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Factors Affecting Virus Photoinactivation by a Series of Phenothiazine Dyes

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ABSTRACT

A series of four phenothiazine dyes, including methylene blue (MB), were previously tested for their ability to photoinactivate viruses in red cell suspensions. One of the dyes, 1,9-dimethyl-3-dimethylamino-7-dimethylaminophenothiazine (1,9-dimethylmethylene blue), exhibited good intracellular and extracellular virucidal activity for several RNA and DNA viruses under conditions that minimally affected red cell properties. In order to understand why the virucidal specificity of 1,9-dimethylmethylene blue was greater than other phenothiazines tested, the physical and chemical properties of the dye were compared to three other closely related analogues (MB, 1,9-dimethyl-3-diethylamino-7-dibutylaminophenothiazine [compound 4-140], 1,9-dimethyl-3-dimethylamino-7-diethylaminophenothiazine [compound 6–136]). All compounds required light and oxygen for virucidal activity and had relatively high singlet oxygen yields (>0.5), but 1,9-dimethylmethylene blue had a singlet oxygen yield approximately 50% greater than that of MB. In addition, the hydrophobicity/hydophilicity of the compounds varied, with the partition coefficients (2-octanol: water) ranging from 0.11 for MB to 3560 for compound 4-140. The dyes had the following affinities for DNA: 1,9-dimethylmethylene blue > compound 6-136 > MB ≈ compound 4-140. This order was similar to the order of activities for photoinactivation of the nonenveloped bacteriophage, R17, by the four compounds. Results with the most hydrophobic compound, 4-140, contrasted with those obtained with 1,9-dimethylmethylene blue. Compound 4-140 had a high affinity for protein and a low affinity for DNA. Although compound 4-140 and light inactivated the nonenveloped bacteriophage R17 poorly, the dye readily photoinactivated enveloped viruses in buffer. However, unlike results with 1,9-dimethylmethylene blue, viral inactivation of enveloped viruses by compound 4-140 was completely inhibited by the presence of red cells and plasma. Thus, the high affinity of 1,9-dimethylmethylene blue for DNA and the dye's efficient singlet oxygen yield suggest viral nucleic acid as a potential target, which could explain the photosensitizer's ability to inactivate viruses without adversely affecting anucleate red cells.

INTRODUCTION

Extensive laboratory testing and careful donor selection have greatly improved the safety of the blood supply. However, some risk of transfusion-transmitted virus infection remains. Most transmission of tested viruses is thought to occur at an early phase of an infection, termed the "window period," when current tests are unable to detect the presence of antibody or virus protein. Based on one recent estimate, the aggregate risk of viral transmission (HIV-1/2, HTLV-I/II, hepatitis B and C) from transfusion of a single blood component in the United States is 1 in 34 000 (1). Many transfused individuals have a greater risk of infection because they receive multiple components. In addition, unknown or emerging viruses may affect the safety of the blood supply in the future. Therefore, pathogen inactivation of blood components remains of interest.

The combination of virucidal dyes and light offers an attractive approach for inactivation of both known and yet to emerge viruses in cellular blood components. By regulating light exposure, photochemical reactions offer more precise control of the extent of unwanted damage to red cells than uncontrolled, chemically reactive antiviral agents. In addition, equilibrium binding of the dye to its target can take place prior to photoactivation, which also helps to limit unwanted damage. Despite the potential advantages of photochemical methods for inactivating viruses in red cells, a number of studies using several different sensitizers have described red cell damage (hemolysis, enhanced ion permeability, IgG-coated red cells, change in red cell surface charge, *etc.*) associated with virucidal phototreatment (2–5). Therefore, a lack of viral specificity by currently used photosensitizers has hampered the practical application of this method in red cells.

In an effort to identify photoactive compounds that cause greater virus inactivation and less red cell damage, a series of phenothiazine derivatives were screened for bacteriophage inactivation and their effect on red cell ion permeability. One compound, 1,9-dimethyl-3-dimethylamino-7-di-

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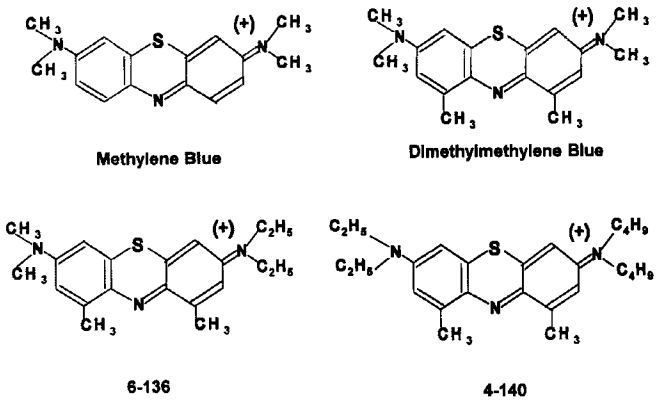

Figure 1. Structures of four phenothiazine dyes. For the sake of simplicity, the chloride counterions are not shown.

methylaminophenothiazine (1,9-dimethylmethylene blue)† appeared to have superior virucidal activity with less red cell damage than the "lead" compound, methylene blue (MB) (6). 1,9-Dimethylmethylene blue and light inactivated a number of RNA and DNA model viruses in red cell suspensions under conditions that caused minimal alteration of red cell ATP and 2,3-diphosphoglycerate (DPG) levels, <1% hemolysis, small changes in osmotic fragility, no IgG binding in 11 of 13 samples, unchanged red cell morphology and small alterations of potassium efflux during 42 days of storage at 1–6°C (6).

In this report, the basis for the improved specificity of 1,9-dimethylmethylene blue for virus inactivation is explored by comparing the dye and three structurally related compounds for virucidal activity, singlet oxygen production, hydrophobicity, spectral characteristics as well as DNA and protein binding affinities.

MATERIALS AND METHODS

Preparation of phenothiazine derivatives

The structures of MB, 1,9-dimethylmethylene blue, 1,9-dimethyl-3-dimethylamino-7-diethylaminophenothiazine (compound 6-136) and 1,9-dimethyl-3-diethylamino-7-dibutylaminophenothiazine (compound 4-140) are given in Fig. 1. Pharmaceutical-grade MB (>95% purity, American Reagent Laboratories, Inc., Shirley, NY) was obtained from Naomi Luban (Childrens National Medical Center, Washington, DC). 1,9-Dimethylmethylene blue was purchased commercially (Polysciences, Warrington, PA). Compounds 6-136 and 4-140 were prepared by the condensation/cyclization of a phenylenediaminethiosulfonic acid derivative with an appropriately substituted aniline under oxidizing conditions (7).

2-Amino-5-diethylaminophenylthiosulfonic acid preparation. Ten grams of *N,N*-diethyl-2-methyl-1,4-phenylenediamine (Aldrich Chemical Co., St. Louis, MO) was added to a mechanically stirred solution of 43.6 g (0.065 mole) of aluminum sulfate octadecahydrate in 100 mL of water. To this solution was added 22.0 g (0.139 mole) of sodium thiosulfate in 80 mL of water followed by 8.8 g (0.063 mole) of zinc chloride dissolved in 12 mL of water. The solution was cooled to 0°C and 5.0 g (0.017 mole) of potassium dichromate in 30 mL of water was added dropwise over a 30 min period. Following this addition, the mixture was allowed to stir for 2 h. During the last 20 min of stirring, the temperature was allowed to rise to 10°C and a viscous material precipitated out of solution. The solid was isolated by filtration and was washed with water followed by acetone. The resulting pink solid weighed 3.1 g (19% yield) and was used in the synthesis of compound 6-136 and 4-140.

Preparation of compound 6-136. A 0.20 g (0.0015 mole) sample of *N, N*-dimethyl-m-toluidine (Aldrich) was added to a solution containing 40 mL methanol, 15 mL water and 0.290 g (0.001 mole) of 2-amino-5-diethylaminophenyl thiosulfonic acid. The solution was heated to reflux temperature, and 1.0 g of 50% silver carbonate on celite (Aldrich) was slowly added over a 15 min period. The resulting mixture was refluxed for 40 min, filtered through a celite pad and the filtrate evaporated. The residue was extracted with methylene chloride and subsequently purified (see below). The final yield was 0.082 g (22%).

Preparation of compound 4-140. Compound 4-140 was prepared using a similar scheme to that used for preparing compound 6-136. *N,N*-dibutyl-*m*-toluidine was prepared by standard alkylating procedures. Briefly, 1.07 g (0.01 mole) of amino-*m*-toluidine (Aldrich), 5.66 g (0.03 mole) butyl iodide and 2.5 g (0.30 mole) of sodium acetate were placed in a stainless steel bomb and heated to 130–140°C for 4 h. The residue was extracted with methylene chloride and the solution was then evaporated to dryness to yield 2.2 g (100%).

Purification of compounds. 1,9-Dimethylmethylene blue, compound 6-136 and compound 4-140 were purified by medium pressure (100 psi) liquid chromatography. A gradient of methylene chloride:methanol (100:0 to 94:6) was used as an eluent for dye bound to silica gel (40μ, Scientific Absorbents Inc., Atlanta, GA). The resulting purified compounds were homogeneous by thin-layer chromatography. The structure of the 1,9-dimethymethylene blue, compound 6-136 and compound 4-140 were confirmed by NMR and their purity was estimated to be >95%.

Red cell preparation

Packed red cells were prepared from units of whole blood (500 ± 50 mL) collected in 70 mL citrate phosphate dextrose in triple-pack container systems (PL146 primary container, Baxter Healthcare Deerfield, IL) by the American Red Cross Blood Services, Holland Laboratory for the Biomedical Sciences. Units were centrifuged at 1471 ***g*** for 4 min and platelet-rich plasma and buffy coats were removed. The packed red cells typically were diluted to approximately 50% (0.50) hematocrit (Hct) with ARC-8, an investigational preservation solution that maintains detectable 2,3-DPG levels beyond 21 day storage at 1–6°C (8,9). The red cell suspension subsequently was leukoreduced using a filter (Leukotrap®-SC RC, Medsep Corporation, Covina, CA) and further diluted to 30% (0.30) Hct with ARC-8.

Addition of virus and phototreatment

Stock suspensions of the mammalian enveloped virus, vesicular stomatitis virus (VSV) or bacteriophages R17 or ϕ6 were diluted 10–100-fold into ARC-8, phosphate-buffered saline (PBS), or leukoreduced red cells. After thorough mixing, one of the four above-mentioned phenothiazine dyes (1 m*M* stocks) was added to obtain a 1 μ*M* solution, and 4 mL samples were aliquoted to polystyrene culture dishes (50 mm bottom diameter) resulting in a sample thickness of 1.9 mm. Samples were agitated at room temperature on a horizontal reciprocal shaker for 15 min in the dark. In general, one culture dish was used as a dark (no intentional illumination) control and the remaining dishes were used for each of the illumination timepoints. For oxygen dependence experiments, irradiated samples were bubbled with nitrogen or air in bubbler tubes.

Samples were placed on an air-cooled transparent plastic stage (agitated 70 cycles/min) and positioned approximately 3 cm between two in-house-fabricated light banks that illuminated samples from above and below. For experiments investigating oxygen dependence, upright bubbler tubes were illuminated by two banks from the side. Each bank contained five cool-white fluorescent bulbs (F20T12-CW,

†*Abbreviations*: compound 4-140, 1,9-dimethyl-3-diethylamino-7-dibutylaminophenothiazine; compound 6-136, 1,9-dimethyl-3-dimethylamino-7-diethylaminophenothiazine; 1,9-dimethylmethylene blue, 1,9-dimethyl-3-dimethylamino-7-dimethylaminophenothiazine; 2,3-DPG, 2,3-diphosphoglycerate; EDTA, ethylenediaminetetraacetic acid; Hct, hematocrit; MB, methylene blue; OD, optical density; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus.

General Electric, Circleville, OH) that provided broadband white light at a fluence rate of 5 mW/cm^2 at the sample surface, as measured by an optical power meter (model 371, United Detector Technology, Hawthorn, CA) equipped with a silicon diode detector and radiometric filter (400–1000 nm, model 115–9, United Detector Technology).

For wavelength dependency studies using 1,9-dimethylmethylene blue, a Polaroid 610 slide projector equipped with a 500 W tungsten lamp (CZX-DAB, Sylvania, GTE Products Corp., Winchester, KY) was utilized with a 700 nm cut-off filter and 660, 580 and 540 nm narrowband pass filters (halfbandwidth 10 nm, Melles Girot, Irvine, CA) to provide fluence rates of 2.5, 4.0 and 2.7 mW/cm^2, respectively.

Source of viruses, cells and virus assays

Bacteriophage ϕ6 and its host, *Pseudomonas phaseolicola* strain HB10Y, was obtained from Leonard Mindich, Public Health Research Institute, New York, NY; bacteriophage R17 and its host, *Escherichia coli* Hfr C, were obtained from David Cook, Cerus Corporation, Concord, CA.

The plaque assays for bacteriophages R17 and ϕ6 utilized standard top agar overlay techniques in petri dishes (10). *Escherichia coli* Hfr C and HB10Y were grown in T-soy broth (Becton Dickenson, Cockeysville, MD) overnight cultures at 37 and 30°C, respectively, to early stationary phase. Then 220 μL of host bacteria and either 100 μL or 1 mL of neat or diluted phototreated or control samples containing bacteriophage were added to 3 mL of 0.8% molten top agar (43°C). The R17 and ϕ6 plaques were evident after overnight incubation at 30°C (R17) or room temperature (ϕ6). Each experiment involving bacteriophage inactivation was performed at least two times for each compound.

The plaque assay for VSV utilized a VERO (isolated from African green monkey kidney, CCL81, ATCC) cell host. Cells were grown in tissue culture flasks using RPMI-1640 medium containing glutamine (Biofluids Incorporated, Rockville, MD) and supplemented with 10% fetal bovine serum (Biofluids Incorporated). Cells were seeded into six well tissue culture plates and allowed to grow to confluence. Control and phototreated samples were serially diluted 10-fold and plated onto confluent VERO cell monolayers and incubated for 45 min with gentle rocking at 37°C for virus adsorption to cells. A semiliquid agar layer (0.1%) was added to each well and infected monolayers were incubated in 5% CO_2/air at 37°C for 1 day. After incubation, the agar layer was removed by aspiration and the monolayer was stained with 0.1% crystal violet in ethanol for at least 15 min. The stain was removed by aspiration, plates were washed with water and plaques were enumerated.

Measurement of oxygen dependence, singlet oxygen production and partition coefficents

The oxygen dependence of ϕ6 inactivation was measured in ARC-8-buffered virus suspensions. Typically, samples were scrubbed with nitrogen bubbles for 15 min prior to and during illumination. Results are expressed as the extent of $\log_{10}$ ϕ6 inactivation observed in nitrogen-bubbled samples relative to the $\log_{10}$ inactivation in air-bubbled samples at the same fluence. The fluence chosen inactivated 4–6 $\log_{10}$ ϕ6 in air-bubbled samples. Totally oxygen-dependent photoinactivation would have a value of 0.0; oxygen-independent inactivation would have a value of 1.0.

The quantum yields of $^1O_2$ formation for 1,9-dimethylmethylene blue, compound 6-136 and compound 4-140 were determined by measuring the $^1O_2$-mediated bleaching of 1,3-diphenylisobenzofuran (11). A cuvette containing a mechanically stirred methanol solution of the phenothiazine dye (optical density [OD][$\lambda_{632.8}$] = 1.000) and the furan $^1O_2$ receptor (OD[$\lambda_{max\,410\,nm}$] ≈ 0.850) was illuminated with an expanded beam (5 mm diameter) of a HeNe laser (5 mW, 632.8 nm) whose intensity, which varied <2% during the course of the experiment, was attenuated using an unpolarized neutral density filter having an OD of 0.8. The 632.8 nm light was absorbed only by the photosensitizer. The rate of furan oxidation was measured spectrophotometrically by the disappearance of the 410 nm band as a function of time for one half-life. Singlet oxygen yields for each dye were calculated by comparing the rate of furan photooxidative bleaching with the rate measured for MB, whose absolute singlet oxygen formation is 0.50 (12). Determination of singlet oxygen yields were performed three times for each compound.

Partition coefficients were determined by the method of Pooler and Valenzeno (13). Each dye was dissolved in methanol and evaporated onto the surface of a flask. The resulting thin film of phenothiazine dye was dissolved by the addition of 5–25 mL of PBS-saturated 2-octanol to yield an OD in the 1.0–5.0 range. The resulting solution was passed through a 0.2 μm cellulose acetate filter and a small fraction of the total volume of the solution was diluted five-fold in methanol and analyzed spectrophotometrically for phenothiazine concentration. Under these conditions, only the monomer peak was observed. Appropriate volumes of 2-octanol-saturated PBS and octanol/dye solution was added to a volumetric flask and the mixture was vigorously agitated for 5 min and allowed to rest at room temperature for 30 min. The two phases were further clarified by centrifugation. The final concentration of the phenothiazine dye in 2-octanol was determined spectrophotometrically by five-fold dilution with methanol. The partition coefficient was determined according to the following equation:

$$P_C = \{[(OD_{Initial})/(OD_{Initial} - OD_{final})] - 1\}(V_{PBS}/V_{2\text{-octanol}}).$$

Partition coefficients were determined at least two times for each compound.

Dialysis

Two- and three-chamber dialysis experiments were performed using cellulose dialysis tubing (Sigma Diagnostics, St Louis, MO) that retained molecules with molecular weights greater than 12 000 Da. In order to reduce nonspecific binding of dyes to the cellulose, sacks were soaked in 1% glacial acetic acid for 1 hr with stirring, transferred to deionized water for 10 min and then incubated for 10 min in a 1% solution of Na_2CO_3 containing 1 m*M* ethylenediamine tetraacetic acid (EDTA). Sacks were then heated to 75°C in a 5% solution of Na_2CO_3 containing 1 m*M* EDTA for 25 min, incubated in deionized water for 15 min, soaked in doubly deionized water heated to 75°C for 15 min and finally washed with deionized water. Treated membranes were stored at 1–6°C in deionized water for up to 1 month before use (method of H. Morrison, personal communication). Treated dialysis tubing was cut to provide a rectangular membrane, which was then placed between two sheets of parafilm with an opening cut similar to the size of the opening of stackable polystyrene dialysis modules (PGC Scientific, Frederick, MD). Two or three modules were assembled using set bolts to construct two or three dialysis chambers. For two-chamber experiments, one chamber contained calf thymus double-stranded DNA (0.5 mg/mL, Sigma) in PBS and another chamber contained dye in PBS. In three-chamber experiments, 5% human serum albumin in PBS (diluted from 25% serum albumin, American Red Cross) and DNA (0.5 mg/mL, Sigma) in PBS were added to the two outer chambers and dye in PBS was added to the center chamber. The resulting dialysis systems were agitated on an orbital shaker (70 rpm) for 24 h at room temperature in the dark before dye absorption from each chamber was measured (14).

RESULTS

Photophysical and chemical properties

The λ_{max}, extinction coefficient (ϵ), $^1O_2$ quantum yield, partition coefficient (P_c) and oxygen dependence for inactivation of bacteriophage R17 for the four phenothiazines are shown in Table 1. All dyes absorb maximally between 649 to 657 nm, with extinction coefficients ranging from 90 000 to 101 000. None of the compounds appreciably inactivates bacteriophage ϕ6 in nitrogen-bubbled suspensions under conditions that inactivate 4–6 $\log_{10}$ of virus in air-bubbled suspensions. Singlet oxygen quantum yields range from 0.50 for MB to 0.76 for 1,9-dimethylmethylene blue. The partition coefficients of the dyes vary over a 10^5-fold range, with

Table 1. Photophysical and chemical properties of four phenothiazine dyes

Dye	λ_{max} (nm)*	ϵ*	ϕ^1O_2†	P_c‡	$\frac{\log_{10}N/N_0(N_2)}{\log_{10}N/N_0(O_2)}$
Methylene blue	651	101 000	0.50	0.11	0.06
1,9-Dimethylmethylene blue	649	90 000	0.76	4.12	0.02
Compound 6-136	651	98 300	0.62	18.4	0.01
Compound 4-140	657	98 900	0.59	3,560	0.05

*Measured in methanol to prevent dimerization.
†Singlet oxygen yields in methanol were determined relative to the published value for MB (12).
‡Partition coefficients were measured in 2-octanol : PBS.

compound 4-140 >> compound 6-136 > 1,9-dimethylmethylene blue > MB.

The results from two-chamber dialysis experiments with the four phenothiazine dyes and DNA are given in Fig. 2, which plots the OD of the tested compound from the DNA containing chamber *versus* the OD from the PBS chamber. The slope of each line is directly proportional to the affinity of the dye to DNA. 1,9-Dimethylmethylene blue binds to DNA the most avidly, with an affinity approximately 10 times that of MB and compound 4-140. Compound 6-136 is intermediate in its affinity for DNA. Similar results were obtained when experiments were conducted in ARC-8 (data not shown).

Table 2 gives the results of three-chamber dialysis experiments. The ranking of dyes most localized in the DNA chamber is: 1,9-dimethylmethylene blue ≈ MB > compound 6-136 > 4-140. In contrast, the order of phenothiazines most localized in the human serum albumin chamber is compound 4-140 >> compound 6-136 ≈ 1,9-dimethylmethylene blue > MB. The concentration of unbound dye (central dialysis chamber) in decreasing order is MB ≈ compound 6-136 >> 1,9-dimethylmethylene blue ≈ compound 4-140.

The spectrum of 1,9-dimethylmethylene blue is shown in Fig. 3 in the presence and absence of 0.5 mg/mL calf thymus DNA. In dilute aqueous 5 μM solutions without DNA, there are two absorption peaks at *ca* 599 and 649 nm (Fig. 3a). With increasing dye concentration (50 μM), the absorption peaks at 599 and 649 shift 4 and 8 nm to the blue and the ratio of the peak amplitude at *ca* 595 nm and 641 nm increases from 0.59 (dilute solutions) to 1.6 (concentrated solutions) (Fig. 3a). Based on the concentration-dependent changes of the absorption spectra, and the behavior of other dyes that form H-aggregates (15), the peaks at *ca* 599 and 649 nm are assigned as the dimer or higher-order aggregates and monomer forms, respectively. In the presence of 0.5 mg/mL of DNA, the concentration of monomer increases, the concentration of dimer decreases and the λ_{max} of the monomer peak is red-shifted approximately 10 nm (Fig. 3b).

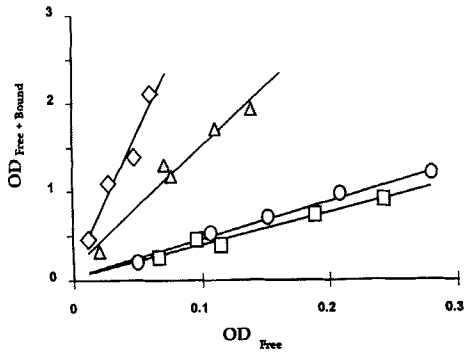

Figure 2. Equilibrium dialysis of phenothiazine dyes with DNA. Several concentrations of dyes were used in two-chamber dialysis experiments with 0.5 mg/mL calf thymus DNA. Squares, MB; circles, compound 4–140; triangles, compound 6–136; diamonds, 1,9-dimethylmethylene blue.

Virus inactivation

Results showing the inactivation of the nonenveloped bacteriophage, R17, in ARC-8 by the four phenothiazines are given in Fig. 4a. 1,9-Dimethylmethylene blue had the greatest virucidal activity followed by compound 6-136, MB and compound 4-140. Qualitatively similar results were obtained with the enveloped bacteriophage ϕ6, except compound 4-140 was considerately more active: 1,9-dimethylmethylene blue ≈ compound 4-140 > compound 6-136 > MB (Fig. 4b).

Because compound 4-140 displayed unexpected activity against enveloped viruses, the dye was compared with 1,9-dimethylmethylene blue for inactivation of enveloped viruses in red cell suspensions. Results with bacteriophage ϕ6 are given in Fig. 5. In contrast to the results obtained in ARC-8, no ϕ6 inactivation was observed using 4 μM compound 4-140 and 13.2 J/cm^2 light in the presence of red cells. However, 6.6. $\log_{10}$ ϕ6 was inactivated using 4 μM 1,9-dimethylmethylene blue and comparable fluences. Experiments using either 1,9-dimethylmethylene blue or compound 4-140 for photoinactivation of VSV in either plasma containing red cell suspensions or washed red cells gave qualitatively sim-

Table 2. Three-chamber dialysis experiments with phenothiazine dyes*

Dye	Serum albumin (%)	PBS (%)	DNA (%)
Methylene blue	29.6	19.6	50.8
1,9-Dimethylmethylene blue	42.0	4.9	53.1
Compound 6-136	44.5	18.9	36.6
Compound 4-140	76.1	6.7	17.2

*Values indicate the percentage of total dye found in each chamber after 24 h dialysis. The initial concentration of each of the phenothiazine dyes in the PBS (center) chamber was 30 μM.

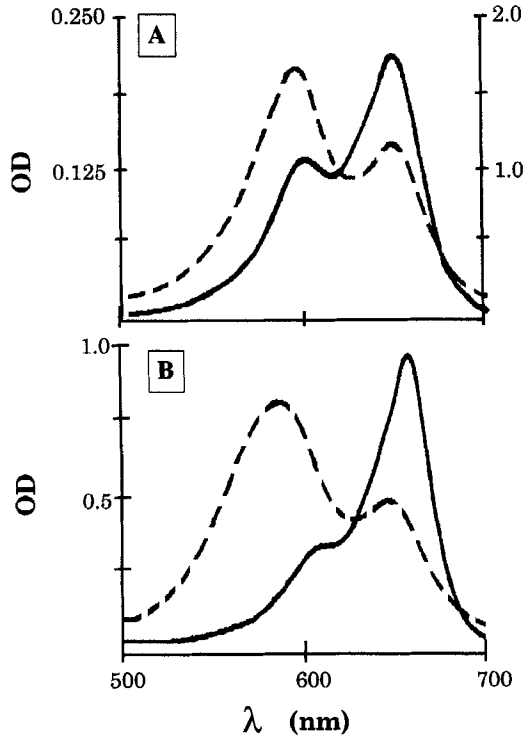

Figure 3. Spectra of aqueous solutions of dimethymethylene blue. (A) In distilled water without DNA; dashed line, 50 μ*M* 1,9-dimethylmethylene blue (0–2.0 OD scale); solid line, 5 μ*M* 1,9-dimethylmethylene blue (0–0.25 OD scale). (B) Dashed line, 30 μ*M* 1,9-dimethylmethylene blue in PBS; solid line, 30 μ*M* 1,9-dimethylmethylene blue and 0.5 mg/mL calf thymus DNA.

ilar results, with no inactivation observed with compound 4-140 under conditions that resulted in >4.4 $\log_{10}$ inactivation with 4 μ*M* 1,9-dimethylmethylene blue and 13.5 J/cm^2 light.

Figure 6 shows the irradiation wavelength dependence on bacteriophage ϕ6 inactivation by dimethymethylene blue using 660, 580 and 540 nm shortband light. The greatest extent of inactivation is observed with 660 nm light, which corresponds to light that can be absorbed primarily by the monomer form of the dye. Irradiation with 580 nm light, which predominantly is absorbed by the dimer form of the dye, results in less inactivation. No significant inactivation was observed using 540 nm light, which is neither absorbed by the dimer nor monomer. Qualitatively similar results on the irradiation wavelength dependence were obtained with the enveloped mammalian virus, VSV (data not shown).

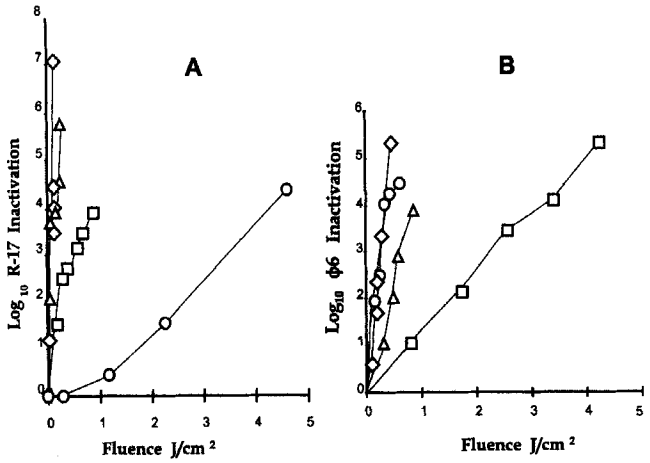

Figure 4. Photoinactivation of nonenveloped and enveloped bacteriophages by phenothiazine dyes. (A) Bacteriophage R17; (B) bacteriophage ϕ6. All experiments were conducted using 1 μ*M* of each dye in ARC-8. Squares, MB; circles, compound 4–140; triangles, compound 6–136; diamonds, 1,9-dimethylmethylene blue.

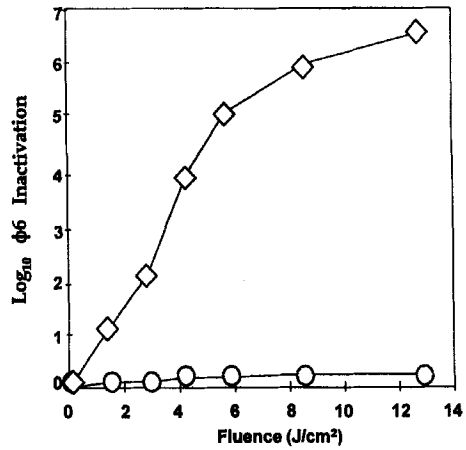

Figure 5. Photoinactivation of bacteriophage ϕ6 by 1,9-dimethylmethylene blue or compound 4–140 in red cell suspensions. Prior to the addition of virus and dye, cells were diluted to 50% Hct (0.5) in ARC-8, leukoreduced by filtration, and further diluted to 30% Hct (0.3) in ARC-8. Diamonds, 4 μ*M* 1,9-dimethylmethylene blue; circles, 4 μ*M* compound 4–140.

DISCUSSION

This study explores the basis for our previous observations of increased specificity of 1,9-dimethylmethylene blue for virus inactivation and low level of accompanying red cell damage by comparing photophysical and chemical properties of 1,9-dimethylmethylene blue and three other structurally related phenothiazines. The four compounds all shared several properties in common, such as red light absorption, similar extinction coefficients and the ability to produce singlet oxygen. In addition, all four compounds required oxygen for substantial bacteriophage ϕ6 inactivation.

The four compounds all have high but different singlet

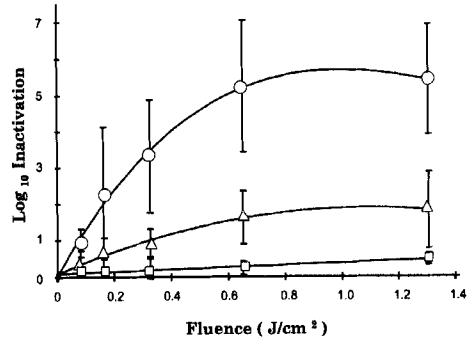

Figure 6. Wavelength dependence on bacteriophage ϕ6 inactivation by 1,9-dimethylmethylene blue. Experiments were conducted using 1 μ*M* of 1,9-dimethylmethylene blue in ARC-8. Circles, 660 nm bandpass filter; triangles, 580 nm bandpass filter; squares, 540 nm bandpass filter.

oxygen yields. Methylation of the phenothiazine ring resulted in a compound, 1,9-dimethylmethylene blue, with a singlet oxygen yield approximately 50% greater than that of MB. Increasing the N-alkyl chain length from all methyl groups (1,9-dimethylmethylene blue) to either methyl and ethyl (compound 6-136) or ethyl and butyl (compound 4-140) moieties reduced the singlet oxygen yield of the dimethylphenothiazine compounds from 0.76 to 0.62 to 0.59, respectively.

Based on two-chamber dialysis experiments (Fig. 2) 1,9-dimethylmethylene blue has an affinity for DNA that is roughly 10 times greater than that of MB. This increase is similar to the 2.6–11.8-fold increase observed when comparing the binding of angelicin and a series of dimethylangelicins to DNA (average fold increase, 7.0 ± 4.0; the increase in binding constant depends on the location of the methyl groups) (16,17). Similarly, the addition of three methyl groups to psoralen is reported to increase the binding constant of 4,5,8′-trimethylpsoralen to nucleic acid by roughly one order of magnitude (18). In contrast to the results obtained by ring methylation, the introduction of larger N-alkyl chains reduces affinity of the dyes for DNA. Larger N-alkyl moieties may sterically hinder DNA intercalation or prevent binding of the dyes to major or minor grooves. In addition, the bulky N-alkyl groups of compound 4-140 greatly increased dye hydrophobicity compared to 1,9-dimethylmethylene blue.

Comparison of the virucidal activity of the four compounds revealed that the extent of R17 inactivation was correlated with a compound's affinity for DNA, with dimethylmethylene blue having the greatest virucidal activity. Further experiments with bacteriophage ϕ6 revealed that the monomer, rather than the dimer form of the dye was, in large part, responsible for photoinactivation. Similar comparative inactivation experiments with the enveloped bacteriophage ϕ6 with the four dyes also revealed that DNA binding was associated with the extent of photoinactivation, except that compound 4-140 was unexpectedly active. Presumably, compound 4-140 may target viral membranes based on its hydrophobicity and low affinity for nucleic acid. The inhibition of compound 4-140 enveloped virus inactivation by washed red cell suspensions suggests that red cell membranes may compete with virus for binding of the dye.

One attractive approach for viral photoinactivation in red cell suspensions is to identify red light-absorbing sensitizers that target nucleic acid, because, unlike viruses, red cells do not contain DNA. The red light-absorbing phenothiazine, MB, served as a logical basis for synthesis of additional dyes because the drug has been used in medicine for the treatment of methemoglobinemia (19), can photoinactivate viruses in media (20,21), plasma (22,23) and red cells (2,3) and associates with DNA (24). Therefore, a series of novel phenothiazine compounds were screened for virus inactivation and potassium leakage from red cells. Based on preliminary screening experiments and subsequent additional studies, 1,9-dimethylmethylene blue appeared to have the greatest potential for virus inactivation with minimal alteration of normal red cell properties during refrigerated storage (6). The finding that 1,9-dimethylmethylene blue has the highest affinity for nucleic acid and the greatest singlet oxygen yield of several closely related compounds is consistent with the hypothesis that compounds that presumably target nucleic acids have the greatest specificity for viral inactivation in red cells. The ring methyl groups of 1,9-dimethylmethylene blue maintain water solubility yet make the compound more amphipathic, improve intracellular virucidal activity (6), maintain a planar structure, improve binding to nucleic acid and increase singlet oxygen yield compared to methylene blue. Analysis of additional compounds of similar structure may identify dyes with even greater specificity.

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