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ELEMENTAL SELENIUM GENERATED BY THE PHOTOBLEACHING OF SELENOMEROCYANINE PHOTOSENSITIZERS FORMS CONJUGATES WITH SERUM MACROMOLECULES THAT ARE TOXIC TO TUMOR CELLS

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Abstract

Elemental selenium generated by the photobleaching of selenomercocyanine dyes forms conjugates with serum albumin and serum lipoproteins that are toxic to leukemia and selected solid tumor cells but well tolerated by normal CD34-positive hematopoietic stem and progenitor cells. Serum albumin and lipoproteins act as Trojan horses that deliver the cytotoxic entity (elemental selenium) to tumor cells as part of a physiological process. They exploit the fact that many tumors have an increased demand for albumin and/or low-density lipoprotein. Se(0)-protein conjugates are more toxic than selenium dioxide, sodium selenite, selenomethionine, or selenocystine. They are only minimally affected by drug resistance mechanism, and they potentiate the cytotoxic effect of ionizing radiation and several standard chemotherapeutic agents. The cytotoxic mechanism of Se(0)-protein conjugates is not yet fully understood. Currently available data are consistent with the notion that Se(0)-protein conjugates act as air oxidation catalysts that cause a rapid depletion of intracellular glutathione and induce apoptosis. Drugs modeled after our Se(0)-protein conjugates may prove useful for the local and/or systemic therapy of cancer.

Keywords

Selenium; subnanoselenium; selenomercocyanine dyes; cancer therapy

INTRODUCTION

The prevailing view is that elemental selenium has little or no biologic activity. We report here that elemental selenium generated by the photobleaching of selenomercocyanine dyes forms conjugates with serum albumin and lipoproteins that are toxic to leukemia and selected solid tumor cells but are well tolerated by normal CD34-positive hematopoietic stem and progenitor cells.

Photosensitizing selenomercocyanine dyes were first synthesized by Günther et al.^{1, 2} to define structure-activity relationships in merocyanine-mediated photodynamic therapy (PDT) and to identify merocyanine photosensitizers with antineoplastic and antiviral properties superior to those of Merocyanine 540 (MC540). Analogues with a selenium atom at the 2-position of the barbiturate are characterized by very high singlet oxygen quantum yields and dramatically

improved antineoplastic and virucidal activities. Initially, the high singlet oxygen quantum yields seemed to offer a plausible and sufficient explanation for the improved cytotoxic and virucidal activity. However, more recent investigations have shown that in addition to this obvious quantitative difference there is also a qualitative difference between the cytotoxic mechanisms of MC540 (or other thiobarbituric and barbituric acid dyes) and its selenobarbituric acid analogues: selone dyes generate a photoproduct that is cytotoxic whereas MC540 and thiobarbituric and barbituric acid analogues generate photoproducts that have no cytotoxic activity.

Like many Type II photosensitizers, second-generation merocyanines are substrates of the singlet oxygen they generate. That is, if solutions of merocyanine dyes are exposed to light in the presence of oxygen, the dyes are oxidized and converted to so-called photoproducts. While the photoproducts of most photosensitizers show very little or no cytotoxic activity, selenomerocyanines generate a photoproduct that is highly cytotoxic to leukemia and selected solid tumor cells. The most surprising aspect of the discovery of a selenomerocyanine-derived cytotoxic photoproduct is that the cytotoxic entity is not a chromophore but selenium in oxidation state zero.

RESULTS AND DISCUSSION

To screen the photoproducts of merocyanine dyes for cytotoxic activity, a panel of merocyanine dyes consisting of MC540, 12 analogues (1 barbituric, 6 thiobarbituric, and 5 selenobarbituric acid analogues) selected from Günther's series of second generation merocyanines^{1,2} were dissolved in culture medium (alpha-medium) supplemented with fetal bovine serum and exposed to cool white fluorescent light (27 W/m²). L1210 leukemia cells were subsequently incubated in the bleached dye solution at 37 °C in the dark for ≥1 hour. At the end of the incubation time, cell suspensions were washed free of excess photoproducts and assayed for surviving cells by in vitro clonal assay. Several combinations of serum concentration, dye concentration, light dose, and incubation time were tried. All produced qualitatively identical results. A dye concentration of 26 μM, a serum concentration of 12%, a fluence of 97.2 kJ/m², and an incubation time of 1 hour were eventually adopted as standard experimental conditions.

All selenobarbituric acid analogues generated photoproducts that were highly toxic to leukemia cells (i.e. killed >99% of clonogenic tumor cells). The presence of an oxidizable selone appeared to be the only requirement for the generation of cytotoxic activity. None of the photoproducts generated by thiobarbituric and barbituric acid analogues was cytotoxic, even if the dyes were structurally identical to selone except for the selenium atom at the 2-position of the barbituric acid. The importance of the selone was underscored by experiments with a bis-(1,3-dibutyl selenobarbituric acid) trimethine oxonol dye.³ The oxonol dye lacked the benzene or naphthalene back ring of our merocyanine dyes but had two (instead of one) selones per molecule. Photoproducts generated by the oxonol dye had twice the potency of photoproducts generated by equimolar concentrations of selenomerocyanines.

Selenomerocyanine-derived cytotoxic photoproducts were active at surprisingly low concentrations. When 99%-inhibitory doses were taken as a basis for comparison and L1210 leukemia cells were used as a standardized target, selenomerocyanine-derived photoproducts were about 35 times more toxic than selenium dioxide, about 50 times more toxic than sodium selenite, and >50 times more toxic than selenomethionine or selenocystine (Figure 1). To achieve significant levels of cytotoxic activity, selone dyes had to be photobleached in the presence of whole serum, serum albumin, or serum lipoproteins (especially low-density lipoprotein). If dyes were photobleached in a protein-free environment and subsequently brought into contact with serum or serum components, little or no cytotoxic activity was

detected. Serum or serum components did not need to be homologous with target cells. Photoproducts generated in the presence of fetal bovine serum killed human leukemia cells as effectively as photoproducts generated in the presence of human serum.

The cytotoxic activity behaved like a macromolecule. It was retained by ultrafiltration membranes with a 30 kDa cut-off, and, like albumin, was precipitated by cold ethanol. Taken together, these results suggested that in order to express their cytotoxic potential, photoproducts had to form high-affinity conjugates with certain serum macromolecules. The observation that carboxymethylated albumin did not support the production of cytotoxic activity suggested that cysteine-34 (CySH-34) or a site close to CySH-34 was involved in the formation of conjugates with albumin.

Reaction Pathways

The photobleaching of merocyanine dyes and the formation of cytotoxic photoproducts were oxygen-dependent processes that were inhibited by quenchers of singlet oxygen. Absorption and fluorescence emission spectroscopy indicated that selone dyes were rapidly converted to their respective barbituric acid analogues. Mass spectra confirmed that the largest molecule in the photobleached dye solution had a mass consistent with the barbituric acid analogue of the original selone dye and did no longer display the characteristic isotope distribution of selenium. If photobleaching took place in a protein-free solvent, characteristic red deposits of elemental selenium formed on the walls of glass or plastic test tubes. Taken together, these results indicated that the oxidation of the selone by singlet oxygen was followed by a substitution of selenium by oxygen and the abstraction of elemental selenium (Scheme 1). Since structure-activity studies had indicated that an oxidizable selone was both necessary and sufficient for the generation of cytotoxic activity, the most plausible explanation of the above data was that elemental selenium rather than a chromophore photoproduct was the cytotoxic entity. Recent pilot experiments with colloidal selenium generated by the reduction of selenium dioxide with ascorbic acid have confirmed that proteinated elemental selenium is cytotoxic. However, so far, colloidal selenium preparations have not been able to match the potency of photochemically generated elemental selenium. Most likely, differences in potency reflect differences in size distribution. Photochemically generated elemental selenium most likely forms molecules of just 8 atoms. Seg molecules would be expected to fit into the 10-Å hydrophobic pit near CySH-34 of albumin. By contrast, colloidal selenium generated by chemical methods typically consists of particles with a mean diameter of ≥ 40 nm. Such large particles could obviously not fit into the hydrophobic pit; they would be larger than the albumin carrier molecule.

Green-Fluorescent Photoproduct-Albumin Conjugates

The primary chromophore photoproduct (barbituric acid analogue of selone dye) was also susceptible to oxidation by singlet oxygen. Therefore, if selone dyes were exposed to light in the presence of oxygen for extended periods of time, the primary chromophore photoproducts were eventually oxidized to secondary photoproducts whose structure has not yet been elucidated. If photobleaching occurred in the presence of albumin, some dyes formed a secondary photoproduct (Scheme 1) that had a strong fluorescence emission peak at about 520 nm. Structure-activity studies showed that the chalcogen of the donor heterocycle was the structural determinant for the green-fluorescent species. Dyes with a sulfur or selenium atom in the donor heterocycle formed the green-fluorescent entity whereas dyes with an oxygen atom in the donor heterocycle did not. The green-fluorescent species behaved like a high-affinity conjugate between a photoproduct and albumin. It was retained by ultrafiltration membranes with a molecular cut-off of 30 kDa, was precipitated by cold ethanol or acetone, and coeluted with serum albumin from a Sephadex G-100 gel filtration column. Carboxymethylation of albumin did not interfere with the formation of green-fluorescent conjugates, suggesting that CySH-34 was not involved. Albumin-depleted serum, lipoproteins,

and immunoglobulins (IgG) did not support the formation of fluorescent conjugates. There was close overlap between the spectral properties of green-fluorescent conjugates and FITC-conjugated molecules. The binding and uptake of fluorescent photoproduct-albumin conjugates was thus readily detected by standard flow cytometers, and, under certain conditions, provided a useful surrogate assay for the binding and uptake of cytotoxic conjugates.

Preclinical Evaluation of Cytotoxic Se(0)-Protein Conjugates:

A 1-hour incubation with micromolar concentrations of photochemically generated Se(0)-protein conjugates was sufficient to reduce leukemia cells by at least 4 orders of magnitude while preserving virtually all normal CD34-positive hematopoietic stem and progenitor cells (Figure 2). Solid tumor cell lines were less sensitive than leukemia cell lines. However, for certain tumors (e.g., breast cancer, neuroblastoma, Ewing's sarcoma), depletions were still large enough to be of potential clinical interest.

Se(0)-protein conjugates were remarkably effective against mutant tumor cell lines that had acquired resistance against a broad range of conventional anti-cancer drugs. For example, melphalan-resistant L1210/L-PAM1 leukemia cells, which are characterized by an elevated content of intracellular glutathione (GSH), were more sensitive to cytotoxic conjugates than their wild-type counterparts. Adriamycin-resistant P388/ADR leukemia cells (elevated P-glycoprotein) and cisplatin-resistant H69-CDDP small cell lung cancer cells (elevated GSH, metallothioneine, and glutathione-S-transferase-pi) were as sensitive as their wild-type counterparts. Adriamycin-resistant HL-60/ADR leukemia cells (MRP-mediated drug efflux) and cisplatin-resistant PC14/CDDP lung cancer cells (reduced drug uptake) were slightly less sensitive than the corresponding wild-type cell lines.

Se(0)-protein conjugates potentiated the anti-tumor effect of ionizing radiation and several established anti-cancer drugs (melphalan, 4-hydroperoxycyclophosphamide, Edelfosine, amifostine, buthionine sulfoximine, and arsenic (III) oxide). A strong antagonistic effect was obtained when tumor cells were simultaneously exposed to cisplatin and Se(0)-protein conjugates. It is interesting to note in this context that cisplatin is known to bind to CySH-34 of albumin, the site involved in the formation of cytotoxic Se(0)-albumin conjugates.

Mechanism of Action

The mechanism of action of cytotoxic Se(0)-protein conjugates is not yet fully understood. Currently available data are consistent with the notion that selenium in oxidation state zero is the cytotoxic entity whereas albumin and lipoproteins act as Trojan horses that deliver the cytotoxic entity to target cells as part of a physiological process. Se(0)-protein conjugates exploit the fact that many tumors have an increased demand for albumin and/or low-density lipoprotein (LDL), and, therefore, an increased capacity to bind and internalize albumin and/or LDL.⁴⁻⁷

Low temperature (e.g. 4 °C) protected cells against the cytotoxic action of Se(0)-protein conjugates and inhibited the uptake of green-fluorescent photoproduct-albumin conjugates. When cells were exposed to fluorescent conjugates and the lysosome-specific probe, Lyso-Tracker Red, many organelles emitted yellow/orange fluorescence, indicating that the lysosome-specific probe and the green-fluorescent photoproduct-albumin conjugates co-localized in the same organelle. Taken together, these data suggest that conjugates are internalized by an endocytotic process and localize, at least initially, in lysosomes.

In sensitive cells, the internalization of Se(0)-protein conjugates led to a rapid depletion (up to 80% in 1 hour) of intracellular GSH, a loss of plasma membrane asymmetry (as indicated by

an increased binding of Annexin V), a loss of mitochondrial potential, mitochondrial swelling, and the activation of several caspases, especially caspases 9 and 13. At this point all data are consistent with the notion that cytotoxic conjugates kill target cells by inducing apoptosis. Se(0)-protein conjugates also promoted the oxidation of 2', 7'-dichlorofluorescein to 2', 7'-dichlorofluorescein. The latter effect was also observed in a simple cell-free system that consisted of Se(0)-protein conjugates, 2', 7'-dichlorofluorescein, and buffer, suggesting that Se(0)-protein conjugates may be acting as air oxidation catalysts.

CONCLUSIONS

Drugs modeled after our Se(0)-protein conjugates may prove useful for the treatment of leukemias and selected solid tumors. The discovery of cytotoxic Se(0)-protein conjugates challenges the widely held view that selenium in oxidation state is biologically inert.

EXPERIMENTAL

Dyes: Merocyanine dyes were synthesized as described by Günther et al.¹ The bis-(1,3-dibutyl selenobarbituric acid) trimethine oxonol dye was synthesized as described by Krieg et al.³ All selone dyes were $\geq 95\%$ pure. All thiobarbituric and barbituric acid analogues were $\geq 98\%$ pure. The lower purity estimates for selone dyes reflects the relative large margin of error of the elemental analysis of selenium.

Cells: Drug-resistant mutant tumor cell lines were gifts from Drs. D.T. Vistica (L1210/L-PAM1), M.J. Egorin (P388/ADR), M.S. Center (HL-60/ADR), and N. Saijo (H69/CDDP). All other tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were propagated in alpha-modified Dulbecco's medium supplemented with 20% (HL-60, HL-60/ADR) or 10 % (all other cell lines) fetal bovine serum. Normal human bone marrow cells were obtained as a mononuclear cell fraction from a commercial source (Poetic BioWhittaker, Rockville MD).

Cytotoxic and Fluorescent Conjugates: Merocyanine dyes were prepared as 1.76 mM stock solutions in ethanol and added to alpha-medium (supplemented with 12% fetal bovine serum) to the desired final concentration (typically $\leq 26 \mu\text{M}$). Unless indicated otherwise, the aqueous dye solution was photobleached by exposing it to light from 2 panels of tubular cool white fluorescent lights for 1 hour. The fluence rate at the sample size was 27 W/m^2 . For selected experiments, selenomerocyanine dyes were photobleached by exposing them to light emitted by two strings of light emitting diodes (LED) with an emission maximum at 612 nm. Both light sources produced cytotoxic conjugates of equal potency. However, the yield of green-fluorescent photoproduct-albumin conjugates was higher when the narrow-band LED light source was used because light emitted by the narrow-band LED light source was only absorbed by the original dye and the primary photoproduct but not by green-fluorescent conjugates. For selected experiments, purified serum components (e.g. albumin, lipoproteins, immunoglobulins, albumin-depleted serum) were used instead of whole serum. Albumin-depleted serum was prepared by affinity chromatography on AffiGel-Blue.

Cytotoxicity Tests: The survival of CD34-positive hematopoietic stem and progenitor cells was assessed by flow cytometric analysis after staining with a suitable fluorescent antibody.

The survival of tumor cells was determined by in vitro clonal assay as described previously.^{8, 9} All data were expressed as means of ≤ 4 determinations $\pm$ standard error. Most standard errors were smaller than the data symbols.

Other Assays: Intracellular glutathione (GSH) was measured with the fluorescent probe, monochlorobimane.¹⁰ The mitochondrial potential was assayed with the J-aggregate forming

lipophilic cation 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide.¹¹ Loss of plasma membrane asymmetry (surface exposure of phosphatidylserine) was detected by the binding of FITC-conjugated Annexin V.¹² Caspase activity was quantified with the Caspase Detection Kit (FITC-VAD-FMK) from Oncogene Research Products (San Diego, CA) and a panel of specific peptide inhibitors.

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FIGURE 1.
Inactivation of L1210 leukemia cells by selenium components and Se(0)-protein conjugates

SCHEME 1.
Formation of cytotoxic and fluorescent conjugates

FIGURE 2.
Preferential inactivation of human tumor cells