BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 13–17 (1997)
ARTICLE NO. RC977762

Haloenol Lactone: A New Synergist of Chemotherapy *in Vitro*

Jiang Zheng,*,†,[1] Gregory T. Wurz,† Timothy B. Cadman,† Michael W. DeGregorio,† A. Daniel Jones,‡ and Bruce D. Hammock*,[1]

**Departments of Entomology and Environmental Toxicology, ‡Facility for Advanced Instrumentation, University of California at Davis, Davis, California 95616; and †Department of Internal Medicine, Division of Hematology & Oncology, University of California at Davis, Sacramento, California 95817*

Received October 29, 1997

Over-expression of glutathione *S*-transferases (GST) has been found to play a significant role in multiple drug resistance in cancer chemotherapy. To combat GST-mediated drug resistance, GST inhibitors are being studied as potential synergists for effective cancer chemotherapy. We have designed and synthesized a haloenol lactone derivative as a mechanism-based inactivator of GST-π isozyme. In the current study, we examined the inhibitory effect of the haloenol lactone compound on GST of a human renal carcinoma cell line UOK130 and found that this compound shows time-dependent GST inhibition in these cancer cells. The enzyme activity lost upon incubation with the haloenol lactone could not be restored by extensive dialysis against buffer. Pretreatment of the cancer cells with 1.0 μM of haloenol lactone increased cytotoxicity induced by cisplatin in the UOK130 cell line. This report further supports the possibility of synergizing alkylating agents in cancer chemotherapy by use of selective GST inhibitors.

A number of alkylating agents used in cancer chemotherapy have been shown to be deactivated by glutathione *S*-transferase (GST). Nitrogen mustards such as melphalan [1-3] and chlorambucil [4] have both been shown to form glutathione (GSH) conjugates. Glutathione *S*-transferases have been found to catalyze the denitrosation of the nitrosourea BCNU by formation of a *S*-nitroso GSH conjugate [5, 6]. These enzymes also catalyze the conjugation of GSH with acrolein [7], a major reactive metabolite of cyclophosphamide.

Over-expression of GST can confer cell resistance to chemotherapeutic drugs. The relationship between drug resistance and expression of GST has been studied extensively in many cancer cell lines. The over-expression of all three classes of GST isozymes, *i.e.* α, μ, and π, has been found in resistance of cultured cells to antineoplastic agents. Glutathione depletors and GST inhibitors have been employed to combat GST-mediated drug resistance to cancer chemotherapy. Buthionine sulfoximine, an inhibitor of GSH synthesis, has been found to resensitize resistant cells to antitumor drugs [8, 9]. The GST inhibitors, ethacrynic acid [10], piriprost [10], indomethacin [11] and gossypol [12], have been used as synergistic agents of chemotherapeutic drugs. Ethacrynic acid has been extensively investigated for possible employment as a synergistic agent of chemotherapeutic drugs. This compound is a widely used diuretic, and it as well as its GSH conjugate has been found to be a reversible inhibitor of GST [13-15]. Pretreatment of mammalian cancer cell lines with ethacrynic acid increases the cytotoxicity of mitomycin [16, 17], chlorambucil [18] and melphalan [19]. However, the diuretic function of ethacrynic acid limits its clinical use for chemotherapy.

Recently, we successfully designed and synthesized a haloenol lactone compound as a mechanism-based inactivator of GST, and this compound was found to selectively and irreversibly inhibit the GST-π isozyme [20]. The enzyme was inhibited by covalent modification of an amino acid residue at the active site by this lactone. Here, we report our recent studies on the inactivation of GST in a human renal carcinoma line (UOK130) by this haloenol lactone derivative and on investigation of the synergistic effect of this compound with cisplatin in these cells.

MATERIALS AND METHODS

Synthesis. Compound **1,** 3-cinnamyl-5(*E*)-bromoethylidenetetrahydro-2-furanone, was synthesized as reported in our earlier paper [20].

Cell culture. UOK130 human renal cancer cells were grown in improved minimum essential medium (IMEM) supplemented with 10% fetal bovine serum. Cells were maintained in Corning T75 flasks and held at 37°C in 5% CO_2 and 95% air.

[1] Correspondence.

0006-291X/97 $25.00

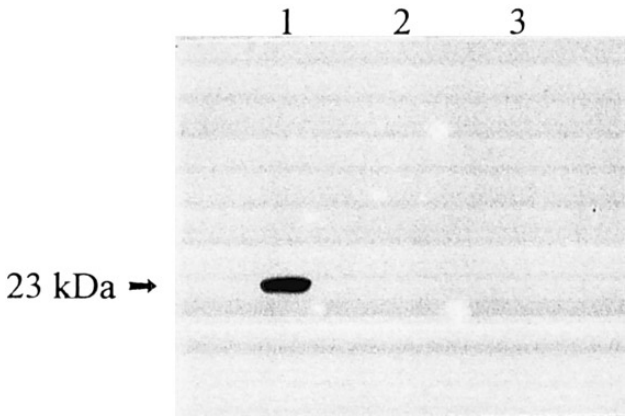


FIG. 1. Immunochemical detection of glutathione *S*-transferase isozymes in UOK130 cell homogenates. Immobilon-P transfer membranes blotted with the cell homogenates were incubated with polyclonal antibodies raised against GST-π (lane 1), GST-μ (lane 2), or GST-α (lane 3), followed by detection of chemiluminescence using ECL Western blotting kits.

Growth inhibition studies. Haloenol lactone **1** was dissolved in a mixture of DMSO and sterile normal saline prior to dilution with culture medium. The final concentration of DMSO was less than 0.1%. Cisplatin stock solution was prepared in sterile normal saline at 1000 μg/ml and diluted as required. Cells were plated in triplicate T25 flasks at 10,000 cells/ml. Twenty-four hours after plating, 500 μl of sterile stock solution were added to the appropriate flasks to achieve final concentrations of 1.0 and 5.0 μM haloenol lactone **1.** Controls received an equal volume of vehicle containing <0.1% DMSO. The cells were incubated at 37°C in 5% CO_2 and 95% air. After four days of continuous exposure to haloenol lactone **1,** the cells were exposed to cisplatin (1.0 μg/ml) for 1 hr. Growth inhibition was determined by cell counts using a hemacytometer and was calculated as percent of the controls. Trypan blue dye exclusion was used to assess viability, which was greater than 95% in all cases.

Kinetics of enzyme inhibition. Cells were lysed by sonication, and supernatants were obtained by centrifugation of the resulting cell lysates at 1000g for 30 min. To 300 μl of 0°C cell supernatant solution containing 3.0 mg/ml protein content was added 5 μl of vehicle or 20 mM compound **1** in ethanol. Aliquots were withdrawn for enzyme activity and the remaining solution was immediately placed in a water bath for incubation at 37°C. Aliquots were withdrawn at 0, 2, 8 and 15 min, and GST activity was determined using spectrophotometric assays described below.

Enzyme assays. Glutathione *S*-transferase activity was measured using glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates according to the method of Habig [21]. The activity of the enzyme was determined in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1.0 mM GSH and 1.0 mM CDNB. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a Shimadzu PC-2101 uv-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Enzyme activities were calculated after correction for nonenzymatic reaction. Specific activities are based on protein concentrations as determined using Bio-Rad protein spectrophotometric assay kits.

Electrophresis and immunoblotting. The protein bands resolved by SDS-polyacrylamide gel electrophoresis (Mini-Protean II, Bio-Rad) using 3.3% stacking and 12% resolving gels were transfered to polyvinylidene difluoride microporous membranes (Immobilon-P transfer membranes, Millipore) by electroblotter (LKB Novablot electrophoretic transfer kit). After 3.0 hr transfer (43 mA), blots were blocked by shaking overnight in 5% non-fat dry milk in PBST buffer. The blotted polyvinylidene difluoride membranes were incubated for 1.0 hr with a 1/500 dilution of polyclonal rabbit antibodies (purchased from Novocastra Laboratories, Newcastle upon Tyne, UK) raised against human GST α, μ, or π isozymes in PBST buffer (0.2 M sodium phosphate containing 0.05% Tween-20, pH 7.4) with 3% non-fat milk. The immunoblots were incubated for 1.0 hr with horseradish peroxidase conjugated goat anti-rabbit IgG antibodies (1/4,000 in PBST buffer, Sigma). The blotted protein bands were detected by chemiluminescence using ECL Western blotting kits (Amersham International plc, England).

RESULTS

Immunoblotting. Glutathione *S*-transferase isozymes in UOK130 cell line were examined by electrophoresis separation and Western blotting using polyclonal antibodies raised against human GST α, μ, and π isozymes. As shown in Figure 1, a single chemiluminescent band with molecular weight of 23 kDa was observed in the membrane blotted with UOK130 cell homogenates, and then incubated with antibodies against human GST-π isozyme, followed by treatment with ECL Western blotting kits. However, no chemiluminescent bands were detected by antibodies against human GST α or μ isozyme.

Inhibition of GST activity. UOK130 cancer cell homogenates (3.0 mg/ml protein content) were incubated with 320 μM haloenol lactone **1** at 37°C, and total GST activity of the resulting homogenate toward CDNB was measured at various times. As shown in Figure 2, a time-dependent GST activity loss was observed after

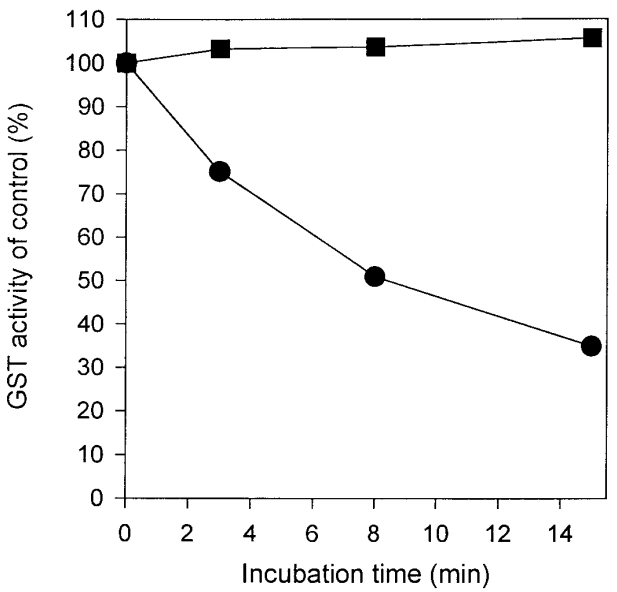


FIG. 2. Time-dependent inhibition of GST activity of UOK130 cell homogenates by haloenol lactone **1.** UOK130 cell homogenates (3.0 mg protein contents/ml) were incubated with vehicle (■) or haloenol lactone 1 (●, 320 μM) at 37°C. The GST activity was determined periodically by the Habig method [21].

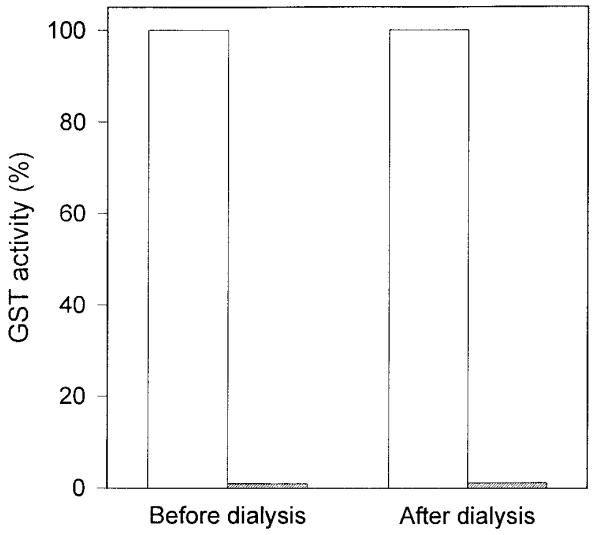


FIG. 3. Irreversible inhibition of GST activity of UOK130 cell homogenates by haloenol lactone **1.** UOK130 cell homogenates (3 mg protein/ml) were incubated with vehicle (open columns) or haloenol lactone **1** (shaded columns, 320 μM) at 37°C for 60 min, followed by 6 changes of dialysis against 0.1 M potassium phosphate buffer (pH 6.5). The GST activity of cell homogenates were determined before and after dialysis.

the homogenates were incubated with compound **1,** and about 30% of total GST activity remained in 15 min.

In separate experiments, the cancer cells (10,000 cells/ml) were incubated with vehicle or compound **1** at concentrations of 1.0 or 5.0 μM (see Method for details). The resulting cells were harvested by centrifugation and lysed by sonication. The specific GST activity of the supernatants were determined by measuring GST activity toward CDNB and the protein content, and 17% and 28% of GST activity was lost relative to the control after exposure of the cells to 1.0 μM and 5.0 μM compound **1,** respectively.

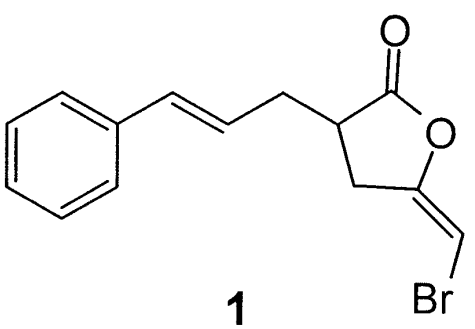
Irreversible inhibition. To determine irreversible enzyme inhibition, the cell homogenates were incubated with 320 μM haloenol lactone **1** or vehicle at 37°C for 60 min, followed by dialysis of 6 changes of 0.1 M potassium phosphate buffer (pH 6.5). The enzyme activity of the resulting proteins toward CDNB and protein contents were measured. The specific enzyme activity was determined by measuring the protein content and GST activity. As shown in Figure 3, more than 99% GST activity lost in 60 min after the cancer cell homogenates were incubated with compound **1** relative to the control. In addition, the extensive dialysis of the homogenates which had been treated with compound **1** did not recover the loss of GST activity, indicating irreversible inhibition of GST by compound **1.**

Synergistic effect on cisplatin-induced cytotoxicity. Cisplatin was used to induce cancer cell death. UOK130 cancer cells (10,000 cells/ml) were preincubated with vehicle or 1.0 μM compound **1,** followed by exposure to cisplatin or vehicle. As shown in Figure 4, the treatment with 1.0 μg/ml cisplatin alone caused neither cell death nor GST inhibition. This cell line is insensitive to cisplatin at low concentration. No cell death was observed after the cells were exposed to compound **1** (1.0 μM) alone. However, 20% GST activity of these cells was inhibited in the presence of 1.0 μM compound **1.** Not surprisingly, cells died only after they were exposed to a combination of compound **1** and cisplatin. As expected, almost the same level of GST activity was lost as that of the cells exposed to compound **1** alone.

DISCUSSION

To develop new drugs to combat GST-mediated drug resistance to cancer chemotherapy, we have successfully designed and synthesized haloenol lactone **1** as a mechanism-based inactivator of GST to synergize alkylating agents in cancer chemotherapy. Haloenol lactone **1** has shown a potent time-dependent inhibitory effect on mouse GST-π, and slower inhibition of α and μ GST isozymes [20]. In an attempt to develop a good *in vitro* system to test the synergistic effect of compound **1** on cytotoxicity induced by alkylating agents, we found predominant levels of GST-π isozyme in UOK130 established from human renal carcinoma (Figure 1). In addition, this cell line is mild resistant to cisplatin. Naturally, we employed this cell line as a model of cell system for our *in vitro* synergy studies. As expected, a time-dependent GST activity loss was observed after the supernatants of UOK130 human cancer cells were exposed to compound **1.** In addition, the GST activity lost in the incubation could not be restored by extensive dialysis. The time-dependent enzyme inhibition along with irreversible inhibition of GST upon exposure to compound **1** indicates that the haloenol lactone inactivates GST through the covalent modification of protein by the lactone other than the formation of simple Michaelis complex as reversible enzyme inhibition. This is consistent with the assumption that compound **1** inactivates GST-π by mechanism-based inhibition.

In addition to the studies of direct exposure of cancer cell supernatants to compound **1,** the human UOK130



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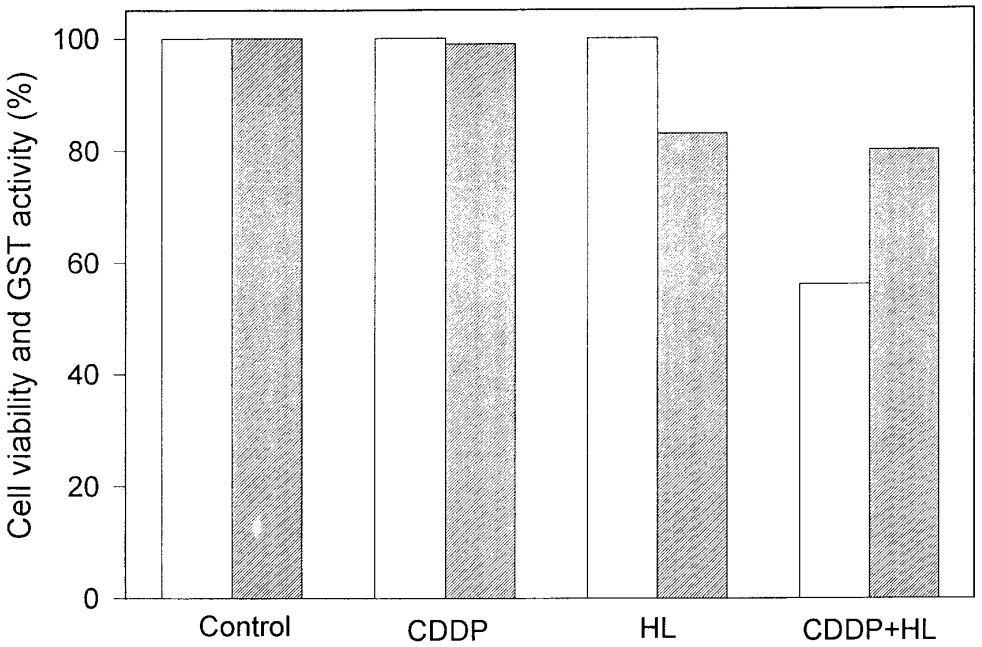

FIG. 4. Cytotoxicity and GST inhibition by cisplatin (CDDP, 1.0 μg/ml) and haloenol lactone **1** (HL, 1.0 μM). UOK130 cells pretreated with vehicle or haloenol lactone **1** at 37°C for 2 days were incubated with vehicle or cisplatin. Cell viability (open columns) was determined by cell counts and the GST activity (shaded columns) of cell homogenates was measured after continuous exposure to haloenol lactone **1** for four days.

cancer cells were incubated with the lactone. As expected, GST activity of cancer cells was inhibited after exposure of the cells to compound **1.** This implies that compound **1** is stable at physiological pH and able to penetrate the cell membranes to attack the target enzyme.

Compound **1** at a concentration of 1.0 μM increased cytotoxicity of cisplatin in the UOK130 cell line with mild resistance to cisplatin. The Western blotting experiments (Figure 1) demonstrated that GST-π is dominant in UOK130 cell line and GST-α and GST-μ are undetectable by the corresponding antibodies. Our early studies have shown compound **1** selectively inactivates GST-π isozyme. The synergistic effect of compound **1** on cisplatin-induced cytotoxicity may be associated with GST-π inactivation by compound **1.** Elevated expression of GST-π has been reported in a variety of human cancer tissues such as gastric cancer, colon cancer, lung cancer, oral cavity cancer, and uterine cancer [22-26]. Over-expression of GST-π has been found in a number of cancer cell lines *in vitro* after exposure to various chemotherapeutic agents including cisplatin [27], adriamycin [28], chlorambucil [29], melphalan [30] and cyclophosphamide [31]. Recently, Ban and co-workers transfected human colon cancer cell line M7609 with GST-π antisense cDNA and found the sensitivity of the transfected cells to adriamycin, cisplatin, melphalan, and etoposide are increased in a range of 2 to 3 fold, respectively [32]. This confirmed that GST-π is directly involved in resistance of cancer cells to alkylating agents. Hayes and Pulford proposed four possible mechanisms responsible for GST-π mediated drug resistance in chemotherapy including (1) the involvement of GST-π in detoxification of alkylating agents through glutathione conjugation; (2) the sequestration of alkylating agents resulting from binding with GST-π, (3) the reduction of lipid peroxides by GST-π, and (4) the reduction of the amounts of DNA peroxides and the repair of DNA [33].

In summary, haloenol lactone **1** showed time-dependent inhibitory effect on GST of human renal cancer cell line UOK130, demonstrating penetration of cellular membranes and irreversible inhibition of GST. *In vitro* studies showed that compound **1** increases cytotoxicity of cisplatin to UOK130 cancer cells.

ACKNOWLEDGMENT

This work was partially supported by an Institutional Research Grant, a program made possible by funding from the American Cancer Society and the Dean, UC Davis School of Medicine.

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