In Vitro Efficacy of Antifungal Treatment Using Riboflavin/UV-A (365 nm) Combination and Amphotericin B

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PURPOSE. To demonstrate the antimicrobial properties of riboflavin/UV-A (365 nm) against fungal pathogens.

METHODS. The antimicrobial properties of riboflavin/UV-A (365 nm), with or without previous treatment with amphotericin B, were tested on three groups of fungi selected from severe cases of keratomycosis: Candida albicans, Fusarium sp, and Aspergillus fumigatus. They were tested by using Kirby-Bauer discs with empty disc (control), riboflavin 0.1% alone (R), UV-A alone (UV-A), riboflavin 0.1% and additional UV-A exposure (R+UV-A), amphotericin B alone (A), amphotericin B and riboflavin 0.1% (A+R), amphotericin B and UV-A (A+UV-A), amphotericin B and riboflavin 0.1% and additional UV-A exposure (A+R+UV-A). The mean growth inhibition zone (GIZ) was measured around the discs.

RESULTS. C. albicans, Fusarium sp, and A. fumigatus did not show any increased GIZ after treatment without previous amphotericin B medication. However, GIZ was significantly greater after pretreatment with amphotericin B and riboflavin/UV-A (A+R+UV-A) for C. albicans (P = 0.0005), Fusarium sp (P = 0.0023) and A. fumigatus (P = 0.0008) compared with A, A+R, and A+UV-A.

CONCLUSIONS. Amphotericin B is believed to interact with fungi membrane sterols to produce aggregates that form transmembrane channels. Given that collagen is one of the principal components of the cornea, it is also probable that amphotericin B may diffuse easily across-linking. Previous treatment with amphotericin B allowed riboflavin/UV-A effectiveness against C. albicans, Fusarium sp, and A. fumigatus. This schema might be used in the future for the treatment of keratomycosis. (Invest Ophtalmol Vis Sci. 2010;51:3950–3953) DOI:10.1167/iovs.09-4013

Infectious keratitis is a medical emergency. Improper management can lead to marked loss of vision. The principal organisms isolated from various etiologies of infectious keratitis are Gram-positive bacteria (e.g., coagulase-negative staphylococci, Staphylococcus aureus), Gram-negative bacteria (e.g., Pseudomonas aeruginosa), protozoa (Acantbamoeba species), viruses (Herpes simplex virus 1), and fungi. Filamentous fungi (Fusarium and Aspergillus) or yeast (primarily Candida albicans) may lead to severe keratomycosis. Globally, the incidence of keratomycoses and systemic mycoses is rising. Fungal keratitis is also difficult to treat. Few prospective studies have evaluated the effectiveness of different therapeutic approaches for fungal keratitis. Polyene antifungal antibiotics, the first-line therapy in fungal keratitis, are not always effective in severe keratomycosis. Echinocandins and triazole derivatives such as voriconazole might be a better choice. Nevertheless, current therapies often are ineffective. Furthermore, there has been an alarming resistance to antimicrobial agents. Emerging patterns of resistance, even to new classes of antimicrobial agents, have stimulated the continuing quest for an agent that provides rapid and complete microbialicidal activity with minimal toxic effects and susceptibility to mechanisms of microbial resistance.

Riboflavin, or vitamin B2, is a naturally occurring compound and an essential human nutrient. Studies demonstrated that riboflavin, when exposed to visible or UV light, may be used to inactivate pathogens. In addition, the antimicrobial activity of UV irradiation includes sporicidal and virucidal effects. This process has proven effective against a wide range of pathogens. This led Martins et al. to propose that the riboflavin/UV-A combination may be useful for the inactivation of pathogens found in corneal infections. Riboflavin/UV-A treatment was effective against nonresistant and multidrug-resistant bacteria but ineffective against C. albicans.

This study aimed to present the results of a series of in vitro experiments conducted in our laboratory to evaluate the antimicrobial effects of riboflavin/UV-A on C. albicans, Fusarium sp, and A. fumigatus and to expand the armamentarium of antimicrobial agents for the management of severe keratomycosis.

MATERIALS AND METHODS

Fungal Pathogens

Three fungal pathogens were selected by corneal scraping from a panel of human clinical ocular fungal pathogens isolated from patients with severe fungal keratitis and identified using routine procedures (culture on specific milieu, macroscopic and microscopic identification, PCR) of the mycology laboratory. The pathogens, maintained in the Mycology Laboratory, were one isolate of C. albicans, one isolate of Fusarium sp, and one isolate of A. fumigatus.

Experimental Design

In a first step, we tested the in vitro effect of riboflavin 1% (R) (Ricrolin, Soof Italia S.r.l., Montegiorgio, Italy) and UV-A light exposure (UV-A) in combination (R+UV-A) on fungal growth using a diffusion susceptibil-
ity assay. In a second step, a pretreatment with amphotericin B 10-μg tablets (A) (Neosensitabs, Rosco Diagnostica, Denmark) for 10 minutes was added to the same previous groups. Each experiment was repeated three times for each isolate.

**In Vitro Testing**

Standardized inocula determined by optical densitometry of *C. albicans* (0.5 McF), *Fusarium* sp (1 McF) and *A. fumigatus* (0.5 McF) were prepared using fungi in the active phase of growth. In accordance with the Clinical and Laboratory Standards Institute recommendations, mould inoculum was prepared from 7-day culture grown to obtain nongerminated conidial suspension. The suspensions were plated using sterile cotton swabs onto Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/mL methylene blue. Immediately after the agar plates were dry, Kirby-Bauer discs were used as reference points to locate the areas in which the riboflavin or the amphotericin B had been instilled and as guides for planimetric assay measurements. Thus, Kirby-Bauer discs were used only as reference points and to focus the instilled and as guides for planimetric assay measurements. Thus, Kirby-Bauer discs were used only as reference points (localization of the instillation and as guides for planimetric assay measurements). Therefore, the UV-A beam, focusing of the UV-A-light).

**Determination of Minimum Inhibitory Concentration (MIC)**

In accordance with the manufacturer’s instructions. For R+UV-A testing, UV-A was applied 20 minutes after riboflavin.

In the second part of the experiments, 10-μg amphotericin B tablets were placed for 20 minutes on the inoculated plates and then removed, and riboflavin (A+R), UV-A (A+UV-A), or riboflavin and UV-A combination (A+R+UV-A) was applied as described.

For the record, the different groups were tested with empty disc (control), riboflavin 0.1% alone (R), UV-A alone (UV-A), riboflavin 0.1% and additional UV-A exposure (R+UV-A), amphotericin B alone (A), amphotericin B and riboflavin 0.1% (A+R), amphotericin B and UV-A (A+UV-A), amphotericin B and riboflavin 0.1%, and additional UV-A exposure (A+R+UV-A).

Plates were then incubated at 37°C for 24 hours for *C. albicans* and 72 hours for *Fusarium* sp and *A. fumigatus*, adapted to the growth speed of each fungus.

**Analysis of Plates**

After incubation, each disc and its surrounding agar area was photographed with a digital camera. Images of each disc and its surrounding agar area were analyzed to measure the area of inhibition zone to the nearest whole centimeter. The mean growth inhibition zone (GIZ) in square centimeters was inversely proportional to the minimum inhibitory concentration of the organisms. The experimental design and the method of analysis are summarized in Figure 1.

**Statistical Analysis**

Descriptive statistics were expressed as mean ± SD of the three repeats. Two-way ANOVA was used for the analysis assuming a theoretically normal population. Multiple comparisons were made post hoc between the different groups to find significant differences. P < 0.05 was determined to be significant.

**Results**

The control, riboflavin, UV-A, and R+UV-A subgroups did not show any area of fungi growth inhibition. Subgroups pretreated with amphotericin B (A, A+R, A+UV-A, and A+R+UV-A) showed a GIZ in all fungi tested from 24 to 72 hours after the treatment was applied (Figs. 2, 3, 4).

On *C. albicans* (Fig. 2), the mean area of GIZ was 3.50 cm² (SD, 0.35) for the A subgroup, 3.34 cm² (SD, 0.22) for the A+R subgroup, 3.42 cm² (SD, 0.35) for the A+UV-A subgroup, and 5.47 cm² (SD, 0.59) for the A+R+UV-A subgroup. The area of GIZ was significantly increased (P = 0.0005) in the A+R+UV-A group compared with the control groups treated with A, A+R, or A+UV-A; the latter was comparable. ***P < 0.001.

On *Fusarium* sp (Fig. 3), GIZ was 3.38 cm² (SD, 0.73) for the A subgroup, 3.46 cm² (SD, 0.10) for the A+R subgroup, 3.26 cm² (SD, 0.49) for the A+UV-A subgroup, and 6.04 cm² (SD, 0.80) for the A+R+UV-A subgroup. The area of GIZ was

**FIGURE 2.** Comparison of the GIZ on *C. albicans* with four regimes of treatment. The mean area of GIZ measured 3.50 cm² (SD, 0.35) for the A subgroup, 3.34 cm² (SD, 0.22) for the A+R subgroup, 3.42 cm² (SD, 0.35) for the A+UV-A subgroup, and 5.47 cm² (SD, 0.59) for the A+R+UV-A subgroup. The area of GIZ was significantly increased (P = 0.0005) in the A+R+UV-A group compared with the control groups treated with A, A+R, or A+UV-A; the latter was comparable. ***P < 0.001.

(A+UV-A), amphotericin B and riboflavin 0.1%, and additional UV-A exposure (A+R+UV-A).

Candida albicans

![Graph showing inhibition zones](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932964/ on 11/26/2018)
A resistance. Recently, riboflavin/UV-A treatment has been confirmed to induce collagen cross-linking in corneal ectasias and UV-A (280–370 nm) may both damage nucleic acids by direct electron transfer, production of singlet oxygen, and UV-A exposure. This mechanism affects a large area of the cornea, it is probable that amphotericin B may diffuse easily after cross-linking. Even if cross-linking with methylglyoxal reduces corneal permeability, riboflavin/UV-A treatment may allow better diffusion of amphotericin B in a horizontal direction, reducing its vertical diffusion into the agar, leading to an overall higher surface concentration of the agent. This mechanism may be helpful to treat keratomycosis. However, the mechanisms of action may be more complex and multifaceted.

**Figure 3.** Comparison of the GIZ on *Fusarium sp* with four regimens of treatment. GIZ measured 3.38 cm² (SD, 0.73) for the A subgroup, 3.46 cm² (SD, 0.10) for the A + R subgroup, 3.26 cm² (SD, 0.49) for the A + UV-A subgroup, and 6.04 cm² (SD, 0.80) for the A + R + UV-A subgroup. The area of GIZ was significantly increased (P = 0.0023) in the A + R + UV-A group compared with the control groups treated with A, A + R, or A + UV-A. **P < 0.01.

**Figure 4.** Comparison of the GIZ on *A. fumigatus* with four regimens of treatment. The GIZ measurements were 3.09 cm² (SD, 0.48) for the A subgroup, 3.19 cm² (SD, 0.32) for the A + R subgroup, 2.92 cm² (SD, 0.48) for the A + UV-A subgroup, and 6.93 cm² (SD, 1.46) for the A + R + UV-A subgroup. The area of GIZ was significantly increased (P = 0.0008) in the A + R + UV-A group compared with the control groups treated with A, A + R, or A + UV-A.

**Discussion**

The work outlined here is directed to the development of riboflavin and UV-A as a novel method for the treatment of fungal keratitis, which is a cause of significant morbidity worldwide and can cause rapid and devastating vision loss. Fungal keratitis continues to be difficult to treat despite the use of topical and systemic antifungal agents and adjuvant surgery, such as corneal transplantation. Medical therapy has been boosted by the use of voriconazole, given topically or by other routes. However, the antimicrobials in use are sometimes problematic because of their toxic effects on the ocular surface and, more important, the emerging and increasing patterns of resistance. Recently, riboflavin/UV-A treatment has been confirmed to induce collagen cross-linking in corneal ectasias and lead to dose-dependent damage that can be expected in human corneas. Coincidently, this approach has also been researched for pathogen inactivation via the byproducts of riboflavin after UV-A exposure. This mechanism affects a large list of pathogens, including parasites and viruses. Riboflavin and UV-A (280–370 nm) may both damage nucleic acids by direct electron transfer, production of singlet oxygen, and generation of hydroxyl peroxide with the formation of hydroxyl radicals. Pathogen DNA/RNA may be affected in the absence of oxygen.

As part of an extensive evaluation of this potential new treatment, we have undertaken an experimental study to assess our assumption that the combined riboflavin/UV-A could have an effect in killing fungal corneal pathogens. Moreover, there is a possibility that the riboflavin already present in the cornea serves as a natural antimicrobial mechanism. Unfortunately, in approximately 50% of cases of keratomycosis, deep fungal penetration develops, often leading to dissemination in the anterior chamber. The combination of riboflavin/UV-A effects, however, can be found only down to approximately 300 μm, which means that clinically they probably have no impact on deep fungal infection.

Martins et al. demonstrated that riboflavin/UV-A treatment did not have any effect against *C. albicans* at either the 0.1% or the 0.5% riboflavin concentration. As we have shown, riboflavin/UV-A treatment was ineffective on *Fusarium sp*. However, our study shows significant in vitro inhibition growth of test isolates using combined riboflavin/UV-A treatment and amphotericin B (A + R + UV-A) compared with the other types of treatment used in this study (R alone, UV-A alone, and R + UV-A). Amphotericin B is a polyene antifungal drug used intravenously for systemic fungal infections and typically for keratomycosis. As with other polyene antifungals, amphotericin B is believed to interact with membrane sterols (ergosterol, a membrane chemical of fungi) to produce an aggregate that forms a transmembrane channel. Intermolecular hydrogen bonding interactions among hydroxyl, carboxyl, and amino groups stabilize the channel in its open form, destroying activity and allowing the cytoplasmic contents to leak out. The efficacy of riboflavin/UV-A treatment previously initiated by amphotericin may be explained here. Riboflavin may enter the fungi by passing through transmembrane channels developed by amphotericin. Conversely, riboflavin/UV-A treatment alone is not effective because riboflavin is blocked by the fungaI-impermeant membranes. We may also hypothesize that the agar consisting of polysaccharides as well as collagen hydrogels might be cross-linked, therefore facilitating amphotericin B diffusion. Because collagen is one of the principal components of the cornea, it is probable that amphotericin B may diffuse easily after cross-linking. Even if cross-linking with methylglyoxal reduces corneal permeability, riboflavin/UV-A treatment may allow better diffusion of amphotericin B in a horizontal direction, reducing its vertical diffusion into the agar, leading to an overall higher surface concentration of the agent. This mechanism may be helpful to treat keratomycosis. However, the mechanisms of action may be more complex and multifaceted.
Given that we were able to demonstrate in vitro activity of the amphotericin B/UV-A/riboflavin against these fungi and that this treatment was safe when the established criteria for the treatment were fulfilled, we suggest it may be possible to treat corneal infections using this approach. Results obtained in vitro do not always correlate with in vivo efficacy; therefore, further tissue culture models and animal studies are under way to test the efficacy of this treatment for infectious keratitis.

References