Effects of glutathione depletion on the cytotoxicity of agents toward a human colonic tumour cell line

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Summary Levels of glutathione (GSH) in tumour tissue may be important in determining the clinical response to certain anticancer agents. Recent reports have suggested that D,L-buthionine-S,R-sulphoximine (BSO), a specific inhibitor of GSH synthesis, may be used to deplete tumour cell GSH and thus increase the therapeutic ratio of these agents. We have previously shown that 1-naphthol is a potential antitumour agent, and that its possible metabolite 1,4-naphthoquinone is thiol reactive and capable of redox cycling. It was therefore of interest to investigate the effect of pretreatment with BSO, on the toxicity of these agents, to tumour cells. For comparison we included three other cytotoxic agents, melphalan, helenalin and menadione, the toxicities of which are reported to be modulated by intracellular GSH. Depletion of GSH using BSO did not effect the toxicity of 1-naphthol, or 1,4-NQ but did produce slight potentiation of the cytotoxicities of menadione, helanalin and melphalan. The lack of effect of BSO on 1-naphthol and 1,4-NQ is not easily explained but if one also considers the modest potentiation of cytotoxicity achieved with the other agents studied, the potential use of BSO in combined chemotherapy is at best rather modest.

Based on our findings that 1-naphthol is selectively toxic to short term organ cultures of human colonic tumour tissue compared to normal colonic tissue from the same patients, we suggested the potential use of 1-naphthol or related compounds in cancer chemotherapy (Cohen *et al.*, 1983; Wilson *et al.*, 1985). Recently we have also shown an antitumour activity of 1-naphthol against Ehrlich ascites tumour cells (Jones *et al.*, 1987) and it therefore is of interest to elucidate its mechanism of toxicity and formation of possible reactive metabolites. 1-Naphthol may be metabolised by a microsomal mixed function oxidase to cytotoxic naphthoquinones, primarily 1,4-naphthoquinone (d'Arcy Doherty *et al.*, 1984*a,b*, 1985). The toxicity of both 1-naphthol and its possible metabolites 1,2-naphthoquinone and 1,4-naphthoquinone, to isolated hepatocytes, is preceeded by a rapid depletion of intracellular glutathione (GSH) (d'Arcy Doherty *et al.*, 1984*b*).

GSH is the major nonprotein thiol in the cell and plays a critical role in cellular defences against oxidative stress, free radicals and alkylating agents (Meister & Anderson, 1979). One of the problems associated with chemotherapy is the wide range of sensitivities to treatment with any or one agent, which is thought to be, in part, due to the differences in sulphydryl levels in tumours. Several recent reports, have therefore considered the potential of modulating intracellular GSH levels in order to increase the chemotherapeutic efficacy of certain antitumour agents, whose toxicity is modulated by GSH (Akman *et al.*, 1985; Arrick *et al.*, 1983; Capranico *et al.*, 1986; Hamilton *et al.*, 1985; Russo *et al.*, 1986; Suzukake *et al.*, 1982, 1983).

The cytotoxic and antitumour effects of certain quinones e.g. adriamycin and menadione, are thought to be related to oxidative stress which arises through the capacity of those compounds to redox cycle (Kappus & Sies, 1981; Thor *et al.*, 1982). Flavoenzymes catalyze a one electron reduction of naphthoquinones to form semiquinone radicals which can readily autoxidise in the presence of molecular oxygen (O_2) forming large amounts of the superoxide anion radical ($O_2^{\cdot-}$), which may then in turn spontaneously, or in a reaction catalysed by superoxide dismutase, dismutate to produce hydrogen peroxide (H_2O_2) which in turn may undergo a metal catalysed reaction forming hydroxyl radical ($OH^{\cdot}$), an extremely powerful oxidant (Bachur *et al.*, 1978; Thor *et al.*, 1982; Wendel *et al.*, 1981; Powis *et al.*, 1981).

This may lead to conditions of oxidative stress, lipid peroxidation, damage to DNA and other vital cellular constituents (Smith *et al.*, 1985). GSH may protect against naphthoquinone mediated oxidative stress in several ways, including direct reaction with the parent naphthoquinone or its semiquinone radicals, or by removing with glutathione peroxidase either H_2O_2 formed or hydroperoxides produced as a result of lipid peroxidation (Nickerson *et al.*, 1963; Wendel *et al.*, 1981). It seems reasonable therefore, to suggest that GSH may play a role in the protection of tumour cells against 1-naphthol or its possible metabolite, 1,4-naphthoquinone. In this study, intracellular GSH was depleted in LoVo cells, a human colonic adenocarcinoma cell line (Drewinko *et al.*, 1976), using DL-buthionine-S-R sulphoximine (BSO), a specific inhibitor of γ-glutamyl cysteine synthetase, the rate limiting enzyme in GSH synthesis (Griffith & Meister, 1979), and the effect on the toxicity of 1-naphthol and its possible metabolite 1,4-naphthoquinone were studied. For comparison, we included menadione (2-methyl-1,4-naphthoquinone) and two alkylating agents, melphalan and helenalin. The chemosensitivity of melphalan and helenalin has previously been shown to be increased in the presence of BSO. Tumour cell lines resistant to the alkylating agent melphalan were found to have elevated GSH and GSH S-transferase levels and sensitivity was restored using BSO, to deplete GSH, in such cell lines (Green *et al.*, 1984; Hamilton *et al.*, 1985). BSO has also been shown to augment the lysis of tumour cells by helenalin, therefore this agent was included as a positive control (Arrick *et al.*, 1983). Menadione, a derivative of vitamin K has been extensively studied, with regard to quinone toxicity and its reactions with GSH (Thor *et al.*, 1982) and is currently undergoing clinical trial with the antimetabolite 5-fluorouracil (Chlebowski *et al.*, 1983).

Depletion of GSH using BSO did not effect the toxicity of 1-naphthol or 1,4-naphthoquinone, as assessed by two end points of toxicity. However moderate potentiation was observed with helenalin, melphalan and menadione. As the effect of BSO on the cytotoxicity of all these agents was not dramatic – the potential use of BSO in combined therapy in the clinic may be limited.

Materials and methods

Cell Culture

LoVo human carcinoma cells (Drewinko *et al.*, 1976)

supplied by Dr Bridget Hill, ICRF London, were grown in monolayer culture in Hams F-12 medium supplemented with 10% foetal calf serum (Flow Labs), 1 mM L-glutamine, penicillin 100 u ml^{-1} and streptomycin 100 μg ml^{-1} (Gibco). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and routinely subcultured each week. Cells in exponential growth phase were used in all experiments.

Drug exposure and cytotoxicity

For assay of protein synthesis inhibition, 200 μl aliquots of a cell suspension of density 5×10^4 cells ml^{-1}, were seeded into 96 wells of a flat-bottomed microtitre plate. The ability of agents to inhibit ^{3}H-leucine incorporation into protein was carried out as previously described (Wilson *et al.*, 1985).

For cell growth determinations, 1.5 ml of a 1.4×10^4 cells ml^{-1} suspension of cells were seeded into 3 cm diameter petriplates. After incubation overnight at 37°C, 5% CO_2, the medium was removed and replaced with complete drug-free medium or medium containing 0.2 mM BSO and again incubated overnight (18 h). Cells were then exposed for 5 h to the cytotoxic agents in fresh medium in the presence or absence of 0.2 mM BSO, as before. After 5 h the medium was then removed by aspiration, replaced with fresh medium and incubated for a further 48 h at 37°C, 5% CO_2, and the cell number estimated by counting an aliquot of trypsinised cells with a Coulter Counter. All experiments were repeated 3–6 times and standard errors calculated.

GSH determination

In cultured cells   Monolayer cultures in 3 cm dishes were washed twice with 0.9% saline and the GSH extracted with 6.5% TCA at 4°C for 10 min. GSH was assayed by the method of Hissin and Hilf (1976) using o-phthalaldehyde (o-PT) and results expressed as nmol GSH 10^{-6} cells.

Following chemical reactions of test agents with GSH   Various concentrations of the chemicals were incubated at 37°C in HEPES (10 mM) (pH 7.5) with 100 μM GSH and the GSH remaining determined using o-P as before.

Results

Effect of BSO on tumour cell GSH

BSO (0.05–0.2 mM) caused a time dependent depletion of GSH (Figure 1) from a starting level of 6.6 ± 0.7 nmol GSH 10^{-6} cells present in control cells. After 24 h exposure to BSO (0.2 mM), a maximum depletion to 12% of control
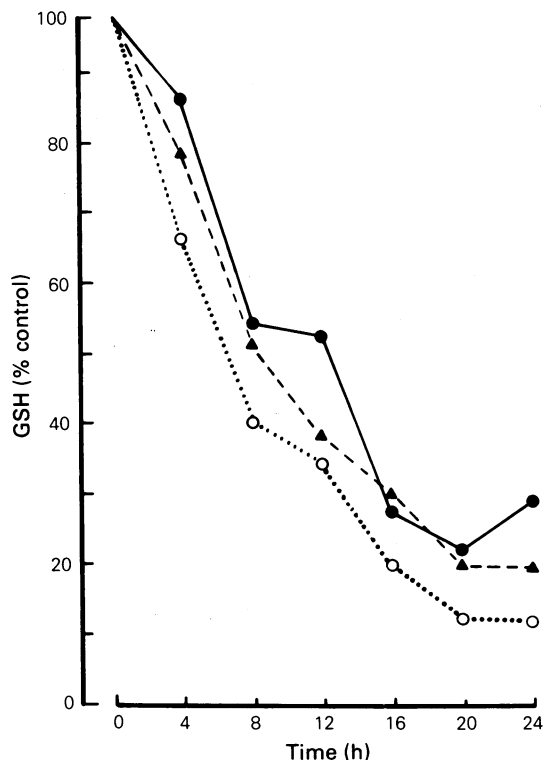
Figure 1 Time course of GSH depletion in LoVo cells, following exposure to BSO. Results are expressed as a percentage of GSH present in untreated cells. ($\bullet$——$\bullet$) 0.05 mM; ($\blacktriangle$----$\blacktriangle$) 0.1 mM and ($\bigcirc$----$\bigcirc$) 0.2 mM BSO.

was obtained with viabilities of $98\% \pm 2.1$ and $95.4 \pm 8.9\%$ as assessed by protein synthesis inhibition and cell numbers, respectively. A concentration of BSO (0.2 mM) was therefore chosen for overnight incubation (18 h) to deplete GSH.

Effect of GSH modulation on the cytotoxicity of the chemicals to LoVo cells

After exposure of LoVo cells to 1-naphthol for 5 h, protein synthesis inhibition was a more sensitive indicator of cytotoxicity than cell numbers, 48 h after exposure (Table I). The opposite effect was observed with melphalan, when the IC_{50} values obtained using cell numbers was one fifth that determined by protein synthesis inhibition (Table I). For all

Table I Effect of BSO on the cytotoxicity of the chemicals to LoVo cells

Chemical	$IC_{50}\,\mu M$ (using ^{3}H-leucine)			$IC_{50}\,\mu M$ (using Cell Number)		
	$-BSO$	$+BSO$	DMF^a	$+BSO$	$-BSO$	DMF^a
1-Naphthol	543 ± 34	540 ± 41	1	950 ± 134	960 ± 154	1
1,4-NQ	13 ± 1.5	13 ± 1.5	1	25.5 ± 6.2	24.5 ± 6.3	1
Menadione	30.4 ± 3.3	23.8 ± 4.8	1.27^b	24.6 ± 6.2	19.5 ± 5.6	1.8^b
Helenalin	3.80 ± 0.2	1.53 ± 0.7	2.48^b	2.6 ± 0.9	1.07 ± 0.6	2.4^b
Melphalan^c	57.8 ± 5.6	43 ± 11	1.38^b	12.7 ± 3.8	6.7 ± 1.7	1.9

^{a}DMF = Dose modification factor; ^{b}Significant difference ($P < 0.05$) between IC_{50} values $\pm$ BSO, using a paired *t*-test; ^{c}Significant difference ($P < 0.05$) between IC_{50} values determined by the two criteria of assessing cytotoxicity, using a paired *t*-test. Each experiment was repeated at least three times and a minimum of four wells per concentration was used in every experiment.

other agents used in this study i.e. menadione, helenalin, and 1,4-NQ, no significant difference in the IC_{50} values, as assessed by either criteria were observed (Table I).

Depletion of GSH using BSO, did not effect the cytotoxicity of 1-naphthol, or 1,4-naphthoquinone as assessed by either method of toxicity, whereas the cytotoxicities as assessed by inhibition of protein synthesis, of menadione, helenalin and melphalan were significantly potentiated due to pretreatment and incubation with BSO (Table I). BSO treatment also potentiated the cytotoxicity of menadione and helenalin, as assessed by cell numbers. A small but not significant effect was observed with melphalan. The maximum modification of an IC_{50} value in the presence of BSO was a 2.5 fold decrease in the helenalin IC_{50} value (Figure 2), all other effects were less than 2 fold.
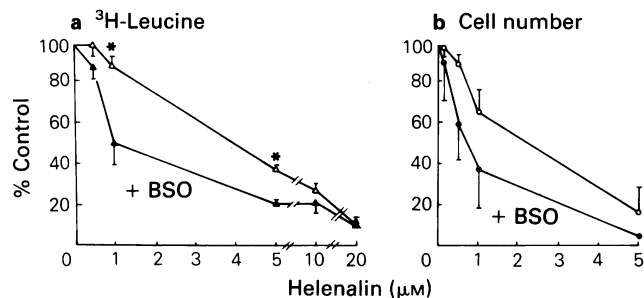

Figure 2 Effect of BSO on the toxicity of helenalin to LoVo cells. (a) Protein synthesis was assessed by incorporation of ³H-leucine and (b) cells numbers were determined 48 h after drug exposure. *Significant at $P<0.05$ paired t-test.

Effects of chemicals on tumour cell GSH

LoVo cells were incubated with equitoxic concentrations (as assessed by protein synthesis inhibition after 5 h exposure) and the GSH levels determined over a 2 h exposure (Figure 3). Within 30 min, 1,4-NQ caused over 95% depletion of GSH in LoVo cells and this level was maintained over the 2 h exposure. Menadione also caused an extensive but insignificant, depletion due to the large variation in the response. Melphalan and 1-naphthol did not deplete GSH, however helenalin actually caused a small but not significant increase in GSH above control value at 30 min which returned to normal at 60 and 120 min (results not shown).

Reactivity of chemicals with GSH in solution

The chemicals were incubated with GSH in buffered solution (Figure 4). 1-Naphthol (100 μM–1 mM) did not react with GSH in solution whereas 1,4-NQ was highly reactive. After 15 min incubation with 100 μM 1,4-NQ, less than 10% of the GSH present at the start of the incubation (100 μM)
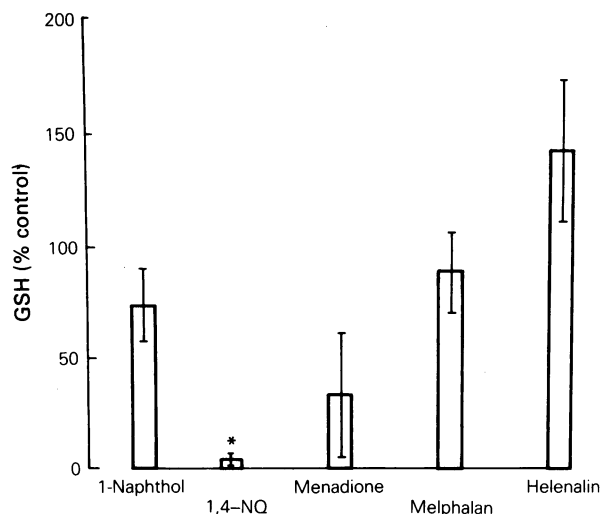

Figure 3 Effect of equitoxic concentrations of cytotoxic chemicals on tumour cell GSH. The GSH levels after 30 min exposure are shown and the results are expressed as percentage GSH present in untreated cells. The concentrations of 1-naphthol, 1,4-NQ, menadione, melphalan and helenalin were 250, 10, 20, 20 and 1 μM respectively. *Significant $P<0.05$ unpaired t-test.

remained (Figure 4). Overall reactivity of the chemicals with GSH in solution followed the order 1,4-NQ > menadione > melphalan ≃ helenalin ≫ 1-naphthol.

Discussion

Our results demonstrate that GSH depletion, using BSO to inhibit GSH synthesis, may affect the cytotoxicity of selected agents to LoVo cells growing in vitro (Table I). Cytotoxicity was assessed by inhibition of both protein synthesis and cell numbers. In a previous study with LoVo cells, little difference was observed in the toxicity of 1-naphthol or 1,4-NQ, when assessed either by inhibition of protein synthesis or by a clonogenic assay (Wilson et al., 1985). The possibility that BSO or the drug treatments altered the uptake of [³H]-leucine or its pool sizes cannot be excluded. However in a similar study with human lung tumour cells, BSO did not affect the uptake of [¹⁴C]-leucine (Brodie & Reed, 1985). The cytotoxicity of 1-naphthol assessed by protein synthesis inhibition or cell number determination after 24 h, was not affected by BSO, indicating that GSH may not be involved in protecting LoVo cells from the toxicity of 1-naphthol. In addition, BSO did not affect the cytotoxicity of 1,4-NQ, a possible metabolite of 1-naphthol. Based on these results, the possible involvement of 1,4-NQ in
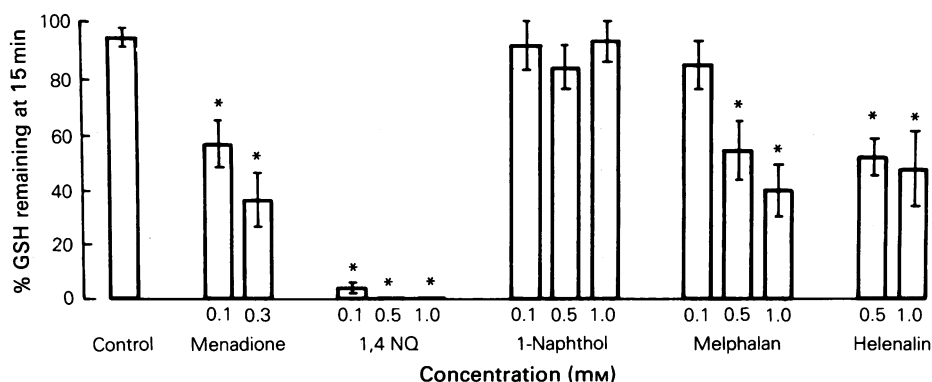
Figure 4 Chemical interaction of agents with GSH in solution. Results are expressed as percentage GSH remaining after 15 min incubation at 37°C. *Significant at $P<0.05$ unpaired t-test.

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Table II Effects of glutathione depletion on the cytotoxicity of antitumour agents

Treatment	Cell line	Measurement of toxicity	Effect	DMF[a] (if given)	Ref.
Helenalin	P815	51 Cr	potentiation	4.7	Arrick *et al.* (1983)
Jatrophone	P815	51 Cr	potentiation	21.3	
Adriamycin	P815	51 Cr	none		Arrick *et al.* (1983)
	ADR resistant	clonogenic	sensitisation		Hamilton *et al.* (1985)
	V79	clonogenic	potentiation	4–10.5	Russo *et al.* (1986)
	A549	clonogenic	none		Russo *et al.* (1986)
Daunorubicin	P388	cell number	none		Romine & Kessel
	P388/ADR resistant		none		(1986)
Bisthiosemi- carbozone B2844	P388 P388/ADR resistant	cell number cell number	potentiation potentiation/ sensitisation	3.4 1.4	Romine & Kessel (1986)
H₂O₂ —preformed	P815 endothelial	51 Cr 51 Cr	none potentiation		Arrick *et al.* (1982) Tsan *et al.* (1985)
—generated	P815 endothelial	51 Cr 51 Cr	potentiation potentiation	3.5	Arrick *et al.* (1982) Tsan *et al.* (1982)
Melphalan (L-PAM)	A1847 LPAM resistant	clonogenic	sensitisation	3.5–10	Green *et al.* (1984)
	L1210 LPam resistant	clonogenic	sensitisation		Somfai-Relle *et al.* (1984)
BCNU[b]	P815	51 Cr	none	0.99	Arrick *et al.* (1982)
Vinblastine		51 Cr	none	1.17	
Cytosine		51 Cr	none	0.74	
Arabinoside (Arac)				0.71	
Maytansine		51 Cr	none	0.91	
Irradiation (DTNB)[c]	lymphoid	trypan blue exclusion	potentiation potentiation		Dethmers & Meister (1981)
5-Fluorouracil			none		
Vincristine			none		
Neocarzino- statin	V79 CCL-210 (normal)	clonogenic clonogenic	protection none		DeGraff *et al.* (1985) Russo *et al.* (1986)

[a]Dose modifying factor; [b]1,3-bis[2-chloroethyl]-1-nitrosourea; [c]5,5-dithiobis(2-nitrobenzoic acid).

the toxicity of 1-naphthol cannot be excluded. The lack of effect of BSO on the toxicity of both 1-naphthol and 1,4-NQ was rather surprising, as with isolated hepatocytes both these compounds caused a depletion of intracellular GSH prior to cell death (d'Arcy Doherty *et al.*, 1984*b*). However with LoVo cells, 1,4-NQ but not 1-naphthol, caused a depletion in GSH (Figure 2). This may be due to differences in the ability of these different cell types to activate these compounds or to deal with the accompanying oxidative stress.

It was of interest that under the same conditions, BSO caused a small but significant potentiation of menadione cytotoxicity but had no effect on the structurally related 1,4-NQ (Table I). One possible explanation for this difference is that the two quinones may exert their toxicity by different mechanisms due to the higher chemical reactivity of 1,4-NQ (Figure 4).

The cytotoxicities of the two alkylating agents in the study, helenalin and melphalan, were potentiated in the presence of BSO (Table I) in agreement with other studies (Arrick *et al.*, 1983; Green *et al.*, 1984; Hamilton *et al.*, 1985; Suzukake *et al.*, 1982). Of the four chemicals used in this study the greatest potentiation of cytotoxicity in the presence of BSO was exhibited by helenalin (Table I and Figure 2). GSH may protect against helenalin cytotoxicity by conjugation, prior to alkylation of target molecules, prevent cross linking or restore critical sulphydryl groups (Hall *et al.*, 1977, 1978). It is of interest to note that in the presence of helenalin (1–10 µM), LoVo cell GSH was not depleted,

suggesting that modulation of GSH may be of importance in determining the toxicity of agents that do not deplete GSH.

Recently a number of studies have investigated the possible relationship between resistance to melphalan (L-PAM mustard), a bifunctional alkylating agent and thiol status of the cells (Suzukake *et al.*, 1983). In this study, a modest potentiation of melphalan toxicity, to LoVo cells was observed in the presence of BSO suggesting a protective role for GSH against the cytotoxic action of melphalan, probably via conjugation reactions. This potentiation of melphalan toxicity was less than that observed by others (Table II) and may be due to a number of possibilities such as the different measures of cytotoxicity used or to lower intracellular levels of GSH in the LoVo cells.

In vitro studies with tumour cells in this laboratory have investigated the involvement of GSH in protection against a range of cytotoxic agents. We observed that the cytotoxicities of menadione, helenalin and melphalan were potentiated due to GSH depletion by BSO, however the effects of BSO were relatively modest (Table I). It is of particular interest to compare these results with other studies in the literature to assess the potential use of BSO in chemosensitising tumour cells (Table II). BSO has been reported to increase drug toxicity, sensitise drug resistant cell lines and also reduce drug toxicity. The majority of effects are clearly very modest and some results actually conflict, possibly due to different effects in different cell lines. Under *in vitro* conditions, it is possible to choose a concentration

and time period of BSO exposure which would cause optimal GSH depletion, prior to incubation with the cytotoxic chemicals. If, under such optimum conditions, only a slight potentiation of toxicity is observed, it is difficult to envisage any great potentiation of toxicity to the tumour tissue occurring *in vivo*, especially as it will be necessary to consider other important factors such as the pharmacokinetics of BSO and the antitumour agent. Furthermore GSH depletion *in vivo* due to administration of BSO will not be confined to tumour tissue therefore potentiation of toxicity to normal tissue may be a limiting factor as was recently observed with the enhanced nephrotoxicity of rats treated with BSO (Kramer *et al.,* 1985).

Our results and those of others suggest that great caution should be exercised in the potential use of BSO in the chemosensitisation of tumours in man.

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