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Evaluating retinal toxicity of intravitreal caspofungin in the mouse eye

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Abstract

Purpose—Caspofungin is a synthetic echinocandin antifungal agent that inhibits the synthesis of β (1,3)-D-glucan, an essential component of the cell wall of susceptible *Aspergillus* and *Candida* species. In this study, we tested for retinal toxicity following intravitreal injection of caspofungin in a mouse model to assess its safety profile for the treatment of fungal endophthalmitis.

Methods—Caspofungin acetate was injected intravitreally in the left eyes of male C57BL/6 mice with final vitreal concentrations corresponding to 0.41 μM, 1.2 μM, 2.5 μM, 4.1 μM and 41. μM (5 mice per cohort). A total of 25 age-matched male C57BL/6 mice injected with balanced salt solution were used as controls (5 controls for each of the 5 different caspofungin acetate concentrations). Electroretinograms (ERG) were recorded 7 weeks after injections and subsequently the injected eyes were examined histologically.

Results—Mice injected with caspofungin at vitreal concentrations from 0.41 to 4.1 μM did not have significant alterations in their ERG waveforms, and their retinas had no detectable morphological changes or loss of cells. At the vitreal concentration of 41. μM, caspofungin reduced the amplitudes of the a-waves, b-waves, and scotopic threshold responses of the ERG and also produced a decrease in the number of cells in the ganglion cell layer.

Conclusion—Caspofungin is a safe antifungal agent at vitreal concentrations of 0.41 – 4.1 μM, in mice and consequently shows promise in the treatment of fungal endophthalmitis in humans. Much higher doses produce toxicity, and should not be used.

Keywords

Antifungal; Caspofungin; Retina; Electroretinogram; Mice

Introduction

Infectious endophthalmitis is an inflammatory response of intra-ocular fluid or tissues to infection that represents one of the most serious and vision-threatening conditions in ophthalmology [1]. According to past studies, mycotic or fungal endophthalmitis accounts for 8.6% to 18.6% of culture-positive endophthalmitis [2–4] with *Candida* spp. and *Aspergillus* spp. being the most frequently isolated organisms [4–7]. The prognosis of fungal endophthalmitis depends on the magnitude of intraocular involvement, the virulence of the organism, and the

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timing and mode of interventions. Because fungal endophthalmitis is a comparatively less common form of endophthalmitis of diverse etiology, it has been difficult to formulate an established treatment plan. Although systemic antifungals have been used in mild fungal endophthalmitis, intravitreal amphotericin B has traditionally been the drug of choice for moderate to severe cases of vitreous involvement or cases non-responsive to systemic treatment 8. Many studies have sought to find alternative antifungal pharmacological agents for intravitreal injections in the treatment of fungal endophthalmitis 9–14. Alternative antifungals would be useful for several reasons: 1) Intravitreal amphotericin B is pro-inflammatory and can cause focal retinal necrosis even at low doses, i.e., 4.1 μg/ml or 8.3 μg/ml 10, 15; 2) Resistance to amphotericin B is an emerging threat 16–18, necessitating alternative therapy or combination therapy; 3) Resistance to other systemically administered antifungals is also increasing; and 4) New antifungals will be required to improve on the spectrum of fungicidal activity.

Caspofungin acetate (1-[(4R,5S)-5-[(2-aminoethyl)amino]-N2-(10,12-dimethyl-1-oxotetradecyl)-4-hydroxy-L-ornithine]-5-[(3R)-3-hydroxy-L-ornithine] pneumocandin B0 diacetate salt) is synthesized from a fermentation product of *Glarea lozoyensis*. It belongs to the echinocandin group of antifungals, and like other members of this group it noncompetitively inhibits UDP-glucose β-(1,3)-D-glucan- β-(3)-D-glucosyltransferase (also referred to as 1,3-β-D glucan synthase), an enzyme that is necessary for the synthesis of an essential component of the cell wall of many fungal species, 1,3-β-D glucan 19–21. Inhibition of glucan synthase destabilizes the integrity of the fungal cell wall, ultimately resulting in cell lysis because of lack of rigidity and inability to resist osmotic pressure 22. We chose to study the retinal toxicity of caspofungin via intravitreal injections because of the following reasons: 1) It is effective against a wide variety of *Candida* spp. (with the exception of *C. parapsilosis* and *C. guilliermondii*) and *Aspergillus* spp. 22 which are the most common causative organisms for fungal endophthalmitis; 2) It is less toxic than amphotericin B 23; and 3) Caspofungin is efficacious for a wide variety of fungal species, but has shown poor intravitreal penetration when administered systemically in both experimental and case studies 24, 25, which necessitates a direct intravitreal administration of this drug. The poor intraocular penetration of caspofungin is most likely due to its high molecular mass (1213 Daltons; the blood-eye barrier is thought to be impermeable to molecules >500 Daltons 26). However, two other studies have shown that systemic caspofungin may be effective in fungal endophthalmitis 27, 28. Although resistance to echinocandins can occur due to mutations in the FKS1 or FKS2 genes, which code for 1,3- β -D-glucan synthase, the presence of a drug efflux pump in the fungal cell wall, or the overexpression of cell wall transport proteins 20, 29–31, the development of resistance to echinocandins has only been sparsely documented in the literature 32–36.

We used pigmented mice as a rodent model for our study, because like rats, the mouse retina (i.e. its scotopic circuit and retinal vascular structure) is very similar to humans. Moreover, the availability of transgenic and knockout animals with known defects opens the possibility for elucidating the exact mechanism of action and toxicity of intravitreally injected drugs 37. Similar to many other studies in the past, electroretinograms and retinal histology were employed as methods to assay for retinal toxicity 9, 10, 12, 13, 15, 37.

Materials and Methods

Animals

50 male C57BL/6 mice, ages 7 to 8 weeks old, at the inception of this study, were used as subjects. Animals were fed *ad libitum* with Purina lab chow and water, and were reared in a room with a 12 h light (<40 lux)/12 h dark cycle. All animal procedures conformed to US Public Health Service and Institute for Laboratory Animal Research guidelines and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

The experimental procedures were in accord with principles of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Caspofungin intravitreal injections

The mice were allowed food and water *ad libitum* before anesthesia. For each dose, the mice were divided into two cohorts of 5 mice each. One cohort was injected intravitreally with balanced salt solution (BSS) and the other was injected with caspofungin acetate dissolved in BSS (C+BSS). All injections were in the left eye. Before the administration of anesthesia, the pupils of the left eyes were fully-dilated using topical instillation of 1% tropicamide and 2.5 % phenylephrine on the cornea. The mice were then anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Under magnification of a zoom stereomicroscope (Nikon sm z800, Nikon Inc., NY, USA), a pilot hole was made with a 30 G needle 0.5 mm behind the limbus, and 1.5 µl of the injectate were delivered slowly with a 35G blunt needle (NF35BL-2, World Precision Instruments, Sarasota, FL, USA) connected to a 100 µl syringe (Nanofil, World Precision Instruments, Sarasota, FL, USA) via Silflex tubing (SifFlex-2, World Precision Instruments, Sarasota, FL, USA). The syringe, which was controlled by a foot pedal-activated microprocessor-based microsyinge pump controller (Micro 4, World Precision Instruments, Sarasota, FL, USA), was used to deliver the injectate at the rate of 170 nl/sec into the intravitreal space of the eye. Eyes were lubricated with methycellulose drops after injection, and Neosporin eye ointment (Bausch & Lomb Incorporated, Tampa, FL, USA) was administered topically after the procedure. The absence of vitreal haemorrhage in the injected eye was confirmed in all animals by planar ophthalmoscopy. All animals developed reversible bilateral cataracts while under anesthesia. The animals were warmed at 37°C till recovery. All animals were mobile and feeding well the day after the procedure. There was no conjunctival redness or post-traumatic cataract in the injected eye, nor was there any residual lens opacity in the contralateral eye for all animals studied.

Dose of caspofungin

Caspofungin acetate (Cancidas, Merck & Co., Inc., Whitehouse Station, N.J., USA) was obtained in vials of 50 mg lyophilized powder and serially diluted in BSS. The doses used in this study ranged from 0.41 µM (0.50 µg/ml) which is a dose that corresponds to the 90th percentile minimum inhibitory concentration (MIC_{90}) for *Aspergillus* species [38], to 100 times that amount (41. µM, 50. µg/ml). Final concentrations were based on a mouse vitreal volume of 20 µl [39]. Intravitreal concentrations of the 5 tested doses were: 41. µM (50. µg/ml, 100x MIC_{90}), 4.1 µM (5.0 µg/ml, 10x MIC_{90}), 2.5 µM (3.0 µg/ml,, 6x MIC_{90}), 1.2 µM (1.5 µg/ml, 3x MIC_{90}) and 0.41 µM (0.50 µg/ml, 1x MIC_{90}).

Electroretinographic recording

Seven weeks after intravitreal injections, electroretinographic (ERG) recordings were carried out according to a protocol described elsewhere 39. Briefly, after overnight dark-adaptation in a ventilated light-tight box, animals were prepared for recording under red illumination [lightemitting diode (LED),> 620 nm]. To obtain consistent and maximal pupillary dilatation without causing ERG amplitude growth during the recording, pupils were fully dilated with a single mydriatic: topical atropine (0.5%) before anesthesia 40. The mice were anesthetized with a single intraperitoneal injection of ketamine (70 mg/kg) and xylazine (7 mg/kg; both drugs from Vedco). Rectal temperature was maintained between 36° and 37°C with an electrically heated blanket (CWE). Each animal was kept in an aluminum Faraday cage for the duration of the recording. The animal's head was held steady to reduce noise originating from respiratory and other movements by using an aluminum head holder with a hole for the upper incisors to fix the upper jaw. This fixation ensured that the jaw remained open throughout the

recording. Moist room air was pumped through a clear polyvinylchloride (PVC) pipe kept close to the open mouth. The head holder also served as the ground. All Animals were kept warm at 37°C until they recovered from anesthesia; data from animals that died during the recording were not considered in this study. Recording sessions lasted up to 30 min. The animals were euthanized soon after recording while still under the influence of anesthesia. Animals that died during anesthesia or while recording ERG's were not included in the study for subsequent analysis. ERGs were recorded differentially between Dawson/Trick/Litzkow nylon/silver electrode fiber electrodes 41 moistened with normal saline and placed on the two eyes. Eyes were covered with contact lenses that were pressure molded from 0.19 mm clear ACLAR film (Ted Pella) for the stimulated eye and 0.7 mm opaque PVC for the nonstimulated eye. Both lenses were placed over a cover of 1.2% methylcellulose in 1.2% saline. The signals were amplified (DC to 500 Hz), digitized at 2 kHz, and sent to the computer for averaging, display and storage, and subsequent analysis. A custom-made LED (λ_{max}, 462 nm; −5.8 to 1.9 log scotopic Troland seconds (sc td s)-based stimulator clocked by an AMD 9513 based timer (USB-4302, Measurement Computing, MA, USA) provided the light stimuli [39]. The intervals between flashes were adjusted so that the response returned to baseline before another stimulus was presented. A digital 60 Hz notch filter was applied off-line. The light-stimulus was calibrated using a calibrated photometer (IL1700, International Light Research) with a filter corrected for human scotopic vision based on the fact that spectral sensitivity of the mouse rods is very similar to the Commission Internationale de I'Eclairage scotopic spectral efficiency 42.

Histology

Eyecup tissue preparation for histological analysis was performed shortly after ERG recordings. The details of tissue preparation and staining methods were similar to those detailed elsewhere 39, 43–46. Each mouse was euthanized by cervical dislocation after ERG recording while the animal was still under the influence of anesthesia. The superior pole of the eye to be sectioned was cauterized for overall eyecup orientation. The eye was enucleated, punctured at the superior limbus with a 26 Ga needle eye, and was immersed in fixative (4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 5 minutes on a Nutator at room temperature (RT). The cornea was then excised, and the enucleated eye was kept in fixative (RT on Nutator) for an additional 10 min. The lens was then removed and fixed for additional 1.25 hrs at RT on Nutator with fresh fixative. The eyecup was rinsed 3 times for 10 minutes each in 0.1 M cacodylate buffer (RT) and subsequently infiltrated with 30% sucrose in 0.1 M cacodylate buffer for 15 −17 hrs at 4°C on a Nutator. The sucrose was then drained, and the eyecups were sectioned close to the optic nerve along the superior/inferior axis. The eyecup halves were then sequentially washed with Tissue Tek_O.C.T. (Sakura Kinetek USA Inc., Torrance, CA) for 0.75 – 1.0 hr; transferred into a casting mold filled with fresh Tissue Tek_O.C.T.; flash-frozen in liquid nitrogen; and then stored at −80°C. Radial cryostat sections (10 μm) were made at −19°C, collected on Superfrost plus slides (VWR international, West Chester, PA, USA) and then stored at −80°C.

The frozen sections were rinsed with Milli-Q water for 1 minute and then stained with 0.1% eosin solution for 2 minutes. The sections were then washed in Milli-Q water for 10 seconds, dehydrated and coverslipped in a fade-retardant mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Burlingame, CA, USA).

The eosin- and DAPI-stained frozen sections were viewed with a fluorescence -microscope (Nikon Eclipse TE 2000-U, Nikon Inc., NY, USA) under 4x and 20x magnification, and images were captured with a digital camera (CoolSNAP cf, Photometrics, Tucson, AZ, USA) under software-controlled uniform conditions of exposure (MetaVue ver 6.7r5, Molecular Devices,

Downingtown, PA, USA). Matching images in the different channels were overlaid by using Adobe Photoshop 6.0 software (Adobe Systems, Inc., Mountain View, CA, USA).

Results

In our experimental design the mice were separated into 5 groups. Each group was divided into age matched cohorts of mice whose eyes were administered intravitreal caspofungin in BSS (caspofungin+BSS) and controls that received intravitreal BSS (BSS). The caspofungin dose across each group was either 1x, 3x, 6x, 10x or 100x the MIC_{90} value for *Aspergillus* species (0.41 μM to 41. μM). Each week for five weeks, injections were performed on a different dose group. After 7 weeks post injections, ERG's were recorded, and histology samples collected from each group, thereby resulting in a total duration of 12 weeks for the entire study. The ERG a- and b-wave amplitudes for the BSS injected control eyes for the different groups were not statistically different from one another [One-way ANOVA: a-wave, $F(4,17) = 0.31$, $p = 0.86$; Scotopic b-wave V_{max} (from Naka-Rushton fit, see below), $F(4,17) = 0.10$, $p = 0.97$; mixed b-wave amplitude (2.3 log sc td s), $F(4,17) = 0.17$, $p = 0.94$]. These observations indicate that our ERG results across the total duration of the study had minimal confounds due to variabilities in the damage caused by the injection procedure, ERG recording settings, differing anesthesia depths, body temperature and levels of dark-adaptation between sessions. Intravitreal injections did not produce observable histological changes to the retina in the BSS injected controls 7-weeks post injection, removing the injection process itself as a significant confound in the interpretation of our results (see Fig. 4).

The raw electroretinograms recorded for those eyes injected intravitreally with 0.41 μM, 1.2 μM, 2.5 μM, and 4.1 μM caspofungin were very similar to those injected with BSS alone. However, the mice that received a dose of 41. μM caspofungin showed reduced amplitudes (Fig. 1). The electroretinograms were analyzed for changes in the photoreceptor-derived, negatively going a-wave measured from the baseline to its trough and for changes in the ON-bipolar derived b-wave, with amplitudes measured from the a-wave trough to the b-wave peak.

To interpret the ERG data in terms of alteration in retinal physiology following caspofungin injection, we plotted the mean b- and a-wave amplitudes of the caspofungin+BSS and BSS injected eyes for each group as a function of stimulus energy, and interpreted their nonlinear-monotonic relationship with a fitted Naka-Rushton function [47] (Fig. 2) as was done in another study [40]:

$$V = \frac{V_{max} * I}{(I + I_{0.5})}$$

where, V = ERG response amplitude, V_{max} = the maximum amplitude of the response, $I_{0.5}$ = flash energy that elicits a half-maximal response, and I= flash energy that elicits the response, V.

For examining the scotopic (rod-driven) b-wave, only those responses to flash energies between −3.5 to 0 log sc td s were used to produce the fit, in order to reduce the effects of the scotopic threshold responses (STRs) in the fit for low energies and to minimize the influence of the cone-driven responses at higher energies [40] (Fig. 2). The parameters of the fit for the capsofungin+BSS and BSS injected cohorts for the scotopic b-wave for each group, along with their coefficient of determination (R^2), are detailed in Table 1. For examining the mixed rod +cone driven b-wave, the average amplitudes in response to a high energy stimulus of 2.3 log sc td s were examined (Fig. 2 top panel, inset; Table 1). The scotopic b-wave V_{max} and $I_{0.5}$ and the mixed rod+cone driven b-wave amplitudes of the caspofungin+BSS injected eyes were

not statistically different from their BSS controls for caspofungin concentrations between 0.41 μM and 4.1 μM. Both the rod-driven b-wave V_{max} and the mixed rod+cone driven b-wave amplitudes for the caspofungin+BSS injected eyes that received the 41. μM dose showed a statistically significant reduction of ~59% compared to the BSS control. The b-wave $I_{0.5}$ for the caspofungin+BSS injected eyes that received the 41. μM dose did not show a statistically significant difference compared to the BSS control. The amplitude-response relationship for the dark-adapted photoreceptor-driven a-wave is shown in the middle panel of Fig. 2. The parameters of the fit for the caspofungin+BSS and BSS injected cohorts for the a-wave for each group, along with their coefficient of determination (R^2), are detailed in Table 1. The scotopic a-wave V_{max} and $I_{0.5}$ of the caspofungin+BSS injected eyes were not statistically different from their BSS controls for caspofungin concentrations between 0.41 μM and 4.1 μM the MIC_{90}. The a-wave V_{max} for the caspofungin+BSS injected eyes that received the 41. μM dose showed a statistically significant reduction of 61% compared to the BSS control. The a-wave $I_{0.5}$ for the caspofungin+BSS injected eyes that received dose of 41. μM did not show a statistically significant difference compared to the BSS control. Because for both the rod-driven b-wave and a-waves, the $I_{0.5}$ values for the caspofungin +BSS injected eye that received a dose of 41. μM were similar to the BSS injected controls, indicating that caspofungin at this concentration must have caused damage to the retina without altering light transmission to the retina (for example, vitreal hemorrhage or cataracts) or altering photoreceptor sensitivity to light [37].

For low flash energies the averaged scotopic threshold responses (STR's) for mice that received 100x caspofungin injection (but not those who received lower doses) showed a statistically significant difference compared to the BSS injected controls at criterion times of 110 ms and 220 ms (t-test, $p<0.05$), indicating that at this concentration there was a likelihood of toxicity to the inner retina, proximal to the bipolar cells.

To investigate the probable cause of reduced a- and b-wave amplitudes for the caspofungin +BSS injected eyes that received 100x caspofungin relative to controls injected only with BSS, we plotted the b-wave amplitudes for the 4 highest flash-energies as a function of the a-wave amplitude (Fig. 3). Although both the b- and a-waves were reduced in the 100x injected eye, their relative ratios were indistinguishable from the controls, indicating that the cause of the reduced a- and b-waves was most likely due to the toxic effect of this drug on the photoreceptors.

There were no signs of retinal haemorrhages or infection in any injected eye. Light microscopic histological examination (Fig. 4) showed no observable retinal abnormality in eyes injected with BSS or caspofungin+BSS injected eyes that received a dose of 4.1 μM or less. The eyes that were injected with the 41. μM dose were conspicuous for a loss of nuclear staining in the ganglion cell layer. The loss of ganglion cells was seen in large areas of the retina (Fig. 5). We did not observe focal areas of necrosis or localized retinal detachment or any observable changes in the photoreceptor outer and inner segments, outer nuclear layer, outer plexiform layer, the inner nuclear layer, or the inner plexiform layer.

Discussion

Caspofungin is known to cause local irritation at the site of injection, histamine release, phlebitis and haemolysis [48], all of which can potentially affect the retina. This study found that caspofungin did not cause statistically significant alterations in the electroretinogram or gross retinal histology for intravitreal concentrations of 4.1 μM or less. There were decreases in ERG amplitudes and a detectable loss of cells in the retinal ganglion cell layer for intravitreal concentrations of 41. μM (at 100 times the MIC_{90}). A caspofungin dose of 4.1 μM, which did not show signs of significant toxicity by our assay, was 10 times higher than the MIC_{90} of

caspofungin for *Aspergillus* spp. [38] or *Candida* spp. [22]. This dose of caspofungin may be sufficient for the therapy of fungal endophthalmitis, especially when used in combination therapy. Even when administered systemically in combination with voriconazole, the low intravitreal levels achieved by caspofungin may have contributed to the treatment of amphotericin-resistant *Aspergillus fumigatus* endophthalmitis [27] and *Candida* endophthalmitis 49. Another advantage of caspofungin is its synergistic action with the azole group of antifungals and amphotericin B (for review, 23).

Intravitreal injection of amphotericin B has traditionally been the treatment of choice for severe fungal endophthalmitis, but amphotericin B is known to produce retinal toxicity at low doses [15, 50], making it the standard for comparing retinal toxicities of other candidate antifungals administered via intravitreal injections. The minimum dose of amphotericin B that produces retinal toxicity in the murine retina remains undetermined. In the rabbit eye intravitreal concentration between 4.1 to 8.3 μg/ml of amphotericin B has been found to produce retinal toxicity [15] making this drug potentially toxic at concentrations that are only 1 times its MIC_{90} for *Aspergillus* spp.(4 μg/ml 38). Our study finds that a non-toxic dose for caspofungin is up to 10 times its MIC_{90} for *Aspergillus* spp. (0.50 μg/ml or 0.41 μM 38) making it a much safer drug than amphotericin B. Our study reports a lower non-toxic dose of the drug compared with another study that found that *in vitro* concentrations up to 50. μg/ml (41. μM) did not show toxic effects of caspofungin on corneal endothelial cells, primary human trabecular meshwork cells, and primary human retinal pigment epithelium (RPE) cells [51]. Another study conducted on a rabbit model of fungal endophthalmitis found no evidence of histological damage to the retina 7 days after intravitreal injection of caspofungin at 500 μg/ml (1000 times its MIC_{90}) [52]. This non-toxic dose of caspofungin in the rabbit retina (as assayed by histology) is 10 times the concentration that produced toxic effects in the mouse, as assayed by ERG and histology 7 weeks following injection. The difference in the caspofungin concentrations that produced damage to the mouse retina, but not to the rabbit retina, could be either because the retinas in the two studies were sampled at different time points or that the mouse eye, with its limited intravitreal space (~1% that of the rabbit), is more sensitive to toxicity. Our study establishes a safe range for retinal toxicity of intravitreally injected caspofungin in mice, which can help guide dosage in humans. The highest non-toxic intravitreal concentration in mice found in our study equates to an injected dose of 20 μg in a human eye, assuming a human intravitreal volume of 4 ml.

To the best of our knowledge there is no information on the pharmacokinetics of intravitreal caspofungin in this animal model or others, and so it is difficult to predict how long this drug remains in ocular tissues in therapeutic concentrations. However, a single intravitreal dose of 100 μg of caspofungin injected into the rabbit vitreous in experimentally induced *Candida* endophthalmitis produced a greater improvement of clinical scores at the end of 3 days, and it reduced *Candida* colony forming units/ml more at the end of 7 days than 50 μg voriconazole, 10 μg of amphotericin B, or 10 μg of itraconazole [52], indicating that caspofungin was retained in the ocular tissues for a sufficiently long time to be therapeutically viable.

Intravitreal injection of caspofungin is likely to be the route of choice in order to deliver therapeutically effective doses of this drug in fungal endophthalmitis. After systemic administration, caspofungin has been reported only in low to undetectable levels in the vitreous, perhaps because of its high molecular weight. The maximum reported vitreal concentration in humans after systemic administration is 0.28 μg/ml (23. μM) in a case of fungal endophthalmitis that was culture positive for *Fusarium* 28. It may not be feasible to administer sufficiently high concentrations of caspofungin systemically to achieve therapeutic intravitreal concentrations because of its hepatotoxicity (for review see 48). Even in cases of endophthalmitis with concomitant systemic fungal infection, direct vitreal injection (but not higher doses of systemic caspofungin) may be the preferred route for delivering therapeutic

doses of caspofungin to the retina because of the possibility of paradoxically reduced systemic efficacy of high doses of systemically administered caspofungin 53, 54.

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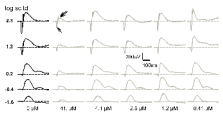

Figure 1. Effects of intravitreal caspofungin on the ERG flash response

Representative ERG responses for intravitreal BSS or varying doses of caspofungin+BSS injected subjects to brief flashes of increasing stimulus energies, as indicated, for the fully dark-adapted condition. Black traces (left), representative intravitreal BSS injected subject; gray traces, representative caspofungin+BSS injected subjects; the intravitreal concentrations of caspofungin are indicated below each column of corresponding ERG traces. Unlabeled single and double arrows indicate the reduced a- and b-waves respectively for a saturating flash of 2.3 log sc td s for the 41. μM concentration of caspofungin.

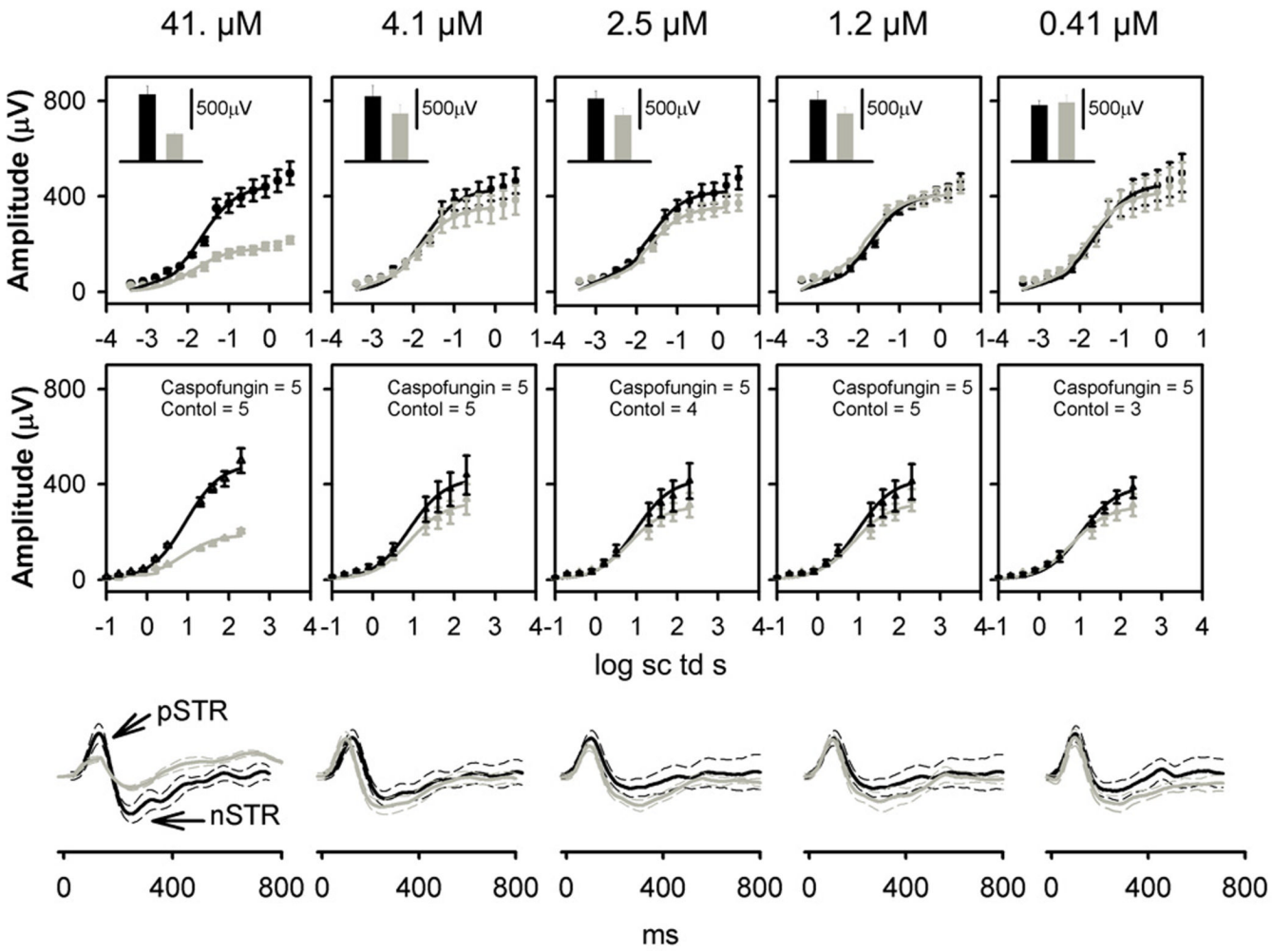

Figure 2. Effects of intravitreal caspofungin on the mean ERG b- and a-wave stimulus-amplitude relationship and scotopic threshold responses

Caspofungin concentrations in µM are indicated at the top of each column. Top panel: Average dark-adapted electroretinogram (ERG) b-wave amplitudes (±S.E.M) for low scotopic energies plotted as a function of stimulus energy for the dark-adapted ERG's. Intravitreal BSS injected controls, black circle; intravitreal caspofungin+BSS injected subjects, gray circle. Black and grey solid curves, best fit Naka-Rushton function for the BSS control and caspofungin+BSS injected eyes, respectively. Inset: bar graph showing average dark-adapted ERG b-wave amplitudes (±S.E.M) for a saturating flash of 2.3 log sc td s. Black bar, BSS injected control; Grey bar, caspofungin+BSS injected eye. Middle panel: Average dark-adapted electroretinogram (ERG) a-wave amplitudes (±S.E.M) plotted as a function of stimulus energy for the dark-adapted ERG's. Intravitreal BSS injected controls, black circle; intravitreal caspofungin+BSS injected subjects, gray circle. Black and grey solid curves, best fit Naka-Rushton function for the BSS control and caspofungin+BSS injected eyes, respectively. Lower panel: Averaged ERG's in response to low flash-energy (−3.7 log sc td s). Intravitreal BSS injected controls, black solid lines; intravitreal caspofungin+BSS injected subjects, gray solid lines. Dashed traces indicate ±1 SEM. p-STR, positive scotopic threshold response; n-STR, negative scotopic threshold response.

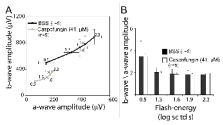

Figure 3. b-wave as a function of a-wave and b-wave to a-wave ratios for the dark-adapted mixed rod-cone ERG's

A. b-wave plotted as a function of a-wave for those eyes that received caspofungin+BSS solution containing 41. μM caspofungin and for the BSS only controls. The numbers alongside the data points indicate the flash-energy in log sc td s. **B.** b-wave to a-wave ratios as a function of the flash-energy.

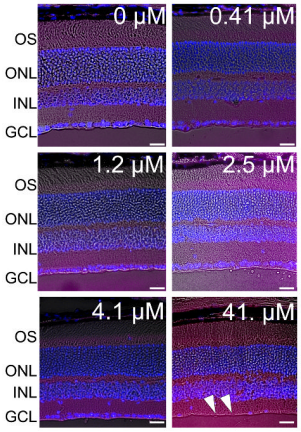

Figure 4. Histopathological examination of radial sections of mice eyes 7 weeks after intravitreal injection of balanced salt solution (BSS) or incrementing concentrations of caspofungin in BSS

Superimposed on a phase contrast image are DAPI stained nuclei in various cell layers (blue) and eosin stained cell membrane (red) prominently seen in the plexiform layers. No retinal abnormalities were noted in the retinae injected with BSS alone or 0.41 μM to 4.1 μM of caspofungin dissolved in BSS. Eyes injected with 41. μM of caspofungin showed loss of cells in the ganglion cell layer (arrowheads). OS, outer and inner segments of the photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; and GCL, ganglion cell layer. Scale bar = 20 μm.

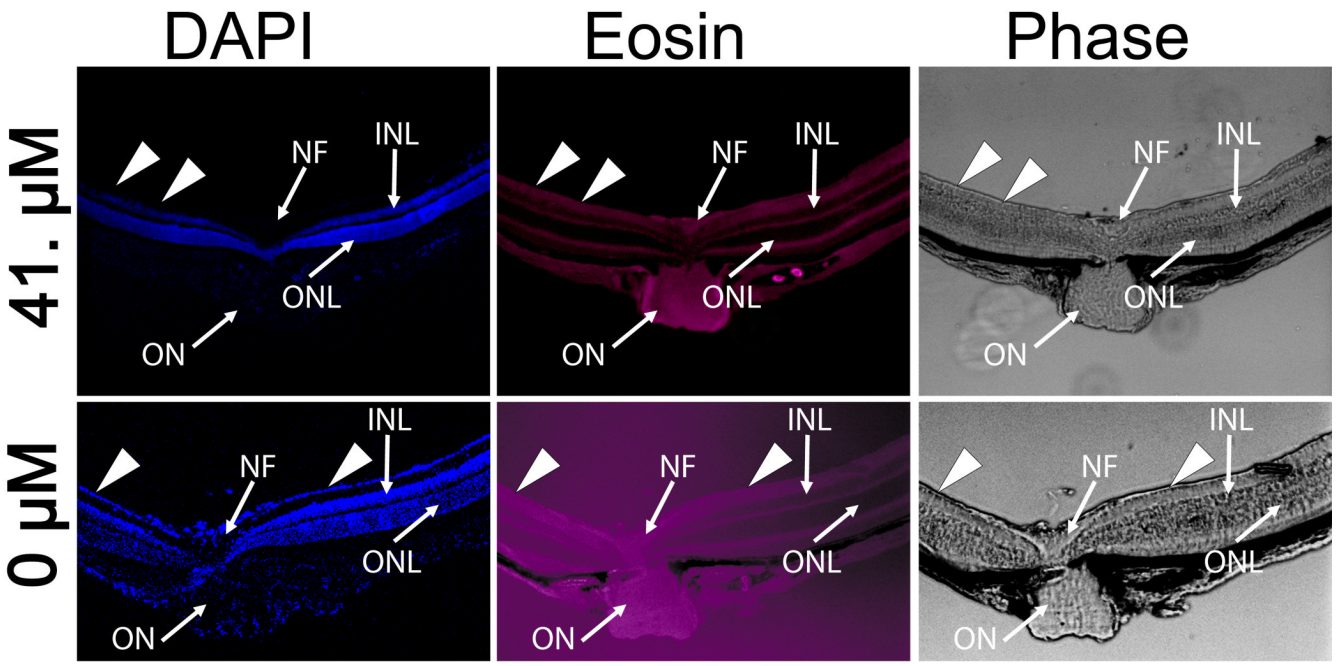

Figure 5. Histopathological examination of radial sections through the optic nerve head of mice eyes 7 weeks after intravitreal injection of balanced salt solution (BSS) or 41. µM (100x MIC_{90}) of caspofungin in BSS

Blue: DAPI stained nuclei in various cell layers; red: eosin stained cell membranes prominently seen in the plexiform layers. No retinal abnormalities were noted in the retinae injected with BSS alone or 0.41 µM to 4.1 µM of caspofungin+BSS. Eyes injected with 41. µM of caspofungin+BSS showed loss of cells in the ganglion cell layer (arrowheads). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer, NF, nerve fiber; and ON, optic nerve. Scale bar = 20 µm.

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Table 1

Summary of ERG results

Mean V_{max} (±S.E.M), mean $I_{0.5}$ (±S.E.M), coefficient of determination (R^2) from Naka-Rushton fit of the a-wave and scotopic b-wave amplitudes over incrementing energies, and mean rod+cone b-wave amplitude (±S.E.M) in response to a high-energy flash (2.3 log sc td s) for different doses of caspofungin

a-wave								
	V_{max}				**$I_{0.5}$**			
	mV	**sem**	**n**	**p**	**log sc td s**	**sem**	**n**	**p**
Control (0 μM) Caspofungin (0.41 μM)	393.18 307.89	44.04 45.57	3 5	0.2611	1.03 0.83	0.08 0.05	3 5	0.0655
Control (0 μM) Caspofungin (1.2 μM)	401.23 320.03	74.66 45.57	5 5	0.3804	0.99 0.88	0.03 0.02	5 5	0.3667
Control (0 μM) Caspofungin (2.5 μM)	403.06 307.89	74.75 50.95	4 5	0.3125	0.92 0.83	0.05 0.03	4 5	0.1284
Control (0 μM) Caspofungin (4.1 μM)	430.37 324.75	81.76 63.62	5 5	0.3378	0.90 0.90	0.02 0.03	5 5	0.8852
Control (0 μM) Caspofungin (41. μM)	488.37 189.01	51.94 9.35	5 5	0.0005 *	0.90 0.80	0.06 0.04	5 5	0.1061

b-wave (scotopic intensities)								
	V_{max}				**$I_{0.5}$**			
	mV	**sem**	**n**	**p**	**logsctds**	**sem**	**n**	**p**
Control (0 μM) Caspofungin (0.41 μM)	441.49 410.36	79.70 62.46	3 5	0.7696	−1.7 −1.8	0.06 0.04	3 5	0.1976
Control (0 μM) Caspofungin (1.2 μM)	403.36 404.59	26.05 26.89	5 5	0.9748	−1.7 −1.8	0.04 0.03	5 5	0.0805
Control (0 μM) Caspofungin (2.5 μM)	418.93 352.68	43.71 32.73	4 5	0.2547	−1.7 −1.7	0.02 0.03	4 5	0.9
Control (0 μM) Caspofungin (4.1 μM)	422.88 350.73	42.55 59.01	5 5	0.3504	−1.9 −1.8	0.06 0.04	5 5	0.2029
Control (0 μM) Caspofungin (41. μM)	435.38 180.18	44.60 16.11	5 5	0.0007*	−1.7 −1.8	0.03 0.05	5 5	0.1247

Mixed b-wave amplitude (2.3 log sc td s)				
	mV	**sem**	**n**	**p**
Control (0 μM)	747.63 780.58	61.19 100.64	3 5	0.8235

Mixed b-wave amplitude (2.3 log sc td s)				
	mV	**sem**	**n**	**p**
Caspofungin (0.41 μM)				
Control (0 μM) Caspofungin (1.2 μM)	817.76 635.12	109.78 84.13	5 4	0.2232
Control (0 μM) Caspofungin (2.5 μM)	834.59 615.05	98.91 92.13	5 5	0.2398
Control (0 μM) Caspofungin (4.1 μM)	865.43 634.36	141.52 114.36	5 5	0.294
Control (0 μM) Caspofungin (41. μM)	887.73 367.45	111.11 16.11	5 5	0.0017*

A p-value <0.05 from Students *t*-test was considered statistically significant and is indicated by *.