Biochemical Pharmacology, Vol. 44, No. 7, pp. 1383–1393, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

DEPLETION OF A DISCRETE NUCLEAR GLUTATHIONE POOL BY OXIDATIVE STRESS, BUT NOT BY BUTHIONINE SULFOXIMINE

CORRELATION WITH ENHANCED ALKYLATING AGENT CYTOTOXICITY TO HUMAN MELANOMA CELLS *IN VITRO*

VESNA JEVTOVIĆ-TODOROVIĆ and THOMAS M. GUENTHNER*
Department of Pharmacology, University of Illinois College of Medicine at Chicago, Chicago, IL 60612, U.S.A.

(*Received* 26 *February* 1992; *accepted* 12 *June* 1992)

Abstract—The existence of a distinct pool of glutathione in the nucleus of cultured human melanoma cells was demonstrated. Melanoma cell nuclei contained 13–35 pmol of glutathione/10^6 nuclei, or approximately 0.4–1.3% of the total cellular glutathione. This nuclear glutathione pool resisted depletion by buthionine sulfoximine, an agent that inhibits glutathione synthesis, but was rapidly and reversibly depleted by subtoxic concentrations of Adriamycin® plus carmustine, two agents that promote oxidation of glutathione without permitting its regeneration through enzymatic reduction of glutathione disulfide. The ability of Adriamycin plus carmustine to deplete this small but significant pool of glutathione in the cell nucleus may explain why these agents potentiate the cytotoxic effects of the DNA-alkylating agent melphalan to a much higher degree than does buthionine sulfoximine at concentrations that are equipotent in depleting cytosolic glutathione.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH†) plays a major role in protecting cells from the cytotoxic effects of many drugs and environmental compounds [1–4]. The role of GSH in protecting tumor cells from the cytotoxic effects of antineoplastic drugs, particularly DNA-alkylating agents, has attracted intense scrutiny; numerous reports have correlated levels of tumor cell GSH, either constitutive or following biochemical manipulation, with the relative susceptibility or resistance of tumor cells to a variety of antitumor agents [5–7]. A particularly attractive paradigm for the enhancement of alkylating agent cytotoxicity has been through the inhibition of GSH synthesis, resulting in lowered whole-cell GSH levels and enhanced susceptibility to alkylating agent cytotoxicity. A compound that has proved most useful in this regard is D,L-buthionine *S,R*-sulfoximine (BSO), a specific inhibitor of the rate-limiting enzyme in GSH biosynthesis, γ-glutamylcysteine synthetase [1, 8]. Treatment of cells with subtoxic concentrations of BSO typically results in depletion of cellular GSH levels to 10–20% of control levels, and a decrease in the LC_{50} for the alkylating agent melphalan (LPAM) by a factor of 1/1.5 to 1/3 [8–20].

Recently, we reported [21] another useful method for rapidly and reversibly depleting GSH from cultured human melanoma cells. Oxidative stress resulting from exposure of cells to a combination of Adriamycin® (ADR) and carmustine (BCNU) at subcytotoxic (1 μM) concentrations depletes cellular GSH, presumably by promoting the oxidation of GSH to glutathione disulfide (GSSG) and preventing the re-reduction of GSSG, due to the selective inhibition of GSSG reductase. In our hands, treatment with ADR + BCNU depletes whole cell GSH to 60–80% of control levels within 1–2 hr. This degree of GSH depletion is similar to that obtained in many laboratories with BSO treatment. However, the enhancement of the cytotoxicity of LPAM observed concomitant with ADR + BCNU-catalyzed depletion of GSH was much greater than any effect ever observed after depletion of GSH by BSO; treatment with ADR + BCNU decreased the LC_{50} for LPAM by factors ranging from 1/19 to 1/55, depending on cell type. These observations suggested that the effects of ADR + BCNU might be attributed to mechanisms additional to a simple depletion of whole-cell GSH levels. One possible explanation for this dramatic potentiation of LPAM cytotoxicity may involve the targeted depletion of GSH in subcellular compartments, specifically the cell nucleus.

It has been demonstrated previously that GSH is not uniformly distributed throughout the cell, and that distinct subcellular GSH pools exist in hepatic cytosol and mitochondria, each with different rates of turnover and depletion [3, 22, 23]. It has also been shown that GSH is present in the cell nucleus [24–27]. The demonstration that nuclear GSH is relatively less susceptible to depletion by synthesis inhibition

* Corresponding author: Thomas M. Guenthner, Ph.D., Department of Pharmacology, University of Illinois College of Medicine at Chicago, 835 South Wolcott Ave., Chicago, IL 60612. Tel. (312) 996-2558; FAX (312) 996-1225.

† Abbreviations: GSH, reduced glutathione; BSO, D,L-buthionine *S,R*-sulfoximine; LPAM, melphalan; ADR + BCNU, Adriamycin® plus carmustine; GSSG, glutathione disulfide; TKM buffer, 50 mM Tris–HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4; LDH, lactate dehydrogenase; and DMF, dose modification factor.

than is cytosolic GSH [24] suggests that nuclear GSH may constitute a kinetically distinct subcellular pool. Because the target site of alkylating agent cytotoxicity is in the cell nucleus (chromosomal DNA), we have postulated that the relatively high degree of potentiation of LPAM cytotoxicity observed after ADR + BCNU treatment may reflect a greater degree of depletion of a nuclear GSH pool by this form of oxidative stress, compared to a relative resistance of this pool to depletion by BSO. Depletion of the nuclear GSH pool may seriously compromise the ability of the cell to protect itself from DNA-directed genotoxins such as LPAM.

In pursuit of mechanisms underlying the enhancement of alkylating agent cytotoxicity that accompanies ADR + BCNU-induced oxidative stress, we have asked the following questions: (1) Can the existence of a nuclear GSH pool that behaves differently from the cytosolic GSH pool be demonstrated? (2) Does this pool respond differently to oxidative stress or inhibition of GSH synthesis when compared to the cytosolic GSH pool? and (3) Can the differences in the relative susceptibilities of the two pools to drug-induced depletion be useful in explaining the relatively potent effects of ADR + BCNU on enhancing LPAM cytotoxicity toward human melanoma?

MATERIALS AND METHODS

ADR was purchased from Adria Laboratories, Columbus, OH. BCNU was purchased from Bristol Laboratories, Evansville, IN. LPAM was a gift from the Burroughs Wellcome Co., Research Triangle Park, NC. All three drugs were obtained in sterile powder form and dissolved just prior to use. Buthionine sulfoximine, fluorodinitrobenzene, iodoacetic acid, GSH, *bis*-benzimide (Hoechst 33258), trypan blue, agar (TC grade agar-agar powder), NADH, and sodium pyruvate were obtained from the Sigma Chemical Co., St. Louis, MO. Fetal calf serum, RPMI-1640 and Eagle's MEM-E cell culture media, glutamine, penicillin, and streptomycin were obtained from Biologos Inc., Naperville, IL. Monobromobimane was purchased from the Calbiochem Co., La Jolla, CA.

Cell culture. Two independently derived human melanoma cell lines were used in this study. The MEL2 cell line was derived from metastatic melanoma by the Division of Surgical Oncology, University of Illinois, in 1986 [28], and is maintained and stored by the Specialized Center for Cancer Research at the University of Illinois. The MM253-4cg human melanoma cell line was a gift from Dr. Peter Parsons, Queensland Institute of Medical Research, Brisbane, Australia [29]. Cells were grown in monolayer culture in MEM-E (MEL2) or RPMI-1640 (MM253-4cg) medium supplemented to 10% (v/v) with fetal calf serum in an atmosphere of 5% CO_2. The doubling time was 2.2 days for the MEL2 cell line and 16 hr for the MM 253-4cg cell line. MEL2 cells from passages 18–26 and MM253-4cg cells from passages 48–53 were used for all experiments.

Drug treatment. Stock solutions of ADR were prepared in 0.9% NaCl. BCNU was dissolved in dimethyl sulfoxide, and LPAM was dissolved in 5% concentrated HCl/95% absolute ethanol. BSO was dissolved in a minimal amount of 0.1 M NaOH solution, pH > 12, which was then diluted into 10 mL of serum-free medium. All stock solutions were prepared immediately before use, and were added to cell culture medium in amounts resulting in less than 1% solvent in the medium. Control culture flasks received appropriate amounts of solvent. Cells were grown in 75 cm^2 flasks to near confluence (2.0 to 4.0×10^6 cells/flask) and exposed to drug-containing or solvent-containing medium for indicated periods of time. BSO was added to the culture medium at the start of the exposure period, and the medium was not subsequently changed before the cells were harvested. Cells were treated with ADR + BCNU according to two different exposure protocols. In one case, drugs were added to the medium, and the medium was not changed prior to cell harvest. In the other case (indicated on figures as "wash out"), cells were exposed to ADR + BCNU for 1 hr, at which time the drug-containing medium was removed and cells were incubated with fresh drug-free medium until they were harvested. Cells were dislodged from the flasks with a trypsin/EDTA solution, and a portion of the resuspended cells was counted after exposure to trypan blue to determine short-term drug toxicity. For ADR + BCNU-treated cells, viability at the time of cell harvest was greater than 95%. For BSO-treated cells, viability ranged from 90 to 97%.

Isolation of nuclei. Suspensions of melanoma cell nuclei were prepared by the method of Blobel and Potter [30]. Harvested cells were resuspended in 0.25 M sucrose in 50 mM Tris–HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4 (TKM), and homogenized with a motor-driven teflon–glass homogenizer. The cell homogenate was layered over a two-step sucrose gradient (0.25 M and 2.3 M in TKM), and centrifuged at 100,000 *g* for 30 min at 4°. The nuclear pellet was collected and resuspended in 1 M sucrose/TKM. This suspension was centrifuged at 3400 *g* for 10 min at 4°; the pellet was collected, resuspended in 0.25 M sucrose/TKM, and washed one more time by centrifugation. The final nuclear preparation was suspended in a volume of 0.25 M sucrose/TKM equivalent to 1 mL per 2×10^6 cells. The number of nuclei obtained from each preparation was determined by fluorometric quantitation of DNA, using the fluorescent dye *bis*-benzimide (Hoechst 33258) [31]. In our hands, 10 μg DNA corresponds to 1.0×10^6 nuclei. The degree of cytosolic contamination in the nuclear pellet was estimated with a standard spectrophotometric assay of the cytosolic marker enzyme lactate dehydrogenase (LDH) [32]. Data obtained from any nuclear preparation that contained LDH activity greater than that of a non-protein-containing blank incubation were not used.

Measurement of GSH levels in whole-cell and nuclear homogenates. For measurement of whole-cell GSH levels, harvested cells were lysed in a 10% perchloric acid solution, and the lysate was centrifuged. GSH levels in the perchloric acid extract were assayed by derivatization with iodoacetic acid and fluorodinitrobenzene followed by ion-exchange

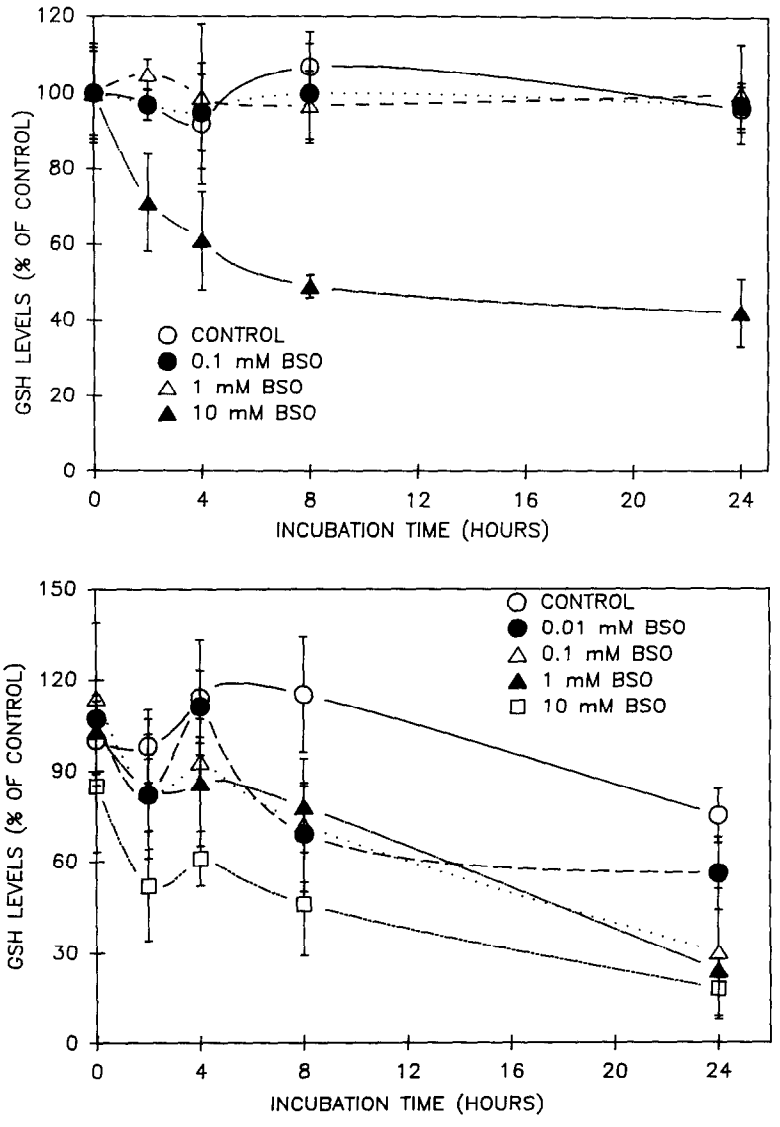

Fig. 1. GSH depletion from cultured human melanoma cells by BSO. MEL2 cells (top panel) and MM253-4cg cells (bottom panel) were grown to near confluence, exposed to the indicated concentrations of BSO, and harvested at intervals thereafter. Cell viability after 8 hr of exposure to drug-containing medium ranged from 90 to 97%. Data are GSH levels in the cells expressed as a percentage of the GSH levels found in control (untreated) cells. Each point represents a mean value obtained from three replicate flasks ± SD. Control (100%) values: 2.12 nmol GSH/10^6 cells for MEL2 (top panel) and 3.07 nmol GSH/10^6 cells for MM253-4cg (bottom panel).

HPLC separation and spectrophotometric quantitation [33]. Total cellular GSH levels were calculated as nanomoles per 10^6 live (trypan blue-excluding) cells. Each data point represents the mean value obtained from at least three determinations, expressed as a percentage of GSH levels in control cells. Nuclear GSH levels are too low to be measured accurately by this method. Therefore, a more sensitive spectrofluorometric method was used [34]. Suspensions of cell nuclei were reacted with 10 mM monobromobimane for 15 min at 37°. Soluble products were extracted with 10% trichloroacetic acid, separated by reverse-phase HPLC, and quantitated by spectrofluorometric detection. GSH levels were calculated as picomoles per microgram DNA. Each point represents the mean value obtained from 4 to 6 determinations, expressed as a percentage of GSH levels in control nuclei.

Cytotoxicity studies. Drug-induced cytotoxicity was measured as inhibition of the formation of clonal colonies in soft agar suspension [21, 35]. Cells were exposed to drugs or solvents for the indicated time periods. They were harvested, counted in the presence of trypan blue, and suspended in semisolid agar containing Eagle's MEM-E medium. A 0.5-mL volume of this suspension was then overlaid onto a

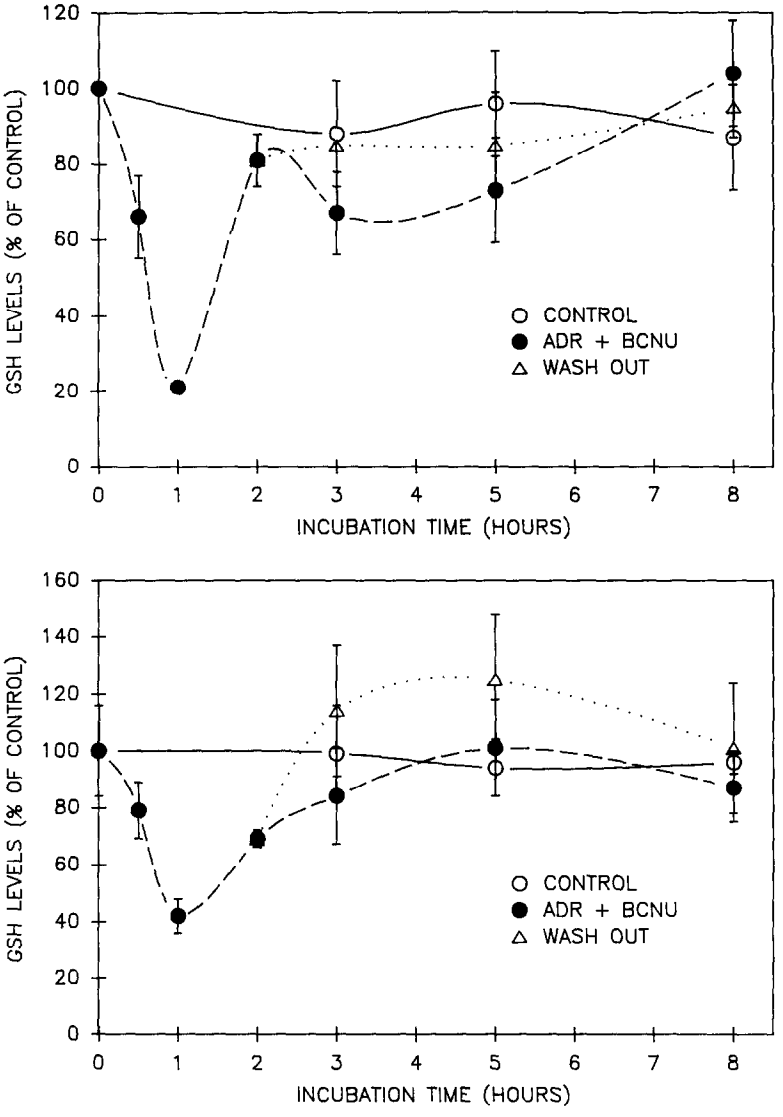

Fig. 2. GSH depletion from cultured human melanoma cells by ADR + BCNU. MEL2 cells (top panel) and MM253-4cg cells (bottom panel) were grown to near confluence, exposed to 1 μM ADR + 1 μM BCNU, and harvested at the times indicated. In some cases, drug-containing medium was replaced with fresh medium after 2 hr of exposure, and cells were harvested as usual thereafter. Cell viability after 8 hr of exposure to drug-containing medium ranged from 93 to 97%. Data are GSH levels in the cells expressed as a percentage of the GSH levels found in control (untreated) cells. Each point represents a mean value obtained from three replicate flasks ± SD. Control (100%) values: 2.93 nmol GSH/10^6 cells for MEL2 (top panel) and 3.39 nmol GSH/10^6 cells for MM253-4cg (bottom panel).

solid agar layer in 24-well microwell plates at a concentration of 10^4 cells per well. Colonies containing more than 50 cells were counted after 2 weeks. Clonogenic efficiency for control cells was always greater than 1%. Each data point represents the mean number of colonies per well, obtained from four replicate wells derived from each of three cell culture flasks.

RESULTS

Comparison of GSH depletion due to BSO exposure with GSH depletion due to ADR + BCNU exposure required that concentrations of BSO and exposure times that resulted in 60%–80% depletion of whole-cell GSH be determined. Figure 1 shows that this degree of GSH depletion was obtained with 8–24 hr of exposure to 10 mM BSO in the case of MEL2 cells (Fig. 1, top), and 0.1 mM BSO in the case of MM253-4cg cells (Fig. 1, bottom). This degree of GSH depletion was equivalent to that obtained with 1 μM ADR + 1 μM BCNU (Fig. 2). The top panel of Fig. 2 shows the time course of ADR + BCNU-promoted depletion of whole-cell

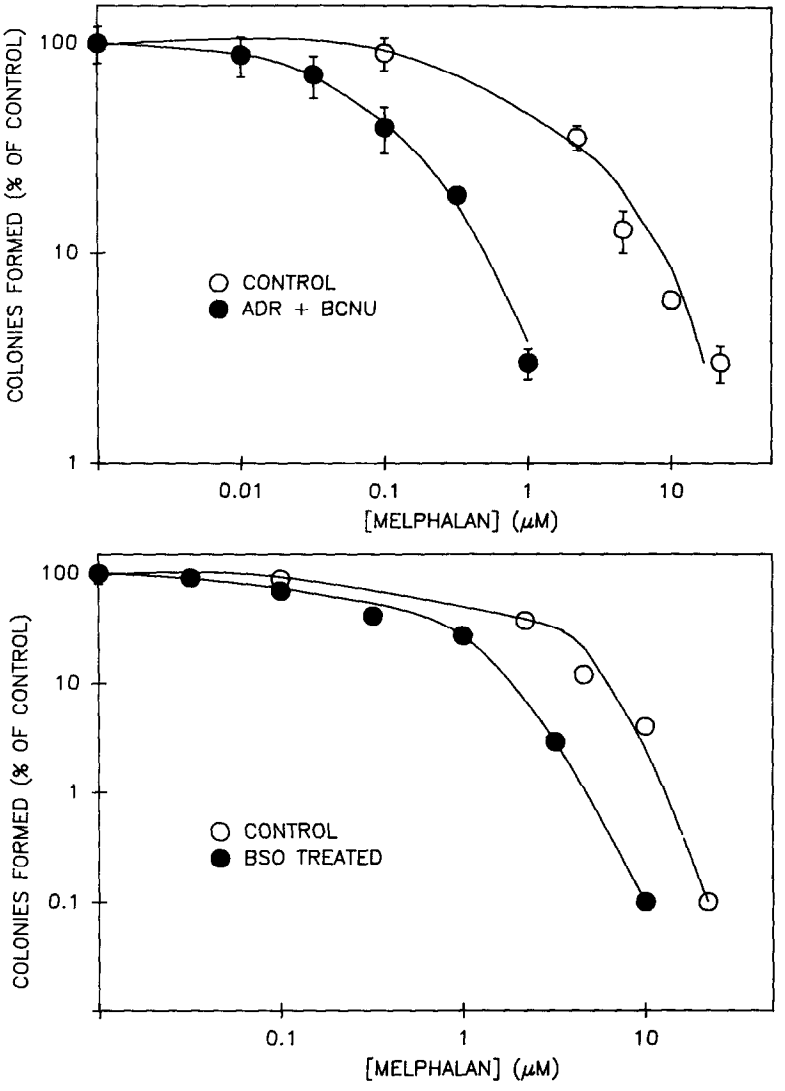

Fig. 3. Effects of GSH-depleting drugs on the cytotoxicity of LPAM to MEL2 cells. Cells were exposed to 1 μM ADR + 1 μM BCNU for 1 hr (top panel) or to 10 mM BSO for 8 hr (bottom panel), and then to LPAM for 1 hr. Cell viability after the 1-hr treatment with LPAM was greater than 90%. Long-term cytotoxicity was measured as decreased clonogenicity of treated cells in soft agar. Clonogenic efficiency for control cells was greater than 1%. Each point represents the mean value obtained from four replicate wells derived from each of three flasks ± SD. Where error bars are not drawn, they are smaller than the radius of the symbol used. Control (100%) values: 308 colonies (top panel) or 343 colonies (bottom panel) per well.

GSH levels from MEL2 cells, and the bottom panel shows depletion of whole-cell GSH from MM253-4cg cells after exposure to ADR + BCNU. Levels of GSH in untreated MEL2 cells ranged from 2.95 to 3.4 nmol/10^6 live cells; levels of GSH in untreated MM253-4cg cells ranged from 2.5 to 3.6 nmol/10^6 live cells. Either ADR or BCNU alone, at 1 μM concentrations, produces less than 10% depletion of whole-cell GSH levels [21].

Figure 3 compares the effects of GSH depletion by ADR + BCNU (top panel) to the effects of a similar degree of GSH depletion by BSO (bottom panel) on the ability of LPAM to inhibit MEL2 cell colony formation in soft agar. A dose modification ratio (DMF; the ratio of the LC_{50} of LPAM in the absence of ADR + BCNU to the LC_{50} in the presence of ADR + BCNU) of 15 was observed for MEL2 cells (Fig. 3, top). At the low (1 μM) concentrations used in these studies, ADR + BCNU have no significant short-term or long-term cytotoxic effects by themselves. The bottom panel of Fig. 3 shows that treatment of the cells with BSO, under conditions that produce a similar depletion of whole-cell GSH levels, produced a much smaller enhancement of the cytotoxicity of LPAM (DMF = 3.2). This degree of enhancement of the cytotoxicity of LPAM by BSO

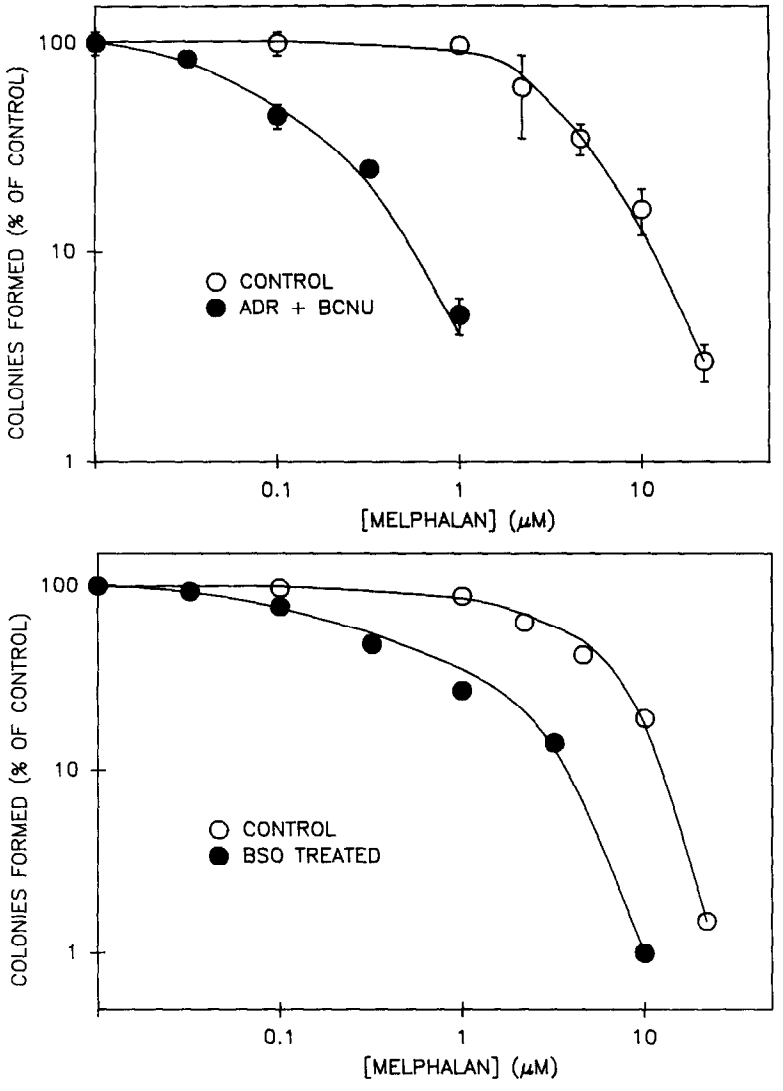

Fig. 4. Effects of GSH-depleting drugs on the cytotoxicity of LPAM to MM253-4cg cells. Cells were exposed to 1 μM ADR + 1 μM BCNU for 1 hr (top panel), or to 10 mM BSO for 8 hr (bottom panel), and then to LPAM for 1 hr. Cell viability after the 1-hr treatment with LPAM was greater than 90%. Long-term cytotoxicity was measured as decreased clonogenicity of treated cells in soft agar. Clonogenic efficiency for control cells was greater than 1%. Each point represents the mean value obtained from four replicate wells derived from each of three flasks ± SD. Where error bars are not drawn, they are smaller than the radius of the symbol used. Control (100%) values: 236 colonies (top panel) or 200 colonies (bottom panel) per well.

was similar to that observed by other investigators [8–20]. Figure 4 shows that the effects of the two methods of GSH depletion of MM253-4cg cells were similar. The top panel of Fig. 4 shows the effects of ADR + BCNU treatment on LPAM cytotoxicity, resulting in a DMF of 35. Depletion of GSH by BSO resulted in a DMF for LPAM cytotoxicity of 7.4 (Fig. 4, bottom).

Because it may be targeted to different degrees by the two methods of GSH depletion, the nuclear GSH pool was examined. Nuclei from solvent-treated MM253-4cg cells, which were regarded as controls for drug-treated cells, contained 13.4 pmol GSH/10^6 cell nuclei. This represents 0.36% of the total cellular GSH. Similar results were obtained with MEL2 cells, which contained 35 pmol GSH/10^6 cell nuclei. This represents 1.25% of the total cellular GSH. No contamination of the isolated nuclear fraction with cellular cytosol, as determined by assay of lactate dehydrogenase activity in the nuclear fraction, was detected for either cell line. This method of measuring cytosolic contamination is capable of detecting a 0.02% contamination of the nuclear fraction with cytosol. Because all data were obtained from nuclear fractions that had no detectable LDH activity, we assume that our level

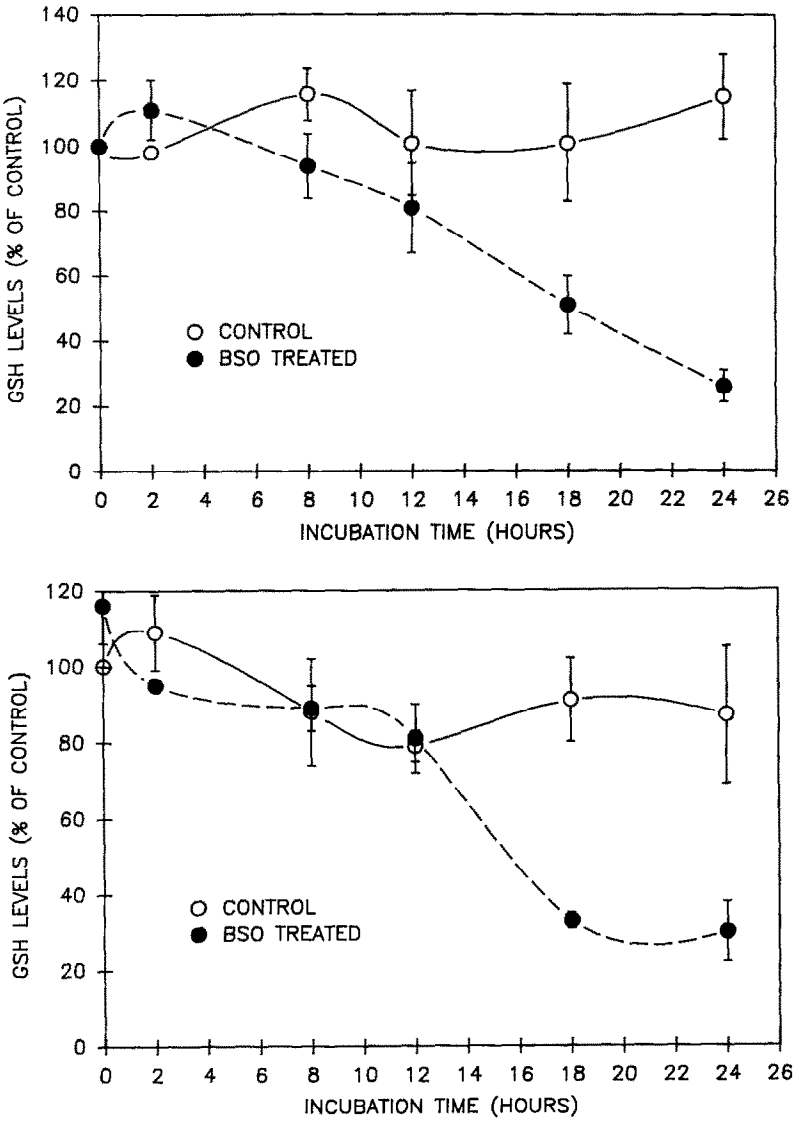

Fig. 5. GSH depletion from melanoma cell nuclei by BSO. MEL2 cells (top panel) and MM253-4cg cells (bottom panel) were grown to near confluence, and exposed to the indicated concentrations of BSO; their nuclei were harvested at intervals thereafter. Cell viability after 8 hr of exposure to drug-containing medium ranged from 90 to 97%. Data are GSH levels in the nuclei expressed as a percentage of the GSH levels found in control (untreated) nuclei. Each point represents a mean value obtained from three replicate flasks ± SD. Control (100%) values: 35 pmol GSH/10^6 nuclei for MEL2 (top panel) and 13.4 pmol GSH/10^6 nuclei for MM253-4cg (bottom panel).

of contamination was lower than 0.02%. Cytosol present at this level of contamination would contribute 0.76 pmol GSH/10^6 cell nuclei, or less than 6% of the observed nuclear GSH.

We observed differences in responses of the nuclear GSH pool and the whole-cell GSH pool (85–90% of which is cytosolic GSH) either to inhibition of GSH synthesis by BSO, or to oxidative stress promoted by ADR + BCNU. Exposure of MEL2 cells to 10 mM BSO resulted in a 50% depletion of whole-cell GSH levels within 8 hr, and a 60% depletion after 24 hr (Fig. 1, top). In contrast, depletion of nuclear GSH following BSO treatment was not significant after 8 hr, and 50% depletion was not seen until 18 hr after exposure (Fig. 5, top). Similar results were observed with MM253-4cg cells. Whole-cell GSH levels were depleted by 30% after 8 hr and by 70% after 24 hr (Fig. 1, bottom), but significant depletion of nuclear GSH levels required 18 hr of exposure (Fig. 5, bottom). On the other hand, nuclear GSH levels were depleted rapidly in both cell lines following exposure to ADR + BCNU. The top panel of Fig. 6 shows that 65% depletion of nuclear GSH levels was observed within 2 hr of treatment of MEL2 cells with ADR + BCNU. Depletion of nuclear GSH lasted for approximately

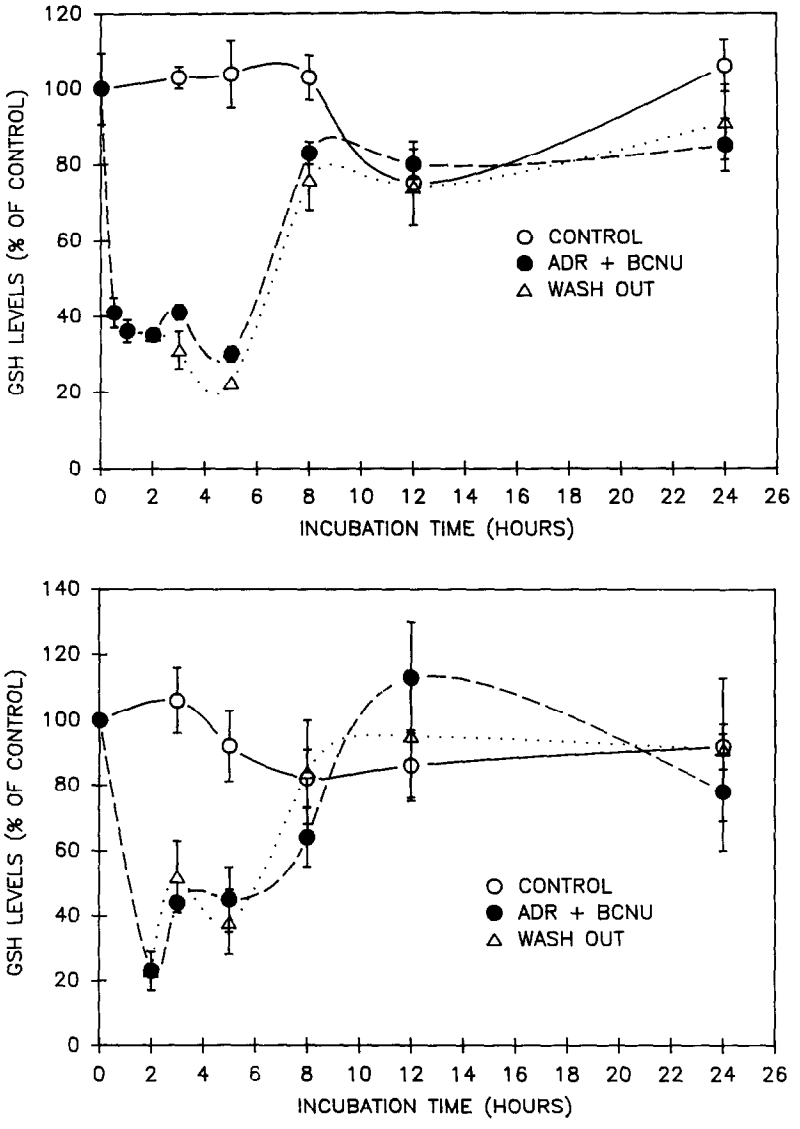

Fig. 6. GSH depletion from melanoma cell nuclei by ADR + BCNU. MEL2 cells (top panel) and MM253-4cg cells (bottom panel) were grown to near confluence, and exposed to 1 μM ADR + 1 μM BCNU; nuclei were harvested at the times indicated. In some cases, drug-containing medium was replaced with fresh medium after 2 hr of exposure, and nuclei were harvested as usual thereafter. Cell viability after 8 hr of exposure to drug-containing medium ranged from 93 to 97%. Data are GSH levels in the nuclei expressed as a percentage of the GSH levels found in control (untreated) nuclei. Each point represents a mean value obtained from three replicate flasks ± SD. Control (100%) values: 21 nmol GSH/10^6 nuclei for MEL2 (top panel) and 13.4 nmol GSH/10^6 nuclei for MM253-4cg (bottom panel).

6 hr, and then returned rapidly to control levels. Similar results were obtained with MM253-4cg cell nuclei (Fig. 6, bottom). Comparison of Figs. 2 and 6 shows that the profile of depletion of nuclear GSH by ADR + BCNU was similar to the profile of depletion of whole-cell GSH levels, but some important differences were evident. Whole-cell GSH levels were also rapidly depleted by ADR + BCNU, and to a similar level as nuclear GSH levels, but the depletion required approximately twice as long to be completed, and lasted only about half as long.

DISCUSSION

Although the presence of GSH in various intracellular organelles has long been recognized [22–27, 36, 37], its compartmentalization into discrete subcellular pools, with different rates of depletion and replenishment, was first directly demonstrated by the characterization of a distinct mitochondrial GSH pool [3, 22, 23, 37]. It is now apparent that, in addition to the cytosolic and mitochondrial GSH pools, a third discrete intracellular GSH pool exists

in the cell nucleus. It has been shown previously [24–27] that GSH is present in the cell nucleus. The compartmentalization of this nuclear GSH into a discrete pool has been suggested by experiments showing differential responses of cytosolic and nuclear GSH levels to BSO treatment [24]. On the other hand, it has also been concluded [25] that levels of GSH in the nucleus are the same as those of GSH in the cytosol, and that no distinct nuclear GSH pool exists. However, in the studies that reached these conclusions, nuclei were isolated by permeabilizing the cell membrane with detergents. These detergents may also affect the nuclear membrane, producing nuclei that are highly permeable to GSH, resulting in an artifactually rapid equilibration of nuclear GSH with the surrounding medium.

The studies presented here provide evidence that this nuclear GSH pool is kinetically distinct from the cytosolic GSH pool. The nuclear pool was depleted less rapidly after inhibition of γ-glutamylcysteine synthetase than was the cytosolic pool. Inhibition of GSH synthesis with the γ-glutamylcysteine synthetase inhibitor BSO resulted in depletion of the cytosolic GSH pool over an 8-hr period. Depletion of the nuclear GSH pool to the same extent required about 24 hr. Just as both pools were depleted by synthesis inhibition, but with different depletion kinetics, both pools were ultimately affected to a similar degree by ADR + BCNU-induced oxidative stress, but again with very different kinetic characteristics. Both the nuclear and cytosolic GSH pools were depleted within 2 hr by ADR + BCNU treatment. However, the amount of time required for recovery of the cytosolic pool to its preexposure level was only about one-sixth the time required for recovery of the nuclear pool. It appears that the nuclear pool turns over more slowly than the cytosolic pool, and is relatively slowly affected by depletion or replenishment of the cytosolic pool.

At this point there are two likely explanations for the slower rate of turnover of the nuclear GSH pool, and its relative lack of dependence on the size of the cytosolic GSH pool. The first is that nuclear GSH may be synthesized in the cytosol and transported into the nucleus. The relative independence of the rates of turnover of the two pools would be explainable if this transport of GSH and equilibration of the two pools were a relatively slow process. An alternative explanation may be that GSH is independently synthesized in the two cell compartments, and that nuclear GSH synthesis is slower, as demonstrated by the slow recovery of the nuclear GSH pool after ADR + BCNU exposure. The slower response of the nuclear pool to the synthesis inhibitor BSO might be explained by a different K_i of a nuclear form of γ-glutamylcysteine synthetase for BSO, or simply by impediments to the rapid intracellular distribution of BSO, resulting in a relatively slow accumulation of BSO in the nucleus. We are presently studying whether a nuclear form of γ-glutamylcysteine synthetase exists, and what effects BSO may have on this enzyme.

These studies also illustrate the toxicological significance of the cell's maintenance of a separate nuclear GSH pool. ADR + BCNU treatment effectively depleted both the cytosolic and nuclear GSH pools. Depletion of the nuclear GSH pool may explain the high degree to which ADR + BCNU treatment enhances the efficacy of the alkylating agent LPAM. The DMF for LPAM of 3.2 to 7.4 in the presence of BSO indicates that loss of cytosolic GSH results in an increase in susceptibility to DNA-directed cytotoxicity. However, depletion of nuclear GSH (by ADR + BCNU) produced a DMF of up to 35 for LPAM, which demonstrates a greatly enhanced vulnerability of the cell to this genotoxic agent following depletion of the nuclear GSH pool.

It appears that the nuclear GSH pool plays a crucial role in protecting the cell from agents whose target site of toxicity is in the nucleus. While cytosolic GSH may represent a "first line" of defense against potentially genotoxic nucleophiles by promoting their conjugation and excretion [1–7], nuclear GSH may play a more direct role in protecting chromosomal DNA, either by deactivation of electrophilic intermediates in the direct vicinity of DNA, by protecting nuclear membranes from the consequences of lipid peroxidation [27], or by maintaining, by preventing oxidation of their thiol groups, the integrity of protective proteins such as DNA repair enzymes [38]. Several studies have attempted to correlate rates of repair of damaged DNA with whole-cell GSH levels. In some cases a positive correlation was observed [39–42], but in several cases [18, 19, 43], a negative or no correlation was observed. In all of these studies, only whole-cell GSH levels were considered, and nuclear GSH levels were not measured. The resistance of nuclear GSH to turnover may ensure that this toxicologically important tool remains stable in the face of relatively large fluctuations in cytosolic GSH levels produced by diurnal regulation [44], dietary stimuli [44, 45], or short-term exposure to xenobiotics [46].

In summary, we have demonstrated the existence of a nuclear GSH pool, whose concentration and rate of turnover are distinct from those of the cytosolic GSH pool. This nuclear GSH pool was more resistant to depletion than the cytosolic GSH pool after BSO inhibition of GSH synthesis (8 hr for 50% depletion of the cytosolic pool vs 18 hr for 50% depletion of the nuclear pool), but was depleted rapidly by ADR + BCNU-induced oxidative stress (60–80% depletion of cytosolic and nuclear pools in 1–2 hr). The nuclear GSH pool may play a significant role in protecting nuclear target sites from attack by electrophilic chemical intermediates, and the ability of ADR + BCNU to deplete nuclear GSH may explain why these agents potentiate the cytotoxic effects of the DNA-alkylating agent LPAM to a high degree. In the search for methods for potentiating the cytotoxicity of antitumor agents, it has often been demonstrated that BSO provides an effective means of depleting GSH from tumor cell cytosol [8–20]. However, the observed potentiation of alkylating agent efficacy *in vivo* has been somewhat disappointing [10, 15, 17, 20]. Direct targeting of the nuclear GSH pool, by ADR + BCNU-induced oxidative stress, for example, appears to more selectively attack what appears to be a very

important GSH-dependent tumor cell defense against antineoplastic agents.

Acknowledgements—Supported by PHS Grant R01 CA51218 and RCDA Award K04 CA01287 (to T.M.G.) from the National Cancer Institute. The authors thank Dr. T. K. Das Gupta, Head, and Dr. C. W. Beattie and Dr. H. A. Briele, of the Specialized Center for Cancer Research, University of Illinois, for the gift of MEL2 cells, for the use of facilities, and for helpful discussions, and Dr. Peter Parsons, Queensland Institute of Medical Research, Brisbane, Australia, for the gift of MM253-4cg cells. We also thank Dr. E. W. Maynert, of this Department, for valuable discussion.

REFERENCES

1. Meister A and Anderson ME, Glutathione. *Annu Rev Biochem* **52**: 711–760, 1983.
2. Nicotera P and Orrenius S, Role of thiols in protection against biological reactive intermediates. *Adv Exp Med Biol* **197**: 41–51, 1986.
3. Reed DR, Glutathione: Toxicological implications. *Annu Rev Pharmacol Toxicol* **30**: 603–631, 1990.
4. Ketterer B and Mulder GJ, Glutathione conjugation. In: *Conjugation Reactions in Drug Metabolism: An Integrated Approach* (Ed. Mulder GJ), pp. 307–364. Taylor & Francis, London, 1990.
5. Arrick BA and Nathan CF, Glutathione metabolism as a determinant of therapeutic efficacy: A review. *Cancer Res* **44**: 4224–4232, 1984.
6. Russo A, Carmichael J, Friedman N, DeGraff W, Tochner Z, Glatstein E and Mitchell JB, The role of intracellular glutathione in antineoplastic chemotherapy. *Int J Radiat Oncol Biol Phys* **12**: 1347–1354, 1986.
7. Mistry P and Harrap KR, Historical aspects of glutathione and cancer chemotherapy. *Pharmacol Ther* **49**: 125–132, 1991.
8. Griffith OW and Meister A, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). *J Biol Chem* **254**: 7558–7560, 1979.
9. Griffith OW and Meister A, Glutathione: Interorgan translocation, turnover, and metabolism. *Proc Natl Acad Sci USA* **76**: 5606–5610, 1979.
10. Somfai-Relle S, Suzukake K, Vistica BP and Vistica DT, Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard. *Biochem Pharmacol* **33**: 485–490, 1984.
11. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34**: 2583–2586, 1985.
12. Richon VM, Schulte N and Eastman A, Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum(II) in murine leukemia L1210 cells. *Cancer Res* **47**: 2056–2061, 1987.
13. Jordan J, Doherty MD and Cohen GM, Effects of glutathione depletion on the cytotoxicity of agents toward a human colonic tumour cell line. *Br J Cancer* **55**: 627–631, 1987.
14. Russo A, Tochner Z, Philips T, Carmichael J, DeGraff W, Friedman N, Fisher J and Mitchell JB, *In vivo* modulation of glutathione by buthionine sulfoximine: Effect on marrow response to melphalan. *Int J Radiat Oncol Biol Phys* **12**: 1187–1189, 1986.
15. Skapek SX, Colvin OM, Griffith OW, Elion GB, Bigner DD and Friedman HS, Enhanced melphalan cytotoxicity following buthionine sulfoximine-mediated glutathione depletion in a human medulloblastoma xenograft in athymic mice. *Cancer Res* **48**: 2764–2767, 1988.
16. Green JA, Vistica DT, Young RC, Hamilton TC, Rogan AM and Ozols RF, Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Res* **44**: 5427–5431, 1984.
17. Kramer RA, Greene K, Ahmed S and Vistica DT, Chemosensitization of L-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine. *Cancer Res* **47**: 1593–1597, 1987.
18. Hansson J, Edgren M, Ehrsson H, Ringborg U and Nilsson B, Effect of D,L-buthionine-*S,R*-sulfoximine on cytotoxicity and DNA cross-linking induced by bifunctional DNA-reactive cytostatic drugs in human melanoma cells. *Cancer Res* **48**: 19–26, 1988.
19. Dorr RT, Reduced thiol content in L1210 cells treated with BSO increases DNA crosslinking by melphalan. *Biochem Biophys Res Commun* **144**: 47–52, 1987.
20. Ozols RF, Louie KG, Plowman J, Behrens BC, Fine RL, Dykes D and Hamilton TC, Enhanced melphalan cytotoxicity in human ovarian cancer *in vitro* and in tumor-bearing nude mcie by buthionine sulfoximine depletion of glutathione. *Biochem Pharmacol* **36**: 147–153, 1987.
21. Jevtović-Todorović V and Guenthner TM, Sensitization of human melanoma cells to melphalan cytotoxicity by adriamycin and carmustine. *J Cancer Res Clin Oncol* **117**: 313–320, 1991.
22. Meredith MJ and Reed DJ, Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* **257**: 3747–3753, 1982.
23. Jocelyn PC and Cronshaw A, Properties of mitochondria treated with 1-chloro-2,4-dinitrobenzene. *Biochem Pharmacol* **34**: 1588–1590, 1985.
24. Edgren M and Révész L, Compartmentalised depletion of glutathione in cells treated with buthionine sulphoximine. *Br J Radiol* **60**: 723–724, 1987.
25. Loh SN, Dethlefsen LA, Newton GL, Aguilera JA and Fahey RC, Nuclear thiols: Technical limitations on the determination of endogenous nuclear glutathione and the potential importance of sulfhydryl proteins. *Radiat Res* **121**: 98–106, 1990.
26. Britten RA, Green JA, Broughton C, Browning PGW, White R and Warenius HM, The relationship between nuclear glutathione levels and resistance to melphalan in human ovarian tumour cells. *Biochem Pharmacol* **41**: 647–649, 1991.
27. Tirmenstein MA and Reed DJ, Characterization of glutathione-dependent inhibition of lipid peroxidation of isolated rat liver nuclei. *Arch Biochem Biophys* **261**: 1–11, 1988.
28. Feucht KA, Walker MJ, Das Gupta TK and Beattie CW, Effect of 17β-estradiol on the growth of estrogen receptor-positive human melanoma *in vitro* and in athymic mice. *Cancer Res* **48**: 7093–7101, 1988.
29. Boon MH and Parsons PG, Cyclophosphamide resistance developed in a human melanoma cell line. *Cancer Treat Rep* **68**: 1239–1246, 1984.
30. Blobel G and Potter VR, Nuclei from rat liver: Isolation method that combines purity with high yield. *Science* **154**: 1662–1665, 1966.
31. Labarca C and Paigen K, A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102**: 344–352, 1980.
32. Bernstein LH and Everse J, Determination of the isozyme levels of lactate dehydrogenase. *Methods Enzymol* **41**: 47–52, 1975.
33. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High-performance liquid chromatography analysis of nanomole levels of

glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
34. Velury S and Howell SB, Measurement of plasma thiols after derivatization with monobromobimane. *J Chromatog* **424**: 141–146, 1988.
35. Bertoncello I, Bradley TR, Webber LM, Hodgson GS and Campbell JJ, Human tumour cell lines established using clonal agar culture. *Aust J Exp Biol Med Sci* **63**: 241–248, 1985.
36. Riley MV and Lehninger AL, Changes in sulfhydryl groups of rat liver mitochondria during swelling and contraction. *J Biol Chem* **239**: 2083–2089, 1964.
37. Jocelyn P, Some properties of mitochondrial glutathione. *Biochim Biophys Acta* **396**: 427–436, 1975.
38. Ziegler R, Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem* **54**: 305–329, 1985.
39. Begleiter A, Grover J, Froese E and Goldenberg GJ, Membrane transport, sulfhydryl levels and DNA cross-linking in Chinese hamster ovary cell mutants sensitive and resistant to melphalan. *Biochem Pharmacol* **32**: 293–300, 1983.
40. Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, Knutsen T, McKoy WM, Young RC and Ozols RF, Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47**: 414–418, 1987.
41. Vistica DT, Cellular pharmacokinetics of the phenylalanine mustards. *Pharmacol Ther* **22**: 379–406, 1983.
42. Hansson J, Edgren M, Lewensohn R and Ringborg U, Melphalan-induced DNA cross-linking in human melanoma cells and phytohaemagglutinin-stimulated lymphocytes in relation to intracellular drug content and cellular levels of glutathione. *Anticancer Res* **7**: 97–104, 1987.
43. Parsons PG, Carter FB, Morrison L and Sr. Regius Mary, Mechanism of melphalan resistance developed *in vitro* in human melanoma cells. *Cancer Res* **41**: 1525–1534, 1981.
44. Lauterburg BH, Smith CV and Mitchell JR, Regulation of hepatic glutathione homeostasis. In: *Drug Metabolism and Drug Toxicity* (Eds. Mitchell JR and Horning MG), pp. 321–330. Raven Press, New York, 1984.
45. Lauterburg BH and Mitchell JR, Regulation of hepatic glutathione turnover in rats *in vivo* and evidence for kinetic homogeneity of the hepatic glutathione pool. *J Clin Invest* **67**: 1415–1424, 1981.
46. Smith MT and Orrenius S, Studies on drug metabolism and drug toxicity in isolated mammalian cells. In: *Drug Metabolism and Drug Toxicity* (Eds. Mitchell JR and Horning MG), pp. 71–98. Raven Press, New York, 1984.