

# Rose Bengal and Riboflavin Mediated Photodynamic Antimicrobial Therapy Against Selected South Florida *Nocardia* Keratitis Isolates

Ethan Adre<sup>1</sup>, Heather Durkee<sup>1,2</sup>, Alejandro Arboleda<sup>1</sup>, Karam Alawa<sup>1</sup>, Jorge Maestre<sup>3</sup>, Keenan J. Mintz<sup>4</sup>, Roger M. Leblanc<sup>4</sup>, Guillermo Amescua<sup>1,5</sup>, Jean-Marie Parel<sup>1,2,5</sup>, and Darlene Miller<sup>3,5</sup>

<sup>1</sup> Ophthalmic Biophysics Center, Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL, USA

<sup>2</sup> Department of Biomedical Engineering, College of Engineering, University of Miami, Coral Gables, FL, USA

<sup>3</sup> Ocular Microbiology Laboratory, Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL, USA

<sup>4</sup> Department of Chemistry, College of Arts and Science, University of Miami, Coral Gables, FL, USA

<sup>5</sup> Anne Bates Leach Eye Hospital, Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL, USA

**Correspondence:** Darlene Miller, University of Miami McKnight Vision Research Center, 1638 NW 10th Avenue, Room 103, Miami, FL 33136, USA.

e-mail: [dmiller@med.miami.edu](mailto:dmiller@med.miami.edu)

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**Purpose:** To examine and compare the efficacy of in vitro growth inhibition using rose bengal and riboflavin photodynamic antimicrobial therapy (PDAT) for *Nocardia* keratitis isolates.

**Methods:** *Nocardia asteroides* complex, *Nocardia amikacinotolerans*, and *Nocardia farcinica* species were isolated from patients with confirmed *Nocardia* keratitis. Isolates were tested against three experimental groups: (1) no photosensitizer/no irradiation, (2) photosensitizer/no irradiation, and (3) photosensitizer/irradiation. Each isolate was prepared in suspension to a concentration of  $1.5 \times 10^8$  CFU/mL. Bacterial suspensions were mixed with water or prepared 0.1% photosensitizer solution for a final bacterial concentration of  $1.5 \times 10^7$  CFU/mL. Aliquots of 1 mL were plated on 5% sheep blood agar. Rose bengal and riboflavin PDAT plates were irradiated for 15 minutes with a 525- or 375-nm custom 6-mW/cm<sup>2</sup> powered light source for a total fluence of 5.4 J/cm<sup>2</sup>. All experimental groups were repeated in triplicate. Plates were incubated in a 35°C non-CO<sub>2</sub> incubator for 96 hours and photographed. Percent inhibition was evaluated using LabVIEW-based software.

**Results:** All strains of *Nocardia* tested with 0.1% rose bengal and irradiated for 15 minutes demonstrated statistically significant inhibition of growth ( $P < 0.05$ ). No other experimental groups displayed any bacterial inhibition.

**Conclusions:** Rose bengal is superior to riboflavin PDAT against selected *Nocardia* isolates. In vivo testing is warranted to investigate the utility of rose bengal PDAT for severe *Nocardia* keratitis.

**Translational Relevance:** In vitro results for three clinical strains of *Nocardia* support the possible use of rose bengal PDAT as a complementary treatment of *Nocardia* keratitis.

## Introduction

*Nocardia* is an aerobic, Gram-positive, partially acid-fast, branching bacteria commonly found in dust,

soil, water, and vegetative matter. Ocular *Nocardia* infections are rare, with a global prevalence below 2%.<sup>1</sup> The majority of *Nocardia* keratitis cases are secondary to trauma,<sup>1–5</sup> contact lens use,<sup>6–12</sup> or iatrogenic<sup>13–15</sup> causes. Given the diversity,<sup>16–19</sup>

regionality,<sup>20</sup> rarity, and inexperience<sup>21</sup> with this organism, it is often misdiagnosed.<sup>3,13,22,23</sup> Initial management consists of multiple courses of topical, periocular, and systemic antimicrobials.<sup>2,3,22,24,25</sup> *Nocardia* keratitis does not respond to typical broad-spectrum antibiotics,<sup>7,25–32</sup> worsens with corticosteroid administration,<sup>33,34</sup> and has demonstrated pocketed resistance to the standard-of-care treatments for *Nocardia*, including amikacin<sup>1,6,10,24,35</sup> and trimethoprim-sulfamethoxazole.<sup>30,31,36</sup> If not identified rapidly and treated properly, *Nocardia* keratitis can result in poor visual prognosis with patients having significant corneal scarring<sup>11,37</sup> or corneal perforation<sup>23</sup> with secondary severe intraocular complications.

In light of this, there is an unmet need for an effective treatment against *Nocardia* keratitis. Photodynamic antimicrobial therapy (PDAT) is a promising alternative treatment option. PDAT is a photochemical reaction that utilizes a photosensitive dye activated by a certain wavelength of light. When the dye is energized to an excited state, it interacts with ambient oxygen to generate reactive oxygen species capable of initiating cell death through a variety of mechanisms.<sup>38</sup> An additional benefit of PDAT in corneal tissues is corneal strengthening via collagen crosslinking.<sup>39</sup> Riboflavin emerged as the first photosensitizing agent used for corneal crosslinking in patients with keratoconus,<sup>39</sup> and rose bengal has since been established as an effective photosensitizer for PDAT.<sup>40–46</sup> Previous in vitro and in vivo studies have demonstrated the efficacy of PDAT against microbial keratitis of multiple etiologies.<sup>41–43,47–49</sup> Further studies have successfully characterized the safety profile of rose bengal PDAT against microbial keratitis in humans, establishing its use as a viable therapy.<sup>40,44–46</sup> This paper reports on and compares the in vitro responses of *Nocardia* corneal isolates against PDAT using two different photosensitizers: rose bengal and riboflavin.

## Materials and Methods

### Organism Selection

Three isolates, *Nocardia asteroides* complex, *Nocardia amikacinintolerans* and *Nocardia farcinica* were chosen for experimentation. These species were among the most commonly isolated *Nocardia* species from Bascom Palmer Eye Institute. Each isolate was located in cold storage based on our ocular microbiology department records from patients with culture positive *Nocardia* recovered from the cornea. Prior to experimentation, each isolate was warmed to room temperature, subbed onto 5% sheep blood agar, and grown

in a 35°C non-CO<sub>2</sub> incubator for 72 hours to ensure maximal growth and reproductive viability and purity of the organisms (Fig. 1).

### Organism Characterization

In vitro antimicrobial susceptibilities of the *Nocardia* strains were determined through minimum inhibitory concentration breakpoints set by the Clinical and Laboratory Standards Institute.<sup>50</sup> Isolates were referred to an outside laboratory (Focus Diagnostics Infectious Disease, Cypress, CA) for identification and susceptibility testing. Intermediate susceptibilities were considered resistant to the accompanying antibiotic. It is important to note that there are no antibiotic susceptibility breakpoints for topical antibiotic therapy.

### Photosensitizer Preparation

Photosensitizing solutions were prepared the day of experimentation and kept in light-protected tubes to prevent photobleaching. Photosensitizing solutions of 0.1% concentration were prepared by mixing a ratio of 1 mg powder to 1 mL sterile water for rose bengal (330000; Sigma-Aldrich, St. Louis, MO) and riboflavin (R7774; Sigma-Aldrich). Photosensitizer concentrations were evaluated post hoc with UV-visible spectroscopy.

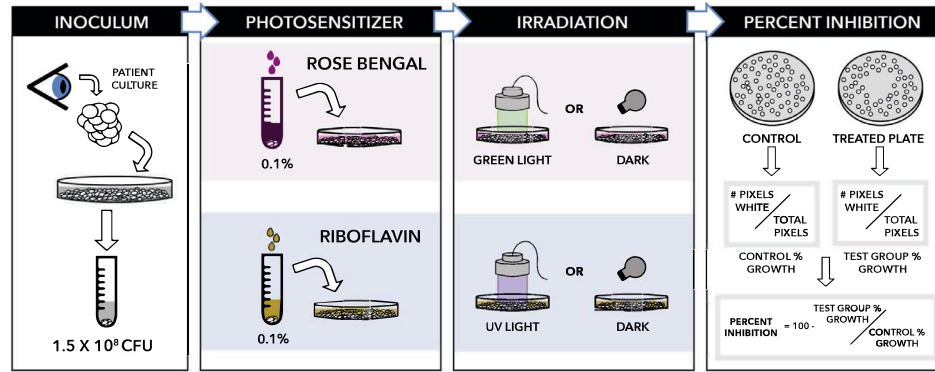
### Organism Preparation for PDAT

Each isolate was grown in 5% sheep blood agar plates for 72 hours before experimentation. Colonies were suspended in sterile water and adjusted to a 0.5 McFarland ( $1.5 \times 10^8$  CFU/mL) solution (Fig. 1).

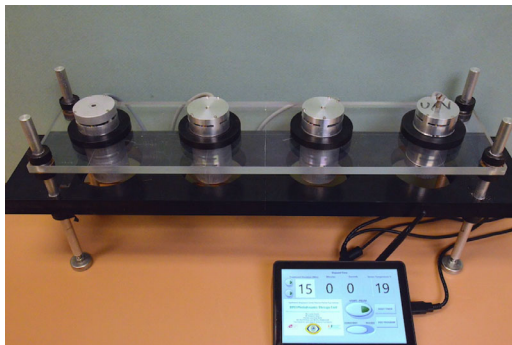
### PDAT

To test the in vitro response of the microorganisms to PDAT, three experimental groups were set for each photosensitizer: (1) growth control (no irradiation, no photosensitizer); (2) rose bengal or riboflavin dark (photosensitizer, no irradiation); and (3) PDAT (photosensitizer with irradiation). Groups were repeated in triplicate with both rose bengal and riboflavin. All experiments were conducted in minimal residual lighting of <1 lux, confirmed with a digital lux meter (LX1010B; Sinometer Instruments, Shenzhen, China).

Isolates were then diluted to a final working concentration of  $1.5 \times 10^7$  CFU/mL with either sterile water or photosensitizer solution. Aliquots of 1 mL of the growth control, rose bengal, or riboflavin solutions were then pipetted to individual 5% sheep blood agar



**Figure 1.** In vitro testing protocol of rose bengal and riboflavin PDAT. Culture-positive isolates are collected and prepared in suspension. Solutions are mixed with either 0.1% rose bengal or riboflavin. Plates are kept in the dark or irradiated with the appropriate light source. Photographs were taken and percent inhibition values were calculated after incubation. (Adapted from Halili et al.<sup>43</sup>)



**Figure 2.** Custom-made in vitro PDAT testing station. This setup allows for simultaneous testing of three sample plates with 525-nm light and an additional single 375-nm lamp on the right. Each light source is computer controlled with custom LabVIEW software.

plates, evenly distributed on the surface, and decanted of the excess solution. Plates in group 3 were irradiated for 15 minutes with either 525-nm light for rose bengal or 375-nm light for riboflavin. The light sources (Fig. 2) were developed in-house with 6-mW/cm<sup>2</sup> power density measured with a power meter (PM200; Thorlabs, Newton, NJ) and sensor (S130C; Thorlabs). The 525- and 375-nm irradiation sources produced a uniform irradiance of 6 mW/cm<sup>2</sup> over a circular surface of 47 mm diameter, and the plates were exposed for 15 minutes, producing a fluence of 5.4 J/cm<sup>2</sup>.<sup>41–43</sup> Previous testing showed that, in this setup, there is no increase in heat during irradiation.<sup>42</sup>

After irradiation, all plates were secured and shielded from light with aluminum foil and incubated at 35°C in a non-CO<sub>2</sub> incubator for 96 hours before observation. Photographs of each plate were taken after 96 hours to evaluate percent inhibition.

Percent growth inhibition on the agar plates was calculated using a custom software in LabVIEW, described previously.<sup>41–43</sup> Briefly, the percent growth

was calculated from a grayscale image:

$$\text{Percent growth} = \frac{\# \text{ White pixels}}{\text{Total \# pixels in central zone}}$$

with control group (1) considered 100% growth. Percent inhibition was then calculated:

$$\text{Percent inhibition} = 100\% - \frac{\text{Experimental group percent growth}}{\text{Growth control percent growth}}$$

ANOVA testing was used to assess the percent inhibition between experimental groups with significance set at  $P < 0.05$ .

## Results

### Nocardia Isolate Susceptibility Profiles

Antimicrobial susceptibility profiles are described in the Table. *Nocardia asteroides* complex was resistant to 8 of the 13 antibiotics tested. *Nocardia amikacini-tolerans* and *Nocardia farcinica* were resistant to 7 of the 13 antibiotics tested. All organisms were resistant to cefepime, ciprofloxacin, clarithromycin, and doxycycline.

### Photosensitizing Solutions

Spectroscopy of each photosensitizer solution measured extinction coefficients for rose bengal (85,410 L mol<sup>-1</sup> cm<sup>-1</sup>) and riboflavin (8140 L mol<sup>-1</sup> cm<sup>-1</sup>) in order to determine the concentrations. Final concentrations of rose bengal and riboflavin solutions were calculated to be 0.12% ± 0.002% and 0.09% ± 0.006%, respectively.

**Table.** Nocardia Isolate Antibiotic Susceptibility Profiles

Antibiotic Class	Antibiotic	<i>Nocardia asteroides</i> Complex	<i>Nocardia</i> <i>amikacinitolerans</i>	<i>Nocardia</i> <i>farcinica</i>
Aminoglycoside	Amikacin	R	R	S
	Tobramycin	S	S	R
Penicillin/cephalosporin	Amoxicillin/ clavulanic	S	S	S
	Ceftriaxone	R	S	R
	Cefepime	R	R	R
Fluoroquinolone	Ciprofloxacin	R	R	R
	Moxifloxacin	R	R	S
Macrolide	Clarithromycin	R	R	R
Tetracycline	Doxycycline	R	R	R
	Minocycline	S	S	R
Carbapenem	Imipenem	R	R	S
Oxazolidinone	Linezolid	S	S	S
Sulfonamide	Trimethoprim/ sulfamethoxazole	S	S	S

R, resistant; S, susceptible.

## PDAT

Photographs of the plates taken at 96 hours are shown in [Figure 3](#). Group 3 (0.1% rose bengal PDAT with 525 nm irradiation) showed 98.8%, 98.3%, and 90.6% inhibition against *Nocardia asteroides* complex, *Nocardia amikacinitolerans*, and *Nocardia farcinica*, respectively. Comparatively, in group 3, riboflavin PDAT with 375-nm irradiation demonstrated less than 0.2% in all tested *Nocardia* strains. In group 2, rose bengal alone and riboflavin alone demonstrated less than 1% inhibition for all *Nocardia* strain. Percent inhibition for all tested strains and experimental groups are shown in [Figure 4](#).

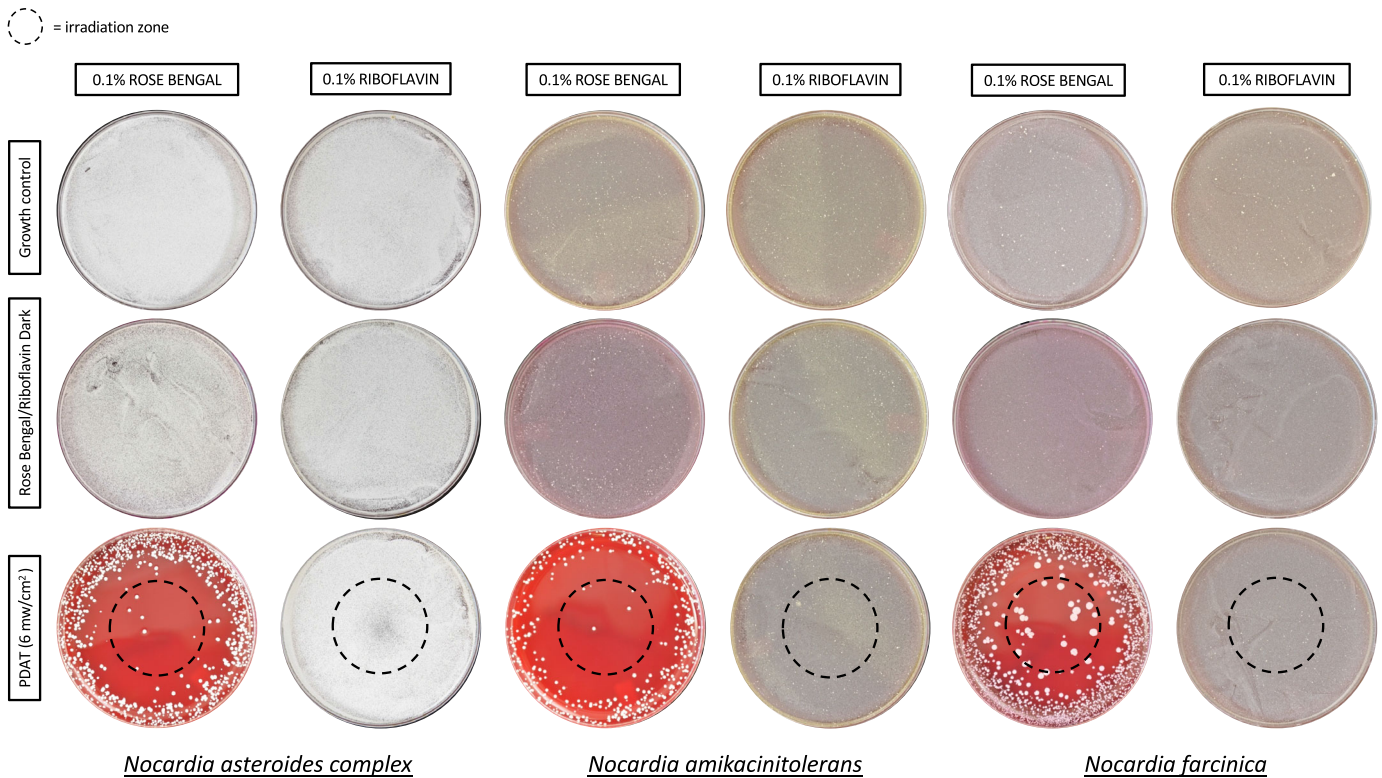
## Statistical Analysis

A one-way ANOVA test determined there was a statistically significant difference among all groups for each species: *Nocardia asteroides* complex,  $F(5,12) = 1638$ ,  $P < 0.001$ ; *Nocardia amikacinitolerans*,  $F(5,12) = 8276$ ,  $P < 0.001$ ; and *Nocardia farcinica*,  $F(5,12) = 31806$ ,  $P < 0.001$ . A Tukey honest significance difference test was conducted post hoc to compare the significance between individual groups. The experimental groups could be divided into two tiers of inhibition, all or none, with no significant overlap. The presence of 0.1% rose bengal with 525-nm irradiation demonstrated the only significant percent inhibition when compared with all other experimental groups tested.

## Discussion

This is the first study to compare in the in-vitro responses of *Nocardia keratitis* isolates to rose bengal and riboflavin PDAT. We found that 0.1% rose bengal PDAT significantly inhibits the growth of *Nocardia asteroides* complex, *Nocardia amikacinitolerans*, and *Nocardia farcinica* isolates. No other experimental groups demonstrated growth inhibition.

This study further supports the use of rose bengal as a photosensitizer for PDAT due to its broad efficacy against multiple bacterial and fungal species. Other studies have found similar results for the in vitro inhibition of PDAT against other microbial keratitis isolates, including *Fusarium*, *Aspergillus*, *Candida*,<sup>41</sup> *Staphylococcus*,<sup>43</sup> and *Pseudomonas*<sup>42</sup> species. These studies also demonstrated significant inhibition using rose bengal as a photosensitizer and limited success using riboflavin. The superior efficacy of rose bengal over riboflavin may be attributed to the greater singlet oxygen yield of rose bengal.<sup>51</sup> Singlet oxygen production of rose bengal may be greater due in part to the increased ability of rose bengal to absorb light. Our measured extinction coefficient of rose bengal ( $85,410 \text{ L mol}^{-1} \text{ cm}^{-1}$ ) was 10 times greater than that for riboflavin ( $8140 \text{ L mol}^{-1} \text{ cm}^{-1}$ ), demonstrating that rose bengal was a better absorber of light and offers the potential for increased production of singlet oxygen. Also, it is well known that, when activated with light, rose bengal exclusively produces singlet



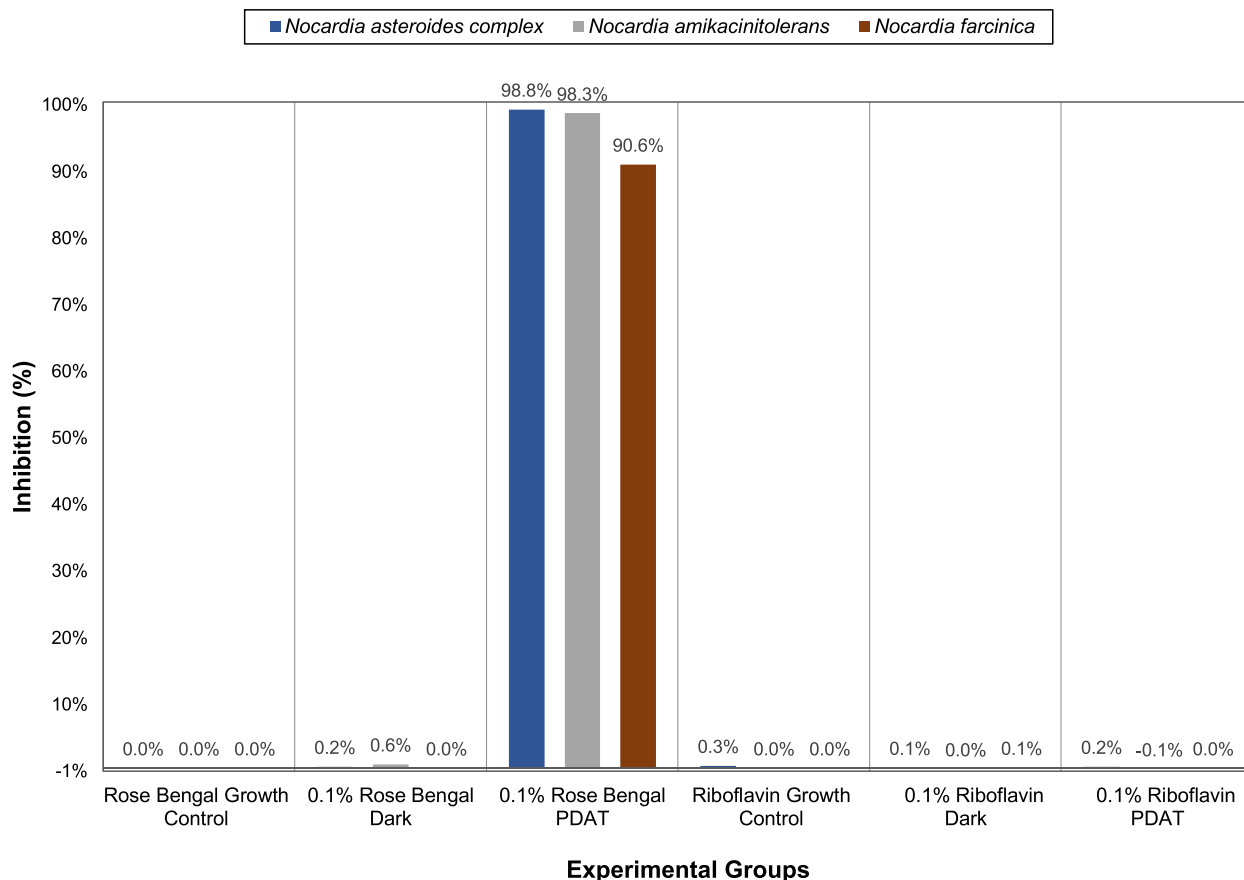
**Figure 3.** *Nocardia* growth after incubation. Representative photographs of each experimental group for three *Nocardia* isolates taken 96 hours after experimentation. The irradiation zone represents the central 47-mm area that corresponds to the diameter of the PDAT lamp.

oxygen (type 2 mechanism) in systems without a donor to facilitate the formation of the superoxide anion, whereas riboflavin produces both singlet oxygen and other reactive oxygen species (ROS) through electron transfer (type 1 mechanism).<sup>51</sup> This difference in ROS production may contribute to the increased effect seen from rose bengal because more singlet oxygen is produced through this photosensitizer.<sup>52</sup> A final potential difference between the efficacies of rose bengal and riboflavin PDAT is that the 375-nm light used to activate the riboflavin is competitively absorbed by bacteria<sup>53</sup>; therefore, less light is available to activate the riboflavin to create ROS and singlet oxygen.

Furthermore, it has been established that *Nocardia* species carry genes coding for catalase<sup>54</sup> and superoxide dismutase,<sup>55,56</sup> which allow them to neutralize free radicals. In vitro experiments have found that *Nocardia* species are able to survive oxidative metabolic bursts from neutrophils through direct activity of these two enzymes.<sup>54–56</sup> If catalase and superoxide dismutase demonstrate greater affinity for neutralizing non-singlet ROS, which are more readily produced with activation of riboflavin,<sup>57</sup> it is conceivable that this would reduce the antimicrobial efficacy of riboflavin PDAT. More studies should be performed to find if these defense mechanisms have an effect and to evaluate

for any strain-specific resistance to PDAT. The inherent free-radical scavenging system of *Nocardia* may play a role in the organism's resistance to riboflavin PDAT.

In this set of *Nocardia* isolates, we found a high resistance to empiric, broad-spectrum antibiotics, including ciprofloxacin and moxifloxacin, in addition to resistance to first-line<sup>35</sup> amikacin treatment in two of the three isolates in this study. Previous studies have characterized antibiotic resistance patterns for *Nocardia*. The reported percent resistance for ciprofloxacin ranged between 45% and 83%,<sup>19,28,30,58,59</sup> moxifloxacin was 60%,<sup>19,28</sup> and amikacin was between 0% and 11.8%.<sup>19,28,30,58,59</sup> Our results align with the general profile of fluoroquinolone resistance but differ with more prominent amikacin resistance. These differences between our susceptibility results and those previously described can be due to a combination of the regionality<sup>32</sup> of *Nocardia* species and strain-specific characteristics.<sup>16,20</sup> Case reports have also noted resistance to amikacin in multiple *Nocardia* species,<sup>4,7,10,25,29</sup> including *Nocardia asteroides complex* and *Nocardia amikacinitorerans*, as examined in our study. For clinicians, this highlights the importance of species identification and susceptibility testing to cater treatment regimen. A single, blanket antibiotic therapy is not effective in treating *Nocardia* keratitis.



**Figure 4.** Percent inhibition of *Nocardia* keratitis isolates growth in response to treatment. The values for each bar represent the average percent inhibition of the three trials for each experimental group.

Overall, this study provides the basis for further experimentation and characterization of *Nocardia* keratitis and its broader response to PDAT. With trends continuing, we will likely discover more antibiotic resistance among other *Nocardia* species and establish greater reason to incorporate alternative antimicrobial strategies such as rose bengal PDAT. The success of this study warrants further examination of the in vivo response of *Nocardia* to rose bengal PDAT. Rose bengal PDAT may even be considered in cases of severe, refractory *Nocardia* keratitis as a last resort before surgical intervention.

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