Biochemical Pharmacology, Vol. 51, pp. 1461–1467, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00087-1

Structurally Modified Trimethine Thiacarbocyanine Dyes

EFFECT OF *N*-ALKYL SUBSTITUENTS ON ANTINEOPLASTIC BEHAVIOR

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ABSTRACT. The effect of dye localization and dye distribution on the antineoplastic behavior of photosensitizers was investigated with a homologous series of trimethine thiacarbocyanine dyes in L1210 leukemia and A549 lung carcinoma cells. These dyes were synthesized with *N*-alkyl groups of different sizes (ethyl to octadecyl) to vary their lipophilic properties without compromising their photophysics. While dyes with smaller *N*-alkyl groups (ethyl to decyl) were already cytotoxic in the dark, longer chain cyanines exhibited antineoplastic activity only after exposure to light. Results from this study indicate that the switch from dark cytotoxicity to phototoxicity occurred when dyes, due to a decrease in cationic character with increasing size of alkyl substituents, were no longer able to cross the plasma membrane. Dark cytotoxicity decreased with increasing size of *N*-alkyl groups and was cell-line independent. On the other hand, photodynamic damage varied by several orders of magnitude depending on the cell line and the length of the alkyl substituents. The most effective photosensitizer was the dioctadecyl dye which achieved a 4- to 5-log reduction of leukemia cells, although it had very modest triplet and singlet oxygen quantum yields of 0.008 and 0.006, respectively. This study also showed that photobiological performance can be improved greatly by optimizing dye binding properties via structural modifications. BIOCHEM PHARMACOL 51;11:1461–1467, 1996.

KEY WORDS. cyanine dyes; cytotoxicity; phototoxicity; cancer cells; drug design

During the past decade, interest in cyanine dyes as potential photodynamic or photochemotherapeutic agents has been growing. MC540†, the most prominent sensitizer of this dye family, was found to effectively photosensitize the removal of leukemia, lymphoma, and neuroblastoma cells from bone marrow grafts while sparing hematopoietic stem cells [1]. MC540 is currently being studied in a phase I clinical trial for the extracorporeal photopurging of leukemia cells from autologous bone marrow grafts of patients with acute nonlymphoblastic and chronic myelogenous leukemia.

Most cyanine dyes, including MC540, have rather poor photosensitizing properties [2]. While cyanine dyes decay efficiently via fluorescence, internal conversion, and photoisomerication from the first excited singlet state to the ground state, intersystem crossing is only a minor deactivation pathway. As a consequence, triplet formation and production of cytotoxic singlet molecular oxygen are very poor. For example, MC540 in methanol has a triplet quantum yield of 0.04 [3] and a singlet oxygen quantum yield of 0.002 to 0.004 [4, 5]. Mechanistic studies, however, have shown that cellular photodamage by MC540 is due mainly to singlet molecular oxygen (Type II mechanism) [6, 7]. To explain the high antineoplastic activity of MC540 despite the low production of cytotoxic singlet oxygen, it has long been speculated that cellular dye localization and/or distribution may be important for the photobiological performance of a sensitizer.

To study the influence of cellular dye solubilization on the antineoplastic behavior of dyes, a homologous series of trimethine thiacarbocyanine dyes was synthesized. These photosensitizers were designed with *N*-alkyl chains of different lengths (ethyl to octadecyl) to gradually modify their lipophilic properties without compromising their photophysics. Previous studies in homogeneous and biomimetic media have confirmed that the photophysical properties of these dyes are not influenced by the size of the *N*-alkyl groups [8, 9]. The trimethine thiacarbocyanine chromophore, on the other hand, was selected because it not only produces sensitizers with the characteristic poor photosensitizing properties of a cyanine dye but also with photophysical properties similar to MC540.

This present work was performed in L1210 leukemia and A549 lung carcinoma cells. We found that the size of *N*-alkyl groups strongly influenced the antineoplastic behavior (dark cytotoxicity/phototoxicity) of these cyanine dyes. Furthermore, dye uptake studies and fluorescence quenching results with CPA suggest that different dye lo-

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† *Abbreviations:* MC540, merocyanine 540; and CPA, *cis*-parinaric acid.

Received 7 September 1995; accepted 21 December 1995.

calization sites and/or dye distribution patterns within the plasma membrane may be the cause of the observed large differences in cellular photodamage induced by long chain thiacarbocyanine dyes.

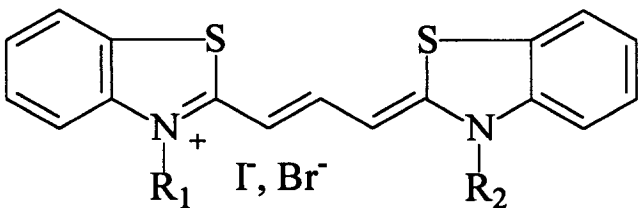
MATERIALS AND METHODS

Materials

The symmetric and asymmetric trimethine thiacarbocyanine dyes in Fig. 1 were prepared as previously described [8, 10, 11]. MC540 was purchased from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. L1210 murine lymphocytic leukemia cells (ATCC CCL 219) and A549 human lung carcinoma cells (ATCC CCL 185) were obtained from the American Type Culture Collection (Rockville, MD). Fetal bovine serum, sodium bicarbonate, glutaraldehyde and minimum essential alpha-medium were from Sigma, streptomycin/penicillin and trypsin/EDTA were from Irvine Scientific (Santa Ana, CA), and HEPES was from Research Organics (Cleveland, OH). Hanks' balanced salt solution was purchased from Life Technologies (Grand Island, NY), 200 proof ethanol was from the Quantum Chemical Corp. (Tuscola, IL), spectroscopic grade ethanol from the Aldrich Chemical Co. (Milwaukee, WI), CPA from Molecular Probes (Eugene, OR), methylcellulose from Fluka (Buchs, Switzerland), and Wright's stain and Giordano buffer were obtained from Ricca (Arlington, TX).

Absorption and Fluorescence

Absorption spectra were recorded on a Perkin Elmer λ4C spectrophotometer (Perkin Elmer, Norwal, CT). Corrected fluorescence emission spectra were measured using a 4800C SLM fluorescence spectrophotometer (SLM Instruments, Urbana, IL).



CY2 $R_1 = R_2 = C_2H_5$;
CY5 $R_1 = R_2 = C_5H_{11}$;
CY6 $R_1 = R_2 = C_6H_{13}$;
CY8 $R_1 = R_2 = C_8H_{17}$;
CY10 $R_1 = R_2 = C_{10}H_{21}$;
CY12 $R_1 = R_2 = C_{12}H_{25}$;
CY18 $R_1 = R_2 = C_{18}H_{37}$;
CY2,18 $R_1 = C_2H_5$; $R_2 = C_{18}H_{37}$;

FIG. 1. Chemical structures of trimethine thiacarbocyanine dyes with corresponding abbreviations.

Cells

L1210 leukemia cells were grown in bicarbonate-buffered alpha-medium supplemented with 10% fetal bovine serum. Cells were harvested in the exponential growth phase. A549 lung carcinoma cells were grown in bicarbonate-buffered alpha-medium containing 15% fetal bovine serum and 1% penicillin/streptomycin. Single cell suspensions of adherent cells were prepared by harvesting from subconfluent monolayers using trypsin/EDTA. Cells were suspended at a density of 1×10^6 cells/mL in HEPES-buffered (10 mM, pH 7.4) minimum essential alpha-medium containing 1% penicillin/streptomycin and supplemented with a 10% fetal bovine serum. Dyes were added from a freshly prepared (200 proof) ethanolic stock solution (final ethanol content: 2%).

For dark cytotoxicity experiments, dye-containing cells were incubated at room temperature without light for the specified time and then were washed once with HEPES-buffered alpha-medium containing 5% fetal bovine serum and 1% penicillin/streptomycin. Surviving cells were determined by *in vitro* clonal assays and by trypan blue exclusion.

For photoirradiation experiments, dye-containing cells were placed in clear polystyrene tubes (15 mm diameter, Corning Glass Works, Corning, NY), preincubated for the specified time in the dark at room temperature, and then irradiated as previously described [3, 12]. The relative fluence rate at the sample site was 27 ± 2 W/m^2. Relative fluence rates were determined by averaging several measurements taken at different sample sites using a calibrated radiometer from United Detector Technology (Hawthorne, CA). Cell suspensions exposed to dye in the dark, cell suspensions without dye exposed to light, and cell suspensions without dye kept in the dark served as controls. After irradiation, cells were washed twice with HEPES-buffered alpha-medium supplemented with 5% fetal bovine serum and then assayed for surviving clonogenic cells.

In Vitro *Clonal Assays*

Clonogenic assays of leukemia cells were performed as described [3, 13]. For A549 cells, a modified literature procedure for anchorage-dependent cells was used [14]: approximately 800 untreated or up to 160,000 treated cells were suspended in 5 mL alpha-medium supplemented with 20% fetal bovine serum containing 1% penicillin/streptomycin and were plated in 60-mm sterile polystyrene culture dishes (2 mm grid; Corning Glass Works). Cultures were incubated for 9–10 days at 37° in a humidified atmosphere of 5% CO_2 in air and then fixed using 0.2 mL of a 25% aqueous glutaraldehyde solution. Fixed cells were stained by adding 2 mL of Wright's stain followed by 2 mL of Giordano buffer. The stain was floated off with a stream of distilled water, the plates were air dried, and colonies were counted using an inverted microscope. The plating effi-

ciency of untreated cells was 60 ± 8% for L1210 cells and 36 ± 3% for A549 cells.

Bone Marrow Transplantation Experiments

Transplantation experiments were performed as previously described [13, 15]. Briefly, groups of 10 lethally irradiated mice were injected with 5×10^6 photosensitized or untreated syngeneic bone marrow cells and monitored for survival for ≥45 days.

Determination of Cellular Dye Concentrations

Dye-containing cells (2-mL samples) were incubated at room temperature in the dark for the specified time, washed once with HEPES-buffered alpha-medium containing 5% fetal bovine serum and 1% penicillin/streptomycin, resuspended in 3 mL ethanol (spectroscopic grade), and lysed by vortexing and sonication. Cell debris was removed by centrifugation. Dye concentrations in the ethanolic supernatant were determined by absorption spectroscopy using a molar extinction coefficient of $1.5 \times 10^5\ M^{-1}\ cm^{-1}$ [8].

Quenching of cis-Parinaric Acid

L1210 and A549 cells were suspended in 10% Hanks' balanced salt solution and incubated with CPA (100 μM) for 15 min at 37°C. Cells were washed once with Hanks' buffer to remove excess CPA and then resuspended in Dulbecco's phosphate-buffered saline (pH 7.4) at a density of 5×10^5 cells/mL. Dyes were added from an ethanolic stock solution as described (*vide supra*) to obtain dye concentrations between 0.2 and 18 μM. Fluorescence emission of CPA was measured at 418 nm using an excitation wavelength of 308 nm. Samples were lightly stirred, and temperature was controlled to 25 ± 1°.

RESULTS

Dark Cytotoxicity

Dark cytotoxicity induced by *N*-alkyl thiacarbocyanine was assessed by exposing all dyes depicted in Fig. 1 to L1210 leukemia cells and to A549 lung carcinoma cells. Dark cytotoxicity was found to be cell-line independent. Clonogenic assays showed an increase in dark cytotoxicity with decreasing size of *N*-alkyl groups and with increasing dye/cell preincubation periods (for a selection of data see Table 1). Short chain cyanines **(CY2, CY5, CY6)** exerted at 20 μM ≥5-log cell kill without requiring a preincubation period. Preincubation, on the other hand, played a role in the inactivation of neoplastic cells by thiacarbocyanines with medium-sized *N*-alkyl groups: while **CY8** eliminated 94% and **CY10** 3% of L1210 cells without preincubation (dye concentration: 20 μM), cellular inactivation increased to 99.2 and 95%, respectively, with a 45-min preincubation period. Dyes with long *N*-alkyl chains **(CY12, CY18)** were not dark cytotoxic, even when dark preincubation periods were extended to 90 min. Complete cellular inactivation (≥5-log cell kill) was also obtained with the asymmetric dye **CY2,18** at 20 μM in the absence of a preincubation procedure. For all cyanine dyes with short to medium size *N*-alkyl groups, dark cytotoxicity was concentration dependent (Fig. 2), and the rank order in cytotoxicity remained always the same. For example, **CY10** at ≤5 μM was no longer cytotoxic (45 min preincubated), while **CY2** concentrations had to be lowered to 1 μM to achieve at least partial, i.e. 22%, cell survival. The determination of cell survival by trypan blue exclusion produced he same cytotoxic pattern as clonogenic assays. Trypan blue exclusion experiments, however, were less sensitive, and they underestimated the cytotoxic effects. This observation has been reported previously [16].

Phototoxicity

Phototoxicity studies were limited to the long-chain dyes **CY12** and **CY18** because dyes with smaller *N*-alkyl groups were dark cytotoxic (*vide supra*). In contrast to the previously described dark cytotoxic behavior, cellular photodamage was cell-line sensitive.

In L1210 leukemia cells, both **CY12** and **CY18** induced light-mediated cellular damage. Dye **CY18** easily removed >4 logs of leukemia cells and was at least as effective as MC540 under the same experimental conditions (Fig. 3). On the other hand, **CY12** with a ≤2-log removal of L1210 cells was significantly less effective than **CY18** (Fig. 3). Figure 3 also shows the concentration dependence of the phototoxic effect: L1210 photodamage decreased from 99.996 to 45% when **CY18** concentrations were lowered

TABLE 1. Effect of (dark) preincubation time on cellular toxicity and cellular dye uptake in L1210 luekemia cells (20 μM total dye concentration, 1×10^6 cells/mL)

Incubation time (min)	Dark toxicity (surviving fraction)				Dye bound to cells (fraction of administered dye)			
	CY2	CY10	CY18	CY18, no serum	CY2	CY10	CY18	CY18, no serum
0	0	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.16 ± 0.04	0.01 ± 0.003	0.01 ± 0.004	0.28 ± 0.07
45	0	0.05 ± 0.04	1.0 ± 0.1	0.7 ± 0.1	0.26 ± 0.03	0.03 ± 0.004	0.02 ± 0.006	0.36 ± 0.02
90	0	$(4 \pm 2) \times 10^{-5}$	1.0 ± 0.1	0.7 ± 0.1	0.31 ± 0.05	0.05 ± 0.005	0.02 ± 0.004	0.38 ± 0.05

Serum content was 10%, unless otherwise indicated. Values are means ± SD and were obtained from ≥2 independent experiments with N ≥ 8 (dark cytotoxicity) or N ≥ 4 (dye binding).

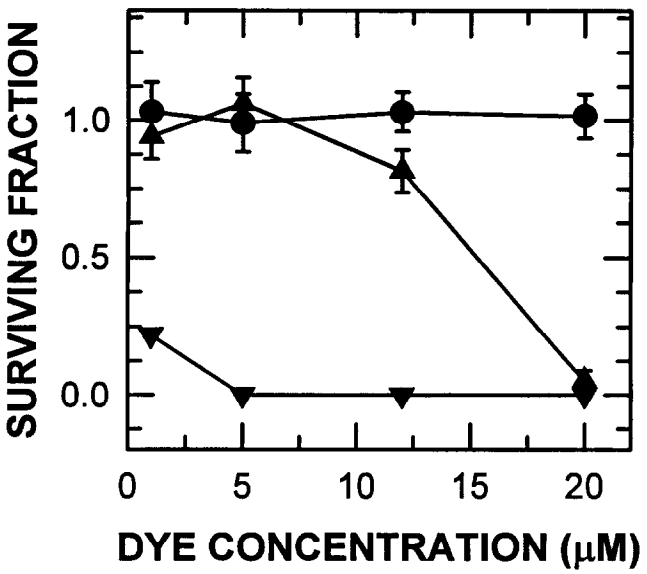


FIG. 2. Effect of dye concentration on dark cytotoxicity in L1210 cells. The preincubation period was 45 min and the cellular concentration was 1 × 10^6 cells/mL. Data points represent mean colony counts of four culture dishes ± SD. The relative fluence rate was 27 ± 2 W/m^2. Key: (▼) CY2; (▲) CY10; and (●) CY18.

from 20 to 5 μM. The photosensitized inactivation of L1210 cells by **CY12** and **CY18** exhibited a biphasic behavior. This lack of first-order kinetics was attributed to the self-sensitized photodecomposition of these dyes (photobleaching) with increasing illumination time. After 90 min of photoirradiation, cellular suspensions no longer contained any dye (as judged by absorption spectrophotometry).

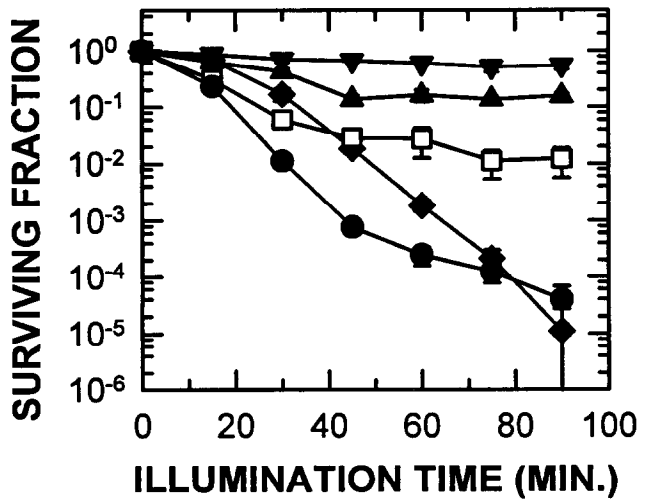


FIG. 3. Representative examples of dye-sensitized photoinactivation of L1210 leukemia cells. The dark preincubation time was 45 min, and the cellular concentration was 1 × 10^6 cells/mL. Data points represent mean colony counts of four culture dishes ± SD. The relative fluence rate was 27 ± 2 W/m^2. Key: (●) CY18, 20 μM; (▲) CY18, 10 μM; (▼) CY18, 5 μM; (□) CY12, 20 μM; and (♦) MC540, 20 μM.

L1210 cellular photoinactivation was affected by the duration of the dark preincubation period before light exposure occurred. As shown in Fig. 4, a period of at least 30 min of dark preincubation at room temperature was necessary to reach maximum photoinactivation capabilities.

Photodamage to leukemia cells sensitized by **CY12** and **CY18** dyes was oxygen dependent (Fig. 5). The photoinactivation of L1210 cells was prevented when photoirradiation occurred in an oxygen-deprived (argon-saturated) environment. The onset of photodamage after 20 min of photoirradiation is attributed to diffusion of oxygen into the polystyrene tubes. On the other hand, increasing oxygen concentration by bubbling air-saturated cellular suspensions with oxygen did not enhance the inactivation of L1210 cells.

In contrast to leukemia cells, A549 lung carcinoma cells were only moderately sensitive to **CY18** and **CY12** photosensitization, e.g. when A549 cells were exposed to light for 90 min at 27 ± 2 W/m^2 (20 μM; 1 × 10^6 cells/mL; 30 min preincubated in the dark), only 60% of the cells were destroyed. Under the same experimental conditions, **CY12** eliminated 98.8% and **CY18** 99.996% of L1210 leukemia cells.

Hematopoietic stem cells were also much less susceptible to **CY18**-mediated photosensitization than L1210 leukemia cells, as shown by murine bone marrow transplantation experiments. Lethally irradiated mice always survived a 45-day observation period whether they were transplanted with 5 × 10^6 untreated or 5 × 10^6 photosensitized syngeneic bone marrow cells (20 μM **CY18**, preincubated 60 min in the dark, irradiated 90 min at 27 ± 2 W/m^2). All animals of the control group receiving no transplant died due to marrow failure (median survival time 12–13 days). From more elaborate bone marrow transplantation experiments per-

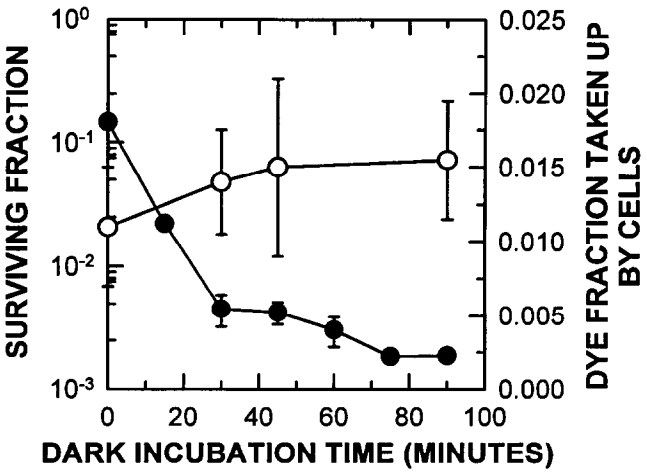


FIG. 4. Effect of dark preincubation time on CY18-sensitized photoinactivation of L1210 cells and on CY18 uptake by L1210 cells. The dye concentration was 20 μM, and the cell concentration was 1 × 10^6 cells/mL. Key: (●) L1210 cells surviving after exposure to 30 min of visible light at a relative fluence rate of 27 ± 2 W/m^2; and (○) fraction of administered dye bound to L1210 cells.

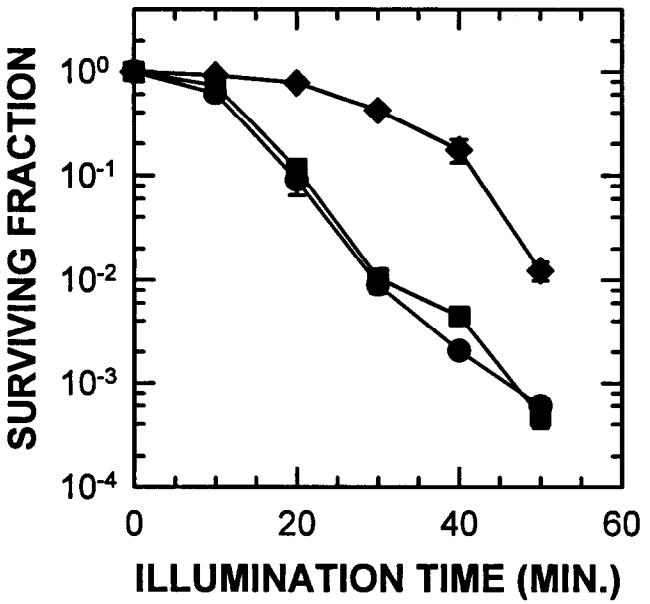


FIG. 5. Representative example of CY18-sensitized photoinactivation of L1210 cells in medium saturated with argon (♦), air (●), or oxygen (■). The dye concentration was 20 μM, and the cellular concentration was 1 × 10^6 cells/mL. Dye and L1210 cells were first incubated for 30 min in the dark and then saturated with argon, oxygen, or air. Data points represent mean colony counts of four culture dishes ± SD. The relative fluence rate was 27 ± 2 W/m^2.

formed under the same experimental conditions and using serial dilutions of syngeneic bone marrow cells [15], the photodamage of **CY18** to normal hematopoietic stem cells was estimated to be ≤2-log reduction under conditions where 4–5 logs of L1210 leukemia cells were eliminated.

Cellular Dye Uptake

To evaluate the effect of cellular dye concentration on dark- and light-induced cytotoxicities, the amount of dye bound to cells was determined. Cellular dye binding was neither cell-line sensitive nor influenced by exposure of cells/dye to short periods (30 sec) of visible light. In contrast to MC540, an equilibrium between cells and medium did not exist as cellular dye accumulation remained unaffected by repeated washes or by temperature (temperature range tested: 0–37°), except for **CY2.** The increase in cellular bound **CY2** with temperature is attributed to the fast disintegration of cells after being exposed to this dye (as observed by trypan blue exclusion).

Cellular dye binding increased with decreasing size of the *N*-alkyl groups. For example, at zero incubation time the fraction of dye taken up by L1210 cells increased from 0.01 for dyes **CY18** (±0.04), **CY12** (±0.001), and **CY10** (±0.003) to 0.05 ± 0.005 for **CY8,** to 0.07 ± 0.014 for **CY6,** to 0.06 ± 0.004 for **CY5,** and to 0.16 ± 0.04 for dye **CY2** (see also Table 1). Furthermore, dye binding also increased by increasing dark preincubation periods (Table 1, Fig. 4). While the amount of dye bound by cells was dose dependent, the dye-specific cell bound dye-fractions remained constant within the measured concentration range of 5–20 μM. Both long-chain dyes **CY12** and **CY18** had, within error limits, the same small cell bound dye-fraction of 0.01 to 0.02 for preincubation periods of 0–90 min.

Cellular dye binding was also strongly dependent on the serum content of the medium. Dye uptake increased dramatically when serum was omitted (Table 1): the fraction of **CY18** bound to L1210 cells increased from 0.015 to 0.025 when the serum content was reduced from 10 to 5%, and it further increased to 0.4 in a serum-free environment (30 min of preincubation).

Quenching of cis-Parinaric Acid

cis-Parinaric acid is a fluorescent fatty acid that is readily incorporated into the plasma membrane of intact cells [17, 18]. Since its structure is very similar to naturally occurring fatty acids, membrane perturbations are believed to be minimal [17]. When CPA-labeled L1210 and A549 cells were exposed to **CY12** and **CY18,** the fluorescence emission of plasma membrane-bound CPA was quenched by these dyes according to the Stern–Volmer relation. Figure 6 shows typical Stern–Volmer plots with intercepts around unity and Stern–Volmer coefficients ($k_q\tau_f$) of (1.67 ± 0.13) × 10^6 M^{-1} for **CY18** in L1210 cells, (9.79 ± 0.45) × 10^5 M^{-1} for **CY12** in L1210 cells, and (2.40 ± 0.31) × 10^5 M^{-1} for **CY18** in A549 cells. Since overall (total sample volume) and not local (plasma membrane volume) dye con-

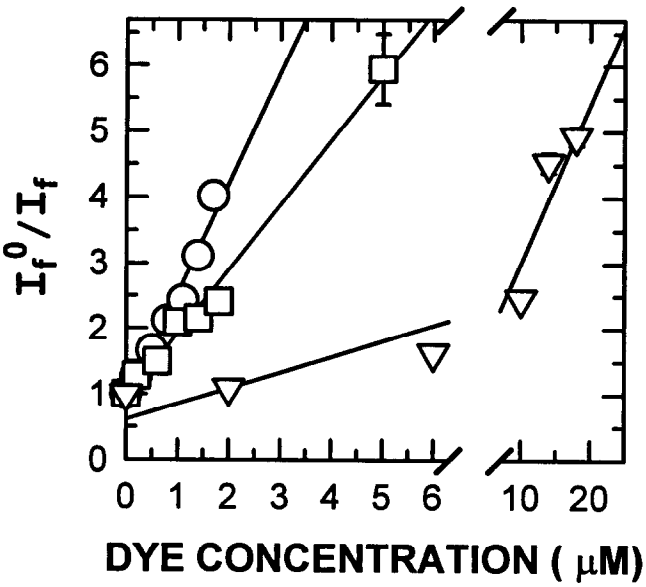


FIG. 6. Fluorescence quenching of membrane bound *cis*-parinaric acid by thiacarbocyanine dyes (Stern–Volmer plot). The *cis*-parinaric acid concentration was 100 μM, and the cellular concentration was 5 × 10^5 cells/mL. Key: (○) CY18 in L1210 leukemia cells, I_f^0/I_f = (0.87 ± 0.13) + (1.67 ± 0.13) × 10^6 M^{-1} [CY18]; (□) CY12 in L1210 cells, I_f^0/I_f = (0.94 ± 0.10) + (9.79 ± 0.45) × 10^5 M^{-1} [CY12]; and (▽) CY18 in A549 lung carcinoma cells, I_f^0/I_f = (0.61 ± 0.33) + (2.40 ± 0.31) × 10^5 M^{-1} [CY18].

centrations were used for these calculations, Stern–Volmer coefficients were larger than generally found in homogeneous solution for dynamic fluorescence quenching. Assuming a fluorescence lifetime τ_f for CPA of ~20 nsec [19] and using a diffusion-controlled rate constant of 3×10^{10} M^{-1} sec^{-1} as an upper limit for the quenching constant k_q, local cellular dye concentrations were at least 300–3000 times higher than the overall concentrations. Similar local dye concentrations were observed previously in synthetic membrane model systems [20, 21].

DISCUSSION

This homologous series of lipophilic cationic thiacarbocyanine dyes exhibited a wide range of dark cytotoxicity ranging from no damage to complete (≥5-log reduction) cellular inactivation. Dark cytotoxicity correlated with the size of both *N*-alkyl substituents. This result was surprising in view of previous studies in unilamellar dimyristoyl phosphatidylcholine vesicles where all dyes within this series (exception: **CY2**) were shown to solubilize near the bilayer surface [9]. On the other hand, the reported results are in agreement with recently published data on cytotoxic effects of short-chain trimethine thiacarbocyanine dyes on human colon cancer cells [22]. These studies, though limited to dyes with *N*-alkyl groups between methyl and pentyl, reported the same relationship between the size of side chains and the dark cytotoxicity of the dyes.

The absence of dark cytotoxicity for long-chain dyes might, at first, be attributed to the very small amount of **CY12** or **CY18** actually bound to cells. However, studies in the absence of serum, where cellular dye uptake is much higher than in the presence of serum, disprove this view. Although L1210 cells in a serum-free environment bound 25–75% more **CY18** than **CY2** in the presence of 10% serum (Table 1), **CY18** induced under these conditions only minimal dark toxicity, i.e. 20–30% leukemia cells were eliminated compared to a removal of >99.999% of cells by **CY2.** This result suggests that long-chain thiacarbocyanine dyes interact differently than analogue small to medium size dyes with neoplastic cells.

Fluorescence quenching experiments with *cis*-parinaric acid identified the plasma membrane as the cellular localization site of **CY12** and **CY18** in both L1210 leukemia and A549 lung cancer cells. Since intercepts around unity were obtained when I_f^0/I_f was plotted versus the overall (total sample volume) dye concentration (Fig. 6), it can be concluded that these dyes were exclusively bound in the plasma membrane. Furthermore, **CY12** and **CY18** not only had similar Stern–Volmer coefficients ($k_q\tau_f$) of (1–2) × 10^6 M^{-1} in L1210 cells, but also identical dye uptake data. Therefore, the >2-log difference in phototoxicity between these two dyes must be attributed either to different dye localization sites and/or different dye distribution patterns within the plasma membrane. Although this phenomenon may also be active in A549 cells, the absence of cellular photodamage in these cells could alternatively be explained with a differential sensitivity of this tumor cell line towards **CY12** and **CY18.**

The exclusive solubilization of **CY12** and **CY18** in the plasma membrane was not expected since cationic dyes do preferentially localize in mitochondria [23–26]. The driving force for this mitochondrial uptake is the net positive charge of the dyes in combination with both plasma and mitochondrial membrane potentials [23]. From this point of view it is conceivable that membrane crossings become more difficult as *N*-alkyl chains increase in size: the lipophilic—and uncharged—side groups become more dominant and are directly competing with the positively charged chromophoric system. This provides a reasonable explanation not only for the exclusive localization of dyes **CY12** and **CY18** in the plasma membrane but also for the observed correlation between chain length, (dark) antineoplastic activity, and cellular dye uptake. On the other hand, the dark cytotoxicity results of the asymmetric dye **CY2,18** seem to be inconsistent with this interpretation. However, it should be taken into account that the positive charge resides with the same probability on the nitrogen carrying the octadecyl group as on the nitrogen bearing the ethyl group. Consequently, the asymmetric dye is more cationic in character than the analogue symmetric dialkyl dye with the same total number of carbons in the alkyl groups, i.e. **CY10,** and hence also more dark cytotoxic.

We previously reported that symmetric trimethine carbocyanine analogues showed a correlation between triplet quantum yields (measured in solution) and photobiological activity [8, 9, 27, *]. This finding and the observation that **CY12** and **CY18** induced photodamage only in the presence of molecular oxygen suggest the involvement of a Type II (singlet molecular oxygen) mechanism. It is therefore surprising that dyes with such unfavorable photosensitizing properties, in particular **CY18** with a triplet quantum yield of 0.008 and a singlet oxygen quantum yield of 0.006 (liposomes) [9], was able to induce such extensive cellular photodamage. In addition, rather a small overall cellular dye concentration was necessary to achieve a 4- to 5-log reduction in L1210 leukemia cells, i.e. for **CY18** approximately 0.2 μM (1% of 20 μM). On the other hand, it should be remembered that local dye concentrations are much larger: according to the CPA fluorescence quenching data (see Results), a 0.2 μM **CY18** concentration in L1210 cells translates into a local dye concentration of ≥600 μM.

This study shows that photodynamic damage may vary several orders of magnitude depending on the cellular localization and/or distribution of dyes. Consequently, dyes with rather poor photophysical properties, such as **CY18** or MC540, can produce significant photobiological damage when properly solubilized. Although the photophysics of a

* Krieg M and Bilitz JM, Cyanine dyes in photodynamic therapy: Photosensitization and structure–activity relationships. *15th IUPAC Symposium on Photochemistry*, 91, 1994.

dye are instrumental for the biological performance of a photosensitizer, cellular localization and/or distribution may ultimately decide on the photobiological effectiveness of a dye. However, this study also shows that the solubilization of dyes in cells and, therefore, antineoplastic activity may be controlled by proper chemical modifications. Furthermore, cellular binding properties and photophysical characteristics can be modified and optimized independently.

The octadecyl analogue **CY18,** with its high antileukemic activity and low hematopoietic stem cell toxicity, is currently evaluated as a possible bone marrow photopurging agent. On the other hand, trimethine carbocyanine dyes with medium to short alkyl-chains may not be suitable as antineoplastic agents as their dark cytotoxic behavior lacks selectivity.

This study was supported by Grant CA50733 from the National Cancer Institute and the MACC Fund.

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