material changing their concentration or the metabolic pattern. Some of these changes related to some bacteria (*Escherichia coli, Clostridium perfringes, Clostridium sordellii, Corynebacterium sp, Enterococcus sp, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus saprophyticus, Streptomyces faecalis*) and fungi (*Candida albicans, Candida parapsilosis, Candida tropicalis*) have already been documented and are mainly related to changes in the concentration of free drugs because of hydrolysis of its conjugates as in the case of the morphine. These changes documented in bacteria and some yeast may lead to misinterpretation. However, there are no systematic studies to date concerning the changes in the concentrations or metabolic patterns of drugs by fungi colonizing cadavers.

Fungi are eukaryotes and as such share similarities with mammalian cells and therefore with mammalian metabolism. Accordingly, in the presented studies, fungi from human decomposed postmortem material were isolated, and morphologically and molecularly identified. The postmortem material used in this study included 252 samples including heart blood (HB) (96), kidney (69), liver (68), and lung (19) which was selected from the autopsy of 105 decomposed bodies collected in the state of Thuringia between the years 2010 and 2012. A group of the samples was processed immediately after necropsy, whereas the other group had previously been stored at -20°C for a least 1 year, and only after this time, it was worked upon. Almost all (98%) of the isolates (156 fungal strains from 62% of the postmortem materials) were identified to the species level using DNA sequencing with Calmodulin, ß-Tubulin, translation elongation factor 1α (TEF), and internal transcribed spacer (ITS) as specific set primers of *Aspergillus, Penicillium, Fusarium, and other* genera, respectively. The most predominant genus was *Candida* (58% - six species) followed by the genera *Penicillium* (10,3% - two species), and *Rhodotorula* (7,1% -

one species). With the isolated strains and the fungi *Cunninghamella elegans (C. elegans)* employed as a positive control (K1), the capability of these strains to metabolize five model drugs *in vitro* -amitriptyline (AT), metoprolol (MET), mirtazapine (MRT), promethazine (PMT) and zolpidem (ZOL) was evaluated. These drugs undergo extensive metabolism activity in humans, and they are representatives of frequently prescribed pharmacological substances in Germany. The analytical techniques employed were gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) employing the same separation and detection settings system for all the metabolites produced in the five model drugs. All of the isolated fungal strains (IFS) were capable of catalyzing the model drugs to a greater or lesser extent. Some fungi showed the ability to yield *in vitro* phase I metabolites also produced in humans and some fungi-specific metabolites not described in mammals. The latter can potentially be used as markers of fungal colonization indicating postmortem fungal metabolism. Therefore, an *in vitro* and *in mortuo* systematic search study for these fungi-specific metabolites in 33 authentic decomposed postmortem blood samples (PBS) positive for one or more model drugs described above (AT, MET, PMZ, ZOL) was conducted. A new *in mortuo* and *in vitro* hydroxyl ZOL metabolite was found in PBS colonized by the fungi *Aspergillus jensenii* and *Mucor circillenoides*. This finding put forward that postmortem fungal metabolism by fungi presents in decomposed postmortem samples (DPS) may happen in real forensic samples.

4 INTRODUCTION

4.1 Postmortem changes and putrefaction

The toxicological analysis on DPS provides a number of very significant challenges to solve questions dealing with unexplained deaths without an apparent cause, homicides, driver fatalities, sudden infant death, and fire deaths among others. The most important pitfalls in postmortem toxicology that must be taken into account for a correct interpretation in a forensic case include postmortem redistribution (PMR) and incomplete distribution in overdose cases, drug stability, evaporation of volatiles, and the process of decomposition. PMR process is defined as the change of drug concentration in PBS taken from the central body cavity (usually HB samples) or peripheral areas (femoral blood samples) and can be affected by the postmortem time interval and the time that elapses from death to taking the postmortem sample. Consequently, the postmortem drug concentration cannot always be used to predict the antemortem state, because of the movement of the drug from a region of higher concentration to a region of a lower concentration [1, 2]. After death, the drugs that are sequestered antemortem in organs are redistributed to the surrounding tissues. The most important biological samples liable to generate PMR are stomach and gastrointestinal tract, lungs, liver, bladder, myocardium, and fat tissues [3]. According to some authors, PMR could be evaluated using two markers. The first of them employs the cardiac to peripheral ratio (C/P) of drug concentrations in PBS [4, 5]. Drugs with high ratios are considered to have a higher potential of PMR; nevertheless, there are some drugs with ratios greater than 1.0 and they are not necessarily susceptible to redistribution. An example of the limitations of this model is tramadol which is not prone to PMR. *Costa et al* evaluated the redistribution of tramadol in 15 cases of fatal intoxication and despite the fact it has low to moderate PMR, in 13 cases a ratio C/P higher one was found [6]. This may be because tramadol does not have neither a large volume of distribution nor a high protein binding. Recently the liver to peripheral ratio (L/P) has been used as a more reliable marker, where values greater than 50 could reflect

an extensive redistribution and values less than 5 would be indicators of a low PMR. Unlike the C/P model, the liver model has the advantage of having a greater magnitude in concentration compared to blood. In order to evaluate PMR, further characteristics of the drug itself must also be taken into consideration. Susceptible drugs to suffer PMR are tricyclic antidepressants, narcotic analgesics, antihistamines, and amphetamines [2, 7]. All of them are lipophilic drugs and have an elevated volume of distribution and a high percentage of protein binding. Also, the knowledge of a drug's stability is fundamental to carry out an appropriate interpretation of concentrations. Storage conditions (time and temperature), the addition of preservatives, type of the collected sample, and type of drug are some of the factors that influence a drug stability.

It could be generally stated that drugs such as amphetamines, some anti-depressant and anti-psychotic drugs, present in postmortem samples are stable changing the concentration in less than 30% in a period less than one year to -20 °C [8]. Other drugs as a cocaine, some benzodiazepines, heroin and morphine are prone to suffer expontaneous degradation. In the matter of benzodiazepines (lorazepam, estazolam, chlordiazepoxide, and ketazolam). *Melo et al.* [9] demonstrated that degradation of these drugs in postmortem blank blood, bile, and vitreous humor are temperature-dependent showing poor stability during six months storage at -20 °C. Another typical example is presented with cocaine, which is spontaneously hydrolyzed by esterase's especially under alkaline conditions to benzoylecgonine. The esterase's activity may continue metabolizing cocaine even after death [10] . This process is more relevant in plasma and is also temperature-dependent [11]. A similar situation occurs with heroin. It is spontaneously and enzymatically hydrolyzed to 6-monoacetylmorphine [12]. Otherwise, the evaporation of volatiles also plays an important role especially with ethanol which is one of the most commonly encountered substances in forensic toxicology. PBS stored with sodium fluoride in a tube with a large headspace showed more susceptibility to losses by oxidation, evaporation, and microbial mechanisms up to 9% whereas vitreous humor is a more stable matrix [13, 14].

The most visible problem dealing with postmortem toxicology is the process of decomposition. It is a common issue in forensic toxicology when decomposed

corpses are only found several days after death. The decomposition process comprises two mechanisms: autolysis and putrefaction. The hydrolytic enzymes present in the body induce the autolytic changes whereas the putrefaction is due to microorganisms. The autolysis process begins a few minutes after death in cells with high concentrations of hydrolytic enzymes (lipases, esterases, helicases) located in high concentration in the pancreas and the gastric mucosa, and more slowly in the cells with lower concentrations of these enzymes like in the heart or liver [7, 15-17]. After the hemolysis comes the invasion of the intestinal flora into tissues and body fluids. Then, rapidly thousands of microorganisms in a short time can quickly produce putrefaction, especially at ambient and elevated temperatures. It is estimated that the gastrointestinal tract includes taxa from about 300 to 1000 different bacteria species with two to four million genes. There are 100 to 1000-fold more anaerobes than aerobes largely *Bacteroides* species (*B*. *thetaiotaomicron, B. vulgatus, B. distasonis,* and *B. fragilis*) which colonize the human body after weaning [18, 19]. In addition to bacteria, fungi also make up a part not only of the gut flora but also of some areas of the skin, mouth, lungs, or urogenital and gastrointestinal tracts [20, 21]. Although the fungi-bacteria ratio is orders of magnitude much smaller, the impact of fungi on human health is especially significant as a source of blooms of microorganisms capable of causing disease in the host and as a potential cofactor in metabolic disorders and inflammatory processes [22]. The most representative fungi present in mucosal surfaces including oral cavity, urogenital system, and gastrointestinal tract belong to *Candida* spp., being *Candida albicans* the fungus most frequently found. Other species of clinical relevance are *C. glabrata, C. rugosa, C. parapsilosis, C. tropicalis, C. dubliniensis, C. krusei, and C. lusitaniae*. [22, 23]. In the skin area, Oyeka *et al* [24] and other authors [23, 25-30] reported the yeast *C. albicans, Rhodotorula rubra,* and *Trichosporon cutaneum*, some dermatophytes such as *Trichphyton rubrum* and *Microsporum gypseum* and nondermatophyte fungi such as *Fusarium*, *Trichosporon cutaneum, Rhizopus stolonifer*, *Mucor* sp., and some species of *Aspergillus* and *Penicillium*. Most of these microbiomes are facultative pathogens, contaminants, or opportunistic saprophytes. Fungi present in oral cavity and lungs, which begin the colonization process through the upper respiratory tract, vary greatly between different

individuals but the most common genera belong to *Candida, Saccharomyces, Cladosporium, Cryptococcus, Fusarium, Pneumocystis, and Aspergillus*. Some of these fungi exist to a low level when the host is healthy and become pathogenic when the host is inmmunocompromised [31]. After death, the immune system stops working, and because of a cadaver is a plentiful source of organic material this natural microbiome, together with fungi present from the place where the corpse is found [32-36], initiates a microbial proliferation process. This colonization process usually begins in the respiratory system and in the gut, at the junction between the small and large intestines. Then comes an invasion in the capillaries of the digestive system spreading first to the liver and spleen, and then into the heart and brain. The colonization is carried out first by anaerobic microbes converting carbohydrates, lipids, and proteins into organic acids and gases because of the depletion of internal oxygen. Due to the formation of gases, the skin is broken and a colonization of aerobics microbes [37] takes effect. Bacteria and fungi are able to breakdown almost 90% [38] of organic matter and their enzymes may contribute to the metabolism and degradation of drugs present in postmortem material changing their concentration or the metabolic pattern as has already been demonstrated by some authors. *Yajima et al.* [39] presented two cases involved in traffic accidents in which the ethanol concentration in postmortem increased because of the presence of *C. albicans, C. parapsilosis, C. tropicalis,* and some species of bacteria (*Corynebacterium sp*. *Escherichia coli*). The results also showed that *C. albicans* generated ethanol more easily in blood diluted by intravenous infusions that included glucose than in undiluted blood. *Robertson et al.* [40] also reported a concentration decrease of nitrobenzodiazepines including flunitrazepam, clonazepam, and nitrazepam in pos-mortem blood due to their reduction to the respective 7-amino metabolites by the presence of gut bacteria such as *Streptococcus faecalis* and *Clostridium perfringens*. Other postmortem changes correspond to the increase of free conjugated drugs (ethanol, morphine, and 6-monoacetylmorphine) because of the hydrolysis of their conjugates by contamination with bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Clostridium sordellii* and some yeast including *C. albicans* in postmortem blood, liver, and urine samples [41-43]. The most recent study about these changes was

reported by Butzbach et al. [44] showing the *in vitro* stability of risperidone and paliperidone, two benzisoxazole antipsychotic drugs in decomposing porcine blood inoculated with bacteria. The result showed the degradation of these drugs to their respective 2-hydroxybenzoyl benzisoxazoles by *Alcaligenes faecalis, Klebsiella pneumonia, Bacillus cereus, Clostridium bifermentans, Proteus mirabilis* and *vulgaris, Clostridium perfringens, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Escherichia coli*. The information above shows how little information there is about drug metabolism in DPS by fungi.

In order to establish an experimental setup for studying postmortem drug metabolism in fungi, an *in vitro* fungal biotransformation model was developed in the author's laboratory [45] using model fungi known to colonize cadavers [32-35, 46] (*Absidia repens, Aspergillus repens, Aspergillus terreus, Gliocladium viride, and Mortierella polycephala*) and five model drugs (Amitriptyline, Metoprolol, Mirtazapine, Promethazine, and Zolpidem). The obtained results showed that some of these model fungi were able of metabolizing the employed model drugs. The main metabolic reactions were demethylation, oxidation, and hydroxylation implying that the concentrations and metabolic patterns of drugs in a postmortem sample may be changed due to colonization by fungi and therefore the analytical results could be misinterpreted or cause false-negative findings. Despite these studies, there is no systematic research to date about what kind of fungi could be present in decomposed postmortem material and which the potential role of these microorganisms could be in the metabolism of xenobiotics.

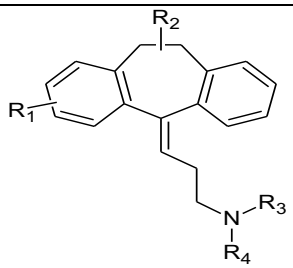
4.2 Model drugs

Postmortem toxicological analysis aims at establishing if the cause of death can be attributed to an overdose, to a subtherapeutic level of drug, to a combination of several drugs, or on the contrary, allows to rule out the contribution of a particular drug or poison to the cause of death. In this regard, the toxicological analysis must not only be directed to the search for abuse drugs but also to the search of pharmaceutical drugs, which besides their wide use, can have dangerous adverse effects. The five model drugs selected in the presented work belong to the groups

of tricyclic, noradrenergic and specific serotonergic antidepressants, adrenergic blocking agents, antihistamines, neuroleptics, and hypnotic drugs and as such, they have important side effects or additive side effects on the central nervous system (sleep disturbance, psychosis, drowsiness, dizziness, depression, seizures) and cardiovascular system (sinus tachycardia, orthostatic hypotension, bradycardia) relevant to establish the cause of death [47-51]. In addition, these drugs undergo extensive mammalian metabolism making it possible to monitor distinct reactions as was demonstrated in a previous work [45] . An overview of the metabolism and analytical identification of the main metabolites of the five model drugs are given in the following section.

4.2.1 Amitriptyline

Amitriptyline (AT) is a tricyclic antidepressant traditionally used to treat symptoms of major depression disorders and anxiety. After AT is absorbed and reaches the general circulation, it is metabolized to more polar compounds through the cytochrome P450 hepatic microsomal enzyme system (CYP450). The bioactivation of the major plasma metabolite (nortriptyline) is catalyzed by the isoenzyme CYP2C19 leading to demethylation of the tertiary amine function [52]. Nortriptyline is further demethylated to produce desmethylnortriptyline metabolite [53]. The formation of hydroxy metabolites is closely linked to the activity of CYP2D6, which has been studied and identified in rats, mice, and human urine [53-56]. Some of the most relevant mammalian metabolites are shown in table 1. Sensitive, specific, and rapid methods based on gas chromatography (GC), mass spectrometry (MS), chemical ionization (CI), liquid chromatography (LC) electrospray ionization (ESI), and tandem mass spectrometry (MS/MS) has been developed for the simultaneous determination of AT and their metabolites [45, 57, 58].



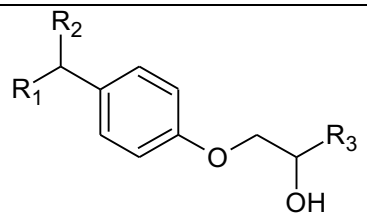
METABOLITE	R_1	R_2	R_3	R_4	Ref
Amitriptyline (AT)	-H	-H	$-CH_3$	$-CH_3$	
AT-M (*N-oxide*)	-H	-H	$-CH_3$	$-CH_3$	[45]
AT-M (nor)	-H	-H	-H	$-CH_3$	[53]
AT-M (demethyl nor)	-H	-H	-H	-H	[53]
AT-M (OH-) (isomer 1 and 2)	-OH	-H	$-CH_3$	$-CH_3$	[56]
AT-M (OH-) (isomer 3 and 4)	-H	-OH	$-CH_3$	$-CH_3$	[53-55]
AT-M (nor-OH-) (isomer 1 and 2)	-OH	-H	-H	$-CH_3$	[56]
AT-M (nor OH-) (isomer 3 and 4)	-H	-OH	-H	$-CH_3$	[53-55]

Table 1. AT and its most relevant mammalian metabolites

4.2.2 Metoprolol

Metoprolol (MET) is a selective ß1-adrenergic blocking agent used as a cardiovascular drug in an effort to reduce cardiovascular events in patients with hypertension, angina, acute myocardial infarction, congestive heart failure, and others coronary heart diseases [59]. MET is absorbed fully after oral administration; nonetheless, its bioavailability is only 40% - 50% due to rapid and extensive metabolism mediated by cytochrome liver enzymes CYP2D6. The drug is excreted from the body primarily by metabolism and only a minor fraction is eliminated as parent drug by the urinary system [60]. The major urinary metabolite corresponds to the product of *O*- demethylation with subsequent oxidation to carboxy metabolite (Table 2). Other metabolites have been described in man, dog, and rat [61, 62]. The identification of MET and its metabolites is assayed by electron-capture detector GC [63, 64] and GC-MS/EI [65] using halogenated derivates. LC methods are also suitable for the simultaneous analysis of MET and its metabolites. *Xu et al* developed an HPLC-UV method with fluorescence detection using plasma and urine

samples [66], while *Rao et al.* worked out a rapid sensitive LC-MS/MS method in rat plasma samples [67].



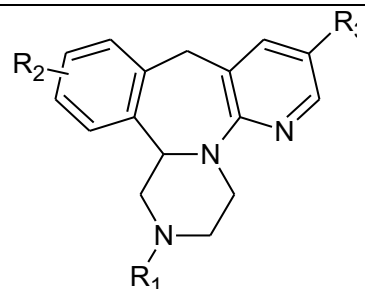
METABOLITE	R_1	R_2	R_3	Ref
Metoprolol (MET)	$-CH_2OCH_3$	-H	$-CH_2NHCH(CH_3)_2$	
MET-M (O-demethyl)	$-CH_2OH$	-H	$-CH_2NHCH(CH_3)_2$	[60-62]
MET-M (Carboxy)	-COOH	-H	$-CH_2NHCH(CH_3)_2$	[61, 62]
MET-M (OH-)	$-CH_2OCH_3$	-OH	$-CH_2NHCH(CH_3)_2$	[60-62]
MET-M (O-demethyl) (OH-)	$-CH_2O$	-OH	$-CH_2NHCH(CH_3)_2$	[61, 68]
MET-M (deaminated)	$-CH_2OCH_3$	-H	-COOH	[61, 62]

Table 2. MET and its most relevant mammalian metabolites

4.2.3 Mirtazapine

Mirtazapine (MRT) is the first noradrenergic and specific serotonergic antidepressant structurally related to piperazinoazepine compounds, which has been used clinically to treat major depressive disorders since 1994. The drug is available as a racemate where both enantiomers possess pharmacological activity [69]. Rapidly absorbed from the gastrointestinal tract following a single oral dose, MRT is eliminated in the urine as products of biotransformation mainly mediated by CYP1A2, CYP2D6 and CYP3A4 isoenzymes [70]. The most relevant metabolites (Table 3) include *N-oxide* formation, *N*-demethylation to produce the nor metabolite of MRT, ring hydroxylation (predominantly at position 8), and glucuronide metabolites. Additionally to the human MRT metabolism, the same metabolites have also been identified in rats, beagle dogs, and horses [71, 72]. Several LC analytical separation techniques have been reported to identify MRT and its two major metabolites in human plasma and serum using fluorescence detection and liquid-liquid extraction (LLE) for sample clean up. [73-76]. The identification could also be

achieved using a muti-target screening analysis and serum human samples [77-80].



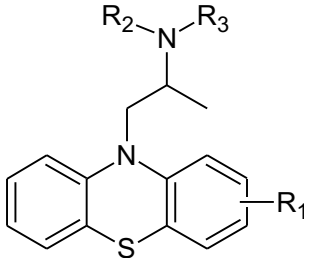
METABOLITE	R_1	R_2	R_3	Ref
Mirtazapine (MRT)	$-CH_3$	-H	-H	
MRT-M (*N-oxide*)	$-CH_3$	-H	-H	[45]
MRT-M (nor)	-H	-H	-H	[71, 72]
MRT-M (OH-) (isomer 1)	$-CH_3$	-OH	-H	[71, 72]
MRT-M (OH-) (isomer 2)	$- CH_3$	-H	-OH	[71, 72]
MRT-M (nor-OH-) (isomer 1)	-H	-OH	-H	[45]
MRT-M (nor-OH-) (isomer 2)	-H	-H	-OH	[45]

Table 3. MRT and its most relevant mammalian metabolites

4.2.4 Promethazine

Promethazine (PMZ) is a first-generation H1 antihistamine and neuroleptic drug of the phenothiazine group approved for use by the Food and Drug Administration in 1951. PMZ is used as an antihistaminic, antiemetic, and sedative drug concerning the treatment of allergic diseases, nausea, and vomiting associated with certain types of anesthetics drugs and sedation [81]. Although the metabolism of PMZ has not been thoroughly investigated in man, in studies with human liver microsomes, it was demonstrated that CYP2D6 and CYP2C9 are the major isoenzymes responsible for the bioactivation of the drug [82, 83]. PMZ is known to be extensively metabolized with less than 0.5% of a dose excreted unchanged in the 72-hour urine. The extensive hepatic metabolic pathways include *S*-oxidation and *N*-demethylation giving promethazine sulfoxide and desmethylpromethazine sulfoxide as major metabolites and desmethylpromethazine as minor metabolite in urine samples (Table 4) [84]. Ring hydroxylation in combination with *S*-oxidation and *N*-oxidation

are additional PMZ metabolites, which have been found in human urine and rabbit liver homogenate samples [45, 85]. PMZ and its metabolites have been measured in urine and plasmas samples by LC-ESI-MS, [86] HPLC ultraviolet detection and electrochemical detectors [81, 84, 87] or MS detection [88]. In most of the above procedures, the samples were worked upon using LLE under basic conditions and solid-phase extraction (SPE).



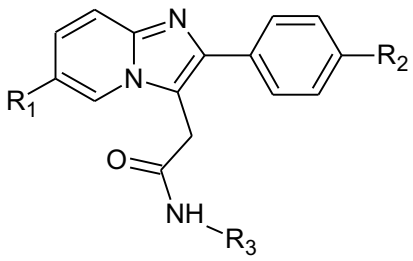
METABOLITE	R_1	R_2	R_3	Ref
Promethazine (PMZ)	-H	$-CH_3$	$-CH_3$	
PMZ-M (*N-oxide*)	-H	$-CH_3$	$-CH_3$	[45]
PMZ-M (*sulfoxide*)	-H	$-CH_3$	$-CH_3$	[81, 84]
PMZ-M (nor)	-H	-H	$-CH_3$	[84]
PMZ-M (*sulfoxide*) *(N-oxide)*	-H	$-CH_3$	$-CH_3$	[45]
PMZ-M (*sulfoxide*) (nor)	-H	$-CH_3$	-H	[84]
PMZ-M (OH-)	-OH	$-CH_3$	$-CH_3$	[45]
PMZ-M (*sulfoxide*) (OH-)	-OH	$-CH_3$	$-CH_3$	[45]
PMZ-M (nor) (OH-)	-OH	-H	$-CH_3$	

Table 4. PMZ and its most relevant mammalian metabolites

4.2.5 Zolpidem

Zolpidem (ZOL) is a nonbenzodiazepine imidazopyridine hypnotic drug derivate used since 1986 in European countries and since 1993 in the United States as a hypnotic agent for the treatment of insomnia, anxiety, sleep disorders, and other neurological diseases [89]. The biotransformation of ZOL is mediated by cytochrome P450 with CYP3A reported as having a dominant role and the isoenzymes CYP1A2 and CYP2D6 with a minor contribution [90, 91]. After oral administration, ZOL is absorbed rapidly and the peak plasma concentrations are reached before 2 hours with an average elimination half-life of 2.6 hours [92]. ZOL is extensively biotransformed to pharmacologically inactive oxidation products that

are eliminated via renal and fecal excretion. The major urinary excretion metabolites (Table 5) are alcohols by oxidation on the phenyl group and furthermore, phenolic hydroxylation to carboxylic acids without the presence of the parent drug [93]. Several published GC-MS/EI and LC-MS/MS methods have been developed to identify and quantify ZOL and its major metabolites in whole blood, urine, and hair samples [94-98]. Most of them use a clean-up step including β-glucoronidase hydrolysis of glucuronide conjugates and SPE or LLE with alcohol or carboxy derivatization reactions.



METABOLITE	R_1	R_2	R_3	Ref
Zolpidem (ZOL)	$-CH_3$	$-CH_3$	$-CH_3$	
ZOL-M (*N-Oxide*)	$-CH_3$	$-CH_3$	$-CH_3$	[45]
ZOL-M (4 -OH)	-H	$-CH_2OH$	$-CH_3$	[91]
ZOL-M (6 -OH)	$-CH_2OH$	-H	$-CH_3$	[91]
ZOL-M (4-COOH)	-H	- COOH	$-CH_3$	[91, 94,
ZOL-M (6-COOH)	- COOH	-H	$-CH_3$	95]
				[91]

Table 5. ZOL and their most relevant mammalian metabolites

4.3 Microbial models used to study a mammalian metabolism

The utilization of microorganisms as microbial models of mammalian metabolism was developed almost four decades ago with the use of a group of fungal and bacterial species on the metabolism of aromatic compounds and their capability to produce hydroxy metabolites in an analogous manner to the mammalian system [99]. Today the use of microbes to mimic the mammalian metabolism of a lot of substances of pharmacological importance is well documented. A variety of microorganisms among bacteria (some species of genera *Actinoplanes,*

Mycobacterium, *Nocardia, Streptomyces*), yeast (some species of genera *Candida*, *Rhodotorula*, *Sacaromices*), and fungi (some species of genera *Actinomucor, Aspergillus, Beauveria, Chaetomiun Cunninghamella, Curvularia, Curvularia, Diplodia, Fusarium, Gliocladium, Heminthosporium, Mortierella, Mucor, Penicillium, Rhizopus, Thamnidium, Verticillium, Whetzelinia*) can be used depending on the type of desired transformations [100]. Because of the similarity between mammalian and microbial CYP450, the pattern of metabolism (phase I - functionalization and phase II – conjugation) found in microbial incubations in most cases are very similar to that observed in *in vitro* and *in vivo* mammalian systems. Furthermore, there are numerous advantages over the of use animal models or mammalian cell cultures; mainly low cost and facility to set up the assay, more easily reproducible, screening with a large number of microbes, easy metabolite identification and characterization because large amount yielding, production of synthetic metabolites involves many steps, and maintenance of microbes cultures is cheaper and simpler than the maintenance of mammalian cell cultures [101, 102]. As eukaryotic organisms, the fungi possess a CYP450 that have proven to be mechanistically similar to the mammalian liver cytochrome P450. In this regard, the most widely fungus used as microbial model of mammalian metabolism corresponds to *C. elegans* which has the ability to produce aromatic and ring hydroxylations, carboxidations, *N-oxydations*, *N*-dealkylations, *N*-acetylations, methylhydroxilations, and epoxidations among others [103-107]. *C. elegans* is a zygomycete fungus of the order Mucorales and the genus *Cunninghamella* [108] found in soil, plant, and animal material especially at Mediterranean and subtropical zones that has been used extensively as a microbial model for mammalian hepatic metabolism of a wide variety of xenobiotics [109]. The genus *Cunninghamella* contains 14 species, being the most common the species *C. elegans*, *C. echinulata, and bertholletiae*; the latter is known as a human and animal pathogen [102]. This fungus has the ability to degrade different class of xenobiotics in a regio- and stereo-selective manner similar to mammalian enzyme systems among which can be mentioned pharmaceutical drugs [105, 106, 110-114], polycyclic aromatic hydrocarbons and pollutants [115-118], drugs of abuse [119], pesticides [109, 120-122], and anabolic androgenic steroids in doping [123-125] by both phase I (oxidative) and phase II (conjugative)

biotransformation mechanisms. Most of these studies have as their main goal the study of mammalian metabolism or the preparation of new active compounds rather than a study of detoxification of xenobiotics. The enzymes involved in the biotransformation of xenobiotics by *C. elegans* include Cytochrome P450 monooxygenase, including the CYP51 family, gluthatione S-transferase, aryl PAPS sulfotransferase, UDP- glucuronosyltransferase and UDP-glucosyltransferase proteins detected in cytosolic or microsomal fractions [108, 126]. In Sabouraud dextrose agar (SDA) *C. elegans* grows relatively fast (fills a petri dish in 2 to 3 days) producing white wooly mycelia becoming grey with age. *C. elegans* strain CBS 167.53 was used in this study as K1 during all the incubation experiments.

5 AIMS AND SCOPE

The process of postmortem decomposition is partly mediated by different microorganisms that are part of normal human microflora [19, 23, 29] or are present at the site of the corpse [32-34]. Some of these microbes may contribute to the metabolism or degradation of drugs present in postmortem material [40, 43, 44, 127]. Despite these findings, postmortem drug metabolism by fungi colonizing corpses has been not yet systematically studied. However, it should be considered that fungi are eukaryotes and as such share similarities with mammalian cells and therefore with mammalian metabolism. In consequence, the main aims of this study were:

- To isolate, maintain, and identify fungi colonizing cadavers from postmortem material (HB, kidney, liver, and lung) collected during the autopsy of decomposed human bodies between 2010 and 2012 in the federal state of Thuringia.

- To evaluate the capability of the previously isolated and identified fungi to metabolize five model drugs (Amitriptyline, Metoprolol, Mirtazapine, Promethazine, and Zolpidem) by incubation experiments and GC-MS/EI and LC-ESI-MS/MS-based analysis and interpretation of mass spectral data for metabolite identification.

- To conduct a systematic search for fungi specific *in vitro* and *in mortuo* metabolites from four model drugs (Amitriptyline, Metoprolol, Mirtazapine, and Zolpidem) using authentic postmortem decomposed blood samples by LC-ESI-MS/MS-based analysis and interpretation of mass spectral data.

6 PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

6.1 Studies on drug metabolism by fungi colonizing decomposing human cadavers. part I: DNA sequence-based identification of fungi isolated from postmortem material [128].

DOI: 10.1007/s00216-013-7250-1

Electronic supplementary material

Anal Bioanal Chem (2013) 405:8443–8450
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RESEARCH PAPER

Studies on drug metabolism by fungi colonizing decomposing human cadavers. Part I: DNA sequence-based identification of fungi isolated from postmortem material

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Abstract Cadavers can be colonized by a wide variety of bacteria and fungi. Some of these microbes could change the concentration or the metabolic pattern of drugs present in postmortem samples. The purpose of this study was to identify fungi from human postmortem material and to further assess their potential role in the metabolism of drugs. Aliquots of 252 postmortem samples (heart blood, liver, kidney, and lung) taken from 105 moderately to severely decomposed bodies were streaked on Sabouraud agar for isolation of fungal species. One part of the samples was worked up immediately after autopsy (group I). The second part had previously been stored at −20 °C for at least 1 year (group II). Identification of the isolates was achieved morphologically by microscopy and molecularly by polymerase chain reaction amplification and sequencing of markers allowing species identification of the respective genera. Depending on the genus, different gene fragments were used: calmodulin for *Aspergillus*, β-tubulin for *Penicillium*, translation elongation factor 1α for *Fusarium*, and the internal transcribed spacer region of the ribosomal DNA for all remaining genera. A total of 156 fungal strains were isolated from 62 % of the postmortem materials. By using these primers, 98 % of the isolates could be identified to the species level. The most common genera were *Candida* (60.0 %—six species), *Penicillium* (10.3 %—two species), *Rhodotorula* (7.1 %—one species), *Mucor* (6.4 %—four species), *Aspergillus* (3.2 %—four species), *Trichosporon* (3.2 %—one species), and *Geotrichum* (3.2 %—one species). Group I samples contained 53 % more fungal species than stored samples suggesting some fungi did not survive the freezing process. The isolated fungi might be characteristic for decomposed bodies. The proposed methodology proved to be appropriate for the identification of fungi in this type of material.

Keywords Fungi · Cadavers · Postmortem material · PCR · Molecular identification

Parts of these results were presented at the 50th annual Meeting of the International Association of Forensic Toxicologists, June 3–8, 2012, Hamamatsu, Japan.

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Introduction

The human body is colonized by a normal microflora consisting in their vast majority of native bacteria of the gastrointestinal tract whose microbial taxa include 300 [1] to 1,000 [2] different species. Some fungi of the genera *Candida*, *Geotrichum*, *Malassezia*, *Trichosporon*, and *Trichophyton* are located especially in the areas of the skin and urogenital and gastrointestinal tracts [3–9]. Another type of fungus such as facultative pathogenic members of the genera *Aspergillus*, *Fusarium*, and *Mucor* [10, 11] may invade the tissue and cause infections in immunocompromised patients. The spores of these fungi enter the human body through the upper respiratory tract [12]. After death and because a corpse is a plentiful

source of organic material, these fungi, and others present from the place where the corpse is found [13–17], begin a colonization process of different body tissues. This colonization is of importance because some of these fungi could change the concentration or the metabolic pattern of drugs or toxic compounds present in postmortem samples as it has already been demonstrated for some bacteria [18–22] and the yeast *Candida albicans* [23] with drugs like opiates, nitrobenzodiazepines, and alcohol. Recently, an in vitro fungal biotransformation model for studying the metabolic capability of fungi colonizing cadavers was developed in the authors' laboratory [24]. The results showed that some of the model fungi used in this study were capable of metabolizing the employed model drugs implying that the concentrations and metabolic patterns of drugs or poisons in a corpse may be changed due to colonization by fungi. However, to the authors' knowledge, the colonization of corpses by fungi has never been studied systematically. Therefore, the main purposes of the present study were to conduct a systematic search for fungi in different postmortem materials collected during autopsy of decomposed bodies, to identify the isolated species, and in a second phase (part II of the communication), to evaluate the potential role of the respective species in the metabolism of xenobiotics.

With the introduction of DNA sequence-based analyses in the systematics of fungi, nearly all fungal groups have experienced an extreme increase in number of species [25–27]. Molecular data revealed that many of the morphologically defined species are in fact species complexes consisting of two or several morphologically similar species [28–30]. As a consequence of the morphological similarity among closely related species in genera such as *Aspergillus*, *Fusarium*, *Mucor*, or *Penicillium*, a correct identification according to the current classification can often only be reached by comparing DNA sequences. A precondition for correct and reliable molecular species identification is the use of an appropriate marker and a comprehensive sequence database ideally including sequences of all known species. In fungi, the internal transcribed spacer (ITS) region of the ribosomal DNA is widely accepted as marker of choice for species identification [31]. However, some genera such as *Aspergillus*, *Fusarium*, and *Penicillium* cannot be reliably identified by ITS because identical ITS sequences are shared among different species. Therefore, alternative and more variable markers such as the calmodulin gene for *Aspergillus*, the β-tubulin gene for *Penicillium*, or the translation elongation factor 1α (TEF) gene for *Fusarium* are sequenced for more reliable identification of species from these genera [26, 32, 33]. Molecular identification is widely performed by using the Basic Local Alignment Search Tool (BLAST) of GenBank (http://www.ncbi.nlm.nih.gov/) that searches for the most similar sequence in the database. GenBank contains a large number of fungal sequences and represents a valuable tool for species identification. However, there is no third party annotation, and misidentifications frequently remain uncorrected. About 20 % of the sequences deposited in GenBank are misidentifications [34–36]. The identification by BLAST is further complicated by an often inconsistent nomenclature and the undersampling of numerous fungal groups. For several genera, public or in-house sequence databases that are curated by specialists were applied because of the more reliable and unambiguous result.

Materials and methods

Chemicals and reagents

Dextrose, protease-peptone, yeast extract, agar, and premixed solution of deoxynucleoside triphosphates (dNTPs) were obtained from Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and magnesium sulfate were purchased from neoLab (Heidelberg, Germany). The primers for partial amplification of the genes β-tubulin (Bt2a/Bt2b), calmodulin (cmd5/cmd6), ITS (ITS1/ITS4), and TEF (A-TEF_F/A-TEF_R) were provided by Eurofins MWG Operon (Ebersberg, Germany). Dap GoldStar® polymerase was obtained from Eurogentec (Köln, Germany). NEB One-Taq® Hot Start DNA polymerase was purchased from New England Biolabs Inc. (MA, USA) and molecular weight marker from life technologies (Darmstadt, Germany). Ingredients for preparation of silver-stained polyacrylamide gels were obtained from Merck (Darmstadt, Germany). The ExoSAP-IT® PCR Product Clean-up Kit was purchased from Affymetrix (Freiburg, Germany). The BigDye® terminator sequencing buffer was provided by Applied Biosystems (Darmstadt, Germany) and the DyeEX 2.0 Spin Kit from Qiagen (Hilden, Germany). All chemicals were of analytical grade or higher.

Postmortem material

A total of 252 postmortem heart blood (HB), kidney, liver, and lung samples were collected during autopsy of 105 moderately (stage 1) to severely (stage 2) decomposed bodies in different cities in the federal state of Thuringia, Germany, between 2010 and 2012. A summary of the main characteristics of the autopsied corpses (74 males and 31 females) included in this study is shown in Table 1. About 75 % were between 40 and 70 years old. From the 105 corpses, 46 % were found in stage 1 (96 samples) and 54 % in stage 2 (156 samples) of decomposition. The main cause of death was natural (32.4 %). In about 44 % of the cases, it was not possible to determine an exact cause of death due to the high degree of decomposition. In none of the cases was a fungal infection diagnosed prior to death.

The first group of 68 samples (HB, kidney, liver, and lung) was prospectively collected from 20 different autopsy cases (group I). These samples were taken under aseptic conditions

Table 1 Summary characteristics of autopsied corpses (n=105), postmortem samples (n=252), and relative number of fungal strains detected

Characteristics	Corpses % total		Isolated fungal strains
	Number	% Total	% Total
Age, years			
<40	10	9.5	7.1
40–49	23	21.9	26.1
50–59	33	31.5	32.0
60–70	22	20.9	24.3
>70	17	16.2	10.2
Sex			
Men	74	70.5	80.1
Women	31	29.5	19.9
Degree of decomposition of the corpse			
Stage 1 (96 samples)	48	45.7	46.1
Stage 2 (156 samples)	57	54.3	53.8
Postmortem material			
Heart blood	96	38.1	26.2
Lung	19	7.5	19.2
Kidney	69	27.4	32.6
Liver	68	27.0	21.8
Cause of death			
Natural	34	32.4	28.8
Accidental	2	1.9	1.9
Homicide	7	6.6	4.2
Suicide (poisoning)	5	4.8	8.3
Suicide (other)	11	10.5	12.1
Undetermined	46	43.8	42.9
Place where the corpse was found			
Home	87	82.8	69.8
Hospital	8	7.6	7.1
Open field	5	4.8	18.6
Water	4	3.8	3.8
Buried	1	0.9	0.6

as follows: A 10-mL sterile hypodermic syringe with a 10-gauge needle was introduced directly in the heart, approximately 5 mL of blood was aspirated, and 1 mL placed in 1.5 mL polypropylene microcentrifuge tubes. Tissue sections (approximately 1–2 cm^2) of kidney, liver, and lung were obtained with a previously flamed scalpel and placed directly into a 1.5-mL polypropylene microcentrifuge tube. Fungal analysis on these samples was started as quickly as possible on the day of collection.

The remaining 184 samples (HB, kidney, liver) from 85 cases had been collected for routine toxicological analysis (group II). They were used for a retrospective microbiological analysis in the present study after completion of routine analysis. These postmortem materials were taken according to the standard operating procedure for all full autopsies in the Institute of Forensic Medicine of Jena University Hospital: after opening the pericardial cavity, the pericardial fluid was removed, and 50 mL of HB was retrieved from the inferior vena cava with a clean ladle. Tissue sections (approximately 30 g) of kidney and liver were obtained using a clean surgical knife. The postmortem materials were placed in separate clean 50-mL plastic containers and stored at −20 °C. At the time of the present study, they had been stored for at least 1 year. During the storage period, 86 of the samples had been thawed once for routine toxicological analysis and refrozen.

Isolation, maintenance, and identification of fungi

The blood samples were streaked on Petri dishes with Sabouraud medium dextrose 10 %, pH 5.2. In the case of tissues, approximately 100 mg of each sample was cut with an aseptic scalpel in a laminar flow and streaked on Petri dishes. Prior to treatment, the samples of group II were thawed at room temperature The dishes were incubated at 25 °C for up to 20 days in darkness, with daily observation until fungal growth was detected. Thereafter, colonies were picked and transferred to fresh dishes up to three times to reach pure cultures. Two dishes for each isolate were maintained in Sabouraud medium at 25 °C. One plate was required for the morphological identification, and the second was used for the DNA extraction with the objective of molecular identification.

DNA extraction

The DNA was extracted according to the procedure described by Cenis [37] with the following modifications: A small amount (approximately 1–2 mm^2) of the isolated yeast colony or the mycelium without agar media was inoculated in a 1.5-mL polypropylene microcentrifuge tube containing Sabouraud medium. The incubation was carried out for 48 h at 25 °C and a shaking velocity of 90 rpm. Immediately, the tubes were centrifuged for 3 min at 9,600×g. The cell pellets (CP) were washed with 300 μL of DNA extraction buffer (DEB) (200 mM Tris/HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5 % SDS) and centrifuged again. The supernatant was discarded, and the CP were resuspended with 300 μL of DEB and scratched with a pipet tip. Then 150 μL of 3 M sodium acetate pH 2.5 was added, and the tubes were incubated for 10 min at 20 °C. After that, the tubes were centrifuged for 5 min at 9,600×g, and the supernatant was transferred in another tube with 300 μL of 2-propanol and gently agitated inverting the tube three to five times. After the precipitation of DNA, it was centrifuged for 20 min at 9,600×g, dried at 55 °C, and resuspended using 50 μL of sterile Aqua bidest.

Polymerase chain reaction amplification

The polymerase chain reaction (PCR) (see Table 2) was carried out in 0.2-mL polypropylene microcentrifuge tubes using a total volume of 25 μL. The primers used for amplification and sequencing are described in Table 3. The PCR was performed in a T3000 ThermoCycler (Biometra, Göttingen, Germany) with the following program: for Dap GoldStar® polymerase, 95 °C for a 3-min initial denaturation step followed by 95 °C for 30 s, annealing temperature for each primer for 1 min, 72 °C for 1 min for 34 cycles. For NEB One-Taq® polymerase, 94 °C for a 3-min initial denaturation step followed by 94 °C for 30 s, annealing temperature for each primer for 1 min, 68 °C for 2 min for 30 cycles, and finally an elongation at 68 °C for 10 min. Isolated DNA from *C. albicans* SC5314 was used as a positive control. Additionally, a blank incubation without DNA extract was performed.

Assessment of PCR product quality

Amplification success was evaluated by electrophoresis on a 8.9 % (*w*/*v*) polyacrylamide gel stained with silver. A volume of 3 μL of PCR-amplified product and 1 μL of Gel Loading Solution (tris–borate buffer with bromophenol blue) was loaded into each lane. A volume of 2 μL of a 100-bp molecular weight marker was run in parallel to approximate PCR-amplified product size.

DNA sequencing and editing

Each PCR-amplified product was washed with ExoSap-IT® PCR Product Clean-up Kit and sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit. The sequence reaction mixture contained 0.5 μL of ready reaction mix, 1 μL of each primer, 1 μL BigDye Terminator v1.1/3.1 Sequencing Buffer, and 2.5 μL PCR product. The PCR program for the sequencing was as follows: an initial step of 96 °C for 10 min followed by 96 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min for 25 cycles. After the sequencing reaction, the products were purified using DyeEX 2.0 Spin Kit and dissolved in 12 μL of formamide. The sequences were visualized using a four-capillary ABI 3130 Genetic Analyzer and edited by ApE-A plasmid Editor Software, version 2.0.37. *Candida glabrata* ATCC2001, *Candida parapsilosis* GA1, and *Aspergillus clavatus* FSU 5160 were used as a sequencing control.

Table 2 PCR setup for DNA amplification. Numbers represent reagent volumes in microliter

Primer	DNA polymerase	DNA extract	Buffer	$MgCl_2$ (50 mM)	dNTPS (10 mM)
0.5 (10 pmol/μL) Bt2a/Bt2b	0.125 NEB One-Taq®	2.5	5.0	–	0.5
0.5 (10 pmol/μL) cmd5/cmd6	0.125 NEB One-Taq®	2.5	5.0	–	0.5
1.0 (10 pmol/μL) ITS1/ITS4	0.15 DAp GoldStar®	1–2.5	2.5	1	1
1.25 (5 pmol/μL) A-TEF_F/A-TEF_R	0.2 DAp GoldStar®	2.5	2.5	1	1+1.3 DMSO

Databases search

Molecular identification of all taxa apart from the genera *Circinella*, *Fusarium*, *Mucor*, and *Trichoderma* was performed by using BLAST of GenBank (http://www.ncbi.nlm.nih.gov/). Species of *Trichoderma* were identified by their ITS sequence in the curated sequence database TrichOKEY 2 (http://www.isth.info/tools/molkey/index.php). For *Fusarium* species, the curated sequence database *Fusarium*-ID (http://isolate.fusariumdb.org/index.php) based on TEF was used. All members of the Mucorales, namely species of *Circinella* and *Mucor*, were identified by using a nonpublic in-house sequence database containing all sequences used by Walther et al. [38].

Results and discussion

A total of 156 fungal strains (Table 4) belonging to 28 species and 15 genera were isolated from 62 % of the postmortem samples. From 69.5 % of the cadavers, at least one fungal strain could be isolated. The distribution of all isolated strains according to genera and type of postmortem material is shown in Fig. 1. *Candida* was the genus with the highest number of isolated species (*C. albicans*, *C. galli*, *C. glabrata*, *C. lipolytica*, *C. parapsilosis*, and *C. tropicalis*) followed by the genera *Mucor* (*M. circinelloides*, *M. hiemalis*, *M. plumbeus*, and *M. racemosus*), and *Aspergillus* (*A. awamori*, *A. fumigatus*, *A. jenseni*, *and A. tubingensis*). Less frequent genera (<2 %) were *Botrytis*, *Chaetomium*, *Circinella*, *Coroliopsis*, *Enterocarpus*, *Fusarium*, and *Trichoderma.* Interestingly, numerous species detected in postmortem material have previously been isolated from clinical samples by other authors, and some of them are known facultative pathogens in immunocompromised patients. Some clinical studies related to the isolated strains in the present investigation are listed in the supplementary material that accompanies the online version of this article, Table S1, Electronic Supplementary Material. The distribution of each *Candida* species in the different postmortem materials is shown in Figure S1, Electronic Supplementary Material.

By using the primer sets given in Table 3, 98 % of the isolates could be identified to the species level. The remaining

Table 3 Primers and their annealing temperature used in this study

Locus	Primer	Primer DNA sequence	Annealing temperature, °C	Bases	Ref
β-Tubulin	Bt2a	5′-GGTAACCAAATCGGTGCTGC TTTC-3′	61.5	24	[44]
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTT GGC-3′		24	
Calmodulin	cmd5	5′-CCGAGTACAAGGAGGCCTTC-3′	53.0	20	[45]
	cmd6	5′-CCGATAGAGGTCATAACGTGG-3′		21	
ITS	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	50.0	19	[46]
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′		20	
TEF	A-TEF_F	5′-CCTTCAAGTACGCYTGGGTTC-3′	52.0	21	[28]
	A-TEF_R	5′-TTCTTGGAGTCACCGGCAA-3′		19	

Table 4 Number of fungal strains isolated from group I and group II samples collected from moderately (stage 1) or severely (stage 2) decomposed corpses

Fungi	Group I (68 samples)								Group II (184 samples)					
	Stage 1 (24 samples)				Stage 2 (44 samples)				Stage 1 (72 samples)			Stage 2 (112 samples)		
	HB	Lung	Kidney	Liver	HB	Lung	Kidney	Liver	HB	Kidney	Liver	HB	Kidney	Liver
Aspergillus awamori												1		
Aspergillus fumigatus		1			1							1		
Aspergillus jenseni									1					
Aspergillus tubingensis												1		
Bjerkandera adusta						1						2		
Botrytis cinerea												2		
Candida albicans		5	4	2		2	5	4	9	7	5	6	6	6
Candida galli			1	1		1			1			1	2	
Candida glabrata						1			2		1		1	1
Candida lipolytica			2	1		1						1	1	
Candida parapsilosis							1	1	2	2	1			
Candida tropicalis							2	1		1	1			
Chaetomium sp.						1								
Circinella minor		1												
Coroliopsis sp.						1								
Enterocarpus sp.						1								
Fusarium solani						1								
Geotrichum candidum								1			2		1	
Mucor circinelloides						2								
Mucor hiemalis									1				1	
Mucor plumbeus						1	2							
Mucor racemosus								1	1					
Penicillium rubens			2				1		1			2	1	
Penicillium crustosum		2	1		1			1	3				2	
Rhodotorula mucilaginosa		1		1		3	1		1	3	1			
Trichoderma harzianum						2								
Trichoderma sp.													1	
Trichosporon asahii						2	1		1					
Total fungal strains, material	0	10	10	5	2	20	13	9	23	13	11	17	16	7
Total fungal strains, group	69								87					

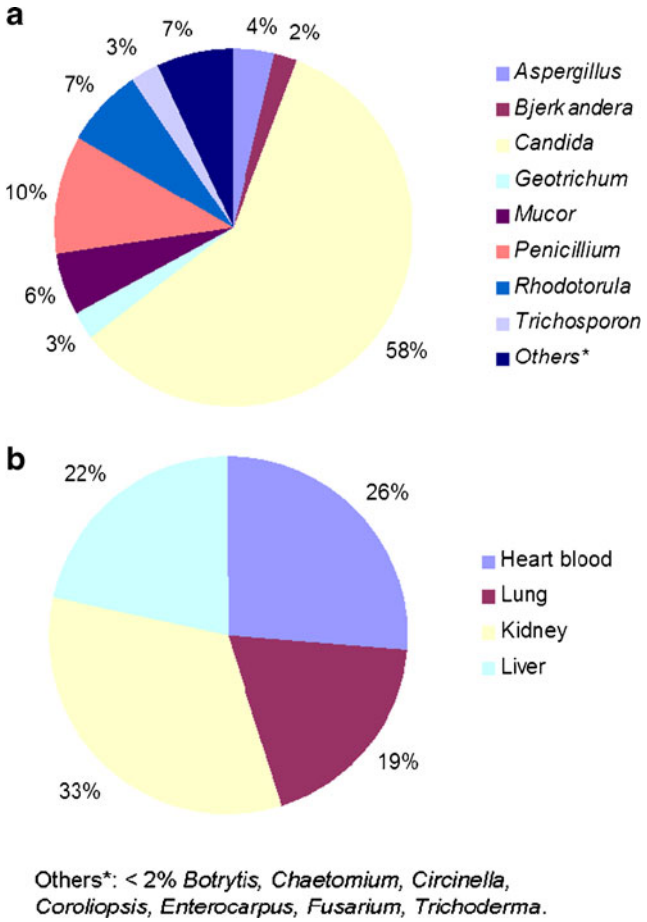

Fig. 1 Distribution of isolated strains (n=156) according to genera (**a**) and type of postmortem material (**b**). Note that lung samples were only collected in the 20 group I cases

three strains belonging to the genera *Coroliopsis*, *Enterocarpus*, and *Trichoderma* could only be identified to the genus level due to the lack of identical or similar reference sequences in GenBank. The decision on the correct identification in GenBank as well as the comparability among different studies is complicated by ongoing changes in the fungal classification and requires the knowledge of the recent publication on the systematics of the respective genera. For example, the morphospecies *Penicillium chrysogenum* is currently known to include at least five species, *P. chysogenum*, *P. flavigenum*, *P. dipodomyis*, *P. nalgiovense*, and *P. rubens*. These species are morphologically very similar. Therefore, the species name "*Penicillium chrysogenum*" of GenBank entries or publications that originate from the time before the new species were recognized can refer to the true *P. chrysogenum* but also to its newly recognized sister species.

In comparison with other studies investigating fungal diversity in postmortem material, the proposed methodology allowed the identification of the isolated strains to the species level in most cases. Sequence comparison of the ITS region as the proposed fungal barcode marker allowed a rapid identification of the majority of isolated strains. For the genera *Aspergillus*, *Penicillium*, and *Fusarium*, the amplification of the genes coding for calmodulin, TEF, and β-tubulin was necessary to assign species names.

Nevertheless, the origin of the fungal strains can only be speculated so far. Basically, there are five ways by which the fungi may have entered the human body: (1) the fungus had already infected the tissue of the living human host, (2) the fungus was a colonizer of the mucosa of the living human host, (3) ungerminated airborne spores of the fungus were already settled in the lungs when the human was alive and germinated after death, (4) the fungus grew into the human body after death, or (5) fungal spores were transported by animals such as maggots or adult insects into the human body and germinated inside. *Candida* species that were most frequent in postmortem material as well as the genera *Geotrichum* and *Trichosporon* were probably already colonizers of the mucosa. The yeast cells of *Candida* or the rod-shaped mitotic spores (arthrospores) of *Geotrichum* and *Trichosporon* may have migrated from the mucosal surfaces into the blood and different tissues before the necropsy was conducted. This phenomenon of postmortem translocation known in bacteria increases with postmortem interval [39], which was not determined in the different cases of this study. The remaining strains isolated do not belong to the natural human microflora and were previously considered to be contaminants. However, contamination is unlikely in the present study (see below).

Fungal strains were most prevalent in lung samples with an average number of 0.52 strains per sample, followed by kidney samples with an average number of 0.50 per sample, liver samples with an average number of 0.36 strains per sample, and HB samples with an average number of 0.22 strains per sample. This is an expected result, because lungs and kidneys have a connection to the environment via the airways and genitourinary tract, whereas the liver and HB are more protected inside the body.

Figures S2 and S3 (Electronic Supplementary Material) show the distribution by group and stage of decomposition of the main isolated genera in each postmortem material. Considering only group I, the diversity distinctly increased from stage 1 to stage 2 (Table 4). Thus, only five species were isolated in total from lung samples from stage 1 cases, while ten species were detected in the same organ in samples from stage 2 cases suggesting that the isolated fungi entered the human body after death.

The genera *Candida* and *Penicillium* predominated in both decomposition stages, while the genera *Aspergillus*, *Mucor*, *Trichoderma*, and *Trichosporon* were prevalent in stage 2 and *Rhodotorula* in stage 1. There are only a few studies addressing the microflora of human cadavers [40–43], and most of them investigated cadavers prior to decomposition. Sidrim et al. [42], the only comparable study on decomposed cadavers, used morphological methods for identification and identified only to

the genus level. In addition, the fungi were isolated from inner and outer surfaces (hair, skin, and sterile swabs collected from the mucosa of the mouth, rectum, and vagina) that are very likely contaminated with spores from the environment, while our samples originated from inside the human body.

Nonetheless, possible environmental contamination is also an issue to be considered in the present study, especially for group II samples, which had been collected by routine techniques without specific measures against microbial contamination from the environment. In the case of environmental contamination from the autopsy room, sample containers, or autopsy instruments, one would have expected detection of the same fungi in different samples from the same case. However, this was not observed. On the contrary, in a considerable percentage of the cases, no fungi could be isolated at all. In other cases, fungi were detected only in one of the collected samples, or different fungi were detected in different samples from the same case, all of which speaks against environmental contamination. In addition to that, precautions against environmental contamination were taken in the prospectively analyzed cases (group I) by using either sterile or flamed instruments during sample collection. At least for this group of samples, environmental contamination can largely be excluded. Nevertheless, further studies on cadavers in the state of decomposition are needed to decide if the species detected in our research are characteristic for postmortem human samples and also for each decomposition stage.

Fungi could be isolated from 40 out of 68 postmortem samples of group I (58 %) and from 75 out of 184 samples (41 %) of group II. The average number of isolated strains per sample was 1.01 and 0.47 in groups I and II, respectively. These results indicate that storage at −20 °C may decrease the number of vital fungal strains in the postmortem samples. Hence, retrospective testing of stored samples for the presence of fungi may lead to considerable underestimation of fungal colonization of the corpse.

As can be seen in Table 1, the percentage of fungal strains detected in subpopulations regarding age, sex, and cause of death more or less reflects the percentage of the respective number of autopsied bodies in the respective subpopulations. This suggests that these parameters have no major influence on fungal colonization of corpses. However, it is remarkable that the 4.8 % of the bodies found in the open field account for almost 20 % of the detected fungal strains. Although the number of corpses in this subpopulation is too small to draw any definitive conclusions, this finding is not surprising considering that there is a distinctly higher proportion of viable fungal elements in the open field than in indoor environments.

Conclusions

Molecular identification using the methodology described in the present study allows reliable identification of the majority of isolates from postmortem material in various decomposition stages. The presence of fungi from the genera *Candida*, *Geotrichum*, and *Trichosporon* belonging to normal human microflora and *Aspergillus*, *Bjerkandera*, *Mucor*, *Penicillium*, and *Rodothorula* as ubiquitous fungi has to be expected in postmortem samples, especially in those with an advanced decomposition stage. This raises the question how far this colonization by fungi could change the concentration or the metabolic pattern of drugs or poisons present in a corpse. This topic will be dealt with in part II of this communication.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Studies on drug metabolism by fungi colonizing decomposing human cadavers. Part I: DNA sequence-based identification of fungi isolated from postmortem material

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Fig. S1. Distribution of the different *Candida species* in each postmortem material

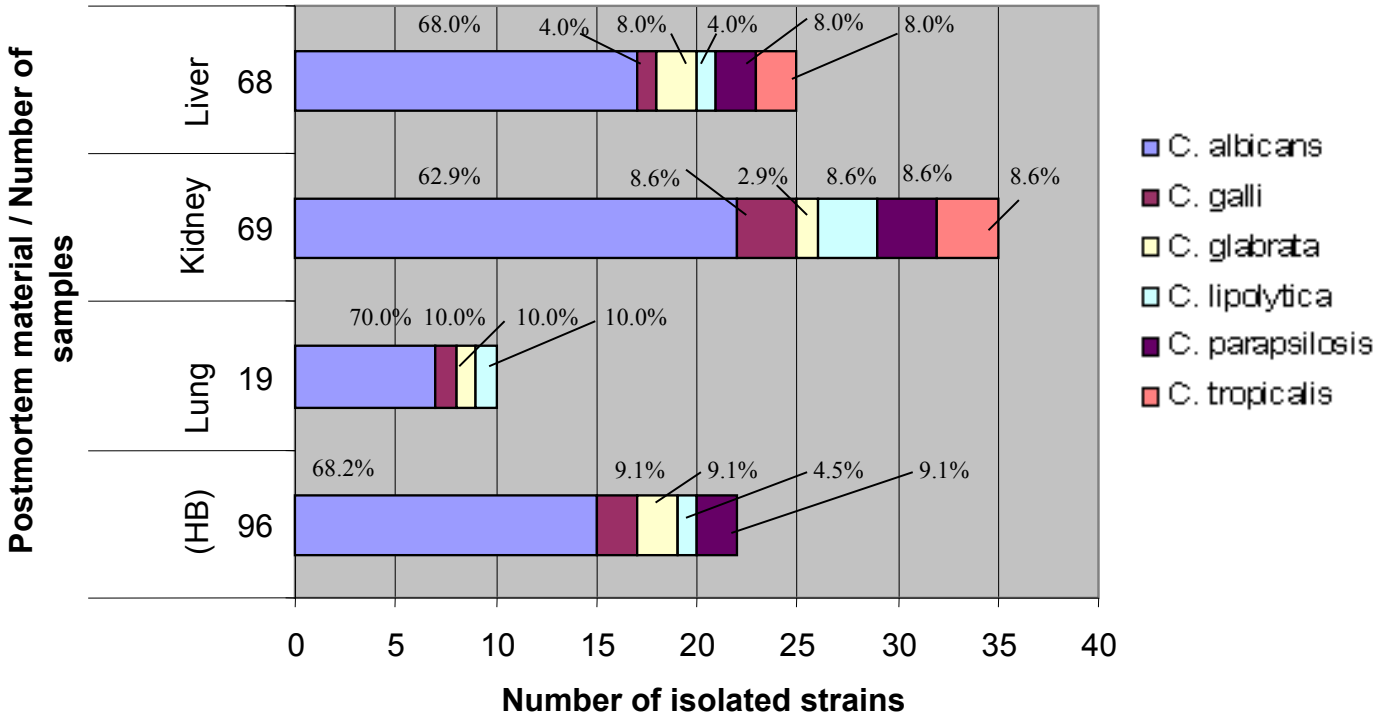

Fig. S2. Group 1: Distribution of the main isolated genera in each postmortem material

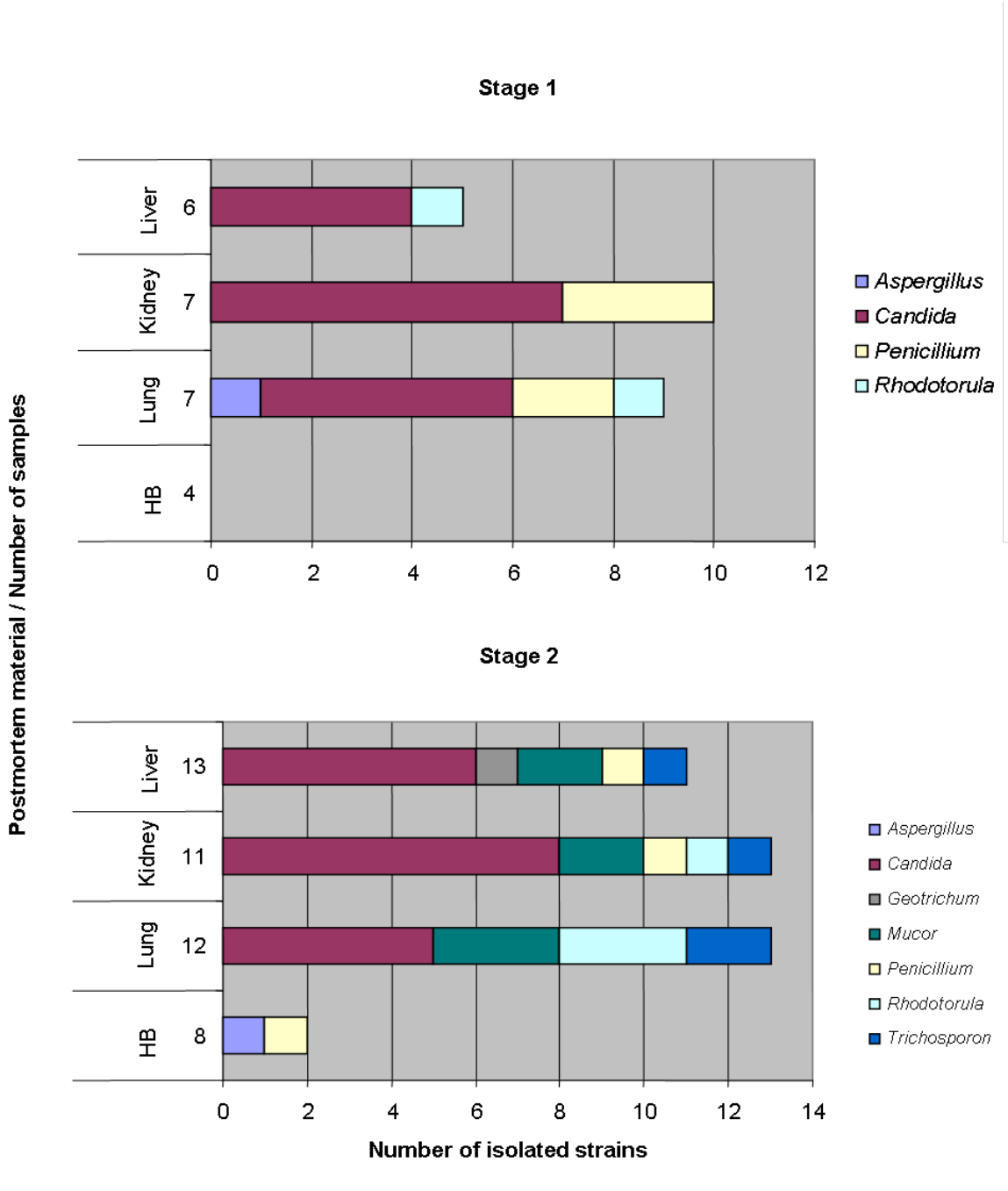

Fig. S3. Group 2: Distribution of the main isolated genera in each postmortem material

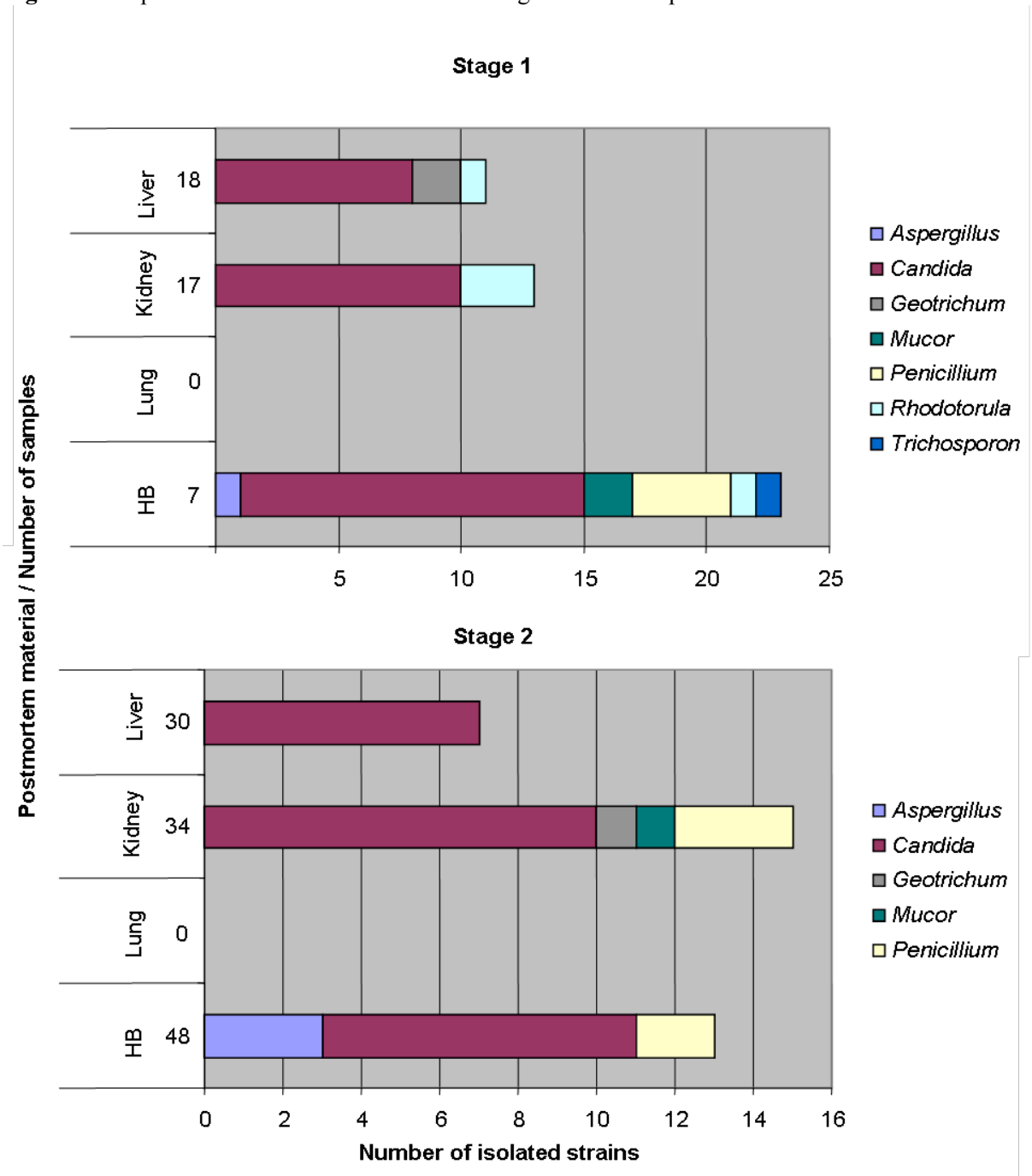

Table S1. Fungal strains isolated from postmortem material (HB, lung, liver and kidney) of decomposed corpses and their relationship with some diseases or surgical procedure in humans samples found in the literature

Genus	Species	Diseases or surgical procedure associated	Biological sample	Identification	Ref
Aspergillus	*awamori*[1] *fumigatus* *jensenii*[2]	invasive aspergillosis / marrow transplant, haematological malignancies	lung, nasal swab, sputum, spleen and kidney, endotracheal aspirate	PCR-IGS region PCR-ELISA morphological features /lactophenol cotton blue	[1-4]
Bjerkandera	*adusta*	Fungs associated chronic cough	sputum	morphological features. PCR-D1/D2 region	[5]
Candida	*albicans* *galli* *glabrata* *lipolytica* *parapsilosis* *tropicalis*	candida infections/ allogeneic blood and marrow transplant, bloodstream infections, nonneutropenic ill patients,	blood, serum, mouthwash, skin and mucosa	API 20C AUX system, BacT/Alert culture system, PCR-RAPD, PCR-ITS, biochemical test and micromorphological identification	[6-11]
Fusarium	*solani*	liver trasnplant	blood, liver	BacT/Alert culture system / morphological features	[12]
Geotrichum	*candidum*	acute leukemia	blood, skin	API 20C system, Yeast Tek system, PCR-D1and D2 domain	[13-14]
Mucor	*circinelloides* *hiemalis* *plumbeus* *racemosus*	diabetes, abdominal surgeries, insect bite, marrow transplant, aplastic anemia	maxillary tissue, blood, skin, liver	micromorphological identification, PCR-ITS/Region	[15-19]
Penicillium	*rubens*[3] *crustosum*	penicilliosis, immunocompromised patient, leukemia	blood, pericardial and Lu tissue	BacT/Alert culture system, micromorphological identification	[20]

Rhodotorula	*mucilaginosa*	fungemia, immunocompromised patient, meningitis	cerebrospinal fluid, blood	biochemical tests	[21-23]
Trichoderma	*harzianum longibrachiatum*	acute lymphoblastic leukemia, Lu infection, renal transplant, inginal abscess	blood, pus, sputum, throat samples	micromorphological identification PCR-ITS region	[24-26]
Trichosporon	*asahii*	neutropenic patients	blood	blood cultures	[27]

[1,2,3] previously classified *as Aspergillus niger*, *Aspergillus versicolor and Penicillium chrysogenum* respectively.

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6.2 Studies on drug metabolism by fungi colonizing decomposing human cadavers. part II: biotransformation of five model drugs by fungi isolated from post-mortem material [129].

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Studies on drug metabolism by fungi colonizing decomposing human cadavers. Part II: biotransformation of five model drugs by fungi isolated from post-mortem material†

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The present study investigated the *in vitro* metabolic capacity of 28 fungal strains isolated from post-mortem material towards five model drugs: amitriptyline, metoprolol, mirtazapine, promethazine, and zolpidem. Each fungal strain was incubated at 25 °C for up to 120 h with each of the five models drugs. *Cunninghamella elegans* was used as positive control. Aliquots of the incubation mixture were centrifuged and 50 μL of the supernatants were diluted and directly analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with product ion scanning. The remaining mixture was analyzed by full scan gas chromatography-mass spectrometry (GC-MS) after liquid-liquid extraction and acetylation. The metabolic activity was evaluated through the total number of detected metabolites (NDM) produced in each model and fungal strains and the percentage of parent drug remaining (%RPD) after up to five days of incubation. All the tested fungal strains were capable of forming mammalian phase I metabolites. Fungi from the normal fungal flora of the human body such as *Candida* sp., *Geotrichum candidum,* and *Trichosporon asahii*) formed up to seven metabolites at %RPD values greater than 52% but no new fungal metabolites (NFM). In contrast, some airborne fungal strains like *Bjerkandera adusta, Chaetomium sp, Coriolopsis sp., Fusarium solani* and *Mucor plumbeus* showed NDM values exceeding those of the positive control, complete metabolism of the parent drug in some models and formation of NFM. NFM (numbers in brackets) were detected in four of the five model drugs: amitriptyline (18), metoprolol (4), mirtazapine (8), and zolpidem (2). The latter NFM are potential candidates for marker substances indicating post-mortem fungal metabolism. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: fungi; drug metabolism; post-mortem material; LC-ESI-MS/MS; GC-MS

Introduction

The post-mortem decomposition process begins almost immediately after death.[1,2] It is partly mediated by microorganisms that are part of the normal human microflora[3–6] or are present at the site of the corpse.[7–9] During the autolysis and putrefaction process, these microbes can invade tissue and body fluids and their enzymes may contribute to metabolism/degradation of xenobiotics present in such post-mortem material.[10] Moriya *et al*. suggested the conversion of morphine glucuronides to free morphine due to the presence of bacterial enzymes.[11] Another study showed how intestinal bacteria decrease the concentration of some nitrobenzodiazepines by reduction to the respective 7-amino metabolites.[12] Recently, Butzbach *et al*. reported complete degradation of risperidone and its main metabolite 9-hydroxyrisperidone in post-mortem blood caused by reductive cleavage of the compounds' isoxazol moieties catalyzed by bacteria present in the samples.[13] However, to date there are no systematic studies dealing with the biotransformation of xenobiotics by fungi colonizing cadavers.

Fungi are eukaryotes and as such share similarities with mammalian cells and therefore with mammalian metabolism. In fact, some of them have been used for the simulation of the mammalian metabolism because of their capability to catalyze metabolic reactions by various enzymes including intracellular cytochrome P450 and extracellullar lignin peroxidase, manganese peroxidase and laccase.[14–16] As demonstrated in Part I of this communication[17] and by other authors, the most common fungi colonizing cadavers belong to the genera *Candida*, *Aspergillus*, *Penicillium*, *Mucor*, *Geotrichum, Fusarium, and Trichosporon*.[18–21] *In vitro* studies with some of these fungi were dedicated to

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bioremediation of soils, synthesis of natural products, and synthesis of drug metabolites of different substrates and showed that fungi may catalyze typical mammalian phase I as well as some phase II metabolic reactions.[22–28] These types of reactions might also occur in cadavers colonized by such fungi. Failure to recognize such changes and their potential relevance in a particular case could lead to false interpretation of drug concentrations and parent drug to metabolite ratios in post-mortem material.

In Part I of this communication, the isolation and identification of 156 fungal strains from authentic post-mortem material in the stage of decomposition was described.[17] The aim of this second part was to evaluate the capability of these strains to metabolize drugs *in vitro*. The experiments were performed employing our previously described approach for studying *in vitro* fungal metabolism using incubation experiments with five model drugs followed by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based analysis and interpretation of mass spectral data.[29]

Materials and methods

Chemicals and reagents

Hydrochlorides of amitriptyline (AT), promethazine (PMZ), and the internal standard cyproheptadine (IS), tartrate of metoprolol (MET), free bases of mirtazapine (MRT), and zolpidem (ZOL) were purchased from Merck (Darmstadt, Germany). Acetonitrile (LC-MS grade), dimethyl sulfoxide (DMSO), methanol, ethyl acetate, acetic acid, hydrochloric acid, iso-propanol, dichloromethane, acetic anhydride, and pyridine were obtained from Merck (Darmstadt, Germany). Glucose, protease-peptone, yeast extract, and agar were obtained from Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and magnesium sulfate were obtained from neoLab (Heidelberg, Germany). All chemicals were analytical grade or higher.

Solutions

Working solutions (1 mM, 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*). A working solution of the IS (20 mg/L) was prepared in methanol. All solutions were stored at 4 °C for up to one month.

Fungal strains

The strain CBS 167.53 of *Cunninghamella elegans* (*C. elegans*) was provided by the CBS-KNAW Fungal Biodiversity Centre of Utrecht, Netherlands. Strains of *Aspergillus awamori, Aspergillus fumigatus, Aspergillus jensenii, Aspergillus tubingensis, Bjerkandera adusta, Botrytis cinerea, Candida albicans, Candida galli, Candida glabrata, Candida lipolytica, Candida parapsilosis, Candida tropicalis, Chaetomium* sp., *Circinella minor, Coriolopsis* sp., *Enterocarpus* sp., *Fusarium solani, Geotrichum candidum, Mucor circinelloides, Mucor hiemalis, Mucor plumbeus, Mucor racemosus, Penicillium rubens, Penicillium crustosum, Rhodotorula mucilaginosa, Trichoderma harzianum, Trichoderma sp. (section: longibrachiatum),* and *Trichosporon asahii* have previously been isolated from post-mortem material in decomposition stage and identified as described in part I of this communication.[17] All the fungal strains were grown on Sabouraud agar plates (glucose 1%) at 25 °C and transferred to fresh plates every month. Fresh cultures were used for all metabolism studies.

Biotransformation procedures

Two loops (0.2 mm diameter) of material of each strain were pre-incubated in triplicates in 10 mL Sabouraud liquid medium (SM pH 5.0) containing 1% glucose at 25 °C and a shaking velocity of 90 rpm. Filamentous fungi were incubated for three days while yeasts such as *Candida*, *Geotrichum, Rhodotorula and Trichosporon* were grown for 12 h. After incubation, 1 mL of AT, MET, MRT, PMZ, or ZOL solution (1 mM) was added to each flask and incubation was continued for another five days (three days for yeasts). For *Candida albicans*, additional incubations were performed in SM buffered to pH 7 and 9 with dipotassium hydrogen phosphate (0.10 M) / NaOH (1.0 M) solution and a daily glucose supplementation of 0.2% in order to extend the length of the growth phase. Additionally, three kinds of controls were conducted simultaneously in all biotransformation experiments. A positive control with the fungus *C. elegans* and the model drugs, a blank incubation control containing the different fungal strains but none of the model drugs to exclude interference from the fungal matrix, and a negative control of each model drug with SM to exclude or account for non-metabolic degradation of the model drugs under the given incubation conditions. From all incubation mixtures, 800 µl samples were drawn every 24 h until 120 h (72 h for the above yeasts) and immediately centrifuged for 3 min at 9600 × *g*.

Sample preparation for LC-MS/MS and GC-MS-based analysis

The supernatants of the fungal incubations were worked up according to Martínez *et al.*[29] A portion of 50 µL plus 10 µL of IS solution was diluted with 940 µL of aqueous ammonium formate solution and 25 µL of this solution were analyzed by LC-MS/MS.

When new fungal metabolites (NFM) had been identified by LC-MS/MS, another portion (750 µL) of the supernatants was worked up for analysis by GC-MS as previously described by Maurer *et al.*[30] with modifications: 5 µL of IS solution was added, and the mixture was adjusted to pH 8–9. After adding 500 µL of ethyl acetate/*iso*-propanol/dichloromethane (60:20:20, *v/v/v*), the vials were capped, left on a rotary shaker and finally centrifuged. Thereafter, 500 µL of the upper organic phase were transferred to a clean autosampler vial and evaporated under a stream of nitrogen at 40 °C. The residue was acetylated with an acetic anhydride/pyridine mixture (3:1, *v/v*) under microwave irradiation (400 W). After evaporation of the excess derivatization reagent under a stream of nitrogen, the derivatized extract was dissolved in 50 µL of methanol and 2 µL were analyzed by GC-MS.

LC-MS/MS analysis

The LC-MS/MS system consisted of LC-20 AD HPLC system (Shimadzu, Jena, Germany) interfaced with a 4000 Q Trap® mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a TurboIonSpray ESI source operated in the positive mode. Nitrogen was used as curtain, source, and collision gas. The system was controlled by Analyst 1.5.1. software which was also used for data analysis. For the chromatographic separation, data acquisition and mass spectrometric evaluation was performed as described by Martínez *et al.*[29] with minor modifications. An Eclipse XDB C18 5 µm (4.1 × 150 mm) column was used with the

Table 1. Model drugs and metabolites detected by LC-MS/MS with their respective pseudomolecular ions (M+H) and retention time (RT) after a biotransformation period of three or five days with *Cunninghamella elegans* (*C. elegans*) and the 28 fungi strains previously isolated from post-mortem material. Metabolites in bold are novel fungi metabolites (NFM) and the main metabolite(s) formed in each model is/are marked with two positive signs (++)

Fig / Ref	model drugs / metabolites	M+H	RT, min	Fungi+														
				C.elegans	1	2	3	4	5*	6	7	8	9	10	11	12	13	14
5a	Amitriptyline (AT)	278	8.32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5b	**Amitriptyline-M (di-HO-)**	**310**	4.01	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
5c	**Amitriptyline-M (dehydro) (isomer 1)**	**276**	4.76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5d	**Amitriptyline-M (dehydro) (isomer 2)**	**276**	5.51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (nor-HO-) (isomer 1)	280	5.83	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
[29]	Amitriptyline-M (HO-) (isomer 1)	294	5.92	++	-	++	+	+	++	-	-	-	+	+	-	-	++	+
[29]	Amitriptyline-M (nor-HO-) (isomer 2)	280	6.10	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
5e	**Amitriptyline-M (HO-, *N-oxide*) (isomer 2)**	**310**	6.02	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 2)	294	6.27	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
[29]	Amitriptyline-M (nor-HO-) (isomer 3)	280	6.49	+	-	-		-	+	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 3)	294	6.58	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+
[29]	Amitriptyline-M (nor-HO-) (isomer 4)	280	6.67	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 4)	294	6.81	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
5f	**Amitriptyline-M (HO-) (isomer 5)**	**294**	6.76	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
5 g	**Amitriptyline-M (HO-) (isomer 6)**	**294**	7.88	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (nor-)	264	8.18	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+
[29]	Amitriptyline-M (*N-oxide*)	294	8.53	+	++	++	++	++	+	++	++	++	++	++	++	++	++	++
6a	Metoprolol (MET)	**268**	5.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[29]	Metoprolol -M (HO-) (isomer 1)	284	3.23	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+
6b	**Metoprolol-M (O-demethyl, *N*-acetyl)**	**296**	4.35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6c	**Metoprolol -M (HO-) (isomer 2)**	**284**	4.61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Metoprolol-M (O-demethyl-)	254	3.38	+	-	++	+	++	++	++	-	-	-	+	-	-	++	++
[29]	Metoprolol-M (Carboxy)	268	3.35	++	-	-	+	-	+	+	-	-	-	-	-	-	+	+
6d	**Metoprolol-M (*N*-dealkyl, *N*-acetyl)**	**268**	5.73	-	++	-	++	-	-	-	-	-	-	-	-	-	++	-
6e	**Metoprolol-M (N-acetyl)**	**310**	6.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7a	Mirtazapine (MRT)	266	5.31	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
7b	**Mirtazapine-M (HO-)**	**282**	3.65	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (nor-HO-) (isomer 1)	268	4.03	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
7c	**Mirtazapine-M (nor-, oxo-)**	**266**	4.11	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (HO-) (isomer 2)	282	4.27	+	-	-	+	-	-	+	-	-	-	-	-	-	+	+
7d	**Mirtazapine-M (nor-HO-) (isomer 1)**	**268**	4.40	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (nor-)	252	5.08	+	-	-	++	+	+	++	-	-	-	-	-	-	+	++
[29]	Mirtazapine-M (HO-) (isomer 3)	282	5.47	++	++	++	++	++	-	+	-	-	++	-	-	-	++	-
7e	**Mirtazapine-M (nor-HO-) (isomer 2)**	**268**	5.77	+	-	-	-	-	+	-	-	-	-	-	-	-		-
[29]	Mirtazapine-M (nor-HO-) (isomer 4)	268	5.92	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Promethazine (PMZ)	**285**	7.61	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
[29]	Prometazine-M (sulfoxide-HO-)	317	4.35	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(Continues)

Table 1. (Continued)

Fig / Ref	model drugs / metabolites	M+H	RT, min	Fungi+														
				C.elegans	1	2	3	4	5*	6	7	8	9	10	11	12	13	14
[29]	Prometazine-M (nor-sulfoxide)	287	5.12	+	+	+	+	-	+	+	-	-	+	+	+	-	+	+
[29]	Promethazine-M (sulfoxide)	301	5.19	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
[29]	Promethazine-M (sulfoxide) (*N-oxide*)	317	5.26	+	-	+	-	-	++	+	-	-	-	-	-	-	-	
[29]	Promethazine-M (nor-HO-)	287	5.87	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Promethazine-M (HO-)	301	6.02	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Prometazine-M (HO-) (*N-oxide*)	317	6.35	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Promethazine-M (nor-)	271	7.46	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+
[29]	Promethazine-M (*N-oxide*)	301	8.01	+	+	+	+	+	-	+	+	-	-	-	+	-	+	-
8a	Zolpidem (ZOL)	308	6.01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[29]	Zolpidem-M (4′ -HO-) isomer 1	324	4.04	++	-	++	++	-	+	++	+	-	++	-	-	+	++	++
8b	**Zolpidem-M (HO-) isomer 3**	**324**	4.33	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
[29]	Zolpidem-M (4′-HOOC-)	338	4.15	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+
[29]	Zolpidem-M (6-HO-) isomer 2	324	5.14	+	-	-	++	++	+	+	++	-	+	-	-	++	+	-
[29]	Zolpidem-M (6-HOOC-)	338	5.40	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
8c	**Zolpidem-M (HO-) isomer 4**	**324**	5.68	-	-	++	++	-	+	+	-	-	-	-	-	-	-	+
[29]	Zolpidem-M (*N-oxide*)	324	6.27	+	++	-	-	+	++	-		-	+	-	-	-	+	+

Fig / Ref	model drugs / metabolites	M+H	RT, min	Fungi+														
				C.elegans	15*	16	17	18	19	20	21	22	23	24	25	26	27	28
5a	Amitriptyline (AT)	278	8.32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5b	**Amitriptyline-M (di-HO-)**	**310**	4.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5c	**Amitriptyline-M (dehydro) (isomer 1)**	**276**	4.76	-	-	-	-	-	+	-	++	+	-	-	-	-	-	-
5d	**Amitriptyline-M (dehydro) (isomer 2)**	**276**	5.51	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
[29]	Amitriptyline-M (nor-HO-) (isomer 1)	280	5.83	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 1)	294	5.92	++	+	++	+	+	++	+	++	+	-	+	-	+	++	-
[29]	Amitriptyline-M (nor-HO-) (isomer 2)	280	6.10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5e	**Amitriptyline-M (HO-, *N-oxide*) (isomer 2)**	**310**	6.02	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 2)	294	6.27	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-
[29]	Amitriptyline-M (nor-HO-) (isomer 3)	280	6.49	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 3)	294	6.58	+	+	+	-	+	+	-	+	+	-	-	-	+	+	-
[29]	Amitriptyline-M (nor-HO-) (isomer 4)	280	6.67	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (HO-) (isomer 4)	294	6.81	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-
5f	**Amitriptyline-M (OH-) (isomer 5)**	**294**	6.76	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
5 g	**Amitriptyline-M (HO-) (isomer 6)**	**294**	7.88	-	++	-	-	+	+	-	-	-	-	-	-	-	-	-
[29]	Amitriptyline-M (nor-)	264	8.18	+	+	+	++	+	++	+	++	++	+	+	+	+	+	+
[29]	Amitriptyline-M (*N-oxide*)	294	8.53	+	+	+	+	++	+	++	++	+	++	++	++	++	+	++
6a	Metoprolol (MET)	**268**	5.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[29]	Metoprolol -M (HO-) (isomer 1)	284	3.23	-	+	-	+	-	+	+	-	-	-	-	-	+	-	-

6b	**Metoprolol-M (O-demethyl, *N*-acetyl)**	**296**	4.35	-	+	-	-	-	-	-	-	-	-	-	-	-	-
6c	**Metoprolol -M (HO-) (isomer 2)**	**284**	4.61	-	-	-	+	-	-	-	-	-	-	-	-	-	-
[29]	Metoprolol-M (O-demethyl-)	254	3.38	+	+	++	+	++	++	++	++	++	++	++	++	+	-
[29]	Metoprolol-M (Carboxy)	268	3.35	++	-	-	++	-	+	-	-	+	-	-	+	++	-
6d	**Metoprolol-M (*N*-dealkyl, *N*-acetyl)**	**268**	5.73	-	+	-	-	-	-	+	-	-	+	++	-	-	-
6e	**Metoprolol-M (N-acetyl)**	**310**	6.31	-	++	-	-	-	-	-	-	-	-	-	-	-	-
7a	Mirtazapine (MRT)	266	5.31	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7b	**Mirtazapine-M (HO-)**	**282**	3.65	+	+	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (nor-HO-) (isomer 1)	268	4.03	+	-	-	-	-	-	+	-	-	-	-	-	-	-
7c	**Mirtazapine-M (nor-, oxo-)**	**266**	4.11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (HO-) (isomer 2)	282	4.27	+	-	+	-	-	++	+	+	+	-	+	-	-	-
7d	**Mirtazapine-M (nor-HO-) (isomer 1)**	**268**	4.40	+	-	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Mirtazapine-M (nor-)	252	5.08	+	-	-	++	-	+	+	+	+	+	+	++	-	-
[29]	Mirtazapine-M (HO-) (isomer 3)	282	5.47	++	++	++	+	++	++	++	++	++	++	++	-	++	++
7e	**Mirtazapine-M (nor-HO-) (isomer 2)**	**268**	5.77	+	-	-	-	-	-	-	+	-	-	-	-	-	-
[29]	Mirtazapine-M (nor-HO-) (isomer 4)	268	5.92	+	-	-	-	-	-	-	-	-	-	+	-	-	-
[29]	Promethazine (PMZ)	**285**	7.61	-	-	+	+	+	+	+	-	+	+	+	+	+	+
[29]	Prometazine-M (sulfoxide-HO-)	317	4.35	+	-	-	-	-	-	-	+	-	-	-	-	-	-
[29]	Prometazine-M (nor-sulfoxide)	287	5.12	+	+	+	-	+	-	+	+	+	+	+	-	-	+
[29]	Promethazine-M (sulfoxide)	301	5.19	++	++	++	++	++	++	++	++	++	++	++	++	++	++
[29]	Promethazine-M (sulfoxide) (*N-oxide*)	317	5.26	+	++	-	-	+	-	-	-	-	-	-	-	-	-
[29]	Promethazine-M (nor-HO-)	287	5.87	+	-	-	-	-	-	+	+	-	-	-	-	-	-
[29]	Promethazine-M (HO-)	301	6.02	+	-	-	-	-	-	+	+	-	-	-	-	-	-
[29]	Prometazine-M (HO-) (*N-oxide*)	317	6.35	+	-	-	-	-	-	-	+	-	-	-	-	-	-
[29]	Promethazine-M (nor-)	271	7.46	+	-	-	+	-	-	+	+	+	+	+	+	-	+
[29]	Promethazine-M (*N-oxide*)	301	8.01	+	-	-	+	+	-	+	+	+	+	+	+	-	+
8a	Zolpidem (ZOL)	308	6.01	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[29]	Zolpidem-M (4′ -HO-) isomer 1	324	4.04	++	+	++	+	-	+	-	+	++	+	+	-	+	-
8b	**Zolpidem-M (HO-) isomer 3**	**324**	4.33	-	++	-	-	-	-	-	-	-	-	-	-	-	-
[29]	Zolpidem-M (4′-HOOC-)	338	4.15	+	-	-	-	-	+	-	-	-	-	+	-	-	-
[29]	Zolpidem-M (6-HO-) isomer 2	324	5.14	+	+	-	+	-	-	-	+	+	+	+	+	++	-
[29]	Zolpidem-M (6-HOOC-)	338	5.40	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8c	**Zolpidem-M (HO-) isomer 4**	**324**	5.68	-	++	-	++	-	++	++	+	+	++	++	-	+	-
[29]	Zolpidem-M (*N-oxide*)	324	6.27	+	-	-	-	-	+	-	+	+	+	+	++	-	-

[+] **1**. *Aspergillus awamori*, **2**. *Aspergillus fumigatus*, **3**. *Aspergillus jenseni*, **4**. *Aspergillus tubingensis*, **5**. *Bjerkandera adusta*, **6**. *Botrytis cinerea*, **7**. *Candida albicans*, **8**. *Candida galli*, **9**. *Candida glabrata*, **10**. *Candida lipolytica*, **11**. *Candida parapsilosis*, **12**. *Candida tropicalis*, **13**. *Chaetomium sp.*, **14**. *Circinella minor* **15**. *Coriolopsis sp.* **16**. *Enterocarpus sp.*, **17**. *Fusarium solani*, **18**. *Geotrichum candidum*, **19**. *Mucor circinelloides*, **20**. *Mucor hiemalis*, **21**. *Mucor plumbeus*, **22**. *Mucor racemosus*, **23**. *Penicillium rubens*, **24**. *Penicillium crustosum*, **25**. *Rhodotorula mucilaginosa*, **26**. *Trichoderma harzianum*, **27**. *Trichoderma sp. (section: longibrachiatum)*, **28**. *Trichosporon asahii*.

* Formation of other novel metabolites (see supplemental material)

following linear gradient of the mobile phases A (aqueous ammonium formate, 50 mM, pH 3.0) and B (0.1 % formic acid in acetonitrile): 10 % B to 60 % B within 10 min. A 3 min equilibration to 10 % B was used between injections. The flow rate was 1.4 mL/min and the injection volume was 25 μL. Positive ionization was performed with the following interface settings: source temperature, 550 °C; ion spray voltage, 5500 V; ion source gas 1 and 2, 35; curtain gas, 20; collision gas, high; declustering potential, 50 V. Information dependent acquisition using the enhanced MS (EMS) mode for the survey scan and the enhanced product ion (EPI) scanning mode for the dependent scan. For EMS, the dynamic fill-time option was used, with a mass range of *m/z* 50–360 at a rate of 1000 u/s. For EPI, the collision energy (CE) was set at 40 V, the collision energy spread at 25 V, and the scan rate at 4000 amu/s. The mass range was the same as for the survey scan. Three EPI experiments were performed and a complete cycle lasted 1.605 s.

GC-MS analysis

The samples were performed on a Shimadzu (Jena, Germany) GC-2010 gas chromatograph combined with an AOC-20s autosampler, an AOC-20i auto-injector and a mass-selective detector GCMS-QP 2010 Plus. The conditions were as follows: splitless injection mode; column, VF-1 ms (Varian, Darmstadt, Germany), 15 m × 0.25 mm inner diameter, 0.25 μm film-thickness; injection port temperature, 270 °C; carrier gas, helium; flow rate, 1 mL/min; column temperature, programmed from 60 to 300 °C at 10 °C /min; initial time 3 min; final time, 5 min. The mass spectrometry conditions for the screening procedure were as follows: full-scan mode (*m/z* 50–550); electron ionization mode; ionization energy, 70 eV; ion source temperature, 200 °C; interface temperature, 280 °C. Data evaluation was performed by semi-automated data evaluation using AMDIS software with a Maurer/Pfleger/Weber (MPW) 2007 target library and deconvolution parameters according to Meyer *et al.*[31]

Evaluation of the metabolic activity

The metabolic activity of each isolated fungal strain in the five model drugs was evaluated after five days of incubation (three days for the yeasts) under the previously established conditions by LC-MS/MS through the following two parameters: Total number of detected metabolites (NDM) and the percentage of remaining parent drug (%RPD) in relation to a negative control of the respective model drugs incubated without fungi (100%) and analyzed in the same way as the incubation samples with the studied fungal strains.

Table 2. Metabolic activity expressed in NDM (total number of detected metabolites) and %RPD (percentage of parent drug remaining) after three or five days of incubation at 25 °C, of fungi isolated from post-mortem material in decomposition stage assayed on the model drugs: Amitriptyline (AT), metoprolol (MET), mirtazapine (MRT), promethazine (PMZ) and zolpidem (ZOL) by ESI-LC-MS/MS

No	*Fungi*	Model drugs									
		AT		MET		MRT		PMZ		ZOL	
		TNM	%RPD	TNM	%RPD	TNM	%RPD	TNM	%RPD	TNM	%RPD
1	***C. elegans***	**10**	**11**	**2**	**26**	**8**	**29**	**9**	**0**	**5**	**7**
2	*Aspergillus awamori*	4	82	1	84	1	85	4	78	1	97
3	*Aspergillus fumigatus*	5	62	1	76	1	64	4	4	2	94
4	*Aspergillus jensenii*	3	81	3	89	3	85	4	32	3	93
5	*Aspergillus tubingensis*	3	92	1	98	2	91	3	46	2	93
6	*Bjerkandera adusta*	20	14	3	72	7	0	3	0	5	76
7	*Botrytis cinerea*	2	80	2	81	3	82	5	0	5	56
8	*Candida albicans*	2	96	0	100	0	100	3	89	2	92
9	*Candida galli*	2	91	0	98	0	96	2	80	0	99
10	*Candida glabrata*	3	93	0	99	1	100	3	91	0	100
11	*Candida lipolytica*	4	95	1	97	0	98	2	84	1	96
12	*Candida parapsilosis*	4	87	0	99	0	99	4	90	0	99
13	*Candida tropicalis*	2	93	0	99	0	99	2	91	2	96
14	*Chaetomium* sp.	6	5	4	75	4	26	4	18	3	38
15	*Circinella minor*	5	69	3	25	2	87	3	70	4	81
16	*Coriolopsis* sp.	5	84	5	49	5	42	3	0	4	66
17	*Enterocarpus* sp.	4	89	1	91	2	90	2	88	1	98
18	*Fusarium solani*	5	69	4	57	2	69	3	9	3	92
19	*Geotrichum candidum*	7	82	1	95	1	97	4	52	0	94
20	*Mucor circinelloides*	9	76	3	92	3	83	1	9	4	82
21	*Mucor hiemalis*	4	96	3	86	4	92	6	72	1	96
22	*Mucor plumbeus*	8	38	3	82	4	60	8	0	4	87
23	*Mucor racemosus*	5	32	2	76	3	51	4	36	4	90
24	*Penicillium rubens*	2	89	2	73	2	94	4	71	4	76
25	*Penicillium crustosum*	3	43	2	89	4	56	4	21	5	85
26	*Rhodotorula mucilaginosa*	2	90	12	86	1	90	3	7	2	98
27	*Trichoderma harzianum*	5	83	3	42	1	91	1	61	3	66
28	*Trichoderma* sp. *(section: longibrachiatum)*	5	89	0	99	1	98	4	84	0	100

Results and discussions

Formation of metabolites

The incubation experiments and analytical methods were based on our previously published model procedure for studying phase I fungal metabolism.[29] For detection of potentially formed phase II metabolites, the mass range of the described LC-MS/MS method would have to be increased. The metabolites of the five model drugs detected in samples incubated with the fungal strains from post-mortem material are listed in Table 1. The formed metabolites could be assigned to the following metabolic reactions: *N*-dealkylation in AT, MET, MRT, and PMZ, followed by *N*-acetylation or *O*-demethylation of MET, *N*-oxidation of AT, PMZ, and ZOL, side chain hydroxylation of AT, MET, and ZOL,

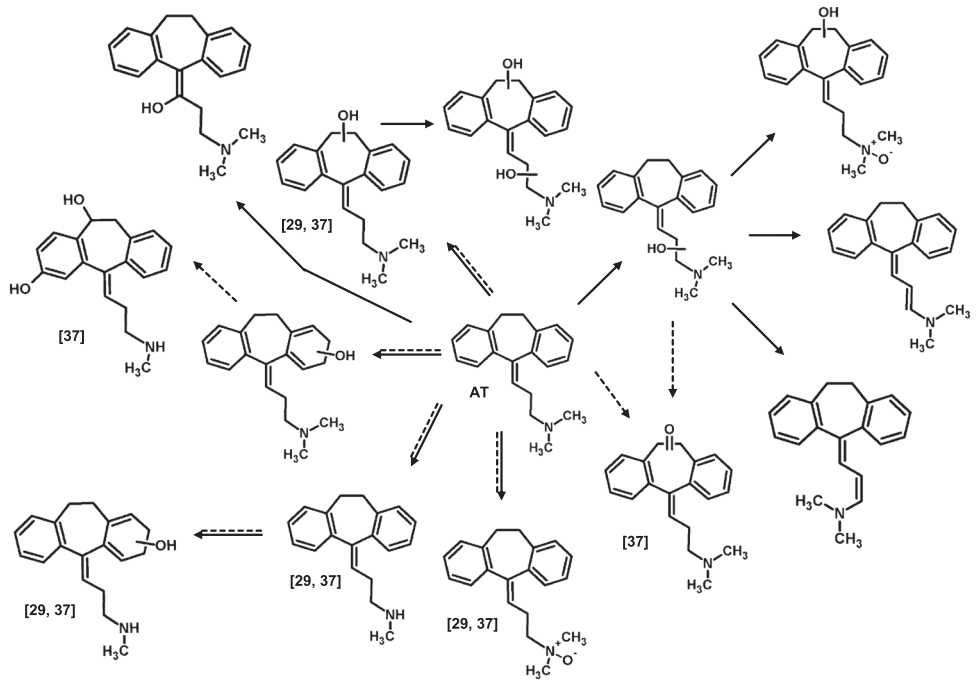

Figure 1. Proposed phase I biotransformation pathways of amitriptyline (AT) incubated with fungi isolated from post-mortem material in decomposition stage in this study (bold lines) compared to those identified in studies on mammalian metabolism (dotted lines).[29,37]

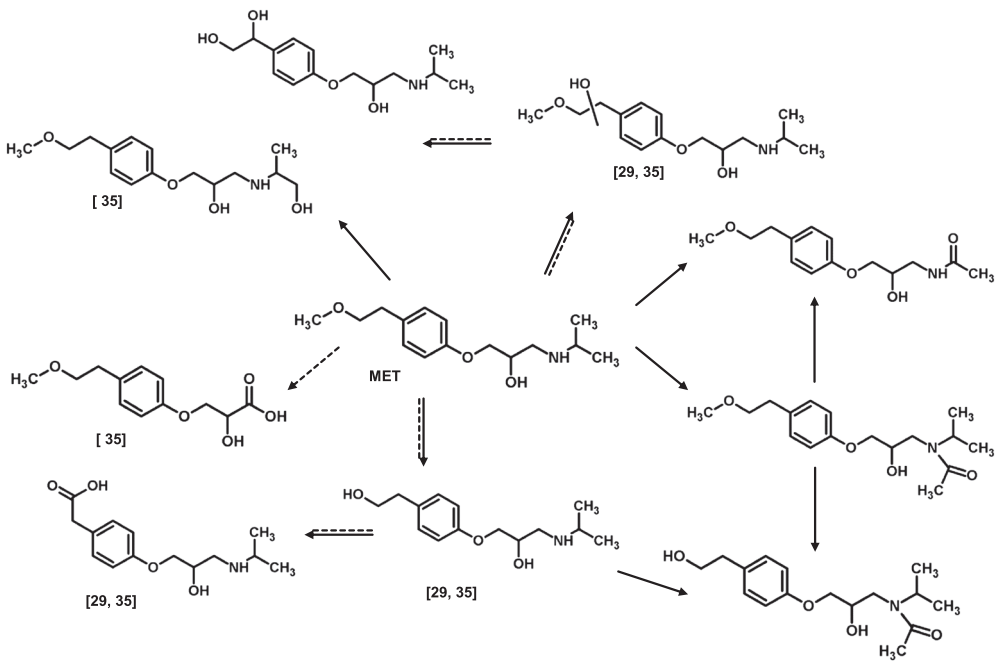

Figure 2. Proposed phase I biotransformation pathways of metoprolol (MET) incubated with fungi isolated from post-mortem material in decomposition stage in this study (bold lines) compared to those identified in studies on mammalian metabolism (dotted lines).[29,35]

followed by oxidation to the respective carboxylic acid in the latter two compounds, as well as sulfoxidation of PMZ, and multiple aromatic or aliphatic oxidations in AT and MRT.

The observed metabolic changes of the model drugs basically follow four patterns of biotransformation: (1) simulation of the mammalian phase I metabolism, i.e. formation of the same metabolites in roughly the same relative abundance (metabolic pattern); (2) simulation of mammalian phase I metabolism with respect to the formed metabolites but with a different metabolic pattern; (3) formation of mammalian metabolites and additional formation of NFM, i.e. metabolites not previously described in mammals; and (4) degradation of the parent drug greater than 95% after five days of incubation, forming metabolites in one of the three previous ways. Examples for patterns 1 and 2 are the

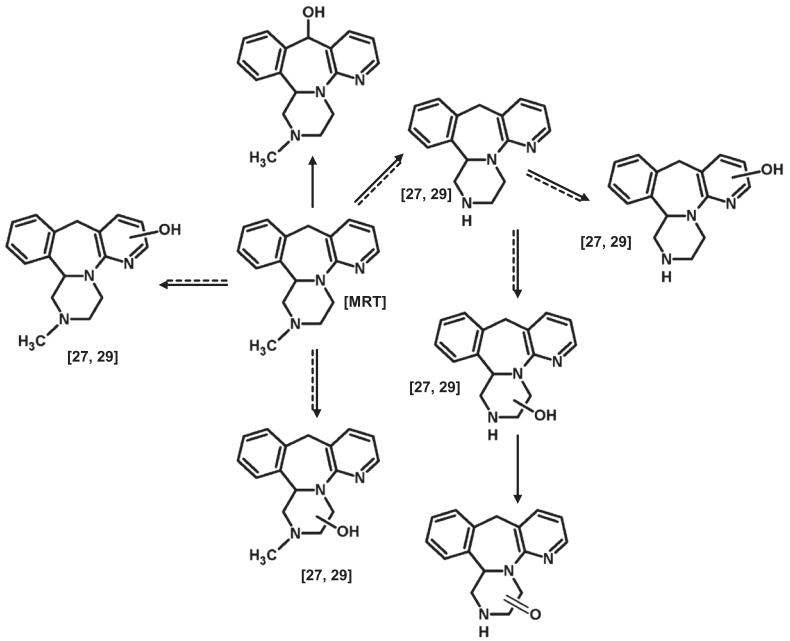

Figure 3. Proposed phase I biotransformation pathways of mirtazapine (MRT) incubated with fungi isolated from post-mortem material in decomposition stage in this study (bold lines) compared to those identified in studies on mammalian metabolism (dotted lines).[27,29]

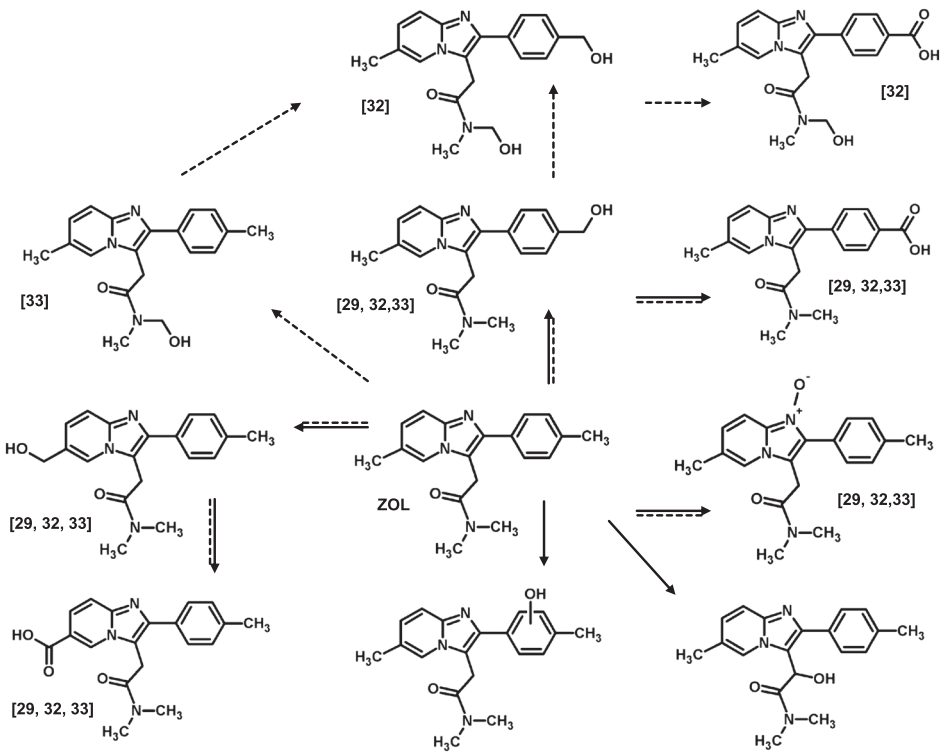

Figure 4. Proposed phase I biotransformation pathways of zolpidem (ZOL) incubated with fungi isolated from post-mortem material in decomposition stage in this study (bold lines) compared to those identified in studies on mammalian metabolism (dotted lines).[29,32,33]

biotransformation of AT and ZOL by the fungi *Circinella minor* and *Chaetomium* sp. respectively. In incubations with *Chaetomium* sp. 4-hydroxy ZOL was the main metabolite and not the isomeric 6-hydroxy ZOL reported to be the major metabolite in humans.[32,33] The simultaneous formation of NFM and mammalian metabolites (pattern 3) can be seen in incubations of *Mucor hiemalis* with MET where besides the mammalian metabolites hydroxy MET and *O*-demethyl MET, *N*-dealkyl-*N*-acetyl MET was formed as NFM as illustrated in Figure 6d. The latter was also a typical metabolite in incubations with all tested strains belonging to the genus *Penicillium* and with the species *Aspergillus awamori*, *Aspergillus jenseni*, *Chaetomium* sp., *Coriolopsis* sp., *Mucor hiemalis,* and *Trichoderma asahii*. As an example for pattern 4, the strains of *Chaetomium* sp., and *Bjerkandera adusta* had degraded AT and MRT by more than 95% after five days of incubation under the described conditions (Table 2). In the case of incubations of *Bjerkandera adusta* and *Coriolopsis* sp. with MRT predominatly NFM were formed (see below).

Metabolic activity

As mentioned above, the metabolic activity was evaluated by the NDM and the %RPD. The first parameter is directly related to the diversity of metabolic phase I reactions catalyzed by the particular fungal enzyme systems responsible for these reactions. A high NDM may reflect a high number of metabolic enzymes and/or

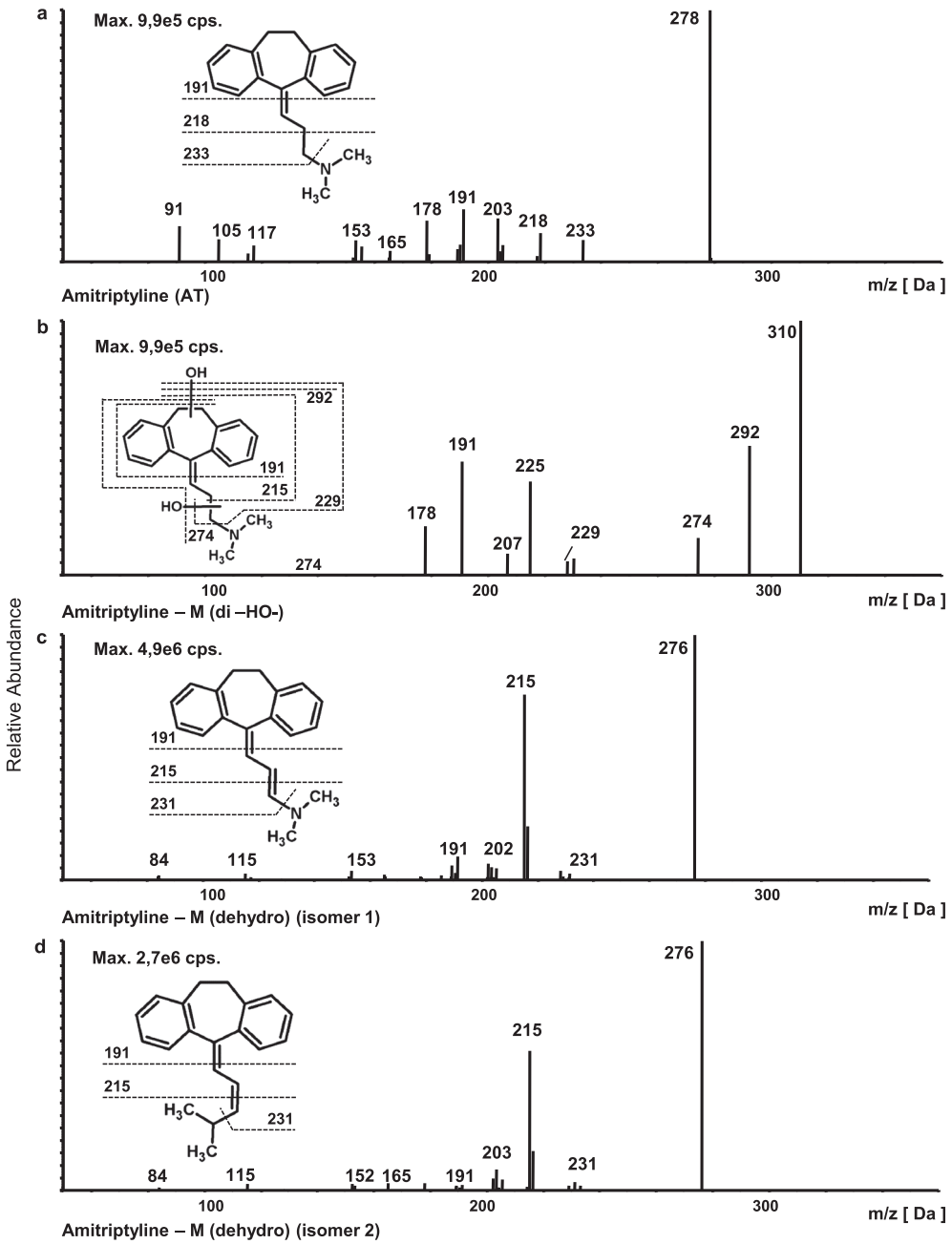

Figure 5. ESI-MS/MS enhanced product ion (EPI) mass spectra of amitriptyline (AT) metabolites, postulated structures, and predominant fragmentation patterns (a–g).

particular enzymes being capable of catalyzing various metabolic reactions. In contrast, the %RPD value represents the efficiency and rate of the catalyzed biotransformation reactions towards the model drugs with percentages close to 0 indicating rapid biotransformation of the model drug by the respective fungus. It is important to note that the formation of many different metabolites is not necessarily associated with high %RPD values and vice versa.

The metabolic activity of each one of the fungal strains tested with the five model drugs is shown in Table 2. Species of *Candida*, the most recurrent genus isolated from post-mortem samples in decomposition stage, formed a maximum of four metabolites in the model drugs AT and PMZ and showed an average %RPD value of ≥95% in all the model drugs. Incubation with *Candida albicans* under different pH conditions (pH 5, 7, and 9) did not result in obvious differences with respect to the number and quantity of formed metabolites. Some other fungi used in this study of which some are opportunistic human pathogens (Table 2), showed considerable metabolic activity with an NDM close to those of the positive control *C. elegans* and %RPD values of ≤40% after five days of incubation. Examples are *Bjerkandera adusta, Chaetomium* sp., *Mucor plumbeus* and *Mucor racemosus* in the model drug AT, *Circinella minor* in the model drug MET, *Aspergillus fumigatus, Rhodotorula mucilaginosa* in the model drug PMZ and *Chaetomium* sp. in the model drug ZOL. Although fungi such as *Fusarium solani* and *Chaetomium* sp. do not form many metabolites of some model drugs, their %RPD values ranged between 49 and 92% and 38 and 87%, respectively. This shows that their enzymes or enzymatic systems combine a high degree of metabolic selectivity with high metabolic capacity. The fungus *Bjerkandera adusta* was the fungus with the highest metabolic activity and capable of forming the most NFM. For the model drugs AT and MET, and MRT, the NDM and %RPD values, respectively, were even higher than in the positive control *C. elegans*. This finding is consistent with a previous reports that *Bjerkandera adusta* has a non-specific enzymatic machinery including lignin peroxidases, manganese peroxidase, and laccase enzymes capable of metabolizing a structurally diverse range of xenobiotic compounds.[16]

Identification of NFM

All metabolites considered in this work as NFM, to the best of our knowledge, have neither been reported in studies dealing with mammalian metabolism nor were they detected in any of the negative controls. For a total of 16 NFM (marked in bold in Table 1) metabolite structures could be postulated in four model drugs: six in AT, four in MET, four in MRT, and two in ZOL. Further NFM were produced from AT and MRT (12 and 4 metabolites, respectively) by *Bjerkandera adusta* and *Coroliopsis* sp. Their

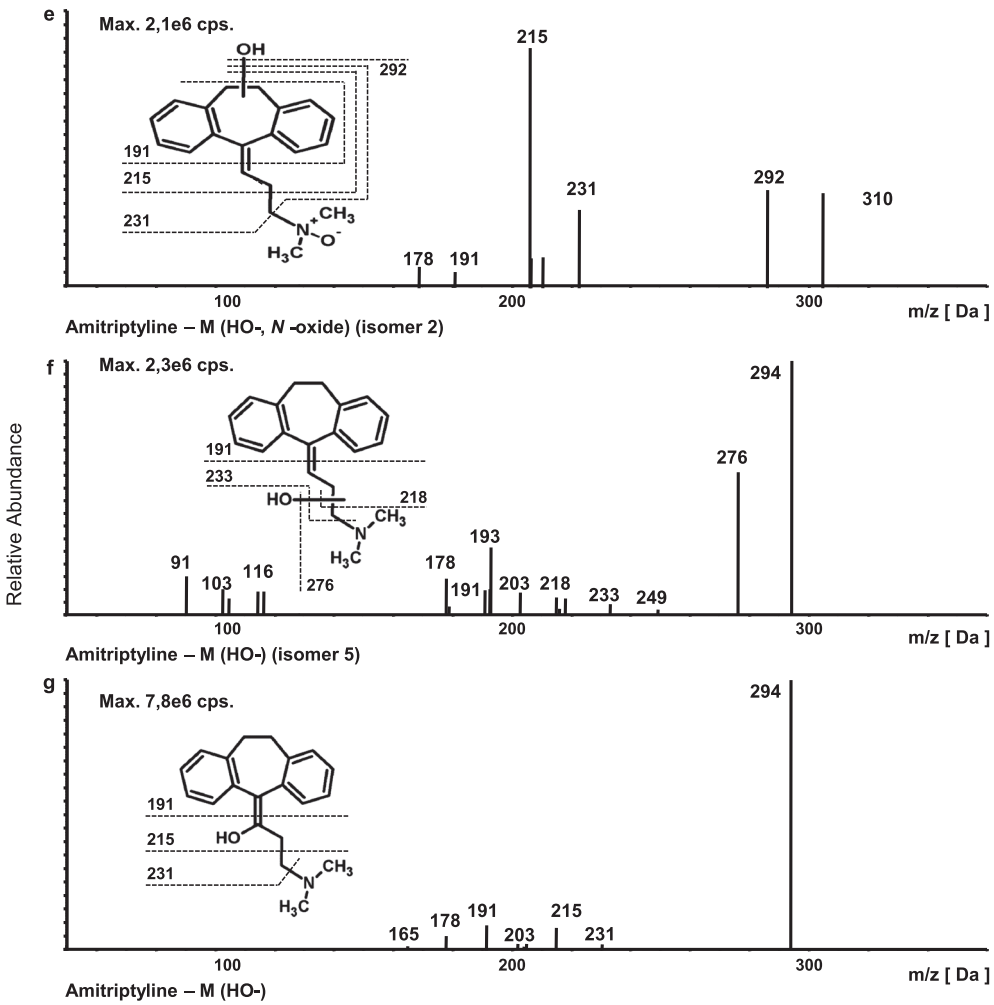

Figure 5. (Continued)

structures could not be fully elucidated with the methods employed in this study. EPI and EI mass spectra of these metabolites are shown in Figures S1–S3 of the Electronic Supplementary Material.

For the model drug PMZ, no NFM were observed, most probably due to the fast biotransformation to its sulfoxide metabolite which was observed in all incubations. The proposed

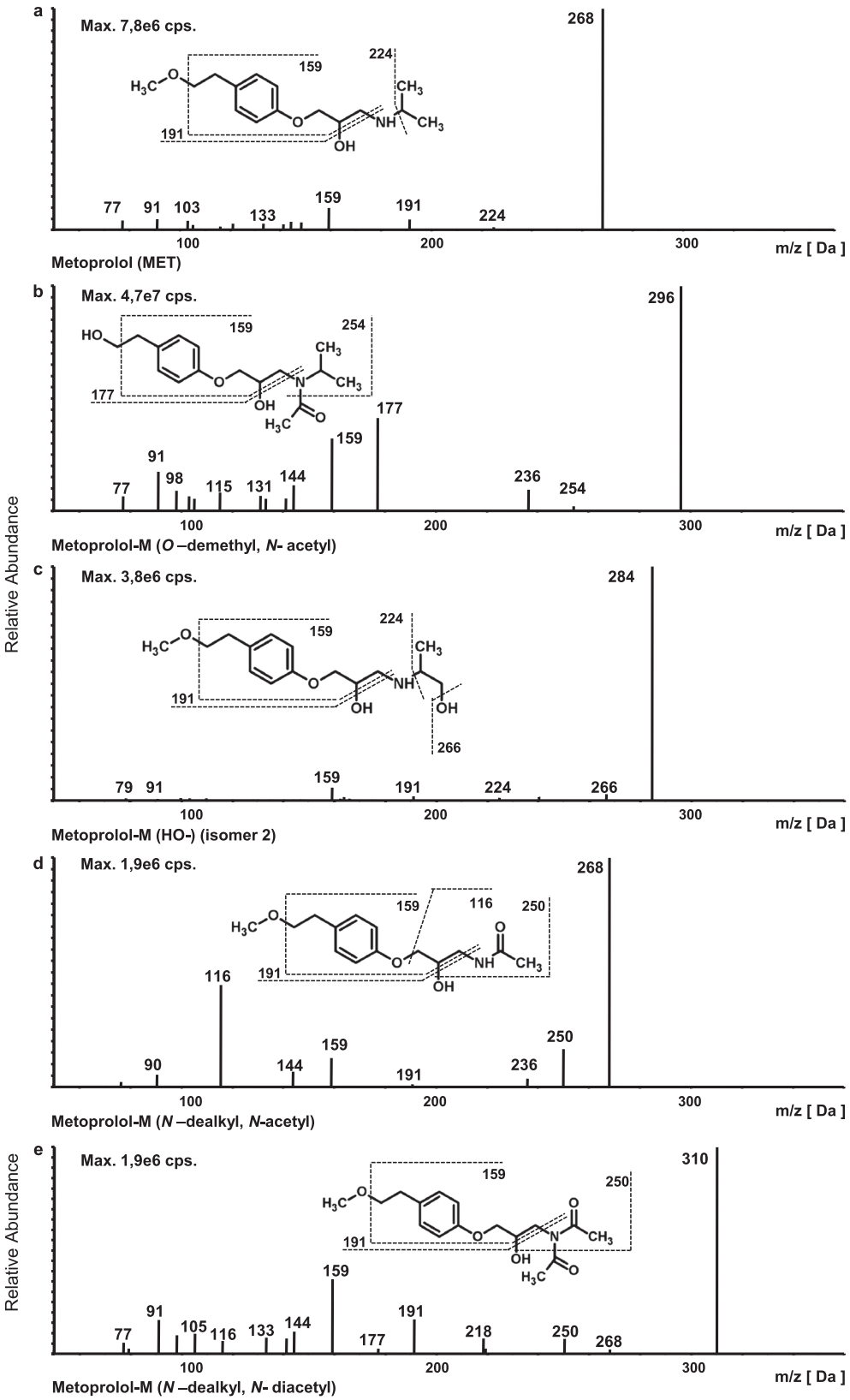

Figure 6. ESI-MS/MS enhanced product ion (EPI) mass spectra of metoprolol (MET) metabolites, postulated structures, and predominant fragmentation patterns (a–e).

biotransformation pathway of each model drug incubated with fungi isolated from post-mortem material (bold lines) compared to those identified in mammalian metabolism (dotted lines) are shown in Figures 1–4. Major NFM of AT (Figure 5a) follow the same fragmentation pattern as described by Martínez *et al*[29]: neutral loss of dimethylamine (Figures 5b, 5c, 5d, 5f, 5 g) or

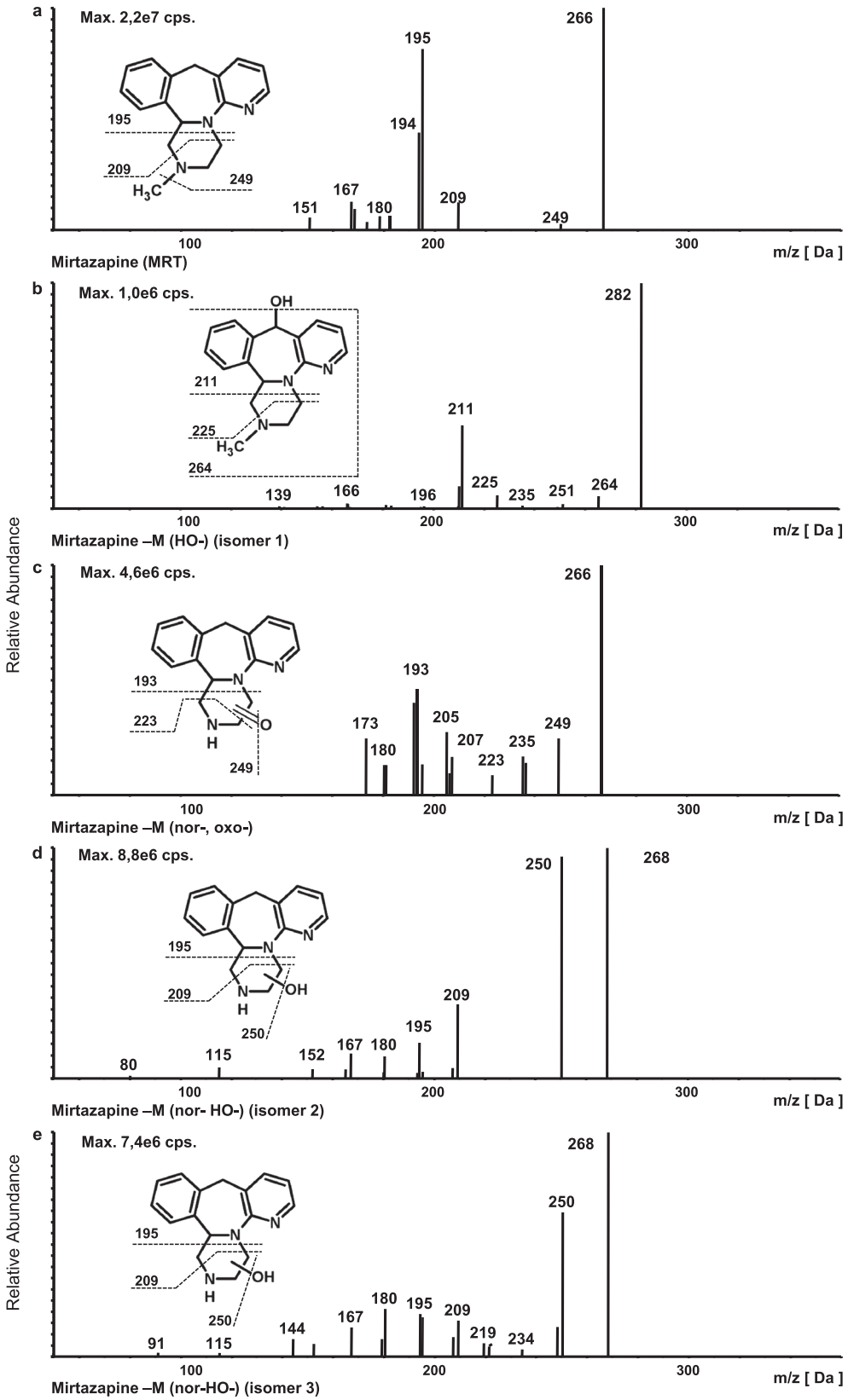

Figure 7. ESI-MS/MS enhanced product ion (EPI) mass spectra of mirtazapine (MRT) metabolites, postulated structures, and predominant fragmentation patterns (a–e).

dimethylhydroxylamine (Figure 5e), α-cleavage of the side chain, and loss of the side chain in all the metabolites. However, unlike in mammalian metabolism some of them have a hydroxy group attached to position 2 (Figures 5b and 5f) or 3 (Figure 5g) of the side chain. Some of them are further characterized by a neutral loss of water from hydroxy groups attached to the aliphatic carbon atoms of the ring system (Figure 5e), the side chain (Figure 5f) or a combination of both (Figure 5b). Possible positions for these aliphatic hydroxylations of AT are the positions 10 and 11 of the ring system (Figure 5b and 5e). An exact assignment of the hydroxylation position was not possible based on the EPI spectra. Regarding hydroxylation of the side-chain, only hydroxylation at positions 2 and 3 leads to stable hydroxy metabolites while hydroxylation in position 1 would finally lead to oxidative deamination. Looking at the fragmentation patterns of the postulated side-chain hydroxy metabolites, one can see prominent water losses in the EPI spectra shown in Figures 5b and 5f. This suggests that the hydroxy groups are located in side-chain position 2 where water loss will lead to formation of a conjugated system throughout the side-chain and ring systems. In contrast, no water loss can be seen in the EPI spectrum shown in Figure 5g. This is in line with hydroxylation in side-chain position 3 yielding an enol from which water loss is much less likely. The dehydro AT metabolites (Figures 5c and 5d) formed by three fungal strains of the genus *Mucor* formally correspond to a dehydrogenation of the side-chain yielding two cis-trans isomeric metabolites. The actual mechanism may, however, be dehydration of a hydroxy metabolite carrying a hydroxy group in position 2 of the side-chain. A similar reaction has previously been described in microbiological biotransformation of the diterpene partheniol by the fungus *Mucor circinelloides*.[34]

The pseudo-molecular ions of the four observed NFM in the EPI spectra of MET metabolites (Figure 6) correspond to *m/z* 296, 284, 268, and 310. These metabolites present fragments ions with *m/z* 254, 224, and 250 in the latter two, respectively. These can be assigned to a neutral loss of an acetyl moiety (Figure 6b), a hydroxypropyl moiety (Figure 6c), water (Figure 6d), and a simultaneous loss of water and an acetyl moiety (Figure 6e). A further fragmentation reaction corresponding to a neutral loss of water and isopropyl acetyl amide leads the formation of the fragment with *m/z* 177 (Figure 6b). Similar reactions with losses of hydroxy isopropylamine and water, acetamide and water, and isopropyl acetamide and water to explain the presence of the common fragment at *m/z* 191 in the EPI spectra shown in Figures 6c, 6d, and 6e, respectively. An additional loss of methanol or water from the methoxyethyl or hydroxyethyl side chain leads to a common fragment at *m/z* 159. These last two fragments were also observed in the fragmentation pattern of the parent drug (Figure 6a). The *N*-dealkyl metoprolol metabolite reported after incubation of metoprolol with the fungus *Cunninghamella*

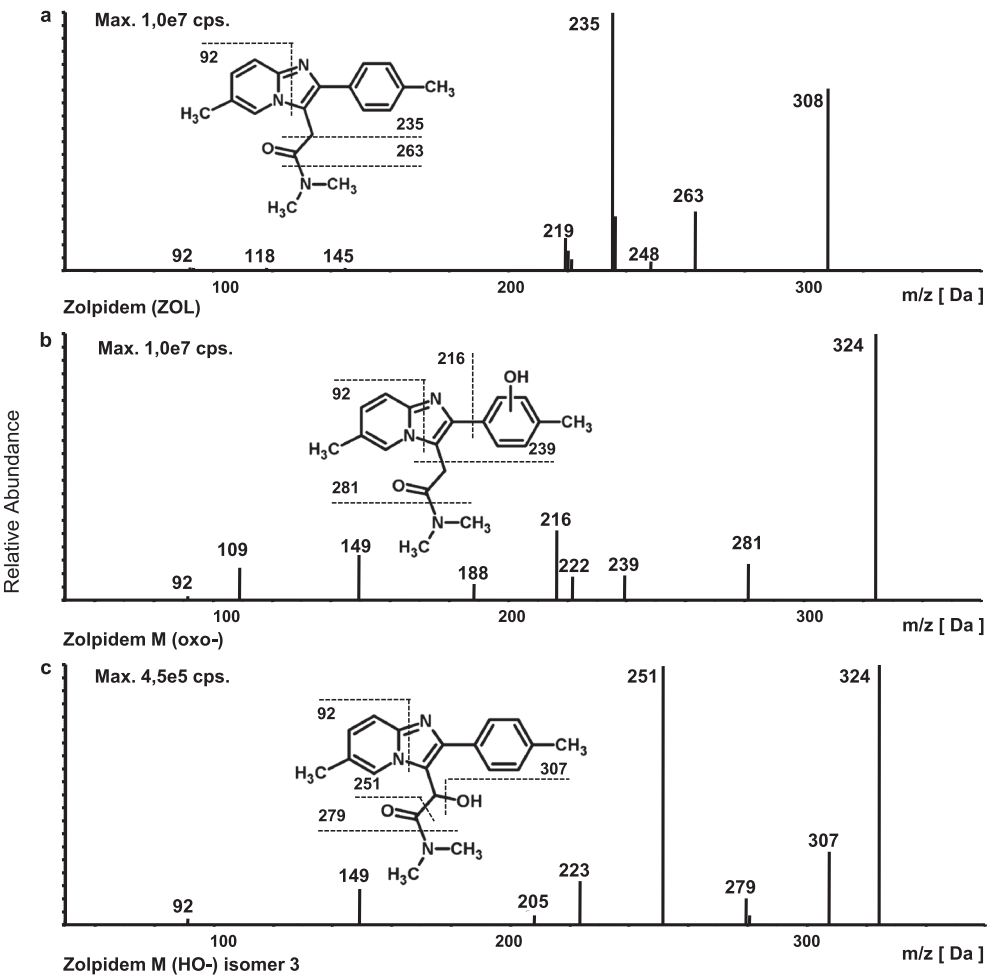

Figure 8. ESI-MS/MS enhanced product ion (EPI) mass spectra of zolpidem (ZOL) metabolites, postulated structures, and predominant fragmentation patterns (a–c).

blakesleeana[35] was not identified in any of the fungal strains studied in the present work. This suggests that the *N*-dealkylation step was either preceded or immediately followed by *N*-acetylation the latter seeming more likely. In any case, these results are in line with the findings of other authors who have previously reported the formation of *N*-acetyl fungal metabolites[22,33] without the detection of the respective *N*-dealkyl metabolites.[10,36]

MRT and its NFM show a cleavage through the piperazine moiety leaving fragments with *m/z* 195 and 209 (Figures 7a, 7d, and 7e) or *m/z* 211 and 225 (Figure 7b) representing the unchanged or hydroxylated tricyclic ring system, respectively. The hydroxy group in the latter metabolite (Figure 7b) is most likely attached to the aliphatic carbon atom of the methylene group between the two aromatic rings because the retention time of this metabolite differs from the hydroxyaryl metabolites previously reported by Martínez *et al*.[29] The metabolites with pseudomolecular ions *m/z* 268 and unchanged tricyclic ring systems (Figures 7d and 7e) can be interpreted as combinations of *N*-demethylation and hydroxylation at the piperazine moiety because they have a prominent neutral loss of water in their EPI spectra. Martínez *et al*.[29] previously reported a human metabolite with the same pseudomolecular ion and fragmentation pattern to the latter two, but with different retention time. This suggests that these isomeric metabolites are NFM. However the sites of ring hydroxylation cannot be further determined by the interpretation of mass spectra. The metabolite with the pseudomolecular ion at *m/z* 266 showed a cleavage through the piperazine moiety leaving fragments at *m/z* 193 and 223 (Figure 7c) compatible with *N*-demethylation and oxidation at the piperazine ring. However, unlike the *N*-demethyl-hydroxy MRT metabolite, it presented a shift of -2 in the pseudomolecular ion and all the fragments. A plausible mechanism for the formation of these metabolites would be hydroxylation at the α or β position of the piperazine ring followed by dehydrogenation to the respective oxo metabolite.

The predominant EPI fragmentation patterns of ZOL (Figure 8a) and its two observed NFM share the following major fragmentation reactions: cleavage of the heterocyclic ring system leading to a common methylpyridinium ion with *m/z* 92 from unchanged heterocycles, neutral loss of dimethylamine, and α-cleavage of the side chain (Figure 8). In the EPI spectrum shown in Figure 8b, a further neutral loss of *N,N*-dimethyl acetamide and cleavage of the tolyl group leading to fragments with *m/z* 239 and *m/z* 216, respectively, can also be observed. Additional characterization of the isomeric metabolite shown in Figure 8c leads to a fragment ion at *m/z* 307 and *m/z* 251 corresponding to a loss of the hydroxy group and α-cleavage next to the carbonyl group leading to a neutral loss of *N,N*-dimethyl formamide.

Conclusions and perspectives

All fungi isolated from post-mortem material in decomposition stage presented metabolic activity with respect to phase I reactions of the five studied model drugs. While this indicates that such fungi could principally contribute to post-mortem drug degradation and metabolite formation, the practical relevance of the presented *in vitro* findings remains unclear at this stage. Further studies are needed to assess the extent of post-mortem fungal metabolism and on whether or not it is a factor to be considered in the interpretation of post-mortem toxicology cases. Some of the metabolites formed by the fungal strains in the present study have never been described in mammalian metabolism. These NFM are potential candidates for marker substances, which can be used in future investigations on post-mortem fungal metabolism events.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Electronic Supplementary Material

Drug Testing and Analysis

Studies on drug metabolism by fungi colonizing decomposing human cadavers. Part II: biotransformation of five model drugs by fungi isolated from post-mortem material†

Jorge A. Martínez-Ramírez,a,b Grit Waltherc,d and Frank T. Peters*

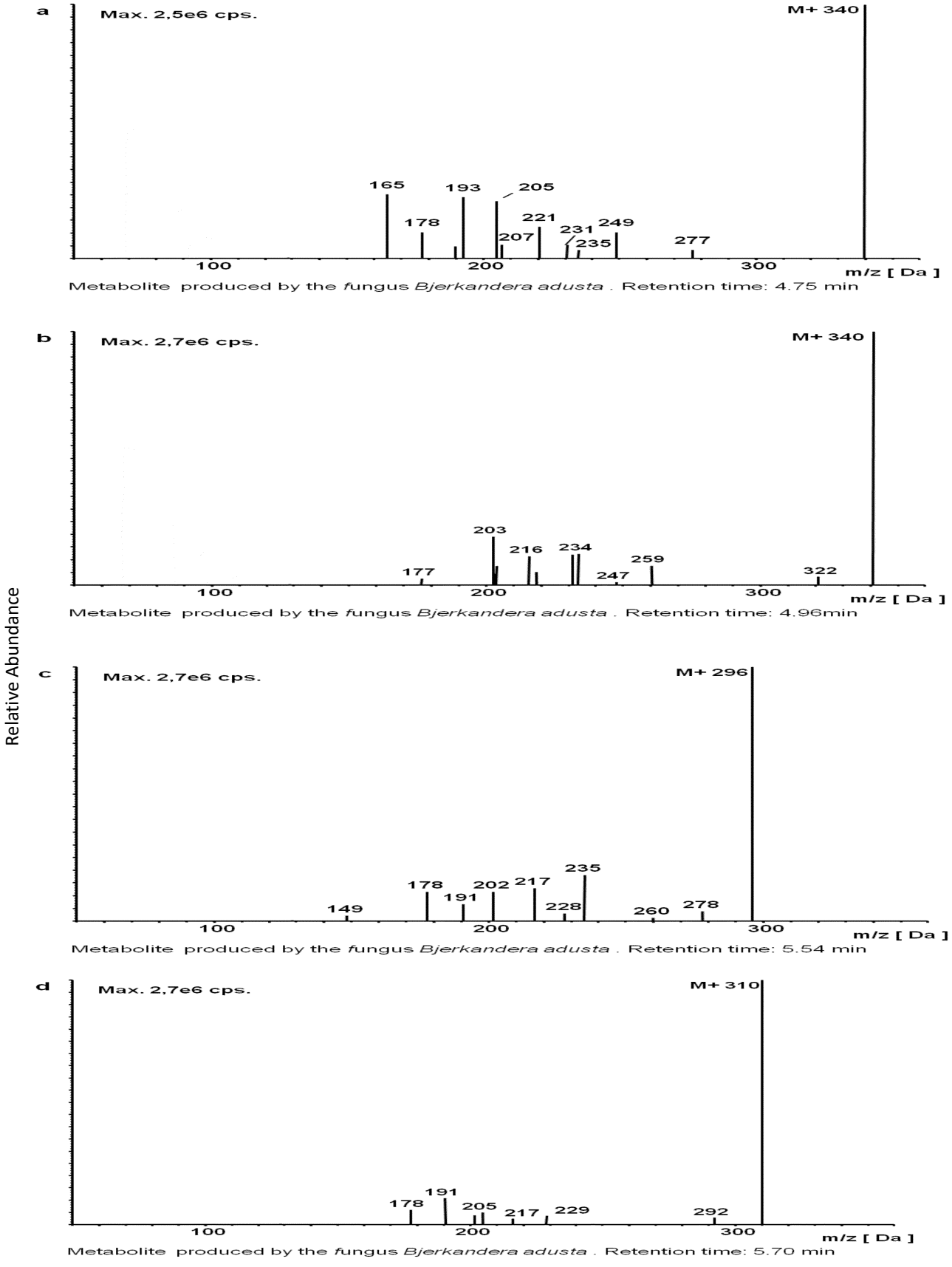


Fig SM1 ESI-MS/MS enhanced product ion (EPI) mass spectra of unidentified AT metabolites (a – l)

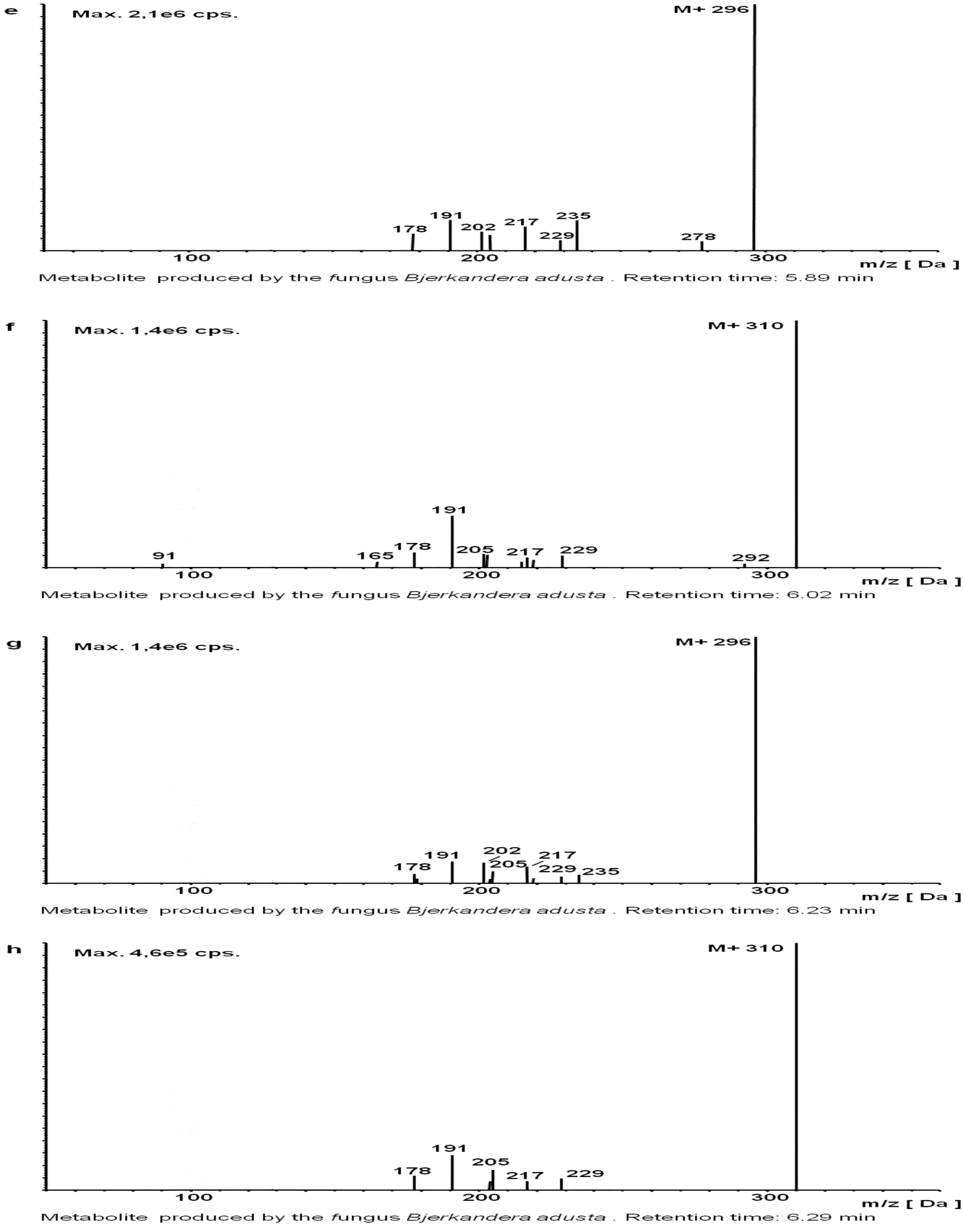


Fig SM1 ESI-MS/MS enhanced product ion (EPI) mass spectra of unidentified AT metabolites (a – l)

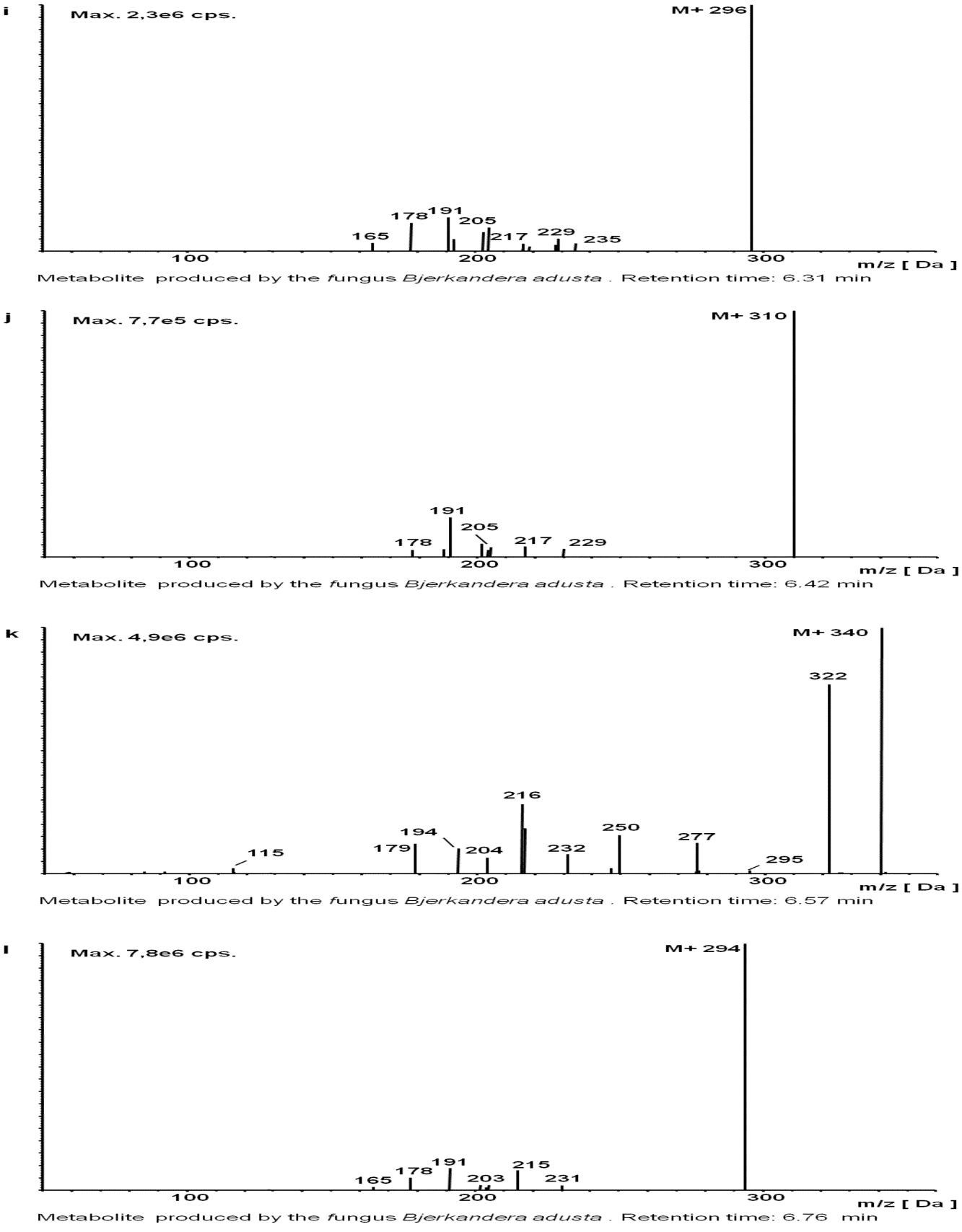
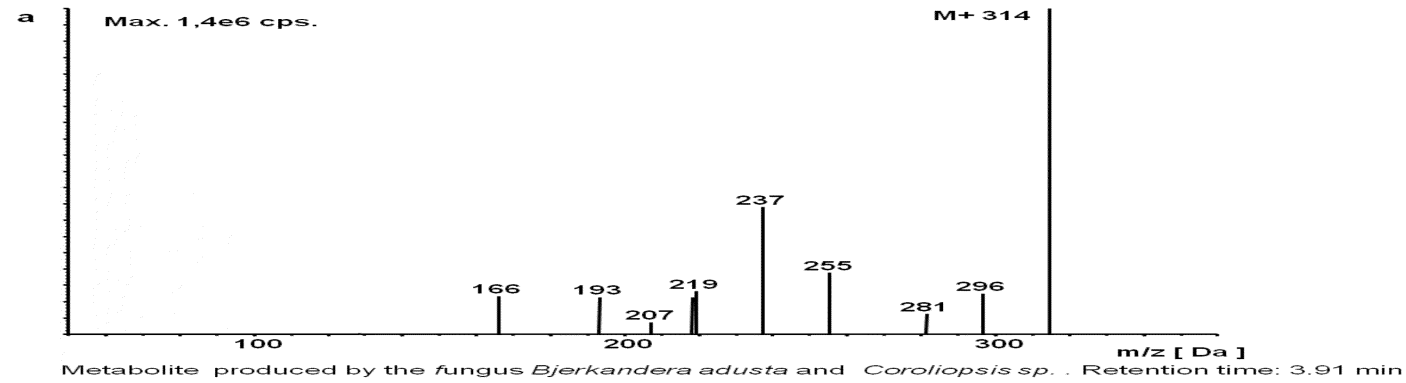
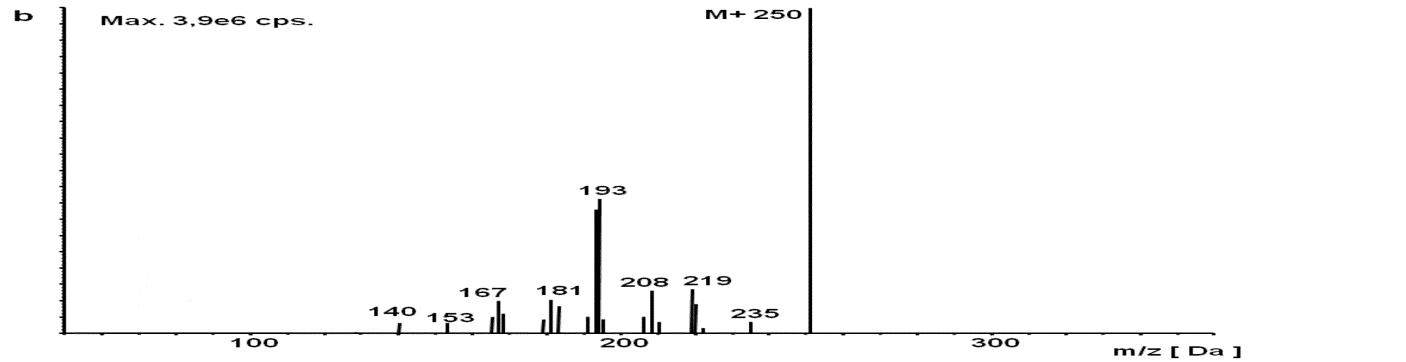


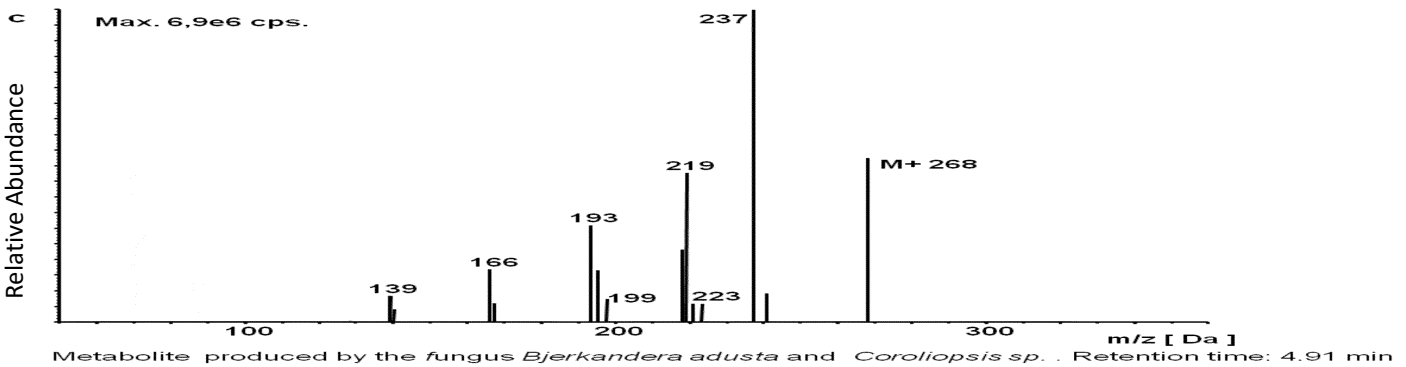
Fig SM1 ESI-MS/MS enhanced product ion (EPI) mass spectra of unidentified AT metabolites (a – l)



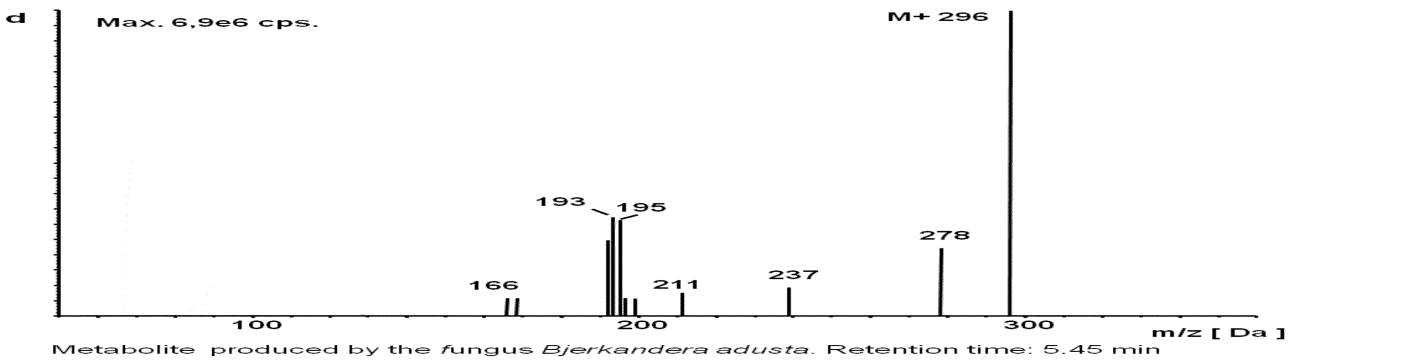
Metabolite produced by the fungus *Bjerkandera adusta* and *Coroliopsis sp.* . Retention time: 3.91 min



Metabolite produced by the fungus *Bjerkandera adusta* and *Coroliopsis sp.* . Retention time: 4.63 min



Metabolite produced by the fungus *Bjerkandera adusta* and *Coroliopsis sp.* . Retention time: 4.91 min



Metabolite produced by the fungus *Bjerkandera adusta*. Retention time: 5.45 min

Fig SM2 ESI-MS/MS enhanced product ion (EPI) mass spectra of unidentified MRT metabolites (a – d)

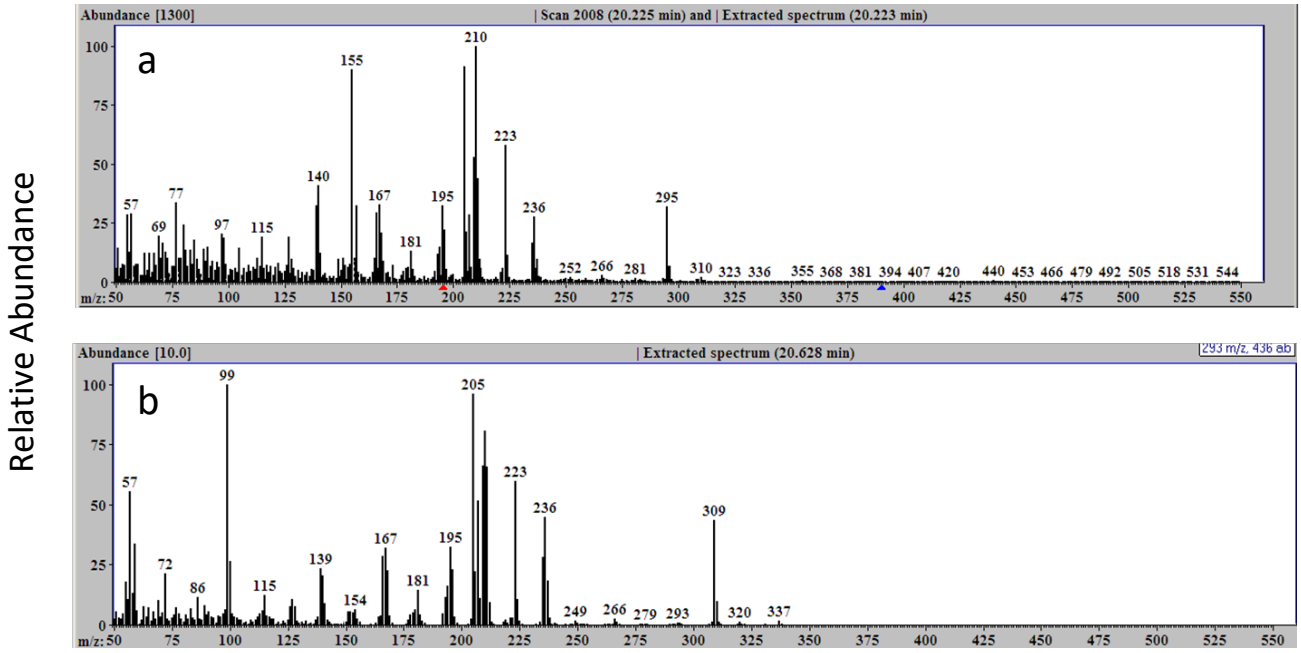


Fig SM3 EI mass spectra of unidentified MRT metabolites (a –b) formed by the fungus *Bjerkandera adusta*

6.3 Search for fungi-specific metabolites of four model drugs in postmortem blood as potential indicators of postmortem fungal metabolism [130].

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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

Table 1
Characteristics of autopsied corpses and postmortem blood samples ($n = 33$).

Characteristics	No. of corpses
Age, years	
<49	4
50–59	10
60–70	9
>70	10
Sex	
Men	17
Women	16
State of the corpses	
Moderate decomposition	16
Severe decomposition	17
Polytrauma	3
Burned	1
Cause of death	
Natural	14
Accidental	7
Drug intoxication	8
Suicide	1
Undetermined	3
Model drugs	
Amitriptyline	6
Metoprolol	10
Mirtazapine	8
Zolpidem	4
Amitriptyline + mirtazapine	1
Metoprolol + zolpidem	1
Metoprolol + mirtazapine	1
Mirtazapine + zolpidem	2

fungi and that many of these fungal species are capable of drug metabolism [17,28]. The metabolites formed are mostly identical with those also described in mammals, particularly humans. However, some of the metabolites detected in the previous study have never been described as mammalian metabolites and might thus be specific for metabolism by fungi. Such metabolites could serve as marker metabolites indicating that fungal metabolism has taken place in corpse. In the present study authentic postmortem species were therefore firstly tested for the presence of such fungi-specific metabolites and secondly for the presence of fungal species capable of forming the respective metabolites.

3.1. Isolation and identification of fungi

Using our previously published procedures [17] at least one fungal species could be isolated and identified in 21 out of 33 (64%) of the PBS included in the study. A total of thirty fungal strains (Table 3) were identified. One belonged to the class of *Ascomycete* the others to 15 species from 8 different genera: *Aspergillus* (2), *Botrytis* (1), *Candida* (8), *Fusarium* (1), *Mucor* (1), *Penicillium* (1), and *Rodothorula* (1). *Candida* was the genus with the highest number of isolated species (*C. albicans*, *C. galli*, *C. glabrata*, *C. lipolytica*, *C. parapsilosis*, *C. tropicalis*, *C. sphaerica* and *C. zeylanoides*) and accounted for 60% of all isolated strains. The total number of isolated strains is likely an underestimate of fungal colonization of the corpse, bearing in mind that all the samples had been stored at least one year at −20 °C. In fact, a previous study [17] showed that the number of strains isolated from postmortem specimen was 50% higher if the specimens were cultivated directly after sampling compared to specimens that had been stored at −20 °C before cultivation. Most of the species identified in the present study had already been isolated from postmortem samples [11,31–33] and from clinical samples [34] by other authors. However, to our knowledge this is the first time that the ubiquitous fungi *Candida sphaerica* (syn. *Kluyveromyces lactis*) and *C. galli* have been found in PBS. Given that these samples were collected by routine toxicological protocols without specific measures against environmental contamination from autopsy room, sample containers, and autopsy instruments, contamination cannot be fully excluded but were found to be unlikely in a similar setting [33]. Moreover, Petri dishes used as negative controls were prepared in parallel with those used for isolation of fungi from PBS to identify any environmental contamination. In none of these negative controls any growth of fungi was observed.

3.2. In vitro *biotransformation and PBS analysis*

The metabolites formed of the 4 model drugs with the isolated strains from PBS (*in vitro* metabolites) and metabolites detected on PBS (*in mortuo* metabolites) are listed in Table 3.

The detected *in vitro* metabolites resulted from *N*-demethylation and *N*-oxidation of AT, *O*-demethylation followed by side chain oxidation and hydroxylation of MET, *N*-demethylation and hydroxylation of MRT, as well as *N*-oxidation and hydroxylation of ZOL. As in our previous study [28], the isolated *Candida* species showed a fairly low metabolic activity. The vast majority of the metabolites detected in the present *in vitro* experiments are identical with known mammalian metabolites. Only the *F. oxysporum* strain isolated from PBS no. 14 formed a fungi-specific MET metabolite hydroxylated at one of the methyl groups of the isopropyl moiety. The same metabolite had previously been

Table 2
LC–ESI–MS/MS chromatographic conditions.

LC analysis			
Column	Eclipse XDB C18 5 (4.1 mm × 150 mm)		
Mobile phase	A: Aqueous ammonium formate, 50 mM, pH 3 B: 0.1% formic acid in acetonitrile		
Gradient	Linear. Program: 10% B to 60% B for 10 min Between injections 3 min equilibration to 10% B		
Flow rate	1.4 mL/min		
Injection volume	25 μL		
ESI–MS/MS analysis			
Ionization	Positive	Curtain gas	20 (nitrogen)
Source temperature	550 °C	Collision gas	High (nitrogen)
Ion spray voltage	5500 V	Declustering potential	50 V
Ion source gas 1 and 2	35 (Nitrogen)		
Data acquisition. Mode IDA (Information dependent acquisition)			
Survey scan	Enhanced MS (EMS) with dynamic fill-time. Rate 1000 u/s.		
Dependent scan	Enhanced product ion (EPI) with collision energy (CE) 40 V and CE spread 25 V. Scan rate 4000 amu/s. Three EPI experiments.		
Mass range	*m/z* 50-360		
Cycle	1.605 s		

Table 3
Fungi isolated from postmortem blood samlples (PBS, $n = 33$) and their metabolites detected by LC–MS/MS *in vitro* (incubation) and *in mortuo* (PBS).

Sample	Model drugs	Concentration[a] (mg/L)	Isolated fungal strains	*In vitro* metabolites	*In mortuo* metabolites
1	AT[b]	0.11	*Botrytis cinerea*	PD, nor and *N*-oxide-AT	PD, nor, *N*-oxide and hydroxy(2)-AT
2		0.19	*Candida albicans*	PD, nor and *N*-oxide-AT	PD, nor, *N*-oxide and hydroxy-AT
3		0.5	*Candida albicans*	PD, nor and *N*-oxide-AT	PD, nor, *N*-oxide and hydroxy(2)-AT
4		0.3	No fungi detected		PD, nor, *N*-oxide and hydroxy-AT
5		0.2	*Rhodutorula mucilaginosa*	PD, nor and *N*-oxide-AT	PD, nor, *N*-oxide and hydroxy(2)-AT
6		0.16	No fungi detected		PD, nor, *N*-oxide and hydroxy(2)-AT
7	MET	0.13	*Candida albicans*	PD	PD and *O*-demethyl-MET
			Rhodutorula mucilaginosa	PD, *O*-demethyl and carboxy-MET	
8		0.4	No fungi detected		PD and *O*-demethyl-MET
9		0.03	No fungi detected		PD and *O*-demethyl-MET
10		0.06	*Candida glabrata*	PD	PD and *O*-demethyl-MET
11		0.2	*Candida parapsilosis*	PD	PD, *O*-demethyl and hydroxy-MET
			Rhodutorula mucilaginosa	PD, *O*-demethyl and carboxy-MET	
12		0.05	*Candida glabrata*	PD	PD
13		0.02	No fungi detected		PD and *O*-demethyl-MET
14		1.12	*Aspergillus fumigatus*	PD and *O*-demethyl-MET	PD and *O*-demethyl-MET
			Fusarium oxysporum	PD, *O*-demethyl, carboxy, hydroxy and novel hydroxy-MET	
15		0.15	No fungi detected		PD, *O*-demethyl and hydroxy-MET
16		0.15	No fungi detected		PD and O-demethyl-MET
17	MRT	0.005	*Candida albicans*	PD	PD
18		0.015	*Candida zeylanoides*	PD	PD
19		6	*Candida lypolitica*	PD	PD, *nor* and hydroxy-MRT
			Candida albicans	PD	
			Candida sphaerica	PD and hydroxy-MRT	
20		0.1	No fungi detected		PD, *nor* and hydroxy-MRT
21		0.05	*Ascomycete*	PD, nor and hydroxy-MRT	PD, *nor* and hydroxy-MRT
22		0.45	*Candida galli*	PD	PD, *nor* and hydroxy-MRT
23		0.15	*Penicillium crustosum*	PD, nor and hydroxy (2)-MRT	PD, *nor* and hydroxy-MRT
24		1.9	*Candida tropicalis*	PD	PD, *nor* -MRT
25	ZOL	0.01	*Candida albicans*	PD and hydroxy (2)-ZOL	PD
26		0.15	No fungi detected		PD and hydroxy (2)-ZOL
27		0.2	*Candida tropicalis*	PD and hydroxy (2)-ZOL	PD and hydroxy-ZOL
			Rhodotorula mucilaginosa	PD, *N*-oxide and hydroxy-ZOL	
			Aspergillus fumigatus	PD, hydroxy and novel hydroxy-ZOL	
28		0.18	*Candida albicans*	PD and hydroxy (2)-ZOL	PD and hydroxy (2) -ZOL
29	AT, MRT	0.2, 0.05	No fungi detected		PD, *nor*, and hydroxy(2)-AT-MRT
30	MET, ZOL	130, 0.12	No fungi detected		PD, O-demethyl-MET, hydroxy-ZOL
31	MRT, ZOL	0.58, 0.14	*Aspergillus jensenii*	PD, nor and hydroxy (2)-MRT-ZOL and novel hydroxy-ZOL	PD, *nor*, and hydroxy-MRT-ZOL and novel hydroxy-ZOL
			Candida parapsilosis	PD (MRT and ZOL)	
32	MET, MRT	0.16, 0.3	*Candida zeylanoides*	PD (MET-MRT)	PD, *O*-demethyl-MET and *nor* -MRT
33	MRT, ZOL	0.1, 0.45	*Mucor circinelloides*	PD, nor and hydroxy (2)-MRT-ZOL and novel hydroxy and carboxy-ZOL	PD, nor–MRT, hydroxy-MRT-ZOL and novel hydroxy-ZOL

[a] Concentrations relate to femoral blood concentrations as determined during routine analysis with exception of nos. 15 and 18 where they relate to heart blood.
[b] *Abbreviations*: AT, amitriptyline; PD, parent drugs; MET, metoprolol; MRT, mirtazapine; ZOL, zolpidem.

isolated from an incubation with a *F. solani* strain isolated from a severely decomposed lung sample [17,28]. The *Aspergillus fumigatus*, *A. jensenii*, and *Mucor circinelloides* strains isolated from PBS nos. 27, 31, and 33, respectively, formed a fungi-specific hydroxy ZOL metabolite. The extracted ion chromatograms of m/z 308 (ZOL) and m/z 324 (isomeric hydroxyl ZOL metabolites) of the incubation of the *Aspergillus jensenii* strain isolated from PBS no. 31, and the enhanced product ion spectrum of the detected fungi-specific metabolite are shown in Fig. 1. This metabolite had previously been identified during *in vitro* experiments with the fungi *Aspergillus fumigatus*, *Bjerkandera adusta*, *Botrytis cinerea*, *Circinella minor*, *Coriolopsis* sp., *Fusarium solani*, *Mucor hiemalis*, *M. plumbeus, M. racemosus, Penicillium rubens*, *P. crustosum*, and *Trichoderma harzianum* [28], but never described in humans.

In all analyzed PBS the parent drugs (PD) of AT, MET, MRT and/or ZOL were detected along with *in mortuo* phase I metabolites, resulting from hydroxylation (all four drugs), *N*-demethylation (AT and MRT), and *N*-oxidation (AT), and *O*-demethylation (MET). With exception of one of the hydroxyl ZOL metabolites detected in PBS nos. 31 and 33, all of the detected metabolites belong to the typical spectrum phase I metabolites of the respective drugs previously described in human blood or plasma samples [35–38]. As compared to the other two hydroxy metabolites of ZOL, the additional hydroxyl metabolite detected in PBS nos. 31 and 33 had a rather low abundance. One explanation for this metabolite not having been described in humans so far might therefore be that it is only a minor metabolite in humans. However, it seems remarkable that this particular metabolite was detected only in two PBS samples from which fungal strains capable of forming this metabolite had been isolated. In the other four samples positive for ZOL, this metabolite was not detectable, although the ZOL concentrations and abundances of the other hydroxy ZOL were

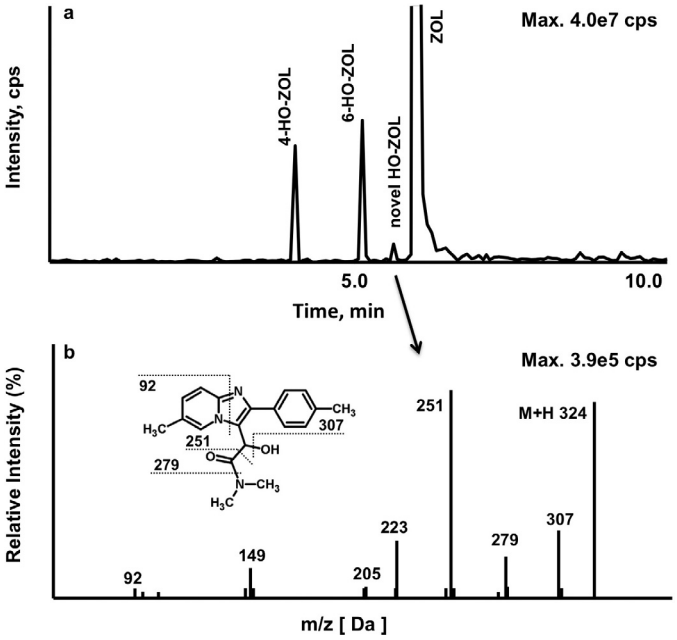

Fig. 1. Extracted ion chromatograms of *m/z* 308 for zolpidem (ZOL) and *m/z* 324 for isomeric hydroxy ZOL metabolites of a diluted supernatant of an incubation of ZOL with the *Aspergillus jensenii* strain isolated from PBS no. 31 (a) and enhanced product ion spectrum of the detected fungi-specific novel HO-ZOL metabolite (b).

similar or even higher than those in the PBS in which only the known major human hydroxy metabolites were detectable (Table 3, samples nos. 25–28). Seen together, these results in our opinion strongly suggest that the additional hydroxy ZOL metabolite is indeed a fungi-specific metabolite formed by the above-mentioned fungal strains detected in PBS samples nos. 27, 31, and 33. It could hence be useful to check for postmortem fungal metabolism of ZOL.

In contrast to the presumably fungi-specific metabolite of ZOL, the presumably fungi-specific hydroxy MET metabolite was not detectable in any of the analyzed PBS from cases involving MET, not even in the one from which a *F. oxysporum* strain capable of forming this metabolite had been isolated. The reason for this discrepancy might be a low postmortem formation rate of this metabolite, *e.g.* due to an insufficient number of fungal cells to produce a detectable amount of this metabolite.

Since all the other metabolites detected in the PBS and incubation samples are human metabolites or identical to those, a differentiation between ante- and postmortem formation of these metabolites is not possible. However, it seems quite possible that these metabolites were at least partly formed during postmortem fungal metabolism, at least in those samples in which metabolically active fungal species were detected.

4. Conclusions

The presented results suggest that postmortem drug metabolism by fungi colonizing decomposing human corpses is not just a theoretical option, but may actually happen in real case scenarios. Since most of the metabolites formed by such fungi are identical to human metabolites, the only way to test for postmortem fungal metabolism is the detection of fungi-specific metabolites such as the new *in mortuo* hydroxy ZOL metabolite found in authentic PBS colonized by fungi capable of its formation. Further studies will be needed to identify fungi-specific metabolites of other toxicologically relevant drugs and to test for their presence in authentic postmortem specimens. In any case, postmortem fungal metabolism should be considered when interpreting postmortem drug concentrations or concentration ratios of PD to metabolites in decomposed specimens.

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7 DISCUSSION

The isolation and identification of fungi from postmortem material were carried out employed postmortem samples of HB (96 samples), kidney (69 samples), liver (68 samples), and lung (19 samples) for a total of 252 samples. The samples were recollected between 2010 and 2012 during the autopsy of 105 moderately (**stage 1** – 96 samples) to severely (**stage 2** – 156 samples) decomposed bodies in different cities in the federal state of Thuringia, Germany. A group of 68 samples (**group I**) from 20 different autopsy cases were collected under aseptic conditions (figure 1).

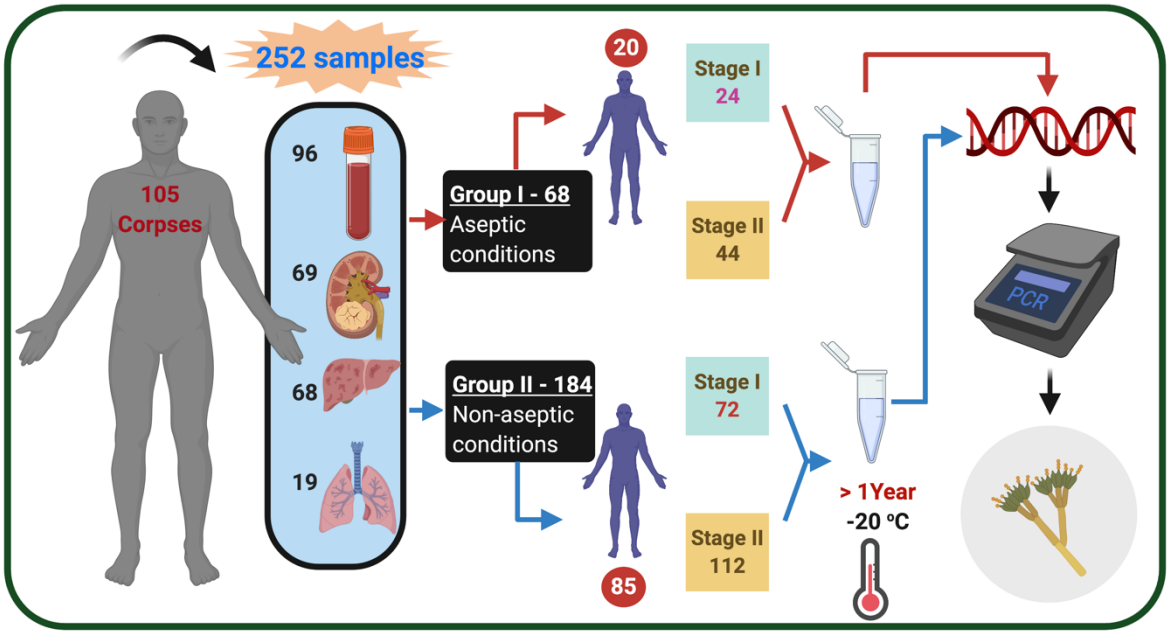

Figure 1. Schematic distribution of postmortem samples used in the study

The isolation of fungi of group I was done immediately after the samples were extracted from the cadaver. The 184 remaining samples, including HB, kidney, and liver (**group II**) from 85 autopsy cases that had already been used once for routine toxicological analysis, were taken with a clean instrument following a routine standard procedure in the Institute of Forensic Medicine of Jena University Hospital. The isolation of fungi of group II was done when the samples had been stored at -20 °C for at least 1 year. The general characteristics of all the samples employed in this study are described in table 1 of the first publication (section 6.1, page 8445)

and a schematic distribution is shown in figure 1. A total of 156-IFS belonging to 28 species and 15 genera were isolated from these DPS [128] (*Aspergillus, Bjerkandera, Botrytis, Candida, Chaetomium, Circinela, Coroliopsis, Enterocarpus, Fusarium, Geotrichum, Mucor, Penicillium, Rhodotorula, Trichoderma, and Trichosporum*). The genera with the greatest number of species were *Candida* with 6 species (*C. albicans, C. galli, C. glabrata, C. lipolytica, C. parapsilosis*, and *C. tropicalis*), *Aspergillus* and *Mucor* with 4 species (*A. awamori, A. fumigatus, A. jenseni*, *A. tubingensis*, *M. circinelloides, M. hiemalis, M. plumbeus, and M. racemosus*), and *Penicillium* with 2 species (*P. rubens, and P. crustosum*). Although the distribution of the isolated strains in HB, lung, kidney, and liver was 26%, 19%, 33%, and 22% respectively, the highest prevalence was seen in lung with an average number of 0.52 strains per sample, followed by kidney, liver, and HB with an average number of 0.50, 0.36, and 0.22 strains per sample. *C. albicans* was the yeast with the mayor prevalence in all PS, being the lung again the matrix with the largest distribution of isolated strains. The percentage of strains isolated from stage I with respect to stage II increased slightly and goes from 46.1% to 53.8%, being *Candida* and *Penicillium* the genera that more predominated in both stages. On the contrary, regarding groups, the number of isolated strains decreased from 58% in group I to 41% in group II. Using specific primers, most of them (98%) could be identified to the species level. The strains *Coroliopsis*, *Enterocarpus,* and *Trichoderma* were only identified to the genus level due to the lack of identical or similar reference sequences in GenBank. Performing a bibliographic search to date, there are two unique studies that could be comparable for their work in the identification of microorganisms in postmortem material. In the first of them, Sidrim et al. [21] carried out a study on decomposed cadavers using samples in three decomposition stages. Nevertheless, the isolates were exclusively identified to the genus level due to the use of morphological methods. In the second study (Schwarz et al) [20] a molecular identification process was carried out using only two classes of primers: *β* – tubulin for *aspergillus* and *penicillium* genera and the internal transcribed spacer (ITS) for the rest microorganisms. A total of 24 fungal strains were isolated and identified to species level. Despite these results and bearing in mind that some genera such as *Aspergillus* and *Penicillium* share similar or identical

region of the ribosomal RNA, specific primers must be used. In this study, three *Aspergillus* and five *Penicillium* species were found using *β* – tubulin primer. The aforementioned results indicate that the authors, Schwarz et al. might have made a misinterpretation of these genera.

In the present study four primers were used (figure 2): Calmodulin primer for *Aspergillus*, *β*–tubulin primer for *Penicillium*, translation elongation factor (TEF 1α) primer for *Fusarium,* and ITS primer for the other genera.

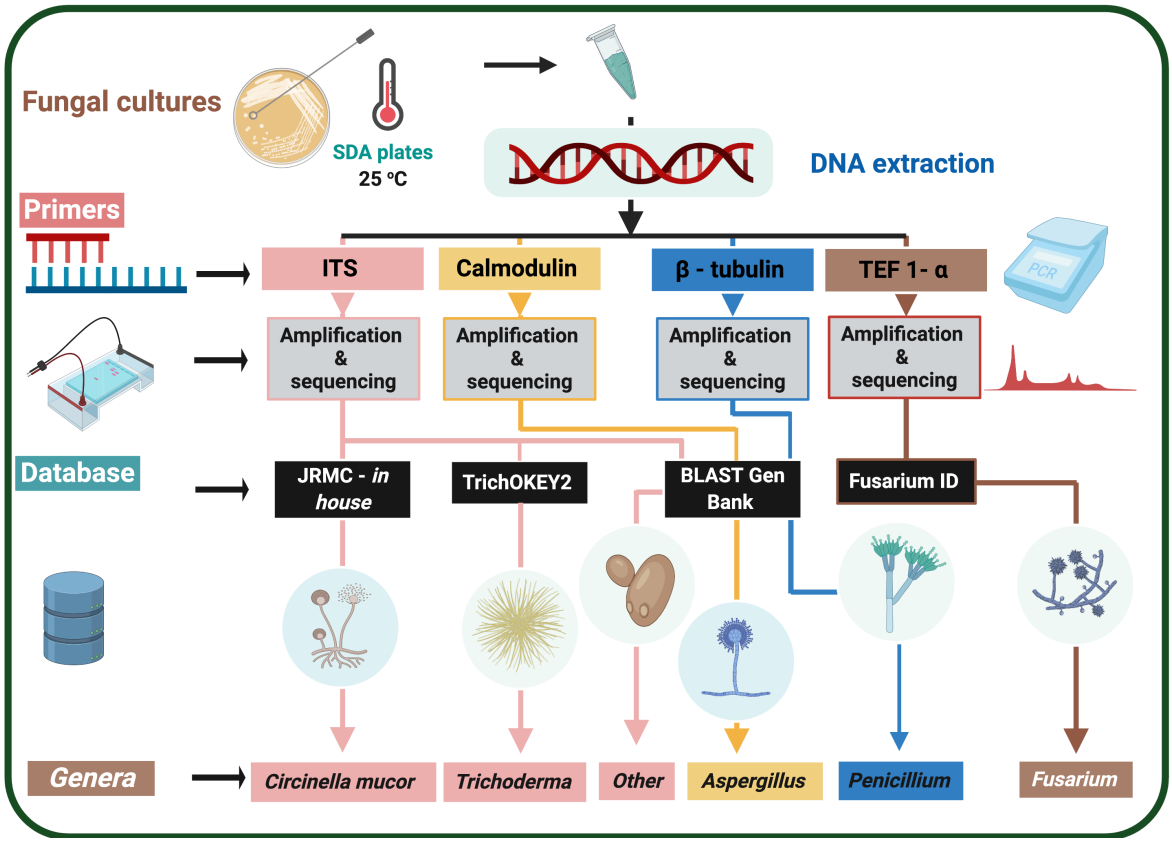

Figure 2. Molecular identification of isolated fungi from postmortem material using four different primers and databases

All the precedent results allow concluding that to date this methodology continues to be appropriate for the identification of fungi to species level in postmortem decomposed material. Many of the fungi identified in this study are facultative pathogens present in immunocompromised patients and have previously been isolated from clinical samples by other authors [131-135]. Although these fungi might be characteristic for DPS, the entrance of the human body can only be

speculated so far by the following 5 ways proposed in the first paper [128]: (1) the fungus had already infected the tissue of the living human host, (2) the fungus was a colonizer of the mucosa of the living human host, (3) ungerminated airborne spores of the fungus were already settled in the lungs when the human was alive and germinated after death, (4) the fungus grew into the human body after death, or (5) fungal spores were transported by animals such as maggots or adult insects into the human body and germinated inside. Morris et al.[136] suggest that bacteria migrate from the mucosal surface into the blood and body tissues before the necropsy was conducted. This phenomenon of postmortem translocation could occur with the cells yeast of *Candida, Geotrichum,* and *Trichosporon*. In lung and kidney samples fungal strains were most prevalent, which could be expected considering that these two organs have a connection to the environment via the airways and genitourinary tract.

Taking into consideration the grade of decomposition (stage I-moderately and stage II-severely) of the samples of group I (collection in aseptic conditions and immediately processed to isolate and identify fungal strains) the proportion and diversity of IFS increased from stage I (**25 IFS**) to stage II (**44 IFS**) (see figures of electronic supplementary material: paper one, section 6.1). This finding allows inferring that as the decomposition process progresses, the fungi gain ground in the colonization process, and their growth becomes exponential until the available organic matter is finished. As previously mentioned, the decomposition process begins a few minutes after death with an autolysis process. After this process. there is a rupture of cell membranes making energy sources more available; after that, an invasion of microorganisms arrives in a short period of time. Fewer strains were isolated in group II, when compared to group I. In principle, this decrease can be attributed to the fact that these postmortem samples were analyzed after a year in which they were stored in freezing conditions. The mechanism by which many fungi did not survive under these conditions can be explained by the rupture of the fungal cell wall. These storage conditions cause the cell wall to swell; and after the thawing of the postmortem material at room temperature or above 30 ^{o}C, the ice crystals formed during the freezing process destroy the membrane.

The culture medium can also play an important role. *Schwarz et al.* [137] isolated and identified fungal strains in all decomposed postmortem material using an SDA medium with an antibiotic. In this work, the percentage of fungi detection was of 62%. The SDA without any antibiotic used in this study is a type of growth medium that allows the isolation, cultivation, and maintenance of a vast amount of fungi especially dermatophytes such as *Microsporum*, *Epidermophyton,* and *Trichophyton* genera which cause superficial and cutaneous mycoses. As heterotrophic microorganisms, they require nitrogen, vitamins, and carbon-like energy sources which come from peptone and dextrose present in the medium. Additionally, this medium is generally adjusted to a pH between 5.0 and 5.5. In this study, it was adjusted to 5.2 to increase the growth of fungi and to suppress bacterial growth in decomposed PS to some extent. Because SDA is a nonselective medium, other genera such as those mentioned above could be isolated and identified, despite the fact that there are selective culture media for them. In this regard, it is recommended for further studies to use another type of fungal culture media that allow the isolation of a greater number of genera and species. An interesting battery to carry out a complete screening in PS in addition to SDA would be the proposed by Scognamiglio *et al*. [138] for clinical samples. One medium with antibiotic, (chloramphenicol and gentamicin) to prevent the growth of bacteria, one medium containing an antibiotic plus a fungicide (cycloheximide) to inhibit the growth of rapidly growing fungi that can overgrow a slower growing fungus, and finally an enriched medium to ensure the growth of fungi that need additional specific nutritional requirements.

The above isolated fungi were assayed with five model drugs (AT, MET, MRT, PMZ, and ZOL) to evaluate the capability of *in vitro* metabolism after five days of incubation in the case of fungi and three days for the yeasts. During the biotransformation procedure three different classes of controls were set up. The fungus *C. elegans* and each model drugs (positive control – **K1**), each IFS without the model drugs (blank incubation control – **K2**) to exclude the response of the matrix and each model drugs with SDA (negative control – **K3**) to make sure that each model drug did not suffer degradation. Each extract was analyzed immediately after incubation period in LC-ESI-MS/MS and GC-MS/EI prior to the acetylation

process. The evaluation of the metabolic activity was carried out with the LC procedure using two parameters: Total number of detected metabolites (NDM) and the percentage of remaining parent drug (%RPD) in comparison to each K3 of the model drugs.

All model drugs were extensively metabolized by incubations *in vitro* with the isolated fungal strains, being AT not only the model with the highest number of metabolites identified but also in which more new fungal metabolites (NFM) were detected (Table 6). A total of 32 NFM were found in the five model drugs studied, 16 of them elucidated with the analytical methodology employed in this study. The rest (12 for AT and 4 MRT produced in incubations with *Bjerkandera adusta* and *Coroliopsis sp*.) could not be completely identified using GC-MS/EI and LC-MS/MS. Their EPI and EI mass spectra can be seen in the electronic supplementary material of section 6. 2. Other authors have not previously identified these metabolites. PMZ was the model drug with the lowest %RPD, being in four fungal strains (*Bjerkandera adusta, Botrytis cinerea, Coriolopsis sp, and Mucor plumbeus*) of 0%. These fungal strains together with *Chaetomium sp* and *Mucor plumbeus* had the highest metabolic capacity either by NDM or %RPD or both.

The low metabolic activity of *Candida* formed a maximum of four metabolites in the model drugs AT and PMZ and their average %RPD value was ≥95%. This finding is relevant because *Candida* was the most recurrent genus isolated from DPS. On the other hand, *Bjerkandera adusta* was the fungus with the highest metabolic activity in the model drugs AT, MET, and MRT, even higher than in the K1. This shows that their enzymes or enzymatic systems combine a high degree of metabolic selectivity with high metabolic capacity. The identification of phase II metabolites by the LC-MS/MS method used in this study and based on a previous author´s work [45] was not possible. The mass range of the enhanced MS scan and survey scan did not set up to high masses. In addition, prior to the gas chromatographic analysis, urine samples were subjected to a hydrolysis process to free the phase I metabolites. With the exception of some cases such as ethyl glucuronide, which is used as a marker for ethanol consumption, the studies of phase II metabolites in toxicology laboratory services do not play a significant role. The interpretation of the

results must be done with the parent drug in blood samples and the phase I metabolites.

Model drugs	NDM	NFM	%RPD	Main reactions
AT	15	6	5 - 96	*N*-dealkylation, *N*-oxidation, aromatic hydroxylation and side chain hydroxylation
MET	6	4	25 - 100	*N*-dealkylation, *N*-acetylation, *O*-demthylation and side chain hydroxylation
MRT	8	4	0 - 100	*N*-dealkylation, ring hydroxylation, and aromatic hydroxylation
PMZ	7	-	0 - 91	*N*-dealkylation, *N*-oxidation, aromatic hydroxylation and sulfoxidation
ZOL	7	2	38 - 100	*N*-oxidation, side chain hydroxylation and later carboxylation

Table 6. Main indicators of metabolization after incubation of the model drugs with 28 IFS using LC-ESI-MS/MS.

In comparison to mammalian metabolism, the observed changes induced by fungal metabolism on the five model drugs followed four basic patterns of biotransformation: (1) simulation of the mammalian phase I metabolism (figure 3), regarding number and proportion of metabolites formed; (2) simulation of the mammalian phase I metabolism (figure 4) but with different metabolic pattern; (3) formation of mammalian metabolites and NFM (figure 5); and (4) high degradation (%RPD < 5%) of the parent drug (figure 6) after incubation period, forming metabolites in one of the three previous ways [129].

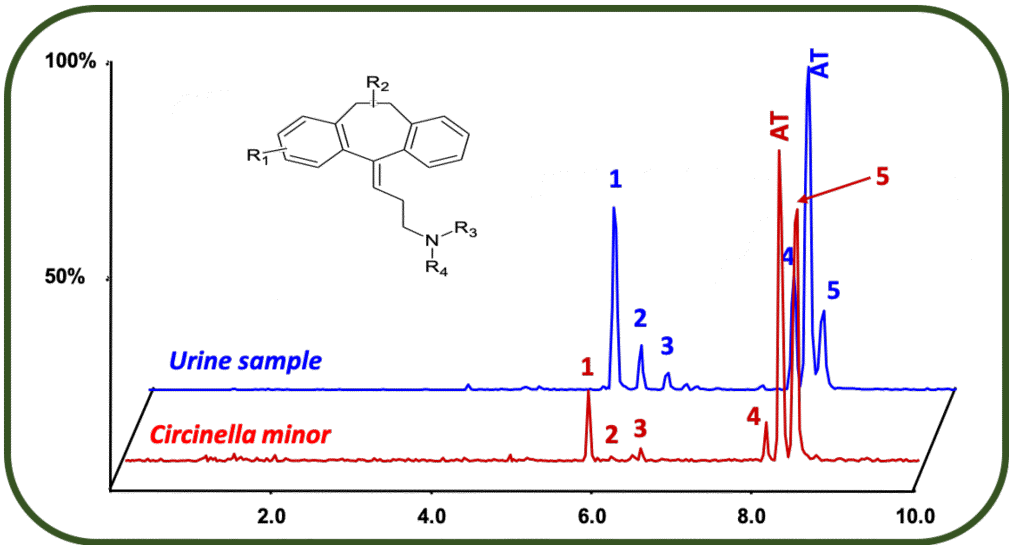

Figure 3. Simulation of the mammalian phase I metabolism of AT incubated with *Circinella minor*. Metabolites (M): 1. Amitriptyline-M (HO-) (isomer 1), 2. Amitriptyline-M (nor-HO-) (isomer 2), 3. Amitriptyline-M (nor-HO-) (isomer 4), 4. Amitriptyline-M (nor), 5. Amitriptyline-M (*N-oxide*)

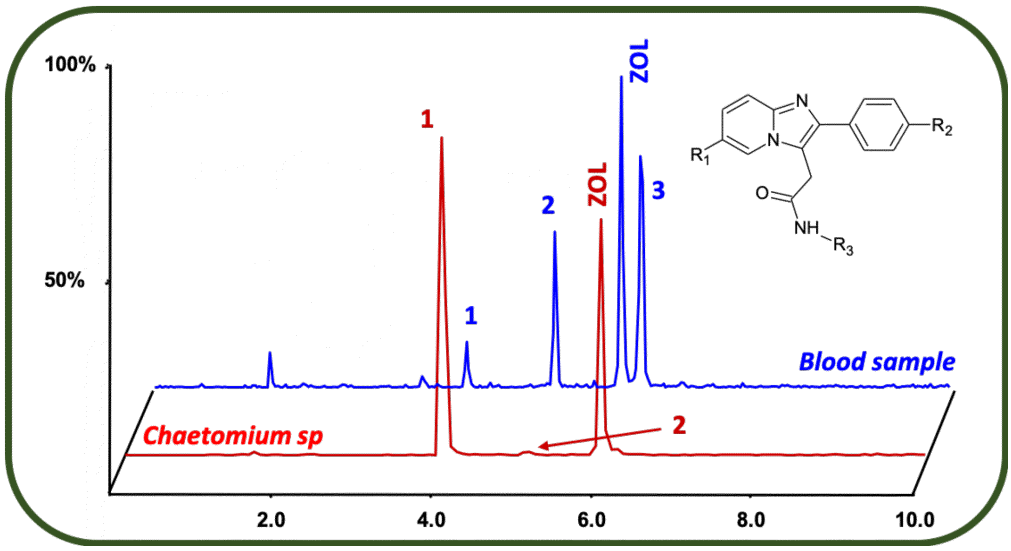

Figure 4. Simulation of the mammalian phase I metabolism with different metabolic pattern of ZOL incubated with *Chaetomium sp*. Metabolites (M): 1. Zolpidem-M (4´-HO-) (isomer 1), 2. Zolpidem-M (6´-HO-) (isomer 2), 3. Zolpidem-M (N-oxide)

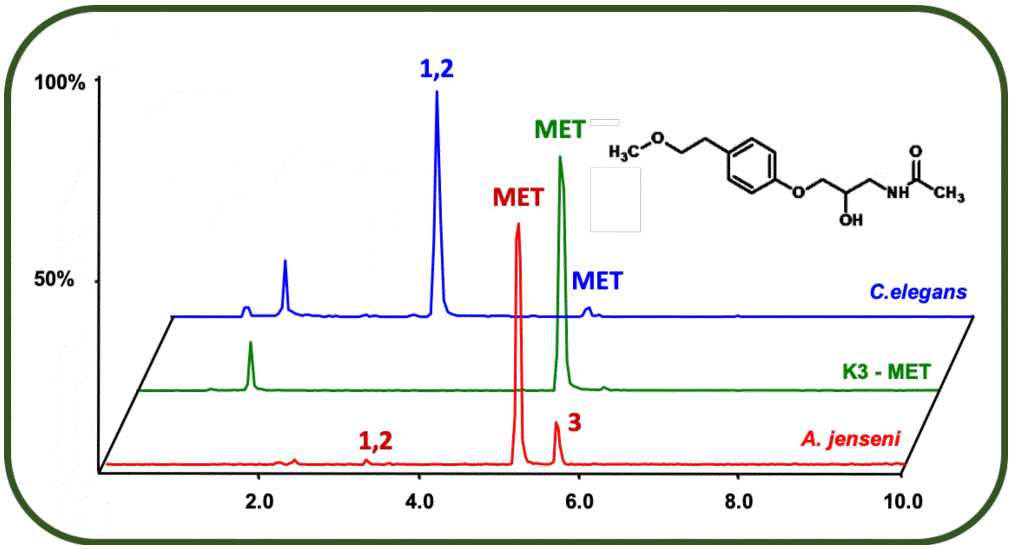

Figure 5. Formation of mammalian phase I metabolites and NFM of MET incubated with A.jenseni and *C. elegans* and K3-MET. Metabolites (M): 1. Metoprolol-M (HO-) (isomer 1), 2. Metoprolol-M (Carboxy), 3. NFM Metoprolol-M (N-dealkyl, N-acetyl)

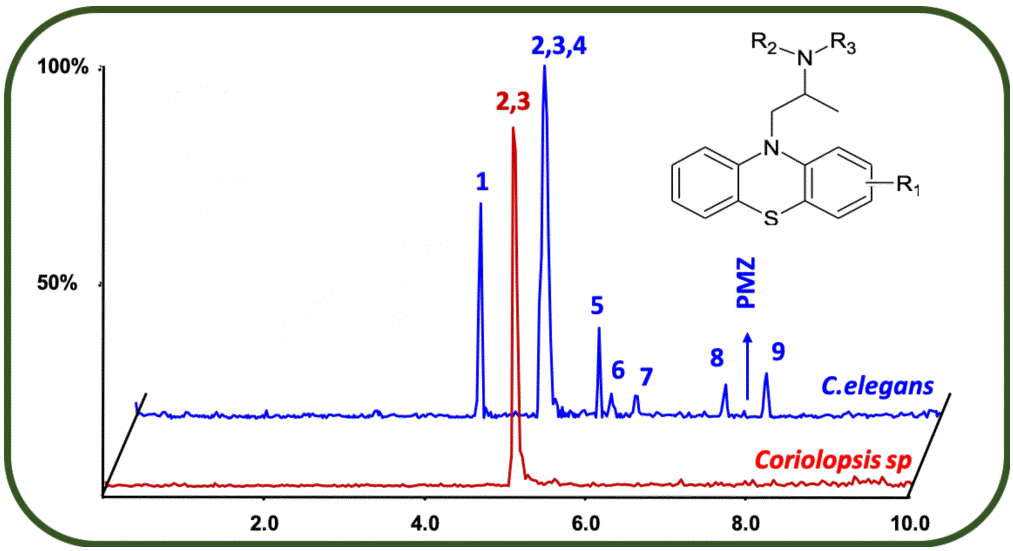

Figure 6. High degradation (%RPD < 5%) of the parent drug of PMZ incubated with *C.elegans* and *Coriolopsis sp*. Main metabolites (M): 1. Promethazine-M (sulfoxide -HO-) 2. Promethazine-M (sulfoxide). Minority metabolites can be seen in section 5.2

The last study presented here (section 5.3) aimed to use the previous NFM as fungi specific metabolites in order to carry out a systematic search in authentic decomposed human blood samples that were previously positives for one or more of the four model drugs studied: AT, MET, MRT, and ZOL. In the model drug PMZ, no NFM were found, and therefore, the model was not included in this latter study.

A total of 33 decomposed PBS (6 for AT, 10 for MET, 8 for MRT, 4 for ZOL, and 5 for more than one of these drugs) were included in the study.

These samples were collected and maintained under the same conditions as those of group II. The general characteristics of all the samples employed in this study are described in Table 1 of section 6.3, page 175 [130]. A total of 30 IFS belonged to the class *Ascomycete* and the genera *Aspergillus, Botrytis, Candida* (genus with the highest number of isolated species -8)*, Fusarium, Mucor, Penillium, and Rodothorula* were identified in the blood samples*. Candida* was the genus with the highest number of isolated species (18), finding that two of them, *C. sphaerica* and *C. galli*, have not been reported in previous studies dealing with PBS. The IFS were subjected to the previous *in vitro* assay procedure and the metabolites produced (*in vitro* metabolites) were compared with metabolites obtained in a PBS (in *mortuo* metabolites). With exception of *Fusarium oxysporum* and the species *A. fumigatus, A. jensenii*, and *Mucor circinelloides* who formed fungi-specific hydroxy-MET and hydroxy ZOL metabolites respectively, the great majority of *in vitro* metabolites are identical to *in mortuo* metabolites. Only in one out of 33 PBS positive to zolpidem a fungus (*Mucor circinelloides*) was jointly detected capable of producing a fungi-specific metabolite and the novel metabolite (hydroxy metabolite).

A total of 16 NFM were postulated in four model drugs: six in AT, four in MET, four in MRT, and two in ZOL which have neither been reported in studies dealing with mammalian metabolism. For the model drug PMZ, no NFM were observed due to the fast biotransformation of its sulfoxide metabolite. Unlike human metabolism, fungi metabolism searches for another alternative pathway of drug detoxification. In this study three different routes could make a difference with mammalian metabolism. In the first place is the formation of hydroxy metabolites, not in the aromatic ring as the human does but inside chains. These types of metabolites were identified in model drugs such as AT and ZOL, with subsequent loss of water when the hydroxy (dehydro metabolites) group is in position 2 or 3 in the case of AT. The second fungi metabolite route is the formation of hydroxy metabolites in non-aromatic rings. Two MRT metabolites were identified, one in the non-aromatic ring of the benzazepine system and the other in a pyrazol ring. Finally, there is the *N*-dealkylation followed by acetylation in the case of metoprolol. The identification of

these classes of metabolites in postmortem material could be an indicator of fungi colonization which must be corroborated by doing a systematic search in the postmortem samples.

These specific metabolites or NFM were used in authentic postmortem samples in order to evaluate postmortem fungal biodegradation. From 33 assayed authentic samples in two decomposed HB samples, a fungi-specific metabolite for ZOL was detected. Also, in these two specific samples (31 and 33), the fungi *Aspergillus jensenii* and *Mucor circillenoides* were isolated. These two fungi had the ability to produce a hydroxy specific metabolite in the model drug ZOL. If this type of metabolites is detected in a postmortem sample, most likely a portion of the human metabolites may have been formed by a fungus and therefore, the metabolic pattern may have been changed. Consequently, these fungi-specific metabolites could be used as a marker to look at future research on post-mortem fungal metabolism.

8 CONCLUSIONS

A systematic search of fungi colonizing corpses and their capability of metabolizing model drugs *in vitro* and *in mortuo* were shown in the three papers of this dissertation. In all kinds of DPS including HB, liver, kidney, and lung in a considerable percentage (**> 62%**) fungi were found, thus demonstrating that these classes of material provide a substrate for the growth of native and facultative microorganisms, despite the normal freezing storage conditions that exist in a forensic toxicology service.

A total of **189** species were isolated from decomposed postmortem material. The most frequently detected fungi belonged to native yeast of the genus *Candida* and facultative pathogenic members of the genera *Penicillium*, *Rhodotorula*, *Mucor*, *Aspergillus*, *Trichosporon*, and *Geotrichum;* all of them identified in their vast majority at level species (**>97%**). According to the experience acquired throughout the present study and the limited literature found, the isolated fungi might be considered characteristic for moderately and severely decomposed postmortem material.

In vitro studies showed that all the model drugs were metabolized for the IFS producing mammalian phase I metabolites following four patterns of biotransformation. A total of **48** different metabolites were found in the five model drugs. These metabolites could produce an overlap with metabolic pathways in mammals. Other metabolites (**15**) formed by IFS in the studied models have never been described in mammalian metabolism and could be considered as NFM. These latter metabolites are potential candidates for marker substances as fungi-specific metabolites and can be used in investigations on postmortem fungal metabolic events.

In the last part of this work, a (**1**) fungi-specific metabolite for ZOL was detected in two authentic decomposed HB samples; concomitantly, the fungi ***Aspergillus jensenii*** and ***Mucor circillenoides*** were also isolated in the same samples which in *in vitro* assays showed the same novel ZOL metabolite. This result suggests that

postmortem drug metabolism by fungi colonizing decomposing human corpses is totally plausible affecting the human pattern metabolism.

All the findings described above allow us to establish three research fronts in future studies. Considering that the human body is colonized by countless (300 to 1000 species) native bacteria of the gastrointestinal tract, more systematic studies on decomposed postmortem material would be addressed in order to isolate and identify this type of microbes. In addition, studying the metabolism of further drug classes by fungi or bacteria colonizing corpses must also be carried out and finally, a systematic search of *in vivo* studies with these microorganisms in authentic postmortem samples must be done.

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10 APPENDICES

10.1 APPENDIX A: Acknowledgments

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10.4 APPENDIX D: Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich- Schiller-Universität bekannt ist,
ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,
mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben:Frank Peters, Jualian Strien and Grit Walther.....,
die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,
dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und
dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers

Bogotá, der 24. November 2020

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Unterschrift

10.5 APPENDIX E: Congress presentations

At the following events I made oral presentations:

- 50th Annual Meeting of the International Association of Forensic Toxicologists, June 3–8, 2012, Hamamatsu, Japan.
- 51st Annual Meeting of the International Association of Forensic Toxicologists (TIAFT). September 2–6, 2013, Funchal, Madeira, Portugal.

10.6 APPENDIX F: Curriculum vitae

CURRICULUM VITAE

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PERSÖNLICHE ANGABEN

Familienstand:	verheiratet
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1977 – 1982:	Grundschule: “Escuela Piloto”
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2009 – 2011:	Diplom Pharmazeut Friedrich Schiller Universität. Jena - Deutschland These: „Entwicklung von Inkubations- und Analysenverfahren zur Untersuchung des Metabolism von Arzneistoffen durch Leichen besiedelnde Mikroorganismen“
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2005 –:	Lehrer. Apothekenabteilung „Universidad Nacional de Colombia“ Instrumentelle Analyse und Forensische Toxikologie
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