Inhibition of Glutathione Synthesis Augments Lysis of Murine Tumor Cells by Sulfhydryl-reactive Antineoplastics

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ABSTRACT GSH plays an important role in cellular defense against a wide variety of toxic electrophiles via the formation of thioether conjugates. We studied the role of GSH in murine tumor cell defense against a novel class of sulfhydryl-reactive antineoplastics, the sesquiterpene lactones (SL). Incubation of P815 mastocytoma cells with any of the four SL tested (vernolepin, helenalin, elephantopin, and eriofertopin) for 1 h resulted in 70–97% depletion of GSH. The importance of GSH resynthesis upon exposure of tumor cells to SL was evaluated with the use of buthionine sulfoximine (BSO), a selective, nontoxic inhibitor of γ-glutamylcysteine synthetase. Inhibition of GSH synthesis with 0.2 mM BSO markedly enhanced SL-mediated cytolysis of four murine tumor cell lines. A 6- to 34-fold reduction in the amount of SL causing 50% lysis was obtained with BSO. Addition of BSO to P815 cells either during or immediately after a 1-h pulse with 10 μg/ml of vernolepin increased cytolysis from <3% to 78–82%. However, a 1.5-h delay in the addition of BSO to such cells, which allowed for substantial resynthesis of GSH, reduced cytolysis to 30%. Recovery of GSH synthetic capacity after BSO treatment correlated with loss of the synergistic effect of BSO on lysis by vernolepin. BSO did not augment cytolysis by six other antineoplastics (doxorubicin, mitomycin C, vinblastine, cytosine arabinoside, maytansine, and 1,3-bis-[2-chloroethyl]-1-nitrosourea [BCNU]). Of these, only BCNU depleted cellular GSH. Lysis by jatrophone, another GSH-depleting antitumor agent, was increased 21-fold by BSO. Since prolonged incubation with BSO alone results in near-complete GSH depletion without loss of cell viability, SL-mediated cytolysis is probably not a result of GSH depletion. We have demonstrated, however, a critical role for GSH synthetic capacity as a determinant of tumor cell susceptibility to cytolysis by SL. GSH also plays an important role in cellular defense against oxidative injury. Vernolepin, acting as a GSH-depleting agent, markedly sensitized tumor cells to lysis by H_2O_2 (>6.5-fold increase with 20 μg/ml of vernolepin). These findings suggest the possibility that the coordinated deployment of sulfhydryl-reactive antitumor agents, BSO, and oxidative injury might constitute an effective chemotherapeutic strategy.

INTRODUCTION

Glutathione, the most abundant nonprotein sulfhydryl of mammalian cells, has been shown to play a critical role in cellular defense against a variety of injurious agents (1–3). We have previously studied the role of the GSH redox cycle in tumor cell defense against oxidative injury. In those studies, interference with the GSH redox cycle augmented in vitro cytolysis of tumor cells by macrophages and granulocytes as well as by a model H_2O_2-delivery system (glucose oxidase plus glucose) (4, 5). Similar manipulations enhanced the antitumor activity of H_2O_2 in vivo (6). GSH also plays a role in protection against toxic electrophiles by thioether formation. In contrast to the cyclic oxidation-reduction of GSH during antioxidant defense, restoration of GSH content after detoxification of electrophiles is dependent upon its resynthesis. In this report, we consider the role of GSH and its synthesis in tumor cell defense against sulfhydryl-reactive antineoplastics, in particular, the sesquiterpene lactones (SL).[1]

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[1] *Abbreviations used in this paper*: Ara-C, cytosine-1-β-D-arabinofuranoside hydrochloride; BCNU, 1,3-bis(2-chloro-

SL are among the natural products that have attracted attention recently as prototypes for the development of novel chemotherapeutic agents (7–11). Some SL inhibit cell growth in vitro, and at higher concentrations, lead to cell death (12–16). High reactivity towards sulfhydryl groups in aqueous buffer is a characteristic feature of SL (17–20), and has led to the hypothesis that their antitumor activity is the result of *S*-alkylation of growth-regulatory or otherwise vital macromolecules (8, 18, 21, 22). Several sulfhydryl-dependent enzymes have been shown to be inhibited by these agents (19, 23–25). Nonetheless, the cellular targets relevant to their antitumor activity in vitro or in vivo have not been identified. Furthermore, it has not been reported whether SL are reactive towards sulfhydryl groups in intact cells.

We studied four SL that are active in vitro (vernolepin, helenalin, elephantopin, and eriofertopin, Fig. 1), and compared them to seven unrelated chemotherapeutic compounds. The effect of each of these agents on the GSH content of murine P815 mastocytoma cells was determined. Buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase (26), permitted us to evaluate the importance of GSH synthesis as a determinant of tumor cell susceptibility to lysis by cytotoxic SL. We found that SL-mediated cytolysis could be augmented by more than an order of magnitude by appropriately timed interference with tumor cell GSH synthesis.

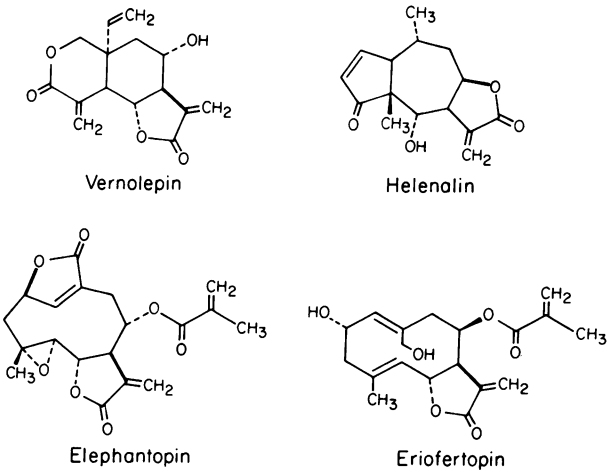

FIGURE 1 Structures of the sesquiterpene lactones in this report.

METHODS

Eagle's minimum essential medium (alpha variant), streptomycin, penicillin, and horse serum were obtained from Flow Laboratories, Rockville, MD. The following were from Sigma Chemical Co., St. Louis, MO: glucose oxidase (type V), Triton X-100, dimethyl sulfoxide, 5-sulfosalicylic acid, 5,5′-dithiobis-(2-nitrobenzoic acid), NADPH, GSH, mitomycin C, Ara-C, and vinblastine sulfate. DL-Buthionine-SR-sulfoximine was from Chemical Dynamics Corp., So. Plainfield, NJ. $Na_2{}^{51}CrO_4$ was obtained from New England Nuclear, Boston, MA. The following compounds were obtained through the courtesy of Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, and Dr. J. D. Douros, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute: helenalin (NSC 85236); vernolepin (NSC 106398); elephantopin (NSC 100046); eriofertopin (NSC 283439); jatrophone (NSC 135037); maytansine (NSC 153858); BCNU (NSC 409962); and doxorubicin hydrochloride (NSC 123127).

Tumors. P815 mastocytoma, YAC-1 lymphoma, and TLX9 lymphoma tumors were maintained by intraperitoneal passage of ascites in histocompatible mice as described (4, 27). For experiments, these tumors were grown in stationary suspension cultures in MEM, supplemented with 100 μg/ml of streptomycin, 100 U/ml penicillin, and 10% heat-inactivated horse serum. J774 cells were maintained in Dulbecco's MEM, 5% fetal bovine serum with antibiotics in spinner culture, and were the kind gift of Mr. G. Healey and Dr. J. Unkeless, The Rockefeller University.

Glutathione depletion. Tumor cells in MEM, 5% horse serum ($1.0 \times 10^6 - 1.7 \times 10^6$/ml) were incubated at 37°C with the various test compounds dissolved in dimethyl sulfoxide, or vehicle alone, for the indicated times. Dimethyl sulfoxide content ranged from 0.1 to 1% and did not affect GSH levels.

Biochemical assays. Total cellular glutathione (GSH plus GSSG) was measured by a minor modification of the method of Tietze as previously described (5, 28). Total glutathione is expressed as nanomoles of the tripeptide per milligram of cell protein and is referred to as GSH in this report. Extracts of cells treated with compounds that we found to cause GSH depletion were mixed with known amounts of GSH and then assayed to rule out the presence of an inhibitor of the Tietze assay, which might have accounted for the observed loss of GSH. The activity of glucose oxidase was measured with the scopoletin assay for H_2O_2 as described (29). Glutathione reductase was assayed by the method of Roos et al. (30). Protein content was determined by the method of Lowry et al. (31) using bovine serum albumin as the standard.

Inhibition of glutathione synthesis with BSO. We have previously reported that with these tumors a maximal rate of GSH depletion, and thus maximal inhibition of synthesis, was achieved with 0.2 mM BSO (5). The rate of GSH depletion in the presence of BSO reflects GSH catabolism, efflux, and dilution by cell division in the absence of synthesis. BSO stock solution (20 mM in H_2O) was stored at 0°C for up to 3 wk before use.

Cytolysis assays. Cells were labeled with $Na_2{}^{51}CrO_4$ as described (27). Cytolysis was studied in three experimental settings: (*a*) 4×10^4 labeled cells were incubated with various concentrations of lytic agent or vehicle alone (0.1–1% dimethyl sulfoxide) in the presence or absence of BSO (0.2 mM) in 0.22 ml of MEM, 5% horse serum at 37°C in 5% CO_2, 95% air for 18 h. (*b*) Cells (1.5×10^6/ml in MEM, 5% horse serum) were incubated with various concentrations of vernolepin or vehicle alone (0.1% dimethyl sulfoxide) plus $Na_2{}^{51}CrO_4$ for 1 h, washed extensively (four centrifugations),

ethyl)-1-nitrosourea; BSO, buthionine sulfoximine; LD_{50}, concentration of lytic agent causing 50% specific release of ^{51}Cr label from the cells; MEM, Eagle's minimum essential medium, alpha variant; SL, sesquiterpene lactone.

and then incubated in MEM, 5% horse serum (1.5×10^5 in 1 ml) for 18 h. BSO (0.2 mM) was added to the medium at various times as indicated in each experiment. (*c*) 4×10^4 labeled cells were incubated with dilutions of glucose oxidase plus vernolepin or vehicle alone (0.1% dimethyl sulfoxide) in the presence or absence of BSO (0.2 mM) in 0.22 ml of MEM, 5% horse serum at 37°C for 5 h.

At the indicated times (18 h for *a* and *b*, 5 h for *c*), supernatant (0.1 ml for *a* and *c*, 0.5 ml for *b*) was removed after centrifugation for gamma counting and the percent specific release was calculated as described (27). The amount of lytic agent causing 50% specific release of ^{51}Cr label (LD_{50}) was determined from a dose-response curve by interpolation. In protocol *b*, if ^{51}Cr release was measured after 26 h rather than 18 h, the LD_{50} was reduced by a factor of 1.2 while the spontaneous release increased by a factor of 1.4. In some experiments the ^{51}Cr release assay was validated by comparison to the trypan blue dye exclusion test as described (4). Each reported data point represents the mean of triplicate determinations.

RESULTS

Depletion of tumor cell GSH by SL. The possible interaction of cellular GSH with SL was first evaluated. Incubation of P815 mastocytoma cells with vernolepin, helenalin, elephantopin, or eriofertopin for 1 h resulted in 70–97% depletion of GSH (Table I). The time course and dose response of GSH depletion by helenalin were examined in greater detail in the experiments illustrated in Fig. 2. Cells were incubated with a range of concentrations of helenalin with and without 0.2 mM BSO, a nontoxic and selective inhibitor of the first of two enzymes responsible for GSH biosynthesis, γ-glutamylcysteine synthetase (26). P815 cells incubated with BSO remain viable (5), and continue to divide in its presence for more than 3 wk (unpublished observations). Depletion of GSH by incubation

TABLE I
Effect of Cytotoxic SL on GSH Content of P815 Cells

SL	μg/ml	% GSH remaining*	n
Vernolepin	10	6.7±2.7	(5)
	25	2.8±0.5	(2)
Helenalin	5	41±6.8	(2)
	10	23±1.2	(3)
	25	5.7±1.6	(3)
Elephantopin	5	39±2.9	(4)
	15	12±0.2	(2)
Eriofertopin	50	29±3.6	(4)

* P815 cells were incubated in MEM, 5% horse serum with the indicated concentrations of sesquiterpene lactones for 1 h. GSH content was determined as described in Methods. Data are from 10 experiments with initial GSH = 29.6±7.6 nmol/mg of protein.

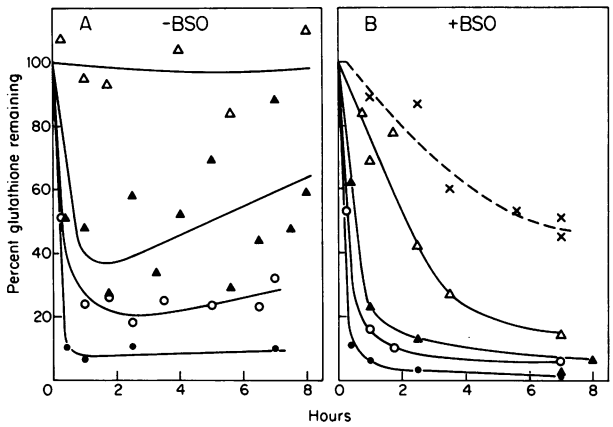

FIGURE 2 Time course and dose response of GSH depletion by helenalin in the absence (A) and presence (B) of BSO. P815 cells were incubated with helenalin at 1 μg/ml (△), 5 μg/ml (▲), 10 μg/ml (○), 25 μg/ml (●), or no helenalin (×). In panel B, the incubation medium also contained 0.2 mM BSO. Data are from five experiments with initial GSH levels of 28.1±6.3 nmol/mg of protein.

of P815 cells with 25 μg/ml of helenalin was both rapid (90% loss within 15 min) and persistent (up to 7 h of coincubation) (Fig. 2A). Incubation of cells with 1 μg/ml of helenalin did not result in detectable GSH depletion. However, the rate of GSH depletion upon incubation with 1 μg/ml of helenalin plus BSO exceeded that observed with BSO alone (Fig. 2B). By inference, 1 μg/ml of helenalin must react with a substantial portion of intracellular GSH, and rapid resynthesis, if unimpeded, maintains GSH content at normal levels. Similarly, the recovery of GSH levels observed with 5 μg/ml of helenalin was abolished by the inclusion of BSO (Fig. 2, closed triangles). By examination of the medium after incubation of cells with helenalin, we determined that depletion of GSH was not due to its release from the cells (not shown).

Effect of BSO on the lysis of tumor cells by SL. We evaluated the sensitivity of ^{51}Cr-labeled tumor cells to lysis by an 18-h coincubation with SL. Fig. 3 illustrates one such experiment in which the lysis of P815 cells by helenalin in the presence or absence of 0.2 mM BSO was measured. Incubation of tumor cells with BSO alone was nontoxic. However, cytolysis by helenalin was greatly enhanced: nonlytic concentrations of helenalin resulted in near-complete lysis when BSO was included (Fig. 3). In Fig. 3, BSO reduced the LD_{50} for helenalin by a factor of 4.7. The effect of BSO on the sensitivity of four tumor cell lines to lysis by SL is documented in Table II. In each experiment, a range of SL concentrations was tested and the LD_{50} was calculated as in Fig. 3. In all cases, the presence of BSO reduced the LD_{50} manyfold. BSO provided the

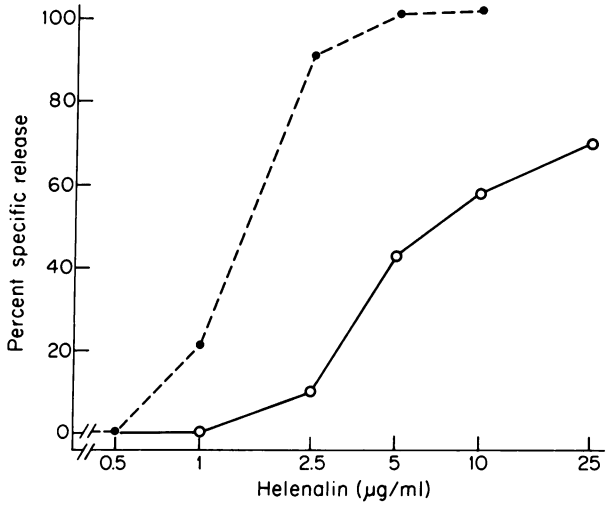

FIGURE 3 Effect of BSO on sensitivity of P815 cells to lysis by helenalin. ^{51}Cr release was measured after an 18-h incubation with the indicated concentrations of helenalin in the absence (○) or presence (●) of 0.2 mM BSO. Points are means of triplicate determinations. SE averaged 2.4%. Spontaneous release of ^{51}Cr label was 22%.

most dramatic synergy with vernolepin, leading to an ~20-fold decrease in the LD_{50} for P815.

It is of interest to note that for these tumors, the rank order of resistance to lysis by vernolepin (J774 > P815 > TLX9 > YAC) does not correspond to GSH content (J774 > YAC > P815 > TLX9) (4). Furthermore, incubation of P815 cells with 10 μg/ml of vernolepin for 1 h before the 18-h assay did not result in increased cytolysis (not shown), in spite of substantial depletion of GSH (>90% in Table I). Thus, in contrast to the importance of GSH resynthesis in protecting tumor cells upon exposure to SL, GSH content prior to exposure to these agents did not influence subsequent lysis.

The synergistic interaction between BSO and vernolepin was further analyzed by considering the kinetics of onset and reversal of their effects. For this, exposure of cells to vernolepin was limited to a 1-h pulse, followed by an 18-h incubation in its absence, at which time lysis was determined. Fig. 4 illustrates the results obtained in one of three such experiments in which lysis in the absence of BSO (open triangles, LD_{50} = 63 μg/ml) was compared to: lysis observed when BSO was present 30 min before and during the 1-h vernolepin incubation (open circles, LD_{50} = 7.3 μg/ml); during this time as well as the subsequent 18-h incubation (closed circles, LD_{50} = 6.8 μg/ml); or during the 18-h incubation only (closed triangles, LD_{50} = 13.8 μg/ml). Thus, addition of BSO need not coincide with but can immediately follow exposure to vernolepin for markedly enhanced cytolysis to result.

Time course of recovery from the effects of vernolepin and BSO. Fig. 5 illustrates the correlation between GSH resynthesis and loss of tumor cell sensitivity to the synergistic effect of BSO added after a 1-h pulse of vernolepin. Recovery of GSH content upon subsequent incubation was rapid, reaching control levels by 3.5 h. Recovery of GSH content could be inhibited by BSO, indicating that *de novo* resynthesis was occurring. A 1.5-h delay in the addition of BSO to cells previously pulsed for 1 h with 10 μg/ml of vernolepin allowed for resynthesis of GSH to ~60% of control, and resulted in roughly a 60% decrease in enhancement of cytolysis, compared to the addition of BSO immediately after the vernolepin pulse. The marked cytolysis observed by treatment of cells with 10 μg/ml of vernolepin followed by an 18-h incubation with BSO was not prevented by the addition of up to 7 mM GSH to the medium shortly after the start of the 18-h incubation (not shown).

TABLE II
Effect of BSO on Sensitivity of Tumor Cells to Lysis by SL

Tumor	SL	LD_{50}, μg/ml* (μM)	BSO-induced relative increase in sensitivity to lysis‡	n
P815	Vernolepin	21±13 (75)	20±8.3	(13)
P815	Helenalin	12±3.8 (47)	6.4±1.6	(4)
P815	Elephantopin	28±4.5 (79)	5.6±2.5	(4)
P815	Eriofertopin	68±24 (195)	6.8±1.8	(5)
YAC	Vernolepin	4.6±2.0 (17)	8.9±2.4	(4)
TLX9	Vernolepin	16±7.4 (59)	19±10	(4)
J774	Vernolepin	47±16 (169)	34±18	(3)

* Lysis of ^{51}Cr-labeled tumor cells after an 18-h incubation with the indicated SL was evaluated as described in Methods. LD_{50} values were calculated by interpolation from curves like those in Fig. 3. Average spontaneous ^{51}Cr release in the presence and absence of BSO, respectively, for the four tumors: P815, 26.6±4.4, 25.7±4.3% (n = 26); YAC, 21.3±4.6, 20.0±3.0% (n = 4); TLX9, 21.8±1.1, 21.0±2.3% (n = 4); J774, 20.1±1.7, 19.9±2.4% (n = 3).
‡ The LD_{50} in the absence of BSO divided by the LD_{50} in the presence of 0.2 mM BSO represents the relative increase in sensitivity to lysis by the various SL.

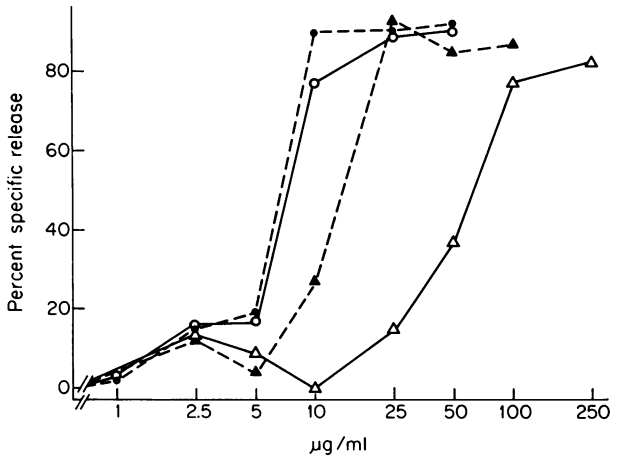

FIGURE 4 Effect of BSO on sensitivity of P815 cells to lysis at 18 h after a 1-h incubation with vernolepin. Cells were pulsed with the indicated concentrations of vernolepin for 1 h and then incubated for 18 h, at which time ^{51}Cr release was measured. BSO was present at 0.2 mM 30 min before and during the 1-h pulse (○), during this time as well as the subsequent 18-h incubation (●), during the 18-h incubation only (▲), or never (△). Points are means of triplicate determinations. SE averaged 1.2%. Spontaneous release was 20–22%.

The reversal of BSO inhibition of γ-glutamylcysteine synthetase is not immediate (5). We therefore compared the time course of recovery from the effects of BSO on cellular GSH content and on sensitivity to vernolepin-mediated cytolysis. As shown in Fig. 6, P815 cells were incubated with BSO for 1 h, washed, incubated for various additional times, and then assayed both for GSH content and for susceptibility to lysis by vernolepin. Gradual recovery of GSH synthesis was evident within 2 to 3 h of the removal of BSO. A net increase in GSH content did not occur until 4 or more h of incubation in the absence of BSO (Fig. 6A). Similarly, ~3 h after a 1-h incubation with BSO, cells began rapidly to recover their resistance to the cytolytic effect of vernolepin (Fig. 6B). Thus, augmentation by BSO of susceptibility to lysis by vernolepin disappeared just as the tumor cells regained their capacity to synthesize GSH. Addition of cycloheximide (10 μg/ml) after removal of BSO did not inhibit recovery, indicating that synthesis of new γ-glutamylcysteine synthetase was not required in order to reverse the effects of BSO (not shown).

GSH depletion by other antitumor agents and the effect of BSO on cytolysis by these agents. We were interested in extending our observations to a variety of antitumor agents, with and without known sulfhydryl reactivity. Many antitumor agents recently derived from plants, in addition to the cytotoxic sesquiterpene lactones, have been shown to possess high reactivity toward sulfhydryl groups (21–23, 32–35). Jatrophone, one such compound, was isolated in 1970 from a plant used in Costa Rica for the treatment of cancer. Incubation of P815 cells with jatrophone resulted in marked depletion of GSH, with only 11% of initial levels remaining after a 1-h incubation with 15 μg/ml (Table III). P815 cells were incubated for 18 h with various concentrations of jatrophone in the presence or absence of BSO, at which time lysis was measured. One of four such experiments is shown in Fig. 7. Inhibition of GSH synthesis resulted in a 21.3±7-fold increase in sensitivity to lysis, relative to cells allowed to synthesize GSH. In contrast, prior depletion of GSH by pulsing cells with 10 μg/ml of vernolepin for 1 h did not sensitize the cells to the lytic effects of jatrophone (not shown).

In a similar manner, we studied six other antitumor agents chosen from three major classes of antineoplastics (alkylating agents, anti-metabolites, and natural products). The effects of these compounds on the GSH content of P815 cells, using a 1-h incubation at

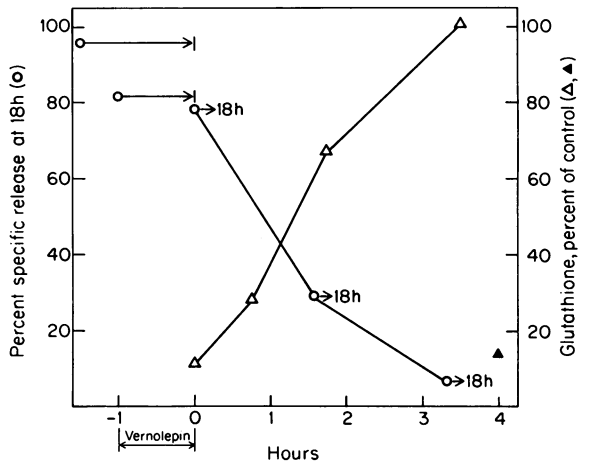

FIGURE 5 Correlation between the rate of GSH resynthesis after a 1-h incubation with vernolepin and the loss over time of synergistic cytolysis when BSO was added after the vernolepin pulse. In the cytolysis assay, cells were pulsed with 10 μg/ml of vernolepin for 1 h and then incubated for 18 h at which time ^{51}Cr release was determined. BSO was present in the medium (0.2 mM) for the indicated time spans (○→), i.e. either it was present during the vernolepin pulse and then washed out or it was added at the indicated times after the pulse treatment for the remainder of the 18-h incubation. SE averaged 1.3% for triplicates. Spontaneous release was <28%. Percent specific release from cells pulsed with vernolepin but never incubated with BSO was <3%. Unlabeled P815 cells were similarly incubated with 10 μg/ml of vernolepin for 1 h, washed, incubated in the presence (▲) or absence (△) of BSO for the indicated times, and then assayed for GSH content. Untreated cells contained 24.4±2.4 nmol GSH/mg of protein (n = 4).

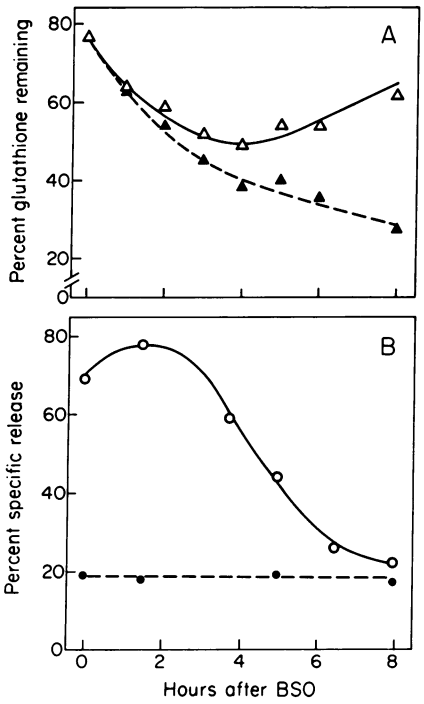

FIGURE 6 Time course of recovery from the effects of BSO. (A) P815 cells were incubated with 0.2 mM BSO for 1 h, washed, and then incubated in the presence (▲) or absence (△) of BSO. At various times GSH content was determined and is here expressed as percent GSH remaining (initial level, 24.2 nmol/mg of protein). (B) Cells were incubated for 1 h in the presence (○) or absence (●) of BSO, washed, and then incubated in its absence. At the indicated times, vernolepin (20 μg/ml) and ^{51}Cr were added for an additional 1 h, cells were washed, and then incubated for 18 h, at which time specific release of the ^{51}Cr label was determined. Spontaneous release was 20%. SE averaged 1%.

concentrations that would be lytic in an 18-h assay, are documented in Table III. Of these, only BCNU was able significantly to reduce the GSH content of the cells. However, unlike jatrophone or SL, cytolysis by BCNU after an 18-h incubation was not affected by the presence of BSO (Table III). The lack of an effect of BSO on cytolysis by the other five agents correlated with their inability to deplete cellular GSH (Table III).

Effect of vernolepin on susceptibility of P815 cells to oxidative cytolysis. Inhibition of the GSH redox cycle sensitizes tumor cells to lysis by H_2O_2 (4, 5). It was therefore of interest to evaluate the effect of vernolepin on lysis of P815 cells by a flux of H_2O_2 generated by the addition of glucose oxidase to the glucose-containing medium. In one such experiment (Fig. 8A), H_2O_2-mediated lysis at 5 h was markedly enhanced in the presence of 10 μg/ml of vernolepin. Fig. 8B illustrates the dose-response of vernolepin-induced sensitization to glucose oxidase-mediated cytolysis. A >6.5-fold increase in susceptibility to lysis by H_2O_2 was achieved with 20 μg/ml of vernolepin (Fig. 8B). Addition of BSO together with the indicated concentrations of vernolepin served to enhance even further the oxidative lysis of these cells (Fig. 8B). Inclusion of BSO alone was without effect (Fig. 8A).

DISCUSSION

Elucidation of the defense mechanisms employed by tumor cells in response to chemotherapeutic agents could be of use in the design of synergistic therapeutic combinations and in the analysis of drug resistance. In this report, we have identified GSH synthesis as an essential component of murine tumor cell defense against the toxic effect of a class of experimental antitumor agents, the SL. These studies extend earlier investigations into the importance of GSH as a determinant of both effectiveness and toxicity of a variety of antineoplastics, including cyclophosphamide (36), L-phenylalanine mustards (37, 38), nitrosoureas (4, 39), doxorubicin (39–42), hydrogen peroxide (4–6), and γ-irradiation (43, 44).

Incubation of P815 mastocytoma cells with any of the four SL studied (vernolepin, helenalin, elephantopin, and eriofertopin) resulted in rapid, dose-dependent depletion of GSH, presumably via adduct formation. Comparison of the rate of GSH depletion by low concentrations of helenalin (1–5 μg/ml) in the presence of BSO, a nontoxic and selective inhibitor of γ-glutamylcysteine synthetase, with that observed with BSO alone or helenalin alone, suggests that a compensatory increase in the rate of GSH synthesis is an early cellular response to these agents.

We evaluated the dose-response of SL-mediated cytolysis, in the presence or absence of BSO, by measurement of ^{51}Cr released after an 18-h coincubation. Incubation of P815 cells with BSO and the resulting depletion of GSH, was without effect on their viability or even their growth (5). In striking contrast, inhibition of GSH synthesis during an 18-h incubation with SL markedly enhanced the lysis of the same cells. A potent synergistic effect of BSO on cytolysis by vernolepin was also observed with each of the other murine tumors tested (YAC, TLX9, and J774).

To characterize further the role of GSH synthetic capacity as a determinant of tumor cell susceptibility to cytolysis by SL, we pulsed P815 cells with vernolepin for 1 h, washed them, and assayed for ^{51}Cr release after an additional 18-h incubation in the absence of vernolepin. Addition of BSO to cells promptly after the vernolepin pulse resulted in enhanced lysis. However, as the interval lengthened between the verno-

TABLE III

Comparison of the Effect of Various Antitumor Agents on GSH Content of P815 Cells with the Effect of BSO on Sensitivity of P815 to Lysis by these Agents

	GSH content after 1 h with indicated concentrations			Lysis at 18 h		
Lytic agent	μg/ml of lytic agent	% GSH remaining*	n	LD_{50}, μg/ml‡ (μM)	BSO-induced relative increase in sensitivity to lysis	n
Doxorubicin	100	96±10	(2)	74±14	1.2±0.8	(3)
	300	101±18	(3)	(127)		
Mitomycin C	5	91±4.7	(2)	5.8±0.6	0.99±0.06	(2)
	10	96±0.5	(3)	(17)		
Vinblastine	100	82±6.0	(2)	30±5.0	0.74±0.11	(2)
				(33)		
Ara-C	300	81±14	(2)	4.9±0.6	0.71±0.10	(2)
				(18)		
Maytansine	50	88±4.6	(3)	36±4.0	0.91±0.09	(3)
				(52)		
BCNU	50	13±2.9	(2)	37±7.0	1.17±0.05	(3)
	100	10±1.6	(2)	(174)		
	200	20±9.3	(2)			
Jatrophone	5	38±2.7	(3)	6.8±1.0	21.3±7.0	(4)
	15	11±1.5	(2)	(22)		

* Data are from five experiments with initial GSH = 25.4±10.5 nmol/mg of protein.

‡ LD_{50} values were calculated by interpolation from dose-response curves. For Ara-C, LD_{25} is reported. S.E. averaged 2.4% for all agents. Average spontaneous ^{51}Cr release in the absence and presence of BSO, respectively, was 23.9±4.2 and 22.3±4.1% (n = 19).

lepin pulse and exposure to BSO, the tumor cells rapidly lost this enhanced susceptibility to lysis, with a time course that correlated with the restoration of GSH levels.

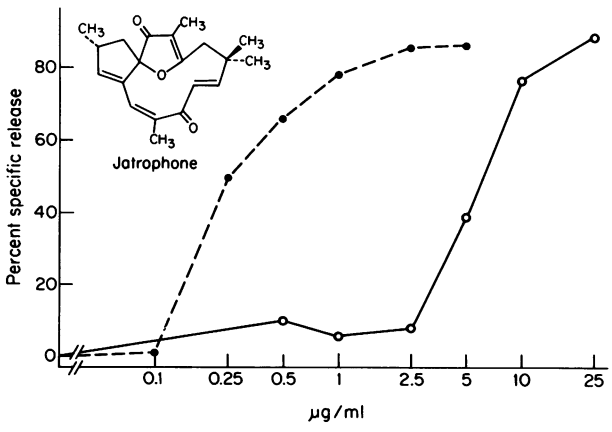

FIGURE 7 Effect of BSO on sensitivity of P815 cells to lysis by jatrophone. ^{51}Cr release was measured after an 18-h incubation with the indicated concentrations of jatrophone in the absence (○) or presence (●) of 0.2 mM BSO. SE averaged 1.9% for triplicates. Spontaneous release was <26%.

BSO probably does not inhibit GSH synthesis by intact cells immediately after it is added to the extracellular medium. This substrate analog must first enter the cell (45) and be phosphorylated by its target enzyme, γ-glutamylcysteine synthetase (46, 47). More cytolysis was evident if BSO was present not only during the 1-h vernolepin treatment, but 30 min before it as well (96% vs. 81%, $p < 0.05$; Fig. 5). Thus, P815 cells appear to be more sensitive to the lytic effects of vernolepin if their GSH synthetic capacity is already inhibited at the onset of vernolepin exposure.

Another aspect of BSO-induced inhibition of GSH synthesis relevant to our analysis is its delayed reversibility. After a 1-h pulse with BSO, GSH synthetic capacity sufficient to replenish cellular GSH was not manifest until 4–5 h of further incubation. Likewise, if a pulse with BSO preceded treatment with vernolepin, a synergistic effect on cytolysis was only seen when the interval between exposures to the two agents was <4–5 h.

The effect of BSO on cytolysis by seven additional antitumor agents was evaluated, and compared to the sulfhydryl-reactivity of the same compounds. With one exception, the relative ability of the cytotoxic

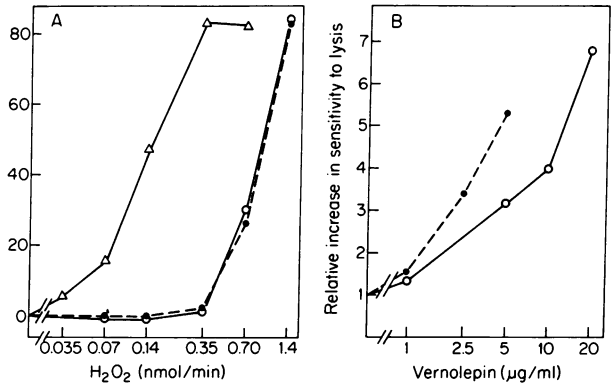

FIGURE 8 Effect of vernolepin on sensitivity of P815 cells to lysis by a flux of H_2O_2. ^{51}Cr release was measured after a 5-h incubation with dilutions of glucose oxidase. (A) Present during the cytolysis assay was 10 μg/ml of vernolepin (Δ), 0.2 mM BSO (●), or neither (○). Points are means of triplicate determinations. SE averaged 1.4%. Spontaneous release of ^{51}Cr was <10%. (B) Vernolepin at the indicated concentrations was included in the 5-h incubation with glucose oxidase in the presence (●) or absence (○) of BSO. LD_{50} were calculated by interpolation from curves like those in panel A, and the relative increase in sensitivity to lysis (LD_{50} (control)/LD_{50} (treated)) was determined. LD_{50} (control) = 0.79 nmol of H_2O_2/min. Spontaneous release averaged 13.1±.8% (n = 8). Addition of vernolepin at concentrations greater than those indicated resulted in significantly elevated spontaneous releases (i.e. lysis in the absence of glucose oxidase).

agent to deplete GSH correlated with its ability to interact synergistically with BSO resulting in increased cytolysis. Five of the compounds tested, including the ansa macrolide maytansine, did not significantly affect cellular GSH content and did not cause increased lysis in the presence of BSO. Conversely, cytolysis by jatrophone, which depleted cellular GSH, was markedly enhanced by BSO. In contrast, the ability of BCNU to deplete GSH, observed by us and others (39, 48), did not translate into increased lysis in the presence of BSO. One explanation might be that a cellular metabolite or degradation product of BCNU mimics the effect of BSO, by inhibiting one of the two enzymes of GSH synthesis or by depleting cysteine, in which case the addition of BSO would be without consequence. Alternatively, the depletion of GSH by BCNU may be of no relevance to either its mechanism of injury or the biochemistry of cellular response and repair.

As a determinant of cytolytic susceptibility, the importance of functional γ-glutamylcysteine synthetase during and after exposure to sesquiterpene lactones surely reflects a critical requirement for GSH synthesis at those times. In contrast, GSH content prior to incubation of cells with vernolepin or jatrophone affords no protection. One possible interpretation is that the amount of vernolepin consumed by reaction with intracellular GSH at the onset of drug exposure is not significant relative to the amount of vernolepin added to achieve lysis.

One can envisage a number of explanations for the importance of GSH in SL-mediated cytolysis. SL did not deplete whole-cell GSH to an extent greater than that obtained by overnight incubation with BSO, which was nontoxic (5). However, it is possible that the GSH in particular organelles, such as mitochondria or the nucleus, was depleted more by SL than by BSO, with toxic consequences. Alternatively, GSH might serve to detoxify SL, either prior to the alkylation of target molecules, or by preventing cross-linking through reaction with a second sulfhydryl-reactive site, in analogy with the biscysteine adducts of helenalin, vernolepin, and elephantopin (17, 20). Finally, a role for GSH in the restoration of critical sulfhydryl groups subsequent to alkylation should be considered. Future experiments will focus on these questions. Finally, the cardinal issue, whether inhibition of tumor cell GSH synthesis will enhance the therapeutic efficacy of SL and similar sulfhydryl-reactive agents in tumor-bearing animals, is under study.

We have previously reported that prior depletion of GSH (5) or inhibition of the GSH redox cycle (4) sensitizes tumor cells to oxidative cytolysis. In this report, we have shown that the capacity for GSH synthesis, regardless of GSH content prior to drug exposure, is a critical determinant of susceptibility to lysis by SL or jatrophone. We have further demonstrated that vernolepin, acting as a GSH-depleting agent, can itself sensitize tumor cells to lysis by H_2O_2. It is conceivable, therefore, that the coordinated deployment of oxidative injury, a SL, and BSO might constitute an effective chemotherapeutic strategy.

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