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A novel, tomographic imaging probe for rapid diagnosis of fungal keratitis

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Abstract

Fungal keratitis is a leading cause of ocular morbidity and blindness in developing countries. Diagnosing fungal keratitis currently relies on a comparative evaluation of corneal biopsy or scraping using a direct microscopy and culture results. These methods not only carry the risk of developing complications due to the invasive tissue sampling but also are largely limited by diagnostic speed and accuracy, making it difficult to initiate timely appropriate antifungal therapy. Therefore, rapid and noninvasive diagnostic tools are a pressing need for improved outcomes for fungal keratitis. Taking advantage of the highly specific fungal cell targeting properties of caspofungin, we have developed a fluorescent chemical probe with high selectivity against fungal pathogens. Utilizing fluorescence imaging technology, we have demonstrated a highly specific and sensitive detection of *Aspergillus* in a fungal keratitis model in mice as early as 5 min post-topical application of the probe. Our results indicate that a fluorescence-mediated platform can be used as a rapid (<10 min) alternative to conventional methods for detecting *Aspergillus,* and potentially other fungi, in fungal infections of the cornea.

Key words: *Aspergillus*, fungal keratitis, diagnosis, *in vivo* fluorescence imaging.

Introduction

Fungal keratitis (FK), an infection of the cornea, is one of the leading causes of ocular morbidity and blindness in developing countries. Major predisposing factors associated with FK include ocular trauma and contact lens wear.[1] Many fungal genera and species that cause FK have been implicated,[2] and the leading causes in most parts of the world are filamentous fungi, including *Aspergillus* spp. and *Fusarium* spp.[3,4] Diagnosis of FK at its early stage is challenging because of its overlap in clinical diagnosis with bacterial keratitis[5] and the low culture positivity for fungal pathogens.[6] Diagnosis and treatment of FK is further complicated because filamentous fungi can penetrate into the stromal layers of the cornea, which renders them inaccessible to corneal scraping for culture identification or therapy.[7] Early diagnosis and initiation of treatment for FK is imperative to prevent visual loss and to minimize the length and cost of treatment. Hence, techniques that allow rapid, sensitive and specific detection of causative fungal pathogens are in pressing need for improved outcome of FK patients.

Caspofungin (CSF), an echinocandin class antifungal, targets the fungal-specific enzyme β-1,3-D-glucan synthase, which is responsible for the biosynthesis of a major fungal cell wall building block. For this reason, CSF binds fungal cells with high specificity[8] resulting in low

overall toxicity. We previously reported that CSF can be modified at the ethylenediamine functionalized position with a reporter BODIPY, while still retaining highly potent antifungal targeting properties.[9] When coupled with the fluorophore label 7-Amino-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One) (DDAO) at the amino group of 3-hydroxyornithine residue, CSF-DDAO can be used as fungal specific probe, whereby drug-bound cells fluoresce and can be visualized by near-infrared (NIR) fluorescence imaging. Furthermore, a liposome-encapsulated CSF-DDAO (L-CSF-DDAO) was developed to facilitate a greater penetration of the probe into the cornea and to minimize its interaction with corneal cell membranes.[10] The high selectivity for fungi combined with lack of ocular toxicity in human[11,12] and in other animal models[13] create a strong diagnostic potential for CSF-DDAO.

The purpose of this work is to establish the L-CSF-DDAO probe as a specific and sensitive diagnostic adjunct for FK using a mouse model of *Aspergillus* keratitis. In conjunction with *in vivo* fluorescence imaging technology, this novel probe can be used as a non-invasive tool that allows rapid detection of *Aspergillus* spp. in the cornea of mice without the need to perform corneal biopsies or scrape for culture and microscopic identification, which carries a potential risk of developing secondary infections.

Methods

Synthesis of CSF-DDAO

Conjugation of CSF with a fluorophore was performed as described previously.[9] Briefly, CSF-DDAO conjugate was produced by incubating 20 mg (15 μmol) of CSF-diacetate and 2.8 mg (8 μmol) of DDAO-isothiocyano derivative in 0.2 ml of dimethylformamide and 4 μl of triethylamine at 50°C during 30 min. Thin liquid chromatography (TLC) analysis in acetonitrile-water (7:1) developing system revealed nearly complete conversion of the original DDAO derivative (Rf = 0.95) to the main reaction product (Rf = 0.6), which was purified by preparative TLC in the same system.

Synthesis of liposomal CSF-DDAO vesicles

To facilitate CSF-DDAO penetrating the stromal layer of the cornea, we also prepared the liposome-encapsulated CSF-DDAO. Liposomal formulation was obtained by dissolving lipids in chloroform at 20 mg/ml in a 4-ml glass vial. CSF-DDAO (4 mM) dissolved in dimethyl sulfoxide (DMSO) was added to the glass vial to produce a 1:50 (mole:mole) ratio of CSF-DDAO to lipid or 75 μl of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 10 μl of CSF-DDAO. The mixture was then dried under nitrogen flow for 5 minutes. After this time, the dried samples were placed under vacuum for a minimum of 3 hours to ensure a complete evaporation of the residual DMSO. The dried CSF-DDAO:lipid mixtures were solubilized in 100 μl of a pH 7 phosphate-buffered saline (PBS) solution with a final drug concentration of 0.4 mM. This solution was then bath sonicated for 30 seconds followed by rapidly freezing in liquid nitrogen. This freeze-thaw cycle was repeated for 20 cycles, upon which time the sample noticeably increased in transparency. The stock solutions of L-CSF-DDAO were stored at room temperature for up to 3 days or at 4°C for up to 1 week until use.

Cell culture

All *Aspergillus* strains (*A. fumigatus* strain R21, *A. flavus* strain DPL9, and *A. niger* strain DPL29) and *Fusarium* strain (*F. solani* strain DPL114) were grown on potato dextrose agar (PDA) slants for 3 days at 37°C and 30°C, respectively. Spores were harvested, washed, and suspended in a sterile saline containing 0.01% Tween 20 to a final concentration of 1×10^8 spores/ml for eye infection.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strain MW2 and *Pseudomonas aeruginosa* strain Xen5 were each streaked out on Lennox broth (LB) agar and grown overnight at 37°C. A single colony was taken to inoculate in LB broth and incubated at 37°C with shaking overnight. Bacterial cells were washed and diluted to a final concentration of 1×10^8 cfu/ml for eye infection.

MEC determination

To evaluate whether the modification of CSF by conjugating fluorophore label or encapsulating into lipid vesicles has any effect on its fungal targeting properties (i.e., the binding affinity), minimum effective concentrations (MECs) of CSF, CSF-DDAO, and L-CSF-DDAO against *A. fumigatus*, *A. flavus*, and *A. niger* were analyzed according to CLSI protocol M38-2A[14]. For echinocandins, the MEC is defined as the lowest concentration of the drug associated with abnormal, truncated hyphal.[8]

Cell labeling

A. fumigatus strain R21, *A. flavus* strain DPL9, and *A. niger* strain DPL29 were used for each of the experiments. One drop of yeast extract peptone dextrose (YPD) broth was placed in the well of a 15-well multi-test slide followed by the addition of 10 μl of saline containing 10^8 conidia of either R21, DPL9, or DPL29. The slide was placed in a sterile Petri dish containing saline soaked gauze to provide a moist environment and incubated in a 37°C incubator for 24 h to facilitate germination and growth of hyphal

elements. A 5-μl quantity of CSF-DDAO or L-CSF-DDAO was added to the well, incubated at room temperature for 5 min, and washed three times with saline to remove excess probe. Saline was aspirated off from each well and 5 μl of Slow Antifade reagent (Life Technologies, Eugene, OR, USA) was added to prolong the fluorescence. Each slide was observed under 20× magnification with Nikon A1Rsi confocal microscope using Nikon Nis-Elements AR software for imaging acquisition.

Experimental animals

Immunocompetent 6–8 week-old female SKH1 hairless mice (Charles River, Wilmington, MA, USA) were used for all *in vivo* experiments. Before the start of the experiment, all corneas of mice were examined individually for any signs of abnormality. All experimental procedures were performed in accordance with National Research Council guidelines and approved by the Rutgers University Research Institutional Animal Care and Use Committee (IACUC).

Fungal keratitis model

Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine+10 mg/kg xylazine. Prior to intrastromal injections, the anesthetized mice were given a drop of Tobramycin Ophthalmic Solution, 0.3% (Bauch+Lomb, Tampa, FL, USA) into the right cornea as prophylaxis to prevent bacterial infection. Establishment of a murine FK model was carried out as described previously.[15,16] In brief, a 30-gauge needle was inserted into the right cornea of each mouse, near the center, to the depth of the superficial stroma. A 33-gauge needle with a 30° bevel was threaded into the stoma and 2 μl of either fungal, bacterial, or PBS suspension was injected intrastromally. For a fungal keratitis model, 20 mice were infected with either 1 × 10^8 spores/ml (5 × 10^6 spores/eye) of *A. fumigatus, A. flavus, A. niger*, or *F. solani*. For a bacterial keratitis model, 10 mice were infected with 1 × 10^8 cfu/ml (5 × 10^6 cfu/eye) of either MRSA, or *P. aeruginosa*. Two mice that served as negative controls were inoculated with the same volume of sterile PBS. Only the right corneas were used for the experiment, with the left corneas used as uninfected control. At 24 h post-infection, mice were applied topically with 5 μl of either the L-CSF-DDAO, or CSF on the infected eye. After 5 minutes of topical application with the conjugate, eyes were irrigated with PBS three times.

In vivo fluorescence imaging

FK was established in a total of 32 mice, as aforementioned. Under vaporized isoflurane anesthesia, the infected and mock-infected eyes of mice were imaged at 5, 30, 50, and 60 min post-application of the probe using the FMT 1500 *in vivo* imager system (PerkinElmer, Waltham, MA, USA) to assess the development of corneal disease and the diagnostic performance of the probes. For tomographic imaging, a field surrounding the eye was selected and a scan was performed using the NIR 680-nm channel. All images were analyzed using the TrueQuant software provided by the same manufacturer. The total amount of the probe was quantified in picomoles as the sum of the background-subtracted fluorescence within a three-dimensional region of interest (ROI) of constant size and shape for each animal using the previously calibrated photochemical properties of the probe. At the last imaging time point, the animals were humanely euthanized via CO_2 narcosis, and both eyes were enucleated and the fungal burdens were enumerated and confirmed.

Table 1. *Aspergillus* MECs.

		MEC (μg ml^{-1})		
Strain	Organism	CSF	CSF-DDAO	L-CSF-DDAO
R21	*A. fumigatus*	0.25	1	1
DPL9	*A. flavus*	0.25	1	1
DPL29	*A. niger*	0.5	1	0.5

Results

Fungal targeting properties of CSF–DDAO probe

As previously reported,[9] modification of CSF had little effect on its antifungal targeting or susceptibility properties. *In vitro* susceptibility testing showed that there was a slight increase in the MEC susceptibility of CSF-DDAO (1, 1, and 1 μg/ml) and L-CSF-DDAO (1, 1, and 0.5 μg/ml), compared to the unlabeled CSF (0.25, 0.25, and 0.5 μg/ml) against *A. fumigatus, A. flavus,* and *A. niger*, respectively (Table 1).

To further assess the sensitivity and specificity of the L-CSF-DDAO probe for fungal elements, *A. fumigatus, A. flavus, or A. niger* cells grown on glass slides and exposed to the probe were imaged by confocal microscopy. The fluorescence intensity of the labeled *Aspergillus* was normalized with respect to the unlabeled control *Aspergillus*. The probe easily detected germlings and hyphal elements of these *Aspergillus* spp. (Fig. 1), whereas individually, CSF and DDAO alone failed to produce detectable fluorescence. The specificity of the L-CSF-DDAO probe was tested against representative Gram negative bacteria *P. aeruginosa* and Gram positive bacteria MRSA under the same labeling and imaging conditions. No fluorescence was detected in any cells.

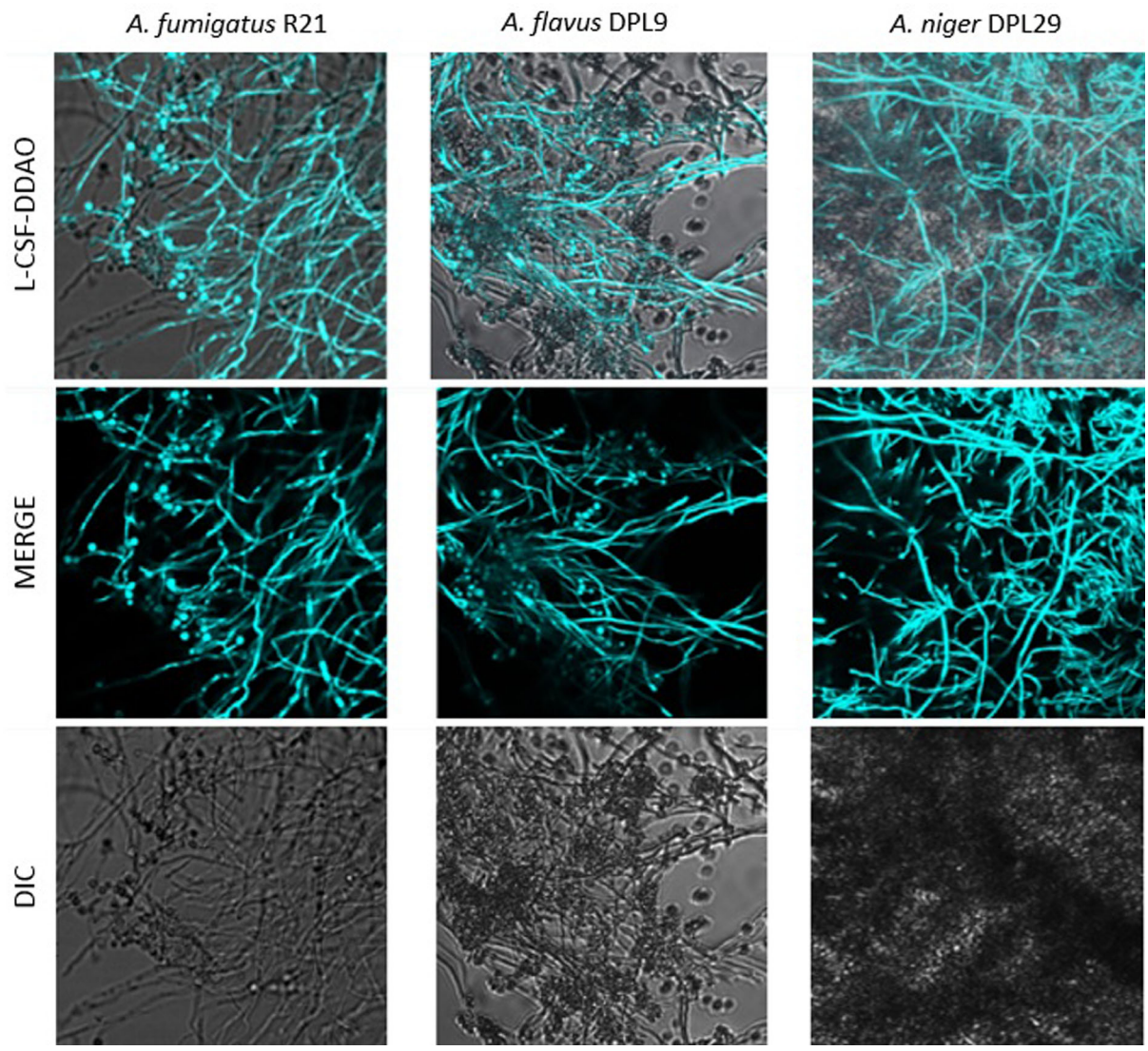

Figure 1. Representative images of of *A. fumigatus* strain R21, *A. flavus* strain DPL9, and *A. niger* strain DPL29 grown on glass slides labeled with L-CSF-DDAO visualized using confocal microscopy. (DIC: differential interference contrast).

Establishment of *Aspergillus* keratitis in SKH1 mice

With an inoculum of 5×10^6 spores of *Aspergillus* per eye, moderate keratitis and heavy neovascularization developed in mice by 24 hours. Although the surface of the corneas remained relatively intact, opaque area remained on the center (Fig. 2).

In vivo fluorescence-mediated tomography imaging

The CSF-DDAO probe was evaluated as a diagnostic tool for FK using a murine keratitis model. The fluorescence signals of uninfected or infected eyes exposed to L-CSF-DDAO probe, DDAO, or CSF were compared at various time points post-exposure (Fig. 3). The quantity of fluorescence detected by the FMT 1500 *in vivo* imaging system in the region of interest is given in picomole levels. Detectable fluorescence was observed from the infected eyes of the mice exposed to L-CSF-DDAO at 5, 10, 30, and 60 min post-application, with an average fluorescence based quantitation of probe molecule at 45 ± 22, 43 ± 20, 44 ± 19, 0 ± 0 picomoles for *A. fumigatus*, 36 ± 38, 6 ± 4, 19 ± 12, 0 ± 0 picomoles for *A. flavus*, and 44 ± 50, 19 ± 18, 9 ± 11, 0 ± 0 for *A. niger*, respectively (Fig. 3). The fluorescence signal in the infected eye applied with the probe gradually decreased as a function of post-exposure time and largely disappeared after 1 h. As expected, no fluorescence was detected in the group exposed to DDAO, CSF, or noninfected mice over the course of this trial. The CSF-DDAO probe could also detect eyes infected with *Fusarium* but detection sensitivity was less and required probe

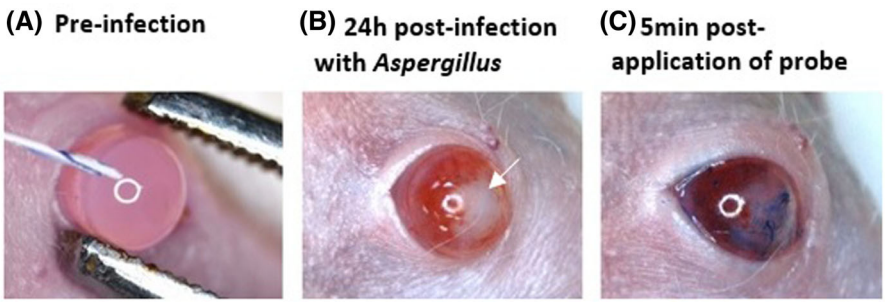

Figure 2. Clinical observation of *Aspergillus* keratitis in mice. Corneas of immunocompetent mouse preinfection (A), 24 h post-infection showing heavy corneal neovascularization surrounding the grayish infiltrates (arrow) (B), 5 min post-application of the L-CSF-DDAO probe followed by irrigation with PBS (C).

application at a twofold higher concentration compared to that of *Aspergillus* keratitis. This is consistent with weaker binding of CSF to *Fusarium* (not shown).

Compared to the L-CSF-DDAO, topical application of CSF-DDAO to infected eyes generated stronger but more prolonged (up to 4 h post-application) fluorescence signals, indicating a longer retention of the probe in the eyes.

Discussion

Accurate and early diagnosis of FK remains a challenge.[17] Given the fact that good clinical outcome of FK largely relies on timely and appropriate antifungal intervention upon early diagnosis, there is a need for novel diagnostic tools that are non-invasive, highly sensitive and specific for ocular fungal infections.

Here we demonstrate the usefulness of a fungal-specific probe coupled with *in vivo* fluorescence imaging as a diagnostic adjunct for FK. We have shown that the L-CSF-DDAO probe is able to detect germinated or hyphal stage fungal cells *in vitro*, while the fungal-targeting properties of the parent molecule were retained. *In vivo* analysis further confirmed the ability of the probe to specifically bind to fungal cells in the infected cornea. As the probe relies upon the echinocandin CSF as its targeting vehicle, it is used at subtherapeutic levels and in a topical form that does not produce measurable systemic drug levels.

In comparison of two different formulations of CSF-DDAO probe, we found that fluorescence signals of L-CSF-DDAO declined rapidly upon application and largely disappeared by 1 h in the infected eyes whereas signals of CSF-DDAO lasted until 4 h post-exposure. The shorter retention of L-CSF-DDAO in the aqueous humor may be due to the uptake of the liposomes by endocytic cells such as macrophages and digestion of the probe in the secondary lysosomes.[10,18] Due to the amphipathic nature of CSF (log *P* value of −2.798),[19] we hypothesize that the intercalated residue of CSF within the lipid bilayer of the liposome may either face inward or outward. This also explains a slightly reduced antifungal potency suggested by MEC increase and fluorescence intensity reduction in *Aspergillus* exposed to L-CSF-DDAO compared to those exposed to CSF-DDAO under the same condition. From the diagnostic perspective, however, the use of L-CSF-DDAO may be more beneficial considering that a shorter interaction with the host cell membrane may limit unspecific binding and mitigate the possible toxicity associated with the drug. It should be noted that the short exposure time (5 min) would not be expected to alter the stability of the CSF-DDAO (or L-CSF-DDAO). The intact chemical entity is needed for efficient targeting and labeling of fungal pathogens, and diminution of response was not observed for at least 1 hour. A detailed stability profile will be part of a product development plan.

Compared to *Aspergillus* keratitis, *Fusarium* keratitis required application of L-CSF-DDAO probe above twofold (0.8 mM) in order to achieve a significant fluorescent signal. This is in accord with reduced binding and a high MEC (>32 μg/ml) observed for *Fusarium* species against echinocandin drugs. For optimal targeting activity, other *Fusarium*-directed probe may be necessary to establish the diagnosis.

To our knowledge, this is the first noninvasive and rapid approach developed to diagnose FK caused by *Aspergillus*. A similar study involving a fluorescent NIR molecular probe was used to successfully target the surfaces of bacterial cells in an *E. coli* thigh infection model.[20] This finding is relevant to the use of L-CSF-DDAO probe for other types of fungal infections. In another study, sensitivity and specificity of laser-scanning in vivo confocal microscopy (LS-IVCM) was used to evaluate microbial keratitis in patients.[21] In spite of the high sensitivity achieved, LS-IVCM relied on the observer's experience and training for an accurate diagnosis of the disease. Moreover, we expect that in the future experiments, this diagnostic platform may be applied for detecting other pathogens of fungal keratitis such as *Candida* spp., in which we previously[9] tested *in vitro* for its sensitivity and specificity to the probe.

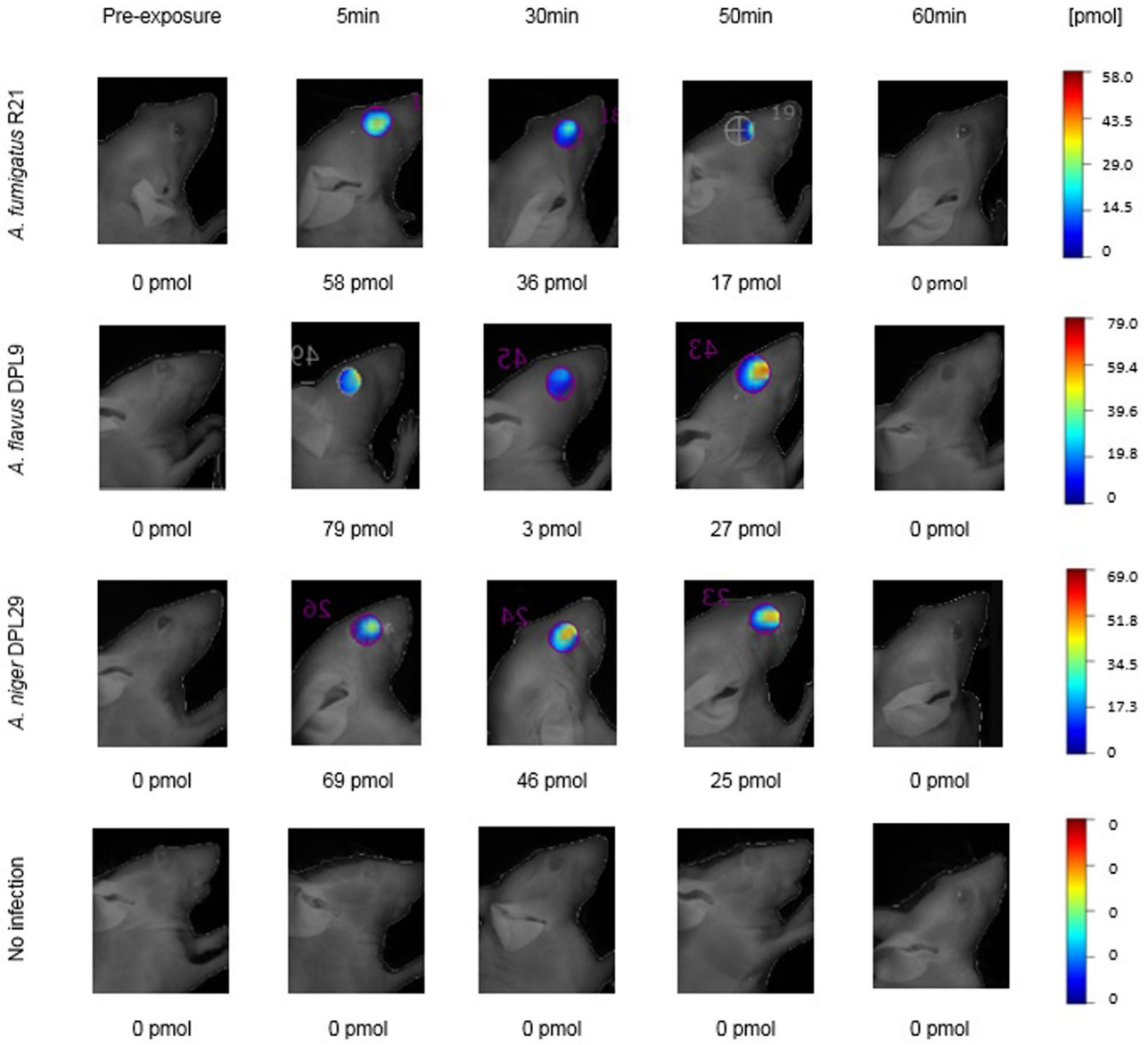

Figure 3. Groups of SKH1 mice (n = 3/group) were infected intrastromally with *A. fumigatus*, *A. flavus*, or *A. niger*. Representative FMT Images were acquired 24 h post-infection at the indicated timepoints (preexposure, 5, 30, 50, and 60 min post-exposure with the L-CSF-DDAO probe) using 680 nm filter. Fluorescence based quantitation for the probe (in picomoles) in the region of interest normalized to the individual mice is shown below each image output and depicted on the intensity map (right), where blue corresponds to the lowest intensity and red to the highest intensity.

With our approach, a portable fluorescent device can potentially be developed and deployed as a point-of-care diagnostic for low-cost, real-time detection, and rapid tracking of causative fungi in the cornea. Such a cost-effective device could potentially be implemented to address high risk populations such as field workers in developing countries where FK is endemic.[22]

In summary, this proof of principle study has demonstrated the usefulness of the novel L-CSF-DDAO probe in combination with *in vivo* fluorescence imaging system for rapid detection of *Aspergillus* in FK. This approach can be developed further to facilitate early diagnosis of FK to permit a prompt and appropriate medical therapy for corneal infections.

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Declaration of interest

D.S.P. receives support from the US National Institute of Allergy and Infectious Diseases; he also receives support from Cidara, Merck, Astellas, Matinas and Scynexis and participates in expert panels for these companies. A provisional patent "METHODS OF DIAGNOSIS USING EVALUATION OF FUNGAL-SPECIFIC FLUORESCENT LABELED ECHINOCANDIN" was filed.

M.H.L., G.W., S.P., and Y.Z. report no potential conflicts.

The authors alone are responsible for the content and the writing of the paper.

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