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Cellular glutathione as a protective agent against 4-hydroperoxycyclophosphamide cytotoxicity in K-562 cells*

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Summary. Exposure of cells of the K-562 erythroleukemia cell line to 4-hydroperoxycyclophosphamide (4-HC), an analog of activated cyclophosphamide, causes a concentration-dependent inhibition of in vitro colony formation by these cells. For investigation of the role of glutathione (GSH) in the metabolism of 4-HC, GSH levels of K-562 cells were modulated by exposing the cells to buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, and/or to GSH ethyl esters. Both the mono- and diethyl esters of GSH were synthesized in our laboratories and their identities were determined by chromatographic methods and fast-atom-bombardment mass spectrometry. An HPLC method including electrochemical detection used for thiol determination was applied for the measurement of GSH esters. Incubation of the cells with BSO depleted GSH levels to approximately 11% of control values and potentiated the cytotoxicity of 4-HC. By contrast, exposure to GSH esters approximately doubled GSH levels and protected the cells against the toxicity of 4-HC. Moreover, when cellular GSH levels were first depleted by BSO exposure and then replenished by incubation with GSH esters, the BSO-associated potentiation of 4-HC cytotoxicity was abolished. The work described herein extends the application of an HPLC method used for thiol determination to the measurement of GSH ethyl esters. In addition, it established that GSH acts as a competitive protecting agent against the in vitro toxicity of 4-HC in the K-562 cell line.

Introduction

A variety of evidence suggests that glutathione (GSH), the major intracellular nonprotein thiol, acts to protect cells against the toxic effects of cyclophosphamide. Thus, depletion of cellular GSH both in vivo and in vitro has been associated with potentiation of the cytotoxicity of cyclophosphamide and its metabolites [10, 20, 27]. On the other hand, the resistance to the drug seen in some cell lines has been attributed to the presence of elevated levels of cellular GSH [8, 18].

In 1985 Anderson et al. [2] reported the synthesis of ethyl esters of GSH. These workers demonstrated that these esters, unlike GSH itself, were readily taken up by cells and that the subsequent hydrolysis trapped the released GSH within the cell. It thus became possible to raise cellular GSH levels experimentally at will in cell lines that otherwise had low or normal levels. These esters represent a new experimental tool for examination of the role of GSH in metabolic processes, such as protection against the cytotoxicity of radiation or chemotherapeutic agents [16, 29, 32]. This report describes a study that used a GSH ethyl ester preparation to increase GSH levels and buthionine sulfoximine (BSO, a specific inhibitor of GSH synthesis [13]) to decrease GSH levels in K-562 cells so as to examine more closely the protective role of cellular GSH against the toxicity of cyclophosphamide.

Materials and methods

Materials. The K-562 cell line, derived from a patient with blastic chronic myelogenous leukemia [17], was obtained from the American Type Culture Collection (Rockville, Md.). 4-Hydroperoxycyclophosphamide (4-HC) was obtained from Dr. O. M. Colvin (The Johns Hopkins Oncology Center, Baltimore, Md.). BSO, GSH, and GSH reductase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Silica gel plates (5 × 20 × 0.25 cm) were purchased from the JT Baker Chemical Co. (Phillipsburg, N. J.).

Cell culture and drug exposure. The K-562 cell line was maintained in logarithmic growth phase in liquid medium consisting of RPMI 1640

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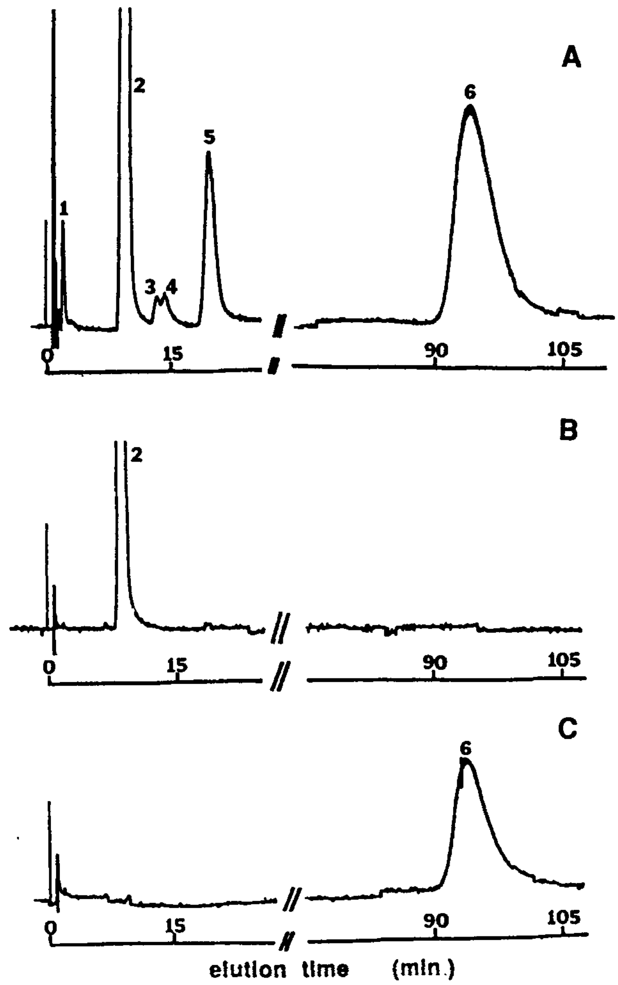

Fig. 1 A–C. HPLC-EC profiles of GSH esters. A Crude product. B, C Products isolated from TLC bands 1 and 2, respectively

(Gibco, Madison, Wis.) with 10% (v/v) fetal calf serum (FCS) (Hyclone, Logan, Utah), 1% (v/v) penicillin: streptomycin (P/S) antibiotics (Gibco), and 1% (v/v) L-glutamine (Gibco). For each experiment, cells were harvested within 2 days of cell passage. Where indicated, the cells (2×10^5/ml) were preincubated with BSO (50 µM) for 18–22 h. The ethyl esters of GSH, synthesized in our laboratories (see below), were first dissolved in 2% NaOH and then incubated with the cells (1×10^6/ml) for 1 h at a concentration of 10 mg/ml. For some experiments, the cells were exposed first to BSO for 17 h and then to GSH esters for 1 h in the presence of BSO. Prior to incubation with 4-HC, BSO and/or GSH esters were removed by washing. The cells (1×10^5/ml) were incubated with 4-HC for 1 h at 37° C in an atmosphere containing 7.5% CO_2. After removal of the drug by washing, the ability of the cells to form colonies in semisolid medium was assayed as previously described [23].

GSH ethyl ester synthesis. The synthesis was performed following the method of Anderson et al. [2]. Thin-layer chromatography (TLC) of the product was carried out on silicic acid plates using a solvent consisting of an *n*-propanol: acetic acid: water mixture (12:1:3, v/v). After drying, the plates were sprayed with 0.2% ninhydrin in ethanol. Bands from TLC plates were scraped and pooled. GSH esters were extracted into the TLC solvent, dried, and redissolved in water containing 0.1% heptafluorobutyric acid (HFBA). The samples were desalted after being loaded on to C_{18} Sep-Pak reverse-phase cartridge columns (Waters Associates), which had been charged with 10 ml 80% acetonitrile/water/0.1% HFBA (v/v), and were then washed and equilibrated with 10 ml 0.1% HFBA/water (v/v) [5, 26]. Next, the eluate was evaporated under N_2. Fast-atom-bombardment (FAB) mass spectra were measured on a Finnigan MAT 212 double-focusing mass spectrometer interfaced to a Finnigan Spectrosystem SS200 data system. FAB ionization was accomplished using an Ion Tech FAB11NF saddle-field fast atom gun (Ion Tech Ltd., Middlesex, England) powered by an Ion Tech B50 power supply operated at 8 kV. Samples were dissolved in glycerol and bombarded with a xenon atom beam. Several scans were recorded over the range of 15–1,000 U at a scan rate of 8 s/decade.

Analytical procedures. The HPLC system with an electrochemical detector (HPLC-EC) for the detection of thiols has previously been described [15] and was used for determinations of reduced cysteine, GSH, and GSH ethyl esters. Total GSH levels were determined by HPLC-EC after reduction with GSH reductase. Briefly, the cell lysate was incubated for 10 min at room temperature in buffer containing 100 mM NaH_2PO_4, 5 mM Na_2EDTA, 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 1.6 units/ml GSH reductase (pH 8). The reaction was stopped by addition of a 1 M perchloric acid solution, and the supernate was analyzed for GSH by HPLC-EC. Oxidized GSH was determined by the subtraction of reduced GSH from total GSH. Total GSH was also measured following the method of Tietze [30]. Nonspecific thiol determination was carried out according to the method of Ellman [12].

Results

Characterization of the GSH ester preparation

Analysis of the GSH ester preparation by HPLC with electrochemical detection (HPLC-EC) indicated that the synthesized material contained two major and three minor thiol components (Fig. 1 A). Peak 1 co-chromatogrammed with authentic GSH and is presumed to represent unreacted starting material. Based on area-under-the-curve (AUC) calculations, this unreacted GSH represented about 1% of the total thiol material. Peaks 2 and 6 were the major components, representing about 48% and 40% of the total thiols, respectively. The minor components, peaks 3, 4, and 5, together accounted for about 11% of the total thiols.

TLC of the GSH ester preparation on silicic acid revealed two ninhydrin-positive bands [retention factor (*Rf*), 0.66 and 0.82] that were distinct from that of the starting material (GSH, *Rf* = 0.27). On elution from the TLC plates, band 1 (*Rf* = 0.66) yielded one peak when subjected to analysis by HPLC-EC (Fig. 1 B); this material co-chromatogrammed with peak 2 from the original ester preparation (Fig. 1 A). When eluted from the silicic acid and subjected to HPLC-EC analysis, band 2 (*Rf* = 0.82), also yielded one peak (Fig. 1 C), which co-chromatogrammed with peak 6 from the original ester preparation (Fig. 1 A). The FAB mass spectra for authentic GSH and the two major reaction products isolated from TLC plates are shown in Fig. 2. The FAB spectrum of authentic GSH (Fig. 2 A) showed a peak at m/z 308, which corresponds to the protonated molecular ion of GSH. This peak was well distinguished from the background peaks attributable to the glycerol matrix. The compounds isolated from TLC bands 1 and 2 yielded FAB-spectra ions at m/z 336 and 364, respectively (Fig. 2 B, C). These ions correspond to the protonated forms of the mono- and diester, respectively. These data suggest that band 1 (peak 2) is the monoester of GSH and band 2 (peak 6) is the diester. However, since the two possible monoesters (glutamyl

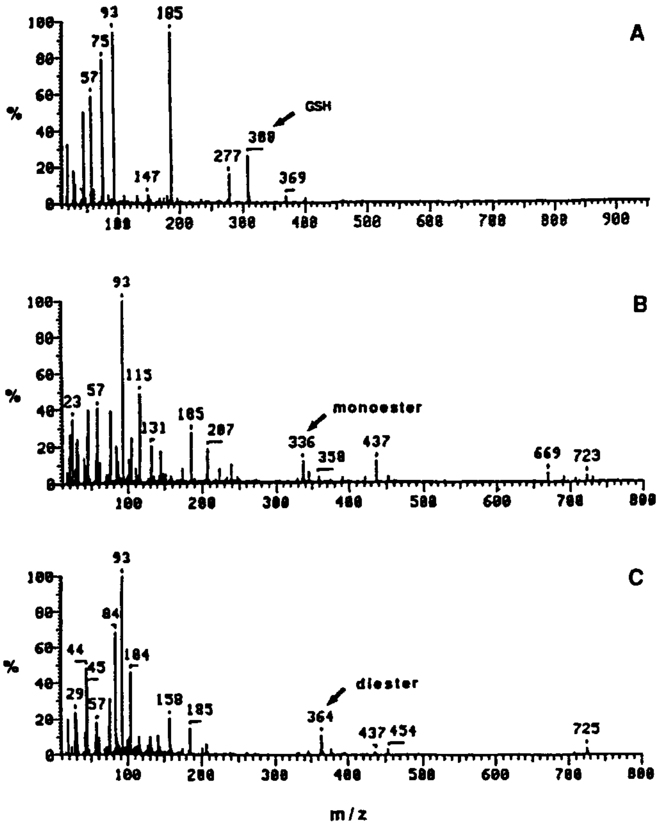

Fig. 2 A–C. FAB mass spectrometric analyses of A GSH and products isolated from TLC bands B 1 and C 2. m/z 308 = MH$^+$ of GSH (A); m/z 336 = MH$^+$ of GSH monoethyl ester (B); and m/z 364 = MH$^+$ of GSH diethyl ester (C)

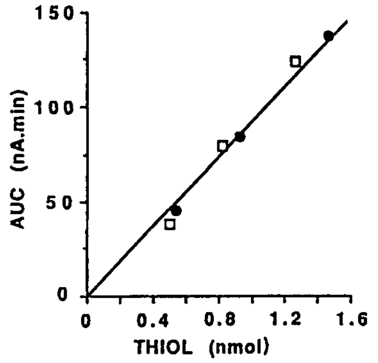

Fig. 3. Quantitation of GSH ethyl esters by HPLC-EC. ●, monoethyl ester; □, diethyl ester

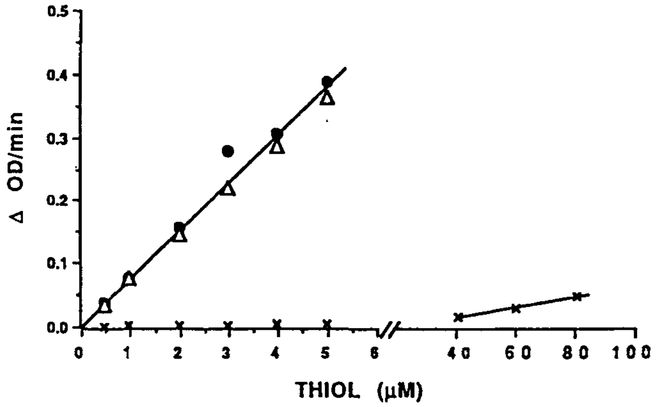

Fig. 4. Determination of GSH and GSH esters by the Tietze assay. ●, GSH; Δ, GSH esters; ×, GSH in the presence of equimolar concentrations of GSH esters

ester and glycyl ester) cannot be distinguished on the basis of their m/z values and the limited fragmentation data in Fig. 2B was insufficient to enable structural assignment, we could not determine which of the two monoesters predominated in the synthesized ester preparation. The minor peaks (3, 4, and 5) detected by HPLC-EC were not found by TLC and could not be further characterized.

Quantitation of GSH esters

For development of quantitative assays for the mono- and diesters of GSH, preparative TLC on silicic acid was performed and the material of bands 1 and 2 was extracted. The purity of the extracted thiol was established by HPLC-EC, and the amount of each purified thiol was determined by assay with Ellman's reagent. Standard curves were developed using HPLC-EC. As shown in Fig. 3, the AUC for both the mono- and the diester was linear, with the amount of thiol being up to 1.5 nmol. The limit of detection, estimated as being 3 times the background signal, was 10 pmol.

Attempts to estimate the GSH esters by the Tietze assay [30], which depends on GSH reductase recycling, were unsuccessful. As shown in Fig. 4, no significant increase in absorption at 412 μm was seen until the concentration of esters exceeded 40 μM. Even this increase can be accounted for by the presence of small amounts of unreacted GSH (ca. 1%) remaining in the synthesized and recrystallized material. It was noteworthy that attempts to inhibit the estimation of GSH by addition of GSH ester at concentrations of up to 100-fold that of GSH were also unsuccessful, indicating that even in the presence of GSH esters, GSH may be measured by the Tietze procedure. These findings also suggest that GSH esters are neither substrates nor inhibitors of GSH reductase.

Modulation of cellular GSH levels

The levels of reduced GSH in K-562 cells grown in logarithmic phase were 4.2 ± 0.3 nmol/10^6 cells (Table 1). The oxidized form of GSH, estimated from the difference between total GSH (measured by HPLC-EC following reduction of the samples with GSH reductase) and reduced GSH, was always <5% of total GSH.

BSO, a specific inhibitor of γ-glutamyl cysteine synthase, and the GSH ethyl ester preparation were used to modulate the cellular levels of GSH in K-562 cells. Exposure of the cells to BSO (50 μM for 18–22 h) depleted GSH levels to 11% ± 6% of control values (Table 1). Exposure of cells to GSH esters (10 mg/ml for 1 h) increased cellular GSH levels to 195% ± 33% of control values. Total low-molecular-weight thiols (GSH, cysteine, and GSH esters) increased to 402% ± 57% of control values (Table 1). Exposure of BSO-treated cells to the GSH ester preparation during the last hour of incubation with BSO brought the GSH levels back to normal values and the total

Table 1. Effect of BSO and/or GSH ethyl esters on intracellular thiol levels

Treatment	Thiol (nmol/10^6 cells)			
	Glutathione	Cysteine	GSH esters	Total thiols
Control (14)	4.2±0.3	<0.1	–	4.2±0.3
BSO (12)	0.5±0.1*	<0.1	–	0.5±0.1*
GSH esters (4)	8.2±0.7*	2.2±0.2	6.4±0.4	16.9±1.2*
BSO + GSH esters (3)	4.2±0.9	1.1±0.6	4.8±0.8	10.2±2.3*

The values given represent the means ± SEM; numbers in parentheses represent the number of separate experiments (for incubation protocols, see Materials and methods)

* Significantly different from control values ($P < 0.05$)

low molecular-weight thiol content to >2 times the normal values (243%±95%; Table 1). Importantly, the treatment regimens used for GSH modulation did not affect cell survival or growth per se.

Effect of variation in cellular GSH levels on 4-HC toxicity

For examination of the role of cellular GSH in the in vitro cytotoxicity of 4-HC, K-562 cells were exposed to the drug following modulation of their GSH levels. The concentration of 4-HC resulting in 50% inhibition of colony formation (IC_{50}) was extrapolated from a log-log transformation of the dose-response curves (Fig. 5 and 6, right panels). In agreement with earlier studies [23, 24], 4-HC caused a concentration-dependent inhibition of colony formation by K-562 cells (IC_{50}, 8 μM; Fig. 5). Preincubation of the cells with BSO, which depleted GSH levels, significantly potentiated the cytotoxic effect of 4-HC (IC_{50}, 5 μM; Fig. 5). In contrast, preincubation with the GSH ethyl ester preparation for enhancement of GSH levels was associated with significant protection against 4-HC cytotoxicity (IC_{50}, 14 μM; Fig. 5).

To determine whether the BSO-mediated potentiation of 4-HC could be reversed by replenishing GSH levels, we exposed K-562 cells to GSH esters during the last hour of BSO incubation. Cellular GSH levels were returned to normal (100%±37%) and the potentiating effect of BSO on 4-HC cytotoxicity was abolished (IC_{50}, 13 μM for both control and BSO + ester groups; Fig. 6).

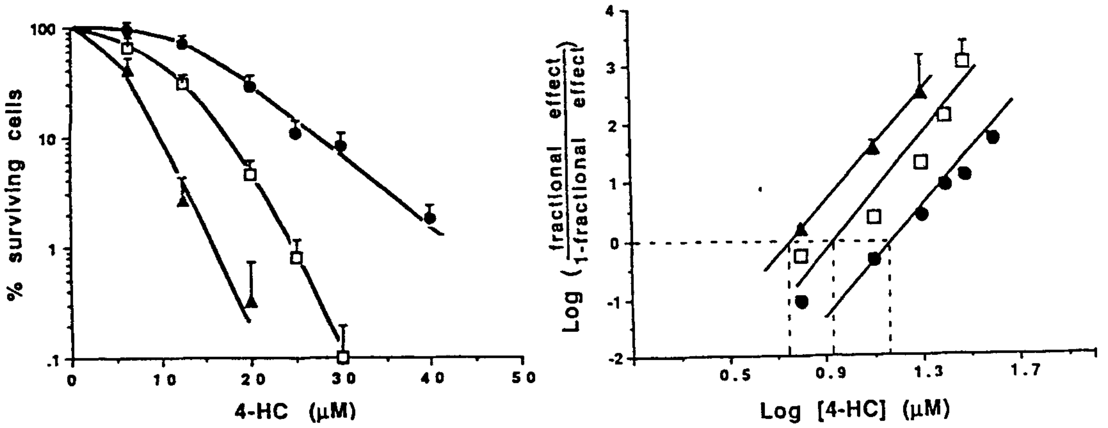

Fig. 5. Effect of BSO or GSH esters on 4-HC toxicity. The cells were exposed first to BSO (50 μM for 18–22 h) or GSH esters (10 mg/ml for 1 h) and then to 4-HC for 1 h. Means of four determinations (+ SEM) are reported. □, control; ▲, BSO; ●, GSH ester

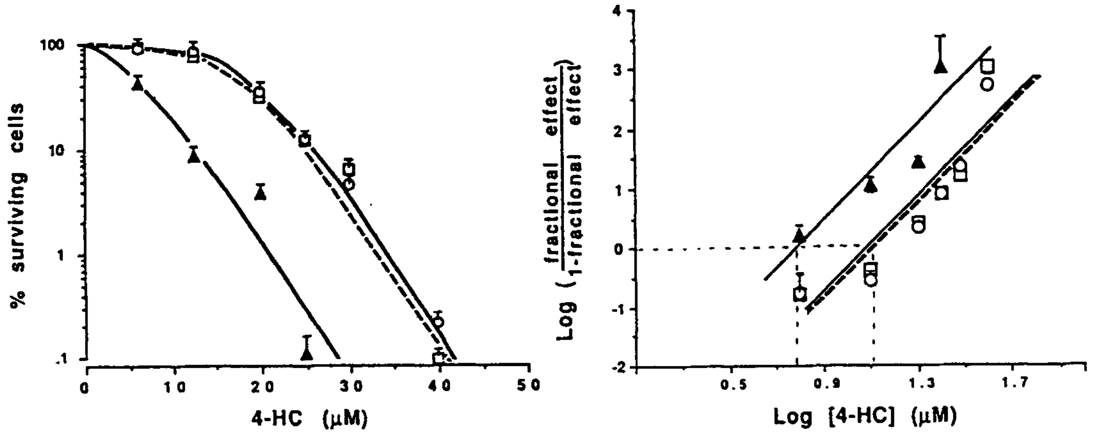

Fig. 6. Effect of BSO and GSH esters on 4-HC toxicity. The cells were exposed to BSO (50 μM for 17 h) and GSH esters (10 mg/ml for 1 h) in the presence of BSO. Next, they were exposed to 4-HC for 1 h. Means of four determinations (+ SEM) are reported. □, control; ▲, BSO; O, BSO + GSH ester

Discussion

It is well known that GSH serves many roles in the cell: it can act as a reductant; as a transport and storage form of cysteine; and as a protective agent against free radicals, reactive oxygen species, and toxic compounds (for review see [19]). Several studies have suggested that an increase in cellular GSH may protect against cyclophosphamide cytotoxicity, whereas a decrease may potentiate the effect of the drug [8, 10, 18, 20, 27]. Also, the addition of low-molecular-weight thiols [cysteine, *N*-acetylcysteine, sodium-2-mercaptoethanesulfonate (mesna), GSH, or GSH esters] to in vivo or in vitro systems seems to protect cells against the toxicity of cyclophosphamide and its metabolites [3, 4, 10, 14, 25, 29, 31].

However, only Crook et al. [10] have attempted to assess the effect of *both* a depletion of and an increase in GSH on cyclophosphamide cytotoxicity in a given cell type. These workers showed that the depletion of GSH by preincubation of K-562 cells with BSO potentiated the in vitro effect of microsomally activated cyclophosphamide on the cells, whereas their preincubation with cysteine increased GSH levels by 60% above normal values and protected these cells against microsomally activated cyclophosphamide. However, since cysteine itself is known to protect against cyclophosphamide cytotoxicity [7, 11, 14], this protection could have resulted either in whole or in part from increased intracellular cysteine levels. Recently, Teicher et al. [29] reported that GSH monoethyl ester protected animals against the lethal effects of cyclophosphamide. Histological evidence indicated that the ester protected the liver from damage, but not the marrow or the implanted FSaII tumor; however, since GSH levels were not reported, this study provides at best indirect evidence that increasing GSH levels with GSH esters can protect against cyclophosphamide toxicity.

Collectively, these studies suggest that low-molecular-weight thiols protect against cyclophosphamide cytotoxicity; however, the role of cellular GSH in the metabolism and efficacy of the drug can be better understood only by studying the effect of careful modulation of cellular GSH levels on the cytotoxicity of cyclophosphamide in a given system. In the present study, we investigated the effect of modulation of cellular GSH levels on the in vitro cytotoxicity of 4-HC in the K-562 cell line by using BSO and GSH esters as specific means for the depletion and increase of GSH, respectively.

To increase GSH levels experimentally in K-562 cells, we synthesized and characterized ethyl esters of GSH. Two products, representing approximately 90% of the GSH ester preparation, were detected by HPLC-EC (peaks 2 and 6, Fig. 1) and identified by FAB mass spectrometry analysis as being the mono- and diethyl esters of GSH (Fig. 2B, C). Importantly, the HPLC-EC system previously used for GSH determination [15] proved to be suitable for quantitation of GSH ethyl esters (Figs. 1, 3). This assay procedure enabled the simultaneous estimation of cysteine, GSH, and the mono- and diethyl esters of GSH.

To establish that GSH protects K-562 cells against the cytotoxicity of 4-HC, we determined the effect of modulation of GSH levels on the inhibition of colony formation mediated by 4-HC. Because depletion of GSH was associated with potentiation of 4-HC and increase in GSH correlated with protection against the drug (Fig. 5), these in vitro studies clearly indicate that GSH protects K-562 cells against the toxicity of 4-HC.

Since the enzymic specificity of BSO for GSH synthesis may not be absolute, it is possible that the potentiation could be due to cellular effects other than GSH depletion. Reversal of the BSO-mediated potentiation of 4-HC by replenishment of GSH with the GSH ester preparation suggests that the effect of BSO on K-562 cells is mediated through depletion of GSH (Fig. 6). Also, the shift of the dose-reponse curves with changes in GSH levels suggests that GSH mediates a competitive detoxification of 4-HC, as suggested by other investigators [7, 11]. Interestingly, when other thiol groups (cysteine, GSH esters) are also measured, the cells reprotected by GSH esters after BSO treatment actually show more than a 2-fold increase in total low-molecular-weight thiols as compared with the controls (Table 1). However, the cells do not show "overprotection". This lack of "overprotection" despite the increase in total low-molecular-weight thiols suggests that GSH is more efficient at protecting cells against 4-HC than are cysteine and the esters.

Although the protective role of GSH against the cytotoxicity of cyclophosphamide is generally accepted, the scientific evidence for this protection is based only on separate studies investigating the effect of either a depletion of or an increase in GSH on the toxicity of the drug. Perhaps with the exception of the study of Crook et al. [10], no study has yet investigated the effect of both specific depletion of and specific increase in GSH levels on cyclophosphamide cytotoxicity in a given system. Collectively, aided by the use of an HPLC-EC system enabling specific measurements of cysteine, GSH, and GSH esters, our findings demonstrate that GSH plays a protective role against 4-HC in the K-562 cell line. Moreover, this protection fits a model of competitive detoxification of 4-HC by GSH.

The actual mechanism by which GSH protects against cyclophosphamide cytotoxicity is not known. Certainly, cyclophosphamide and three of its metabolites have been shown to react with low-molecular-weight thiols such as cysteine, *N*-acetylcysteine, sodium-2-mercaptoethanesulfonate (mesna), or GSH [1, 6, 7, 11, 21, 25, 34]. The reversible formation of 4-thio derivatives by substitution of the hydroxyl group of 4-hydroxycyclophosphamide has been reviewed [34]. However, since several synthetic representatives of 4-thiocyclophosphamide have been shown to undergo rapid hydrolysis [22], the role of the reversible formation of 4-thio adducts as a mode of deactivation of the drug has been questioned [33]. Upon the opening of the oxazaphosphorine ring and the release of phosphoramide mustard, the chloroethyl groups react readily with nucleophilic centers, and substitution of the chlorides of phosphoramide mustard by low-molecular-weight thiols has been described [9, 21, 34]. Another metabolite of cyclophosphamide, acrolein, which has been suggested to be responsible for the urotoxicity of the drug, can also react with sulfhydryl groups [1, 6]. Such reaction is exploited in clinical treatments by the administration of mesna to patients receiving cyclophosphamide therapy so as to protect them against cyclophosphamide-induced hemorrhagic cystitis [6]. Thus, the direct conjugation and inactivation of one or more metabolites of 4-HC with GSH is possible and would explain the competing detoxification of 4-HC by GSH observed in the present studies.

As a final note, our in vitro clonogenic assay does not measure the selective antitumor effect of 4-HC, but only its cytotoxicity. Thus, it is possible that in vivo depletion of GSH by BSO could result in an increase in the overall toxicity of cyclophosphamide and, consequently, in a decrease in the therapeutic index, as has been suggested [28].

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