Aqueous Humor Concentrations of Topically Administered Caspofungin in Rabbits

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Abstract
Background: The echinocandin caspofungin (CAS) is a novel antifungal drug with fungicidal in vitro activity against all Candida spp., which are the most frequent cause of fungal keratitis. Penetration of CAS through the cornea into the aqueous humor after topical administration was investigated. Methods: A CAS solution with a concentration of 7 mg/ml was applied onto each rabbit’s cornea. Drug application after corneal epithelium abrasion was processed in different time intervals: single application with aqueous humor sampling after 1 and 2 h. In addition, after continuous application of CAS every 30 min, aqueous humor concentrations of CAS after 1, 2 and 5 h were analyzed by liquid-chromatography tandem mass spectrometry. Results: Topical administration of CAS without corneal epithelium abrasion resulted in no detectable amounts of the drug in the aqueous humor. However, with corneal abrasion, after a single application, levels of 2.16 ± 1.57 µg/ml (n = 6) were reached after 1 h and then decreased to 1.76 ± 0.88 µg/ml (n = 2) after 2 h. After serial application every 30 min, the following intracameral levels of CAS were detected: after 1 h, 2.11 ± 1.09 µg/ml (n = 6); after 2 h, 4.94 ± 1.80 µg/ml (n = 5), and after 5 h, 3.45 ± 2.11 µg/ml (n = 6). Conclusion: In the aqueous humor, therapeutic drug levels can be reached that cover the MICs of most fungi after epithelial abrasion. To achieve a sustained high level of CAS as an effective antifungal therapy for corneal keratitis, CAS should be administered topically every 30 min after removal of the corneal epithelium.

Introduction
Once considered rare, the number of cases of fungal eye infections has increased during the last decade. Keratitis is the most frequent presentation [1], but the orbit, lids and lacrimal apparatus, conjunctiva, sclera and intraocular structures may also be involved. Comprehensive reviews of fungal diseases have been published by Behrens-Baumann [2] and Klotz et al. [3].

A representative of a new class of antifungal drugs is caspofungin (CAS), an echinocandin which has antifungal activity in vitro against all Candida spp., including strains resistant to fluconazole or voriconazole. In randomized clinical trials, CAS has been shown to be as effective as amphotericin B for the treatment of different invasive candidiases and oropharyngeal candidiasis [4–6]. Since CAS has a high molecular weight of 1,093.5 Da and is highly protein bound, corneal penetration across
the normal healthy cornea is reduced. Goldblum et al. [5] described topical CAS treatment in an animal model of Candida albicans keratitis to investigate the clinical implications of this drug in ocular diseases.

The aim of this study was to evaluate whether topical CAS can reach levels above the MIC₉₀ for C. albicans (0.06 μg/ml) in the aqueous humor after the removal of the corneal epithelium. To prove and underline the clinical outcome, we measured anterior chamber concentrations of CAS after topical treatment. Topical CAS treatment would potentially enable more effective treatment of keratomycosis with higher concentrations and possibly fewer side effects. Beside the fact that the infected site in fungal keratomycosis – the cornea – is the primary target of the drug, penetration through the infected tissue into deeper ocular structures is assumed to subsequently deliver effective pharmaceutical concentrations to the primary target.

**Material and Methods**

**Animals**

Twelve male, inbred albino rabbits weighing approximately 2.5 kg each were used. No pretreatment was performed. All animals were provided by an authorized breeding center (Harlan Winkelmann, Germany) and were kept in individual cages under well-defined and standardized conditions (humidity- and temperature-controlled rooms with a cycle of 13 h of light and 11 h of darkness). They received standard dry food and water ad libitum. All eyes were initially examined by an ophthalmologist with a handheld slitlamp. Only animals with no signs of ocular pathology were included.

To keep the number of animals needed down, we allowed sampling aqueous humor in the same animal and eye multiple times after full recovery of the epithelium. Other prerequisites were at least 10 days recovering from previous sampling and drug administration, and no sign of ocular pathology was revealed by handheld slitlamp examination. This procedure also assured the full realignment of the anterior chamber and eliminated a potential drug concentration factor due to aqueous humor removal.

All experiments were conducted in accordance with the Statement for the Use of Animals in Ophthalmic and Visual Research of the Association for Research in Vision and Ophthalmology and were approved by the German federal and local ethics committees.

**Administration of the Antifungal Agent**

CAS (caspofungin diacetate) as commercially available as powder (Cancidas®, 70-mg packages) was diluted in sterile normal saline to a final concentration of 7 mg/ml (0.9% NaCl). One drop containing 20 μl was administered to the cornea. We applied CAS at different time intervals to the cornea in unanesthetized animals. Prior to the administration, one set of animals received a corneal epithelium abrasion under local anesthesia with a hockey knife. Aqueous humor samples were taken with a 25-gauge needle. For this procedure, all animals were anesthetized with intragluteal injection of xylazine (3 mg/kg body weight) and ketamine (60 mg/kg body weight).

**Treatment Protocol**

In all cases, only one eye of each animal was treated. We administered one drop of CAS. The animals in the first experimental setting received a single drop of CAS every 30 min for 6 h without prior corneal epithelium abrasion. Since no aqueous humor concentration of CAS was detected, in all further experiments, treatment groups always underwent a corneal epithelium abrasion prior to the administration of the eyedrops. To answer the question of whether CAS accumulates in the anterior chamber, one set of animals received only a single CAS treatment, and aqueous humor samples were taken after 1 and 2 h following this single treatment. In addition, a second set of animals received continuous CAS eyedrops every 30 min, and samples were taken after 1, 2 and 5 h, respectively.

Before aqueous humor sampling, corneas were rinsed with sterile normal saline (0.9% NaCl) solution to avoid any contamination of the samples with remaining CAS from the corneal surface or conjunctiva.

**Quantification of CAS Levels by Liquid-Chromatography Tandem Mass Spectrometry**

We used an online-extraction method with an Alliance HT, an HLB-Oasis (2.0×10, 60 μm) and an Atlantis (3.0×50, 5 μm) column (Waters, Milford, Mass., USA), combined with a Quattro Micro (Waters), a triple-stage quadrupole instrument with electrospray interface. Standards and quality control samples were prepared in drug-free serum. 100 μl acetonitrile containing amphotericin B (internal standard) was added to the 50-μl sample, vortexed and centrifuged. The supernatant was decanted into an autosampler vial. The autosampler injected 20 μl supernatant onto the extraction column with 1 ml/min 0.1% acetic acid for 0.5 min. After activation of the switching valve, analytes were eluted with acetonitrile/1.0% acetic acid 7.3 vol/vol (40°C, 0.8 ml/min). Elution times of CAS and the internal standard were 2.53 and 2.72 min, respectively. Run time per sample was 4 min.

**Statistical Analysis**

All data were analyzed with SPSS software package and expressed as means ± standard deviation.

**Results**

Application of CAS on intact corneal epithelium every 30 min did not result in any detectable amount of the drug in the anterior chamber after 6 h (4 samples were tested).

A single application of CAS with prior corneal epithelium abrasion resulted in a peak of 2.16 ± 1.57 μg/ml (n = 6) after 1 h and a concentration of 1.76 ± 0.88 μg/ml after 2 h, respectively (fig. 1).

Continuous administration of CAS every 30 min led to an accumulative aqueous humor concentration of the...
antifungal agent. The highest levels were detected 2 h after the beginning of the treatment, compared to untreated controls. The following concentrations were reached by continuous CAS treatment (µg/ml): 2.11 ± 1.09 after 1 h (n = 6), 4.94 ± 1.80 after 2 h (n = 5) and 3.45 ± 2.11 after 5 h (n = 6; fig. 2).

In comparing CAS-treated with untreated corneas (both series with corneal epithelium abrasion), no toxic side effects on the cornea were found after histopathological evaluation. The treatment regimen for this series was the continuous administration of CAS drops every 30 min for 6 h (data not shown).

**Discussion**

Although fungal keratitis with subsequent fungal endophthalmitis is still rare, it remains an important clinical problem in ophthalmology. Potentially devastating consequences can result from this infection, and only limited therapeutic options are currently available, such as systemically or intravitreally administered antifungal agents. Until the early nineties, intravitreal injection of amphotericin B was still the treatment of choice in problematic cases. Unfortunately, side effects limited the intravenous use of this drug in many cases, as compared to the rather marginal intravitreal levels achieved after intravenous administration. However, it was O’Day and Head [1] who later demonstrated that the triazole agent fluconazole was able to achieve significant levels of this drug in the vitreous after systemic administration. This newer antifungal agent was therefore the first showing intraocular penetration due to lower protein binding and improved water solubility. However, fluconazole lacked a broad spectrum of coverage against many of the commonly encountered organisms found to cause fungal endophthalmitis [7].

To address the increasing evidence of fungal infections in general, CAS – an antifungal cell wall synthesis inhibitor of the novel class of echinocandins – was introduced. Effective against Candida and Aspergillus spp., it is thought that it should be administered only intravenously, due to its high molecular weight. In this study, we proved that for healthy eyes without any manipulation, topical administration of CAS would not result in any detectable amounts of intracameral CAS. Because of the chemical characteristics of CAS, this finding was not surprising.

However, this situation might be different in infected eyes. To investigate if substances with higher molecular weight could also penetrate the cornea under certain pathological circumstances, a corneal epithelium abrasion was performed prior to the instillation of CAS. The blood-eye barrier is considered to be critical for intraocular deposition of antifungal drugs. In inflamed eyes, breakdown and permeability of this barrier occur, and pharmacokinetics are altered under this specific condition [8]. Clinical investigations regarding the efficacy of

![Fig. 1. Aqueous humor (AH) concentrations (means ± SD) after single topical CAS application in rabbit eyes after corneal epithelium abrasion. Data shown after 1 (n = 6) and 2 h (n = 2).](image1)

![Fig. 2. Aqueous humor (AH) concentrations (means ± SD) after frequent topical CAS application every 30 min in rabbit eyes after corneal epithelium abrasion. Data shown after 1 (n = 6), 2 (n = 5) and 5 h (n = 6).](image2)
CAS have been conducted for oropharyngeal, esophageal and invasive candidiases [9]. Only a few studies were conducted using CAS in cases of ocular fungal infections. Goldblum et al. [5] reported the efficacy of topical CAS treatment in the management of C. albicans keratitis in a rabbit model. Clinical outcome by slitlamp scores, histopathology and quantitative fungal recovery rates were determined, suggesting that the topical 0.5% CAS is as safe and efficacious as topical 0.15% amphotericin B. For clinical reasons, combined treatment studies of antifungals and corticosteroids in rabbits have also been conducted [10]. Several studies were undertaken to evaluate ocular penetration of antifungals in healthy and infected rabbit eyes [11]. The observed ocular penetration of CAS in a rabbit model of uveitis with an impaired blood-eye barrier revealed that therapeutic levels of CAS within the eye may be obtained after systemic treatment.

Although antifungal effectiveness of CAS in infectious corneal and eye diseases does not depend on intracameral concentration alone, still further fulminant progression of infection can be prevented. The source and the spread of pathogens depend on various other aspects such as paradoxical biofilm-derived growth and formation of Candida as found in contact lens wearers, and therefore topical administration of antifungal drugs with corneal penetration can be an important treatment factor [12, 13].

In our study of topical ocular treatment with CAS in noninfected eyes, we demonstrated that, after removal of the corneal epithelium, the drug can penetrate into the anterior chamber of the eye and reach significant therapeutic levels to cover the MIC of relevant fungal species. However, no detectable CAS concentrations were found in unaffected eyes without corneal epithelium manipulation. Therefore, topical CAS for deep keratomycosis seems promising, if affected eyes undergo a corneal epithelium abrasion prior to the topical treatment. In this scenario, even topically applied drugs with a higher molecular weight, as shown in this study, will reach significant levels in the anterior chamber of the eye.

In addition to single reported cases of successful systemic CAS treatment of ocular fungal infections, further animal studies are urgently needed to validate and further improve the promising topical CAS treatment approach in fungal keratitis.

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