Aus dem Zentrum für Innere Medizin der Universität zu Köln

Klinik I für Innere Medizin

Direktor: Universitätsprofessor Dr. med. M. Hallek

Die Konzentration von Posaconazol in Kompartimenten des peripheren Bluts

Inaugural-Dissertation zur Erlangung der Würde eines

doctor rerum medicinalium

der Hohen Medizinischen Fakultät der Universität zu Köln

vorgelegt von

Fedja Farowski

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Promoviert am 4. Mai 2011

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Die vorliegende Arbeit, sowie alle dieser Arbeit zugrunde liegenden Experimente, wurden im Labor für Therapeutisches Drug Monitoring, Institut für Pharmakologie des Klinikums der Universität zu Köln von November 2008 bis August 2010 in der Arbeitsgruppe von Dr. Carsten Müller von mir unter seiner Anleitung durchgeführt.

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GLOSSARY

AC	alveolar cell(s)
AM	alveolar macrophage(s)
ANC	absolute neutrophil count
AMB	amphotericin B deoxycholate
BSI	bloodstream infection(s)
C/E	ratio between cellular and extracellular concentration
CMV	cytomegalovirus
CSF	cerebrospinal fluid
ELF	epithelial lining fluid
EMEA	European medicines agency
FDA	food and drug administration
GvHD	graft-versus-host disease
HBSS	Hanks' balanced salt solution
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HSCT	haematopoetic stem cell transplantation
IA	invasive aspergillosis
IFI	invasive fungal infection(s)
IPA	invasive pulmonary aspergillosis
ISP	pneumatically assisted electrospray / ion spray

LAMB	liposomal amphotericin B
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLOQ	lower limit(s) of quantitation
PBMC	peripheral blood mononuclear cell(s)
PP	professional phagocyte(s)
PMN	polymorphonuclear leucocyte(s)
RBC	red blood cell(s)
ROS	reactive oxygen species
TDM	therapeutic drug monitoring
TISP	turbo ion spray

1. INTRODUCTION / OBJECTIVE

1.1. INVASIVE FUNGAL INFECTIONS

Invasive fungal infections (IFIs) are opportunistic infections mainly affecting immunocompromised hosts, such as patients undergoing cancer chemotherapy, solid organ or bone marrow transplantation, or patients who are infected with the human immunodeficiency virus (HIV). As a result of the increasing amount of immunocompromised patients the incidence of IFI has also increased. The most common pathogens accountable for probably more than 95% of all IFI are *Aspergillus* spp. and *Candida* spp. [59, 71]. Despite new targeted antifungal therapies the attributable mortality of IFI is still very high, ranging from 5 to 71% for invasive candida infections (candidiasis) and from 60 to 90% for invasive aspergillosis (IA) [31, 53, 118].

1.1.1. ASPERGILLOSIS

Aspergillus spp. are ubiquitous soil-dwelling fungi, often found in walls and dust. Upon inhalation, *Aspergillus* conidia (spores) may grow as hyphae at body temperature in the terminal airways and alveoli, causing invasive pulmonary aspergillosis (IPA) [51]. Profound and prolonged neutropenia, with an absolute neutrophil count of less than 500/µL for more than 10 days, is the single most important risk factor for IA. The risk for invasive aspergillosis in patients with acute leukemia or myelodysplastic syndrome during induction chemotherapy is 5 to 24% [25]. The probability of developing a post-engraftment IA within one year after undergoing haematopoetic stem cell transplantation (HSCT) is almost 10%. In this case the very late onset (> 6 month after transplantation) is associated with chronic graft-versus-host disease (GvHD) and cytomegalovirus (CMV) disease [25, 57]. Among the patients undergoing solid organ transplantation, those receiving heart-lung or small intestine are the most compromised, displaying an IA incidence rate of 11-14% [21, 57]. The incidence

rate of IA in patients with severe burns or in patients who are immunocompromised due to therapy with CD52 antibodies is 4-7% [6, 8, 47, 100, 104]. In patients with AIDS and without antiviral therapy an incidence rate for IA of 12% has been observed [54].

The overall case fatality rate of 1941 patients with IA, who participated in 50 clinical studies published after 1995, was 58% [53]. While IPA is the most common form of IA (80-90%) there are many other manifestations of IA, e.g. cutaneous, airway and disseminated aspergillosis. Cerebral aspergillosis, a form of disseminated aspergillosis, occurs in 10-20% of all cases of IA and is associated with a high mortality among immunocompromised patients [25].

1.1.2. CANDIDIASIS

Invasive candidiasis is caused by fungi of the genus *Candida*. The risk for acquiring invasive candidiasis is driven by several factors. Therefore some authors developed a scoring system to identify high risk patients. Candida infections are usually accompanied either by recent gastrointestinal surgery, extremes of age (< 1 year, or > 65 years), or haematologic malignancy/neutropenia. The individual risk further depends on a multitude of additional factors like the presence of an indwelling catheter, the administration of broad spectrum antibacterial drugs, prolonged intensive care unit stay, parenteral nutrition, mucosal colonisation, and renal failure [84].

Candida spp. are the fourth most common cause for nosocomial bloodstream infections (BSI) in the United States of America, surpassed only by staphylococci and enterococci [118]. Despite modern antifungal therapies an attributable mortality rate of 49% has been reported for nosocomial candidemia [37]. Among 2019 patients with proven candidemia, enrolled between 1st July 2004 and 5th March 2008 in 23 North American centers, the most commonly identified pathogen was *C. albicans* (45.6%); collectively, non-*C. albicans*

Candida species were more frequently isolated from blood culture (54.4%). The majority of the other species identified included *C. glabrata* (26.0%), *C. parapsilosis* (15.7%), *C. tropicalis* (8.1%), and *C. krusei* (5.2%) [43]. The crude 12-week mortality of these patients was 35.2%, while 29.9% of the patients were lost to follow-up.

1.2. ANTIFUNGAL DRUGS (A HISTORY)

1.2.1. AMPHOTERICIN B AND ITS LIPID-BASED FORMULATIONS

Amphotericin B deoxycholate (AMB) was licensed based on open label non-comparative studies more than 50 years ago in 1959. Clinical experience has proven that it is a reliable antifungal agent, but a toxic compound. For many years the treatment options for IFIs were limited. The introduction of lipid-based formulations offered possible ways to reduce toxicity compared to the conventional formulation [2, 41]. AMB exists in various lipid formulations all over the world. The three most common are Ambisome®, Abelcet®, and Amphocil/Amphotec®. Apart from the other formulations, Ambisome® is a true liposomal formulation of amphotericin B (LAMB). LAMB has been approved by the food and drug administration (FDA) of the USA in 1997.

Since the liposomal formulation of AMB does not reduce its *in vitro* activity against clinically relevant fungal isolates [22], most susceptibility testing is performed using the deoxycholate formulation. AMB exhibits a broad spectrum of activity, including most strains of *Candida* spp., *Aspergillus* spp., zygomycetes, and dimorphic fungi [86]. However, infections caused by *Aspergillus terreus* [98], as well as *Scedosporium* spp. [99] cannot successfully be treated with AMB. The polyene resistance of *C. lusitaniae* has been overestimated, since currently only about 20% of *C. lusitaniae* isolates are resistant against AMB [40, 95].

The broad antifungal activity of AMB is reflected in animal models of fungal infections, where LAMB proved to be effective against candidiasis [108, 121], aspergillosis [14, 15, 35, 52, 58, 65, 101, 102, 109], fusariosis [66], and zygomycosis [44]

The clinical efficacy of LAMB for the empirical treatment of persistent fever during neutropenia [112, 115, 117] and proven IFI [19, 46, 50, 79] has been

assessed in various randomized, double-blind, multicenter trials [19, 46, 50, 79, 112, 115, 117]. These trials compared the efficacy and/or safety of LAMB 3 mg/kg/d with either alternative formulations of AMB, different concentrations of LAMB (10 mg/kg/d), caspofungin, or micafungin.

The clinical efficacy and/or safety of LAMB for the empirical treatment of persistent fever during neutropenia was evaluated in three randomized, double-blind, multicenter trials [112, 115, 117]. Patients, who had received chemotherapy for cancer or underwent HSCT, with an absolute neutrophil count (ANC) of less than 500 /µL and persistent fever (>38.0°C) despite antibacterial therapy for at least 3 [117], 4 [115], or 5 [112] days were eligible for these trials. In all three trials an intravenous LAMB dosage of 3.0 mg/kg/d was administered. Dosage adjustments with regard to efficacy and toxicity were allowed in two of the trials [112, 115], while the remaining trial disposed of an alternative LAMB dosage group of 5.0 mg/kg/d [117]. The clinical efficacy [114, 115, 117] of LAMB was similar to that of conventional AMB at 0.6 mg/kg/d [112], ABLC at 5.0 mg/kg/d [117], caspofungin at 50 mg/d (70 mg/d on day 1) [115], and micafungin at 100 mg/d [50]. In terms of the safety distinct differences were demonstrated. The nephrotoxicity (serum creatinine >2 times baseline value) of LAMB was significantly reduced compared to AMB [112, 117] and ABLC [117], while it was still significantly elevated compared to caspofungin [115].

With the approval of new azoles (i.e. voriconazole and posaconazole) and echinocandins (i.e. caspofungin, micafungin, and anidulafungin) the antifungal armamentarium expanded markedly, offering new treatment options for IFI. Due to these increased treatment options clinicians have the opportunity to choose from a broad variety of drugs. The first priority when choosing a drug will usually be its efficacy for the particular case. Since efficacies of two or more

drugs may be indistinguishable, safety properties might tip the scale for one drug.

1.2.2. FLUCYTOSINE, 5-FLUOROCYTOSINE

In 1971 flucytosine (5-fluorocytosine, 5-FC), a halogenated pyrimidine was approved by the FDA. It is only indicated in the treatment of serious infections caused by susceptible strains of *Candida* and/or *Cryptococcus* [107]. Due to a rapid development of resistance towards 5-FC it should only be used in combination with other antifungals. Today, its role is limited to the use in combination with an AMB formulation for the treatment cryptococcal meningitis [85].

1.2.3. EARLY AZOLES

The target of all azole antifungals is the cytochrome P450-dependent lanosterol 14α-demethylase enzyme (CYP51 or Erg11p), thus they inhibit the synthesis of ergosterol, which is the primary sterol in the fungal cell membrane. Consequently ergosterol will be depleted [62], influencing fungal cell membrane stability and the function of membrane-associated proteins [110]. There are two groups of azoles in clinical use: the imidazoles (clotrimazole, econazole, ketoconazole, and miconazole) and the triazoles (fluconazole, itraconazole, posaconazole, and voriconazole). Due to their inferior safety profile compared to that of the triazoles the use of imidazoles is limited to the treatment of superficial fungal infections [12]. Hence, they were not the focus of this work.

In January 1990 fluconazole was approved by the FDA. It is licensed for treatment of vaginal candidiasis, oropharyngeal and oesophageal candidiasis, and cryptococcal meningitis, as well as the prophylaxis of candidiasis in patients undergoing bone marrow transplantation receiving cytotoxic chemotherapy and/or radiation therapy [74]. Mainly in non-neutropenic patients it

demonstrated a high efficacy and was non-inferior compared to AMB [1, 63, 78, 80].

Itraconazole administered orally was first approved by the FDA in 1992, followed by an intravenous formulation, which has been approved in 1999 [45]. In immunocompromised and non-compromised patients, itraconazole is approved for the treatment of blastomycosis, histoplasmosis, and aspergillosis refractory to AMB. Additionally it is also approved for the treatment of onychomycosis in non-immunocompromised patients.

1.2.4. NEW AZOLES

In May 2002 voriconazole was approved by the FDA [75]. It is available for oral and intravenous administration. The intravenous formulation contains sulfobutyl ether beta-cyclodextrin sodium, which acts as a solubilizer for the otherwise low soluble drug. Voriconazole is indicated for the treatment of invasive aspergillosis, candidemia in non-neutropenic patients, oesophageal candidiasis, as well as the following *Candida* infections: disseminated infections in skin and infections in abdomen, kidney, bladder wall, and wounds. Furthermore it is also indicated for the treatment of serious fungal infections caused by *Scedosporium apiospermum* (the asexual form of *Pseudallescheria boydii*) and *Fusarium* spp. including *Fusarium solani* in patients intolerant of or refractory to other therapies.

Posaconazole is a new generation triazole, which is available as suspension for oral administration. Posaconazole has been approved in Europe in October 2005 [87] and in the US by the FDA in September 2006 [88]. As shown in various *in vitro* studies, posaconazole is active against a broad spectrum of geographically diverse and clinically important fungal pathogens, like many common yeasts and moulds [26, 34, 72, 73]. In effect posaconazole was the only

azole that exhibited consistent activity against zygomycetes, although it was less active than amphotericin B [86].

Binding models for posaconazole and itraconazole, other than fluconazole and voriconazole, revealed that they not only ligate the iron atom of the CYP51 haem cofactor but also occupy a specific channel within the CYP51 enzyme [119]. Due to these two different binding motifs, the activity of posaconazole is suspected to be less affected by mutations occurring in and around the active site of CYP51. However, it has been shown that a single nucleotide substitution may lead to posaconazole resistance [56, 60, 119].

Isavuconazole is the active component of the water-soluble azole BAL8557, a new-generation azole with broad antifungal activity against *Candida albicans*, non-*albicans Candida* spp., fluconazole-resistant *Candida* spp., *Aspergillus* spp. and Zygomycetes, e.g., *Mucor*, *Rhizopus* and *Absidia* spp. [91]. As opposed to itraconazole, voriconazole and posaconazole, oral and intravenous preparations of BAL8557 are highly water-soluble, rendering the addition of cyclodextrins or other excipients to enhance solubility unnecessary. After administration, rapid cleavage of BAL8557 into isavuconazole and an inactive cleavage product occurs. Phase I and II clinical trials showed good toleration of BAL8557 [89, 90].

1.2.5. ECHINOCANDINS

Echinocandin antifungals are a recent addition to the antifungal armamentarium. They act as noncompetitive inhibitors of the 1,3-ß-D-glucan synthesis, hence inhibiting the production of a main component of the cell wall in many medically important fungi. Consequently, the fungal cell walls become less stable and unable to resist osmotic pressure, which ultimately leads to cell lysis. Currently, three antifungals of the echinocandin class are commercially available.

The first echinocandin to gain approval from the FDA was caspofungin. Approval was granted for the following indications: Empiric treatment of presumed fungal infections in patients with febrile neutropenia, treatment of oesophageal candidiasis, candidemia and other *Candida* spp. infections, as well as the treatment of invasive aspergillosis in patients who are refractory or intolerant to other therapies [61].

Micafungin has been approved by the European Medicines Agency (EMEA) in April 2008 and by the United States food and drug administration in March 2005 for the treatment of patients with oesophageal candidiasis, invasive candidiasis and the prophylaxis of *Candida* infections in patients undergoing HSCT [3, 4].

Anidulafungin is the latest antifungal agent of this class and has been approved by the FDA for the treatment of oesophageal candidiasis, candidemia and other *Candida* spp. infections [76]. The EMEA approved anidulafungin for the treatment of invasive candidiasis in adult non-neutropenic patients [77].

1.3. THERAPEUTIC DRUG MONITORING (TDM)

Therapeutic drug monitoring (TDM) aims at optimizing the benefits and risks of pharmacotherapy, specifically for drugs exhibiting significant pharmacokinetic variability [42, 96]. Especially the pharmacokinetics of triazole antifungals is not well predictable. Voriconazole for example is known for its high interindividual and intraindividual variability of the plasma concentrations [83, 103], which is among others held responsible for different therapeutic outcomes [67]. Itraconazole displays a nonlinear pharmacokinetic with an extensive variability [38]. Even different generic formulations of itraconazole may vary in their bioavailability to a clinically significant extent [69]. The bioavailability of posaconazole depends among others on concomitant food intake and is also subject to an interindividual variability [97], while the elimination of posaconazole is increased by diarrhea [48, 97]. In addition, a reduced bioavailability of posaconazole was described for neutropenic patients [105]. Therefore it is currently discussed whether TDM of triazoles may increase their clinical efficacy [42, 48].

For the treatment with echinocandins therapeutic drug monitoring is not a standard and it is unknown whether TDM in this setting has the potential to increase the efficacy of those regimens.

However, an understanding of the pharmacokinetics of a measured substance is crucial for the interpretation of their serum concentrations. Information about tissue distribution and compartmental concentrations of antifungals is often limited. For voriconazole it was shown that the therapeutic concentrations may be achieved in cerebrospinal fluid (CSF), synovial fluid, and the epithelial lining fluid (ELF) of the lung [9, 24, 55]. Posaconazole has been successfully quantified in ELF and alveolar cells of healthy volunteers and lung transplant recipients [16, 18], and it may transgress to the CSF in case of a distrusted blood-brain-barrier [82].

Even less is known about antifungal concentrations in different compartments of the peripheral blood, i.e. peripheral blood mononuclear cells (PBMCs), polymorphonuclear leucocytes (PMNs), and red blood cells (RBCs). But especially the PBMCs and PMS are important pillars of the host defence against invasive fungal infections and interactions with these cells might well influence the efficacy of antifungal drugs. The sole information in this regard stems from exclusively laboratory-based experiments on the interaction of fluconazole and voriconazole with PMNs. It was shown that both agents were characterized by rapid intracellular uptake and elution. While fluconazole concentrations were only twice as high as in the surrounding medium [68], voriconazole concentrations were 8.5-fold increased [5]. These results were explained by the hydrophobic properties of voriconazole. The uptake of the two triazoles was not altered by changes in environmental temperature, pH or cell viability, suggesting intracellular penetration by a passive mechanism. Until recently, no data on the intracellular concentration of other antifungals in different cells of the peripheral blood has been published.

1.4. OBJECTIVE

Information about the intracellular concentrations of antifungals in different cells of the peripheral blood is limited, although interactions with these cells might well influence their efficacy. Hence, the primary objective of this work was to develop a feasible method to quantitate the intracellular concentrations of the most clinically relevant antifungal drugs.

The secondary objective was to use the previously developed method to quantitate the intracellular concentration of posaconazole in different compartments of the peripheral blood using samples from patients receiving posaconazole.

2. METHODS

This is a cumulative dissertation; the methods of this works were published in:

Farowski, F., et al. (2010). Quantitation of azoles and echinocandins in compartments of peripheral blood by liquid chromatography-tandem mass spectrometry. Antimicrob Agents Chemother. 54(5): 1815-9 (Appendix 1)

3. RESULTS

This is a cumulative dissertation; the results of this works were published in:

Farowski, F., et al. (2010). Quantitation of azoles and echinocandins in compartments of peripheral blood by liquid chromatography-tandem mass spectrometry. Antimicrob Agents Chemother. 54(5): 1815-9 (Appendix 1)

Farowski, F., et al. (2010). Intracellular concentrations of posaconazole in different compartments of peripheral blood. Antimicrob Agents Chemother. 54(7): 2928-31 (Appendix 2)

4. DISCUSSION

4.1. DETERMINATION OF ANTIFUNGAL CONCENTRATIONS

Within the scope of this work a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitation of echinocandins and triazoles, i.e. anidulafungin, caspofungin, isavuconazole, micafungin, posaconazole, and voriconazole, in different compartments of the peripheral blood was developed. This method can be used to measure this selection of antifungals by only one single method, hence replacing various other methods currently used in parallel, and thus optimising the cost-effectiveness of antifungal therapeutic drug monitoring, while ensuring a rapid turnaround (<1 day) regardless of the variety of antifungals currently in use [33].

In view of the small sample volumes (~10^7 cells; i.e. 3-4 µL) for the determination of intracellular concentrations only a mass spectroscopy method was feasible, because methods using UV detection would require much larger sample volumes (i.e. 100-1000 µL).

For the triazoles the lower limits of quantitation (LLOQ) were calculated (LLOQ=10σ/S´; σ: standard deviation; S´: slope of the calibration curve) because they were not within the range of the calibration standards. The calculated LLOQs of the triazoles were 4.5, 10, and 4.2 ng/mL for isavuconazole, posaconazole, and voriconazole, respectively. The LLOQs of the echinocandins were reached at 64, 108, and 160 ng/mL for anidulafungin, caspofungin, and micafungin, respectively. However, since the injection volume (20 µL) is relatively small compared to the extraction volume of 100 µL, a dry freezer could be facilitated to constrain the extract, and therefore improve the sensitivity of the method [33].

Various LC-MS/MS methods for the determination of isavuconazole [89, 90], posaconazole [49, 92], and voriconazole [29, 122], as well as

caspofungin [11, 30, 81] and anidulafungin [27, 28] have been published. However, the presented method is the only published LC-MS/MS method for the determination of micafungin. A previous high-performance liquid chromatographic (HPLC) method for the quantitation of micafungin in plasma showed an LLOQ of 50 ng/mL using a 100 µL sample [64, 120]. Due to the larger sample volume (100 vs. 30 µL) within the cited method a lower LLOQ was achieved. In another HPLC assay for the determination of micafungin an LLOQ of 100 ng/mL, but no sample volume was reported [36]. Hence the precision of this method may not be compared with that of other methods. Despite efforts to enhance the signal intensity of micafungin, an intensity comparable to those of the other antifungals was not achieved. There was also a tailing of micafungin during the chromatography. Only a complete change of the gradient programme might improve the peak quality of micafungin, but would probably result in a significant loss in the peak quality of the other analytes [33].

The number of methods for the simultaneous determination of azole antifungals is increasing. Since the publication of the method presented in this work two more LC-MS/MS methods for the simultaneous determination of azoles (i.e. fluconazole, itraconazole, posaconazole and voriconazole) in serum and plasma have already been published [10, 111]. Like in the presented method, the sample preparation was also done by protein precipitation. The chromatographic conditions differ slightly, because different columns were used and these methods do not include echinocandins. The slightly higher LLOQs in these methods might be explained by the smaller injection volumes (5 µL vs. 20 µL).

Compared to a previously reported method for the determination of isavuconazole in plasma and urine, the method presented here was less sensitive, but required a smaller sample volume (30 µL vs. 50 µL) [89, 90]. Previously published methods for the determination of voriconazole in

blood [29] and aqueous humor [122], as well as the method presented here were comparable regarding their sensitivity.

Quite apart from the azoles, the method presented here is the first LC-MS/MS method for the simultaneous determination of echinocandins in one sample. Two LC-MS/MS methods for the quantitation of anidulafungin in human plasma were described previously [27, 28]. Despite the very small injection volume (5 µL vs. 20 µL), the LLOQ for the cited methods was only slightly higher (100 ng/mL vs. 64 ng/mL) than that of the method presented here.

The quantitation of caspofungin in human plasma samples by LC-MS/MS was previously described by Chavez-Eng [11], Egle [30], and Rochat [81]. In the method published by Chavez-Eng similar product ions were acquired for both caspofungin and its internal standard. Rochat and Egle used an ion transition with different product ions (m/z 547.4 → 137.1 for caspofungin; m/z 548.0 → 62.2 for the internal standard of caspofungin). Chavez-Eng´s LC-MS/MS deployed pneumatically assisted electrospray (ion spray, ISP) and turbo ion spray (TISP) interfaces with injection volumes of 50 and 75 µL, respectively. Following a solid phase extraction, with a sample volume of 1 mL plasma, the eluent was evaporated and reconstituted in 100 µL mobile phase. Owed to the large sample volume, the LLOQs of this method were 10 ng/mL and 2.5 ng/mL using the ISP and TISP interface, respectively [11]. Rochat presented two different precipitation methods for the sample preparation, with sample volumes of 500 µL and 100 µL plasma, achieving LLOQs of 5 and 40 ng/mL, respectively. The method presented here also deployed a precipitation method for the sample preparation; however, due to the smaller sample volume (30 µL) the LLOQ was higher (108 ng/mL). The method described by Egle consists of a column-switching technique with inline extraction of caspofungin. The LLOQ of this method was 200 ng/mL using a 5 µL plasma sample [30, 33].

4.2. INTRACELLULAR CONCENTRATIONS OF POSACONAZOLE

Little is known about the intracellular concentration of antifungals within cells of the peripheral blood. The best studied cells in this context are alveolar cells (AC), since these cells, i.e. pulmonary epithelial cells and alveolar macrophages (AM), phagocytise inhaled conidia. Hence they constitute the first line of defence against pulmonary fungal infections. Prior to this work the intracellular concentration of posaconazole was only known for these cells. In a study with 25 healthy subjects who received 14 doses of posaconazole (400 mg twice daily) before undergoing bronchoscopy at different time intervals, the ratios between the concentration within the alveolar cells and the plasma concentration (AC/plasma) was determined. In this study the AC/plasma ratio of posaconazole ranged from 27.3 ± 18.0 to 44.3 ± 44.2 [18]. In a similar study with lung transplant recipients, who also received posaconazole (400 mg twice daily), the AC/plasma ranged from 27.3 to 63.6 [16]. The majority of the alveolar cells recovered in this study were from the monocyte/macrophage class [16]. In our study, the mean ratio between the cellular and extracellular, i.e. plasma, concentration (C/E) of posaconazole within PBMCs (22.5 ± 22.1) was numerically lower than the mean AC/plasma ratio in the quoted studies, although this difference was not significant [32].

Information about intracellular azole concentrations within cells of the peripheral blood only stem from exclusively laboratory-based experiments on the interaction of fluconazole [68] and voriconazole [5] with PMNs. In these experiments PMNs were incubated with radiolabelled fluconazole and voriconazole, respectively in Hanks' balanced salt solution (HBSS). The uptake and elution of both representatives of the azole class was rapid and independent of pH and temperature, suggesting a passive transport mechanism. After in vitro incubation the intracellular concentrations of fluconazole and voriconazole were significantly increased (2.2-fold, and 8.5-fold, respectively)

compared to the surrounding medium. However, in another *in vitro* study it was shown that the uptake of itraconazole by AMs depended on the composition of the surrounding medium, with alveolar macrophage C/E ratios of 88, 18, and 3 for 0%, 5% and 100% serum, respectively [70]. Hence, the previously quoted PMN C/E ratios for fluconazole and voriconazole might not concur with their C/E ratios *in vivo*. Therefore, the peripheral blood cells used in the presented work were isolated directly from whole blood samples of patients receiving posaconazole. The mean posaconazole PMN C/E ratio determined by this method was 7.66 ± 6.50. Assuming that these results and the C/E ratios of fluconazole and voriconazole were comparable, we would have anticipated a higher PMN C/E ratio for posaconazole, since the greater uptake of voriconazole compared to fluconazole was explained by its higher hydrophobicity [5] and posaconazole is even more hydrophobic than voriconazole. However, the fact that the posaconazole C/E ratio is not higher than that of voriconazole is in accordance with the assumption that the intracellular uptake might be abased by higher amounts of serum within the surrounding medium. To which extend interindividual compositions of the whole blood samples might have influenced the C/E ratio was not studied.

In addition to the intracellular concentration of posaconazole in PBMCs and PMNs, the concentration within RBCs was also assessed, since in volume they represent the majority of the peripheral blood cells. The posaconazole C/E ratio of the RBCs was 0.09 ± 0.05. Previously only the intracellular concentration of itraconazole within *in vitro* incubated RBCs from New Zealand white rabbits has been quantified. However, also in this case the surrounding medium only consisted of serum-free HBSS containing the lipophilic itraconazole, which might explain the high C/E ratio of 90.4 ± 10.1 [70].

As mentioned before, the intracellular concentrations of many other antifungals are only studied within alveolar cells. Among them are anidulafungin and

micafungin, 24 hours after the administration of the drug the AC/plasma C/E ratios were 33.4 [23] and 6.2 [113], respectively. Due to their structural resemblance this difference of C/E ratios between both substances is striking. However, the samples were obtained at different numbers of doses; while the micafungin samples were obtained after a single or the last dose, the anidulafungin samples were obtained after the third dose. The authors of the respective studies did not comment on these differences. The maximal C/E ratios of itraconazole, posaconazole, and voriconazole in alveolar cells were 5.4 (12 hours after the last dose) [17], 44.3 (5 hours after the last dose) [18], and 6.5 (12 hours after the last dose) [23], respectively.

4.3. POSSIBLE EFFECTS OF HIGH INTRACELLULAR ANTIFUNGAL CONCENTRATIONS

The varying posaconazole concentrations within different compartments of the peripheral blood might in part contribute to the varying posaconazole concentrations that are observed in plasma [32]. The intracellular uptake of the azoles appears to be passive [5, 68] and dependent on the composition of the extracellular medium [70]. Hence, the intracellular and consequently the extracellular concentrations would instantly change upon entering different body compartments with different extracellular medium compositions. For example, since the protein concentration within the lymphoid tissue (3-5 g/L) is much lower than in the blood (60-80 g/L), lymphocytes within lymphoid tissue, or the Peyer's patches, might take up the azoles and release them at the sites of inflammation or within the peripheral blood. Furthermore, about 50% of the neutrophil granulocytes adhere to endothelial walls, especially of the lung and spleen vessels, from where they may be rapidly mobilised [39].

Apart from possible effects on the pharmacodynamics and pharmacokinetics the intracellular concentrations may well contribute to the intracellular killing of some fungi. Especially for drugs acting against pathogens which are primarily eliminated by professional phagocytes (PP), intracellular concentrations may be of importance. High intracellular concentrations may result in an optimal exposure of the pathogen to the drug.

Hence, the high intracellular concentration of posaconazole might contribute to its superior prophylactic efficacy compared to other antifungal drugs, i.e. fluconazole [20, 106] and itraconazole [20]. Voriconazole, which displays lower intracellular concentrations than posaconazole, was not superior compared to fluconazole [116].

It was shown that intracellular voriconazole in monocyte-derived macrophages augments the killing of intracellular *Candida glabrata*, *C. krusei* and *C. parapsilosis* [7]. The intracellular killing of *Cryptococcus neoformans* by PMNs and PBMCs was also improved by voriconazole. This effect could be enhanced even further by the addition of granulocyte-macrophage colony stimulating factor GM-CSF [13].

However, intracellular voriconazole concentrations were not assessed in these studies. Further studies on this area may take heed of the varying uptake with respect to the surrounding medium and different cells types [32].

Another study examined the resistance of Zygomycetes, *Rhizopus oryzae*, *Rhizopus microsporus* and *Cunninghamella bertholletiae* versus PMN-derived reactive oxygen species (ROS) with and without antifungals, such as posaconazole and voriconazole [94]. The hyphae were equally resistant against PMNs, whether alone or in combination with antifungals. In fact, a trend towards an attenuated hyphal damage was observed for the combination of voriconazole and PMNs. The authors suggested that this might be caused by a potential immunomodulatory effect of the antifungal.

The efficacy of PPs depend on their functional capacities, such as actively migrating to the site of infection (chemotaxis), engulfing pathogens (phagocytosis), releasing ROS (oxidative burst), and killing of phagocytosed pathogens. Even the reduction of just one of these capacities leads to a suppressed immunologic response [93]. In patients with severe infections, not caused by fungi, the use of antifungals might impair the elimination of the actual causal pathogen. The “perfect” antifungal would exhibit maximal efficacy against the pathogen, without inhibiting the functional capacities of the PPs. Hence, further studies should evaluate possible effects on the functional capacities of PPs, as a result of the intracellular accumulation of antifungals more thoroughly.

5. SUMMARY / ZUSAMMENFASSUNG

5.1. SUMMARY

The introduction of new antifungals led to a considerable improvement of the therapeutic options against invasive fungal infections (IFI). However, despite targeted therapies, the amount of patients failing to respond to their treatment is high. Unreliable pharmacokinetics, with vast inter- and intra-individual variances of the plasma concentrations, may be one cause for treatment failures. With regard to the question whether cells of the peripheral blood are a relevant compartment for the pharmacokinetics of antifungal drugs a method for the quantitation of those drugs (anidulafungin, caspofungin, isavucoazole, micafungin, posaconazole and voriconazole) within different compartments of the peripheral blood was developed. It was shown that the intracellular concentration of posaconazole in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leucocytes (PMNs) was greatly increased (22.5- and 7.66-fold respectively) compared to the plasma concentration. The intracellular concentrations of antifungals are of particular interest, since they target pathogens, which are eliminated by professional phagocytes (PP) or may even persist intracellularly. High intracellular concentrations might result in an optimal exposition to the drug and thus improve killing.

5.2. ZUSAMMENFASSUNG

Neue Antimykotika haben zu einer deutlichen Verbesserung der therapeutischen Möglichkeiten invasiver Mykosen (*invasive fungal infections*, IFIs) geführt. Dennoch zeigen viele Patienten kein Ansprechen auf eine gezielte antimykotische Therapie. Die Gründe hierfür werden unter anderem in einer unverlässlichen Pharmakokinetik mit einer hohen inter- und intraindividuellen Variabilität der Plasmaspiegel gesucht. Im Hinblick auf die Fragestellung, ob die Zellen des peripheren Blutes ein relevantes Kompartiment für die Pharmakokinetik von Antimykotika darstellen könnten, haben wir eine Methode zur Bestimmung verschiedener Antimykotika (Anidulafungin, Caspofungin, Isavuconazol, Micafungin, Posaconazol und Voriconazol) in unterschiedlichen Kompartimenten des peripheren Blutes entwickelt. Messungen der intrazellulären Konzentration von Posaconazol zeigten, dass die Konzentration von Posaconazol sowohl innerhalb der mononukleären Zellen des peripheren Blutes (PBMCs), als auch innerhalb der polymorphkernigen Leukozyten (PMNs) gegenüber Plasmakonzentration 22.5-fach, bzw. 7.66-fach erhöht war. Für therapeutische Substanzen, die sich gegen Pathogene richten, die primär durch professionelle Phagozyten eliminiert werden oder gar die Fähigkeit zur intrazellulären Persistenz haben, sind die intrazellulären Konzentrationen von besonderem Interesse. Einerseits können hohe intrazelluläre Konzentrationen des Medikamentes eine optimale Exposition des Erregers hinsichtlich seiner therapeutischen Wirksamkeit erreichen, andererseits können sie toxisch auf die Zelle wirken und so die erfolgreiche Eliminierung des Erregers beeinträchtigen.

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7. PREVIOUS PUBLICATIONS

Farowski, F., et al. (2010). Quantitation of azoles and echinocandins in compartments of peripheral blood by liquid chromatography-tandem mass spectrometry. Antimicrob Agents Chemother. 54(5): 1815-9 (Appendix 1)

Farowski, F., et al. (2010). Intracellular concentrations of posaconazole in different compartments of peripheral blood. Antimicrob Agents Chemother. 54(7): 2928-31 (Appendix 2)

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Quantitation of Azoles and Echinocandins in Compartments of Peripheral Blood by Liquid Chromatography-Tandem Mass Spectrometry▽†

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A rapid turnaround is a prerequisite of therapeutic drug monitoring (TDM). For antifungals, this need is still unmet, since hardly any method has been established to simultaneously quantitate concentrations of different antifungal classes. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed allowing quantitation of anidulafungin (ANF), caspofungin (CSF), isavuconazole (ISC), micafungin (MCF), posaconazole (PSC), and voriconazole (VRC). Quantitation was successful with diluted plasma samples, peripheral blood mononuclear cells (PBMC), polymorphonuclear leukocytes (PMN), and erythrocytes (RBC). A triple quadrupole mass spectrometer in selected reaction monitoring mode was used with positive electrospray ionization. Cells and calibration standards were extracted with acetonitrile containing internal standard. Internal standards were a CSF derivate for echinocandins and itraconazole for triazoles. Chromatographic separation of the supernatant was achieved by a gradient method facilitating a BetaBasic C4 column. Analytes were quantified in a single 8-min run. Calibration curves were linear and fitted using least squares with a weighting factor of the reciprocal concentration. Limits of detection (ng/ml) were ANF, 8.3; CSF, 31.5; ISC, 1.5; MCF, 97.7; PSC, 3.3; and VRC, 1.4. The lower limits of quantitation (ng/ml) were ANF, 64; CSF, 108; ISC, 4.5; MCF, 160; PSC, 10; and VRC, 4.2. Intraday precisions ranged from 6.3% to 8.8% for azoles and 8.8% to 15.4% for echinocandins. Intraday and interday accuracies (percent bias) of all analytes were within 13.8%. The method was established as standard practice for the quantitation of intracellular antifungal concentrations and optimizes TDM by applying a rapid single method for 6 antifungals.

Therapeutic drug monitoring (TDM) aims at optimizing the benefits and risks of pharmacotherapy, specifically for drugs exhibiting significant pharmacokinetic variability (18, 34).

TDM of antifungals is increasingly recommended to tailor therapy for individual patients (25, 28). Itraconazole (ITC) plasma concentration correlates with prophylactic efficacy (15). Voriconazole (VRC) exhibits nonlinear pharmacokinetics likely caused by saturation of its P450-dependent clearance (19). Recently, correlations of efficacy and toxicity with seemingly difficult-to-predict VRC plasma levels have been described (4, 21, 25, 35, 41). Posaconazole (PSC) demonstrates linear pharmacokinetics, but with a saturation of absorption effect above a daily dose of 800 mg (10). There is an exposure-dependent response rate for invasive aspergillosis (38), and while FDA reports suggest a possible correlation of lower plasma levels and failure of antifungal prophylaxis (30), no cutoff PSC plasma concentration could be defined for breakthrough fungal infections (8). For isavuconazole (ISC), phase III clinical trials are currently ongoing, and it might be too early to assess the role of TDM for this drug.

Relative to the azoles, echinocandins are larger and structurally different molecules; hence, we expect different patterns of distribution. Currently, three antifungals of the echinocandin class, i.e., anidulafungin (ANF), caspofungin (CSF), and micafungin (MCF), are commercially available (1, 22, 27). Common to all echinocandins is their low oral bioavailability, high protein binding, and relatively low urinary excretion of the parent drug. The relation between echinocandin blood levels and treatment outcome is currently undefined.

Some drugs may achieve different concentrations in different compartments and tissues. Recently, a 33-fold increased PSC level, i.e., for the maximum concentration of drug (C_{max}) and the area under the curve (AUC), was found for alveolar cells compared to plasma concentrations (7). Peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN) are important pillars of host defense against invasive fungal infections (IFI). After phagocytosis, intracellular antifungal concentrations may well influence their lytic capability. For example, it is known that intracellular voriconazole in monocyte-derived macrophages augments the killing of intracellular *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis* (3), and synergy of itraconazole cocultured with macrophages on the killing of *Blastomyces dermatitidis* has been described (5). However, little is known about the inter-

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action of antifungal drugs with peripheral blood cells. The only data published refer to fluconazole (FLC) and VRC interacting with PMN, both undergoing rapid intracellular uptake and elution. While FLC concentrations were twice as high as those in the surrounding medium (26), VRC concentrations were 8.5-fold (2). ISC and PSC are expected to behave likewise, but this has not been proven so far. The same holds true for the echinocandins.

Since plasma concentrations, especially of PSC and VRC, show substantial variability (16, 36, 37), their intracellular concentrations might more accurately correlate with their efficacy. We therefore developed a feasible method of quantitating most clinically relevant antifungal drugs in different compartments of the peripheral blood.

TABLE 1. Monitored transitions, retention times, and selected internal standards[a]

Compound	Mass transition (*m/z*)	Collision energy (eV)	Retention time (min)	Selected internal standard
ANF	1,140.8 → 1,122.5	18	4.6	CIS
CSF	547.4 → 137.1	34	2.3	CIS
CIS	548.0 → 62.2	20	2.3	NA
ISC	438.2 → 224.0	28	5.9	AIS
AIS	705.2 → 392.1	40	5.8	NA
MCF	1,270.9 → 1,172.4	25	4.0	CIS
PSC	701.3 → 683.2	34	4.4	AIS
VRC	350.1 → 127.0	38	3.7	AIS

[a] ANF, anidulafungin; CSF, caspofungin; CIS, caspofungin internal standard; ISC, isavuconazole; AIS, azole internal standard; ITC, itraconazole; MCF, micafungin; PSC, posaconazole; VRC, voriconazole.

MATERIALS AND METHODS

Chemicals and reagents. ANF was kindly provided by the Central Research Division, Pfizer Inc. (Groton, CT). CSF and CSF internal standard (CIS) were kindly provided by Merck Research Laboratories (Rahway, NJ). CIS, which is a derivate of CSF, was used as an internal standard for all echinocandins. ISC was kindly provided by Basilea Pharmaceutical AG (Basel, Switzerland). MCF was kindly provided by Astellas Pharma Inc. (Osaka, Japan). PSC was kindly provided by the Chemical Research Division, Schering-Plough Research Institute (Kenilworth, NJ). VRC was kindly provided by the Central Research Division, Pfizer Inc. (Sandwich, United Kingdom). The internal standard ITC for the azole antifungals (AIS) was purchased from Jannsen Research Foundation (Beerse, Belgium). Methanol and acetonitrile were purchased from Roth (Karlsruhe, Germany). Histopaque 1077 and Histopaque 1119 were both purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Formic acid, sodium chloride, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). All reagents were of analytical or high-performance liquid chromatography (HPLC) grade. Deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA), was used. HPLC eluent A was water with 0.1% formic acid. Acetonitrile was used as HPLC eluent B.

Materials and equipment. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a triple-stage quadruple mass spectrometer (TSQ Quantum; Thermo Finnigan, San Jose, CA), working in selected reaction monitoring (SRM) mode with positive electrospray ionization (ESI). The system was equipped with a Surveyor quaternary narrow-bore LC pump, a Surveyor autosampler, fitted with a temperated tray and column oven. The Finnigan Xcalibur software (version 1.3), installed on a personal computer, was used for instrument control and data acquisition. The MS/MS conditions were optimized using the Quantum Tune Master software (Thermo Finnigan). The HPLC column used was a BetaBasic C4 (100 by 3.0 mm; Thermo Hypersil-Keystone, Dreieich, Germany). Attached to the HPLC column was a guard column (10 by 3.0 mm; Thermo Hypersil-Keystone, Dreieich, Germany).

Standards. Stock standard solutions of all analytes and the internal standards were prepared in either methanol-water (50:50 [vol/vol]) for the echinocandins or methanol for the azoles. The concentrations were 64 mg/liter for ANF, 108 mg/liter for CSF, 40 mg/liter for CIS, 50 mg/liter for ISC, 30 mg/liter for AIS, 80 mg/liter for MCF, 124 mg/liter for PSC, and 96 mg/liter for VRC. The stock solutions of ANF, CSF, ISC, MCF, PSC, and VRC were spiked into plasma samples that were diluted 10 times with phosphate-buffered saline (PBS) to obtain working standard solutions containing the analytes. The calibration standards (CS) were prepared by further diluting the working standards with diluted plasma, since the preparation of CS in cell samples was not considered feasible. Diluted plasma samples were considered to be the most reliable matrix condition. The calibration curves consisted of eight standards containing the concentrations listed in Table S1 in the supplemental material. All CS solutions were stored at −20°C until analyzed.

Sample processing. Whole blood was collected in two EDTA-treated tubes (16 ml). Blood was diluted with PBS to a volume of 24 ml and carefully layered onto a double-discontinuous Ficoll-Hypaque density gradient (Histopaque 1077 and 1119; Sigma-Aldrich, Munich, Germany) with equal volumes and spun at 850 × *g* for 30 min at 20°C. After centrifugation, the plasma was drawn from the upper layer, diluted 10 times with PBS, and stored at −20°C until used. PBMC and PMN were collected on the corresponding layers at densities ranging from 1.024 to 1.077 g/ml and 1.077 to 1.119 g/ml, respectively. A volume of 500 μl RBC was collected from the bottom of the tube. To ensure that the PBMC and PMN cells fractions were free of RBC, a hypotonic lysis with phosphate buffer (pH 7.4) was performed just before the washing steps. The cells were washed twice with PBS (pH 7.4, 150 mM). The absence of antifungals in the washing solution was checked by taking 500 μl of each solution. Counts were determined in a Neubauer chamber, and the cell pellets were stored at −20°C until analysis. The volume of each pellet was calculated based on the total cell count and the mean cell volume, i.e., 0.4 pl for PBMC, 0.334 pl for PMN, and 0.09 pl for RBC. The isolated PBMC and PMN cells (~10^7 cells, i.e., 3 or 4 μl) were extracted with 100 μl acetonitrile containing internal standard by sonication for 10 min, vortexing for 1 min, and centrifugation for 10 min at 4°C and 15,000 × *g*. The calibration standards and diluted plasma samples, 30 μl for each preparation, were treated analogously. For the extraction of the RBC, 1 ml, instead of 100 μl, acetonitrile containing the internal standard was used.

Chromatography. Aliquots containing 20 μl of the clear supernatants were injected onto the BetaBasic C4 column (100 by 3.0 mm, 5 μm) with its corresponding guard column (10 by 3.0 mm). The temperature of the column was maintained at 30°C, and the tray temperature in the autosampler was kept at 4°C. The system was equilibrated until the stability of total ion current (TIC) was reached for a minimum of 2 h. Subsequent to sample injection, the autosampler syringe and the injection needle were repeatedly rinsed with methanol-water (20:80 [vol/vol]). A gradient program was worked out for HPLC separation with a constant flow rate of 250 μl/min. The initial composition of eluents A and B was 50% each. Within 3.2 min, the fraction of eluent B was increased from 50% to 75% and increased further to 90% within 0.3 min. This composition of 10% eluent A and 90% eluent B remained constant for 3.3 min, before the composition was restored to 50% eluent A and 50% eluent B within 1.2 min. The retention times (RT) were 2.3 min for CSF and CIS, 4.0 min for MCF and VRC, 4.4 min for PSC, 4.6 min for ANF, 5.8 min for AIS, and 5.9 min for ISC (Table 1). The total duration of each run was 8 min.

Mass spectrometric conditions. The detection was performed by a TSQ Quantum mass spectrometer fitted with an ESI source operating in positive ion and selected reaction monitoring mode. The following settings resulted in the best ion yield: capillary voltage, 3.4 kV; and desolvation temperature of heated capillary, 350°C. Nitrogen was used as sheath and auxiliary gas, set to 37 and 4 (arbitrary units), respectively. The mass spectrometer operated with a full width at half maximum (FWHM) of 0.7 Th for Q1 and Q3. The detection of the analytes was performed in SRM mode with a scan width of 0.5 Th for all channels. The argon collision gas pressure was set to 1.5 mtorr. The collision energy was set to 18 eV for ANF, 34 eV for CSF, 20 eV for CIS, 28 eV for ISC, 40 eV for AIS, 25 eV for MCF, 34 eV for PSC, and 38 eV for VRC. The following SRM transitions of $[M+H]^+$ precursor ions to product ions were selected for each analyte (Table 1): *m/z* 1,140.8 → 1,122.5 for ANF; *m/z* 547.4 → 137.1 for CSF; *m/z* 548.0 → 62.2 for CIS; *m/z* 438.2 → 224.0 for ISC; *m/z* 705.2 → 392.1 for AIS; *m/z* 1270.9 → 1,172.4 for MCF; *m/z* 701.3 → 683.2 for PSC; and *m/z* 350.1 → 127.0 for VRC. The scan time (dwell time) for the SRM channels was set to 200 ms for all analytes.

Integration of peaks. The Thermo Finnigan processing software LCquan (version 1.3) was used to integrate the peaks using the Interactive Chemical Information System (ICIS) peak detection and integration algorithm, which has advanced peak detection efficiency at low MS signal levels. To improve graphical appearance and hence the peak detection, the boxcar smoothing algorithm was applied to reduce the noise levels. All chromatograms were reviewed and, if necessary, reintegrated manually. However, if the change of the peak area was

TABLE 2. LOD and LLOQ[a]

Compound	LOD (ng/ml)	LLOQ (ng/ml)
ANF	8.3	64
CSF	31.5	108
ISC	1.5	4.5*
MCF	97.7	160
PSC	3.3	10*
VRC	1.4	4.2*

[a] Limit of detection (LOD = $3.3\sigma/S'$; σ, standard deviation; S', slope of the calibration curve) and lower limit of quantification (LLOQ) for ANF, CSF, ISC, MCF, PSC, and VRC. *, estimated: LLOQ = $10\sigma/S'$.

negligible (<1%) compared to the method setting, the integration was reset to the method setting.

Calibration and calculation. At the end of each sample queue (maximum of 30 samples), the calibration curves were recorded using a minimum of three sets of eight different known concentrations of analytes (excluding blank values) (see Table S1 in the supplemental material). A weighted (1/concentration) linear least-squares regression of the peak area ratios of analyte to internal standard versus the concentration was used to obtain the calibration curves. These curves were then used to calculate the concentrations of the analytes.

Method validation. The validation of the quantitation method was performed with respect to linearity, intra- and interday accuracy, and precision. The accuracy was calculated by the difference between observed and nominal concentrations expressed as percent bias. The precision was evaluated as the standard deviation of the observed concentration divided by the mean concentration expressed as percent relative standard deviation (RSD). The lower limit of detection (LOD) was calculated using the equation LOD = $(3.3\sigma)/S'$, where σ is the standard deviation for the calibration curve and S' is its slope (20). For echinocandin antifungals, the lower limit of quantitation (LLOQ) was defined as the lowest concentration within the calibration range with an RSD of less than 20%. The LLOQ of the azoles was not covered by the calibration range; hence, it was determined using the equation LLOQ = $(10\sigma)/S'$, thus implying a signal-to-noise (S/N) ratio of 10 for the LLOQ. The stability of samples of all concentrations was investigated after four freeze-and-thaw cycles. During each cycle, the samples were thawed at room temperature for 1 h and again frozen at −20°C overnight. The recovery of the analytes from diluted blank plasma was assessed by comparing the peak intensities of the extracted samples in replicates of three with those of the unextracted standards, representing 100% extraction efficiency.

RESULTS

The calibration curves of all analytes were linear and fitted by using least squares with a weighting factor of the reciprocal concentration ($1/x$). The correlation coefficients of these curves were 0.99 or better. The precisions and accuracies were evaluated over the entire concentration range (see Table S2 in the supplemental material). The accuracies were within ±13.8% for ISC, ±10.9% for PSC, and ±12.2% for VRC. The intraday precisions (RSD) were within ±8.8%, ±6.3%, and ±6.9% for ISC, PSC, and VRC, respectively, while the interday assays of the azoles (i.e., ISC, PSC, and VRC) showed a variability of up to 12.8%.

The estimated LLOQs for the azoles were 4.5, 10, and 4.2 ng/ml for ISC, PSC, and VRC, respectively. For ANF, CSF, and MCF, the LLOQ was reached at 64, 108, and 160 ng/ml, respectively. The LODs and LLOQs are shown in Table 2. Above the LLOQ, the accuracies of the echinocandins were within ±11.7%, ±11.9%, and ±11.1% for ANF, CSF, and MCF, respectively. The intraday precisions (RSD) of the echinocandins above the LLOQ were ±15.4% for ANF, ±8.1% for CSF, and ±8.0% for MCF. The respective intraday and interday assays of all analytes are summarized in Table S2 in the supplemental material. In all stability tests (see Table S3 in the supplemental material), no significant decrease in drug concentrations was substantiated, i.e., mean changes in concentrations of the analytes were within the variability of the method.

The mean extraction efficiencies from diluted plasma were 94.0% for ANF, 68.0% for CSF, 78.0% for CIS, 89.5% for ISC, 93.7% for MCF, 98.1% for AIS, 93.3% for PSC, and 91.0% for VRC (see Table S4 in the supplemental material).

Figure 1 shows a chromatogram of diluted pooled blank plasma (Institute of Transfusion Medicine, Cologne, Germany) spiked with 5 μg/ml ANF, 5 μg/ml CSF, 5 μg/ml CIS, 1 μg/ml ISC, 1 μg/ml AIS, 5 μg/ml MCF, 1 μg/ml PSC, and 1 μg/ml VRC in one sample. The signals are clearly separated and well shaped. A chromatogram of pooled blank plasma is shown in Fig. S1 in the supplemental material.

Clinical application. The quantitation method is currently in use to analyze samples collected as part of the Cologne biobank protocol (University of Cologne Ethics Committee; 08-160) on *Improving Diagnosis of Severe Infections in Immunocompromised Patients* (ISI) (9). Figures S2 to S4 in the supplemental material show representative chromatograms of PSC in PBMC, PMN, and RBC extracted from whole-blood samples from a patient receiving PSC at 200 mg three times a day (t.i.d.).

DISCUSSION

We describe a sensitive LC-MS/MS method to determine concentrations of ANF, CSF, ISC, MCF, PSC, and VRC in different compartments of the peripheral blood. This method can be used to measure this selection of antifungals by only one method, hence replacing various other methods currently used in parallel and thus optimizing the cost effectiveness of antifungal therapeutic drug monitoring, while ensuring a rapid turnaround (<1 day), regardless of the variety of antifungals currently in use. However, therapeutic drug monitoring is not a standard for the treatment with echinocandins, and it is unknown whether TDM in this setting has the potential to increase the efficacy of those regimens.

The lower limit of quantitation (LLOQ) was reached at 64, 108, and 160 ng/ml for ANF, CSF, and MCF, respectively. However, since the injection volume (20 μl) is relatively small compared to the extraction volume of 100 μl, a dry freezer could be facilitated to constrain the extract and therefore improve the sensitivity of the method.

Pascual et al. and Ballesta et al. both used radiometric assays to determine the intracellular uptake and elution of FLC and VRC, respectively (2, 26). Conte et al. as well as Nicasio et al. described methods (7, 23) which were modified for measuring PSC and MCF in bronchoalveolar lavage fluid and alveolar macrophages (7, 23). Although those authors presented a higher sensitivity, such low concentrations are actually not required for the purpose of our method. Our method was optimized with regard to the injection and sample volumes. In a previous report, an LC-MS/MS method developed for ISC in plasma and urine was also more sensitive than the method presented here (31, 32), but, compared to our method, a larger sample volume was required (50 μl versus 30 μl). Compared with published LC-MS/MS procedures for VRC in blood (13) and aqueous humor (40), the sensitivity of our method is similar to that of the published methods.

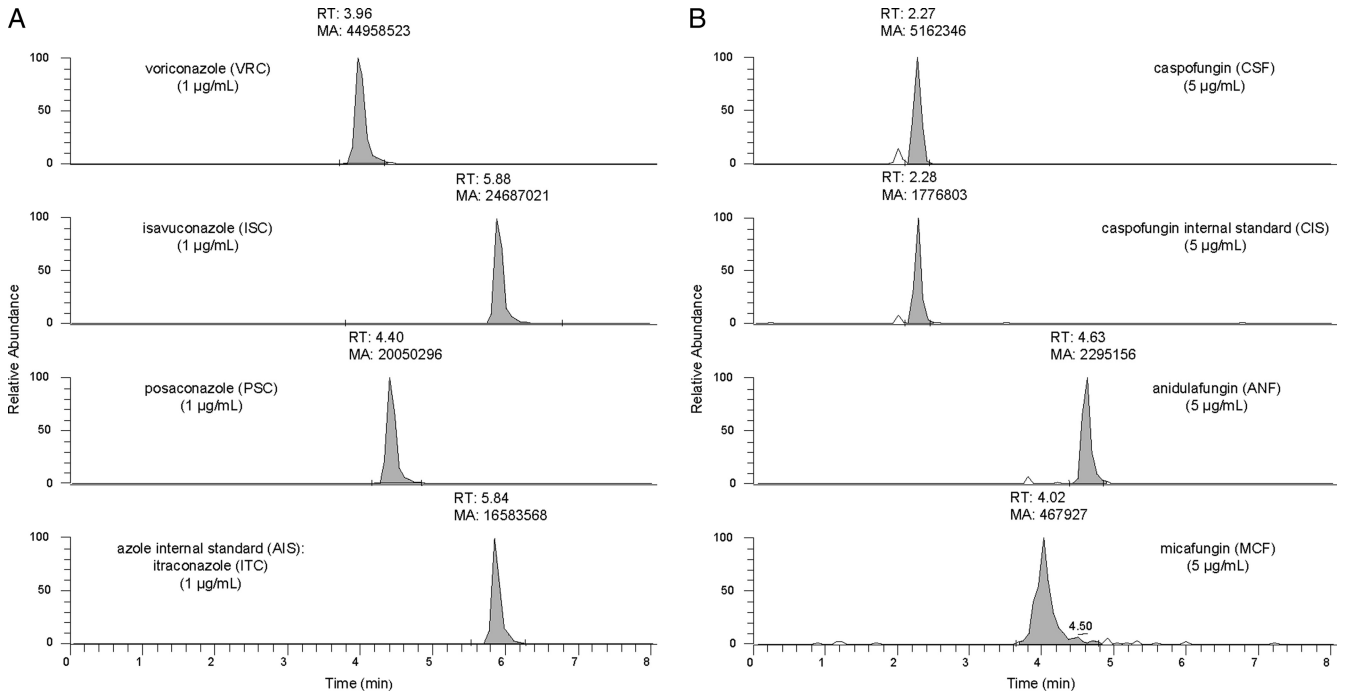

FIG. 1. Chromatogram of azole and echinocandin antifungals. (A) Azole antifungals voriconazole (VRC), isavuconazole (ISC), and posaconazole (PSC) and the azole internal standard (AIS) itraconazole (ITC), with 1 μg/ml each; (B) echinocardin antifungals caspofungin (CSF), anidulafungin (ANF), and micafungin (MCF) and the caspofungin internal standard (CIS), with 5 μg/ml each in spiked diluted plasma, demonstrating clearly separated and well-shaped peaks for all analytes.

At present, no LC-MS/MS method for the determination of MCF has been published. A previous HPLC method for quantitation of MCF in plasma showed an LLOQ of 50 ng/ml using a 100-μl sample (24, 39). Despite efforts to enhance the signal intensity of MCF, we were not able to achieve the same signal intensities as that for the other molecules. There is also a tailing of MCF during chromatography (Fig. 1). Only a complete change of the gradient program would significantly improve the peak quality of MCF but would probably result in a significant loss in the peak quality of the other analytes. Two LC-MS/MS methods for ANF in human plasma, using either efavirenz (11) or loradine (12) as an internal standard, were described previously. However, of the internal standards used, efavirenz and loradine, neither is a member of the echinocandin class nor has a molecular mass (316 and 383 g/mol, respectively) similar to that of ANF (1,140 g/mol). Their chemical structures and physicochemical properties evidently differ largely from those of ANF. We used as internal standards structurally similar compounds, which mimic the properties of the analytes more closely.

The quantitation of CSF in human plasma samples by LC-MS/MS was previously described by Chavez-Eng et al. (6), Egle et al. (14), and Rochat et al. (29). In the method published by Chavez-Eng et al., similar product ions were acquired for both CSF and CIS. Rochat et al. and Egle et al. used an ion transition with different product ions (m/z 547.4 → 137.1 for CSF; m/z 548.0 → 62.2 for CIS). Chavez-Eng et al.'s LC-MS/MS method deployed pneumatically assisted electrospray (ion spray [ISP]) and turbo ISP (TISP) interfaces with injection volumes of 50 and 75 μl, respectively. Following a solid-phase extraction, with a sample volume of 1 ml plasma, the eluent was evaporated and reconstituted in a 100-μl mobile phase. Owing to the large sample volume, the LLOQs of this method were 10 ng/ml and 2.5 ng/ml using the ISP and TISP interface, respectively (6). Rochat et al. presented two different precipitation methods for the sample preparation, with sample volumes of 500-μl and 100-μl plasma samples, achieving LLOQs of 5 and 40 ng/ml, respectively. Like Rochat et al., we also deployed a precipitation method for the sample preparation; however, due to the fact that our sample volume was smaller (30 μl), our LLOQ was greater (108 ng/ml). The method described by Egle et al. consists of a column-switching technique, with inline extraction of CSF. The LLOQ of this method was 200 ng/ml using a 5-μl plasma sample. This column-switching method was too specific for our aim, and we had to make concessions with regard to sensitivity, while we developed a rapid method suitable for quantitating 6 antifungals in different compartments of the peripheral blood with a single method.

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Intracellular Concentrations of Posaconazole in Different Compartments of Peripheral Blood[▽]

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Therapeutic drug monitoring (TDM) of antifungal plasma concentrations is increasingly recommended. However, data on antifungal concentrations in the other compartments of the peripheral blood are limited. Hence, we collected 23 blood samples from 14 patients receiving posaconazole for prophylaxis of fungal infections. These samples were separated by double-discontinuous Ficoll-Hypaque density gradient centrifugation. The intracellular posaconazole concentrations of the obtained cells, i.e., the peripheral blood mononuclear cells (PBMCs), polymorphonuclear leukocytes (PMNs), and red blood cells (RBCs), were determined by liquid chromatography-tandem mass spectrometry. The intracellular concentrations of the PBMCs and PMNs were significantly higher than those of surrounding media ($P < 0.001$). The ratios between the intracellular and extracellular concentrations (C/E) were 22.5 ± 21.2, 7.66 ± 6.50, and 0.09 ± 0.05 for the PBMCs, PMNs, and RBCs, respectively. Posaconazole reaches high concentrations within human PBMCs and PMNs and is, to a lesser extent, present in RBCs. The high intracellular concentrations might contribute to posaconazole efficacy and distribution.

Posaconazole is an orally administered broad-spectrum triazole antifungal which has recently been approved for prophylaxis and treatment of opportunistic invasive fungal infections in immunocompromised patients. Posaconazole exposure depends on prandial status and drug-drug interactions, leading to intra- and interindividual variability (7, 12). Depending on the dosing group, a 38 to 68% interindividual variability of the pharmacokinetic parameters has been observed (14). The clinical significance of this finding is currently unclear (15).

The intestinal absorption is likely to be influenced by graft-versus-host disease and mucositis. Ullmann et al. also described a reduced bioavailability of posaconazole in neutropenic patients (16). Due to the extensive pharmacokinetic variability of posaconazole, therapeutic drug monitoring (TDM) has been discussed as a possible means of ensuring a sufficient exposure (9, 13). While TDM usually is performed by measurement of serum or plasma concentrations, little is known about the concentrations in other compartments of the peripheral blood, i.e., peripheral blood mononuclear cells (PBMCs), polymorphonuclear leukocytes (PMNs), and red blood cells (RBCs). PBMCs and PMNs are important pillars of host defense against invasive fungal diseases (IFD). Interactions with these cells might well influence the efficacy of antifungal drugs.

In a recently published trial by Conte et al., a 33-fold increase of the maximum concentration (C_{max}) and area under the concentration-time curve (AUC) of posaconazole in alveolar macrophages (AM) compared to those of plasma was shown (4). However, up to now, no data on the intracellular concentration of posaconazole in PBMCs, PMNs, and RBCs have been published.

The aim of the present work was to determine the intracellular concentrations of posaconazole in different compartments of the peripheral blood using a previously published liquid chromatography-tandem mass spectrometric assay (6).

In keeping with the established literature, we use the term "intracellular" throughout this article, although we are aware that the term "cell associated" may be more accurate.

MATERIALS AND METHODS

Patients. Whole blood obtained from patients ($n = 14$) receiving posaconazole at 200 mg three to four times per day was collected as part of the Cologne biobank protocol (08-160; University of Cologne Ethics Committee) on improving diagnosis of severe infections in immunocompromised patients (ISI) (5). All patients had hematological malignancies, were hospitalized in the University Hospital of Cologne, and required treatment with posaconazole for the prevention of invasive fungal infections. Patients were approached in the first half of 2009 upon recovery from severe neutropenia. Written informed consent was obtained prior to blood sampling. All samples were at trough levels and collected during the posaconazole steady-state phase.

Posaconazole assay. Blood samples were processed and analyzed as described previously (6). In brief, whole blood was collected in two EDTA salt-containing tubes (2 by 8 ml) and separated by double-discontinuous Ficoll-Hypaque density gradient (Histopaque 1077 and 1119; Sigma-Aldrich, Munich, Germany) centrifugation. The cells were collected at the corresponding layers and washed twice with phosphate-buffered saline (PBS). During the second washing step, a hypertonic lysis was implemented for the PBMC and PMN fractions in order to eliminate contaminating red blood cells (RBCs). Afterwards, the cells were counted in a Neubauer chamber. The volume of each pellet was calculated based on the total cell count and the mean cell volume; i.e., 0.4 pl for PBMCs, 0.334 pl for PMNs, and 0.09 pl for RBCs. The isolated PBMCs and PMNs were extracted

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TABLE 1. Mean posaconazole concentrations and ratios between the intracellular and extracellular concentrations[a]

Blood compartment	Mean PSC concn ± SD (ng/ml)	C/E ratio ± SD
PBMCs	12,764 ± 14,057[b]	22.5 ± 21.2
PMNs	4,031 ± 3,692[b]	7.66 ± 6.50
RBCs	50.8 ± 46.18[b]	0.09 ± 0.05
Plasma	603.2 ± 475.9	

[a] PBMCs, peripheral blood mononuclear cells; PMNs, polymorphonuclear leukocytes; RBCs, red blood cells; PSC, posaconazole; C/E, ratio between intracellular and extracellular concentrations.
[b] $P < 0.001$ compared to plasma (ANOVA and Dunnett T3 test).

with 100 μl acetonitrile containing itraconazole as an internal standard by sonication for 10 min, vortexing for 1 min, and centrifugation for 10 min at 4°C and 13,000× *g*. The RBCs (500 μl) were treated analogously with 1,000 μl acetonitrile containing the internal standard. The samples, i.e., the supernatants, were analyzed by liquid chromatography-tandem mass spectrometry, facilitating a method developed for the quantitation of antifungals in different compartments of the peripheral blood as previously described (6). The lower limit of quantitation (LLOQ) and the limit of detection (LOD) for posaconazole were 10 ng/ml, and 3.3 ng/ml, respectively. The calibration range was 10 ng/ml to 6,200 ng/ml. For the calibration standard, the accuracy (percent bias) ranged from −10.9% to 1.1%, and the precision (percentage of coefficient of variation; relative standard deviation [RSD]) ranged from 2.4% to 12.8%.

Statistical analysis was performed using IBM SPSS Statistics 17.0 computer software (SPSS Inc., IL). Values were compared with analysis of variance (ANOVA), using a logarithmic approach to improve the homogeneity of variance; intergroup differences were confirmed by a Bonferroni post hoc test, with multiple test corrections, as well as a Dunnett T3 test. *P* values lower than 0.05 were considered statistically significant. Figures were computed with GraphPad Prism 5.02 computer software (GraphPad Software Inc., CA).

RESULTS

Twenty-three samples obtained from 14 subjects receiving posaconazole for the prophylaxis of fungal infections were analyzed. The mean age (±standard deviation [SD]) of the 14 subjects was 54.8 ± 14.3 years; 9 subjects were male, and 5 were female, with mean (±SD) leukocyte, neutrophil, and RBC counts of 5.4 ± 5.9 × 10^6 cells/ml, 1.7 ± 1.9 × 10^6 cells/ml, and 3.2 ± 0.5 × 10^9 cells/ml, respectively. All subjects had hematological malignancies, including acute myelogenous leukemia (7 patients), non-Hodgkin lymphoma (4 patients), acute lymphoblastic leukemia (1 patient), aplastic anemia (1 patient), and chronic myelogenous leukemia (1 patient). The mean (±SD) weight of the subjects was 73.7 ± 10.8 kg. All subjects received posaconazole at 200 mg, three times ($n = 15$) to four times ($n = 8$) per day, resulting in a mean daily dose of 8.9 ± 2.1 mg/kg.

The mean posaconazole concentrations in PBMCs, PMNs, RBCs, and plasma are shown in Table 1. While posaconazole concentrations within the PBMCs and PMNs were significantly increased compared to the plasma concentration ($P < 0.001$; ANOVA and post hoc tests), the concentration within the RBCs was significantly decreased compared to those in all other compartments ($P < 0.001$; ANOVA and post hoc tests). The posaconazole concentration in PBMCs was also significantly increased ($P = 0.01$; ANOVA and post hoc tests) compared to that in the PMNs. For mean ratios between intracellular and extracellular concentrations (C/E) for the PBMCs, PMNs, and RBCs, refer to Table 1. The intracellular posaconazole concentrations at different extracellular concentrations are shown in Fig. 1. There was only a moderate relationship between PMN and plasma levels, which may have been caused by an outlier among the four samples with plasma levels of >1,000 ng/ml.

DISCUSSION

Posaconazole was present in all compartments of the peripheral blood; however, the concentrations within these compartments varied significantly. While the intracellular posaconazole concentrations within the PBMCs and PMNs was significantly increased (22.5-fold and 7.66-fold), the concentration within the RBCs was only 9% of that in the plasma.

The intracellular uptake of two representatives of the azole class, i.e., fluconazole (FLC) and voriconazole (VRC), by PMNs has been studied using radiometric assays (1, 10). Both drugs were characterized by a rapid intracellular uptake and

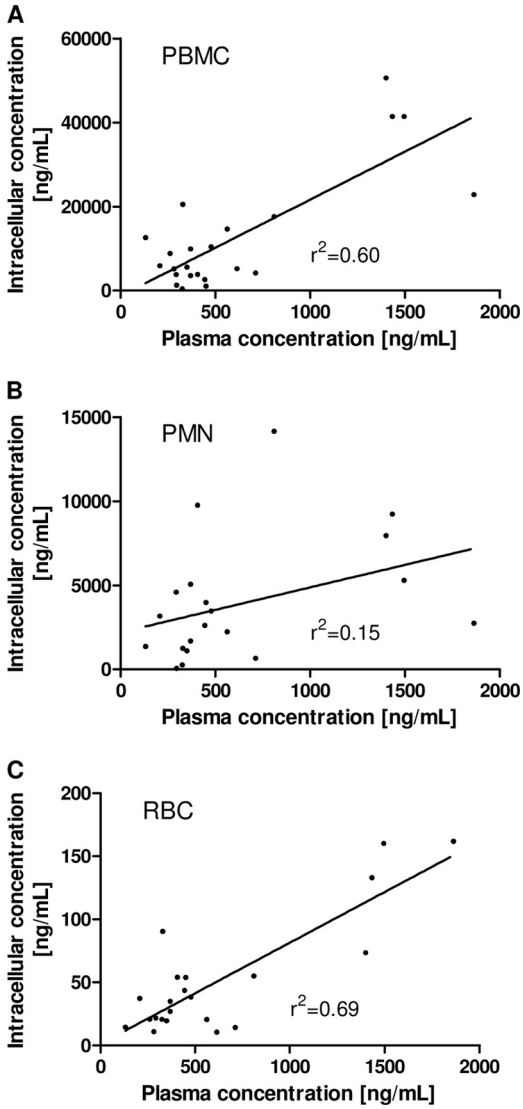

FIG. 1. PBMC-associated posaconazole (A; $n = 23$), PMN-associated posaconazole (B; $n = 20$), and RBC-associated posaconazole (C; $n = 22$) at different plasma concentrations.

elution. After *in vitro* incubation of PMNs with FLC (10) or VRC (1), the intracellular concentrations of these drugs were significantly increased (2.2-fold, and 8.5-fold, respectively) compared to those in the surrounding medium. The uptake was reversible, and independent of pH and temperature, suggesting a passive transport mechanism. The PMN C/E ratio of posaconazole was comparable with that of voriconazole (7.66 versus 8.5, respectively) (1), and higher than that observed for fluconazole (7.66 versus 2.2, respectively) (10). It was previously suspected that the greater uptake of voriconazole compared to that of fluconazole was caused by its higher hydrophobicity (1). While the greater hydrophobicity of posaconazole compared to that of voriconazole should allow an even higher intracellular uptake, it is also a much larger molecule, with a molecular weight about twice as high as that of voriconazole. We observed an almost equal uptake of these two drugs (1), which might be explained by the different medium compositions that were examined. We obtained the cells from whole blood, whereas Ballesta et al. (1) and Pascual et al. (10) incubated the cells in Hanks' balanced salt solution (HBSS). It may be speculated that highly liposoluble posaconazole accumulates in cell organelles and membranes without major involvement of active transport mechanisms; a similar model was discussed for the intracellular uptake of voriconazole (1).

The uptake of itraconazole by alveolar macrophages (AM) was previously studied using cells obtained from New Zealand White rabbits, which were infected with *Mycobacterium bovis* (11). The uptake was both rapid and substantial, with a C/E ratio of 87.9 ± 5.3 for the alveolar macrophages and 90.4 ± 10.1 for RBCs in serum-free medium. Beyond that, it was also shown that the uptake depended on the medium, with alveolar macrophage C/E ratios of 18 for 5% serum and 3 for 100% serum. The effect of different medium compositions on the uptake of itraconazole by RBCs was not studied (11). The observations on the uptake of itraconazole by alveolar macrophages are consistent with those made by Conte et al. (3), who studied intracellular levels of itraconazole in alveolar macrophages of healthy subjects. They demonstrated an overall itraconazole AM/plasma ratio of 3.4 ± 1.9 in healthy subjects after the administration of 10 doses of itraconazole (200 mg, twice daily).

In a study with 25 healthy subjects who received 14 doses of posaconazole (400 mg, twice daily) before undergoing bronchoscopy at different time intervals, the AM/plasma ratios ranged from 27.3 ± 18.0 to 44.3 ± 44.2 (4). In our study, the mean C/E ratio of the PBMCs was numerically lower than the mean AM/plasma ratio in the quoted study (22.5 versus 33, respectively) (4), although this difference was not significant.

To our knowledge, no data on the intracellular concentration of posaconazole exist, and apart from the above-cited animal study of itraconazole (11), no data on the intracellular concentration of azole antifungals in RBCs exist. The marked difference between the intracellular concentration of itraconazole in RBCs as reported by Perfect et al. (11) and the intracellular posaconazole concentration in our study might also have been caused by the different media used.

The various posaconazole concentrations within the different compartments of the peripheral blood might in part contribute to the various posaconazole concentrations that are observed in the plasma. The intracellular uptake of the azoles appears to be passive (1, 10) and dependent on the composition of the extracellular medium (11). Hence, the intracellular and, consequently, the extracellular concentrations would instantly change upon entering different body compartments with different extracellular medium compositions. For example, since the protein concentration within the lymphoid tissue (3 to 5 g/liter) is much lower than in the blood (60 to 80 g/liter), lymphocytes within lymphoid tissue, or the Peyer's patches, might take up the azoles and release them at the sites of inflammation or within the peripheral blood. Furthermore, about 50% of the neutrophil granulocytes adhere to endothelial walls, especially those of the lung and spleen vessels, from where they may be rapidly mobilized (8).

Apart from possible effects on the pharmacodynamics and pharmacokinetics, the intracellular concentrations may well contribute to the intracellular killing of some fungi. It was shown that, e.g., intracellular voriconazole in monocyte-derived macrophages augments the killing of intracellular *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis* (2). However, the intracellular voriconazole concentration was not assessed in that study. Further studies of this area may take heed of the varied uptake with respect to the surrounding medium and the different cells types. They may determine the intracellular concentrations by a quantitative assay.

We conclude that (i) administration of posaconazole oral solution results in significantly increased intracellular concentrations in PBMCs and PMNs compared to those in plasma and that (ii) posaconazole is, though to a lesser extent, present in RBCs. These intracellular concentrations might influence the distribution of posaconazole, possibly in part explaining its superior prophylactic efficacy compared to that of fluconazole and itraconazole. Future studies should explore a possible difference in plasma levels between neutropenic and nonneutropenic patients.

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8.3. CURRICULUM VITAE

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Name	Fedja Farowski
Date of Birth	Nov. 4th, 1976
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University education

1997-2004	Diploma student in Biochemistry at the University of Bielefeld. Final examinations in Biochemistry, Inorganic Biochemistry, Gene Technology and Immunology.

Research Activities & Professional Career

2004 (8 month)	Diploma thesis at the medical polyclinic of Bonn: "The influence of oestrogen receptor α and β on signalling cascades in the myocardium of mice."
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