

# 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 after cross-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 Ophthalmol Vis Sci.* 2010;51:3950–3953) DOI:10.1167/iops.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 (*Acanthamoeba* species), viruses (Herpes simplex virus 1), and fungi.<sup>1</sup> 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.<sup>2</sup> Fungal keratitis is also difficult to treat.<sup>3</sup> 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. Echinocandin and triazole derivatives such as voriconazole might be a better choice. Nevertheless, current therapies often are ineffective.<sup>4</sup> 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 microbicidal activity with minimal toxic effects and susceptibility to mechanisms of microbial resistance.<sup>5</sup>

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.<sup>6,7</sup> In addition, the antimicrobial activity of UV irradiation includes sporicidal and virucidal effects.<sup>8</sup> This process has proven effective against a wide range of pathogens.<sup>9</sup> This led Martins et al.<sup>10</sup> 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, Sooft 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-

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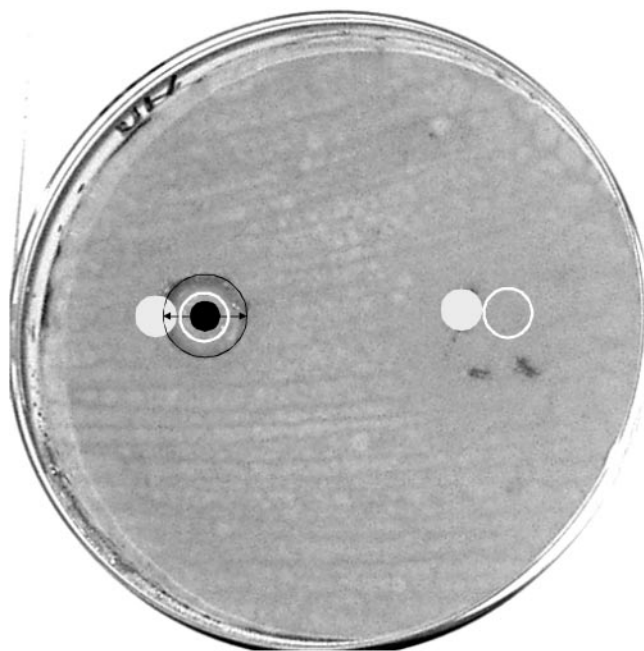
ity assay. In a second step, a pretreatment with amphotericin B 10- $\mu$ g tablets (A) (Neosensitabs, Rosco Diagnostica, Denmark) for 10 minutes was added to the same previous groups. Each experiment was repeated three times for each isolate.

**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  $\mu$ g/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 UV-A light. For riboflavin testing, 40  $\mu$ L riboflavin drops were placed next to the reference paper discs. For UV-A testing, the beam of the UV-A light (UV-X; Peschke Meditrade GmbH, Zürich, Switzerland) was directed next to the reference paper discs for 1 hour (spot, 3 mW/cm<sup>2</sup> and 11 mm; distance from the UV-A-source, 5 cm; surface, 95 mm<sup>2</sup>), in accordance with the manufacturer’s instructions. For R+UV-A testing, UV-A was applied 20 minutes after riboflavin.

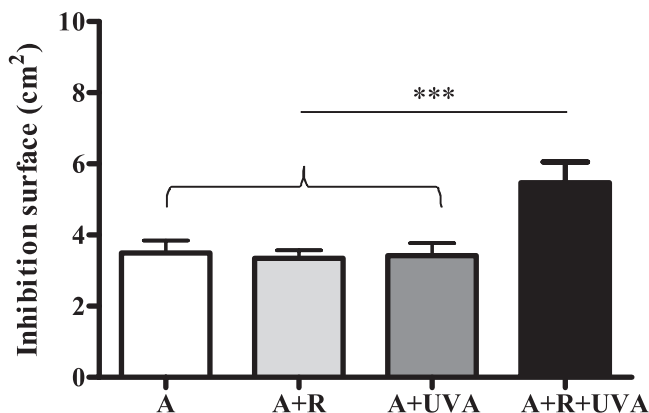
In the second part of the experiments, 10- $\mu$ g amphotericin B tablets were placed for 20 minutes on the inoculated plates and then removed, and riboflavin (A+R), UV-A (A+UV-A), or riboflavin and UV-A combination (A+R+UV-A) was applied as described.

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



**FIGURE 1.** Experimental design and analysis of plates.<sup>1</sup> Kirby-Bauer discs were used as reference points (localization of the instillation and the UV-A beam, focusing of the UV-A-light). *Black dot*: area for amphotericin B tablets applied for 10 minutes and then removed. *White circle*: riboflavin (40  $\mu$ L drop and 20 minutes of diffusion) and irradiation areas (for 1 hour after riboflavin instillation). *Black circle*: GIZ after a period of incubation. For example, in this picture, the GIZ on *Fusarium* sp is greater with a previous treatment with amphotericin B. *Left*: amphotericin B, riboflavin/UV-A. *Right*: riboflavin/UV-A.

*Candida albicans*



**FIGURE 2.** Comparison of the GIZ on *C. albicans* with four regimens of treatment. The mean area of GIZ measured 3.50 cm<sup>2</sup> (SD, 0.35) for the A subgroup, 3.34 cm<sup>2</sup> (SD, 0.22) for the A+R subgroup, 3.42 cm<sup>2</sup> (SD, 0.35) for the A+UV-A subgroup, and 5.47 cm<sup>2</sup> (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).

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**

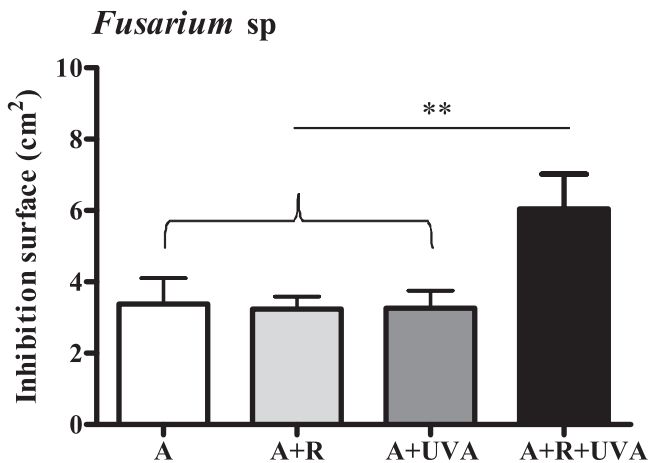
Descriptive statistics were expressed as mean  $\pm$ SD of the three repeats. Two-way ANOVA was used for the analysis assuming a theoretically normal population. Multiple comparisons were made post hoc between the different groups to find significant differences.  $P < 0.05$  was determined to be significant.

**RESULTS**

The control, riboflavin, UV-A, and R+UV-A subgroups did not show any area of fungi growth inhibition. Subgroups pre-treated 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 cm<sup>2</sup> (SD, 0.35) for the A subgroup, 3.34 cm<sup>2</sup> (SD, 0.22) for the A+R subgroup, 3.42 cm<sup>2</sup> (SD, 0.35) for the A+UV-A subgroup, and 5.47 cm<sup>2</sup> (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 being comparable.

On *Fusarium* sp (Fig. 3), GIZ was 3.38 cm<sup>2</sup> (SD, 0.73) for the A subgroup, 3.46 cm<sup>2</sup> (SD, 0.10) for the A+R subgroup, 3.26 cm<sup>2</sup> (SD, 0.49) for the A+UV-A subgroup, and 6.04 cm<sup>2</sup> (SD, 0.80) for the A+R+UV-A subgroup. The area of GIZ was



**FIGURE 3.** Comparison of the GIZ on *Fusarium sp* with four regimens of treatment. GIZ measured 3.38 cm<sup>2</sup> (SD, 0.73) for the A subgroup, 3.46 cm<sup>2</sup> (SD, 0.10) for the A+R subgroup, 3.26 cm<sup>2</sup> (SD, 0.49) for the A+UVA subgroup, and 6.04 cm<sup>2</sup> (SD, 0.80) for the A+R+UVA subgroup. The area of GIZ was significantly increased ( $P = 0.0023$ ) in the A+R+UVA group compared with the control groups treated with A, A+R, or A+UVA.  $**P < 0.01$ .

significantly increased ( $P = 0.0023$ ) in the A+R+UVA group compared with the control groups treated with A, A+R, or A+UVA, the latter being comparable.

On *A. fumigatus* (Fig. 4), the GIZ measurements were 3.09 cm<sup>2</sup> (SD, 0.48) for the A subgroup, 3.19 cm<sup>2</sup> (SD, 0.32) for the A+R subgroup, 2.92 cm<sup>2</sup> (SD, 0.48) for the A+UVA subgroup, and 6.93 cm<sup>2</sup> (SD, 1.46) for the A+R+UVA subgroup. The area of GIZ was significantly increased ( $P = 0.0008$ ) in the A+R+UVA group compared with the control groups treated with A, A+R, or A+UVA.

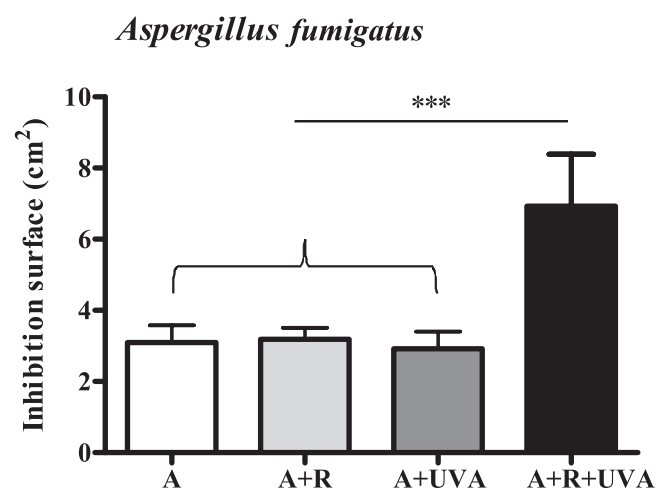
## DISCUSSION

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.<sup>1,2</sup> Fungal keratitis continues to be difficult to treat despite the use of topical and systemic antifungal agents and adjuvant surgery, such as corneal transplantation.<sup>1,2</sup> Medical therapy has been boosted by the use of voriconazole, given topically or by other routes.<sup>3,11</sup> 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.<sup>4</sup> Recently, riboflavin/UV-A treatment has been confirmed to induce collagen cross-linking in corneal ectasias<sup>12</sup> and to lead to dose-dependent damage that can be expected in human corneas.<sup>13</sup> Coincidentally, 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.<sup>14</sup> Riboflavin and UV-A (280–370 nm) may both damage nucleic acids by direct electron transfer, production of singlet oxygen, and generation of hydrogen peroxide with the formation of hydroxyl radicals. Pathogen DNA/RNA may be affected in the absence of oxygen.<sup>15,16</sup>

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.<sup>17,18</sup> The combination riboflavin/UV-A effects, however, can be found only down to approximately 300 μm,<sup>19</sup> which means that clinically they probably have no impact on deep fungal infection.

Martins et al.<sup>10</sup> 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 *A. fumigatus* and *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 topically 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.<sup>20,21</sup> 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.<sup>20</sup> Conversely, riboflavin/UV-A treatment alone is not effective because riboflavin is blocked by the fungal-impermeant membranes.<sup>20</sup> We may also hypothesize that the agar consisting of polysaccharides as well as collagen hydrogels might be cross-linked, therefore facilitating amphotericin B diffusion.<sup>22</sup> Because collagen is one of the principal components of the cornea, it is probable that amphotericin B may diffuse easily after cross-linking.<sup>22</sup> Even if cross-linking with methylglyoxal reduces corneal permeability,<sup>23</sup> 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 4.** Comparison of the GIZ on *A. fumigatus* with four regimens of treatment. The GIZ measurements were 3.09 cm<sup>2</sup> (SD, 0.48) for the A subgroup, 3.19 cm<sup>2</sup> (SD, 0.32) for the A+R subgroup, 2.92 cm<sup>2</sup> (SD, 0.48) for the A+UVA subgroup, and 6.93 cm<sup>2</sup> (SD, 1.46) for the A+R+UVA subgroup. GIZ area was significantly increased ( $P = 0.0008$ ) in the A+R+UVA group compared with the control groups treated with A, A+R or A+UVA.  $***P < 0.001$ .

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,<sup>24</sup> 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

1. Thomas PA, Geraldine P. Infectious keratitis. *Curr Opin Infect Dis*. 2007;20:129-141.
2. Srinivasan M. Fungal keratitis. *Curr Opin Ophthalmol*. 2004;15:321-327.
3. Jones A, Muhtaseb M. Use of voriconazole in fungal keratitis. *J Cataract Refract Surg*. 2008;34:183-184.
4. Thomas PA. Current perspectives on ophthalmic mycoses. *Clin Microbiol Rev*. 2003;16:730-797.
5. Shukla PK, Kumar M, Keshava GB. Mycotic keratitis: an overview of diagnosis and therapy. *Mycoses*. 2008;51:183-199.
6. Ruane PH, Edrich R, Gamp D, Keil SD, Leonard RL, Goodrich RP. Photochemical inactivation of selected viruses and bacteria in platelet concentrates using riboflavin and light. *Transfusion*. 2004;44:877-885.
7. Tsugita A, Okada Y, Uehara K. Photosensitized inactivation of ribonucleic acids in the presence of riboflavin. *Biochim Biophys Acta*. 1965;103:360-363.
8. Norval M. The effect of ultraviolet radiation on human viral infections. *Photochem Photobiol*. 2006;82:1495-1504.
9. Tang HM, Hamblin MR, Yow CM. A comparative in vitro photoinactivation study of clinical isolates of multidrug-resistant pathogens. *J Infect Chemother*. 2007;13:87-91.
10. Martins SA, Combs JC, Noguera G, et al. Antimicrobial efficacy of riboflavin/UV-A combination (365 nm) in vitro for bacterial and fungal isolates: a potential new treatment for infectious keratitis. *Invest Ophthalmol Vis Sci*. 2008;49:3402-3408.
11. Sponzel W, Chen N, Dang D, et al. Topical voriconazole as a novel treatment for fungal keratitis. *Antimicrob Agents Chemother*. 2006;50:262-268.
12. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg*. 2003;29:1780-1785.
13. Wollensak G, Spoerl E, Reber F, Seiler T. Keratocyte cytotoxicity of riboflavin/UV-A-treatment in vitro. *Eye*. 2004;18:718-722.
14. Goodrich RP. The use of riboflavin for the inactivation of pathogens in blood products. *Vox Sang*. 2000;78(suppl 2):211-215.
15. Baier J, Maisch T, Maier M, Engel E, Landthaler M, Baumler W. Singlet oxygen generation by UV-A light exposure of endogenous photosensitizers. *Biophys J*. 2006;91:1452-1459.
16. Hiraku Y, Ito K, Hirakawa K, Kawanishi S. Photosensitized DNA damage and its protection via a novel mechanism. *Photochem Photobiol*. 2007;83:205-212.
17. Das S, Samant M, Garg P, Vaddavalli PK, Vemuganti GK. Role of confocal microscopy in deep fungal keratitis. *Cornea*. 2009;28:11-13.
18. Vemuganti GK, Garg P, Gopinathan U, et al. Evaluation of agent and host factors in progression of mycotic keratitis: a histologic and microbiologic study of 167 corneal buttons. *Ophthalmology*. 2002;109:1538-1546.
19. Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr Opin Ophthalmol*. 2006;17:356-360.
20. Baginski M, Czub J, Sternal K. Interaction of amphotericin B and its selected derivatives with membranes: molecular modeling studies. *Chem Rec*. 2006;6:320-332.
21. Resat H, Baginski M. Ion passage pathways and thermodynamics of the amphotericin B membrane channel. *Eur Biophys J*. 2002;31:294-305.
22. Ahearne M, Yang Y, Then KY, Liu KK. Non-destructive mechanical characterisation of UV-A/riboflavin crosslinked collagen hydrogels. *Br J Ophthalmol*. 2008;92:268-271.
23. Stewart JM, Schultz DS, Lee OT, Trinidad ML. Collagen cross-links reduce corneal permeability. *Invest Ophthalmol Vis Sci*. 2009;50:1606-1612.
24. Spoerl E, Mrochen M, Sliney D, Trokel S, Seiler T. Safety of UV-A-riboflavin cross-linking of the cornea. *Cornea*. 2007;26:385-389.