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Effective photosensitization and selectivity *in vivo* of *Candida albicans* by meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate

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Abstract

Background and Objective—The fungus *Candida albicans* commonly causes mucosal and cutaneous infections in patients with impaired immunity. We investigated the effectiveness of the photosensitizer meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363) in the photodynamic treatment (PDT) of *C. albicans* infection *in vitro* and its selectivity in an animal model.

Materials and Methods—The efficacy of TMP-1363 in PDT of *C. albicans* *in vitro* was compared to that of methylene blue (MB) using a colony forming unit (CFU) assay. *In vivo* infection in the mouse was established by inoculation of *C. albicans* yeast in the intradermal space of the ear pinna. Two days post-infection, 0.3 mg mL⁻¹ TMP-1363 was administered topically. Thirty minutes after TMP-1363 application, the ears were irradiated at 514 nm using a fluence of 90 J cm⁻² delivered at an irradiance of 50 mW cm⁻². The ears were excised 2 hours post-irradiation, homogenized and the organism burden was determined by a CFU assay. *In vivo* wide field and confocal fluorescence imaging assessed the localization of the photosensitizer in relationship to *C. albicans*.

Results—Photosensitization with TMP-1363 resulted in a greater than three-log increase in killing of *C. albicans* *in vitro* compared to MB. *In vivo* fluorescence imaging demonstrated a high degree of selective labeling of *C. albicans* by TMP-1363. PDT of infection using TMP-1363 resulted in a significant reduction in CFU/ear relative to untreated controls. Infected ears subjected to PDT displayed complete healing over time with no observable damage to the pinna.

Conclusion—Our *in vitro* and *in vivo* findings support TMP-1363-mediated PDT as a viable therapeutic approach for the photodynamic treatment of candidiasis.

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Keywords

Photodynamic therapy; *Candida albicans*; TMP-1363; *In vivo* imaging; Confocal fluorescence microscopy

INTRODUCTION

Candida species colonize the epithelial surfaces of the body in the majority of the human population [1]. Clinical candidiasis commonly occurs when colonization progresses to infection following impairment of innate and/or adaptive host defenses [2]. Patients undergoing therapy for cancer [3], those suffering from HIV/AIDS [1], primary immunodeficiencies [4], diabetes [5], neonatal and elderly populations [1], and patients with indwelling catheters [6] are among the specific groups at risk of developing infection from *Candida*. As a result of its widespread colonization of mucosal and cutaneous epithelium, the bulk of *Candida* infections are located at these sites [1] and significantly impair the quality of life. Mucosal *C. albicans* infections in particular are a significant infectious disease problem and are often difficult to eradicate because of the high frequency of acquired resistance to conventional antifungal agents [7]. Photodynamic therapy (PDT) is a readily applicable alternative to control cutaneous and mucocutaneous *C. albicans* infection [8–10].

Previous studies demonstrated that filamentous forms and biofilms of *C. albicans* were sensitive to PDT using Photofrin as a photosensitizer [11,12]. In contrast, early stationary phase yeast forms of *C. albicans* and *Candida glabrata* were not adversely affected by Photofrin-based PDT [11]. We recently reported that PDT mediated by the cationic porphyrin photosensitizer meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363; Frontier Scientific, Logan, UT) is highly effective *in vitro* against the yeast form of these two pathogenic *Candida* species [13,14]. TMP-1363 is also effective in antibacterial PDT *in vitro* in the presence of serum [8], and a closely related tetra-cationic porphyrin TMAP⁴⁺ has potent *in vitro* activity in PDT of *C. albicans* [15]. The cationic phenothiazine photosensitizer methylene blue (MB) [16–18] has also been used in PDT of *C. albicans* *in vitro*. However, there has been only limited testing of the effectiveness of PDT against *C. albicans* infection *in vivo* [19–21] and no reports describing TMP-1363 *in vivo* against *Candida*.

In a recent study, we established an intradermal *C. albicans* infection in the mouse ear pinna to study *Candida* morphogenesis *in vivo* [22]. In this model, *C. albicans* establishes local microabscesses like those seen in the kidney after tail vein inoculation with the fungus [23]. Similar models have been used previously to study cutaneous parasite infection [24] and wound healing [25]. The infected ear pinna model also offers several advantages in terms of convenience of imaging infection, topical application of photosensitizer and PDT irradiation.

Here we describe the superior efficacy of TMP-1363 against *C. albicans* *in vitro* relative to MB. We also report exquisitely specific labeling of *C. albicans* organisms by TMP-1363 *in vivo* following topical application on the infected ear and significant reduction of fungal burden following PDT using a short 30-minute drug-light interval. Our results justify further optimization of topical TMP-1363-mediated PDT in anti-fungal applications.

METHODS

Organisms and animal model

C. albicans was grown overnight to early stationary phase in yeast extract-peptone-dextrose (YPD) broth (Difco, Detroit, MI). To establish infection, 3×10^7 *C. albicans* yeast forms in a 30 μ L volume of phosphate buffered saline were injected intradermally in the ears of anesthetized female BALB/c mice. The evaluation of PDT response *in vivo* was performed using *C. albicans* strain SC5314 [26]. *In vivo* co-localization studies of *C. albicans* with photosensitizer TMP-1363 used *C. albicans* strain YAW3 constitutively expressing green fluorescent protein (GFP). *C. albicans* YAW3 was generously provided by Drs. James Konopka and Amy Warena (State University of New York, Stony Brook) [27]. Mice used in the co-localization experiments were maintained on chlorophyll-free chow to minimize endogenous fluorescence [28].

In vitro PDT treatment conditions

C. albicans SC5314 was grown overnight to early stationary phase in YPD broth (Difco). Organisms were washed twice in sterile dH₂O and adjusted to an O.D._{600 nm} of 1.0. Cells were incubated with photosensitizer for 30 min at a concentration of 10 μ M for both TMP-1363 and clinical grade methylene blue (MB; American Regent, Shirley, NY). Cells were pelleted by centrifugation, the supernatant containing photosensitizer was removed and the cells were resuspended in the original volume of dH₂O. Two mL of cell suspension was placed in a 6-well tissue culture dish (Becton Dickinson, Franklin Lakes, NJ) and irradiated with a fluence of 2.4 J cm⁻² of visible light from a 48 cm x 48 cm light box equipped with a bank of fluorescent lamps (Sylvania GRO-LUX, 15 W, Model: F15T8/GRO). The irradiance at the surface of the light box was 4.0 mW cm⁻², and the spectrum of the light was such that approximately 67% of the power was emitted within the range of 575–700 nm. The fluence of 2.4 J cm⁻² corresponded to a 10 minute irradiation time. Untreated organisms and organisms treated with photosensitizer but shielded from light were used as controls. Organism killing was determined by a CFU assay as described previously [13]. To compare photosensitizer efficacy quantitatively, we used the measured output spectrum of the fluorescent lamps and the absorption spectra to compute the relative numbers of photons absorbed by each photosensitizer under the conditions of our experiments. These are shown for TMP-1363 and MB in Figure 1. The number of photons emitted by the lamps in 1-nm wavelength intervals in the range 400–750 nm was computed, and the number absorbed by each photosensitizer on a per molecule basis was calculated using the expression,

$$N_{abs} = \sum_{\lambda=400nm}^{750nm} N_{in}(\lambda)(1 - 10^{-OD(\lambda)}) \quad (1)$$

where N_{abs} is the number of photons absorbed and $N_{in}(\lambda)$ is the number of photons emitted by the lamps at wavelength λ . The optical density (OD) is that of the photosensitizer.

Confocal imaging of photosensitizer uptake by *C. albicans*

Uptake of cationic photosensitizer TMP-1363 was examined in both yeast and filamentous forms. *C. albicans* SC5314 was grown overnight to early stationary phase in YPD broth (Difco), and washed twice with sterile dH₂O. For photosensitizer uptake by yeast forms, cells were adjusted to 1×10^7 cells mL⁻¹ in M199 tissue culture medium (Invitrogen Corp., Carlsbad, CA). To induce filamentation, early stationary phase yeast forms were adjusted to 5×10^5 cells mL⁻¹ in M199 medium. Two mL of the respective cell suspensions was added to 6-well culture dishes containing sterile 25-mm circular No. 1 thickness glass coverslips.

Cultures were incubated at 37⁰ C for 2 h to allow cells to adhere to the coverslip [11]. Medium 199 was removed, replaced with 2 mL 10 µg mL⁻¹ of TMP-1363 in dH₂O, and cells were incubated at room temperature for 10 min. Coverslips were then washed gently with dH₂O to remove excess TMP-1363. Confocal microscopy was performed with a Nikon TE-2000 inverted microscope equipped with custom laser scanning confocal fluorescence imaging capability [11,29]. Images were acquired using 639 nm excitation and a 60× 1.4-NA oil immersion objective, providing an optical section thickness of 0.8 µm.

In vivo PDT treatment conditions

Two days post-infection, a topical application of 0.3 mg mL⁻¹ TMP-1363 in 50 µL of either a glycerol/water (2:3) or ethanol/glycerol/water (2:2:1) vehicle was applied to the dorsal surface of the ear. Control ears were infected and left untreated. 30 min after photosensitizer application, the ear was cleaned by gentle wiping with a cotton swab soaked in phosphate buffered saline. The dorsal surface of the ear was irradiated with 514 nm light from an argon-ion laser (Coherent Inc., Santa Clara, CA) delivered through a GRIN-lens-terminated multimode fiber (OZ Optics, Ottawa, ON, Canada). The treatment fluence was 90 J cm⁻², and the irradiance was 50 mW cm⁻².

Assay of in vivo phototoxicity

Two hours post-irradiation, the mice were sacrificed, and the ears excised and placed in a 2 mL microfuge tube containing 0.5 mL 0.25% trypsin and 0.38 g L⁻¹ EDTA (Invitrogen, Carlsbad, CA). Ear tissue was dissociated using a mechanical tissue homogenizer (VWR International Inc., West Chester, PA) to release organisms. The organism burden per ear was determined by a CFU assay. Serial dilutions of the ear tissue homogenate in PBS were plated onto YPD agar (Difco) plates containing 50 µg mL⁻¹ gentamicin and 10 µg mL⁻¹ chloramphenicol to suppress growth of bacteria colonizing the ear surface, and plates were incubated at 37°C for 48 h. Data were expressed as CFU/ear.

Statistics

For the comparison of methylene blue (MB) and TMP-1363 in PDT of *C. albicans in vitro*, each experimental group was assayed in duplicate and the experiments were performed three times. The colony forming unit data to assess phototoxicity of TMP-1363 against *C. albicans in vivo* was collected from three ears for each of the three treatment groups described above. For both *in vitro* and *in vivo* studies, pair-wise comparisons of means $\pm$ standard deviations between treatment groups were made using the *t* test (two-sample *t*-test, Origin 8.0, OriginLab, Northampton, MA, USA). The assessment of the underlying populations was conducted assuming them to be independent and by considering the variances between the two samples to be equal or unequal. The latter assumption did not influence the results at the *p* < 0.05 level.

Imaging and spectroscopy

In vivo fluorescence imaging was used to assess TMP-1363 distribution throughout the infected ear. Prior to imaging, hair on the ears was removed by chemical depilation. To examine photosensitizer distribution throughout the entire ear, single, large-field-of-view (FOV) images of TMP-1363 emission were acquired using a stereofluorescence microscope (Model SMZ1500, Nikon Instruments, Melville, NY) equipped with an X-Cite illumination source (EXFO, Mississauga ON, Canada). TMP-1363 fluorescence was acquired using a custom filter cube (HQ560/120×; 635 DCXR; HQ645LPm, Chroma Technology, Rockingham, VT) and a 12-bit TE-cooled CCD (CoolSNAPHQ, Roper Scientific, Trenton, NJ). Images were acquired using a 0.5× objective with a 0.75× magnification zoom that corresponded to FOVs of 25.2 mm × 19 mm.

To confirm the presence of TMP-1363 in the imaged field, fluorescence spectroscopy measurements were performed using a bifurcated fiber-optic bundle accessory to a fluorescence spectrophotometer (Cary Eclipse, Varian Inc., Palo Alto, CA). The end of the fiber bundle was placed in direct contact with the mouse ear, and the emission spectra were recorded with excitation at 420 nm.

In vivo confocal fluorescence imaging at subcellular resolution was performed by placing the anesthetized mouse on the stage of an inverted microscope in a supine position, so that the ventral side of the ear was facing downward for imaging. Images were acquired using a 10×, 0.45 NA objective and a 100 μm-diameter pinhole, which provided an optical section thickness of approximately 6 μm as determined by fluorescence edge response measurements. Images were acquired at 16 bits with a lateral resolution of 1 μm per pixel. GFP was excited at 488 nm, and emission was detected using a combination of 500 nm long pass and 515/30 nm bandpass filters (Chroma). TMP-1363 fluorescence was excited at 639 nm and detected using a combination of 647 nm (Semrock, Rochester, NY) and 655 nm (Chroma Technology) long pass filters. For imaging both *C. albicans* and TMP-1363 in the same optical section, we performed sequential image acquisitions of the same field of view using 488 and 639 nm excitation. GFP-labeled *C. albicans* organisms were good candidates for co-localization analysis in that GFP was not excited at 639 nm but was excited very efficiently at 488 nm. Similarly, there was no detectable TMP-1363 fluorescence excited at 488 nm. Thus, images from identical fields of view were comprised exclusively of GFP or TMP-1363 fluorescence when excited by 488 or 639 nm light, respectively.

Co-localization analysis

The extent of co-localization between the fluorescence of GFP-expressing *C. albicans* (green channel) and that of TMP-1363 (red channel) images was determined using the Intensity Correlation Analysis tool in ImageJ (<http://rsb.info.nih.gov/ij/>), a statistical approach based on a cross-correlation analysis introduced by Manders *et al.* [30]. This analysis yielded coefficients that quantified the co-localized fraction of the signal in the two channels above a specified intensity threshold. The co-localization coefficients for each channel were calculated using Equation 2,

$$M_{red} = \frac{\sum_i R_i \delta_{rg}(i)}{\sum_i R_i} \quad M_{green} = \frac{\sum_i G_i \delta_{gr}(i)}{\sum_i G_i}, \quad (2)$$

where M_{red} and M_{green} are the co-localization coefficients, R_i and G_i represent red and green intensities of the i^{th} pixel, and $\delta_{rg}(i)$ and $\delta_{gr}(i)$ are Kronecker deltas that represent intensity independent co-localization weights. Thus, for every i^{th} pixel, $\delta_{rg}(i) = \delta_{gr}(i) = 0$, if there is a red intensity or green intensity but not both, and $\delta_{rg}(i) = \delta_{gr}(i) = 1$, only if there is both a red and green intensity associated with the i^{th} pixel. Therefore, M_{red} and M_{green} may have values that vary from 0 to 1, the former corresponding to non-overlapping images and the latter reflecting 100% co-localization between both images.

RESULTS AND DISCUSSION

We compared the efficacy of PDT mediated by the porphyrin-based photosensitizer TMP-1363 with that of the phenothiazine MB against *C. albicans in vitro*. As shown in Figure 2, upon irradiation at a fixed fluence of 2.4 J cm⁻², photosensitization with TMP-1363 resulted in greater than three logs more killing of *C. albicans* than MB at the

same incubation concentration. This representation, however, may underestimate the photodynamic efficacy of TMP-1363 because, as illustrated in Figure 1, it is excited less efficiently by our irradiation source than MB. Based on the output spectrum of the irradiation light source and the absorption spectra of the sensitizers, we used Equation 1 to determine that, for equivalent durations of irradiation, the number of photons absorbed by TMP-1363 was ~ 10-fold lower than that absorbed by the equivalent concentration of MB. Because both photosensitizers generate reactive oxygen species at high yields, it is likely that differences in cellular uptake and/or intracellular localization account for the significantly greater PDT efficacy of TMP-1363.

Our previous *in vivo* imaging study with GFP-labeled *C. albicans* in the infected mouse ear model revealed that the morphologies of *C. albicans* range from yeast to multicellular hyphal filaments [22]. Therefore, a rigorous evaluation of photosensitizer localization demanded that the uptake of TMP-1363 by *C. albicans* be examined in both the yeast and the hyphal form of the organism. TMP-1363 was incubated with both of these forms of *C. albicans* and its subcellular localization imaged using confocal fluorescence microscopy. As shown in Figure 3, bright intracellular fluorescence from TMP-1363 was observed after a 10-minute incubation with a concentration of $10 \mu\text{g mL}^{-1}$. A series of $0.8 \mu\text{m}$ -thick optical sections through a field of *C. albicans* illustrated in Figure 3(c–f), confirmed that the sensitizer fluorescence did not originate solely with cell-surface-bound-material but was distributed throughout the cell. The punctate appearance of intracellular fluorescence, most evident in Fig. 3(a), suggests a degree of organelle compartmentalization of TMP-1363 in *C. albicans*. These findings *in vitro* provided strong rationale for the further evaluation of TMP-1363 *in vivo*.

Figure 4 illustrates TMP-1363 localization in the *C. albicans*-infected mouse ear pinna model. To begin the investigation, TMP-1363 was solubilized in a glycerol:water (2:3) vehicle. The addition of glycerol to water-soluble TMP-1363 was intended to enhance the association of the photosensitizer with the pinna. TMP-1363 was applied topically to the entire ear. We evaluated TMP-1363 fluorescence using large field of view stereofluorescence imaging, *in vivo* spectroscopy, and confocal fluorescence imaging. As shown in the stereofluorescence image of Figure 4(a), we observed strong fluorescence in selected areas distributed over large areas of the ear, consistent with specific localization to infected areas. To confirm that the fluorescence signal originated from TMP-1363, we used a bifurcated fiber bundle attachment to a fluorometer to perform *in vivo* fluorescence spectroscopy of the infected ear. At 420 nm excitation, the fluorescence emission spectrum, an example of which is shown in Figure 4(b), was dominated overwhelmingly by TMP-1363. In order to visualize the distribution of TMP-1363 in the infected tissue at high spatial resolution, the mouse ear was imaged *in vivo* using confocal microscopy. Images like that shown in Figure 4(c) revealed that the intense fluorescence from TMP-1363 appeared highly localized to individual *C. albicans* cells. This highly selective localization was persistent up to at least 6 hours after the topical application.

In a recently reported study [22], we used a *C. albicans* strain transfected with yeast GFP [27] placed under the control of the constitutively active *ADHI* promoter to image *C. albicans* morphogenesis during the progression of an intradermal infection in the ear *in vivo*. Using this model system, we rigorously evaluated the extent of selectivity of TMP-1363 for *C. albicans* following topical application *in vivo*. GFP fluorescence from *C. albicans* was excited efficiently by 488 nm light, and in the absence of TMP-1363 there was no detectable endogenous fluorescence under conditions of 639 nm excitation (data not shown). TMP-1363 fluorescence was not detected with excitation at 488 nm but was excited well at 639 nm. Figures 5(a) and 5(b) show *in vivo* images from identical fields of view in an infected ear that are comprised exclusively of GFP or TMP-1363 fluorescence when excited

by 488 or 639 nm light, respectively. Figure 5(c) is a merged image of the two channels. Strong pixel co-localization of GFP and TMP-1363 fluorescence is depicted by the yellow/orange color. The panel of images in Figures 5(d–e) demonstrates on a magnified scale the extent of co-localization between the GFP and TMP-1363 fluorescence in a region of interest indicated by the box superimposed on the image shown in Figure 5(c). In the merged image of Figure 5(f), a high degree of overlap is evident between pixels that display both GFP fluorescence and TMP-1363 labeling.

Analysis of the data shown in Figure 5 using the Mander's coefficient approach as described in Methods and summarized in Equation (2) yielded co-localization coefficients $M_{red} = 0.99$ and $M_{green} = 0.89$. Thus, 99% of pixels exhibiting red TMP-1363 fluorescence also contained a green signal from GFP-expressing *C. albicans*, and 89% of the pixels positive for GFP had a TMP-1363 signal, as well. Taken together, the data suggest that TMP-1363 exhibits high affinity for *C. albicans* relative to host tissue and may serve not only as a selective PDT agent but also as an excellent fluorescent contrast agent for detection of *C. albicans* infection.

Based on the highly selective staining of *C. albicans* with TMP-1363 in the context of infection, we subjected *C. albicans*-infected tissue to PDT. One of the challenges in the topical administration of photosensitizer is the barrier posed by stratum corneum. Recent studies by Boiy *et al.* [31,32] investigated the penetration of the photosensitizer hypericin into mouse skin using different vehicles, and reported significant, vehicle-dependent differences in penetration depths. Based on our own initial findings and their results, we evaluated PDT efficacy *in vivo* following the topical administration of 50 μ L of 0.3 mg mL⁻¹ TMP-1363 in two vehicles: glycerol:water (2:3) and ethanol:glycerol:water (2:2:1), each applied once for 30 min. Infected ears were then irradiated at 514 nm for 90 J cm⁻² with an irradiance of 50 mW cm⁻². Shown in Figure 6, the statistical analysis of organism levels recovered from infected control animals demonstrated a consistent level of infection with *C. albicans*, as reflected by a low standard deviation in CFU recovered from tissue homogenates. Significantly reduced organism levels ($p < 0.05$) were observed following PDT using the ethanol:glycerol:water formulation compared to untreated controls, resulting in an ~ 50-fold reduction in CFU/ear relative to untreated control mice. PDT using TMP-1363 in ethanol:glycerol:water resulted in a significant reduction in organism burden ($p < 0.05$) compared to PDT using the photosensitizer in the glycerol:water formulation.

To address the issue of potential phototoxicity to host tissue, we monitored an infected mouse ear pinna subjected to PDT with TMP-1363 in ethanol:glycerol:water using the same treatment parameters. Again, the TMP-1363 formulation was applied topically to the dorsal surface of the ear, and the entire dorsal surface was irradiated. The panel of photographs shown in Figure 7 taken over 19 days post-PDT demonstrated initial scar formation and subsequent healing following PDT, including recovery of hair growth.

In summary, we demonstrated that tetra-cationic, porphyrin-based photosensitizer TMP-1363 displayed substantial efficacy against *C. albicans* both *in vitro* and *in vivo*. The *in vitro* efficacy of TMP-1363 in PDT of early stationary phase yeast forms is particularly striking in comparison to MB, especially given the relatively inefficient excitation of TMP-1363 under the conditions of our experiments. *In vivo*, fluorescence microscopy and image analysis demonstrated that TMP-1363 had a remarkably strong selectivity for *C. albicans* in the context of infection. We suggest that the phototoxicity of TMP-1363 for *C. albicans*, coupled with selectivity for the fungus, resulted in significant organism reduction using a PDT dose that allowed apparently normal healing of infected tissue. These collective observations indicate that topical application of the photosensitizer TMP-1363 in the appropriate formulation could be effective in PDT of focal *C. albicans* infections in humans.

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Figure 1. Absorption spectra of TMP-1363 and methylene blue (MB)

Upper panel: The normalized emission from the light box used for the *in vitro* PDT experiments. Lower panel: Absorption spectra of TMP-1363 (solid line) and MB (dashed line) at equimolar concentrations. The primary absorption bands of MB occur at wavelengths that coincide with significant emission from the irradiation lamp source. In contrast, TMP-1363's peak absorption occurs outside of the range optimally excited by the light box.

Figure 2. Comparison of methylene blue (MB) and TMP-1363 in PDT of *C. albicans* in vitro
C. albicans SC5314 was grown overnight to early stationary phase in YPD. Organisms were adjusted to O.D._{600 nm} = 1.0, and incubated with photosensitizer for 30 min at a concentration of 10 μ M for both TMP-1363 and MB. Cells were irradiated with a fluence of 2.4 J cm⁻². Organisms either untreated, or treated with photosensitizer but shielded from light, were used as controls. Organism killing was determined by a CFU assay. Data represent the mean $\pm$ S.D. of three experiments performed in duplicate. A significant difference in organism killing was observed in cells treated with TMP-1363 and irradiated compared to cells treated with MB and irradiated; $p < 0.001$).

Figure 3. TMP-1363 fluorescence in *C. albicans* in vitro

Yeast (a) and filamentous (b) forms of *C. albicans* accumulate TMP-1363 following a 10 min incubation ($10 \mu\text{g mL}^{-1}$). The field of view (FOV) in (a) and (b) is $100 \mu\text{m} \times 100 \mu\text{m}$. (c-f) A series of optical sections through a field of *C. albicans* confirming the intracellular localization of TMP-1363. The FOV is $50 \mu\text{m} \times 50 \mu\text{m}$. All images were acquired using an excitation wavelength of 639 nm and a $60\times$ 1.4-NA oil-immersion objective. The optical section thickness was $0.8 \mu\text{m}$.

Figure 4. TMP-1363 localization in the *C. albicans*-infected mouse ear pinna model

(a) Stereofluorescence image illustrating the distribution of TMP-1363 fluorescence following topical application to the dorsal surface of the mouse ear pinna. (b) Fluorescence emission acquired from the infected ear exhibited a spectrum consistent with TMP-1363. The emission was acquired with excitation at 420 nm using a bifurcated fiber bundle attachment to a Varian Cary Eclipse fluorometer. Shown in the inset is the spectrum of TMP-1363 in aqueous solution. (c) Confocal fluorescence imaging of the ear *in vivo* revealed that intense fluorescence from TMP-1363 appeared highly localized to individual *C. albicans* cells and persisted for at least 6 h after topical administration.

Figure 5. Selectivity of TMP-1363 for *C. albicans* in vivo

(a–c) *in vivo* confocal fluorescence images from the same FOV of an infected ear illustrating (a) GFP-labeled *C. albicans* and (b) the localization of TMP-1363. The merged image in (c) displays the overlay of the two images. The area depicted in these images is $1.6 \text{ mm} \times 1.6 \text{ mm}$, constructed from a montage of four individual $800 \mu\text{m} \times 800 \mu\text{m}$ fields. (d–f) Magnified view of a $250 \mu\text{m} \times 250 \mu\text{m}$ region of interest (ROI) superimposed on the merged image and shown as a white box in (c). All images were acquired using a $10\times 0.45\text{-NA}$ objective. The optical section thickness was $6 \mu\text{m}$. GFP and TMP-1363 images were acquired using 488 nm and 639 nm excitation, respectively.

Figure 6. PDT of *C. albicans* infection *in vivo* using topical TMP-1363

Mouse ears were inoculated intradermally with 3×10^7 *C. albicans*, with 3 ears in each experimental group. Two days post-injection, 50 μ L of a topical solution of 0.3 mg mL⁻¹ TMP-1363 in a glycerol:water (2:3) or ethanol:glycerol:water (2:2:1) vehicle was applied to the entire ear. Excess photosensitizer was removed 30 min after application and the entire ear was irradiated (514 nm, 50 mW cm⁻², 90 J cm⁻²). Infected, control ears received neither TMP-1363 nor light. Two hours post-irradiation, the ears were excised and homogenized, and the organism burden was determined by a colony forming unit (CFU) assay. TMP-1363 in the ethanol:glycerol:water vehicle resulted in a 50-fold reduction in CFU/ear relative to untreated controls. CFUs in both PDT groups were significantly reduced relative to controls (*, $p < 0.05$). Use of the ethanol:glycerol:water formulation also resulted in significantly enhanced reduction in organism burden relative to the glycerol:water vehicle ($p < 0.05$).

Figure 7. Mouse ear pinna following PDT using topical TMP-1363

The ear was inoculated intradermally with 3×10^7 *C. albicans* and photographed immediately (Day 0). Two days following initiation of infection, 0.3 mg mL^{-1} TMP-1363 in an ethanol:glycerol:water (2:2:1) vehicle was applied topically to the dorsal surface of the ear. The ear was irradiated (514 nm , 50 mW cm^{-2} , 90 J cm^{-2}) 30 min after application. The mouse was monitored for 19 days following PDT.