Modulation of Cytotoxicity of Menadione Sodium Bisulfite versus Leukemia L1210 by the Acid-soluble Thiol Pool

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ABSTRACT

We investigated the mechanism of antitumor activity of the water-soluble derivative of menadione, menadione sodium bisulfite (vitamin K3), versus murine leukemia L1210. Vitamin K3 in concentrations >27 µM, caused time- and concentration-dependent depletion of the acid-soluble thiol (GSH) pool. Maximal GSH depletion to 15% of control occurred at 45 µM vitamin K3. Vitamin K3-mediated GSH depletion and vitamin K3-mediated growth inhibition were abrogated by coincubation with 1 mM cysteine or 1 mM reduced glutathione but not by 1 mM ascorbic acid or 180 µM α-tocopherol. Low concentrations of vitamin K3 (9–27 µM) elevated both the GSH pool and the total glutathione pool, the latter to a greater degree. Vitamin K3 also caused an increased rate of superoxide anion generation by L1210 maximal at 45 µM vitamin K3 (300% of control), and a concentration-dependent depletion of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) and total nicotinamide adenine dinucleotide phosphate (NADP) pools. Forty–fifty % depletion of the NADPH pool occurred after exposure to ≥ 27 µM vitamin K3 and 100% occurred at 36 µM vitamin K3. 27 µM vitamin K3 is a nonontoxic concentration of vitamin K3. Loss of NADPH and total NADP was prevented by coincubation with 1 mM cysteine but not by coincubation with ascorbic acid or α-tocopherol. We conclude that tumor cell growth inhibition by vitamin K3 is modulated by acid-soluble thiols and may be caused by GSH pool and/or NADPH depletion. Tolerance of partial NADPH depletion by L1210 cells may indicate that a threshold level of NADPH loss of >50% is necessary for toxicity. NADPH depletion may be a toxic effect common to quinone drugs. Equitoxic concentrations of vitamin K3, phylloquinone, lapachol, dichlorolapachol, and doxorubicin caused L1210 NADPH pools to deplete to 30 ± 10 (SD), 60 ± 10, 60 ± 11, and 80 ± 12% of control, respectively. In contrast, GSH depletion may not be a common mechanism of toxicity. Of these quinones, only vitamin K3 caused significant GSH depletion when studied in equitoxic concentrations.

INTRODUCTION

The naphthoquinone derivative menadione (2-methyl-1,4-naphthoquinone) has been shown to inhibit the growth of tumor cells in vitro (1–6). In the human tumor stem cell soft agar cloning assay (7), menadione causes inhibition of clonal growth of a wide variety of tumor cell types (3). Its anticancer activity with this assay is comparable or superior to currently used standard chemotherapeutic agents, such as doxorubicin. Menadione is in use in early trials in patients with advanced cancer (2) with the antimetabolite 5-fluorouracil. The mechanism of tumor cell growth inhibition by menadione is not known. Due to its quinone structure, menadione participates as a powerful oxidation-reduction agent in oxygen-consuming electron transfer reactions catalyzed by microsomal enzymes (8–12). Menadione semiquinone free radical is generated during reduction of the quinone (9, 10); this radical is short lived, producing superoxoide radicals (9, 10, 12, 13). The consequences of menadione reduction have been invoked as the cause(s) of menadione toxicity in normal hepatocytes (8, 13) and neutrophils (14), and menadione is thought to be responsible for: (a) depletion of NAD(P)H with subsequent depletion of mitochondrial ATP (15); (b) superoxide and peroxy radical generation, causing peroxidative damage (13, 16, 17); and (c) depletion of cellular GSH (13, 14). The data presented in this paper suggest that menadione potently depletes tumor cell GSH and NADP pools and that acid-soluble thiols are important modulators of menadione antitumor activity.

MATERIALS AND METHODS

Chemicals and Reagents. L-Cysteine, vitamin K3, ascorbic acid, NADPH, NADP*, reduced glutathione, superoxide dismutase (EC 1.15.1.1, from bovine blood), glutathione reductase (EC 1.6.4.2), and all reagents for the assay for pyridine nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol/polyethylene glycol 100: succinate was purchased from Eastman Chemical Co. (Kingsport, TN). This derivative of α-tocopherol is soluble in boiling water at concentrations ≤ 50 mM. DTNB was purchased from K and K Laboratories (Hollywood, CA). Lapachol [1,4-naphthoquinone-2-hydroxy-3-(methyl-2-butenyl), NSC 11905] and dichlorolapachol [2-hydroxy-3-(3,3-dichloroallyl)-1,4-naphthoquinone, NSC 125771] were received as gifts from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Doxorubicin was obtained from Adria Laboratories (Columbus, OH). Phylloquinone (Aqua Mephyton) was obtained from Merck, Sharpe, and Dohme (Westpoint, PA). All chemicals were used as received.

Cell Culture. Leukemia L1210, a murine leukemia maintained in long term liquid suspension culture, was used in all experiments. L1210 cells were maintained in 15-m1 tissue culture flasks (Falcon Plastics, Oxnard, CA) at an initial concentration of 100,000 cells/ml using Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 15% heat-inactivated (56°C for 30 min) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) at 37°C in an atmosphere containing 5% CO2 and 95% air. Cultures were fed twice weekly and were in logarithmic growth phase at the time of all experimental studies.

Growth inhibition by the drugs reported in these studies were observed by the dose-response method described previously (18). Briefly experiments were carried out in 15-m1 tissue culture flasks. Supplemented Roswell Park Memorial Institute Medium 1640, drug(s), and cells in logarithmic growth were added to the flasks and incubated at 37°C in...

The abbreviations used are: GSH, total-acid soluble thiols, reduced form; vitamin K3, menadione sodium bisulfite; GSSG, total acid soluble thiols, oxidized form; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); SOD, superoxide dismutase (EC 1.15.1.1).
an atmosphere of 5% CO₂ with 95% air. Cells were added at an initial concentration of 100,000 cells/ml. Drugs were diluted in Dulbecco's phosphate-buffered saline (Grand Island Biological Co.) (19). Each experimental point was derived from triplicate cultures. After 16 h of incubation, cells were washed free of drug with two washes of Roswell Park Memorial Institute Medium 1640 and incubated in fresh supplemented media. Cells were fed with new media on day 4. Cell counts were performed on days 4 and 7 on a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

**Acid-soluble Thiols.** Acid-soluble thiol pools were determined for aliquots of 10 to 50,000,000 cells by modification of the method of Beutler et al. (20). Cells were cooled on ice for 5 min and harvested by centrifugation at 280 x g at 4°C. After removal of aliquots for protein assay by the method of Bradford (21), the resultant cell pellets were layered onto 600 μl of 0.14 M m-phosphoric acid containing 4.6 mM EDTA and 3.47 mM sodium chloride, overlaid with 500 μl of silicone fluid DC550 (Dow Chemical, Midland, MI)/light mineral oil, 84:16, in 1.5-ml Eppendorf centrifuge tubes. Tubes were centrifuged at 15,000 x g for 10 s in a microcentrifuge (Fisher Scientific, Tustin, CA), allowing 100% of the cells to migrate into the m-phosphoric acid layer, with less than 2% contamination by supernatant (22, 23). Supernatant and oil layers were removed and cell pellets were dispersed in the m-phosphoric acid solution by gentle homogenization with a precooled glass rod. Particulate debris was removed by centrifugation at 15,000 x g for 10 min. Aliquots (500 μl) of supernatant were mixed with 1.5 ml of a reaction mixture containing DTNB (Ellman's reagent; 0.05 mg/ml) in 0.25% sodium citrate and 0.3 M dibasic sodium phosphate. The absorbance of the reaction mix at 412 nm was determined on a Model 25 double beam spectrophotometer (Beckman Instruments, Fullerton, CA).

Total glutathione content of 10 to 20 million log phase growth cells were determined by a modification of the method of Akerboom and Sies (24). Cell suspensions were placed in Eppendorf centrifuge tubes containing 500 μl of ice-cold 0.6 M perchloric acid overlaid with 500 μl of DC550 silicone fluid:mineral oil (84:16) and centrifuged at 15,000 x g for 15 s, after which the oil layers were removed. The acid extracts were neutralized with 1 N KOH in 0.3 M N-morpholinosopropansulfonic acid buffer. KC10₄ precipitates were removed by centrifugation and 50-μl aliquots of the supernatants were placed in cuvettes containing 1 ml of 0.1 M potassium phosphate buffer (pH 7.0), 0.7 μM NADPH, 20 μM DTNB (Ellman's reagent), and 0.2 unit of glutathione reductase (Sigma type III, from yeast). Glutathione content per cell suspension was assayed by the method of Bradford (21), using bovine serum albumin type V as standard.

**Superoxide Generation.** Superoxide generation by L1210 cells were assayed by the method of Smith et al. (12). One million cells per assay were suspended in 1.35 ml of Krebs-Henseleit buffer plus or minus SOD (0.02 mg/ml) and warmed at 37°C for 2 min. Fifty μl of cytochrome c (Sigma type VI, from horse heart; 50 mg/ml) plus or minus 150 μl of various concentrations of vitamin K₃ were added and the suspensions were incubated for an additional 30 min at 37°C. After incubation, suspensions were cooled on ice for 5 min, and 1-ml aliquots were layered over 500 μl of a mixture of DC550 silicone fluid:mineral oil (84:16) in 1.5-ml Eppendorf tubes and centrifuged at 15,000 x g for 10 s to remove 100% of the cells. Absorbance at 550 nm of the supernatant was measured by the method of Akerboom and Sies (24). Cells were cooled on ice for 5 min and harvested by centrifugation at 280 x g for 10 min at 4°C. Cells were then washed three times in ice-cold Dulbecco's phosphate-buffered saline (19) supplemented with 10 μM glucose. After cells were washed, NADPH pools were determined on cells which were extracted with 300 μl of ice-cold 0.5 μl KOH in 50% ethanol, heated for 5 min at 90°C, recooled to 0°C, and neutralized with 0.5 μl triethanolamine in 0.4 M dibasic potassium phosphate. NAPDH in the neutralized alkaline extracts was converted to NAPDH⁺ prior to assay by addition of 10 μM GSSG and 0.2 unit of glutathione reductase (Sigma type III, from yeast) and incubation at 37°C for 30 min. The extracts were then acidified with ice-cold 2 M m-phosphoric acid, centrifuged at 15,000 x g for 5 min to remove protein, and reneutralized with 2 N KOH in 0.3 M N-morpholinosopropansulfonic acid. NAPDH⁺ pools were determined on cells extracted with 0.2 M m-phosphoric acid. After centrifugation at 1500 x g and neutralization of the supernatants with 1 N KOH in 0.15 M N-morpholinosopropansulfonic acid, NAPDH⁺ content was determined by measuring the rate of increase of absorbance of 570 nm light due to reduction of thiazolyl blue in the cycling system containing 300 μl of the cell extracts, 0.1 N Tris-HCl (pH 7.8), 5 mM glucose 6-phosphate, 5 mM MgSO₄, 0.8 mM phenazine methosulfate, 0.2 mM thiazolyl blue, and 0.3 unit glucose-6-phosphate dehydrogenase (Sigma type IX, from yeast). Each assay was carried out at 25°C in cuvets containing 1.2 ml total volume. NAPDH⁺ content was determined by addition of authentic NAPDH⁺ standard to each assay. This assay was linear in the range 50-2000 pmol. The lower limit of sensitivity was 10 pmol for acid-extracted samples and 100 pmol for alcoholic alkali-extracted samples.

**RESULTS**

Incubation with vitamin K₃ caused a time- and concentration-dependent depletion of the GSH pool (Charts 1 and 2). The GSH pool reached a minimum of 15% of control after 16 h of exposure to 72 μM vitamin K₃. The concentration-response relationship for GSH depletion was steep, with 16 h exposure to up to 27 μM vitamin K₃ producing no depletion and ≥45 μM vitamin K₃ producing maximal depletion of 85%. Low concentrations of vitamin K₃ (<27 μM) actually enhanced the GSH pool, by as much as 2-fold.
MODULATION OF REDUCED GLUTATHIONE BY VITAMIN K₃

Low concentrations of vitamin K₃ also elevate the total glutathione pool (Chart 2). The percentage of elevation of the total glutathione pool at 9–27 µM vitamin K₃ was greater than the percentage of elevation of the GSH pool. Also the total glutathione pool remained at levels equivalent to those of control cells after exposure to higher concentrations of vitamin K₃ (>36 µM), despite depletion of the GSH pool.

L1210 cells readily repleted the GSH pool after a short term (1 h) incubation with vitamin K₃. Within 3 h after removal from a 1-h exposure to medium containing 72 µM vitamin K₃, the GSH pool recovered to 90 ± 5% (SD) of control (n = 3) from a minimum of 60 ± 4% of control. However, L1210 cells showed only a limited capacity to replete the GSH pool after a prolonged (16-h) exposure to vitamin K₃. Five h after removal of cells from a 16-h exposure to medium containing 36 µM vitamin K₃, GSH levels recovered to only 30 ± 13% of control from 10 ± 2%. No further recovery of GSH was observed beyond 5 h; the viability of the cell began to deteriorate at that point.

Vitamin K₃ more readily depleted the L1210 GSH pool than did equitoxic concentrations of some other quinones. Exposure to 72 µM for 1 h vitamin K₃ reduced the GSH pool to 20 ± 18% of control, whereas 53 µM lapachol (110 ± 9% of control), dichlorolapachol (90 ± 10% of control), and 37 µM doxorubicin (110 ± 14% of control), all equitoxic at the noted concentrations to L1210 growing in suspension culture (data not shown), did not reduce the GSH pool at all. The equitoxic concentration of phylloquinone (670 µM) actually raised the GSH pool to 150 ± 21% of control. These data suggest that GSH depletion may be a more important component of the antitumor activity of vitamin K₃ than are these other quinones.

Coincubation with 1 mM cysteine restored the vitamin K₃-mediated depletion of the L1210 GSH pool (Table 1); coincubation with 1 mM reduced glutathione partially restored the GSH pool. However, reduced glutathione was somewhat unstable in aerobic tissue culture medium with up to 43% oxidation of glutathione occurring in 30 min in medium in the absence of cells, with or without the presence of vitamin K₃. Coincubation with the nonthiol reducing agents ascorbic acid and α-tocopherol did not prevent vitamin K₃-mediated GSH depletion (Table 1).

Growth Inhibition. The effect of 16 h exposure to vitamin K₃ on L1210 growth in liquid suspension culture was evaluated. Exposure for 16 h was chosen to assure maximal depletion of the GSH pool. Vitamin K₃ inhibited L1210 growth in concentrations >36 µM (Chart 3). The dose concentration-response relationship was quite steep with concentrations <36 µM having no effect.

Coincubation of L1210 cells with vitamin K₃ plus 1 mM cysteine or 1 mM reduced glutathione abrogated vitamin K₃-mediated growth inhibition (Table 2). However, nontoxic concentrations of ascorbic acid and α-tocopherol did not prevent vitamin K₃-mediated growth inhibition.

Superoxide Generation. Ohnishi et al. have shown that microsomes plus NAD(P)H may reduce vitamin K₃ to a semiquinone, which has a short lifetime and may produce superoxide (9, 10). In some cells, superoxide generation has been correlated with GSH depletion (27, 28, 29). In L1210 cells, vitamin K₃ caused almost a 400% stimulation of superoxide detected extracellularly.
Effect of reducing agents on vitamin K₃-mediated inhibition of L1210 growth

L1210 cells were grown in liquid suspension culture in the continuous presence of 36 μM vitamin K₃ ± reducing agent (see "Materials and Methods"). Shown are the means ± SD of the percentage of control (i.e., no vitamin K₃ exposure) cell count at 96 h of a representative experiment. None of these reducing agents alone affected L1210 growth at all at the concentrations shown.

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>% of normal growtha</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Ascorbic acid (0.7 mw)</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>α-Tocopherol (0.2 mw)</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Cysteine (1 mw)</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>Glutathione (1 mw)</td>
<td>100 ± 3</td>
</tr>
</tbody>
</table>

* Cell count of control cells at 96 h was 4.2 ± 0.4 million.

Table 2

Effect of vitamin K₃-reducing agents on NADPH/NADP⁺ pools in L1210 cells

From 10 to 20 million L1210 cells per data point were incubated with the agents shown for 16 h at 37°C in a 5% CO₂:95% air atmosphere, after which pyridine nucleotides were measured by the thiazolyl blue cycling assay of Bernofsky and Swan (20). Proteins were assayed by the method of Bradford (21). Shown are the means ± SE of the combined data from 4–7 separate experiments.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>NADPH⁺</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.2 ± 0.04</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Vitamin K₃</td>
<td>9 μM</td>
<td>0.2 ± 0.04</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>Vitamin K₃:α-tocopherol</td>
<td>27 μM</td>
<td>0.1 ± 0.06</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Vitamin K₃:cysteine</td>
<td>36 μM</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Vitamin K₃:ascorbic acid</td>
<td>27 μM:1 mm</td>
<td>0.2 ± 0.07</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Vitamin K₃:α-tocopherol</td>
<td>27 μM:0.2 mm</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Significantly different than control (P < 0.05 by paired t test).

Table 3

DISCUSSION

The GSH pool is an important determinant of tumor cell viability (30–32). Exposure of leukemia L1210 cells by vitamin K₃ causes marked alterations in the GSH pool; the concentration effect curve has a biphasic shape. Exposure to low concentrations (9–27 μM) of vitamin K₃ is associated with up to 2-fold elevation of the GSH pool, while higher concentrations of vitamin K₃ (>36 μM) cause marked depletion of the GSH pool. Total glutathione is also increased by a low concentration of vitamin K₃ to a further degree than is GSH and is unaffected by higher vitamin K₃ concentrations. These data suggest that vitamin K₃ has two effects on thiol metabolism in L1210 cells: (a) to stimulate new glutathione synthesis; and (b) cause a shift in the GSH:GSSG ratio favoring GSSG. At low concentrations of vitamin K₃, new thiol synthesis predominates; thus both GSH and GSSG pools are raised. At higher concentrations, thiol synthesis cannot keep up with rapid oxidation to GSSG; thus the GSH pool is depleted.

In L1210 leukemia, vitamin K₃ depletes the GSH pool to a significantly greater degree than do equitoxic concentrations of its analogues phylloquinone, lapachol, and dichlorolapachol or the benzanthroquinone doxorubicin (37 μM) lowered the NADPH pool to 80 ± 12% of control.

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α-tocopherol neither prevented vitamin K₃-mediated GSH depletion nor protected L1210 cells from vitamin K₃ toxicity.

It is of interest that reduced glutathione protected the cells from vitamin K₃ toxicity in that, unlike cysteine, the tripeptide glutathione does not stimulate the L1210 GSH pool (Table 1). This suggests the possibility that vitamin K₃-mediated cytotoxicity may occur at or near the cell surface, in proximity to extracellular glutathione. Morrison et al. have made a similar suggestion in hepatocytes (33). Although we cannot entirely exclude the possibility that glutathione inhibits entry of vitamin K₃ into the cell by covalently forming vitamin K₃-glutathione thioether adducts, the fact that intracellular GSH pool depletion occurs in the presence of the vitamin K₃-reduced glutathione combination (Table 1) suggests that the cell is subject to some vitamin K₃ effect, despite the presence of reduced glutathione. Further work on the site of vitamin K₃ toxicity is clearly necessary.

There are several mechanisms by which cellular metabolism of vitamin K₃ may cause a shift in the GSH:GSSG ratio favoring GSSG: (a) vitamin K₃ semiquinone radical may reduce molecular oxygen, causing superoxide and hydrogen peroxide formation (10, 12, 13, 16). Peroxide generation could consume reduced glutathione by the glutathione peroxidase reaction; (b) reduction of vitamin K₃ utilizes NAD(P)H cofactor (15, 27), causing a depletion of the NAD(P)H pool. NADPH is also a cofactor for reduction of oxidized glutathione (28, 34); loss of NADPH could impair glutathione reduction; (c) vitamin K₃ may bind reduced glutathione covalently at position 3 via thio ether formation (16).

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