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

J. Jordan, M. d'Arcy Doherty & G.M. Cohen

Toxicology Unit, Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK.

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 potent 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, melphanal, 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, helenalin and melphanal. 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., 1984a,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., 1984b).

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 (O₂), forming large amounts of the superoxide anion radical (O₂⁻). which may then in turn spontaneously, or in a reaction catalysed by superoxide dismutase, dismutate to produce hydrogen peroxide (H₂O₂) which in turn may undergo hydroperoxidase or hydroperoxide radical (OH⁻), 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 semi-quinone radicals, or by removing with glutathione peroxidase either H₂O₂ 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 sulphoxime (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, melphanal and helenalin. The chemosensitivity of melphanal and helenalin has previously been shown to be increased in the presence of BSO. Tumour cell lines resistant to the alkylating agent melphanal 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 (Chlebowsk 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, melphanal 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 units ml⁻¹ and streptomycin 100 μg ml⁻¹ (Gibco). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ 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 x 10⁴ cells ml⁻¹, were seeded into 96 wells of a flat-bottomed microtitre plate. The ability of agents to inhibit ³H-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 x 10⁴ cells ml⁻¹ suspension of cells were seeded into 3 cm diameter petriplates. After incubation overnight at 37°C, 5% CO₂, 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% CO₂, and the cell number estimated by counting an aliquot of trypan stained 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 was obtained with viabilities of 98% ± 2.1 and 95.4 ± 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 IC₅₀ values obtained using cell numbers was one fifth that determined by protein synthesis inhibition (Table I). For all

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**Table 1** Effect of BSO on the cytotoxicity of the chemicals to LoVo cells

| Chemical      | IC₅₀ μM (using ³H-leucine) | IC₅₀ μM (using Cell Number) |
|---------------|----------------------------|-----------------------------|
|               | −BSO (+BSO) DMF*           | +BSO −BSO DMF*              |
| 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*| 24.6 ± 6.2 19.5 ± 5.6 1.8*  |
| Helenalin     | 3.80 ± 0.2 1.53 ± 0.7 2.48*| 2.6 ± 0.9 1.07 ± 0.6 2.4*   |
| Melphalan     | 57.8 ± 5.6 43 ± 11 1.38*   | 12.7 ± 3.8 6.7 ± 1.7 1.9    |

*DMF = Dose modification factor; *Significant difference (P<0.05) between IC₅₀ values ± BSO, using a paired t-test; **Significant difference (P<0.05) between IC₅₀ 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 IC50 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 IC50 value in the presence of BSO was a 2.5 fold decrease in the helenalin IC50 value (Figure 2), all other effects were less than 2 fold.

Effects of chemicals on tumour cell GSH
LoVo cells were incubated with equitoxic concentrations (as assessed by protein synthesis inhibition after 3h exposure) and the GSH levels determined over a 2h 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 2h 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) 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 24h, 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

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**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.

**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.

**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.
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., 1984b). 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 sulphhydryl 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 possibility of 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

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

| Treatment     | Cell line | Measurement of toxicity | Effect   | DMF* (if given) | Ref.       |
|---------------|-----------|-------------------------|----------|-----------------|-----------|
| Helenalin     | P815      | 51 Cr                   | potentiating | 4.7            | Arrick et al. (1983) |
| Jatrophosphate| P815      | 51 Cr                   | potentiating | 21.3           | Arrick et al. (1983) |
| Adriamycin    | P815      | 51 Cr                   | none      | 4-10.5         | Russo et al. (1986) |
|               | ADR resistant | V79        | clonogenic  | sensitisation  | Hamilton et al. (1985) |
|               |           | A549        | clonogenic  | potentiating   | Russo et al. (1986) |
| Daunorubicin  | P388      | cell number            | none      | 3.4            | Romine & Kessel (1986) |
|               | P388/ADR resistant | cell number | potentiating | 1.4            | Romine & Kessel (1986) |
| Bisthiosemi-carbozone | P388 | cell number            | potentiating | none         | Arrick et al. (1982) |
| B2344         |           | cell number            | potentiating | none         | Tann et al. (1985) |
| H$_2$O$_2$—preformed | P815 | endothelial           | none      | 0.99           | Arrick et al. (1982) |
|               |           | 51 Cr                   | none      | 3.5            | Arrick et al. (1982) |
|               |           | 51 Cr                   | none      | 3.5–10         | Green et al. (1984) |
| Melphan (L-PAM) | A1847 L-PAM resistant | clonogenic  | sensitisation | none         | Somfai-Rele et al. (1984) |
|               | L120      | L-PAM resistant       | clonogenic | sensitisation |           |
| 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) | 51 Cr   | none                   | 0.71      |                |           |
| Maytansine    | 51 Cr     | none                   | 0.91      |                |           |
| Irradiation (DTNB)$^c$ | lymphoid | trypan blue exclusion | potentiating | none         | Dethmers & Meister (1981) |
| 5-Fluourouracil | 51 Cr | none                   | none      |                |           |
| Vincristine   | 51 Cr     | none                   | none      |                |           |
| Necarizino-statin | V79   | clonogenic             | protection| 0.8            | DeGraff et al. (1985) |
|               | CCL-210 (normal) | clonogenic | none      |                | Russo et al. (1986) |

*Dose modifying factor; $^b$1,3-bis[2-chloroethyl]-1-nitrosourea; $^c$5,5-dithiobioc(2-nitrobenzoic acid).
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 tissues 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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