Lasers in Surgery and Medicine

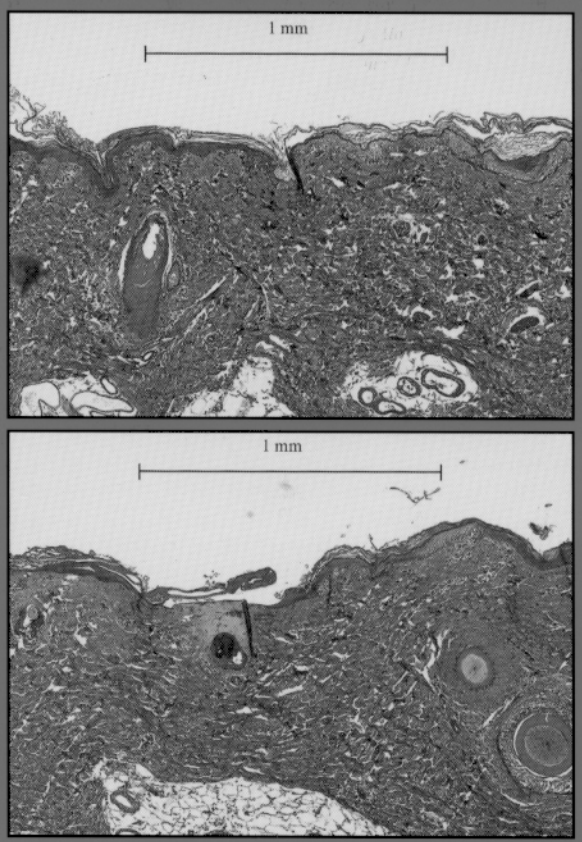
The Official Journal of the ASLMS
American Society for Laser Medicine and Surgery, Inc.

SPECIAL ISSUE: Dermatologic Laser Surgery
Guest Editor: Brian Biesman

Sub-lethal Lasers on the Expression of P16 Melanoma Cells

Rose Bengal–Photosensitizer; Potential Photodynamic Therapy(PDT) Agent

Cooling Efficiency: Epidermal Protection



Volume 32, Number 2, 2003

WILEY-LISS

ISSN 0196-8092

Topical Rose Bengal: Pre-Clinical Evaluation of Pharmacokinetics and Safety

Eric Wachter, PhD,[1]* Craig Dees, PhD,[1] Jay Harkins,[1] Timothy Scott, PhD,[1] Mark Petersen, DVM,[2] Rusty E. Rush, MS,[3] and Amy Cada, PhD[4]
[1]*Provectus Pharmaceuticals, Inc., Knoxville, Tennessee 37931*
[2]*College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37916*
[3]*Springborn Laboratories, Inc., Spencerville, Ohio 45887*
[4]*Therimmune Research Corporation, Gaithersburg, Maryland 20879*

Background and Objectives: Rose bengal (RB) is a potent photosensitizer that has largely been overlooked as a potential photodynamic therapy (PDT) agent. In this study, the feasibility of topical delivery of RB to the epidermis has been evaluated.
Study Design/Materials and Methods: Topical formulations of RB were assessed on murine and rabbit skin for pharmacokinetic properties, cutaneous toxicity, and photosensitization.
Results: Hydrophilic formulations ($\leq$1% RB) exhibited rapid, selective, uniform delivery to the epidermis, with no significant acute cutaneous toxicity in normal skin. Illumination (532 nm) elicited no acute phototoxicity for light intensities $\leq$100 mW/cm^2 at a light dose of 100 J/cm^2; use of higher intensities resulted in superficial thermal damage. Repeat treatment of rabbit skin (weekly for four weeks) elicited minor phototoxicity only at the highest concentration (1% RB).
Conclusions: These results indicate that RB is safe for PDT treatment of skin disorders, exhibiting negligible effects in normal skin. Lasers Surg. Med. 32:101–110, 2003. © 2003 Wiley-Liss, Inc.

Key words: psoriasis; actinic keratosis; photodynamic therapy; photosensitizer; dermatology; laser

INTRODUCTION

Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium, or RB) is a well known type-II photosensitizer exhibiting facile photocatalytic conversion of triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$*) [1–7]. It has an extremely large cross-section in the green ($E_M = 99{,}800$ $\underline{M}^{-1}$ cm^{-1} at 549 nm in water) [4] that is only mildly affected by local environment [6]. RB has an intersystem crossing quantum yield approaching unity ($\Phi_{isc} = 0.98$) and a high singlet oxygen yield ($\rho(^1O_2^*) > 0.75$), indicating that RB is capable of highly efficient $^1O_2$* production upon irradiation with green light [8]. Its long history of safe use as a systemic diagnostic of hepatic function [9–21] as well as a topical ophthalmic diagnostic [22–28] suggests that, in marked contrast to many photodynamic therapy (PDT) agents, RB should have minimal potential for side effects, such as prolonged photosensitivity.

Since RB readily photobleaches [29], its photodynamic effects may be self-limiting. This is particularly relevant for treatment of many dermatologic conditions, such as psoriasis and actinic keratosis, since precise light dosimetry is impractical over the large surface areas typically involved in these diseases: a PDT regimen that exhibits self-limiting effects would avoid the need for complex light dosimetry.

The combination of photodynamic potential, substantial regulatory precedent, minimal known side-effects, and likelihood of self-limiting therapeutic effect, motivated us to evaluate key pharmacokinetic and safety aspects of topical RB with green light activation, where the minimally-penetrating nature of such green light matches a desire to restrict photodynamic action to the epidermis.

MATERIALS AND METHODS

Reagents and Vehicles

RB was obtained from Sigma Chemicals (St. Louis, MO) or Akorn Inc. (Decatur, IL). Carboxymethylcellulose, U.S.P. medium viscosity sodium salt (CMC), dimethylsulfoxide (DMSO), ethanol, isopropanol, and propylene glycol (PG) were purchased from Sigma Chemicals (St. Louis, MO). Saline (sodium chloride, 0.9% U.S.P.), was obtained from Abbott Laboratories (North Chicago, IL) or B. Braun (Burns Veterinary Supply, Farmers Branch, TX). AquaGel

Abbreviations: CMC, carboxymethylcellulose; D, density; DE, dermis; DMSO, dimethylsulfoxide; δT, temperature rise; ED, epidermis; H&E, hematoxylin and eosin; LoD, limit of detection; LSM, laser scanning microscopy; NF, National Formulary; $^1O_2$*, singlet oxygen; PDT, photodynamic therapy; PG, propylene glycol; PI, penetration index; RB, rose bengal; SC, stratum corneum; T_{max}, maximum surface temperature; U, uniformity; *w/v*, weight-to-volume.

Eric Wachter, Craig Dees, and Timothy Scott have disclosed a potential conflict of interest with this study.

*Correspondence to: Eric Wachter, PhD, Provectus Pharmaceuticals, Inc., 7327 Oak Ridge Highway, Knoxville, TN 37931.
E-mail: wachter@provectuscorp.com

Accepted 27 September 2002
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.10138

TABLE 1. Rose Bengal (RB) Cutaneous Penetration Results, 30 Minutes Topical Application to Murine Skin

Formulation		Penetration index			
Composition	Properties	*N*	PI_{SC}	PI_{ED}	PI_{DE}
1% RB in saline	Hydrophilic aqueous liquid	7	2.4	2.5	0.0
1% RB in saline + 2% CMC	Hydrophilic aqueous hydrogel	2	2.5	2.8	0.0
1% RB in AquaGel	Hydrophilic aqueous hydrogel	2	2.0	2.3	0.0
1% RB in LiquaGel	Hydrophilic aqueous hydrogel	3	2.2	2.0	0.0
1% RB in PG	Moderately lipophilic liquid	4	1.5	0.0	0.0
1% RB in LiquaDerm A	Lipophilic liquid	1	1.5	1.0	0.0
1% RB in Dermabase	Emulsion (oil-in-water)	1	1.5	2.0	0.0
1% RB in DMSO	Universal penetrant	3	3.0	3.0	3.0

N indicates number of replicate treatments conducted for each formulation. No local or systemic adverse effects were noted for any tests.

was purchased from Parker Laboratories (Minneapolis, MN). LiquaDerm A, LiquaGel, and Dermabase were purchased from Paddock Laboratories (Fairfield, NJ). Additional RB hydrogel formulations (ca. 0.2% Carbomer 934P NF with 5% PG U.S.P.) were prepared by Midwest Institute of Research and Technology (Edmund, OK). All materials were used as received. Topical formulations are summarized in Table 1.

Animals and Animal Husbandry

Animal housing and care were based on standards established by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and guidelines set forth in the Guide for the Care and Use of Laboratory Animals, NIH Publication No. 96-03, 1996.

Pharmacokinetic Studies

Topical delivery of RB was evaluated in hairless female mice (Crl:SKH-1-hrBR, Charles River Laboratories, Wilmington, MA). Mice were anesthetized via continuous inhalation using 2% Isofluorane (Baxter Healthcare Corporation, Deerfield, IL). A single patch of skin on one flank was cleaned with ethanol, and an absorbent patch (2 cm × 2 cm section of Kimwipe, Kimberly-Clark, Roswell, GA) applied (for all vehicles except Dermabase and saline-CMC, wherein no patch was used). The patch was saturated with one of the study formulations, then covered with an occlusive dressing (Handiwrap, Dow, Indianapolis, IN or Tegaderm, 3M, St. Paul, MN). After 30 minutes contact, the covering and patch were removed, the skin blotted dry with a gauze pad, and the animal euthanized by carbon dioxide inhalation. Tissue specimens from the treated area, and of untreated skin, were immediately collected and processed by frozen sectioning. A portion of the resultant slides underwent H&E staining, while the remainder were processed without counterstaining.

Additional pharmacokinetic data were obtained using tissue and blood samples from New Zealand white rabbits (collected at the end of a four-week multiple-treatment regimen with topical RB, as described in greater detail below). Specimens of treated skin were collected upon sacrifice 24 hours after final topical administration of RB in carbomer hydrogel, then processed by frozen sectioning (as described above for murine specimens). Blood samples collected 24 hours prior to final treatment with RB and at 1, 4, and 24 hours following such treatment were centrifuged for 15 minutes at 3,000 rpm. The resultant separated plasma was collected and analyzed for RB content using fluorimetry (Cytofluor 2350, Millipore, Bedford, MA); the limit of detection (LoD) for this assay was ca. 20 ng RB/ml plasma.

Microscopy and Fluorescence Microscopy

An Olympus BX60 microscope (Olympus America, Inc. Melville, NY) was used to examine and photograph processed tissue sections (skin and underlying tissue) using transmitted light brightfield observation (halogen illumination). Specimens were photographed using Fujichrome 64 Professional T slide film (Fuji Photo Film Co., Ltd, Tokyo, Japan). Additionally, specimens that were not counterstained during processing were uniformly illuminated with green light and the resultant fluorescence photographed using a BX-FLA reflected light fluorescence attachment fitted with a U-MWG fluorescence filter cube (510–550 nm excitation, >590 nm emission, Olympus). This approach allowed RB present in tissue to be readily imaged (as orange RB fluorescence on an otherwise dark background). Control sections (having received no applied RB nor H&E counterstaining) exhibited no detectable autofluorescence under such illumination.

Additional imaging was performed using a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss Micro-Imaging, Inc., Thornwood, NY). Excitation at 543 nm using a 40×/1.30 Plan-NEOFLUAR oil objective, coupled with confocal detection using a 560 nm long-pass detection filter, allowed sub-cellular distribution of RB to be imaged against an otherwise non-fluorescent background.

Histologic Assessment of RB Penetration

Fluorescence micrographs were examined to assess RB staining; micrographs were scored by two blinded readers.

A scale of 0 (low) to 3 (high) was used to assign uniformity (U) and density (D) scores for RB staining of each specimen. Individual scores for U and D were assigned for stratum corneum (SC), viable epidermis (ED, comprising the Malpighian layers), and dermis (DE). Separate scores were then combined into a single metric (penetration index, PI) for each of these three layers:

$$PI_{SC}=(U_{SC}+D_{SC})/2$$

where PI_{SC} is the penetration index for stratrum corneum, based on uniformity (U_{SC}) and density (D_{SC}) scoring for the stratum corneum. Similar indices were calculated for viable epidermis (PI_{ED}) and dermis (PI_{DE}). Comparison with H&E stained sections facilitated identification of major tissue structures.

Acute Dermal Toxicity

Topical RB alone (0.0001–0.01% RB in saline) or in conjunction with green light photoactivation was administered once to male New Zealand White SPF rabbits (Myrtle's Rabbitry, Thompson Station, TN). Animals were individually housed in stainless steel cages and acclimatized for a minimum of eight days, at which point a section of the mid-dorsal back of each rabbit was shaved and the animal randomly assigned to one of eight treatment groups (Table 2). At this point rabbits were approximately 9 weeks of age with body weights ranging from 1.8 to 2.1 kg. The following day, animals were anesthetized and placed in ventral recumbency on a flat surface. A 7.0-cm diameter circle was delineated within the shaved area, and 625 μl of aqueous agent (i.e., saline or RB in saline) applied evenly to the delineated area using a glass rod. The application site was allowed to air dry for 15 minutes, and the animals then maintained on the flat surface for an additional 1,000 seconds; a subset of animals were illuminated with green light during this latter period (as described below). Animals were subsequently returned to their cages and observed for recovery from anesthesia. Upon recovery, animals were fitted with a collar to prevent access to the test site. The collar remained in place for approximately 24 hours, at which point it was removed and the test site rinsed with gauze moistened in deionized water.

General health/mortality checks were performed twice daily, and detailed clinical observation of each animal was performed daily. Any post-treatment abnormalities at the test site were recorded. Individual body weights were recorded at randomization and on days 7 and 13 following treatment, and final body weights recorded on the day of scheduled sacrifice (day 3 or 14). Blood collected from each animal immediately prior to sacrifice was evaluated for a comprehensive range of hematology, coagulation, and biochemistry parameters. Urine was also collected from each animal at necropsy (day 3 or 14). For each treatment group, 2 of the 5 animals were sacrificed at day 3, and the remainder sacrificed at day 14 post-treatment. All animals were subjected to complete gross necropsy, fresh organ weights were obtained and selected tissues and organs were retained at necropsy; these tissues and organs were processed for histopathologic and microscopic examination.

Dermal Toxicity Upon Repeated Treatment

A similar dermal toxicity study was conducted over a 4-week period to assess the effects of weekly treatment with topical RB alone (0.001–1% RB in carbomer hydrogel) or upon photoactivation with green light. Male and female New Zealand White rabbits (Covance Research Products, Denver, CO) were randomly assigned to one of seven treatment groups (Table 3). Following acclimatization, all animals had an area shaved (approximately a 10-cm diameter circle, starting behind the scapular region and extending posteriorly) and delineated with marker 2–48 hours prior to treatment. An additional area (approximately 5 cm × 10 cm, posterior to the circular area) was shaved to serve as an untreated control area. Each area was re-shaved as necessary throughout the duration of the study. At initial treatment, rabbits were approximately 12 weeks of age with body weights ranging from 2.2 to 2.8 kg.

Immediately prior to treatment, each animal was anesthetized, and the left half of the 10 cm diameter circular shaved area rubbed lightly with electrode preparation

TABLE 2. Treatment Group Assignment and Effects of a Single Treatment of Rabbit Skin With Topical RB (Saline Formulation); All Effects Were Limited to the Immediate Treatment Site

Treatment group	*N*	Intensity (mW/cm^2)	Light dose (J/cm^2)	δT (°C)	Treatment effect
Saline	5	0	0	—	None
0.0001% RB	5	0	0	—	None
0.001% RB	5	0	0	—	None
0.01% RB	5	0	0	—	None
Saline + laser	5	100	100	9.4	Transient telogenization (day 3)
0.0001% RB + laser	5	100	100	10.8	Transient telogenization (day 3)
0.001% RB + laser	5	100	100	10.7	Transient telogenization (day 3)
0.01% RB + laser	5	100	100	12.6	Transient telogenization (day 3)

δT represents maximum change in skin surface temperature during illumination. Transient telogenization was noted in all laser-treated animals sacrificed at day 3 but was absent in animals sacrificed at day 14.

TABLE 3. Treatment Group Assignment, Penetration Index Values, and Effects of Repeated Treatment of Rabbit Skin With Topical RB (Carbomer Hydrogel Formulation)

Treatment group	N	Penetration index		Intensity (mW/cm^2)	Light dose (J/cm^2)	δT (°C)	Treatment effect
		PI_{ED}	PI_{DE}				
Sham (no treatment)	10	0.1 ± 0.2	0.0 ± 0.0	0	0	—	None
1.0% RB alone	10	1.6 ± 0.5	0.0 ± 0.0	0	0	—	Minor skin irritation
Vehicle + laser	7	0.3 ± 0.3	0.0 ± 0.0	50	50	6.0 ± 2.1	None
0.001% RB + laser	8	0.2 ± 0.3	0.0 ± 0.0	50	50	6.3 ± 0.5	None
0.01% RB + laser	8	1.0 ± 0.2	0.0 ± 0.0	50	50	7.0 ± 0.1	None
0.1% RB + laser	8	1.6 ± 0.5	0.0 ± 0.0	50	50	7.8 ± 1.2	Minor skin irritation
1% RB + laser	10	1.5 ± 0.3	0.0 ± 0.0	50	50	9.0 ± 1.2	Minor skin irritation

Skin samples for determination of penetration index were obtained 24 hours after final treatment. All treatment effects were limited to the immediate treatment site. Statistically significant differences in treatment effects relative to the sham group were observed only for the 1% RB + Laser group.

paper to abrade the outer layers of skin. Test or control agent was evenly applied topically to the entire treatment area (6 ml total volume, 0.075 ml/cm^2) using a glass rod and allowed to remain in contact with the skin for 30 minutes; the posterior 5 cm × 10 cm strip of skin remained untreated in all groups. Animals in the sham treatment group did not receive any topical treatment but were otherwise treated identically. After the 30-minute contact period, the treatment area was repeatedly wiped with water saturated gauze to remove as much applied agent as possible, then wiped with dry gauze. A subset of animals were then illuminated with green light (as described below). Following treatment, all animals were fitted with a collar to prevent access to the test area, returned to their cages, and observed for recovery from anesthesia. In contrast with the acute dermal toxicity study, the collar remained in place for the duration of the study (except during repeat treatment of the test area on study days 8, 15, and 22).

General health/mortality checks were performed twice daily, and detailed clinical observation of each animal was performed daily. Any post-treatment abnormalities at the test site were individually rated according to the following scales:

Erythema: 0 = not present; 1 = slight; 2 = moderate; 3 = severe;

Edema: 0 = not present; 1 = slight; 2 = pronounced; and

Desquamation (sloughing or defoliation): 0 = not present; 1 = slight; 2 = moderate; 3 = severe.

Any incidence of bleeding, scabbing, fissuring, or ulceration was also noted. Individual body weights were recorded prior to randomization, on each treatment day (study days 1, 8, 15, and 22), and prior to necropsy (study day 23). Blood was collected from each animal 24 hours prior to the final treatment (i.e., on study day 21), and approximately 1, 4, and 24 hours after the final treatment (i.e., on study days 22 and 23); these were evaluated for hematology, coagulation, and biochemistry parameters, along with plasma RB content. Urine was collected at necropsy. Upon sacrifice (on study day 23), all animals underwent complete gross necropsy, fresh organ weights were obtained, and selected tissues and organs retained for histopathologic and microscopic examination.

Skin Illumination With Green Light

For murine studies, a 2-cm^2 area of skin (untreated or freshly treated with topical RB, as described above) was illuminated with green laser light (532 nm, Verdi-5W laser, Coherent Laser Group, Santa Clara, CA) projected onto the skin as a uniform, circular field using a multimode optical fiber fitted with a microlens diffuser. Thermal images of illuminated areas were continuously recorded using an infrared camera (Thermacam PM-380, Inframetrics, North Billerica, MA). This allowed estimation of maximum surface temperature (T_{max}) and temperature rise (δT, the difference between initial surface temperature and T_{max}) at the site of illumination. Intensities of 50–400 mW/cm^2 were evaluated. All mice were anesthetized throughout this procedure using Isofluorane inhalation.

Similar methods were used to illuminate a 20-cm^2 section of rabbit skin (freshly treated with saline- or RB-saline, as described above) for acute dermal toxicity studies. Illumination was performed at an intensity of 100 mW/cm^2, with a 100 J/cm^2 light dose. All animals were anesthetized throughout this procedure (using Domitor, 0.4 mg/kg). For studies of dermal toxicity upon repeat treatment, an 80-cm^2 section of rabbit skin (freshly treated with vehicle-alone or RB-hydrogel, as described above) was illuminated at an intensity of 50 mW/cm^2, using a 50 J/cm^2 light dose. Animals were anesthetized throughout this procedure (using ketamine, 35 mg/kg; xylazine, 5 mg/kg; and butorphanol, 0.1 mg/kg).

Adverse Effect Monitoring

The occurrence of any adverse effects (such as erythema, edema, or serious irritation) was noted immediately post-treatment and at frequent, regular intervals thereafter. Any animal exhibiting significant adverse effects was immediately euthanized.

RESULTS

RB Penetration Into Normal Skin

Murine skin. Results summarized in Table 1 illustrate a range of formulation performance in murine skin. Example data are shown in Figure 1. With the exception of DMSO, the hydrophilic formulations appeared to exhibit better delivery of RB to the epidermis, with no detectable delivery to dermis or underlying tissues. Inclusion of inert thickening agents in hydrophilic formulations (such as in the CMC and other hydrogel formulations) appeared to have no significant effect on delivery of RB to the epidermis. In contrast, DMSO yielded dense, uniform staining of all tissue layers, while the lipophilic formulations exhibited markedly reduced delivery of RB to the epidermis. In an additional subset of animals sacrificed 24 hours post-application, only DMSO exhibited significant staining of any tissue; all other formulations exhibited minimal or no RB residue, presumably due to normal grooming of the site by animals and their cagemates. No other evidence of migration of RB within the epidermis or into the dermis was noted for non-DMSO formulations; due to the intense staining of all tissues with DMSO, it was impossible to assess whether drug migration occurred over a 24-hour period. No special precautions were taken to avoid exposure of test areas to ambient light, and no adverse effects were noted for any of the tested formulations.

Rabbit skin. Results similar to those for murine skin were obtained upon treatment of rabbit skin with the RB-hydrogel formulation. The density of RB staining, at 24 hours after application, increased with the concentration of the agent (as evidenced by trends in the penetration index scores), and was confined to the epidermis and hair follicles (Table 3); no staining was observed in the dermis or underlying tissues.

Sub-cellular distribution. High resolution imaging of skin specimens using confocal laser scanning microscopy allowed the sub-cellular distribution of RB to be imaged, as illustrated in Figure 2. Localization of RB within the epidermis, with patches of high concentration in the stratum corneum, is comparable to that observed using conventional fluorescence microscopy (Fig. 1). Sub-cellular localization of RB within cell walls and internal membranes, along with exclusion from nuclei, is also evident.

Pharmacokinetic Results

Plasma from rabbits receiving a total of four weekly treatments with topical RB exhibited no detectable levels of RB in any treatment group at any time point (i.e., at 24 hours prior to, or at 1, 4, and 24 hours after, application of the final dose of RB).

Acute Toxicity of Topical RB in Rabbit Skin

All animals recovered from the anesthetic with no adverse effects. No mortality, notable clinical abnormalities, dermal findings, or statistically significant or toxicologically meaningful differences in mean body weights or body weight gain were noted during the 14-day observation period. Moreover, no toxicologically significant clinical

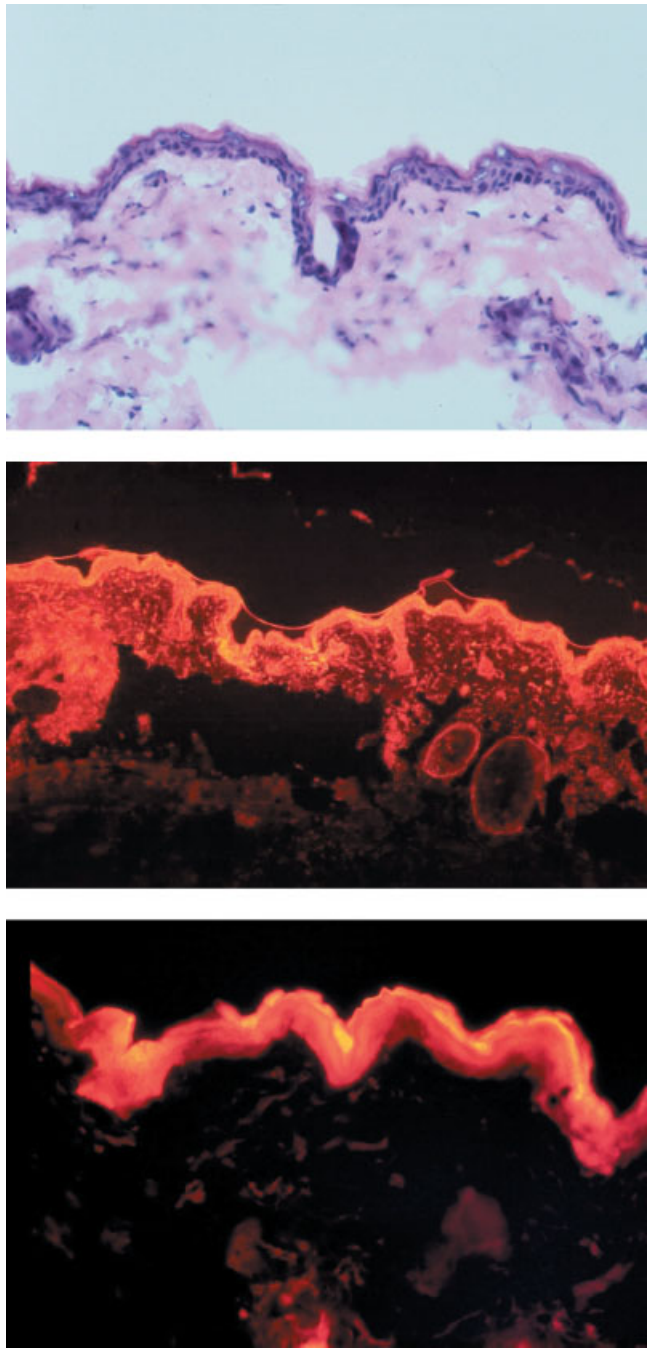


Fig. 1. Tissue sections illustrating Rose bengal (RB) penetration into normal murine skin, magnification 25×. **Top**: Control section, H&E stained, under brightfield observation; lightly stained stratum corneum is evident atop the thicker, darkly stained viable epidermis. **Middle**: Fluorescence micrograph of skin treated with 1% RB in dimethylsulfoxide (DMSO); bright staining of all skin layers is evident. **Bottom**: Fluorescence micrograph of skin treated with 1% RB in saline; uniform staining of epidermis is clearly demarcated from unstained dermis.

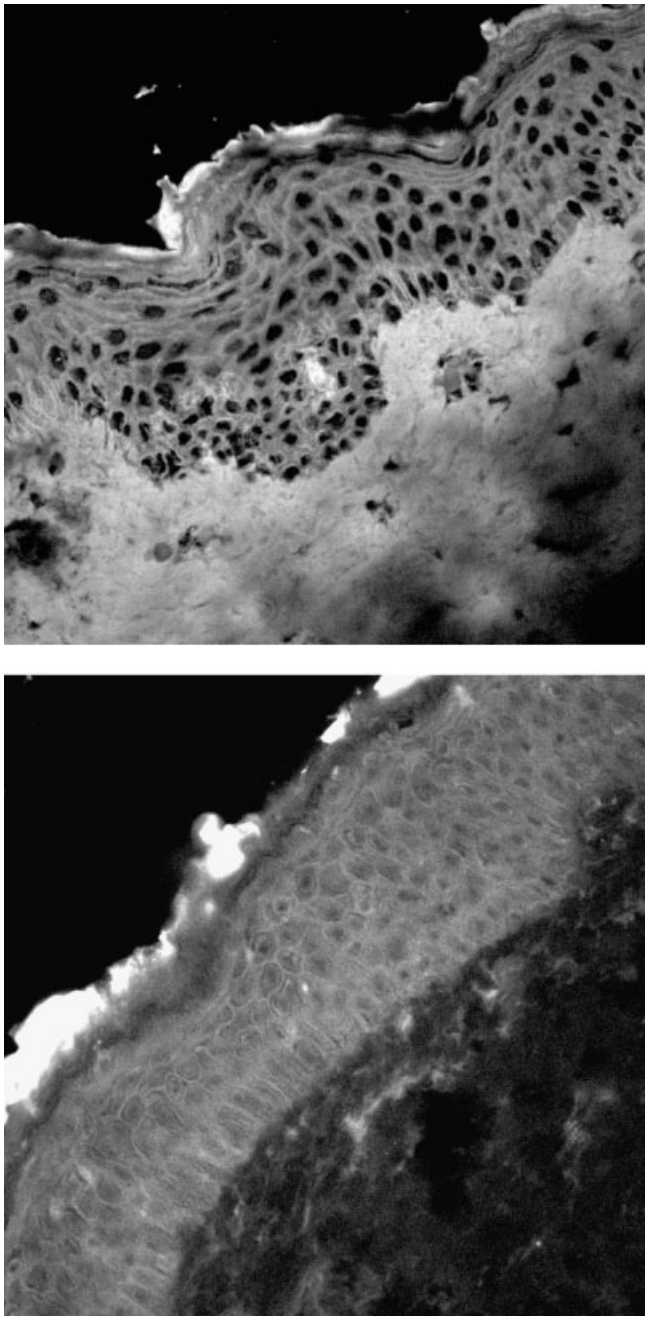


Fig. 2. Confocal LSM images of a fresh frozen skin sections, image size 230 μm × 230 μm. **Top**: Control section, H&E stained—fluorescence emission of eosin is visible throughout the epidermis, with some areas of dermis also exhibiting staining. This pattern is comparable to that seen in Figure 1. Note that hematoxylin staining is not visible in the LSM image, and that no significant nuclear staining is apparent from the eosin signature. **Bottom**: Typical staining pattern following topical application of RB in saline. As is evident in comparable sample in Figure 1, RB is localized within the epidermis, with patches of high concentration in the stratum corneum. Subcellular localization within cellular membranes and exclusion from nuclei is also evident.

pathology abnormalities were observed, nor were any treatment-related abnormalities observed at gross necropsy (including toxicologically significant differences in absolute or relative organ weights). For those animals not exposed to laser illumination, no significant gross or microscopic changes were observed in any of the tissues or organs examined. However, as described below, microscopic examination indicated that skin exposed to laser illumination underwent transient telogenization.

Toxicity of Topical RB Upon Repeated Treatment

All animals recovered from the anesthetic with no adverse effects. One male animal (receiving vehicle alone plus light treatment) died during blood collection on study day 21; this was judged to be unrelated to treatment. Otherwise, no statistically significant or toxicologically meaningful differences in mortality, clinical abnormalities, body weights or body weight changes, food consumption, ophthalmology, organ weights, clinical pathology, urinalysis, gross pathology, or microscopic changes in tissues were noted during the 23-day observation period.

Minor skin irritation (erythema and desquamation) was noted for some animals treated with 0.1% RB when combined with laser illumination, and for 1% RB alone or when combined with laser illumination; differences from the sham group were statistically significant only for the group treated with 1% RB combined with laser illumination. These results are summarized in Figures 3 and 4. No correlation was observed between irritation and skin abrasion with electrode preparation paper. No animals exhibited edema.

Effects of Illumination With Green Light

Thermal response of murine and rabbit skin upon laser illumination is shown in Figures 5 and 6. Figure 5 illustrates skin surface temperature of rabbit skin as a function of cumulative light dose upon continuous illumination with 532 nm light at 100 mW/cm^2. Temperature increased in a monotonic manner throughout the illumination period, approaching equilibrium upon delivery of the full light dose (i.e., 100 J/cm^2). No significant differences were noted in skin surface temperature trends for animals within a given treatment group. As evidenced by the data in Figure 5 and Table 2, δT was comparable for animals treated with low concentrations of RB (i.e., 0.0001 and 0.001% RB) and for control animals (i.e., treated with saline); animals treated with 0.01% RB exhibited both a faster rate of increase and larger maximum increase in skin surface temperature.

Rabbits in the repeat-treatment study exhibited similar trends, as evidenced by the data in Table 3. As observed for rabbits in the acute study, δT correlated with concentration of RB, exhibiting fastest rate of increase and largest magnitude of increase for animals treated with 0.1 and 1% RB, respectively.

Data for the murine experiments (Table 4 and Fig. 6) illustrate that, for a given topical formulation, δT is directly proportional to light intensity. As seen in the rabbit experiments, topical delivery of concentrated RB (which, at the concentrations tested on mice, strongly absorbs light at

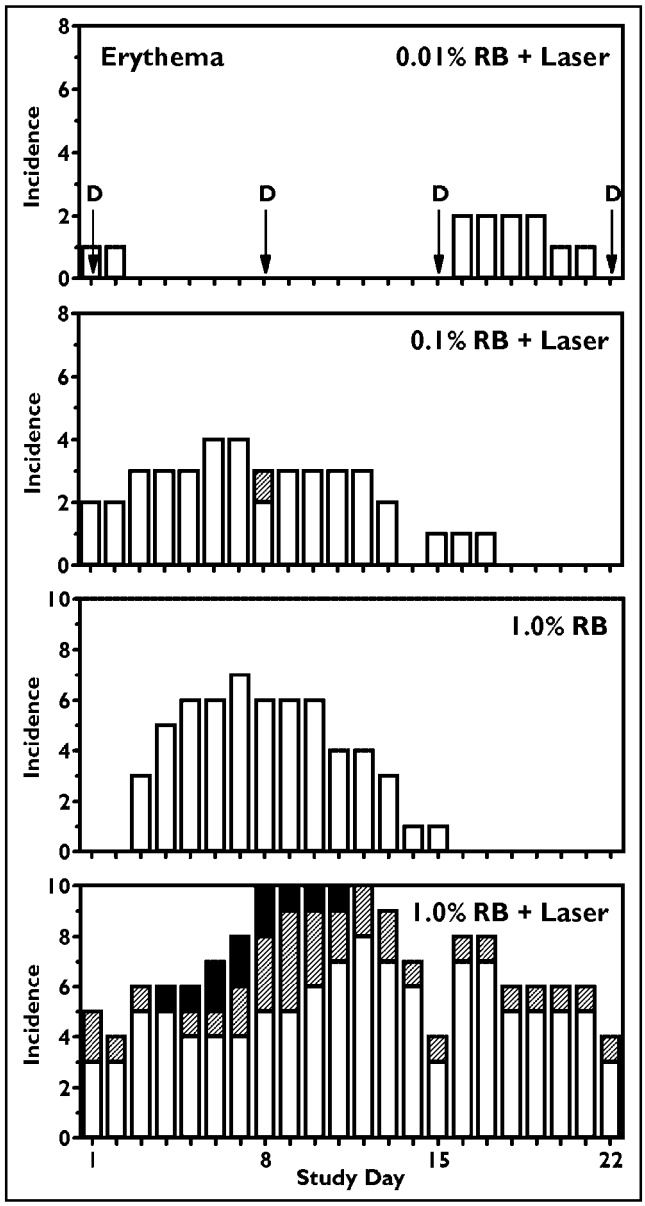


Fig. 3. Incidence of skin irritation (erythema) observed upon repeated treatment of rabbit skin with topical RB (carbomer hydrogel formulation). Stacked bars illustrate total number of animals per treatment group per day exhibiting any incidence of slight (irritation score = 1, white bar), moderate (score = 2, crosshatched bar), or severe (score = 3, black bar) erythema. All effects were limited to the immediate treatment site. Treatment performed on each day indicated by "D."

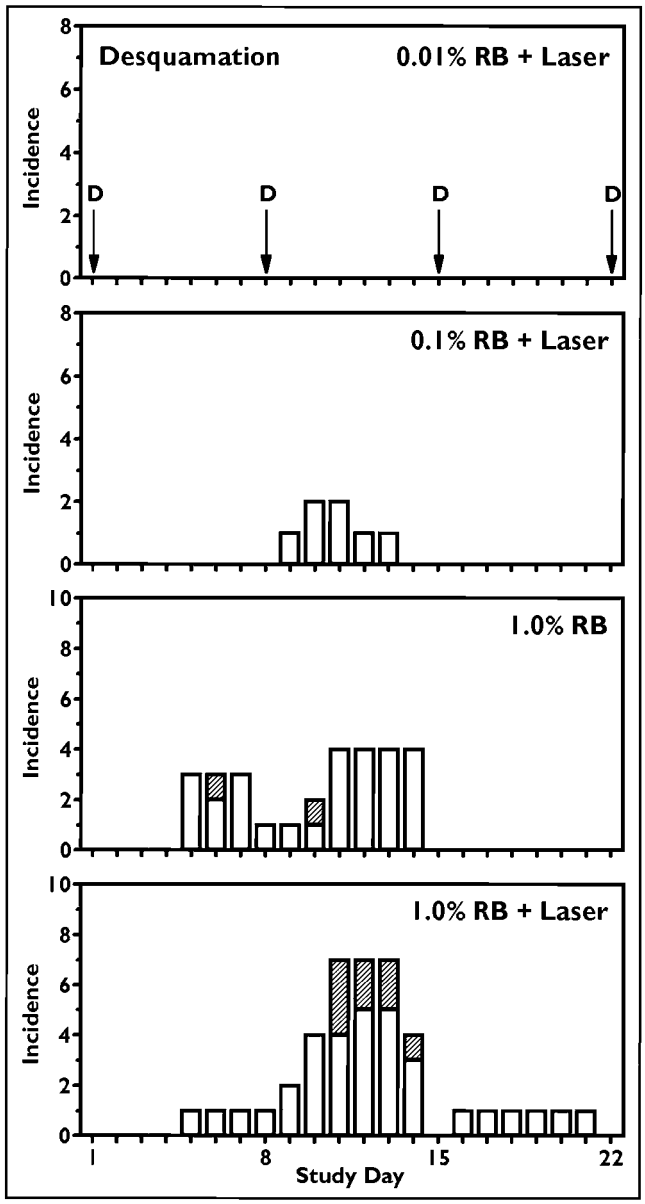


Fig. 4. Incidence of skin irritation (desquamation) observed upon repeated treatment of rabbit skin with topical RB; white bar, slight (irritation score = 1), crosshatched bar, moderate (score = 2). There was no incidence of severe effects. All effects were limited to the immediate treatment site.

532 nm) to the skin increased δT. Because the optical absorbance of RB present within the epidermis appears to be relatively large for applied concentrations in the range of 0.1% RB and higher, similar thermal response was noted for such concentrations (for example, 100 J/cm^2 at 100 mW/cm^2 yielded $\delta T = 6.9^\circ C$ for 1% RB in Dermabase, while $\delta T = 5.7^\circ C$ for the same formulation at 0.1% RB).

Comparison of thermal effects in control areas of murine and rabbit skin (Fig. 6) illustrates that rabbit skin (i.e., treated with vehicle only) exhibits a greater change in temperature than murine skin upon illumination with light at 532 nm. This may result from the lower metabolic rate of rabbits relative to mice, yielding lower blood flow, and resultant lower heat transfer, from the skin of rabbits.

Adverse effects are summarized in Tables 2–4; any noted effects were limited to the immediate laser treatment site. In murine skin (Table 4), adverse effects were

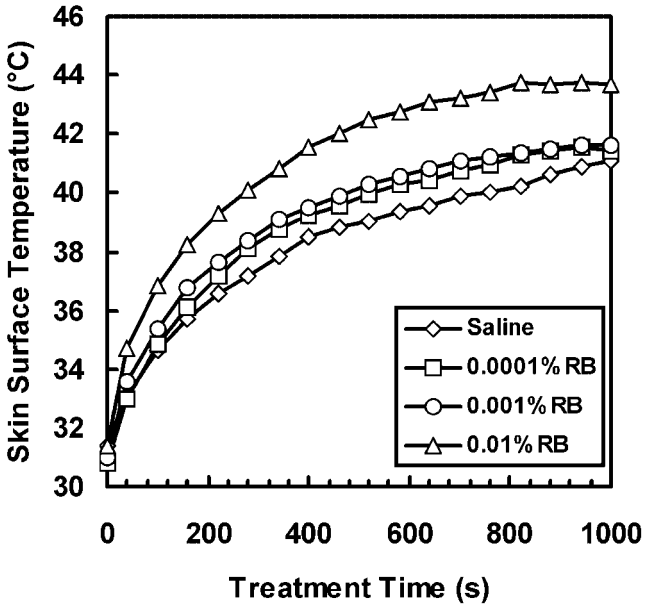


Fig. 5. Skin surface temperature measurements for rabbit skin during illumination with 532 nm light at 100 mW/cm^2. Diamonds, control skin treated with saline only; squares, circles, and triangles represent skin treated with RB in saline at 0.0001, 0.001, and 0.01% w/v, respectively. Each data point comprises an average response (N = 5) for all rabbits illuminated under the respective conditions; maximum standard deviation from average response at any time point was 1.2°C. Illumination was discontinued at 1,000 seconds (i.e., 100 J/cm^2).

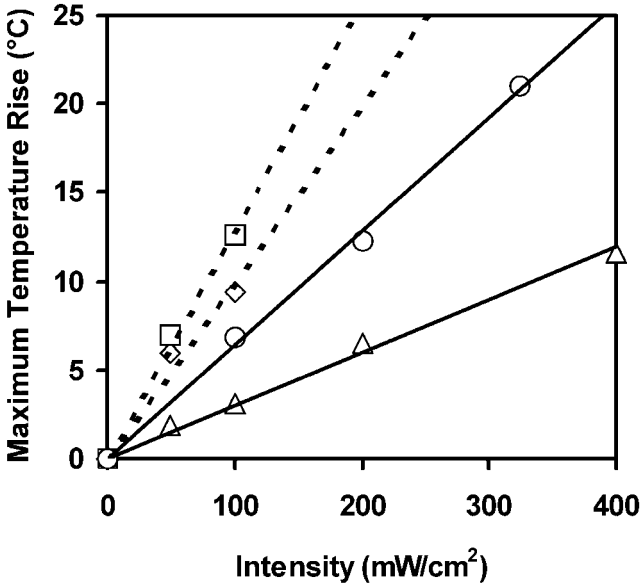


Fig. 6. Thermal response of murine and rabbit skin upon illumination at 532 nm. Triangles, untreated murine skin; circles, murine skin treated with 1% RB in Dermabase; diamonds, rabbit skin treated with vehicle only; squares, rabbit skin treated with 0.01% RB. Linear least squares fit, forced through origin, shown as solid lines for murine data, dashed lines for rabbit data.

only observed for conditions where $\delta T > 10°C$ (i.e., intensity > 100 mW/cm^2). For acute studies on rabbit skin (Table 2), the only observed effect was transient telogenization (induction of hair follicle resting phase) in animals sacrificed at day 3 following laser illumination. Animals sacrificed at day 14 exhibited normal hair growth with no apparent structural changes to hair follicles. For those rabbits receiving repeat treatment (Table 3), minor skin irritation was noted in some animals at the highest concentrations of RB (i.e., 0.1 and 1% RB); this effect was of statistical significance only in the 1% RB + laser group, and appeared to elicit increased tolerance after two treatments. Otherwise, remaining rabbits exhibited no symptoms related to treatment and no rabbits exhibited evidence of pain or other discomfort following laser illumination.

DISCUSSION

RB Penetration Into Normal Skin

The observed trends for delivery of RB to the epidermis from an aqueous vehicle are consistent with standard transdermal delivery models for hydrophilic agents [30]; such agents must pass through the desiccated stratum corneum before they can reach significant concentration in viable regions of the epidermis and beyond. Thus, it is not surprising that the hydrating effects of an aqueous vehicle markedly enhance transport through the stratum corneum. The addition of thickening agents that do not interact with RB, such as CMC [31], may enhance RB delivery to the epidermis by improving contact between the skin surface and the applied surface reservoir of hydrated RB. It appears that topical RB does not penetrate beyond the epidermis; lack of detectable levels of RB in the dermis (as evidenced by fluorescence microscopy for all formulations except DMSO), combined with a sharp, clear demarcation between uniformly high levels of RB in the epidermis and the RB-free dermis (see, for example, Figures 1 and 2), support this observation.

The apparent absence of significant RB penetration beyond the epidermis is further substantiated by the plasma assay data, which indicate that, at most, negligible amounts of RB are present in plasma at any time point following topical application (i.e., all measurements were below the LoD of 20 ng RB/ml plasma). For example, a 2.2 kg rabbit should have approximately 120 ml of blood, and approximately 60 ml of which comprises plasma. Since the fluorimetric assay can detect RB whenever this 60 ml of plasma contains >1.2 μg RB, the lack of measurable plasma levels upon application of 1% RB to an 80 cm^2 patch of skin makes it clear that less than 0.002% of the applied RB (assuming 6 ml of 1% RB, comprising a total applied dose of 60 mg RB) is present in plasma at any time point. Moreover, it is likely that the high efficiency of hepatic excretion of RB [9–15] assures that any RB that might cross

TABLE 4. Effects of Illumination of Murine Skin With Green Light; All Effects Were Limited to the Immediate Treatment Site

Formulation	Intensity (mW/cm^2)	Light dose (J/cm^2)	δT (°C)	Treatment effect
None (control)	100	100	3.1	None
None (control)	200	100	6.5	None
0.1% RB in Dermabase	100	100	5.7	None
0.1% RB in Dermabase	200	100	13.1	Moderate Localized Erythema
1% RB in Dermabase	100	100	6.9	None
1% RB in Dermabase	200	100	12.3	Moderate Localized Erythema
1% RB in Dermabase	325	1,250	22	Localized Thermal Burn

into the highly vascularized dermis is rapidly eliminated from the bloodstream.

Further evidence supporting an absence of significant RB migration beyond the epidermis comes from repeat examination of the prepared tissue sections, which were found to exhibit no evidence of RB migration upon repeated microscopic examination over a period of several months storage at room temperature. Frozen sectioning avoids washout of RB during tissue processing, and results in a semi-solid mounted specimen. Such specimens might be expected to exhibit some evidence of migration of RB within remaining tissue fluids and within the surrounding film of preservation compound. The lack of such migration suggests that topically applied RB is persistently associated with certain cellular components (such as cell membranes) in the epidermis [25,31–35]. Confocal LSM examination of these samples corroborates this conjecture, showing that epidermal RB is primarily confined to cell walls and internal membranes, wherein the amphipathic properties of RB may yield favorable partitioning.

Dermal and Systemic Toxicity

The lack of significant dermal toxicity in mice and rabbits is consistent with prior safe, widespread use of RB as a systemic hepatic and topical ophthalmic diagnostic. The current studies show that even upon repeated treatment of the same area of skin, the topically applied agent is benign over a very wide range of concentrations. Topical administration to a substantial fraction of the total skin surface elicited no evidence of systemic effects, apparently due to a favorable combination of confinement to the epidermis and rapid hepatic excretion upon any systemic uptake. It is notable that in its former use as a hepatic diagnostic, an intravenous dose of ca. 1.5 mg RB/kg body weight elicited no significant systemic toxicity nor prolonged photosensitization. This suggests that use of RB as a topical PDT agent for dermatology is unlikely to elicit significant local or systemic adverse effects in humans.

Effects of Illumination

Niemz [36] points out that irreversible hyperthermic damage will occur when tissue is heated to *ca.* 46–48°C (i.e., $\delta T = 10–15$°C) for more than 500 seconds. This corresponds closely to the thermal conditions and duration of exposure experienced by RB-treated murine skin when illuminated with 100 J/cm^2 at $\geq$200 mW/cm^2 (i.e., 500 seconds duration of illumination) and by rabbit skin treated with 1% RB then illuminated with 50 J/cm^2 at 50 mW/cm^2 (i.e., 1,000 seconds duration of illumination). The observed erythema in murine and rabbit skin is consistent with such damage. When heated above 55°C (i.e., $\delta T \geq 20$°C) for more than 10 seconds, severe damage is expected (as observed for murine skin illuminated at 325 mW/cm^2). Heating to $\leq$45°C (i.e., $\delta T \leq 10$°C) is not expected to result in permanent tissue damage. The transient telogenization noted in illuminated rabbit skin (comparable for both RB-treated skin and saline-only controls) appears to result from such low-grade thermal effects. In contrast, no such effect was noted in non-illuminated skin, supporting the position that this effect is of thermal origin rather than due to intrinsic toxicity or phototoxicity of topical RB. Hence, all significant observed effects appear to be thermal in origin, and may be avoided by using moderate light intensities (i.e., 50–100 mW/cm^2 or less) and agent concentrations (i.e., 0.1% RB or less).

The authors acknowledge that this preclinical study does not attempt to address the potential therapeutic efficacy of topical RB for treatment of specific dermatologic conditions, such as psoriasis or actinic keratosis, for which no suitable animal model exists. However, prior work by the authors [37,38] indicates that RB can be used under equivalent illumination conditions to successfully (and selectively) treat a variety of cancerous tumors implanted in mice, including renal adenocarcinomas, hepatomas, and human breast tumors. Thus, the absence of significant dermal toxicity or phototoxicity in normal skin upon irradiation with moderate intensity (i.e., $\leq$100 mW/cm^2) green light, even at high light doses (i.e., 100 J/cm^2), combined with the rapidity, selectivity, and uniformity of topical RB delivery to the epidermis, suggests that dermatologic use of topical RB may avoid the pain, erythema, and other side effects typically noted in normal skin upon photoactivation of many other PDT agents. Such relative safety would be consistent with the long history of safe use of RB for various diagnostic applications.

ACKNOWLEDGMENTS

The many valuable suggestions of Peter Bjerring, MD, DSc, University Hospital, Aarhus, DK, are greatly

appreciated. The assistance of Dave Piston, Vanderbilt University Medical Center, in obtaining the images shown in Figure 2, is also greatly appreciated.

REFERENCES

1. Rose bengal sodium. In U.S.P. 24/NF 19, The United States Pharmacopeia/The National Formulary. Rockville, MD: United States Pharmacopeial Convention, Inc. 2000:2213.
2. Ito T, Kobayashi K. A survey of in vivo photodynamic activity of xanthenes, thiazines, and acridines in yeast cells. Photochem Photobiol 1977;26:581–587.
3. Bezman SA, Burtis PA, Izod TPJ, Thayer MA. Photodynamic inactivation of *E. coli* by rose bengal immobilized on polystyrene beads. Photochem Photobiol 1978;28:325–329.
4. Houba-Herin N, Calberg-Bacq CM, Piette J, Van de Vorst A. Mechanisms for dye-mediated photodynamic action: Singlet oxygen production, deoxyguanosine oxidation, and phage inactivating efficiencies. Photochem Photobiol 1982;36:297–306.
5. Paczkowski J, Lamberts JJM, Paczkowska B, Neckers DC. Photophysical properties of rose bengal and its derivatives. J Free Rad Biol Med 1985;1:341–351.
6. Neckers DC. Rose Bengal. J Photochem Photobiol 1989;47:1–29.
7. Kochevar IE, Lambert CR, Lynch MC, Tedesco AC. Comparison of photosensitized plasma membrane damage caused by singlet oxygen and free radicals. Biochim Biophys Acta 1996; 1280:223–230.
8. Lee PC, Rodgers MAJ. Laser flash photokinetic studies of rose bengal sensitized photodynamic interactions of nucleotides and DNA. Photochem Photobiol 1987;45:79–86.
9. Delprat GD, Epstein NN, Kerr WJ. A new liver function test: The elimination of rose bengal when injected into the circulation of human subjects. Arch Intern Med 1924;34:533–541.
10. Kerr WJ, Delprat GD, Epstein NN, Dunievitz M. The rose bengal test for liver function: Studies on the rates of elimination from the circulation in man. JAMA 1925;85:942–946.
11. Epstein NN, Delprat GD, Kerr WJ. The rose bengal test for liver function: Further studies. JAMA 1927;88:1619–1623.
12. Taplin GV, Meredith OM, Kade H. The radioactive (I131-tagged) rose bengal uptake-excretion test for liver function using external gamma-ray scintillation counting techniques. J Lab Clin Med 1955;45:665–678.
13. Taplin GV, Meredith OM, Kade H. Development of a radioisotope tracer test for the differential diagnosis of jaundice (the rose bengal I131 hepatogram). J Louisianna Med Soc 1957;109:255–266.
14. Taplin GV, Meredith OM, Winter CC, Johnson D. Rose bengal and iodopyracet as radiodiagnostic agents in liver and kidney diseases. Ann NY Acad Sci 1959;78:872–893.
15. Nordyke RA, Blahd WH. Blood disappearance of radioactive rose bengal—rapid simple test of liver function. JAMA 1959;170:1159–1164.
16. Nordyke RA. Biliary tract obstruction and its localization with radioiodinated rose Bengal. Am J Gastroenterol 1960;33:563–573.
17. Eyler WR, Schuman BM, Du Sault LA, Hinson RE. The radioiodinated rose bengal livers scan as an aid in the differential diagnosis of jaundice. Am J Roentgenol 1965;94: 469–476.
18. Eyler WR, Schuman BM, Du Sault LA, Hinson RE. Rose bengal I-131 liver scan. An aid in the differential diagnosis of jaundice. JAMA 1965;194:990–992.
19. Nordyke RA. Surgical vs. nonsurgical jaundice: Differentiation by a combination of rose bengal I131 and standard liver-function tests. JAMA 1965;194:949–953.
20. Robengatope, rose bengal sodium I131 injection USP. Squibb no. J3-381C, 1977.
21. Sodium rose bengal I-131 injection. In USP XXI, The United States Pharmacopeia, Twenty-First Revision. Rockville, MD: United States Pharmacopeial Convention, Inc. 1985.
22. Sjögren H. Zur Kenntnis der Keratoconjunctivits sicca (Keratitis filiformis bei Hypofunktion der Tränendrüsen). Acta Ophthalmol 1933;11(Suppl 2):1–151.
23. Marsh RJ, Fraunfelder FT, McGill JI, Phil D. Herpetic corneal epithelial disease. Arch Ophthalmol 1976;94:1899–1902.
24. Wilson FM. Rose bengal staining of epibulbar squamous neoplasms. Ophthalmic Surg 1976;7:21–23.
25. Pavan-Langston D. Manual of ocular diagnosis and therapy. 2nd edition. Boston: Little Brown & Co. Inc.; 1985. p 76–77.
26. Feenstra RP, Tseng SC. What is actually stained by rose bengal? Arch Ophthalm 1992;110:984–993.
27. Akorn, Inc. Barnes-Hind Rose Bengal Strips, 1.3 mg, Part No. 8820.
28. Chauvin Pharmaceuticals, Ltd. Minims® rose bengal eye drops, rose bengal 1%.
29. Tonogai Y, Ito Y, Iwaida M, Tati M, Ose Y, Sato T. Studies of the toxicity of coal-tar dyes. I. Photodecomposed products of four xanthene dyes and their acute toxicity to fish. J Toxicol Sci 1979;4:115–125.
30. Wester RC, Maibach HI. Penetration enhancement by skin hydration. In: Smith EW, Maibach HI, editors. Percutaneous Penetration Enhancers. Boca Raton, FL: CRC Press; 1995. p 21–28.
31. Tseng SC, Zhang SH. Interaction between rose bengal and different protein components. Cornea 1995;14:427–435.
32. Allen MT, Lynch M, Lagos A, Redmond RW, Kochevar IE. A wavelength dependent mechanism for rose bengal-sensitized photoinhibition of red cell acetylcholesterase. Biochim Biophys Acta 1991;1075:42–49.
33. Giulivi C, Sarcansky M, Rosenfeld E, Boveris A. The photodynamic effect of rose bengal on proteins of the mitochondrial inner membrane. Photochem Photobiol 1990;52: 745–751.
34. Chodosh J, Dix RD, Howell RC, Stroop WG, Tseng SC. Staining characteristics and antiviral activity of sulforhodamine B and lissamine green B. Invest Ophthalmol Vis Sci 1994;35:1046–1058.
35. Lee YC, Park CK, Kim MS, Kim JH. In vitro study for staining and toxicity of rose Bengal on cultured bovine corneal endothelial cells. Cornea 1996;15:376–385.
36. Niemz MH. Laser-tissue interactions, fundamental, and applications. Berlin: Springer; 1996. p 77–80.
37. Dees C, Harkins J, Scott TC, Wachter EA. Immune-mediated regression of tumors and production of anti-tumor immunity induced by chemoablative or photodynamic therapy. Counsel of Research Workers in Animal Disease, Chicago, IL, USA. 2001: abstract.
38. Dees C, Harkins J, Scott T, Wachter E. Use of halogenated xanthenes to specifically target diseased tissue. 29th Annual Meeting of the American Society for Photobiology, Chicago, IL, USA. 2001: abstract.