Rhodamine Dyes as Potential Agents for Photochemotherapy of Cancer in Human Bladder Carcinoma Cells

Christopher R. Shea, Norah Chen, Joanne Wimberly, and Tayyaba Hasan

Wellman Laboratories of Photomedicine, Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114

ABSTRACT

The phototoxicity in vitro of rhodamine 123 and tetrabromo rhodamine 123 (TBR) was compared, in order to assess their photochemotherapeutic potential. Exposure to 514.5-nm radiation from an argon ion laser caused phototoxicity in MGH-U1 bladder carcinoma cells previously treated with either dye at 10 μM for 30 min. As assessed by colony formation and cellular morphology, TBR was markedly more phototoxic than rhodamine 123, reflecting increased intersystem crossing of TBR to the triplet manifold via spin-orbital coupling induced by the heavy bromine atoms. Photoresponses of TBR very efficiently generated singlet oxygen (O2) in solution; furthermore, irradiation of TBR-treated cells was significantly more toxic when performed in the presence of deuterium oxide, an enhancer of damage caused by O2. Retention of fluorescence in TBR-treated cells was enhanced by irradiation, indicating that a stable photoproduc may be formed in reaction with cellular components.

INTRODUCTION

Photochemotherapy is the combination of non-ionizing radiation and photosensitizing drugs to treat cancer (1) or benign disorders (2). In this method, systemically administered drugs are locally activated by light at a desired site, allowing toxicity from drug action in other organs to be reduced or eliminated. The photosensitizer most widely studied for treatment of cancer by this modality is HPD (1), but its disadvantages, such as prolonged cutaneous phototoxicity, have prompted the exploration of new photochemotherapeutic agents (3–6).

R123, a specific fluorescent stain for living mitochondria, is an interesting candidate molecule for photochemotherapy of cancer. In vitro, R123 is taken up and retained by carcinoma cells preferentially and impairs their proliferation (9) by mechanisms requiring for cell killing and growth inhibition (13–20) indicate, however, that R123 is a relatively weak phototoxin. We hypothesized that its potency might be increased by substitution of halogen for hydrogen atoms in order to enhance intersystem crossing to the triplet state, and potentially the photochemical generation of singlet oxygen (O2) (21), a toxic, reactive species important in the mechanism of photosensitization by HPD (22) and many other agents (23). Accordingly, we have compared the phototoxicity in vitro of R123 and TBR and investigated the chemical and biological mechanisms of phototoxicity sensitized by TBR.

MATERIALS AND METHODS

Cells. MGH-U1 cells, a human transitional cell bladder carcinoma line (24), were maintained as adherent, exponentially growing stock monolayer cultures in 100-mm-diameter plastic Petri dishes in McCoy's Medium 5A (Gibco Laboratories, Grand Island, NY) supplemented with 5% (by volume) heat-inactivated fetal bovine serum (Gibco), at 37°C in a humidified, 95% air:5% CO2 atmosphere. For experiments, cells were trypsinized and replated into 35- or 100-mm-diameter dishes at appropriate numbers as given below.

Radiation Source. The 514.5-nm emission from an argon ion laser (Coherent, Inc., Palo Alto, CA) was passed sequentially through a timed-closure shutter, a quartz lens, a 1-mm-diameter quartz fiberoptic, and a ×20 microscope objective to form a 50- or 100-mm-diameter spot, within which individual Petri dishes were placed. Irradiance at the monolayer was measured by a thermal diode detector; spatial uniformity of irradiance was ±10%, as measured by a silicon diode array (EG&G Electro-optics, Salem, MA). There was no detectable heating of the cultures under the conditions used.

Chemicals. Unless otherwise specified, chemicals were dissolved in DPBS (Gibco) containing 0.49 mm MgCl2·H2O and 0.9 mm CaCl2, pH 7.2, at the following final concentrations: histidine (Sigma Chemical Co., St. Louis, MO), 10 μM; RNO (Aldrich Chemical Co., Milwaukee, WI), 10 μM; R123 (Eastman Kodak, Rochester, NY), 10 μM; EB (Sigma), 1 μM; deuterium oxide (Sigma), 90% by volume; fluorescein diacetate (FDA) (Sigma), 24 μM; RB (Sigma), 10 μM; and TBR, 10 μM. TBR was prepared from laser-grade R123 by the method of Chery et al. (25) (Fig. 1) and was found to be pure by thin-layer chromatography with three different solvent systems. TBR was stored in the dark at ~70°C at a stock concentration of 400 μM in methanol; both TBR and R123 solutions had 2.5% (by volume) methanol at their final concentrations, as did the dye-free control solutions (DPBS:methanol). Absorption spectroscopy was performed with a diode array spectrophotometer (model 8451 A; Hewlett-Packard, Sunnyvale, CA) using 1-cm-pathlength quartz cuvets; the absorption spectra of R123 and TBR were determined in DPBS:methanol and in n-butyl alcohol at 10 μM, and intracellular spectra were determined in cell suspensions after treatment with 100 μM dye for 30 min, trypsinization, washing, and resuspension of cells in DPBS. These samples were referenced against cells undergoing treatment with DPBS without dye.

Morphology and Vital Staining Properties of Cells at 24 h after Irradiation. Cells forming a confluent monolayer (~105 cells/35-mm dish) were incubated at 37°C for 30 min in 1 ml/dish of solution (DPBS:methanol, R123, or TBR), washed, covered with fresh DPBS, either kept in the dark or irradiated at room temperature (0.1–100 J/cm2 at 100 mW/cm2), and reincubated in complete medium. Twenty-four h after treatment, cells were examined and photographed at ×400 in situ by phase-contrast and fluorescence microscopy, the latter using excitation by 400–490 nm emission >520 nm. Cells were then washed, trypsinized, centrifuged, resuspended, treated with EB and FDA in complete medium, and examined by fluorescence microscopy. Three aliquots per dish were assessed for percentage of dead (EB-positive, fluorescing orange) and living (FDA-positive, fluorescing green) cells after 5–10 min (26). Further experiments compared TBR versus TBR in 90% D2O.

Colony Formation. This assay was run a minimum of 3 times at each radiant exposure. Petri dishes 100 mm in diameter were seeded with 200–300 cells/dish in complete medium, incubated for 48 h, treated with 5 ml of dye (10 μM TBR or R123) or DPBS:methanol for 30 min at 37°C, rinsed once with DPBS, either kept in the dark or irradiated at room temperature (0.1–40 J/cm2 at 25 mW/cm2), and returned to the incubator in complete medium. Eight days after irradiation, the dishes were rinsed, fixed with methanol, and stained with 0.1% aqueous...
crystal violet solution. Colonies consisting of 50 or more cells were scored as positive. In each experimental run, the colony count for each treatment group was summed from triplicate cultures, and the surviving fraction was calculated by normalization to the total colony count in untreated triplicate cultures. In total, 138 dishes were evaluated.

### Intracellular Dye Concentration
Multiple Petri dishes 100 mm in diameter were seeded with $7 \times 10^6$ cells/dish 24 h before dye treatment (10 $\mu$M for 30 min). At 1 and 24 h after dye treatment, dishes were rinsed 3 times with DPBS, cell counts were performed after trypsinization, and the dye was extracted in 6 ml of fluorometric-grade n-butyl alcohol. The extraction of both TBR and R123 was found to go to completion. Absorption spectroscopy was used to determine dye content per cell by comparison of extracts with standard dye solutions.

### Singlet Oxygen Generation
The change in absorbance at 440 nm of the RNO oxidation product was used to quantify the photosensitized generation of $^1$O$_2$ (27). One-ml aliquots of 10 $\mu$M TBR, R123, or RB solution containing histidine and RNO were placed into 15-mm-diameter wells, either kept in the dark or irradiated (0.1–40 J/cm$^2$ at 100 mW/cm$^2$), and studied by absorption spectroscopy (350 to 650 nm). The quantum yield of $^1$O$_2$ generation ($\phi$) for each dye was calculated after correction for the molar extinction coefficient at 514.5 nm (6.0, 4.1, and 2.2 x 10$^7$ liters mol$^{-1}$ cm$^{-1}$ in DPBS:methanol, for TBR, R123, and RB, respectively).

## RESULTS

### Rhodamine Dye Absorption Spectra
In DPBS:methanol, the absorption maximum of R123 was at 500 nm; the intracellular absorption spectrum was red-shifted by 14 nm to a maximum of 514 nm (Fig. 2). In contrast, the intracellular absorption maximum of TBR (516 nm) was red-shifted by only 2 nm from the maximum in DPBS:methanol (514 nm). Thus both dyes absorbed very efficiently at the 514.5-nm argon ion laser emission used for irradiation in this study.

### Intracellular Rhodamine Dye Localization
As expected, R123 localized selectively within mitochondria, forming a bright pattern of discrete, threadlike fluorescence. Fluorescence of TBR was partially localized to mitochondria (identified by phase-contrast viewing) but there was also diffuse cytoplasmic as well as punctate and globular nonmitochondrial fluorescence; as with R123, TBR fluorescence spared the nuclei. On viewing, TBR fluorescence faded much more rapidly than that of R123, i.e., marked photobleaching occurred. R123 fluorescence within mitochondria was fainter but still readily seen 24 h after washing; in contrast, cellular TBR fluorescence was virtually undetectable by 24 h.

### Cellular Morphology at 24 h after Irradiation
Cells treated with light alone, in the absence of dye, appeared unchanged 24 h after radiant exposures of up to 100 J/cm$^2$, as did R123-treated cells irradiated with up to 10 J/cm$^2$. Exposure of R123-treated cells to 20–100 J/cm$^2$ induced generalized, cytoplasmic vacuolation (foamy change) in ~20% of them (Fig. 3); R123 photosensitization did not induce ballooning or blebbing at any radiant exposure studied. In contrast, >90% of TBR-treated cells 24 h after irradiation showed foamy change at a threshold of only 0.3 J/cm$^2$ (Fig. 4A) and loss of adherence, blebbing, and ballooning at 2 J/cm$^2$ and above. Treatment with TBR plus irradiation enhanced the retention of intracellular fluorescence at 24 h (Fig. 4B), compared to unirradiated cells; such photo-binding was not seen with R123.

### Vital Staining at 24 h after Irradiation
Irradiation of undyed cells with up to 100 J/cm$^2$ caused no lethality by 24 h, assessed by vital staining with EB-FDA; similarly, treatment with R123 caused no significant lethality by 24 h in cells either kept in the dark or irradiated with up to 100 J/cm$^2$ (Fig. 5). Treatment with TBR, in contrast, caused approximately 40% lethality by 24 h after exposure to 1 J/cm$^2$, and >90% lethality at 5 J/cm$^2$. The addition of 90% D$_2$O during irradiation with 0.5 J/cm$^2$ caused a doubling of lethality in TBR-treated cells (Table 1). Exposure to TBR in the absence of irradiation caused <10% lethality, compared to treatment with DPBS alone.

### Colony Formation
Photosensitization by both dyes caused radiant-exposure-dependent inhibition of colony formation, but
RHODAMINE-DYE PHOTOSENSITIZATION

Fig. 4. Effect at 24 h of TBR (10 μM for 30 min) and exposure to 0.3 J/cm². In A, intracytoplasmic vacuolation is prominent in >90% of cells. In B, in the same field, TBR fluorescence is bright, globular, and punctate and is localized in the vacuole-free region of the cytoplasm. Cells treated with TBR and kept in the dark for 24 h show no fluorescence (data not shown).

Fig. 5. Lethality at 24 h following treatment with light plus either TBR or R123 (10 μM for 30 min). Cells staining positively with EB but not with FDA were considered dead. Each entry represents the mean ± SE (bars) of 3–7 experiments. To accommodate the marked difference between the two dyes in dose-response relationship, the radiant exposures are displayed on a logarithmic scale. Closed symbols, data for TBR; open symbols, data for R123.

Table 1 Effect of 90% D₂O on viability 24 h after exposure to 10 μM TBR for 30 min and irradiation with 0.5 J/cm²

<table>
<thead>
<tr>
<th>D₂O</th>
<th>% of viable cells</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>36.56 ± 7.80</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Absent</td>
<td>79.14 ± 2.56</td>
<td></td>
</tr>
</tbody>
</table>

TBR (Fig. 6) did so >90 times more efficiently than did R123 (Fig. 7). To accommodate this large difference in dose-response relationship, the scales for radiant exposure in Figs. 6 and 7 differ by 2 orders of magnitude. Light alone caused no inhibition at radiant exposures up to 40 J/cm² (Fig. 7). R123 inhibited colony formation in the absence of irradiation by 9.2 ± 9.2% (SE) compared to untreated controls; the corresponding figure for TBR was 21.4 ± 3.4%. Mean efficiency of colony formation in untreated control cultures was ~35%.

Singlet Oxygen Generation. Fig. 8 shows results of the RNO assay with TBR and R123. The data are calculated by assuming stoichiometric reaction of O₂ to form the RNO oxidation product and are normalized to the known φO₂ = 0.75 of RB (21). φO₂ of TBR and R123, defined as the slope of linear portion of the plots, was ~0.7 and <0.01, respectively.
Dye Extraction. As shown in Table 2, immediate cellular uptake of TBR was approximately 5 times greater than that of R123. This relative uptake ratio of TBR:R123 was confirmed independently by the absorbance measurements of intracellular dye in situ in cell suspensions (Fig. 2). By 24 h, however, <4% of initial TBR remained in cells, compared to ~20% retention of R123, confirming the qualitative finding of reduced fluorescence of TBR in situ at 24 h, by microscopy. Thus the clearance rate from cells was greater for TBR than R123.

**DISCUSSION**

R123 is a valuable probe of living mitochondria, in part because it is not strongly photosensitizing. The low photosensitizing potency of R123 is explained by its very high quantum yield of fluorescence (ΦF ~0.9) and correspondingly low quantum yield of the triplet state (ΦT ~0.1) (28, 29), and by its relatively short (~200 ns) triplet lifetime (29). Our data confirm that R123 is a relatively weak phototoxin in vitro, a conclusion supported by most other published reports (13–17); reports of R123 phototoxicity at much lower radiant exposures reflect conditions of R123 treatment that caused significant dark toxicity (18, 19). Powers et al. (20) reported partial response of rat gliomas in vivo treated with systemic R123 and interstitial argon laser irradiation; cells at the periphery of the treated tumors were unaffected, a finding that may reflect both the limitations imposed by tissue optics in their model and the intrinsically low phototoxicity of R123.

In contrast, all our data show TBR to be a much more potent phototoxin than R123, by approximately 2 orders of magnitude. With both dyes, the colony formation assay is more sensitive than either phase-contrast microscopy or fluorescence microscopy. Clonogenic assays are often more sensitive than nonclonogenic assays (30), because they may detect both cytostatic and cytotoxic effects (31), as well as the delayed expression of toxicity (32). Other studies indicate that both these explanations hold true for rhodamine dyes, as we discuss in detail elsewhere (33). Briefly, R123 phototoxicity appears to cause largely cytostatic and TBR largely cytotoxic effects; and the expression of phototoxicity of TBR but not of R123 does tend to increase at progressively longer times after irradiation, depending on the radiant exposure. In the present study, even cells showing striking vacuolation at 24 h retain some functional integrity of the plasma membrane and of intracellular esterases able to cleave FDA to fluorescein, as assayed by differential EB-FDA staining (26). Conversely, most cells that have lost these functions display extreme morphological changes indicative of necrosis. These findings suggest that with both dyes the plasma membrane is not the site of primary phototoxic injury but rather is affected as a terminal event at high radiant exposures; this interpretation is supported by the lack of detectable rhodamine dye fluorescence in the plasma membrane of unirradiated cells.

Oseroff et al. (14), using the same concentration of TBR, incubation time, assay (EB), and cell line as in our present study, previously reported that TBR photosensitization caused <20% lethality 24 h after exposure to 900 J/cm² of broadband spectrum light. After correction for the ~50-nm absorption spectrum of TBR, these data indicate an approximately 100-fold lower phototoxicity of TBR compared to our results. It is difficult to explain such a large difference. The possibility of a systematic error in radiometry is suggested by the internal discordance of 2 orders of magnitude, between the calculated radiant exposures, irradiance, and irradiation times given in a description of experiments on another dye in the same paper (14).

It is likely that bromination of R123 to form TBR increases its intrinsic phototoxicity by permitting more efficient intersystem crossing to the triplet manifold via spin-orbital coupling, thereby generating O₂⁺ by energy transfer to ground-state triplet O₂ (21, 34, 35). In support of this mechanism, the triplet state of TBR is quenched by O₂ (36), and TBR phototoxicity is potentiated by D₂O (Table 1). The lifetime of O₂ is about 10 times longer in D₂O than in H₂O (37); as with most photosensitizers, actual cellular phototoxicity of TBR is potentiated by D₂O to a lesser extent, for O₂-mediated reactions occurring in hydrophobic environments such as membranes cannot be modulated by D₂O, and other modes of decay of O₂ exist besides reaction with H₂O (38). Alternatively, D₂O may lengthen the triplet-state lifetime of TBR itself (39), favoring reaction from this manifold via O₂ generation or other mechanisms. Our RNO data indicate a >70-fold higher φO₂ of TBR than of R123 in solution and have been confirmed by experiments using direct detection of the 1.27-μm phosphorescence signal of O₂ to rule out free-radical processes in the RNO assay (40). The measured φO₂ of TBR implies that φT ≥ 0.7, a value 7 times greater than that shown for R123 (29). Furthermore, the triplet lifetime of TBR in air is reportedly more than 10 times longer than that of R123 (29, 36). The greater triplet yield and longer triplet lifetime of TBR should combine to produce a >70-fold greater φO₂ of TBR than of R123, in excellent agreement with our data.

By definition, quantum yields are calculated after correction for the number of photons absorbed; cellular phototoxicity sensitized by different dyes, however, may also depend on absolute differences in the amount of light absorbed. The concentration of TBR within cells at the time of irradiation is approximately 5 times that of R123, and this fact is probably responsible for part of the much greater phototoxicity induced by TBR as compared to R123. Differences in absorption maxima of the two dyes, however, are unlikely to contribute greatly to their differential phototoxicity, especially because the intracellular R123 absorption spectrum (Fig. 2A) is red-shifted close to the wavelength of laser irradiation, while the TBR absorption maximum is shifted slightly away from this wavelength. Similar red shifts have been demonstrated in the R123 absorption spectrum in the presence of coupled, isolated mitochondria (41), and in the R123 fluorescence emission spectrum in L1210 cells (42).

The intracellular localization of R123 is strictly mitochondrial, in response to the negative intramitochondrial electric potential (43). Bromination of R123 to form TBR alters its localization somewhat, perhaps because of steric shielding of the positive charge, or dispersal of the charge due to the negative inductive effects of the bromine atoms. Differences in localization of the two dyes may bear upon their relative phototoxicity, by affecting the total uptake into cells and sensitizing intracellular sites that have different susceptibility to toxic photoreactions. The more rapid clearance rate of TBR from unirradiated cells may likewise be related to its partially extramitochondrial

---

**Table 2: Dye concentration (fmol/cell) 1 or 24 h after incubation**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>TBR</th>
<th>R123</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.18 ± 0.05</td>
<td>0.22 ± 0.005</td>
</tr>
<tr>
<td>24</td>
<td>0.04 ± 0.005</td>
<td>0.04 ± 0.005</td>
</tr>
</tbody>
</table>

* C. R. Shea and T. Hasan, unpublished data.
localization. Laser irradiation enhances the retention of fluoro-
usence within TBR-treated cells, possibly by a specific pho-
toaddition reaction in situ; e.g., photoinduced homolytic cleav-
age of C–Br bonds may generate reactive free radical(s) able to
form a stable photoproduc covalently linked to intracellular
components. Such photooaddition reactions may contribute to
the phototoxicity of TBR.

In summary, bromination of R123 to form TBR markedly
increases its phototoxicity, through enhanced O₂ generation
and possibly also the formation and retention of a stable pho-
toproduc; on the other hand, bromination also modifies the
uptake, localization, and retention of the dye. TBR may prove
useful in the photochemotherapy of superficial malignancies
that may be appropriately treated by 514.5-nm irradiation (44,
45). Basic studies are being continued to elucidate the chemical
and biological mechanisms of TBR phototoxicity, including the
ultrastructural aspects of cellular injury, and to assess its ther-
apeutic potential.

ACKNOWLEDGMENTS

We thank Dr. Chi-Wei Lin for his generous gift of MGH-U1 cells
and Frederick H. Long for expert engineering help.

REFERENCES

1. Dougherty, T. J., Weishaupt, K. R., and Boyle, D. G. Photodynamic sensi-
tizers. In: V. T. DeVita, S. Hellman, and S. Rosenberg (eds.), Cancer: Princi-
ple and Practice of Oncology, Ed. 2. pp. 2277–2279. Philadelphia: J. B. Lippincott,
1985.
2. Parrish, J. A., Fitzpatrick, T. B., and Tanenbaum, L. Phototherapy of
tumors with oral methoxsalen and longwave ultraviolet light. N. Engl. J.
3. Kessel, D. Localization and photosensitization of murine tumors in vivo
4. Arztopden, J., Gulati, S. C., and Clarkson, S. D. Comparison of the cytotoxic
effects of merocyanine-540 on leukemia cells and normal human bone mar-
5. Spikes, J. D. Phthalocyanines as photosensitizers in biological systems and
use of mono-aspartyl chlorin (NPe6) for photodynamic therapy.
994, 1980.
rhodamine 123 uptake by carcinoma cells. Cancer Res., 45: 6093–6095,
1985.
mine-123 selectively reduces clonal growth of carcinoma cells in vitro.
Rhodamine 123 inhibits biochemical function in isolated rat mitochondria.
oma activity in vivo of rhodamine-123, a mitochondrial-specific dye. Science
and Whitmore, W. F., Jr. Anticancer activity of rhodamine 123 against a murine
13. Powers, S. K., Pribil, S., Gillespie, G. Y., and Watkins, P. J. Laser photo-
chemotherapy of rhodamine-123 sensitized human glioma cells in vitro.
L. Intramitochondrial dyes allow selective in vitro photolysis of carcinoma
15. Castro, D. J., Saxton, R. E., Fetterman, H. R., Castro, D. J., and Ward,
P. H. Rhodamine-123 as a new photochemosensitizing agent with the argon
laser: “nonthermal” and thermal effects on human squamous carcinoma cells
P. H. Rhodamine-123 as a chemosensitizing agent for argon laser therapy.
Rhodamine Dyes as Potential Agents for Photochemotherapy of Cancer in Human Bladder Carcinoma Cells

Christopher R. Shea, Norah Chen, Joanne Wimberly, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/49/14/3961