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Evaluation of Diethyl-3-3'-(9,10-anthracenediyl)bis Acrylate as a Probe for Singlet Oxygen Formation during Photodynamic Therapy

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Abstract

The cell-permeable anthracene analog diethyl-3-3'-(9,10-anthracenediyl)bis acrylate (DADB) was recently identified as a highly selective probe for singlet oxygen ($^1\text{O}_2$). Now, we show that DADB can be used to monitor $^1\text{O}_2$ formation in cell culture during photodynamic therapy. An atypical property of DADB is that fluorescence emission is decreased upon oxidation. Using photosensitizers that target specific organelles, we determined that DADB could detect $^1\text{O}_2$ whether formed in ER, mitochondria or lysosomes. DADB fluorescence was not, however, significantly altered when the photosensitizing agent was the palladium bacteriopheophorbide termed WST11, an agent reported to produce mainly oxygen radicals upon irradiation in an aqueous environment, whereas singlet oxygen was formed in organic solvents.

INTRODUCTION

Photodynamic therapy (PDT) involves the use of photosensitizing agents that selectively localize in neoplastic tissues and their vasculature (1,2). Subsequent irradiation results in formation of reactive oxygen species, with cytotoxic consequences. For most photosensitizing agents, the initial cytotoxic product formed is $^1\text{O}_2$, with other ROS formed downstream: $\cdot\text{O}_2^-$, $\cdot\text{OH}$ and H_2O_2 (3–5). One method for the monitoring of $^1\text{O}_2$ formation involves the detection of the 1270 nm signal, using a detector cooled to 77 K. This requires equipment not routinely available in many laboratories. Electron spin resonance (ESR) can also be used in conjunction with appropriate spin traps, but this can be unwieldy for studies involving cell culture. A variety of fluorescent probes have been identified that can detect different ROS, but the specificity is often less than is advertised (6–8). We reported that the fluorescent probe 3'-*p*-(aminophenyl) fluorescein (APF) initially designed to detect $\cdot\text{OH}$ (9), could also detect $^1\text{O}_2$ in the context of PDT[†] (10). This lack of selectivity would, however, preclude the use of APF as a specific $^1\text{O}_2$ probe.

A more selective probe for $^1\text{O}_2$ detection is singlet oxygen sensor green (SOSG), although this agent is said to be unable to penetrate cell membranes (11). Ogilby's group reported a procedure for promoting cellular uptake of SOSG, but reported that the ability of the probe to detect $^1\text{O}_2$ formation during PDT was unreliable (12). It is possible that this problem arose from their choice of the photosensitizing agent, the tetra cationic porphyrin TMPyP.

This agent was reported to concentrate in nuclei (13) and the extent of penetration of SOSG into nuclei remains unknown.

A recent report on a new cell-permeable probe for $^1\text{O}_2$ prompted the present study (14). This anthracene derivative, termed diethyl-3-3'-(9,10-anthracenediyl)*bis* acrylate (DADB), was shown to be both highly selective for $^1\text{O}_2$ and readily able to cross the plasma membrane. Unlike many other ROS probes, the fluorescence of DADB is reduced by the formation of an endoperoxide when exposed to $^1\text{O}_2$.

In this study, we examined the ability of DADB to detect ROS formed by a group of photosensitizing agents with different sites of subcellular localization. We also compared the effects of two lysosomal photosensitizers with differing patterns of ROS formation: the chlorin NPe6 (15) and the palladium bacteriopheophorbide WST11 (16). NPe6 is known to have a high $^1\text{O}_2$ yield upon irradiation (17), whereas WST11 produces mainly oxygen radicals upon irradiation in a relatively aqueous environment (18).

MATERIALS AND METHODS

Drugs and chemicals

Tissue culture media were purchased from Sigma–Aldrich (St. Louis, MO). *N*-aspartyl chlorin e6 (NPe6) was provided by Prof Kevin M. Smith, Louisiana State University. BPD (benzoporphyrin derivative, Verteporfin) was purchased from VWR, meso-tetrahydroxyphenyl porphine (mTHPC, Foscan) was obtained from Frontier Scientific and WST11 was prepared as described in (19). The synthesis of DADB was carried out as indicated in (14) with a minor alteration: the reaction mixture (dibromoanthracene + ethyl acrylate) was maintained at 110°C for only 8 h without affecting the yield of product.

Cells and cell culture

Maintenance of the murine hepatoma Hepa 1c1c7 cell line has been reported (20).

PDT Protocols

Cells were cultured on 22 mm glass coverslips. PDT studies were carried out after incubation with 1 μM WST11 for 16 h, 0.5 μM BPD for 1 h, 20 μM NPe6 for 1 h or 0.5 μM mTHPC for 16 h, all at 37°C. The medium was then replaced and the cells were irradiated. The light source was a 600 W quartz-halogen lamp with IR radiation attenuated by a 10 cm layer of water and further limited by interference filters (± 10 nm). Irradiation took place at a temperature of 15°C to inhibit initiation of apoptosis during this step. Concentrations of the photosensitizers were adjusted so as to produce a 90% photokilling by a 270 mJ sq cm⁻¹ light dose. The wavelengths employed were 750 nm (WST11), 690 nm (BPD) and 660 nm (mTHPC and NPe6). During the final hour of the photosensitizer loading incubation, DADB was added (10 μM). Phototoxicity was assessed by clonogenic assays as described in (21). Fluorescence images were acquired directly after irradiation.

Phase-contrast and fluorescence microscopy

Images were acquired with a Nikon E-600 microscope using a Rolera EMCCD camera (QImaging, Surrey, BC, Canada). DADB fluorescence centered at 525 nm was determined using 360–400 nm excitation. A 600 nm low-pass filter was inserted into the emission path to eliminate fluorescence emission from the photosensitizing agents. The acquisition time for each fluorescence image was 100 ms. The resulting images were acquired and analyzed using METAMORPH software. Thresholding was uniformly applied so that the software determined pixel intensity in cytoplasmic loci only, and not in the dark background or nuclei. Thresholding also removed a few very bright punctate spots that may represent

undissolved fragments of the probe. Data are reported in terms of average pixel density $\pm$ SD for images involving at least 20 cells. Additional studies were carried out to assess the variation of this average value in replicate images.

Photobleaching of DADB

To assess the stability of DADB to irradiation during fluorescence microscopy, we evaluated the photobleaching effects of the exciting illumination. Two successive 100 ms exposures were used to determine whether there was a loss of fluorescence after a 100 ms exposure to exciting light on the stage. As an additional test, the cells loaded with DADB were exposed to exciting light for 15 s, and an image was then acquired. We also examined the ability of photosensitizers to alter DADB fluorescence during image acquisition. This was carried out by assessing the change in pixel brightness during two subsequent 100 ms image acquisitions when the cells contained both DADB and a photosensitizing agent.

RESULTS

DADB: labeling pattern

Incubation of 1c1c7 cells with 10 μ M DADB for 60 min led to a labeling pattern that appears to involve intracellular membranes (Fig. 1). We could not distinguish any specific organelle population in the labeling pattern, although nuclear labeling was negligible.

Intrinsic DADB photobleaching

Mammalian cells are known to form ROS, including $^1\text{O}_2$, during UV irradiation (22). To examine the effect of light used in image acquisition on DADB, we used cells that had been incubated with the probe alone. No significant decrease in the fluorescence signal was observed after a 100 ms exposure interval (c.f. Fig. 2a,b; Fig. 4). A significant level of DADB photobleaching was, however, observed if cells were exposed to the UV excitation source on the microscope stage for 15 s before image acquisition (Fig. 2c; Fig. 4).

Effects of varying the ROS on DADB photobleaching

Both NPe6 and WST11 localize in lysosomes, but produce different ROS upon irradiation (23). An LD₉₀ PDT dose led to a barely detectable effect on DADB fluorescence when WST11 was employed (Fig. 3). In contrast, a significant photobleaching of DADB was observed when PDT utilized NPe6, an agent known to produce a substantial $^1\text{O}_2$ yield upon irradiation (15).

DADB photobleaching after PDT with other photosensitizers

Photodynamic therapy under LD₉₀ conditions with two other photosensitizing agents also resulted in a significant degree of DADB photobleaching (Fig. 4). In this study, we examined mTHPC, an agent that targets ER and Golgi (24) and BPD, an agent that targets mitochondria (25). Results obtained with WST11 and NPe6 are also shown in this figure.

Statistical considerations

The gray error bars shown in Fig. 4 represent the variation in pixel intensity in the cytoplasmic region of cells in the microscope field. The size of the error bars indicate that there are subcellular regions that differ considerably in brightness in all images. This variation can readily be observed in a magnified image of a portion of Fig. 1b, shown in panel c. These error bars do not reflect the variation in the mean value for pixel intensity for replicate determinations. When the mean pixel density was measured in triplicate samples, the average value of pixel intensity showed a much smaller variation (open bars shown in Fig. 4).

DISCUSSION

In this study, we demonstrate that DADB can monitor the formation of $^1\text{O}_2$ by several photosensitizing agents regardless of their site of initial subcellular localization. A high degree of specificity of DADB for singlet oxygen detection was indicated in (14), as was the ability of this agent to penetrate cell membranes. The loss of DADB fluorescence upon irradiation in a cell-free system containing a photosensitizing agent established the ability of this agent to interact with $^1\text{O}_2$ (14), but did not provide an estimate of sensitivity in the context of PDT. Using BPD, mTHPC or NPe6, we observed a somewhat similar loss of DADB fluorescence at equitoxic PDT doses (Fig. 4). Qualitative comparisons are readily feasible, *e.g.*, demonstrating that an LD₉₀ PDT dose with the lysosomal targeting photosensitizer NPe6 produces substantially more $^1\text{O}_2$ than did a comparable PDT dose with WST11. The latter also targets lysosomes (23), but was expected to produce mainly oxygen radicals (18).

Other procedures for $^1\text{O}_2$ are available, *e.g.*, detection of the 1270 nm $^1\text{O}_2$ signal (26). Geiger (27) has described a chromatographic procedure that measures the conversion of cholesterol to a product that is uniquely formed by the action of $^1\text{O}_2$. The development of a cell-permeable fluorescent probe, if adequately sensitive, would place $^1\text{O}_2$ measurements within the reach of laboratories with conventional fluorescence microscopy facilities. Quantitative aspects of the analysis depend on how well $^1\text{O}_2$ generated at different subcellular loci can interact with DADB and how well DADB can compete with biological substrates that can undergo oxidation.

We observed a broad spectrum of pixel intensities within a single field. The standard deviation value was as large as 30% of the mean in untreated cells and cells that had been photosensitized and irradiated (Fig. 4). The areas selected for the thresholding program show a considerable variation in pixel intensity, but the mean pixel intensity measurement appears to provide a reproducible indication of the level of probe oxidation. An alternative approach is discussed in (14): GC/MS analysis could provide an indication of DADB oxidation free from errors implicit in the thresholding process.

These data indicate that DADB can successfully be used to as a fluorescence probe for singlet oxygen formation in live cells. As the wavelength of excitation can also elicit DADB photobleaching in the absence of a photosensitizing agent, care must be taken so that the exposure times are sufficiently small so that this problem is avoided.

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Figure 1. Phase-contrast (a) and fluorescence images (b and c) showing the localization of DADB in 1c1c7 cells following a 1 h incubation with a 10 μ M DADB concentration. Panel (c) is an enlargement of the lower left portion of panel (b).

Figure 2.

Fluorescence intensity of intracellular DADB as a function of the time of exposure of cells to illumination on the microscope stage (380–400 nm). Panels (a and b) represent two sequential exposures of 100 ms each, to test for photobleaching during image acquisition. Panel (c) showed the image captured by a 100 ms exposure after 15 s of irradiation on the microscope stage.

Figure 3.
Effect of ROS formation on DADB photobleaching: (a) cells following an LD₉₀ PDT dose using NPe6; (b) a similar study with WST11.

Figure 4.

DADB oxidation by $^1\text{O}_2$ as indicated by pixel brightness. All acquisitions involved a 100 ms exposure interval. Gray bars: mean pixel intensity of DADB in a typical experiment involving control cells and cells after PDT. C1, C2 = two sequential images of the same field to test for photobleaching during acquisition; C3 = an image acquired after a 15 s exposure of cells to the excitation beam on the microscope stage; NPe6, WST11, BPD, mTHPC = images of cells after LD₉₀ PDT doses with specified photosensitizers. Open bars represent the mean $\pm$ SD for three separate experiments where only the mean pixel intensity of the images is considered.