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Photochemically Generated Elemental Selenium Forms Conjugates with Serum Proteins that Are Preferentially Cytotoxic to Leukemia and Selected Solid Tumor Cells

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Abstract

The objective of this study was to determine if and how photoproducts contribute to the anti-tumor effect of merocyanine-mediated PDT. A panel of barbituric, thiobarbituric and selenobarbituric acid analogues of Merocyanine 540 was photobleached, and the resulting photoproducts were characterized by absorption, fluorescence emission, mass, energy dispersive X-ray, and X-ray photoelectron spectroscopy, and tested for cytotoxic activity against tumor cell lines and freshly explanted bone marrow cells. While all dyes were readily photobleached, only photoproducts of selone dyes showed cytotoxic activity. One-hour incubations with micromolar concentrations of selone-derived photoproducts were sufficient to reduce leukemia/lymphoma cells $\geq 10,000$ fold while preserving virtually all normal CD34-positive bone marrow cells. Of 6 multi-drug resistant tumor cell lines tested, 5 were as sensitive or more sensitive to photoproducts than the corresponding wild-type lines. Physicochemical characterizations of the cytotoxic activity indicated that it consisted of conjugates of subnano particles of elemental selenium and (lipo)proteins. The discovery of cytotoxic Se-protein conjugates provides a rare example of photoproducts contributing substantially to the anti-tumor effect of PDT and challenges the long-held view that Se in oxidation state zero is biologically inert. Agents modeled after our Se-protein conjugates may prove useful for the treatment of leukemia.

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INTRODUCTION

Photosensitizing selenomercocyanine dyes were first synthesized by Günther *et al.* (1, 2) as part of an effort to define structure-activity relationships in merocyanine-mediated photodynamic therapy (PDT) and to develop more efficient and more selective photosensitizers for the ex vivo purging of autologous hematopoietic stem cells grafts and the inactivation of viruses in blood components (3–11). Three structural modifications of the prototype dye, Merocyanine 540 (MC540) were found to have major beneficial effects. **1)** Replacing oxygen (O) in the donor heterocycle by sulfur (S) or selenium (Se) made dyes resistant to degradation by plasma and serum (12). **2)** Expanding the back ring from benzene to naphthalene improved cytotoxic and virucidal activity by enhancing the dyes' affinity for cellular and viral binding sites (1, 2). **3)** Replacing S by Se at the 2-position of the barbiturate improved singlet oxygen quantum yields by almost 2 orders of magnitude and dramatically enhanced cytotoxic and virucidal activity (1, 2).

Initially, all data obtained with second-generation merocyanines seemed to support the notion that all dyes killed target cells by the same mechanism: singlet oxygen-mediated oxidative damage to the plasma membrane (13, 14). The high singlet oxygen quantum yield of selenobarbituric acid analogues offered a plausible explanation for the improved cytotoxic activity of selone dyes. Only when thione and selone dyes were compared at different temperatures did it become obvious that there was a qualitative difference between the cytotoxic mechanisms of selone and thione dyes. While thione dyes were always more effective at 5 °C than at room temperature (because cellular defense mechanisms were impaired at low temperatures (15), selone dyes always performed better at room temperature.

Like many Type II photosensitizers, merocyanine dyes are substrates of the singlet oxygen they generate. When exposed to light in the presence of oxygen, they are oxidized ('photobleached') and converted to so-called photoproducts. When MC540 or its barbituric and thiobarbituric acid analogues are photobleached under typical PDT conditions, they form photoproducts that are not cytotoxic. The same appears to be true for most non-merocyanine photosensitizers. As we report here, selenobarbituric acid analogues of MC540 are rare exceptions. They generate a photoproduct that is highly cytotoxic if allowed to combine with certain proteins or lipoproteins.

This communication reports on 1) an initial physical, chemical, and biological characterization of selenomercocyanine-derived photoproducts, 2) the surprise finding that the cytotoxic photoproduct appears to be Se in oxidation state zero, and 3) an initial preclinical evaluation of photochemically generated Se-protein conjugates as potential anti-cancer agents.

MATERIALS AND METHODS

Materials

Structural analogues of MC540 (Fig. 1) were synthesized as described by Günther *et al.* (1) and labeled MC1 through MC57. The nomenclature reflects the order in which analogues were tested in our laboratory. It has no structural or functional connotations. The bis-(1,3-dibutyl selenobarbituric acid) trimethine oxonol dye was synthesized as described by Krieg *et al.* (16). The purity of dyes was assessed by thin layer chromatography, absorption and fluorescence emission spectroscopy, and elemental analysis. Purity was $\geq 95\%$ for selone dyes and $\geq 98\%$ for thione and oxone dyes. The lower purity estimate for selone dyes primarily reflects the relatively large margin of error in the elemental analysis of Se. Merocyanine 540 (MC540) was from Eastman Kodak (Rochester, NY), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid hemisodium salt (HEPES) from Research Organics (Cleveland, OH), methylcellulose (4000 cPs) from Fluka (Buchs, Switzerland), recombinant granulocyte/macrophage colony stimulating factor (murine or human sequence) from R&D Systems, Minneapolis, MN), FITC anti mouse CD34 antibody and FITC rat IgG2a from Serotec (Raleigh, NC), and FITC anti human CD34 antibody and FITC mouse IgG1^K from BD Biosciences Pharmingen (San Diego, CA). Albumin-depleted fetal bovine serum was prepared by affinity chromatography of fetal bovine serum on Affi Gel Blue (Bio-Rad, Hercules, CA). All other reagents were from Sigma (St. Louis, MO).

Cells

Wild-type L1210 and P388 murine leukemia, K562 human leukemia, Daudi human lymphoma, MCF7 human breast cancer, D283 human medulloblastoma, and SK-ES-1 human Ewing's sarcoma cells were obtained from the American Type Culture Collection (Manassas, VA). Wild-type HL-60 human leukemia cells were a gift from Dr. Robert C. Gallo. Adriamycin-resistant HL-60/ADR cells (drug resistance based on MRP-mediated drug efflux (17)) were a gift from Dr. Melvin S. Center. Wild-type H69 human small cell lung cancer cells and cisplatin-resistant mutant H69/CDDP cells (characterized by elevated glutathione-S-transferase- π , metallothionein, and glutathione (18)) were gifts from Dr. Nagahiro Saijo. Melphalan-resistant mutant L1210/L-PAM₁ and L1210/L-PAM₂ murine leukemia cells (characterized by elevated levels of intracellular glutathione (19)) were gifts from Dr. David T. Vistica. Adriamycin-resistant P388/ADR murine leukemia cells (overexpression of P-glycoprotein) were a gift from Dr. Merrill J. Egorin. The B1 human neuroblastoma cell line was a gift from Drs. Joel Shilyansky and James T. Casper. Wild-type PC14 and cisplatin-resistant PC14/CDDP lung adenocarcinoma cells (drug resistance based on reduced drug uptake (20)) were supplied by one of the authors (I.T.). All cell lines were propagated in alpha-modified Dulbecco's medium (alpha-medium) supplemented with 10% (L1210, L1210/L-PAM₁, L1210/L-PAM₂, P388, P388/ADR, PC14, PC14/CDDP, MCF7, B1, D283, H69, H69/CDDP, and SK-ES-1) or 20% (Daudi, HL-60, HL-60/ADR) fetal bovine serum. The medium used for mass cultures of melphalan-resistant mutant L1210 leukemia cells contained melphalan (2.5 $\mu\text{g/ml}$) and 2-mercaptoethanol (50 μM). Cells were, however, transferred to melphalan-free medium 24 hours before being harvested for in vitro cytotoxicity experiments. All cells used for cytotoxicity experiment were harvested in exponential growth phase.

Normal murine bone marrow cells were obtained from female C57BL/6 × DBA/2 F1 mice (6–24 weeks old; Jackson Laboratory, Bar Harbor, ME or Taconic, Germantown, NY) under protocols approved by the institutional animal care and use committee. Mouse bone marrow cells used for the analysis of CD34-positive cells were enriched for mononuclear cells by density centrifugation on Histopaque. Normal human bone marrow cells (mononuclear cell fraction) were obtained from Poietic BioWhittaker (Gaithersburg, MD) or from healthy volunteer donors for allogeneic bone marrow transplants after informed consent had been obtained under protocols approved by the institutional review board.

Photoproducts

Unless indicated otherwise, photoproducts were prepared in alpha-medium supplemented with 12% fetal bovine serum. The appropriate merocyanine or oxonol dye was added as a freshly prepared 1.76 mM stock solution in ethanol to the desired final concentration (typically $\leq 26 \mu\text{M}$). Dilute dye solutions were placed into clear 15-mL polybutadiene styrene tubes (Nunc, Naperville, IL) that rotated at approximately 30 rpm between two banks of tubular fluorescent lights (5 lights per bank; F20T12.CW; General Electric, Cleveland, OH) for 60 min. The fluence rate at the sample site was 27 W/m^2 as determined by a model S351A power meter (United Detector Technology, Hawthorne, CA) equipped with a model 262 detector and a radiometric filter number 1158. When dye concentrations in excess of $26 \mu\text{M}$ were required, dye was added in two or more increments ($\leq 26 \mu\text{M}$) spaced 60 min apart. Illumination times were increased proportionally to ensure adequate photobleaching.

The selenomercyanine dye MC54 (Fig. 1) was the preferred source of photoproducts for the majority of experiments because in addition to the cytotoxic photoproduct, it also generated a fluorescent photoproduct that provided the basis of a surrogate assay for the binding/uptake of cytotoxic photoproduct by target cells. Furthermore, as an agent for PDT, MC54 had been studied more extensively than other selone dyes because it offered the best combination of antineoplastic activity, stability, and solubility in water. While MC54 generated less cytotoxic activity than the selenooxonol dye, it was easier to synthesize with good yield and good purity.

If the main objective of an experiment was a spectrophotometric analysis of photoproducts, HEPES buffer (10 mM, pH 7.4) was used instead of alpha-medium to avoid interference by colored components of the tissue culture medium. For selected experiments, MC54 was photobleached in a custom built (AgSpace Technologies International, Cross Plains, WI) light source equipped with two parallel arrays of eight light emitting diodes (LEDs) having a peak emission at 612 nm (Toshiba Ultra Bright LED, Model TLOH156P; Toshiba Corp., Tokyo, Japan; operated at 21 V and 40 mA) and two separately operated parallel arrays of eight LEDs having a peak emission at 574 nm (Toshiba Ultra Bright LED, Model TLGE158P; operated at 24 V and 40 mA).

Cytotoxicity assays

The cytotoxic effect of merocyanine- and oxonol-derived photoproducts was assessed by in vitro clonal assay as described previously (3, 5). Control cells were incubated and washed with medium that contained the appropriate concentration of serum and vehicle but no dye or photoproducts. All incubations with photoproducts were performed in the dark to rule out photochemical processes. In a few experiments, cytotoxicity was assessed by trypan blue exclusion or by staining with propidium iodide ($1 \mu\text{g/mL}$) followed by flow cytometric analysis.

Flow cytometric analyses

Flow cytometric analyses were performed as described previously using a FACScan (Becton-Dickinson, Mountainview, CA) flow cytometer equipped with an argon-ion laser (15 mW, 488 nm line) (21, 22). The fluorescence emission of cell-associated photoproduct-albumin conjugates or cell-associated FITC anti-CD34-antibodies was measured through a 525/30 nm bandpass filter and expressed in arbitrary units (channel number) on a logarithmic scale. A 584/42 nm bandpass filter was used for the identification of propidium iodide-positive cells. Data were analyzed using the CellQuest (Becton-Dickinson) software package (21).

Absorption and fluorescence spectroscopy

Ground state absorption spectra were recorded with a Perkin-Elmer (Norwalk, CT) Lambda 4C UV/VIS spectrophotometer (modified for the analysis of turbid samples) or a Hitachi (Tokyo, Japan) U-3310 UV/VIS spectrophotometer. Corrected steady-state fluorescence emission spectra were recorded with a model LS-50 (Perkin-Elmer) or a model F-4500 (Hitachi) fluorescence spectrophotometer.

Mass spectroscopy, inductively coupled plasma mass spectroscopy, X-ray photoelectron spectroscopy, and scanning electron microscopy

For the analysis of photoproducts, electrospray ionization spectrometry was performed by M-Scan Inc. (West Chester, PA) using a VG Bio-Q (VG Biotech/Fisons, Altrincham, UK) instrument with quadrupole analyzer. Cesium iodide was used to calibrate the instrument. Sample aliquots of 30 μ l were injected into the instrument source and eluted with 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 10 μ L/min. A Sciex Q-Star/Pulsar ESI-MS/MS instrument (Perkin Elmer, Norwalk, CT) was used for attempts to perform negative ion electrospray ionization spectrometry of albumin-stabilized elemental selenium. Samples were diluted in 50% acetonitrile, and 1- μ l sample aliquots were introduced via a nanospray needle. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed by the Center for Chemical Characterization and Analysis at Texas A & M University (College Station, TX). X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) were performed by Materials and Chemistry Laboratory, Inc. (Oak Ridge, TN) using two instruments, a JEOL (Tokyo, Japan) 840 scanning electron microscope equipped with a KeveX (San Carlos, CA) EDS system and a Hitachi (Tokyo, Japan) S-5000 scanning electron microscope equipped with a Noran (Middleton, WI) Voyager EDS system. Samples that were to be analyzed with the JEOL 840 instrument were deposited on carbon tape and coated with carbon and gold. Samples that were to be analyzed with the Hitachi S-5000 instrument were deposited on beryllium slabs and left uncoated. The Hitachi S-5000 instrument was equipped with a backscatter electron detector. Heavy atoms backscatter electrons more strongly than light atoms, which make Se appear brighter than calcium, oxygen, or carbon on backscatter images.

RESULTS

Cytotoxic activity of photobleached dyes

To screen photosensitizing dyes for their capacity to generate cytotoxic photoproducts, MC540, 13 structural analogues selected from Günther's (1) series of second-generation merocyanine dyes, and one selenooxonol dye (Fig. 1) were dissolved in serum-supplemented alpha-medium and photobleached by exposure to cool white fluorescent light. L1210 leukemia cells were subsequently suspended in the photobleached dye solutions and incubated at 37 °C in the dark. At the end of the incubation period, cells were washed with

dye- and photoproduct-free alpha-medium supplemented with 5% fetal bovine serum and probed for surviving colony-forming cells.

Several combinations of serum concentration, dye concentration, light dose, and incubation time were tried in pilot experiments. All gave qualitatively identical results. A dye concentration of 26 μM , a fetal bovine serum concentration of 12%, a fluence of 97.2 kJ/m^2 , a cell concentration of $10^6/\text{mL}$, and an incubation time of 1 hour were eventually adopted as standard conditions, a) because they were similar to those used in preclinical and phase I/II clinical applications of merocyanine-PDT for the extracorporeal purging of autologous hematopoietic stem cell grafts (3–9, 23) and b) because for most cell lines, they generated levels of cytotoxic activity that were within the range of sensitivity of the clonal assay.

As Table 1 shows, all selenobarbituric acid analogues generated photoproducts that were toxic to L1210 leukemia cells. When prepared and tested under standard conditions, photobleached solutions of all selenobarbituric acid analogues reduced the concentration of colony-forming leukemia cells by $\geq 98\%$. By contrast, MC540 and its barbituric (MC47) and thiobarbituric (MC1, MC4, C6, MC7, MC9, MC10, MC17, and MC49) acid analogues generated photoproducts that showed no cytotoxic activity, even if dyes were structurally identical to selone dyes except for the oxygen or sulfur atom at the 2-position of the barbiturate. All selenobarbituric acid analogues (MC54, MC55, MC56, and MC57) generated cytotoxic photoproducts of similar potency regardless of the structure of the aromatic back ring. Thus, an oxidizable selone appeared to be the only structural requirement for cytotoxic activity. The importance of the selone was underscored by experiments with the bis-(1,3-dibutyl selenobarbituric acid) trimethine oxonol dye (16). The oxonol dye lacked the benzene or naphthalene back ring of the merocyanine dyes but contained two selones instead of a single selone (Fig. 1). In agreement with its higher selone content, the oxonol dye generated cytotoxic photoproducts that had twice the potency of photoproducts generated by equimolar concentrations of selenomerocyanines. Temperature was critical for cytotoxic activity. Very little or no activity was detected when cells were incubated with photoproducts on ice.

Since selone dyes had higher singlet oxygen quantum yields than thione and oxone dyes, selone dyes were photobleached more rapidly than their thione and oxone analogues. This raised the question whether lack of cytotoxic activity was simply the result of inadequate photobleaching. To address this question, light doses were increased 4-fold (to 388.8 kJ/m^2) for the photobleaching of MC540 but reduced 4-fold (to 24.3 kJ/m^2) for the photobleaching of MC54. Under these conditions, MC540 was bleached 88%, and MC54 was bleached 85%. Despite virtually identical degrees of photobleaching, only the photoproducts of the selone dye (MC54) showed cytotoxic activity.

Selenomerocyanine-derived cytotoxic photoproducts were active at remarkably low concentrations. When 99%-inhibitory doses were taken as a basis for comparison, MC54-derived photoproducts were about 35–50 times more toxic than selenium dioxide (selenious acid) or sodium selenite and $>>50$ times more toxic than seleno-DL-methionine or seleno-DL-cystine (Fig. 2).

No cytotoxic activity was detected unless selone dyes were photobleached in the presence of serum or certain serum constituents (Table 1). Fetal bovine serum, human serum, bovine serum albumin, human low-density lipoprotein (LDL), and human high-density lipoprotein (HDL) supported the production of cytotoxic activity whereas human IgG (which did not bind to L1210 target cells) did not.

When MC54 was photobleached in the presence of carboxymethylated bovine albumin (S-carboxymethyl-albumin; less than 0.02 mole sulfhydryl per mole of albumin and no more

than 1.5 moles of S-carboxymethyl-cysteine per mole of albumin), no cytotoxic activity was detected within the tested dose range. This suggested that cysteine-34 (CySH-34), the lone thiol of albumin, or a site close to CySH-34 played a role in the generation of cytotoxic activity.

We tried to corroborate this finding with cysteinylated bovine serum albumin (cysteinyl-albumin; less than 0.02 mole sulfhydryl per mole of albumin). However, results were inconclusive because the commercial preparation of cysteinyl-albumin was highly cytotoxic (>95% reduction of clonogenic L1210 cells) in the absence of photoproducts. Additional evidence for a role of CySH-34 in the formation of cytotoxic activity was derived from experiments that exposed tumor cells simultaneously to cytotoxic photoproducts and a non-cytotoxic concentration (6 $\mu\text{g/mL}$) of cisplatin, a drug known to bind to CySH-34. Cisplatin protected tumor cells from the cytotoxic effects of MC54-derived photoproducts, most likely by competing with cytotoxic photoproducts for the same site.

Serum or an appropriate serum (lipo)protein had to be present *during* the photobleaching process to support the generation of cytotoxic activity. When selone dyes were first photobleached in ethanol and subsequently mixed with serum- or albumin-containing medium, very little or no cytotoxic activity was detected.

To support the production of cytotoxic activity, serum or serum components did not need to be derived from the same species as the target cells. A comparison of MC54-derived photoproducts generated in the presence of human serum or fetal bovine serum showed that both preparations were equally effective at killing human K562 leukemia cells.

When cytotoxic photoproduct preparations were subjected to ultrafiltration with a molecular weight cut-off of 30 kDa, the cytotoxic activity was retained in the large molecular weight fraction. When proteins in cytotoxic photoproduct preparations were precipitated by the addition of 4 volumes of cold ethanol, the cytotoxic activity was recovered in the protein precipitate. Thus, in both experiments, the cytotoxic activity behaved like a macromolecule or a conjugate between a small photoproduct and a macromolecule. ICP-MS confirmed that in cytotoxic photoproduct preparations, virtually all Se present was associated with the macromolecular fraction. However, attempts to determine the oxidation state of Se by X-ray photoelectron spectroscopy (a quantitative spectroscopic surface analysis technique that measures the elemental composition of materials and the electronic state of elements) were not successful because the concentration of Se was too low relative to the concentration of protein and buffer salts.

When glutathione, sodium azide, or alpha-tocopherol (1 mM) were present during the photobleaching process, the production of cytotoxic activity was inhibited. The inhibitory effect of the quenchers/scavengers thus was consistent with (but not strictly diagnostic of) a singlet oxygen-mediated process.

Cytotoxic photoproducts of potency equal to that achieved with the white light source were also obtained with the 574 nm and the 612 nm narrow-band LED light sources, indicating that the photochemical reaction was indeed driven by chromophores that absorbed in the green/orange region of the visible spectrum.

Cytotoxic photoproduct preparations were stable if stored at $-80\text{ }^{\circ}\text{C}$. No loss of activity was noticed over a period of 6 months (Fig. 3a). Preparations that were stored at $-20\text{ }^{\circ}\text{C}$ (Fig. 3b) or $5\text{ }^{\circ}\text{C}$ (data not shown) showed a gradual loss of cytotoxic activity. Macroscopic precipitates suggestive of elemental Se were noticed in samples that had lost cytotoxic activity while stored at $5\text{ }^{\circ}\text{C}$ for several months. Precipitates were insoluble in water and ethanol. X-ray photoelectron spectroscopy (XPS) of precipitates that had been harvested by

centrifugation and washed with water and ethanol confirmed the presence of Se. The oxidation state of the Se could not be unequivocally established, as a sodium peak adjacent to the Se 3d peak interfered with the analysis. However, scanning electron microscopy combined with energy dispersive X-ray spectroscopy (a variant of XPS that separately identifies x-rays that are characteristic of each element's unique atomic structure) clearly showed that the precipitates consisted of Se in oxidation state zero (Fig. 4). Secondary electron images (Fig. 4a) and corresponding EDS spectra taken with the JEOL 840/KeveX instrument showed two distinct regions that were evident throughout the sample, a calcium (Ca)-rich region and a sponge-like Se-rich region. A small oxygen (O) signal was consistently associated with the Ca-rich region but not with the Se-rich region (Fig. 4c, 4d). Backscatter micrographs taken with the Hitachi S-5000 instrument confirmed that the sponge-like material consisted of a heavier atom (Se) than the crystals that constituted the Ca-rich regions (Fig. 4b). EDS spectra obtained with the Hitachi S-5000/Voyager system confirmed that Se-rich regions contained only trace amounts of O at variable O:Se ratios (data not shown). By contrast, the Ca-rich regions showed strong O and carbon (C) signals at constant O:C:Ca ratios, indicating that the Ca-rich regions consisted of calcium carbonate and the Se-rich region consisted of elemental Se.

Analysis of photoproduct formation by absorption spectroscopy, fluorescence emission spectroscopy, and mass spectroscopy

All merocyanine dyes were photobleached when exposed to white light in the presence of oxygen. When the selenobarbituric acid analogue MC54 (Fig. 1) was dissolved in HEPES buffer (10 mM, pH 7.4) supplemented with fetal bovine serum (12%) and then exposed to white light (27 W/m²) for up to 60 min, the absorption peak of the intact dye (apex of monomer at 625 nm) disappeared rapidly, and a blue-shifted peak with a monomer apex at 598 nm emerged in its place (Fig. 5a). Whereas the intact dye was weakly fluorescent with an emission maximum at 635 nm, the blue-shifted peak of the primary chromophore photoproduct was more intensely fluorescent with an emission maximum at 613 nm (Fig. 5b). These modest blue-shifts of the absorption and fluorescence emission spectra were consistent with a minor disruption of the conjugation such as a singlet oxygen-mediated oxidation of the selone and a subsequent substitution of Se by O. The spectral characteristics (peak absorption wavelength, peak fluorescence emission wavelength) of the primary chromophore photoproduct were indeed indistinguishable from the spectral characteristics of dye MC47 (Fig. 1), the authentic barbituric acid analogue of MC54 (1, 2). The higher fluorescence quantum yield of the primary chromophore photoproduct was also consistent with a replacement of the original selone dye by its oxone analogue (1).

All selenomerocyanine dyes showed the same initial photobleaching pattern regardless of the structure of their aromatic back rings. They all generated primary chromophore photoproducts that were characterized by moderately blue-shifted absorption and fluorescence emission spectra and enhanced fluorescence quantum yields. While these changes were consistent with a replacement of the original selone dyes by their oxone analogues, an authentic oxone analogue for direct comparisons was only available for MC54.

The first step of the photobleaching process (replacement of the original selone dye by its oxone analogue) also took place in organic solvent (ethanol), provided the solvent contained some water (with dissolved O₂) to support the production of singlet oxygen. Mass spectroscopic analyses of photoproducts generated in ethanol showed that the primary chromophore photoproduct of MC54 had a mass consistent with the calculated mass of the dye's oxone analogue (Fig. 5e, 5f). The mass spectrum of intact MC54 showed the typical isotope distribution of Se (M 74, 0.9%; M 76, 9.0%; M 77, 7.6%; M 78, 23.5%; M 80, 49.8%; M 82, 9.2%) (Fig. 5c, 5d). By contrast, the mass spectrum of the primary

chromophore photoproduct showed no evidence of Se (Fig. 5f) and matched replacement of Se by O.

When aqueous solutions of MC54 were photoirradiated for prolonged periods of time in the presence of suitable proteins, the primary chromophore photoproduct (oxone analogue) was gradually replaced by a secondary chromophore photoproduct that was characterized by a small absorption peak at 499 nm (Fig. 5a) and a strong fluorescence emission peak at 522 nm (Fig. 5b). These major (90–100 nm) blue-shifts of the absorption and fluorescence emission spectra were indicative of a major disruption of the conjugation such as an oxidation of the polymethine chain.

As Table 1 shows, the structural requirements for the generation of the green-fluorescent species were different from those for the generation of cytotoxic activity. Whereas only selone dyes were able to generate photoproducts with cytotoxic activity, selone, thione, and oxone dyes were able to generate the green-fluorescent species. Whereas the structure of the back ring was irrelevant for the generation of cytotoxic activity, only dyes with S or Se in the donor heterocycle were able to generate the green-fluorescent entity.

Protein requirements were also different (Table 1). At least three serum constituents (albumin, LDL, HDL) supported the generation of cytotoxic activity, whereas only one - albumin - supported the production of the green-fluorescent species (Table 1). Carboxymethylation of albumin interfered with the generation of cytotoxic activity, but neither carboxymethylation nor cysteinylolation interfered with the generation of the green-fluorescent photoproduct (Table 1). This suggested that CySH-34 did not play a role in the formation of the green-fluorescent entity.

The green-fluorescent species was retained by ultrafiltration membranes with a cut-off of 30 kDa and was co-precipitated with serum albumin by the addition of 4 volumes of cold ethanol or acetone. When MC54 was photobleached in the presence of fetal bovine serum and subsequently chromatographed on a Sephadex G-100 gel filtration column, the green-fluorescent material co-eluted with the albumin monomer/dimer peaks. Taken together, these results indicated that the green-fluorescent species was a conjugate between a chromophore photoproduct and serum albumin and that the formation of cytotoxic and green-fluorescent conjugates involved different sites of the albumin molecule.

The formation of green-fluorescent photoproduct-albumin conjugates was a light-dependent process even if the primary chromophore photoproduct (i.e. authentic MC47) was used as a starting material. No green-fluorescent products formed when MC47 was incubated with albumin in the dark. Displacement of oxygen in the buffer by argon, or the addition of sodium azide, alpha-tocopherol, or glutathione (1 mM) inhibited the formation of green-fluorescent photoproduct-albumin conjugates, suggesting that green-fluorescent photoproduct-albumin conjugates were also the product of a singlet oxygen-mediated photochemical process.

The green-fluorescent material was resistant to 5 M guanidine hydrochloride and extraction with chloroform:methanol (2:1). Boiling the material in sodium dodecyl sulfate (2%) and dithiothreitol (100 mM) for 3 min reduced the green-fluorescent peak by 58%.

Unlike cytotoxic conjugates, green-fluorescent photoproduct-albumin conjugates were stable under a wide range of conditions. No loss of fluorescence was noticed when samples were stored at room temperature, 5 °C, –20 °C, or –80 °C for up to 6 months. Green-fluorescent conjugates were, however, moderately sensitive to light exposure. This became evident when photobleaching was extended beyond 1 hour (97.2 kJ/m²) in an attempt to generate green-fluorescent photoproduct-albumin conjugates that were free of trace amounts

of original dye and primary chromophore photoproduct. When white light was used for this purpose, green-fluorescence yields peaked after about 4 hours of exposure (388.8 kJ/m^2) and then progressively decreased to 23% of the peak value after 25 hours ($2,430 \text{ kJ/m}^2$) of exposure. When the 612 nm narrow-band LED light source (which did not emit in the 490 nm range) was used for the same purpose, peak fluorescence yields achieved after 4 hours of exposure were about 70% higher than those achieved with white light and remained at this level for at least 25 hours of continued exposure.

When tumor cells were incubated with photobleached (in the presence of serum or serum albumin) MC54 under conditions that were cytotoxic for target cells, the target cells bound/internalized substantial amounts of green-fluorescent material that were readily detected with the flow cytometer using standard FITC settings. The amount of cell-associated green-fluorescent material was proportional to conjugate concentration and incubation time. Low temperatures, which protected cells against the cytotoxic effect of Se-protein conjugates also inhibited the uptake of green-fluorescent material. Spectroscopic analyses performed on detergent extracts (2% Triton X-100) of conjugate-stained tumor cells confirmed the flow cytometry data.

Fluorescent conjugates prepared with carboxymethylated albumin were bound/internalized by target cells at the same rate as fluorescent conjugates prepared with native albumin. The failure of carboxymethylated albumin to support the formation of cytotoxic conjugates (Table 1) could thus not be explained by a lack of binding/uptake of the carrier protein.

Since the formation of fluorescent and cytotoxic photoproduct-protein conjugates was independently regulated, it was possible to generate conjugate preparations that were cytotoxic and fluorescent, cytotoxic but not fluorescent, or fluorescent but not cytotoxic. Side-by-side comparisons of cytotoxic conjugates prepared from MC54 and MC56 (Fig. 1) indicated that at the concentrations used, the presence of green-fluorescent material did neither enhance nor reduce the activity of cytotoxic conjugates. Preparations that were both cytotoxic and fluorescent were used for the majority of experiments because the binding/uptake of green fluorescent photoproduct-albumin conjugates provided a convenient surrogate assay for the binding/uptake of cytotoxic conjugates. On the other hand, MC56-derived preparations that were cytotoxic but not fluorescent were preferred for experiments that involved the use of green-fluorescent probes such as FITC-labeled antibodies.

Reaction pathways

Fig. 6 summarizes our current understanding of the formation of cytotoxic and green-fluorescent photoproduct-protein conjugates using as a specific example the selone dye, MC54. 1) A portion of the singlet oxygen generated by the photoirradiation of the selone dye oxidizes the selone. 2) The resulting selone oxide is unstable, and the Se atom is substituted by O. Selenium in oxidation state zero and the barbituric acid analogue of the original dye are produced as primary photoproducts. 3) If the elemental Se is generated in the presence of serum or suitable serum (lipo)proteins, cytotoxic conjugates are formed. 4) If the chalcogen in the donor heterocycle of the original dye is S or Se, green-fluorescent conjugates are formed with serum albumin by an oxygen-dependent photochemical process.

The stoichiometry of the formation of cytotoxic and green-fluorescent conjugates is not yet fully understood. Experiments with different dye:albumin ratios indicated that dye:albumin molar ratios between 1:1 and 2:1 were saturating for the formation of green-fluorescent photoproduct-albumin conjugates. For the formation of cytotoxic conjugates with albumin, a dye:albumin ratio of about 4.5:1 was saturating. Ratios in excess of 4.5:1 were not explored because of the limited solubility of MC54 in water and concerns about the damaging effects of high concentrations of ethanol on proteins and cells. Taking into consideration the

importance of CySH-34 for the formation of cytotoxic conjugates and the fact that the thiol content of commercial preparations of serum albumin is usually ≤ 0.7 M/M (24), one plausible explanation for a saturating dye:protein ratio of about 4.5:1 is that mercaptalbumin molecules bound 6 or 8 atoms of Se, most likely as cyclic hexamers or octamers - two known stable configurations of elemental Se (25) - whereas non-mercaptalbumin molecules bound no Se.

We tried to use mass spectroscopy to corroborate the proposed stoichiometry of cytotoxic Se-albumin conjugates. However, pilot experiments conducted by the institutional MALDI-TOF facility using cytotoxic conjugates prepared under standard conditions showed only native albumin and no evidence of Se or Se-albumin conjugates. We therefore also prepared protein-stabilized elemental Se by an established method (the reduction of selenite by ascorbic acid in the presence of bovine serum albumin) and deliberately used high Se:protein molar ratios of 66:1 and 80:1, respectively to maximize the chances of detecting Se-albumin conjugates. The product was stable, readily passed through $0.1 \mu\text{m}$ membrane filters, and had the typical red color of elemental Se, yet the analysis by electrospray ionization spectrometry (performed by M-Scan on a Sciex Q-Star/Pulsar instrument) showed only native albumin and no evidence of Se or Se-albumin conjugates. It thus appears that Se-protein conjugates are not amenable to this type of mass spectroscopic analysis.

Role of cytotoxic photoproducts in selone dye-mediated PDT

How much photoproducts contribute to the cytotoxic action of selone-mediated PDT depended on experimental conditions such as temperature, exposure time, serum concentration, the capacity of target cells to bind and internalize cytotoxic photoproducts, and the target cells' intrinsic sensitivity to cytotoxic photoproducts. When selone photosensitizers were used under conditions that are typically employed for the extracorporeal purging of hematopoietic stem cell grafts, the contribution of cytotoxic photoproducts was surprisingly large. For example, when simulated autologous remission marrow grafts were exposed to MC54 ($13 \mu\text{M}$) and white light (16.2 kJ/m^2) in the presence of 12% serum, L1210 leukemia were depleted about 8-fold if the process took place at 5°C (no uptake of cytotoxic photoproducts) but more than 16,000-fold if it took place at room temperature, which allowed the uptake of photoproducts. In other words, much of the dramatically improved anti-tumor activity of selone photosensitizers that originally had been attributed to an enhanced singlet oxygen-mediated oxidation of plasma membrane constituents was in fact attributable to the uptake of a cytotoxic photoproduct that was directed at intracellular targets.

Cytotoxic Se-protein conjugates as potential anti-cancer agents

Micromolar concentrations of MC54-derived Se-protein conjugates were cytotoxic to leukemia and lymphoma cells (Fig. 7a, 7b). An incubation time of 1 hour was sufficient to deplete most in vitro clonogenic leukemia cells by ≥ 5 orders of magnitude while causing little or no damage to normal CD34-positive murine and human bone marrow cells. Normal granulocyte/macrophage progenitors (CFU-GM) showed intermediate sensitivity to cytotoxic conjugates (Fig. 7a, 7b). As a group, solid tumor cells were less sensitive to cytotoxic conjugates than leukemia and lymphoma cells (Fig. 7c). However, for most solid tumor cell lines, therapeutic indices were still large enough to be of potential therapeutic interest (Fig. 7c, 7d).

Tumor cells died quickly after exposure to selenomercocyanine-derived cytotoxic photoproducts. For example, when L1210 leukemia cells were exposed to MC54-derived photoproducts ($26 \mu\text{M}$) under standard conditions, 82% of cells were scored as 'dead' at the end of the 1-hour incubation period based on their staining reaction with propidium iodide

(PI). Two hours post incubation, 98% of cells were PI-positive. Similar results were obtained with trypan blue exclusion assays. This rapid onset of cell death was in marked contrast to our experience with MC540-mediated PDT. In MC540-mediated PDT, typically >90% of tumor cells maintain plasma membrane integrity for at least 3 hours post treatment, even if clonal assays indicate a ≥ 4 log depletion of in vitro clonogenic cells (26).

For logistical reasons, the flow cytometric quantitation of CD34-positive cells typically began ≥ 4 hours after the cells' exposure to cytotoxic conjugates. Absolute cell counts and trypan blue exclusion tests were performed on sample aliquots while the flow cytometric analysis was in progress. They showed no significant decrease in total cell numbers and no difference in cell viability between treated and untreated samples of normal bone marrow.

Surprisingly, melphalan-resistant mutant L1210/L-PAM₁ and L1210/L-PAM₂ leukemia cells were more sensitive to cytotoxic conjugates than their wild-type counterparts (Fig. 7a). The higher sensitivity to cytotoxic conjugates was not linked to the mutant cell lines' drug resistance mechanism (elevated levels of intracellular glutathione), because when mutant cells were grown in medium supplemented with DL-buthionine-[S,R]- sulfoximine to inhibit glutathione biosynthesis and to restore glutathione levels to wild-type levels, sensitivity to cytotoxic conjugates was not reduced to wild-type levels but further enhanced. Flow cytometric binding/uptake measurements showed that mutant L1210 cells bound/internalized green-fluorescent photoproduct-albumin conjugates and fluorescein-labeled bovine serum albumin at a higher rate than wild-type cells. This suggested that the enhanced sensitivity to cytotoxic conjugates by mutant cells was the result of enhanced conjugate binding/uptake.

When investigations were extended to four additional drug-resistant mutant tumor cell lines, one mutant cell line (cisplatin-resistant H69/CDDP small cell lung cancer; elevated glutathione-S-transferase- π , metallothioneine, and glutathione) was more sensitive (Fig. 7d), and one (adriamycin-resistant HL60/ADR leukemia; MRP-mediated drug efflux) was less sensitive (Fig. 7f) than the corresponding wild-type line. The remaining two mutant cell lines, the adriamycin-resistant P388/ADR leukemia cell line (overexpression of P-glycoprotein) (Fig. 7e) and the cisplatin-resistant PC14/CDDP lung adenocarcinoma cell line (reduced drug uptake) (data not shown) were as sensitive as the corresponding wild-type cells. The enhanced sensitivity of H69/CDDP cells correlated with enhanced binding/uptake of green-fluorescent photoproduct-albumin conjugate whereas the reduced sensitivity of HL60/ADR cells correlated with reduced binding/uptake.

DISCUSSION

This study produced two surprise findings: 1) Selone photosensitizers generate photoproducts that are remarkably cytotoxic and remarkably selective when allowed to combine with an appropriate protein carrier. 2) The cytotoxic entity appears to be Se in oxidation state zero. The discovery of selone-derived cytotoxic photoproducts provides a rare example of photoproducts contributing substantially to the anti-tumor effect of PDT rather than competing with it. It may also provide the basis of a novel class of anti-cancer agents.

The finding that the cytotoxic entity is Se in oxidation state zero was entirely unexpected, as biological activities of Se had always been attributed to organic Se or inorganic Se in oxidation states -2 , $+4$, or $+6$ (27). We suspect that the potent cytotoxic activity of photochemically generated Se-zero is directly related to its exceptionally small size. The traditional view that elemental Se is biologically inert is largely based on experiments with

bulk material or colloidal preparations with particle diameters of ≥ 400 nm. By contrast, the postulated cyclic Se hexamers and octamers (25) have diameters of ≤ 1 nm.

Size may be important for three reasons. 1) Small particles can act as unobtrusive passengers on carrier proteins. Since they do not interfere with the binding and uptake of proteins by cells, they can use such proteins as Trojan horses to get access to intracellular targets. 2) Only small particles have access to the hydrophobic pit of albumin and/or CySH-34 inside the hydrophobic pit (24). 3) Size may profoundly affect the chemical properties of elemental Se, as at the low end of the nanometer scale, the physical, chemical, and biological properties of matter are often fundamentally different from those of bulk material, because quantum forces that govern the behavior of individual atoms and molecules begin to have a significant impact on the properties of matter. Evidence for such a size-dependent change of the chemical properties of elemental Se was recently reported by Huang *et al* (28) who noticed that Se particles with mean diameters of 10–140 nm (larger than our photochemically generated Se-zero but smaller than conventional colloidal Se) have the capacity to scavenge free radicals. As we will report elsewhere in more detail, our cytotoxic Se(0)-protein conjugates have pro-oxidant rather than anti-oxidant properties, suggesting that the chemical properties of elemental Se undergo further changes as particle size is reduced to ≤ 1 nm.

Very small particles of elemental Se that form conjugates with soluble proteins behave like solutes rather than particles. Therefore, they are not amenable to standard particle sizing methods such as those based on light scattering. Furthermore, Se entities with dimensions of ≤ 1 nm are below the resolution of most electron microscopes that identify Se by energy-dispersive X-ray analysis. Therefore, our estimate of a particle size of ≤ 1 nm had to be based on indirect evidence such as the stoichiometry of the reaction, the molecular modeling of Se₆ and Se₈ based on known atomic properties of Se (25), and the failure to detect Se particles by electron microscopy.

Cytotoxic activity was only observed when selenone dyes were photobleached in the presence of a suitable protein or lipoprotein. The role of the (lipo)protein was two-fold. It stabilized the elemental Se, and it preferentially delivered the elemental Se to target cells. Selenium particles differ from many typical colloids in that they are not surrounded by a hydration shell and do not carry an electric charge capable of inducing mutual repulsion. Because they are essentially naked, they can contact each other. Contact readily leads to bond interchange, linking, and, eventually, the fusion of small particles into larger particles (29, 30). Fusion into large particles can be prevented by performing the reaction in the presence of a protein that covers Se particles with a protective coat. In fact, varying the protein concentration is one way to control particle size when elemental Se is generated by the chemical reduction of Se⁴⁺ with high protein concentrations favoring the formation of smaller particles and low protein concentrations favoring the formation of large particles (28). What is remarkable about our photochemical method is that it generates extremely small particles even in the presence of low concentrations of protein.

Just about any protein should be capable of stabilizing colloidal Se. However, as our data show, to support the generation of cytotoxic conjugates with photochemically generated elemental Se, the protein component needs to meet certain structural and functional requirements such as having a hydrophobic pit and/or a free thiol group, and being bound and internalized in significant amounts by target cells. All native proteins used in this study happened to contain at least one free thiol. The distinction between non-covalent binding to a hydrophobic pit and covalent binding to a free thiol inside the pit is not trivial because agents that block the free thiol also sterically hinder access to the hydrophobic pit (24). Furthermore, elemental Se is known to bind to free thiols, but the bond - although covalent -

is not very stable. Therefore, the gradual loss of cytotoxic activity upon prolonged storage and the concomitant formation of macroscopic precipitates of elemental Se does not rule out that the large precipitates originated from much smaller entities that were at one time covalently bound to CySH-34.

Three of the (lipo)proteins used in our study - albumin, LDL, and HDL - supported the generation of cytotoxic conjugates. All three have been used in preclinical models and/or clinical trials as macromolecular carriers for the targeted delivery of imaging agents or therapeutic agents (31–39). Albumin plays a key role in the energy and nitrogen metabolism of tumor cells. Most tumor cells bind, internalize, and degrade albumin more efficiently than normal cells (31, 39). One survey of 382 breast cancer patients showed that serum albumin that had been sequestered from the circulation represented on average 18.5% of the cytosolic protein content of tumor tissue (40). Animal experiments and clinical trials with intravenously administered methotrexate-albumin conjugates have shown that drug-albumin conjugates accumulate preferentially in tumors and can deliver therapeutically effective drug doses despite the presence of excess native albumin in plasma (32, 35, 37, 38). Concerns that drug-albumin conjugates would accumulate primarily in liver or kidneys or would non-specifically target all rapidly proliferating cells have not been borne out. Under certain circumstances, LDL may target tumors even more effectively than albumin. Melanomas, for examples, have been shown to accumulate 28 times more LDL than the corresponding normal tissue (34).

The mechanism of action of cytotoxic Se-protein conjugates is only partially understood. As we will report elsewhere in more detail, incubation of sensitive cells with Se-protein conjugates causes a rapid and extensive (up to 80% after 1 hour of exposure) depletion of intracellular glutathione, a loss of plasma membrane asymmetry as indicated by the increased binding of Annexin V, a loss of mitochondrial potential, mitochondrial swelling, and the activation of several caspases. Se-protein conjugates also promote the oxidation of 2', 7'-dichlorofluorescein to 2', 7'-dichlorofluorescein. The latter effect can be reproduced in a simple aqueous buffer that contains neither cells nor cell extract, suggesting that Se-protein conjugates indeed have pro-oxidant properties.

The discovery of cytotoxic conjugates of elemental Se and protein challenges the widely held view that elemental Se is biologically inert and it may provide the basis of a new class of anti-cancer agents. The data presented here are meant to serve as a proof of principle. The next challenge will be to develop a simple and inexpensive chemical alternative to our currently used photochemical approach, one that is not based on the destruction of an expensive seleno dye and lends itself to easy scale-up.

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Figure 1.
Structures of selected merocyanine dyes and one Se-oxonol dye.

Figure 2.

Cytotoxic activity of photobleached selenomercocyanine dye (MC54) and selected selenium compounds. L1210 leukemia cells were suspended in HEPES-buffered alpha-medium supplemented with 12% fetal bovine serum and MC54-derived photoproducts or Se compounds as indicated, incubated at 37 °C for 1 hour, washed, and then assayed for surviving in vitro clonogenic cells. Data points reflect mean colony counts of 4 replicate culture dishes $\pm$ standard errors. Most error bars are smaller than the data symbols.

Figure 3.

Stability of MC54-derived cytotoxic photoproduct-protein conjugates. Material that was stored at $-80\text{ }^{\circ}\text{C}$ (a) was stable for at least 6 months. Material that was stored at $-20\text{ }^{\circ}\text{C}$ (b) showed a gradual decline of cytotoxic activity. Fetal bovine serum served as carrier protein, and L1210/L-PAM₁ leukemia cells were used to assess cytotoxic potency. Data points reflect mean colony counts of 4 replicate culture dishes $\pm$ standard errors. Most error bars are smaller than the data symbols.

Figure 4.

Scanning electron micrographs of crystalline material found after prolonged storage of cytotoxic photoproducts. (a) Secondary electron image obtained with the JEOL 840 instrument. (b) Backscatter image obtained with the Hitachi S-5000 instrument. The sponge-like material backscatters electrons more effectively (brighter image) than the surrounding material, indicating that the sponge-like material was made up of a heavier atom (Se) than the surrounding material. (c and d) EDS spectra of Se-rich and Ca-rich regions obtained with JEOL 840/KeveX instrument. A light gold coating of the sample (to prevent charging effects) explains the gold (Au) signals.

Figure 5.

Analysis of the photobleaching of the selenomercocyanine dye MC54 by absorption spectroscopy (a), fluorescence emission spectroscopy (b), and mass spectroscopy (c–f). Panel a shows absorption spectra of native and photobleached MC54 (2.6 μ M) dissolved in HEPES buffer (10 mM; pH 7.4) supplemented with 12% fetal bovine serum. Panel b shows normalized fluorescence emission spectra of intact MC54, its primary chromophore photoproduct (oxone analogue of MC54), and the conjugate of the secondary chromophore photoproduct with albumin. Photobleaching was performed in HEPES buffer supplemented with bovine serum albumin. Panels c and d show the mass spectrum of intact MC54: a (M+H) pseudomolecular ion cluster centered on m/z 662.1, a (M+Na) adduct ion cluster centered on m/z 684.1, and a (M–H+2 Na) adduct ion cluster centered on m/z 706.1. The isotope distribution pattern (d) of these clusters was consistent with a Se-containing compound. Panels e and f show the mass spectrum of the primary chromophore photoproduct of MC54 generated in 90% ethanol: a (M+H) pseudomolecular ion cluster centered on m/z 598.2, a (M+Na) adduct ion cluster centered on m/z 620.2, and a (M–H+2 Na) adduct ion cluster centered on m/z 642.2. The isotope distribution pattern (f) of these clusters was consistent with a compound that did *not* contain Se, and their masses were consistent with the calculated mass of the oxone analogue of MC54.

Figure 6.
Reaction pathways for the formation of cytotoxic and green-fluorescent conjugates.

Figure 7.

Preferential inactivation of murine leukemia cells (a), human leukemia/lymphoma cells (b), human solid tumor cells (c), and selected drug-resistant mutant tumor cell lines (d–f) by cytotoxic Se-protein conjugates. All tumor cell lines were more sensitive to cytotoxic Se-protein conjugates than normal murine or human CD34-positive bone marrow cells. Most tumor cell lines were also more sensitive than normal murine (mCFU-GM) or human (hCFU-GM) granulocyte/macrophage progenitors (A–C). Melphalan-resistant L1210/L-PAM₁ and L1210/L-PAM₂ leukemia cells (A) and cisplatin-resistant H69/CDDP small cell lung cancer cells (d) were more sensitive than the corresponding wild-type cells. Adriamycin-resistant P388 leukemia cells (e) were as sensitive as the corresponding wild-type cells. Only one mutant cell line, the adriamycin-resistant HL-60/ADM leukemia (f), was less sensitive than wild type HL-60 cells. Data points reflect mean colony counts of 4 replicate culture dishes or mean numbers of CD34-positive bone marrow cells of 4 determinations $\pm$ standard errors. Most error bars are smaller than the data symbols

Table 1

Structural requirements and protein requirements for generation of cytotoxic and green-fluorescent photoproducts

Dye	Descriptor 1	Descriptor 2	Cytotox Act	Green Fluor
MC540	Benzoxazole	Thiobarbituric	No	No
MC1	Naphth[1,2- <i>d</i>] oxazole	Thiobarbituric	No	No
MC4	Naphth[2,1- <i>d</i>] oxazole	Thiobarbituric	No	No
MC6	Benzothiazole	Thiobarbituric	No	Yes
MC7	Benzoselenazole	Thiobarbituric	No	Yes
MC9	Naphth[2,1- <i>d</i>] thiazole	Thiobarbituric	No	Yes
MC10	Naphth[2,3- <i>d</i>] oxazole	Thiobarbituric	No	No
MC17	Naphth[1,2- <i>d</i>]thiazole	Thiobarbituric	No	Yes (weak)
MC47	Naphth[2,1- <i>d</i>] thiazole	Barbituric	No	Yes
MC49	Benzothiazole	Thiobarbituric	No	Yes
MC54	Naphth[2,1- <i>d</i>]thiazole	Selenobarbituric	Yes	Yes
MC55	Naphth[1,2- <i>d</i>] thiazole	Selenobarbituric	Yes	Yes
MC56	Benzoxazole	Selenobarbituric	Yes	No
MC57	Benzothiazole	Selenobarbituric	Yes	Yes
Se-oxonol	Selenobarbituric	Selenobarbituric	Yes	No
Solvent/protein				
Fetal bovine serum			Yes	Yes
Bovine serum albumin			Yes	Yes
Human serum albumin			Yes	Yes
Carboxymethylated bovine serum albumin			No	Yes
Cysteinylated bovine serum albumin			Not done	Yes
Human immunoglobulin G (IgG) [†]			No	No
Human low-density lipoprotein			Yes	No
Human high-density lipoprotein			Yes	No
Albumin-depleted fetal bovine serum			Yes	No
HEPES buffer			No	No
Ethanol			No	No

[†] IgG did not bind to L1210 target cells. Dyes (26 μ M) were dissolved in tissue culture medium containing 12% fetal bovine serum and were photobleached for 1 hour. Target L1210 cells were incubated with photobleached dyes for 2 hours. A "yes" for cytotoxic activity indicates a $\geq 98\%$ depletion of in vitro clonogenic L1210 leukemia cells, a "No" a $\leq 17\%$ depletion of L1210 leukemia cells. A "Yes" for green fluorescence indicates a fluorescence emission peak height of ≥ 210 arbitrary units (AU), a "No" a fluorescence emission signal of ≤ 30 AU. Under standard screening conditions, dye MC17 generated a weakly fluorescent signal of 75 AU.