

# Changes in Glutathione Content and Resistance to Anticancer Agents in Human Stomach Cancer Cells Induced by Treatments with Melphalan *in Vitro*<sup>1</sup>

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## ABSTRACT

A clone of a human gastric carcinoma cell line was used to determine whether cells which had survived a treatment with Melphalan would express altered survival responses when treated again with this agent 1 week or more later. Cells were treated for 1 h each week with 2  $\mu\text{g}/\text{ml}$  (99% lethal dose). After the first Melphalan treatment, the cells exhibited a 10-fold reduction in sensitivity to Melphalan. This was preceded by a 2-fold increase in intracellular glutathione content. By the end of 10 weekly treatments, the cells were 50 times more resistant than controls (based on changes in survival fractions). They also demonstrated collateral resistance to Actinomycin D, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, galactitol, and X-rays, but showed no change in sensitivity to 5-fluorouracil, bleomycin, and Adriamycin. The resistance to Melphalan was not reversible when treatment was withheld for 4 weeks on two different occasions.

The results suggest that treatment with a high dose of Melphalan either selects an existing population of cells with a high GSH content or induces mutations leading to increased GSH content or both, and this results in the expression of greater Melphalan resistance at the time of other treatments. Furthermore, Melphalan treatment stimulates a 50% increase in GSH content in resistant cells in just 6 h, an 85% increase in 36 h, and a 150% increase in 72 h. L-Buthionine sulfoximine partially reversed the expression of resistance to Melphalan by inducing a 60% reduction in intracellular glutathione content.

## INTRODUCTION

In recent studies we reported that multiple single treatments with MeCCNU<sup>3</sup> induced changes in sensitivity in a multiclonal human astrocytoma mixture model *in vitro* (1). Sensitive and resistant clones, which had been mixed (50:50) and grown together, were treated for 1 h each week with single LD<sub>90</sub> doses of MeCCNU. After only three such treatments the sensitive cells in the mixture were killed, leaving behind a population that was almost 100% resistant to further exposures to MeCCNU. The loss of the sensitive cells from the mixture after each weekly treatment was easily detected by flow cytometry histograms, since the clones had different DNA indices (1).

Of equal interest was that similar weekly exposures to MeCCNU of the unmixed sensitive astrocytoma clone produced a linear decrease in survival at first. But after the third weekly treatment these cells became progressively more resistant to MeCCNU. This induced resistance to MeCCNU was accompanied by collateral resistance to 1-3-bis-2-chloro-

ethylnitrosourea and galactitol but increased sensitivity to Adriamycin, with no change in response to ionizing radiation (2).

These experiments raised several questions about the induction of drug resistance and about how the change in drug sensitivity might be preceded and predicted by other changes at the biochemical level in the cell, for example, a change in quantity of glutathione, which is related to some forms of drug resistance (3, 4). The purpose of this report is to describe the results of experiments related to this question in a human gastric cancer clone (AGS-6) which was treated weekly for 1 h with an LD<sub>90</sub> dose of Melphalan and assayed for changes in survival, changes in glutathione levels, and the appearance of multidrug resistance.

## MATERIALS AND METHODS

**Cell and Culture Techniques.** The AGS-6 cells are a clone of human gastric carcinoma originally isolated from an untreated patient and established as a permanent cell line (5). The cells are maintained *in vitro* in Ham's F10 medium supplemented with 20% newborn calf serum, in a 5% CO<sub>2</sub> humidified incubator, at 37°C. Under these conditions the doubling time is 19 h.

**Fractionated Dose Model.** Cells were seeded into replicate culture flasks and the cells were subcultured as necessary to maintain them in exponential growth, as described previously (1). Some cells were treated weekly for 1 h with 2  $\mu\text{g}$  Mel/ml and then plated for survival determinations. Comparison cultures either served as untreated controls or were treated only once to assure that the inherent Melphalan sensitivity was not changing during the experiment. Following treatments (and survival determinations), the remaining cells were placed into stock flasks, were allowed to recover and grow, and were fed or subcultured as needed; they were treated again the next week. All determinations were performed in triplicate. Immediately before each weekly treatment, control and previously treated cells were assayed for cell kinetics changes via flow microfluorometry and for changes in GSH levels. These assays were also performed in triplicate.

At various times during the 23-week experiment, cells were preserved by freezing, for further testing of sensitivity of the previously treated cells to other drugs and radiation.

**Survival Determinations.** The effects of drugs and radiation on survival were determined on exponentially growing cells in the fractionated dose model. Replicate flasks or Petri dishes were used in each survival study, and each experiment was performed at least 3 times. In all cases survival was determined by the ability of treated cells to form colonies. After the treatments, the cell cultures were washed twice with Puck's solution A and trypsinized (0.25% for 5 min), and known numbers of single cells were plated into Petri dishes and incubated 10-12 days for colony formation. Colonies were stained and counted. A cell was considered to have retained reproductive capacity (viability) if it gave rise to a colony of 50 or more cells.

**Drug Solutions.** The drug solutions were always prepared immediately before use to prevent loss of activity due to decay. All anticancer drugs were first dissolved in the appropriate solvent and then diluted to final treatment concentrations in medium (ACT D, Gal, 5FU, Bleo, Adria) or in saline (MeCCNU).

**Radiation of Cells.** Cells grown in 60-mm Petri dishes were irradiated at room temperature with single graded doses of 0 (control), 200, 400, 600, 800, and 1000 rad, using a therapy X-ray unit (operated at 200

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<sup>3</sup> The abbreviations used are: MeCCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; GSH, glutathione; LD<sub>90</sub>, lethal dose to 99% of the treated population; ACT D, Actinomycin D; Gal, galactitol; Bleo, bleomycin; Adria, Adriamycin; 5FU, 5-fluorouracil; BSO, L-buthionine sulfoximine; D<sub>0</sub>, measure of the slope of the survival curve; Mel, Melphalan; MR, Melphalan-resistant.

kVp, 20 mA, 0.2-mm copper filter, half value layer (HVL) of 0.55-mm copper) whose output was 183.4 rad/min. All Petri dishes were irradiated within the 98% isodose area for the beam.

**Flow Microfluorometry Techniques.** Cell cultures were washed with Puck's solution A, and cells were detached by pepsin (0.5%) and fixed by addition of ethanol to a final concentration of 70%. Cells were treated with RNase (0.1%), stained with a 1:1 mixture of ethidium bromide-mithramycin for 20 min according to the method of Zante *et al.* (6), and processed on a Coulter Profile flow cytometer (laser tuned to an excitation wavelength of 488 nm). The fractions of cells in G<sub>1</sub>, S, and G<sub>2</sub>-M were calculated by the method described by Guseman and Bryant (7). With this procedure, we routinely obtain coefficients of variation ranging from 2 to 4%.

**Glutathione Determinations.** Total reduced GSH was measured by the fluorometric method of Cohn and Lyle (8), as modified by Mokrasch and Teschke (9), on a Turner Model 110 spectrofluorometer with a primary filter for 365 nm and a secondary filter for 415 nm and longer wavelengths. GSH was measured in control cells and after Melphalan or BSO treatments.

## RESULTS

**Effects of Weekly Fractions of Melphalan on Survival.** In the experiments reported here, the AGS-6 clone received 1-h exposures to 2  $\mu$ g Mel/ml each week, for an accumulated total dose of 30  $\mu$ g. It can be seen in Fig. 1 that the first treatment at week 1 killed about 99% of the cells. When the survivors of this treatment were exposed to Melphalan again 1 week later, the survival fraction was almost 10 times higher. During the next several weeks, repeated weekly treatments of previously treated cells with Melphalan killed even fewer cells, reaching a survival value at the 10th week that was 50-fold higher than the normal sensitivity of AGS-6 cells (Fig. 1). During weeks 14 to 18, and again in weeks 19 to 23, the cells were not treated but only fed and subcultured as needed. This was done to determine whether the altered sensitivity of the cells to Melphalan would return to the pretreatment values if the cells were not treated weekly. Reexposure of the previously treated cells to Melphalan at week 18 and again at week 23 showed that the cells were still more resistant than controls (Fig. 1).

**Effects of Weekly Melphalan Dose Fractions on Glutathione.** Intracellular GSH was assayed once each week, immediately

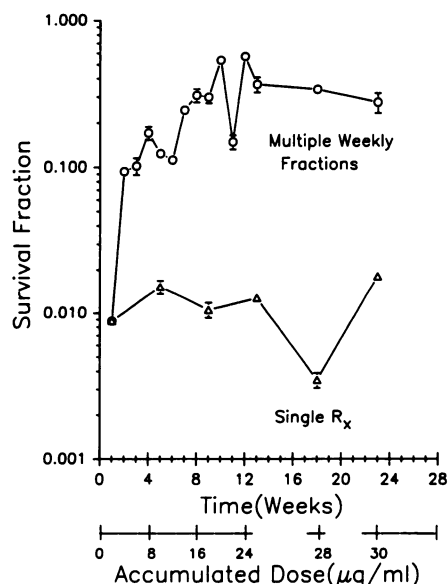


Fig. 1. Effects of Melphalan on survival in AGS-6 cells. O, dose accumulated at 2  $\mu$ g/ml for 1 h/week;  $\Delta$ , single exposures to the same dose, at times shown. Whenever SE bars are not shown, they were smaller than the symbol.

before treatment, in control and treated populations, to determine the GSH relationship to Melphalan sensitivity. Fig. 2 shows that control cell GSH values were about 7 nmol/ $10^6$  cells at the start of the experiment and ranged from 4 to 11 nmol/ $10^6$  cells through the 23rd week. In the treated population, however, the GSH almost doubled, from 7 nmol/ $10^6$  cells before the start of treatment to 13 nmol/ $10^6$  cells just 7 days after the first Melphalan treatment. This 2-fold increase in GSH values coincided with the 10-fold increase in resistance expressed by the cells treated at that time with the second dose fraction of Melphalan. GSH values continued to increase after each of the first three Melphalan treatments and then fluctuated between 10 and 20 nmol/ $10^6$  cells through the rest of the experiment, staying always about 2–3 times higher than control samples assayed at the same time.

At 10 weeks (when the GSH levels and Melphalan resistance were at their highest values), additional samples were taken and GSH levels were obtained twice daily for 4 days and on day 7 after treatment. This was done to determine if the Melphalan treatment itself was driving the GSH values up even higher during the days between treatments. (GSH assays were also performed on other matched replicates of resistant and control cells which received no additional exposure to Melphalan.) In Fig. 3 it can be seen that the GSH values for controls were uniform, fluctuating only about 25% above 0-h values. As expected, the GSH values for the resistant cells (no additional Melphalan exposure) were higher than controls. The GSH values in this group were increased 25% 6 h after the experiment started and were 77% higher on the third day, before returning to 0-h baseline values on day 7. However, the resistant cells which received a Melphalan exposure at 0 h had a 50% increase in GSH values at 6 h; it increased to 85% by 36 h and to 150% by day 3, before decreasing to baseline values at day 7.

**Effects on the Cell Cycle.** Flow cytometry-derived cell cycle phase distributions obtained at the start of each week's treatment indicated no differences between previously treated and control cells, nor was there a change in DNA content of the treated cells throughout the experiment (data not shown).

**Partial Reversal of Melphalan Resistance by BSO.** By week 10, the cells had received ten 2- $\mu$ g/ml treatments with Melphalan and were 40–50 times more resistant than controls

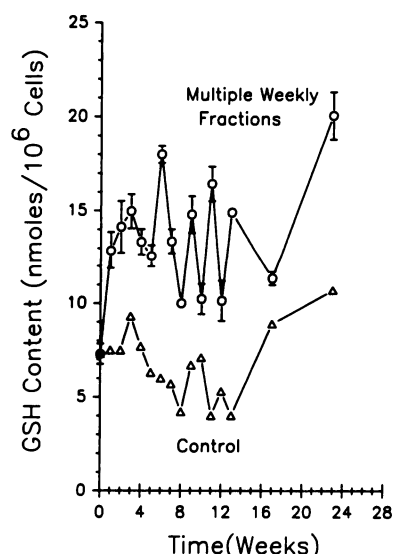


Fig. 2. GSH content of AGS-6 cells determined immediately before treatment. O, cells accumulating multiple weekly Melphalan dose fractions;  $\Delta$ , cells receiving no Melphalan treatments.

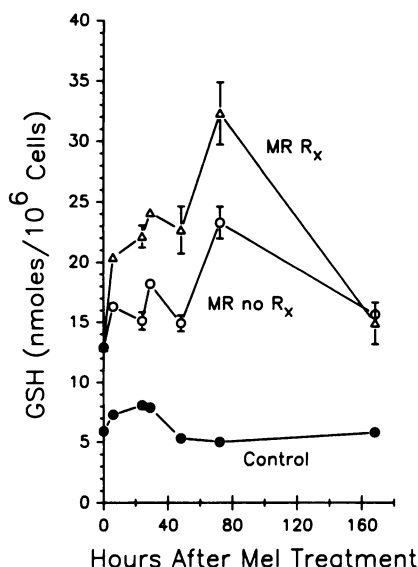


Fig. 3. Changes in GSH content in control cells (●), MR cells which received no additional Melphalan treatment during this experiment (○), and MR cells treated for 1 h with 2  $\mu\text{g}$  Melphalan/ml at 0 h ( $\Delta$ ).

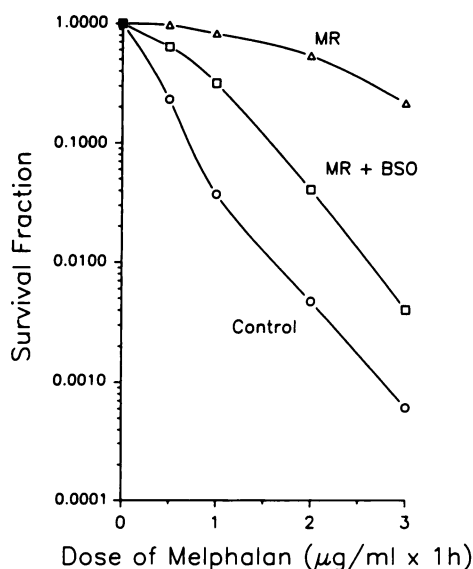


Fig. 4. Survival responses of AGS-6 cells to Melphalan treatment. ○, control cells;  $\Delta$ , MR cells;  $\square$ , MR cells treated first with 50  $\mu\text{M}$  BSO and then with Melphalan;  $\blacksquare$ , survival fraction resulting from BSO exposure only.

(based on changes in survival fractions). Experiments were performed to determine whether a BSO-induced reduction in GSH values would reverse the Melphalan resistance. Melphalan dose-survival responses were obtained (a) on control cells, (b) on MR cells, and (c) on MR cells after they received 50  $\mu\text{M}$  BSO for 48 h to reduce the GSH levels. As expected, the MR cells were more resistant than controls to all doses of the agent tested (Fig. 4). The  $D_0$  for the control cells was 0.4  $\mu\text{g}$  Mel/ml  $\times$  1 h, and for the Melphalan-resistant cells it was almost 2.8-fold higher (Table 1). The BSO treatment did not alter the doubling times or cell cycle phase distributions, nor did it kill any cells by itself during the 48-h exposure; however, it did cause GSH values to decrease by 60% in the MR cells (Table 1), and this resulted in a partial reversal in Melphalan resistance in the MR cells, as far as  $D_0$  values are concerned. However, the shoulder of the survival curve remained (Fig. 4).

**Multidrug Resistance in Melphalan-treated Cells.** Melphalan-resistant cells were tested for the expression of multidrug resistance at 10, 18, and 23 weeks of the experiments, compared

Table 1 Effects of a 48-h BSO treatment on GSH, cell cycle phase distributions, and sensitivity to Melphalan

Group <sup>a</sup>	GSH (nmol/10 <sup>6</sup> cells)	$D_0$ ( $\mu\text{g}/\text{ml} \times 1 \text{ h}$ )	$G_1$ (%)	S (%)	$G_2\text{-M}$ (%)
Control	8.2	0.4	38.2	43	18.6
Mel-resistant (no BSO)	11.7	1.1	32.1	49	18.6
Mel-resistant (with BSO)	5.0	0.5	36.1	45	18.6

<sup>a</sup> Control, never treated with BSO or Mel; Mel-resistant (no BSO), treated with ten 1-h weekly fractions of 2  $\mu\text{g}$  Mel/ml, no BSO; Mel-resistant (with BSO), Mel-resistant cells given 48-h exposure to 50  $\mu\text{M}$  BSO.

to control sensitivities. Since the week in which the survival data were obtained made no difference in sensitivity to X-rays or to a particular drug, the results were averaged and are shown in Fig. 5. It can be seen that the Melphalan-resistant cells also had developed collateral resistance to ACT D, MeCCNU, Gal, and X-rays. Comparisons of  $D_0$  values (Table 2) show that  $D_0$  for ACT D went from 0.11  $\mu\text{g}/\text{ml} \times 1 \text{ h}$  in controls to 0.16  $\mu\text{g}/\text{ml} \times 1 \text{ h}$  in the Melphalan-resistant cells (a 45% increase in resistance). For MeCCNU,  $D_0$  went from 5.5  $\mu\text{g}/\text{ml} \times 1 \text{ h}$  in controls to 8  $\mu\text{g}/\text{ml} \times 1 \text{ h}$  in resistant cells (45% increase); for Gal,  $D_0$  was increased 40% in resistant cells. Only a 10% increase in resistance was observed for X-rays. Minimal or no change in sensitivity was observed between control and Melphalan-resistant cells for Bleo, Adria, or 5FU.

## DISCUSSION

When cancer cell populations are treated with anticancer agents (whether *in vivo* or *in vitro*), large changes may occur in the cell populations. Many cells are killed by the treatment, while others are not affected at all, either because they are resistant or because of biochemical, cell cycle, or extracellular environmental sanctuaries. As soon as these environmental conditions are reversed, the cells may express sensitivity again. Other cells, however, are sublethally damaged and, depending upon the accuracy and completeness of the recovery process, it may be assumed that the drug sensitivity of some of these surviving cells could be substantially altered; this may have great influence on the rest of that patient's therapy.

We designed experiments to test whether human gastric cancer cells which had survived treatments with Melphalan would express altered survival responses when treated again with this agent. Our results show that after only one exposure to an LD<sub>99</sub> dose of Melphalan, the surviving cell population was 10 times more resistant (higher survival values) to the next Melphalan treatment 1 week later; this resistance eventually increased almost 50-fold after the cells had received the 10th weekly treatment. The initial resistance expressed to Melphalan at the time of the second treatment was preceded by a 2-fold elevation in GSH values; the GSH levels remained between 2 and 3 times higher than controls for the rest of the experiment. Similar results associating elevated intracellular GSH and resistance to X-rays and Melphalan, Adriamycin, cisplatin, and other drugs have been reported for *in vivo* and *in vitro* studies (3, 4, 10–16, 26). Furthermore, the inducibility of glutathione S-transferases and the manipulation of GSH levels by BSO and various mutagens (17–20, 27) have also been reported.

Of additional concern was the demonstration of collateral resistance of the Melphalan-treated cells to all other doses of Melphalan tested and to Act D, MeCCNU, and galactital (Fig. 5, Table 2). The fact that the Melphalan-treated cells were only 10% more resistant to X-rays than controls may not be of great concern; however, it is rare to show such a change in X-ray

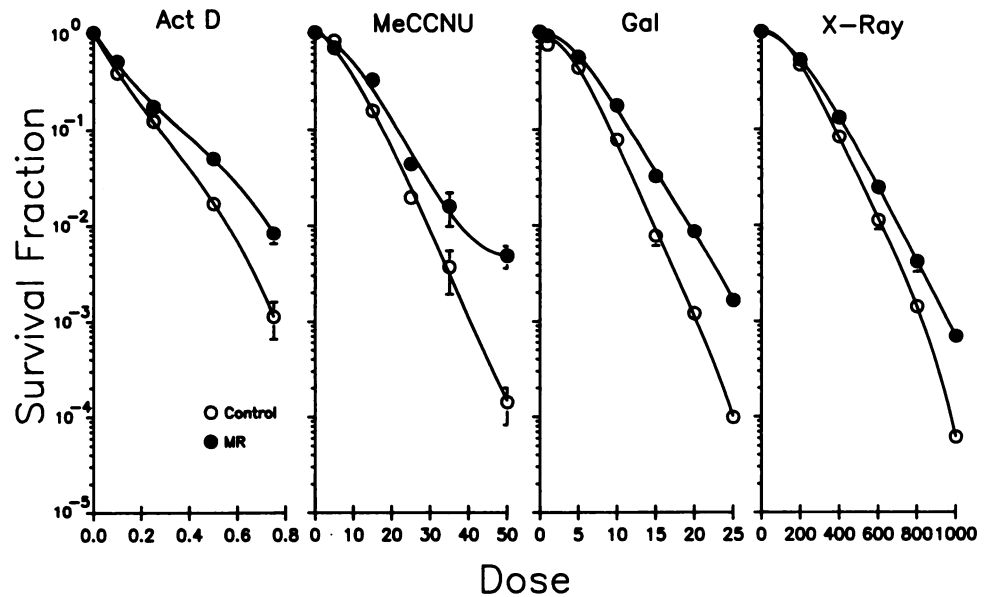


Fig. 5. Survival responses of control cells (O) and Melphalan-resistant cells (●) to ACT D, MeCCNU, and Gal (doses in  $\mu\text{g}/\text{ml} \times 1 \text{ h}$ ) and to X-rays (dose in rad).

Table 2. Multidrug resistance in Melphalan-resistant cells

	$D_{50}$ <sup>a</sup>						
	ACT D	MeCCNU	Gal	X-ray	5FU	Bleo	Adria
Control	0.11	5.5	2.5	100	280	8	0.45
Mel-resistant	0.16	8.0	3.5	110	270	8	0.45
Change (%)	45	45	40	10	3.7	0	0

<sup>a</sup>  $D_{50}$ , in  $\mu\text{g}/\text{ml} \times 1 \text{ h}$  for drugs, in rad for X-rays.

sensitivity. These results were in general agreement with other published results on previously treated cells (1, 2, 21–23). However, the lack of effect on Adriamycin sensitivity in Melphalan-resistant gastric cancer cells (Table 2) was an unexpected finding. Work by Teicher *et al.* (22) and our own previous studies (2) have both shown cross-resistance to alkylating agents and greater sensitivity to Adriamycin in the same cell lines. More recently, Lee and Sieman (4) have presented evidence of correlation of high GSH content and increased resistance to Adriamycin. This could be a cell- or cancer-specific response and would be especially important in the treatment of gastric cancers. For those patients who have gastric cancer that cannot be totally removed by surgical excision, the prognosis remains dismal. Until the introduction of Adriamycin, previously used chemotherapeutic regimens produced no significant improvement in survival. At this time, our results do not explain this discrepancy. There are, however, many causes of resistance.

When the weekly treatments with Melphalan were halted between weeks 14 and 18 and again between weeks 19 and 23, the drug-induced resistance to Melphalan did not revert to the original sensitivity. The survival responses of cells retreated at weeks 18 and 23 were still at least 15 times higher than the average age- or time-matched control responses to Melphalan (Fig. 1), and the GSH values in the resistant population remained higher than in controls (Fig. 2). The resistance to Melphalan could be reversed, however, by treatment with BSO, a specific inhibitor of  $\gamma$ -glutamyl cysteine synthetase and GSH synthesis (24, 25). By week 10, the treated cells had accumulated a total dose of 20  $\mu\text{g}$  Mel/ml (in 2- $\mu\text{g}/\text{ml}$  increments), and the cells were 50 times more resistant than controls (Fig. 1). Exposure of the resistant cells to BSO reduced the GSH values to 40% of control values and partially reversed the Melphalan resistance at all doses (Fig. 4, Table 1). This is in agreement with other *in vivo* and *in vitro* reports (3, 10, 16, 17, 26, 27).

In conclusion, these results show that a single treatment with a high dose of Melphalan ( $LD_{99}$ ) can alter the sensitivity expressed by a cancer cell population to the next treatment with the same agent 1 week later. The 10- to 50-fold increases in resistance, preceded by 1.5- to 3-fold elevations in GSH levels, show that the higher GSH levels were associated with resistance to Melphalan. This suggests the possibility that Melphalan itself may have selected populations of cells with high GSH levels during the first exposure and that this resulted in the initial expression of resistance. However, we showed that additional exposures to Melphalan modulated GSH values to even higher levels in a matter of hours (Fig. 3), and treatments at these times might be proven to be even less effective in cell killing than the weekly dose fractions. The fact that BSO can partially reverse the resistance to Melphalan in the human gastric cancer cell model in this study may be important clinically, especially since these cells were treated in a way similar to the weekly dose fractions used in patients. However, the reduction of GSH in normal cells such as bone marrow or gastrointestinal tissue must be considered, since BSO followed by Melphalan could result in increased toxicity to the patient.

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